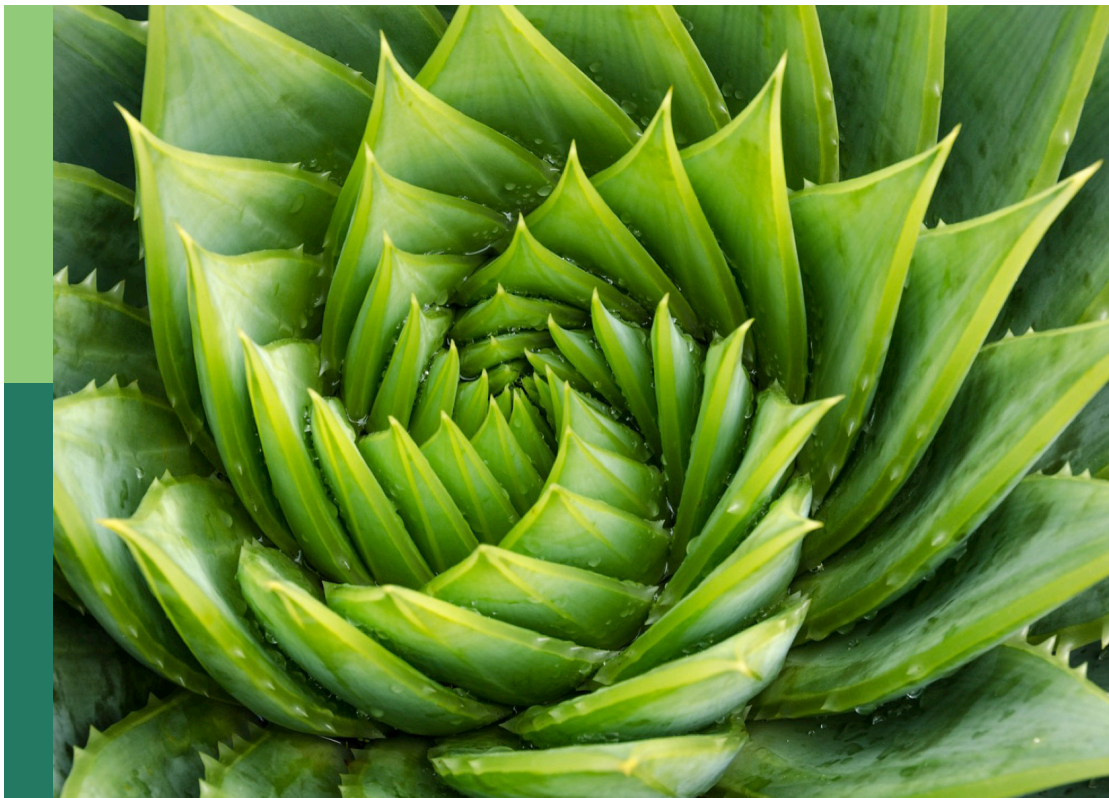
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Growth regulators and biostimulants: Upcoming opportunities

Edited by
Paromik Bhattacharyya, Karel Dolezal, Manoj Gajanan Kulkarni and Shubhpriya Gupta

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Growth regulators and biostimulants: Upcoming opportunities

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Editorial: Growth regulators and biostimulants: upcoming opportunities

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Editorial on the Research Topic
Growth regulators and biostimulants: upcoming opportunities

Plant growth regulators (PGR) and biostimulants are known to regulate plant growth and development, boost plant metabolism, and enhance nutrient uptake, resulting in healthier plants with better yields. However, their mode of action to promote plant growth is different. Plant growth regulators are defined as synthetic compounds, phytohormone derivatives such as [e.g., 2, 4-Dichlorophenoxy Acetic acid), alpha-Naphthalene Acetic Acid, 6-benzylaminopurine, thidiazuron and 6-furfurylaminopurine or kinetin] that mimic naturally occurring plant hormones [e.g., indole-3-acetic acid, *trans*-Zeatin, *cis*-zeatin, dihydrozeatin DZ, isopentenyladenine] (Gianfagna, 1995; Scacchi et al., 2009; Gupta and Chaturvedi, 2022). Growth regulators do not hold nutritive value and function by either suppressing or boosting plant growth and development by directly influencing plant hormones at low doses (Rademacher, 2015). On the other hand, biostimulants usually are complex mixtures containing organic (example, extracts of seaweed, vermicompost leachate, protein hydrolysates, humic substances, smoke-water), microbial (fungi and bacteria) and/or inorganic (Si, Se) (Brown and Saa, 2015; Colla and Rouphael, 2015; Du Jardin, 2015; Gupta et al., 2021; Shahrajabian et al., 2023) constituents. They enhance plant growth and health by stimulating natural processes at a minute quantity rather than directly controlling plant growth (Rouphael and Colla, 2018; EBIC, 2020).

Plant growth regulators are required in low quantities and have fewer impurities than conventional fertilizers. Furthermore, biostimulants are derived from natural resources (Gupta and Van Staden, 2021). Therefore, there is a minimal risk regarding toxicity and safety to humans and the environment (Kisvarga et al., 2022). Growth regulators and biostimulants are becoming increasingly popular among farmers and consumers worldwide because they can help increase yields and improve soil health with low dependence on synthetic fertilizers and

pesticides. There is a paradigm shift around the world post COVID-19 and an increase in demand for organic food among consumers as a preventive health measure both from developed and developing countries (Ćirić et al., 2020; Śmiglak-Krajewska and Wojciechowska-Solis, 2021; Brata et al., 2022; Wang et al., 2022). With a growing global population and increasing concerns about environmental degradation there is a growing demand for sustainable agriculture practices and the adoption of GLOBALGAP (GLOBAL Good Agricultural Practices) policies (Kleemann et al., 2014; Mook and Overdevest, 2021). Furthermore, the market for growth regulators and biostimulants is expected to grow and expand into new areas, including Asia and Africa (Markets and Markets, 2022; Markets and Markets, 2023). Growth regulators and biostimulants are likely to become more significant in agriculture over the next few years due to technological advancements and growing demand for sustainable agriculture.

In 2022, PGR and biostimulants market was valued at circa (i.e., approx., ca.) USD 2.9 billion and ca. USD 3.5 billion respectively (Markets and Markets, 2022; Markets and Markets, 2023). For PGR, it is projected to reach ca. USD 4.5 billion with a CAGR (Compound Annual Growth Rate) of 7.4% by 2028 (Markets and Markets, 2023) and ca. USD 6.2 billion and 11.8% for biostimulants by 2027 (Markets and Markets, 2022). Therefore, both small and big companies like, Isagro (Italy), Arysta (Japan), BASF (Germany), Syngenta (Switzerland), Bio AG Alliance (US), FMC Corporation (US), Valagro (Italy), Kelpak (South Africa), Biolchim (Italy), Acadian (Canada), Koppert (Netherlands), Biostadt (India), Italpollina (Italy) and many more are pushing into growth regulators and biostimulants and are significantly investing in research (Corsi et al., 2022, Markets and Markets, 2022; Critchley et al., 2021; Moyo et al., 2021). With the growing popularity of growth regulators and biostimulants, many countries are developing regulations to ensure the safety and effectiveness of these products (Du Jardin, 2015; García-Sánchez et al., 2022). Furthermore, as regulatory frameworks advance, there will be opportunities for PGR and biostimulants companies to develop innovative products that satisfy regulatory requirements and fulfill the needs of farmers (Caradonia et al., 2019; Norrie et al., 2021).

Recent technological developments have enabled more efficient growth regulators and biostimulants to be developed. For example, the application of nanotechnology can enhance the efficacy and delivery of these products suggesting that growth regulators and biostimulants will become more significant in agribusiness in the coming years. The research papers in the focus Research Topic on "*Growth Regulators and Biostimulants: Upcoming Opportunities*" highlight the importance of growth regulators and biostimulants in agriculture and their role in improving crop yield in sustainably under biotic and abiotic stress. In this Research Topic, Rathore and Kumar studied the dynamics of phosphorus and biostimulants [Amino Booster G (the amino acid solution) and V-Hume (the humic acid solution)] on the agro-morphological traits, essential oil yield, and chemical constituents of German chamomile (*Matricaria chamomilla*). They found that the amino acid and humic acid solution positively affects plant growth, flower yield, and essential oil composition. Li et al. discovered that exogenous application of trehalose to maize (*Zea mays*) generated more significant carbon and nitrogen metabolic activity, increased chlorophyll content, and enhanced dry matter accumulation in roots and shoots as compared to the effect of chitosan, humic acid and gamma-aminobutyric acid. Parmar et al. reviewed the metabolites produced by microalgae biostimulants and their effects on plant growth, productivity, and tolerance against stressors, as well as different modes of application of microalgae metabolites. Furthermore, the authors emphasized the circular economy model of microalgae-mediated bioremediation coupled with biorefinery approaches for generating high-value metabolites and enhancing the sustainability of microalgae biomass production. Mohammed et al. demonstrated that the seed presoaking and irrigation of broad bean (*Vicia faba*) and sunflower (*Helianthus annuus*) with *Sargassum polycystum* aqueous extract improved the germination, growth and antioxidant activity of plants. Raj et al. discovered that biostimulants like vermicompost, biofertilizer, and liquid seaweed extract enhanced basil (*Ocimum basilicum*) yield and quality without using harmful agrochemicals.

Wang et al. explained the molecular mechanisms of melatonin and its function in promoting adventitious root formation in cucumber (*Cucumis sativus*) seedlings grown under shade. The authors found that melatonin significantly increased adventitious root formation in the cucumber hypocotyl by controlling the expression of genes involved in hormone synthesis, signaling, and cell wall biogenesis, as well as by raising levels of auxin, cytokinin, jasmonic acid, salicylic acid, and abscisic acid.

Plant growth regulators can be used to alleviate the detrimental effects of biotic and abiotic stress and improve crop yield and quality. Singh et al. showed that various hormones (such as ABA, cytokinin, GA, and IAA) differently control flowering in saffron by regulating floral integrator [*FT* (*FLOWERING LOCUS T*) and *LFY* (LEAFY)], repressor [*SVP* (*SHORT VEGETATIVE PHASE*) and *TFL1-2* (*TERMINAL FLOWER1*)] genes, and homeotic [*PISTILLATA*, *SEPETALLA*, and *DL* (*DROPPING LEAF*)] gene expression. Otari et al. found that MS medium fortified with 2.0 mg/l BAP + KIN and 0.5 mg/l IAA + IBA + NAA produced the best shoot and root development results, respectively, in Indian Pennywort (*Bacopa floribunda*). The role of cytokinin in defense or stress priming and the maintenance of photosynthesis was meticulously reviewed by Hudeček et al.. Debnath and Ghosh reviewed in-depth study on the phenotypic variation in micropropagated berry plants and the role of DNA methylation in these variations. Pandey et al. showed that foliar spray or seed soaking treatment of a novel natural plant growth enhancer, "calliterpenone," (a phyllocladane diterpenoid) isolated from *Callicarpa macrophylla* enhanced crop productivity of wheat (*Triticum aestivum*), rice (*Oryza sativa*) potato (*Solanum tuberosum*), chickpea (*Cicer arietinum*), tomato (*Solanum lycopersicum*), and onion (*Allium cepa*).

In this Research Topic, the study by Zhang et al. found that Ca^{2+}-treated pears can suppress the production of stone cells which affects pear quality, and provided insight into its molecular mechanism. Liu et al. discovered that among carbon dioxide, ethylene, nitrogen, and wounding, carbon dioxide treatment was

the best to induce the formation of heartwood in 6-year-old Indian sandalwood (*Santalum album*) trees.

Kaushal et al. reviewed the molecular pathways activated by microbial biostimulants (plant growth-promoting rhizobacteria, PGPR) in plants facing abiotic and biotic stress. Morcillo et al. reviewed the effect of cell-free microbial culture filtrates on beneficial soil microbes, plant growth promotion and in combating stress tolerance. Ali et al. reported that the application of stress-tolerant *Bacillus* sp. improved the length of shoots, roots, and number of roots in saffron (*Crocus sativus*). del-Canto et al. showed that the inoculation of common bean (*Phaseolus vulgaris*) with drought and salinity-tolerant indigenous rhizobium strains improved drought tolerance and yield compared to nitrogen fertilization.

Plant growth regulators and biostimulants have great potential to improve agricultural yield, making them a great option for farmers and growers looking to increase productivity and profitability in a sustainable manner. The use of plant growth regulators and biostimulants can help reduce the environmental impact of chemical fertilizers by increasing nutrient uptake, improving soil health and fertilizer management practices. With advancements in technology and growing demand for sustainable agriculture, there are numerous opportunities for PGR and biostimulants. For example, precision agriculture techniques can be used to improve the efficiency and effectiveness of PGR and biostimulants. These techniques can aid in boosting crop quality and yield. In addition, the use of biostimulants and PGR is spreading beyond conventional crops like grains and vegetables to include ornamental, medicinal, and fruit-producing plants. Plant growth regulators and biostimulants will likely play a crucial role in improving the productivity of crops and minimizing environmental impact by prioritizing sustainable agricultural practices.

Author contributions

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

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Trehalose Outperforms Chitosan, Humic Acid and Gamma-Aminobutyric Acid in Promoting the Growth of Field Maize

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Despite the fact that there are many distinct types of plant growth regulators (PGRs), the diverse ways in which they regulate plant development are rarely compared. In this study, four PGRs (trehalose, chitosan, humic acid and gamma-aminobutyric acid) were selected and sprayed folially, and plant samples were collected while maize was at vegetative leaf stages 6, 10, and 14 (V6, V10, and V14, respectively) to reveal the effects of different PGRs on photosynthesis, dry matter accumulation, oxidative stress, carbon and nitrogen metabolism, hormone levels, and gene expression of maize. Results showed that 100 mg/L PGRs did not induce oxidative damage or repair activities in maize. Trehalose significantly increased chlorophyll content at V6 and promoted dry matter (roots and shoots) accumulation at V6 and V10. The activities of carbon and nitrogen metabolizing enzymes were significantly enhanced by trehalose treatment, which promoted the accumulation of sucrose and soluble sugar, but did not affect the biosynthesis of auxin and gibberellin at V6. Changes in carbon and nitrogen metabolism enzymes are regulated by transcription of related synthetic genes. Lower starch content and higher sucrose content in trehalose-treated maize leaves are important biological characteristics. Further analysis revealed that the effect of trehalose on the metabolic activity of maize was a short-term promoting effect (0–12 days), while the effect on maize growth was a long-term cumulative effect (13–28 days). Overall, exogenous trehalose induced stronger carbon and nitrogen metabolic activity, higher photosynthetic capacity and more dry matter accumulation than chitosan, humic acid and gamma-aminobutyric acid.

Keywords: plant growth regulator, endogenous hormones, carbon assimilation, nitrogen metabolism, maize

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INTRODUCTION

Global climate change is gradually transforming agricultural production and development patterns. Maize is an important source of food, feed and biofuel and the most widely grown cereal crop in global agricultural production (Zhang et al., 2017). The Food and Agriculture Organization (FAO) has projected that the global population will grow from 7.6 billion at present to 9 billion by 2050 (Bahar et al., 2020). Rapid population growth has led to increased demand for food,

and the imbalance between supply and demand has gradually become an acute problem. How to improve plant growth, promote crop yield and reduce pollution has become an important research focus of scientists. Sustainable field management practices, targeted gene editing techniques and application of nitrogen-fixing microorganisms and plant growth regulators (PGRs) are particularly effective strategies (Siddiqui et al., 2022). PGRs are natural products of small molecules that improve plant growth at very low concentrations, including trehalose, chitosan, humic acid, and gamma-aminobutyric acid, among others (Zhang et al., 2022).

Trehalose is a non-reducing disaccharide that can be synthesized by all organisms except vertebrates (Paul, 2007). It acts as a molecular stabilizer in higher plants and as a carbon source in lower organisms (Elbein et al., 2003). Application of exogenous trehalose and endogenous overexpression of trehalose can enhance the resistance of plants to drought, salt damage, low temperature and other stresses by promoting carbohydrate synthesis, increasing the activity of antioxidant enzymes, regulating changes in hormone levels and reducing ROS levels (Ali and Ashraf, 2011; Abdallah et al., 2016; Zulfiqar and Ashraf, 2021). Accordingly, the combined application of trehalose and salicylic acid to sweet basil (*Ocimum basilicum* L.) was more effect than application of trehalose alone in improving drought resistance, promoting plant growth and increasing dry matter accumulation (Zulfiqar et al., 2021). Chitosan is an abundant natural biopolymer and a product of chitin deacetylation, and the complex diversity of structure and function of chitosan expands its application in agriculture. Chitosan nanomaterials can inhibit the incidence of *Fusarium oxysporum* (Elsherbiny et al., 2022) or increase maize yield by increasing chlorophyll content and leaf area index (Kumaraswamy et al., 2021). Chitosan oligomers have also been shown to improve the resistance of maize to cadmium stress (Qu et al., 2019). Chitosan and calcium carbonate can mitigate the negative effects of salt stress on wheat (Sadak and Talaat, 2021). Silicon and chitosan have been shown to improve maize resistance to salt stress (Younas et al., 2021). Humic acid is the main extractable part of humic compounds with hormone-like activity [e.g., auxin (IAA) and gibberellin (GA)] and is a natural chelating agent (Nikbakht et al., 2008). Foliar spraying of humic acid improved rapeseed oil yield and quality by promoting nutrient uptake and transport (Nasiri et al., 2021). Humic acid application improved soybean seedling tolerance (Matuszak-Slamani et al., 2017). The four-carbon non-protein amino acid gamma-aminobutyric acid is distributed among animals, plants and microorganisms (Kinnersley and Lin, 2000). Because gamma-aminobutyric acid production can be rapidly induced under biotic and abiotic stresses in response to various environmental signals. Recent studies have shown that gamma-aminobutyric acid is involved in signal transduction and regulation of carbon and nitrogen metabolism balance in higher plants, reduces oxidative damage in plants, improves plant stress resistance and mediates the interaction between plants and microorganisms (Ramesh et al., 2017).

However, the majority of their conclusions are based on laboratory experiments, with only a small amount of research on the effects of field circumstances on maize growth. The vegetative growth period is a time during which plants are particularly sensitive to environmental changes. Accordingly, we investigated photosynthesis, carbon and nitrogen metabolism, endogenous hormones, oxidative stress and key metabolic genes of maize at three growth stages [vegetative leaf stages 6, 10, and 14 (V6, V10, and V14, respectively)] under foliar-spraying of PGRs. Pearson correlation analysis is effective in revealing complex metabolic regulatory activities. In addition, the application of principal component analysis and partial least squares discriminant analysis (PCA and PLS-DA) were utilized to better distinguish the significant differences between different treatments. Thus, this study was undertaken to evaluate the effects of different PGRs on maize growth and reveal their differential regulatory mechanisms, and the findings have significant implications for the use of PGRs in agricultural production.

MATERIALS AND METHODS

Experimental Design

The experiment site was located in Ping yuan New District, Xinxiang City, Henan Province, China (113°40′42″E, 34°47′55″N). The soil type in this area is fluvo-aquic soil, and the average annual rainfall is 645 mm, mainly concentrated in the 3 months of July, August and September. The annual evaporation is 1,450 mm, and the annual average temperature is 14.4°C. The maize variety selected for the present study was "Jun dan 20" (cultivated by the Institute of Agricultural Sciences of Jun xian County, Henan Province, China). The amount of fertilizer applied during the season was based on the recommended amount of fertilizer for summer maize, consisting of N (210 kg/hm^2), P_2O_5 (75 kg/hm^2) and K_2O (90 kg/hm^2). The experimental plot was divided into subplots, each 6 m × 8 m, with a total of 15 subplots. The experimental treatment was based on a random block design.

Untreated water was the control (because water was not expected to negatively affect plants) treatment. The other treatments included addition of the PGRs trehalose (Sigma Aldrich Shanghai Trading Co., Ltd., Shanghai, China), chitosan (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China), humic acid (Sigma Aldrich Shanghai Trading Co., Ltd.) and gamma-aminobutyric acid (Beijing Solai Bao Technology Co., Ltd., Beijing, China), respectively. Each treatment included three replicates. The maize sowing date was June 8, 2021, the sowing depth was 7 cm, and each plot was planted in 10 rows. The field management measures after sowing were conducted in accordance with the local farmland production practices. The 100 mg/L PGR treatments were applied by foliar spraying. The spraying was conducted before sunset in the evening, and 0.1% Tween 20 was added to the solvent. Plots were sprayed on June 18, June 23 and June 28, three times in total. Each plant was sprayed with 10 mL (in order to ensure the consistency and effectiveness of the treatment).

The sampling points of plant samples were June 30 (V6), July 16 (V10) and August 3 (V14), and these three growth points were selected because they are the key nodes in the vegetative growth of maize (Bender et al., 2013). A portion of

the collected maize leaves (the middle of the leaf, with the main veins removed) were stored at –20°C for the subsequent detection of physiological and biochemical indexes; the other portion was stored at –80°C for subsequent assays of the composition of samples. Additionally, five whole plants were collected for the calculation of the accumulation of maize biomass. Roots were collected from a soil volume of 30 × 30 × 35 cm (length × width × height).

Detection Methods

Photosynthetic Index

Complete functional leaves of similar shape and size were selected, and a SPAD 502 DL Plus instrument (Konica Minolta, Tokyo, Japan) was used to measure the leaves at the center of maize plants under different PGR treatments at V6, V10 and V14.

Oxidative Stress Indicators

For all assays of oxidative stress indicators, 0.1-g samples of maize leaves were added to 1 mL of PBS buffer (Phosphate buffered saline, 0.01 M, PH 7.2–7.4), homogenized with a pre-cooled mortar on ice and centrifuged at 10,000 × *g* for 10 min at 4°C, and the resulting supernatant was collected for later use.

Determination of Malondialdehyde Content

First, 1 mL of 0.5% thiobarbituric acid was added to the supernatant, which was mixed well and placed in boiling water for 10 min. The content of lipid peroxide was estimated by measuring the absorbance at 532 nm and the absorbance value at 600 nm; the difference between the two values was used for the calculation of malondialdehyde (MDA) content.

Determination of the Total Antioxidant Capacity

First, 0.18 mL of FRAP working solution (0.3 M acetate buffer:10 mmol/L TPTZ:20 mmol/L $FeCl_3$, 10:1:1, *V*:*V*:*V*) was added to the supernatant, which was then incubated in a water bath at 37° for 10 min. Then, the absorbance at 593 nm was measured, and the depth of the color reflected the total antioxidant capacity (T-AOC) of the plant. Distilled water was used to determine the zero value, and the result was recorded as units per gram (U/g).

Carbon and Nitrogen Metabolites

The soluble sugar, sucrose, NO_3^- and starch contents were measured using the methods of Hu et al. (2021). Free amino acid (FAA) content was determined by utilizing the principle that amino acids and ninhydrin can be quantified when they are co-heated. In short, fresh plant leaves were washed, cut into pieces and mixed evenly. Then, 0.1-g samples were added to 10 mL of distilled water and placed in a boiling water bath for 20 min. Supernatants were transferred to 20-mL scale test tubes, and a small amount of distilled water was continually added after repeated extraction of a constant volume. Then, 0.5 mL of NaCN buffer and ninhydrin hydrate was successively added, and after 12 min in a boiling water bath, 5 mL of 95% ethanol was added. The absorbance value was measured at a 570 nm wavelength for the calculation of the amino acid concentration.

Carbon and Nitrogen Metabolism Enzymes

The enzymes involved in carbon metabolism, including sucrose synthase (SS), ADPG pyrophosphorylases (AGPases), sucrose phosphate synthase (SPS), isocitrate dehydrogenase (IDH), phosphoenolpyruvate carboxylase (PEPC) and 1,5-ribulose diphosphate carboxylase (Rubisco) were measured using the corresponding assay kits following the manufacturer's instructions (Beijing Chejeter Technology Co., Ltd., Beijing, China). The activities of enzymes related to nitrogen assimilation, including glutamate dehydrogenase (GDH), nitrate reductase (NR), glutamate synthase (GS), and glutamine synthase (GOGAT), were determined according to the assay kit instructions (Qi Yi Biological Technology Co., Ltd., Shanghai, China).

Endogenous Plant Hormones

The contents of IAA and GA were determined using an ELISA kit (Qi Yi Biological Technology Co., Ltd.). First, 0.1-g samples of maize leaves were added to 1 mL of PBS buffer to prepare the homogenate. Samples were centrifuged at 8,000 × *g* for 10 min, and 10 μL of supernatant was collected and added to 40 μL of sample buffer. Then, 100 μL of horseradish peroxidase (HPR)-labeled detection antibody was added, and samples were incubated in a water bath at 37° for 60 min. Substrate for the chromogenic reaction assay was added after the samples were washed, and after incubation for 15 min away from light, stop solution was added. The hormone content was calculated by measuring the absorbance at 450 nm.

Quantitative Reverse Transcription PCR

The extraction of RNA was accomplished with a plant total RNA extraction kit. RNA extraction quality and concentration were assessed by spectrophotometry and agarose gel electrophoresis. The reverse transcription program was as follows: 42°C for 15 min and 95°C for 3 min. RNA was reverse transcribed, and gene expression was detected using the SYBR Green kit (Tian gen Biotech Co., Ltd., Beijing, China). Quantitative reverse transcription PCR (RT-qPCR) assays were performed on Applied Biosystems ABI ViiATM7 platform (Applied Biosystems, Waltham, MA, United States). The upstream and downstream primers were designed using Primer Premier 5.0 software (Premier Biosoft Int., Palo Alto, CA, United States), and the sequence information of the target gene was obtained from NCBI[1] (**Supplementary Table 1**). The following PCR amplification program was used: Pre-denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 20 s and extension at 72°C for 32 s. β-*actin* was used as an internal reference gene, and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Data Analysis

The data presented in all graphs are mean ± standard error (SE) values, and statistical analysis was conducted with SPSS Statistics 23 (IBM Corp., Armonk, NY, United States). One-way ANOVA

[1]https://www.ncbi.nlm.nih.gov

was used to determine significant differences between treatments, and the significance level applied was $P < 0.05$.

RESULTS

Oxidative Stress State

First, to assess whether foliar spraying of PGRs can alter the growth status of planted maize, levels of oxidative damage and T-AOC were analyzed.

Trehalose, chitosan, humic acid, and gamma-aminobutyric acid did not significantly change the content of MDA, and T-AOC of maize compared with the control conditions at V6, V10 and V14 (**Table 1**).

Photosynthetic Capacity and Plant Traits

We detected changes induced by PGRs in the chlorophyll content of maize leaves at three growth stages (V6, V10, and V14). However, we found that compared with the control conditions at V6, only the chlorophyll content after trehalose treatment was significantly increased by 6.85%, while chitosan, humic acid, and gamma-aminobutyric acid treatments induced no significant differences. There was no significant difference between the groups treated with PGRs at V10 and V14 (**Supplementary Figure 1**).

In contrast with the chlorophyl content results, growth of the maize plants was significantly promoted by PGR treatments. The trehalose and gamma-aminobutyric acid treatments were most significant at V6 and V10. Compared with the control conditions at V6, the dry weight of root tissue (**Figure 1A**), shoot tissue (**Figure 1B**) and the whole plant (**Figure 1D**), were significantly increased under trehalose treatment by 1.52, 1.04, and 1.12 times, respectively, and the dry weight of root tissue, shoot tissue and the whole plant was significantly increased under the gamma-aminobutyric acid treatment by 0.78, 0.71, and 0.72 times, respectively. All PGR treatments improved the growth of maize compared to the control conditions at V10. In addition, we analyzed the change in the maize root-to-shoot ratio (**Figure 1C**) under various PGR treatments. In short, our results showed that trehalose is the PGR that leads to the most significant accumulation of maize dry matter.

Carbon Metabolism Enzymes and Carbon Metabolites

Carbon metabolic activity is related to changes in plant photosynthetic capabilities. Compared with control conditions at V6, all PGRs promoted varying degrees of carbon metabolite accumulation. Among metabolite response variables, the sucrose content (**Figure 2A**), soluble sugar content (**Figure 2C**) and sucrose-to-starch ratios (**Figure 2D**) of trehalose treatment were significantly increased by 2.4, 0.89, and 1.63 times, respectively, but the change in starch content (**Figure 2B**) was not significant. Sucrose, starch and soluble sugar contents and the sucrose-to-starch ratios under chitosan treatment were significantly improved by 2.18, 0.78, 0.79, and 0.8 times, respectively. The sucrose, starch and soluble sugar contents under treatment with humic acid were significantly improved by 1.15, 0.93, and 0.63 times, respectively. Changes under gamma-aminobutyric acid treatment were basically consistent with those under humic acid treatment.

The synthesis of carbon metabolites is rigorously regulated by enzyme activity or content (**Supplementary Figure 2**). Rubisco, SPS, SS and PEPC activity levels were significantly improved by trehalose, chitosan and gamma-aminobutyric acid treatments. Humic acid treatment significantly improved AGPase and PEPC activity. The activity of IDH was improved in all PGRs, but only trehalose treatment induced significant differences at V6, V10 and V14.

Nitrogen Metabolism Enzymes and Nitrogen Metabolites

The effects of PGR treatments on the accumulation of nitrogen metabolites were weak overall, but differed individually. Compared with the control conditions at V6, the NO_3^- content

TABLE 1 | Effects of PGR treatments on oxidative stress in maize.

Indexes	Treatment	Growth period		
		V6	V10	V14
MDA (nmol/g FW)	Control	28.64 ± 2.73	23.52 ± 1.54	25.89 ± 2.10
	Trehalose	30.37 ± 1.65	24.79 ± 3.48	29.41 ± 0.50
	Chitosan	26.64 ± 0.66	22.88 ± 3.27	27.98 ± 3.30
	Humic acid	26.24 ± 2.55	27.61 ± 2.19	30.07 ± 4.79
	Gamma-aminobutyric acid	25.42 ± 1.60	23.77 ± 2.94	23.21 ± 4.75
T-AOC (U/g FW)	Control	16.57 ± 1.37	11.50 ± 1.00	11.13 ± 0.04
	Trehalose	14.73 ± 2.00	11.18 ± 1.43	11.47 ± 2.35
	Chitosan	16.50 ± 1.72	10.74 ± 0.24	12.38 ± 1.42
	Humic acid	15.88 ± 2.27	9.83 ± 0.61	10.36 ± 0.68
	Gamma-aminobutyric acid	17.03 ± 2.14	11.74 ± 1.35	9.03 ± 0.58

Statistical analysis was used to compare differences in means between PGR treatments shown. Within each stage, treatments labeled with different lowercase letters were significantly different ($P < 0.05$; $n = 3$), and the lack of such letters indicates the lack of a significant difference. V6, V10, and V14 represent the 6-, 10-, and 14-leaf stages of vegetative growth, respectively. MDA, malondialdehyde; T-AOC, total antioxidant capacity; FW, fresh weight.

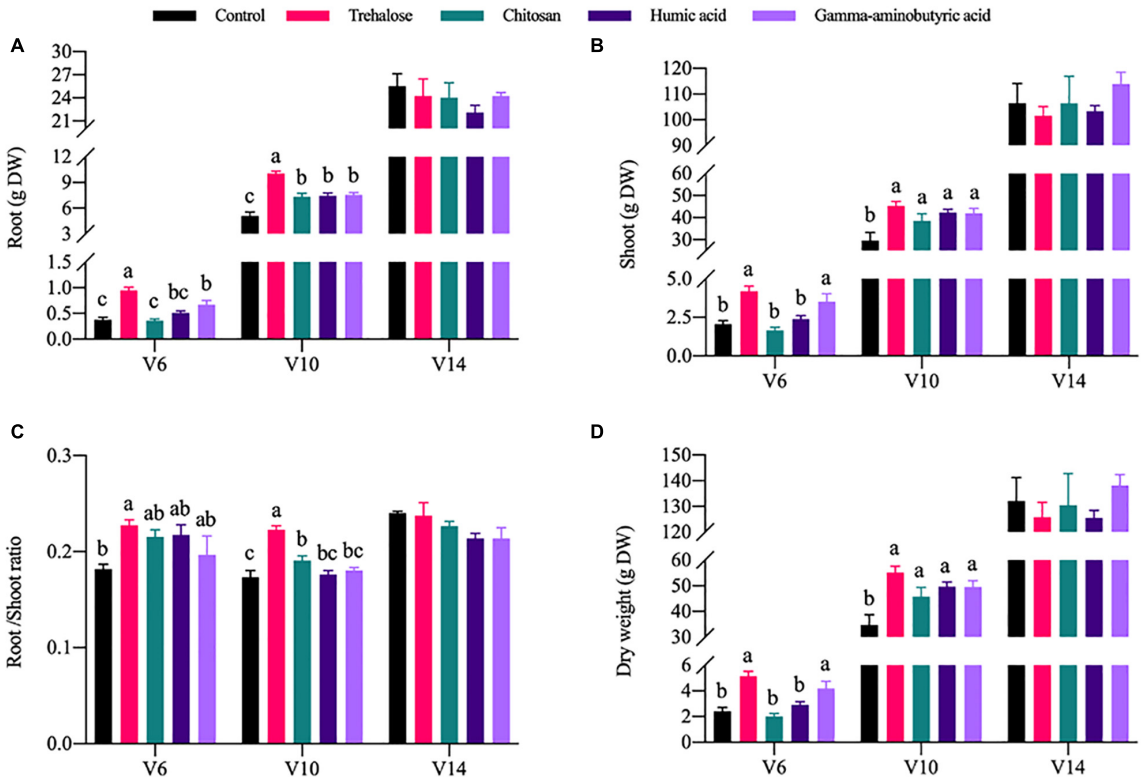


FIGURE 1 | Effects of PGRs on maize growth at different growth stages. Comparisons were made between PGRs treatments with different colors in the same growth period; significant differences are indicated by different lowercase letters in the same figure panel, and no letter indicates no significant difference ($P < 0.05$, $n = 5$). V6, V10, and V14 represent the 6-, 10-, and 14-leaf stages of vegetative growth, respectively. DW, dry weight of the plant. **(A)** Root. **(B)** Shoot. **(C)** Root/shoot ratio. **(D)** Dry weight.

under humic acid treatment was significantly increased by 0.78 times. Compared with the control conditions at V10, NO_3^- content under trehalose treatment was significantly increased by 0.52 times. Other PGRs induced no significant difference under V6 and V10 (**Figure 3A**). Compared with the control conditions at V6, the content of FAA under chitosan treatment was significantly decreased by 0.18 times. There were no significant differences at V6 and V10 in the other PGR treatments (**Figure 3B**).

In contrast with the accumulation of nitrogen metabolites, the activity of nitrogen metabolic enzymes was actively regulated by PGRs. Compared with the control conditions at V6, the activity levels of NR, GOGAT and GS under trehalose treatment were significantly increased by 0.54, 0.57, and 0.28 times, respectively. The NR, GOGAT and GDH activity levels under chitosan treatment were increased by 0.44, 0.68, and 0.17 times, respectively. Other PGR treatments induced no significant difference compared with the control conditions (**Supplementary Figure 3**).

Plant Hormone

Plant hormones are another important regulator of plant growth. GA and IAA differed in their response to treatments. The changes in GA and IAA induced by the application of PGRs were concentrated earlier (at V6 and V10) and later (at V10 and V14), respectively (**Supplementary Figure 4**).

Compared with the control conditions at V6, the content of GA was significantly increased by 0.1 times under treatment with chitosan, and there was no significant difference for other PGR treatments. In addition, all PGR treatments did not alter the level of IAA at V6. Compared with the control conditions at V10, humic acid resulted in significant increases of 0.16 and 0.1 times in GA and IAA, respectively, but gamma-aminobutyric acid only significantly increased GA content. Compared with the control conditions at V14, the effects of PGRs on the synthesis of GA and IAA were not significantly different, but the accumulation of IAA differed between treatments with different PGRs.

Pearson Correlation Analysis

We performed a Pearson correlation analysis (**Figure 4**) of the indicators at V6 to clarify the interaction between different indicators and elucidate changes in maize growth in response to PGRs.

There was a strong positive correlation between carbon and nitrogen metabolism activities (including carbon metabolism enzymes PEPC, Rubisco and SPS and nitrogen metabolism enzymes NR, GOGAT, and GS) in response to the application of PGRs. This interaction occurs not only between carbon metabolites and sucrose (sucrose-to-starch ratio and sucrose,

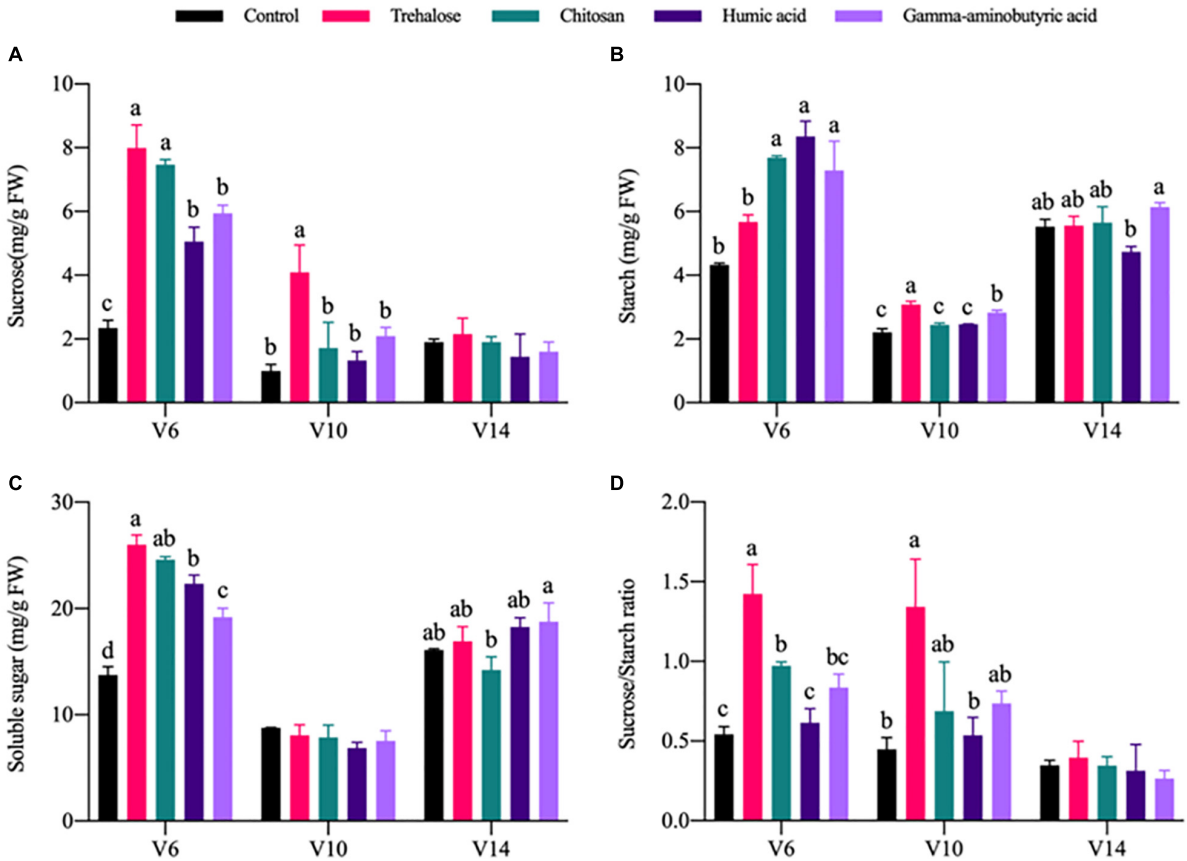


FIGURE 2 | Effects of PGRs on carbon metabolite accumulation in maize at different growth stages. Statistical analysis was used to compare differences in the mean among the PGR treatments shown in different colors. Treatments labeled with different lowercase letters were significantly different ($P < 0.05$; $n = 3$), and the lack of such letters indicate no significant difference among treatments at each stage. V6, V10, and V14 represent the 6-, 10-, and 14-leaf stages of vegetative growth, respectively. FW, fresh weight. **(A)** Sucrose. **(B)** Starch. **(C)** Soluble sugar. **(D)** Sucrose/starch ratio.

soluble sugars, and sucrose contents), but also between carbon metabolites and carbon and nitrogen metabolizing enzymes (sucrose and PEPC, Rubisco, SPS, SS, IDH, NR, GOGAT, and GS). In addition, we also observed a negative correlation between MDA and T-AOC. There were weak correlations among the contents of FAA and GDH, GA and IAA, but a significant positive correlation was observed between IAA and FAA contents.

Principal Component Analysis and Partial Least Squares Discriminant Analysis

To more comprehensively reveal the biological variation and metabolic activity characteristics of maize leaves treated with different PGRs at V6, but not at V10 and V14, we conducted principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) with MetaboAnalyst 5.0[2] (**Supplementary Figure 5**).

Before performing multivariate data analysis, the following operations were performed: sample normalization (normalization based on summation), data transformation ($\log_{10}$ transformation) and data scaling (Pareto scaling) (**Supplementary Figure 5A**). In the PCA model, PC1 explained 50.2% of the variance in the data, and PC2 explained another 15.8%. The score scatter plot clearly showed an obvious separation between the control group and the PGR treatment groups (**Supplementary Figure 5B**), and the loading of the scatter plot indicated that most of the indicators (except FAA, IAA, and GDH) in carbon and nitrogen metabolic enzyme activities were responsible for the differentiation of the treatment groups based on their individual characteristics (**Supplementary Figure 5C**). However, the PCA model is based on the grouping with the highest variation in the sample, which ignores the distinct effects of the various PGRs. Therefore, we chose to use PLS-DA, a supervised multivariate analysis, to test separation between treatments. Data preprocessing was consistent with PCA. The reliability of the model was verified by 100 permutation tests and cross-validation, which was crucial for subsequent analysis (**Supplementary Figures 5D,E**). In PLS-DA, PC1 and PC2 explained 27.6 and 35% of the variance in the data, respectively (**Supplementary Figure 5F**). The

[2]https://www.metaboanalyst.ca

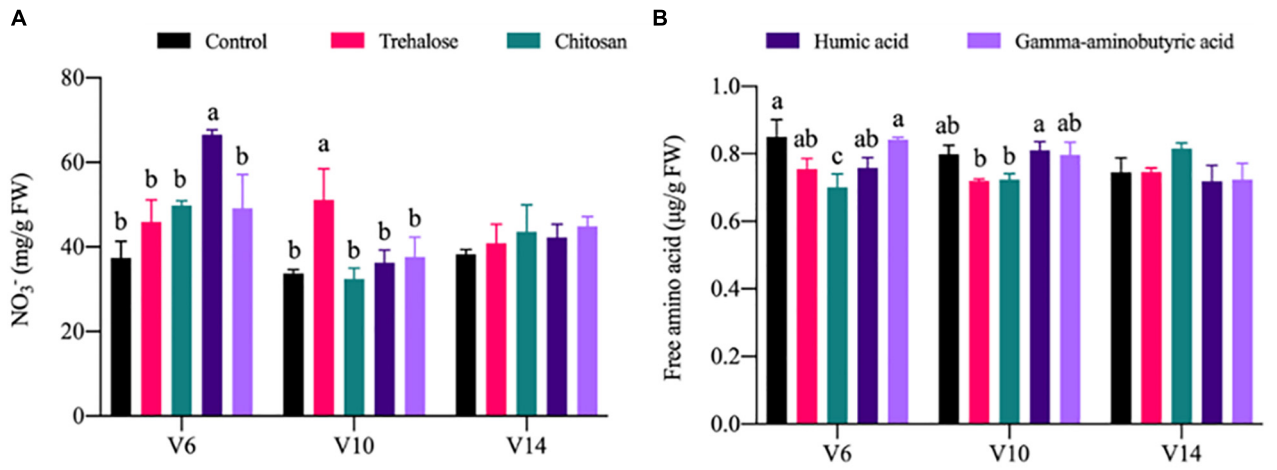


FIGURE 3 | Effects of PGRs on nitrogen metabolite accumulation in maize at different growth stages. Statistical analysis was used to compare means of PGR treatments shown in different colors; significantly different treatments within each stage are labeled with different lowercase letters ($P < 0.05$; $n = 3$), and the lack of such letters indicates there was no significant difference. V6, V10, and V14 represent the 6-, 10-, and 14-leaf stages of vegetative growth, respectively. FW, fresh weight. **(A)** NO_3^-. **(B)** Free amino acid.

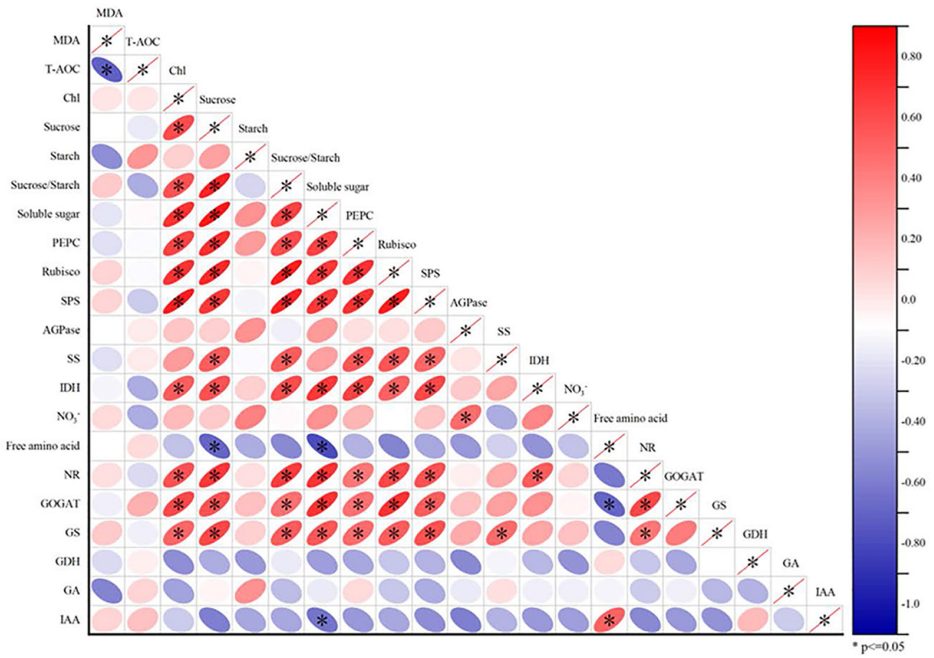


FIGURE 4 | Correlation analysis of maize leaf oxidative stress (MDA, T-AOC), carbon assimilation (Chl, sucrose, starch, sucrose/starch ratio, soluble sugar, PEPC, Rubisco, SPS, AGPase, and SS), organic acid metabolism (IDH), nitrogen metabolism (NO_3^-, free amino acid, NR, GOGAT, GS, and GDH) and endogenous hormones (IAA and GA). The color of the ellipse in each small square represents the degree of correlation between the two indicators, and the orientation of the ellipse indicates a correlation is positive or negative. *$P < 0.05$ indicates statistically significant correlations.

score plot divided all treatments into three regions: (i) Control group, (ii) trehalose treatment, (iii) chitosan, humic acid, and gamma-aminobutyric acid treatments combined. To further identify the variables underlying the differences in partitions, we utilized variable importance (VIP) in projections. VIP describes the importance of each variable to the model, with its partition contribution, and variables for which VIP > 1.5 and $P < 0.05$ (one-way ANOVA) were considered significant. Based on the above analysis, we found that starch content (VIP = 2.30, $P < 0.01$) and sucrose content (VIP = 1.83, $P < 0.01$) were the only two significant indicators in the PLS-DA model (**Supplementary Figure 5G**).

Reverse Transcription Quantitative PCR

A more in-depth method was used to analyze the expression changes of key genes in the carbon and nitrogen metabolism pathway by RT-PCR at V6 (**Supplementary Figure 6**). Compared with control conditions, *rca1*, *fgs1*, *sps1*, *pep1* and *NR* were significantly up-regulated by trehalose and chitosan treatment by 0.88 and 0.9, 1.67, and 0.7, 1.27 and 10.7 and 1.85 and 1.1 times, respectively. In addition, trehalose treatment also up-regulated *GRMZM2G057910* expression. However, most gene expression changes were not significant in plants treated with humic acid and gamma-aminobutyric acid, except that *fgs1* and *sps1* were up-regulated by humic acid treatment by 0.32 and 0.54 times, respectively, and *sps1* and *pep1* were up-regulated by gamma-aminobutyric acid treatment by 0.67 and 1.6 times, respectively. Moreover, all PGR treatments significantly down-regulated the expression of *gdh1*.

To better reveal the trend in gene expression under PGR treatment, a heat map was rendered (**Figure 5**). The heat map reveals a clear difference between the effects of trehalose and other PGRs. This difference is reflected in the darker red color indicating a higher change in gene expression, and although other PGRs were also significantly up-regulated, the degree of up-regulation was weaker than that observed under trehalose treatment.

DISCUSSION

Plant Oxidative Stress Levels and Growth Status

Generally, plants under stress exhibit an increase in the activity of antioxidant enzymes and the content of osmotic regulators, thereby increasing their resistance to adverse conditions (Reddy et al., 2004; Zulfiqar and Ashraf, 2022). MDA is generally regarded as a biomarker of disruptions of homeostasis in plants (Sofo et al., 2004). T-AOC is an indicator of total antioxidant capacity of plants. However, we did not detect significant changes between them, indicating that plant oxidative stress levels were not changed or were changed very little by PGR treatments (**Table 1**).

Chlorophyll is one of the most important parameters determining the photosynthesis and yield formation of C_4 crops (Guidi et al., 2019). **Supplementary Figure 1** shows that the chlorophyll content of the trehalose treatment group was increased significantly by 11.6%, compared with the control conditions at V6. This may be explained by trehalose treatment protecting the integrity of chromosomal and chloroplast membrane structures in maize leaves (Islam and Mohammad, 2021), thereby improving the absorption and utilization of light energy by plants. This was consistent with the observed increase in biomass of maize treated with trehalose (**Figure 1**). However, we also observed that gamma-aminobutyric acid treatment significantly increased plant biomass, although chlorophyll content did not change significantly. This pair of contrasting results suggested that changes in chlorophyll content might not be a comprehensive indicator of stress response induced by PGRs.

The root-to-shoot ratio (**Figure 1C**) is an important parameter of plant growth coordination, and only trehalose treatment significantly increased the root-to-shoot ratio of maize at V6 and V10. In contrast, gamma-aminobutyric acid did not significantly increase the root-to-shoot ratio at V6, but significantly increased the root-to-shoot ratio at V10. According to Lin et al. (2017) exogenous trehalose increased the root-shoot ratio of tobacco, especially under low nitrogen conditions. These results suggest that more complex metabolic activity might be involved in the regulation of maize growth and development.

Comprehensive Analysis of Carbon and Nitrogen Metabolic Activities and Endogenous Hormones

Carbon and nitrogen are the most abundant elements in cells and have irreplaceable roles in regulating organ development, material metabolism and grain formation (Xu et al., 2020). Carbon assimilation provides both the carbon skeleton and energy for nitrogen metabolism.

Photosynthesis is the basis of dry matter production and is limited by the intensity of the carbon metabolism in the internal environment of plants (Stitt, 1986). Carbon metabolic intensity is limited by two factors: (1) The accumulation and transport capacity of photosynthates; (2) the content or activity of carbon metabolic enzymes. Sucrose is the final product of photosynthesis and the most abundant soluble storage carbohydrate in most plants (Deng et al., 2019). SPS and SS are the rate-limiting enzymes in sucrose synthesis and the key enzymes in the conversion of sucrose to starch, respectively. In addition, Rubisco is a key enzyme in the Calvin cycle, catalyzing the fixation of net CO_2 in all photosynthetic organisms (Atkinson et al., 2020). Compared with the control conditions, trehalose treatment significantly increased sucrose accumulation at V6 and V10, which was related to the increase of SPS, SS and Rubisco enzyme activities; correlation analysis revealed significant positive correlations between them (**Figure 2A** and **Supplementary Figures 2A–C**). Yu et al. (2021) found that a short-term treatment with 100 mM trehalose resulted in a large drop in SS and a considerable increase in SPS, and that the activities of SS and SPS were regulated by SnRK. Our results demonstrated a strong positive correlation between SS and sucrose synthesis, despite the fact that SS is responsible for sucrose decomposition in general. The following are a few explanations for this discrepancy: (1) The trehalose concentration used in this investigation was 100 mg/L ($<$ 100 mM). (2) SnRK1 regulates the breakdown of SS, which is a passive process controlled by sucrose levels. During periods of excess carbohydrate synthesis, leaves divert hexose to starch synthesis to maintain a high level of light energy utilization and avoid photoinhibition (McKinley et al., 2018). AGPase regulates the first rate-limiting step in starch biosynthesis (Yoon et al., 2021). Compared with the control conditions, the starch content under chitosan, humic acid and gamma-aminobutyric acid treatments was significantly increased at V6. In contrast, the starch content under trehalose treatment did not change significantly at V6 (**Figure 2B**). This was basically consistent with the trend in

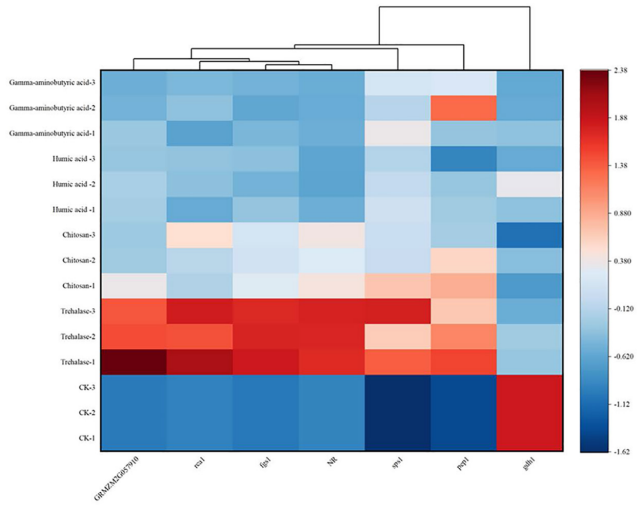


FIGURE 5 | Heat map of differentially expressed genes in maize leaves treated with plant growth regulators (PGRs) at the six-leaf stage of vegetative growth (V6) stage. Columns and rows in the heatmap represent genes and samples, respectively. The sample names are displayed to the left of the heatmap. The color scale indicates fold changes in gene expression. Blue and red indicate decreases and increases in transcript abundance, respectively. A cluster analysis of differentially expressed genes is shown above the figure. Heat maps were drawn with Origin software (2021b) based on normalized *Z*-scores.

AGPase activity, although starch content and AGPase activity were not positively correlated (**Figure 4** and **Supplementary Figure 2D**). The sucrose-to-starch ratio is considered an important factor regulating plant growth and yield formation (Li et al., 2018). In our experiment, the sucrose-to-starch ratio was significantly increased by trehalose and chitosan (**Figure 2D**). Pearson correlation analysis revealed that the sucrose-to-starch ratio was positively regulated by sucrose, but not starch; this was consistent with the observed trends in sucrose content and the sucrose-to-starch ratio (**Figures 2A,D**, **4**). PCA and PLS-DA joint analysis revealed the accumulation of sugar and starch was an important control target of PGR, and sucrose and starch accumulation treatment group could be divided into three parts: (1) The control conditions, with low starch and sucrose; (2) the trehalose treatment, with high sugar and low starch; (3) other PGR treatment, with low sugar and high starch (**Supplementary Figure 5**). The sucrose availability signal is regulated by trehalose-6-phosphate (Tre6P), and high levels of Tre6P inhibit the mobilization of starch, thereby regulating the contents of sucrose and starch (Hamilton et al., 2021). Exogenous trehalose may increase sucrose synthesis and decrease starch synthesis by promoting Tre-6P (positive feedback), in contrast with the negative feedback caused by the accumulation of endogenous trehalose. Plants utilize sucrose during the day and store starch for metabolism at night (Liu et al., 2019), which may reflect a rapid remobilization of photosynthates, which plays an important role in promoting plant growth under trehalose treatment.

In particular, PEPC catalyzes the formation of oxaloacetate from phosphoenolpyruvate (PEP) in the presence of Mg^{2+}, and IDH regulates the synthesis of organic acid anions, providing 2-oxoglutaric acid (2-OG) for ammonia assimilation (Cai et al., 2022). The activity of PEPC was significantly increased under all PGR treatments at V6. Among treatments, only trehalose significantly increased the activity of IDH at V6 (**Supplementary Figures 2E,F**). Changes in IDH activity may result in a drop in NAD and NADPH content, causing a disruption in cell redox levels (Foyer et al., 2011). Thus, trehalose is the only one of the four PGRs that regulates tricarboxylic acid cycle flux throughout the whole growth cycle (V6, V10, and V14). Furthermore, the changes in carbon assimilation activities induced by PGRs may further affect primary nitrogen metabolism. NO_3^-, as the major form of nitrogen available to most higher plants, is reduced to ammonium salts by NR, which is assimilated into amino acids through the GDH and GS-GOGAT pathways. NO_3^- is catalyzed by NR, which is the rate-limiting step in nitrogen assimilation (Han et al., 2022). In general, the application of PGRs did not significantly change NO_3^- content, except humic acid did significantly increase its accumulation at V6. However, the activities of NR, GOGAT, and GS were significantly increased by trehalose. Compared with trehalose treatment, chitosan only significantly increased the activities of NR and GOGAT (**Figure 3A** and **Supplementary Figures 3A–C**). Ozfidan-Konakci et al. (2018) reported that humic acid enhanced nitrogen uptake by plants and promoted cell elongation and division. Lin et al. (2017) described the promoting effect of trehalose on nitrogen metabolism. Correlation analysis revealed a significant positive correlation between the activity levels of carbon metabolism enzymes (PEPC, Rubisco, SPS, and SS) and nitrogen metabolism enzymes (NR, GOGAT, and GS) (**Figure 4**). The accumulation of plant biomass and the synthesis of carbon metabolites were also further improved (**Figures 1**, **2** and **Supplementary Figure 2**). Therefore, the application of trehalose and chitosan appears to accelerate the transformation and assimilation of NO_3^-. The concentration of FAA is regulated by both plant growth

and nitrogen accumulation status (Atilio and Causin, 1996). Chitosan significantly reduced the accumulation of FAA in leaves, but there was no significant difference in leaf FAA content under other PGR treatments at V6 (**Figure 3B**). There was a negative correlation between FAA and sucrose and soluble sugar accumulation (**Figure 4**). However, the importance of increased sucrose for improved plant growth is demonstrated by the results (**Figures 1**, **2**, **4** and **Supplementary Figure 5**). Accordingly, this might reveal why the effect of the chitosan treatment was weaker than that of the trehalose in terms of carbon and nitrogen metabolic intensity. GDH mainly exists in mitochondria and participates in detoxification of ammonium and replenishment of glutamate pools in plants (Ashraf et al., 2018). All PGR treatments tended to inhibit GDH activity, and chitosan had the most significant inhibition effect at V6 (**Supplementary Figure 3D**). This might be caused by chitosan enhancing the flow of ammonium into the GS-GOGAT cycle by inhibiting excessive accumulation of glutamate, as high concentrations of glutamate can inhibit root growth in rice and cucumber (Kim et al., 2010). GDH participates in the regulation of ammonium ion concentration and limits ammonium poisoning. However, we did not detect the generation of oxidative stress (**Table 1**). Therefore, GDH and FAA may tend to be inhibited under normal conditions under the application of PGRs, which may further enhance nitrogen metabolic activity.

Changes in endogenous hormones are another important strategy driving plant growth. Plant hormones regulate all developmental activities (Lin et al., 2020). IAA and GA control different processes, but cooperate in the formation and development of source and sink organs (Duan et al., 2018; Siddiqui et al., 2022). At the Overall level, compared with the control conditions, the application of PGRs did not significantly change the contents of GA and IAA. At the individual level, there were significant differences in the application of different PGR treatments compared with control conditions. Generally, the effects of PGRs on GA were mainly concentrated at V6 and V10, while the effects on IAA were mainly concentrated at V10 and V14 (**Supplementary Figure 4**). Although trehalose and chitosan significantly upregulated carbon and nitrogen metabolism, they did not significantly change the content of GA and IAA in leaves. In addition, no significant interaction between carbon and nitrogen metabolic activities and hormone levels was revealed by the Pearson correlation analysis (**Figure 4**).

Thus, PGR treatment had a short-term promoting effect on maize metabolic activity until V6 (0–12 days), and a long-term cumulative effect of metabolites associated with maize growth until V10 (13–28 days). Over the longer time period until V14 (29–46 days), no significant changes in carbon and nitrogen metabolic activity were observed. This may be explained by the effect of the PGR treatments being diminished or by the metabolic activity being converted into a potential capacity for improvement of grain yield or quality at maturity. One important strategy for using PGRs to improve plant growth status is through regulation of the intensity of carbon and nitrogen metabolic activity, rather than by changing endogenous hormone levels.

Comprehensive Analysis of Key Genes in the Carbon and Nitrogen Metabolism Pathway

Gene expression levels in key metabolic pathways are associated with changes in enzyme activity or content (**Supplementary Figure 6**). *GRMZM2G057910* encodes IDH, a key enzyme in the tricarboxylic acid cycle that is responsible for catalyzing the oxidative decarboxylation of isocitrate (Vega, 2020). Compared with the control conditions, the expression level of *GRMZM2G057910* in the trehalose treatment group was significantly up-regulated. The gene *rca1* encodes Rubisco in plants (Erdal, 2019), and activation of Rubisco enzymes is associated with high net photosynthesis. Compared with the control conditions, the *rca1* expression levels in the trehalose and chitosan treatment groups were significantly increased. The gene *sps1* controls the synthesis of SPS enzymes, thereby actively participating in the synthesis and distribution of carbon assimilation products. The application of PGRs significantly up-regulated *sps1* expression. Compared with the control conditions, except humic acid, other PGR treatments significantly up-regulated the expression level of *pep1*, the gene that encodes PEPC. The up-regulation of carbon metabolism function genes increased the levels of related enzymes in the synthetic pathways, resulting in a net accumulation of carbon metabolites (sucrose, starch and soluble sugar) (**Figure 2**), which provided sufficient material and energy sources for plant biological activities. NR is encoded by the *NR* gene and involved in the synthesis of amino acids and proteins through the GS/GOGAT cycle and GDH pathway. Additionally, its activity and content can affect the accumulation of ammonium. Trehalose and chitosan significantly up-regulated the expression of *NR* genes. The gene *fgs1* encodes a protein related to GOGAT synthesis. Compared with the control conditions, the expression of *fgs1* in the trehalose, chitosan and humic acid treatment groups was significantly increased. GDH is encoded by *gdh1* and has a dual function in regulating the concentration of ammonium ions in plants. All PGR treatments significantly decreased the expression of *gdh1*. The expression patterns of nitrogen metabolism function genes were basically consistent with the changes in enzyme activities (**Supplementary Figure 3**). The increase in nitrogen metabolism enzyme activity indicates the enhancement of nitrogen absorption and assimilation ability. Therefore, our results revealed the positive effects of PGR application on functional genes associated with carbon and nitrogen metabolism. Compared with humic acid and gamma-aminobutyric acid, trehalose and chitosan had the most significant promoting effects. Our results were consistent with the previously reported effects of chitosan and trehalose enhancing carbon and nitrogen assimilation activities (Li et al., 2022). The heat map in **Figure 5** shows the differential expression of carbon and nitrogen metabolism genes, clarifying the changes induced by PGR treatments more intuitively. Trehalose treatment increased the expression of more functional genes than did the chitosan treatment. In addition, cluster analysis results also indicated that the expression of *gdh1* was different from that of other genes, which might be owing to

GDH supplying 2-ketoglutarate to carbon-limited tissues to participate in carbon cycling, rather than the reassimilation of excess ammonium (Dubois et al., 2003). However, carbon limitation did not occur under the PGR treatments. On the contrary, a rapid nitrogen assimilation process may lead to the occurrence of ammonium toxicity.

RT-qPCR and various analytical methods clarified the effects of PGRs on maize growth. The differential regulation of carbon and nitrogen metabolism by trehalose, chitosan, humic acid and gamma-aminobutyric acid is a critical factor affecting the morphology of maize. Compared with chitosan, humic acid and gamma-aminobutyric acid treatments, trehalose treatment showed a stronger promoting effect on maize growth and development. However, there was a time limit to this enhancement. Nonetheless, this study fills a gap in the present understanding of the effects of different PGRs on maize growth and has applied value in enhancing agricultural production. In addition, environmental factors such as light, rainfall and soil texture, among others, brought about by different geographical locations and climate changes may limit the application of PGRs, so a more comprehensive analysis is merited.

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/s.

AUTHOR CONTRIBUTIONS

TG provided help and advice on sample processing. WZ provided guidance and support on the experimental design. BL completed the supplemental experimental content and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.889615/full#supplementary-material

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Dynamics of Phosphorus and Biostimulants on Agro-Morphology, Yield, and Essential Oil Profile of German Chamomile (*Matricaria chamomilla* L.) Under Acidic Soil Conditions of the Western Himalaya

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German chamomile (*Matricaria chamomilla* L.) is a promising and easy to cultivate crop under suitable nutrient supply conditions, but acidic soils of Indian western Himalayas limit the availability of phosphorus to the plant and reduce flower production. Thus, a field experiment was conducted for two consecutive seasons (2018–2019 and 2019–2020) to study the effect of phosphorus dynamics and biostimulant application on the agro-morphological traits, essential oil (EO) yield, and chemical constituents of German chamomile in the mid hills of the western Himalayan region. The experiment consisted of 12 treatments, four phosphorus fertilizer levels (0, 30, 60 and 90 kg ha^{-1}) and three biostimulants levels (control, amino acid at 5 mL L^{-1}, and humic acid at 10 mL L^{-1}). The experiment was replicated three times in a factorial complete randomized block design (FRBD). Agro-morphological and yield characteristics were significantly higher in phosphorus at 90 kg ha^{-1} and humic acid application compared to the control. Dry flower and EO yield was 17.87 and 26.76% higher with the 90 kg ha^{-1} phosphorus application while 2.45 and 5.79% higher in humic acid at 10 mL L^{-1} compared to the control. The EO constituents *viz*., chamazulene was 12.04 and 8.85% higher in phosphorus at 90 kg ha^{-1} and humic acid at 10 mL L^{-1} application compared to the control. On the other hand, α-bisabolol oxide B and α-bisabolol oxide A were decreased with increase in phosphorus application. This study presents novel facts, elucidation, and explanation for farmers and industrialists to produce German chamomile in acidic soils by integrating biostimulants with phosphorus fertilization and getting maximum yield and quality EO.

Keywords: German chamomile, triple superphosphate, amino acid, humic acid, bisabolol oxide

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INTRODUCTION

Medicinal and aromatic plants (MAPs) have served as the root of traditional medicine around the world since time immemorial (Gurib-Fakim, 2006). They are used extensively in herbal medicines and other sectors such as food, cosmetics, and perfumery (Zouaoui et al., 2020). German chamomile (*Matricaria chamomilla* L.) is globally one of the important MAPs (family: *Asteraceae*) used in a

diverse range of food, cosmetics, and perfumery sectors and has been used traditionally as a herbal medicine for thousands of years in Greece, Egypt, and Rome (Mann and Staba, 2002), but the major confront in the agriculture system is to boost crop production with a sustainable approach for short- and long term-goal achievements (Fallah et al., 2020).

It is more regrettable that long-term continuous disproportionate fertilizer application has reduced soil organic matter and quality, thus declining agricultural production and enhancing environmental pollution (Guo et al., 2010). Hence, all the aforesaid issues have become significant concerns at present (Chaudhry et al., 2009). The nutrient elements, *viz.*, nitrogen (N) and phosphorus (P) are particularly significant in MAPs, because they are involved in the structures of the precursors in EO, and in enzymes and energy-carrying molecules such as ATP (Dehsheikh et al., 2020). Thus, the use of plant nutrients cannot be neglected, but the source of these can be modified from chemical to organic origin (derived from plant and animal wastes) to achieve the sustainable agriculture goal (Fallah et al., 2018).

The soil pH values in the mid-hill Himalayan region range from 4 to 6 (acidic) because of high rainfall and highly weathered soils. In acidic soils, P becomes unavailable to plants as it gets fixed in the soil by sesquioxides, eventually leading to P deficiency. Thus, P becomes the most limiting nutrient in the crop production system (Johan et al., 2021). The reduced level of available P can adversely affect plant height, lateral plant spread, flower number, flower size, biomass, pollen production, etc. (Jiang et al., 2017). P is next to N in plant macronutrient supply, and P fertilizers are primary sources that can be applied to increase P availability to plant roots, thus enhancing crop growth, development, and production. In flowering plants, P has a major role in the flower development and reproduction stages (Kumar et al., 2006). With German chamomile being a flowering plant, it is imperative to study the effect of P on morphological and EO quality traits. The application of a certain amount and type of P fertilizer has a straight influence on the flowering, weight of flowers, and EO yield of German chamomile (Omidbeygi, 1995).

Biostimulants are natural preparations (organic substances and/or microorganisms) that increase plant nutrient utilization efficiency and abiotic stress tolerance and improve crops' quality without causing adverse side effects (Rouphael et al., 2018). They include humic and fulvic acids, salicylic acid, phenols, amino acids, proteins, enzymes, and micronutrients (Jardin Du, 2015). Humic acid is a hormone-like substance used for plant nutrition and improves nutrient absorption and plant growth (El-Gohary et al., 2019). It also has a positive effect on cell membrane functions by biosynthesis of nucleic acids, ion absorption, and respiration (Yang et al., 2004). Humic acid improves plant hormones and responsiveness, because it inhibits indole acetic acid oxidase activity, leading to increased IAA hormone activity and encouraging plant growth (El-Gohary et al., 2019). Other than this, among natural biostimulants, amino acid-based biostimulants with high content of free amino acids are prepared by enzymatic hydrolysis. Moreover, the increase in customers' consciousness regarding healthy products favors the enhancement and significance of organic production, and farmers are also keen on using natural stimulants to improve crop quality (Drobek et al., 2019). Effects of the foliar application of humic acid (De Hita et al., 2020) and amino acids (Vassilev, 2016) were studied on some morphological, physiological, and biochemical characteristics of cucumber (*Cucumis sativus* L.) and sunflower (*Helianthus annuus* L.). There is a dearth of information on German chamomile in response to P and biostimulants from the high precipitation region of the western Himalaya hence an attempt has been made to study the effect of P and biostimulants on German chamomile.

MATERIALS AND METHODS

Experiment Site

A field experiment was conducted for 2 repeated years, during 2018–2020 in the experimental farm of CSIR-IHBT (Council of Scientific and Industrial Research- Institute of Himalayan Bioresource Technology), Palampur, Himachal Pradesh (HP), India situated 1,325 m above mean sea level (amsl) altitude (32°11′39"N latitude and 76°56′51"E longitude). The climate of the region was subtropical, and the soil was characterized as clayey loam with acidic pH (5.34 ± 0.06), low in available P, i.e., 5.3 ± 0.35 P_2O_5 kg ha^{-1}, low organic carbon (0.28 ± 0.03%), medium in available nitrogen (234.24 ± 2.01 kg ha^{-1}), and high in available potassium (292.09 ± 3.44 K_2O kg ha^{-1}). Weather parameters, *viz.,* minimum and maximum temperature (°C), relative humidity (RH%), and average bright sunshine (BSS) hours during crop growth season, were acquired from "Crop weather outlook", an agro-meteorological advisory (Anonymous, 2020) and are illustrated in **Figure 1**. The maximum temperature (34°C) was recorded in May and the minimum temperature (2°C) in December. During 2018–2019, mean RH was maximum (73%) in February and minimum (33%) in May. The received total rainfall in German chamomile growth season during 2018–2019 was 557 mm, with the maximum in February and the lowest in December, while the received average daily BSS was 7 h. The maximum (32°C) and minimum (2°C) temperatures were recorded in May and February, respectively. Mean RH was maximum (81%) in January and minimum (45%) in May. The received total rainfall in German chamomile growth season during 2019–2020 was 803 mm, with the maximum in March and the lowest in February, while the received average daily BSS was 6 h. The total precipitation of the region during crop growth duration was lesser during the first growth year while it was higher in the second growth year. Moreover, the precipitation during the first growth year was higher in February (320 mm), i.e., the time period when plants were in the vegetative stage; while during the second growth year, the precipitation was higher from March to April (317 mm), i.e., the period when plants were blooming.

Factors and Experiment Details

The design used was a factorial randomized block design (FRBD) with three replications under field conditions. The experiment consists of 12 treatments with two factors, i.e., four phosphorus (P_2O_5) fertilizer levels, *viz.*, P1 0 kg ha^{-1} (control), P2 30 kg ha^{-1}, P3 60 kg ha^{-1}, and P4 90 kg ha^{-1}, and three biostimulant

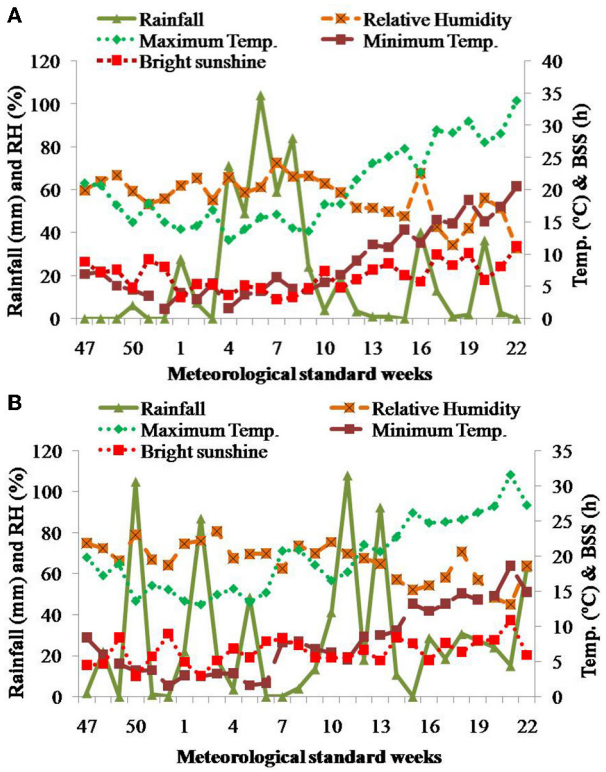


FIGURE 1 | Mean weather conditions during the crop growth seasons **(A)** 2018–2019 (Upper graph) and **(B)** 2019–2020 (Lower graph) at the experimental cultivation site in Palampur, HP, India. BSS, bright sunshine hour; RH, relative humidity.

levels, B1: control (distilled water), B2: amino acid at 5 mL L^{-1} and B3: humic acid at 10 mL L^{-1}. Treatment combinations of different levels of treatments factors were 12 allocated randomly in each block replicated thrice in the prepared field. The biostimulants are commercially available in the market under the brand name Amino Booster G (the amino acid solution) and V-Hume (the humic acid solution), the total phosphorus content in the amino acid and the humic acid was 0.03 and 0.12%, respectively; these were purchased for experiment execution. Well-decomposed farmyard manure (FYM at 15 t ha^{-1}) was applied 2 months before seed sowing; for better uniformity and distribution of plants in the field, the seeds were mixed with sand. The seed sowing of accession I (α-bisabolol oxide A-rich chemotype) was conducted in equidistant rows with a spacing of 40 cm in each experimental plot (size: 4 × 2.25 m). During both crop-growing years, the recommended dose of fertilizer (1/3 nitrogen and complete potassium) was given through urea (N 46%) and muriate of potash (K_2O) as pre-sowing fertilization; the remaining N was applied during the crop elongation and flower initiation stages in equal amounts. The P fertilizer used in the experiment was triple super phosphate (TSP) ($CaH_4P_2O_8$) and mixed in the soil before seed sowing. Biostimulants were applied by foliar application by dissolving in distilled water, and concentrations of 5 and 10 mL L^{-1} of the amino acid and humic acid, respectively, were prepared. The concentrations of the biostimulants and distilled water (control) were then sprayed before the flower bud formation stage on the treated plants and the control plants, respectively. As the flowering of German chamomile is not synchronous, spraying of the treatments was carried out fortnightly (after the first spray) from the end of the spring season (March) to the summer season (May) during both years. Weeding and irrigation procedures were carried out as per crop requirement.

