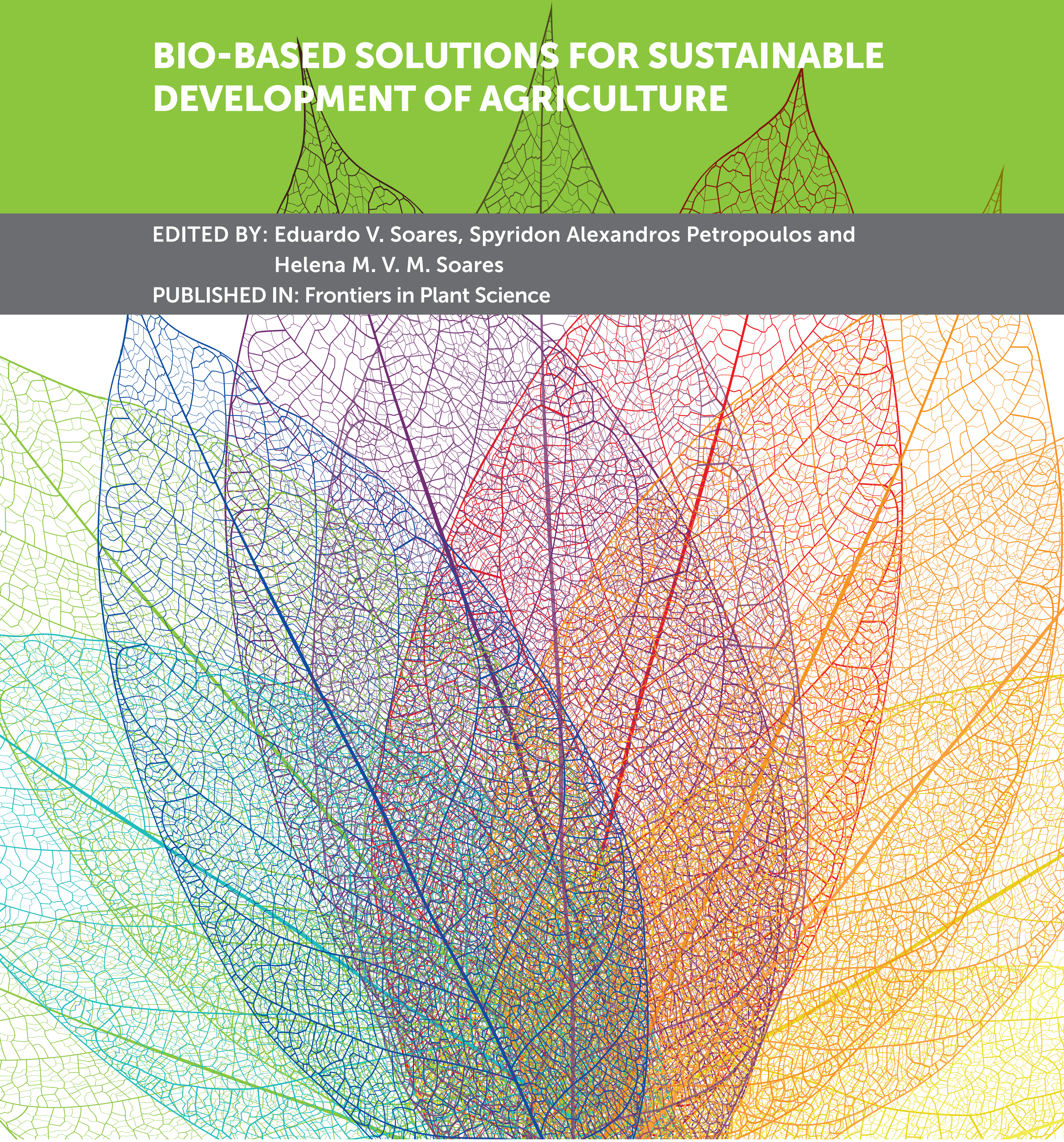

BIO-BASED SOLUTIONS FOR SUSTAINABLE DEVELOPMENT OF AGRICULTURE

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-651-6
DOI 10.3389/978-2-83250-651-6

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BIO-BASED SOLUTIONS FOR SUSTAINABLE DEVELOPMENT OF AGRICULTURE

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Citation: Soares, E. V., Petropoulos, S. A., Soares, H. M. V. M., eds. (2022). Bio-based Solutions for Sustainable Development of Agriculture. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-651-6

Table of Contents

Frontiers in Plant Science

TYPE Editorial
PUBLISHED 17 October 2022
DOI 10.3389/fpls.2022.1056140

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SPECIALTY SECTION
This article was submitted to Plant Nutrition, a section of the journal Frontiers in Plant Science

RECEIVED 28 September 2022
ACCEPTED 06 October 2022
PUBLISHED 17 October 2022

CITATION
Soares EV, Petropoulos SA and Soares HMVM (2022) Editorial: Bio-based solutions for sustainable development of agriculture.
Front. Plant Sci. 13:1056140.
doi: 10.3389/fpls.2022.1056140

Editorial: Bio-based solutions for sustainable development of agriculture

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KEYWORDS

biofertilizers, biostimulants, environmental stress, inhibition of phytopathogens (biocontrol), nutrient use efficiency, mycorrhizal fungi, microbial inoculants, microbial siderophores

Editorial on the Research Topic
Bio-based solutions for sustainable development of agriculture

The modern agricultural sector is facing major challenges related to the increase of global population and food security that threatens many regions of the world. Considering that we expect an increase by 20% of global population until 2050, the agricultural industry has to cope with the increasing demands for food. However, despite the rapid evolution of cropping techniques and farming systems that have substantially improved crop performance over the years, the ongoing climate crisis, the ever-expanding anthropogenic activities and the irrational use of natural resources has put new obstacles since plants have to grow under unfavourable conditions, which severely affect the yield and quality of the marketable product.

Therefore, modern science has to provide new pillars to support the farming sector and identify alternative and sustainable agronomic practices and shift from agrochemicals to bio-based solutions to confront the current and emerging challenges (Ferreira et al., 2019a; Ferreira et al., 2019b; Petropoulos, 2020; Soares, 2022). In this context, Liu et al. evaluated the use of green manuring as an alternative practice to chemical control of goosegrass (*Eleusine indica* L.) in paddy fields. For this purpose, the authors tested a very common species for green manuring, namely milk vetch (*Astragalus sinicus* L.), in a series of experiments including the use of aqueous extracts, decomposed liquid material containing milk vetch powder and, finally, pot experiments where milk vetch powder was added in the growing medium. The obtained results showed great potential for incorporating green manure of milk vetch in soil as a sustainable weed control practice. The biocontrol of phytopathogens was examined by Minchev et al. who designed two synthetic microbial communities (SynComs), composed of bacteria (including, *Bacillus* spp. and *Pseudomonas* spp.) and fungi (*Trichoderma* spp. and *Rhizophagus irregularis*) with complementary biocontrol modes of action. The efficacy of the consortia to control shoot (*Botrytis cinerea*) and root (*Fusarium oxysporum*) pathogens was compared. The results showed that microbial consortia (SynComs) are more versatile (widest protection) than individual microbial inoculants, as they allow the efficient and simultaneous control of both pathogens, under different application methods. In the same line, Soudani et al. studied the tolerance of tomato induced by essential oil produced by *Artemisia absinthium* (AEO), against phytopathogens. A protective effect on tomato seedlings, grown under hydroponics conditions after previous treatment of the seeds with AEO, against *Fusarium oxysporum* sp. *oxysporum radicis lycopersici* (*Fol*) was observed together with: the decrease of various plant disease effects (e.g. fresh weight loss, chlorosis and tissue necrosis); the increase of plant defenses (reactive oxygen species production); callose deposition on seeds surface, and metabolomics and transcriptomics induction modifications that all together resulted in a long term tolerance against the tested fungus. On the other hand, Tran et al. evaluated the efficacy of rhizobacterial strains of *Streptomyces* against *Fusarium verticillioides*, the causal agent of the disease in maize crop known as Fusarium ear rot. The authors showed that *Streptomyces* spp. presented two modes of action against *F. verticillioides.* A direct antagonism, observed *in vitro* and *in vivo* studies, which is characterized by the inhibition of fungal growth and the repression of the production of the mycotoxin fumonisin, as well as the transient modification of expression profiles of the genes *AUX1*, *ARF1*, and *ARF2* (associated with biosynthesis pathway of auxin), and *AN1* (a gibberellic acid-related gene). In addition, a slight induction of the expression of genes associated with salicylic and jasmonic acid biosynthesis and pathogenesis-related proteins was recorded. Moreover, *Streptomyces* spp. also promoted plant growth even when infected with the fungus. Entomopathogenic fungi (EPF), besides their classical action as insect killers, can also perform other functions, such as colonizing various host plants (endophytic fungi), antagonizing phytopathogens and promoting plant growth (biofertilizers), which opens a new opportunity for its use in a sustainable agriculture. Bamisile et al. reviewed the mechanisms involved in plant growth promotion and defence against diseases by endophytic EPF and presented the current knowledge, challenges and limitations associated with the use of these fungi as an alternative to classical agrochemicals of synthetic origin.

In the study of Wang et al., the biostimulatory activity of zaxinone mimics was tested in three vegetable crops, namely tomato, pepper and squash grown under saline conditions. The obtained results showed that the specific compound increased plant growth and yield parameters (fruit size and total fruit weight) when applied at low concentrations (3 μM), while crop performance was higher for zaxinone mimics compared to humic acid, which is commonly used as biostimulant. De Paula et al. evaluated the effect of endophytic and rhizospheric plant growth promoting bacteria (PGPB) on a forage crop, namely *Paspalum atratum*, under *in vivo* and *in vitro* conditions. The *in vitro* tests allowed the isolation of 116 strains of bacteria, while 43 of them showed positive results in regards to nitrogen fixation, phosphate solubilisation and indole acetic acid biosynthesis. Further *in vivo* studies with 8 selected strains indicated 3 strains that significantly improved plant growth of *P. atratum.* The effect of plant growth promoting bacteria, as well as arbuscular mycorrhiza fungi (AMF) was also tested on soybean plants by Ngosong et al. aiming to evaluate its effects under nutrients restriction. The authors suggested that inoculation with PGPB and/or AMF significantly increased root nodulation and acid phosphatase activities, while they also increased the number of effective root nodules resulting in higher crop yield and grain content in macronutrients (carbohydrates, proteins) and minerals (Fe and Zn). However, they also noted that these effects were more profound when PGPB and AMF were integrated with mineral fertilizers. Another strategy, developed by Yadav et al. was based on the inoculation of four plant growth-promoting rhizobacteria, isolated from the rhizosphere of chickpea, to solubilize zinc from the rhizosphere of wheat. As a consequence, a concomitant improvement of wheat growth was observed together with an increase of zinc content in the grains and an over-expression of several TaZIP transport genes in the roots. Enriching the Fe content of plants is an important aspect both for the purpose of increasing crop productivity and for overcoming iron deficiency (the main form of micronutrients malnutrition) and, thus, improving human health. Lurthy et al. reviewed the impact of rhizosphere microbiota (highlighting the influence of plant-microorganism interaction) on increasing the iron content (biofortification) of comestible plant parts. The authors proposed a more holistic approach, based on the characteristics of plants and microorganisms, in order a better assimilation of Fe by plants can be achieved. The efficacy of a fertilizer resultant from a nitrified liquid anaerobic digestate supplemented with mineral nutrients (P, S and/or B) was evaluated by Weimers et al. for the production of leafy vegetables grown on peat-based growing media and compared to standard mineral fertilizers. Results demonstrated that plants fertilized with the non-supplemented mineral digestate evidenced S and B deficiency and early P deficiency, whereas plants fertilized with the supplemented mineral digestate resulted in sufficient plant tissue contents for all elements (except for S) and similar marketable yields comparatively to those fertilized with standard mineral fertilizer. A two-year field experiment study was performed by Yang et al. where the effect of various combinations of organic fertilizers (OF; corresponding to 30%, 50% and 70% of the total nitrogen applied) were assessed for improving the wheat yields with simultaneous minimization of nitrogen leaching and compared with the controlled-release urea (CU) effect. Whereas CU fertilization resulted in a rapid release of nitrogen in the first two months, a slow release of nitrogen combined with a significant residual effect was observed for the OF fertilizers combinations. Moreover, superior grain yield and nitrogen uptake was observed using two fertilizer combinations (30% OF+70%CU and 50%OF+50%CU) comparatively to the urea treatment.

Chen et al. evaluated the physiological and mechanistic functions of arginine on the growth of apple (*Malus hupehensis*) under nitrogen deficiency aiming to increase the tolerance of plants to nitrogen deficiency and, thus, to reduce the use of nitrogenous fertilizers. Under low nitrogen stress, external arginine supplementation, besides providing nitrogen to plants, also promoted the absorption and use of phosphorus, nitrogen and potassium, increasing the overall plant photosynthetic capacity and the antioxidant capacity of the plants. Modifications in the synthesis and metabolism of amino acids (namely, glutamate and ornithine) were also observed and they were reflected in the urea and Krebs cycles. Singh et al. examined the potential of water-stress alleviating effects obtained from biochar incorporation into the soil on maize crop. The authors tested two biochar materials (hardwood and softwood) and three irrigation levels [100%, 70% and 40% of evapotranspiration (ETc)] and suggested that hardwood affected soil properties (bulk density and soil porosity), while moderate water stress (70% ETc) recorded similar values to the control treatment (100% ETc) for the evaluated growth parameters and crop performance. On the other hand, Li et al. evaluated the importance of the PGPB rhizobacteria *Rahnella aquatili*s JZ-GX1 in promoting salt tolerance of the plant *Robinia pseudoacacia*. It was shown that plant seedlings, under salt stress (100 mM NaCl), exposed (through the roots) to bacterium presented an increase in fresh weight and root development compared to untreated ones. The exposure of plant seedlings to volatile organic compounds, produced by the bacterium, led to a reduction in oxidative stress indicators and an increase in enzymatic antioxidant defences and proline (an osmoprotectant) content in plant leaves. The authors proposed that 2,3-butanediol can be one of the relevant signalling compounds in the enhancement of salt tolerance in plants. In their work, Ahmad et al. started by revising the impact caused by heat stress on plants at various plant levels (cellular, organellar and plant as a whole) during different stages of plant growth as well as their natural tolerance mechanisms to combat it. Subsequently, the role of PGPB on the mediation of thermotolerance in plants was highlighted, emphasizing their role on the regulation of various enzymes, phytohormones and metabolites that contribute to induce heat tolerance in plants.

In summary, this Research Topic comprises a collection of 16 articles that offer new and updated knowledge about biofertilizers, biocontrol and improved resilience to environmental stressors. The information presented can be useful in the future development of bio-based products that are expected to be used, as an alternative to current agrochemicals, in modern and more sustainable agriculture.

Author contributions

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding

ES and HS are grateful to the Portuguese Foundation for Science and Technology (FCT) for financial support, funded by national funds through the FCT/MCTES (PIDDAC), under the scope of the strategic funding of UIDB/04469/2020 (Centre of Biological Engineering, University of Minho) and UIDB/50006/2020 (Associated Laboratory for Green Chemistry - Clean Technologies and Processes, LAQV-REQUIMTE) unit, respectively.

Acknowledgments

The authors thank all the peer reviewers who took time to review for this Research Topic.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ferreira, C. M. H., López-Rayo, S., Lucena, J. J., Soares, E. V., and Soares, H. M. V. M. (2019a). Evaluation of the efficacy of two new biotechnological-based freeze-dried fertilizers for sustainable fe deficiency correction of soybean plants grown in calcareous soils. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.01335

Ferreira, C. M. H., Soares, H. M. V. M., and Soares, E. V. (2019b). Promising bacterial genera for agricultural practices: An insight on plant growth-promoting properties and microbial safety aspects. *Sci. Total Environ.* 682, 779–799. doi: 10.1016/j.scitotenv.2019.04.225

Petropoulos, S. A. (2020). Practical applications of plant biostimulants in greenhouse vegetable crop production. *Agronomy* 10, 10–13. doi: 10.3390/agronomy10101569

Soares, E. V. (2022). Perspective on the biotechnological production of bacterial siderophores and their use. *Appl. Microbiol. Biotechnol.* 106, 3985–4004. doi: 10.1007/s00253-022-11995-y

frontiers in Plant Science

REVIEW
published: 30 September 2021
doi: 10.3389/fpls.2021.741804

Model Application of Entomopathogenic Fungi as Alternatives to Chemical Pesticides: Prospects, Challenges, and Insights for Next-Generation Sustainable Agriculture

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Specialty section:
This article was submitted to
Plant Pathogen Interactions,
a section of the journal
Frontiers in Plant Science

Received: *15 July 2021*
Accepted: *06 September 2021*
Published: *30 September 2021*

Citation:
Bamisile BS, Akutse KS,
Siddiqui JA and Xu Y (2021) Model
Application of Entomopathogenic
Fungi as Alternatives to Chemical
Pesticides: Prospects, Challenges,
and Insights for Next-Generation
Sustainable Agriculture.
Front. Plant Sci. 12:741804.
doi: 10.3389/fpls.2021.741804

In the past few decades, the control of pests and diseases of cultivated plants using natural and biological measures has drawn increasing attention in the quest to reduce the level of dependence on chemical products for agricultural production. The use of living organisms, predators, parasitoids, and microorganisms, such as viruses, bacteria, and fungi, has proven to be a viable and sustainable pest management technique. Among the aforementioned, fungi, most importantly the insect-pathogenic species, have been in use for more than 150 years. These include the most popular strains belonging to the genera *Beauveria*, *Metarhizium*, *Isaria*, *Hirsutella*, and *Lecanicillium*. Their application is usually through an inundative approach, which inherently involves exposure of the fungal spores to unfavorable humidity, temperature, and solar radiation conditions. These abiotic factors reduce the persistence and efficacy of these insect-pathogenic fungi. Despite these limitations, over 170 strains have been formulated as mycopesticides and are available for commercial use. In the last few decades, numerous studies have suggested that these species of entomopathogenic fungi (EPF) offer far more benefits and have broader ecological functions than hitherto presumed. For instance, aside from their roles as insect killers, it has been well established that they also colonize various host plants and, hence, provide other benefits including plant pathogen antagonism and plant growth promotion and serve as sources of novel bioactive compounds and secondary metabolites, etc. In this light, the potential of EPF as alternatives or perhaps as supplements to chemical pesticides in plant protection is discussed in this review. The paper highlights the numerous benefits associated with endophytic fungal entomopathogen and host plant associations, the mechanisms involved in mediating plant defense against pests and pathogens, and the general limitations to the use of EPF in plant protection. A deeper understanding of these plant host-fungus-insect relationships could help unveil the hidden potentials of fungal endophytes, which would consequently increase the level of acceptance and adoption by users as an integral part of pest management programs and as a suitable alternative to chemical inputs toward sustainable crop production.

Keywords: biological control, plant-fungi interactions, integrated pest management, mutualism, plant nutrients, mycopesticides

INTRODUCTION

Insect pests, plant pathogens, and unfavorable growing conditions induce biotic and abiotic stresses in crop plants. These factors are responsible for huge plant productivity losses (up to 26% crop losses, valued at over $470 billion worldwide; Culliney, 2014). To ensure optimum productivity of cultivated plants, agriculturists depend heavily on chemical insecticides and inorganic fertilizers to combat these problems (Skinner et al., 2014). The overdependence on synthetic fertilizers for improving the growth of plants is another constraint faced by agriculturists. This is because the overreliance on these chemicals poses several side effects to users, non-target organisms, and the environment (Skinner et al., 2014; Fadiji and Babalola, 2020b).

As the population of the world is expected to increase to approximately 9.1 billion by 2050 (Liu et al., 2017a), efforts are in place to ensure sustainable agricultural production. However, excessive usage and overdependence on synthetic pesticides and fertilizers, climatic changes, poor land management, and mass urbanization are some of the factors affecting these efforts (Smith et al., 2016). The potential application of entomopathogenic fungi (EPF) as biocontrol agents against herbivores represents an environmentally sustainable alternative insect pest management approach (West and Gwinn, 1993). EPF are known for their ability to infect insects leading to disease in proper conditions, where they directly colonize the insect body by penetrating its cuticles. To date, more than 700 species from approximately 90 different genera have been established as insect-pathogenic fungi (Khachatourians and Qazi, 2008). These include the most popular strains belonging to the genera *Beauveria*, *Metarhizium*, *Isaria*, *Hirsutella*, and *Lecanicillium* (Inglis et al., 2001; Khachatourians and Qazi, 2008). Among them, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *Isaria fumosorosea* Wize, *Metarhizium anisopliae* (Metschnikoff) Sorokin, and *Lecanicillium lecanii* (Zimmerman) Viegas are the most commonly studied fungal species (Li et al., 2011; Chen et al., 2015). These entomopathogenic fungal strains are commonly studied for their potential use as biological control agents in mitigating crop losses due to insect pests (Hunter, 2005). *Beauveria bassiana* and *M. anisopliae* are the most widely distributed species and are commonly found on and have been isolated from infected insects in both temperate and tropical regions throughout the world (Zimmermann, 2007a). Several of the EPF species, for example, *I. fumosorosea* and *I. farinosa*, can infect multiple hosts without showing any of the numerous harmful effects associated with chemical pesticides and, therefore, are considered safe and environmentally friendly (Gao et al., 2017). These EPF, aside from naturally regulating insect populations by causing epizooties, have also been established to play additional multitrophic roles. They have the ability to colonize different host plants and exist in the form of fungal endophytes (Vega et al., 2009; Qayyum et al., 2015; Bamisile et al., 2018b; Wakil et al., 2020), act as rhizosphere colonizers (Hu and St Leger, 2002), serve as plant pathogen antagonists (Ownley et al., 2004, 2010; Kim et al., 2008; Jaber and Ownley, 2018), plant growth promoters/biofertilizers (Kabaluk and Ericsson, 2007; Elena et al., 2011; Sasan and Bidochka, 2012; Liao et al., 2014; Lopez and Sword, 2015; Jaber and Enkerli, 2017), and as sources of novel bioactive compounds and multiple secondary metabolites (Tadych et al., 2009; Hu et al., 2016; Al-Ani et al., 2021). In addition, they can also play an essential role in the biotransformation of steroids and flavonoid glycosides (Dymarska et al., 2017, 2018). Several studies have reported different insect-pathogenic fungal species as natural colonizers/endophytes of many economically important crops, including maize, coffee, potato, cotton, beans, Jimson weed, tomato, and chickpea (Jones, 1994; Arnold and Lewis, 2005; Vega et al., 2008; Qayyum et al., 2015; Wakil et al., 2020). Similarly, the potential for establishing these EPF strains as endophytes in different plant species using various artificial inoculation methods has also been previously discussed (Bamisile et al., 2018a; Sinno et al., 2020).

These numerous attributes of endophytic EPF ensure that, in addition to their conventional application as insect killers, they can also be adopted as beneficial plant growth-promoting microorganisms, and they have shown great potential thus far as biofertilizers. These endophytic fungal species are believed to serve as alternatives to systemic fertilizers, as well as an efficient and eco-friendly approach toward food security (Glick, 2014). In organic farming systems, the level of utilization of fungal endophytes as a means of improving yields and protecting plants from damage is increasing (Shrivastava et al., 2010). Various species of endophytic fungi have been underlined for their potential as indirect biocontrol agents in large-scale agricultural applications (Lacey and Neven, 2006). The use of biotechnology for crop improvement through inoculation of plants with modified fungal strains would therefore reduce toxicity to humans, livestock, and the environment. The genes of fungal endophytes could be genetically transformed through the removal of detrimental genes or otherwise by the addition of new beneficial genes (Adeleke and Babalola, 2021). Endophytes could then be used as surrogate hosts to transform crops genetically. Using this method, the endophyte of ryegrass has been genetically transformed and successfully applied as a deterrent to herbivores (Murray et al., 1992). Similarly, in the quest to improve endophytic resources, efforts are being made toward the discovery of novel metabolic compounds that cannot otherwise be synthesized using chemical methods. It is therefore imperative to have a clear understanding of the biology of plants and the ecology of the fungal communities to explore the richness of beneficial fungal endophytes under different cropping systems. In addition, to make fungal entomopathogens readily available and easy to use, as they are considered as biocontrol agents with a non-resistance and non-contaminant advantage over synthetic pesticides, many insect-pathogenic fungal strains have been formulated as bioinsecticides (Fang et al., 2014) and, thus, are currently considered an alternative management method for many insect pests of economic value. Due to the aforementioned attributes and many other prospects, the level of acceptance and adoption of fungal entomopathogens/endophytes is rapidly increasing, and thus, research into their biology, ecology, and mode of action is attracting more public and scientific interest (Dong et al., 2016; Hu et al., 2016). In this light, the current review discusses the available knowledge on EPF utilization and mechanisms as biological agents for plant growth promotion and pest and disease control, thereby exploring the prospects and limitations toward potential adoption as alternatives to synthetic pesticides.

ENTOMOPATHOGENIC FUNGI AS ALTERNATIVES TO CHEMICAL PESTICIDES: A REALITY OR MYTH?

Entomopathogenic fungi have been in active use for the management of a plethora of economic pests of crop plants for approximately 200 years now. *Beauveria bassiana* was first isolated and identified approximately 170 years ago (Zimmermann, 2007b), while *Beauveria brongniartii* (Saccardo) Petch and *M. anisopliae* have also been in use for over 110 and 130 years, respectively (Zimmermann, 2007a). These fungal species together with other known hypocrealean fungi, such as *I. fumosorosea*, *M. brunneum*, *M. robertsii*, and *Hirsutella thompsonii* Fisher, are commonly used against a broad range of arthropod pests (Dara, 2019b). They are mostly applied through inundative approaches (Bamisile et al., 2018b; Jaber and Araj, 2018) and have been reported to be effective against several insects of different feeding guilds including aphids, locusts, thrips (Gulzar et al., 2021), grubs (Wakil et al., 2017; Yasin et al., 2019), moths (Ali et al., 2015; Tahir et al., 2019), mites, mosquitoes, whiteflies, and tephritid fruit flies (Dong et al., 2016; Bamisile et al., 2020; Canassa et al., 2020; Usman et al., 2020). Additionally, EPF have been found to be pathogenic against phytopathogenic nematodes and other soil-borne pests (Pocasangre et al., 2000).

The management of economic pests using insect-pathogenic fungi, therefore, serves as an effective and sustainable alternative to chemical control. Despite the enormous benefits of EPF, the exposure of fungal spores to unfavorable climatic conditions in the field reduces their efficiency and level of general utilization (Dong et al., 2016). However, utilization of EPF through inoculation as fungal endophytes rather than using an inundative approach can help significantly reduce the negative effects due to abiotic stressors (Vega, 2018). For instance, it has been reported that *B. bassiana* can offer longer protection to the host plant when existing as an endophyte *in planta*. The fungus can persist in the host tissues over a long duration. This possibility has been reported in citrus (Bamisile et al., 2020), jute, *Corchorus olitorius* (Biswas et al., 2012), and radiata pine, *Pinus radiata* (Brownbridge et al., 2012), where endophytic colonization of the hosts was found to be sustained up to 2, 3, and 9 months post-fungal treatment of seedlings, respectively. Several studies also reported similar effects and properties in different plant species, including coffee, fava bean, and common bean (Posada et al., 2007; Jaber and Enkerli, 2016; Dash et al., 2018). In this regard, there are significant pieces of evidence to confirm that EPF can be successfully introduced as fungal endophytes in plants using different artificial inoculation methods (Bamisile et al., 2018a) and could consequently be used as substitutes for chemical pesticides. In addition, some previous studies have reported systemic colonization of treated plants without any symptomatic effects. This implies that treatment of a specific organ or part of the plant (leaf, stem, or root) irrespective of the artificial inoculation method used can result in endophytic colonization of the entire plant and confer systemic resistance to the host plant against herbivores (Mantzoukas et al., 2015; Jaber and Enkerli, 2016; Dash et al., 2018).

Successful endophytic colonization of plants provides multiple benefits, including plant growth promotion, protection against insect pests, induction of systemic resistance, antagonization of plant pathogenic fungi, bacteria, and nematodes, and suppression of the negative effects of abiotic stressors on host plants (Kabaluk and Ericsson, 2007; Kim et al., 2008; Vega et al., 2009; Ownley et al., 2010; Elena et al., 2011; Sasan and Bidochka, 2012; Liao et al., 2014; Vega, 2018). In addition to the aforementioned benefits of EPF/endophytes, in the past few decades, fungal endophytes have garnered more attention, as well as broader biotechnological and industrial relevance, due to their uniqueness as sources of secondary metabolites. Their ability to secrete novel biochemical compounds arguably provides an edge over chemical pesticides. Furthermore, they serve medical/pharmaceutical purposes as antimicrobial, antidiabetic, antitumor, and immune suppressing agents (Gouda et al., 2016; Yadav, 2018). This aspect is discussed further in the section "*endophytic fungi as good sources of pharmaceutical products*." As a rich source of natural products, it is worth noting that in the last 20 years, these organisms have been isolated from various plants for industrial and agricultural purposes (Fadiji and Babalola, 2020a).

Many studies have underlined the important roles played by endophytic fungi, and their non-pesticidal properties are of huge importance in fungal evolution and survival in plants and in soil environments in the absence of arthropod hosts (Dara, 2019b). Many studies have provided evidence that confirms that these beneficial microbes can improve the soil structure and microbiome, and nutrient and water absorption in plants, induce systemic resistance, and serve as probiotics that antagonize harmful microorganisms (Sasan and Bidochka, 2012; Jaber and Salem, 2014; Behie et al., 2015; Jaber and Alananbeh, 2018). Another interesting aspect of the use of endophytic fungi as insect pest biocontrol agents is that some of the notorious pests that have developed resistance or otherwise successfully evaded chemical pesticide treatment have been shown to be successfully controlled using these EPF/endophytes.

Examples of such pests are stem borers, which have been reported to be susceptible following treatment of coffee (Posada and Vega, 2006) and sorghum plants (Tefera and Vidal, 2009) with endophytic *B. bassiana*.

In recent years, with the increasing interest in sustainable food production systems, biological agents, including beneficial microbes, biostimulants, and other biocontrol agents, have been adopted and are now gaining more popularity. Considering the unique attributes of endophytic fungi, most of these species are currently being explored for crop production in both small- and large-scale farming and in home and community gardens (Dara, 2019b). With reference to the aforementioned benefits and many more that are still emerging and the numerous prospects of endophytic EPF, the possibility of becoming a suitable replacement for inorganic chemicals is rapidly becoming a reality.

FORMULATION OF ENTOMOPATHOGENIC FUNGI AS MYCOPESTICIDES

Multiple genera of hypocrealean fungi that have been found effective against various species of arthropod pests are considered an integral component of integrated pest management (IPM) strategies in maintaining pest control efficacy, mitigating the risk of inorganic pesticide resistance, and offering environmentally sustainable pest suppression (Dara, 2019b). To achieve these objectives, many fungal entomopathogen-based biopesticides have been formulated over the years, as they are believed to be suitable and direct replacements of the commonly used synthetic insecticides. Although the history of mycoinsecticide and mycoacaricide development dates back to the early 1960s (de Faria and Wraight, 2007), it is of note that the frequencies of applications and timings of most of these mycoinsecticides are similar to those of conventional insecticides (Wraight et al., 2000; Shah and Pell, 2003). The most common mycopesticides are products formulated from *B. bassiana*, *M. anisopliae*, *B. brongniartii*, and *I. fumosorosea*. For instance, in the last three decades, a good number of *M. anisopliae*- and *B. bassiana*-based mycoinsecticides have been commercialized and registered in various countries (Zimmermann, 2007a,b; more detail is provided in **Table 1**; see also Wraight et al., 2001; de Faria and Wraight, 2007).

The spores of *B. bassiana* and *B. brongniartii* have been successfully formulated as mycopesticides in many countries, and several of these products have passed the registration requirements and are therefore currently widely used for biocontrol of pest insects in various countries (Wraight et al., 2001; de Faria and Wraight, 2007). *Metarhizium anisopliae* strains have also been developed and commercialized against several pests and many disease vectors (Akutse et al., 2020). Similarly, *L. lecanii* has been reported to naturally control aphid and scale insect populations in tropical and subtropical regions and thus has been studied and formulated for use as a mycoinsecticide. According to Shah and Pell (2003), *L. lecanii* was the first fungus to be developed as inundative mycoinsecticide for use in medium- to large-scale farming in glasshouses. The active ingredients from two different isolates were formulated into two products: "Vertalec" against aphids and "Mycotal" for the control of whiteflies and thrips (Shah and Pell, 2003). Both products have been registered in numerous countries in Europe and beyond, including the Netherlands, Finland, Denmark, France, Norway, Turkey, Spain, and the United Kingdom. Since their introduction in 1981, strong efficacy against a broad range of aphid species has been reported (Yeo et al., 2003). Another popular product that has been developed for use is "Mycotrol," which is a mycoinsecticide formulated from *B. bassiana* (Bradley et al., 1992). The product was registered in 1999 for use against aphids, grasshoppers, thrips, whiteflies, and many other insect pests affecting trees and field crops. Similarly, another mycopesticides, "Green Muscle," was developed in Africa to control the outbreak of the desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Arcidridae), between 1985 and 1989. The research project was undertaken by research institutions in the United Kingdom, the Netherlands, the Republic of Benin, and Niger. The mycoinsecticide was made of dried conidia of *Metarhizium anisopliae* var. *acridum*, which is often mixed with kerosene or diesel oil before application (Bateman et al., 1998). The product was reported to cause up to 90% mortality in treated grasshoppers and locusts within 2–3 weeks post-treatment, while no side effects on non-target organisms were recorded (Lomer et al., 2001). Before this period, around the early 1980s in Russia (then USSR), a *B. bassiana*-based mycopesticide named "Boverin" was applied over thousands of hectares for the control of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), and the codling moth, *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae; Hussey and Tinsley, 1981). Several other similar products are available for use in glasshouses, as well as by organic farmers in the United States and beyond. Examples of such formulations are "BotaniGard" and "Mycotrol-O." All of these products are considered suitable replacements for synthetic insecticides.

BENEFITS AND SAFETY CONCERNS OF BIOPESTICIDES

In recent years, the numerous benefits that could be derived from mycopesticide utilization in place of chemical insecticides have been highlighted by many authors (Wraight et al., 2001; Shah and Pell, 2003; de Faria and Wraight, 2007; Zimmermann, 2007a,b). More advanced scientific investigations on how to manage the various setbacks encountered and improve the efficiency of these microbial organisms are still emerging. However, the most common questions raised, which have been attracting the attention of several researchers and mycopesticide users, are concerns about the safety risks of biopesticides and their secondary metabolites. Zimmermann (2007a) analyzed the safety issues related to *Beauveria* spp. use as mycopesticides, where the author highlighted the biological properties, history, geographical distribution, host range, mode of action, and toxin-producing capabilities of the fungal species. The potential side effects on non-target organisms, such as predators,

TABLE 1 | Some of the common mycopesticides that have been formulated and registered for use as alternatives to chemical pesticides.

Source	Trade name of product	Name of manufacturing company	Country of origin	Target insect pests	Reference
B. bassiana	Bauveril	Laverlam S.A., Colombia	Colombia, Dominican Republic	Coleoptera (Curculionidae, Scarabaeidae), Lepidoptera (Castniidae)	de Faria and Wraight, 2007
	Bio-Power	Stanes	India	Stem borers, cut worms, root grubs, leafhoppers, whiteflies, aphids, thrips and mealy bugs.	de Faria and Wraight, 2007; Zimmermann, 2007a
	BotaniGard ES BotaniGard 22 WP	Laverlam International (formerly Emerald BioAgriculture)	United States	Grasshoppers, whiteflies, thrips, aphids and many other insect pests	Shah and Pell, 2003; de Faria and Wraight, 2007
	Boverol	Fytovita	Czech Republic	Coleoptera (Chrysomelidae)	de Faria and Wraight, 2007; Zimmermann, 2007a
	Boverin	Biodron	Russia	Colorado potato beetle; *Leptinotarsa decemlineata*, and the codling moth; *Cydia pomonella*	Zimmermann, 2007a
	Conidia	Hoechst Schering AgrEvo	Columbia	Coleoptera (Curculionidae)	de Faria and Wraight, 2007; Zimmermann, 2007a
	Mycotrol ES Mycotrol-O	Laverlam International (formerly Emerald BioAgriculture)	United States	Grasshoppers, whiteflies, thrips, aphids, and many other insect pests	Shah and Pell, 2003
	Naturalis	Intrachem	Italy	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hymenoptera (Formicidae), Diptera (Tipulidae), Hemiptera (Lygaeidae, Cercopidae, Cicadellidae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae)	Zimmermann, 2007a
	Naturalis-L	Andermatt Biocontrol Troy Biosciences Inc.	Switzerland United States	Coleoptera (Chrysomelidae, Curculionidae), Hemiptera (Miridae, Cicadellidae, Aleyrodidae, Aphididae, Psyllidae), Lepidoptera, Thysanoptera (Thripidae)	de Faria and Wraight, 2007; Zimmermann, 2007a
	No tradename	Anhui Heibaogong Pest Control Co., Ltd	China	Unknown	Website[1]
	No tradename	Shandong Ruyi Biotechnology Co., Ltd	China	*Plutella xylostella* (Linnaeus)	Website[1]
	No tradename	Hebei Zhongbao green crop Technology Co., Ltd	China	Thrips	Website[1]
	Ostrinil	Arysta (formerly NPP, Calliope)	France	Lepidoptera (Crambidae)	Wraight et al., 2001; de Faria and Wraight, 2007; Zimmermann, 2007a
	Proecol	Probioagro S.A., Venezuela	Venezuela	Lepidoptera (Noctuidae)	Wraight et al., 2001; de Faria and Wraight, 2007; Zimmermann, 2007a
	Racer BB	SOM Phytopharma	India	Lepidoptera (Noctuidae)	de Faria and Wraight, 2007; Zimmermann, 2007a
	Trichobass-L Trichobass-P	AMC Chemical/Trichodex	Spain	Coleoptera (Curculionidae, Scarabaeidae), Lepidoptera (Castniidae, Pieridae), Hemiptera (Aleyrodidae), Thysanoptera (Thripidae)	de Faria and Wraight, 2007; Zimmermann, 2007a

(Continued)

TABLE 1 | Continued

Source	Trade name of product	Name of manufacturing company	Country of origin	Target insect pests	Reference
B. brongniartii	Beauveria Schweizer	Lbu (formerly Eric Schweizer Seeds)	Switzerland	European cockchafer and other scarab beetles species	Shah and Pell, 2003
	Betel	Arysta (formerly NPP, Calliope)	France	Coleoptera (Scarabaeidae)	Zimmermann, 2007a
	Biolisa-Kamikiri	Nitto Denko	Japan	Coleoptera (Cerambycidae)	Wraight et al., 2001; de Faria and Wraight, 2007; Zimmermann, 2007a
	Engerlingspilz	Andermatt Biocontrol AG	Switzerland	European cockchafer and other scarab beetles species	Shah and Pell, 2003
	Melocont-Pilzgerste	Agrifutur-Kwizda	Italy Austria		Zimmermann, 2007a
I. farinosa	Paecilomin		Russia	Apple moth, Siberian pine caterpillar, and larch caterpillar	Weng et al., 2019
I. fumosorosea	NoFly™ WP	Natural Industries Inc.	United States	Whiteflies, aphids, thrips, psyllids, mealybugs, and fungus gnats	Weng et al., 2019
L. lecanii	Mycotal	Koppert Biological Systems	Netherlands	Whiteflies and thrips	Shah and Pell, 2003
	Vertalec	Koppert Biological Systems	Netherlands	Aphids	Shah and Pell, 2003
M. anisopliae	Achieve	Real IPM	Mozambique	*Tetranynchus urticae*	Akutse et al., 2020
	BioBlast	EcoScience	United States	Isoptera (Kalotermitidae, Rhinotermitidae, Termopsidae)	de Faria and Wraight, 2007; Zimmermann, 2007b
	Bio-Cane	Granules Becker-Underwood	Australia	Sugarcane pest; grayback canegrub	Zimmermann, 2007b, Website[2]
	Bio-Catch-M	Stanes	India	Hemiptera (Aleyrodidae, Aphididae)	de Faria and Wraight, 2007; Zimmermann, 2007b
	Bio-Green Granules	Becker-Underwood	Australia	Coleoptera (Scarabaeidae)	de Faria and Wraight, 2007; Zimmermann, 2007b
	Bio-Magic	Stanes	India	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Cercopidae)	de Faria and Wraight, 2007; Zimmermann, 2007b
	BioPath	EcoScience	United States	Blattodea (Blattellidae, Blattidae)	Zimmermann, 2007b
	Campaign	Real IPM	Ghana	Mealybugs	Akutse et al., 2020
			Uganda	Thrips, fruit flies, mealybugs	
	Cobican	Probioagro	Venezuela	Coleoptera (Scarabaeidae), Hemiptera (Cercopidae, Aphididae)	de Faria and Wraight, 2007; Zimmermann, 2007b
	Gran Met-P	Kwizda/Agrifutur	Austria/Italy	Coleoptera (Scarabaeidae, Curculionidae, Nitidulidae)	de Faria and Wraight, 2007; Zimmermann, 2007b
	Green Guard SC	Becker-Underwood	Australia	Orthoptera (Acrididae)	Zimmermann, 2007b
	Green Guard ULV	Becker-Underwood	Australia	Orthoptera (Acrididae)	Zimmermann, 2007b
	Green Muscle	CABI Bioscience/NPP	United Kingdom/ France	Locust and grasshopper	Shah and Pell, 2003
	Mazao achieve	Real IPM	Kenya	*T. urticae*	Akutse et al., 2020
	Mazao campaign	Real IPM	Kenya	Mealybugs	Akutse et al., 2020
	Mazao supreme	Real IPM	South Africa	Aphids	Akutse et al., 2020
	Metaquino		Brazil	Sugarcane spittle bug; *Mahanarva postica*	Zimmermann, 2007b
	Metarhizium Schweizer	Lbu (formerly Eric Schweizer Seeds)	Switzerland	Coleoptera (Scarabaeidae)	Zimmermann, 2007b
	Metathripol	ICIPE	Kenya	Thrips	Website[3]

(Continued)

TABLE 1 | Continued

Source	Trade name of product	Name of manufacturing company	Country of origin	Target insect pests	Reference
	No tradename	Chongqing Zhongda Biotechnology Development Co., China	China	Orthoptera (Acrididae)	Nong et al., 2015
	No tradename	Jiangxi Tianren Ecology Corp., China	China	Lepidoptera (Noctuidae)	Nong et al., 2015
	No tradename	Beijing Sangbai Biotechnology Co., Ltd., China	China	Blattaria	Nong et al., 2015
	Pacer	SOM Phytopharma	India	Isoptera	Zimmermann, 2007b
	Real metarhizium 69	Real IPM	South Africa	Mealybugs, thrips, leafminers	Akutse et al., 2020
			Zambia	Fruit flies, mealybugs	
	Real metarhizium 78	Real IPM	Canada	*T. urticae*, plant growth regulator	Akutse et al., 2020
	Real metarhizium OD	Real IPM	Zimbabwe	Biofertilizer	Akutse et al., 2020
	Real metarhizium SC	Real IPM	Tanzania	Mealybugs	Akutse et al., 2020
	Taenure Granular Bioinsecticide	Novozymes Biologicals (formerly Earth BioSciences)	United States	Coleoptera (Curculionidae, Scarabaeidae), Diptera (Ephydridae, Mycetophilidae, Sciaridae, Tipulidae), Thysanoptera (Thripidae)	Zimmermann, 2007b, Website[4]
	TAE-001 Technical Bioinsecticide	Novozymes Biologicals (formerly Earth BioSciences)	United States	Coleopterans; Elateridae, Curculionidae	Zimmermann, 2007b
	Tick-Ex EC	Novozymes Biologicals (formerly Earth BioSciences)	United States	Acari (Ixodidae), Coleoptera (Scarabaeidae)	Zimmermann, 2007b
	Tick-Ex G	Novozymes Biologicals (formerly Earth BioSciences)	United States	Acari (Ixodidae), Coleoptera (Scarabaeidae)	Zimmermann, 2007b

[1] *Website: http://www.chinapesticide.org.cn/hysj/index.jhtml (In Chinese).*
[2] *Website: https://www.bioscregister.com/Becker_Underwood/Supplier/sid2909.htm*
[3] *Website: https://sitem.herts.ac.uk/aeru/bpdb/Reports/1980.htm*
[4] *Website: http://www.epa.gov/oppbppd1/biopesticides/ingredients/*

parasitoids, pollinators, arthropods, and vertebrates (birds, fish, amphibians, and reptiles), and human health were also discussed. The author suggested that, to date, no serious side effects have been ascribed to the use of the two *Beauveria* strains and hence concluded that both *B. bassiana* and *B. brongniartii* are relatively safe for use as mycopesticides (Zimmermann, 2007a). Other recent studies have also confirmed the low-risk status of common mycoinsecticides and therefore proposed their use as alternatives to chemical insecticides for the management of agricultural pests and disease vectors (Zimmermann, 2007a,b). The safety of these products to users and the environment has been well-assessed (Haas-Costa et al., 2010). In addition, the mycotoxins produced by them are considered very unlikely to enter the food chain (Hu et al., 2016).

ENTOMOPATHOGENIC FUNGI MEDIATING PLANT DEFENSE AGAINST INSECT PESTS

Many species of insect-pathogenic fungi have been characterized for their ability to colonize and become established as fungal endophytes in plants. *Beauveria bassiana*, *M. anisopliae*, and other hypocrealean fungi are known to colonize many plants endophytically (Vega et al., 2008). Several studies have demonstrated the colonization potential of various EPF using different artificial inoculation techniques. Endophytic fungi can be inoculated into plants using foliar application, soil drench, flower treatment, stem injection, seed soaking, etc. (Lopez and Sword, 2015; Muvea et al., 2015; Greenfield et al., 2016; Bamisile et al., 2018b; Rondot and Reineke, 2018; Ramos et al., 2020). The inoculation method may depend on the part of the plant targeted for endophytic colonization or the type of insects to be controlled, that is, root eater, stem borer, or leaf chewing insect (Bamisile et al., 2018a). However, irrespective of the inoculation method used, many of the recent studies have revealed several tritrophic interactions that exist among the inoculated plants, the endophytic EPF, and the herbivores feeding on the endophyte-challenged plants (Akello et al., 2008; Vega et al., 2008; Reddy et al., 2009; Dash et al., 2018).

Fungal endophytes generally live part or their lifecycle within the tissues of living hosts without causing any noticeable disease symptoms (Suryanarayanan, 2013; Hardoim et al., 2015). They can colonize any part of the host, including the embryo of seeds. As the seedlings germinate and develop during the early growth stages, the endophytes also increase in abundance (Shade et al., 2017). Endophytes depend on their plant hosts for nutrition and provide indirect defense against herbivores associated with the hosts (Backman and Sikora, 2008; Tadych et al., 2009). The endophytic fungi involved in this fungus-plant interaction have been described as plant-defending mutualists (Saikkonen et al., 2004), where the fungi mediate adaptive protection against insect pests of the host plant (White et al., 2002). Several recent studies have demonstrated that endophyte-challenged crop plants are less likely to be attacked by insect pests (Lopez and Sword, 2015; Muvea et al., 2015; Rondot and Reineke, 2018; Ramos et al., 2020). In addition, evidence of endophytic fungi reducing the productivity of herbivores feeding on colonized plants is readily available (Vega et al., 2009; Lopez et al., 2014; Dash et al., 2018; Dara, 2019b). Fungal endophytes are believed to serve as bodyguards for their host plants against primary herbivore pests (Vidal and Jaber, 2015; Jaber and Ownley, 2018). Under moderate to high levels of herbivore attack, plants colonized with endophytic fungi can generally outperform endophyte-free plants (Clay, 1997).

MODE OF ACTION AGAINST INSECT PESTS

When present in the form of fungal endophytes in plants, EPF induce indirect detrimental effects on pests through various non-pathogenic mechanisms, such as antibiosis, antixenosis, and induced systemic resistance (ISR; Hartley and Gange, 2009). In this case, the fungus provides indirect defense against the host pests in exchange for carbohydrate energy resources derived from the plant (Wang and Qiu, 2006). The fungal endophytes, which provide indirect defense against their hosts' primary enemies, may have been derived from different origins, including mutualistic root endophyte associations and the evolution of EPF into plant-associated endophytes (Vega et al., 2008). This plant-fungus mutualist interaction has been found to increase the rate of water and nutrient absorption as well as providing protection from insect pests, birds, and mammals (Lekberg and Koide, 2005). The mechanisms by which endophytic fungi minimize insect herbivore damage in their host plants are numerous, including pest avoidance or deterrence (Latch et al., 1985), reduction in feeding (Knoch et al., 1993), survival (Lacey and Neven, 2006), oviposition (Clay, 1990), and growth and developmental rate (Valenzuela-Soto et al., 2010).

As new findings are emerging periodically, various scientific studies are ongoing, and many others are still to come. Several questions have been raised, and different authors have identified various research gaps in very recent publications. In this light, the mechanisms underlying the pathogenicity-related activities of endophytic fungi against insect pests might not have been fully explored (Vidal and Jaber, 2015; Vega, 2018). In general, endophytic fungi promote host protection against primary pests by stimulating the production of plant defensive compounds, which have been characterized as having numerous bioactivities and functions (Carroll, 1988). Fungal endophyte-challenged plants exhibit feeding deterrence or antibiosis against their primary insect pests due to the synthesis of secondary metabolites by endophytic fungi. Colonized plants are less favorable to herbivores and indirectly affect the fecundity, fitness, and longevity of pests (Akello and Sikora, 2012; Akutse et al., 2013; Muvea et al., 2014; Mantzoukas et al., 2015; Dash et al., 2018; Jaber and Ownley, 2018). Endophytic fungi belonging to the genera *Beauveria* and *Metarhizium* spp. are commonly known for their ability to synthesize different arrays of secondary metabolites, which have been reported to exhibit antibacterial, antifungal, and insecticidal properties. These compounds include bassianolides, bassianolone, beauvericin, and oosporein, which are synthesized by *B. bassiana*. Similarly, cytochalasins, destruxins, serinocyclins, etc., are key compounds derived from *Metarhizium* spp. (Krasnoff et al., 2007). For instance, a hexa-cyclodepsipeptidic mycotoxin known as destruxin A (DA), synthesized by *M. anisopliae*, has been revealed to exhibit insecticidal and immunosuppressing activities (Fan et al., 2013; Ravindran et al., 2016).

Following endophytic colonization of plants, the fungus alters the nutrient content of the host to favor the production of secondary metabolites. Alterations in the chemical composition of the host plant inhibit the rate of herbivory and oviposition by insects (Clay, 1990). The detrimental effects of endophytic fungi and their metabolites on insect pests are different from those of the fungal infections caused by herbivore exposure to conidia or blastospores. The fungus grows as mycelia inside the plant, while infective structures are not produced inside the plant tissues as opposed to the hemolymph of the infected host insect. As a result, mycosis does not generally occur due to herbivores feeding on colonized plant tissues (Qayyum et al., 2015). However, there are a few records of mycosis in insect cadavers (Powell et al., 2009; Ramakuwela et al., 2020). The most common examples are in chewing insects, where insects feeding on colonized plants can easily be exposed to EPF emerging from wounded plant tissues. Following exposure, conidiation and infection could occur epiphytically, resulting in the dead insect showing mycosis. Powell et al. (2009) opined mycosis could have resulted from the exposed insect consuming an intact and sufficient amount of hyphae. There are a few available reports of mycosis in pests that fed upon host plants endophytically colonized by *B. bassiana*, such as Akello et al. (2008), Vidal and Jaber (2015), Klieber and Reineke (2016), and Ramakuwela et al. (2020). The negative effects of defense chemicals induced by fungal endophytes are more evident in generalist pests than in specialist pests. This is because generalists are more susceptible to endophytic fungal-mediated specific and qualitative defenses (Smith and Read, 2010). Koricheva et al. (2009) suggested that the negative effects of fungal infection on generalist pests could indirectly benefit specialist chewing insects. A similar finding has also revealed significant detrimental effects on generalist mesophyll feeders, while in contrast, phloem feeders were found to be less susceptible to fungal defense (Gehring and Bennett, 2009).

FUNGAL ENDOPHYTE-PATHOGEN INTERACTIONS MEDIATING HOST RESISTANCE AGAINST PLANT PATHOGENS AND DISEASES

Endophytic fungi protect their host plants against pathogens by engaging similar mechanisms as those used in inducing plant resistance against herbivores (Jaber and Ownley, 2018). The secondary metabolites produced by these wide ranges of fungal endophytes have been found to exhibit antifungal and antibacterial potential, which help host plants evade damage/disease caused by phytopathogenic microorganisms (Gunatilaka, 2006).

In general, endophytic fungi mediate plant disease antagonism by inducing systemic plant resistance. The endophytes of the upper parts of grasses and some other beneficial plants have been well characterized for these activities (Jaber and Enkerli, 2016; Dash et al., 2018). Many species of endophytic EPF have been reported for their antibiotic and herbicidal properties. In addition, studies have revealed that these endophytic fungi may also influence plant pathogen associations by reducing their diversity and abundance (Tadych et al., 2009). Most importantly, for *B. bassiana* and *L. lecanii*, in addition to their well-known biological control activities against insect pests, both fungi have been revealed to possess antimicrobial and plant pathogen antagonism potential (Ownley et al., 2010; Jaber, 2018; Jaber and Ownley, 2018). For instance, the ability of *B. bassiana* to antagonize plant disease-causing pathogens in tomato, squash, cotton, grapevine, and many other economic crops has been reported (Ownley et al., 2004, 2008, 2010; Vega et al., 2009; Jaber and Ownley, 2018; Vega, 2018). The available reports have provided evidence of *Beauveria* sp. inhibiting various plant pathogens, including *Fusarium oxysporum*, *Botrytis cinerea*, *Septoria* sp., *Gaeumannomyces graminis*, *Pythium* sp., and *Rhizoctonia solani* (Renwick et al., 1991; Flori and Roberti, 1993; Veselý and Koubova, 1994; Bark et al., 1996; Lee et al., 1999). The level of infection by Zucchini yellow mosaic virus in squash was reduced following treatment of seedlings with *B. bassiana* (Jaber and Salem, 2014). Ownley et al. (2004) also reported a reduction in the severity of damping-off disease caused by *R. solani* in tomato. Aside from reports on *B. bassiana* and *L. lecanii*, other insect-pathogenic fungal strains have also been found to exhibit antagonistic properties against various arrays of phytopathogenic organisms. For example, *M. anisopliae* was found to minimize the spread of Dutch elm disease (DED), a vascular wilt disease caused by the ascomycete fungus, *Ophiostoma ulmi* Buisman (Gemma et al., 1984). Additionally, *M. brunneum* reduced the activities of *Fusarium culmorum* Smith – the causal agent of CRR in wheat (Jaber, 2018) and sweet pepper (Jaber and Alananbeh, 2018). In addition to the aforementioned reports, there are many other pieces of evidence of EPF interactions with phytopathogenic fungi (Kim et al., 2008; Sasan and Bidochka, 2012; Jaber, 2015; Jaber and Araj, 2018; Barra-Bucarei et al., 2020; Canassa et al., 2020).

MECHANISMS OF FUNGAL ENDOPHYTE-INDUCED PLANT DEFENSE AGAINST PATHOGENS

The mechanisms involved in fungal endophyte-induced plant defense against plant pathogens may not have been fully elucidated (Ganley et al., 2008); however, numerous modes of action through which this plant-endophyte mutualism helps hosts build resistance against pathogens have been proposed (Ownley et al., 2008, 2010; Gao et al., 2010; Jaber, 2018; Jaber and Ownley, 2018). The utilization of metabolites, which is the most popular and widely discussed indirect plant disease management strategy employed by the endophytic EPF in their hosts, is highlighted many times in this paper. The secretion of these unique biochemical compounds by endophytes helps to inhibit the evasion of harmful foreign microbes (Kusari et al., 2012). Some of the important metabolites, such as alkaloids, flavonoids, peptides, phenols, polyketides, quinones, steroids, and terpenoids, have been discovered from fungal endophytes and characterized in terms of their antimicrobial activities (Mousa and Raizada, 2013; Lugtenberg et al., 2016). The ability of these bioactive compounds to inhibit phytopathogens has been exclusively explored, as a good number of previous studies have identified novel metabolites from fungal endophytes that are suitable for commercial purposes (Suryanarayanan, 2013). Ongoing and future studies should focus on similar directions to explore the potential of these endophytes in phytopathogen and disease management. Fungal endophytes are unique for their ability to colonize the internal tissues of plants, an advantage they hold over many other biocontrol agents. In addition, the promotion of plant growth and initiation of systemic plant resistance are some of the other identified possible indirect mechanisms engaged by endophytic fungi. Several previous studies have established the possibility of fungal endophytes mediating systemic plant resistance and growth promotion in their hosts and hence reducing the damage caused by phytopathogenic microorganisms (Ganley et al., 2008). A systemic resistance strategy was observed in *B. bassiana*-treated pumpkin and cotton plants against zucchini yellow mosaic virus (Jaber and Salem, 2014) and *Xanthomonas axonopodis* pv. *malvacearum* (Ownley et al., 2008), respectively.

Generally, in response to attacks from parasites, pathogens, and other biotic and abiotic stressors, two different kinds of induced resistance patterns can be mediated by plants, namely, systemic acquired resistance (SAR) and ISR (Choudhary et al., 2007). SAR is activated upon exposure of the host to virulent or avirulent pathogens and other non-pathogenic microbes. This pathway is enhanced by the accumulation of the plant hormone, salicylic acid, and other pathogenesis-related proteins in the plant. Salicylic acid activates the SAR genes and prepares the plants for impending attack by a variety of pathogens in a quick and effective manner. The other pathway, ISR, is activated by the jasmonic acid and ethylene pathways following the activities of non-pathogenic microbes. Jasmonic acid production and pathogenesis-related protein activation are closely related to wounding in plants. Largely, the production of oxidative enzymes, such as polyphenol oxidases, peroxidases, and lipoxygenases, is involved in the latter pathway, while the production of antifungal pathogenesis-related proteins, including chitinases, 1, 3-glucanases, and thaumatins, is involved in the salicylate-induced pathway. Enzymes are directly involved in lysing foreign cells, cell wall strengthening, and cell death (Gao et al., 2010; Dara, 2019b).

Entomopathogenic fungal endophytes activate the production of plant defense proteins in their colonized host. This implies that the induced systemic responses produced by fungal endophytes are related to the enhancement of genes that are expressed in pathogenesis (Fadiji and Babalola, 2020a). Their ability to increase the production of pathogenesis-related proteins and other defense enzymes has been demonstrated by Karthiba et al. (2010) in rice and Senthilraja et al. (2013) in peanut, following dual treatment of both plants with *B. bassiana* and *Pseudomonas fluorescens*. The significant growth improvement recorded in the colonized plants was noticeable in plants resistance to the pathogen as well as an increase in the overall accumulation of peroxidase and polyphenol oxidase in the rice plants. Similar significant increases in the levels of catalase, chitinases, lipoxygenase, glucanase, phenolics, peroxidase, polyphenol oxidase, superoxide dismutase, and phenylalanine ammonia-lyase accumulated were recorded in the treated peanut seedlings. Similar findings were reported for date palm leaves following inoculation with *B. bassiana* and *Lecanicillium dimorphum* (Cordycipitaceae, Hypocreales; Gómez-Vidal et al., 2009). Furthermore, a strain of *B. bassiana* modified the gene expression levels across the phytoalexin, pathogenesis, salicylic acid, and jasmonic acid signaling pathways in *Arabidopsis*. As a result, the rate of *Sclerotinia sclerotiorum* (Libert) de Bary infection was significantly reduced in colonized *Arabidopsis* plants (Raad et al., 2019). The regulation of photosynthesis and energy metabolism-related proteins has also been reported. On the other hand, disease suppression *via* mycoparasitism, competition, and antibiosis have all been identified as some of the direct strategies employed by fungal endophytes against phytopathogens (Ownley et al., 2010; Jaber, 2018; Jaber and Ownley, 2018). For instance, while conducting both *in vitro* and *in vivo* observations of *Lecanicillium* spp. activities against *Pythium ultimum*, a ubiquitous soil-borne pathogen that causes damping-off and root rot infections in various plants, evidence of mycoparasitism between the two microorganisms was reported by Benhamou and Brodeur (2001). In another related study, Ownley et al. (2008) also found and reported a similar interaction between *Pythium myriotylum* and *B. bassiana*.

The ability of fungal endophytes to compete with disease-causing pathogens is another unique direct mechanism used to inhibit the colonization of their hosts by foreign microbes (Martinuz et al., 2013). Endophytic fungi are known to colonize their host, thereby hoarding the available nutrients and space and in turn limiting the activities of pathogens (Rodriguez et al., 2009). For instance, the ability of *B. bassiana* and *M. brunneum* to exhibit competition and antibiosis against *F. culmorum* upon subculturing both fungi in a dual plate assay with the fungal plant pathogen was revealed (Jaber and Alananbeh, 2018). Clear zones of inhibition across the interphase with the phytopathogenic fungus were observed, which provided evidence of competition for the available resources. The suppression or total removal of the endophytic fungi colonizing a plant through the application of fungicides, for instance, would allow the invasion of the plant tissues by other foreign microbes, as demonstrated in the study conducted by Mohandoss and Suryanarayanan (2009).

The mechanisms of competition utilized by fungal endophytes involve systematic colonization of parts of the host where foreign microbes could potentially colonize and, as a result, prevent further attack by the pathogen. In addition, fungal endophytes can also initiate a direct attack on pathogens or their propagules, a mechanism commonly known as mycoparasitism (Ownley et al., 2008). Endophytic fungi are known to produce lyase, which effectively aids the evasion of the pathogen and destruction of the pathogen cell walls. This potential mechanism was demonstrated by Grosch et al. (2006) using three different strains of *Trichoderma* sp., which were able to penetrate the hyphae of *R. solani*. Many species of endophytic fungi are known to exhibit predatory behaviors against plant pathogens. The activity is common under nutrient-deficient conditions, and the mechanism is generally termed microbial predation. For instance, Gao et al. (2010) indicated in their report the potential of *Trichoderma* sp. to produce an array of enzymes known to attack the cell walls of fungal pathogens. Similarly, *B. bassiana* has the capability of improving plant growth and causing a reduction in disease severity, even in the presence of plant pathogens. The mechanisms for causing a reduction in the activities of the pathogens were related to competition for space and parasitism and ISR in the *B. bassiana*-colonized plants (Ownley et al., 2008). Similar observations have been reported for *M. anisopliae* and *B. bassiana* in strawberry plants against *B. cinerea* and *Rhizopus* spp. (Dara, 2019a). Both fungi also offered protection for strawberry plants against *Macrophomina phaseolina* (Tassi) Goid, the causal organism of seedling blight (Dara et al., 2018), and treated corn plants against *Fusarium graminearum* Schwabe (Rivas-Franco et al., 2019).

PLANT-ENDOPHYTIC FUNGI SYMBIOSIS AND HOST GROWTH PROMOTION

The findings of the various greenhouse and field trials on fungal endophytes have revealed multiple additional roles of EPF, besides the well-publicized roles as insect killers and plants pathogen antagonists (Vega et al., 2009; Vega, 2018). In addition to the ability of fungal endophytes to induce systemic resistance against herbivores and pathogens, other benefits, such as enhancing drought resistance, inducing tolerance to heavy metals, improving plant fitness under environmental extremes, and promoting general plant growth (biofertilizers), have been mentioned (Tadych et al., 2009; Vega et al., 2009).

Many previous studies and some new publications have provided evidence of the ability of endophytic EPF to promote plant growth, either when existing naturally or when artificially introduced into host plants using various kinds of artificial inoculation techniques (Kabaluk and Ericsson, 2007; Elena et al., 2011; Sasan and Bidochka, 2012; Liao et al., 2014; Lopez and Sword, 2015; Jaber and Enkerli, 2016, 2017; Dash et al., 2018; Bamisile et al., 2020). The capacity of fungal endophytes to colonize plant tissues, establishing a strong symbiotic association with their hosts, has now been well established by various researchers (Kumar et al., 2017). This beneficial association between the two organisms results in plant growth enhancement and improvement of the host's ability to withstand abiotic and biotic stressors (Saravanakumar and Samiyappan, 2007).

Fungal endophytes are now commonly applied for crop and yield improvement, as they are generally considered eco-friendly, affordable, and renewable sources of nutrients to plants (Kumar et al., 2017). In addition, endophytic fungi also serve as a close alternative to chemical fertilizers when acting as biofertilizers, thereby reducing the heavy dependence on these synthetic compounds (Pal et al., 2015). Many species of endophytic EPF, including *M. anisopliae*, *M. brunneum*, *M. robertsii*, *B. bassiana*, *Purpureocillium lilacinum*, and several others, have been acknowledged for their plant growth promotion potential (Jaber and Enkerli, 2017; Bamisile et al., 2018a,b; Jaber, 2018). The ability to improve plant nutrient uptake, root hair density, and dry weight has been reported for *M. anisopliae*. This is evident in the improved growth and enhancement of root hair density recorded in switch grass and common beans (Sasan and Bidochka, 2012; Behie et al., 2015) and the increase in plant dry weight (biomass) recorded in mung bean, *Vigna radiata* (Rekadwad et al., 2016) following artificial inoculation of the plants with a conidial suspension of the fungus. In addition, the fungus also improved the plant height, root length, root and shoot dry weight of treated tomato seedlings (Elena et al., 2011; Qayyum et al., 2015), foliar biomass, leaf collar formation, and total yield in corn plants (Kabaluk and Ericsson, 2007; Liao et al., 2014) and significantly promoted root development in peanuts (Liu et al., 2017b). Similarly, another fungal species belonging to the genus *Metarhizium*, *M. robertsii*, has also been shown to improve growth in switch grass, corn, wheat, sorghum, tomato, and common beans (Reddy et al., 2009; Elena et al., 2011; Sasan and Bidochka, 2012; Liao et al., 2014). *Metarhizium brunneum* has also been reported as a plant biomass and yield promoter and was also found to improve the nitrogen and phosphate contents, as well as the efficiency of water utilization in colonized plants (Dara, 2019b). This important property of endophytic fungi has also been demonstrated for *B. bassiana*, *B. brongniartii*, *L. lecanii*, *I. fumosorosea*, and several other endophytic insect-pathogenic fungal species (Lopez and Sword, 2015; Jaber and Enkerli, 2017; Dash et al., 2018; Bamisile et al., 2019).

In addition to growth and plant yield promotion, the mutual interaction between the plants and their fungal colonizers also initiates protection for the hosts against unfavorable environmental conditions, such as drought, frost, and heavy metals (Gao et al., 2010). The host defense mechanism against phytopathogenic microorganisms is also enhanced *via* this same interaction. The overall increase in plant growth could also mediate vigor enhancement and resistance to various kinds of biotic and abiotic stressors (Kuldau and Bacon, 2008).

MECHANISMS BY WHICH ENDOPHYTIC FUNGI ACT AS PLANT GROWTH PROMOTERS/BIOFERTILIZERS

Fungal endophytes promote plant growth and host resistance to environmental stressors using various modes of action (Yadav, 2018). The mode of action utilized by these endophytic fungi could be in the form of direct or indirect mechanisms. The ability of fungal endophytes to improve plant growth due to acquisition of nutrients or production of growth-promoting phytohormones is considered a direct mechanism (Hiruma et al., 2018). Fungal endophytes directly improve the rate of growth and development of their hosts by secreting plant growth-promoting hormones, which in turn contributes to improvement of host nutrition using bidirectional transfer of nutrients. The health status of host plants is also improved by protection against phytopathogens (Shen et al., 2019). The rate of phytohormone synthesis mediated by these fungal endophytes varies from plant to plant, with a significant level of effect on the growth, development, morphology, and structure of the hosts (Bamisile et al., 2018a). Concerning plant growth promotion, endophytic fungi are believed to utilize similar mechanisms as rhizobacteria. Several bioactive compounds have been identified to be closely linked to growth promotion in endophytically colonized plants, including auxins (Dutta et al., 2014), gibberellic acid (Khan et al., 2014), cytokinins, and ethylene (Kang et al., 2012). Indole acetic acid (IAA) and the rest of these biocompounds regulate plant physiology, including plant cell division, differentiation and extension, root and xylem development, seed and tuber germination, overall vegetative growth, metabolite biosynthesis, and formation of pigments and photosynthesis (Gao et al., 2010). The insect-pathogenic fungus, *M. robertsii*, has been demonstrated to promote *Arabidopsis* seedling growth (Liao et al., 2017), where the fungus boosted lateral root growth and root hair development using what the authors described as an auxin (IAA)-dependent mechanism. In addition, the fungus activated IAA-regulated gene expression in IAA-deficient mutants and consequently reduced the root hair defects in the mutants. Other strains belonging to *Metarhizium* sp. and *Beauveria* sp. were also found to synthetize auxins (Liao et al., 2017).

Another growth-promoting mechanism is the ability of endophytic fungi to increase the rate of nutrient transport genes in their colonized hosts. This was demonstrated in the study of Behie et al. (2012), where the insect-pathogenic fungus *M. robertsii* was found to transfer nitrogen from the larvae of *Galleria mellonella* Linnaeus to the plant. Similar findings were also reported by Behie and Bidochka (2014), where this same fungus, *M. robertsii*, together with other strains of *B. bassiana*, *M. brunneum*, and *M. guizhouense*, improved the productivity of soybean, wheat, green bean, and switch grass plants through the transfer of insect-derived nitrogen to the host plants. The overall improvement in plant growth due to plant-fungal symbiosis was evident in the ability of the plant to supply photosynthates to the fungi in exchange for the provided insect-derived nitrogen, as demonstrated by Behie et al. (2017).

Many species of plant root colonizing insect-pathogenic fungi, due to their microbial activities, can change the bioavailability of many soil nutrients, hence making them readily available for plant use. This ability involves the mineralization of elements such as nitrogen, iron, potassium, and phosphorus. The latter element, which is the second most essential nutrient (after nitrogen) for plant growth, could be converted from insoluble phosphate into soluble forms and made readily available for plant uptake, a process known as phosphate solubilization (Adhikari and Pandey, 2019; Tandon et al., 2020). Several species of EPF have now been implicated in the production of different forms of organic acids and siderophores, which are small molecular compounds that are known for their ability to make iron readily available for plants (Yadav, 2018).

To this end, several other available reports on endophytic fungus-plant interactions have indicated that plant growth promotion is generally due to the fixation of nutrients, bioactive metabolite production, and synthesis of plant growth-promoting hormones/phytohormones in colonized plants (Behie et al., 2012, 2017).

ADDITIONAL BENEFICIAL APPLICATIONS OF ENDOPHYTIC FUNGI

Fungal endophytes play significant roles in IPM programs and have been found to be able to influence plant activities in many ways. As we have discussed in the previous sections, research into endophytes is attracting more interest due to their roles in biocontrol, plant growth promotion, and their potential application in the near future as a replacement/alternative to chemical pesticides and inorganic fertilizers (Shen et al., 2019). However, aside from the aforementioned activities, there are still many more roles and attributes that have been linked to these essential endophytic microorganisms. In fact, many authors have put forward suggestions that the role of fungal endophytes in plant fitness is far from completely defined and should be fully investigated (Vidal and Jaber, 2015; Vega, 2018; Fadiji and Babalola, 2020b).

ENVIRONMENTALLY SAFE ALTERNATIVES TO CHEMICAL PESTICIDES

The safety of users, other humans, animals, plants, natural enemies, pollinators, and the general ecosystem are the major public concerns related to the application of EPF for the biological control of pests and phytopathogens. Several studies have been conducted in line with ecotoxicological assessments of various EPF (Siegel and Shadduck, 1990; Goettel and Jaronski, 1997; Vestergaard et al., 2003). Studies were conducted to examine the potential side effects resulting from the application of fungal entomopathogens. Roberts (1977) conducted the first study on the detrimental effects of *M. anisopliae* on fish. Following conidia application to waterbodies, the author found no significant effects on the mortality of the examined fishes. Similar observations were recorded in studies conducted to examine the negative effects of *M. anisopliae* on the northern leopard frog, *Rana pipiens* Schreber, and African clawed frog, *Xenopus laevis* Daudin (Peveling and Demba, 2003). Another strain of *M. anisopliae* var. *acridum* formulated into a mycoinsecticide commercially known as green muscle for desert locust control was also tested against the fringe-toed lizard, *Acanthodactylus dumerili* Milne-Edwards. However, no negative effect of the fungus was recorded on the treated lizards following inhalation of conidia, oral exposure, and feeding with mycosed *S. gregaria* locusts. In contrast, *A. dumerili* was found to be greatly affected by the synthetic insecticide fipronil (Peveling and Demba, 2003).

Similarly, toxicity assessments of different strains of EPF have been carried out on several bird species, where birds were reared on EPF-infected insects or directly fed with fungal spores deposited in their feeds. For instance, the ring-necked pheasants, *Phasianus colchicus* Linnaeus were exposed to *B. bassiana* conidia (Johnson et al., 2002), chickens were fed *B. brongniartii*-infected white grubs, and American sparrowhawks, *Falco sparverius* Linnaeus were equally fed spores of *B. bassiana* (Althouse et al., 1997). In the aforementioned studies, histopathological changes were not reported in any of the treated birds, whereas no significant differences were reported among the control and treated samples with regard to the growth, body mass, and survival of birds (Zimmermann, 2007a).

Toxicity tests on *B. bassiana* conducted on rats and other vertebrates also confirmed the non-toxicity of the fungus (Goettel and Jaronski, 1997). Intramuscular injection of *B. bassiana* into mice indicated that the fungus could only survive for 3 days inside the rodents (Semalulu et al., 1992).

Another study that was conducted by Zimmermann (1992) to investigate the vertical movement of wet and dry spores of *M. anisopliae* also confirmed that the possibility of contamination of the groundwater by the fungus is very low. When insect-pathogenic fungi and all kinds of mycopesticides are applied for biocontrol, the water bodies and the atmosphere are arguably the clear destinations for drifting formulations. However, according to Weng et al. (2019), until now, there has been no available record of the negative effects of EPF from water and the atmosphere on human health. The reason for this is the inability of fungal spores to persist or proliferate in the atmosphere for a long duration (Shah and Pell, 2003). Milner et al. (2002) also concluded that the possibility of *Metarhizium*-based biopesticides posing negative effects on aquatic living organisms is relatively low. Most mycotoxins that are commonly known as environmental or food chain pollutants have now been found to be produced as a result of plant infection by fungal phytopathogens, rather than endophytic colonization of the plant by the fungal entomopathogens (Oyedele et al., 2017; Weng et al., 2019). Common examples are the mycotoxins produced by *Fusarium* spp., *Aspergillus* spp., and other fungal phytopathogens, which have been found to contaminate the environment through the crops and products they infect (Oyedele et al., 2017; Mallebrera et al., 2018).

Several recent studies have also presented reports on the ability of endophytic fungi to initiate the production of phytohormones and other bioactive compounds related to plant growth promotion, thereby improving the overall development and growth of colonized host plants. As a result, we can now anticipate a potential decline in the level of dependence on synthetic fertilizers, which are notorious for ecosystem pollution due to their residual effects and tendencies to enter the food chain. It is also noteworthy that during the development and registration of any EPF for use as a mycopesticide, the fungus is extensively examined for safety against many beneficial non-target organisms. In this light, there is an overall tendency of biological control agents to be safer for use than chemical products. There is therefore a strong need to create awareness and inform policy and regulatory authorities on the safety and advantages of using biopesticides compared to their synthetic counterparts.

COMPATIBILITY WITH OTHER BIOCONTROL AGENTS

The compatibility of several species of EPF with many other biological control agents, especially the associated natural enemies of targeted pests, such as predators (Canassa et al., 2019) and parasitoids (Akutse et al., 2014; Gathage et al., 2016; Jaber and Araj, 2018), has widely been reported. For instance, the potential management of green peach aphids, *Myzus persicae* Sulzer (Hemiptera: Aphididae) in sweet pepper using the parasitoids of green peach aphids, *Aphidius colemani* Viereck (Hymenoptera: Braconidae) in combination with *B. bassiana* and *M. brunneum* was demonstrated by Jaber and Araj (2018). Similarly, according to Akutse et al. (2014), the pea leafminer, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) can be controlled using two parasitoid species, *Diglyphus isaea* Walker (Hymenoptera: Eulophidae) and *Phaedrotoma scabriventris* Nixon (Hymenoptera: Braconidae), in combination with different fungal isolates, including *B. bassiana* and *H. lixii*. The potential utilization of two isolates of *B. bassiana* and *M. robertsii* in combination with the predatory mite, *Phytoseiulus persimilis* Athias-Henrio (Acarina: Phytoseiidae) for the management of the two spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae) on strawberry plants in the greenhouse (Canassa et al., 2019) and in the strawberry field (Canassa et al., 2020) has also been demonstrated. Similarly, studies have revealed the possibility of applying *L. lecanii* in combination with an aphid alarm pheromone and sublethal doses of the insecticide imidacloprid as part of an autodissemination strategy to enhance the efficacy of the fungus for aphid biocontrol (Hartfield et al., 2001). Another typical generalist insect-pathogenic fungal species that has been tested for compatibility with other biocontrol agents is *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales). The fungus was applied in an autodissemination technique for the management of the diamondback moth, *Plutella xylostella* Linnaeus (Lepidoptera: Yponomeutidae) in combination with semiochemicals. The host-specific semiochemical attracted the insects into an inoculation device, where the moths were exposed to the conidia of *Z. radicans* (Pell et al., 1993). The combined use of *B. bassiana*, *M. anisopliae* and different chemical fungicides in harvested strawberry was also to be found suitable for the management of *B. cinerea* and *Rhizopus* sp. (Dara, 2019a).

Most biocontrol approaches benefit from being used together, therefore, to improve the efficacy of specific biological control approaches, it is imperative to apply them in an integrative manner in combination with other cultural or conventional measures, as the synergy would benefit both biocontrol agents and significantly suppress pest populations. In addition, classical and inoculation methods could also be applied in combination with conservative methods in the quest to increase the efficiency of both approaches (Pell et al., 2001).

ENDOPHYTIC FUNGI AS GOOD SOURCES OF PHARMACEUTICAL PRODUCTS

Endophytic fungi are not just biocontrol and plant growth-promoting agents, but they have now been established as good sources of various arrays of medicinal or pharmaceutical products. The endophytic microbes constitute an important source for drug discovery, and their plant sources are being extensively explored for new chemical compounds for therapeutic purposes (Tadych et al., 2009; Fadiji and Babalola, 2020a). Fungal endophytes act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids, that serve as potential candidates with antimicrobial, anti-insect, anticancer and many more properties (Gouda et al., 2016). A variety of products derived from bioactive secondary metabolites belonging to different endophytic fungal species has now been developed for use as antibiotic agents such as cephalosporin and penicillin (Tadych et al., 2009). These biocompounds have also been explored for their antimalarial, antiarthritis, anticancer, antidiabetic, antiviral, antituberculosis, anti-inflammatory, and immunosuppressive potentials (Tadych et al., 2009; Fadiji and Babalola, 2020a). The products are isolated and utilized in their raw forms or otherwise formulated to produce different drugs for the treatment of many health conditions (Hu et al., 2016).

Data collected over the last four decades have listed over 70 novel secondary metabolites derived from *Isaria* sp. alone (Weng et al., 2019). For instance, a non-ribosomal peptide metabolite known as beauvericin, which is a cyclic hexadepsipeptide mycotoxin isolated from a strain of *I. fumosorosea*, has been found to possess insecticidal, antibacterial, antiviral, and cytotoxic properties and is considered valuable for the formulation of new pesticides (Lu et al., 2016). Additionally, fumosorinone, a terpene compound isolated from the same fungal species, could act as a classic non-competitive inhibitor of protein tyrosine phosphatase 1B (PTP1B), indicating that the compound could function in medicine for the clinical treatment of diabetes (type II) and other related metabolic disorders (Liu et al., 2015). This same compound has also been related to cytotoxicity against human cancer lines (Chen et al., 2018). Peroxyergosterol is a biocompound isolated from another strain of *I. fumosorosea* and has been tested for various bioactivities, including its cytotoxicity against cancer cells (Sheu et al., 2000), apoptosis of human leukemia cells (Takei et al., 2005), and potential production of vitamin A (Zhang et al., 2013).

In addition, there are several reports of the isolation and identification of many other biochemical compounds of medicinal importance from other fungal species, including *B. bassiana*, *B. brongniartii*, and *M. anisopliae* (Zimmermann, 2007a,b). To this end, endophytic microbes are now commonly utilized in the mass production of drugs, enzymes, antibodies, supplements, and riboflavin, among many other industrial products (Latz et al., 2018). Isolated microorganisms are of huge importance in the fields of medicine, agriculture, and industry (Sahay et al., 2017).

ENTOMOPATHOGENIC FUNGI AS ALTERNATIVES TO CHEMICAL PESTICIDES: WHAT ARE THE CHALLENGES?

The huge importance of insect-pathogenic endophytic fungi and their derived biocompounds to agriculture, industry, and medicine cannot be overemphasized. However, despite their numerous attributes and functions, several problems affecting their successful application as biological control agents have been identified. One of the major challenges is the difficulties in isolation and identification of fungal endophytes. As many fungal endophyte strains have been found to be unculturable, measuring and identifying the endophyte community structure and diversity has been a difficult task (Fadiji and Babalola, 2020b). Even though, very recently, scientists in advanced countries have found alternative ways to isolate and identify novel fungal strains, especially by employing various cultivation-independent techniques. However, there is every possibility that a larger percentage of fungal isolation and identification efforts still depend heavily on traditional culture methods with selective media. The adverse effects of geographical location, vegetation type, and human disturbance on fungal entomopathogen distribution are another problem. The irregular localization or biodiversity of fungal entomopathogens in soils as a result of geographic and climatic conditions has been reported as a major disadvantage. For instance, in a study conducted across the Qinghai-Tibet Plateau and Gansu Corridor of China in 2016, it was reported that the likelihood of isolating novel strains of fungal entomopathogens is higher in areas characterized as remote and less disturbed by human activities (Dong et al., 2016). The soil types, vegetation or landscapes, habitat fragmentation and alteration, and climatic conditions are some of the determining factors that are also related to endophytic fungal richness and diversity in the soil. The negative influence of environmental conditions, such as temperature, humidity, and solar radiation, on fungal entomopathogen virulence and persistence in the field has also been investigated (Zimmermann, 2007b). Another limiting factor is the rapid decline in the level of efficacy of EPF over a short duration. As a result, fungal-based mycopesticides are generally not highly regarded as alternatives to chemical pesticides among users. The possibility of posing unwanted residual effects on predators, parasitoids, pollinators, and other non-target organisms has also been mentioned. This activity has been examined in some insect-pathogenic fungal species with a broad spectrum and wide host range, such as *B. bassiana* and *B. brongniartii* (Goettel et al., 1990). Some previous studies investigated various possible adverse effects on beneficial insects, earthworms, honeybees, vertebrates, and plants (Vestergaard et al., 2003). Although most of the studies were conducted in the laboratory and only a few were field trials, many of these studies argued that EPF could be used with little or no side effects on non-target organisms (Goettel et al., 1990; Vestergaard et al., 2003). Nevertheless, as with every general principle, there could be some exceptions across species and perhaps even among isolates. There are possibilities that different isolates within the same species can perform very differently even on the same host. For instance, insect host range, fungal infection levels, rate of germination, and temperature optima can vary among fungal species and isolates (Zimmermann, 2007a). To increase the adoption of these mycopesticides, it is also important to develop a "new paradigm" for applying these entomopathogens as opposed to the "old paradigm" of application in ways similar to their synthetic chemical counterparts.

OVERVIEW OF THE RESEARCH ADVANCES IN THE LAST FEW DECADES AND INSIGHTS FOR NEXT-GENERATION SUSTAINABLE AGRICULTURE

Several of the previous studies on fungal endophytes and other related studies have focused on co-culturing methods in an *in vitro* dual plate assay examine the antagonistic effects of endophytic fungi against some targeted pathogens. Many of the highlighted studies only indicated endophytic fungal antagonistic effects on the target plant pathogens without necessarily conducting comprehensive assessments of the physiological changes in the colonized plants. Another strategy that is commonly adopted is to compare the treated and untreated seedlings following artificial inoculation of plants with pathogens with respect to the rate of survival, colonization rate, and disease severity index (Jaber, 2018). The mechanisms by which endophytic fungi mediate changes in host physiology and volatile levels also have yet to be fully explored. The available data are limited and have shown inconsistencies under various environmental conditions (Fontana et al., 2009). However, in addition to the aforementioned descriptive studies, in the last few decades, a good number of emerging studies have been conducted to further explore the ecology of fungal endophyte-plant host specificity and their multitrophic effects (Hartley and Gange, 2009). During that period, the molecular mechanisms related to fungal endophyte-induced host plant defense were an area of increasing focus and research interest (Zheng and Dicke, 2008).

Since the beginning of the biotechnology revolution, scientific research has been focused on genetic engineering of fungal endophytes with the sole aim of improving plant yields and their defense systems (Clay, 1994). With the introduction of gene modification procedures in EPF (Wang and St Leger, 2007) and the progress of RNAi technology, studies are now targeted at constructing recombinant fungal strains with enhanced virulence (Chen et al., 2015). Genetic engineering could therefore provide useful strategies to increase fungal virulence or enhance fungal resistance to different stress factors. Over time, the utilization of recombinant endophytic genes as biocontrol agents has become popular and of huge importance. Recombinant endophytic organisms produce anti-pest proteins for insect pest management, and they can also successfully colonize host plants (Fadiji and Babalola, 2020b). However, it is worth noting that recombinant endophytic fungi with enhanced virulence against insects may represent a risk for pollinators and beneficial insects (natural enemies). In the efforts to understand the chemical pathways that are applicable in biotechnological applications, the transfer of genes from associated endophytic fungi to the genome of their hosts toward the production of secondary metabolites has been one of the principles used for explaining the multiple origins of chemical defenses within the phylogeny of different plant species (Wink, 2008). In recent times, advances in microbial biotechnology have translated into the biotransformation of many chemicals in the quest of reducing environmental pollution. Novel techniques such as bioremediation, waste management, and composting represent forms of technological advancement from the crude method of metabolite synthesis involving only ethanol and butanol. In recent times, various scientists have focused on exploring the world of microorganisms, plants, and animals for their potential utilization in the production of novel medicinal products (Gouda et al., 2016; Latz et al., 2018). It is now evident that products derived from natural sources are less expensive and user- and ecosystem-friendly (Fadiji and Babalola, 2020a).

The integrated use of EPF, such as *B. bassiana*, in combination with other chemical pesticides has been investigated. There are suggestions that the adoption of the combination would help improve resistance management strategies and reduce ecosystem pollution due to excessive use of inorganic insecticides (Al-Ani et al., 2021). In the past few decades, the combined application of biological control agents through an autodissemination strategy has also recorded a level of success. For EPF in particular, this strategy has proven successful for many strains when using EPF in combination with semiochemicals and other insect natural enemies (Vega et al., 2000).

Over the years, examination of plant-endophyte symbiosis has gone beyond culture media assays, as there are pressing needs to analyze many other non-culturable endophytic fungal species using culture-independent methods (Adeleke and Babalola, 2021). In this light, more comprehensive methods, such as microscopic observation of fluorescent mycelia and confocal scanning electron micrographs enabled by green fluorescent protein (GFP) labeling, have recently been employed (Sasan and Bidochka, 2012; Behie et al., 2015). The latter approach would enable observation of intercellular and intracellular endophytic localization of the endophytic fungi in the treated plants (Behie et al., 2015).

To gain more insights into the molecular mechanisms associated with plant responses to endophytic fungal colonization, metagenomic analysis of different plant organs for prospective fungal colonizers can be conducted. The analysis would help to examine the functions, structures, and phylogenetic construction of genetic relatedness in the microbial genomes from long reads of metagenome sequence data (Adeleke and Babalola, 2021). For studies related to genome structure and features, molecular techniques that are now commonly used include polymerase chain reaction (PCR), DNA sequencing, DNA microarrays, and RT-PCR (Chen et al., 2015; Wang et al., 2020), among many others. For studies related to endophyte-induced secondary compounds, phytohormones, and enzymes, several molecular analyses are commonly being used. The most common technologies used include liquid chromatography-mass spectrometry (LC-MS-MS; Wang et al., 2020), gas chromatography-mass spectrometry (GC-MS/MS; Elbanhawy et al., 2019), high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; Cotes et al., 2020), nuclear magnetic resonance spectroscopy (NMR), headspace solid-phase microextraction (HS-SPME), Fourier transform infrared spectroscopy (FTIR), and proteomics (Al-Ani et al., 2021).

Similarly, different types of high-throughput equipment are currently used for fungal DNA sequencing, notably next-generation sequencing methods such as 454 pyrosequencing and Illumina sequencing. This method can enhance the understanding of fungal microbiomes. The latest advances in microbiology research ensure that the discovery, isolation, and identification of novel genetic traits are easier while providing greater insights into the underlying mechanisms of plant-microbe interactions. All microbial communities could be examined from the internal tissues of the plants, with a special focus on the novel genes responsible for host growth improvement, phytohormone synthesis, cellular metabolism, and nitrogen fixation (Hardoim et al., 2015). Scientists, through metagenomics analysis of the internal tissues of plants, can now detect the specific genes related to plant growth promotion and other physiological functions (Igiehon and Babalola, 2017). Additionally, through knowledge gained with metagenomics, the various studies related to fungal endophytes and other microorganisms are made simpler and more accurate. The application of omics technology has advanced studies on plant-microbe interactions to the level of genomics, proteomics, and transcriptomics (Akinola and Babalola, 2020). With the enormous research progress and technological advancements made in the past couple of decades, endophytic fungi and their bioactive compounds are arguably suitable for adoption as replacements of inorganic fertilizers and chemical pesticides if carefully explored by researchers and embraced by policymakers (Fadiji and Babalola, 2020b).

CONCLUSION

The latest advances in microbiological research have helped to establish the importance of microorganisms in the fields of medicine, industry, and agriculture. An in-depth understanding of the roles of these beneficial microorganisms will enhance the exploitation of their ecosystem services and their successful adoption and optimum utilization in agriculture, especially as plant growth- and crop yield-promoting agents. Concerns about the negative effects of synthetic chemical pesticides have also driven attention toward developing eco-friendly pest management techniques. The various species of insect-pathogenic fungi, fungal endophytes, and other beneficial microorganisms that could function as biocontrol agents are now generally considered sustainable pest management options for incorporation into IPM programs or as a substitute/supplement for chemical pesticides. Overall, the potential applications of mycopesticides as alternatives to chemical pesticides are promising; however, there is still much work to be done to fully explore their services. Based on the available pieces of evidence, most EPF-based pesticides are considered to be relatively safe for use and could effectively mitigate the abuse of synthetic pesticides. Nevertheless, with respect to future registrations of new fungal strains, it is imperative to conduct pathogenicity/toxicity-related tests in non-target organisms, as well as for vertebrates, to avoid potential risks. It has, however, been suggested that all risks cannot be excluded; nevertheless, efforts should be put in place to ensure that existing precautionary measures during production and application are taken to avoid harmful reactions. There are no specific criteria that guarantee the acceptance or adoption of fungal biocontrol agents, but efforts are warranted to promote the use of bioproducts from these microorganisms due to their numerous advantages. The various underlying problems that need to be solved will not only be addressed by laboratory or field trials but also at the policy and regulatory levels. In addition to scientific aspects, economic, social, and political limitations must also be addressed to fully explore the potential uses of these microorganisms.

AUTHOR CONTRIBUTIONS

BB and KA designed the review outline. The manuscript was written by BB and JS. KA and YX reviewed the manuscript. All authors have read and agreed to the final version of the manuscript.

FUNDING

We acknowledge funding support received through Laboratory of Lingnan Modern Agriculture Project (NZ2021022) and the UK's Foreign, Commonwealth & Development Office (FCDO; B2329ADFID-FAW and B2291A-FCDO-BIOPESTICIDE) through the International Centre of Insect Physiology and Ecology (*icipe*).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.741804/full#supplementary-material

REFERENCES

Adeleke, B. S., and Babalola, O. O. (2021). Biotechnological overview of agriculturally important endophytic fungi. *Hortic. Environ. Biotechnol.* 62, 507–520. doi: 10.1007/s13580-021-00334-1

Adhikari, P., and Pandey, A. (2019). Phosphate solubilization potential of endophytic fungi isolated from *Taxus wallichiana* Zucc. roots. *Rhizosphere* 9, 2–9. doi: 10.1016/j.rhisph.2018.11.002

Akello, J., Dubois, T., Coyne, D., and Kyamanywa, S. (2008). Endophytic *Beauveria bassiana* in banana (*Musa* spp.) reduces banana weevil (*Cosmopolites sordidus*) fitness and damage. *Crop Prot.* 27, 1437–1441. doi: 10.1016/j.cropro.2008.07.003

Akello, J., and Sikora, R. (2012). Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. *Biol. Control* 61, 215–221. doi: 10.1016/j.biocontrol.2012.02.007

Akinola, S. A., and Babalola, O. O. (2020). The importance of adverse soil microbiomes in the light of omics: implications for food safety. *Plant Soil Environ.* 66, 421–430. doi: 10.17221/118/2020-PSE

Akutse, K. S., Fiaboe, K. K., Van den Berg, J., Ekesi, S., and Maniania, N. K. (2014). Effects of endophyte colonization of *Vicia faba* (Fabaceae) plants on the life–history of leafminer parasitoids *Phaedrotoma scabriventris* (Hymenoptera: Braconidae) and *Diglyphus isaea* (Hymenoptera: Eulophidae). *PLoS One* 9:e109965. doi: 10.1371/journal.pone.0109965

Akutse, K. S., Maniania, N., Fiaboe, K., Van den Berg, J., and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecol.* 6, 293–301. doi: 10.1016/j.funeco.2013.01.003

Akutse, K., Subramanian, S., Maniania, N., Dubois, T., and Ekesi, S. (2020). Biopesticide research and product development in Africa for sustainable agriculture and food security – experiences from the International Centre of Insect Physiology and Ecology (icipe). *Front. Sustainable Food Syst.* 4:152. doi: 10.3389/fsufs.2020.563016

Al-Ani, L. K. T., Aguilar-Marcelino, L., Salazar-Vidal, V. E., Becerra, A. G., and Raza, W. (2021). "Role of useful fungi in agriculture sustainability," in *Recent Trends in Mycological Research*. ed. A. N. Yadav (Cham: Springer), 1–44.

Ali, K., Wakil, W., Zia, K., and Sahi, S. T. (2015). Control of *Earias vittella* (Lepidoptera: Noctuidae) by *Beauveria bassiana* along with *Bacillus thuringiensis*. *Int. J. Agric. Biol.* 17, 773–778. doi: 10.17957/IJAB/14.0009

Althouse, C., Petersen, B., and McEwen, L. (1997). Effects on young American kestrels (*Falco sparverius*) exposed to *Beauveria bassiana* bioinsecticide. *Bull. Environ. Contam. Toxicol.* 59, 507–512. doi: 10.1007/s001289900507

Arnold, A. E., and Lewis, L. C. (2005). "Ecology and evolution of fungal endophytes, and their roles against insects," in *Insect-Fungal Associations: Ecology and Evolution*. eds. F. E. Vega and M. Blackwell (New York: Oxford University Press), 74–96.

Backman, P. A., and Sikora, R. A. (2008). Endophytes: an emerging tool for biological control. *Biol. Control* 46, 1–3. doi: 10.1016/j.biocontrol.2008.03.009

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., Afolabi, O. G., Hussain, M., et al. (2018a). Prospects of endophytic fungal entomopathogens as biocontrol and plant growth promoting agents: An insight on how artificial inoculation methods affect endophytic colonization of host plants. *Microbiol. Res.* 217, 34–50. doi: 10.1016/j.micres.2018.08.016

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., and Wang, L. (2018b). Fungal endophytes: beyond herbivore management. *Front. Microbiol.* 9:544. doi: 10.3389/fmicb.2018.00544

Bamisile, B. S., Dash, C. K., Akutse, K. S., Qasim, M., Ramos Aguila, L. C., Wang, F., et al. (2019). Endophytic *Beauveria bassiana* in foliar-treated citrus Limon plants acting as a growth suppressor to three successive generations of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). *Insects* 10:176. doi: 10.3390/insects10060176

Bamisile, B. S., Senyo Akutse, K., Dash, C. K., Qasim, M., Ramos Aguila, L. C., Ashraf, H. J., et al. (2020). Effects of seedling age on colonization patterns of citrus Limon plants by endophytic *Beauveria bassiana* and *Metarhizium anisopliae* and their influence on seedlings growth. *J. Fungi* 6:29. doi: 10.3390/jof6010029

Bark, Y. G., Lee, D. G., Kang, S., and Kim, Y. (1996). Antibiotic properties of an entomopathogenic fungus, *Beauveria bassiana*, on *Fusarium oxysporum* and *Botrytis cinerea*. *Korean J. Plant Pathol.* 12, 245–250.

Barra-Bucarei, L., France Iglesias, A., Gerding González, M., Silva Aguayo, G., Carrasco-Fernández, J., Castro, J. F., et al. (2020). Antifungal activity of *Beauveria bassiana* endophyte against *Botrytis cinerea* in two solanaceae crops. *Microorganisms* 8:65. doi: 10.3390/microorganisms8010065

Bateman, R., Neethling, D., and Oosthuizen, F. (1998). *Green Muscle-Handbook for Central and Southern Africa*. Berkshire: LUBILOSA-Lutte Biologique Contre les Locustes et les Sauteriaux Collaborative Research Programme Asco, 12

Behie, S. W., and Bidochka, M. J. (2014). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Appl. Environ. Microbiol.* 80, 1553–1560. doi: 10.1128/AEM.03338-13

Behie, S. W., Jones, S. J., and Bidochka, M. J. (2015). Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. *Fungal Ecol.* 13, 112–119. doi: 10.1016/j.funeco.2014.08.001

Behie, S. W., Moreira, C. C., Sementchoukova, I., Barelli, L., Zelisko, P. M., and Bidochka, M. J. (2017). Carbon translocation from a plant to an insect-pathogenic endophytic fungus. *Nat. Commun.* 8:14245. doi: 10.1038/ncomms14245

Behie, S., Zelisko, P., and Bidochka, M. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science* 336, 1576–1577. doi: 10.1126/science.1222289

Benhamou, N., and Brodeur, J. (2001). Pre-inoculation of Ri T-DNA transformed cucumber roots with the mycoparasite, *Verticillium lecanii*, induces host defense reactions against *Pythium ultimum* infection. *Physiol. Mol. Plant Pathol.* 58, 133–146. doi: 10.1006/pmpp.2001.0322

Biswas, C., Dey, P., Satpathy, S., and Satya, P. (2012). Establishment of the fungal entomopathogen *Beauveria bassiana* as a season long endophyte in jute (*Corchorus olitorius*) and its rapid detection using SCAR marker. *BioControl* 57, 565–571. doi: 10.1007/s10526-011-9424-0

Bradley, C. A., Black, W. E., Kearns, R., and Wood, P. (1992). "Role of production technology in mycoinsecticide development," in *Frontiers in Industrial Mycology*. ed. G. F. Leatham (Boston, MA: Springer), 160–173.

Brownbridge, M., Reay, S. D., Nelson, T. L., and Glare, T. R. (2012). Persistence of *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte following inoculation of radiata pine seed and seedlings. *Biol. Control* 61, 194–200. doi: 10.1016/j.biocontrol.2012.01.002

Canassa, F., Esteca, F. C., Moral, R. A., Meyling, N. V., Klingen, I., and Delalibera, I. (2020). Root inoculation of strawberry with the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* reduces incidence of the twospotted spider mite and selected insect pests and plant diseases in the field. *J. Pest. Sci.* 93, 261–274. doi: 10.1007/s10340-019-01147-z

Canassa, F., Tall, S., Moral, R. A., de Lara, I. A., Delalibera, I. Jr., and Meyling, N. V. (2019). Effects of bean seed treatment by the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* on plant growth, spider mite populations and behavior of predatory mites. *Biol. Control* 132, 199–208. doi: 10.1016/j.biocontrol.2019.02.003

Carroll, G. (1988). Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* 69, 2–9. doi: 10.2307/1943154

Chen, X., Li, L., Hu, Q., Zhang, B., Wu, W., Jin, F., et al. (2015). Expression of dsRNA in recombinant *Isaria fumosorosea* strain targets the TLR7 gene in *Bemisia tabaci*. *BMC Biotechnol.* 15:64. doi: 10.1186/s12896-015-0170-8

Chen, C., Xue, T., Fan, P., Meng, L., Wei, J., and Luo, D. (2018). Cytotoxic activity of Shp2 inhibitor fumosorinone in human cancer cells. *Oncol. Lett.* 15, 10055–10062. doi: 10.3892/ol.2018.8593

Choudhary, D. K., Prakash, A., and Johri, B. (2007). Induced systemic resistance (ISR) in plants: mechanism of action. *Indian J. Microbiol.* 47, 289–297. doi: 10.1007/s12088-007-0054-2

Clay, K. (1990). Fungal endophytes of grasses. *Annu. Rev. Ecol. Syst.* 21, 275–297. doi: 10.1146/annurev.es.21.110190.001423

Clay, K. (1994). "The potential role of endophytes in ecosystems," in *Biotechnology of Endophytic Fungi of Grasses*. eds. C. W. Bacon and J. F. White Jr, (Boca Raton, Florida: CRC Press), 73–86.

Clay, K. (1997). *Fungal Endophytes, Herbivores and the Structure of Grassland Communities*. London: Blackwell Science

Cotes, B., Thoming, G., Amaya-Gomez, C. V., Novak, O., and Nansen, C. (2020). Root-associated entomopathogenic fungi manipulate host plants to attract herbivorous insects. *Sci. Rep.* 10:22424. doi: 10.1038/s41598-020-80123-5

Culliney, T. W. (2014). "Crop losses to arthropods," in *Integrated Pest Management*. eds. D. Pimentel and R. Peshin (Dordrecht: Springer), 201–225.

Dara, S. K. (2019a). Five shades of gray mold control in strawberry: evaluating chemical, organic oil, botanical, bacterial, and fungal active ingredients. *EJ Entomol. Biol.* https://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=30729 (Accessed July 13, 2021).

Dara, S. K. (2019b). Non-entomopathogenic roles of entomopathogenic fungi in promoting plant health and growth. *Insects* 10:277. doi: 10.3390/insects10090277

Dara, S. S., Dara, S. S., and Dara, S. K. (2018). Preliminary report on the potential of *Beauveria bassiana* and *Metarhizium anisopliae* sl in antagonizing the charcoal rot causing fungus *Macrophomina phaseolina* in strawberry. *EJ. Entomol. Biol. Plant Health* 5:7

Dash, C. K., Bamisile, B. S., Keppanan, R., Qasim, M., Lin, Y., Islam, S. U., et al. (2018). Endophytic entomopathogenic fungi enhance the growth of *Phaseolus vulgaris* L. (Fabaceae) and negatively affect the development and reproduction of *Tetranychus urticae* Koch (Acari: Tetranychidae). *Microb. Pathog.* 125, 385–392. doi: 10.1016/j.micpath.2018.09.044

de Faria, M. R., and Wraight, S. P. (2007). Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biol. Control* 43, 237–256. doi: 10.1016/j.biocontrol.2007.08.001

Dong, T., Zhang, B., Jiang, Y., and Hu, Q. (2016). Isolation and classification of fungal whitefly entomopathogens from soils of Qinghai-Tibet Plateau and Gansu Corridor in China. *PLoS One* 11:e0156087. doi: 10.1371/journal.pone.0168785

Dutta, D. K., Puzari, K. C., Gogoi, R., and Dutta, P. (2014). Endophytes: exploitation as a tool in plant protection. *Braz. Arch. Biol. Technol.* 57, 621–629. doi: 10.1590/S1516-8913201402043

Dymarska, M., Grzeszczuk, J., Urbaniak, M., Janeczko, T., Plaskowska, E., Stepien, L., et al. (2017). Glycosylation of 6-methylflavone by the strain *Isaria fumosorosea* KCH J2. *PLoS One* 12:e0184885. doi: 10.1371/journal.pone.0184885

Dymarska, M., Janeczko, T., and Kostrzewa-Suslow, E. (2018). Glycosylation of 3-hydroxyflavone, 3-methoxyflavone, quercetin and baicalein in fungal cultures of the genus *Isaria*. *Molecules* 23:2477. doi: 10.3390/molecules23102477

Elbanhawy, A. A., Elsherbiny, E. A., Abd El-Mageed, A. E., and Abdel-Fattah, G. M. (2019). Potential of fungal metabolites as a biocontrol agent against cotton aphid, *Aphis gossypii* Glover and the possible mechanisms of action. *Pestic. Biochem. Physiol.* 159, 34–40. doi: 10.1016/j.pestbp.2019.05.013

Elena, G. J., Beatriz, P. J., Alejandro, P., and Lecuona, R. (2011). *Metarhizium anisopliae* (Metschnikoff) Sorokin promotes growth and has endophytic activity in tomato plants. *Adv. Biol. Res.* 5, 22–27.

Fadiji, A. E., and Babalola, O. O. (2020a). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Front. Bioeng. Biotechnol.* 8:467. doi: 10.3389/fbioe.2020.00467

Fadiji, A. E., and Babalola, O. O. (2020b). Exploring the potentialities of beneficial endophytes for improved plant growth. *Saudi J. Biol. Sci.* 27, 3622–3633. doi: 10.1016/j.sjbs.2020.08.002

Fan, J.-Q., Chen, X.-R., and Hu, Q.-B. (2013). Effects of destruxin A on hemocytes morphology of *Bombyx mori*. *J. Integr. Agric.* 12, 1042–1048. doi: 10.1016/S2095-3119(13)60482-7

Fang, W., Lu, H.-L., King, G. F., and Leger, R. J. S. (2014). Construction of a hypervirulent and specific mycoinsecticide for locust control. *Sci. Rep.* 4:7345. doi: 10.1038/srep07345

Flori, P., and Roberti, R. (1993). Treatment of onion bulbs with antagonistic fungi for the control of *Fusarium oxysporum* f. sp. cepae. *Difesa delle Piante* 16, 5–12.

Fontana, A., Reichelt, M., Hempel, S., Gershenzon, J., and Unsicker, S. B. (2009). The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites of *Plantago lanceolata* L. *J. Chem. Ecol.* 35, 833–843. doi: 10.1007/s10886-009-9654-0

Ganley, R. J., Sniezko, R. A., and Newcombe, G. (2008). Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *For. Ecol. Manag.* 255, 2751–2760. doi: 10.1016/j.foreco.2008.01.052

Gao, F.-K., Dai, C.-C., and Liu, X.-Z. (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *Afr. J. Microbiol. Res.* 4, 1346–1351. doi: 10.5897/AJMR.9000480

Gao, T., Wang, Z., Huang, Y., Keyhani, N. O., and Huang, Z. (2017). Lack of resistance development in *Bemisia tabaci* to *Isaria fumosorosea* after multiple generations of selection. *Sci. Rep.* 7:42727. doi: 10.1038/srep42727

Gathage, J. W., Lagat, Z. O., Fiaboe, K. K. M., Akutse, K. S., Ekesi, S., and Maniania, N. K. (2016). Prospects of fungal endophytes in the control of *Liriomyza leafminer* flies in common bean *Phaseolus vulgaris* under field conditions. *BioControl* 61, 741–753. doi: 10.1007/s10526-016-9761-0

Gehring, C., and Bennett, A. (2009). Mycorrhizal fungal–plant–insect interactions: the importance of a community approach. *Environ. Entomol.* 38, 93–102. doi: 10.1603/022.038.0111

Gemma, J. N., Hartmann, G. C., and Wasti, S. S. (1984). Inhibitory interactions between *Ceratocystis ulmi* and several species of entomogenous fungi. *Mycologia* 76, 256–260. doi: 10.2307/3793101

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Goettel, M. S., and Jaronski, S. T. (1997). Safety and registration of microbial agents for control of grasshoppers and locusts. *Mem. Entomol. Soc. Can.* 129, 83–99. doi: 10.4039/entm129171083-1

Goettel, M. S., Poprawski, T., Vandenberg, J., Li, Z., and Roberts, D. W. (1990). "Safety to nontarget invertebrates of fungal biocontrol agents," in *Safety of Microbial Insecticides*. eds. M. Laird, L. A. Lacey and E. W. Davidson (Boca Raton, FL: CRC Press), 209–231.

Gómez-Vidal, S., Salinas, J., Tena, M., and Lopez-Llorca, L. V. (2009). Proteomic analysis of date palm (*Phoenix dactylifera* L.) responses to endophytic colonization by entomopathogenic fungi. *Electrophoresis* 30, 2996–3005. doi: 10.1002/elps.200900192

Gouda, S., Das, G., Sen, S. K., Shin, H.-S., and Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. *Front. Microbiol.* 7:1538. doi: 10.3389/fmicb.2016.01538

Greenfield, M., Gómez-Jiménez, M. I., Ortiz, V., Vega, F. E., Kramer, M., and Parsa, S. (2016). *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. *Biol. Control* 95, 40–48. doi: 10.1016/j.biocontrol.2016.01.002

Grosch, R., Scherwinski, K., Lottmann, J., and Berg, G. (2006). Fungal antagonists of the plant pathogen *Rhizoctonia solani*: selection, control efficacy and influence on the indigenous microbial community. *Mycol. Res.* 110, 1464–1474. doi: 10.1016/j.mycres.2006.09.014

Gulzar, S., Wakil, W., and Shapiro-Ilan, D. I. (2021). Combined effect of Entomopathogens against *Thrips tabaci* Lindeman (Thysanoptera: Thripidae): laboratory, greenhouse and field trials. *Insects* 12:456. doi: 10.3390/insects12050456

Gunatilaka, A. L. (2006). Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J. Nat. Prod.* 69, 509–526. doi: 10.1021/np058128n

Haas-Costa, J., Alves, L. F. A., and Daros, A. A. (2010). Safety of *Beauveria bassiana* (Bals.) Vuill. to *Gallus domesticus* L. *Braz. Arch. Biol. Technol.* 53, 465–471. doi: 10.1590/S1516-89132010000200027

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., et al. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320. doi: 10.1128/MMBR.00050-14

Hartfield, C., Campbell, C., Hardie, J., Pickett, J., and Wadhams, L. (2001). Pheromone traps for the dissemination of an entomopathogen by the damson-hop aphid *Phorodon humuli*. *Biocontrol Sci. Tech.* 11, 401–410. doi: 10.1080/09583150120055817

Hartley, S. E., and Gange, A. C. (2009). Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. *Annu. Rev. Entomol.* 54, 323–342. doi: 10.1146/annurev.ento.54.110807.090614

Hiruma, K., Kobae, Y., and Toju, H. (2018). Beneficial associations between Brassicaceae plants and fungal endophytes under nutrient-limiting conditions: evolutionary origins and host-symbiont molecular mechanisms. *Curr. Opin. Plant Biol.* 44, 145–154. doi: 10.1016/j.pbi.2018.04.009

Hu, Q., Li, F., and Zhang, Y. (2016). Risks of mycotoxins from mycoinsecticides to humans. *Biomed. Res. Int.* 2016:3194321. doi: 10.1155/2016/3194321

Hu, G., and St Leger, J. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl. Environ. Microbiol.* 68, 6383–6387. doi: 10.1128/AEM.68.12.6383-6387.2002

Hunter, D. M. (2005). Mycopesticides as part of integrated pest management of locusts and grasshoppers. *J. Orthoptera Res.* 14, 197–201. doi: 10.1665/1082-6467(2005)14[197:MAPOIP]2.0.CO;2

Hussey, N., and Tinsley, T. (1981). "Impressions of insect pathology in the People's Republic of China," in *Microbial Control of Pests and Plant Diseases, 1970–1980*. ed. H. D. Burges (London: Academic Press), 785–795.

Igiehon, N. O., and Babalola, O. O. (2017). Biofertilizers and sustainable agriculture: exploring arbuscular mycorrhizal fungi. *Appl. Microbiol. Biotechnol.* 101, 4871–4881. doi: 10.1007/s00253-017-8344-z

Inglis, G. D., Goettel, M. S., Butt, T. M., and Strasser, H. (2001). "Use of hyphomycetous fungi for managing insect pests" in *Fungi as Biocontrol Agents*. eds. T. M. Butt, C. Jackson and N. Magan (Wallingford, UK: CABI), 23–69.

Jaber, L. R. (2015). Grapevine leaf tissue colonization by the fungal entomopathogen *Beauveria bassiana* sl and its effect against downy mildew. *BioControl* 60, 103–112. doi: 10.1007/s10526-014-9618-3

Jaber, L. R. (2018). Seed inoculation with endophytic fungal entomopathogens promotes plant growth and reduces crown and root rot (CRR) caused by *Fusarium culmorum* in wheat. *Planta* 248, 1525–1535. doi: 10.1007/s00425-018-2991-x

Jaber, L. R., and Alananbeh, K. M. (2018). Fungal entomopathogens as endophytes reduce several species of *Fusarium* causing crown and root rot in sweet pepper (*Capsicum annuum* L.). *Biol. Control* 126, 117–126. doi: 10.1016/j.biocontrol.2018.08.007

Jaber, L. R., and Araj, S.-E. (2018). Interactions among endophytic fungal entomopathogens (Ascomycota: Hypocreales), the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae). *Biol. Control* 116, 53–61. doi: 10.1016/j.biocontrol.2017.04.005

Jaber, L. R., and Enkerli, J. (2016). Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. *Biol. Control* 103, 187–195. doi: 10.1016/j.biocontrol.2016.09.008

Jaber, L. R., and Enkerli, J. (2017). Fungal entomopathogens as endophytes: can they promote plant growth? *Biocontrol Sci. Tech.* 27, 28–41. doi: 10.1080/09583157.2016.1243227

Jaber, L. R., and Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biol. Control* 116, 36–45. doi: 10.1016/j.biocontrol.2017.01.018

Jaber, L. R., and Salem, N. M. (2014). Endophytic colonisation of squash by the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) for managing *Zucchini yellow mosaic virus* in cucurbits. *Biocontrol Sci. Tech.* 24, 1096–1109. doi: 10.1080/09583157.2014.923379

Johnson, D. L., Smits, J. E., Jaronski, S. T., and Weaver, D. K. (2002). Assessment of health and growth of ring-necked pheasants following consumption of infected insects or conidia of entomopathogenic fungi, *Metarhizium anisopliae* var. acridum and *Beauveria bassiana*, from Madagascar and North America. *J. Toxicol. Environ. Health A* 65, 2145–2162. doi: 10.1080/00984100290071847

Jones, K. (1994). Aspects of the biology and biological control of the European corn borer in North Carolina. doctoral dissertation. Raleigh: North Carolina State University.

Kabaluk, J. T., and Ericsson, J. D. (2007). *Metarhizium anisopliae* seed treatment increases yield of field corn when applied for wireworm control. *Agron. J.* 99, 1377–1381. doi: 10.2134/agronj2007.0017N

Kang, J. W., Khan, Z., and Doty, S. L. (2012). Biodegradation of trichloroethylene by an endophyte of hybrid poplar. *Appl. Environ. Microbiol.* 78, 3504–3507. doi: 10.1128/AEM.06852-11

Karthiba, L., Saveetha, K., Suresh, S., Raguchander, T., Saravanakumar, D., and Samiyappan, R. (2010). PGPR and entomopathogenic fungus bioformulation for the synchronous management of leaffolder pest and sheath blight disease of rice. *Pest Manag. Sci.* 66, 555–564. doi: 10.1002/ps.1907

Khachatourians, G. G., and Qazi, S. S. (2008). "Entomopathogenic fungi: biochemistry and molecular biology," in *Human and Animal Relationships*. eds. A. A. Brakhage and P. F. Zipfel (Berlin, Heidelberg: Springer), 33–61.

Khan, A. L., Waqas, M., Kang, S.-M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., et al. (2014). Bacterial endophyte *Sphingomonas* sp LK11 produces gibberellins and IAA and promotes tomato plant growth. *J. Microbiol.* 52, 689–695. doi: 10.1007/s12275-014-4002-7

Kim, J. J., Goettel, M. S., and Gillespie, D. R. (2008). Evaluation of *Lecanicillium longisporum*, Vertalec® for simultaneous suppression of cotton aphid, *Aphis gossypii*, and cucumber powdery mildew, *Sphaerotheca fuliginea*, on potted cucumbers. *Biol. Control* 45, 404–409. doi: 10.1016/j.biocontrol.2008.02.003

Klieber, J., and Reineke, A. (2016). The entomopathogen *Beauveria bassiana* has epiphytic and endophytic activity against the tomato leaf miner *Tuta absoluta*. *J. Appl. Entomol.* 140, 580–589. doi: 10.1111/jen.12287

Knoch, T., Faeth, S., and Arnott, D. (1993). Fungal endophytes: plant mutualists via seed predation and germination. *Bull. Ecol. Soc. Am.* 74:313

Koricheva, J., Gange, A. C., and Jones, T. (2009). Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. *Ecology* 90, 2088–2097. doi: 10.1890/08-1555.1

Krasnoff, S. B., Keresztes, I., Gillilan, R. E., Szebenyi, D. M., Donzelli, B. G., Churchill, A. C., et al. (2007). Serinocyclins A and B, cyclic heptapeptides from *Metarhizium anisopliae*. *J. Nat. Prod.* 70, 1919–1924. doi: 10.1021/np070407i

Kuldau, G., and Bacon, C. (2008). Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. *Biol. Control* 46, 57–71. doi: 10.1016/j.biocontrol.2008.01.023

Kumar, M., Saxena, R., and Tomar, R. S. (2017). "Endophytic microorganisms: promising candidate as biofertilizer," in *Microorganisms for Green Revolution*. eds. D. Panpatte, Y. Jhala, R. Vyas, and H. Shelat (Singapore: Springer), 77–85.

Kusari, S., Hertweck, C., and Spiteller, M. (2012). Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chem. Biol.* 19, 792–798. doi: 10.1016/j.chembiol.2012.06.004

Lacey, L. A., and Neven, L. G. (2006). The potential of the fungus, *Muscodor albus*, as a microbial control agent of potato tuber moth (Lepidoptera: Gelechiidae) in stored potatoes. *J. Invertebr. Pathol.* 91, 195–198. doi: 10.1016/j.jip.2006.01.002

Latch, G., Christensen, M., and Gaynor, D. (1985). Aphid detection of endophyte infection in tall fescue. *N. Z. J. Agric. Res.* 28, 129–132.

Latz, M. A., Jensen, B., Collinge, D. B., and Jørgensen, H. J. (2018). Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. *Plant Ecolog. Divers.* 11, 555–567. doi: 10.1080/17550874.2018.1534146

Lee, S., Yeo, W., Jee, H., Shin, S., and Moon, Y. (1999). Effect of entomopathogenic fungi on growth of cucumber and *Rhizoctonia solani*. *J. For. Sci.* 62, 118–125.

Lekberg, Y., and Koide, R. (2005). Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. *New Phytol.* 168, 189–204. doi: 10.1111/j.1469-8137.2005.01490.x

Li, S.-J., Xue, X., Ahmed, M. Z., Ren, S.-X., Du, Y.-Z., Wu, J.-H., et al. (2011). Host plants and natural enemies of *Bemisia tabaci* (Hemiptera: Aleyrodidae) in China. *Insect Sci.* 18, 101–120. doi: 10.1111/j.1744-7917.2010.01395.x

Liao, X., Lovett, B., Fang, W., and St Leger, R. J. (2017). *Metarhizium robertsii* produces indole-3-acetic acid, which promotes root growth in *Arabidopsis* and enhances virulence to insects. *Microbiology* 163, 980–991. doi: 10.1099/mic.0.000494

Liao, X., O'Brien, T. R., Fang, W., and Leger, R. J. S. (2014). The plant beneficial effects of *Metarhizium* species correlate with their association with roots. *Appl. Microbiol. Biotechnol.* 98, 7089–7096. doi: 10.1007/s00253-014-5788-2

Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M. J., et al. (2017a). Inner plant values: diversity, colonization and benefits from endophytic bacteria. *Front. Microbiol.* 8:2552. doi: 10.3389/fmicb.2017.02552

Liu, S.-F., Wang, G.-J., Nong, X.-Q., Liu, B., Wang, M.-M., Li, S.-L., et al. (2017b). Entomopathogen *Metarhizium anisopliae* promotes the early development of peanut root. *Plant Prot. Sci.* 53, 101–107. doi: 10.17221/49/2016-PPS

Liu, L., Zhang, J., Chen, C., Teng, J., Wang, C., and Luo, D. (2015). Structure and biosynthesis of fumosorinone, a new protein tyrosine phosphatase 1B inhibitor firstly isolated from the entomogenous fungus *Isaria fumosorosea*. *Fungal Genet. Biol.* 81, 191–200. doi: 10.1016/j.fgb.2015.03.009

Lomer, C., Bateman, R., Johnson, D., Langewald, J., and Thomas, M. (2001). Biological control of locusts and grasshoppers. *Annu. Rev. Entomol.* 46, 667–702. doi: 10.1146/annurev.ento.46.1.667

Lopez, D. C., and Sword, G. A. (2015). The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea*). *Biol. Control* 89, 53–60. doi: 10.1016/j.biocontrol.2015.03.010

Lopez, D. C., Zhu-Salzman, K., Ek-Ramos, M. J., and Sword, G. A. (2014). The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. *PLoS One* 9:e103891. doi: 10.1371/journal.pone.0104342

Lu, C.-L., Lin, H.-I., Chen, B.-F., and Jow, G.-M. (2016). Beauvericin-induced cell apoptosis through the mitogen-activated protein kinase pathway in human nonsmall cell lung cancer A549 cells. *J. Toxicol. Sci.* 41, 429–437. doi: 10.2131/jts.41.429

Lugtenberg, B. J., Caradus, J. R., and Johnson, L. J. (2016). Fungal endophytes for sustainable crop production. *FEMS Microbiol. Ecol.* 92:fiw194. doi: 10.1093/femsec/fiw194

Mallebrera, B., Prosperini, A., Font, G., and Ruiz, M. J. (2018). In vitro mechanisms of Beauvericin toxicity: A review. *Food Chem. Toxicol.* 111, 537–545. doi: 10.1016/j.fct.2017.11.019

Mantzoukas, S., Chondrogiannis, C., and Grammatikopoulos, G. (2015). Effects of three endophytic entomopathogens on sweet sorghum and on the larvae of the stalk borer *Sesamia nonagrioides*. *Entomol. Exp. Appl.* 154, 78–87. doi: 10.1111/eea.12262

Martinuz, A., Schouten, A., and Sikora, R. A. (2013). Post-infection development of *Meloidogyne incognita* on tomato treated with the endophytes *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* strain G12. *BioControl* 58, 95–104. doi: 10.1007/s10526-012-9471-1

Milner, R. J., Lim, R. P., and Hunter, D. M. (2002). Risks to the aquatic ecosystem from the application of *Metarhizium anisopliae* for locust control in Australia. *Pest Manag. Sci.* 58, 718–723. doi: 10.1002/ps.517

Mohandoss, J., and Suryanarayanan, T. (2009). Effect of fungicide treatment on foliar fungal endophyte diversity in mango. *Sydowia* 61, 11–24.

Mousa, W. K., and Raizada, M. N. (2013). The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. *Front. Microbiol.* 4:65. doi: 10.3389/fmicb.2013.00065

Murray, F. R., Latch, G. C., and Scott, D. B. (1992). Surrogate transformation of perennial ryegrass, *Lolium perenne*, using genetically modified *Acremonium* endophyte. *Mol. Gen. Genet. MGG* 233, 1–9. doi: 10.1007/BF00587554

Muvea, A., Meyhöfer, R., Maniania, N., Poehling, H.-M., Ekesi, S., and Subramanian, S. (2015). Behavioral responses of *Thrips tabaci* Lindeman to endophyte-inoculated onion plants. *J. Pest. Sci.* 88, 555–562. doi: 10.1007/s10340-015-0645-3

Muvea, A. M., Meyhöfer, R., Subramanian, S., Poehling, H.-M., Ekesi, S., and Maniania, N. K. (2014). Colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*. *PLoS One* 9:e108242. doi: 10.1371/journal.pone.0108242

Nong, X., Zhang, Y., Wang, Y. (2015). Advances in registration and formulation techniques of *Metarhizium* biological insecticides. *Acta Phytophylacica Sinica* 42, 702–714.

Ownley, B. H., Griffin, M. R., Klingeman, W. E., Gwinn, K. D., Moulton, J. K., and Pereira, R. M. (2008). *Beauveria bassiana*: endophytic colonization and plant disease control. *J. Invertebr. Pathol.* 98, 267–270. doi: 10.1016/j.jip.2008.01.010

Ownley, B. H., Gwinn, K. D., and Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl* 55, 113–128. doi: 10.1007/s10526-009-9241-x

Ownley, B. H., Pereira, R. M., Klingeman, W. E., Quigley, N. B., and Leckie, B. M. (2004). "*Beauveria bassiana*, a dual purpose biocontrol organism, with activity against insect pests and plant pathogens," in *Emerging Concepts in Plant Health Management*. eds. R. T. Lartey and A. J. Caesar (Kerala, India: Research Signpost), 255–269.

Oyedele, O. A., Ezekiel, C. N., Sulyok, M., Adetunji, M. C., Warth, B., Atanda, O. O., et al. (2017). Mycotoxin risk assessment for consumers of groundnut in domestic markets in Nigeria. *Int. J. Food Microbiol.* 251, 24–32. doi: 10.1016/j.ijfoodmicro.2017.03.020

Pal, S., Singh, H., Farooqui, A., and Rakshit, A. (2015). Fungal biofertilizers in Indian agriculture: perception, demand and promotion. *J. Eco-friendly Agric.* 10, 101–113.

Pell, J., Eilenberg, J., Hajek, A., and Steinkraus, D. (2001). "Biology, ecology and pest management potential of Entomophthorales," in *Fungi as Biocontrol Agents: Progress, Problems and Potential*. eds. T. M. Butt, C. Jackson, and N. Magan (Wallingford, UK: CABI), 71–154.

Pell, J., Macaulay, E., and Wilding, N. (1993). A pheromone trap for dispersal of the pathogen *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) amongst populations of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Biocontrol Sci. Tech.* 3, 315–320. doi: 10.1080/09583159309355286

Peveling, R., and Demba, S. A. (2003). Toxicity and pathogenicity of *Metarhizium anisopliae* var. acridum (Deuteromycotina, Hyphomycetes) and fipronil to the fringe-toed lizard *Acanthodactylus dumerili* (Squamata: Lacertidae). *Environ. Toxicol. Chem.* 22, 1437–1447. doi: 10.1002/etc.5620220704

Pocasangre, L., Sikora, R., Vilich, V., and Schuster, R. (2000). Survey of banana endophytic fungi from Central America and screening for biological control of the burrowing nematode (*Radopholus similis*). *InfoMusa* 9, 3–5.

Posada, F., Aime, M. C., Peterson, S. W., Rehner, S. A., and Vega, F. E. (2007). Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycol. Res.* 111, 748–757. doi: 10.1016/j.mycres.2007.03.006

Posada, F., and Vega, F. E. (2006). Inoculation and colonization of coffee seedlings (*Coffea arabica* L.) with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycoscience* 47, 284–289. doi: 10.1007/S10267-006-0308-6

Powell, W. A., Klingeman, W. E., Ownley, B. H., and Gwinn, K. D. (2009). Evidence of endophytic *Beauveria bassiana* in seed-treated tomato plants acting as a systemic entomopathogen to larval *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Entomol. Sci.* 44, 391–396. doi: 10.18474/0749-8004-44.4.391

Qayyum, M. A., Wakil, W., Arif, M. J., Sahi, S. T., and Dunlap, C. A. (2015). Infection of *Helicoverpa armigera* by endophytic *Beauveria bassiana* colonizing tomato plants. *Biol. Control* 90, 200–207. doi: 10.1016/j.biocontrol.2015.04.005

Raad, M., Glare, T. R., Brochero, H. L., Müller, C., and Rostás, M. (2019). Transcriptional reprogramming of *Arabidopsis thaliana* defence pathways by the entomopathogen *Beauveria bassiana* correlates with resistance against a fungal pathogen but not against insects. *Front. Microbiol.* 10:615. doi: 10.3389/fmicb.2019.00615

Ramakuwela, T., Hatting, J., Bock, C., Vega, F. E., Wells, L., Mbata, G. N., et al. (2020). Establishment of *Beauveria bassiana* as a fungal endophyte in pecan (*Carya illinoinensis*) seedlings and its virulence against pecan insect pests. *Biol. Control* 140:104102. doi: 10.1016/j.biocontrol.2019.104102

Ramos, Y., Taibo, A. D., Jiménez, J. A., and Portal, O. (2020). Endophytic establishment of *Beauveria bassiana* and *Metarhizium anisopliae* in maize plants and its effect against *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) larvae. *Egypt. J. Biol. Pest Control* 30, 1–6. doi: 10.1186/s41938-020-00223-2

Ravindran, K., Akutse, K. S., Sivaramakrishnan, S., and Wang, L. (2016). Determination and characterization of destruxin production in *Metarhizium anisopliae* Tk6 and formulations for *Aedes aegypti* mosquitoes control at the field level. *Toxicon* 120, 89–96. doi: 10.1016/j.toxicon.2016.07.016

Reddy, N. P., Khan, A. P. A., Devi, U. K., Sharma, H. C., and Reineke, A. (2009). Treatment of millet crop plant (*Sorghum bicolor*) with the entomopathogenic fungus (*Beauveria bassiana*) to combat infestation by the stem borer, *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae). *J. Asia Pac. Entomol.* 12, 221–226. doi: 10.1016/j.aspen.2009.06.001

Rekadwad, B. N., Khobragade, C. N., Jadhav, V. G., and Kadam, S. U. (2016). Enhancing growth of *Vigna radiata* in the presence of *Pseudomonas aeruginosa* biopolymer and *Metarhizium anisopliae* spores. *Adv. Agric.* 2016:4314958. doi: 10.1155/2016/4314958

Renwick, A., Campbell, R., and Coe, S. (1991). Assessment of in vivo screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. *Plant Pathol.* 40, 524–532. doi: 10.1111/j.1365-3059.1991.tb02415.x

Rivas-Franco, F., Hampton, J. G., Morán-Diez, M. E., Narciso, J., Rostás, M., Wessman, P., et al. (2019). Effect of coating maize seed with entomopathogenic fungi on plant growth and resistance against *Fusarium graminearum* and *Costelytra giveni*. *Biocontrol Sci. Tech.* 29, 877–900. doi: 10.1080/09583157.2019.1611736

Roberts, D. W. (1977). "Isolation and development of fungus pathogens of vectors," in *Biological Regulation of Vectors*. ed. J. E. Briggs (Wallingford, UK: CABI), 85–93.

Rodriguez, R., White, J. Jr., Arnold, A., and Redman, R. (2009). Fungal endophytes: diversity and functional roles. *New Phytol.* 182, 314–330. doi: 10.1111/j.1469-8137.2009.02773.x

Rondot, Y., and Reineke, A. (2018). Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects. *Biol. Control* 116, 82–89. doi: 10.1016/j.biocontrol.2016.10.006

Sahay, H., Yadav, A. N., Singh, A. K., Singh, S., Kaushik, R., and Saxena, A. K. (2017). Hot springs of Indian Himalayas: potential sources of microbial diversity and thermostable hydrolytic enzymes. *3 Biotech* 7:118. doi: 10.1007/s13205-017-0762-1

Saikkonen, K., Wäli, P., Helander, M., and Faeth, S. H. (2004). Evolution of endophyte–plant symbioses. *Trends Plant Sci.* 9, 275–280. doi: 10.1016/j.tplants.2004.04.005

Saravanakumar, D., and Samiyappan, R. (2007). ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. *J. Appl. Microbiol.* 102, 1283–1292. doi: 10.1111/j.1365-2672.2006.03179.x

Sasan, R. K., and Bidochka, M. J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *Am. J. Bot.* 99, 101–107. doi: 10.3732/ajb.1100136

Semalulu, S., MacPherson, J., Schiefer, H., and Khachatourians, G. (1992). Pathogenicity of *Beauveria bassiana* in mice. *J. Veterinary Med. Ser. B* 39, 81–90. doi: 10.1111/j.1439-0450.1992.tb01141.x

Senthilraja, G., Anand, T., Kennedy, J., Raguchander, T., and Samiyappan, R. (2013). Plant growth promoting rhizobacteria (PGPR) and entomopathogenic fungus bioformulation enhance the expression of defense enzymes and pathogenesis-related proteins in groundnut plants against leafminer insect and collar rot pathogen. *Physiol. Mol. Plant Pathol.* 82, 10–19. doi: 10.1016/j.pmpp.2012.12.002

Shade, A., Jacques, M.-A., and Barret, M. (2017). Ecological patterns of seed microbiome diversity, transmission, and assembly. *Curr. Opin. Microbiol.* 37, 15–22. doi: 10.1016/j.mib.2017.03.010

Shah, P., and Pell, J. (2003). Entomopathogenic fungi as biological control agents. *Appl. Microbiol. Biotechnol.* 61, 413–423. doi: 10.1007/s00253-003-1240-8

Shen, F.-T., Yen, J.-H., Liao, C.-S., Chen, W.-C., and Chao, Y.-T. (2019). Screening of rice endophytic biofertilizers with fungicide tolerance and plant growth-promoting characteristics. *Sustainability* 11:1133. doi: 10.3390/su11041133

Sheu, J. H., Chang, K. C., and Duh, C. Y. (2000). A cytotoxic 5 alpha, 8 alpha-epidioxysterol from a soft coral *Sinularia* species. *J. Nat. Prod.* 63, 149–151. doi: 10.1021/np9903954

Shrivastava, G., Rogers, M., Wszelaki, A., Panthee, D. R., and Chen, F. (2010). Plant volatiles-based insect pest management in organic farming. *Crit. Rev. Plant Sci.* 29, 123–133. doi: 10.1080/07352681003617483

Siegel, J., and Shadduck, J. (1990). *Safety of Microbial Insecticides to Vertebrates: Humans*. Boca Raton, FL: CRC Press

Sinno, M., Ranesi, M., Gioia, L., d'Errico, G., and Woo, S. L. (2020). Endophytic fungi of tomato and their potential applications for crop improvement. *Agriculture* 10:587. doi: 10.3390/agriculture10120587

Skinner, M., Parker, B. L., and Kim, J. S. (2014). "Role of entomopathogenic fungi in integrated pest management," in *Integrated Pest Management*. ed. D. P. Abrol, 169–191.

Smith, P., House, J. I., Bustamante, M., Sobocka, J., Harper, R., Pan, G., et al. (2016). Global change pressures on soils from land use and management. *Glob. Chang. Biol.* 22, 1008–1028. doi: 10.1111/gcb.13068

Smith, S. E., and Read, D. J. (2010). *Mycorrhizal Symbiosis. 3rd Edn.* London: Academic Press.

Suryanarayanan, T. S. (2013). Endophyte research: going beyond isolation and metabolite documentation. *Fungal Ecol.* 6, 561–568. doi: 10.1016/j.funeco.2013.09.007

Tadych, M., White, J. F., and Moselio, S. (2009). "Endophytic microbes," in *Encyclopedia of Microbiology*. ed. M. Schaechter (Elsevier), 431–442.

Tahir, M., Wakil, W., Ali, A., and Sahi, S. T. (2019). Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates against larvae of the polyphagous pest *Helicoverpa armigera*. *Entomol. Gen.* 38, 225–242. doi: 10.1127/0171-8177/2019/0460

Takei, T., Yoshida, M., Ohnishi-Kameyama, M., and Kobori, M. (2005). Ergosterol peroxide, an apoptosis-inducing component isolated from *Sarcodon aspratus* (Berk.) S. Ito. *Biosci. Biotechnol. Biochem.* 69, 212–215. doi: 10.1271/bbb.69.212

Tandon, A., Fatima, T., Shukla, D., Tripathi, P., Srivastava, S., and Singh, P. C. (2020). Phosphate solubilization by Trichoderma koningiopsis (NBRI-PR5) under abiotic stress conditions. *J. King Saud Univ. Sci.* 32, 791–798. doi: 10.1016/j.jksus.2019.02.001

Tefera, T., and Vidal, S. (2009). Effect of inoculation method and plant growth medium on endophytic colonization of sorghum by the entomopathogenic fungus *Beauveria bassiana*. *BioControl* 54, 663–669. doi: 10.1007/s10526-009-9216-y

Usman, M., Gulzar, S., Wakil, W., Wu, S., Piñero, J. C., Leskey, T. C., et al. (2020). Virulence of entomopathogenic fungi to *Rhagoletis pomonella* (Diptera: Tephritidae) and interactions with entomopathogenic nematodes. *J. Econ. Entomol.* 113, 2627–2633. doi: 10.1093/jee/toaa209

Valenzuela-Soto, J. H., Estrada-Hernández, M. G., Ibarra-Laclette, E., and Délano-Frier, J. P. (2010). Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting *Bacillus subtilis* retards whitefly *Bemisia tabaci* development. *Planta* 231, 397–410. doi: 10.1007/s00425-009-1061-9

Vega, F. E. (2018). The use of fungal entomopathogens as endophytes in biological control: a review. *Mycologia* 110, 4–30. doi: 10.1080/00275514.2017.1418578

Vega, F. E., Dowd, P. F., Lacey, L. A., Pell, J. K., Jackson, D. M., and Klein, M. G. (2000). "Dissemination of beneficial microbial agents by insects," in *Field Manual of Techniques in Invertebrate Pathology*. L. A. Lacey and H. K. Kaya (Dordrecht: Springer), 153–177.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., et al. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecol.* 2, 149–159. doi: 10.1016/j.funeco.2009.05.001

Vega, F. E., Posada, F., Aime, M. C., Pava-Ripoll, M., Infante, F., and Rehner, S. A. (2008). Entomopathogenic fungal endophytes. *Biol. Control* 46, 72–82. doi: 10.1016/j.biocontrol.2008.01.008

Veselý, D., and Koubova, D. (1994). In vitro effect of entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Beauveria brongniartii* (Sacc.) Petch on phytopathogenic fungi. *Ochrana Rostlin* 30, 113–120.

Vestergaard, S., Cherry, A., Keller, S., and Goettel, M. (2003). "Safety of hyphomycete fungi as microbial control agents," in *Environmental Impacts of Microbial Insecticides*. eds. H. M. T. Hokkanen and A. E. Hajek (Dordrecht: Springer), 35–62.

Vidal, S., and Jaber, L. R. (2015). Entomopathogenic fungi as endophytes: plant–endophyte–herbivore interactions and prospects for use in biological control. *Curr. Sci.* 109, 46–54. doi: jstor.org/stable/24905690

Wakil, W., Tahir, M., Al-Sadi, A. M., and Shapiro-Ilan, D. (2020). Interactions between two invertebrate pathogens: an endophytic fungus and an externally applied bacterium. *Front. Microbiol.* 11:522368. doi: 10.3389/fmicb.2020.522368

Wakil, W., Yasin, M., and Shapiro-Ilan, D. (2017). Effects of single and combined applications of entomopathogenic fungi and nematodes against *Rhynchophorus ferrugineus* (Olivier). *Sci. Rep.* 7:5971. doi: 10.1038/s41598-017-05615-3

Wang, F., Nong, X., Hao, K., Cai, N., Wang, G., Liu, S., et al. (2020). Identification of the key genes involved in the regulation of symbiotic pathways induced by *Metarhizium anisopliae* in peanut (*Arachis hypogaea*) roots. *3 Biotech* 10:124. doi: 10.1007/s13205-020-2105-x

Wang, B., and Qiu, Y.-L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* 16, 299–363. doi: 10.1007/s00572-005-0033-6

Wang, C., and St Leger, R. J. (2007). A scorpion neurotoxin increases the potency of a fungal insecticide. *Nat. Biotechnol.* 25, 1455–1456. doi: 10.1038/nbt1357

Weng, Q., Zhang, X., Chen, W., and Hu, Q. (2019). Secondary metabolites and the risks of *Isaria fumosorosea* and *Isaria farinosa*. *Molecules* 24:664. doi: 10.3390/molecules24040664

West, C., and Gwinn, K. (1993). "Role of Acremonium in drought, pest, and disease tolerances of grasses." in *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*. eds. D. E. Hume, G. C. M. Latch and H. S. Easton (Palmerston North, New Zealand: Plenary papers), 131–140.

White, J. F., Belanger, F., Meyer, W., Sullivan, R. F., Bischoff, J. F., and Lewis, E. A. (2002). Clavicipitalean fungal epibionts and endophytes-Development of symbiotic interactions with plants. *Symbiosis* 33, 201–213.

Wink, M. (2008). Plant secondary metabolism: diversity, function and its evolution. *Nat. Prod. Commun.* 3:1934578X0800300801. doi: 10.1177/1934578X0800300801

Wraight, S., Carruthers, R., Jaronski, S., Bradley, C., Garza, C., and Galaini-Wraight, S. (2000). Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silverleaf whitefly, *Bemisia argentifolii*. *Biol. Control* 17, 203–217. doi: 10.1006/bcon.1999.0799

Wraight, S., Jackson, M., and De Kock, S. (2001). "Production, stabilization and formulation of fungal biocontrol agents," in *Fungi as Biocontrol Agents: Progress, Problems and Potential*. eds. T. M. Butt, C. Jackson and N. Magan (Wallingford, UK: CABI), 253–287.

Yadav, A. N. (2018). Biodiversity and biotechnological applications of host-specific endophytic fungi for sustainable agriculture and allied sectors. *Acta Sci. Microbiol.* 1, 1–5.

Yasin, M., Wakil, W., Ghazanfar, M. U., Qayyum, M. A., Tahir, M., and Bedford, G. O. (2019). Virulence of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against red palm weevil, *Rhynchophorus ferrugineus* (Olivier). *Entomol. Res.* 49, 3–12. doi: 10.1111/1748-5967.12260

Yeo, H., Pell, J. K., Alderson, P. G., Clark, S. J., and Pye, B. J. (2003). Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. *Pest Manag. Sci.* 59, 156–165. doi: 10.1002/ps.622

Zhang, Y., Han, Y., Liu, X., and Liang, Z. (2013). Screening of vitamin A-praducing fungal resources in soil. *Guizhou Agric. Sci.* 10, 99–101.

Zheng, S.-J., and Dicke, M. (2008). Ecological genomics of plant-insect interactions: from gene to community. *Plant Physiol.* 146, 812–817. doi: 10.1104/pp.107.111542

Zimmermann, G. (1992). Laborversuche zum Versickerungsverhalten des insektenpathogenen Pilzes *Metarhizium anisopliae* (Metsch.) Sorok. in Standardböden und Sand. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 44, 19–23.

Zimmermann, G. (2007a). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Sci. Tech.* 17, 553–596. doi: 10.1080/09583150701309006

Zimmermann, G. (2007b). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Tech.* 17, 879–920. doi: 10.1080/09583150701593963

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 October 2021
doi: 10.3389/fpls.2021.753332

Volatile Organic Compounds of the Plant Growth-Promoting Rhizobacteria JZ-GX1 Enhanced the Tolerance of *Robinia pseudoacacia* to Salt Stress

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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: 04 August 2021
Accepted: 22 September 2021
Published: 14 October 2021

Citation:
Li P-S, Kong W-L, Wu X-Q and Zhang Y (2021) Volatile Organic Compounds of the Plant Growth-Promoting Rhizobacteria JZ-GX1 Enhanced the Tolerance of Robinia pseudoacacia to Salt Stress. Front. Plant Sci. 12:753332. doi: 10.3389/fpls.2021.753332

Salt stress is one of the major abiotic stresses that affects plant growth and development. The use of plant growth-promoting rhizobacteria to mitigcate salt stress damage in plants is an important way to promote crop growth under salt stress conditions. *Rahnella aquatilis* JZ-GX1 is a plant growth-promoting rhizobacterial strain, but it is not clear whether it can improve the salt tolerance of plants, and in particular, the role of volatile substances in plant salt tolerance is unknown. We investigated the effects of volatile organic compounds (VOCs) from JZ-GX1 on the growth performance, osmotic substances, ionic balance and antioxidant enzyme activities of acacia seedlings treated with 0 and 100 mm NaCl and explored the VOCs associated with the JZ-GX1 strain. The results showed that compared to untreated seedlings, seedlings exposed to plant growth-promoting rhizobacterium JZ-GX1 via direct contact with plant roots under salt stress conditions exhibited increases in fresh weight, lateral root number and primary root length equal to approximately 155.1, 95.4, and 71.3%, respectively. *Robinia pseudoacacia* seedlings exposed to VOCs of the JZ-GX1 strain showed increases in biomass, soil and plant analyser development values and lateral root numbers equal to 132.1, 101.6, and 166.7%, respectively. Additionally, decreases in malondialdehyde, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) contents and increases in proline contents and superoxide dismutase, peroxidase and glutathione reductase activities were observed in acacia leaves. Importantly, the sodium-potassium ratios in the roots, stems, and leaves of acacia exposed to VOCs of the JZ-GX1 strain were significantly lower than those in the control samples, and this change in ion homeostasis was consistent with the upregulated expression of the (Na^+, K^+)/H^+ reverse cotransporter RpNHX1 in plant roots. Through GC-MS and creatine chromatography, we also found that 2,3-butanediol in the volatile gases of the JZ-GX1 strain was one of the important signaling substances for improving the salt tolerance of plants. The results showed that *R. aquatilis* JZ-GX1 can promote the growth and yield of *R. pseudoacacia* under normal and salt stress conditions. JZ-GX1 VOCs have good potential as protectants for improving the salt tolerance of plants, opening a window of opportunity for their application in salinized soils.

Keywords: *Rahnella aquatilis*, volatile organic compounds, salt stress tolerance, *Robinia pseudoacacia*, 2,3-butanediol

INTRODUCTION

In natural environments, plants are usually exposed to various abiotic stresses, such as drought, salinity, and extreme temperatures. Salt stress is one of the major abiotic factors affecting plant growth (Thi et al., 2016). A number of methods have been developed to reduce the severe effects of salt stress on plants. At present, the improvement of salinized soil is mainly carried out through measures such as adjusting water resources, applying chemical fertilizers and growing salt-tolerant plants, but these methods have certain limitations, may require large energy inputs, are costly, are not sustainable for the environment and ecosystem (Bresler et al., 1982), and cannot fundamentally improve soil quality conditions or increase plant biomass (Li et al., 2019). In the last decade, the use of plant growth-promoting rhizobacteria (PGPR) has been shown to have significant advantages over other methods due to their beneficial effects on plant growth and stress tolerance. PGPR are economical, environmentally friendly, easy to detect and inexpensive (Batchelor et al., 1997). Therefore, PGPR have attracted widespread attention (Sheikh et al., 2016; Cheng et al., 2017; Baek et al., 2020).

It has been reported that PGPR can promote plant growth or increase plant stress resistance through the production of nonvolatile substances. For example, growth hormones, cytokinin, 1-aminocyclopropane-1-carboxylic acid deaminase, and iron are synthesized to promote the uptake of nutrients by roots (Loper and Schroth, 1981; Glick et al., 1999; Timmusk et al., 1999). In recent years, there has been increased interest in studying volatile organic compounds (VOCs) released by PGPR, and these VOCs play a significant role in promoting the growth and development of plants and resisting adverse environmental conditions (Kanchiswamy et al., 2015; Sharifah et al., 2019).

The effect of VOCs on plant growth was first identified by Ryu et al. (2003). *Bacillus subtilis* enhances vegetable growth, photosynthesis, iron uptake and disease resistance through the release of volatile chemicals (Zhang et al., 2007, 2008a, 2009). Under salt stress conditions, *Arabidopsis thaliana* treated with *B. subtilis* GB03 VOCs exhibited higher biomass and less sodium ion accumulation than control plants (Zhang et al., 2010), and this treatment promoted Na^+ transfer from the ground to the roots by regulating the activity of the *A. thaliana* Na^+ transporter protein HKT1 (Zhang et al., 2008b). Volatile gas production by interroot bacteria confers systemic tolerance to abiotic stresses by regulating the production of proline, antioxidants and hormones and reducing the accumulation of sodium ions in plants (Zhang et al., 2010; Liu and Zhang, 2015; Sharifi and Ryu, 2017). The main biologically active volatile compounds reported in microorganisms are 2,3 butanediol (Ryu et al., 2003; Farag et al., 2013), adipic acid, butyric acid (Farag et al., 2013), dimethylhexadecylamine (Batchelor et al., 1997; Nilsson et al., 2001), and tridecane (Lee et al., 2012). Of these compounds, 2,3 butanediol is the most widely reported (Ryu et al., 2003; Ji and Huang, 2011).

Rahnella aquatilis JZ-GX1 is a strain of plant-promoting bacteria that was isolated from the rhizosphere of *Pinus massoniana* in our previous study (Li and Wu, 2014). A previous study showed that JZ-GX1 significantly increased the germination rate, germination potential, fresh weight, primary root length and stem length of tomato seeds under salt stress, and it is a moderately salinophilic bacterium with good growth-promoting function (Li et al., 2021). However, it is not clear whether VOCs from this strain can promote plant growth under salt stress. Current studies on the interaction between plant growth-promoting rhizobacterial VOCs and plants have mostly focused on herbaceous plant species, with *A. thaliana* being the most commonly studied, and no reports on woody plant species have been published. Therefore, the present study was carried out using the woody plant species *Robinia pseudoacacia* as the study material with the aim of (i) understanding whether VOCs produced by *R. aquatilis* JZ-GX1 under salt stress conditions affect the tolerance of woody plants to salt stress; (ii) revealing how VOCs produced by *R. aquatilis* JZ-GX1 under salt stress conditions affect the tolerance of woody plants to salt stress; and (iii) exploring whether the VOC 2,3-butanediol produced by *R. aquatilis* JZ-GX1 under salt stress conditions is a signaling compound that affects plant salt tolerance. This study aimed to explore the mechanisms by which JZ-GX1 regulates plant salt stress responses to provide evidence that microorganisms producing 2,3-butanediol can enhance the salt tolerance of plants.

MATERIALS AND METHODS

Test Strain and Culture Medium

Rahnella aquatilis JZ-GX1 is a plant growth-promoting bacterium that was isolated from the rhizosphere soil of 28-year-old *P. massoniana* in Nanning, Guangxi, and is currently stored in the Type Culture Preservation Center of China (CCTCC, No: M 2012439; Li and Wu, 2014).

LB solid medium was prepared as follows: 10 g peptone, 5 g yeast powder, 10 g sodium chloride, 15–20 g agar, pH 7.2. Additionally, 1/2-strength MS medium was purchased from Beijing Rong Shi De Biomedical Technology Co. Based on preliminary results (unpublished) and the study by Zhang et al. (2018) on soil salinity grading, the NaCl concentrations of the above media were set at 0 and 100 mm. The activated test strain was placed in the media and shaken at 28°C and 200 rpm until it reached the logarithmic phase of growth. The seed medium was prepared as follows: 10 g glucose, 1 g yeast powder, 2 g peptone, 6 g $(NH_4)_2SO_4$, 10 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgSO_4$, pH 7.2. The fermentation medium was prepared as follows: 60 g glucose, 20 g peptone, 5 g yeast powder, 0.5 g KH_2PO4, pH 7.0.

Plant Material and Treatment

Plant Material Sources

The seeds of *R. pseudoacacia* used in the experiment were obtained from the Southern Forestry Seed Inspection Center of the State Forestry Administration, China.

Plant Material Treatments

Undamaged seeds that were uniform in size and fullness were surface-sterilized (soaked in 70% ethanol for 2 min and 5% NaClO for 15 min), washed thoroughly with sterile distilled water and air-dried to remove any surface water. The seeds were then sown in water agar Petri dishes and vernalized for 2 days at 4°C in the dark. Then, the seeds were placed in a growth chamber followed by a light incubator and subsequently incubated at 28°C in the dark for germination prior to use.

Experimental setup with bacteria in direct contact with plant roots: Germinated seedlings were arranged in special square plastic plates (3 plants per plate) that contained 0 mm or 100 mm NaCl, and the seedlings were treated with a 1×10^7 cfu/ml bacterial suspension 5 cm from the tip of the primary root. The experiment was replicated six times with 18 seedlings in each replicate.

Experimental setup with bacteria not in direct contact with plant roots: Germinated seedlings were transplanted into glass vials that contained two sections (first section: LB solid medium; second section: 1/2-strength MS solid medium). A control group (CK; 5 µl sterilized PBS buffer) and a test group (JZ-GX1; 5 µl JZ-GX1 suspension) were established. An experiment to verify whether 2,3-butanediol, which is one JZ-GX1 VOC, plays an important role in improving plant salt tolerance was performed by transplanting germinated seedlings into one side of a dichotomous dish (100 × 15 mm) containing a central partition (plate I: LB solid medium; plate II: 1/2-strength MS solid medium). The treatments included (i) 5 µl sterile water; (ii) 5 µl JZ-GX1 suspension; and (ii) different concentrations of 2,3-butanediol.

The medium salt concentration was set to 100 mm NaCl for all the cultures. The glass vials and plates were sealed with paraffin film and placed in a light incubator. The light incubator was set to a cycle of 12 h of light and 12 h of darkness at a temperature of 25 ± 4°C and a relative humidity of 65 ± 10% (Velázquez-Becerra et al., 2011). The experiments were independently performed eight times.

Quantification of Biomass and Root Growth

The fresh weight of the seedlings was measured by an analytical balance after 10 days of direct contact between *R. aquatilis* JZ-GX1 and plant roots. The length of the primary roots was measured with a straightedge, and the number of lateral roots was determined immediately after harvest. After 14 days of exposure to *R. aquatilis* JZ-GX1 VOCs, the fresh weight of the seedlings was measured with an analytical balance, the length of the primary roots was measured with a straightedge, the number of lateral roots was determined immediately after harvesting, and plant soil and plant analyser development (SPAD) values were measured with a SPAD 502 Plus chlorophyll metre. The experiments were performed with three replicates with ten seedlings per replicate.

Determination of MDA Content

Acacia leaves (0.5 g) were weighed and exposed to JZ-GX1 VOCs for 14 d, cut and put into a mortar. Ten milliliters of 5% trichloroacetic acid and a small amount of quartz sand were added, the leaf pieces were ground into a homogenate and centrifuged at 3000 rpm for 10 min, and the supernatant was aspirated as the extraction solution. Then, 1 ml of supernatant was transferred to a test tube, and 1 ml of 0.67% TBA solution was added and shaken well (1 ml of distilled water was added as a control). Then, the test tube was placed into boiling water for 30 min, removed from the bath to cool, and centrifuged at 3000 rpm for 10 min. Finally, the supernatant was collected to measure its absorbance at 532, 450, and 600 nm with TBA solution as a reference. $C\ (\mu mol/L) = 6.45 \times A532 - A600 - 0.56 \times A450$ (Li, 2000). The experiments were performed in triplicate with twenty seedlings per replicate.

Determination of Proline Content

Robinia pseudoacacia leaves (0.1 g) were weighed after 14 d of exposure to JZ-GX1 VOCs. Three replicates were established for each treatment, and the plant proline content was measured after different treatments using a kit (Cominbio, Suzhou, China). The experiments were performed in triplicate with twenty seedlings per replicate.

Ion Determination

The plant tissues were dried at 70°C after 14 d of exposure to JZ-GX1 VOCs. The dried plant tissues were finely ground and passed through a 1mm sieve, and 1g of plant tissues was accurately weighed and placed in a 100 ml triangular flask. Thirty milliliters of mixed acid (HNO_3: $HClO_4$: H_2SO_4; 8: 1: 1, V/V) was added, and a curved-neck funnel was placed at the mouth of the flask and left overnight. The following day, temperature-controlled decoction was performed in a fume hood with six adjustable electric furnaces. The solution in the triangular flask was maintained at a low boil until a large amount of brown nitrogen dioxide NO_2 gas was released. When the brown gas disappeared, the furnace temperature was increased to dehydrate the silica until white smoke was produced. If the solution was still cloudy, 5 ml an acid mixture was added, and heating was continued until the solution became clear and showed white smoke. After cooling, 20 ml of deionized water was added, and the solution was filtered through filter paper into a 100 ml volumetric flask. Then, the triangular flask and filter residue were washed with preheated 1% hydrochloric acid solution until there was no Fe^{3+} reaction. The volume was fixed with deionized water, and the solution was shaken well and measured (Lin et al., 2016). A sodium-potassium ion standard solution was prepared, and the sodium-potassium ion content in the standard and sample was determined with an FP6450 flame photometer. A standard curve was drawn to calculate the sodium-potassium concentrations of the samples. The experiments were performed with three replicates of twenty seedlings per replicate.

Assays for Hydrogen Peroxide (H_2O_2), Superoxide Anion (O_2^-), Glutathione Reductase and Antioxidant Enzymes

Leaf samples (0.1 g) were ground in liquid nitrogen and homogenized in an ice bath using 1 ml of the extraction solution from the kit. Centrifuge, remove supernatant and place on ice for measurement.

The concentration of H_2O_2 was determined by measuring the absorbance of the titanium–hydroperoxide complex (Sui et al., 2007). The superoxide anion reacts with hydroxylamine hydrochloride to produce NO_2^-, NO_2^- in the presence of p-aminobenzenesulfonic acid and naphthylamine to form a red azo compound with a characteristic absorption peak at 530 nm. The O_2^- generation was expressed as the content per gram of fresh leaf mass (Sui et al., 2007). The activity of superoxide dismutase (SOD) was determined with SOD assay kit and was presented as U/g FW. One unit of SOD activity is the amount of extract that gives 50% inhibition in reducing xanthine monitored at 560 nm (Sunjeet et al., 2021). peroxidase (POD) activity was measured by using a POD assay kit based on the POD-catalyzed oxidation of a specific substrate by H_2O_2 witvh characteristic light absorption at 470 nm and expressed as U/g FW. One unit of POD activity is the amount of enzyme, which causes the decomposition of 1 μg substrate per minute in 1 mg fresh sample at 37°C. Similarly, the activity of CAT was measured with a CAT assay kit and was presented as U/g FW. One unit of CAT activity is the amount of enzyme which causes the decomposition of 1 μmol H_2O_2 per minute in 1 mg fresh sample at 37°C (Sunjeet et al., 2021). The glutathione reductase (GSH) content was determined with a glutathione assay kit according to the DTNB [5, 5, − dithiobis (2-nitrobenzoic acid)] method. The absorbance was measured at 412 nm, and GSH content was expressed as μmol/g FW.

The contents of H_2O_2, O_2^-, SOD, POD, CAT and GSH were measured with the corresponding assay kits (Cominbio, Suzhou, China) based on the manufacturer's protocols. All experiments were performed with three replicates of ten seedlings per replicate.

Detection of 2,3-Butanediol Production by *R. aquatilis* JZ-GX1

The colonies were inoculated into seed medium and incubated at 28°C and 200 rpm until they reached the logarithmic growth phase. Then, the samples were inoculated into fermentation medium at 1% inoculum and incubated for 12 h.

Indirect detection: Ethylene coumarin is a precursor substance of 2,3-butanediol. To detect ethylene coumarin by a colorimetric method, a certain amount of fermentation broth was collected and diluted in the following reaction system (10 ml reaction system: 10% NaOH: 0.5% creatine: 5% 1-naphthol:deionized water = 1:1:1:7), and the treatment setup included (i) an ethylene coumarin standard; (ii) JZ-GX1 fermentation broth; and (iii) H_2O. After the reaction solution was shaken and mixed, 25 μl the diluted fermentation solution was added immediately, the effect of the volume of the fermentation solution on the reaction system was ignored, and the colour change after the reaction was observed.

Direct detection: An appropriate amount of fermentation supernatant was collected, ethyl acetate was used as the extractant, and the extract and fermentation solution volume ratio was 1:1. GC-MS detection as performed with a DB-5MS chromatographic column (30 m*0.25 mm*0.25 μm), and the column temperature cycle was as follows: held at 50°C for 1 min, ramped up to 90°C at a rate of 6°C/min and held for 10 min, and finally ramped up to 230°C at a rate of 20°C/min. The inlet temperature was 240°C, the injection volume was 1 μl, the carrier gas flow rate was 1.0 ml/min, and the carrier gas was high-purity helium. The TraceMS mass spectrometry conditions were as follows: EI+ bombardment source, full scan mode, scan mass range of 30–500 amu, emission current of 200 μA, and electron energy of 70 eV. The mass spectrometry detection library was the NIST98 library (Yang, 2013). Our experiments were carried out independently three times.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total plant RNA was isolated using an RNA kit (Beijing Zhuangmeng International Biogene Technology Co., Ltd.) according to the manufacturer's instructions. cDNA samples were prepared using HiScript II Q Select RT SuperMix for qPCR (China). The expression of genes related to ion absorption, migration and compartmentalization was determined by qRT-PCR with an ABI 7500 (Applied Biosystems, United States), and atpD was used as an internal control (Jie et al., 2017). Two genes associated with Na^+ ion uptake, movement and compartmentalization were identified in the study by Jie et al. (**Table 1**). The relative changes in gene expression were calculated by the 2−ΔΔCT method. The RT-PCR assay consisted

TABLE 1 | Primers used in the RT-qPCR analysis.

Gene name	Primers (5′-3′)	Annealing temperature
actin	CCCAAATCATGTTTGAGACCTTCA CATAGATTGGCACAGTGTGACTCA	57
RpSOS1	AAGGTTGGAATYTGSWTGTTA AATWGMRCTTTSCTSCCACAG	60
RpNHX1	CTATGGAGAYATACATGCAGT AAGCTGCWCTRTTKACCTTCAA	53

of three independent experiments with three replicates of each experiment.

Reproducibility of Results and Statistical Analysis

The data were subjected to analysis of variance and Duncan's multiple comparison using SPSS 17.0 software, and the mean values plus standard errors and significance levels were calculated. Different letters indicate significant differences between control and JZ-GX1 inoculated plants in the control or treatment groups ($p < 0.05$).

RESULTS

Rahnella aquatilis JZ-GX1 Enhances Plant Tolerance to Salt Stress

To test whether the JZ-GX1 strain has a protective effect on plants under salt stress conditions when in direct contact with the plant root system, we performed growth assays using *R. pseudoacacia*. At day 10 of treatment, the plant size of seedlings cocultured with JZ-GX1 was slightly larger than that of the untreated control plants (**Figure 1A**). The fresh weight of seedlings treated with JZ-GX1 for 10 days was measured (**Figure 1B**). The results showed that the fresh weight of JZ-GX-treated seedlings under non-salt stress conditions significantly increased by 41.1% compared with that of seedlings under control conditions, and the fresh weight of JZ-GX1-treated seedlings grown on salt media was 2.55 times heavier than that of untreated seedlings.

To investigate the effect of JZ-GX1 on plant root development under salt stress conditions, we determined the number of lateral roots and the length of the main root (**Figures 1C**,**D**). Under non-salt stress conditions, the JZ-GX1 treatment increased the number of lateral roots and the length of primary roots by approximately 123.5 and 94.6%, respectively, compared to the untreated control. Under salt stress (100 mm), coculture with JZ-GX1 increased the number of lateral roots and the length of primary roots by approximately 95.4 and 71.3%, respectively.

Compared to acacia plants exposed to the water control, acacia plants exposed to JZ-GX1 volatiles showed robust growth when *R. aquatilis* JZ-GX1 did not directly contact the plant root system under either non-salt stress (0 mm) or salt stress (100 mm) conditions (**Figure 2A**). Fourteen days after treatment, plants exposed to JZ-GX1 volatiles clearly showed enhanced biomass on medium containing 0 mm and 100 mm NaCl (**Figure 2B**), and the chlorophyll content SPAD value increased by 45.4% (**Figure 2C**). This suggests that JZ-GX1 volatiles can promote photosynthesis through chlorophyll accumulation and enhance photosynthetic growth under salt stress conditions. JZ-GX1 volatiles were also found to reduce the primary root length but increase the number

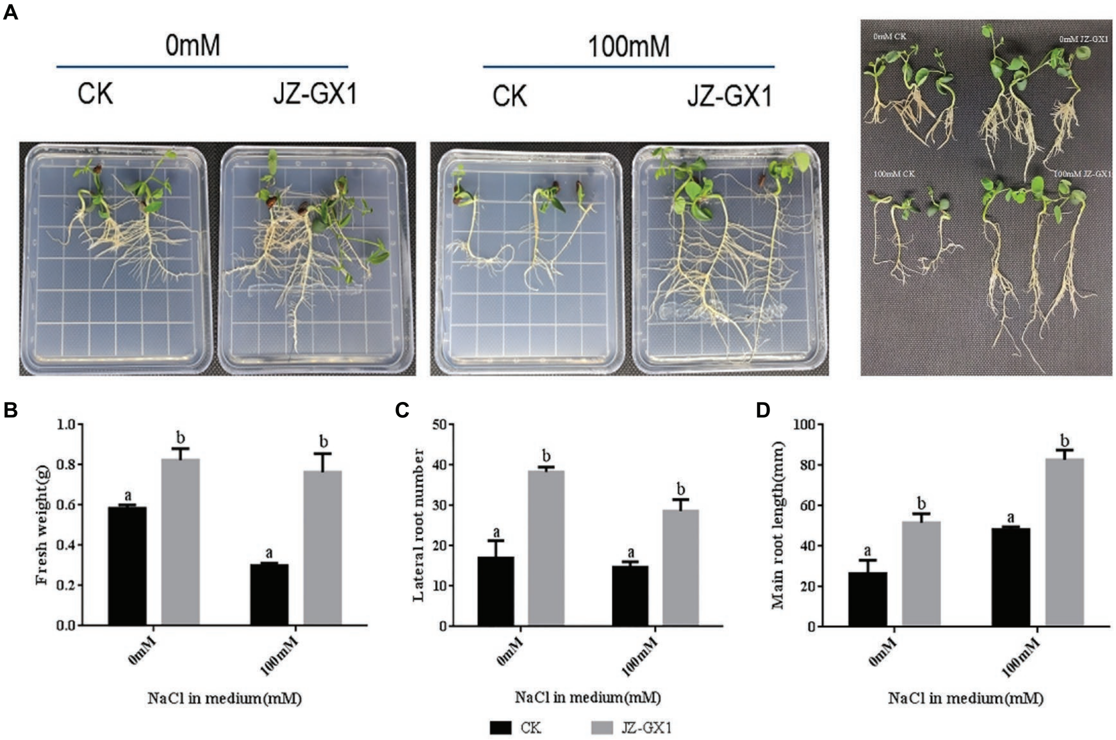

FIGURE 1 | Effect of *Rahnella aquatilis* JZ-GX1 on the growth of *Robinia pseudoacacia*. **(A)** phenotype; **(B)** fresh weight; **(C)** lateral root number; **(D)** primary root length. Different letters indicate significant differences between treatments ($p < 0.05$), and the same applies below.

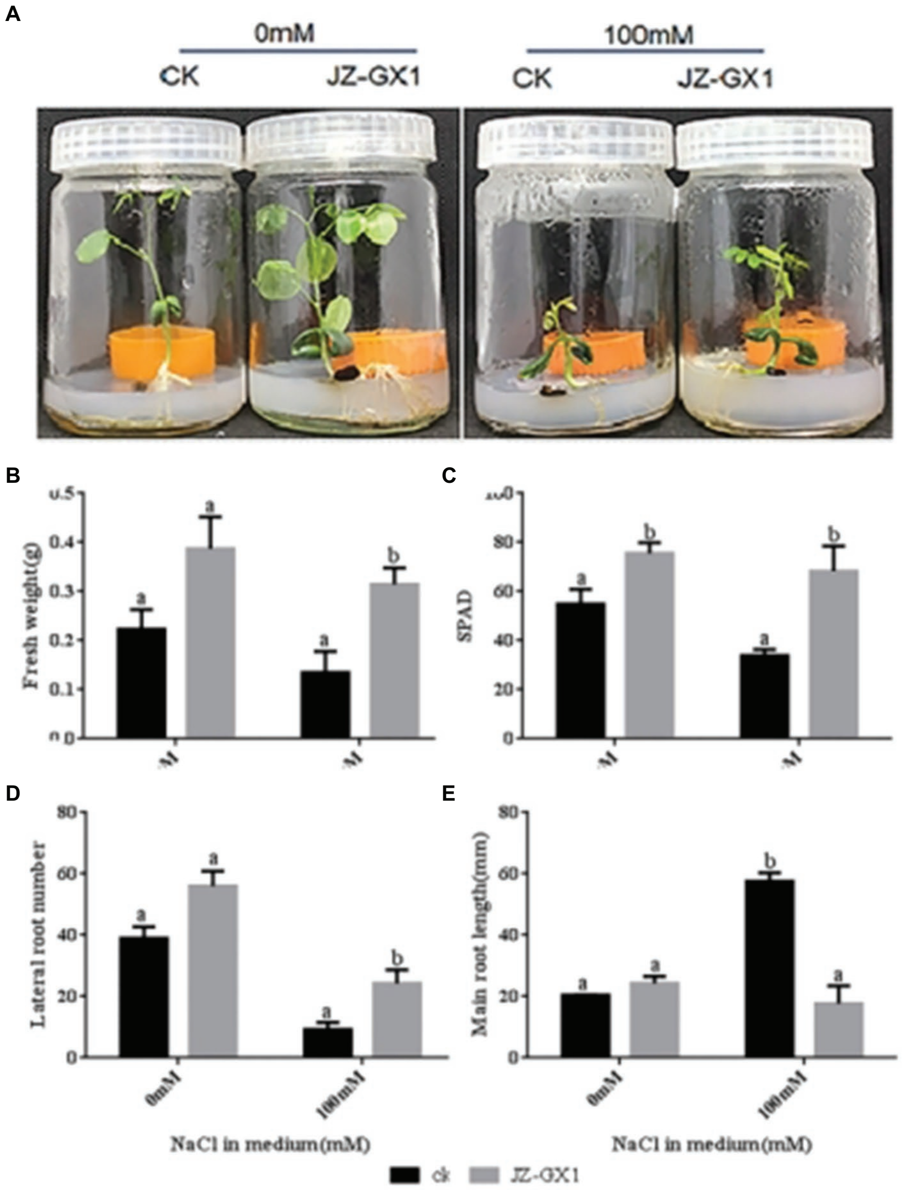

FIGURE 2 | Effect of volatile gas from *R. aquatilis* JZ-GX1 on the growth of *R. pseudoacacia*. **(A)** phenotype; **(B)** fresh weight; **(C)** soil and plant analyser development value; **(D)** lateral root number; **(E)** primary root length.

of lateral roots in acacia, which was 2.7 times higher under salt stress conditions than under the control water treatment conditions (**Figures 2D**,**E**). Therefore, JZ-GX1 can help plants alleviate salt stress by affecting the root structure and improving water uptake efficiency.

Enhancement of the Osmoregulatory Ability of Acacia by *R. aquatilis* JZ-GX1

To study the changes in lipid peroxidation biomarkers after treatment with JZ-GX1 volatile compounds, we measured the level of malondialdehyde (MDA). The 100 mm NaCl treatment increased the MDA content of the leaves of noninoculated and inoculated JZ-GX1 plants by 75.9 and 41.0%, respectively, compared to the 0 mm NaCl treatment. Under normal and salt stress conditions, the MDA content of acacias cocultured with JZ-GX1 for 14 days was reduced by approximately 27.1 and 58.5%, respectively, compared to that of untreated acacias. These results suggest that the plant growth-promoting rhizobacterium *R. aquatilis* JZ-GX1 reduced membrane oxidative damage in plants under salt stress. Proline is an amino acid that acts as an osmoprotectant and as a hydroxyl radical scavenger under various abiotic stress conditions (Hayat et al., 2012). In our study, *R. aquatilis* JZ-GX1 volatiles significantly increased the accumulation of proline in the plants (**Figure 3**).

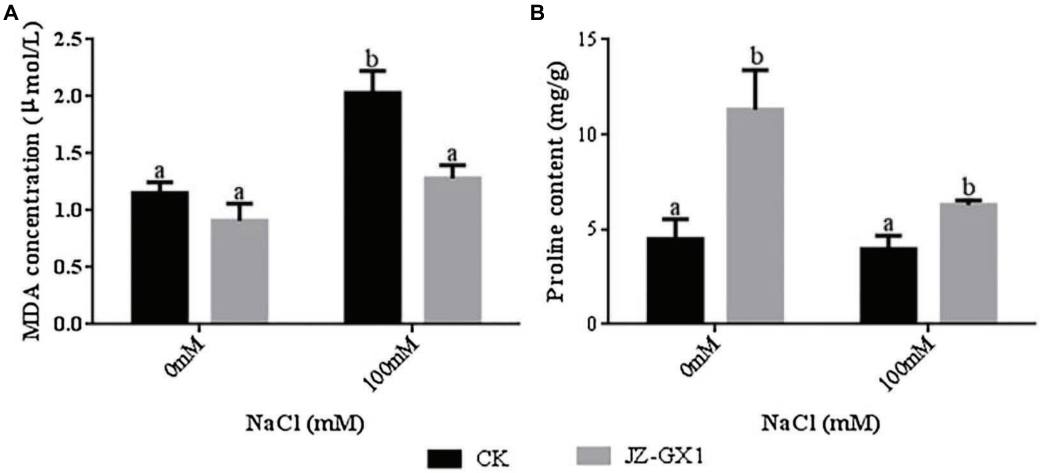

FIGURE 3 | Effect of volatile gases of *R. aquatilis* JZ-GX1 on the malondialdehyde (MDA) content and proline osmoregulatory substance content in *R. pseudoacacia*. **(A)** MDA; **(B)** proline.

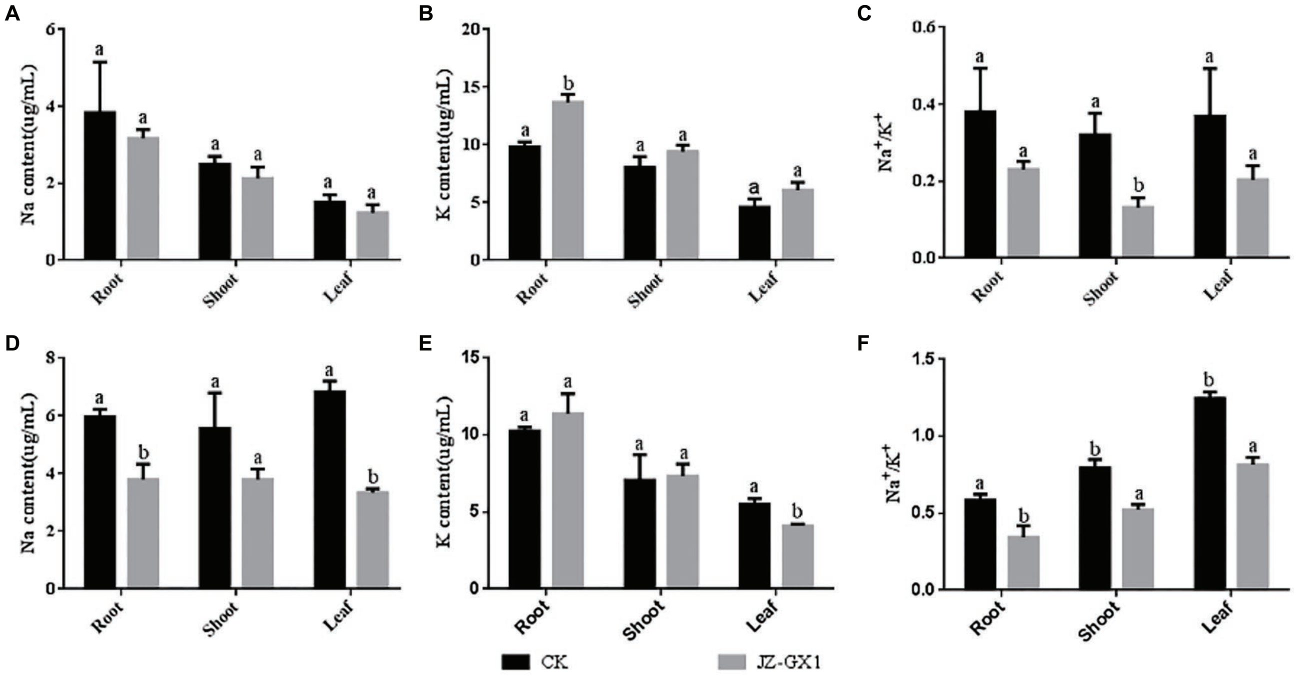

FIGURE 4 | Effect of volatile gases produced by *R. aquatilis* JZ-GX1 on the distribution of Na$^+$ and K$^+$ contents in *R. pseudoacacia*. **(A)** 0 mm Na$^+$ content; **(B)** 0 mm K$^+$ content; **(C)** 0 mm Na$^+$/K$^+$; **(D)** 100 mm Na$^+$ content; **(E)** 100 mm K$^+$ content; **(F)** 100 mm Na$^+$/K$^+$.

Rahnella aquatilis JZ-GX1 Regulates Na$^+$ and K$^+$ Homeostasis

Fourteen days after exposure, under non-salt stress, the Na$^+$ accumulation in the roots, stems and leaves of *R. pseudoacacia* treated with volatile gases produced by *R. aquatilis* JZ-GX1 was not significantly different from that in the control samples, and the Na + distribution in the roots, stems and leaves showed the same trend, specifically following the order of leaves > roots > stems (**Figure 4A**). Under salt stress conditions, the total Na$^+$ accumulation in acacias exposed to JZ-GX1 was 40.5% of the total accumulation in non-exposed plants (**Figure 4D**), indicating that the volatile gas produced by the JZ-GX1 strain reduced Na$^+$ accumulation in acacia roots, stems, and leaves. The Na$^+$ distribution under the CK treatment followed the order of leaves > roots > stems, but the Na$^+$ distribution under the treatment with the volatile gas from

JZ-GX1 followed the order of roots = stem > leaves. Additionally, the Na^+ content in the aboveground and belowground parts was significantly lower after treatment with volatile gas from JZ-GX1 than after the CK treatment, indicating that the transport of Na^+ from the roots to the aboveground parts was restricted under salt stress conditions.

In addition to reducing Na^+ levels, under salt stress and non-salt stress conditions, JZ-GX1 exposure increased the total K^+ content in *R. pseudoacacia* (**Figures 4B,E**). Specifically, with elevated NaCl (100 mm) in the growth medium, an 11.1% increase in K^+ accumulation in the roots and a 4.7% increase in the stems were observed in the presence of JZ-GX1 exposure, while K^+ levels decreased by approximately 24.4% in the shoots (**Figure 4E**).

Exposure to volatile gas from *R. aquatilis* JZ-GX1 reduced the Na^+/K^+ level in *R. pseudoacacia* and leaves under both salt stress and non-salt stress conditions, helping plants resist salt stress (**Figures 4C**,**F**).

We further determined the genes associated with ion uptake, mobility and compartmentalization in acacia plants. In the root system, volatile gas produced by JZ-GX1 under salt stress conditions downregulated the expression of the RpSOS1 gene, which decreased by 9.0% compared to the control condition, while the expression of the RpNHX1 gene in inoculated plants was 99.2% higher than that in noninoculated plants after treatment with 100 mm NaCl (**Figure 5**).

Enhancement of the Antioxidant Capacity of Acacia by *R. aquatilis* JZ-GX1

Salt stress significantly increased the H_2O_2 content (1.83-fold) and O_2^- production rate (2.44-fold) in seedlings compared to non-salt stress conditions. The H_2O_2 content and O^{2-} production rate of JZ-GX1-inoculated seedlings were significantly reduced by 15.9 and 12.7%, respectively, under salt stress compared to those of the uninoculated control (**Figure 6**). This indicates that inoculation with JZ-GX1 can effectively reduce the accumulation of reactive oxygen species in seedlings under salt stress and alleviate oxidative stress.

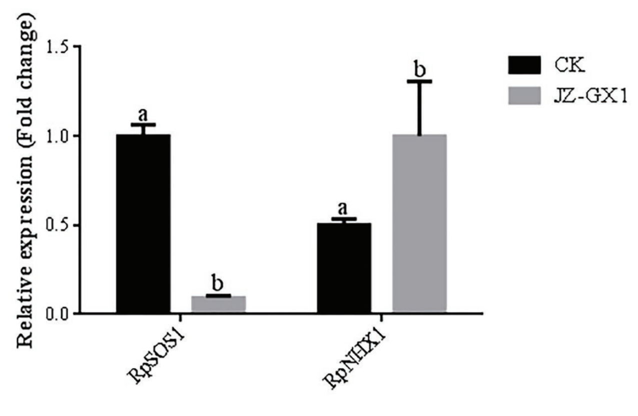

FIGURE 5 | Effect of volatile gas produced by *R. aquatilis* JZ-GX1 on the expression of RpSOS1 and RpNHX1 in *R. pseudoacacia* root systems.

With increasing salt concentrations, the SOD activities of the leaves and roots of seedlings inoculated and not inoculated with JZ-GX1 increased (**Figures 7A**,**B**). In the roots, 100 mm NaCl treatment increased the SOD activity of noninoculated and inoculated seedlings by 47.7 and 20.8%, respectively, compared to 0 mm NaCl treatment. In the leaves, the SOD activity of seedlings inoculated with JZ-GX1 was increased by 6.6 and 33.8%, respectively, under 100 mm NaCl treatment conditions compared to that under nonstressed conditions. Although in the root system, the leaf SOD activity of seedlings inoculated with JZ-GX1 was significantly lower than that of noninoculated seedlings under 100 mm NaCl conditions, the leaf SOD activity of seedlings inoculated with JZ-GX1 was significantly higher than that of noninoculated plants under both 0 and 100 mm NaCl conditions (31.5 and 65.0% higher than the latter, respectively).

In the roots, the POD activity of seedlings increased significantly with increasing salt concentrations. The POD activity of seedlings inoculated with JZ-GX1 decreased by 176.5% under nonstressed conditions and increased by 17.4% under stressed conditions compared with nonstressed conditions (**Figure 7C**). In leaves, the POD activity of seedlings without and with JZ-GX1 inoculation increased with increasing salt concentrations. The POD activity of seedlings without JZ-GX1 inoculation was 55.9% higher under the 100 mm NaCl condition than under the nonstressed condition. The POD activity of seedlings inoculated with JZ-GX1 was 505.0% higher under the 100 mm NaCl condition than under the nonstressed condition. Although the POD activity of inoculated seedlings was reduced under non-salt stress conditions compared with that of seedlings not inoculated with JZ-GX1, the POD activity of seedlings inoculated with JZ-GX1 was significantly increased by 52.7% under salt stress conditions (**Figure 7D**).

Under salt stress conditions, the CAT activities of the roots and leaves of JZ-GX1-inoculated seedlings were reduced, and only the CAT activities of the leaves were reduced in noninoculated plants (**Figures 7E**,**F**). Under non-salt stress conditions, inoculation with JZ-GX1 greatly increased the CAT activity of the seedling roots. In contrast, the CAT activity of the roots and leaves of seedlings inoculated with JZ-GX1 decreased significantly by 182.5 and 396.3%, respectively, under the 100 mm NaCl treatment compared with those without inoculation.

In the roots, the GSH content was reduced by 206.3 and 108.8% in seedlings inoculated with JZ-GX1 and uninoculated seedlings, respectively, under 100 mm NaCl treatment conditions compared to nonstressed conditions. The GSH content of the noninoculated plants did not change significantly with increasing salt concentrations (**Figure 7G**). The root GSH content of the plants inoculated with JZ-GX1 was significantly higher than that of the noninoculated plants after treatment with 0 and 100 mm NaCl (44.9 and 112.5% higher than the latter, respectively). In the leaves, the GSH content of seedlings not inoculated with JZ-GX1 increased significantly with increasing salt concentrations. The GSH content of seedlings inoculated with JZ-GX1 decreased by 9.3% under 100 mm NaCl treatment conditions compared with that under nonstressed conditions. The leaf GSH content of the seedlings inoculated with JZ-GX1

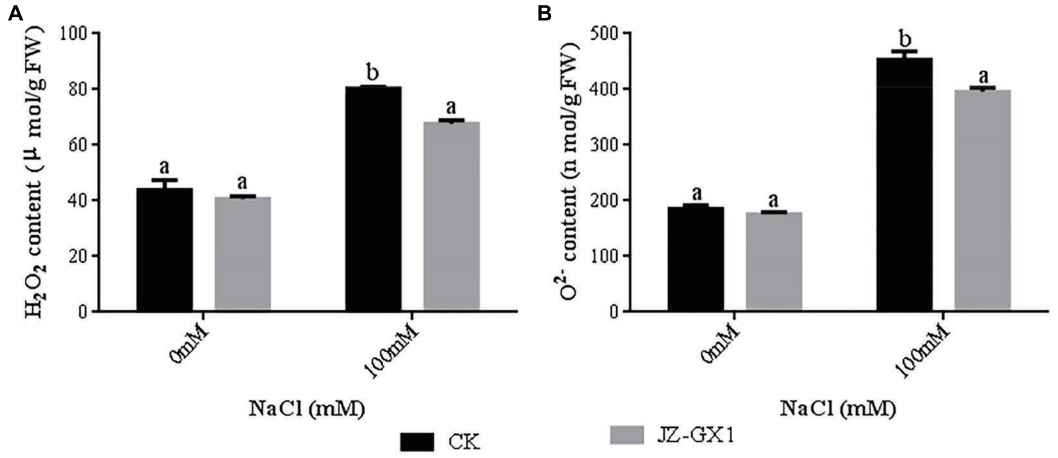

FIGURE 6 | Effect of volatile gases produced by *R. aquatilis* JZ-GX1 on the H_2O_2 content of *R. pseudoacacia*. **(A)** H_2O_2 content; **(B)** O_2^- content.

was significantly higher than that of the noninoculated seedlings under the 0 mm NaCl condition (53.2% higher than the latter) but lower than that of noninoculated plants under the 100 mm NaCl condition (**Figure 7H**).