Plant Growth, Pigment, and Yield Determination

Morphological features, *viz*., plant height (in cm) and numbers of branches were recorded at crop harvest (full flowering) by placing a quadrant (25 cm^2) in two sites in the sampling rows of each plot. Growth-contributing attributes such as photosynthetic photon flux density (PPFD, in $\mu mol^{-1}s^{-1}$ m^2) and leaf area index (LAI) were measured with an LI-191R line quantum sensor (measures PAR) and Handheld Laser LAI Meter CI-110/120 (CID, Bio-Science, United States), respectively. The major flowering flush started from mid-April to May; flower plucking was carried out with a 2-week interval. The number of flowers was evaluated by placing a quadrant (25 cm^2) in two sites in the core region row of the plot, and flower drying (up to constant weight) was conducted at room temperature, i.e., 20 to 25°C during April and 25 to 30°C during May; then, cumulative dry flower yield was calculated. Fresh leaf samples (1 g) were taken, washed with distilled water, homogenized with acetone (80%), and centrifuged at 5,000 rpm for 5 min, and the absorbance (OD) of the supernatant was recorded at λ = 663, 646, and 470 nm with a spectrophotometer (T90 + UV spectrophotometer; PG instruments Ltd.) for chlorophyll (Chl a and Chl b) and carotenoid determination. Chlorophyll (a and b) and carotenoid (in mg g^{-1}) content was determined (Lichtenthaler and Buschmann, 2001).

Chl a = 12.21 OD_{663}-2.81 OD_{646},
Chl b = 20.13 OD_{646}-5.03 OD_{663}, and
Carotenoids = (1,000 OD_{470}-3.27 Chl a−104 Chl b)/229.

Identification and Determination of Essential Oil Components

Fresh flowers were harvested from the net plot area of each treatment, dried at room temperature, and hydrodistilled in triplicates for 4 h in a Clevenger-type apparatus (European Pharmacopoeia, 2007). EO content was calculated as volume (in mL) of EO obtained per weight (in g) of flowers, and the EO was dried with anhydrous sodium sulfate (Na_2SO_4). The EO was then stored at 4°C in a glass container before analysis. EO yield was calculated by multiplying the EO content with the specific gravity (0.92) of EO and total dry flower yield of all the harvests. GC and GC/MS analyses were executed in triplicates with a flame ionization detector (FID) on a Shimadzu GC 2010 gas chromatograph and QP2010 (Shimadzu Corp., Tokyo, Japan) fitted with an AOC 5000 auto-injector.

TABLE 1 | Analysis of variance for effect of cropping year, phosphorus and biostimulant on growth and yield traits of *M. chamomilla*.

Sources of variations	Df	Plant height	Number of branches	Number of flowers	Flower diameter (cm)	Flower yield (t ha^{-1})	Essential oil yield (kg ha^{-1})
Year	1	NS	NS	**	**	**	**
Phosphorus	3	**	**	**	**	**	**
Biostimulant	2	**	**	**	**	**	**
Year × Phosphorus	3	NS	**	NS	NS	**	**
Y × Biostimulant	2	NS	NS	NS	NS	*	*
Phosphorus × Biostimulant	6	**	NS	NS	**	NS	NS
Year × Phosphorus × Biostimulant	6	NS	NS	NS	NS	NS	NS

** and ** represent significant relationship at p = 0.05 and p = 0.01, respectively. ns, not–significant; Df, degree of freedom.*

TABLE 2 | Growing years, phosphorus and biostimulant application influences plant growth and yield contributing attributes in *M. chamomilla* at harvest.

Treatment	Plant height (cm)	Number of branches per plant	PAR	LAI
Growing years				
2018–2019	75.46	22.36	575.81	3.10
2019–2020	74.62	22.25	571.81	2.92
SEm (±)	0.78	0.15	1.21	0.01
LSD ($p = 0.05$)	NS	NS	3.43	0.03
Phosphorus levels				
Control	67.90	21.04	559.89	2.84
30 kg ha^{-1}	70.74	21.78	574.22	2.97
60 kg ha^{-1}	77.66	23.27	577.33	2.98
90 kg ha^{-1}	83.87	23.13	583.78	3.24
SEm (±)	1.10	0.22	1.70	0.02
LSD ($p = 0.05$)	3.13	0.62	4.85	0.04
Biostimulants				
Control (0 mL L^{-1})	69.79	21.43	570.00	2.98
Amino acid (5 mL L^{-1})	72.10	22.32	578.08	3.03
Humic acid (10 mL L^{-1})	83.24	23.17	573.33	3.03
SEm (±)	0.95	0.19	1.48	0.01
LSD ($p = 0.05$)	2.71	0.53	4.20	0.04

SEm ±, standard error mean; LSD, least significant difference (p = 0.05); NS, not significant; PAR, photosynthetically active radiations; LAI, leaf area index.

The auto-injector consisted of a 30-m long ZB-5 MS capillary column with a 0.25-mm i.d. and a 0.25-μm thick film (SGE International, Ringwood, Australia). Ten μL EO was dissolved in 2 mL of dichloromethane and auto-injected in split mode with 2 μL volume. N (carrier gas) was used at a 1.05 mL min^{-1} flow rate; the temperature of the oven was maintained at 70°C for 3 min and subsequently risen to 220°C for 5 min at a rate of 4°C min^{-1}. The maintained temperature of the injector was 220 °C while that of the detector was 250°C. The settings of temperature lineup, injection volume, and carrier gas utilized to execute GC and GC/MS were similar to the detailed procedure for *Rosmarinus officinalis* L. in Rathore et al. (2022). To identify components from areas of GC peaks, a series of hydrocarbons was used for retention index (RI) determination without the use of any correction factor. EO components were identified by matching the experimental RIs with the RIs reported in the literature (Adams, 2017; Rathore and Kumar, 2021). Moreover, the identification was also conducted by comparing and matching the minimum mass spectral fragmentation pattern of the components with the NIST library (Stein, 2005).

Statistical Analysis

Once the morphological and chemical composition evaluation was completed, data were analyzed with the analysis of variance (ANOVA) technique for factorial RBD. Least significant testing was completed by Fisher's least significant difference (LSD) test and regarded as statistically significant at $p = 0.05$. Analysis of variance for effect of cropping year, phosphorus and biostimulant on growth and yield traits of *M. chamomilla* was also studied (**Table 1**). EO constituents were subjected to multivariate principal component analysis to determine the expression of treatments in EO constituents with software (PCA software PAST 3).

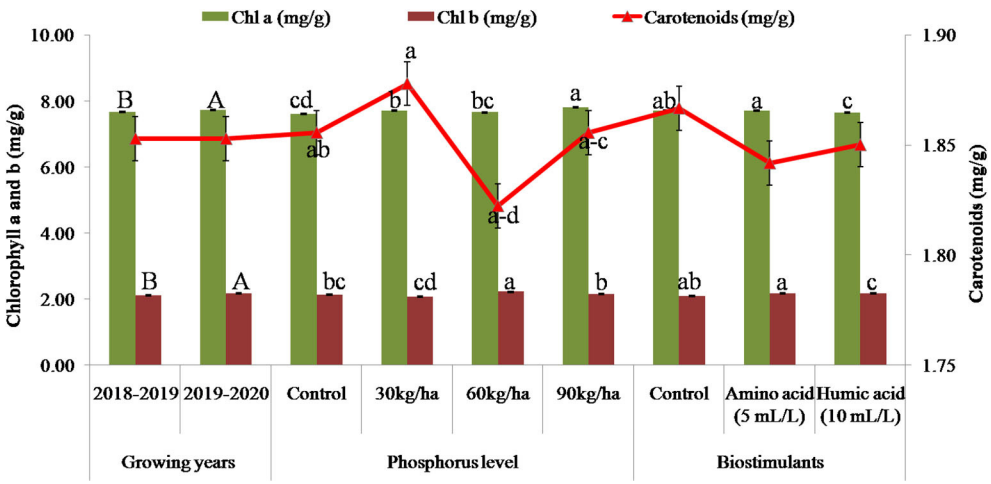


FIGURE 2 | Growing years, phosphorus and biostimulant application affected the chlorophyll and carotenoids (mg g^{-1}) concentration at harvest in *M. chamomilla*.

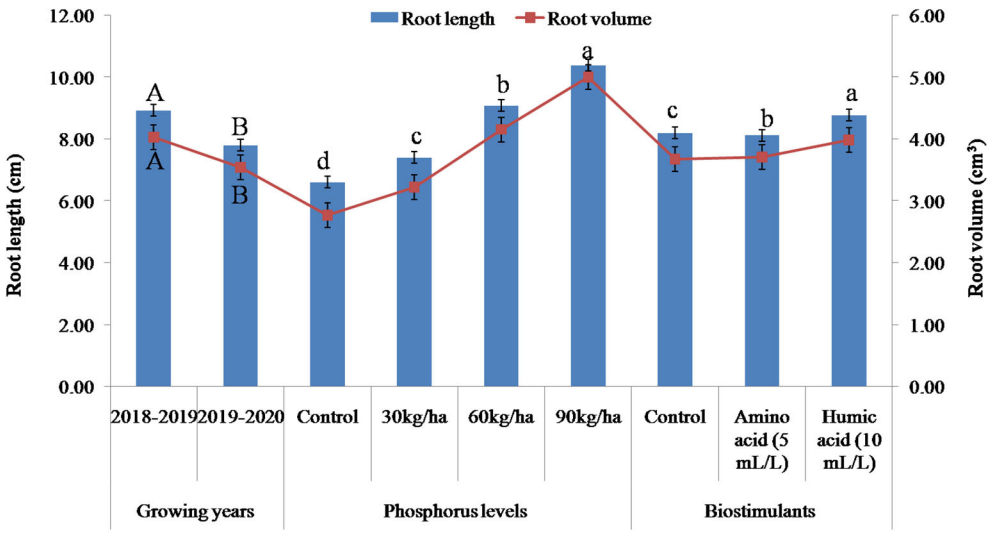


FIGURE 3 | Growing years and phosphorus and biostimulant application affected root length (in cm) and root volume (in cm^3) at harvest in *M. chamomilla*.

RESULTS

Plant Growth and Pigment Investigation

At harvest, plant growth attributes, *viz.*, plant height and number of branches per plant, were not significantly affected by growth years, but photosynthetically active radiation (PAR) (575.81 μ mol s^{-1} m^{-1}) and LAI (3.1) were significantly higher during 2018–2019 than during 2019–2020 (**Table 2**). Significantly higher concentrations of Chl a and Chl b were recorded during 2019–2020; while carotenoids were not significantly affected by the growing years (**Figure 2**). Similarly, significantly higher plant height (83.8 cm), PAR (583.78 μ mol s^{-1} m^{-1}), and LAI (3.24) were recorded in P at 90 kg ha^{-1} compared with the control, while the number of branches was significantly higher in P at 60 kg ha^{-1} compared with the control but remained at par with 90 kg ha^{-1} P application (**Table 2**). Likewise, Chl a was significantly higher in P at 90 kg ha^{-1} as compared to the control, while Chl b and carotenoids were significantly higher in the P at 60 and 30 kg ha^{-1} applications as compared to the control (**Figure 2**). Significantly higher plant height (83.24 cm) and number of branches per plant (23.17) were recorded in the humic acid application compared to the control. PAR (μ mol s^{-1} m^{-1}) accumulation was measured as difference in PAR above plant canopy and below plant canopy; usually much flourished plant canopies contribute to more PAR accumulation by plants. The PAR (578.08 μ mol s^{-1} m^{-1}) accumulation by the plants was significantly higher in the amino acid compared to the other biostimulant applications, and LAI (3.03) was significantly higher in the humic acid application compared to control the

but remained at par with the amino acid application (**Table 2**). The concentration of Chl a (7.7) and carotenoids (1.87) was significantly higher in the control than in other biostimulant treatments, while Chl a was significantly higher in the amino acid application (**Figure 2**).

The application of biostimulants significantly affected PAR and LAI. Significantly higher PAR (578.08 μ mol s^{-1} m^{-1}) was recorded in the amino acid than in the humic acid and control, while LAI (3.03) was significantly higher in the humic acid application than in the control but remained at par with the amino acid application (**Table 2**). In the crop harvest stage, root length and root volume were significantly affected by the growing years and P and biostimulant applications (**Figure 3**). Significantly higher root length (8.93 cm) and root volume (4.03 cm^3) were recorded in 2018–2019 than in 2019–2020. Likewise, P at 90 kg ha^{-1} and humic acid recorded significantly higher root length (10.38 cm) and root volume (5 cm^3) than the control (**Figure 3**).

Yield Attributes, Essential Oil (EO) Content, and Yield

At crop harvest, the number of flowers per plant (71.56), dry flower weight (6.8 g $plant^{-1}$), flower diameter (2.29 cm), disc floret height (89.69 mm), dry flower yield (2.46 t ha^{-1}), EO content (0.79%), EO yield (17.59 kg ha^{-1}) were significantly higher during 2019–2020, while the number of ray florets was not significantly affected by the crop-growing years (**Table 3**). The number of flowers per plant (75.24), flower diameter (2.3 cm), disc floret height (92.33 mm), and dry flower yield (2.63 t ha^{-1}), EO yield (19.17 kg ha^{-1}) were significantly higher in P at 90 kg ha^{-1} than in the control. Similarly, dry flower weight (7.05 g $plant^{-1}$) and EO content (0.81%) were significantly higher in P at 90 kg ha^{-1} but remained at par with the 60 kg ha^{-1} P application. Likewise, the number of ray florets was also significantly higher in P at 90 kg ha^{-1} than in the control but remained at par with the 60 and 30 kg ha^{-1} P applications. The application of biostimulants significantly affected the yield attributes. The foliar application of humic acid recorded a significantly higher number of flowers (71.3), dry flower weight (6.72 g $plant^{-1}$), and EO yield (17.25 kg ha^{-1}) than the amino acid and control. Similarly, dry flower yield and EO yield were also significantly higher in humic acid than in the control but remained at par with the amino acid application, while flower diameter was significantly higher in the amino acid but remained at par with the humic acid application (**Table 3**).

Regression and Correlation Analysis

Regression equations were illustrated among the independent variables, i.e., P and biostimulant applications, and dependent variables, i.e., flower yield and EO yield (**Figure 4**). The flower and EO yields in German chamomile increased with increase in P application, and the highest yield was observed at 90 kg P ha^{-1}. The application of P developed a strong linear relationship with flower yield and EO yield using equations y = 2.172 + 0.005x, (r^2 = 0.979) and y = 14.07 + 0.06x, (r^2 = 0.972) at P = 0.01), respectively (**Figure 4A**). The application of P from 0 to 30 kg ha^{-1} and from 30 to 60 kg ha^{-1} recorded a considerable increase in flower yield and EO yield, but this increase was much slighter

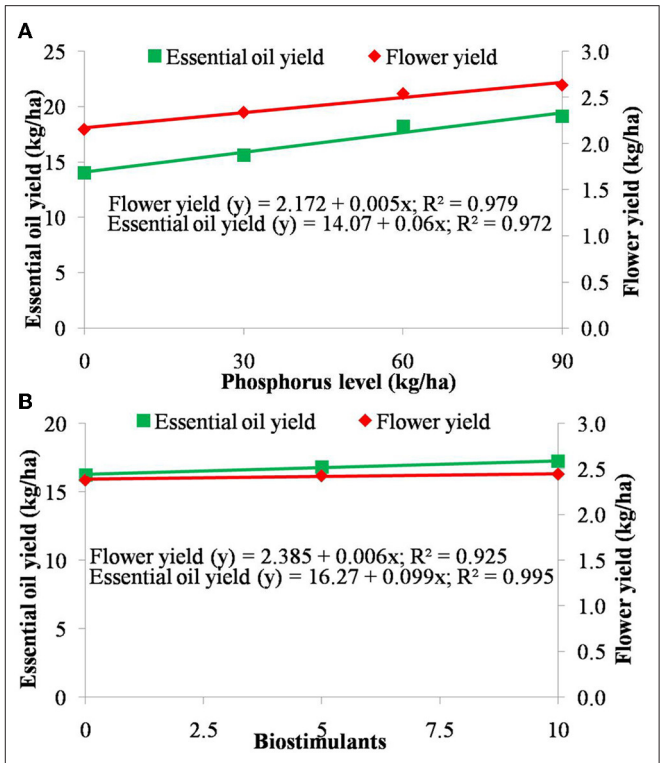


FIGURE 4 | Regression equation between independent variable, **(A)** levels of phosphorus (upper graph) and **(B)** biostimulant and dependent variables (lower graph) i.e., total flower yield (in kg ha^{-1}) and essential oil yield (in kg ha^{-1}). The levels of phosphorus and biostimulants are represented in the primary *X* axis. Total essential oil yield and flower yield are presented in the primary *Y* axis and secondary *Y* axis, respectively.

from 60 to 90 kg ha^{-1} P application. Similarly, flower yield and EO yield increased with the application of the biostimulants and developed a strong relationship, i.e. y = 2.385 + 0.006x, (r^2 = 0.925) and y = 16.27 + 0.099x, (r^2 = 0.995) respectively (**Figure 4B**).

The correlation matrix recorded a significant (P = 0.01) correlation of EO yield with number of branches (r = 0.73), number of flowers (r = 0.97), flower diameter (r = 0.84), flower yield (in t ha^{-1}) (r = 0.97), EO content (r = 0.96) and a significant correlation with plant height (cm) (r = 0.69) at P = 0.05 significance level (**Figure 5**). Similarly, EO content recorded a significant (P = 0.01) correlation with number of branches (r = 0.91), number of flowers (r = 0.91), flower diameter (cm) (r = 0.91), and flower yield (t ha^{-1}) (r = 0.88), and showed a significant (P = 0.05) correlation with plant height (r = 0.62). Also, flower yield showed a significant (P = 0.01) correlation with number of branches (r = 0.75), number of flowers (r = 0.96), and flower diameter (cm) (r = 0.77). Moreover, a significant (P = 0.01) correlation was observed between flower diameter and number of flowers (r = 0.82), while a positive correlation was observed with number of branches (r = 0.5) and plant height (r = 0.43). Number of flowers showed a significant correlation with number of

TABLE 3 | Growing years, phosphorus and biostimulant application influences yield attributes and yield in *M. chamomilla* at crop harvest.

Treatment	Number of flowers	Dry flower weight (g plant^{-1})	Flower diameter (cm)	Number of ray florets per flower	Disc floret height (mm) per flower	Dry flower yield (t ha^{-1})	E. oil content (%)	E. oil yield (kg ha^{-1})
Growing years								
2018–2019	67.63	6.26	2.12	22.94	88.31	2.38	0.74	15.96
2019–2020	71.56	6.80	2.29	22.72	89.69	2.46	0.79	17.59
SEm (±)	0.40	0.03	0.01	0.19	0.18	0.00	0.00	0.07
LSD ($p = 0.05$)	1.15	0.10	0.02	NS	0.52	0.01	0.01	0.21
Phosphorus levels								
Control	62.49	5.85	2.09	21.56	82.22	2.16	0.72	14.04
30 kg ha^{-1}	67.02	6.30	2.19	23.00	89.89	2.34	0.74	15.63
60 kg ha^{-1}	73.62	6.93	2.25	23.11	91.56	2.54	0.80	18.23
90 kg ha^{-1}	75.24	7.05	2.30	23.67	92.33	2.63	0.81	19.17
SEm (±)	0.57	0.05	0.01	0.26	0.26	0.01	0.00	0.10
LSD ($p = 0.05$)	1.63	0.14	0.03	0.75	0.74	0.02	0.01	0.29
Biostimulants								
Control (0 mL L^{-1})	67.82	6.36	2.17	22.46	89.17	2.38	0.75	16.25
Amino acid (5 mL L^{-1})	69.66	6.52	2.24	22.96	88.67	2.43	0.77	16.81
Humic acid (10 mL L^{-1})	71.30	6.72	2.21	23.08	89.17	2.44	0.78	17.25
SEm (±)	0.50	0.04	0.01	0.23	0.23	0.00	0.00	0.09
LSD ($p = 0.05$)	1.41	0.12	0.03	NS	NS	0.01	0.01	0.25

SEm ±, standard error mean; LSD, least significant difference (p = 0.05); NS, not significant.

branches ($r = 0.8$), and number of branches further showed a significant correlation with plant height ($r = 0.75$) at the $P = 0.01$ significance level.

Determination of Essential Oil Components

The major EO constituents (more than 5% area) in EO were (Z)-β-farnesene, germacrecene D, α-bisabolol oxide B, bisabolone oxide A, α-bisabolol oxide A, and en-in-dicycloether, while artemisia ketone and chamazulene were reported as minor constituents (>2% area contribution) (**Table 4**). The concentration of (Z)-β-farnesene, germacrecene D, and en-in-dicycloether was significantly higher in 2019–2020, while that of α-bisabolol oxide B, bisabolone oxide A, chamazulene, and α-bisabolol oxide A was significantly higher in 2018–2019. Likewise, the application of biostimulants recorded a significantly higher concentration of (Z)-β-farnesene and germacrecene D with the 30 kg ha^{-1} P application, while α-bisabolol oxide B and α-bisabolol oxide A were significantly higher in the control. On the other hand, chamazulene and en-in-dicycloether were significantly higher with the 90 kg ha^{-1} P application compared to the other P levels. Similarly, the application of biostimulants significantly affected the EO constituents and recorded significantly higher (Z)-β-farnesene, germacrecene D, chamazulene, α-bisabolol oxide A, and en-in-dicycloether in the humic acid application than the amino acid and control. On the other hand, α-bisabolol oxide B and bisabolone oxide A were significantly higher in the control and amino acid applications than the other treatments while later remaining at par with the control (**Table 4**).

Principal Component Analysis (PCA)

The dependent variables such as flower yield (t ha^{-1}) and EO yield (kg ha^{-1}) and major EO constituents, *viz.*, α-bisabolol oxide B, bisabolone oxide A, chamazulene, α-bisabolol oxide A, and en-in-dicycloether were subjected to principal component (PC) analysis to discover the relationship between growing years and the P and biostimulant applications (**Figure 6**). The PC analysis revealed that 80.62% of the total variations were explained by PC1 and PC2. Among all the variables, flower yield, EO yield, bisabolone oxide A, chamazulene, and en-in-dicycloether showed a positive association, while α-bisabolol oxide B and bisabolol oxide A showed a negative association in PC1. Similarly, in PC2, flower yield, EO yield, α-bisabolol oxide B, bisabolone oxide A, chamazulene, and bisabolol oxide A showed a positive association, while only en-in-dicycloether showed a negative association. The analysis through PC separated the P3, P4 (phosphorus levels), and B2 (amino acid application) treatments in PC1 and PC2 by showing a positive contribution in PCs, flower yield, EO yield, bisabolone oxide A, and chamazulene. The findings of the present study showed that the first three PCs were extremely informative, with eigen values of 3.8, 1.9, and 0.8 and thus contributed about 92% of the overall variance of the dependent variables. The observed score plot could be divided into six distinct clusters (**Figure 6**). Cluster I included treatments, *viz.*, P3 and P4; cluster II included B2 and B3; cluster V included B1 and P2; clusters III, IV, and VI corresponded to 2019–2020, P1, and 2018–2019 treatments, respectively. Cluster I included higher ranges of flower yield (2.54–2.63 t ha^{-1}), EO yield (18.23–19.17 kg ha $^{-1}$), and en-in-dicycloether (8.39–9.36%) in P3 and P4 phosphorus levels.

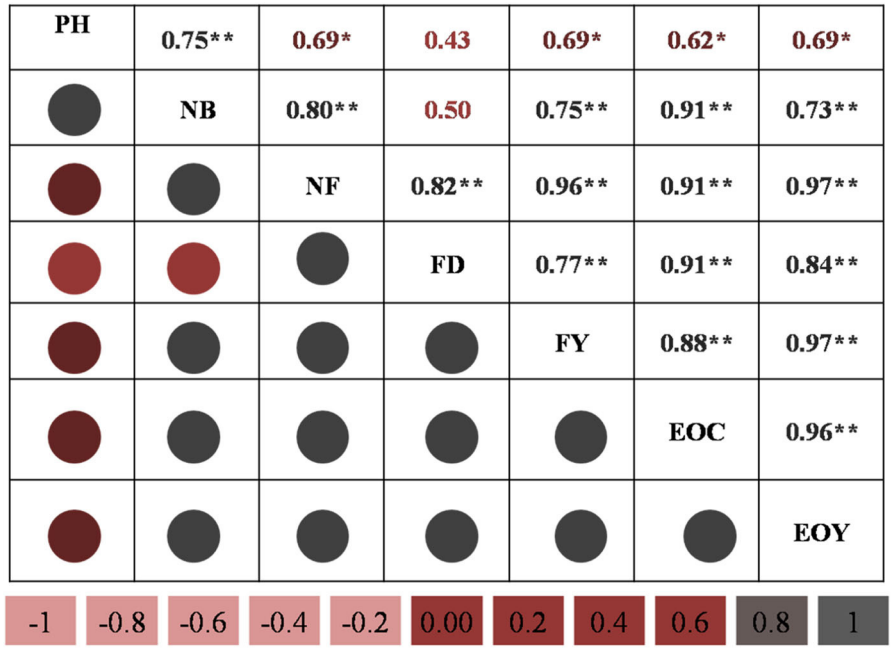


FIGURE 5 | Correlation analysis of growth and yield parameters. PH, plant height; NB, number of branches; NF, number of flowers; FD, flower diameter (in cm); FY, dry flower yield (in t ha^{-1}); EOC, essential oil content (in %); EOY: essential oil yield. The mean values of the 2-year pooled data of the corresponding treatments are used; * and ** indicate that the corresponding values are significant at $p = 0.05$ and $p = 0.01$, respectively.

Cluster II comprised the lowest bisabolone oxide A (11.1–12.37%) and intermediate ranges of flower yield (2.43–2.44 t ha^{-1}) and EO yield (16.81–17.25 kg ha^{-1}) in B2 and B3 biostimulant applications. Additionally, cluster II included the 11.49–11.73, 2.67–2.71, 41.67–42.29, and 7.87–8.35 ranges of α-bisabolol oxide B, chamazulene, bisabolol oxide A, and en-in-dicycloether, respectively. Cluster III included a single treatment level, i.e., the 2019–2020 growing year of the crop, which consisted of the lowest α-bisabolol oxide B (10.02%) and bisabolone oxide A (11.33%) range and the highest en-in-dicycloether (11.9%) concentration. Similarly, cluster IV again comprised of a single treatment level, i.e., P1 (no phosphorus application) with the lowest flower yield (2.16 t ha^{-1}), EO yield (14.04 kg ha^{-1}), and chamazulene (2.41%) and the highest bisabolol oxide A (44.77%) and intermediate range of en-in-dicycloether (6.06%) concentration. Cluster V included B1 and P2 levels of the biostimulant and P applications; flower yield, bisabolone oxide A, and bisabolol oxide A had the lowest range, i.e., 2.34–2.38 t ha^{-1}, 11.15–12.23%, and 39.98–41.42% in cluster V. Cluster VI included a single treatment level, i.e., the 2018–2019 growing year of the crop that consisted of the highest ranges of α-bisabolol oxide B (13.91 %) and chamazulene (2.76%), and the lowest range of flower yield (2.38 t ha^{-1}) and en-in-dicycloether (3.19%) was observed. In the end, a significant disparity in flower yield and EO yield was recorded in the different growing years and P and biostimulant applications under the open field conditions of western Himalayan foothills (**Table 5**).

DISCUSSION

Plant Growth and Pigment Investigation

The use and integration of plant biostimulants with fertilizers are increasing in traditional agriculture systems with an aim to enhance crop production. PAR accumulation, LAI, root length, and root volume were significantly affected by crop growth years, and variations can be attributed to diverse climatic conditions during crop growth years. The PAR at harvest was higher during the first growing year, which might be because of lesser precipitation during harvest months, i.e., end of March to mid-May on meteorological standard weeks (MSWs) 13 to 20 (**Figure 1A**) during the first growth year, while precipitation was higher at harvesting time in the second growing year on MSWs 13 to 20 (**Figure 1B**). Similarly, during the first crop-growing year, the higher LAI can be synchronized with lesser precipitation and higher solar radiation in terms of BSS, while higher precipitation and lesser BSS in the second crop growing year contributed to lesser LAI (**Figure 1**). Similar findings with higher PAR and LAI were reported in lower cloud cover and higher solar radiation in forest crop species of the Amazon forest (Li et al., 2018). The higher root attributes in the first growing year might be attributed to lesser average precipitation in the crop growing period, because enhanced root growth can be inferred as an adaptive strategy to deal with lower water availability due to lesser precipitation during growing season by increasing absorptive root surface (Metcalfe et al., 2008). Similar to the present findings, the production of roots in *Artemisia barrelieri* Besser increased with a 30% rainfall reduction (Padilla et al., 2015). In contrast

TABLE 4 | Growing years, phosphorus and biostimulant application influences essential oil composition in *M. chamomilla*.

Treatment	Ocimene <(E)-β->	Artemisia ketone	Camphor	β-farnesene	Germacrene D	Bicyclogermacrene	α-farnesene	Spathulenol	Epi-α-Cadinol	α-bisabolol oxide B	Bisabolone oxide A	Chamazulene	α-bisabolol oxide A	En-in-dicycloether
Growing years														
2018–2019	0.18	2.64	0.79	5.87	0.79	1.56	0.91	0.97	0.95	13.91	12.47	2.76	42.99	3.19
2019–2020	0.94	2.95	0.52	8.22	8.22	0.96	0.50	0.45	0.62	10.02	11.33	2.47	40.59	11.90
SEm (±)	0.18	0.06	0.02	0.08	0.04	0.02	0.03	0.04	0.04	0.08	0.09	0.04	0.14	0.09
LSD ($p = 0.05$)	0.50	0.16	0.06	0.24	0.11	0.05	0.07	0.12	0.12	0.21	0.25	0.10	0.39	0.26
Phosphorus levels														
Control	0.41	2.47	0.54	6.72	4.90	1.05	0.70	0.54	0.77	13.26	11.60	2.41	44.77	6.06
30 kg ha^{-1}	1.10	3.64	0.59	8.71	5.16	1.14	0.85	0.64	0.98	11.72	11.15	2.67	39.98	6.36
60 kg ha^{-1}	0.46	2.51	0.85	5.81	3.90	1.74	0.74	0.72	0.76	11.45	12.75	2.66	41.40	8.39
90 kg ha^{-1}	0.28	2.58	0.65	6.94	4.07	1.13	0.53	0.95	0.63	11.42	12.12	2.74	41.01	9.36
SEm(±)	0.25	0.08	0.03	0.12	0.05	0.03	0.04	0.06	0.06	0.11	0.13	0.05	0.19	0.13
LSD ($p = 0.05$)	NS	0.22	0.09	0.33	0.15	0.07	0.10	0.17	0.17	0.30	0.36	0.15	0.55	0.37
Biostimulants														
Control (0 mL L^{-1})	0.46	3.28	0.59	7.20	4.42	1.14	0.59	0.71	0.81	12.67	12.23	2.47	41.42	6.42
Amino acid (5 mL L^{-1})	0.44	2.68	0.85	6.61	4.37	1.60	0.76	0.70	0.78	11.49	12.37	2.67	41.67	7.87
Humic acid (10 mL L^{-1})	0.79	2.43	0.53	7.32	4.73	1.05	0.76	0.72	0.76	11.73	11.10	2.71	42.29	8.35
SEm(±)	0.22	0.07	0.03	0.10	0.05	0.02	0.03	0.05	0.05	0.09	0.11	0.04	0.17	0.11
LSD ($p = 0.05$)	NS	0.19	0.08	0.29	0.13	0.06	0.09	NS	NS	0.26	0.31	0.13	0.47	0.32

SEm ±, standard error mean; LSD, least significant difference ($p = 0.05$); NS, not significant.

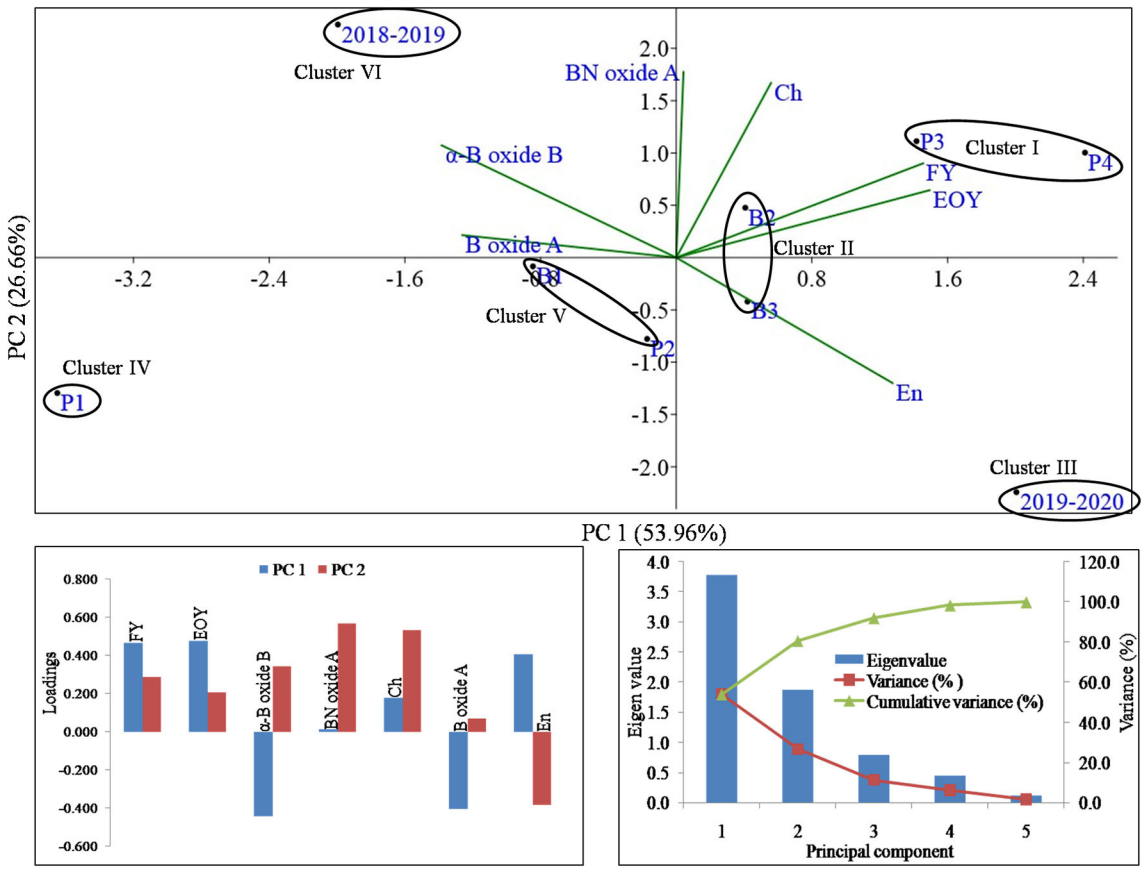


FIGURE 6 | The multivariate analyses of mean value of growth parameters, yield and major compounds of essential oil were conducted through principal component analysis. PC1 and PC2 jointly explained the variations of 80.62%. The loading values of variables and treatment combinations are presented as vectors in the space of the PCA. The eigenvalues and loading scores of the variables with PC1 and PC2 are presented in the bottom left and right corner, respectively. FY, dry flower yield (t ha^{-1}); EOY, essential oil yield (Kg ha^{-1}); α-B oxide B, α-bisabolol oxide B; BN oxide A, bisabolone oxide A; Ch, chamazulene; B oxide A, bisabolol oxide A; En, en-in-dicycloether.

TABLE 5 | Clusters variability in dependent variables in *M. chamomilla* affected by growing years, phosphorus and biostimulant application.

Variables	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI
Flower yield	2.54–2.63	2.43–2.44	2.46	2.16	2.34–2.38	2.38
Essential oil yield	18.23–19.17	16.81–17.25	17.59	14.04	15.63–16.25	15.96
α-bisabolol oxide B	11.42–11.45	11.49–11.73	10.02	13.26	11.72–12.67	13.91
Bisabolone oxide A	12.12–12.75	11.10–12.37	11.33	11.60	11.15–12.23	12.47
Chamazulene	2.66–2.74	2.67–2.71	2.47	2.41	2.47–2.67	2.76
Bisabolol oxide A	41.01–41.40	41.67–42.29	40.59	44.77	39.98–41.42	42.99
En-in-dicycloether	8.39–9.36	7.87–8.35	11.90	6.06	6.36–6.42	3.19

to the present findings, precipitation has no significant effect on root length and biomass allocation in *Erodium oxyrhynchum* M.Bieb. (Chen et al., 2019), while Cheng et al. (2006) reported an increase in root length as moisture in soil profile increased. The application of P at 90 kg ha^{-1} remained at par with 60 kg P ha^{-1} and recorded significantly higher plant height, number of branches, PAR, and LAI. This may be because of the fact that the soil of the experimental plot was acidic, and that the amount of P in the soil was very low, hence making it unavailable for root uptake and contributed to low plant height and number of branches in the control plots compared with the P-applied plots (Zheng, 2010). The findings are in accordance with Sonmez (2018), who reported higher plant height in P application than control in anise (*Pimpinella anisum* L.). Erbas et al. (2017) also recorded significantly higher plant growth with the application of 100 kg P ha^{-1} in lavandin. On the

contrary, the P application did not significantly affect plant height and number of branches in rose geranium (*Pelargonium graveolens* L.) (Sedibe and Allemann, 2012). Similar to the present findings, P application increased the chlorophyll content in basil (Ramezani et al., 2009) and African marigold (Rathore et al., 1985) and the LAI (Maurya, 1989) in coriander genotypes. Similarly, the application of monopotassium phosphate at 0 to 3 g L^{-1} in *Rosa multiflora* Thunb. increased leaf chlorophyll content, root dry weight, total root length, fine root length, and surface area (Ma et al., 2021). P application increased root length and root volume, which corroborates the finding in wheat, where increased availability of P due to application of biochar increased root length by 1.4–1.8 times (Song et al., 2020). Likewise, the application of humic acid (75 mg kg^{-1}) and P fertilizer (120 mg P_2O_5 kg^{-1} air-dried soil) in corn recorded higher root volume and other root attributes compared with the control (Purwanto et al., 2021).

A significant influence of biostimulants on agromorphological characteristics was observed, which is comparable to previous studies on lavandin (Erbas et al., 2017), garden thyme (*Thymus vulgaris* L.) (Kwiatkowski et al., 2020), and German chamomile (Mazrou et al., 2021). Similar to the present findings, increased plant height and number of branches in Dutch fennel (*Foeniculum vulgare* Mill.) (Mohamed, 2020) and increased photosynthetic pigments and LAI in basil (Amer et al., 2021) by foliar application of humic acid were observed. The beneficial influence of applying biostimulants on chlorophyll was due to their capacity to provide components, *viz*., betaines, phytohormones, polymers, and nutrients; thus, their synergetic action enhances endogenous cytokinins synthesis, which produced a protective effect on chloroplast (Zavaleta-Mancera et al., 2007) and, accordingly, affected the chlorophyll content.

Yield Attributes and Essential Oil Content and Yield

The crop growth years influenced the yield attributes, which were significantly higher in the second growing year. This might be because of better accumulation of photosynthetically active radiation, which was correlated to improved growth in the second growth year and increased yield attributes. The yield attributes, *viz*., number of flowers, dry weight of flower, and flower diameter, were higher in the second crop growth year because of favorable prevailing weather conditions with reference to precipitation. The precipitation was higher during the second year between March to April, (**Figure 1B**) when plants were in the full flowering stages compared to first year when there was a considerable decrease in rainfall during the same period (**Figure 1A**), which reduced flowering attributes. The favorable precipitation condition in the 2nd year contributed positively to plant yield attributes, as the roots of German chamomile are shallow and capable of taking water from soil when precipitation is higher. At the same time, during the dry spell of the 1st growing year, there was less water absorption that eventually decreased the plant yield attributes. Similar to the present findings, higher rainfall contributed to more flower yield in chamomile in a typical Mediterranean climate with hot, dry summers and cold, humid winters (Guo et al., 2010). The yield attributes, *viz*., number of flowers per plant, flower diameter, disc floret height, dry flower yield, and EO yield, were significantly higher with P at 90 kg ha^{-1} that with the control. The higher yield attributes with P application were due to P's involvement in the floral structure development (Ma et al., 2001) of German chamomile, and similar improvement in floral characteristics was reported in lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) (Erbas et al., 2017), *Calendula officinalis* (Steward and Lovett-Doust, 2003), *Rosa multiflora* (Ma et al., 2021), and *Pimpinella anisum* (Meena et al., 2015). The highest dry flower and EO yields were produced with 90 kg ha^{-1} of P application (**Table 3**), because P influences several metabolic measures such as photosynthesis, chlorophyll content, carbohydrate, protein, and oil syntheses, thus contributing in EO yield increase (Erbas et al., 2017). Corroborating the current findings, P application increased the yield attributes, *viz*., seeds number per umbel, 1,000-seed weight, seed yield, and EO content in anise (*Pimpinella anisum* L.) (Sonmez, 2018), and EO yield in German chamomile with 150 kg ha^{-1} of phosphate fertilizer (TSP) with zinc application (Jeshni et al., 2015). On the contrary, the application of higher doses of P fertilizer (300 kg ha^{-1}) showed a negative influence on German chamomile yield because the higher P application induced Zn deficiency in soil, thus creating an imbalance of nutrient supply (Jeshni et al., 2015). Moreover, the application of other nutrient fertilizers like N (Emongor and Chweya, 1992; Guo et al., 2010) also affected the performance of German chamomile. The application of NPK at 100:60:40 kg ha^{-1} recorded the highest growth and yield attributes of German chamomile (Gandomi et al., 2021). The application of NPK at 100:60:40 kg ha^{-1} recorded the highest growth and yield attributes of German chamomile (Upadhyay et al., 2016). Likewise, the application of micronutrients, i.e., zinc fertilizer ($ZnSO_4H_2O$) at 30 kg ha^{-1} and irrigation at 50% of field capacity, improved the EO yield and EO components of German chamomile (Jeshni et al., 2015). Additionally, the application of N fertilizer and the cultivation of legume stubble catch crops improved the performance of German chamomile. The cultivation of Dukat (*Anethum graveolens* L.) decreases fertilizer rate from 90 to 60 kg N ha^{-1} without decreasing anthodium yield. EO yield was highest after applying 60 kg N ha^{-1} (Andrzejewska and Woropaj-Janczak, 2014). In the present findings, biostimulant application significantly increased yield attributes, i.e., number of flowers, dry flower weight, and EO yield. Previous studies on humic acid influence were related to increased nutrient absorption and translocation, which facilitated the stimulation of plasma membrane H^+-ATPases by transferring released free energy by ATP hydrolysis, thus generating electrochemical potential through the membrane and supplied nutrients (Jardin Du, 2015). Additionally, the ATPase membrane pump causes cell wall loosening and elongation, and expansion of cells, resulting in increased growth and chlorophyll content in plants (Abdelgawad et al., 2020). Moreover, humic acid is also known to induce modifications in gene expression and metabolites (primary and secondary) of plants (Baia, D. C., et al., 2020), which are involved in a variety of physiological processes (such as photosynthesis, metabolism, and Krebs cycle) (Carletti et al., 2018; Sofi et al.,

2018). In ajowan, the application of humic acid with a chemical fertilizer recorded the highest EO content and main chemical constituents including thymol and *p*-cymene content (Chiyaneh et al., 2022). The increase in EO yield in German chamomile by the humic acid application may be due to stimulation of metabolic reactions and some enzymatic responses which are accountable for biosynthesis of EO and its constituents (Burbott and Loomis, 1969). Additionally, humic acid is a hormone-like substance and increases the levels of other internal plant hormones, *viz*., auxin, cytokinin, and gibberellin, which stimulate the division and elongation of plants cells (Abdel-Mawgoud et al., 2007) and improve plant growth (Abdel-Mawgoud et al., 2007). Applying supplementary biostimulants through amino acids facilitates the defense mechanisms in plants, prevents water loss, stimulates photosynthesis, determines metabolic processes' pace and path, and regulates internal enzymes and hormones (Kocira et al., 2015), which might have contributed to the improved characteristics of German chamomile compared to the control.

Determination of Essential Oil Components

The crop growing years also showed a significant effect on EO constituents where (Z)-β-farnesene, germacrecene D, and en-in-dicycloether were higher in 2019–2020, while α- bisabolol oxide B, bisabolone oxide A, chamazulene, and α-bisabolol oxide A were higher in 2018–2019. In the present findings, α-bisabolol oxide A was the major EO constituent, followed by α-bisabolol oxide B and (Z)-β-farnesene in both growing years, which was in accordance with previous reports on German chamomile (Sharafzadeh and Alizadeh, 2011). During both years, some quantitative and qualitative differences in some components (α-bisabolol oxide A, α-bisabolol oxide B, and (Z)-β-farnesene) were observed, but the components did not lose their main characteristic, that is, richness. Even the concentrations of the three main components significantly differed according to variations in the weather parameters of both years but were close to each other. Similar to the present findings, a wide range of variability was observed in quality traits of fennel during growing years, as the studied traits might be influenced by environmental conditions (Lal, 2008). The EO production in aromatic crops may be influenced in a positive or negative manner by the amount and type of fertilizers (Ramezani et al., 2009; Said-Al Ahl et al., 2016). Similarly, P fertilization affected the composition of EO differently in lavender (Chrysargyris et al., 2016) and fennel (*Foeniculum vulgare* Mill.) (Kapoor et al., 2004), but there was no effect on sage (*Salvia officinalis* L.) (Rioba et al., 2015) and sweet basil (Chimura et al., 1993). In the present study, the major EO constituents were (Z)-β-farnesene, germacrecene D, α-bisabolol oxide B, bisabolone oxide A, α-bisabolol oxide A, and en-in-dicycloether, while artemisia ketone and chamazulene were reported as minor constituents. Earlier studies had also reported a similar EO profile of German chamomile with bisabolol oxide A as a major EO constituent (Orav et al., 2010; Can et al., 2012).

The EO composition of German chamomile in the present findings was in accordance with the standard ISO 19332:2008, which described α-bisabolol oxide A as 35–50% and chamazulene as 2–5% compound concentration in the EO supply. In aromatic crops, P is the primary macronutrient of plants and has an imperative function in the synthesis and assimilation of EO, where farnesyl pyrophosphate (FPP) is compressed to geranyle diphosphate (GPP) and linalool diphosphate, which are the precursor compounds of EO production (Sangwan et al., 2001). In the present study, the application of P at 90 kg ha^{-1} recorded significantly higher chamazulene and en-in-dicycloether, which is similar to the finding of Mikhak et al. (2017). On the other hand, chamazulene content was higher in the control plots (no phosphorus) but lower in the P treatments (Ubessi et al., 2021). Similar to the present findings, higher α- bisabolol oxide B and α-bisabolol oxide A content in control was also reported by Ubessi et al. (2021), while increase in α- bisabolol oxide B and α-bisabolol oxide A content was recorded with increase in P applications (Karami et al., 2006; Jeshni et al., 2015). According to the European Pharmacopoeia, there are two types of EO, one is rich in bisabolol oxides (between 29 and 81%) and the other in α-bisabolol (between 10 and 65%). Similarly, the EO constituents, *viz*., (Z)-β-farnesene, germacrecene D, chamazulene, α-bisabolol oxide A, and en-in-dicycloether were significantly higher in the humic acid application, while α-bisabolol oxide B and bisabolone oxide A were significantly higher in the control and amino acid applications. Comparable to the present findings, earlier studies also recorded variations in the chemical constituent profile of chamazulene and bisabolol contents with application of biostimulants, *viz*., salicylic acid (Ghasemi et al., 2016; Rathore and Kumar, 2021), in German chamomile. Moreover, the use of certain biostimulants such as humic acid, glycyrrhizic acids, salicylic acid, and their nanocomplexes in basil (*O. basilicum* L.) influences the biochemical attributes (Amer et al., 2021). These biostimulants may change the secondary metabolites' pathway, affect the plastid and chlorophyll levels and tolerance to stress conditions, and ultimately result in manipulation of the quantity and quality of EO (Ahmed, A. M., 2014). Humic acid improves nutrient accessibility for plants and is essential for the development and division of glandular trichomes, secretory ducts, and EO channels (Salehi et al., 2019). Moreover, the application of a biostimulant may be related to nutrient availability, and its direct role in plants by foliar application enhances the photosynthetic activity of enzymes and precursors of EOs, counting isoprenes, and phenylpropanes (Rehman et al., 2016). Rezaei-Chiyaneh et al. (2021) reported that biostimulant application in aromatic crops, *viz*., black cumin (*Nigella sativa* L.) and fenugreek (*Trigonella foenum-graecum* L.), enhanced EO quantity and quality. Additionally, EO profile varies according to climate, geographical features, and date of collection (Swamy and Sinniah, 2015). Accordingly chamazulene, bisabolone oxide, (α)-bisabolol, β-farnesene, bisabolol oxides A and B, and en-in-dicycloether were the major EO constituents of EO with biostimulant application under the lower altitude conditions of Iran (Ghasemi et al., 2016), while α-bisabolol oxide A, α-bisabolol oxide B, cis-β-farnesene, and bisabolone oxide were major constituents under the arid saline conditions of Egypt (Omer et al., 2013). The use of amino acid hydrolysates was also studied on maize and showed auxin-like activity and improved crop performance (Ugolini et al., 2014). The application of amino acids decreased chamazulene content and increased α-bisabolol oxide A content in German chamomile

(El-Din and Abd El-Wahed, 2005). Other findings on amino acid application recorded higher α-bisabolol oxide B, α-bisabolol oxide A, and bisabolone oxide at higher doses, while at lower doses of application, (Z)-β-farnesene was higher (Omer et al., 2013). The present study can represent a facet for the accomplishment of sustainable means in German chamomile cultivation, particularly by taking into consideration the possible measures of reducing expenses on chemicals and increasing the opportunities for long-term sustainable plant nutrition by integrating chemical and sustainable sources of organic origin for crop production. The results validated that utilization of biostimulants directly increases the yield and influences the EO profile of German chamomile, which is particularly significant to farmers and straightforwardly indicates monetary benefits. Successively, from the customers' viewpoint, it is now more essential that the execution of this agronomic practice in the field offers their produce with an improved therapeutic and industrial perspective. The findings of the present study showed that German chamomile cultivation could be effectively initiated with the combination of P fertilization and biostimulant application under the acidic soil conditions of the western Himalayas. The hypothesis assumes that P and biostimulant application in acidic soils is fully justified considering German chamomile production and the economic concerns of growers.

CONCLUSION

The present study aims to improve the availability of P to plants by applying a readily available source of P fertilizer in combination with foliar biostimulant applications for direct absorption by German chamomile plants. The results suggested that the combination of a synthetic P nutrient source and biostimulants can positively affect plant growth, flower yield, and EO composition. According to research, optimal utilization of P fertilizer and biostimulants improved the performance of German chamomile concerning flower production, biomass of flowers, and EO yield. The present research suggested that the utilization of P fertilizers and plant-based biostimulants is efficient for crop improvement in acidic soils, thus contributing to increased yield goal of growers. Considering the different reactions of P and biostimulants on agronomic and quality traits of German chamomile under stressed acidic conditions, it is recommended that these can be utilized in acidic soils of high rainfall regions. The lowest chamazulene and bisabolone oxide A content was obtained with the control, which means that when the availability of P is low, the yield attributes and chamazulene and bisabolone oxide A content will severely decrease. Similarly, the biostimulants also increased the content of chamazulene, α-bisabolol oxide A, (Z)-β-farnesene, and en-in-dicycloether in the EO of German chamomile. Detailed understanding of the effects of P and biostimulants will significantly provide benefits to various cropping systems and eventually lead to improved environmental steadiness by increasing crop yield and quality and reducing chemical inputs slowly and steadily for long-term benefits.

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

SR: data curation, methodology, visualization, validation, investigation, formal analysis, software, and writing—original draft and review and editing. RK: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Induction of heartwood formation in young Indian sandalwood (*Santalum album* L.) by gas elicitors

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Induction of heartwood formation in 6-year-old Indian sandalwood (*Santalum album* L.) trees by treatment with carbon dioxide, ethylene, nitrogen, and wounding was investigated. All treatments induced fragrant heartwood formation upward and downward from the drill hole. The amount of heartwood formed above and below the drill hole depended on the treatment in the order nitrogen>carbon dioxide>ethylene>wounding, whereas the radial extension proportion was, in order, nitrogen>carbon dioxide>ethylene=wounding. Based on the chemical analysis (GC–MS) and evaluation of the essential oil quality and heartwood properties, heartwood induced by carbon dioxide showed the maximum similarities to naturally formed heartwood, which included the same color, similar chemical composition, reasonable oil content, and quality essential oil, whereas ethylene, nitrogen, and wounding treatment showed fewer similarities to natural heartwood. The results suggest that carbon dioxide is a promising candidate gas elicitor for inducing heartwood formation in young *S. album*.

KEYWORDS

carbon dioxide, ethylene, heartwood formation, gas elicitor, *Santalum album*

Introduction

In most hardwood tree species, the trunk wood can be divided into living sapwood and dead heartwood. Heartwood lies in the inner layers of the secondary xylem, has ceased to contain living cells, and is characterized by the accumulation of extractives (oils, phenols, gels, pigments, other heartwood substances, etc.). Sapwood contains living cells (ray parenchyma and axial parenchyma) and is characterized by a high content of reserve materials (Anon, 1957; IAWA, 1964; Galibina et al., 2020). These differences in composition result in the significant variations in color, density, durability, odor, moisture content, and permeability (Taylor et al., 2002; Tae et al., 2017). As a result, the applications as well as economic values of heartwood and sapwood differ markedly (Johannes and Peer, 2008; Miranda et al., 2017; Tae et al., 2017; Cui et al., 2020).

Sandal (*Santalum album* L.), commonly known as East Indian Sandalwood, is the most famous valuable tree species belonging to the Santalaceae family and is highly valued for its fragrant heartwood, which has various applications in the perfumery, religion, cosmetic, wood carving, and pharmacological industries

(Nicolas et al., 2011; Misra and Dey, 2013; Haque and Coury, 2018; Mohankumar et al., 2019; Pullaiah et al., 2021). *S. album* was first introduced into China in 1962 (Li, 2003) and has exhibited encouraging growth performance in most planting areas in the recent decades in southern China (Liu et al., 2011, 2016). However, very few sandal plantations formed aromatic heartwood naturally when they were young (<10 years old), and the quality of essential oil distilled from those young sandals was quite poor (Liu et al., 2011, 2012). Therefore, induction of fragrant heartwood formation in young sandalwood plantations has great importance in large-scale cultivation practices because the values of sandalwood at harvest will depend largely on the volume and the quality of heartwood. Several studies have shown that some chemical elicitors, such as $CuSO_4$ (Kadambi, 1954), ethrel (Li and Chen, 1994), paraquat (Radomiljac, 1998), benzyladenine (Liu et al., 2013), and H_2O_2 (Li et al., 2021), can induce heartwood formation in young sandals. However, all these chemical elicitors were used as liquid solutions, and the induced heartwood was generally formed around the injection holes; moreover, the amount was quite low.

Stem gases play the important roles related to many physiological or metabolic processes in trees, such as aerobic respiration, the tricarboxylic acid cycle, secondary metabolism, and heartwood extractive accumulation, which can further regulate heartwood formation (Carrodus, 1967; Eklund, 2000; Spicer and Holbrook, 2007; Johannes and Peer, 2008). For instance, when branches from *Acacia mearnsii* containing only sapwood were exposed to a carbon dioxide (CO_2) atmosphere, heartwood flavonoids specific for *A. mearnsii* were induced (Carrodus, 1971). Stem filling with ethylene (C_2H_4) can induce discolored heartwood formation in *Pinus sylvestris*, and the chemical composition of induced the heartwood was quite similar to that of naturally formed heartwood (Nilsson et al., 2002). Johannes and Peer (2008) noted that oxygen could cause red heartwood formation in European beech (*Fagus sylvatica*) by affecting the activities of microorganisms in standing stems and suggested that there is a threshold for oxygen concentration to trigger red heartwood formation.

Compared to liquid elicitors, gaseous elicitors seem to induce a larger amount of heartwood because pressurized gases can permeate almost everywhere in stems or branches (Nilsson et al., 2002). Therefore, it is necessary to test whether gas elicitors can induce fragrant heartwood formation in young sandals. The aim of this study was to determine whether and how gaseous elicitors (CO_2, C_2H_4, and N_2) influence fragrant heartwood formation in young sandal.

Materials and methods

Experimental site and tree selection

The field experiment was conducted in a sandalwood experimental and demonstration base of the Research Institute of Tropical Forestry, Chinese Academy of Forestry, which is located approximately 15 km north of Zhaoqing City, Guangdong Province, southern China (112°36′ E, 22°54′ N). The mean temperature is 23°C, and the annual rainfall is 1,700 mm. The plantation was first established in April 2004 in a laterite soil type with a sandalwood spacing of 3 × 3 m, and the main hosts were *Caesalpinia sappan* L. (Caesalpiniaceae), *Clausena lansium* (Lour.) Skeels (Rutaceae), *Ligustrum lucidum* Ait. (Oleaceae), and *Murraya exotica* L. (Rutaceae), one of which was planted between two sandalwoods with a spacing of 3 × 3 m. A preliminary study showed that this area was suitable for sandalwood growth and that the trees formed heartwood naturally at an early age (Liu et al., 2011).

A total of thirty sandals (*S. album*) without natural heartwood formed were selected randomly from a 6-year-old sandalwood plantation planted in the above sandalwood experimental and demonstration base. The presence of natural heartwood was determined by the color and odor of wood chips drilled through the central bottom of each sandal using a hand drill. The average height was 5.14 ± 0.43 m, and the average diameter on breast height (DBH) was 8.52 ± 0.41 cm. The selected trees were then divided into three gas treatment groups, one wounding group and one control group, and each treatment consisted of 6 individuals.

Gas treatment

Holes measuring 10 mm in diameter were drilled (without drilling through the trunk) using a hand drill at 1-m height above ground. The depth of each hole was calculated as the diameter minus 1 cm (Figure 1). After drilling, a rubber stopper equipped with a stainless steel tube was sealed on the entrance of each hole, so that a specific environment based on the gas treatment was created through the stem of each sandal, and thus, the volume of each hole was calculated. Drilling and tube insertion were conducted in April, so that the wounds had enough time to heal before treatment began in September, just before the onset of heartwood formation. Pressurized gases filled in different steel cylinders were released by a pressure reducing valve to maintain a steady flow (the air pressure was ~0.2 MPa), and a flowmeter equipped with a metal clip was placed between the valve and the rubber stopper to control the volume of gas filling the stem (Figure 1). Before filling, we opened the rubber stopper and released the corresponding gases from deep in each hole for 5 min to ensure that the residual gases were exhausted. Then, we closed the rubber stopper and sealed it with paraffin. A total of two volumes of gases (determined and controlled by a flowmeter and a digital timer) were filled into each hole and then stopped with a metal clip. Preliminary tests with ethylene showed that ethylene-filled trunks can still maintain a 15% concentration of the gas after 7 days, so this procedure was repeated one time a week for 1 year. A total of three types of gases were tested in this experiment: carbon dioxide (99.999%, CO_2), ethylene (99.9%,

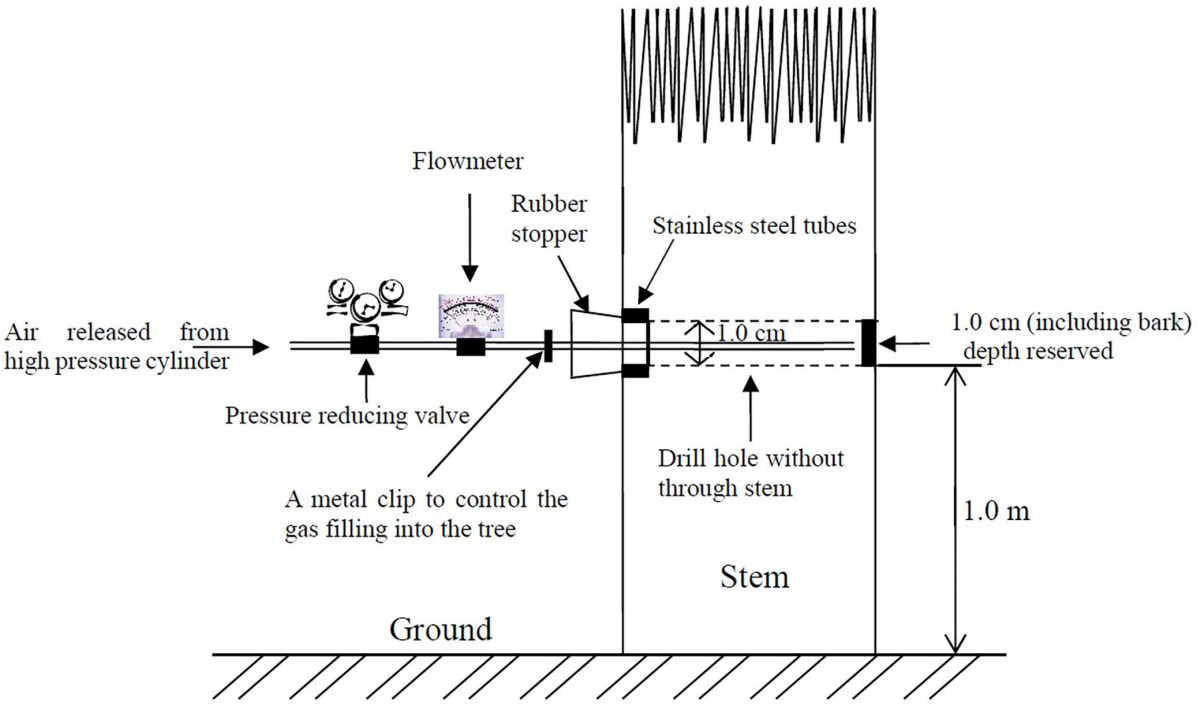


FIGURE 1
Schematic map for treatment of sandal with different gases.

C_2H_4), and nitrogen (99.999%, N_2). Nitrogen was applied to create a hypoxic environment, wounding group consisted of sandals that remained untouched during the whole experiment after the holes drilled, and another control group kept the same (without drilling hole), just to observe whether heartwood formed naturally during the experiment.

Sample collection and heartwood extension determination

An increment borer (Haglof, C300, 5.15 mm inner diameter, Sweden) was applied to draw two core samples from each tree at approximately 1 cm above the hole and at right angles to each other. The presence of heartwood and the length of heartwood and sapwood were determined and recorded. To determine the maximum extension distance of heartwood in each tree, we repeated the core sample collection procedure every 5 cm above or below the hole until no heartwood was observed. Using the above data, the whole extension area upward and downward from the hole can be simulated by drawing software (SigmaPlot, Version 13.0). All the cores were sealed separately with parafilm and then stored in a cooled insulated container before being transported to the laboratory, where the essential oil content and oil composition were determined.

Heartwood property determination

The basic density and relative water content of the induced heartwood were determined immediately after core sample collection. The basic density (bone-dry weight per unit of fresh volume) was determined using the water displacement method, and the dry weight was obtained after drying at 105°C to constant weight. The water content (WC, express as percentage) was calculated as the percentage of water content per fresh weight (Searle and Owen, 2005).

Essential oil extraction and content determination

Sapwood was removed from the collected cores using a knife, and the remaining induced fresh heartwood was weighed (FHW) and then ground into fine particles. Essential oil was extracted from the heartwood powder using the solvent (diethyl ether) extraction method (Howes et al., 2004). The diethyl ether solution was dried over anhydrous sodium sulfate and evaporated on a rotary evaporator under reduced pressure to give a yellow oil, and then, the weight of oils for each individual was measured separately (OW). Finally, the essential oil content was calculated by the dry weight (oil content of each dry heartwood, DW) and fresh weight (oil content of each fresh

heartwood, FW) separately according to the following formula (Haffner, 1994):

$$\text{Essential oil content (DW)\%} = \frac{\textit{Oil weight } (OW)}{\textit{Fresh heartwood weight } (FHW) \times (100 - WC)} \times 100$$

$$\text{Essential oil content (FW)\%} = \frac{\textit{Oil weight } (OW)}{\textit{Fresh Heartwood weight } (FHW)} \times 100$$

where:

WC = water content of the induced heartwood.

Essential oil composition determination

The essential oil composition extracted from the induced heartwood was determined by gas chromatography–mass spectrometry (GC–MS) according to the methodology by Howes et al. (2004) and Liu et al. (2011). The solvent (diethyl ether) was obtained from Aldrich, China. The GC–MS analysis was performed on a Finnigan TRACE GC-2000-MSTM (USA) instrument equipped with a DB-5 column (Agilent, USA, 30 m length, 0.25 mm inner diameter, and 0.25-μm film thickness). The injection port temperature was 220°C, and the oven initial temperature was 45°C. The oven temperature was ramped from 45°C at 3°C /min to 220°C and held for 5 min (total run time 63 min). The carrier gas was helium with a flow rate of 1.0 ml/min, and no split was used. All oils were diluted to 1.0% (v/v) with diethyl ether prior to analysis. The total injection volume was 1 μl. The mass spectra were fitted with an EI source operated at 70 eV with a source temperature of 200°C, and the mass spectra were recorded in the range m/z 35–335 amu at 1 scan/0.75 s. Each compound (peak) was identified either from retrieving the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) standard mass spectra libraries (NIST2005, NIST2005s, NIST2014, and NIST2014s) or by comparing retention indices and/or mass spectra with published data (Verghese et al., 1990; Howes et al., 2004; Shellie et al., 2004; Jones et al., 2006; Sciarrone et al., 2011). The relative content of each compound was estimated according to the relative area through an area normalization method.

Statistical analysis

Analysis of variance (ANOVA) was performed on each dataset using the statistical program SPSS 18.0 (SPSS I, 2004) after examining the data graphically to confirm normality of distribution and homogeneity of variances. Duncan's multiple range test was used to compare the differences between treatment means ($\alpha = 0.05$).

Results

Heartwood formation and extension

All treated sandals formed fragrant heartwood 1 year after treatment (Figure 2), but the longitudinal extension range (estimated by the distance upward and downward from the drill hole), radial extension proportion (expressed as the maximum width of heartwood relative to the whole wood), and heartwood color differed greatly among treatments (Figures 2, 3). The greatest upward and downward extensions of induced heartwood from the drill holes were observed in trees treated with nitrogen (N_2), which showed the significant differences when compared to the other treatments (Figure 3, $p < 0.05$), and the corresponding extension ranges were 16 and 17 cm, respectively. The maximum radial extension proportion of induced fragrant heartwood was 56% on average; however, the color of induced heartwood seems to be paler or lighter than that of naturally formed heartwood (Brand et al., 2012) (Figure 2). Carbon dioxide (CO_2) induced less extended heartwood than N_2, the corresponding upward and downward extension ranges were 11 and 12 cm, respectively, and the radial extension proportion of induced heartwood was 45% on average, but the color of induced heartwood was quite similar to that of naturally formed heartwood. The ethylene (C_2H_4) and wounding treatments induced the least amount heartwood, and the upward and downward extensions from the drill holes were quite similar (~7–8 and 4–5 cm, respectively). The radial extension proportions of C_2H_4-induced and wounding-induced heartwood were 24 and 22%, respectively. The amount of fragrant heartwood induced by C_2H_4 was slightly higher than that induced by wounding in both the longitudinal extension range and radial extension proportion; however, no significant difference was observed (Figure 3). The color of heartwood induced by C_2H_4 was slightly darker than that of naturally formed heartwood, but the wounding treatment exhibited quite similar color to the naturally formed heartwood of sandal (Figure 2). However, no heartwood was observed in all the control group at the same height (Figure 2, CK), which may indicate that 7-year-old sandalwood can hardly form heartwood naturally at the height of 1 m, and thus, the following indices such as heartwood properties, essential oil content, and oil composition cannot be determined and compared in this study.

Heartwood properties

The results of the different gas treatments on the properties of induced heartwood are shown in Table 1. The ANOVA results showed that there was no significant difference among the gas treatments in either the water content ($p = 0.747$) or basic density ($p = 0.996$) of the induced heartwood in young sandals



FIGURE 2
Core samples induced by different gas treatments (CO_2, wounding, C_2H_4, and N_2). Cores were taken from 1 cm above the drill hole; CK corresponds to the control group of young sandals at the same height. Ruler = 10 cm.

($\alpha = 0.05$). Treatment with C_2H_4 resulted in the highest water content and basic density (Table 1).

Essential oil content

Great differences were observed in the essential oil content ($p = 0.003$) of the induced heartwood among the gas treatments according to the ANOVA, and the color of the extracted essential oil was different in shade (Figure 4). Treatment with C_2H_4 resulted in a statistically higher content than all the other treatments (Figure 5), and the corresponding essential oil contents were 13.11% in fresh weight and 17.25% in dry weight. The CO_2 and N_2 treatments induced less essential oil content than the treatment with C_2H_4, and the essential oil content did not differ significantly from each other. The wounding treatment resulted in the lowest essential oil content, only 2.26% in fresh weight and 5.02% in dry weight.

Essential oil composition

The GC–MS analysis revealed that 38 peaks were detected in all the essential oils extracted from heartwood induced by the

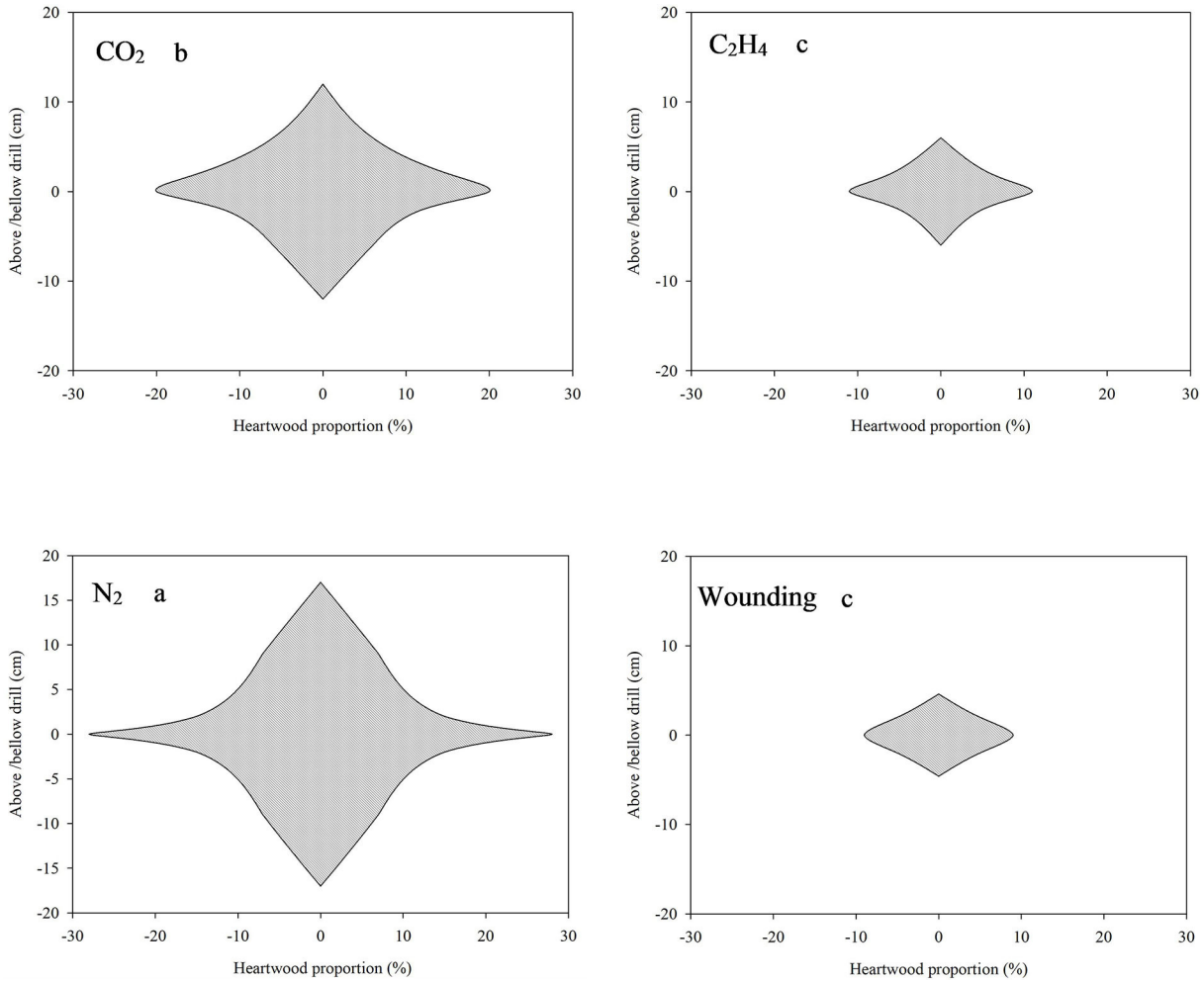


FIGURE 3
Longitudinal and radial extension of heartwood induced by different gas treatments. Longitudinal extension is expressed as the distance above/below the drill hole (cm), and radial extension proportion is expressed as the width of induced heartwood relative to the whole wood (%). Different letters indicate significant differences at $p < 0.05$.

TABLE 1 Effects of different gas treatments on wood property of induced heartwood in young sandals*.

Treatments	Water content (%)	Basic density (g.cm^{-3})
CO_2	27.40 ± 1.87a	0.587 ± 0.080b
C_2H_4	30.95 ± 4.35a	0.603 ± 0.027b
N_2	28.04 ± 8.94a	0.595 ± 0.087b
Wounding	26.61 ± 6.03a	0.592 ± 0.146b

*Data are shown as means ± standard deviation. Values with the same letters are not significantly different from each other according to Duncan's multiple comparison (α = 0.05).

different gas treatments, of which 31 components were identified and confirmed. Details on the constituents identified from the essential oils treated with the different gases are listed in Table 2, and the total ion chromatogram of each treatment is shown in Figure 6. Significant differences ($p < 0.01$) were observed among the different gas treatments in the content of the main characteristic compounds of the essential oil *viz.* α-santalene, α-bergamotene, epi-β-santalene, β-santalene, α-santalol, and β-santalol according to the ANOVA (Table 2, Figure 6).