2,3-Butanediol Is an Important Chemical Signal for the Enhancement of Salt Tolerance in *R. pseudoacacia* by *R. aquatilis* JZ-GX1

The results of the creatine chromogenic test showed that the supernatant of JZ-GX1 became pink after the addition of creatine chromogenic solution, while the blank control culture retained its original colour (**Figure 8**), indicating that strain JZ-GX1 could produce acetoin. Acetoin produces a precursor substance of 2,3-butanediol; therefore, these results indirectly indicated that the JZ-GX1 strain can produce 2,3-butanediol. GC–MS analysis showed that 2,3-butanediol could be produced by *R. aquatilis* JZ-GX1 by comparing the peak emergence time with the database (**Figures 9, 10**).

To explore the contribution of 2,3-butanediol to the growth of *R. pseudoacacia* under salt stress conditions, different concentrations of 2,3-butanediol were used to test whether it could enhance plant growth and salt tolerance. The results showed that under salt stress conditions, the fresh weight of acacia increased significantly when incubated with 10 μm 2,3-butanediol compared with the control. The biomass increased at 2,3-butanediol concentrations of 1, 2, 5, 20 and 50 μm, but the difference was not significant. Plants exposed to the volatile gas of *R. aquatilis* JZ-GX1 showed the highest fresh weight (**Figure 11**).

Summarizing the Main Mechanisms of Salt Tolerance in Plants Induced by *R. aquatilis* JZ-GX1 VOCs

With increasingly saline soils in the future (Otlewska et al., 2020), the effective use of saline soils is important. Exposure to JZ-GX1 VOCs modulates chlorophyll; alters root morphology; reduces MDA, superoxide anion and hydrogen peroxide contents; increases the proline osmoregulatory substance content; reduces intracellular sodium accumulation; and increases the antioxidant capacity to enhance stress tolerance under salt stress conditions in plants. Additionally, JZ-GX1 VOCs induce the differential expression of the (Na^+, K^+)/H^+ reverse cotransporter RpNHX1. 2,3-Butanediol is also involved in VOC-induced plant resistance to salt stress (**Figure 12**).

DISCUSSION

Studies on plant growth-promoting rhizobacterial VOCs and plant resistance have included various strains of *Agrobacterium*, *Azospirillum*, *Bacillus*, *Pseudomonas* and *Rhizobium* (Ahmad et al., 2014). Among these strains, *Bacillus* and *Pseudomonas* strains are the most frequently studied (Ge et al., 2014). This study reports the effect of VOCs of *Rahnella* on plant salinity tolerance for the first time.

Some strains of PGPR play a crucial role in helping plants cope with adverse conditions, including salt stress (Zhang et al., 2010; Bresson et al., 2013). In this study, we showed that the plant growth-promoting rhizobacterium *R. aquatilis* JZ-GX1 improved plant growth and salt tolerance by promoting aboveground and root development when in contact with plant roots. VOCs emitted by JZ-GX1 enter the atmosphere as vapors and thus come into contact with plants due to their significantly high vapor pressure and low molecular weight (Dimkpa et al., 2009). Both plant biomass and SPAD values were significantly increased in plants grown on medium containing 100 mm NaCl compared to the control medium. In addition, it is noteworthy that, in general, seedlings of the control-treated plants had only primary and secondary lateral roots, but seedlings treated with the JZ-GX1 strain produced tertiary and even quaternary lateral roots at the seedling stage, and the number of lateral roots was also 2.7 times higher than that under the control treatment. Patten and Glick (2002) reported that the promotion

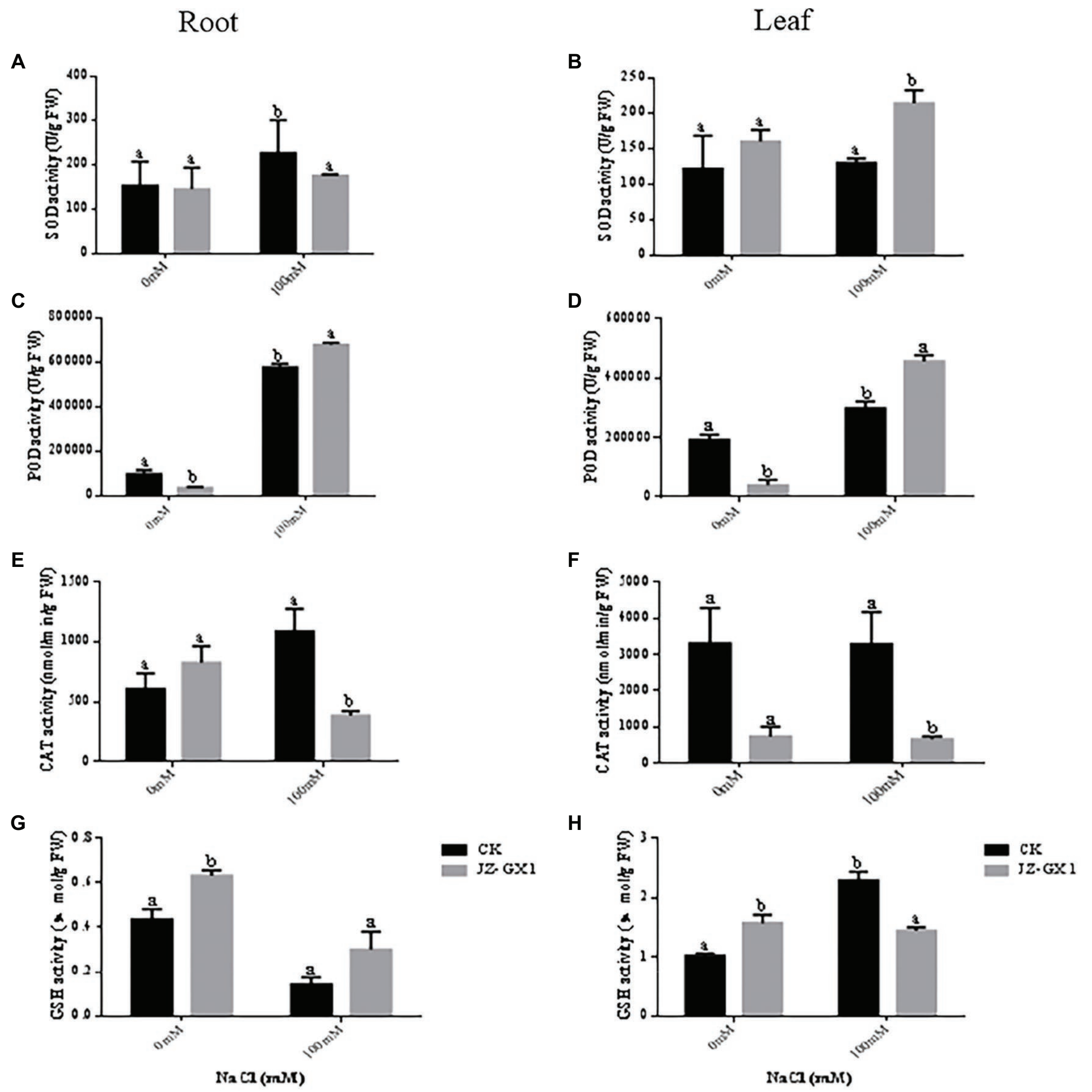

FIGURE 7 | Effect of volatile gases produced by *R. aquatilis* JZ-GX1 on the superoxide dismutase (SOD), peroxidase (POD), CAT and glutathione reductase (GSH) activities in the leaves and roots of *R. pseudoacacia*. **(A)** Root SOD activity; **(B)** leaf SOD activity; **(C)** root POD activity; **(D)** leaf POD activity; **(E)** root CAT activity; **(F)** leaf CAT activity; **(G)** root GSH activity; **(H)** leaf GSH activity.

of lateral root growth is one of the main indicators of the beneficial effects of PGPR and that rapid root establishment through lateral root proliferation is beneficial to seedlings because it increases the ability of seedlings to anchor themselves to the substrate and obtain water and nutrients (Hommel et al., 2016). Better colonization of plant roots could also provide an advantage for bacteria by allowing them to obtain more root secretions and carbon sources (Yuan et al., 2020). In the natural environment, plant growth-promoting rhizobacterial VOCs diffusing through inter-root soil pores are mainly sensed by roots (Tahir et al., 2017). Thus, *R. aquatilis* JZ-GX1 appears to affect the root structure through changes in root development, which may enhance plant tolerance to salt stress. Since bacterial cells do not contact plant roots, VOCs produced by *R. aquatilis* JZ-GX1 under salt stress conditions play a role in improving the salt tolerance of plants.

The salt tolerance of plants depends largely on the amount of Na absorbed by the plant, the distribution of Na in the plant (Porcel et al., 2016), and the amount of Na^+/K^+ in the cytoplasm (Shabala and Cuin, 2008). Studies on wheat (*Triticum aestivum*; Talaat and Shawky, 2011) and *Tribulus terrestris* (*Medicago truncatula*; Garcia et al., 2017) revealed that Na^+ accumulation in plants inoculated with arbuscular mycorrhizae was lower than that in noninoculated plants, and the proportion of Na allocated to aboveground parts was lower than that in noninoculated plants (Evelin et al., 2012; Porcel et al., 2016). The results of this experiment also showed that the volatile gas produced by *R. aquatilis* JZ-GX1 significantly increased K^+ uptake by acacia under stress conditions. The reason for this may be, on the one hand, that the volatile gas produced by JZ-GX1 increases the number of phytometric roots and increases the contact area, thus effectively improving the ability of the prickly ash to absorb mineral nutrients from the culture substrate. On the other hand, K can be selectively absorbed by plants as an isotonic substance and transported to various plant organs and tissues, helping plants avoid the absorption of more Na^+. The JZ-GX1 VOCs limit Na^+ distribution in leaves and Na^+ translocation to aboveground parts of seedlings, reducing plant Na^+/K^+ to maintain a stable ionic equilibrium. Blocking the translocation of Na^+ from the root system to aboveground parts is an important strategy by which plants protect photosynthetic organs from Na^+ toxicity (Zhu et al., 2016). A series of membrane transporter proteins responsible for K^+ and Na^+ uptake, movement and compartmentalization in plants plays a key role in reducing the excessive accumulation of Na elements (Wang et al., 2017). The cytoplasmic membrane Na^+/H^+ reverse cotransporter SOS1 and the vesicular membrane $(Na^+, K^+)/H^+$ reverse cotransporter NHX1 are responsible for excreting Na^+ from the cell or compartmentalizing it in vesicles, respectively (Qiu et al., 2002; Deinlein et al., 2014). These transporters represent two major mechanisms by which low Na^+ levels are maintained in the cytosol (Deinlein et al., 2014). Because of the positive effects of JZ-GX1 in reducing plant Na^+ contents, increasing K^+ contents, and reducing leaf Na^+/K^+ contents, we further examined the expression of these 2 genes involved in K^+ and Na^+ uptake, transport, and compartmentalization. The results showed that in roots, JZ-GX1 VOCs upregulated the expression of RpNHX1 under stress conditions, which was in agreement with the results of (Porcel et al., 2016) obtained in rice (*O. sativa*) plants. Upregulation of RpNHX1 expression in roots promotes Na^+ efflux from the intracellular space to the soil or plastid space, and the compartmentalization of Na^+ in vesicles facilitates osmoregulation by cells. In fact, OsNHX1-4, which is located in the vesicles in rice, functions as a reverse vesicular $(Na^+, K^+)/H^+$ cotransporter. Therefore, further studies on the expression of other genes encoding NHX proteins are needed to more clearly elucidate the contribution of these genes to Na^+ compartmentalization in different regions of inoculated plants. In addition, JZ-GX1 VOC treatment reduced the expression of RpSOS1 in the root system, which differed from the results of Estrada et al. obtained in rice. Estrada et al. (2013) studied maize (*Zea mays*) inoculated with different arbuscular mycorrhizal fungi (AMF), including *Rhizophagus irregularis*, *Claroideoglomus etunicatum* and *Septoglomus constrictum*, and found that AMF could better regulate the expression of ZmSOS1 in the root systems of host plants. We speculate that the use of different plants and strains in the experiment is the reason for this inconsistent result.

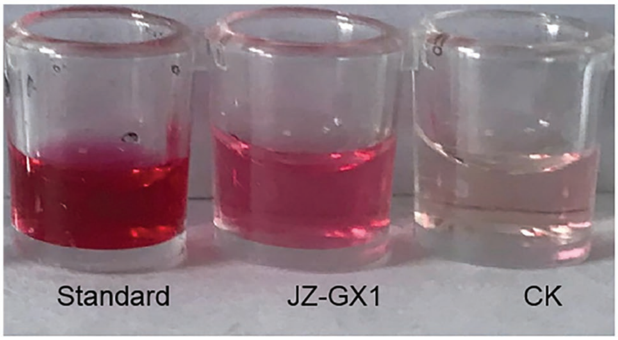

FIGURE 8 | Determination of the 2,3-butanediol production ability of *R. aquatilis* JZ-GX1. (standard: 2,3-butanediol standard; JZ-GX1: JZ-GX1 fermentation solution; CK: H2O).

Salt stress induces excessive production of ROS in plant cells, leading to membrane lipid peroxidation and disrupting the cell membrane integrity (Gill and Tuteja, 2010). In particular, very low concentrations of the superoxide anion hydrogen peroxide are required by plants for intracellular signal transduction processes. However, hydrogen peroxide overproduction inhibits the growth of plant roots (Cerny et al., 2018). In this study, the H_2O_2 content and O_2^- production rate were higher in acacia seedlings under salt stress conditions,

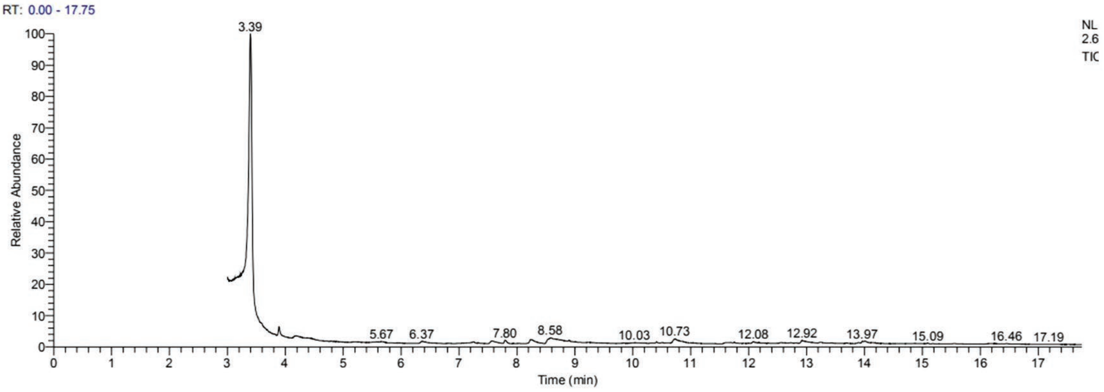

FIGURE 9 | Spectrum of 2,3-butanediol identification by GC-MS in the fermentation broth of *R. aquatilis* JZ-GX1.

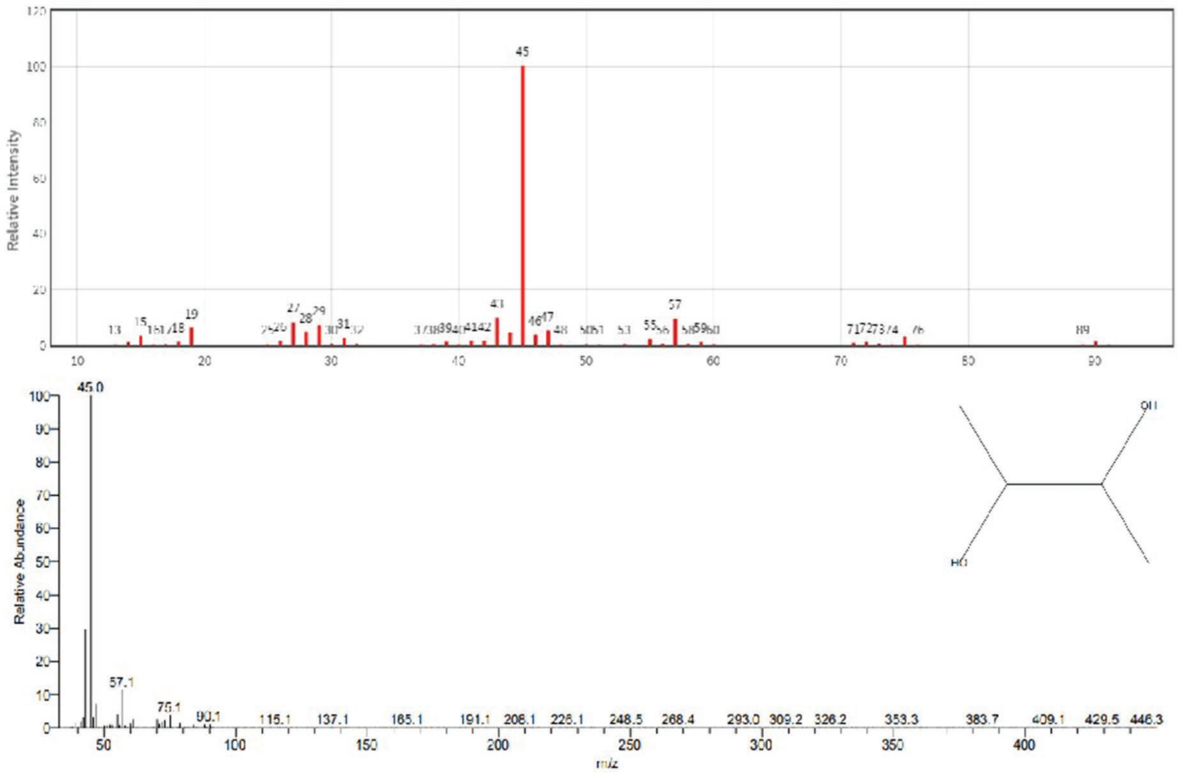

FIGURE 10 | Gas chromatography–mass spectrometry analysis of the fermentation broth of *R. aquatilis* JZ-GX1.

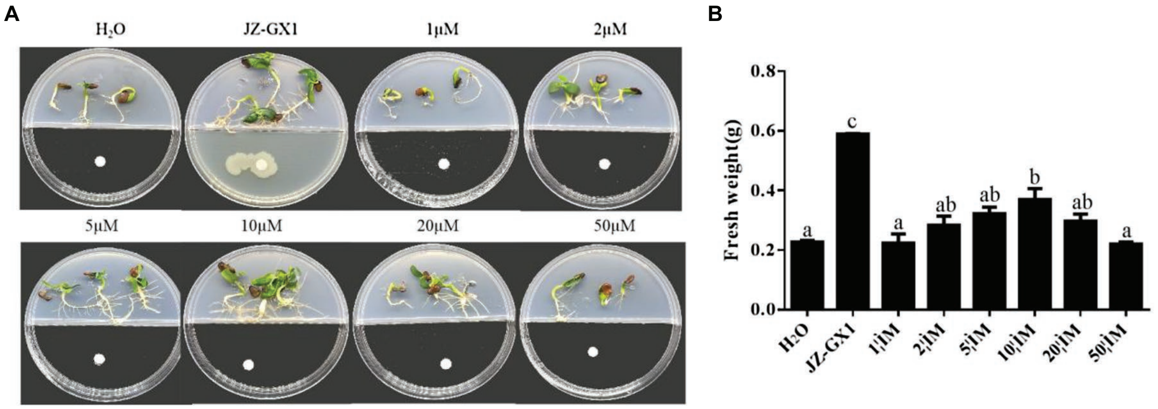

FIGURE 11 | Effect of different concentrations of 2,3-butanediol on *R. pseudoacacia* under salt stress conditions. **(A)** Phenotype; **(B)** fresh weight.

indicating that the cells were under oxidative stress and that the cell membranes were damaged. In contrast, the H_2O_2 and O_2^- levels of inoculated seedlings were consistently lower than those of uninoculated seedlings under salt stress conditions. This is in agreement with Alexander et al. (2020), who showed that the application of *Stenotrophomonas maltophilia* BJ01 led to a decrease in the reactive oxygen levels in peanuts (*Arachis hypogaea*) under salt stress conditions. Enzymatic antioxidant systems (e.g., POD, SOD, and CAT) and nonenzymatic components (e.g., GSH and proline) play key roles in the induction, elimination, detoxification or neutralization of toxic levels of ROS (Liebthal et al., 2018). SOD is the first line of defense against damage caused by ROS (Apel and Hirt, 2004). The results of the experiment showed that the SOD activity in the leaves of the inoculated plants was significantly higher than that of

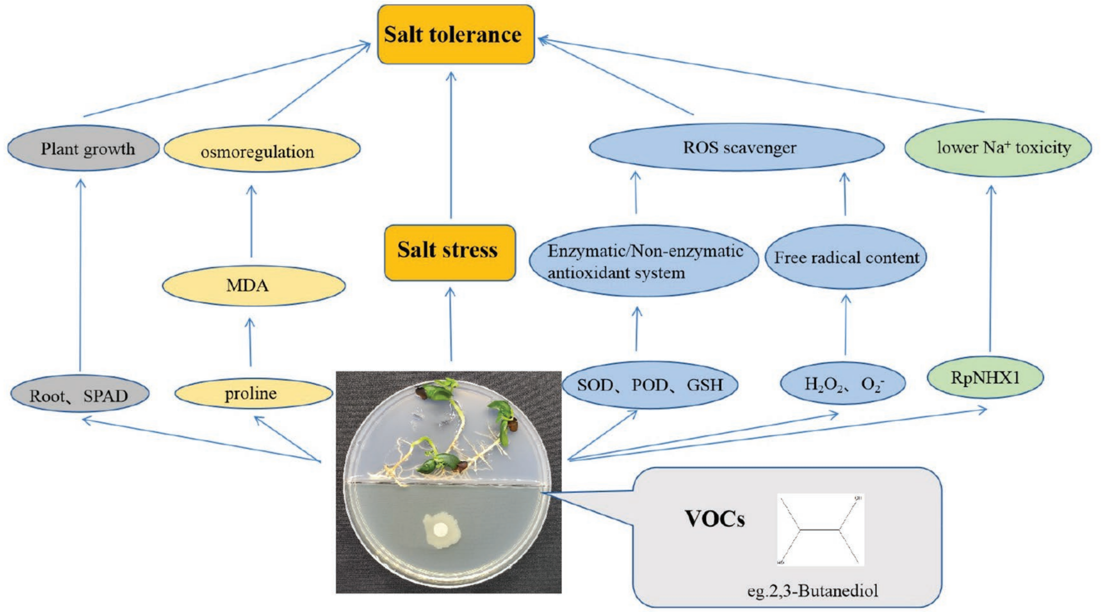

FIGURE 12 | Main pathways of salt tolerance in plants induced by *R. aquatilis* JZ-GX1 volatile organic compounds.

the noninoculated plants at a concentration of 100 mm NaCl, reflecting that the ability of the inoculated plants to scavenge O_2^- under salt stress conditions was higher than that of the noninoculated plants. After SOD catalyses O_2^- via a disproportionation reaction to H_2O_2 and O_2^-, other ROS-scavenging enzymes are required to quickly and efficiently remove H_2O_2 (Li et al., 2020). POD, CAT and GSH can all play a role in this process (Li et al., 2012). Inoculation with JZ-GX1 under salt stress conditions significantly increased the activity of POD in plant leaves and the content of GSH in the root system. The effect on CAT activity was not consistent with our expectations, as JZ-GX1 decreased CAT activity under stress conditions. However, overall, plants inoculated with JZ-GX1 had a stronger ability to scavenge reactive oxygen species than did noninoculated plants, and therefore, their MDA content was lower than that of noninoculated plants, although their CAT activity was also lower. Furthermore, the improvement of salt tolerance in plants by inoculation with JZ-GX1 did not cause an increase in the activity of all antioxidant enzymes. Thus, our results suggest that VOCs from the plant growth-promoting rhizosphere bacterium *R. aquatilis* JZ-GX1 can jointly regulate the antioxidant capacity of plants to prevent the oxidative damage caused by reactive oxygen species through both enzymatic and nonenzymatic systems. In addition, we found that volatile 2,3-butanediol helps protect plants from abiotic stresses and is the "chemical language" used by *R. aquatilis* JZ-GX1 VOCs to interact with their plant partners, which is consistent with results Sharifi and Ryu (2018). Volatile 2,3-butanediol helps protect plants from abiotic stresses. Treatment with the *Pseudomonas aeruginosa* O6 mutant, which is unable to synthesize 2,3-butanediol, did not improve drought stress tolerance in Arabidopsis compared with the wild-type strain. Notably, the effect of JZ-GX1 VOCs was better than the optimal concentration of pure 2,3-butanediol (10 μm), suggesting the presence of other unknown gases with biological activity inducing plant resistance. The production and accumulation of substances has an impact on the signaling pathways induced by high-salt stress conditions and helps mitigate the effects of high salt concentrations (Faisal and John, 2020). Thus, 2,3-butanediol can be used as a promising tool for improving plant stress resistance.

CONCLUSION

In conclusion, we observed a significant plant growth-promoting effect of *R. aquatilis* JZ-GX1 VOCs on *R. pseudoacacia* seedlings under salt stress conditions. *R. aquatilis* JZ-GX1 can help counteract stress through several mechanisms, mainly those involving the plant root conformation, the sue of osmoregulatory substances, reactive oxygen species scavenging and the reduction of sodium toxicity, as well as volatile 2,3-butanediol, which helps protect plants from abiotic stress. Our findings provide an effective and sustainable approach for the development of new microbial resources and the bioprotection of plants under salt stress conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

P-SL completed the data analysis and the first draft of the paper. P-SL and W-LK completed the experimental research. YZ participated in the experimental results analysis. X-QW directed the experimental design, data analysis, paper writing, and revision. All authors read and agreed on the final version of the text.

FUNDING

This work was supported by the National Key Research and Development Program of China (2017YFD0600104) and the Priority Academic Program Development of the Jiangsu Higher Education Institutions (PAPD).

ACKNOWLEDGMENTS

We thank Prof. Fang-Yuan Yu at Nanjing Forestry University for providing the *R. pseudoacacia* seeds.

REFERENCES

Ahmad, M., Zahir, Z. A., Nazli, F., Akram, F., Arshad, M., and Khalid, M. (2014). Effectiveness of halo-tolerant, auxin producing *Pseudomonas* and *Rhizobium* strains to improve osmotic stress tolerance in mung bean (*Vigna radiata* L.). *Braz. J. Microbiol.* 44, 1341–1348. doi: 10.1590/s1517-83822013000400045

Alexander, A., Singh, V. K., and Mishra, A. (2020). Halotolerant PGPR Stenotrophomonas maltophilia BJ01 induces salt tolerance by modulating physiology and biochemical activities of Arachis hypogaea. *Front. Microbiol.* 11:568289. doi: 10.3389/fmicb.2020.568289

Apel, K., and Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701

Baek, D., Rokibuzzaman, M., Khan, A., Min, C. K., and Chung, Y. R. (2020). Plant-growth promoting *Bacillus oryzicola* YC7007 modulates Stress-response gene expression and provides protection from salt stress. *Front. Plant Sci.* 10:1646. doi: 10.3389/fpls.2019.01646

Batchelor, S. E., Cooper, M., Chhabra, S. R., Glover, L. A., Stewart, G. S. A. B., Williams, P., et al. (1997). Cell densityregulated recovery of starved biofilm populations of ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* 63, 2281–2286. doi: 10.1128/aem.63.6.2281-2286.1997

Bresler, E., Mcneal, B. L., and Carter, D. L. (1982). Saline and sodic soils. *Advanced* 4, 1095–1107.

Bresson, J., Varoquaux, F., Bontpart, T., Touraine, B., and Vile, D. (2013). The PGPR strain *Phyllobacterium brassicacearum* STM196 induces a reproductive delay and physiological changes that result in improved drought tolerance in *Arabidopsis*. *New Phytol.* 200, 558–569. doi: 10.1111/nph.12383

Cerny, M., Habánová, H., Berka, M., Luklová, M., and Brzobohatý, B. (2018). Hydrogen peroxide: its role in plant biology and crosstalk with signalling networks. *Int. J. Mol. Sci.* 19:2812. doi: 10.3390/ijms19092812

Cheng, Z., Zhu, L., Xie, Y., and Li, F.,Xin, F., Ma, Z., and Wang, J. (2017). *Bacillus licheniformis* SA03 confers increased saline–alkaline tolerance in chrysanthemum plants by induction of abscisic acid accumulation. *Front. Plant Sci.* 8:1143. doi:10.3389/fpls.2017.01143

Deinlein, U., Stephan, A. B., Horie, T., Luo, W., Xu, G., and Schroeder, J. I. (2014). Plant salt-tolerance mechanisms. *Trends Plant Sci.* 19, 371–379. doi: 10.1016/j.tplants.2014.02.001

Dimkpa, C., Weinand, T., and Asch, F. (2009). Plant rhizobacteria interactions alleviate abiotic stress conditions. *Plant Cell Environ.* 32, 1682–1694. doi: 10.1111/j.1365-3040.2009.02028.x

Estrada, B., Aroca, R., Maathuis, F. J. M., Barea, J. M., and Ruiz-Lozano, J. M. (2013). Arbuscular mycorrhizal fiungi native from a mediterranean saline area enhance maize tolerance to salinity through improved ionhomeostasis. *Plant Cell Environ.* 36, 1771–1782. doi: 10.1111/pce.12082

Evelin, H., Giri, B., and Kapoor, R. (2012). Contribution of Glomus intraradices inoculation to nutrient acquisition and mitigation of ionic imbalance in NaCl-stressed *Trigonella foenum-graecum*. *Mycorrhiza* 22, 203–217. doi: 10.1007/s00572-011-0392-0

Faisal, Z., and John, T. (2020). Hydrogen sulfide in horticulture: emerging roles in the era of climate change - sciencedirect. *Plant Physiol. Biochem.* 155, 667–675. doi: 10.1016/j.plaphy.2020.08.010

Farag, M. A., Zhang, H., and Ryu, C. M. (2013). Dynamic chemical communication between plants and bacteria through airborne signals: induced resistance by bacterial volatiles. *J. Chem. Ecol.* 39, 1007–1018. doi: 10.1007/s10886-013-0317-9

Garcia, K., Chasman, D., Roy, S., and Ane, J. M. (2017). Physiological responses and gene co-expression network of mycorrhizal roots under K^+ deprivation. *Plant Physiol.* 173, 1811–1823. doi: 10.1104/pp.16.01959

Ge, C. H., Meng, A. J., Ma, Y. R., Yang, X. H., Wang, X. Y., and Sun, J. S. (2014). Screening, identification and fermentation characteristics of a strain of cucumber root-perimeter promoting bacteria. *Biotechnol. Bull.* 30, 94–99.

Gill, S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930. doi: 10.1016/j.plaphy.2010.08.016

Glick, B. R., Patten, C. N., Holguin, G., and Penrose, D. M. (1999). "Overview of plant growth-promoting bacteria," in *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria* (London: Imperial college Press), 1–13.

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal. Behav.* 7, 1456–1466. doi: 10.4161/psb.21949

Hommel, R., Siegwolf, R., Zavadlav, S., Arend, M., Schaub, M., Galiano, L., et al. (2016). Impact of interspecific competition and drought on the allocation of new assimilates in trees. *Plant Biol.* 8, 785–796. doi: 10.1111/plb.12461

Ji, X. J., and Huang, H. (2011). Microbial 2,3-butanediol production: a state of the art review. *Biotechnol. Adv.* 29, 351–364. doi: 10.1016/j.biotechadv.2011.01.007

Jie, C., Zhang, H., Zhang, X., and Ming, T. (2017). Arbuscular mycorrhizal symbiosis alleviates salt stress in black locust through improved photosynthesis, water status, and k^+/Na^+ homeostasis. *Front. Plant Sci.* 8:1739. doi: 10.3389/fpls.2017.01739

Kanchiswamy, C. N., Malnoy, M., and Maffei, M. E. (2015). Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci.* 6:151. doi: 10.3389/fpls.2015.00151

Lee, B., Farag, M. A., Park, H. B., Kloepper, J. W., Lee, S. H., and Ryu, C. M. (2012). Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a c13 volatile produced by paenibacillus polymyxa. *PLoS One* 7:e48744. doi: 10.1371/journal.pone.0048744

Li, H. S. (2000). *Principles and Techniques of Plant Physiological and Biochemical Experiments*. Beijing: Higher Education Press, 260–261.

Li, E., Hu, H. R., Li, J. N., Du, G. H., and Liu, F. H. (2019). Research progress on endophytic fungi to enhance plant resistance to salt stress. *Biotechnol. Bull.* 35, 169–178. doi: 10.13560/j.cnki.biotech.bull.1985.2019-0499

Li, P. S., Kong, W. L., and Wu, X. Q. (2021). Salt tolerance mechanism of the rhizosphere bacterium JZ-GX1 and its effects on tomato seed germination and seedling growth. *Front. Microbiol.* 8:657238. doi: 10.3389/fmicb.2021.657238

Li, K., Pang, C. H., Ding, F., Sui, N., and Wang, B. S. (2012). Overexpression of suaeda salsa stroma ascorbate peroxidase in arabidopsis chloroplasts enhances salt tolerance of plants. *S. Afr. J. Bot.* 78, 235–245. doi: 10.1016/j.sajb.2011.09.006

Li, H., Wang, H., Wen, W., and Yang, G. (2020). The antioxidant system in Suaeda salsa under salt stress. *Plant Signal. Behav.* 15:1771939. doi: 10.1080/15592324.2020.1771939

Li, G. E., and Wu, X. Q. (2014). Phytase characterization of the highly efficient phytate-degrading bacterium *Rahnella aquatilis* JZ-GX1. *J. Central South Univ. Fores. Sci. Technol.* 6, 90–93.

Liebthal, M., Maynard, D., and Dietz, K. J. (2018). Peroxiredoxins and redox signaling in plants. *Antioxid. Redox. Signal.* 28, 609–624. doi: 10.1089/ars.2017.7164

Lin, C., Liu, Y., Wu, G., and Njeri, K. V.,Shen, Q., Nan, Z., and Zhang, R. (2016). Induced maize salt tolerance by rhizosphere inoculation of *Bacillus amyloliquefaciens* SQR9. *Physiol. Plant.* 158, 34–44. doi:10.1111/ppl.12441

Liu, X. M., and Zhang, H. (2015). The effects of bacterial volatile emissions on plant abiotic stress tolerance. *Front. Plant Sci.* 6:774. doi: 10.3389/fpls.2015.00774

Loper, J. E., and Schroth, M. N. (1981). Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. *Phytopathology* 76, 386–389.

Nilsson, P., Olofsson, A., Fagerlind, M., Fagerstrom, T. E., Rice, S., Kjelleberg, S., et al. (2001). Kinetics of the AHL regulatory system in a model biofilm system: How many bacteria constitute a "quorum"? *J. Mol. Biol.* 309, 631–640. doi: 10.1006/jmbi.2001.4697

Otlewska, A., Migliore, M., Pien, D. S., Manfredini, A., and Pinzari, F. (2020). When salt meddles between plant, soil, and microorganisms. *Front. Plant Sci.* 11:553087. doi: 10.3389/fpls.2020.553087

Patten, C. L., and Glick, B. R. (2002). Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Appl. Environ. Microbiol.* 68, 3795–3801. doi: 10.1128/AEM.68.8.3795-3801.2002

Porcel, R., Aroca, R., Azcon, R., and Ruiz-Lozano, J. M. (2016). Regulation of cation transporter genes by the arbuscular mycorrhizal symbiosis in rice plants subjected to salinity suggests improved salt tolerancedue to reduced Na^+ root-to-shoot distribution. *Mycorrhiza* 26, 673–684. doi: 10.1007/s00572-016-0704-5

Qiu, Q. S., Guo, Y., Dietrich, M. A., Schumaker, K. S., and Zhu, K. (2002). Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. U. S. A.* 99, 8436–8441. doi: 10.1073/pnas.122224699

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Wei, H. X., Pare, P. W., et al. (2003). Bacterial volatiles promote growth in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4927–4932. doi: 10.1073/pnas.0730845100

Shabala, S., and Cuin, T. A. (2008). Potassium transport and plant salt tolerance. *Physiol. Plant.* 133, 651–607. doi: 10.1111/j.1399-3054.2007.01008.x

Sharifah, F. S., Lilia, C. C., Elvis, T. C., Fong, Y. C., Peter, M. M., and Eladl, G. E. (2019). Soil bacterial diffusible and volatile organic compounds inhibit Phytophthora capsica and promote plant growth. *Sci. Total Environ.* 69, 267–280. doi: 10.1016/j.scitotenv.2019.07.061

Sharifi, R., and Ryu, C. M. (2017). Chatting with a tiny belowground member of the holobiome: communication between plants and growth-promoting rhizobacteria. *Adv. Bot. Res.* 82, 135–160. doi: 10.1016/bs.abr.2016.09.002

Sharifi, R., and Ryu, C. M. (2018). Sniffing bacterial volatile compounds for healthier plants. *Curr. Opin. Plant Biol.* 44, 88–97. doi: 10.1016/j.pbi.2018.03.004

Sheikh, H. H., Kausar, H., and Saud, H. M. (2016). Plant growth-promoting rhizobacteria enhance salinity stress tolerance in okra through ROS-scavenging enzymes. *Biomed. Res. Int.* 9, 284–347. doi: 10.1155/2016/6284547

Sui, N., Li, M., Liu, X. Y., Wang, N., Fang, W., and Meng, Q. W. (2007). Response of xanthophyll cycle and chloroplastic antioxidant enzymes to chilling stress in tomato over-expressing glycerol-3-phosphate acyltransferase gene. *Photosynthetica* 45, 447–454. doi: 10.1007/s11099-007-0074-5

Sunjeet, K., Gaojie, L., Jing, J. Y., Xin, F. H., and Hou, H. (2021). Effect of salt stress on growth, physiological parameters, and ionic concentration of water dropwort (oenanthe javanica) cultivars. *Front. Plant Sci.* 12:660409. doi: 10.3389/fpls.2021.660409

Tahir, H., Qin, G., Wu, H., Waseem, R., Alwina, H., Wu, L., et al. (2017). Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* syst2. *Front. Microbiol.* 8:171. doi: 10.3389/fmicb.2017.00171

Talaat, N. B., and Shawky, B. T. (2011). Influence of arbuscular mycorrhizae on yield, nutrients, organic solutes, and antioxidant enzymes of two wheat cultivars under salt stress. *J. Planut Nutr. Soil Sci.* 174, 283–291. doi: 10.1002/jpln.201000051

Thi, H., and Thach, T.,Thuy, N.,Brett, W.,Penelope, W., Sean, B., and Sagadevan, M. (2016). Improvement of salinity stress tolerance in rice: challenges and opportunities. *Agronomy* 6:54. doi:10.3390/agronomy6040054

Timmusk, S., Nicander, B., Granhall, U., and Tillberg, E. (1999). Cytokinin production by Paenibacillus polymyxa. *Soil Biol. Biochem.* 31, 1847–1852. doi: 10.1016/S0038-0717(99)00113-3

Velázquez-Becerra, C., Macías-Rodríguez, L. I., López-Bucio, J., Altamirano-Hernández, J., Flores-Cortez, I., and Valencia-Cantero, E. (2011). A volatile organic compound analysis from arthrobacter agilis identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and medicago sativa morphogenesis in vitro. *Plant Soil* 18, 329–340. doi: 10.1007/s11104-010-0583-z

Wang, Y., Lv, J., Dan, C., Zhang, J., Qi, K., Rui, C., et al. (2017). Genome-wide identification, evolution, and expression analysis of the kt/hak/kup family in pear. *Genome* 61, 755–765. doi: 10.1139/gen-2017-0254

Yang, T. W. (2013). *Efficient Synthesis and Fermentation Control of 2,3-Butanediol by Bacillus Amyloliquefaciens.* Jiangnan University.

Yuan, R. W., Liu, L., Zhang, R., and Fan, S. Y. (2020). Progress in the mechanism of interactions between plant inter-root secretions and soil microorganisms. *Chin. Agro. Bull.* 36, 26–35.

Zhang, R. G., Fan, H. M., Guo, J. Y., Zhang, S. M., Xu, M. G., and She, D. (2018). Spatial distribution and influencing factors of soil salinization in agricultural fields in Weibei, Shaanxi. *Northwest J. Agri.* 27, 440–450.

Zhang, H. M., Kim, M. S., Krishnamachari, V. K., Payton, P. T., Sun, Y., Grimson, M., et al. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. *Planta* 226, 839–851. doi: 10.1007/s00425-007-0530-2

Zhang, H., Kim, M. S., Yan, S., Dowd, S. E., and Paré, P. W. (2008a). Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. *Mol. Plant-Microbe Interact.* 21, 737–744. doi: 10.1094/MPMI-21-6-0737

Zhang, H., Murzello, C., Sun, Y., Kim, M. S., and Paré, P. W. (2010). Choline and osmotic-stress tolerance induced in *Arabidopsis* by the soil microbe *Bacillus subtilis* (GB03). *Mol. Plant-Microbe Interact.* 23, 1097–1104. doi: 10.1094/MPMI-23-8-1097

Zhang, H., Sun, Y., Xie, X., Kim, M. S., Dowd, S. E., and Paul, W. (2009). A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. *Plant J.* 58, 568–577. doi: 10.1111/j.1365-313X.2009.03803.x

Zhang, H., Xie, X., Kim, M. S., Kornyeyev, D. A., and Paré, P. W. (2008b). Soil bacteria augment Arabidopsis photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. *Plant J.* 56, 264–273. doi: 10.1111/j.1365-313X.2008.03593.x

Zhu, M., Shabala, L., Cuin, T. A., Huang, X., Zhou, M., Munns, R., et al. (2016). Nax loci affect SOSi-like Na^+/H^+ exchanger expression and activity in wheat. *J. Exp. Bot.* 67, 835–844. doi: 10.1093/jxb/erv493

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 05 November 2021
doi: 10.3389/fpls.2021.756368

Microbial Consortia for Effective Biocontrol of Root and Foliar Diseases in Tomato

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The use of beneficial microorganisms for the biological control of plant diseases and pests has emerged as a viable alternative to chemical pesticides in agriculture. Traditionally, microbe-based biocontrol strategies for crop protection relied on the application of single microorganisms. However, the design of microbial consortia for improving the reliability of current biological control practices is now a major trend in biotechnology, and it is already being exploited commercially in the context of sustainable agriculture. In the present study, exploiting the microbial library of the biocontrol company Koppert Biological Systems, we designed microbial consortia composed of carefully selected, well-characterized beneficial bacteria and fungi displaying diverse biocontrol modes of action. We compared their ability to control shoot and root pathogens when applied separately or in combination as microbial consortia, and across different application strategies that imply direct microbial antagonism or induced systemic plant resistance. We hypothesized that consortia will be more versatile than the single strains, displaying an extended functionality, as they will be able to control a wider range of plant diseases through diverse mechanisms and application methods. Our results confirmed our hypothesis, revealing that while different individual microorganisms were the most effective in controlling the root pathogen *Fusarium oxysporum* or the foliar pathogen *Botrytis cinerea* in tomato, the consortia showed an extended functionality, effectively controlling both pathogens under any of the application schemes, always reaching the same protection levels as the best performing single strains. Our findings illustrate the potential of microbial consortia, composed of carefully selected and compatible beneficial microorganisms, including bacteria and fungi, for the development of stable and versatile biological control products for plant protection against a wider range of diseases.

Keywords: arbuscular mycorrhiza, biocontrol, disease suppression, microbial consortia, SynCom, *Trichoderma*, plant-growth promoting rhizobacteria, induced systemic resistance

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *10 August 2021*
Accepted: *04 October 2021*
Published: *05 November 2021*

Citation:
Minchev Z, Kostenko O, Soler R and Pozo MJ (2021) Microbial Consortia for Effective Biocontrol of Root and Foliar Diseases in Tomato. Front. Plant Sci. 12:756368. doi: 10.3389/fpls.2021.756368

INTRODUCTION

A plethora of soil-borne microorganisms live associated with plant roots, and although some are detrimental, others provide important benefits to the host plant, from improved nutrition through growth and protection against multiple abiotic and biotic stresses (Bakker et al., 2018). Nowadays soil microbes are considered key players in modern crop management programs aiming to increase sustainability in agriculture (Barea, 2015; Trivedi et al., 2017; Compant et al., 2019). The use of plant beneficial microorganisms as biological control agents (BCAs) of pests and diseases emerges as a viable alternative to the abusive use of agrochemicals (Ab Rahman et al., 2018; Rändler-Kleine et al., 2020). A strong increase in registered microbial biocontrol agents worldwide in recent years serves as good evidence (van Lenteren et al., 2017). Yet, while the use of insects and mites to control pests is well established and used in practice for decades, microbes to control pests and diseases are in an earlier developmental phase (Mitter et al., 2019).

The ability of microorganisms to control pests and diseases has been well documented, but the variability of results often recorded under field conditions is one of the major challenges for wider adoption in agriculture (Trivedi et al., 2017; Mitter et al., 2019). Originally, biocontrol research focused on the application of single microorganisms (Sarma et al., 2015; Trivedi et al., 2020). The inoculant's functionality and persistence are strongly influenced by their complex interactions within the soil microbiota and the environment (Barea et al., 2005; Trivedi et al., 2020; Pozo et al., 2021). Inconsistent or ineffective performance of single strain inoculants can be related to limited competitiveness against indigenous microbes and the varying environmental conditions (Trivedi et al., 2020). It has been proposed that a way to overcome these issues is by combining different strains to cover a wider range of target organisms and conditions (Faust, 2019; Mitter et al., 2019). Yet, successful examples of better performance for microbial consortia are comparatively limited and usually relate to growth or yield promotion (Bradáčová et al., 2019).

Plant microbiome engineering and the design of synthetic microbial communities (SynComs) to improve crop productivity and resilience is a major research topic in this decade (Arif et al., 2020; Liu et al., 2020; Trivedi et al., 2020). SynComs may improve the stability of biocontrol practices as microbial consortia are expected to deal better than single-strain inoculants with the large diversity of environmental challenges encountered in practice (Sarma et al., 2015; Arif et al., 2020; Pozo et al., 2021). Besides acquiring this plasticity, the consortium can combine diverse modes of action, likely providing better pest or disease control than single microorganisms with their specific abilities (Sarma et al., 2015). Yet, most SynComs studies focus exclusively on bacteria, whereas fungi are major biocontrol agents (Pozo et al., 2021). Including fungi in the consortia would likely expand the range of functions and potential colonization niches of these mixed inoculants (Srivastava et al., 2010; Pozo et al., 2021). Thus, combining both bacteria and fungi in SynComs design is expected to result in a multifunctional and more resilient product for biocontrol; this is the basis of this study.

Diving deeper mechanistically, two main groups of biocontrol mechanisms are described: (i) those with direct effects on the attacker and (ii) those with indirect, usually plant-mediated effects. Direct effects are mostly based on microbial antagonism through antibiosis, competition for nutrients or colonization niches, and/or parasitism (Whipps, 2001). Indirect mechanisms reducing pathogen proliferation, aggressiveness, or damage commonly involve plant-mediated effects. Beneficial microorganisms can improve the plant nutritional status, leading to damage compensation and tolerance, and stimulate the plant immune system, priming plant defenses and leading to induced systemic resistance (ISR) to diverse aggressors (Pieterse et al., 2014; Barea, 2015; Pineda et al., 2015; Gruden et al., 2020; De Kesel et al., 2021). Among rhizospheric microorganisms, plant growth-promoting rhizobacteria (PGPR), *Trichoderma* spp., and arbuscular mycorrhizal fungi (AMF) have been shown to effectively protect plants against diverse pests and diseases through different mechanisms (Pozo and Azcón-Aguilar, 2007; Barea et al., 2013; Pieterse et al., 2014; Barea, 2015; Pineda et al., 2015).

Plant growth-promoting rhizobacteria have been shown to control plant pathogens through antibiosis, reduction of pathogen virulence, competition for iron, plant growth promotion, and ISR (Lugtenberg and Kamilova, 2009; Barea, 2015). Most reported PGPR antagonists are from the genera *Bacillus* and *Pseudomonas* (Haas and Défago, 2005; Santoyo et al., 2012).

Regarding fungi, *Trichoderma* spp. is the most widely used BCA in agriculture, and many *Trichoderma*-based products are available in the market (Woo et al., 2014). These fungi are extremely efficient not only for the control of fungal pathogens mainly through direct antagonism but also stimulating plant defenses (Harman et al., 2004; Martínez-Medina et al., 2014; Woo et al., 2014). Finally, AMF is commercialized as biostimulants in agriculture. These obligate biotrophs improve plant nutrient uptake and tolerance/resistance to multiple stresses, being able to protect the host plant against diverse pathogens and pests (Jung et al., 2012; Sanmartín et al., 2020; Rivero et al., 2021). AMF does not produce antibiotics, but compete with the pathogens for nutrients and colonization sites and boosts the defensive capacity of plants, leading to ISR (Pozo and Azcón-Aguilar, 2007; Jung et al., 2012).

In this study, we test the hypothesis that microbial consortia are more versatile than individual microbial inoculants, displaying an extended functionality in the biocontrol of a wider range of plant diseases and application methods through the combination of diverse modes of action. For that, we designed different SynComs by carefully selecting diverse and well-characterized microbial biocontrol agents, including *Bacillus* spp., *Pseudomonas* spp., *Trichoderma* spp., and the AMF *Rhizophagus irregularis* and compared the ability to control root and shoot pathogens when applied individually or in combinations as SynComs. Using different inoculation methods and two agronomically relevant pathosystems (tomato plants challenged with *Fusarium oxysporum* or *Botrytis cinerea* as root and shoot pathogens, respectively), we demonstrate the advantages of targeting microbial consortia as versatile products for efficient biocontrol of diverse plant diseases.

MATERIALS AND METHODS

Microbial Consortia Design

A careful selection of beneficial microorganisms to create synthetic microbial consortia was performed focusing on the main groups of rhizospheric beneficial microorganisms such as PGPR, mycoparasitic fungi, and AMF. An extensive literature review on biocontrol studies of known BCAs was performed, taking also into account as potential candidates the microbial strains available at Koppert Biological Systems. The most relevant studies considered are summarized in **Supplementary Table 1**.

As a result, we chose two *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237, formerly known as *Bacillus subtilis* UMAF6614 and UMAF6639, respectively (Magno-Perez-Bryan et al., 2015), and *Pseudomonas chlororaphis* MA 342 and *Pseudomonas azotoformans* F30A (Abuamsha et al., 2011a; Levenfors et al., 2014). From fungi, we selected *Trichoderma harzianum* strains T22 and ESALQ1306 (Geraldine et al., 2013; Coppola et al., 2019), and for the ISR bioassay, we included additionally the AMF *R. irregularis* MUCL 57021.

Microbe Growing Conditions and Inoculum Preparation

Bacillus amyloliquefaciens strains were grown on tryptone soya agar (TSA, Oxoid, Basingstoke, United Kingdom) for 24 h at 28°C. After that, a single colony from TSA culture was inoculated in 25 ml of Difco sporulation medium (DSM; Nicholson and Setlow, 1990) and incubated for 48 h at 28°C in a rotatory shaker (200 rpm). Spores were quantified using a Bürker-Türk counting chamber, then centrifuged at 5,000 rpm for 15 min, and after discarding the supernatant, the pellet containing the spores was resuspended in sterile tap water to a final concentration of 1×10^7 spores/ml.

Pseudomonas azotoformans and *P. chlororaphis* were grown on TSA for 24 h at 28°C. Liquid pre-culture was prepared using tryptone soya broth (TSB, Oxoid, Basingstoke, United Kingdom) inoculated with a single bacterial colony from TSA culture and incubated overnight at 28°C with rotary shaking at 200 rpm. After that, 1 ml of pre-culture was inoculated in 25 ml of TSB medium and placed in a rotatory shaker (200 rpm) at 28°C. After 150 min of incubation, with bacterial growth in exponential phase, the cell concentration was calculated measuring the O.D. (620 nm) of the bacterial culture on Shimadzu UVmini-1240 Spectrophotometer. The bacterial culture was centrifuged at 5,000 rpm for 15 min, and after discarding the supernatant, the pellet containing the bacterial cells was resuspended in sterile tap water to a final concentration of 1×10^7 colony forming unit (cfu)/ml.

Trichoderma harzianum strains were cultured on potato dextrose agar (PDA, Difco, Le Pont de Claix, France) for 7 days at room temperature. Spores were collected from sporulating plates in sterile tap water, and the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1×10^7 spores/ml.

Rhizophagus irregularis was grown in a monoxenic culture on a minimal (M) medium and using *Agrobacterium rhizogenes*-transformed carrot (*Daucus carota*) roots as a host root (St-Arnaud et al., 1996). To extract the AMF spores, citrate buffer 0.01 M (pH = 6) was added to a sporulating AMF culture in a proportion of 3:1 (v/v) and placed in a rotary shaker for 1 h to dissolve the agar. AMF spores were recovered from the solution using sieves of different sizes (250 and 53 μm) and resuspended in sterile tap water at final concentrations of 1,000 spores/ml.

Pathogenic Fungi, Growing Conditions, and Inoculum Preparation

Two major fungal pathogens causing important crop losses worldwide were tested: *F. oxysporum* f.sp. radicis-lycopersici as soil pathogen and the necrotrophic shoot pathogen *B. cinerea* strain B05.10.

Fusarium oxysporum was grown on PDA at 25°C for 4 days. For spore production, 25 plugs of 4 mm diameter with new growing mycelia were removed from the PDA plates and transferred to 500 ml Erlenmeyer containing 200 ml of Czapek Dox Broth (Oxoid, Basingstoke, United Kingdom) and placed in a rotary shaker (110 rpm) at room temperature. After 4 days of incubation, the liquid culture was filtered using a sterile miracloth filter, and the spore concentration was quantified using a Bürker-Türk counting chamber. The resulting spore suspension was centrifuged at 9,500 rpm for 15 min and after discarding the supernatant, the pellet containing the spores was resuspended in sterile tap water to a final concentration of 1×10^8 spores/ml.

Botrytis cinerea was cultured on PDA at 20°C. Spores were collected from sporulating 14 days old plates in potato dextrose broth (PDB, Difco, Le Pont de Claix, France), and the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1×10^6 spores/ml.

In vitro Antagonism Assay

The antagonistic activity of the individual strains *Bacillus amyloliquefaciens* CECT 8238 and CECT 8237, *P. azotoformans, P. chlororaphis*, and *T. harzianum* T22 and ESALQ1306 were initially evaluated *in vitro*, in confrontation assays against *F. oxysporum* and *B. cinerea*. For *Trichoderma*, one PDA plug (4 mm) of *Trichoderma* culture and one of the pathogen cultures were placed on PDA plates with 4 cm of distance from each other. For *Bacillus* and *Pseudomonas*, 10 μl drop of TSB liquid culture grown overnight was used instead of PDA plugs. As a control, a plug of the pathogen culture was placed in the Petri dish without any antagonist. All treatments were replicated three times. All plates were incubated at 25°C for 7 days. The radius of the pathogen colony in the confrontation plates was measured and compared to the radius of the pathogen colony in the control plates.

In planta Bioassays

Biocontrol potential was tested *in planta* through several bioassays including diverse inoculation methods and targeting different pathogens. This strategy allows testing *in vivo* different modes of action ranging from direct antagonism to indirect plant-mediated effects. Thus, we tested through seed inoculation suppression of the root pathogen *F. oxysporum* and ISR against the foliar pathogen *B. cinerea*, and suppression of *B. cinerea* by foliar spray application.

Microbial Treatments

In all bioassays, individual microorganisms and different synthetic consortia were tested (**Supplementary Table 2**). All microorganisms tested individually were applied at 1 × 10^7 cfu or spores/plant in the seed application and at 1 × 10^7 cfu or spores/ml in the foliar application. For the AMF treatments, a suspension of 1,000 spores of *R. irregularis* was applied per plant. Regarding the consortia, the first microbial consortium, SynCom1, was composed of one strain from each genus (*Bacillus amyloliquefaciens* CECT 8238, *P. azotoformans* F30A, *and T. harzianum* T22). The second one, SynCom2, was composed of all selected microorganisms (*Bacillus amyloliquefaciens* CECT 8238 and CECT 8237, *P. azotoformans* F30A, *P. chlororaphis* MA 342, and *T. harzianum* T22 and ESALQ1306). Both consortia were tested at two doses: A—the same amount of each microorganism in both consortia (1 × 10^7 cfu each, that is a total of 3 × 10^7 cfu per seed or ml for SynCom1, 6 × 10^7 cfu per seed or ml for SynCom2) or B—same total cfu per consortia (3.33 × 10^6 cfu per microorganism in SynCom1 or 1.67 × 10^6 cfu in SynCom2, for a total of 1 × 10^7 cfu per seed or ml in both).

Substrate, Seed Surface Sterilization, and Plant Growing Conditions

Solanum lycopersicum cv. Money maker seeds (Vreeken's Zaden, Dordrecht, Netherlands) were surface-sterilized by immersion in 5% sodium hypochlorite solution for 1 min followed by at least three washing steps in sterile water for 10 min each. The surface sterilized seeds were dried in a laminar flow cabinet and used for the experiments. The growing substrate was gamma-irradiated nutrient poor peat soil (BVB, Netherlands). All experiments were performed in a growing chamber at Koppert B.V. (Berkel en Rodenrijs, Netherlands) under controlled conditions (25°C:23°C day:night with photoperiod 16 h:8 h light:dark and 60% of relative humidity).

Bioassay: Suppression of *Fusarium oxysporum in planta*

Rectangular plastic containers of 18 cm × 13 cm × 6 cm (length × width × height) were filled with 300 g of soil previously moistened with tap water (300 ml/1,000 g of soil) and infected with 1 × 10^6 conidia/g of soil *F. oxysporum* f.sp. radicis-lycopersici conidia. The *F. oxysporum* conidia were carefully mixed through the soil by hand. Then, 12 seeds were sown in each container in a regular grid and inoculated with the microbial treatments (**Supplementary Table 2**) by pipetting the microbial suspension to each seed. Finally, the seeds were covered with sterile vermiculite to avoid desiccation and undesired contaminations. We included two control treatments: a "non-diseased control" using the same soil and conditions but without the addition of *F. oxysporum* and microbial treatments, and a "disease control" using the same pathogen-infected soil but without beneficial microbes. Each treatment was replicated five times. We used a randomized complete block design. Each treatment was randomly assigned to each block. Plant survival was evaluated 15 days after sowing by counting the number of healthy tomato plantlets in each container.

Bioassay: Suppression of *Botrytis cinerea in planta*

Tomato seeds were sown in pots filled with 250 ml of soil (one seed per pot). Plants were grown for 7 weeks and watered two times per week with water and once per week with Long Ashton nutrient solution (Hewitt, 1966). The individual and the consortia treatments described above (**Supplementary Table 2**) were applied to one fully developed leaf by spraying its surface until runoff. The disease control treatment was treated similarly, applying the same amount of sterile water but lacking any BCA microbial propagules. Each treatment was replicated six times. Treated leaves were detached after the application, using a scalpel, and used for the bioassay. Each leaflet of the detached leaves was inoculated with one 4 μl drop of *B. cinerea* conidia suspension (1 × 10^6 conidia/ml). The leaves were placed in six sealed boxes with high humidity at 20°C, locating one replicate from each treatment in each box. About 60 h after infection, the diameter of the resulting necrotic lesions was measured using a digital caliper.

Bioassay: Induced Systemic Resistance Against *Botrytis cinerea*

Tomato seeds were sown in pots containing 250 ml of soil (one seed per pot) and the microbial treatments (**Supplementary Table 2**) were applied by pipetting the microbial suspension to the seeds. In this experiment, the AMF *R. irregularis* was also included, both, individually and in the consortia. A disease control treatment was included where the seeds only received water without any BCA microbial addition. Each treatment was replicated 12 times. We used a randomized complete block design. Plants were watered two times per week with water and once per week with Long Ashton nutrient solution (Hewitt, 1966) but with reduced phosphorous concentration (50% of the standard concentration) to ensure mycorrhizal establishment. After 5 weeks, one fully developed leaf from each plant was detached using a scalpel, and each leaflet was inoculated with one 4 μl drop of *B. cinerea* conidia suspension (1 × 10^6 conidia/ml). The leaves were placed in 12 sealed boxes with high humidity at 20°C and locating one replicate from each treatment in each box. About 48 h after infection, the diameter of the necrotic lesions was measured using a digital caliper.

Bioassay: Strains-Compatibility

Rectangular plastic containers of 18 cm × 13 cm × 6 cm (length × width × height) were filled with 300 g of soil previously moistened with tap water (300 ml/1,000 g of soil). Then, 12 surface-sterilized tomato seeds were sown in each container in a regular grid. The seeds were inoculated with the different microbial treatments (**Supplementary Table 2**) by pipetting the microbial suspension to each seed. Each microbial strain (except *R. irregularis*) was initially inoculated at 1 × 10^7 cfu/plant, resulting in a total concentration of 4 × 10^5 cfu/g of soil for each strain (12 plants/300 g of soil). Finally, the seeds were covered with sterile vermiculite to avoid desiccation and undesired contaminations. We included a control treatment without any microbial inoculation. Each treatment was replicated five times. We used a randomized complete block design.

Microbial colonization was evaluated 15 days after sowing using methods described in the next section.

Quantification of Microbes and Root Mycorrhizal Colonization

For the different bacteria and *Trichoderma*, we estimated for each genus the number of colony forming units (cfu) per gram of rhizospheric soil. For this, 1 g of rhizospheric soil was sampled, diluted in 9 ml of sterile tap water, and homogenized in a horizontal shaker at 350 rpm for 1 h. Serial dilutions were plated on PDA + igepal (11 ml/L) + tetracycline (50 μg/ml) when targeting *Trichoderma* and on TSA + natamycin (0.1 g/L) when targeting bacteria. The plates were then incubated at 25°C and cfu were counted after 24 h for bacteria and after 48 h for *Trichoderma*. In consortia treatments, *Bacillus* spp., *Pseudomonas* spp., and *Trichoderma* spp. were distinguished morphologically, as they are well-characterized strains in the Koppert collection (**Supplementary Figures 1A,B**). Microbial identity was confirmed in representative colonies from each type by PCR using specific primers for *Trichoderma, Bacillus,* or *Pseudomonas* spp. For treatments including AMF, mycorrhizal colonization was estimated by ink staining fungal structures within the roots. For that, roots were washed and sampled upon harvesting and cleared in 10% KOH, and the AMF structures were stained with 5% ink in 2% acetic acid (Vierheilig et al., 2005). The percentage of root length colonized by the AMF was quantified using the gridline intersection method (Giovannetti and Mosse, 1980) under a light microscope.

Statistical Analysis

Data were analyzed using R statistical language, version 4.0.5 (R Core Team, 2021), and figures were produced using the package ggplot2 (Wickham, 2009). The effect of microbial treatments (single strains and synthetic communities) on the necrotic lesions caused by *B. cinerea*, microbial colonization after single and combined inoculations, and the effect of single strains on *B. cinerea* and *F. oxysporum* radial growth was assessed using a general linear model with blocks as an error term and microbial treatments as a fixed effect. To examine whether microbial treatments influenced the probability of the tomato seedlings to survive to the soil pathogenic fungus *F. oxysporum*, a generalized linear model with binomial distribution and logit link function and blocks as an error term was performed. *Post hoc* comparisons among microbial treatments were based on the Tukey honestly significant difference (HSD). Model validation was performed graphically by inspecting the residuals and fitted values (Zuur and Ieno, 2016).

RESULTS

The First Step: Consortia Design

Upon a thorough literature review, we selected bacterial and fungal groups/genera with well-documented potential to control plant pathogens, trying to compile diverse mechanisms including antibiosis, competition for iron and other nutrients, and colonization sites, mycoparasitism, and induction of plant resistance. Strains from the selected groups available at the Koppert microbial collection were: *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237, *P. chlororaphis* MA 342, and *P. azotoformans* F30A, *T. harzianum* strains T22 and ESALQ1306, and the AMF *R. irregularis* MUCL 57021 (**Supplementary Table 1**). Two synthetic communities were designed, one combining one strain for each genera (SynCom1), and another in which all selected microbes were included (SynCom2).

Exploring *in vitro* Antagonistic Activity Against Soil and Leaf Pathogens

As a first screening to move into the biocontrol potential of the selected individual strains, their antagonistic activity was tested in an *in vitro* dual confrontation assay. All selected BCA strains decreased *F. oxysporum* radial growth compared to the control plates ($p < 0.05$; **Supplementary Figure 2A**). Both *T. harzianum* strains showed the strongest antifungal activity, with about 80% reduction of the pathogen radial growth ($p < 0.05$; **Supplementary Figure 2A**). Similarly, all individual strains reduced *B. cinerea* radial growth compared to the control, and *T. harzianum* T22 was the most effective strain with a 90% reduction of pathogen growth ($p < 0.05$; **Supplementary Figure 2B**).

Assessing the Potential to Directly Suppress Soil Diseases *in planta*

The research was scaled up using a tomato-*Fusarium*-soil system, comparing the biocontrol activity of the individual microbial strains and the differently designed consortia (SynCom1, SynCom2). The pathogen fully compromised plant survival, as no plants survived in the disease control, while almost 100% survival was found in the absence of the pathogen (non-diseased control) (**Figure 1A**). None of the individual bacterial strains significantly increased plant survival compared to the disease control. In contrast, both *T. harzianum* strains and all of the SynComs were able to efficiently suppress *F. oxysporum*, increasing plant survival above 80% ($p < 0.05$, **Figure 1A**). In fact, plant survival in the *T. harzianum* and consortia treatments reached the levels of the non-diseased control ($p < 0.05$, **Figure 1A**). These results not only show the potential of *T. harzianum* but also indirectly the compatibility/tolerance of the other isolates as this high protection level was maintained in the consortia treatments (**Figures 1A,B**).

Assessing the Potential to Directly Suppress Foliar Diseases *in planta*

The antagonistic potential of single strains and consortia against the foliar pathogen *B. cinerea* was also tested *in planta*, applying the BCA treatments by spraying the leaves before *B. cinerea* infection. Among single microbial treatments, *P. chlororaphis*, *P. azotoformans*, and *T. harzianum* T22 were able to reduce the area of the necrotic lesion caused by *B. cinerea* by 56, 45, and 38%, respectively, compared to the control treatment ($p < 0.05$, **Figure 2A**). Remarkably, all the microbial consortia treatments

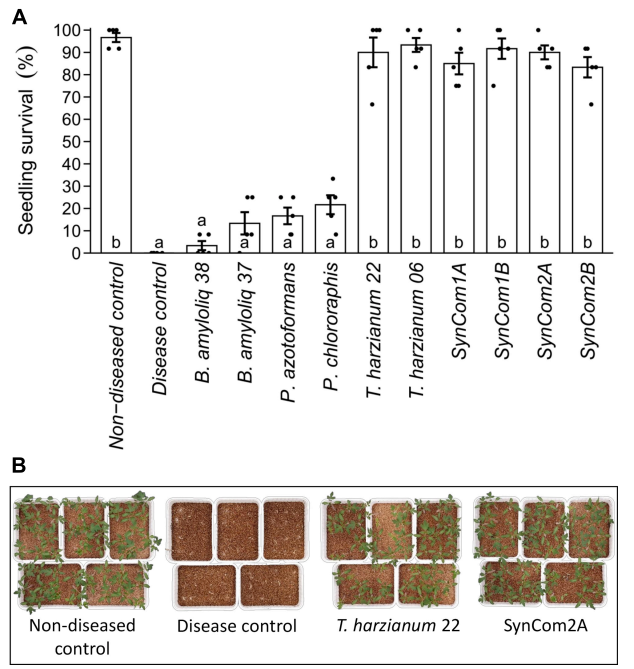

FIGURE 1 | Effect of microbial inoculation on disease caused by the soil-borne pathogen *Fusarium oxysporum.* **(A)** Survival of tomato plants after 15 days of growth in *F. oxysporum*-infected soil. Seeds were either water-inoculated ("disease control") or inoculated with the individual or consortia treatments (see **Supplementary Table 2**). A "non-diseased control" was also included, where water-inoculated seeds were sown in soil without *F. oxysporum*. Single strains were inoculated at 1 × 10^7 cfu/plant and the consortia were inoculated at the same concentration for each microorganism (SynCom1A, SynCom2A) or at 1 × 10^7 cfu/plant total microbial concentration (SynCom1B, SynCom2B). Bars represent predicted mean ± SE of the probability of seedling survival based on a generalized linear model with binomial distribution and logit link function. Black dots represent raw data points. Treatments not sharing a letter in common are significantly different based on the Tukey honestly significant difference (HSD) test ($p < 0.05$, $n = 5$). **(B)** Survival of plant seedlings in *F. oxysporum*-infected soil. Pictures illustrate plant survival in non-diseased and disease control, *Trichoderma harzianum,* and SynCom2A treatments.

reduced *B. cinerea* lesion area by about 50% as compared to the disease control, reaching up to a 70% reduction in SynCom2B ($p < 0.05$, **Figure 2A**). The higher antagonistic effect against *B. cinerea* was therefore achieved by *P. chlororaphis* (56%) and the SynCom2B (**Supplementary Figure 3**).

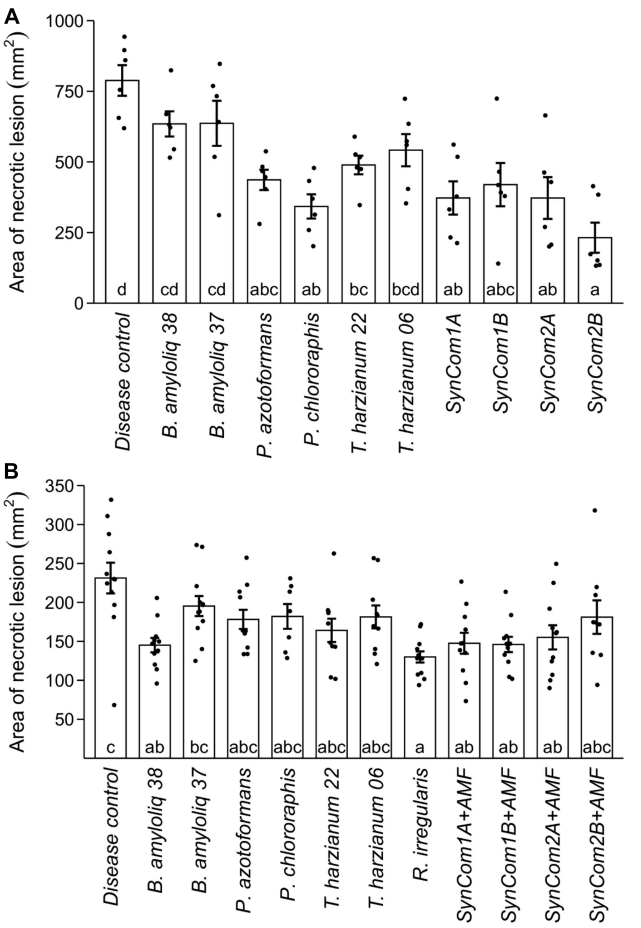

FIGURE 2 | Effect of microbial inoculation on disease caused by the foliar pathogen *Botrytis cinerea.* **(A)** Area of necrotic lesions in plants pre-treated by foliar spray with the different treatments (see **Supplementary Table 2**). Water-treated plants (no BCA treatment) were included as disease control. **(B)** Area of necrotic lesions in plants inoculated at sowing either with water (disease control) or with the different microbial treatments (see **Supplementary Table 2**) to determine ISR. Single strains were inoculated at 1 × 10^7 cfu/ml in **(A)** and cfu/plant in **(B)**, and the consortia were inoculated at the same concentration for each microorganism (SynCom1A, SynCom2A) or at 1 × 10^7 cfu/ml in **(A)** and cfu/plant in **(B)** as total microbial concentration (SynCom1B, SynCom2B). +AMF indicates consortia co-inoculated with 1,000 spores/plant of *Rhizophagus irregularis.* Bars represent means ± SE and black dots represent raw data. Treatments not sharing a letter in common are significantly different based on the general linear model and Tukey honestly significant difference (HSD) test [$p < 0.05$, $n = 6$ in **(A)**, $n = 12$ in **(B)**].

Moving Into Plant-Mediated Control: Inducing Systemic Resistance

In addition to the direct antagonistic effect of the foliar application against *B. cinerea*, we evaluated the capacity of the microbial treatments to activate plant systemic resistance. We tested the potential plant-mediated effects by avoiding direct contact between the BCAs and the pathogen. In this experiment, the AMF *R. irregularis* was included both individually and in the consortia due to the reported capacity of AMF to induce ISR and their current interest as inoculants in agriculture. Among the individual treatments, only *Bacillus amyloliquefaciens* CECT 8238 and *R. irregularis* were able to induce ISR against *B. cinerea*, reducing the area of the necrotic lesions by 38 and 44%, respectively, as compared to the control treatment ($p < 0.05$, **Figure 2B**). The consortia also achieved significant plant-mediated protection against *B. cinerea,* with SynCom1A, SynCom1B, and SynCom2A reducing lesions by 33–37% as compared to the control ($p < 0.05$, **Figure 2B**). Again, a similar reduction in disease symptoms was achieved by the consortia and the best performing individual treatments in this pathosystem.

TABLE 1 | Effects of the microbial treatments tested in the different *in planta* bioassays.

Microbial treatment	**Suppression *Fusarium oxysporum***	**Suppression *Botrytis cinerea***	**ISR against *Botrytis cinerea***
Bacillus amyloliquefaciens CECT 8238	o	o	+
Bacillus amyloliquefaciens CECT 8237	o	o	o
Pseudomonas azotoformans	o	+	o
Pseudomonas chlororaphis	o	+	o
Trichoderma harzianum T22	+	+	o
Trichoderma harzianum ESALQ1306	+	o	o
Rhizophagus irregularis	nt	nt	+
SynCom1A	+	+	+
SynCom1B	+	+	+
SynCom2A	+	+	+
SynCom2B	+	+	o

"+" and "o" indicates statistically different effect from the control treatment and no effect, respectively, based on the Tukey honestly significant difference (HSD). "nt" indicates that the microbial treatment was not tested.

Taking into account all the bioassays performed, SynComs was more versatile than the individual strains, showing effective biocontrol across the different pathosystems and inoculation methods, as summarized in **Table 1**.

Microbial Compatibility

The repeated success of the consortia across all the *in planta* bioassays supports the strains compatibility. In fact, the SynComs performance in biocontrol was not significantly different from that achieved by the best performing BCA strain in any of the experiments. We further investigated the compatibility of the components in a new experiment aiming to compare the colonization of each microorganism in the single or SynCom treatments after interacting in the tomato rhizosphere for 15 days. The absence in the soil of indigenous species from any of the inoculated genera (*Bacillus*, *Pseudomonas*, and *Trichoderma*) was confirmed in the control treatment plates (**Supplementary Figure 1A**). Each microbial strain (except *R. irregularis*) was initially inoculated at a total concentration of 4 × 10^5 cfu/g of soil for each strain (both in the individual microbial treatments and in consortia).

Bacillus spp. abundance at the end of the experiment was similar to that initially inoculated both in single strain and SynCom1 treatments (**Figure 3A**). In SynCom2 treatments, both *Bacillus* strains were co-inoculated, and the abundance of *Bacillus* spp. in the soil was even higher, around 6 × 10^5 cfu/g of soil (**Figure 3A**). These results confirm the successful establishment of both *Bacillus amyloliquefaciens* strains, both when inoculated individually and in consortia.

In the single strain treatments, *Pseudomonas* spp. abundance increased compared to the initial inoculation (up to 1.5 × 10^6 and 1.2 × 10^6 cfu/g of soil in *P. azotoformans* and *P. chlororaphis*, respectively) (**Figure 3B**), evidencing the good colonization ability of *Pseudomonas* spp. Remarkably, *Pseudomonas* spp. abundance in soil increased more than four times in SynCom1 (containing *P. azotoformans*) compared to the individual *P. azotoformans* treatment (around 6.5 × 10^6 cfu/g of soil) (**Figure 3B**). Regarding SynCom2 treatments in the absence of AMF *Pseudomonas* spp., abundance was 2.7 × 10^6 cfu/g of soil, corresponding to the sum of both inoculated *Pseudomonas* species in SynCom2, whereas in SynCom2 + AMF, their abundance was more than double (6.9 × 10^6 cfu/g of soil), pointing to a potential positive effect of AMF presence in this consortium (**Figure 3B**).

Trichoderma spp. abundance in the individual treatments was 9.3 × 10^5 and 6.3 × 10^5 cfu/g of soil in *T. harzianum* T22 and ESALQ1306, respectively, which in the case of T22 is more than double of the concentration inoculated (**Figure 3C**). Regarding the consortia, *Trichoderma* spp. abundance was in a similar range than the individual inoculations: 1 × 10^6 cfu/g of soil in SynCom1 (where only T22 was present) and around 1.5 × 10^6 cfu/g of soil in SynCom2 treatments equivalent to the sum of both *Trichoderma* strains co-inoculated in this consortium (**Figure 3C**).

Finally, the percentage of root length colonized by *R. irregularis* was 1.2% when applied individually (**Figure 3D**). Root colonization was similar in both consortia treatments, (SynCom1 + AMF and SynCom2 + AMF) (**Figure 3D**), confirming that mycorrhizal colonization was not significantly affected when inoculated in consortia. The low percentages are common in the early stages of the colonization (only 2 weeks upon AMF inoculation). To compare the treatments in more advanced stages of the mycorrhizal symbiosis, mycorrhizal colonization was quantified in the roots of the ISR bioassay, corresponding to plants growing with the AMF for 5 weeks. Mycorrhizal colonization reached 40% in the individual treatment, and these levels remained unaltered in both SynCom1 and SynCom2 treatments at any of the tested doses (**Supplementary Figure 4**).

DISCUSSION

In the present study, by combining well-characterized and compatible microorganisms, including bacteria and fungi, we demonstrated the potential of microbial consortia to effectively control fungal pathogens with different lifestyles through direct and plant-mediated disease suppression and using different application methods. Our findings pinpoint the design of synthetic microbial consortia for biocontrol of plant pathogens as a potential strategy to extend the functionality and versatility of microbial biological control.

A Dilemma to Face

Across the different experiments, different individual microorganisms were the most effective in the different scenarios, depending on the type of pathogen or the strategy used for its control. Remarkably, the consortia effectively controlled all pathogens in all different bioassays, both through direct antagonism by seed or foliar application, or inducing plant systemic resistance against foliar pathogens by seed inoculation (results summarized in **Figure 4**). The bioprotection achieved by the consortia was always similar to that of the best performing

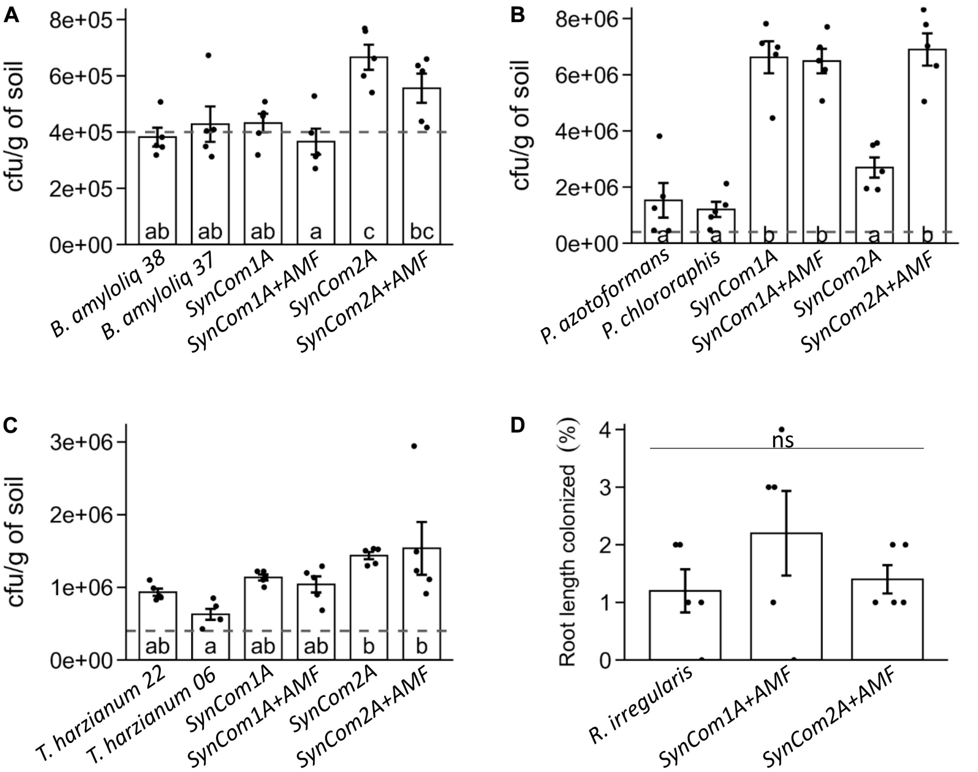

FIGURE 3 | Rhizospheric soil colonization by **(A)** *Bacillus* spp., **(B)** *Pseudomonas* spp., and **(C)** *Trichoderma* spp., expressed as cfu/g of soil, and **(D)** mycorrhizal colonization by *Rhizophagus irregularis* represented as the percentage of root length colonized by the fungus. Plants were inoculated at sowing with the individual or consortia treatments (see **Supplementary Table 2**) and grown for 15 days. +AMF indicates consortia co-inoculated with 1,000 spores/plant of *R. irregularis.* Bars represent mean ± SE. Dashed lines represent the initial concentration inoculated for each microorganism (4×10^5 cfu/g of soil). Treatments not sharing a letter in common are significantly different based on the general linear model and Tukey honestly significant difference (HSD) test ($p < 0.05$, $n = 5$).

single strains. Although no significant synergism was detected, no negative interactions were observed, in contrast to some studies reporting positive and negative effects by the combination of BCAs (Freeman et al., 2004; Abo-Elyousr et al., 2009; Elliott et al., 2009; Ruano Rosa et al., 2014). Our results illustrate the topical dilemma of selecting single beneficial microbes versus SynComs for biological control. Strictly from the potential efficacy point of view, SynComs offered the widest protection after comparing the single components and several consortia across the soil and foliar threats and through direct and indirect actions. Yet, the efficacy was not higher than that of the best performing single strain and, in most cases, more than one individual microbe provided effective control. Considering the current high costs and outstanding long process for registering microbial products, targeting single strain or SynCom products is a tough dilemma to face from the commercial point of view. Nevertheless, the advantage of SynComs as a more versatile tool may become more apparent under field conditions, considering the variability of growing conditions and the uncertainty of the potential challenges to be faced—what pathogens or pests would be threatening the crop. We postulate that in the field, under commercial conditions, the benefits for the SynComs would further differentiate to the individual components. Thorough validation of results in field conditions will give the answer.

Selecting a Potentially Powerful Pool as Step One

For the design of the synthetic microbial consortia, we selected different strains aiming to combine different mechanisms for biocontrol from the production of diverse antimicrobial metabolites through mycoparasitism to ISR. *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237 have been shown to promote plant growth and effectively control diverse microbial pathogens through direct antagonism or indirectly through ISR (Romero et al., 2007; García-Gutiérrez et al., 2012, 2013; Magno-Perez-Bryan et al., 2015). *P. chlororaphis* MA342 has been described to effectively control seed and soil pathogens *via* direct antagonism (Tombolini et al., 1999; Abuamsha et al., 2011a) and protecting against leaf pathogens through seed priming (Abuamsha et al., 2011b). *P. azotoformans* F30A effectively enhance plant emergency and growth (Levenfors et al., 2014) and can also induce ISR to leaf pathogens (Sang et al., 2014; Bouaoud et al., 2018). *T. harzianum* strain T22 is one of the best characterized and commercialized *Trichoderma* strains. It effectively antagonizes soil pathogens (Wilson et al., 2008; Percival et al., 2011; Roberti et al., 2015; Fatouros et al., 2018) and can trigger ISR against diverse above- and belowground attackers (Tucci et al., 2011; Vitti et al., 2016; Coppola et al., 2017, 2019; Debode et al., 2018; Pocurull et al., 2020; Alınç et al., 2021).

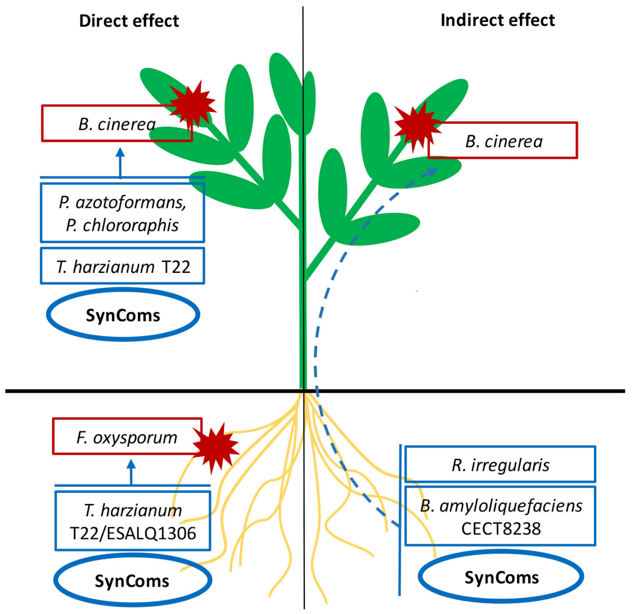

FIGURE 4 | Summary of the microbial treatments showing suppressive effects on *Botrytis cinerea* and *Fusarium oxysporum* through direct antagonism (arrows) or the induction of plant of systemic resistance (dashed arrow) after foliar spray and seed application.

Besides promoting plant growth, *T. harzianum* ESALQ1306 has been shown to highly reduce *Sclerotinia sclerotiorum* disease severity through parasitism and to induce ISR against spider mites (Geraldine et al., 2013; De Oliveira et al., 2018; Barroso et al., 2019; Canassa et al., 2020). In contrast, *R. irregularis* is not a direct antagonist of plant pathogens, but is able to induce ISR against root and foliar pathogens (Martínez-Medina et al., 2011; Sanchez-Bel et al., 2016; Bidellaoui et al., 2019; Campo et al., 2020; Sanmartín et al., 2020). All in all, we selected a potentially powerful pool of microbes, already well characterized in multiple aspects. A number of them are being already exploited commercially either under development into microbial products, or, like *T. harzianum*, already commercialized as BCA by Koppert Biological Systems all over the world from vegetable and ornamental to field and row crops.

Single Strains Versus SynComs, Variable Outcomes so Far

Most studies focusing on the use of microbial consortia for disease control are looking for synergistic or additive effects, aiming to achieve a higher pest or disease control than their components. While some of these studies have indeed reported positive effects (Guetsky et al., 2001, 2002; Srivastava et al., 2010; Singh et al., 2013; Ruano Rosa et al., 2014; Sylla et al., 2015), many others showed similarly or even less effective in disease control when applying consortia as compared to the application of the individual microbes (Freeman et al., 2004; Abo-Elyousr et al., 2009; Elliott et al., 2009; Ruano Rosa and López Herrera, 2009). However, most of these studies focused on one model system. In contrast, we intended to extend the scope by including an array of target diseases—soil and foliar—and possible mechanisms—direct and indirect control *via* ISR. The SynComs performed consistently well across the different pathosystems. Yet, differences between the SynComs and the individual components were relatively mild in terms of efficacy/degree of control.

Exploring the Compatibility of the Components of the SynComs

Microbial compatibility is a key factor when designing a microbial consortium, essential for the successful establishment and functionality of the included microorganisms and the success of SynCom products. In our study, the conservation of the biocontrol effectiveness in the SynComs to the same levels as the best performing individual isolates supported the compatibility between the coexisting microorganisms. We further tested their compatibility in our consortia by assessing the microbial survival in a plant–soil-based experiment, and we did not find any negative interaction between them. Instead, *Bacillus* and *Trichoderma* performed in the consortia as good as when individually inoculated, and *Pseudomonas* even benefited from the combination with the other organisms, as they performed better in the SynComs than when inoculated alone. It is important to note that *R. irregularis* was neither negatively affected in early nor late symbiosis stages by the presence of *Trichoderma* spp., as demonstrated by the similar mycorrhizal colonization in roots inoculated with the AMF alone or as part of the consortia. This is remarkable, as the compatibility of *Trichoderma* spp. with mycorrhizal fungi is frequently questioned because of the high mycoparasitic potential of these biocontrol fungi. In fact, *Trichoderma* is able to parasite AMF *in vitro* (Rousseau et al., 1996), but other studies proved their compatibility under more realistic scenarios (i.e., rhizospheric soil) as observed here (Martínez-Medina et al., 2011). Even more, *Trichoderma*-AMF synergistic effects have been reported (Poveda et al., 2019). Although microbe compatibility remains poorly studied, understanding the compatibility between groups or key BCA genera is required for informed decisions in the selection of suitable candidates for SynComs development in biocontrol programs in agriculture.

Overall, our findings highlight the potential multifunctionality of SynComs for biological control. Combining compatible beneficial microorganisms with complementary effects on different targets, direct and indirect mechanisms of control and/or effective under different conditions will lead to the development of biocontrol products with increased versatility. To became commercial products, consistency of the outcomes needs to be tested and finally validated across multiple field trials in the geographical regions where is aimed to be used. This is a key step for the successful application of this sustainable technology in agriculture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MP, RS, OK, and ZM designed the experiments. ZM performed all the experimental work. OK and ZM analyzed the data. RS and OK critically revised the manuscript. MP and ZM wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 765290. OK and RS are supported by Koppert Biological Systems. MP is supported by the grant RTI2018-094350-B-C31 from the Spanish National R&D Plan of the Ministry of Science, Innovation and Universities (MICIU) and the European Regional Development Fund (ERDF).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.756368/full#supplementary-material

REFERENCES

Ab Rahman, S. F. S., Singh, E., Pieterse, C. M. J., and Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Sci.* 267, 102–111. doi: 10.1016/j.plantsci.2017.11.012

Abo-Elyousr, K. A. M., Hashem, M., and Ali, E. H. (2009). Integrated control of cotton root rot disease by mixing fungal biocontrol agents and resistance inducers. *Crop Protect.* 28, 295–301. doi: 10.1016/j.cropro.2008.11.004

Abuamsha, R., Salman, M., and Ehlers, R. U. (2011a). Differential resistance of oilseed rape cultivars (*Brassica napus* ssp. *oleifera*) to *Verticillium longisporum* infection is affected by rhizosphere colonisation with antagonistic bacteria, *Serratia plymuthica* and *Pseudomonas chlororaphis*. *BioControl* 56, 101–112. doi: 10.1007/s10526-010-9308-8

Abuamsha, R., Salman, M., and Ehlers, R. U. (2011b). Effect of seed priming with *Serratia plymuthica* and *Pseudomonas chlororaphis* to control *Leptosphaeria maculans* in different oilseed rape cultivars. *Eur. J. Plant Pathol.* 130, 287–295. doi: 10.1007/s10658-011-9753-y

Alınç, T., Cusumano, A., Peri, E., Torta, L., and Colazza, S. (2021). *Trichoderma harzianum* strain t22 modulates direct defense of tomato plants in response to *Nezara viridula* feeding activity. *J. Chemical Ecol.* 1, 1260–1263. doi: 10.1007/s10886-021-01260-3

Arif, I., Batool, M., and Schenk, P. M. (2020). Plant microbiome engineering: expected benefits for improved crop growth and resilience. *Trends Biotechnol.* 38, 1385–1396. doi: 10.1016/j.tibtech.2020.04.015

Bakker, P. A. H. M., Pieterse, C. M. J., de Jonge, R., and Berendsen, R. L. (2018). The Soil-Borne Legacy. *Cell* 172, 1178–1180. doi: 10.1016/j.cell.2018.02.024

Barea, J. M. (2015). Future challenges and perspectives for applying microbial biotechnology in sustainable agriculture based on a better understanding of plant-microbiome interactions. *J. Soil Sci. Plant Nutrit.* 15, 261–282. doi: 10.4067/S0718-95162015005000021

Barea, J. M., Pozo, M. J., Azcón, R., and Azcón-Aguilar, C. (2005). Microbial co-operation in the rhizosphere. *J. Exp. Bot.* 56, 1761–1778. doi: 10.1093/jxb/eri197

Barea, J.-M., Pozo, M.-J., Azcón, R., and Azcón-Aguilar, C. (2013). "Microbial interactions in the rhizosphere," in *Molecular microbial ecology of the rhizosphere*, ed. B. Dehority (Hoboken, NJ: John Wiley and Son), 29–44. doi: 10.1051/rnd:19970701

Barroso, F. M., Muniz, P. H. P. C., Milan, M. D., Santos, W. S., dos, Ferreira, N. C., et al. (2019). Growth promotion of parsley (*Petroselinum crispum* L.) using commercial strains of *Trichoderma* spp. *J. Agricult. Sci.* 11:493. doi: 10.5539/jas.v11n4p493

Bidellaoui, B., Segarra, G., Hakkou, A., and Trillas, M. I. (2019). Beneficial effects of *Rhizophagus irregularis* and *Trichoderma asperellum* strain T34 on growth and fusarium wilt in tomato plants. *J. Plant Pathol.* 101, 121–127. doi: 10.1007/s42161-018-0159-y

Bouaoud, Y., Troulet, C., Foughalia, A., Berge, O., Aissat, K., and Bardin, M. (2018). A multi-criteria approach for the selection of efficient biocontrol agents against *Botrytis cinerea* on tomato in Algeria. *BioControl* 63, 299–311. doi: 10.1007/s10526-017-9851-7

Bradáčová, K., Florea, A. S., Bar-Tal, A., Minz, D., Yermiyahu, U., Shawahna, R., et al. (2019). Microbial consortia versus single-strain inoculants: an advantage in PGPM-assisted tomato production? *Agronomy* 9, 1–23. doi: 10.3390/agronomy9020105

Campo, S., Martín-Cardoso, H., Olivé, M., Pla, E., Catala-Forner, M., Martínez-Eixarch, M., et al. (2020). Effect of root colonization by arbuscular mycorrhizal fungi on growth, productivity and blast resistance in rice. *Rice* 13:42. doi: 10.1186/s12284-020-00402-7

Canassa, F., D'Alessandro, C. P., Sousa, S. B., Demétrio, C. G. B., Meyling, N. V., Klingen, I., et al. (2020). Fungal isolate and crop cultivar influence the beneficial effects of root inoculation with entomopathogenic fungi in strawberry. *Pest Manage. Sci.* 76, 1472–1482. doi: 10.1002/ps.5662

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Coppola, M., Cascone, P., Chiusano, M. L., Colantuono, C., Lorito, M., Pennacchio, F., et al. (2017). *Trichoderma harzianum* enhances tomato indirect defense against aphids. *Insect Sci.* 00, 1–9. doi: 10.1111/1744-7917.12475

Coppola, M., Diretto, G., Digilio, M. C., Lorito, M., and Rao, R. (2019). Transcriptome and metabolome reprogramming in tomato plants by *Trichoderma harzianum* strain T22 primes and enhances defense responses against aphids. *Front. Physiol.* 10:1–21. doi: 10.3389/fphys.2019.00745

De Kesel, J., Conrath, U., Flors, V., Luna, E., Mageroy, M. H., Mauch-Mani, B., et al. (2021). The induced resistance lexicon: do's and don'ts. *Trends Plant Sci.* 2021, 685–691. doi: 10.1016/j.tplants.2021.01.001

De Oliveira, J. B., Muniz, P. H. P. C., Peixoto, G. H. S., De Oliveira, T. A. S., Duarte, E. A. A., Rodrigues, F., et al. (2018). Promotion of seedling growth and production of wheat by using *Trichoderma* spp. *J. Agricult. Sci.* 10:267. doi: 10.5539/jas.v10n8p267

Debode, J., Tender, C., De, Cremelie, P., Lee, A. S., Kyndt, T., et al. (2018). *Trichoderma* -inoculated miscanthus straw can replace peat in strawberry cultivation, with beneficial effects on disease control. *Front. Plant Sci.* 9:1–15. doi: 10.3389/fpls.2018.00213

Elliott, M., Shamoun, S. F., Sumampong, G., James, D., Masri, S., and Varga, A. (2009). Evaluation of several commercial biocontrol products on European and North American populations of *Phytophthora ramorum*. *Biocontrol Sci. Technol.* 19, 1007–1021. doi: 10.1080/09583150903243870

Fatouros, G., Gkizi, D., Fragkogeorgi, G. A., Paplomatas, E. J., and Tjamos, S. E. (2018). Biological control of *Pythium, Rhizoctonia* and *Sclerotinia* in lettuce: association of the plant protective activity of the bacterium *Paenibacillus alvei* K165 with the induction of systemic resistance. *Plant Pathol.* 67, 418–425. doi: 10.1111/ppa.12747

Faust, K. (2019). Microbial consortium design benefits from metabolic modeling. *Trends Biotechnol.* 37, 123–125. doi: 10.1016/j.tibtech.2018.11.004

Freeman, S., Minz, D., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., et al. (2004). Trichoderma biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *Eur. J. Plant Pathol.* 110, 361–370. doi: 10.1023/B:EJPP.0000021057.93305.d9

García-Gutiérrez, L., Romero, D., Zeriouh, H., Cazorla, F. M., Torés, J. A., de Vicente, A., et al. (2012). Isolation and selection of plant growth-promoting rhizobacteria as inducers of systemic resistance in melon. *Plant Soil* 358, 201–212. doi: 10.1007/s11104-012-1173-z

García-Gutiérrez, L., Zeriouh, H., Romero, D., Cubero, J., de Vicente, A., and Pérez-García, A. (2013). The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defence responses. *Microb. Biotechnol.* 6, 264–274. doi: 10.1111/1751-7915.12028

Geraldine, A. M., Lopes, F. A. C., Carvalho, D. D. C., Barbosa, E. T., Rodrigues, A. R., Brandão, R. S., et al. (2013). Cell wall-degrading enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma* spp. *Biol. Control* 67, 308–316. doi: 10.1016/j.biocontrol.2013.09.013

Giovannetti, M., and Mosse, B. (1980). Giovannetti and mosse 1980.pdf. *New Phytol.* 84, 489–500.

Gruden, K., Lidoy, J., Petek, M., Podpeèan, V., Flors, V., Papadopoulou, K. K., et al. (2020). Ménage à trois: unraveling the mechanisms regulating plant–microbe–arthropod interactions. *Trends Plant Sci.* 25, 1215–1226. doi: 10.1016/j.tplants.2020.07.008

Guetsky, R., Shtienberg, D., Elad, Y., and Dinoor, A. (2001). Combining biocontrol agents to reduce the variability of biological control. *Biol. Control* 91, 621–627. doi: 10.1094/PHYTO.2001.91.7.621

Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., and Dinoor, A. (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Biol. Control* 92, 976–985. doi: 10.1094/PHYTO.2002.92.9.976

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307–319. doi: 10.1038/nrmicro1129

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species - Opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Hewitt, E. J. (1966). *Sand and water culture methods used in the study of plant nutrition*, 2nd Edn. Farnham Royal: Commonwealth Agricultural Bureaux, doi: 10.1017/S0014479700021852

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., and Pozo, M. J. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *J. Chem. Ecol.* 38, 651–664. doi: 10.1007/s10886-012-0134-6

Levenfors, J., Welch, C. F., Fatehi, J., Wikstrom, M., Rasmussen, S., and Hökeberg, M. (2014). *Fluorescent pseudomonad of the species Pseudomonas azotoformans* for enhancement of plant emergence and growth. Geneva: World Intellectual Property Organization.

Liu, H., Brettell, L. E., Qiu, Z., and Singh, B. K. (2020). Microbiome-mediated stress resistance in plants. *Trends Plant Sci.* 25, 733–743. doi: 10.1016/j.tplants.2020.03.014

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Magno-Perez-Bryan, M. C., Martinez-Garcia, P. M., Hierrezuelo, J., Rodriguez-Palenzuela, P., Arrebola, E., Ramos, C., et al. (2015). Comparative genomics within the bacillus genus reveal the singularities of two robust *Bacillus amyloliquefaciens* biocontrol strains. *Mol. Plant Microbe Interact.* 28, 1102–1116. doi: 10.1094/MPMI-02-15-0023-R

Martínez-Medina, A., Alguacil, M., del, M., Pascual, J. A., and Van Wees, S. C. M. (2014). Phytohormone profiles induced by *Trichoderma* isolates correspond with their biocontrol and plant growth-promoting activity on melon plants. *J. Chem. Ecol.* 40, 804–815. doi: 10.1007/s10886-014-0478-1

Martínez-Medina, A., Roldán, A., Albacete, A., and Pascual, J. A. (2011). The interaction with arbuscular mycorrhizal fungi or *Trichoderma harzianum* alters the shoot hormonal profile in melon plants. *Phytochemistry* 72, 223–229. doi: 10.1016/j.phytochem.2010.11.008

Mitter, B., Brader, G., Pfaffenbichler, N., and Sessitsch, A. (2019). Next generation microbiome applications for crop production - limitations and the need of knowledge-based solutions. *Curr. Opin. Microbiol.* 49, 59–65. doi: 10.1016/j.mib.2019.10.006

Nicholson, W. L., and Setlow, P. (1990). "Sporulation, germination and outgrowth," in *Molecular biological methods for Bacillus*, eds C. R. Harwood and S. M. Cutting (New York, NY: John Wiley and Sons), 391–450.

Percival, G. C., Smiley, E. T., and Fox, R. T. V. (2011). Root collar excavation with *Trichoderma* inoculations as a potential management strategy for honey fungus (*Armillaria mellea*). *Arboricult. J.* 33, 267–280.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Pineda, A., Soler, R., Pozo, M. J., Rasmann, S., and Turlings, T. C. J. (2015). Above-belowground interactions involving plants, microbes and insects. *Front. Plant Sci.* 6:1–3. doi: 10.3389/fpls.2015.00318

Pocurull, M., Fullana, A. M., Ferro, M., Valero, P., Escudero, N., Saus, E., et al. (2020). Commercial formulates of *Trichoderma* induce systemic plant resistance to *Meloidogyne incognita* in tomato and the effect is additive to that of the Mi-1 . 2 resistance gene. *Front. Microbiol.* 10:1–10. doi: 10.3389/fmicb.2019.03042

Poveda, J., Hermosa, R., Monte, E., and Nicolás, C. (2019). *Trichoderma harzianum* favours the access of arbuscular mycorrhizal fungi to non-host Brassicaceae roots and increases plant productivity. *Sci. Rep.* 9, 1–11. doi: 10.1038/s41598-019-48269-z

Pozo, M. J., and Azcón-Aguilar, C. (2007). Unraveling mycorrhiza-induced resistance. *Curr. Opin. Plant Biol.* 10, 393–398. doi: 10.1016/j.pbi.2007.05.004

Pozo, M. J., Zabalgogeazcoa, I., Vazquez, de Aldana, B. R., and Martinez-Medina, A. (2021). Untapping the potential of plant mycobiomes for applications in agriculture. *Curr. Opin. Plant Biol.* 60:102034. doi: 10.1016/j.pbi.2021.102034

R Core Team (2021). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Rändler-Kleine, M., Wolfgang, A., Dietel, K., Junge, H., Cernava, T., and Berg, G. (2020). "How microbiome approaches can assist industrial development of biological control products," in *Integrative Biological Control*, Vol. 20, eds Y. Gao, H. Hokkanen, and I. Menzler-Hokkanen (Berlin: Springer), 201–215. doi: 10.1007/978-3-030-44838-7_13

Rivero, J., Lidoy, J., Llopis-Giménez, Á, Herrero, S., Flors, V., and Pozo, M. J. (2021). Mycorrhizal symbiosis primes the accumulation of antiherbivore compounds and enhances herbivore mortality in tomato. *J. Exp. Bot.* 72, 5038–5050. doi: 10.1093/jxb/erab171

Roberti, R., Bergonzoni, F., Finestrelli, A., and Leonardi, P. (2015). Biocontrol of *Rhizoctonia solani* disease and biostimulant effect by microbial products on bean plants. *Microbiol. Ital.* 44, 49–61. doi: 10.6092/issn.2465-311X/5742

Romero, D., De Vicente, A., Zeriouh, H., Cazorla, F. M., Fernández-Ortuño, D., Torés, J. A., et al. (2007). Evaluation of biological control agents for managing cucurbit powdery mildew on greenhouse-grown melon. *Plant Pathol.* 56, 976–986. doi: 10.1111/j.1365-3059.2007.01684.x

Rousseau, A., Benhamou, N., Chet, I., and Piché, Y. (1996). Mycoparasitism of the extramatrical phase of *Glomus intraradices* by *Trichoderma harzianum*. *Biochem. Cell Biol.* 86, 434–443.

Ruano Rosa, D., and López Herrera, C. J. (2009). Evaluation of *Trichoderma* spp. as biocontrol agents against avocado white root rot. *Biol. Control* 51, 66–71. doi: 10.1016/j.biocontrol.2009.05.005

Ruano Rosa, D., Martín Pérez, R., Bonilla, N., Cazorla, F. M., De Vicente, A., and López Herrera, C. J. (2014). Biological control of avocado white root rot with combined applications of *Trichoderma* spp. and rhizobacteria. *Eur. J. Plant Pathol.* 138, 751–762. doi: 10.1007/s10658-013-0347-8

Sanchez-Bel, P., Troncho, P., Gamir, J., Pozo, M. J., Camañes, G., Cerezo, M., et al. (2016). The nitrogen availability interferes with mycorrhiza-induced resistance against *Botrytis cinerea* in tomato. *Front. Microbiol.* 7:1–16. doi: 10.3389/fmicb.2016.01598

Sang, M. K., Kim, E. N., Han, G. D., Kwack, M. S., Jeun, Y. C., and Kim, K. D. (2014). Priming-mediated systemic resistance in cucumber induced by *Pseudomonas azotoformans* GC-B19 and *Paenibacillus elgii* MM-B22 against *Colletotrichum orbiculare*. *Phytopathology* 104, 834–842. doi: 10.1094/PHYTO-11-13-0305-R

Sanmartín, N., Pastor, V., Pastor-Fernández, J., Flors, V., Pozo, M. J., and Sánchez-Bel, P. (2020). Role and mechanisms of callose priming in mycorrhiza-induced resistance. *J. Exp. Bot.* 71, 2769–2781. doi: 10.1093/jxb/eraa030

Santoyo, G., Orozco-Mosqueda, M. C., Govindappa, M., Santoyo, G., Orozco-mosqueda, M. C., and Govindappa, M. (2012). Mechanisms of biocontrol and plant growth- promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: a review. *Biocontr. Sci. Technol.* 22, 855–872. doi: 10.1080/09583157.2012.694413

Sarma, B. K., Yadav, S. K., Singh, S., and Singh, H. B. (2015). Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy. *Soil Biol. Biochem.* 87, 25–33. doi: 10.1016/j.soilbio.2015.04.001

Singh, A., Sarma, B. K., Upadhyay, R. S., and Singh, H. B. (2013). Compatible rhizosphere microbes mediated alleviation of biotic stress in chickpea through enhanced antioxidant and phenylpropanoid activities. *Microbiol. Res.* 168, 33–40. doi: 10.1016/j.micres.2012.07.001

Srivastava, R., Khalid, A., Singh, U. S., and Sharma, A. K. (2010). Evaluation of arbuscular mycorrhizal fungus, fluorescent *Pseudomonas* and *Trichoderma harzianum* formulation against *Fusarium oxysporum* f. sp. *lycopersici* for the management of tomato wilt. *Biol. Control* 53, 24–31. doi: 10.1016/j.biocontrol.2009.11.012

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., and Fortin, J. A. (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. *Mycol. Res.* 100, 328–332. doi: 10.1016/S0953-7562(96)80164-X

Sylla, J., Alsanius, B. W., Krüger, E., and Wohanka, W. (2015). Control of *Botrytis cinerea* in strawberries by biological control agents applied as single or combined treatments. *Eur. J. Plant Pathol.* 143, 461–471. doi: 10.1007/s10658-015-0698-4

Tombolini, R., Van Der Gaag, D. J., Gerhardson, B., and Jansson, J. K. (1999). Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA 342 on barley seeds visualized by using green fluorescent protein. *Appl. Environ. Microbiol.* 65, 3674–3680. doi: 10.1128/aem.65.8.3674-3680.1999

Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., and Singh, B. K. (2020). Plant–microbiome interactions: from community assembly to plant health. *Nat. Rev. Microbiol.* 18, 607–621. doi: 10.1038/s41579-020-0412-1

Trivedi, P., Schenk, P. M., Wallenstein, M. D., and Singh, B. K. (2017). Tiny Microbes, Big Yields: enhancing food crop production with biological solutions. *Microb. Biotechnol.* 10, 999–1003. doi: 10.1111/1751-7915.12804

Tucci, M., Ruocco, M., Masi, L. D. E., Palma, M. D. E., and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp . on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/J.1364-3703.2010.00674.X

van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J., and Urbaneja, A. (2017). Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl* 63, 39–59. doi: 10.1007/s10526-017-9801-4

Vierheilig, H., Schweiger, P., and Brundrett, M. (2005). An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. *Physiol. Plant.* 125, 393–404. doi: 10.1111/j.1399-3054.2005.00564.x

Vitti, A., Pellegrini, E., Nali, C., Lovelli, S., Sofo, A., Valerio, M., et al. (2016). Trichoderma harzianum T-22 induces systemic resistance in tomato infected by *Cucumber mosaic virus*. *Front. Plant Sci.* 7:1–11. doi: 10.3389/fpls.2016.01520

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487–511. doi: 10.1093/jexbot/52.suppl_1.487

Wickham, H. (2009). *ggplot2: Elegant graphics for data analysis*, 1st Edn. New York, NY: Springer-Verlag, doi: 10.1007/978-0-387-98141-3

Wilson, P. S., Ketola, E. O., Ahvenniemi, P. M., Lehtonen, M. J., and Valkonen, J. P. T. (2008). Dynamics of soilborne *Rhizoctonia solani* in the presence of *Trichoderma harzianum*: effects on stem canker, black scurf and progeny tubers of potato. *Plant Pathol.* 57, 152–161. doi: 10.1111/j.1365-3059.2007.01706.x

Woo, S. L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., et al. (2014). *Trichoderma*-based products and their widespread use in agriculture. *Open Mycol. J.* 8, 71–126. doi: 10.2174/1874437001408010071

Zuur, A. F., and Ieno, E. N. (2016). A protocol for conducting and presenting results of regression-type analyses. *Methods Ecol. Evolut.* 7, 636–645. doi: 10.1111/2041-210X.12577

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Plant Science

ORIGINAL RESEARCH
published: 25 November 2021
doi: 10.3389/fpls.2021.755733

Streptomyces Strains Promote Plant Growth and Induce Resistance Against *Fusarium verticillioides* via Transient Regulation of Auxin Signaling and Archetypal Defense Pathways in Maize Plants

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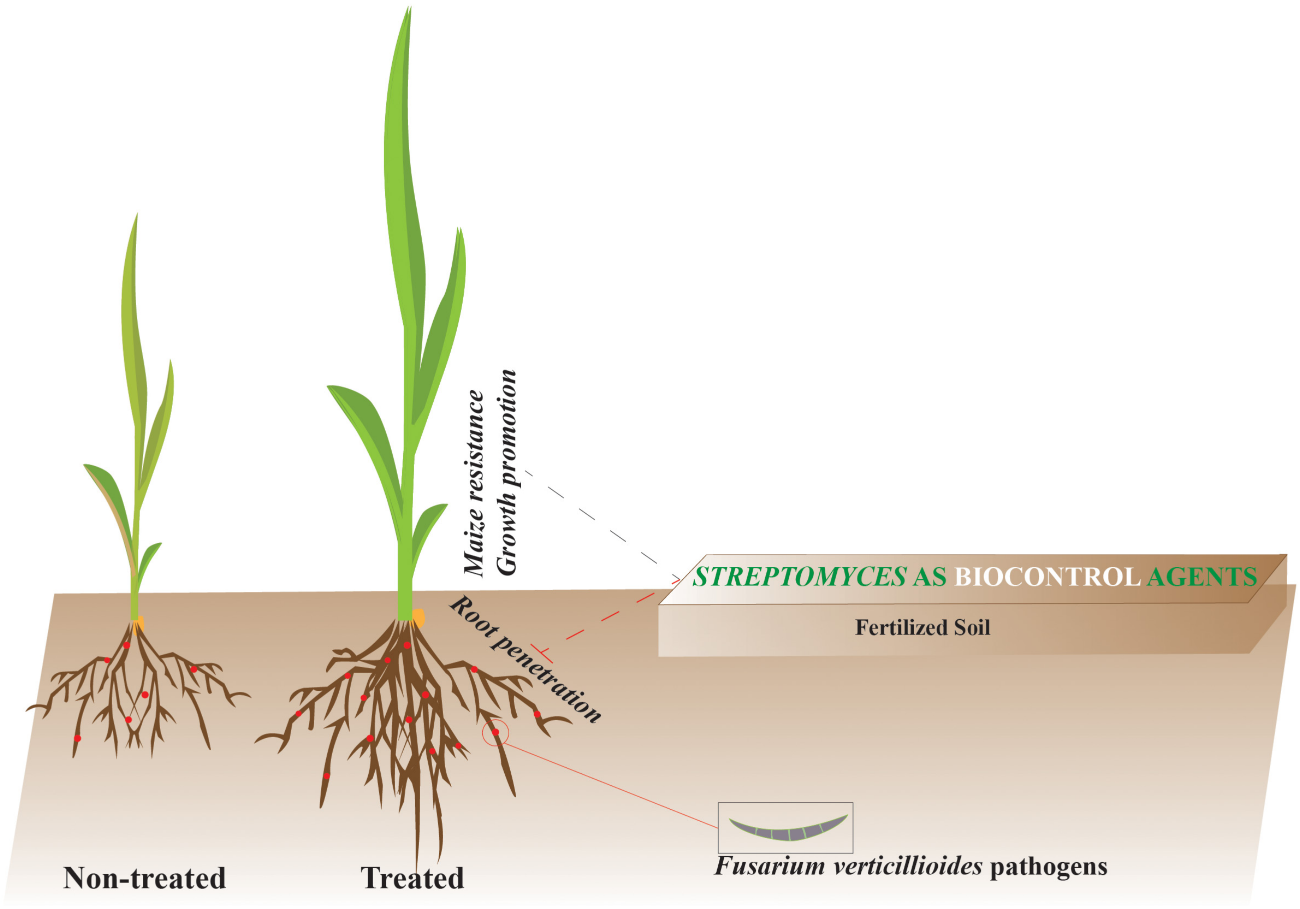
Specialty section:
This article was submitted to Plant Symbiotic Interactions, a section of the journal Frontiers in Plant Science

Received: *20 August 2021*
Accepted: *01 November 2021*
Published: *25 November 2021*

Citation:
Tran TM, Ameye M, Devlieghere F, De Saeger S, Eeckhout M and Audenaert K (2021) Streptomyces Strains Promote Plant Growth and Induce Resistance Against Fusarium verticillioides via Transient Regulation of Auxin Signaling and Archetypal Defense Pathways in Maize Plants. Front. Plant Sci. 12:755733. doi: 10.3389/fpls.2021.755733

Driven by climate change, *Fusarium* ear rot (FER) caused by *Fusarium verticillioides* occurs frequently in maize worldwide. In parallel, legislative regulations and increasing environmental awareness have spurred research on alternative FER biocontrol strategies. A promising group of bacterial control agents is *Streptomyces* species due to their metabolic versatility. However, insights into the molecular modes of action of these biocontrol agents are often lacking. This study aims at unraveling the biocontrol efficacy of *Streptomyces* rhizobacterial strains against *F. verticillioides*. We first assessed the direct antagonism of four *Streptomyces* strains ST02, ST03, ST07, and ST08. Then, a profile of 16 genes associated with intrinsic plant defense signaling was assessed in maize plants. Both *in vitro* and *in vivo* data showed that the biocontrol strain ST03 perfectly suppressed the growth of *F. verticillioides*. High inhibition efficacy was also observed for extracellular compounds in the supernatant secreted by this strain. Especially, for maize cobs, the biocontrol strain ST03 not only inhibited the proliferation of *F. verticillioides* but also significantly repressed fungal fumonisin production 7 days after inoculation. On maize plants, the direct antagonism was confirmed by a significant reduction of the fungal DNA level in soils when co-applied with *F. verticillioides* and strain ST03. In terms of its action on plants, strain ST03 induced downregulation of auxin responsive genes (*AUX1*, *ARF1,* and *ARF2*) and gibberellic acid (GA)-related gene *AN1* even in the absence of *F. verticillioides* at early time points. In leaves, the biocontrol strain induced the expression of genes related to salicylic acid (SA), and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-mediated pathways, and pathogenesis-related proteins in the presence or absence of the pathogen. Interestingly, the biocontrol strain significantly promoted plant growth even in the presence of *F. verticillioides*. All of which demonstrated that the *Streptomyces* strain ST03 is a promising FER biocontrol and a growth-promoting candidate.

Keywords: auxin, fumonisins, *Fusarium* ear rot (FER), maize, mycotoxins, *Streptomyces*, Vietnam



GRAPHICAL ABSTRACT | *Streptomyces* strains promote plant growth and induce resistance against *Fusarium verticillioides* via transient regulation of auxin signaling and archetypal defense pathways in plants.

INTRODUCTION

Fusarium verticillioides is a hemibiotrophic pathogen and the primary causal agent of the disease *Fusarium* ear rot (FER) in maize worldwide (Bacon et al., 2008). The disease not only reduces the productivity of maize and the nutritive value of grains, but also exerts a severe impact on human and animal health since grains infected with *F. verticillioides* can be contaminated with its toxic secondary metabolites, particularly fumonisin B_1 (FB_1), FB_2, and FB_3 (Pitt and Miller, 2016). Vulnerable parts of the maize cob such as silks (Reid et al., 1999) and insect feeding sites are the main points of entry for this pathogen (Parsons and Munkvold, 2010; Blandino et al., 2015). In some cases, the presence of *F. verticillioides* can be traced back to the seedling stage during which the fungus infects and resides asymptomatically followed by moving upwards toward the cob during anthesis and ultimately leading to FER (Oren et al., 2003; Suárez-Moreno et al., 2019). To date, no registered FER-resistant maize inbred lines are available on the market.

In the context of increasing plant disease pressure, plant pathologists face a challenging decision between the continuity of using fungicides and negative side-effects of the use of agrochemicals in crop protection (Lamichhane et al., 2016). Overreliance on fungicides is posing a threat to the environment, soil ecosystems, and human health. Therefore, research has turned to sustainable and eco-friendly alternative control measures. Rhizobacteria *Streptomyces* spp. Are well-known as not only plant growth-promoting rhizobacteria (PGPR) but also promising biocontrol agents, resulting in increased yields of agricultural crops (Dimkpa et al., 2009). From a crop protection point of view, *Streptomyces* spp. have been demonstrated to be effective against a broad spectrum of fungal phytopathogens, e.g., *Fusarium* tomato wilt (Abbasi et al., 2019), *Fusarium* head blight (FHB) (Tan et al., 2020), *Fusarium* banana wilt (Zhu et al., 2020), *Ralstonia* tomato wilt (Shen et al., 2021), and cucumber *Phytophthora* damping-off (Sadeghi et al., 2017). The effectiveness of the biocontrol can be attributed to the metabolic diversity of anti-fungal compounds and antibiotics, and their bioactive and adaptable capability in the agro-ecological relevant niche of the soil (Tan et al., 2020). Together with direct antagonism against the pathogens, several Rhizobacteria can interact with the plant defense system. They have been shown to trigger the salicylic acid (SA)- and/or jasmonic acid (JA)/ethylene (ET) mediated defense pathways in plants (Conn et al., 2008; Kurth et al., 2014; Tan et al., 2020; Vergnes et al., 2020).

Developing a biocontrol agent (BCA) for FER management requires an extensive understanding of the molecular mechanisms involved in the tripartite interaction between plant, pathogen, and BCA. A thorough understanding of phytohormone-mediated plant defense response is needed. The key phytohormones involved in the plant defense response are salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and auxin (Aux) (Tzin et al., 2017). Pathogenesis-related proteins (PRs) [e.g., *PR1*, and *PR10*, 1,3-β-glucanase (*PR2*), chitinase (*PR3*)] (Nasser et al., 1990), and benzoxazinoids (BXs) e.g., 2-4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Ding et al., 2015) are responsible for plant resistance against pathogens.

To the best of our knowledge, there is little information available on the biological control of FER in maize by *Streptomyces*. Therefore, this current study aims to (i) unravel the antagonistic effect of *Streptomyces* strains to *F. verticillioides* and (ii) further insight into the molecular modes of action of the biocontrol strain in the induction of plant resistance against this fungal phytopathogen.

MATERIALS AND METHODS

Preparation of *Fusarium* and *Streptomyces* Strains

In this study, an *F. verticillioides* strain F01.12 (accession number MZ559332) isolated by Tran et al. (2021a) was used. In addition, five other *Fusarium* strains comprising *F. proliferatum* and *F. mangiferae* were isolated from maize grains in the central highlands of Vietnam (Tran et al., 2021b), *F. oxysporum*, and *F. solani* isolated from onion in Dalat, Vietnam (Le et al., 2020) and *F. graminearum* PH1 isolated from wheat (Gale et al., 2005) were also used. Each fungus was cultured on a PDA plate (Potato Dextrose Agar, 40 g L^{-1}) (Sigma Aldrich, Overijse, Belgium) for 7 days at 25°C before performing assays. To prepare a spore suspension of *F. verticillioides*, we placed a 7-day-old PDA plate of this fungus into a cabinet equipped with near-UV lights (12 h light/12 h darkness) for 7 days. The spore suspension was then collected and diluted with sterile water until a final concentration of 10^7 conidia mL^{-1}.

We used four rhizobacterial strains ST02, ST03, ST07, and ST08 isolated from soil in Ben Tre, Vietnam. For the identification of these strains, we amplified the recombinase A (recA) gene by using a primer pair of *recAF* and *recAR* as described by Guo et al., 2008 (**Supplementary Table 1**). The sequences were deposited to the NCBI gene bank, representing MZ614615 for strain ST02, MZ614616 for strain ST03, MZ614619 for strain ST07, and MZ614615 for strain ST08. For inoculation, each strain was grown on a TSA plate (Tryptic Soy Agar, 30 g L^{-1}) (Sigma Aldrich, Overijse, Belgium) for 5 days. Three rhizobacterial colonies were then transferred into a 50-mL Falcon tube containing 20 mL TSB (tryptic soy broth, 30 g L^{-1}) (Sigma Aldrich, Overijse, Belgium) and incubated for 7 days at 200 g, and 28°C. The Falcon tubes were then centrifuged at 10000 *g* for 10 min and the cell suspension was obtained by discarding the supernatant through a four-fold Mira cloth followed by re-suspending the cell pellets in 5 mL sterile water. A ten-fold serial dilution was made to determine the colony-forming unit (CFU) of each cell suspension (5×10^6 CFU mL^{-1}). The cell-free supernatant was concentrated 10 times using a nitrogen flow at 1 atm in a water bath at 40°C for 2 h.

In vitro Antagonistic Assay on Agar Medium

In agar plates, we used two different models: (1) a diffusion assay and (2) a volatile assay. For the diffusion assay, we used a four-hole TSA plate (Φ 90 mm) (**Supplementary Figure 1A**). In each plate, a 7-day-old agar plug of each pathogen (Φ 5 mm) was placed in the center, and 50 μL of each rhizobacterial cell suspension or supernatant was filled in each hole. The plate was incubated at 28°C and the radius of the fungal colony was measured. In addition, to evaluate the effects of the volatile compounds produced by rhizobacterial strains we performed the volatile assay in which a TSA plate was divided into two parts by making an agar-free channel in the middle (Φ 5 mm) (**Supplementary Figure 1B**). Water and blank TSB were used as control.

In vitro Antagonistic Assay on Liquid Medium

In this assay, we used a six-well plate containing 4.5 mL TSB per well (**Supplementary Figure 1C**). A ST03 cell suspension (5×10^7 CFU mL^{-1}) or a 10-time-concentrated supernatant was injected to each well at different volumes (μL) of 0 (0%), 31.25 (0.6%), 62.5 (1.3%), 125 (2.5%), 250 (5%), and 500 μL (10%).

TSB was subsequently added to each well till 5 mL. A 7-day-old agar plug of *Fusarium verticillioides* (Φ 5 mm) was then transferred to each well. The plate was inoculated at 28°C for 7 days. To analyze the fungal growth on the surface, an image was taken using a custom-build multispectral phenotyping platform as described by Tan et al. (2020). This platform is equipped with a 6 Mp-16 bit camera mounted on a Cartesian coordinate robot and allows 6-μm-high-resolution multispectral imaging. The multispectral camera can visualize the surface growth of the fungus. Finally, biomass from each well was collected followed by freeze-dried and weighted.

In vivo Antagonistic Assay on Maize Cobs

This bioassay was employed using baby maize cobs (70 mm) (Baby corn, Excel Fruits, Thailand). In each treatment, five maize cobs were disinfected with 1% NaOCl solution, washed twice with sterile water, and then placed in a one-well plate equipped with a moist Whatman paper (**Supplementary Figure 1D**). Subsequently, we made a hole in the middle of each cob (depth ∼ 10 mm) using a sterile 10 μL pipette tip. A mixture consisting of 5 μL spore suspension of *Fusarium verticillioides* (10^7 conidia mL^{-1}) and 5 μL cell suspension (5×10^6 CFU mL^{-1}) [FV + ST03 treatment)/or cell-free supernatant of rhizobacterial strain ST03 (FV + Sup) treatment] was loaded to each hole. Each maize ear was inoculated at 28°C for 7 days. Five infected maize cobs were treated with sterile water as a positive control (FV + W) and five *F. verticillioides* non-infected cobs were treated with sterile water as a negative control (W + W). The cobs were then divided into two equal parts using a scalpel. To determine the damage level caused by the pathogen, we analyzed images taken by the multispectral camera using the RGB module. Finally, each maize cob was thoroughly ground using liquid nitrogen for quantification of FB_1, FB_2, and FB_3 by an LC-MS/MS method (De Boevre et al., 2012).

In vivo Antagonistic Assay on Maize Seedlings

In this assay, we used two hybrid maize lines *Bt/GT* NK7328 (supplied by Syngenta company, Vietnam) and CP888 (supplied by C. P. company, Vietnam) as they are predominantly planted in the central highlands of Vietnam (Tran et al., 2021c). Maize seeds were disinfected with 1% NaOCl solution and germinated in a rectangular plastic pot (20 × 16 × 6 cm) with vermiculite substrate (Vermex, Soprema, Belgium) for 5 days. For the biocontrol treatment (FV + ST03 treatment), five germinated seeds were infected with *F. verticillioides* by soaking in a fungal suspension (10^7 conidia mL^{-1}) for 1 h, and then sowed in five glass tubes (a seed per a tube) containing soil inoculated with the ST03 cell suspension. To inoculate the soil with *Streptomyces*, 3 g polymer gel (DCM Aquaperla, Grobbendonk, Belgium) absorbed in 300 mL tap water was mixed with 2 kg non-sterile fine sand (Voss chemie-Benelux, Lier, Belgium) (De Zutter et al., 2021). This mixture was then inoculated with the ST03 cell suspension in a concentration of 10^6 CFU g^{-1} soil 3 days before planting. The positive control comprised five *F. verticillioides* infected seeds in soil non-inoculated with the ST03 cell suspension (FV + W treatment). For the negative control, five mock seeds treated with sterile water were planted in non-inoculated soil with the strain ST03 (W + W treatment), and five mock seeds were planted in soil pre-inoculated with ST03 (W + ST03 treatment). Every 2 days plants were watered with 10 mL tap water. To assess the direct antagonism of strain ST03, the soil was collected after 2 weeks of planting and the DNA levels of *F. verticillioides* were qualified as described below.

To measure the impact of ST03 on the plant growth when co-applied with *F. verticillioides* in maize seedlings, the length and fresh biomass of leaves and roots were measured at 16 days after inoculation (dai). Chlorophyll Index (ChlIdx) was also assessed using the multispectral camera as described by Tan et al. (2021). Meanwhile, to uncover the impact of the *Streptomyces* strain ST03 on the expression of genes related to plant defense response, plants of each treatment were harvested at four different time points at dai 1, 2, 4, and 8 (five plants per each time-point) (**Supplementary Figure 1E**). Roots and leaves from each plant were extracted for RNA as described below to quantify the gene expression using RT-qPCR.

RNA Extraction and RT-qPCR

Total RNA from roots and leaves was extracted using TRizol reagent (Sigma Aldrich, Belgium) according to the manufacturer's instructions. RNA concentration was determined using a Quantus fluorometer (Promega, Netherlands). cDNA of each RNA template was synthesized using the iScript^{TM} kit (Bio-Rad, Belgium) and diluted five times with nuclease-free water. The quantitative reverse transcription PCR (RT-qPCR) assay was performed using a CFX96 Tough Real-time PCR Detection System (Bio-Rad, Belgium). Each PCR reaction contained 6.3 μL Gotagq®PCR master mix (Promega, Netherlands), 2 μL cDNA, 0.6 μL each primer (5 μM), and 0.2 μL CXR dye (Promega, Netherlands), and 2.3 μL nuclease-free water. The thermal program was set up as follows: 95°C for 3 min; 39 cycles of 95°C for 10 s, and 60°C for 30 s, followed by a melting curve acquisition from 65 to 95°C with the rate of 0.5°C s^{-1}. The primers used for all target genes are shown in **Supplementary Table 1**. Elongation factor 1α (*EF-1α*) and β tubulin (β-*TUB*) primers were used as housekeeping genes. Gene expression analysis was done using qBase^{+} software (Biogazelle, Zwijnaarde, Belgium). Fold change was computed by diving the CNRQ values (calibrated normalized relative quantities) of the treated samples by values of the control samples. In each gene, four biological replicates and two technical replicates were done.

Quantification of Fumonisins by LC-MS/MS

Quantification of fumonisin B_1, B_2, and B_3 in each maize ear was performed using a Waters Acquity HPLC-Quattro Premier XE mass spectrometer (Waters, Milford, MA, United States) in positive electrospray ionization (ESI^{+}) mode. Chromatographic separation was performed using a Symmetry C18 (150 mm × 2.1 mm, i.d. 5 μm) column with a guard column (10 mm × 2.1 mm i.d.) of the same material

(Waters, Zellik, Belgium). A clean-up procedure was carried out prior to injecting samples into the device. Briefly, 100 μL de-epoxy-deoxynivalenol (DOM) (50 ng μL^{-1}) were added to a Falcon tube containing 5 g of each ground sample as an internal standard. Each sample was extracted with 10 mL ethyl acetate/folic acid (99/1, v/v) by agitating for 15 h on an overhead shaker. The Falcon tubes were centrifuged at 4000 *g* for 15 min. Each upper layer was transferred into a new tube and evaporated using a nitrogen flow at 40°C, 1 atm. The pellet was re-dissolved in 200 μL injection solvent {consisting of mobile phase A [water/methanol/acetic acid (94/5/1, v/v/v) + 5 mM ammonium acetate] and mobile phase B [methanol/water/acetic acid (97/2/1, v/v/v) + 5 mM ammonium acetate] with a ratio of A/B (60/40, v/v)}; and the mixture was defatted with 200 μL hexane and subsequently filtrated using a centrifuge filter at 10000 *g* for 5 min. Finally, each filtrate was conveyed into an HPLC vial. The liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method was described by De Boevre et al. (2012). Five biological replicates were done per treatment.

Quantification of *Fusarium verticillioides* DNA in Soil by Quantitative PCR

A quantitative PCR (qPCR) assay was conducted in a CFX96 Touch Real-Time PCR Detection System (BIO-RAD, Temse, Belgium) as described by Tran et al. (2021a). Briefly, total genomic DNA extraction was performed from 100 mg for each soil sample, and 50 mg for pure *F. verticillioides* mycelia using the E.Z.N.A® Soil DNA Kit (VWR International, Leuven, Belgium). DNA concentration was quantified with a Quantus fluorometer (Promega, Leiden, Netherlands). Each reaction mixture contained 6.3 μL Gotag® qPCR Master Mix (Promega, Leiden, Netherlands), 2 μL DNA template, 0.6 μL each primer *FVer* (5 μM), 0.2 μL CXR reference dye (Promega, Leiden, Netherlands), and nuclease-free water up to a total volume of 12 μL. The PCR thermal cycling program was set-up as follows: 95°C for 3 min; 39 cycles of 95°C for 10 s, and 60°C for 30s, followed by a melting curve acquisition from 65 to 95°C. A standard curve was generated based on C_t (threshold cycle) values by using tenfold serial dilutions of the pure *F. verticillioides* DNA (ranging from 1 ng to 1×10^{-5} ng/μL). The amount of fungal DNA in samples was calculated from C_t values using the standard curve. Each sample calculation was performed twice. Five biological replicates were undertaken per treatment.

Statistical Analysis

All heat maps and boxplots were generated using the R software v.4.0.2 with the packages ggplot2 and gplots[1]. The normal distribution of data was tested using the Shapiro–Wilk test. The one-way ANOVA test followed by a *post hoc* Tukey test was used in a case of the normal distribution, otherwise, a non-parametric Kruskal–Wallis test and a *post hoc* Dunn's test were applied. As for statistical analyses in gene expression, fold change (FC) was used in the case of up-regulation, while for down-regulation log [FC] base 2 (Log_2FC) was used. All analyses were tested at a significance level of $\alpha = 0.05$.

RESULTS

In vitro Direct Antagonism of *Streptomyces* Strains Against *Fusarium* Species

In this study, all the rhizobacterial strains were identified as *Streptomyces* species. The *in vitro* bioassays showed an effective and stronger direct antagonism of the strain ST03 against six *Fusarium* pathogens compared to the other rhizobacterial strains (**Figure 1A**). It is clear that in the *in vitro* diffusion assay, an early and significantly inhibitory indication was observed against *F. verticillioides*, *F. proliferatum*, *F. oxysporum,* and *F. graminearum* when co-applied with the strain ST03 at 2 dai and at 3 dai for *F. mangiferae* and *F. solani* in comparison to the mock control. Surprisingly, *F. verticillioides*, *F. proliferatum,* and *F. oxysporum* were thoroughly inhibited by the strain ST03 from 2 dai onward since their colony radii remained unchanged between 2 and 7 dai. It was coherent when its inhibition efficacy against these three pathogens was highest and significant at 7 dai compared to the other bacterial strains (**Figure 1B** and **Supplementary Figure 2**). The results also indicated absolute but a later inhibition of *F. mangiferae*, *F. solani,* and *F. graminearum* when co-inoculated with the strain ST03 at 5 dai (**Figure 1A**), which explained lower inhibition efficacy at 7 dai, of 65 ± 1%, 56 ± 2%, and 51 ± 2%, respectively (**Figure 1B** and **Supplementary Figure 2**). It was significantly higher than the inhibition efficacy of the other strains ST02, ST07, and ST08. An obvious example is that at 7 dai the inhibition efficacy against *F. graminearum* by the strain ST03 was 51 ± 2%, while this was not significantly effective for the other ones, for example, ST02 (3 ± 2%, $p < 0.001$), ST07 (6 ± 8%, $p < 0.001$), and ST08 (4 ± 2%, $p < 0.001$) (**Supplementary Figure 2**). Remarkably, the antagonistic efficacy of the strain ST03 reached above 50% at 5 dai for all the pathogens except for *F. solani* (**Supplementary Figure 2**). We, therefore, used this strain ST03 for in-depth studies.

To assess the impact of volatile compounds produced by strain ST03, we used an *in vitro* volatile bioassay. Even though this assay did not indicate the role of volatile metabolites for the antagonism against the pathogens, it strengthened the diffusion-based antagonism since the inhibition zones were more transparent for each pathogen compared to the mock control (**Figure 1C**). At 11 dai, *F. verticillioides*, *F. proliferatum*, and *F. oxysporum* did not grow onto the other side inoculated with strain ST03, whereas for the mock control plates the fungi colonized the other side from the channel by approx. 25 mm. As similar to the diffusion assay, the inhibition efficacy was less for *F. mangiferae*, *F. solani*, and especially for *F. graminearum* (**Figure 1C**).

The high antagonistic efficacy of strain ST03 was proven in the other *in vitro* assay using a liquid medium. It was confirmed by a significant decrease in biomass production by *F. verticillioides* when co-inoculated with the cell suspension of strain ST03, ranging from 2.5 to 10% (**Figure 2A**). The fungal growth was remarkably suppressed when co-applied with 2.5% of strain ST03 equivalently with 1.25×10^6 CFU mL^{-1} because there was a significant reduction of dried biomass by 59% compared

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[1] https://cran.r-project.org/

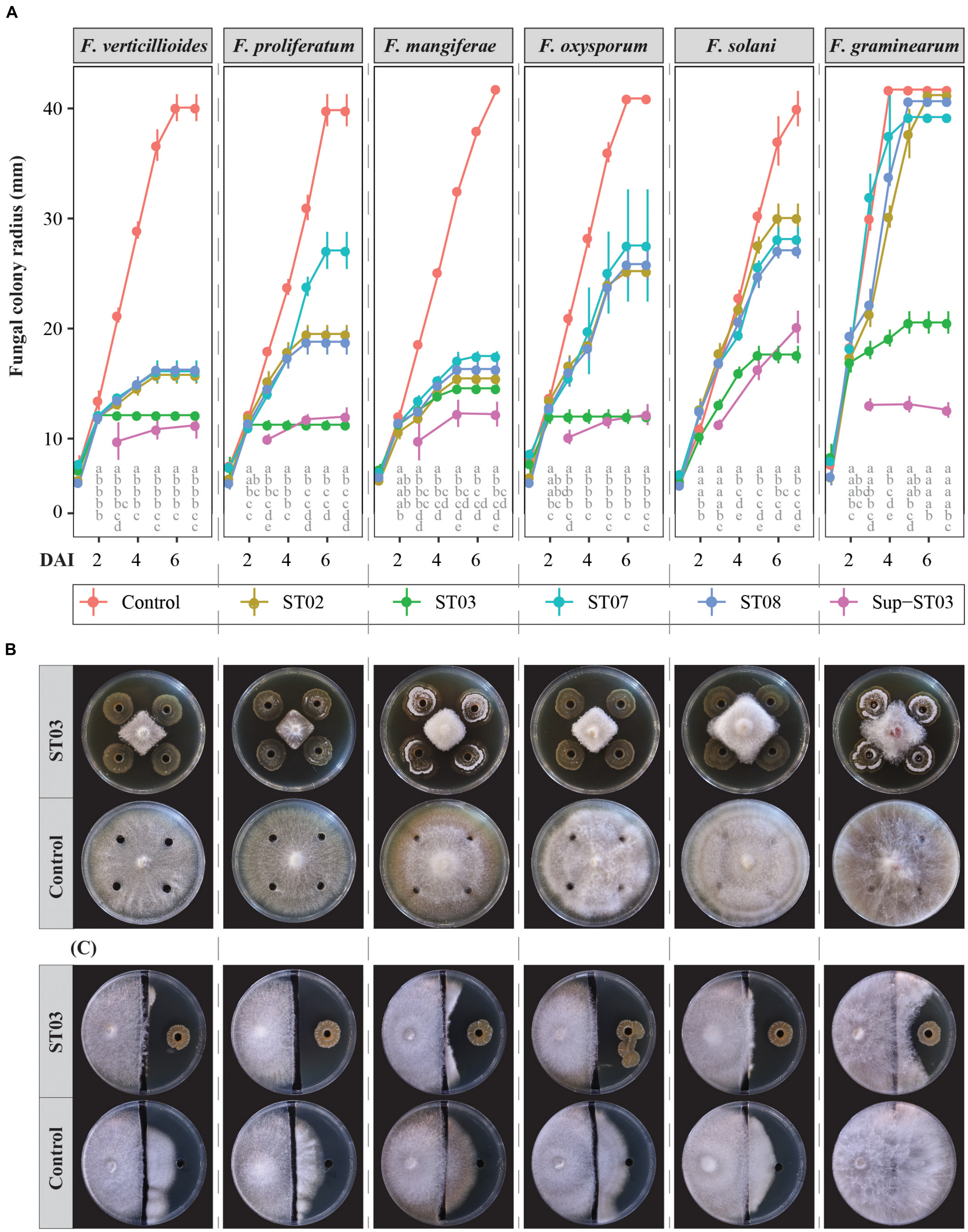

FIGURE 1 | *In vitro* antagonism of four *Streptomyces* strains (ST02, ST03, ST07, and ST08) against six *Fusarium* species. **(A)** Fungal colony radii (mm) by days of inoculation (dai) between 1 and 7 dai. Four cell suspensions of ST02, ST03, ST07, and ST08, respectively, and one 10-time-concentrated supernatant of the strain ST03 (Sup-ST03) were used. Water or tryptic soy broth were used as the mock controls. Different letters by columns pinpoint a significant difference between treatments at each time point using an ANOVA test and a *post hoc* Turkey test at α = 0.05. **(B)** An *in vitro* diffusion assay of the ST03 cell suspension at 7 dai against 6 *Fusarium* species as **(A)**. **(C)** An *in vitro* volatile assay of the strain ST03 at 11 dai. Four biological replicates were done per treatment. The experiment was repeated twice.

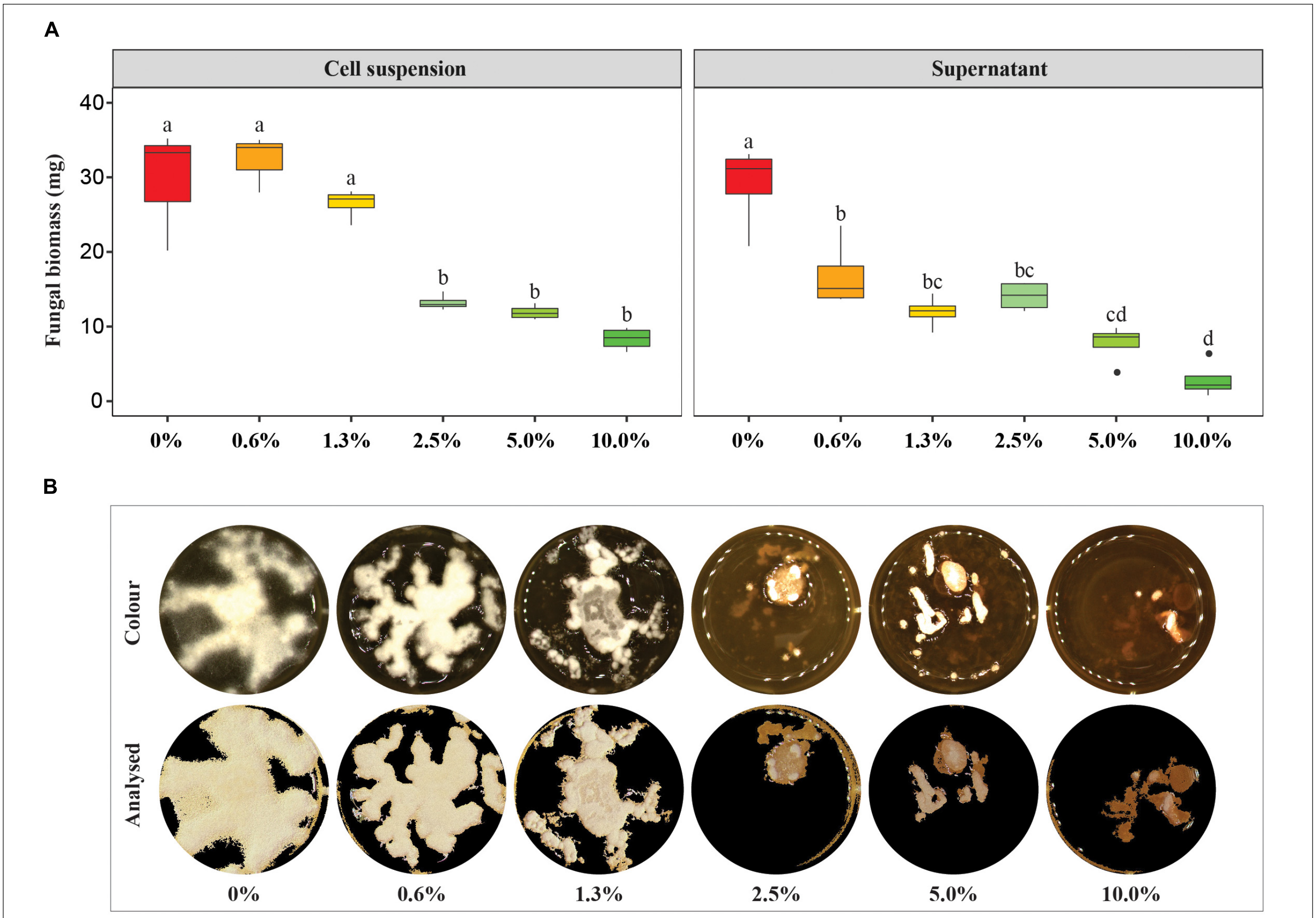

FIGURE 2 | Impact of the *Streptomyces* strain ST03 on the production of biomass of *Fusarium verticillioides* in liquid medium. **(A)** Dried *F. verticillioides* biomass (g) by treatments. An initial concentration of the cell suspension was 5×10^7 CFU mL^{-1}, the supernatant was concentrated 10 times before application. Different letters above each box depict the significant difference between treatments using an ANOVA test and a *post hoc* Turkey test at a significant level of $\alpha = 0.05$. A mock control (0% treatment) was inoculated with tryptic soy broth (TSB). **(B)** Growth surface of *F. verticillioides* in tryptic soy broth supplemented with the supernatant at different concentrations ranging 0 – 10% at 7 days of inoculation. Four biological replicates were done per treatment. The experiment was repeated twice.

to the mock control (0% treatment), specifically 13 ± 1 and 32 ± 9 mg, respectively ($p = 0.021$). This trend also occurred in the treatments co-inoculated with 5% (12 ± 1 mg, $p = 0.017$) and 10% (8 ± 2 mg, $p = 0.005$) of the cell suspension, but the change was not different from the treatment 2.5% (**Figure 2A**).

We hypothesized whether extracellular compounds secreted by strain ST03 in the supernatant are involved in the interaction with the pathogens. To explore this, we concentrated the supernatant 10 times after separating from the cell and applied both *in vitro* bioassays in the same way with the cell suspension. As indicated in the plate diffusion assay (**Figure 1A**), the supernatant of strain ST03 had a significantly effective antagonism against six *Fusarium* species. It was clear that the growth of all fungi were strongly inhibited when co-applied with the supernatant at 3 dai by their smaller radii compared to the mock controls. For example, the colony radius for *F. verticillioides* was 10 ± 2 cm vs. 21 ± 1 cm ($p < 0.001$), *F. proliferatum* (10 ± 0.5 cm vs. 18 ± 0.3 cm, $p < 0.001$), and *F. graminearum* (13 ± 1 cm vs. 30 ± 1 cm, $p < 0.001$). Surprisingly, an absolute inhibition was observed at 3 dai for all the pathogens with the exclusion of *F. solani* when the fungi did not grow further after 3 days of being inoculated with the supernatant (**Figure 1A**). This phenomenon resulted in a high inhibition efficacy against these pathogens of over 60% at 5 dai and over 70% at 7 dai (**Supplementary Figure 2**).

In addition, the antifungal activity of this supernatant was demonstrated in the liquid assay (**Figure 2**). The fungus *F. verticillioides* was significantly inhibited when co-applied with 0.6% of the supernatant at 7 dai by a significant decrease in dried biomass compared to the mock control (0%), given 17 ± 5 mg vs. 29 ± 6 mg, respectively ($p = 0.016$) (**Figure 2A**). Although no significant change in the biomass production of the fungus in a range of between 0.6 and 2.5% was observed, more suppression was observed at 5% (8 ± 3 mg dried biomass, $p < 0.010$), and 10% (3 ± 2 mg dried biomass, $p < 0.001$) than 2.5% (14 ± 2 mg) (**Figure 2A**). Moreover, it was clear that the growth surface of the fungus was remarkably smaller in the treatments 2.5, 5, and 10% compared to the mock control (**Figure 2B**).

Moreover, an inhibition efficacy calculated at a concentration of 10% was 90 ± 8%.

In conclusion, the *in vitro* antagonistic bioassays show that of the four *Streptomyces* strains, strain ST03 had the highest inhibition efficacy against all the surveyed *Fusarium* pathogens. The data also indicate the antifungal potential of the extracellular compounds in the supernatant produced by this strain.

In vivo Direct Antagonism of *Streptomyces* Strain ST03 Against *Fusarium verticillioides*

To confirm the antagonistic efficacy of strain ST03 from the *in vitro* parts, we performed two *in vivo* bioassays on maize cobs and maize seedlings infected with *F. verticillioides*. We focused on *F. verticillioides* since it is the main causal agent of FER and predominantly occurs in maize fields worldwide.

First, we assessed the impact of strain ST03 when co-applied with *F. verticillioides* on maize cobs (**Figure 3**). Data illustrated that the infection of the fungus to maize cobs was significantly counteracted when co-inoculated with the cell suspension or supernatant of strain ST03 (**Figure 3**). By using the multispectral camera, a high infection level of *F. verticillioides* on maize cobs (red area) (FV + W) was observed with a larger lesion area when compared to the negative control (W + W) (p = 0.001) (**Figures 3A,B**). However, it significantly reduced when co-applied with the cell suspension (FV + ST03) or supernatant (FV + Sup) of strain ST03 by smaller surface areas (p = 0.008), respectively (**Figure 3B**).

For the *in vivo* bioassay on maize seedlings, we checked the direct antagonism of the biocontrol strain ST03 by quantification of the *F. verticillioides* DNA in soil surrounding roots upon 14 days of infection. We planted *F. verticillioides*-infected CP888 seeds into two types of soil with and without pre-inoculation with the biocontrol strain ST03 (**Figure 4A**). qPCR data indicate that a higher level of *F. verticillioides* DNA was present in soil without pre-inoculation with strain ST03 (FV + W), compared to soil that was pre-inoculated (p = 0.009) (**Figure 4B**). This was consistent with the phenotypic characteristics of roots and lower levels of infection were observed in plants treated with the biocontrol strain compared to the non-treated plants (**Figure 4A**). All evidence shows that strain ST03 could suppress *F. verticillioides* through a direct antagonism upon infection on maize seedlings.

To sum up, both *in vivo* bioassays on maize cobs and plants demonstrate the direct antagonism of the biocontrol strain ST03 against *F. verticillioides*. In addition to cell suspension, the extracellular compounds in the supernatant secreted by this strain also show a strong inhibition against *F. verticillioides*.

Impact of *Streptomyces* Strain ST03 on Fumonisin Production When Co-applied With *Fusarium verticillioides* on Maize Cobs

The *in vivo* antagonistic bioassay on maize cobs indicated the inhibition efficacy of both cell suspension and supernatant of the strain ST03 by the reduction of the necrosis area (**Figure 3**). To evaluate the impact on fumonisin production by *F. verticillioides* we measured FBs in these cobs. Data show that levels of FB_1, FB_2, and FB_3 in maize cobs treated with the cell suspension (FV + ST03) or the supernatant (FV + Sup) was significantly lower than in the non-treated maize cobs (FV + W) (**Figure 5**). A significantly lower FB_1 median level was found in maize cobs treated with the cell suspension compared to the non-treated maize cobs, given 9 ± 5 $\mu g\ kg^{-1}$ and 80 ± 10 $\mu g\ kg^{-1}$, respectively (p = 0.012). A similar result was observed for FB_2, amounting 0 ± 4 $\mu g\ kg^{-1}$ (in FV + ST03 treatment) and 92 ± 25 $\mu g\ kg^{-1}$ (in FV + W treatment) (p = 0.009), and for FB_3 (0 ± 2 $\mu g\ kg^{-1}$ vs. 22 ± 3 $\mu g\ kg^{-1}$, p = 0.013). Similar results were also observed for the maize cobs treated with the supernatant (FV + Sup) except for FB_2 (**Figure 5**). Collectively, the biocontrol strain was capable of suppressing the production of fumonisins by *F. verticillioides*.

Impact of *Streptomyces* Strain ST03 on Plant Growth When Co-applied With *Fusarium verticillioides* on Maize Seedlings

After uncovering the direct antagonism of the biocontrol strain on maize seedlings by the reduction of the DNA level of *F. verticillioides* in soil (**Figure 4**), we explored whether the growth of plants was impacted. To examine this, we used two hybrid maize lines CP888 and *Bt/GT* NK7328 predominantly planted in the central highlands of Vietnam (Tran et al., 2021c). For line CP888, the growth of plants infected with the fungus (FV + W) was negatively affected with significantly lower fresh leaf and root biomass weight when compared to the control non-infected plants (**Figure 6A**). Moreover, a reduction of chlorophyll index was observed in the infected plants versus the control plants (**Figure 6B**). For line *Bt/GT* NK7328, although the plants were less infected compared to line CP888, their root length was also significantly shorter than the control NK7328 plants, of 10.3 ± 0.3 and 11.8 ± 0.3 cm, respectively (p = 0.029) (**Figure 6A**).

However, when co-applied with the biocontrol strain ST03 (FV + ST03), the growth of plants was significantly stimulated compared to the infected and the control plants of both maize lines. For example, for line CP888, the leaf length of plants co-applied with strain ST03 was 22.5 ± 1.4 cm (FV + ST03) versus 13.0 ± 1.0 cm of the infected plants (FV + W) (p = 0.029). Likewise, for *Bt/GT* NK7328 plants the length was 39.4 ± 1.0 cm (FV + ST03) vs. 30.2 ± 3.1 cm (FV + W) (p = 0.029) (**Figure 6A**). In both cultivars, the growth of the plants when co-applied with the biocontrol strain was also better than the growth of the control plants (W + W). It was evidenced by higher fresh biomass and leaf length (**Figure 6A**). Chlorophyll index was higher in the leaves of CP888 plants when co-applied with strain ST03, given 2.3 ± 0.1 (FV + ST03) vs. 1.4 ± 0.1 (FV + W) (p = 8e-4) or vs. 1.6 ± 0.1 (W + W) (p = 0.008) (**Figure 6B**).

We hypothesized whether this biocontrol strain ST03 acts as a plant growth-promotion agent on top of its direct antagonism. To investigate this, we examined growth indicators of plants that were applied with strain ST03 alone (W + ST03). Data revealed that the plant growth of both lines was significantly promoted

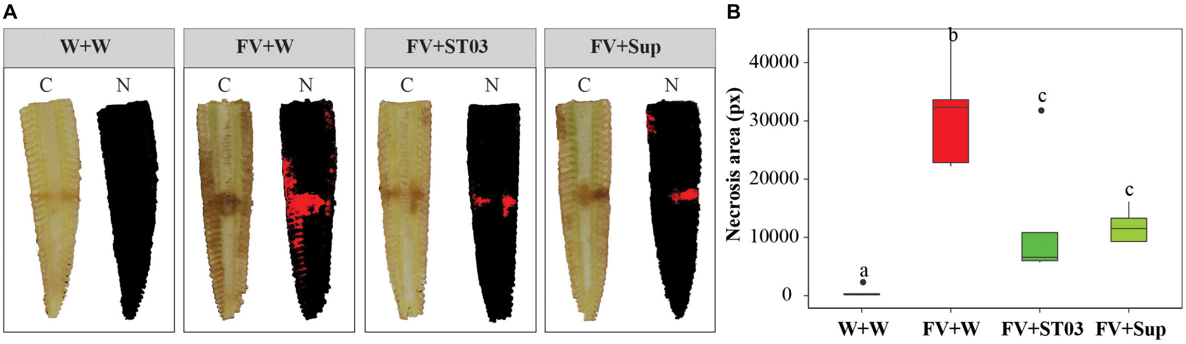

FIGURE 3 | Impact of the *Streptomyces* strain ST03 when co-applied with *Fusarium verticillioides* on maize cobs. **(A)** Imaging inside maize cobs by treatments at 7 days using a multispectral camera. Color images **(C)**, and necrosis images in red color (N). **(B)** Necrosis area (px) by treatments. Negative maize cob control was inoculated with sterile water (W + W), positive maize cob control comprised *F. verticillioides* and sterile water (FV + W), two biocontrol treatments included *F. verticillioides* and the *Streptomyces* cell suspension (FV + ST03)/or the supernatant of the strain ST03 (FV + Sup). Different letters above each box depict the significant difference between treatments using a Kruskal–Wallis test followed by a *post hoc* Dunn's test at a significant level of α = 0.05. Five biological replicates were performed per treatment. The experiment was repeated twice.

with the presence of ST03 strain compared to the control plants (W + W). For example, in *Bt/GT* NK7328 line, leaf length was longer at 40.1 ± 0.5 (W + ST03) vs. 28.4 ± 3.1 cm (W + W) ($p = 0.029$), and a higher leaf biomass of 2.1 ± 0.1 vs. 1.3 ± 0.1 g ($p = 0.029$) (**Figure 6A**). Similar results were observed in plants of line CP888 (**Figure 6A**). The chlorophyll index of plants applied with strain ST03 was significantly higher than that of the control plants, given 2.2 ± 0.1 and 1.6 ± 0.1, respectively ($p = 0.008$) (**Figure 6B**). Nevertheless, the primary root length of plants applied with the biocontrol strain was shorter compared to the control. It was true in both lines CP888 [of 9.5 ± 0.2 (W + ST03) vs. 11 ± 0.6 (W + W), ($p = 0.145$) and *Bt/GT* NK7328 (of 10.0 ± 0.6 vs. 11.8 ± 0.3, ($p = 0.028$)]. Higher fresh root biomass was observed in plants applied with strain ST03 compared to the control plants (**Figure 6A**) since root hair formation was induced as shown in **Figure 4A**.

From the aforementioned data, we can conclude that the *Streptomyces* strain ST03 not only hampered the growth of *F. verticillioides* but also stimulated the growth of plants.

Impact of *Streptomyces* Strain ST03 on Intrinsic Plant Defense Response and Plant Growth Promoting Hormones When Co-applied With *Fusarium verticillioides* on Maize Seedlings

Concerning the action of this biocontrol strain on modulating plant growth promoting hormones, the involvement of genes associated with the metabolic pathways of auxin/indole-3-acetic acid (Auxin/IAA), abscisic acid (ABA), and gibberellic acid (GA) were examined. In the literature, the timely activation of key genes in the biosynthesis pathways of salicylic acid (SA), jasmonic acid (JA), 1,4-benzoxazine-3-ones (BXs), and pathogenesis-related proteins (PRs) plays a dominant role in maize resistance to fungal plant pathogens (Bacon et al., 2007; Ding et al., 2015; Wang et al., 2016). Thus, we further uncovered modes of action of the biocontrol strain ST03 on these secondary metabolism-related genes.

The Action on Key Genes in the Biosynthesis of Auxin, Gibberellic Acid, and Abscisic Acid

To assess the impacts of the biocontrol strain on the biosynthesis of the typical plant growth hormones Auxin/IAA, GA, and ABA, we verified the expression profiles of the genes encoding the auxin responsive factors 1 and 2 (*ARF1* and *ARF2)*, the auxin transporter-like protein (*AUX1)* (for auxin signaling pathway), the glycine-rich protein (*ABI)* (for ABA signaling pathway), and the anther ear 1 (*AN1)* (for GA signaling pathway). These genes were selected because of their important biological function in the biosynthesis pathways of these phytohormones. For example, *ARF1* and *ARF2* encode two auxin transcription factors, while *AUX1* encodes an auxin influx transporter (Zhang et al., 2019). Gene *ABI* plays a regulatory role in ABA signaling by encoding a glycine-rich protein (Ding et al., 2015). Finally, *AN1* is capable of regulating the GA phytohormone biosynthesis in maize (Zhang et al., 2019).

In roots, at early time points 1 and 2 dai, a clear downregulation of *AUX1*, *ARF1*, and *ARF2* was observed in plants applied with strain ST03 only (W + ST03 treatment) (**Figure 7**). However, later at 4 and 8 dai, *ARF2* was significantly up-regulated, respectively representing 2.7-fold ($p = 0.029$) and 5.5-fold ($p = 0.029$), while *ARF1* and *AUX1* returned to the basal levels compared to the control plants. Similar trends were observed when co-applied with the pathogen (FV + ST03 treatment). Especially, at 2 dai, there was a concomitant downregulation of *AUX1* ($\text{Log}_2\text{FC} = -2.7$, one-tailed $p = 0.065$), *ARF1* ($\text{Log}_2\text{FC} = -3.4$, $p = 0.029$), and *ARF2* ($\text{Log}_2\text{FC} = -9.7$, $p = 0.029$). By contrast, when applied with *F. verticillioides* alone, at 1 and 2 dai, the expression levels of *AUX1*, *ARF1,* and *ARF2* were not different from the control plants. Comparable to the other treatments, *ARF2* was upregulated at 4 (2.7-fold, $p = 0.029$) and 8 (3.9-fold, $p = 0.029$) dai. Interestingly, similar results were observed in leaves in all the treatments.

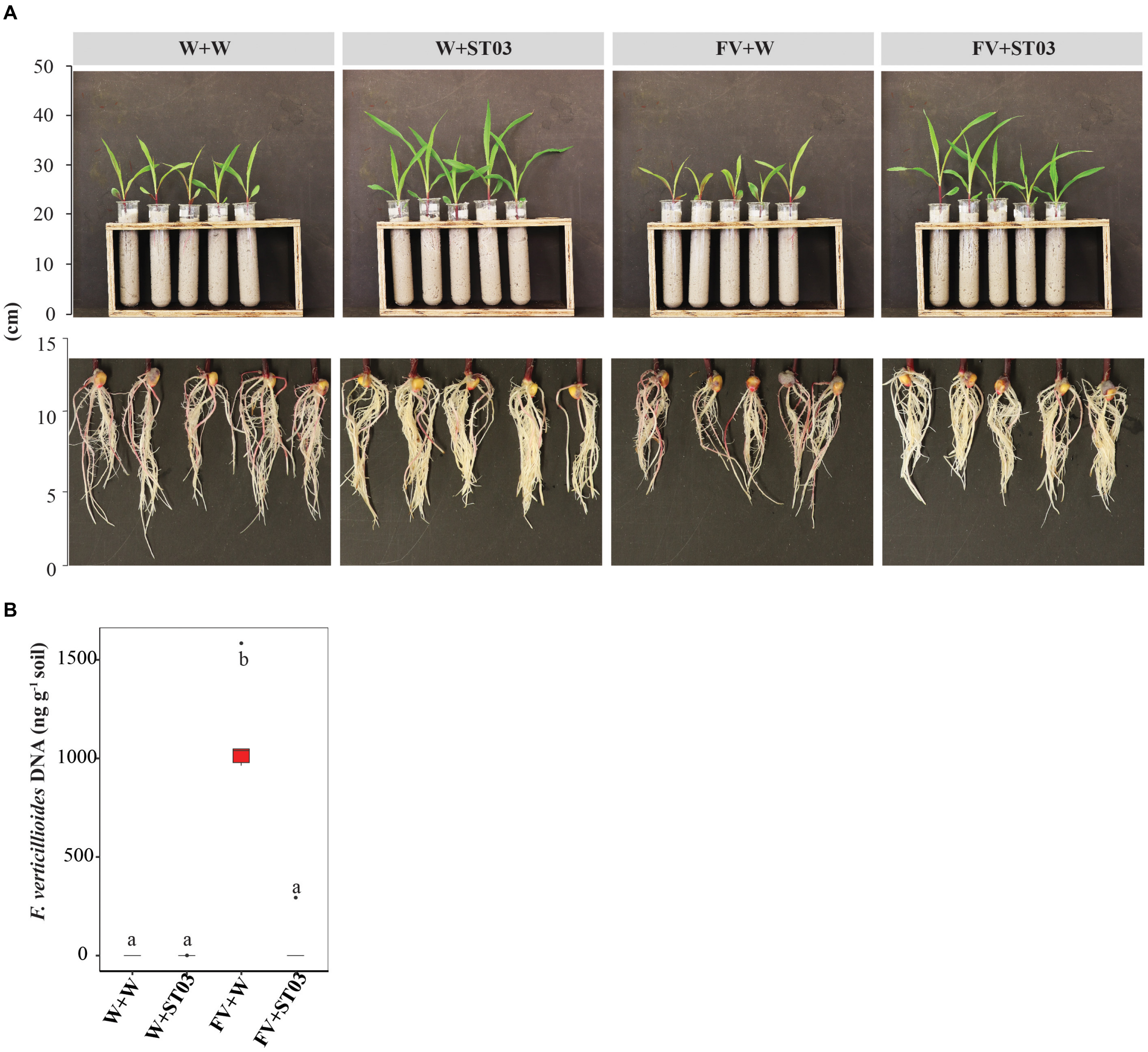

FIGURE 4 | *In vivo* direct antagonism of the *Streptomyces* strain ST03 on maize seedlings based on levels of *Fusarium verticillioides* DNA in soil. **(A)** Plants and roots at 14 days after inoculation. **(B)** The fungal DNA content (ng g^{-1}) in soil by treatments using a qPCR method. *F. verticillioides*-infected maize seeds were planted in soil pre-inoculated with the biocontrol strain ST03 (FV + ST03) and planted in soil mock-inoculated with sterile water (FV + W); *F. verticillioides* non-infected maize seeds were planted in soil pre-inoculated with the biocontrol strain (W + ST03) and planted in mock soil (W + W). A hybrid CP888 maize line was used. Different letters above each box depict the significant difference between treatments using a Kruskal–Wallis test followed by a *post hoc* Dunn's test at a significant level of $\alpha = 0.05$. Five biological replicates were performed per treatment. The experiment was repeated twice.

For the GA biosynthesis-related gene *AN1*, in roots, at 2 dai, a very small downregulation was observed at this gene [$\text{Log}_2\text{FC} = -1$, $p = 0.1$] when applied with the biocontrol strain (**Figure 7**). Later *AN1* was induced at 8 dai (4.7-fold, $p = 0.029$). In the concomitant presence of *F. verticillioides* and the biocontrol strain, a strong suppression of *AN1* was observed at 2 dai ($\text{Log}_2\text{FC} = -5.7$, one-tailed $p = 0.065$), subsequently, at 8 dai, this gene returned to the basal level as in the control plants. When applied with *F. verticillioides* alone, the expression level of *AN1* was not different from the control plants at all time points. In leaves, similar trends of *AN1* expression were obtained from roots for all treatments after 1 dai.

For the profile of ABA-related gene *ABI* in roots when co-applied with the pathogen (FV + ST03), at the earlier time-points 1 and 2 dai *ABI* was triggered 8.8-fold ($p = 0.029$) and 2.2-fold (one-tailed $p = 0.057$) respectively and returned to the basal level. A similar trend was observed at the singular inoculation of *F. verticillioides*. Similar observations were obtained in leaves by treatments.

In conclusion, the findings indicate that the preinoculation of biocontrol strain ST03 in the soil resulted in a downregulation of the auxin responsive genes *ARF1*, *ARF2*, and *AUX1*, and the GA-related gene *AN1* at earlier time-points even in absence of *F. verticillioides*. Afterward, the expression of these genes

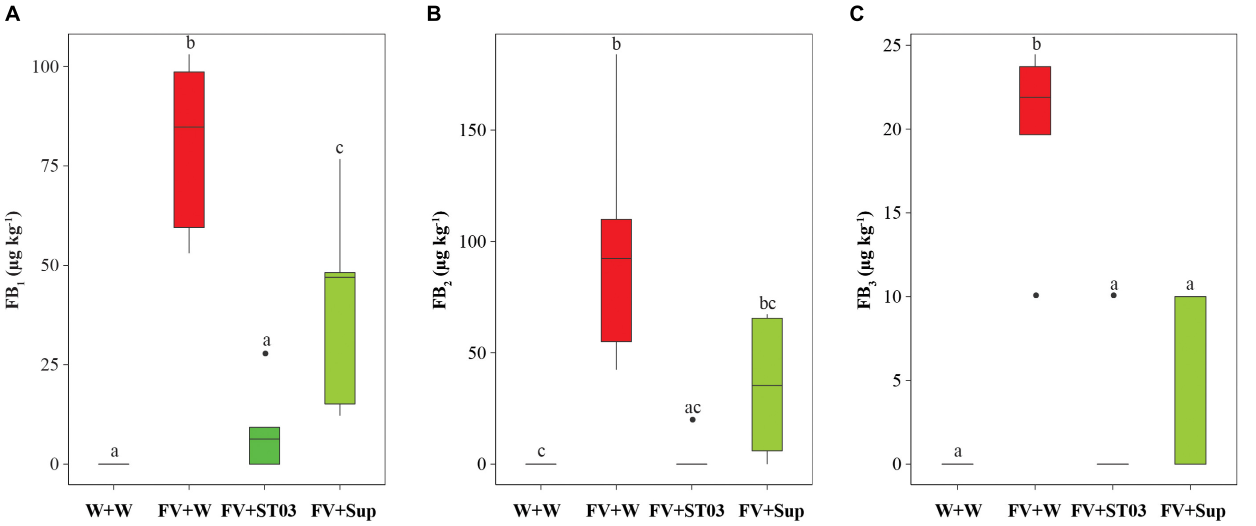

FIGURE 5 | Impact of the *Streptomyces* strain ST03 on the production of fumonisin B$_1$ (FB$_1$) **(A)**, FB$_2$ **(B)**, and FB$_3$ **(C)** by *Fusarium verticillioides* on maize cobs. The negative maize cob control was inoculated with sterile water (W + W), positive maize cob control comprised *F. verticillioides* and sterile water (FV + W), the two biocontrol treatments included *F. verticillioides* and the *Streptomyces* cell suspension (FV + ST03) or the supernatant of the strain ST03 (FV + Sup). Different letters above each box depict the significant difference using a Kruskal–Wallis test followed by a *post hoc* Dunn's test at a significant level of α = 0.05. Five biological replicates were performed per treatment. The experiment was repeated twice.

was similar to the control treatment or upregulated from 4 dai onward, particularly, *ARF2*. By contrast, the singular infection of *F. verticillioides* did not impact the expression levels of these genes.

The Action on Key Genes in the Biosynthesis of Salicylic Acid, Jasmonic Acid, and Pathogenesis-Related Proteins

To assess the action on the SA, JA, and PRs biosynthesis, we used genes encoding the hallmark enzymes phenylalanine ammonialyase (*PAL)* (for SA pathway), allene oxide synthase (*AOS*), and lipoxygenases (*LOX3* and *LOX10)* (for JA pathway), and PR proteins (*PR1*, *PR2*, *PR3*, and *PR10)* (for PRs biosynthesis). The activation of these genes has been demonstrated to be involved in the defense responses of maize to biotic stresses (Lanubile et al., 2010; Tzin et al., 2017; Zhang et al., 2019).

In roots, for plants applied with train ST03 alone (W + ST03), the expression level of *PAL* remained unchanged compared to the mock control plants at all time points (**Figure 7**). Similar results were observed in plants co-applied with the pathogen (FV + ST03) with the exception at 4 dai where a small but significant induction of *PAL* (3.3-fold, p = 0.028) was observed. In leaves, at early time-points 1 and 2 dai, *PAL* seemed to be upregulated in all the treatments (**Figure 7**). Notably, at 2 dai, the gene expression levels were high of respectively, 5.4-fold (in W + ST03, one-tailed p = 0.15), 13.4-fold (in FV + ST03, p = 0.029), and 4.7-fold (in FV + W, p = 0.029).

Turning to the effects on *PR1*, *PR2*, *PR3,* and *PR10* genes, in roots, at 2 dai, the singular presence of the biocontrol strain resulted in a strong induction of *PR2* (6.1-fold, p = 0.028), *PR3* (6.5-fold, p = 0.028), and *PR10* (5.2-fold, p = 0.028) when compared to the mock control plants (**Figure 7**). The expression levels of *PR2* and *PR10* subsequently reduced till the basal levels at 4 dai onward. Remarkably, the concomitant application of *F. verticillioides* and the biocontrol strain ST03 resulted in a strong induction of the four PRs encoding genes at 2 and 4 dai. For example, at 4 dai, the expression levels were 6.3-fold of *PR1* (p = 0.028), 108.2-fold of *PR2* (p = 0.029), 11.3-fold of *PR3* (p = 0.029), and 3.6-fold of *PR10* (p = 0.029) compared to the mock control treatment (**Figure 7**). Similar results were observed for plants applied with the pathogen alone, indicating that *F. verticillioides* triggers the PR genes.

In leaves, when applied with the biocontrol strain alone, the expression of *PR1*, *PR2*, *PR3*, and *PR10* was observed at the basal levels as in the control plants. When in co-inoculation of *F. verticillioides* and the biocontrol strain, at 1 dai, a small induction of *PR1*, *PR3*, and *PR10* was present, particularly, there was a significantly clear upregulation of *PR3* (8.8-fold, p = 0.029) compared to the mock control treatment. Remarkably, at 2 dai, and 4 dai, these genes were still upregulated together with the concomitant upregulation of the *PR2* gene. Later, at 8 dai, all the PRs genes were regulated toward the basal levels as in the control treatment. Similar trends were observed when applied with the pathogen alone (**Figure 7**). Nonetheless, at 2 dai, data show that the expression levels of *PR2*, *PR3*, and *PR10* was lower in plants applied with the pathogen than in plants co-applied with the biocontrol strain and *F. verticillioides*, representing *PR2* [8.1-fold (FV + W) vs. 83.8-fold (FV + ST03)], *PR3* (3.7-fold vs. 17-fold), and *PR10* (7-fold vs. 37-fold) (**Figure 7**).

As for the expression of JA pathway-related genes, in roots, at 1 and 2 dai, a very small induction of *AOS*, but not of *LOX3* and *LOX10* was observed when applied with the biocontrol strain alone (**Figure 7**). Then, at 4 dai, *AOS* was suppressed

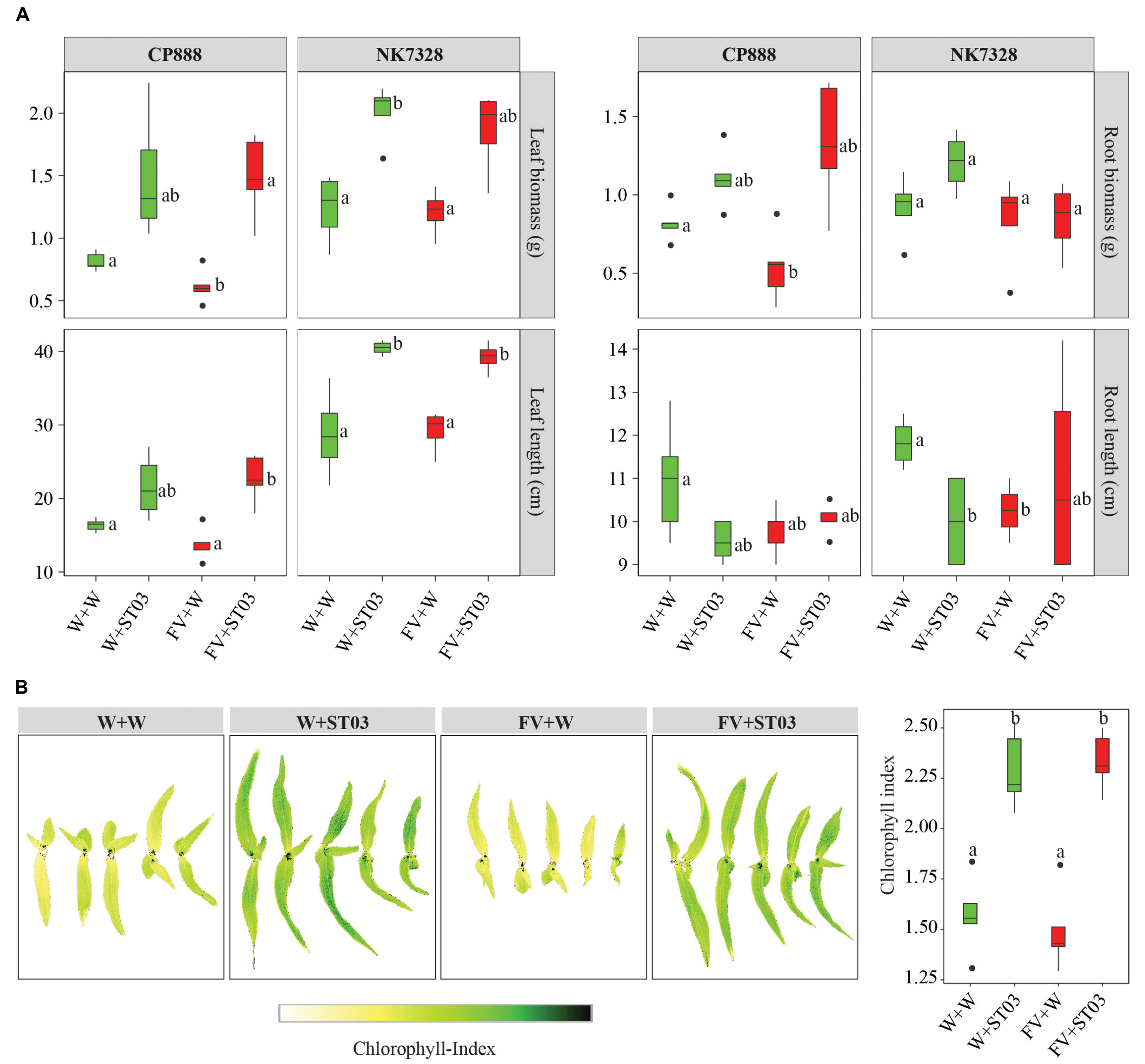

FIGURE 6 | (A) Impact of the *Streptomyces* strain ST03 on plant growth using two hybrid maize cultivars CP888 and *Bt/GT* NK7328 at 14 days after planting. **(B)** Chlorophyll level by treatments on CP888 plants. Chlorophyll content was assessed using a multispectral camera; white is a low chlorophyll index (ChlIdx) and dark green is high. *F. verticillioides*-infected maize seeds were planted in soil pre-inoculated with the biocontrol strain ST03 (FV + ST03) and planted in mock-inoculated with sterile water soil (FV + W); *F. verticillioides* non-infected maize seeds were planted in soil pre-inoculated with the biocontrol strain (W + ST03), or planted in mock soil (W + W). Different letters above each box depict the significant difference between treatments by cultivars using a Kruskal–Wallis test followed by a *post hoc* Dunn's test at a significant level of α = 0.05. Five biological replicates were done per treatment. The experiment was repeated twice.

with Log_2FC levels of respectively, –2.8 ($p = 0.029$), while *LOX3* showed a small upregulation (4.4-fold, $p = 0.029$). When co-applied with the pathogen (FV + ST03), at 2 dai, an upregulation of *AOS* (5.6-fold, $p = 0.029$) and *LOX3* (1.9-fold, $p = 0.029$) was observed. *LOX3* was upregulated (3.4-fold, $p = 0.029$) at 4 dai, and then was downregulated at 8 dai ($\text{Log}_2\text{FC} = -1.8$, $p = 0.029$). Similar trends were observed in plants applied with the pathogen alone (**Figure 7**).

In leaves, at 1 dai, only *AOS* was slightly induced by the biocontrol strain (3.1-fold, one-tailed $p = 0.25$) (**Figure 7**). Subsequently, the small induction of *AOS* (2-fold, one-tailed $p = 0.15$) continued to be observed at 2 dai besides induction of *LOX10* (5.8-fold, $p = 0.029$). At 4 dai, both *AOS* and *LOX10* were down-regulated with Log_2FC values of –1.4 (one-tailed $p = 0.06$) and –2.3 (one-tailed $p = 0.05$), respectively. At 8 dai, they were modulated back to the basal levels as in the control treatment. Similar trends were observed in plants co-applied with *F. verticillioides*. In contrast, in presence of *F. verticillioides* alone, the expression levels of *AOS*, *LOX3*, and *LOX10* were not different from the control treatment.

To sum up, the data show that the biocontrol strain ST03 slightly induced *PAL* (SA pathway), *AOS* and *LOX3* (JA

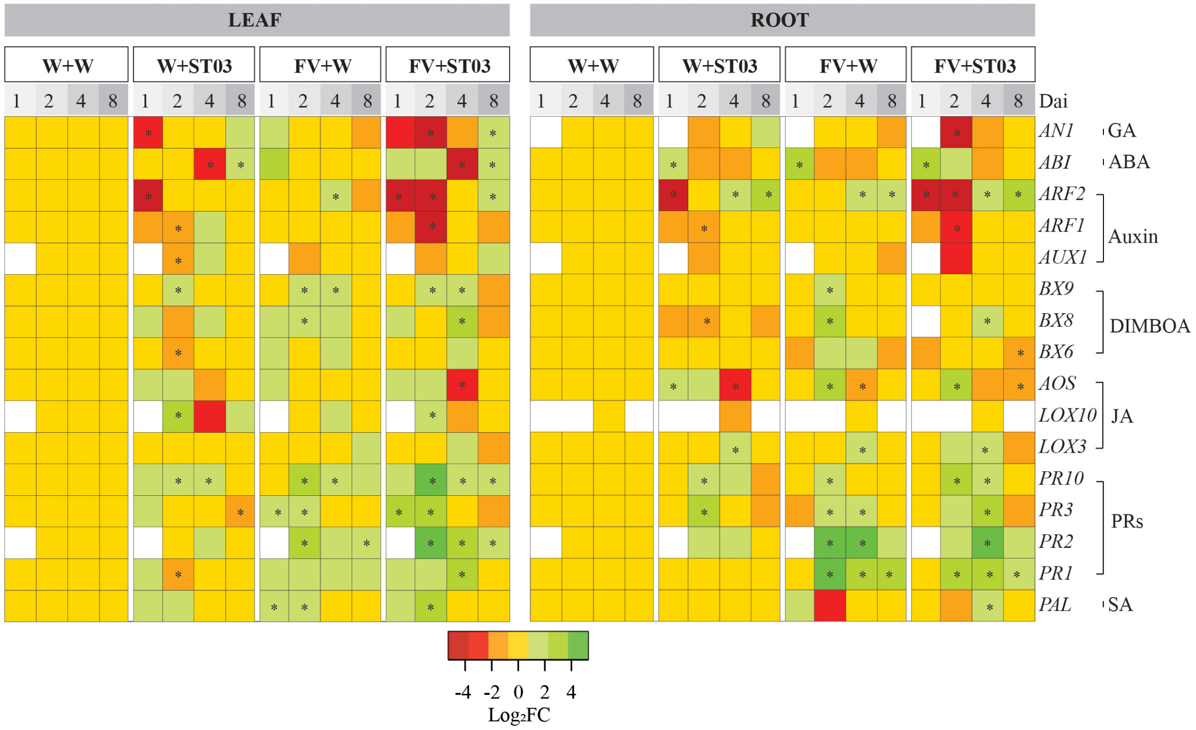

FIGURE 7 | Heat map of the differential gene expression levels in leaf and root by treatments at four-time points. This work applied *Bt/GT* NK7328-maize line. The colors indicate the FC changes (FC) of gene expression in log base 2. Up-regulation of each gene is shown in green (increased abundance), down-regulation is shown in red (decreased abundance), no change is shown in yellow, and absence of expression is indicated in white. Salicylic acid (SA); jasmonic acid (JA); 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA); abscisic acid (ABA); gibberellic acid (GA); auxin/indole-3-acetic acid (Aux/IAA). dai, days after inoculation. Defense response was monitored at 1, 2, 4, and 8 dai. *F. verticillioides*-infected maize seeds were planted in soil pre-inoculated with the biocontrol strain ST03 (FV + ST03) and planted in mock-inoculated with sterile water soil (FV + W); *F. verticillioides* non-infected maize seeds were planted in soil pre-inoculated with the biocontrol strain (W + ST03); or planted in mock soil (W + W). The final concentration of *Streptomyces* ST03 in soil was 10^6 CFU g^{-1}. At each time point, four biological replicates were done per treatment. The experiment was repeated twice. Symbol (*) depicts the significant difference between treatments and the control mock plants using a one-tailed Kruskal–Wallis test at a significant level of $\alpha = 0.05$.

pathway), and the PRs-related genes (*PR1*, *PR2*, *PR3*, and *PR10*) even in absence of a pathogen. Secondly, root infection by *F. verticillioides* resulted in a strong induction of PRs-related genes in both roots and leaves.

The Action on Key Genes in the Biosynthesis of 1,4-Benzoxazine-3-Ones

To determine the effect of the biocontrol strain ST03 on the biosynthesis of BXs defensive secondary metabolites, we examined three key BXs responsive genes *BX6*, *BX8*, and *BX9* in the leaves and roots of plants. The induction of these genes might increase the resistance of maize plants against *F. verticillioides*.

In roots, the expression of BXs-related genes remained unchanged in plants applied with the biocontrol strain alone (W + ST03) (**Figure 7**) except for a slight down-regulation of the *BX8* at 2 dai ($Log_2FC = -1.8$, $p = 0.029$) compared to the mock control plants. A similar expression was observed in plants that were co-applied with the pathogen (FV + ST03). By contrast, at 2 dai, these genes were found at upregulated expression levels in plants applied with the pathogen alone (FV + W), especially for *BX8* with the 5.5-fold change ($p = 0.029$), demonstrating that *F. verticillioides* could induce the BXs-mediated resistance response of maize roots (**Figure 7**).

Considering the responses of leaves, we observed a small but non-significant regulation of the BXs-related genes in plants when applied with the *Streptomyces* strain only compared to the mock control plants. On the contrary, a slight induction of these BXs-related genes appeared in the leaves of the plants when co-applied with the biocontrol strain and *F. verticillioides*. Specifically, at 2 dai, the expression level of *BX9* significantly increased (2.8-fold, $p = 0.029$), and notably, a concomitant upregulation of *BX6*, *BX8*, and *BX9* was present at 4 dai, of which *BX8* showed a significant expression level with 6.5-fold change ($p = 0.029$) compared to the mock control plants (**Figure 7**). At 2 dai the BXs-related genes *BX8* (4.7-fold, $p = 0.029$) and BX9 (2.5-fold, $p = 0.029$) were slightly triggered upon infection of *F. verticillioides*.

In conclusion, the *BX6*, *BX8*, and *BX9* expression data show that the biocontrol strain ST03 did not induce these genes in both leaves and roots in the absence of the pathogen. While a small induction was observed in leaves when in concomitant presence of *F. verticillioides*. The BXs pathway in maize seedlings was also slightly triggered by a singular *F. verticillioides* infection.

DISCUSSION

Fusarium ear rot is a severe disease in maize mainly caused by *F. verticillioides*, which not only reduces crop production and grain quality but also poses a health risk due to the production of fumonisins, which are possibly carcinogenic to humans. This fungal phytopathogen increasingly occurs in maize fields worldwide. To the best of our knowledge, no FER-resistant maize inbred lines are registered on market. Together with the strict legislation of agrochemicals, searching for rhizobacteria-based BCAs is a sustainable and eco-friendly strategy. Prior reports have documented that BCAs mainly suppress fungal crop pathogens through direct antagonism and indirect induction of the plant systemic resistance (Pineda et al., 2010; Khan et al., 2020; Raymaekers et al., 2020). Yet, to date, molecular insights into the intricate interaction of *F. verticillioides – Streptomyces* – maize are scarce.

This study demonstrated that the *Streptomyces* strain ST03 not only directly antagonizes the pathogen but also interferes with plant defense systems and promotes the growth of plants. Moreover, the *in vitro* data show a wide spectrum of direct antagonism of this biocontrol strain ST03 to other fungal crop diseases, e.g., FHB in wheat caused by *F. graminearum*, *Fusarium* basal rot (FBR) in onion caused by *F. oxysporum*.

In agreement with prior studies *Streptomyces* spp. can produce antibiosis-mediated compounds, e.g., fungal cell wall-degrading enzymes (such as 1,3-β-glucanase, and chitinase) (Getha and Vikineswary, 2002), volatile compounds (Jones and Elliot, 2017), and other toxic extracellular metabolites that can defeat the pathogen in a distance due to their rapid diffusion (Getha and Vikineswary, 2002; Faheem et al., 2015). The cell-free supernatant – a reservoir of extracellular compounds excreted by the strain ST03, showed high efficacy against the pathogens. The cell-free supernatant inhibited the proliferation of *F. verticillioides* in both *in vitro*, and *in vivo* bioassays.

Apart from the direct antagonism, the strain ST03 hampered the mycotoxin production of *F. verticillioides*. The levels of FB_1, FB_2, and FB_3 were significantly reduced in the *F. verticillioides* infected cobs treated with the *Streptomyces* ST03 cell suspension or cell-free supernatant compared to the non-treated cobs. A possibility for the reduction of fumonisins is the inhibition of fungal growth by strain ST03. In agreement with Nguyen et al. (2020), *Streptomyces* spp. not only inhibit the growth of *F. verticillioides*, but also reduce fumonisin production via interfering with the fungal metabolic pathways, particularly mycotoxigenesis. Notwithstanding, the underlying mechanisms on the actions on mycotoxigenesis of *F. verticillioides* by *Streptomyces* spp. are often lacking. A study by Strub et al. (2019) has indicated that *Streptomyces* sp. suppresses the fumonisin biosynthesis-involved genes when co-cultured with *F. verticillioides.* In addition, although there is no report on biodegradation of fumonisins by *Streptomyces* spp., biodegradation of aflatoxin B_1 by this microbial community has been reported (Harkai et al., 2016). Several studies have shown an effective biocontrol potential of *Streptomyces* spp. in suppression of the growth of *F. graminearum*, *Aspergillus flavus,* and their mycotoxin production (Boukaew et al., 2020; Colombo et al., 2020).

Interestingly, the biocontrol strain ST03 is also a plant growth-promoting candidate. Data show that the physiological and developmental index, e.g., leaf length, leaf biomass, and chlorophyll content were significantly stimulated when applied with the biocontrol strain alone or when co-applied with the pathogen. Numerous studies have demonstrated that actinobacteria can produce plant growth hormones, especially indole-3-acetic acid (IAA) (Goudjal et al., 2013; Lin and Xu, 2013). We hypothesize that the production of IAA by *Streptomyces* plays a pivotal role in the rhizosphere colonization and plant growth promotion of plants. IAA is well-known as an important signaling molecule in plant-microbe interaction (Spaepen and Vanderleyden, 2011). In the present work, it is possible that IAA produced by the *Streptomyces* strain ST03 resulted in the suppression of auxin pathway-related genes *ARF1*, *ARF2,* and *AUX1* in plants (**Graphical Abstract**), which could not only be a strategy used by ST03 to circumvent the plant defense system, contributing to better bacteria-plant-root interaction (Spaepen and Vanderleyden, 2011) but especially this suppression might also decrease susceptibility of plants to the pathogen (Navarro et al., 2006). A report by Ding et al. (2008) showed that repression of auxin/IAA signaling enhances the resistance of rice to *Xanthomonas oryzae* pv. *Oryzae.* This is because the IAA is an elicitor of the biosynthesis of expansins that loosen the plant cell wall, but this loosening can facilitate root penetration of the pathogen (Ding et al., 2008). Thus, inhibition of expansins activity is a result of the repression of auxin signaling.

In addition to the suppression of auxin signaling, the *AN1* expression data show that the biocontrol strain suppresses the GA biosynthesis pathway. A possibility is that *Streptomyces* strain ST03 is also capable of producing GA beside IAA, resulting in suppression of GA signaling in plants. Toumatia et al. (2016) reported that a *Streptomyces* strain has the ability to produce both IAA and GA, which promote the growth of wheat seedlings. Even though the roles of bacterial GA in plant-microbe communication are not fully understood yet, numerous studies have evidenced that GA producing microorganisms are categorized as plant growth-promoting rhizobacteria (Bottini et al., 2004).

Upon inoculation of plants with the biocontrol strain ST03, we observed a change in root architecture, mainly as an increase in lateral roots and root hairs and shortening of the root length (**Figures 4A**, **6A**). Similar modes of action have been reported for plant growing rhizobacteria on roots (Dobbelaere et al., 1999). Apart from bacterial IAA biosynthesis, bacterial activation of, ACC (1-aminocyclopropane-1-carboxylate) deaminase is also responsible for plant growth promotion through reduction of ethylene (Spaepen and Vanderleyden, 2011).

Together with direct antagonism and the action on auxin and GA signaling, our findings indicate that the biocontrol strain ST03 slightly induces *PAL* (in SA pathway), *AOS,* and *LOX3* (in JA pathway) and PRs-related genes (*PR1*, *PR2*, *PR3,* and *PR10*) even in the absence of a pathogen. Prior studies have also shown that rhizobacteria can induce the archetypal defense pathways in plants related to SA and/or JA (Conn et al., 2008; Kurth et al., 2014; Tan et al., 2020; Vergnes et al., 2020). Notably, the accumulation of SA and JA correlate to the strong activation of the PRs-related genes *PR1*, *PR2*, *PR3*, and *PR10* using a tobacco-based model plant (Niki et al., 1998). *PR2* and *PR3* encoding glucanase and chitinase respectively, which are the two foremost antifungal components, play crucial roles in the plant defense system (Van Loon, 1997).

In addition, BXs and their derivatives such as 2,4–dihydroxy–1,4–benzoxazin–3–one (DIBOA), 2,4–Dihydroxy–7–methoxy–2H–1,4–benzoxazin–3(4H)–one (DIMBOA), and 6–methoxybenzoxazolin–3–one (MBOA) are the key defense secondary metabolites in maize against pathogens and insects (Niemeyer, 2009; Lanubile et al., 2017). Even though the biosynthesis pathways of such antimicrobial metabolites in maize have been well documented (Cotton et al., 2019), the study of the action of actinobacteria on the BXs biosynthesis pathways in maize remains poorly understood. In this current work, the *Streptomyces* strain ST03 did not induce the BXs biosynthesis-related genes, but when co-applied with *F. verticillioides*, a small amount of induction of *BX6*, *BX8*, and *BX9* was observed in the leaves. Ding et al. (2015) demonstrated that resistance of maize to *Bipolaris maydis*, a pathogen causing corn leaf blight, strongly correlates to up-regulation of BXs gene family (*BX1* and *BX8*). Also, BXs-dependent resistance of maize toward *Spodoptera littoralis*, a leaf feeder, has been reported (Maag et al., 2016). In agreement with Tran et al. (2021c), *F. verticillioides* induced the upregulation of the BXs genes in leaves of line *Bt/GT* NK7328. This finding might be helpful for one who wants to develop FER-resistant maize lines associated with these genes.

CONCLUSION

Our data demonstrated the *Streptomyces* strain ST03 as a promising and effective biocontrol and plant growth-promoting candidate toward good management of *Fusarium* ear rot in maize. We uncovered two modes of action of strain ST03 against *F. verticillioides* comprising (1) direct antagonism and (2) modulating plant defense system via transient regulation of auxin signaling and archetypal defense pathways in plants. This strain could thus be used to develop a biofertilizer formulation toward sustainable agriculture.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MZ614615; https://www.ncbi.nlm.nih.gov/genbank/, MZ614616; https://www.ncbi.nlm.nih.gov/genbank/, MZ614619; and https://www.ncbi.nlm.nih.gov/genbank/, MZ614620.

AUTHOR CONTRIBUTIONS

TT: conceptualization, performing, formal analysis, writing, and original draft. MA: reviewing and editing. SD: supervision, reviewing, and editing. FD: supervision, reviewing, and editing. ME: supervision, reviewing, and editing. KA: supervision, conceptualization, reviewing, and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by VLIR-UOS (No. VN2017TEA452A103) and the Hercules Foundation (No. AUGent/15/17).

ACKNOWLEDGMENTS

We acknowledge Nguyen Thi Dieu Hanh, a senior lecturer at Industrial University of Ho Chi Minh City, Vietnam, who provided support in the isolation of *Streptomyces* spp. We would like to thank Mario Van de Velde, Christ'l Detavernier, and Frédéric Dumoulin, senior technicians at the Centre of Excellence in Mycotoxicology and Public Health, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.755733/full#supplementary-material

Supplementary Figure 1 | (A) *In vitro* diffusion assay. **(B)** *In vitro* volatile assay using tryptic soy agar. **(C)** *In vitro* assay using tryptic soy broth. **(D)** *In vivo* assay on maize cobs. **(E)** Workflow diagram of *in planta* assay.

Supplementary Figure 2 | *In vitro* inhibition efficacy (%) of four *Streptomyces* strains (ST02, ST03, ST07, and ST08) against six *Fusarium* species. The inhibition efficacy was calculated as a formula: (Rm – Rb)/Rm × 100 where Rm is a fungal radius of a mock control, and Rb is a fungal radius of a biocontrol treatment. Different letters by columns pinpoint a significant difference between treatments at each time point using an ANOVA test and a *post hoc* Turkey test at $\alpha = 0.05$. Four cell suspensions of ST02, ST03, ST07, and ST08, respectively, and one 10-time-concentrated supernatant of the strain ST03 (Sup-ST03) were used. Water or tryptic soy broth were used as the mock controls. Four biological replicates were done per treatment. The experiment was repeated twice.

Supplementary Table 1 | Primers of target genes and reference genes used in this study.

REFERENCES

Abbasi, S., Safaie, N., Sadeghi, A., and Shamsbakhsh, M. (2019). Streptomyces strains induce resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 3 in tomato through different molecular mechanisms. *Front. Microbiol.* 10:1505. doi: 10.3389/fmicb.2019.01505

Bacon, C. W., Glenn, A., and Yates, I. (2008). *Fusarium verticillioides*: managing the endophytic association with maize for reduced fumonisins accumulation. *Toxin Rev.* 27, 411–446. doi: 10.1080/15569540802497889

Bacon, C. W., Hinton, D. M., Glenn, A. E., Macías, F. A., and Marin, D. (2007). Interactions of *Bacillus mojavensis* and *Fusarium verticillioides* with a benzoxazolinone (BOA) and its transformation product, APO. *J. Chem. Ecol.* 33, 1885–1897. doi: 10.1007/s10886-007-9347-5

Blandino, M., Scarpino, V., Vanara, F., Sulyok, M., Krska, R., and Reyneri, A. (2015). Role of the European corn borer (*Ostrinia nubilalis*) on contamination of maize with 13 *Fusarium mycotoxins*. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 32, 533–543. doi: 10.1080/19440049.2014.966158

Bottini, R., Cassán, F., and Piccoli, P. (2004). Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. *Appl. Microbiol. Biotechnol.* 65, 497–503. doi: 10.1007/s00253-004-1696-1

Boukaew, S., Petlamul, W., and Prasertsan, P. (2020). Efficacy of *Streptomyces philanthi* RL-1-178 culture filtrate against growth and aflatoxin B_1 production by two aflatoxigenic fungi on maize seeds. *Eur. J. Plant Pathol.* 156, 1041–1051. doi: 10.1007/s10658-020-01955-5

Colombo, E. M., Kunova, A., Gardana, C., Pizzatti, C., Simonetti, P., Cortesi, P., et al. (2020). Investigating useful properties of four *Streptomyces* strains active against *Fusarium graminearum* growth and deoxynivalenol production on wheat grains by qPCR. *Toxins* 12:560. doi: 10.3390/toxins12090560

Conn, V., Walker, A., and Franco, C. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 21, 208–218. doi: 10.1094/MPMI-21-2-0208

Cotton, T. A., Pétriacq, P., Cameron, D. D., Al Meselmani, M., Schwarzenbacher, R., Rolfe, S. A., et al. (2019). Metabolic regulation of the maize rhizobiome by benzoxazinoids. *ISME J.* 13, 1647–1658. doi: 10.1038/s41396-019-0375-2

De Boevre, M., Di Mavungu, J. D., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., et al. (2012). Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 29, 819–835. doi: 10.1080/19440049.2012.656707

De Zutter, N., Ameye, M., Debode, J., De Tender, C., Ommeslag, S., Verwaeren, J., et al. (2021). Shifts in the rhizobiome during consecutive in planta enrichment for phosphate-solubilizing bacteria differentially affect maize P status. *Microb. Biotechnol.* 14, 1594–1612. doi: 10.1111/1751-7915.13824

Dimkpa, C., Weinand, T., and Asch, F. (2009). Plant–rhizobacteria interactions alleviate abiotic stress conditions. *Plant Cell Environ.* 32, 1682–1694. doi: 10.1111/j.1365-3040.2009.02028.x

Ding, X., Cao, Y., Huang, L., Zhao, J., Xu, C., Li, X., et al. (2008). Activation of the indole-3-acetic acid–amido synthetase GH3-8 suppresses expansin expression and promotes salicylate-and jasmonate-independent basal immunity in rice. *Plant Cell* 20, 228–240. doi: 10.1105/tpc.107.055657

Ding, X., Yang, M., Huang, H., Chuan, Y., He, X., Li, C., et al. (2015). Priming maize resistance by its neighbors: activating 1, 4-benzoxazine-3-ones synthesis and defense gene expression to alleviate leaf disease. *Front. Plant Sci.* 6:830. doi: 10.3389/fpls.2015.00830

Dobbelaere, S., Croonenborghs, A., Thys, A., Broek, A. V., and Vanderleyden, J. (1999). Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. *Plant Soil* 212, 153–162. doi: 10.1023/A:1004658000815

Faheem, M., Raza, W., Zhong, W., Nan, Z., Shen, Q., and Xu, Y. (2015). Evaluation of the biocontrol potential of *Streptomyces goshikiensis* YCXU against *Fusarium oxysporum* f. sp. *niveum*. *Biol. Control* 81, 101–110. doi: 10.1016/j.biocontrol.2014.11.012

Gale, L. R., Bryant, J., Calvo, S., Giese, H., Katan, T., O'Donnell, K., et al. (2005). Chromosome complement of the fungal plant pathogen *Fusarium graminearum* based on genetic and physical mapping and cytological observations. *Genetics* 171, 985–1001. doi: 10.1534/genetics.105.044842

Getha, K., and Vikineswary, S. (2002). Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f. sp. *cubense* race 4: indirect evidence for the role of antibiosis in the antagonistic process. *J. Ind. Microbiol. Biotechnol.* 28, 303–310. doi: 10.1038/sj/jim/7000247

Goudjal, Y., Toumatia, O., Sabaou, N., Barakate, M., Mathieu, F., and Zitouni, A. (2013). Endophytic actinomycetes from spontaneous plants of Algerian Sahara: indole-3-acetic acid production and tomato plants growth promoting activity. *World J. Microbiol. Biotechnol.* 29, 1821–1829. doi: 10.1007/s11274-013-1344-y

Guo, Y., Zheng, W., Rong, X., and Huang, Y. (2008). A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. *Int. J. Syst. Evol. Microbiol.* 58, 149–159. doi: 10.1099/ijs.0.65224-0

Harkai, P., Szabó, I., Cserháti, M., Krifaton, C., Risa, A., Radó, J., et al. (2016). Biodegradation of aflatoxin-B1 and zearalenone by *Streptomyces* sp. collection. *Int. Biodeterior. Biodegradation* 108, 48–56. doi: 10.1016/j.ibiod.2015.12.007

Jones, S. E., and Elliot, M. A. (2017). Streptomyces exploration: competition, volatile communication and new bacterial behaviours. *Trends Microbiol.* 25, 522–531. doi: 10.1016/j.tim.2017.02.001

Khan, N., Bano, A., Ali, S., and Babar, M. A. (2020). Crosstalk amongst phytohormones from planta and PGPR under biotic and abiotic stresses. *Plant Growth Regul.* 90, 189–203. doi: 10.1007/s10725-020-00571-x

Kurth, F., Mailänder, S., Bönn, M., Feldhahn, L., Herrmann, S., Große, I., et al. (2014). Streptomyces-induced resistance against oak powdery mildew involves host plant responses in defense, photosynthesis, and secondary metabolism pathways. *Mol. Plant Microbe Interact.* 27, 891–900. doi: 10.1094/MPMI-10-13-0296-R

Lamichhane, J. R., Dachbrodt-Saaydeh, S., Kudsk, P., and Messéan, A. (2016). Toward a reduced reliance on conventional pesticides in European agriculture. *Plant Dis.* 100, 10–24. doi: 10.1094/PDIS-05-15-0574-FE

Lanubile, A., Luca, P., and Adriano, M. (2010). Differential gene expression in kernels and silks of maize lines with contrasting levels of ear rot resistance after *Fusarium verticillioides* infection. *J. Plant Physiol.* 167, 1398–1406. doi: 10.1016/j.jplph.2010.05.015

Lanubile, A., Maschietto, V., Borrelli, V. M., Stagnati, L., Logrieco, A. F., and Marocco, A. (2017). Molecular basis of resistance to *Fusarium* ear rot in maize. *Front. Plant Sci.* 8:1774. doi: 10.3389/fpls.2017.01774

Le, D., Ameye, M., De Boevre, M., De Saeger, S., Audenaert, K., and Haesaert, G. (2020). Population, virulence and mycotoxin profile of *Fusarium* spp. associated with basal rot of *Allium* spp. in Vietnam. *Plant Dis.* 105, 1942–1950. doi: 10.1094/PDIS-08-20-1850-RE

Lin, L., and Xu, X. (2013). Indole-3-acetic acid production by endophytic *Streptomyces* sp. En-1 isolated from medicinal plants. *Curr. Microbiol.* 67, 209–217. doi: 10.1007/s00284-013-0348-z

Lin, Y., Zhang, C., Lan, H., Gao, S., Liu, H., Liu, J., et al. (2014). Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. *PLoS One* 9:e95445. doi: 10.1371/journal.pone.0095445

Maag, D., Köhler, A., Robert, C. A., Frey, M., Wolfender, J. L., Turlings, T. C., et al. (2016). Highly localized and persistent induction of Bx1-dependent herbivore resistance factors in maize. *Plant J.* 88, 976–991. doi: 10.1111/tpj.13308

Nasser, W., De Tapia, M., and Burkard, G. (1990). Maize pathogenesis-related proteins: characterization and cellular distribution of 1, 3-β-glucanases and chitinases induced by brome mosaic virus infection or mercuric chloride treatment. *Physiol. Mol. Plant Pathol.* 36, 1–14. doi: 10.1016/0885-5765(90)90087-E

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., et al. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312, 436–439. doi: 10.1126/science.1126088

Nguyen, P.-A., Strub, C., Lagrée, M., Bertrand-Michel, J., Schorr-Galindo, S., and Fontana, A. (2020). Study of in vitro interaction between *Fusarium verticillioides* and *Streptomyces* sp. using metabolomics. *Folia Microbiol.* 65, 303–314. doi: 10.1007/s12223-019-00725-z

Nicolaisen, M., Suproniené, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H., and Justesen, A. F. (2009). Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *J. Microbiol. Methods* 76, 234–240. doi: 10.1016/j.mimet.2008.10.016

Niemeyer, H. M. (2009). Hydroxamic acids derived from 2-hydroxy-2 H-1, 4-benzoxazin-3 (4 H)-one: key defense chemicals of cereals. *J. Agric. Food Chem.* 57, 1677–1696.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., and Ohashi, Y. (1998). Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol.* 39, 500–507. doi: 10.1093/oxfordjournals.pcp.a029397

Oren, L., Ezrati, S., Cohen, D., and Sharon, A. (2003). Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Appl. Environ. Microbiol.* 69, 1695–1701. doi: 10.1128/AEM.69.3.1695-1701.2003

Parsons, M., and Munkvold, G. (2010). Associations of planting date, drought stress, and insects with *Fusarium* ear rot and fumonisin B1 contamination in California maize. *Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess.* 27, 591–607. doi: 10.1080/19440040903456337

Pineda, A., Zheng, S.-J., van Loon, J. J., Pieterse, C. M., and Dicke, M. (2010). Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends Plant Sci.* 15, 507–514. doi: 10.1016/j.tplants.2010.05.007

Pitt, J. I., and Miller, J. D. (2016). A concise history of mycotoxin research. *J. Agric. Food Chem.* 65, 7021–7033. doi: 10.1021/acs.jafc.6b04494

Raymaekers, K., Ponet, L., Holtappels, D., Berckmans, B., and Cammue, B. P. (2020). Screening for novel biocontrol agents applicable in plant disease management–a review. *Biol. Control* 144:104240. doi: 10.1016/j.biocontrol.2020.104240

Reid, L., Nicol, R., Ouellet, T., Savard, M., Miller, J., Young, J., et al. (1999). Interaction of *Fusarium graminearum* and *F. moniliforme* in maize ears: disease progress, fungal biomass, and mycotoxin accumulation. *Phytopathology* 89, 1028–1037. doi: 10.1094/PHYTO.1999.89.11.1028

Sadeghi, A., Koobaz, P., Azimi, H., Karimi, E., and Akbari, A. R. (2017). Plant growth promotion and suppression of *Phytophthora drechsleri* damping-off in cucumber by cellulase-producing *Streptomyces*. *Biocontrol* 62, 805–819. doi: 10.1007/s10526-017-9838-4

Shen, T., Lei, Y., Pu, X., Zhang, S., and Du, Y. (2021). Identification and application of *Streptomyces microflavus* G33 in compost to suppress tomato bacterial wilt disease. *Appl. Soil Ecol.* 157:103724. doi: 10.1016/j.apsoil.2020.103724

Spaepen, S., and Vanderleyden, J. (2011). Auxin and plant-microbe interactions. *Cold Spring Harb. Perspect. Biol.* 3:a001438. doi: 10.1101/cshperspect.a001438

Strub, C., Dieye, C., Nguyen, P., Constancias, F., Durand, N., Guendouz, S., et al. (2019). Transcriptomes of the interaction between *Fusarium verticillioides* and a Streptomyces strain reveal the fungal defense strategy under the pressure of a potential biocontrol agent. *Fungal Biol.* 125, 78–88. doi: 10.1016/j.funbio.2019.11.007

Suárez-Moreno, Z. R., Vinchira-Villarraga, D. M., Vergara-Morales, D. I., Castellanos, L., Ramos, F. A., Guarnaccia, C., et al. (2019). Plant-growth promotion and biocontrol properties of three *Streptomyces* spp. isolates to control bacterial rice pathogens. *Front. Microbiol.* 10:290. doi: 10.3389/fmicb.2019.00290

Tan, J., Ameye, M., Landschoot, S., De Zutter, N., De Saeger, S., De Boevre, M., et al. (2020). At the scene of the crime: new insights into the role of weakly pathogenic members of the *Fusarium* head blight disease complex. *Mol. Plant Pathol.* 21, 1559–1572. doi: 10.1111/mpp.12996

Tan, J., De Zutter, N., De Saeger, S., De Boevre, M., Tran, T. M., van der Lee, T., et al. (2021). Presence of the weakly pathogenic *Fusarium poae* in the *Fusarium* head blight disease complex hampers biocontrol and chemical control of the virulent *Fusarium graminearum* pathogen. *Front. Plant Sci.* 12:641890. doi: 10.3389/fpls.2021.641890

Toumatia, O., Compant, S., Yekkour, A., Goudjal, Y., Sabaou, N., Mathieu, F., et al. (2016). Biocontrol and plant growth promoting properties of *Streptomyces mutabilis* strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization. *S. Afr. J. Bot.* 105, 234–239. doi: 10.1016/j.sajb.2016.03.020

Tran, M. T., Ameye, M., Phan, L. T.-K., Devlieghere, F., De Saeger, S., Eeckhout, M., et al. (2021a). Impact of ethnic pre-harvest practices on the occurrence of *Fusarium verticillioides* and fumonisin B1 in maize fields from Vietnam. *Food Control* 120:107567. doi: 10.1016/j.foodcont.2020.107567

Tran, M. T., Ameye, M., Phan, L. T.-K., Devlieghere, F., De Saeger, S., Eeckhout, M., et al. (2021b). Post-harvest contamination of maize by *Fusarium verticillioides* and fumonisins linked to traditional harvest and post-harvest practices: a case study of small-holder farms in Vietnam. *Int. J. Food Microbiol.* 339:109022. doi: 10.1016/j.ijfoodmicro.2020.109022

Tran, M. T., Ameye, M., Landschoot, S., Devlieghere, F., De Saeger, S., Eeckhout, M., et al. (2021c). Molecular insights into defense responses of Vietnamese maize varieties to *Fusarium verticillioides* isolates. *J. Fungi* 7:724. doi: 10.3390/jof7090724

Tzin, V., Hojo, Y., Strickler, S. R., Bartsch, L. J., Archer, C. M., Ahern, K. R., et al. (2017). Rapid defense responses in maize leaves induced by *Spodoptera exigua* caterpillar feeding. *J. Exp. Bot.* 68, 4709–4723. doi: 10.1093/jxb/erx274

Van Loon, L. (1997). Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant Pathol.* 103, 753–765. doi: 10.1023/A:1008638109140

Vergnes, S., Gayrard, D., Veyssière, M., Toulotte, J., Martinez, Y., Dumont, V., et al. (2020). Phyllosphere colonization by a soil *Streptomyces* sp. promotes plant defense responses against fungal infection. *Mol. Plant Microbe Interact.* 33, 223–234. doi: 10.1094/MPMI-05-19-0142-R

Wang, Y., Zhou, Z., Gao, J., Wu, Y., Xia, Z., Zhang, H., et al. (2016). The mechanisms of maize resistance to *Fusarium verticillioides* by comprehensive analysis of RNA-seq data. *Front. Plant Sci.* 7:1654. doi: 10.3389/fpls.2016.01654

Zhang, X., Wang, B., Zhao, Y., Zhang, J., and Li, Z. (2019). Auxin and GA signaling play important roles in the maize response to phosphate deficiency. *Plant Sci.* 283, 177–188. doi: 10.1016/j.plantsci.2019.02.011

Zhu, Z., Tian, Z., and Li, J. (2020). A *Streptomyces morookaensis* strain promotes plant growth and suppresses *Fusarium* wilt of banana. *Trop. Plant Pathol.* 46, 175–185. doi: 10.1007/s40858-020-00396-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Plant Science

REVIEW
published: 03 December 2021
doi: 10.3389/fpls.2021.744445

Importance of the Rhizosphere Microbiota in Iron Biofortification of Plants

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Increasing the iron content of plant products and iron assimilability represents a major issue for human nutrition and health. This is also a major challenge because iron is not readily available for plants in most cultivated soils despite its abundance in the Earth's crust. Iron biofortification is defined as the enhancement of the iron content in edible parts of plants. This biofortification aims to reach the objectives defined by world organizations for human nutrition and health while being environment friendly. A series of options has been proposed to enhance plant iron uptake and fight against hidden hunger, but they all show limitations. The present review addresses the potential of soil microorganisms to promote plant iron nutrition. Increasing knowledge on the plant microbiota and plant-microbe interactions related to the iron dynamics has highlighted a considerable contribution of microorganisms to plant iron uptake and homeostasis. The present overview of the state of the art sheds light on plant iron uptake and homeostasis, and on the contribution of plant-microorganism (plant-microbe and plant-plant-microbe) interactions to plant nutritition. It highlights the effects of microorganisms on the plant iron status and on the co-occurring mechanisms, and shows how this knowledge may be valued through genetic and agronomic approaches. We propose a change of paradigm based on a more holistic approach gathering plant and microbial traits mediating iron uptake. Then, we present the possible applications in plant breeding, based on plant traits mediating plant-microbe interactions involved in plant iron uptake and physiology.

Keywords: iron, biotic interactions, plant–microbe interaction, microbiota, plant nutrition, iron biofortification, rhizosphere

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *20 July 2021*
Accepted: *29 October 2021*
Published: *03 December 2021*

Citation:
Lurthy T, Pivato B, Lemanceau P and Mazurier S (2021) Importance of the Rhizosphere Microbiota in Iron Biofortification of Plants. Front. Plant Sci. 12:744445. doi: 10.3389/fpls.2021.744445

INTRODUCTION

More than 820 million people are suffering from chronic undernourishment, and two billion from micronutrient deficiencies (hidden hunger) worldwide (FAO et al., 2019). People from lower-middle-income countries suffer from hunger (undernourishment) and do not have access to a varied diet (malnutrition). This is leading to micronutrient deficiencies (MNDs) in micronutrients such as iron (Fe), Zinc (Zn) and vitamin A. Overcoming undernourishment and overcoming malnutrition represent two of the main "Sustainable Development Goals" of the United Nations Development Program UNDP 2015 (UNDP, 2015).

Iron deficiency—the main case of MND in the world—has serious effects on human health, such as microcytic anemia, impaired immune function and poor endocrine function (Bailey et al., 2015;

Wakeel et al., 2018; World Health Organization, 2021). Iron plays an essential role in the physiology of living organisms, e.g., in DNA synthesis, respiration, and photosynthesis (Aisen et al., 2001).

Meat is the main source of iron in food, with 20–60% of Fe in the form of haemoproteins that are easily assimilable by the human body (Cross et al., 2012). However, a significant fraction of the world human population does not have access to meat and thus suffers from iron deficiency. This deficiency also occurs in developed countries, especially among young ladies (Beck et al., 2014). In plant-based diets in developing countries, iron is supplied by plant products, especially grains (cereals and legumes). However, these grains contain low levels of Fe, and even more include anti-nutritional compounds such as phytates which hamper Fe assimilation (Gómez-Galera et al., 2010). Yet, the iron concentration in grains has decreased over the years because of intensified agriculture, e.g., with the introduction of semi-dwarf, high-yielding cultivars of wheat (Fan et al., 2008). At the same time, meat consumption is decreasing in developed countries with the growing concern for environmental (higher environmental footprint of animal proteins than of plant proteins) and diet issues. Thus, increasing Fe content and assimilability in plant products represents a major challenge for human nutrition and health.

To address this challenge, food fortification, which consists in artificially supplementing food with micronutrients (iron, zinc, vitamins), has been proposed as an option. The Food Fortification Initiative[1] created a network of governmental and private agencies in several developing countries to promote the iron fortification of wheat flour. As a result, the consumption of Fe-fortified wheat flour has increased from 18% in 2004 to 27% in 2007, allievating iron deficiency for 540 million people (White and Broadley, 2009). Promising results were also obtained in India, the Philippines and Rwanda with rice, pearl millet and beans supplemented with iron (Finkelstein et al., 2017, 2019). However, there are limitations to this approach. Costs are important, supplementation may modify food taste and is not always well accepted, and finally fortified food hardly reaches poor people with limited or no access to commercial channels. Iron fertilization is a common agricultural practice also used to mitigate plant iron deficiency but not considered so far overlooked to increase staple food quality. Three main groups of Fe fertilizers are used: inorganic Fe compounds, synthetic Fe, and organic Fe complexes (Abadía et al., 2011; Zanin et al., 2019). In addition to their high cost, the possible incorporation of these ligands into edible parts of the plant (Abadía et al., 2011) may represent a problem. These limitations also apply to new nano-chelates under development (Yuan et al., 2018). Indeed, the increasing use of nanoparticles raises concerns for human health or the environment (Soares and Soares, 2021). In short, efficient Fe fertilizers have several drawbacks: they are expensive, their efficiency is variable, and they can be incorporated in the host plant including its edible parts. They do not represent sustainable options for increasing the iron content of agricultural products, even if foliar applications of iron may be of interest in specific cases (e.g., increasing the iron content of rice and barley grains, Slamet-Loedin et al., 2015).

Another option relies on iron biofortification, the promotion of the iron content of plants, especially their edible parts such as leaves (e.g., lettuce or spinach), roots (e.g., carrot or cassava), fruits (e.g., peach or apple), and grains (e.g., rice, wheat, maize). This biofortification relies on better plant iron nutrition and on the modulation of iron homeostasis. This option was reported to be more efficient, sustainable and cheaper than micronutrient fortification (Murgia et al., 2012; World Health Organization, 2017). According to the FAO, "biofortified crop varieties are those which have been nutritionally enhanced using conventional plant breeding or modern biotechnology, especially recombinant DNA techniques. However, by far the most widely adopted biofortified crop varieties have been those developed through conventional crop breeding" (FAO, 2019, 2021). Approximately 90 iron-biofortified crop varieties have been released in different part of the world (FAO, 2019; Singh and Prasanna, 2020) and help fight hidden hunger, but much progress remains to be made. Iron biofortification represents a major challenge because Fe is not readily available for living organisms, including plants, in most cultivated soils despite its abundance: Fe is the fourth element in the Earth's crust (Lemanceau et al., 2009). Low iron availability is particularly acute in alkaline soils which represent about 30% of the world's croplands and up to 40% of arable surfaces (Chen and Barak, 1982; Sullivan and Gadd, 2019). Iron availability depends on the soil properties and more specifically on the soil pH and redox potential (Robin et al., 2008; Colombo et al., 2014). Immobilization of iron in the form of scarcely soluble complexes formed between Fe^{3+} and hydroxides, oxyhydroxides, and oxides, increases with the soil pH (Robin et al., 2008). The low availability of iron may lead to plant growth depression and even to iron deficiency chlorosis (IDC), interveinal chlorosis, which ultimately negatively impact crop yield (Briat et al., 2015) and quality (their Fe content).

Soil microorganisms have long been known to contribute to plant iron nutrition (Marschner, 1995). This beneficial effect was first showed by comparing the iron contents of plants grown in sterile and non-sterile soils. The iron content of sunflower, maize (Masalha et al., 2000), rape and red clover (Rroço et al., 2003; Jin et al., 2006) was significantly lower when they were grown in sterile rather than non-sterile soil; sunflower even suffered from chlorosis in sterile soil. A high occurrence of oligotrophic bacteria in lupine rhizosphere was associated with an increased concentration of Fe, Cu, Mn and Zn in plant shoots, suggesting that these bacteria may contribute to plant iron and more generally to plant mineral nutrition (De Santiago et al., 2019). The promotion of iron nutrition in a range of plant species by various microbial strains (e.g., root symbionts) and metabolites has been reported in a series of studies listed in **Table 1**. Interactions between plants may also facilitate their iron nutrition. Intercropping cereal and legume plants can notably improve their iron content (Zuo et al., 2000; Gunes et al., 2007; Zuo and Zhang, 2009; Xue et al., 2016). Thus, maize-peanut intecropping improved Fe nutrition of peanut

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[1] http://www.ffinetwork.org/

TABLE 1 | Microorganisms and microbial metabolites mediating the plant iron status.

Microorganisms and/or microbial metabolites	Application modes	Plants	Effects on the plant iron status	Additional observations	Effects on plant genes	Mechanism(s) proposed by authors	References
Acinetobacter calcoaceticus O-13; *Bacillus simplex* K-10	Bacterial suspension	Potato	Plant [Fe]/Fe(1)↗(2)	Tryptophan addition enhance iron uptake		Sid.(3) iron mobilization	Mushtaq et al., 2021
N_2 fixer and/or auxin producer mutants of *Azospirillum brasilence* FP2	Bacterial suspension	Maize	Plant [Fe]/Fe↗, modif. Fe distrib(3).	Root ethylene production↘(4), root auxin and DIMBOA(5) production↗, metabolic partitioning of carbon differed		Regulation of hormone signaling and cellular iron transport	Housh et al., 2021
Gluconacetobacter diazotrophicus PAL5; *Azospirillum brasilense* REC3	Bacterial suspension	Strawberry	Plant [Fe]/Fe↗	Phenolic compounds content↘, chlorophyll↗		Sid. iron mobilization	Delaporte-Quintana et al., 2020
Pseudomonas spp.; *Enterobacter* spp.; *Bacillus sporothernodurans*	Bacterial suspension	Sunflower	Plant [Fe]/Fe↗	Sid.(6) production↗, phytohormone production↗, phosphate solubilization↗, HCN(7) production↗		Sid. iron mobilization	Pourbabaee et al., 2018
Burkholderia cepacia JFW16	Bacterial suspension	Milkvetch	Plant [Fe]/Fe↗	Rhizosphere acidification, root FR(8)↗, flavin release, sid. and phytohormone production↗	*FRO2* expr.(9)↗, *IRT1* expr.↗, *AHA2* expr.↗, *FIT1* expr.↗	Promotion of iron mobilization by acidification, strategy I iron uptake, and hormonal regulation	Zhou et al., 2018
Pseudomonas fluorescens ATCC13525	Bacterial suspension	Tomato	Plant [Fe]/Fe↗		*IRT1* expr.↗, *FRO2* expr.↗, *NRAMP3* expr.↗	Promotion of strategy I iron uptake, and redistribution	Nagata, 2017
Burkholderia terricola LMG20594; *Pseudomonas brassicacearum* NFM421; *B. pyrrocinia* LMG14191; *P. mandelii* NBRC103147; *Herbaspirillum huttiense* NBRC10252	Bacterial suspension	Lentil, pea	Plant [Fe]/Fe↗	Rhizosphere acidification, sid. production↗, phytohormone production↗		Iron uptake	Reza, 2017
Paenibacillus polymyxa BFKC01	Bacterial suspension	Arabidopsis	Plant [Fe]/Fe↗	Root FR↗	*FRO2* expr.↗, *IRT1* expr.↗, *FIT1* expr.↗ MYB72 expr.↗	Promotion of iron uptake by modulation of the expression of strategy I key genes and of ISR key genes	Zhou et al., 2016
Rhizobium leguminosarum bv.(10) *phaseoli*; *Pseudomonas* spp. Avm	Bacterial suspension	Common bean	Plant [Fe]/Fe↗, modif. Fe distrib.	Wild variety more efficient in Fe uptake than cultivated variety after microbial inoculation		Promotion of iron uptake	Carrillo-Castañeda et al., 2005
Bacillus subtilis CPA; *Bacillus* sp. AHP3; *Pseudomonas chlororaphis* PR29; *Glomus fasciculatum* (consortium)	Bacterial and fungal suspension	Wheat	Plant [Fe]/Fe↗	Grain protein content↗ superoxide dismutase (SOD)↗ catalase (CAT)↘, chlorophyll↗ Metabolome modification		Promotion of nutrient yield by metabolic regulation and ROS scavenging activity	Yadav et al., 2020
Arthrobacter sulfonivorans DS-68; *Enterococcus hirae* DS-163	Bacterial coating	Wheat	Plant [Fe]/Fe↗, seed [Fe]/Fe↗, modif. Fe assimil.(11)	Anti-nutritional factor↘		Promotion of iron uptake	Singh et al., 2018

(Continued)

TABLE 1 | (Continued)

Microorganisms and/or microbial metabolites	Application modes	Plants	Effects on the plant iron status	Additional observations	Effects on plant genes	Mechanism(s) proposed by authors	References
Bacillus subtilis BHHU10, *Trichoderma harzianum* TNHU27, and *Pseudomonas aeruginosa* PJHU15 (consortium)	Bacterial and fungal coating	Pea	Modif. Fe assimil.	Phenolics, flavonoids, ascorbic acid and protein content↗		Promotion of ROS scavenging activity in plants	Jain et al., 2014
Pseudomonas fluorescens C7R12; pyoverdine of *P. fluorescens* C7R12	Bacterial suspension; apo-siderophore	*Arabidopsis*	Root [Fe]/Fe↘, shoot [Fe]/Fe↗	Changes in plant hormone production, Incorporation of Fe-pyoverdine suggested by ^{15}N-labeling and immunodetection	Numerous modifications evidenced in a transcriptomic study	Sid. promotion of iron mobilization in the rhizosphere including the apoplast, of strategy I iron uptake, and regulation of hormone signaling	Trapet et al., 2016
B. subtilis GBO3	Bacterial suspension; Bacterial VOCs(12)	*Arabidopsis*	Plant [Fe]/Fe↗	Rhizosphere acidification, root FR↗	*FRO2* expr.↗, *IRT1* expr.↗, *FIT1* expr.↗	Promotion of iron mobilization by acidification and of strategy I iron uptake	Zhang et al., 2009
Bacillus amyloliquefaciens BF06	Bacterial VOCs	*Arabidopsis*	Plant [Fe]/Fe↗	Root FR↗, Fe^{2+} production↗, Production of VOCs implied (2R or 3R-butanediol)	*FRO2* expr.↗, *IRT1* expr.↗, *FIT1* expr.↗	Promotion of strategy I iron uptake through gene expression modulation	Wang et al., 2017
Arthrobacter sulfonivorans DS-68; Arthrobacter sp. DS-179	Liquid bacterial culture coating	Wheat	Plant [Fe]/Fe↗	Organic acid production↗	*ZIP* expr.↗	Promotion of iron uptake and translocation through organic acid production and stimulation of iron transporters	Singh et al., 2017
B. subtilis GBO3	Liquid bacterial culture	Cassava	Shoot [Fe]/Fe↗			Promotion of the plant iron status through the regulation of the plant iron metabolism including hormone signaling	Freitas et al., 2015
Paenibacillus cookie JGR8; *Pseudomonas pseudoalcaligenes* JGR2; *Bacillus megaterium* JGR9	Liquid bacterial culture	Lesser bullrush	Shoot [Fe]/Fe↗ for strain JGR2, modif. Fe distrib.	Sid. production↗, phytohormone production↗, phosphate solubilization↗		Sid. promotion of iron accumulation and translocation; relationship between sid. production and phosphate solubilization	Ghosh et al., 2014
Chryseobacterium spp. C138	Liquid bacterial culture	Tomato	Plant [Fe]/Fe↗			Fe-sid. used as a source of iron under iron deficiency	Radzki et al., 2013
P. putida MTCC 103, Enterobacteria	Liquid bacterial culture	Rice	Plant [Fe]/Fe↗, seed [Fe]/Fe↗	Variation of peroxidase activity		Promotion of iron solubilization, uptake and translocation related to sid. production	Sharma et al., 2013
R. leguminosarum PR1; *Pseudomonas* sp. PGERs17	Liquid bacterial culture	Lentil	Plant [Fe]/Fe↗	Nodulation↗, leghaemoglobin↗		Fe-sid. used as a source of iron under iron deficiency	Mishra et al., 2011
Trichoderma asperellum T34	Fungal conidia	Cucumber	Shoot [Fe]/Fe↗			Fe-sid. used as a source of iron under iron deficiency	De Santiago et al., 2013

(Continued)

TABLE 1 | (Continued)

Microorganisms and/or microbial metabolites	Application modes	Plants	Effects on the plant iron status	Additional observations	Effects on plant genes	Mechanism(s) proposed by authors	References
T. asperellum T34	Fungal conidia	White lupin	Shoot [Fe]/Fe↗	Peroxidase activity↗, catalase activity↗		Sid. promotion of iron accumulation and translocation under iron deficiency; promotion of ROS scavenging activity is implied	De Santiago et al., 2009
Hymenoscyphus ericae	Fungal suspension	Heather	Plant [Fe]/Fe↗	Variation in results depending on calcium addition		Sid. iron mobilization	Leake et al., 1990
Glomus etunicatum WV579A, *G. diaphanum* WV579B, *G. intraradices* WV894	Fungal cultures	Maize	Root [Fe]/Fe↗	Variation in results depending on soil pH and fungal strain		Sid. iron mobilization	Clark and Zeto, 1996
Glomus mossae and rhizosphere microorganisms	Fungal spores, root pieces and soil	Peanut, sorghum	Plant [Fe]/Fe↗ modif. Fe distrib.	Plant phosphate↗		Increased soil exploration	Caris et al., 1998
Glomus mossae, G. albidum, G. fasciculatum, G. macrocarpum.	Fungal spores propagated in sterile soil	Galleta grass	Plant [Fe]/Fe↗	use of ^{59}Fe		Sid. iron mobilization and transport into mycorrhizal plants	Cress et al., 1986
Glomus intraradices	Commercial inoculant	Maize	Shoot [Fe]/Fe↗ Shoot [Fe]/Fe↘	Variation in results with amount of micronutrients and P added		Increased soil exploration	Liu et al., 2000
Arbuscular mycorrhiza fungi inoculant	Commercial inoculant	Chickpea	Plant [Fe]/Fe↗	No effect of mineral N fertilization		Increased soil exploration	Farzaneh et al., 2011
Glomus intraradices, G. mosseae, G. aggregatum, G. etunicatum	Commercial inoculant	Sorghum	Plant [Fe]/Fe↗	Plant biomass↗, chlorophyll↗, Plant S↗, ROS↘	*DMAS2* exp.↗, *NAS2* exp.↗, *YS1* exp.,↗	Promotion of strategy II iron uptake (PS↗), and of ROS scavenging activity	Prity et al., 2020
Glomus intraradices, G. mosseae, G. aggregatum, G. etunicatum	Fungal spores (mix)	Alfalfa	Plant [Fe]/Fe↗	Plant biomass↗, chlorophyll↗, plant S↗, root FR↗, ROS↘	*FRO* expr.↗, *SULTR (1;1, 1;2,1;3, 3;1)* expr.↗	Promotion of iron mobilization in the rhizosphere including the root apoplast, and of ROS scavenging activity	Rahman et al., 2020
Glomus intraradices, G. mosseae, G. aggregatum, G. etunicatum	Fungal spores (mix)	Sunflower	Plant [Fe]/Fe↗	Plant biomass↗, chlorophyll↗, root FR↗, ROS↘, CAT↗, SOD↗	*FRO1* expr.↗, *IRT1* expr.↗, *ZIP1* expr.↗,	Promotion of iron mobilization and uptake, and ROS scavenging activity	Kabir et al., 2020
Rhizophagus irregularis DAOM197198	Fungal spores	Maize	Shoot [Fe]/Fe↗	Genes implied in strategy II were not induced	*OPT8* expr.↗, *NAS* expr.↗	Selective induction of putative iron transporters	Kobae et al., 2014
Rhizophagus irregularis DAOM197198	Fungal spores	Chicory	Root [Fe]/Fe↗	Root exploration volume↗, phosphatase production↗		Increased soil exploration, phosphatase activities implied	Labidi et al., 2012
Desferrioxamine B, sid. of *Streptomyces* obtained commercially	Fe-siderophore	Wheat	Plant [Fe]/Fe↘, modif. Fe distrib.	Variation of phytosiderophore production		Sid. inhibition of iron uptake via PS chelation	Sadrarhami et al., 2021

(Continued)

TABLE 1 | (Continued)

Microorganisms and/or microbial metabolites	Application modes	Plants	Effects on the plant iron status	Additional observations	Effects on plant genes	Mechanism(s) proposed by authors	References
3 pyoverdines, sids of *P. fluorescens* C7R12; *Pseudomonas* sp. B4214; *Pseudomonas* sp. D426	Fe-siderophore	Pea	Plant [Fe]/Fe↗, modif. Fe distrib.	Effects on the plant Fe status varying with pea cv.(13) and sids, modifications of the plant ionome		Fe-sid. used as a source of iron under iron deficiency	Lurthy et al., 2020
Azotochelin, sid. of *Azotobacter vinelandii* obtained commercially	Fe-siderophore	Soybean	Plant [Fe]/Fe↗			Sid. iron mobilization	Ferreira et al., 2019
Pyoverdine, sid. of *P. fluorescens* ATCC13525	Fe-siderophore	Tomato	Plant [Fe]/Fe↗,	chlorophyll↗	*FRO2* expr.↗, *IRT1* expr.↗	Fe-sid. used as a source of iron under iron deficiency	Nagata et al., 2013
Pyoverdine, sid. of *P. fluorescens* C7R12	Fe-siderophore	*Arabidopsis,* tobacco, barley, wheat, fescue, rye grass	Plant [Fe]/Fe↗	Incorporation of Fe-pyoverdine suggested by ^{15}N-labeling		Fe-sid. used as a source of iron under iron deficiency	Shirley et al., 2011
Pseudomonas spp. sid.	Fe-siderophore	Red clover	Plant [Fe]/Fe↗	chlorophyll↗		Sid. iron mobilization, Fe-sid. used as a source of iron under iron deficiency	Jin et al., 2010
Pyoverdine, sid. of *P. fluorescens* C7R12	Fe-siderophore	*Arabidopsis*	Plant [Fe]/Fe↗	An *IRT*1 mutant still incorporated Fe-pyoverdine, incorporation of Fe-pvd suggested by ^{15}N-labeling and immunodetection		Fe-sid. used as a source of iron under iron deficiency using a non-reductive uptake mechanism	Vansuyt et al., 2007
Aerobactin, sid. of *Citrobacter diversus*	Fe-siderophore	Soybean	Plant [Fe]/Fe↗	Fe^{2+} production↘		Fe-sid. used as a source of iron under iron deficiency using a non-reductive uptake mechanism	Chen et al., 2000
Hydroxamate, sid. mixture from *Penicillium chrysogenum*	Fe-siderophore	Cucumber, maize	Plant [Fe]/Fe↗	Fe^{2+} production↗		Sid. iron mobilization	Hördt et al., 2000
Rhizoferrin, sid. of *Rhizopus arrhizus*	Fe-siderophore	Tomato	Modif. Fe distrib., root [Fe]/Fe↗	chlorophyll↗		Sid. iron mobilization	Yehuda et al., 2000
Ferrioxamine B, sid. of *Streptomyces* spp. obtained commercially	Fe-siderophore	Onion	Root [Fe]/Fe↗	Root FR unchanged		Sid. iron mobilization	Manthey et al., 1996
Rhizoferrin, sid. of *Rhizopus arrhizus*	Fe-siderophore	Tomato	Modif. Fe distrib., root [Fe]/Fe↗			Fe-sid. used as a source of iron under iron deficiency	Shenker et al., 1995
Pseudobactin, syn.(14) pyoverdine, sid. of *Pseudomonas putida* WCS358	Fe-siderophore	Barley	Modif. Fe distrib., root [Fe]/Fe↗	No Fe exchange between pyoverdine and phytosiderophore		Fe-sid. used as a source of iron under iron deficiency	Duijff et al., 1994
Ferrioxamine B, sid. of *Streptomyces* spp. obtained commercially	Fe-siderophore	Cucumber	Plant [Fe]/Fe↗	Siderophore in the xylem		Fe-sid. used as a source of iron, uptake through the transpiration stream and translocation	Wang et al., 1993

(Continued)

TABLE 1 | (Continued)

Microorganisms and/or microbial metabolites	Application modes	Plants	Effects on the plant iron status	Additional observations	Effects on plant genes	Mechanism(s) proposed by authors	References
Ferrioxamine B, sid. of *Streptomyces* spp. obtained commercially	Fe-siderophore	Cotton, maize	Root [Fe]/Fe↗	Fe removal from chelate around the root, chlorophyll↗		Sid.-mediated iron uptake	Bar-Ness et al., 1992
Ferrioxamine B, sid. of *Streptomyces* spp. obtained commercially	Fe-siderophore	Pine	Plant [Fe]/Fe↘ in mycorrhizal plant			Sid.-mediated iron uptake	Leyval and Reid, 1991
Ferrichrome A, sid. of *Ustilago sphaerogena* ATCC 12421; ferrioxiamine B, sid. of *U. sphaerogena* ATCC 12421	Fe-siderophore	Oat	Plant [Fe]/Fe↗			Fe-sid. used as a source of iron under iron deficiency using a specific sid. uptake mechanism	Crowley et al., 1988
Agrobactin, sid. of *Agrobacterium tumefaciens* B6	Fe-siderophore	Pea, bean	Shoot [Fe]/Fe↗	Chlorophyll↗		Fe-sid. used as a source of iron	Becker et al., 1985a
Pseudobactin, syn. pyoverdine, sid. of *Pseudomonas aureofaciens* (now commonly referred to as *Pseudomonas chlororaphis*) ATCC15926	Fe-siderophore	Pea, maize	Shoot [Fe]/Fe↘	Chlorophyll↘		Sid. competitive bidding of iron	Becker et al., 1985b

(1)*[Fe]/Fe, iron concentration and/or iron amount;* (2) ↗*, increase;* (3)*Modif. Fe distrib., modified Fe distribution;* (4) ↘*, decrease;* (5)*DIMBOA, (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one);* (6)*Sid(s)., siderophore(s);* (7)*HCN, hydrogen cyanide;* (8)*FR, ferric reductase;* (9)*expr., expression;* (10)*bv., biovar;* (11)*Modif. Fe assimil., modified Fe assimilability;* (12)*VOC(s), volatile organic compound(s);* (13)*cv., cultivar;* (14)*syn., synonym.*

(Zuo et al., 2000), while wheat-chickpea intercropping increased the Fe concentration in wheat seeds (Gunes et al., 2007). Interestingly, the rhizosphere microbiota of these associated plant species differed from the rhizosphere microbiota of these same plants cultivated separately (Sun et al., 2009; Zhang et al., 2012; Wahbi et al., 2016a; Taschen et al., 2017). A more complex rhizosphere bacterial network was recently shown in pea-wheat intercropping (Pivato et al., 2021). Thus, we can hypothesize that the rhizosphere microbiota accounts for the increased iron uptake by intercropped plants.

On the basis of a range of studies published lately, we argue that iron biofortification is a relevant option to alleviate MND. This option requires better knowledge of the organisms and mechanisms that promote plant iron uptake and homeostasis. The present overview of the state of the art sheds light on plant iron uptake and homeostasis, and on the plant-microorganisms interactions (plant-microbe and plant-plant-microbe) that impact these processes. Then, we describe different strategies of iron fortification of plants, with a special focus on biofortication, and we finally discuss promising prospects based on the monitoring of the dynamic interplay between plants and their rhizosphere microbiota, including microbes from the surrounding soil, attached to and influenced by the roots, plus from the roots themselves (endophytes).

BIOLOGICAL LEVERS TO PROMOTE PLANT IRON UPTAKE AND REGULATE IRON HOMEOSTASIS

Valuing Plant Genetic Resources to Improve Iron Nutrition

Plant Iron Physiology

The forms of iron available to plants are the ferric iron cation (Fe^{3+}), or ferric-ion chelates (Fe^{3+}-chelates), and the ferrous iron cation (Fe^{2+}) (**Figure 1**). Two main strategies of root iron acquisition are described: strategy I (the reduction-based strategy), and strategy II (the chelation strategy) (Curie and Briat, 2003; Curie et al., 2009; Kobayashi and Nishizawa, 2012; Connorton et al., 2017). Strategy I is found in non-graminaceous monocots and dicots. It relies on the reduction of Fe^{3+} by a ferric reduction oxidase (encoded by a *FRO* gene), and the incorporation of the resulting Fe^{2+} into the root by an iron-regulated transporter (encoded by an *IRT* or a *RIT* gene). The pH is decreased in the rhizosphere (Hinsinger et al., 2003), as a result of proton extrusion by plasma membrane proton pumps (encoded by an *AHA* gene); this acidification increases Fe^{3+} solubility.

Strategy II is found in grasses. It relies on the excretion of phytosiderophores (PSs, structural derivatives of mugineic acid) by a transporter of mucigenic acid (encoded by a *TOM* gene) that chelates Fe^{3+} (Fe^{3+}-PS) before incorporation into the root by an oligopeptide transporter belonging to the Yellow Stripe/Yellow Stripe Like family (YS/YSL) (Curie et al., 2009).

Differentiating plant species according to their iron uptake strategy has its own limitations, as both strategies are found in

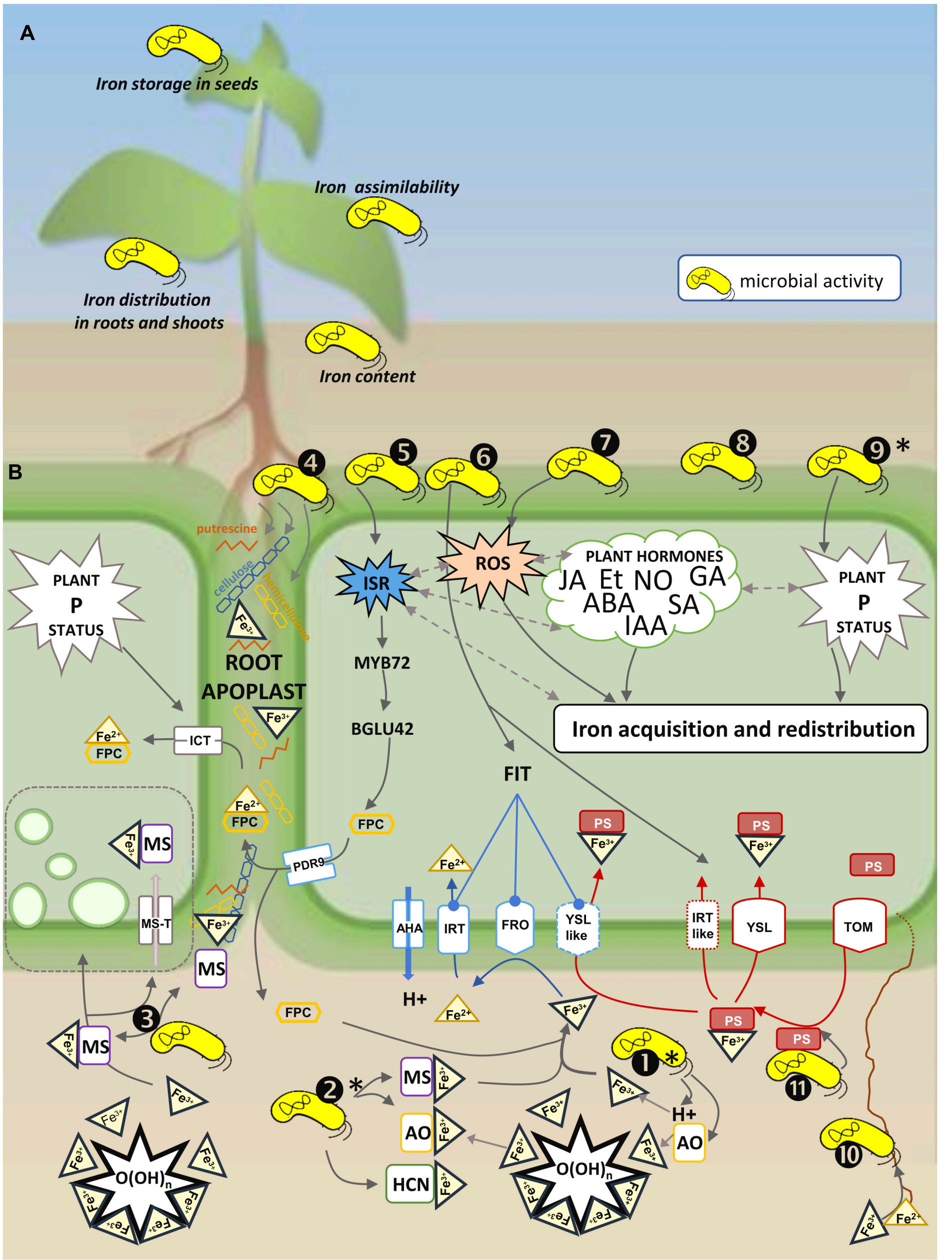

FIGURE 1 | Schematic representation of different aspects of microbial regulation of the plant iron status. **(A)** Types of effects of microorganisms on the plant iron status: (i) content, (ii) root and/or shoot distribution, (iii) assimilability, and (iv) iron storage in the seeds (see **Table 1** for further information). **(B)** Microbial activities involved in the active strategy of plant iron uptake and homeostasis. Plant transporters and enzymes mediating iron uptake are represented in blue for dicots and non-graminaceous monocots, and in red for grasses. In iron uptake strategy I (reductive strategy), protons are extruded by H^+-ATPases (e.g., AHA in *Arabidopsis*), Fe^{3+} is reduced by plant ferric reductases (e.g., FRO2 in *Arabidopsis*) to Fe^{2+} which is internalized in root cells by a specific transporter (e.g., IRT1 in *Arabidopsis* or IRT-like in rice and barley). Excretion of root fluorescent phenolic compounds (FPC) *via* ABC transporters (e.g., PDR9 in *Arabidopsis*) contributes to strategy I iron uptake and more specifically to re-mobilization of root apoplastic iron which is internalized via IRT1. In iron uptake strategy II (chelating strategy), Fe^{3+} is chelated by phytosiderophores (PS) (e.g., secreted via TOM1 in rice) and the Fe-PS complex is internalized by a specific transporter (e.g., YSL in rice or YSL-like in peanut). A non-reductive mechanism controlled by the plant phosphorus (P) status implies a putative specific iron chelate transporter (ICT) possibly involved in the internalization of chelates formed by Fe^{3+} reduced and complexed by FPC (Fe^{2+}-FPC). Numbers represent the types of microbial activities: ❶Acidification through production of protons (H^+), organic acids (OA) and hydrogen cyanide (HCN), and ❷Chelation by OA, HCN or microbial siderophores (MS) contributes to solubilization of ferric iron (Fe^{3+}) immobilized in O(OH)n. Acidification and chelation increase bioavailability of Fe^{3+} to plants. ❸Fe^{3+}-MS complexes are suspected to be internalized by a non-reductive process (MS transport, MS-T) with possible endocytosis (represented by green vesicles). ❹Rhizosphere microorganisms can metabolize cellulose, hemicellulose and putrescine, or produce putrescine, and thus modify molecules involved in root apoplastic iron storage and re-mobilization. ❺Microorganisms triggering induced systemic plant resistance (ISR) influence FPC production; FPCs contribute to plant iron uptake by re-mobilizing root apoplastic

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FIGURE 1 | iron through the modulation of *MYB72* (root transcription factor) and B*GLU42* (beta-glucosidase) expression. ❻Microorganisms influence the expression of key genes of plant iron uptake strategies I and II. ❼Microorganisms influence the plant reactive oxygen species (ROS) status, and this modulates the plant iron status. ❽Microorganisms influence the plant hormone (JA, jasmonic acid; Et, ethylene; ABA, abscisic acid; NO, nitric oxide; GA, gibberellin; SA, salicylic acid; IAA, indole acetic acid) status (directly by synthesizing or metabolizing them, or indirectly by inducing plant defense responses), and this modulates the plant iron status. ❾Microorganisms influence the plant P status, which is implied in the regulation of the plant iron status. ❿Fungal root symbionts extend the volume of soil explored, and this improves acquisition of nutrients including iron. ⓫Phytosiderophore scavenging by soil bacteria influences strategy II plant iron uptake. Slash-dotted arrows indicate interrelations between various components of plant physiology influencing plant iron acquisition and redistribution. (*) influence on plant P bioavailability.

rice, barley and peanut (Ishimaru et al., 2006; Pedas et al., 2008; Xiong et al., 2013). In *Arabidopsis* (a strategy I plant), chelating agents (e.g., coumarins) may contribute to iron nutrition in addition to the reduction strategy, especially in alkaline environments (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). These fluorescent phenolic compounds (FPCs) are synthesized *via* the phenylpropanoid pathway and secreted *via* an ABC transporter (e.g., PDR9 in *Arabidopsis*). Chlorotic phenotypes of *IRT*1 and *FRO*2 mutants were not restored by FPCs from plant exudates, suggesting that strategy I is implied in the uptake of Fe^{3+}chelated to PC (Fourcroy et al., 2016). However, based on a review of results obtained under phosphate-(Pi-) deficient conditions, Tsai and Schmidt (2017) hypothesized the existence of an auxiliary IRT1-independent iron chelate transporter (ICT) that would bypass the IRT1 uptake system and internalize the Fe-FPC complex under Pi-deficient conditions. Flavins, another family of phenolic compounds, also increased iron solubilization in the rhizosphere of other plant species (e.g., barrel medic, sugar beet) than *Arabidopsis* (Rodríguez-Celma et al., 2013; Sisó-Terraza et al., 2016). Iron solubilization by phenolic compounds has also been reported in rice, a strategy II plant (Bashir et al., 2010; Ishimaru et al., 2011). Besides PSs and FPCs, organic acids (e.g., citrate or succinate) chelate Fe^{3+} (Fe^{3+}-OC) and contribute to plant iron nutrition (Abadía et al., 2011; Adeleke et al., 2017). Synthetic ferric chelates (Fe^{3+}-SC)—e.g., Fe-EDDHA or Fe-EDTA –, may provide iron to strategy I plants; entire chelates have been found in roots and shoots (Orera et al., 2009, 2010). These small hydrophilic molecules are suspected to use the transpiration stream as the driving force of entry (Abadía et al., 2011). Iron uptake by the leaves follows similar mechanisms as those described in the roots (Malhotra et al., 2019). Thus, even if strategies I and II remain essential pathways for iron acquisition by plants, they are not necessarily exclusive and may be complemented by additional mechanisms.

Iron is a central cofactor of enzymatic reactions involving electron transfer in essential metabolic pathways such as respiration or photosynthesis (Balk and Schaedler, 2014). Yet, its redox properties may also induce toxic effects. Free Fe^{2+} catalyzes the Fenton reaction in which reactive oxygen species (ROS) characteristic of oxidative stress are generated, and ROS may cause irreparable damage to cellular components when they are present in excessive concentrations (Winterbourn, 1995). The intracellular concentrations and forms of iron are therefore tightly regulated at the cellular level. The mechanisms involved in iron homeostasis have many common features in strategy I and strategy II plants (Connorton et al., 2017; Grillet and Schmidt, 2019; Kobayashi et al., 2019). *In planta*, chelation of Fe^{2+} to Nicotianamine (NA), and Fe^{3+} to citrate, allows iron mobilization and the control of its high reactivity (Curie et al., 2009; Connorton et al., 2017). In strategy II plants, ferric iron is additionally present in the form of Fe^{3+}-PS (Zhang et al., 2019). Iron is also stored in unreactive forms such as ferritins that represent a major iron pool in plants mostly found in chloroplasts and mitochondria. They behave like a buffer that stores iron to avoid overload and the resulting ROS formation, and releases it when needed. To avoid oxidative stress, iron storage in vacuoles also contributes to iron homeostasis. Candidate transporters for moving cytosolic iron into vacuoles are members of the IRT, FPN/IREG (ferroportin/iron regulated) and VIT (vacuolar iron transporter) families (Morrissey et al., 2009; Roschzttardtz et al., 2009; Vert et al., 2009). VIT transporters are particularly important for vacuolar iron storage in seeds (Roschzttardtz et al., 2009; Zhang et al., 2012). In seeds, iron is mainly found under an insoluble form poorly available for nutrition because it is complexed with phosphate bound to inositol in phytates (Mary et al., 2015). Iron remobilization from the vacuoles is mediated by members of the natural resistance-associated macro-phage protein family (NRAMP) (Curie et al., 2000; Nevo and Nelson, 2006). In addition to ferritins and vacuoles, the root apoplast appears as a third level of iron storage by plants (Curie and Mari, 2017). Bienfait et al. (1985) demonstrated that a pool of 500–1,000 nanomoles of Fe *per* gram fresh weight could be formed in the root apoplast where it is adsorbed on the cell wall whose net charge is negative (Shomer et al., 2003). The cell wall composition, more particularly the respective proportions of celluloses, hemicelluloses, pectins, and lignins, varies depending upon plant genotypes, and influences the amount of adsorbed iron (Chen, 2014; Shi et al., 2018). The plant capacity to store iron in its root apoplast is positively correlated to the amount of hemicelluloses in the cell wall and would be a key determinant in the IDC tolerance of graminaceous plant species such as maize (Shi et al., 2018). IDC tolerance in soybean is also associated with an increased amount of root apoplastic iron (Longnecker and Welch, 1990). Remobilization of precipitated apoplastic iron relies on phenolic compounds (Jin et al., 2007; Bashir et al., 2011; Ishimaru et al., 2011; Lei et al., 2014), putrescine synthesis, and decreased cell wall suberization (Zhu et al., 2016; Curie and Mari, 2017).

Proteins involved in iron acquisition—F6′H1, PDR9, and members of the HA, FRO, and IRT family—also mediate cellular iron trafficking (Connorton et al., 2017). Members of the oligopeptide transporter (OPT) protein family (e.g., YS/YSL transporters) are also key determinants of iron transport *in planta* (Su et al., 2018; Grillet and Schmidt, 2019; Kumar et al., 2019). This transporter family is particularly important for the transport of Fe to the seeds (Grillet et al., 2014; Curie and Mari, 2017; Su et al., 2018; Kumar et al., 2019).

Plant Breeding

The identification of plant traits mediating plant iron uptake and iron homeostasis *in planta* offers opportunities for plant breeders to promote iron nutrition and content in agricultural products (Waters and Sankaran, 2011).

Conventional plant selection and new breeding strategies are both applied to enhance iron acquisition, storage and nutritional availability in edible parts of crops. There exists a natural genetic variation in the level of expression of the mechanisms regulating iron uptake and homeostasis among plant genotypes. The efficiency of plant iron nutrition is highly variable across plant species (Hansen et al., 2006). The level of tolerance or the susceptibility to IDC highly differs according to plant species and even to cultivars (e.g., Gildersleeve and Ocumpaugh, 1989; Zribi and Gharsalli, 2002; Mahmoudi et al., 2009; Helms et al., 2010). The Fe levels in grains can vary significantly depending on cultivars (e.g., from 10 to 160 mg/kg in maize, 15–360 mg/kg in wheat, 23–105 mg/kg in pea and 34–157 mg/kg in bean) (White and Broadley, 2005, 2009). Assimilability of Fe for human beings correlates positively with the iron content in edible parts of crops (Welch et al., 2000) and varies with the forms of iron. The most assimilable forms of iron in plants are Fe^{2+}-nicotianamine (Fe^{2+}-NA) and Fe^{3+}-ferritin (Zielińska-Dawidziak, 2015; Beasley et al., 2019). In addition, iron nutritional availability is decreased by antinutrient molecules (e.g., phytates and tannins) that hamper its assimilation. Sufficient iron availability in food products is only possible when the concentration of these molecules is low (Sandberg, 2002; Delimont et al., 2017). Conventional breeding has led to the selection of cultivars showing better resistance to iron stress (i.e., IDC tolerant) and a higher iron content in edible parts, but also a decreased content of antinutrients (reviewed in Garcia-Oliveira et al., 2018). This was the case in species displaying high natural variability in their iron content (e.g., bean and pearl millet) (Manwaring et al., 2016; Lockyer et al., 2018). The selection of IDC-tolerant cultivars has further improved yields under iron stress conditions. However, the corresponding selection process relies on a long and costly screening of inbred lines.

Taking that limitation into account, transgenesis has been proposed as an option to promote plant iron nutrition and content by overexpressing or silencing genes mediating plant iron acquisition, transport and/or storage. The corresponding strategy has been followed through the targeting of one gene or several ones in combination and has led to genotypes with an increased iron content (i.e., from <2 to 6-fold) (Kawakami and Bhullar, 2018; Connorton and Balk, 2019). However, transgenesis raises public concerns (Lassoued et al., 2018). Furthermore, positive effects recorded in controlled conditions may be lost in field conditions because iron bioavailability varies among soils (Gregory et al., 2017). For example, iron uptake by soybean was increased by overexpressing *FRO* in controlled iron stress conditions (Vasconcelos et al., 2006) but not in high-calcareous soil environments (Kocak, 2014); even more, this genetic transformation appeared to be deleterious under non-iron stressed conditions due to toxic effects of the iron overload (Vasconcelos et al., 2006).

More generally, results from cultivars obtained from conventional and new breeding strategies vary depending upon soil iron bioavailability (Gregory et al., 2017; Garcia-Oliveira et al., 2018; Lockyer et al., 2018; Connorton and Balk, 2019). Alternative strategies based on QTL (quantitative trait loci) identification and on genome-wide association (GWAS) have been proposed to identify putative traits and genes mediating plant iron nutrition, and include them in plant breeding programs. The first step of this strategy confirmed the importance of genes implied in (i) iron uptake strategies I and II, (ii) the synthesis of phenolic compounds, and (iii) iron homeostasis. They further underlined the multigenic character of traits related to the plant iron status and the crucial importance of environmental conditions (Garcia-Oliveira et al., 2018; Connorton and Balk, 2019). Gene expression profiling of soybean plants sensitive or tolerant to IDC pinpointed key roles for phenylpropanoids (Waters et al., 2018). The major contribution of iron storage in the root apoplast and of fluorescent phenolics to remobilize this extracytoplasmic iron was confirmed and represents potential breeding targets (Curie and Mari, 2017; Waters et al., 2018). The complex and interregulated mechanisms of plant iron uptake and homeostasis has also been emphasized. A key role has been given to (i) phosphorus known to be in close relation with the iron status (e.g., Vansuyt et al., 2003; Tsai and Schmidt, 2017; Shi et al., 2018; Filiz and Kurt, 2019), (ii) ISR through the root-specific transcription factor MYB72 and beta-glucosidase BGLU42 (Zamioudis et al., 2014), and (iii) hormone signaling, especially IAA, Et, NO and ABA signaling (Lei et al., 2014; Li et al., 2015; Curie and Mari, 2017; Filiz and Kurt, 2019). The complex interrelations between the plant iron status, the P status, defense reactions and hormone signaling make the promotion of plant nutrition via plant breeding a difficult task.

Plant-Microbe Interactions Mediating Iron Uptake and Homeostasis

Impact of Rhizosphere Microbiota on Iron Availability

The rhizosphere microbiota impacts the physico-chemical properties of the root environment by acidifying the soil through the release of organic acids and protons, and chelating iron with organic acids and siderophores (**Figure 1B❶**, **❷**). These modifications prompt iron extraction from the soil matrix and thus modify its solubility and availability for the host plant.

Solubilization of iron in the rhizosphere is promoted by acidification. Protons are released during microbial activities such as nitrification (Kuypers et al., 2018). Protons may also be released from carboxylic groups when the pH of the soil solution is higher than the pKa of organic acids exuded by microorganisms (Glasauer et al., 2003). The protons released by microbial and plant activities acidify the rhizosphere (Hinsinger et al., 2003; Norton and Ouyang, 2019). Iron initially bound in scarcely soluble minerals (e.g., hematites, goethites) and amorphous solids [e.g., $Fe(OH)_3$] is replaced by protons at the sorption sites and released in the soil solution (**Figure 1B❶**; Albrecht-Gary and Crumbliss, 1998; Glasauer et al., 2003). Acidification also results from phosphate solubilization (Sharma A. et al., 2013). Organic acids are produced by rhizosphere bacteria that solubilize phosphates (e.g., *Pseudomonas*, *Bacillus*, *Rhizobium,* and *Enterobacter*) (Werra et al., 2009; Adeleke et al., 2017). Fe and P are often sequestered in soils together in low-solubility minerals like strengite or phosphosiderite. Thus, increased solubility of iron is associated with increased solubility of P (Marschner et al., 2011; Rijavec and Lapanje, 2016).

Solubilization of iron in the rhizosphere is also promoted by its chelation with organic acids, and by siderophores that scavenge ferric iron immobilized in scarcely soluble or insoluble forms and make it available to plants (**Figure 1B❷**; Kraemer, 2004; Jin et al., 2010; Ferret et al., 2014). The great majority of aerobic microorganisms synthesize small molecules with a high affinity for ferric iron—called siderophores—for their nutrition in iron stress conditions. Microbial siderophores (MSs) present high but variable affinity for Fe^{3+}, and are also diverse in size and chemical composition (Budzikiewicz, 2004; Hider and Kong, 2010; Saha et al., 2016; Khan et al., 2019). It has long been known that plants use iron chelated to MSs for their nutrition in Fe-limiting conditions; in particular, pyoverdines (pvds), a major class of siderophores produced by fluorescent pseudomonads, show a high affinity for ferric iron (reviewed by Crowley, 2006; Vansuyt et al., 2007; Jin et al., 2010; Shirley et al., 2011; Nagata et al., 2013; Radzki et al., 2013; Trapet et al., 2016). Rhizoferrin, ferrocrocin, fusigen, and coprogen, all produced by fungal root symbionts, also display high affinity for ferric iron (Winkelmann, 2017; Haselwandter et al., 2020). Microorganisms are expected to be highly competitive for Fe compared to plant roots because they can (i) use Fe bound to phytosiderophores (PSs) (microbial siderophores like pyoverdine have much higher affinity for Fe than PSs do), (ii) decompose PSs, and (iii) acquire iron more efficiently (**Figure 1B⓫**; Marschner et al., 2011; Sadrarhami et al., 2021). However, while Fe-pvds are more stable than Fe-PSs, they do not depress plant iron nutrition but, even more, promote it (Vansuyt et al., 2007; Jin et al., 2010; Shirley et al., 2011) in contrast with the early report of Becker et al. (1985b). The mechanisms underlying the beneficial effect of microbial siderophores on plant nutrition remain to be elucidated, even if some insights have been given (Vansuyt et al., 2007; Gonzàlez-Guerrero et al., 2016). Organic acids present much lower affinity for iron than siderophores do, but in circumneutral and alkaline environments such as calcareous soils, organic acids may be deprotonated and thus act as metal-complexing agents (Dehner et al., 2010). Hydrogen cyanide (HCN) produced by microorganisms may also contribute to iron mobilization by chelation (Frey et al., 2010; Rijavec and Lapanje, 2016).

Availability of soil nutrients, including Fe, can also be enhanced by increasing the volume of soil explored (**Figure 1B❿**). This is achieved by root fungal symbionts which greatly extend the scope of the roots through their fine hyphae. Increases in plant iron content have been ascribed to a better access to soil nutrients via fungal networks (Caris et al., 1998; Liu et al., 2000; Farzaneh et al., 2011). In addition, iron is transported into root cells by endosymbionts (Gonzàlez-Guerrero et al., 2016).

Plant Iron Physiology Modulation by the Rhizosphere Microbiota

Rhizosphere microorganisms modulate plant iron uptake mechanisms. The expression of genes involved in strategies I and II is modulated in the presence of microorganisms (**Figure 1B❻**; Zhang et al., 2009; Nagata et al., 2013; Nagata, 2017; Kobae et al., 2014; Zhou et al., 2016, 2018; Wang et al., 2017; Kabir et al., 2020). Rhizosphere acidification and ferric reductase activity (implied in strategy I) and plant PS synthesis (implied in strategy II) are enhanced (Reza, 2017; Wang et al., 2017; Zhou et al., 2018; Prity et al., 2020). The production of plant phenolics known to impact plant iron uptake and remobilization (**Figure 1B❺**; Fourcroy et al., 2016; Curie and Mari, 2017; Waters et al., 2018) and iron assimilability in food due to their antinutrient properties (Delimont et al., 2017) is also modified. Fluorescent pseudomonads induce the ISR (Van Loon et al., 2008; Berendsen et al., 2015) which regulates the expression of the root-specific transcription factor MYB72 and the MYB72-controlled beta-glucosidase BGLU42 (Zamioudis et al., 2014). These factors control the synthesis and excretion of iron-mobilizing FPCs in *Arabidopsis* (Palmer et al., 2013). Coumarins improve plant performance by eliciting microbe-assisted iron nutrition (Harbort et al., 2020). The concentration and composition of phenolic compounds in edible parts of plants is regulated by associated microorganisms (e.g., Basha et al., 2006; Lavania et al., 2006; Baslam et al., 2011; Jain et al., 2014; Singh et al., 2014; Baker et al., 2015).

Microorganisms modulate plant hormone signaling, which in turn impacts the plant iron physiology by modulating iron acquisition and homeostasis (**Figure 1B❽**; Lei et al., 2014; Li et al., 2015; Filiz and Kurt, 2019). Plant hormone signaling is under the control of microorganisms through the elicitation of the induced systemic response (ISR), a plant response interrelated with the plant iron deficiency response (Zamioudis et al., 2014; Romera et al., 2019). Changes in the plant indole acetic acid (IAA) and iron contents are observed concomitantly after bacterial inoculation, suggesting that microorganisms impact together hormone signaling and iron nutrition (Zhou et al., 2016, 2018; Housh et al., 2021). Emission of volatile organic compounds (VOCs) by *Bacillus amyloliquefaciens* promotes plant iron nutrition in *Arabidopsis,* and this promotion requires nitric oxide (NO) regulation (Wang et al., 2017), suggesting that the beneficial effect of the rhizosphere bacterial strain is regulated by plant hormone signaling. Rhizosphere microorganisms may either synthesize or degrade phytohormones [i.e., abscisic acid (ABA); IAA; gibberellic acid (GA); cytokinins (CKs); salicylic acid (SA); ethylene (Et); NO] and therefore modulate phytohormone concentrations (Horchani et al., 2011; Bakker et al., 2014; Egamberdieva et al., 2017; Ravanbakhsh et al., 2018). For example, the concentration of ethylene, a key regulator of root apoplastic iron remobilization under Fe shortage (Curie and Mari, 2017), is regulated by microbial 1-aminocyclopropane-1 carboxylic acid (ACC) deaminase that degrades the Et precursor (Ravanbakhsh et al., 2018).

The influence of microorganisms on the plant antioxidant defense has been associated to an increase of the plant iron content in a series of studies performed on sorghum, sunflower and alfalfa (**Figure 1B❼**; Kabir et al., 2020; Prity et al., 2020; Rahman et al., 2020). According to these authors, the promotion of ROS-scavenging activities by arbuscular mycorrhizal fungi (AMF) is part of the mechanisms involved in alleviation of Fe-deficiency symptoms.

Rhizosphere microorganisms can modify the plant iron status *via* their influence on the plant P status (**Figure 1B❾**; Tsai and Schmidt, 2017; Shi et al., 2018; Filiz and Kurt, 2019). Phosphate solubilizers and AMF have long been described to promote plant P nutrition and growth (Brown, 1974; Smith et al., 2011). More recently, microbial promotion of P nutrition was showed to impact iron partitioning in the roots and shoots of *Thypha angustifolia* (Ghosh et al., 2014), and to enhance iron nutrition in chicory through an AMF (*Glomus irregulare* syn. *Rhizophagus irregularis*; Labidi et al., 2012).

Other activities of rhizosphere microorganisms may also influence iron storage in the root apoplast and its remobilization. The hemicellulose composition of the root cell wall influences the amount of stored Fe, while putrescine, a diamine excreted by the roots, is involved in the iron remobilization process (**Figure 1**; Zhu et al., 2016; Shi et al., 2018). Since cellulose and hemicellulose are degraded by microbial activities, iron storage in the root apoplast is likely to be impacted by the corresponding microorganisms (**Figure 1B❹**; Lasa et al., 2019). Metatranscriptomic data indicate that the proportion of cellulose degraders is increased in the rhizosphere of cereals (Turner et al., 2013). Also, putrescine is one of the most commonly used substrate by wheat rhizosphere microorganisms (Gałązka et al., 2019): microbial degradation of this diamine (e.g., by pseudomonads) is thus likely to regulate plant remobilization of apoplastic iron (Kuiper et al., 2001; Song et al., 2015; Liu et al., 2018).

In sum, MSs play a key role in plant physiology related to iron uptake and homeostasis (**Table 1** and **Figures 1**, **2**) through (i) phosphorus solubilization and thus the plant P status (Sharma A. et al., 2013), (ii) elicitation of plant defense reactions through Microbial Associated Molecular Patterns (MAMPs) inducing ISR (De Vleesschauwer et al., 2006; Höfte and Bakker, 2007; Van Loon et al., 2008), (iii) plant hormone signaling and the synthesis of fluorescent root phenolics via ISR (Pieterse et al., 2014; Zamioudis et al., 2014), and (iv) the expression of genes mediating iron uptake and homeostasis (**Table 1**). The importance of microbial siderophores in the rhizosphere is also evidenced by results showing that their synthesis and activities are enhanced in the rhizosphere. Protein families related to siderophore production increased in barley root- and rhizosphere-associated bacterial taxa (Bulgarelli et al., 2015), and sequences encoding bacterial siderophore synthesis were highly enriched within bacterial endophytes in rice roots (Sessitsch et al., 2012).

In addition, discussion is running on the possible contribution of microbial siderophores to the remobilization of root apoplastic iron and in a non-reductive process of iron uptake by plants (**Figure 1B❸**). A large-scale transcriptomic study in *Arabidopsis* suggests that remobilization of root apoplastic iron is promoted by the pyoverdine synthesized by *P. fluorescen*s strain C7R12 (Trapet et al., 2016). Plants grown in iron-deficient conditions in the presence of apo-pyoverdine (a siderophore uncomplexed with iron) exhibited a phenotype similar to that of plants grown in iron-containing medium and incorporated more iron than the untreated plants did. In these conditions, pyoverdine repressed the expression of root genes related to ABA signaling (Trapet et al., 2016), suggesting that the MS may promote the remobilization of root apoplastic iron (which indeed implies ABA regulation) (Lei et al., 2014; Curie and Mari, 2017). Possible non-reductive uptake of bacterial ferrisiderophores would represent an additional and major influence of microorganisms on the plant iron status. Various findings support the existence of a transport system whereby the plant would internalize bacterial ferrisiderophores in the form of integral ferric chelates. Pyoverdines of fluorescent pseudomonads chelated to iron (Fe^{3+}-pvd) contribute to the plant iron nutrition of both dicots (strategy I) and graminaceous monocots (strategy II) more efficiently than the synthetic ferric chelate Fe^{3+}-EDTA does (Vansuyt et al., 2007; Jin et al., 2010; Shirley et al., 2011). The stability constant of the Fe^{3+}-pvd complex is significantly higher (10^{32}) than that of Fe^{3+}-EDTA (10^{25}) or Fe-PS (10^{18}) (Vansuyt et al., 2007; Shirley et al., 2011). This suggests that dissociation and ligand exchange between Fe^{3+}-pvd and Fe^{3+}-PS might not be the sole process accounting for the enhanced iron nutrition by Fe-MS and suggests incorporation of Fe^{3+}-pvd by the roots. The presence of pvd *in planta* was confirmed by measurements of ^{15}N-labeled Fe^{3+}-pvd and by immunodetection using anti-pyoverdine antibodies (Vansuyt et al., 2007; Trapet et al., 2016). The use of *IRT*1 knock-out mutants indicated that this membrane transporter of Fe^{2+} (IRT1) is not involved in iron uptake from Fe^{3+}-pvd in strategy I plants (Vansuyt et al., 2007). Thus, Fe-pvd clearly contributes to plant iron nutrition, with evidence of the presence of pvd *in planta* but no proof of the direct uptake of the entire Fe^{3+}-pvd complex. The description of a non-reductive iron uptake system in a phytoplankton organism supports a possible incorporation of bacterial ferrisiderophores kept throughout evolution (Kazamia et al., 2018). This hypothesis is also supported by the presence of vesicles in the roots of *Arabidopsis* supplemented with Fe-pvd (Lemanceau et al., 2009); these vesicles mediate the internalization of ferrisiderophores by endocytosis in diatoms (Kazamia et al., 2018).

Plant-Plant-Microbe Interactions Mediating Plant Iron Nutrition and Homeostasis

The plant iron status is modulated by plant-plant interactions that also involve microbial interactions in non-sterile growing conditions. Intercropping, in which at least two plant species are grown together in the same field, is proposed as a means to increase crop yield and quality in low-input agricultural systems by valuing beneficial plant-plant interactions. Several studies

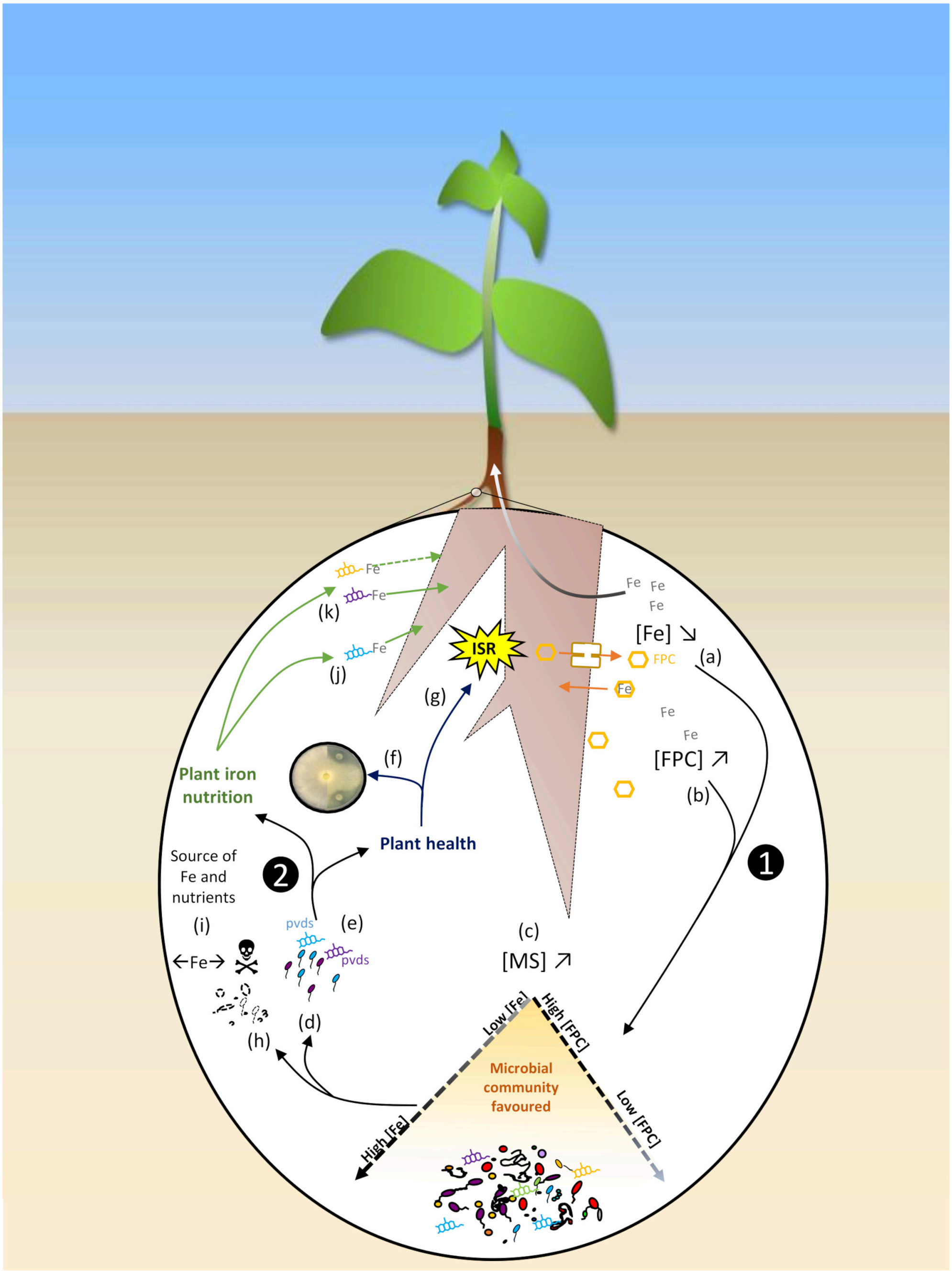

FIGURE 2 | Schematic representation of the feedback loop summarizing the iron dynamics in the rhizosphere as regulated by plant-microbe interactions. ❶The plant iron status shapes the rhizosphere microbiota. Iron bioavailability ([Fe]↘) is decreased in the rhizosphere due to plant Fe uptake (a) (Robin et al., 2006, 2007), and plant excretion of root fluorescent phenolic compounds (FPCs) is enhanced in calcareous soils ([FPC]↗), with low iron availability ([Fe]↘) (b) (Jin et al., 2010; Stringlis et al., 2018). Production of microbial siderophores is consequently increased ([MS]↗) (c) (Jin et al., 2010), and pseudomonads adapted to iron stresss conditions are favored (d) (Robin et al., 2007) thanks to the synthesis of specific pyoverdines (pvds) (e) (Robin et al., 2007; Stringlis et al., 2018). ❷In return, these populations positively influence plant health and iron nutrition. Specific pvds of rhizosphere pseudomonads display high antagonistic activities by competing against phytopathogens for iron (f) (Robin et al., 2007; Gu et al., 2020), and others trigger plant induced systemic resistance (ISR) (g) (Stringlis et al., 2018). Fungal phytopathogens (*Fusarium*) can be counter-selected by FPCs (h) (Stringlis et al., 2018). Specific microbial populations are counter-selected by iron competition (←Fe→), by FPC toxicity (☠), or microbial antagonism; these populations represent a source of iron (and of other nutriments) when metabolized (i). A siderophore produced by a pseudomonad strain recruited in the rhizosphere of an iron-stressed plant can also favor plant iron nutrition (j) (Jin et al., 2010), and distinct pvds of different strains of *Pseudomonas* differently favor plant iron nutrition (k) (Lurthy et al., 2020), suggesting that plant iron nutrition is impacted differently depending on the pseudomonads recruited in the rhizosphere.

reviewed by Xue et al. (2016) and Dai et al. (2019) reported increased tolerance to IDC of legumes and non-legume dicots in alkaline conditions when they were associated with a cereal in intercropping systems. Intercropping can also modulate plant iron distribution (Xue et al., 2016). Intercropping with grass was even more efficient than adding iron chelates on the yields of blueberries cultivated in sub-alkaline soil (Michel et al., 2019). An increased iron content of peanut grains (1.43-fold) was recorded when peanut was intercropped with maize in calcareous soil (Zuo and Zhang, 2009). Intercropping with oat was as good as in-furrow amendment with chemical Fe chelate (FeDDHA) for alleviating soybean IDC on calcareous soils (Kaiser et al., 2014). However, variations were observed depending on environmental conditions, and Fe amendment was sometimes more reliable. Better knowledge of the biotic interactions involved is therefore required to increase the reproducibility of the results so as to develop these environmentally friendly cropping systems.

Regarding iron uptake, three mechanisms of facilitation may account for the enhancement of iron nutrition in dicots in the presence of graminaceous crop plants.

The first mechanism would rely on the extraction of iron by chelation with PSs from grasses that would increase iron availability to dicots, as shown when intercropping olive (Cañasveras et al., 2014) and citrus rootstocks (Cesco et al., 2006) with grasses. Intercropping impacted PS production and expression of the *FRO* and *IRT* genes implied in the strategy I iron uptake system. However, this trend lacks consistency across studies (Dai et al., 2019).

A second mechanism would rely on a non-reductive mechanism used by dicots to incorporate Fe-PSs formed with PSs excreted by grasses. Fe-PSs from a strategy II plant (maize) were internalized by a strategy I plant (peanut) (Xiong et al., 2013) via a membrane transporter belonging to the YS/YSL family of Fe-PS transporters (Curie et al., 2000, 2009).

Finally, the third mechanism enhancing iron uptake in dicots intercropped with maize would rely on the remobilization of apoplastic iron by root phenolic compounds. Under Fe deficiency, maize was unable to remobilize its pool of root apoplastic iron, contrary to bean (Bienfait et al., 1985). In addition, Fe-deficient bean plants mobilized iron from the root apoplast of other plants grown in their presence (Bienfait et al., 1985). In alkaline conditions, increased synthesis of root fluorescent phenolics (Waters et al., 2018) could contribute to the mobilization of rhizosphere iron by dicots. Therefore, the non-used root apoplastic iron pool of maize roots could be remobilized by an associated dicotyledonous crop. This could partly account for the better iron nutrition of legumes grown together with maize (Xue et al., 2016; Dai et al., 2019).

The rhizosphere microbiota also contributes to the better efficiency of plant species cultivated together. In cereal-legume intercropping, symbiotic interactions between the legume species and nitrogen-fixing microorganisms decrease competition for soil nitrogen, and the resulting resource partitioning promotes nitrogen nutrition of the cereal. In addition to limited interspecific competition for N acquisition in cereal-legume intercropping, other processes such as soil N enrichment or high N restitution through below-ground legume residues benefit N acquisition by the cereal (Hauggaard-Nielsen et al., 2009; Fustec et al., 2010). More generally, plant-plant interactions impact root exudation, and this affects soil rhizosphere microbiota (Vora et al., 2021) and favors colonization by AMF (Ingraffia et al., 2019). On the other hand, improved mycorrhization increased the Fe content in wheat intercropped with faba bean, but did not increase it in mono-cropped faba bean (Ingraffia et al., 2019). According to these authors, the enhancement of plant Fe uptake modulated by AMF depends on soil physico-chemical properties. The mycorrhizosphere of associated plants, formed by AMF-colonized roots and hyphae, increases microbiota functionalities (Wahbi et al., 2016b). Intercropping impacts the abundance, diversity, activity and co-occurrence network of rhizosphere microbial communities (Li et al., 2016, 2018; Wahbi et al., 2016a; Duchene et al., 2017; Taschen et al., 2017; Gao et al., 2019; Zaeem et al., 2019; Liu et al., 2021; Pivato et al., 2021). This is in agreement with the well-known positive relationship between plant and microbial diversity (Spehn et al., 2000; Carney and Matson, 2005; Qiao et al., 2012; Ahmad et al., 2013). In controlled conditions, the pea-wheat association did not harbor a mixture of the two rhizospheres, but rather a new bacterial community with more Actinobacteria and a decreased abundance of α-Proteobacteria and Acidobacteria (Taschen et al., 2017). In another study in field conditions, bacterial networks were impacted by pea-wheat intercropping, but bacterial diversity and structure were not, suggesting a more complex bacterial network and more complex interactions (Pivato et al., 2021). The observed changes in the microbial community diversity and its increased complexity may account for the beneficial effects observed in intercropping. Compared to maize and peanut cultivated independently, comparable microbial communities have been observed whether the roots were separated or not: *Bacillus*, *Brevibacillus*, and *Paenibacillus* were mainly increased in the rhizosphere of maize, while *Burkholderia*, *Pseudomonas*, and *Rhizobium* were mainly increased in the rhizosphere of peanut. In these conditions, the availability of nutrients (N and P) was increased (Li et al., 2018), even if no correlation was found with the changes observed in the microbial community.

More generally, the higher microbial diversity associated with higher plant diversity results in better plant fitness, resilience to stress (De Vries et al., 2018), and positive effects of intercropping (Sun et al., 2009). Various studies suggesting a better iron nutrition of strategy I plants grown in association with a cereal have been reported (reviewed by Xue et al., 2016; Dai et al., 2019). Despite the well-known impact of intercropping on the rhizosphere microbiota and evidence of the role of microorganisms (e.g., AMF) in enhancing plant nutrition in association, data allowing us to evaluate the role played by the plant microbiota are missing. Additional data on the plant iron content will also be required because up to now the effect of intercropping has been mostly evaluated by visually recording IDC symptoms. Therefore, knowledge integrating plant-plant, plant-microorganism and microbe-microbe interactions is sorely lacking.

CONSEQUENCES FOR THE DEVELOPMENT OF IRON BIOFORTIFICATION STRATEGIES

Microorganisms modulate iron bioavailability nearby and within the roots, as weel as plant iron uptake and homeostasis (**Figure 1**). Optimizing the biotic interactions that mediate plant iron uptake and homeostasis opens onto stimulating prospects for plant iron biofortification. The importance of microorganisms in plant nutrition including iron nutrition is widely acknowledged, but up to now they have been mainly used as biofertilizers and applied to plants in different formulations containing one or several microorganisms. However beneficial effects of microbial inoculation are often offset by a lack of consistency due to poor survival of the introduced strains (Singh and Prasanna, 2020; French et al., 2021).

Current research is now shifting its focus on the monitoring of rhizosphere microbiota on the basis of increasing knowledge of the plant-microbe feedback. The impact of the rhizosphere microbiota on iron availability and plant iron physiology is part of dynamic processes that are themselves influenced by plant-microbe interactions. Monitoring plant-microbe interactions mediating plant iron nutrition and homeostasis requires to decipher the complexity of the corresponding interactions. It is now well established that plants shape the composition of their microbiota via rhizodeposition including root exudation (Badri et al., 2013; Lemanceau et al., 2017a; Canarini et al., 2019; Jones et al., 2019). In turn, the rhizosphere microbiota impacts plant nutrition, growth and health. This feedback loop is modulated by the plant genotype and by the soil physico-chemical and biological properties (Lemanceau et al., 2017b; Rodriguez et al., 2019). These reciprocal interactions are well illustrated by the iron dynamics in the rhizosphere (**Figure 2**). Two series of studies report that the Fe-chelating ability of the rhizosphere microbiota is modified by the plant iron status. The first one was conducted on transgenic tobacco deregulated in ferritin, hence hyperaccumulation of iron *in planta* and iron depletion of the corresponding rhizosphere. This depletion resulted in the selection of pseudomonad populations highly adapted to iron-stressed conditions thanks to the synthesis of efficient siderophores (Robin et al., 2006, 2007). The second series was conducted with clover grown in Fe-deficient conditions; this plant synthesized more phenolic compounds, hence the selection of a higher occurrence of siderophore-producing bacteria (Jin et al., 2010). In both cases, the plant contributed to decrease rhizosphere iron availability. This led to an increased level of iron competition that favored the microbial communities most adapted to these iron stress conditions thanks to their siderophores (**Figure 2**; Robin et al., 2006, 2007; Jin et al., 2007, 2010), while depleting those susceptible to low iron availability. The plant metabolites released in iron stress conditions (e.g., phenolic compounds like scopoletin) may even have a biocidal effect on susceptible populations (Gnonlonfin et al., 2012). Microbial populations recruited by the host plant in turn impact plant nutrition, growth, and health (**Figure 2**). Thus, plant iron nutrition was promoted by siderophores synthesized by a *Pseudomonas* strain originating from the rhizosphere of Fe-deficient clover (Jin et al., 2010). Also, a siderophore from a pseudomonad strain highly represented in the rhizosphere of a pea cultivar tolerant to IDC significantly improved iron nutrition of this plant (Lurthy et al., 2020). Similarly, two strains (*P. simiae* WCS417 and *P. capeferrum* WCS358) highly tolerant to the antimicrobial effect of root phenolics promoted *Arabidopsis* growth *via* siderophore production (**Figure 2**; Berendsen et al., 2015; Stringlis et al., 2018). The biomass of the microbes counter-selected by iron competition and phenolics represents a potential pool of iron and other nutrients. Thus, in addition to iron stored in ferritins, vacuoles and the root apoplastic compartment, the root microbiota could be used as an additional level of iron storage by plants. Regarding plant health, major phytopathogens are controlled by iron competition in the rhizosphere. Siderophores with a high affinity for iron and retrieved from the rhizosphere of ferritin-overexpressing transgenic tobacco displayed a higher antagonistic activity against the phytopathogenic oomycete *Pythium aphanidermatum* (**Figure 2**; Robin et al., 2007). Root FPCs synthesized through a MYB72-dependent pathway selectively inhibited the soil-borne fungal pathogens *Fusarium oxysporum* and *Verticillium dahlia* (**Figure 2**; Stringlis et al., 2018). In addition, plant protection was promoted by the above mentioned *P. simiae* WCS417 and *P. capeferrum* WCS358, inducers of plant systemic resistance (ISR; Stringlis et al., 2018).

The influence of the crosstalk between the host plant and its associated microbiota on plant iron nutrition (**Figure 2**) stresses the importance of considering the plant together with its microbiota in biofortification strategies. Progress in the knowledge of the interactions between eukaryotic organisms and their associated microbiota has led to the emergence of the holobiont concept, defined as the host and its associated microbes (Vandenkoornhuyse et al., 2015). Because of the importance of their associated microbiota, in terms of abundance, diversity and beneficial effects for the host plant, plants can no longer be considered as stand-alone entities (Dessaux et al., 2016). According to this concept, the genome interacting with its environment is no more restricted to the plant genome but is extended to that of the holobiont (hologenome) (Theis et al., 2016). Therefore, we propose to consider holobiont genetic resources for improving the plant iron status. Including the plant microbiota and its transmission by seeds in breeding programs has been proposed (Gopal and Gupta, 2016; Wei and Jousset, 2017; Berg and Raaijmakers, 2018). More recently, Wille et al. (2019) presented a comprehensive review of the plant-microbe interactions implied in resistance to root diseases in grain legumes and discussed possible consequences for breeding strategies. They especially proposed to consider the entire plant holobiont in resistance breeding strategies. The same principle should be applied to iron biofortification. In that prospect, plant traits included in breeding programs should comprise traits modulating plant-microbe interactions beneficial for the plant iron status. These traits represent promising new breeding targets. Among them, three types stand out and require special attention (i) the synthesis pathways of fluorescent phenolics and plant defense responses sharing common key components, (ii) plant regulation of iron storage in the root apoplast, and (iii) putrescine synthesis mediating apoplastic iron remobilization. The targeted plant traits should also include those involved in the recruitment of functional microbial genes (Lemanceau et al., 2017b) mediating siderophore production, synthesis, or degradation of specific molecules related to the plant iron physiology (e.g., cellulose, hemicellulose, putrescine, plant hormones). Particular attention should be paid to microbial siderophores because they represent a major contribution of microorganisms to the plant iron status, although the mechanisms involved are not all known yet. Recent results show that effects of Fe-MS on the plant iron status vary depending on plant genotype and MS structure (Lurthy et al., 2020). Therefore, the high level of specificity between the plant and its microbiota should be taken into account. Beyond plant-microorganism interactions, crop biodiversity and plant-plant interactions represent major levers for improving the resistance and resilience of canopies and reducing their dependence on synthetic inputs, to ultimately ensure crop sustainability (Wezel et al., 2014; Dubey et al., 2020). Increasing crop biodiversity relies on the association of plants cultivated in intercropping. The challenge is to find out plant associations and practices that favor processes of ecological facilitation in intecropping. This occurs when the association optimizes the development of both species (e.g., improved resource availability) and minimizes any negative interactions that might occur between the two species (Callaway, 1995). To allow this facilitation process to occur and thus promote the functioning and performance of intercropping, the choices of plant species and cultivars to be grown in association, together with the cropping practices (seeding density and pattern, level of nitrogen fertilization) are key to success (Andersen et al., 2007; Neumann et al., 2007; Bedoussac et al., 2015). When these conditions are met, intercropping allows better nutrition of each associated plant species thanks to the facilitation process (Duchene et al., 2017) and the use of fertilizers can be reduced (Bedoussac and Justes, 2010). Research is ongoing to optimize biotic interactions that promote plant nutrition. Given the impact of the plant species, but also of its genotype, on the rhizosphere microbiota, characterizing the effect of different cultivars of a plant species grown in association on the microbial community is a key step for identifying the best performing cultivars in the association. However, the mechanisms underlying the positive effects of these cropping systems on plant-microbe interactions remain largely untapped, and further studies are required to better understand and exploit the interplay of these biotic interactions.

CONCLUSION AND PROSPECTS

Iron amounts in soils are above plant needs but are not readily available in most agricultural soils. Consequently, increasing soil iron bioavailability to enhance plant and ultimately human nutrition represents a major challenge. The soil microbiota has a great impact on iron bioavailability in the rhizosphere and on plant iron physiology. This should open avenues for plant iron biofortification strategies that will value these biotic interactions. The entire plant holobiont should be considered in biofortification strategies, and the plant traits included in breeding programs should comprise traits modulating plant-microbe interactions beneficial for the plant iron status. These traits will include the synthesis of root phenolics and the regulation of apoplastic iron storage and remobilization. Plant traits mediating the recruitment of microbial genes involved in the synthesis or degradation of specific molecules related to the plant iron physiology (e.g., cellulose, hemicellulose, putrescine, plant hormones) have to be investigated. Special attention should be paid to the interactions with microbial siderophores, which strongly impact the plant iron status; many of the mechanisms involved still have to be identified. Recent findings highlight the specificity of biotic interactions, the role of the environment, and the interconnexion between plant iron nutrition and other parameters that also influence the quantity and the quality of vegetal products—plant health, the P status, and the ionome. This leads us to think that important headways should be made possible by the development of integrative approaches. In addition to iron biofortification, these approaches will take into account plants and their extended genotype formed by each plant and its specific microbiota; this holobiotic organism will be more prone than the plant alone to adapt to environmental stresses. Intercropping appears promising to implement these strategies.

More options for iron biofortification could be brought by emerging research perspectives. Most of the findings on the microbial influence on plant iron come from studies focusing on soil and rhizosphere interactions. Yet, the phyllosphere and spermosphere microbiota, whose influence has long been underestimated, also influence the plant iron physiology (Lemanceau et al., 2017a). Iron is absorbed by the leaves, and a signal originating from the shoots and involving IAA appears to elicit root-to-shoot iron translocation (Kabir et al., 2013; Garnica et al., 2018). The importance of the shoot microbiota on these components of the plant iron dynamics remains to be explored. The spermosphere microbiota is at least partly inherited from parent plants (Lemanceau et al., 2017a). Therefore, it is essential to evaluate the role of the corresponding microorganisms. Studies on the plant microbiota mostly provide taxonomic descriptions of plant-associated microorganisms. Therefore, results are deeply influenced by the soil microbiota reservoir which varies according to the soil physical and chemical properties (Dequiedt et al., 2009; Ranjard et al., 2013; Xue et al., 2018). Lemanceau et al. (2017b) have proposed the principle of a functional plant-genotype-specific core microbiota shared whatever the soil in which the corresponding genotype is cultivated. This proposal relies on the fact that plant-beneficial microbial traits (e.g., production of siderophores, hormones, antibiotic molecules, and HCN) can be found in distinct microbial taxa. Finally, it is important to widen the objectives and develop more integrative studies. Potential trade-offs may indeed occur. Breeding programs focused on plant health promotion could be detrimental to plant growth and also impact the plant iron status, and *vice versa*. The dynamics of iron in the rhizosphere also modulates plant health (**Figure 2**). Seemingly, increasing plant iron content also more globally impacts the plant ionome (Cohen et al., 1998). This could be favorable to other essential micronutrients like Zn, but could also lead to the accumulation of toxic elements due to the variable specificity of plant iron transporters (Rajkumar et al., 2010; De Valença et al., 2017). Another trade-off probably involves phenolic antioxidant compounds. Research about them is ongoing for improving human health, but they may also act as antinutrients by decreasing iron assimilability. The selection of new genotypes should no longer be oriented toward the production of plants harboring specific characters like enhanced iron content or resistance to a given pathogen. Plant improvement strategies should rather consider the extended genotype formed by the plant and its specific microbiota, and search for combinations allowing the holobiont to quickly adapt to a range of severe biotic and abiotic stresses likely to occur for a particular crop. We should rather tend toward the search for "ideoholotypes."

AUTHOR CONTRIBUTIONS

SM and PL initiated the review. All authors contributed and approved the final manuscript.

FUNDING

This work was supported by the POSiTiF project of the Plant2Pro Carnot Institute and by the "Conseil Régional de Bourgogne".

ACKNOWLEDGMENTS

We are grateful to Annie Buchwalter for correcting the English text.

REFERENCES

Abadía, J., Vázquez, S., Rellán-Álvarez, R., El-Jendoubi, H., Abadía, A., Alvarez-Fernández, A., et al. (2011). Towards a knowledge-based correction of iron chlorosis. *Plant Physiol. Biochem.* 49, 471–482. doi: 10.1016/j.plaphy.2011.01.026

Adeleke, R., Nwangburuka, C., and Oboirien, B. (2017). Origins, roles and fate of organic acids in soils: a review. *S. Afr. J. Bot.* 108, 393–406. doi: 10.1016/j.sajb.2016.09.002

Ahmad, I., Cheng, Z., Meng, H., Liu, T., Wang, M., Ejaz, M., et al. (2013). Effect of pepper-garlic intercropping system on soil microbial and bio-chemical properties. *Pak. J. Bot.* 45, 695–702.

Aisen, P., Enns, C., and Wessling-Resnick, M. (2001). Chemistry and biology of eukaryotic iron metabolism. *Int. J. Biochem. Cell. Biol.* 33, 940–959. doi: 10.1016/s1357-2725(01)00063-2

Albrecht-Gary, A. M., and Crumbliss, A. L. (1998). Coordination chemistry of siderophores: thermodynamics and kinetics of iron chelation and release. *Met. Ions Biol. Syst.* 35, 239–327.

Andersen, M. K., Hauggaard-Nielsen, H., Høgh-Jensen, H., and Jensen, E. S. (2007). Competition for and utilisation of sulfur in sole and intercrops of pea and barley. *Nutr. Cycl. Agroecosyst.* 77, 143–153. doi: 10.1007/s10705-006-9053-7

Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., and Vivanco, J. M. (2013). Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J. Biol. Chem.* 288, 4502–4512. doi: 10.1074/jbc.M112.433300

Bailey, R. L., West, K. P. W. Jr., and Black, R. E. (2015). The epidemiology of global micronutrient deficiencies. *Ann. Nutr. Metab.* 66, 22–33. doi: 10.1159/000371618

Baker, C. J., Mock, N. M., Smith, J. M., and Aver'Yanov, A. A. (2015). The dynamics of apoplast phenolics in tobacco leaves following inoculation with bacteria. *Front. Plant. Sci.* 6:649. doi: 10.3389/fpls.2015.00649

Bakker, P. A. H. M., Ran, L., and Mercado-Blanco, J. (2014). Rhizobacterial salicylate production provokes headaches! *Plant Soil* 382, 1–16. doi: 10.1007/s11104-014-2102-0

Balk, J., and Schaedler, T. A. (2014). Iron cofactor assembly in plants. *Annu. Rev. Plant Biol.* 65, 125–153. doi: 10.1146/annurev-arplant-050213-035759

Bar-Ness, E., Hadar, Y., Chen, Y., Römheld, V., and Marschner, H. (1992). Iron uptake by plants from microbial siderophores: a study with 7-nitrobenz-2 oxa-1,3-diazole-desferrioxamine as fluorescent ferrioxamine B analog. *Plant Physiol.* 99, 1329–1335. doi: 10.1104/pp.99.4.1329

Basha, S. A., Sarma, B. K., Singh, D. P., Annapurna, K., and Singh, U. P. (2006). Differential methods of inoculation of plant growth-promoting rhizobacteria induce synthesis of phenylalanine ammonia-lyase and phenolic compounds differentially in chickpea. *Folia Microbiol. (Praha).* 51, 463–468. doi: 10.1007/bf02931592

Bashir, K., Ishimaru, Y., and Nishizawa, N. K. (2010). Iron uptake and loading into Rice Grains. *Rice.* 3, 122–130. doi: 10.1007/s12284-010-9042-y

Bashir, K., Ishimaru, Y., Shimo, H., Kakei, Y., Senoura, T., Takahashi, R., et al. (2011). Rice phenolics efflux transporter 2 (PEZ2) plays an important role in solubilizing apoplasmic iron. *J. Soil. Sci. Plant. Nutr.* 57, 803–812. doi: 10.1080/00380768.2011.637305

Baslam, M., Garmendia, I., and Goicoechea, N. (2011). Arbuscular mycorrhizal fungi (AMF) improved growth and nutritional quality of greenhouse-grown lettuce. *J. Agr. Food. Chem.* 59, 5504–5515. doi: 10.1021/jf200501c

Beasley, J. T., Hart, J. J., Tako, E., Glahn, R. P., and Johnson, A. A. T. (2019). Investigation of nicotianamine and 2' deoxymugineic acid as enhancers of iron bioavailability in caco-2 cells. *Nutrients.* 11:1502. doi: 10.3390/nu11071502

Beck, K. L., Conlon, C. A., Kruger, R., and Coad, J. (2014). Dietary determinants of and possible solutions to iron deficiency for young women living in industrialized countries: a review. *Nutrients.* 6, 3747–3776. doi: 10.3390/nu6093747

Becker, J. O., Hedges, R. W., and Messens, E. (1985b). Inhibitory effect of pseudobactin on the uptake of iron by higher plants. *Appl. Environ. Microbiol.* 49, 1090–1093. doi: 10.1128/aem.49.5.1090-1093.1985

Becker, J. O., Messens, E., and Hedges, R. W. (1985a). The influence of agrobactin on the uptake of ferric iron by plants. *FEMS Microbiol. Ecol.* 31, 171–175. doi: 10.1111/j.1574-6968.1985.tb01145.x

Bedoussac, L., and Justes, E. (2010). The efficiency of a durum wheat-winter pea intercrop to improve yield and wheat grain protein concentration depends on N availability during early growth. *Plant Soil* 330, 19–35. doi: 10.1007/s11104-009-0082-2

Bedoussac, L., Journet, E.-P., Hauggaard-Nielsen, H., Naudin, C., Corre-Hellou, G., Jensen, E. S., et al. (2015). Ecological principles underlying the increase of productivity achieved by cereal-grain legume intercrops in organic farming. A review. *Agron. Sustain. Dev.* 35, 911–935. doi: 10.1007/s13593-014-0277-7

Berendsen, R. L., van Verk, M. C., Stringlis, I. A., Zamioudis, C., Tommassen, J., Pieterse, C. M., et al. (2015). Unearthing the genomes of plant-beneficial *Pseudomonas* model strains WCS358, WCS374 and WCS417. *BMC Genomics* 16:539. doi: 10.1186/s12864-015-1632-z

Berg, G., and Raaijmakers, J. M. (2018). Saving seed microbiomes. *Int. Soc. Microbial. Ecol. J.* 12, 1167–1170. doi: 10.1038/s41396-017-0028-2

Bienfait, H. F., Briel, W., and van den Mesland-Mul, N. T. (1985). Free space iron pools in roots: generation and mobilization. *Plant Physiol.* 78, 596–600. doi: 10.1104/pp.78.3.596

Briat, J.-F., Dubos, C., and Gaymard, F. (2015). Iron nutrition, biomass production, and plant product quality. *Trends Plant Sci.* 20, 33–40. doi: 10.1016/j.tplants.2014.07.005

Brown, M. E. (1974). Seed and root bacterization. *Annu. Rev. Phytopathol.* 12, 181–197. doi: 10.1146/annurev.py.12.090174.001145

Budzikiewicz, H. (2004). Siderophores of the Pseudomonadaceae sensu stricto (fluorescent and non-fluorescent *Pseudomonas* spp.). *Fortschr. Chem. Org. Naturst.* 87, 81–237. doi: 10.1007/978-3-7091-0581-8_2

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weimn, A., Dröge, J., Pan, Y., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Callaway, R. M. (1995). Positive interactions among plants. *Bot. Rev.* 61, 306–349. doi: 10.1007/BF02912621

Canarini, A., Kaiser, C., Merchant, A., Richter, A., and Wanek, W. (2019). Root exudation of primary metabolites: mechanisms and their roles in plant responses to environmental stimuli. *Front. Plant. Sci.* 10:157. doi: 10.3389/fpls.2019.00157

Cañasveras, J. C., del Campillo, M. C., Barrón, V., and Torrent, J. (2014). Intercropping with grasses helps to reduce iron chlorosis in olive. *J. Soil Sci. Plant. Nutr.* 14, 554–564. doi: 10.4067/S0718-95162014005000044

Caris, C., Hördt, W., Hawkins, H. J., Römheld, V., and George, E. (1998). Studies of iron transport by arbuscular mycorrhizal hyphae from soil to peanut and sorghum plants. *Mycorrhiza* 8, 35–39. doi: 10.1007/s005720050208

Carney, K. M., and Matson, P. A. (2005). Plant communities, soil microorganisms, and soil carbon cycling: does altering the world belowground matter to ecosystem functioning? *Ecosystems* 8, 928–940. doi: 10.1007/s10021-005-0047-0

Carrillo-Castañeda, G., Muñoz, J. J., Peralta-Videa, J. R., Gomez, E., and Gardea-Torres, J. L. (2005). Modulation of uptake and translocation of iron and copper from root to shoot in common bean by siderophore-producing microorganisms. *J. Plant. Nutr.* 28, 1853–1865. doi: 10.1080/01904160500251340

Cesco, S., Rombolà, A. D., Tagliavini, M., Varanini, Z., and Pinton, R. (2006). Phytosiderophores released by graminaceous species promote 59Fe-uptake in citrus. *Plant Soil* 287, 223–233. doi: 10.1007/s11104-006-9069-4

Chen, H. (2014). "Chemical composition and structure of natural lignocellulose," in *Biotechnology of Lignocellulose: Theory And Practice*, ed. H. Chen (Dordrecht: Springer), 25–71. doi: 10.1007/978-94-007-6898-7_2

Chen, L., Dick, W. A., and Streeter, J. G. (2000). Production of aerobactin by microorganisms from a compost enrichment culture and soybean utilization. *J. Plant. Nutr.* 23, 2047–2060. doi: 10.1080/01904160009382164

Chen, Y., and Barak, P. (1982). Iron nutrition of plants in calcareous soils. *Adv. Agron.* 35, 217–240. doi: 10.1016/S0065-2113(08)60326-0

Clark, R. B., and Zeto, S. K. (1996). Mineral acquisition by mycorrhizal maize grown on acid and alkaline soil. *Soil Biol Biochem.* 28, 1495–1503. doi: 10.1016/S0038-0717(96)00163-0

Cohen, C. K., Fox, T. C., Garvin, D. F., and Kochian, L. V. (1998). The role of iron-deficiency stress responses in stimulating heavy-metal transport in plants. *Plant Physiol.* 116, 1063–1072. doi: 10.1104/pp.116.3.1063

Colombo, C., Palumbo, G., He, J.-Z., Pinton, R., and Cesco, S. (2014). Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *J. Soil. Sedim.* 14, 538–548. doi: 10.1007/s11368-013-0814-z

Connorton, J. M., and Balk, J. (2019). Iron biofortification of staple crops: lessons and challenges in plant genetics. *Plant Cell Physiol.* 160, 1447–1456. doi: 10.1093/pcp/pcz079

Connorton, J. M., Balk, J., and Rodríguez-Celma, J. (2017). Iron homeostasis in plants – a brief overview. *Metallomics* 9, 813–823. doi: 10.1039/C7MT00136C

Cress, W. A., Johnson, G. V., and Barton, L. L. (1986). The role of endomycorrhizal fungi in iron uptake by Hilaria jamesii. *J. Plant Nutr.* 9, 3–7. doi: 10.1080/01904168609363465

Cross, A. J., Harnly, J. M., Ferrucci, L. M., Rish, A., Mayne, S. T., and Sinha, R. (2012). Developing a heme iron database for meats a.ccording to meat type, cooking method and doneness level. *Food Nutr. Sci.* 3, 905–913. doi: 10.4236/fns.2012.37120

Crowley, D. E. (2006). "Microbial siderophores in the plant rhizosphere," in *Iron Nutrition in Plants and Rhizospheric Microorganisms*, eds L. L. Barton and J. Abadia (Dordrecht: Springer), 169–198. doi: 10.1007/1-4020-4743-6_8

Crowley, D. E., Reid, C. P., and Szaniszlo, P. J. (1988). Utilization of microbial siderophores in iron acquisition by oat. *Plant Physiol.* 87, 680–685. doi: 10.1104/pp.87.3.680

Curie, C., Alonso, J. M., Jean, M. L., Ecker, J. R., and Briat, J. F. (2000). Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem. J.* 347, 749–755. doi: 10.1042/bj3470749

Curie, C., and Briat, J.-F. (2003). Iron transport and signaling in plants. *Annu. Rev. Plant Biol.* 54, 183–206. doi: 10.1146/annurev.arplant.54.031902.135018

Curie, C., and Mari, S. (2017). New routes for plant iron mining. *New Phytol.* 214, 521–525. doi: 10.1111/nph.14364

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jan, M., et al. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103, 1–11. doi: 10.1093/aob/mcn207

Dai, J., Qiu, W., Wang, N., Wang, T., Nakanishi, H., and Zuo, Y. (2019). From Leguminosae/Gramineae intercropping systems to see benefits of intercropping on iron nutrition. *Front Plant. Sci.* 10:605. doi: 10.3389/fpls.2019.00605

De Santiago, A., García-López, A. M., Quintero, J. M., and Delgado, A. (2013). Effect of *Trichoderma asperellum* strain T34 and glucose addition on iron nutrition in cucumber grown on calcareous soils. *Soil. Biol. Biochem.* 57, 598–605. doi: 10.1016/j.soilbio.2012.06.020

De Santiago, A., Perea-Torres, F., Avilés, M., Moreno, M. T., Carmona, E., and Delgado, A. (2019). Shifts in microbial community structure influence the availability of Fe and other micronutrients to lupin (*Lupinus albus* L.). *Appl. Soil Ecol.* 144, 42–50. doi: 10.1016/j.apsoil.2019.06.018

De Santiago, A., Quintero, J. M., Avilés, M., and Delgado, A. (2009). Effect of *Trichoderma asperellum* strain T34 on iron nutrition in white lupin. *Soil Biol. Biochem.* 41, 2453–2459. doi: 10.1016/j.soilbio.2009.07.033

De Valença, A. W., Bake, A., Brouwer, I. D., and Giller, K. E. (2017). Agronomic biofortification of crops to fight hidden hunger in sub-Saharan Africa. *Glob. Food Secur.* 12, 8–14. doi: 10.1016/j.gfs.2016.12.001

De Vleesschauwer, D., Cornelis, P., and Höfte, M. (2006). Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances Rhizoctonia solani susceptibility in rice. *Mol. Plant. Microbe Interact.* 19, 1406–1419. doi: 10.1094/MPMI-19-1406

De Vries, F. T., Griffiths, R. I., Bailey, M., Craig, H., Girlanda, M., Gweon, H. S., et al. (2018). Soil bacterial networks are less stable under drought than fungal networks. *Nat. Commun.* 9:3033. doi: 10.1038/s41467-018-05516-7

Dehner, C. A., Awaya, J. D., Maurice, P. A., and DuBois, J. L. (2010). Roles of siderophores, oxalate, and ascorbate in mobilization of iron from hematite by the aerobic bacterium *Pseudomonas mendocina*. *Appl. Environ. Microbiol.* 76, 2041–2048. doi: 10.1128/AEM.02349-09

Delaporte-Quintana, P., Lovaisa, N. C., Rapisarda, V. A., and Pedrza, R. O. (2020). The plant growth promoting bacteria *Gluconacetobacter diazotrophicus* and *Azospirillum brasilense* contribute to the iron nutrition of strawberry plants through siderophores production. *Plant. Growth Regul.* 91, 185–199. doi: 10.1007/s10725-020-00598-0

Delimont, N. M., Haub, M. D., and Lindshield, B. L. (2017). The impact of tannin consumption on iron bioavailability and status: a narrative review. *Curr. Dev. Nutr.* 1, 1–12. doi: 10.3945/cdn.116.000042

Dequiedt, S., Thioulouse, J., Jolivet, C., Saby, N. P. A., Lelievre, M., Maron, P. A., et al. (2009). Biogeographical patterns of soil bacterial communities. *Environ. Microbiol. Rep.* 1, 251–255. doi: 10.1111/j.1758-2229.2009.00040.x

Dessaux, Y., Grandclément, C., and Faure, D. (2016). Engineering the rhizosphere. *Trends Plant. Sci.* 21, 266–278. doi: 10.1016/j.tplants.2016.01.002

Dubey, R. K., Tripathi, V., Prabha, R., Chaurasia, R., Singh, D. P., Rao, Ch S, et al. (2020). "Belowground microbial communities: key players for soil and environmental sustainability," in *Unravelling the Soil Microbiome. SpringerBriefs in Environmental Science*, (Cham: Springer), doi: 10.1007/978-3-030-15516-2_2

Duchene, O., Vian, J.-F., and Celette, F. (2017). Intercropping with legume for agroecological cropping systems: complementarity and facilitation processes and the importance of soil microorganisms. A review. *Agri. Ecosyst. Environ.* 240, 148–161. doi: 10.1016/j.agee.2017.02.019

Duijff, B. J., De Kogel, W. J., Bakker, P. A. H. M., and Schippers, B. (1994). Influence of pseudobactin 358 on the iron nutrition of barley. *Soil Biol. Biochem.* 26, 1681–1688. doi: 10.1016/0038-0717(94)90321-2

Egamberdieva, D., Wirth, S. J., Alqarawi, A. A., Abd-Allah, E., and Hashem, A. (2017). Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. *Front. Microbiol.* 8:2104. doi: 10.3389/fmicb.2017.02104

Fan, M.-S., Zhao, F.-J., Fairweather-Tait, S. J., Poulton, P. R., Dunham, A. J., and MacGrath, S. P. (2008). Evidence of decreasing mineral density in wheat grain over the last 160 years. *J. Trace Elem. Med. Bio.* 22, 315–324. doi: 10.1016/j.jtemb.2008.07.002

FAO (2019). *Biofortification: A Food-Systems Solution to Help End Hidden Hunger*. Rome: FAO. Available online at: http://www.fao.org/3/ca8711en/ca8711en.pdf (accessed June 2, 2021).

FAO (2021). *Biofortification: A Food-Systems Solution to Help End Hidden Hunger. Corrigendum*. Rome: FAO. Available online at: http://www.fao.org/3/CA8711EN/Corrigendum-ca8711en.pdf (accessed October 7, 2021).

FAO, IFAD, UNICEF, WFP, and WHO (2019). *The State of Food Security and Nutrition in the World 2019. Safeguarding Against Economics Slowdowns and Downturns*. Rome: FAO. Available online at: http://www.fao.org/3/ca5162en/ca5162en.pdf (accessed October 7, 2021).

Farzaneh, M., Vierheilig, H., Lössl, A., and Kaul, H. P. (2011). Arbuscular mycorrhiza enhances nutrient uptake in chickpea. *Plant Soil Environ.* 57, 465–470. doi: 10.17221/133/2011-PSE

Ferreira, C. M. H., Sousa, C. A., Sanchis-Pérez, I., Lopez-Rayo, S., Barros, M. T., Soares, H. M. V. M., et al. (2019). Calcareous soil interactions of the iron(III) chelates of DPH and Azotochelin and its application on amending iron chlorosis in soybean (*Glycine max*). *Sci. Total Environ.* 647, 1586–1593. doi: 10.1016/j.scitotenv.2018.08.069

Ferret, C., Sterckeman, T., Cornu, J.-Y., Gangloff, S., Schalk, I., and Geoffroy, V. A. (2014). Siderophore-promoted dissolution of smectite by fluorescent *Pseudomonas*. *Environ. Microbiol. Rep.* 6, 459–467. doi: 10.1111/1758-2229.12146

Filiz, E., and Kurt, F. (2019). FIT (Fer-like iron deficiency-induced transcription factor) in plant iron homeostasis: genome-wide identification and bioinformatics analyses. *J. Plant Biochem. Biotechnol.* 28, 143–157. doi: 10.1007/s13562-019-00497-0

Finkelstein, J. L., Fothergill, A., Hackl, L. S., Hass, J. R., and Mehta, S. (2019). Iron biofortification interventions to improve iron status and functional outcomes. *Proc. Nutr. Soc.* 78, 197–207. doi: 10.1017/S0029665118002847

Finkelstein, J. L., Haas, J. D., and Mehta, S. (2017). Iron-biofortified staple food crops for improving iron status: a review of the current evidence. *Curr. Opin. Biotech.* 44, 138–145. doi: 10.1016/j.copbio.2017.01.003

Fourcroy, P., Sisó-Terraza, P., Sudre, D., Saviron, M., Reyt, G., Gaymard, F., et al. (2014). Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency. *New Phytol.* 201, 155–167. doi: 10.1111/nph.12471

Fourcroy, P., Tissot, N., Gaymard, F., Briat, J.-F., and Dubos, C. (2016). Facilitated Fe nutrition by phenolic compounds excreted by the *Arabidopsis* ABCG37/PDR9 transporter requires the IRT1/FRO2 high-affinity root Fe2+ transport system. *Mol. Plant.* 9, 485–488. doi: 10.1016/j.molp.2015.09.010

Freitas, M. A., Medeiros, F. H. V., Carvalho, S. P., Guilherme, L. R. G., Teiseira, W. D., Zhang, H., et al. (2015). Augmenting iron accumulation in cassava by the beneficial soil bacterium Bacillus subtilis (GBO3). *Front. Plant Sci.* 6:596. doi: 10.3389/fpls.2015.00596

French, E., Kaplan, I., Iyer-Pascuzzi, A., Nakatsu, C. H., and Enders, L. (2021). Emerging strategies for precision microbiome management in diverse agroecosystems. *Nat. Plants* 7, 256–267. doi: 10.1038/s41477-020-00830-9

Frey, B., Rieder, S. R., Brunner, I., Plotze, M., Koetzsh, S., Lapanje, A., et al. (2010). Weathering-associated bacteria from the *Damma glacier* forefield: physiological capabilities and impact on granite dissolution. *Appl. Environ. Microbiol.* 76, 4788–4796. doi: 10.1128/AEM.00657-10

Fustec, J., Lesuffleur, F., Mahieu, S., and Cliquet, J. B. (2010). Nitrogen rhizodeposition of legumes. *A review. Agron. Sustain. Dev.* 30, 57–66. doi: 10.1051/agro/2009003

Gałązka, A., Grzęda, E., and Jończyk, K. (2019). Changes of microbial diversity in rhizosphere soils of new quality varieties of winter wheat cultivation in organic farming. *Sustainability* 11:4057. doi: 10.3390/su11154057

Gao, L., Liu, X. M., Du, Y. M., Zong, H., and Shen, G. M. (2019). Effects of tobacco-peanut relay intercropping on soil bacteria community structure. *Ann. Microbiol.* 69, 1531–1536. doi: 10.1007/s13213-019-01537-9

Garcia-Oliveira, A. L., Chander, S., Ortiz, R., Menkir, A., and Gedil, M. (2018). Genetic basis and breeding perspectives of grain iron and zinc enrichment in cereals. *Front. Plant Sci.* 9:937. doi: 10.3389/fpls.2018.00937

Garnica, M., Bacaicoa, E., Mora, V., San Francisco, S., Baigorri, R., Zamarreño, A. M., et al. (2018). Shoot iron status and auxin are involved in iron deficiency-induced phytosiderophores release in wheat. *BMC Plant Biol.* 18:105. doi: 10.1186/s12870-018-1324-3

Ghosh, U. D., Saha, C., Maiti, M., Lahiri, S., Ghosh, S., Seal, A., et al. (2014). Root associated iron oxidizing bacteria increase phosphate nutrition and influence root to shoot partitioning of iron in tolerant plant *Typha angustifolia*. *Plant Soil* 381, 279–295. doi: 10.1007/s11104-014-2085-x

Gildersleeve, R. R., and Ocumpaugh, W. R. (1989). Greenhouse evaluation of subterranean clover species for susceptibility to iron-deficiency chlorosis. *Crop Sci.* 29, 949–951. doi: 10.2135/cropsci1989.0011183X002900040023x

Glasauer, S., Weidler, P. G., Langley, S., and Beveridge, T. J. (2003). Controls on Fe reduction and mineral formation by a subsurface bacterium. *Geochim. Cosmochim. Acta.* 67, 1277–1288. doi: 10.1016/S0016-7037(02)01199-7

Gnonlonfin, G. J. B., Sanni, A., and Brimer, L. (2012). Review scopoletin – a coumarin phytoalexin with medicinal properties. *Crit. Rev. Plant Sci.* 31, 47–56. doi: 10.1080/07352689.2011.616039

Gómez-Galera, S., Rojas, E., Sudhakar, D., Zhu, C., Pelacho, A. M., Capelle, T., et al. (2010). Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Res.* 19, 165–180. doi: 10.1007/s11248-009-9311-y

Gonzàlez-Guerrero, M., Escudero, V., Saéz, A., and Tejada-Jimenéz, M. (2016). Transition metal transport in plants and associated endosymbionts: arbuscular mycorrhizal fungi and rhizobia. *Front. Plant Sci.* 7:1088. doi: 10.3389/fpls.2016.01088

Gopal, M., and Gupta, A. (2016). Microbiome selection could Spur next-generation plant breeding strategies. *Front. Microbiol.* 7:1991. doi: 10.3389/fmicb.2016.01971

Gregory, P. J., Wahbi, A., Adu-Gyamfi, J., Heiling, M., Gruber, M., Joy, E., et al. (2017). Approaches to reduce zinc and iron deficits in food systems. *Glob. Food Sec.* 15, 1–10. doi: 10.1016/j.gfs.2017.03.003

Grillet, L., and Schmidt, W. (2019). Iron acquisition strategies in land plants: not so different after all. *New Phytol.* 224:16005. doi: 10.1111/nph.16005

Grillet, L., Mari, S., and Schmidt, W. (2014). Iron in seeds – loading pathways and subcellular localization. *Front. Plant Sci.* 4:535. doi: 10.3389/fpls.2013.00535

Gu, S., Wei, Z., Shao, Z., Friman, V. P., Cao, K., Yang, T., et al. (2020). Competition for iron drives phytopathogen control by natural rhizosphere microbiomes. *Nat Microbiol.* 5, 1002–1010. doi: 10.1038/s41564-020-0719-8

Gunes, A., Inal, A., Adak, M. S., Alpaslan, M., Bagci, E. C., Erol, T., et al. (2007). Mineral nutrition of wheat, chickpea and lentil as affected by intercropped cropping and soil moisture. *Nutr. Cycl. Agroecosyst.* 78, 83–96. doi: 10.1007/s10705-006-9075-1

Hansen, N. C., Hopkins, B. G., Ellsworth, J. W., and Jolley, V. D. (2006). "Iron nutrition in field crops," in *Iron Nutrition in Plants and Rhizospheric Microorganisms*, eds L. L. Barton and J. Abadia (Dordrecht: Springer), 23–59. doi: 10.1007/1-4020-4743-6_2

Harbort, C. J., Hashimoto, M., Inoue, H., Niu, Y., Guan, R., Rombolà, A. D., et al. (2020). Root-secreted coumarins and the microbiota interact to improve iron nutrition in *Arabidopsis*. *Cell Host Microbe* 28, 825–837.e6. doi: 10.1016/j.chom.2020.09.006

Haselwandter, K., Haas, H., Häninger, G., and Winkelmann, G. (2020). Siderophores in plant root tissue: tagetes patula nana colonized by the arbuscular mycorrhizal fungus *Gigaspora margarita*. *Biometals.* 33, 137–146. doi: 10.1007/s10534-020-00238-0

Hauggaard-Nielsen, H., Gooding, M., Ambus, P., Corre-Hellou, G., Crozat, Y., Dahlmann, C., et al. (2009). Pea–barley intercropping forefficient symbiotic N2-fixation, soil N acquisition and use of other nutrients in European organic cropping systems. *Field Crop. Res.* 113, 64–71. doi: 10.1016/j.fcr.2009.04.009

Helms, T. C., Scott, R. A., Schapaugh, W. T., Goos, R. J., Franzen, D. W., and Schlegel, A. J. (2010). Soybean iron-deficiency chlorosis tolerance and yield decrease on calcareous soils. *Agron. J.* 102, 492. doi: 10.2134/agronj2009.0317

Hider, R. C., and Kong, X. (2010). Chemistry and biology of siderophores. *Nat. Prod. Rep.* 27, 637–657. doi: 10.1039/b906679a

Hinsinger, P., Plassard, C., Tang, C., and Jaillard, B. (2003). Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: a review. *Plant Soil* 248, 43–59. doi: 10.1023/A:1022371130939

Höfte, M., and Bakker, P. A. H. M. (2007). "Competition for iron and induced systemic resistance by siderophores of plant growth promoting rhizobacteria," in *Microbial Siderophores*, eds A. Varma and S. B. Chincholkar (Berlin: Springer), 121–133. doi: 10.1007/978-3-540-71160-5_6

Horchani, F., Prévot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., et al. (2011). Both plant and bacterial nitrate reductases contribute to nitric oxide production in *Medicago truncatula* nitrogen-fixing nodules. *Plant Physiol.* 155, 1023–1036. doi: 10.1104/pp.110.166140

Hördt, W., Römheld, V., and Winkelmann, G. (2000). *Fusarinines* and dimerum acid, mono- and dihydroxamate siderophores from *Penicillium chrysogenum*, improve iron utilization by strategy I and strategy II plants. *Biometals* 13, 37–46. doi: 10.1023/a:1009234612486

Housh, A. B., Powell, G., Scott, S., Anstaett, A., Gerheart, A., Benoit, M., et al. (2021). Functional mutants of *Azospirillum brasilense* elicit beneficial physiological and metabolic responses in Zea mays contributing to increased host iron assimilation. *Int. Soc. Microbial. Ecol. J.* 15, 1505–1522. doi: 10.1038/s41396-020-00866-x

Ingraffia, R., Amato, G., Frenda, A. S., and Giambalvo, D. (2019). Impacts of arbuscular mycorrhizal fungi on nutrient uptake, N2 fixation, N transfer, and growth in a wheat/faba bean intercropping system. *PLoS One* 14:e0213672. doi: 10.1371/journal.pone.0213672

Ishimaru, Y., Kakei, Y., Shimo, H., Bashir, K., Sato, Y., Sato, Y., et al. (2011). A rice phenolic efflux transporter is essential for solubilizing precipitated apoplasmic iron in the plant stele. *J. Biol. Chem.* 286, 24649–24655. doi: 10.1074/jbc.M111.221168

Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., et al. (2006). Rice plants take up iron as an Fe3+-phytosiderophore and as Fe2+. *Plant J.* 45, 335–346. doi: 10.1111/j.1365-313X.2005.02624.x

Jain, A., Singh, A., Chaudhary, A., Singh, S., and Singh, H. B. (2014). Modulation of nutritional and antioxidant potential of seeds and pericarp of pea pods treated with microbial consortium. *Food Res. Int.* 64, 275–282. doi: 10.1016/j.foodres.2014.06.033

Jin, C. W., He, Y. F., Tang, C. X., Wu, P., and Zheng, S. J. (2006). Mechanisms of microbially enhanced Fe acquisition in red clover (*Trifolium pratense* L.). *Plant Cell Environ.* 29, 888–897. doi: 10.1111/j.1365-3040.2005.01468.x

Jin, C. W., Li, G. X., Yu, X. H., and Zheng, S. J. (2010). Plant Fe status affects the composition of siderophore-secreting microbes in the rhizosphere. *Ann. Bot.* 105, 835–841. doi: 10.1093/aob/mcq071

Jin, C. W., You, G. Y., He, Y. F., Tang, C., Wu, P., and Zheng, S. J. (2007). Iron deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover. *Plant Physiol.* 144, 278–285. doi: 10.1104/pp.107.095794

Jones, P., Garcia, B. J., Furches, A., Tuskan, G. A., and Jacobson, D. (2019). Plant host-associated mechanisms for microbial selection. *Front. Plant Sci.* 10:862. doi: 10.3389/fpls.2019.00862

Kabir, A. H., Debnath, T., Das, U., Prity, S. A., Haque, H., Rahman, M. M., et al. (2020). Arbuscular mycorrhizal fungi alleviate Fe-deficiency symptoms in sunflower by increasing iron uptake and its availability along with antioxidant defense. *Plant Physiol. Biochem.* 150, 254–262. doi: 10.1016/j.plaphy.2020.03.010

Kabir, A. H., Paltridge, N. G., Roessner, U., and Stangoulis, J. C. R. (2013). Mechanisms associated with Fe-deficiency tolerance and signaling in shoots of *Pisum sativum*. *Physiol Plant.* 147, 381–395. doi: 10.1111/j.1399-3054.2012.01682.x

Kaiser, D. E., Lamb, J. A., Bloom, P. R., and Hernandez, J. A. (2014). Comparison of field management strategies for preventing iron deficiency chlorosis in soybean. *Agron. J.* 106, 1963–1974. doi: 10.2134/agronj13.0296

Kawakami, Y., and Bhullar, N. K. (2018). Molecular processes in iron and zinc homeostasis and their modulation for biofortification in rice. *J. Integr. Plant. Biol.* 60, 1181–1198. doi: 10.1111/jipb.12751

Kazamia, E., Sutak, R., Paz-Yepes, J., Dorrell, R. G., Roch, F., Mach, H., et al. (2018). Endocytosis-mediated siderophore uptake as a strategy for Fe acquisition in diatoms. *Sci. Adv.* 4:eaar4536. doi: 10.1126/sciadv.aar4536

Khan, A., Singh, J., Upadhayay, V. K., Singh, A. V., and Shah, S. (2019). "Microbial biofortification: a green technology through plant growth promoting microorganisms," in *Sustainable Green Technologies for Environmental Management*, eds S. Shah, V. Venkatramanan, and R. Prasad (Cham: Springer), 255–269. doi: 10.1007/978-981-13-2772-8

Kobae, Y., Tomioka, R., Tanoi, K., Kobayashi, N. I., Ohmori, Y., Nishida, S., et al. (2014). Selective induction of putative iron transporters, OPT8a and OPT8b, in maize by mycorrhizal colonization. *Soil Sci. Plant Nutr.* 60, 843–847. doi: 10.1080/00380768.2014.949854

Kobayashi, T., and Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. *Annu. Rev. Plant Biol.* 63, 131–152. doi: 10.1146/annurev-arplant-042811-105522

Kobayashi, T., Nozoye, T., and Nishizawa, N. K. (2019). Iron transport and its regulation in plants. *Free Radical Bio. Med.* 133, 11–20. doi: 10.1016/j.freeradbiomed.2018.10.439

Kocak, K. (2014). *Evaluation of Iron Deficiency Chlorosis in Soybeans (Glycine max). ETD Collection for University of Nebraska - Lincoln*. 24. Available online at: https://digitalcommons.unl.edu/dissertations/AAI3618676/ (accessed June 23, 2021).

Kraemer, S. M. (2004). Iron oxide dissolution and solubility in the presence of siderophores. *Aquat. Sci.* 66, 3–18. doi: 10.1007/s00027-003-0690-5

Kuiper, I., Bloemberg, G. V., Noreen, S., Thomas-Oates, J., and Lugtengerg, B. (2001). Increased uptake of putrescine in the rhizosphere inhibits competitive root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol. Plant. Microbe Interact.* 14, 1096–1104. doi: 10.1094/MPMI.2001.14.9.1096

Kumar, A., Kaur, G., Goel, P., Bhati, K. K., Kaur, M., Shukla, V., et al. (2019). Genome-wide analysis of oligopeptide transporters and detailed characterization of yellow stripe transporter genes in hexaploid wheat. *Funct. Integr. Genomics.* 19, 75–90. doi: 10.1007/s10142-018-0629-5

Kuypers, M. M., Marchant, A. K., and Kartal, B. (2018). The microbial nitrogen-cycling network. *Nat. Rev. Microbiol.* 16, 263–276. doi: 10.1038/nrmicro.2018.9

Labidi, S., Ben Jeddi, F., Tisserant, B., Debiane, D., Rezgui, S., Grandmougin-Ferjani, A., et al. (2012). Role of arbuscular mycorrhizal symbiosis in root mineral uptake under CaCO3 stress. *Mycorrhiza.* 22, 337–345. doi: 10.1007/s00572-011-0405-z

Lasa, A. V., Mašínová, T., Baldrian, P., and Fernández-López, M. (2019). Bacteria from the endosphere and rhizosphere of Quercus spp. use mainly cell wall-associated enzymes to decompose organic matter. *PLoS One* 14:e0214422. doi: 10.1371/journal.pone.0214422

Lassoued, R., Smyth, S. J., Phillips, P. W. B., and Hesseln, H. (2018). Regulatory uncertainty around new breeding techniques. *Front. Plant Sci.* 9:1291. doi: 10.3389/fpls.2018.01291

Lavania, M., Chauhan, P. S., Chauhan, S. V. S., Singh, H. B., and Nautiyal, C. S. (2006). Induction of plant defense enzymes and phenolics by treatment with plant growth-promoting rhizobacteria *Serratia marcescens* NBRI1213. *Curr. Microbiol.* 52, 363–368. doi: 10.1007/s00284-005-5578-2

Leake, J. R., Shaw, G., and Read, D. J. (1990). The biology of mycorrhiza in the Ericaceae. XVI. Mycorrhiza and iron uptake in *Calluna vulgaris* (L.) Hull in the presence of two calcium salts. *New Phythol.* 114, 651–657. doi: 10.1111/j.1469-8137.1990.tb00436.x

Lei, G. J., Zhu, X. F., Wang, Z. W., Dong, F., Dong, N. Y., and Zheng, S. J. (2014). Abscisic acid alleviates iron deficiency by promoting root iron reutilization and transport from root to shoot in *Arabidopsis*. *Plant Cell Environ.* 37, 852–863. doi: 10.1111/pce.12203

Lemanceau, P., Barret, M., Mazurier, S., Mondy, S., Pivato, B., Fort, T., et al. (2017a). Chapter Five - Plant communication with associated microbiota in the spermosphere, rhizosphere and phyllosphere. *Adv. Bot. Res.* 8, 101–133. doi: 10.1016/bs.abr.2016.10.007

Lemanceau, P., Bauer, P., Kraemer, S., and Briat, J.-F. (2009). Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. *Plant Soil.* 321, 513–535. doi: 10.1007/s11104-009-0039-5

Lemanceau, P., Blouin, M., Muller, D., and Moënne-Loccoz, Y. (2017b). Let the core microbiota be functional. *Trends Plant Sci.* 22, 583–595. doi: 10.1016/j.tplants.2017.04.008

Leyval, C., and Reid, C. P. P. (1991). Utilization of microbial siderophores by mycorrhizal and non-mycorrhizal pine roots. *New Phytol.* 119, 93–98. doi: 10.1111/j.1469-8137.1991.tb01011.x

Li, G., Xu, W., Kronzucker, H. J., and Shi, W. (2015). Ethylene is critical to the maintenance of primary root growth and Fe homeostasis under Fe stress in *Arabidopsis*. *J. Exp. Bot.* 66, 2041–2054. doi: 10.1093/jxb/erv005

Li, Q., Chen, J., Wu, L., Luo, X., and Li, N. (2018). Belowground interactions impact the soil bacterial community, soil fertility, and crop yield in maize/peanut intercropping systems. *Int. J. Mol. Sci.* 19:622. doi: 10.3390/ijms19020622

Li, Q., Yang, A., and Zhang, W.-H. (2016). Efficient acquisition of iron confers greater tolerance to saline-alkaline stress in rice (Oryza sativa L.). *J. Exp. Bot.* 67, 6431–6444. doi: 10.1093/jxb/erw407

Liu, A., Hamel, C., Hamilton, R. I., Ma, B. L., and Smith, D. L. (2000). Acquisition of Cu. Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. *Mycorrhiza* 9, 331–336. doi: 10.1007/s005720050277

Liu, Y., Yang, H., Liu, Q., Zhao, X., Xie, S., Wang, Z., et al. (2021). Effect of two different sugarcane cultivars on rhizosphere bacterial communities of sugarcane and soybean upon intercropping. *Front. Microbiol.* 11:596472. doi: 10.3389/fmicb.2020.596472

Liu, Z., Beskrovnaya, P., Melnyk, R. A., Hossain, S. S., Khorasani, S., O'Sullivan, L. R., et al. (2018). A genome-wide screen identifies genes in rhizosphere-associated *Pseudomonas* required to evade plant defenses. *mBio* 9, e00433-18. doi: 10.1128/mBio.00433-18

Lockyer, S., White, A., and Buttriss, J. L. (2018). Biofortified crops for tackling micronutrient deficiencies – what impact are these having in developing countries and could they be of relevance within Europe? *Nutr. Bull.* 43, 319–357. doi: 10.1111/nbu.12347

Longnecker, N., and Welch, R. M. (1990). Accumulation of apoplastic iron in plant roots: a factor in the resistance of soybeans to iron-deficiency induced chlorosis? *Plant Physiol.* 92, 17–22. doi: 10.1104/pp.92.1.17

Lurthy, T., Cantat, C., Jeudy, C., Declerck, P., Gallardo, K., Barraud, C., et al. (2020). Impact of bacterial siderophores on iron status and ionome in pea. *Front. Plant Sci.* 11:730. doi: 10.3389/fpls.2020.00730

Mahmoudi, H., Koyro, H.-W., Debez, A., and Abdelly, C. (2009). Comparison of two chickpea varieties regarding their responses to direct and induced Fe deficiency. *Environ. Exp. Bot.* 66, 349–356. doi: 10.1016/j.envexpbot.2009.03.013

Malhotra, H., Pandey, R., Sharma, S., and Bindraban, P. S. (2019). Foliar fertilization: possible routes of iron transport from leaf surface to cell organelles. *Arch. Agron. Soil Sci.* 66, 279–300. doi: 10.1080/03650340.2019.1616288

Manthey, J. A., Tisserat, B., and Crowley, D. E. (1996). Root responses of sterile-grown onion plants to iron deficiency. *J. Plant. Nutr.* 19, 145–161. doi: 10.1080/01904169609365113

Manwaring, H. R., Bligh, H. F. J., and Yadav, R. (2016). The challenges and opportunities associated with biofortification of pearl millet (*Pennisetum glaucum*) with elevated levels of grain iron and zinc. *Front. Plant Sci.* 7:1944. doi: 10.3389/fpls.2016.01944

Marschner, H. (1995). *Mineral Nutrition of Higher Plants*, 2nd Edn. London: Academic Press, 889.

Marschner, P., Crowley, D., and Rengel, Z. (2011). Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis – model and research methods. *Soil Biol. Biochem.* 43, 883–894. doi: 10.1016/j.soilbio.2011.01.005

Mary, V., Ramos, M. S., Gillet, C., Socha, A., Giraudat, J., Agorio, A., et al. (2015). Bypassing iron storage in endodermal vacuoles rescues the iron mobilization defect in the natural resistance associated-macrophage protein3natural resistance associated-macrophage protein4 Double Mutant. *Plant Physiol.* 169, 748–759. doi: 10.1104/pp.15.00380

Masalha, J., Kosegarten, H., Elmaci, Ö, and Mengel, K. (2000). The central role of microbial activity for iron acquisition in maize and sunflower. *Biol. Fertil. Soils* 30, 433–439. doi: 10.1007/s003740050021

Michel, L., Beyá-Marshall, V., Rombolà, A. D., Pastenes, C., and Covarrubias, J. I. (2019). Evaluation of FE-heme applications or intercropping for preventing iron deficiency in blueberry. *J. Soil Sci. Plant. Nutr.* 19, 117–126. doi: 10.1007/s42729-019-0017-9

Mishra, P. K., Bisht, S. C., Ruwari, P., Joshi, G. P., Singh, G., Bisht, J. T., et al. (2011). Bioassociative effect of cold tolerant *Pseudomonas* spp. and Rhizobium leguminosarum-PR1 on iron acquisition, nutrient uptake and growth of lentil (*Lens culinaris* L.). *Eur. J. Soil Biol.* 47, 35–43. doi: 10.1016/j.ejsobi.2010.11.005

Morrissey, J., Baxter, I. R., Lee, J., Li, L., Lahner, B., Grotz, N., et al. (2009). The ferroportin metal efflux proteins function in iron and cobalt homeostasis in *Arabidopsis*. *Plant Cell.* 21, 3326–3338. doi: 10.1105/tpc.109.069401

Murgia, I., Arosio, P., Tarantino, D., and Soave, C. (2012). Biofortification for combating 'hidden hunger' for iron. *Trends Plant Sci.* 17, 47–55. doi: 10.1016/j.tplants.2011.10.003

Mushtaq, Z., Asghar, H. N., Zahir, Z. A., and Massood, M. (2021). The interactive approach of rhizobacteria and L-tryptophan on growth, physiology, tuber characteristics, and iron concentration of potato (*Solanum tuberosum* L.). *J. Plant Growth Regul.* doi: 10.1007/s00344-021-10395-2

Nagata, T. (2017). Effect of *Pseudomonas fluorescens* inoculation on the improvement of iron deficiency in tomato. *Plant Root.* 11, 1–9. doi: 10.3117/plantroot.11.1

Nagata, T., Oobo, T., and Aozasa, O. (2013). Efficacy of a bacterial siderophore, pyoverdine, to supply iron to *Solanum lycopersicum* plants. *J. Biosci. Bioengi.* 115, 686–690. doi: 10.1016/j.jbiosc.2012.12.018

Neumann, A., Schmidtke, K., and Rauber, R. (2007). Effects of crop density and tillage system on grain yield and N uptake from soil and atmosphere of sole and intercropped pea and oat. *Field Crop. Res.* 100, 285–293. doi: 10.1016/j.fcr.2006.08.001

Nevo, Y., and Nelson, N. (2006). The NRAMP family of metal-ion transporters. *Biochim. Biophys. Acta - Mol. Cell Res.* 1763, 609–620. doi: 10.1016/j.bbamcr.2006.05.007

Norton, J., and Ouyang, Y. (2019). Controls and adaptive management of nitrification in agricultural soils. *Front. Microbiol.* 10:1931. doi: 10.3389/fmicb.2019.01931

Orera, I., Abadía, A., Abadía, J., and Álvarez-Fernández, A. (2009). Determination of o,oEDDHA – a xenobiotic chelating agent used in Fe fertilizers – in plant tissues by liquid chromatography/electrospray mass spectrometry: overcoming matrix effects. *Rapid Commun. Mass Spectro.* 23, 1694–1702. doi: 10.1002/rcm.4056

Orera, I., Rodríguez-Castrillón, J. A., Moldovan, M., García-Alonso, J. I., Abadía, A., Abadía, J., et al. (2010). Using a dual-stable isotope tracer method to study the uptake, xylem transport and distribution of Fe and its chelating agent from stereoisomers of an Fe(iii)-chelate used as fertilizer in Fe-deficient Strategy I plants. *Metallomics* 2, 646–657. doi: 10.1039/C0MT00018C

Palmer, C. M., Hindt, M. N., Schmidt, H., Clemens, S., and Guerinot, M. L. (2013). MYB10 and MYB72 are required for growth under iron-limiting conditions. *PLoS Genet.* 9:e1003953. doi: 10.1371/journal.pgen.1003953

Pedas, P., Ytting, C. K., Fuglsang, A. T., Jahn, T. P., Schjoerring, J. K., and Husted, S. (2008). Manganese efficiency in barley: identification and characterization of the metal ion transporter HvIRT1. *Plant Physiol.* 148, 455–466. doi: 10.1104/pp.108.118851

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D., Van Wees, S. C. M., and Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Pivato, B., Semblat, A., Guégan, T., Jacquiod, S., Martin, J., Deau, F., et al. (2021). Rhizosphere bacterial networks, but not diversity, are impacted by pea-wheat intecropping. *Front. Microbiol.* 12:674556. doi: 10.3389/fmicb.2021.674556

Pourbabaee, A. A., Shoaibi, F., Emami, S., and Alikhani, H. A. (2018). The potential contribution of siderophore producing bacteria on growth and Fe ion concentration of sunflower (*Helianthus annuus* L.) under water stress. *J. Plant Nutr.* 41, 619–626. doi: 10.1080/01904167.2017.1406112

Prity, S. A., Sajib, S. A., Rahman, M. M., Haider, S. A., and Kabir, A. H. (2020). Arbuscular mycorrhizal fungi mitigate Fe deficiency symptoms in sorghum through phytosiderophore-mediated Fe mobilization and restoration of redox status. *Protoplasma* 257, 1373–1385. doi: 10.1007/s00709-020-01517-w

Qiao, Y. J., Li, Z. H., Wang, X., Zhu, B., Hu, Y. G., and Zeng, Z. H. (2012). Effect of legume-cereal mixtures on the diversity of bacterial communities in the rhizosphere. *Plant. Soil Environ.* 58, 174–180. doi: 10.17221/351/2011-PSE

Radzki, W., Gutierrez Mañero, F. J., Algar, E., Lucas Garcia, J. A., Garcia-Villaraco, A., and Ramos Solano, B. (2013). Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. *Antonie Leeuwenhoek* 104, 321–330. doi: 10.1007/s10482-013-9954-9

Rahman, M. A., Parvin, M., Das, U., Ela, E. J., Lee, S. H., Lee, K. W., et al. (2020). Arbuscular mycorrhizal symbiosis mitigates iron (Fe)-deficiency retardation in alfalfa (*Medicago sativa* L.) through the enhancement of Fe accumulation and sulfur-assisted antioxidant defense. *Int. J. Mol. Sci.* 21:2219. doi: 10.3390/ijms21062219

Rajkumar, M., Ae, N., Prasad, M. N. V., and Freitas, H. (2010). Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends Biotechnol.* 28, 142–149. doi: 10.1016/j.tibtech.2009.12.002

Ranjard, L., Dequiedt, S., Chemidlin Prévost-Bouré, N., Thioulouse, J., Saby, N. P. A., Lelievre, M., et al. (2013). Turnover of soil bacterial diversity driven by wide-scale environmental heterogeneity. *Nat. Commun.* 4:1434. doi: 10.1038/ncomms2431

Ravanbakhsh, M., Sasidharan, R., Voesenek, L. A. C. J., Kowalchuk, G. A., and Jousset, A. (2018). Microbial modulation of plant ethylene signaling: ecological and evolutionary consequences. *Microbiome* 6:52. doi: 10.1186/s40168-018-0436-1

Reza, M. (2017). *Assessing the Effect of Siderophore Producing Bacteria for the Iron Nutrition in Lentil and Pea.* Ph.D. thesis. Saskatchewan, SK: University of Saskatchewan, 130.

Rijavec, T., and Lapanje, A. (2016). Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. *Front. Microbiol.* 7:1785. doi: 10.3389/fmicb.2016.01785

Robin, A., Mazurier, S., Mougel, C., Vansuyt, G., Corberand, T., Meyer, J.-M., et al. (2007). Diversity of root-associated fluorescent pseudomonads as affected by ferritin overexpression in tobacco: diversity of fluorescent pseudomonads in rhizosphere. *Environ. Microbiol.* 9, 1724–1737. doi: 10.1111/j.1462-2920.2007.01290.x

Robin, A., Mougel, C., Siblot, S., Vansuyt, G., Mazurier, S., and Lemanceau, P. (2006). Effect of ferritin overexpression in tobacco on the structure of bacterial and pseudomonad communities associated with the roots: structure of rhizosphere bacterial and pseudomonad communities. *FEMS Microbiol. Ecol.* 58, 492–502. doi: 10.1111/j.1574-6941.2006.00174.x

Robin, A., Vansuyt, G., Hinsinger, P., Meyer, J.-M., Briat, J.-F., and Lemanceau, P. (2008). Chapter 4 Iron dynamics in the rhizosphere. *Adv. Agron.* 99, 183–225. doi: 10.1016/S0065-2113(08)00404-5

Rodriguez, P. A., Rothballer, M., Chowdhury, S. P., Nussbaumer, T., Gutjahr, C., and Falter-Braun, P. (2019). Systems biology of plant-microbiome interactions. *Mol. Plant* 12, 804–821. doi: 10.1016/j.molp.2019.05.006

Rodríguez-Celma, J., Lin, W. D., Fu, G. M., Abadía, J., López-Millán, A. F., and Schmidt, W. (2013). Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by *Arabidopsis* and *Medicago truncatula*. *Plant Physiol.* 162, 1473–1485. doi: 10.1104/pp.113.220426

Romera, F. J., García, M. J., Lucena, C., Martínez-Medina, A., Aparicio, M. A., Ramos, J., et al. (2019). Induced systemic resistance (ISR) and Fe deficiency responses in dicot plants. *Front. Plant Sci.* 10:287. doi: 10.3389/fpls.2019.00287

Roschzttardtz, H., Conéjéro, G., Curie, C., and Mari, S. (2009). Identification of the endodermal vacuole as the iron storage compartment in the *Arabidopsis* Embryo. *Plant Physiol.* 151, 1329–1338. doi: 10.1104/pp.109.144444

Rroço, E., Kosegarten, H., Harizaj, F., Imani, J., and Mengel, K. (2003). The importance of soil microbial activity for the supply of iron to sorghum and rape. *Eur. J. Agron.* 19, 487–493. doi: 10.1016/S1161-0301(02)00185-5

Sadrarhami, A., Grove, J. H., and Zeinali, H. (2021). The microbial siderophore desferrioxamine B inhibits Fe and Zn uptake in three spring wheat genotypes grown in Fe-deficient nutrient solution. *J. Plant Nut.* 44, 2299–2309. doi: 10.1080/01904167.2021.1899205.

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S., and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environ. Sci. Pollut. Res.* 23, 3984–3999. doi: 10.1007/s11356-015-4294-0

Sandberg, A.-S. (2002). Bioavailability of minerals in legumes. *Br. J. Nutr.* 88, 281–285. doi: 10.1079/BJN/2002718

Schmid, N. B., Giehl, R. F. H., Döll, S., Mock, H. P., Strehmel, N., Scheel, D., et al. (2014). Feruloyl-CoA 6′-Hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in *Arabidopsis*. *Plant Physiol.* 164, 160–172. doi: 10.1104/pp.113.228544

Schmidt, H., Günther, C., Weber, M., Sporlein, C., Losher, S., Böttcher, C., et al. (2014). Metabolome analysis of *Arabidopsis thaliana* roots identifies a key ketabolic pathway for iron acquisition. *PLoS One* 9:e102444. doi: 10.1371/journal.pone.0102444

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T. M., et al. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Mol. Plant Microbe. Interact.* 25, 28–36. doi: 10.1094/MPMI-08-11-0204

Sharma, A., Shankhdhar, D., and Shankhdhar, S. C. (2013). Enhancing grain iron content of rice by the application of plant growth promoting rhizobacteria. *Plant Soil Environ.* 59, 89–94. doi: 10.17221/683/2012-PSE

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus.* 2:587. doi: 10.1186/2193-1801-2-587

Shenker, M., Chen, Y., Ghirlando, R., Oliver, I., Helmann, M., and Hadar, Y. (1995). Chemical structure and biological activity of a siderophore produced by Rhizopus arrhizus. *Soil Sci. Soc. Am. J.* 59, 837. doi: 10.2136/sssaj1995.03615995005900030029x

Shi, R., Melzer, M., Zheng, S., Benke, A., Stich, B., and von Wiren, N. (2018). Iron retention in root hemicelluloses causes genotypic variability in the tolerance to iron deficiency-induced chlorosis in maize. *Front. Plant Sci.* 9:557. doi: 10.3389/fpls.2018.00557

Shirley, M., Avoscan, L., Bernaud, E., Vansuyt, G., and Lemanceau, P. (2011). Comparison of iron acquisition from Fe–pyoverdine by strategy I and strategy II plants. *Botany.* 89, 731–735. doi: 10.1139/b11-054

Shomer, I., Novacky, A. J., Pike, S. M., Yermiyahu, U., and Kinraide, T. B. (2003). Electrical potentials of plant cell walls in response to the ionic environment. *Plant Physiol.* 133, 411–422. doi: 10.1104/pp.103.024539

Singh, A., Jain, A., Sarma, B. K., Upadhyray, R. S., and Signh, H. B. (2014). Rhizosphere competent microbial consortium mediates rapid changes in phenolic profiles in chickpea during Sclerotium rolfsii infection. *Microbiol. Res.* 169, 353–360. doi: 10.1016/j.micres.2013.09.014

Singh, D., and Prasanna, R. (2020). Potential of microbes in the biofortification of Zn and Fe in dietary food grains. A review Agron. *Sustain. Devl.* 40:15. doi: 10.1007/s13593-020-00619-2

Singh, D., Geat, N., Rajawat, M. V. S., Mahajan, M. M., and Prasanna, R. (2017). Deciphering the mechanisms of endophyte-mediated biofortification of Fe and Zn in wheat. *J. Plant Growth Regul.* 37, 174–182. doi: 10.1007/s00344-017-9716-4

Singh, D., Geat, N., Rajawat, M. V. S., Prasanna, M. V., Kar, A., Singh, A. M., et al. (2018). Prospecting endophytes from different Fe or Zn accumulating wheat genotypes for their influence as inoculants on plant growth, yield, and micronutrient content. *Ann. Microbiol.* 68, 815–833. doi: 10.1007/s13213-018-1388-1

Sisó-Terraza, P., Rios, J. J., Abadía, J., Abadía, A., and Álvarez-Fernández, A. (2016). Flavins secreted by roots of iron-deficient Beta vulgaris enable mining of ferric oxide via reductive mechanisms. *New Phytol.* 209, 733–745. doi: 10.1111/nph.13633

Slamet-Loedin, I. H., Johnson-Beebout, S. E., Impa, S., and Tsakirpaloglou, N. (2015). Enriching rice with Zn and Fe while minimizing Cd risk. *Front. Plant Sci.* 6:121. doi: 10.3389/fpls.2015.00121

Smith, S. E., Jakobsen, I., Grønlund, M., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiol.* 156, 1050–1057. doi: 10.1104/pp.111.174581

Soares, E. V., and Soares, H. M. V. M. (2021). Harmful effects of metal(loid) oxide nanoparticles. *Appl. Microbiol. Biotechnol.* 105, 1379–1394. doi: 10.1007/s00253-021-11124-1

Song, C., Mazzola, M., Cheng, X., Oetjen, J., Alexandrov, T., Dorrestein, P., et al. (2015). Molecular and chemical dialogues in bacteria-protozoa interactions. *Sci. Rep.* 5, 12837. doi: 10.1038/srep12837

Spehn, E. M., Joshi, J., Schmid, B., Alphei, J., and Körner, C. (2000). Plant diversity effects on soil heterotrophic activity in experimental grassland ecosystems. *Plant Soil* 224, 217–230. doi: 10.1023/A:1004891807664

Stringlis, I. A., Yu, K., Feussner, K., de Jonge, R., Van Bentum, S., Van Verk, M. C., et al. (2018). MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proc. Natl. Acad. Sci. U.S.A.* 115, E5213–E5222. doi: 10.1073/pnas.1722335115

Su, H., Chu, Y., Bai, J., Gong, L., Huang, J., Xu, W., et al. (2018). Genome-wide identification and comparative analysis for OPT Family genes in *Panax ginseng* and eleven flowering plants. *Molecules.* 24, 15. doi: 10.3390/molecules24010015

Sullivan, T. S., and Gadd, G. M. (2019). Chapter Three - Metal bioavailability and the soil microbiome. *Adv. Agron.* 155, 79–120. doi: 10.1016/bs.agron.2019.01.004

Sun, Y. M., Zhang, N. N., Wang, E. T., Yuan, H. L., Yang, J. S., and Chen, W. X. (2009). Influence of intercropping and intercropping plus rhizobial inoculation on microbial activity and community composition in rhizosphere of alfalfa

(*Medicago sativa* L.) and Siberian wild rye (*Elymus sibiricus* L.). *FEMS Microbiol. Ecol.* 70, 218–226. doi: 10.1111/j.1574-6941.2009.00752.x

Taschen, E., Amenc, L., Tournier, E., Deleporte, P., Malagoli, P., Fustec, J., et al. (2017). Cereal-legume intercropping modifies the dynamics of the active rhizospheric bacterial community. *Rhizosphere* 3, 191–195. doi: 10.1016/j.rhisph.2017.04.011

Theis, K. R., Dheilly, N. M., Klassen, J. L., Brucker, R. M., and Baines, J. F. (2016). Getting the hologenome concept right: an eco-evolutionary framework for hosts and their microbiomes. *mSystems* 1, e00028–16. doi: 10.1128/mSystems.00028-16

Trapet, P., Avoscan, L., Klinguer, A., Pateyron, S., Citerne, S., Chervin, C., et al. (2016). The *Pseudomonas* fluorescens siderophore pyoverdine weakens *Arabidopsis thaliana* defense in favor of growth in iron-deficient conditions. *Plant Physiol.* 171, 675–693. doi: 10.1104/pp.15.01537

Tsai, H.-H., and Schmidt, W. (2017). One way. Or another? Iron uptake in plants. *New Phytol.* 214, 500–505. doi: 10.1111/nph.14477

Turner, T. R., Ramakrishnan, K., Walshaw, J., Heavens, D., Alston, M., Swarbreck, D., et al. (2013). Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. *Int. Soc. Microbial. Ecol. J.* 7, 2248–2258. doi: 10.1038/ismej.2013.119

UNDP (2015). *Sustainable Development Goals. In: United Nations Development Programme*. New York, NY: UNDP. Available online at: https://www.undp.org/content/undp/en/home/sustainable-development-goals.html (accessed July 2, 2021).

Van Loon, L. C., Bakker, P. A., van der Heijdt, W. H., Wendehenne, D., and Pugin, A. (2008). Early responses of tobacco suspension cells to rhizobacterial elicitors of induced systemic resistance. *Mol. Plant Microbe Interact.* 21, 1609–1621. doi: 10.1094/MPMI-21-12-1609

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Van, A. L., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Vansuyt, G., Robin, A., Briat, J.-F., Curie, C., and Lemanceau, P. (2007). Iron acquisition from Fe-pyoverdine by *Arabidopsis thaliana. Mol. Plant Microbe Interact.* 20, 441–447. doi: 10.1094/MPMI-20-4-0441

Vansuyt, G., Souche, G., Straczek, A., Briat, J.-F., and Jaillard, B. (2003). Flux of protons released by wild type and ferritin over-expressor tobacco plants: effect of phosphorus and iron nutrition. *Plant Physiol. Biochem.* 41, 27–33. doi: 10.1016/S0981-9428(02)00005-0

Vasconcelos, M., Eckert, H., Arahana, V., Graef, G., Grusak, M. A., and Clemente, T. (2006). Molecular and phenotypic characterization of transgenic soybean expressing the Arabidopsis ferric chelate reductase gene. *FRO2. Planta.* 224, 1116–1128. doi: 10.1007/s00425-006-0293-1

Vert, G., Barberon, M., Zelazny, E., Séguéla, M., Briat, J.-F., and Curie, C. (2009). *Arabidopsis* IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta* 229, 1171–1179. doi: 10.1007/s00425-009-0904-8

Vora, S. M., Ankati, S., Patole, C., Podile, A. R., and Archana, G. (2021). Alterations of primary metabolites in root exudates of intecropped Cajanus cajan-Zea mays modulate the adaptation and proteome of Ensifer (*Sinorhizobium*) fredii NGR234. *Microb. Ecol.* doi: 10.1007/s00248-021-01818-4. [Epub ahead of print].

Wahbi, S., Maghraoui, T., Hafidi, M., Sanguin, H., Oufdou, K., Prin, Y., et al. (2016a). Enhanced transfer of biologically fixed N from faba bean to intercropped wheat through mycorrhizal symbiosis. *Appl. Soil Ecol.* 107, 91–98. doi: 10.1016/j.apsoil.2016.05.008

Wahbi, S., Prin, Y., Thioulouse, J., Sanguin, H., Baudoin, E., Maghraoui, T., et al. (2016b). Impact of wheat/faba bean mixed cropping or rotation systems on soil microbial functionalities. *Front. Plant Sci.* 7:1364. doi: 10.3389/fpls.2016.01364

Wakeel, A., Farooq, M., Bashir, K., and Ozturk, L. (2018). "Micronutrient malnutrition and biofortification: recent advances and future perspectives," in *Plant Micronutrient Use Efficiency*, eds M. A. Houssain, T. Kamiya, L. S. Phan Tran, and T. Fujiwara (Amsterdam: Elsevier), 225–243. doi: 10.1016/B978-0-12-812104-7.00017-4

Wang, J., Zhou, C., Xiao, X., Xie, Y., Zhu, L., and Ma, Z. (2017). Enhanced iron and selenium uptake in plants by volatile emissions of *Bacillus amyloliquefaciens* (BF06). *Appl. Sci.* 7, 85. doi: 10.3390/app7010085

Wang, Y., Brown, H. N., Crowley, D. E., and Szaniszlo, P. J. (1993). Evidence for direct utilization of a siderophore, ferrioxamine B, in axenically grown cucumber. *Plant Cell Environ.* 16, 579–585. doi: 10.1111/j.1365-3040.1993.tb00906.x

Waters, B. M., Amundsen, K., and Graef, G. (2018). Gene expression profiling of iron deficiency chlorosis sensitive and tolerant soybean indicates key roles for phenylpropanoids under alkalinity stress. *Front. Plant Sci.* 9:10. doi: 10.3389/fpls.2018.00010

Waters, B. M., and Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: genes and physiological processes from a biofortification perspective. *Plant Sci.* 180, 562–574. doi: 10.1016/j.plantsci.2010.12.003

Wei, Z., and Jousset, A. (2017). Plant breeding goes microbial. *Trends Plant Sci.* 22, 555–558. doi: 10.1016/j.tplants.2017.05.009

Welch, R. M., House, W. A., Beebe, S., and Cheng, Z. (2000). Genetic selection for enhanced bioavailable levels of iron in bean (*Phaseolus vulgaris* L.) seeds. *J. Agr. Food Chem.* 48, 3576–3580. doi: 10.1021/jf0000981

Werra, P., de, Péchy-Tarr, M., Keel, C., and Maurhofer, M. (2009). Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Appl. Environ. Microbiol.* 75, 4162–4174. doi: 10.1128/AEM.00295-09

Wezel, A., Casagrande, M., Celette, F., Vian, J. F., Ferrer, A., and Peigné, J. (2014). Agroecological practices for sustainable agriculture. A review. *Agron. Sustain. Dev.* 34, 1–20. doi: 10.1007/s13593-013-0180-7

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets - iron, zinc, copper, calcium, magnesium, selenium and iodine: research review. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

White, P., and Broadley, M. (2005). Biofortifying crops with essential mineral elements. *Trends Plant Sci.* 10, 586–593. doi: 10.1016/j.tplants.2005.10.001

Wille, L., Messmer, M. M., Studer, B., and Hohmann, P. (2019). Insights to plant–microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. *Plant Cell Environ.* 42, 20–40. doi: 10.1111/pce.13214

Winkelmann, G. (2017). A search for glomuferrin; a potential siderophore of arbuscular fungi of the genus Glomus. *Biometals* 30, 559–564. doi: 10.1007/s10534-017-0026-x

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol. Lett.* 8, 969–974. doi: 10.1016/0378-4274(95)03532-X

World Health Organization (2017). *Nutritional Anaemias: Tools for Effective Prevention and contro*. Geneva: WHO, 96. Available online at: https://www.who.int/publications/i/item/9789241513067 (accessed June 2, 2021).

World Health Organization (2021). *Micronutrient Deficiencies, Iron Deficiency Anaemia. In: Nutrition: Nutrition topics*. Geneva: WHO.

Xiong, H., Kakei, Y., Kobayashi, T., Guo, X., Nakazano, M., Takahashi, H., et al. (2013). Molecular evidence for phytosiderophore-induced improvement of iron nutrition of peanut intercropped with maize in calcareous soil. *Plant Cell Environ.* 36, 1888–1902. doi: 10.1111/pce.12097

Xue, P.-P., Carrillo, Y., Pino, V., Minasny, B., and Mc Bratney, A. B. (2018). Soil properties drive microbial community structure in a large scale transect in South Eastern Australia. *Sci. Rep.* 8:17725. doi: 10.1038/s41598-018-30005-8

Xue, Y., Xia, H., Christie, P., Zhang, Z., Li, L., and Tang, C. (2016). Crop acquisition of phosphorus, iron and zinc from soil in cereal/legume intercropping systems: a critical review. *Ann. Bot.* 117, 363–377. doi: 10.1093/aob/mcv182

Yadav, R., Ror, P., Rathore, P., Kumar, S., and Ramakrishna, W. (2020). *Bacillus subtilis* CP4, isolated from native soil in combination with arbuscular mycorrhizal fungi promotes biofortification, yield and metabolite production in wheat under field conditions. *J. Appl. Microbiol.* 131, 339–359. doi: 10.1111/jam.14951

Yehuda, Z., Shenker, M., Hadar, Y., and Chen, Y. (2000). Remedy of chlorosis induced by iron deficiency in plants with the fungal siderophore rhizoferrin. *J. Plant Nutr.* 23, 1991–2006. doi: 10.1080/01904160009382160

Yuan, J., Chen, Y., Li, H., Lu, J., Zhao, H., Liu, M., et al. (2018). New insights into the cellular responses to iron nanoparticles in *Capsicum annuum. Sci. Rep.* 8:3228. doi: 10.1038/s41598-017-18055-w

Zaeem, M., Nadeem, M., Pham, T. H., Ashiq, W., Ali, W., Gilani, S. S. M., et al. (2019). The potential of corn-soybean intercropping to improve the soil health status and biomass production in cool climate boreal ecosystems. *Sci Rep.* 9:13148. doi: 10.1038/s41598-019-49558-3

Zamioudis, C., Hanson, J., and Pieterse, C. M. J. (2014). β-Glucosidase BGLU42 is a MYB72-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in Arabidopsis roots. *New Phytol.* 204, 368–379. doi: 10.1111/nph.12980

Zanin, L., Tomasi, N., Cesco, S., Varanini, Z., and Pinton, R. (2019). Humic substances contribute to plant iron nutrition acting as chelators and biostimulants. *Front. Plant Sci.* 10:675. doi: 10.3389/fpls.2019.00675

Zhang, H., Sun, Y., Xie, X., Kim, M.-S., Dowd, S. E., and Paré, P. W. (2009). A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. *Plant J.* 58, 568–577. doi: 10.1111/j.1365-313X.2009.03803.x

Zhang, L., Xing, J., and Lin, J. (2019). At the intersection of exocytosis and endocytosis in plants. *New Phytol.* 224, 1479–1489. doi: 10.1111/nph.16018

Zhang, Y., Xu, Y.-H., Yi, H.-Y., and Gong, J.-M. (2012). Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. *Plant J.* 72, 400–410. doi: 10.1111/j.1365-313X.2012.05088.x

Zhou, C., Guo, J., Zhu, L., Xiao, X., Xie, Y., Zhu, J., et al. (2016). *Paenibacillus polymyxa* BFKC01 enhances plant iron absorption via improved root systems and activated iron acquisition mechanisms. *Plant Physiol. Biochem.* 105, 162–173. doi: 10.1016/j.plaphy.2016.04.025

Zhou, C., Zhu, L., Ma, Z., and Wang, J. (2018). Improved iron acquisition of *Astragalus sinicus* under low iron-availability conditions by soil-borne bacteria *Burkholderia cepacia*. *J. Plant. Interact.* 13, 9–20. doi: 10.1080/17429145.2017.1407000

Zhu, X. F., Wang, B., Song, W. F., Zheng, S. J., and Shen, R. F. (2016). Putrescine aleviates iron deficiency via NO-dependent reutilization of root cell-wall Fe in *Arabidopsis*. *Plant Physiol.* 170, 558–567. doi: 10.1104/pp.15.01617

Zielińska-Dawidziak, M. (2015). Plant ferritin—a source of iron to prevent its deficiency. *Nutrients* 7, 1184–1201. doi: 10.3390/nu7021184

Zribi, K., and Gharsalli, M. (2002). Effect of bicarbonate on growth and iron nutrition of pea. *J. Plant Nutr.* 25, 2143–2149. doi: 10.1081/PLN-120014066

Zuo, Y. M., Zhang, F. S., Li, X. L., and Cao, Y. P. (2000). Studies on the improvement in iron nutrition of peanut by intecropping maize on a calcareous soil. *Plant Soil.* 50, 1071–1078. doi: 10.1080/00380768.2004.10408576

Zuo, Y., and Zhang, F. (2009). Iron and zinc biofortification strategies in dicot plants by intercropping with gramineous species. A review. *Agron. Sustain. Dev.* 29, 63–71. doi: 10.1051/agro:2008055

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Plant Science

ORIGINAL RESEARCH
published: 24 December 2021
doi: 10.3389/fpls.2021.802137

Combining Organic Fertilizer With Controlled-Release Urea to Reduce Nitrogen Leaching and Promote Wheat Yields

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Soil deterioration, low nitrogen use efficiency (NUE), and environmental risks caused by excessive chemical N fertilizer use are key factors restricting sustainable agriculture. It is extremely critical to develop effective N management strategies that consider both environmental and agronomic benefits. From 2017 to 2019, a field experiment was conducted to assess the effects of combinations of organic fertilizers (OF, provided at 30, 50, and 70% of the total applied N) and controlled-release urea (CU) on the NUE, N leaching and wheat yield compared with the effects of urea and CU. The results suggested that OF released N slowly in the early stage and showed a significant residual effect, while CU released N quickly in the first 2 months. The OF substitutes with 30–50% CU increased wheat yield by 4.2–9.2%, while the 70%OF+30%CU treatment showed no significant difference relative to the urea treatment. The average maximum apparent NUE recovery (50.4%) was achieved under the 50%OF+50%CU treatment, but the partial factor productivity was not affected by the N type. As the OF application rate increased, the total carbon content increased, and the total N value decreased. The NO_3^--N and NH_4^+-N concentrations in the OF+CU treatments were lower before the jointing stage but higher from the grain-filling to mature stages than those in the urea treatment. NO_3^--N and NH_4^+-N were mainly concentrated in the 0–60-cm layer soil by OF substitution, and N leaching to the 60–100-cm soil layer was significantly reduced. Hence, the results suggest that the combination of 30–50% OF with CU synchronizes absorption with availability due to a period of increased N availability in soils and proved to be the best strategy for simultaneously increasing wheat production and reducing N leaching.