Treatment with C_2H_4 obtained the highest number of identified ingredients (29 constituents) from the essential oil, followed by treatment with CO_2 and N_2, both of which had 25 identified constituents. The wounding treatment obtained the lowest number of identified ingredients with only 23 components identified. Although great variations in chemical composition and relative content were observed among the different treatments, the most dominant compounds in all gas treatments were found nearly in the same order: α-santalol > β-santalol > α-*trans*-bergamotenol > epi-β-santalol (Table 2, Figure 5).

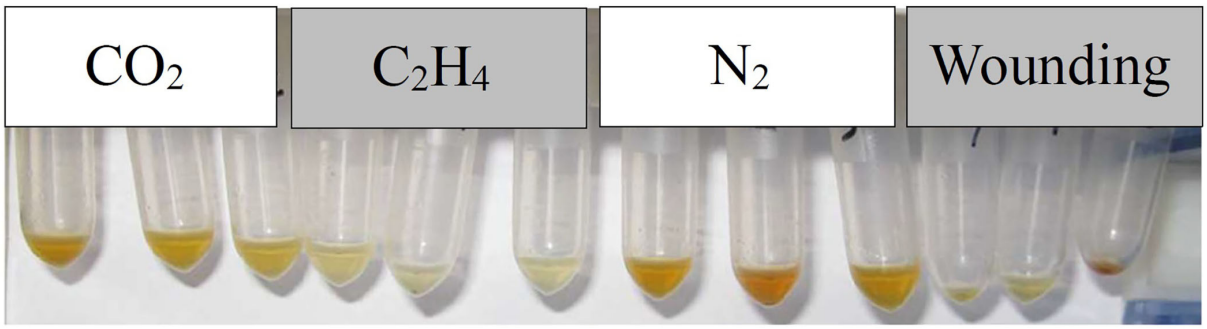


FIGURE 4
Essential oil extracted from heartwood induced by different gases in young sandals.

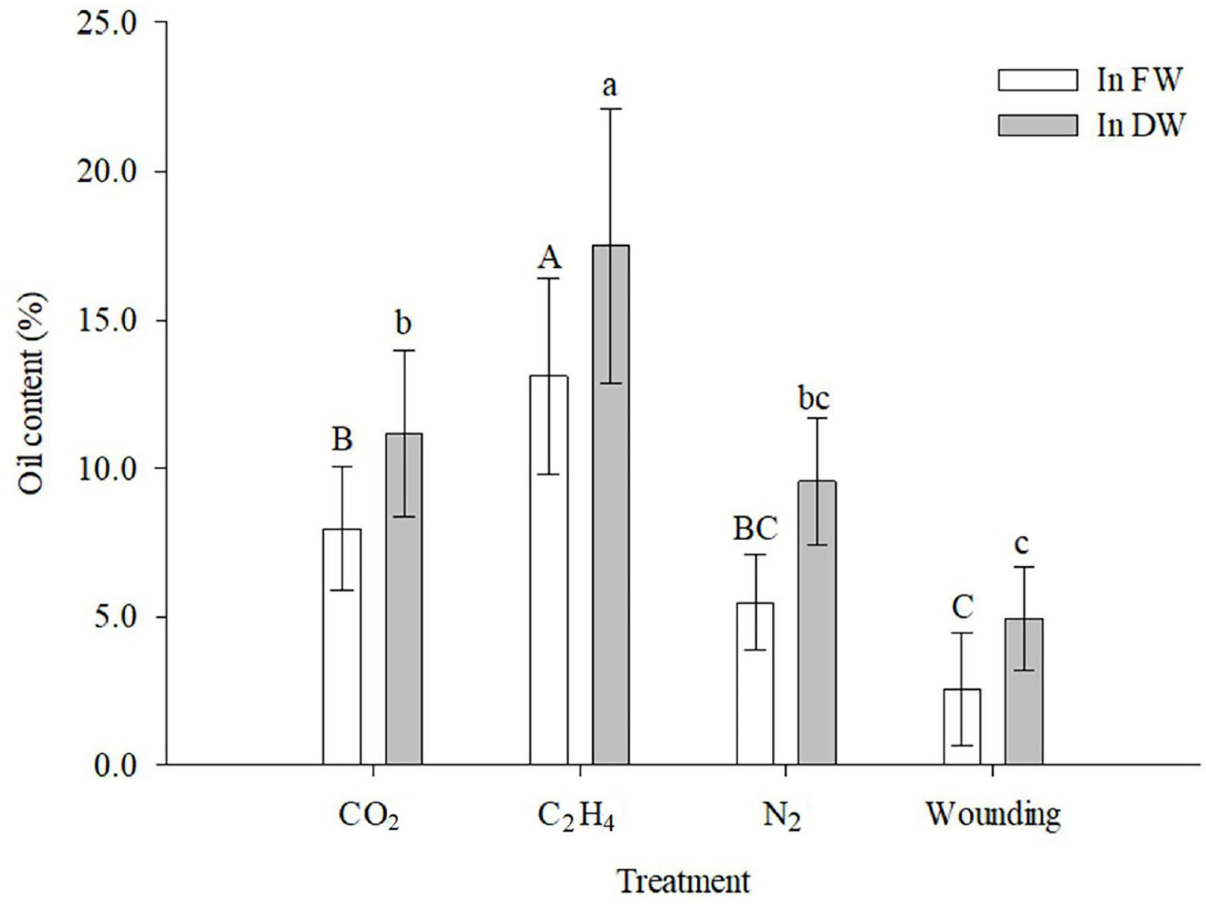


FIGURE 5
Effects of different gas treatments on essential oil content. Data are shown as means ± standard deviation. Values with the same superscript letters are not significantly different according to Duncan's multiple comparisons test ($\alpha = 0.05$).

The quality of the sandalwood essential oil was characterized by the presence of a high content of sesquiterpene alcohols and the corresponding sesquiterpenes. The ANOVA results showed that there were significant differences in the contents of total santalols ($p = 0.015$), total santalenes ($p = 0.003$), total sesquiterpenes ($p = 0.039$), and total sesquiterpene alcohols ($p = 0.002$). Treatment with CO_2 was most favorable for santalol and sesquiterpene alcohol synthesis, whereas treatment with

TABLE 2 Chemical composition of essential oil extracted from heartwood induced by differnet gas treatments.

Retention time (min)	Compound name	Molecular formula	Relative content(%)			
			CO_2	C_2H_4	N_2	Wounding
9.45	Isopropyl acetate*	$C_5H_{10}O_2$	1.83 ± 1.45b	4.56 ± 3.66a	1.14 ± 0.56b	–
9.96	3-Ethoxy-2-butanone*	$C_6H_{12}O_2$	0.70 ± 0.34b	2.13 ± 1.75a	0.48 ± 0.23b	0.37 ± 0.45b
11.01	Acetal*	$C_6H_{14}O_2$	0.71 ± 0.36b	2.04 ± 1.57a	0.47 ± 0.22b	0.32 ± 0.44b
19.06	Nononal	$C_9H_{18}O$	–	0.37 ± 0.36	0.13 ± 0.06	0.14 ± 0.07
19.75	Glycerol	$C_3H_8O_3$	0.75 ± 0.71	0.83 ± 0.79	0.32 ± 0.16	–
24.00	Decanal	$C_{10}H_{20}O$	0.06 ± 0.01	0.14 ± 0.07	0.10 ± 0.06	0.17 ± 0.17
27.89	n-Docosane	$C_{22}H_{46}$	0.07 ± 0.04	0.31 ± 0.11	0.10 ± 0.07	0.25 ± 0.18
28.68	n-Eicosane	$C_{20}H_{42}$	0.19 ± 0.08	0.83 ± 0.31	0.27 ± 0.20	0.68 ± 0.47
30.29	α-Sinensal	$C_{15}H_{22}O$	–	0.62 ± 0.38	–	–
33.12	Dodecanal*	$C_{12}H_{24}O$	0.18 ± 0.12c	1.21 ± 0.52a	0.80 ± 0.59b	0.74 ± 0.42b
34.13	α-Santalene*	$C_{15}H_{24}$	0.89 ± 0.14ab	1.26 ± 0.36a	0.55 ± 0.19bc	0.42 ± 0.07c
34.77	α-Bergamotene*	$C_{15}H_{24}$	0.93 ± 0.12a	1.02 ± 0.18a	0.43 ± 0.11b	0.31 ± 0.04b
35.13	Epi-β-Santalene*	$C_{15}H_{24}$	0.83 ± 0.16a	1.17 ± 0.31a	0.44 ± 0.13b	0.39 ± 0.05b
35.64	β-Santalene*	$C_{15}H_{24}$	1.25 ± 0.24ab	1.80 ± 0.49a	0.72 ± 0.21bc	0.53 ± 0.04c
36.37	α-Curcumene	$C_{15}H_{22}$	–	0.47 ± 0.20	–	–
37.34	2,4-Di-tert-butylphenol*	$C_{14}H_{22}O$	0.29 ± 0.23b	1.41 ± 0.75a	–	1.17 ± 0.81a
38.98	Teresantalol*	$C_{10}H_{16}O$	1.05 ± 0.51a	1.18 ± 0.64a	0.91 ± 0.28a	0.43 ± 0.20b
39.66	Lauric acid hydride*	$C_{24}H_{46}O_3$	0.75 ± 0.47b	1.47 ± 0.85a	0.78 ± 0.16b	0.88 ± 0.70b
40.00	Farnesol	$C_{15}H_{26}O$	0.54 ± 0.18	0.79 ± 0.37	0.57 ± 0.25	0.56 ± 0.15
43.64	α-Santalol*	$C_{15}H_{24}O$	43.09 ± 2.10a	31.05 ± 5.03b	37.56 ± 1.30a	38.09 ± 2.91a
44.13	*α-trans*-Bergamotenol	$C_{15}H_{24}O$	6.65 ± 0.91	5.91 ± 2.58	6.84 ± 0.71	5.25 ± 1.36
44.44	Epi-β-Santalol	$C_{15}H_{24}O$	3.92 ± 1.13	2.38 ± 0.25	3.81 ± 0.17	3.46 ± 0.14
44.97	β-Santalol*	$C_{15}H_{24}O$	18.91 ± 2.73a	12.67 ± 3.57b	16.88 ± 2.32ab	16.39 ± 0.20ab
45.19	Heptacosane	$C_{27}H_{56}$	–	1.25 ± 0.69	–	–
46.45	*cis*-Lanceol	$C_{15}H_{24}O$	–	1.48 ± 0.33	–	–
51.97	Oxabicyclo[4.1.0]heptane,2,2,6-trimethyl-1-(3-methyl-1,3-butadienyl)-5-methylene-	$C_{15}H_{22}O$	2.36 ± 0.29	1.60 ± 1.46	3.08 ± 1.16	2.77 ± 1.70
53.69	Hexadecanic acid	$C_{16}H_{32}O_2$	2.48 ± 0.69	5.35 ± 3.83	3.58 ± 1.25	3.32 ± 0.86
56.60	Dioctyl phthalate	$C_{24}H_{38}O_4$	–	–	–	0.25 ± 0.20
58.48	Linolelaidic acid-methyl ester	$C_{19}H_{34}O_2$	0.32 ± 0.31	–	0.28 ± 0.15	–
58.86	*trans*-9-Octadecenoic acid	$C_{18}H_{34}O_2$	0.64 ± 0.39	1.34 ± 0.67	0.91 ± 0.24	–
59.68	Octadecanoic acid	$C_{18}H_{36}O_2$	0.81 ± 0.18	2.75 ± 1.09	1.47 ± 0.49	1.02±0.38
Total			90.23	89.38	82.27	77.91

Data are shown as means ± standard deviation. "–" means trace amount or not detected. Constitutes with "*" mean that there exists statistically different among all the gas treatment, and values with the same letters are not significantly different from each other according to Duncan's multiple comparison ($\alpha = 0.05$).

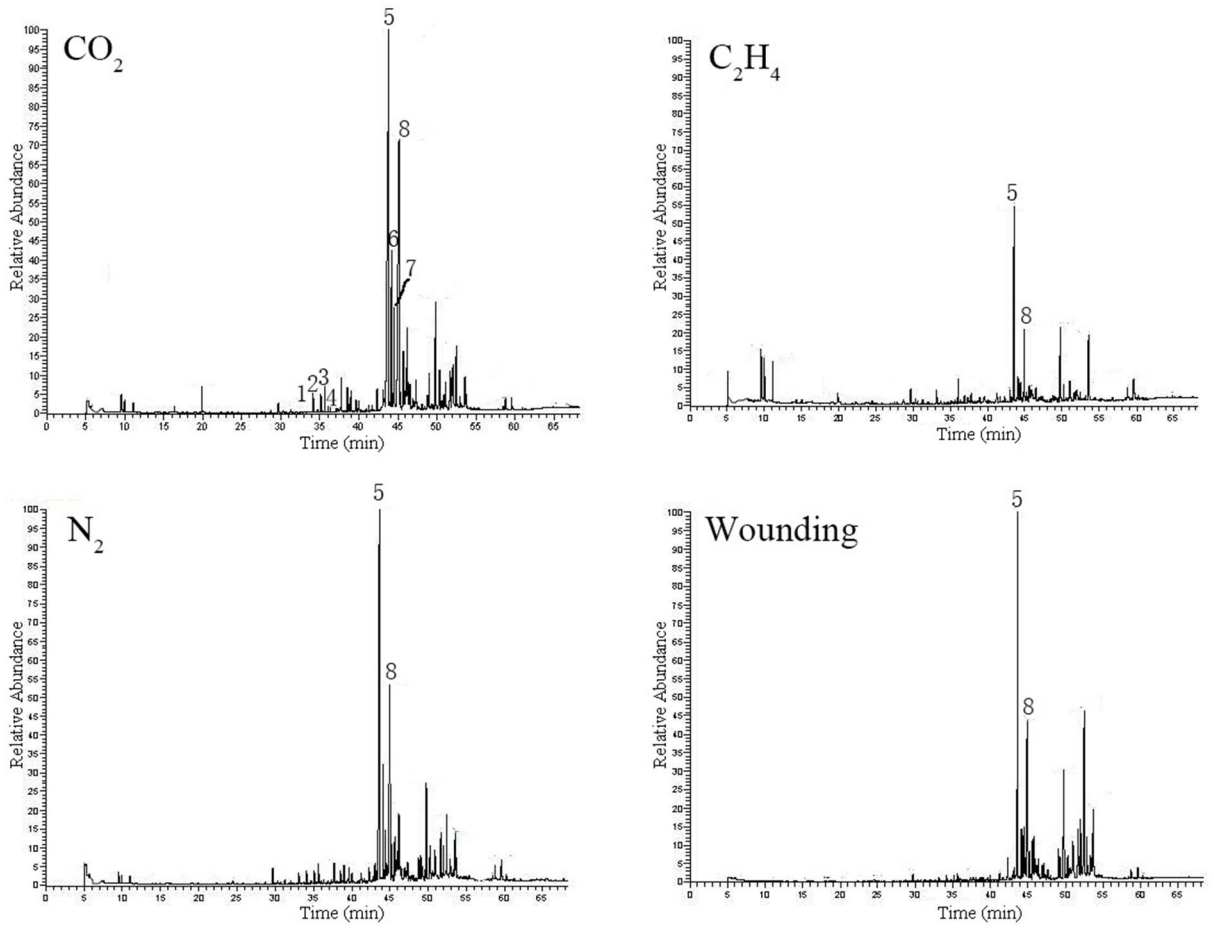


FIGURE 6
Total ion chromatogram of essential oil extracted from sandal induced by gas elicitors. 1: α-santalene 2: α-bergamotene 3: epi-β-santalene 4: β-santalene 5: α-santalol 6: α-*trans*-bergamotenol 7: epi-β-santalol 8: β-santalol.

C_2H_4 was helpful for santalene and sesquiterpene accumulation (Table 3).

Essential oil quality

The current International Organization for Standardization standard for *S. album* oil (ISO 3518:2002) requires that the essential oil contains both 41–55% α-santalol and 16–24% β-santalol (ISO, 2002), whereas the recommended quality for sandalwood oil to traded is α-santalol $\geq$43% and β-santalol $\geq$18% (Howes et al., 2004). Based on the above quality standard, only the treatment with carbon dioxide met the ISO standard for *S. album* oil and can also be traded on the international market. The contents of α-santalol and β-santalol were 43.09 and 18.91% on average, respectively (Table 2). This may also mean that stem filling with CO_2 can induce or accelerate young sandals to form quality *S. album* oils, whereas the *S. album* oil produced by the other treatments (N_2, C_2H_4, and wounding) did not meet the current ISO standards and cannot be traded on the international market.

Discussion

Heartwood formation is a complicated biochemical process during tree growth and development, which includes the death of parenchyma, gas accumulation, lack of water transport, desiccation, embolism, pit closure, ethylene production, nutrient recycling, etc. (Taylor et al., 2002), and many hypotheses or theories, such as the pipe model (Shinozaki et al., 1964), homeostasis (Gartner, 1991), heartwood-inducing substances (Bamber, 1976; Cui et al., 2020), phytohormone stress (Nilsson et al., 2002), metabolism (Cui et al., 2021, 2022), pit membrane degradation (Crombie et al., 1985), and microbial infection (Johannes and Peer, 2008), have been proposed to interpret the mechanism of heartwood formation. However, it seems

TABLE 3 ANOVA results of major ingredient group of essential oil induced by different gas treatments.

Treatment	Relative content (%)*			
	Total santalols	Total santalenes	Total sesquiterpenes	Total sesquiterpene alcohols
CO_2	65.92 ± 5.25 a	2.97 ± 0.51 b	3.90 ± 0.51 b	72.56 ± 6.13 a
C_2H_4	46.10 ± 6.83 c	4.23 ± 1.15 a	5.25 ± 1.08 a	53.49 ± 8.54 c
N_2	58.25 ± 3.56 b	1.71 ± 0.53 c	2.15 ± 0.53 c	65.09 ± 3.89 b
Wounding	57.94 ± 3.37 b	1.35 ± 0.16 c	1.66 ± 0.18 c	63.19 ± 2.62 b

*Data are shown as means ± standard deviation. Values with the same superscript letters are not significantly different from each other according to Duncan's multiple comparison ($\alpha = 0.05$).

that the heartwood formation mechanism varies greatly by species (Bieniasz and Tulik, 2020), and too many factors can affect heartwood formation. In this study, the holes drilled in the trunk interrupted part of the xylem vessels, and water transportation driven by transpiration from the roots to the canopy was then affected. Consequently, the water potential in peripheral vessels was increased, and the filling with pressurized gases then penetrated the water-filled vessels through peripheral inter-vessel pits. As a result, cavitation or air embolism was occasionally formed in xylem vessels. With the continuous filling of pressurized gases, cavitation or air embolism occurred repeatedly, the pit membranes were damaged and subsequently degraded, and the heartwood (dead sapwood) was finally formed. Therefore, all the gas treatments (including wounding) in this experiment formed colored heartwood (Figure 2), and similar results were also found in *Populus tremuloides* (Sperry et al., 1991).

The heartwood extension area differed greatly among the gas treatments (Figure 3), which may be due to the different functions of these gases in live woody tissues. As an inert gas, nitrogen is rarely involved in primary and secondary metabolic reactions; it just entered the water-filled vessels and penetrated into the surrounding tissue through the frequent closure of pits, and finally, a large amount of pit membrane was damaged and degraded, which resulted in a larger area of induced heartwood. In the case of carbon dioxide and ethylene, both were involved in metabolic reactions related to heartwood formation. For instance, carbon dioxide can induce heartwood formation in *Acacia mearnsii* by synthesizing flavonoids (Carrodus, 1971), and ethylene has been widely applied to accelerate heartwood formation in many species, such as *Azadirachta indica* (Shah et al., 1981), *Dalbergia odorifera* (Cui et al., 2019), and *Pinus sylvestris* (Nilsson et al., 2002). Under such situations, gases filling the trunk were partly depleted, and thus, the area of induced heartwood was less than that under nitrogen treatment. The reason that ethylene treatment induced less heartwood than carbon dioxide may be attributed to the greater depletion of ethylene in the heartwood formation of young sandal than carbon dioxide. Due to the lack of continuous filling of pressurized gases, cavitation or embolism rarely occurs, so the wounding treatment induced the least amount of heartwood (Figure 3); a similar phenomenon was also observed in *P. sylvestris* (Nilsson et al., 2002).

It is well-known that ethylene can increase the activities of phenylalanine ammonia-lyase, peroxidase, and terpene synthase, which are the important enzymes for polyphenol and terpene biosynthesis, and discolored heartwood is ultimately induced (Nilsson et al., 2002). In this experiment, significantly higher contents of phenols (Table 2), santalenes (Table 3), and sesquiterpenes (Table 3) were observed in the essential oil extracted from the induced heartwood treated with ethylene, which may indicate that ethylene accelerates the formation of sandal heartwood by stimulating the activities of phenylalanine ammonia-lyase, peroxidase, and terpene synthase. In the case of carbon dioxide, the accumulation of santalols (Table 2) and sesquiterpene alcohols (Table 3) was significantly stimulated, which may be explained by the consensus that high concentrations of carbon dioxide were helpful for the synthesis of ring structures in flavonoids and stilbenes (Carrodus, 1971; Higuchi, 1997). Although nitrogen does not participate in the metabolism of sandal heartwood formation, it causes hypoxia and thus may alter metabolism toward less oxygen-demanding reactions, which can also induce heartwood formation. Wounding treatment induced a small amount of heartwood, which may be attributed to the release of the stress ethylene (Nilsson et al., 2002; Liu et al., 2013). However, further study is needed to explore or confirm this hypothesis.

Oil yields of sandal heartwood are typically 3–8% through hydro-distillation (Jones and Plummer, 2007), whereas in the ethylene treatment, the essential oil content extracted from the induced heartwood was 13.11% in average (Figure 3), which was far higher than that from naturally formed heartwood. The extraction methods used in this study may be one of the reasons. Generally, solvent extraction method yielded more oil than the traditional hydro-distillation (Zhang et al., 2012; Liu et al., 2017), and this may due to the solvent can dissolve more substances than hydro-distillation. In this study, ethylene treatment got the highest oil content than all the other treatment, which may indicate that several ingredients with high boiling point were produced during heartwood formation, and these constitutes formed in the induced heartwood were extracted by the solvent

(diethyl ether). As a result, higher content of essential oil was calculated and then observed. However, these ingredients cannot be isolated or detected through gas chromatography (GC), so we cannot find these substances in the total ion chromatogram (Table 2, Figure 6). Another may be related to the particular heartwood formation model of sandal (Type-III, Santalum) (Celedon and Bohlmann, 2017), which was quite different from Type-I (*Robinia*-type) and Type II (*Juglans*-type). The phenols and terpenes stimulated by ethylene may partly accumulate in the newly formed heartwood (Celedon et al., 2016) and then be extracted into the essential oils; therefore, a higher oil content was detected. Further studies focus on this issue were strongly demanded.

Although great differences were found in the above indices among the different treatments, the properties of induced heartwood were only slightly different (Table 1), which may indicate that the properties of sandal were relatively stable and less affected by external elicitors. Similar results were also observed in *D. odorifera* when induced by different plant growth regulators (Cui et al., 2021).

Based on the above quantitative and qualitative data, we suggest that carbon dioxide is an effective gas elicitor in the induction of heartwood formation in young sandal, and ethylene, nitrogen, and wounding are the potential regulators of heartwood formation in sandal, whereas in the case of *P. sylvestris* and *Fagus sylvatica*, ethylene and oxygen are the most important regulators (Nilsson et al., 2002; Johannes and Peer, 2008). However, the mechanism may differ greatly, and the mechanism underlying this regulation has not been investigated well. Thus, studies focusing on the metabolism and molecular mechanism of induction of heartwood formation are greatly necessary, as there are still too many unknown events or processes that occur during heartwood formation in trees.

Conclusion

This study clearly demonstrated that gas elicitors played the important roles in controlling heartwood formation of young sandal. Stem filling with carbon dioxide can induce young sandal to form quality fragrant heartwood, which is consistent with naturally formed heartwood in color, properties, chemical composition, and essential oil quality. There may exist a link between carbon dioxide and the biosynthesis of santalol or sesquiterpenoids. Carbon dioxide is a promising candidate gas elicitor for inducing heartwood formation in young *S. album*.

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

XL and DX conceived the ideas and designed the methodology. QZ collected the data. ZH analyzed the data. XL wrote the manuscript. DX supervised the project. All authors contributed critically to the drafts and gave final approval for publication.

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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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Ca^{2+} mediates transcription factor *PuDof2.5* and suppresses stone cell production in pear fruits

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Stone cells are sclerenchyma cells formed by deposition of lignin, which is the most significant factor limiting the quality of pears. Ca^{2+} was known to inhibit stone cells in pear fruits, but the underlying molecular mechanism remains unclear. Our study revealed that exogenous $CaCl_2$ (Ca^{2+}) treatment of "Nanguo" pear (*Pyrus ussuriensis*) suppressed the synthesis of lignin and stone cell production. We further analysed the transcriptomes using RNA-seq, identified a transcription factor, *PuDof2.5*, and its targets gene *PuPRX42-like* (lignin polymerase gene) expression decreased in $CaCl_2$-treated samples, which are involved in suppressing lignin biosynthesis in pear fruit. *PuDof2.5* was found to bind directly to the *PuPRX42-like* promoter and induced its transcription. Taken together, our results revealed that Ca^{2+} modulated the key lignin biosynthetic transcription factor *PuDof2.5* to suppress stone cell production in pear fruits.

KEYWORDS

pear, Ca^{2+}, *PuDof2.5*, *PuPRX42-like*, lignin, stone cell

Introduction

The Nanguo pear (*Pyrus ussuriensis* Maxim) is one of the most popular pears in northern China, and it is deeply loved by consumers for its excellent taste and flavor (Wei et al., 2017). The stone cell content of the Nanguo pear is relatively high, which reduces fruit quality and results in rough flesh texture, lowering its economic value. Therefore, minimizing the stone cell content is essential to improve Nanguo pear quality.

Stone cells are formed from parenchymal cells by deposition of lignin (Rogers and Campbell, 2004). Stone cell production is closely correlated with lignin synthesis, transfer, and deposition (Barros et al., 2015). The biosynthetic pathway of lignin has been studied in pear fruits (Cai et al., 2010). The process starts with the formation of phenylacrylic acid (PA) by the enzyme phenylalanine ammonia lyase (PAL) from L-Phe (L-phenylalanine); cinnamate 4-hydroxylase (C4H) and 4-hydroxycinnamate-CoA ligase (4CL) catalyze the conversion of PA, cinnamyl alcohol dehydrogenase (CAD), and other key enzymes form lignin monomers; finally, the monomers are converted to lignin complexes by peroxidase (PRX) and laccase (LAC).

The key role of PRX genes in plant cell wall lignification has been proved (Shigeto and Tsutsumi, 2016). In Arabidopsis (*Arabidopsis thaliana*), cell-specific downregulation of the peroxidase *AtPRX64* causes a marked delay in the formation of the casparian band, a lignin-based paracellular diffusion barrier in plants (Lee et al., 2019). Overexpression of *FaPRX27* in strawberry fruit, supporting peroxidase involved in the root and fruit lignification (Ring et al., 2013). In the loss-of-function *Arabidopsis* mutant, *PRX17* showed lower lignin content in leafs, while overexpression of *PRX17* showed the opposite phenotype (Cosio et al., 2017). These findings suggested a broader role of *PRXs* genes in lignin biosynthesis.

The DNA-binding One Zinc Finger (Dof) is a family of plant-specific transcription factors that bind to the promoter of their targeted genes at the consensus sequence AAAG (Yanagisawa and Schmidt, 1999). These transcription factors also participate in various developmental processes and respond to environmental stimuli in plants (Khaksar et al., 2019). To date, Dof TF-regulated lignin production has been demonstrated in several plants (Rogers et al., 2005; Li et al., 2019; Cao et al., 2020). In *Arabidopsis*, the double mutants *VDof1* and *VDof2* showed enhanced lignin deposition, suggesting VDof1 and VDof2 as negative regulators of lignin deposition (Ramachandran et al., 2020). However, PuDof2.5, which regulates the stone cell biosynthesis mechanism in Nanguo pear, is not fully understood.

Calcium is a secondary messenger that participates in cell signaling pathways and is closely related to cell wall development, which plays an important role in regulating fruit quality (Wójcik et al., 2014). Modulation of calcium has been reported in terms of the fruit quality of several pear species (Sajid et al., 2019; Zudaire et al., 2019; Dalzochio et al., 2021). Meanwhile, Ca^{2+} also regulates the lignin synthesis process. For example, exogenous application of.5% $CaCl_2$ in "Niitaka" pear fruits can decrease PRX enzyme activity to inhibit lignin synthesis (Lee et al., 2007). In "Whangkeumbae" pear, $CaCl_2$ significantly reduced PAL, CAD, and PRX enzyme activities, and the expression levels of *PpCAD1* and *PpCAD2* were downregulated (Lu et al., 2015). Ca^{2+} regulates lignin biosynthetic molecular mechanism in "Nanguo" pear remains unclear.

Several studies have indicated that Ca^{2+} can decrease stone cell content. However, most of them only focused on changes in lignin content and related gene expression. In the present study, we characterized the role of *PuDof2.5* in pear fruits. *PuDof2.5* was lowly expressed in fruits after Ca^{2+} treatment. Finally, stone cells were decreased by inhibiting the expression of lignin synthesis gene *PuPRX42-like*. These findings provide a better understanding of the molecular mechanism by which Ca^{2+} inhibits stone cell production in pear fruits.

Materials and methods

Plant materials and treatments

Nanguopear (n = 65) trees in the orchard at the Shenyang Agricultural University (Shenyang, China) were used as test materials in this study. The trees were treated with 5 g/L $CaCl_2$, which was sprayed on flowers and fruits at full bloom (FB) and 15 days after full bloom (DAFB) twice. Trees were treated with an equal volume of water as the control. Fruit samples were harvested 20, 35, 50, 65, 80, and 110 DAFB in 2021 and 25, 30, 35, 50, 80, and 125 DAFB in 2020. Fresh fruits were harvested for histochemical analysis and stored at −80°C for further analysis. Three replicates were maintained per treatment with 20 fruits per biological replicate.

Ca^{2+} localization and paraffin section analysis

Localization of free Ca^{2+} in Ca^{2+}-treated and control fruits was observed by fluorescence imaging (Wang et al., 2018). The middle flesh was collected with a blade, washed twice with a HEPES buffer, and incubated with Fluo-3/AM at 4°C for 2 h. Then, all the samples were washed with the HEPES buffer at 25°C. Finally, Fluo-3 fluorescence was scanned with a confocal laser scanning microscope (TCS Sp8; Leica, Germany) using a 488-nm laser light to excite the dye and a 525–530-nm long-pass emission filter to read.

Stone cells were observed in paraffin sections by following previous methods (Liu et al., 2021). The pear fruits were fixed with FAA (formaldehyde, acetic acid, and ethanol), followed by safranin and green staining. Finally, the tissue section was mounted with neutral balsam and observed under a light microscope (Nikon, DS-U3).

Analysis of stone cell, lignin content, and lignin histochemistry

The stone cell and lignin contents of the pear fruits were determined 20, 35, 50, 65, 80, and 110 DAFB. The content of stone cells was determined with a previous method (Xue et al., 2019). The calculation method is as follows: stone cell content (mg/g) = stone cell dry weight (mg)/flesh weight (g). Lignin content was determined with a previous method (Anderson et al., 2015) and calculated as follows: lignin content ($mg.g^{-1}$) = lignin content (mg)/dry weight (g).

Fresh pears were used as the material and were cut and sliced by hand at six developmental stages (20, 35, 50, 65, 80, and 110 DAFB) to confirm the histochemical location of lignin.

The sections were placed in 1% phloroglucinol solution and treated with 30% HCl for 5 min. Images were captured with a handheld camera.

RNA-sequencing

Total RNA was isolated from $CaCl_2$-treated and control fruit flesh 35 DAFB (three biological replicates for each treatment), and a library was constructed and sequenced on an Illumina HiSeq 2000 system at LC-Bio Technology Co., Ltd. (Hangzhou, China). We used the fragments per kb per million reads method to calculate differentially expressed genes. Raw RNA-seq data have been deposited on the NCBI Sequence Read Archive (SRA) under accession number PRJNA 797117.

RNA extraction and gene expression analysis

The cetyltrimethylammonium bromide (CTAB) method was used to extract total RNA from $CaCl_2$-treated and untreated fruit flesh (Jaakola et al., 2001), and cDNA synthesis and gene expression analysis were conducted as previously described (Zhang et al., 2019). qRT-PCR was performed on a 7500 Real-time PCR system (Applied Biosystems, Foster City, United States) using SYBR Green Kit (Takara, Tokyo, Japan). The primers used in gene expression analysis are listed in Supplementary Table S1.

PRX enzyme activity

The PRX activity in $CaCl_2$-treated and control fruit flesh was measured as previously described (Alici and Arabaci, 2016). One PRX unit is defined as the amount of enzyme that causes an absorbance change of 0.001 per minute under the test conditions.

Subcellular localization

The *PuPRX42-like* and *PuDof2.5* coding regions were fused with GFP to form the *Pro35S:GFP-PuPRX42-like* and *Pro35S:GFP-PuDof2.5* constructs. Respectively, the construct was co-infiltrated with the mCherry-labeled membrane marker (PM Marker) and nuclear marker (mCherry marker) into onion bulb samples by *Agrobacterium tumefaciens*-mediated infiltration, the onion bulb samples were cultured in an MS medium for 48 h after infiltration. GFP fluorescence was observed under a confocal microscope (TCS Sp8; Leica, Germany). For green fluorescence observation, the excitation wavelength was 488 nm and the emission wavelength was 520–540 nm. For red fluorescence observation, the excitation wavelength was 561 nm and the emission wavelength was 610–630 nm. *Pro35S:GFP* was used as a control. All the assays were repeated at least 3 times.

Functional analysis

To overexpress *PuDof2.5* and *PuPRX42-like* in the Nanguo pear fruits, CDS regions were separately cloned into a pRI101 vector to form *Pro35S:pRI101-PuDof2.5* and *Pro35S: pRI101-PuPRX42-like*. These plasmids were transformed into *A. tumefaciens* strain GV3101, and preparation of infiltration buffer and fruit infiltration were performed as previously described (Li et al., 2016). Briefly, the infiltration buffer was taken with a 1-ml sterile syringe and injected into on-tree Nanguo pear fruits 20 DAFB. For each fruit, one side was used for infiltrating target constructs, and the other side for infiltrating empty pRI101 as control. The infiltrated fruits were harvested 6 day after the infiltration, and the fruit flesh around the infiltrated area was sampled for further use.

GUS analysis

The *PuPRX42-like* promoter sequence (1,011 bp) was cloned upstream of the GUS gene in the pBI101 vector to obtain the reporter construct. Meanwhile, the *PuDof2.5* CDS was introduced into the pRI101 vector to generate the effector construct. The *A. tumefaciens* strain GV1301 harboring the reporter and effector vectors were infiltrated into *N. benthamiana* leaves (Li et al., 2017). Histochemical staining was performed with X-gluc, and GUS activity was determined as previously described (Li et al., 2016). Stained leaves were viewed with an optical microscope, the infiltration was repeated three times (three biological replicates).

ChIP-PCR analysis

The *PuDof2.5* CDS was cloned into the pRI101-3xflag vector (Yue et al., 2020) and transformed into an *A. tumefaciens* GV1301 strain. The pear fruits were infected as previously described (Li et al., 2020). ChIP assay was performed using the EpiQuik Plant ChIP Kit (56383; Cell Signaling Technology, Danvers, MA, United States) following the manufacturer's instructions. Chromatin fragmentation was performed using an Uibra Cell VCX 150PB sonicator (Sonics and Materials Inc., Newtown, CT, United States). The specific for the ChIP-PCR assay was prepared as previously method (Li et al., 2020). Each ChIP assay was repeated three times, and enriched DNA fragments in each ChIP sample were used as one biological replicate for qPCR. Six regions of the *PuDof2.5* promoter were

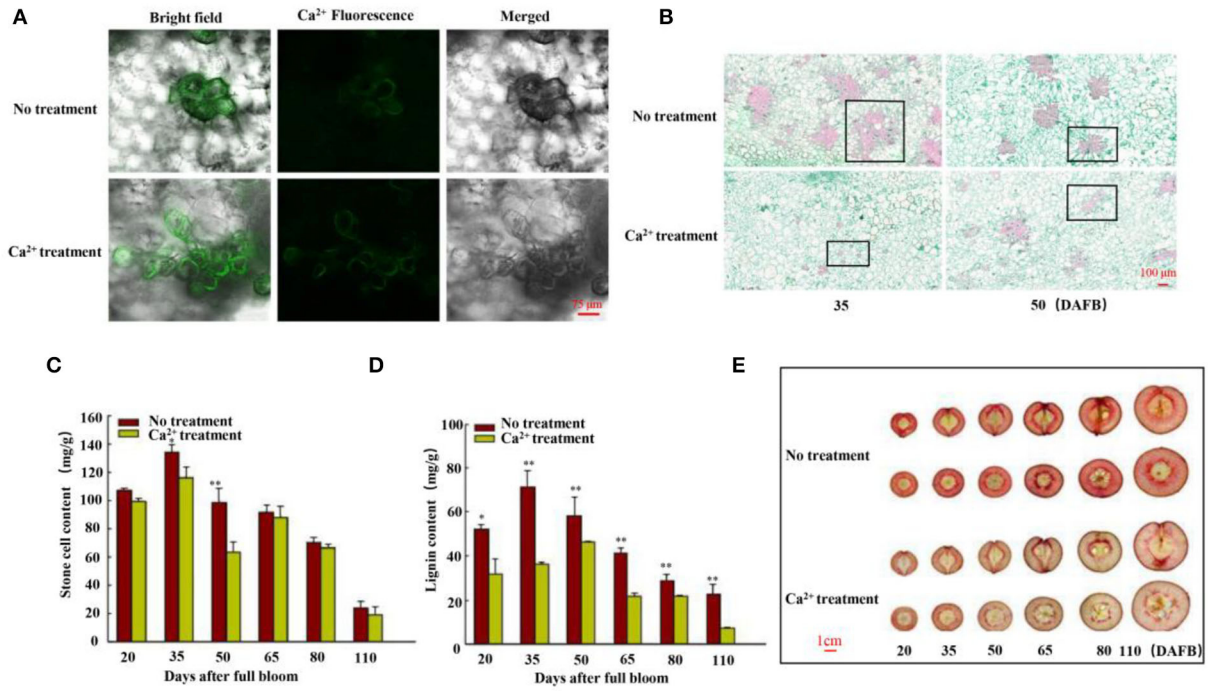


FIGURE 1
Free Ca^{2+}, stone cell morphology, and lignin and stone cell contents. **(A)** Free Ca^{2+} in the flesh cells of fruits was detected using Fluo-3/AM 35 days after full bloom (DAFB). Scale bar 75 μm. **(B)** Morphology of stone cells was observed using paraffin sections of fruits 35 and 50 DAFB. Scale bar 100 μm. **(C)** Stone cell production and **(D)** lignin content of fruits were measured at six stages (20, 35, 50, 65, 80, and 110 DAFB). **(E)** Staining of sections with phloroglucinol-HCl (scale bar 1 cm). Data are shown as means ± SE (n = 3). Statistical significance was determined by Student's *t*-test: ** $P < 0.01$.

analyzed to assess the enrichment; the primers used are listed in Supplementary Table S1.

Yeast one-hybrid assays

The *PuDof2.5* CDS sequences were inserted into the pGADT7 vector at the *Nde*I and *Eco*RI restriction sites to generate a prey construct. The *PuPRX42-like* promoter was cloned into the pAbAi vector using the *Sac*I and *Sma*I restriction sites to produce the bait constructs. The specific operation for the Y1H assay was performed as previously described (Ji et al., 2021). All the primers used in this study are listed in Supplementary Table S1.

Results

Calcium suppresses lignin and stone cell contents in pear fruits

The Nanguo fruits on the tree were treated with 5 $g \cdot L^{-1}$ $CaCl_2$ in 2020 and 2021. Fruits were harvested 35 days after full bloom (DAFB). The fluo-3/AM staining revealed that flesh cells had higher free Ca^{2+} in $CaCl_2$-treated fruits than in control fruits (Figure 1A).

Then, the lignin and stone cell contents of the fruits were measured at six fruit developmental stages, and the findings were consistent with the paraffin section results (Figures 1B–D, Supplementary Figure S2). We further stained the harvested fruits (25, 30, 35, 50, 80, and 125 DAFB in 2020 and 20, 35, 50, 65, 80, and 110 DAFB in 2021) to confirm the results. Hand-cut sections were prepared by phloroglucinol-HCl staining. The lignin-specific histochemical staining indicated that the level of lignification in the control fruits was higher than in the $CaCl_2$-treated fruits (Figure 1E, Supplementary Figure S1), indicating fewer stone cell primordia in the $CaCl_2$-treated fruits. Thus, the findings collectively indicate that the $CaCl_2$ treatment significantly inhibited lignin biosynthesis and stone cell production in Nanguo pear.

PuPRX42-like gene assists calcium in inhibiting lignin biosynthesis

To explore how $CaCl_2$ inhibits lignin production from 20 to 125 DAFB in Nanguo pear fruits, we measured the PAL,

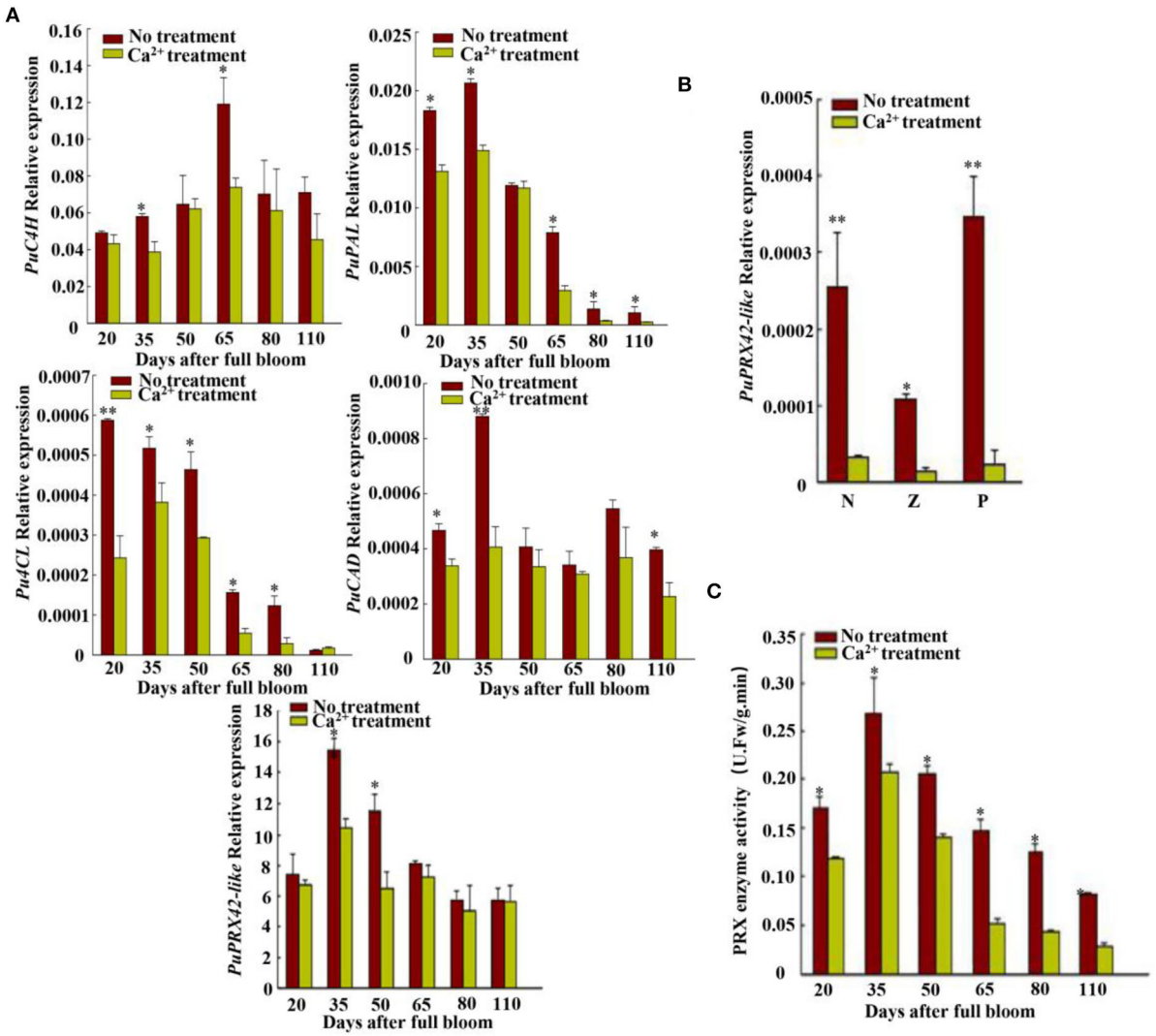


FIGURE 2
Synthetic lignin relative gene expression and PRX enzyme activity analyses. **(A)** Expressions of lignin biosynthesis genes *PuC4H*, *PuPAL*, *Pu4CL*, *PuCAD*, and *PuPRX42-like* were measured at six fruit developmental stages; *PuC4H*, cinnamate 4-hydroxylase gene; *PuPAL*, phenylalanine ammonia lyase gene; *Pu4CL*, 4-coumarate gene; *PuCAD*, cinnamyl alcohol dehydrogenase gene; *PuPRX*42-like, peroxidase gene. **(B)** *PuPRX42-like* expression in different pear tissues. N, flesh close to the core; Z, flesh in the middle; P, flesh close to the fruit skin. **(C)** PRX enzyme activity at six developmental stages. Data are shown as mean ± SE. Statistical significance was determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$.

C4H, 4CL, CAD, and PRX activities of the $CaCl_2$-treated and control fruit samples (Supplementary Figure S3). The $CaCl_2$ treatment significantly inhibited PRX activity (Figure 2C), and PRX activity showed the highest correlation with lignin and stone cell contents (Table 1). Subsequently, the expression of lignin biosynthesis genes *PuPRX42-like*, *PuPAL*, *PuC4H*, *Pu4CL*, and *PuCAD* was analyzed by qRT-PCR (Figure 2) to identify the key genes that contribute to the lower lignin accumulation in $CaCl_2$-treated fruits. The expression level of *PuPRX42-like* was lower in the $CaCl_2$-treated fruits than in the control fruits and consistent with PRX activity (Figures 2A,C).

Furthermore, a transient expression assay was conducted to explore the putative function of *PuPRX42-like* in lignin production. The recombinant plasmid (*35S::PuPRX42-like*-OE) was then transiently transformed into *Agrobacterium* and infiltrated into Nanguo pear fruits on the tree 30 DAFB using the empty vector (pRI101) as the control; The expression of *PuPRX42-like* was significantly higher in *PuPRX42-like*-OE fruits than in the control fruits, consistent with lignin content (Figures 3A,C,D,E). These results indicate that *PuPRX42-like* is important for lignin biosynthesis in pear fruits. *PuPRX42-like* CDS was tagged with GFP and transformed into onion bulb cells

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TABLE 1 Correlation analysis between stone cell, lignin content, and lignin-related enzyme activity.

	Lignin	Stone cell	PAL	C4H	4CL	CAD	POD
Lignin	1	0.789**	0.684**	0.626**	0.579**	0.517**	0.910**
Stone cell		1	0.749**	0.716**	0.639**	0.620**	0.856**

PAL, PAL enzyme activity; C4H, C4H enzyme activity; 4CL, 4CL enzyme activity; CAD, CAD enzyme activity; POD, POD enzyme activity. Statistical significance was determined by Student's t-test: ** $P < 0.01$.

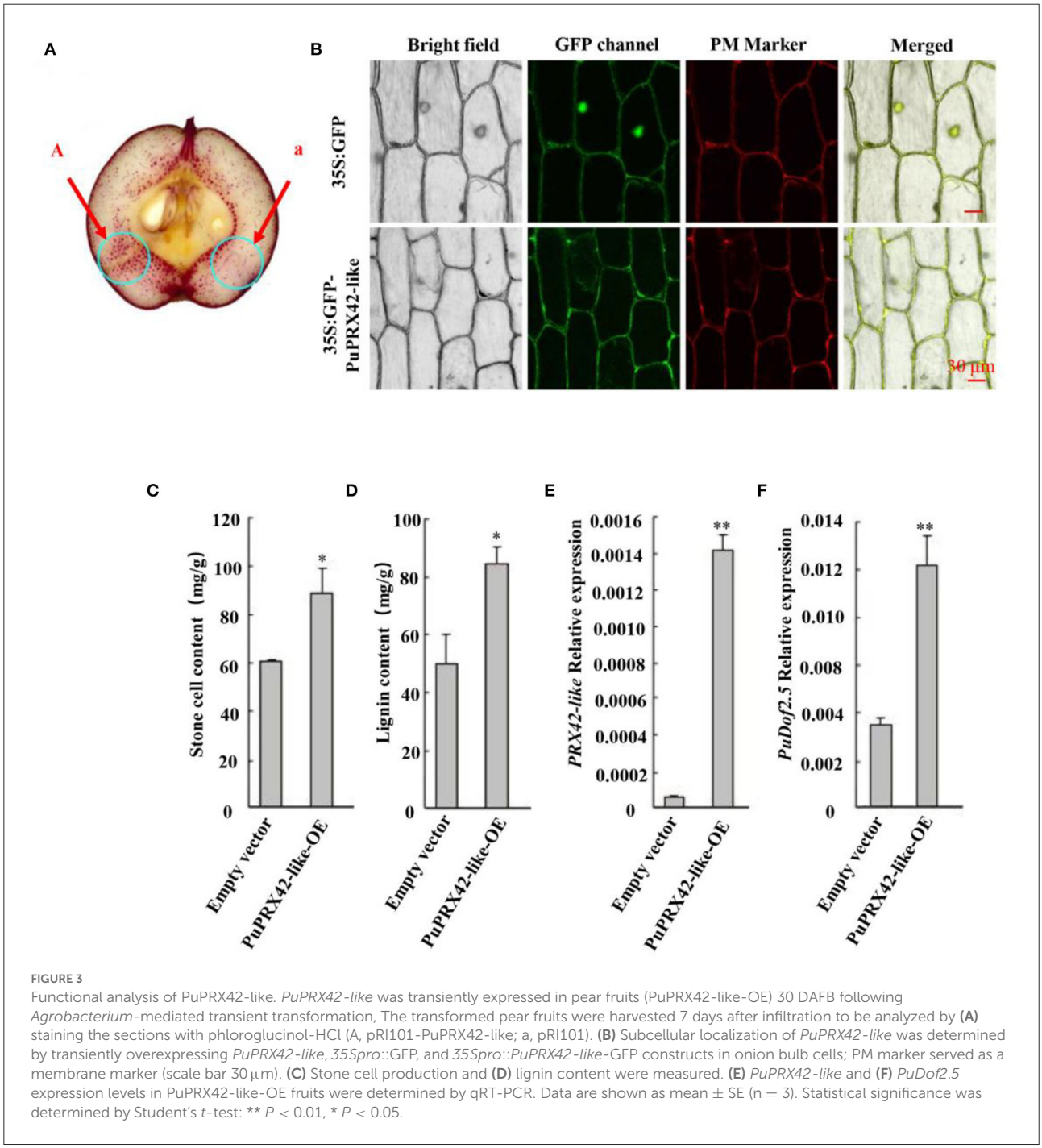


FIGURE 3
Functional analysis of PuPRX42-like. *PuPRX42-like* was transiently expressed in pear fruits (PuPRX42-like-OE) 30 DAFB following *Agrobacterium*-mediated transient transformation, The transformed pear fruits were harvested 7 days after infiltration to be analyzed by **(A)** staining the sections with phloroglucinol-HCl (A, pRI101-PuPRX42-like; a, pRI101). **(B)** Subcellular localization of *PuPRX42-like* was determined by transiently overexpressing *PuPRX42-like*, *35Spro*::GFP, and *35Spro*::*PuPRX42-like*-GFP constructs in onion bulb cells; PM marker served as a membrane marker (scale bar 30 μm). **(C)** Stone cell production and **(D)** lignin content were measured. **(E)** *PuPRX42-like* and **(F)** *PuDof2.5* expression levels in PuPRX42-like-OE fruits were determined by qRT-PCR. Data are shown as mean ± SE (n = 3). Statistical significance was determined by Student's *t*-test: ** $P < 0.01$, * $P < 0.05$.

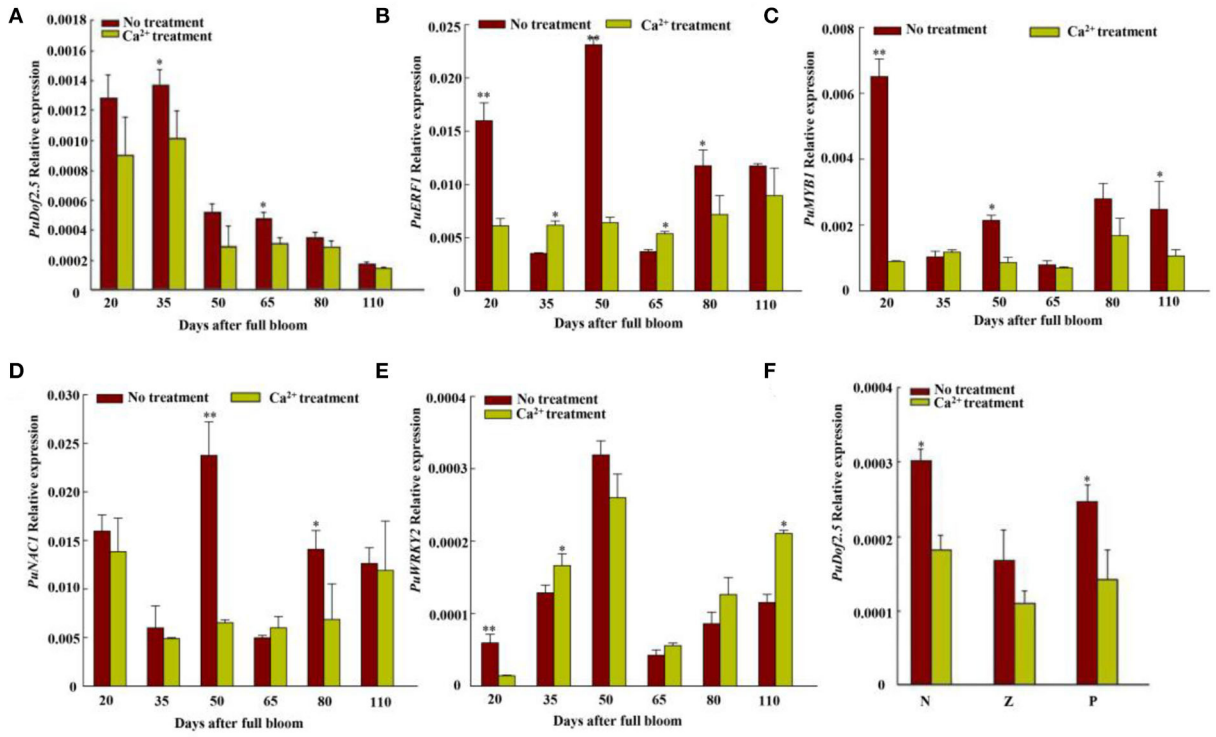


FIGURE 4
Transcriptome-filtered transcription factor expression level analyses of Nanguo pear fruits. **(A)** Expression level of *PuDof2.5* at six fruit developmental stages after Ca^{2+} treatment. **(B)** Expression level of *PuERF1* at six fruit developmental stages after Ca^{2+} treatment. **(C)** Expression level of *PuMYB1* at six fruit developmental stages after Ca^{2+} treatment. **(D)** Expression level of *PuNAC1* at six fruit developmental stages after Ca^{2+} treatment. **(E)** Expression level of *PuWRKY2* at six fruit developmental stages after Ca^{2+} treatment. **(F)** *PuDof2.5* expression in different pear tissues. N, flesh close to the pit; Z, flesh in the middle; P, flesh close to the fruit skin. Data are shown as mean ± SE (n = 3). Statistical significance was determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$.

following the *Agrobacterium tumefaciens* mediation to further explore its subcellular localization. The analysis of onion bulb cells showed that PuPRX42-like was localized on the membranes (Figure 3B).

PuDof2.5 was the key transcription factor after calcium-treated pear fruits

We compared the transcriptomes of $CaCl_2$_treated and control fruits harvested 35 DAFB, and RNA-seq data had different transcription factors, *PuDof2.5*, *PuWRKY2*, *PuNAC1*, *PuERF1*, and *PuMYB1* (Figure 4). Among them, all genes expressions were downregulated after Ca^{2+} treatment, but only *PuDof2.5* expression trend was the same as stone cell change at six fruit developmental stages; these were confirmed by qRT-PCR. We also analyzed *PuDof2.5* expression in different tissues and found a high expression level in flesh close to the core. Furthermore, we identified cis-elements (AAAG) of Dof transcription factor in the promoter of *PuPRX42-like* (1,500 bp) and hypothesized the role of *PuDof2.5* in regulating *PuPRX42-like* expression.

Furthermore, the *PuDof2.5* CDS was cloned into the pRI101 vector under the control of the 35S promoter to build a 35S: *PuDof2.5* overexpression construct (PuDof2.5-OE). The vector was transformed into *Agrobacterium* and infiltrated into on-tree pear fruits. pRI101 alone was used as control, and qRT-PCR confirmed higher expression of *PuDof2.5* in *PuDof2.5*-OE fruits than in control fruits (Figure 5). We detected that the stone cell and lignin contents in *PuDof2.5*-OE fruits were significantly increased. Notably, the expression level of *PuPRX42-like* was also high in the *PuDof2.5*-OE fruits, suggesting that *PuDof2.5* might play a role in suppressing stone cells by regulating the expression of *PuPRX42-like*.

PuDof2.5 binds to the promoter of *PuPRX42*-like

Furthermore, a yeast one-hybrid (Y1H) assay was conducted to investigate the binding of *PuDof2.5*. The results showed that

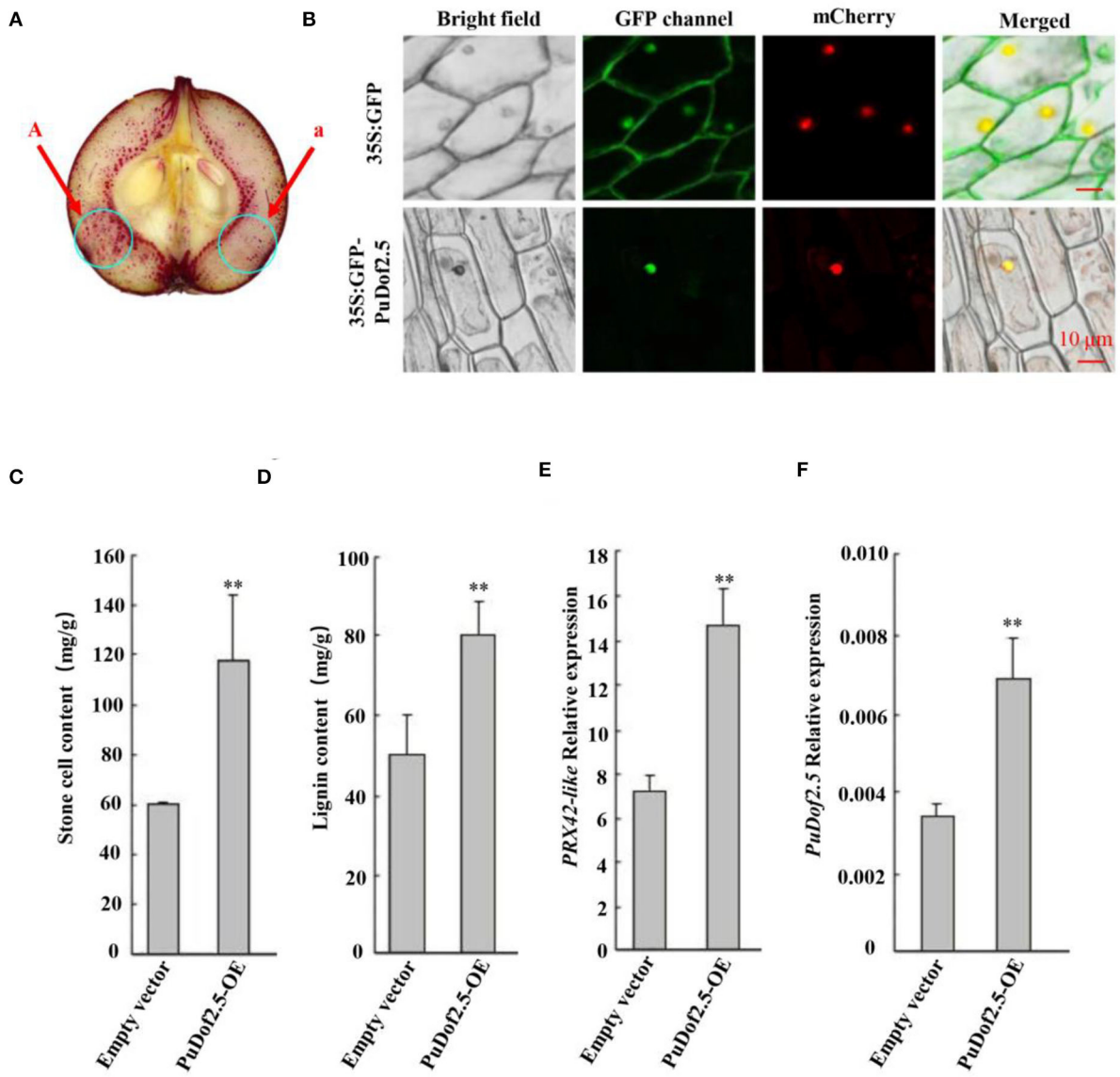


FIGURE 5
Functional analysis of *PuDof2.5*. **(A)** staining the sections with phloroglucinol-HCl (A, pRI101-PuDof2.5; a, pRI101). **(B)** Subcellular localization of *PuDof2.5* was determined by transiently overexpressing *PuDof2.5*, *35Spro*::GFP, and *35Spro*:: *PuDof2.5*-GFP constructs in onion bulb cells; PM marker served as a membrane marker (scale bar 10 μm). **(C)** Stone cell content and **(D)** lignin content were measured. **(E)** *PuPRX42-like* and **(F)** *PuDof2.5* expression levels in PuDof2.5-OE fruits were determined by qRT-PCR. Data are shown as mean ± SE (n = 3). Statistical significance was determined by Student's *t*-test: ** $P < 0.01$.

PuDof2.5 bound to the *PuPRX42-like* promoter (Figure 6A). To investigate whether *PuPRX42-like* is a direct target of *PuDof2.5*, ChIP-qPCR assays were performed. The analysis confirmed that *PuDof2.5* could bind to regions of *PuPRX42-like* promoters (Figure 6B). Next, we investigated the *PuDof2.5* regulation to the *PuPRX42-like* promoter by β-glucuronidase (*GUS*) transactivation assay. There was significantly increased activity of the *PuPRX42-like* promoter compared to the empty vector (Figures 6C,D), suggesting that *PuDof2.5* is a transcriptional activator of *PuPRX42-like*. Taken together, the results suggested that *PuDof2.5* directly binds to the *PuPRX42-like* promoter to activate its expression.

Discussion

Stone cells are a restrictive factor of Nanguo pear fruit quality (Yan et al., 2014). The formation of stone cells is closely related to the deposition of lignin in pear fruits (Li et al., 2007). However, although previous studies have described that Ca^{2+} induces the biosynthesis of lignin in

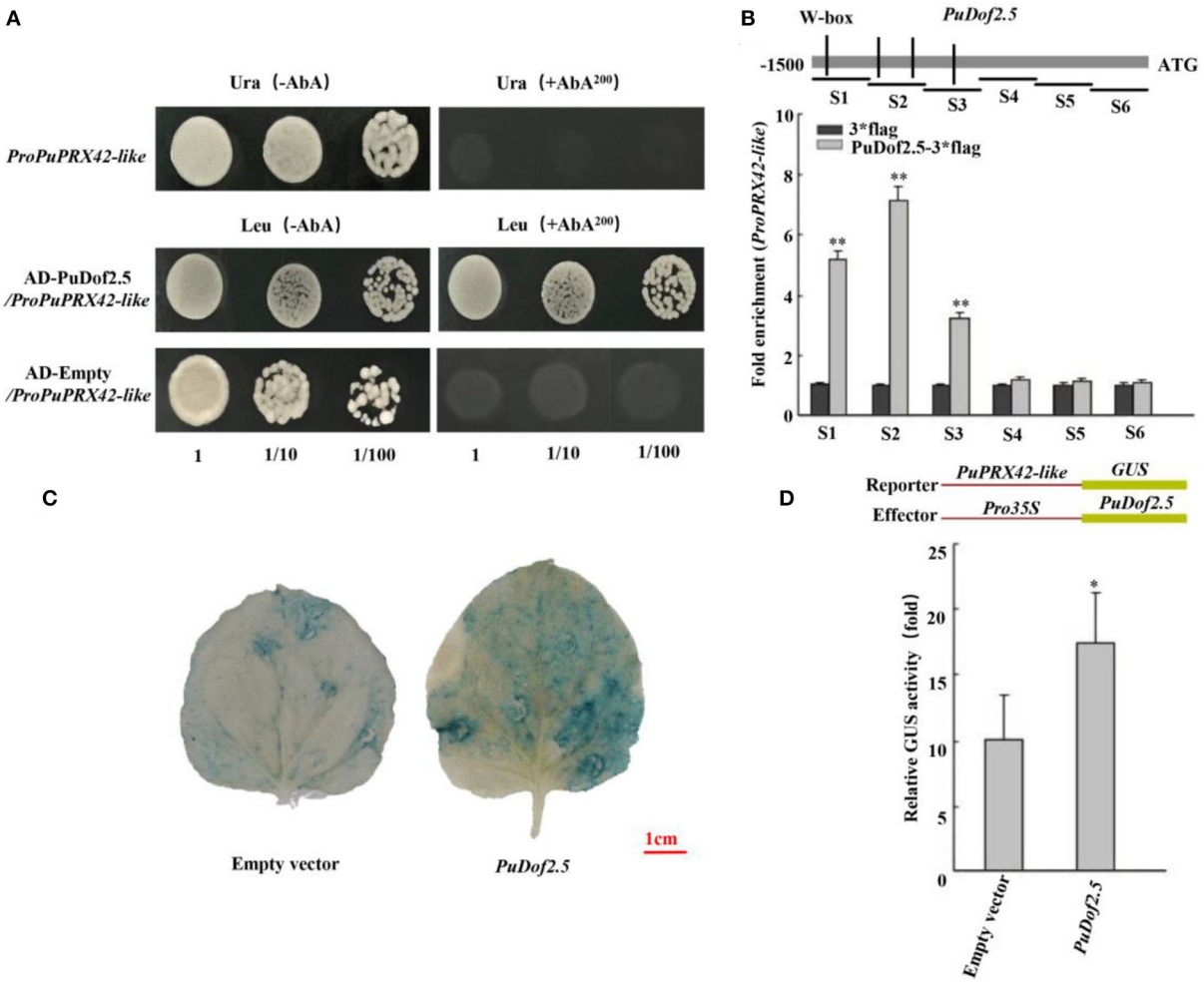


FIGURE 6
PuDof2.5 regulates *PuPRX42-like* transcription by promoter binding. **(A)** Y1H analysis of *PuDof2.5* binding to the promoter of *PuPRX42-like*. AbA is a yeast growth inhibitor, and the basal concentration was 200 ng/ml. The empty vector and the *PuPRX42-like* promoter were used as negative controls. **(B)** ChIP-qPCR analysis to determine how *PuDof2.5* directly binds to the *PuPRX42-like* promoter. Eluted DNA was used in the quantitative PCR (qPCR) performed to analyze the six fragments (S1–S6). Immunoprecipitate from cells transiently expressing the vector alone was used as a negative control. **(C)** Histochemical GUS (β-glucosidase) staining. **(D)** GUS activity analysis to determine how *PuDof2.5* promotes the activity of the *PuPRX42-like* promoter. Data are shown as mean ± SE (n = 3). Statistical significance was determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$.

fruit species. $CaCl_2$ significantly inhibited lignin biosynthesis enzyme and PAL activities to decrease lignin content in apple fruits (Zhao and Wang, 2015). The studies only investigated changes in stone cell production after exogenous Ca^{2+} treatment and the expression profile of genes involved in lignin biosynthesis and signal transduction. The detailed mechanism by which Ca^{2+} regulates lignin biosynthesis to suppress stone cell production in pear fruits was unclear. Here, we determined that Ca^{2+}-inhibited PuDof2.5 induces the expression of a lignin biosynthetic gene (*PuPRX42-like*) by directly binding to its promoter. Our findings suggest that Ca^{2+} has an essential role in reducing stone cell accumulation in pear fruits.

Calcium reduces stone cell production by reducing lignin accumulation

The texture of pear fruits depends on stone cell mass density and content (Wang et al., 2021). In this study, stone cells developed as clusters, presenting a radial shape throughout the fruit flesh in control fruits, which is consistent with earlier reports (Li et al., 2017). However, Ca^{2+} significantly suppressed stone cell mass content and density (Figure 1B). Higher free Ca^{2+} levels were observed in the Ca^{2+}-treated fruits, whereas the levels were lower in the control pear fruits (Figure 1A). The phloroglucinol staining revealed lignin deposition in the flesh of pear fruits and lignified stone cells; the detailed analysis

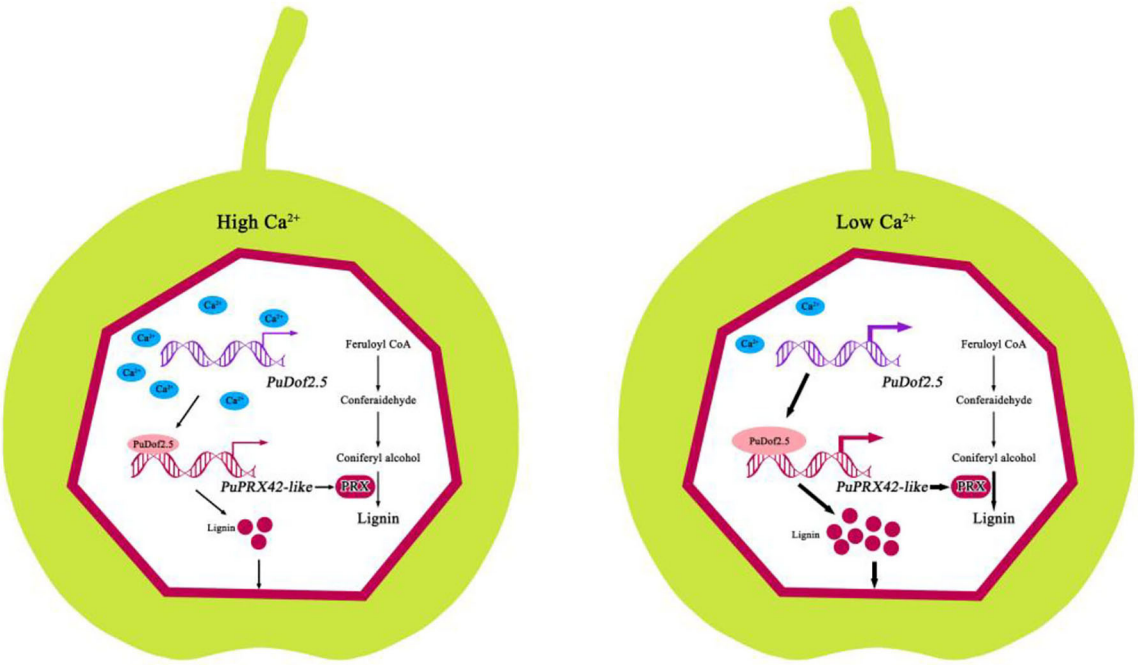


FIGURE 7
Model of the mechanism by which Ca^{2+} suppresses stone cell production in pear fruits. At high levels of endogenous Ca^{2+}, *PuDof2.5* downregulated the transcription of lignin biosynthetic gene *PuPRX42-like* by directly binding to the promoter, decreasing lignin accumulation and stone cell production. At low levels of endogenous Ca^{2+}, *PuDof2.5* upregulated the transcription of lignin biosynthetic s *PuPRX42-like*, increasing lignin accumulation and stone cell production.

revealed that lignin accumulated rapidly from 20 to 50 DAFB (Figure 1D). Meanwhile, the control fruits were stained darker than the Ca^{2+}-treated fruits (Figure 1E), consistent with stone cell content (Figure 1C). A similar result was reported in "Whangkeumbae" (*Pyrus pyrifolia*) pear (Lu et al., 2015). Based on these findings, we proposed that Ca^{2+} regulates lignin accumulation by inhibiting the expression of lignin biosynthesis genes. Together, the studies provided detailed information that Ca^{2+} decreased stone cell content by inhibiting lignin accumulation in pear fruits.

PuPRX42-like is important for Ca^{2+}-induced lignin biosynthesis

Studies have proven the role of various enzymes in lignin synthesis. Among PAL, C4H, CAD, 4CL and PRX (Tao et al., 2009), PRX is the key enzyme to catalyze monolignol polymerization into lignin (Dean and Eriksson, 1994; Cheng et al., 2017). The present study analyzed the activities of PRX, PAL, C4H, CAD, and 4CL related to lignin biosynthesis after Ca^{2+} treatment (Supplementary Figure S3). Interestingly, the Ca^{2+} treatment only reduced PRX enzyme activity, and it is consistent with lignin and stone cell contents (Figures 1C,D, 2C, Supplementary Figure S3). An earlier finding with the same results in "Niitaka" pear fruit (Lee et al., 2007) suggested that the PRX enzyme acts a key factor in lignin accumulation to form stone cell. The correlation analysis between lignin synthesis enzyme (PRX, PAL, C4H, CAD, and 4CL) with lignin and stone cell contents also supported this observation (Table 1).

Previous studies have revealed that *PuPRXs* regulated PRX activity and lignin biosynthesis in pear, and that the high expression level of *PuPRX2* promotes lignin accumulation in *PuRBOHF*-overexpressing pear calli (Wang et al., 2021). In our present study, *PuPRX42-like* expression levels were consistent with PRX activity changes (Figures 2A,C). Meanwhile, the overexpression of *PuPRX42-like* significantly accelerated lignin accumulation and stone cell production. PuPRX42-like was localized on the plasma membrane, including sites where lignification occurred. To sum up, the results indicated that *PuPRX42-like* was a key point in the regulation of lignin biosynthesis.

PuDof2.5 binds to *PuPRX42*-like promoter and regulates lignin biosynthesis

Dofs are plant-specific transcription factors that act as either transcriptional activators or repressors to influence plant

developmental processes (Licausi et al., 2013), i.e., *OsDof15* promoted rice root elongation response to salt stress, and overexpression of *GhDof1* resulted in significant cold tolerance (Su et al., 2017). Meanwhile, overexpression of *AtDof2.1* promoted rice leaf senescence (Zhou et al., 2021). Nonetheless, the role of Dof in regulating lignin biosynthesis in pear fruits is not well-known. We observed that Ca^{2+} significantly suppressed the expression of *PuDof2.5* (Figure 4A). Moreover, *PuDof2.5* is a transcriptional activator that binds to the lignin biosynthetic gene *PuPRX42-like* promoter, directly upregulating its expression (Figures 5A,B,D). Overexpression of *PuDof2.5* increased lignin and stone cell contents and upregulated the expression level of *PuPRX42-like*. Thus, our findings propose that *PuDof2.5* acted as a useful marker to respond to Ca^{2+} treatment.