Keywords: controlled-release urea, organic fertilizer, nitrogen leaching, nitrogen use efficiency, wheat yield

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Specialty section:
This article was submitted to Plant Nutrition,
a section of the journal
Frontiers in Plant Science

Received: *26 October 2021*
Accepted: *02 December 2021*
Published: *24 December 2021*

Citation:
Yang X, Zhang C, Ma X, Liu Q, An J, Xu S, Xie X and Geng J (2021) Combining Organic Fertilizer With Controlled-Release Urea to Reduce Nitrogen Leaching and Promote Wheat Yields.
Front. Plant Sci. 12:802137.
doi: 10.3389/fpls.2021.802137

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the third most cultivated cereal in the world, and optimizing fertilization practices is the main factor controlling the maintenance of wheat yields and protecting the environment (Hao et al., 2020). China is the largest producer and consumer of agricultural chemicals, amounting to 30% of global fertilizer use (Akhtar et al., 2020).

This booming chemical fertilizer consumption has made it possible to produce sufficient amounts of food to feed the increasing population of China (Liu et al., 2013). However, the overuse of chemical fertilizers (especially N) and improper fertilization methods have led to diminishing returns of production and other adverse effects (Humber et al., 2016). Earlier N fertilizer application increases the risk of N loss from the root zone by leaching and denitrification. Split- or late-season applications of N fertilizer are common approaches to improve wheat yield and NUE. Biomass was greater under split N application (applied at the tillering and flag leaf stages) than under full N application at tillering (Velasco et al., 2012). Although split fertilizer application requires additional labor, this fertilization method is typically used on crops under traditional agriculture practices (Hickman et al., 2014). However, this fertilization method is currently inappropriate in China, as the worker population is aging and labor shortages for crop-production activities are becoming increasingly severe (Zhou et al., 2017). Thus, balancing the benefits derived from fertilizers with the associated environmental issues is ultimately indispensable.

When urea is used as basal fertilization, the available N is rapidly mineralized for wheat, but less than half of the total N is effectively utilized and leftover has a negative ecological effect by leaching (Galloway et al., 2008). Controlled-release urea (CU) is designed to disperse N at rates that are synchronous with the N requirements of plants and is used as a beneficial, mitigating fertilizer alternative to reduce environmental pollution (Liu et al., 2019). Some studies have shown that CU decreases residual NO_3^--N in the deep soil layer, decreases N losses resulting from ammonia volatilization, and increases the amount of N taken up by plants (Tang et al., 2021). Additionally, a one-time application of CU was found to save more labor and time than a conventional urea application. Although many CU-associated advantages have been confirmed by a considerable amount of research, large-scale CU applications to cereal crops have been limited, especially in developing countries. Most coating materials are derived from petroleum resources, which are expensive and nonrenewable (Doran, 2002). Moreover, coating shells may pose potential pollution risks to the soil environment.

To lessen the negative environmental and economic impacts of chemical fertilizer use, combining chemical fertilizers with organic fertilizers (OFs) is an essential strategy for sustainable agriculture (Muhammad et al., 2020). The raw materials used for OF preparation are abundant, and the cost of these materials is low. The application of OF can alleviate soil degradation and rebuild healthy soil. A previous study found that OF substitutions reduced the soil bulk density compared with that of non-OF fertilized soil (Blanco-Canqui et al., 2015), promoted soil fertility by increasing the contents of organic matter and other mineral nutrients (Meade et al., 2011), and accelerated the activities of beneficial microorganisms (Yadav et al., 2000). The decomposition of OF releases mainly N, which can increase wheat yields where the soil N supply is limited (Palmer et al., 2017). However, organic carbon sequestration potential can be changed by many factors, such as soil conditions and climate. If organic fertilizer is applied alone, the nutrients are released slowly, and the release period is long (Garzon et al., 2011), leading to the slow and variable short-term effects of OF applications on wheat yields. Thus, most farmers adhere to the use of synthetic fertilizers rather than OF to preserve their crop yields.

Partially substituting N fertilizer with OF makes it possible to take advantage of both the total nutrients and available nutrients, thus promoting biological activities in soils and the physicochemical characteristics of soils. Considering the cost and security of CU and the residual effect of OF, the combined application of OF with CU is hypothesized to improve wheat yields by releasing N synchronously with the N requirements of plants. The major objectives of the 2-year field experiment conducted in this study were to evaluate the effects of different fertilization treatments on wheat yield and NUE, to assess the environmental pollution risk induced by the leaching of inorganic N, and to identify the optimum ratio and feasibility of the applied OF and CU combinations. The experimental results provide a theoretical basis for scientific fertilization practices that consider both environmental and agronomic benefits in winter wheat fields.

MATERIALS AND METHODS

Site and Material Descriptions

A 2-year field experiment was established from October 2017 to June 2019 in the Yimeng Mountain area, Linyi, Shandong Province, China (35°7′4″N, 118°16′50″E). This area is a typical representative northern rocky mountain area with gravel, shallow soil, and low soil water storage capacity, and the soil depth is generally below 80 cm. The total precipitation amounts in 2018 and 2019 were 966.3 mm and 864.6 mm, respectively, and the average temperatures were 14.83°C and 14.43°C during the first and second growing seasons, respectively (**Figure 1**). The study area has cold winters and hot summers, and the rainy season generally spans from June to August (Yang et al., 2021). Before this study, the field was previously managed as a winter wheat/summer maize rotation system and had been continuously cultivated for more than 6 years. The annual N-P_2O_5-K_2O fertilization rate was 400–300–350 kg ha^{-1}, and no straw returning measures were adopted in this region. By request, the farmer did not implement summer fertilization of maize (N-P_2O_5-K_2O was 210–150–180 kg ha^{-1}) to reduce soil mineral N levels before beginning experiments.

The soil texture is classified as silty clay-loam, and the clay, sand and silt contents are 17.6, 15.8, and 66.6%, respectively. The chemical properties of the cultivated soil layer (0–20 cm) were as follows: total N, 0.88 g kg^{-1}; organic matter, 12.5 g kg^{-1}; total carbon, 10.1 g kg^{-1}; NO_3^--N, 68.4 mg kg^{-1}; NH_4^+-N, 39.5 mg kg^{-1}; available phosphorus, 35.0 mg kg^{-1}; available potassium, 104.5 mg kg^{-1}; and pH, 6.3.

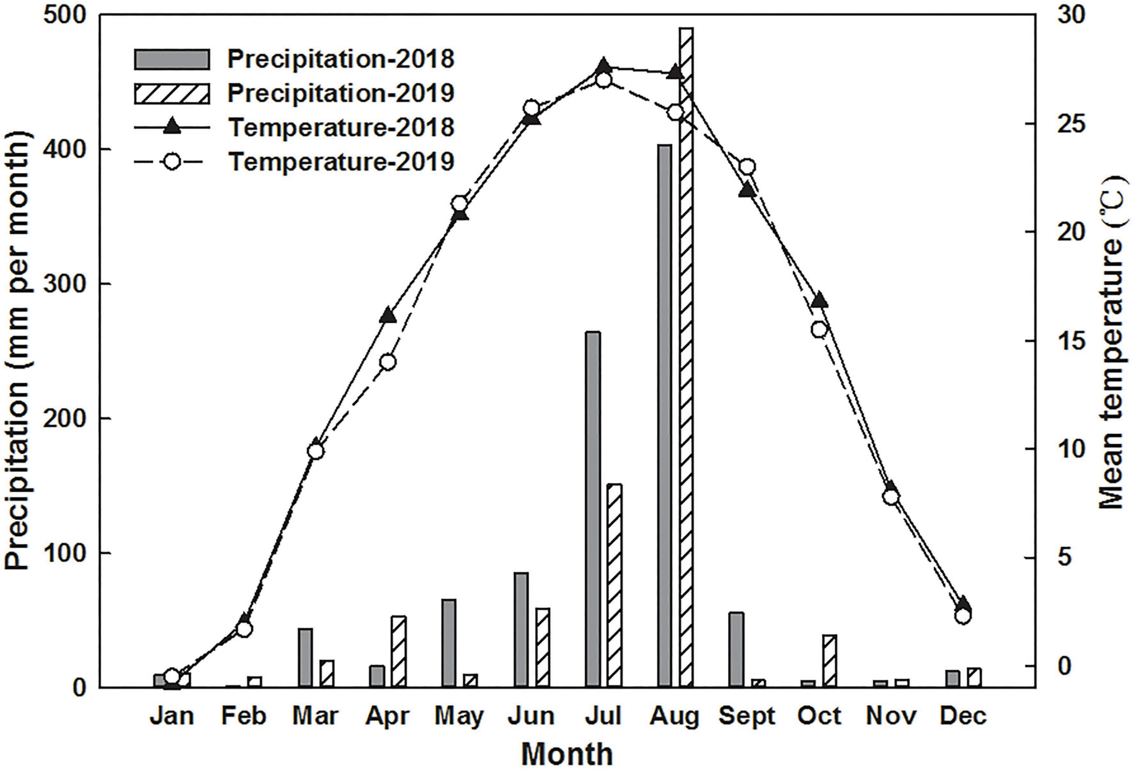

FIGURE 1 | Variations in precipitation and air temperature.

The chemical fertilizers applied in the study area included concentrated superphosphate (P_2O_5, 46%), potassium chloride (K_2O, 60%), urea (N, 46%), CU, sulfur-coated urea with a polymer coating, and 35% N, with a longevity of approximately 3 months. The morphology of the material before and after burial in soil was characterized by SEM and is shown in **Supplementary Figure S1**. The raw organic fertilizer material was cassava (cassava was used to produce ethanol, and the remaining waste residue was sufficiently decomposed to form organic fertilizer), which contained 60% organic matter with average nutrient contents of 2.5% N, 1.2% P_2O_5, 2.1% K_2O and a pH of 6.8. FTIR spectra of organic fertilizer are shown in **Supplementary Figure S2**. All fertilizers were provided by Jin Yimeng Group CO., LTD, Shandong, China.

Experimental Design and Field Management

The treatments were arranged in a randomized block design and repeated in triplicate. Six treatments were created: no N fertilizer (CK); urea providing 100% of the chemical N (urea); CU providing 100% of the chemical N (CU); organic fertilizer providing 30% of the N and CU providing 70% of the N (30%OF+70%CU); organic fertilizer providing 50% of the N and CU providing 50% of the N (50%OF+50%CU); and organic fertilizer providing 70% of the N and CU providing 30% of the N (70%OF+30%CU). All the fertilization treatments contained the same amount of N-P_2O_5-K_2O (180–150–150 kg ha^{-1}) according to the conventional recommended fertilizer rate in the local region. Before seeds were planted, organic and chemical fertilizers (except urea) were deposited as basal applications, and all fertilizers were incorporated into the soil at a depth of 10–15 cm. Urea (40%) was applied before planting, and urea (60%) was applied during the jointing period. The length and width of each experimental plot were 6 m and 5 m, respectively, and longitudinal and lateral protection areas of 0.5 m were placed around the plots to reduce marginal effects.

The cropping system was a winter wheat–summer maize rotation system, and the wheat and maize cultivars were "Jimai 22" and "Zhengdan 958," respectively. Summer maize underwent similar treatments as wheat; in contrast, the amount of N-P_2O_5-K_2O was 210–150–180 kg ha^{-1}. Wheat was sown on October 4, 2017, and October 5, 2018, and harvested on June 5, 2018 and May 26, 2019, immediately followed by the sowing of maize in mid-June. Before planting winter wheat, the field was ploughed to a depth of 15–20 cm and rotary-cultivated to prepare the wheat seed bed. Seeds (150 and 165 kg ha^{-1}) were planted in each plot in 2017 and 2018 at sowing depths of approximately 2–3 cm at a row spacing of 15 cm and subsequently flood irrigated. Depending on rainfall events, the field was flood-irrigated again in spring. As the soil moisture content was high in 2019, more wheat seeds were sown to ensure that the seedlings emerged evenly.

Sampling and Analysis

Determination of the Yield, N Uptake, NUE and Economic Benefit Analysis of Wheat

Ten representative wheat plants were collected from each plot by cutting the aboveground portion at the seedling stage, jointing stage, grain-filling stage, and mature stage in 2019. At the mature stage of each year, the grain yield of wheat was determined using a 4 m^2 (2×2 m) area in the center of each plot (adjusted to a 13% water content), and the thousand-grain weight was randomly calculated from the harvested grain. The straw biomass (stems and leaves) and grain were separated, and each component was dried at 105°C for half an hour and then dried to a constant weight at 75°C. Then, the samples were ground and passed through a 1-mm mesh screen for the following analyses. The total N contents of the straw and grain samples were obtained by digestion with H_2SO_4-H_2O_2-miscible liquids and determined by a Kjeldahl nitrogen analyzer (Douglas et al., 1980). The plant N uptake was calculated based on the dry matter weight and N concentration of each plant part.

The fertilizer NUE included the apparent recovery N use efficiency (ARNUE, %), agronomic nitrogen use efficiency (ANUE, kg kg^{-1}) and partial factor productivity (PFP, kg kg^{-1}). These data were calculated as described by Yang et al. (2021) using the following formulas:

$$ARNUE(\%) = \frac{N - N0}{F} \times 100 \quad (1)$$

$$ARNUE = \frac{Y - Y0}{F} \quad (2)$$

$$PFP = \frac{Y}{F} \quad (3)$$

In the above equations, N and Y represent the N uptake and grain yield obtained from N-treated plants, respectively, $N0$ and $Y0$ represent the N uptake and grain yield obtained from non-N-treated plants, respectively, and F represents the application rate of N.

The price of fertilizers was as follows: OF—62.5 US \$ ha^{-1}, CU—515.3 \$ ha^{-1}, potassium chloride—470 \$ ha^{-1}, urea—360 \$ ha^{-1}, potassium chloride—470 \$ ha^{-1}, and concentrated superphosphate—563 \$ ha^{-1}. The wheat grain yield was 406.9 \$ $ha^{-1,}$ and the labor cost of fertilization one time was 72.5 \$ ha^{-1}. The total revenue (\$ ha^{-1}) was the product of wheat grain yield and its price, and the net profit was calculated as the total revenue minus the total cost.

Release Characterization of CU

The N release characteristics of CU were detected by the method described by Geng et al. (2015). Briefly, 25 mesh bags (10×8 cm) containing 10 g CU granules were buried in the soil before sowing, and 3 bags were randomly selected each month, rinsed with distilled water to remove the loosely adhered soil, and dried. The N release rate from CU was calculated by measuring the weight loss.

Soil Sampling and Analysis

Five soil samples were collected randomly from each plot and thoroughly mixed to produce a composite sample. Soil samples were collected with a soil corer at the seedling stage (on November 5, 2017, and November 11, 2018), jointing stage (on March 18, 2018, and March 16, 2019), grain-filling stage (on May 4, 2018, and May 2, 2019) and mature stage. The cultivated horizon of the 0–20-cm soil layer was sampled in both years, and soil samples were collected at depths of 0–100 cm at an increment of 20 cm at the seedling and mature stages in 2019. Then, the samples were air-dried and passed through 2-mm and 0.25-mm mesh sieves. The soil NO_3^--N and NH_4^+-N were extracted with 0.01 mol L^{-1} KCl (the ratio of solution to soil was 10:1) and analyzed through a flow injection autoanalyzer (Bran-Luebbe, Norderstedt, Germany). The total N and total soil C concentrations were determined through combustion with an automatic elemental analyzer (Vario Micro Cube elemental analyser, Germany).

Statistical Analyses

The raw data were preconditioned using Microsoft Excel 2017, and then the data were submitted to normalization tests and variance homogeneity tests. One-way ANOVA was used to assess the significant differences in total N, total C, NH_4^+-N, and NO_3^--N. Two-way ANOVA was adopted to determine the effect of the interaction of year and N treatments on yield, yield component, and NUE. ANOVA and mean separation tests (Duncan's multiple range test, at the 5% probability level) were performed using Statistical Analysis System package version 9.2 (2010, SAS Institute Cary, NC). All of the illustrations were drawn in SigmaPlot version 12.0 (MMIV Systat Software, Inc., San Jose, CA).

RESULTS

Biomass and Grain Yield of Wheat

The statistical analysis showed that the total biomass strongly increased following N fertilization (**Table 1**), but the N sources had no obvious effect on the straw biomass. The total biomass, straw biomass, and grain yield showed significant differences between the 2 years, and these indexes were also affected by the interaction of N sources and year. The total biomass measured following the urea treatment was lower than those following the other N fertilization treatments in 2018, and the highest total biomass occurred in association with the 50%OF+50%CU treatment in 2019, but no obvious differences were observed among the other treatments. The effect of N application on the grain yield was greater than that on the straw biomass. Specifically, the straw biomass measured following the CK and urea treatments showed no remarkable differences in the first year, and no significant differences were detected

TABLE 1 | Grain yields and straw biomass of wheat under different N application treatments.

Year	Treatment	Total biomass kg ha^{-1}	Straw biomass kg ha^{-1}	Grain yield kg ha^{-1}	Thousand grain weight/g	Increase rate of grain vs. urea/%
2018	CK	14878.3c	7818.3c	7060.0c	40.6a	−8.41
	Urea	16144.8b	8436.3bc	7708.5b	40.4a	0
	CU	18376.9a	1036.0ab	8340.8a	39.7a	8.2
	30%OF+70%CU	18980.8a	10565.4a	8415.4a	40.0a	9.17
	50%OF+50%CU	18606.7a	10313.4a	8293.3ab	39.5a	7.59
	70%OF+30%CU	18782.8a	10954.4a	7828.4b	38.5a	1.56
2019	CK	15647.8c	8168.3b	7479.4c	43.4a	−9.75
	Urea	20190.1b	11902.6a	8287.5b	40.62b	0
	CU	21346.5ab	12850.4a	8496.0ab	41.7ab	2.52
	30%OF+70%CU	21247.3ab	12615.1a	8632.2a	40.9b	4.16
	50%OF+50%CU	22835.2a	13850.6a	8984.7ab	40.8b	8.41
	70%OF+30%CU	22291.4ab	13831.9a	8459.5ab	41.4ab	2.08
Source of variation						
N source		0.2817*	0.7959^{ns}	0.2429*	0.1328*	
Year		0.3293*	0.1672*	0.2367*	0.6251^{ns}	
N source × Year		0.1411*	0.01*	0.1631*	0.6978^{ns}	

*The means followed by the same letter within a row are not significantly different at $p < 0.05$. "ns" means no significant difference. *significant at 5%.*

among any N fertilization treatments in the second year. The grain yield decreased progressively with the OF dose in 2018, and the highest yield was observed in association with the 50%OF+50%CU treatment in 2019, reaching 8984.7 kg ha^{-1}, 1.6–9.2% higher than that associated with the urea treatment; no significant differences were observed between the CU and OF+CU treatments. The thousand-grain weights varied from 38.5–42.4 g, and no pronounced difference was observed among treatments. (However, in 2019, the CK treatment resulted in a higher thousand-grain rate than the urea treatment.)

Nitrogen Uptake and N Use Efficiency

The total biomass was higher in 2019 than in 2018, as was the total N uptake by the wheat plants (**Figure 2**). The combined OF and CU treatments showed some superiority in increasing N uptake compared with the urea and CU treatments, and the highest plant N uptake value appeared in the 50%OF+50%CU treatment in 2019. The ARNUE and ANUE were significantly affected by the N source, year, and the interaction of N source × year, while the PFP was not influenced by the interaction of N source × year (**Table 2**). The PFP values were not affected by the N fertilizer types, maintaining a wide range from 42.8–49.9 kg grain per kg N. However, the ARNUE was increased by combining OF and CU and was 29.2–58.9% higher in the OF+CU treatments than in the urea treatment. The ARNUE associated with the CU treatment was higher than those measured under the 50%OF+50%CU and 70%OF+30%CU treatments in 2018, but no significant differences were exhibited between the CU treatment and the OF+CU treatments in 2019. The maximum ARNUE values were 42.5% (CU) in 2018 and 66.7% (50%OF+50%CU) in 2019. No significant differences in ANUE were identified among the OF+CU treatments, but the values measured under the 50%OF+50%CU treatment were all higher than those associated with the urea treatment in both years of study.

Economic Benefit Analysis of Wheat

Urea was used as basal and topdressing fertilizer, leading to double the labor cost of fertilization than the other fertilizers (**Table 3**). The total revenue and net profit of CK were the lowest in both years. No obvious difference of total revenue was exhibited among the CU, 30%OF+70%CU and 50%OF+50%CU treatments, but it was significant higher in the three treatments than that in the 70%OF+30%CU and urea treatments in 2018; meanwhile, the total revenues of the 50%OF+50%CU and 30%OF+70%CU treatments were higher than that of the urea treatment in 2019. Similarly, the net profit of the 50%OF+50%CU treatment was prominently higher than that of the urea treatment by 11.9 and 12.7%, respectively, in 2018 and 2019, but there was no significant difference between the CU and OF treatments.

Biomass and Total N Uptake Dynamics of Wheat Plants

The biomass (**Figure 3**) and total N uptake (**Figure 4**) increased with increasing growth period, and the two indicators under the CK treatment exhibited the lowest values in each stage. Specifically, the biomass ranged from 579.5 to 681.0 kg ha^{-1}, and no significant difference was found among all N-applied treatments (except CU, which was higher than that in 50%OF+50%CU) in the seedling stage. Although there was 117 d from the seedling stage to the jointing stage, the wheat biomass did not increase much, as it was winter, and the weather was cold. In the grain-filling stage, biomass was significantly increased by OF application, and the values in the OF treatments were higher than those in the CU and urea treatments. Moreover, the values in the CU treatment were larger than those in the urea treatment. At the mature stage, the highest and lowest plant biomass appeared in the 50%OF+50%CU and urea treatments, which reached 22835.2 and 20190.1 kg ha^{-1}, respectively. Total N uptake dynamics showed similar trends with biomass.

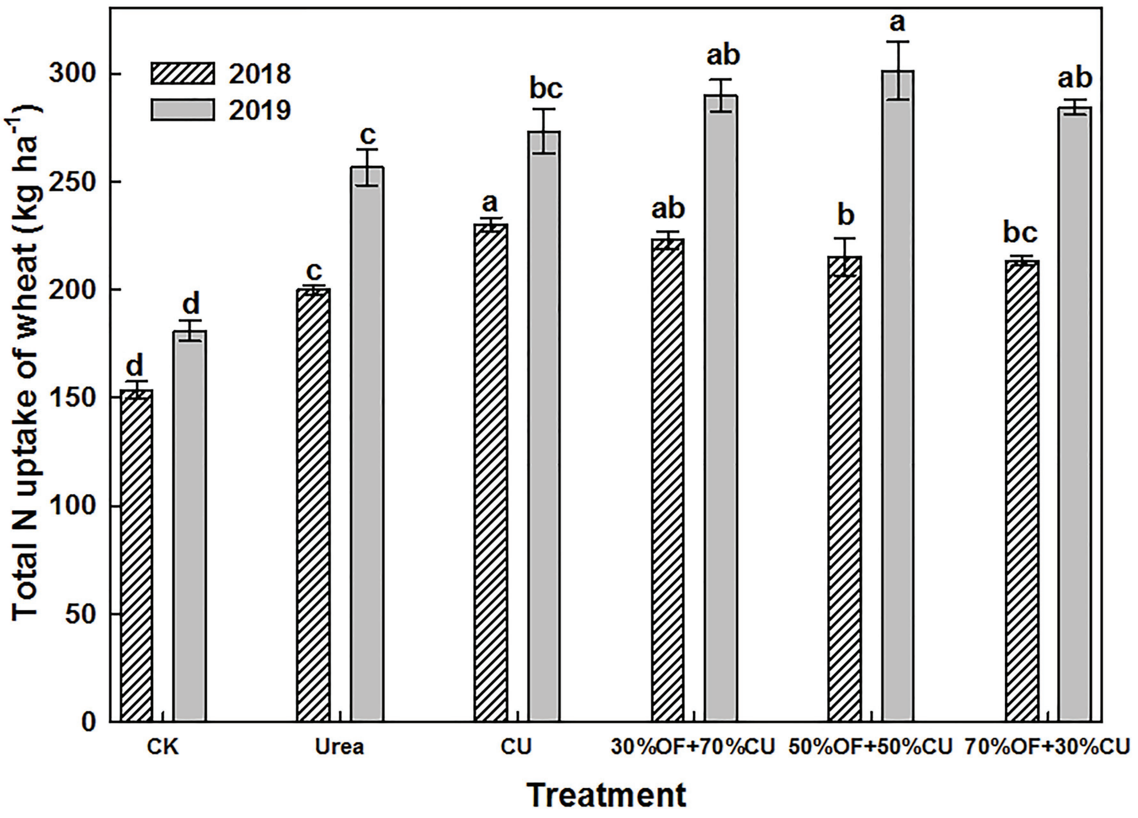

FIGURE 2 | The total N uptake of wheat plants. The error bars indicate the standard errors ($n = 3$). The different lowercase letters above error bars indicate significant differences at the $p < 0.05$ level for each treatment in the same year.

TABLE 2 | Nitrogen use efficiencies of wheat plants measured under different N application treatments.

Year	Treatment	ARNUE/%	PFP kg kg^{-1}	ANUE kg kg^{-1}
2018	Urea	25.73c	42.83a	3.60b
	CRU	42.47a	46.34a	7.67a
	30%OF+70%CRU	38.49ab	46.38a	8.09a
	50%OF+50%CRU	34.15b	46.07a	6.85a
	70%OF+30%CRU	33.22b	43.49a	4.27b
2019	Urea	41.97b	46.04a	4.49b
	CRU	51.28ab	47.2a	5.65ab
	30%OF+70%CRU	60.36a	47.96a	6.41ab
	50%OF+50%CRU	66.69a	49.92a	8.03a
	70%OF+30%CRU	57.41ab	47.0a	5.45ab
Source of variation				
N source		0.2819*	0.3509*	0.1741*
Year		0.5495*	0.2384*	0.0835*
N source × Year		0.1594*	0.8890^{ns}	0.3227*

*ARNUE, apparent recovery nitrogen use efficiency; PFP, partial factor productivity; ANUE, agronomic nitrogen use efficiency. *significant at 5%.*

Release Characterization of CU

The N within CU was released quickly in the first 2 months of the study period, with N releases of 14.3, 42.2, and 52.2% measured on the 10th, 30th and 60th days after burial in soil, respectively; only 13.2% of the total N was released from the 60th to the 150th day. Then, the release rate gradually increased from the 150th to the 220th day, and 94.4% of N was released at the time of harvest (**Figure 5**).

Temporal Variations in the Soil Total Carbon Content and Soil Bulk Density

The lowest total carbon level was observed in the CK treatment at each ontogenetic stage in both years (**Figure 6**). The total carbon content increased with an increasing OF application rate in the first year, but the 50%OF+50%CU treatment yielded the highest value in 2019. The total carbon content associated with the urea treatment was lower than those of the other N application treatments, and CU showed no significant difference from 30%OF+70%CU from the seedling stage to the grain-filling stage. Nevertheless, the total carbon content was lower in the CU treatment than in the OF+CU treatments at the mature stage. No obvious difference in soil bulk density was exhibited among all treatments (**Supplementary Figure S3**).

Temporal Variation in the Soil Total Nitrogen Content

Generally, in 2018, the total N content showed a tendency of first increasing from the seedling to the jointing sage

TABLE 3 | Average cost, total revenue, and net profit of wheat under different N application treatments (\$ ha^{-1}).

Treatment	Fertilizer cost	Labor cost of fertilization	Other costs	Total revenue		Net profit	
				2018	2019	2018	2019
CK	301.4	72.5	700	2872.7c	3043.4c	1798.8b	1969.5c
Urea	397.4	145	700	3136.6b	3372.2b	1894.2ab	2129.8b
CU	474.9	72.5	700	3393.9a	3457.0ab	2146.5a	2209.6ab
30%OF+70%CU	479.7	72.5	700	3424.2a	3512.4a	2172.0a	2260.3ab
50%OF+50%CU	483.0	72.5	700	3374.5a	3655.9a	2119.0a	2400.4a
70%OF+30%CU	486.3	72.5	700	3185.4b	3442.2ab	1926.6ab	2183.4ab

Other costs include seeds, machinery, pesticides, insecticides, irrigation, and other materials and expenses.

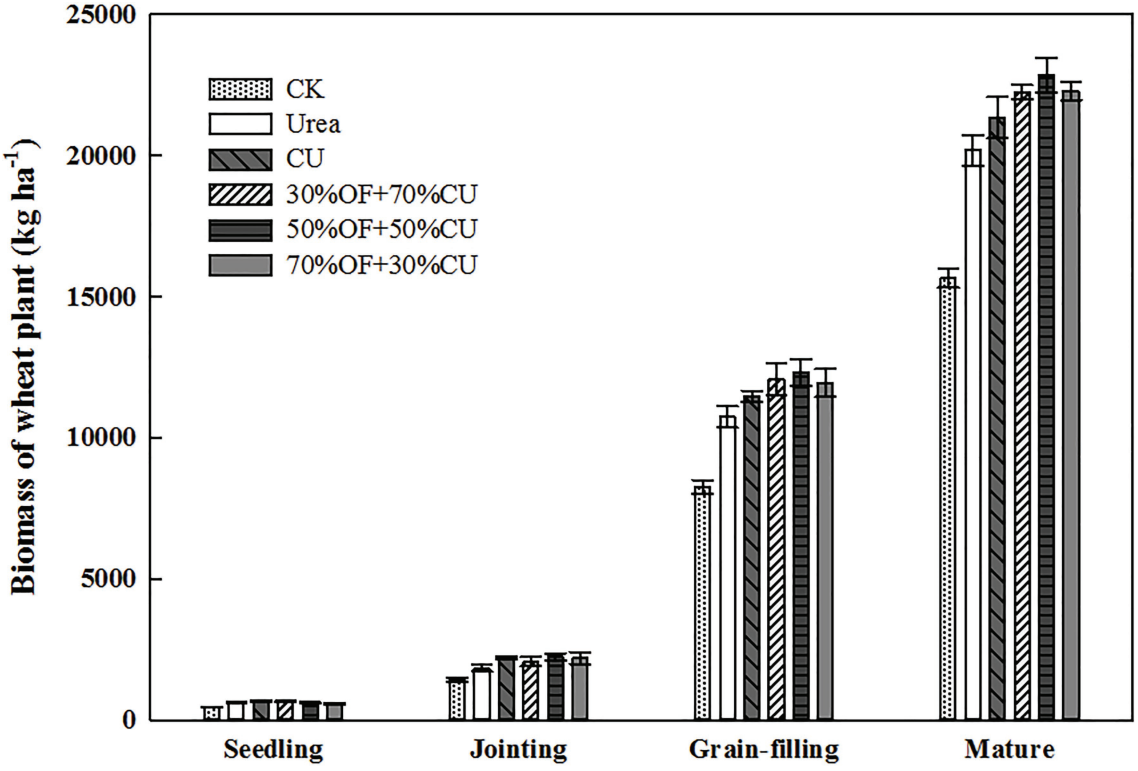

FIGURE 3 | Biomass dynamics of wheat plants during the growing season.

and then decreasing; in 2019, it displayed a roughly downward trend (**Figure 7**). Specifically, in 2018, the total N content measured in the urea treatment was higher than those measured in other treatments in the seedling stage but was lower beginning in the jointing stage. The values decreased with the increasing OF substitution rate in the OF+CU treatments, and 70%OF+30%CU showed no significant difference from CK in the seedling stage. At harvest, the highest value was observed under the 30%OF+70%CU treatment, and no significant difference was found among 50%OF+50%CU, 30%OF+70%CU, or CU. In 2019, the lowest values were still exhibited in association with the CK treatment at each stage of growth, and the greatest levels appeared under the CU, 70%OF+30%CU and 50%OF+50%CU treatments at the seedling, jointing, and grain-filling stages, respectively. In addition, no significant difference was observed among the OF + CU treatments at harvest.

Temporal Variations in the Soil NO_3^--N and NH_4^+-N Contents

In all treatments, the temporal variations in the NO_3^--N and NH_4^+-N concentrations showed downward trends throughout the growth stages (**Figure 8**), and these concentrations were markedly improved by N fertilization compared with the CK treatment. The levels of inorganic N differed significantly among the N-applied treatments. The highest NO_3^--N and NH_4^+-N values were yielded under the urea treatment at the seedling stage, reaching 111.9 mg kg^{-1} and 55.0 mg kg^{-1}, respectively.

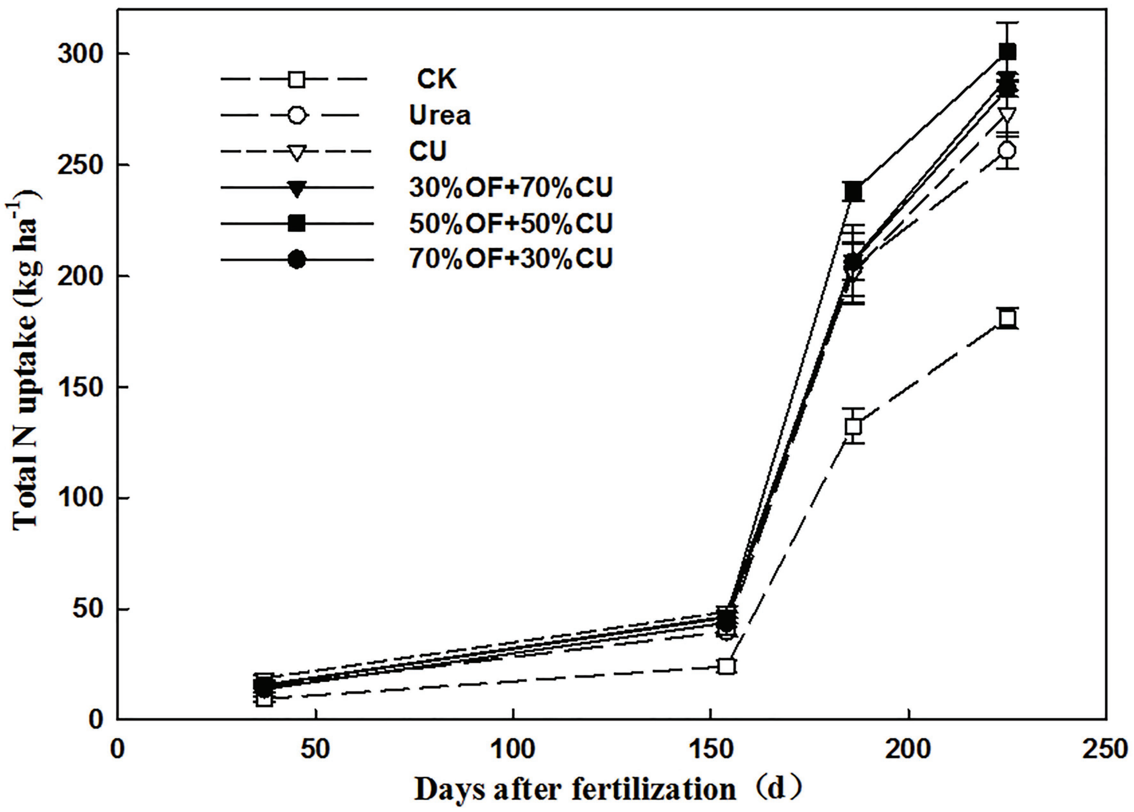

FIGURE 4 | Total N uptake of wheat plants during the growing season.

However, these values dropped dramatically from the jointing stage and were significantly lower than the values measured under other N-applied treatments. Under the CU treatment, the NO_3^--N and NH_4^+-N values were lower than those measured under the OF+CU treatments, except in the seedling stage. The NO_3^--N and NH_4^+-N concentrations were significantly increased by OF application, especially from the grain-filling to mature stages.

Leaching of Nitrate Nitrogen and Ammonium Nitrogen With Time in the 0–100-cm Soil Layer Two Years After Fertilization

The lowest NO_3^--N level was observed under the CK treatment in each soil layer, regardless of the growth stage (**Figures 9A,B**). In addition, the NO_3^--N level exhibited a general downward trend as the soil depth increased under the CK treatment, except for the peak observed in the 60–80-cm layer. The NO_3^--N concentrations measured under all treatments were higher at the seedling stage than at maturity. In contrast, these values were lower in the OF+CU treatments than in the urea treatment 37 days after fertilization in the 0–40-cm soil layer, but the opposite results appeared at maturity. In the seedling stage, the greatest values in the 60–100-cm soil layers were detected under the urea treatment, and the value measured under the urea treatment was also higher in the 80–100-cm soil layer at maturity. Moreover, at maturity, the amounts of nitrate N in the 0–100-cm soils were higher in the OF+CU treatments than in treatments that used CU alone. The 50%OF+50%CU treatment presented a higher level in the 0–60-cm soil layer than those of 30%OF+70%CU and 70%OF+30%CU.

With wheat growth, the NH_4^+-N content decreased from the seedling stage (**Figure 10A**) to maturity (**Figure 10B**). Under each treatment, the variation trend of ammonium N was roughly consistent with that of nitrate N. By using urea, the soil NH_4^+-N content in the seedling stage increased at depths of 0–100 cm but was lower at the 0–80-cm depths at maturity. No pronounced difference was found among OF+CU treatments at 0–60-cm depths; nevertheless, all these treatments yielded higher values at 0–40-cm depths at maturity than those measured under the CU treatment.

DISCUSSION

Combined Effects of OF and CU on Wheat Yield and Nitrogen Use Efficiency

Excessive chemical N fertilization and improper fertilizer application practices intensify N losses and result in low N utilization rates (Pinochet et al., 2018), which can be attributed to the asynchrony between wheat N deficits and N fertilizer applications (Osterholz et al., 2017). In the current experiment,

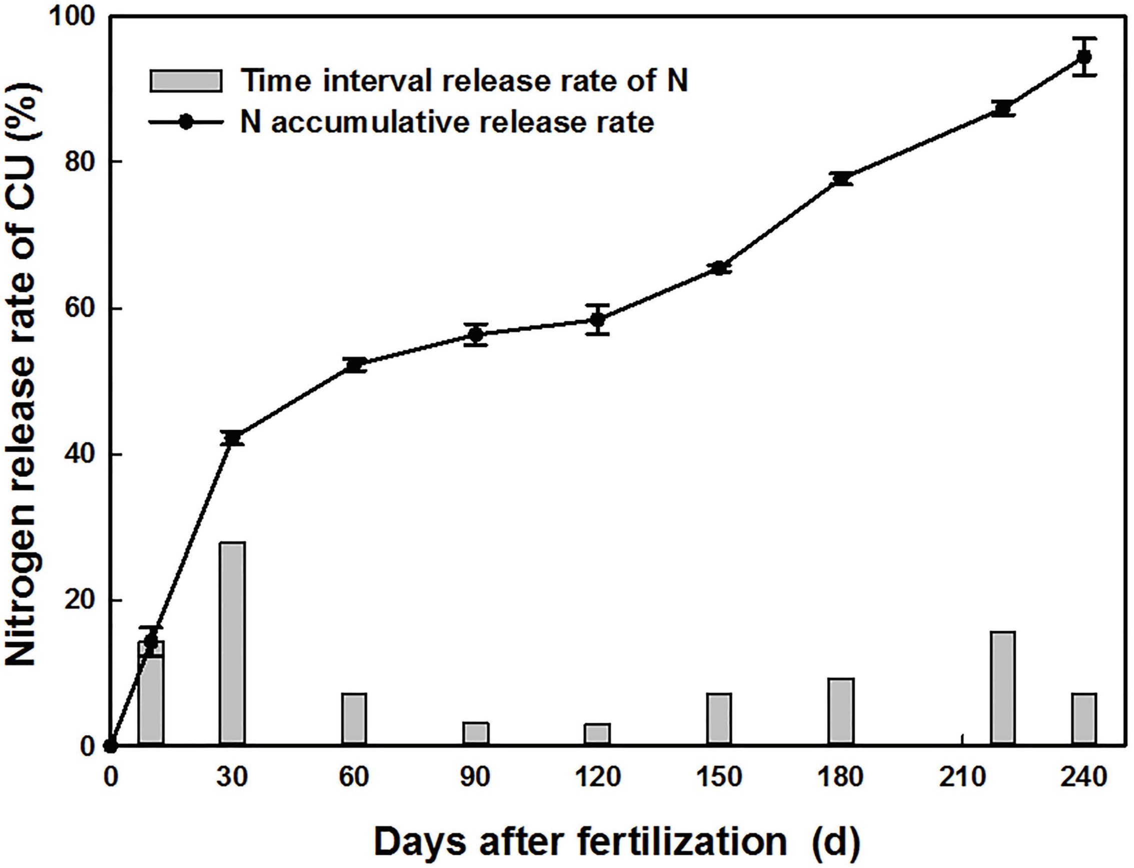

FIGURE 5 | Release characteristics of CU in soil.

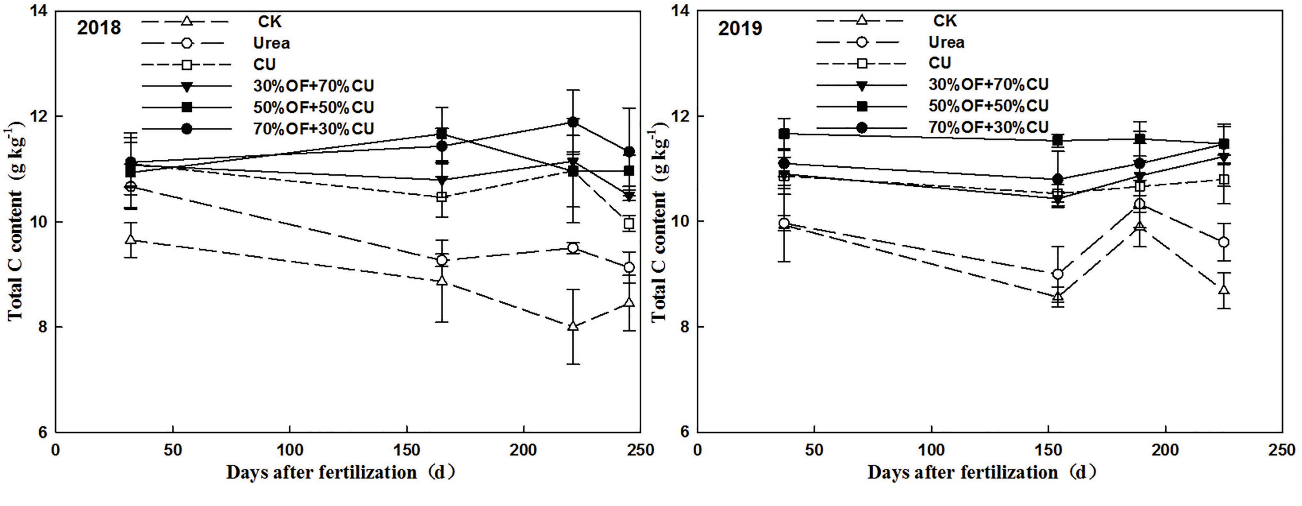

FIGURE 6 | Variations in the total carbon concentrations of the 0–20-cm topsoil.

the contribution of the grain weight to the total biomass was remarkable compared with the contribution of the straw weight. The ARNUE was higher in 2019 than in 2018 due to the higher initial fertility of the tested soil; otherwise, the differences in precipitation between the two years also contributed to the difference in results; this result was consistent with the observations reported in previous research (Geng et al., 2016). Compared with CU, the rapid hydrolysis of urea resulted in

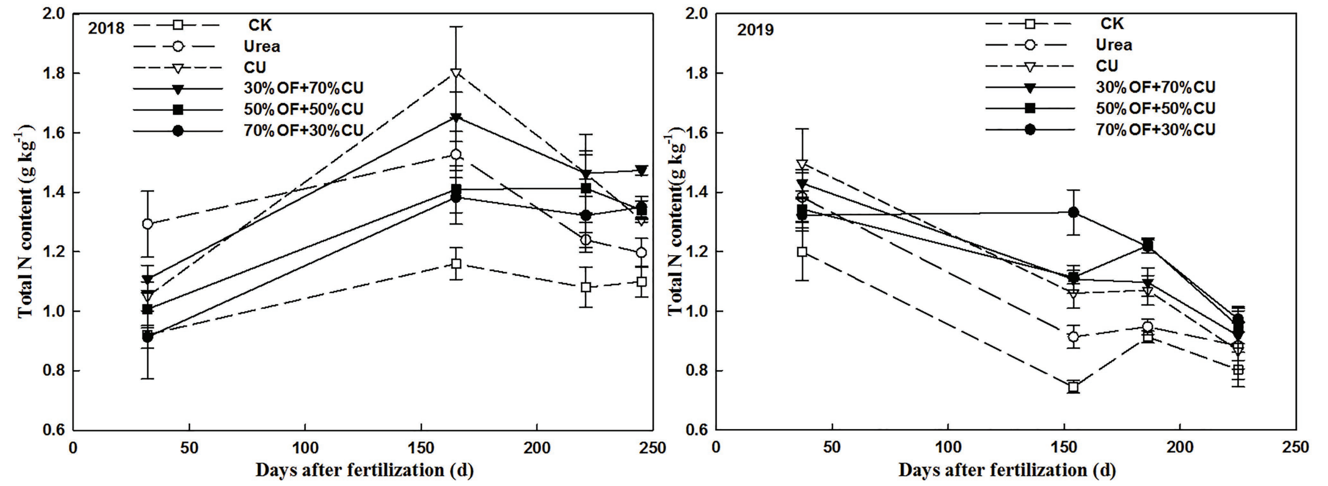

FIGURE 7 | Variations in the total N concentrations of the 0–20-cm topsoil.

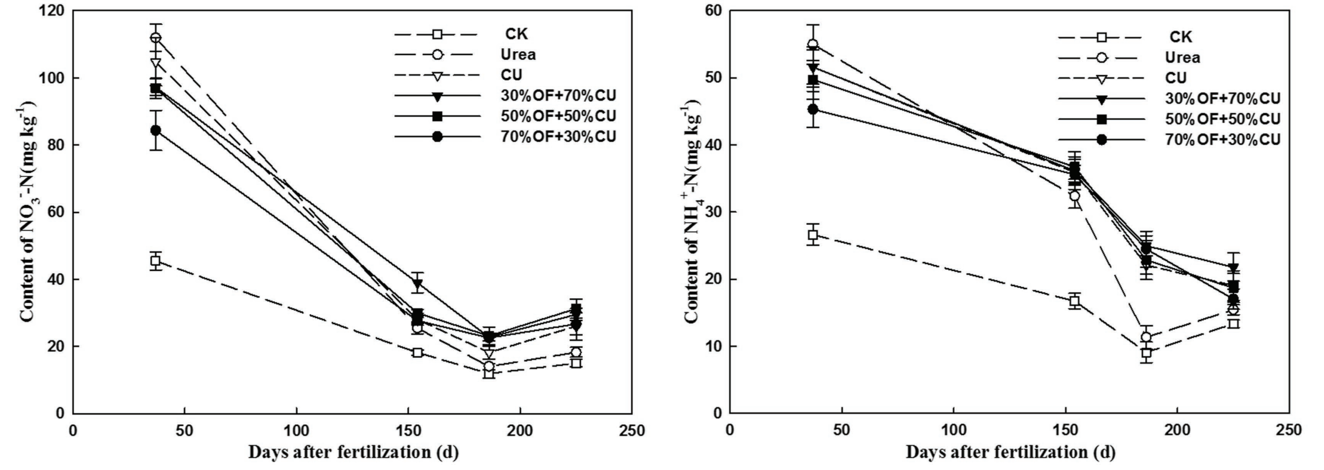

FIGURE 8 | Variations in NO_3^--N and NH_4^+-N in the 0–20-cm topsoil in 2018.

a higher concentration of inorganic N in the early stage, but this treatment could not produce a sustained N supply until the grain-filling stage (Varinderpal-Singh et al., 2020).

At the early growth stages, the poor absorption and interception abilities of wheat roots restricted N assimilation and may have accelerated the amount of reactive N escaping or leaching from fertilizer N (Oka et al., 2012). Optimal wheat yields depend on the agronomic capacity to synchronize the N availability in soils with the N demands of plants (Clunes and Pinochet, 2020). Wheat can even be successfully grown without N fertilizer use at sowing in soils with indigenous N supplies (Liu et al., 2017). The N within CU was released quickly in the first 2 months of the study and reached 42.2% in the first month, suggesting that the N supply from CU was sufficient for wheat growth at the early ontogenetic stage. The continuous release of N in the later stages also played an important role in increasing the yield, thus increasing the NUE. Moreover, the sulfur polymer-coated films partially degraded, signifying that the CU coating materials did not cause secondary pollution to the soil environment.

The functional groups of OF did not change extensively before or after the composting process, and the absorption peaks observed at 1636 and 3,416 cm^{-1} can be attributed to the stretching vibrations of the C=C and O-H bonds in the aromatic rings (Kumar et al., 2013). The presence of the C-O(H) group resulted in the observed peak at 1034 cm^{-1}, and CH_2 and C-H scissoring vibrations were observed at 2923 and 1,401 cm^{-1}. Intricate functional groups made the residual effects of OF application visible, and the continuous supply of N led to higher nutrient availabilities for wheat growth. Most of the N in OF existed in organic forms, resulting in a slow release of available N, and failed to meet the N requirement of wheat plants, thus leading to lower biomass in the early growth stage. Then, the organic matter from OF gradually decomposed and mineralized more nutrients, such as N, P and K, since the

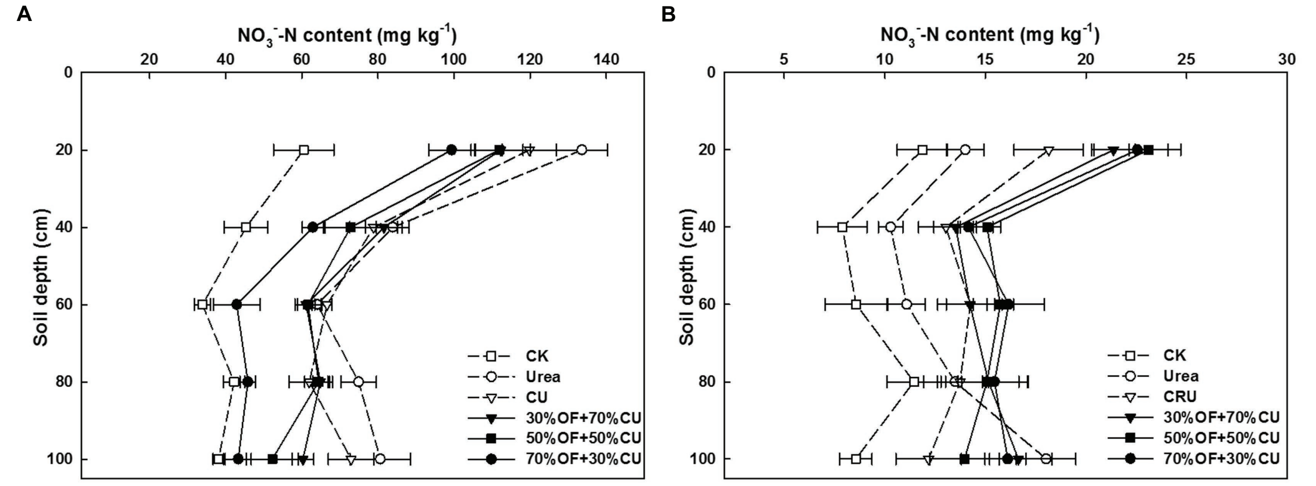

FIGURE 9 | The distributions of soil NO_3^--N in the 0–100-cm soil profiles at the seedling stage **(A)** and mature stage **(B)** in 2019.

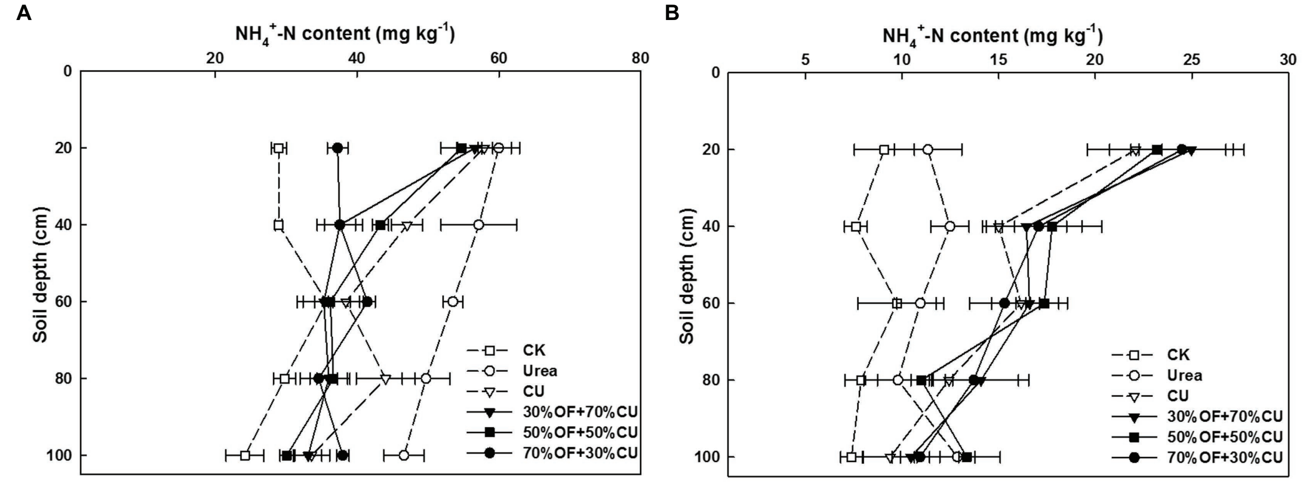

FIGURE 10 | The distributions of soil NH_4^+-N in the 0–100-cm soil profiles at the seedling stage **(A)** and mature stage **(B)** in 2019.

extension of the fertilization period and increased rainfall (Jiang et al., 2020). It continued supplying nutrients to the wheat during the later growth stage or next year of production. Therefore, the increased production is not only caused by N but also depends on the content of organic matter in the soil.

Higher organic carbon contents were yielded by applying OF than by applying conventional urea; these results were analogous with the findings of Sihi et al. (2017). When the amount of organic carbon in soil increases, wheat grain yield production increases concurrently. Integrated fertilization methods using both CU and OF were shown to be effective for improving wheat productivity. Compared with urea, the 70% OF substitution did not further promote the wheat yield compared with the wheat yield measured under the urea-only fertilization treatment. A high OF application rate results in an apparent increasing effect on the wheat yield but a weaker increasing effect than that induced by urea application and an even lower effect than that observed through CU application. These results may be attributed to the residual effect of OF application; the residual effect was found to maintain crop yields for several years in long-term experiments (Shen et al., 2007), while a high OF ratio was not an ideal model for increasing crop yields in short-term management studies. Under field conditions, crop growth depends mainly on available mineral nutrients, and the yield-increasing effect of organic fertilizer was not significant in the studied growth season.

Although the use of OF has contributed prominently to environmental sustainability and increased crop production (Osterholz et al., 2017), OF has not been used extensively in China. The incorporation of OF with CU presents a viable alternative to standard chemical N fertilizer, as the slow and variable short-term effects of OF could be averted and could be made full use of the advantages of CU. Thus, the uptake of N induced by substituting OF could be increased, supporting the achievement of higher biomass and grain yields. Although OF combined with CU spend more input on fertilizer, the 50%OF+50%CU treatment achieved higher net profit by 11.9–12.7% compared with the urea treatment due to its higher grain yield. More importantly, top dressing is avoided, and manual labor is reduced by the combination of OF and CU, as the worker population is aging and labor shortages for crop-production activities are becoming increasingly severe. The interannual variation in precipitation is large both temporally and spatially, leading to different rates of decomposition and mineralization of OF, thus affecting wheat yield and the distribution of NO_3^--N in soil. However, the combined application of OF with CU showed an increasing trend with different increase amplitudes in both years and promoted net profit. The interaction effect will be universal across regions and years, and this result is beneficial for updating the extension policy of OFs in China.

Combined Effects of OF and CU on Nitrogen Leaching

Agriculture intensification and expansion demand high doses of chemical N fertilizer in cultivated lands (Fan et al., 2021), and the unprecedented input of N resulting from these fertilizer applications would increase N availability and cycling and, subsequently, NO_3^--N leaching from agroecosystems (Galloway et al., 2008). The rapid hydrolysis of urea resulted in higher inorganic nitrogen contents being measured at the seedling stage, but root growth was slow at this time, and the wheat plants had small interception areas, thus limiting N uptake from fertilizer (Wu and Ma, 2015). Moreover, high soil NO_3^--N and NH_4^+-N concentrations enhance soil fertility but also increase the risk of harmful nitrate leaching to the groundwater. The distributions of soil NO_3^--N and NH_4^+-N in the 0–100-cm soil profiles confirmed this phenomenon.

Otherwise, the "luxury" N consumption that occurs when N is taken up by wheat is higher than the critical N concentration required before the reproductive growth stages (Liu et al., 2017), leading to straw biomass not differing prominently among N-treated management practices. For the same N input, the N in the substituted OF must be mineralized, resulting in the slowed release of mineral N (Gutser et al., 2005). Contemporarily, the N supplied by CU was released according to the N demands of wheat, and more mineral N was maintained in the topsoil, thus feeding plants and decreasing the leaching of N (Geng et al., 2016).

Large NO_3^--N accumulation and leaching in the soil profile occurs as mineralized N well exceeds the N demands of crops (Yang et al., 2020). In the current study, NO_3^--N and NH_4^+-N were mainly concentrated in the 0–60-cm soil layers in the OF substitution treatments, preventing N from leaching into deeper layers, and their appearance was enhanced as the rate of OF application increased. This may have been because OF can alter N cycling by providing nutrients to N cycling-related soil microbial communities (Thangarajan et al., 2013), improving the physicochemical properties of soil (Muhammad et al., 2020); this process may temporarily immobilize mineral N in the soil and contribute to reduced leaching losses. Otherwise, OF fertilization augmented soil organic carbon, and higher organic matter contents increased the abiotic sorption and biotic degradation processes of synthetic chemicals, resulting in lower chemical leaching (Levanon et al., 1993). In China, soil productivity has declined dramatically, and the environmental impact of reactive N has been evident for some time, as more synthetic N is applied (Hartmann et al., 2015). The economic net benefits in OF treatments were improved by 11.87–12.68% compared with urea, but OF had a residual effect and was beneficial for improving the quality of cultivated land, and the increment of wheat yield was more obvious over time. Meanwhile, for the same N input, the total N contents in topsoil of the OF substitution treatments were higher than those in the urea treatments, which indicated that more N was leached into the environment or deep soil of the urea treatment. Considering the environmental and agronomic benefits together, the combination of 30–50% OF with CU was the most conducive for enhancing wheat production.

CONCLUSION

Combining OF and CU could regulate the continuous release of N from fertilizers to match the corresponding N uptake of wheat. The 30%OF+70%CU and 50%OF+50%CU treatments showed some superiority in increasing the grain yield and N uptake compared with the urea treatment, with increase rates of 4.2–9.2% and 6.7–17.3%, respectively. The net profit of the 50%OF+50%CU treatment was prominently higher than that of the urea treatment by 11.9–12.7%. NO_3^--N and NH_4^+-N were mainly concentrated in the 0–60-cm soil layer and reduced N leaching to the 60–100-cm soil layer by the combined application of OF and CU. The application of OF is beneficial to the soil of the Yimeng Mountain area, not only in enhancing wheat productivity while mitigating the environmental pollution induced by N but also due to its residual effect. Undoubtedly, more research *via* long-term field experiments is still necessary.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XY: methodology, software, and writing. CZ: resources and investigation. XM: investigation. QL: conceptualization and formal analysis. JA: supervision. SX and XX: edit and revise.

JG: project administration and validation. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the Natural Science Foundation of China (Grant no. 42007091/42077061/41977067/41977262), Project of Introducing and Cultivating Young Talent in the Universities of Shandong Province (Soil Erosion Process and Ecological Regulation), and the Natural Science Foundation of Shandong Province of China (ZR2020QC163).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.802137/full#supplementary-material

Supplementary Figure S1 | SEM images of the surface morphology of CU films. Panels A1, A2, and A3 show the CU surfaces before fertilization at 5 μm, 10 μm, and 50 μm, respectively. Panels B1, B2 and B3 show the CU surfaces after burial in soil at 5 μm, 10 μm, and 50 μm, respectively.

Supplementary Figure S2 | FTIR spectra of organic fertilizer.

Supplementary Figure S3 | Bulk density of the 0–20-cm topsoil in 2019.

REFERENCES

Akhtar, K., Wang, W. Y., Ren, G. X., Khan, A., Enguang, N., Khan, A., et al. (2020). Straw mulching with inorganic nitrogen fertilizer reduces soil CO_2 and N_2O emissions and improves wheat yield. *Sci. Total Environ.* 741:140488. doi: 10.1016/j.scitotenv.2020.140488

Blanco-Canqui, H., Hergert, G. W., and Nielsen, R. A. (2015). Cattle manure application reduces soil compactibility and increases water retention after 71 years. *Soil Sci. Soc. Am. J.* 79, 212–223. doi: 10.2136/sssaj2014.06.0252

Clunes, J., and Pinochet, D. (2020). Effect of slow-release nitrogen on the nitrogen availability in an andisol and the critical nitrogen concentration in wheat. *Agron. J.* 112, 1250–1262. doi: 10.1002/agj2.20131

Doran, J. W. (2002). Soil health and global sustainability: translating science into practice. *Agric. Ecosyst. Environ.* 88, 119–127. doi: 10.1016/S0167-8809(01)00246-8

Douglas, L. A., Riazi, A., and Smith, C. J. (1980). A semi-micro method for determining total nitrogen in soils and plant material containing nitrite and nitrate. *Soil Sci. Soc. Am. J.* 44, 431–433. doi: 10.2136/sssaj1980.03615995004400020047x

Fan, C. C., Zhang, W., Chen, X., Li, N., Li, W., Wang, Q., et al. (2021). Residual effects of four-year amendments of organic material on N_2O production driven by ammonia-oxidizing archaea and bacteria in a tropical vegetable soil. *Sci. Total Environ.* 781:146746. doi: 10.1016/j.scitotenv.2021.146746

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., and Freney, J. R. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* 320, 889–892. doi: 10.1126/science.1136674

Garzon, E., Gonzalez-Andres, F., García-Martínez, V. M., and Paz, J. M. (2011). Mineralization and nutrient release of an organic fertilizer made by flour, meat, and crop residues in two vineyard soils with different pH levels. *Commun. Soil Sci. Plant Anal.* 42, 1485–1496. doi: 10.1080/00103624.2011.581719

Geng, J. B., Chen, J. Q., Sun, Y. B., Zheng, W. K., Tian, X. F., Yang, Y. C., et al. (2016). Controlled release urea improved nitrogen use efficiency and yield of wheat and corn. *Agron. J.* 108, 1666–1673. doi: 10.2134/agronj2015.0468

Geng, J. B., Sun, Y. B., Zhang, M., Li, C. L., Yang, Y. C., Liu, Z. G., et al. (2015). Long-term effects of controlled release urea application on crop yields and soil fertility under rice-oilseed rape rotation system. *Field Crop Res.* 184, 65–73. doi: 10.1016/j.fcr.2015.09.003

Gutser, R., Ebertseder, T., Weber, A., Schraml, M., and Schmidhalter, U. (2005). Short-term and residual availability of nitrogen after long-term application of organic fertilizers on arable land. *J. Plant Nutr. Soil Sci.* 168, 439–446. doi: 10.1002/jpln.200520510

Hao, T. X., Zhu, Q. C., Zeng, M. F., Shen, J. B., Shi, X. J., Liu, X. J., et al. (2020). Impacts of nitrogen fertilizer type and application rate on soil acidification rate under a wheat-maize double cropping system. *J. Environ. Manage.* 270:110888. doi: 10.1016/j.jenvman.2020.110888

Hartmann, T. E., Yue, S., Schulz, R., He, X., Chen, X., Zhang, F. S., et al. (2015). Yield and N use efficiency of a maize-wheat cropping system as affected by different fertilizer management strategies in a farmer's field of the North China plain. *Field Crops Res.* 174, 30–39. doi: 10.1016/j.fcr.2015.01.006

Hickman, J. E., Palm, C. A., Mutuo, P., Melillo, J. M., and Tang, J. W. (2014). Nitrous oxide (N_2O) emissions in response to increasing fertilizer addition in maize (*Zeamays* L.) agriculture in western Kenya. *Nutr. Cycl. Agroecosyst.* 100, 177–187. doi: 10.1007/s10705-014-9636-7

Humber, J., Dwyer, J. M., Andrey, A., and Arlettaz, R. (2016). Impacts of nitrogen addition on plant biodiversity in mountain grasslands depend on dose, application duration and climate: a systematic review. *Glob. Chang. Biol.* 22, 110–120. doi: 10.1111/gcb.12986

Jiang, Y. J., Lei, T., Du, W., Liang, C. L., Li, H. D., and Lv, J. L. (2020). Substituting chemical fertilizer nitrogen with organic manure and comparing their nitrogen use efficiency and winter wheat yield. *J. Agric. Sci.* 158, 262–268. doi: 10.1017/S0021859620000544

Kumar, D. S., Kumar, P. S., Rajendran, N. M., and Anbuganapathi, G. (2013). Compost maturity assessment using physicochemical, solid state spectroscopy, and plant bioassay analysis. *J. Agric. Food Chem.* 61, 11326–11331. doi: 10.1021/jf4034943

Levanon, D., Codling, E. E., Meisinger, J. J., and Starr, J. L. (1993). Mobility of agrochemicals through soil from two tillage systems. *J. Environ. Qual.* 22, 155–161. doi: 10.2134/jeq1993.00472425002200010020x

Liu, Z. J., Chen, Z. J., Ma, P. Y., Meng, Y., and Zhou, J. B. (2017). Effects of tillage, mulching and N management on yield, water productivity, N uptake and residual soil nitrate in a long-term wheat-summer maize cropping system. *Field Crop Res.* 213, 154–164. doi: 10.1016/j.fcr.2017.08.006

Liu, J. L., Yang, Y. C., Gao, B., Li, Y. C., and Xie, J. Z. (2019). Bio-based elastic polyurethane for controlled-release urea fertilizer: fabrication, properties, swelling and nitrogen release characteristics. *J. Clean. Prod.* 209, 528–537. doi: 10.1016/j.jclepro.2018.10.263

Liu, X. J., Zhang, Y., Han, W. X., Tang, A. H., Shen, J. L., Cui, Z. L., et al. (2013). Enhanced nitrogen deposition over China. *Nature* 494, 459–462. doi: 10.1038/nature11917

Meade, G., Lalor, S., and Cab, E. T. M. (2011). An evaluation of the combined usage of separated liquid pig manure and inorganic fertiliser in nutrient programmes for winter wheat production. *Eur. J. Agron.* 34, 62–70. doi: 10.1016/j.eja.2010.10.005

Muhammad, Q., Huang, J., Waqas, A., Li, D. C., Liu, S. J., Zhang, L., et al. (2020). Yield sustainability, soil organic carbon sequestration and nutrients balance under long-term combined application of manure and inorganic fertilizers in acidic paddy soil. *Soil Tillage Res.* 198:104569. doi: 10.1016/j.still.2019.104569

Oka, M., Shimoda, Y., Sato, N., Inoue, J., Yamazaki, T., Shimomura, N., et al. (2012). Abscisic acid substantially inhibits senescence of cucumber plants (*Cucumis sativus*) grown under low nitrogen conditions. *J. Plant Physiol.* 169, 789–796. doi: 10.1016/j.jplph.2012.02.001

Osterholz, W. R., Rinot, O., Liebman, M., and Castellano, M. J. (2017). Can mineralization of soil organic nitrogen meet maize nitrogen demand? *Plant Soil* 415, 73–84. doi: 10.1007/s11104-016-3137-1

Palmer, J., Thorburn, P. J., Biggs, J. S., Dominati, E. J., Probert, M. E., Meier, E. A., et al. (2017). Nitrogen cycling from increased soil organic carbon contributes both positively and negatively to ecosystem services in wheat agro-ecosystems. *Front. Plant Sci.* 8:731. doi: 10.3389/fpls.2017.00731

Pinochet, D., Clunes, J., Gauna, C., and Contreras, A. (2018). Reasoned fertilization of potato in response to nitrogen supply in Andisols. *J. Soil Sci. Plant Nutr.* 18, 790–803. doi: 10.4067/S0718-95162018005002301

Shen, M. X., Yang, L. Z., Yao, Y. M., Wu, D. D., Wang, J., Guo, R., et al. (2007). Long-term effects of fertilizer managements on crop yields and organic carbon storage of a typical rice-wheat agroecosystem of China. *Biol. Fertil. Soils* 44, 187–200. doi: 10.1007/s00374-007-0194-x

Sihi, D., Dari, B., Sharma, D. K., Pathak, H., Nain, L., and Sharma, O. P. (2017). Evaluation of soil health in organic vs. conventional farming of basmati rice in North India. *J. Plant Nutr. Soil Sci.* 180, 389–406. doi: 10.1002/jpln.201700128

Tang, L., Li, S. Q., and Shen, Y. F. (2021). Response of maize yield and nitrogen leaching to combining controlled-release urea and normal urea under different surface mulching. *J. Sci. Food Agr.* 101, 5520–5528. doi: 10.1002/jsfa.11201

Thangarajan, R., Bolan, N. S., Tian, G., Naidu, R., and Kunhikrishnan, A. (2013). Role of organic amendment application on greenhouse gas emission from soil. *Sci. Total Environ.* 465, 72–96. doi: 10.1016/j.scitotenv.2013.01.031

Varinderpal-Singh, A., Kaur, S., Singh, J., Kaur, A., and Gupta, R. K. (2020). Rescheduling fertilizer nitrogen topdressing timings for improving productivity and mitigating N_2O emissions in timely and late sown irrigated wheat (*Triticum aestivum* L.). *Arch. Agron. Soil Sci.* 67, 647–659. doi: 10.1080/03650340.2020.1742327

Velasco, J. L., Rozas, H. S., Echeverra, H. E., and Barbieri, P. A. (2012). Optimizing fertilizer nitrogen use efficiency by intensively managed spring wheat in humid regions: effect of split application. *Can. J. Plant Sci.* 92, 847–856. doi: 10.4141/cjps2011-146

Wu, W., and Ma, B. L. (2015). Integrated nutrient management (INM) for sustaining crop productivity and reducing environmental impact: a review. *Sci. Total Environ.* 512–513, 415–427. doi: 10.1016/j.scitotenv.2014.12.101

Yadav, R. L., Dwivedi, B. S., Prasad, K., Tomar, O. K., Shurpali, N. J., and Pandey, P. S. (2000). Yield trend, and changes in soil organic-C and available NPK in a long-term rice-wheat system under integrated use of manures and fertilizers. *Field Crop Res.* 68, 219–246. doi: 10.1016/S0378-4290(00)00126-X

Yang, X. Y., Geng, J. B., Liu, Q. J., Zhang, H. Y., Hao, X. D., Sun, Y. B., et al. (2021). Controlled-release urea improved rice yields by providing nitrogen in synchrony with the nitrogen requirements of plants. *J. Sci. Food Agr.* 101, 4183–4192. doi: 10.1002/jsfa.11056

Yang, Y. J., Lei, T., Du, W., Liang, C. L., Li, H. D., and Lv, J. L. (2020). Substituting chemical fertilizer nitrogen with organic manure and comparing their nitrogen use efficiency and winter wheat yield. *J. Agric. Sci.* 158, 262–268. doi: 10.1017/S0021859620000544

Zhou, Z. J., Shen, Y. Z., Du, C. W., Zhou, J. M., Qin, Y. S., and Wu, Y. J. (2017). Economic and soil environmental benefits of using controlled-release bulk blending urea in the North China plain. *Land Degrad. Dev.* 28, 2370–2379. doi: 10.1002/ldr.2767

Conflict of Interest: CZ and XM were employed by company Jinyimeng Group Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 January 2022
doi: 10.3389/fpls.2021.772086

Arginine Increases Tolerance to Nitrogen Deficiency in *Malus hupehensis* via Alterations in Photosynthetic Capacity and Amino Acids Metabolism

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *07 September 2021*
Accepted: *20 December 2021*
Published: *14 January 2022*

Citation:
Chen Q, Wang Y, Zhang Z, Liu X, Li C and Ma F (2022) Arginine Increases Tolerance to Nitrogen Deficiency in Malus hupehensis via Alterations in Photosynthetic Capacity and Amino Acids Metabolism. Front. Plant Sci. 12:772086. doi: 10.3389/fpls.2021.772086

Arginine plays an important role in the nitrogen (N) cycle because it has the highest ratio of N to carbon among amino acids. In recent years, there has been increased research interest in improving the N use of plants, reducing the use of N fertilizer, and enhancing the tolerance of plants to N deficiency. Here, the function of arginine in the growth of apple (*Malus hupehensis*) under N deficiency was explored. The application of 100 μmol L^{-1} arginine was effective for alleviating N-deficiency stress. Exogenous arginine promoted the absorption and use of N, phosphorus (P), and potassium (K) under low N stress. The net photosynthetic rate, maximal photochemical efficiency of photosystem II, and chlorophyll content were higher in treated plants than in control plants. Exogenous arginine affected the content of many metabolites, and the content of many amino acids with important functions was significantly increased, such as glutamate and ornithine, which play an important role in the urea cycle. Half of the metabolites were annotated to specialized metabolic pathways, including the synthesis of phenolic substances, flavonoids, and other substances with antioxidant activity. Our results indicate that arginine promotes the plant photosynthetic capacity and alters amino acid metabolism and some antioxidants including phenolic substances and flavonoids to improve the tolerance of apple to N deficiency, possibly through the improvement of arginine content, and the absorption of mineral.

Keywords: arginine, *Malus*, nitrogen, metabolite, amino acid

INTRODUCTION

Nitrogen (N) is an essential element for plant growth. In addition to being absorbed and utilized by plants, a large amount of N in soil is lost in the form of NO_2, NO_x, N_2O, N_2, dissolved organic N (DON), and particulate organic N (PON) (Zhang et al., 2015), which can lead to a gradual reduction of available N in soil. The use of N fertilizer can increase the proportion of N in soil and crop yields. Although the large amount of N fertilizer used in the agricultural industry increases the economic output of farmers, it can also lead to various problems such as water eutrophication and soil acidification (Guo et al., 2010; Liu et al., 2021). Therefore, improving the tolerance of plants to N-deficient conditions is an effective approach for mitigating soil N loss and environmental contamination caused by the excessive application of N fertilizer.

Nitrogen is involved in nearly all metabolic processes in plants. Although some plants use N-fixing microorganisms to obtain N, most plants meet their own growth and development needs by absorbing N from the soil. Therefore, low N availability in the soil can affect the metabolism, resource allocation, growth, and development of plants (Xu et al., 2012; Hsieh et al., 2018). The level of N absorbed by the plant directly affects the activity of enzymes involved in N absorption and transport in plants, and the activity of these enzymes is often closely related to chlorophyll (chl) synthesis. Therefore, chlorophyll is degraded when plants are faced with N deficiency, and plants often exhibit symptoms of leaf chlorosis and experience reductions in photosynthetic rate (Wang et al., 2019; Wen et al., 2019). Some plants also exhibit a decrease in height, the number of nodes, and leaf area (Chen et al., 2018). Previous studies have shown that N affects the activity of enzymes involved in the Calvin cycle, and a limited N supply reduces the CO_2 assimilation capacity and decreases the light quantum yield (Φ) (Sun et al., 2016). A low N supply also affects the electron transport efficiency and maximum photochemical efficiency of PSII (F_v/F_m) (Luisa et al., 2018). Liu et al. (2020) found that the F_v/F_m of *Malus hupehensis* decreases markedly under N deficiency. In maize, Lu and Zhang (2000) also found that N deficiency affects plant PSII.

There are two forms of soil N uptake by plants: inorganic N (nitrates and ammonium salts) and organic N, mainly in the form of amino acids (Torgny et al., 2009). NO_3^- uptake in plants is primarily achieved by NO_3^- transporters, a family of transporters that can be divided into the low-affinity system and the high-affinity system based on their transport activity. The low-affinity transport system is mainly mediated by the NRT1 (nitrate transporter 1) protein, whereas the high-affinity transport system is mainly mediated by the NRT2 protein (Fan et al., 2017; Asif et al., 2019). Ammonium transport is also carried out by ammonium transporters (AMTs) (Bai et al., 2010). The transport of nitrates and ammonium salts has been extensively studied, but plants can also absorb and use organic N directly. Amino acid transporters are currently classified into three major groups: ATFs (amino acid transporter superfamily), APCs (amino acid-polyamine-choline transporter superfamily), and UMAMITs (usually multiple acids move in and out transporter superfamily). They are responsible for the absorption and transport of different types of amino acids (Pratelli and Pilot, 2014; Dinkeloo et al., 2018). For example, AtAAP5 (amino acid permease 5) plays an important role in the uptake of arginine and lysine at common concentrations in soil (Svennerstam et al., 2011). In addition, amino acid transporters exhibit some common characteristics, such as broad expression patterns and substrate specificity (Yang et al., 2020).

Arginine has the highest ratio of N to carbon among the 21 amino acids. The anabolism of arginine involves many metabolites and processes. Glutamic acid is the initial substrate for arginine synthesis (Winter et al., 2015); it thus provides a means of N storage and transport and has important functions in plants. Previous studies have shown that arginine metabolism is closely related to N metabolism, and many of the pathways in arginine metabolism show tissue specificity in plants (Gao et al., 2009). Ornithine is both a product and metabolic intermediary of arginine metabolite (Urbano-Gámez et al., 2020). These amino acids also play an important role in N utilization and the urea cycle in plants. Arginine succinate synthase (ASS), a key enzyme in arginine synthesis, has been reported to cause ASS1-deficient cell death in animals (Locke et al., 2016). Arginine can also undergo decarboxylation by arginine decarboxylase (ADC) and be converted to guanidine butylamine and then putrescine and other polyamines (Patel et al., 2017). Nitric oxide synthase catalyzes the conversion of arginine to citrulline and NO, and arginase (ARG) catalyzes the decomposition of arginine to ornithine and urea; urea is the substrate that links arginine metabolism and the urea cycle (Cao et al., 2010). The activity of these enzymes controls the direction of arginine metabolism.

Recent studies have focused on the function of arginine and related genes. Exogenous arginine treatment delays fruit coloring, inhibits fruit ripening, and increases the activity of antioxidant enzymes in strawberry fruits (Lv et al., 2020). Arginine also increases the N content in tomato (Wang et al., 2021) and NO content in wheat seedlings (Karpets et al., 2018). Overexpression of *OsARG* in rice (cv. Kitaake) increases the number of grains per plant under N deficiency (Ma et al., 2013). Infection of mature *Arabidopsis thaliana* with *Botrytis cinerea* up-regulates *ARGAH2* expression (Brauc et al., 2011). T-DNA insertional mutants of *argah1-1* and *argah2-1* and double mutants of *argah1argah2* show increased tolerance to abiotic stress, including water stress, salt stress, and low temperature, which is accompanied by the accumulation of NO and polyamines. Overexpression of arginase reduces tolerance to abiotic stress (Teresita et al., 2008; Shi et al., 2013). The *adc*-silenced line is more susceptible than parental WT plants to infection by *Botrytis cinerea* (Chávez-Martínez et al., 2020). Salt stress up-regulates the expression of *GHASS1* in cotton (Wang et al., 2016), and its expression peaks after 1 day of salt stress, suggesting that *GHASS1* might be involved in the early response of cotton to salt stress.

Research on arginine in *Malus* is still in an incipient stage. Arginine is an N storage substance that can alleviate N-deficiency stress, delay senescence, and reduce the use of N fertilizer. The aim of this study was to characterize changes in the physiological parameters and exogenous substances of *Malus hupehensis* Rehd. under N deficiency and explore the physiological functions and mechanisms of arginine in plants under low N stress. Overall, the results of our study have implications for the apple industry, especially the development of the green economy, environmental protection, and sustainability.

MATERIALS AND METHODS

Materials and Growth Conditions

The experiment was carried out at Northwest A&F University, Yangling (34° 15′ N, 108° 4′ E) Shaanxi, China. Seeds of *M. hupehensis* were collected in Pingyi (35° 70′ N, 117° 25′ E), Shandong Province. The seeds were disinfected and stored in sand for 2 months at 4°C to break dormancy. The germinated seeds were seeded in a nursery (12 cm × 12 cm). Plants of similar size were used for hydroponics and grown in 8–10 nurseries. Hoagland nutrient solution was the source of nutrients during hydroponics. The two N concentrations applied were 5 mmol L^{-1} and 0.2 mmol L^{-1}. N was provided exclusively in the form of NO_3^-. Arginine was added 3 days before N-deficiency treatment to facilitate the adaptation of plants to the experimental environment. The temperature was maintained at 23–25°C in the daytime and 15–18°C at night. The sodium lamp provided light during the 14/10 h light/dark photoperiod (photon flux density was 160 mmol m^{-2} s^{-1}). Oxygen was added to the nutrient solution to maintain the oxygen concentration at 8.0–8.5 mg L^{-1} through the dissolved oxygen controller (FC-680; Corporation of Super, Shanghai, China). The nutrient solution was renewed every 5 days.

Hydroponics Screening

The plants were pre-cultivated for 15 days to permit adaptation to hydroponics conditions. To determine the optimal concentration of exogenous arginine, the plants were divided into seven treatments at 20 days: half-strength nutrient solution (5 mmol L^{-1}) and N-deficiency treatment with 20, 100, 500, 2500, and 10000 μmol L^{-1} arginine. Arginine was supplemented throughout the entire experiment. The net photosynthetic rate (*Pn*) was measured at Day 0, 5, 10, 15, 20, and 25 after treatment; the dry weight, fresh weight, and plant height of all groups were measured at 25 days after treatment; and the phenotypic characteristics of the plants were recorded.

According to the optimal arginine concentration, the plants were divided into four treatments: control (1/2 Hoagland solution containing 5 mmol L^{-1} N, CK), control + arginine (1/2 Hoagland solution containing 5 mmol L^{-1} N with 100 μmol L^{-1} arginine, CKA), N-deficiency solution (1/2 Hoagland solution containing 0.2 mmol L^{-1} N, LN), and LN + arginine (1/2 Hoagland solution containing 0.2 mmol L^{-1} N with 100 μmol L^{-1} arginine, LNA). The physiological indexes of each treatment were measured.

Growth Measurements

The shoot height (SH) was measured from the top of the stem to the stem base; the leaf number was counted per plant at 20 days after treatment. On the first and last day of the treatments, the plants were divided into leaves, stems, and roots for weighing. The samples were then washed with tap water, distilled water, and double water, followed by fixation at 105°C for 30 min and drying at 72°C until a constant weight was achieved (at least 72 h). The

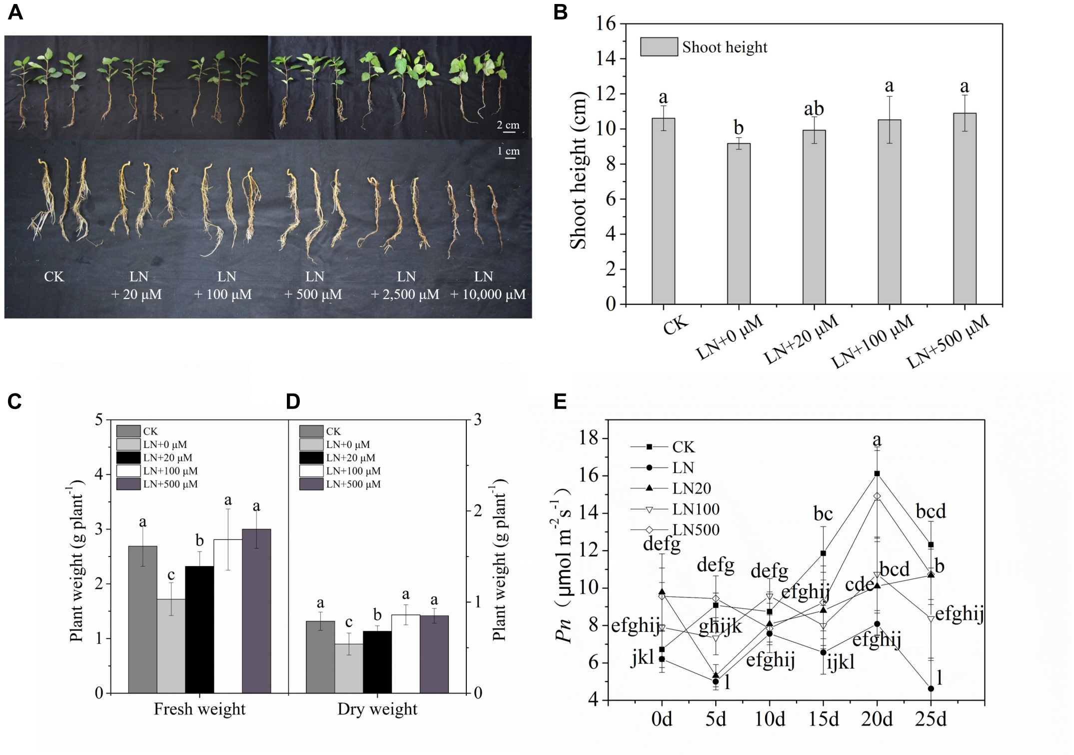

FIGURE 1 | Effect of N deficiency on the growth parameters of *Malus hupehensis*. Whole plant and underground phenotypes **(A)**, shoot height (cm) **(B)**, fresh weight (g^{-1} plant) **(C)**, dry weight (g^{-1} plant) **(D)**, and net photosynthetic rate (*Pn*) **(E)** from 0 to 25 days every 5 days. CK, half-strength nutrient solution; LN, N-deficiency treatment; LN + 20 μmol L^{-1}, N-deficiency treatment plus 20 μmol L^{-1} arginine; LN + 100 μmol L^{-1}, N-deficiency treatment plus 100 μmol L^{-1} arginine; LN + 500 μmol L^{-1}, N-deficiency treatment plus 500 μmol L^{-1} arginine; LN + 2500 μmol L^{-1}, N-deficiency treatment plus 2500 μmol L^{-1} arginine; and LN + 10000 μmol L^{-1}, and N-deficiency treatment plus 10000 μmol L^{-1} arginine. The picture was taken on the third d of the experiment). Values are means of ten replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

fresh weight and dry weight were calculated from the weight of the leaves, stems, and roots before and after drying, respectively.

Measurement of Malondialdehyde Content and Root Activity

The malondialdehyde (MDA) content was measured per the instructions provided in an MDA reagent kit (Suzhou Comin Biotechnology Co., Ltd., China). Root activity was measured using triphenyl tetrazolium chloride (TTC) following the method of Joseph et al. (2010).

Determination of Nitrogen, Potassium, and Phosphorus Concentrations

The tissues in the four treatments were collected at 20 days after treatment to estimate the N, P, and K concentrations. After quick-freezing, tissue (0.1 g) was ground and digested

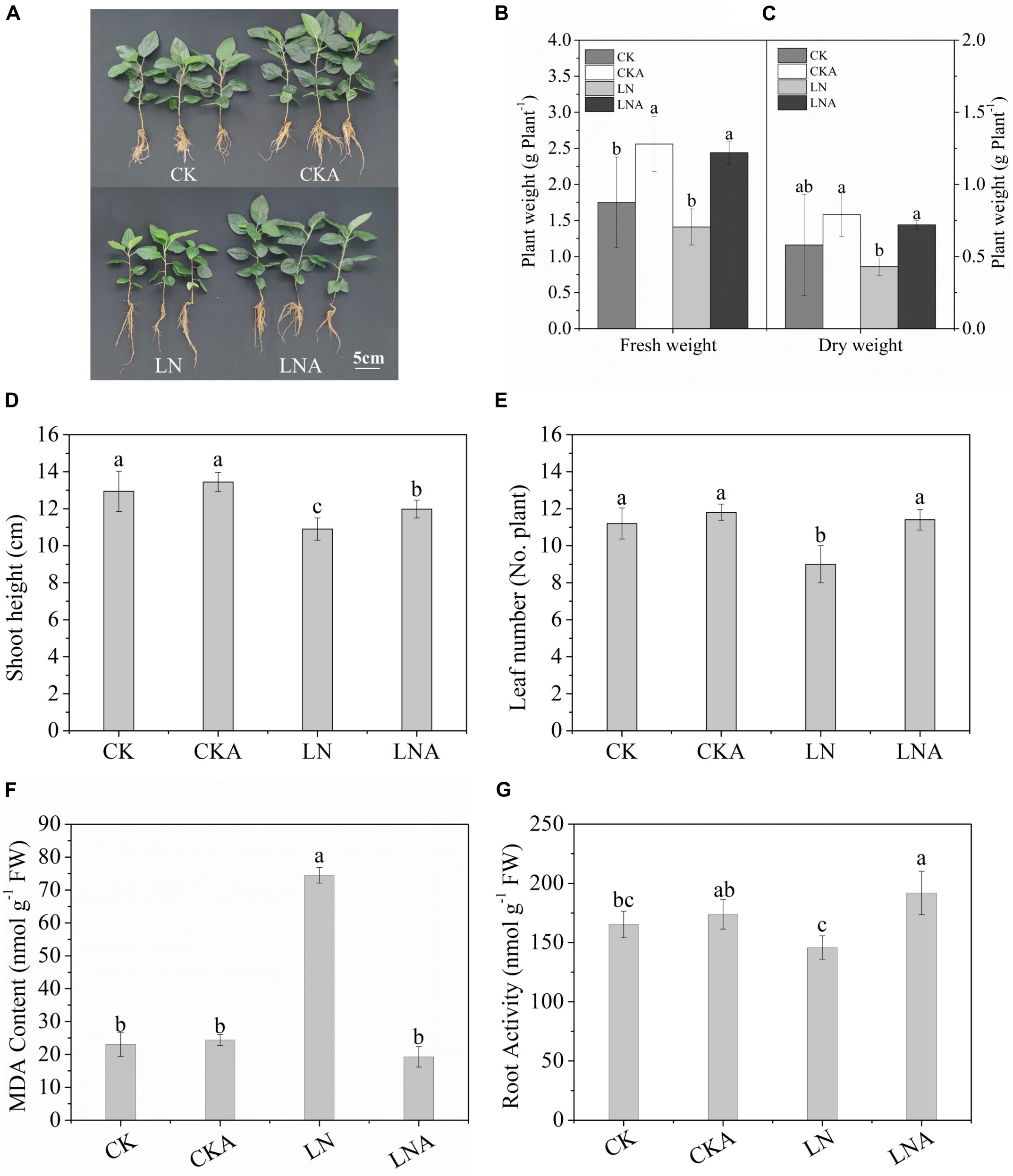

FIGURE 2 | Analysis of the growth parameters of CK, CKA, LN, and LNA at 20 days. We measured the phenotype **(A)**, fresh weight (g^{-1} plant) **(B)**, dry weight (g^{-1} plant) **(C)**, shoot height (cm) (No. plant) **(D)**, and the number of leaves **(E)** of apple plants from these four treatments. The content of MDA after 20 days **(F)**. Root activity of CK, CKA, LN, and LNA after 20 days of treatment **(G)**. Half-strength nutrient solution (CK); half-strength nutrient solution + 100 μmol L^{-1}arginine (CKA); N-deficiency solution (LN); and N-deficiency solution + 100 μmol L^{-1} arginine (LNA). Values are means of five replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

with concentrated sulfuric acid (H_2SO_4, AR, 98%) and hydrogen peroxide (H_2O_2, GR, ≥30%). The N and P content was measured by an AutoAnalyzer 3 continuous-flow analyzer (AA3; SEAL Analytical, Norderstedt, Germany), and the K content was measured by a flame photometer (M410; Sherwood Scientific, Cambridge, United Kingdom) according to the method of Li et al. (2013).

Determination of Nutrient Uptake Fluxes, Transport, Accumulation, and Partitioning

The formulas for nutrient uptake fluxes (TN) are as follows (Kruse et al., 2007):

$$TN_r = RGR \times DW_r \times C_r$$

$$TN_s = RGR \times DW_s \times C_s$$

$$TN_l = RGR \times DW_l \times C_l$$

where, RGR is the relative growth rate, DW is the dry weight, and C is the concentration of elements in each tissue. The uptake flux was expressed in units of milligrams per plant per day or micrograms per plant per day.

The transportation of N, P, and K was defined as the total amount of element transported to leaf and stem tissue per gram root DW per day. The accumulation of N, P, and K in the roots was defined as the total amount of nutrient absorbed into root per gram root DW per day. The formula is as follows (Kruse et al., 2002):

$$\text{transfer/accumulation} = (M_2 - M_1) \times (LnW_2 - LnW_1)/(W_2 - W_1)/(T_2 - T_1)$$

where, M represents the total content of elements, W represents the dry weight of roots, and T is the treatment time.

The element content of each part (root, stem, and leaf) is the product of the dry weight of each part and the element concentration.

Enzyme Activity Assays

The tissues of four treatments were collected at 20 days after treatment. The samples were ground into powder with liquid N and stored at −80°C. Samples were weighed (0.1 g) for each enzyme activity measurement. The activity of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) was measured per the instructions

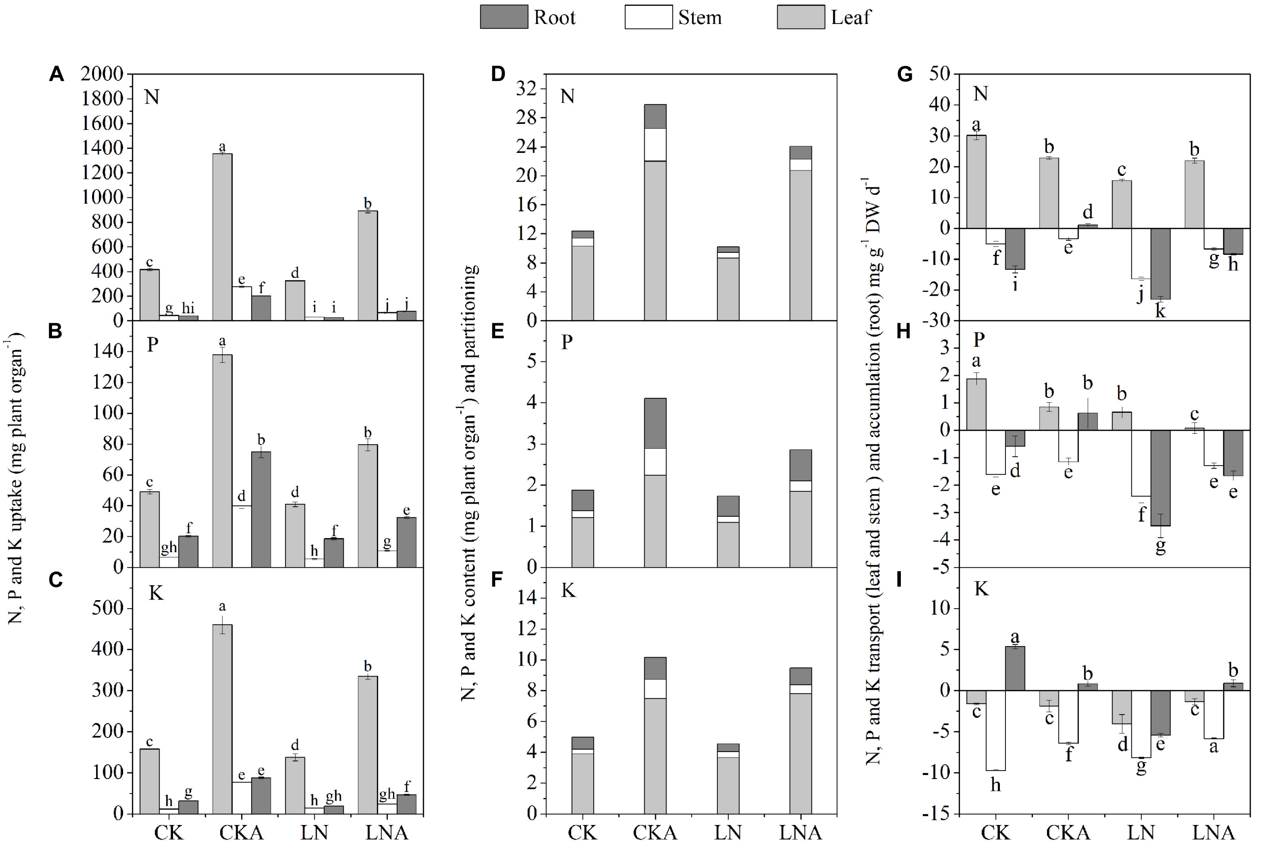

FIGURE 3 | Effects of arginine on N, P, and K in *M. hupehensis* under low N. The uptake of total N **(A)**, K **(B)**, and P **(C)** in the leaves, stems, and roots. The accumulation and partitioning of N **(D)**, P **(E)**, and K **(F)** and transport of N **(G)**, P **(H)**, and K **(I)** in the leaves, stems, and roots. Values are means of three replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

provided by corresponding kits (Suzhou Comin Biotechnology Co., Ltd., China). Absorbance of NR and GOGAT was recorded at 340 nm. Absorbance of NIR and GS was recorded at 540 nm. The standard was based on the instructions provided by the kits. NR activity was expressed as catalytic reduction of 1 nmol NADH per min per g of fresh weight. NiR activity was expressed as the reduction of 1 μmol NO_2^- per g tissue per hour. GS activity was expressed as 1 μmol of γ-glutamyl hydroxamic acid produced per gram of tissue per ml of reaction system per hour. GOGAT activity was expressed as 1 nmol of NADH consumed per gram of tissue per minute.

Measurement of Photosynthetic Characteristics

Chl was extracted with 80% acetone at 0 and 20 days. The total chl, chl *a*, chl *b*, and carotenoid (car) content was measured spectrophotometrically following the procedure of Arnon (1949) with minor modifications.

The *Pn*, stomatal conductance (*gs*), intercellular carbon dioxide concentration (*Ci*), and transpiration rate (*Tr*) were measured with a portable photosynthesis system (CIRAS3) from 9:00 am to 10:00 am. All measurements were performed at 1000 μmol photons m^{-2} s^{-1} and a constant airflow rate of 500 μmol s^{-1}. The cuvette CO_2 concentration was set to 400 μmol CO_2 mol^{-1} air, with a vapor pressure deficit of 2.0–3.4 kPa. Measurements were taken from the fully extended leaves located at the same position from five randomly selected plants in each group.

Chl fluorescence was measured by a Dual-PAM-100 Chlorophyll Fluorometer (Walz, Germany). After 30 min of dark adaptation, the minimum fluorescence (F_0) and maximum Chl fluorescence yield (F_m) were measured. F_v/F_m was calculated as $(F_m-F_0)/F_m$.

RT-PCR Analysis

Total RNA was extracted from leaves using a Wolact® Plant RNA Isolation Kit (Vicband, HongKong, China) per the manufacturer's instructions. qRT-PCR was performed on a LightCycler 480 (Roche, Indianapolis, IN, United States) Real-Time System using SYBR Premix Ex Taq II (Takara, Kyoto, Japan) following a previously described method (Gao et al., 2020). Sequences of the primers for *MdAMT1;2, MdAMT2;1*, *MdFd-GOGAT*, and *MdNADH-GOGAT* were designed with Primer Premier 5 software (Biosoft International, Palo Alto, CA, United States) (**Supplementary Table 1**). *MdMDH* was used as an internal reference gene.

Metabolomic Analysis

Biological samples were crushed using a mixer mill (MM 400, Retsch). Extraction and detection methods were adjusted following the method of Chen et al. (2013). Briefly, 100 mg of powder was dissolved with 1.2 mL of 70% methanol solution. The solution was vortexed for 30 s every 30 min a total of 6 times. The sample was extracted at 4°C overnight, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was filtrated through a 0.22-μm organic strainer before UPLC-MS/MS (UPLC, SHIMADZU Nexera X2; MS, Applied Biosystems 4500 Q TRAP) analysis. The UPLC was equipped with an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. The gradient program was as follows: 0–9 min, 95% A to 5% A and 5% B to 95% B with a linear gradient and hold for 1 min; 5% A to 95% A and 95% B to 5% B

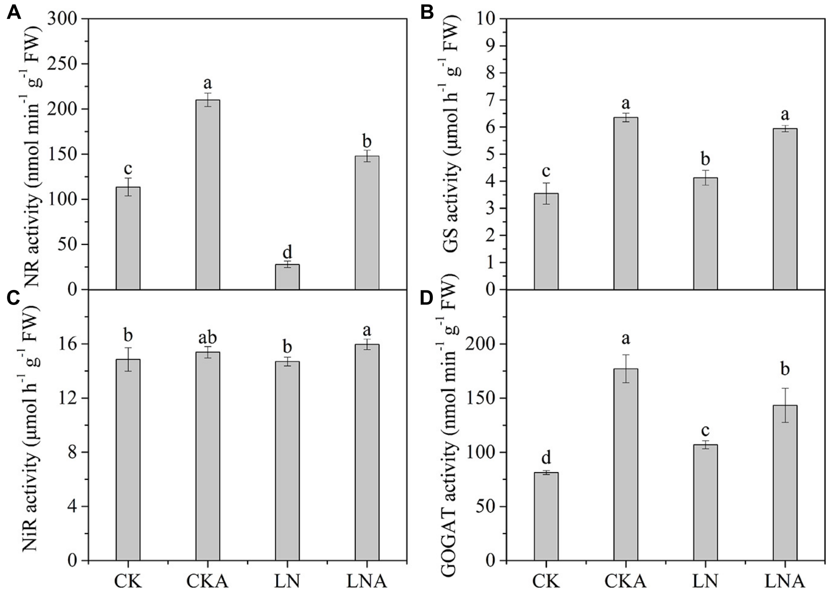

FIGURE 4 | The activity of enzymes in the leaves of CK, CKA, LN, and LNA after 20 days. NR activity **(A)**, GS activity **(B)**, NiR activity **(C)**, and GOGAT activity **(D)**. Values are means of five replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

in 1.10 min and hold for 2.9 min. The flow velocity was 0.35 mL min^{-1}, the column temperature was 40°C, and the injection volume was 4 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), AB4500 Q TRAP UPLC/MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 550°C; ion spray voltage (IS), 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 50, 60, and 25.0 psi, respectively; and the collision-activated dissociation (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol L^{-1} polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (N) set to medium. DP and CE for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Determination of Amino Acids

The amino acids were extracted and detected by LC-MS following the method of Jin et al. (2019). Fresh tissue was ground in liquid N, 500 mg of sample powder was weighed in tubes, and 1 mL of 50% ethanol (containing 0.1 mol L^{-1} HCl) was added to tubes to extract amino acids. The supernatant was transferred to a new tube after centrifugation at 13,000 *g* and 4°C for 10 min. The liquid was filtered through a 0.22-μm organic strainer. The filtrate was diluted 20 times with methanol (AR, 98%) to a 1-mL volume. The LC-MS analysis was carried out on an Inertsil OSD-4C18 column (150 mm × 3.0 mm; packing size, 3.5 μm) at 25°C; 0.5% formic acid water (solvent A) and methanol (solvent B) were used as the mobile phase at a flow rate of 0.3 mL min^{-1}. The content was calculated according to the standard curve.

Statistical Analysis

Data on the content of metabolites detected were used for principal component analysis (PCA), hierarchical cluster analysis, and orthogonal partial least squares-discriminant

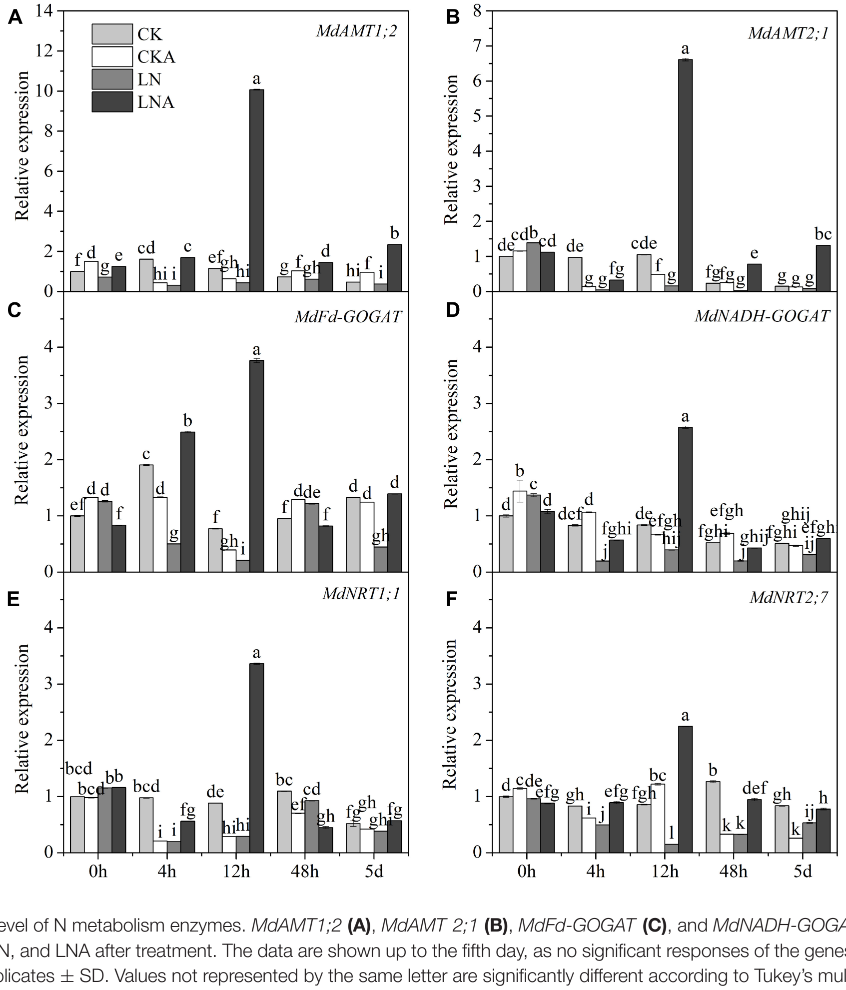

FIGURE 5 | The expression level of N metabolism enzymes. *MdAMT1;2* **(A)**, *MdAMT 2;1* **(B)**, *MdFd-GOGAT* **(C)**, and *MdNADH-GOGAT* **(D)**, *MdNRT1;1* **(E)** and *MdNRT2;7* **(F)** in CK, CKA, LN, and LNA after treatment. The data are shown up to the fifth day, as no significant responses of the genes were observed after 5 days. Values are means of three replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

analysis in R[1] to analyze the accumulation of metabolites in response to arginine and N deficiency (Wang et al., 2015). The Pearson correlation coefficients were calculated in R to verify the repeatability between classes. Significantly regulated metabolites were filtered using the following criteria: VIP $\geq$ 1 and Log2FC (fold change) $\geq$ 2 or Log2FC $\leq$ 0.5. To avoid overfitting, a permutation test (200 permutations) was performed. To clarify the role of arginine in response to N-deficiency stress, the identified metabolites were annotated using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. MSEA (metabolite sets enrichment analysis) was then conducted on the pathways with significantly regulated metabolites, and the significance of these pathways was determined by hypergeometric tests. Annotated metabolites were mapped to the KEGG Pathway database.

The data were statistically analyzed by one-way ANOVA. Tukey's multiple range test was used to determine significant differences between means ($P < 0.05$) using SPSS 25.0 software (IBM Corp., Armonk, NY, United States).

RESULTS

Hydroponics Screening

The growth rate of apple was significantly inhibited under N-deficiency conditions. The growth of apple treated with 2500 and 10000 μmol L^{-1} arginine was severely inhibited, as indicated by the lower plant length, wilted leaves, absence of root growth, and even death on the 3rd d with 10,000 μmol L^{-1} arginine and 6th d with 2,500 μmol L^{-1} arginine (**Figure 1A**) under N-deficient solutions. Apple treated with 100 and 500 μmol L^{-1} arginine had better growth performance, indicating that this concentration of arginine alleviated the inhibition induced by N-deficiency stress (**Figure 1**).

Under N deficiency, the dry weight and *Pn* decreased sharply. However, the dry weight was 59.26% higher in apple plants treated with 100 μmol L^{-1} arginine than under N deficiency (**Figure 1D**). Exogenous application of 100 or 500 μmol L^{-1} arginine resulted in higher *Pn* values during treatments compared with the control (**Figure 1E**). Based on these results, 100 μmol L^{-1} arginine was the optimal concentration used in subsequent experiments.

Exogenous Arginine Enhanced the Growth of *Malus hupehensis* Under Nitrogen Deficiency

The growth of *M. hupehensis* plants was inhibited significantly under N deficiency, and growth was restored via exogenous arginine application (**Figure 2A**). The dry weight and fresh weight of *M. hupehensis* plants were higher under exogenous arginine application compared with LN (**Figures 2B,C**); the growth of plants was also enhanced by arginine application under a normal nutrient supply. The SH and number of leaves were lower under N deficiency compared with when a normal level

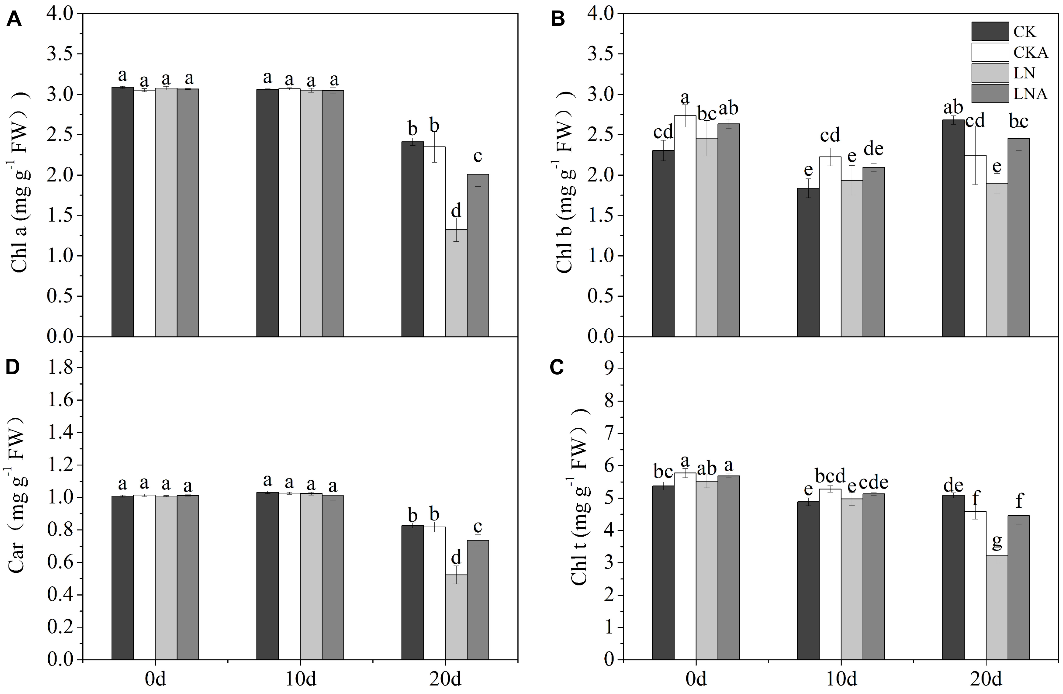

FIGURE 6 | Analysis of chlorophyll. Chlorophyll *a* **(A)**, chlorophyll *b* **(B)**, total chlorophyll **(C)**, and carotenoids **(D)**. Values are means of five replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

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[1] www.r-project.org/

of N was supplied (**Figures 2D,E**). There were no significant differences observed between CK and CKA.

Root vitality of *M. hupehensis* was significantly affected by N deficiency. Root vitality was higher for plants under LNA compared with plants under LN (**Figure 2G**).

The MDA content was higher in LN than in LNA, and no clear differences were observed among CK, CKA, and LNA (**Figure 2F**). This indicated that arginine alleviates oxidative damage to the cell membrane.

These results indicated that exogenous arginine enhanced aboveground growth and root activity under N deficiency and alleviated the damage to cells caused by N-deficiency.

Concentration, Transport, Accumulation, and Partitioning of Nitrogen, Potassium, and Phosphorus

Nutrient concentrations in different tissues were altered at 20 days after N-deficiency treatment. Arginine increased the total N concentration of the leaves, stems, and roots under stress (**Supplementary Figures 1A–C**). In the leaves and roots (**Supplementary Figures 1D,F**), the total K concentration was higher under LNA than under LN, but in the stems (**Supplementary Figure 1E**), the total K concentration was higher under LN compared with CK. The total P concentration in the stems and roots increased after the application of arginine under N stress (**Supplementary Figures 1H,I**). The leaf P content was lower under LNA than under LN (**Supplementary Figure 1G**).

The uptake, accumulation, and partitioning of N, P, and K were affected under N deficiency. The uptake of N, P, and K was decreased under N deficiency in *M. hupehensis*. The use of arginine significantly alleviated this inhibition (**Figures 3A–C**).

The content of N, P, and K was highest in leaves and lowest in stems. The application of arginine significantly increased the content of N, P, and K in the roots, stems, and leaves of *M. hupehensis*, especially the stems. Compared with LN, arginine also increased the N, P, and K content in tissues of *M. hupehensis* under low N stress (**Figures 3D–F**).

Under N deficiency, arginine increased the accumulation of N, P, and K in the roots, as well as the rate of transport of these nutrients in the stems compared with LN. The N and P transport rate to leaves was decreased, and the N and K transport rate was increased (**Figures 3G–I**). These results indicated that exogenous arginine treatment had a significant effect on nutrient transport, accumulation, and partitioning under N-deficiency stress.

Changes in Nitrogen Metabolism-Related Enzyme Activity and Gene Expression

Given the observed effects of arginine on the absorption and distribution of nutrients, we measured the activity of these four enzymes. The activity of these four enzymes was significantly inhibited under N-deficiency stress, and exogenous arginine alleviated this inhibition (**Figure 4**). The ammonium transporter genes *MdAMT1;2* and *MdAMT2;1* and the ammonium assimilation genes *MdFd-GOGAT* and *MdNADH-GOGAT* were significantly up-regulated at 12 h after low N stress with arginine application (**Figure 5**); the NO_3^- transporter genes *MdNRT1;1* and *MdNRT2;7* were up-regulated 3.3 and 2.3 times, respectively, at 12 h, and no significant response was observed after 12 h.

Changes in Photosynthetic Parameters and Chlorophyll

The chl *a*, chl *b*, car, and total chl content under N deficiency decreased significantly at 20 days after treatment; chl *b* was most affected by N deficiency (**Figure 6B**). The chl *a*, chl *b*, car, and total chl content was higher under LNA than under LN (**Figure 6**).

The *Pn*, *gs*, and *Tr* of plants decreased at 20 days after low N treatment (**Figures 7A–C**). However, *Pn* was higher under LNA than under LN. Under normal nutrient conditions, *Pn* was higher under CK than under CKA at 20 days (**Figure 7A**). Starting on the fifth day, the *Tr* of LNA was higher than that of LN, and *gs* was higher under LNA than under LN on the 10 days (**Figures 7B,C**). Lower *Ci* aided the ability of plants to cope with stress, and *Ci* was high under LN (**Figure 7D**), indicating that plants were under greater nutrient stress; *Ci* was low under CK, CKA, and LNA.

F_v/F_m measurements indicated that F_v/F_m was significantly lower under LN than under CK and LNA, but no significant differences between CKA and CK were observed (**Figure 7E**). The application of exogenous arginine alleviated damage to the photosynthetic system under low N stress but had no effect under normal N conditions.

Differential Metabolite Profiling Analysis

We analyzed the differential metabolites of *M. hupehensis* plants. PCA was used to conduct an unsupervised clustering analysis of the detected metabolites. The replications of the different treatments were clustered, and there was clear separation among the four treatments, indicating that the results were reliable. LN and CK were significantly separated along the first principal component (PC1), accounting for 23.29% of the total variance. LN and LNA were significantly separated along the second principal component (PC2), accounting for 18.45% of the total variance (**Figure 8B**). Differences between CKA and LNA along PC1 and PC2 were small (**Figure 8B**).

A total of 59, 58, 22, and 37 differential metabolites were identified in the four comparison groups (CK vs. CKA, CK vs. LN, CKA vs. LNA, and LN vs. LNA, respectively) (**Figure 8A**). In the CK vs. LN and LN vs. LNA comparisons, 18 and 23 metabolites were up-regulated, and 40 and 14 metabolites were down-regulated, respectively (**Supplementary Tables 2, 3**). A total of 43 down-regulated and 16 up-regulated metabolites were identified between CK and CKA, and 10 down-regulated and 12 up-regulated metabolites were identified between CKA and LNA (**Figure 8A**). Venn diagram analysis showed that only one differential metabolite was present in all four comparison groups (**Figure 8C**).

The differential metabolites detected under N deficiency were annotated to several pathways through KEGG analysis. Most of the metabolites were annotated to the metabolism

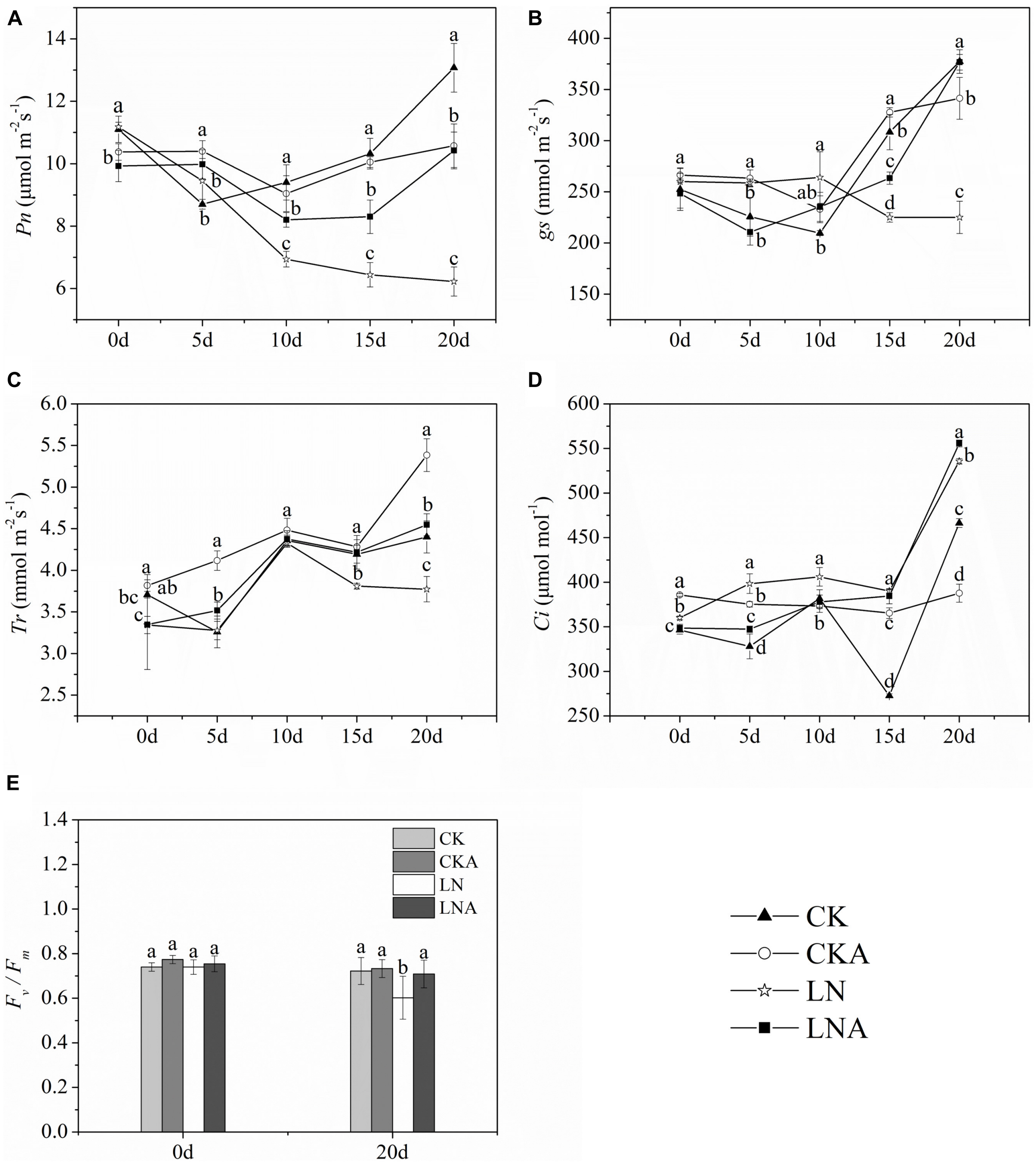

FIGURE 7 | Changes in the photosynthetic system of *M. hupehensis*. Net photosynthetic rate (*Pn*, **A**), stomatal conductance (*gs*, **B**), transpiration rate (*Tr*, **C**), intercellular CO_2 concentration (*Ci*, **D**), and F_v/F_m **(E)** of CK, CKA, LN, and LNA after 20 days. Values are the means of ten replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

and biosynthesis of specialized metabolites, which accounted for 82.14% and 60.71% of total metabolites. Approximately, 1/3 of the metabolites were related to amino acid synthesis (**Figure 9A**). N deficiency also affected lysine synthesis and degradation, as well as histidine, arginine, serine, threonine, glycine, alanine, proline, and ornithine synthesis (**Figure 9A**). Some metabolites were involved in carbon and sulfur metabolism, the TCA (Tricarboxylic Acid Cycle) cycle, and carbon fixation in photosynthetic organisms, which are important for plant growth. Nicotinate and nicotinamide, oxidative, phosphorylation, anthocyanin, and 2-oxocarboxylic acid metabolism and synthesis were also related to metabolites under N deficiency.

Thirty-seven differential metabolites were detected in the LN and LNA comparison: a total of 23 up-regulated metabolites, including 14 amino acids and derivatives, 2 nucleotides and derivatives, 3 flavonoids, 2 alkaloids, 1 phenolic acid, and 1 organic acid, and a total of 14 down-regulated metabolites, including 2 amino acids, 3 flavonoids, 1 phenolic acid, 2 lipids, 3 terpenoids, and 3 alkaloids (**Supplementary Tables 3, 4**). Most were annotated to the metabolism and biosynthesis of specialized metabolites, which accounted for 70.83% and 50% of total

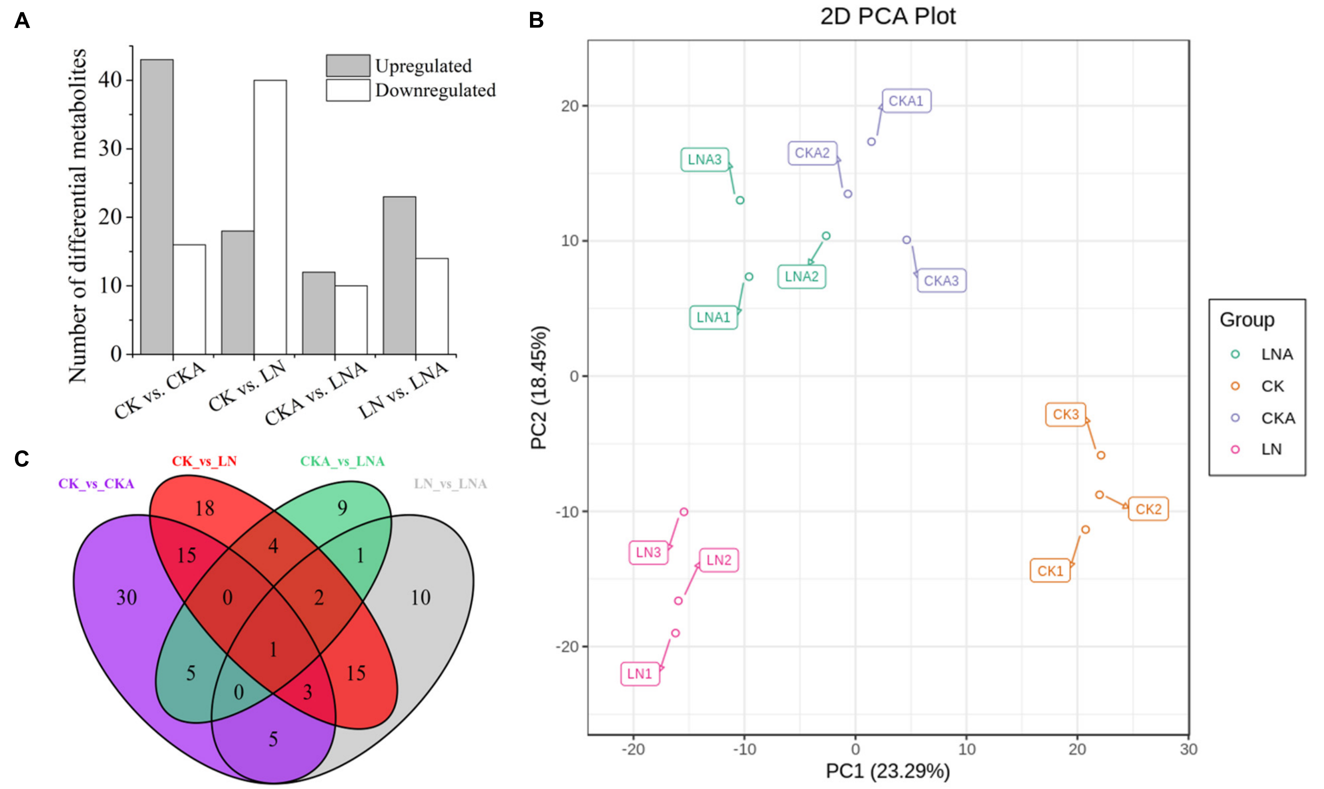

FIGURE 8 | Differential metabolite analysis. Number of differential metabolites for the comparisons CK vs. CKA, CK vs. LN, CKA vs. LNA, and LN vs. LNA **(A)**. PCA score diagram of each group. PC1 represents the first principal component, and PC2 represents the second principal component **(B)**. Venn diagram of the common metabolites and differential metabolites among the four groups **(C)**. Significantly regulated metabolites were those with fold change $\geq$ 2 or fold change $\leq$ 0.5 and VIP $\geq$ 1.

metabolites, respectively. Metabolites related to indole alkaloid, linoleic acid, and caffeine were detected in LNA (**Figure 9B**).

Next, we compared the differential metabolites between CK vs. LN and LN vs. LNA. A total of 17 metabolites detected in both showed opposite expression patterns, including 12 amino acids (L-serine, 5-aminovaleric acid, pipecolic acid, L-asparagine, L-ornithine, L-citrulline, L-cysteinyl-L-glycine, L-arginine, N-monomethyl-L-arginine, homoarginine, glutathione-reduced form, and L-homomethionine), 3 flavonoids (delphinidin-3-O-arabinoside, malvidin-3-O-arabinoside, and kaempferol-3-O-rhamnosyl (1→2) glucoside), 1 organic acid (3-ureidopropionic acid), 1 phenolic acid (4-O-methylgallic acid), 1 alkaloid (diethanolamine), 2 terpenoids (1-oxo-siaresinolic acid, myrianthic acid), and 1 lipid (LysoPC 20:4). These metabolites were up-regulated in LNA and down-regulated in LN, with the exception of terpenoids (**Supplementary Table 3**). Other metabolites were only related to synthesis under N deficiency (**Supplementary Table 2**), suggesting that exogenous arginine did not affect these pathways.

Exogenous Arginine Altered the Content of Endogenous Amino Acids

The content of arginine, glutamic acid, proline, serine, threonine, leucine, and valine was decreased (**Figures 10A–C,E–H**). However, the application of arginine restored the synthesis of these amino acids. The content of amino acids was higher in *M. hupehensis* growing under normal N conditions after arginine application. The content of histidine showed the opposite pattern: histidine content was high under LN but low when 100 μmol L^{-1} arginine was added at 20 days after N deficiency (**Figure 10D**). Under normal N conditions, the content of histidine was similar to that of other amino acids. The content of isoleucine significantly increased only in CKA (**Figure 10I**).

DISCUSSION

Nitrogen plays a prominent role in plant growth and development. Thus, the challenge of improving N use efficiency while reducing the use of N fertilizer has become a major focus of research. Arginine is known to be an important component of the N pool, and previous studies have shown that arginine is involved in the response to stress, including both biotic and abiotic stresses. The aim of this study was to investigate the role of arginine in mediating N-deficiency stress. Consistent with previous studies (Ji et al., 2018; Conceição et al., 2021), we found that the effect of arginine was dose-dependent. The photosynthetic rate of plants was higher under N deficiency

FIGURE 9 | KEGG classification of differential metabolites. CK vs. LN **(A)** and LN vs. LNA **(B)**. The ordinate shows the KEGG metabolic pathways, and the abscissa shows the number of metabolites annotated to these pathways and the proportion of the number of metabolites to the total number of metabolites annotated.

when 100 μmol L^{-1} arginine was applied; these plants also exhibited high SH, dry weight, and numbers of leaves. These results indicate that arginine can alleviate N-deficiency stress.

Nitrogen, potassium, and phosphorus are three essential elements for plant growth. N has been shown to compose macromolecules such as nucleic acids, nucleoprotein, chl, and protein; thus, more N is required compared with other elements during plant growth and development (Wen et al., 2020). The absorption of N and P is coupled and affects plant growth; N and K are important for ensuring plant yields (Clemente et al., 2013; Yan et al., 2015). In our study, the level of P and K was affected by arginine. Increased K and P content was observed

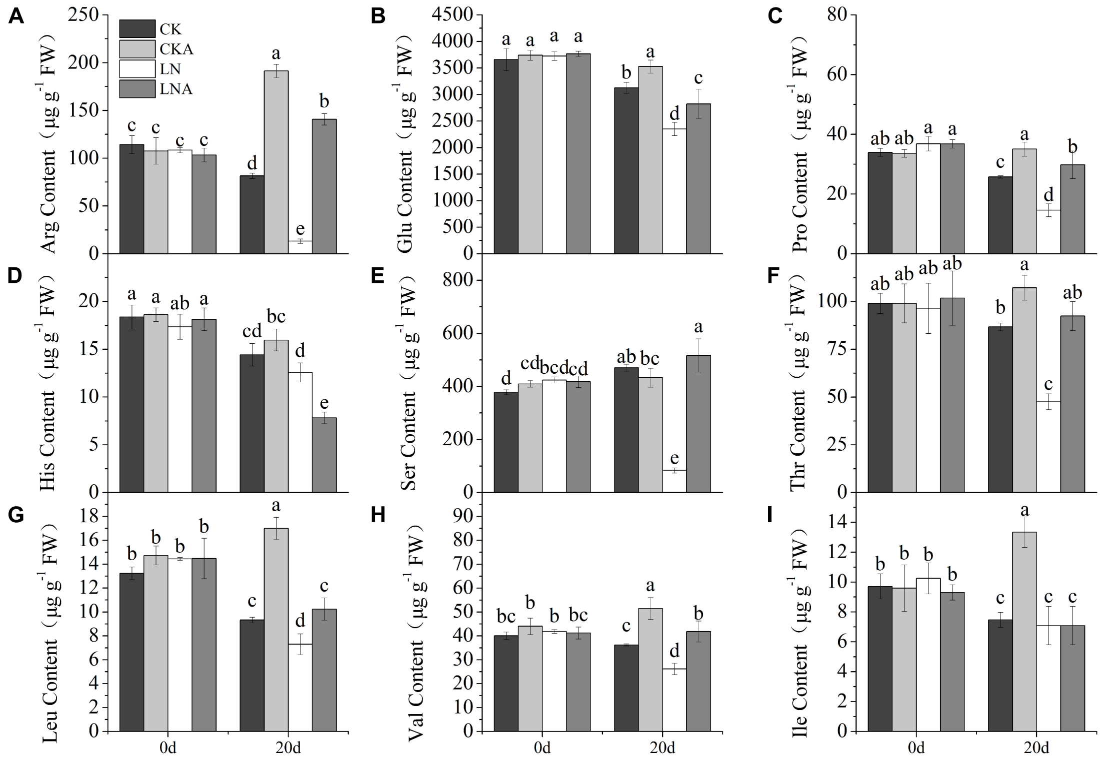

FIGURE 10 | The content of amino acids. Arginine **(A)**, glutamic acid **(B)**, proline **(C)**, histidine **(D)**, serine **(E)**, threonine **(F)**, leucine **(G)**, valine **(H)**, and L-isoleucine **(I)** in CK, CKA, LN, and LNA on the 20th day of treatment. Values are means of five replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

following arginine application. Under N-deficiency conditions, N absorption was inhibited, and N accumulated in the roots. However, the use of arginine under N deficiency enhanced the absorption and content of N, as well as the accumulation of N in the leaves and stems. Arginine is closely related to the storage and re-mobilization of N. Arginine dihydrolase AgrE/ArgZ of Anabaena, coupled with bifunctional proline oxidase PutA, mediates the conversion of arginine to glutamate, which provides a more direct route linking the arginine and ammonia pool (Zhang and Yang, 2019). After the genes controlling arginase synthesis were silenced in A*rabidopsis,* the nitric oxide synthase activity and increase in the NO content caused a decrease in N in plants (Flores et al., 2008). The total N concentration and content in the four treatments increased under arginine application. Arginine also facilitated nutrient transport and partitioning under N deficiency. NR and NiR are important enzymes in the process of nitrate reduction (Jung et al., 2021). GS and GOGAT are important enzymes involved in the process of ammonium assimilation (Esen and Öztürk Ürek, 2014). However, N transformation and key metabolic enzyme genes were up-regulated after arginine application under N deficiency at 12 h. Enzyme activity also increased at 20 days. There was a difference in the timing of the responses of genes and enzyme activity. Our results indicate that arginine can not only be transformed into N for plant absorption and use but can also promote the absorption and use of limited N, P, and K by plants.

Photosynthesis is sensitive to environmental changes; thus, the photosynthetic activity of plants can be altered by stress, including N deficiency. Most N is located in the chloroplasts of leaves and participates in the synthesis of photosynthetic-related substances. Approximately, 80% of the N in C3 plants is in the chloroplasts (Makino et al., 2003; Hiroyuki and Amane, 2018). In poplar, *Pn* is directly proportional to the N content (Luo and Zhou, 2019). In our experiment, the *Pn* of plants in low N environments was significantly decreased; *gs* and *Tr* also decreased under N deficiency. The application of exogenous arginine significantly alleviated the effect of N deficiency on plant photosynthesis and increased *Pn*. This effect may stem from the high N to carbon ratio in arginine. Arginine also plays an important role in amino acid metabolism. These amino acids and proteins make up the components of the plant's photosynthetic machinery (Ma et al., 2013; Huo et al., 2020). Therefore, under N deficiency, the application of exogenous arginine increases *Pn*, *gs*, and *Tr*, which increases the rapidity and efficiency of CO_2 assimilation and carbohydrate production under low N stress.

The content of chl *b* was significantly affected by arginine. Previous studies have shown that chl *b* functions in the light-harvesting complex (LHC) component of plants, plays an important role in regulating the size of the photosynthetic antenna, and maintains the stability of LHCII (Yamasato et al., 2005). This indicated that arginine helps reverse the negative effect of N deficiency by maintaining the content of chl *b*. The

F_v/F_m results also lead to the same conclusion. These results indicate that exogenous arginine can alleviate the degradation of chl and reduce the damage to PSII to increase photosynthetic efficiency caused by N deficiency, which enhances plant growth.

Arginine plays an important role in many biological processes. It can be broken down into intermediates that participate in the Krebs cycle (Vance et al., 2017). It also participates in the synthesis of amino acids, γ-aminobutyric acid (GABA), and NO (Majumdar et al., 2016). The content of most of the amino acids decreased under N deficiency, and N deficiency had a significant effect on amino acid synthesis. This effect was alleviated by supplementation of arginine, which increased the content of glutamate family amino acids such as the content of arginine, glutamate, proline, and ornithine. Proline is an essential amino acid that contributes to a variety of physiological and molecular responses in plants under stress (Saleem et al., 2020). Glutamate plays an important role in the synthesis of amino acids. It is a precursor in the synthesis of ornithine, as well as an intermediate in the synthesis of proline. Arginine is also a precursor in ornithine synthesis and a substrate of polyamine synthesis. The reactions of amino acids are also related to the TCA cycle, suggesting that arginine can affect the carbohydrate supply (Majumdar et al., 2015). These results were consistent with the metabolite profiling analysis. The fact that the content of amino acids changed significantly after arginine supplementation indicated that arginine is a key biochemical hub in amino acid metabolism.

Arginine affected specialized metabolites, and this might explain why the tolerance of plants to N deficiency increased. Metabolomic fingerprinting and KEGG analysis identified several significantly regulated metabolites, which were mainly divided into four groups: flavonoids, alkaloids, phenols, and amino acid derivatives. Phenols, alkaloids, and terpenoids are the main chemical substances involved in plant defense (Nguyen et al., 2020). Anthocyanins are flavonoids, which are water-soluble plant pigments with strong antioxidant activity (Meng et al., 2019). Gil et al. (2000) found that the remaining phenolic compounds only accounted for 28% of the total antioxidant activity after removing the anthocyanin fraction from pomegranate juice, indicating that anthocyanins can contribute important antioxidant effects. Phenols have been shown to be antioxidants that can remove excess reactive oxygen in cells; they also provide various benefits to human health (Shay et al., 2015; Zhang et al., 2019). In this study, the content of delphinidin-3-O-arabinoside, malvidin-3-O-arabinoside, and kaempferol-3-O-rhamnosyl (1→2) glucoside was up-regulated under LNA but down-regulated under LN. The alkaloids diethanolamine and caffeine were significantly up-regulated. The latter is known to play a role in the tolerance of viruses, bacteria, and pest insects; it is thus often used as an anti-herbivory and allelopathic agent (Kim et al., 2006, 2010). Phenols including 4-O-methylgallate were up-regulated more than 5,000 times under LNA. They are the major product of gallic acid metabolism *in vivo*. Gallic acid has antioxidant activity. A sharp increase in gallic acid was detected after arginine supplementation under N deficiency, suggesting that phenolic acid plays an important role in mediating the effects of arginine under N deficiency. The jasmonic acid content significantly increased under N deficiency and was not induced by arginine. This indicated that the absorption of exogenous arginine by *M. hupehensis* can help plants produce more antioxidant substances under N deficiency to reduce the damage caused by reactive oxygen species.

In sum, there are two possible mechanisms by which arginine alleviated the effects of N deficiency. First, arginine can promote the absorption and use of N, P, and K to compensate for the lack of N; it also can be converted into N at the same time, thereby inhibiting chl degradation to improve the efficiency of the photosynthetic system and plant growth. Second, arginine is a biochemical hub in amino acid synthesis and metabolism. Consequently, the application of exogenous arginine altered the content of a large number of amino acids in apple plants. Many intermediates participate in the urea cycle during the conversion and synthesis of these amino acids. Application of arginine affects the content of antioxidant metabolites, such as phenols, alkaloids, terpenoids, and flavonoids, which play a key role in reducing the peroxidation damage experienced by apple plants under N deficiency. The results of this study provided new insights into the mechanisms by which arginine alleviates the effects of N-deficiency stress. Additional studies are needed to further our understanding of these mechanisms.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

FM, CL, and QC conceived and designed the experiments. QC performed the experiments, analyzed the data, and wrote the manuscript. YW, ZZ, and XL prepared the materials and obtained the experimental data. All authors read and approved the manuscript.

FUNDING

This work was supported by the China's Agricultural Research System of MOF and MARA (CARS-27).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.772086/full#supplementary-material

Supplementary Figure 1 | Concentration of total nitrogen, phosphorus, and potassium. Nitrogen in the leaf **(A)**, stem **(B)**, and root **(C)**; total potassium in the leaf **(D)**, stem **(E)**, root **(F)**, total phosphorus in the leaf **(G)**, stem **(H)**, and root **(I)** after 20 days of treatment of the four groups. Values are means of three replicates ± SD. Values not represented by the same letter are significantly different according to Tukey's multiple-range test ($P < 0.05$).

REFERENCES

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts: polyphenoloxidase in Beta vulgaris. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1

Asif, I., Dong, Q., Madeeha, A., Wang, X. R., Gui, H. P., Zhang, H. H., et al. (2019). Untangling the molecular mechanisms and functions of nitrate to improve nitrogen use efficiency. *J. Sci. Food Agric.* 100, 904–914. doi: 10.1002/jsfa.10085

Bai, T. H., Li, C. Y., Ma, F. W., Feng, F. J., and Shu, H. R. (2010). Responses of growth and antioxidant system to root-zone hypoxia stress in two *Malus* species. *Plant Soil* 327, 95–105. doi: 10.1007/s11104-009-0034-x

Brauc, S., Vooght, E. D., Claeys, M., Geuns, J., Höfte, M., and Angenon, G. (2011). Overexpression of arginase in *arabidopsis thaliana* influences defence responses against botrytis cinerea. *Plant Biol.* 14, 39–45. doi: 10.1111/j.1438-8677.2011.00520.x

Cao, F. Q., Werner, A. K., Dahncke, K., Romeis, T., Liu, L. H. and Witte, C. P. (2010). Identification and characterization of proteins involved in rice urea and arginine catabolism. *Plant Physiol.* 154, 98–108. doi: 10.1016/j.aninu.2016.04.005

Chávez-Martínez, A. I., Ortega-Amaro, M. A., Torres, M., Serrano, M., and Jiménez-Bremont, J. F. (2020). *Arabidopsis* adc-silenced line exhibits differential defense responses to Botrytis cinerea and *Pseudomonas* syringae infection. *Plant Physiol. Biochem*. 156, 494–503. doi: 10.1016/j.plaphy.2020.09.035

Chen, C. T., Lee, C. L., and Yeh, D. (2018). Effects of Nitrogen, Phosphorus, Potassium, Calcium, or Magnesium Deficiency on Growth and Photosynthesis of Eustoma. *HortScience* 53, 795–798. doi: 10.21273/HORTSCI12947-18

Chen, W., Gong, L., Guo, Z. L., Wang, W. S., Zhang, H. Y., Liu, X. Q., et al. (2013). A Novel Integrated Method for Large-Scale Detection, Identification, and Quantification of Widely Targeted Metabolites: application in the Study of Rice Metabolomics. *Mol. Plant* 6, 1769–1780. doi: 10.1093/mp/sst080

Clemente, J. M., Martinez, H. E. P., Alves, L. C., and Lara, M. C. (2013). Effect of N and K doses in nutritive solution on growth, production and coffee bean size. *Soil Sci. Plant Nutr.* 60, 279–285. doi: 10.1590/s0034-737x2013000200018

Conceição, V. J., Mello, S. C., Carvalho, M. E. A., Gaziola, S., and Azevedo, R. A. (2021). Exogenous arginine modulates leaf antioxidant enzymes and hydrogen peroxide content in tomato plants under transient heat stresses. *Bragantia* 80:e2621. doi: 10.1590/1678-4499.20200493

Dinkeloo, K., Boyd, S., and Pilot, G. (2018). Update on amino acid transporter functions and on possible amino acid sensing mechanisms in plants. *Semin. Cell Dev. Biol*. 74, 105–113. doi: 10.1016/j.semcdb.2017.07.010

Esen, M., and Öztürk Ürek, R. (2014). Nitrate and iron nutrition effects on some nitrate assimilation enzymes and metabolites in Spirulina platensis. *Turk. J. Biol.* 38, 690–700. doi: 10.3906/biy-1405-37

Fan, X., Naz, M., Fan, X., Xuan, W., Miller, A. J., and Xu, G. (2017). Plant nitrate transporters: from gene function to application. *J. Exp. Bot*. 68, 2463–2475. doi: 10.1093/jxb/erx011

Flores, T., Todd, C. D., Tovar-Mendez, A., Dhanoa, P. K., Correa-Aragunde, N., Hoyos, M. E., et al. (2008). Arginase-negative mutants of *Arabidopsis* exhibit increased nitric oxide signaling in root development. *Plant Physiol.* 147, 1936–1946. doi: 10.1104/pp.108.121459

Gao, H. J., Yang, H. Q., and Wang, J. X. (2009). Arginine metabolism in roots and leaves of apple (*Malus* domestica Borkh.): the tissue-specific formation of both nitric oxide and polyamines. *Sci. Hortic.* 119, 147–152. doi: 10.1016/j.scienta.2008.07.034

Gao, T. T., Zhang, Z. J., Liu, X. M., Wu, Q., Chen, Q., Liu, Q. W., et al. (2020). Physiological and transcriptome analyses of the effects of exogenous dopamine on drought tolerance in apple. *Plant Physiol. Biochem*. 148, 260–272. doi: 10.1016/j.plaphy.2020.01.022

Gil, M. I., Tomas-Barberan, F. A., Hess-Pierce, B., Holcroft, D. M., and Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.* 48, 4581–4589. doi: 10.1021/jf000404a

Guo, J. H., Liu, X. J., Zhang, Y., Shen, J. L., Han, W. X., Zhang, W. F., et al. (2010). Significant acidification in major Chinese croplands. *Science* 327, 1008–1010. doi: 10.1126/science.1182570

Hiroyuki, I., and Amane, M. (2018). Impacts of autophagy on nitrogen use efficiency in plants. *Soil Sci. Plant Nutr.* 64, 100–105. doi: 10.1080/00380768.2017.1412239

Hsieh, P. H., Kan, C. C., Wu, H. Y., Yang, H. C., and Hsieh, M. H. (2018). Early molecular events associated with nitrogen deficiency in rice seedling roots. *Sci. Rep*. 8:12207. doi: 10.1038/s41598-018-30632-1

Huo, L. Q., Guo, Z. J., Wang, P., Zhang, Z. J., Jia, X., Sun, Y. M., et al. (2020). *MdATG8i* functions positively in apple salt tolerance by maintaining photosynthetic ability and increasing the accumulation of arginine and polyamines. *Environ. Exp. Bot*. 172:103989. doi: 10.1016/j.envexpbot.2020.103989

Ji, N., Min, D., Li, F., Shao, S., and Zhang, X. (2018). Role of nitric oxide synthase pathway in arginine-induced disease resistance in postharvest tomato fruit. *Food Sci*. 39, 250–257.

Jin, X., Liu, T., Xu, J., Gao, Z., and Hu, X. (2019). Exogenous GABA enhances muskmelon tolerance to salinity-alkalinity stress by regulating redox balance and chlorophyll biosynthesis. *BMC Plant Biol*. 19:48. doi: 10.1186/s12870-019-1660-y

Joseph, A. B., David, J. B., and Peter, J. F. (2010). Stomata: key players in the earth system, past and present. *Curr. Opin. Plant Biol.* 13, 232–239. doi: 10.1016/j.pbi.2010.04.013

Jung, Y. J., Park, J. S., Go, J. Y., Lee, H. J., Kim, J. Y., Lee, Y. J., et al. (2021). Research status of transcription factors involved in controlling gene expression by nitrate signaling in higher plants. *J. Plant Biotechnol*. 48, 124–130. doi: 10.5010/JPB.2021.48.3.124

Karpets, Y. V., Kolupaev, Y. E., Lugovaya, A. A., Shvidenko, N. V., and Yastreb, T. O. (2018). Effects of Nitrate and L-Arginine on Content of Nitric Oxide and Activities of Antioxidant Enzymes in Roots of Wheat Seedlings and Their Heat Resistance. *Russ. J. Plant Physiol*. 65, 908–915. doi: 10.1134/S1021443718050096

Kim, Y. S., Choi, Y. E., and Sano, H. (2010). Plant vaccination: stimulation of defense system by caffeine production in planta. *Plant Signal. Behav*. 5, 489–493. doi: 10.4161/psb.11087

Kim, Y. S., Uefuji, H., Ogita, S., and Sano, H. (2006). Transgenic tobacco plants producing caffeine: a potential new strategy for insect pest control. *Transgenic Res*. 15, 667–672. doi: 10.1007/s11248-006-9006-6

Kruse, J., Hetzger, I., Hänsch, R., Mendel, R. R., Walch-Liu, P., Engels, C., et al. (2002). Elevated $p\mathrm{CO}_2$ favours nitrate reduction in roots of wild type tobacco (*Nicotiana tabacum* cv. Gat.) and significantly alters N-metabolism in transformants lacking functional nitrate reductase in the roots. *J. Exp. Bot.* 379, 2351–2367. doi: 10.1093/jxb/erf094

Kruse, J., Kopriva, S., Hansch, R., Krauss, G. J., Mendel, R. R., and Rennenberg, H. (2007). Interaction of sulfur and nitrogen nutrition in tobacco (*Nicotiana tabacum*) plants: significance of nitrogen source and root nitrate reductase. *Plant Biol.* 9, 638–646. doi: 10.1055/s-2007-965434

Li, C., Wei, Z., Liang, D., Zhou, S., Li, Y., Liu, C., et al. (2013). Enhanced salt resistance in apple plants overexpressing a *Malus* vacuolar $\mathrm{Na}^+/\mathrm{H}^+$ antiporter gene is associated with differences in stomatal behavior and photosynthesis. *Plant Physiol. Biochem.* 70, 164–173. doi: 10.1016/j.plaphy.2013.05.005

Liu, X. M., Gao, T. T., Zhang, Z. J., Tan, K. X., Jin, Y. B., Zhao, Y. J., et al. (2020). The mitigation effects of exogenous dopamine on low nitrogen stress in *Malus hupehensis*. *J. Integr. Agric*. 19, 2709–2724. doi: 10.1016/s2095-3119(20)63344-5

Liu, Y., Wang, H., Jiang, Z., Wang, W., Xu, R., Wang, Q., et al. (2021). Genomic basis of geographical adaptation to soil nitrogen in rice. *Nature* 590, 600–605. doi: 10.1038/s41586-020-03091-w

Locke, M., Ghazaly, E., Freitas, M. O., Mitsinga, M., Lattanzio, L., Lo Nigro, C., et al. (2016). Inhibition of the polyamine synthesis pathway is synthetically lethal with loss of argininosuccinate synthase 1. *Cell Rep.* 16, 1604–1613. doi: 10.1016/j.celrep.2016.06.097

Lu, C., and Zhang, J. (2000). Photosynthetic CO_2 assimilation, chlorophyll fluorescence and photoinhibition as affected by nitrogen deficiency in maize plants. *Plant Sci*. 151, 135–143. doi: 10.1016/S0168-9452(99)00207-1

Luisa, B. G., Carolina, S., Katherine, P., Leonardo, C., María, R., Vilbett, B., et al. (2018). Nitrogen physiology of contrasting genotypes of Chenopodium quinoa Willd. (*Amaranthaceae*). *Sci. Rep*. 8:17524. doi: 10.1038/s41598-018-34656-5

Luo, J., and Zhou, J. (2019). Growth performance, photosynthesis, and root characteristics are associated with nitrogen use efficiency in six poplar species. *Environ. Exp. Bot*. 164, 40–51. doi: 10.1016/j.envexpbot.2019.04.013

Lv, J. H., Pang, Q. P., Chen, X. Q., Li, T., Fang, J. G., Lin, S. Y., et al. (2020). Transcriptome analysis of strawberry fruit in response to exogenous arginine. *Planta* 252:82. doi: 10.1007/s00425-020-03489-w

Ma, X. F., Cheng, Z. J., Qin, R. Z., Qiu, Y., Heng, Y. Q., Yang, H., et al. (2013). *OsARG* encodes an arginase that plays critical roles in panicle development and grain production in rice. *Plant J.* 73, 190–200. doi: 10.1111/j.1365-313x.2012.05122.x

Majumdar, R., Barchi, B., Turlapati, S. A., Gagne, M., Minocha, R., Long, S., et al. (2016). Glutamate, Ornithine, Arginine, Proline, and Polyamine metabolic interactions: the pathway is regulated at the post-transcriptional level. *Front. Plant Sci.* 7:78. doi: 10.3389/fpls.2016.00078

Majumdar, R., Minocha, R., and Minocha, S. (2015). "Ornithine: at the crossroads of multiple paths to amino acids and polyamines," in *Amino Acids In Higher Plants*, ed. J. P. F. D'Mello. (Boston, MA: CABI Publishers), 156–176. doi: 10.1079/9781780642635.0156

Makino, A., Sakuma, H., Sudo, E., and Mae, T. (2003). Differences between maize and rice in N-use efficiency for photosynthesis and protein allocation. *Plant Cell Physiol.* 44, 952–956. doi: 10.1093/pcp/pcg113

Meng, L. S., Zhu, J. Y., Ma, Y., Sun, X. Y., Li, D. N., Li, L., et al. (2019). Composition and antioxidant activity of anthocyanins from Aronia melanocarpa cultivated in Haicheng, Liaoning, China. *Food Biosci.* 30:100413. doi: 10.1016/j.fbio.2019.100413

Nguyen, M. T., Nguyen, V. T., Le, V. M., Trieu, L. H., Lam, T. D., Bui, L. M., et al. (2020). Assessment of preliminary phytochemical screening, polyphenol content, flavonoid content, and antioxidant activity of custard apple leaves (*Annona squamosa* Linn.). *IOP Conf. Ser. Mater. Sci. Eng.* 736:062012. doi: 10.1088/1757-899x/736/6/062012

Patel, J., Ariyaratne, M., Ahmed, S., Ge, L., Phuntumart, V., Kalinoski, A., et al. (2017). Dual functioning of plant arginases provides a third route for putrescine synthesis. *Plant Sci.* 262, 62–73. doi: 10.1016/j.plantsci.2017.05.011

Pratelli, R., and Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. *J. Exp. Bot.* 65, 5535–5556. doi: 10.1093/jxb/eru320

Saleem, M. H., Fahad, S., Khan, S. U., Din, M., Ullah, A., El Sabagh, A., et al. (2020). Copper-induced oxidative stress, initiation of antioxidants and phytoremediation potential of flax (*Linum usitatissimum* L.) seedlings grown under the mixing of two different soils of China. *Environ. Sci. Pollut. Res.* 27, 5211–5221. doi: 10.1007/s11356-019-07264-7

Shay, J., Elbaz, H. A., Lee, I., Zielske, S. P., Malek, M. H., and Huttemann, M. (2015). Molecular mechanisms and therapeutic effects of (-)-Epicatechin and other polyphenols in cancer, inflammation, diabetes, and neurodegeneration. *Oxid. Med. Cell. Longev.* 2015:181260. doi: 10.1155/2015/181260

Shi, H., Ye, T., Chen, F., Cheng, Z., Wang, Y., Yang, P., et al. (2013). Manipulation of arginase expression modulates abiotic stress tolerance in *Arabidopsis*: effect on arginine metabolism and ROS accumulation. *J. Exp. Bot.* 64, 1367–1379. doi: 10.1093/jxb/ers400

Sun, J., Ye, M., Peng, S., and Li, Y. (2016). Nitrogen can improve the rapid response of photosynthesis to changing irradiance in rice (*Oryza sativa* L.) plants. *Sci. Rep.* 6:31305. doi: 10.1038/srep31305

Svennerstam, H., Jämtgård, S., Ahmad, I., Danell, H. K., Näsholm, T., and Ganeteg, U. (2011). Transporters in *Arabidopsis* roots mediating uptake of amino acids at naturally occurring concentrations. *New Phytol.* 191, 459–467. doi: 10.1111/j.1469-8137.2011.03699.x

Teresita, F., Christopher, D. T., Alejandro, T. M., Preetinder, K. D., Natalia, C. A., Mary Elizabeth, H., et al. (2008). Arginase-negative mutants of *Arabidopsis* exhibit increased nitric oxide signaling in root development. *Plant Physiol.* 147, 1936–1946.

Torgny, N., Knut, K., and Ulrika, C. (2009). Uptake of organic nitrogen by plants. *New Phytol.* 182, 31–48. doi: 10.1111/j.1469-8137.2008.02751.x

Urbano-Gámez, J. A., El-Azaz, J., Ávila, C., de la Torre, N. F., and Cánovas, F. M. (2020). Enzymes Involved in the Biosynthesis of Arginine from Ornithine in Maritime Pine (*Pinus pinaster* Ait.). *Plants* 9:1271. doi: 10.3390/plants9101271

Vance, L. A., Melissa, K. S., and Adrian, B. (2017). "Cellular and physiological effects of arginine in seniors," in *Nutrition and Functional Foods for Healthy Aging*, ed. R. R. Watson (Cambridge, Massachusetts: Academic Press), 317–336. doi: 10.2174/1389557043403305

Wang, H., Sun, Y., Feng, X., Zhang, Y., Yang, J., Ma, M., et al. (2016). Cloning and expression analysis of argininosuccinate synthetase gene *GhASS1* from gossypium hirsutum. *Sci. Agric. Sin.* 49, 1242–1253. doi: 10.3864/j.issn.0578-1752.2016.07.003

Wang, Q., Liu, C. H., Huang, D., Dong, Q. L., Li, P. M., and Ma, F. W. (2019). High-efficient utilization and uptake of N contribute to higher NUE of 'Qinguan' apple under drought and N-deficient conditions compared with 'Honeycrisp'. *Tree Physiol.* 39, 1880–1895. doi: 10.1093/treephys/tpz093

Wang, S., Tu, H., Wan, J., Chen, W., Liu, X., Luo, J., et al. (2015). Spatio-temporal distribution and natural variation of metabolites in citrus fruit. *Food Chem.* 199, 8–17. doi: 10.1016/j.foodchem.2015.11.113

Wang, T. Q., Liu, Q. Q., Wang, N. W., Dai, J., Lu, Q. F., Jia, X., et al. (2021). Foliar arginine application improves tomato plant growth, yield, and fruit quality via nitrogen accumulation. *Plant Growth Regul.* 95, 421–428. doi: 10.1007/s10725-021-00752-2

Wen, B. B., Li, C., Fu, X. L., Li, D. M., Li, L., and Chen, X. D. (2019). Effects of nitrate deficiency on nitrate assimilation and chlorophyll synthesis of detached apple leaves. *Plant Physiol. Biochem.* 142, 363–371. doi: 10.1016/j.plaphy.2019.07.007

Wen, B. B., Xiao, W., Mu, Q., Li, D. M., Chen, X. D., Wu, H. Y., et al. (2020). How does nitrate regulate plant senescence? *Plant Physiol. Biochem.* 157, 60–69. doi: 10.1016/j.plaphy.2020.08.041

Winter, G., Todd, C. D., Trovato, M., Forlani, G., and Funck, D. (2015). Physiological implications of arginine metabolism in plants. *Front. Plant Sci.* 6:534. doi: 10.3389/fpls.2015.00534

Xu, G. H., Fan, X. R., and Miller, A. J. (2012). Plant Nitrogen Assimilation and Use Efficiency. *Annu. Rev. Plant Biol.* 63, 153–182. doi: 10.1146/annurev-arplant-042811-105532

Yamasato, A., Nagata, N., Tanaka, R., and Tanaka, A. (2005). The N-terminal domain of chlorophyllide a oxygenase confers protein instability in response to chlorophyll B accumulation in *Arabidopsis*. *Plant Cell* 17, 1585–1597. doi: 10.1105/tpc.105.031518

Yan, Z. B., Kim, N. Y., Han, W. X., Guo, Y. L., Han, T. S., Du, N. Z., et al. (2015). Effects of nitrogen and phosphorus supply on growth rate, leaf stoichiometry, and nutrient resorption of *Arabidopsis thaliana*. *Plant Soil* 388, 147–155. doi: 10.1007/s11104-014-2316-1

Yang, G., Wei, Q., Huang, H., and Xia, J. (2020). Amino Acid Transporters in Plant Cells: a Brief Review. *Plants* 9:967. doi: 10.3390/plants9080967

Zhang, C., Yao, X., Ren, H., Chang, J., and Wang, K. (2019). RNA-Seq reveals flavonoid biosynthesis-related genes in Pecan (*Carya illinoinensis*) Kernels. *J. Agric. Food Chem.* 67, 148–158. doi: 10.1021/acs.jafc.8b05239

Zhang, H., and Yang, C. (2019). Arginine and nitrogen mobilization in cyanobacteria. *Mol. Microbiol.* 111, 863–867. doi: 10.1111/mmi.14204

Zhang, X., Davidson, E. A., Mauzerall, D. L., Searchinger, T. D., Dumas, P., and Shen, Y. (2015). Managing nitrogen for sustainable development. *Nature* 528, 51–59. doi: 10.1038/nature15743

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 25 February 2022
doi: 10.3389/fpls.2022.777771

Modulation in Biofertilization and Biofortification of Wheat Crop by Inoculation of Zinc-Solubilizing Rhizobacteria

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Specialty section:
This article was submitted to Plant Nutrition,
a section of the journal
Frontiers in Plant Science

Received: 15 September 2021
Accepted: 10 January 2022
Published: 25 February 2022

Citation:
Yadav RC, Sharma SK, Varma A, Rajawat MVS, Khan MS, Sharma PK, Malviya D, Singh UB, Rai JP and Saxena AK (2022) Modulation in Biofertilization and Biofortification of Wheat Crop by Inoculation of Zinc-Solubilizing Rhizobacteria. Front. Plant Sci. 13:777771.
doi: 10.3389/fpls.2022.777771

Zinc is an important micronutrient needed for the optimum growth and development of plants. Contrary to chemical zinc fertilizers, the use of zinc-solubilizing bacteria is an environmentally friendly option for zinc enrichment in edible parts of crops. This study was conducted with the objective of selecting potential zinc-solubilizing rhizobacteria from the rhizosphere of chickpea grown in soils of eastern Uttar Pradesh and further assessing their impact on the magnitude of zinc assimilation in wheat crops. Among 15 isolates, CRS-9, CRS-17, CRS-30, and CRS-38 produced net soluble zinc in broth to the tune of 6.1, 5.9, 5.63, and 5.6 μg ml^{-1}, respectively, in zinc phosphate with the corresponding pH of 4.48, 5.31, 5.2, and 4.76. However, the bacterial strains CRS-17, CRS-30, CRS-38, and CRS-9 showed maximum zinc phosphate solubilization efficiency of 427.79, 317.39, 253.57, and 237.04%, respectively. The four bacterial isolates were identified as *Bacillus glycinifermentans* CRS-9, *Microbacterium oxydans* CRS-17, *Paenarthrobacter nicotinovorans* CRS-30, and *Bacillus tequilensis* CRS-38 on the basis of morphological and biochemical studies and 16S rRNA gene sequencing. Bacterial inoculants significantly colonized the roots of wheat plants and formed a biofilm in the root matrix. These strains significantly increased seed germination (%) and vigor indices in wheat grown under glasshouse conditions. After 30 days of sowing of wheat under microcosm conditions, eight zinc transporter (*TaZIP*) genes were expressed maximally in roots, with concomitant accumulation of higher zinc content in the bacterially treated plant compared to the absolute control. Out of the four strains tested, two bacteria, *B. tequilensis* CRS-38 and *P. nicotinovorans* CRS-30, improved seed germination (%), vigor indices (2–2.5 folds), plant biomass, grain yield (2.39 g plant^{-1}), and biofortificated grains (54.25 μg g^{-1}Zn) of wheat. To the best of our knowledge, this may be the first report on the presence of zinc solubilization trait in *B. glycinifermentans* CRS-9, *M. oxydans* CRS-17, and *P. nicotinovorans* CRS-30.

Keywords: rhizobacteria, wheat, zinc phosphate, biofortification, zinc-solubilizing rhizobacteria

INTRODUCTION

The most important challenge to researchers, scientists, and policymakers is to ensure food and nutritional security for the burgeoning population, which may reach 9.8 billion by the year 2050. Food security entails increasing agricultural productivity and improvement in product quality while reducing the adverse footprints of cultivation practices in natural resources and the environment. Among the 17 sustainable development goals (SDGs), SDGs 1–3 are directly related to sustainable agricultural growth and aim to eliminate poverty and hunger, and ensure good health and well-being, respectively. More than half of the global population suffers from micronutrient malnutrition in developed and developing countries (Bouis, 2003). Among various micronutrients, zinc (Zn) is required for the optimal growth and development of all the organisms (Hafeez et al., 2013). In plants, Zn plays an important role in carbohydrate and auxin metabolism (Alloway, 2004, 2008a,b), protein synthesis, and assimilation of other major nutrients, and it also acts as a significant antioxidant (Singh et al., 2018c). Currently, Zn deficiency is common in plants, human beings, and animals. Zn deficiency in plants causes a reduction in photosynthesis, flowering and fruit development, synthesis of carbohydrate and phytohormones, shoot and root development, and leaf size; it induces chlorosis and susceptibility to heat, light, and fungal infections; it affects water uptake and delays crop maturity, leading to decrease in crop yield and nutritional quality of grains (Alloway, 2004; Tavallali et al., 2010). Zinc deficiency results in yellowing of leaves and stunted growth in wheat plants. Zinc deficiency in humans is due to consumption of zinc-deficient food including wheat grown in Zn-deficient soils. Globally, the widespread occurrence of Zn-deficiency in crops is due to the low solubility of Zn rather than low Zn availability in soil (Iqbal et al., 2010). More than 50% of Indian soils have been estimated to be deficient in Zn (Ramesh et al., 2014a). The total content of zinc in soil ranges from 50–300 $\mu g\ g^{-1}$ soil. Cereal crops grown on these soils are zinc-deficient. Urgent corrective measures are required to eliminate Zn deficiency in edible crops to ensure proper Zn nutrition.

In general, plants have several mechanisms to overcome Zn deficiency. Among them, Zn finger transcription factors are important for the development of floral tissues, flowering, fertilization, and fruiting (Epstein and Bloom, 2005). Zn also plays a vital role in the regulation of uptake and translocation of Zn in plants and modulates several biological pathways during environmental stress. Furthermore, genetic biofortification is time-consuming, and it may not be successful in some different soil environments (Cakmak, 2008). The agronomic strategy of biofortification using zinc as a fertilizer, crop rotation (White and Broadley, 2009; Dimpka and Bindraban, 2016), and application of organic manures (Aghili et al., 2014; Helfenstein et al., 2016) and microbial inoculants (Saravanan et al., 2007a,b; Ramesh et al., 2014b; Wang et al., 2014; Gandhi and Muralidharan, 2016) are gaining momentum. In this context, using plant growth-promoting rhizobacteria (PGPR) with zinc-solubilizing ability as rhizobacterial inoculants is a cost-effective and eco-friendly alternative for zinc biofertilization. Zinc solubilizing bacteria alone or with organic materials may enhance the bioavailability of native and applied zinc in plants through different mechanisms.

Several plant growth-promoting microorganisms (PGPMs) including rhizobacteria have been reported to be effective zinc solubilizers (Fasim et al., 2002; Saravanan et al., 2007a; Abaid Ullah et al., 2015; Singh et al., 2018a,b). These bacteria colonize the rhizosphere and intensify zinc bioavailability by solubilizing complex zinc compounds, thereby improving plant growth and development. Bacteria are endowed with various zinc-solubilizing mechanisms, and acidification is one of the key processes (Ramesh et al., 2014a; Hussain et al., 2015; Naz et al., 2016; Yadav et al., 2020). In soil, bacteria produce organic acids, which sequester zinc cations, resulting in a reduction in pH of nearby soil. Moreover, anions are able to chelate zinc and increase the solubility of zinc (Jones and Darrah, 1994; Yadav et al., 2020). Solubilization probably also takes place through other mechanisms, viz. production of siderophores and proton, oxido-reductive systems in cell membranes, and chelated ligands (Wakatsuki, 1995; Chang et al., 2005; Saravanan et al., 2011). Moreover, several bacilli have been reported to cause modulation of growth- and yield-contributing factors, along with zinc biofortification of wheat and soybean crops (Sharma et al., 2012; Khande et al., 2017; Yadav et al., 2020). Globally, wheat (*Triticum aestivum* L.) is an important crop, being a source of food, feed, fiber, and fuel (Singh et al., 2019a,b). As against cereals like rye and barley, the importance of bread wheat in human nutrition has jumped up because of changes in its use over the last two millennia, and this has led to an exponential increase in wheat production worldwide including in India. In general, wheat contains 75–80% carbohydrates, 9–18% protein, fiber, many vitamins (especially B group of vitamins), calcium, iron, and many other macro- and micro-nutrients (Igrejas and Branlard, 2020). In the Indian subcontinent, more than one billion people depend on diets containing cereals (rice and wheat), pulses, and oilseeds that are deficient in Zn (Prasad et al., 2010). Keeping the significance of zinc biofortification of wheat and possible role of Zn-solubilizing rhizobacteria in view, this investigation aimed to characterize potential zinc solubilizing rhizobacterial isolates from chickpea rhizosphere and their application in zinc biofortification of wheat crop. Rhizobacteria isolated from chickpea rhizosphere were used in this study, with the assumption that these bacteria are also associated with the wheat crop, because farmers grow either chickpea or wheat crop in the same piece of land during the *Rabi* season (December to April).

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and Cultural Conditions

General media, glucose, zinc oxide, zinc carbonate, zinc phosphate, hydrogen peroxide, nicotinamide adenine dinucleotide, etc., were purchased from HiMedia Pvt., Ltd., Mumbai (India). Analytical-grade chemicals and standards were procured from Sigma–Aldrich (St. Louis, MO, United States). Bovine serum albumin and high-performance liquid chromatography (HPLC)-grade solvents and chemicals were procured from E. Merck (Mumbai, India), while molecular-grade chemicals were purchased from Banglore GeNi (Bangaluru, India), BioRAD (Gurugram, Haryana, India), and Agilent (Mumbai, India). PCR and qPCR primers were synthesized from Eurofin Private Limited, Bangaluru (India).

In this study, 15 rhizobacterial isolates were retrieved from the rhizosphere of chickpea grown in different agroclimatic regions of eastern Uttar Pradesh, India (**Supplementary Table 1**). These isolates were designated as CRS-9 (CRS–chickpea rhizosphere soil), CRS-17, CRS-26, CRS-30, CRS-37, CRS-38, CRS-42, CRS-43, CRS-45, CRS-47, CRS-50, CRS-54, CRS-55, CRS-57, and CRS-77, and cultured in a nutrient broth medium at 28^{o}C for 28–48 h with shaking at 120 rpm and preserved in 20% glycerol stock at −80^{o}C in deep freezer.

Selection of Potential Zinc-Solubilizing Rhizobacteria

Screening of the fifteen (15) rhizobacterial isolates was performed for zinc solubilization using a tris-minimal agar medium supplemented with D-glucose and with different insoluble zinc compounds as per the methods of Fasim et al. (2002), with slight modification (Sharma et al., 2011). Specifically, the tris-minimal medium was amended separately with zinc oxide [ZnO] (1.244 g l^{-1}), zinc phosphate [Zn_3 $(PO_4)_2$] (1.9882 g l^{-1}), and zinc carbonate [$ZnCO_3.2H_2O$] (1.728 g l^{-1}), equivalent to the concentration of 0.1% Zn as sole zinc source to find out the ability of rhizobacterial isolates to solubilize zinc oxide, zinc phosphate, and zinc carbonate. After autoclaving (at 121^{o}C for 15 min), the culture medium was transferred to sterilized Petri plates. Freshly grown bacterial cultures were spot-inoculated in triplicates in the medium using sterile toothpicks, followed by incubation of the plates at 28°C for 7 days in the dark to observe Zn solubilization in the form of a clear halo zone around colonies. After 7 days, colony diameter and the diameter of the halo zone (mm) formed around the colony were measured. Afterward, the same plates were flooded with a methyl red solution to observe acid production by bacteria. Change in color of a clear zone from yellow to red following methyl red solution indicates a positive reaction (Garcia and Isenberg, 2007). The extent of zinc solubilization by different isolates was ensured by measuring the zone of solubilization around the colonies. Zinc solubilization index (ZSI) and zinc solubilization efficiency (ZSE) of the isolates were determined (Vazquez et al., 2000; Ramesh et al., 2014b). Isolates showing maximum ZSE were considered to be potential zinc solubilizers.

Quantitative Assay for Zinc Solubilization

Potential zinc solubilizing rhizobacterial isolates were further tested for their ability to release soluble zinc in a liquid medium and their effect on the pH of the medium. The isolates were inoculated in a tris-minimal broth medium supplemented with 0.1% Zn as zinc phosphate. For each isolate, 50 ml of the liquid tris-minimal broth medium supplemented with 0.1% Zn as zinc phosphate was taken in a 100-ml Erlenmeyer flask and autoclaved. One ml aliquot of each culture with a cell load of 10^8cfu/ml was inoculated in each flask. A tris-minimal broth medium supplemented with zinc phosphate but without bacterial inoculation served as the absolute control. Each treatment was replicated thrice. All the flasks were incubated at 28^{o}C in an orbital shaker at 120 rpm for 10 days. After 10 days of incubation, samples were withdrawn, and aliquots of the liquid broth medium were centrifuged at 10,000 rpm for 8–10 min to remove cell debris, and a clear supernatant was collected and fed directly to an atomic absorption spectrophotometer for determination of released soluble zinc (μg Zn $ml^{-1)}$ in the clear broth, and the pH of each culture and the uninoculated broth was measured (Ramesh et al., 2014b).

Analysis of Gluconic Acid Produced by Zinc-Solubilizing Bacteria Using High-Performance Liquid Chromatography

Gluconic acid is the pre-dominant acid produced during bacterial Zn solubilization. In this study, gluconic acid was quantified using an HPLC system (Separon SGX C18 column; Shimadzu, Kyoto, Japan) equipped with a quaternary pump, an auto-sampler, a DAD detector, and a degasser and according to Larcher et al. (2009) but with slight modifications (Sunithakumari et al., 2016). Specifically, the rhizobacterial isolates were tested for production of gluconic acid. They grew in 50-ml of the tris-minimal broth medium supplemented with 0.1% Zn as zinc phosphate for 10 days. After incubation, the bacterial cultures were centrifuged (12,000 rpm, 20 min, and 4°C), and the supernatant was passed through 0.2-μm membrane filters. A filter-sterilized culture filtrate was collected and analyzed using the HPLC system. Elution was performed with an isocratic flow consisting of acetonitrile:water (30:70v/v) with a flow rate of 1 ml/min at 210 nm using a UV/VIS detector. Retention time was 2.4 min with 10 μl of injection. The detection limit of the instrument is up to 0.5 mg kg^{-1}, and standard gluconic acid is detected in the range of 50 mg kg^{-1}. Pure gluconic acid was purchased from Sigma-Aldrich (Mumbai, India) and used as a reference. Thus, the gluconic acid present in the culture filtrate was determined by comparing the retention time and peak area of the sample with the standards of gluconic acid.

Screening of Plant Growth-Promoting Attributes in Zinc-Solubilizing Rhizobacteria

Plant growth promotion attributes of the isolates, such as phosphate solubilization, potash solubilization, production of siderophore, indole-3-acetic acid (IAA), and ammonia, were also assessed (Dinesh et al., 2018). Phosphate solubilization was determined using a Pikovaskya medium containing 0.1% tri-calcium phosphate, and the release of phosphorus by phosphate solubilization was studied by the method described by Olsen and Sommers (as cited in Penrose and Glick, 2003). However, the K solubilization test was performed according to Rajawat et al. (2016). The chrome azurol S (CAS) agar assay described by Schwyn and Neilands (1987) was followed to screen the bacterial isolates for siderophore production. The test isolates were spot inoculated on CAS agar plates and incubated at 30 ± 2°C for 5 days. Development of yellow-orange halo zones around the growing colonies is an indication of siderophore production. IAA production by rhizobacterial isolates was estimated as per the method of Ahmad et al. (2020). Bacterial isolates were grown for 3 days in a Luria Bertani (LB) broth at 28 ± 2°C and were centrifuged at 8,000 rpm for 30 min. The supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowaski reagent, and incubated in the dark at room temperature for 20 min followed by measurement of absorbance at 530 nm using pure IAA (Sigma-Aldrich, United States) as standard. Ammonia content was detected by adding 1 ml of a Nessler reagent to 72-h old cultures grown in peptone broth. Positive samples showed a yellowish-brown color. Bacterial isolates were screened for ACC deaminase production following the method of Bal et al. (2013).

Characterization of Zinc-Solubilizing Rhizobacteria

Morphological and biochemical characterizations of the four potential zinc-solubilizing isolates were performed as per the methods described in *Bergey's Manual of Determinative Bacteriology*. Biochemical characterization includes Gram-reaction, catalase, oxidase, nitrate, H_2S, citrate utilization, Voges Proskauer's, esculin hydrolysis, methyl red, indole, ONPG, lysine, ornithine, urease, phenylalanine, malonate, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, and lactose utilization. Identification of the potential isolates was performed using 16S rRNA gene sequence similarity as per the method described by Singh et al. (2016). Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA-X), and 16S rRNA gene sequences were submitted to the NCBI GenBank[1].

Evaluation of Zinc Solibilizing Rhizobacteria

Planting Materials and Growth Conditions

Microcosm experiments were carried out on wheat (*cv.* HD-2967) as a test crop in a glasshouse situated at Research Farm of ICAR-Indian Institute of Seed Sciences (ICAR-IISS), Mau, Uttar Pradesh, India (25°53′ 56.99″N 83°29′18.29″E, elevation 74 m) during the winter season (2019 and 2020) to investigate the impact of zinc-solubilizing rhizobacteria on wheat growth and development. The wheat seeds used in the experiments were procured at ICAR-IISS, Mau, Uttar Pradesh. The weather conditions during the growing period were: mean temperature 22–25°C, relative humidity of 70–75% with an 11/13-h photoperiod.

Preparation of Rhizobacterial Inoculants

Four potential rhizobacterial strains, *Bacillus glycinifermentans* (CRS-9), *Microbacterium oxydans* (CRS-17), *Paenarthrobacter nicotinovorans* (CRS-30), and *Bacillus tequilensis* (CRS-38) were used for the evaluation of their effects on plant growth, rhizosphere properties, zinc acquisition, and mobilization. The bacterial formulations were prepared using a sterile saline solution and employing the methods of Singh et al. (2016). The colony forming unit (CFU) count of the formulations was adjusted to 2×10^8 cfu ml^{-1} at the time of application.

Root Colonization and Formation of Biofilm

Bio-primed seeds (20 ml kg^{-1} seeds) were sown in small pots (4 in^2 × 5 in^2) containing a sterile soil mixture (sand:soil:vermiculite in 1:1:1 ratio) under glasshouse conditions. After 15 days of sowing, plants were uprooted gently and washed in running tap water. Root samples were fixed with an osmium tetraoxide solution (HiMedia) and 2.5% glutaraldehyde (HiMedia) according to Singh et al. (2021). After fixation, the roots were dehydrated using a gradient of ethyl alcohol (5, 10, 20, 50, 70, 90, and 100%) and dried under vacuum. Thereafter, the samples were coated with gold (20 nm) and visualized under a scanning electron microscope (S-3400N; Hitachi, Chiyoda-ku, Tokyo, Japan).

Effects on Seed Germination and Vigor Indices

Effects of seed inoculation on germination and vigor indices of the plants grown in pots after 15 and 30 days of sowing, respectively, were recorded. To see the effects of seed inoculation on germination and vigor indices, seeds were surface-sterilized (1% NaOCl for 60 s) and subsequently rinsed with sterile distilled water thrice to remove NaOCl. The seeds were bioprimed (20 ml kg^{-1} seeds), incubated overnight at 28°C, and sown in pots containing experimental soil under glasshouse conditions. For the germination test, a set of experiments was conducted following the protocols of the International Seed Testing Association (ISTA, 2003; Singh et al., 2016). Specifically, for the seed germination test, 500 seeds were taken and sown in pots (20 seeds pot^{-1}) containing a sterile soil mixture. The germinated seeds were counted after 15 days of sowing, and the percent seed germination was calculated. To see the effects of seed inoculation on vigor indices (vigor indexes I and II), bioprimed seeds were sown in pots (5 seeds pot^{-1}) under glasshouse conditions. After 30 days of sowing, vigor indexes I and II were calculated according to Singh et al. (2016).

Effects of Seed Inoculation on Plant Growth, Yield, and Zn Content

Experimental Setup

The glasshouse experiment was laid out with six treatments and 10 replications each in a complete randomized block design (CRBD). Treatments were: T_1: the absolute control (without zinc phosphate + without bacteria), T_2: $Zn_3(PO_4)_2$ (without bacteria), T_3: $Zn_3(PO_4)_2$ + CRS-9, T_4: $Zn_3(PO_4)_2$ + CRS-17, T_5: $Zn_3(PO_4)_2$ + CRS-30, and T_6: $Zn_3(PO_4)_2$ + CRS-38. Soil for the microcosm experiment was collected from the Research Farm of ICAR-IISS, Mau. Characteristics of the experimental soil were: pH 7.7, OC.37%, available N 313.85 kg ha^{-1}, available P 79.36 kg ha^{-1}, available K 599.64 kg ha^{-1}, and DTPA-Zn 6.6 mg kg^{-1}. The soil was mixed with $Zn_3(PO_4)_2$ (2g kg^{-1}) as Zn source, and each pot contained 3 kg of experimental soil.

Initially, four bioprimed seeds were sown in each pot, and, after germination, two plants per pot were maintained each throughout the experimentation. No additional fertilizers were applied during the experimentation. Moisture in the pots was maintained at field capacity by adding sterile distilled water as

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[1] https://www.ncbi.nlm.nih.gov

and when needed. The experiment was carried out in two sets. In the first set, five replicates of wheat plants were harvested after 30 days and utilized for recording the observations. However, the remaining five replicates were maintained up to the harvest stage, and data were recorded at maturity.

Analysis of Plant Samples

After 30 days of sowing, plants were harvested from the first set of experiments and data were recorded. Shoot length, root length, and dry weight of roots and shoots were recorded using standard agronomic procedures. For estimation of Zn content, plant samples (roots and shoots) were dried, ground to fine powder, and digested in a di-acid mixture containing nitric acid and perchloric acid (5:4 v/v) at 320^{o}C for 1 h, and Zn content (μg Zn g^{-1} plant material) was measured using an atomic absorption spectrophotometer. For further analyses, plants were sampled from the second set of experiments after 120 days of sowing and brought to the laboratory. Roots and shoots were separated and washed in running tap water. Thereafter, the total length and fresh and dry weight of the shoots and roots were recorded. Seed yield (g plant^{-1}) was also recorded. Zinc content in the shoot, root, and grain samples was estimated using the above-described method.

Expression Analyses of ZIP Transporter Genes

Quantitative real time-PCR (qPCR) analysis was performed to elucidate the expression and accumulation of transcript of ZIP transporter genes in different parts of wheat after 30 days of sowing, as per the method described by Singh et al. (2021). From the first set of experiments, root, shoot, and leaf samples were collected from each treatment in triplicates, brought to the laboratory, and quick-frozen in liquid nitrogen to isolate total RNA. RNA extraction was performed using an RNA isolation kit (Agilent, Mumbai, India) following the instructions of the manufacturer. cDNA synthesis was performed using a cDNA synthesis kit (BioRAD, Gurugram, Haryana, India) following the protocols of the manufacturer. The quality and quantity of cDNA were analyzed using Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, United States). The housekeeping genes *actin* and *SuccDH* were used as an endogenous standard to normalize the quantitative expression data of *TaZIP1, TaZIP3, TaZIP5, TaZIP6, TaZIP7, TaZIP10, TaZIP13,* and *TaZIP15* genes in wheat. Gene expression was analyzed using gene-specific primers (**Supplementary Table 2**). A BioRAD Real-Time PCR system (MJ MiniOpticon System; BioRAD) was used for expression analyses, and relative transcription levels were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

The glasshouse experiments were repeated twice for two consecutive years (2019 and 2020), and a pool analysis was performed. To test the significance of zinc-solubilizing rhizobacterial strains, a one-way analysis of variance was performed using Monitab 170. Means were separated by least significant differences (LSDs) at 95% significance level ($p \leq 0.05$). A principal component analysis of strains based on variables was performed using SPSS version 16.0. Data were compared by Duncan's multiple range test (DMRT) at $p \leq 0.05$. Statistical software Origin version 9.0 and Microsoft Excel (Window 10) were used to prepare graphs.

RESULTS

Screening of Zinc-Solubilizing Rhizobacteria

The zinc solubilization efficacy of rhizobacterial isolates was determined by measuring the diameter of halo zones formed in a growth medium supplemented with three different zinc compounds, viz. zinc oxide, zinc phosphate, and zinc carbonate. Among 15 rhizobacterial isolates, 11, viz., CRS-9, CRS-17, CRS-30, CRS-38, CRS-42, CRS-43, CRS-45, CRS-47, CRS-55, CRS-57, and CRS-77 showed varying degrees of zinc solubilization (zone ranging from 12.33 to 25.67 mm) (**Table 1**). However, all the fifteen rhizobacterial isolates did not solubilize zinc oxide and zinc carbonate. Out of the 11 zinc-solubilizing bacteria, four isolates, i.e., CRS-9 (21.33 mm), CRS-17 (25.67 mm), CRS-30 (24.33 mm), and CRS-38 (23.67 mm) were selected as potential zinc solubilizing rhizobacteria, and these isolates were chosen for further study (**Supplementary Figure 1**). Based on ZSI and ZSE, the most efficient bacterial isolates were CRS-17, CRS-38, CRS-30, and CRS-9 (**Table 1**). Furthermore, the rhizobacterial strains, viz., CRS-17, CRS-30, CRS-38, and CRS-9 showed maximum zinc phosphate solubilization efficiency (427.79, 317.39, 253.57, and 237.04%, respectively) (**Table 1**). The isolates, namely, CRS-17, CRS-30, CRS-9, and CRS-38 produced net soluble zinc a 6.1, 5.9, 5.63, and 5.6 μg ml^{-1}, respectively, in zinc phosphate, and in broth, with corresponding pH decline to 4.5, 5.3, 5.2, and 4.8, respectively, from pH 6.7 (**Table 1**). Considering both qualitative and quantitative assays, the study indicated that all the four isolates are efficient zinc solubilizers. In general, the pH declined because of inoculation from 6.7 to 4.5 in zinc phosphate as against the absolute control. Maximum release of soluble zinc occurred in the liquid medium supplemented with zinc phosphate at the lowest mean pH value of 4.5, suggesting that solubilization is dependent on reduction of pH either by organic acid production or proton extrusion by bacteria (**Table 1**). Furthermore, the production of gluconic acid as one of the organic acids was measured with the assumption that it was a predominant organic acid that plays a crucial role in zinc solubilization.

Production of Gluconic Acid by Zinc Solubilizing Rhizobacteria

Production of gluconic acid in the presence of zinc phosphate by the bacterial isolates used in the study was determined by HPLC. The retention time for the gluconic acid standard was 2.187 min (**Figure 1**). It was observed that all the isolates produced gluconic acid in the medium supplemented with zinc phosphate, as depicted by peak and retention times. Peak height and area of gluconic acid in the chromatograph of *P. nicotinovorans* CRS-30 were found to be more compared to the other isolates. HPLC results revealed that a significantly higher amount of gluconic acid was produced by CRS-30 (189.41 μg ml^{-1}) followed by CRS-38 (174.66 μg ml^{-1}) and CRS-9 (153.29). However, the least

TABLE 1 | Efficacy of rhizobacterial isolates in insoluble solubilize zinc compounds under laboratory conditions.

Isolates	Zinc oxide	Zinc carbonate	Zinc phosphate *	Halo zone (mm)	ZSI	ZSE (%)	Zinc concentration (μg ml)	pH
CRS-9	–	–	++++	21.33 ± 1.05	11.37 ± 0.35	237.037	5.63 ± 0.21	5.2
CRS-17	–	–	++++	25.67 ± 2.15	10.28 ± 0.75	427.778	6.10 ± 0.14	4.5
CRS-26	–	–	–	–	–	–	–	–
CRS-30	–	–	++++	24.33 ± 1.33	10.84 ± 0.42	317.391	5.90 ± 0.26	5.3
CRS-37	–	–	–	–	–	–	–	–
CRS-38	–	–	++++	23.67 ± 1.65	11.87 ± 0.76	253.571	5.60 ± 0.37	4.7
CRS-42	–	–	+++	16.33 ± 1.01	10.04 ± 0.86	204.13	4.27 ± 0.45	4.9
CRS-43	–	–	+++	20.00 ± 0.95	10.98 ± 0.56	230.68	4.57 ± 0.25	4.8
CRS-45	–	–	+++	13.33 ± 0.45	9.67 ± 0.67	166.63	4.07 ± 0.33	4.7
CRS-47	–	–	+++	13.33 ± 0.66	8.22 ± 0.35	222.17	4.17 ± 0.30	5.1
CRS-50	–	–	–	–	–	–	–	
CRS-54	–	–	–	–	–	–	–	–
CRS-55	–	–	+++	14.67 ± 0.25	10.09 ± 0.27	176.11	4.37 ± 0.20	5.0
CRS-57	–	–	+++	12.33 ± 0.48	9.81 ± 0.63	148.02	3.73 ± 0.15	4.7
CRS-77	–	–	+++	17.67 ± 0.67	10.45 ± 0.25	212.12	4.60 ± 0.41	5.0

**+++, moderated activity; ++++, strong activity, -, no activity; ZSI, zinc solubilization index; ZSE, zinc solubilization efficiency; data are mean ± standard deviation (n = 5).*

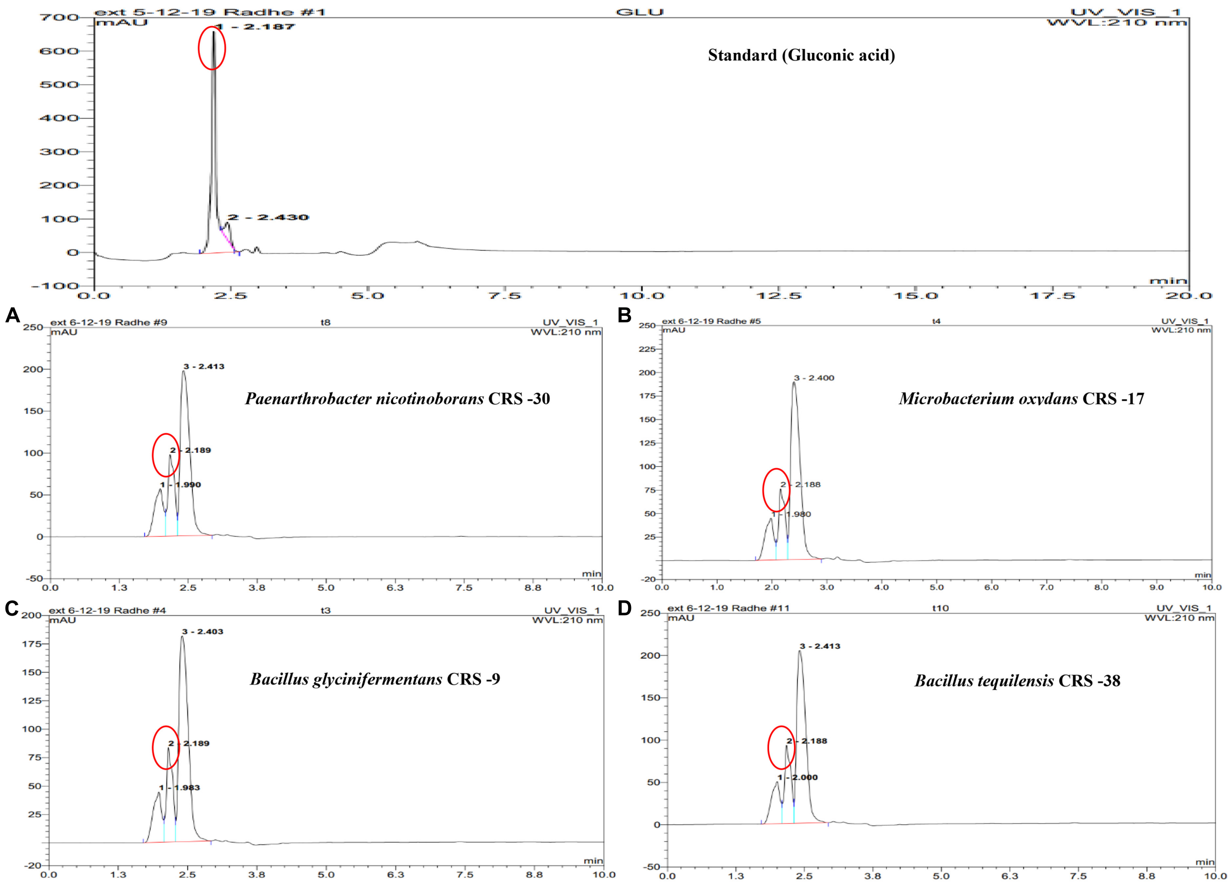

FIGURE 1 | High-performance liquid chromatography (HPLC) chromatogram depicting the peaks of gluconic acid secreted by zinc-solubilizing rhizobacteria. Chromatograph of gluconic acid as standard (top) for a comparative study on its retention time (2.18 min) with the selected strains. Chromatographs of the samples of strains **(A)** CRS-30, **(B)** CRS-17, **(C)** CRS-9, and **(D)** CRS-38 of the 10-day cultured supernatant. Peak identified as gluconic acid is indicated through circle.

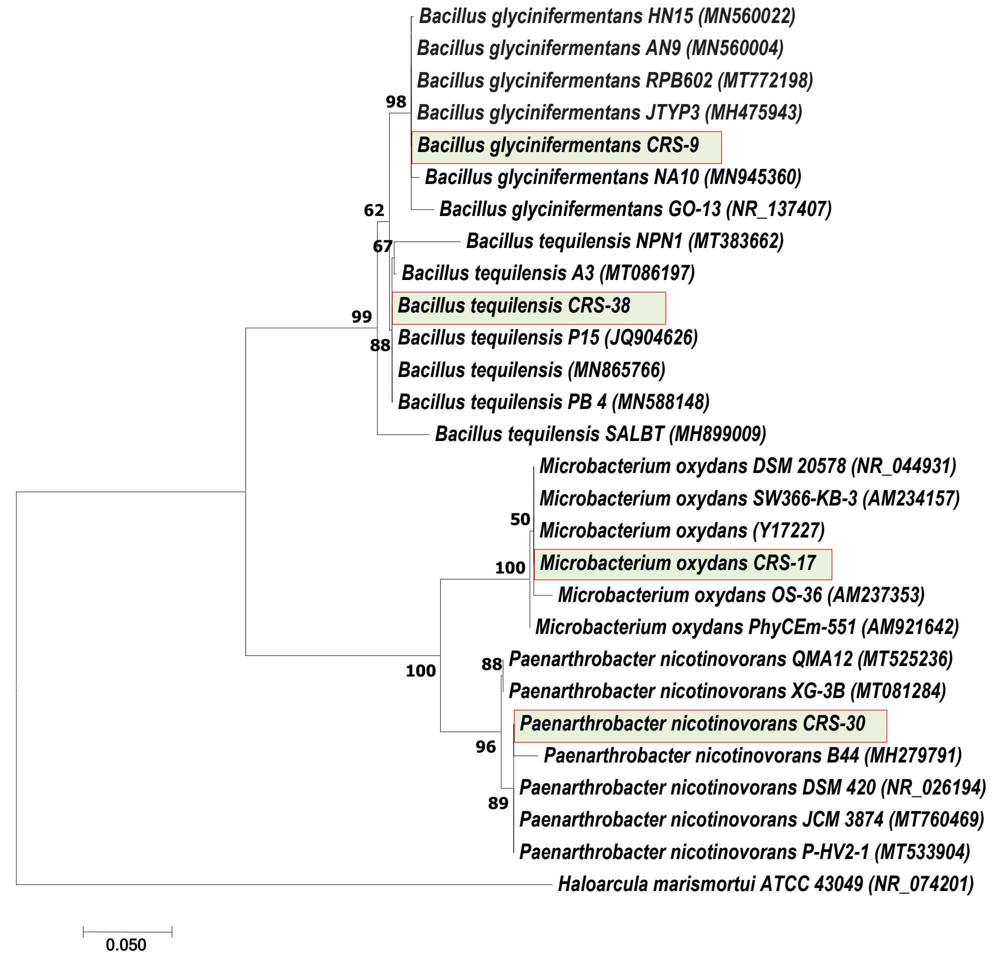

FIGURE 2 | Neighbor-joining tree derived with CLUSTAL W and MEGA 5.0 by analysis of 16S rRNA gene sequences. The numbers at nodes indicate bootstrap support values, as calculated with MEGA 5.0. Bacterial strains used in this study are presented in highlighted box.

amount of gluconic acid (144.4 μg ml^{-1}) was recorded for CRS-17.

Plant Growth-Promoting Traits

The selected zinc-solubilizing rhizobacterial isolates were further characterized for PGP traits, *viz.*, P solubilization, K solubilization, IAA production, ACC deaminase activity, siderophore production, and ammonia production (**Supplementary Table 3**). All the four isolates, CRS-9, CRS-17, CRS-30, and CRS-38, exhibited strong PGP traits and solubilize P and K except CRS-17, which lacks potassium solubilization ability. Similarly, all the isolates were found to produce varying levels of IAA in culture filtrate wherein CRS-30 showed highest IAA production (17.16 μg ml^{-1}) followed by CRS-38 (8.61 μg ml^{-1}), CRS-9 (6.16 μg ml^{-1}), and CRS-17 (6.43 μg ml^{-1}). Except for CRS-38, the other three isolates were found to be negative for ACC deaminase activity. All the bacterial isolates produced siderophore except CRS-9, and only two isolates, viz., CRS-9 and CRS-38, produced ammonia in the respective media (**Supplementary Table 3**).

Biochemical and Molecular Characterization of Isolates

Four rhizobacterial isolates, viz., CRS-9, CRS-17, CRS-30, and CRS-38, were characterized on a cultural basis as per *Bergey's Manual of Determinative Bacteriology* (**Supplementary Table 4**). All the strains tested showed different colony morphology, were Gram-positive, and rod-shaped, and two of them (CRS-9 and CRS-17) formed endospores. All the strains showed different reactions for catalase, oxidase, nitrate, H_2S, citrate utilization, Voges Proskauer's, esculin hydrolysis, methyl red, indol, ONPG, lysine, etc. (**Supplementary Table 4**).

Based on 16S rRNA gene sequencing, these isolates were identified as *Bacillus glycinifermentans* CRS-9 (MH497203; 1,412 bp, 99.5% similarity), *Microbacterium oxydans* CRS-17 (MH497204; 1,368 bp, 100% similarity), *Paenarthrobacter nicotinovorans* CRS-30 (MH497212; 1,378 bp, 99.1% similarity), and *Bacillus tequilensis* CRS-38 (MH497217; 1,407 bp, 99.7% similarity) (**Figure 2**). Percent similarity, E-score, query coverage, etc., are presented in **Supplementary Table 5**. All the cultures were deposited in the National Agriculturally

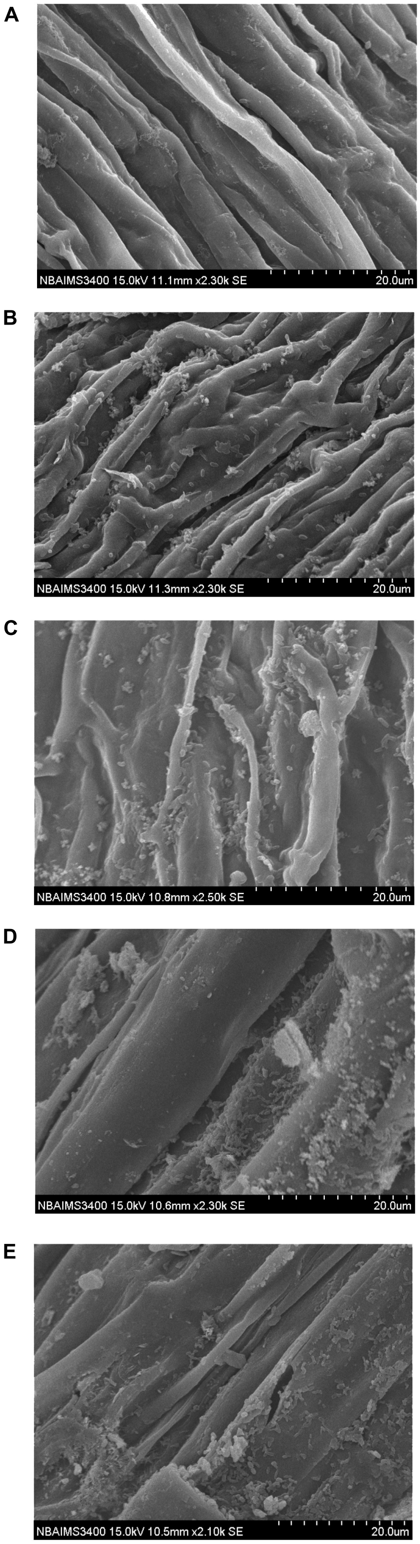

FIGURE 3 | Scanning electron microphotographs showing root colonization and biofilm formation by selected strains under glasshouse conditions, **(A)** Absolute control, **(B)** *Bacillus glycinifermentans* CRS-9, and **(C)** *Microbacterium oxydans* CRS-17, **(D)** *Paenarthrobacter nicotinovorans* CRS-30, and **(E)** *Bacillus tequilensis* CRS-38.

Important Microbial Culture Collection (NAIMCC), a microbial resource center of ICAR-NBAIM, Kushmaur, India (**Supplementary Table 5**).

Root Colonization and Biofilm Formation

Upon inoculation, the bacterial strains start colonizing radicals and grow along with the primary and secondary roots. Scanning electron microphotographs clearly indicated that the selected strains have a potential to colonize wheat roots. All the strains were found to colonize and formed biofilm in wheat roots. Results indicated that bacterial strains produced an ample amount of extracellular polysaccharides (EPSs) which facilitate the initial attachment and biofilm cells that are embedded in the EPS matrix along with root surface (**Figure 3**).

Effects of Inoculation on Seed Germination and Vigor Indices

The results revealed that maximum seed germination (in%) was recorded in seeds treated with CRS-38 (89.10%) followed by CRS-30 (86.25%). However, least germination was recorded for the absolute control (76.29%) after 30 days of sowing (**Table 2**). Similarly, a significant increment in vigor index I was recorded in plants inoculated with CRS-38 (4,868.26) followed by CRS-30 (4,249.25), and CRS-17 (4,075.14) compared to the other treatments and absolute control (2,237.84). More or less similar trends were recorded in the case of vigor index-II (**Table 2** and **Supplementary Figure 2**).

Plant Experiments

Of the three zinc sources tested, only zinc phosphate was solubilized by the selected rhizobacterial isolates. Hence, zinc phosphate was selected as a source of zinc in plant experiments under microcosm conditions. Based on zinc phosphate solubilization and multiple PGP traits, the four potential rhizobacterial strains were selected for plant experiments. In these experiments, each treatment was replicated ten times.

After 30 Days of Plant Growth

Plant Growth Attributes

The first set of plant experiments showed that all the strains considerably increased the parameters of root and shoots as compared to plants from absolute control (**Figures 4A–F**). Maximum shoot length was recorded for *B. tequilensis* CRS-38-inoculated plants; *B. glycinifermentans* CRS-9, *P. nicotinovorans* CRS-30, and *M. oxydans* CRS-17 showed minor differences (**Figure 4A**). Inoculation with *B. tequilensis* CRS-38 significantly increased the fresh and dry weight of shoots compared to that with absolute control and control + Zn_3 $(PO_4)_2$. All the other isolates significantly increased the fresh and dry weight of shoots of the inoculated plants compared to the absolute control, but this increase was not significant when compared to control + Zn_3 $(PO_4)_2$ (**Figures 4B,C**). Seed treatment with *B. glycinifermentans* CRS-9 and *P. nicotinovorans* CRS-30 resulted in a substantial increase in root length. However, *M. oxydans* CRS-17 and *B. tequilensis* CRS-38 seem to increase the root length of the plants significantly compared to the absolute control and control + $Zn_3 (PO_4)_2$. Maximum root length was observed in plants that emerged from the seeds treated with CRS-17 followed by those treated with CRS-38, and minimum root length was recorded for plants that emerged from the seeds treated with CRS-9 compared to other inoculants (**Figure 4D**). Inoculation with *B. glycinifermentans* CRS-9 and *M. oxydans* CRS-17 significantly increased the fresh and dry root weight of the plants compared to that with the absolute control and control + $Zn_3 (PO_4)_2$. A non-significant increase was observed in the fresh and dry root weight of plants inoculated with *P. nicotinovorans* CRS-30 and *B. tequilensis* CRS-38 compared to the absolute control and control + $Zn_3 (PO_4)_2$ (**Figures 4E,F**).

Zinc Content

Inoculation with different zinc-solubilizing rhizobacterial strains significantly increased the zinc content in shoots and roots of the inoculated plants as compared to the absolute control plants (**Figures 5A,B**). Maximum zinc content was found in shoots of the plants inoculated with *B. tequilensis* CRS-38 (64.26 $\mu g\ g^{-1}$) followed by those inoculated with *P. nicotinovorans* CRS-30 (45.25 $\mu g\ g^{-1}$), *M. oxydans* CRS-17 (33.33 $\mu g\ g^{-1}$), and *B. glycinifermentans* CRS-9 (31.26 $\mu g\ g^{-1}$). However, least zinc content was recorded in shoots of the control plants (24.1 $\mu g\ g^{-1}$) after 30 days of sowing (**Figure 5A**).

The zinc content of the roots was significantly higher than that of the shoots across the treatments (**Figure 5B**). The highest zinc content in the roots was recorded in plants treated with *Bacillus tequilensis* CRS-38 (77.50 $\mu g\ g^{-1}$) followed by those treated with *B. glycinifermentans* CRS-9 (71.46 $\mu g\ g^{-1}$), *P. nicotinovorans* CRS-30 (71.25 $\mu g\ g^{-1}$), and *M. oxydans* CRS-17 (57.1 $\mu g\ g^{-1}$). Least zinc content was found in roots of the absolute control plants (42.5 $\mu g\ g^{-1}$) and plants supplemented with $Zn_3 (PO_4)_2$ (44.25 $\mu g\ g^{-1}$) after 30 days of sowing (**Figure 5B**).

Expression of ZIP Transporter Genes

In this investigation, 9 *TaZIP* transporters genes were taken for an expression study, and out of the 9 *TaZIP* transporters, 8 *TaZIP* transporter genes (*TaZIP1, TaZIP3, TaZIP5, TaZIP6, TaZIP7, TaZIP10, TaZIP13,* and *TaZIP15*) were found to be expressed in different parts of the plants after 30 days of sowing. The results revealed that significantly higher expression of all the 8 *TaZIP* transporters was recorded in the bacteria-inoculated plants supplemented with $Zn_3 (PO_4)_2$ compared to the $Zn_3 (PO_4)_2$ and absolute control plants (**Figure 6**). Interestingly, the expression of all the 8 *TaZIP* transporters genes was significantly higher in the roots than in shoots and leaves of the same plants across the treatments. In general, the expression of the 8 *TaZIP* transporters genes was higher in leaves than in stems but lower in roots of the same plants. Furthermore, it was observed that plants treated with bacterial inoculant CRS-38 + $Zn_3(PO_4)_2$ showed maximum transcript level followed by CRS-30 + $Zn_3 (PO_4)_2$. However, least expression was recorded in untreated control plants followed by plants amended with $Zn_3 (PO_4)_2$ alone (**Figure 6**).

TABLE 2 | Effect of seed inoculation on germination and vigor indices of wheat grown in pots under glasshouse conditions after 30 days of sowing.

Treatments	Germination (%)	Vigor index-I	Vigor index-II
Absolute control	76.29 ± 1.20^d	$2{,}237.84 \pm 6.50^f$	50.56 ± 1.96^f
$Zn_3(PO4)_2$	77.05 ± 1.33^d	$2{,}568.33 \pm 8.25^e$	56.67 ± 2.50^e
$Zn_3(PO4)_2$ + CRS-9	82.76 ± 1.25^c	$3{,}565.58 \pm 10.02^d$	105.96 ± 2.33^c
$Zn_3(PO4)_2$ + CRS-17	82.05 ± 1.66^c	$4{,}075.15 \pm 11.25^c$	113.39 ± 1.75^b
$Zn_3(PO4)_2$ + CRS-30	86.25 ± 1.50^b	$4{,}249.25 \pm 9.66^b$	90.56 ± 2.25^d
$Zn_3(PO4)_2$ + CRS-38	88.10 ± 1.96^a	$4{,}868.99 \pm 8.50^a$	137.44 ± 3.36^a

Data are mean ± standard deviation (n = 5), and values within a column followed by a different letter are significantly different at $p < 0.05$.

In Harvest Stage (120 Days of Sowing)

Plant Growth Attributes

Harvesting of the wheat crop was done after 120 days of sowing, and data for shoot and root length, fresh and dry weight, Zn content in roots, shoots, and grains, and yield $plant^{-1}$ were recorded (**Figure 7**). Significantly higher shoot length was recorded for plants inoculated with *B. tequilensis* CRS-38 + $Zn_3 (PO_4)_2$ (62.5 cm) followed by *B. glycinifermentans* CRS-9 + $Zn_3 (PO_4)_2$ (58.25 cm), and *M. oxydans* CRS-17 + $Zn_3 (PO_4)_2$ (55.62 cm). Least shoot length was recorded for the absolute control (48.33 cm) and plants supplemented with $Zn_3(PO_4)_2$ alone (49.1 cm) after 120 days of sowing (**Figure 7A**). Similarly, all the bacterial isolates showed a significant increase in root length of the plants whereas *B. glycinifermentans* CRS-9 + $Zn_3 (PO_4)_2$ showed maximum increment in root length (39.25 cm) followed by *P. nicotinovorans* CRS-30 + $Zn_3 (PO_4)_2$ (36.25 cm), *B. tequilensis* CRS-38 + $Zn_3 (PO_4)_2$ (31.35 cm), and *M. oxydans* CRS-17 + $Zn_3 (PO_4)_2$ (31.33 cm) when compared to the root length of the absolute control plants (25.66 cm) after 120 days of sowing (**Figure 7D**).

Similar trends were recorded for shoot and root fresh weight. Significantly higher fresh biomass of shoots and roots was recorded in plants inoculated with *B. tequilensis* CRS-38 + $Zn_3(PO_4)_2$ (3.1 and 2.38 g $plant^{-1}$, respectively) after 120 days of sowing (**Figure 7B**). Moreover, maximum dry weight accumulation in shoots was observed in plants inoculated with *B. tequilensis* CRS-38 + $Zn_3(PO_4)_2$ (2.19 g $plant^{-1}$). Least shoot dry weight was recorded for absolute control plants (1.05 g $plant^{-1}$). However, maximum root dry weight was observed in *B. glycinifermentans* CRS-9 + $Zn_3(PO_4)_2$ and *B. tequilensis* CRS-38 + $Zn_3(PO_4)_2$ (0.94 and.93 g $plant^{-1}$, respectively) compared to the other treatments after 120 days of sowing (**Figures 7C,F**).

Zinc Content

The zinc content of shoots, roots, and grains was recorded in plants inoculated with different zinc-solubilizing rhizobacterial isolates after 120 days of sowing (**Figure 8**). Significantly higher Zn content in shoots was recorded in plants inoculated with *P. nicotinovorans* CRS-30 + $Zn_3 (PO_4)_2$ (54.25 $\mu g\ g^{-1}$) followed by *B. tequilensis* CRS-38 + $Zn_3 (PO_4)_2$ (43.5 $\mu g\ g^{-1}$), and *M. oxydans* CRS-17 + $Zn_3 (PO_4)_2$ (38.62 $\mu g\ g^{-1}$) (**Figure 8A**). However, maximum Zn content in roots was observed in plants inoculated with *B. tequilensis* CRS-38 + $Zn_3 (PO_4)_2$ (61.25 $\mu g\ g^{-1}$), while it was least with the absolute control (20.33 $\mu g\ g^{-1}$)

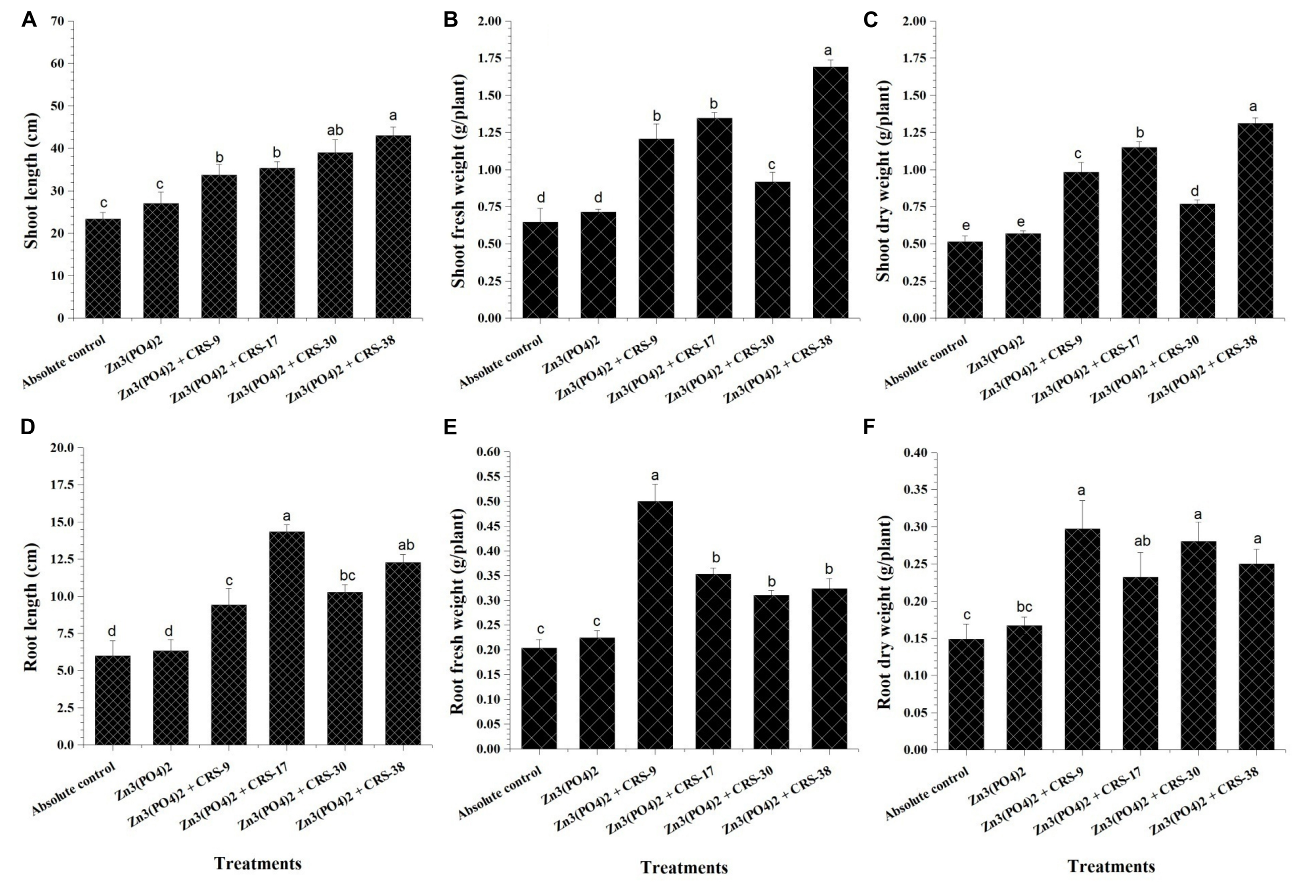

FIGURE 4 | Effect of zinc solubilizing rhizobacteria on growth parameters of plants harvested after 30 days (first set). **(A)** Shoot length, **(B)** Fresh weight of shoots **(C)** Dry weight of shoots, **(D)** Root length **(E)** Fresh weight of roots and **(F)** Dry weight of roots in comparison with absolute control. Data are mean (*n* = 5) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

after 120 days of sowing (**Figure 8B**). In general, plant roots have higher Zn content than shoots across the treatments including the absolute control.

Similar to Zn content in the shoot, significantly higher Zn content in grain was observed in the plant inoculated with *B. tequilensis* CRS-38 + Zn_3 $(PO_4)_2$ (53.25 μg g^{-1}) followed by *P. nicotinovorans* CRS-30 + Zn_3 $(PO_4)_2$ (49.82 μg g^{-1}), *M. oxydans* CRS-17 + Zn_3 $(PO_4)_2$ (46.98 μg g^{-1}) and *B. glycinifermentans* CRS-9 + Zn_3 $(PO_4)_2$ (44.50 μg g^{-1}) as against the absolute control (33.76 μg g^{-1}) and the plant supplemented with Zn_3 $(PO_4)_2$ alone (36.35 μg g^{-1}) (**Figure 8C**). The significant increase in zinc content of grains of the inoculated plants showed the importance of bacterial inoculants across the treatments. These results indicate the efficacy of the selected zinc solubilizing-rhizobacteria in enhancing the bioavailability of zinc and mobilizing it toward wheat grains. Inoculation with *B. glycinifermentans* CRS-9, *M. oxydans* CRS-17, *P. nicotinovorans* CRS-30, and *B. tequilensis* CRS-38 significantly increased grain yield compared to that with the absolute control and supplementation with Zn_3 $(PO_4)_2$ alone. Maximum grain yield was recorded for plants inoculated with *B. tequilensis* CRS-38 + Zn_3 $(PO_4)_2$ (2.39 g $plant^{-1}$) followed by *P. nicotinovorans* CRS-30 + Zn_3 $(PO_4)_2$ (2.33 g $plant^{-1}$) compared to the other treatments (**Figure 8D**).

The position of different plant growth attributes, such as fresh and dry weight of root and shoot, and Zn content as influenced by rhizobacterial strains in the four zones of biplot of PCA are depicted in **Figure 9**. The PCA comprising two principal components (PC1 74.76% and PC2 17%) accounted for 91.76% of the variance (**Figure 9A**). The interpretation among the different plant growth parameters and Zn content was more evident through the projection of PC1 and PC2. All plant growth attributes occupied a position solely in the right upper part of the biplots and showed a positive correlation among them. However, they were negatively correlated with root zinc content. Furthermore, it was observed that rhizobacterial strains + Zn_3 $(PO_4)_2$ formed a separate group, and that Zn_3 $(PO_4)_2$ alone and the absolute control formed separate groups in both mid stage (30 DAS) and harvest stage. However, two separate clusters formed during the mid-stage and harvest stage. PC1 and PC2 indicated that CRS-38 and CRS-30 showed a highly significant correlation compared to the other two rhizobacterial strains in the mid stage

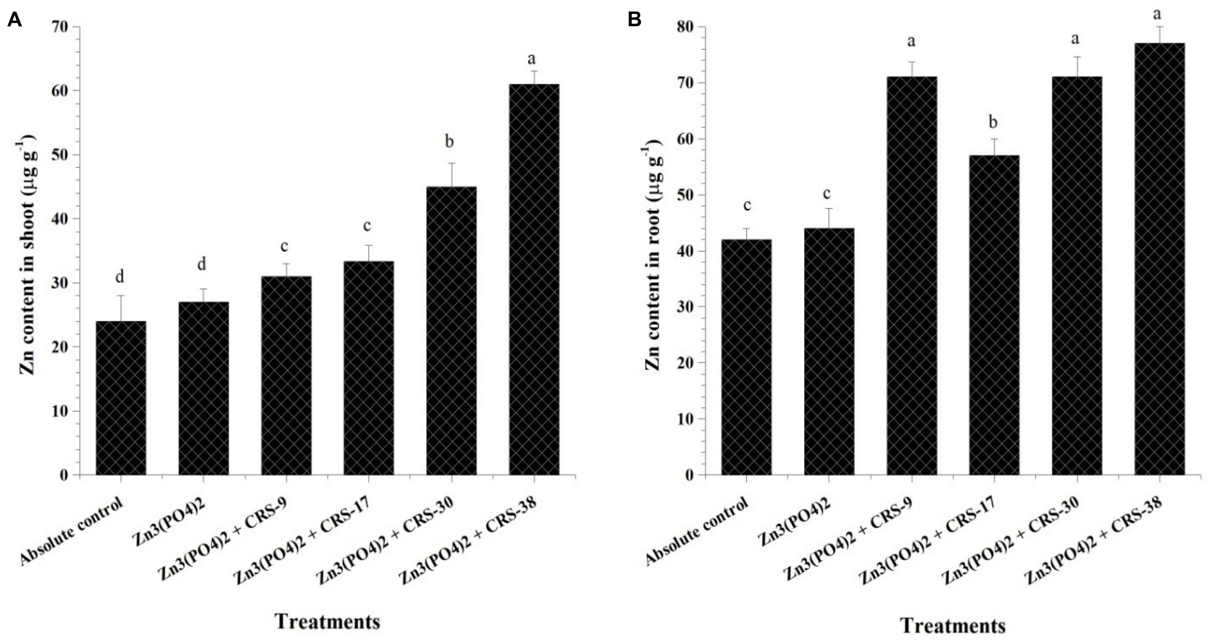

FIGURE 5 | Estimation of zinc content in **(A)** shoots and **(B)** roots of inoculated and un-inoculated plants harvested after 30 days. Data are mean ($n = 5$), and vertical bar represents standard deviation. Data with different letters show significant difference in column data in the randomized block design test at $p < 0.05$ under Duncan's multiple range test.

with respect to PC2. In contrast, CRS-38 and CRS-30 showed higher significance than the other two rhizobacterial strains in the harvest stage with respect to PC1 (**Figure 9B**). It is evident that the rhizobacterial strains did not express their potential completely in the mid stage, and the full potential of the bacterial strains was recorded only in the harvest stage. However, factors responsible for this differential effect of rhizobacteria on wheat crops need to be identified.

DISCUSSION

Zinc is an essential micronutrient required for the overall growth and development of a plant. Zinc deficiency is prevalent globally in crops, leading to considerable economic losses. Alleviation of zinc deficiency through the application of zinc fertilizers may not be environmentally friendly and cost-effective. Many strategies are available that help in enhancing zinc levels in plants without harming the environment (Cakmak, 2008; White and Broadley, 2011). One such strategy is harnessing the potential of rhizobacteria that can mobilize unavailable zinc and increase assimilation of zinc, and accelerate growth and yield of plants. Rhizobacteria play an important role in environmental geo-cycling processes, such as solubilization of immobilized metal ion forms, which can be suitably taken up by plants. Zinc-solubilizing rhizobacteria can be used as an alternative strategy for enhancing zinc uptake by plants because of their ability to solubilize Zn through secretion of organic acids, protein extrusion, and production of chelating agents (Nahas, 1996; Seshadri et al., 2002). Thus, nowadays, scientific interest has increased in utilization of zinc-solubilizing rhizobacteria because of their role in increasing zinc uptake in plants. Therefore, the main focus of this study was to characterize potential zinc-solubilizing rhizobacterial isolates and their application for overall plant growth, with special reference to zinc biofortification in wheat.

In this study, different rhizobacteria were isolated from chickpea rhizospheric soils of the Indo-Gangetic plains of Northern India. These bacteria were evaluated for their ability to solubilize zinc using different zinc compounds. Out of 15 rhizobacterial isolates, only 11 showed zinc-solubilizing potential in a medium supplemented with zinc phosphate Zn_3 (PO_4). The variation in the diameter of halo zone formation indicated a variation in the degree of solubilization. Various studies have reported variation in solubilization by bacterial strains (Sharma et al., 2011; Ramesh et al., 2014a; Khande et al., 2017). In this investigation, the isolates showed varying levels of ZSI and ZSE ranging from 8.22 to 11.87 and 148 to 427%, respectively. These findings are corroborated by the study of Ramesh et al. (2014b), wherein they found ZSE of up to 175% by strains of *Bacillus aryabhattai*. In this study, it was found that different rhizobacterial strains produced a varying level of zinc content in a liquid medium that ranged from 2.47–6.1 µg/ml. The results obtained in this study are consistent with the findings of Sharma et al. (2011), wherein they showed that the zinc concentration of different *Bacillus* spp. ranged between 2.23 and 4.87 µg/ml. This study showed that zinc-solubilizing rhizobacterial isolates were able to decrease pH from 7 to 4.5. The reduction in pH of the liquid media suggested that these rhizobacterial isolates produce organic acids. This finding is in consonance with reports by other workers (Saravanan et al., 2004) who reported a reduction in

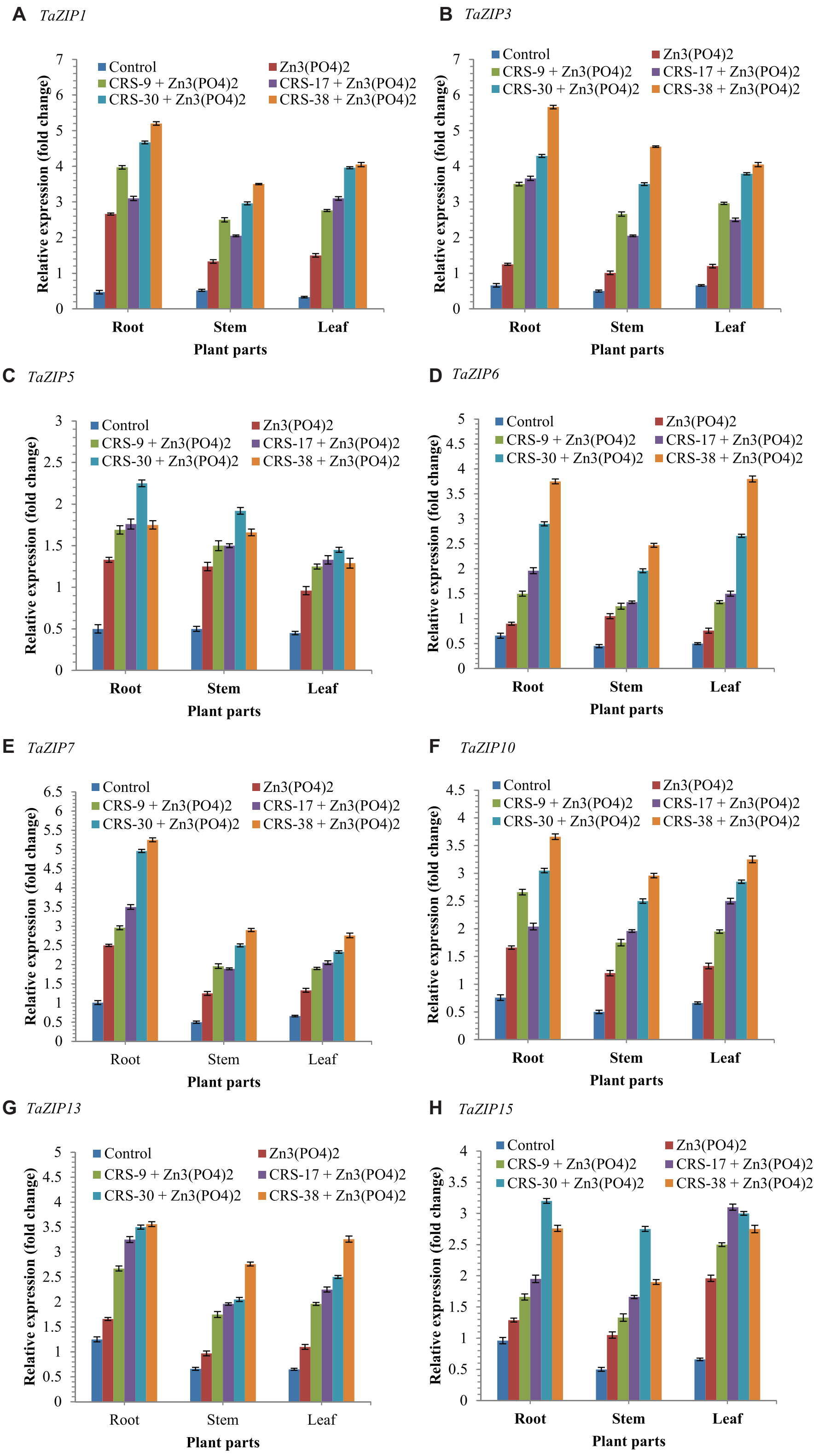

FIGURE 6 | Effects of seed bioinoculation on expression profile of TaZIP transporter genes (fold change) in the wheat root, shoot and leaves grown at 30 days of sowing. **(A)** Relative expression (fold change) of *TaZIP1*, **(B)** Relative expression (fold change) of *TaZIP3*, **(C)** Relative expression (fold change) of *TaZIP5*, **(D)** Relative expression (fold change) of *TaZIP6*, **(E)** Relative expression (fold change) of *TaZIP7*, **(F)** Relative expression (fold change) of *TaZIP10*, **(G)** Relative expression (fold change) of *TaZIP13*, and **(H)** Relative expression (fold change) of *TaZIP15*.

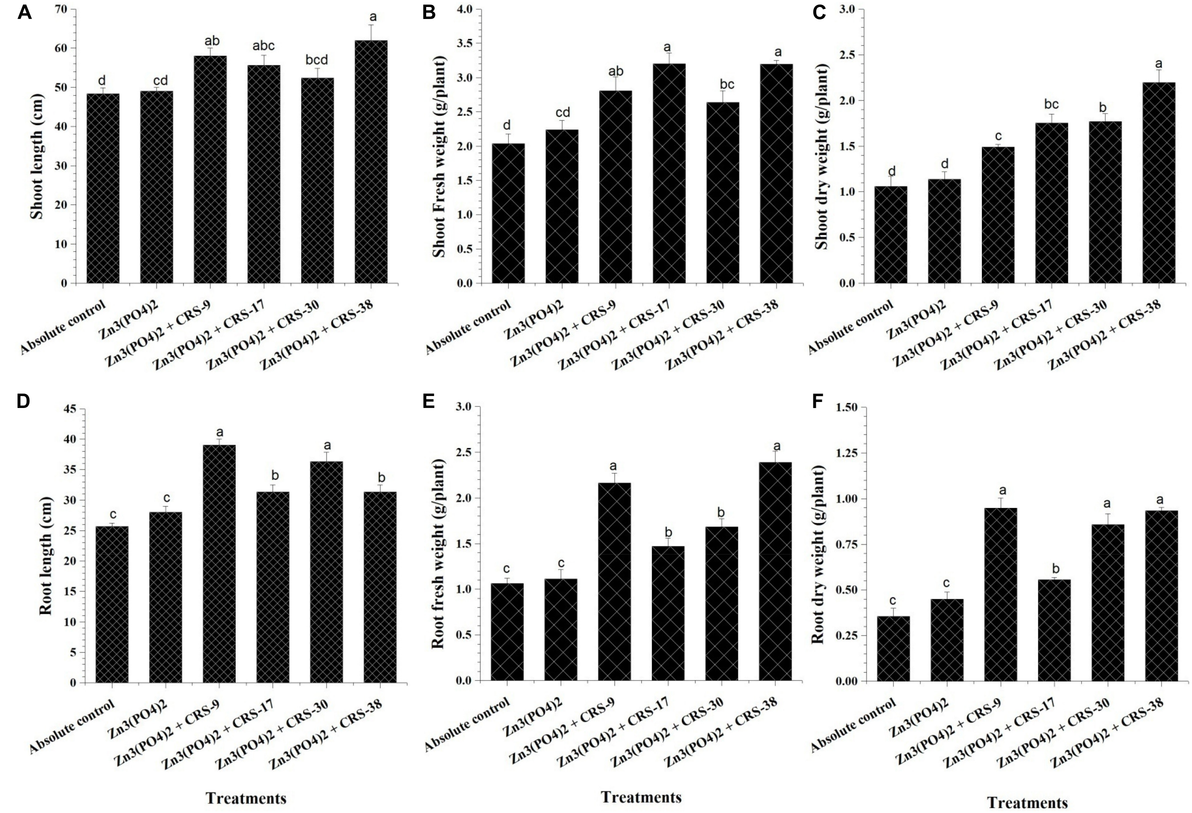

FIGURE 7 | Effect of zinc-solubilizing rhizobacterial inoculation on growth parameters of plants harvested after 120 days (second set). **(A)** Shoot length, **(B)** fresh weight of shoots, **(C)** dry weight of shoots, **(D)** root length, **(E)** fresh weight of roots, **(F)** dry weight of roots in comparison with absolute control. Data are mean ($n = 5$), and the vertical bar represents standard deviation. Data with different letters show a significant difference in column data in the randomized block design test at $p < 0.05$ under Duncan's multiple range test.

the pH of liquid media by rhizobacteria owing to their ability to produce different organic acids. Similar observations have been reported by several other workers (Desai et al., 2012; Kumari et al., 2016) who reported that gluconic acid is the main organic acid besides other organic acids in solubilization of insoluble minerals.

Promising zinc-solubilizing bacterial isolates, viz., CRS-9, CRS-17, CRS-30, and CRS-38 were identified as *B. glycinifermentans*, *M. oxydans*, *P. nicotinovorans,* and *B. tequilensis,* respectively. Scanning electron microphotographs clearly showed that all the four strains tested colonized wheat roots and developed biofilm in the root matrix. These bacterial genera have also been reported to colonize plant rhizosphere and increase plant growth (Çakmakçı et al., 2017; Singh et al., 2018a). Various species of *Bacillus* have been extensively studied for their potential plant growth-promoting activity (Mumtaz et al., 2017; Dinesh et al., 2018). In this study, *B. tequilensis* CRS-38 and *B. glycinifermentans* CRS-9 have been found to possess significant zinc solubilization activity and PGP traits. However, to the best of our knowledge, this is the first report on zinc solubilization and PGPR activity by *B. glycinifermentans*, although the selected rhizobacteria have been previously isolated from Ohio soil and soybean food in Korea (Zeigler, 2016). Similarly, species belonging to *Microbacterium* have also been found to exhibit multiple plant growth-promoting characteristics (Ouertani et al., 2020). In this study, *M. oxydans* CRS-17 has shown numerous plant growth-promoting activities (**Supplementary Table 3**) and has also been found to solubilize zinc in its insoluble form (**Table 1**). Reports have shown that the plant growth-promoting strain of *M. oxydans* increases the overall biomass of rape plants grown on metal-contaminated soil (Ren et al., 2019). However, reports on zinc solubilization by *M. oxydans* are lacking elsewhere. *Paenarthrobacter* spp. has been reported to produce siderophores and indole acetic acid, and fix nitrogen. *P. nicotinovorans* (formerly known as *Arthrobacter nicotinovorans*) was previously known for its ability of P solubilization, IAA production, and ACC deaminase activity, and enhances P uptake and plant biomass in maize (Pereira and Castro, 2014). In this study, *P. nicotinovorans* CRS-30 also showed multiple PGPR traits along with solubilization of zinc, phosphorus, and potassium (**Supplementary Table 3**).

To the best of our knowledge, the three strains, *B. glycinifermentans* CRS-9, *M. oxydans* CRS-17, and *P. nicotinovorans* CRS-30 utilized in this study have not yet been reported for their zinc-solubilizing ability, zinc biofortification, and contribution in promoting growth of wheat

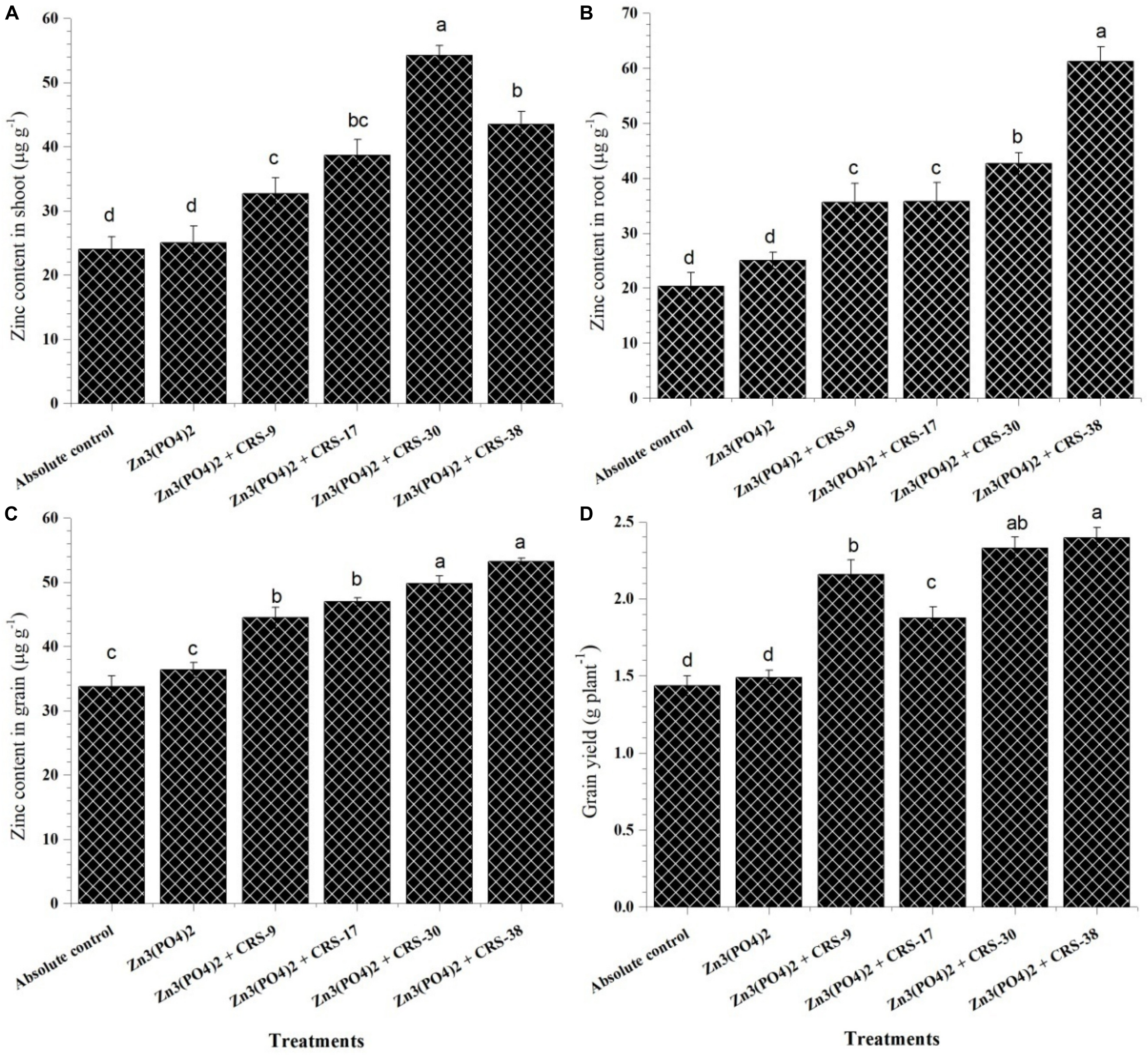

FIGURE 8 | Estimation of zinc content in **(A)** shoots, **(B)** roots, **(C)** grains, and **(D)** grain yield of inoculated and un-inoculated plants harvested after 120 days (second set). Data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0 : 05$ under Duncan's multiple range test.

plants. Inoculation of wheat plants with these strains resulted in a significant positive difference in root and shoot length, biomass, and zinc content compared to that with the absolute control. At 30 DAS, *B. glycinifermentans* CRS-9 and *P. nicotinovorans* CRS-30 were found to significantly increase zinc concentration in the shoots, while the maximum increase in zinc content in the roots was recorded for inoculation with *B. tequilensis* CRS-38 followed by that with *P. nicotinovorans* CRS-30 and *B. glycinifermentans* CRS-9. However, zinc concentration in the shoots and roots was maximally increased by *P. nicotinovorans* CRS-30 and *B. tequilensis* CRS-38 at the time of harvesting (120 DAS). *B. glycinifermentans* CRS-9, *P. nicotinovorans* CRS-30, and *B. tequilensis* CRS-38 were found to be promising when the zinc content of wheat grains was analyzed at the time of harvest (120 DAS). These observations support that these PGPR genera have significantly contributed to enhancement of the bioavailability of zinc in the shoots, roots, and grains of the wheat plant and provide it with more available zinc as against absolute control.

The ZIP (Zn-regulated, iron-regulated transporter-like protein) transporter family is one of the key gene families regulating the uptake, transport, and accumulation of Zn and Fe in plants (Singh et al., 2017, 2018a,b). It is a widely studied transporter playing an important role in several developmental processes, such as plant growth, uptake, and translocation of key microelements, tissues differentiation, biofortification, etc., in plant systems (Velmourougane et al., 2017). Although the ZIP protein family has been widely and perhaps systematically studied in *Arabidopsis*, a model plant, and many other plant species, the significance of this family in wheat upon inoculation of *B. glycinifermentans* CRS-9, *M. oxydans* CRS-17, and

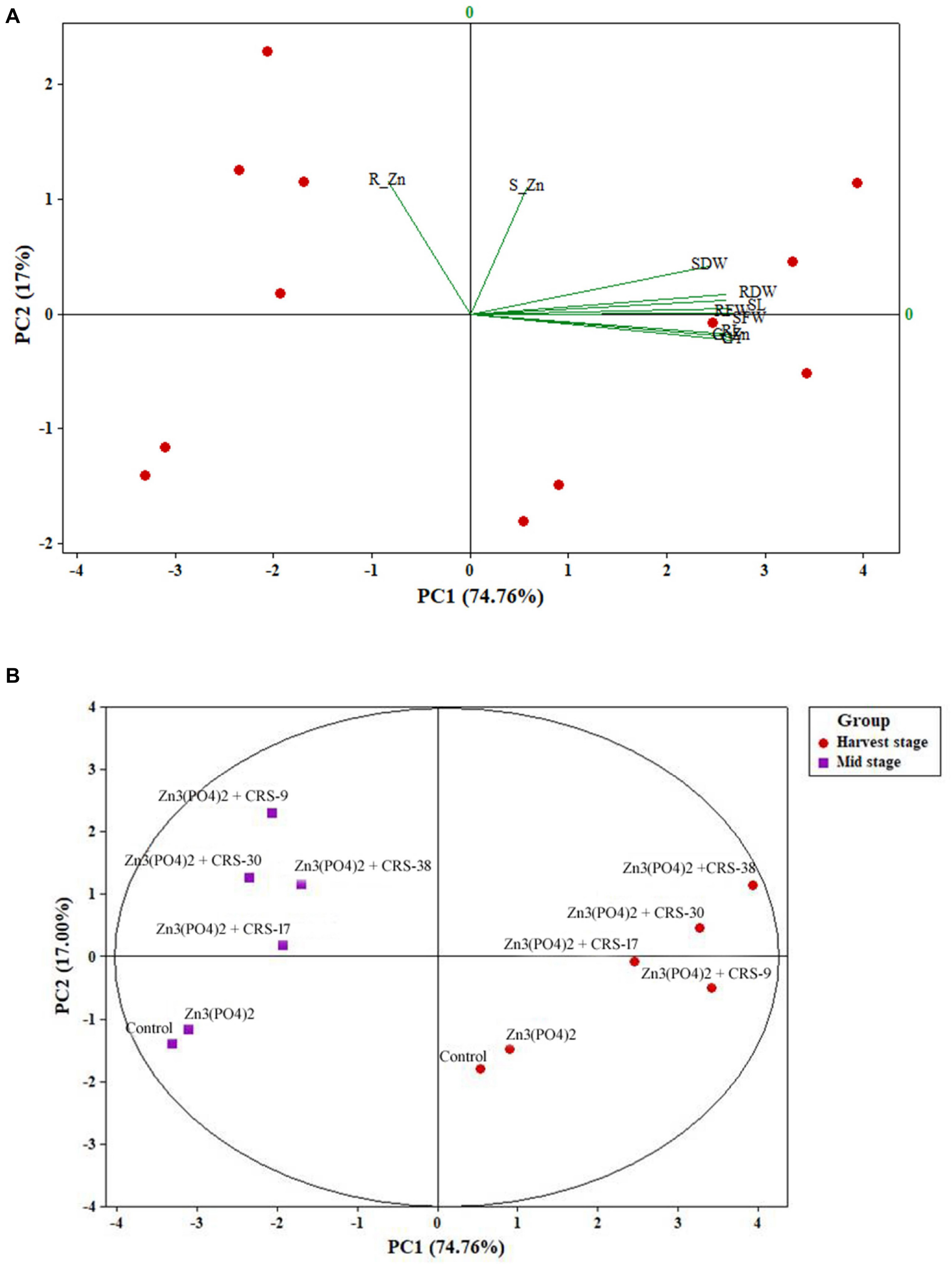

FIGURE 9 | (A) Grouping of variables of wheat crops based on principal component scores (PC1 and PC2) and **(B)** grouping of rhizobacterial strains based on principal component scores (PC1 and PC2) derived from variables (SWD, RWD, RL, SL, RFW, R_Zn, S_Zn, and G_Zn) of wheat crop after 30 and 120 days (harvest stage) of wheat crop sowing.

P. nicotinovorans CRS-30 is not well-understood at present. The results of glasshouse experiments with *B. glycinifermentans* CRS-9, *M. oxydans* CRS-17, and *P. nicotinovorans* CRS-30 clearly showed the significance of these inoculants on uptake, translocation, and bioaccumulation of Zn in wheat. The expression analysis clearly showed that the *TaZIPs* genes were significantly upregulated and highly expressed in the roots, and that nine *TaZIP* genes were up-regulated in the roots, stems, and leaves of plants bioprimed with selected microbial inoculants in the presence of zinc phosphate after 30 days of sowing.

Enhanced zinc content in the roots and shoots as against absolute plants is in line with the previous reports wherein inoculation of plants with PGPR led to enhanced yield, plant growth, and improved nutrition. Besides the increase in the overall yield of the plant, PGPR have been reported to be useful in countering nutrient deficiencies and have received attention for their use as biofertilizers. Ramesh et al. (2014b) reported increased mobilization of zinc by zinc-solubilizing *Bacillus aryabhattai* in wheat and soybean. Furthermore, the application of PGPR boosted the translocation of zinc toward wheat grains, and this is because of the ability of rhizobacteria to successfully execute plant-microbe interactions, viz., induction of physiological processes, mineralization, and solubilization (Lucas et al., 2014; Wang et al., 2014). Zinc solubilization is important in improving plant growth and cannot be overlooked. Zinc solubilization by PGPR is a comparatively new approach, but not many strains have yet been reported for this activity. This investigation explored the potential of *B. tequilensis* and *P. nicotinovorans* for their use as biofertilizers to overcome zinc deficiency in wheat crops.

CONCLUSION

Our study on zinc-solubilizing, plant growth-promoting rhizobacteria in wheat revealed that their inoculation is an effective method for enhancing the growth of wheat and maintaining nutritional quality. Out of four rhizobacteria, *B. tequilensis* CRS-38 and *P. nicotinovorans* CRS-30 inoculation improved the expression of eight *TaZIP* transporter genes in the roots after 30 days of wheat growth and increased grain yield and grain with zinc in wheat crop. The zinc solubilization potential of *B. glycinifermentans* CRS-9, *M. oxydans* CRS-17, and *P. nicotinovorans* CRS-30 has been reported for the first time in this study. These bacteria could be used as bio-input for improving productivity while combating nutrient deficiency in wheat as an option both in conjunction with and without chemical fertilizers, and such practice would help in achieving the objectives of SDG1, SDG2 and SDG3 of the United Nations by 2030.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SS, AV, PS, and AS conceptualized the idea for this research study and corrected base manuscript. RY designed the experiments, and conducted and developed the first draft of research manuscript. DM and US conducted the transporter gene expression experiments. MR and MK analyzed the data using different kinds of software and interpreted the data thereafter. All authors read and gave consent to the final draft of manuscript.

FUNDING

We would gratefully acknowledge the Indian Council of Agricultural Research, Ministry of Agriculture and Farmers Welfare, Government of India, for providing financial support for the study.

ACKNOWLEDGMENTS

We would like to thank Director, ICAR-NBAIM, Mau, Uttar Pradesh, India for extending facilities to carry out this study. We would also like to thank Brijesh K. Yadav for providing technical support during experimentation and soil analyses. The technical assistance rendered by Alok Upadhyay is also acknowledged with thanks. We would also further like to thank J. C. Tarafdar, UGC-Emeritus Professor, for editing the English language of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.777771/full#supplementary-material

REFERENCES

Abaid Ullah, M., Hassan, M. N., Jamil, M., Brader, G., Shah, M. K. N., and Sessitsch, A. (2015). Plant growth promoting rhizobacteria: an alternate way to improve yield and quality of wheat (*Triticum aestivum*). *Int. J. Agric. Biol.* 17, 51–60.

Aghili, F., Gamper, H. A., Eikenberg, J., Khoshgoftarmanesh, A. H., Afyuni, M., Schulin, R., et al. (2014). Green manure addition to soil increases grain zinc concentration in bread wheat. *PLoS One* 9:101487. doi: 10.1371/journal.pone.0101487

Ahmad, E., Sharma, S. K., and Sharma, P. K. (2020). Deciphering operation of tryptophan-independent pathway in high indole-3-acetic acid (IAA) producing *Micrococcus aloeverae* DCB-20. *FEMS Microbiol. Lett.* 367:fnaa190. doi: 10.1093/femsle/fnaa190

Alloway, B. J. (2004). *Zinc in Soil and Crop Nutrition*. Belgium: International Zinc Association.

Alloway, B. J. (2008a). "Micronutrients and crop production: an introduction," in *Micronutrient Deficiencies in Global Crop Production*, ed. B. J. Alloway (Dordrecht: Springer), 1–39. doi: 10.1007/978-1-4020-6860-7_1

Alloway, B. J. (2008b). *Zinc in Soils and Crop Nutrition*. Brussels: International Zinc Association, 1–135.

Bal, H. B., Nayak, L., Das, S., and Adhya, T. K. (2013). Isolation of ACC deaminase producing PGPR from rice rhizosphere and evaluating their plant growth promoting activity under salt stress. *Plant Soil* 366, 93–105. doi: 10.1007/s11104-012-1402-5

Bouis, H. E. (2003). Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost. *Proc. Nutr. Soc.* 62, 403–411. doi: 10.1079/pns2003262

Cakmak, I. (2008). Enrichment of cereal grains with zinc: agronomic or genetic biofortification. *Plant Soil* 302, 1–17. doi: 10.1007/s11104-007-9466-3

Çakmakçı, R., Turan, M., Kıtır, N., Güneş, A., Nikerel, E., Özdemir, B. S., et al. (2017). "The role of soil beneficial bacteria in wheat production: a review," in *Wheat Improvement, Management and Utilization*, Vol. 24, eds R. Wanyera and J. Owuoche (London: Intech), 115–149.

Chang, H. B., Lin, C. W., and Huang, H. J. (2005). Zinc-induced cell death in rice (*Oryza sativa* L.) roots. *Plant Growth Regul*. 46, 261–266. doi: 10.1007/s10725-005-0162-0

Desai, S., Kumar, P., Sultana, U., Pinisetty, S., and Reddy, G. (2012). Potential microbial candidate strains for management of nutrient requirements of crops. *Afr. J. Microbiol. Res.* 6, 3924–3931.

Dimpka, C. O., and Bindraban, P. S. (2016). Fortification of micronutrients for efficient agronomic production. *Ann. Rev. Agron. Sustain. Dev.* 36:7.

Dinesh, R., Srinivasan, V., Hamza, S., Sarathambal, C., Gowda, S. A., Ganeshamurthy, A. N., et al. (2018). Isolation and characterization of potential Zn solubilizing bacteria from soil and its effects on soil Zn release rates, soil available Zn and plant Zn content. *Geoderma* 321, 173–186. doi: 10.1016/j.geoderma.2018.02.013

Epstein, E., and Bloom, A. J. (2005). *Mineral Nutrition of Plants: Principles and Perspectives*, 2nd Edn. Sunderland, MA: Sinauer Associates.

Fasim, F., Ahmed, N., Parsons, R., and Gadd, G. M. (2002). Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. *FEMS Microbiol. Lett.* 213, 1–6. doi: 10.1111/j.1574-6968.2002.tb11277.x

Gandhi, A., and Muralidharan, G. (2016). Assessment of zinc solubilizing potentiality of *Acinetobacter* sp. isolated from rice rhizosphere. *Eur. J. Soil Biol.* 76, 1–8. doi: 10.1016/j.ejsobi.2016.06.006

Garcia, L. S., and Isenberg, H. D. (2007). *Clinical Microbiology Procedures Handbook*, 2nd Edn. Washington, DC: ASM Press.

Hafeez, F. Y., Abaid-Ullah, M., and Hassan, M. N. (2013). "Plant growth-promoting rhizobacteria as zinc mobilizers: a promising approach for cereals biofortification," in *Bacteria in Agrobiology: Crop Productivity*, eds D. K. Maheshwari, M. Saraf, and A. Aeron (Berlin: Springer), 217–235. doi: 10.1007/978-3-642-37241-4_9

Helfenstein, J., Müller, I., Grüter, R., Bhullar, G., Mandloi, L., Papritz, A., et al. (2016). Organic wheat farming improves grain zinc concentration. *PLoS One* 11:e0160729. doi: 10.1371/journal.pone.0160729

Hussain, A., Arshad, M., Zahir, Z. A., and Asghar, M. (2015). Prospects of zinc solubilizing bacteria for enhancing growth of maize. *Pak. J. Agric. Sci.* 52, 915–922.

Igrejas, G., and Branlard, G. (2020). "The importance of wheat," in *Wheat Quality For Improving Processing and Human Health*, eds G. Igrejas, T. M. Ikeda, and C. Guzmán (Cham: Springer), 1–7. doi: 10.4324/9780429267505-1

Iqbal, U., Jamil, N., Ali, I., and Hasnain, S. (2010). Effect of zinc-phosphate-solubilizing bacterial isolates on growth of *Vigna radiata*. *Ann. Microbiol.* 60, 243–248. doi: 10.1007/s13213-010-0033-4

ISTA (2003). *Seed Testing International (ISTA News Bulletin) No. 126*.

Jones, D. L., and Darrah, P. R. (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant Soil* 166, 247–257. doi: 10.1007/bf00008338

Khande, R., Sharma, S. K., and Sharma, M. P. (2017). Zinc solubilizing *Bacillus* strains that modulate growth, yield and zinc biofortification of soybean and wheat. *Rhizosphere* 4, 126–138. doi: 10.1016/j.rhisph.2017.09.002

Kumari, S., Padma Devi, S. N., and Vasandha, S. (2016). Zinc solubilizing bacterial isolates from the agricultural field of Coimbatore, Tamil Nadu, and India. *Curr. Sci.* 110, 196–205. doi: 10.18520/cs/v110/i2/196-205

Larcher, R., Nicolini, G., Roman, T., Bertoldi, D., and Puecher, C. (2009). Determination of gluconic acid in wine using high pressure liquid chromatography with pulsed amperometric detection. *Vitis* 48, 201–204.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lucas, J. A., García-Cristobal, J., Bonilla, A., Ramos, B., and Gutierrez-Manero, J. (2014). Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. *Plant Physiol. Biochem.* 82, 44–53. doi: 10.1016/j.plaphy.2014.05.007

Mumtaz, M. Z., Ahmad, M., Jamil, M., and Hussain, T. (2017). Zinc solubilizing *Bacillus* spp. potential candidates for biofortification in maize. *Microbiol. Res.* 202, 51–60. doi: 10.1016/j.micres.2017.06.001

Nahas, E. (1996). Factors determining rock phosphate solubilization by microorganisms isolated from soil. *World J. Microbiol. Biotechnol*. 12, 567–572. doi: 10.1007/BF00327716

Naz, I., Ahmad, H., Khokhar, S. N., Khan, K., and Shah, A. H. (2016). Impact of zinc solubilizing bacteria on zinc contents of wheat. *Am. Euras. J. Agric. Environ. Sci.* 16, 449–454. doi: 10.3390/plants9101375

Ouertani, R., Ouertani, A., Mahjoubi, M., Bousselmi, Y., Najjari, A., Cherif, H., et al. (2020). New plant growth-promoting, chromium-detoxifying microbacterium species isolated from a tannery wastewater: performance and genomic insights. *Front. Bioeng. Biotechnol*. 38:521. doi: 10.3389/fbioe.2020.00521

Penrose, D. M., and Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant* 118, 10–15. doi: 10.1034/j.1399-3054.2003.00086.x

Pereira, S. I., and Castro, P. M. (2014). Phosphate-solubilizing rhizobacteria enhance Zea mays growth in agricultural P-deficient soils. *Ecol. Eng*. 1, 526–535. doi: 10.1016/j.ecoleng.2014.09.060

Prasad, J., Karmakar, S., Kumar, R., and Mishra, B. (2010). Influence of integrated nutrient management on yield and soil properties in maize-wheat cropping system in an Alfisol of Jharkhand. *J. Indian Soc. Soil Sci.* 58, 200–204.

Rajawat, M. V. S., Singh, S., Tyagi, S. P., and Saxena, A. K. (2016). A modified plate assay for rapid screening of potassium-solubilizing bacteria. *Pedosphere* 26, 768–773. doi: 10.1016/s1002-0160(15)60080-7

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014a). Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. *Appl. Soil. Ecol.* 73, 87–96. doi: 10.1016/j.apsoil.2013.08.009

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014b). Plant growth-promoting traits in *Enterobacter cloacae* subsp. *dissolvens* MDSR9 isolated from soybean rhizosphere and its impact on growth and nutrition of soybean and wheat upon inoculation. *Agric. Res*. 3, 53–66. doi: 10.1007/s40003-014-0100-3

Ren, X. M., Guo, S. J., Tian, W., Chen, Y., Han, H., Chen, E., et al. (2019). Effects of plant growth-promoting bacteria (PGPB) inoculation on the growth, antioxidant activity, Cu uptake, and bacterial community structure of rape (*Brassica napus* L.) grown in Cu-contaminated agricultural soil. *Front. Microbiol*. 27:1455. doi: 10.3389/fmicb.2019.01455

Saravanan, V. S., Kumar, M. R., and Sa, T. M. (2011). "Microbial zinc solubilization and their role on plants," in *Bacteria in Agrobiology: Plant Nutrient Management*, ed. D. Maheshwari (Berlin: Springer), 47–63. doi: 10.1007/978-3-642-21061-7_3

Saravanan, V. S., Madhaiyan, M., and Thangaraju, M. (2007a). Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacter diazotrophicus*. *Chemosphere* 66, 1794–1798. doi: 10.1016/j.chemosphere.2006.07.067

Saravanan, V. S., Kalaiarasan, P., Madhaiyan, M., and Thangaraja, M. (2007b). Solubilization of insoluble zinc compounds by *Gluconacetobacter diazotrophicus* and the detrimental action of zinc ion (Zn^{+2}) and zinc chelates on root knot nematode *Meloidogyne incognita*. *Lett. Appl. Microbiol*. 44, 235–241. doi: 10.1111/j.1472-765X.2006.02079.x

Saravanan, V. S., Subramoniam, S. R., and Raj, S. A. (2004). Assessing *in vitro* solubilization potential of different zinc solubilizing bacterial (ZSB) isolates. *Braz. J. Microbiol.* 35, 121–125.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem*. 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Seshadri, S., Ignacimuthu, S., and Lakshminarsimhan, C. (2002). Variations in hetetrophic and phosphate solubilizing bacteria from Chennai, southeast coast of India. *Indian J. Mar. Sci.* 31, 69–72.

Sharma, S. K., Johri, B. N., Ramesh, A., Joshi, O. P., and Sai Prasad, S. V. (2011). Selection of plant growth-promoting *Pseudomonas* spp. that enhanced productivity of soybean-wheat cropping system in central India. *J. Microbiol. Biotechnol*. 21, 1127–1142. doi: 10.4014/jmb.1012.12018

Sharma, S. K., Sharma, M. P., Ramesh, A., and Joshi, O. P. (2012). Characterization of zinc-solubilizing *Bacillus* isolates and their potential to influence zinc assimilation in soybean seeds. *J. Microbiol. Biotechnol*. 22, 352–359. doi: 10.4014/jmb.1106.05063

Singh, U. B., Malviya, D., Khan, W., Singh, S., Karthikeyan, N., Imran, M., et al. (2018c). Earthworm grazed-*Trichoderma harzianum* biofortified spent mushroom substrates modulate accumulation of natural antioxidants and bio-fortification of mineral nutrients in tomato. *Front. Plant Sci.* 9:1017. doi: 10.3389/fpls.2018.01017

Singh, D., Geat, N., Rajawat, M. V. S., Prasanna, R., Kar, A., Singh, A., et al. (2018a). Prospecting endophytes from different Fe or Zn accumulating wheat genotypes for their influence as inoculants on plant growth, yield, and micronutrient content. *Ann. Microbiol.* 68, 815–833.

Singh, D., Geat, N., Rajawat, M. V. S., Mahajan, M. M., Prasanna, R., Singh, S., et al. (2018b). Deciphering the mechanisms of endophyte-mediated biofortification of Fe and Zn in wheat. *J. Plant Growth Regul.* 37, 174–182. doi: 10.1007/s00344-017-9716-4

Singh, D., Rajawat, M. V. S., Kaushik, R., Prasanna, R., and Saxena, A. K. (2017). Beneficial role of endophytes in biofortification of Zn in wheat genotypes varying in nutrient use efficiency grown in soils sufficient and deficient in Zn. *Plant Soil* 416, 107–116.

Singh, S., Singh, U. B., Trivedi, M., Malviya, D., Sahu, P. K., Roy, M., et al. (2021). Restructuring the cellular responses: connecting microbial intervention with ecological fitness and adaptiveness to the maize (*Zea mays* L.) grown in saline–sodic soil. *Front. Microbiol.* 11:568325. doi: 10.3389/fmicb.2020.568325

Singh, U. B., Malviya, D., Singh, S., Kumar, M., Sahu, P. K., Singh, H. V., et al. (2019a). *Trichoderma harzianum*- and methyl jasmonate-induced resistance to *Bipolarissorokiniana* through enhanced phenylpropanoid activities in bread wheat (*Triticumaestivum* L.). *Front. Microbiol.* 10:1697. doi: 10.3389/fmicb.2019.01697

Singh, U. B., Malviya, D., Singh, S., Pradhan, J. K., Singh, B. P., Roy, M., et al. (2016). Bio-protective microbial agents from rhizosphere eco-systems trigger plant defense responses provide protection against sheath blight disease in rice (*Oryza sativa* L.). *Microbiol. Res.* 192, 300–312. doi: 10.1016/j.micres.2016.08.007

Singh, U. B., Singh, S., Malviya, D., Karthikeyan, N., Imram, M., Chaurasia, R., et al. (2019b). Integration of anti-penetrant tricyclazole, signaling molecule salicylic acid and root associated *Pseudomonas fluorescens* enhances suppression of *Bipolaris sorokiniana* in bread wheat (*Triticum aestivum* L.). *J. Plant Pathol.* 101, 943–954. doi: 10.1007/s42161-019-00296-5

Sunithakumari, K., Devi, S. P., and Vasandha, S. (2016). Zinc solubilizing bacterial isolates from the agricultural fields of Coimbatore, Tamil Nadu, India. *Curr. Sci.* 110, 196–205.

Tavallali, V., Rahemi, M., Eshghi, S., Kholdebarin, B., and Ramezanian, A. (2010). Zinc alleviates salt stress and increases antioxidant enzyme activity in the leaves of pistachio (*Pistaciavera L. 'Badami'*) seedlings. *Turk. J. Agric. For.* 34, 349–359.

Vazquez, P., Holguin, G., Puente, M. E., Lopez-Cortes, A., and Bashan, Y. (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biol. Fertil. Soils* 30, 460–468.

Velmourougane, K., Prasanna, R., Singh, S., Chawla, G., Kumar, A., Saxena, A. K., et al. (2017). Modulating rhizosphere colonization, plant growth, soil nutrient availability and plant defense enzyme activity through *Trichoderma viride-Azotobacter chroococcum* biofilm inoculation in chickpea. *Plant Soil* 421, 157–174. doi: 10.1007/s11104-017-3445-0

Wakatsuki, T. (1995). Metal oxidoreduction by microbial cells. *J. Ind. Microbiol.* 14, 169–177. doi: 10.1007/BF01569900

Wang, Y., Yang, X., Zhang, X., Dong, L., Zhang, J., and Wei, Y. (2014). Improved plant growth and Zn accumulation in grains of rice (*Oryza sativa* L.) by inoculation of endophytic microbes isolated from a Zn hyperaccumulator, *Sedum alfredii* H. *J. Agric. Food Chem.* 62, 1783–1791. doi: 10.1021/jf404152u

White, P. J., and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in humane diets: iron, zinc copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84. doi: 10.1111/j.1469-8137.2008.02738.x

White, P. J., and Broadley, M. R. (2011). Physiological limits to zinc biofortification of edible crops. *Front. Plant Sci.* 2:80. doi: 10.3389/fpls.2011.00080

Yadav, R. C., Sharma, S. K., Ramesh, A., Sharma, K., Sharma, P. K., and Varma, A. (2020). "Contribution of zinc-solubilizing and-mobilizing microorganisms (ZSMM) to enhance zinc bioavailability for better soil, plant, and human health," in *Rhizosphere Microbes*, eds S. K. Sharma, U. B. Singh, P. K. Sahu, H. V. Singh, and P. K. Sharma (Singapore: Springer), 357–386. doi: 10.1007/978-981-15-9154-9_14

Zeigler, D. R. (2016). Genome sequence of *Bacillus glycinifermentans* TH008, isolated from Ohio soil. *Genome Announc.* 25:4. doi: 10.1128/genomeA.01573-15

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 23 March 2022
doi: 10.3389/fpls.2022.770179

Liquid Anaerobic Digestate as Sole Nutrient Source in Soilless Horticulture—Or Spiked With Mineral Nutrients for Improved Plant Growth

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Specialty section:
This article was submitted to Plant Nutrition, a section of the journal Frontiers in Plant Science

Received: *03 September 2021*
Accepted: *07 February 2022*
Published: *23 March 2022*

Citation:
Weimers K, Bergstrand K-J, Hultberg M and Asp H (2022) Liquid Anaerobic Digestate as Sole Nutrient Source in Soilless Horticulture—Or Spiked With Mineral Nutrients for Improved Plant Growth. Front. Plant Sci. 13:770179. doi: 10.3389/fpls.2022.770179

Digestate from biogas production high in plant-available macro- and micro-nutrients could replace mineral fertilizer in protected (soilless) horticulture. Previous uses of digestate have shown that low concentrations of plant-available phosphorus (P) and sulfur (S) may be limiting factors for growth when using digestate as the sole fertilizer. In this study, digestate collected from a municipal biogas plant in Sweden was nitrified in a moving-bed biofilm reactor prior to its use as fertilizer. A greenhouse pot trial with pak choi grown in peat-based growing medium was established to assess the (i) macro- and micro-nutrient availability in the digestate, with particular focus on P and S and (ii) the effect of amending the digestate solution with nutrients considered to be lacking [P, S, magnesium (Mg), manganese (Mn), boron (B), and molybdenum (Mo)]. The results showed that plants fertilized with raw digestate suffered from S and B deficiency and early P deficiency. Supplementing the digestate with nutrients originating from mineral salts resulted in sufficient plant tissue concentrations of all elements except S. The marketable yield was similar to that achieved using standard mineral fertilizer and the dry matter yield was 17% higher. In the light of the present results, the use of nitrified digestate in soilless plant production seems like a fruitful way forward to recycle organic nutrients from waste streams. In the case where a strict organic protocol is not needed, amendment with inorganic nutrients may be a way to increase the utilization of organically derived nutrients.

Keywords: bio-based society, biogas residues, hydroponics, nutrient solution, organic fertilizer, phosphorus, sulfur

INTRODUCTION

Anaerobic digestion of organic residues in biogas plants produces renewable energy and a residue containing nutrients essential to plant growth which is known as digestate. The digestate can be used as a plant fertilizer directly or after further processing, contributing to the closing of global energy and nutrient cycles (Alburquerque et al., 2012; Möller and Müller, 2012). According to Jain et al. (2019, and references therein), digestate could replace 5–7% of the inorganic macro nutrient fertilizer currently used globally if the potential of the biogas industry were fully utilized. Digestate is generally spread directly onto fields, like manure (Odhner et al., 2015). However, due to the high share of plant-available macro- and micro-nutrients, e.g., the nitrogen (N) content in a digestate from a mixed feedstock may typically be around 0.5% of the dry weight as was the case in the digestate used in this study (see "Materials and Methods" section), digestate also has the potential to replace mineral fertilizers in protected horticulture (soilless systems). This would increase the nutrient composition requirements for digestate because, unlike fertilizers in soil-based systems, fertilizers in soilless systems must provide the crop with all essential macro- and micro-nutrients at sufficient levels during the whole cropping cycle. To date, research on digestate fertilizers in protected horticulture has been scarce and the results are conflicting. The nutrient use efficiency and harvest when digestate was used compared to commercial fertilizers have been reported to be both higher and lower due, e.g., to different origins of the digestates (Guilayn et al., 2019) but also often dependent on the control treatment used in different studies (Liedl et al., 2004a,b; Ronga et al., 2019; Pelayo Lind et al., 2020). Other findings where results conflict are, e.g., the benefit of the nitrification of the digestate (Uchimura et al., 2014; Pokhrel et al., 2019; Cheong et al., 2020), availability of phosphorus (P) in the digestate (Stoknes et al., 2018), and the effects of adding inorganic nutrients to the digestate (Liu et al., 2009, 2011).

During anaerobic digestion, biogas, primarily composed of methane (CH_4), and carbon dioxide (CO_2), is produced through bacterial degradation of organic matter. The resulting digestate is a complex matrix of partially degraded organic matter, inorganic compounds, and microbial biomass (Möller, 2015) in proportions depending on the composition of the biomass feedstock and process parameters, e.g., operating temperature and average retention time (Alburquerque et al., 2012; Risberg, 2015). According to a review by Möller and Müller (2012), most plant nutrients in the raw feedstock are retained during the digestion process, and digestate normally contains all essential macro- and micro-nutrients in varying proportions, reflecting those in the feedstock. Due to mineralization and carbon removal during microbial anaerobic digestion, digestate is characterized by high ammonium nitrogen (NH_4-N) to total nitrogen ratio, alkaline pH (7.3–9.0), and increased solubilization of essential plant nutrients (Möller and Müller, 2012).

The alkaline pH in digestate can decrease the bioavailability of P and calcium (Ca), due to the formation of insoluble, complex Ca-P compounds, mainly hydroxylapatite (HAp, $Ca_5(PO_4)_2OH$) (Güngör et al., 2007; Marti et al., 2008). The amount of HAp formed in relation to more reactive plant-available compounds, such as struvite ($NH_4MgPO_4{\cdot}6H_2O$) and simple calcium phosphates, is partly determined by the amount of magnesium $(Mg)^{2+}$ and Ca^{2+} present in the solution (Nelson et al., 2003; Hjorth et al., 2010; Vanden Nest et al., 2021). Multiple studies have shown that low levels of available P are a factor limiting growth when digestate alone is used as fertilizer (Svensson et al., 2004; Liu et al., 2011; Abubaker et al., 2012; Losak et al., 2014; Stoknes et al., 2018; Pokhrel et al., 2019). To deal with low P availability, some researchers have supplemented digestate nutrient solutions with mineral P, resulting in increased yields of lettuce and kohlrabi (Liu et al., 2011; Losak et al., 2014).

Anaerobic digestion has also been shown to decrease the sulfur (S) content of the feedstock through emissions of hydrogen sulfide (H_2S) and other volatile S-containing compounds (Massé et al., 2007; Peu et al., 2011; Wahid et al., 2018; Fontaine et al., 2020). Hydrogen sulfide has corrosive properties and must be removed from the biogas stream using one of the varieties of techniques available (Moestedt et al., 2013). In large-scale plants, S-containing gases are commonly removed through the addition of iron (Fe) salts to the digester, resulting in the precipitation of dissolved sulfides with ferric or ferrous iron, which limits the formation of H_2S (Moestedt et al., 2013). Iron sulfides are insoluble in water and strongly decrease the plant availability of S.

In addition to P, S, and Ca, other nutrients in digestate may also be present in insufficient levels or forms not available to plants depending on the composition of the feedstock to the biogas plant and process parameters. For example, low levels of potassium (K) in digestate have been reported, as well as significant losses of the plant available micronutrients manganese (Mn), zinc (Zn), and copper (Cu) during digestion (Bloomfield and McGrath, 1982; Massé et al., 2007; Marcato et al., 2009; Abubaker et al., 2012; Zirkler et al., 2014). The yield of lettuce and tomatoes has been found to increase after the addition of Fe and Mg, respectively, to digestate (Liedl et al., 2004b; Liu et al., 2011).

When digestate is used as fertilizer in systems lacking nitrifying microbiota from the soil, the high NH_4^+-N content in the digestate can result in NH_4^+ toxicity, with negative impacts on growth and biomass production. The toxicity to NH_4^+ is mainly found when soilless- or various hydroponic production systems are used (Liedl et al., 2004a; Neal and Wilkie, 2014; Ronga et al., 2019). Accordingly, the bacterial oxidation of NH_3 (in equilibrium with NH_4^+) to nitrate (NO_3^-) in bioreactors prior to application or in integrated biofilters in the system is recommended and is reported to result in yields similar to those produced using commercial fertilizers in e.g., tomato, lettuce, and pak choi (Stoknes et al., 2018; Pelayo Lind et al., 2020).

In the present study, the plant availability of macro- and micro-nutrients in liquid anaerobic digestate was determined. The digestate was collected from a municipal biogas plant and nitrified in a moving bed biofilm reactor (MBBR) prior to use as fertilizer in the production of pak choi grown in limed peat. Plant growth, quality, and physiological parameters were studied and compared with those of pak choi plants produced with standard fertilization using mineral nutrients only. Special attention was given to the plant uptake of P and S. The experiments were designed to compare the growth performance of plants fertilized with an anaerobic digestate to plants in a mineral fertilizer made to resemble the nutrient composition in the digestate. In another comparison, the anaerobic digestate was amended with mineral nutrients: P, S, magnesium (Mg), Mn, boron (B), and molybdenum (Mo), considered low when compared to the concentrations in commercial mineral fertilizer. The questions to be answered by the study were if the minerals, especially P and S, were taken up as effectively by the plants from the organic fertilizer as from the mineral one; or if a less effective uptake of these elements was the case, due to the reasons discussed above. Also, the overall performance of the plants i.e., growth and mineral content was a question of interest since the presence of several organic compounds may interfere with the plant's physiology and biochemistry as well as on the availability of nutrients. The hypothesis, based on the intention to measure the plant-available nutrients, is that the plants given the organic fertilizer will perform equally well as the ones receiving a mineral fertilizer, despite the different origin of the fertilizers and the different complexity of the matrices in the solutions.

MATERIALS AND METHODS

Plant Material and Growing Conditions

A greenhouse study was conducted between May and June at the Department of Biosystems and Technology, Swedish University of Agricultural Science, Alnarp, Sweden. On April 30, pak choi (*Brassica rapa,* ssp. *chinensis*, "Joy Choi," Olssons Frö AB, Helsingborg, Sweden) seeds were sown in a plug tray, with one seed per plug in the same growing media as described below. The emerging plantlets were sub-irrigated and fertilized with half-strength commercial inorganic fertilizer for soilless production (0.5 + 0.5 g L^{-1}, respectively, of Calcinit^{TM} and Kristalon^{TM} Indigo; Yara, Oslo, Norway). Two weeks after sowing, the plantlets were transferred to 2-L pots with trays. A growing medium of peat moss (0–25 mm, H2-4, H5-7; SW Horto AB, Sweden) with 5.5 kg m^{-3} dolomite lime [$CaMg(CO_3)_2$], (54% liming effect compared to pure CaO, Björka mineral AB, Sweden) giving a pH (H_2O) of 6.1, was used in all treatments. The dry bulk density was 284 g L^{-1} and the porosity was 72%. To get the same amount of growing medium to each pot, it was weighed to give two L based on the peat bulk density (EN13040:2007). The pots were kept in a greenhouse compartment where the temperature was set to 18°C and the roof ventilation was opened at 20°C. The greenhouse shading screen was closed when the outdoor light intensity was above 1,200 W m^{-2} s^{-1}. Only natural light was provided, giving a weekly mean of 150 MJ (PAR) m^{-2} (Priva Intégro v. 730 + Priva Office, Priva, De Lier, the Netherlands). The plants were irrigated with tap water according to need, which was every seventh day at the beginning of the experiment and once a day by the end of the experiment. Water was manually slowly added into each pot until the drained water covered the trays with 5 mm water. The water was later sought up by the plants and no water was lost through drainage. The plants were harvested on 19 June, 51 days after sowing.

Fertilization Strategy

The N requirement of *B. rapa* "Joi Choi" was calculated using an estimated shoot fresh matter (FM) yield of 250 g per plant, 30% weight addition for root FM, 95% water content, and 3.5% N content in dry matter (DM), resulting in an estimated N assimilation of 570 mg $plant^{-1}$ for the whole cultivation time. It was assumed that 15% of the added N remained unavailable to the plants (30% of the original digestate consisted of non-mineralized N, as can be seen by the Kjeldahl analysis, an assumption was made that half of this could be mineralized during the nitrification process and later in the growing medium), resulting in an estimated N requirement of 650 mg $plant^{-1}$. Thus, the common basis for the four different fertilizer solutions used in the experiment was the total N addition to the treatments, with all receiving a total of 650 mg N $plant^{-1}$. The plant-available N content in the peat was 20 mg L^{-1}. Beginning 3 days after planting, the plants were fed a nutrient solution every second or third day for a total of 13 occasions. The nutrient dose was increased stepwise during the cultivation period, with a starting dose that was half the final dose. The nutrient solutions were stored at 5°C before being used as fertilizers.

Mineral Fertilizer

A slightly modified version of Sonneveld and Straver's (1994) nutrient solution, formulated to optimize the growth of Asiatic vegetables including pak choi in hydroponic systems, was used as a reference (M2 in **Table 1**; Sonneveld and Straver, 1994; Bergstrand and Hultin, 2014). The relative proportions of the nutrients by weight, with the total N set to one, were: NO_3-N 0.93, NH_4-N 0.07, P 0.2, K 1.37, Ca 0.64, Mg 0.15, S 0.18, Fe 0.012, Mn 0.003, Zn 0.0017, B 0.00014, Cu 0.0002, and Mo 0.00024 (Sonneveld and Straver, 1994; Bergstrand and Hultin, 2014). The solution was diluted to give a final concentration of 250 mg N L^{-1} to match the concentrations in the digestate treatment. The final amounts added to the treatments are shown in **Table 2**.

Anaerobic Digestate

Biogas digestate was collected at the Karpalund municipal biogas plant in southern Sweden in February. The feedstock entering the biogas plant consisted of 37% organic household waste, 29% manure (2/3 pig manure and 1/3 cattle manure), 21% slaughter waste, 5% fat from grease separators, 8% other food waste, and < 0.3% iron chloride and iron sludge as processing aids. The average temperature during digestion was 44°C; and the retention time in the reactor was 50 days.

After sieving through a 0.8 mm mesh, the nutrient content in the digestate was analyzed by an accredited laboratory (Eurofins Environment Testing, Sweden AB, Lidköping) using the Kjeldahl and Devarda methods for the total-N, the Kjeldahl method for NH_4-N (Standard Methods 4500-N mod.) (APHA, 1998), silver nitrate titration for Cl, and, for the remaining substances, extraction with aqua regia (HNO_3 + 3 HCl) and determination of concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES), in accordance with International Organisation for Standardisation (ISO) 11466. The nutrient content in the digestate per kg^{-1} (fresh weight) was as follows: in g kg^{-1} total-N 5.3, NH_4-N 3.7, P.25, K 1.5, Ca 0.7, Mg 0.045, S 0.28, Na 0.8, and Cl 1.8 and in mg kg^{-1}: Fe 325, Zn 9.25, Mn 5.25, Cu 3.25, B.6, Mo 0.1225, and Co 0.05. The total solids content was 2.5% and the pH was 8.1.

The digestate was sieved through a 0.8 mm mesh and nitrified in an aerated small-scale MBBR prior to the experiment, in order to lower the NH_4-N/NO_3-N ratio, as described by Bergstrand et al. (2020). A 120 L plastic container served as a reactor. It was filled with 70 L distilled water and 18 L biofilm carriers (K3, AnoxKaldnes, Lund, Sweden) taken from an ongoing nitrification with the same digestate as used in this experiment. The digestate was loaded automatically into the reactor in addition, portioned to keep the NH_4^+-N concentration below 2 mg L^{-1}. The pH was kept at 5.6–5.8 by the addition of the raw digestate or K_2CO_3 when the pH fell below 5.6. A Hach DR1900 spectrophotometer was used to monitor the concentrations of NH_4^+, nitrite (NO_2^-) and NO_3^- during the nitrification process (Hach Lange tests LCK 303 for [NH_4^+], LCK

TABLE 1 | Fertilizer treatments in the soilless production of pak choi and the main variables tested.

Treatment			Variable tested
Digestate treatments	D1	Nitrified digestate.	
	D2	Nitrified digestate + P, Mg, S, Mn, B, and Mo, to resemble the nutrient composition of M2.	Compared to D1: The effect of added mineral nutrients on plant growth.
Mineral treatments	M1	Mineral nutrient solution designed to mimic the total nutrient composition of D1.	Compared to D1: The plant availability of nutrients in the nitrified digestate.
	M2	Standard mineral nutrient solution, designed for optimal growth.	Compared to D2: The plant availability of added mineral nutrients in the nitrified digestate.
Negative control	W	Water	Negative control.

TABLE 2 | Total amounts of nutrients (mg) supplied to each plant during the cultivation time, as nitrified digestate (D1 and D2) or mineral fertilizer (M1 and M2), in the different treatments (in total, 2.6 l of respective nutrient solution per plant).

	Treatment				Growing medium
	Plain digestate	**Amended digestate**	**Mineral digestate equivalent**	**Standard mineral solution**	**$CaMg(CO_3)_2$ (liming)**
	D1	D2	M1	M2	
NH_4-N	230	230	214	43	
NO_3-N	420	420	432	605	
Tot N_{min}	650	650	646	648	
K	1,241	1,241	1,243	885	
P	97	**128**	101	128	
Ca	144	**170**	36	413	2,391
Mg	10	**41**	10	100	1,450
S	54	**115**	54	116	
Cl	327	327	331	0	
Na	145	145	24	101	
Fe	59.1	59.1	59.1	8.05	
Mn	0.95	**2.26**	0.96	2.26	
Zn	1.68	1.68	1.68	1.08	
B	0.11	**0.89**	0.11	0.89	
Cu	0.59	0.59	0.59	0.13	
Mo	0.02	**0.16**	0.02	0.16	
Ni	0.02	0.02	0	0	
pH	7.7	7.6	7.6	5.9	6.1

The pH of the nutrient solutions and growing medium (pH-H_2O). The total amount of Ca and Mg in the growing medium of each pot, provided by the dolomite lime. The numbers highlighted in bold show which minerals were increased by addition to the digestate in D2.

342 for [NO_2^-], and LCK 340 for [NO_3^-]) (Hach, Loveland, CO, United States). The maximum conversion of NH_4^+ to NO_3^- (11.7 g N m^{-3} d^{-1}) was found on day 28 of the process. The nitrification process lasted for 51 days. At the end of the process, water and concentrated un-nitrified digestate was added to reach a final N_{min} concentration of 250 mg N_{min} L^{-1}, resulting in 35% NH_4^+-N and 65% NO_3^- -N. The occurrence of NO_2^- was checked throughout the nitrification process and in the final solution, it was below the detection limit,0.6 mg L^{-1}. The final fertigation solution was odorless, clear in appearance, and light brown in color.

Experimental Set-Up

The experiment was set up as a completely randomized design consisting of five treatments (including the negative control) with eight replicate pots per treatment with one plant per pot. The pots were randomly placed on a greenhouse table and re-randomized twice during the experiment. Four treatments were compared: nitrified digestate (D1); nitrified digestate with the addition of minerals to resemble the nutrient levels in the standard mineral nutrient solution used in the experiment (D2); a mineral nutrient solution designed to mimic the nutrient levels in the nitrified digestate (M1); standard mineral nutrient solution (M2). In addition, a negative control without fertilization was included (W). The treatments and variables tested are listed in **Table 1**. The treatments were formulated based on their mineral N (N_{min}, NO_3-N + NH_4-N) content, and all pots (except the negative control) received the same amount of N_{min} (650 mg $plant^{-1}$). Minerals were added to the digestate treatment D2 in the form of the mineral salts $MgSO_4 \times 7\ H_2O$, $CaSO_4 \times 2\ H_2O$, H_3PO_4, $MnSO_4 \times H_2O$, H_3BO_3, and $Na_2MoO_4 \times 2\ H_2O$. The final concentration was set to equal the concentration in treatment M2. The total amount of nutrients added to the treatments, analyzed by the accredited laboratory (Eurofins, Kristianstad, Sweden) with the standard methods as described above, is shown in **Table 2**.

The mineral nutrient solutions (M1 and M2) were mixed separately for each treatment and diluted to 250 mg N L^{-1}. Sodium hydroxide (NaOH) was used to adjust the pH in M2.

Analysis

The following data were collected on growing days 45 and 46 using the youngest mature leaf of each plant: (1) Chlorophyll content, using an MC-100 Chlorophyll Meter from Apogee Instruments, Logan, UT, United States and (2) chlorophyll fluorescence, measured with a Pocket PEA Chlorophyll Fluorimeter from Hansatech Instruments, Norfolk, United Kingdom. The chlorophyll content was measured using the method described by Parry et al. (2014). The chlorophyll fluorescence, expressed as the maximum yield of photosystem II (Fv/Fm), was measured after 20 min of dark adaption. The maximum yield of photosystem II (Fv/Fm) was calculated as (Fm – F0)/Fm, where Fm is the maximum fluorescence and F0 is the minimum fluorescence (Maxwell and Johnson, 2000).

The following data were collected after the harvest of each plant: (1) fresh and dry weight (after 3 days at 60°C) of shoots, (2) number of leaves > 10 mm in length, (3) total area of leaves > 10 mm in length, measured with an LI-3100 Area Meter from Li-Cor, Lincoln NE, United States, (4) concentrations of minerals in the shoot plant sap, (5) total mineral content in the shoot DM, and (6) nutrient content, electrical conductivity (EC), and pH in the used growing medium. Measurements 4–6 were performed by an accredited laboratory (LMI AB, Helsingborg, Sweden), using inductively coupled plasma- optical emission spectrometry (ICP-OES) to determine the concentrations of elements. The leaves for the sap samples were taken in the morning from fully turgid plants. The samples were kept in closed plastic bags in the dark until they were analyzed. The plant sap was sampled by pressing the sap out of 100 g of the oldest but fully vital leaves and analyzed after filtration. The nutrient content of the growing medium was measured in Spurway extract, a weak acetic acid (0.018 mol L^{-1}) solution (Spurway and Lawton, 1949).

The uptake efficiency of fertilizer P and S (PUE and SUE) was calculated using the partial balance method, i.e., as the ratio of nutrients removed by crop harvest (nutrient content in shoots) to fertilizer nutrients supplied (Fixen et al., 2015). The uptake efficiency of digestate fertilizer P and S as compared to the readily available orthophosphate in the mineral solutions was determined as the ratio of the nutrients taken up from the nitrified digestates to the rate of nutrients taken up from the respective mineral control (D1/M1, D2/M2 denoted $PUE_{D/M}$ and $SUE_{D/M}$).

Statistical Analysis

One-way ANOVA and Tukey's honestly significant difference (HSD) test for differences of means, with the CI set to 95%, were used for the statistical analysis of the data from the experiment. Normality was tested by the Anderson-Darling test for normality and homoscedasticity was tested by Levene's test. Transformation to the square root of the response factor was needed for one set of results, shoot FW in **Table 3**. The software used was Minitab Express version 15.1.

RESULTS

Plant Growth: Visual Observations

At harvest, the plants in all fertilized treatments had reached a height of about 30 cm and showed no signs of nutrient deficiency (**Figure 1**). There were no obvious visible differences between the plants in the different treatments. The plants in the unfertilized negative control, only irrigated with tap water, had grown to about 10 cm in height at harvest and showed severe symptoms of nutrient deficiency.

Plant Yield and Physiological Parameters

The plain digestate (D1) and its mineral equivalent (M1) resulted in the same DM yield, the same FM yield, and the same chlorophyll content (**Table 3**). However, D1 resulted in lower FM yield than the standard mineral nutrient solution (M2). The addition of P, S, Mg, Ca, Mn, B, and Mo to the digestate, giving the D2 treatment, was found to increase the FM yield by 10%, but it did not increase the DM yield significantly. It was also found to decrease the chlorophyll content. The supplemented digestate (D2) performed as well as M2 with respect to FM yield and outperformed it with respect to DM yield (17% higher).

The chlorophyll fluorescence (calculated as Fv/Fm) did not differ between the fertilizer treatments at harvest (**Table 3**). There were also no differences in the total leaf area between the treatments or in the number of leaves between digestate and mineral treatments (**Table 3**).

TABLE 3 | Growth and quality parameters at harvest of the pak choi grown in a soilless system fertilized with anaerobic digestate (D1, D2) or mineral nutrient solution (M1, M2).

Treatment	Shoot fresh weight (g) (*n* = 8)	Shoot dry weight (g) (*n* = 4)	Chlorophyll content (CCI) (*n* = 8)	Leaf number (*n* = 4)	Leaf area (dm^2) (*n* = 4)	Chlorophyll fluorescence (Fv/Fm) (*n* = 8)	Water content (%) (*n* = 4)
D1	368 ± 16 b	22.4 ± 0.8 ab	29.8 ± 4.0 a	19.5 ± 1.7 ab	30.6 ± 1.9 a	0.81 ± 0.01 a	93.7 ± 0.2 a
D2	402 ± 22 a	24.4 ± 0.9 a	24.2 ± 2.0 b	17.8 ± 0.4 ab	30.5 ± 1.3 a	0.81 ± 0.01 a	94.1 ± 0.2 a
M1	385 ± 11 ab	23.1 ± 2.1 ab	31.0 ± 4.1 a	19.6 ± 0.6 a	29.6 ± 1.6 a	0.80 ± 0.01 a	94.0 ± 0.5 a
M2	393 ± 21 a	20.9 ± 0.5 b	22.3 ± 2.2 b	17.5 ± 0.6 b	28.3 ± 1.9 a	0.81 ± 0.01 a	94.4 ± 0.2 a
W	10 ± 3 c	1.3 ± 0.5 c	24.1 ± 4.6 b	6.3 ± 1.0 c	1.8 ± 0.2 b	0.72 ± 0.06 b	89.4 ± 1.9 b

Means, given with SD, within each column that do not share a letter are statistically different ($P < 0.05$).

FIGURE 1 | One representative plant from each treatment at harvest. D1, digestate; D2, digestate with amendments; M1, mineral nutrient solution designed to have the same nutrient composition as D1; M2, mineral nutrient solution designed for optimal growth.

TABLE 4 | Nutrient uptake efficiency of phosphorous (P) and sulfur (S) (PUE and SUE), calculated as the ratio of nutrients taken up by crop (content in shoots) to fertilizer nutrients applied (*n* = 4 ± SD).

Treatment	Total amount applied (mg/plant)	Total shoot uptake (mg/plant)	PUE and SUE,%	Concentration in shoots at harvest (g/kg)	$PUE_{D/M}$ and $SUE_{D/M}$
		Phosphorus			
D1	97	63 ± 3.1 c	65 ± 3.0 c	2.80 ± 0.75 c	78%
M1	101	84 ± 0.8 b	83 ± 0.8 a	3.65 ± 0.36 b	
D2	128	95 ± 4.8 a	75 ± 3.8 b	3.92 ± 0.27 b	93%
M2	128	102 ± 7.0 a	80 ± 5.4 ab	4.89 ± 0.31 a	
		Sulfur			
D1	54	36 ± 3.2 c	67 ± 5.9 c	1.62 ± 0.11 d	71%
M1	54	51 ± 0.6 b	95 ± 1.2 a	2.24 ± 0.25 c	
D2	115	84 ± 2.9 a	73 ± 2.5 bc	3.45 ± 0.17 b	94%
M2	115	89 ± 5.2 a	77 ± 4.4 b	4.26 ± 0.27 a	

Means within each column that do not share a letter are statistically different ($P < 0.05$). P ($PUE_{D/M}$) and S ($SUE_{D/M}$) uptake efficiency from the nitrified digestate (D) compared to the mineral solutions (M), were determined as the ratio of the nutrient recovered from the nitrified digestate to the ratio of nutrient recovered from respective mineral control (i.e., D1/M1, D2/M2).

Nutrient Uptake

The concentration of P in the shoots in D1 was significantly lower than that in its mineral equivalent, M1. In D1, 65% of the applied P was found in the shoots at harvest, compared with 83% in M1, which corresponded to the P uptake from the digestate/uptake from the mineral fertilizer ($PUE_{D/M}$) of 75% (**Table 4**). The addition of P to the digestate solution significantly increased the shoot P concentration from 2.8 g (D1) to 3.9 g kg^{-1} (D2) (**Table 5**). This resulted in P recovery similar to that in M2 and a $PUE_{D/M}$ value of 93% (**Table 4**). The nutrient solutions with the highest P content (D2 and M2) resulted in the highest *P*-values for the shoots and plant sap (**Tables 5**, **6**). The P content in the nutrient solution did not reflect the P content in the growing medium at harvest (**Table 7**). The M2 treatment resulted in significantly lower residual plant-available P levels in the growing medium at harvest than D2 (1.8 mg L^{-1} compared with 3.3 mg L^{-1}).

The total uptake of S and shoot-tissue S concentrations were significantly lower in D1 than in M1 (**Table 4**), with recovery SUE of 65 and 95% in D1 and M1, respectively. The corresponding $SUE_{D/M}$ in D1 was 71%. The addition of $CaSO_4$ and $MgSO_4$ to the digestate nutrient solution significantly increased the shoot S concentration from 1.62 g kg^{-1} (in D1) to 3.45 g kg^{-1} (in D2) (**Table 5**). It also resulted in an SUE similar to that in M2 (**Table 4**). The nutrient solutions with the highest S content (D2 and M2) resulted in the highest plant-tissue and plant-sap S concentrations (**Tables 5**, **6**). The growing media did not differ significantly in S content at harvest (**Table 7**).

Overall, the shoot concentrations increased for all nutrients that were added to the digestate, comparing D1 and D2 (**Table 5**). The exceptions were Ca and Mg, for which the concentrations were already high in all growing media due to the liming material content. The shoot concentrations in the amended digestate treatment (D2) came closer to the mineral equivalent (M2), but P and S concentrations were still slightly higher with the mineral fertilizer. The shoot sap concentrations of the nutrients at harvest showed similar results to the total shoot concentration, on comparing both D1–D2 and D2–M2 (**Table 6**).

DISCUSSION

Phosphorus

Phosphorus Use Efficiency as Influenced by Magnesium and Calcium

The total P content in the digestate used in the experiments was in the same range as that normally found in mineral nutrient solutions designed for high yielding soilless production (N:P molar ratio of 6.7:1). For comparison, the commonly used Hoagland lettuce solution for hydroponics has an N:P ratio of 7:1 (Smith et al., 1983) and the mineral reference solution in this trial, M2, had an N:P ratio of 5:1. However, a fraction of the P in the digestate was not readily plant-available, as the $PUE_{D/M}$ in D1 was 75% of that in M1, where P was added to the pots as plant-available orthophosphate (HPO_4^{2-}). The Phosphorus Use Efficiency (PUE) value was also lower than that in previous studies on soil, where the PUE close to that of TripleSuper-P has been observed in laboratory incubation trials and pot experiments with digested (but not nitrified as in the present study) animal slurries, energy crops, and mixtures of the two (Loria and Sawyer, 2005; Bachmann et al., 2016, 2011; Vanden Nest et al., 2021). The PUE in the digestate treatment in this trial was also lower than that determined in a hydroponic trial by Pelayo Lind et al. (2020), in which pak choi plants were fertilized with nitrified digestate. In that study, the P shoot concentrations in the digestate treatments were similar to those in the mineral fertilizer reference treatment, but the N:P ratio in the digestate was 3.5:1 and P was probably present in excess amounts.

TABLE 5 | Concentration of nutrients in shoot dry matter at harvest of pak choi grown in soilless system fertilized with anaerobic digestate (D1, D2) or mineral fertilizers (M1, M2).

	g/kg						
Treatment	**N**	**P**	**K**	**S**	**Ca**	**Mg**	**Na**
D1	22 ± 6.2 b	2.8 ± 0.1 c	43 ± 1.4 ab	1.6 ± 0.1 d	15 ± 0.9 abc	7.3 ± 0.4 abc	6.6 ± 0.4 b
D2	23 ± 1.6 ab	3.9 ± 0.3 b	37 ± 2.4 bc	3.5 ± 0.2 b	14 ± 0.5 bc	6.7 ± 0.2 c	6.9 ± 0.5 b
M1	27 ± 3.0 a	3.7 ± 0.4 b	47 ± 4.9 a	2.2 ± 0.3 c	14 ± 0.7 c	7.2 ± 0.3 bc	3.9 ± 0.5 c
M2	27 ± 1.8 a	4.9 ± 0.3 a	36 ± 1.6 c	4.3 ± 0.3 a	17 ± 0.8 ab	7.6 ± 0.3 abc	7.0 ± 0.3 a
W	14 ± 2.1 c	0.7 ± 0.3 d	9.4 ± 1.7 d	2.2 ± 0.3 c	16 ± 2 ab	8.1 ± 1.2 ab	9.0 ± 1.5 a

	mg/kg					
Treatment	**Mn**	**Fe**	**Zn**	**B**	**Cu**	**Mo**
D1	86 ± 11 bc	49 ± 5.1 a	42 ± 4.7 a	10 ± 0.6 b	4.1 ± 0.4 ab	1.0 ± 0.2 c
D2	116 ± 1.3 a	52 ± 4.4 a	42 ± 2.1 a	33 ± 1.1 a	4.4 ± 0.6 ab	3.7 ± 0.3 b
M1	46 ± 3.9 d	53 ± 6.3 a	32 ± 3.3 b	8.6 ± 2.3 b	5.6 ± 1.1 a	1.5 ± 0.2 c
M2	95 ± 24 abc	55 ± 3.9 a	46 ± 3.5 a	35 ± 3.2 a	3.6 ± 0.7 b	3.4 ± 0.6 b
W	71 ± 7.8 cd	44 ± 7.6 a	43 ± 2.6 a	8.9 ± 0.7 b	3.4 ± 0.9 b	6.2 ± 0.5 a

Means within each column that do not share a letter are statistically different (P < 0.05; n = 4 ± SD).

TABLE 6 | Concentrations (mg L^{-1}) of macro- and micro-nutrients in plant sap at harvest of pak choi grown in soilless system fertilized with anaerobic digestate (D1, D2) or mineral fertilizers (M1, M2).

	mg L^{-1}							
Treatment	**NH_4-N**	**NO_3-N**	**P**	**K**	**S**	**Ca**	**Mg**	**Na**
D1	6.3 ± 0.7	69 ± 38	86 ± 10 b	2,800 ± 183 a	77 ± 12 b	1,103 ± 159	560 ± 62	290 ± 28 a
D2	5.8 ± 1.0	88 ± 90	185 ± 31 a	2,800 ± 392 a	318 ± 46 a	1,133 ± 158	603 ± 57	338 ± 39 a
M1	6.3 ± 0.8	56 ± 13	120 ± 12 b	2,625 ± 457 a	115 ± 20 b	923 ± 237	523 ± 112	170 ± 22 b
M2	5.4 ± 0.5	37 ± 18	183 ± 25 a	1,725 ± 150 b	283 ± 17 a	1,123 ± 100	613 ± 57	303 ± 55 a
	n.s.	*n.s.*				*n.s.*	*n.s.*	

Treatment	**Cl**	**Mn**	**Fe**	**Zn**	**B**	**Cu**	**Mo**
D1	2,200 ± 141 a	6.8 ± 1.4 b	0.93 ± 0.06	3.1 ± 0.32	0.09 ± 0.01 b	0.2 ± 0.03	0.16 ± 0.01 c
D2	2,150 ± 265 a	10.4 ± 2.2 a	1.17 ± 0.05	3.2 ± 0,33	2.38 ± 0.36 a	0.24 ± 0.03	0.41 ± 0.03 a
M1	1,750 ± 359 a	3.3 ± 1.0 c	0.96 ± 0.10	1.9 ± 0,17	0.18 ± 0.10 b	0.2 ± 0.02	0.20 ± 0.04 c
M2	945 ± 247 b	9.1 ± 1.5 ab	1.72 ± 0.96	3.3 ± 1.76	1.88 ± 0.30 a	0.3 ± 0.17	0.32 ± 0.01 b
			n.s.	*n.s.*		*n.s.*	

Means within each column that do not share a letter are statistically different (P < 0.05; n = 4 ± SD). n.s., no significant differences were found within the columns.

The reason for the low PUE obtained for the digestate used in the present study might be its low content of Mg and relatively high content of Ca, with a P:Mg, Ca:Mg, and Ca:P molar ratio of about 4, 9, and 2, respectively. The amount of Mg and Ca:Mg ratio is partly decisive for whether more struvite or HAp is formed (Vanden Nest et al., 2021). Despite its low water solubility, struvite is an efficient P fertilizer, resulting in crop PUE comparable to that of water-soluble mineral P fertilizers (e.g., reviewed by Möller et al., 2018 and reported by Vanden Nest et al., 2021). Hydroxylapatite, on the other hand, has low P solubility and plant availability, e.g., a study comparing TripleSuper-P, struvite, and HAp for their P-fertilizing properties in soil found 6.0, 5.4, and 0.7% P recovery, respectively, in the shoots of ryegrass and fescue (Achat et al., 2014). Studies on struvite precipitation in wastewater have revealed that an increased Ca content severely restricts struvite precipitation in favor of HAp formation when the Ca:Mg molar ratio exceeds 1–2.5 (Yan and Shih, 2016; Daneshgar et al., 2018; Liu and Wang, 2019). In manure compost, the formation of HAp is reported to occur when the molar Ca:P ratio exceeds 2 (Toor et al., 2005). For a range of organic fertilizers, including digestate, Vanden Nest et al. (2021) observed a significant negative correlation between P plant availability and molar Ca:P ratio exceeding 2–3, and attributed this to the formation of HAp. Accordingly, it is likely that the low Mg content and relatively high Ca content in the digestate in this study enabled the formation of HAp, with negative impacts on PUE. Two previous studies on the speciation of P precipitates in digestate with relatively low Ca:Mg molar ratio (1.2–1.3) found that, although struvite constituted the major fraction, HAp was present in considerable concentrations. Güngör et al. (2007) found that 78.2% of P in the 25–53 μm size fraction of a dairy manure digestate was present as struvite, and 21.8% as HAp (Ca:P = 2.1), while Marti et al. (2008) found that 58% of precipitated P in anaerobic digestate from a pilot plant was present as struvite and 15% as calcium phosphates, forming mainly HAp (Ca:PO_4 = 1.6).

Phosphorus Use Efficiency as Affected by Fe Supplementation

The addition of Fe-salts for desulfurization in anaerobic digesters can negatively influence plant P availability, as Fe can form insoluble precipitates with P (Krogstad et al., 2005; Möller et al., 2018). Accordingly, increasing the Fe:P ratio has been observed to negatively influence the PUE of organic fertilizers (Vanden Nest et al., 2021). In a trial on P recovery from wastewater, Yan and Shih (2016) concluded that when ferric ions (Fe^{3+}) were added at an Fe:Mg molar ratio of 1:5 and in concentrations above 100 ppm, the formation of struvite crystals was negatively affected at pH 7.5 and pH 9.0, probably largely due to the precipitation of ferric phosphates. In the present study, the Fe concentration in the raw digestate was 325 mg Fe L^{-1} digestate and the Fe:Mg molar ratio was 3:1. However, due to the reducing conditions in the biogas process, and an Fe:S molar ratio of about 1:1.5, a large proportion of the Fe added in this study was most probably precipitated as Fe^{2+} with sulfide and present as low-solubility Fe-S compounds (Yekta et al., 2014).

Phosphorus Use Efficiency as Affected by Nitrification in a Moving Bed Biofilm Reactor

The precipitation of P as plant-unavailable forms might have continued during storage, due to the slightly alkaline pH (8.1) of the digestate. However, the nitrification treatment in the MBBR decreased the pH to levels where the P solubility is at its maximum (pH 5.5–6.5) for an extended period of time (Hjorth et al., 2010). This potentially allowed for the solubilization of compounds that would otherwise not solubilize within the timeframe of the experiment. Moreover, during MBBR treatment, P speciation is under the influence of microbial processes such as immobilization/mobilization and mineralization. However, the fate of P during nitrification treatments in small-scale reactors or integrated biofilters in hydroponic systems is not well documented and needs further investigation.

TABLE 7 | Plant-available nutrients in mg L^{-1} growing medium.

	mg L^{-1}								
Treatment	**N-Kjeldal**	**NH4-N**	**P**	**K**	**S**	**Ca**	**Mg**	**Na**	**Cl**
D1	2.13 ± 0.52 ab	2.0 ± 0.8ab	2.5 ± 0.6 ab	20.8 ± 5.1ab	3.0 ± 1.4	345 ± 5.8 a	243 ± 5.0 b	51.3 ± 2.3 a	<6
D2	2.45 ± 0.59 a	2.3 ± 0.5a	3.3 ± 0.5 a	22.5 ± 1.7a	4.8 ± 1.0	335 ± 17.3 ab	258 ± 22.2ab	38.3 ± 1.3 b	<6
M1	2.40 ± 0.34 a	2.3 ± 0.5a	1.5 ± 0.6 b	18 ± 2.9ab	3.5 ± 1.0	315 ± 5.8 b	265 ± 17.3ab	26.5 ± 2.7 c	<6
M2	1.16 ± 0.22 b	1.0 ± 0.0b	1.8 ± 0.5 b	15.8 ± 1.3b	4.8 ± 0.5	358 ± 18.9 a	278 ± 9.6 a	26.0 ± 5.0 c	<6
		.			**n.s.**				

Treatment	**Mn**	**Fe**	**B**	**pH**	**EC (mS cm^{-1})**
D1	0.31 ± 0.05 ab	0.45 ± 0.06 b	0.13 ± 0.01 ab	6.7 ± 1.1 a	0.25 ± 0.10
D2	0.41 ± 0.06 a	0.41 ± 0.18 b	0.13 ± 0.001 a	6.5 ± 0.1 ab	0.25 ± 0.06
M1	0.21 ± 0.03 b	0.95 ± 0.40 a	0.12 ± 0.001 b	6.3 ± 0.0 b	0.25 ± 0.05
M2	0.29 ± 0.08 b	0.49 ± 0.06 ab	0.13 ± 0.005 ab	6.6 ± 0.13 a	0.20 ± 0.00
					n.s.

One part of the growing medium was extracted in six parts of 0.018 mol L^{-1} acetic acid for one h and analyzed. pH, and electrical conductivity (EC) in the growing medium at the harvest of pak choi grown in soilless system fertilized with anaerobic digestate (D1, D2) or mineral fertilizers (M1, M2). Means within each column that do not share a letter are statistically different ($P < 0.05$; n = 4 ± SD). n.s., no significant differences were found within the columns.

Risk of Phosphorus Deficiency

The lower uptake of P in treatment D1 resulted in a plant tissue P concentration of 0.28%, which is just within the recommended range (0.3–0.5%) for the optimal growth of *B. oleracea* crops (broccoli, cabbage, cauliflower) (Magnusson et al., 2006). Accordingly, P was not limiting for growth in the digestate treatment. However, the value was just on the verge of potential P shortage, so there may be a risk of P deficiency when using a digestate with a similar or higher N:P ratio than that in this study. The result is in accordance with the findings by Stoknes et al. (2018), who observed the risk of P limitation in tomatoes even after maximizing the P levels by using digestate solids as the growing medium (N:P ratio 1.4:1) and the whole digestate instead of the liquid fraction as the nutrient solution (N:P ratio 6:1). Losak et al. (2014) also observed P limitation of growth when using digestate with an N:P ratio of 6:1 as fertilizer. However, that trial was performed in a soil low in P, where P fixation could be expected (Menezes-Blackburn et al., 2016).

Effect of Phosphorus Addition

A straightforward approach to avoid limitations on plant growth and quality when using a digestate with a low content of plant-available P as fertilizer is to supplement the digestate with inorganic P [e.g., phosphoric acid (H_3PO_4)]. However, the plant availability of added P is difficult to predict due to the complex matrix in the digestate together with the potentially suboptimal and unstable pH after nitrification due to ongoing nitrification/denitrification processes. Still, active nitrification bacteria will decrease pH when NH_4^+ is present but at the same time, anaerobic pockets may form in the growing medium giving suitable circumstances for pH-increasing denitrification (Kremen et al., 2005). The slightly alkaline pH of the digestate nutrient solution in this study (7.6), the relatively high content of Ca (Ca:P ratio 2.1), and the low content of Mg (Ca:Mg ratio 9), posed a risk of precipitation of the added P to poorly soluble compounds such as HAp (Daneshgar et al., 2018; Vanden Nest et al., 2021). However, the recovery of the added P in this study was > 100% (adding 31 mg of extra P per pot resulted in an average increase in shoot P uptake of 32 mg) (**Table 3**), showing that the (bio)chemical properties of the digestate did not negatively influence the plant availability of the added P. Competing ligands in the digestate, such as the organic ligands citrate and oxalate, which influence the extent to which P ions can bind to metal ions, might have influenced the high recovery (Hinsinger, 2001).

The decrease in digestate pH after application to the pots was probably an important factor for the high P recovery rate. In D2, the growing medium pH was 6.5 at harvest (**Table 7**). The solubility of P is at its maximum around this pH, as the concentrations of aluminum (Al) and Fe ions on one hand, and Ca ions on the other, are minimized (Lindsay, 1979). There may also have been synergistic effects of P and the other added nutrients. For example, Mo fertilizer is reported to increase P accumulation in the shoots of *B. napus* (Liu et al., 2010). The significantly increased shoot P concentration and P recovery after the addition of P confirm the results obtained by Liedl et al. (2004b), who reported the positive effects of adding H_3PO_4 to a pig-slurry digestate. The direct addition of K_2HPO_4 into diluted digestate has also been reported to be successful (Liu et al., 2011).

Sulfur

Low Sulfur Content

The total S content relative to N in the digestate was lower than that needed for most crops; the N:S ratio was 12:1, which was twice as high as that in the inorganic control (6:1). In comparison, a hydroponic lettuce solution recipe recommended by various fertilizer companies has an N:S ratio of 7:1 (AkzoNobel, Eurofins Agro, Nmi, Sqm, and Yara, 2016). For *Brassica* crops, which have high S demand and are sensitive to S deficiency (Haneklaus et al., 2007), the optimal ratio is lower. For the *B. oleracea* crops, cabbage, broccoli, and cauliflower, the recommended N:S ratio in the aboveground parts at harvest range between 2:1 and 7.5:1 (Magnusson et al., 2006). If all nutrients are supplied with the fertilizer, a similar ratio of plant-available N and S in the fertilizer is required.

Sulfur (S) and N are both introduced into biogas reactors mainly as a constituent of proteins (Straka et al., 2007). The reactor feedstock in this study consisted of 21% protein-rich slaughterhouse waste and relatively protein-rich pig- and cattle manure and organic household waste. However, due to S losses during anaerobic digestion caused by H_2S volatilization and/or iron sulfide precipitation, feedstock with a relatively high S content can still result in a digestate with an N:S ratio that is too high to meet the needs of crops in soilless systems (Massé et al., 2007; Peu et al., 2011; Wahid et al., 2018; Fontaine et al., 2020). The S content may also be decreased by volatilization during storage and handling, as digestate can contain potentially volatile S compounds not precipitated with the added Fe (Möller and Müller, 2012).

Sulfur Recovery

The use efficiency of S in the digestate fertilizer was 71% of that in the mineral equivalent $SUE_{D/M}$ (**Table 4**). Considering the reported S-speciation in the digestate, this is a remarkably high value. Yekta et al. (2014) investigated the chemical speciation of S in the digestate from five industrial biogas reactors in southern Sweden which, like the Karpalund reactor, digest mixtures of different organic wastes and use Fe-salts as process additives. They found that the S speciation in the digestate was dominated by insoluble iron sulfides (27–57%), followed by reduced organic S (22–46%) and zero-valent S (6–16%). Inorganic sulfate (SO_4^{2-}), i.e., the form in which plant roots can assimilate S was found in some samples, but only at low concentrations (Yekta et al., 2014). It has been reported that SO_4^{2-} only makes up 3–8% of the total S in the digestate from anaerobic fermentation of cover crops and straw mixtures (Suzuki, 1999; Fontaine et al., 2020). The amount of iron sulfides entering the Karpalund biogas reactor and the amount leaving the reactor with the digestate used in this study are not known. However, due to the small particle size of iron sulfides, some were probably retained in the digestate.

The mobilization and mineralization of organic S and oxidation of sulfide and elemental S to SO_4^{2-} are enzyme-driven processes dependent on microbial activity (Edwards, 1998; Suzuki, 1999). The 71% $SUE_{D/M}$ obtained in this study shows that the microflora in the MBBR biofilter, which was transferred to the peat substrate with the digestate, was probably capable of the net mineralization of organic S and oxidizing sulfide and elemental S to SO_4^{2-}. However, it seems unlikely that the oxidation of iron sulfides at any relevant rate took place during the experiment, considering its short duration and the absence of soil microflora (Freney, 1967).

This result is in sharp contrast to the findings in other studies on digestate application to soil, where the S recovery rate has been found to be very low and similar to unfertilized controls, even with the digestate with a relatively high S level and low C:S ratio (Assefa et al., 2013; Fontaine et al., 2020). This has been attributed to high SO_4^{2-} immobilization after the application of digestate to soil at C:S ratios which are usually related to net S mineralization (<200) (Fontaine et al., 2020).

The high SUE observed in this study supports the findings by Pelayo Lind et al. (2020) of the high uptake of S by pak choi plants grown with nitrified digestate as fertilizer in a hydroponic setup. In that study, the digestate-fed plants outperformed the inorganic control in the uptake of S. Similar to the digestate in this trial, the digestate was from a large-scale biogas plant using iron chloride as a process additive and was nitrified by MBBR. However, their digestate had a lower N:S ratio (6.7:1) than the presently used digestate, probably due to plant-based feedstock with less N. This lower ratio resulted in higher total S application, which explains the higher S uptake compared to that in the present study. However, the reason for the higher uptake may also lie in the hydroponic setup, as the peat growing medium and pot culture in this study allowed for a larger microbial community and thus higher S immobilization, although microbial growth was probably limited by low C availability.

Risk of Sulfur Deficiency

The low total S content and the presence of non-plant-available S in the digestate resulted in low plant-tissue concentrations of S in D1 (0.16%) (**Table 5**). According to Teuber et al. (2020), an adequate S supply is reflected by tissue concentrations of S between 0.17 and 0.40% for most crops. For *Brassica* crops, which are considered particularly sensitive to S deficiency (Haneklaus et al., 2007), the optimum range is higher. For example, Haneklaus et al. (2007) recommended 0.75% for *Brassica* vegetable crops and Magnusson et al. (2006) recommended an S content of 0.4–1.3% in plant tissues for the optimal growth of *B. oleracea* crops. Low levels of S in *Brassica* crops have been found to result in lower yields and lower concentrations of valuable S-containing metabolites such as glucosinolates (Scherer, 2001). Accordingly, the S content in the D1 plants can be considered very low and in the deficiency range, with potential negative effects on yield and quality. The low S availability in the digestate treatments might also explain the lower N concentrations observed in the plants in these treatments, as S interacts closely with N uptake in plants (Eriksen et al., 2001).

Doubling the total S content in the digestate by adding $MgSO_4$ and $CaSO_4$ to D2 (**Table 2**) significantly increased shoot-tissue concentration levels of S from 0.16 to 0.36%, which is close to the minimum level recommended by Magnusson et al. (2006) (0.4–1.3%). The recovery of the added S in the shoots was 79% (adding 61 mg of extra S per pot resulted in an average increase in S uptake by shoots of 48 mg). Considering this, a higher S addition rate (e.g., tripling the total S content in the digestate) would have been more beneficial. The sap concentration of S (**Table 6**), reflecting the vacuolar content of S, was also largely increased by S addition to the digestate, indicating elevated cell S status (Marschner, 1978).

Effects of Adding Magnesium, Manganese, Boron, and Molybdenum

No correlation was found between the Mg content in nutrient solution and Mg plant uptake, which was probably a result of the high Mg levels in the growing medium due to its dolomite content.

The addition of the micronutrients B, Mn, and Mo to the digestate resulted in significant increases in the shoot mineral content of these micronutrients, e.g., the B concentration increased from 10 to 33 mg kg^{-1} in supplemented plants. For most dicotyledonous species, the critical deficiency range for B is 20–70 mg kg^{-1} (Broadley et al., 2012). For the *B. oleracea* species broccoli and cauliflower, 30–100 mg kg^{-1} B in the shoots has been recommended for optimal growth (Magnusson et al., 2006). Based on these values, the D1 plants suffered from B deficiency and the supplemented D2 plants were just within the range recommended for optimal growth. One of the most rapid responses to B deficiency is the inhibition of root elongation, which results in stubby and bushy roots (Broadley et al., 2012). The low tissue concentrations of B in D1 and M1 (10.1 and 8.6 mg kg^{-1}, respectively) may therefore explain the distinctly shorter (but not bushy) roots observed in those treatments (data not shown). However, no aboveground symptoms of B deficiency were detected. Further, there were no differences in DM yield between treatments D1 and D2. This was unexpected, as inhibited shoot growth is a typical early symptom of B deficiency (Broadley et al., 2012). However, the fresh matter yield was significantly higher in D2, which could be explained by an increase in root volume when B was supplied at sufficient levels, allowing for the increased uptake of water. The tissue concentrations of Mn and Mo were above the threshold level for deficiency (10–20 mg kg^{-1} for Mn and 0.1–1.0 mg kg^{-1} for Mo) in both D1 and D2 (Broadley et al., 2012), showing that these nutrients were present in sufficient levels in the digestate.

General Observations

The sap concentration of all elements added to the digestate (including P and S) increased in D2 compared to D1 (**Table 6**). In contrast to the total tissue concentration, the sap nutrient concentration is believed to reflect the vacuolar concentration, indicating a larger cellular nutrient buffer when increased, as was the case comparing D1–D2 (Marschner, 1978). The amended digestate in D2 also had the same or higher sap nutrient concentrations compared to the mineral equivalent M2. This could indicate an increased deliverance of nutrients from D2 compared to M2 at the end of the culture time.

The overall good performance by the plants given the digestate, despite the lower uptake efficiency of P and S, may indicate the positive effects of digestates on plant growth beyond the nutrient effect such as the presence of suggested biostimulants such as auxin-like compounds (Scaglia et al., 2017) and humic substances (Guilayn et al., 2020). As a result of the anaerobic digestion of the feedstock, digestates contain a complex mixture of partially degraded organic matter and inorganic compounds, including monosaccharides, free amino acids, fatty acids, polypeptides, nucleic acids, vitamins, phytohormones, as well as compounds of higher molecular weight (Möller and Müller, 2012; Scaglia et al., 2017). The same compounds, when derived from other organic sources, have been reported to act as bio-stimulants on plant growth (du Jardin, 2015).

The chlorophyll fluorescence is often used as a measurement of possible physiological stress affecting plants. All the fertilized treatments had an Fv/Fm at 0.81, showing that they probably were not stressed by any of the treatments or at least all to the same degree. An average value for unstressed vascular plants of various origins has been found to be 0.83 (Demmig and Björkman, 1987).

CONCLUSION

As hypothesized, the growth performance in the digestate resembled the growth in the mineral solutions made to mimic the digestate. This was also the case when the digestate was amended with nutrients to resemble a commercial nutrient solution. This suggests that the availability of the nutrients in the digestate is high and that the digestate fully can substitute the mineral fertilizer and with the minor addition of selected nutrients perform even better.

This study made the promising finding that after the addition of macro- and micro-nutrients, nitrified digestate can be used successfully as a fertilizer in the production of leafy vegetables on peat-based growing media. Supplementing the digestate with nutrients increased the FM yield of pak choi by 10% and resulted in similar marketable yields as when using standard mineral fertilizer. Further, plants fertilized with the supplemented digestate had a 17% higher dry weight than plants treated with standard mineral fertilizer. This weight increase was probably a result of P, S, and/or B addition, as shoot-tissue concentrations of these nutrients were low (in the deficiency range for S and B) in plants fertilized with the not amended digestate. However, this study was not replicated over time and thus indicative. More research is needed to identify the factors determining the recovery of P and S in digestate fertilizers. Since struvite is reported to be a readily plant-available P source in organic fertilizers, it would be interesting in future studies ahead to investigate supplementation of digestate with struvite recovered from other waste streams, e.g., sewage sludge or industrial effluents, thus contributing to recirculation of P and closing of global nutrient cycles.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KW and HA conceived and designed the experiments. KW performed the experiments and analyzed the data. KW wrote the first draft with HA as the main co-author. KW, HA, K-JB, and MH contributed to the writing and approved the submitted version.

FUNDING

This research was supported by the FORMAS (grant no. 2018-01845) to MH and the SLU Partnership Alnarp (grant no. 118119) to HA.

REFERENCES

Abubaker, J., Risberg, K., and Pell, M. (2012). Biogas residues as fertilisers – effects on wheat growth and soil microbial activities. *Appl. Energy* 99, 126–134. doi: 10.1016/j.apenergy.2012.04.050

Achat, D. L., Sperandio, M., Daumer, M. L., Santellani, A. C., Prud'Homme, L., Akhtar, M., et al. (2014). Plant-availability of phosphorus recycled from pig manures and dairy effluents as assessed by isotopic labeling techniques. *Geoderma* 232, 24–33. doi: 10.1016/j.geoderma.2014.04.028

AkzoNobel, Eurofins Agro, Nmi, Sqm, and Yara. (2016). *Nutrient Solutions for Greenhouse Crops*. Available online at: https://www.yara.nl/siteassets/toolbox/nutrient-solutions/nutrient-solutions-for-greenhouse-crops-dec2017.pdf/ (accessed December 15.20).

Alburquerque, J. A., de la Fuente, C., Ferrer-Costa, A., Carrasco, L., Cegarra, J., Abad, M., et al. (2012). Assessment of the fertiliser potential of digestates from farm and agroindustrial residues. *Biomass Bioenergy* 40, 181–189. doi: 10.1016/j.biombioe.2012.02.018

APHA (1998). *Standard Methods for the Examination of Water and Wastewater*. (Washington, DC: American Public Health Association).

Assefa, K. M., Tucher, S. V., and Schmidhalter, U. (2013). Biogas residues as source of sulfur to pak choi Brassica rapa var. chinensis (L.). *J. Soil Sci. Environ. Manage.* 4, 163–168. doi: 10.5897/JSSEM13.0409

Bachmann, S., Uptmoor, R., and Eichler-Löbermann, B. (2016). Phosphorus distribution and availability in untreated and mechanically separated biogas digestates. *Sci. Agric.* 73, 9–17. doi: 10.1590/0103-9016-2015-0069

Bachmann, S., Wentzel, S., and Eichler-Löbermann, B. (2011). Codigested dairy slurry as a phosphorus and nitrogen source for *Zea mays* L. and *Amaranthus cruentus* L. *J. Plant Nutr. Soil Sci.* 174, 908–915. doi: 10.1002/jpln.201000383

Bergstrand, K.-J., Asp, H., and Hultberg, M. (2020). Utilizing anaerobic digestates as nutrient solutions in hydroponic production systems. *Sustainability* 12:10076. doi: 10.3390/su122310076

Bergstrand, K.-J., and Hultin, S. (2014). Development of strategies for hydroponic cultivation in vertical systems. *Acta Hortic.* 1034, 149–156. doi: 10.17660/ActaHortic.2014.1034.17

Bloomfield, C., and McGrath, S. P. (1982). A comparison of the extractabilities of Zn, Cu, Ni and Cr from sewage sludges prepared by treating raw sewage with the metal salts before or after anaerobic digestion. *Environmental Pollut.* 3, 193–198. doi: 10.1016/0143-148X(82)90060-X

Broadley, M., Brown, P., Cakmak, I., Rengel, Z., and Zhao, F. (2012). "Function of nutrients: micronutrients," in *Marschner's Mineral Nutrition of Higher Plants*, 3rd Edn, Chap. 7, eds P.Marschner, (Cambridge,MA: Academic Press). 191–248.

Cheong, J. C., Lee, J. T. E., Lim, J. W., Song, S., Tan, J. K. N., Chiam, Z. Y., et al. (2020). Closing the food waste loop: food waste anaerobic digestate as fertilizer for the cultivation of the leafy vegetable, xiao bai cai (Brassica rapa). *Sci. Total Environ.* 715:136789. doi: 10.1016/j.scitotenv.2020.136789

Daneshgar, S., Buttafava, A., Capsoni, D., Callegari, A., and Capodaglio, A. G. (2018). Impact of pH and ionic molar ratios on phosphorous forms precipitation and recovery from different wastewater sludges. *Resources* 7:71. doi: 10.3390/resources7040071

Demmig, A. B., and Björkman, O. (1987). Photon yield of O2 evoulution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* 170, 489–504. doi: 10.1007/BF00402983

du Jardin, P. (2015). Plant bio stimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Edwards, P. J. (1998). *Sulfur Cycling, Retention, and Mobility in Soils: a Review. General Technical Report NE-250*. (Newton Square, PA: United States Department of Agriculture Forest Service, Northeastern Research Station).

Eriksen, J., Nielsen, M., Mortensen, J. V., and Schjørring, J. K. (2001). Redistribution of sulphur during generative growth of barley plants with different sulphur and nitrogen status. *Plant Soil* 230, 239–246. doi: 10.1023/A:1010328115203

Fixen, P., Brentrup, F., Bruulsema, T., Garcia, F., Norton, R., and Zingore, S. (2015). "Nutrient/fertilizer use efficiency: measurement, current situation and trends," in *Managing Water and Fertilizer for Sustainable Agricultural Intensification*, eds P. Drechsel, P. Heffer, H. Magen, R. Mikkelsen, and D. Wichelns (Paris: International Fertilizer Industry Association). 8–38.

Fontaine, D., Feng, L., Labouriau, R., Møller, H. B., Eriksen, J., and Sørensen, P. (2020). Nitrogen and sulfur availability in digestates from anaerobic co-digestion of cover crops, straw and cattle manure. *J. Soil Sci. Plant Nutr.* 20, 621–636. doi: 10.1007/s42729-019-00151-7

Freney, J. R. (1967). Oxidation of sulphur in soils. *Miner. Depos.* 2, 181–118. doi: 10.1007/BF00201914

Guilayn, F., Benbrahim, M., Rouez, M., Crest, M., Patureau, D., and Jimenez, J. (2020). Humic-like substances extracted from different digestates: first trials of lettuce biostimulation in hydroponic culture. *Waste Manage.* 104, 239–245. doi: 10.1016/j.wasman.2020.01.025

Guilayn, F., Jimenez, J., Martel, B., Rouezc, M., Crest, M., and Patureaua, D. (2019). First fertilizing-value typology of digestates: a decision-making tool for regulation. *Waste Manage.* 86, 67–79. doi: 10.1016/j.wasman.2019.01

Güngör, K., Jürgensen, A., and Karthikeyan, K. G. (2007). Determination of phosphorus speciation in dairy manure using XRD and XANES spectroscopy. *J. Environ. Qual.* 36, 1856–1863. doi: 10.2134/jeq2006.0563

Haneklaus, S., Bloem, E., and Schnug, E. (2007). "Sulfur interactions in crop ecosystems," in *Sulfur in Plants An Ecological Perspective*, eds M. J. Hawkesford and L J. De Kok (AA Dordrecht: Springer), 7–58. doi: 10.1007/978-1-4020-5887-5_2

Hinsinger, P. (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant soil* 237, 173–195.

Hjorth, M., Christensen, K. V., Christensen, M. L., and Sommer, S. G. (2010). Solid—liquid separation of animal slurry in theory and practice. a review. *Agron. Sustain. Dev.* 30, 153–180. doi: 10.1051/agro/2009010

Jain, S., Newman, D., Nzihou, A., Dekker, H., Le Feuvre, P., Richte, H., et al. (2019). *Global Potential of Biogas. (Doctoral dissertation)*. (London: World Biogas Association).

Kremen, A., Bear, J., Shavit, U., and Shaviv, A. (2005). Model demonstrating the potential for coupled nitrification denitrification in soil aggregates. *Environ. Sci. Technol.* 39, 4180–4188. doi: 10.1021/es048304z

Krogstad, T., Sogn, T. A., Asdal, Å, and Sæbø, A. (2005). Influence of chemically and biologically stabilized sewage sludge on plant-available phosphorous in soil. *Ecol. Eng.* 25, 51–60. doi: 10.1016/j.ecoleng.2005.02.009

Liedl, B. E., Cummins, M., Young, A., Williams, M. L., and Chatfield, J. M. (2004a). Hydroponic lettuce production using liquid effluent from poultry waste bioremediation as a nutrient source. *Acta Hortic.* 659, 721–728. doi: 10.17660/ActaHortic.2004.659.93

Liedl, B. E., Cummins, M., Young, A., Williams, M. L., and Chatfield, J. M. (2004b). Liquid effluent from poultry waste bioremediation as a potential nutrient source for hydroponic tomato production. *Acta Hortic.* 659, 647–652. doi: 10.17660/ActaHortic.2004.659.83

Lindsay, W. L. (1979). *Chemical equilibria in soils*. (Chichester: John Wiley and Sons Ltd).

Liu, H., Hu, C., Sun, X., Tan, Q., Nie, Z., and Hu, X. (2010). Interactive effects of molybdenum and phosphorus fertilizers on photosynthetic characteristics of seedlings and grain yield of Brassica napus. *Plant Soil* 326, 345–353. doi: 10.1007/s11104-009-0014-1

Liu, W. K., Lianfeng, D., and Qichang, Y. (2009). Biogas slurry added amino acids decreased nitrate concentrations of lettuce in sand culture. *Acta Agric. Scand. B Soil Plant Sci.* 59, 260–264. doi: 10.1080/09064710802029551

Liu, W. K., Yang, Q. C., Du, L. F., Cheng, R. F., and Zhou, W. L. (2011). Nutrient supplementation increased growth and nitrate concentration of lettuce cultivated hydroponically with biogas slurry. *Acta Agric. Scand. B Soil Plant Sci.* 61, 391–394. doi: 10.1080/09064710.2010.482539

Liu, X., and Wang, J. (2019). Impact of calcium on struvite crystallization in the wastewater and its competition with magnesium. *Chemical Engineering Journal* 378, 122121. doi: 10.1016/j.cej.2019.122121

Loria, E. R., and Sawyer, J. E. (2005). Extractable soil phosphorus and inorganic nitrogen following application of raw and anaerobically digested swine manure. *Agron. J.* 97, 879–885. doi: 10.2134/agronj2004.0249

Losak, T., Hlusek, J., Zatloukalova, A., Musilova, L., Vitezova, M., Skarpa, P., et al. (2014). Digestate from biogas plants is an attractive alternative to mineral fertilisation of kohlrabi. *J. Sustain. Dev. Energy Water Environ. Sys.* 2, 309–318. doi: 10.13044/j.sdewes.2014.02.0025

Magnusson, M., Rölin, Å, and Ögren, E. (2006). *Förslag Till Riktvärden För Jord- Och Plantanalyser I Ekologisk Grönsaksodling. Hushållningssällskapet*. Available online at: http://www.margaretamagnusson.se/PDF/PUBLICERING/FOURAPPORT2006.pdf (accessed January 12, 2022).

Marcato, C.-E., Pinelli, E., Cecchi, M., Winterton, P., and Guiresse, M. (2009). Bioavailability of Cu and Zn in raw and anaerobically digested pig slurry. *Ecotoxicol. Environ. Saf.* 72, 1538–1544. doi: 10.1016/j.ecoenv.2008.12.010

Marschner, W. J. H. (1978). Effect of silicon on manganese tolerance of bean plants (*Phaseolus vulgaris* L.). *Plant Soil* 50, 287–303.

Marti, N., Bouzas, A., Seco, A., and Ferrer, J. (2008). Struvite precipitation assessment in anaerobic digestion processes. *Chem. Eng. J.* 141, 67–74. doi: 10.1016/j.cej.2007.10.023

Massé, D. I., Croteau, F., and Masse, L. (2007). The fate of crop nutrients during digestion of swine manure in psychrophilic anaerobic sequencing batch reactors. *Bioresour. Technol.* 98, 2819–2823. doi: 10.1016/j.biortech.2006.07.040

Maxwell, K., and Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *J. Exp. Bot.* 51, 659–668. doi: 10.1093/jxb/51.345.659

Menezes-Blackburn, D., Paredes, C., Zhang, H., Giles, C. D., Darch, T., Stutter, M., et al. (2016). Organic acids regulation of chemical–microbial phosphorus transformations in soils. *Environ. Sci. Technol.* 50, 11521–11531. doi: 10.1021/acs.est.6b03017

Moestedt, J., Nilsson Påledal, S., and Schnürer, A. (2013). The effect of substrate and operational parameters on the abundance of sulphate-reducing bacteria in industrial anaerobic biogas digesters. *Bioresour. Technol.* 132, 327–332. doi: 10.1016/j.biortech.2013.01.043

Möller, K. (2015). Effects of anaerobic digestion on soil carbon and nitrogen turnover, N emissions, and soil biological activity. a review. *Agron. Sustain. Dev.* 35, 1021–1041. doi: 10.1007/s13593-015-0284-3

Möller, K., and Müller, T. (2012). Effects of anaerobic digestion on digestate nutrient availability and crop growth: a review. *Eng. Life Sci.* 12, 242–257. doi: 10.1002/elsc.201100085

Möller, K., Oberson, A., Bünemann, E. K., Cooper, J., Friedel, J. K., Glæsner, N., et al. (2018). Improved phosphorus recycling in organic farming: navigating between constraints. *Adv. Agron. Elsevier* 147, 159–237. doi: 10.1016/bs.agron.2017.10.004

Neal, J., and Wilkie, D. A. C. (2014). Anaerobic digester effluent as fertilizer for hydroponically grown tomatoes. *J. Undergrad. Res.* 15, 1–5. doi: 10.1016/j.heliyon.2020.e05745

Nelson, N. O., Mikkelsen, R. L., and Hesterberg, D. L. (2003). Struvite precipitation in anaerobic swine lagoon liquid: effect of pH and Mg:P ratio and determination of rate constant. *Bioresour. Technol.* 89, 229–236. doi: 10.1016/s0960-8524(03)00076-2

Odhner, P., Sernhed, K., Svensson, S.-E., and Juhlin, M. (2015). *Biogödsel i Skåne en Inventering och Marknadsanalys*. Alnarp: Swedish University of Agricultural Sciences.

Parry, C., Blonquist, J. M. Jr., and Bugbee, B. (2014). In situ measurement of leaf chlorophyll concentration: analysis of the optical/absolute relationship. *Plant Cell Environ.* 37, 2508–2520. doi: 10.1111/pce.12324

Pelayo Lind, O., Hultberg, M., Bergstrand, K.-J., Larsson-Jönsson, H., Caspersen, S., and Asp, H. (2020). Biogas digestate in vegetable hydroponic production: ph dynamics and ph management by controlled nitrification. *Waste Biomass Valorization* 12, 123–133. doi: 10.1007/s12649-020-00965-y

Peu, P., Sassi, J.-F., Girault, R., Picard, S., Saint-Cast, P., Béline, F., et al. (2011). Sulphur fate and anaerobic biodegradation potential during co-digestion of seaweed biomass (Ulva sp.) with pig slurry. *Bioresour. Technol.* 102, 10794–10802. doi: 10.1016/j.biortech.2011.08.096

Pokhrel, B., Sorensen, J. N., Moller, H. B., and Petersen, K. K. (2019). Processing methods of organic liquid fertilizers affect nutrient availability and yield of greenhouse grown parsley. *Renew. Agric. Food Sys.* 34, 430–438. doi: 10.1017/S1742170517000771

Risberg, K. (2015). Quality and function of anaerobic digestion residues. (Ph D dissertation). *Swedish University of Agricultural Sciences*, (Uppsala: Acta Universitatis Agriculturae Sueciae). 40, 1652-6880.

Ronga, D., Setti, L., Salvarani, C., De Leo, R., Bedin, E., Pulvirenti, A., et al. (2019). Effects of solid and liquid digestate for hydroponic baby leaf lettuce (*Lactuca sativa* L.) cultivation. *Sci. Hortic.* 244, 172–181. doi: 10.1016/j.scienta.2018.09.037

Scaglia, B., Pognani, M., and Adani, F. (2017). The anaerobic digestion process capability toproduce biostimulant: the case study of the dissolved organic matter (DOM) vs. auxin like property. *Sci. Total Environ.* 589, 36–45. doi: 10.1016/j.scitotenv.2017.02.223

Scherer, H. W. (2001). Sulphur in crop production — invited paper. *Eur. J. Agron.* 14, 81–111. doi: 10.1016/S1161-0301(00)00082-4

Smith, G. S., Johnston, C. M., and Cornforth, I. S. (1983). Comparison of nutrient solutions for growth of plants in sand culture. *New Phytol.* 94, 537–548. doi: 10.1111/j.1469-8137.1983.tb04863.x

Sonneveld, C., and Straver, N. B. (1994). *Nutrient Solution for Vegetables and Flowers Grown in Water or Substrates. Research Station for Floriculture and Glasshouse Vegetables*. (Aalsmeer: Voedingspolossingen Glastijnbouw). 8.

Spurway, C. H., and Lawton, K. (1949). *Soil Testing: a Practical System of Soil Fertility Diagnosis. East Lansing: Agriculture Experiment Station*. East Lansing, MI: Michigan State University.

Stoknes, K., Wojciechowska, E., Jasińska, A., Gulliksen, A., and Tesfamichael, A. A. (2018). Growing vegetables in the circular economy; cultivation of tomatoes on green waste compost and food waste digestate. *Acta Hortic.* 1215, 389–396. doi: 10.17660/ActaHortic.2018.1215.71

Straka, F., Jenicek, P., Zabranska, J., Dohanyos, M., and Kuncarova, M. (2007). "Anaerobic fermentation of biomass and wastes with respect to sulfur and nitrogen contents in treated materials," in Proceedings *of the 11th International Waste Management and Landfill Symposium, Sardinia, 2007*, (Cagliari). 33.

Suzuki, I. (1999). Oxidation of inorganic sulfur compounds: chemical and enzymatic reactions. *Can. J. Microbiol.* 45:9.

Svensson, K., Odlare, M., and Pell, M. (2004). The fertilizing effect of compost and biogas residues from source separated household waste. *J. Agric. Sci.* 142, 461–467. doi: 10.1017/S0021859604004514

Teuber, O., Samarappuli, D., and Berti, M. (2020). Nitrogen and sulfur fertilization in Kale and Swede for grazing. *Agronomy* 10:619. doi: 10.3390/agronomy10050619

Toor, G. S., Peak, J. D., and Sims, J. T. (2005). Phosphorus speciation in broiler litter and turkey manure produced from modified diets. *J. Environ. Qual.* 34, 687–697. doi: 10.2134/jeq2005.0687

Uchimura, K., Sago, Y., Kamahara, H., Atsuta, Y., and Daimon, H. (2014). "Treatment of anaerobic digestion effluent of sewage sludge using soilless cultivation," in Proceedings *of the The Irago Conference 2013*, (Aichi), 59–63. doi: 10.1063/1.4866619

Vanden Nest, T., Amery, F., Fryda, L., Boogaerts, C., Bilbao, J., and Vandecasteele, B. (2021). Renewable P sources: p use efficiency of digestate, processed animal manure, compost, biochar and struvite. *Sci. Total Environ.* 750:141699. doi: 10.1016/j.scitotenv.2020.141699

Wahid, R., Feng, L., Cong, W.-F., Ward, A. J., Møller, H. B., and Eriksen, J. (2018). Anaerobic mono-digestion of lucerne, grass and forbs – influence of species and cutting frequency. *Biomass Bioenergy* 109, 199–208. doi: 10.1016/j.biombioe.2017.12.029

Yan, H., and Shih, K. (2016). Effects of calcium and ferric ions on struvite precipitation: a new assessment based on quantitative X-ray diffraction analysis. *Water Res.* 95, 310–318. doi: 10.1016/j.watres.2016.03.032

Yekta, S. S., Svensson, B. H., Björn, A., and Skyllberg, U. (2014). Thermodynamic modeling of iron and trace metal solubility and speciation under sulfidic and ferruginous conditions in full scale continuous stirred tank biogas reactors. *Appl. Geochem.* 47, 61–73. doi: 10.1016/j.apgeochem.2014.05.001

Zirkler, D., Peters, A., and Kaupenjohann, M. (2014). Elemental composition of biogas residues: variability and alteration during anaerobic digestion. *Biomass Bioenergy* 67, 89–98. doi: 10.1016/j.biombioe.2014.04.021

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 29 March 2022
doi: 10.3389/fpls.2022.804104

Essential Oils Prime Epigenetic and Metabolomic Changes in Tomato Defense Against *Fusarium oxysporum*

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *10 November 2021*
Accepted: *07 February 2022*
Published: *29 March 2022*

Citation:
Soudani S, Poza-Carrión C, De la Cruz Gómez N, González-Coloma A, Andrés MF and Berrocal-Lobo M (2022) Essential Oils Prime Epigenetic and Metabolomic Changes in Tomato Defense Against Fusarium oxysporum. Front. Plant Sci. 13:804104. doi: 10.3389/fpls.2022.804104

In this work, we studied the direct and indirect plant protection effects of an *Artemisia absinthium* essential oil (AEO) on tomato seedlings against *Fusarium oxysporum* sp. *oxysporum radicis lycopersici* (*Fol*). AEO exhibited a toxic effect *in vitro* against *Fol*. Additionally, tomato seedlings germinated from seeds pretreated with AEO and grown hydroponically were protected against *Fol*. Plant disease symptoms, including, water and fresh weight loss, tissue necrosis, and chlorosis were less pronounced in AEO-treated seedlings. AEO also contributed to plant defenses by increasing callose deposition and the production of reactive oxygen species (ROS) on seed surfaces without affecting seed germination or plant development. The essential oil seed coating also primed a durable tomato seedling defense against the fungus at later stages of plant development. RNA-seq and metabolomic analysis performed on seedlings after 12 days showed that the AEO treatment on seeds induced transcriptomic and metabolic changes. The metabolomic analysis showed an induction of vanillic acid, coumarin, lycopene, oleamide, and an unknown metabolite of m/z 529 in the presence of *Fol*. The *StNRPD2* gene, the second largest component of RNA polymerases IV and V directly involved in *de novo* cytosine methylation by RNA-directed DNA methylation (RdDM), was highly induced in the presence of AEO. The host methionine cycle (MTC) controlling trans-methylation reactions, was also altered by AEO through the high induction of S-adenosyl methionine transferases (SAMts). Our results suggest that AEO treatment could induce *de novo* epigenetic changes in tomato, modulating the speed and extent of its immune response to *Fol*. The EO-seed coating could be a new strategy to prime durable tomato resistance, compatible with other environmentally friendly biopesticides.

Keywords: *Fusarium*, tomato, essential oil, plant priming, seed coating, *Artemisia absinthium*, biopesticide, epigenetics

INTRODUCTION

Worldwide policies to decrease dependence on toxic pesticides means that new environmentally friendly biopesticides need to be developed. These natural compounds will also help protect the aquatic and agroforestry environment, soil health and biodiversity. According to European Commission policy, the development of new strategies and natural sources of biopesticides is one of the challenges that must be addressed to diminish levels of pesticide residue in food and feed and the corresponding risks for human health (European Commission, 2021; Montanarella and Panagos, 2021). Aromatic plants, studied for their fungistatic, insecticidal, larvicidal, and bactericidal compounds, are currently one of the main sources of natural compounds in the biosphere. The biopesticide potential of the millions of compounds acting directly to protect crops is growing exponentially in tandem with agricultural requirements around the globe (Montanarella and Panagos, 2021).

The plant defense response is accompanied by an extensive transcriptional reprogramming of defense-related genes. The well-known processes which enable systemic acquired resistance (SAR) (Durrant and Dong, 2004), pathogen triggered immunity response (PTI) and enhanced triggered immunity (ETI) (Macho and Zipfel, 2014; Nishimura and Dangl, 2014; Zipfel and Oldroyd, 2017), form part of the priming process in plants. Priming is "the physiological state that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells." A transgenerational and non-transgenerational or short term-inheritance of defense-related priming, improving the stability of this priming effect, has recently been shown in plants (Molinier et al., 2006; Pastor et al., 2013; Gully et al., 2019).

Essential oils (EOs) can act as priming molecules both in biotic and abiotic plant stress responses (Bertrand et al., 2021). EOs can be an effective and sustainable tool to control seedborne diseases (Klein et al., 2017; Spadaro et al., 2017). Seed priming prior to sowing is a promising strategy insofar as it makes seed more tolerant to disease and increases the yield and quality of high-value crops such as tomato. Seed priming alleviates stress at germination thus increasing seedling emergence and successful establishment of the seedling. Successful germination determines the vigor of seedling growth at later stages (Acharya et al., 2020). To date, few studies have been conducted on the transcriptomic and metabolomic responses of tomato seeds coated with essential oil to combat phytopathogens or on the assumed "*de novo*" molecular synthesis of immunity-related compounds for priming effect of such treatments (Kaplan et al., 2006; Ben-Jabeur et al., 2015; Rocha et al., 2019).

In this work we studied the transcriptomic and metabolomic responses of tomato seeds coated with an antifungal EO against the phytopathogen *Fusarium oxysporum*. The interaction between tomato and *Fusarium oxysporum f.* sp. *lycopersici* (*Fol*) serves as a model to study the molecular basis of disease resistance and susceptibility. Gene-for-gene interactions in this system have provided the basis for the development of tomato cultivars resistant to *Fusarium* wilt disease. *Fol* is one of the phytopathogens with the greatest impact on the agroforestry environment, producing damping-off disease and reducing tomato (*Solanum lycopersicum*) productivity worldwide (Windels, 2000; Carvalho et al., 2006; Berrocal-Lobo and Molina, 2008; Ma et al., 2013; Edel-Hermann and Lecomte, 2019).

Artemisia absinthium is a medicinal plant distributed principally throughout temperate regions in Europe, Asia, and Africa (Bora and Sharma, 2011). *Artemisia* species are current used in industry making them good candidates as a source of new biopesticides to protect plants (Bailen et al., 2013; Kundu et al., 2021). As part of our ongoing project on the sustainable production of biopesticides, thujone-free Spanish populations of *Artemisia absinthium* were domesticated, giving rise to a new plant variety (candial) with *cis*-epoxyocimene, chrysanthenol, chrysanthenyl acetate, *trans*-caryophyllene, and linalool as the main components of its essential oil. *(−)-cis*-Chrysanthenol has been identified as the chemical indicator of the antifungal effects of *A. absinthium* (candial) oil (Arraiza, 2017). However, the indirect effects of this EO against pathogenic fungi (such as plant priming) are still unknown.

Here we describe new metabolites and molecular changes associated with the short- and long-term protection of tomato plants germinated from AEO-coated seeds against the high impact phytopathogenic fungus *Fol*. The effects of the EO are discussed based on metabolomic and RNA-seq analysis. This work proposes new uses of EOs as a source of environmentally friendly biopesticides and biotechnological tools.

MATERIALS AND METHODS

Biological Material

Flowering plants of *Artemisia absintium* var. Candial were harvested in 2019 and their essential oil (AEO) extracted by vapor pressure in a stainless steel semi-industrial plant equipped with two 3,000 L vessels as described (Julio et al., 2015).

Untreated tomato seeds (*Solanum lycopersicum* L., *var. marmande*), were kindly provided by Ramiro Arnedo S.A (La Rioja, Spain). Seeds were dried, stored, and maintained at 4°C until use. *Fusarium oxysporum* sp. *oxysporum radicis lycopersici* (*Fol*) was provided by CECT (# 2715), Valencia, Spain. The fungus was grown on potato dextrose broth medium (PDB) at 28°C for 8 days in darkness as previously described in Berrocal-Lobo et al. (2002). Spores were collected in sterile water, filtered, quantified with a Neubauer chamber and stored in 20% (v/v) glycerol at −80°C until use.

Fungicidal Activity of *Artemisia absinthium* Essential Oil Against *Fusarium oxysporum* sp. *oxysporum radicis lycopersici* Phytopathogen

The assay to determine AEO fungicidal activity was prepared as previously described with little modification (Bailen et al., 2013). Briefly, the conidial concentration of a potato dextrose agar (PDA) was measured using a Neubauer chamber and diluted to a final concentration of the suspension being determined as 10^5 spores/ml. Spore susceptibility was tested in a 96-well plate using 100 μl Roswell Park Memorial Institute medium (RPMI) to support spore viability, 80 μl DMSO (1%) was used as a negative control (solvent- and drug-free); 80 μl Amphotericin B (5 μg/ml) as positive control. AEO was tested at different concentrations (5, 50, 500, and 1,000 μg/ml). After 24 h of incubation, antifungal activity was determined by an MTT (Thiazolyl blue tetrazolium bromide staining) assay measuring spore viability, proliferation, and cytotoxicity (Berrocal-Lobo et al., 2009). Menadione 1 mM and 25 μl RPMI medium were added per well incubated for 3 h at 37°C. and then removed prior to the addition of 200 μl isopropanol acid (95% isopropanol with 1 M HCl) After 30 min, the resulting-colored solution was quantified by measuring absorbance at 630 nanometers using a multi-well spectrophotometer and the Gen2.01 program. Data was analyzed with the Stat-graphics Centurion 18 program, using a Variance check ($p > 0.05$) and a non-parametric Kruskal-Wallis test.

The mycelial growth inhibition test was performed in 12-well plates. Ethanol was used as a negative control (2% final concentration). 1,950 μl PDA medium, 10 μl of MTT (5 mg/ml), and 40 μl ethanol were mixed in a sterile Falcon tube, shaken in a vortex, and then poured into each well. AEO stock was prepared at 50 mg/ml and serially diluted (0.1, 0.25, 0.5, and 1 mg/ml). Four replicates were used for each treatment. The plates were covered with aluminum foil and incubated at 27°C for 5 days (Morales-Sánchez et al., 2021). The fungal growth was quantified with the ImageJ program by measuring two perpendicular diameters of the grown area (cm^2) which was calculated using the formula:

$$\boldsymbol{Area} = Diameter \text{ x}\pi$$

Data was analyzed using the Stat graphics Centurion 18 program, using a Variance check ($p > 0.05$) and the non-parametric Kruskal-Wallis test.

Plant Growth Conditions

Tomato seeds were germinated, and seedlings grown for 12 days in an Aralab chamber (Lisbon, Portugal), at 50% humidity (v/v), temperature of 24°C during the day and 18°C during the night, with a 16-h light/8-h dark photoperiod and light intensity of 150 $\mu E{\cdot}m^{-2}$ per second for all experiments. Seeds were germinated in sterilized distilled water on paper filter discs in 12 well plates with 12 seeds per well. Plants were irrigated with regular water supply.

Absinthium Essential Oil Seed Coating

A 10 mg/ml AEO solution was prepared in 100% ethanol (Sigma-Aldrich, St. Louis, MO, United States) and serially diluted (1.0 and 0.5 mg/ml) for seed coating. Fresh tomato seeds previously stored for 24 h at 4°C were dipped in the solution one by one and then air dried on sterilized aluminum foil under sterile conditions until use. Control seeds were treated with corresponding 100% ethanol dilutions to AEO dilutions in sterilized water for each assay.

Plant Inoculations

Coated seeds were kept in constant contact with the fungus in a hydroponic system containing 500 μl of *Fol* (10^6 spores/ml) in water. Control samples were treated with 500 μl of sterilized distilled water with the corresponding dilution of glycerol. Inoculated plants were placed in the growth chamber (Aralab S.L, Lisbon, Portugal) under the growth conditions specified above. Infection was monitored by measuring the disease parameters specified for each assay.

Disease Symptoms

Disease symptoms of tomato seedlings (*var. marmande*) were measured considering the different seed germination start and finish times (between 4 and 9 days, respectively) and growth stages. Disease symptoms observed after 7 days were rated as follows: "0" normal seed germination and root emergence; "1" delayed seed germination and root emergence compared to 0; "2" germinated seeds with roots shorter than 2 cm compared to control; "3" germinated seeds with roots shorter than 1 cm; "4" no germination and seeds covered in fungus. Disease symptoms observed after 12 days were rated as follows: "0" no disease symptoms on seedlings; "1" delayed growth observed for shoots and roots with no apparent necrosis or chlorosis; "2" light chlorosis and necrosis on aerial part, including main leaves and shoots; "3" high chlorosis on main leaves and necrosis on shoots; "4" failed seedlings. Trypan blue staining was performed as previously described to detect seedling tissue cell death and necrosis (Lichtenthaler and Wellburn, 1983). Briefly, trypan blue solution is prepared: 10 ml lactic acid (85% w:w), 10 ml phenol (TE balanced buffer, pH 7.5–8.0), 10 ml glycerol (≥99%), 10 ml distilled water, 40 mg trypan blue (final concentration of 10 mg/ml). Seedlings were stained for 20 min and rinsed with 100% ethanol overnight and preserved in 60% glycerol until microscopy observation. Diamino-benzidine (DAB) staining performed as described in the literature was used to stain reactive oxygen species (ROS) production on coated and inoculated seeds (Livak and Schmittgen, 2001). Briefly, seeds and seedlings were placed in ethanol (100%) for 24 h. Tissue was treated with DAB solution (1 mg/ml) for 2 min under vacuum and covered with aluminum foil for 2 h at room temperature. The DAB solution was removed and Ethanol (100%) was added for 2 h before placing in glycerol 60%. DAB staining was performed at 30 min, 1 h, 24 h, 7, and 12 dpi. Callose deposition was measured by aniline blue staining of seedlings. Briefly, seeds or leaves previously treated with ethanol 100% for 24 h were stained in darkness using a 0,1 mg/ml water solution of aniline blue (Sigma-Aldrich, St. Louis, MO, United States) at for 30 min. Tissue was then rinsed with distilled water and placed in 60% glycerol at 4°C and mounted on microscopy slides. DAB and trypan blue were detected by bright light and aniline blue was detected using a DAPI/UV filter by fluorescence microscopy using a stereomicroscope (A292/21 Microscopy iScope IS.3153-PLFi/6 with Fluorescence—IS.3153-PLi/6,nEWF 10x/22, with Plan Fluarex PLFi, 4×, 10×, 20×, 40×, and 100× oil lenses including fluorescence: Blue, Green, UV-DAPI, and Red filters, Microsercon SLU, Madrid, Spain) with a charge-coupled device (CCD) digital cooled camera (A292/21 Euromex 20 MP USB 3.0 with 1 inch CMOS sensor), to obtain digital photos. Image processing and quantification of aniline blue (callose) and DAB signals was performed using

ImageJ Software and specific plug-in tools for DAB detection (Schneider et al., 2012).

To measure water content and loss, the fresh and dry weight of seedling tissue was measured, fresh plants were oven-dried at 85°C for 2 days and weighed. The moisture content of individual samples was calculated as follows.

$$\boldsymbol{WC = \left(FW - \frac{DW}{FW}\right) \times 100}$$

Where WC is the water content of individual plants or seedlings, FW is the fresh weight and DW is dry weight.

Total chlorophyll A, B and carotenoid content was determined according to Lichtenthaler and Wellburn (1983), with little modification. Briefly, 500 mg of fresh leaves were collected in a sterilized Eppendorf with borosilicate glass boils in liquid nitrogen. Samples were then ground in 5 ml of acetone (90%) and centrifuged at 3,000 g for 10 min. The supernatant was collected, and the absorbance of samples was measured using a spectrophotometer (Hach DR 2000, Hach Co., Loveland, CO, United States) at three wavelengths: 662, 644, and 470 nm. Pigment content was then calculated following authors' specifications and expressed as mg 100 g^{-1}, related to fresh weight (fw).

$$Ca = (13,96 \times A_{665}) - (6,88 \times A_{650})$$

$$Cb = (24,96 \times A_{650}) - (7,32 \times A_{665})$$

$$Ct = \frac{(1000 \times A_{470}) - (2,05 \times Ca) - (114,8 \times Cb)}{245}$$

Where Ca is Chlrolophyl a, Cb is Chlrolophyl b, and Ct is Carotenoids.

Statistical Analysis

The Stat Graphics Centurion XVI.II program (Stat Point Technologies, Inc., Warrenton, VA, United States) was used for all data analysis relating to plant growth and disease parameters. A one-way analysis of variance (ANOVA) and Duncan's mean comparison test were performed for all experiments and *t*-tests with a significance level of 0.05%. In the case of non-homogeneous variance, a non-parametric Kruskal–Wallis test was used.

RNA Quantitative Real-Time-PCR Analyses

Quantitative Reverse Transcription-PCR analysis was performed for RNA-seq data validation. Total RNA was isolated from frozen tomato tissue, separately analyzing roots and aerial parts corresponding to shoots. TRIzol Reagent (Invitrogen®, Carlsbad, CA, United States) was used according to the manufacturer's protocol along with chloroform. RNA samples were then treated with High pure RNA isolation kitto remove trace amounts of genomic DNA (Roche, Manheimm, Germany). RNA samples were analyzed to check quantity using a NanoDrop (UV-Vis ACTG Gene UVS—99. 200 a 850 nm) and quality was checked using Qubit 4.0 (Thermo Fisher Scientific, Madrid, Spain). RNA samples were visualized in 1% agarose gel before next step staining with GelRed (Nippon, Japan). First-strand cDNA synthesis was primed using a hexanucleotide random primer, and cDNA was synthesized using a First-Strand Synthesis Kit (Amersham-Pharmacia, Rainham, United Kingdom) according to the manufacturer's protocol. A 1.5 μl aliquot of the first-strand synthesis reaction was used as the template for PCR amplification. The program consisted of 3 min at 95°C, 40 cycles of: 30 s at 95°C, 30 s at 60°C, with a final extension step consisting of 7 min at 72°C and dissociation melting curves. The quantitative real-time (qRT-PCR) experiments were performed using a SYBR® Green qPCR master mix (Nzytech, Lisbon, Portugal) with reactions at a final volume of 10 μl per well. Samples were run in a DNA Engine One-Step QRT-PCR machine (Thermo Fisher Scientific, United States). Gene-specific primers were designed using the Primer Express 2.0 program (Applied Biosystems, Foster City, CA, United States), and minimal self-hybridization and dimer formation of primers were determined using the Oligo 6.0 program (Molecular Biology Insights, West Cascade, CO, United States). Primers with annealing temperatures of 58–60°C that amplified products with lengths of about 150 bp were selected and then verified for specificity using a Basic Local Alignment Search Tool (BLAST). The amplification efficiency for each pair of oligonucleotides was calculated as recommended by the manufacturer (Bio-Rad, Hercules, CA, United States) selecting only oligonucleotides with efficiencies above 90% for assays. Gene specific primers used for quantitative real-time PCRs are detailed in **Supplementary Table 1**. Data was acquired using the One-Step PCR Applied Biosystem Analysis software (Version 2.01), and changes in transcript levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Data points were compared using *t*-tests. Three independent biological replicates from different assays were used with three technical replicates in each experiment.

Construction of RNA-Seq Libraries

Total RNA from three independent biological replicates was extracted as detailed previously. For each sample, 1 μg of total RNA was used to construct the Illumina sequencing libraries according to the manufacturer's instructions (TruSeq Stranded mRNA LT Sample Prep Kit). Libraries were sequenced using the Illumina HiSeq 2500 platform (Biomarker Technologies) and 150 bp paired-end reads were generated.

Analysis of RNA-Seq Data

About 4 Gb of high-quality 150-bp paired-end reads were generated from each library and the quality of the clean reads was checked using the $Q < 20$ threshold. To reduce analysis bias, artifacts such as low-quality reads, adaptor sequence, contaminant DNA, and PCR duplicates were removed using Cutadapt.[1] Trimmed reads were mapped to the reference genome with HISAT2 splice-aware aligner (Pertea et al., 2016). The tomato reference genome and gene model annotation files were

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[1] https://pypi.org/project/cutadapt/

downloaded from the genome website browser (SGN release version SL2.50).[2] Known genes and transcripts were assembled using String Tie with aligned reads (Kovaka et al., 2019) based on the reference genome model (SL2.50). After assembly, gene/transcript abundance was calculated in the read count and normalized values obtained, i.e., FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Kilobase Million) for each sample using the feature counts function of the Bioconductor (Huber et al., 2015) package R subread (Liao et al., 2019) (strand Specific = 0, is Paired End = TRUE, require Both Ends Mapped = TRUE, primary Only = TRUE, ignore Dup = TRUE). Differentially expressed genes (DEGs) between samples were identified using the DESeq2 package (Love et al., 2014) with standard parameters (fold-change was ≥ 1 and FDR-adjusted P-value < 0.05). Average gene expressions in the three biological replicates were used for DEG identification (**Supplementary Tables 2, 3**).

Gene Ontology Enrichment Analysis and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis

Panther GO[3] was used for Gene Ontology (GO) enrichment. The GO enrichment analysis provided all the GO terms which were significantly enriched in the DEGs relative to the genomic background, and DEGs were filtered according to cellular components, molecular functions and biological processes. Kyoto Encyclopedia of Genes and Genomes (KEGG)[4] is a main pathway-related database. Based on the comparison of the DEGs to the genomic background, pathway enrichment analysis pinpointed the enriched pathways.

Validation of RNA-Seq by Quantitative Real-Time-PCR

To validate RNA sequencing reading data, 1 μg total RNA was reverse transcribed into cDNA following the previously described protocol for first strand synthesis using oligo (dT) primers. A Quantitative real-time PCR (qRT-PCR) was performed as previously described under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The fluorescence signal was monitored automatically in each cycle. Relative expression levels of specific mRNAs were measured as previously described using the $2^{(-\Delta\Delta Ct)}$ analysis method (Livak and Schmittgen, 2001), and expression values were normalized using the β-Actin gene. A regression line was calculated to analyze the correlation between Log_2 RNA-seq readings and quantitative real-time PCR Ct results from 12 independent RNA samples and five genes for each tissue (**Supplementary Figure 1**). Three independent biological replicates were analyzed for each sample. Primers used in this study are listed in **Supplementary Table 1**.

Metabolomic Analysis

Tomato Extract Preparation

Treated and untreated tomato seedlings (12 days old, 12 seedlings per replica with three biological replicas) were frozen in liquid N and then extracted with MeOH. Extracts were filtered and kept at −20°C until HPL-MS analysis. For GC-MS, the MeOH extracts were partitioned with dichloromethane (DCM), filtered and the solvent evaporated prior to GC-MS analysis.

Gas Chromatography Coupled With Mass Spectrometry Analysis of Essential Oil and Tomato Extracts

The essential oil and DCM fractions of MeOH tomato extracts were analyzed by gas chromatography coupled with mass spectrometry (GCMS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 μl) were performed using an AOC-20i and equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 μm film thickness) Teknokroma TRB-5 (95%) Dimetil- (5%) diphenylpolisiloxane. Working conditions were as follows: split ratio (20:1), injector temperature 300°C, temperature of the transfer line connected to the mass spectrometer 250°C, initial column temperature 70°C, then heated to 290°C, at 6°C/min intervals. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those found in the Wiley 229 and NIST Mass Spectral Database. All extracts (4 μg/μl) were dissolved in 100% DCM for injection. Pure compounds (salicylic acid, chlorogenic acid, and methyl jasmonate from Sigma-Aldrich) were injected and analyzed under the same conditions just described.

LCMS Analysis of Tomato Extracts

Methanolic tomato extracts were analyzed by liquid chromatography coupled with mass spectrometry (HPLC-MS) in a Shimadzu apparatus equipped with an LC- 20AD pump and a CTO-10AS VP column oven coupled to a mass spectrometer with a simple quadrupole analyzer LCMS-2020 QP, with an electrospray ionization source (ESI). An ACE 3 C18 column (150 mm × 4.6 mm, 3 μm particle size) with an ACE3 C18 analytical pre-column was used for separation. The compounds were eluted with Methanol (LC-MS grade) (MeOH): MiliQ water with 1% acetic acid 5% MeOH for 5 min, followed by a gradient 5:100% MeOH for 30 min, 100% MeOH for 10 min and 100:5% MeOH for 8 min, with a flow rate of 0.5 ml/min. The nitrogen flow (drying gas for solvent evaporation) was 15 L/min. Electrospray capillary potential was + 4.50 kV and a Full Scan was used in positive mode (m/z 100–700) with a potential of 1.40 kV and a capillary temperature of 250°C. Stock solutions of extracts were injected at 0.25 mg/ml with a 5 μl injection through an automatic injector (SIL-20A XR). All extracts (0.25 μg/μl) were dissolved in 100% MeOH for injection. Pure compounds (lycopene, carotene, salicylic acid, chlorogenic acid, and methyl jasmonate from Sigma-Aldrich) were injected at 0.2 mg/ml and analyzed under the same conditions as described above.

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[2] https://solgenomics.net

[3] http://www.pantherdb.org

[4] http://www.genome.jp/kegg/

TABLE 1 | Chemical composition of the *Artemisia absinthium* var. candial essential oil tested.

Compound	Retention time (min)	Area (≥1%)
Linalool	6.451	2.03
(−)-(*Z*)-Epoxyocimene	7.088	34.85
(*E*)-Epoxyocimene	7.303	2.37
Camphor	7.447	1.97
(−)-*cis*-Chrysanthenol	7.765	9.04
Chrysanthenyl Acetate	9.930	8.40
trans-Caryophyllene	13.546	4.74
Germacrene-D	14.868	2.41
β-Selinene	14.990	1.45
Dihydrochamazulene	15.520	3.37
Dihydrochamazulene isomer	17.672	1.03
Neointermedeol	18.526	1.20
Chamazulene	19.906	5.01
Geranyl-α-terpinene	24.667	3.30
Geranyl-α-terpinene isomer	24.791	3.24

RESULTS

Artemisia absinthium Essential Oil

Table 1 shows the chemical composition of *A. absinthium* var. Candial (AEO). The oil was characterized by *cis*-epoxyocimene (35%), followed by *cis*- chrysanthenol (9,04%), chrysanthenyl acetate (8,40%), chamazulene (5,01%), and *t*-caryophyllene (4,74%). The composition was like the one previously reported for other crops, highlighting the chemical stability of this plant variety (Julio et al., 2015).

Fungicidal Activity of *Artemisia absinthium* Essential Oil Against *Fusarium oxysporum* sp. *oxysporum radicis lycopersici* Phytopathogen

We tested increasing concentrations of AEO (0, 0.005, 0.05, 0.5, and 1 mg/ml) to test spore germination rate. AEO exhibited strong fungicidal activity against *Fol* spores *in vitro* with a significant spore germination inhibition rate of 44.25% ± 1.72, compared to controls at 0.5 mg/ml, with an EC50 of 109.91 as shown in **Table 2**. Moderate or no fungicidal effects on spore germination were observed at concentrations below 0.005 mg/ml.

Artemisia absinthium Essential Oil Effect on Seed Germination and Tomato Seedling Growth in Presence of *Fusarium oxysporum* sp. *oxysporum radicis lycopersici*

To test for physiological effects of the AEO coating, seeds were treated with 0.5 mg/ml and 1 mg/ml of AEO and germinated in presence (+), or absence (−), of *Fol* (10^6 spores/ml). Germination rates were determined at 7 dpi, before seed germination was complete, and after 12 days once germination was finished.

TABLE 2 | *In vitro* analysis of toxic activity of AEO on *Fol* spores.

AEO (μg/ml)	
5	83.58 ± 4.74
50	45.43 ± 0.41
500	44.25 ± 1.72
1000	30.62 ± 2.69
EC_{50}	109.91 (63.02–191.68)

Different concentrations of AEO (μg/ml), were tested on Fol spores (10^5 spores/ml), by MTT assay (Abs 630 nm).

Figure 1A (−) shows that, in absence of the fungus, there were no significant differences in seed germination rate between AEO treated seeds with 0.5 mg/ml (light blue bars), 1 mg/ml (dark blue bars), or control watered seedlings (white bars).

However, **Figure 1A** (+), shows that in presence of *Fol* (+), the fungus severely inhibited germination in non-coated seeds (white bars), compared to the AEO treated ones with 0.5 mg/ml (light blue bars) and 1 mg/ml (dark blue bars). This shows that AEO contributed to seedling germination both at 7 and at 12 dpi in presence of *Fol*. Based on these results, the 0.5 mg/ml concentration of AEO, was chosen for further assays.