Our results indicated that Ca^{2+} induced stone production in pear fruits (Figure 7). When endogenous Ca^{2+} levels are high, *PuDof2.5* expression declines, and *PuDof2.5* suppresses the transcription of lignin biosynthetic gene *PuPRX42-like* by direct promoter binding; thus, PRX enzyme activity is reduced, lignin biosynthesis is inhibited, and stone cell production is decreased. When endogenous Ca^{2+} levels decline, *PuDof2.5* expression is enhanced, which accelerates PRX enzyme activity and the transcription of *PuPRX42-like*, leading to a burst of stone cells. Taken together, the results provide a novel insight into the mechanism by which Ca^{2+} regulates stone cell accumulation to improve pear quality.

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

XL and GD designed this study. HZ, TW, SG, and MX performed the experiments. XL, GD, and HZ wrote the manuscript. All the authors read and approved the final version of the manuscript.

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Conflict of interest

Author TW was employed by Xinjiang Production and Construction Corps.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.976977/full#supplementary-material

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Calliterpenone, a natural plant growth promoter from a medicinal plant *Callicarpa macrophylla*, sustainably enhances the yield and productivity of crops

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The global population is rising at an alarming rate, which is threatening food and nutritional security. Although chemical fertilizers and pesticides are important for achieving food security, their excessive usage critically affects soil health and adds up residues in the food chain. There is an increasing interest in identifying eco-friendly farm inputs that can improve crop productivity through sustainable agricultural practices. One of the most common approaches to reducing chemical inputs in agriculture is the use of plant growth regulators (PGRs). Here, we demonstrate the benefits of a natural and novel plant growth enhancer "calliterpenone," isolated from *Callicarpa macrophylla,* a medicinal plant, for increasing crop productivity in six crops, *viz.*, rice, wheat, potato, tomato, chickpea, and onion. Results revealed that the application of calliterpenone (foliar spraying or seed soaking) enhanced the yield of rice (28.89%), onion (20.63%), potato (37.17%), tomato (28.36%), and chickpea (26.08%) at 0.001 mM and of wheat (27.23%) at 0.01 mM concentrations in comparison to control. This enhancement in yield was reflected through improvements in its growth attributes, *viz.*, spike length, tillers plant^{-1}, seeds spike^{-1}, plant height, and biomass. Furthermore, the exogenous application of calliterpenone could increase the endogenous level of indole-3-acetic acid (IAA) in all tested crops and decrease the content of abscisic acid (ABA) in a few. Trials conducted at farmers' fields showed an overall ~12% increase in rice yield (mean of 11 farmers' fields ranging from 3.48 to 19.63%) and ~10% increase in wheat yield (ranging from 3.91 to 17.51%). The 0.001 mM of calliterpenone was the best effective dose for most crops except wheat,

where a concentration of 0.01 mM was found to be the most optimal. This study indicates that calliterpenone is a natural plant growth promoter that can be used in boosting the yields of multiple crops and would be an important input component of organic farming.

KEYWORDS

calliterpenone, plant growth regulators, IAA, ABA, yield contributing traits, sustainable crop production, medicinal plants

Introduction

The green revolution considerably enhanced food grain production by employing high-yielding varieties (HYVs), adequate fertilizers, and modern farming practices (Swaminathan and Bhavani, 2013). However, since agricultural land resources are shrinking day by day and the population is rapidly increasing, it is projected to grow by over 8 billion by 2025 (Hinrichsen and Rowley, 1999). Hence, an urgent need is felt to enhance crop productivity to sustain this ever-increasing population. Although chemical fertilizers and pesticides are important for achieving food security, their excessive usage critically affects soil health and environmental quality (Tilman, 1998). Therefore, an eco-friendly and sustainable approach must be explored to meet the increasing population demand from a limited cultivation area. One of the common approaches to reducing chemical inputs in agriculture is the use of plant growth regulators (PGRs) such as gibberellins, auxins, and cytokinins (Masroor et al., 2006; Tiwari et al., 2011; Giannakoula et al., 2012; Choudhury et al., 2013; Rastogi et al., 2013; Kurubar et al., 2017; Zhang et al., 2017; McMillan et al., 2020; Chen et al., 2021). PGRs are important for enhancing plant growth and productivity in intensive agriculture, especially for high-value crops. Gibberellins (especially gibberellic acid) are important PGRs used for increasing the economic yields of various crops. These have been found effective in rice (Gavino et al., 2008; Tiwari et al., 2011; Prajapati et al., 2021), wheat (Pavlista et al., 2014; Amram et al., 2015; Zhang et al., 2017; Dawar et al., 2021), potato (Thornton et al., 2013), tomato (Choudhury et al., 2013), cotton (Copur et al., 2010), sweet pepper (Maboko and DuPlooy, 2015), *Pyrethrum* (Singh et al., 2011), peppermint (Khanam and Mohammad, 2017), onion (Ghani et al., 2021), and chickpea (Rafique et al., 2021). However, the higher cost of gibberellins has restricted their use to high-value crops only. CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP) isolated "calliterpenone", a novel and natural plant growth-promoter from *Callicarpa macrophylla* (Singh et al., 2004, 2009), which could be useful in increasing the growth and yield of several crop plants.

Callicarpa macrophylla Vahl (Family Verbenaceae) is a valuable medicinal plant effective for treating many diseases such as fever, diarrhea, diabetes, tumor, polydipsia, and dysentery (Pandey et al., 2014). This erect shrub is widely distributed over sub-Himalayan and Indo-Gangetic regions of India (Chopra et al., 1986; Manandhar, 2002). About 20 species of *Callicarpa* are found in South Asia and China (Jones and Kinghorn, 2008). Various compounds including carbohydrates, amino acids, lipids, flavonoids, diterpenes, benzenoids, phytosterols, triterpenes, phenylpropanoids, and sesquiterpenes have been reported from *Callicarpa* (Tellez et al., 2000).

Calliterpenone (16α, 17-dihidroxy phyllocladane-3-one) (Figure 1), has a similar substitution pattern similar to the ent-kaurenoid compound "abbeokutone" (16α, 17-dihidroxy kaurane-3-one), the precursor of gibberellins in the biosynthetic pathway (Liu et al., 2003; Bottini et al., 2004). The structural relationship of calliterpenone to gibberellic acid prompted us to evaluate its plant growth-promoting activities. It was found that calliterpenone and its mono-acetate induced better plant growth than well-established PGRs such as gibberellic acid and many cytokinins (Singh et al., 2004, 2011; Goel et al., 2007; Bose et al., 2013). Also, as calliterpenone is obtained from a natural resource, this could be a beneficial product for organic farming. The organic food market's projected compound annual growth rate (CAGR) is likely to grow by 16.15% during 2017–2021 globally due to rising incomes and growing consumer awareness concerning the health benefits of consuming organic food. The projections for the growth of organic farm produce in India are about 25%. The market for PGRs was around USD 2.11 billion in 2017 and may grow to a value of USD 2.93 billion by 2022, at a CAGR of 6.8% (TechSci Research, 2017).

It has also been found that the calliterpenone has the potential to alleviate the growth-retarding effects of allelochemicals (e.g., *Artemisia annua* seed extract) (Anaya et al., 2003; Singh et al., 2004) and promote plant growth. Calliterpenone improved the yield of flowers and the content of pyrethrin in *Pyrethrum* flowers (Singh et al., 2011). In

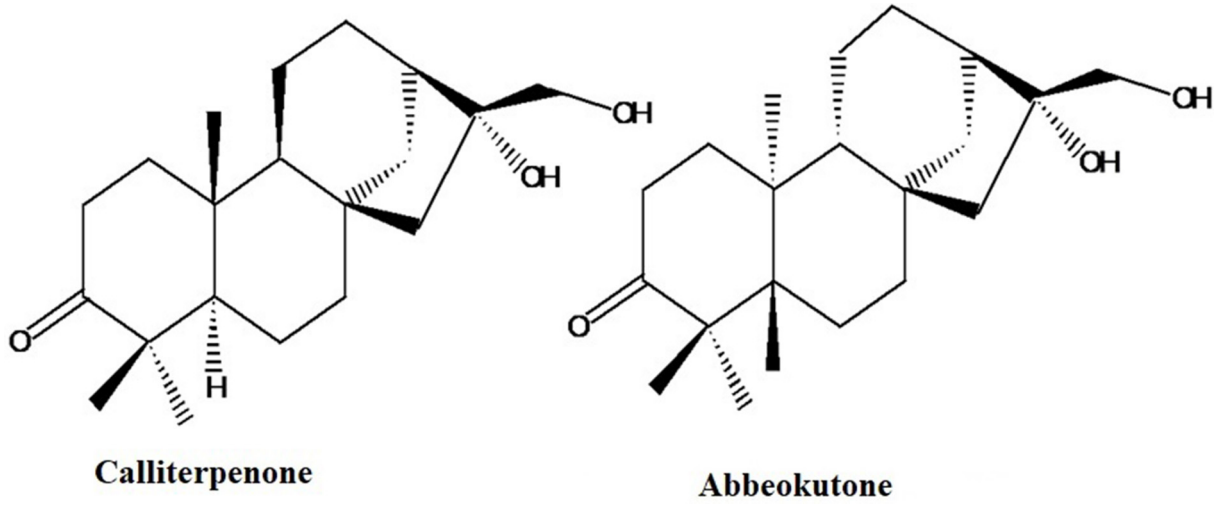


FIGURE 1
Structure of calliterpenone and abbeokutone.

Mentha arvensis, calliterpenone has been found to induce sprouting, increase branching, herb yield (Haider et al., 2009), oil content, and trichome density (Bose et al., 2013). Calliterpenone could also enhance the number of cells of *Bacillus*, *Rhizobium*, and *Cyanobacteria* (Kalra et al., 2010; Maji et al., 2013; Patel et al., 2014) in the growth medium and significantly improved the growth as well as multiplication of *in vitro* shoot cultures of *Rauwolfia serpentina* (Goel et al., 2007). Recently, Zafar and Sangwan (2019) studied physiological and chemical changes related to the growth-enhancing effects of calliterpenone on *Mentha arvensis*. They found that application of calliterpenone significantly increases chlorophyll content, total sugars, protein content, moisture content, and activity of invertase enzyme, along with considerable enhancement in phenotypic growth parameters (number of branches, plant height, internodal length, and the total numbers of fresh sprouting). These findings indicate that calliterpenone is an efficient and economic plant growth promoter. Considering the importance of calliterpenone, efficient and economically viable process technology for isolation of calliterpenone from leaves of *C. macrophylla* was developed and patented worldwide (Singh et al., 2004, 2009, 2016). Although its plant growth-promoting effect on medicinal and aromatic plants is well known, no information is available on its efficacy in food crops. This study is our effort to establish its growth-promoting potential in major groups of field crops, *viz.*, cereals, vegetables, and pulses, which will surely help in its commercialization as plant growth promoters. Therefore, this study was undertaken to evaluate the effects of calliterpenone on some agriculturally important food crops to enhance their yield and productivity in an eco-friendly manner without increasing the additional chemical load on soil.

Materials and methods

Site description

The site of this study was the experimental farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India, geographically located at 26.05°N latitude, 80.05°E longitude, and an altitude of 120 m above mean sea level, experiencing a semiarid subtropical climate with an annual rainfall of 900 mm, with hot summers and cold winters being the main characteristics of the region. The soil characteristics of the field are sandy loam in texture with pH 7.95, EC 0.18 dS m^{-1}, organic carbon 0.40%, nitrogen 162.75 kg ha^{-1}, phosphorus 15.02 kg ha^{-1}, and potassium 161.12 kg ha^{-1}.

The farmers' field trials were conducted in the fields of 11 farmers in the districts of Lucknow (26.05°N, 80.05°E), Sitapur (27.58°N, 80.66°E), and Raebareli (26.23°N, 81.24°E) in Uttar Pradesh, India.

Plant materials and experimental design

Field experiments were conducted on food crops, *viz.*, wheat (*Triticum aestivum*), rice (*Oryza sativa)* potato (*Solanum tuberosum)*, chickpea (*Cicer arietinum*), tomato (*Solanum lycopersicum*), and onion (*Allium cepa*). The experimental material consisted of ten treatment combinations of each crop (Table 1) arranged in an RBD (randomized block design) with three replications in two consecutive crop seasons during 2016–2018. The individual plot size of each treatment and

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TABLE 1 Detail of treatments used in various crops.

Treatments	Calliterpenone concentration and application method	Crops					
		Rice	Wheat	Tomato	Potato	Onion	Chickpea
Control	Treated with water	✓	✓	✓	✓	✓	✓
ST1	Seed treatment with 0.1 mM	✓	✓	✓	✓		✓
ST2	Seed treatment with 0.01 mM	✓	✓	✓	✓		✓
ST3	Seed treatment with 0.001 mM	✓	✓	✓	✓		✓
STA1	Seed treatment + 1 spray with 0.1 mM		✓		✓		✓
STA2	Seed treatment +1 spray with 0.01 mM		✓		✓		✓
STA3	Seed treatment + 1 spray with 0.001 mM		✓		✓		✓
STB1	Seed treatment + 2 spray with 0.1 mM		✓		✓		
STB2	Seed treatment + 2 spray with 0.01 mM		✓		✓		
STB3	Seed treatment + 2 spray with 0.001 mM		✓		✓		
SRT1	Seed + root treatment with 0.1 mM	✓					
SRT2	Seed + root treatment with 0.01 mM	✓					
SRT3	Seed + root treatment with 0.001 mM	✓					
SRTA1	Seed + root treatment + 1 spray with 0.1 mM	✓		✓			
SRTA2	Seed + root treatment + 1 spray with 0.01 mM	✓		✓			
SRTA3	Seed + root treatment + 1 spray with 0.001 mM	✓		✓			
SRTB1	Seed + root treatment + 2 spray with 0.1 mM			✓			
SRTB2	Seed + root treatment + 2 spray with 0.01 mM			✓			
SRTB3	Seed + root treatment + 2 spray with 0.001 mM			✓			
SP1	Spray with 0.1 mM						✓
SP2	Spray with 0.01 mM						✓
SP3	Spray with 0.001 mM						✓
RT1	Root treatment with 0.1 mM					✓	
RT2	Root treatment with 0.01 mM					✓	
RT3	Root treatment with 0.001 mM					✓	
RTA1	Root treatment + 1 spray with 0.1 mM					✓	
RTA2	Root treatment + 1 spray with 0.01 mM					✓	
RTA3	Root treatment + 1 spray with 0.001 mM					✓	
RTB1	Root treatment + 2 spray with 0.1 mM					✓	
RTB2	Root treatment + 2 spray with 0.01 mM					✓	
RTB3	Root treatment + 2 spray with 0.001 mM					✓	

spacing between rows and plants varied from crop to crop. For tomato, potato, and onion, the plot size was 4 m^2 (4 rows of 2 m, with 50 cm spacing between rows). However, plant-to-plant spacings were 50 cm, 15 cm, and 10 cm for tomato, potato, and onion, respectively. In the case of chickpea, 4 m^2 plot size consisted of 5 rows of 2 m, spaced 40 cm apart between rows and 10 cm apart between the plants, while in rice, 7 m^2 plots (10 rows of 3.5 m with a spacing of 20 and 10 cm between rows and plants, respectively) and in wheat, 3.5 m^2 plots (3.5 m row length of 5 rows having 20 cm spacing between rows and 5 cm between plants). Crop varieties used for the experiments were Omkar-283 (tomato), Chipsona (potato), Punjab selection (onion), DCP-92-3 (chickpea), Tilakchandan (rice), and HD-2967 (wheat). For trials conducted at farmers' fields, the plot size of each farmer was 500 m^2. The doses of fertilizers (N:P:K kg ha^{-1}) applied in different crops were rice (120:60:40), wheat (150:60:40), tomato (180:100:60), potato (120:80:80), onion (100:50:50), and chickpea (20:40:20). The total amount of potassium and phosphorus were applied as basal dose at the time of planting through muriate of potash (MOP) and single super phosphate (SSP), respectively. Nitrogen was supplemented through urea in three splits, i.e., half as basal dose at the time of planting, and the rest of nitrogen was top-dressed in two equal split doses in 30- and 55-days old crops. The other agronomic practices, *viz.*, irrigation, weeding, insecticides, and pesticides, were adopted as and when required to have a good crop stand.

Extraction and isolation of calliterpenone

Calliterpenone was extracted from the leaves of the *C. macrophylla* plant as described earlier (Singh et al., 2009, 2016). Two hundred grams of cleaned and dried leaves were extracted with 6 L of 1% alkaline water for 3 h in a Clevenger-like glass apparatus (10 L capacity). The resulting extract was reduced to about one-third volume and was further extracted in dichloromethane (3 × 200 ml). The pooled dichloromethane extract was concentrated to a viscous residue. The residue was purified by celite filtration, i.e., absorbed over 2 g celite and passed through a bed prepared from 20 g celite in a vacuum filtration funnel fitted with a glass sintered disc. It was first eluted with 10% ethyl acetate in hexane (600 ml) and then with 50% ethyl acetate in hexane (1 L). This 1 L of 50% ethyl acetate hexane elution was dried under vacuum to produce calliterpenone (0.46%), having 84% purity as estimated by HPLC as described by Verma et al. (2009). For pilot scale extraction, 15 kg of dried leaves were processed similarly in a stainless still distillation tank, and for extraction, dichloromethane continuous solvent–solvent extraction technology was used. It was further purified by column chromatography.

Dose and method of calliterpenone treatment

A stock solution (0.1 mM) of calliterpenone was made by dissolving 3.2 mg of calliterpenone in 1 ml of absolute alcohol followed by 100 ml of water. To prepare 0.01 mM and 0.001 mM solutions of calliterpenone, 10 ml of stock solution was taken in volumetric flasks, and the volume was made up to 100 ml and 1,000 ml by adding distilled water, respectively. Three different concentrations (0.1, 0.01, and 0.001 mM) of calliterpenone were used; seed soaking (6 h), root treatment of seedlings before transplanting (3 h), and foliar spraying at 30 days and 60 days after planting (DAP). However, the methods of application varied from crop to crop (Table 1). The foliar application was carried out at 30 DAP and 60 DAP by spraying calliterpenone (~0.25 L/m^2) on plants uniformly to the point of run-off using a Knapsack sprayer with constant flow. Utmost care was taken while spraying to ensure that no run-off from the leaves reaches the soil that may possibly affect soil microbial activity. Control plots were treated with the same volume of water. The selection of various doses of calliterpenone is based on our initial evaluation of doses of calliterpenone for their growth-promoting activities, wherein the most suitable concentrations were recorded as 0.001–0.1 mM (w/v basis) while increasing the concentration growth inhibitory activities were observed in some instances (Bagchi et al., 1997; Singh et al., 2004). Based on the results of the main field experiments of this study, 0.001 mM dose for rice and 0.01 mM for wheat were selected for assessment of its effects on farmers' fields trials; the applications were made at the time of sowing as seed treatment followed by a foliar spray at 30 DAP.

Data collection on yield and yield attributing traits

Data were collected for each treatment from 10 randomly selected representative plants avoiding border row plants for plant height (cm), tillers plant^{-1}, panicle length or spike length (cm), seeds panicle^{-1} or seeds spike^{-1}, grain yield plant^{-1} (g), grain yield plot^{-1} (kg), biological yield plant^{-1} (g), harvest index (%), and test weight (g). However, data on days to 50% flowering were recorded on a plot basis; during the crop standing stage, plants were monitored daily to observe the initiation of the flowering, and when 50% of plants in each plot flowered the date was noted and calculated the number of days from the date of sowing. At the time of physiological maturity, plant height and number of tillers plant^{-1} were measured; the number of tillers of 10 plants were counted and averaged to calculate tillers plant^{-1}. After harvesting, plants were separated into straw and panicles and panicle length was measured. The panicles were hand-threshed and the total numbers of seeds were counted and averaged to determine the number of seeds panicle^{-1}. The ratio of grain yield to total dry matter of the plant (biological yield) was considered as harvest index and expressed in percentage. It was calculated by the following formula given by Donald and Hamblin (1976):

$$\text{Harvest index (\%)} = \frac{\text{Grain yield}}{\text{Biological yield}} \text{ x } 100$$

To calculate test weight filled seeds were separated from unfilled seeds and 1,000 seeds were counted and weighed. In order to calculate biological yield plant^{-1}, the whole plants above the ground level were harvested, sun-dried, and weighed; on the other hand, to determine grain yield plant^{-1}, the total quantity of the grains obtained after threshing was cleaned and weighed. For calculating the yield of farmers' fields, 10 samples from treatment and control plots were harvested using 1 × 1 m quadrat excluding border rows and averaged.

Standard solution and sample preparation for residue analysis

For residue analysis, plant samples (rice leaves, straw, grains, husk, and soil) were collected from the plants on which morphological data were recorded. Samples (in triplicate) that received maximum concentration treatment were considered for the study. After harvesting the plants, straws, leaves, and grains were separated. Furthermore, husks were removed from grains

by hand hulling. Samples were grounded to a fine powder, and a total of 1.0 g of the powdered tissue was extracted in 10 ml of 2% alkaline water (200 mg NaOH in 10 ml water) for 2 h, followed by portioning of the extracts using dichloromethane. The dichloromethane extracts were then dried and later dissolved in methanol. Before HPLC injection, solutions were filtered through 0.45 μm membranes (Millipore, Billerica, MA). For extraction of calliterpenone, the HPLC analysis was performed as per the method described by our group (Verma et al., 2009) using an LC-10A HPLC system (Shimadzu, Japan) with acetonitrile:water (45:55) as mobile phase and detection was made at 220 nm using a Waters Spherisorb ODS-2 column (250 × 4.6 mm I.D., 10 μm).

Measurement of endogenous indole-3-acetic acid (IAA) and abscisic acid (ABA) content

The quantitative determinations of ABA and IAA contents were carried out using Phytodetek-IAA and ABA immunoassay kit (Agdia, Elkhart, IN) following the manufacturer's protocol. Phytohormone extraction from leaf samples of different crops was performed as described by Barnawal et al. (2016). Leaf samples frozen in liquid nitrogen were grounded to a fine powder. A total of 0.5 g of the powdered leaf tissue in 8 ml of extraction solution consisting of methanol (80%), butylated hydroxytoluene (BHT,100 mg L^{-1}), and citric acid monohydrate (0.5 g L^{-1}) was continuously stirred in the dark at 4°C for overnight. The solution was then centrifuged at 4°C for 20 min at 1,000 g. Later, the supernatant was dried under a vacuum. The dry residue was then dissolved in a solution containing 900 μl of Tris-buffered saline [T.B.S. (pH 7.8)] and 100 μL of methanol (100%). The concentrations of ABA and IAA in the filtrate were determined with ABA/IAA enzyme immunoassay test kit and expressed based on fresh weight (FW).

Statistical analysis

The data were compiled using mean values of randomly selected plants in each treatment and employed for statistical analyses. The analysis of variance (ANOVA) for RBD was performed as per the methodology suggested by Panse and Sukhatme (1985). Treatments were statistically compared through critical differences at the $P \leq 0.05$ level of significance by Fisher's *F*-test.

Results

The experimental findings concerning various growth and yield parameters in tested crops, including cereals (rice, wheat), vegetables (tomato, potato, onion), and pulse crop (chickpea), have been illustrated in the following sections:

Effect of calliterpenone on plant growth, yield, and yield contributing traits in rice

The data relating to the effect of calliterpenone on yield contributing traits and yield in rice is given in Table 2 and Figure 2A, and the percentage of the yield increase over control in Supplementary Table S1. All the characteristics were significantly different except for days to 50% flowering and test weight. A maximum yield increase of 28.89% was observed in treatment ST2 (seed treatment with 0.01 mM), followed by 23.26% in ST3 (seed treatment with 0.001 mM), significantly higher than control. However, treatments like SRTA2 (seed treatment + root treatment + 1 spray with 0.01 mM), SRT1 (seed treatment + root treatment with 0.1 mM), and SRTA1 (seed treatment + root treatment + 1 spray with 0.1 mM) showed significantly lower yields than control, i.e., – 25.28, – 15.71, and – 10.78%, respectively.

Early flowering is a desirable characteristic. The days to 50% flowering ranged from 117.67 to 119.33 days, and there were no significant differences among treatments. Treatment SRT3 (182.33 cm) followed by SRTA3 (seed treatment + root treatment + 1 spray with 0.001 mM) with 178.60 cm plants resulted in significantly taller plants than control. However, ST3-treated plants were a little shorter (173.23 cm) than the control (175.33 cm).

More effective tillers, large panicles, and the number of seeds $panicle^{-1}$ are generally responsible for higher yields in rice. SRT2 (10.60) followed by ST2 (10.57), SRT1 (10.50), and SRT3 (10.43) led to higher tillering, while SRTA2 (8.77), SRTA1 (9.47), SRTA3 (9.77), and ST1 (9.50) had fewer tillers than control (9.93). The panicle size was significantly larger in plants receiving the treatments such as SRTA3 (30.51 cm), ST3 (29.77 cm), SRT1 (29.77 cm), SRT3 (29.77 cm), SRT2 (29.52 cm), and ST2 (29.50 cm) than the control (28.87 cm). The maximum number of seeds $panicle^{-1}$ was observed in ST3 (175.33) followed by ST2 (172.80), SRTA3 (169.87), and SRT2 (168.87), which were significantly higher than in control (162.17). However, treatments SRTA1 with 158.10 and ST1 (seed treatment with 0.1 mM) with 158.70 produced fewer seeds $panicle^{-1}$ than in control.

Maximum and significantly higher biological yield $plant^{-1}$ was observed in SRTA1 (101.97 g), followed by SRT1 (99.27 g), and SRT3 (97.33 g). However, ST3 (73.87 g) had a lower biological yield $plant^{-1}$ than control (80.60 g). Grain yield $plant^{-1}$ was maximum in SRTA1 (27.51 g), followed by SRT3 (27.30 g), SRTA2 (24.83 g), and ST2 (24.56 g), significantly higher than control (21.36 g). However, SRTA3 (20.33 g) resulted in minimum grain yield $plant^{-1}$.

TABLE 2 Effect of calliterpenone on yield and yield contributing traits in rice.

Treatments		Days to 50% flowering	Plant height (cm)	Tillers plant^{-1}	Panicle length (cm)	Seeds panicle^{-1}	Biological yield plant^{-1} (g)	Grain yield plant^{-1}(g)	Harvest index (%)	Test weight (g)
Control		118.33	175.33	9.93	28.87	162.17	80.60	21.36	26.57	18.67
ST1		119.33	177.17	9.50	29.22	158.70	83.40	23.11	27.70	18.97
ST2		118.67	177.97	10.57	29.50	172.80	84.40	24.56	29.13	19.07
ST3		118.67	173.23	10.00	29.77	175.53	73.87	23.59	31.95	18.70
SRT1		118.00	178.13	10.50	29.77	168.67	99.27	23.16	23.54	19.00
SRT2		118.33	176.47	10.60	29.52	168.87	84.97	23.44	27.53	19.10
SRT3		117.67	182.33	10.43	29.77	162.00	97.33	27.30	28.25	18.33
SRTA1		118.33	175.17	9.47	28.70	158.10	101.97	27.51	26.96	18.53
SRTA2		118.67	176.90	8.77	29.43	163.90	89.57	24.83	27.72	19.30
SRTA3		119.33	178.60	9.77	30.51	169.87	82.07	20.33	25.13	18.17
F ratio		1.19	3.41	4.00	4.61	4.73	3.48	3.85	2.76	0.86
F prob.		0.36	0.01	0.01	0.00	0.02	0.01	0.01	0.03	0.58
C.V.		0.70	1.29	5.20	1.40	2.85	9.62	8.40	8.54	3.63
C.D. at 5%		1.72	3.93	0.89	0.71	8.12	14.49	3.45	4.02	1.18
S.E.M.		0.52	1.73	0.42	0.35	4.14	6.58	1.65	1.68	0.37
Range	Min	117.67	173.23	8.77	28.70	158.10	73.87	20.33	23.54	18.17
	Max	119.33	182.33	10.60	30.51	175.53	101.97	27.51	31.95	19.30

C.V., coefficient of variation; C.D., critical difference; S.E.M., standard error of mean. Other abbreviations are as shown in Table 1.

Maximum harvest index was recorded in ST3 (31.95%) followed by ST2 (29.13%), SRT3 (28.25%), SRTA2 (27.72%), ST1 (27.70%), and SRT2 (27.53%), significantly higher than control (26.57%). However, SRT1 (23.54%) had a minimum harvest index. In the case of test weight, there were no significant differences among treatments.

Effect of calliterpenone on plant growth, yield, and yield contributing traits in wheat

The data relating to the effect of calliterpenone on yield and yield contributing traits in wheat is presented in Table 3 and Figure 2B, and the percent yield increase over control in Supplementary Table S1. ANOVA revealed significant differences for most of the characters, except harvest index and test weight. Maximum yield increase over control 27.23% was observed in STA1 (seed treatment + 1 spray with 0.1 mM) followed by 25.71% in STA2 (seed treatment + 1 spray with 0.01 mM), 21.29% in STB1 (seed treatment + 2 spray with 0.1 mM), 21.16% in ST1 (seed treatment with 0.1 mM), 19.57% in STB3 (seed treatment + 2 spray with 0.001 mM), 16.59% in STB2 (seed treatment + 2 spray with 0.01 mM), 14.58% in STA3 (seed treatment + 1 spray with 0.001 mM), and 12.50% in ST3 (seed treatment with 0.001 mM).

The treatment ST1 (seed treatment with 0.1 mM) induced early flowering (69.33 days) than control (70.67 days). On the other hand, STA1 (seed treatment + 1 spray with 0.1 mM), STA2 (seed treatment + 1 spray with 0.01 mM), STA3 (seed treatment + 1 spray with 0.001 mM), and STB3 (seed treatment + 2 spray with 0.001 mM) resulted in late flowering (71.00 days) than control. Maximum plant height of 108.67 cm was recorded in STA1, followed by 108.53 cm in ST2 (seed treatment with 0.01 mM), 107.43 cm in STB2, and 106.83 cm in ST3, which were significantly higher than control (104.47 cm). The plant height was recorded the least in STA3 (102.89 cm).

Significantly higher tillering over control was recorded in ST2 (4.97), ST1 (4.90), STB1 (4.83), ST3 (4.83), STA2 (4.83), and STA3 (4.80). All the treatments resulted in significantly larger spikes than control (9.65 cm) except STA1 (10.00 cm). The length of the spike was observed to be maximum in STA3 (10.87 cm) followed by ST2 (10.85 cm), STA2 (10.82 cm), ST3 (10.78 cm), STB1 (10.47 cm), STB3 (10.30 cm), ST1 (10.27 cm), and STB2 (10.27 cm). The treatments STA2 (58.67) followed by STA1 (57.73) led to a significantly more number of seeds spike^{-1} than the control (52.60). However, a significantly lower number of seeds spike^{-1} were recorded in STA3 (46.33).

The treatments ST1 (32.97 g), STA2 (32.27 g), and STA1 (32.11 g) resulted in significantly higher biological yield plant^{-1} than control (27.52 g). The maximum grain yield plant^{-1} was recorded in STA2 (10.95 g), followed by ST1 (10.88 g) and

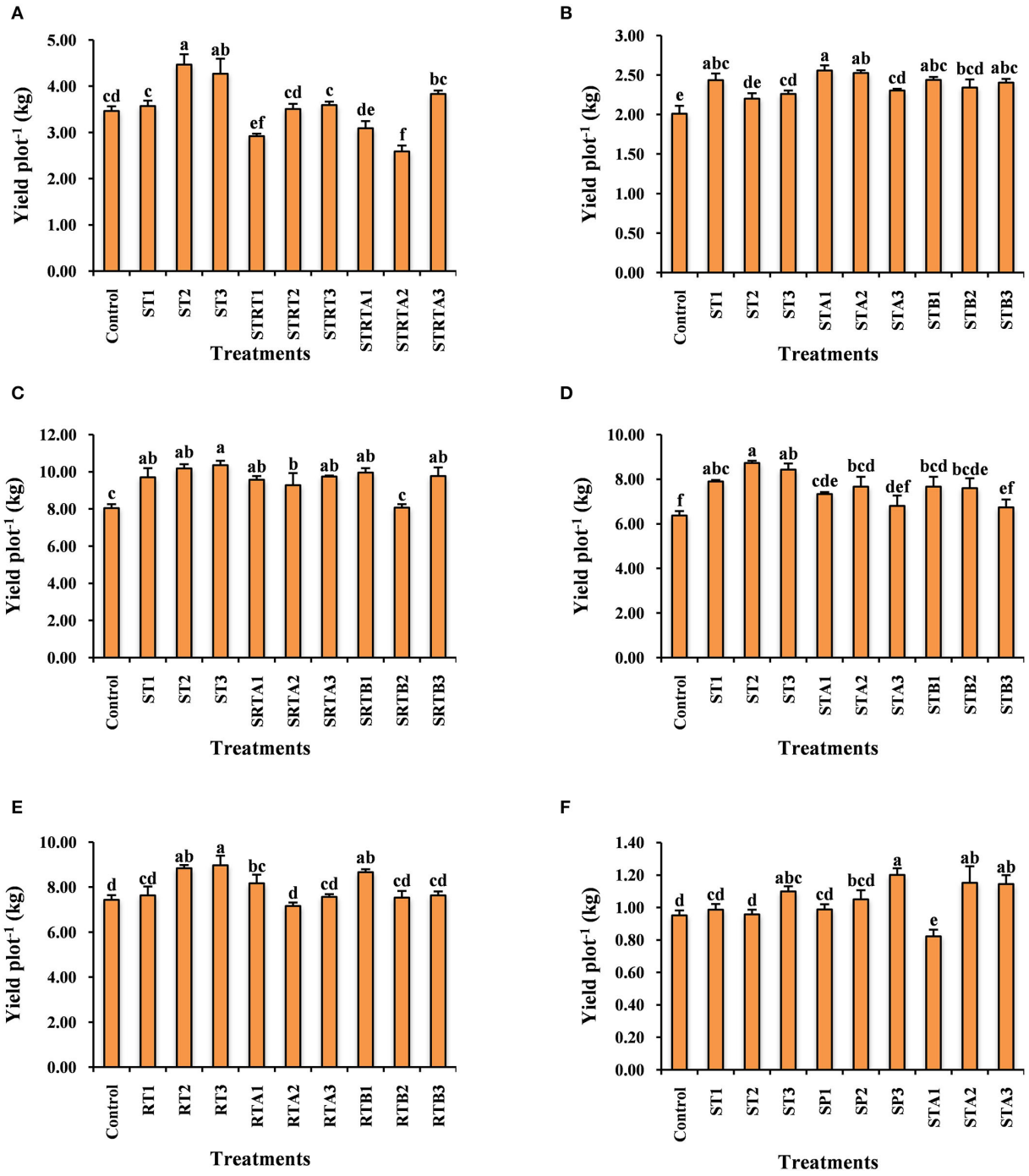


FIGURE 2
Effect of calliterpenone on yield enhancement in rice **(A)**, wheat **(B)**, tomato **(C)**, potato **(D)**, onion **(E)**, and chickpea **(F)**. Statistical analysis was carried out taking means of three replicates; different letters above bars showed significant differences as compared to control through critical difference ($P \leq 0.05$). ST1, seed treatment with 0.1 mM; ST2, seed treatment with 0.01 mM; ST3, seed treatment with 0.001 mM; STA1, seed treatment + 1 spray with 0.1 mM; STA2, seed treatment + 1 spray with 0.01 mM; STA3, seed treatment + 1 spray with 0.001 mM; STB1, seed treatment + 2 spray with 0.1 mM; STB2, seed treatment + 2 spray with 0.01 mM; STB3, seed treatment + 2 spray with 0.001 mM; SRT1, seed treatment + root treatment with 0.1 mM; SRT2, seed treatment + root treatment with 0.01 mM; SRT3, seed treatment + root treatment with 0.001 mM; SRTA1, seed treatment + root treatment + 1 spray with 0.1 mM; SRTA2, seed treatment + root treatment + 1 spray with 0.01 mM; SRTA3, seed treatment + root treatment + 1 spray with 0.001 mM; SRTB1, seed treatment + root treatment + 2 spray with 0.1 mM; SRTB2, seed treatment + root treatment + 2 spray with 0.01 mM; SRTB3, seed treatment + root treatment + 2 spray with 0.001 mM; SP1, spray with 0.1 mM; SP2, spray with 0.01 mM; SP3, spray with 0.001 mM; RT1, root treatment with 0.1 mM; RT2, root treatment with 0.01 mM; RT3, root treatment with 0.001 mM; RTA1, root treatment + 1 spray with 0.1 mM; RTA2, root treatment + 1 spray with 0.01 mM; RTA3, root treatment + 1 spray with 0.001 mM; RTB1, root treatment + 2 spray with 0.1 mM; RTB2, root treatment + 2 spray with 0.01 mM; RTB3, root treatment + 2 spray with 0.001 mM.

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TABLE 3 Effect of calliterpenone on yield and yield contributing traits in wheat.

Treatments		Days to 50% flowering	Plant height (cm)	Tillers plant^{-1}	Spike length (cm)	Seeds spike^{-1}	Biological yield plant^{-1}(g)	Grain yield plant^{-1}(g)	Harvest index (%)	Test weight (g)
Control		70.67	104.47	4.60	9.65	52.60	27.52	8.73	31.74	44.78
ST1		69.33	104.33	4.90	10.27	57.53	32.97	10.88	32.93	43.08
ST2		70.33	108.53	4.97	10.85	53.93	29.74	9.67	32.46	43.60
ST3		70.67	106.83	4.83	10.78	55.60	30.02	10.00	33.29	45.04
STA1		71.00	108.67	4.43	10.00	57.73	32.11	10.54	32.71	42.89
STA2		71.00	104.90	4.83	10.82	58.67	32.27	10.95	34.03	43.85
STA3		71.00	102.47	4.80	10.87	46.33	30.18	9.91	32.83	44.38
STB1		70.33	104.87	4.83	10.47	48.20	28.29	8.49	30.08	42.31
STB2		70.67	104.00	4.57	10.27	52.20	29.83	8.75	29.32	43.64
STB3		71.00	107.43	4.60	10.30	51.47	28.94	8.93	30.94	42.61
F ratio		3.60	10.52	4.95	5.76	4.03	2.61	2.52	1.01	0.90
F prob.		0.01	0.00	0.00	0.00	0.01	0.04	0.05	0.47	0.54
C.V.		0.67	1.05	2.85	2.83	6.62	6.31	10.39	8.05	3.84
C.D. at 5%		0.81	1.91	0.23	0.51	6.07	3.27	1.73	4.75	2.87
S.E.M.		0.36	1.28	0.12	0.27	2.82	1.42	0.73	1.46	0.11
Range	Min	69.33	102.47	4.43	9.65	46.33	27.52	8.49	29.32	42.31
	Max	71.00	108.67	4.97	10.87	58.67	32.97	10.95	34.03	45.04

C.V., coefficient of variation; C.D., critical difference; S.E.M., standard error of mean; Other abbreviations are as shown in Table 1.

STA1 (10.54 g). However, lower grain yield plant^{-1} than control (8.73 g) was recorded in STB1 (8.49 g). In the context of harvest index and test weight, it was observed that there were no significant differences among treatments.

Effect of calliterpenone on the yield of vegetable crops tomato, potato, and onion

All the treatments in tomatoes (Figure 2C, Supplementary Table S1) resulted in significantly greater yields than control, except SRTB2 (seed treatment + root treatment + 2 sprays with 0.01 mM). The maximum yield increase of 28.63% was observed in ST3 followed by 26.59% in ST2, 23.77% in SRTB1 (seed treatment + root treatment + 2 spray with 0.1 mM), 21.39% in SRTB3 (seed treatment + root treatment + 2 spray with 0.001 mM), 21.10% in SRTA3, 20.55% in ST1, 18.94% in SRTA1 and 15.34% in SRTA2.

Most of the treatments showed significantly higher yields than the control, except STA3 and STB3. The maximum yield increase in potato (Figure 2D, Supplementary Table S1) was noticed in ST2 (37.17%) followed by ST3 (32.46%), ST1 (24.46%), STA2 (20.42%), STA1 (15.18%), STB1 (13.86%), and STB2 (12.87%).

In the case of onion (Figure 2E, Supplementary Table S1), considerably higher yields over control were observed in RT3 (root treatment with 0.001 mM) (20.63%) followed by RT2 (root treatment with 0.01 mM) (18.83%) and RTB1 (root treatment + 2 spray with 0.1 mM) (16.59%).

Effect of calliterpenone on a pulse crop, chickpea

Chickpea is the most important pulse crop; however, its productivity is relatively low compared to cereals. With the use of calliterpenone, significantly higher yields (Figure 2F, Supplementary Table S1) were observed in treatments SP3 (spray with 0.001 mM) (26.08%) followed by STA2 (21.02%), STA3 (20.21%), ST3 (15.35%), and SP2 (spray with 0.01 mM) (10.35%).

Effect of calliterpenone on endogenous IAA and ABA contents

The application of calliterpenone modulated the plant's endogenous hormonal levels by increasing IAA and decreasing ABA contents (Figure 3). The maximum increases in IAA level were noticed with the concentration of 0.1 mM followed by 0.01 and 0.001 mM compared to control plants. However, a maximum reduction in ABA content was observed in 0.001 mM followed by 0.01 and 0.1 mM concentrations than control.

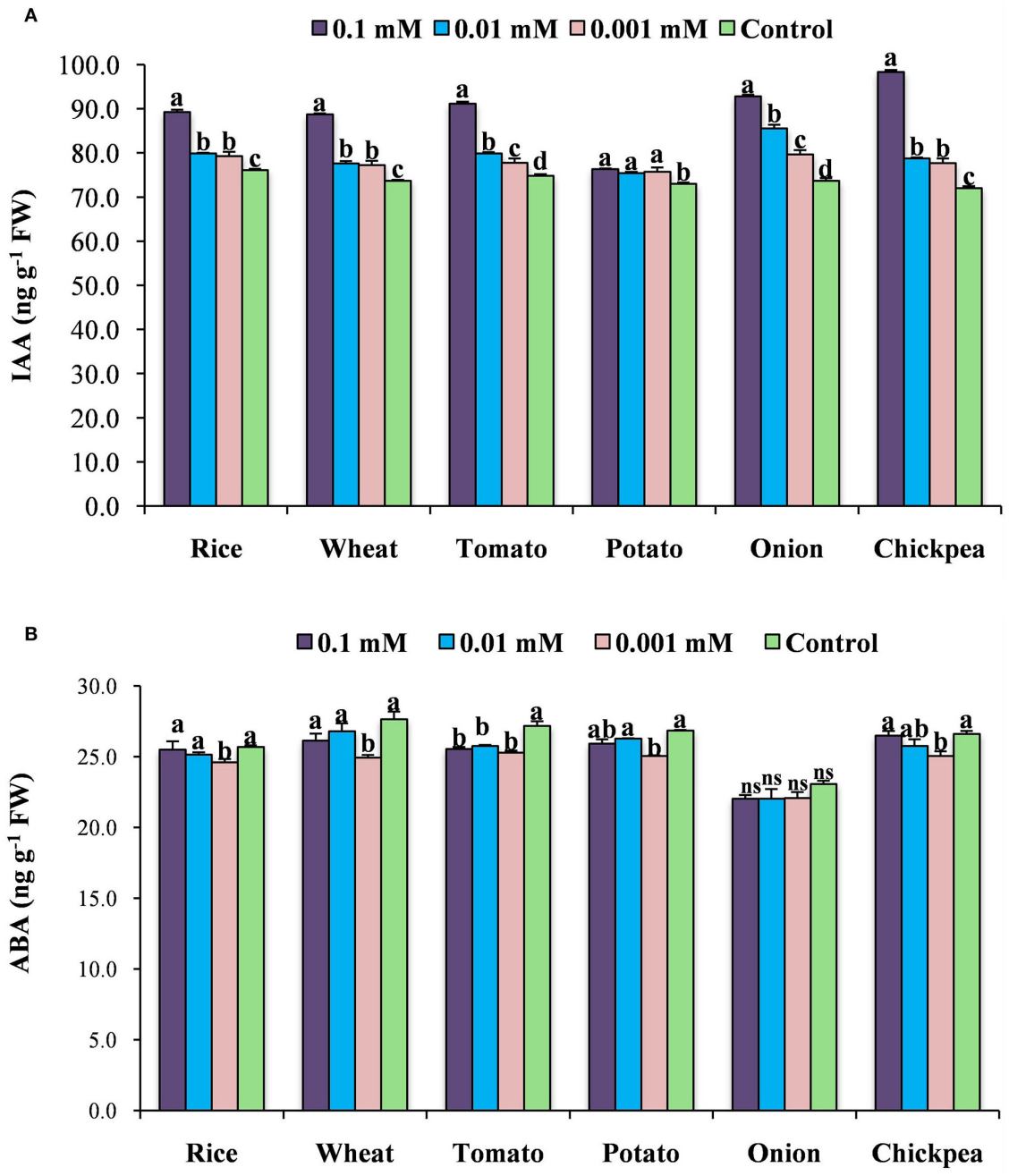


FIGURE 3
Effect of calliterpenone on endogenous IAA content **(A)** and ABA content **(B)** in six crops. Statistical analysis was carried out taking means of three replicates; different letters above bars showed significant differences as compared to control (Duncan's multiple range test $P \leq 0.05$).

Performance of calliterpenone in rice and wheat crops grown in farmers' fields

Results of 11 farmers' field trials from 3 districts (Lucknow, Sitapur, and Raebareli) showed that the application of calliterpenone resulted in an overall 11.93% increase in rice grain yield ranging from 3.48 to 19.63% (Table 4). This yield increase resulted from improvements in growth and yield contributing traits, *viz*., plant height (0.39%), tillering (3.34%), and panicle length (2.59%). In the case of wheat, an overall

TABLE 4 Effect of calliterpenone on growth and yield of rice and wheat at farmers' field.

Crops	Characters	% increase over control	Range	*df*	*t* value	*p* value
Rice	Plant height	0.39	−0.40 to 1.19	20	0.47	0.65
	Tillers plant^{-1}	3.35	1.39 to 5.71	20	2.26	0.04
	Panicle length	2.59	0.43 to 5.33	20	4.42	0.00
	Grain yield	11.95	3.48 to 19.63	20	4.32	0.00
Wheat	Plant height	2.791	0.75 to 4.38	20	2.22	0.04
	Tillers plant^{-1}	3.791	2.22 to 5.75	20	1.53	0.14
	Spike length	2.423	0.49 to 3.50	20	1.80	0.09
	Grain yield	9.708	3.91 to 17.51	20	2.02	0.05

TABLE 5 Most effective concentration of calliterpenone in various crops.

Crops	Effective concentration (mM)	Increased yield (%)
Rice	0.001	28.89
Wheat	0.01	25.71
Tomato	0.001	28.63
Potato	0.001	32.46
Onion	0.001	20.63
Chickpea	0.001	26.08

increase of 9.71% in grain yield, which ranged from 3.91 to 17.51% in different fields, was observed and an improvement in other growth and yield contributing traits, *viz.*, plant height 2.79%, tillering 3.79%, and spike length 2.42%, were also recorded.

Most effective doses

The effective doses of calliterpenone are presented in Table 5, which revealed that 0.001 mM of calliterpenone was the best effective dose for most crops except wheat, where a concentration of 0.01 mM was found to be the most optimal. The most appropriate stage for its application was found to be seed treatment, followed by a foliar spray at 30 DAP.

Safety studies

To test the formulation for its safety, the residue analysis of calliterpenone was carried out in the samples of rice for leaves, straw, grains, husk, and soil for all the treatments, and no traces of calliterpenone were recorded in any sample.

Discussion

Plant growth regulators play a vital role in sustainable crop production by increasing plant morphological characteristics, including plant height, biomass, and fruiting. Organically produced foods are remarkably healthier, more attractive, and usually preferred by consumers (Hajšlová et al., 2005), but organic cultivation restricts the use of chemically synthesized plant growth hormones. Calliterpenone, a natural plant growth enhancer, therefore, might be a useful product for organic farming.

The plant growth-promoting activities of calliterpenone were found better than many cytokinins and gibberellic acid when tested during *in vitro* experiments (Singh et al., 2004; Goel et al., 2007). It has been reported earlier that calliterpenone possesses growth-promoting activities of microbes (Kalra et al., 2010; Patel et al., 2014), besides improving the yield and productivity of many plants (Haider et al., 2009; Singh et al., 2010, 2011; Bose et al., 2013; Maji et al., 2013; Zafar and Sangwan, 2019).

Seed yield is a very complex characteristic, which is the end product of different growth and developmental processes, influenced by several yield components (Chen et al., 2019). Generally, a higher number of effective tillers, large spikes, and more fertile seeds are closely associated with higher seed yield (Tiwari et al., 2011). It was clear that treating plants with calliterpenone results in increased plant height, tillering, spike length, and seeds spike^{-1} over the control, which might be associated with the growth-promoting properties of calliterpenone in stimulating cell division, elongation, and enlargement, which could have increased the total biomass of the plants (Bora and Sarma, 2006; Khassawneh et al., 2006).

Plant growth regulators have the potential to improve the actual crop yields once the crop growth is stimulated and the photosynthates are accumulated in the harvested products (Setia and Setia, 1990). A plant's improved vegetative growth and vigor are mostly responsible for enhanced seed yield because several photosynthesizing sites are affected by the initial growth stages.

On the contrary, we found that although plant biomass increased at higher concentrations, seed yield was reduced. Earlier workers have also reported similar effects of calliterpenone on various crops (Goel et al., 2007; Haider et al., 2009; Singh et al., 2011). It was also found that foliar spraying of calliterpenone at 30 DAP was more effective than at 60 DAP. This effect may be due to the availability of plant growth substances at an appropriate time when the meristematic tissues of plants are maximally active. During active growth, dividing cells need more mobilization of nutrients triggered by the timely availability of phytohormones (Shah et al., 2006). However, active plant growth starts declining by 60 DAP; therefore, the spray of plant growth substance might have a limited role to play. Similarly, higher and/or repeated doses may cause desensitization of receptors, leading to no effect on plant growth. This higher dose-dependent receptor desensitization may be responsible for the terminating cell-signaling response at relatively higher doses of calliterpenone. However, after applications at doses below a threshold level, such receptors may again become sensitive (Xiao et al., 1999).

Foliar application of phytohormones affects the growth and yield of crop plants. It has been demonstrated that foliar treatments of IAA with appropriate amounts promoted plant growth and enhanced the yield of several crops like *Brassica* (Mir et al., 2020), *Guizotiaabys sinica* (Talukdar et al., 2022), *Linum usitatissimum* (Rastogi et al., 2013), and *Epipremnum aureum* (Di Benedetto et al., 2015), whereas its suboptimal application leads to a reduction in crop yield (Rastogi et al., 2013; Di Benedetto et al., 2015). In our studies, maximal benefits of the application of calliterpenone could be achieved with seed treatments. Cellular IAA-ABA interaction extensively regulates plant growth and development (Emenecker and Strader, 2020). Therefore, here we determined the content of IAA and ABA in calliterpenone-treated plants. Exogenous application of calliterpenone on the selected crops could influence the endogenous IAA and ABA levels (increased IAA and decreased ABA contents compared to control plants). Guzmán-Téllez et al. (2014) suggested that increased levels of endogenous IAA might result in an inhibitory effect; therefore, the optimal endogenous levels must be maintained for obtaining enhancement effects on various growth parameters. Similarly, owing to the endogenous accumulation of IAA, a significant increase in plant growth and biomass was observed, though the effect of these changes on seed yield is uncertain. Consequently, we found that the optimal level of IAA and ABA for maximum yield was a 0.001 mM concentration in most of the crops except wheat, where a concentration of 0.01 mM was found to be the most optimal. Recent studies have also shown that the exogenous application of hormones considerably increases endogenous IAA content and decreases ABA content in wheat plants (Yang et al., 2011; Cai et al., 2014).

Plant growth regulators have enormous potential for increasing crop yields, while their deployment and evaluation must be carefully designed in terms of optimal concentration, application levels, and species specificity (Khan and Mazid, 2018). The results of this study showed that the efficacy of calliterpenone, though not crop-specific, varies with the concentration, time of application, and methods of application. In rice, a maximum yield increase (28.89%) was observed through seed treatment with 0.01 mM calliterpenone followed by seed treatment with 0.001 mM (23.26%), both statistically at par and significantly higher than control. It was also observed that seed treatment followed by root treatment and a foliar spray at 30 DAP produced a lower yield than seed treatment with the same dose because seed treatment following root treatment and a foliar spray negatively impacted important yield components, *viz.*, tillers plant^{-1} and seeds panicle^{-1}. Though the concentration of 0.01 mM gave higher yields, we recommend a 0.001 mM dose of calliterpenone for rice seed treatment as it is more economical compared to 0.01 mM while producing at par yields. Similarly, in wheat, 0.1 mM concentration of calliterpenone applied through seed treatment and a foliar application at 30 DAP resulted in the highest yield (27.23%), followed by seed treatment and foliar application at 30 DAP with a 0.01 mM concentration (25.71%), which was at par with each other. This enhancement in yield was mainly associated with the greater number of seeds spike^{-1} compared to other treatments. Hence, we recommend a 0.01 mM concentration of calliterpenone in wheat applied through seed treatment followed by a foliar spray at 30 DAP. Tomato, potato, and onion are the three most consumed and produced vegetable crops in India. Production of these vegetables over the past few years has dramatically increased, making India the second largest vegetable producer in the world (Gulati et al., 2022). This achievement was made possible through improved varieties, modern farming practices, and the use of efficient fertilizers; besides this, PGRs are also becoming important in vegetable farming. In vegetable crops such as tomato, potato, and onion, the yield was significantly improved by applying calliterpenone with a 0.001 mM concentration. Seed treatment was found to be more effective in tomatoes and potatoes, whereas root treatment produced the maximum yield in onions. Moreover, the application of calliterpenone at earlier stages produced higher yields compared to later stages of crop growth, which is in conformity with the previous findings (Naeem et al., 2001; Shah et al., 2006). In chickpeas, foliar spray with 0.001 mM was found to be more effective than the treatment of the same dose through other application methods, so we recommend a 0.001 mM dose of calliterpenone as a foliar spray in chickpeas. These findings are in agreement with several recent studies that also found that foliar sprays of PGRs at limited concentrations significantly improve the growth as well as yield attributes in rice (Prajapati et al., 2021), wheat (Zhang et al., 2017; Dawar et al., 2021), potato (Thornton et al., 2013), tomato (Choudhury et al., 2013), onion (Ghani et al., 2021), and chickpea (Rafique et al., 2021).

Conclusion

The plant growth-enhancing effects of calliterpenone (a phyllocladane diterpenoid) isolated from a medicinal plant *Callicarpa macrophylla* have been earlier demonstrated in many medicinal and aromatic plants, *viz.*, *Pyrethrum*, *Mentha arvensis*, *Rauwolfia serpentina*, and *Artemisia annua* and found better than many well-established growth promoters such as gibberellins, auxins, and cytokinins. This encouraged us to study its effects on agriculturally important field crops (cereals: rice and wheat; vegetables: tomato, potato, and onion; and pulses: chickpea). Our results clearly illustrated that calliterpenone significantly enhances crop yields at a lower concentration of 0.001 mM in most crops except wheat, for which 0.01 mM was the most effective. Moreover, the exogenous application of calliterpenone could increase the endogenous level of IAA in all tested crops and reduce the content of ABA in many crops. Therefore, calliterpenone can be effectively used in augmenting the crop yields of agricultural/horticultural crops. Cost-effective and ready-to-use formulations of calliterpenone with a higher degree of purity enhance its potential for commercialization as a natural growth-promoting compound valuable for increasing crop yield and productivity. It will not only improve crop productivity and help in the upliftment of the socio-economic status of farmers, but it could also be helpful in solving world food problems. We firmly believe that it will sustainably increase crop yields and could be a vital input component of organic farming.

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

AK conceived the experiment and planned the methodology along with HS. AS and ST isolated the compound calliterpenone. PP did the experiment, collected data, and wrote the manuscript. AA helped in statistical analyses. SP and AT helped in manuscript preparation. AK corrected and finalized the manuscript. All authors reviewed and agreed on the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the study 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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Supplementary material

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Corrigendum: Calliterpenone, a natural plant growth promoter from a medicinal plant *Callicarpa macrophylla*, sustainably enhances the yield and productivity of crops

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KEYWORDS

calliterpenone, plant growth regulators, IAA, ABA, yield contributing traits, sustainable crop production, medicinal plants

A Corrigendum on

Calliterpenone, a natural plant growth promoter from a medicinal plant *Callicarpa macrophylla*, sustainably enhances the yield and productivity of crops.

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In the published article, there was an error in the legend for Figure 1: "Structure of calliterpenone and gibberellic acid". The corrected legend appears below. There was also an error in Figure 1, Abbeokutone was mislabelled as "Gibberellic acid".

The corrected Figure 1 appears below.

In the published article, a compound name was misspelled in the Introduction. ["abeoketone"]. This sentence previously stated: "Calliterpenone (16α, 17-dihidroxy phyllocladane-3-one) (Figure 1), has a similar substitution pattern similar to the ent-kaurenoid compound "abeoketone" (16α, 17-dihidroxy kaurane-3-one), the precursor of gibberellins in the biosynthetic pathway (Liu et al., 2003; Bottini et al., 2004)."

The correct sentence appears as follows:

10.3389/fpls.2022.1055825

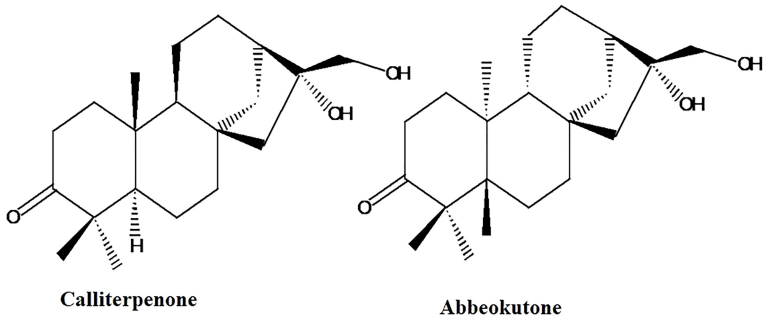


FIGURE 1
Structure of calliterpenone and abbeokutone.

"Calliterpenone (16α, 17-dihidroxy phyllocladane-3-one) (Figure 1), has a similar substitution pattern similar to the entkaurenoid compound "abbeokutone" (16α, 17-dihidroxy kaurane-3-one), the precursor of gibberellins in the biosynthetic pathway (Liu et al., 2003; Bottini et al., 2004)."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Cleaner production technologies for the amelioration of soil health, biomass and secondary metabolites in *Ocimum basilicum* L. under Indian Western Himalaya

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Ocimum basilicum L. and its derived products are primarily consumed by humans; hence, agrochemical use seems inappropriate for its cultivation. However, farmers are accustomed to using rampant inorganic fertilizers to augment crop productivity, which has unintendedly engendered severe environmental perturbations. Concomitantly, farmers will soon have to confront the challenges of growing crops under suboptimal conditions driven by global climate change. Consequently, to develop a clean, sustainable, and resilient production technology, field experiments spanning over two years (2020 and 2021) were conducted, comprising three biostimulants, *viz*., vermicompost (0, 4, and 8 Mg ha^{-1}), biofertilizer (uninoculated and inoculated), and liquid seaweed extract (without and at 7 ml L^{-1}) in the Indian western Himalaya for the first time. Soil health indicators, leaf photosynthetic pigments, gaseous exchange, mineral contents, essential oil (EO) quantity, and composition were evaluated. Soil microbial respiration (SMR), microbial biomass carbon (MBC), organic carbon (OC), dehydrogenase (DHA), alkaline phosphatase (ALP), and β-glucosidase activities were increased by 36.23, 83.98, 30.61, 42.69, 34.00, and 40.57%, respectively, when compared with the initial soil status. The net photosynthetic rate (Pn) was significantly increased with the highest (8 Mg ha^{-1}) and moderate (4 Mg ha^{-1}) vermicompost dosages by 13.96% and 4.56%, respectively, as compared with the unfertilized control (0 Mg ha^{-1}). Likewise, the biofertilizer and seaweed extract also enhanced Pn by 15.09% and 10.09%, respectively. The crop's key EO constituents, *viz*., methyl chavicol and linalool, were significantly improved with the highest and moderate vermicompost rates of 2.71, 9.85%, and 1.18, 5.03%, respectively. Similarly, biofertilization and seaweed application also boosted methyl chavicol and linalool by 3.29, 8.67%, and 1.93, 3.66%,

respectively. In both years, significantly higher herbage (8.86 and 11.25 Mg ha^{-1}) and EO yield (113.78 and 154.87 kg ha^{-1}) were recorded with a congregate treatment of the highest vermicompost dose, biofertilizer, and liquid seaweed extract. In conclusion, the integrated use of biostimulants having complementary properties can sustainably maximize the quantity and quality of *O. basilicum* and concomitantly ameliorate soil health. This study can inspire scientific communities and industries to develop second-generation biostimulant products, delivering better sustainability and resilience for a renaissance in agriculture.

KEYWORDS

biostimulants, essential oil, leaf gaseous exchange, methyl chavicol, plant growth promoting rhizobacteria, soil health, sustainable production

Introduction

Global consumption of primary nutrient fertilizers, *viz*., nitrogen (N), phosphate (P_2O_5), and potash (K_2O) applied in agriculture is expected to increase annually by ~1.5, 2.2, and 2.4%, respectively, and eventually reach up to ~202 million Mg by the end of 2020 (FAO, 2017). India, being the largest consumer of chemical fertilizers, consumes ~16% N, ~19% P_2O_5, and ~15% K_2O nutrients every year compared to the total world's consumption (Parween et al., 2017). Although chemical fertilizers augment crop productivity, their extensive and imprudent use has unfortunately prompted soil health degradation (Banerjee et al., 2019), eutrophication, climate change (Frank et al., 2019), and consequently declined agricultural productivity (Adegbeye et al., 2019). In addition, shortages of synthetic chemical fertilizers like urea, ammonium nitrate, calcium ammonium nitrate, mono, di-ammonium phosphates, and single, triple-superphosphates are also expected in the near future, as they are all made from non-renewable resources (Blouin et al., 2019). Concomitantly, their production process also emits harmful greenhouse gases (GHG_S) like methane (CH_4), oxides of carbon (CO and CO_2), nitrogen (NO_x), and sulfur (SO_2), even a poisonous volatile hydrogen fluoride gas (HF) (Hao et al., 2020). Meanwhile, ~80% and 25%–75% of N and P_2O_5 fertilizers get lost in the environment, and this loss is encouraged by excessive precipitation/rainfed agroecosystems (Choudhary and Kumar, 2019). Likewise, ~1% of chemical pesticides reach their target sites, and the remaining amount resides in the environment. These worries about using synthetic inorganic agrochemicals compelled scientific communities to develop alternative, environment-friendly, and safer methods to improve crop growth and agricultural yields in a sustainable manner. In this context, organic farming, which defies and abates agrochemical use, has sparked exorbitant consumer attention and scientific interest. Nonetheless, the main hitch in organic farming is its lower and erratic yield compared to conventional farming. The judicious application of biostimulants, which are compatible with organic farming, can act as a panacea, truncating this apparent yield gap between organic and conventional agriculture systems. Moreover, global climate change has also aggravated irregularities in crop yields. This could be reconciled with the use of biostimulants, as they can regulate and alter the plant's physiological processes to confront or alleviate stressors and consequently assure yield stability.

Sweet basil (*Ocimum basilicum* L.) is an annual, essential oil (EO) producing medicinal aromatic plant (MAP), predominantly cultivated for direct human consumption and chemical use is inappropriate for its production (Maham et al., 2018). The crop ranks second after spearmint; methyl chavicol and linalool are the key constituents attributed to the crop's characteristic aroma (Tahami et al., 2017). It is cultivated during the rainy season in tropical areas throughout the world. India has occupied ~60% (3,000 ha) of the world's area (5,000 ha), generating ~70% of EO (350 Mg) annually, contrary to the total world's production (~500 Mg) which is still short for its growing demand (Absar et al., 2016). Furthermore, globally, ~3,000 forms of plant EO are traded (~40,000–60,000 Mg $annum^{-1}$) with an estimated commercial market value of ~US $700 million which is also forecasted to expand in the coming years (Chouhan et al., 2019). The basil EO market is also estimated to increase with a 6.2% cumulative average growth rate (CAGR), thereby reaching a value of US$ 285.69 million in 2027. Sweet basil EO is widely used either directly as methyl chavicol or after its conversion into anethole in the food and flavoring industries for the preparation of baked goods, condiments, vinegars, oils, cheese, jams, teas, ice creams, sausages, meat products, salad dressings, liqueurs, and

beverages. Some pharmaceutical industries also use basil EO for making gripe water. Furthermore, for millennia, people have used basil leaves and their EO to treat several common ailments like headaches, colds, digestive problems, and bloating. Also, in the conventional medicine system, basil is prescribed as a stimulant, fever reducer, antimalarial, and anti-flatulent (Tavallali et al., 2018). Meanwhile, the worldwide basil leaf market is also predicted to rise with a 1.3% CAGR, reaching up to 62 million by 2026 (Sipos et al., 2021), thus indicating its significant consumption.

Considering the perils of using inorganic fertilizers and the worth of MAPs, the use of organic biostimulants, *viz*., compost, biofertilizers, and seaweed extracts can be recommended as an alternative solution for their sustainable production (Bhattacharyya et al., 2021; Gupta et al., 2021a; Huang et al., 2021; Kulkarni et al., 2021). These can provide almost all essential nutrients for crop growth and also provide micronutrients, *viz*., copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn) at optimal levels with better acquisition. Additionally, these can supply organic carbon (OC), which improves soil edaphic conditions and ultimately increases crop yield. Among composts, vermicompost is considered the best fertilizer, having higher humic substances (HSs), being homogenous, microbiologically active, and less phytotoxic (Soobhany, 2019). Moreover, vermicomposting is a greener and cleaner process that involves the bio-oxidation and stabilization of organic waste due to the interactions between earthworms and microorganisms. Likewise, nowadays propensity of using biofertilizers is prevailing as a suitable alternative to counteract the adverse environmental impacts exerted by synthetic agrochemicals (Gupta et al., 2021b). They are composed of microbial living cells which can augment plant growth by making some of the vital nutrients available from their unavailable forms through regulation of biogeochemical cycles (C, N, P, K, S), production of plant growth regulators (PGRs) like indole-3-acetic acid (IAA), gibberellic acid (GA), and cytokinin (CK), and discharge of biological materials such as vitamin B, nicotinic acid, pantothenic acid, and biotin into the soil (Mącik et al., 2020). Additionally, the seaweed extracts possess macro and micronutrients including some PGRs such as IAA, CK, abscisic acid, amino acids, and vitamins which can improve plant growth, soil microecology, and fertilizer use efficiency indicating it as a potential fertilizer/biostimulant (Jung and Kim, 2020). Besides these, a recent study claimed that the presence of some secondary metabolites in seaweed extracts like sulfabenzamide, 1-phosphatidyl-1D-myoinositol, and dodecanamide has proven bioactivities related to plant growth promotion and disease resilience (Vaghela et al., 2022). Also, the presence of quaternary ammonium compounds like glycine, betaine, and choline chloride in seaweed extracts may impart resilience to crops against various stressors, especially under rainfed conditions because of their osmolytic properties (Singh et al., 2016b). Furthermore, the production process of seaweed extract is eco-friendlier and does not entail any harmful field emissions (Singh et al., 2018; Sudhakar et al., 2019).

Owing to the plant growth promoting (PGP) characteristics and biostimulatory response of vermicompost, biofertilizer, and seaweed extracts (Wozniak et al., 2020), we hypothesized that their congregate use could remarkably augment sweet basil productivity compared to sole application and also increase EO synthesis holistically by altering the physiological and biochemical processes of the plant. As plant growth-promoting rhizobacteria (PGPR) present in biofertilizers are heterotrophic in nature and continuously require a source of carbon and energy, adding vermicompost can decisively augment PGPR growth and metabolic activities by providing readily available nutrients, eventually leading to enhanced crop productivity. Concomitantly, the seaweed extract can promote the growth of PGPR and also establish unison with their host plant by inducing a mimicking effect. Furthermore, the current prevailing voyage of investigating the complementary properties of applying combinations of different biostimulant categories has demonstrated their additive/synergistic effect on improving crop productivity (Gupta et al., 2021a; Sani and Yong, 2021). While, some combination treatments like PGPR (*Pseudomonas fluorescens* and *Bacillus licheniformis*) and smoke-derived compound (karrikinolide) showed antagonistic effects also (Papenfus et al., 2015; Salomon et al., 2017). Nevertheless, these investigations were predominantly performed on non-MAP crops like onion, groundnut, grapevine, lettuce, okra, etc., under controlled/semi-controlled conditions; albeit, their evaluations under open field conditions are scanty and remain a discerning task (Rouphael and Colla, 2018; Rouphael and Colla, 2020). As crops confront combined multifaceted stressors in open fields, which are difficult to mimic in a controlled environment; also, the biostimulants screened in controlled environments do not always enact as expected under field conditions (Rouphael et al., 2018). Moreover, field-scale evaluation of biostimulants on growth, productivity, secondary metabolite accumulation, and composition, especially in MAPs, is still a major backdrop.

Based on the available literature, the synergistic and additive properties between biostimulant categories are fascinating and indicative of their intricate biostimulatory mechanisms in determining plant growth, performance, and resilience. Consequently, it is indispensable to decipher the sole and combined effects of biostimulants to invent the next generation of biostimulant products having harmonious properties for improved crop growth, yield, quality, and resilience. Additionally, with improved mechanistic clarity, it will be possible to design deliberate combinations of non-microbial and microbial biostimulants that would interact synergistically to provide the required results in terms of acceptable yield and quality products in a sustainable manner. Therefore, accentuating the inextricable and serious collateral problems of using chemical fertilizers, coupled with their forthcoming shortages, and the context of sustainable

production of MAPs, the current study was designed to evaluate the synergistic/additive effect of diverse biostimulants on growth, productivity, secondary metabolite accumulation, and composition in sweet basil. The salient objectives of the study were: (i) to analyze the effect of biostimulants on the physiological performance of sweet basil under field conditions; (ii) to elicit the amount and key constituents of sweet basil's EO and elucidate its plausible mechanism; and (iii) to evaluate the effect of organic biostimulant amendments on soil physical, chemical, and biological properties.

Materials and methods

Experimental details, cultivation, and treatment procedures

A field study was conducted for two consecutive cropping years (2020 and 2021) at the experimental farm of CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India (elevation: 1,328 m above mean sea level; latitude: 32°11′39′′ N; longitude: 76°56′51′′ E). The first factor was composed of three levels of vermicompost (0, 4, and 8 Mg ha^{-1}); the second factor was comprised of two levels of biofertilizer (uninoculated, or inoculated), and the third factor consisted of two levels of seaweed extract (control, and foliar application at 7 ml L^{-1}) (Table 1). The experimental plot consisted of three blocks, each 25 m long and contained 12 plots of 3.6 m × 3.0 m. In each plot, 48 plants were grown within 45 cm × 50 cm spacing, eight rows of 6 plants $plot^{-1}$. The composition and attributes of biostimulants used in the current study are provided in Table 2.

The Indian sweet basil seeds of accession IHBT/OC-1, having 98% vigor, were manually planted in pots comprising sand, soil, and farmyard manure in a 1:1:1 ratio within a depth of 1–2 cm during the first week of June in both years. Seeds were germinated 7–14 days after sowing, and thirty-day-old seedlings (30 DOS) having an average root length of 11.93 cm and a shoot length of 15.76 cm (Supplementary Figure S1A) were used for transplantation. The corresponding dose of vermicompost was added by manually spreading it on the topsoil surface of the corresponding plots 30 days before transplanting. The biofertilizer was prepared with two PGPRs strains, i) *Arthrobacter psychrochitiniphilus* IHB B 13602 (KU160185) isolated from the rhizosphere of agricultural land situated at the cold desert region of the Lahaul valley and ii) *B. altitudinis* IHBT 705 (CP074101) harnessed from the native agricultural field of Palampur, Himachal Pradesh, India. These PGPR strains were selected based on their multiple PGP traits like IAA synthesis (27.30 ± 1.21, 59.91 ± 1.17 μg ml^{-1}), siderophore production (20.00 ± 1.32, 15.00 ± 0.92 mm), phosphate solubilization (62.82 ± 1.03, 66.25 ± 1.01 μg ml^{-1}), and ACCD (1-aminocyclopropane-1-carboxylate deaminase) activity (Table 2). The preparation of biofertilizer was carried out by growing them in sterile tryptone soya broth (TSB) at 28°C (180 rpm; 24 h). Afterward, the bacterial cells were pelleted down by centrifugation at 6,000 rpm by using an Eppendorf 5804R benchtop refrigerated centrifuge for 10 min, followed by dissolution into sterile deionized water and mixing with a sterile carrier material (biochar). Thereafter, to make a consortium, both were mixed at a 1:1 ratio; the biofertilizer thus formed has 1.8×10^8 CFU ml^{-1} bacterial cells. Biofertilizer was applied at two stages: i) at the seedling transplanting stage (30 DOS; 6–8 leaves stage); ii) at the first weeding event; 20 days after transplanting (DAT) after the establishment of plants in the main field.

TABLE 1 Abbreviation and treatment details.

Sr. No.	Treatment code	First factor (organic manure)	Second factor (biofertilizer)	Third factor (sea weed extract)	Abbreviation
1	T1	No added manure	–	–	V–
2	T2	No added manure	–	+	S+
3	T3	No added manure	+	–	B+
4	T4	No added manure	+	+	B+S+
5	T5	Vermicompost at 4 Mg ha^{-1}	–	–	V4
6	T6	Vermicompost at 4 Mg ha^{-1}	–	+	V4S+
7	T7	Vermicompost at 4 Mg ha^{-1}	+	–	V4B+
8	T8	Vermicompost at 4 Mg ha^{-1}	+	+	V4B+S+
9	T9	Vermicompost at 8 Mg ha^{-1}	–	–	V8
10	T10	Vermicompost at 8 Mg ha^{-1}	–	+	V8S+
11	T11	Vermicompost at 8 Mg ha^{-1}	+	–	V8B+
12	T12	Vermicompost at 8 Mg ha^{-1}	+	+	V8B+S+

V, vermicompost; V–, unfertilized control; V4, vermicompost at 4 Mg ha^{-1}; V8, vermicompost at 8 Mg ha^{-1}; B, biofertilizer; S, seaweed extract; (–), without factor; (+), with factor.

TABLE 2 Physicochemical attributes and composition of organic manure (vermicompost), carrier material (biochar), biofertilizer, and liquid seaweed extract (Sagarika, IFFCO, India) used during the experiment.

#Organic manure (Vermicompost)						
Attributes	pH	EC (m mhos cm^{-1})	OC (%)	N (%)	P (%)	K (%)
Value	6.9 ± 0.10	7.1 ± 0.20	27.53 ± 0.16	1.49 ± 1.21	0.41 ± 0.33	2.30 ± 0.60
#Carrier material (biochar)						
Attributes	pH	EC (m mhos cm^{-1})	OC (%)	Bulk density (Mg cm^{-3})		WHC (%)
Value	6.1 ± 0.12	3.6 ± 0.13	5.30 ± 0.23	0.71 ± 0.41		8.9 ± 0.19
#Biofertilizer						
	Arthrobacter psychrochitiniphilus IHB B 13602 (KU160185)				*Bacillus altitudinis* IHBT 705 (CP074101)	
PGP attributes	PS (μg ml^{-1})	IAA (μg ml^{-1})	SP (mm)	ACCD activity	PS (μg ml^{-1})	IAA (μg ml^{-1}) SP (mm) ACCD activity
Value	62.82 ± 1.03	27.30 ± 1.21	20 ± 1.32	+	66.25 ± 1.01	59.91 ± 1.17 15 ± 0.92 +
*Seaweed extract (Sagarika, IFFCO, India; 28% w/w *Kappaphycus alvarezii* and *Sargassum swartzii*)						
Attributes	N (%)	P (%)	K (%)	S (%)	Auxin (ppm)	Cytokinin (ppm) Gibberellin (ppm)
Value	0.12–0.30	0.05–0.19	14.00–18.00	1.25–2.25	400–600	200–400 500–800

#Results of composition of organic manure (vermicompost), and carrier material (biochar) and biofertilizer attributes are presented as means of three replicates (n = 3) ± standard deviation.
*Whereas the composition of seaweed extract was provided by the manufacturer (IFFCO, India) on the label of the product.
pH, potential of hydrogen; EC, electrical conductivity; OC, organic carbon; N, available nitrogen; P, available phosphorous; K, available potassium; S, available sulfur; WHC, water holding capacity; PGP attributes, plant growth promoting attributes; PS, inorganic phosphate solubilization; IAA, indole-3-acetic acid production; SP, siderophore production (qualitative); ACCD, 1-aminocyclopropane-1-carboxylate deaminase activity.

The first inoculation of biofertilizer was achieved through root dipping (Verma et al., 2016) of disinfected seedlings (Supplementary Figure S1B) for 15 min; sterile jaggery was used as a sticking agent, whilst booster inoculation was facilitated through a hoeing procedure in well-established plants by exposing the rhizospheric region of each plant and stuffing it with ~5 g biofertilizer, followed by covering it with soil to increase the bacterial rate and to ensure the infection of the new roots (Anli et al., 2020). Foliar application of liquid seaweed extract (28% w/w; Sagarika, IFFCO, India) derived from *Kappaphycus alvarezii* (red algae) and *Sargassum swartzii* (brown algae) at 7 ml L^{-1} was done using a Knapsack sprayer with a constant flow until complete canopy runoff during a cloudless sunny day (Elansary et al., 2016) at vegetative (15 DAT) and flower initiation stage (40 DAT). The crop was completely grown under rainfed conditions, with only two intermittent hand-weeding events at 20 and 35 DAT, respectively. The soil of the experimental area was characterized as silty clay with low OC (0.49 ± 0.11%), acidic pH (5.3 ± 0.02), deficient in P_2O_5 (7.60 ± 0.87 kg ha^{-1}), moderate in available N (139.10 ± 2.39 kg ha^{-1}), and rich in available K_2O (318.00 ± 3.19 kg ha^{-1}) (Supplementary Table S1). The climate of the region was subtropical. Weekly mean weather conditions like rainfall, temperature (maximum and minimum), relative humidity, and bright sunshine hours for the entire experimental duration were retrieved from the crop weather outlook agro-meteorological advisory (Anonymous, 2022) and have been represented in Figure 1. All considered climatic conditions varied during both experimental years, and notably, total rainfall was ~16.83% higher in the second year (Figure 1B) as compared with the first cropping season (Figure 1A).

Assessment of soil health

Soil samples before and after the experiment were collected from each plot with five core points (0–30 cm depth) and the rhizosphere of five plants from each plot, respectively. At each sampling event, 10–20 g of soil was collected, and the specimens were mixed to get a composite sample depicting the whole field. Soil biological, physicochemical, and enzymatic properties were analyzed. To assess the biological features such as soil microbial respiration (SMR), microbial biomass carbon (MBC), bacterial population count (BPC), and soil enzymatic activities some soil subsamples were put in zip-lock plastic bags and immediately kept inside a cold room (8°C), rest samples were shade dried, sieved with a 2 mm mesh and kept at room temperature until analysis. The OC was evaluated by adding potassium dichromate solution ($K_2Cr_2O_7$; 10 ml, 0.5 M), and deionized water (150 ml) to 0.5 g of homogenized soil. Diphenylamine was added followed by titration using 1 N ammonium iron (III) sulfate hexahydrate and expressed in percentage (OC%). The soil texture was determined by the hydrometer method while, pH was detected with soil and water (1:2) suspension by a glass electrode pH meter. A macro Kjeldahl method was adopted for the determination of available N by digesting 0.5 g of soil in a Kjeldahl digestion tube with concentrated sulfuric acid (10 ml), and the N concentration was determined by an N analyzer (Kelplus classic DX VA, Pelican Instruments Pvt. Ltd., India). Available phosphorous (P) was determined with a soil solution extracted with 0.5 M sodium bicarbonate ($NaHCO_3$) at 470 nm using a spectrophotometer (T90+ UV/vis, PG Instruments Limited, UK). At the same time, the amount of exchangeable K was quantified with 1 N extractable ammonium acetate

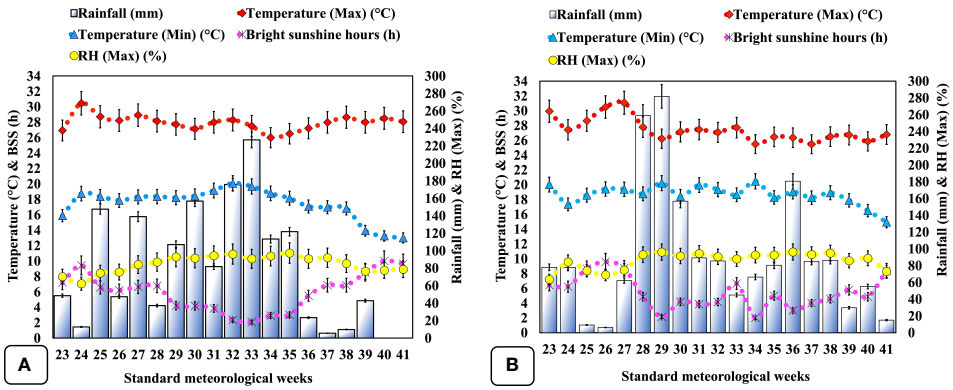


FIGURE 1
Climatogram representing weekly mean meteorological data during the period of the experiment (2020–2021) at Palampur, Himachal Pradesh, India. **(A)** weather data of the first cropping year (June–November, 2020); **(B)** weather data of the second cropping year (June-November, 2021). BSS, bright sunshine hours; RH (Max), maximum relative humidity.

(NH_4OAc) by flame atomic emission spectrometry (Raj et al., 2021). Soil micronutrients *viz.*, Mn, Cu, Zn, and Fe were quantified using an atomic absorption spectrophotometer as per the standard protocols (Jones, 1998), while the BPC was performed by serial dilution up to 10 folds by taking 1 g of soil into a sterile test tube containing 9 ml of sterile 0.9% normal saline and expressed as CFU g^{-1} soil (Raj et al., 2019).

The MBC and SMR were quantified using a modified fumigated and alkali absorption extraction techniques, respectively (Dehsheikh et al., 2020). Uniformly, soil (5 g) was fumigated with chloroform at 27°C for 24 h, followed by the addition of potassium sulfate (0.5 M; 25 ml) to make a soil suspension. The suspension was agitated for 30 min, and then filtered, followed by pH calibration (6.5–8.5). Afterwards, 10 ml of potassium dichromate (0.05 M) and 200 ml of deionized water were added, followed by titration with ferric ammonium sulfate hexahydrate (0.5 N) and diphenylamine. Simultaneously, the same procedure was repeated in non-fumigated soils as well. In addition, the soil dry weight was determined by placing 5 g of soil inside a hot air oven at 105°C. The MBC was estimated as per the standard equation and expressed as mg C 100 g^{-1} dry soil. To determine SMR, 10 g of homogenized soil was dispersed in a glass beaker having a lid, and the tube containing 5 ml of NaOH (0.25 M) solution was placed inside each flask to capture the CO_2, followed by incubation at 27°C for 48 h. The remaining NaOH solution was titrated with HCl (0.25 M), $BaCl_2$ (0.5 M), and phenolphthalein. Simultaneously, 10 g of soil was dried at 105°C, and SMR was calculated using a standard equation and expressed as mg CO_2 g^{-1} dry soil day^{-1}.

Soil enzymatic activities like dehydrogenase (DHA), urease, alkaline phosphatase (ALP), and β-glucosidase were accessed as per the standard procedures (Mukherjee et al., 2021) with appropriate modifications. DHA was estimated by taking uniformly soil (5 g) in a 50 ml flask, followed by the addition of calcium carbonate (0.1 g), 3% triphenyl tetrazolium chloride (TTC), and 4 ml of deionized water. The suspension was mixed gently by tapping and allowed to incubate at 37°C for 24 h. Then, for extraction, 40 ml of acetone was added. This was followed by the mixing of the content and then filtering. Furthermore, additional acetone was added and the volume was raised to 50 ml. Optical density (OD) was taken at 485 nm using a spectrophotometer for triphenyl formazan (TPF) in samples using a standard calibration curve and DHA was expressed as µg TPF g^{-1} dry soil h^{-1}. For determination of urease activity, soil (5 g) was taken in a flask (100 ml) and 2.5 ml of urea solution (0.08 M) was poured over. Then, the flask was stoppered and allowed to incubate at 37°C for 2 h, followed by the addition of 50 ml of potassium chloride solution (1 N KCl in 0.01 N HCl) and vigorously shaken for 30 min. Then the solution was filtered, and the clean filtrate (1 ml) was pipetted into another flask (50 ml), followed by the addition of deionized water (9 ml), sodium salicylate/sodium hydroxide solution (5 ml), and 2 ml of sodium dichloro-isocyanurate solution. The contents of flasks were incubated at room temperature for 30 min, followed by quantification of ammonium (NH_4^+) using a spectrophotometer at 690 nm, and urease activity was expressed as µg N-NH_4^+ g^{-1} dry soil h^{-1}.

For determination of ALP uniformly, 1 g of soil was taken in a flask (50 ml), followed by the addition of 0.25 ml toluene, 4 ml modified universal buffer (MUB) (pH 11), and 1 ml p-nitrophenyl phosphate solution (PNP). The flask was stoppered, followed by the mixing of all contents, and allowed to incubate at 37°C for 1 h. Afterward, 0.5 M calcium chloride (1 ml) and 0.5 M sodium hydroxide (4 ml) were added and again mixed to form a suspension. The suspension was then filtered, the filtrate's OD was measured at 400 nm, and the amount of soil ALP activity was expressed as µg PNP g^{-1} dry soil h^{-1}. The β-glucosidase activity was assessed by taking uniformly soil (1 g) in a test tube followed

by the addition of toluene (0.25 ml). Thereafter, 4 ml of MUB (pH 6) and 1 ml of p-nitrophenyl-β-D-glucoside (25 mM PNG) were added, followed by proper mixing. The suspension was incubated at 37°C for 1 h, followed by the addition of 1 ml of calcium chloride (0.5 M) and tris solution (4 ml) followed by filtration, and OD was taken at 400 nm. A calibration curve of standard p-nitrophenyl-β-D-glucoside (PNG) was prepared and the activity was expressed as μg PNG g^{-1} dry soil h^{-1}.

Plant sampling

Non-peripheral net plants (22) were considered and selected for various observations in all treatments to avoid the border effect. Five plants from each plot were tagged for observation of growth attributes like plant height, plant spread, and primary branches at 50 and 100 DAT. Leaf, stem, and inflorescence weight, number of inflorescences, average length of inflorescence, herbage yield, EO content, and yield were recorded at harvest. In addition, a few healthy leaves were also taken at 50 and 100 DAT to determine the photosynthetic pigments and leaf mineral concentration, respectively.

Estimation of photosynthetic pigments, mineral nutrients, and gaseous exchange

Uniformly, 1 g of fresh leaf sample was washed with deionized water. It was followed by homogenization with 80% acetone and centrifugation at 6,000×*g* for 5 min to collect the supernatant. The OD of the collected supernatant was measured at 663, 646, and 470 nm for Chl_a, Chl_b, and carotenoids, respectively, using a spectrophotometer. Acetone (80%) was used as a blank for the procedure, and concentrations of Chl_a, Chl_b, and carotenoids (mg g^{-1}) were calculated according to formulas previously described by Rathore and Kumar (2021). Additionally, dried pulverized leaves (0.5 g) were digested with concentrated H_2SO_4 and a mixture of concentrated H_2SO_4:$HClO_4$ (5:1) for estimation of N and P, respectively. The contents of N were estimated with an N analyzer while P was quantified by a spectrophotometer.

Three individual plants were selected from each plot for the measurement of gas exchange-associated characteristics like net photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r), intracellular CO_2 (C_i), and leaf vapor pressure deficit (VPD_{leaf}) using a portable leaf chamber of 2 cm × 3 cm (6 cm^2) having a red-blue LED light source and an infrared gas analyzer (LICOR-6400 XT®, LI-COR Biosciences, Lincoln, NE, USA). Before all measurements, the instrument was warmed up for ~30 min and an auto program was run to determine the light dependence of CO_2 gaseous exchange in leaves with ten log points, and a light intensity curve was established by giving PAR ranging from 0 to 2,000 μmol photons m^{-2} s^{-1} (Supplementary Figure S2). During both years, physiological parameters were measured during the flower initiation stage every day from 9:00 am to 11:00 am when the sky was clear. The third fully expanded leaf was placed inside the chamber by ensuring it was touched with a thermocouple from the underside. Chamber conditions were set as CO_2 at 400 mmol mol^{-1} provided by a 12 g CO_2 cartridge (LI-COR Bioscience, Lincoln, NE, U.S.A.) using a CO_2 mixer with a flow rate of 500 μmol s^{-1}, relative humidity of 50%–60%, and PAR was set at 1,300 mmol m^{-2} s^{-1} as determined by light calibration curve (Supplementary Figure S2). The block temperature and average leaf area were set at 25°C and 2.5 cm^2, respectively. Data were considered and logged when flow rate, CO_2, and H_2O were stable to quantify the leaf gaseous exchange parameters. Additionally, intrinsic water use efficiency (WUE_{int}) and the ratio of intracellular CO_2 to ambient CO_2 were calculated as P_n/T_r and C_i/C_a, respectively.

Essential oil extraction and yield determination

A uniform weight (1,000 g) of 24 h shade-dried aboveground parts (leaf and inflorescence) excluding stems from every plot was hydro-distilled for 3 h in the Clevenger apparatus. The extracted EO was treated with anhydrous sodium sulfate (Na_2SO_4) and kept in a sealed amber glass vial at 4°C for further analysis. The EO weight (g $1{,}000^{-1}$g) of aboveground dried parts was used to compute the EO content (% w/w). The EO yield was estimated using the following formula and expressed in kg ha^{-1}.

$$\text{EO yield} = \text{herbage yield} \times \text{EO content} \times \text{specific gravity (0.9)} \times 10\ (\text{conversion factor}).$$

Quantification of essential oil constituents

Quantitative evaluation of EO compounds was done by taking three biological replicates with three technical repeats on a single quadrupole gas chromatograph-mass spectrometer (GC-MS) through a flame ionization detector (FID) (GC QP2010 SE, Shimadzu Corp., Tokyo, Japan) fitted with an AOC 5000 Plus auto-injector comprised of Zebron ZB-5 MS capillary column (length: 30 m; internal diameter: 0.25 mm; thickness: 0.25 μm). The extracted EO (10 μl) was dissolved in dichloromethane (2 ml), followed by injection in split mode (2 μl each). Nitrogen gas was used as a carrier at a 1.5 ml min^{-1} flow rate. The temperature was set to 70°C for 3 min, then increased at a rate of 4°C min^{-1} for 5 min, with injector and detector temperatures of 280°C and 300°C, respectively. The mass spectrometer was operated at 70 eV ionization energy and the reading speed (50–500 m/z) was set at 1 $scan^{-1}$. The GC peak

was used to compute the number of volatile constituents and their amounts, which were then sorted by the order of GC elution. The retention index (RI) was calculated without correction factors using a series of hydrocarbons. The constituents of EO were recognized by a comparison of the experimental RI_s with RI_s described in the literature (Adams, 2017). Additionally, the constituents were also recognized by comparing the components' lowest mass spectral fragmentation pattern with the NIST library (Stein, 2005).

Statistical analysis

Datasets of two years were pooled and used to evaluate the effects of the cropping year and used biostimulants on growth, yield, EO amount and composition, leaf nutrient uptake, and gaseous exchange using a four-factor analysis of variance (ANOVA). The results were represented as the mean of three replications ($n = 3$) ± SE. Data were analyzed by comparing means using Fisher's LSD (least significant difference) at a $P = 0.05$ confidence level (SYSTAT Software Inc., Chicago, Illinois, USA). A co-relationship between crop traits was also determined by using Pearson's coefficient (PAST-4).

Results

Soil health condition

A remarkable amelioration in soil health contributing attributes was observed after the end of the two-year experiment as compared with the initial values (Figures 2A–C; Supplementary Table S1). Interestingly, a modest shift in soil pH from 5.3 to 5.7, a slight increase in cation exchange capacity (CEC), and electrical conductivity (EC) by 8.5 and 28.57%, respectively, were observed, indicating the positive effects of using organic biostimulants. The soil bulk density was reduced from 1.39 to 1.27 Mg cm^{-3}. Conversely, no change in soil texture was observed, while OC was increased from 0.49 to 0.64%. In terms of soil nutrient availability, available macro and micronutrients *viz*., N, P, K, Mn, Fe, Cu, and Zn were increased by 28.90, 64.20, 13.62, 7.22, 4.63, 9.52, and 8.47%, respectively, compared with the initial values. Likewise, SMR, MBC, DHA, urease, ALP, and β-glucosidase activities were enhanced by 36.23, 83.98, 42.69, 8.24, 34.00, and 40.31%, respectively.

Growth attributes

Significantly higher plant height (36.42, 70.69 cm) and primary branches (5.96, 10.22) at 50, and 100 DAT, respectively, were recorded during the second year compared with the first (Table 3). Among the vermicompost levels, significantly higher plant height (36.46, 69.69 cm) and the branches (6.77, 10.87) were recorded with the highest dose (8 Mg ha^{-1}) when compared with moderate (4 Mg ha^{-1}) and control (0 Mg ha^{-1}) at 50 and 100 DAT, respectively. Likewise, biofertilizer significantly benefited all treated plants, leading to a 7.21 11.19% enhancement in plant height and branching by 39.96 24.32% at 50 and 100 DAT, respectively. On the contrary, foliar application of liquid seaweed extract at

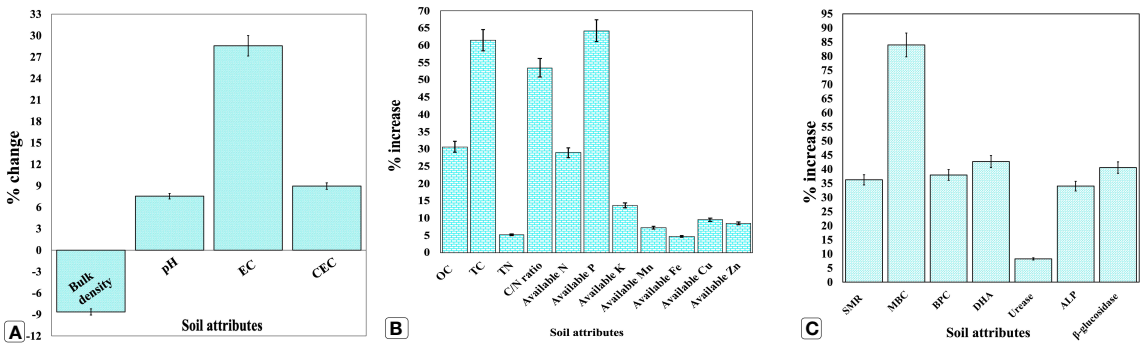


FIGURE 2
Effect of organic manure, biofertilizer, and seaweed extract on soil health indicators after two consecutive years. **(A)** changes in soil edaphic conditions; **(B)** increases in nutrient availability; **(C)** Increase in biological and enzymatic conditions. Results are the means of three replications ($n = 3$) ± SD. OC, organic carbon (%); TC, total carbon (g kg^{-1}); TN, total nitrogen (g kg^{-1}); C/N ratio: ratio of carbon to nitrogen; N, available nitrogen (kg ha^{-1}), P, available phosphorus (kg ha^{-1}); K, available potassium (kg ha^{-1}); Mn, available manganese (mg kg^{-1}); Fe, available iron (mg kg^{-1}); Cu, available copper (mg kg^{-1}); Zn, available zinc (mg kg^{-1}); SMR, soil microbial respiration (CO_2 g^{-1} dry soil day^{-1}); MBC, soil microbial biomass carbon (mg C 100 g^{-1} dry soil); BPC, bacterial population count (× 10^7 CFU g^{-1} soil); DHA, dehydrogenase activity (μg TPF g^{-1} dry soil h^{-1}); Urease: urease activity (μg $NH4^+$-N g^{-1} dry soil h^{-1}); ALP, alkaline phosphatase activity (μg PNP g^{-1} dry soil h^{-1}); β-glucosidase, β-glucosidase activity (μg PNG g^{-1} dry soil h^{-1}); TPF, triphenyl formazan; PNP, p-nitrophenyl phosphate; PNG, p-nitrophenyl β-glucoside.

TABLE 3 Effect of cropping year, organic manure, biofertilizer, and seaweed extract on growth attributes in *O. basilicum* at different growing intervals 50 DAT, and at harvest (100 DAT).

Treatments	Plant height (cm)		Primary branches plant^{-1}		Plant spread N–S (cm)		Plant spread E–W (cm)	
	50 DAT	100 DAT	50 DAT	100 DAT	50 DAT	100 DAT	50 DAT	100 DAT
Cropping year								
2020	31.73^{b}	54.19^{b}	4.79^{b}	8.04^{b}	12.27^{b}	25.39^{b}	13.68^{b}	25.10^{b}
2021	36.42^{a}	70.69^{a}	5.96^{a}	10.22^{a}	14.36^{a}	40.42^{a}	18.18^{a}	43.06^{a}
SEm	0.39	0.33	0.08	0.16	0.38	0.38	0.57	0.23
LSD (*P = 0.05*)	1.10	0.94	0.23	0.47	1.08	1.09	1.63	0.67
Vermicompost								
V–	32.21^{bc}	56.29^{c}	4.08^{c}	7.29^{c}	11.05	27.08^{c}	13.24^{c}	29.17^{c}
V4	33.54^{b}	61.35^{b}	5.28^{b}	9.24^{b}	13.54	31.84^{b}	15.95^{b}	32.90^{b}
V8	36.46^{a}	69.69^{a}	6.77^{a}	10.87^{a}	15.35	39.80^{a}	18.59^{a}	40.17^{a}
SEm	0.47	0.40	0.10	0.20	0.46	0.47	0.70	0.29
LSD (*P = 0.05*)	1.35	1.15	0.28	0.57	NS	1.32	1.99	0.81
Biofertilizer								
B–	32.89^{b}	59.13^{b}	4.56^{b}	8.14^{b}	12.45^{b}	29.98^{b}	14.52^{b}	31.73^{b}
B+	35.26^{a}	65.75^{a}	6.20^{a}	10.12^{a}	14.17^{a}	35.84^{a}	17.33^{a}	36.43^{a}
SEm	0.39	0.33	0.08	0.16	0.38	0.38	0.57	0.23
LSD (*P = 0.05*)	1.10	0.94	0.23	0.47	1.08	1.09	1.63	0.67
Seaweed extract								
S–	33.74	60.91^{b}	5.03^{b}	8.47^{b}	12.97	31.31^{b}	15.61^{ab}	33.07^{b}
S+	34.40	63.98^{a}	5.72^{a}	9.79^{a}	13.66	34.50^{a}	16.25^{a}	35.09^{a}
SEm	0.39	0.33	0.08	0.16	1.63	0.38	0.57	0.23
LSD (*P = 0.05*)	NS	0.94	0.23	0.47	NS	1.09	1.63	0.67

Results are the mean of three replications *(n = 3)* of two years pooled data, different lowercase letters in the same column of each treatment are significantly different at *(P = 0.05)*. DAT, days after transplantation; N–S, plant spread towards north to south direction; E–S, plant spread towards east to west direction; SEm, standard error of mean; LSD, least significant difference *(P = 0.05)*; NS, non-significant; V–, unfertilized control; V4, vermicompost at 4 Mg ha^{-1}; V8, vermicompost at 8 Mg h^{-1}; B, biofertilizer; S, seaweed extract at 7 ml L^{-1}; (–), without factor; (+): with factor.

7 ml L^{-1} showed a significant effect on plant height and branches at 100 DAT only.

Leaf photosynthetic pigments and gaseous exchange

Concentrations of photosynthetic pigments Chl_a, Chl_b, and carotenoids were significantly enhanced by 2.43, 5.14, and 3.52%, respectively, in the second year when compared with the first (Figure 3A; Supplementary Table S2). Vermicompost at 4 and 8 Mg ha^{-1} significantly increased the Chl_a by 3.27, 10.57%, Chl_b by 4.74, 7.58%, and carotenoids by 4.00, 4.44%, respectively as compared with the unfertilized control (0 Mg ha^{-1}). Similarly, biofertilizer and seaweed extracts have also significantly increased Chl_a, Chl_b, and carotenoids by 16.97, 16.12, 12.90%, and 4.68, 8.06, and 6.69%, respectively. The moderate and highest vermicompost dosages also significantly influenced the rate of photosynthesis by 4.56 and 13.96%, respectively, when compared with the unfertilized control. On the contrary, biofertilizer and seaweed extract significantly enhanced both P_n and WUE_{int} by 15.09, 5.90%, and 4.46, 7.58%, respectively (Figure 3B; Supplementary Table S2).

Yield attributes and biomass yield

The herbage yield (leaf + inflorescence) was significantly higher (6.86 Mg ha^{-1}) in the second year in contrast with the first year (6.01 Mg ha^{-1}) (Figure 4A; Supplementary Table S3). The vermicompost amendment with the highest and modest dose significantly increased biomass yield by 69.85 and 31.60%, respectively, compared with the unfertilized control. Similarly, biofertilizer and seaweed extract significantly augmented herbage yield by 49.37 and 24.75%, respectively. A reverse trend was observed in the leaf+inflorescence/stem ratio for the ascending dose of vermicompost. Conversely, only biofertilizer significantly improved the ratio by 7.95%, whilst the foliar application of seaweed extract had no significant effect.

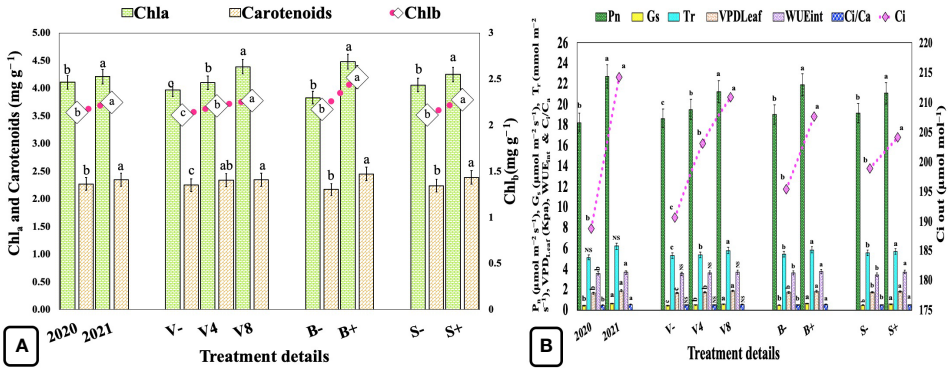


FIGURE 3
Effect of cropping year, organic manure, biofertilizer, and seaweed extract on leaf photosynthetic pigments and gaseous exchange in sweet basil. **(A)** Effect on photosynthetic pigments **(B)** Effect on photosynthetic characteristics. Data are pooled over two years, and results are represented as the means of three replications (*n=3*) ± SE, bars with different letters are significantly different at $P = 0.05$. NS, non-significant; V–, unfertilized control (no added manure); V4, vermicompost at 4 Mg ha^{-1}; V8, vermicompost at 8 Mg ha^{-1}; B–, uninoculated; B+, inoculated; S–, without seaweed extract; S+, foliar spray of seaweed extract at 7 ml L^{-1}; Chl_a, chlorophyll a; Chl_b, chlorophyll b; P_n, net photosynthetic rate; G_s, stomatal conductance; C_i, CO_2 mole fraction in the leaf intercellular air spaces; T_r, transpiration rate; VPD_{Leaf}, leaf vapor pressure deficit; WUE_{int}, intrinsic water use efficiency; C_i/C_a, ratio of intracellular CO_2 to ambient CO_2.

Mineral concentration (N and P%) in leaves

Leaf N and P contents were significantly boosted by 3.29 and 6.46%, respectively, in the second year as compared with the first. The highest and moderate doses of vermicompost have significantly enhanced N and P concentrations by 11.41, 18.67%, and 2.80, 7.82%, respectively when compared with the unfertilized control. Similarly, seaweed extract and biofertilizer also significantly increased N and P contents by 4.56, 5.84%, and 9.53, 17.66%, respectively (Figure 4B).

Essential oil percentage, composition, and yield

The EO content and yield were significantly higher in the second year (1.05%, 69.03 kg ha^{-1}) when compared with the first (0.97%, 55.03 kg ha^{-1}), respectively (Figure 4C; Supplementary Table S3). Among factors, a significantly higher EO concentration (1.03%) was observed in plants treated with 8 Mg ha^{-1} vermicompost, compared with control (0 Mg ha^{-1}) but remained statistically at par with the moderate dose (4 Mg ha^{-1}). Likewise, biofertilization and foliar application of seaweed extract also significantly affected EO content and yield by 45.35, 67.95%, and 18.48, 31.33%, respectively. The major constituents of EO, methyl chavicol and linalool, significantly increased by 2.84 and 5.68%, respectively, in the second year as compared with the first year (Figure 4C; Supplementary Table S4). The highest and moderate rates of vermicompost also significantly increased the concentration of methyl chavicol and linalool by 2.71, 9.85%, and 1.18, 5.03%, respectively, when compared with the unfertilized control. Similarly, the biofertilization and seaweed extract also enhanced their concentrations by 3.29, 8.67%, and 1.93, 3.66%, respectively.

Correlations of traits and interaction effect of biostimulants (V × B × S)

The matrix of correlation (Figure 5) was significant ($P = 0.01$) and showed a positive correlation of EO yield with herb yield (r = 0.91), EO content (r = 0.94), dry matter content (r = 0.97), leaf + inflorescence/stem ratio (r = 0.81); number of inflorescence plants^{-1} (r = 0.94); average length of panicle (r = 0.95); net rate of photosynthesis (r = 0.72). Furthermore, the methyl chavicol content was also positively correlated at $P = 0.01$ with all chosen traits, except for dry matter content, which was significant at $P = 0.05$ (r = 0.62). Similarly, the herbage yield was positively correlated ($P = 0.01$) with all considered attributes, excluding the net photosynthetic rate, which was significant at $P = 0.05$ (r = 0.67). Biomass and EO yield were both significantly influenced by the interaction effect of all three involved factors, *viz.*, vermicompost (V), biofertilizer (B), and liquid seaweed extract (S) during both years (Figures 6A, B). In both years, the maximum herb and EO yield were recorded as 8.86, 11.25 Mg ha^{-1} and 113.78, 154.87 kg ha^{-1}, respectively in T12 (V8B+S+) receiving congregate treatment of all three biostimulants (Figure 7).

Discussion

Assuring sustainable production of salubrious crop products for environmental safety and socio-economic issues is not only a critical topic for agriculture, ecology, and the environment, but

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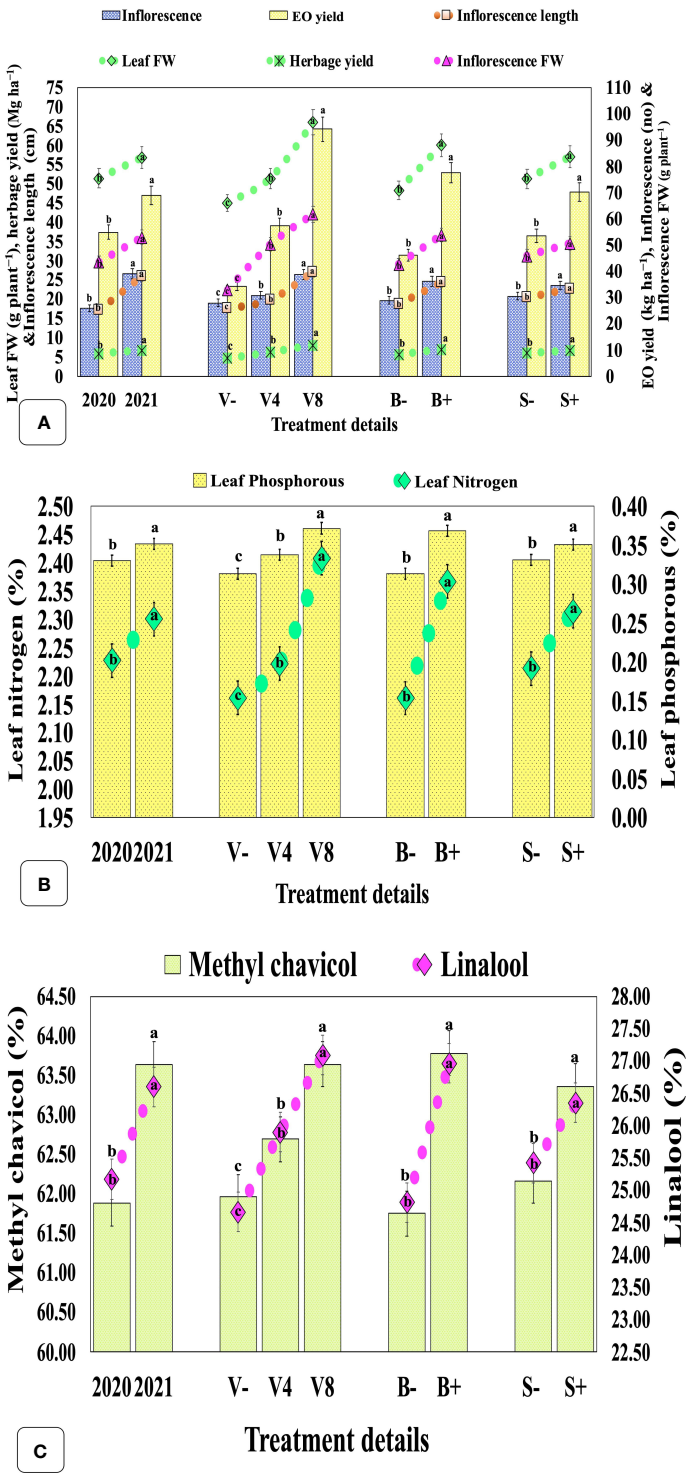


FIGURE 4
Effect of cropping year, organic manure, biofertilizer, and seaweed extract on yield, leaf mineral content, and secondary metabolites in sweet basil. **(A)** effect on yield, and associated attributes; **(B)** effect on leaf nitrogen and phosphorous contents; **(C)** effect on EO, methyl chavicol, linalool content. Data are pooled over two years, and results are represented as the means of three replications ($n = 3$) ± SE, bars with different letters are significantly different at $P = 0.05$. V–, unfertilized control (no added manure); V4, vermicompost at 4 Mg ha^{-1}; V8, vermicompost at 8 Mg ha^{-1}; B–, uninoculated; B+, inoculated; S–, without seaweed extract; S+, foliar spray of seaweed extract at 7 ml L^{-1}; EO, essential oil; FW, fresh weight.

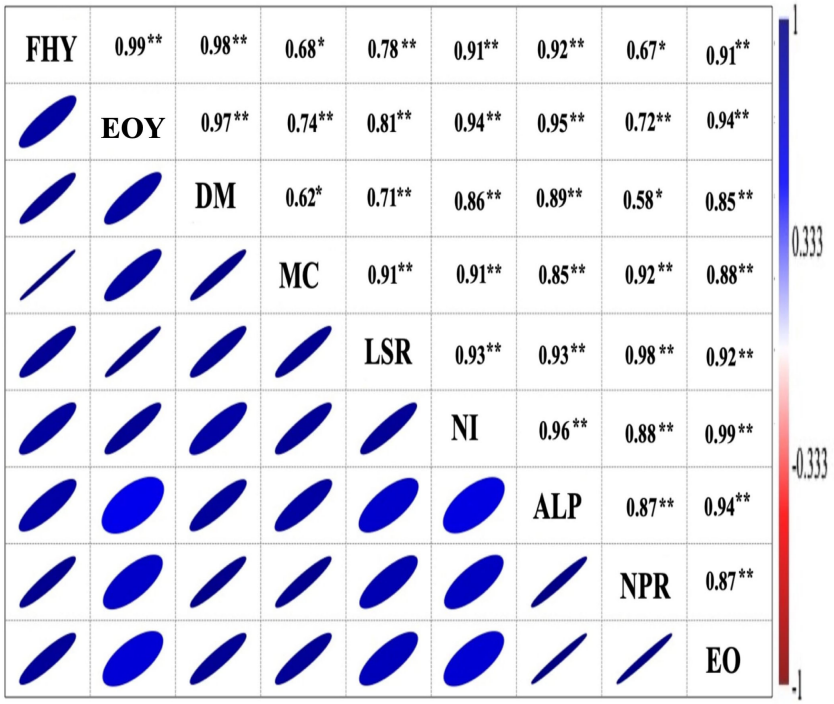


FIGURE 5
Correlation analysis between photosynthesis, yield parameters, and sweet basil's marker compound. FHY, fresh herb yield; EOY, essential oil yield (kg ha^{-1}); DM, dry matter $plant^{-1}$; MC, methyl chavicol (%); LSR, leaf + inflorescence/stem ratio; NI, number of inflorescence $plant^{-1}$; ALP, average length of panicle (cm); NPR, net photosynthetic rate (μmol m^{-2} s^{-1}); EO, essential oil (%). The mean values of three biological replicates of the corresponding treatments (12) were (N3:N:2:N:2 = 12) used, * and ** indicate the significant differences between corresponding values at $P = 0.05$ and $P = 0.01$, respectively.

also pondered by farmers, researchers, policymakers, and stakeholders. However, farmers are accustomed to using a lot of energy, such as chemical fertilizers, to augment plant growth and productivity, which has increased CO_2 concentration, temperature, GHG emissions, environmental pollution, and unintentionally deteriorated soil health, including crop product quality. Moreover, organic farming practices are the only way to keep the soil in good shape and increase agricultural production in the long term. However, the biggest disadvantage of organic farming is its lower and irregular output when compared with conventional farming. Consequently, rigorous research is needed to understand how biostimulants may be employed to alleviate nutrient limitations by boosting nutrient absorption, and ultimately cutting short the apparent yield gap between organic and traditional output. Furthermore, the sole application of biostimulants cannot meet the requirements for a viable alternative to mineral fertilizers. Therefore, the present investigation was performed to develop innovative and robust yet sustainable production technologies for the cleaner production of Indian sweet basil by using a combinatorial application of a diverse range of biostimulants under the rainfed conditions of Indian western Himalaya. The findings of the current investigation not only revealed the beneficial effects of used organic biostimulants on sweet basil growth, physiological performance, biomass, EO yield, and composition but also meliorated soil health.

Soil health

Soil health is an intricate result of a complex interaction between the soil's physical, biological, and chemical components. Agronomical measures like soil management can have a significant impact on these components (Zhang et al., 2020; Hu et al., 2021). Despite the important influence of organic biostimulant amendments on soil quality, only a few studies are available that incorporate all these three soil quality indicators. Therefore, in the current investigation, we tried to decipher the effect of biostimulants on all three soil components. In terms of soil physical properties, a modest increase in soil pH was noticed (Figure 2A; Supplementary Table S1), making it more favorable for soil processes like nutrient availability and microbial activity, as the pH range between 5.5 and 8.0 provides favorable optimal circumstances for almost all soil activities. The present results

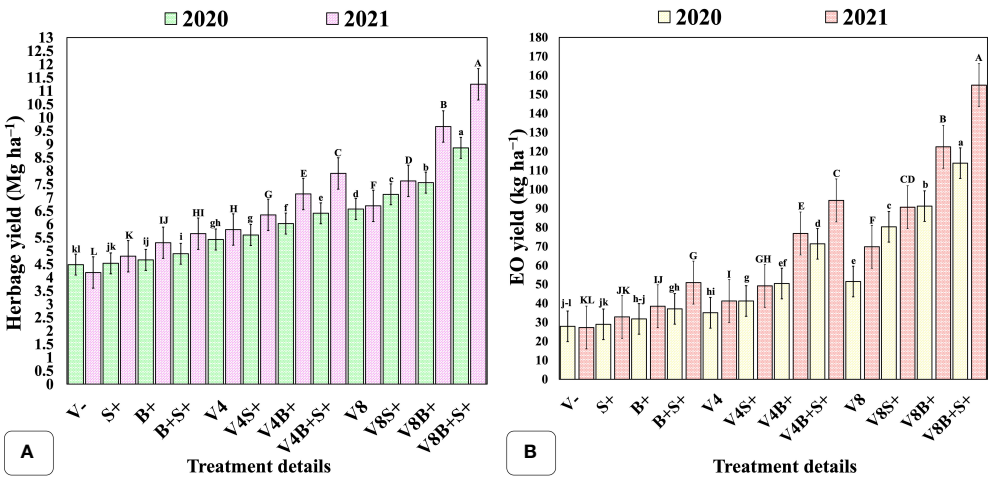


FIGURE 6
Interaction effect of organic manure, biofertilizer, and seaweed extract on yield components of sweet basil. **(A)** herbage yield (Mg ha^{-1}); **(B)** EO yield (kg ha^{-1}). Data are represented as the means of three replications ($n = 3$) ± SE, bars with different letters are significantly different at $P = 0.05$. V–, unfertilized control (no added manure); V, vermicompost; V4, vermicompost at 4 Mg ha^{-1}; V8, vermicompost at 8 Mg ha^{-1}; B, biofertilizer; S, seaweed extract; (–), without factor; (+), with factor. Other abbreviations are provided in Table 1.

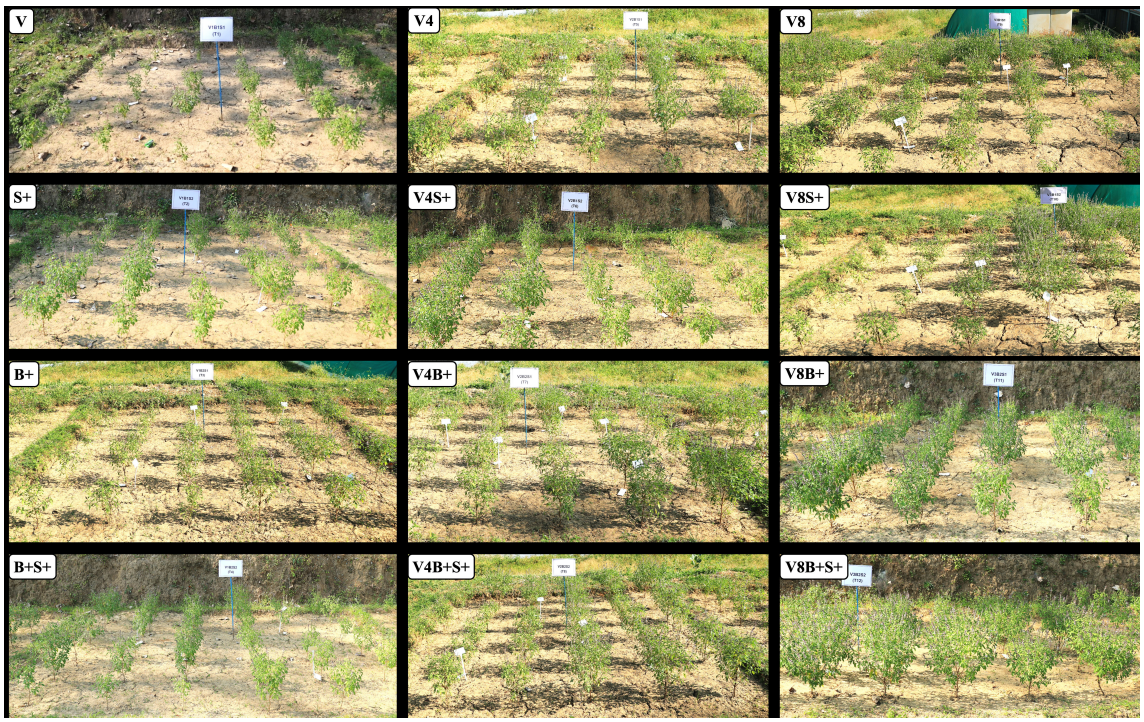


FIGURE 7
Treatment-wise representative experimental plots of *O. basilicum* in the Indian western Himalaya. V, vermicompost; V–: unfertilized control (no added manure); V4, vermicompost at 4 Mg ha^{-1}; V8, vermicompost at 8 Mg ha^{-1}; B, biofertilizer; S, seaweed extract; (–), without factor; (+), with factor. Other abbreviations are provided in Table 1.

are in congruence with previous findings reporting an increase in soil pH towards neutrality by adding vermicompost and phosphate solubilizing bacteria (PSB) through organic compound creation during the decomposition of complex organic molecules and mineralization (Goswami et al., 2017). Additionally, the soil pH might have increased due to the presence of sodium ions (Na^+) in seaweed extract (Lötze and Hoffman, 2016). In contrast, soil bulk density declined, possibly due to the addition of organic carbon (OC) (Figure 2B), which might have increased the pore space and thereby the soil aggregation (Gourav et al., 2019). Interestingly, a slight increase in soil EC and CEC was also noticed, which is accountable for the large negative charge of biological materials accumulated as of biostimulant amendments; being critical for nutrient retention and its availability to plants (Choudhary et al., 2021).

Among soil biological properties, SMR and MBC denote the estimation of CO_2 liberated by soil microflora in the respiration process and the extent of carbon fixed in soil microorganisms' cell structures, respectively. Both are connoted by the soil microbial population and their activity (Parastesh et al., 2019). A significant enhancement in SMR and MBC can be attributed to the increased BPC (Figure 2C), as organic amendments can act as substrates, supply soluble nutrients to soil microflora, and eventually augment the relative abundance of the soil bacterial population (Sarma et al., 2018). Accounting for the soil enzymatic traits, the increased DHA could be attributed to enhanced SMR and BPC (Figure 2C) by organic additives, resulting in improved soil metabolic activities (Hamdi et al., 2019). Likewise, ALP catalyzes the hydrolysis of insoluble organic phosphomonoester to inorganic P, releasing orthophosphates that could be easily digested by plants and soil microbes. Moreover, it was already discovered that vermicompost and bioinoculants could boost soil ALP activity due to increased inorganic soluble phosphates (Hussain et al., 2016). Similarly, the mineralization and mobilization of NPK by biofertilizers might have augmented the soil-accessible NPK (Figure 2B; Supplementary Table S1), as microbial biostimulants actively participate in the mineralization of fixed nutrients into accessible ones by accelerating the rate of biogeochemical cycling. Moreover, vermicompost contains HSs, C, P, and N, and a diverse range of PGPRs (Pathma and Sakthivel, 2013), which could also be the source of the observed increase in soil-accessible NPK after the end of the two-year experiment. Furthermore, organic materials such as vermicompost, seaweed extracts, and bioinoculants have demonstrated a significant increase in soil health in some earlier investigations (Verma et al., 2016; Gupta et al., 2019; Trivedi et al., 2022) due to improved microbial community structure and soil enzymatic activities, which are in coherence with the current study.

Growth and yield

All growth-contributing attributes were significantly higher in the second year (Table 3) as compared with the first. This might be due to biostimulant amendments for two consecutive years leading to a significant improvement in soil health (Figures 2A–C; Supplementary Table S1), as they contain a considerable amount of macro and micronutrients, including organic matter (Table 2). Furthermore, despite receiving ~16.83% higher rainfall in the second year (Figure 1B), the crop yield was consistent or even far better than the first year. This might be attributable to the stress alleviation properties of biostimulants delivering better resilience to crops to confront various environmental stressors. The PGPR strains used as biofertilizers in the current investigation have the potential to demonstrate ACCD activity (Table 2), which alleviates the stressors by preventing the formation of ethylene under waterlogging conditions (Barnawal et al., 2012). Also, seaweed extracts are well-documented as stress alleviators because of the osmolytic properties of glycine, betaine, and choline chloride in their composition (Singh et al., 2016b). The biofertilizer can stimulate plant growth through biological nitrogen fixation (BNF), regulation of PGRs and expression of nitrate transporter genes (*NRT1.1*, *NRT2*, and *NAR2.2*), solubilization of insoluble compounds like calcium di, tri-phosphates, and siderophore production (Van Gerrewey et al., 2020; Song et al., 2021). Interestingly, a recent study demonstrated that CK, a PGR released by beneficial microbes, augments crop productivity, as microbial-produced CKs could promote plant metabolism, leading to increased vigor and also recruit disease-protective microbiome, finally increasing the overall benefit to the host plant (Gupta et al., 2022). Moreover, the combined application of vermicompost and biofertilizer might have enhanced nutrient acquisition, plant growth-promoting enzymes, and soil microbial diversity and ultimately increased the overall productivity of the crop (Misra et al., 2019). Current findings are consistent with some former investigations, reporting a significant increase in crop biomass using organic manure in *O. basilicum* (Cabanillas et al., 2013) and biofertilizer in *Mentha arvensis* L. (Singh et al., 2019).

Additionally, seaweed extracts can also boost crop growth. Still, the exact process remains elusive; it is assumed that the plausible activity of seaweed extracts is due to the presence of macro/micronutrients, PGRs, betaines, and phenolic compounds in their composition (Table 2). In a prior study, foliar spraying with *K. alvarezii* and *Gracilaria edulis* seaweed saps significantly increased the yield and quality of maize. This improvement was attributed to higher photosynthetic capacity, higher net assimilation rate, and the impact of various PGRs as well as other substances present in these seaweed saps (Singh et al., 2016b). Similarly, a recent root transcriptome analysis

revealed that the soil drench with seaweed extract (*K. alverazii*) in *Zea mays* L. upregulates genes related to root growth, PGR signaling, stress responses, plant nutrition, and transport, including DNA repair under drought conditions (Kumar et al., 2020). Sequentially, a recent leaf transcriptomic study revealed that the foliar spray of seaweed extract (*K. alverazii*) induces the photosynthesis and starch biosynthesis associated genes in *Z. mays*, leading to higher biomass production (Trivedi et al., 2021). Owing to these previously reported observations, it was speculated that in the present study, sweet basil might have experienced a similar effect like enhanced photosynthetic pigments (Figure 3A), rate of photosynthesis (P_n) (Figure 3B), and eventually produced higher crop biomass after foliar application with liquid seaweed extract.

Leaf photosynthetic pigments, mineral nutrients, and gas exchange

All biostimulants significantly enhanced the concentrations of photosynthetic pigments as compared with the unfertilized control (Figure 3A). Similar findings were reported by others in *Amaranthus hybridus* L. after foliar application with some natural biostimulants like smoke-water, karrikinolide, vermicompost leachate, Kelpak®, and eckol (Ngoroyemoto et al., 2019) and also in *Vigna unguiculata* (L.) Walp after treatment with seaweed extract (Kelpak®) and vermicompost leachate under drought stress (Voko et al., 2022). Likewise, biofertilizer application can also increase the number of photosynthetic pigments as there is a well-established link between total chlorophyll content and increased iron acquisition facilitated by PGPRs through iron-containing enzymes and siderophores (Eshaghi Gorgi et al., 2021). Furthermore, the higher leaf N concentrations (Figure 4B) might have enhanced the content of chlorophyll (Figure 3A), as thylakoid N is directly proportional to the chlorophyll content. Additionally, manganese (Mn) plays a pivotal role in plant metabolisms like enzyme activation, nitrate reduction, amino acid, chlorophyll, and protein synthesis, including photosynthesis and phytohormone regulation (Ghannadnia et al., 2014). Therefore, the increased concentration of photosynthetic pigments can also be ascribed to the improved status of Mn in soil (Figure 2B; Supplementary Table S1).

Similarly, biofertilizers can also influence the photosynthetic process by altering the auxin pool, which aids the plant's root system and allows better acquisition of mineral nutrients, leading to the accumulation of higher photosynthetic pigments and gaseous exchange (Liu et al., 2019; Samani et al., 2019). However, the association between photosynthesis and PGPRs is commonly given only in an indirect way, albeit, in the current investigation, a direct and pronounced favorable effect of biofertilizers on photosynthesis-associated characteristics was observed (Figure 3B). As a result, the quinone acceptors (Q_a) are substantially oxidized and their excitation energy is used in electron transport, resulting in the increased synthesis of energy molecules (ATP and NADPH). These energy molecules are further used for carbon assimilation during the C_3 cycle and eventually increase crop biomass (Rozpądek et al., 2015). Additionally, an increase in N and P content in leaves treated with seaweed extract might be due to the considerable increase in transcript abundance of genes related to N and P assimilation in leaves, including increased nitrate and phosphate transporters and nitrate reductase activity in roots, as earlier reported in *Z. mays* (Trivedi et al., 2018a; Trivedi et al., 2021). Furthermore, CO_2 produced during SMR can also enhance the rate of photosynthesis (P_n). Therefore, the increased SMR (Figure 2C) can also be ascribed as a putative reason for the enhanced net photosynthetic activity observed in the current study.

Essential oil content, yield and composition

The observed variabilities in EO content and composition as of the exogenous application of biostimulants in the current investigation are in agreement with earlier studies that agronomic practices (Choudhary and Kumar, 2019; Rathore and Kumar, 2021), genomic makeup, environmental factors, soil edaphic conditions, and ontology (Esmaeili et al., 2018) all can influence the quantity and quality of EO in MAPs. Furthermore, plants require micronutrients, especially Zn and Cu, for cell division, photosynthesis, electron transport chain (ETC), chlorophyll, protein, and auxin synthesis. Additionally, these act as metal constituents of several enzymes and regulatory cofactors involved in saccharide metabolism (Tavallali et al., 2018). The higher availability of micronutrients in soil (Figure 2B; Supplementary Table S1) could be attributed to the observed increased EO content under organic biostimulant amendments (Figure 4C). Similarly, multiple studies have already reported that the organic amendment and bioinoculant can increase EO in various MAP_s like *Dracocephalum moldavica* L. (Vafadar-Yengeje et al., 2019), *D. kotschyi* Boiss. (Fallah et al., 2020), and *O. basilicum* (Yilmaz and Karik, 2022). Furthermore, both N and P are critical for the production and activation of enzymes involved in various biochemical reactions. Therefore, the increased N and P contents in the current investigation (Figure 4B) due to organic biostimulants might have enhanced EO content in sweet basil (Figure 4C; Supplementary Table S4); as the formation of EO-carrying cells, channels, glandular trichomes, and secretory ducts are all aided by these two moieties (Rostaei et al., 2018). Moreover, N may boost electron transport rates (ETR), RuBisCO activity, and photosynthesis, which provides ATP and carbon substrate for isoprene synthesis and ultimately increases the accumulation of EO (Machiani et al., 2018). Alike, P being a component of EO precursors (isopentyl diphosphate

and dimethylallyl diphosphate) can also enhance EO production (Dehsheikh et al., 2020). The observed increase in EO yield reported in the current investigation might be attributed to the increased number of glandular trichomes and antioxidants, as some posterior studies have already claimed these factors are responsible for the increase in EO yield after combined treatment of plant beneficial microbes in MAPs like sweet basil (Gupta and Pandey, 2015) and *Pelargonium graveolens* L'Hér (Gupta et al., 2016).

The biosynthetic pathways of EO components are well-recognized to be extensive and complex. Despite extensive research that has been conducted to elucidate the plausible mechanism of biosynthesis, the exact relation between biosynthetic routes of various constituents remains elusive. Nonetheless, it is well known that terpenoid biosynthesis is dependent on primary metabolisms like photosynthesis and oxidative pathways for carbon and energy supply, and thus the plants inoculated with biofertilizer can produce more primary metabolites through boosting photosynthesis and other metabolic activities, which can eventually enhance the secondary metabolite accumulation (Banchio et al., 2010). Furthermore, the activation/inactivation of enzymes involved in EO biosynthetic pathways like mevalonate (MVA), methylerythritol phosphate (MEP), and shikimic acid could also cause alterations in constituents of EO (Gupta et al., 2020). Interestingly, in the current investigation, methyl chavicol and linalool content were increased with all three amended biostimulants (Figure 4C). On the contrary, only biofertilizer application has exorbitantly boosted α-Bergamotene (Supplementary Table S4), which has the highest binding affinity for Angiotensin-Converting Enzyme 2 (ACE2) protein among all available antiviral medicines for SARS CoV2 (Duru et al., 2021). The plausible mechanism behind this could be the alterations in expression patterns of the genes involved in the putative biosynthetic pathway of terpenoid biosynthesis. Moreover, not only the application of live cultures but also their metabolites can alter the secondary metabolite pathways in MAPs; as a prior study demonstrated an increase in withanolide A content along with growth in *Withania somnifera* (L.) Dunal after inoculation with cell pellets and metabolites of PGPRs like *B. subtilis* and *Streptomyces* sp. at 10 ml plant^{-1} pot^{-1} under a poly greenhouse condition (Singh et al., 2016a). Sequentially, another study also showed ~1.5-fold augmentation in bacoside A production in *Bacopa monnieri* (L.) Pennell inoculated with chitinolytic microbes as of alteration in the biosynthetic pathway of bacoside A (Gupta et al., 2017). In addition, it was hypothesized that the observed increase in EO and secondary metabolite production might be linked to elicitors (PGRs and other biologically active chemicals) produced by biostimulants, which eventually stimulated the secondary metabolite synthesis *via* an induced systemic resistance (ISR) mechanism, leading to activation of the host plant's chemical defense cascades like jasmonic acid (JA) and ethylene (ETH), as many studies have successfully demonstrated the occurrence of ISR in MAPs after biostimulation (Egamberdieva et al., 2015; Chamkhi et al., 2021).

Interaction effect of biostimulants

The current investigation demonstrated a synergistic effect of used biostimulants and reported the maximum herb and EO yields were observed when the crop was treated with the congregate application of all three biostimulants (Figures 6A, B). These findings are in congruence with some posterior studies demonstrating a synergistic/additive effect of the combined application of a diverse array of biostimulants for augmenting plant growth, production, and even biochemical composition in crops like lettuce (Rouphael et al., 2017; Rasouli et al., 2022), green amaranth (Ngoroyemoto et al., 2020), onion (Gupta et al., 2021a), and wheat (Najafi Vafa et al., 2022). Moreover, the observed beneficial effects of the combinatory use of biostimulants in the current study might be attributable to the increased photosynthetic pigments, physiological performance, and enhanced N and P contents in the leaves of *O. basilicum* (Figures 3A–C). Additionally, the plausible action mechanism of enhanced crop performance might be associated with the improved soil health status (Figures 2A–C) coupled with enhanced bacterial abundance and nutrient use efficiency (Figure 4B) driven by the synergistic action of vermicompost, biofertilizer, and seaweed extract. Concurrent observations were reported in a previous investigation in which a combined application of *Trichoderma*-based biostimulant at 50 g L^{-1} and seaweed extract at 2 g L^{-1} derived from *Ascophyllum nodosum* interacted synergistically and augmented the growth, nutritional and functional quality of organically grown tomatoes. In addition, they also reported a significant increase in soil fertility by fostering the growth of rhizospheric microbial populations, thereby increasing nutrient use efficiency, plant growth, and levels of antioxidant enzymes (Sani et al., 2020).

Elucidation of plausible bio-stimulatory mechanism

The plausible implication of biostimulants on several agronomical, biochemical, physiological, and molecular processes governing plant growth, productivity, quality, and resilience was adopted by Sani and Yong (2021) and illustrated in Figure 8A. The bio-stimulatory effect of biofertilizer could be explained by several mechanisms: i) improving soil pH, EC, CEC, WHC, nutrient availability, plant uptake, and assimilation; (ii) modification of root architecture; (iii) enhancing the physiological performance of plants; (iv) bolstering the antioxidant defense system; (v) PGR production and regulation; (vi) Upregulation of nutrient assimilation and transporters genes such as *AMT*, *NRT1.1*, *NRT2*, *NAR2.2*, *Pht1*, *PT2-1*, *DULTR4.2*, and *SULTR4.1*; and (vii)

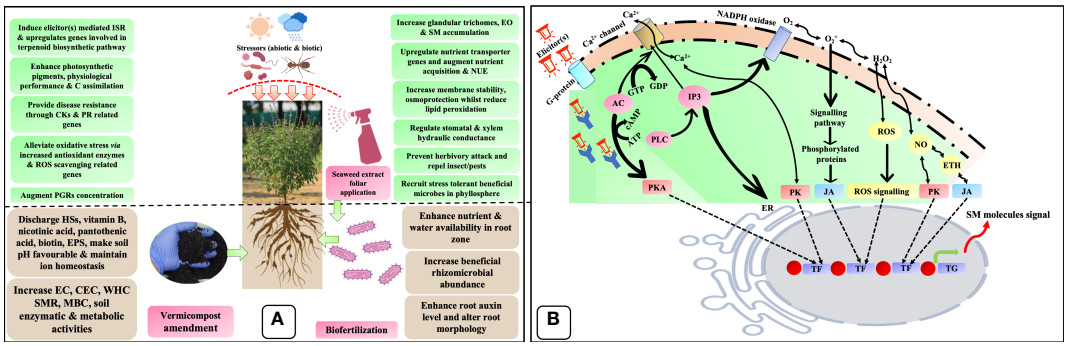


FIGURE 8
Hypothetical bio-stimulatory action mechanisms of biostimulants. **(A)** Plausible mode of action of biostimulants (vermicompost, biofertilizer, and seaweed extract) upon interaction with plants and their growing environment; **(B)** Plausible bio-stimulatory signaling pathways for elicitation of secondary metabolite synthesis. HSs, humic substances; EPS, exopolysaccharide; PGRs, plant growth regulators; CKs, cytokinins; PR, pathogenesis-related; C, carbon; ISR, induce systemic resistance; EO, essential oil; NUE, nutrient use efficiency; EC, electrical conductance; CEC, cation exchange capacity; WHC, water holding capacity; SMR, soil microbial respiration; MBC, microbial biomass carbon; G-protein, guanine nucleotide-binding proteins; AC, adenylate cyclase; IP3, inositol trisphosphate; ER, endoplasmic reticulum; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; GTP, guanosine triphosphate; GDP, guanosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PLC, phospholipase C; PKA, proteins kinase A; PK, proteins kinase; H_2O_2, hydrogen peroxide; OH, hydroxy free radical; $^1O_2$, singlet oxygen; ROS, reactive oxygen species; NO, nitric oxide; ETH, ethylene; TFs, transcriptional factors; TG, target gene; SMs, secondary metabolites.

alteration of rhizomicrobiome through the production of enzymes and organic compounds. Likewise, the seaweed extract can augment plant growth and development through hormonal homeostasis, upregulation of nutrient transporter genes, stimulation of photosynthesis, and increased stress tolerance due to antioxidant stimulation and decreased lipid peroxidation, including reactive oxygen species (ROS) levels (Trivedi et al., 2018b). Additionally, the presence of polysaccharides, betaines, polyamines, phenolic compounds, and phytohormones in seaweed extracts may influence several signaling pathways and gene expression, therefore, imparting a positive effect on plants. Furthermore, seaweed extract induces bacteria-plant signaling *via* stimulatory effects, activates bacterial genes (*nodC*), and recruits beneficial microflora in the phyllo and rhizosphere. Likewise, vermicompost's bio-stimulatory action is due to the presence of HSs, PGRs, PGPRs, and other advantageous substances in its composition. The biostimulatory activity of HSs has been attributed to numerous mechanisms (i) increasing WHC and CEC, neutralizing soil pH, and meliorating the soil structure; (ii) improving P solubility by preventing its precipitation and also preventing leaching, thereby augmenting the availability of nutrients, (iii) act like auxin and improve lateral root induction thereby, trigger the plasma membrane H^{+-}ATPase activity, and (iv) stimulates nitrate assimilation *via* upregulation of the target genes/enzymes. Additionally, HSs can decrease hydrogen peroxide (H_2O_2) and lipid peroxidation, which consequently raises the proline concentration and recruits beneficial stress-resilient microbial communities in the rhizosphere (Xu et al., 2020).

Furthermore, it is well known that the plant G-proteins (guanine nucleotide-binding proteins) are associated with the response to biostimulants and other cellular processes related to growth, hormonal signaling, and defensive responses. The elicitor(s) produced by biostimulants serve as ligands and are identified by PRR (pattern-recognition receptors) to create a ligand–receptor complex. In this manner, a plant cell cognizes the signal produced by the elicitor(s) (Yakhin et al., 2017). Upon reception, the transduction signal produces many secondary messengers, *viz*., calcium ion (Ca^{2+}), JA, salicylic acid (SA), nitric oxide (NO), and ROS, leading to enhanced secondary metabolite (SM) accumulation in MAPs (Figure 8B). When elicitor(s) bind to membrane receptors like plant G-protein, the transmembrane domain is activated and GTP is degraded to GDP *via* GTPase activity. This signaling event activates adenylate cyclase (AC) activity and converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). As a result, phosphorylated protein kinase A (PKA) stimulates transcriptional factors (TFs) of the target gene (TG). Furthermore, inositol trisphosphate (IP3) is produced by phospholipase C (PLC) activity stimulated by G-protein activation. This secondary messenger increases intracellular Ca^{2+} by activating Ca^{2+} channel efflux and its binding to a particular receptor on the endoplasmic reticulum (ER). The augmented intracellular Ca^{2+} concentrations cause phosphorylation of certain proteins like proteins kinase (PK), which regulates gene expression by activating TFs. Additionally, activated PLC is also implicated in generating oxidative bursts by producing H_2O_2, hydroxy free radical (OH), singlet oxygen ($^1O_2$), and superoxide anion (O_2) through swapping between O_2 and H_2O_2 depending on NADPH oxidase and peroxidase activity in the plasma membrane. Consequently, H_2O_2

generation in the plasma membrane leads to stimulation of ER through increased cytosolic Ca^{2+} concentration. Furthermore, these molecules also trigger secondary messengers like JA, SA, NO, PK, ETH, and ROS that can modify the epigenetic pathways regulating gene expression. Likewise, various secondary messengers regulate the expression of genes by transforming the methylation state of DNA into a hypo-methylation state to permit access to TFs and RNA polymerases to the promoter region. Moreover, secondary messengers can also relax the structure of chromatin by shifting it to a euchromatin state, which also allows access of TFs and RNA polymerases to the promoter region. These epigenetic state transitions mediated by secondary messengers allow gene expression flexible in the presence of elicitor(s). In this manner, the plant cell genome incorporates elicitor(s) signals *via* epigenomic variations and thereby increases the production of secondary metabolites for its defense (Chamkhi et al., 2021).

Conclusion

The pursuit of cleaner crop production is to improve nutrient availability and nutrient use efficiency while preserving soil health and producing quality crop products to slacken reliance on agrochemicals. In this regard, the current study unequivocally alludes that the congregate use of vermicompost, biofertilizer and seaweed extract can be practiced by MAP growers and stakeholders for the cleaner and environment-friendly production of Indian sweet basil EO. In essence, the current field experiment suggests that the combinatorial application of organic biostimulants having harmonizing properties not only significantly enhanced crop yield but also improved soil health. Based on two years of crop productivity, averred maximum EO (~135 kg ha^{-1}) was recorded, when the crop was treated with a congregate dose of vermicompost (8 Mg ha^{-1}), biofertilizer, and foliar application of liquid seaweed extract (7 ml L^{-1}). This production technology can be considered inexpensive and environmentally sustainable, having low carbon footprints and no harmful field emissions. Therefore, it can be concluded that the combination of microbial and non-microbial biostimulant categories can act synergistically and maximize the soil health and quality production of sweet basil EO, particularly under the rainfed conditions of the Indian western Himalayas. Nevertheless, more rigorous field evaluations are required to evaluate and legitimize the interaction effects of the diverse range of biostimulants, especially on MAPs in a range of agroclimatic conditions. In addition, deciphering the combinative effects of biostimulants on functional rhizomicrobiomes would allow us to have a better comprehension of the soil–microbe–plant nexus. Moreover, the exact mechanism of biostimulation is not fully understood and further investigations are required to discern the fine-tuning between biostimulants and the host plant by evaluating the global changes in the abundance of mRNA transcripts using a comparative high-throughput RNA-seq approach.

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

YR: Conceptualization, methodology, investigation, formal analysis, data acquisition, and curation, writing—original draft, and writing—review and editing. NA: Methodology. AP: Investigation and supervision. RK: Funding acquisition, conceptualization, investigation and supervision, validation, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.976295/full#supplementary-material

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Transcriptome and hormone Analyses reveal that melatonin promotes adventitious rooting in shaded cucumber hypocotyls

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Melatonin, a multi-regulatory molecule, stimulates root generation and regulates many aspects of plant growth and developmental processes. To gain insight into the effects of melatonin on adventitious root (AR) formation, we use cucumber seedings subjected to one of three treatments: EW (hypocotyl exposed and irrigated with water), SW (hypocotyl shaded and irrigated with water) and SM (hypocotyl shaded and irrigated with 100 μM melatonin). Under shaded conditions, melatonin induced significant AR formation in the hypocotyl. To explore the mechanism of this melatonin-induced AR formation, we used transcriptome analysis to identify 1296 significant differentially expressed genes (DEGs). Comparing SM with SW, a total of 774 genes were upregulated and 522 genes were downregulated. The DEGs were classified among different metabolic pathways, especially those connected with the synthesis of secondary metabolites, with hormone signal transduction and with plant-pathogen interactions. Analyses indicate exogenous melatonin increased contents of endogenous auxin, jasmonic acid, salicylic acid, cytokinin and abscisic acid levels during AR formation. This study indicates melatonin promotes AR formation in cucumber seedings by regulating the expressions of genes related to hormone synthesis, signaling and cell wall formation, as well as by increasing the contents of auxin, cytokinin, jasmonic acid, salicylic acid and abscisic acid. This research elucidates the molecular mechanisms of melatonin's role in promoting AR formation in the hypocotyl of cucumber seedings under shaded conditions.

KEYWORDS

melatonin, cucumber, hypocotyl shade, adventitious roots, hormones, transcriptome

Introduction

Cucumber plants have poor ability to take up water and minerals because of their weak root regeneration and shallow roots system, which seriously reduces yield. The hypocotyl of cucumber seedlings has the ability to generate AR (Qi et al., 2019; Qi et al., 2020), therefore measures that stimulate adventitious root development are crucial for promoting plant growth and development and thus for increasing crop yield and so making better use of scarce agricultural land.

Adventitious roots (ARs) can emerge from a hypocotyl, a stem, a leaf or some other plant organ, with emergence regulated by environmental signals and/or by hormones (Sukumar et al., 2013). Many hormones have been shown to play important roles in the induction of AR, among these auxin is the main one that promotes AR initiation (Li et al., 2009; Da Costa et al., 2013; Pacurar et al., 2014).It is reported that auxin synthesis is necessary for AR formation, with the number of ARs increasing with auxin concentration (Boerjan and W., 1995; Ahkami et al., 2013). Conversely, reduced auxin transport or reduced auxin signaling decreases AR development (Vidoz et al., 2010). In addition to auxin, cytokinin (CK), jasmonic acid (JA), salicylic acid (SA) and ethylene are also involved in regulating AR development, and they interact with one another. Studies have shown that CK inhibits AR formation (Hutchison et al., 2006; Mao et al., 2019), over-expression of cytokinin oxidase (*CKX*) increases AR formation in transgenic tobacco (Werner et al., 2001). It is reported that SA works synergistically with auxin to induce AR development in cucumber (Dong et al., 2020) and Arabidopsis (Pasternak et al., 2019). Moreover, ethylene is another important hormone that interacts with auxin during root development; with ethylene inducing AR formation by regulating auxin transport (Vidoz et al., 2010). Moreover, the crosstalk between ethylene and JA signaling has been shown to be critical for AR formation in Arabidopsis (Fattorini et al., 2018).

Melatonin is a natural molecule that exists widely in plants (Pelagio-Flores et al., 2012), which is structurally related to indole-3-acetic acid (IAA). Initially, the structural similarity between melatonin and IAA prompted researchers to investigate its function in greater depth (Hernández-Ruiz et al., 2004). In plants, melatonin may act as a growth regulator or new hormone (Arnao and Hernández-Ruiz, 2014; Arnao and Hernandez-Ruiz, 2019). A growing number of studies have reported the possible physiological roles of melatonin and their mechanisms. They have shown that melatonin can perform a number of roles, such as protecting the photosynthetic system and related subcellular structures (Zhao et al., 2016; Altaf et al., 2021), promoting seed germination (Zhang et al., 2014; Hwang and Back, 2020) and providing effective defenses against both biotic and abiotic stresses (Zhang et al., 2017; Li et al., 2018). Moreover, with auxin-like activity, melatonin is able to stimulate root initiation and induce root growth. In recent years, a large number of studies have confirmed that applications of exogenous melatonin can promote root development, especially the development of lateral roots and AR. For example, exogenous melatonin can promote the emergence of lateral roots and AR in lupin (Arnao and Hernández-Ruiz, 2007) and Arabidopsis (Pelagio-Flores et al., 2012), and can promote AR formation in tomato (Wen et al., 2016) and apple (Mao et al., 2020).

Previous studies have shown that melatonin is an important modulator of gene expression related to hormone production, as well as of the metabolism of auxin, CK, JA, SA and ethylene and other endogenous hormones (Arnao and Hernandez-Ruiz, 2018). For example, melatonin regulates root development by increasing the level of auxin and inducing the expression of genes related to auxin signal transduction, transport and synthesis (Chen et al., 2009; Wen et al., 2016; Liang et al., 2017; Mao et al., 2020). However, applications of exogenous melatonin at high concentration, inhibit root growth by inhibiting auxin transport and synthesis, and is accompanied by a decrease in endogenous IAA (Wang et al., 2016). Under copper stress, melatonin promotes root development in melon by inhibiting the expression of JA synthesis-related genes (Hu et al., 2020). In Arabidopsis, melatonin promotes the expressions of IAA and ethylene synthesis-related genes, whereas it inhibits the expressions of brassinosteroid, JA and CK synthesis-related genes, thereby integrating hormone signals to further mediate the expressions of zinc finger proteins and calmodulin-like proteins, so as to promote lateral roots development (Yang et al., 2021a). Moreover, melatonin also upregulates SA-related genes, leading to an increase in SA, while melatonin synthesis enzyme serotonin N-acetyltransferase mutants exhibited lowered levels of SA (Lee et al., 2015). Though these studies demonstrate a link between melatonin and hormones, the relationship between melatonin and these hormones in the formation of AR in cucumber seedlings is unknown.