Seedling root and shoot length was determined after 7 and 12 days in presence (+) and absence (−) of *Fol* (10^6 spores/ml) from pre-treated seeds with an AEO coating of 0.5 mg/ml (0.5), or 1 mg/ml (1). **Figure 1B** (−) shows that, at control plants, after 7 days, AEO did not affect shoot growth (dark green bars) or root growth (brown bars), in absence of the fungus (−). However, **Figure 1B** (+) shows that, in presence of the fungus (+), AEO raises plant tolerance to *Fol* as determined by increased root and shoot length, compared to controls. **Figure 1C** represents the percent of growth inhibition of shoots (dark green bars) and roots (light green bars) compared to controls in presence of the fungus (+). Growth inhibition of roots and shoots was higher for inoculated plants in absence of AEO.

Figure 1D (−) shows that after 12 days, into control non-inoculated plants (−), AEO did not affect shoot growth (dark green bars) or root growth (brown bars). Furthermore, as shown in **Figure 1D**, in presence of the fungus (+), an increase in root and shoot length happens, in AEO coated seedlings, showing that AEO contributes to plant tolerate to *Fol*. **Figure 1E** shows the percent of growth inhibition of shoots (dark green bars) and roots (light green bars) compared to controls in presence of the fungus (+). The inhibition observed on growth, was higher on inoculated plants in absence of AEO, in roots but was not observed in shoots.

Artemisia absinthium Essential Oil Effect on Tomato Seedlings Disease Parameters

The disease parameters of the seedlings were studied under hydroponic conditions in the presence and absence of *Fol*. Disease ratios were measured considering the different stages of germination and development of tomato at 7 and 12 days. After 7 days, disease ratios were measured completion of seed germination and after 12 days during heterotrophic growth.

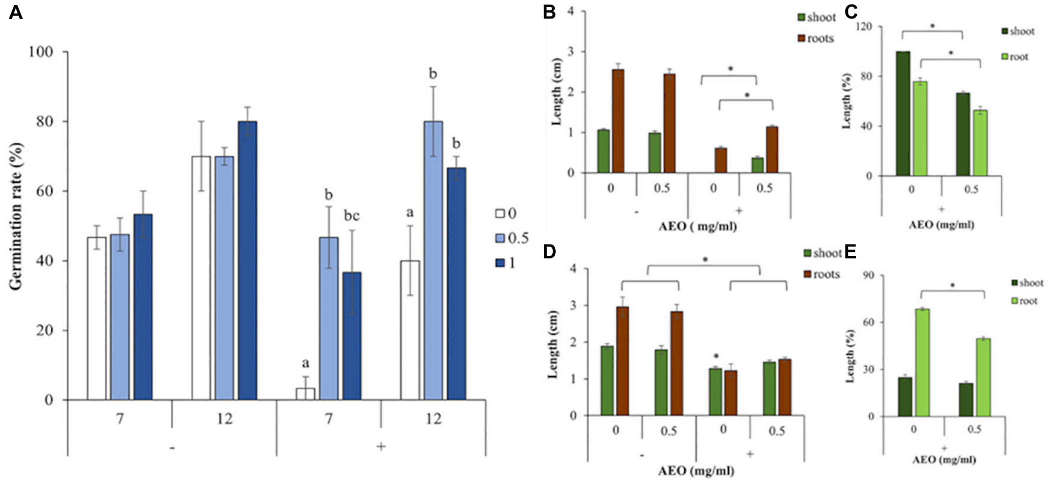

FIGURE 1 | AEO and *Fol* effects on germination rate and growth of tomato seedlings. **(A)** Germination rate measured in non-inoculated (–), or inoculated (+) seedlings, with *Fol* (10^6 spores/ml), after seed coating with AEO at 0.5 mg/ml (light blue bars), AEO at 1 mg/ml (dark blue bars), or watered controls (white bars), according to section "Materials and Methods". **(B)** Shoot length (green bars), and root length (brown bars), measured in non-inoculated (–), and inoculated (+) with *Fol* (10^6 spores/ml), on 7-day-old seedlings, in absence of AEO ("0"), or presence of AEO 0.5 mg/ml (0.5). **(C)** Percent of growth inhibition of shoots (green bars), and roots (brown bars), 7 dpi with *Fol* (+), related to non-inoculated seedlings, in presence of AEO 0.5 mg/ml (0.5) or in watered controls. **(D)** Shoot length (green bars), and root length (brown bars), measured in non-inoculated (–), and inoculated (+) with *Fol* (10^6 spores/ml), on 12-day-old seedlings, in absence of AEO ("0"), or presence of AEO 0.5 mg/ml (0.5). **(E)** Percent of growth inhibition of shoots (green bars), and roots (brown bars), 12 dpi with *Fol* (+), related to non-inoculated seedlings, in presence of AEO 0.5 mg/ml (0.5) and in watered controls. The * denotes a statistically significant difference using variance check (P-value $\leq$ 0.05) and Duncan test, between bracket samples at **(B,C)**, or (P-value $\geq$ 0.05) and Kruskal-Wallis test at **(D,E)**. The * on **(D)** denotes a statistically significant difference related to all the other treatments.

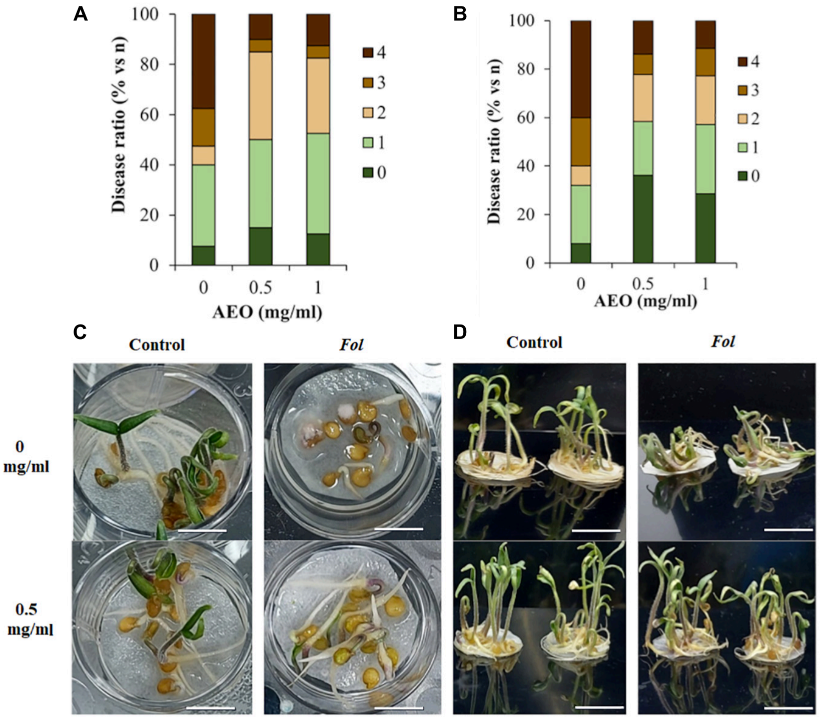

FIGURE 2 | Disease ratio under AEO treatment of seedling inoculated with *Fol*. The effect of AEO (at 0 and 0.5 mg/ml) protecting tomato against *F. solani*. **(A,C)** Disease ratio 7 days after inoculation (+) with *Fol* (10^6esp/ml), measured as percent of plants with different disease ratios, where: 0: no symptoms and normal germination (shoots and roots longer than 2 cm); 1: Delayed germination, showing radicle with no shoot; 2: Shoot and root measuring less than 2 cm; 3: Necrotic germinated seeds; 4: necrotic ungerminated seeds. **(B,D)** Disease ratio 12 days after inoculation (+) with *Fol* where: 0: no symptoms; 1: growth inhibition of shoots and roots and leaf chlorosis; 2: leaf necrosis and chlorosis; 3: leaf chlorosis and root necrosis; 4: decayed seedlings. Bars: (1 cm).

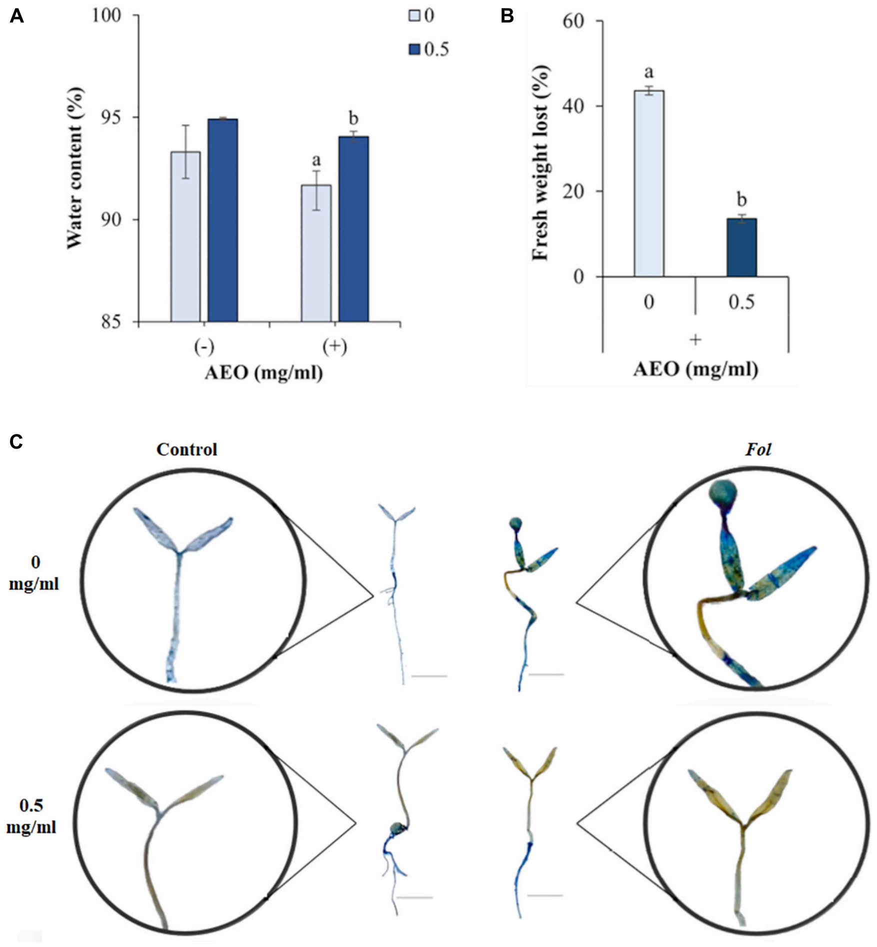

FIGURE 3 | Disease symptoms produced by Fol in AEO treated tomato seedlings. **(A)** Water content (WC), **(B)** Fresh weight loss (FWL), **(C)** Trypan blue (TB) staining showing necrosis induced by *Fol* (10^6esp/ml). Measurements taken after 12 dpi. Amplification of TB staining of seedlings are shown in circles. AEO treatment at concentrations of 0 (Controls at the top) and 0.5 mg/ml (at the bottom), and in absence (at the left) and in presence (at the right) of *Fol*. Bars (1 cm).

A quantitative analysis of disease symptoms indicated that the AEO treatment diminished the disease ratio both at 7 dpi (**Figures 2A,C**) and 12 dpi (**Figures 2B,D**).

AEO increased plant water content (15%, **Figure 3A**), and reduced fresh weight loss (FWL) (30%, **Figure 3B**). AEO also reduced other disease symptoms such as leaf necrosis (**Figure 3C**) and pigment content measured as total content of chlorophylls A and B (**Figures 4A,B**), and carotenoids (**Figure 4C**). Levels of all 3 pigments increased after 12 dpi compared to the corresponding controls into AEO treated seedlings (**Figure 4D**).

Artemisia absinthium Essential Oil Seed Coating Effect on Reactive Oxygen Species Production and Callose Deposition on Seeds After Germination

To specifically determine how AEO might protect seeds and seedlings against *Fol*, a kinetic of seed response to AEO treatment (0.5 mg/ml) was performed. Coated seeds were stained with DAB and aniline blue to determine the reactive oxygen production (ROS) of seeds throughout the germination process and the effect of AEO on the callose deposition process, respectively. Staining intensity was measured after 30 min, 1 h and at 1, 4, 7, and 12 days after the coating treatment in the presence and absence of Fol, AEO or both treatments. As shown in **Figure 5A**, the AEO coating treatment increased callose deposition during the germination period, between days 4 and 7. Callose quantification (see section "Materials and Methods"), confirmed the observed increase in callose deposition. The increase in callose was maintained up to 12 days in the AEO treated, inoculated and non-inoculated seedlings (**Figure 5B**). A high level of ROS production was also observed before germination in all seeds, as was a reduction in ROS production during seed germination (between 4 and 7 days) in control and AEO treated seeds in absence of the fungus (**Figure 6A**). The ROS level was high, on infected with *Fol*, even after the germination state. However, ROS levels in control seeds, seeds treated with AEO and AEO pre-treated and infected seeds, decreased once the germination process started (**Figure 6A**). The quantification of DAB confirmed those

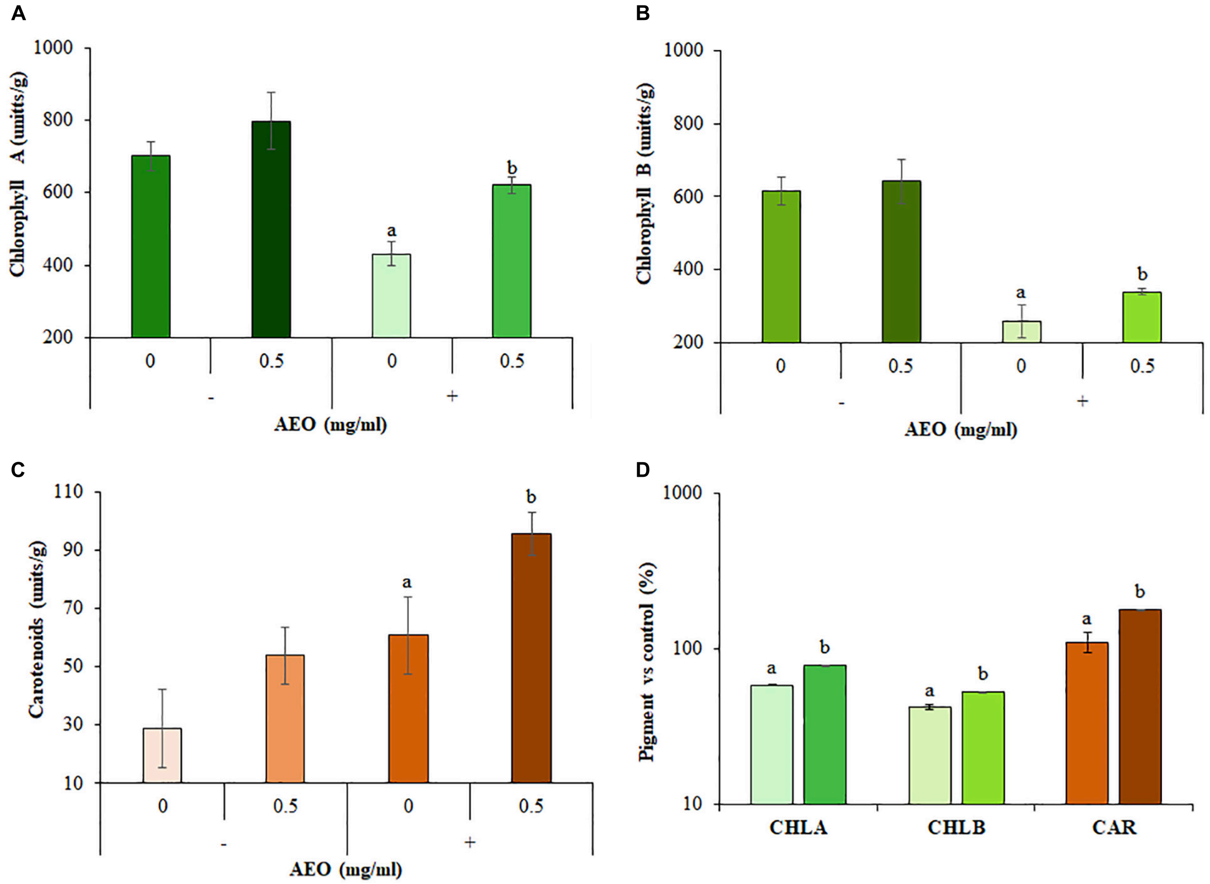

FIGURE 4 | Pigment content of AEO treated seedlings. **(A)** Total content of Chlorophyll A (CHLA). **(B)** Total content of Chlorophyll B (CHLB). **(C)** Total content of carotenoids. **(D)** Pigment increases compared to respective controls. Absorbance (see section "Materials and Methods") by leave tissue was measured after 12 days on non-inoculated (–) and inoculated (+) seedlings with *Fol* (10^6 sp/ml), pretreated with AEO at concentrations of 0 (Control) and 0.5 mg/ml. Total pigment content is expressed in (unit/g).

data (**Figure 6B**). A similar staining analysis was performed on seedling leaves but no ROS or callose depositions were observed in that tissue (data not shown).

Analysis of Seedling Response to *Artemisia absinthium* Essential Oil by RNA-Seq and Metabolomics

While the short-term compounds involved in tomato's recognition of *Fol* have been described, (Kaplan et al., 2006; Berrocal-Lobo and Molina, 2008; de Lamo and Takken, 2020) studies characterizing the long-term and "*de novo*" synthetized molecular compounds involved in plant tomato defense against *Fol* are still scarce. This work focuses on characterizing the effect that AEO has on tomato's long-term defense response to *Fol*. RNA-seq sequencing of plant genome after 12 days of inoculation with the fungus enabled us to determine the longer-term transcriptional changes produced by AEO in terms of the immunity response of tomato seedlings (see section "Materials and Methods"). A volcano Plot performed based on DEG analysis allowed us to determine that the number of genes transcriptionally upregulated or downregulated was substantially higher in shoots than in roots, 1,061 being induced in shoots compared to 526 in roots, 1,174 genes repressed in shoots compared to 323 in roots (**Figures 7A–D**). DEG analysis of shoot tissue showed a significant number of genes that are transcriptionally induced or repressed by the AEO treatment, 925 genes upregulated and 1,059 repressed, respectively (**Figure 8A**). A small number of genes was sufficient to perform a DEG analysis of the roots. The KEGG analysis determining pathway enrichment (see section "Materials and Methods") enabled us to identify the metabolomic pathways involved in the biosynthesis of secondary metabolites, linolenic acid, phenylpropanoids, monoterpenoids (McGarvey and Croteau, 1995), amino acid degradation, and plant-pathogen interactions, i.e., the main pathways induced as part of the long-term defense response of tomato seedlings (**Figure 8B**, left panel). Significant inhibition of other metabolic pathways was repressed, including the biosynthesis of secondary metabolites, the primary metabolism of carbon and glyoxylate and dicarboxylate metabolism, showing a transcriptional plant response to AEO affecting some specific metabolic pathways but not others (**Figure 8B**,

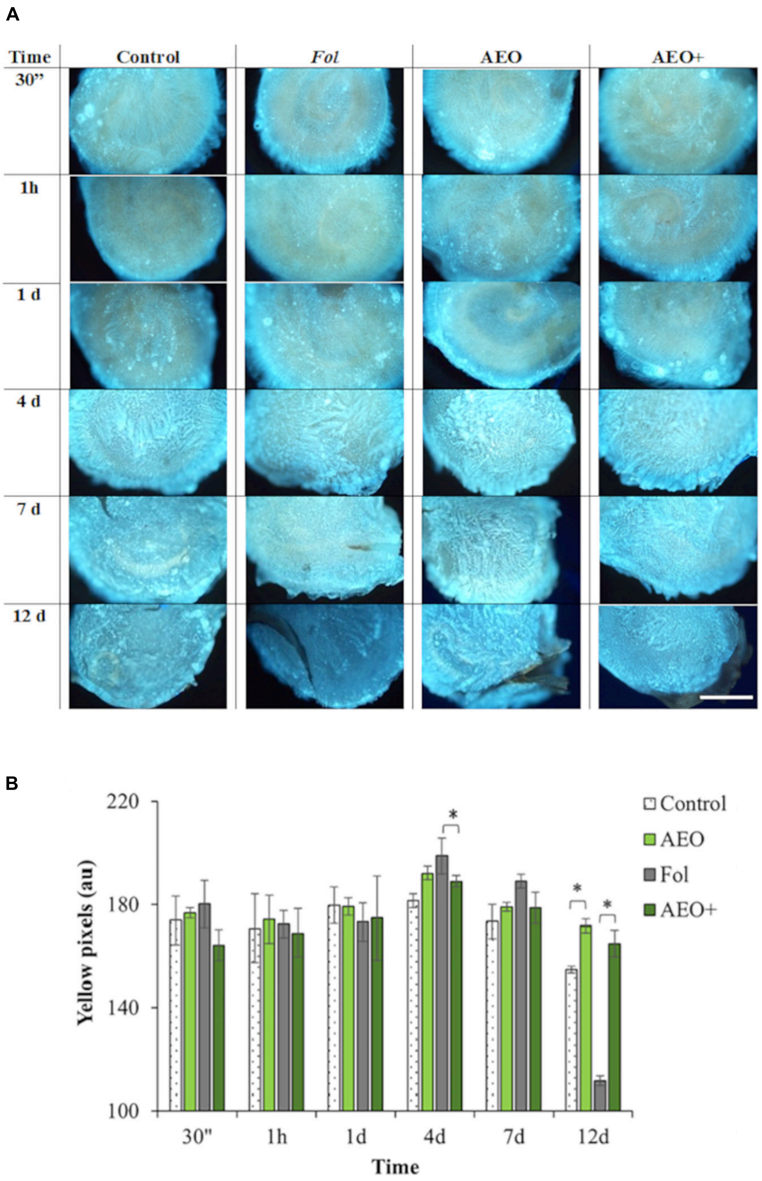

FIGURE 5 | Kinetic of callose deposition on seeds. Kinetic of callose deposition. The * denotes a statistically significant difference using variance check (*P*-value $\geq$ 0.05) and Kruskal–Wallis test. Tomato seeds were coated with 0.5 mg/ml of AEO. **(A)** Production of callose on seed surfaces was followed by fluorescence staining of callose with aniline blue (see section "Materials and Methods") after 30 min, 1 h, 24 h, 4, 7, and 12 days (see section "Materials and Methods"), on non-inoculated (–) and inoculated (+) seeds with *Fol* (10^6 sp/ml), pretreated with AEO concentrations of 0 (Controls) and 0.5 mg/ml. **(B)** Histogram showing quantification of yellow pixels produced by callose measured with Image J program (see section "Materials and Methods"). Bars (1 mm).

right panel). Raw data were submitted, *GEO accession number GSE186754.*

Metabolomic Effects of *Artemisia absinthium* Essential Oil on Tomato Seedlings

The mass ions of 12-day-old tomato seedlings analyzed by LC-MS showed induced metabolites of mass ions compatible with vanillic acid (179, M+H), coumarin (147, M+H), lycopene (537, M+H), and an unknown metabolite of m/z 529 that could be related to lycopene (**Table 3**). All these compounds were induced in the presence of AEO and *Fol*, with the compound of m/z (529) giving the strongest response (**Figure 9**). GCMS analysis of the dichloromethane fraction of the MeOH extracts (**Figure 10**) showed a strong induction of the lipid oleoamide also in the presence of AEO plus *Fol*.

DISCUSSION

In this work we have demonstrated the antifungal effects of *A. absinthium* var. candial vapor pressure essential oil (AEO) against *Fusarium oxysporum* conidia. Previous reports have

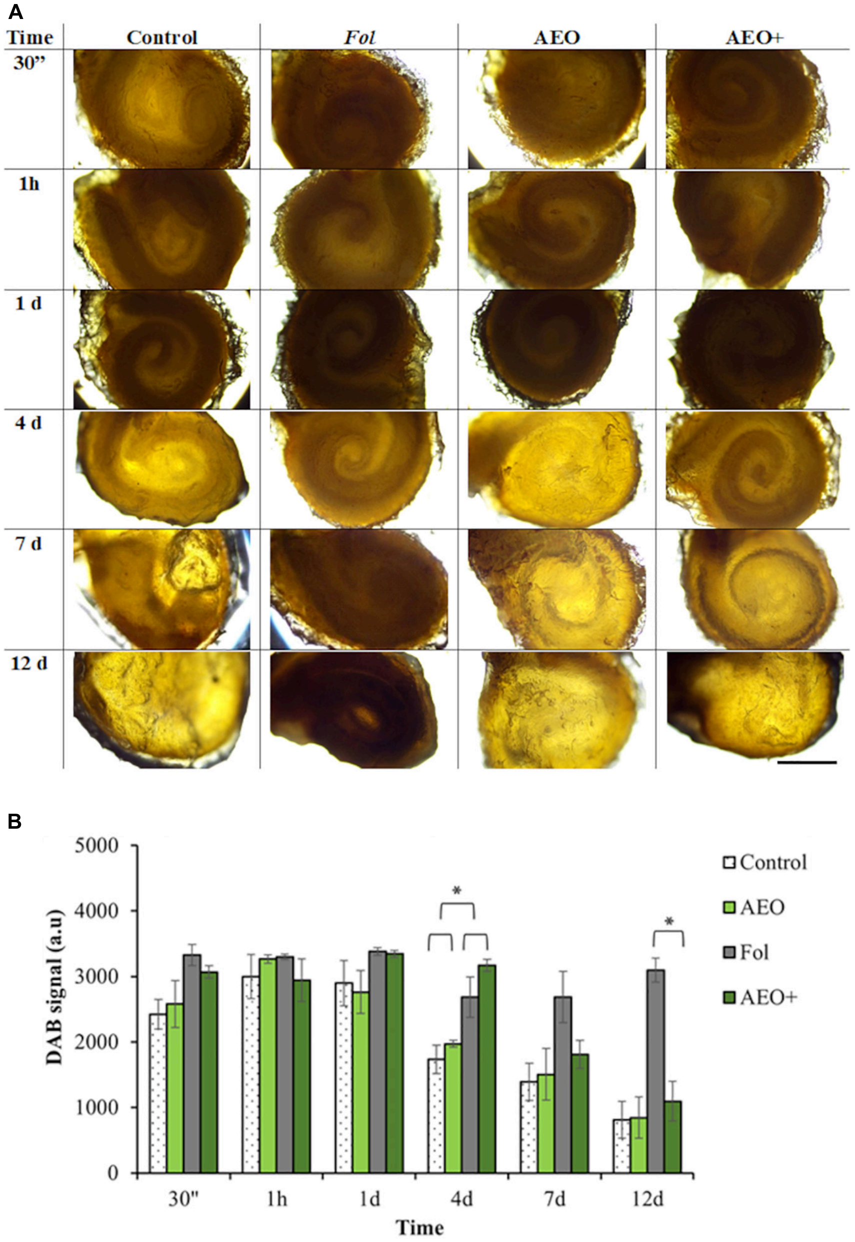

FIGURE 6 | Kinetic of Reactive Oxygen Species Production (ROS) on seeds. In day 4, the * denotes a statistically significant difference using variance check (P-value $\leq$ 0.05) and Duncan test. In day 12, the * denotes a statistically significant difference using variance check (P-value $\geq$ 0.05) and Kruskal–Wallis test. Tomato seeds were coated with 0.5 mg/ml of AEO. **(A)** Production of ROS on seed surfaces was followed by DAB staining of ROS production (see section "Materials and Methods"), after 30 min, 1 h, 24 h, 4, 7, and 12 days, on non-inoculated (–) inoculated (+) seeds with *Fol* (10^6 sp/ml), pretreated with AEO at concentrations of 0 (Controls) and 0.5 mg/ml. Bars (100 pixel). **(B)** Histogram showing quantification of DAB signal measured with Image J program (see section "Materials and Methods"). Bars (1 mm).

shown that *A. absinthium* var. candial collected in previous years (2008–2013), was moderately antifungal against the mycelium of *Fusarium* species (*F. moniliforme and F. solani*), with stronger effects against *Botrytis cinerea*. Fractionation of the AEO resulted in the identification of *(—)-cis*-chrysanthenol as the main antifungal compound, followed by linalool (Julio et al., 2015).

The coating of tomato seeds with a vapor pressure essential oil from *A. absinthium* var. candial (AEO) protected seed germination and seedling growth against *Fol*. Our results indicate that AEO protected seeds by directly affecting the fungus but also by the induction of a long-term response in terms of ROS production and callose deposition after germination. These effects were not detected in untreated seeds, where callose and

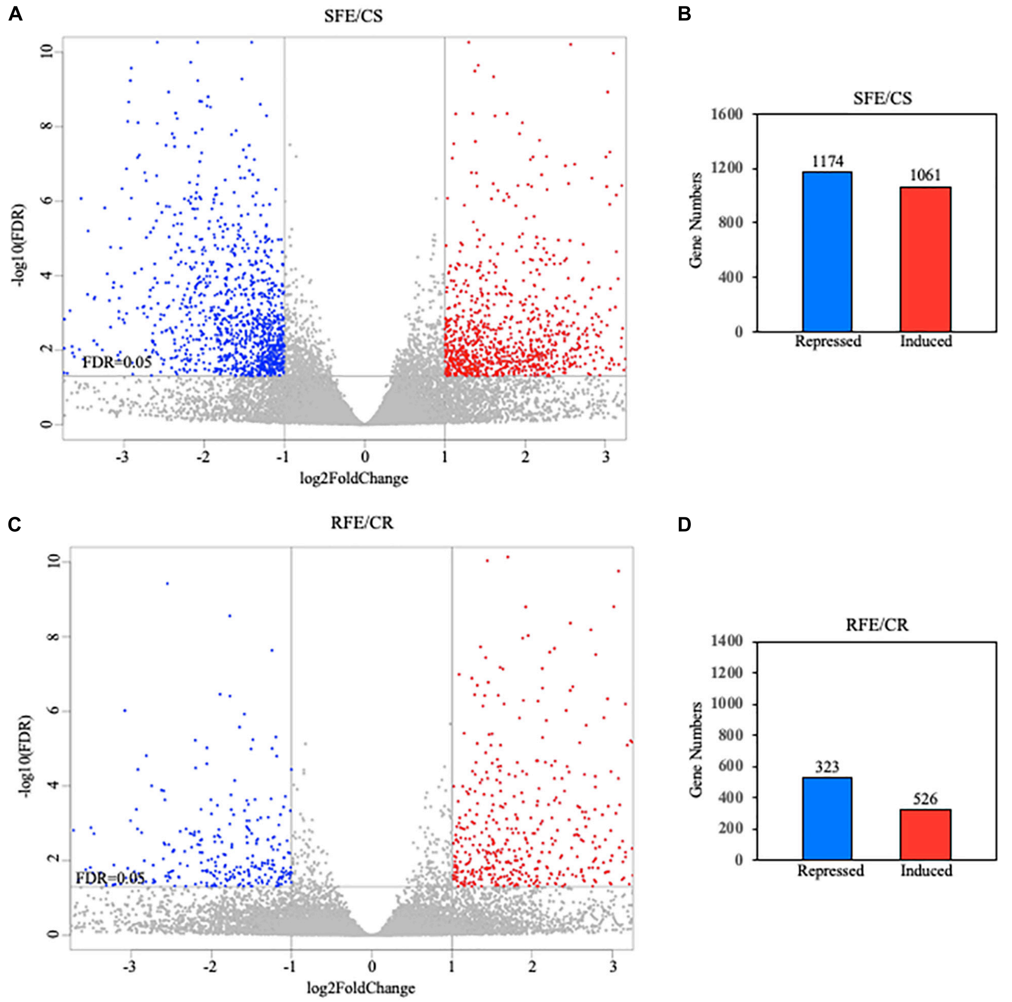

FIGURE 7 | Comparison of RNA-seq data between shoot and root assays. **(A,C)** Volcano plots made on shoots (upper) and roots (lower). Up-and downregulated DEGs are reported as red and blue dots, respectively. No DEG contribution represented as gray dots. **(B,D)** Number of induced or transcriptionally repressed genes corresponding to volcano analysis.

ROS production increased during germination and decreased in the absence of the fungus. There are previous results describing effects of coated seed priming on tomato (Król et al., 2015; Gonçalves et al., 2021).

A long-term response from AEO seed treatment was detected, which maintained higher levels of ROS and callose up to 12 days. Few studies describe callose deposition on tomato seed in the presence of phytopathogens (Zhang et al., 2015; Crespo et al., 2017) and this is the first work to date that describes callose deposition on tomato seeds induced by *Fol* and an essential oil. Our results indicate that AEO might penetrate the seeds once the testa is broken inducing molecular modifications in seeds and undifferentiated embryonic cells, contributing to the long-term tolerance observed in seedlings after 12 days. Our RNA-seq results suggest that the AEO coating treatment might induce specific "*de novo*" molecular changes detected at transcriptional level after 12 days in contact with the fungus. However, the molecular mechanism that might be involved in these effects remain unknown.

This work measures several of the hormones involved in the activation of the short-term signaling pathways related to tomato plant defense (Hernández-Aparicio et al., 2021) such as salicylic acid, jasmonate, methyl-jasmonate, and ethylene. However, the signal levels were not changed significantly compared to controls (data not shown), indicating that they are not the principal molecules involved in the long-term tolerance of tomato seedlings responding to *Fol*. The RNA-seq analysis showed that some other genes related to plant defense responses might also contribute to this tolerance, including genes involved in fatty acid metabolism, peroxidases, terpene synthases (Sun et al., 2016; Zhou and Pichersky, 2020), methyltransferases, and enzymes involved in gene silencing. Transcriptomic analysis showed enriched transcriptional induction on specific secondary metabolism pathways. The terpene synthases were transcriptionally induced. Lycopene and carotenoid synthesis

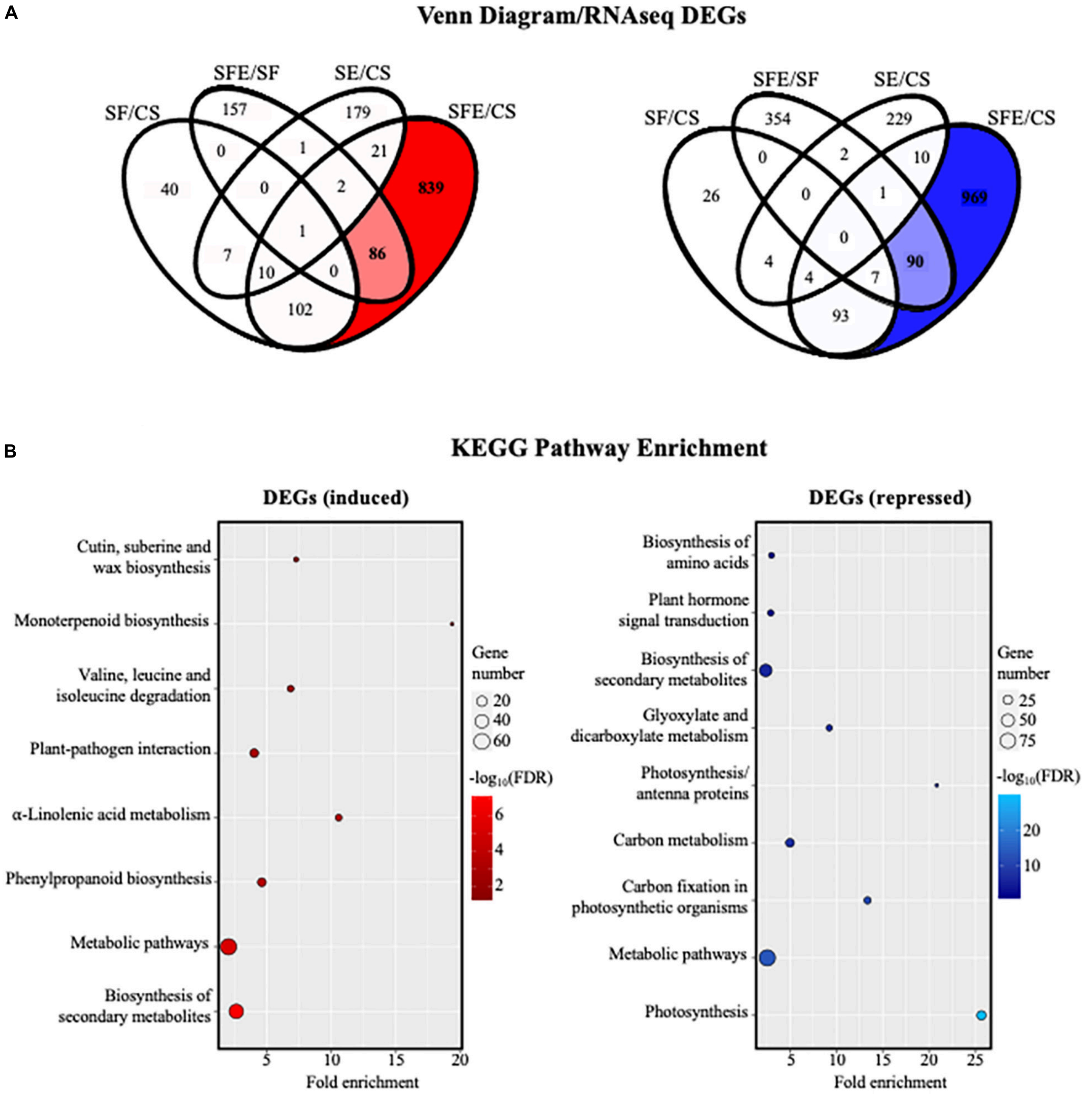

FIGURE 8 | Analyses of RNA-seq data from seedlings (shoots). **(A)** Identification of differentially expressed genes (DEGs) between different types of treatments using Venn diagram software (VENNY $^{2.1}$; https://bioinfogp.cnb.csic.es/tools/venny/index.html). Correspondences are (CS, control plants; SE, plants treated with AEO 0.5 mg/ml; SF, seeds inoculated with *Fol* 10^6 sp/ml; and SFE, AEO coated seeds 0.5 mg/ml inoculated with *Fol*. **(B)** KEGG pathway enrichment analysis of DEGs obtained from Venn diagram in RNA-seq. Gene number corresponds to the number of target genes in each pathway. Fold enrichment is the ratio (observed/expected) of the number of DEGs in a certain pathway. FDR-value is corrected *P*-value. The color and size of the dots represents the range of the $-\log_{10}$(FDR)-value and the number of DEGs mapped to the indicated pathways, respectively. Top enriched pathways are shown in the figure.

was also transcriptionally induced by AEO in our DEG analysis as previously described for wheat (Colasuonno et al., 2017).

The metabolomic analysis showed that the coating of tomato seeds with a vapor pressure essential oil from *A. absinthium* var. *candial* (AEO) induced several metabolites in tomato seedlings when infected with *Fol*, including the polar compounds vanillic acid and coumarin and the apolar ones lycopene, a metabolite of M+ 528, and the fatty acid derivative oleamide, these latter two being the most highly induced compounds. These results agree with the transcriptional induction of carotenoids and fatty acid metabolic pathways detected by RNA-seq analysis, and the increase in total chlrolophyl A and B and carotenoids found in the

TABLE 3 | LC-MS m/z adducts of main metabolites detected in tomato seedlings extracts analyzed by LC-MS.

Retention time (min)	[M+H]+ m/z	Identification
2.93	179	Vanillic acid
3.49	147	Coumarin
32.10	537	Lycopene
34.53	529	Lycopene-related

pigment analysis. These results might suggest that AEO treatment induces *de novo* changes that remained in the presence of the fungus for long-term plant tolerance.

Vanillic acid is a phenolic allelochemical reportedly present in tomato plants (Méndez and Brown, 2011) which significantly improves salinity tolerance and plant growth performance when externally applied to tomato seedlings (Parvin et al., 2020). Coumarin is an antioxidant, antimicrobial, and growth promoter in plants (Saleh et al., 2015; Nduche et al., 2019; Santra and Banerjee, 2020) and also mitigates salt stress in tomato plants (Parvin et al., 2020). Lycopene is a red carotenoid pigment of M+ (536) found in fruits and vegetables, including tomatoes (Stahl and Sies, 1996). Carotenoids are involved in photosynthesis and photoprotection in plants (Tao et al., 2007). Lycopene has an antimicrobial effect against bacteria and fungi such as *Candida albicans* by inducing apoptosis via ROS production and mitochondrial dysfunction (Choi and Lee, 2015). However, this is the first report on the induction of an apolar compound of M+ (528) in tomato plants treated with AEO and *Fol*. Oleamide (an oleic acid derivative) content increased with UV-B2 treatment in an olive cultivar (Celeste Dias et al., 2018). The role played by oleamide in plants remains unclear, but could be involved in growth/development regulation, stress response, and pathogen interactions (Kim et al., 2013) as shown here.

Additional assays are needed to determine the specific genetic modifications that enable the transcriptional and metabolic changes responsible for the long-term tolerance of tomato observed in this work. The possibility that epigenetic modifications are taking place in seeds resulting in "*de novo*" molecular modification for long-term tolerance of aerial parts opens new perspectives for the use of priming tools. The RNAseq analysis, also detected an inhibition of genes related to redox stress associated to ROS production during photosynthetic electron transport in the chloroplast. This response probably relates chloroplast stress with immunity response to *Fol*. Similarly, mitochondrial stress induced plant resistance, through chromatin changes, against phytopathogenic fungi and bacteria in Arabidopsis (López Sánchez et al., 2021). Therefore the stress produced in organelles involved into primary metabolism, might contribute to plant immunity. Furthermore, the interactions between metabolomics, transcriptomics, redox regulation, and epigenetics as shown here, are under current study in other plant systems (Shen et al., 2016). Future assays will be necessary to dilucidated our hypotheses. Our RNA-seq results, confirmed by quantitative real time PCR, show that *NRPD2* (López et al., 2011) mediating in *de novo* cytosine methylation by RNA-directed DNA methylation pathway (RdDM), is highly induced after 12 days of treatment with AEO in *Fol* infected seedlings (**Supplementary Data**). *NRPD2* was involved in efficient immunity response to *Botrytis cinerea* (López et al., 2011) and *Pseudomonas syringae pv tomato DC3000* in *Arabidopsis thaliana* (Zhang et al., 2021), and was necessary for reactive oxygen species (ROS) production, and activation of jasmonic acid and salicylic acid signaling pathways. Alterations in chromatin structure were necessary for efficient resistance to that fungus (Walley et al., 2008). To our knowledge, this is the first report relating NRPD2 to tomato immunity response regulation to *Fol*. *NRPD2* was also highly induced at transcriptional level by cucumber mosaic virus (CMV) in *Arabidopsis* being essential for plant immunity and demonstrating the importance of the induction of that gene during plant defense response (Kanazawa et al., 2011). In line with our results, a previous work demonstrated in *Arabidopsis* that DNA methylation is involved in immunity against *Fol* (Le et al., 2014) since RdDM-related mutants showed

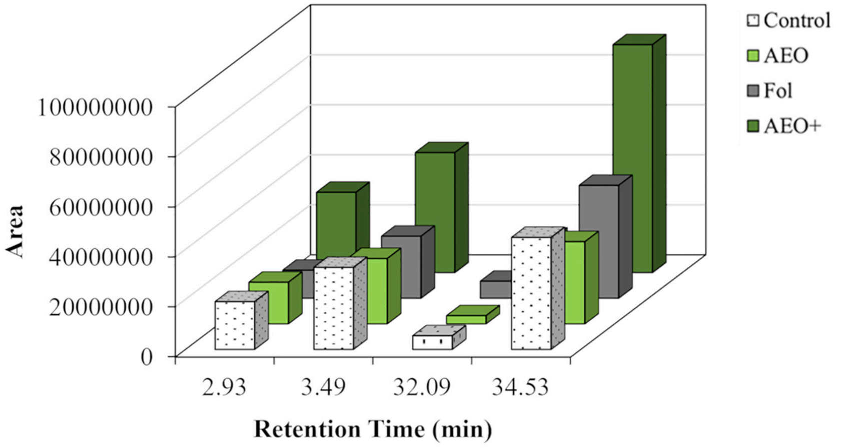

FIGURE 9 | Metabolomic analysis in AEO tomato seedlings. Metabolite induction is represented by arbitrary units. Correspondences are (CS, control plants; SE, plants treated with AEO 0.5 mg/ml; SF, seeds inoculated with *Fol* 10^6 sp/ml; and SFE, AEO coated seeds 0.5 mg/ml, inoculated with *Fol*).

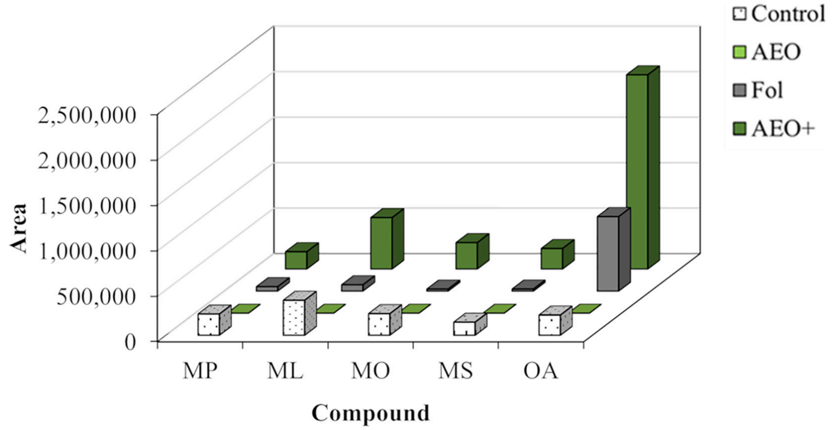

FIGURE 10 | GC-MS analysis of the DCM-soluble fraction of AEO tomato seedlings. The analyzed compounds are palmitic acid methyl ester (MP), linoleic acid methyl ester (ML), oleic acid methyl ester (MO), stearic acid methyl ester (MS), and oleoamide (OA). CS, corresponds to control plants; SE, plants treated with AEO 0.5 mg/ml; SF, seeds inoculated with *Fol* 10^6 sp/ml; and (SFE, AEO coated seeds 0.5 mg/ml, inoculated with *Fol*).

enhanced susceptibility to this fungus, suggesting that *de novo* methylation process contributes to *Fol* immunity. In addition, in this work the WRKY33 transcription factor, involved in the epigenetic control of plant defense against necrotrophs (Espinas et al., 2016; Ramirez-Prado et al., 2018; Alvarez-Venegas et al., 2019), was also induced specifically by AEO in presence of *Fol* in this work (**Supplementary Data**). This transcription factor showed increased levels on H3K4me3 on its promoter during *Botrytis cinerea* tomato infection (Crespo-Salvador et al., 2018). We also observed a significant increase in two S-adenosyl-L-methionine-dependent methyltransferases, one induced by real time PCR more than one hundred times (**Supplementary Data**), on AEO infected tomato seedlings after 12 days of treatment. *S-adenosylmethionine transferases (*SAMts*)* are responsible for maintaining the plant's methionine cycle (MTC) in the plant (Mäkinen and De, 2019). MTC connects ethylene and methylation pathways, where SAMs are DNA cytosine methylation markers for transcriptional RNA silencing (RGS), (Pooggin, 2013). The overexpression of SAMs in tomato increased tolerance to ROS stress (Gong et al., 2016) and prevented ROS accumulation in Arabidopsis (Jang et al., 2012). Our results detected very high levels of 1-aminocyclopropane-1-carboxylate oxidase 2 (ACCO), specifically involved in MTC cycle, for the synthesis of ethylene.

Considering that our RNA sequencing results indicate that MTC cycle is altered by AEO primed tomato plants in presence of *Fol*, a molecular analysis including histone epigenetic marks and effects on the offspring will be necessary to characterize this *de novo* long-term tolerance, produced by *A. absinthium* AEO.

CONCLUSION

This work demonstrates that the essential oil from *Artemisia absinthium* var. candial primed tolerance in tomato seedlings against phytopathogens such as *Fusarium oxysporum* sp., protecting not only seed germination, but also seedling growth and producing long term effects on plant tolerance after germination by inducing metabolomic and transcriptomic changes. Therefore, this work demonstrates that seed priming might be a useful tool to induce "*de novo*" non-transgenerational epigenetic changes in crops, modulating later responses of aerial parts of the plant, allowing crops to grow and improve tolerance against high impact phytopathogens.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: GSE186754).

AUTHOR CONTRIBUTIONS

SS performed all the assays characterizing the physiological response of tomato seeds and seedlings to AEO and *Fol* including sampling for RNA-seq and metabolomic analysis. CP-C designed and carried out the RNA-seq analysis, including DEG, KEG, primer design, and reposition of public data. ND performed RNA extractions and QRT-PCR analysis for RNA-seq confirmation. AG-C and MA performed AEO isolation and chemical characterization and metabolomic analysis and metabolite chemical characterization. MB-L coordinated this work and designed the experiments to characterize tomato responses to AEO and *Fol* and drafted the manuscript. All authors contributed to critical reading and writing of the manuscript and drew up their corresponding figures or tables.

FUNDING

This work was supported by the Ministry of Science, Spain, European Hub on New Challenges in the Field of Essential Oils

(EOHUB), and PID2019-106222RB-C31, MCIU, Spain. SS was funded by an international Ph.D. fellow from the UPM-Algerian Government from MHESR, Decree No. 14–196. ND was funded by a Ph.D. fellow DIN2018-009928 from MCIU, Spain. CP-C was supported by an I+D+i contract, Fucovasa, Spain.

ACKNOWLEDGMENTS

We specially want to acknowledge the contribution of our friend Paz Arraiza making this work and collaboration possible (RIP). We acknowledge Ramiro S.L for his kind contribution to the seeds, Ruben Muñoz for chromatographic analysis and Luis Andrés Agustín, Microsercon S.L for technical support and microscopy facilities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.804104/full#supplementary-material

Supplementary Table 2 | List of selected genes induced by AEO in presence of the fungus.

Supplementary Table 3 | List of selected genes repressed by AEO in presence of the fungus.

REFERENCES

Acharya, P., Jayaprakasha, G. K., Crosby, K. M., Jifon, J. L., and Patil, B. S. (2020). Nanoparticle-mediated seed priming improves germination, growth, yield, and quality of watermelons (*Citrullus lanatus*) at multi-locations in Texas. *Sci. Rep.* 10:5037. doi: 10.1038/s41598-020-61696-7

Alvarez-Venegas, R., De-la-Peña, C., and Casas-Mollano, J. A. (2019). *Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications: Transcriptional Regulation and Chromatin Remodelling in Plants: Second Edition*. Cham: Springer International Publishing.

Arraiza, M. P. (2017). *The Basics of Industrial Application*. Al Sharjah: Bentham Science Publishers.

Bailen, M., Julio, L. F., Diaz, C. E., Sanz, J., Martínez-Díaz, R. A., Cabrera, R., et al. (2013). Chemical composition and biological effects of essential oils from *Artemisia absinthium* L. cultivated under different environmental conditions. *Ind. Crops Prod.* 49, 102–107. doi: 10.1016/J.INDCROP.2013.04.055

Ben-Jabeur, M., Ghabri, E., Myriam, M., and Hamada, W. (2015). Thyme essential oil as a defense inducer of tomato against gray mold and *Fusarium* wilt. *Plant Physiol. Biochem.* 94, 35–40. doi: 10.1016/j.plaphy.2015.05.006

Berrocal-Lobo, M., and Molina, A. (2008). Arabidopsis defense response against *Fusarium oxysporum*. *Trends Plant Sci.* 13, 145–150. doi: 10.1016/j.tplants.2007.12.004

Berrocal-Lobo, M., Molina, A., Rodríguez-Palenzuela, P., García-Olmedo, F., and Rivas, L. (2009). *Leishmania donovani*: thionins, plant antimicrobial peptides with leishmanicidal activity. *Exp. Parasitol.* 122, 247–249. doi: 10.1016/j.exppara.2009.03.019

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of Ethylene-Response-Factor1 in arabidopsis confers resistance to several necrotrophic fungi. *Plant J.* 29, 23–32. doi: 10.1046/j.1365-313x.2002.01191.x

Bertrand, C., Gonzalez-Coloma, A., and Prigent-Combaret, C. (2021). Plant metabolomics to the benefit of crop protection and growth stimulation. *Adv. Bot. Res.* 98, 107–132.

Bora, K. S., and Sharma, A. (2011). The genus Artemisia: a comprehensive review. *Pharm. Biol.* 49, 101–109. doi: 10.3109/13880209.2010.497815

Carvalho, A. O., Souza-Filho, G. A., Ferreira, B. S., Branco, A. T., Araújo, I. S., Fernandes, K. V., et al. (2006). Cloning and characterization of a cowpea seed lipid transfer protein cDNA: expression analysis during seed development and under fungal and cold stresses in seedlings' tissues. *Plant Physiol. Biochem.* 44, 732–742. doi: 10.1016/j.plaphy.2006.09.011

Celeste Dias, M., Pinto, D. C. G. A., Correia, C., Moutinho-Pereira, J., Oliveira, H., Freitas, H., et al. (2018). UV-B radiation modulates physiology and lipophilic metabolite profile in *Olea europaea*. *J. Plant Physiol.* 222, 39–50. doi: 10.1016/J.JPLPH.2018.01.004

Choi, H., and Lee, D. G. (2015). Lycopene induces apoptosis in *Candida albicans* through reactive oxygen species production and mitochondrial dysfunction. *Biochimie* 115, 108–115. doi: 10.1016/J.BIOCHI.2015.05.009

Colasuonno, P., Marcotuli, I., Lozito, M. L., Simeone, R., Blanco, A., and Gadaleta, A. (2017). Characterization of aldehyde oxidase (AO) genes involved in the accumulation of carotenoid pigments in wheat grain. *Front. Plant Sci.* 8:863. doi: 10.3389/fpls.2017.00863

Crespo, E. F., Navarro, J. A., Soriano, M. S., Finiti, I., García-Agustín, P., Pallás, V., et al. (2017). Hexanoic acid treatment prevents systemic MNSV movement in cucumis melo plants by priming callose deposition correlating SA and OPDA accumulation. *Front. Plant Sci.* 8:1793. doi: 10.3389/fpls.2017.01793

Crespo-Salvador, Ó., Escamilla-Aguilar, M., López-Cruz, J., López-Rodas, G., and González-Bosch, C. (2018). Determination of histone epigenetic marks in *Arabidopsis* and tomato genes in the early response to *Botrytis cinerea*. *Plant Cell Rep.* 37, 153–166. doi: 10.1007/S00299-017-2218-9

de Lamo, F. J., and Takken, F. L. W. (2020). Biocontrol by *Fusarium oxysporum* using endophyte-mediated resistance. *Front. Plant Sci.* 11:37. doi: 10.3389/FPLS.2020.00037

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/ANNUREV.PHYTO.42.040803.140421

Edel-Hermann, V., and Lecomte, C. (2019). Current Status of *Fusarium oxysporum* formae speciales and races. *Phytopathology* 109, 512–530. doi: 10.1094/PHYTO-08-18-0320-RVW

Espinas, N. A., Saze, H., and Saijo, Y. (2016). Epigenetic control of defense signaling and priming in plants. *Front. Plant Sci.* 7:1201. doi: 10.3389/fpls.2016.01201

European Commission (2021). *The Common Agricultural Policy at a Glance*. Available online at: https://ec.europa.eu/info/food-farming-fisheries/key-policies/common-agricultural-policy/cap-glance_en#title (accessed October 26, 2021).

Gonçalves, D. C., Tebaldi de Queiroz, V., Costa, A. V., Lima, W. P., Belan, L. L., Moraes, W. B., et al. (2021). Reduction of *Fusarium* wilt symptoms in tomato seedlings following seed treatment with *Origanum vulgare* L. essential oil and carvacrol. *Crop Protect.* 141:105487. doi: 10.1016/J.CROPRO.2020.105487

Gong, B., Wang, X., Wei, M., Yang, F., Li, Y., and Shi, Q. (2016). Overexpression of S-adenosylmethionine synthetase 1 enhances tomato callus tolerance to alkali stress through polyamine and hydrogen peroxide cross-linked networks. *Plant Cell Tissue Organ Cult.* 124, 377–391. doi: 10.1007/S11240-015-0901-5/FIGURES/8

Gully, K., Celton, J. M., Degrave, A., Pelletier, S., Brisset, M. N., and Bucher, E. (2019). Biotic stress-induced priming and de-priming of transcriptional memory in *Arabidopsis* and apple. *Epigenomes* 3:3. doi: 10.3390/epigenomes3010003

Hernández-Aparicio, F., Lisón, P., Rodrigo, I., Bellés, J. M., and López-Gresa, M. P. (2021). Signaling in the tomato immunity against *Fusarium oxysporum*. *Molecules* 26:1818. doi: 10.3390/MOLECULES26071818

Huber, W., Carey, V. J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B. S., et al. (2015). Orchestrating high-throughput genomic analysis with bioconductor. *Nat. Methods* 12, 115–121. doi: 10.1038/NMETH.3252

Jang, S. J., Wi, S. J., Choi, Y. J., An, G., and Park, K. Y. (2012). Increased polyamine biosynthesis enhances stress tolerance by preventing the accumulation of reactive oxygen species: T-DNA mutational analysis of *Oryza sativa* lysine decarboxylase-like protein 1. *Mol. Cells* 34, 251–262. doi: 10.1007/s10059-012-0067-5

Julio, L. F., Burillo, J., Giménez, C., Sanz, J., Martínez-Díaz, R. A., Cabrera, R., et al. (2015). Chemical and biocidal characterization of two cultivated *Artemisia absinthium* populations with different domestication levels. *Ind. Crops Prod.* 76, 787–792. doi: 10.1016/J.INDCROP.2015.07.041

Kanazawa, A., Inaba, J. I, Kasai, M., Shimura, H., and Masuta, C. (2011). RNA-mediated epigenetic modifications of an endogenous gene targeted by a viral vector—a potent gene silencing system to produce a plant that does not carry a transgene but has altered traits. *Plant Signal. Behav.* 6:1090. doi: 10.4161/PSB.6.8.16046

Kaplan, B., Davydov, O., Knight, H., Galon, Y., Knight, M. R., Fluhr, R., et al. (2006). Rapid transcriptome changes induced by cytosolic Ca2+ transients reveal ABRE-related sequences as Ca2+-responsive cis elements in *Arabidopsis*. The plant cell online. *Plant Cell* 18, 2733–2748. doi: 10.1105/tpc.106.042713

Kim, S. C., Faure, L., and Chapman, K. D. (2013). Analysis of fatty acid amide hydrolase activity in plants. *Methods Mol. Biol.* 1009, 115–127. doi: 10.1007/978-1-62703-401-2_12

Klein, J. D., Firmansyah, A., Panga, N., Abu-Aklin, W., Dekalo-Keren, M., Gefen, T., et al. (2017). Seed treatments with essential oils protect radish seedlings against drought. *AIMS Agric. Food* 2, 345–353. doi: 10.3934/AGRFOOD.2017.4.345

Kovaka, S., Zimin, A. V., Pertea, G. M., Razaghi, R., Salzberg, S. L., and Pertea, M. (2019). Transcriptome assembly from long-read RNA-seq alignments with StringTie2. *Genome Biol.* 20, 1–13. doi: 10.1186/S13059-019-1910-1

Król, P., Igielski, R., Pollmann, S., and Kepczyñska, E. (2015). Priming of seeds with methyl jasmonate induced resistance to hemi-biotroph *Fusarium oxysporum* f.sp. lycopersici in tomato via 12-oxo-phytodienoic acid, salicylic acid, and flavonol accumulation. *J. Plant Physiol.* 179, 122–132. doi: 10.1016/j.jplph.2015.01.018

Kundu, A., Dutta, A., Mandal, A., Negi, L., Malik, M., Puramchatwad, R., et al. (2021). A comprehensive in vitro and in silico analysis of nematicidal action of essential oils. *Front. Plant Sci.* 11:614143. doi: 10.3389/fpls.2020.614143

Le, T. N., Schumann, U., Smith, N. A., Tiwari, S., Au, P. C., Zhu, Q. H., et al. (2014). DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in *Arabidopsis*. *Genome Biol.* 15:458. doi: 10.1186/S13059-014-0458-3

Liao, Y., Smyth, G. K., and Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res.* 47:e47. doi: 10.1093/NAR/GKZ114

Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592. doi: 10.1042/bst0110591

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

López, A., Ramírez, V., García-Andrade, J., Flors, V., and Vera, P. (2011). The RNA silencing enzyme RNA polymerase V is required for plant immunity. *PLoS Genet.* 7:e1002434. doi: 10.1371/JOURNAL.PGEN.1002434

López Sánchez, A., Hernández Luelmo, S., Izquierdo, Y., López, B., Cascón, T., and Castresana, C. (2021). Mitochondrial stress induces plant resistance through chromatin changes. *Front. Plant Sci.* 12:704964. doi: 10.3389/FPLS.2021.704964/FULL

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. doi: 10.1186/S13059-014-0550-8

Ma, L. J., Geiser, D. M., Proctor, R. H., Rooney, A. P., O'Donnell, K., Trail, F., et al. (2013). Fusarium pathogenomics. *Annu. Rev. Microbiol.* 67, 399–416. doi: 10.1146/annurev-micro-092412-155650

Macho, A. P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Mol. Cell* 54, 263–272. doi: 10.1016/j.molcel.2014.03.028

Mäkinen, K., and De, S. (2019). The significance of methionine cycle enzymes in plant virus infections. *Curr. Opin. Plant Biol.* 50, 67–75. doi: 10.1016/J.PBI.2019.03.002

McGarvey, D. J., and Croteau, R. (1995). Terpenoid metabolism. *Plant Cell* 7, 1015–1026. doi: 10.1105/TPC.7.7.1015

Méndez, J., and Brown, S. A. (2011). Phenols and coumarins of tomato plants. *Can. J. Bot.* 49, 2097–2100. doi: 10.1139/B71-295

Molinier, J., Ries, G., Zipfel, C., and Hohn, B. (2006). Transgeneration memory of stress in plants. *Nature* 442, 1046–1049. doi: 10.1038/nature05022

Montanarella, L., and Panagos, P. (2021). The relevance of sustainable soil management within the European Green Deal. *Land Use Policy* 100:104950. doi: 10.1016/j.landusepol.2020.104950

Morales-Sánchez, V., Díaz, C. E., Trujillo, E., Olmeda, S. A., Valcarcel, F., Muñoz, R., et al. (2021). Bioactive metabolites from the endophytic fungus aspergillus sp. SPH2. *J. Fungi* 7, 1–12. doi: 10.3390/jof7020109

Nduche, M., Nkaa, F., and Onyebinime, A. (2019). Phytochemical screening and antimicrobial activity of *Carica papaya* L, *Citrus paradisi* L, *Citrus sinensis* L, and *Vernonia amygdalina* Del. Cent. *J. Dairy Vet. Sci.* 9, 1–8. doi: 10.19080/JDVS.2019.09.555768

Nishimura, M. T., and Dangl, J. L. (2014). Paired plant immune receptors. *Science* 344, 267–268. doi: 10.1126/science.1253849

Parvin, K., Nahar, K., Hasanuzzaman, M., Bhuyan, M. H. M. B., Mohsin, S. M., and Fujita, M. (2020). Exogenous vanillic acid enhances salt tolerance of tomato: insight into plant antioxidant defense and glyoxalase systems. *Plant Physiol. Biochem.* 150, 109–120. doi: 10.1016/J.PLAPHY.2020.02.030

Pastor, V., Luna, E., Mauch-Mani, B., Ton, J., and Flors, V. (2013). Primed plants do not forget. *Environ. Exp. Bot.* 94, 46–56. doi: 10.1016/j.envexpbot.2012.02.013

Pertea, M., Kim, D., Pertea, G. M., Leek, J. T., and Salzberg, S. L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT. StringTie and Ballgown. *Nat. Protoc.* 11, 1650–1667. doi: 10.1038/NPROT.2016.095

Pooggin, M. M. (2013). How can plant DNA viruses evade siRNA-directed DNA methylation and silencing? *Int. J. Mol. Sci.* 14, 15233–15259. doi: 10.3390/IJMS140815233

Ramirez-Prado, J. S., Abulfaraj, A. A., Rayapuram, N., Benhamed, M., and Hirt, H. (2018). Plant immunity: from signaling to epigenetic control of defense. *Trends Plant Sci.* 23, 833–844. doi: 10.1016/j.tplants.2018.06.004

Rocha, I., Ma, Y., Souza-Alonso, P., Vosátka, M., Freitas, H., and Oliveira, R. S. (2019). Seed coating: a tool for delivering beneficial microbes to agricultural crops. *Front. Plant Sci.* 10:1357. doi: 10.3389/FPLS.2019.01357

Saleh, A. M., Madany, M. M. Y., and González, L. (2015). The effect of coumarin application on early growth and some physiological parameters in faba bean (*Vicia faba* L.). *J. Plant Growth Regul.* 34, 233–241. doi: 10.1007/S00344-014-9459-4

Santra, H. K., and Banerjee, D. (2020). "Natural products as fungicide and their role in crop protection," in *Natural Bioactive Products in Sustainable Agriculture*, eds J. Singh and A. Yadav (Cham: Springer), 131–219. doi: 10.1007/978-981-15-3024-1_9

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Shen, Y., Issakidis-Bourguet, E., and Zhou, D. X. (2016). Perspectives on the interactions between metabolism, redox, and epigenetics in plants. *J. Exp. Bot.* 67, 5291–5300. doi: 10.1093/JXB/ERW310

Spadaro, D., Herforth-Rahmé, J., and van der Wolf, J. (2017). Organic seed treatments of vegetables to prevent seedborne diseases. *Acta Hortic.* 1164, 23–31. doi: 10.17660/ACTAHORTIC.2017.1164.3

Stahl, W., and Sies, H. (1996). Lycopene: a biologically important carotenoid for humans? *Arch. Biochem. Biophys.* 336, 1–9. doi: 10.1006/ABBI.1996.0525

Sun, P., Schuurink, R. C., Caissard, J. C., Hugueney, P., and Baudino, S. (2016). My way: noncanonical biosynthesis pathways for plant volatiles. *Trends Plant Sci.* 21, 884–894. doi: 10.1016/j.tplants.2016.07.007

Tao, N., Hu, Z., Liu, Q., Xu, J., Cheng, Y., Guo, L., et al. (2007). Expression of phytoene synthase gene (Psy) is enhanced during fruit ripening of Cara Cara navel orange (*Citrus sinensis* Osbeck). *Plant Cell Rep.* 26, 837–843. doi: 10.1007/S00299-006-0289-0

Walley, J. W., Rowe, H. C., Xiao, Y., Chehab, E. W., Kliebenstein, D. J., Wagner, D., et al. (2008). The chromatin remodeler SPLAYED regulates specific stress signaling pathways. *PLoS Pathog.* 4:e1000237. doi: 10.1371/journal.ppat.1000237

Windels, C. E. (2000). Economic and social impacts of fusarium head blight: changing farms and rural communities in the northern great plains. *Phytopathology* 90, 17–21. doi: 10.1094/PHYTO.2000.90.1.17

Zhang, Y., Li, D., Zhang, H., Hong, Y., Huang, L., Liu, S., et al. (2015). Tomato histone H2B monoubiquitination enzymes SlHUB1 and SlHUB2 contribute to disease resistance against *Botrytis cinerea* through modulating the balance between SA- and JA/ET-mediated signaling pathways. *BMC Plant Biol.* 15:252. doi: 10.1186/s12870-015-0614-2

Zhang, Y., Shi, C., Fu, W., Gu, X., Qi, Z., Xu, W., et al. (2021). *Arabidopsis* MED18 interaction with RNA pol IV and V subunit NRPD2a in transcriptional regulation of plant immune responses. *Front. Plant Sci.* 12:692036. doi: 10.3389/fpls.2021.692036

Zhou, F., and Pichersky, E. (2020). More is better: the diversity of terpene metabolism in plants. *Curr. Opin. Plant Biol.* 55, 1–10. doi: 10.1016/j.pbi.2020.01.005

Zipfel, C., and Oldroyd, G. E. D. (2017). Plant signalling in symbiosis and immunity. *Nature* 543, 328–336. doi: 10.1038/nature22009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORIGINAL RESEARCH
published: 22 April 2022
doi: 10.3389/fpls.2022.853746

Biochar Implications Under Limited Irrigation for Sweet Corn Production in a Semi-Arid Environment

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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *13 January 2022*
Accepted: *21 March 2022*
Published: *22 April 2022*

Citation:
Singh M, Singh S, Parkash V, Ritchie G, Wallace RW and Deb SK (2022) Biochar Implications Under Limited Irrigation for Sweet Corn Production in a Semi-Arid Environment. Front. Plant Sci. 13:853746. doi: 10.3389/fpls.2022.853746

The integration of biochar and deficit irrigation is increasingly being evaluated as a water-saving strategy to minimize crop yield losses under reduced irrigation in arid and semi-arid regions such as West Texas. A 2-year (2019 and 2020) open-field study evaluated the effect of two types of biochar amendments (hardwood and softwood) and three irrigation rates [100, 70, and 40% crop evapotranspiration (ET_C) replacement] on the physiology, plant growth, and yield of sweet corn in semi-arid West Texas. All experimental units were replicated four times in a split-plot design. The chlorophyll content (Chl_{SPAD}) in 40% ET_C dropped significantly compared to 100% ET_C and 70% ET_C during the reproductive phase. Although water stress under 40% ET_C decreased photosynthesis (P_n) to limit transpiration (E) by stomatal closure, it improved intrinsic water use efficiency (iWUE). The above-mentioned gas exchange parameters were comparable between 100% ET_C and 70% ET_C. Both biochar treatments increased Chl_{SPAD} content over non-amended plots, however, their effect on gas exchange parameters was non-significant. All growth and yield-related parameters were comparable between 100% ET_C and 70% ET_C, but significantly greater than 40% ET_C, except water productivity (WP). Both deficit irrigation treatments improved WP over full irrigation in 2019, but in 2020, the WP gains were observed only under 70% ET_C. Hardwood biochar decreased soil bulk density and increased soil porosity, but it had a marginal effect on the water retention characteristics. These results suggest that 70% ET_C can be used as an alternative to full irrigation to save water with a minimal yield penalty for sweet corn production in the West Texas region. The hardwood biochar application improved the vegetative biomass significantly but yield marginally during the first 2 years of application. A long-term study is required to test the effect of biochar under deficit irrigation beyond 2 years.

Keywords: water productivity, water holding capacity, drought stress, semi-arid, physiology, photosynthesis, plant available water

INTRODUCTION

The High Plains region of Texas in the United States is water-stressed like many arid and semi-arid regions of the world (Nielsen-Gammon et al., 2020). Global climate change, increasing population, and water use have exacerbated the uncertainty of water availability in the future and increased the vulnerability to drought events. Drought is the most critical abiotic stress, which impairs the plant's physiological processes, growth, and causes heavy yield losses (Seleiman et al., 2021). Therefore, the availability of an irrigation source is necessary to ensure future food security. The average yearly reference evapotranspiration (ET_o) of 1,500 mm in the Lubbock County of West Texas is higher than the 469 mm of average rainfall (TAMU, 2021). This necessitates the need for supplemental irrigation to meet crop water requirements and maintain high-crop yields. The Ogallala aquifer is the primary source of water on the Texas High Plains and more than 90% of the water derived from this aquifer is being used for irrigation purposes (McGuire, 2017). The high water extraction rate has caused a serious decline in the water levels, especially in West Texas (Daher et al., 2019). Hence, strategies promoting the efficient use of irrigating water are required to sustain the water resources and ensure food security in the future.

Crop yields are generally the highest under optimal water application, but crops can adapt and produce reasonably under limited water (FAO, 2002). Deficit irrigation (DI), a strategy of applying less water than the evapotranspiration demands, is generally employed to increase water productivity (WP). However, water stress can induce several physiological and biochemical changes in the plant, ultimately affecting its morphology. Water stress reduces stomatal conductance (g_s), which in turn moderates the transpiration (E) and leaf gas exchange (Seleiman et al., 2021). Transpiration plays an important role in regulating metabolic activities by moderating the leaf temperature (Sterling, 2005). The reduction in gas exchange decreases CO_2 assimilation. The loss of turgor due to moisture stress retards cell elongation and division causing reduced leaf expansion. Thus, water stress can adversely affect net photosynthesis by reducing the leaf-level photosynthesis (P_n) and decreasing the leaf area. The reduction in net photosynthesis ultimately reduces biomass production and yield. However, plants can adjust to a maintain high P_n with a moderate reduction in the g_s, and P_n is generally less sensitive to water stress than g_s (Liu et al., 2005; Pazzagli et al., 2016; Parkash, 2020). Thus, the leaf-level intrinsic water use efficiency (iWUE), defined as the ratio of P_n and g_s, is expected to increase under moderate water stress. It is important to quantify iWUE gains at different DI levels and evaluate if these are translated into WP at the crop level. A few researchers have investigated the feasibility of DI in sweet corn (*Zea mays* L. var. *rugosa*) in terms of yield and biomass production (Stone et al., 2001; Farsiani et al., 2011; Saberi et al., 2012; Motazedian et al., 2019) but studies assessing the DI effects on sweet corn physiology are lacking.

Sweet corn (*Zea mays* L. var. *rugosa*) is a warm-season crop relished for its tastefulness, high-sugar concentration, soft kernels, and thin shell (Oktem et al., 2003). Sweet corn is considered sensitive to water stress due to its shallow root system (Laboski et al., 1998). Oktem (2008) observed a 6, 22, and 37% decline in the sweet corn yield with 10, 20, and 30% reduction in the irrigation compared to full irrigation, respectively. Ertek and Kara (2013) suggested that 85% of full irrigation could serve as an alternative without a significant decrease in the sweet corn yield. These conflicting results suggest that water stress can lead to dramatic fluctuations in the sweet corn yield, especially in drought-prone semi-arid regions like the Texas High Plains.

The plant responses to water stress vary with climate and soil (Singh et al., 2021). Soil organic amendments have been suggested to improve the soil's physiochemical and microbial properties (Ding et al., 2016; Blanco-Canqui, 2017; Atkinson and Aitkenhead, 2018). Biochar, a carbon-rich product of pyrolysis of organic matter is increasingly being studied as a soil amendment to mitigate drought stress. Previous literature suggests that biochar application generally decreases the bulk density, and increases soil porosity and water retention (Blanco-Canqui, 2017), although responses vary with biochar feedstock, pyrolysis conditions, and soil type (Spokas et al., 2012).

Reviewing the available reports, Blanco-Canqui (2017) noted an increase in plant available water with biochar application in 72% of the cases. Several recent studies revealed the improvement in yield and WP of various crops with integrated use of biochar application and DI (Ali et al., 2018; Faloye et al., 2019; Singh et al., 2019; Alfadil et al., 2021). In a greenhouse tomato experiment, using 25 t ha^{-1} wheat straw biochar, Agbna et al. (2017) observed significant improvement in transpiration and photosynthesis rate and obtained comparable yield between water-stressed and full irrigation treatments. Gavili et al. (2019) observed an increase in stomatal conductance, water use efficiency (WUE), growth, and yield of greenhouse-grown drought-stressed soybean plants treated with 1.25% (w/w) cattle manure biochar compared to untreated plants. Similarly, an open-field study conducted by Langeroodi et al. (2019) suggests that soil amendment using 10 t ha^{-1} maize straw biochar improved chlorophyll content, seed yield, and WUE of pumpkin subjected to DI on a silt loam soil in a semi-arid climate. Contrarily, Ramlow et al. (2019) observed no significant effect of broadcasting 25 t ha^{-1} woody biochar on biomass and yield of maize under full or limited irrigation. Liu et al. (2017) observed a negative effect of 0.74% (w/w) birch wood biochar on biomass and WUE of potato plants raised in pots filled with sandy loam soil. The results obtained in the above-mentioned studies with different biochars in various crops subjected to DI suggest that biochar may influence crop physiology and growth depending on factors like biochar feedstock and preparation procedure, crop type, soil characteristics, and climatic conditions. Therefore, investigation of such effects and the mechanisms involved is necessary before implementing the use of any biochar material in crop production.

Currently, no report investigating the use of biochar for mitigating water stress in sweet corn is available in the literature. Field studies investigating the interactive effects of DI and biochar application on crop plants are lacking in semi-arid regions like the Texas High Plains. We hypothesized that using DI in sweet corn can save water, and biochar can alleviate the negative effect of water stress and help maintain crop productivity. The combined use of DI and biochar can be a part of water and food sustainability approaches needed in the semi-arid Texas High Plains. The objective of this study is to evaluate the effect of two biochar types and DI levels on physiology, growth, yield, and WP of sweet corn.

MATERIALS AND METHODS

Site Description

The field experiments were conducted in 2019 and 2020 at the Quaker Research Farm, Texas Tech University, Lubbock, TX (33° 36′ 18″ N, -101° 54′ 26″W, and 992 m above sea level). The trials were carried out on the same experimental plots during both years. The climate of the experimental site is semi-arid with an average annual rainfall of 469 mm, mostly concentrated from May to October. The average annual high and low temperatures are 23.3 and 7.8 °C, respectively. The average annual evapotranspiration is 1,501 mm, far exceeding the average annual rainfall (TAMU, 2021). The soil of the experimental site is described as Amarillo sandy clay loam (fine-loamy, mixed, super active, thermic Aridic Paleustoll).

Land Preparation and Planting

The seedbeds were prepared with a tractor-mounted disk plow. The seeds of a sweet corn hybrid, Remedy, were planted using a four-row planter at the rate of 7.4 kg/ha maintaining a 100 cm spacing between the rows. The planting was done on 5 May 2019 and 2020. However, in 2020, replanting was done on 15 May due to poor germination. The plant density obtained after crop establishment was 4.1 and 3.3 plants m^{-2} in 2019 and 2020, respectively. Mechanical weeding was performed once when the crop was in the knee-high stage in both years. Thereafter, manual weeding was done as needed. The field was irrigated using a subsurface drip irrigation system laid at the depth of 30 cm under each bed and 100 cm apart. All experimental plots received equal amounts of fertilizer based on the soil test recommendations. The fertilizers were applied through a drip irrigation system with 112 kg N/ha at 4 weeks after sowing and 56 kg N/ha at 8 weeks after planting in 2019, and 6 weeks after planting with 90 kg N/ha in 2020 using URAN 32 (32-0-0, Nitrogen Fertilizer Solution, Nutrien Ag Solution, Loveland, Colorado).

Biochar Properties and Application

Two types of biochar prepared from different feedstock, hardwood-oak, and softwood-pine used in this experiment were acquired from Wakefield$^{\text{TM}}$ BioChar. The hardwood biochar was prepared through slow pyrolysis at 350°C for 24 h whereas the softwood biochar was prepared at 500°C for 15 min. The physical and chemical characteristics of the two biochars are described in **Table 1**. Both biochars were spread in the respective field plots at 13 Mg ha^{-1} and incorporated into the soil using a tractor-mounted rotary tiller once on 9 April 2019, approximately 1 month before sowing.

Experimental Design and Treatments

A split-plot design was used for randomizing the irrigation and biochar treatment combinations. Three irrigation treatments, 100, 70, and 40% of crop evapotranspiration (ET_c) replacement were main plots and the biochar treatments, hardwood, softwood, and control (no biochar) were randomized as subplots. Each treatment combination was replicated four times accounting for 36 experimental units. The field is comprised of three irrigation zones corresponding to each irrigation treatment with independent irrigation control. Each irrigation zone consisted of 12 plots of 7.6 m length and 8 m width. The plots within the irrigation zone were separated by 0.9 m wide alleys.

The irrigation application was based on the ET_c requirement calculated as a product of reference evapotranspiration (ET_o) and stage-specific crop coefficients (K_c). The ET_o was computed from the weather data using the Penman–Monteith method (Zotarelli et al., 2010). The weather data were recorded by a weather station (Davis instruments 6152, Wireless Vantage Pro2, Davis Instruments Corporation, Hayward, California) installed near the experimental site. The K_c values for the sweet corn were used as K_c initial = 0.40 [0–20 days after planting (DAP)], K_c crop development = 0.80 (20–45 DAP), K_c mid = 1.15 (45–70 DAP), K_c late = 1.00 (70–80 DAP) (Brouwer and Heibloem, 1986). The irrigation water needs were calculated as a difference of ET_c and precipitation. Irrigation was applied once a week to restore the ET_c for the previous week. A water meter was installed for each zone to measure the volume of applied water. The electrical conductivity (EC) and pH of irrigation water were 2.2 mmhos/cm and 7.65, respectively.

Soil Sampling and Analyses

At the beginning of the experiment in 2019, the core and bulk soil samples were collected from the experimental field within the 0–30 cm depth before the biochar application and planting. The samples were analyzed to determine the physical properties at 0–30 cm depth (10 cm increment) while the chemical properties were determined for 0–10 cm depth (**Table 2**). The bulk soil samples were air-dried at room temperature and crushed to pass through a 2 mm sieve. The particle size analysis was conducted using the hydrometer method (Gee and Bauder, 1986) and USDA textural classification (Soil Science Division Staff, 2017).

To assess the effect of biochar treatments on soil properties, soil core and bulk samples were collected from each experimental plot at the end of the growing season in 2020. The bulk soil samples were collected with an auger at 0–10 cm depth from three sample points in each plot. For determining the soil pH and EC, bulk samples were air-dried and ground to pass through a 2 mm sieve. Soil (15 g) was suspended in the deionized water in a 1:1 (w/w; soil/deionized water) ratio using 50 ml conical tubes. The samples in the conical tubes were mixed thoroughly using the Eberbach's benchtop fixed-speed reciprocal shaker for 15 min and left overnight at room temperature. The samples were centrifuged at 10,000 rpm for 5 min to collect the supernatant aqueous solution in another set of fresh conical tubes. After calibration with multiple standard solutions for the instrument, the soil EC and pH were measured using the Orion Star pH/Conductivity Portable Meter in the aqueous solution.

The undisturbed core soil samples were collected in 5 cm × 5 cm stainless steel cores at 0–5 and 5–10 cm soil depths. The cores were pushed into the soil using a core sampler with a

TABLE 1 | Characteristics of hardwood and softwood biochars.

Characteristics	Hardwood-oak	Softwood-pine
Total organic matter	82.07 % wt.	95.12 % wt.
Total carbon	62.96 % wt.	88.01 % wt.
Total ash	17.93 % wt.	4.88 % wt.
pH	8.6	7.4
Nitrogen	0.64 % wt.	0.59 % wt.
Total phosphate	3.52 mg/kg	4.53 mg/kg
Potassium	2,960 mg/kg	614 mg/kg
Sulfur	0.011 % wt.	0.031 % wt.
Hydrogen	2.09 % wt.	0.40 % wt.
Oxygen	16.37 % wt.	6.09 % wt.
Calcium	64,900 mg/kg	4,128 mg/kg
Copper	1.72 mg/kg	3.57 mg/kg
Iron	1,770 mg/kg	595 mg/kg
Magnesium	4,540 mg/kg	1,225 mg/kg
Manganese	1,040 mg/kg	234 mg/kg
Zinc	23.2 mg/kg	4.59 mg/kg
Surface area correlation	17.74 m^2/g	375.76 m^2/g

slide hammer (AMS, Inc.). The measurements of the soil water retention curves, that is the functional relationship between the soil matric potential and soil water content, were made on these core samples under 7 different pressures (0 (saturation), -10, -33, -250, -500, -1,000, and -1,500 kPa) following the pressure plate apparatus procedure described by Saini et al. (2020). The soil bulk density was determined using the core method (Blake and Hartge, 1986) and calculated as the ratio of the dry soil mass (oven-dried at 105°C for 24 h) to the bulk soil volume (volume of the core). The soil porosity was calculated by dividing the volume of water held at saturation by the bulk soil volume (volume of the core). The plant available water (PAW) was calculated as the difference between the soil water content at field capacity (at -33 kPa) and the permanent wilting point (at -1,500 kPa), which were obtained from the soil water retention curve.

Gas Exchange and Chlorophyll

The physiological responses of sweet corn to irrigation and biochar treatments were assessed by measuring the g_s, E, and P_n using a portable photosynthesis system (Model LI-6800, LI-COR Biosciences, Lincoln, NE, United States). All physiological measurements were recorded using two young fully expanded leaves from two randomly chosen plants within each experimental plot. The portable photosynthesis system was used at a steady state by keeping 1,500 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR), 400 μmol mol^{-1} reference CO_2 concentration, 700 μmol^{-1} air flow rate, 65% of relative humidity, and switching off the temperature control. These gas exchange measurements were done at 37, 60, and 80 DAP in 2019 and 38, 56, and 81 DAP in 2020. The iWUE was calculated as the ratio of P_n and g_s. The chlorophyll was determined using the SPAD 502 Plus chlorophyll meter (Spectrum Technologies, Inc.). The chlorophyll measurements were recorded at 30, 52, and 80 DAP in 2019 and 24, 55, and 81 DAP in 2020. The physiological observations were recorded between 10:00 and 14:00 h because the plants were well-lighted and fully active during this time.

TABLE 2 | Soil's physical and chemical properties in 2019, Lubbock, TX.

Property	Unit	Soil depth		
		0–10 cm	10–20 cm	20–30 cm
% Sand	%	61.8	60.8	59.3
% Silt	%	27.1	29.1	30.9
% Clay	%	11.1	10.1	9.8
Bulk density	g cm^{-3}	1.461	1.749	1.728
Saturated water content (0 kPa)	% v/v	0.499	0.392	0.394
Field capacity (-33 kPa)	% v/v	0.223	0.225	0.226
Permanent wilting point (-1,500 kPa)	% v/v	0.157	0.191	0.192
Organic matter %	%	0.8	-	-
pH		7.5	-	-
NO3 N	ppm	3	-	-
Phosphorus	ppm	38	-	-
Potassium	ppm	488	-	-

Growth and Yield

The leaf area index (LAI) was measured using a ceptometer (Model: AccuPAR LP-80, Decagon Devices Inc.) from two sites per plot during solar noon. The ceptometer was placed close to the plant base parallel to the rows. The instrument was calibrated based on the coordinates of the experimental location and date. The device measured the PAR and intercepted the PAR non-destructively and used an in-built equation to calculate the LAI.

A total of five plants were selected randomly in each plot to measure the plant height from the soil surface to the tip of the tassels at 80 DAP in 2019 and 69 DAP in 2020. An area of 7.6 m^2 was hand-harvested from each plot on 31 July (87 DAP) in 2019 and 10 August (87 DAP) in 2020 to determine the ear yield and total fresh plant biomass. The representative ear and plant samples from each plot were weighed and oven-dried at 70°C to a constant weight to determine the moisture content. The resulting moisture content was used to calculate the ear and total plant dry weights.

The harvest index (HI) was calculated using equation (1).

$$\text{HI} = \frac{\text{Fresh ear yield}}{\text{Total aboveground fresh weight}} \tag{1}$$

The WP was calculated using equation (2).

$$\text{WP} = \frac{\text{Fresh ear yield}}{\text{Irrigation} + \text{Rainfall}} \tag{2}$$

Statistical Analysis

We conducted the analysis of variance (ANOVA) with a split-plot design in the R version 3.5.2 using the Agricolae package version 1.2-8 to analyze the collected data. Data were analyzed separately for each year. The least significant difference (LSD) test at a 5% significance level was used to compare the treatment means. The SigmaPlot software version 14 (Systat Software, San Jose, CA) was used to make figures.

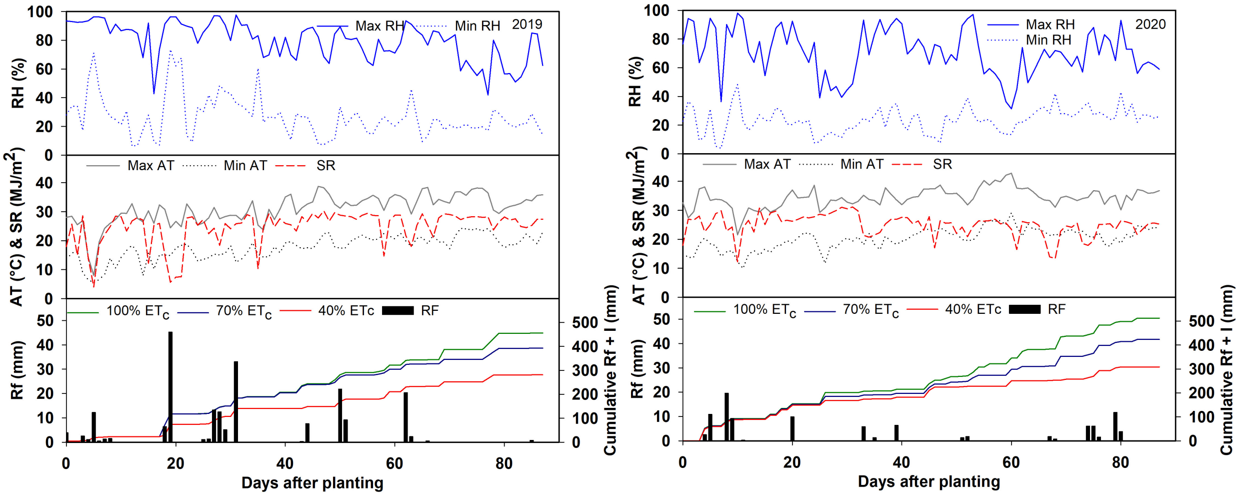

FIGURE 1 | Daily maximum (max) and minimum (min) relative humidity (RH), daily maximum and minimum air temperature (AT), daily average solar radiation (SR), rainfall (Rf), and cumulative rainfall plus irrigation (RF + I) during the 2019 and 2020 growing seasons.

TABLE 3 | Irrigation amount, rainfall, reference evapotranspiration (ET_o), and water-saving for irrigation treatments in the 2019 and 2020 growing season.

Irrigation treatments	Irrigation amount (mm)		Rainfall (mm)		ET_o		% water-saving	
	2019	2020	2019	2020	2019	2020	2019	2020
100% ET_c	252	412	203	101	572	614	–	–
70% ET_c	189	323	203	101	572	614	24	22
40% ET_c	78	208	203	101	572	614	69	50

TABLE 4 | Effect of biochar treatments on soil pH, electrical conductivity (EC), bulk density, and porosity.

	EC (μS cm^{-1})	pH	Bulk density (g cm^{-3})		Porosity	
Soil depth →	0–10 cm	0–10 cm	0–5 cm	5–10 cm	0–5 cm	5–10 cm
Control (B_0)	1,139 a	8.14 a	1.51 a	1.52 a	0.466 a	0.464 b
Hardwood (B_H)	1,147 a	8.16 a	1.45 b	1.49 a	0.480 a	0.482 a
Softwood (B_S)	1,018 a	8.11 a	1.51 a	1.51 a	0.470 a	0.471 ab

Different letters within a column indicate significant differences ($P \leq 0.05$) among treatments.

RESULTS

Atmospheric Conditions and Irrigation

The prevailing weather conditions during the sweet corn growing season in 2019 and 2020 are described in **Figure 1**. The average relative humidity was 53.2 % in 2019 and 47.3% in 2020. The average temperature during the growing season was 24.3 and 27.3°C in 2019 and 2020, respectively. Overall, the 2020 growing season was drier and hotter compared to 2019. In 2020, trial plants were exposed to hot and dry spells at 50–65 DAP (**Figure 1**). The daily average solar radiation was recorded as 24.7 MJm^{-2} in 2019 and 24.9 MJ/m^{-2} in 2020. As an output of the above-described weather conditions, the reference evapotranspiration (ET_o) in 2020 was higher compared to 2019 (**Table 3**). The total rainfall during the growing season was 203 mm in 2019 and 101 mm in 2020. Therefore, the irrigation requirements in 2020 were substantially higher than in 2019 (**Table 3**). All the plots received an equal amount of initial irrigation to ensure good crop establishment. In 2019, the irrigation treatments began 43 days after planting due to continuous rainfall in the early season (**Figure 1**). In 2020, the irrigation treatments were started at 26 DAP.

Biochar Effects on the Soil Properties

The biochar application did not affect the soil pH and EC (**Table 4**). Hardwood biochar application reduced the soil bulk density by 4% at 0–5 cm depth and improved soil porosity over non-amended plots by 3.9% at 5–10 cm soil depth (**Table 4**). However, softwood biochar did not affect the bulk density and porosity. The measured volumetric water content was higher in the hardwood biochar treatment than softwood and non-amended plot at all pressures. The magnitude of the differences was greater at 5–10 cm soil depth (**Figure 2**). As shown in **Figure 3**, the hardwood biochar soil samples held more water at saturation, field capacity, and permanent wilting point, but the differences were significant only for the saturated water content. The plant available water was higher in the biochar treatments compared to the non-amended plots but the differences were non-significant.

Deficit Irrigation and Biochar Effects on the Physiological Parameters

The effect of DI and biochar on Chl_{SPAD}, g_s, E, P_n, and iWUE of sweet corn is presented in **Figures 4–8**. The interactions among irrigation and biochar treatments were non-significant for all the measured physiological parameters. The differences in Chl_{SPAD} among the irrigation treatments became significant toward the end of the growing season in both years (**Figure 4**). In both years, the Chl_{SPAD} recorded under 100% ET_c at 80 DAP was at par with 70% ET_c but significantly greater than 40% ET_c. Both biochar treatments increased the Chl_{SPAD} over non-amended during both years (**Figure 4**). The differences in Chl_{SPAD} due to the biochar treatments were significant at 52 and 80 DAP in 2019 and 81 DAP in 2020.

The 40% ET_c treatment reduced the g_s significantly at 80 DAP in 2019, and 56 and 81 DAP in 2020 compared to other irrigation treatments (**Figure 5**). Consequently, the plants in the 40% ET_c also recorded a significant reduction in E compared to 100% ET_c during both years (**Figure 6**). The P_n responses to the irrigation treatments followed a similar pattern as E during both years but the magnitude of the decline in P_n due to DI was much lower compared to E (**Figure 7**). For instance, compared to 100% ET_c, the E under 40% ET_c was reduced by 14 and 36% at 60 and 80 DAP in 2019, respectively. However, the decrease of 9 and 14% in P_n under the 40% ET_c on the same days was much lower than the decrease in E. Consequently, the iWUE under 40% ET_c increased by 23 and 15% compared to 100% ET_c in 2019 and 2020, respectively, toward the end of the growing season (**Figure 8**). The 70% ET_c maintained a statistically similar g_s, E, P_n, and iWUE as 100% ET_c during both years. The biochar treatments did not have any significant effect on the gas exchange parameters during both years except at 60 DAP in 2019 when the plants in the hardwood biochar treatment recorded significantly higher g_s (**Figure 5**) and E compared to the control plots (**Figure 6**).

Deficit Irrigation and Biochar Effects on Plant Growth and Yield

The interactions among the irrigation and biochar treatments for the measured plant growth and yield parameters were non-significant during both years. In 2019, 40% ET_c decreased the plant height significantly whereas the height of 70% ET_c plants was comparable to 100% ET_c (**Table 5**). In 2020, both DI treatments (70 and 40% ET_c) decreased the plant height significantly by 5 and 16 cm compared to full irrigation (100% ET_c). The biochar treatments did not have a significant effect on the plant height in 2019 but the hardwood biochar increased the plant height significantly over the non-amended plots in 2020. The irrigation and biochar treatment had a non-significant effect on LAI in both years.

The 70% ET_c produced the highest aboveground vegetative dry biomass and total aboveground dry biomass followed by 100 and 40% ET_c in 2019 (**Table 5**). However, in 2020, these values were in the order of 100, 70, and 40% ET_c. In 2020, both DI treatments reduced the aboveground vegetative dry biomass significantly but the 70% ET_c produced comparable total aboveground dry biomass as 100% ET_c. The biochar treatments had a non-significant effect on these parameters in 2019, but the hardwood biochar application significantly increased the aboveground vegetative dry biomass compared to the control in 2020. The 40% ET_c decreased the ear yield by 17%, whereas 70% ET_c increased the yield by 9% compared to 100% ET_c in 2019 (**Table 5**). In 2020, the ear yield values in 70 and 40% ET_c were 3 and 44% lower than 100% ET_c, respectively. The biochar treatments did not affect the ear yield during both years. On average, the ear yield in 2020 dropped by 47% compared to 2019. However, the total aboveground dry biomass was higher, and the aboveground vegetative dry biomass was more than double in 2020 compared to 2019. The HI differences due to the irrigation treatments were non-significant in 2019. However, 100 and 70% ET_c treatments recorded significantly higher HI compared to 40% ET_c in 2020 (**Table 5**). The biochar treatments did not affect HI during both years.

In 2019, 70 and 40% ET_c treatments improved the WP significantly over 100% ET_c by 26 and 35%, respectively. However, in 2020, the 70% ET_c treatment improved the WP by 18% whereas 40% ET_c reduced the WP by 7% compared to 100% ET_c. The biochar treatments did not affect the WP during both years. Overall, the WP was approximately double in 2019 than in 2020.

DISCUSSION

Effect of Biochar on Soil pH and Water Retention Characteristics

Biochar acts as a liming agent and often increases the soil pH due to its alkaline nature. However, we did not observe the effect of biochar on soil pH, which was expected because the pH of both biochars used in this study was comparable to the soil pH. The biochar application generally decreases the bulk density of soils and increases the porosity with a greater effect in coarse-textured soils (Blanco-Canqui, 2017). Our results indicate a 4% decrease in the bulk density at 0–5 cm soil depth with 13 Mg ha^{-1} hardwood biochar application, but no biochar effect at 5–10 cm depth in the sandy clay loam soil profile. Zheng et al. (2016) reported a 4 and 7 % decrease in the bulk density of sandy loam soil with 20 and 40 Mg ha^{-1} application of wheat straw biochar, respectively. However, Rogovska et al. (2016) observed no change in the bulk density after the hardwood biochar application at 9.9 and 18.4 Mg ha^{-1} in loam, clay loam, and silty clay loam soil. An increase in the soil porosity at 5–10 cm soil depth with hardwood biochar application resulted in enhanced soil water retention characteristics in the hardwood plots compared to control (**Figure 2**). However, the statistical analyses indicated that

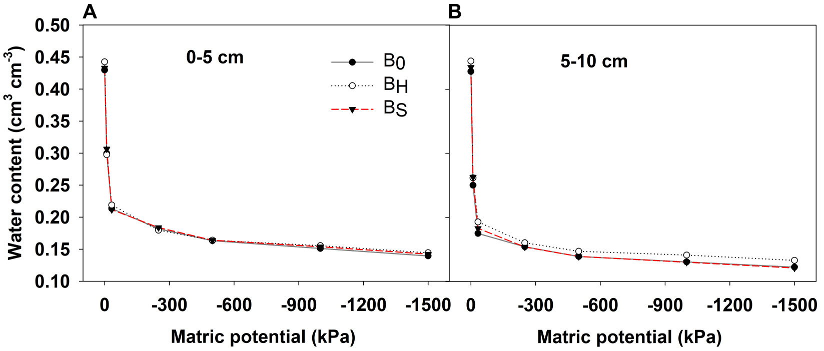

FIGURE 2 | Measured soil water retention curves at **(A)** 0–5 cm and **(B)** 5–10 cm soil depths for biochar treatments after harvest in 2020.

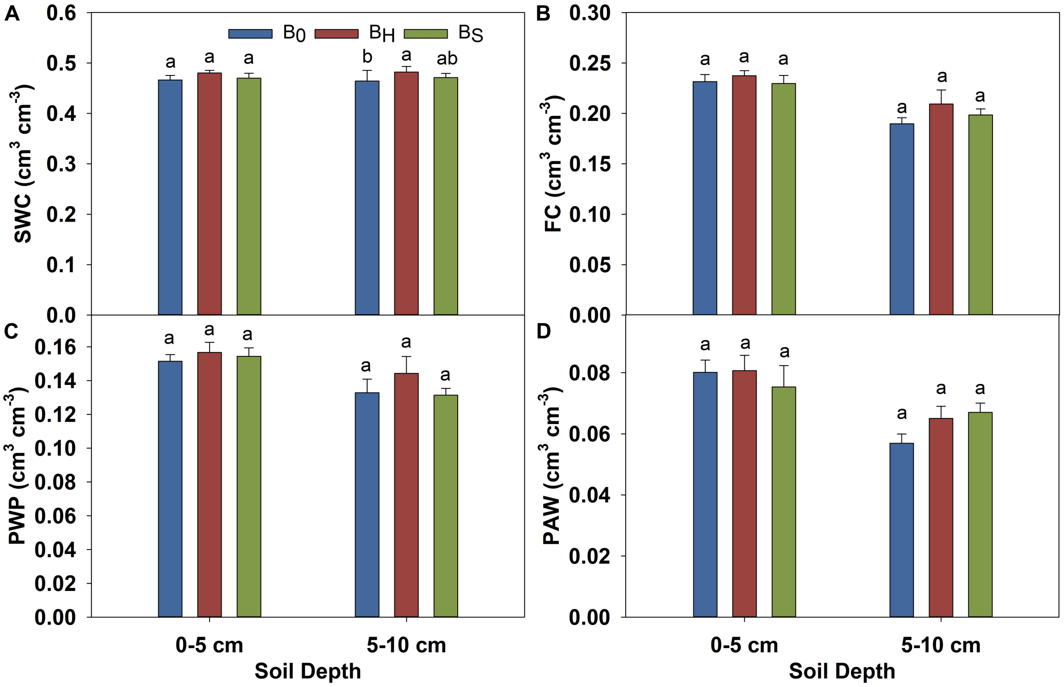

FIGURE 3 | Effect of biochar on the volumetric water content of soil at **(A)** saturated water content (0 kPa), **(B)** field capacity (-33 kPa), **(C)** the permanent wilting point (-1,500 kPa), and **(D)** plant available water after harvest in 2020. The error bars represent ± standard error. Different letters indicate significant differences ($p \leq 0.05$) among treatments.

the hardwood biochar effect was significant only for saturated water content. Notably, saturated water content provided a measure of the total porosity of the soil. The differences in the soil water content at field capacity, permanent wilting point, and plant available water among the biochar treatments were non-significant. The effect of the biochar application at different rates on soil water retention has yielded contrasting results in different studies. For instance, similar to our results, Moragues-Saitua et al. (2017) also observed no increase in water retention with *Miscanthus* sp. biochar application (10 and 20 Mg ha^{-1}) on sandy loam soil. Contrarily, Ma et al. (2016) observed a significant increase in the field capacity and plant available water with 7.8 Mg ha^{-1} application of maize straw and peanut hull biochar.

The investigation of the irrigation levels and application of two types of biochar in sweet corn revealed that the interactive effects were non-significant for any of the examined parameters. The irrigation levels affected all the parameters significantly whereas the biochar effects were significant only on Chl$_{SPAD}$, plant height, and vegetative dry biomass. The results of this study and their implications for sweet corn production in semi-arid climate are discussed with a focus on the main effects.

Sweet Corn Physiology, Biomass, and Yield as Affected by Deficit Irrigation

Water stress generally has an adverse effect on the gas exchange, plant growth, and biomass but its effect on chlorophyll content

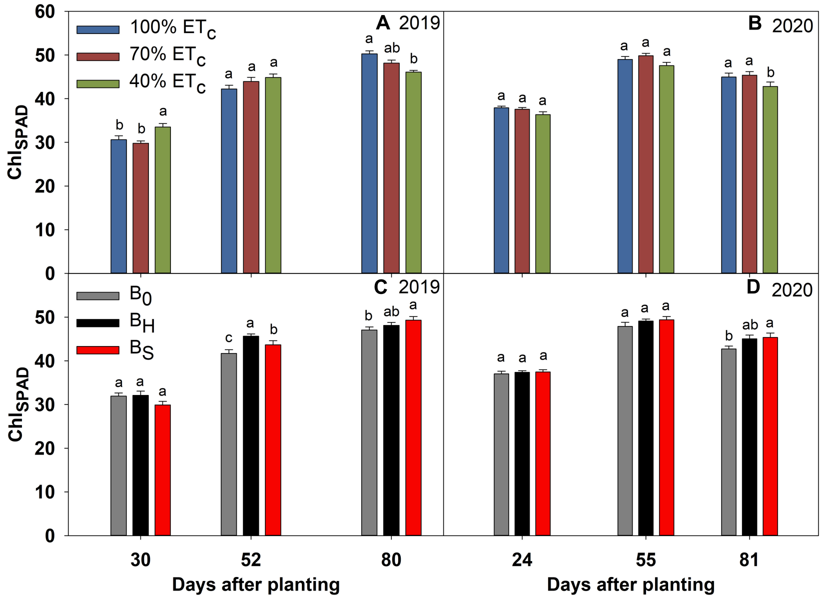

FIGURE 4 | Chlorophyll (Chl_{SPAD}) of sweet corn under deficit irrigation **(A,B)** and biochar application **(C,D)** during the 2019 and 2020 growing seasons. The error bars represent ± standard error. Different letters indicate significant differences ($p \leq 0.05$) among treatments on a measurement day in **Figures 4–8**.

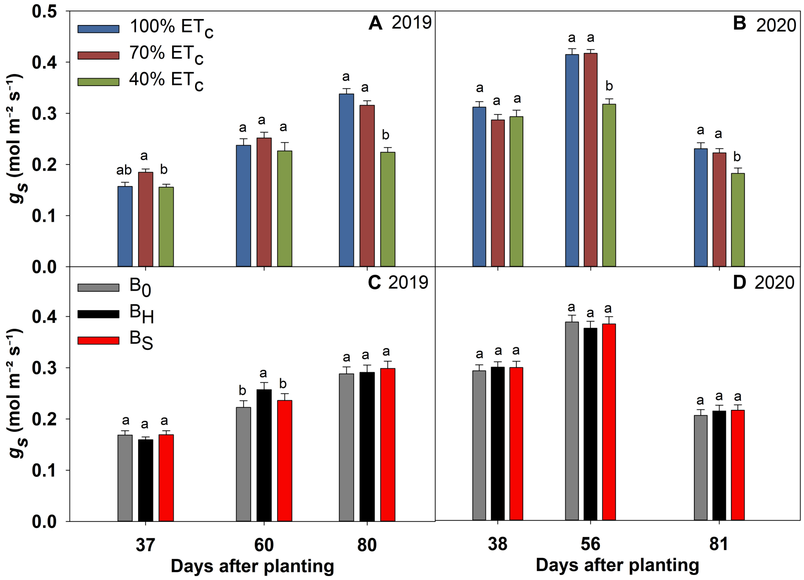

FIGURE 5 | Stomatal conductance (g_s) of sweet corn under deficit irrigation **(A,B)** and biochar application **(C,D)** during the 2019 and 2020 growing seasons.

is conflicting. Some researchers report an increase (Ramírez et al., 2014; Gavili et al., 2019) while others report a decrease (Mansouri-Far et al., 2010; Langeroodi et al., 2019), and still, others observe no change (Pandey et al., 2000) in the chlorophyll levels under water stress. The increased chlorophyll concentration due to moisture stress is mainly attributed to the increased concentration of nitrogen (N) and magnesium (Mg) with a corresponding reduction in the plant biomass due to the essential roles these elements play in chlorophyll synthesis (Gavili et al., 2019). However, our results indicate a significant drop in Chl_{SPAD} at higher water stress (40% ET_c) only toward the end of the growing season. This agrees with the results of Mansouri-Far et al. (2010) who observed a significant decline in the leaf chlorophyll content by withholding irrigation at the reproductive stage (R3) of maize. Brevedan and Egli (2003) also observed a decline in the chlorophyll levels in soybean exposed to water stress at the seed filling stage. The imposition of water deficit causes a reduction in the uptake of N and Mg leading

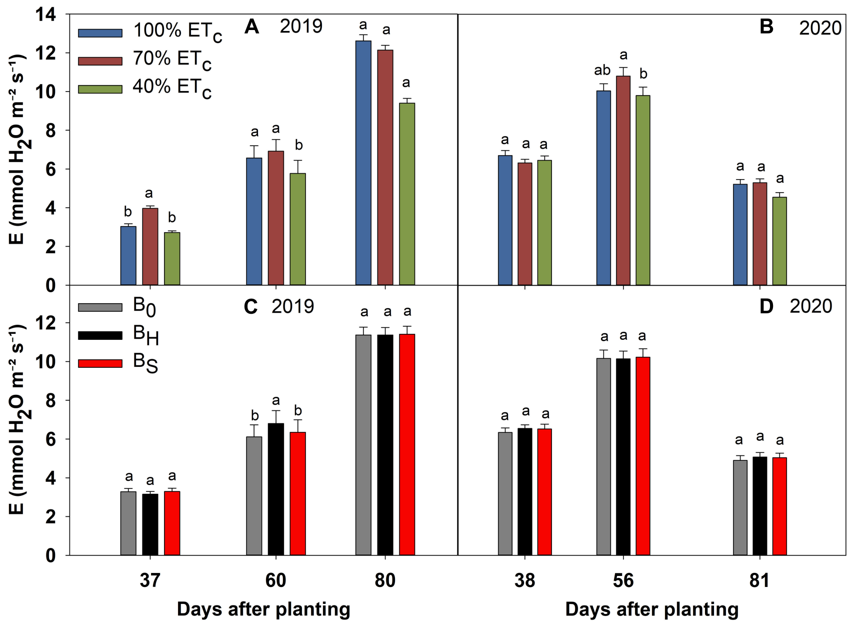

FIGURE 6 | Transpiration (E) of sweet corn under deficit irrigation **(A,B)** and biochar application **(C,D)** during the 2019 and 2020 growing seasons.

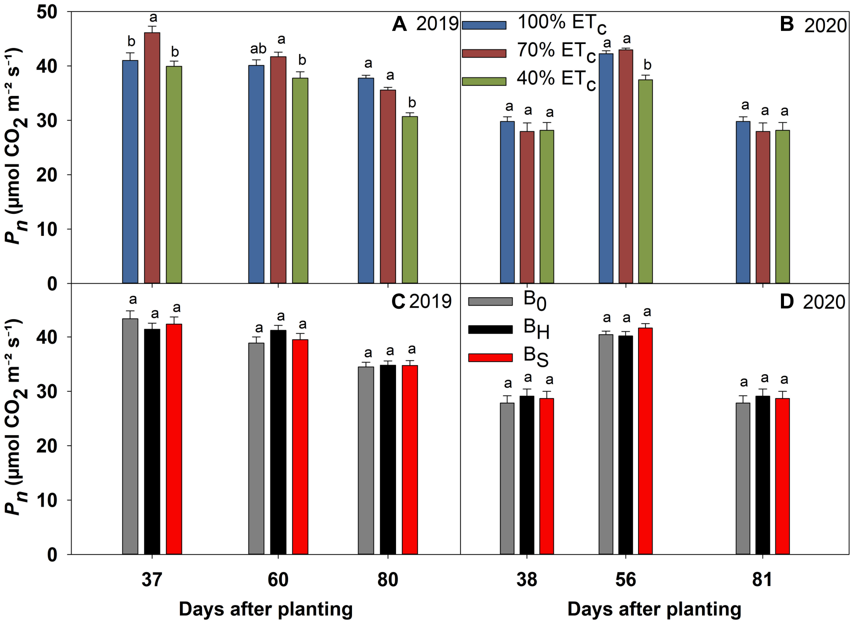

FIGURE 7 | Photosynthesis (P_n) of sweet corn under deficit irrigation **(A,B)** and biochar application **(C,D)** during the 2019 and 2020 growing seasons.

to a reduction in chlorophyll synthesis and its concentration in the leaves (Peuke and Rennenberg, 2011; Bista et al., 2018). Nevertheless, Chl_{SPAD} under mild water stress of 70% ET_c remained comparable to full irrigation (100% ET_c). Langeroodi et al. (2019) also observed a minimal decline in the chlorophyll content index at mild water stress of 60% maximum allowable depletion (MAD) of available water compared to 45% MAD but reported a significant drop with further increase in water stress.

In response to water stress, plants close stomata to reduce transpiration losses. The reduction in g_s means reduced gas exchange and a consequent decrease in CO_2 assimilation (Chaves et al., 2002). In this study, a significant decline in P_n under 40% ET_c indicates that E losses were prevented by stomatal closure at the expense of CO_2 intake. The reduction in P_n decreased the biomass accumulation and yield in 40% ET_c compared to 100% ET_c. Higher g_s values do not necessarily mean greater P_n and

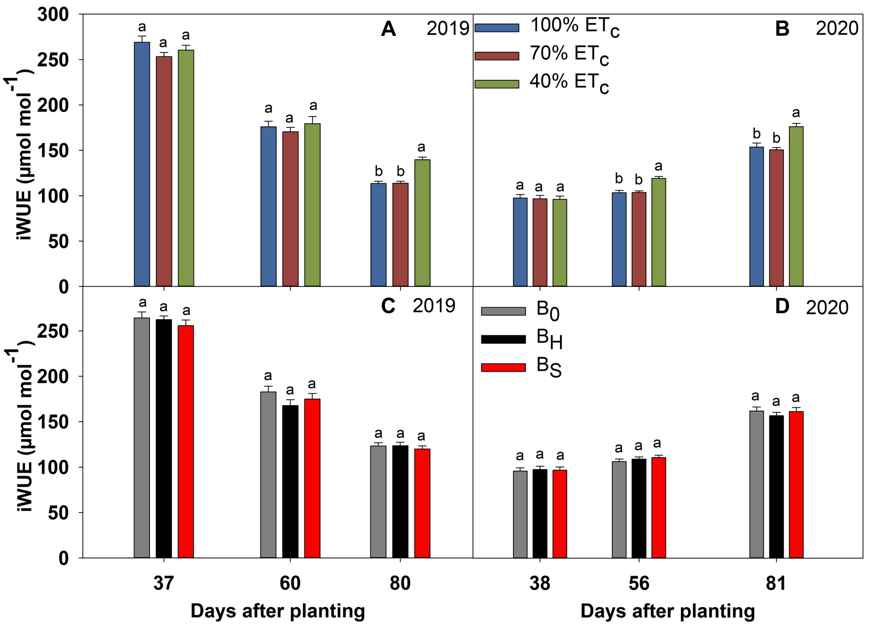

FIGURE 8 | Intrinsic water use efficiency (iWUE) of sweet corn under deficit irrigation **(A,B)** and biochar application **(C,D)** during the 2019 and 2020 growing seasons.

can be less water-efficient (Álvarez and Sánchez-Blanco, 2013). The decreased g_s in response to water stress limiting E losses may still be high enough to maintain satisfactory internal CO_2 levels (Langeroodi et al., 2019), and thus more water-efficient. Our results indicate that the magnitude of P_n reduction under 40% ET_c was lower than the reduction in g_s and E, thus achieving higher iWUE with 40% ET_c compared to 100% ET_c. The 70% ET_c maintained similar g_s, P_n, and E as 100% ET_c during the whole growing seasons of both years indicating that sweet corn plants can adapt to mild water stress without adverse effects on physiology. As a result, the total aboveground dry biomass and ear yield were not significantly different between 100 and 70% ET_c. Furthermore, both DIs (40 and 70% ET_c) maintained comparable LAI as 100% ET_c but the reduction in the plant height was significant for 70% ET_c in 2020 and 40% ET_c in both years. It may be attributed to a reduction in the internodal length or increased biomass allocation toward the leaves under water deficit to maintain high-net photosynthesis. Kirda et al. (2005) reported that the effect of 50% DI on the maize plant height and LAI was non-significant. Ertek and Kara (2013) reported a 3.8, 14, and 23% decline in fresh ear yield of sweet corn reducing the water use by 30, 45, and 60%, respectively, based on ET_c. Hirich et al. (2012) suggested that a 25% water deficit during the vegetative stage can maintain the sweet corn productivity whereas Oktem (2008) recorded a significant reduction in the yield at a 20% water deficit during the growing season. Our results suggest that 70% ET_c can be used for sweet corn production in West Texas with minimal yield reduction whereas 40% ET_c reduces the yield significantly. The WP improved significantly under 70% ET_c due to the 62 and 89 mm irrigation water-saving without any significant yield reductions over 100% in 2019 and 2020, respectively. The WP for 40% ET_c improved over full irrigation in 2019 due to a 175-mm reduction in water use and improvement in iWUE. Although the iWUE for 40% ET_c was significantly higher than the 100% ET_c in 2020, it did not result in a high WP due to the following reasons.

The weather conditions during the growing season in 2020 characterized by 3°C higher average air temperature, 3% lower average RH, and 103 mm lower rainfall were more stressful compared to 2019. In 2020, the crop experienced a hot and dry spell at 50–65 DAP, which coincided with the anthesis and pollination events. This had an adverse effect on the kernel formation confirmed by the presence of numerous empty kernels in many ears. The high temperatures during flowering inhibit pollen viability and germination, which may cause kernel abortion (Bakhtavar et al., 2015; Li and Howell, 2021). Previous studies have reported heat stress sensitivity of kernel formation in maize and a reduction in the kernel number due to high temperatures around silking ultimately reducing the yield (Niu et al., 2021; Wang et al., 2021). Consequently, a major reduction in yield occurred in 2020 compared to 2019. The adverse effect on yield was more pronounced in 40% ET_c, wherein heat stress was accompanied by higher water stress. The reduction in yield caused higher biomass partitioning toward the vegetative parts resulting in higher vegetative dry biomass and lower HI in 2020 than 2019. Farré and Faci (2009) observed a reduction in HI of maize only when the flowering stage was exposed to water stress. Compared to the other irrigation treatments, the HI dropped significantly under more stressed 40% ET_c in 2020 due to a greater reduction in the yield caused by poor kernel formation. This resulted in a significant decline in WP of 40% ET_c in 2020. Overall, sweet corn accumulated 1,260 heat units in 2019 and 1,524 in 2020. The higher heat units resulted in higher total aboveground dry biomass accumulation in 2020.

TABLE 5 | Effect of deficit irrigation and biochar application on growth and yield parameters, and water productivity (WP) of sweet corn.

Treatment	Plant height	Leaf area index	Aboveground vegetative dry biomass	Total aboveground dry biomass	Yield	Harvest index	WP
	cm		kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	(%)	kg ha^{-1} mm^{-1}
2019							
Irrigation (I)							
100% ET_c	138.0 a	4.61 a	1,165 ab	3,450 b	12,648 a	65.1 a	27.8 b
70% ET_c	139.0 a	4.61 a	1,350 a	4,239 a	13,726 a	64.1 a	35.0 a
40% ET_c	130.8 b	4.29 a	1,027 b	3,122 b	10,527 b	66.5 a	37.6 a
Biochar (B)							
B_0	134.3 a	4.42 a	1,114 a	3,409 a	12,075 a	65.5 a	33.0 a
B_H	136.6 a	4.63 a	1,234 a	3,700 a	12,427 a	65.3 a	33.8 a
B_S	136.8 a	4.45 a	1,194 a	3,702 a	12,398 a	64.8 a	33.7 a
Interaction (I × B)	ns	ns	ns	ns	ns	ns	ns
2020							
Irrigation (I)							
100% ET_c	143.4 a	4.62 a	2,804 a	5,230 a	7,707 a	39.7 a	15.0 b
70% ET_c	138.3 b	4.79 a	2,460 b	4,830 a	7,495 a	40.7 a	17.7 a
40% ET_c	127.1 c	4.30 a	2,371 b	3,663 b	4,320 b	31.4 b	14.0 b
Biochar (B)							
B_0	134.3 b	4.49 a	2,428 b	4,434 a	6,461 a	37.9 a	15.4 a
B_H	137.8 a	4.78 a	2,645 a	4,716 a	6,626 a	37.0 a	15.9 a
B_S	136.8 ab	4.44 a	2,561 ab	4,573 a	6,435 a	37.0 a	15.4 a
Interaction (I × B)	ns	ns	ns	ns	ns	ns	ns

Different letters in a column within a factor indicate significant differences ($P \leq 0.05$) among treatments. 'ns' represents non-significant difference/interaction.

Sweet Corn Physiology, Biomass, and Yield as Affected by Biochar Application

The biochar application was expected to alleviate the adverse effect of water stress on the sweet corn physiology, growth, and yield, and improve WP by improving the soil properties. However, our hypothesis was only partially true. The hardwood biochar application decreased soil bulk density and increased porosity, but it did not increase PAW. The hardwood biochar increased the Chl_{SPAD}, improved g_s and E at mid-stage in 2019, but did not affect the P_n significantly. The plant height and vegetative biomass were improved by the biochar application but it did not have much impact on the yield and WP. These results are in consensus with Vaccari et al. (2015) who reported an improvement in the tomato plant growth with biochar application without any fruit yield gains. Ramlow et al. (2019) reported an increase in soil moisture with woody biochar without alleviating the effect of water stress. Nevertheless, several reports illustrating the potential of biochar to mitigate water stress in crops are available in the literature (Faloye et al., 2019; Singh et al., 2019; Alfadil et al., 2021).

The increased Chl_{SPAD} due to biochar application may be attributed to the increased N and Mg concentrations in the leaves. The higher concentrations of N and Mg in hardwood biochar compared to softwood biochar may have resulted in greater Chl_{SPAD} values for hardwood biochar at most of the measurement days. Although we did not measure the nutrient concentration in the leaves, chlorophyll concentration is closely correlated with the N concentration and used as a tool to determine the N status of the plants (Oppong Danso et al., 2020).

The increase in the vegetative dry biomass may correspond to the enhanced nutrient uptake by plants due to biochar application, though the differences were significant only in 2020, wherein hardwood biochar plots produced significantly higher vegetative dry biomass compared to control. Langeroodi et al. (2021) reported an increased concentration of N, P, K, and Mg in the sunflower (*Helianthus annuus* L.) shoots by paper sludge biochar application. The concentrations of most macro- and micronutrients were higher in hardwood biochar compared to softwood (**Table 1**). Based on the 2-year data, the hypothesis that biochar may improve the water status of the sweet corn plants under water stress was hardly true as it did not alter the gas exchange parameters significantly. Although hardwood biochar application increased the g_s and E at mid-stage during 2019, it did not cause a significant improvement in the P_n and ear yield.

CONCLUSION

This research investigated the implications of using biochar under water-limited conditions in a semi-arid environment for enhancing water and food sustainability. Soil amendment with hardwood biochar marginally affected the physical properties of soil by decreasing bulk density and improving saturated water content, but it did not improve plant available water significantly. The moderate DI (70% ET_c) was found to be the most water-efficient among irrigation treatments. This moderate reduction in water use maintained the plant physiology, growth, and yield similar to 100% ET_c for 2 consecutive years. The hardwood biochar application at the rate of 13 Mg ha^{-1} increased the chlorophyll content, plant height, and vegetative dry biomass, marginally affecting the gas exchange but did not alter the yield and WP. The results of this 2-year study suggest that biochar application provided only limited benefits for sweet corn production under DI. However, for better understanding, its potential benefits under limited irrigation in the long run, its effect on chemical and biological properties along with crop production need to be investigated in long-term studies. The 70% ET_c can be recommended as an alternative to 100% ET_c for water-efficient sweet corn production in West Texas.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SS and MS contributed to the conception and design of the study. MS performed the experiment, analyzed the data, prepared figures, and wrote the manuscript. VP helped in data collection. SS supervised the study. SS, GR, SKD, and RWW provided guidance on data collection and revised the manuscript. All authors approved the submitted manuscript.

ACKNOWLEDGMENTS

We would like to thank the Department of Plant Soil Science, Texas Tech University, for providing necessary support for this study. We are thankful to Lelton Howel and his crew, Reagan Anders, Bishwoyog Bhattarai, Azeezahmed Shaik, Atinderpal Singh, Arjun Kafle, Puneet Kaur Mangat, and Kamalpreet Kaur for their help with field and laboratory work.

REFERENCES

Agbna, G. H. D., Dongli, S., Zhipeng, L., Elshaikh, N. A., Guangcheng, S., and Timm, L. C. (2017). Effects of deficit irrigation and biochar addition on the growth, yield, and quality of tomato. *Sci. Hortic.* 222, 90–101. doi: 10.1016/j.scienta.2017.05.004

Alfadil, A. A., Shaghaleh, H., Alhaj Hamoud, Y., Xia, J., Wu, T., Hamad, A. A. A., et al. (2021). Straw biochar-induced modification of the soil physical properties enhances growth, yield and water productivity of maize under deficit irrigation. *Commun. Soil Sci. Plant Anal.* 52, 1954–1970. doi: 10.1080/00103624.2021.1901913

Ali, A. B., Haofang, Y., Hong, L., Yan You, W., Elshaikh, N. A., Hussein, G., et al. (2018). Enhancement of depleted loam soil as well as cucumber productivity utilizing biochar under water stress. *Commun. Soil Sci. Plant Anal.* 50, 49–64. doi: 10.1080/00103624.2018.1547389

Álvarez, S., and Sánchez-Blanco, M. J. (2013). Changes in growth rate, root morphology and water use efficiency of potted Callistemon citrinus plants in response to different levels of water deficit. *Sci. Hortic.* 156, 54–62. doi: 10.1016/j.scienta.2013.03.024

Atkinson, C. J., and Aitkenhead, M. (2018). How good is the evidence that soil-applied biochar improves water-holding capacity? *Soil Use Manag.* 34, 177–186. doi: 10.1111/sum.12413

Bakhtavar, M. A., Afzal, I., Basra, S. M. A., Ahmad, A.-U.-H., and Noor, M. A. (2015). Physiological strategies to improve the performance of spring maize (*Zea mays* L.) planted under early and optimum sowing conditions. *PLoS One* 10:e0124441. doi: 10.1371/journal.pone.0124441

Bista, D. R., Heckathorn, S. A., Jayawardena, D. M., Mishra, S., and Boldt, J. K. (2018). Effects of drought on nutrient uptake and the levels of nutrient-uptake proteins in roots of drought-sensitive and -tolerant grasses. *Plants* 7:28. doi: 10.3390/plants7020028

Blake, G. R., and Hartge, K. H. (1986). "Bulk density," in *Methods of Soil Analysis*, ed. A. Klute (Madison: Soil Science Society of America), 363–375.

Blanco-Canqui, H. (2017). Biochar and soil physical properties. *Soil Sci. Soc. Am. J.* 81, 687–711. doi: 10.2136/sssaj2017.01.0017

Brevedan, R. E., and Egli, D. B. (2003). Short periods of water stress during seed filling, leaf senescence, and yield of soybean. *Crop Sci.* 43, 2083–2088. doi: 10.2135/cropsci2003.2083

Brouwer, C., and Heibloem, M. (1986). *Irrigation Water Management: Irrigation Water Needs*. Washington, DC: USDA.

Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osório, M. L., et al. (2002). How plants cope with water stress in the field? Photosynthesis and growth. *Ann. Bot.* 89, 907–916. doi: 10.1093/aob/mcf105

Daher, B., Lee, S.-H., Kaushik, V., Blake, J., Askariyeh, M. H., Shafiezadeh, H., et al. (2019). Towards bridging the water gap in Texas: a water-energy-food nexus approach. *Sci. Total Environ.* 647, 449–463. doi: 10.1016/j.scitotenv.2018.07.398

Ding, Y., Liu, Y., Liu, S., Li, Z., Tan, X., Huang, X., et al. (2016). Biochar to improve soil fertility. A review. *Agron. Sustain. Dev.* 36:36.

Ertek, A., and Kara, B. (2013). Yield and quality of sweet corn under deficit irrigation. *Agric. Water Manag.* 129, 138–144. doi: 10.1016/j.agwat.2013.07.012

Faloye, O. T., Alatise, M. O., Ajayi, A. E., and Ewulo, B. S. (2019). Effects of biochar and inorganic fertiliser applications on growth, yield and water use efficiency of maize under deficit irrigation. *Agric. Water Manag.* 217, 165–178. doi: 10.1016/j.agwat.2019.02.044

FAO (2002). *Water Reports: Deficit Irrigation Practices*. Rome: FAO.

Farré, I., and Faci, J. M. (2009). Deficit irrigation in maize for reducing agricultural water use in a Mediterranean environment. *Agric. Water Manag.* 96, 383–394. doi: 10.1016/j.agwat.2008.07.002

Farsiani, A., Ghobadi, M.-E., and Jalali-Honarm, S. (2011). The effect of water deficit and sowing date on yield components and seed sugar contents of sweet corn (*Zea mays* L.). *Afr. J. Agric. Res.* 6, 5769–5774.

Gavili, E., Moosavi, A. A., and Kamgar Haghighi, A. A. (2019). Does biochar mitigate the adverse effects of drought on the agronomic traits and yield components of soybean? *Ind. Crops Product.* 128, 445–454. doi: 10.1016/j.indcrop.2018.11.047

Gee, G., and Bauder, J. (1986). "Particle-size analysis," in *Methods of Soil Analysis. Part 1. Agron. Monogr. 9*, ed. A. Klute (Madison, WI: ASA and SSSA), 383–411.

Hirich, A., Rami, A., Laajaj, K., Choukr-Allah, R., Jacobsen, S., El Youssfi, L., et al. (2012). Sweet corn water productivity under several deficit irrigation regimes applied during vegetative growth stage using treated wastewater as water irrigation source. *World Acad. Sci. Eng. Technol.* 61, 840–847.

Kirda, C., Topcu, S., Kaman, H., Ulger, A. C., Yazici, A., Cetin, M., et al. (2005). Grain yield response and N-fertiliser recovery of maize under deficit irrigation. *Field Crops Res.* 93, 132–141. doi: 10.1016/j.fcr.2004.09.015

Laboski, C. A. M., Dowdy, R. H., Allmaras, R. R., and Lamb, J. A. (1998). Soil strength and water content influences on corn root distribution in a sandy soil. *Plant Soil* 203, 239–247.

Langeroodi, A. R. S., Campiglia, E., Mancinelli, R., and Radicetti, E. (2019). Can biochar improve pumpkin productivity and its physiological characteristics under reduced irrigation regimes? *Sci. Hortic.* 247, 195–204. doi: 10.1016/j.scienta.2018.11.059

Langeroodi, A. R. S., Mancinelli, R., and Radicetti, E. (2021). Contribution of biochar and arbuscular mycorrhizal fungi to sustainable cultivation of sunflower under semi-arid environment. *Field Crops Res.* 273:108292. doi: 10.1016/j.fcr.2021.108292

Li, Z., and Howell, S. H. (2021). Heat stress responses and thermotolerance in maize. *Int. J. Mol. Sci.* 22:948. doi: 10.3390/ijms22020948

Liu, C., Liu, F., Ravnskov, S., Rubaek, G. H., Sun, Z., and Andersen, M. N. (2017). Impact of wood biochar and its interactions with mycorrhizal fungi, phosphorus fertilization and irrigation strategies on potato growth. *J. Agron. Crop Sci.* 203, 131–145. doi: 10.1111/jac.12185

Liu, F., Jensen, C. R., Shahanzari, A., Andersen, M. N., and Jacobsen, S.-E. (2005). ABA regulated stomatal control and photosynthetic water use efficiency of potato (*Solanum tuberosum* L.) during progressive soil drying. *Plant Sci.* 168, 831–836. doi: 10.1016/j.plantsci.2004.10.016

Ma, N., Zhang, L., Zhang, Y., Yang, L., Yu, C., Yin, G., et al. (2016). Biochar improves soil aggregate stability and water availability in a mollisol after three years of field application. *PLoS One* 11:e0154091. doi: 10.1371/journal.pone.0154091

Mansouri-Far, C., Modarres Sanavy, S. A. M., and Saberali, S. F. (2010). Maize yield response to deficit irrigation during low-sensitive growth stages and nitrogen rate under semi-arid climatic conditions. *Agric. Water Manag.* 97, 12–22. doi: 10.1016/j.agwat.2009.08.003

McGuire, V. L. (2017). *Water-Level and Recoverable Water in Storage Changes, High Plains Aquifer, Predevelopment to 2015 and 2013–15*. Reston: US Geological Survey.

Moragues-Saitua, L., Arias-González, A., and Gartzia-Bengoetxea, N. (2017). Effects of biochar and wood ash on soil hydraulic properties: a field experiment involving contrasting temperate soils. *Geoderma* 305, 144–152. doi: 10.1016/j.geoderma.2017.05.041

Motazedian, A., Kazemeini, S. A., and Bahrani, M. J. (2019). Sweet corn growth and GrainYield as influenced by irrigation and wheat residue management. *Agric. Water Manag.* 224:105748. doi: 10.1016/j.agwat.2019.105748

Nielsen-Gammon, J. W., Banner, J. L., Cook, B. I., Tremaine, D. M., Wong, C. I., Mace, R. E., et al. (2020). Unprecedented drought challenges for texas water resources in a changing climate: what do researchers and stakeholders need to know? *Earth's Future* 8:e2020EF001552.

Niu, S., Du, X., Wei, D., Liu, S., Tang, Q., Bian, D., et al. (2021). Heat stress after pollination reduces kernel number in maize by insufficient assimilates. *Front. Genet.* 12:728166. doi: 10.3389/fgene.2021.728166

Oktem, A. (2008). Effect of water shortage on yield, and protein and mineral compositions of drip-irrigated sweet corn in sustainable agricultural systems. *Agric. Water Manag.* 95, 1003–1010. doi: 10.1016/j.agwat.2008.03.006

Oktem, A., Simsek, M., and Oktem, A. G. (2003). Deficit irrigation effects on sweet corn (*Zea mays* saccharata Sturt) with drip irrigation system in a semi-arid region: I. Water-yield relationship. *Agric. Water Manag.* 61, 63–74. doi: 10.1016/s0378-3774(02)00161-0

Oppong Danso, E., Yakubu, A., Arthur, E., Sabi, E. B., Abenney-Mickson, S., and Andersen, M. N. (2020). Effect of rice straw biochar and irrigation on growth, dry matter yield and radiation-use efficiency of maize grown on an Acrisol in Ghana. *J. Agron. Crop Sci.* 206, 296–307. doi: 10.1111/jac.12383

Pandey, R. K., Maranville, J. W., and Chetima, M. M. (2000). Deficit irrigation and nitrogen effects on maize in a Sahelian environment: II. Shoot growth, nitrogen uptake and water extraction. *Agric. Water Manag.* 46, 15–27. doi: 10.1016/s0378-3774(00)00074-3

Parkash, V. (2020). *Production of Cucumber under Deficit Irrigation and Eggplant under Salinity Stress: Assessment of Physiology, Growth, Yield and Water Use Efficiency*. Lubbock, TX: Texas Tech University.

Pazzagli, P. T., Weiner, J., and Liu, F. (2016). Effects of CO2 elevation and irrigation regimes on leaf gas exchange, plant water relations, and water use efficiency of two tomato cultivars. *Agric. Water Manag.* 169, 26–33. doi: 10.1016/j.agwat.2016.02.015

Peuke, A. D., and Rennenberg, H. (2011). Impacts of drought on mineral macro- and microelements in provenances of beech (*Fagus sylvatica* L.) seedlings. *Tree Physiol.* 31, 196–207. doi: 10.1093/treephys/tpr007

Ramírez, D. A., Yactayo, W., Gutiérrez, R., Mares, V., De Mendiburu, F., Posadas, A., et al. (2014). Chlorophyll concentration in leaves is an indicator of potato tuber yield in water-shortage conditions. *Sci. Hortic.* 168, 202–209. doi: 10.1016/j.scienta.2014.01.036

Ramlow, M., Foster, E. J., Del Grosso, S. J., and Cotrufo, M. F. (2019). Broadcast woody biochar provides limited benefits to deficit irrigation maize in Colorado. *Agric. Ecosyst. Environ.* 269, 71–81. doi: 10.1016/j.agee.2018.09.017

Rogovska, N., Laird, D. A., and Karlen, D. L. (2016). Corn and soil response to biochar application and stover harvest. *Field Crops Res.* 187, 96–106. doi: 10.1016/j.fcr.2015.12.013

Saberi, A., Kiani, A., Mosavat, S., and Halim, R. (2012). The effect of different sowing patterns and deficit irrigation management on yield and agronomic characteristics of sweet corn. *Afr. J. Biotechnol.* 11, 13882–13887.

Saini, R., Singh, A., and Deb, S. K. (2020). Effect of seed meals on weed control and soil physical properties in direct-seeded pumpkin. *Sustainability* 12:5811. doi: 10.3390/su12145811

Seleiman, M. F., Al-Suhaibani, N., Ali, N., Akmal, M., Alotaibi, M., Refay, Y., et al. (2021). Drought stress impacts on plants and different approaches to alleviate its adverse effects. *Plants* 10:259. doi: 10.3390/plants10020259

Singh, M., Saini, R. K., Singh, S., and Sharma, S. P. (2019). Potential of integrating biochar and deficit irrigation strategies for sustaining vegetable production in water-limited regions: a review. *HortScience Horts* 54:1872. doi: 10.21273/hortsci14271-19

Singh, M., Singh, P., Singh, S., Saini, R. K., and Angadi, S. V. (2021). A global meta-analysis of yield and water productivity responses of vegetables to deficit irrigation. *Sci. Rep.* 11:22095. doi: 10.1038/s41598-021-01433-w

Soil Science Division Staff (2017). "Examination and description of soil profiles," in *Soil Survey Manual: USDA Handbook 18*, eds C. Ditzler, K. Scheffe, and H. C. Monge (Washington, DC: Government Printing Office).

Spokas, K. A., Cantrell, K. B., Novak, J. M., Archer, D. W., Ippolito, J. A., Collins, H. P., et al. (2012). Biochar: a synthesis of its agronomic impact beyond carbon sequestration. *J. Environ. Qual.* 41, 973–989. doi: 10.2134/jeq2011.0069

Sterling, T. M. (2005). Transpiration: water movement through plants. *J. Nat. Resour. Life Sci. Educ.* 34, 123–123. doi: 10.2134/jnrlse.2005.0123

Stone, P. J., Wilson, D. R., Reid, J. B., and Gillespie, R. N. (2001). Water deficit effects on sweet corn. I. Water use, radiation use efficiency, growth, and yield. *Austral. J. Agric. Res.* 52, 103–113. doi: 10.1071/ar99146

TAMU (2021). *Historic ETo Reference [Online]*. College Station, TX: TAMU.

Vaccari, F., Maienza, A., Miglietta, F., Baronti, S., Di Lonardo, S., Giagnoni, L., et al. (2015). Biochar stimulates plant growth but not fruit yield of processing tomato in a fertile soil. *Agric. Ecosyst. Environ.* 207, 163–170. doi: 10.1016/j.agee.2015.04.015

Wang, Y., Sheng, D., Zhang, P., Dong, X., Yan, Y., Hou, X., et al. (2021). High temperature sensitivity of kernel formation in different short periods around silking in maize. *Environ. Exp. Bot.* 183:104343. doi: 10.1016/j.envexpbot.2020.104343

Zheng, J., Chen, J., Pan, G., Liu, X., Zhang, X., Li, L., et al. (2016). Biochar decreased microbial metabolic quotient and shifted community composition four years after a single incorporation in a slightly acid rice paddy from southwest China. *Sci. Total Environ.* 571, 206–217. doi: 10.1016/j.scitotenv.2016.07.135

Zotarelli, L., Dukes, M. D., Romero, C. C., Migliaccio, K. W., and Morgan, K. T. (2010). *Step by Step Calculation of the Penman-Monteith Evapotranspiration (FAO-56 Method)*. Gainesville, FL: Institute of Food and Agricultural Sciences University of Florida.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 29 April 2022
doi: 10.3389/fpls.2022.829421

Astragalus *sinicus* Incorporated as Green Manure for Weed Control in Corn

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Astragalus sinicus L. (milk vetch), one of the most widespread green manure species, is widely planted in the temperate zone. *Eleusine indica* L. (goosegrass), a serious annual weed in the world, has evolved resistance to some non-selective herbicides. The use of milk vetch as green manure for weed control in paddy fields was proposed. Aqueous extracts of milk vetch are known to exert a different level of phytotoxicity on weeds and crops. Phytotoxic substances contained in green manure were released into the soil by leaching at the initial stage and decomposition at the later stage after the return of green manure. Considering the need for searching new sustainable strategies for weed control, a question arises: "if milk vetch could be applied in goosegrass control, which stage is the most important to control goosegrass after milk vetch returned to the field, and at the same time, will the subsequent crop, corn (*Zea mays* L.), be affected by the side effects from milk vetch phytotoxicity?" In this study, the potential of milk vetch for goosegrass control was approached by repeated laboratory experiments, which include the aqueous extract experiment, decomposed experiment, and pot experiment. The effects of milk vetch returning to the field on maize were simulated by a pot experiment. The extract of milk vetch could significantly inhibit the germination of goosegrass at 2% concentration, and the inhibition enhanced with the increase of concentration. In the decomposed liquid experiment, decay time within 15 days, with the increase of decay days or concentration, goosegrass inhibition effect of decomposed liquid was enhanced. When decay time was more than 15 days, the inhibition ability of the decomposed liquid to goosegrass decreased. According to the RI accumulated value, aqueous extract and decomposed liquid have a "hormesis effect" on the germination and growth of goosegrass. Pot experiment proved that the addition of 1–10% (w/w) of milk vetch significantly reduced the germination and growth of goosegrass. On the contrary, the comprehensive analysis showed that the participation of milk vetch was conducive to the growth of corn. Our results constitute evidence that the incorporation of milk vetch into the soil could be a feasible practice to reduce weed infarctions in the corn-based cropping system.

Keywords: milk vetch, goosegrass suppression, allelopathy, corn growth, phytotoxicity

Edited by:
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Specialty section:
This article was submitted to
Plant Physiology,
a section of the journal
Frontiers in Plant Science

Received: *05 December 2021*
Accepted: *01 April 2022*
Published: *29 April 2022*

Citation:
Liu S, Ma Z, Zhang Y, Chen Z, Du X and Mu Y (2022) Astragalus sinicus Incorporated as Green Manure for Weed Control in Corn. Front. Plant Sci. 13:829421. doi: 10.3389/fpls.2022.829421

INTRODUCTION

Goosegrass is an annual gramineous weed, which is a worldwide weed harmful to crop growth in agricultural production (Zhang et al., 2021). In some suitable areas, it becomes a malignant weed due to its strong fecundity and resistance (An et al., 2014). In recent years, the widespread and excessive use of synthetic herbicides for weed control has led to many problems such as the development of herbicide-resistant weed species, damage to soil microecology, and environmental pollution. There is an urgent need to find other forms of weed control that can reduce the use of synthetic herbicides (Tesio and Ferrero, 2010). Milk vetch is a biennial herb of the leguminous family astragalus, and an important green manure crop. Previous studies showed that applying milk vetch could improve rice yield and reduce the use of chemical fertilizers (Chen et al., 2020; Zhou G. P. et al., 2020). Researchers found that the returning of milk vetch changed soil microbes and has the greatest potential to improve crop productivity as well as increase in corn yield (Tao et al., 2017). Studies have shown that planting milk vetch in winter fallow fields can improve soil fertility, reduce compound fertilizer application, improve soil physical and chemical properties, and preserve soil and water (Yang et al., 2014; Zhou et al., 2019; Zhou X. et al., 2020). In addition, intercropping of milk vetch could improve the quality, yield, and pest resistance of target crops (Bista and Dahal, 2018). Researchers found that incorporating milk vetch in paddy fields could significantly reduce the density of weed seed banks and significantly increase the species diversity and evenness of weed seed banks, which indicated that milk vetch returning to the field has the potential to control weed (Tang et al., 2016). Researchers found that the incorporation of milk vetch can significantly suppress the germination of summer weeds in paddy fields (Utami et al., 2020). Therefore, the research focus of milk vetch is on its effects on crop growth, soil physical and chemical properties, diseases, and insect pests, while there are a few studies on its inhibitory effect on weeds, especially on dryland weed control. Meanwhile, the physiological mechanism of how milk vetch inhibits weed growth is still unclear. There are many studies on the effect of returning leguminous crops on weeds. Researchers based their findings on field tests that faba bean (*Vicia faba* L.) could exert weed control when used as green manure, and the weed inhibition ability was highest at the time of faba bean incorporation into the field (Álvarez-Iglesias et al., 2018). However, He et al. (2020) found that corn seed germination and the lengths of radicle and plumule were lower than those of the control, particularly with 1% faba bean aqueous extract concentrations. Moreover, incorporating faba bean as green manure, the aboveground biomass of corn at the heading stages was negatively reduced by the whole plant, aboveground part, and stem (He et al., 2020). Milk vetch and spring corn rotation is an important tillage system. Previous studies have revealed that incorporating milk vetch into the soil can suppress weed infarctions. It is the premise of scientific and rational tillage to fully study the phytotoxicity effect of green manure on the main economic crops. Thus, it is necessary to further study whether the return of milk vetch to the field affects the corn-based tillage system. In this study, three goosegrass control laboratory experiments were designed with milk vetch as the material, based on previous studies and field observations, to find out the optimal decay time of allelochemicals in the decomposition process of milk vetch, and to clarify the impact of milk vetch returning on the growth of corn. It provides a theoretical basis for rational utilization and development of milk vetch in agricultural production.

MATERIALS AND METHODS

Plant Material

Milk vetch (*Astragalus sinicus* L.) seeds for the experiment were provided by Royal Garden Greening Engineering Co., Ltd. In July 2020, milk vetch was seeded in an artificial climate incubator (temperature 23 ± 2 (temperature %, 12 h/12 h light–dark cycle) and kept the soil moist. In September of the same year, milk vetch was harvested, air-dried, and crushed under natural conditions, sealed, and stored at room temperature for later use. Goosegrass (*Eleusine indica* L.) seeds were collected in the Experimental Base of South China Agricultural University (23°14’18.42″.42re c°38’8.0642re collected in the Experime“Black pearl waxy corn.”

Experiment and Treatment Design

This study includes milk vetch aqueous extract experiment, decomposed liquid experiment, and pot experiment. In the aqueous extract experiment, we used dry milk vetch powder as the material, weighed 20 g of powder, put in a bottle, added 500 ml sterilized ultra-pure water, and soaked in a shaker for 48 h with 200 rpm. The aqueous extract was filtered by filter paper and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered and the 0.22 μm microporous membrane was used to remove bacteria. The concentration of the undiluted aqueous extract was set as 100% and diluted with sterilized ultra-pure water into 2, 5, 30, and 80% as the treatment solution. We used 2.2% sodium hypochlorite to disinfect the goosegrass seeds for 10 min, and adopted the double-layer filter paper method, inoculated with 30 seeds of goosegrass into Petri dishes, treated them with the treatment solution. Sterile water treatment was used as a control (CK), each treatment was set up 5 replicates. Placed the inoculated Petri dishes in an artificial climate incubator (KES) at a temperature of 26 ± 2°C, a relative humidity of 75%, and a light–dark cycle of 12 h/12 h. Replenished water and recorded the germination number every day. The emergence of the radicle was taken as the indicator of germination. When the germination rate remained unchanged for 3 days, the recording was stopped.

In the decomposed experiment, we used milk vetch powder as material, mixed it according to the following ratio: powder, fresh paddy soil, and ultra-pure water (1:1:30 by weight) and put them into a plastic bottle with an inner lid. Soil solution without powder was used as the control (CK), and placed in a constant temperature shaker at 28°C for 200 rpm for 2, 3, 7, 15, 20, 30, and 42 days to obtain the decomposed liquid. The decomposed liquid from 15 days ago was used for the test immediately after decomposition, and the decomposed liquid from the subsequent time points (i.e., 20, 30, and 42 days) was stored in a -20ed in asequtor and was used for the test together after the last decomposition. The decomposed liquid and the control were filtered by filter paper and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered by 0.22 μm microporous membrane, and the obtained decomposed liquid was diluted with sterilized ultra-pure water into 2, 5, 30, and 80% concentration gradient treating solution. The next steps were similar to the aqueous extract experiment.

In the pot experiment, according to the dry weight yield of milk vetch 11.25 t/hm and the soil bulk density of about 1.3 g/cm^3, assuming that the tillage depths of no-tillage and rotary tillage are 1 and 6 cm, respectively, the milk vetch straw ratio is around 1.44–8.66%. We used milk vetch powder as material, mixed it well according to the mass ratio of powder:substance (Jiffy, consists of peat soil, coconut bran, vermiculite, and perlite) = 1:100, 3:100, 6:100, and 10:100, and the mixture was put in the flowerpot, and matrix soil without powder was used as the control (CK). Inoculated 30 goosegrass seeds or nine corn seeds into the flowerpot, each treatment was set up five replicates. The seeded pots were placed on the terrace, the average temperature during the experiment being 24°C, and the relative humidity being 85%. To ensure the authenticity of the results, all the management and environmental condition were maintained as consistent as possible.

Data Collection

In the germination stage of the aqueous extract and decomposed liquid experiment, we determined six germination indicators, namely, germination potential, germination rate, germination index, vital index, plant height, and fresh weight. Ten seedlings with uniform growth were selected from each treatment to measure plant height. A total of 20 plants were randomly selected from the five replicates for three replicates to determine the fresh weight of the plants (The number of samples was reduced by a certain proportion, and the final result was multiplied by the corresponding multiple after weighing when the sample was insufficient, same as the goosegrass of pot experiment). In the pot experiment, the seed germination rate of corn and goosegrass was recorded 8 days after inoculation, because no new seeds germinated after 8 days. Fifteen days after inoculation, 10 seedlings of goosegrass and 5 seedlings of corn with uniform growth were selected from each treatment to measure plant height. Five corn plants with uniform growth were selected from each treatment to measure fresh weight. At the same time, biochemical indicators, photosynthetic characteristics, and morphologic photographs of goosegrass and corn were recorded. Germination-related indicators were calculated according to the following formula:

$$Gp - \frac{number\ of\ germinated\ seeds\ at\ 2\ d}{number\ of\ tested\ seeds} \times 100\%;$$

$$Gr - \frac{number\ of\ normal\ germinated\ seeds}{number\ of\ tested\ seeds} \times 100\%;$$

$$Vi - s \times Gi, (s\ means\ plant\ height\ here);$$

$$Gi - \sum\left(\frac{Gt}{Dt}\right), \left(Gt\ : Germination\ number\ at\ different\ day,\right.$$

$$\left. Dt\ : The\ statistical\ number\ of\ days\right);$$

Gr: Germination rate; Gp: Germination potential; Gi: Germination index; Vi: Vital index.

The allelopathic effects of milk vetch were evaluated using means of response index (RI). When T ≥ C, RI = 1-C/T; T < C, RI = T/C-1 (C: result of CK; T: result of treatment). RI > 0, indicating allelopathic promotion; RI < 0, indicating allelopathic inhibition; absolute value was consistent with the degree of allelopathy (Williamson and Richardson, 1988). RI accumulate values of each indicator were calculated and the allelopathic effects of milk vetch were evaluated comprehensively. RI accumulate values reached -6, indicating that germination and growth of weeds were totally inhibited. In the second-leaf stage of corn in the pot experiment, the last fully expanded leaf of goosegrass and corn seedlings was taken to determine the protective enzyme activity, content of soluble protein, and malondialdehyde (MDA) content. Superoxide dismutase (SOD) activity was measured by the nitroblue tetrazolium (NBT) reduction method, peroxidase (POD) activity was measured by the colorimetric method, and catalase (CAT) activity was measured by the guaiacol method (Zhang et al., 2017). Soluble protein content was measured by Coomassie's brilliant blue staining (Goharrizi et al., 2021). MDA content was measured by the TBA method and the root activity was measured by the 2,3,5-Triphenyte-trazoliumchloride (TTC) method (Xiao et al., 2021). Photosynthesis and fluorescence-related indexes, namely, net photosynthetic rate (Pn), transpiration rate (E), stomatal conductance (gtc), intercellular carbon dioxide concentration (Ci), instant water use efficiency (iWUE), and maximal quantum yield of PSII (Fv/Fm), PSII actual photochemical quantum efficiency (φPSII), electron transport rate (ETR), photochemical quenching (qP), and non-photochemical quenching (NPQ), were measured by an LI-6,800 photosynthesis measurement system (*LI*-COR, United States). The last fully expanded leaf of 3 uniform corn seedlings at the fourth-leaf stage was selected from 5 replicates of each treatment and the parameters related to photosynthesis and fluorescence were measured. The chlorophyll content was expressed by the Soil and Plant Analyzer Development (SPAD) value, which was collected by SPAD-502 plus (Konica Minolta, Inc.). The last fully expanded leaf of a fourth-leaf corn seedling was selected from each of the 5 replicates of each treatment, and the SPAD values of the upper, middle, and lower parts of the leaf were measured, and their average value was calculated as the final SPAD value. Root and leaf area analysis was performed using GXY-A, a scanner produced by Zhejiang Tuopu Yunnong Technology Co., Ltd. One corn seedling was selected from each of the five replicates of each treatment, and all roots and leaves were cut and scanned by GXY-A. Root morphologic parameters were analyzed by WinRHIZO, and leaf area was calculated by Photoshop. Both are well known for image processing.

Statistical Analysis

The data of experiments were analyzed by one-way ANOVA. We verified normality and homogeneity of variance using the Shapiro–Wilk test and Levene's test, respectively. Significant differences were further compared using the *post hoc* Fisher LSD test. SPSS 24 was used for significance analysis of the data, which were expressed as mean ± standard deviation. GraphPad Prism 7 was used to plot.

RESULTS AND DISCUSSION

Effect of Milk Vetch Aqueous Extract on Goosegrass

With the increase in the concentration of the milk vetch–leached liquor (**Figure 1**), the inhibitory effect of the aqueous extract on Gp, Gr, and Gi was enhanced. Low concentration leached liquor ($\leq$30%) can improve the plant height of goosegrass, while the vital index was improved with less than 5% aqueous extract treatment. The fresh weight of goosegrass did not show an obvious rule. Researchers studied the allelopathic effects of eucalyptus leachates on three tree species and found that foliage + litter and foliage leachates even increased fresh weight and dry weight of *Leucaena leucocephala* and *Schefflera octophylla*, respectively (Song et al., 2019). When the concentration of milk vetch aqueous extract reached 80% (**Supplementary Table 1**), all the goosegrass seeds in the test could not germinate so the RI value reached -6. The variety of goosegrass germination indicators were dose dependent. Many studies reveal that the allelopathic effect of a plant extract or a leachate was dependent of its concentration (Chen B. M. et al., 2018; Eladel et al., 2019; Ojija et al., 2019). According to the RI accumulate value, milk vetch leached liquor has a "hormesis effect" on germination and growth of goosegrass. Only from the point of view of germination, the results of the aqueous extract experiment were consistent with the previous studies on the effects of *Hyptis suaveolens* (L.) Poit on the *Parthenium hysterophorus* I. and *Senna uniflora* (Mill.) H.S. Irwin (Kumari and Prasad, 2018). Allelopathic substance affects different plant receptors in different directions, some for germination and some for growth. Returning eucalyptus leaves as green manure can inhibit the germination of representative weeds in the field (Puig et al., 2013). In the aqueous extract experiment, depending on the RI value of germination and growth indicators of goosegrass, we can conclude that milk vetch aqueous extract mainly affected the germination process of goosegrass. We did not find significant negative effects of aqueous extract on plant height and the fresh weight of goosegrass seedlings. Aqueous extract within 30% even increased the plant height of goosegrass, and 2 and 30% concentration can also increase the fresh weight.

Effect of Different Decay Time Decomposed Liquid of Milk Vetch on Goosegrass

The release of allelochemicals through decomposition is an important way for green fertilizer to exert the effect of weed control. The germination-related indicators (**Figure 2**), namely, germination potential, germination rate, and germination index were significantly improved in 2 days decomposed liquid of $\leq$ 5%, and 3 days decomposed liquid of 2%. All the germination-related indicators were inhibited in 7 and 15 days decomposed liquids of all the concentration treatments. The 15 days decomposed liquid had the highest goosegrass-inhibited potential among all the decay times. When the decay time was greater than 15 days and less than 30 days, germination-related indicators went up gradually at low concentrations treatment ($\leq$5%). When it was 42 days, decomposed liquids less than 30% could significantly improve all the indicators of goosegrass. None of the seeds germinated at 80% decomposed liquid except those with 2 and 42 days treatment. The vital index of goosegrass seeds was affected by the interaction of germination index and plant height. The low concentration decomposed liquids of 2 and 45 days treatment can significantly improve the vital index of goosegrass, while 2% decomposed liquids of 3, 7, 20, and 30 days can improve the vital index. A 15 days decomposed liquid can significantly inhibit the vital index of goosegrass among all the concentration treatments. The change of plant height and fresh weight of the goosegrass seedlings have a similar tendency. On 2, 3, and 42 days, both plant height and fresh weight increased when the concentration of decomposed liquids was less than 30%, but with the increase in concentration, plant height and fresh weight were significantly inhibited. The plant height and fresh weight were increased by 7, 20, and 30 days decomposition solution at low concentration ($\leq$5%), but plant height and fresh weight were significantly inhibited with the increase of concentration. Plant height and fresh weight have an insignificant change in 15 days decomposed liquid of low concentration treatment but were significantly inhibited at high concentration treatment ($\geq$30%). In the field of allelopathy research, we often see that the law of allelopathy among plants is summarized as low promotion and high inhibition, which simply means the "hormesis effect." *Carex thunbergii* extracts increased the germination rate of *Lolium perenne* at low concentrations and showed an allelopathic inhibition effect at a high concentration (Zhang et al., 2019). In conclusion, the phytotoxicity potential of milk vetch decomposed liquid on goosegrass varied with the decay time, which reached the peak at 15 days and was dose dependent. In the allelopathic effect experiment of soybean straw decomposition on mung bean, researchers found that in four periods (0, 7, 14, and 21 days), 14 days of decomposition could produce a variety of chemical substances and significantly inhibit mung bean, whereas decomposition for 21 days reduced the inhibitory effect (Azhar et al., 2018). The decomposition patterns and the persistence of allelopathic chemicals are different in

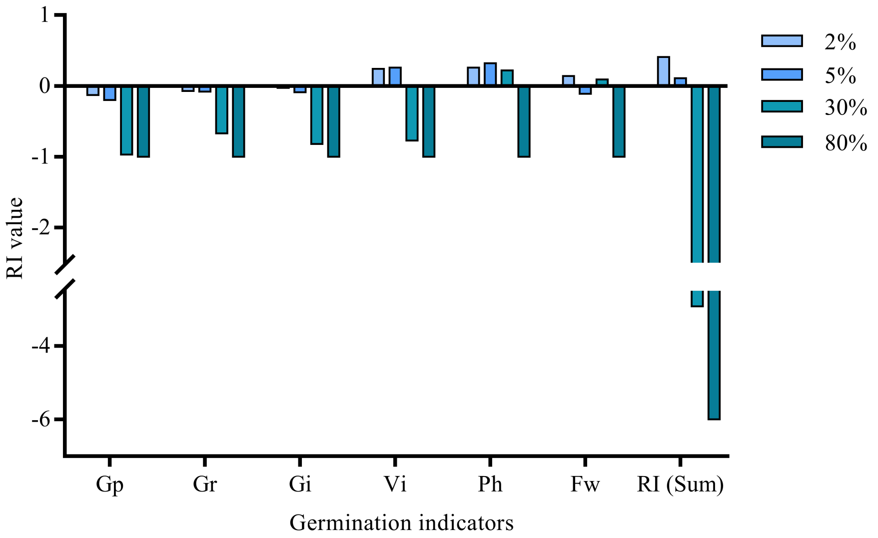

FIGURE 1 | RI value of milk vetch aqueous extract on germination and growth of goosegrass. Gr, Germination rate; Gp, Germination potential; Gi, Germination index; Vi, Vital index; Ph, Plant height; Fw, Fresh weight; RI (Sum), The sum of the respond index value of Gp, Gr, Gi, Vi, Ph, and Fw.

different plants. Researchers found a rule in the cereal rye cover crop decomposition studies that the phenolic acids released from cereal rye increased in soils during the first 3–7 days after cereal rye termination and then decreased to initial concentrations after 56 days (Otte et al., 2020). The allelopathic ability increased with the increase of decomposition time before 15 days of decomposition, and the allelopathic inhibition ability decreased after 15 days of decomposition, which was similar to a previous research. As shown in **Figure 3**, the intensity of the allelopathy of the decomposed liquid was determined by the concentration and the decay time.

Effect of Simulation of Milk Vetch Simulated Returning to the Field on the Germination and Growth of Goosegrass

Different Ratios of Milk Vetch Straw and Soil Treatments on Goosegrass Morphologic Properties

Only from the weed control perspective (**Figure 4**), the significant weed inhibition effect can be seen intuitively with the input of milk vetch straw in different straw and soil ratios. The germination rate, plant height, and fresh weight of goosegrass (**Figure 5**) were significantly inhibited by the incorporation of milk vetch straw. A 1:100 straw-to-soil ratio has an insignificant effect on plant height of goosegrass, with the ratio going up, plant height was significantly inhibited. Fresh weight of goosegrass was inhibited significantly among all the ratios treatment of the experiment. The germination and growth-related indicators of goosegrass did not improve in the pot experiments even in the lowest straw proportion treatment. In a study of weed control on the corn field, researchers found that the incorporation of faba bean as green manure can reduce the germination, root and bud elongation, and aboveground biomass of major weeds in the corn fields, and a higher proportion of faba bean treatment showed stronger weed inhibition potential (Álvarez-Iglesias et al., 2018). Submerged macrophytes (*Ceratophyllum demersum* L.) could allelopathically inhibit the growth of *Chlorella vulgaris* Beij., the effects of which were concentration-dependent (Dong et al., 2019). The return of milk vetch to the field reduced the occurrence of goosegrass and inhibited the growth of the germinated weeds, which is worth further study. The researchers used the *eucalyptus globulus* leaves as green manure and isolated two allelopathic substances after returning to the field, which inhibited the germination and growth of weed (Puig et al., 2018).

Different Ratios of Milk Vetch Straw and Soil Treatment on Photosynthesis-Related Properties of Goosegrass

Compared to the CK, different straw and soil ratio treatments can significantly inhibit the net photosynthesis rate (Pn) and instant water use efficiency (iWUE) of goosegrass. Allelochemicals cannot only inhibit the growth establishment but also significantly decrease the photosynthetic rate of lettuce (Nasrollahi et al., 2018). Treatments of 3:100 and 6:100 ratios significantly reduced the transpiration rate **(E)** of goosegrass, while 1:100 and 10:100 treatments did not significantly reduce the transpiration rate, but slightly decreased. Different straw and soil ratio treatments can decline the stomatal conductance (gtc) of goosegrass, and in the high straw and soil ratio treatments, the change reached a significant level. Different straw and soil ratio treatments can improve the intercellular carbon dioxide concentration (Ci) of goosegrass, and in 1:100 and 10:100 treatments, the change of intercellular carbon dioxide concentration reached a significant level. Chen S. et al. (2018) reported that the decomposing leaf litter of *Leucaena leucocephala* can decrease the photosynthetic rate, transpiration rate, and stomatal conductance, and continuously increase the intercellular carbon dioxide concentration, which was consistent

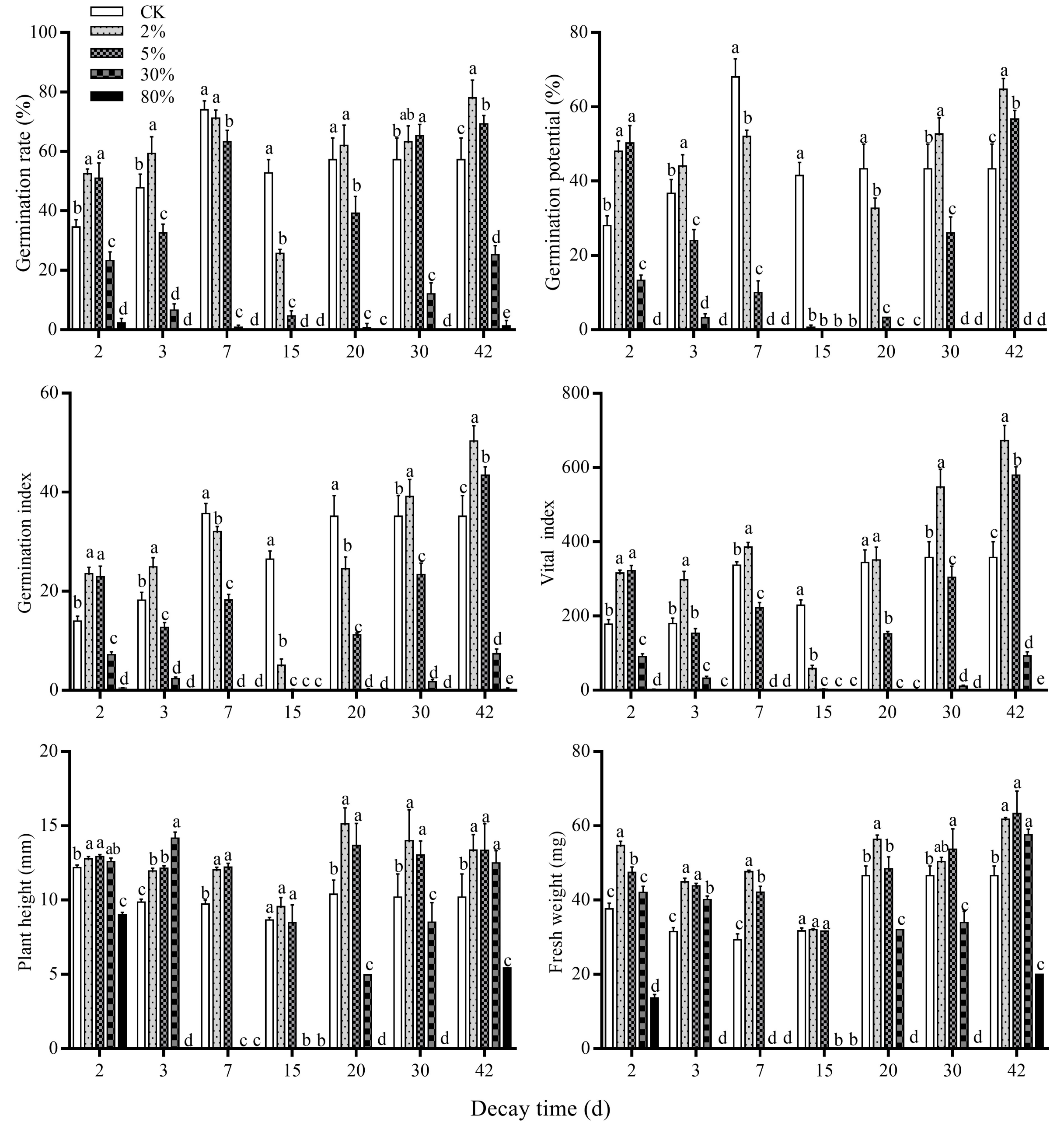

FIGURE 2 | Effect of different decay time milk vetch decomposed liquids on germination and growth of goosegrass. Different letters above each group of the bar chart denote significant difference among different treatments of decomposed liquid at $P < 0.05$ by LSD test, respectively. Bars represent 10, 3, and 5 repetitions ± SD in plant height, fresh weight, and germination-related parameters, respectively.

with this study. Researchers speculated that in plants under stress, the photosynthesis characteristic was inhibited, which leads to a lower net photosynthetic rate and higher intercellular carbon dioxide concentrations (Bao et al., 2020).

Chlorophyll fluorescence serves as a sensitive indicator of the functional state of photosynthetic apparatus in chloroplasts (Kalmatskaya et al., 2019). Different milk vetch straw and soil ratios have no significant impact on the maximal quantum yield of PSII (Fv/Fm). The highest value of PSII actual photochemical quantum efficiency (φPSII) and electron transport rate (ETR) were recorded in the 6:100 treatment. Compared to the CK, the φPSII and ETR of goosegrass were significantly suppressed, except for the 6:100 treatment. The lowest photochemical quenching (qP) value was recorded in the 1:100 treatment, while the highest value was recorded in the 6:100 treatment, and the rest treatments had no significant effect compared with CK.

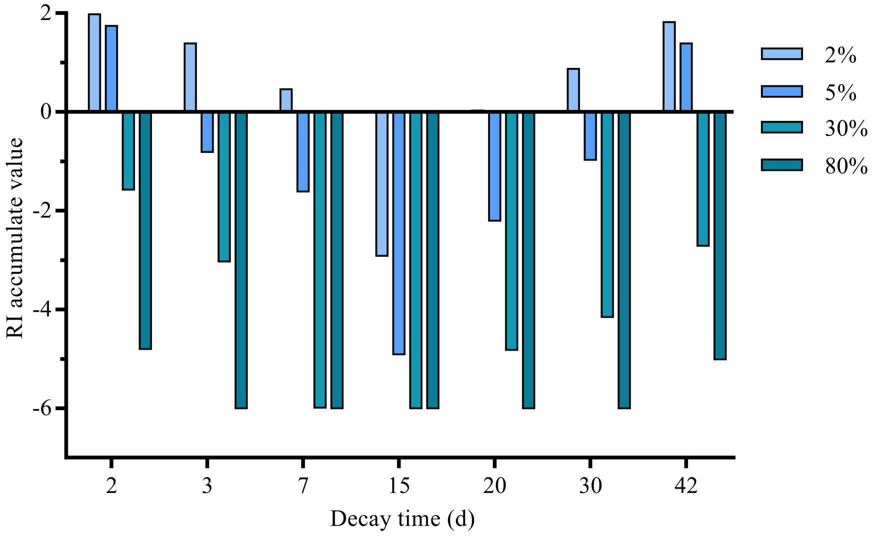

FIGURE 3 | RI accumulated value of milk vetch decomposed liquids on goosegrass. RI: Respond index.

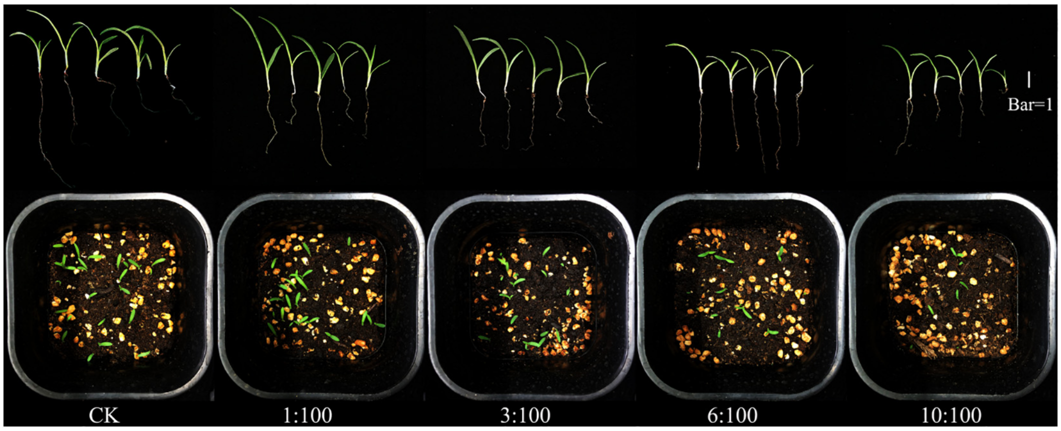

FIGURE 4 | Effect of different milk vetch straw and soil ratios on goosegrass.

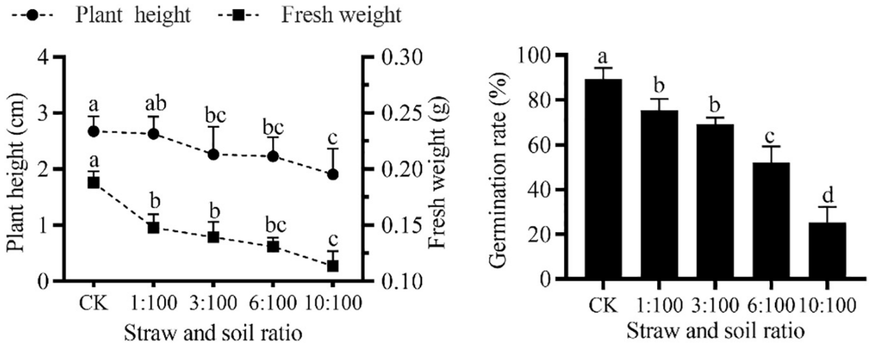

FIGURE 5 | Effect of different ratios of milk vetch straw and soil on germination and growth of goosegrass. All data were subjected to analysis of variance with *post hoc* LSD test. Different letters denote significant difference among different treatments at $P < 0.05$. Symbols represent 10, 3, and 5 repetitions ± SD in plant height, fresh weight, and germination rate, respectively.

Wang et al. (2018) on the study of *Dracontomelon duperreanum* leaf litter impacted the photosynthesis of *Microcystis aeruginosa* found that the growth rate of *Microcystis aeruginosa* in response to different extract concentrations was consistent with changes in the photosynthesis efficiency (alpha), maximal relative electron transport rate, and maximal quantum yield of PSII. Compared with our study, although it was slightly different from previous studies in some indicators, it could be found that the photosynthesis of goosegrass was adversely affected in general.

When plants live under stress, it is often accompanied by an increase in non-photochemical dissipation of excitation energy, NPQ (Doğru and Çakirlar, 2020). The lowest non-photochemical quenching (NPQ) value was recorded in CK, while the highest value was recorded in 6:100 treatment, and the rest treatments were significantly improved compared with CK. The non-photochemical quenching significantly improved in all treatments, which indicated that the plant's photosynthetic system has been adversely affected (**Supplementary Table 2**). According to previous studies, researchers hold the view that high NPQ values indicate a disadvantaged condition where plants are living in Kalmatskaya et al. (2019) and Gao et al. (2020).

Different Ratios of Milk Vetch Straw and Soil Treatment on the Biochemical Properties of Goosegrass

The SOD activity of goosegrass seedlings was significantly declined at 1:100 treatment and gradually improved with the increase of straw ratio. SOD activity increased significantly when the percentage of straw was more than 6% (**Figure 6A**). The change tendency of POD and CAT activity were synchronous, which were significantly improved at all straw and soil ratio treatments. Compared to other treatments, POD activity at 6:100 treatment was slightly declined. CAT activity at a high ratio treatment (≥6:100) was relatively lower than the rest of the treatment (**Figures 6B,C**). Researchers of the studies on allelopathic effect of *Bidens pilosa* leachates on *Pteris multifida* found that superoxide dismutase (SOD) and catalase (CAT), as well as guaiacol peroxidase enhanced with the increase in leachate concentration (Zhang et al., 2016). Phytotoxic substances (cinnamic acid) can induce the production and accumulation of ROS, decrease root and shoot length, fresh and dry weights, and photosynthetic pigments (Yadav et al., 2019). In this study, we did not measure the ROS content, but from the change in antioxidant enzyme activity, we suspected that decomposing liquid may increase the ROS content in plants. All of the treatments can significantly improve the soluble protein except for the 3:100 treatment (**Figure 6D**). A 6:100 straw and soil ratio treatment can increase the MDA content of goosegrass, but the level did not reach a significant level. The rest treatment can significantly decrease the MDA content of goosegrass except for the 1:100 treatment (**Figure 6E**). The photosynthesis-related indexes and chlorophyll content of the 6:100 treatment were relatively poor, which may be related to MDA content. The change in the protective enzyme system means that peroxides and superoxides were produced during the decomposition of milk vetch. According to the change of POD, CAT, and MDA, we speculated that the content of MDA was mainly affected by peroxide. As for why the change of MDA content was not parallel with the milk vetch proportion, a previous study found that the release of allelochemicals by the interaction of green manure and microorganism is an important process of green manure returning to the field. Ping et al. (2015) found that different proportions of corn straw returned to the soil can affect the community composition of soil microbes, but the signatures (PLFAs) were non-linear with straw and soil ratios, which may be associated with the non-linear change in peroxide-related enzyme activity as well as MDA content in our study. Coumarins are widely distributed substances in plant species that promote phytotoxic effects, allowing them to be exploited as herbicides less harmful to the environment. The researchers found that coumarin can inhibit crop germination, reducing its biomass, and root and shoot growth. Subsequent studies have shown that coumarin is cytogenotoxic due to its damage to the cell cycle and the occurrence of chromosomal abnormalities. However, it does not cause lipid peroxidation (Govêa et al., 2020), most of Govêa et al.'s findings are consistent with this study.

Effect of Simulation of Milk Vetch Simulated Returning to the Field on the Germination and Growth of Corn

Different Ratios of Milk Vetch Straw and Soil Treatments on the Morphologic Properties of Corn

Many studies have reported that the incorporation of milk vetch can improve the yield of the main crop and the productivity and sustainability of soil (Yang et al., 2013). Before returning green manure to the field for weed control, it is necessary to assess the duration of the early effects of green manure incorporation into the soil and its short-term effects on germination and growth of the main crop and some accompanying weeds (Puig et al., 2013; Álvarez-Iglesias et al., 2018). The effect of green manure allelopathy in corn must be taken into account for putative field management of green manure for weed control. Consequently, a pot experiment on the phytotoxic effects of green manure was designed to establish a relay planting of corn after the incorporation of milk vetch into the soil. Root morphogenesis at the seedling stage is very important to growth and yield at the later stage. Compared to the CK, all the treatments can improve the root system of corn except for the total length of corn at 10:100 condition and branch points of corn at 3:100 condition. Incorporating a certain percentage of milk vetch into the soil can significantly improve all the root systems of corn except total length and branch points (**Supplementary Table 3**). Root growth and development were closely related to reactive oxygen species (ROS), the reduced ROS content resulted in defective root growth of cucumber (Huang et al., 2020). Whether corn root growth is exactly affected by ROS and the influence of certain milk vetch ratios for root growth needs further study. Visually, adding vetch straw can promote the growth of corn seedlings (**Figure 7**). All the treatments can significantly improve the plant height of corn (**Figure 8**). Application of green manure together with out-of-season corn crop promoted the highest plant height and soybean yield-related

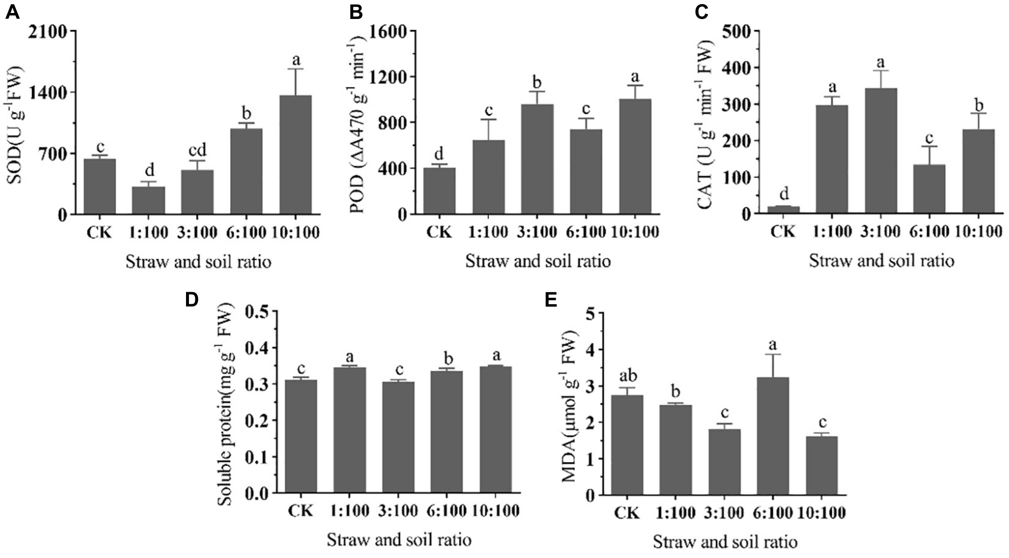

FIGURE 6 | Effect of different ratios of milk vetch straw and soil on SOD activity **(A)**, POD activity **(B)**, CAT activity **(C)**, soluble protein **(D)**, and MDA **(E)** content of goosegrass. All data were subjected to analysis of variance with *post hoc* LSD test. Different letters denote significant difference among different treatments at $P < 0.05$. Bar chart represents mean values of 5 replicates ± SD.

FIGURE 7 | Effect of different ratios of milk vetch straw and soil on the growth of corn.

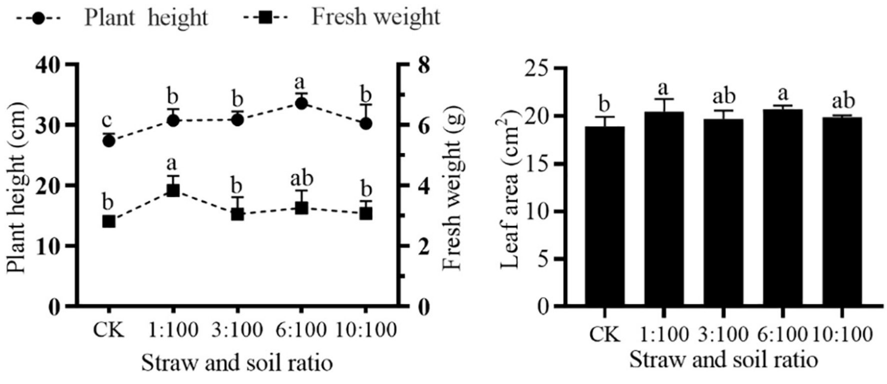

FIGURE 8 | Effect of different ratios of milk vetch straw and soil on plant height, fresh weight, and leaf area of corn. All data were subjected to analysis of variance with *post hoc* LSD test. Different letters denote significant difference among different treatments at $P < 0.05$. Symbols represent 5 repetitions ± SD.

traits in succession (Genovesi et al., 2019). The fresh weight of corn could be increased by different milk vetch proportion treatments, and the change level reached a significant level at 1:100 ratio treatment. All the treatments can improve the leaf area of corn, and the change reaches a significant level at 1:100 and 6:100 treatments. The return of green manure provides a

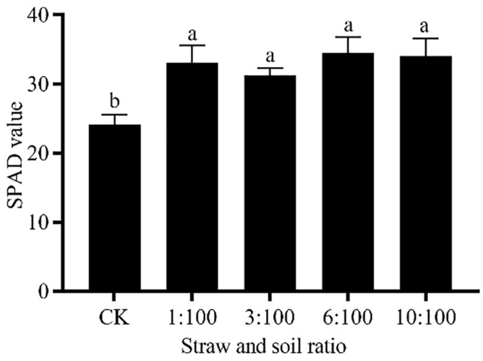

FIGURE 9 | Effect of different ratios of milk vetch and soil on SPAD value of corn. All data were subjected to analysis of variance with *post hoc* LSD test. Different letters denote significant difference among different treatments at $P < 0.05$. Bar chart represents 5 replicates ± SD.

breeding ground for subsequent crops, which will be a benefit to the growth and development of crops. Previous studies can also prove this point. Crop straw combined with green manure crops improved the germination rate, growth process of corn plants, and contributed to the better establishment of generative organs, elements of the structure of the yield (Sendetsky, 2018). In conclusion, milk vetch returning was a possible practice, which has the potential to improve the growth of corn seedlings.

Different Ratios of Milk Vetch and Soil Treatment on Chlorophyll Content and Photosynthesis-Related Properties of Corn

The incorporation of milk vetch was beneficial to all the photosynthesis properties of corn except for transpiration rate at 1:100 treatment and instant water use efficiency at 10:100 treatment. The net photosynthetic rate (Pn), transpiration rate (E), and stomatal conductance (gtc) of maize were significantly improved when the ratio of milk vetch was more than 1:100. Different milk vetch and soil ratios can improve the intercellular carbon dioxide concentration (Ci) of corn, but the change did not reach a significant level. Instant water use efficiency (iWUE) can be improved when the milk vetch proportion is less than 6%, and the change in 1:100 treatment reached a significant level. Not only green manure, crop stalks can also play a similar role. Researchers found that an appropriate ratio of corn straw in the substrate could significantly improve the photosynthetic parameters and chlorophyll content of tomato seedlings (Chen S. et al., 2018).

The participation of milk vetch can improve the PSII actual photochemical quantum efficiency (φPSII), electron transport rate (ETR), and photochemical quenching (qP). The change of φPSII and ETR reached a significant level among all the treatments, while the change of qP at 1:100 and 10:100 treatment reached a significant level. the maximal quantum yield of PSII (Fv/Fm) was improved at 3:100 and 6:100 treatment, and the change at 3:100 reached a significant level. All the treatments can decline the non-photochemical quenching (NPQ) of corn, and the changes reached a significant level. The lowest value of NPQ was recorded at 1:100 and 10:100 treatment (**Supplementary Table 4**). Milk vetch is a kind of green fertilizer with high nitrogen (N) nutrition, studies have found that the use of milk vetch can improve soil nutrition, especially N nutrition (Bimantara et al., 2020). The high-N condition was conducive to the photosynthesis parameters and maximal quantum yield of PSII (Yousuf et al., 2020). Considering that corn is a high-N plant, from the current experimental results, the high proportion of milk vetch is still beneficial to corn growth, and the follow-up experiments should be conducted around the straw returning

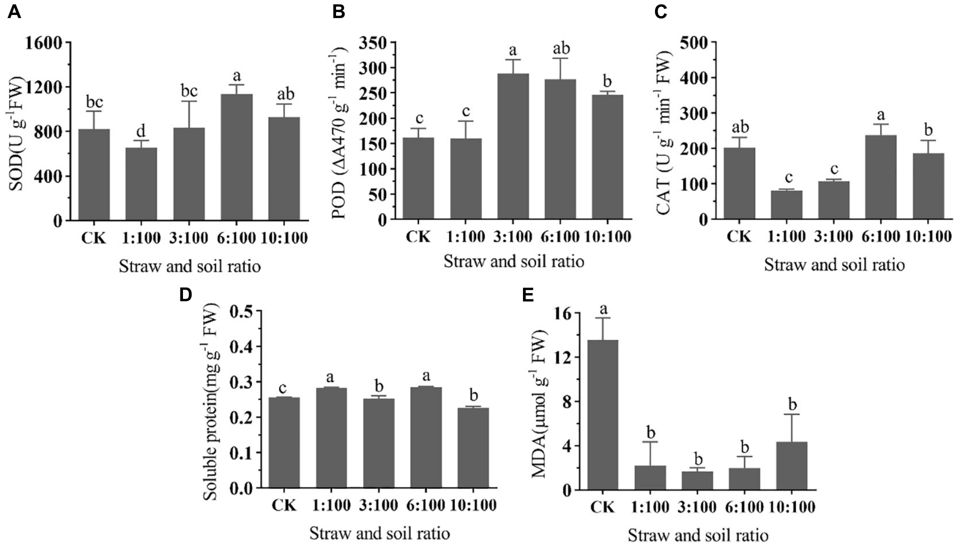

FIGURE 10 | Effect of different ratios of milk vetch and soil on SOD activity **(A)**, POD activity **(B)**, CAT activity **(C)**, soluble protein **(D)**, and MDA **(E)** content of goosegrass. All data were subjected to analysis of variance with *post hoc* LSD test. Different letters denote significant difference among different treatments at $P < 0.05$.

threshold to prevent possible damage to corn (**Figure 9**). Incorporating the milk vetch as green manure showed a positive tendency on promoting the chlorophyll content of corn. In the study of corn straw on the growth of tomato seedlings, researchers found that the total chlorophyll and photosynthesis parameters were highest.

Different Ratios of Milk Vetch and Soil Treatment on Biochemical Properties of Corn

The SOD and CAT activities (**Figures 10A,C**) decreased under the condition of low milk vetch proportion and increased with the increase of proportion. SOD activity reached a peak under 6:100 proportion. The SOD activity significantly improved at 6:100, while CAT activity significantly declined at low proportions (≤3:100). POD activity (**Figure 10B**) stands still at 1:100 treatment, and then significantly improved as the milk vetch proportion was greater than 3:100. In a similar study of "Effect of the aqueous extract of *Juglans. regia* leaves on the seed germination and seedling enzymatic activity of *Scutellaria baicalensis* in Shangluo," the researcher found that a low concentration of aqueous extract promoted the protective enzyme activities of *Scutellaria baicalensis* seedling; but with the increasing concentration of aqueous extract, the promoting role gradually decreased (Peng, 2011). Previous studies on the effect of silicon application on sugarcane disease resistance found that silicon application can increase the activity of SOD and decrease the activity of POD in the sugarcane seedling stage, which resulted in the increase of MDA and H_2O_2, induced the resistance response, and finally enhanced the ability of disease resistance in the following growth stage (Deng et al., 2020). Garlic allelochemicals (aqueous extraction) act as plant biostimulants to enhance auxin biosynthesis and transportation, resulting in root growth promotion. Additionally, antioxidant enzymes activities regulations indicate activation of the defense responses in tomato seedlings resulting in better growth and development (Hayat et al., 2020), which is consistent with this study.

The 1:100 and 6:100 treatments significantly increased the content of soluble protein in corn (**Figure 10D**), while the content of soluble protein significantly declined at 10:100 treatment. As for the decrease of soluble protein content caused by the 10:100 treatment, it is speculated that the negative effect may be caused by high green manure content. Researchers found that four plant leaf extracts exhibited beneficial allelopathic effects on the onion plant protein content (Qari et al., 2020). All of the treatments can significantly decrease the content of corn MDA (**Figure 10E**). When the milk vetch proportion reached 10:100, MDA content tends to increase. Researchers of the previous studies about the allelopathic effect of co-culture pepper and garlic reported that a low garlic/pepper ratio tends to induce protective enzyme systems, reduce MDA content, and finally promote pepper growth, but a high garlic/pepper ratio resulted in a high concentration of garlic root exudates, which have deleterious effects on membrane lipid and inhibited protective enzyme activities (Ding et al., 2016). The tendency of protective enzyme systems activities, soluble protein, and MDA content in 10:100 treatment should be paid more attention. Further study should optimize the milk vetch returning proportion to provide a suitable condition for corn production.

This study provides positive evidence that milk vetch incorporated into the soil as green manure has the potential to inhibit the growth of weeds. Planting corn immediately after milk vetch is returned to the field, can suppress the emergence of weeds and promote the growth of corn. Combined with the weed control effect and the growth of corn, this study suggested that the ratio of milk vetch and soil should be within 10:100, and the ratio of 6:100 is the best under laboratory conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SL and YM: conceptualization. SL: experimental method and design, software, original draft, and writing. YZ, ZC, and XD: data curation and revision of the manuscript. YM and ZM: funding acquisition, supervision, and validation. All authors have read and agreed to the published version of this manuscript.

FUNDING

This project was supported by Natural Science Foundation of Guangdong Province (2018A0303130194) and Bayer Crop Science supporting project (2021 Grants4AG).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.829421/full#supplementary-material

REFERENCES

Álvarez-Iglesias, L., Puig, C. G., Revilla, P., Reigosa, M. J., and Pedrol, N. (2018). Faba bean as green manure for field weed control in maize. *Weed Res.* 58, 437–449. doi: 10.1111/wre.12335

An, J., Shen, X., Ma, Q., Yang, C., Liu, S., et al. (2014). Transcriptome profiling to discover putative genes associated with paraquat resistance in goosegrass (*Eleusine indica* L.). *PLoS One* 9:e99940. doi: 10.1371/journal.pone.0099940

Azhar, M., Muhammad, N., Farhan, K., Yousaf, S., Tasawer, A., et al. (2018). Identification of phytotoxins in different plant parts of *Brassica napus* and their influence on mung bean. *Environ. Sci. Pollut. Res.* 25, 18071–18088. doi: 10.1007/s11356-018-2043-x

Bao, Y., Wang, J. X., Chen, C., and Yu, X. M. (2020). Effects of NaCl and NaHCO_3 stress on photosynthesis and chlorophyll fluorescence characteristics of *Hemerocallis fulva* 'Golden Doll'. *Agricult. Biotechnol.* 9, 18–25. doi: 10.19759/j.cnki.2164-4993.2020.03.006

Bimantara, U., Sabri, U., Kautsar, T., and Cheng, H. (2020). Incorporation of winter grasses suppresses summer weed germination and affects inorganic nitrogen in flooded paddy soil. *Soil Sci. Plant Nutrit.* 66, 389–397. doi: 10.1080/00380768.2020.1725914

Bista, B., and Dahal, S. (2018). Cementing the organic farming by green manures. *Int. J. Appl. Sci. Biotechnol.* 6, 87–96. doi: 10.3126/ijasbt.v6i2.20427

Chen, B. M., D'Antonio, C. M., Molinari, N., and Peng, S. L. (2018). Mechanisms of influence of invasive grass litter on germination and growth of coexisting species in California. *Biol. Invas.* 20, 1881–1897. doi: 10.1007/s10530-018-1668-5

Chen, F. Q., Liu, K. H., Xie, Z. Q., Liu, M., and Chen, C. (2018). Effects of decomposing leaf litter of *Leucaena leucocephala* on photosynthetic traits of *Cynodon dactylon* and *Medicago sativa*. *New For.* 49, 667–679. doi: 10.1007/s11056-018-9651-7

Chen, J. R., Qin, W. J., Chen, X. F., Cao, W. D., Qian, G. M., et al. (2020). Application of Chinese milk vetch affects rice yield and soil productivity in a subtropical double-rice cropping system. *J. Integ. Agricult.* 19, 2116–2126. doi: 10.1016/S2095-3119(19)62858-3

Chen, S., Song, S., Zhao, C., Zhao, J., and Li, Y. (2018). Effects of mixed corn stalk substrates on growth and photosynthesis of tomato seedlings. *Acta Horticult.* 1227, 463–470. doi: 10.17660/actahortic.2018.1227.58

Deng, Q. Q., Wu, J., Chen, J. W., and Shen, W. K. (2020). Physiological mechanisms of improved smut resistance in sugarcane through application of silicon. *Front. Plant Sci.* 11:130. doi: 10.3389/FPLS.2020.568130

Ding, H. Y., Cheng, Z. H., Liu, M. L., Hayat, S., and Feng, H. (2016). Garlic exerts allelopathic effects on pepper physiology in a hydroponic co-culture system. *Biol. Open* 5, 631–637. doi: 10.1242/bio.016451

Doğru, A., and Çakirlar, H. (2020). Effects of leaf age on chlorophyll fluorescence and antioxidant enzymes activity in winter rapeseed leaves under cold acclimation conditions. *Braz. J. Bot.* 43, 11–20. doi: 10.1007/s40415-020-00577-9

Dong, J., Chang, M. Y., Li, C. H., Dai, D. J., and Gao, Y. N. (2019). Allelopathic effects and potential active substances of *Ceratophyllum demersum* L. on *Chlorella vulgaris* Beij. *Aquat. Ecol.* 53, 651–663. doi: 10.1007/s10452-019-09715-2

Eladel, H., Battah, M., Dawa, A., Abd-Elhay, R., and Anees, D. (2019). Effect of rice straw extracts on growth of two phytoplankton isolated from a fish pond. *J. Appl. Phycol.* 31, 3557–3563. doi: 10.1007/s10811-019-01893-8

Gao, S., Liu, X. N., Liu, Y., Cao, B. L., Chen, Z. J., et al. (2020). Photosynthetic characteristics and chloroplast ultrastructure of welsh onion (*Allium fistulosum* L.) grown under different LED wavelengths. *BMC Plant Biol.* 20:78. doi: 10.1186/s12870-020-2282-0

Genovesi, G., Rasera, G. P., Moreski, H. M., Felipe, D. F., and Mannigel, A. R. (2019). Parameters of soybean cultivated in succession with intercropping system of out-of-season corn with green manure. *J. Agricult. Stud.* 7, 272–281. doi: 10.5296/jas.v7i4.15404

Goharrizi, K. J., Meru, G., Ghotbzadeh, K. S., Heidarinezhad, A., and Salehi, F. (2021). Short-term cold stress affects physiological and biochemical traits of pistachio rootstocks. *South Afr. J. Bot.* 141, 90–98. doi: 10.1016/J.SAJB.2021.04.029

Govêa, K. P., Pereira, R. S. T., De Assis, M. D. O., Alves, P. I., Brancaglion, G. A., et al. (2020). Allelochemical activity of eugenol-derived coumarins on *Lactuca sativa* L. *Plants* 9, 533–550. doi: 10.3390/plants9040533

Hayat, S., Ahmad, H., Nasir, M., Khan, M. N., Ali, M., et al. (2020). Some physiological and biochemical mechanisms during seed-to-seedling transition in tomato as influenced by garlic allelochemicals. *Antioxidants* 9, 235–255. doi: 10.3390/antiox9030235

He, Z. B., Yao, L. H., Zhang, X. F., Li, Y., Wang, D. K., et al. (2020). Faba bean organs differed in their effects on maize seed germination rate and soil microbial activities as well as their decomposition patterns in a regosol soil. *J. Soil Sci. Plant Nutrit.* 20, 367–379. doi: 10.1007/s42729-019-00117-9

Huang, C. Z., Xu, L., Sun, J. J., Zhang, Z. H., Fu, M. L., et al. (2020). Allelochemical p-hydroxybenzoic acid inhibits root growth via regulating ROS accumulation in cucumber (*Cucumis sativus* L.). *J. Integr. Agricult.* 19, 518–527. doi: 10.1016/S2095-3119(19)62781-4

Kalmatskaya, O. A., Karavaev, V. A., and Tikhonov, A. N. (2019). Slow induction of chlorophyll a fluorescence excited by blue and red light in *Tradescantia* leaves acclimated to high and low light. *Photosyn. Res.* 142, 265–282. doi: 10.1007/s11120-019-00663-4

Kumari, J. A., and Prasad, P. R. C. (2018). Assessing the allelopathy and autotoxicity effects of *Parthenium hysterophorus* I., *Senna uniflora* (Mill.) H.S. Irwin and barneby and *Hyptis suaveolens* (L.) Poit. *Russ. J. Biol. Invas.* 9, 290–298. doi: 10.1134/S2075111718030086

Nasrollahi, P., Razavi, S. M., Ghasemian, A., and Zahri, S. (2018). Physiological and biochemical responses of lettuce to thymol, as allelochemical. *Russ. J. Plant Physiol.* 65, 598–603. doi: 10.1134/S1021443718040167

Ojija, F., Arnold, S. E. J., and Treydte, A. C. (2019). Bio-herbicide potential of naturalised *Desmodium uncinatum* crude leaf extract against the invasive plant species *Parthenium hysterophorus*. *Biol. Invas.* 21, 3641–3653. doi: 10.1007/s10530-019-02075-w

Otte, B. A., Rice, C. P., Davis, B. W., Schomberg, H. H., Mirsky, S. B., et al. (2020). Phenolic acids released to soil during cereal rye cover crop decomposition. *Chemoecology* 30, 25–34. doi: 10.1007/s00049-019-00295-z

Peng, X. B. (2011). Effect of the aqueous extract of J. Regia leaves on the seed germination and seedling enzymatic activity of S. Baicalensis in Shangluo. *Med. Plant* 2, 1–4. doi: 10.37376/1571-000-052-009

Ping, L., Yin, H. L., Zhong, Q. Y., Yan, P. X., Fei, T., et al. (2015). Effects of application of corn straw on soil microbial community structure during the maize growing season. *J. Basic Microbiol.* 55, 22–32. doi: 10.1002/jobm.201300744

Puig, C. G., Gonçalves, R. F., Valentão, P., Andrade, P. B., Reigosa, M. J., et al. (2018). The consistency between phytotoxic effects and the dynamics of allelochemicals release from *Eucalyptus globulus* leaves used as bioherbicide green manure. *J. Chem. Ecol.* 44, 658–670. doi: 10.1007/s10886-018-0983-8

Puig, C. G., Lorena, A., Reigosa, M. J., and Pedrol, N. (2013). *Eucalyptus globulus* leaves incorporated as green manure for weed control in maize. *Weed Sci.* 61, 154–161. doi: 10.1614/WS-D-12-00056.1

Qari, S. H., Khalil, A. H., Abdelfattah, N. A. H., and Shehawy, A. A. (2020). Effect of different plant extracts and nanoparticles on *Thrips tabaci* (Lind.) (*Thysanoptera: Thripidae*) under field conditions and their allelopathic potential on the onion, *Allium cepa* L. Using bioassays and RAPD analysis. *Egyp. J. Biol. Pest Cont.* 30:2161. doi: 10.1186/s41938-020-00216-1

Sendetsky, V. M. (2018). Growth and development of corn plants depending on the use of straw and green manure crops. *Agrology* 1, 281–285. doi: 10.32819/2617-6106.2018.13007

Song, Q., Qin, F., He, H., Wang, H., and Yu, S. (2019). Allelopathic potential of rain leachates from *Eucalyptus urophylla* on four tree species. *Agrofor. Syst.* 93, 1307–1318. doi: 10.1007/s10457-018-0240-8

Tang, S., Wang, Y. Q., Zhang, Z., Wu, L. M., Huang, J., et al. (2016). Effects of multi-year milk vetch application on weed seedbank density and biodiversity in paddy field. *Chin. J. Ecol.* 35, 1730–1736. doi: 10.13292/j.1000-4890.201607.031

Tao, J. M., Liu, X. D., Liang, Y., Niu, J. J., Xiao, Y. H., et al. (2017). Maize growth responses to soil microbes and soil properties after fertilization with different green manures. *Appl. Microbiol. Biotechnol.* 101, 1289–1299. doi: 10.1007/s00253-016-7938-1

Tesio, F., and Ferrero, A. (2010). Allelopathy, a chance for sustainable weed management. *Int. J. Sust. Dev. World Ecol.* 17, 377–389. doi: 10.1080/13504509.2010.507402

Utami, A. I., Bimantara, P. O., Umemoto, R., Sabri, R. K., Kautsar, V., et al. (2020). Incorporation of winter grasses suppresses summer weed germination and affects inorganic nitrogen in flooded paddy soil. *Soil Sci. Plant Nutrit.* 66, 389–397. doi: 10.1080/00380768.2020.1725914

Wang, X. X., Szeto, Y. T., Jiang, C. C., Wang, X. J., Tao, Y., et al. (2018). Effects of Dracontomelon duperreanum leaf litter on the growth and photosynthesis of Microcystis aeruginosa. *Bull. Environ. Contam. Toxicol.* 100, 690–694. doi: 10.1007/s00128-018-2289-5

Williamson, G. B., and Richardson, D. (1988). Bioassays for allelopathy: Measuring treatment responses with independent controls. *J. Chem. Ecol.* 14, 181–187. doi: 10.1007/BF01022540

Xiao, F., Zhao, Y., Wang, X. R., Liu, Q., and Ran, J. (2021). Transcriptome analysis of needle and root of Pinus massoniana in response to continuous drought stress. *Plants* 10, 769–782. doi: 10.3390/PLANTS10040769

Yadav, V., Singh, N. B., Singh, H., Singh, A., and Hussain, I. (2019). Putrescine affects tomato growth and response of antioxidant defense system due to exposure to cinnamic acid. *Int. J. Veg. Sci.* 25:110. doi: 10.1080/19315260.2018.1508110

Yang, B. J., Huang, G. Q., Xu, N., and Wang, S. B. (2013). Effects of different multiple cropping systems on paddy field weed community under long term paddy-upland rotation. *Chin. J. Appl. Ecol.* 24, 2533–2538. doi: 10.13287/j.1001-9332.2013.0501

Yang, Z., Zheng, S., Nie, J., Liao, Y., and Xie, J. (2014). Effects of long-term winter planted green manure on distribution and storage of organic carbon and nitrogen in water-stable aggregates of reddish paddy soil under a double-rice cropping system. *J. Integr. Agricul.* 13, 1772–1781. doi: 10.1016/S2095-3119(13)60565-1

Yousuf, T. A., Shanawaz, B. S., and Altaf, A. (2020). Low nitrogen stress regulates chlorophyll fluorescence in coordination with photosynthesis and Rubisco efficiency of rice. *Physiol. Mol. Biol. Plants* 26, 89–94. doi: 10.1007/s12298-019-00721-0

Zhang, C., Yu, C. J., Zhang, T. J., Guo, W. L., and Tian, X. S. (2021). Transcriptomic analysis reveals the transcription factors involved in regulating the expression of EPSPS gene, which confers glyphosate resistance of goosegrass (*Eleusine indica*). *J. Integr. Agricult.* 20, 2180–2194. doi: 10.1016/S2095-3119(21)63682-1

Zhang, J. Y., Zhou, Z. Z., and Ye, X. X. (2019). "Allelopathic effects of extracts from *Carex thunbergii* on three Gramineae species," in *The 9th symposium on allelopathy of plants in China*, Shaanxi.

Zhang, K. M., Shen, Y., Fang, Y. M., and Liu, Y. (2016). Changes in gametophyte physiology of Pteris multifida induced by the leaf leachate treatment of the invasive Bidens pilosa. *Environ. Sci. Pollut. Res.* 23, 3578–3585. doi: 10.1007/s11356-015-5589-x

Zhang, X. Q., Liang, Y. J., Zhu, K., Wu, C. X., Yang, L. T., et al. (2017). Influence of inoculation of *Leifsonia xyli subsp*. Xyli on photosynthetic parameters and activities of defense enzymes in sugarcane. *Sugar Tech.* 19, 394–401. doi: 10.1007/s12355-016-0479-1

Zhou, G. P., Cao, W. D., Bai, J. S., Xu, C. X., Zeng, N. H., et al. (2020). Co-incorporation of rice straw and leguminous green manure can increase soil available nitrogen(N) and reduce carbon and N losses:an incubation study. *Pedosphere* 30, 661–670. doi: 10.1016/S1002-0160(19)60845-3

Zhou, X., Liao, Y. L., Lu, Y. H., Rees, R. M., Cao, W. D., et al. (2020). Management of rice straw with relay cropping of Chinese milk vetch improved double-rice cropping system production in southern China. *J. Integr. Agricult.* 19, 2103–2115. doi: 10.1016/S2095-3119(20)63206-3

Zhou, X., Lu, Y. H., Liao, Y. L., Zhu, Q. D., Cheng, H. D., et al. (2019). Substitution of chemical fertilizer by Chinese milk vetch improves the sustainability of yield and accumulation of soil organic carbon in a double-rice cropping system. *J. Integr. Agricult.* 18, 2381–2392. doi: 10.1016/S2095-3119(18)62096-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Plant Science

REVIEW
published: 23 May 2022
doi: 10.3389/fpls.2022.794782

What Did We Learn From Current Progress in Heat Stress Tolerance in Plants? Can Microbes Be a Solution?

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Temperature is a significant parameter in agriculture since it controls seed germination and plant growth. Global warming has resulted in an irregular rise in temperature posing a serious threat to the agricultural production around the world. A slight increase in temperature acts as stress and exert an overall negative impact on different developmental stages including plant phenology, development, cellular activities, gene expression, anatomical features, the functional and structural orientation of leaves, twigs, roots, and shoots. These impacts ultimately decrease the biomass, affect reproductive process, decrease flowering and fruiting and significant yield losses. Plants have inherent mechanisms to cope with different stressors including heat which may vary depending upon the type of plant species, duration and degree of the heat stress. Plants initially adapt avoidance and then tolerance strategies to combat heat stress. The tolerance pathway involves ion transporter, osmoprotectants, antioxidants, heat shock protein which help the plants to survive under heat stress. To develop heat-tolerant plants using above-mentioned strategies requires a lot of time, expertise, and resources. On contrary, plant growth-promoting rhizobacteria (PGPRs) is a cost-effective, time-saving, and user-friendly approach to support and enhance agricultural production under a range of environmental conditions including stresses. PGPR produce and regulate various phytohormones, enzymes, and metabolites that help plant to maintain growth under heat stress. They form biofilm, decrease abscisic acid, stimulate root development, enhance heat shock proteins, deamination of ACC enzyme, and nutrient availability especially nitrogen and phosphorous. Despite extensive work done on plant heat stress tolerance in general, very few comprehensive reviews are available on the subject especially the role of microbes for plant heat tolerance. This article reviews the current studies on the retaliation, adaptation, and tolerance to heat stress at the cellular, organellar, and whole plant levels, explains different approaches, and sheds light on how microbes can help to induce heat stress tolerance in plants.

Keywords: climate change, heat stress, heat stress effect on plants, heat tolerant PGPRs, sustainable agriculture

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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *14 October 2021*
Accepted: *21 April 2022*
Published: *23 May 2022*

Citation:
Ahmad M, Imtiaz M, Shoib Nawaz M, Mubeen F and Imran A (2022) What Did We Learn From Current Progress in Heat Stress Tolerance in Plants? Can Microbes Be a Solution? Front. Plant Sci. 13:794782. doi: 10.3389/fpls.2022.794782

INTRODUCTION

Crop growth is the function of temperature, soil fertility, and water status; all at optimum satisfaction. Temperature is the key determining factor of the vegetation of a particular region (Argosubekti, 2020) and a balance between optimal temperature range and time of incidence is important to regulate plant growth. A slight change/ deviation in the atmospheric temperature, can disrupt normal biological, structural, and molecular processes in plants, which ultimately results in stunted growth and reduced yield (Argosubekti, 2020). Heat stress is an increase in environmental temperature (above the upper limit of threshold) for a certain period that can cause irreversible plant damage. The level of crop adaptation and productivity affected by heat stress, however, depends upon the temperatures and the phase of plant development.

The onset of industrial revolution and massive mechanization has shown a gradual increase in CO_2 emissions (30–150%) and greenhouse effect over the past 250 years (Friedlingstein et al., 2010) leading to a persistent rise in temperature during the last two centuries. The global land and ocean surface temperature increased by around 0.85°C from 1880 till 2012 and an annual rise of at least 0.2°C per decade is further anticipated (Change, 2014) while the greenhouse gases will further add 1.1–1.5°C to it. The National Aeronautics and Space Administration (NASA) released a comparative data of average global temperature from with a baseline of 1951–1980 (**Figure 1**) showing that average monthly temperature has increased $>1°C$ during the past decade (2000–2020) and $>1.5°C$ during the past 5 years (2015–2020). With 1.5–2.0°C change in the global mean temperature, the possibility of suffering from record-breaking, high-impact extreme climate disasters increase significantly, which ultimately cause a significant yield reduction and food supply chain around the globe (Chen et al., 2018; Kong et al., 2018). The IPCC (Intergovernmental Panel on Climate Change) reports that the world's major staple crops and food production have a great impact of climate change (Easterling et al., 2007; Porter et al., 2014) where an increase of one Celsius atmospheric temperature costs a decrease of 6% in crop yield (Asseng et al., 2015). In China, heat stress in the last decade resulted in rice yield losses of 5.18 million tons (Tian et al., 2009) while in South-East Asia upto 14%. In wheat, about 7 million ha in developing countries and 36 million ha in the temperate region had been affected by heat stress in 2001 (Reynolds et al., 2001) causing a yield reduction of 19 million tons (Lobell and Field, 2007) while USDA (United States Department of Agriculture) reported ≈5.5% yield reduction in wheat (Lobell et al., 2011).

Agricultural and global food production is one of the most fragile sectors of society prone to climate uncertainty and changes (Meinke et al., 2006). Detailed multi-locational field analyses have shown that heat and drought stress are more closely linked. Increased heat imposes the drought stress hence, both stresses should be considered together rather than dealing individually. Zia et al. (2021) highlighted the impact of drought along with possible eco-friendly strategies to improve plant growth under drought stress, but the eco-friendly heat-stress management strategies is urgently needed. The plants are substantially affected by high day, low night or high air and surface temperatures. Each cultivar has a temperature optima (threshold) for a significant vegetative and reproductive growth (Zinn et al., 2010; Hatfield and Prueger, 2015) while growth ceases above or below the threshold (Kaushal et al., 2016). The lower threshold temperatures may vary from plant to plant, but is usually lower for most of the tropical, temperate, and cold season crops (Miller et al., 2001). Exposure to high temperature, e.g., 35°C significantly decreases pollen viability (Dupuis and Dumas, 1990), replication of amyloplast and cell division and growth rate ultimately decreasing the size and the total number of the grains (Commuri and Jones, 2001; Rangan et al., 2014). **Table 1** describes in detail the growth destructive temperatures of different crops. The higher degree of heat stress (45°C or above) disrupts cellular homeostasis, induces extreme growth retardation, and severely affects the biological activity of proteins due to aggregation or misfolding rendering cells unable to defend themselves (Sarkar et al., 2013; Reddy et al., 2016). The deposition of improper-folded proteins may be permanent that alter the functioning of the cells.

The heat stress causes direct and indirect damage to multiple plant functions resulting in morphophysiological changes, hampering different growth phases and metabolic processes (Wu et al., 2018) and ultimately yield reduction (Mcclung and Davis, 2010; Grant et al., 2011). The detail the impact of heat stress on various plant functions at multiple levels has been described in **Figure 2**. The direct effect includes; protein denaturation and misfolding, increased membrane fluidity, the inactivation of chloroplast and mitochondrial enzymes, inhibition of protein synthesis and degradation, and loss of membrane integrity (Howarth, 2005). Indirect impact includes the changes in the pathogen behavior and disease pattern. Each pathogen has an optimum temperature for its replication and virulence (Velásquez et al., 2018), e.g., *Globodera pallida* nematode infect potato at 15°C (Jones et al., 2017), *papaya ringspot virus* (*PRSV*) infect papaya at 26–31°C (Mangrauthia et al., 2009). It has been predicted that many diseases will migrate into new geographical areas as temperatures rise, where they will encounter new hosts (Etterson and Shaw, 2001), cause severe and frequent epidemics (Ma et al., 2015), improve their survival under heat or desiccation, or become dormant for many years (Turkensteen et al., 2000; Ritchie et al., 2013). Change in the temperature also changes the pathogen behavior (Roberts et al., 2018), e.g., new pathogen strains adapted to high temperatures are being reported which are more active, more virulent, and widely transmitted worldwide (Hovmøller et al., 2008; Milus et al., 2009), e.g., *Phytophthora infestans* (cause late blight in potato and tomato) (Mariette et al., 2016) and *Puccinia striiformis* (rust fungus in wheat). Even pathogens can cause an outbreak by transient variations in temperature, e.g., soybean rust can be developed even after 1-h of exposure at 37°C, although, the optimum temperature for disease development is 12–25°C (Bonde et al., 2012).

Plant endophytic bacteria have a symbiotic relationship with their host plant, dwelling within it for the bulk of their life cycle and having the capacity to colonize the plant's interior tissues *via* penetrating the seed and root.

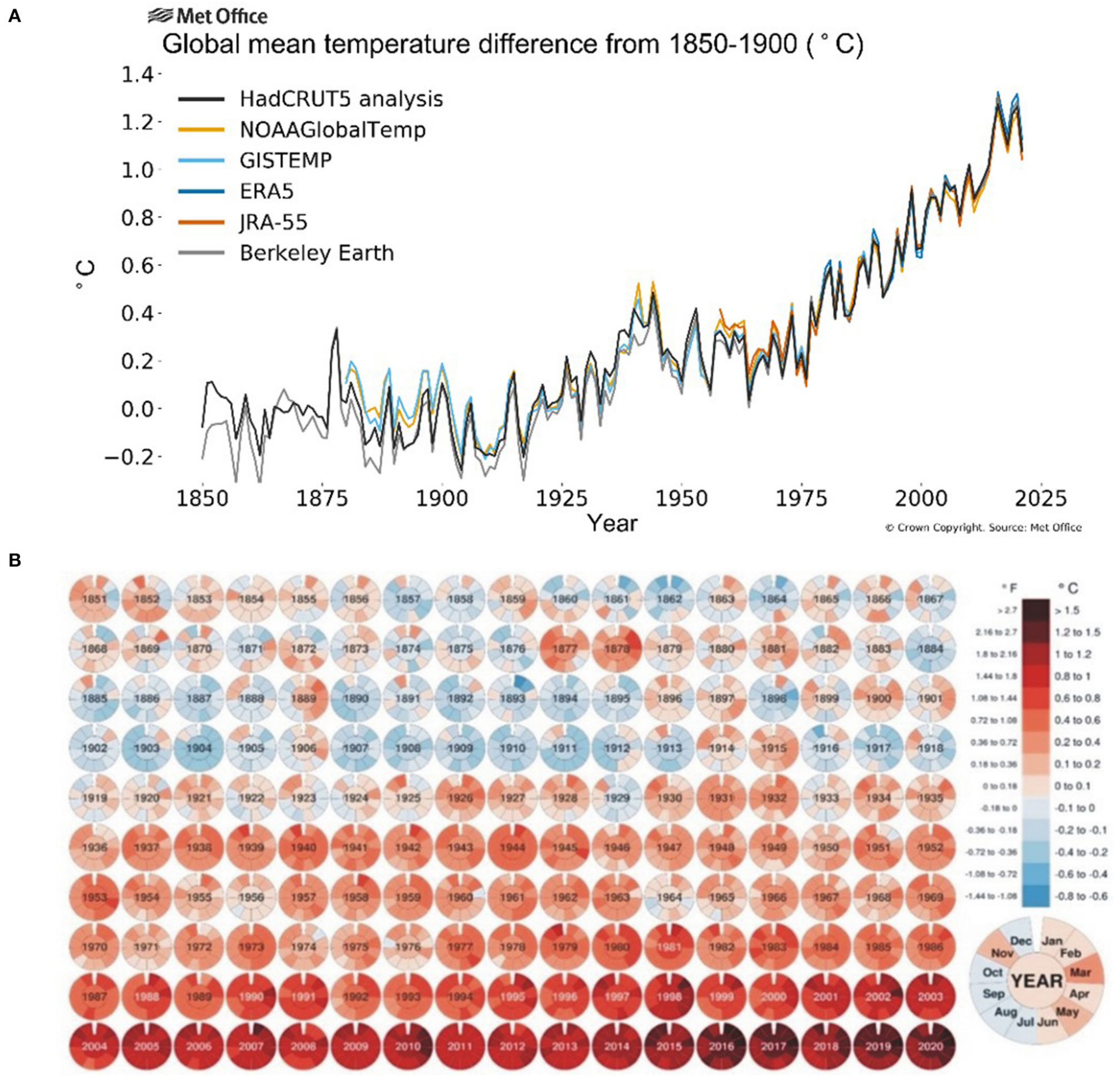

FIGURE 1 | Average global temperatures from 1850 to 2025 compared to a baseline average from 1850 to 1900 **(A)** source: Berkeley Earth (2019); and monthly global mean temperature 1851–2020 compared to 1850–1900 averages **(B)** source: Visual Capitalists (2019).

These endophytic microbiomes fixed the atmospheric nitrogen, produced phytohormones, solubilized the inorganic phosphorus, zinc, potassium and calcium, produced exopolysaccharides and iron chelating siderophore compounds under heat stress condition which ultimately enhance the plant growth (Hakim et al., 2021). On the other hand, there are also some beneficial fungi, i.e., arbuscular mycorrhiza fungi (AMF) that is also help plants under heat stress condition. These AMF form symbiotic relationship with plant roots and improve photosynthetic process, increase nutrients uptake, secondary metabolites accumulation, regulate the oxidative defense system, and maintain the osmotic balance in plants (Begum et al., 2019). Plant genetic engineering and maker assisted selection breeding and multiple approaches have been published multiple reviews on heat stress and their management which are costly and time consuming. On the other hand, PGPR is cost-effective, eco-friendly, and ecologically safe solution to induce heat stress tolerance. So, current review will give a quick rundown of the current knowledge about the methods and pathways that PGPR use to promote tolerance in plants under heat stress. It will also

TABLE 1 | Growth destructive temperature of different crops.

Plants	Exposure time	Destructive temperature (°C)	References
Cereal crops			
(*Triticum aestivum*) Wheat	10–15 min	45–50	CIMMYT, 2020
(*Zea mays*) Maize, Corn	10	49–51	Argosubekti, 2020
(*Oryza sativa*) Rice	10–15 min	38–45	Sarsu, 2018
Cash crops			
(*Gossypium hirsutum)* Cotton	30 min	40–45	Cotton Info, 2018
(*Saccharum officinarum*) Sugarcane	20 min	50–55	Damayanti and Putra, 2010
Oilseed crops			
(*Brassica napus*) Rapeseed	10 min	49–50	Argosubekti, 2020
(*Brassica juncea)* Mustard	7 days	40–45	Argosubekti, 2020
Vegetables			
(*Solanum tuberosum*) Potato	1 h	42.5	Argosubekti, 2020
(*Allium cepa*) onion	–	30–35	Ikeda et al., 2019
(*Cucurbita pepo*) Squash	10 min	49–51	Argosubekti, 2020
Fruits			
(*Citrus aurantium*) Sour orange	15–30 min	50.5	Argosubekti, 2020
(*Vitis vinifera*) Grape	–	65	Argosubekti, 2020
(*Solanum lycopersicum*) Tomato fruits	–	45	Argosubekti, 2020

discuss the heat stress response factors and genes and how PGPR regulate the expression of such genes to modify plant response under heat stress.

PLANT RESPONSE TO HIGH TEMPERATURE

The response of plants toward heat stress varies with the degree and duration and the plant's developmental stage (Ruelland and Zachowski, 2010). These heat stress disorders may be recurrent or persistent in nature or both, therefore, plants have evolved different mechanisms to cope with them (Bäurle, 2016). The heat stress response (HSR) is an evolutionarily conserved mechanism that describes the plant's adaptation and induction of thermotolerance by either by activating the defense mechanisms to stop the disruption at the transcriptomic level in the cell or by the activation of heat shock proteins (HSPs), epigenetic pathways, and DNA methylation (Lämke and Bäurle, 2017). **Table 2** describes few major effects of heat stress on plants.

Morphological Response During Early and Reproductive Growth

Of all the growth phases, seed germination is the most adversely affected phase by heat stress, but, the response varies in different crops or even within varieties (Sita et al., 2017). The germination is significantly reduced above 45°C because of denaturation and embryonic cells (Cheng et al., 2009), along with severe impact on seedlings potency, the development of radicle and plumule, and seedling growth (Toh et al., 2008; Sita et al., 2017). At later stage, the effect varies with the time, length, and severity of stress (Fahad et al., 2016) but mainly heat stress decreases the cell water contents, the cell size, the plant size, growth and biomass, net assimilation rate (NAR) and relative growth rate (RGR) (Ashraf and Hafeez, 2004; Wahid and Close, 2007). Other visual symptoms include leaves and twigs scorching, growth inhibition, damaged leaves, early leaf senescence, discoloration of fruits/plants (Vollenweider and Günthardt-Goerg, 2005), reduced number of floret and spikes (Prasad et al., 2006; Fahad et al., 2016), decrease in the internodal length (Siddiqui et al., 2015; Gray and Brady, 2016), plant height, total biomass, and the number of panicles (Modarresi et al., 2010). At the reproductive stage, one degree rise in temperature is detrimental because it degenerates mitochondria and proteins, loss of seed mass, quality, viability and vigor (Nahar, 2013; Balla et al., 2019).

Physiological Responses

Water and Nutrient Uptake

The heat usually correlates with water because it cause dehydration in plant tissues especially in tropical and subtropical ecosystems (Giri, 2013; Giri et al., 2017). High temperature is lethal under both insufficient (Giri et al., 2017) or sufficient soil water because it affect the water conductivity and root permeability (Giri, 2013) which ultimately restrict the water and nutrients supply to the plant causing starvation and reduced growth (Wahid et al., 2007; Huang et al., 2016). The exact basis of the crop nutrient relationships under heat is not clearly known but overall, it lowers the photosynthesis and water viscosity and the activity of enzymes, e.g., nitrate reductase that is involved in the breakdown of nutrients, recycling, absorption, and accessibility to the plants (Kumarathunge et al., 2019).

TABLE 2 | Effect of heat stress on different stages of plant growth in major cereals.

Plants	Developing stage	Temperature	Major effect	References
(*Triticum aestivum*) Wheat	60 days after sowing	30/25°C day/night	Reduce leaf size, elongation of booting stage, heading stage, pollination process.	Djanaguiraman et al., 2010
	Maturity and Grain filling	37/28°C (day/night), 20 days	Decrease the number of spikelets and reduce final yield. Early maturation and grain filling. Decreased grain weight.	Rahman et al., 2009
	Vegetative stage	25–42.5°C	Reduce CO_2 concentration within plants.	Djanaguiraman et al., 2011
(*Oryza sativa*) Rice	Panicle stage	Above 33°C, 10 days	Reduced the rates of pollen and spikelet fertility.	Hurkman et al., 2009
	Reproductive stage	32°C (night temperature)	Enhance pollen sterility, decrease grain size and weight which leading to yield reduction.	Suwa et al., 2010
(*Zea mays*) Maize, Corn	Reproductive stage	35/27°C (day/night), 14 days	Suppress the production of cellulose and hemicellulose which leading to a reduction in photosynthate supply in plants	Yin et al., 2010
	During pre-anthesis and silking onwards	33–40°C, 15 days	Decrease plant growth and ear formation rate.	Zhang et al., 2013

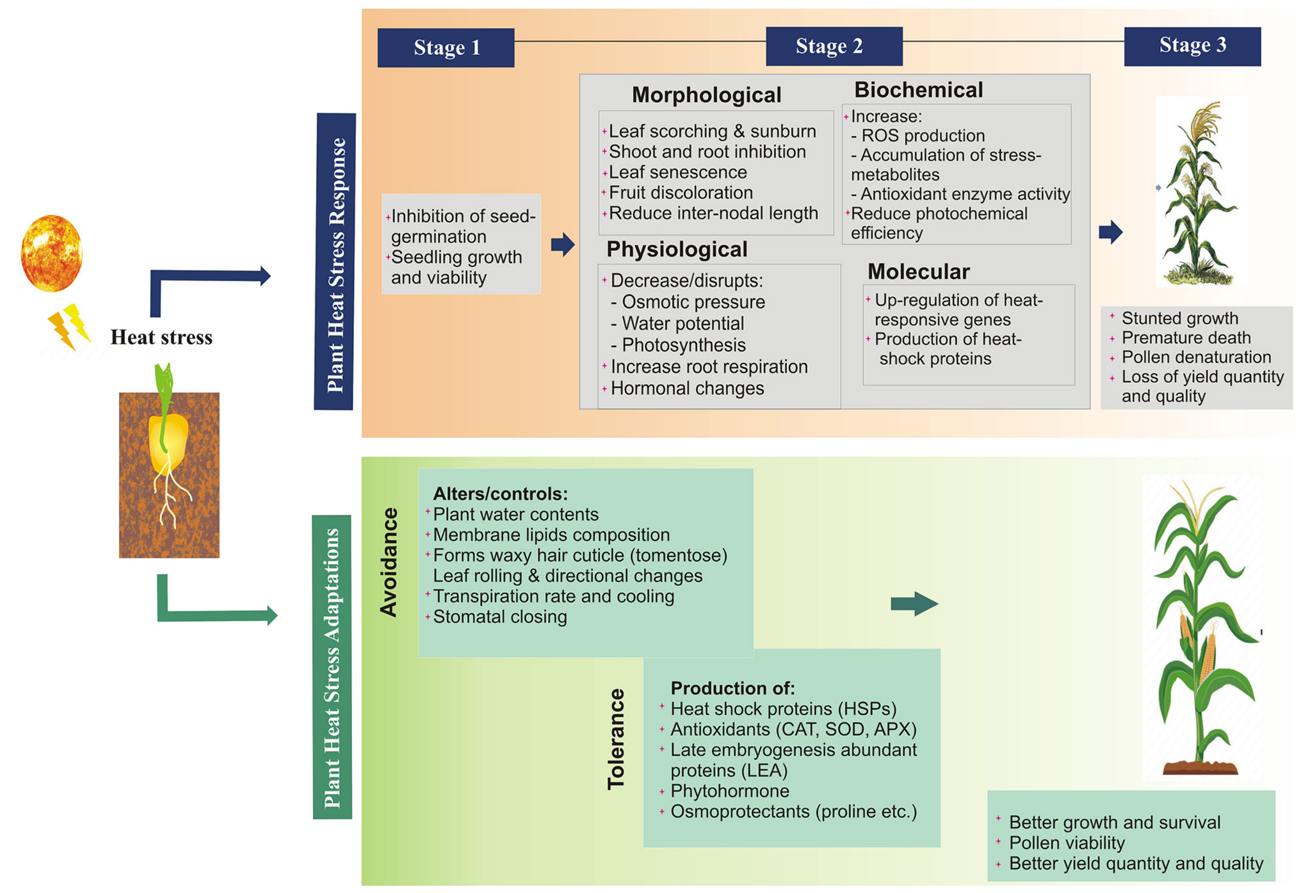

FIGURE 2 | Heat stress responses and adaptation mechanisms in plants at different levels.

Photosynthesis

Photosynthesis is among the most thermosensitive pathways in plants which is adversely affected due to the heat-induced reduced water contents, numbers of leaves, premature senescence and cell death (Hu et al., 2020). The major impact is the heat-induced injury to the photosynthetic machinery, i.e., stroma and thylakoid lamellae (Kmiecik et al., 2016; Sun and Guo, 2016). This disruption inhibits the thylakoid activity and the functional efficiency of photosystem II (Morales et al., 2003). Other plant processes that hamper photosynthesis under thermal stress include the reduction of photosynthetic pigments (Marchand et al., 2005), leaf moisture, transpiration rate, low CO_2 concentration and supply to photosystem due to stomatal closure (Ashraf and Hafeez, 2004), reduction of amino acids, rubisco binding proteins (Li et al., 2018), carbohydrate depletion and plant malnutrition due to reduced activity of ADP-glucose pyro-phosphorylase, sucrose phosphate synthase, and invertase (Djanaguiraman et al., 2009). The reduction in chlorophyll pigment is linked with the activity of thylakoid membranes, lipid peroxidation of chloroplast, and reduced photochemistry (F_v/F_m ratio) of photosystem II, which ultimately reduce the overall photosynthesis in crop plants (Mohammed and Tarpley, 2010). Reduction of chlorophyll may be due to the reduced (about 60–90%) biosynthesis under high or low temperature, or increased chlorophyll-pigments degradation, or an accumulative effect of the two (Hemantaranjan et al., 2014).

Oxidative Damage

Heat exposure stimulates oxidative stress due to the generation of activated, extremely reactive, and toxic oxygen species. The reaction of reactive oxygen species (ROS) includes supper oxide radical (O_2^-) singlet oxygen (O_2), hydroxide ions (hydroxyl) radical (OH^-), and hydrogen peroxide (H_2O_2) (Marutani et al., 2012; Suzuki et al., 2012). ROS causes lipid membrane to lose the ability to regulate substance exchange across the cell membrane (Suzuki et al., 2012). Oxidative stress enhance peroxidation and further damage the proteins, lipids, carbohydrates, and DNA contributing to premature aging (Savicka and Škute, 2010). Continuous thermal stress increases the ROS deposition in the plasma membranes with cell membrane depolarization, which leads to the activation of the RBOHD enzyme (respiratory burst oxidase homolog protein-D) that produces ROS and initiates programmed cell death signal (Mittler et al., 2011). The elevated temperature raises the O_2 content in the root by 68%, and leaf malondialdehyde (MDA) content by 27% at the early and 58% at later stages (Medina et al., 2021). Though, the plant has a special mechanism to avoid unnecessary reactive oxygen species (ROS) by the production of specific antioxidants.

Respiration

Heat stress affects mitochondrial functions by influencing respiration. The respiration often rises with increasing temperature, but at a certain duration of photoperiod, the respiration process declines due to damage to the respiratory system (Prasad et al., 2008; Rasmusson et al., 2020). In the heat-prone varieties under heat stress (35/25°C day/night) the respiration rate in the flag leaf of wheat was considerably higher compared to that of the control (23/18°C day/night) (Akter and Islam, 2017). The solubility of O_2 and CO_2 as well as Rubisco's kinetics, loss of respiratory carbon due to heat enhances the ROS and reduces ATP production and respiration in plants (Cossani and Reynolds, 2012).

Crop Yield Response

HS induces significant yield reduction mainly due to decrease in the number, size and quality of grain, starch synthesis and accumulation, protein concentration, pod, fiber content, and breakdown of the Ca content (van Es, 2020). HS usually accelerates the rate of grain-filling but shortens the grain-filling time leading to significant decrease in grain length, width, and weight, grain quality (Högy et al., 2013; Lamaoui et al., 2018) reduced accumulation of storage compounds (Hurkman et al., 2013) and increased male sterility (Suwa et al., 2010). HS also increase proteinogenic amino acid and maltose content and decrease the concentrations of starch, sugars, raffinose, sucrose and lipids (Vasseur et al., 2011). A slight increase in temperature (1–1.5°C) reduces the harvest index and yield up to 10% in different crops (**Table 3**) (Tubiello et al., 2007; Ahamed et al., 2010; Hatfield et al., 2011). Heat-susceptible cultivars show more yield losses compared with thermo-tolerant cultivars (Ahamed et al., 2010; Hussain et al., 2019).

PLANT HEAT-ADAPTATION STRATEGIES

Plant HS adaptation involves a variety of strategies and various processes including basal heat tolerance (BHT), acquired heat tolerance (AHT), and avoidance (Fitter and Hay, 2012; Bäurle, 2016). *BHT* is the natural capacity of plants to tolerate heat while *AHT* (also called priming or acclimation), is acquired tolerance *via* short pre-exposure to heat (Yeh et al., 2012). A sudden heat exposure results in short-term reaction, i.e., leaves orientation, osmotic modification, evaporation, and adjustments in cell membrane structure (Bäurle, 2016; Zhongming and Wei, 2021) whereas, under long-term exposure, multiple adaptation mechanisms work synergistically to minimize the impact of heat stress. Heat tolerance involves the activation of some important immunity pathways, including ions transporters, proteins late embryogenesis abundant (LEA), phytohormones, antioxidant-resistance, and factors implicated in transduction signaling and transcriptional control (Li, 2020; Li et al., 2021). AHT is distinguished between short-term acquired tolerance (SAT) and long-term acquired tolerance (LAT) and establish a molecular stress memory state that protect themselves from acute heat damage and death (Sani et al., 2013). The stress-sensitive feature is characterized by an early signal that can be in the form of anionic and osmotic effects or alterations in membrane fluidity which restore homeostasis and sustain defective proteins and membranes (Kumarathunge et al., 2019). Plants HS *avoidance* includes long-lasting developmental morphological and physiological adaptations or short-lived accommodation strategies (**Figure 2**), e.g., intensive transpiration from leaves prevents damage by lowering the temperature of the leaves by 6°C or even 10–15°C than the normal temperature (Fitter and Hay, 2012; Li, 2020). Other plant varieties have special features that enable them to escape from warm conditions, e.g., having heat-sensitive buds, leaf abscission, annual desert buds, and by completing the entire regenerative period during the winter (Fitter and Hay, 2012). These adaptations are correlated to each other and increases the net process of photosynthesis at HS (especially for C4 and CAM plants) (Sarieva et al., 2010; Li, 2020).

Antioxidant System

Plants have evolved a series of detoxification systems to fight against oxidative damage. These systems include peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) which show mitigating effects against different kinds of stress including HS (Suzuki et al., 2012; Caverzan et al., 2016). These antioxidants prevent the excessive free radicals and repair the damage effects that function as a catalyst to change the dismutation reaction from SOD anion to H_2O_2 and O_2 molecules. They also affect other physiological phenomena including biosynthesis of lignin, enzyme catabolism, defense against injury and insect/pathogen attack, and physiological damage caused by temperature stress (Wani et al., 2016; Devireddy et al., 2021).

Production of Metabolites and Hormones

The production of a wide range of metabolites of low molecular mass including soluble carbohydrates, amino acids, variety of sugars, and sugar alcohols and phenolics have been linked to stabilization of cellular membranes, protection of protein structures, maintenance of the cell water balance, and buffering of the cellular redox potential under abiotic stress in plants (Kumarathunge et al., 2019). Accumulation of different osmoprotectants (e.g., proline, glycine betaine, etc.) and carotenoids (e.g., xanthophlls, terpenoids, etc.) under extreme temperature participate directly in the osmotic adjustment and show positive correlation with more negative leaf osmotic potential and production of protective pigments (Wani et al., 2016; Li et al., 2021). Glycine betaine maintains the activity of Rubisco by preventing its thermal inactivation (Devireddy et al., 2021). Sucrose and other carbohydrates act as antioxidant (Lang-Mladek et al., 2010; Devireddy et al., 2021) as well as regulate carbon allocation and sugar signaling consequently protecting the pollen viability. Plant growth regulators are also involved in enhancing plants' ability to tolerate stress, e.g., ABA, SA, IAA or CK (Ding et al., 2010; Hsu et al., 2010; Devireddy et al., 2021).

TABLE 3 | Yield losses reported in different crops due to heat stress.

Plants	Yield loss (%age)	References
Cereal crops		
(*Triticum aestivum*) Wheat	18–30	Balla et al., 2011; Djanaguiraman et al., 2020; Dubey et al., 2020
(*Zea mays*) Maize, Corn	42	
(*Oryza sativa*) Rice	50	Li et al., 2010; Da Costa et al., 2021; Xu et al., 2021
Cash crops		
(*Gossypium hirsutum*) Cotton	50	Zafar et al., 2018; Majeed et al., 2021
(*Saccharum officinarum*) Sugarcane	20–40	Zhao and Li, 2015; Hussain et al., 2018
Vegetables		
(*Solanum tuberosum*) Potato	12–35	Rykaczewska, 2015; Momčilović, 2019
(*Allium cepa*) onion	20–50	Kandil et al., 2011; Ratnarajah and Gnanachelvam, 2021

Heat Shock Factors, Proteins and CRISPR Technology

Many stress-inducible transcriptions factors (known as heat shock factors; HSF, e.g., Hsf6A, DREB1A, OsMYB55), stress-related genes, and proteins (HSP) are synthesized and overexpressed to induce thermotolerance (Lamaoui et al., 2018). The HS response is preceded by HSF that acts further on the transcription of HSP mRNA. HSFs are components of a complex signaling system that control responses not only to high temperatures but many other abiotic stresses. They are transcriptional activators of HS genes and bind specifically to heat shock sequence elements throughout the genome. **Figure 3** describes in detail the mechanism of HSP/HSF activation under heat stress in the plant cell. A rapid increase in temperature up to 5–10°C, causes the plant to trigger the "heat-responsive genes" which translate into a special protein called heat shock proteins (HSPs). HSPs protect the plants by activating the chaperons and proper folding of proteins, inhibit the denaturation and aggregation of intercellular proteins and maintain their function by proper folding of proteins (Baniwal et al., 2005). HSPs are miscellaneous, highly diverse and normally present in living cells but under heat stress, different small and large HSPs are produced which help in (i) folding of newly synthesized proteins (Hsp60 and Hsp70), (ii) translocation the proteins between the cellular membrane and from organelle to organelle (Hsp70), (iii) prevent from deterioration, self-aggregation, improper folding, and the production of the polymeric compound of proteins (sHsp, Hsp70, Hsp90, Hsp100), (iv) the breakdown of defective proteins by proteolytic (Hsp70, Hsp100), (v) activation of signaling molecules, transduction, transcriptional factors and transcription (Hsp70, Hsp90), (vi) production of oligomeric complexes of high molecular weight that act as a cellular matrix for unfolded protein stabilization (Hsp20 or small Hsp (sHSP) (Hasanuzzaman et al., 2013).

CRISPR/Cas9 is a genome editing tool, widely use to edit the eukaryotic genome specially in plants The transient expression of this system is very useful to activate or limit the desired targeted gene in plants (LeBlanc et al., 2018). Recently, the scientist explained the heat-shock inducible CRISPR/Cas9 system for genome editing and demonstrate its effectiveness in rice. They combined the promoter of heat shock protein of rice *U3* and soybean to induce the HS-CRISPR/Cas9 system *via* Cas9 and single guider RNA (Nandy et al., 2019). In another study, the Enhanced response to ABA1 (ERA1) gene present in *Arabidopsis thaliana* was targeted, that encode the farnesyltransferase and upregulate the abscisic acid (ABA) signaling under heat and drought stress. By using CRISPR/Cas9 system, they knock in the *ERA1* gene into rice genome and enhanced the ABA in rice under heat and water deficit condition (Ogata et al., 2020).

MANAGING AGRICULTURE UNDER HEAT STRESS

By proper agronomic management, such as water, the methods and quantity for fertilization, the sowing time and method, the addition of protectants, plants can be grown effectively under heat stress (Ortiz et al., 2008). The usual practices are as follows:

- *Mulching* is done to preserve optimum moisture in soil, because it reduces soil evaporation (Chen et al., 2015), improves nitrogen and water efficiency (Singh et al., 2011), prevents yield loss (Chakraborty et al., 2008; Głab and Kulig, 2008) in temperate and tropical regions under thermal stress (Akter and Islam, 2017).
- Proper *sowing time* is very important for crops as early or late plantation shortens the heading and maturity period, improves pathogen infestation, eventually affecting economic yield and grain quality (Khichar and Niwas, 2007; Al-Karaki, 2012; Hakim et al., 2012; Hossain and Da Silva, 2012).
- Increasing *crop nutrition* (e.g., N, Ca, K, Mg, B, Se, and Mn) alter the stomatal function, activate physiological and molecular processes, improve tissue water potential, decrease ROS toxicity by increasing the antioxidant concentration stimulate heat stress tolerance (Waraich et al., 2012; Mengutay et al., 2013). The post-anthesis foliar application also improve grain proteins, slows down the ABA synthesis, improves cytokinin production, stimulates the photosynthesis and accumulation of assimilates (Dias and Lidon, 2010; Singh et al., 2011).

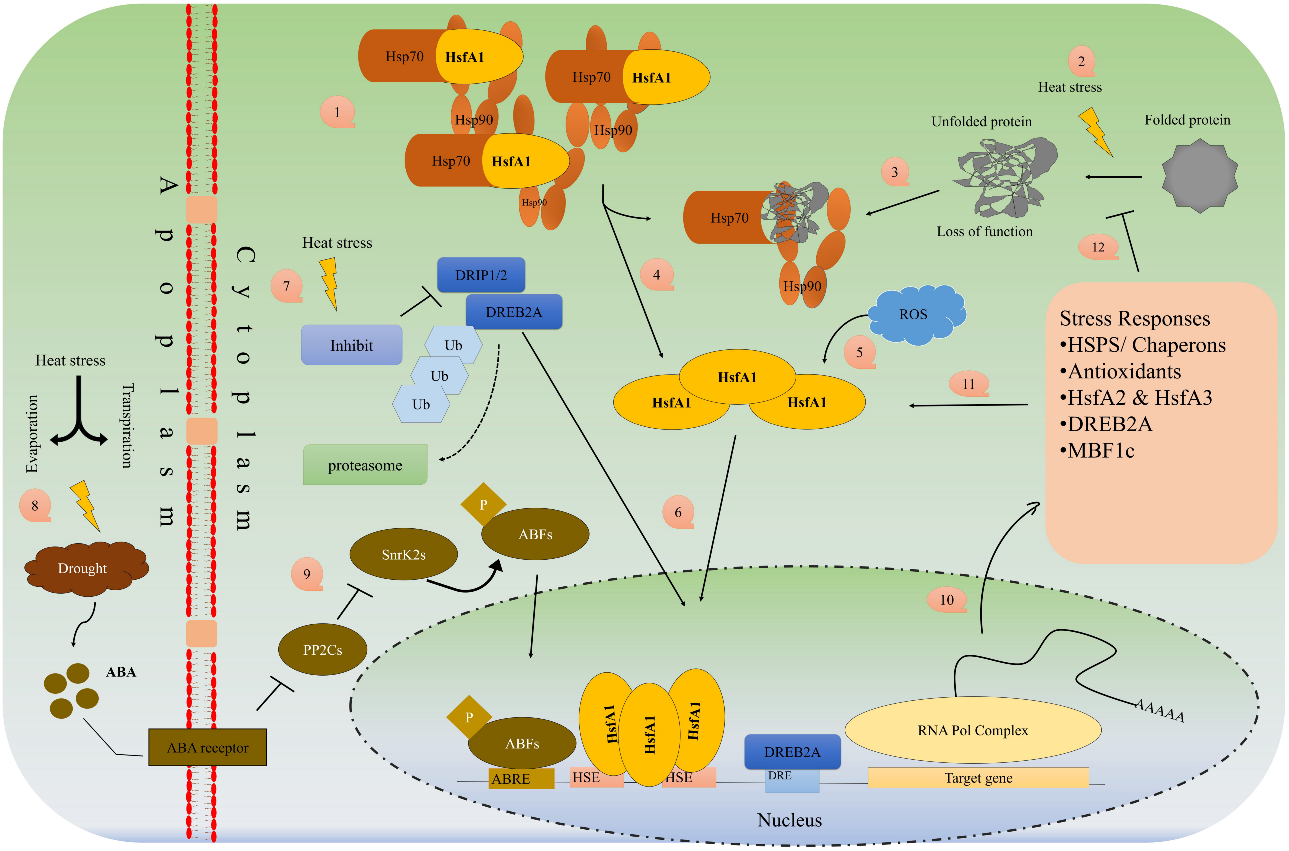

FIGURE 3 | Schematic overview of HSP/HSF's pathway activation under heat/drought stress.

- The exogenous application of *growth promoters* [e.g., phytohormones, osmoregulatory, receptor molecules, polyamines along with spermidine, putrescine, spermine, putrescine, alpha-tocopherol (vitamin E), arginine etc.] regulate the ROS and enhance the efficiency of the antioxidant thus protect plants under HS (Farooq et al., 2011; Sharma and Chahal, 2012; Hemantaranjan et al., 2014; Uprety and Reddy, 2016).
- Exogenous *calcium application* activates MDA that enhances the activity of guaiacol peroxidase, CAT, SOD, which ultimately induce heat resistance in plants. It can also protect chlorophyll from solar radiation destruction and sustain stomatal functioning (Dias and Lidon, 2009; Waraich et al., 2012). Activation of different enzymatic, e.g., CAT, SOD, ascorbate peroxidase (APX), and non-enzymatic antioxidants, e.g., tocopherol, and ascorbic acid have a significant effect on oxidative management under multiple stresses (Balla et al., 2007).
- Plant treatment with reactive short-chain leaf volatiles (*RSLVs* also known as oxylipins; a group of C4–C9 straight chain carbonyls categorized by an alpha, β-unsaturated carbonyl bond) show high thermotolerance (Yamauchi, 2018).
- Co-polymers of Poly (N-isopropyl acrylamide) (Dimitrov et al., 2007) or poly acrylic acid (PAA) have been used as thermo-tolerant nanoparticle to transport specific chemicals within the plant to develop resistant against HS (Xu et al., 2015).
- The use of natural *bio-stimulants*, e.g., amino acids, microorganisms, fruit extracts, seaweeds, inorganic materials, and chitin or engineered, e.g., plant hormones, phenols, salts, chemical substances, and other elements with plant regulating properties (Calvo et al., 2014; Van Oosten et al., 2017).
- Transfer of heat shock regulatory proteins (HSFs, HSPs) or development of heat shock transcriptional elements (HSEs) enhance gene expression within minute and induced heat tolerance in plants.

MICROBE THERAPY FOR HEAT TOLERANCE

Plants are home to large collection of microbes collectively known as plant microbiome (Liu and Tan, 2017; Álvarez-Pérez et al., 2019) which is highly dynamic in nature and show significant shift in the composition in response to external stimuli and environmental stresses, e.g., heat (Santos-Medellín et al., 2017; Timm et al., 2018). This shift is not a passive plant reaction, but a deliberate response because both plants and microbes have been coevolved since millions of years (Durán et al., 2018; Kwak et al., 2018). Plants use precise combinations of chemical stimuli under abiotic, or pathogen-induced stress which trigger specific microbes to overexpress particular proteins or enzymes for inducing stress tolerance (Bakker et al., 2018; Kumar and Verma, 2018; Liu and Brettell, 2019; Hakim et al., 2021). Overall, the plant microbiome controls nutrient availability, root growth, plant yield and modulates resistance to stresses (Chialva et al., 2018; Lu et al., 2018).

The microbial application (inoculation) is cheap, eco-friendly, low-input, and time-saving strategy as compared to the development of stress-tolerant crop variety or germplasm screening (Shrivastava and Kumar, 2015). Plant growth-promoting microbes (PGPM) require optimum condition for maximum efficiency (e.g., production of phytohormones, nitrogen fixation, and solubilization of nutrients P, Zn, Ca, Fe, etc.). Even though stress factors (salinity, drought, heat, and heavy metals) decrease their efficacy, but some PGPR modify themselves for maximum efficiency under stress. Species of *Enterobacter, Acetobacter,* and *Pseudomonas* solubilize the phosphorus 74%, 75%, and 80% respectively at normal (30–32 ± 2°C) and high temperature (38–40 ± 2°C) (Kachhap et al., 2015). Similarly, the *Acetobacter* spp. produced 100%, *Enterobacter* spp. produced 82% and *Pseudomonas* spp. produced 50% IAA both at high and low temperatures. While high temperature reduced the efficacy of nitrogen fixation in rhizobia. High temperature reduced the symbiotic relationship between plant roots and microbes which ultimately decreased the rate of nitrogen fixation. Heat stress inhibits microbial adherence on root hair and root nodules (Alexandre and Oliveira, 2013) and also disturb the molecular signals between microbes and root hair which leads to forming a weak symbiotic relationship between partners (Sadowsky, 2005). However, some rhizobial species from *Acacia* have thermotolerance up to 44°C which enable them to fix nitrogen at high temperature and give benefit to plant (Zahran et al., 1994).

PGPM are stable under chilling and heat stress (Barka et al., 2006; Ali et al., 2011) but the molecular and physiological changes connected to this stress management are poorly understood. Inoculation studies reports that systemic effects are involved during heat/chilling stress that change metabolic and regulatory function of plant supporting both growth and stress management (Abd El-Daim et al., 2019). **Table 4** describes the role of PGPR in plant under stress condition. Decrease in the ROS production by seed treatment with *Bacillus amyloliquefaciens* and *Azospirillum brasilense* has also been reported under HS (Abd El-Daim et al., 2014). A recent review published the meta-analysis of microbe-mediated thermotolerance in plants and their mechanisms from 39 published research articles (Dastogeer et al., 2022). They have reported a significant decrease in accumulation of MDA and H_2O_2 in colonized plants showing lower oxidation activity but a corresponding increase in the activities of catalase, peroxidase, glutathione reductase under heat stress. However, the activities of SOD, ascorbate oxidase, ascorbate peroxidase and proline were variable. The overall impact of microbial colonization was more pronounced under heat stress.

Mechanistic Interpretation of Microbe Therapy

A variety of PGPM have been known to induce heat stress using various mechanisms (Dodd and Pérez-Alfocea, 2012; Ramadoss et al., 2013). For instance, *Pseudomonas putida* produces heat shock protein that plays a vital role in increasing plant thermal tolerance (Ali et al., 2011) (**Figure 4**). Many of PGPM activate structural changes in plants that impart tolerance to heat stress, a phenomenon known as induced systemic tolerance (Yang et al., 2009). Apart from inducing direct stress tolerance, several plant-beneficial traits exhibited by these bacteria support plant growth in a synergistic manner under stress (Etesami and Beattie, 2017; Imran et al., 2021). They benefit plants either directly, through the phytohormones production, nutrient mobilization, and nitrogen fixation or indirectly by triggering the signaling cascades in the host plant. *Burkholderia phytofirmans* strain *PsJN* is a well-reported PGPR that enhances heat tolerance in tomatoes, cold tolerance in grapevine, water stress tolerance in wheat, salt, and freezing tolerance in *Arabidopsis*. The same bacterium also has the antifungal property that protects the plant from biotic stress (Issa et al., 2018) revealing that a single bacterium can induce multiple benefits in same or different hosts (Imran et al., 2021).

Role of Microbial Phytohormones Under HS

Phytohormones are the key regulators of plant development, and the plants have a natural ability to synthesize, perceive and respond to these hormones. This response is modulated upon exposure to external/internal stimuli (Khan et al., 2014; Hakim et al., 2021), e.g., decreased levels of auxin, cytokinin, and gibberellin and increased ABA level under HS (Wu et al., 2016). One of the mechanisms of PGPM-mediated plant growth improvement is the phytohormone production trait (Etesami et al., 2015) which affect the metabolism of endogenous (Hashem et al., 2016; Sorty et al., 2016) and ultimately play a key role in modulating the plant's response under stress, uptake etc. (Spaepen et al., 2008; Khan et al., 2011). These facts validate that the phytohormones-producing PGPM reverse the adverse effects of heat stress.

Auxin (IAA) is the most important hormone, imperative for cell division, differentiation that controls seed germination, roots development, and apical dominance (Maheshwari et al., 2015). The majority of the rhizospheric microbes (>80%) synthesize and release IAA in the rhizosphere which elongates plant roots, increases the number of root hairs for enhanced uptake of water and nutrients under normal (Oleńska et al., 2020) as well as stress (Imran et al., 2015; Nawaz et al., 2020; Zia et al., 2021). IAA producing *Ochrobactrum* sp. and *Pseudomonas* sp. (volcanos isolates) have shown improved root and shoot length, fresh weight, and biomass of maize under high temperature (40°C), drought (up to 60% Poly Ethylene Glycol 6000), and salt (500 mM NaCl) (Mishra et al., 2017).

Gibberellins (GAs) regulate plant developmental processes such as embryogenesis, leaf expansion, stem elongation,

TABLE 4 | Role of PGPR in plant under stress condition.

Bacterial strain	Crop	Role in plant	References
1-ACC Deaminase			
Klebsiella sp.	*Triticum aestivum* (Wheat)	*Klebsiella* sp. SBP-8 protects the plants against adverse effects of salt and heat stress; reduce stress-induced ethylene and regulation of ion transporters	Singh et al., 2015
Bacillus cereus	*Triticum aestivum* (Wheat)	Increase plant growth (root, shoot fresh and dry weight, chlorophyll contents) under heat stress	Ali et al., 2011
Pseudomonas putida			
2-Exopolysaccharides			
Bacillus cereus	*Solanum lycopersicum* (Tomato)	Increase the number of flowers and fruits Increase chlorophyll, proline, and antioxidants	Mukhtar T. et al., 2020
Bacillus amyloliquefaciens UCMB5113	*Triticum aestivum* (Wheat)	Increase HSP26 and chlorophyll content Accumulate GABA and modulate metabolic pathways	Abd El-Daim et al., 2018
Azospirillum brasilense NO40			
Pseudomonas sp. AKM-P6	*Sorghum bicolor* (Sorghum)	Enhance tolerance of sorghum seedlings to elevated temperatures	Ali et al., 2011
Rhizobium sp. (Cajanus)	*Leguminosae* (Legume)	Heat shock protein (Hsp) of 63-74 kDa	Sutherland, 2001
Pseudomonas sp. PsJN	*Solanum tuberosum* (Potato)	Promote growth	Bensalim et al., 1998
Bacillus aryabhattai H26-2 and *Bacillus siamensis* H30-3	*Brassica oleracea var. capitata* (Cabbage)	Leaf abscisic acid (ABA) content and reduced stomatal opening after stresses treatments, Biocontrol activity against soft rot	Abd El-Daim et al., 2018
Bradyrhizobium diazoefficiens USDA110	*Glycine max* (Soybean)	Survival in starvation	Nishihata et al., 2018
Shinorizobium meliloti	*Medicago sativa* (Alfalfa)	Affect symbiosis during heat stress	Ogden et al., 2019

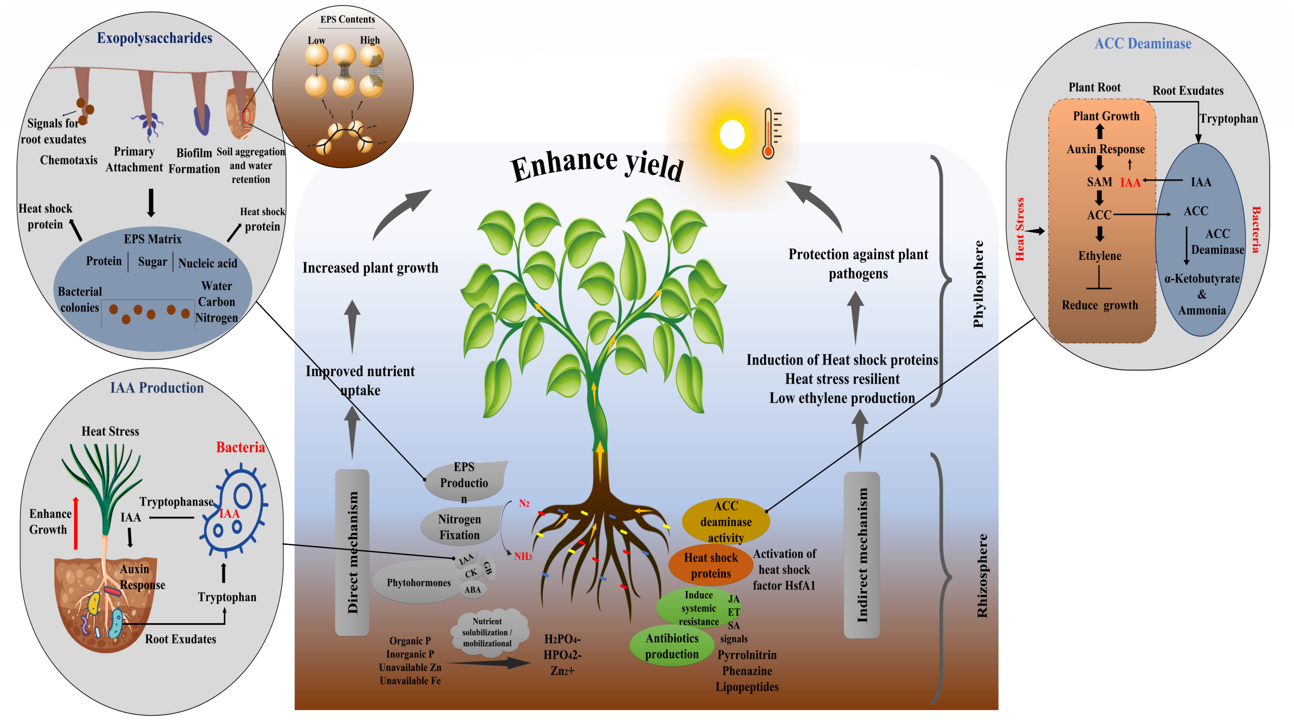

FIGURE 4 | Mechanism of PGPR for growth promotion and a(biotic) stress tolerance.

flowering, and fruit ripening (Binenbaum et al., 2018) while abscisic acid (ABA) regulates cell division and elongation, seed dormancy and germination, embryo maturation, floral induction, and responses to stresses (Finkelstein, 2013). PGPM capable to synthesize gibberellins stimulate plant growth and stress tolerance by modulating the endogenous levels of GAs and ABA. For instance, inoculation with GAs producing *Serratia nematodiphila* and *B. tequilensis* increase the endogenous synthesis of GA4 and ABA while reduced the salicylic and jasmonic acids and improved plant biomass under HS (Kang et al., 2015). Phytohormones-producing endophytic fungi (*Phoma* and *Penicillium* sp.) and *Bacillus* spp. also modulate the level of endogenous abscisic acid, salicylic acid, and jasmonic acid under multiple stresses and improve thermotolerance (Waqas et al., 2012). Inoculation with a multiple hormone (IAA, CK, JA, SA, GAs, and ABA) producing *B. aryabhattai* strain significantly improved nodule number, overall plant growth, and increased stress tolerance of soybean to drought and high temperatures (38°C) (Park et al., 2017).

Cytokinins are involved in processes such as seed germination, apical dominance, roots development, nodule organogenesis, development of vascular tissues, flower and fruit, and plant-pathogen interactions (Osugi and Sakakibara, 2015). Different microbes (such as *Bacillus*, *Escherichia*, *Agrobacterium*, *Methylobacterium*, *Proteus*, *Pseudomonas*, and *Klebsiella*) inhabiting plant rhizosphere are capable to synthesize and release CK in the rhizosphere which stimulate plant growth under stress including heat (Liu et al., 2013). Exogenous application of INCYDE-F (an inhibitor of CK-oxidase/dehydrogenase) in *Arabidopsis* increased the contents of CK trans-zeatin and cis-zeatin in roots and IAA in all tissues after HS. It further reduced the level of ABA in leaves and ethylene in apices of roots which shows that inhibition of CK-degradation helped the *Arabidopsis* to cope with HS (Prerostova et al., 2020).

Ethylene is a gaseous hormone involved in abscission, senescence, reproductive development, and a(biotic) stress response (Liu et al., 2021). Pollen development is the most thermosensitive stage during reproduction, therefore, regulation of ethylene signaling in reproductive tissues is critical to gain reproductive success (Jegadeesan et al., 2018). Various abiotic and biotic stressors enhanced the levels of ethylene in plant tissues which is detrimental for plants. *Enterobacter* sp. SA187-induced thermo-tolerance to wheat in field has been reported (Shekhawat et al., 2021) which is mediated by the ethylene signaling *via* the TF EN13 and constitutive H3K4me3 modification of HS memory genes, generating robust thermotolerance in plants.

Biosynthesis of ethylene is undertaken by two transcripts (i) *PsACS* [encode enzymes that convert S-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC)] and (ii) PsACO (encode enzymes that convert ACC to ethylene (Savada et al., 2017). Various microbes, i.e., *Methylobacterium*, *Bacillus*, *Alcaligenes*, *Enterobacter*, *Pseudomonas*, *Azospirillum*, *Rhizobium*, and *Bradyrhizobium* have an enzyme "1-aminocyclopropane-1-carboxylate (ACC) deaminase," which metabolize ACC (an immediate precursor of ethylene) into α-ketobutyrate and ammonia ultimately lowering down the ethylene levels and detrimental impact on plants under stress including HS (Saleem et al., 2007). *Burkholderia phytofirmans, Pseudomonas frederiksbergensis, P. vancouverensis*, *P. putida,* and *B. cereus* are few examples of heat resilient microbes that utilize the ACC-deaminase enzyme to reduce the endogenous ethylene levels in plants produced under a range of different stresses including salinity (Liu et al., 2021), drought (Danish et al., 2020), and heat (Mukhtar S. et al., 2020).

Role of Microbial Exopolysaccharides Under HS

Exopolysaccharides (EPSs) are bacterial extracellular polymers that form 3D structure of complex compound (sugars, enzymes, polysaccharides, lipids, nucleic acids, extracellular DNA structural proteins) which is used in energy exchange mechanism in response to environmental signals (Mishra, 2013) and have a direct role in heat stress tolerance (Nishihata et al., 2018; Ogden et al., 2019). These EPSs are involved in cell aggregation, adhesion, water retention, building a protective barrier and supplying nutrients. They contain high-molecular-weight polymers that enable bacteria to cling with the soil particles *via* hydrogen bonding, van der wall forces, anionic and cationic bonding, and keep them alive. The inoculation with heat-resilient, ESPs-producing *B. cereus* increased root and shoot length, chlorophyll and proline contents, water intake, number of flowers and fruits in tomato under high temperature (Mukhtar T. et al., 2020). HS induces changes in EPSs production as well as other cellular proteins, i.e., HSPs which prevents protein aggregation, misfolding, and target abnormally folded proteins for degradation (Parsell and Lindquist, 1993). Nguyen et al. (2014) reported that *Pseudomonas* sp. strain PsJN produced ESPs at high temperature and enhanced growth of the potato. Application of *Bacillus, Pseudomonas* and *Azospirillum* spp. (Ali et al., 2011; Abd El-Daim et al., 2019; Da Jeong Shin et al., 2019) have been shown to induce metabolic and regulatory proteins modulation to develop heat stress tolerance along with the cold and drought stresses. Apart from this, EPS act as chemoattractant and help to develop microbial biofilm matrix on the root surface that protect root surfaces from any damage under any kind of stress including HS.

Microbe-Induced HS Tolerance and Antioxidant Activity

Plant growth-promoting rhizobacteria not only produce phytohormones but also help plants by modulation of different genes under heat stress. Heat stress upregulates the genes involved in autophagy (e.g., *SlWRKY33b* and *SlATG5* genes) that are harmful to plants. But the application of *Bacillus cereus* sp. isolate (SA1) and humic acid (HA) down-regulate the expression of these genes which not only give relief but provide thermotolerance to the tomato plants (Khan et al., 2020) and increase the uptake of potassium (K) phosphorus (P), and iron (Fe). *B. cereus* SA1 enhanced the activity of antioxidants (CAT, APX, SOD) as well as the efficacy of chlorophyll pigments. The inoculation also resulted in a reduced level of abscisic acid (ABA) and enhanced the level of salicylic acid (SA). The treated plant showed a reduced level of glutathione (GSH). It has been shown that thermotolerant bacteria (*Bacillus* spp. *actinobacterium Kocuria* sp.) and cyanobacteria (*Calothrix elenkinii* and *Anabaena laxa*) show plant growth-promoting activities (30–50% root and shoot length, biomass, and dry weight) and stimulate peroxidase (5–10%) and endoglucanase activities in a range of different plants (Kumar et al., 2013).

Thermotolerant *P. putida* strain *AKMP7* inoculated to wheat plants increased the plant biomass and dry weight, enhanced root and shoot length, increase the number of tillers and spikelet, and enhance grain formation (Ali et al., 2011). The inoculated bacteria prevent the plant from cellular injury, enhance the antioxidant enzymatic activities (SOD, APX, CAT), and improve cellular metabolism, e.g., the level of protein, proline contents, cellular sugar, amino acid, and starch, and the efficiency of chlorophyll under heat stress condition. *AKMP7* formed biofilm on plant roots which help plants to combat adverse heat stress conditions.

CONCLUSIONS AND FUTURE PROSPECTS

Global warming has become a critical challenge to food security, causing severe yield losses of major crops worldwide. Given the expanding needs for global food supply as well as the extreme pressure of population growth and climate change trajectories, strategies should primarily be focused on the right investigation on different abiotic stresses especially on heat stress that becomes a key problem in the last decade. The plants are more vulnerable to high temperatures because of their sessile nature. They exhibit physio-morphological, biochemical, and molecular adaptations against heat, but further investigations are required to understand the thermotolerance mechanism active in different plant species. The plants themselves produce antioxidants, reduce the stomatal conductance, activate heat-responsive genes, and produce heat shock proteins, but on the other hand, exogenous or foliar application of chemicals like calcium chloride ($CaCl_2$), salicylic acid, bio-stimulants, nanoparticles, and osmoprotectants are useful in sustainable agriculture. Furthermore, heat-sensitive genes can be targeted through CRISPR-Cas9 to develop heat-insensitive crops in the future. The heat resilient microbes produces phytohormones, ethylene, ACC deaminase, antioxidant enzymes, and HSP under heat stress enabling plants to maintain their growth under stress. A recent review publishes the meta-analysis of 39 published studies in support of PGPR-mediated thermotolerance in plants. This supports that microbe-mediated solutions are more sustainable than developing heat-tolerant varieties as microbes are usually present in the soil and rhizosphere and can form associations with homologous as well as non-homologous hosts. So, a single heat resilient microbe-inoculum may be effective in more than one crop or plants. Furthermore, the microbes are multi-purpose and exhibit many other traits of plant interest apart from giving tolerance against heat stress making the microbe-therapy more effective than chemical or genetic engineering or breeding approaches. However, the molecular mechanisms involved in this tolerance may be studied in detail in different crops prone to heat stress. As plants can't live in isolation in any environment, they have a direct impact of environment and also interact with the microbes present in soil. Whereas microbes also interact with other microbes and to the environment. So, this tri-partite association is very important for the stable functioning of the plants and microbes and over all agricultural sustainability in any given environment.

AUTHOR CONTRIBUTIONS

MA collected data, made illustrations and figures, and wrote the initial draft. MI, FM, and MS helped in the analysis and writing. AI conceptualized and finalized the review. All authors contributed to the article and approved the submitted version.

REFERENCES

Abd El-Daim, I., Bejai, S., Fridborg, I., and Meijer, J. (2018). Identifying potential molecular factors involved in Bacillus amyloliquefaciens 5113 mediated abiotic stress tolerance in wheat. *Plant Biol.* 20, 271–279. doi: 10.1111/plb.12680

Abd El-Daim, I. A., Bejai, S., and Meijer, J. (2014). Improved heat stress tolerance of wheat seedlings by bacterial seed treatment. *Plant Soil* 379, 337–350. doi: 10.1007/s11104-014-2063-3

Abd El-Daim, I. A., Bejai, S., and Meijer, J. (2019). Bacillus velezensis 5113 induced metabolic and molecular reprogramming during abiotic stress tolerance in wheat. *Sci. Rep.* 9, 1–18. doi: 10.1038/s41598-019-52567-x

Ahamed, K.-U., Nahar, K., Fujita, M., and Hasanuzzaman, M. (2010). Variation in plant growth, tiller dynamics and yield components of wheat (*Triticum aestivum* L.) due to high temperature stress. *Adv. Agric. Botanics* 2, 213–224.

Akter, N., and Islam, M. R. (2017). Heat stress effects and management in wheat. A review. *Agron. Sustain. Dev.* 37, 37. doi: 10.1007/s13593-017-0443-9

Alexandre, A., and Oliveira, S. (2013). Response to temperature stress in rhizobia. *Critic. Rev. Microbiol.* 39, 219–228. doi: 10.3109/1040841X.2012.702097

Ali, S. Z., Sandhya, V., Grover, M., Linga, V. R., and Bandi, V. (2011). Effect of inoculation with a thermotolerant plant growth promoting *Pseudomonas putida* strain AKMP7 on growth of wheat (*Triticum* spp.) under heat stress. *J. Plant Interact.* 6, 239–246. doi: 10.1080/17429145.2010.545147

Al-Karaki, G. N. (2012). Phenological development-yield relationships in durum wheat cultivars under late-season high-temperature stress in a semiarid environment. *ISRN Agron.* 2012, 456856. doi: 10.5402/2012/456856

Álvarez-Pérez, S., Lievens, B., and Fukami, T. (2019). Yeast–bacterium interactions: the next frontier in nectar research. *Trends Plant Sci.* 24, 393–401. doi: 10.1016/j.tplants.2019.01.012

Argosubekti, N. (2020). "A review of heat stress signaling in plants," in *IOP Conference Series: Earth and Environmental Science*. Bristol: IOP Publishing, 012041.

Ashraf, M., and Hafeez, M. (2004). Thermotolerance of pearl millet and maize at early growth stages: growth and nutrient relations. *Biol. Plant.* 48, 81–86. doi: 10.1023/B:BIOP.0000024279.44013.61

Asseng, S., Ewert, F., Martre, P., Rötter, R. P., Lobell, D. B., Cammarano, D., et al. (2015). Rising temperatures reduce global wheat production. *Nat. Clim. Change* 5, 143–147. doi: 10.1038/nclimate2470

Bakker, P. A., Pieterse, C. M., de Jonge, R., and Berendsen, R. L. (2018). The soil-borne legacy. *Cell.* 172, 1178–1180. doi: 10.1016/j.cell.2018.02.024

Balla, K., Bed,o, Z., and Veisz, O. (2007). Heat stress induced changes in the activity of antioxidant enzymes in wheat. *Cereal Res. Commun.* 35, 197–200. doi: 10.1556/CRC.35.2007.2.8

Balla, K., Karsai, I., Bónis, P., Kiss, T., Berki, Z., Horváth, Á., et al. (2019). Heat stress responses in a large set of winter wheat cultivars (*Triticum aestivum* L.) depend on the timing and duration of stress. *PLoS One* 14, e0222639. doi: 10.1371/journal.pone.0222639

Balla, K., Rakszegi, M., Li, Z., Bekes, F., Bencze, S., and Veisz, O. (2011). Quality of winter wheat in relation to heat and drought shock after anthesis. *Czech J. Food Sci.* 29, 117–128. doi: 10.17221/227/2010-CJFS

Baniwal, S., Bharti, K., Chan, K., Fauth, M., Ganguli, A., Kotak, S., et al. (2005). Heat stress response in plants: A complex game with chaperones and more than twenty heat stress transcription factors. *J. Biosci.* 29, 471–487. doi: 10.1007/BF02712120

Barka, E. A., Nowak, J., and Clément, C. (2006). Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, Burkholderia phytofirmans strain PsJN. *Appl. Environ. Microbiol.* 72, 7246–7252. doi: 10.1128/AEM.01047-06

Bäurle, I. (2016). Plant heat adaptation: priming in response to heat stress. *F1000Research* 5, F1000 Faculty Rev-694. doi: 10.12688/f1000research.7526.1

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., et al. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Front. Plant Sci.* 10, 1068. doi: 10.3389/fpls.2019.01068

Bensalim, S., Nowak, J., and Asiedu, S. K. (1998). A plant growth promoting rhizobacterium and temperature effects on performance of 18 clones of potato. *Am. J. Potato Res.* 75, 145–152.

Binenbaum, J., Weinstain, R., and Shani, E. (2018). Gibberellin localization and transport in plants. *Trends Plant Sci.* 23, 410–421. doi: 10.1016/j.tplants.2018.02.005

Bonde, M., Nester, S., and Berner, D. (2012). Effects of daily temperature highs on development of *Phakopsora pachyrhizi* on soybean. *Phytopathology* 102, 761–768. doi: 10.1094/PHYTO-01-12-0011-R

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Caverzan, A., Casassola, A., and Brammer, S. P. (2016). Antioxidant responses of wheat plants under stress. *Genet. Mol. Biol.* 39, 1–6. doi: 10.1590/1678-4685-GMB-2015-0109

Chakraborty, D., Nagarajan, S., Aggarwal, P., Gupta, V., Tomar, R., Garg, R., et al. (2008). Effect of mulching on soil and plant water status, and the growth and yield of wheat (*Triticum aestivum* L.) in a semi-arid environment. *Agric. Water Manag.* 95, 1323–1334. doi: 10.1016/j.agwat.2008.06.001

Change, I. C. (2014). *Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Geneva: IPCC, 1454.

Chen, J., Zhou, X., Wang, W., Liu, B., Lv, Y., Yang, W., et al. (2018). A review on fundamental of high entropy alloys with promising high–temperature properties. *J. Alloys Compd.* 760, 15–30. doi: 10.1016/j.jallcom.2018.05.067

Chen, Y., Liu, T., Tian, X., Wang, X., Li, M., Wang, S., et al. (2015). Effects of plastic film combined with straw mulch on grain yield and water use efficiency of winter wheat in Loess Plateau. *Field Crops Res.* 172, 53–58. doi: 10.1016/j.fcr.2014.11.016

Cheng, L., Zou, Y., Ding, S., Zhang, J., Yu, X., Cao, J., et al. (2009). Polyamine accumulation in transgenic tomato enhances the tolerance to high temperature stress. *J. Integr. Plant Biol.* 51, 489–499. doi: 10.1111/j.1744-7909.2009.00816.x

Chialva, M., Salvioli Di Fossalunga, A., Daghino, S., Ghignone, S., Bagnaresi, P., Chiapello, M., et al. (2018). Native soils with their microbiotas elicit a state of alert in tomato plants. *New Phytol.* 220, 1296–1308. doi: 10.1111/nph.15014

Commuri, P., and Jones, R. (2001). High temperatures during endosperm cell division in maize: a genotypic comparison under in vitro and field conditions. *Crop Sci.* 41, 1122–1130. doi: 10.2135/cropsci2001.4141122x

Cossani, C. M., and Reynolds, M. P. (2012). Physiological traits for improving heat tolerance in wheat. *Plant Physiol.* 160, 1710–1718. doi: 10.1104/pp.112.207753

Da Costa, M. V. J., Ramegowda, Y., Ramegowda, V., Karaba, N. N., Sreeman, S. M., and Udayakumar, M. (2021). Combined drought and heat stress in rice: responses, phenotyping and strategies to improve tolerance. *Rice Sci.* 28, 233–242. doi: 10.1016/j.rsci.2021.04.003

Da Jeong Shin, S.-J. Y., Hong, J. K., Weon, H.-Y., Song, J., and Sang, M. K. (2019). Effect of Bacillus aryabhattai H26-2 and B. siamensis H30-3 on growth promotion and alleviation of heat and drought stresses in Chinese cabbage. *Plant Pathol. J.* 35, 178. doi: 10.5423/PPJ.NT.08.2018.0159

Damayanti, T., and Putra, L. (2010). Hot water treatment of cutting-cane infected with Sugarcane streak mosaic virus (SCSMV). *J. ISSAAS* 16, 17–25. doi: 10.5423/20113256683

Danish, S., Zafar-Ul-Hye, M., Mohsin, F., and Hussain, M. (2020). ACC-deaminase producing plant growth promoting rhizobacteria and biochar mitigate adverse effects of drought stress on maize growth. *PLoS ONE* 15, e0230615. doi: 10.1371/journal.pone.0230615

Dastogeer, K. M., Zahan, M. I., Rhaman, M. S., Sarker, M. S., and Chakraborty, A. (2022). Microbe-mediated thermotolerance in plants and pertinent mechanisms-a meta-analysis and review. *Front. Microbiol.* 13, 833566. doi: 10.3389/fmicb.2022.833566

Devireddy, A. R., Tschaplinski, T. J., Tuskan, G. A., Muchero, W., and Chen, J.-G. (2021). Role of reactive oxygen species and hormones in plant responses to temperature changes. *Int. J. Mol. Sci.* 22, 8843. doi: 10.3390/ijms22168843

Dias, A., and Lidon, F. (2009). Evaluation of grain filling rate and duration in bread and durum wheat, under heat stress after anthesis. *J. Agron. Crop Sci.* 195, 137–147. doi: 10.1111/j.1439-037X.2008.00347.x

Dias, A. S., and Lidon, F. C. (2010). Bread and durum wheat tolerance under heat stress: a synoptical overview. *Emir. J. Food Agric.* 2010, 412–436. doi: 10.9755/ejfa.v22i6.4660

Dimitrov, I., Trzebicka, B., Müller, A. H., Dworak, A., and Tsvetanov, C. B. (2007). Thermosensitive water-soluble copolymers with doubly responsive reversibly interacting entities. *Prog. Polym. Sci.* 32, 1275–1343. doi: 10.1016/j.progpolymsci.2007.07.001

Ding, W., Song, L., Wang, X., and Bi, Y. (2010). Effect of abscisic acid on heat stress tolerance in the calli from two ecotypes of Phragmites communis. *Biol. Plant.* 54, 607–613. doi: 10.1007/s10535-010-0110-3

Djanaguiraman, M., Annie Sheeba, J., Durga Devi, D., and Bangarusamy, U. (2009). Cotton leaf senescence can be delayed by nitrophenolate spray through enhanced antioxidant defence system. *J. Agron. Crop Sci.* 195, 213–224. doi: 10.1111/j.1439-037X.2009.00360.x

Djanaguiraman, M., Narayanan, S., Erdayani, E., and Prasad, P. V. (2020). Effects of high temperature stress during anthesis and grain filling periods on photosynthesis, lipids and grain yield in wheat. *BMC Plant Biol.* 20, 1–12. doi: 10.1186/s12870-020-02479-0

Djanaguiraman, M., Prasad, P., and Al-Khatib, K. (2011). Ethylene perception inhibitor 1-MCP decreases oxidative damage of leaves through enhanced antioxidant defense mechanisms in soybean plants grown under high temperature stress. *Environ. Exp. Bot.* 71, 215–223. doi: 10.1016/j.envexpbot.2010.12.006

Djanaguiraman, M., Prasad, P. V., and Seppanen, M. (2010). Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. *Plant Physiol. Biochem.* 48, 999–1007. doi: 10.1016/j.plaphy.2010.09.009

Dodd, I. C., and Pérez-Alfocea, F. (2012). Microbial amelioration of crop salinity stress. *J. Exp. Bot.* 63, 3415–3428. doi: 10.1093/jxb/ers033

Dubey, R., Pathak, H., Chakrabarti, B., Singh, S., Gupta, D. K., and Harit, R. (2020). Impact of terminal heat stress on wheat yield in India and options for adaptation. *Agric. Syst.* 181, 102826. doi: 10.1016/j.agsy.2020.102826

Dupuis, I., and Dumas, C. (1990). Influence of temperature stress on in vitro fertilization and heat shock protein synthesis in maize (*Zea mays* L.) reproductive tissues. *Plant Physiol.* 94, 665–670. doi: 10.3389/fpls.2015.00999

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell* 175, 973–983.e914. doi: 10.1016/j.cell.2018.10.020

Easterling, W. E., Aggarwal, P. K., Batima, P., Brander, K. M., Erda, L., Howden, S. M., et al. (2007). Food, fibre and forest products. *Clim. Change* 2007, 273–313.

Etesami, H., Alikhani, H. A., and Hosseini, H. M. (2015). Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. *MethodsX.* 2, 72–78. doi: 10.1016/j.mex.2015.02.008

Etesami, H., and Beattie, G. A. (2017). “Plant-microbe interactions in adaptation of agricultural crops to abiotic stress conditions,” in *Probiotics and Plant Health* (Singapore: Springer), 163–200. doi: 10.1007/978-981-10-3473-2_7

Etterson, J. R., and Shaw, R. G. (2001). Constraint to adaptive evolution in response to global warming. *Science* 294, 151–154. doi: 10.1126/science.1063656

Fahad, S., Hussain, S., Saud, S., Hassan, S., Ihsan, Z., Shah, A. N., et al. (2016). Exogenously applied plant growth regulators enhance the morpho-physiological growth and yield of rice under high temperature. *Front. Plant Sci.* 7, 1250. doi: 10.3389/fpls.2016.01250

Farooq, M., Bramley, H., Palta, J. A., and Siddique, K. H. (2011). Heat stress in wheat during reproductive and grain-filling phases. *Crit. Rev. Plant Sci.* 30, 491–507. doi: 10.1080/07352689.2011.615687

Finkelstein, R. (2013). Abscisic acid synthesis and response. *Am. Soci. Plant Biol.* 11, 166. doi: 10.1199/tab.0166

Fitter, A. H., and Hay, R. K. (2012). *Environmental Physiology of Plants.* New York: Academic press.

Friedlingstein, P., Houghton, R., Marland, G., Hackler, J., Boden, T. A., Conway, T., et al. (2010). Update on CO 2 emissions. *Nat. Geosci.* 3, 811–812. doi: 10.1038/ngeo1022

Giri, A. (2013). *Effect of Acute Heat Stress on Nutrient Uptake by Plant Roots.* Toledo: The University of Toledo.

Giri, A., Heckathorn, S., Mishra, S., and Krause, C. (2017). Heat stress decreases levels of nutrient-uptake and-assimilation proteins in tomato roots. *Plants* 6, 6.

Głab, T., and Kulig, B. (2008). Effect of mulch and tillage system on soil porosity under wheat (*Triticum aestivum*). *Soil Tillage Res.* 99, 169–178. doi: 10.1016/j.still.2008.02.004

Grant, R., Kimball, B., Conley, M., White, J., Wall, G., and Ottman, M. J. (2011). Controlled warming effects on wheat growth and yield: field measurements and modeling. *Agron. J.* 103, 1742–1754. doi: 10.2134/agronj2011.0158

Gray, S. B., and Brady, S. M. (2016). Plant developmental responses to climate change. *Dev. Biol.* 419, 64–77. doi: 10.1016/j.ydbio.2016.07.023

Hakim, M., Hossain, A., Da Silva, J.,a,.T., Zvolinsky, V., and Khan, M. (2012). Protein and starch content of 20 wheat (*Triticum aestivum* L.) genotypes exposed to high temperature under late sowing conditions. *J. Sci. Res.* 4, 477–477. doi: 10.3329/jsr.v4i2.8679

Hakim, S., Naqqash, T., Nawaz, M. S., Laraib, I., Siddique, M. J., Zia, R., et al. (2021). Rhizosphere engineering with plant growth-promoting microorganisms for agriculture and ecological sustainability. *Front. Sustain. Food Syst.* 5, 16. doi: 10.3389/fsufs.2021.617157

Hasanuzzaman, M., Nahar, K., Alam, M., Roychowdhury, R., and Fujita, M. (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *Int. J. Mol. Sci.* 14, 9643–9684. doi: 10.3390/ijms14059643

Hashem, A., Abd Allah, E. F., Alqarawi, A. A., Al-Huqail, A. A., Wirth, S., and Egamberdieva, D. (2016). The interaction between arbuscular mycorrhizal fungi and endophytic bacteria enhances plant growth of Acacia gerrardii under salt stress. *Front. Microbiol.* 7, 1089. doi: 10.3389/fmicb.2016.01089

Hatfield, J. L., Boote, K. J., Kimball, B., Ziska, L., Izaurralde, R. C., Ort, D., et al. (2011). Climate impacts on agriculture: implications for crop production. *Agron. J.* 103, 351–370. doi: 10.2134/agronj2010.0303

Hatfield, J. L., and Prueger, J. H. (2015). Temperature extremes: effect on plant growth and development. *Weather Clim. Extrem.* 10, 4–10. doi: 10.1016/j.wace.2015.08.001

Hemantaranjan, A., Bhanu, A. N., Singh, M., Yadav, D., Patel, P., Singh, R., et al. (2014). Heat stress responses and thermotolerance. *Adv. Plants Agric. Res* 1, 1–10. doi: 10.15406/apar.2014.01.00012

Högy, P., Poll, C., Marhan, S., Kandeler, E., and Fangmeier, A. (2013). Impacts of temperature increase and change in precipitation pattern on crop yield and yield quality of barley. *Food Chem.* 136, 1470–1477. doi: 10.1016/j.foodchem.2012.09.056

Hossain, A., and Da Silva, J. T. (2012). Phenology, growth and yield of three wheat (*Triticum aestivum* L.) varieties as affected by high temperature stress. *Not. Sci. Biol.* 4, 97–109. doi: 10.15835/nsb437879

Hovmøller, M. S., Yahyaoui, A. H., Milus, E. A., and Justesen, A. F. (2008). Rapid global spread of two aggressive strains of a wheat rust fungus. *Mol. Ecol.* 17, 3818–3826. doi: 10.1111/j.1365-294X.2008.03886.x

Howarth, C. (2005). “Genetic improvements of tolerance to high temperature,” in *Abiotic Stresses–Plant Resistance Through Breeding and Molecular Approaches,* eds M. Ashraf, P. J. C. Harris (New York: The Haworth Press), 277–300.

Hsu, S.-F., Lai, H.-C., and Jinn, T.-L. (2010). Cytosol-localized heat shock factor-binding protein, AtHSBP, functions as a negative regulator of heat shock response by translocation to the nucleus and is required for seed development in Arabidopsis. *Plant Physiol.* 153, 773–784. doi: 10.1104/pp.109.151225

Hu, S., Ding, Y., and Zhu, C. (2020). Sensitivity and responses of chloroplasts to heat stress in plants. *Front. Plant Sci.* 11, 375. doi: 10.3389/fpls.2020.00375

Huang, Y.-C., Niu, C.-Y., Yang, C.-R., and Jinn, T.-L. (2016). The heat stress factor HSFA6b connects ABA signaling and ABA-mediated heat responses. *Plant Physiol.* 172, 1182–1199. doi: 10.1104/pp.16.00860

Hurkman, W. J., Tanaka, C. K., Vensel, W. H., Thilmony, R., and Altenbach, S. B. (2013). Comparative proteomic analysis of the effect of temperature and fertilizer on gliadin and glutenin accumulation in the developing endosperm and flour from *Triticum aestivum* L. cv. Butte 86. *Proteome Sci.* 11, 1–15. doi: 10.1186/1477-5956-11-8

Hurkman, W. J., Vensel, W. H., Tanaka, C. K., Whitehand, L., and Altenbach, S. B. (2009). Effect of high temperature on albumin and globulin accumulation in the endosperm proteome of the developing wheat grain. *J. Cereal Sci.* 49, 12–23. doi: 10.1016/j.jcs.2008.06.014

Hussain, H. A., Men, S., Hussain, S., Chen, Y., Ali, S., Zhang, S., et al. (2019). Interactive effects of drought and heat stresses on morpho-physiological attributes, yield, nutrient uptake and oxidative status in maize hybrids. *Sci. Rep.* 9, 1–12. doi: 10.1038/s41598-019-40362-7

Hussain, S., Khaliq, A., Mehmood, U., Qadir, T., Saqib, M., Iqbal, M. A., et al. (2018). *Sugarcane Production Under Changing Climate: Effects of Environmental Vulnerabilities on Sugarcane Diseases, Insects and Weeds*. Vienna: Intech Open.

Ikeda, H., Kinoshita, T., Yamamoto, T., and Yamasaki, A. (2019). Sowing time and temperature influence bulb development in spring-sown onion (*Allium cepa* L.). *Sci. Hortic.* 244, 242–248. doi: 10.1016/j.scienta.2018.09.050

Imran, A., Hakim, S., Tariq, M, Nawaz, M. S., Laraib, I., Gulzar, U., et al. (2021). Diazotrophs for lowering nitrogen pollution crises; looking deep into the roots. *Front. Microbiol.* 12, 637815. doi: 10.3389/fmicb.2021.637815

Imran, A., Mirza, M. S., Shah, T. M., Malik, K. A., and Hafeez, F. Y. (2015). Differential response of kabuli and desi chickpea genotypes toward inoculation with PGPR in different soils. *Front. Microbiol.* 6, 859. doi: 10.3389/fmicb.2015.00859

Issa, A., Esmaeel, Q., Sanchez, L., Courteaux, B., Guise, J.-F., Gibon, Y., et al. (2018). Impacts of Paraburkholderia phytofirmans strain PsJN on tomato (*Lycopersicon esculentum* L.) under high temperature. *Front. Plant Sci.* 9, 1397. doi: 10.3389/fpls.2018.01397

Jegadeesan, S., Chaturvedi, P., Ghatak, A., Pressman, E., Meir, S., Faigenboim, A., et al. (2018). Proteomics of heat-stress and ethylene-mediated thermotolerance mechanisms in tomato pollen grains. *Front. Plant Sci.* 9, 1558. doi: 10.3389/fpls.2018.01558

Jones, L. M., Koehler, A. K., Trnka, M., Balek, J., Challinor, A. J., Atkinson, H. J., et al. (2017). Climate change is predicted to alter the current pest status of *Globodera pallida* and *G. rostochiensis* in the United Kingdom. *Glob. Change Biol.* 23, 4497–4507. doi: 10.1111/gcb.13676

Kachhap, S., Chaudhary, A., and Singh, S. (2015). Response of plant growth promoting rhizobacteria (pgpr) in relation to elevated temperature conditions in groundnut (*Arachis hypogaea* L.). *Ecoscan* 9, 771–778.

Kandil, A., Attia, A., Sharief, A., and Leilh, A. (2011). Response of onion (*Allium cepa* L.) yield to water stress and mineral biofertilization. *Acta. Agron. Hung.* 59, 361–370. doi: 10.1556/AAgr.59.2011.4.7

Kang, S.-M., Khan, A. L., Waqas, M., You, Y.-H., Hamayun, M., Joo, G.-J., et al. (2015). Gibberellin-producing Serratia nematodiphila PEJ1011 ameliorates low temperature stress in *Capsicum annuum* L. *Eur. J. Soil Biol.* 68, 85–93. doi: 10.1016/j.ejsobi.2015.02.005

Kaushal, N., Bhandari, K., Siddique, K. H., and Nayyar, H. (2016). Food crops face rising temperatures: an overview of responses, adaptive mechanisms, and approaches to improve heat tolerance. *Cogent Food Agric.* 2, 1134380. doi: 10.1080/23311932.2015.1134380

Khan, A. L., Hamayun, M., Kim, Y. H., Kang, S. M., Lee, J. H., and Lee, I. J. (2011). Gibberellins producing endophytic Aspergillus fumigatus sp. LH02 influenced endogenous phytohormonal levels, isoflavonoids production and plant growth in salinity stress. *Process Biochem.* 6, 440–447. doi: 10.1016/j.procbio.2010.09.013

Khan, M. A., Asaf, S., Khan, A. L., Jan, R., Kang, S.-M., Kim, K.-M., et al. (2020). Extending thermotolerance to tomato seedlings by inoculation with SA1 isolate of Bacillus cereus and comparison with exogenous humic acid application. *PLoS ONE* 15, e0232228. doi: 10.1371/journal.pone.0232228

Khan, M. I. R., Asgher, M., and Khan, N. A. (2014). Alleviation of salt-induced photosynthesis and growth inhibition by salicylic acid involves glycinebetaine and ethylene in mungbean (*Vigna radiata* L.). *Plant Physiol. Biochem.* 80, 67–74. doi: 10.1016/j.plaphy.2014.03.026

Khichar, M., and Niwas, R. (2007). Thermal effect on growth and yield of wheat under different sowing environments and planting systems. *Indian J. Agric. Sci.* 41, 92–96.

Kmiecik, P., Leonardelli, M., and Teige, M. (2016). Novel connections in plant organellar signalling link different stress responses and signalling pathways. *J. Exp. Bot.* 67, 3793–3807. doi: 10.1093/jxb/erw136

Kong, B., Li, Z., Wang, E., Lu, W., Chen, L., and Qi, G. (2018). An experimental study for characterization the process of coal oxidation and spontaneous combustion by electromagnetic radiation technique. *Process Saf. Environ.* 119, 285–294. doi: 10.1016/j.psep.2018.08.002

Kumar, A., and Verma, J. P. (2018). Does plant-microbe interaction confer stress tolerance in plants: a review?. *Microbiol. Res.* 207, 41–52. doi: 10.1016/j.micres.2017.11.004

Kumar, M., Prasanna, R., Bidyarani, N., Babu, S., Mishra, B. K., Kumar, A., et al. (2013). Evaluating the plant growth promoting ability of thermotolerant bacteria and cyanobacteria and their interactions with seed spice crops. *Sci. Hortic.* 164, 94–101. doi: 10.1016/j.scienta.2013.09.014

Kumarathunge, D. P., Medlyn, B. E., Drake, J. E., Tjoelker, M. G., Aspinwall, M. J., Battaglia, M., et al. (2019). Acclimation and adaptation components of the temperature dependence of plant photosynthesis at the global scale. *New phytol.* 222, 768–784. doi: 10.1111/nph.15668

Kwak, M.-J., Kong, H. G., Choi, K., Kwon, S.-K., Song, J. Y., Lee, J., et al. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nat. Biotechnol.* 36, 1100–1109. doi: 10.1038/nbt.4232

Lamaoui, M., Jemo, M., Datla, R., and Bekkaoui, F. (2018). Heat and drought stresses in crops and approaches for their mitigation. *Front. Chem.* 6, 26. doi: 10.3389/fchem.2018.00026/full

Lämke, J., and Bäurle, I. (2017). Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biol.* 18, 1–11. doi: 10.1186/s13059-017-1263-6

Lang-Mladek, C., Popova, O., Kiok, K., Berlinger, M., Rakic, B., Aufsatz, W., et al. (2010). Transgenerational inheritance and resetting of stress-induced loss of epigenetic gene silencing in Arabidopsis. *Mol. Plant.* 3, 594–602. doi: 10.1093/mp/ssq014

LeBlanc, C., Zhang, F., Mendez, J., Lozano, Y., Chatpar, K., Irish, V. F., et al. (2018). Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. *Plant J.* 93, 377–386.

Li, N., Euring, D., Cha, J. Y., Lin, Z., Lu, M., Huang, L.-J., et al. (2021). Plant hormone-mediated regulation of heat tolerance in response to global climate change. *Front. Plant Sci.* 11, 2318. doi: 10.3389/fpls.2020.627969/full

Li, X., Cai, C., Wang, Z., Fan, B., Zhu, C., and Chen, Z. (2018). Plastid translation elongation factor Tu is prone to heat-induced aggregation despite its critical role in plant heat tolerance. *Plant Physiol.* 176, 3027–3045. doi: 10.1104/pp.17.01672

Li, Z., Peng, T., Xie, Q., Han, S., and Tian, J. (2010). Mapping of QTL for tiller number at different stages of growth in wheat using double haploid and immortalized F 2 populations. *Genetic.* 89, 409. doi: 10.1007/s12041-010-0059-1

Li, Z.-G. (2020). "Mechanisms of plant adaptation and tolerance to heat stress," in *Plant Ecophysiology and Adaptation Under Climate Change: Mechanisms and Perspectives II* (Berlin: Springer), 39–59.

Liu, C.-H., Siew, W., Hung, Y.-T., Jiang, Y.-T., and Huang, C.-H. (2021). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase gene in pseudomonas azotoformans is associated with the amelioration of salinity stress in tomato. *J. Agric. Food Chem.* 69, 913–921. doi: 10.1021/acs.jafc.0c05628

Liu, F., Xing, S., Ma, H., Du, Z., and Ma, B. (2013). Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. *Appl. Microbiol. Biotechnol.* 97, 9155–9164. doi: 10.1007/s00253-013-5193-2

Liu, H., and Brettell, L. E. (2019). Plant defense by VOC-induced microbial priming. *Trends Plant Sci.* 24, 187–189. doi: 10.1016/j.tplants.2019.01.008

Liu, Z., and Tan, V. Y. F. (2017). "Relative error bounds for nonnegative matrix factorization under a geometric assumption," in *2017 IEEE International Conference on Acoustics, Speech and Signal Processing (ICASSP)*, 2552–2556.

Lobell, D. B., and Field, C. B. (2007). Global scale climate–crop yield relationships and the impacts of recent warming. *Environ. Res. Lett.* 2, 014002. doi: 10.1088/1748-9326/2/1/014002/meta

Lobell, D. B., Schlenker, W., and Costa-Roberts, J. (2011). Climate trends and global crop production since 1980. *Science* 333, 616–620. doi: 10.1126/science.1204531

Lu, T., Ke, M., Lavoie, M., Jin, Y., Fan, X., Zhang, Z., et al. (2018). Rhizosphere microorganisms can influence the timing of plant flowering. *Microbiome* 6, 1–12. doi: 10.1186/s40168-018-0615-0

Ma, L., Qiao, J., Kong, X., Zou, Y., Xu, X., Chen, X., et al. (2015). Effect of low temperature and wheat winter-hardiness on survival of Puccinia striiformis f. sp. tritici under controlled conditions. *PLoS ONE* 10, e0130691. doi: 10.1371/journal.pone.0130691

Maheshwari, D. K., Dheeman, S., and Agarwal, M. (2015). "VPhytohormone-producing PGPR for sustainable agriculture," in *Bacterial Metabolites in Sustainable Agroecosystem* (Berlin: Springer), 159–182.

Majeed, S., Rana, I. A., Mubarik, M. S., Atif, R. M., Yang, S.-H., Chung, G., et al. (2021). Heat stress in cotton: a review on predicted and unpredicted growth-yield anomalies and mitigating breeding strategies. *Agronomy* 11, 1825. doi: 10.3390/agronomy11091825

Mangrauthia, S. K., Shakya, V. P. S., Jain, R., and Praveen, S. (2009). Ambient temperature perception in papaya for papaya ringspot virus interaction. *Virus Genes* 38, 429–434. doi: 10.1007/s11262-009-0336-3

Marchand, F. L., Mertens, S., Kockelbergh, F., Beyens, L., and Nijs, I. (2005). Performance of High Arctic tundra plants improved during but deteriorated after exposure to a simulated extreme temperature event. *Glob. Chang Biol.* 11, 2078–2089. doi: 10.1111/j.1365-2486.2005.01046.x

Mariette, N., Androdias, A., Mabon, R., Corbiere, R., Marquer, B., Montarry, J., et al. (2016). Local adaptation to temperature in populations and clonal lineages of the Irish potato famine pathogen Phytophthora infestans. *Ecol. Evol.* 6, 6320–6331. doi: 10.1002/ece3.2282

Marutani, Y., Yamauchi, Y., Kimura, Y., Mizutani, M., and Sugimoto, Y. (2012). Damage to photosystem II due to heat stress without light-driven electron flow: involvement of enhanced introduction of reducing power into thylakoid membranes. *Planta* 236, 753–761. doi: 10.1007/s00425-012-1647-5

Mcclung, C. R., and Davis, S. J. (2010). Ambient thermometers in plants: from physiological outputs towards mechanisms of thermal sensing. *Curr. Biol.* 20, R1086–R1092. doi: 10.1016/j.cub.2010.10.035

Medina, E., Kim, S.-H., Yun, M., and Choi, W.-G. (2021). Recapitulation of the function and role of ROS generated in response to heat stress in plants. *Plants* 10, 371. doi: 10.3390/plants10020371

Meinke, H., Nelson, R., Kokic, P., Stone, R., Selvaraju, R., and Baethgen, W. (2006). Actionable climate knowledge: from analysis to synthesis. *Clim. Res.* 33, 101–110. doi: 10.3354/cr033101

Mengutay, M., Ceylan, Y., Kutman, U. B., and Cakmak, I. (2013). Adequate magnesium nutrition mitigates adverse effects of heat stress on maize and wheat. *Plant soil* 368, 57–72. doi: 10.1007/s11104-013-1761-6

Miller, P., Lanier, W., and Brandt, S. (2001). *Using Growing Degree Days to Predict Plant Stages*. Bozeman: Montana State University-Bozeman, 994–2721.

Milus, E. A., Kristensen, K., and Hovmøller, M. S. (2009). Evidence for increased aggressiveness in a recent widespread strain of *Puccinia striiformis* f. sp. tritici causing stripe rust of wheat. *Phytopathology* 99, 89–94. doi: 10.1094/PHYTO-99-1-0089

Mishra, J. (2013). "Microbial exopolysaccharides," in *The Prokaryotes–Applied Bacteriology and Biotechnolgy*, ED E. Rosenberg. Berlin: Springer-Verlag.

Mishra, S. K., Khan, M. H., Misra, S., Dixit, V. K., Khare, P., Srivastava, S., et al. (2017). Characterisation of Pseudomonas spp. and *Ochrobactrum* sp. isolated from volcanic soil. *Antonie Van Leeuwenhoek* 110, 253–270. doi: 10.1007/s10482-016-0796-0

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., et al. (2011). ROS signaling: the new wave? *Trends Plant Sci.* 16, 300–309. doi: 10.1016/j.tplants.2011.03.007

Modarresi, M., Mohammadi, V., Zali, A., and Mardi, M. (2010). Response of wheat yield and yield related traits to high temperature. *Cereal Res. Commun.* 38, 23–31. doi: 10.1556/CRC.38.2010.1.3

Mohammed, A. R., and Tarpley, L. (2010). Effects of high night temperature and spikelet position on yield-related parameters of rice (*Oryza sativa* L.) plants. *Eur. J. Agron.* 33, 117–123. doi: 10.1016/j.eja.2009.11.006

Momčilović, I. (2019). Effects of heat stress on potato productivity and nutritive quality. *Hrana i ishrana* 60, 43–48. doi: 10.5937/hraIsh1902043M

Morales, D., Rodríguez, P., Dell'amico, J., Nicolas, E., Torrecillas, A., and Sánchez-Blanco, M. J. (2003). High-temperature preconditioning and thermal shock imposition affects water relations, gas exchange and root hydraulic conductivity in tomato. *Biol. Plant.* 47, 203. doi: 10.1023/B:BIOP.0000022252.70836.fc

Mukhtar, S., Zareen, M., Khaliq, Z., Mehnaz, S., and Malik, K. (2020). Phylogenetic analysis of halophyte-associated rhizobacteria and effect of halotolerant and halophilic phosphate-solubilizing biofertilizers on maize growth under salinity stress conditions. *J. Appl. Microbiol.* 128, 556–573. doi: 10.1111/jam.14497

Mukhtar, T., Smith, D., Sultan, T., Seleiman, M. F., Alsadon, A. A., Ali, S., et al. (2020). Mitigation of heat stress in *Solanum lycopersicum* L. by ACC-deaminase and exopolysaccharide producing Bacillus cereus: effects on biochemical profiling. *Sustainability* 12, 2159. doi: 10.3390/su12062159

Nahar, K. (2013). Castor bean (*Ricinus communis* L.)-a biofuel plant: morphological and physiological parameters propagated from seeds in Bangladesh. *Asian Bus. Rev.* 2, 64–66.

Nandy, S., Pathak, B., Zhao, S., and Srivastava, V. (2019). Heat-shock-inducible CRISPR/Cas9 system generates heritable mutations in rice. *Plant Dir.* 3, e00145. doi: 10.1002/pld3.145

Nawaz, M. S., Arshad, A., Rajput, L., Fatima, K., Ullah, S., Ahmad, M., et al. (2020). Growth-stimulatory effect of quorum sensing signal molecule n-acyl-homoserine lactone-producing multi-trait *Aeromonas* spp. on wheat genotypes under salt stress. *Front. Microbiol.* 11, 553621. doi: 10.3389/fmicb.2020.553621

Nguyen, H. T., Razafindralambo, H., Blecker, C., N'yapo, C., Thonart, P., and Delvigne, F. (2014). Stochastic exposure to sub-lethal high temperature enhances exopolysaccharides (EPS) excretion and improves Bifidobacterium bifidum cell survival to freeze–drying. *Biochem. Eng. J.* 88, 85–94. doi: 10.1016/j.bej.2014.04.005

Nishihata, S., Kondo, T., Tanaka, K., Ishikawa, S., Takenaka, S., Kang, C.-M., et al. (2018). Bradyrhizobium diazoefficiens USDA110 PhaR functions for pleiotropic regulation of cellular processes besides PHB accumulation. *BMC Microbiol.* 18, 1–17. doi: 10.1186/s12866-018-1317-2

Ogata, T., Ishizaki, T., Fujita, M., and Fujita, Y. (2020). CRISPR/Cas9-targeted mutagenesis of OsERA1 confers enhanced responses to abscisic acid and drought stress and increased primary root growth under nonstressed conditions in rice. *PLoS ONE* 15, e0243376. doi: 10.1371/journal.pone.0243376

Ogden, A. J., Mcaleer, J. M., and Kahn, M. L. (2019). Characterization of the Sinorhizobium meliloti HslUV and ClpXP protease systems in free-living and symbiotic states. *J. Bacteriol.* 201, e00498–e00418. doi: 10.1128/JB.00498-18

Oleńska, E., Małek, W., Wójcik, M., Swiecicka, I., Thijs, S., and Vangronsveld, J. (2020). Beneficial features of plant growth-promoting rhizobacteria for improving plant growth and health in challenging conditions: A methodical review. *Sci. Total Environ.* 2020, 140682. doi: 10.1016/j.scitotenv.2020.140682

Ortiz, R., Sayre, K. D., Govaerts, B., Gupta, R., Subbarao, G., Ban, T., et al. (2008). Climate change: can wheat beat the heat? *Agric. Ecosyst. Environ.* 126, 46–58. doi: 10.1016/j.agee.2008.01.019

Osugi, A., and Sakakibara, H. (2015). QandA: How do plants respond to cytokinins and what is their importance? *BMC Biol.* 13, 1–10. doi: 10.1186/s12915-015-0214-5

Park, H., Weier, S., Razvi, F., Peña, P. A., Sims, N. A., Lowell, J., et al. (2017). Towards the development of a sustainable soya bean-based feedstock for aquaculture. *Plant Biotechnol. J.* 15, 227–236. doi: 10.1111/pbi.12608

Parsell, D., and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27, 437–496.

Porter, J. R., Xie, L., Challinor, A. J., Cochrane, K., Howden, S. M., Iqbal, M. M., et al. (2014). *Food Security and Food Production Systems. IPPC*. Available online at: https://www.ipcc.ch/site/assets/uploads/2018/02/WGIIAR5-Chap7_FINAL.pdf (accessed May 9, 2022).

Prasad, P., Boote, K., Allen Jr, L., Sheehy, J., and Thomas, J. (2006). Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field Crops Res.* 95, 398–411. doi: 10.1016/j.fcr.2005.04.008Get

Prasad, P., Staggenborg, S., and Ristic, Z. (2008). *Impacts of Drought and/or Heat Stress on Physiological, Developmental, Growth, and Yield Processes of Crop Plants. Response of Crops to Limited Water: Understanding and Modeling Water Stress Effects on Plant Growth Processes* 1, 301–355.

Prerostova, S., Dobrev, P. I., Kramna, B., Gaudinova, A., Knirsch, V., Spichal, L., et al. (2020). Heat acclimation and inhibition of cytokinin degradation positively affect heat stress tolerance of Arabidopsis. *Front. Plant Sci* 11, 87. doi: 10.3389/fpls.2020.00087

Rahman, M. A., Chikushi, J., Yoshida, S., and Karim, A. (2009). Growth and yield components of wheat genotypes exposed to high temperature stress under control environment. *Bangl. J. Agr. Res.* 34, 360–372. doi: 10.3329/bjar.v34i3.3961

Ramadoss, D., Lakkineni, V. K., Bose, P., Ali, S., and Annapurna, K. (2013). Mitigation of salt stress in wheat seedlings by halotolerant bacteria isolated from saline habitats. *SpringerPlus* 2, 6. doi: 10.1186/2193-1801-2-6

Rangan, P., Subramani, R., Kumar, R., Singh, A. K., and Singh, R. (2014). Recent advances in polyamine metabolism and abiotic stress tolerance. *Biomed. Res. Int.* 2014, 239621. doi: 10.1155/2014/239621

Rasmusson, L. M., Buapet, P., George, R., Gullström, M., Gunnarsson, P. C., and Björk, M. (2020). Effects of temperature and hypoxia on respiration, photorespiration, and photosynthesis of seagrass leaves from contrasting temperature regimes. *ICES J. Marine Sci.* 77, 2056–2065. doi: 10.1093/icesjms/fsaa093

Ratnarajah, V., and Gnanachelvam, N. (2021). Effect of abiotic stress on onion yield: a review. *Adv. Technol.* 2021, 147–160.

Reddy, P. S., Chakradhar, T., Reddy, R. A., Nitnavare, R. B., Mahanty, S., and Reddy, M. K. (2016). "Role of heat shock proteins in improving heat stress tolerance in crop plants," in *Heat Shock Proteins and Plants*. (Berlin: Springer), 283–307.

Reynolds, M., Calderini, D., Condon, A., and Rajaram, S. (2001). "Physiological basis of yield gains in wheat associated with the LR19 translocation from *Agropyron elongatum*," in *Wheat in a Global Environment* (Berlin: Springer), 345–351.

Ritchie, F., Bain, R., and Mcquilken, M. (2013). Survival of sclerotia of R hizoctonia solani AG 3 PT and effect of soil-borne inoculum density on disease development on potato. *J. Phytopathol.* 161, 180–189. doi: 10.1111/jph.12052

Roberts, K. E., Hadfield, J. D., Sharma, M. D., and Longdon, B. (2018). Changes in temperature alter the potential outcomes of virus host shifts. *PLoS Pathog.* 14, e1007185.

Ruelland, E., and Zachowski, A. (2010). How plants sense temperature. *Environ. Exp. Bot.* 69, 225–232. doi: 10.1016/j.envexpbot.2010.05.011

Rykaczewska, K. (2015). The effect of high temperature occurring in subsequent stages of plant development on potato yield and tuber physiological defects. *Am. J. Potato Res.* 92, 339–349. doi: 10.1007/s12230-015-9436-x

Sadowsky, M. J. (2005). "Soil stress factors influencing symbiotic nitrogen fixation," in *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment* (Berlin: Springer), 89–112.

Saleem, M., Arshad, M., Hussain, S., and Bhatti, A. S. (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *J. Ind. Microbiol. Biot.* 34, 635–648. doi: 10.1104/pp.126.3.1281

Sani, E., Herzyk, P., Perrella, G., Colot, V., and Amtmann, A. (2013). Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. *Genome Biol.* 14, 1–24. doi: 10.1186/gb-2013-14-6-r59

Santos-Medellín, C., Edwards, J., Liechty, Z., Nguyen, B., and Sundaresan, V. (2017). Drought stress results in a compartment-specific restructuring of the rice root-associated microbiomes. *MBio* 8, e764. doi: 10.1128/mBio.00764-17

Sarieva, G., Kenzhebaeva, S., and Lichtenthaler, H. (2010). Adaptation potential of photosynthesis in wheat cultivars with a capability of leaf rolling under high temperature conditions. *Russ. J. Plant Physl.* 57, 28–36. doi: 10.1134/S1021443710010048

Sarkar, N. K., Kundnani, P., and Grover, A. (2013). Functional analysis of Hsp70 superfamily proteins of rice (*Oryza sativa*). *Cell Stress Chaperon* 18, 427–437. doi: 10.1007/s12192-012-0395-6

Sarsu, F. (2018). "Screening protocols for heat tolerance in rice at the seedling and reproductive stages," in *Pre-Field Screening Protocols for Heat-Tolerant Mutants in Rice* (Berlin: Springer), 9–24.

Savada, R. P., Ozga, J. A., Jayasinghege, C. P., Waduthanthri, K. D., and Reinecke, D. M. (2017). Heat stress differentially modifies ethylene biosynthesis and signaling in pea floral and fruit tissues. *Plant Mol. Biol.* 95, 313–331. doi: 10.1007/s11103-017-0653-1

Savicka, M., and Škute, N. (2010). Effects of high temperature on malondialdehyde content, superoxide production and growth changes in wheat seedlings (*Triticum aestivum* L.). *Ekologija* 56, 26–33. doi: 10.2478/v10055-010-0004-x

Sharma, N., and Chahal, K. (2012). Protectant potential of Marigold oil (*Tagetes erecta* L.) and its fractions against *Tribolium castaneum* (Herbst). *Ann. Plant. Protect Sci.* 20, 294–297.

Shekhawat, K., Saad, M. M., Sheikh, A., Mariappan, K., Al-Mahmoudi, H., Abdulhakim, F., et al. (2021). Root endophyte induced plant thermotolerance by constitutive chromatin modification at heat stress memory gene loci. *EMBO Rep.* 22, e51049. doi: 10.15252/embr.202051049

Shrivastava, P., and Kumar, R. (2015). Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi J. Biol. Sci.* 22, 123–131. doi: 10.1016/j.sjbs.2014.12.001

Siddiqui, M. H., Al-Khaishany, M. Y., Al-Qutami, M. A., Al-Whaibi, M. H., Grover, A., Ali, H. M., et al. (2015). Morphological and physiological characterization of different genotypes of faba bean under heat stress. *Saudi J. Biol. Sci.* 22, 656–663. doi: 10.1016/j.sjbs.2015.06.002

Singh, B., Pathak, K., Verma, A., Verma, V., and Deka, B. (2011). Effects of vermicompost, fertilizer and mulch on plant growth, nodulation and pod yield of French bean (*Phaseolus vulgaris* L.). *Veg. Crop. Res. Bull.* 74, 153–165. doi: 10.2478/v10032-011-0013-7

Singh, R. P., Jha, P., and Jha, P. N. (2015). The plant-growth-promoting bacterium Klebsiella sp. SBP-8 confers induced systemic tolerance in wheat (*Triticum aestivum*) under salt stress. *J. Plant Physiol.* 184, 57–67. doi: 10.1016/j.jplph.2015.07.002

Sita, K., Sehgal, A., Hanumantharao, B., Nair, R. M., Vara Prasad, P. V., Kumar, S., et al. (2017). Food Legumes and Rising Temperatures: Effects, Adaptive Functional Mechanisms Specific to Reproductive Growth Stage and Strategies to Improve Heat Tolerance. *Front. Plant Sci.* 8, 1658. doi: 10.3389/fpls.2017.01658

Sorty, A. M., Meena, K. K., Choudhary, K., Bitla, U. M., Minhas, P. S., and Krishnani, K. K. (2016). Effect of plant growth promoting bacteria associated with halophytic weed (Psoralea corylifolia L) on germination and seedling growth of wheat under saline conditions. *Appl. Biochem. Biotechnol.* 180, 872–882. doi: 10.1007/s12010-016-2139-z

Spaepen, S., Dobbelaere, S., Croonenborghs, A., and Vanderleyden, J. (2008). Effects of Azospirillum brasilense indole-3-acetic acid production on inoculated wheat plants. *Plant Soil.* 312, 15–23. doi: 10.1007/s11104-008-9560-1

Sun, A.-Z., and Guo, F.-Q. (2016). Chloroplast retrograde regulation of heat stress responses in plants. *Front. Plant Sci.* 7, 398. doi|: 10.3389/fpls.2016.00398/full

Sutherland, I. W. (2001). Microbial polysaccharides from Gram-negative bacteria. *Int. Dairy J.* 11, 663–674. doi: 10.1016/S0958-6946(01)00112-1

Suwa, R., Hakata, H., Hara, H., El-Shemy, H. A., Adu-Gyamfi, J. J., Nguyen, N. T., et al. (2010). High temperature effects on photosynthate partitioning and sugar metabolism during ear expansion in maize (*Zea mays* L.) genotypes. *Plant Physiol. Bioch.* 48, 124–130. doi: 10.1016/j.plaphy.2009.12.010

Suzuki, N., Koussevitzky, S., Mittler, R., and Miller, G. (2012). ROS and redox signalling in the response of plants to abiotic stress. *Plant Cell Environ.* 35, 259–270. doi: 10.1111/j.1365-3040.2011.02336.x

Tian, F., Li, B., Ji, B., Zhang, G., and Luo, Y. (2009). Identification and structure–activity relationship of gallotannins separated from Galla chinensis. *LWT-Food Sci. Technol.* 42, 1289–1295. doi: 10.1016/j.lwt.2009.03.004

Timm, C. M., Carter, K. R., Carrell, A. A., Jun, S.-R., Jawdy, S. S., Vélez, J. M., et al. (2018). Abiotic stresses shift belowground Populus-associated bacteria toward a core stress microbiome. *MSystems* 3. doi: 10.1128/mSystems.00070-17

Toh, S., Imamura, A., Watanabe, A., Nakabayashi, K., Okamoto, M., Jikumaru, Y., et al. (2008). High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. *Plant Physiol.* 146, 1368–1385. doi: 10.1104/pp.107.113738

Tubiello, F. N., Soussana, J.-F., and Howden, S. M. (2007). Crop and pasture response to climate change. *Proc. Natl. Acad. Sci.* 104, 19686–19690. doi: 10.1073/pnas.0701728104

Turkensteen, L., Flier, W., Wanningen, R., and Mulder, A. (2000). Production, survival and infectivity of oospores of Phytophthora infestans. *Plant Physiol.* 49, 688–696. doi: 10.1046/j.1365-3059.2000.00515.x

Uprety, D. C., and Reddy, V. (2016). "Mitigation technologies to control high-temperature stress in crop plants," in *Crop Responses to Global Warming*. (Berlin: Springer), 117–125.

van Es, S. W. (2020). Too hot to handle, the adverse effect of heat stress on crop yield. *Physiol. Plant.* 169, 499–500. doi: 10.1111/ppl.13165

Van Oosten, M. J., Pepe, O., De Pascale, S., Silletti, S., and Maggio, A. (2017). The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol.* 4, 5. doi: 10.1186/s40538-017-0089-5

Vasseur, F., Pantin, F., and Vile, D. (2011). Changes in light intensity reveal a major role for carbon balance in Arabidopsis responses to high temperature. *Plant Cell Environ.* 34, 1563–1576. doi: 10.1111/j.1365-3040.2011.02353.x

Velásquez, A. C., Castroverde, C. D. M., and He, S. Y. (2018). Plant–pathogen warfare under changing climate conditions. *Curr Biol.* 28, R619–R634. doi: 10.1016/j.cub.2018.03.054

Vollenweider, P., and Günthardt-Goerg, M. S. (2005). Diagnosis of abiotic and biotic stress factors using the visible symptoms in foliage. *Environ. Pollut.* 137, 455–465. doi: 10.1016/j.envpol.2005.01.032

Wahid, A., and Close, T. (2007). Expression of dehydrins under heat stress and their relationship with water relations of sugarcane leaves. *Biol. Plant.* 51, 104–109. doi: 10.1007/s10535-007-0021-0

Wahid, A., Gelani, S., Ashraf, M., and Foolad, M. R. (2007). Heat tolerance in plants: an overview. *Environ. Exp. Bot.* 61, 199–223. doi: 10.1016/j.envexpbot.2007.05.011

Wani, S. H., Kumar, V., Shriram, V., and Sah, S. K. (2016). Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *Crop J.* 4, 162–176. doi: 10.1016/j.cj.2016.01.010

Waqas, M., Khan, A. L., Kamran, M., Hamayun, M., Kang, S.-M., Kim, Y.-H., et al. (2012). Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. *Molecules* 17, 10754–10773. doi: 10.3390/molecules170910754

Waraich, E., Ahmad, R., Halim, A., and Aziz, T. (2012). Alleviation of temperature stress by nutrient management in crop plants: a review. *J. Soil Sci. Plant Nut.* 12, 221–244. doi: 10.4067/S0718-95162012000200003

Wu, C., Cui, K., Wang, W., Li, Q., Fahad, S., Hu, Q., et al. (2016). Heat-induced phytohormone changes are associated with disrupted early reproductive development and reduced yield in rice. *Sci. Rep.* 6, 1–14. doi: 10.1038/srep34978

Wu, H.-C., Bulgakov, V. P., and Jinn, T.-L. (2018). Pectin methylesterases: cell wall remodeling proteins are required for plant response to heat stress. *Front. Plant Sci.* 9, 1612. doi: 10.3389/fpls.2018.01612

Xu, Y., Chu, C., and Yao, S. (2021). The impact of high-temperature stress on rice: challenges and solutions. *Crop J.* 9, 963–976. doi: 10.1016/j.cj.2021.02.011

Xu, Z., Liu, S., Liu, H., Yang, C., Kang, Y., and Wang, M. (2015). Unimolecular micelles of amphiphilic cyclodextrin-core star-like block copolymers for anticancer drug delivery. *Chem. Commun.* 51, 15768–15771. doi: 10.1039/C5CC02743H

Yamauchi, Y. (2018). "Integrated chemical control of abiotic stress tolerance using biostimulants," in *Plant, Abiotic Stress and Responses to Climate Change*, 133–143.

Yang, J., Kloepper, J. W., and Ryu, C.-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci.* 14, 1–4. doi: 10.1016/j.tplants.2008.10.004

Yeh, C.-H., Kaplinsky, N. J., Hu, C., and Charng, Y.-Y. (2012). Some like it hot, some like it warm: phenotyping to explore thermotolerance diversity. *Plant Sci.* 195, 10–23. doi: 10.1111/nph.14212

Yin, Y., Li, S., Liao, W., Lu, Q., Wen, X., and Lu, C. (2010). Photosystem II photochemistry, photoinhibition, and the xanthophyll cycle in heat-stressed rice leaves. *J. Plant Physiol.* 167, 959–966. doi: 10.1016/j.jplph.2009.12.021

Zafar, S. A., Noor, M. A., Waqas, M. A., Wang, X., Shaheen, T., Raza, M., et al. (2018). "Temperature extremes in cotton production and mitigation strategies," in *Past, Present and Future Trends in Cotton Breeding.* eds M. R. Rahman and Y. Zafar (New York: Intechopen), 65-91.

Zahran, H., Räsänen, L. A., Karsisto, M., and Lindström, K. (1994). Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. *World J. Microbiol. Biotechnol.* 10, 100–105. doi: 10.1007/BF00357572

Zhang, X., Cai, J., Wollenweber, B., Liu, F., Dai, T., Cao, W., et al. (2013). Multiple heat and drought events affect grain yield and accumulations of high molecular weight glutenin subunits and glutenin macropolymers in wheat. *J. Cereal Sci.* 57, 134–140. doi: 10.1016/j.jcs.2012.10.010

Zhao, D., and Li, Y.-R. (2015). Climate change and sugarcane production: potential impact and mitigation strategies. *Int. J. Agron.* 2015, 547386. doi: 10.1155/2015/547386

Zhongming, Z., and Wei, L. (2021). *How to Beat the Heat: Memory Mechanism Allows Plants to Adapt to Heat Stress.* Science Daily.

Zia, R., Nawaz, M., Yousaf, S., Amin, I., Hakim, S., Mirza, M., et al. (2021). Seed inoculation of desert-PGPR induce biochemical alterations and develop resistance against water stress in wheat. *Physiol. Plant.* 172:990–1006. doi: 10.1111/ppl.13362

Zinn, K. E., Tunc-Ozdemir, M., and Harper, J. F. (2010). Temperature stress and plant sexual reproduction: uncovering the weakest links. *J. Exp. Bot.* 61, 1959–1968. doi: 10.1093/jxb/erq053

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 16 June 2022
doi: 10.3389/fpls.2022.874858

Evaluation of the Biostimulant Activity of Zaxinone Mimics (MiZax) in Crop Plants

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Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: 13 February 2022
Accepted: 02 May 2022
Published: 16 June 2022

Citation:
Wang JY, Jamil M, Hossain MG, Chen G-TE, Berqdar L, Ota T, Blilou I, Asami T, Al-Solimani SJ, Mousa MAA and Al-Babili S (2022) Evaluation of the Biostimulant Activity of Zaxinone Mimics (MiZax) in Crop Plants. Front. Plant Sci. 13:874858. doi: 10.3389/fpls.2022.874858

Global food security is a critical concern that needs practical solutions to feed the expanding human population. A promising approach is the employment of biostimulants to increase crop production. Biostimulants include compounds that boost plant growth. Recently, mimics of zaxinone (MiZax) were shown to have a promising growth-promoting effect in rice (*Oryza sativa*). In this study, we investigated the effect of MiZax on the growth and yield of three dicot horticultural plants, namely, tomato (*Solanum lycopersicum*), capsicum (*Capsicum annuum*), and squash (*Cucurbita pepo*) in different growth environments, as well as on the growth and development of the monocot date palm (*Phoenix dactylifera*), an important crop in the Middle East. The application of MiZax significantly enhanced plant height, flower, and branch numbers, fruit size, and total fruit yield in independent field trials from 2020 to 2021. Importantly, the amount of applied MiZax was far less than that used with the commercial compound humic acid, a widely used biostimulant in horticulture. Our results indicate that MiZax have significant application potential to improve the performance and productivity of horticultural crops.

Keywords: biostimulant, apocarotenoids, zaxinone, zaxinone mimics (MiZax), green pepper (*Capsicum annuum*), squash (*Cucurbita pepo*), tomato (*Solanum lycopersicum*), date palm (*Phoenix dactylifera*)

INTRODUCTION

Ensuring food security is a global issue challenged by different factors, such as climate change, environmental pollution, and, particularly, the rapid growth of the human population (Shaheen et al., 2017). According to the report of the United Nations Food and Agriculture Organization (FAO), food production must increase to double to feed the ever-increasing human population around the world by 2050 (FAO: The World Needs 70% More Food by 2050).[1] Inarguably, enhancing the yield of crops is one of the solutions to fulfill the goal. Naturally, the growth of crop plants relies on their ability to get nutrients from the soil, which is inevitably affected by

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[1] https://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=4778

unfavorable growth conditions, such as drought, salinity, or biotic stresses (Parida and Das, 2005; Takahashi et al., 2020; Iqbal et al., 2021). Usually, these conditions negatively affect the plant's health and development, which could reduce the final yield (Pandey et al., 2017). Moreover, climate changes decrease the green land area for food production, while the increase of freshwater usage of the human population also influences crops irrigation (Zhao et al., 2017; Raza et al., 2019). To minimize these adverse impacts, one of the strategies to help the plants to overcome unfavorable growth problems is the usage of biostimulants, which accelerate not only plants' life cycle, but also maximize fruit production (Bulgari et al., 2015).

Biostimulants are recognized as compounds that enhance plant growth and performance (Du Jardin, 2015; Yakhin et al., 2017), including the specialized metabolites from carotenoid biosynthesis. Carotenoids are isoprenoid pigments that provide precursors for the evolutionary-conserved plant hormones such as abscisic acid (ABA) and strigolactones (SLs) (Al-Babili and Bouwmeester, 2015; Hou et al., 2016; Wang et al., 2021a), as well as apocarotenoid signaling molecules, such as anchorene and zaxinone (Fiorilli et al., 2019; Jia et al., 2019; Wang et al., 2019; Ablazov et al., 2020). Due to the instability of authentic metabolites or restricted natural sources, several ABA and SL analogs have been developed in the past few years and some of them have been tested in the field (Samejima et al., 2016; Screpanti et al., 2016; Jamil et al., 2019, 2020, 2022a,b; Kountche et al., 2019; Vaidya et al., 2019). The further case is zaxinone, a candidate of novel apocarotenoid-derived phytohormones, which is required for normal rice growth and development (Wang et al., 2019). Zaxinone (chemical structures shown in **Figure 1A**) exerts its function likely through promoting sugar metabolism and regulating the homeostasis of SLs and cytokinins in rice roots (Wang et al., 2019, 2021b). In addition, the application of

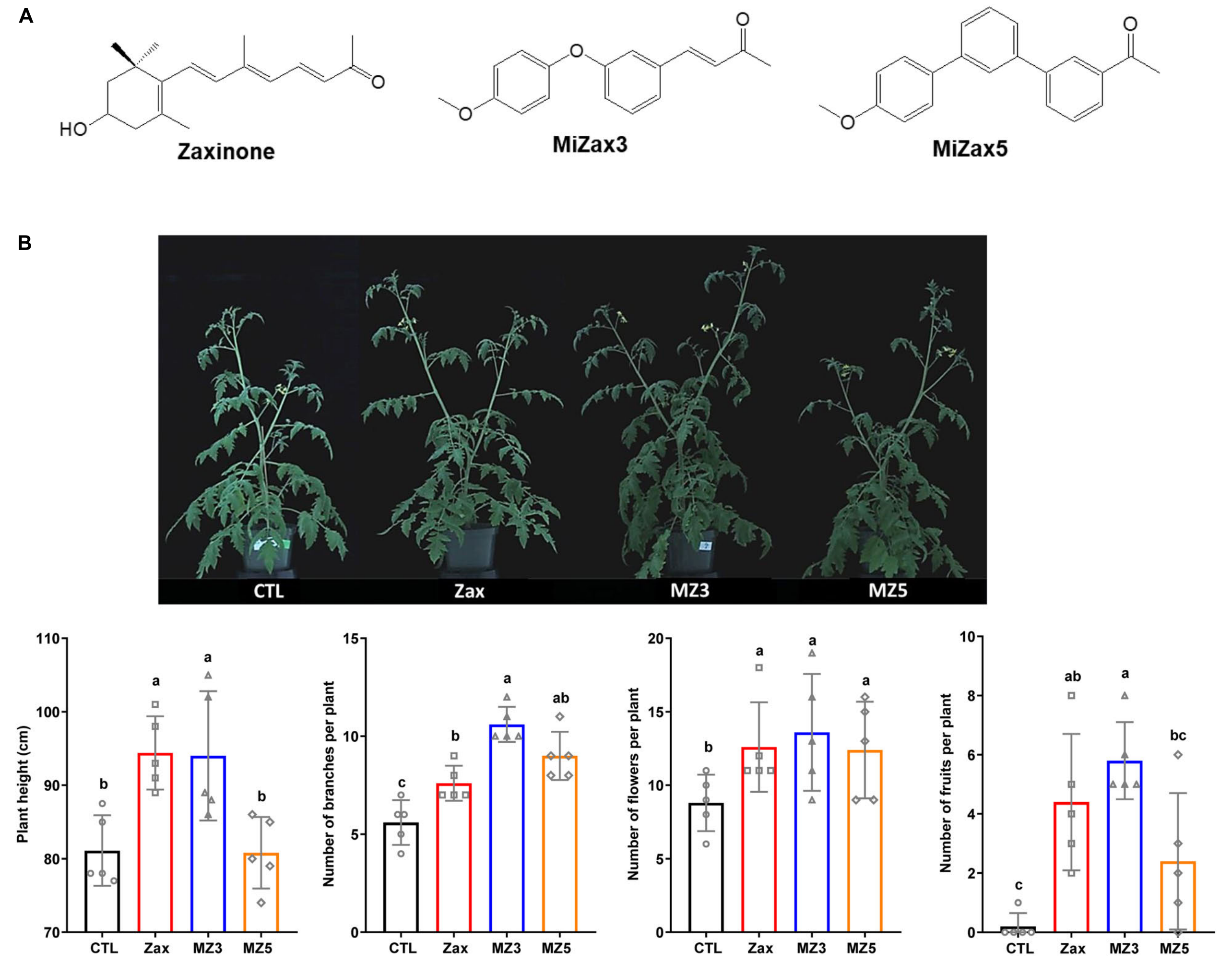

FIGURE 1 | (A) Chemical structures of zaxinone, MiZax3, and MiZax5. **(B)** Foliar application of Zax, MZ3, and MZ5 at 5 μM on tomato plants under greenhouse conditions in 2019. Each data point represents one plant (*n* = 5). Data represent mean ± SD. Statistical analysis was performed using one-way ANOVA and the Tukey's *post hoc* test. Different letters denote significant differences ($p < 0.05$). Symbols (○, CTL; □, Zax; △, MiZax3; and ◇, MiZax5) used over error bars represent each replicates in the investigated group. CTL, control; Zax, zaxinone; MZ3, MiZax3; MZ5, MiZax5.

zaxinone increased wild-type rice (*Oryza sativa*) performance, which indicated its biostimulant activity (Wang et al., 2019). Therefore, we have developed mimics of zaxinone (MiZax), which were supposed to be tested and used as biostimulants. After screening a number of mimics, we identified MiZax3 and MiZax5 (chemical structures shown in **Figure 1A**) as two compounds exerting similar biological activities of zaxinone with respect to rice grown hydroponically and in the soil systems (Wang et al., 2020). However, the growth-promoting activities of MiZax on the horticultural crops still remain elusive. It is plausible that introducing these biostimulants might bring a positive impact on yield, quality, and overall economic benefit to horticultural crops.

In this report, we tested zaxinone and MiZax on tomato [*Solanum lycopersicum* (*S. lycopersicum*)] plants under greenhouse conditions by foliar application. Moreover, to extend its practical application, we conducted four independent open field studies with three different fruit crops, namely, green pepper [*Capsicum annuum* (*C. annuum*)], squash [*Cucurbita pepo* (*C. pepo*)], and date palm [*Phoenix dactylifera* (*P. dactylifera*)] during the planting year 2020–2021 in the desert climate of Kingdom of Saudi of Arabia. Application of MiZax significantly increased the plant height and branches of date palm, as well as the flower numbers, fruit size, and total fruit yield of green pepper and squash. Notably, we observed these effects with MiZax amounts that are far less than that used for the commercial biostimulant humic acid, a common used growth regulator with positive effects on sustainable agriculture (Canellas et al., 2015 and references therein). Our results indicate that MiZax are potential biostimulants promoting the growth and yield of horticultural plants under different environmental conditions and, hence, they contribute to ensuring global food security.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato seeds (*S. lycopersicum cv*. Moneymaker) were sown in a 24-well plastic tray to raise nursery. 1-week-old uniform seedlings were then transferred to 3 L plastic pots. Each pot was sprayed with biostimulants at 5 μM concentration twice per week. The pots were irrigated with nutrient solution when needed. Data on tomato growth, yield, and yield components were collected.

For raising seedlings, green pepper seeds (*C. annuum* L. var. California Wonder) were sown on 10 October 2019 in Jiffy-7 peat pellets (44 mm) containing a peat-based substrate (peat moss) (manufactured by Norway). Before sowing, seeds were

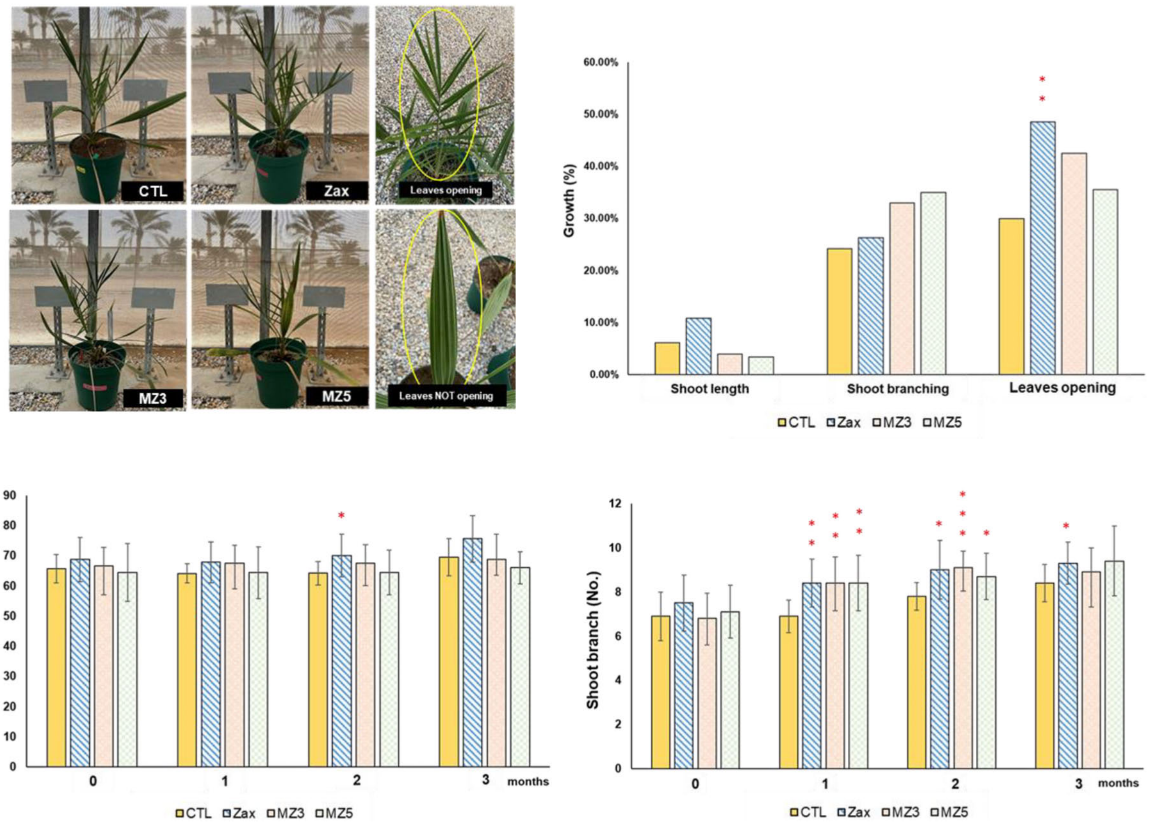

FIGURE 2 | Effect of foliar application of Zax, MZ3, and MZ5 at 5 μM on date palm plants under open field condition, performed in 2021. Data represent mean ± SD. $n = 10$. Statistical analysis was performed using the two-tailed Student's *t*-test. Asterisks indicate statistically significant differences as compared to CTL ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). CTL, control; Zax, zaxinone; MZ3, MiZax3; MZ5, MiZax5.

soaked into water overnight and pelleted for 2 h. Pellets were then placed into the trays with seeds into hole and covered very well. After the appearance of first true leaves, plants were watered as required. After 5 weeks, the seedlings were transplanted into the well-prepared plot to initiate the experiment.

Squash (*C. pepo*) seeds of the variety Camila 625 F1 Hybrid (Emerald Seeds Company, United States) were obtained from the local agricultural market at Jeddah, Saudi Arabia.

Field Trials at the King Abdulaziz University Station

The field experiments were conducted at the Agricultural Research Station, Hada Al-Sham (21°48′3″ N, 39y43′25″ E), King Abdulaziz University (KAU), Jeddah, Saudi Arabia to evaluate the performance of plant biostimulants (zaxinone and MiZax) and humic acid applications on growth and yield of green pepper under freshwater and salty water and squash under freshwater. The soil texture of the experimental site was classified as sandy loam, soil pH 7.8, and EC 1.79 d Sm^{-1}. The dominant climate of the area is arid, with high temperatures and long photoperiods during summer season (Mousa and Al-Qurashi, 2018).

Directing seeding was used to plant the squash seeds with the distance between rows of 1 m and 0.5 m between each two adjacent plants in the same row. The squash plants were irrigated with low saline water (3.51 dSm^{-1}) until end of growing season. The squash experiment followed a randomized complete block design (RCBD) with 3 replicates. Seedling of the green peppers were planted in plots each of 2.8 m × 1.8 m and the planting distance was 70 cm × 60 cm. The experiment was laid on a split-plot design where three levels of water salinity were assigned to the main plots, while mimics of zaxinone (MiZax3 and MiZax5) and humic acid were randomly distributed over the subplots following RCBD with 3 replicates. The water salinity of underground water was 3.51 d Sm^{-1} and used as control

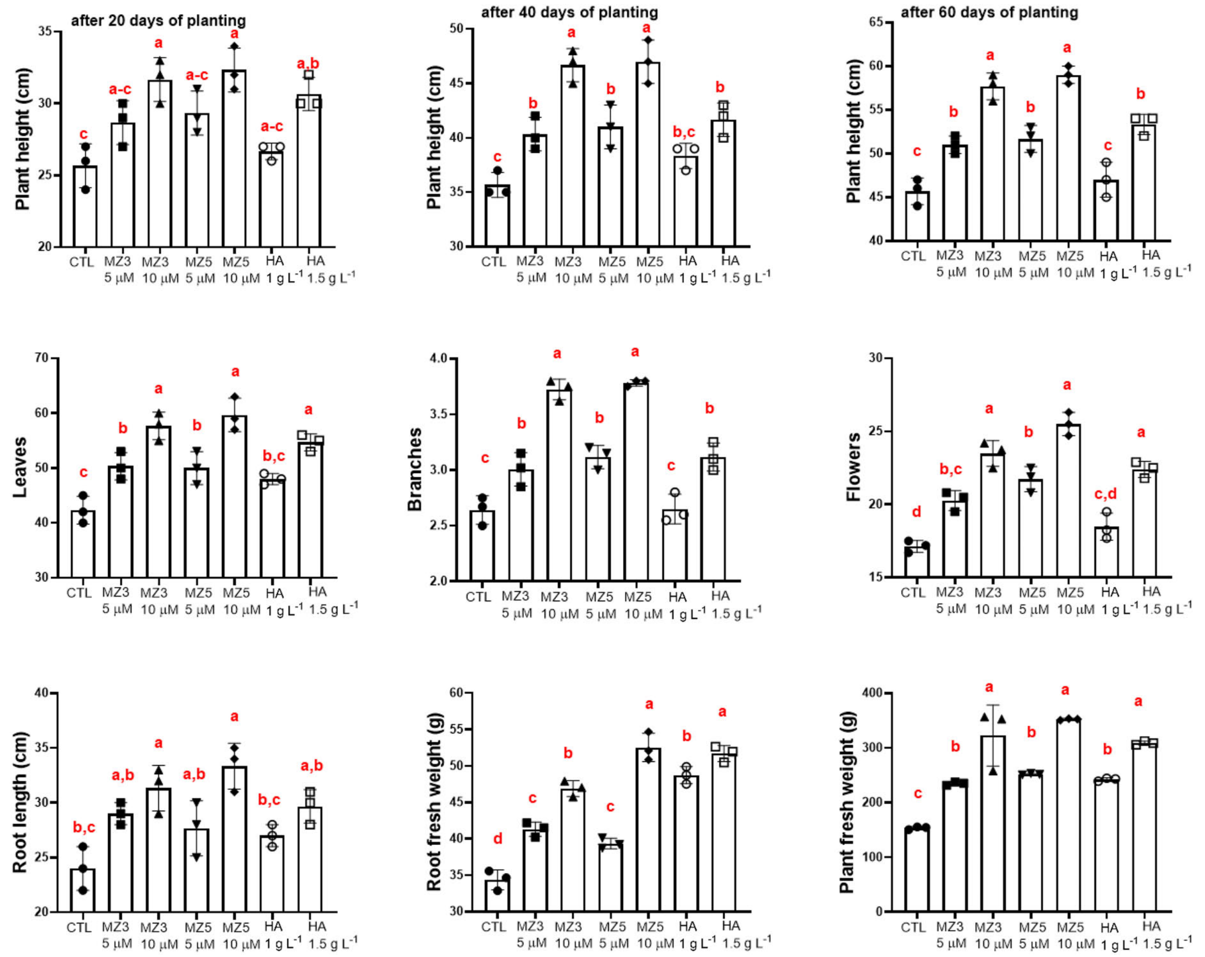

FIGURE 3 | Plant phenotypical evaluation of mimics of zaxinone (MiZax) effect on green pepper from the field of the King Abdulaziz University (KAU), performed in 2020. Each data point represents the average from 15 plants of three plots ($n = 3$). Data represent mean ± *SD*. Statistical analysis was performed using one-way ANOVA and the Tukey's *post hoc* test. Different letters denote significant differences ($p < 0.05$). Symbols (●, CTL; ■, 5 μM MZ3; ▲, 10 μM MZ3; ▼, 5 μM MZ5; ♦, 10 μM MZ5; ○, 1 g l^{-1} HA; and □, 1.5 g l^{-1} HA) used over error bars represent each replicates in the investigated group. CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

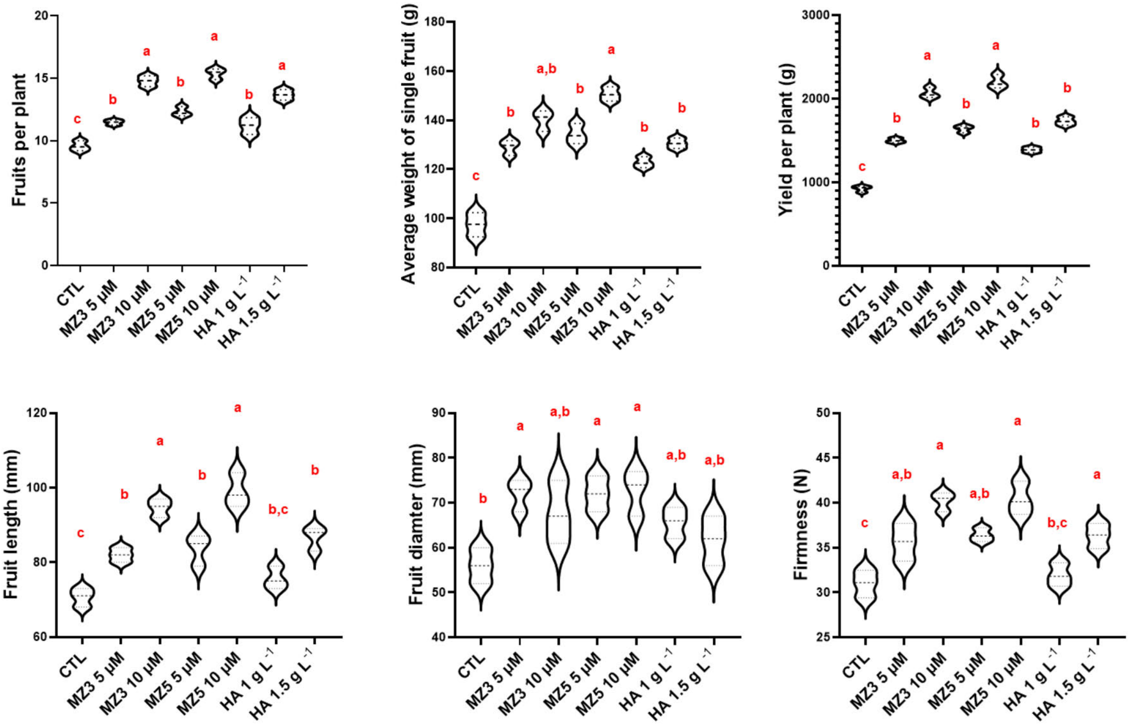

FIGURE 4 | MiZax effect on green pepper fruit production from the field of the KAU, performed in 2020. The data represents as the distribution of 15 plants from three plots (*n* = 3). Data represent mean ± *SD*. Statistical analysis was performed using one-way ANOVA and the Tukey's *post hoc* test. Different letters denote significant differences ($p < 0.05$). CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

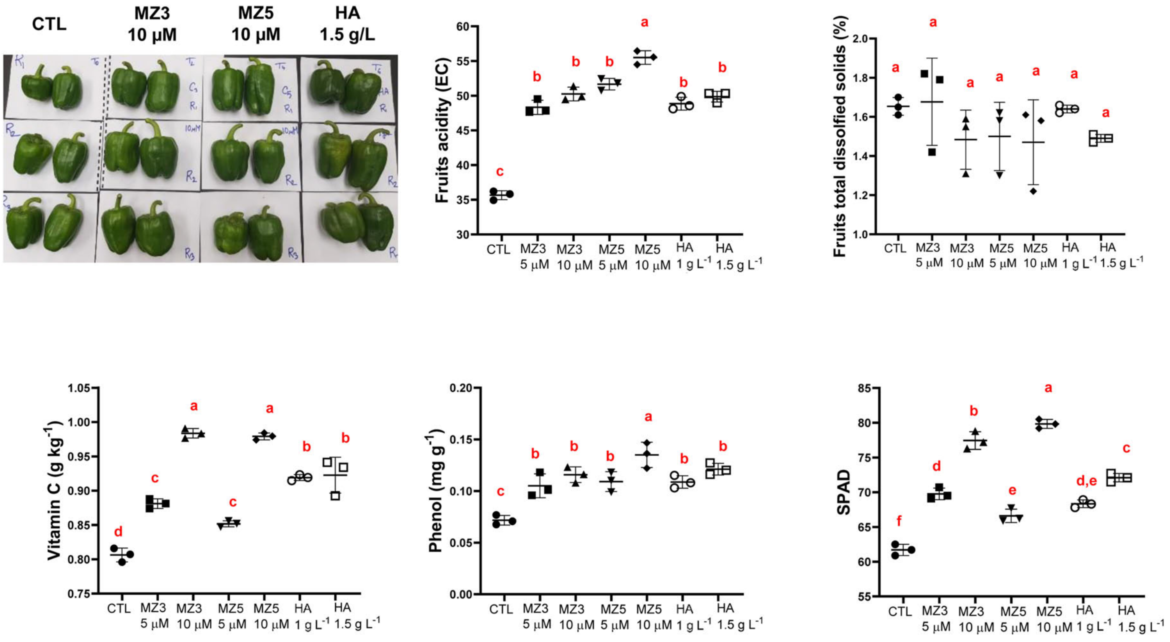

FIGURE 5 | Biochemical analysis of fruits upon MiZax treatment on green pepper from the field of the KAU, performed in 2020. Represented harvested fruits from CTL, 5 μM MZ3, 5 μM MZ5, and 1.5 g l^{-1} HA application were shown here. Each data point represents the average from 15 plants of three plots (*n* = 3). Data represent mean ± *SD*. Statistical analysis was performed using ANOVA and the Tukey's *post hoc* test. Different letters denote significant differences ($p < 0.05$). Symbols (•, CTL; ■, 5 μM MZ3; ▲, 10 μM MZ3; ▼, 5 μM MZ5; ◆, 10 μM MZ5; ○, 1 g l^{-1} HA; and □, 1.5 g l^{-1} HA) used over error bars represent each replicates in the investigated group. CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

treatment. NaCl was added weekly to the other tanks to raise the water salinity to 8.04 and 11.71 d Sm^{-1} during the season. For watering squash and green peppers, a drip irrigation system was instilled to supply the plants with their water requirements twice per day for 10 min. The plants were weekly fertilized with nitrogen, phosphorus, and potassium (NPK) fertilizer (20:20:20) during vegetative growth and 10:10:40 (N:P:K) at flowering and fruit setting and maturity stages. The biostimulants MiZax3 and MiZax5 at 5 and 10 μM and humic acid at 1 and 1.5 g L^{-1} were foliar applied once per week and for 8 weeks to the plants and after 15 days of transplantation for green peppers and at the second true leaves for squash. Two control treatments received either acetone (as MiZax3 and MiZax5 dissolvent) at 1 ml per L or only water (dissolvent of humic acid). All the recommended agriculture practices for both the crops were applied and kept uniform across the treatments throughout the growing season. The growth and yield parameters of squash, including plant height (cm), numbers of leaves per plant, fresh and dry mass per plant (g), sex ratio (male: female), fruits per plant, and yield per plant (kg), were assessed. For green peppers, we measured plant height (cm) after 20, 40, and 60 days of transplanting, branches, leaves, and flowers per plant at end of season, roots length (cm) and mass (g), plant fresh and dry mass (g), fresh fruits per plant, and yield of fresh fruits per plant (kg). Pepper fruits quality were measured, including weight of single fruit (g), fruit length and diameter (mm), total soluble solid (TSS) (%), vitamin C (mg g^{-1}), firmness (N), acidity (EC), and total phenol (mg g^{-1}). Soil plant analysis development estimations were taken utilizing SPAD meter (Konica Minolta 502, Tokyo, Japan) from 9:00 to 11:00 a.m. in fully extended third to fifth leaf.

Field Trial of the King Abdullah University of Science and Technology Experimental Station

A field experiment at the King Abdullah University of Science and Technology (KAUST) (22°18’08.6″N, 39°06’40.0″E)

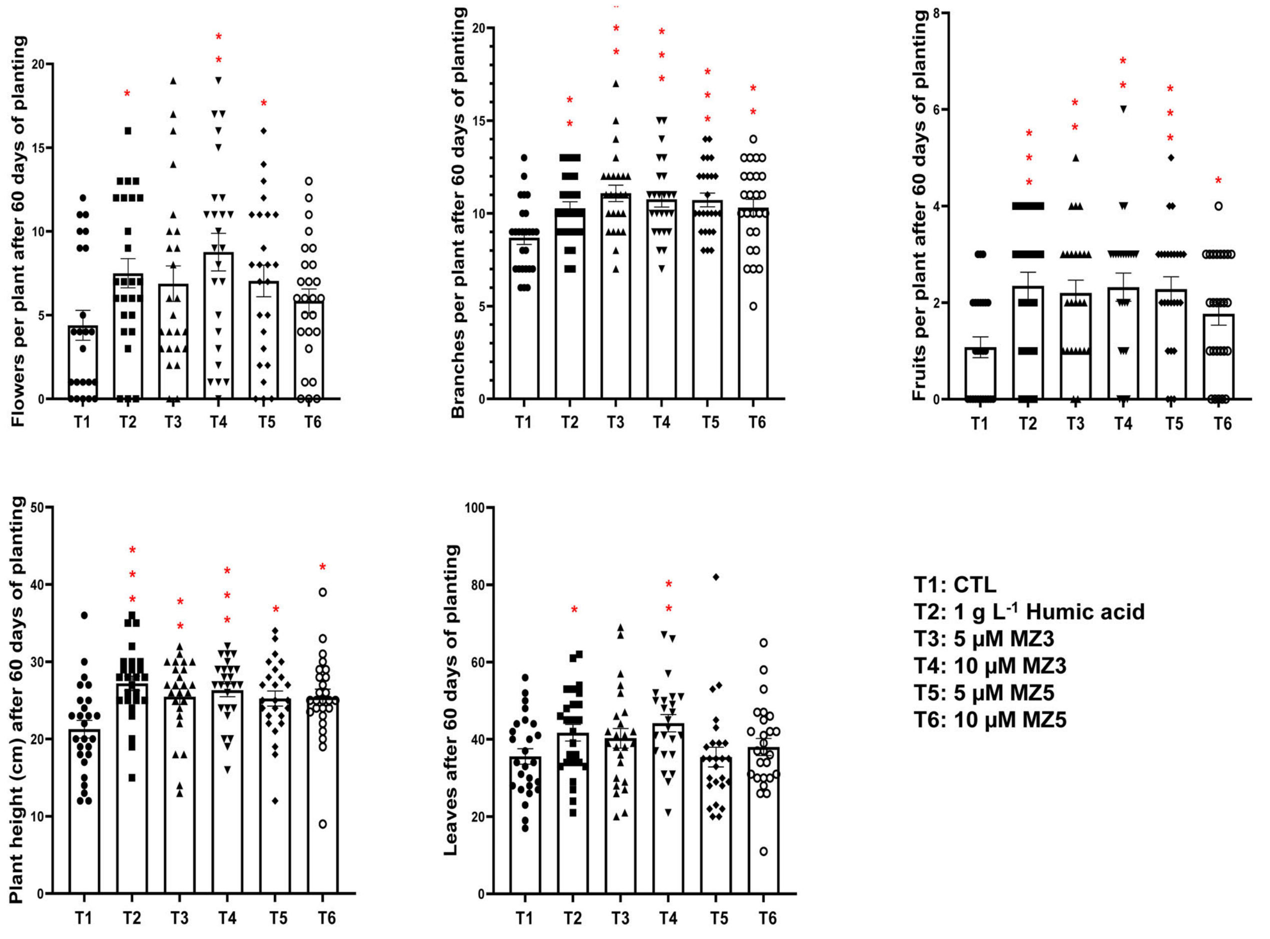

FIGURE 6 | Plant phenotypical evaluation of MiZax treatment on green pepper from the King Abdullah University of Science and Technology (KAUST) field, performed in 2021. Each data point represents one plant ($n \geq 25$). Data represent mean ± *SD*. Statistical analysis was performed using the two-tailed Student’s *t*-test. Asterisks indicate statistically significant differences as compared with CTL ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Symbols (●, CTL; ■, 1.5 g l^{-1} HA; ▲, 5 μM MZ3; ▼, 10 μM MZ3; ◆, 5 μM MZ5; and ○, 10 μM MZ5) used over error bars represent each replicates in the investigated group. CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

was conducted to evaluate the growth-promoting activity of MZ3 and MZ5 on green pepper (*C. annuum* L. var. California Wonder). After three successive plowing, the field was divided into plots and treatments were allocated by following randomized complete block design (RCBD) with three replications. The plot size was 3 m × 1.2 m and the planting distance was 50 cm × 60 cm. 4-week-old uniform-sized green pepper seedlings were transferred (8 plants per plot) into the field. MiZax3 and MiZax5 were sprayed at 5 and 10 μM concentration with 1 week interval up to 8 weeks. Humic acid was applied at 1 g l^{-1} and untreated plots were included to compare the treatments effect. The plots were irrigated and fertilized when needed and all the other agronomic practices were adopted uniformly by following the standard procedures. Data on plant growth, yield, and yield components were collected.

The field experiment of date palm (*P. dactylifera cv.* ajwa) was conducted at the KAUST study field to investigate the effect of zaxinone and MiZax. Compounds were dissolved in acetone and prepared in a final volume mixed with 0.5% Tween-20. Zaxinone, MiZax3, and MiZax5 were sprayed at 5 μM concentration once per week on the 8-week-old uniform date palm plants. The pots were irrigated with nutrient solution once per week. Data on the shoot growth, branches, and leaves opening were collected.

Statistical Analysis

Data are represented as mean and their variations as SD. The statistical significance was determined by one-way ANOVA with the Tukey's multiple comparison test, using a probability level of $p < 0.05$ or the two-tailed Student's *t*-test to denote significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). All the statistical elaborations were performed using GraphPad Prism version 8.3.0.

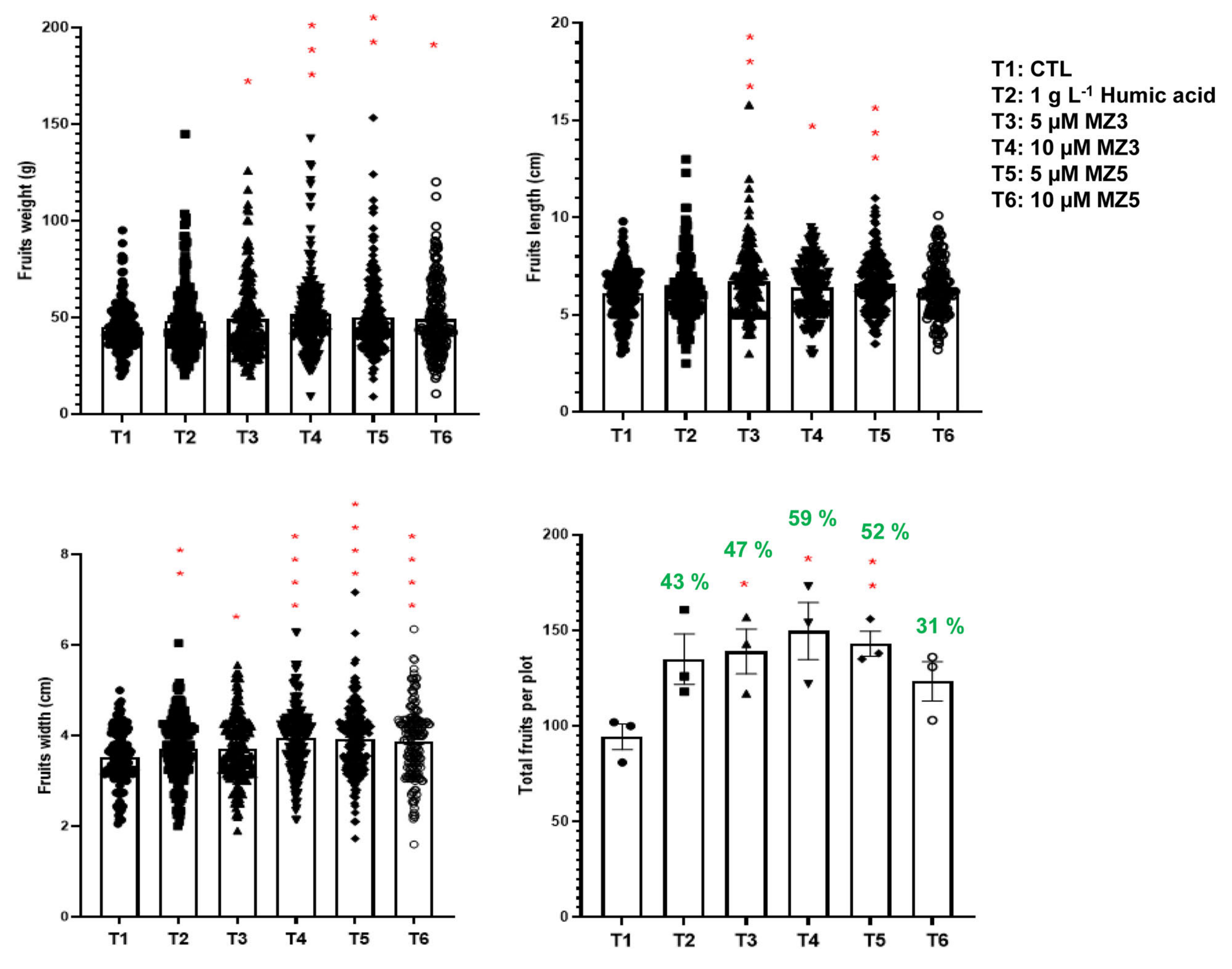

FIGURE 7 | MiZax effect on green pepper fruit production from the KAUST field, performed in 2021. Each data point represents one plant ($n \geq 166$). Data represent mean ± *SD*. Statistical analysis was performed using the two-tailed Student's *t*-test. Asterisks indicate statistically significant differences as compared to CTL ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). Symbols (●, CTL; ■, 1.5 g l^{-1} HA; ▲, 5 μM MZ3; ▼, 10 μM MZ3; ◆, 5 μM MZ5; and ○, 10 μM MZ5) used over error bars represent each replicates in the investigated group. CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

RESULTS AND DISCUSSION

Zaxinone and Mimics of Zaxinone Enhanced Tomato and Date Palm Plant Growth

Zaxinone and its mimics MiZax3 and MiZax5 showed to promote the growth on the monocot plant rice (Wang et al., 2020). Hence, we hypothesized that these compounds might show the same activity in dicots. To test this hypothesis, we sprayed 5 μM of each of the three stimulants, namely, zaxinone, MiZax3, and MiZax5, to 3-week-old tomato plants for 8 weeks in the greenhouse. Interestingly, we observed striking growth-promoting activities of MiZax3 on plant height, branching, number of flowers, and fruits (**Figure 1B**). Zaxinone and MiZax5 showed a similar, but less pronounced effects, compared to MiZax3.

Next, we applied the three stimulants to plantlets of date palm, an important regional fruit plant for sustaining current desert agriculture in the Middle East (Xiao et al., 2019), to investigate their growth-triggering effect on a monocot tree. Although date palms are slow-growing plants, zaxinone remarkably increased the number of branches, plant height, and leaves opening after 2 months of treatment, while MiZax3 and MiZax5 tended to promote growth (**Figure 2**). Moreover, these compounds seemed continuously to promote shoot branches at least 1 month after the final application, especially zaxinone led to more leaves opening (**Supplementary Figure 1**).

These results revealed that the application of MiZax and zaxinone in dicot crop plants and the monocot tree date palm has the same bioactivity as in the cereal plant rice. However, zaxinone was also shown to be a positive regulator on SL and ABA biosynthesis in the dicot plant *Arabidopsis* (Ablazov et al., 2020), which may inhibit root growth and development and indicate that the effect of this compound may differ depending on the plant species. Indeed, both the tomato and date palm contain *ZAXINONE SYNTHASE* (*ZAS*) gene (s) and can form a symbiosis with arbuscular mycorrhizal fungi (AMF). In contrast, *ZAS* is missing in most of the non-mycorrhizal plant, such as *Arabidopsis thaliana* (Wang et al., 2019), which indicates a role of *ZAS* in regulating growth as well as in AM symbiosis. A further point that should be considered is the method of application. Foliar application may lead to different effects, compared with adding the compound to the medium in hydroponic culture as it was done with *Arabidopsis* (Ablazov et al., 2020). Our results also indicate that MiZax3, MiZax5, and zaxinone may differ in

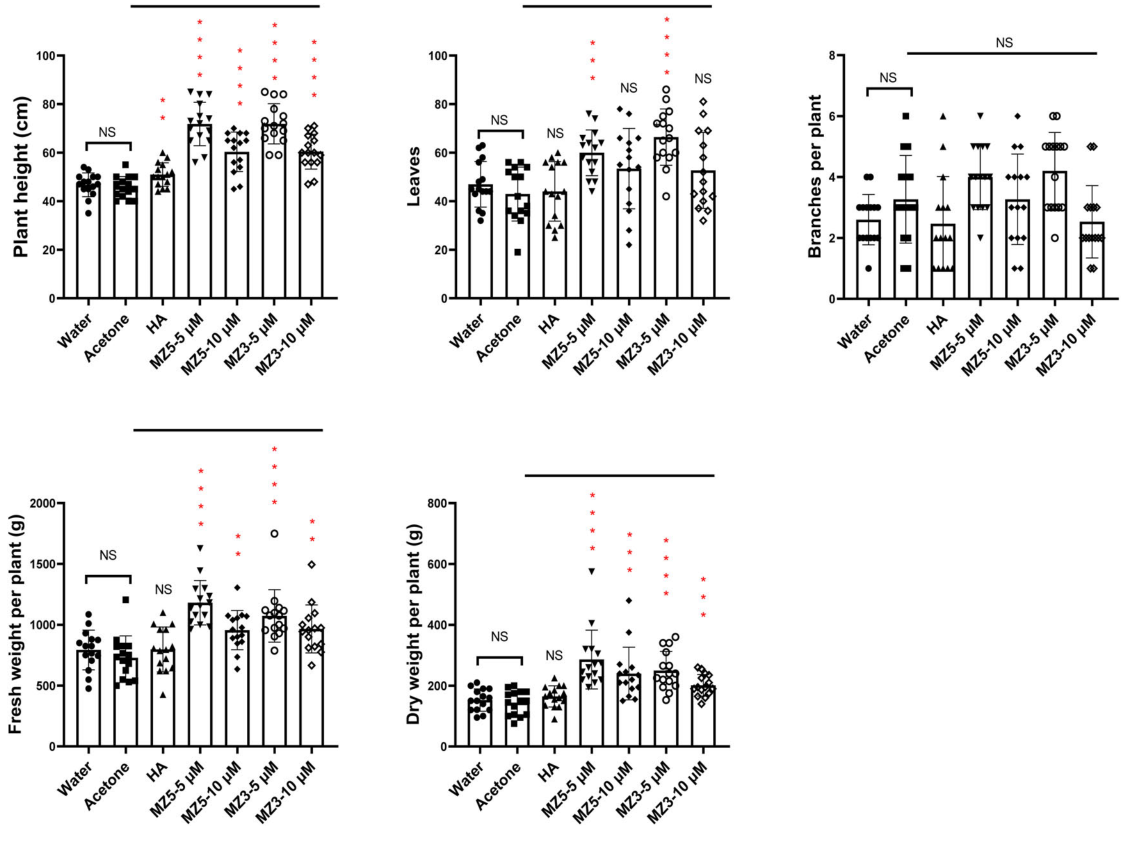

FIGURE 8 | Plant phenotypical evaluation of MiZax on squash from the field of the KAU, performed in 2021. MiZax effect on crop production from the field of the KAU in 2021 (n = 15). Statistical analysis was performed using the two-tailed Student's *t*-test. Asterisks indicate statistically significant differences as compared to CTL (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Symbols (●, CTL; ■, Acetone; ▲,1.5 g L^{-1} HA; ▼, 5 μM MZ5; ◆, 10 μM MZ5; ○, 5 μM MZ3; ◇ 10 μM MZ3) used over error bars represent each replicates in the investigated group. CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

particular activities. In summary, MiZax3 and MiZax5 are good candidates for new biostimulants (Yakhin et al., 2017) that can be used to boost tomato and rice plant performance and increase the growth of date palm plantlets under controlled conditions.

Field Application of Boosted Growth and Yield of Green Pepper Under in Normal Environment and Salty Soil

To explore the possibility of using MiZax on field application, we tested two concentrations (5 and 10 μM) of MiZax3 and MiZax5 on the valuable crop, green pepper, in comparison to the commercial compound humic acid. We observed that MiZax significantly triggered shoot growth from 20 to 60 days after planting in the Agricultural Research Station of the King Abdulaziz University (KAU) (**Figure 3**) and clearly boosted the development of flowers, leaves, and branching (**Figure 3**) in a concentration-dependent manner. Moreover, the root length, root biomass, and plant fresh weight of pepper plants were also increased after MiZax and humic acid application (**Figure 3**). Surprisingly, MiZax showed, at a concentration of at 5 μM, a comparable effect to 1.5 g l^{-1} of humic acid. Moreover, the performance of MiZax increased, when applied at 10 μM concentration. These positive results were also observed in salty conditions under which MiZax maintained and increased the development of pepper plants (**Supplementary Figure 2**), indicating that MiZax also increased salt tolerance.