To better understand how melatonin induces AR formation in the hypocotyls of cucumber seedlings under shaded conditions, we first analyze the effects of melatonin on AR formation and hormone content in cucumber hypocotyls. Then, combined with transcriptomics analysis, we explore the mechanism of AR formation induced by exogenous melatonin under shaded hypocotyl conditions. This research aimed to explore the effects of melatonin on AR development in cucumber seedlings, and to provide a basis for revealing the mechanism of melatonin in regulating AR formation in cucumber seedlings.

Materials and methods

Plant materials

Seeds of cucumber (*Cucumis sativus* L.) cultivar 'Xinyan 4' were soaked in 55°C water for 12 h to trigger germination, then placed in a growth chamber under conditions of photoperiod (day/night, 12/12 h), light intensity (300 μmolm-2 s-1), temperature (28/18°C) and relative humidity (75%). When the second true leaf was fully expanded, seedlings were divided randomly into three groups. Each group was subjected to one of three treatments: EW (hypocotyl exposed to air, irrigated with water), SW (hypocotyl shaded with sand, irrigated with water) and SM (hypocotyl shaded with sand, irrigated with 100 μM melatonin) and then cultivated for three days. The hypocotyls were then isolated and analyzed for hormone content and subjected to transcriptome analysis. Each treatment was repeated three times, with 180 seedlings per treatment.

Morphological observation of adventitious roots

Three days after treatment, adventitious root development in the hypocotyl was determined under a stereomicroscope. Ten seedlings were collected 12 days after treatment and the numbers of AR in the hypocotyl were counted.

Determination of hormone content

Hormone determination of the freeze-dried hypocotyl was carried out with three biological replicates by UPLC-MS/MS (LCMS-6500 system, Sciex) according to the method described previously (Flokova et al., 2014; Cui et al., 2015; Guo et al., 2021). Briefly, hormone was extracted by crushing the frozen sample in a mixer mill (MM 400, Retsch) for 1.0 min at 30 Hz under freezing conditions. Next, 50 mg of the frozen powder was extracted with a methanol: water: formic acid mix (15:4:1, v:v:v) and injected into a QTRAP 6500+ LC-MS/MS system (Sciex, USA). Multiquant 3.0.3 software (Sciex) was used to analyze the data.

RNA extraction and library construction

The RNA was extracted from the hypocotyl tissue (three replicates per treatment). The RNA-Seq was commissioned to Metware Biotechnology Co. Ltd. (Wuhan, China). Sequencing libraries were constructed using Illumina's NEBNext UltraTM RNA Library Prep Kit (NEB, USA). Firstly, magnetic beads with oligo (dT) were used to enrich mRNA, and the mRNA were broken into fragment by adding fragmentation buffer. Then added M-MuLV Reverse Transcriptase, random hexamer primer, RNase H and DNA Polymerase I to synthesize double-stranded cDNA. Library was constructed after purification and amplification. Sequencing was carried out on the Illumina HiSeq platform.

Transcriptomic data analysis

Clean data were produced by removing reads of low-quality, containing poly-N and adapter sequences from raw reads. HISAT2 tools was used to map the clean data to the cucumber reference genome sequence (http://cucurbitgenomics.org/organism/20). The gene expression levels were calculated as fragments per kb of transcript per million reads (FPKM) values. We used the DESeq2 package to conduct differentially expressed genes (DEGs) analyses. Genes with $|\log_2 FC| \geq 1$ and padj ≤ 0.05 were considered to be DEGs. KEGG pathway analysis and GO analysis were carried out for all DEGs.

Quantitative real-time PCR validation

The qRT-PCR was carried out on a Step One Plus Real-Time Fluorescent Quantitative PCR system (ABI, USA) using a SYBR Premix EX Taq kit (Takara, Japan). Quantification was evaluated using the $2^{-(\Delta\Delta Ct)}$ method. Specific primers of 16 genes randomly selected from the DEGs were listed in Supplementary Table S2.

Results

Effects of melatonin on adventitious root development

After three days of treatment, AR primordia on hypocotyls could be seen in the SW and SM treatments, with SM showing more AR primordia than SW (Figure 1). The rooting rate in the SM treatments was significantly higher than in the SW treatments after 12 days, while no ARs appeared in the EW treatment (Figure 1). The number of AR in the SM treatment was 18, that is 2.25-fold higher than in the SW treatment (Figure 1). The results suggest that exogenous melatonin promotes AR development in the shaded hypocotyls of cucumber seedlings.

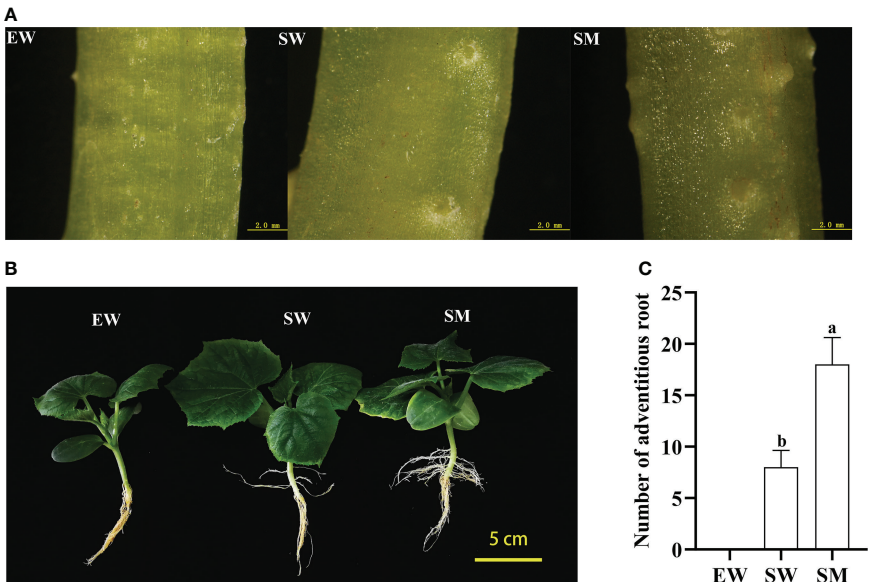


FIGURE 1
Effects of melatonin on adventitious root (AR) development of cucumber seedings under hypocotyl shading conditions. **(A)** Observation of AR primordium development with stereoscope after 3 days under EW, SW, and SM treatment (scale bar = 2 mm). **(B)** Observation of AR morphological after 12 days under EW, SW, and SM treatment (scale bar = 5 cm). **(C)** Number of AR after 12 days under EW, SW, and SM treatment. EW (hypocotyl exposed and irrigated with water); SW (hypocotyl shaded and irrigated with water); and SM (hypocotyl shaded and irrigated with 100 μM melatonin). Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$).

Differentially expressed genes induced in hypocotyls by melatonin under shaded conditions

Three cDNA libraries were obtained by sequencing RNA extracted from the EW, SW and SM hypocotyls. Each library consisted of more than 7.1 Gb of clean data. The Q20 percentage was more than 95%, and the error rate was less than 0.04%, indicating high sequencing quality (Table 1).

To verify the reliability of the RNA-Seq results, 16 DEGs were randomly selected for qRT-PCR verification. The qRT-PCR data were consistent with the RNA-seq data (Figure 2), the correlation coefficient is $R^2 = 0.8283$ (Supplementary Figure S1). These results validate the reliability of the RNA-seq data.

To elucidate the molecular regulation mechanism of exogenous melatonin on AR formation in cucumber seedlings, DEGs were analyzed in two comparison groups (EW vs. SW and SW vs. SM). Hierarchical cluster analysis was carried out to observe the overall expression pattern of DEGs, with red and green bands representing high and low genes expression levels, respectively (Figure 3). A total of 2760 DEGs were detected in EW and SW, with 1379 genes upregulated and 1381 genes downregulated relative to those in EW. A total of 1296 DEGs were detected in SW and SM, with 774 genes upregulated and 522 genes downregulated relative to those in SW (Figure 3). These DEGs were induced by exogenous melatonin and so are important candidate genes for further study.

We carried out a KEGG enrichment analysis of the DEGs to identify the main pathways enriched in the melatonin-induced AR development process. Between EW and SW, the top 20 enriched KEGG pathways were mainly related to biosynthesis of secondary metabolites, metabolic pathways, photosynthesis, MAPK signaling pathways and phenylpropanoid biosynthesis (Figure 3). Between SW and SM, the major enriched KEGG pathways being biosynthesis of secondary metabolites, metabolic pathways, plant hormone signal transduction, plant-pathogen interaction and phenylpropanoid biosynthesis (Figure 3). We also conducted a

TABLE 1 Summary of sequence data.

Sample	Clean Reads	Clean Base (G)	Error Rate (%)	Q20(%)	GC Content (%)
EW	49380905	7.41	0.04	95.58	43.35
SW	48687753	7.3	0.04	95.55	42.94
SM	47758435	7.16	0.04	95.66	42.26

EW (hypocotyl exposed and irrigated with water), SW (hypocotyl shaded and irrigated with water) and SM (hypocotyl shaded and irrigated with 100μM melatonin).

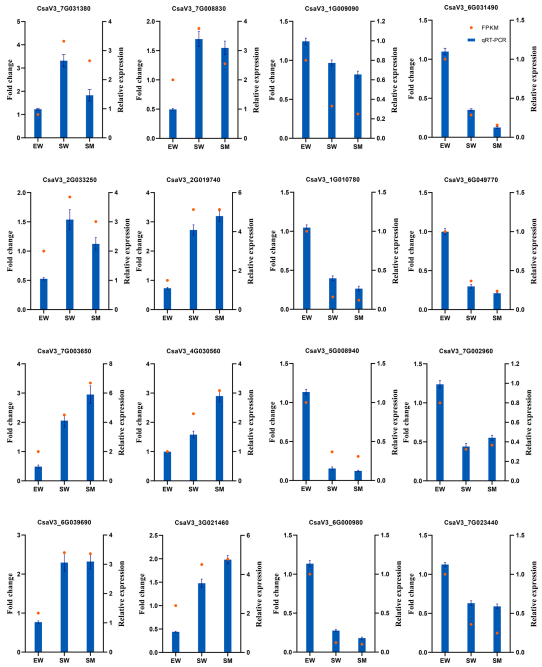


FIGURE 2
qRT-PCR validation of DEGs detected by RNA-Seq.

GO enrichment analysis of the DEGs to identify the main enriched biological process categories. Between EW and SW, DEGs were significantly enriched in the secondary metabolic process, photosynthesis, response to water deprivation and other biological process categories (Supplementary Figure S2). Between SW and SM, DEGs were mainly categorized into the secondary metabolic process, regulation of hormone levels, cell wall biogenesis, phenylpropanoid biosynthetic process, response to fungus and other biological process categories (Supplementary Figure S2). From the above, it is clear that DEGs induced by melatonin were significantly enriched in regulation of hormone levels and hormone signal transduction. So, we focused on the effects of melatonin treatment on hormone contents and their related genes.

Contents of auxin and cytokinin and expression analyses of related genes

We found that irrigation with water and melatonin and with shaded hypocotyl the auxin content was significantly affected and also the expressions of the auxin-related genes, involved in auxin synthesis (*TDC, YUCCA* and *TAA*), decomposition (*DAO*), transport (*PIN7*) and signaling (*SAUR*, *AUX/IAA* and *GH3*). The contents of IAA and indole-3-acetyl-L-aspartic acid (IAA-Asp) in SW increased significantly, while indole-3-acetyl-L-valine methyl ester (IAA-Val-Me) and 3-Indoleacetonitrile (IAN) were significantly decreased in SW relative to in EW. Whereas compared with SW, the contents of IAA-Val-Me and IAN increased in SM 3.4-time and 3.1-times, respectively. Moreover, the contents of indole-3-carboxylic acid (ICA) and of indole-3-carboxaldehyde (ICAld) in SM also increased significantly (Figure 4). From the transcriptome analysis, we found that the expressions of tryptophan decarboxylase (*TDC*) (CsaV3_1G036910), *PIN7*(CsaV3_3G000190), small auxin-up RNA (*SAUR71*) (CsaV3_1G009830), *SAUR36* (CsaV3_6G001030), *SAUR21* (CsaV3_7G000720) and 2-oxoglutarate-dependent dioxygenase *(DAO)* (CsaV3_7G032850) were significantly upregulated in SW relative to in EW. Also, the expressions of these genes (except *SAUR21*) in SM were upregulated further relative to those in SW (Figure 4). However, compared with that in EW, the expressions of Gretchen Hagen (*GH3.17*) (CsaV3_4G002130), *SAUR32*(CsaV3_4G031250), Flavin-containing monooxygenase (*YUCCA*) (CsaV3_3G017920 and CsaV3_6G012670) and tryptophan aminotransferase (*TAA*) (CsaV3_1G001250 and CsaV3_1G001250) were significantly downregulated in SW. Among these, *GH3.17* was upregulated, while *SAUR32* was downregulated in SM relative to those in SW. *YUCCA* and *TAA* which did not change significantly in SM (Figure 4, Supplementary Table S1). The SM treatment significantly induced the expressions of *DAO* (CsaV3_4G000540 and CsaV3_7G032860) but inhibited the expression of auxin/indole-3-acetic acid (*AUX/IAA26*) (CsaV3_3G048460) (Figure 4, Supplementary Table S1).

The content of CK and the expressions of genes related to CK synthesis (*IPT*), conjugation (*UGT76C1*), decomposition (*CKX*) and signal transduction (*AHP*) were also regulated by melatonin under shaded conditions. Compared with that in EW, the contents of 2-Methylthio-cis-zeatin riboside (2MeScZR) and cis-Zeatin riboside (cZR) were significantly increased in SW, while that of N6-isopentenyladenine (IP) was significantly decreased. The level of 2-Methylthio-cis-zeatin (2MeScZ) in SM was increased 5.97-times relative to that in SW (Figure 4D). The expressions of cytokinin dehydrogenase (*CKX*) (CsaV3_2G001450 and CsaV3_4G036030) in SW were downregulated relative to those in EW, while in the SM treatment these genes were significantly upregulated. In SW, however, compared with in EW, the expressions of histidine kinase (*AHP6*) (CsaV3_4G001620) and UDP-glycosyltransferase (*UGT76C1*) (CsaV3_1G035800) were upregulated, the expressions of these genes in SM were further upregulated relative to those in SW (Figure 4C, Supplementary Table S1). Lastly, the expressions of *tRNA-IPT* (CsaV3_3G013770) and *CKX* (CsaV3_5G006200) were significantly inhibited under shaded conditions, while *AHP1* (CsaV3_1G039330) was induced by melatonin (Figure 4C, Supplementary Table S1).

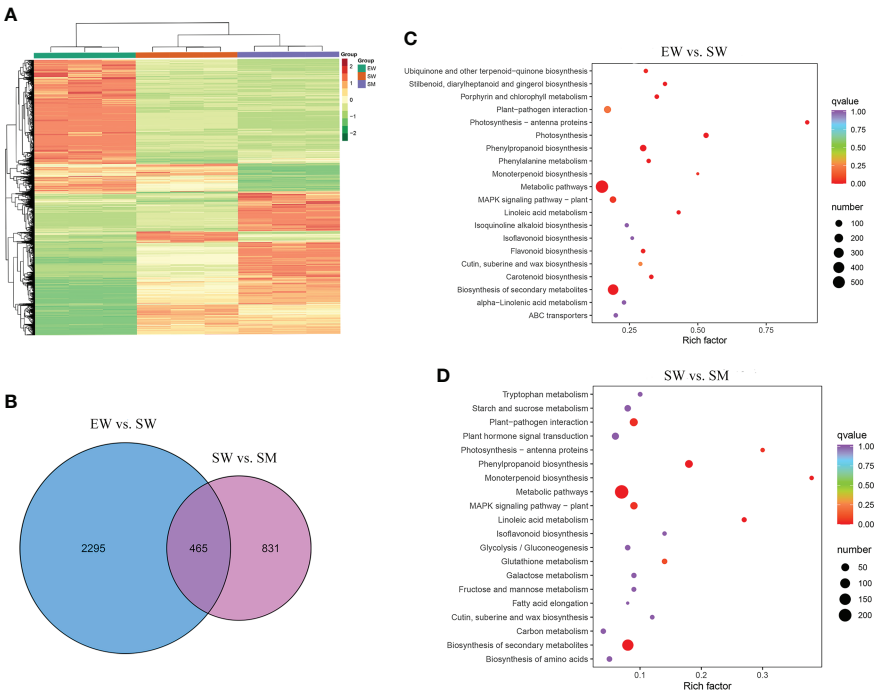


FIGURE 3
The differentially expressed genes (DEGs) induced by melatonin under hypocotyl shading conditions. **(A)** Hierarchical clustering of DEGs. **(B)** Venn diagram of unique and common DEGs. **(C, D)** KEGG pathways enrichment analysis of DEGs. EW (hypocotyl exposed and irrigated with water), SW (hypocotyl shaded and irrigated with water) and SM (hypocotyl shaded and irrigated with 100 μM melatonin).

Contents of salicylic acids and jasmonic acids and expression analyses of related genes

We found that melatonin induced significant accumulations of salicylic acid 2-O-β-glucoside (SAG) and SA under shaded conditions, with contents of SA increasing by 10.9-times relative to that in SW (Figure 5B). However, the content of SAG in SW was significantly decreased, while SA did not change significantly compared with in EW (Figure 5B). Melatonin also regulated the expressions of genes related to SA synthesis (*CM*, *PPA-ATs* and *PAL*), conjugation (*UGT74F2*) and signaling (*PR*). Compare with EW, the SW treatment significantly upregulated the expressions of chorismate mutase (*CM*) (CsaV3_5G038370), prephenate aminotransferase (*PPA-ATs*) (CsaV3_4G000360) and UDP-glycosyltransferase (*UGT74F2*) (CsaV3_6G015940), while the expressions of these genes in SM were upregulated further relative to in SW (Figure 5A, Supplementary Table S1). In addition, the SM treatment significantly upregulated the expressions of *PR* (CsaV3_7G007550 and CsaV3_7G007610) and of transcription factor *TGA* (CsaV3_3G039040, CsaV3_4G004890 and CsaV3_4G030930), while it downregulated the expression of phenylalanine ammonia-lyase (*PAL*) (CsaV3_6G036550) relative to in SW (Figure 5A, Supplementary Table S1).

Under shaded conditions, melatonin significantly increased the contents of dihydrojasmonic acid (H2JA), N-[(-)-Jasmonoyl]-(L)-valine (JA-Val), jasmonoyl-L-isoleucine (JA-ILE) and cis(+)-12-Oxophytodienoic acid (OPDA). It also significantly promoted accumulations of JA, N-[(-)-Jasmonoyl]-(l)-phenalanine (JA-Phe) and 3-oxo-2-(2-(Z)-Pentenyl) cyclopentane-1-butyric acid (OPC-4) (Figure 5D). Irrigation with water and melatonin and with shaded hypocotyl the expressions of genes related to JA synthesis (*LOX*) and signaling (*JAZ* and *MYC2*) was significantly affected. Transcriptome analysis showed that compared with in EW, the expressions of eight lipoxygenase (*LOX*) genes (CsaV3_2G005360, CsaV3_2G006370, CsaV3_2G006420, CsaV3_4G023820, CsaV3_4G023830, CsaV3_4G023840, CsaV3_4G023880 and CsaV3_4G023910) were upregulated in SW and the expressions of these genes were even further upregulated in SM (Figure 5C, Supplementary Table S1). However, compared with in EW, the SW treatment downregulated the expressions of jasmonate ZIM-domain proteins (*JAZ*) (CsaV3_1G041270) and did not significantly change those in SM (Supplementary Table S1). In addition, two *LOX* (CsaV3_2G023200 and CsaV3_4G023890) and one transcription factor *MYC2* (CsaV3_6G014370) were significantly upregulated in SM, relative to in SW (Figure 5C).

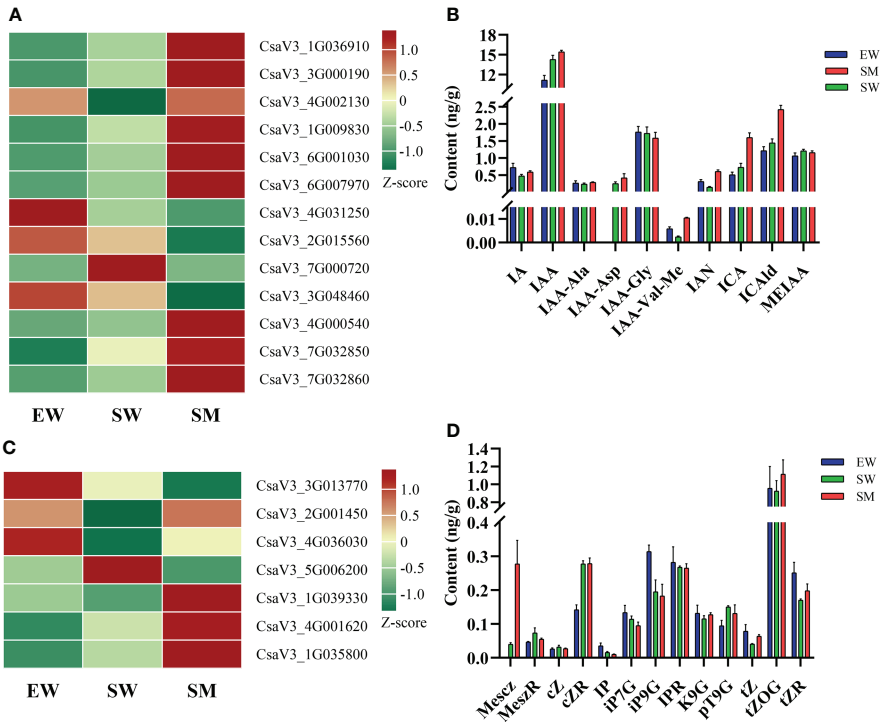


FIGURE 4
Effects of melatonin on genes and hormones related to auxin and cytokinin in cucumber seedings. **(A, B)** Effects of melatonin on the expression of auxin-related genes and auxin content. **(C, D)** Effects of melatonin on the expression of cytokinin-related genes and cytokinin content. EW (hypocotyl exposed and irrigated with water), SW (hypocotyl shaded and irrigated with water) and SM (hypocotyl shaded and irrigated with 100 μM melatonin).

Contents of ethylene and abscisic acid and expression analyses of related genes

The content of 1-Aminocyclopropanecarboxylic acid (ACC) in SW decreased significantly relative to in EW, but it did not change significantly in SM (Figure 6B). ACC synthase (ACS) and ACC oxidase (ACO) are key enzymes in ethylene synthesis. We found that, compared with in EW, the expressions of ethylene-responsive transcription factor (*ERF1*) (CsaV3_3G022370 and CsaV3_7G027380) in SW were upregulated. The expressions of these genes were even further upregulated in SM (Figure 6A). However, the expressions of *ACO* (CsaV3_3G012240 and CsaV3_3G023650) in SW were significantly downregulated, and these genes did not change significantly in SM (Supplementary Table S1). However, compared with in SW, the expressions of *ACO* (CsaV3_1G005350 and CsaV3_3G012290) and *ACS* (CsaV3_6G000890) were significantly upregulated in SM, while S-adenosylmethionine synthase (*SAMs*) (CsaV3_6G039940) was significantly downregulated (Figure 6A).

The content of ABA was significantly decreased by shading but significantly increased by melatonin under shading (Figure 6D). The expressions of genes related to ABA synthesis (*ABA2*), metabolism (*ABA 8′-hydroxylase, β-glucosidase*) and signal transduction (*CDPK*) were also regulated by melatonin. We found that the expressions of β-glucosidase *(β-Glu)* (CsaV3_1G042500, CsaV3_1G042530 and CsaV3_1G042540) and *ABA 8′-hydroxylase* (CsaV3_7G008610) were significantly upregulated by shading, and the expressions of these genes (except of *ABA 8′-hydroxylase*) were further upregulated by melatonin under shaded conditions, while *ABA 8′-hydroxylase* was inhibited by melatonin (Figure 6C, Supplementary Table S1). However, the expression of *β-Glu* (CsaV3_1G042490) was downregulated by shading, while it was upregulated by melatonin under shaded. Melatonin also significantly induced the expressions of short-chain dehydrogenase (*ABA2*) (CsaV3_5G002010) and calcium dependent protein kinase (*CDPK*) (CsaV3_3G038220 and CsaV3_6G038920) under shaded conditions (Figure 6C).

Expressions of cell wall formation-related genes

We found that in SM, compared with in EW, the expressions of *expansin* (CsaV3_7G001490 and CsaV3_7G001530), nine peroxidase *(POD)* (CsaV3_2G014160, CsaV3_4G023640, CsaV3_4G023650,

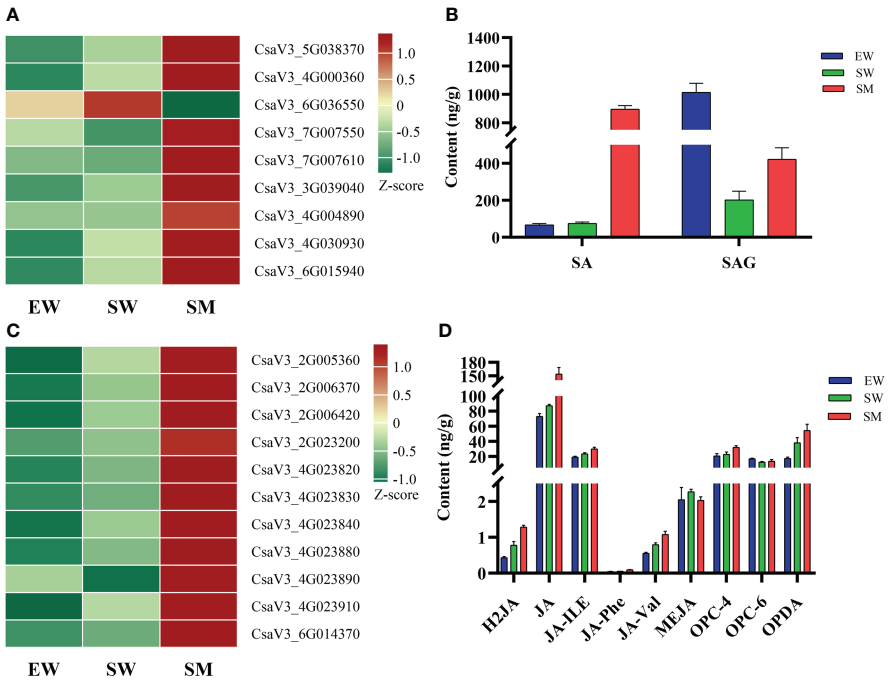


FIGURE 5
Effects of melatonin on genes and hormones related to salicylic acid and jasmonic acid in cucumber seedings. **(A, B)** Effects of melatonin on the expression of salicylic acid-related genes and salicylic acid content. **(C, D)** Effects of melatonin on the expression of jasmonic acid-related genes and jasmonic acid content. EW (hypocotyl exposed and irrigated with water), SW (hypocotyl shaded and irrigated with water) and SM (hypocotyl shaded and irrigated with 100 μM melatonin).

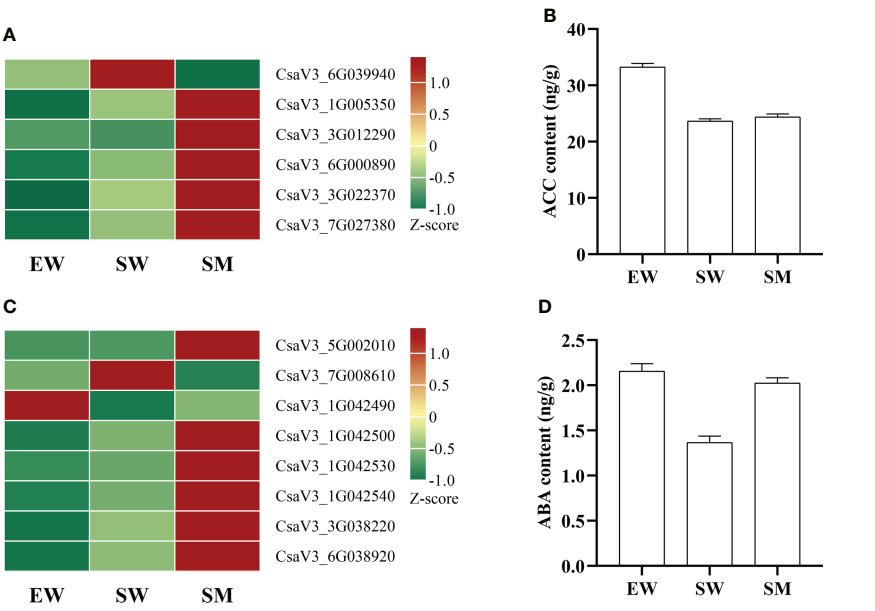


FIGURE 6
Effects of melatonin on genes and hormones related to ethylene and abscisic acid in cucumber seedings. **(A, B)** Effects of melatonin on the expression of ethylene-related genes and ethylene content. **(C, D)** Effects of melatonin on the expression of abscisic acid-related genes and abscisic acid content. EW (hypocotyl exposed and irrigated with water), SW (hypocotyl shaded and irrigated with water) and SM (hypocotyl shaded and irrigated with 100 μM melatonin).

CsaV3_4G023680, CsaV3_5G012840, CsaV3_6G005630, CsaV3_6G043090, CsaV3_6G043930, CsaV3_7G005720) and cellulose synthase (*CesA*) (CsaV3_1G033150) were upregulated. The SM treatment further upregulated the expressions these genes (except *CesA*) (Supplementary Figure S3, Supplementary Table S1). The SM treatment upregulated the expressions of *expansin* (CsaV3_7G001510 and CsaV3_7G001520), of ten *POD* (CsaV3_7G030390, CsaV3_7G030400, CsaV3_7G030420, CsaV3_1G030170, CsaV3_2G016300, CsaV3_4G023590, CsaV3_4G023660, CsaV3_4G023670, CsaV3_6G006890 and CsaV3_7G003750) and cellulose synthase-like *(CSL)* (CsaV3_7G011490), and downregulated the expressions of xyloglucan glycosyltransferase (CsaV3_5G037390) and *CesA* (CsaV3_2G009180 and CsaV3_6G007440) significantly (Supplementary Figure S3, Supplementary Table S1).From these results, it is clear that exogenous melatonin significantly affected the expressions of genes related to cell wall formation under hypocotyl shading conditions.

Discussion

The formation of AR is affected by water, minerals, wounding and hormones. Many studies have shown that hormones contribute greatly to AR formation (Hutchison et al., 2006; Vidoz et al., 2010; Da Costa et al., 2013; Dong et al., 2020). Melatonin treatments could regulate endogenous hormone contents and their related genes (Arnao and Hernandez-Ruiz, 2018) and so stimulate root generation, giving rise to new lateral and adventitious roots (Arnao and Hernández-Ruiz, 2007; Pelagio-Flores et al., 2012; Mao et al., 2020). Our results show that exogenous melatonin significantly promotes AR formation in cucumber seedings under hypocotyl shading conditions. We measured hormones levels in the hypocotyls among the three treatments and found that melatonin significantly induced the accumulation of the hormones - auxin, CK, SA, JA and ABA. The GO and KEGG analyses showed that DEGs induced by melatonin were significantly enriched in regulation of hormone levels and plant hormone signal transduction, suggesting that hormones and hormone signal transduction may play important roles in cucumber AR formation.

Melatonin and auxin are tryptophan-derived compounds, previous studies have shown that melatonin may increase auxin contents or exhibit auxin-like activity during root development. For example, melatonin regulates root development in apple (Mao et al., 2020), tomato (Wen et al., 2016), rice (Liang et al., 2017) and Arabidopsis (Yang et al., 2021b) by inducing the expression of genes related to auxin synthesis, transport and signaling, as well as increasing auxin contents. However, other studies have shown that melatonin can regulate root development independently of auxin signaling in Arabidopsis (Pelagio-Flores et al., 2012; Zia et al., 2019). In this study, we found that exogenous melatonin induced AR development in cucumber seedlings may be related to auxin. It has been reported that, transport and accumulation of auxin are necessary for root development, PIN proteins are responsible for auxin transport, *pin1, pin3* and *pin7* mutants show statistically significant reductions in AR formation (Sukumar et al., 2013), while down regulation of the *PIN5* promotes lateral root development (Ren et al., 2019). In this study, it was found that compared with EW, the SW treatment induced the expressions of tryptophan decarboxylase (*TDC*) (CsaV3_1G036910) and *PIN7* (CsaV3_3G000190), whereas it inhibited the expressions of genes related to auxin synthesis, such as *YUCCA* (CsaV3_3G017920 and CsaV3_6G012670) and *TAA*(CsaV3_1G001250) (Figure 4A, Supplementary Table S1); and decreased the level of IAN, while it increased the levels of IAA and IAA-Asp (Figure 4B). Which may be related to the upregulation of the auxin transport gene *PIN*, resulting in local accumulations of IAA. The melatonin (SM) treatment further activated the expression of *TDC* (CsaV3_1G036910) and *PIN7* (CsaV3_3G000190) (Figure 4A) under hypocotyl shading conditions. It also increased the contents of IAN significantly (Figure 4B), whereas the content of IAA did not increase significantly in SM. This may be related to the upregulation of *DAO* (CsaV3_4G000540 and CsaV3_7G032860), which convert IAA to bound state. It is clear that the increased level of IAA induced by hypocotyl shading is required for the formation of AR (Figure 1, Figure 4B), while exogenous melatonin significantly promoted the accumulation of IAN under hypocotyl shading conditions (Figure 4B), which may be the factor inducing the significant increase in AR. Auxin signaling plays a vital role in the transition of protocambial cells to AR-initiating cells (Chen et al., 2016), auxin induced primary auxin responsive genes include *AUX/IAA*, *SAUR* and *GH3*. In the absence of auxin, Aux/IAAs protein interacts with ARFs and prevents ARFs from binding to the promoter of auxin-reponsive genes. In the presence of auxin, the Aux/IAA protein is degraded through the ubiquitination pathway, then ARFs are released from inhibition and can regulate the expressions of auxin-responsive genes (Weijers and Jürgens, 2004). *SAUR* are highly induced in response to auxin, which regulate various aspects of plant development, such as: root initiation, cell division and cell differentiation. Studies show that SAURs may function as positive regulators of cell expansion, which plays vital roles in IAA-mediated adventitious rooting in petunia cuttings (Yang et al., 2019). *GH3* controls auxin homeostasis in plants, *GH3.3, GH3.5, GH3.6* are required for AR initiation in the Arabidopsis hypocotyl (Gutierrez et al., 2012), *GH3.17* is necessary to control meristem size of the root (Di Mambro et al., 2019). The expression of *SAUR71* (CsaV3_1G009830) and *SAUR36* (CsaV3_6G001030) were upregulated, *GH3.17* (CsaV3_4G002130) was downregulated by hypocotyl shading, and the expressions of these genes were upregulated by melatonin under hypocotyl shading conditions (Figure 4A). In addition, melatonin inhibited the expression of *AUX/IAA26* (CsaV3_3G048460) under hypocotyl shading conditions (Figure 4A, Supplementary Table S1). These results

show that hypocotyl shading treatment promoted signal transduction of auxin, and the application of exogenous melatonin under hypocotyl shading conditions further induced auxin signaling, which may promote the formation and elongation of AR. This observation is consistent with the effects of auxin in root induction and elongation (Guan et al., 2019; Qi et al., 2019). From the results, it is clear that the induction effect of melatonin on AR formation in the cucumber seedling hypocotyl may be related to auxin, and there may be interactions between melatonin and auxin. Similar results have been obtained in studies with lupin (Arnao and Hernández-Ruiz, 2007), tomato (Wen et al., 2016) and apple (Mao et al., 2020).

Cytokinin can regulate cell division, vascular development and root development (Werner et al., 2001; Sakakibara, 2006). It has been reported that, exogenous applications of CK inhibits both lateral (Hutchison et al., 2006; Li et al., 2006) and adventitious root formation (Mahonen et al., 2006) in Arabidopsis. Isopentenyltransferase (IPT) is a key enzyme in CK synthesis, while CK dehydrogenase (CKX) catalyzes the oxidative decomposition of CK (Sakakibara, 2006). Over-expression of IPT can significantly inhibit the development of primary and lateral roots, while over-expressing CKX significantly promotes AR formation in Arabidopsis seedlings (Werner et al., 2001). Transcriptome data analysis found that the expressions of *CKX* (CsaV3_2G001450 and CsaV3_4G036030) were downregulated by hypocotyls shading (Supplementary Table S1), whereas the content of IP was decreased (Figure 4D), which may be related to the upregulation of *UGT76C1* (CsaV3_1G035800) and glycosylate free-state IP to the bound state. However, melatonin downregulated the expression of *tRNA-IPT* (CsaV3_3G013770) and upregulated the expressions of *CKX* (CsaV3_2G001450 and CsaV3_4G036030) under hypocotyl shading conditions (Figure 4C, Supplementary Table S1); while the level of 2MeScZ was increased significantly in SM (Figure 4D). It has been reported that, 2MeS-CKs (unbound methylthiolated CKs) is less cytotoxic and more resistant to CKX than classical CKs, which may be responsible for continuous tissue proliferation during plant-pathogen interactions (Gibb et al., 2020). In this study, the level of 2MeScZ in SM was significantly increased relative to that in SW, which may be related to the stronger resistance of 2MeScZ to *CKX* and the reduction of decomposition, resulting in local accumulations of 2MeScZ. In addition, KEGG enrichment analysis found the plant-pathogen interaction pathway was significantly enriched in the SW and SM comparison groups (Figure 3D). This may be related to pathogenic bacterial infection during AR formation. The accumulation of 2MeScZ in hypocotyls, can mediate plant defenses, promote tissue proliferation around the site of infection and ease deleterious effects on AR development, thereby facilitating the formation of AR. *AHPs* plays a vital role in CK signal transduction, most *AHPs* are positive regulators of CK signal transduction (Hutchison et al., 2006), whereas *AHP6* is a negative regulator of CK signal transduction (Mahonen et al., 2006). In Arabidopsis, reduced AHP function in ahp1,2,3,4,5 mutants resulted in a short primary root but enhanced AR development. Additionally, AHP2, AHP3 and AHP5 are particularly important and AHP1 and AHP4 may play minor roles in these processes (Hutchison et al., 2006). *ahp6* mutants grown on media with increasing concentrations of CK, produce fewer ARs in Arabidopsis (Mahonen et al., 2006). In our study, we found that compared with that in EW, the SW treatment induced the expression of *AHP6* (CsaV3_4G001620), while SM treatment activated *AHP6* expression further (Figure 4C, Supplementary Table S1). This suggests that melatonin inhibited CK signaling, which may enhance AR development.

Salicylic acid affects seed germination, root elongation and AR development, which is also involved in defense against pathogens. Studies have shown that SA and auxin act synergistically to regulate AR development (Pasternak et al., 2019; Dong et al., 2020). Exogenous SA promoted AR formation in wheat under flooding condition (Koramutla et al., 2022), whereas Arabidopsis *eds5-1* and *eds5-2* mutants lacking SA synthesis produced fewer AR than the wild type. This suggests SA is a positive regulator of AR development (Gutierrez et al., 2012). In Arabidopsis, melatonin upregulated SA-related genes, resulting in an increased level of SA (Lee et al., 2015). In our study, we found that melatonin significantly upregulated the expression of SA synthetase *CM* (CsaV3_5G038370) and *PPA-ATs* (CsaV3_4G000360) (Figure 5A, Supplementary Table S1), thereby significantly increasing the level of SA in the hypocotyl (Figure 5B). Moreover, UDP-glycosyltransferase *UGT74F2* (CsaV3_6G015940) was also upregulated by melatonin, which glycosylated SA to SAG, resulting in a significant increase in SAG in the SM plants. These results indicate that the significant increase in AR induced by exogenous melatonin may be related to increased levels of SA. This is consistent with the effect of SA in cucumber hypocotyl AR induction (Dong et al., 2020). On the other hand, in the process of AR emergence, the young plant is susceptible to affection by soil-borne pathogens. KEGG enrichment analysis found that the plant-pathogen interaction pathway was significantly enriched in the SW and SM comparison groups (Figure 3D). This may be related to a pathogenic bacterial infection during AR formation. To ensure plant growth in an unfavorable environment, SA plays a vital role in regulating the growth-defense balance. The expression of pathogenesis-related gene 1(PR1) is considered a marker for activating SA-mediated systemic acquired resistance. It has been reported that, SA inhibited lateral root formation, enhanced adventitious rooting, promoted cell wall lignification and plasmodesmal closure by inducing the expressions of PR1 and PR2, which can directly prevent pathogens from invading roots and so facilitate plants growth under adverse conditions (Bagautdinova et al., 2022). In our study, we found that under hypocotyl shading conditions, exogenous melatonin significantly induced the expressions of *PR1* (CsaV3_7G007550 and CsaV3_7G007610) and of transcription factors *TGA* (CsaV3_3G039040, CsaV3_4G004890 and

CsaV3_4G030930) (Figure 5A, Supplementary Table S1). These can mediate systemic acquired resistance, effectively resisting pathogen infection during AR formation and so facilitating AR development.

It is reported that JA serves as a signal to activate rooting, thereby promoting AR formation in tobacco (Fattorini et al., 2009), pea (Rasmussen et al., 2015), petunia (Lischweski et al., 2015) and Arabidopsis (Fattorini et al., 2018). LOX is a key enzyme in JA synthesis. We found that most LOX genes were upregulated by hypocotyl shading (Figure 5C, Supplementary Table S1), thereby significantly increasing the content of Jasmonoyl-L-isoleucine (JA-ILE) (Figure 5D). Meanwhile, melatonin significantly further upregulated the expressions of these genes under hypocotyl shading conditions (Figure 5C, Supplementary Table S1), as well as promoting the accumulation of JA-ILE and JA (Figure 5D). JAZ proteins are key repressors of JA signaling. MYC2, a bHLH TF, is considered to be a master regulator of the JA signaling (Fernandez-Calvo et al., 2011). In our study, it was found that the expression of *JAZ* (CsaV3_1G041270) was significantly downregulated by hypocotyl shading, and the expression of *MYC2* (CsaV3_6G014370) was significantly upregulated by melatonin under hypocotyl shading conditions (Figure 5C, Supplementary Table S1). These findings indicate that the formation of ARs induced by hypocotyl shading may be related to the accumulation of JA-ILE and enhanced signaling. Meanwhile, the significant accumulation of JA-ILE and JA, accompany the upregulation of *MYC2* induced by exogenous melatonin may further activate the expression of JA-responsive genes, thereby significantly promoting AR formation, in accordance with the finding that JA acts as a accelerator of AR formation in tea plants (Fan et al., 2021).

Ethylene interacts with auxin, gibberellin and ABA to regulate AR formation in tomato (Vidoz et al., 2010) and rice (Steffens and Sauter, 2006). ACS and ACO are key synthase genes of ethylene, which are upregulated during AR formation in petunia (Lischweski et al., 2015). In Arabidopsis, *ACS* and *ACO* are significantly regulated by melatonin (Yang et al., 2021a). Our Transcriptome data analysis showed that the expressions of *ACO* (CsaV3_3G012240 and CsaV3_3G023650) were downregulated by hypocotyl shading (Supplementary Table S1), thereby the level of ACC in SW decreased significantly (Figure 6B). However, the melatonin upregulated the expressions of *ACO* (CsaV3_1G005350 and CsaV3_3G012290) and *ACS* (CsaV3_6G000890), while it downregulated the expression of *SAMs* (CsaV3_6G039940) under hypocotyl shading conditions (Figure 6A, Supplementary Table S1). Also, the ACC content did not change significantly (Figure 6B). Many ethylene response factors (*ERFs*) are common mediators of root developmental. It has been found that ERFs are upregulated during AR development in cuttings (Druege et al., 2016). In our study, we found melatonin significantly enhanced the expressions of *ERF1* (CsaV3_3G022370 and CsaV3_7G027380) under hypocotyl shading conditions (Figure 6A, Supplementary Table S1). The above results show that the significant increase in AR in our cucumber seedlings may well be related to the enhanced expression of ethylene synthesis and response factor genes.

Earlier studies found that ABA can promote AR formation in cucumber (Li et al., 2016) and mung bean seedlings (Li et al., 2014) under stress conditions. ABA 8'-hydroxylase catalyzes the oxidative degradation of ABA, while β-glucosidase (β-Glu) can hydrolyze ABA gluconate into bioactive ABA. Studies on tea plants showed that ABA content was higher in the easy-to-root cultivar, which indicate that ABA is involved in the occurrence of AR (Fan et al., 2021). In our study, the expression of *β-Glu* (CsaV3_1G042500, CsaV3_1G042530 and CsaV3_1G042540) were significantly upregulated by hypocotyl shading and melatonin further upregulated these genes under hypocotyl shading conditions, *while ABA 8'-hydroxylase* was inhibited by melatonin (Figure 6A, Supplementary Table S1). Thereby the level of ABA in SM increased significantly (Figure 6D), which will contribute to the occurrence of AR. Additionally, ABA regulates stomatal transpiration and also root hydrophilicity. One prominent response of roots to ABA sensitivity is their ability to perceive differences in water potential and translate this into growth towards water, which depends on ABA signaling kinase SnRK2.2 activity (Dietrich et al., 2017). Calcium dependent protein kinase (CDPK) is a positive effector of ABA signaling transduction and plays a key role between ABA signal perception and response. In our study, we found that exogenous melatonin significantly induced the expressions of *CDPK* (CsaV3_3G038220 and CsaV3_6G038920) under hypocotyl shading conditions (Figure 6C, Supplementary Table S1). The upregulation of *CDPK* could phosphorylate downstream target proteins to respond to ABA signals, and so improve the absorptive capacity for water. This too will facilitate the production of AR. These findings indicate that ABA may be involved in melatonin-induced AR development in our cucumber seedlings.

Cellulase-like synthases (CSL) are the main load-bearing components of cell walls. AtCSLD3 is very important for the synthesis of primary cell wall in the root hair region and plays vital roles in root hair growth. In *AtCSLD3* deficient plants, the growth of root hairs is affected (Xuan et al., 2001). Peroxidase (POD) induces cell wall loosening and cell elongation by regulating the elongation and cross-linking of cell wall components (Passardi et al., 2004). From our analyses, we found that melatonin significantly induced *POD* expression under hypocotyl shading conditions (Supplementary Figure S3, Supplementary Table S1). In melon, the expression of POD was also upregulated by melatonin during lateral root development (Hu et al., 2020). The *expansin* can restore the expansion of heat-inactivated cell walls under acidic conditions, which loosen the cell wall, thereby affecting the extensibility of the cell wall (McQueen-Mason and S., 1992). In transgenic tobacco plants over-expressing *TaEXPB23*, the number of lateral roots was significantly increased (Han et al., 2014). In our study, we found exogenous melatonin significantly induced the

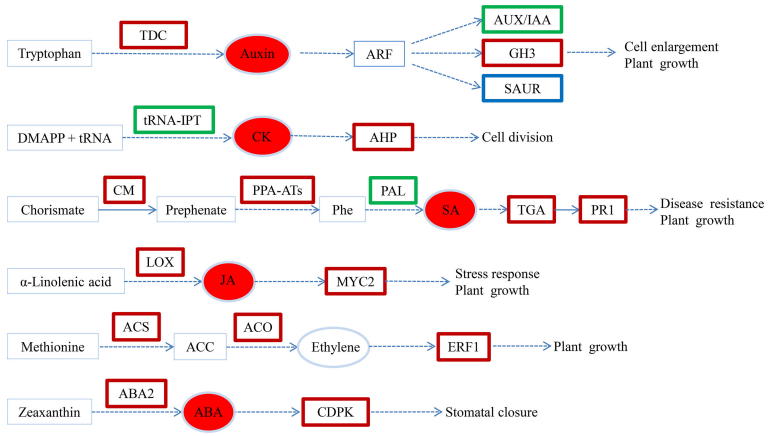


FIGURE 7
Transcriptional changes in hormone biosynthesis and signaling pathways in SM compared to SW. Upregulated, downregulated and both up- and downregulated genes are boxed in red, green and blue, respectively. The solid arrow indicates a direct step, and the broken arrow indicates an indirect step. SW (hypocotyl shaded and irrigated with water), SM (hypocotyl shaded and irrigated with 100 μM melatonin).

expression of *expansin* under hypocotyl shading conditions (Supplementary Figure S3, Supplementary Table S1). The upregulation of *expansin* will lead to the loosening of the cell wall where the new lateral roots appear, which will reduce the mechanical resistance of the stem tissue, thereby aiding the emergence of new ARs. Therefore, exogenous melatonin regulates the formation of cell walls by upregulating the expression of *CSL*, *POD* and *expansin* under hypocotyl shading conditions, thereby significantly inducing AR formation in our cucumber seedlings hypocotyl.

Conclusion

Hypocotyl shading was necessary to induce AR formation, while exogenous melatonin application significantly promoted AR formation in cucumber seedlings under hypocotyl shading conditions. To gain a deeper understanding of the underlying regulatory mechanisms, RNA-seq and hormone analyses were carried out. Our results show that exogenous melatonin induced the expressions of auxin, SA, JA, ethylene and ABA synthesis and signaling related genes. It also inhibited the expressions of CK signaling genes, as well as significantly increasing the auxin, CK, JA, SA and ABA contents (Figure 7). In the process of AR formation, melatonin also regulated the expressions of genes related to cell wall formation and induced the loosening of cell walls, thereby facilitating the emergence of AR. Finally, melatonin promoted the formation of a large numbers of AR in the cucumber seedling hypocotyls.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA717318.

Author contributions

YW, ZZ and LZ carried out experimental work. LL, XH, YZ and LH performed the data analyses. YW wrote the manuscript. HZ and ML designed the experiments and assisted with writing the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1059482/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Relationship between RNA-seq and qRT-PCR expression data (log2 fold change).

SUPPLEMENTARY FIGURE 2
GO enrichment analysis of DEGs.

SUPPLEMENTARY FIGURE 3
Effects of melatonin on the expression of cell wall formation-related genes in cucumber seedings.

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Phenotypic variation and epigenetic insight into tissue culture berry crops

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Berry crops, a nutrient powerhouse for antioxidant properties, have long been enjoyed as a health-promoting delicious food. Significant progress has been achieved for the propagation of berry crops using tissue culture techniques. Although bioreactor micropropagation has been developed as a cost-effective propagation technology for berry crops, genetic stability can be a problem for commercial micropropagation that can be monitored at morphological, biochemical, and molecular levels. Somaclonal variations, both genetic and epigenetic, in tissue culture regenerants are influenced by different factors, such as donor genotype, explant type and origin, chimeral tissues, culture media type, concentration and combination of plant growth regulators, and culture conditions and period. Tissue culture regenerants in berry crops show increased vegetative growth, rhizome production, and berry yield, containing higher antioxidant activity in fruits and leaves that might be due to epigenetic variation. The present review provides an in-depth study on various aspects of phenotypic variation in micropropagated berry plants and the epigenetic effects on these variations along with the role of DNA methylation, to fill the existing gap in literature.

KEYWORDS

antioxidants, clonal fidelity, DNA methylation, *in vitro* culture, vegetative growth, somaclonal variation

Introduction

Berry crops, also called small fruits, are composed of plants in a number of genera including *Actinidia* (hardy kiwi), *Amelanchier* (Juneberry/Saskatoon, serviceberry), *Aronia* (*Aronia melanocarpa*), *Fragaria* (strawberry), *Hippophae* (sea buckthorn), *Lonicera* (edible honeysuckle/Haskap), *Prunus* (chokecherry), *Ribes* (currants, gooseberries), *Rubus* (blackberries, raspberries and their hybrids), *Sambucus* (elderberries), *Schisandra* (schisandra), *Shepherdia* (silver buffalo berry), *Vaccinium* (blueberries, cranberries, lingonberries, bilberries, and huckleberries), *Viburnum* (highbush cranberry), and *Vitis* (grapes) (Debnath et al., 2012; Debnath, 2016;

Debnath, 2018). Although grapes are the most important berry crop worldwide, the production of strawberries, blueberries, raspberries, and cranberries is also a profitable agricultural enterprise (Debnath, 2016). These produce small- to moderate-sized edible fruits on perennial vines, herbs, or shrubs, and can be consumed in fresh, juice, dried, or processed form. The fruits and leaves are a good source of health-promoting bioactive compounds (Vyas et al., 2015; Abeywickrama et al., 2016; Debnath and An, 2019; Bhatt and Debnath, 2021) and are believed to fight against cardiovascular and degenerative diseases (Isaak et al., 2017; Debnath, 2018; Debnath and Arigundam, 2020; Debnath and Goyali, 2020; Hewage et al., 2020). Berry crops are genetically heterozygous and are generally propagated vegetatively to maintain genetic integrity and fruit quality, and for achieving quick fruit-bearing condition (Debnath, 2018). Micropropagation, also called *in vitro* propagation, is a multi-billion-dollar industry across the world for the propagation of various crop plants. It has been long used in berry crops for their quick and mass propagation in commercial cultivation. Despite the tremendous advantage of this propagation system in berry crop plants, clonal fidelity or trueness-to-type is of significant importance for its commercial application. *In vitro*-derived variations can be heritable (genetic) or epigenetic, that arise without alteration of DNA sequences but due to gene activation or silencing. Epigenetic variations can be unstable and may disappear following transfer from culture media or after a few clonal or sexual generations (Kaeppler et al., 2000), although heritable epigenetic changes are also available that are valuable for enhanced crop production and improvement (Kakoulidou et al., 2021). However, propagation strategies have been developed to minimize variations in micropropagated berry plants (Debnath and Goyali, 2020). Although a number of DNA-based molecular markers have been used to monitor the clonal fidelity in berry crops including raspberry (Debnath, 2014), lingonberry (Arigundam et al., 2020), blueberry (Goyali et al., 2015; Debnath, 2017a), and strawberry (Debnath, 2009a), tissue culture-induced epigenetic variation such as DNA methylation has been studied so far only in blueberry (Ghosh et al., 2017; Goyali et al., 2018; Ghosh et al., 2021a), strawberry (Cao et al., 2021), and lingonberry (Sikdar et al., 2022). The present review deals with in-depth variations during *in vitro* culture of berry crops along with their analysis at morphological, biochemical, and molecular levels.

In vitro propagation

The theoretical concept of *in vitro* culture was explained by Gottlieb Haberlandt in his lecture at the German Academy of Sciences based on his experiments on single-cell culture (Haberlandt, 1902). Haberlandt experimented with leaf and other actively differentiating cells that failed to give rise to artificial embryos. He further added that cultivating vegetative cells under optimal hormonal influence can be established as a new approach for plant propagation. Although he did not succeed in his experiments, his practice of regeneration of artificial embryos from vegetative cells supported the concept of totipotency (Thorpe, 2007). Haberlandt (1902) was successful at tuning the survival of *in vitro*-grown tissue, while cell division under *in vitro* conditions was first observed by Hannig (1904) and regeneration from callus tissue was first documented by Simon (1908). However, Gautheret (1934) demonstrated the first true plant tissue cultures using cambial tissue of *Acer pseudoplatanus*.

Regeneration of plants *via* tissue culture is dependent on two primary concepts, which are totipotency and developmental plasticity. According to Skoog and Miller (1957), totipotency can be explained as the ability of a plant cell to differentiate, proliferate, and eventually grow into a plantlet under optimal culture conditions and under hormonal influence. In general, cells from young tissues and meristems are totipotent in nature; however, differentiated cells also occasionally exhibit totipotency (Debnath, 2007a). On the other hand, plasticity is the capability of the plant tissues to adjust their metabolism, growth pattern, and development in order to survive under various environmental conditions. Plant tissue culture, which is also known as *in vitro* cell culture, is an important area of basic and applied science studies. Due to the availability of several *in vitro* propagation techniques, since approximately mid-1960s to 1980s, these techniques have gained a huge popularity to solve various biological, agricultural, horticultural, and forestry problems (Thorpe, 2007).

During the *in vitro* culture process, an individual cell, tissue, or organ of a plant can be used as an explant. Explants are then cultured in an artificial medium containing macro- and micronutrients, a carbohydrate source, vitamins, plant growth regulators (PGRs), and a chelating agent depending on media type. Under the optimum hormonal stimuli and appropriate environment, explants develop into an identical copy of the source plant under aseptic conditions, which is called a clone (Altman and Loberant, 2010). Clones can be regenerated during *in vitro* propagation *via* organogenesis by forming either shoot or root meristems and/or somatic embryogenesis (SE) where shoot and root meristems form simultaneously (Steward et al., 1970). Organogenesis follows the pathway of shoot proliferation from a pre-existing bud followed by adventitious shoot regeneration (Steward et al., 1970). In a tissue culture system, plants produce hundreds of identical copies within a short period. The commercial use of micropropagation techniques includes the maintenance of pathogen-free germplasm, production of nuclear stock, and yearlong production of clones of hybrid and parental lines (Debnath, 2014). In recent years, for the large-scale commercial micropropagation of elite varieties, industries have implemented micropropagation techniques. Consequently, many tissue culture laboratories were set up around the globe especially in developing countries due to cheaper labor costs (Kumar and Reddy,

2011). *In vitro* propagation of blueberry was first reported in the early 1970s using rhizome explants without the help of any PGR (Barker and Collins, 1963) in White's medium (White, 1943). However, the first report on the use of micropropagation techniques for commercial purposes was in the 1970s by Boxus (1974) in strawberry. Some other major discoveries were chemical and hormonal regulation of plant regeneration (Skoog and Miller, 1957), the application of *in vitro* propagation techniques in basic and applied science (Komamine et al., 1992), regeneration of virus-free plantlets, haploid culture (Nitsch and Nitsch, 1969), plantlet formation from protoplast culture (Cocking, 1960), secondary metabolite production (Kaul and Staba, 1965), and cell culture in a liquid medium in a bioreactor (Noguchi et al., 1977). Skoog and Miller (1957) hypothesized the effect of auxin–cytokinin on plant morphogenesis. The authors concluded from their experiment on tobacco pith culture that the auxin–cytokinin ratio is the deciding element for shoot and root formation. Skoog and Miller (1957) hypothesized that the culture medium supplemented with a higher concentration of cytokinin gives rise to shoot, and an increased concentration of auxin induces root formation, whereas a balanced ratio of auxin and cytokinin leads to the formation of a callus.

Micropropagation in berry crops is being used for year-round propagation of virus-free (indexed) clones, which can be used as a first step in a nuclear stock crop production system (Debnath, 2018). Softwood or rhizome cuttings of desirable clones can be used to establish new blueberry stands as they are comparatively easy to root; however, their flowering ability makes the establishment process very slow for plantings (Debnath, 2014). With the intervention of micropropagation techniques, this problem can be largely avoided (Smagula and Lyrene, 1984). Many of the small fruit crops have been traditionally produced *via* plant tissue culture techniques to attain rapid fruit-bearing condition and to maintain genetic fidelity (Debnath, 2018). Especially in the case of lowbush blueberry, micropropagated plants develop similar spreading behaviors as seedlings. In addition to that, they exhibit consistent productivity behaviors similar to the rooted cuttings (Frett and Smagula, 1983). Furthermore, *in vitro* propagation methods match the traditional way of introducing new desirable characters into the progeny and multiplying them in a short period of time (Meiners et al., 2007). Propagation *in vitro* has been reviewed in berry crops (Debnath, 2018; Debnath and Keske, 2018; Debnath and Arigundam, 2020; Debnath and Goyali, 2020).

There are three ways of propagation *in vitro*: axillary shoot proliferation, adventitious shoot regeneration, and SE. Shoot proliferation from axillary buds is one of the most common pathways of organogenesis to attain true-to-type clones from the donor plants due to low chances of genetic alterations (Debnath, 2018). It does not involve cell differentiation; rather, it follows the natural pathway of plantlet formation from pre-existing meristems (Novikova and Zaytseva, 2018). Meristem tissues are comparatively less prone to mutations or genetic alteration than unorganized tissues such as callus; however, the chance of changes occurring during *in vitro* culture still persists (Baránek et al., 2015). Berry crop micropropagation *via* axillary shoot proliferation has been reported by various authors both on semisolid media (Chauhan et al., 2019; de Oliveira Prudente et al., 2019; Kopper et al., 2020; Kryukov et al., 2022) and in liquid media using bioreactor systems (Arencibia et al., 2013a; Arencibia et al., 2013b; Debnath, 2017a; Arigundam et al., 2020; Ghosh et al., 2021a; Kryukov et al., 2022).

De novo adventitious shoot regeneration can occur two ways: directly without any involvement of callus, or indirectly *via* callus formation. However, direct shoot regeneration is the more preferable technique for mass propagation as the chances of somaclonal variance are scarce (Debnath, 2018; Novikova and Zaytseva, 2018). During the indirect regeneration process, shoots originate from the surface of the callus tissue and thus have a better chance to be genetically transformed (Arencibia et al, 2013b). According to Ammirato (1985), adventitious shoot regeneration can initiate from unipolar organs either through shoot or with root meristem (organogenesis; Figure 1, Ghosh et al, 2021a) or from bipolar organs simultaneously containing shoot and root meristem (SE; Figure 2, Ghosh et al., 2018). The adventitious shoot regeneration technique has been successfully employed for mass propagation of berry crops using semisolid (Qiu et al, 2018; Schuchovski et al., 2020) and liquid media in bioreactors (Debnath, 2014; Arigundam et al., 2020). While working with blackberry (*Rubus fruticosus*), blueberry (*V. corymbosum*), and kiwiberry (*Actinidia argute*), Hunková et al. (2022) regenerated shoots from petioles and internodal segments; Verma et al. (2022) reported plant regeneration through adventitious shoot formation from hypocotyl and leaf explants of *Lycium barbarum* (Goji), on a semisolid medium.

SE is explained as the process of formation of embryos from differentiating somatic cells. Somatic embryos and zygotic embryos are remarkably similar both temporally and morphologically, although they develop independent to the physical constraints of the maternal tissues (Zimmerman, 1993). Somatic embryos are bipolar and contain radicals, hypocotyl, cotyledons, and the usual embryonic organs (von Arnold et al., 2002). Similar to zygotic embryos, somatic embryos also develop globular-shaped, heart-shaped, and torpedo-shaped embryos followed by formation of shoot and root meristem simultaneously (Zimmerman, 1993). Like organogenesis, SE can be direct from an explant or indirect, involving callus formation (Williams and Maheswaran 1986). Microspores, ovules, embryos (zygotic and somatic), and seedlings are a few examples of explants that are commonly used in direct embryogenesis (von Arnold et al., 2002). The morphological differences between direct and indirect SE are still not clear. However, according to an older hypothesis, direct SE starts from pre-destined embryonic cells while indirect SE is induced from undetermined unorganized callus tissues.

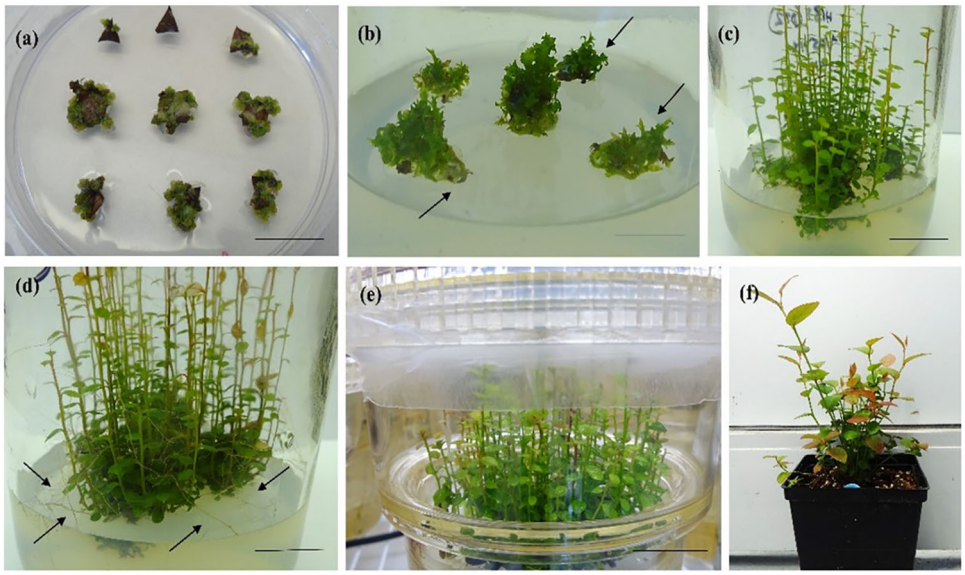


FIGURE 1
In vitro organogenesis in half-high blueberry cv. "Patriot". **(A)** Callus formation after 4 weeks of culture on a semisolid medium (SSM) with 2.3 μM ZEA (bar = 2 cm), **(B)** bud initiation from callus (arrows) after 8 weeks of culture on SSM with 9.2 μM zeatin (bar = 2 cm), **(C)** shoot regeneration after 12 weeks of culture on SSM with 9.2 μM zeatin (bar = 2 cm), **(D)** shoot elongation and root formation (arrows) after 16 weeks of culture on SSM with 9.2 μM zeatin (bar = 2 cm), **(E)** shoot elongation after 16 weeks of culture in a temporary immersion bioreactor (TIB) containing a liquid medium with 9.2 μM zeatin (bar = 3 cm), and **(F)** 1-year-old hardened-off plants in a greenhouse (bar = 8 cm).

However, calluses developed during the indirect SE process can be either embryogenic or non-embryogenic (von Arnold et al., 2002).

The initial description of SE was reported on carrot callus culture (Steward et al., 1958), and since then, carrot has been used as the principal model system to study the early regulatory and morphogenic processes occurring during SE (Zimmerman, 1993). SE is a unique developmental pathway that is well recognized as a plantlet regeneration pathway from cell culture systems (Zimmerman, 1993). There are few available detailed reports on SE in blueberries (Ghosh et al., 2017; Ghosh et al., 2018). However, studies are available on SE in grapes (Nakajima and Matsuta, 2003; Dhekney et al., 2016; Forleo et al., 2021), strawberry (Biswas et al., 2009), *Arbutus unedo* (strawberry tree; Martins et al, 2022) and *L. barbarum* (Verma et al., 2022) on semisolid media. Reports on SE in berry crops are scarce in a bioreactor system.

Liquid media and bioreactors for propagating berry crops

Micropropagation of berry crops using liquid media in a bioreactor system is one of the most developed techniques of mass propagation. Over time, automated bioreactors have become an important tool for the success of large-scale commercial production of tissue culture plants (Debnath, 2014). Haberlandt (1902) first used sucrose supplemented in Knop's liquid medium for propagating individual bract cells of Lamium purpureum (Preil, 2005). Kohlenbach (1959) used mesophyll cells of Macleaya as an explant to develop cell-forming organs, somatic embryos, and cell clusters 60 years after Haberlandt's experiment. Liquid media have been used in stationary and temporary immersion bioreactors (TIBs) to induce organogenesis and SE (Ziv, 2005). Paek et al. (2005) described bioreactors as a sterilized, independent unit that works on the principal of inflow and outflow of the liquid medium. Automated bioreactors are capable of easily managing the microenvironment conditions such as aeration, agitation, and dissolved oxygen level during the intensive culture process (Paek et al., 2005). The environment of the culture room is also responsible for determining the microenvironment inside the bioreactor (Morini and Melai, 2003). Bioreactors also provide a better control of the gaseous exchange of plants, pH of the medium, and temperature (Levin and Tanny, 2004). Keeping the above-mentioned factors in mind, two types of bioreactors have been developed: (a) agitated and (b) non-agitated bioreactors (Debnath, 2011a).

Levin and Vasil (1989) introduced bioreactor systems for mass propagation of various horticultural crops: ever since, agitated and non-agitated bioreactors have been used successfully for various ornamental and vegetative crops such

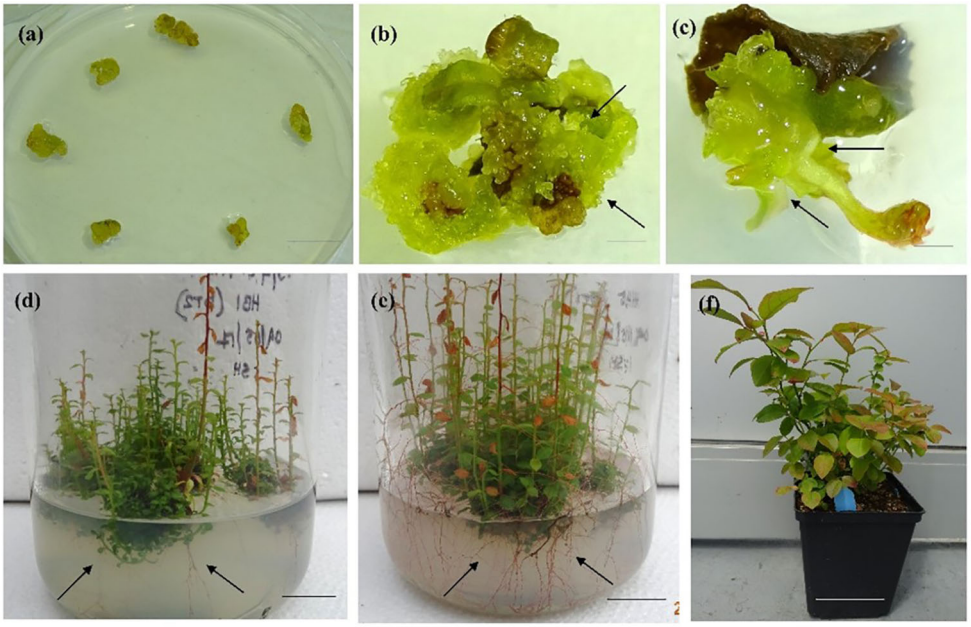


FIGURE 2
In vitro propagation *via* somatic embryogenesis in half-high blueberry cv. "Northblue". **(A)** Young leaf cultured on a medium with 9 μM TDZ, **(B)** globular embryo development (arrows) after 4 weeks of culture in a 9 μM TDZ-supplemented medium, **(C)** germination of somatic embryos and shoot and root apex development (arrow) after 10 weeks of culture with 9 μM TDZ (scale bar = 0.5 cm), **(D)** root elongation (arrows) in a nutrient medium containing 2.3 μM TDZ, after 4 weeks of culture in a glass jar (scale bar = 2 cm), **(E)** development of rooting system (arrows) in a nutrient medium with 2.3 μM TDZ after 6 weeks of culture in a glass jar (scale bar = 2 cm), and **(F)** 1-year-old hardened-off plants in a greenhouse (scale bar = 5 cm).

as oriental lily (Lian et al., 2003) and potato (Piao et al., 2003). Micropropagation of berry plants using liquid medium in bioreactors has been investigated in few berry crops (Debnath, 2007; Debnath, 2008; Debnath, 2009a; Debnath, 2009b; Debnath, 2011a; Debnath, 2011b; Arencibia et al., 2013a; Arencibia et al., 2013b; Debnath, 2014; Debnath, 2017a; Arencibia-Rodríguez et al., 2018; Arigundam et al., 2020; Kryukov et al., 2022). A two-step procedure for cloudberry (*Rubus chamaemorus* L.) micropropagation using a liquid medium in plastic airlift bioreactors was reported by Debnath (2007). Strawberry shoot culture was obtained in commercially available TIBs using leaves from five cultivars as explants in Murashige and Skoog (MS) medium supplemented with 9 μM thidiazuron (TDZ) and 2.5 μM indole-3-butyric acid (IBA) (Hanhineva et al., 2005). Debnath (2008) optimized the shoot regeneration and proliferation protocol in strawberry cv. "Bounty" using a liquid medium in a TIB system coupled with a semisolid medium (SSM) with 2–4 μM TDZ. Similarly, micropropagation of two lowbush blueberry genotypes and one cultivar was established using TIB in combination with a SSM and 1, 2, or 4 μM zeatin (ZEA) (Debnath, 2009b). Adventitious shoots of three lowbush blueberry genotypes "PB1," "QB1," and "QB2" were regenerated in a liquid medium supplemented with 1.2–2.3 μM thidiazuron (TDZ) in a bioreactor (Debnath, 2011b). Arencibia et al. (2013b) reported micropropagation of three raspberry cultivars "Meeker," "Amity," and "Heritage" using shoot tip cultures (~5 cm) in a liquid media in a TIB supplemented with various sucrose concentrations. *In vitro* multiplication of raspberry cultivars "Festival," "Heritage," and "Latham" has been reported by Debnath (2014) in TDZ-supplemented media. Adventitious shoot regeneration of wild lingonberry clones were obtained using stationary bioreactors and TIBs with liquid media supplemented with 9.1 μM TDZ and 1.8 μM ZEA (Arigundam et al., 2020). Organogenesis in half-high blueberry cv. "Patriot" is shown in Figure 1 using a semisolid and a liquid medium in a TIB (Ghosh et al., 2021a). Clapa et al. (2021) micropropagated raspberry cultivars "Maravilla" and "Willamette" in TIB containing a liquid MS medium supplemented with 0.5 mg/L 6-benzyladenine (BA). Kryukov et al. (2022) used bioreactor micropropagation in strawberry cultivar "Murano" and grapevine cultivar "Traminer Pink" in combination with a semisolid medium. Shoots were proliferated in the semisolid medium followed by proliferation in a liquid medium containing 2 mg/L BA. The authors reported a 300-fold fresh mass increase after a 6-week cycle in TIB with a propagation increase rate of approximately 5 and 20 for grape and strawberry, respectively (Kryukov et al., 2022).

The epigenome of plants

Plant studies have provided a significant contribution in epigenetic research. In 1929, Heitz observed the difference between euchromatin and heterochromatin based on several cytological analyses (Heitz, 1929). Carl von Linne in the 18th century described "peloric" mutants with changed floral symmetry that was due to silenced epialleles mimicking the DNA sequence of the expressed alleles, as described by Cubas et al. (1999). A year after, Soppe et al. (2000) showed that epialleles can make changes in developmental switches. In *Arabidopsis thaliana*, the FWA gene is prominent evidence of this phenomenon. Paramutation in maize and tomato is another epigenetic phenomenon, which displays non-Mendelian epigenetic inheritance (Chandler et al, 2000). Later, paramutation has also been detected in mammals, flies, and plants (Cao et al., 2021). Understanding the paramutation phenomenon led several scientists who had been using plants as a model system to study epigenetic regulations. With the discovery of transposable elements in maize in the 1940s by McClintock and others, various connections were made between genetic and epigenetic regulations (Lisch, 2009). Mobilization of transposable elements has also been used as a technique to introduce epigenetic mutations in plant systems. Usually, engineered transgenes are used as silenced marker genes to screen mutations in epigenetic regulators and that is how the use of transgenic techniques in plant development proved to be advantageous in epigenetic research (Pikaard and Scheid, 2014).

Molecular components of epigenetic regulation in plants

More than 130 protein-encoding genes have been found to control epigenetic regulators in plants to date (Pikaard and Scheid, 2014). During plant development, epigenetic regulations play an important role as they help the plant to maintain the stability and integrity of their gene expression profiles. There are several reported studies focusing on alteration of DNA methylation and histone modifications during the de-differentiation pathway in the tissue culture system (Ruffoni and Savona, 2013). Although there is an ever-developing flow of information on epigenetic modifiers, based on current knowledge, they are discussed in the following sections.

DNA methylation

DNA methylation is one of the most studied epigenetic modifications. DNA methylation is a chromatin modification that does not alter genetic sequence but regulates gene expression by suppressing the transcription factor and DNA association (Smith and Meissner, 2013). DNA methylation can be present in various forms depending on the targeted nucleotide during the modification process (Ratel et al., 2006). It is a post-replicative mechanism and generates several methylated bases such as 5-methylated cytosine (5-mC), N6-methyladenine (6-mA), and N4-methylcytosine (4-mC) (Wion and Casadesús, 2006). 5-mC is available mostly in higher plants and mammals, unlike 6-mA and 4-mC, which are predominant in bacteria, protists, and lower eukaryotes (Wion and Casadesús, 2006). In the case of plants, cytosine methylation usually takes place within CG, CHG, or CHH motifs (H = A/T/C). However, in the case of mammals, promoters are usually present in CpG islands, although CpG islands are not easily detectable in plants. Cytosine methylation takes place randomly within the protein-coding regions of highly expressive genes, at their differentially regulated promoters in plants (Zilberman et al., 2007), and they also occur mainly in the repetitive parts of the genome such as in transposable elements and silenced rRNA gene repeats (Pikaard and Scheid, 2014).

Various DNA cytosine methylation enzymes such as methyltransferases (METS) and domain rearranged methyltransferases (DRMs) mediate the DNA methylation process (Rival et al., 2008). DNA methyltransferases facilitate the formation of newly methylated cytosines at the previously unmethylated cytosines or maintain a preexisting methylation pattern (Li and Zhang, 2014). In eukaryotes, there are three families of DNA methyltransferases that are conserved, and these three families are present in modern plants and are homologs of mammalian DNA methyltransferases such as Dnmt1, Dnmt2, and Dnmt3 (Li and Zhang, 2014). DNA methyltransferase 1 (MET 1), which is a plant equivalent of Dnmt1, carries out CG motif maintenance and contributes to *de novo* methylation of CG contexts (Zhang et al., 2018). On the other hand, plant homologs of Dnmt2 process the methylation activity of transfer RNA (Goll et al., 2006). DRMs, which are the plant equivalent of mammalian Dnmt3 groups, primarily carry out *de novo* cytosine methylation. Among others, DRM 2 catalyzes cytosine methylation of all CG, CGH, and CHH contexts and predominantly takes part in RNA-directed DNA methylation pathways (Pikaard and Scheid, 2014). In contrast to mammals, plants have an exclusive family of cytosine methyltransferase, which binds to the methylated histones at their chromodomains. Chromomethylase 1 (CMT 2) plays an important role in maintaining CHH methylation at the central part of the large transposons while CMT 3 is one such enzyme, which is mainly responsible for CHG maintenance methylation (Zemach et al., 2013). CMT 3 also takes part in the balancing act between repressive DNA methylation and histone modification by dimethylating histone 3 protein on lysine 9 (H3K9me2), thus maintaining the cross talk between both epigenetic states in plants (Law and Jacobsen, 2010).

Histone modifications

Similar to any other organism, plants have several enzymes that post-translationally modify various histone proteins affecting gene regulation. In plants, these enzymes are generally encoded by large gene families (Berr et al., 2011). Histone modifications usually occur in two ways, namely, acetylation and methylation. Histone acetylation and histone methylation are reversible epigenetic marks, as enzymes like histone acetyltransferase (HAT) mediate "writer" activities, and histone deacetylase (HDAC) acts as an "eraser" of the acetylation phenomenon; similarly, histone lysine methyltransferases (HKMTs) also reverse the methylation phenomenon by methylating a specific lysine on histones, thus promoting or inhibiting the transcription process (Cheng, 2014). Plants have several HAT and HDAC gene families (Chen and Tian, 2007). The genes included in the HAT family can be classified into five subfamilies depending on their structure and substrate profiles (Earley et al, 2007). However, only two HDAC gene families have been identified to date: HDA1 and HDA6. While the function of HDAC1 is still unclear, HDAC6 is responsible for interacting with MET1, as well as the maintenance of CG and CHG methylation. HDAC6 is also responsible for transposon silencing, repression of rRNA genes, and nucleolar dominance (Pikaard and Scheid, 2014). Aufsatz et al., 2007 also found that HDAC6 actively takes part in seed maturation, flowering time control, and stress responses in plants.

In vitro propagation and epigenetic variation

Plant tissue culture techniques have been developed and improved over time for numerous plant species. Many researchers have incorporated micropropagation techniques into many basic and applied aspects of plant science for years (Lee and Phillips, 1987). According to Cassells and Curry (2001), there are few well-described physiological, genetic, and epigenetic problems that are associated with a plant cultured *in vitro* such as recalcitrance, hyperhydricity, and somaclonal variation (Miguel and Marum, 2011). Variation that emerged in tissue culture plants has been termed "somaclonal variation" (Larkin and Scowcroft, 1981). Due to its origin, somaclonal variation is also referred to as tissue culture-induced variation (TCIV). Mutations are referred to as heritable genetic variations that emerged without any intervention by genetic recombination or segregation (Van Harten, 1998). Mutations can be induced either physically, chemically, or by tissue culture (Predieri, 2001; Roux, 2004). However, mutagenesis due to tissue culture is different because of physical and chemical agents, and is unclear in the long term (Larkin, 1998). *In vitro* propagation techniques such as callus induction, embryo formation, and regeneration can be exceptionally stressful to the plant cells (Lörz et al, 1988). TCIV can occur due to two reasons: epigenetic (developmental) and genetic (heritable variation) causes (Lörz et al, 1988). Epigenetic variations are more often transient and inheritable even though the plant material is asexually propagated (Duncan, 1996). However, these variations can last for many generations, and phenotypic variants involve changes in the expression of specific genes (Hartman and Kester, 1983). In multicellular organisms, genetic and epigenetic mechanisms are involved in coordinated gene expression. DNA methylation, chromatin modification, and non-coding RNA biosynthesis mediate epigenetic regulation (Bond and Finnegan, 2007).

Rejuvenation is described as the restoration of juvenile traits in an adult plant as a result of an epigenetic phenomenon. Many woody perennial plant species lose the ability to rejuvenate with increased maturity, which can impose a major problem in regard to vegetative propagation (Welander, 1983). However, according to Huang et al. (2012), a plant's capability to rejuvenate can be gained during the tissue culture process. During the tissue culture process, plant cells go through callus formation (dedifferentiation) and plantlet regeneration (redifferentiation), which create a highly stressful condition for the plant material; thus, normal cellular regulation gets disrupted (Guo et al., 2007). It has already been determined that the degree of altered DNA methylation level is related to differentiation, and during dedifferentiation and redifferentiation, DNA methylation levels change drastically (Huang et al., 2012). There are a few studies available on DNA methylation during the tissue culture process. Goyali et al. (2015) observed that tissue culture-regenerated lowbush blueberry plants showed increased levels of antioxidant than their softwood cutting counterparts. Later, molecular analysis with simple sequence repeat (SSR) markers confirmed no genetic changes in the regenerants, which further led the authors to consider the involvement of epigenetic factors (Goyali et al., 2015). The first report of epigenetic variation through tissue culture-induced altered DNA methylation patterns was found in blueberry callus culture (Ghosh et al., 2017). A higher percentage of total DNA methylation was detected in callus tissues than in leaf tissues of three lowbush blueberry clones as well as in a hybrid genotype produced using the methylation-sensitive amplification polymorphism (MSAP) technique (Ghosh et al., 2017). It was also found that methylation events in *in vitro*-cultured calli were polymorphic compared to leaf samples from greenhouse-grown plants (Ghosh et al., 2017). TCIV arises due to changes in the microenvironment of the tissue culture system such as continuous subculturing and tissue culture-induced stress, and categorically includes molecular and physiological changes in the regenerants (Figure 3). Evidently, the occurrence of epigenetic variations such as DNA methylation is much more common in the tissue culture system and is thought to be one of the main causes of TCIV (reviewed by Ghosh et al., 2021b). Global methylation analysis using the MSAP technique detected altered DNA methylation patterns in tissue cultured plantlets of the lowbush blueberry clone "QB 9C" and the cultivar "Fundy" than

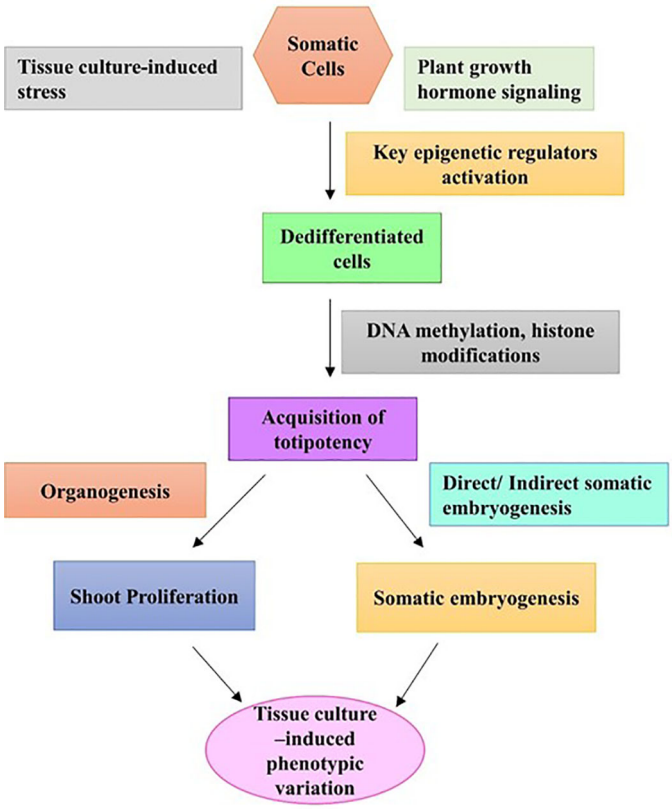


FIGURE 3
A hypothesized pathway of tissue culture-induced phenotypic variation due to activation of key epigenetic regulators.

in softwood cutting plants (Goyali et al., 2018). Shoots of two highbush blueberry cultivars, "Patriot" and "Chippewa", derived from semisolid media and bioreactor systems, showed higher levels of methylation when compared with their greenhouse-grown donor plants. Among these two tissue culture systems, explants from the bioreaction system showed increased levels of methylation variation and polymorphism than explants derived from the semisolid media (Ghosh et al., 2021a). Cao et al. (2021) studied genomic DNA methylation using whole genome bisulfite sequencing (WGBS) in wild strawberry (*F. nilgerrensis*) at various tissue culture stages, starting from use of the shoot tip explants to out planting and acclimation. The authors found that the most obvious methylation changes occurred in the transposable element region of the genome, and the global methylation levels alternately increased and decreased during the tissue culture process. Additionally, during the dedifferentiation and redifferentiation stages of the tissue culture process, differentially methylated regions were detected, which were mostly transposable elements. This finding suggests that dedifferentiation and redifferentiation processes were involved in activation or silencing of transposable elements (Cao et al., 2021).

Sikdar et al. (2022) demonstrated the interaction between cytosine methylation and secondary metabolites in tissue-culture shoots, lingonberry plants, and cutting propagated plants under *in vitro* and *ex vitro* conditions. Through the MSAP assay, the authors observed more methylation in tissue culture shoots and plants than in the respective cutting propagated donor plants, although higher levels of secondary metabolites were observed in cutting propagated plants (Sikdar et al., 2022).

Enhanced vegetative growth

Culture *in vitro* has significant influences on the growth and morphology of the resulting plants. Increased vegetative growth, branching, and rhizome production are often noted in tissue culture (TC) plants compared to conventional cutting propagated plants in berry crops. The variation in morphology between cutting propagated and tissue culture plants of berry crops has been reviewed elsewhere (Debnath et al., 2012; Debnath and Arigundam, 2020; Debnath and Goyali, 2020). Strawberry TC plants produced more crowns, leaves, runners, and berries than conventional runner cutting plants, which might be due to the plant growth regulator treatment used in culture media during *in vitro*

culture (Debnath, 2009a; Debnath, 2017b). TC strawberries were more resistant to frost damage than the runner cutting plants (Rancillac and Nourrisseau, 1988). Likewise, Dalman and Malata (1997) observed that "Senga Sengana" TC strawberry plants were better for overwintering than those of runner propagated plants, although the opposite was true for the cultivar "Mari", whereas for the cultivar "Jonsok", there was no difference between the *in vitro* and *ex vitro* propagated plants. These results indicate that enhanced vegetative growth, berry production, and the development of TC plants over conventional cutting propagated plants, are genotype dependent. TC red raspberry plants produced longer and more numerous canes and more berries than those of root cutting propagated plants in the cultivar "Festival" but not in "Latham" (Debnath, 2014). Increased vegetative growth and rhizome production in TC plants were also reported in lingonberry (Debnath, 2005, 2006) and blueberry (Debnath, 2007). Cranberry TC plants had more runners, uprights, and leaves per upright than those of cutting propagated (CP) plants (Debnath, 2008). Micropropagated lowbush blueberry plants produced less flower buds than conventionally propagated plants (Jamieson and Nickerson, 2003).

Working with wild and cultivated lowbush blueberries over 5 years, Goyali et al. (2013) reported that TC plants produced a higher number of stems and branches with denser and larger shoots than the CP plants. Under field conditions, TC plants had longer canes with more berries and higher yields than the root cutting plants for the red raspberry cultivar "Festival" but not for "Latham" (Debnath, 2014). However, Naing et al. (2019) reported that meristem-derived micropropagated plants did not differ from those of conventionally propagated strawberry plants in terms of growth and fruit quality. The authors proposed that a low level of cytokinin (0.5 mg L^{-1} kinetin) in the culture medium might have produced phenotypically similar plants during micropropagation (Naing et al., 2019). Similarly, Arigundam et al. (2020) did not find any phenotypic difference between micropropagated and cutting propagated lingonberry plants. Cutting propagated lingonberry plants produced more vigorous growth but with less number of shoots, leaves, and rhizomes per plant compared to leaf and node culture-derived TC plants under greenhouse conditions (Sikdar et al, 2022). The juvenile characteristics of TC plants may be the reason for increased rhizome production, which is of great help for the quick establishment and early production of berry crops under field conditions (Debnath et al., 2012).

Variation in bioactive compounds

Micropropagation of *Vaccinium* berry plants has been an area of interest for plant scientists due to possible implications for improving phytochemical properties. This technique has been used in several medicinal plant species to elevate the levels of phenolic components with antioxidant activities (AAs) to satisfy industrial pharmaceutical needs (Giri and Zaheer, 2016). It was found earlier that micropropagated berries have a higher level of antioxidants in comparison to conventionally propagated blueberries (Debnath and Goyali, 2020). However, micropropagated lingonberry plants had less chlorophyll a and b contents in the leaves compared to cutting propagated plants (Arigundam et al., 2020). The reason behind the different levels of bioactive compounds during plant tissue culture can be due to the use of PGR in the growth media, which may be involved in the up- and downregulation of genes engaged in secondary metabolite production pathways (Zifkin et al., 2012).

Blueberries are popularly known as a "super fruit" because of their high levels of *in vitro* antioxidant properties due to the ample presence of polyphenolic compounds (Kalt et al., 2020). However, the antioxidant capacities of these phenolic compounds were not directly accessible under *in situ* conditions due to their low bioavailability (Williamson and Clifford, 2010). In blueberries, the antioxidant capacity depends on the presence of polyphenolic and flavonoid compounds, their redox potential, and structures (Prior et al., 1998). It was previously proven that there was a strong and positive correlation between the total phenolic (TPC) and anthocyanin content (TAC) and AA in blueberries (Goyali et al., 2013, 2015). However, the relationship between TPC and AA was much more stronger than TAC and AA (Moyer et al., 2002). Moreover, Ghosh et al. (2018) reported that in half-high blueberry plants, TPC and AA were strongly related in comparison to the total flavonoid content (TFC) and AA. Nonetheless, quantification of total AA in a plant is a complex process as it is influenced by the linkage of different phytochemicals and works synergistically or antagonistically in the presence of various environmental factors (Hassimotto et al., 2005).

It was proven that the type of cytokinin and concentration in the culture media affects the biosynthesis pathways of secondary metabolites (Debnath and Goyali, 2020). Phenolic compounds are the most commonly found secondary metabolites in plants, and are derivatives of phenylalanine. Cytokinin concentration is positively correlated with the level of transcription of gene-encoding enzymes such as ammonia lyase, chalcone synthase, chalcone isomerase, and dihydroflavonol reductase involved in the flavonoid biosynthesis pathway, and is thus indirectly correlated to the TAC in Arabidopsis (Deikman and Hammer, 1995). In addition, various environmental factors like low concentrations of nutrients and light increase phenylalanine ammonia lyase activity, which is an important regulatory factor of plant metabolic pathways (Taiz and Zeiger, 2006). It was found that the lowbush blueberry clone "QB9C" and the cultivar "Fundy" were affected by micropropagation techniques and "QB9C" fruits showed a higher content of secondary metabolites and AA than "Fundy" regenerants (Goyali et al., 2013). Later, Goyali et al. (2015) reported increased levels of TPC, TAC, TFC, total proanthocyanidins (TPAC), and AA in the tissue culture regenerants of the lowbush blueberry clone "QB9C" and the cultivar "Fundy", in comparison to stem cutting counterparts. Georgieva et al. (2016) studied the TPC, AA, and

ferric-reducing capacities of *in vitro*- and *ex vitro*-grown lingonberry, bilberry, raspberry, and strawberry fruit extracts. The authors found a high content of AAs in all three types of *in vitro*-grown berries in comparison to berries collected from the *ex vitro* condition. Bioreactor- and agar-gelled media-derived strawberries of the cultivar "Bounty" exhibited a higher content of TAC and AA than strawberries grown using conventional runner cuttings. Even though there was a significant difference in the AAs of the fruits collected from two different pathways of tissue culture and conventional cuttings, the inter-simple sequence repeat (ISSR) marker assay did not show any heterogeneities in their amplification profiles. These results confirmed the clonal fidelity of the micropropagated plants, yet suggest the occurrence of somaclonal variation as a reason for increased TAC and AA levels (Debnath, 2009a). Similar results were also reported in lingonberry wild clones, where the clonal fidelities of TC plants were confirmed by expressed sequence tag (EST)–SSR, EST–polymerase chain reaction (PCR), and ISSR markers; however, variation was observed between the chlorophyll contents of TC and CP plants (Arigundam et al., 2020).

Detection of DNA methylation in tissue culture-derived berry plants

There are various technologies available to evaluate the role of epigenetic changes in a crop improvement program such as the use of modern genome editing tools like CRISPR/Cas9, zinc finger nucleases, transcription activator like-nucleases, epigenetic recombinant inbred lines, and RNA-directed DNA methylation (Arencibia et al., 2019). However, the study of global DNA methylation can be employed in a small-scale tissue culture set up to understand the alteration of the epigenetic status of regenerants. DNA methylation studies can be approached from various standpoints as there are different methods available to detect tissue culture-induced DNA methylation and localization of methylated cytosine in a particular region of the genome. Among them, the most popular are detection by MSAP, high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), and WGBS. MSAP is based on the sensitivity of two restriction enzymes (*Msp*I and *Hpa*II) to identify methylated cytosine bases (Cedar et al., 1979). This is a modification of the amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995), which was developed by Reyna-Lopez et al, (1997) to detect DNA methylation in dimorphic fungi. The MSAP technique has been used in tissue culture regenerants to detect global methylation in only a few berry crops including blueberry (Ghosh et al., 2017; Goyali et al., 2018; Ghosh et al, 2021a), grapes (Baránek et al., 2010), and lingonberry (Sikdar et al., 2022). The genomic DNA level of methylated cytosine can be detected by enzymatic means, although this is not as sensitive as the HPLC method because its resolution is restricted to the cleavage sites of the endonucleases. The HPLC technique quantifies DNA methylation *via* fractionation of four main bases (A, T, G, and C) (Fraga and Esteller, 2002). When options are available, HPCE proves to be more beneficial than HPLC as it is faster, cheaper, and comparatively more sensitive (Fraga et al., 2000). These methods have not been used in berry crops to date, but have been used to detect cytosine methylation in tissue culture regenerants in various other crops such as oil palm (Jaligot et al., 2000), *Acca sellowiana* (Fraga et al., 2012), triticale (Machczyńska et al., 2014), and apple (Li et al, 2002). Because of these benefits, enzymatic methods are commonly used, as they do not involve complex equipment or skilled labor. Enzymatic isoschizomer-based methods do not provide any details on the role of methyl cytosine in cell and molecular biology. However, these details can be determined with the use of bisulfite modification of methylated DNA. There are various methods available based on the treatment of sodium bisulfite to detect methylated cytosine located at specific locations in DNA (Fraga et al., 2000). Sodium bisulfite converts unmethylated cytosine to uracil, whereas methylated cytosine stays the same (Furiuchi et al., 1970). WGBS provides genome-wide methylation profiling without any interference; however, not much information is available on the minimum required coverage and other factors such as susceptibility, precision, and cost of the assay (Ziller et al., 2015). WGBS has been used in wild strawberry (Cao et al., 2021) and in blueberry tissue culture (Ghosh et al., unpublished results).

Conclusions

Micropropagation techniques for the improvement and commercial production of berry plants are currently used as alternative methods to satisfy market demand. In plants, gene regulation is related to the level of DNA methylation, and this epigenetic mechanism is intricately linked to growth and development, as well as *in vitro* processes such as organogenesis and SE. It is important to further explore the epigenomes of berry plants to see if an epigenetic footprint remains within the epigenome of regenerated plants due to different culture environments. This can be done by identifying genes subjected to differential methylation, identifying regions of differential methylation, and by identifying the hypo/hypermethylated gene sets involved in various biological and molecular functions under *in vitro* and *in vivo* conditions using advance methylation detection techniques. In a crop improvement program, it is important to employ genome editing tools such as CRISPR/Cas9 to detect the occurrence of undesirable traits due to epigenetic variation during the tissue culture process as well as to apply various bioinformatic

tools to predict the inheritance of altered epigenetic patterns in regenerants. Enhanced vegetative growth and/or bioactive components due to *in vitro* culture and propagation are of significant importance for early field establishment and improved berry production, and for increased health-promoting factors in berry crop production and improvement programs.

Author contributions

Conceptualization, writing, reviewing and editing by SD. Writing and reviewing by AG. Both authors agree with the final version of the manuscript. The authors declare that the content of this paper has not been published or submitted for publication elsewhere. All authors contributed to the article and approved the submitted version.

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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Cell-free microbial culture filtrates as candidate biostimulants to enhance plant growth and yield and activate soil- and plant-associated beneficial microbiota

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In this work we compiled information on current and emerging microbial-based fertilization practices, especially the use of cell-free microbial culture filtrates (CFs), to promote plant growth, yield and stress tolerance, and their effects on plant-associated beneficial microbiota. In addition, we identified limitations to bring microbial CFs to the market as biostimulants. In nature, plants act as metaorganisms, hosting microorganisms that communicate with the plants by exchanging semiochemicals through the phytosphere. Such symbiotic interactions are of high importance not only for plant yield and quality, but also for functioning of the soil microbiota. One environmentally sustainable practice to increasing crop productivity and/or protecting plants from (a)biotic stresses while reducing the excessive and inappropriate application of agrochemicals is based on the use of inoculants of beneficial microorganisms. However, this technology has a number of limitations, including inconsistencies in the field, specific growth requirements and host compatibility. Beneficial microorganisms release diffusible substances that promote plant growth and enhance yield and stress tolerance. Recently, evidence has been provided that this capacity also extends to phytopathogens. Consistently, soil application of microbial cell-free culture filtrates (CFs) has been found to promote growth and enhance the yield of horticultural crops. Recent studies have shown that the response of plants to soil application of microbial CFs is associated with strong proliferation of the resident beneficial soil microbiota. Therefore, the use of microbial CFs to enhance both crop yield and stress tolerance, and to activate beneficial soil microbiota could be a safe, efficient and environmentally friendly approach to minimize shortfalls related to the technology of microbial inoculation. In this review, we compile information on microbial CFs and the main constituents

(especially volatile compounds) that promote plant growth, yield and stress tolerance, and their effects on plant-associated beneficial microbiota. In addition, we identify challenges and limitations for their use as biostimulants to bring them to the market and we propose remedial actions and give suggestions for future work.

KEYWORDS

plant-microbe interaction, biostimulants, crop yield, stress tolerance, volatile organic compounds, soil- and plant-associated microbiota

Introduction

Plant' growth and development are influenced by microorganisms occurring in the phytosphere that communicate with plants by exchanging chemical signals (Hartmann et al., 2014). Some of these microorganisms can benefit host plants in a variety of ways, a scenario of utmost interest when searching for new and efficient agricultural approaches based on manipulation of plant-associated microbiota. Beneficial microorganisms can directly promote plant growth through mechanisms involving production of bioactive compounds (e.g. phytohormones, volatile compounds, peptides, etc.), dinitrogen fixation, solubilization of minerals and organic material and enhancement of water and nutrient uptake and use (Tsavkelova et al., 2006a; Rodríguez et al., 2007; Francis et al., 2010). These microorganisms can also indirectly promote plant growth by antagonism/antibiosis against pathogens, alleviation of stress caused by environmental pollutants or other stressful abiotic conditions (e.g. drought and salinity), or by triggering in the host plant enhanced defense capacities against pathogen attack.

A decline in natural resources and the environmental damage caused by practices relying on the excessive and inappropriate application of fertilizers and depletion of soil and water resources have become major limitations in conventional agriculture. A more sustainable and eco-friendly agriculture requires increases in product yield quality, while reducing the negative environmental impact of agrochemicals on soil fertility and biodiversity; potential solutions may be fostered by microbial-based approaches (Calvo et al., 2014). The aim of this review was to compile information on current and emerging microbial-based fertilization practices, particularly the use of microbial inoculants, microbial-derived compounds and microbial culture filtrates (CFs), to promote plant growth, yield and stress tolerance, and their effects on plant-associated beneficial microbiota. In addition, we identify challenges and limitations to bring microbial CFs to the market as biostimulants compliant with scientific requirements of the official regulations for fertilizer products.

Soil inoculation of beneficial microorganisms: limitations of a widely used practice to enhance crop yield and/or protect plants from (a)biotic stresses in an eco-friendly manner

One environmentally safe and sustainable practice to promote plant growth, increase crop yield and/or enhance stress tolerance is based on the inoculation of soil with plant growth promoting microorganisms (PGPM) (Miransari, 2011; Calvo et al., 2014; Ahmad et al., 2018; Backer et al., 2018; Fiorentino et al., 2018; Zhong et al., 2019; Noceto et al., 2021). Microbial inoculants consist of one or a reduced number of microbial strains which are grown separately or in mixed culture fermentation, concentrated and then formulated with an appropriate carrier into the final product form. Despite its undisputable success (Li et al., 2022), the technology based on PGPM inoculation has faced a number of limitations and inconsistences that are summarized in Table 1. First, the efficiency of inoculation depends on soil pH, temperature and nutrient content, interaction with the crop species (host specificity), competition with native strains and compatibility between the microbial inoculants (Svenningsen et al., 2018; Emmanuel and Babalola, 2020). Second, development of multi-strain bioinoculants on a large-scale level is difficult since each co-inoculant requires specific culture media and physical-chemical conditions (Reddy and Saravanan, 2013). Third, inoculation with beneficial microorganisms without a proper carrier or formulation may result in a rapid decline in the applied microbial population, as the inoculated microbes must compete with the often better-adapted native microbial community (Bashan et al., 2014; Cardinale et al., 2015; Parnell et al., 2016). For instance, the abundance in soil of some well-known beneficial microbes such as *Bacillus amyloliquefaciens* FZB42 and *Trichoderma harzianum* falls below detection limits just a few weeks after application (Papavizas, 1982; Kröber et al., 2014; Oskiera et al., 2017). Although seed coating with beneficial

TABLE 1 Limitations of soil inoculation of PGPM and application of microbial-derived compounds and microbial culture filtrates.

	PGPM inoculation	Application of microbial-derived compounds	Application of microbial culture filtrates
Efficiency depends on environmental factors, host compatibility and competition with native microbes	x		
Efficiency depends on microbial culture composition and age	x	x	x
Difficulty to develop multi-strain bioinoculants	x		
Manner of application: necessity for a proper carrier or formulation	x	x	x
Pathogenicity risk to indigenous microbial communities and/or plants	x		
Biostimulant effect depends on plant nutritional status	x		
Scaling-up: difficulty to culture plant-associated microbes in reactors	x	x	x
Long and difficult process to isolate, identify and purify the beneficial compound		x	
Dose-dependence response		x	x
Complex synergic and cooperative interactions between different compounds to promote plant growth		x	
Contradictory effect on different plant species		x	
Antagonistic effects on native microbiota		x	
High production cost		x	x

"X" highlights the limitation of each microbial-derived method.

microorganisms may be a suitable option to maintain microbial survival in soil over a longer period, an appropriate coating requires a delicate balance between coating materials, microbe and compatible chemistry, which is not always easy to obtain (Glare et al., 2012; Parnell et al., 2016). Moreover, an adequate delivery system for microorganisms in the soil is also required, which represents a major challenge to industry since it involves mass production, formulation, and application of the beneficial microbes (Ravensberg, 2011; Glare et al., 2012; Vassilev et al., 2020). Fourth, some PGPM including some *Pseudomonas* spp. are opportunistic pathogens (Belimov et al., 2007; Sitaraman, 2015) and thus their use is associated with a pathogenicity risk. Fifth, the growth-promoting effect of inoculating beneficial microorganisms strongly depends on the nutritional status of the plant in relation to the extent that they can be rendered ineffective in promoting plant growth (Hoeksema et al., 2010; Pineda et al., 2013). Thus, plants respond better to mycorrhizal inoculation when grown in soils with high microbial diversity and when subjected to phosphorous limitation (Hoeksema et al., 2010). For instance, *Colletotrichum tofieldiae* promotes growth in *Arabidopsis thaliana* only under phosphorus deficiency conditions (Hiruma et al., 2016). Inoculation with the growth-promoting rhizobacterium *B. amyloliquefaciens* GB03 can have deleterious effects on plant growth under phosphate deficiency conditions, due to an activation of the phytohormone-mediated immune response modulated by a phosphate-starvation response (Morcillo et al., 2020). Sixth, the PGPM inoculation efficiency largely depends upon production of bioactive compounds by the inoculated microbes, which in turn strongly depends on abiotic and biotic environmental contexts. Therefore, it can never be guaranteed that inoculation of a particular microbe will result in the production of compounds with plant growth-promoting or stress tolerance-conferring properties. Seventh, most of soil and plant-associated microorganisms cannot be cultured in reactors. Eighth, inoculation of non-native, allochthonous microorganisms is known to produce strong shifts in microbial communities (Schmidt et al., 2014; Diagne et al., 2018; Berg et al., 2021), with unpredictable and unwanted effects (Hart et al., 2018). For instance, non-native mycorrhizal fungal commercial inoculants may lead to undesirable promotion of exotic over native plant species (Burkle and Belote, 2015; Middleton et al., 2015; Hart et al., 2018).

Some of the constraints of the classic single and multi-strain bioinoculation approach can be circumvented by holistic approaches based on the use of SynComs (for **Syn**thetic microbial **Com**munities), which has emerged as a new paradigm not only to better understand plant-microbe interactions, but also to benefit from them

(Castrillo et al., 2017; Durán et al., 2018; Kwak et al., 2018; Carrión et al., 2019; Marin et al., 2021). SynComs are based on the use of metagenomic tools to determine the structure and potential function of plant-associated microbial communities, followed by the isolation and co-culturing of multiple locally adapted native microorganisms. Establishment and survival of inoculated SynComs in the field are higher than that of single or multi-strain bioinoculations, as SynComs are capable of competing with the pre-existing microbiota present in the plant or soil (Liu et al., 2022; Shayanthan et al., 2022). However, despite the obvious theoretical advantages of application of SynComs designed "à la carte" to mimic the role of a particular microbiome, this technology still has some constraints, including technical limitations in the correct metagenomic identification of the isolated microorganisms (Liu et al., 2020), development of SynComs inoculants at a large-scale industrial level, dependence upon the nutritional status of the plant, maintenance of the stability and function of SynComs over time under changing environmental conditions in open field, etc.

Application of microbial-derived compounds: A step to minimize shortfalls related to PGPM inoculation technology

Depending on environmental conditions, microorganisms can release diffusible compounds including phytohormones, siderophores, proteins, peptides, sugar-derived molecules, amino acids, exopolysaccharides, organic acids and volatile compounds that alter metabolism, enhance photosynthesis, promote plant growth, confer resistance to (a)biotic stresses and cause massive lateral root formation, thus improving the root´s exploratory capacity for nutrients and predisposing plants for colonization and infection by microbes (Ryu et al., 2003; Arkhipova et al., 2005; Tsavkelova et al., 2006b; Spaepen et al., 2007; Berg, 2009; Contreras-Cornejo et al., 2009; Ortíz-Castro et al., 2009; Chanclud and Morel, 2016; Saha et al, 2016; Sánchez-López et al, 2016; Egamberdieva et al., 2017; García-Gómez et al., 2019; Morcillo and Manzanera, 2021). Some of these compounds are capable of activating soil microbial activity (Rodríguez-Morgado et al., 2017; Macias-Benitez et al, 2020). To address limitations related to PGPM inoculation technology, the application of small quantities of microbial bioactive compounds in pure form, either as alternatives, supplements or complements to microbial cells, has been proposed as a possible approach for improving crop productivity and stress tolerance while reducing agrochemical use (Kanchiswamy et al., 2015; Naamala and Smith, 2021). This approach offers reliability and the easy control of the quantity and quality of a compound of interest (Table 1). Furthermore, compared with the PGPM inoculation technology, the use of pure microbial compounds can benefit a broader range of crops and minimize pathogenicity risk (Table 1). Although the stability of many of these compounds depends on abiotic environmental factors (temperature, salt concentration in soil, pH, etc.) and biotic factors (they could be used by native microorganisms as nutrient source), their rapid perception by plants can prime them for growth promotion. However, there are quite a number of limitations associated with the use of microbe-derived compounds including time-consuming processes of isolation, identification and purification of bioactive compounds, dose-dependence of the response, complex synergic and cooperative interactions between different compounds to promote plant growth, contradictory effects of the same compound on different plants, antagonistic effects on beneficial microbiota, etc. (Naamala and Smith, 2021) (Table 1).

Application of cell-free microbial culture filtrates: A sustainable and environmentally friendly approach to activate the soil- and plant-associated beneficial microbiota and cope with constraints related to PGPM inoculation and application of microbial-derived compounds?

Some of the limitations related to the use of microbe-derived compounds could be circumvented by the use of cell-free filtrates of beneficial bacterial and fungal cultures, which are mixtures of phytohormones, siderophores, proteins, peptides, amino acids, exopolysaccharides, organic acids, volatile compounds, etc. derived from broth cultures processed through centrifugation or filtration (i.e. micro/ultra/nanofiltration and inverse osmosis) for cell removal (Pellegrini et al., 2020). Agronomic studies have provided evidence that application of these complex cocktails is an efficient approach to promote plant growth and enhance yield and stress tolerance in a wide range of crops while reducing the use of agrochemicals. Notably, recent studies have shown that cell-free CFs of phytopathogens can also be used to enhance yield and stress tolerance (Baroja-Fernández et al., 2021 and unpublished results). Table 2 summarizes details of the studies on effects of microbial CF application. Most of these studies indicated that phytohormones occurring in the microbial CFs (especially indole acetic acid (IAA)) are major determinants of the response of plants to these extracts. However, some studies indicated that microbial amino acids, peptides, extracellular proteins, lipopeptides and siderophores could also play important roles in the response of plants to fungal CFs. For instance, Buensateai et al. (2013) showed that application of *Bacillus* sp. strain CaSUT007 CFs enriched in extracellular proteins increased root and shoot

TABLE 2 Studies on effects of microbial CF application on plant.

Microbial species		Culture medium	Plant species	Application manner	Effect on plant	Mechanism/ mode of action	Reference
Bacterial species							
Azotobacter vinelandii	Beneficial	Specific medium	*Solanum lycopersicum*	Root irrigation with culture supernatants	Increase shoot dry weight and fruit production	CFs contain auxins, gibberellins and cytokinin-like substances	Azcón and Barea (1975)
Azotobacter beijerinckii							
Streptomyces olivaceoviridis	Beneficial	Starch-casein medium	*Triticum aestivum*	Pretreatment of wheat grain with CFs	Enhance growth vigor and crop yield	CFs contain auxins, gibberellins and cytokinin-like substances	Aldesuquy et al. (1998)
Streptomyces rimosus							
Streptomyces rochei							
Streptomyces atroolivaceus	Beneficial	MBGM	*Triticum aestivum*	Pretreatment of wheat grain with CFs	Increases the shoot length, fresh and dry mass, root fresh and dry mass but suppresses the depth of the root system	Effects probably caused by activity of plant growth regulators	El-Shanshoury (1989)
Azospirillum brasilense	Beneficial	NFb	*Oryza sativa*	CFs applied in hydroponic medium	Enhance root growth and development	CFs contain IAA	El-Khawas and Adachi (1999)
Klebsiella pneumoniae		NFDM					
Bacillus amyloliquefaciens (FZB24, FZB42, FZB45)	Beneficial	GNB	*Zea mays*	Coleoptiles incubated with CFs	Enhance length growth	CFs contain IAA	Idris et al. (2004)
Bacillus subtilis FZB37							
***Bacillus amyloliquefaciens* KPS46**	Beneficial	GNB	*Glycine max*	Pretreatment of seeds with CFs	Increases root and shoot length and plant biomass	IAA and extracellular proteins	Buensanteai et al (2008)
Streptomyces coelicolor	Beneficial	GYMA broth	*Triticum aestivum*	Seed coating with CFs	Improve plant growth under water-stress conditions	CFs contain IAA	Yandigeri et al. (2012)
Streptomyces olivaceus							
Streptomyces geysiriensis							
***Methylobacterium* spp.**	Beneficial	AMS	*Triticum aestivum*	Pretreatment of seeds with CFs	Enhances seed germination and seedling growth	CFs contain CKs	Meena et al. (2012)
***Bacillus* sp. CaSUT007**	Beneficial	GNB	*Manihot esculenta*	Preteatment of stakes with CFs	Increases root and shoot lengths	IAA and extracellular proteins	Bulgarelli et al. (2013)
***Bacillus subtilis* EA-CB0575**	Beneficial	TSB, SBM	*Musa* spp.	Pretreatment of germinated seeds with CFs	Increases shoot length and dry weight	CFs contain Lipopeptides and siderophores	Posada et al. (2016)
Burkholderia seminalis	Beneficial	Specific medium	*Solanum lycopersicum*	*In vitro* application of CFs	Increases seed germination	CFs contain IAA	Tallapragada et al. (2015)

(Continued)

TABLE 2 Continued

Microbial species		Culture medium	Plant species	Application manner	Effect on plant	Mechanism/ mode of action	Reference
Enterococcus faecium	Neutral	LB	*Cucumis melo*	Soil irrigation with CFs	Increases shoot and root lengths, plant fresh weight, and chlorophyll content	CFs contain IAA and GAs	Lee et al. (2015)
***Streptomyces* sp.**	Beneficial	TYB	*Solanum lycopersicum*	Soil irrigation with CFs	Enhances plant growth	Effects probably caused by IAA production	Kaur et al. (2019)
***Azospirillum brasilense* (*Ab–V5, Ab–V6*)**	Beneficial	DYGS	*Glycine max*	Spraying of leaves or seeds	Increase root nodulation and root development	Indolic compounds	Rondina et al. (2020)
Pectobacterium carotovorum	Pathogenic	LB	*Arabidopsis thaliana*	Soil irrigation with CFs	Increase shoot and root biomass but not production	Unkwnon	Ávila and Poveda (2022)
Pseudomonas syringa							
Fungal species							
Piriformospora indica	Beneficial	Specific medium	*Zea mays*	Root irrigation with CFs	Enhances shoot but not root growth	Unknown	Varma et al. (1999)
Piriformospora indica	Beneficial	M+ medium	*Arabidopsis thaliana*	*In vitro* application of CFs	Induces root branching	Production of a diffusible factor that is probably IAA	Sirrenberg et al. (2007)
Sebacina vermifera	Beneficial	MYP	*Panicum virgatum*	Pretreatment of seeds with CFs	Enhances seed germination and biomass production	Unknown	Ghimire et al. (2009)
***Cladosporium* sp. MH-6**	Beneficial	Czapek's broth	*Cucumis sativus*	*In vitro* application of CFs	Increases shoot and root lengths and plant dry and fresh weight	CFs contain GAs	Hamayun et al. (2010)
***Penicillium minioluteum* LHL09**	Beneficial	Czapek's broth	*Oryza sativa (waito-C)/ Glycine max*	*In vitro* application of CFs	Promotes plant growth and nitrogen assimilation, with and without sodium chloride-induced salinity	CFs contain GAs	Khan et al. (2011)
Piriformospora indica	Beneficial	*Aspergillus*minimal medium	*Helianthus annus L*	Root irrigation with CFs	Increases plant growth and seed production. Increases oil content in seeds	Unknown	Bagde et al. (2011)
Shimizuomyces paradoxus	Pathogenic	PDB	*Brassica napus*	Pretreatment of seeds with CF and foliar spray	Increases seed germination and seedling growth	Unknown	Sung et al. (2011)
***Penicillium citrinum* KACC43900**	Beneficial	Czapek's broth	*Carex kobomugi*	Injection/foliar spray with CFs	Increases leaf blade length, chlorophyll and carotenoids contents and photosynthesis	Unknown	Hwang et al. (2011)
Fusarium oxysporum	Pathogenic	CYA	*Cajanus cajan*	Pretreatment of seeds with CF	Increases seed germination and promotes plant growth	Unknown	Jalander and Gachande (2012)
***Penicillium* sp.**	Beneficial	Czapek's broth	*Suaeda japonica*	*In vitro* application of CFs	Increases plant lenght	CFs contain GAs	You (2012)
Trichoderma virens	Beneficial	Richard's solution	*Capsicum annuum*	Seed coating with CFs	Enhance seed germination and plant vigor	Unknown	Rahman et al. (2012)
Trichoderma pseudokoningii							

(Continued)

TABLE 2 Continued

Microbial species		Culture medium	Plant species	Application manner	Effect on plant	Mechanism/ mode of action	Reference
Trichoderma harzianum							
***Penicillium* sp. PNF2**	Beneficial	PDB	*Sesamum indicum*	Pretreatment of seeds with CFs	Increases shoot length and fresh weight	CFs contain IAA	Radhakrishnan et al. (2013)
Fusarium oxysporum	Pathogenic	LGN	*Solanum lycopersicum*	Pretreatment of seeds with CFs	Inhibit seed germination and plant growth	CFs contain toxins	Raithak and Gachande (2013)
Alternaria solani							
***Penicillium* spp.**	Beneficial/ Pathogenic	ME	*Triticum aestivum*	Pretreatment of seeds with CFs	Increases seed germination and plant biomass	Unknown	Khokhar et al. (2013)
Piriformospora indica	Beneficial	*Aspergillus*minimal medium	*Aristolochia elegans*	Soil irrigation with CFs	Increases shoot and root length and fresh and dry weight	Unknown	Bagde et al. (2013)
***Trichoderma* spp.**	Beneficial	ME	*Cicer arietinum*	Pretreatment of seeds with CFs	Increases seed germination and promote plant growth	Unknown	Ali et al. (2014)
***Penicillium* spp. (*NICS01, DFC01*)**	Beneficial	PDB	*Sesamum indicum*	Pretreatment of seeds with CFs	Increase shoot and root length and fresh and dry weight	Amino acids	Radhakrishnan et al., 2014
***Pseudomonas* sp.**	Beneficial	King´s B medium	*Coriandrum sativum*	Pretreatment of seeds with CF and foliar spray	Increases fresh and dry weight and oil production	Unknown	Hegazi et al. (2015)
Alternaria alternata	Pathogenic	Czapek's broth	*Triticum aestivum*	Pretreatment of seeds with CF and foliar spray	Increase seed germination and plant growth	Unknown	Bhajbhuje (2015)
Alternaria solani							
Penicillium nordicum	Pathogenic	CYA	*Sorghum bicolor*	Pretreatment of seeds with CFs	Inhibit seed germination	CFs contain mycotoxin	Vankudoth et al. (2015)
***Penicillium citrinum*,**							
Penicillium chrysogenum							
Penicillium commune							
Penicillium verrucosum							
Penicillium camemberti							
Penicillium digitatum							
Penicillium oxalicum	Beneficial	PDB	*Pennisetum glaucum*	Pretreatment of seeds with CFs	Increases seed germination and seedling vigor	Unknown	Murali and Amruthesh (2015)
Fusarium tricinctum RSF-4L	Pathogenic	Czapek's broth	*Oryza sativa L. cv. Dongjin*	*In vitro* application of CFs	Increase shoot and root lengths, plant fresh weight, and chlorophyll content	CFs contain IAA	Khan et al. (2015)
Alternaria alternata RSF-6L							

(Continued)

TABLE 2 Continued

Microbial species		Culture medium	Plant species	Application manner	Effect on plant	Mechanism/ mode of action	Reference
Purpureocillium lilacinum	Beneficial	Specific medium	*Solanum lycopersicum*	Pretreatment of seeds with CFs/ Soil irrigation with CFs	Increases seed germination and promotes plant growth	CFs contain IAA	Cavello et al. (2015)
Fusarium oxysporum	Pathogenic	Czapek's broth	*Triticum aestivum Hordeum vulgare Solanum tuberosum*	Pretreatment of seeds with CFs	Inhibit seed germination and plant growth	Unknown	Ogórek (2016)
Fusarium sulphureum							
Gibberella avenacea							
Gibberella intrincans							
***Trichoderma harzianum* WKY1**	Beneficial	Czapek's broth	*Shorgum*	Soil irrigation with CFs	Increase shoot and root lengths, plant fresh weight, and total phenol content	CFs contain IAA	Saber et al. (2017)
***Aspergillus fumigatus* TS1**	Beneficial	Czapek's broth	*Oryza sativa (waito-C)*	Application to the apical meristem	Enhance chlorophyll content, root-shoot length, and biomass production	CFs contain IAA and GAs	Bilal et al. (2018)
Fusarium proliferatum BRL1							
Fusarium solani	Pathogenic	Richard's solution	*Solanum lycopersicum Brassica rapa Raphanus sativus Trigonella melongena*	Pretreatment of seeds with CFs	Enhances seed germination	Unknown	Parveen et al. (2019)
Trichothecium roseum					Inhibit seed germination	CFs contain mycotoxins	
Aspergillus niger							
Cladoporium herbarum							
Alternaria alternata							
Penicillium chrysogenum							
Penicillium expansum							
***Trichoderma* spp.**	Beneficial				Enhance seed germination	Unknown	
Trichoderma asperellum							
Trichoderma harzianum							
Piriformospora indica	Beneficial	CM	*Cichorium intybus*	Foliar spray	Enhances growth and morpho-physiological traits	Unknown	Rashnoo et al. (2020)
Trichoderma harzianum,	Beneficial	MS	*Capsicum annuum*	Soil irrigation with CFs and DEs	Stimulate root growth and enhance fruit yield	CFs produce changes in plant-associated microbiota	Baroja-Fernández et al. (2021)
Alternaria alternata	Pathogenic						
Penicilium aurantiogriseum							

(Continued)

TABLE 2 Continued

Microbial species		Culture medium	Plant species	Application manner	Effect on plant	Mechanism/ mode of action	Reference
Gibberella intermedia	Beneficial	Czapek's broth	*Oryza sativa (waito-C)*	Application to the apical meristem	Increases shoot growth	CFs contain GAs	Khalmuratova et al. (2021)
Fusarium oxysporum	Pathogenic	PDB	*Arabidopsis thaliana*	Soil irrigation with CFs	Increase shoot and root biomass but not production	Unkwnon	Ávila and Poveda (2022)
Pythium irregulare							
Rhizoctonia solani							
Chaetomium globosum	Beneficial	ME	*Cichorium intybus*	Soil irrigation with CFs	Increase of biomass, shoots and roots length, and leaf area	Increases phenylalanine pathway and chicoric acid	Spinelli et al. (2022)
Minimedusa polyspora						Increases phenylalanine pathway and 4-OH-benzoate	
Geotrichum candidum	Neutral	PDB	*Vigna radiata*	Pretreatment of seeds with CFs	Increases seed germination and promotes plant growth	CFs contain IAA	George et al. (2019)
Saccharomyces cerevisiae	Beneficial	Sucrose	*Coreandrum sativum*	Pretreatment of seeds with CF and foliar spray	Increases fresh and dry weight and oil production	Unknown	Hegazi et al. (2015)

IAA, Indole-3-acetic acid; GAs, Gibberellins; AMS, Ammonium mineral salt medium supplemented with methanol; CM, Complex medium; CYA, Czapek yeast medium; GNB, Glucose-enriched nutrient broth; GYMA broth, Glucose, yeast extract, malt extract; LB, Luria-Bertani broth; LGN, Liquid glucose nitrate medium; MBGM, Modified bouillon glycerol medium; ME, Malt extract broth; MS, Murashige and Skoog medium; MYP broth, Malta Yeast Peptone broth; PDB, Potato Dextrose Broth; NFb, Nitrogen-free malate; NFDM, Nitrogen-free dextrose; SBM, Sporulation Bacillus Medium; TSB, Trypticase Soy Broth; TYB, Tryptone-Yeast Extract Broth. Specific medium, see publication for details.

lengths and total biomass of cassava stalks. Furthermore, Posada et al. (2016) showed that application of *B. subtilis* EA-CB0575 CFs enhanced dry weight of banana plants by the action of lipopeptides and siderophores. Moreover, Buensanteai et al. (2008) showed that extracts of *B. amyloliquefaciens* strain KPS46 promoted soybean growth through the actions of the antibiotic surfactin and proteins secreted by the bacterium, including auxin biosynthetic enzymes, proteins related to phosphate solubilization and nitrogen metabolism, antifungal lipopeptides and proteins related to protection against oxidative stress. Also, CFs enriched in amino acids secreted by several *Penicillium* spp. enhanced shoot and root length as well as the biomass of sesame plants under well-irrigated and drought conditions (Radhakrishnan et al., 2014).

Baroja-Fernández et al. (2021) have recently shown that soil application of CFs of beneficial and phytopathogenic fungi cultured in Murashige & Skoog (MS) medium promoted root growth, enhanced fruit yield and altered composition of fruits of pepper plants. In the same study, the authors found that CFs of the different fungal species possessed volatile organic compounds (VOCs) that, once distilled and applied to soil, promoted responses similar to those triggered by direct application of the fungal CFs. These findings indicated that (i) CFs of both beneficial and phytopathogenic fungi can be used to improve crop yield and (ii) VOCs mediate the crops' responses to fungal CF application. Some bioactive VOCs present in the fungal CFs are shown in Table 3. As further discussed below, it is conceivable that some these compounds (particularly acetic acid) are involved in the crop response to soil application of CFs. Notably, high-throughput sequencing analyses revealed that soil application of fungal CFs and distillates (DEs) promoted similar changes in the soil microbiota, and promoted the proliferation of the same beneficial microbial taxa (Baroja-Fernández et al., 2021) (Table 4). Collectively, the findings of Baroja-Fernández et al. (2021) indicated that (i) CFs of both beneficial and fungal phytopathogens can be used to activate the soil and plant-associated beneficial microbiota, and (ii) microbial VOCs mediate the plants' responses to soil application of fungal CFs through mechanisms involving stimulation of the beneficial soil microbiota as schematically illustrated in Figure 1.

TABLE 3 List of potentially relevant bioactive VOCs present in at least one of the fungal CFs used by Baroja-Fernández et al. (2021) and their effects on plant.

VOCs	Effect on plant	Reference
1-butanol-3-methyl	Enhances fresh shoot and root biomass and chlorophyll content in Arabidopsis and increases both root length and thickness in *Agave salmiana*.	Camarena-Pozos et al. (2019)
	Increases size, fresh weight and total chlorophyll content in Arabidopsis	Lee et al. (2019a)
1-butanol-2-methyl	Increases size, fresh weight and total chlorophyll content in Arabidopsis	Lee et al. (2019a)
1-hexanol	Promotes growth of Arabidopsis	Blom et al. (2011)
benzaldehyde	Increases the volatile oil accumulation in *Atractylodes lancea*	Zhou et al. (2016)
2-phenylethyl alcohol	Enhances fresh shoot and root biomass, chlorophyll content, in Arabidopsis and increases both root length and thickness in *A. salmiana*	Camarena-Pozos et al. (2019)
acetic acid	Enhances drought tolerance in Arabidopsis, rapeseed, maize, rice and wheat plants	Kim et al. (2017)
	Increases root biomass and photosynthesis in mung bean	Rahman et al. (2019)
acetoin	Increases root length, shoot length and dry weight in *L. sativa* seedlings	Fincheira et al. (2017)
	Induces stomatal closure in Arabidopsis and *Nicotiana benthamiana*	Wu et al. (2018)
2,3-butanediol	Promotes growth of Arabidopsis	Ryu et al. (2003)
	Induces stomatal closure in Arabidopsis and *N. benthamiana*	Wu et al. (2018)
	Increases root length, shoot length and dry weight in *L. sativa* seedlings	Fincheira et al. (2017)
2-heptanone	Promotes the growth of Arabidopsis seedlings	Jiang et al. (2019)
2-nonanone	Increases root length, shoot length and dry weight in *L. sativa* seedlings	Fincheira et al. (2017)
acetophenone	Elicits the increase of biomass in Arabidopsis	Camarena-Pozos et al. (2019)
cis-thujopsene	Induces lateral root formation of Arabidopsis seedlings and poplar plantlets	Ditengou et al. (2015)

Challenges and limitations of the microbial CF technology

Despite having great potential as a strategy for improving productivity in a sustainable and eco-friendly manner, the technology based on microbial CF application is still at its infancy and faces important challenges and limitations before it can be widely used (Table 1). First, one challenging aspect of the microbial CF-based technology is the manner of application of the extracts, especially in cases in which bioactivity of CFs is based on compounds with high evaporation rates such as VOCs. Most studies on the effect of application of microbial CFs on plants are based on seed coating and soil applications of CFs (Table 2), but other means of delivery, should be explored to develop appropriate and durable methods that can be used in the field. Second, another challenging aspect of the microbial CF-based technology is the scaling up from lab-scale shake flasks to stirred tanks-based pilot-scale production (Figure 2). This also applies to the technologies of soil inoculation of beneficial microorganisms and application of microbial-derived compounds. Overcoming this limitation is not an easy task due to marked differences in hydromechanical properties and nutrients/oxygen gradients between flasks and large fermentors. For successful scaling up, key parameters affecting heat, momentum and mass transfer should be considered (Trujillo-Roldán et al., 2013). Moreover, some physical parameters should be combined to obtain dimensionless numbers intended to be kept constant during the scaling up process. Third, there are no studies on techno-economic viability of large-scale production of microbial CFs involving (1) propagation of the microbial strain until desired inoculum concentration is reached, (2) fermentation of the microbial strains in large, industrial size fermentors until desired cell concentration is reached and (3) microbial cell removal. For the first two steps, some techno-economic models have been developed for typical liquid biofertilizer production plants (Pérez-Sánchez et al., 2018). These models can be used to investigate the main factors that affect the production process, in order to optimize plant productivity and reliability, and also to reduce costs. Fourth, the limitations to microbial CF use are closely related to the downstream processes for production. However, there are no studies on the formulation and shelf life of microbial CFs and on allocation of fitness costs for resources for the large-scale synthesis of these extracts. Clearly, the formulation of new products ready to be commercialized requires further scientific and industrial up-scaling studies. Fifth, the effects

TABLE 4 List of microbial species whose populations are enriched by the soil application of fungal CFs and DEs used by Baroja-Fernández et al. (2021).

Microbial species	Mechanism/mode of action	Reference
Bacterial species		
Burkholderia arboris	Solubilizes phosphate and produces IAA and siderophores	Zhang et al. (2022)
Burkholderia silvatlantica	Has ACC deaminase activity Fixes N_2	Onofre-Lemus et al. (2009)
		Perin et al. (2006)
Caballeronia udeis	Solubilizes phosphate and produces siderophores	Puri et al. (2020)
Duganella ginsengisoli	Produces IAA	Goodwin (2022)
Pseudomonas brassicacearum	Has ACC deaminase activity	Belimov et al. (2007)
Pseudomonas mediterranea	Solubilizes organic phosphate and produces siderophores, proteases, ammonia and IAA	Gu et al. (2020)
Pseudomonas Knackmussii	Solubilizes phosphate and produces IAA and siderophores	Rabhi et al. (2018)
Rhodanobacter glycinis	Synthesizes osmolytes and biocontrol-related substances	Lee et al. (2019b)
Fungal species		
Candida subhashii	Biological control of plant pathogenic fungi	Hilber-Bodmer et al. (2017)
Geotrichum candidum	Produces phytohormones and reactive oxygen species	Waqas et al. (2017)
	Solubilizes phosphate	Wu et al. (2012)
	Produces IAA, ammonia and polyamines	Fu et al. (2016)
	Produces IAA and siderophores and has ACC deaminase activity	George et al. (2019)
***Pseudogymnoascus* spp.**	Solubilizes phosphate	Abdel-Ghany et al. (2019)

of CFs on plants and associated microbiota may vary depending on the plant and microbial species and ecotypes as well as on media composition, age and growth conditions of the microbial culture. Thus, whereas Khokhar et al. (2013) reported that application of CFs of several *Penicillium* spp. cultured for 15 days at 20 °C in malt extract broth exerted a positive effect on wheat germination and growth, Vankudoth et al. (2015) reported that application of CFs of the same fungal species cultured for 12 days at 27 °C in CYA broth exerted a negative effect on sorghum germination. CFs of the phytopathogen *Fusarium oxysporum* grown in Czapek-Dox and potato dextrose liquid media exerted a negative effect on seed germination and growth of cucumber and garden cress (*Lepidium sativum* L.) plants (Melo and Piccinin, 1999; Ogórek, 2016), whereas application of CFs of *F. oxysporum* cultured for 5 days at 25 °C in CYA broth enhanced pigeonpea (*Cajanus cajan* L.) seed germination and growth (Jalander and Gachande, 2012). Application of CFs of the fungal phytopathogen *A. alternata* cultured in Czapek broth and MS media promoted growth of rice and wheat plants and enhanced pepper fruit yield (Bhajbhuje, 2015; Khan et al., 2015; Baroja-Fernández et al., 2021), whereas application of CFs of the same species cultured in Richard´s solution exerted a negative effect on germination of seeds of several crop plants (Parveen et al., 2019). In many instances, the growth inhibitory effect of the CFs of phytopathogens was due to toxins released by the microorganism to the culture medium (Raithak and Gachande, 2013; Vankudoth et al., 2015; Parveen et al., 2019). Sixth, above threshold levels, many microbial compounds are toxic to plants. Therefore, excess application of microbial CFs has the potential to exert a negative effect on plants. However, after due assessment of the dose-response effect on specific crops, microbial CFs can be safely managed. Seventh, although Baroja-Fernández et al. (2021) showed that application of CFs of diverse microorganisms resulted in activation of beneficial soil and plant-associated microbiota without significant changes in the relative abundance of populations of pathogenic microbial species, it is important to ensure that these results can be extrapolated to other CFs in different soil types and environmental scenarios. Eighth, CFs based on co-cultivation of various microorganisms might be an efficient approach to obtain widely range of bioactive compounds. Nevertheless, this practice faces similar problems to those of multi-microbial bioinoculants, since each co-inoculant requires specific culture conditions (Reddy and Saravanan, 2013). Nineth, there are few studies on the mechanisms and modes of action of microbial CFs on plants.

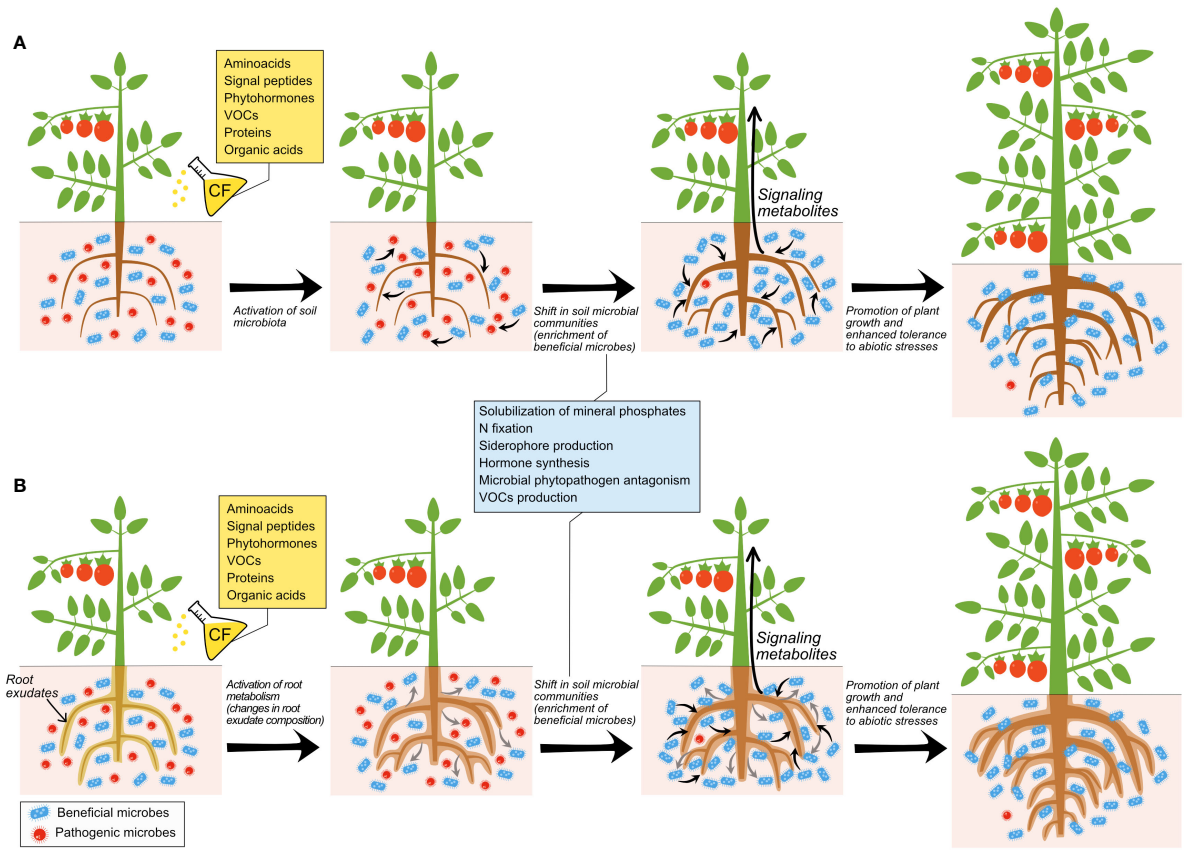


FIGURE 1
Different scenarios of responses of plants and microbiota to soil application of cell-free microbial CFs. **(A)** illustrates direct action of microbial CF compounds on roots and/or on beneficial microorganisms, which release compounds that exert antagonistic effects on pathogenic microorganisms and/or promote plant growth. **(B)** illustrates direct action of microbial CF compounds on the root exudate composition, which modulates rhizosphere microbiota by impoverishing populations of pathogenic microorganisms and enriching those of beneficial microorganisms, which in turn promote plant growth and enhance the capacity of plants to adapt to their environment.

The necessity of identifying the mechanisms and modes of action of cell-free microbial CFs for their use as biostimulants

In our opinion, the exploitation of microbial CFs as biostimulants (defined by the European Biostimulant Industry Council (https://biostimulants.eu/) as "*substances and/or microorganisms whose function when applied to plants or to soil is to stimulate natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and crop quality*" (du Jardin, 2015)) is only just evolving and its broad potential is now beginning to be demonstrated. The majority of studies describing the positive effect(s) of the application of microbial CFs on plant growth and yield did not identify modes or mechanisms of action of these extracts (Table 1). At most, some of these studies proposed that phytohormones and other growth promoting compounds released by microbes in their culture media are involved in the response of plants to microbial CFs. This may result in the assumption by many that these complex, multicomponent mixtures are "magic potions" or "snake oil" not compliant with scientific requirements of the official regulations for fertilizer products (Yakhin et al., 2017). The identification of mechanisms and modes of action of microbial CFs on plants and the characterization of the biological functions and ecological roles of their bioactive components, based on reasonable scientific hypotheses rather than a try-it-and-see approach, could be important not only to develop novel products able to increase yields in crops in a sustainable and environmentally benign manner, but also to obtain clues for the biotechnological design of more productive and efficient crops.

Baroja-Fernández et al. (2021) showed that VOCs are important mediators in the response of plants and plant-associated microbiota to soil application of microbial CFs. However, the bioactive VOCs produced by fungal cultures and

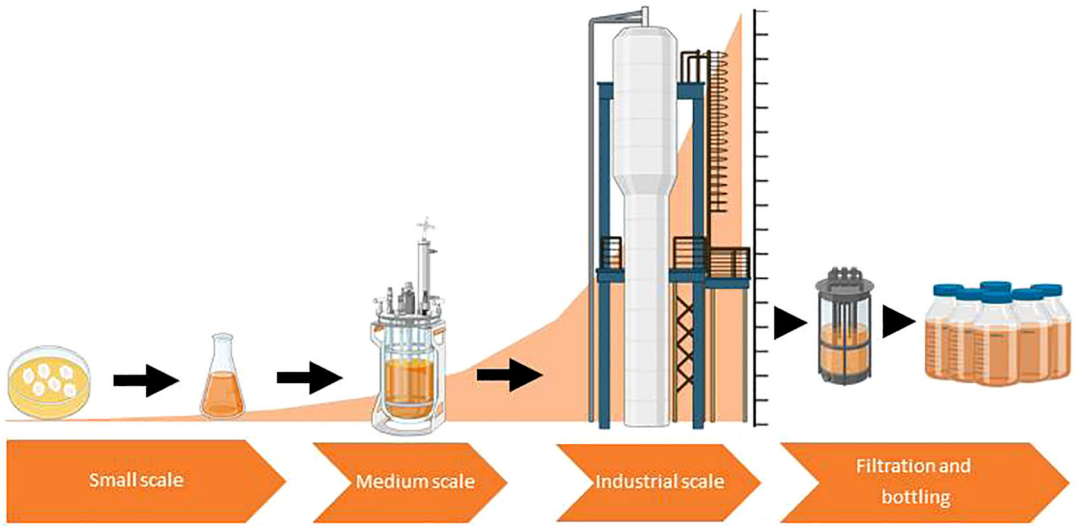


FIGURE 2
Scheme illustrating the scaling-up process of cell-free microbial culture filtrates (CFs) production to industrial level. Figure was created using BioRender.com.

their mechanisms and modes of action remain unverified. Furthermore, whether the effect exerted by soil application of VOCs on plants is direct or mediated by changes induced in the composition of the soil microbiota (or both) still needs to be determined, as does whether the effect exerted by soil application of VOCs on the plant-associated microbiota is direct or mediated by changes induced in the root metabolism (Figure 1). That VOCs isolated from CFs of diverse beneficial and phytopathogenic microorganisms promoted similar growth and fruit yield and quality responses in crops indicate that plants respond similarly to a wide range of bioactive microbial VOCs. Alternatively, it is likely that many microorganisms produce the same growth promoting VOCs. One of such compounds could be acetic acid, which is present in soils, where microorganisms produce it in response to biotic and abiotic stresses (Adeleke et al., 2017). Recent studies have shown that soil application of acetic acid, but not other organic acids, enhances drought tolerance in Arabidopsis and important crops including maize, wheat, rice and cassava (Kim et al., 2017; Utsumi et al., 2019) and tolerance to bacterial wilt disease (Wang et al., 2021). In Arabidopsis, the enhancement of drought resistance promoted by soil application of acetic acid involves JA signaling and an ON/OFF switching epigenetic mechanism dependent on histone deacetylase HDA6 (Kim et al., 2017). However, the biochemical and molecular mechanisms underlying the enhancement of drought tolerance promoted by soil application of acetic acid in crops remain to be elucidated.

Several lines of evidence indicate that the manner of application of microbial volatile compounds and/or plant growth conditions are important determinants of the biochemical and molecular mechanisms triggered in plants. Application of these compounds *via* the air promotes plant growth and metabolic and developmental changes, enhances photosynthesis and improves nutrient and water acquisition (Ryu et al., 2003; Zhang et al., 2008; Zhang et al., 2009; Ezquer et al., 2010; Gutiérrez-Luna et al, 2010; Garnica-Vergara et al., 2016; Sánchez-López et al., 2016; García-Gómez et al., 2019; García-Gómez et al., 2020). In *Arabidopsis*, these responses are associated with changes in the transcriptome, proteome, metabolome, hormonome and redox-proteome through mechanisms involving long-distance communication between roots and the aerial part of the plant and proteostatic regulation of central metabolic pathways in the plastidial compartment (Zhang et al., 2007; Zhang et al., 2008; Zhang et al., 2009; Sánchez-López et al., 2016; Ameztoy et al., 2019; García-Gómez et al., 2020; Ameztoy et al., 2021; Gámez-Arcas et al., 2022). However, Baroja-Fernández et al. (2021) showed that no such mechanisms operate in crops irrigated with VOC-containing microbial CFs and DEs. Needless to say, further work will be necessary to understand how plants respond to microbial volatiles.

The discovery that soil application of VOCs from diverse microorganisms can enhance crop yield and promote similar changes in the soil microbiota extends knowledge on the mechanisms modulating the physiology of the plant and their interaction with plant-associated microbes, raising questions on their ecological significance and biotechnological applications. Because microbes respond to microbial VOCs, it is likely that the fungal VOC-promoted enrichment of the soil beneficial microbiota is due to direct action of these compounds on the microorganisms, which in turn determine plant growth and metabolism through emission of semiochemicals. These compounds trigger the production of molecules by roots that, once transported to the

aerial part of the plant, act as long-distance signals that promote growth and enhance yield (Figure 1A). In line with this presumption, Yuan et al. (2017) showed that exposure of soil to VOCs of *B. amyloliquefaciens* NJN-6 altered the composition of soil microbial communities. Compounds secreted by roots in their exudates attract beneficial microorganisms which, in turn, strongly influence plant fitness and enhance the capacity of plants to adapt to environmental changes and stress (Baudoin and Benizri, 2003; Badri and Vivanco, 2009; Badri et al., 2013; Bulgarelli et al., 2013; Schulz-Bohm et al., 2017; Sasse et al., 2018; Zhalnina et al., 2018; Huang et al., 2019; Chen et al., 2020; Vílchez et al., 2020; He et al., 2022). Therefore, it is conceivable that enrichment in the plant-associated beneficial microbiota triggered by application of fungal CFs and DEs is due to an alteration of composition of root exudates (Figure 1B). This capacity of root exudates to influence the soil microbiota is not static since the composition of root exudates is not uniform over time (Zhalnina et al., 2018) and depends on the plant species or genotype (Bulgarelli et al., 2012; Bodenhausen et al., 2014; Bouffaud et al., 2014; Zhong et al., 2019). For instance, it has been shown that soybean genotype significantly influences the structure and composition of its associated rhizosphere and affects connections in rhizobacterial networks (Zhong et al., 2019).

Additional remarks

According to the Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products, a microbial plant biostimulant may contain micro-organisms belonging to the *Azotobacter Rhizobium*, *Azospirillum* genera and mycorrhizal fungi, including dead or empty-cell micro-organisms and non-harmful residual elements of the media on which the microorganisms were cultured (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R1009). Although regulations are of great importance for guaranteeing food security and preserving environmental integrity, the stringency and exclusivity of the list of microorganisms that can be used for the production of biostimulants may strongly limit the potential benefits of these products. As shown in the review, growing evidence has been compiled demonstrating that application of cell-free CFs of beneficial and phytopathogenic microorganisms is an efficient approach to promote plant growth and enhance yield and stress tolerance in a wide range of crops while reducing the use of agrochemicals. Therefore, it may be appropriate to enlarge the list of microorganisms that can be used for the production of cell-free CF-based biostimulants according to EU regulation on fertilising products, assuming scientific evidence can demonstrate that these products are safe for both the environment and consumers.

Authors contributions

RL, EB-F and J-PR conceptualized the topic and wrote the manuscript. All authors contributed in the literature search and discussions, and reviewed and approved the final manuscript.

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Conflict of interest

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Plant hormone cytokinin at the crossroads of stress priming and control of photosynthesis

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To cope with biotic and abiotic stress conditions, land plants have evolved several levels of protection, including delicate defense mechanisms to respond to changes in the environment. The benefits of inducible defense responses can be further augmented by defense priming, which allows plants to respond to a mild stimulus faster and more robustly than plants in the naïve (non-primed) state. Priming provides a low-cost protection of agriculturally important plants in a relatively safe and effective manner. Many different organic and inorganic compounds have been successfully tested to induce resistance in plants. Among the plethora of commonly used physicochemical techniques, priming by plant growth regulators (phytohormones and their derivatives) appears to be a viable approach with a wide range of applications. While several classes of plant hormones have been exploited in agriculture with promising results, much less attention has been paid to cytokinin, a major plant hormone involved in many biological processes including the regulation of photosynthesis. Cytokinins have been long known to be involved in the regulation of chlorophyll metabolism, among other functions, and are responsible for delaying the onset of senescence. A comprehensive overview of the possible mechanisms of the cytokinin-primed defense or stress-related responses, especially those related to photosynthesis, should provide better insight into some of the less understood aspects of this important group of plant growth regulators.

KEYWORDS

priming, cytokinin, photosynthesis, stomata, ROS, stress, chlorophyll fluorescence

Introduction

The crisis of feeding the world's rapidly growing population with annual growth of around 80 million (Roser et al., 2013) is compounded by a plethora of conflicting issues, such as limited water availability or insufficient allocation of agricultural land. Moreover, various biotic and abiotic stresses resulting from long-term exposure to high temperatures and osmotic stress significantly limit crop yields. Water stress and salinity are considered to be the biggest challenges of present-day agriculture (Abhinandan et al., 2018), leading to poor seed

germination rates, poor seedling emergence and poor stand establishment, thereby significantly limiting global crop production (Reed et al., 2022). Sustainable agriculture under the pressure of climatic change is one of the priorities of developed countries across the globe. The crop productivity is expected to have increased by approximately 60-100% by 2050; achieving such a goal without damaging the agricultural soil is challenging (Lau et al., 2022). Environmental stresses may be prevented by optimizing plant growth conditions and applying plant growth regulators (Yakhin et al., 2016). Seed priming – a process that involves seed imbibition with small amount of water to allow distribution of the priming agent – is recognized as an innovative and affordable technology to counteract harmful effects of abiotic stress by enhancing plant defense responses (Arun et al., 2022).

Similar to immunization in animals and humans, where an infectious agent or vaccine helps the immune system develop immunity to a disease, plants can also be immunized. This type of "acquired physiological immunity" in plants is now referred to as priming and can generally be described as the ability to elicit a faster and/or stronger defense response after a prior exposure to a biotic or an abiotic stress condition (Bruce et al., 2007; Hilker and Schmülling, 2019). Both biotic and abiotic stressors can elicit an adaptive response of the plant immune system, including changes at the epigenetic level, transcriptional reprogramming, and changes in protein phosphorylation (Conrath, 2011; Mauch-Mani et al., 2017). In general, priming allows plants to remember past stress events and prepare them for future attacks (Tsuda et al., 2013; Bjornson et al., 2014; Choi et al., 2014; Campos et al., 2016; de Zelicourt et al., 2016; Ariga et al., 2017; Chen et al., 2021). Linked multilevel processes generate a stress imprint or stress memory of a given priming event so that when a plant encounters the same specific condition, it can reach a primed state of enhanced and faster responsiveness and effectively regulate the relevant defense signaling cascade(s) (Conrath et al., 2006). Some excellent reviews address the molecular mechanisms underlying stress memory and priming formation upon various stress conditions in different plant species (Conrath, 2011; Conrath et al., 2015).