Consistent with the observation from plant physiological results about growth and development, the application of MiZax enhanced fruit production and total yield (**Figure 4**). In addition,

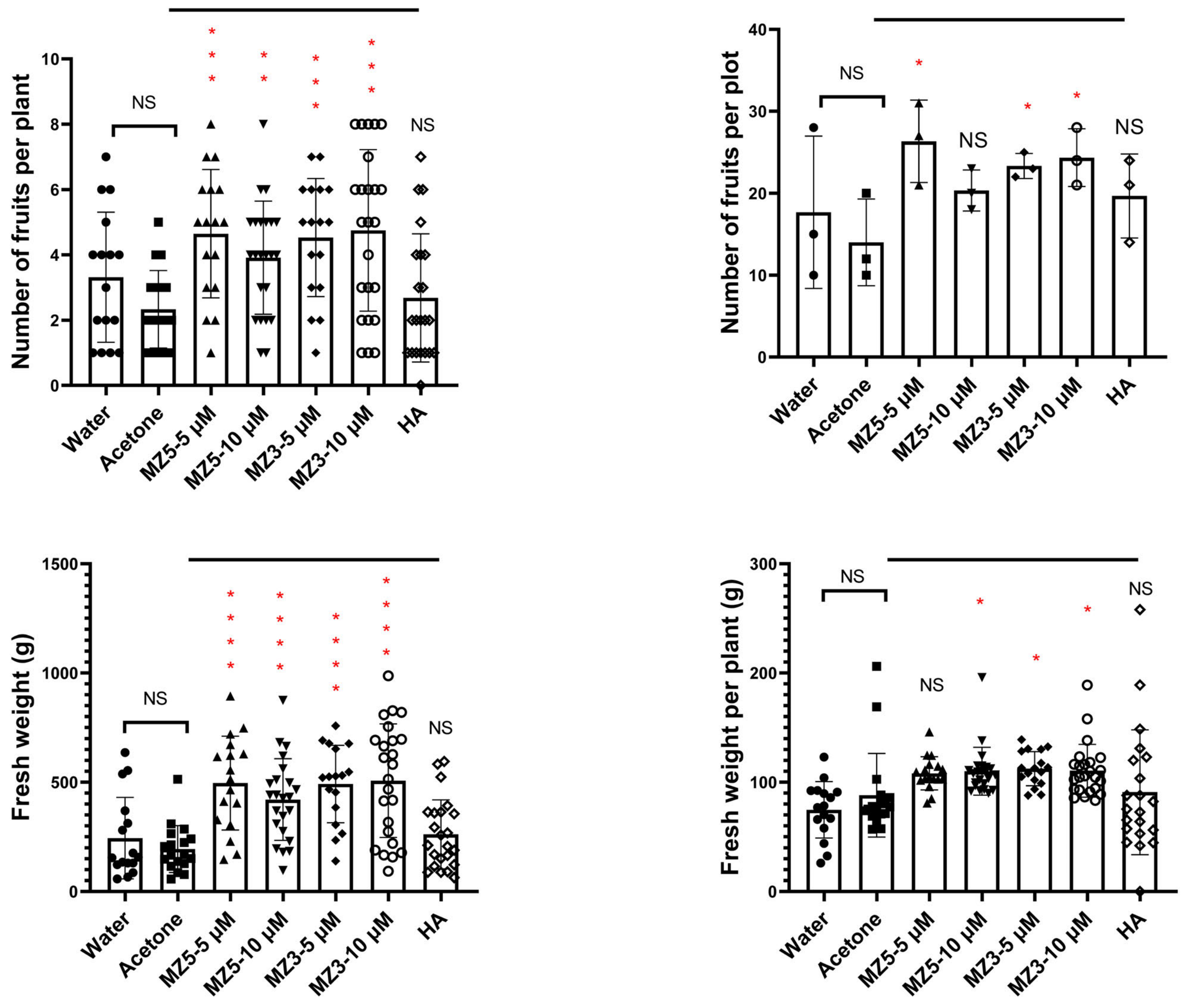

FIGURE 9 | MiZax effect on squash fruit production from the field of the KAU, performed in 2021 ($n \geq 16$). Statistical analysis was performed using the two-tailed Student's *t*-test. Asterisks indicate statistically significant differences as compared to CTL (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Symbols (●, CTL; ■, Acetone; ▲, 5 μM MZ5; ▼, 10 μM MZ5; ◆, 5 μM MZ3; ○, 10 μM MZ3; and ◇, 1.5 g l^{-1} HA) used over error bars represent each replicates in the investigated group. CTL, Control; HA, humic acid; MZ3, MiZax3; MZ5, MiZax5.

the length and width, as well as the firmness of fruits were also enhanced, compared with the control group (**Figure 4**), even under salty fields, except the diameter under high salt condition (**Supplementary Figure 3**). To assess the quality of fruits harvested in the KAU field, we measured the content of vitamin C and phenols and quantified the acidity and total dissolved solids. As shown in **Figure 5** and **Supplementary Figure 4**, except for the total dissolved solids, all the biochemical parameters were increased in all the treatments. Although there was no superior difference between MiZax and humic acid, the amount of MiZax used was less than that of humic acid ($\sim$3.5 vs. 1 g l^{-1}), which represents a big advantage of MiZax. Moreover, the leaf single-photon avalanche diode (SPAD) values were higher after applying the high concentration of MiZax treatments (**Figure 5** and **Supplementary Figure 4**). This result indicates that MiZax could increase the levels of chlorophyll and might enhance the photosynthetic activities, probably by increasing leaf numbers. A positive effect of zaxinone on photosynthesis in rice was recently reported (Wang et al., 2021b). Moreover, the increased fruit size suggests that MiZax might also enhance source-to-sink allocation, as zaxinone triggers primary metabolism in rice plants (Wang et al., 2021b).

To verify our findings in the KAU field, we further conducted another trial in 2021 at the King Abdullah University of Science and Technology (KAUST) experimental field, a warmer and more moisture place next to the Red Sea. Following the study design in the KAU, we compared 1 g l^{-1} of humic acid to 5 and 10 μM of MiZax. Constantly, the plant height, branches, flowers, and fruits of the green pepper were substantially increased after 60 days of planting (**Figure 6**), while the number of leaves showed high variability, which was due to windy climate. Furthermore, the weight, length, and width of harvested fruits were also considerably enhanced by MiZax (**Figure 7**), compared to humic acid and untreated control plot. Most importantly, MiZax caused 31–59% increase in total yield per plant (**Figure 7**). However, we observed in this trial that the lower concentration of MiZax5 had a better effect than 10 μM, which might suggest that higher concentrations of MiZax5 could not be optimal for plant growth and development. Although MiZax did not disturb the arbuscular mycorrhiza fungi spore germination (Wang et al., 2020), the safe application range of MiZax should be further studied.

Mimics of Zaxinone Treatment Increased Squash Production in the Field

Finally, to explore the suitability to other horticultural crops, we performed a trial on squash in the KAU field. Again, MiZax significantly boosted the height and biomass of squash plants, while humic acid failed to promote growth (**Figure 8**). Although no difference in the branches, the leaves number was observed following the low concentration of MiZax treatments (**Figure 8**). Surprisingly, only MiZax application significantly triggered the fruit production and weight, no matter per plant or plot, whereas humic acid did not show expected activity (**Figure 9**). We also observed that the low concentration of both MiZax had better bioactivity than the higher one, which indicates that MiZax should be applied in lower amounts in future studies. Additionally, the acetone mock did not show any difference compared to the water control group (**Figures 8**, **9**), which suggests that the solvent used to dissolve MiZax did not play a significant role in these studies.

CONCLUSION

In conclusion, based on our three independent field studies, the amount of applied MiZax was much lower (at the micromolar or mg level) than the commercial biostimulant humic acid with a better bioactivity in promoting crop plant growth and development. Thus, MiZax are potential biostimulants enhancing the performance and production of horticultural plants (monocots and dicots).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SA-B, IB, SA-S, and MM proposed the concept. JYW, MJ, and Md GH designed the experiments. JYW, MJ, Md GH, G-TEC, and LB conducted the experiments. JYW, MJ, Md GH, IB, MM, and SA-B analyzed and discussed the data. TO and TA synthesized and provided MiZax3 and MiZax5. JYW, MJ, and SA-B wrote the manuscript. All the authors have read, edited, and approved the final version of the manuscript.

FUNDING

This study was supported by baseline funding given to SA-B from the King Abdullah University of Science and Technology (KAUST).

ACKNOWLEDGMENTS

We sincerely thank Dr. Justine Braguy, Dr. Yağız Alagöz, and Dr. Abrar Felemban for their assistance in the KAUST field study. We also thank the field work team at the KAU Agricultural Research Station for their help to conduct the field trials at the KAU. We appreciate the support from the members of the KAUST Plant Growth Core Laboratory and the Bioactives Laboratory.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.874858/full#supplementary-material

REFERENCES

Ablazov, A., Mi, J., Jamil, M., Jia, K.-P., Wang, J. Y., Feng, Q., et al. (2020). The Apocarotenoid Zaxinone Is a Positive Regulator of Strigolactone and Abscisic Acid Biosynthesis in Arabidopsis Roots. *Front. Plant Sci.* 11:578. doi: 10.3389/fpls.2020.00578

Al-Babili, S., and Bouwmeester, H. J. (2015). Strigolactones, a Novel Carotenoid-Derived Plant Hormone. *Annu. Rev. Plant Biol.* 66, 161–186. doi: 10.1146/annurev-arplant-043014-114759

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., and Ferrante, A. (2015). Biostimulants and crop responses: a review. *Biol. Agric. Hortic.* 31, 1–17. doi: 10.1080/01448765.2014.964649

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., et al. (2015). Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* 196, 15–27. doi: 10.1016/j.scienta.2015.09.013

Du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14.

Fiorilli, V., Wang, J. Y., Bonfante, P., Lanfranco, L., and Al-Babili, S. (2019). Apocarotenoids: old and new mediators of the arbuscular mycorrhizal symbiosis. *Front. Plant Sci.* 10:1186. doi: 10.3389/fpls.2019.01186

Hou, X., Rivers, J., León, P., McQuinn, R. P., and Pogson, B. J. (2016). Synthesis and function of apocarotenoid signals in plants. *Trends Plant Sci.* 21, 792–803. doi: 10.1016/j.tplants.2016.06.001

Iqbal, Z., Iqbal, M. S., Hashem, A., AbdAllah, E. F., and Ansari, M. I (2021). Plant defense responses to biotic stress and its interplay with fluctuating dark/light conditions. *Front. Plant Sci.* 12:297. doi: 10.3389/fpls.2021.631810

Jamil, M., Kountche, B. A., Haider, I., Wang, J. Y., Aldossary, F., Zarban, R. A., et al. (2019). Methylation at the C-3' in D-ring of strigolactone analogs reduces biological activity in root parasitic plants and rice. *Front. Plant Sci.* 10:353. doi: 10.3389/fpls.2019.00353

Jamil, M., Kountche, B. A., Wang, J. Y., Haider, I., Jia, K.-P., Takahashi, I., et al. (2020). A new series of carlactonoic acid based strigolactone analogs for fundamental and applied research. *Front. Plant Sci.* 11:434. doi: 10.3389/fpls.2020.00434

Jamil, M., Wang, J. Y., Yonli, D., Ota, T., Berqdar, L., Traore, H., et al. (2022a). *Striga hermonthica* suicidal germination activity of potent strigolactone analogs: evaluation from laboratory bioassays to field trials. *Plants* 11:1045. doi: 10.3390/plants11081045

Jamil, M., Wang, J. Y., Yonli, D., Patil, R. H., Riyazaddin, M., Gangashetty, P., et al. (2022b). A new formulation for Strigolactone suicidal germination agents, towards successful *Striga* management. *Plants* 11:808. doi: 10.3390/plants11060808

Jia, K.-P., Dickinson, A. J., Mi, J., Cui, G., Xiao, T. T., Kharbatia, N. M., et al. (2019). Anchorene is a carotenoid-derived regulatory metabolite required for anchor root formation in Arabidopsis. *Sci. Adv.* 5:eaaw6787. doi: 10.1126/sciadv.aaw6787

Kountche, B. A., Jamil, M., Yonli, D., Nikiema, M. P., Blanco-Ania, D., Asami, T., et al. (2019). Suicidal germination as a control strategy for *Striga hermonthica* (Benth.) in smallholder farms of sub-Saharan Africa. *Plants People Planet* 1, 107–118. doi: 10.1002/ppp3.32

Mousa, M. A. A., and Al-Qurashi, D. A. (2018). Growth and yield of cowpea (Vigna unguiculata L.) cultivars under water deficit at different growth stages. *Legume Res.Int. J.* 41, 702–709. doi: 10.1007/s12298-021-01089-w

Pandey, P., Irulappan, V., Bagavathiannan, M. V., and Senthil-Kumar, M. (2017). Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. *Front. Plant Sci.* 8:537. doi: 10.3389/fpls.2017.00537

Parida, A. K., and Das, A. B. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotoxicol. Environ. Saf.* 60, 324–349. doi: 10.1016/j.ecoenv.2004.06.010

Raza, A., Razzaq, A., Mehmood, S. S., Zou, X., Zhang, X., Lv, Y., et al. (2019). Impact of climate change on crops adaptation and strategies to tackle its outcome: a review. *Plants* 8:34. doi: 10.3390/plants8020034

Samejima, H., Babiker, A. G., Takikawa, H., Sasaki, M., and Sugimoto, Y. (2016). Practicality of the suicidal germination approach for controlling Striga hermonthica. *Pest Manag. Sci.* 72, 2035–2042. doi: 10.1002/ps.4215

Screpanti, C., Fonné-Pfister, R., Lumbroso, A., Rendine, S., Lachia, M., and De Mesmaeker, A. (2016). Strigolactone derivatives for potential crop enhancement applications. *Bioorganic Med. Chem. Lett.* 26, 2392–2400.

Shaheen, S., Ahmad, M., and Haroon, N. (2017). *Edible Wild Plants: an Alternative Approach to Food Security*. Germany: Springer.

Takahashi, F., Kuromori, T., Urano, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2020). Drought stress responses and resistance in plants: from cellular responses to long-distance intercellular communication. *Front. Plant Sci.* 11:556972. doi: 10.3389/fpls.2020.556972

Vaidya, A. S., Helander, J. D., Peterson, F. C., Elzinga, D., Dejonghe, W., Kaundal, A., et al. (2019). Dynamic control of plant water use using designed ABA receptor agonists. *Science* 366:eaaw8848. doi: 10.1126/science.aaw8848

Wang, J. Y., Alseekh, S., Xiao, T., Ablazov, A., Perez de Souza, L., Fiorilli, V., et al. (2021a). Multi-omics approaches explain the growth-promoting effect of the apocarotenoid growth regulator zaxinone in rice. *Commun. Biol.* 4:1222. doi: 10.1038/s42003-021-02740-8

Wang, J. Y., Haider, I., Jamil, M., Fiorilli, V., Saito, Y., Mi, J., et al. (2019). The apocarotenoid metabolite zaxinone regulates growth and strigolactone biosynthesis in rice. *Nat. Commun.* 10:810. doi: 10.1038/s41467-019-08461-1

Wang, J. Y., Jamil, M., Lin, P.-Y., Ota, T., Fiorilli, V., Novero, M., et al. (2020). Efficient mimics for elucidating zaxinone biology and promoting agricultural applications. *Mol. Plant* 13, 1654–1661. doi: 10.1016/j.molp.2020.08.009

Wang, J. Y., Lin, P.-Y., and Al-Babili, S. (2021b). On the biosynthesis and evolution of apocarotenoid plant growth regulators. *Semin. Cell Dev. Biol.* 109, 3–11. doi: 10.1016/j.semcdb.2020.07.007

Xiao, T. T., Raygoza, A. A., Pérez, J. C., Kirschner, G., Deng, Y., Atkinson, B., et al. (2019). Emergent protective organogenesis in date palms: a morpho-devo-dynamic adaptive strategy during early development. *Plant Cell* 31, 1751–1766. doi: 10.1105/tpc.19.00008

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Zhao, C., Liu, B., Piao, S., Wang, X., Lobell, D. B., Huang, Y., et al. (2017). Temperature increase reduces global yields of major crops in four independent estimates. *Proc. Nat. Acad. Sci. U. S. A.* 114, 9326–9331. doi: 10.1073/pnas.1701762114

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 28 July 2022
DOI 10.3389/fpls.2022.884716

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SPECIALTY SECTION
This article was submitted to
Plant Symbiotic Interactions,
a section of the journal
Frontiers in Plant Science

RECEIVED 26 February 2022
ACCEPTED 05 July 2022
PUBLISHED 28 July 2022

CITATION
de Paula AF, Cruz FPN, Dinato NB, de Andrade PHM, de Moraes ACP, Junior WB, Bernardi ACC, Vigna BBZ, Fávero AP and Lacava PT (2022) Endophytic and rhizospheric bacteria associated with *Paspalum atratum* and its potential for plant growth promotion with different phosphate sources. *Front. Plant Sci.* 13:884716. doi: 10.3389/fpls.2022.884716

Endophytic and rhizospheric bacteria associated with *Paspalum atratum* and its potential for plant growth promotion with different phosphate sources

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The genus *Paspalum* belongs to the family Poaceae and has several species that are native to Brazil. The *Paspalum* Germplasm Bank (GB) of the Brazilian Agricultural Research Corporation comprises approximately 450 accessions from 50 species. Among these accessions, *Paspalum atratum* (BGP 308) has economic potential for forage purposes. However, the endophytic and rhizospheric microbial communities within this accession and their ability to promote plant growth remain unknown. The present study aimed to isolate the endophytic and rhizospheric bacteria associated with *P. atratum* and to assess their potential for plant growth improvement, so-called plant growth-promoting bacteria (PGPB). For the *in vitro* tests, the ability of nitrogen-fixing bacteria (NFB), phosphate solubilization (PS) and indoleacetic acid (IAA) production were evaluated. A total of 116 endophytic and rhizosphere bacteria were obtained from the isolation. In the *in vitro* tests, 43 (37.00%) of these isolates showed positive NFB, PS, and IAA results. These isolates were identified by 16S rDNA sequencing. The phosphate solubilization index (PSI) ranged from 2 to 3.61, all 43 strains performed biological nitrogen fixation and the IAA production ranged from 12.85 to 431.41μgml^{-1}. Eight of these 43 isolates were evaluated *in vivo* in a greenhouse using *P. atratum* caryopsis. The pots were filled with soil prepared with three different phosphate sources and one control without phosphate. After growth, the plants were submitted to morphological, bromatological and chemical determination. Data were analyzed using analysis of variance (ANOVA) and principal component analysis (PCA). In the *in vivo* test, treatments 105 (*Pseudomonas* sp.) and 458 (*Pseudomonas* sp.) were the most significant for the crystalline phosphate source, 109 (*Bacillus* sp.) for the sedimentary phosphate source and, as for the soluble phosphate source most treatments that received bacterial isolates had higher phosphorus content in the dry matter than the uninoculated soluble phosphate control. The 105FCR (crystalline phosphate+*Pseudomonas* sp.), 109FSE (sedimentary phosphate+*Bacillus* sp.), and 110 FSE (sedimentary phosphate+*Enterobacter* sp.) treatments showed the best results for plant growth promotion. This work made it possible to determine the bacterial community associated with *P. atratum* (BGP308) and to obtain new potential plant growth-promoting strains.

KEYWORDS

bacterial community, genetic resources, biological nitrogen fixation, indoleacetic acid, phosphate solubilization

Introduction

Pasture is the main form of animal nutrition used for herds. Due to the potential for growth in different soils and climate conditions and requiring little management (Rodrigues et al., 2014), the genus *Urochloa* currently constitutes the majority of Brazilian pastures used for animal feed. The main problem in regard to *Urochloa* spp. is exposure to ecological imbalances owing to its low genetic variability (Compant et al., 2010). However, *Paspalum* is an important and highly diverse genus in the Poaceae family in the Americas (Novo et al., 2016), occurring throughout Brazil, Bolivia, Paraguay, Argentina, Chile and Uruguay (Zuloaga and Morrone 2005; Novo et al., 2019). Therefore, *Paspalum* spp. could potentially replace pastures composed of *Urochloa* spp. or could occupy areas where these grasses do not grow. Brazil is one of the largest meat producers and exporters globally; therefore, there is a need to research new sources and ways to carry out animal nutrition. According to the *Brazilian Association of Meat Exporting Industries* (ABIEC), Brazil exported approximately 8.50 million tons of beef in 2020 and 4.38 million tons in the first half of 2021, significantly contributing to Brazil's economy (ABIEC, 2021).

Another critical point is that with the increase in cultivated areas, agrochemical use and environmental impacts also increase. Thus, alternative nonpolluting and more economical methods of promoting plant growth have gained greater attention (Coelho et al., 2019; Xiang et al., 2020).

Phosphate fertilizers are among the most commonly used agrochemicals in agriculture, and the lack of adequate levels of these fertilizers is a limiting factor for plant growth (Crous et al., 2019). Therefore, plants usually receive soluble phosphorus through industrialized fertilizers. In addition, some phosphate rocks can be applied for direct soil fertilization (Ahemad and Kibret, 2014). First, however, it is necessary to transform this phosphorus into a soluble form for plants. Phosphate-solubilizing bacteria (PSB) transform this phosphorus by chelation, ion exchange, and organic acid production (Khan et al., 2009; Afzal et al., 2019). Among the types of insoluble phosphate rocks are the sedimentary rocks of Arad (33.0% P_2O_5) and the crystalline rocks of Cajati/SP (5.0% P_2O_5) (Alves and Hagni, 2008; Ramos et al., 2009).

Paspalum accessions from the germplasm bank of Embrapa Pecuaria Sudeste have been evaluated for different uses, such as forage (Marcón et al., 2018) and turf (Souza et al., 2020). The studies developed with *Paspalum* plants from this GB have included characterization regarding shade stress (Barro et al., 2012), water stress (De Pezzopane et al., 2017), insect tolerance (Gusmão et al., 2016), and cryopreservation for new hybrid production (Dinato et al., 2018). However, studies related to the endophytic and rhizospheric bacterial microbiota diversity associated with this germplasm collection are still scarce.

Among the *Paspalum* accessions evaluated in the Brazilian Agricultural Research Corporation breeding program, BGP 308 from *P. atratum* Swallen is a promising accession for becoming a forage cultivar and for composing the preliminary studies of the endophytic and rhizospheric microbiota.

The analysis of the bacterial diversity associated with this species can indicate new microorganisms to be used for plant growth promotion in the forage *plant P. atratum*. Thus, to collaborate with works that seek alternative and sustainable ways to use phosphorus to avoid the environmental and economic impacts caused by the industrial process of obtaining industrial phosphorus.

The objective of this study was to search for phosphate-solubilizing bacteria, evaluate their ability to solubilize phosphate rocks, and promote plant growth.

The present study is the first report to examine phosphate rock in the nutrition of *P. atratum* plants, intermediated by cultivable endophytic and rhizospheric bacteria.

Materials and methods

The work was divided into steps *in vitro* and *in vivo*. In the *in vitro* stage, there was isolation, identified and evaluated of the functional capacity of the plant growth-promoting bacterial strains. The three main characterizations performed were for phosphate solubilization (PS), nitrogen-fixing bacteria (NFB) and indoleacetic acid (IAA) production. In the *in vivo* stage, *P. atratum* plants were characterized in a morphological, nutritional and mineral manner (emphasizing phosphate solubilization), when inoculated or not with plant growth-promoting bacterial strains.

Isolation of endophytic and rhizospheric bacteria

Samples (rhizospheric soils, roots, and leaves) from an adult plant of *P. atratum* BGP 308 (BRA 030078/VRcMmSv 14,525) were collected in August 2016 (dry season) and January 2017 (rainy season). Access BGP 308 belongs to the *Paspalum* germplasm bank, located at Brazilian Agricultural Research Corporation[1] (21°57′42″S, 47°50′28″W, 860 m), São Carlos, SP, Brazil.

The endophytic bacterial community was isolated according to Araújo et al. (2014) and Bogas et al. (2015). Plant tissues were superficially disinfected by serial washes in 70% ethanol (EtOH) for 2 minutes, followed by 3% sodium hypochlorite for 3 minutes, 1 minute in 70% EtOH, and two rinses with sterile distilled water. Plant tissues were incubated in phosphate buffered saline (PBS) for 2 h at 28°C/200 rpm. Aliquots of 100 μl of decimal dilutions were inoculated in duplicate in plates containing tryptone soya agar (TSA) supplemented with Benlate ($50\,\mu g\,ml^{-1}$) to prevent fungal growth.

The isolation of rhizospheric bacteria was performed according to Mohite (2013) with modifications, in which the temperature used was 28°C, and aliquots of 100 μl of decimal dilutions were inoculated in duplicate in plates containing tryptic soy agar (TSA) supplemented with Benlate ($50\,\mu g\,ml^{-1}$). Bacterial cultures were preserved in tryptone soya broth (TSB) supplemented with glycerol (1:1) at −80°C until further study.

Strain identification

Total DNA was extracted according to Aljanabi and Martinez (1997). The 16S gene was amplified using the primers V3F (5′-ACTCCTACGGGAGGCAGCAG-3′) and V6R (5′ ACAGCC ATGCANCACCT 3′; Yang et al., 2016). Polymerase chain reaction (PCR) containing 60 ng of genomic DNA, 25 μl of Thermo Scientific PCR Master Mix (1.25 U of Taq polymerase enzyme, 1 × PCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 50 mM $MgCl_2$, and 1.25 mM dNTP) and 3 pmol of each primer was performed for the selected isolates. The reaction conditions consisted of an initial 95°C step for 3 min, followed by 31 cycles of 95°C for 30 s., 60°C for 30 s., 72°C for 1 min. and a final extension of 10 min at 72°C in a BioRad T100 thermocycler. The amplicons were examined by 0.7% agarose gel electrophoresis and purified by using a QIAquick (Qiagen) kit. The sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and sequenced using an ABI 3730 DNA Analyzer. Sequences were analyzed by Sequencing Analysis 5.3.1 software using the Base Caller KB, and the low-quality sequences were visualized and edited using BioEdit software (Hall, 1999). The final sequence was subjected to BLASTn bacterial identification (Altschul et al., 1997; Maidak et al., 2000; Garrity et al., 2004).

The phylogenetic tree was obtained by multiple alignments of *P. atratum* endophytes and 16S rRNA sequences were retrieved from NCBI using the algorithm ClustalW and generated using MEGA X software (Kumar et al., 2018) using the maximum likelihood method in combination with a general time reversible model with 1,000 bootstrap replications as branch support (Saitou and Nei, 1987; Tamura et al., 2011). *Halobacterium* sp. (NR_113428.1) sequence was used as an outgroup.

In vitro evaluation of plant growth-promoting bacteria

Bacteria isolated from *P. atratum* were qualitatively/quantitatively screened in triplicate for their ability to solubilize inorganic calcium phosphate. Strains were incubated in nutrient agar supplemented with $Ca_3(PO4)_2$ for 96 h at 28°C (Rodríguez and Fraga, 1999; Verma et al., 2001). As a result, the phosphate solubilization index (PSI) was calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter and was classified as low ($PSI < 2$), medium ($2 < PSI < 3$), and high ($PSI > 3$; Silva Filho and Vidor, 2000; Pande et al., 2017).

The nitrogen-fixing bacteria (NFB) assay was carried out by growing the strains in a semisolid nitrogen-free medium twice for 72 h/28°C (Döbereiner et al., 1995). For the quantification of auxin production, the strains were grown in the broth tryptone de soy 10% medium supplemented with L-tryptophan for 72 h/28°*Ca.* This method was initially proposed by Bric et al. (1991) and adapted as a quantitative method (Husen, 2016).

In vivo PGPB assay

Caryopsis of *P. atratum* were germinated and inoculated with eight different strains that presented $PSI \geq 2.0$. The experiment was conducted in a greenhouse at the Brazilian Agricultural Research Corporation (see footnote 1), São Carlos,

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[1] https://www.embrapa.br/en/pecuaria-sudeste

SP, Brazil. A randomized block design with three replications was used in a factorial scheme of 8 × 3 + 1 with pots containing 0.5 l and 4.5 l of soil.

Soil preparation and treatments

The soil was prepared with three different sources of phosphate plus the control without phosphate, totaling 36 treatments and 108 pots. The three sources of P used were (1) soluble phosphate—triple granulated superphosphate (46.0% P_2O_5 soluble in neutral ammonium citrate + water), (2) sedimentary phosphate—Arad phosphate rock concentrate (33.0% P_2O_5 total), and (3) crystalline phosphate—Cajati phosphate rock concentrate (5.0% P_2O_5 total) with a dose of 200 $mg\,kg^{-1}$ P or 458 $mg\,kg^{-1}$ P_2O_5.

The chemical characteristics of the soil were determined according to Van Raij et al. (2001). Based on the soil analysis results, dolomitic limestone (total neutralizing power ratio, TNPR = 70%) was added to achieve a base saturation of 60% prior to transplanting. Then, the three phosphorus sources were applied at the transplanting of the seedlings, and the pots were fertilized with K_2SO_4 (60% K_2O) until K reached 3% of the cation exchange capacity (CEC).

Germination, seedling transplantation, and inoculations

The caryopses of the spikelets were removed and subjected to the disinfection process in a closed desiccator using the protocol described by Quesenberry et al. (2010). The caryopses were inserted into 16 × 100 mm test tubes containing Murashige and Skoog (MS) medium according to the Orbovic and Grosser (2006) seed germination protocol. After 14 days, the seedlings were removed from the test tube and inserted into a Falcon tube with 15 ml of bacterial suspension for 30 min at 28°C. The liquid MS medium was standardized at 10^9 CFU/ml and used in this step. Thus, seedlings were transplanted into 500 ml pots containing limed soil without correction of nutrients. Five more inoculations were performed: in 500 ml pots, they received two more inoculations (15 and 30 days after transplant), and in 4.5 kg pots, they received three more inoculations (45, 60, and 75 days after transplant). Finally, the 6 ml volume of the standardized bacterial suspension was inoculated into the soil close to each plant's root. The control was inoculated with phosphate buffered saline (PBS) solution without the bacterial isolate.

Morphological, nutritional, and mineral analysis

The seedlings remained in the 500 ml pots for 43 days and were transplanted into the 4.5 kg pots for the four treatments. At 50 days after transplanting, the aerial parts of the plants were cut 15 cm from ground level and measured.

The aerial part samples were placed in an oven with forced circulation at 60°C for 72 h. After drying the samples and determining the dry leaf weight, the material was crushed in a Wiley mill with 1 mm sieves. The collected material was stored in a plastic bottle.

Thirty-two descriptors were evaluated: EP (phosphorus extract), EN (nitrogen extract), ECa (calcium extract), CP (crude protein), MM (mineral matter), LIG (lignin), Ca (calcium), Mg (magnesium), P (phosphorus), K (potassium), S (sulfur), Mn (manganese), Zn (zinc), N (nitrogen), SPAD (SPAD index), AFW (aerial fresh weight), ADW (aerial dry weight), LW (leaf width), NL (number of leaves), NT (number of tillers), ANT (presence of anthocyanin), LA (leaf area), EZn (zinc extract), DIV (*in vitro* digestibility), FDN (neutral fiber detergent), Ll (leaf length), PHe (plant height), Fe (iron), FDA (acid detergent fiber), MS (dry matter), EE (ether extract) and Cu (copper). Dry matter, crude protein, neutral detergent fiber, acid detergent fiber, lignin, and *in vitro* digestibility were determined using a near-infrared spectrometer (NIRS; Büchi Labortechnik, 2007) with a calibrated curve for *Paspalum*.

Based on Nogueira et al. (1998), the total nutrient content was determined. Nitrogen was determined in the extract of sulfuric digestion by the semimicro Khjeldhal method. The determination of K was made in the extract of nitro-perchloric digestion and determined by flame photometry. The other macronutrients (P, Ca, Mg, and S) and micronutrients (Cu, Fe, Mn, and Zn) were determined in the same nitro-perchloric extract and determined by induced plasma spectrometry (ICP–OES).

Statistical analysis

Thirty-two traits (morphological, nutritional, and mineral) were analyzed. Data were analyzed using analysis of variance (ANOVA) and principal component analysis (PCA) with SAS® 9.3 software (SAS Institute Inc, 2011).

Results

Isolation of endophytic and rhizospheric bacteria

In the rainy season isolation, the bacterial population ranged from 02×10^{-1} cfu gm^{-1} (leaf) to 43×10^{-3} cfu gm^{-1} (root), whereas in the dry season isolation, the bacterial population ranged from 05×10^{-1} cfu gm^{-1} (leaf) to 35×10^{-2} cfu gm^{-1} (rhizosphere; Table 1). In the rainy season, the soil had a pH of 5.4 (water) and 4.8 ($CaCl_2$), and in the dry season, it was 5.6 (water) and 5.0 ($CaCl_2$).

A total of 116 bacterial isolates were collected from *P. atratum* BGP 308, where 43 (37.00%) were obtained from rhizospheric

TABLE 1 Isolation of endophytic and rhizospheric bacteria from soil samples.

Samples	Isolation medium	Dilution	Amount of sample (ml)	Dilution factor (D)	Number of colony (24 h)	Mean cfu per 10 mg sample
Rhizosphere	TSA	10^{-2}	0.1	10^{2}	40	40×10^{-2}
Root	TSA	10^{-3}	0.1	10^{3}	43	43×10^{-3}
Leaf	TSA	10^{-1}	0.1	10^{1}	2	02×10^{-1}

soils, 42 (36.20%) from roots, and 31 (26.70%) from leaves. A total of 74 (63.70%) strains were isolated in the rainy season and 42 (36.20%) in the dry season.

Identification and evaluation of the functional capacity of the plant growth-promoting bacterial strains

Among the 116 strains obtained, 43 (37.00%) showed positive NFB, SF, and IAA results. From these, the strains that belonged to the genera *Enterobacter* (46.50%), *Pseudomonas* (32.50%), and *Pantoea* (13.90%) were the most abundant (Figure 1). On the other hand, *Bacillus*, *Microbacterium*, and *Micrococcus* strains represented only 6.90%. The phylogenetic analysis showed highly significant support (>98%) for the groups formed of samples from each genus and its correspondent reference sequences for the genus. Moreover, nodes forming a group with all the samples from genera *Enterobacter*, *Pantoea* and *Pseudomonas* and another group with all the samples from genera *Bacillus*, *Microbacterium* and *Micrococcus* also showed significant support (>90%), showing the relation among genera identified in this study (Figure 1).

The phosphate solubilization index (PSI) ranged from 2 to 3.61 (Table 2). Strains 103, 89, and 102, which are root endophytes and belong to the *Enterobacter* genus, showed the best results, with PSI values of 3.61, 3.58, and 3.56, respectively. All 43 strains in Table 2 performed biological nitrogen fixation, characterized by a semisolid nitrogen-free medium. The IAA production ranged from 12.85 to 431.41 μg ml^{-1}. The endophytic leaf strain 170 (*Pantoea* sp.) showed the highest IAA production (431.41 μg ml^{-1}), followed by 18 *Enterobacter* spp.; endophytes from roots presented 105.05–263.74 μg ml^{-1} of IAA production.

All strains belonging to *Microbacterium*, *Micrococcus*, *Pantoea*, *Bacillus*, and *Enterobacter* were endophytes, while the *Pseudomonas* strains were either endophytic or rhizospheric. Among the 43 strains selected for the *in vitro* tests, six were isolated from the rhizosphere, 27 from the root, and 10 from the leaf (Table 3).

Plant growth-promotion assay

In the plant growth promotion assay, the selected strains belonged to the rhizosphere, root and leaf. The PSI ranged from 2 to 3.61, and all were positive for NFB and IAA. The rhizospheric bacteria selected for the *in vivo* test were Isolates 25 (*Pseudomonas* sp.) and 458 (*Pseudomonas* sp.), and the endophytic bacteria were Isolates 103 (*Enterobacter* sp.), 105 (*Pseudomonas* sp.), 109 (*Bacillus* sp.), 110 (*Enterobacter* sp.), 161 (*Pseudomonas* sp.), and 170 (*Pantoea* sp.).

The triple interaction [Source of Phosphorus (3) vs. Isolate (9) vs. Cut (3)] for Phosphorus extract (P) was significant with p-value = 0.0023.

The P extract showed that in treatments with crystalline phosphate, the mean phosphorus content in dry matter ranged from 0.90 to 3.82 kg ha^{-1}, considering treatments and cuts (Figure 2). The treatments that received strains 105 (*Pseudomonas* sp.) and 458 (*Pseudomonas* sp.) had a higher phosphorus content than the control crystalline phosphate in the first cut and showed a drop in the second third cut. The treatment containing Strain 110 (*Enterobacter* sp.) presented a high level of phosphorus in the second cut compared to the control. Within this treatment, P extract increased in the second cut, and decreased it in the first and third cut. Despite the numerical variations, in the phosphorus content, there was no statistical difference ($p > 0.05$). In treatments with sedimentary phosphate, the mean phosphorus content in dry matter ranged from 0.89 to 11.82 kg ha^{-1}, considering treatments and cuts. The treatment that received the bacterial isolate 109 (*Bacillus* sp.) had a higher phosphorus content than the sedimentary phosphate control in the first cut ($p > 0.05$). Within this treatment, the phosphorus content was higher in the first cut, followed by the second and third cut ($p \leq 0.05$). The treatment that received strain 103 (*Enterobacter* sp.) also showed an increase in phosphorus content in the first cut. In the other treatments there were no ($p > 0.05$) significant changes in the phosphorus content in the cuts.

In treatments with soluble phosphate, the mean phosphorus content in dry matter ranged from 3.14 to 24.00 kg ha^{-1}, considering treatments and cuts. Regardless of treatment, the phosphorus content was higher in the first cut ($p \leq 0.05$). In this cut, the lowest phosphorus content in the dry matter was in the treatment that received the strain 103 (*Enterobacter* sp.) and the highest was in the treatment that received the strain 161 (*Pseudomonas* sp.) with 11.65 and 24.00 kg ha^{-1}, respectively. The treatment that received the bacterial isolate 110 (*Enterobacter* sp.) showed a significant difference ($p \leq 0.05$) between the three cuts, for the phosphorus content, with 21.66, 7.73 and 3.98 kg ha^{-1}, respectively. The phosphorus content, in cut1, for the control of uninoculated soluble phosphate was 15.21 kg ha^{-1}. In cuts 2 and 3, the maximum phosphorus content reached was 7.73 kg ha^{-1}, regardless of the treatments.

The results of the principal component analysis (PCA) showed that Component 1 (PRIN1 = 65.31%) and Component 2

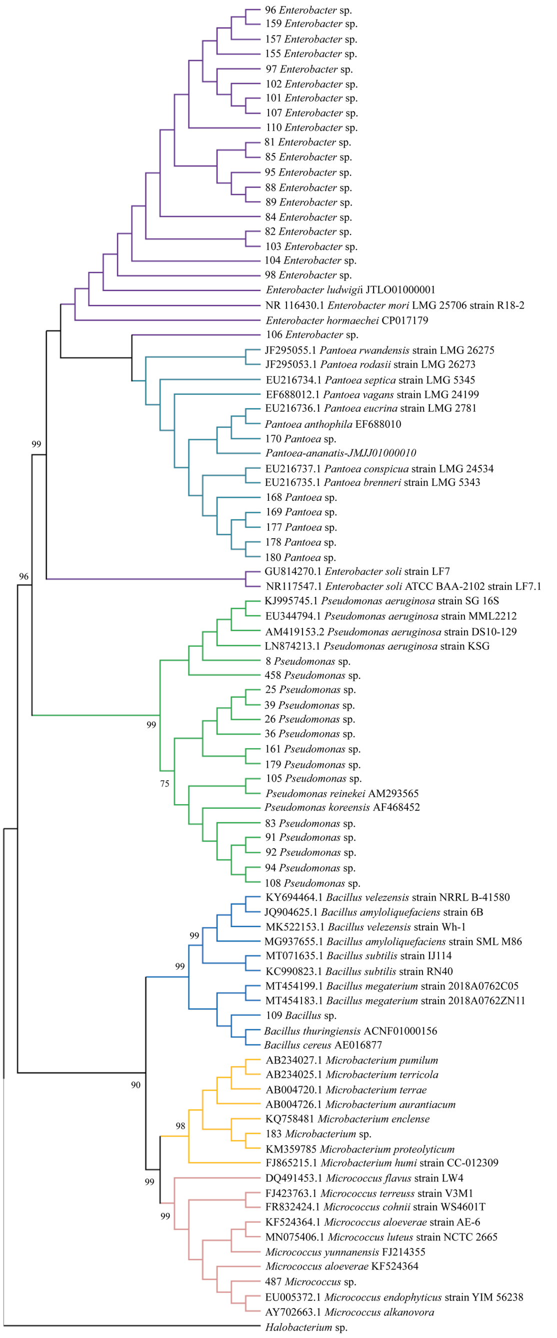

FIGURE 1
Consensus tree obtained from a maximum likelihood phylogenetic analysis using the general time reversible model (bootstrap with 1,000 replicates) based on 670bp of the partial 16S rDNA gene.

(PRIN2 = 8.52%) explained 73.83% of the variance observed. A total of 22 variables were significant (correlation > 50%) in the discrimination of treatments (Table 4) among the 32 variables used.

When evaluating the phosphate source used in the experiment, the analyzed variables divided the treatments into two groups (Figure 3), directed by Principal Components 1 (PRIN1) and 2 (PRIN2). The treatments that received soluble phosphate are distributed on the left side of the graph. In contrast, the treatments without phosphate (control) and those receiving sedimentary and crystalline phosphate are distributed on the right side. For the most significant morphological, mineral and bromatological variables, the principal component analysis showed that Principal Component 1 had significant associations with the SPAD index (SPAD), potassium (K), zinc (Zn), crude protein (CP), lignin (LIG) and nitrogen (N) (Figure 2). Furthermore, these variables were responsible for grouping the nine treatments (103 FSO, 105 FSO, 109 FSO, 110 FSO, 161 FSO, 170 FSO, 25 FSO, 458 FSO, and CAFSO) that received the soluble phosphate source in Group 1.

Principal Component 2 showed strong associations with aerial fresh weight (AFW), aerial dry weight (ADW), leaf width (LW), number of leaves (NL), number of tillers (NT), anthocyanin (ANT), area leaf (LA), phosphorus extract (EP), calcium extract (ECa), calcium (Ca), magnesium (Mg), phosphorus (P), sulfur (S), manganese (Mn), nitrogen (EN) and mineral matter (MM) (Figure 2). These variables grouped the 27 treatments (103FCR, 103FSE, 103SF, 105FCR, 105FSE, 105SF, 109FCR, 109FSE, 109SF, 110FCR, 110FSE, 110SF, 161FCR, 161FSE, 161SF, 170FCR, 170FSE, 170SF, 25FCR, 25FSE, 25SF, 458FCR, 458FSE, 458SF, CAFCR, CAFSE and CASF) that did not receive the soluble phosphate source in Group 2.

Tables 5, 6 describe the order of descriptors that most contributed to the morphological variation observed in Principal Component 1 (PRIN 1) of the principal component analysis (PCA) of all treatments evaluated in this work. Analyzing the most important descriptors (SPAD, K, Zn, CP, LIG and N), it can be observed that the treatments that received soluble phosphate presented lower values (Tables 5, 6) for these descriptors, and this located these treatments in group 1 (Figure 3). The inverse of values for these descriptors are represented in the procedures that they located in group 2.

In Figure 4, the principal component graph shows the two groups and treatments closest to those receiving phosphorus from the soluble phosphate source. The group on the right side of the graph belongs to treatments with soluble phosphate. In contrast, the group to the left and central part of the graph belongs to treatments containing sedimentary phosphate, crystalline phosphate, and no phosphate (control). Any treatment that received sedimentary and crystalline phosphate was not observed, composing the group of those that received the soluble phosphate. Nevertheless, the ones that came closest were the 105FCR (crystalline phosphate + *Pseudomonas* sp.), 109FSE (sedimentary phosphate + *Bacillus* sp.), and 110FSE (sedimentary phosphate + *Enterobacter* sp.) treatments.

Discussion

In the production of pastures for animal nutrition, there is great concern regarding the cultivation of plants. For cultivation, plants obtain most of their nutrients from some industrialized source. In 2018, Brazil consumed 852.4 thousand tons of triple

TABLE 2 Molecular identification of the genera of the 43 bacterial strains that showed positive results for the phosphate solubilization index (PSI), nitrogen-fixing bacteria (NFB), and indole acetic acid (IAA) production.

Sample	Bacterial genus	Strains code	Accession number (GenBank)	PSI	NFB	IAA (μg.ml^{-1})
Root	*Enterobacter* spp.	103	MK521286	3.61	+	165.29
Root	*Enterobacter* spp.	89	MK521276	3.58	+	222.62
Root	*Enterobacter* spp.	102	MK521285	3.56	+	134.54
Leaf	*Pantoea* spp.	169	MK521301	3.41	+	66.04
Leaf	*Micrococcus* spp.	487	MK521314	3.38	+	47.38
Leaf	*Pantoea* spp.	180	MK521306	3.36	+	61.09
Root	*Enterobacter* spp.	84	MK521273	3.28	+	164.52
Leaf	*Pantoea* spp.	178	MK521304	3.18	+	52.33
Root	*Enterobacter* spp.	101	MK521284	3.10	+	133.37
Root	*Enterobacter* spp.	106	MK521289	3.10	+	105.05
Leaf	*Pantoea* spp.	177	MK521303	3.10	+	59.32
Leaf	*Pantoea* spp.	170	MK521302	3.06	+	431.41
Root	*Enterobacter* spp.	85	MK521274	2.99	+	204.32
Leaf	*Pantoea* spp.	168	MK521300	2.97	+	28.04
Root	*Enterobacter* spp.	88	MK521275	2.88	+	148.75
Root	*Enterobacter* spp.	155	MK521296	2.88	+	263.74
Root	*Enterobacter* spp.	107	MK521290	2.87	+	131.33
Root	*Enterobacter* spp.	110	MK521293	2.85	+	87.6
Root	*Enterobacter* spp.	104	MK521287	2.81	+	166.95
Root	*Enterobacter* spp.	82	MK521271	2.77	+	187.87
Root	*Enterobacter* spp.	81	MK521270	2.76	+	216.78
Root	*Enterobacter* spp.	98	MK521283	2.75	+	187.73
Root	*Enterobacter* spp.	97	MK521282	2.7	+	135.85
Root	*Pseudomonas* spp.	83	MK521272	2.69	+	23.25
Root	*Pseudomonas* spp.	94	MK521279	2.65	+	25.24
Rhizosphere	*Pseudomonas* spp.	458	MK521308	2.62	+	65.12
Root	*Pseudomonas* spp.	91	MK521277	2.62	+	21.64
Root	*Enterobacter* spp.	157	MK521297	2.62	+	142.23
Root	*Pseudomonas* spp.	92	MK521278	2.61	+	21.71
Root	*Bacillus* spp.	109	MK521292	2.58	+	18.26
Leaf	*Pseudomonas* spp.	161	MK521299	2.55	+	72.15
Root	*Pseudomonas* spp.	108	MK521291	2.47	+	14.54
Root	*Enterobacter* spp.	95	MK521280	2.46	+	205.29
Root	*Enterobacter* spp.	159	MK521298	2.45	+	158.29
Root	*Enterobacter* spp.	96	MK521281	2.44	+	88.12
Leaf	*Pseudomonas* spp.	179	MK521305	2.39	+	12.85
Leaf	*Microbacterium* spp.	183	MK521307	2.23	+	20.79
Rhizosphere	*Pseudomonas* spp.	8	MK521261	2.20	+	16.09
Root	*Pseudomonas* spp.	105	MK521288	2.18	+	35.85
Rhizosphere	*Pseudomonas* spp.	26	MK521263	2.02	+	27.75
Rhizosphere	*Pseudomonas* spp.	25	MK521262	2.00	+	44.85
Rhizosphere	*Pseudomonas* spp.	36	MK521264	2.00	+	24.39
Rhizosphere	*Pseudomonas* spp.	39	MK521265	2.00	+	27.33

superphosphate (largest consumer on a global scale) and 2,437.7 thousand tons of ammonium phosphate (third consumer on a global scale). For nitrogen, phosphorus, and potassium (NPK) fertilizer, Brazil consumed 15507.1 thousand tons and imported 13338.7 thousand tons, the largest importer of NPK on a worldwide scale (International Fertilizer Industry Association, 2021). Microorganisms can provide nutrients from alternative and nonindustrialized sources to plants. The main focus of this work was to address phosphate solubilization by plant growth-promoting bacteria (PGPB).

TABLE 3 Bacterial genus, number of strains, origin and isolation period of strains of BGP 308 from *P. atratum* that were positive for phosphate solubilization, biological nitrogen fixation, and indole acetic acid production.

Bacterial genus	Origin and isolation period of strains					
	Rhizosphere (Dry)	Rhizosphere (Rainy)	Root (Dry)	Root (Rainy)	Leaf (Dry)	Leaf (Rainy)
Bacillus spp.	–	–	1	–	–	–
Enterobacter spp.	–	–	20	–	–	–
Microbacterium spp.	–	–	–	–	1	–
Micrococcus spp.	–	–	–	–	–	1
Pantoea spp.	–	–	–	–	6	–
Pseudomonas spp.	5	1	6	-	2	-

Traces means no microorganism was selected for a given condition.

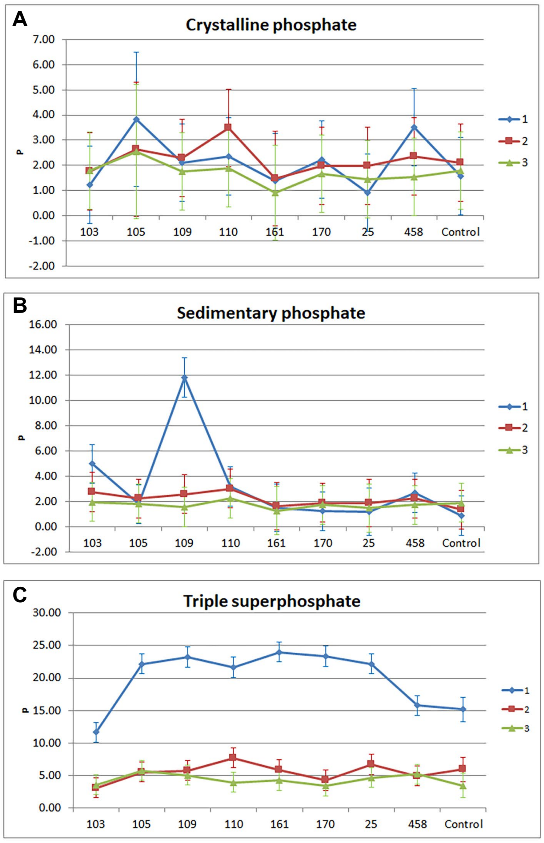

FIGURE 2
Triple interaction (source of phosphorus vs. isolate vs. cut). Treatments that received crystalline **(A)**, sedimentary **(B)**, and soluble **(C)** phosphate as a source of phosphorus.

TABLE 4 Contribution of morphological descriptors for principal component analysis.

Variable	Variable	PCR1	PCR2
Aerial fresh weight	AFW	0.965	0.082
Aerial dry weight	ADW	0.962	0.059
Calcium extract	ECa	0.954	0.029
Nitrogen extract	EN	0.954	0.117
Leaf area	LA	0.942	0.085
Number of tillers	NT	0.928	−0.111
Magnesium	Mg	0.904	0.254
Number of leaves	NL	0.892	−0.095
Phosphorus extract	EP	0.889	−0.088
Sulphur	S	0.885	0.235
Phosphorus	P	0.857	0.177
Calcium	Ca	0.792	0.223
Mineral matter	MM	0.61	0.488
Leaf width	LW	0.578	0.185
Presence of anthocyanin	ANT	0.557	−0.387
Manganese	Mn	0.512	−0.021
Zinc extract	EZn	0.405	0.172
In vitro digestibility	DIV	0.279	−0.424
Neutral fiber detergent	FDN	0.179	−0.804
Leaf length	Ll	0.029	0.505
Plant height	PHe	−0.053	0.399
Iron	Fe	−0.147	0.364
Acid detergent fiber	FDA	−0.23	−0.613
Dry matter	MS	−0.232	−0.664
Ether extract	EE	−0.373	0.169
Copper	Cu	−0.433	0.018
Lignin	LIG	−0.587	0.51
Zinc	Zn	−0.658	−0.135
Crude protein	CP	−0.67	0.53
Nitrogen	N	−0.67	0.532
SPAD index	SPAD	−0.805	0.17
Potassium	K	−0.877	−0.058

Studies involving the endophytic and rhizospheric microbiota of *Paspalum* accessions are scarce. Zhao et al. (2021) isolated endogenous *Enterobacter* strains from *Paspalum vaginatum* and evaluated their influence on promoting salt tolerance in the plant. Pérez and Martínez (2016) isolated and identified endophytic bacteria that is resistant to mercury associated with *P. arundinaceum* in Mina Santa Cruz, Bolivar., Colombia, aiming to obtain plant growth-promoting strains with potential for phytoremediation of mercury-contaminated soil. Amaral et al. (2021) isolated and

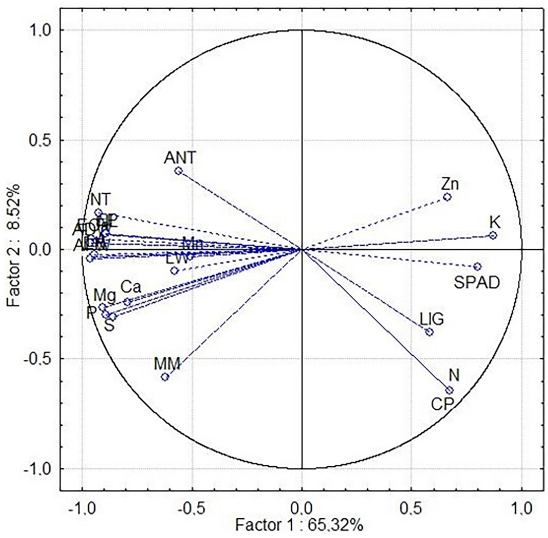

FIGURE 3
Principal component analysis of the 22 descriptors evaluated. Correlation between the evaluated descriptors. Phosphorus extract (EP), nitrogen extract (EN), calcium extract (ECa), crude protein (CP), mineral matter (MM), lignin (LIG), calcium (Ca), magnesium (Mg), phosphorus (P), potassium (K), sulfur (S), manganese (Mn), zinc (Zn), nitrogen (N), SPAD index (SPAD), aerial fresh weight (AFW), aerial dry weight (ADW), leaf width (LW), number of leaves (NL), number of tillers (NT), presence of anthocyanin (ANT), and leaf area (LA).

characterized plant growth-promoting rhizobacteria (PGPR) from 10 genotypes of *Paspalum* and evaluated the effect of inoculation on *P. regnellii*, *P. atratum*, and *P. malacophyllum*. The genotypes were also collected from the *Paspalum* germplasm bank at the Brazilian Agricultural Research Corporation; however, the genotype of *P. atratum* studied was cv. *Pojuca* BGP 098. For studies involving phosphate solubilization in *Paspalum* with potential for forage, this work is the first to examine phosphate rock in the nutrition of *P. atratum* plants, intermediated by cultivable endophytic and rhizospheric microorganisms.

Based on the present study results, the bacterial population was higher in the rhizosphere and the root. The rhizosphere exhibits different physical and chemical characteristics than nonrhizospheric soil, as plants can produce root exudates, which provide bacterial nutrition and make this environment favorable for bacterial colonization (Wu et al., 2018). In the case of endophytic bacteria, the root is the main entrance way for microorganisms in plants (White et al., 2014). Thus, it is also expected that the roots present a greater population of microorganisms than other plant segments, such as stems and leaves. Abedinzadeh et al. (2019) found similar data, in which the population size was $3.4 \pm 2.12 \times 10^6$ and $6.8 \pm 1.20 \times 10^3$ for rhizospheric and endophytic bacteria in maize plants.

Among the 116 strains isolated, 43 showed positive results for NFB, SF, and IAA. The 43 strains belonged to Proteobacteria, Firmicutes, and Actinobacteria. Genetic diversity studies have reported that these phyla are both endophytic and rhizospheric (Rosenblueth and Martínez-Romero, 2006; Pisa et al., 2011; Prabha et al., 2018) and generally present strains with potential for plant growth promotion.

In this work, through *in vitro* tests, the main strains that showed the potential to promote plant growth belonged to *Bacillus*, *Enterobacter*, *Microbacterium, Micrococcus*, *Pantoea*, and *Pseudomonas* (Amaral et al., 2021). The main PGPB isolated from *Paspalum* genotypes were *Acinetobacter*, *Bacillus*, *Cupriavidus*, *Dyadobacter*, *Enterobacter*, *Paraburkholderia*, *Pseudomonas*, and *Rhizobium*.

In the *in vivo* test, treatments were separated into two groups. Group 1 received alternative phosphate sources, and Group 2 received a soluble phosphate source. The most significant morphological descriptor for Group 1 was SPAD (SPAD index). The mineral descriptor was potassium (K), and as for the results of the bromatological analysis, the crude protein (CP) content was the most significant. These descriptors showed lower values in treatments that received the soluble phosphate source. For Group 2, the most significant morphological descriptors were aerial fresh weight (AFW) and aerial dry weight (ADW), the mineral was calcium extract (ECa), and the bromatological descriptor was nitrogen extract (EN). Phosphorus was the 11th most significant descriptor in Principal Component 1 and the 12th most significant in Principal Component 2; thus, it had equal importance for both groups.

The *in vitro* tests showed that the PSI ranged from 2 to 3.61, and the phosphate-solubilizing bacteria belonged to *Bacillus*, *Enterobacter*, *Microbacterium*, *Micrococcus*, *Pantoea*, and *Pseudomonas*. These genera are already described in the literature as phosphate-solubilizing bacteria. De Assumpção et al. (2009) found *Pseudomonas* sp. with 5.3 and 8.3 of PSI and *Pantoea* sp. with PSI = 6.0. Suleman et al. (2018), when prospecting bacteria with the potential for phosphate solubilization for wheat plants, found that the best results were with *Enterobacter*, presenting 2.2–5.8 of PSI. Similarly, the strain with the highest PSI also belonged to the *Enterobacter* genus in the present study.

Regarding the *in vivo* test in a greenhouse, the treatments with crystalline phosphate plus Isolates 105 (*Pseudomonas* sp.) and 458 (*Pseudomonas* sp.) showed available phosphorus in the initial period of plant development. However, the treatment that received the 110 (*Enterobacter* sp.) strain showed higher phosphorus content in the second cut, suggesting that the 110 strain needs a more extended period to make the phosphorus available to the plant. In the literature, there is a search for an alternative source of phosphorus for plant nutrition, with Arad rock phosphate being one of those rocks with potential for this purpose. Gatiboni et al. (2003) used natural phosphates from Arad as a source of phosphorus for white clover (*Trifolium repens*) and ryegrass (*Lolium multiflorum*) pastures. Gatiboni et al. (2003) observed that the use of natural phosphate from Arad was effective in moderate to high soil and that liming increased the efficiency of superphosphate and decreased the efficiency of rock phosphate as a source of phosphorus. Guedes

TABLE 5 Values of extracts and morphological descriptors evaluated on average.

TRAT	EP	EN	ECa	AFW	ADW	LW	NL	NT	ANT	LA	SPAD
103FCR	4.77	143.39	53.00	45.58	7.77	47.47	76.00	21.00	0.00	1728.29	94.66
103FSE	18.41	117.97	65.46	38.18	6.34	45.15	64.33	16.67	0.67	1572.39	98.48
103FSO	9.73	229.95	101.51	80.61	12.24	47.61	102.67	29.00	0.00	2819.05	78.35
103SF	7.77	197.23	92.91	57.63	10.02	47.37	90.33	24.00	0.00	2075.62	92.30
105FCR	9.00	242.43	139.71	67.77	13.23	48.40	75.00	26.00	0.00	2520.89	93.10
105FSE	5.90	128.34	55.66	38.06	6.70	42.62	57.33	18.00	0.00	1460.29	91.15
105FSO	33.49	267.02	170.67	86.24	15.65	46.88	116.00	39.67	2.00	3250.76	79.17
105SF	6.94	162.80	78.76	49.25	8.80	44.34	80.67	24.67	0.67	1877.44	89.00
109FCR	6.18	168.98	73.43	51.32	9.38	43.97	92.67	26.00	0.00	2025.18	92.75
109FSE	15.99	187.61	100.17	55.86	10.12	47.98	81.33	21.33	0.00	2401.46	94.24
109FSO	34.11	257.14	144.78	85.97	14.12	48.31	105.00	31.33	1.33	2975.36	77.53
109SF	3.18	136.65	40.71	41.58	7.12	47.39	73.33	18.67	0.33	1265.03	96.55
110FCR	7.73	152.25	83.83	48.22	8.49	43.36	81.00	23.67	0.00	1657.16	93.17
110FSE	8.51	177.87	88.64	65.35	9.81	48.32	97.67	27.33	0.67	2482.98	82.77
110FSO	33.38	237.84	150.67	71.60	12.61	48.42	113.00	28.33	2.33	2513.11	80.52
110SF	6.90	170.86	78.08	52.62	8.98	43.92	75.67	23.00	1.33	1937.38	91.54
161FCR	3.79	95.33	44.01	34.25	5.31	43.24	57.50	18.00	0.50	1247.10	85.35
161FSE	4.47	132.72	46.03	43.94	7.05	43.61	77.00	21.00	0.50	1702.60	95.25
161FSO	34.12	258.69	156.58	82.58	13.55	47.19	111.00	32.67	0.00	2878.92	81.07
161SF	7.30	178.25	84.54	55.00	9.25	47.27	78.50	22.00	0.50	2141.04	98.53
170FCR	5.90	154.12	69.07	48.89	8.32	46.08	90.33	25.67	0.00	1721.76	88.71
170FSE	4.92	123.40	48.91	46.76	6.68	42.53	63.33	17.33	0.00	1590.86	93.22
170FSO	31.04	271.82	149.72	82.53	14.90	49.22	131.00	38.00	1.67	2813.21	82.25
170SF	5.32	126.78	63.86	38.22	6.88	45.64	80.67	22.00	0.67	1379.32	92.59
25FCR	4.33	109.01	39.24	39.29	5.90	40.08	73.67	20.00	0.33	1324.59	89.14
25FSE	4.59	133.15	60.82	39.23	6.67	44.39	73.00	21.00	0.00	1328.45	94.39
25FSO	33.61	259.85	167.92	78.28	14.31	45.54	99.33	30.67	1.33	2961.34	77.20
25SF	6.38	175.93	81.28	53.41	9.49	48.62	75.00	20.67	0.00	2114.69	98.42
458FCR	7.44	183.05	91.76	52.41	9.68	45.13	77.33	22.67	0.67	1786.17	94.45
458FSE	6.68	163.90	78.93	52.02	8.87	43.85	79.67	23.67	1.33	2039.63	84.69
458FSO	25.92	229.01	117.09	74.05	12.72	48.32	109.33	29.33	1.00	2522.91	85.12
458SF	9.12	192.69	92.46	58.94	10.59	48.10	80.33	26.00	0.33	2017.38	95.50
CAFCR	5.46	138.77	56.43	43.30	7.32	46.94	73.00	20.33	0.00	1598.96	98.06
CAFSE	4.18	104.83	38.57	35.93	5.70	42.27	76.67	16.00	0.00	1228.91	94.01
CAFSO	24.67	285.21	167.37	76.06	14.86	48.34	104.00	35.50	0.50	2681.90	83.67
CASF	5.48	146.56	53.94	50.84	8.16	48.84	82.67	22.00	1.00	1722.92	90.53

Phosphorus extract (EP), nitrogen extract (EN), calcium extract (ECa), aerial fresh weight (AFW), aerial dry weight (ADW), leaf width (LW), number of leaves (NL), number of tillers (NT), presence of anthocyanin (ANT), leaf area (LA) and SPAD index (SPAD).

et al. (2012), evaluating the use of natural Arad phosphate and liming in two tropical grass species in degraded Amazon soil, observed better results in *Megathyrsus maximus* than in *Urochloa brizantha*, noting that the success in fertilization was dependent on the cultivated species and soil acidity. These studies sought to explore the gradual capacity of natural rocks to release phosphorus. However, they only used Arad's natural phosphate in crops without studying the endophytic and rhizospheric microbiota of the host plant. In the present study, the primary method was the optimization of the phosphorus contained in the phosphate rock through selected microorganisms isolated from soil and plant tissue of *P. atratum*. Another characteristic observed in *P. atratum* was the acidic soil; both in the rainy and dry seasons, the soil pH of the soil was not higher than 5.6. As mentioned by Guedes et al. (2012), this feature facilitates the solubilization of phosphate from natural rocks.

Principal component analysis showed that the 110FCR (*Enterobacter* sp.) and 458FCR (*Pseudomonas* sp.) treatments were very close. The 105FCR (*Pseudomonas* sp.) treatment stood out, being the treatment with crystalline phosphate that came closest to the treatments that received soluble phosphate. The literature shows that the main phosphate solubilizers are *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*,

TABLE 6 Values of mineral and bromatological descriptors evaluated on average.

TRAT	MM	Ca	Mg	P	S	Mn	K	Zn	LIG	CP	N
103FCR	9.16	9.26	10.22	0.80	4.96	63.98	11.15	14.80	3.77	12.46	19.93
103FSE	8.33	7.04	7.96	0.63	4.11	71.27	14.18	13.32	3.39	11.68	18.69
103FSO	9.63	10.67	14.17	3.17	6.79	95.04	8.49	8.55	2.95	11.97	19.16
103SF	8.87	8.07	9.66	0.78	4.42	58.83	11.20	14.58	2.88	11.44	18.31
105FCR	8.77	10.21	11.53	0.68	5.03	67.23	10.15	11.81	6.18	11.14	17.83
105FSE	8.63	8.33	9.63	0.88	4.69	96.74	14.56	17.56	4.40	11.99	19.19
105FSO	9.02	10.50	12.71	1.95	5.86	76.82	7.14	9.41	2.04	10.12	16.19
105SF	8.49	8.73	9.40	0.78	4.67	78.90	10.99	12.54	2.80	11.48	18.36
109FCR	8.04	7.81	9.27	0.66	4.08	61.19	10.45	13.92	2.80	11.25	18.00
109FSE	8.57	9.56	12.17	1.43	6.47	73.56	8.93	11.59	2.89	11.44	18.31
109FSO	9.53	9.97	11.79	2.16	5.79	83.02	7.35	8.33	1.97	10.95	17.52
109SF	8.36	5.61	6.96	0.45	3.28	62.85	9.15	8.67	3.75	12.07	19.32
110FCR	8.69	9.82	10.06	0.92	4.73	69.86	10.80	14.49	3.51	11.14	17.83
110FSE	8.80	8.99	10.28	0.86	4.54	69.27	9.35	15.71	3.30	11.29	18.07
110FSO	9.17	11.70	14.84	2.37	6.30	88.98	8.70	8.05	2.35	11.57	18.51
110SF	8.25	8.76	9.75	0.77	4.26	80.24	10.38	14.44	3.11	11.94	19.10
161FCR	8.27	8.22	7.90	0.71	4.09	109.41	12.81	12.77	4.15	11.15	17.84
161FSE	8.85	6.78	8.47	0.65	4.41	62.53	11.84	13.30	4.55	11.98	19.17
161FSO	9.37	11.02	13.54	2.18	6.40	120.32	7.29	9.46	2.38	11.43	18.29
161SF	9.13	9.09	9.91	0.79	4.77	49.85	9.61	12.88	5.90	12.04	19.26
170FCR	8.23	8.25	9.28	0.71	4.33	76.29	11.45	17.27	3.37	11.53	18.45
170FSE	8.60	7.57	8.84	0.76	4.17	71.28	13.78	14.96	4.96	11.85	18.95
170FSO	9.09	8.88	11.51	1.71	6.12	76.42	7.03	7.78	2.13	10.81	17.30
170SF	8.85	9.36	9.33	0.78	4.44	63.97	11.76	14.64	2.90	11.56	18.50
25FCR	8.54	7.08	8.03	0.79	3.90	70.75	13.53	13.18	3.52	12.19	19.50
25FSE	8.95	8.62	9.28	0.64	4.35	58.06	9.41	11.27	4.21	11.64	18.63
25FSO	8.75	11.08	13.82	2.09	6.66	89.59	7.16	9.45	1.80	10.86	17.37
25SF	8.28	8.62	10.21	0.68	4.51	70.72	11.38	17.95	3.30	11.71	18.74
458FCR	9.08	9.12	9.83	0.75	4.88	75.66	9.69	12.98	3.86	11.59	18.55
458FSE	8.65	8.82	10.22	0.77	4.64	84.32	10.50	18.55	3.06	11.38	18.20
458FSO	9.42	8.82	12.22	1.95	5.74	71.30	7.71	10.68	2.57	11.28	18.05
458SF	8.09	8.59	10.79	0.86	4.55	85.10	10.46	16.38	2.02	11.28	18.05
CAFCR	8.49	7.89	8.86	0.76	4.15	43.95	12.02	14.16	3.62	11.98	19.17
CAFSE	8.69	7.28	8.03	0.78	3.73	51.14	11.87	14.85	3.22	11.81	18.89
CAFSO	9.03	10.17	12.82	1.58	6.26	118.00	7.28	11.60	2.33	11.24	17.98
CASF	8.21	6.77	8.40	0.70	4.00	64.40	12.24	16.71	2.54	11.54	18.46

Mineral matter (MM), calcium (Ca), magnesium (Mg), phosphorus (P), sulfur (S), manganese (Mn), potassium (K), zinc (Zn), lignin (LIG), crude protein (CP) and nitrogen (N).

Beijerinckia, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Mesorhizobium*, *Microbacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, and *Serratia* (Bhattacharyya and Jha, 2012; Gouda et al., 2018). There is a search to optimize phosphate rock from Cajati as an alternative source of phosphorus. Lemos et al. (2013) sought to use the rock in the diet of Nellore cattle, as Bernardi and Oliveira (2021) sought to associate the phosphate rock of Cajati with zeolite minerals to nourish the alfalfa crop.

In treatments with sedimentary phosphate, the 109FSE (*Bacillus* sp.) treatment was the closest to the treatments with soluble phosphate and presented the highest phosphorus content in the first cut. One hypothesis to explain the higher content of phosphorus in the first cut is that there was great solubilization of sedimentary phosphate during the initial periods of the plant, thus depleting almost all available phosphorus sources in the first cut and, consequently, reducing these in dry matter in the second and third cut. This fact shows the importance of topdressing after grazing.

The results observed in treatments with soluble phosphate that included the bacterial isolates also suggest that the source of phosphorus was depleted during the initial stages of plant development. Of the eight treatments that received the strains, seven showed higher phosphorus content than the soluble

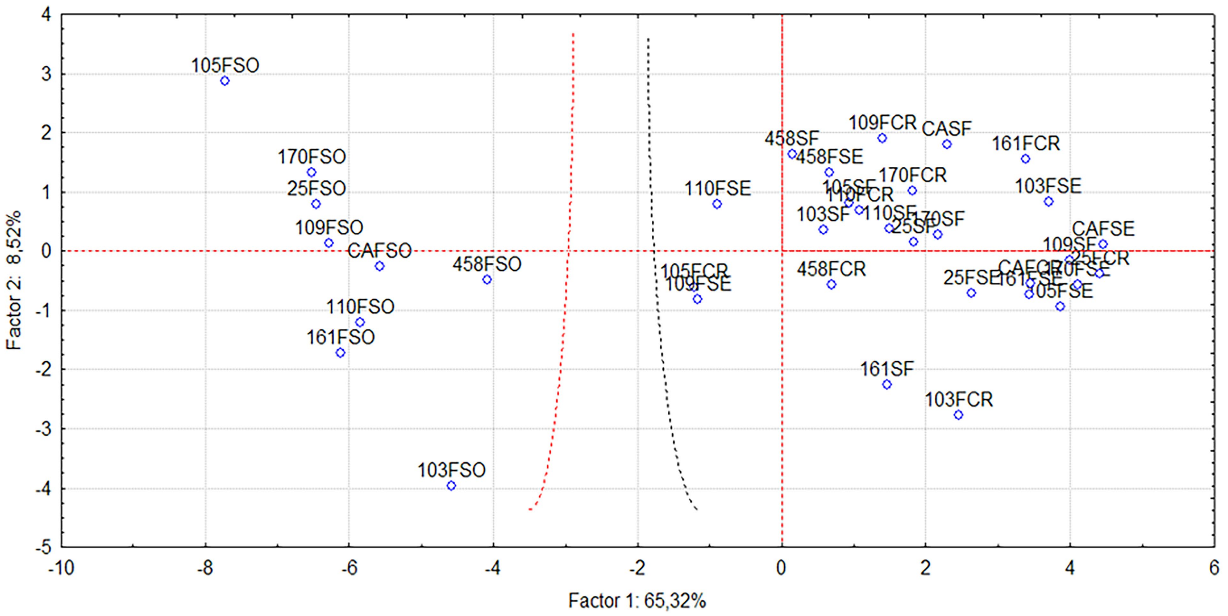

FIGURE 4
Biplot graph resulting from the 36 treatments obtained by principal component analysis considering the 22 descriptors for Principal Components 1 and 2. Control (CA), crystalline phosphate (FCR), sedimentary phosphate (FSE), phosphate-free (SFO), and soluble phosphate (FSO). The number that precedes the treatment refers to the strain code.

phosphate control dry matter. The exception was treatment 103FSO (*Enterobacter* sp.). Therefore, future studies should explore the possibility of using less soluble phosphorus by inoculating phosphate-solubilizing bacteria, seeking to optimize the use of soluble phosphorus in agriculture. When looking for bacteria with the potential for phosphate solubilization in peas, Oteino et al. (2015) conducted an experiment involving *Bacillus* sp. and *Pseudomonas* sp. They used soluble phosphate as a control and tricalcium phosphate ($Ca_3(PO_4)_2$) insoluble in the treatments. As a result, it was observed that the strains increased the phosphorus content in the plant compared to the insoluble control. Nevertheless, no treatment equaled or surpassed the phosphorus content present in the dry matter of the plants treated with soluble phosphate. Similar to the results presented by Oteino et al. (2015), no alternative treatments had equal or higher phosphorus levels as seen in treatments that received the soluble phosphate source. However, a significant difference was that treatments with soluble phosphate also received the bacteria, indicating the potential of these microorganisms to optimize the soluble phosphate in the plant.

During the *in vitro* tests, using the NFB medium, there was an expectation of finding *Azorhizophilus paspali* (*Azotobacter paspali*), a nitrogen-fixing bacteria, found by Döbereiner et al. (1995) when developing the culture medium (Baldani et al., 2014). Batista et al. (2018), when using NFB medium, observed that the main strains with potential for biological nitrogen fixation belonged to *Bacillus* and *Burkholderia*. As in these studies, no strains of *Azotobacter* were obtained in the present study. However, the growth of other microorganisms in the NFB medium was justified because the medium was not highly selective; therefore, strains with the potential to use malic acid as a carbon source and with a pH of 6.8 can grow (Baldani et al., 2014). This method characterized the strains with potential for nitrogen fixation in this work. The cultivable bacterial community with the potential for nitrogen fixation will undoubtedly increase by using another culture medium for isolation or biochemical characterization.

The bromatological descriptors crude protein (CP) and nitrogen (N) content correlated with the morphological descriptor index SPAD in the plant. The CP descriptor was calculated by multiplying the nitrogen content by 6.25 (Druzian et al., 2012; De Medeiros et al., 2015). The SPAD index was generated by the SPAD-502 chlorophyll meter (Soil Plant Analysis Development), which indirectly measures the leaf chlorophyll content without destroying the leaf (D'Oliveira et al., 2020), and the chlorophyll concentration positively correlated with the nitrogen content (Benati et al., 2021).

Among the most significant descriptors to assess the variation between treatments and plant growth promotion, crude protein content was significantly crucial in Group 1 treatments. The 161SF (*Pseudomonas* sp.), 103FSE (*Enterobacter* sp.), 25SF (*Pseudomonas* sp.), CAFCR, and 109SF (*Bacillus* sp.) were the ones with the highest SPAD index. On the other hand, the 103FCR (*Enterobacter* sp.), 25FCR (*Pseudomonas* sp.), 109SF (*Bacillus* sp.), 161SF (*Pseudomonas* sp.), and 105 FSE (*Pseudomonas* sp.) treatments presented the highest levels of CP and N.

Leite et al. (2001) studied the growth and chemical composition of *P. atratum* cv. Pojuca grass in soil with satisfactory nutrients and nitrogen fertilization during the rainy season. The researchers found CP contents between 6.90 and 12.11%. By sampling the nutritional contents of three cultivars, Porto et al. (2009) found CP contents of 11.1, 11.9, and 9.4% for Tanzania grass (*M. maximus*), Stargrass (*Cynodon*), and marandu grass (*U. brizantha* cv. Marandu), respectively.

Crude protein contents lower than 7% in the dry matter limit animal nutrition (De Abreu et al., 2006). All treatments studied in the present work had a protein content greater than 7%, ranging from 10.12 to 12.46%, values similar to those found by Leite et al. (2001) for the Pojuca cultivar (*P. atratum*) and by Porto et al. (2009) for the cultivars Capim-tanzânia, Grama-estrela, and Capim-marandu. The leaf protein content in the Pojuca cultivar ranges from 8 to 10% (Karia and de Andrade, 2001). The values found in this work were also superior to the results obtained by Lopes et al. (2010) for *U. brizantha*, *U. decumbens*, *U. humidicola,* and *U. Ruziziensis,* which ranged from 6.4 to 7.5% CP in dry matter.

In the present study, none of the treatments received nitrogen fertilization. Nevertheless, many of them had similar or superior CP results compared to other studies that evaluated cultivars already on the market. Even the phosphate-free control (CSF), which did not receive any phosphorus source or bacterial inoculum, showed CP results superior to those found by Leite et al. (2001). This fact shows the potential of this genotype as a forage plant.

The main potential NFB were 25 (*Pseudomonas* sp.), 103 (*Enterobacter* sp.), 105 (*Pseudomonas* sp.), 109 (*Bacillus* sp. and 161 (*Pseudomonas* sp.). The 103FSO (*Enterobacter* sp.) treatment stood out due to high levels of CP and N in the dry matter. The Isolate 103 (*Enterobacter* sp.) also showed good levels of CP and N in treatments with a sedimentary and crystalline phosphate source, making it a good candidate for biological nitrogen fixation investigation. Potassium was another significantly important mineral descriptor observed in Group 1 treatments. In the K^+ format, potassium regulates the osmotic potential and activates enzymes involved in respiration and photosynthesis in the plant (Taiz et al., 2016). Therefore, potassium was among the most significant descriptors in the principal component analysis. The treatments that presented the lowest K content received the soluble phosphate source. The treatments that showed the highest K content were 105FSE (*Pseudomonas* sp.), 103FSE (*Enterobacter* sp.), 170FSE (*Pantoea* sp.), 25FCR (*Pseudomonas* sp.), and 161FCR (*Pseudomonas* sp.), followed by the control treatments CASF, CAFCR, and CAFSE. The microbiological modification technique enables the direct application of rocks in agriculture. Citric and oxalic acids produced by microorganisms release potassium from biotite, a common mineral in the silicate class (Van Straaten, 2010).

The plants inoculated with the bacterial Isolates 105FCR (*Pseudomonas* sp.), 109FSE (*Bacillus* sp.), 110FSE (*Enterobacter* sp.), 103SF (*Enterobacter* sp.), 458SF (*Pseudomonas* sp.), and 458FCR (*Pseudomonas* sp.) showed growth similar to those treated with a soluble phosphate source. Therefore, they can be selected for future plant growth-promotion experiments.

Conclusion

A total of 116 cultivable endophytic and rhizospheric strains were isolated from rhizospheric soil samples, roots, and leaves of *P. atratum*.

As for the capacity of the plant growth-promoting bacterial strains, 43 (37.00%) strains showed positive NFB, SF, and IAA results and belonged to *Enterobacter* (46.50%), *Pseudomonas* (32.50%), and *Pantoea* (13.90%), and *Bacillus*, *Microbacterium*, and *Micrococcus* strains represented 6.90%.

The phosphate solubilization index (PSI) ranged from 2 (*Pseudomonas* spp.) to 3.61 (*Enterobacter* spp.) and the IAA production ranged from 12.85 (*Pseudomonas* spp.) to 431.41 (*Pantoea* spp.) $\mu g\, ml^{-1}$.

In the *in vivo* test, treatments 105 (*Pseudomonas sp.*) and 458 (*Pseudomonas sp.*) were the most significant for the crystalline phosphate source, 109 (*Bacillus sp.*) for the sedimentary phosphate source and, as for the soluble phosphate source most treatments that received bacterial isolates had higher phosphorus content in the dry matter than the uninoculated soluble phosphate control.

For the morphological, mineral and bromatological variables, the principal component analysis showed that Principal Component 1 had significant associations with the SPAD index (SPAD), potassium (K), zinc (Zn), crude protein (CP), lignin (LIG) and nitrogen (N). While, principal Component 2 showed strong associations with the other 16 descriptors.

These diverse cultivable bacterial genera have the potential to promote plant growth, and the 105FCR (crystalline phosphate + *Pseudomonas* sp.), 109FSE (sedimentary phosphate + *Bacillus* sp.), and 110 FSE (sedimentary phosphate + *Enterobacter* sp.) treatments showed the best results in the plant growth promotion assay.

Other treatments showed isolated characteristics of interest for one or another descriptor analyzed, such as dry weight, potassium, and nitrogen content in the leaves.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MK521286, MK521276, MK521285, MK521301, MK521314, MK521306, MK521273, MK521304, MK521284, MK521289, MK521303, MK521302, MK521274, MK521300, MK521275, MK521296, MK521290, MK521293, MK521287, MK521271, MK521270, MK521283, MK521282, MK521272,

MK521279, MK521308, MK521277, MK521297, MK521278, MK521292, MK521299, MK521291, MK521280, MK521298, MK521281, MK521305, MK521307, MK521261, MK521288, MK521263, MK521262, MK521264, and MK521265.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

This work was supported by grants from the São Paulo Research Foundation, FAPESP (Proc. No. 2020/11315-6).

Conflict of interest

ND, WJ, AB, BV, and AF were employed by Embrapa Pecuária Sudeste.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abedinzadeh, M., Etesami, H., and Alikhani, H. A. (2019). Characterization of rhizosphere and endophytic bacteria from roots of maize (*Zea mays* L.) plant irrigated with wastewater with biotechnological potential in agriculture. *Biotechnol. Rep.* 21:e00305. doi: 10.1016/j.btre.2019.e00305

ABIEC (2021). *Associação Brasileira das Indústrias Exportadoras de Carnes.* Available at: http://abiec.com.br/exportacoes/# (Accessed July, 2022).

Abreu, E. M. A.De, Fernandes, A. R., Martins, A. R. A., and Rodrigues, T. E. (2006). Produção de forragem e valor nutritivo de espécies forrageiras sob condições de pastejo, em solo de várzea baixa do Rio Guamá. *Acta Amaz.* 36, 11–18. doi: 10.1590/S0044-59672006000100003

Afzal, I., Shinwari, Z. K., Sikandar, S., and Shahzad, S. (2019). Plant beneficial endophytic bacteria: mechanisms, diversity, host range and genetic determinants. *Microbiol. Res.* 221, 36–49. doi: 10.1016/j.micres.2019.02.001

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *J. King Saud Univ. Sci.* 26, 1–20. doi: 10.1016/j.jksus.2013.05.001

Aljanabi, S. M., and Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res.* 25, 4692–4693. doi: 10.1093/nar/25.22.4692

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Alves, P. R., and Hagni, R. D. (2008). The carbonatite-hosted apatite deposit of Jacupiranga, Southeast Brazil - styles of mineralisation, ore characterisation and association with mineral processing. *Australas. Inst. Min. Metall. Publ. Ser.* 381–389.

Amaral, M. B., Ribeiro, T. G., Alves, G. C., Coelho, M. R. R., Matta, F. D. P., Baldani, J. I., et al. (2021). The occurrence of rhizobacteria from paspalum genotypes and their effects on plant growth. *Sci. Agric.* 79, 1–10. doi: 10.1590/1678-992x-2020-0240

Araújo, W. L., Quecine, M. C., Lacava, P. T., Aguilar-Vildoso, C. I., Marcon, J., Lima, A. O. S., et al. (2014). *Micro-organismos Endofíticos: Aspectos Teóricos e Práticos de Isolamento e Caracterização*. Santarém: UFOPA.

Baldani, J. I., Reis, V. M., Videira, S. S., Boddey, L. H., and Baldani, V. L. D. (2014). The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant Soil* 384, 413–431. doi: 10.1007/s11104-014-2186-6

Barro, R. S., Varella, A. C., Lemaire, G., De Medeiros, R. B., De Saibro, J. C., Nabinger, C., et al. (2012). Forage yield and nitrogen nutrition dynamics of warm-season native forage genotypes under two shading levels and in full sunlight. *Rev. Bras. Zootec.* 41, 1589–1597. doi: 10.1590/S1516-35982012000700006

Batista, B. D., Lacava, P. T., Ferrari, A., Teixeira-Silva, N. S., Bonatelli, M. L., Tsui, S., et al. (2018). Screening of tropically derived, multi-trait plant growth-promoting rhizobacteria and evaluation of corn and soybean colonization ability. *Microbiol. Res.* 206, 33–42. doi: 10.1016/j.micres.2017.09.007

Benati, J. A., Nava, G., and Mayer, N. A. (2021). Spad index for diagnosis of nitrogen status in 'esmeralda' peach. *Rev. Bras. Frutic.* 43, 1–6. doi: 10.1590/0100-29452021093

Bernardi, C. D., and Oliveira, C. D. (2021). Improved alfalfa phosphate utilization using zeolite amendments in low pH soil. *J. Soil Sci. Plant Nutr.* 21, 1307–1317. doi: 10.1007/s42729-021-00441-z

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9

Bogas, A. C., Ferreira, A. J., Araújo, W. L., Astolfi-Filho, S., Kitajima, E. W., Lacava, P. T., et al. (2015). Endophytic bacterial diversity in the phyllosphere of Amazon *Paullinia cupana* associated with asymptomatic and symptomatic anthracnose. *Springerplus* 4:258. doi: 10.1186/s40064-015-1037-0

Bric, J. M., Bostock, R. M., and Silverstone, S. E. (1991). Rapid *in situ* assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol.* 57, 535–538. doi: 10.1128/aem.57.2.535-538.1991

Büchi Labortechnik, A. (2007). NIRCal, Quick Guide number 93119-93123. *NIRWare Software Suite Quick Guide. Version B*, 1–3.

Coelho, F. E., Lopes, L. C., Cavalcante, R. M., Corrêa, G. C., and Leduc, A. O. (2019). Brazil unwisely gives pesticides a free pass. *Science* 365, 552–553. doi: 10.1126/science.aay3150

Compant, S., Van Der Heijden, M. G. A., and Sessitsch, A. (2010). Climate change effects on beneficial plant-microorganism interactions. *FEMS Microbiol. Ecol.* 73, 197–214. doi: 10.1111/j.1574-6941.2010.00900.x

Crous, K. Y., Wujeska-Klause, A., Jiang, M., Medlyn, B. E., and Ellsworth, D. S. (2019). Nitrogen and phosphorus retranslocation of leaves and stemwood in a mature Eucalyptus forest exposed to 5 years of elevated CO2. *Front. Plant Sci.* 10, 1–13. doi: 10.3389/fpls.2019.00664

D'Oliveira, P. S., Suzuki, L. S., Da Costa Carneiro, J., Machado, J. C., Prates, J. F., Rocha, D. A. S. D. C., et al. (2020). Use of SPAD index in elephant grass pre-breeding. *Semin. Agrar.* 41, 1047–1052. doi: 10.5433/1679-0359.2020v41n3p1047

De Medeiros, S. R., Gomes, R. C., and Bungenstab, D. J. (2015). *Nutrição de bovinos de corte Fundamentos e aplicações*. Campo Grande: Embrapa.

De Assumpção, L. C., Lacava, P. T., Dias, A. C. F., De Azevedo, J. L., and Menten, J. O. M. (2009). Diversidade e potencial biotecnológico da comunidade bacteriana endofítica de sementes de soja. *Pesqui. Agropecuária Bras.* 44, 503–510. doi: 10.1590/S0100-204X2009000500010

Dinato, N. B., Santos, I. R. I., Leonardecz, E., Burson, B. L., Quarín, C. L., de Paula, A. F., et al. (2018). Storage of bahiagrass pollen at different temperatures. *Crop Sci.* 58, 2391–2398. doi: 10.2135/cropsci2018.03.0164

Döbereiner, J., Baldani, V. L. D., and Baldini, J. I. (1995). *Como Isolar e identificar bactérias diazotróficas de plantas não-leguminosas*. Brasília: Embrapa-SPI.

Druzian, J. I., Machado, B. A. S., De Souza, C., Fraga, L. M., De Duran, V. A., De Burghgrave, U. S., et al. (2012). Influência da dieta suplementada com óleo de soja na composição centesimal e perfil lipídico de tilápias do Nilo (*Oreochromis niloticus*). *Rev. Inst. Adolfo Lutz* 71, 85–92.

Garrity, G. M., Bell, J. A., Lilburn, T. G., and Lansing, E. (2004). *Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology*. New York, Berlin, Heidelberg: Springer.

Gatiboni, L. C., Kaminski, J., Rheinheimer, D. S., and Brunetto, G. (2003). Superphosphate and rock phosphates as phosphorus sources for grass-clover pasture on a limed acid soil in southern Brazil. *Commun. Soil Sci. Plant Anal.* 34, 2503–2514. doi: 10.1081/CSS-120024782

Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H.-S., and Patra, J. K. (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. *Microbiol. Res.* 206, 131–140. doi: 10.1016/j.micres.2017.08.016

Guedes, E. M. S., Fernandes, A. R., Lobato, A. K.Da, Guedes, R. S., de Ávila, W. S., and da Silva, S. P. (2012). Natural phosphate and liming improves phosphorus extraction in two tropical grasses grown in degraded Amazon soil. *J. Food Agric. Environ.* 10, 1165–1168.

Gusmão, M. R., Valério, J. R., Matta, F. P., Souza, F. H. D., Vigna, B. B. Z., Fávero, A. P., et al. (2016). Plant resistance warm-season (C4) Turfgrass genotypes resistant to spittlebugs (Hemiptera: Cercopidae). *J. Econ. Entomol. Adv.* 109, 1914–1921. doi: 10.1093/jee/tow135

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95 / 98 / NT. *Nucleic Acids Symp. Ser.* 41, 95–98.

Husen, E. (2016). Screening of soil bacteria for plant growth promotion activities *in vitro*. *Indones. J. Agric. Sci.* 4, 27–31. doi: 10.21082/ijas.v4n1.2003.27-31

International Fertilizer Industry Association (2021). Available at https://www.ifastat.org/databases/plant-nutrition (Accessed February 23, 2021).

Karia, C. T., and de Andrade, R. P. (2001). Cultivo do Capim Pojuca. *Embrapa Cerrados* 50, 1–2.

Khan, A. A., Jilani, G., Saleem Akhtar, M., Naqvi, M. S., and Rasheed, M. (2009). A phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *J. Agric. Biol. Sci.* 1, 48–58. doi: 10.5923/j.re.20120201.10

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Leite, G. G., Silveira, L. F.Da, Fernandes, F. D., and Gomes, A. C. (2001). *Crescimento e Composição Química do Capim P. atratum cv.* Pojuca. Planaltina.

Lemos, G. C., Costa, R. M., Neto, M. G., and Malafaia, P. (2013). Desempenho ponderal de bovinos Nelore suplementados com fontes alternativas de fósforo. *Pesqui. Vet. Bras.* 33, 188–192. doi: 10.1590/S0100-736X2013000200009

Lopes, F. C. F., Paciullo, D. S. C., Mota, E. F., Pereira, J. C., Azambuja, A. A., Motta, A. C. S., et al. (2010). Composição química e digestibilidade ruminal in situ da forragem de quatro espécies do gênero Brachiaria. *Arq. Bras. Med. Veterinária e Zootec.* 62, 883–888. doi: 10.1590/s0102-09352010000400018

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Saxman, P. R., Stredwick, J. M., et al. (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res.* 28, 173–174. doi: 10.1093/nar/28.1.173

Marcón, F., Urbani, M. H., Quarin, C. L., and Acuña, C. A. (2018). Agronomic characterization of *P. atratum* Swallen and *P. lenticulare* Kunth. *Trop. Grasslands-Forrajes Trop.* 6, 70. doi: 10.17138/tgft(6)70-81

Mohite, B. (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J. Soil Sci. Plant Nutr.* 13, 638–649. doi: 10.4067/s0718-95162013005000051

Nogueira, A. R. A., Machado, P. L. O. A., Carmo, C. A. F. S., and Ferreira, J. R. (1998). Manual de Laboratório: Solo, água, nutrição vegetal, nutrição animal e alimentos. São Carlos: EMBRAPA - CPPSE, 72p.: il. Available at: https://www.infoteca.cnptia.embrapa.br/infoteca/bitstream/doc/44394/1/ManualdeLaboratorios.pdf

Novo, P. E., Francisco, J., Valls, M., Galdeano, F., Honfi, A. I., Espinoza, F., et al. (2016). Interspecific hybrids between *Paspalum plicatulum* and *P. oteroi*: a key tool for forage breeding. *Sci. Agric.* 73, 356–362. doi: 10.1590/0103-9016-2015-0218

Novo, P. E., Galdeano, F., Espinoza, F., and Quarin, C. L. (2019). Cytogenetic relationships, polyploid origin, and taxonomic issues in Paspalum species: inter-and intraspecific hybrids between a sexual synthetic autotetraploid and five wild apomictic tetraploid species. *Plant Biol.* 21, 267–277. doi: 10.1111/plb.12931

Orbovic, V., and Grosser, J. W. (2006). "Citrus" in *Agrobacterium Protocols*. ed. K. Wang (Nova Iorque: Humana Press Inc), 177–189.

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., et al. (2015). Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. *Front. Microbiol.* 6, 1–9. doi: 10.3389/fmicb.2015.00745

Pande, A., Pandey, P., Mehra, S., Singh, M., and Kaushik, S. (2017). Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. *J. Genet. Eng. Biotechnol.* 15, 379–391. doi: 10.1016/j.jgeb.2017.06.005

Pérez, A., and Martínez, D. B. Z. (2016). Bacterias endófitas asociadas a los géneros Cyperus y Paspalum en suelos contaminados con mercurio. *Rev. U.D.C.A Actual. & Divulg. Científica* 19, 67–76.

Pezzopane, C.De, Lima, A. G., Cruz, P. G., Beloni, T., Fávero, A. P., and Santos, P. M. (2017). Evaluation and strategies of tolerance to water stress in Paspalum germplasm. *Trop. Grasslands-Forrajes Trop.* 5, 153–162. doi: 10.17138/tgft(5)153-162

Pisa, G., Magnani, G. S., Weber, H., Souza, E. M., Faoro, H., Monteiro, R. A., et al. (2011). Diversity of 16S rRNA genes from bacteria of sugarcane rhizosphere soil. *Brazilian J. Med. Biol. Res.* 44, 1215–1221. doi: 10.1590/S0100-879X2011007500148

Porto, P. P., Deresz, F., Tadeu, G., César, F., Lopes, F., Cecato, U., et al. (2009). Produção e composição química do leite, consumo e digestibilidade de forragens tropicais manejadas em sistema de lotação intermitente. *Rev. Bras. Zootec.* 38, 1422–1431. doi: 10.1590/S1516-35982009000800005

Prabha, R., Singh, D. P., Verma, M. K., Sahu, P., and Kumar, P. (2018). Bacterial diversity in rhizosphere of *Paspalum scrobiculatum* L. (kodo millet) is revealed with shotgun metagenome sequencing and data analysis. *Data Br.* 20, 1653–1657. doi: 10.1016/j.dib.2018.09.006

Quesenberry, K. H., Dampier, J. M., Lee, Y. Y., Smith, R. L., and Acuña, C. A. (2010). Doubling the chromosome number of bahiagrass *via* tissue culture. *Euphytica* 175, 43–50. doi: 10.1007/s10681-010-0165-4

Ramos, S. J., Faquin, V., Rodrigues, C. R., Silva, C. A., and Boldrin, P. F. (2009). Biomass production and phosphorus use of forage grasses fertilized with two phosphorus sources. *Rev. Bras. Cienc. do Solo* 33, 335–343. doi: 10.1590/S0100-06832009000200011

Rodrigues, R. C., Sousa, T. V. R., Melo, M. A. A., Araújo, J. S., Lana, R. P., Costa, C. S., et al. (2014). Agronomic, morphogenic and structural characteristics of tropical forage grasses in Northeast Brazil. *Trop. Grasslands-Forrajes Trop.* 2, 214–222. doi: 10.17138/TGFT(2)214-222

Rodríguez, H., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* 17, 319–339. doi: 10.1016/S0734-9750(99)00014-2

Rosenblueth, M., and Martínez-Romero, E. (2006). Bacterial Endophytes and their interactions with hosts. *Mol. Plant-Microbe Interact.* 19, 827–837. doi: 10.1094/mpmi-19-0827

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425. doi: 10.1093/oxfordjournals.molbev.a040454

SAS Institute Inc. (2011). *Base SAS® 9.3 Procedures Guide: Statistical Procedures*. SAS Institute Inc: Cary, NC.

Silva Filho, G. N., and Vidor, C. (2000). Solubilização de fostatos por microrganismos na presença de fontes de carbono. *Rev. Bras. Ciência do Solo* 24, 311–319. doi: 10.1590/s0100-06832000000200008

Souza, F. H. D., Gusmão, M. R., Cavallari, M. M., and Barioni, W. (2020). Characterization of the potential of native grasses for use as lawns. *Ornam. Hortic.* 26, 109–120. doi: 10.1590/2447-536X.v26i1.2067

Suleman, M., Yasmin, S., Rasul, M., Yahya, M., Atta, B. M., and Mirza, M. S. (2018). Phosphate solubilizing bacteria with glucose dehydrogenase gene for phosphorus uptake and beneficial effects on wheat. *PLoS One* 13, e0204408–e0204428. doi: 10.1371/journal.pone.0204408

Taiz, L., Zeiger, E., Moller, I. M., and Murphy, A. (2016). *Fisiologia e desenvolvimento vegetal. 6th Edn.* ed. P. L. de Oliveira (São Paulo).

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Van Raij, B., Andrade, J. C., Cantarella, H., and Quaggio, J. A. (2001). Análise química para avaliação da fertilidade de solos tropicais. *Campinas Inst. Agronômico* 775, 26–32. doi: 10.1016/j.mrfmmm.2015.03.010

Van Straaten, P. (2010). "Rochas e minerais como fertilizantes alternativos na agricultura: uma experiência internacional" in *Agrominerais para o Brasil* (Rio de Janeiro: CETEM/MCT), 235–264.

Verma, S. C., Ladha, J. K., and Tripathi, A. K. (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J. Biotechnol.* 91, 127–141. doi: 10.1016/S0168-1656(01)00333-9

White, J. F., Torres, M. S., Somu, M. P., Johnson, H., Irizarry, I., Chen, Q., et al. (2014). Hydrogen peroxide staining to visualize intracellular bacterial infections of seedling root cells. *Microsc. Res. Tech.* 77, 566–573. doi: 10.1002/jemt.22375

Wu, Z., Liu, Q., Li, Z., Cheng, W., Sun, J., Guo, Z., et al. (2018). Environmental factors shaping the diversity of bacterial communities that promote rice production. *BMC Microbiol.* 18, 51–11. doi: 10.1186/s12866-018-1174-z

Xiang, T., Malik, T. H., and Nielsen, K. (2020). The impact of population pressure on global fertiliser use intensity, 1970–2011: an analysis of policy-induced mediation. *Technol. Forecast. Soc. Change* 152, 119895. doi: 10.1016/j.techfore.2019.119895

Yang, B., Wang, Y., and Qian, P. Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinform.* 17, 135. doi: 10.1186/s12859-016-0992-y

Zhao, X., Chen, X., Li, Z., Zhang, J., and Liu, T. (2021). An evaluation of the effects of the plant endophyte Enterobacter on the salt tolerance of bermudagrass. *Acta Prataculturae Sin.* 30, 127–136. doi: 10.11686/cyxb2020317

Zuloaga, F. O., and Morrone, O. (2005). Revisión de las Especies de Paspalum para América del Sur Austral (Argentina, Bolivia, sur del Brasil, Chile, Paraguay, y Uruguay). *Econ. Bot.* 59, 304. doi: 10.1663/0013-0001(2005)059[0304:dfabre]2.0.co;2

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 26 September 2022
DOI 10.3389/fpls.2022.934339

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SPECIALTY SECTION
This article was submitted to Plant Symbiotic Interactions, a section of the journal Frontiers in Plant Science

RECEIVED 02 May 2022
ACCEPTED 29 August 2022
PUBLISHED 26 September 2022

CITATION
Ngosong C, Tatah BN, Olougou MNE, Suh C, Nkongho RN, Ngone MA, Achiri DT, Tchakounté GVT and Ruppel S (2022) Inoculating plant growth-promoting bacteria and arbuscular mycorrhiza fungi modulates rhizosphere acid phosphatase and nodulation activities and enhance the productivity of soybean (*Glycine max*). *Front. Plant Sci.* 13:934339.
doi: 10.3389/fpls.2022.934339

Inoculating plant growth-promoting bacteria and arbuscular mycorrhiza fungi modulates rhizosphere acid phosphatase and nodulation activities and enhance the productivity of soybean (*Glycine max*)

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Soybean [*Glycine max* (L.) Merrill] cultivation is important for its dual role as rich source of dietary protein and soil fertility enhancer, but production is constrained by soil nutrient deficiencies. This is often resolved using chemical fertilizers that exert deleterious effects on the environment when applied in excess. This field study was conducted at Nkolbisson-Yaoundé in the agro-ecological zone V of Cameroon to assess the performance of soybean when inoculated with plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without NPK fertilizer addition. Ten treatments (Control, PGPB, AMF, PGPB+AMF, PGPB+N, PGPB+PK, PGPB+N+PK, PGPB+AMF+N, PGPB+AMF+PK, and PGPB+AMF+N+PK) were established in a randomized complete block design with three replicates. Mycorrhizal colonization was only observed in AMF-inoculated soybean roots. In comparison to control, sole inoculation of PGPB and AMF increased the number of root nodules by 67.2% and 57%, respectively. Co-application of PGPB and AMF increased the number of root nodules by 68.4%, while the addition of NPK fertilizers significantly increased the number of root nodules by 66.9–68.6% compared to control. Acid phosphatase activity in soybean rhizosphere ranged from 46.1 to 85.1 mg h^{-1} kg^{-1} and differed significantly across treatments ($p < 0.001$). When compared to control, PGPB or AMF or their co-inoculation, and the addition of NPK fertilizers increased the acid phosphatase activity by 45.8%, 27%, 37.6%, and 26.2–37.2%, respectively. Sole inoculation of PGPB or AMF and their integration with NPK fertilizer increased soybean yield and grain contents (e.g., carbohydrate, protein, zinc, and iron) compared to the control ($p < 0.001$). Soil phosphorus correlated significantly ($p < 0.05$) with soybean grain protein ($r = 0.46$) and carbohydrate ($r = 0.41$) contents. The effective root nodules correlated significantly ($p < 0.001$) with acid phosphatase ($r = 0.67$) and soybean yield ($r = 0.66$). Acid phosphatase correlated significantly ($p < 0.001$) with soybean grain yield ($r = 0.63$) and carbohydrate ($r = 0.61$) content. Effective root nodules correlated significantly with carbohydrate ($r = 0.87$, $p < 0.001$), protein ($r = 0.46$, $p < 0.01$), zinc ($r = 0.59$, $p < 0.001$), and iron ($r = 0.77$, $p < 0.01$) contents in soybean grains. Overall, these findings indicate strong relationships between farm management practices, microbial activities in the rhizosphere, and soybean performance.

KEYWORDS

fertilizer, N_2-fixation, P-solubilization, phosphatase, rhizosphere

Introduction

Soybean [*Glycine max* (L.) Merrill] plays crucial roles in food and nutrition security due to its high nutrient contents, while its ability to biologically fix atmospheric nitrogen in symbiosis with Rhizobia enhances the productivity of agricultural systems. Soybean production in Cameroon has been increasing since 2010, and it is the second most cultivated legume after peanuts, with the rapid development of cultivated areas from 6,705 ha in 2008 to 15,020 ha in 2018 (WWF, 2014; Nyahnone, 2017; Nzossié and Bring, 2020). Macroeconomic data show that Cameroon imports an average of 20,000 tons of soybeans worth approximately CFAF 10 billion a year (WWF, 2014), and GMO soybean meal worth CFAF 14 billion (Nyahnone, 2017). Hence, there is a challenge to increase domestic supply to meet agro-industrial demand, which is indicative of the enthusiasm of farmers for soybean production. Wendt and Atemkeng (2004) reported soybean yield ranging between 448 and 709 kg/ha across the first and second planting seasons, with a significant effect of soil nutrients (especially magnesium content) on soybean yield.

Poor soil fertility is a major constraint for crop production in Cameroon with nitrogen (N) and phosphorus (P) as the main limiting elements (Tening et al., 2013; Ngosong et al., 2019; Nanganoa et al., 2020). Soil nutrient deficiencies are commonly resolved using chemical NPK fertilizers that are deleterious to the environment and humans when applied in excess, which has necessitated alternative management practices that can foster crop productivity without jeopardizing sustainability (Ntambo et al., 2017; Mahmud et al., 2020; Mndzebele et al., 2020). A promising alternative to increase crop performance is the use of beneficial microbes to enhance soil fertility, plant nutrition, and protection (Korir et al., 2017; Tchakounté et al., 2020). Despite the widely demonstrated importance of soil beneficial microbes in fostering biotic interactions in the rhizosphere and improving crop performance, biofertilizers have not been fully incorporated in farming systems relative to chemical fertilizers. Although proficient microbes can sustainably improve plant nutrition and protection (Bender et al., 2016; Venneman et al., 2017; Bello et al., 2018), microbial products are still largely untapped in Africa. Hence, there is a need to develop local microbial biofertilizers that can be harnessed to enhance the grain yield and nutrient contents of soybeans (Sogut, 2016; Marro et al., 2020; Zhang et al., 2020).

Besides the high nutrient contents and income generation potential of soybean cultivation, they additionally improve soil nitrogen *via* symbiotic biological N_2 fixation (Alam et al., 2015; Kalayu, 2019; Soumare et al., 2020). Indigenous or inoculated plant growth-promoting bacteria (PGPB) can improve crop yield *via* biological N_2 fixation, solubilization of inorganic phosphate, or production of phytohormones (Backer et al., 2018; Rosenblueth et al., 2018; Tchakounté et al., 2018; Bechtaoui et al., 2020). Some microbes mediate crop growth *via* secretion of metabolites, drought tolerance, and protection against pests and diseases (Radhakrishnan et al., 2017). Phosphate-solubilizing bacteria can convert inorganic or organically bound phosphate into bioavailable hydrogen-phosphate ions ($H_2PO_4^-$ or HPO_4^{2-}) through solubilization and mineralization processes (Behera et al., 2017). These microbes facilitate the conversion of complex forms of N and P to simple available forms for root uptake to enhance crop growth and yield (Kang et al., 2014, 2015; Kuan et al., 2016). Some *Bacillus* spp. release ammonia from nitrogenous organic matter in the soil or have *nifH* gene that produces nitrogenase for N_2 fixation to supply plants and enhance yield (Ding et al., 2005; Hayat et al., 2010; Kuan et al., 2016). Iron chelation by *Bacillus* spp. *via* siderophore production facilitates solubilization of iron from minerals and organic compounds in the rhizosphere by binding Fe^{3+} in complex substances and reducing them to Fe^{2+} for plant uptake (Walker and Connolly, 2008; Nadeem et al., 2012).

The bacteria biofertilizer used in this study comprised a consortium of symbiotic *Rhizobium* for N_2 fixation *via* root nodules to directly support the plants, and non-symbiotic PGPB to freely fix N_2 in the rhizosphere for uptake by soybean roots. In addition, inoculation of arbuscular mycorrhiza fungi (AMF) was intended to boost soybean performance by indirectly modulating soil enzyme activities associated with the processes of N_2 fixation, P solubilization, and mineralization, or by directly supplying the plants with N and P *via* its hyphal transport network. Arbuscular mycorrhiza fungi (AMF) exert significant positive effects on N_2 fixation *via* direct or indirect interactions with PGPB through nutrient transport and crop protection (Daniel et al., 2020; Novais et al., 2020). Moreover, microbial activities in the rhizosphere and plant performance can be enhanced by incorporating appropriate amounts of chemical NPK fertilizers in combination with microbial inoculants (Islam et al., 2017; Ntambo et al., 2017; Herliana et al., 2019). Chemical NPK fertilizers were applied in combination with PGPB and/or AMF to assess the possibility of boosting the potential of biofertilizers to enhance soybean performance within the nexus of integrated soil fertility management (Vanlauwe et al., 2010; Kanomanyanga et al., 2021). Hence, this study aims at evaluating microbial dynamics in the soybean rhizosphere, and soybean productivity as influenced by the application of locally produced biofertilizer, with or without the addition of chemical NPK fertilizers. It was hypothesized that inoculating PGPB and AMF will enhance microbial activities in the rhizosphere, including root nodulation and acid phosphatase, and increase the soybean grain yield and nutrient contents.

Materials and methods

Experimental site and setup

The experiment was conducted from April to July 2021 at the Institute of Agricultural Research for Development (IRAD) Nkolbisson, Yaoundé, Cameroon. The site is located in agro-ecological zone V of Cameroon, which is a humid forest zone situated between Latitude 03° 8’ 71.2” N and Longitude 11° 45’ 38.0” E. The area has an equatorial climate with a mean annual temperature of 23.5°C (ranging between 16°C and 31°C), and 1,600 mm rainfall that occurs in a bimodal configuration such that the first and second cropping seasons are separated by a 4-month dry season, which lasts from mid-March to early July and from late August to mid-November, respectively (Ambassa-Kiki and Nill, 1999). However, the duration of each season presently varies and the second season rains are erratic due to global climate change dynamics.

Rice was previously cultivated on the field site from 2016 to 2019 and fallowed for 1 year in 2020 before this study. The field experiment was laid out as a randomized complete block design with ten treatments and three replicates each, giving a total of thirty experimental units. The treatments include T1 – control (no input), T2 – plant growth-promoting bacteria (PGPB), T3 – Arbuscular mycorrhiza fungi (AMF), T4 – PGPB+AMF, T5 – PGPB+N, T6 – PGPB+PK, T7 – PGPB+N+PK, T8 – PGPB+AMF+N, T9 – PGPB+AMF+PK, and T10 – PGPB+AMF+N+PK. Each experimental plot measured 3.2 m × 4 m (12.8 m^2) with a 1 m buffer zone between plots and a 1.5 m buffer between the replicate blocks.

The experimental site was cleared and weed regrowth was allowed for 2 weeks, and the emerging weeds were sprayed with a systemic herbicide (Roundup 360SL, Belgium; comprising active components glyphosate) at a rate of 129.6 kg in 360 L of water ha^{-1}. Three days after the application of herbicide, all plots were tilled manually to produce raised beds of approximately 30 cm high (**Figure 1**), and soybean was planted 1 week after herbicide spray. Three soybean seeds (Panorama 357 variety) were planted per hole at approximately 4 cm depth and 10 cm intra-row and 40 cm inter-row spacing, making 7 rows per plot. Thinning was done after germination to two vigorous plants per hole, giving a total of 382,813 plants ha^{-1}. Synthetic insecticide K-Optimal (SCPA SIVEX International France; comprising active components Lambda—cyhalothrine 15 g L^{-1} + Acetamipride 20 g L^{-1}) and fungicide Monchamp 72 WP (Mancozeb 60% + Metalaxyl 12%) were applied at 3 weeks after sowing to control insect pests and fungal infections. Each was applied at the rate of 150 ml in 15 L of knapsack sprayer, which is equivalent to 2 L ha^{-1} each. The field was regularly monitored and weeding was done manually.

Microbial inoculation

Plant growth-promoting bacteria

The bacterial inoculant used in this study consisted of a consortium of symbiotic and non-symbiotic plant growth-promoting bacteria—PGPB (**Table 1**). The non-symbiotic PGPB included the following organisms: (03) *Arthobacter* sp., (03) *Bacillus* sp., (01) *Lysinibacillus* sp., (03) *Paenibacillus* sp., and (01) *Sinomonas* sp., which were isolated from the rhizosphere of maize plants in Cameroon (Tchakounté et al., 2018); and (01) *Kosakosania radicincitans* isolated from the phyllosphere

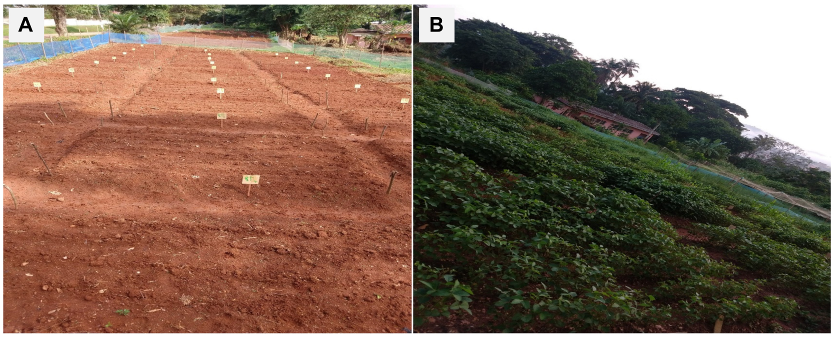

FIGURE 1
Experimental site with tilled soil beds before sowing **(A)** and soybean plants at full podding **(B)**.

of winter wheat in Germany and deposited in NCBI as DSM 16656^T GenBank: CP018016.1, CP018017.1, CP018018.1 (Ruppel and Merbach, 1995; Becker et al., 2018). The symbiotic bacterium (*Bradyrhizobium japonicum*) was obtained from the Soil Microbiology Laboratory of the Biotechnology Center of the University of Yaoundé I, Cameroon.

For the production of microbial inoculant consortium, a colony of symbiotic *B. japonicum* was collected from an inoculum stock and transferred into a 500-ml flask containing 100 ml sterilized yeast mannitol broth (YMB), and incubated in a shaker at 28°C at 200 rpm for 48 h. From a stock culture of each of the non-symbiotic plant growth-promoting bacteria, a pure colony was collected and transferred into a 500-ml flask containing 100 ml sterilized nutrient broth (Standard nutrient broth I, Carl Roth, Germany), and incubated at 28°C for 24–48 h. The individually cultured symbiotic and non-symbiotic plant growth-promoting bacteria were assembled into a microbial consortium in a 5 L container and sugar was added (1:1) to serve as an adjuvant because the *B. japonicum* used in this study is not sticky as compared to the non-symbiotic microbes. The soybean seeds were immersed in the microbial inoculant consortium (e.g., 1 kg of soybean seeds per 100 ml of biofertilizer inoculum) and thoroughly mixed. The seeds were removed from the inoculum and allowed to air-dry for 1 h before planting (Atieno et al., 2012).

Arbuscular mycorrhiza fungi

The composite inoculant product used for this experiment was obtained from the Regional Biocontrol and Applied Microbiology Laboratory of IRAD, Nkolbisson, Cameroon. It comprised the three most dominant arbuscular mycorrhiza strains (*Enthophospora infrequens*, *Scutellospora cerradessis,* and *Gigaspora gigantea*) identified in the soybean rhizosphere of six high-intensity soybean-producing areas across the five different agro-ecological zones of Cameroon. In total, 300 g of mycorrhiza inoculum stock was used to produce a bulk inoculum using a sterilized mixture of fine and coarse sand substrates (1:1), sterilized at 121°C for 1 h in an autoclave (model PTS-B100L). Three polypropylene bags were filled with 20 kg each of sterilized substrates, and highly mycotrophic *Sorghum bicolor* plants were planted and maintained in the greenhouse for 3 months with regular irrigation, before subjecting the plants to water stress for 1 month to stimulate sporulation by mycorrhiza. The suitability of the mycorrhiza inoculum was determined by the spore density, which was determined from a 100 g sample of the homogenized inoculant product by immersing in 300 ml distilled water in a 1,000-ml beaker, and the mixture was stirred and allowed to stand for 15 s. Four sieves were arranged in decreasing order of mesh size (710, 225, 125, and 45 μm) and used to filter the supernatant of each mixture. The contents of the last three sieves were collected, washed, and the number of observed mycorrhiza spores was counted on a square graduated petri-dish using a stereo microscope (WILD M2B, Germany). The observed spore density of 670 spores 100 g^{-1} of inoculum was considered suitable for use as AMF inoculum. The AMF inoculum was air-dried and used for field inoculation at 20 g per soybean stand (containing approximately 134 spores). The inoculum was applied by placing the 20 g inoculum at about 80 mm depth in the planting hole before planting soybean seeds.

Application of chemical fertilizers

So far there is no specific fertilizer recommendation for soybean production on the study site. Therefore, the application of nitrogen as urea, phosphorus as triple superphosphate (TSP), and potassium as muriate of potash (MOP) was based on general fertilizer recommendations for soybean. Urea was applied at the rate of 40 kg N ha^{-1} (Uko et al., 2002) as two split doses of 20 kg N ha^{-1} each 2 weeks after planting and beginning of seed development at the R5 stage. Phosphorus was applied at 30 kg P_2O_5 ha^{-1} (Lamptey et al., 2014) and potassium at 40 kg K_2O ha^{-1} (Islam et al., 2017) 2 weeks after planting. All fertilizers were applied by ringing at approximately 5 cm from plants to

TABLE 1 Plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi, their functional traits, and potential roles in the consortium of biofertilizers to enhance soil fertility and performance of soybean plants.

S/N	Beneficial microbes		Microbial isolates	Genus	Family	Phylum	nifH gene	Siderophore production	Phosphorus solubilization	Biocontrol activity	Reason for selection and inclusion in the inoculant consortium
1	PGPB	Symbiotic	NKa11	*Bradyrhizobium*	Rhizobiaceae	Proteobacteria	√	/	/	/	N_2 fixation
2			*DSM 16656^{T}	*Kosakonia*	Enterobacteriaceae	Proteobacteria	√	√	√	√	N_2 fixation, Siderophore production and P solubilization
3		Non-symbiotic	V64 (*MN128891), V84 (*MN128892), and V127	*Arthrobacter*	Micrococcaceae	Actinobacteria	/	√	√	/	Siderophore production and P solubilization
4			VA9, V22, and V65	*Bacillus*	Bacillaceae	Firmicutes	/	√	√	/	Siderophore production and P solubilization
5			V47	*Lysinibacillus*	Bacillaceae	Firmicutes	√	/	/	/	N-Fixation
6			VA7	*Paenibacillus*	Paenibacillaceae	Firmicutes	/	√	√	/	P solubilization and N_2 Fixation
7			V12 and V18	*Paenibacillus*	Paenibacillaceae	Firmicutes	√	/	√	/	P solubilization and N_2 Fixation
8			V4	*Sinomonas*	Micrococcaceae	Actinobacteria	/	√	√	/	Siderophore production and P solubilization
9	Endophytic fungi		*Enthophospora infrequens*, *Scutellospora cerradessis*, and *Gigaspora gigantea*	Mycorrhiza	Acaulosporaceae -Gigasporaceae	Glomeromycota	/	/	√	√	P solubilization and mobilization

*NCBI accession reference.

avoid burns and minimize nutrient loss through leaching and volatilization.

Data collection

Soil properties

Pre-planting soil was sampled for the entire experimental site after clearing and laying out but before tillage, while post-planting soil was sampled for each plot at harvest. An auger was used to collect three pre-planting soil samples randomly using a Z-pattern at 0–15 cm depth and bulked to form a composite sample. Three post-planting soil samples were also collected randomly from each treatment plot at 0–15 cm depth using an auger, and thoroughly mixed to form a composite sample. All soil samples were air-dried at room temperature and stored in polybags before analysis. The soil samples were crushed and sieved through a 2-mm sieve for the determination of soil's physical and chemical properties.

The soil particle size distribution was determined using the pipette sampling method with sodium hexametaphosphate as a dispersing agent, and the textural class was assigned according to USDA textural triangle (Van Reeuwijk, 1992). The soil pH was determined potentiometrically in water (H_2O) and 1 N potassium chloride (KCl) solutions after 24 h in soil suspension (soil/liquid 1:2.5 w/v) using a glass electrode pH meter. The exchangeable bases (Ca^{2+}, Mg^{2+}, K^+, and Na^+) were extracted using 1 N ammonium acetate ($NH_4CH_3CO_2$) solution at pH 7. Calcium (Ca) and magnesium (Mg) were determined by the titration method using Eriochrome Black T (EBT or Erio T) as an indicator while potassium (K) and sodium (Na) were determined using the flame photometer (Rowell, 1994). Exchange acidity was extracted with 1 N KCl and determined by titrating the extract with 0.01 N NaOH, using a phenolphthalein indicator (Van Reeuwijk, 1992). Effective cation exchange capacity (ECEC) was determined by the summation of exchangeable bases and exchange acidity. The total soil nitrogen (N) was determined by the macro Kjeldahl digestion method (Bremner and Mulvaney, 1982). Plant available phosphorus (P) in the soil was determined by the Bray II method (Van Reeuwijk, 1992), and organic carbon was determined by the wet oxidation method (Kalra and Maynard, 1991).

Mycorrhiza colonization

An assessment of AMF root colonization was conducted according to Begoude et al. (2016). Briefly, 1–2 cm segments of root samples were placed in 5% KOH solution for 24 h at room temperature and rinsed three times with water on a fine sieve. Root samples were acidified in 10% HCl (v/v) for 15 min and stained with 0.01% (w/v) fuchsine acid for 24 h at room temperature. Root segments were randomly selected from the stained samples and three replicates of 10 roots per slide were assessed for the occurrence of AMF structures (e.g., vesicles, arbuscules, and hyphae) using an optical microscope (Biological compound microscope with replaceable LED light, OMAX 40X-2500X Trinocular, Germany) (**Figure 2**). The mycorrhizal frequency (F%) was given as the ratio of colonized root fragments to the total number of observed root fragments.

Quantification of rhizosphere acid phosphatase activity

Five plants were randomly selected from the center of each plot at full podding for assessment of acid phosphatase activity. A spade was used to dig approximately 5 cm around each plant at the depth of 20 cm, and 1 g root adhering soil was collected from each plant and bulked to form a composite sample of 5 g, from which 1 g was taken into a microcentrifuge tube and 0.5 ml of 100 mM phosphate buffer was added. P-nitrophenyl phosphate (p-NPP, 10 mM) in 100 μl solution was used as substrate. The final volume of the reaction mixture was adjusted to 1 ml by adding the required amount of distilled water. The tube was vortexed (2 min) at room temperature and incubated at 37°C for 60 min in shake condition (100 rpm). After the incubation, samples were centrifuged at 10,000 rpm (5 min) and the clear supernatants were transferred into clean test tubes and 2 ml of 1 M NaOH was added. The yellow filtrate was analyzed using a colorimeter (Klett colorimeter, Clinical model, 800-3, 115 VAC) at λ = 430 nm. The concentration of soluble protein in the supernatant was determined using the Bradford reactive procedure, and the specific activity of soil acid phosphatase was estimated using the formula:

$$\text{Protein content} = \frac{10 \times C}{17800} \tag{1}$$

Where, 10 = constant, C = concentration of soluble protein, and 17,800 is the molecular extension coefficient of phosphatase. All derived values were then converted to mg h^{-1} kg^{-1} soil by multiplying by 100,000.

Root nodulation

Five plants were randomly selected from the second border rows of each plot at full podding and assessed for nodulation parameters. The nodulation rating was assessed according to Tamiru et al. (2012) as follows:

$$\text{Nodulation rating} = \frac{(a \times 10) + (b \times 5) + (c \times 1) + (d \times 0)}{\text{Total number of plants}} \tag{2}$$

Where the number of plants showing tap root nodulation (a), plants with nodules in secondary roots but close to tap root (b), plants with scattered nodulation (c), and plants without nodulation (d) were evaluated.

The nodule volume was also assessed according to Tamiru et al. (2012), where nodules of the five sampled plants were

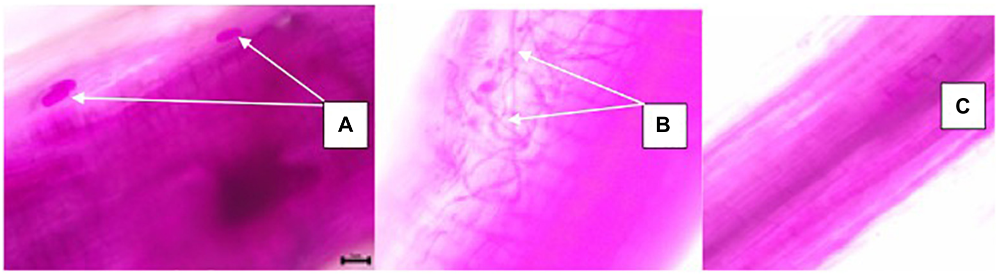

FIGURE 2
Microscopic identification of mycorrhiza structures in soybean roots; Vesicles **(A)**, Hyphae **(B)**, and un-infected roots **(C)** as influenced by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers.

immersed in a 50 ml plastic cylinder containing 30 ml of water. The amount of water displaced after immersion of the nodules was recorded and the mean was considered as nodule volume per plant. For the effectiveness of root nodules, all the nodules from the five sampled plants per treatment were dissected using a sharp knife, and a hand lens was used to observe the internal nodule color. The presence of pink or reddish coloration was considered as effective and used to distinguish nodules that are actively fixing nitrogen from the inactive nodules (Ngeno et al., 2012).

Soybean grain yield

Soybean plants were harvested at physiological maturity and the weight of 1,000 randomly selected grains from each plot maintained at 10% moisture content was reported as a thousand grain weight. Soybean grain yield was obtained by adjusting the moisture level to 10% according to the following formula and converted to tons ha^{-1}:

$$Adjusted\ yield = \frac{100 - MC}{100 - 10} \times unadjusted\ yield \quad (3)$$

Where MC is the moisture content of soybean seeds at the time of measurement, and 10 is the percentage standard moisture content of soybean seeds at harvest.

Nutrient contents of soybean grains

The proximate (e.g., protein and carbohydrate) composition of soybean grains were determined according to the official method described by the Association of Official and Analytical Chemist (AOAC, 2005). Each sample was analyzed in triplicates and values were presented in percentages. The Anthrone standard method (David, 1978) was used to estimate the content of carbohydrates where different volumes of glucose solution from 200 μg mol^{-1} stock solution were pipetted and made up to 1 ml with distilled water. In total, 15 tubes were used with tube 1 considered as blank, tubes 2–9 were used to construct a standard curve, and tubes 10–15 were used for unknown samples. Anthrone (5 ml) was added to each tube and vortexed to thoroughly mix and allowed to cool. The tubes were covered with marble/caps and incubated at 90°C for 17 min and allowed to cool at room temperature, and optical density was measured at 620 nm against the blank sample. The amount of glucose in the unknown sample was determined by plotting a standard curve of A620 on Y-axis and μg glucose on X-axis.

Crude protein was determined using the Kjeldahl digestion method by measuring the nitrogen content of the soybean grain samples and multiplying it by a factor of 6.25, based on the fact that protein contains approximately 16% nitrogen. Approximately 2 g of crushed soybean grain powder was weighed into a Kjeldahl flask and 25 ml of concentrated sulfuric acid, 0.5 g of copper sulfate, 5 g of sodium sulfate, and a speck of selenium tablet acid were added. Heat was first applied in a fume cupboard slowly to prevent undue frothing. The digestion continued for 45 min until the digestate became clear pale green. The digestate was transferred into a 100 ml volumetric flask and this was made up to the mark with distilled water. The Kjeldahl distillation apparatus used for distillation was steamed up and 10 ml of the digest was added into the apparatus *via* a funnel and allowed to boil. Sodium hydroxide (10 ml) was added from the measuring cylinder so that ammonia was not lost. It was later distilled into 50 ml of 2% boric acid containing screened methyl red indicator. The contents of the collecting flask (50 ml) were titrated with sulfuric acid standard volumetric solution using a burette and the amount of titrant used was read. When colorimetric end-point detection was applied, the end-point was reached when the color of the solution changed from green to red. The burette reading was estimated to the nearest 0.01 ml. To confirm that the reagents were free from nitrogen, a blank test was conducted (e.g., performing digestion, distillation, and titration) using only reagents without adding soybean material. The nitrogen content of soybean grains was calculated as follows:

$$\mathrm{MN} = \frac{(Va - Vb) \times CHCl \times MN}{mvz} \times 1,000 \quad (4)$$

where Va = volume of standard HCl solution when titrating sample, Vb = volume of standard HCl solution when titrating blank, CHCl = concentration of HCl (mol L^{-1}), MN = nitrogen molar mass (g mol^{-1}), and mvz = weight of sample (g).

$$\text{Percentage crude protein} = \%\ \text{Nitrogen x } 6.25 \qquad (5)$$

The mineral content of zinc (Zn) and iron (Fe) were determined using atomic absorption spectrometry according to the standard method (AOAC, 2005). Samples were ashed at 550°C and boiled with 10 ml of 20% hydrochloric acid in a beaker and then filtered into a 100 ml standard flask. This was made up to the mark with distilled water. The mineral content of Zn and Fe was determined from the resulting solution using Atomic Absorption Spectrophotometer at 510, 213.86, 766.5, 213.6 nm. Different electrode lamps were used for each mineral and the equipment was run for standard solutions of each mineral before and during determination to ascertain the efficiency. All values were expressed in mg 100 g^{-1}.

$$Zn/Fe\ (mg/100g) = \frac{Absorbency\ (ppm) \times dilution\ factor \times volume\ of\ extract\ (mL)}{Weight\ of\ sample} \qquad (6)$$

Data analysis

All statistical analyses were done using SPSS (Ver. 23), and data sets were analyzed for normality and homogeneity using Kolmogorov–Smirnov and Levene's tests, respectively. Data on soil chemical properties, root nodulation, acid phosphatase activity, grain yield, and nutrient contents were subjected to analysis of variance (ANOVA, $p < 0.05$) to test the effects of treatments as categorical predictors. Significantly different means were separated using Tukey's HSD test (Tukey's HSD, $p < 0.05$). Where applicable, correlation ($p < 0.05$) was performed to determine the degree of association between the dependent and independent variables.

Results

Soil chemical properties

The baseline soil analysis before establishing the experiment indicates an acidic soil with very low soil nutrient contents (e.g., N, P, Ca, Mg, K, Na) and cation exchange capacity (Table 2). Only the organic C content (1.4–2.4%) is suitable, which results in a quite wide pre-experiment C/N ratio of approximately 20 units. The post-planting soil Ca and Mg contents differed significantly across the experimental treatments. When compared to pre-planting soil, treatments

TABLE 2 Soil properties as influenced by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers.

Treatments	pH (H_2O)	pH (KCl)	Total N	Organic C	Organic M	C/N	Bray P	Ca	Mg	K	Na	ECEC
			(%)				(mg kg^{-1})	cmol kg^{-1}				
Pre-plant	4.30 ± 0.17a	3.23 ± 0.06ab	0.09 ± 0.02a	2.16 ± 0.27a	3.73 ± 0.47a	20.66 ± 2.93a	5.56 ± 1.02a	2.40 ± 0.13d	4.13 ± 1.62b	0.76 ± 0.12a	0.03 ± 0.00b	17.70 ± 1.55a
Control	4.37 ± 0.15a	3.10 ± 0.10b	0.10 ± 0.03a	2.36 ± 1.05a	4.06 ± 1.81a	24.16 ± 11.47a	4.23 ± 0.32a	6.51 ± 0.85abc	1.60 ± 0.92b	1.42 ± 1.21a	0.42 ± 0.27ab	22.19 ± 6.75a
PGPB	4.27 ± 0.12a	3.40 ± 0.17ab	0.07 ± 0.02a	2.45 ± 0.59a	4.23 ± 1.01a	41.55 ± 24.04a	4.80 ± 0.31a	5.71 ± 0.53abc	1.76 ± 0.57b	2.73 ± 2.78a	0.14 ± 0.03ab	22.08 ± 6.41a
AMF	4.37 ± 0.23a	3.50 ± 0.10a	0.11 ± 0.03a	2.40 ± 0.83a	4.13 ± 1.43a	23.95 ± 15.35a	5.88 ± 0.64a	7.26 ± 0.83a	2.18 ± 0.81b	2.50 ± 1.39a	0.15 ± 0.09ab	20.48 ± 7.92a
PGPB+AMF	4.43 ± 0.06a	3.20 ± 0.10ab	0.09 ± 0.04a	2.36 ± 0.47a	4.06 ± 0.80a	30.24 ± 17.61a	5.45 ± 0.43a	6.81 ± 1.08ab	1.89 ± 1.45b	1.93 ± 0.76a	0.34 ± 0.10ab	22.61 ± 1.29a
PGPB+N	4.23 ± 0.25a	3.20 ± 0.10ab	0.11 ± 0.02a	2.32 ± 0.47a	3.99 ± 0.61a	21.99 ± 1.93a	5.17 ± 0.86a	4.78 ± 0.37abc	2.48 ± 0.64b	1.59 ± 1.10a	0.23 ± 0.13ab	21.33 ± 0.88a
PGPB+PK	4.27 ± 0.21a	3.33 ± 0.15ab	0.11 ± 0.06a	1.46 ± 0.36a	2.53 ± 0.81a	14.75 ± 5.46a	8.56 ± 0.99a	5.21 ± 0.88abc	3.44 ± 3.45b	1.02 ± 0.39a	0.15 ± 0.15ab	18.56 ± 1.62a
PGPB+NPK	4.23 ± 0.1a	3.30 ± 0.10ab	0.10 ± 0.01a	1.81 ± 0.12a	3.13 ± 0.21a	18.74 ± 1.07a	9.34 ± 0.36a	5.15 ± 0.67abc	1.81 ± 1.01b	0.50 ± 0.30a	0.10 ± 0.11ab	13.23 ± 5.18a
PGPB+AMF+N	4.23 ± 0.3a	3.33 ± 0.15ab	0.05 ± 0.04a	2.12 ± 0.71a	3.66 ± 1.22a	64.28 ± 52.65a	8.95 ± 4.76a	5.79 ± 1.21abc	2.56 ± 1.76b	0.69 ± 0.13a	0.06 ± 0.02b	24.29 ± 13.47a
PGPB+AMF+PK	4.37 ± 0.1a	3.43 ± 0.12ab	0.08 ± 0.02a	1.81 ± 0.53a	3.13 ± 0.91a	23.63 ± 10.69a	7.47 ± 3.49a	4.35 ± 1.01bc	2.86 ± 2.48b	0.56 ± 0.06a	0.03 ± 0.00b	17.72 ± 3.85a
PGPB+AMF+NPK	4.27 ± 0.40a	3.20 ± 0.10ab	0.06 ± 0.02a	2.36 ± 0.32a	4.06 ± 0.56a	40.77 ± 15.13a	8.58 ± 2.00a	4.11 ± 1.40c	8.29 ± 2.80a	0.62 ± 0.24a	0.03 ± 0.03b	16.88 ± 1.47a

Data (Mean ± SD) within columns with different letters are significantly different (Tukey's HSD, $p < 0.05$).

with or without microbial and chemical fertilizer application increased the soil calcium content significantly ($F_{9,20}$ = 3.78, $p < 0.05$, **Table 2**), which ranged from 2.40 to 7.26 cmol kg^{-1}, with the highest in the sole mycorrhiza treatment and the lowest in PGPB+AMF+NPK. The post-planting magnesium content ranged from 1.60 to 8.30 cmol kg^{-1} of soil, and it was significantly higher in the PGPB+AMF+NPK treatment ($F_{9,20}$ = 3.43, $p < 0.05$, **Table 2**).

Mycorrhiza colonization and nodulation of soybean roots

The soybean roots were colonized by mycorrhiza at the rate of 51.1–56.7%, which was only observed in plant roots that were inoculated with AMF, but there was no significant difference between the treated plots ($p > 0.05$, **Figure 3**). Hence, the addition of PGPB or chemical NPK fertilizer to the AMF treatments did not influence the rate of colonization of soybean roots by AMF. The number of root nodules ranged from 2.9 to 9.1 with the highest in PGPB treatments followed by the sole AMF as compared to the control ($F_{9,20}$ = 37.53, $p < 0.001$; **Figure 4A**). The inoculation of PGPB and AMF increased the number of root nodules by 67.2% and 57%, respectively, as compared to the control, with the PGPB performing 10.2% more than AMF in relation to the control. Meanwhile, the integration of PGPB and AMF increased the number of root nodules by 68.4% compared to the control. Furthermore, the treatments with co-application of biofertilizers (PGPB and AMF) and mineral NPK fertilizer significantly increased the number of root nodules at the range of 66.9–68.6% compared to the control, but there was no significant difference between the treated plots (**Figure 4A**). A similar trend of results was also observed for the number of effective nodules ($F_{9,20}$ = 28.85, $p < 0.001$; **Figure 4B**). The highest nodulation rating occurred in PGPB treatments compared to the control ($F_{9,20}$ = 8.36, $p < 0.05$; **Supplementary Table 1**). Nodule volume ranged from 0.16 to 0.40 ml per plant and differed significantly ($F_{9,20}$ = 22.15, $p < 0.05$; **Supplementary Table 1**) with the highest in PGPB treatments. The nodule dry weight ranged from 0.017 to 0.038 g with the highest in PGPB treatments compared to the control ($F_{9,20}$ = 43.38, $p < 0.05$; **Supplementary Table 1**). Overall, no additional effect of mineral NPK fertilizer application was detected in all the measured root nodulation parameters.

Acid phosphatase activity in the soybean rhizosphere

The acid phosphatase activity in the soybean rhizosphere ranged from 46.1 to 85.1 mg h^{-1} kg^{-1}, which differed significantly across treatments ($F_{9,20}$ = 13.25, $p < 0.001$; **Figure 5**), with the lowest phosphatase activity recorded in the soybean roots of the control without any microbial or mineral fertilizer application, while the highest occurred in the sole PGPB treatment as compared to all the other treatments. All the microbial treatments almost doubled the acid phosphatase activity in the soybean rhizosphere as compared to the control, with a 45.8% and 27% increase in acid phosphatase activity for PGPB and AMF, respectively, as compared to the control, while the PGPB performed 18.8% more than AMF in relation to the control. Meanwhile, the integration of PGPB and AMF increased the acid phosphatase activity by 37.6% compared to the control, but this was 8.2% lower than the sole PGPB application. Furthermore, the treatments with co-application of biofertilizers (PGPB and AMF) and mineral NPK fertilizer significantly increased the acid phosphatase activity in the range of 26.2–37.2% compared to the control, but there was no significant difference between the NPK fertilizer–treated plots (**Figure 5**). Meanwhile, the co-application of biofertilizers (PGPB and AMF) and mineral NPK fertilizer significantly reduced the acid phosphatase activity by 13.7–26.5% as compared to the sole PGPB treatment.

Soybean grain yield and nutrient contents

The soybean grain yield ranged between 0.50 and 1.16 tons ha^{-1} and increased significantly in all applied treatments compared to the non-treated control ($F_{9,20}$ = 8.83, $p < 0.001$; **Figure 6**). The soybean grain yield increased significantly by 46.1–57.1% for all treated plots (sole PGPB or AMF and their combination with NPK fertilizer) in relation to the control, but there was no significant difference between the treated plots. In contrast to soybean grain yield that was not influenced by the integrated application of biofertilizers (PGPB or AMF) and chemical NPK fertilizers, the nutrient contents of soybean grains increased significantly in the integrated biofertilizer and NPK fertilizer treatments as compared to the sole application of biofertilizers (**Table 3**). All treatments, including biofertilizers alone and their combinations with chemical NPK fertilizers, significantly increased the contents of carbohydrate, protein, zinc, and iron in the soybean grains (**Table 3**). The carbohydrate content ranged between 20.1 and 22.7% ($F_{9,20}$ = 5926.53, $p < 0.001$) and protein ranged between 31 and 39.9% ($F_{9,20}$ = 3977.2, $p < 0.001$) across treatments, with the highest in PGPB+AMF+NPK treatment, as compared to the lowest values in the control (**Table 3**). Inoculating PGPB and AMF with or without NPK fertilizers significantly enhanced the protein content of soybean ($F_{9,20}$ = 3977.18, $p < 0.001$; **Table 3**), which ranged from 31 to 39.9% with the highest in PGPB+AMF+NPK treatment and the lowest in the control. The zinc content ranged between 2.7 and 4.9 mg 100 g^{-1} ($F_{9,20}$ = 1842.11, $p < 0.001$; **Table 3**) and iron ranged between 16.8 and 19.9 mg 100 g^{-1} across treatments, again with the

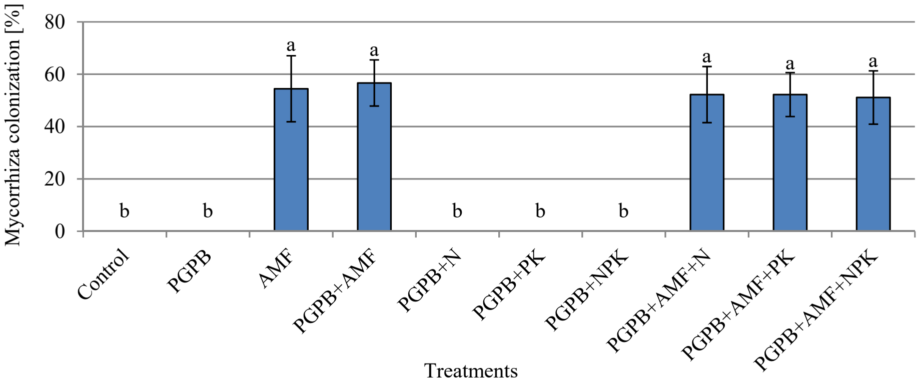

FIGURE 3

Mycorrhiza colonization of soybean roots affected by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers. Data (Mean ± SD) with different letters are significantly different (Tukey's HSD, $p < 0.05$).

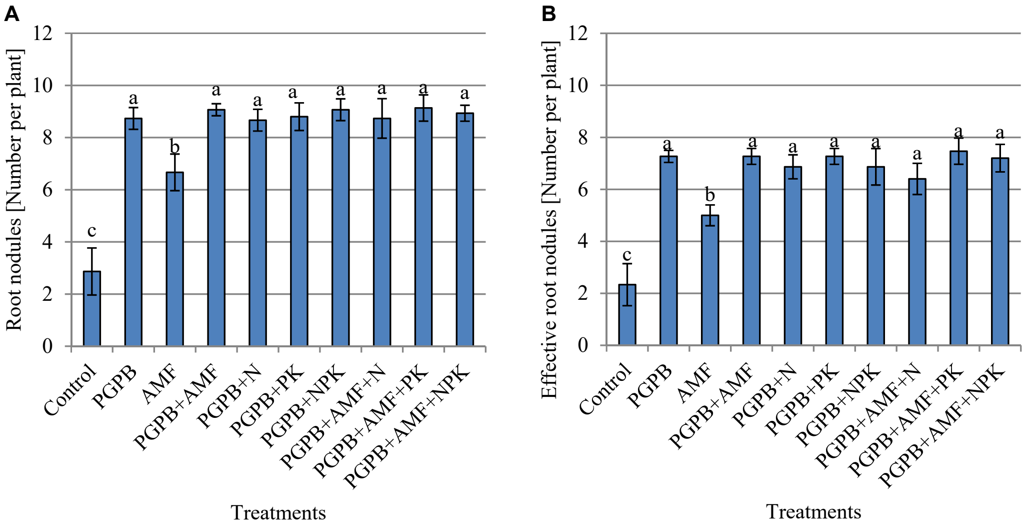

FIGURE 4

The total number of root nodules per plant **(A)** and the number of effective root nodules per plant **(B)** as affected by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers. Data (Mean ± SD) with different letters are significantly different (Tukey's HSD, $p < 0.001$).

highest in PGPB+AMF+NPK addition, as compared to the lowest in the control.

Correlation of soil parameters and soybean performance

The soil phosphorus correlated significantly ($p < 0.05$; Table 4) with protein ($r = 0.46$) and carbohydrate ($r = 0.41$) contents in soybean grains. The effective root nodules correlated significantly ($p < 0.001$) with acid phosphatase ($r = 0.67$; Figure 7A) and soybean yield ($r = 0.66$; Figure 7B). Acid phosphatase correlated significantly ($p < 0.001$) with soybean yield ($r = 0.63$; Figure 7C) and carbohydrate ($r = 0.61$; Table 4) contents in the grains. The effective root nodules correlated significantly with carbohydrate ($r = 0.87$, $p < 0.001$), protein ($r = 0.46$, $p < 0.01$), zinc ($r = 0.59$, $p < 0.001$), and iron ($r = 0.77$, $p < 0.01$) contents in soybean grains (Table 4). These correlations indicate higher root nodulation with increased acid phosphatase activity in the rhizosphere of soybean plants, and the influence of symbiotic N_2 fixation and plant available phosphorus in soil on the nutrient contents of soybean grains. Overall, these results highlight strong relationships between farm management practices, rhizosphere microbial activities, and soybean grain bio-fortification.

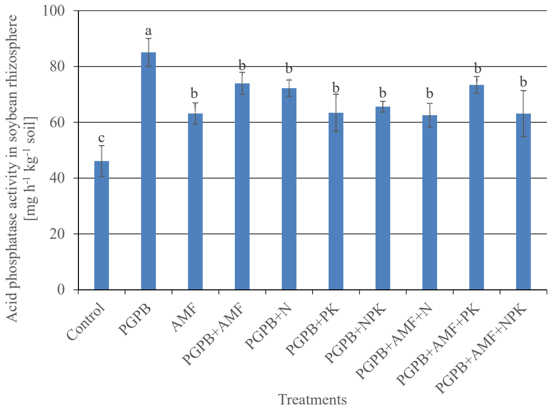

FIGURE 5
Acid phosphatase activity in the rhizosphere of soybean plants affected by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers. Data (Mean ± SD) with different letters are significantly different (Tukey's HSD, $p < 0.001$).

Discussion

Mycorrhization and root nodulation

Although soil N and P did not differ significantly across treatments, increased microbial activities (root nodulation, mycorrhization, and acid phosphatase) in the rhizosphere resulting from the influence of inoculated microorganisms probably increased plant nutrient uptake, which resulted in greater yield (Rawat et al., 2020). The fact that mycorrhiza colonization was not observed in plants that were not inoculated with AMF likely reflects low native AMF presence in soil, while a consistent level of AMF colonization for all inoculated treatments reflects positive plant–AMF symbiosis, especially under low native AMF status. However, the addition of PGPB and NPK fertilizer did not affect the rate of AMF colonization compared to the sole AMF treatment, indicating a strong boost of the inoculated AMF to compensate for low native AMF presence, irrespective of other inputs. Chen et al. (2018) and Powell and Rillig (2018) also described such a low AMF colonization rate as an indication of low soil health status. This could be attributed to intensive and unsustainable farm management practices such as chemical inputs and soil tillage under rotational cropping systems that may affect the soil biota (Wakam et al., 2015; Begoude et al., 2016). The observed high root colonization in AMF inoculated plants demonstrates the capacity of the inoculated mycorrhiza to compete with other rhizosphere microbiota and survive, which is an important characteristic of efficient bio-inoculants in enhancing soil fertility and productivity.

The high root nodulation achieved with inoculation of the PGPB consortium comprising *Bradyrhizobium* suggests successful symbiosis between the inoculated indigenous rhizobia and the soybean roots (Korir et al., 2017; Soumare et al., 2020). Accordingly, improved soybean root nodulation and N_2 fixation were reported with the inoculation of *Bradyrhizobium* species and fertilizer addition (Ulzen et al., 2016; Leggett et al., 2017; Dabesa and Tana, 2021). Moreover, the high root nodulation in soybean rhizosphere inoculated with PGPB comprising symbiotic *Bradyrhizobium* and non-symbiotic bacteria species is consistent with Jabborova et al. (2021) who reported higher root nodulation and nitrogen-fixing capacity following co-inoculation of *B. japonicum* and *Pseudomonas putida* compared to uninoculated plants. Additionally, Akley et al. (2022) reported a positive residual effect of *Bradyrhizobium* inoculation on soybean root nodulation after three cropping seasons. Nonetheless, root nodulation by soybean plants was not favored by inoculation of mycorrhiza, which is not consistent with other reports on the contribution of mycorrhiza to the N_2 fixing process (Daniel et al., 2020; Novais et al., 2020). The poor root nodulation in the control can be attributed to a low density of native N_2 fixing symbiotic rhizobium and low soil phosphorus content (Rosenblueth et al., 2018; Soumare et al., 2020). Although high plant available N in the soil can reduce or inhibit the symbiotic effectiveness of introduced rhizobia strains (Kasper et al., 2019), the addition of N to PGPB-inoculated soybean plants did not reduce root nodulation. However, the present results do not support our hypothesis that co-inoculating plant growth-promoting bacteria and mycorrhiza will enhance soybean root nodulation. This may be due to nutrient deficiencies especially low soil phosphorus as Püschel et al. (2017) reported that the contribution of AMF to N_2 fixation by rhizobium can be affected by environmental factors and nutrient availability including soil phosphorus.

Acid phosphatase activity

Soil phosphatase enzymes can be produced by plant roots or rhizosphere microorganisms and they play key roles in catalyzing reactions associated with organic matter decomposition and P cycling, while their quick response to changes in soil management is considered a useful biological indicator (Chodak and Nikliñska, 2012; Rejsek et al., 2012; Behera et al., 2017). The low acid phosphatase activity recorded in the rhizosphere of non-inoculated soybean plants is likely the contributions of soybean roots and the indigenous soil microbial community, while high phosphatase activity in the inoculated plants reflects the additional influence of the inoculated microbes that are involved in mineralizing organic to inorganic P (Rawat et al., 2020), or solubilizing inorganic phosphates (Tchakounté et al., 2020). The high acid phosphatase activity in sole PGPB treatment compared to the

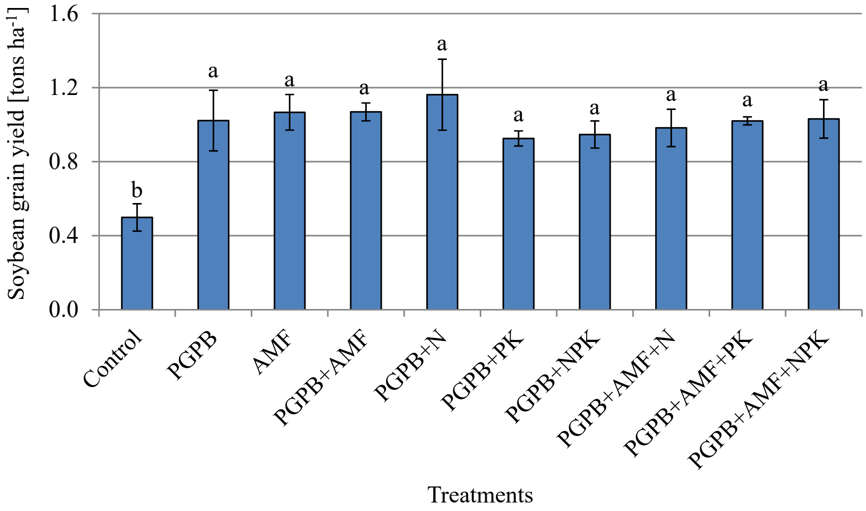

FIGURE 6
Soybean grain yield (tons ha^{1}) affected by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers. Data (Mean ± SD) with different letters are significantly different (Tukey's HSD, $p < 0.001$).

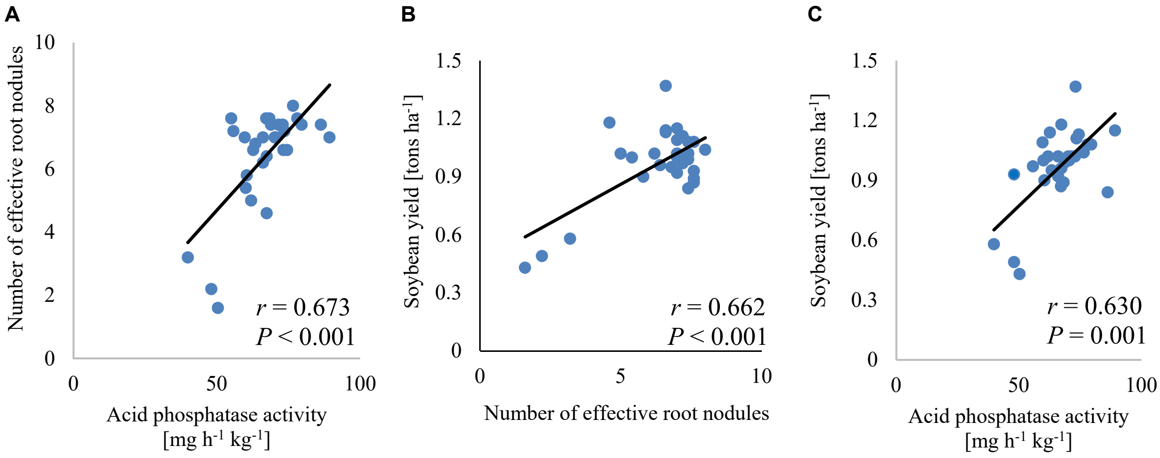

FIGURE 7
Correlation of the number of effective root nodules and acid phosphatase activity **(A)**, soybean yield and number of effective root nodules **(B)**, or acid phosphatase activity **(C)**.

control and sole AMF treatments highlights the potential of inoculated plant growth-promoting bacteria. Some microbes in the inoculant biofertilizer used in this study were isolated from the rhizosphere of maize plants in Cameroon, with demonstrated ability to solubilize rock phosphate, fix N_2, and produce siderophores (Tchakounté et al., 2018, 2020). Therefore, acid phosphatase activity in the rhizosphere can be explored as a possible mode of action of the inoculated microbes to induce nutrient dynamics that may enhance crop productivity. The superior acid phosphatase activity in the soybean rhizosphere inoculated with PGPB comprising symbiotic *Bradyrhizobium* and non-symbiotic bacteria species is in line with Jabborova et al. (2021) study which reported a higher acid phosphatase activity following co-inoculation of *B. japonicum* and *P. putida*. The increase in phosphatase activity following mycorrhiza inoculation could have been due to the production of glycoproteins (e.g., glomalin-related soil proteins) that increased microbial and enzymatic activities (Agnihotri et al., 2022). The observed decrease in acid phosphatase activity in the rhizosphere of PGPB-inoculated soybean plants and amended with NPK is in line with the demonstrated ability of NPK fertilizers to reduce soil microbial functions and acid phosphatase activity as reported by Mndzebele et al. (2020). These results are consistent with the second hypothesis that

TABLE 3 Nutrient contents of soybean grains as affected by inoculation of plant growth-promoting bacteria (PGPB) and arbuscular mycorrhiza fungi (AMF), with or without the addition of nitrogen (N), phosphorus (P), and potassium (K) fertilizers.

Treatments	**Carbohydrate**	**Protein**	**Zinc**	**Iron**
	%		**mg 100 g^{-1}**	
Control	20.14 ± 0.02f	31.02 ± 0.16h	2.74 ± 0.03h	16.75 ± 0.03f
PGPB	22.23 ± 0.02e	32.38 ± 0.06f	3.21 ± 0.01g	18.32 ± 0.03e
AMF	22.24 ± 0.02e	32.06 ± 0.06g	3.19 ± 0.02g	18.23 ± 0.02e
PGPB+AMF	22.33 ± 0.02d	32.75 ± 0.06e	3.33 ± 0.03f	18.43 ± 0.02d
PGPB+N	22.30 ± 0.02d	38.65 ± 0.19c	3.46 ± 0.07e	18.49 ± 0.03d
PGPB+PK	22.30 ± 0.02d	32.65 ± 0.10e	4.74 ± 0.02c	19.64 ± 0.08b
PGPB+NPK	22.37 ± 0.02c	38.33 ± 0.10d	4.73 ± 0.02c	19.71 ± 0.02b
PGPB+AMF+N	22.34 ± 0.02cd	39.60 ± 0.10b	3.64 ± 0.03d	18.60 ± 0.01c
PGPB+AMF+PK	22.41 ± 0.01b	38.85 ± 0.04c	4.82 ± 0.03b	19.72 ± 0.08b
PGPB+AMF+NPK	22.67 ± 0.02a	39.92 ± 0.04a	4.92 ± 0.05a	19.90 ± 0.02a

Data (Mean ± SD) within columns with different letters are significantly different (Tukey's HSD, $p < 0.001$).

TABLE 4 Correlation of nutrient value of soybean grains with the number of effective root nodules, acid phosphatase activity in the rhizosphere, and plant available phosphorus in soil.

Nutrient contents	**Number of effective nodules**	**Acid phosphatase activity**	**Plant available phosphorus in soil**
of soybean grains	*r* values	*r* values	*r* values
Carbohydrate	0.87***	0.61***	0.41*
Protein	0.46**	ns	0.46*
Zinc	0.59***	ns	ns
Iron	0.77**	ns	ns

Values are significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; ns, not significant.

inoculating plant growth-promoting bacteria and arbuscular mycorrhiza fungi will enhance acid phosphatase activity in the rhizosphere of soybean plants, although their co-inoculation did not produce greater effects.

Soybean grain yield and nutrient contents

The soybean grain yield in the control plots for this study is within the range of 448–709 kg ha^{-1} that was previously reported by Wendt and Atemkeng (2004), across the first and second planting seasons in the humid forest ecosystem of Cameroon. However, treatment applications in this study increased the soybean grain yield relative to the control and the previously reported yield by Wendt and Atemkeng (2004). Inoculating PGPB and AMF with or without NPK fertilizer significantly increased soybean yield as compared to the control, which supports the third hypothesis that PGPB and AMF will increase soybean grain yield and nutrient contents (Thioub et al., 2017). Księżak and Bojarszczuk (2022) also reported higher soybean yield following inoculation with *Bradyrhizobium* and fertilizer addition. This increased soybean yield is consistent with the role of inoculated PGPB that comprise some *Bacillus* strains in nutrient solubilization and siderophore production as reported by Tchakounté et al. (2018, 2020). This is supported by Radhakrishnan et al. (2017) who highlighted the role of *Bacillus* strains in mediating crop growth *via* secretion of metabolites, drought tolerance, and protection against pests and diseases. The inoculated microbes in this study likely facilitated the conversion of complex forms of essential nutrients (e.g., P and N) to simple available forms for uptake by plant roots leading to enhanced growth and yield (Kang et al., 2015; Kuan et al., 2016). Furthermore, the secretion of phosphatase and organic acids by *Bacillus* spp. probably facilitated the conversion of inorganic phosphate into plant available phosphate for root uptake (Kang et al., 2014, 2015). Additionally, the inoculated microbes may have released ammonia from nitrogenous organic matter in the soil or their *nifH* genes produced nitrogenase for N_2 fixation and uptake by plant roots to enhance growth and yield as reported for *Bacillus* spp. (Ding et al., 2005; Hayat et al., 2010; Kuan et al., 2016). The inoculation of mycorrhiza likely enhanced the production of glycoproteins that improved soil quality *via* increased aggregation and carbon sequestration, water-holding capacity, nutrient storage and availability, microbial and enzymatic activities, and production of extracellular polysaccharides, which increased crop performance (Agnihotri et al., 2022). The role of microbial inoculation in the present study is supported by correlations of soybean grain yield with microbial activities in the rhizosphere such as acid phosphatase and root nodulation, which is consistent with Lamptey et al. (2014). The superior performance of soybean plants that were inoculated with the consortium of PGPB comprising *Bradyrhizobium* is supported by Leggett et al. (2017), who reported improved soybean growth, nodulation and N_2 fixation, and grain yield following inoculation with *Bradyrhizobium*. Meanwhile, the lack of grain yield increase when NPK fertilizers were added to PGPB or AMF indicates that the additional NPK inputs were not enough to cause a significant difference in this study. This finding opens up avenues for further investigation on the appropriate NPK fertilizer amounts to integrate with microbial inoculants.

The higher nutrient content in soybean grains inoculated with PGPB comprising symbiotic *Bradyrhizobium* and non-symbiotic bacteria species is in line with Jabborova et al. (2021) study which reported superior plant nutrients following inoculation of *B. japonicum* and *P. putida* compared to uninoculated plants. Akley et al. (2022) also reported a positive residual effect of *Bradyrhizobium* inoculation on soybean yield after three cropping seasons. The high nutrient contents in soybean grains from plants inoculated with PGPB and AMF, with NPK fertilizer addition in this study indicates soil nutrient enhancement and their effective uptake by plants that eventually accumulated in the grains. Similar results were reported by Sogut (2016), Marro et al. (2020), and Zhang et al. (2020). Beneficial microorganisms may directly enhance nutrient uptake and accumulation or indirectly stimulate other biochemical processes that are involved in the mobilization and deposition of nutrients in the grains (Lamptey et al., 2014; Marro et al., 2020). Overall, the improved nutrient contents in soybean grains following co-inoculation of plant growth-promoting bacteria and AMF with or without NPK fertilizer addition likely results from a combination of increased biochemical processes as reported by Sheteiwy et al. (2021), and supports the concept of integrated soil fertility management (Vanlauwe et al., 2010). These results confirm the positive impact of microbial inoculation and fertilizers on grain yield and protein content of maize (Martins et al., 2017) and soybeans (Marro et al., 2020; Alemayehu and Tana, 2021; Księżak and Bojarszczuk, 2022). Yasmin et al. (2020) also reported higher nutrient contents (e.g., N, P, K, Ca, Mg) in sweet potato roots following inoculation with *Klebsiella* sp. and N fertilizer addition. Nitrogen is an important component that is responsible for several physiological and biochemical processes in plants, being a structural constituent of chlorophyll molecules, proteins, enzymes, and nucleic acids (Nunes-Nesi et al., 2010). Hence, plants with proper nutrition *via* the application of chemical and biofertilizers likely had higher chlorophyll content with increased photosynthesis and production of photoassimilates, grain filling, and chemical composition, which increased soybean yield and nutritive contents. Ramesh et al. (2014) and Khande et al. (2017) reported that *Bacillus* species substantially influenced the mobilization of zinc and its concentration in soybean, which can be utilized as bio-inoculants for bio-fertilization and bio-fortification. Iron chelation by *Bacillus* spp. *via* siderophore production facilitates iron solubilization from minerals and organic compounds in the rhizosphere by binding Fe^{3+} in complex substances and reducing them to Fe^{2+} for uptake by plants (Walker and Connolly, 2008; Nadeem et al., 2012). These reports support the potential of our locally produced inoculant biofertilizer comprising three *Bacillus* strains with the potential to produce siderophore that likely increased mobilization of zinc for uptake by soybean plants.

Conclusion

The absence of mycorrhizal colonization in non–AMF-inoculated plants compared to the inoculated ones demonstrates very low content of native AMF strains in the Yaoundé field site in Cameroon, but there was no significant difference between the AMF-inoculated treatments. The successful mycorrhizal colonization of soybean roots for plants inoculated with AMF coupled with the significantly higher soybean yield highlights the potential of AMF inoculation to boost soybean productivity as compared to the control. Sole inoculation of PGPB or AMF-enhanced root nodulation and acid phosphatase activities in the rhizosphere of soybean plants, but their co-inoculation was not significantly different from the sole inoculations. The inoculation of PGPB and AMF increased soybean grain yield and bio-fortification, while NPK fertilizer addition enhanced soybean grain bio-fortification. Overall, these results open up important pathways for further investigation on local strategies for sustainable integrated soil fertility management to boost the productivity of soybean.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

CN conceived the study, performed data analysis and literature searches, and prepared the first manuscript draft. BT participated in conceiving the study, established the field experiment, performed literature searches, and reviewed the manuscript. MO produced the inoculant formulations of plant growth-promoting bacteria, contributed in establishing the field experiment and literature searches, and reviewed the manuscript. CS participated in conceiving the study and coordinated the establishment and management of the field experiment. RN participated in the field establishment and literature searches and read the manuscript draft. MN participated in literature searches and read the manuscript draft. DA participated in data processing and analysis and literature searches and read the manuscript draft. GT isolated and plant growth-promoting bacteria and coordinated the production of microbial inoculum, and read the manuscript draft. SR coordinated the study and read the manuscript draft. All authors read the draft manuscript and approved the final manuscript.

Funding

This study was funded by the German Ministry of Education and Research (BMBF) through the MicBioTec (01DG20008) research grant.

Acknowledgments

We express gratitude to the Faculty of Agriculture and Veterinary Medicine of the University of Buea, and the Ministry of Higher Education of Cameroon for supporting the corresponding author. We also appreciate the support of Ndakwe Abigail Tifuh of the Rhizobiology Laboratory of the University of Buea for producing the PGPB consortium.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.934339/full#supplementary-material

References

Agnihotri, R., Sharmaa, M. P., Prakash, A., Ramesh, A., Bhattacharjya, S., Patra, A. K., et al. (2022). Glycoproteins of arbuscular mycorrhiza for soil carbon sequestration: review of mechanisms and controls. *Sci. Tot. Environ.* 806:150571. doi: 10.1016/j.scitotenv.2021.150571

Akley, E. K., Rice, C. W., Adotey, N., Ampim, P. A. Y., Prasad, P. V. V., Danquah, E. O., et al. (2022). Residual *Bradyrhizobium* inoculation effects on soybean performance and selected soil health parameters. *Agron. J.* 114, 1627–1641. doi: 10.1002/agj2.21037

Alam, F., Bhuiyan, M. A. H., Alam, S. S. A., Waghmode, T. R., Kim, P. J., and Lee, Y. B. (2015). Effect of *Rhizobium* sp. BARIRGm901 inoculation on nodulation, nitrogen fixation and yield of soybean *(Glycine max)* genotypes in gray terrace soil. *Biosci. Biotechnol. Biochem.* 79, 1660–1668. doi: 10.1080/09168451.2015.1044931

Alemayehu, D., and Tana, T. (2021). Response of Soybean (*Glycine max* L. (Merrill)) to *Bradyrhizobium* inoculation, lime, and phosphorus applications at Bako, Western Ethiopia. *Int. J. Agron.* 2021:6686957. doi: 10.1155/2021/6686957

Ambassa-Kiki, R., and Nill, D. (1999). Effects of different land management techniques on selected topsoil properties of a forest ferralsol. *Soil Tillage Res.* 59, 259–264. doi: 10.1016/S0167-1987(99)00067-7

AOAC (2005). *Official Methods of Analysis of the Association of Analytical Chemists*, 18th Edn. Gathersburg, MD: AOAC International.

Atieno, M., Herrmann, L., Okalebo, R., and Lesueur, D. (2012). Efficiency of different formulations of *Bradyrhizobium japonicum* and effect of co-inoculation of *Bacillus subtilis* with two different strains of *Bradyrhizobium japonicum*. *World J. Microbiol. Biotechnol.* 28, 2541–2550. doi: 10.1007/s11274-012-1062-x

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and road map to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1465–1473. doi: 10.3389/fpls.2018.01473

Bechtaoui, N., Raklami, A., Benidire, L., Tahiri, A., Göttfert, M., and Oufdou, K. (2020). Effects of PGPR co-inoculation on growth, phosphorus nutrition and phosphatase/phytase activities of faba bean under different phosphorus availability condition. *Polish J. Environ. Stud.* 29, 1557–1565. doi: 10.15244/pjoes/110345

Becker, M., Patz, S., Becker, Y., Berger, B., Drungowski, M., Bunk, B., et al. (2018). Comparative genomics reveal a flagellar system, a type VI secretion system and plant growth-promoting gene clusters unique to the endophytic bacterium *Kosakonia radicincitans*. *Front. Microbiol.* 9:1997. doi: 10.3389/fmicb.2018.01997

Begoude, D. A. B., Sarr, P. S., Mpon, T. L. Y., Owona, D. A., Kapeua, M. N., and Araki, S. (2016). Composition of *Arbuscular mycorrhizal* fungi associated with cassava (*Manihot esculenta* Crantz) cultivars as influenced by chemical fertilization and tillage in Cameroon. *J. Appl. Biosci.* 98, 9270–9283. doi: 10.4314/jab.v98i1.4

Behera, B. C., Yadav, H., Singh, S. K., Mishra, R. R., Sethi, B. K., Dutta, S. K., et al. (2017). Phosphate solubilization and acid phosphatase activity of *Serratia* sp. isolated from mangrove soil of Mahanadi River Delta, Odisha, India. *J. Genet. Eng. Biotechnol.* 15, 169–178. doi: 10.1016/j.jgeb.2017.01.003

Bello, S. K., Yusuf, A. A., and Cargele, M. (2018). Performance of cowpea as influenced by native strain of rhizobia, lime and phosphorus in Samaru, Nigeria. *Symbiosis* 75, 167–176. doi: 10.1007/s13199-017-0507-2

Bender, S. F., Wagg, C., and van der Heijden, M. G. (2016). An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol. Evol.* 31, 440–452. doi: 10.1016/j.tree.2016.02.016

Bremner, J. M., and Mulvaney, C. S. (1982). "Nitrogen-Total," in *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties*, eds A. L. Page, R. H. Miller, and D. R. Keeney (Madison, WI: American Society of Agronomy), 595–624. doi: 10.2134/agronmonogr9.2.2ed.c31

Chen, M., Arato, M., Borghi, L., Nouri, E., and Reinhardt, D. (2018). Beneficial services of arbuscular mycorrhizal fungi–from ecology to application. *Front. Plant Sci.* 9:1270. doi: 10.3389/fpls.2018.01270

Chodak, M., and Niklińska, M. (2012). Development of microbial biomass and enzyme activities in mine soils. *Polish J. Environ. Stud.* 21:569.

Dabesa, A., and Tana, T. (2021). Response of Soybean (*Glycine max* L. (Merrill)) to *Bradyrhizobium* inoculation, lime, and phosphorus applications at Bako, Western Ethiopia. *Int. J. Agron.* 2021:6686957.

Daniel, M. M., Ezekiel, M. N., Methuselah, M. N., and John, M. M. (2020). *Arbuscular mycorrhizal* fungi and *Bradyrhizobium* co-inoculation enhances nitrogen fixation and growth of green grams (*Vigna radiata* L.) under water stress. *J. Plant Nutr.* 43, 1036–1047. doi: 10.1080/01904167.2020.1711940

David, T. P. (1978). *An Introduction to Practical Biochemistry*, 2nd Edn. London: Mc Graw-Hill, 183–184.

Ding, Y., Wang, J., Liu, Y., and Chen, S. (2005). Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. *J. Appl. Microbiol.* 99, 1271–1281. doi: 10.1111/j.1365-2672.2005.02738.x

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60, 579–598. doi: 10.1007/s13213-010-0117-1

Herliana, O., Harjoso, T., Anwar, A. H. S., and Fauzi, A. (2019). The effect of *Rhizobium* and N fertilizer on growth and yield of black soybean (*Glycine max* (L) Merril). *IOP Conf. Ser. Earth Environ. Sci.* 255:2015. doi: 10.1088/1755-1315/255/1/012015

Islam, M. S., Ahmed, M., Hossain, M. S., Akter, H., and Aktar, S. (2017). Response of soybean to *Rhizobium* biofertilizer under different levels of phosphorus. *Prog. Agric.* 28, 302–315. doi: 10.3329/pa.v28i4.36370

Jabborova, D., Kannepalli, A., Davranov, K., Narimanov, A., Enakiev, Y., Syed, A., et al. (2021). Co-inoculation of rhizobacteria promotes growth, yield, and nutrient contents in soybean and improves soil enzymes and nutrients under drought conditions. *Sci. Rep.* 11:22081. doi: 10.1038/s41598-021-01337-9

Kalayu, G. (2019). Phosphate solubilizing microorganisms: promising approach as biofertilizers. *Int. J. Agron.* 2019:4917256. doi: 10.1155/2019/4917256

Kalra, Y. P., and Maynard, D. G. (1991). *Methods Manual for Forest Soil and Plant Analysis.* Information Report NOR-X-319. Edmonton, AB: Forestry Canada, 116.

Kang, S.-M., Radhakrishnan, R., Lee, K.-E., You, Y.-H., Ko, J.-H., Kim, J.-H., et al. (2015). Mechanism of plant growth promotion elicited by Bacillus sp. LKE15 in oriental melon. *Acta Agric. Scand. B Soil Plant Sci.* 65, 637–647. doi: 10.1080/09064710.2015.1040830

Kang, S.-M., Radhakrishnan, R., You, Y.-H., Joo, G.-J., Lee, I.-J., Lee, K.-E., et al. (2014). Phosphate solubilizing *Bacillus megaterium* mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote mustard plant growth. *Indian J. Microbiol.* 54, 427–433. doi: 10.1007/s12088-014-0476-6

Kanomanyanga, J., Jiao, X., Mudare, S., Mabasa, S., and Tang, L. (2021). Meta-analysis of the effects of *Rhizobia* inoculants and phosphorus fertilizer on soybean nodulation in Africa. *Afr. J. Plant Sci.* 15, 206–224. doi: 10.5897/AJPS2021.2168

Kasper, S., Christoffersen, B., Soti, P., and Racelis, A. (2019). Abiotic and biotic limitations to nodulation by leguminous cover crops in South Texas. *Agriculture* 9:209. doi: 10.3390/agriculture9100209

Khande, R. K., Sharma, S. K., Ramesh, A., and Sharma, M. P. (2017). Zinc solubilizing *Bacillus* strains that modulate growth, yield and zinc biofortification of soybean and wheat. *Rhizosphere* 4, 126–138. doi: 10.1016/j.rhisph.2017.09.002

Korir, H., Mungai, N. W., Thuita, M., Hamba, Y., and Masso, C. (2017). Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Front. Plant Sci.* 8:141. doi: 10.3389/fpls.2017.00141

Księżak, J., and Bojarszczuk, J. (2022). The effect of mineral N fertilization and *Bradyrhizobium japonicum* seed inoculation on productivity of soybean (*Glycine max* (L.) Merrill). *Agriculture* 12:110. doi: 10.3390/agriculture12010110

Kuan, K. B., Othman, R., Rahim, K. A., and Shamsuddin, Z. H. (2016). Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. *PLoS One* 11:e0152478. doi: 10.1371/journal.pone.0152478

Lamptey, S., Ahiabor, B. D. K., Yeboah, S., and Asamoah, C. (2014). Response of soybean (*Glycine max*) to Rhizobial inoculation and phosphorus application. *J. Exp. Biol. Agric. Sci.* 2, 73–77.

Leggett, M., Diaz-Zorita, M., Koivunen, M., Bowman, R., Pesek, R., Stevenson, C., et al. (2017). Soybean response to inoculation with *Bradyrhizobium japonicum* in the United States and Argentina. *Agron. J.* 109, 1031–1038. doi: 10.2134/agronj2016.04.0214

Mahmud, K., Makaju, S., Ibrahim, R., and Missaoui, A. (2020). Current progress in nitrogen fixing plants and microbiome research. *Plants* 9:97. doi: 10.3390/plants9010097

Marro, N. A., Cofréa, M. N., Grilli, G., Alvarez, C., Labuckas, D. O., Maestri, D., et al. (2020). Soybean yield, protein content and oil quality in response to interaction of *Arbuscular mycorrhizal* fungi and native microbial populations from mono and rotation-cropped soils. *Appl. Soil Ecol.* 152:8. doi: 10.1016/j.apsoil.2020.103575

Martins, M. R., Jantalia, C. P., Reis, V. M., Döwich, I., and Polidoro, L. C. (2017). Impact of plant growth-promoting bacteria on grain yield, protein content, and urea-15 N recovery by maize in a Cerrado Oxisol. *Plant Soil* 422, 239–250. doi: 10.1007/s11104-017-3193-1

Mndzebele, B., Ncube, B., Fessehazion, M., Mabhaudhi, T., Amoo, S., Plooy, C., et al. (2020). Effects of cowpea-amaranth intercropping and fertiliser application on soil phosphatase activities, available soil phosphorus, and crop growth response. *Agronomy* 10:79. doi: 10.3390/agronomy10010079

Nadeem, S. M., Shaharoona, B., Arshad, M., and Crowley, D. E. (2012). Population density and functional diversity of plant growth promoting rhizobacteria associated with avocado trees in saline soils. *Appl. Soil Ecol.* 62, 147–154. doi: 10.1016/j.apsoil.2012.08.005

Nanganoa, L. T., Ngome, F. A., Suh, C., and Basga, S. D. (2020). Assessing soil nutrients variability and adequacy for the cultivation of maize, cassava, and sorghum in selected agroecological zones of Cameroon. *Int. J. Agron.* 2020:8887318. doi: 10.1155/2020/8887318

Ngeno, J., Chemining'wa, G. N., Muthomi, J. W., and Shibairo, S. I. (2012). Effect of *Rhizobium* inoculation and nitrogen fertilizer application on growth, nodulation and yield of two garden pea genotypes. *J. Anim. Plant Sci.* 15, 2147–2156.

Ngosong, C., Bongkisheri, V., Tanyi, C. B., Nanganoa, L. T., and Tening, A. S. (2019). Optimizing nitrogen fertilization regimes for sustainable maize (*Zea mays* L.) production on the volcanic soils of Buea Cameroon. *Adv. Agric.* 2019:4681825. doi: 10.1155/2019/4681825

Novais, C. B., Sbrana, C., Jesus, C. E., Rouws, L. F. M., Giovannetti, M., Avio, L., et al. (2020). Mycorrhizal networks facilitate the colonization of legume roots by a symbiotic nitrogen-fixing bacterium. *Mycorrhiza* 30, 389–396. doi: 10.1007/s00572-020-00948-w

Ntambo, M. S., Isaac, S. C., Taruvinga, A., Hafeez, S., Anwar, T., Sharif, R., et al. (2017). The effect of *Rhizobium* inoculation with nitrogen fertilizer on growth and yield of soybeans (*Glycine max* L.). *Int. J. Biosci.* 10, 163–172. doi: 10.12692/ijb/10.3.163-172

Nunes-Nesi, A., Fernie, A. R., and Stitt, M. (2010). Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Mol. Plant* 3, 973–996. doi: 10.1093/mp/ssq049

Nyahnone, T. J. (2017). *Le Développement De La Culture Du Soja Dans Les Fronts D'extensions Cotonniers du Nord-Cameroun: Le cas du Mayo-Rey.* Mémoire De Master De Géographie, Université de Ngaoundéré, Yaounde, 135.

Nzossié, E. J. F., and Bring, C. (2020). *Soybean (Glycine max (L.) Merr.) Production in the Cameroonian Cotton Basin between the Dynamics of Structuring an Agricultural Value Chain and Sustainability Issues.* London: Intechopen, 93981.

Powell, J. R., and Rillig, M. C. (2018). Biodiversity of arbuscular mycorrhizal fungi and ecosystem function. *New Phytol.* 220, 1059–1075. doi: 10.1111/nph.15119

Püschel, D., Janoušková, M., Voøíšková, A., Gryndlerová, H., Vosátka, M., and Jansa, J. (2017). Arbuscular mycorrhiza stimulates biological nitrogen fixation in two *Medicago* spp. through improved phosphorus acquisition. *Front. Plant Sci.* 8:390. doi: 10.3389/fpls.2017.00390

Radhakrishnan, R., Hashem, A., and Abd Allah, E. F. (2017). *Bacillus*: a biological tool for crop improvement through bio-molecular changes in adverse environments. *Front. Physiol.* 8:667. doi: 10.3389/fphys.2017.00667

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014). Zinc solubilizing *Bacillus cereus* and related species modulates growth, yield and zinc biofortification of soybean and wheat seeds cultivated in central India. *Appl. Soil Ecol.* 73, 87–96. doi: 10.1016/j.apsoil.2013.08.009

Rawat, P., Das, S., Shankhdhar, D., and Shankhdhar, S. C. (2020). Phosphate-solubilizing microorganisms: mechanism and their role in phosphate solubilization and uptake. *J. Soil Sci. Plant Nutr.* 21, 49–68. doi: 10.1007/s42729-020-00342-7

Rejsek, K., Vronova, V., Pavelka, M., and Formanek, P. (2012). Acid phosphomonoesterase (E.C. 3.1.3.2.) location in soil. *J. Plant Nutr. Soil Sci.* 175:196. doi: 10.1002/jpln.201000139

Rosenblueth, M., Orrillo, O. E., López, L. A., Rogel, M. A., Hernández, R. B. J., Romero, M. J. C., et al. (2018). Nitrogen fixation in cereals. *Front. Microbiol.* 9:1794. doi: 10.3389/fmicb.2018.01794

Rowell, D. L. (1994). *Soil Science: Methods and Applications*. London: Addison Wesley Longman Singapore Publishers (Pte) Ltd.

Ruppel, S., and Merbach, W. (1995). Effects of different nitrogen sources on nitrogen fixation and bacterial growth of *Pantoea agglomerans* and *Azospirillum* sp. in bacterial pure culture: an investigation using $^{15}N_2$ incorporation and acetylene reduction measures. *Microbiol. Res.* 150, 409–418. doi: 10.1016/S0944-5013(11)80023-6

Sheteiwy, M. S., Elgawad, H. A., Xiong, Y.-C., Macovei, A., Brestic, M., Skalicky, M., et al. (2021). Inoculation with *Bacillus amyloliquefaciens* and mycorrhiza confers tolerance to drought stress and improve seed yield and quality of soybean plant. *Physiol. Plant.* 172, 2153–2169. doi: 10.1111/ppl.13454

Sogut, T. (2016). *Rhizobium* inoculation improves yield and nitrogen accumulation in soybean (*Glycine max*) cultivars better than fertilizer. *N. Z. J. Crop Hortic. Sci.* 342, 115–120. doi: 10.1080/01140671.2006.9514395

Soumare, A., Diedhiou, A. G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., et al. (2020). Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. *Plants* 9:1011. doi: 10.3390/plants9081011

Tamiru, S., Mohan, L. P., and Angaw, T. (2012). Effects of inoculation by *Bradyrhizobium japonicum* strains on nodulation, nitrogen fixation, and yield of soybean (*Glycine max* L. Merill) varieties on nitisols of Bako, Western Ethiopia. *Int. Sch. Res. Notices* 2012:261475. doi: 10.5402/2012/261475

Tchakounté, T. G.-V., Berger, B., Patz, S., Henri, F., and Ruppel, S. (2018). Community structure and plant growth-promoting potential of cultivable bacteria isolated from Cameroon soil. *Microbiol. Res.* 214, 47–59. doi: 10.1016/j.micres.2018.05.008

Tchakounté, V. T. G., Berger, G., Patz, S., Becker, M., Ková, V. T., Novák, O., et al. (2020). The response of maize to inoculation with *Arthrobacter sp.* and *Bacillus* sp. in phosphorus-deficient, salinity-affected soil. *Microorganisms* 8:1005. doi: 10.3390/microorganisms8071005

Tening, A. S., Foba-Tendo, J. N., Yakum-Ntaw, S. Y., and Tchuenteu, F. (2013). Phosphorus fixing capacity of a volcanic soil on the slope of mount Cameroon. *Agric. Biol. J. North Am.* 4, 166–174. doi: 10.5251/abjna.2013.4.3.166.174

Thioub, M., Ewusi-Mensah, N., Sarkodie-Addo, J., and Adjei-Gyapong, J. (2017). Arbuscular mycorrhizal fungi inoculation enhances phosphorus use efficiency and soybean productivity on a Haplic Acrisol. *Soil Tillage Res.* 192, 174–186. doi: 10.1016/j.still.2019.05.001

Uko, A. E., Oko, B. F. B., and Ndon, B. A. (2002). Yield response of soybeans to levels of nitrogen and potassium fertilizer in the humid tropics. *Glob. J. Agric. Sci.* 1, 101–109. doi: 10.4314/gjass.v1i2.2189

Ulzen, J., Abaidoo, R. C., Mensah, N. E., Masso, C., and AbdelGadir, A. H. (2016). *Bradyrhizobium* inoculants enhance grain yields of soybean and cowpea in northern Ghana. *Front. Plant Sci.* 7:1770. doi: 10.3389/fpls.2016.01770

Van Reeuwijk, L. P. (1992). *Procedures for Soil Analysis*, 3rd Edn. Wageningen: International Soil Reference and Information Centre (ISRIC).

Vanlauwe, B., Bationo, A., Chianu, J., Giller, K. E., Merckx, R., Mokwunye, U., et al. (2010). Integrated soil fertility management: operational definition and consequences for implementation and dissemination. *Outlook Agric.* 39, 17–24. doi: 10.5367/000000010791169998

Venneman, J., Audenaert, K., Verwaeren, J., Baert, G., Boeckx, P., Moango, A. M., et al. (2017). Congolese rhizospheric soils as a rich source of new plant growth-promoting endophytic *Piriformospora* Isolates. *Front. Microbiol.* 8:212. doi: 10.3389/fmicb.2017.00212

Wakam, L. N., Nanhou, A. P., Fokom, R., and Tchameni, S. N. (2015). Effects of nitrogen levels and arbuscular mycorrhizal fungi on biomass production, mineral nutrition, sugar and total phenolic content of two *Zea mays* Cultivars. *Am. J. Exp. Agric.* 9, 1–11. doi: 10.9734/AJEA/2015/18088

Walker, E. L., and Connolly, E. L. (2008). Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. *Curr. Opin. Plant Biol.* 11, 530–535. doi: 10.1016/j.pbi.2008.06.013

Wendt, J., and Atemkeng, M. (2004). Soybean, cowpea, groundnut, and pigeonpea response to soils, rainfall, and cropping season in the forest margins of Cameroon. *Plant Soil* 263, 121–132. doi: 10.1023/B:PLSO.0000047731.35668.e0

WWF (2014). *The Growth of Soy: Impacts and Solutions*. Gland: WWF International.

Yasmin, F., Othman, R., and Maziz, M. N. H. (2020). Yield and nutrient content of sweet potato in response of plant growth-promoting rhizobacteria (PGPR) inoculation and N fertilization. *Jordan J. Biol. Sci.* 13, 117–122.

Zhang, R., Wang, C., Teng, W., Wang, J., Lyu, X., Dong, S., et al. (2020). Accumulation and distribution of fertilizer nitrogen and nodule-fixed nitrogen in soybeans with dual root system. *Agronomy* 10:397. doi: 10.3390/agronomy10030397

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