While defense priming is typically associated with bacterial and fungal pathogens or other biotic stressors, an altered response to abiotic stressors (also called hardening) is no less important. Previous exposure to a mild abiotic stress including heat, cold, osmotic or water stress can also improve resistance of various environmental stresses (Thomashow, 2010; Amooaghaie and Tabatabaie, 2017; Fan et al., 2018; Lemmens et al., 2019). Priming with plant hormones (hormopriming) is another popular and effective approach how to improve overall plant development (Iqbal et al., 2006; Nawaz et al., 2013; Chipilski et al., 2021; Vedenicheva et al., 2022), with cytokinins (CKs) emerging as a potentially interesting new group of hormopriming regulators that have been repeatedly shown to influence several developmental processes, including photosynthesis and senescence (Sobol et al., 2014; Vylíčilová et al., 2016; Hönig et al., 2018; Kučerová et al., 2020). In this review, we focus on possible CK effects in stress priming, with particular emphasis on the maintenance of photosynthesis, which is likely the backbone of the adaptive response mediated by this plant hormone.

Cytokinin priming to increase crops growth and yield

It has been recently reported that priming of wheat seeds with benzylaminopurine (BAP) and kinetin (KIN) applied by spraying the wheat plants during the grain filling stage resulted in up to 14% higher productivity, higher fresh and dry weight, and chlorophyll content index of flag leaves (Chipilski et al., 2021). In addition to the positive impact on photosynthesis, another way in which CKs can influence plant fitness and yield is their natural ability to suppress oxidative stress, which has been also repeatedly associated with defense priming in plants (Kerchev et al., 2020). The offspring of CK-treated plants during the grain filling stage showed a lower accumulation of stress markers in field conditions demonstrating the transgenerational effect of this stress imprint (Chipilski et al., 2021). The CK-primed enhanced protection against oxidative damage was demonstrated on 15-day-old seedlings of wheat (cv. Geya-1) and these results positively correlated with a 25% lower average accumulation of malondialdehyde and hydrogen peroxide in 5-day-old seedlings of such wheat. In addition, the exogenous CK application in field conditions enhances the wheat seed viability after a low-temperature storage, which is an essential feature for practical agriculture (Chipilski et al., 2021). Moreover, KIN improved tomato shoot lengths after seed priming (Nawaz et al., 2013). As outlined above, the CK treatment can reasonably influence seed germination. This may be correlated with increased activity of hydrolytic enzymes in developing seeds, which can neutralize the impact of seed aging (Nawaz et al., 2013).

CK priming becomes particularly important when dealing with the consequences of climate change, such as temperature changes, increased solar radiation, drought, or salinity. The priming of rye seeds (cv. Boguslavka) with zeatin resulted in significant changes of endogenous CK pools in both shoots and roots of 7-day-old seedlings (Vedenicheva et al., 2022). Also, rye plants grown from CK-primed seeds were more resistant to hyperthermia stress than untreated control (Vedenicheva et al., 2022). Two cultivars of hexaploid spring wheat (*Triticum aestivum* L.) seeds were pre-soaked in BAP and KIN solutions, and the primed and non-primed seeds of salt-intolerant MH-$_{97}$, as well as salt-tolerant Inqlab-91, were compared. KIN was effective in increasing the germination rate in the salt-intolerant cultivar and early seedling growth when compared with hydropriming under salt stress. Furthermore, the KIN-primed seeds showed a consistent promoting effect in the field and improved growth and grain yield in both cultivars under salt stress (Iqbal et al., 2006). The authors explain the priming effect by a complex crosstalk regulatory mechanism involving levels of active CK, abscisic acid (ABA) and indoleacetic acid (IAA) in developing plant leaves. CKs applied on the seeds can enhance the future shoot regeneration efficiency, as it activates the dedication of the shoot progenitor at later stages and allows chromatin to maintain shoot identity genes (Fathy et al., 2022; Wu et al., 2022).

Many CK-based compounds have already found application in micropropagation techniques and protection of plants against various types of abiotic stress, some were also tested experimentally in in field

trials (Koprna et al., 2016; Plíhalová et al., 2016). Regarding seed priming, it may be interesting to note that the application of some CK-based derivatives, such as 2-substituted-6-anilino-9-heterocyclylpurine derivatives, has already been patented for seed dressing/coating of various crops, mainly cereals (Zatloukal et al., 2015). Their application led to increase of yield and quality of the agricultural product in harmful conditions (Zatloukal et al., 2015). Purine based (CK) derivatives were applied on maize, winter wheat and rapeseed. Other CK derivatives derived from urea were applied to fight stress caused by drought, heat or cold stress and salinity (Nisler et al., 2015). Such urea-based derivatives were applicated on winter wheat *Triticum aestivum* cv Hereward and spring barley (*Hordeum vulgare*), malting variety Bojos or winter oilseed rape treated by picking with a 50 μM solution of urea-based CK derivatives (Nisler et al., 2015). Since even seed coating where the active substances remain on the surface of the seed can cause such an impact on the crop productivity as described above, soaking the seed in the priming agents (although more technically demanding) may produce earlier and more consistent responses. Thus, it can have a more significant impact on crop yield.

Mechanisms of cytokinin-mediated effects on photosynthesis

The reduction in photosynthetic capacity is associated with the reduced growth in many plant species exposed to stressful environments, demonstrating a direct relation between the photosynthetic capacity and crop yield (Moradi and Ismail, 2007; Naeem et al., 2010; Wu et al., 2012; Wu et al., 2019). Among the various biochemical processes, photosynthesis is highly sensitive to any environmental stress, with the photosynthetic apparatus being one of the most stress-sensitive plant components. Thus, photosynthetic capacity determined through the gaseous exchange (von Caemmerer and Farquhar, 1981) and chlorophyll fluorescence (Roháček, 2002; Lazár, 2015) measurements provides an excellent way to analyze the effects of stress on plants. In addition, monitoring photosynthetic variables by non-invasive techniques can be used to assess whether priming effects have been achieved and to determine the potential fitness benefits of the process. Optimization of the photosynthesis through transcriptomic reprogramming of target components of photosynthetic protein complexes or specific regulation of chlorophyll-related and other metabolic processes could, on the other hand, provide us with a powerful tool to increase yield under certain stress conditions, resulting in an overall improvement of agricultural production.

Priming, due to an inherently relatively high stability of photosystems and their high recovery response, offers a role in reducing the severity of stress and can therefore promote a rapid and complete recovery of plant physiological functions. Although different priming methods undoubtedly improve photosynthetic parameters and overall photosynthetic efficiency of plants exposed to various environmental stresses (Vincent et al., 2020; Sorrentino et al., 2021; Aswathi et al., 2022; Johnson and Puthur, 2022), the control mechanisms that govern the photosynthetic protection are still poorly understood. Recently, a major role in both the direct and indirect regulation of the photosynthetic protection under stress conditions has been attributed to various plant hormones (Müller and Munné-Bosch, 2021), which have long been known as effective priming agents (Kauss and Jeblick, 1995; Mur et al., 1996; Ton and Mauch-Mani, 2004; Hirao et al., 2012; Wang et al., 2014; Ji et al., 2021). We observed that CKs and their derivatives have important protective effects on the photosynthetic apparatus, which is manifested by the upregulation of photosystem components PSII (and more rarely PSI), upregulation of Calvin cycle components (RuBisCO, glyceraldehyde-3-phosphate dehydrogenase, etc.) and maintenance of chlorophyll through the downregulation of chlorophyll catabolism (chlorophyll b reductase coded by *NOL*, *NYE1*, *NYE2*, etc.; Vylíčilová et al., 2016) and the upregulation of chlorophyll biosynthesis, presumably through upregulation of protochlorophyllide oxidoreductase (Kusnetsov et al., 1998). This leads to a significantly better plant fitness and a delayed onset of senescence.

CKs are non-volatile plant hormones that reside mostly within plant vascular tissues and are unable to provide defense responses in neighboring plants (Dervinis et al., 2010). However, this does not seem to lessen their potential as effective priming agents. Apart from the relatively decent knowledge about their mode of action under optimal developmental and growth conditions, their role in plant defense priming related especially to photosynthesis is still a mosaic of individual findings. CKs play a central role in the chloroplast development and function and in chlorophyll biosynthesis (Cortleven and Schmülling, 2015). They are known to regulate many genes associated with photosynthesis (Brenner and Schmülling, 2012) and protect the photosynthetic machinery and productivity of plants exposed to various stresses (Chernyad'ev, 2009). CKs appear to act in the protection of photosynthesis at both levels, light and dark photosynthetic reactions, including the control of gas exchange.

Regarding the control of gas exchange by CKs, they are often considered as an ABA antagonist. Generally, exogenous CKs can inhibit ABA-induced stomatal closure in diverse species (Tanaka et al., 2006). The increased CK concentration in the xylem sap promotes the opening of stomata and reduces sensitivity to ABA (Daszkowska-Golec and Szarejko, 2013). Wild-type tomato leaves treated with CK showed enhanced transpiration and increased numbers of stomata per leaf area than untreated leaves (Farber et al., 2016). Mohammadi et al. (2015) demonstrated that BAP foliar application can significantly increase stomatal conductance in wheat under drought stress. An increase in internal CO_2 concentration and water use efficiency (WUE) or increase in CO_2 assimilation rate, stomatal conductance and transpiration was recorded under salt stress in eggplant after seedlings' exposition to BAP (Wu et al., 2012) or in *Panax ginseng* plants at a later growth stage after inserting the stem base into BAP solution (Li and Xu, 2014). The naturally occurring zeatin-type bases, ribosides and *O*-glucosides supplied to the leaf in xylem sap regulated transpiration *in planta* in oat (Badenoch-Jones et al., 1996). Wheat priming by seed pretreatment with *cis*-zeatin or *trans*-zeatin significantly increased stomatal conductance, photosynthetic efficiency, shoot biomass with grain yield upon salt and drought stress (Alharby et al., 2020). Applying synthetic cytokinin, KIN, to *Tradescantia albiflora* leaves

induced stomatal opening (Pharmawati et al., 1998). Seed priming with KIN alleviated the adverse effect of salt stress on gas exchange characteristics of wheat leading to improving growth and grain yield (Iqbal and Ashraf, 2005). Similarly, foliar spray of KIN on salinized mulberry plants increased the net photosynthetic rate (P_n), WUE, carboxylation efficiency and leaf yield (Das et al., 2002). KIN could act as an effective priming agent also upon waterlogging stress, as reduced levels of reactive oxygen species (ROS), better water status, osmotic adjustment and an increased P_n, WUE, improved growth and biomass under waterlogging were detected in primed mungbean plants (Islam et al., 2022a). Foliar-applied KIN remarkably improved maize performance by modulating growth, gas exchange and water related parameters under drought stress (Islam et al., 2022b).

The mechanism of direct action of CK on guard cells may involve the induction of membrane hyperpolarization by stimulation of the electrogenic H^+-pump (Pospíšilová, 2003). The internal cytosolic free calcium concentration may mediate interactions between CK and ABA (Hare et al., 1997). In Kentucky Bluegrass, BAP has been proposed to promote stomatal opening through its effect on ABA balance leading to improved photosynthetic recovery from drought (Hu et al., 2012). CKs may promote stomatal opening also by scavenging H_2O_2 in guard cells as demonstrated in *Vicia faba* plants (Song et al., 2006). In addition, a higher carboxylation efficiency (P_n/C_i) was observed in *Anthurium* plants sprayed with KIN along with its possible effects on gas exchange and antioxidant enzyme activities (de Moura et al., 2018). Farber et al. (2016) proposed that CKs can act indirectly in stomata movement – CK levels reduced during adaptation to water deficiency suppress growth and reduce stomatal density, both of which reduce transpiration, thereby increasing the tolerance to drought. Priming by exogenous application of BAP to nutrient solution upregulated the RuBisCO large subunit content in some leaves of wheat plants (Criado et al., 2009). An incubation of wheat leaves in BAP solution reduced the degradation of the large and small subunits of RuBisCO (Zavaleta-Mancera et al., 2007). Foliar spraying of rice by synthetic CK (*N*-2-(chloro-4-pyridyl)-*N*-phenyl urea) reversed the drought mediated suppression of RAF1 and the RuBisCO activase proteins, implied in the assembly and activation of RuBisCO complex (Gujjar et al., 2020). Thus, CKs may also enhance photosynthesis at the molecular level by modulating the abundance of proteins related to stomatal conductance, chlorophyll content, and RuBisCO activity (Gujjar et al., 2020).

CKs may also protect the primary photochemistry processes of photosynthesis. An improved photosynthetic performance at donor and acceptor sides of the photosystem II reaction centre (RCII) was detected in wheat plants sprayed with BAP, which simultaneously increased their endogenous zeatin levels. A higher effective and maximal quantum yields of PSII photochemistry in the light (ΦPSII) and dark (F_v/F_m) adapted state, better transfers of electrons beyond Q_A, enhanced electron transport rate and lower relative variable fluorescence intensity at the J-step were detected in the BAP primed wheat (Yang et al., 2018). In drought stressed maize seedlings, exogenous BAP priming regulated transient rise of fluorescence and increased the electron donation capacity of PSII (Shao et al., 2010). Application of BAP to the nutrient solution alleviated the detrimental effect of salt stress on primary photochemistry of eggplant by increasing F_v/F_m, F_v'/F_m' (maximal quantum yield of PSII photochemistry in light adapted state), ΦPSII and increase of q_p (parameter of photochemical quenching) at the expense of non-photochemical quenching (NPQ) leading to a lower dissipation of excitation energy in the PSII antennae (Wu et al., 2012).

The protection of primary photochemistry by CKs can be attributed to their positive effect on the maintenance of chlorophyll pigments and the integrity of chloroplast membranes. Priming with BAP prior to water stress resulted in increased content of xanthophyll cycle pigments and their degree of de-epoxidation in four drought-exposed plant species (Haisel et al., 2006). Exogenously applied BAP significantly reduced the senescence-induced decrease in Chl, car, xanthophyll content and in the Chl/car ratio which was reflected in a lower impairment of PSII function in barley segments (Janečková et al., 2019). Seed priming by BAP significantly increased the content of photosynthetic pigments (chl a+b), car and carbohydrates in a salt stressed soybean (Mangena, 2020). Other mechanisms by which CKs maintain PSII functionality could be to stabilize Chl-protein complexes, both Light-harvesting complex II (LHCII) and RCII, as demonstrated during dark-induced senescing Arabidopsis leaves floating in BAP solution (Oh et al., 2005). In line with this, several genes encoding the light-harvesting chlorophyll a/b-binding proteins and various proteins of PSII are regulated by CKs in Arabidopsis (Brenner and Schmülling, 2012; Vylíčilová et al., 2016). CKs may also protect the cell membranes and the photosynthetic machinery from oxidative damage. Exogenous BAP reduced levels of ROS and enhanced the activity of antioxidant enzymes (CAT, APX) in dark senescing wheat leaves (Zavaleta-Mancera et al., 2007). Exogenously applied BAP alleviated the harmful effects of salt stress by increasing photosynthetic efficiency and activity of antioxidant enzymes (SOD, POD, APX, CAT) and by reducing malonaldehyde contents and $O_2^{\cdot-}$ production in eggplant (Wu et al., 2012). Foliar application of KIN to *Vigna radiata* plants regulated antioxidant enzyme activities and reduced the increase in total peroxide, leading to suppression of the adverse effect of salt stress (Chakrabarti and Mukherji, 2003).

In addition to naturally occurring CKs or the notorious synthetic derivatives, a new avenue opens up for the possibility of studying the priming of the photosynthetic apparatus using purposefully modified CK derivatives. Exogenous KIN and its synthetic derivatives significantly protected lipid membranes from the negative effects of accumulated ROS in detached wheat leaves in the dark (Mik et al., 2011). Synthetic purine based halogenated CK derivatives increased PSII photochemistry efficiency, chlorophyll and carotenoid levels, and abundance of some LHCII components during Arabidopsis leaf senescence (Vylíčilová et al., 2016). Aromatic CK arabinosides (BAPAs) presumably act as a new type of priming agents that promote the plant innate immunity (PAMP-like responses) and positively affect leaf longevity (Bryksová et al., 2020). BAPAs treatment in detached Arabidopsis leaves indicated its action as a priming agent for the PTI (PAMP-triggered immunity) response (Bryksová et al., 2020) and at the same time markedly reduced the high light-induced cell membrane damage, accumulation of lipid hydroperoxides as well as PSII photoinhibition (Kučerová et al., 2020).

Cytokinin crosstalk with other phytohormones in priming

At first glance, the role of CKs in plant defense and priming may seem auxiliary compared to other plant hormones and volatile compounds. The traditional plant defense response related to the hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are interlinked in a complex network regulating plant immunity (Conrath, 2006; Mishina and Zeier, 2007; Pieterse et al., 2009; Engelberth et al., 2011; Fragnière et al., 2011; Pieterse et al., 2014). These compounds and their volatile derivatives are well-documented as priming agents in many physiological processes (Farmer and Ryan, 1990; Engelberth et al., 2011; Song and Ryu, 2018; Avramova, 2019; Zhao et al., 2020; Singewar et al., 2021). In last decades, the participation of CKs in biotic (Choi et al., 2011; Cortleven et al., 2019) and abiotic (Barciszewski et al., 2000; Wani et al., 2016) stress responses has also been demonstrated; however, less is known about the molecular aspects of CKs in priming. Similarities in the defense mechanisms mediated by CKs and other hormones or Green Leaf Volatiles (GLVs) suggest that CKs play a more important role in the priming process than previously thought.

CKs are essential in orchestrating an immune response, most likely through the crosstalk mechanisms with other hormonal pathways. Importantly, CKs are significantly involved in plant-pathogen interactions through crosstalk with the SA pathway (reviewed in Choi et al., 2011). The CK transcription factor ARR2 confers resistance to *Pseudomonas syringae* in Arabidopsis *via* interaction with SA response factor TGA3 and binding to the *PR1* promoter. In addition to the direct activation of defense responses by ARR2, CK pretreatment led to a hyperactivation of *PR1* transcription after pathogen inoculation (Choi et al., 2010). It was also shown that high concentrations of CK primed defense responses in Arabidopsis seedlings against the *Hyaloperonospora arabidopsidis* (Argueso et al., 2012). CK treatment alone resulted in slightly increased SA-related defense pathway gene expression. However, following the pathogen inoculation, it was further potentiated. The CK-responsive gene expression was diminished in *eds16* mutant plants with impaired SA biosynthesis. Thus, this crosstalk mechanism may help plants fine-tune the defense responses against biotrophic pathogens and adjust the effects of CK on plant immunity (Argueso et al., 2012).

In addition to the well-documented crosstalk with the SA pathway, the connection of the CK pathway with the JA pathway has also been reported. CKs can prime an immune response against herbivory through a crosstalk with the JA pathway, resulting in reduced emission of GLVs (Schäfer et al., 2015). GLVs were among the first well-documented compounds to effectively prime a plant's defense system (Engelberth et al., 2004). *In planta* exposure to GLV *cis*-3-hexenyl acetate caused a rapid accumulation of JA and linolenic acid (LNA) in poplar leaves after herbivory (Frost et al., 2008). In line with this, Dervinis et al. (2010) reported higher JA and LNA levels after the CK pretreatment following wounding, while the CK treatment alone did not alter the JA and LNA levels in unwounded leaves. In addition, wounding and CK priming resulted in reduced larval weight gain (Dervinis et al., 2010).

Elevated levels of JA were also measured in a recent study on a CK-mediated resistance against brown planthopper in rice (Zhang et al., 2022). Using the CRISPR-Cas9 genome-editing approach, the authors generated two independent rice knock-out lines of cytokinin oxidase/dehydrogenase 1 (*OsCKX1*). Consequently, the mortality dropped to 30% in the case of *OsCKX1* knock-out plants upon an infestation by brown planthopper, compared to the 90% wild-type mortality upon infestation. A simple CK treatment positively regulated JA biosynthetic and an expression of JA-responsive genes. In addition, the CK-mediated resistance against the brown planthopper infestation was diminished in JA-deficient mutant *og1* (Zhang et al., 2022). Although previous studies have shown an antagonistic relationship between these two hormones, in the case of biotic stress defense responses and priming, the roles of CK and JA may be rather synergistic (Zhang et al., 2022).

The mechanisms of a crosstalk between GLV and CK pathways are much less understood but offer a significant potential for further study. It is tempting to speculate that there should be a regulatory mechanism to maintain a balance in leaf mining processes during herbivory. The existence of "green islands" with elevated levels of zeatins, isopentenyl adenine and other CKs has been reported during leaf-mining by galling insects (Giron et al., 2007). Colonization of plants by endophagous organisms thus requires a hormonal crosstalk, with GLVs playing a possible role in the process due to their volatile nature, which allows them to prime defense responses in places where CKs cannot overcome the natural barriers given by vascular constraints. Interestingly, after the treatment of *Nicotiana attenuata* leaves with the oral secretion of *Manduca sexta*, the CK pathway suppressed GLV esters emission (Schäfer et al., 2015). While JA concentrations are positively correlated with the activity of the CK pathway (Dervinis et al., 2010; Schäfer et al., 2015), the release of GLV esters was negatively correlated, suggesting that CKs control the balance between these two oxylipin classes (Schäfer et al., 2015).

As outlined by Schäfer et al. (2015), the activation of the classic CK signaling pathway after a CK application does not exclude the possibility of an alternative CK pathway involved in plant defense and priming. Priming enables plants to retain a "stress memory" and the priming state can last for multiple generations (Holeski et al., 2012; Hilker and Schmülling, 2019). For this reason, CK priming may also trigger various epigenetic modifications. CK-induced phenotypic changes in canola, such as increased surface area of petals, jagged edges of petals, and altered vasculature of flowers, are carried forward to the next generation of non-primed plants (Zuñiga-Mayo et al., 2018). This phenomenon can be achieved through the alterations of DNA methylation status. *S*-Adenosyl-L-homocysteine hydrolase (SAHH) appears to play a major role in this process (Li et al., 2008). Exogenous CK application induced the expression of the three cytosine DNA methyltransferase genes, *MET1, CMT3*, and *DRM2*, suggesting an important role of CKs in promoting DNA methylations in Arabidopsis (Li et al., 2008). Interestingly, downregulation of *SAHH* caused DNA hypomethylation, increased levels of CKs, and resistance against various plant viruses in tobacco (Masuta et al., 1995).

In addition, our aromatic CK derivatives that have been synthesized and tested retain certain properties of CK responses, such as delayed senescence and defense against pathogens, while the classical CK signaling pathway is only negligibly affected (Bryksová et al., 2020; Kučerová et al., 2020); therefore, an alternative mode of action for CK priming *via* the classical MAPK signaling cascade in

PAMP-triggered immunity (PTI) responses and underlying epigenetic modifications cannot be ruled out. Experiments with these aromatic CK derivatives confirmed the synergistic relationship between CK and JA in defense priming, as the levels of JA and its metabolites were significantly elevated (Bryksová et al., 2020). However, a negative correlation was found between the senescence activity of aromatic CK derivatives and ET production (Kučerová et al., 2020). An ET production is generally known to be stimulated by exogenous CK treatment (Cary et al., 1995; Zdarska et al., 2015). Together with the JA pathway, ET regulates the herbivory-induced responses (Onkokesung et al., 2010). Interestingly, no stimulation of ET production was observed in aromatic CK derivative-treated wheat and Arabidopsis leaves (Kučerová et al., 2020).

While the effects of CK priming under biotic stresses are relatively well documented, mechanistic studies on CK priming in relation to abiotic stresses are just beginning to emerge. Previous works are mainly devoted to CK's role in priming against heat stress and drought (Cheikh and Jones, 1994; Kang et al., 2012; Sobol et al., 2014; Wu et al., 2016; Wu et al., 2017). Kang et al. (2012) found that sensor histidine kinase (AHK) mutant lines, mainly *ahk2* and *ahk3*, show significant tolerance to dehydration. CK crosstalk with ABA may be significantly involved in dehydration tolerance, as *ahk* mutants have an increased level of expression of ABA-upregulated genes (Kang et al., 2012). CK pretreatment with KIN was able to further increase the survival rate of *ahk* single mutant lines after subsequent dehydration stress (Kang et al., 2012). This phenomenon can be explained by the interplay of increased expression of ABA-upregulated genes and a wide range of CK effects (including priming) in plant defense may not be linked to the classical CK pathway, as previously discussed.

Conclusion and perspectives

In the last decade, many studies have pointed to the practical use of plant growth regulators that can mitigate the negative effects of biotic and abiotic stress conditions and improve overall crop condition and yield. Hormopriming is widely accepted as an

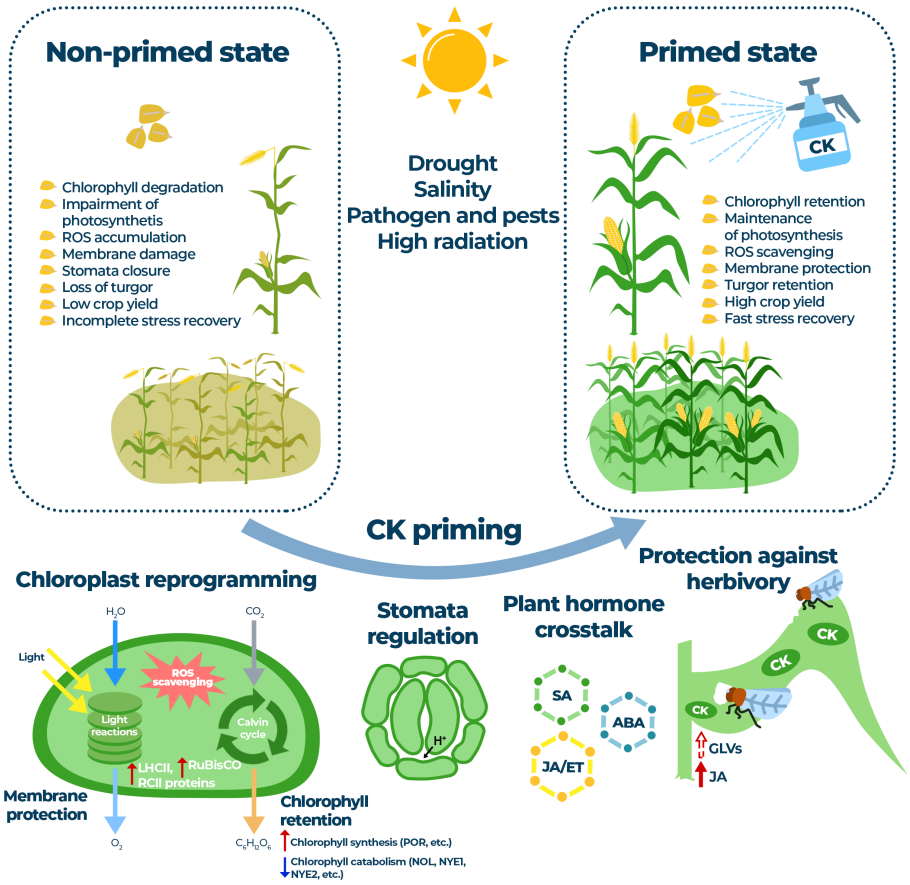


FIGURE 1
A simplified model showing the beneficial effects of cytokinin priming on photosynthesis and plant regulatory processes under stress conditions. Cytokinins can protect the light and dark reactions of photosynthesis by processes such as chlorophyll retention (upregulation of chlorophyll biosynthesis and downregulation of chlorophyll catabolism; cytokinin upregulated genes coding for *POR*, Protochlorophyllide oxidoreductase and downregulated genes coding for *NOL*, Chlorophyll b reductase; *NYE1*, Non-yellowing 1, and *NYE2*, Non-yellowing 2, are shown). Other cytokinin regulated processes include upregulation of LHCII and RCII components, upregulation of RuBisCO activity and activation of ROS scavenging system leading to membrane protection. Another important mechanism of action of cytokinins during stress and recovery is the desirable crosstalk of cytokinins with traditional plant stress hormones, potentially including volatile substances (GLVs). During herbivory, so-called "green islands" with elevated levels of CKs are formed; crosstalk mediated by volatile compounds and other phytohormones can initiate defense priming. Thus, the application of cytokinins can regulate plant response to various abiotic and biotic stresses, leading to balanced photosynthetic function, improved stress tolerance and increased crop yield.

efficient way to create memory imprints in seeds or young developing plants that can later, under limiting or stressful conditions, help shift the endogenous hormonal balance towards defense responses. Given the persistent social barriers to genetically modified crops, targeted modification of stress-related pathways through treatment with low amounts of highly bioactive substances offers an interesting alternative for agricultural use. The plant hormone CK is emerging as a promising new type of priming agents, the use of which appears to be closely linked to the control of the photosynthetic apparatus (Figure 1). Given the growing number of studies linking CK to defense or stress priming on the one hand and maintenance of photosynthesis on the other, more clear understanding on the mechanisms of a CK action on a photosynthetic and gas exchange machinery will be necessary for the more comprehensive understanding of CK-controlled stress protective responses. So far, mainly aromatic CKs BAP or KIN have been reported as priming agents with several possibilities of use. The application of natural CKs and many of their derivatives offers numerous advantages due to their negligible cytotoxicity to human cells and low risks to the environment resulting from their high biological activity in plants. However, although natural and some synthetic CKs show satisfactory results in terms of retarding senescence or maintaining balanced photosynthetic activity, they also show some negative effects on root development, and optimizing their effective use in field conditions remains a challenge. Therefore, new further SAR studies will be required to carefully promote the desirable traits of CK-based growth regulators to offer more practical future applications in sustainable agriculture.

Author contributions

MH and VN wrote the original draft of the manuscript. VN designed the figure with help of OP. OP and VN conceived the idea and made the final adjustments. All authors contributed to the article and approved the submitted version.

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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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Physiological and molecular insight of microbial biostimulants for sustainable agriculture

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Increased food production to cater the need of growing population is one of the major global challenges. Currently, agro-productivity is under threat due to shrinking arable land, increased anthropogenic activities and changes in the climate leading to frequent flash floods, prolonged droughts and sudden fluctuation of temperature. Further, warm climatic conditions increase disease and pest incidences, ultimately reducing crop yield. Hence, collaborated global efforts are required to adopt environmentally safe and sustainable agro practices to boost crop growth and productivity. Biostimulants appear as a promising means to improve growth of plants even under stressful conditions. Among various categories of biostimulants, microbial biostimulants are composed of microorganisms such as plant growth-promoting rhizobacteria (PGPR) and/or microbes which stimulate nutrient uptake, produce secondary metabolites, siderophores, hormones and organic acids, participate in nitrogen fixation, imparts stress tolerance, enhance crop quality and yield when applied to the plants. Though numerous studies convincingly elucidate the positive effects of PGPR-based biostimulants on plants, yet information is meagre regarding the mechanism of action and the key signaling pathways (plant hormone modulations, expression of pathogenesis-related proteins, antioxidants, osmolytes etc.) triggered by these biostimulants in plants. Hence, the present review focuses on the molecular pathways activated by PGPR based biostimulants in plants facing abiotic and biotic challenges. The review also analyses the common mechanisms modulated by these biostimulants in plants to combat abiotic and biotic stresses. Further, the review highlights the traits that have been modified through transgenic approach leading to physiological responses akin to the application of PGPR in the target plants.

KEYWORDS

PGPR, biostimulant, antioxidants, phytohormones, signaling, stress tolerance, crop productivity, systemic resistance

Introduction

Agriculture is presently facing several challenges due to shortage of cultivable land, fluctuating weather conditions, increased incidence of pests and pathogens and rising weed infestations. To increase crop productivity, chemical fertilizers are used indiscriminately (Rouphael and Colla, 2020; Hendriksen, 2022). However, extreme usage of chemicals causes detrimental effects on the soil microorganisms, human and the environment leading to decreased water holding capacity, loss of soil fertility, imbalances in soil nutrients, and increased salinity levels (Wan et al., 2021; Jin et al., 2022). To meet the rising demand for food, boosting crop productivity is imperative. Hence there is a need for a green, efficient, sustainable and economically productive system to improve agronomic traits of crops (Drobek et al., 2019; Cataldo et al., 2022). Currently, biostimulants have emerged as one of the most potent and promising tools for enhancing the growth and productivity of crops naturally, simultaneously addressing the issues related to chemical fertilizers. Biostimulants/bioeffectors/bioprotectors or biobased products are different classes of organic or inorganic compounds which consists of bioactive substances or microorganisms and when applied on target plants promote its growth and productivity (Shahrajabian et al., 2021; Franzoni et al., 2022; Monteiro et al., 2022). Over the time, various researchers have classified biostimulants into nine broad categories including seaweeds and plant extracts, complex organic materials (obtained from sewage sludge extracts, composts, manure urban and agro-industrial waste products), humic substances, antitranspirants (kaolin and polyacrylamide), chitin and chitosan derivatives, elements (Al, Co, Se, Na, and Si), hydrolyzed proteins, nitrogen-containing compounds and microbial inoculants (Colla and Rouphael, 2020; Teklić et al., 2021; Franzoni et al., 2022; Monteiro et al., 2022). Out of this wide category, microbial-based biostimulants including plant growth-promoting bacteria (*Bacillus, Serratia, Arthrobacter, Pseudomonas, Rhodococcus, Enterobacter, Ochrobactrum, Acinetobacter, Azospirillum, Rhizobium, Streptomyces* and *Stenotrophomonas*) have surfaced as highly valuable and inexpensive agricultural input for improving plant yield (Orozco-Mosqueda et al., 2020; Baltazar et al., 2021; Miceli et al., 2021; Ayed et al., 2022; Fadiji et al., 2022). These microbiome based biostimulants trigger plant growth through solubilization of minerals (Zn, P and K), nitrogen fixation, production of phytohormones like indole-3-acetic acid (IAA), abscisic acid (ABA), ethylene (ET), cytokinin (CK), jasmonic acid (JA), secondary metabolites (siderophores, N-acyl homoserine lactone, lipopeptides, rhamnolipids, cyclic lipopeptides), enzymes (chitinases, cellulose, protease, glucanase etc.), volatile organic compounds (VOCs) (fatty acids and derivatives, hydrocarbons (alkanes, alkenes and alkynes), carbohydrates, (acids, alcohols, lactones, aldehydes, benzenoids, etc.) terpenoids, nitrogen (metalloid, amides, amines and imines), volatile inorganic compounds (HCN, H_2S, NH_3, CO_2, CO, and NO) and lipopolysaccharides (Caulier et al., 2019; Lephatsi et al., 2021). These components modulate root morphology including biomass of roots, its surface area and newly formed lateral roots, shoot length, leaf area, soil structure (nutrient, water holding capacity, porosity and water filtration), improve nutrient and mineral acquisition (N, P, Fe, Zn, Mn etc.) and photosynthetic capacity of a plant. Further, they also enhance biotic and abiotic stress tolerance by activating genes responsible for antioxidant defense system, production of phenolics, enzymes, amino acids and organic acids (Backer et al., 2018; Rouphael and Colla, 2018; Hamid et al., 2021; Aremu et al., 2022; Lin and Jones, 2022; Vocciante et al., 2022), but the exact functionality at cellular and biomolecular mechanisms are yet to be deciphered (Lephatsi et al., 2021; Othibeng et al., 2022). To elucidate the mechanism of action of biostimulants on plants, combined potential of molecular tools, proteomics, transcriptomics and metabolomics have been harnessed by several researchers (Franzoni et al., 2022). Although progress has been made in understanding the physiological and biochemical aspect of plant-microbe interactions under stress, but the core mechanism and elucidation of molecular interactions are still in infancy. Thus, the present review comprehends the physiological and biochemical modulations along with the signaling components of PGPR based biostimulants in imparting abiotic (major detrimental stresses like temperature, drought and salinity) and biotic stress tolerance in plants. An in-depth understanding of the biochemical and molecular mechanisms triggered by the microbial biostimulants particularly PGPR based will aid in designing and developing novel bioformulations for sustainable agriculture.

Role of PGPR-based biostimulants in combating abiotic stress

Drought and heat stress

About 60% of the world's region falls under arid and semi-arid areas and depends mainly on irrigated agriculture (Swain et al., 2017). With climate change, it is expected that there will be a decrease in rainfall, a rise in temperature, an increase in atmospheric CO_2 and severe alternations in weather conditions leading to frequent floods and droughts (Torres and Henry, 2018). In upcoming years, agriculture will be increasingly challenged by water scarcity, and plants will experience drought and heat stress leading to compromised productivity. Drought stress occurs under low humidity levels in soil, air and high ambient temperature (Lipiec et al., 2013; Kaya et al., 2020; Kosar et al., 2021; Mansoor et al., 2021), while heat stress can be described as an increase in temperature beyond a threshold measure hampering the normal development of a plant. It is observed that the combined action of both stresses restrict the physiological (photosynthesis, respiration, etc.), biochemical and cellular metabolism of plants such as cell membrane fluidity, integrity, elasticity, water potential, stomatal conductance, the structure of amino acids, proteins, nucleic acids, enzymes etc. (Rana et al., 2021; Mitra et al., 2021; Murali et al., 2021a; Noor et al., 2022; Shaffique et al., 2022). In order to adapt under environmental stress plants regulate their diverse molecular signaling pathways such as phytohormones, stress responsive proteins, antioxidants machinery, and osmolytes (Kosar et al., 2021). While understanding the physiological response of plants, it is crucial that both drought and heat stresses must be considered together as the physiological responses are closely interlinked and dependent (Dreesen et al., 2012). Numerous studies have elucidated the positive impact of PGPR functioning as a biostimulant on plants challenged by heat

and drought (Rashid et al., 2022; Yasmin et al., 2022). Basically, drought and thermotolerance is a complex mechanism, however, microbial metabolites including organic acids, sugars, trehalose, choline, amino acids, proline, glycine betaine, polyamines, exopolysaccahrides (EPS), production of heat shock proteins (HSPs), dehydrins, VOCs, ACC-deaminase, phytohormones etc. play a vital role in imparting drought and heat tolerance [Figures 1A, B; (Ahluwalia et al., 2021; Ansari et al., 2021; Yasmin et al., 2021a; Notununu et al., 2022; Kour and Yadav, 2022; Shaffique et al., 2022)].

1-Amino Cyclopropane-1-Carboxylate (ACC) deaminase produced by several PGPRs help in combating drought stress experienced by its host plant by interfering with the ethylene biosynthesis pathway leading to lowering of ethylene concentration thereby counteracting stress signals. In one of the studies, the application of *B. licheniformis* K11 capable of producing auxin and ACC deaminase reduced the negative impact of drought in pepper without the use of agrochemicals (Lim and Kim, 2013). Similarly, improved growth was noticed in pea and maize under drought conditions on treating with ACC deaminase producing strain

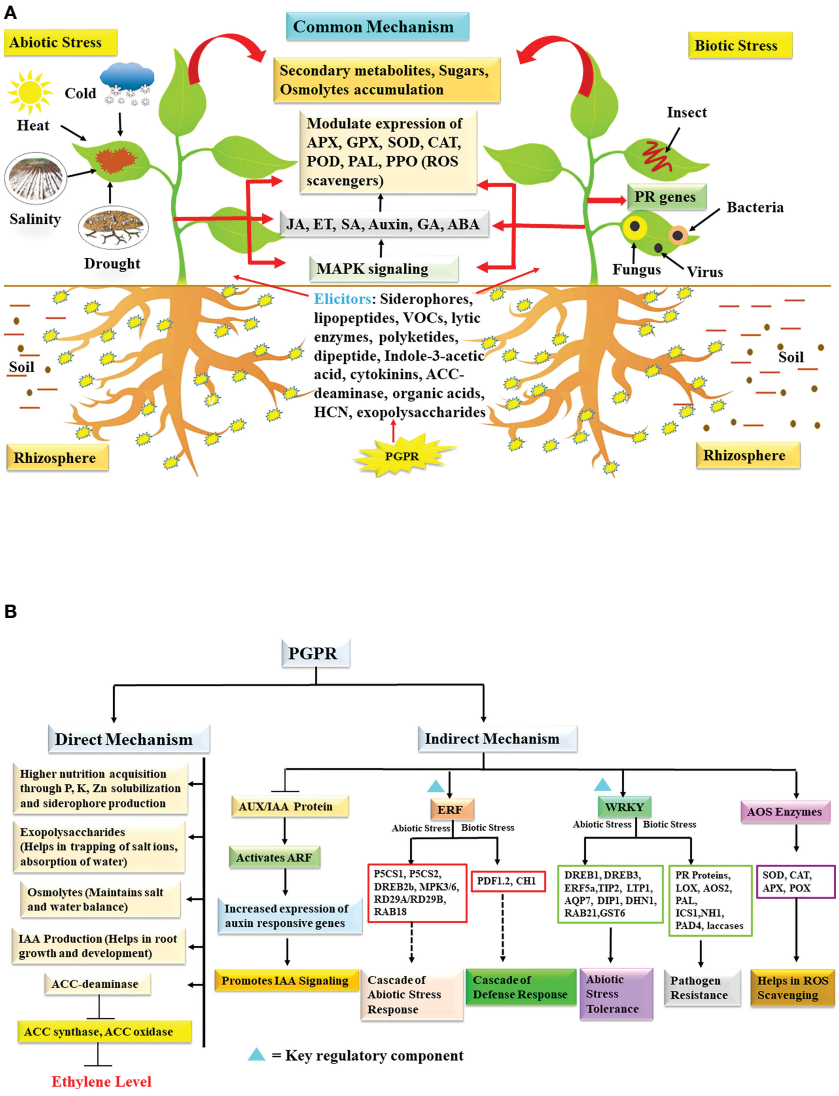


FIGURE 1
(A) Schematic representation of PGPR induced stress tolerance mechanism in plant challenged by abiotic and biotic stresses. Different elicitors released by PGPRs modulates endogenous phytohormones which in turn influences secondary metabolites, osmolytes production, activity of antioxidant enzymes and PR proteins. These combined metabolic pathways imparts stress tolerance and promotes plant growth under stressed environment. **(B)** PGPR-based direct and indirect mechanism involved in activating cascade of abiotic and biotic stress signaling in plants. The activation events are represented by arrows, inhibition process is represented by bar while dashed arrows represent signaling cascade. IAA, indole-3-acetic acid; ACC, 1-Amino Cyclopropane-1-Carboxylate; AUX/IAA, auxin/indole-3-acetic acid; ARF, auxin response factor; ERF, ethylene response factor; P5CS1, Δ^1 - pyrroline-5-carboxylate synthase1; P5CS2, Δ^1 - pyrroline-5-carboxylate synthase 2; DREB2b/DREB1/DREB3, drought-responsive element binding protein 2b, drought-responsive element binding protein 1, drought-responsive element binding protein 3; MPK3/MPK6, mitogen–activated protein kinase 3, mitogen-activated protein kinase 6; RD29A/RD29B, response-to-desiccation 29A, response-to-desiccation 29B; PDF1.2, protodermal factor 1.2; CH1, chitinase; WRKY, W-box domain binding transcription factor; TIP2, tonoplast intrinsic protein 2; LTP1, lipid transfer protein 1; AQP7, aquaporin 7; GST6, glutathione S-transferase; DIP1, dehydration stress-inducible protein 1; DHN1, dehydrin 1; RAB21, responsive to ABA protein 21; PR, pathogenesis related proteins; LOX, lipoxygenase; AOS2, allene oxide synthase 2; PAL, phenylalanine ammonia lyase; ICS1, isochorismate synthase 1; NH1, *Arabidopsis* NPR1 homolog 1; PAD4, p hytoalexin-deficient 4; AOS, antioxidant scavenging; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; POX, peroxidases; ROS, reactive oxygen species.

Pseudomonas spp., *Enterobacter cloacae*, *Achromobacter xylosoxidans* and *Leclercia adecarboxylata* primarily due to reduced ethylene accumulation as compared to untreated plants (Arshad et al., 2008; Danish et al., 2020). Additionally, these bacteria are capable of supplying nitrogen by sequestering and degrading ACC to α-ketobutyrate using ACC deaminase (Gupta and Pandey, 2019) thereby promoting better vegetative growth of plants.

Production of reactive oxygen species (ROS) is a common phenomenon observed under drought conditions causing damage to cells (Cruz de Carvalho, 2008) and antioxidant enzymes such as catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO) scavenge ROS preventing stress related injury thereby imparting stress tolerance [Figures 1A, B; (Zandalinas et al., 2018)]. These antioxidants also promote faster recovery from water limitation and dehydration compared to the control plants (Laxa et al., 2019). Hence, the potential of PGPR in enhancing the production of antioxidants is a desirable attribute. Several studies illustrate the beneficial trend of antioxidant enzymes under severe drought, as was noticed in mentha (Chiappero et al., 2019; Asghari et al., 2020) and in tomato (Mekureyaw et al., 2022). Noticeably, seeds of Pearl millet treated with *Bacillus amyloliquefaciens* (MMR04) enhanced expression of ascorbate peroxidase (APX) and superoxide dismutase (SOD) genes leading to the enhanced concentration of SOD and APX and decreased level of malondialdehyde (MDA) compared to the untreated lot (Murali et al., 2021b).

Accumulation of proline is one of the common mechanisms involved in imparting drought tolerance. Proline helps to maintain protein structure and activity thereby supporting membrane integrity (Kishor et al., 2005). Also, proline has multiple roles as it can act as a chelator of metals, a signaling molecule, and a defense molecule triggering the production of a series of antioxidants (Hayat et al., 2012). It has been observed that *Bacillus subtilis* (HAS31), *Pseudomonas* strains apart from increasing antioxidants (SOD, CAT and POD) activity also significantly enhanced the total soluble sugars and proline in potato and sweet corn (Batool et al., 2020; Zarei et al., 2020).

PGPRs have an inherent capability to produce plant growth hormones like auxin, gibberellins (GA), JA, salicylic acid (SA) and ABA. These hormones may be directly responsible for triggering a series of responses in the host (plant) to combat stress conditions. The role of SA in imparting drought stress has been illustrated to alter nitrogen metabolism, triggering the production of antioxidants and accumulation of glycine betaine thus conferring protection against stress (Khan et al., 2022). An interesting research showed that under heat-stress conditions inoculation of *Bacillus tequilensis* SSB07 strain in soybean enhanced the endogenous level of JA, SA, and reduced the level of ABA content (Kang et al., 2019a). All the treated plants had better biomass and photosynthetic pigment compared to the control. Two PGPR strains *Bacillus* sp. WM13-24 and *Pseudomonas* sp. M30-35, promoted the growth of ryegrass subjected to drought stress by modulating auxin distribution and ABA content in the plant (He et al., 2021).

Several scientific investigations illustrated that microbial applications induce the expression of specific genes related to drought response. Treating *Arabidopsis* and soybean with *Paenibacillus polymyxa* CR1 upregulated dehydration-responsive genes (*RD29A* and *RD29B*), which equipped the plants to face drought conditions (Liu et al., 2020). Similarly, application of *Bacillus subtilis* strain GOT9 triggered up-regulation of numerous drought stress related genes particularly *Response-to-desiccation 29B, 20* (*RD29B, RD20), RAB18* (encodes dehydrin protein), *9-cis-epoxycarotenoid dioxygenase (NCED3)*) in *Arabidopsis* and *BrDREB1D*, BrWRKY7 and *BraCSD3* in *Brassica* thereby minimizing the physiological damage. Enhanced expression of ABA inducible genes clearly showed that GOT9 increased ABA accumulation in plant and hence provided drought tolerance (Woo et al., 2020). Inoculation of *Pseudomonas putida* GAP-P45 in *Arabidopsis* modulated several important polyamine biosynthetic genes (*arginine decarboxylase (ADC), agmatine iminohydrolase (AIH), N- carbamoyl putrescine amidohydrolase (CPA), spermidine synthase (SPDS), spermine synthase (SPMS)* and *S-adenosylmethionine decarboxylase (SAMDC)* thereby impacting cellular polyamine levels. The increased level of free cellular spermidine and putrescine positively correlated with water stress (Sen et al., 2018). In transgenic *Arabidopsis* overexpressing *ABA stress ripening 6 (OsASR6)* (auxin activated) gene increased the expression of auxin-responsive genes, *small auxin up-regulated family (SAUR32)*, Ser/Thr protein kinase (PINOID), and *auxin response factor 5 (ARF5)* auxin transcription factor leading to greater root density and biomass. These effects of *OsASR6* expression were found to mimic the beneficial effects of PGPRs in rice (Agarwal et al., 2019). PGPR strain *Streptomyces* mitigated drought stress in tomato and regulated the expression of transcription factors *ethylene response factor 1 (ERF1)* and *WRKY70* [Figure 1B; (Abbasi et al., 2020)]. In another study, expression of ABA independent genes, i.e. *drought-responsive element binding protein (DREB2* and *DREB1-2)* significantly enhanced due to PGPR (*Bacillus* sp.) inoculation in *Brassica* under water scarcity indicating *Bacillus*-mediated priming for drought tolerance (Bandeppa et al., 2019). Pepper treated with *B. licheniformis* K11 upregulated genes *Cadhn*, *VA*, *sHSP* and *CaPR10* leading to higher production of dehydrin-like protein, vacuolar H^+-ATPase, small heat shock protein and pathogenesis-related protein which helped in in survival of plants under severe drought conditions (Lim and Kim, 2013). An interesting study was carried out wherein it was elucidated that *Pseudomonas putida* modulates the expression of important stress-responsive miRNAs in response to drought and salt stresses (Jatan et al., 2019).

All these studies illustrate that PGPRs influence expression of heat/drought related genes there by triggering production of series of antioxidants, osmolytes, proline and several key biomolecules which may contribute in mitigating heat and drought stress. Also, several hormones produced by PGPRs as illustrated in Figure 1A trigger cascade of biochemical reaction in host which enable plants to tie over drought stress.

Cold stress

Low temperatures limit agriculture productivity in temperate ecosystems. In these areas, the plants constantly face chilling stress that leads to 51-82% annual yield loss (Hwarari et al., 2022). It generally affects the critical processes of plants such as ROS homeostasis, energy metabolism (electron transport chain), photosynthesis efficiency, cell wall structure, fluidity, root hydraulic

conductance and structure of biomolecules (enzymes, proteins and nucleic acids) (Kazemi-Shahandashti and Maali-Amiri, 2018; Tiryaki et al., 2018; Zhou et al., 2021). In order to survive under prolonged low temperatures, the plants exhibit altered gene and transcription factor expression, leading to changes in membrane lipids, proteins, osmolytes levels, phytohormones, phenolic content and reactive oxygen scavenging enzymes (Brüggemann et al., 1999; Saltveit, 2000; Ait Barka et al., 2006; Amini et al., 2021; Guo et al., 2021; Ritonga et al., 2021; Saleem et al., 2021a; Eom et al., 2022; Hwarari et al., 2022; Wei et al., 2022).

The application of PGPR to improve crop productivity is a sustainable and safer means compared to chemical inputs. It has been found that treatment of *Burkholderia phytofirmans* strain *PsJN* (*Bp PsJN*) on *Arabidopsis thaliana* subjected to cold stress prevented disruption of plasmalemma, exhibited cell wall strengthening of mesophyll cells (Su et al., 2015). Inoculation of *Vitis vinifera* with PGPR strain *PsJN* exhibited better CO_2 fixation, increased content of phenolics, proline and starch (Ait Barka et al., 2006). In another study, *PsJN* improved cold tolerance in *Vitis vinifera* wherein higher accumulation of proline, MDA and aldehydes (ALD) were observed along with higher expression of phenylalanine ammonia lyase (PAL) and stilbene synthase (STS) genes in primed plants compared to non-treated plants (Theocharis et al., 2012). Similarly, another finding revealed that under chilling stress treatment of tomato plants with psychrotolerant *Pseudomonas vancouverensis* (OB155) and *P. frederiksbergensis* (OS261) microbes minimized the stress effect by increasing the level of proline, antioxidant enzymes [(SOD, APX and glutathione (GSH)] (Subramanian et al., 2016). Further, *Pseudomonas fragi*, *P. chloropaphis*, *P. fluorescens* and *Brevibacterium frigoritolerans* inoculants also improved the growth of beans by regulating the activities of SOD, CAT, POX, APX and GR during low temperature stress [Figure 1A; (Tiryaki et al., 2018]. Subsequently, further research reflected the key role of *Bacillus* spp. in wheat under cold stress. Under cold stress, the bacterial treatment significantly reduced the level of ABA, ET, MDA by directly targeting the *ABA- response element* (*ABARE*), *Ethylene response factor* (*ERF*) and *4-Hydroxy-2-nonenal* (*4-HNE*; encodes α, β- unsaturated aldehyde during lipid peroxidation) genes, but at the same time the expression of *Δ^1- pyrroline-5-carboxylate synthase* (*P5CS*), expansin (*expA1*), cytokinin (*CKX2*) and auxin (*ARF*) increased (Zubair et al., 2019). Another study evaluated the role of *Rhizobium* inoculation (RI) in legumes model plant i.e. *Medicago truncatula* against cold stress. Compared to the control plant, the treated plant showed a significant increase in the SOD, CAT, APX, ascorbate, reduced glutathione, proline, soluble sugars and glycine betaine, whereas POD, *lipoxygenase* (*LOX*) activity and nitro-oxidative damage was notably reduced. Moreover, RI also stimulated the nitrogen (N) uptake in cold stress seedlings by enhancing the activity of nitrate reductase (NR) enzyme (Irshad et al., 2021). In addition, *Streptomyces* sp. 506 (TOR3209) played a distinct function in boosting tolerance to cold stress in tomato. Expression profile of TOR3209 treated stressed plants showed an increased level of HY5 (bZIP) mediated ABA signaling genes [(*zeaxanthin epoxidase* (*ZEP1*), *9-cis-epoxycarotenoid dioxygenase* (*NCED1*), carotenoid dioxygenase and carotene beta-hydroxylase), *dehydrin* (*TASI4*) (that triggers accumulation of soluble sugars, proline). Besides this, TOR3209 also reduced the photosynthetic damage by modulating the activities of RUBISCO (Ribulose 1, 5-bisphosphate carboxylase/oxygenase), NAD-MDH and NADP-MDH (malate dehydrogenases) enzymes suggesting that main mechanism of imparting cold tolerance is by ABA pathway (Ma et al., 2022). Over and above, other finding highlights the role of cold active PGPR in rice growth and development. It was observed that strains of *Pseudomonas*, *Enterobacter*, *Stenotrophomonas* genera inoculation ameliorated the effect of cold in rice by increasing the accumulation of metabolites (proline and soluble sugars), protein content, nutrients (N, P and K), antioxidants (SOD, POD and CAT) (Expósito et al., 2022).

These studies illustrate that most of the cold tolerance by PGPRs are due to cross-interlinkage of antioxidants, soluble sugars, proteins, proline, phenolics, phytohormones etc. Further scientific investigations will provide better insight into PGPR mediated cold tolerance in plants but nevertheless, there are credential evidences which indicate that the right microbial strains can boost productivity in the temperate ecosystem.

Salinity stress

High salt concentration leads to osmotic stress that interferes with physiology, biochemical functioning (photosynthesis, stomatal conductance, enzyme activities, water and nutrient uptake), growth and yield of crops (Yasmin et al., 2021b; Choudhary et al., 2022). Microbial application triggers various mechanisms for improving plant growth under salinity stress (Sarkar et al., 2018). It generally includes the production of ACC-deaminase, EPS, phytohormones (auxin, CK and SA), antioxidant enzymes, VOCs, synthesis of osmoprotectant metabolites (proline, trehalose, alanine, glycine, glutamic acid, serine, threonine, aspartate, choline, betaine and organic acids), regulation of ion affinity transporters that in turn maintains ionic, osmotic, water homeostasis, thus resulting in improved plant growth under salt stress [Figure 1A; (Tewari and Arora, 2018; Abbas et al., 2019; Kumar et al., 2019; Bhat et al., 2020; Sunita et al., 2020; Choudhary et al., 2022; Gamalero and Glick, 2022; Kumawat et al., 2022)].

PGPR based compounds modulate the phytohormones, antioxidants and osmolytes levels in plants for their growth under stress conditions (Choudhary et al., 2022). A study demonstrated that under salt stress *Rhodopseudomonas palustris* G5 treated cucumber seedlings showed higher expression of SOD, POD, PPO and soluble sugars (Ge and Zhang, 2019) compared to untreated plants. Another ACC- deaminase producing endophytic strain *Pseudomonas* spp. OFT5 confers salt tolerance to tomato by reducing ethylene production (Win et al., 2018). Inoculating paddy with halotolerant *Curtobacterium albidum* strain SRV4 improved plant growth under various salinity levels by significantly increasing SOD, CAT, POX, APX expression, and by maintaining Na^+/K^+ homeostasis. This study showed that EPS production by strain reduced sodium ions availability to the plant and hence overcome effect of salinity stress (Vimal et al., 2019). Furthermore, *Planomicrobium* sp. MSSA-10 regulated antioxidants, phenolics and nutrients mobilization pathways in pea and promoted its growth in saline conditions. It was observed that bio-inoculant treatment increased total phenolics, POD, CAT and nutrients (N, P and K) uptake for reducing negative effects of salt [Figure 1B; (Shahid et al., 2018)]. Additionally, it was observed that salt tolerant *Bacillus pumilus* FAB10 significantly

decreased antioxidant enzymes like SOD, CAT, glutathione reductase (GR) activities, proline and MDA content in wheat at different salt concentrations (Ansari et al., 2019). Furthermore, endophytic bacteria *Curtobacterium* sp. SAK1 treatment reduced the effect of salt stress in soybean by lowering endogenous ABA, JA, ROS, PPO and POD levels, whereas glutathione (cellular antioxidant) concentrations were found to be higher (Khan et al., 2019c). Another research team observed that inoculation of soybean with five halotolerant strains i.e. *Arthrobacter woluwensis* (AK1), *Microbacterium oxydans* (AK2), *Arthrobacter aurescens* (AK3), *Bacillus megaterium* (AK4) and *Bacillus aryabhattai* (AK5) conferred salt tolerance by elevating the expression of SOD, glutathione synthase (GSH) and enhancing K^+ uptake. Besides the antioxidants, microbial inoculation significantly reduced Na^+ ion concentration, ABA level, but increased the expression of IAA related gene i.e. *auxin resistant 1* (*GmLAX3*) and salt tolerant gene (*Soybean salt tolerance 1*) (*GmST1*) (Khan et al., 2019b). In addition to this, it was found that halotolerant bacteria *Leclercia adecarboxylata* MO1 improved tomato growth under salt stress by significantly increasing sugars (sucrose, glucose and fructose), organic acids (citric acid and malic acid), amino acids (serine, glycine, methionine and proline) and simultaneously decreasing endogenous ABA level (Kang et al., 2019b). Similarly, PGPR *Pseudomonas* PS01 imparted salt tolerance in *Arabidopsis* by modulating the expression of stress related genes. Results illustrate that PS01 inoculation in salt stressed plants increased expression of *lipoxygenase (LOX2)* (related to JA synthesis), while decreased *APX2*, *GLY17* (ROS scavenging and detoxification). No significant change was observed in the expression of *RD29A* and *RD29B* (ABA signaling genes) (Chu et al., 2019). One more study figured out that in rice inoculation with halotolerant *Glutamicibacter* sp. YD01 increased the expression of antioxidants such as *OsPOX1*, *OsFeSOD*, *OsGR2*, abiotic stress related genes (*OsWRKY1*, *OsDREB2*), *OsHKT1* (related to ionic balance) and downregulated *OsERF1* (related to ethylene production), thus enhancing their tolerance to salt stress (Ji et al., 2020). In a different fascinating research it was found that *Pseudomonas pseudoalcaligenes* (SR16) and *Bacillus subtilis* (SR3) also provide salt stress tolerance to hydroponically grown soybean seedlings. The strain SR16 effectively reduced 100mM NaCl stress by increasing the total protein, proline content, and activities of various antioxidants (SOD, CAT, APX, POD, PAL and PPO) (Yasmin et al., 2020). Recent findings suggest that soil application of *Kosakonia sacchari* improved mung bean performance by reducing the level of oxidative stress markers such as proline, MDA, H_2O_2 content, antioxidants like APX, CAT, SOD and GR. In contrast, the level of antioxidant metabolites i.e. ascorbic acid and glutathione increases in the foliage of treated plants which in turn reduced the toxicity of NaCl (Shahid et al., 2021). Moreover, inoculation of salt-tolerant PGPR *Acinetobacter johnsonii* provided tolerance to maize by downregulating SOD, CAT, proline and MDA content. It was observed that rhizobacterial inoculation improved dehydrogenase, alkaline phosphatase, acid phosphatase, urease and enzyme activity in the soil. Improved microbes mediated soil enzyme activities plays an important role in balancing nutrient profile, plant growth under salt stress (Shabaan et al., 2022). Additional evidence demonstrates role of halotolerant *Bacillus* strains (NMCN-1 and LLCG23) in mitigating 200mM salinity stress in wheat. Application of both inoculants significantly downregulated the expression of *ABA- response element* (*ABARE*), *4-Hydroxy-2-nonenal* (*4-HNE*), whereas the *P5CS* gene was found to be upregulated. This clearly illustrates that these halophilic microbes regulate key stress signaling pathways (ABA synthesis, MDA and proline production) that subsequently lowered the effect of stress (Ayaz et al., 2022). From the cited literature (Table 1), it is evident that PGPR could alleviate salt stress by improving soil health, nutrient uptake, hormone production, antioxidant activity, and stress- responsive genes.

From the above studies it is clear that bacterial production of ACC deaminase (lowers ethylene concentration), enhanced production of a range of antioxidants (scavenges ROS) and higher production of proline (signaling molecule) in stress affected plants are some of the common mechanisms operate in PGPR treated plants to enable them combat temperature, drought and salinity stress. Hence, application of PGPR based biostimulants with proven PGP traits could be an ecofriendly sustainable means to boost crop productivity under single or combined abiotic stress.

Role of PGPR-based biostimulants in imparting tolerance against disease (biotic stress)

Plant resistance against pathogens is generally based upon two mechanisms i.e. induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR is mainly mediated by beneficial microorganisms through root colonization, root immunity modulation and production of certain elicitors like siderophores, polysaccharides, VOCs, plant hormones, enzymes etc. whereas SAR is defined as the plant's acquired or adaptive resistance (Olowe et al., 2020; Hamid et al., 2021). Both ISR and SAR resistance mechanism is effective against wide group of pests and pathogens (Vlot et al., 2021; Meena et al., 2022; Yu et al., 2022). Though numerous studies reported that PGPR modulates various physiological, biochemical and molecular processes in plants and helps their survival under pathogens attack (Olowe et al., 2020; Castiglione et al., 2021; Yu et al., 2022), but the core mechanism of action is not fully understood. The results obtained through systematic studies indicate that induction of resistance against multiple pathogens including virus, fungi, bacteria rely on combined mechanisms that may work simultaneously (Yu et al., 2022). It includes induction of specific defense response genes/ enzymes like ROS scavengers/antioxidants such as (CAT, APX, guaiacol peroxidase (GPX), GR, POD and SOD), accumulation of phytohormones (JA, ET, SA, GA and auxin), glucanases, chitinases, sugars, osmolytes, pathogenesis related proteins (PR) and secondary metabolites which in turn are directly involved in controlling growth and proliferation of pathogen [Figures 1A, B; (Baxter et al., 2014; Pieterse et al., 2014; Conrath et al., 2015; Camejo et al., 2016; Li et al., 2016; Guo et al., 2019; Ebrahimi et al., 2020; Olowe et al., 2020; da Silva et al., 2021; Luo et al., 2022)].

From the wide group of PGPR, *Bacillus* sp. has been considered as an excellent agent for controlling pathogens attack in various plants such as tomato, banana, tobacco, rice, wheat, cucumber, watermelon, cotton (Saechow et al., 2018; Gamez et al., 2019; Wu et al., 2019; Fu et al., 2020; Jiao et al., 2020; Dimopoulou et al., 2021; Kazerooni et al.,

TABLE 1 PGPRs along with their mode of action in combating abiotic stress.

PGPR Strain	Host Plant	Stress	Mechanism of action	References
Pseudomonas aeruginosa	Sorghum	Heat	Increased proline, chlorophyll, sugar, amino acids, and protein content	Ali et al. (2009)
Rhizobium, Pseudomonas	Maize	Salinity	Decreased electrolyte leakage and increased proline, relative water content	Bano and Fatima (2009)
Pseudomonas putida	Wheat	Heat	Reduced membrane injury and the level of antioxidant enzymes such as SOD, APX and CAT	Ali et al. (2011)
Pseudomonas fluorescens, Bacillus subtilis	Green Gram	Water	Enhanced activity of *CAT1* and POD	Saravanakumar et al. (2011)
Pseudomonas chlororaphis	*Arabidopsis thaliana*	Drought	Up-regulation of genes such as *NIT1* (associated with plant growth regulators), *Atcor15a* (associated with ABA), *RD21a*, KIC (calcium binding protein)	Cho et al. (2011)
Pseudomonas koreensis, Pseudomonas fluorescens, Pseudomonas jessenii	Wheat	Cold	Increased relative water content, anthocyanin, proline, total phenolics, starch and reduced electrolyte leakage	Mishra et al. (2011)
Bacillus licheniformis	Pepper	Drought	Increased content of stress proteins i.e. *Cadhn*, *VA*, *sHSP* and *CaPR*-10	Lim and Kim (2013)
Bacillus pumilus, Bacillus firmus	Potato	Salinity/ Drought	Enhanced mRNA expression related to ROS scavenging enzymes (SOD, GR, CAT, DHAR and APX) and proline level	Gururani et al. (2013)
Bacillus megaterium, Bacillus subtilis	Wheat/ Barley	Cold	Significant reduction in the level of ROS and antioxidant enzyme (SOD, POD and CAT)	Turan et al. (2013)
Pseudomonas aeruginosa	Mung bean	Drought	Increased expression of CAT, POX, SOD and drought responsive genes (*DREB2A*, *CAT1* and *DHN*)	Sarma and Saikia (2014)
Bacillus amyloliquefaciens, Azospirillum brasilense	Wheat	Heat	Increased level of DHAR (Dehydroascorbate reductase), MDHAR (Mono-dehydroascorbate reductase) and GR whereas decreased APX and modulated expression of *SAMS1*, *HSP17*.8	Abd El-Daim et al. (2014)
Enterobacter sp.	Arabidopsis	Salinity	Up-regulated expression of salt stress responsive genes such as *DREB2b*, *RD29A*, *RD29B*, and RAB18, proline biosynthesis genes (*P5CS1* and *P5CS2*), MPK signaling genes (*MPK3* and *MPK6*)	Kim et al. (2014)
	Tomato		Enhanced expression of APX	
Proteus penneri, Pseudomonas aeruginosa, Alcaligenes faecalis	Maize	Drought	Increased relative water content, sugar, decreased proline, antioxidant enzymes (SOD, POD and CAT)	Naseem and Bano (2014)
Burkholderia phytofirmans	*Arabidopsis thaliana*	Salinity	Enhancement of the proline and transcript level of ABA signaling genes (*RD29*, *RD29B), APX2* (antioxidant related), *GYLI7* (glyoxylate pathway), decreased expression of *LOX2* (related to JA)	Pinedo et al. (2015)
Bacillus megaterium, Enterobacter sp.	Okra	Salinity	Increased ROS scavenging enzymes (CAT, SOD, APX, GR and DHAR)	Habib et al. (2016)
Bacillus pumilus	Rice	Salinity	Augmented activity of antioxidants such as (SOD, POD and CAT)	Khan et al. (2016)
Dietzia natronolimnaea	Wheat	Salinity	Enhanced expression of salt stress tolerant (*TaST*), Salt Overly Sensitive (SOS) related genes (*SOS1* and *SOS4*), antioxidant enzymes genes (*APX, MnSOD, CAT, POD, GPX* and *GR*) and ABA signaling (*TaABARE* and *TaOPR1)*	Bharti et al. (2016)
Pseudomonas frederiksbergensis, Flavobacterium glaciei, Pseudomonas vancouverensis	Tomato	Cold	Increased proline content and, enhanced antioxidant enzymes such as SOD, APX and GSH	Subramanian et al. (2016)
Bacillus aryabhattai	Soybean	Heat	Enhanced levels of phytohormones IAA, JA, GA, ABA, antioxidants (CAT and SOD), but CK decreased	Park et al. (2017)
Bacillus megaterium	Arabidopsis	Salinity	Enhanced level of *CYP94B3* (responsible of JA-Ile catabolism), MDHAR and ATP synthase	Erice et al. (2017)
Bacillus subtilis	Wheat	Salinity	Decreased proline, MDA content whereas SA and water storage capacity enhanced	Lastochkina et al. (2017)
Bacillus amyloliquefaciens	*Arabidopsis thaliana*	Salinity	Up-regulation of genes such as *GST* and *POX* (antioxidant responsive), *ACS7*, *ACS2*, *ACS8*, and *ACS*11(ET signaling), *LOX* (JA signaling) downregulated *NCED3, NCED4, ABI1* and *MARD1* (ABA signaling)	Liu et al. (2017)

(Continued)

TABLE 1 Continued

PGPR Strain	Host Plant	Stress	Mechanism of action	References
Pseudomonas chlororaphis, Pseudomonas extremorientalis	Tomato	Salinity	Decreased H_2O_2, APX, GR level with simultaneous increase in SOD and CAT activity	Egamberdieva et al. (2017)
Azospirillum brasilense, Herbaspirillum seropedicae	Maize	Drought	Decreased expression of *ZmVP14* (involved in the biosynthesis of ABA), proline, ET content but MDA level increased	Curá et al. (2017)
Pseudomonas putida	Finger Millet	Drought	Increased activities of SOD, CAT, APX and GPX antioxidants	Chandra et al. (2018)
Paraburkholderia phytofirmans	Tomato	Heat	Augmented chlorophyll content, gas exchange, expression of *APX2* and *CAT1*. No significant change was observed in SOD, *CHI3*, *TIV1*, *Frk2, Hxk1*, *Hxk2*, *RbcL* and *RbcS* level	Issa et al. (2018)
Ochrobactrum pseudogrignonense, Pseudomonas sp., *Bacillus subtilis*	Black gram/ Pea	Drought	Downregulated expression of *ACO*, increased proline content and, antioxidant enzymes (CAT and POD)	Saikia et al. (2018)
Pseudomonas fragi, Pseudomonas chloropaphis, Pseudomonas fluorescens, Brevibacterium frigoritolerans	Bean	Cold	Decreased MDA, ROS (O_2 and H_2O_2) and POX level whereas SOD, CAT, APX and GR activity increased	Tiryaki et al. (2018)
Arthrobacter woluwensis	Soybean	Salinity	Upregulated expression of salt stress response genes such as *GmLAXs* and *GmST*, Low level of ABA and JA. Significant change in the activities of PPO and POD was also observed	Khan et al. (2019a)
Ochrobactrum pseudogrignonense	Wheat	Salinity	Increased activity of APOX, GR, SOD and germin-like proteins, whereas no significant change in the level of POX and CAT	Chakraborty et al. (2019)
Bacillus pumilus	Wheat	Salinity	Reduced antioxidant enzyme (CAT, SOD, GR) activities, proline and MDA content	Ansari et al. (2019)
Bacillus velezensis	Wheat	Heat/ Cold/ Drought	Modulated various stress related proteins, antioxidant activity, amino acids metabolic pathways and accumulation of γ-aminobutyric acid (GABA)	Abd El-Daim et al. (2019)
Pseudomonas fluorescens, Bacillus amyloliquefaciens	Peppermint	Drought	Increased total phenolic content, antioxidant enzymes (SOD and POX), reduced proline and MDA content	Chiappero et al. (2019)
Bacillus sp.	Guinea grass	Drought	Reduced proline accumulation GR activity and increased APX level	Moreno-Galván et al. (2020)
Cupriavidus necator, Pseudomonas fluorescens	Maize	Drought	Increased nitrogen and phosphorous use efficiency	Pereira et al. (2020)
Azotobacter chroococcum, Azospirillum brasilense	Peppermint	Drought	Augmented ABA, SOD, proteins, soluble sugars, phenolic, flavonoid and oxygenated monoterpenes, but other antioxidant enzymes GPX and CAT activity decreased	Asghari et al. (2020)
Kocuria rhizophila	Maize	Salinity	Increased antioxidant enzyme (APX, GPX and GR), proline and expression of *ZmGR1, ZmAPX* (encoding antioxidants), *ZmNHX1*, *ZmNHX2, ZmNHX3, ZmWRKY58* and *ZmDREB2A* (salt tolerance genes), whereas decreased MDA	Li et al. (2020)
Bacillus cereus	Soybean	Heat	Increased APX, SOD, GSH, proline, expression of *GmLAX3*, *GmAKT2* (genes involved in the regulation of the ABA). Decreased MDA content and expression of *GmHSP* (heat shock protein)	Khan et al. (2020)
Bacillus cereus	Tomato	Heat	Increased proline content, and antioxidant enzymes (SOD, POD and CAT)	Mukhtar et al. (2020)
Bacillus cereus, Serratia marcescens, Pseudomonas aeruginosa	Wheat	Salinity	Decreased antioxidant enzymes (SOD, CAT and POX), non-enzymatic antioxidants (GSH, AsA, and α-TOC)	Desoky et al. (2020)
Bacillus sp.	Rye grass	Drought	Increased proline, antioxidant enzymes (CAT and POD), decreased MDA, relative membrane permeability and H_2O_2 accumulation	He et al. (2021)
Bacillus sonorensis, Bacillus cereus, Bacillus subtilis, Bacillus safensis, Bacillus paramycoides, Bacillus tequilensis, Brevibacillus sp.	Cotton	Salinity	Increased absorption of K^+, while decreased absorption of Na^+ and, maintenance of the proline content, Chlorophyll Content Index (CCI), Relative Water Content (RWC) and Relative Electrolyte Leakage (EL)	Saleem et al. (2021b)
Pseudomonas putida, *Alcaligenes* sp., *Klebsiella* sp., *Pseudomonas cedrina*	Alfalfa	Salinity	Reduced proline, MDA and H_2O_2 level	Tirry et al. (2021)

(Continued)

TABLE 1 Continued

PGPR Strain	Host Plant	Stress	Mechanism of action	References
Bacillus megaterium, Bacillus licheniformis	Wheat	Drought	Increased proline, and antioxidant enzymes (SOD, CAT, APX, POD and GR)	Rashid et al. (2022)
Bacillus subtilis, Bacillus pumilus	Cotton	Salinity	Modulated the ascorbate, aldarate, glyoxylate, dicarboxylate metabolism pathways, and pentose, glucuronate interconversions pathway	Akbar et al. (2022)
Bacillus subtilis, Pseudomonas sp.	Brinjal	Salinity	Increased level of free polyamines (spermine, spermidine, puterscine), expression of *psbD*, GR, GST and Protease I/II whereas lipases level decreased	Mokabel et al. (2022)
Bacillus thuringiensis PM25	Maize	Salinity	Increased antioxidants (APX, POD, SOD, AsA), total soluble sugars, proteins, flavonoids, osmolytes (free amino acids, glycine betaine and proline)	Ali et al. (2022)
Bacillus butanolivorans	Pepper	Drought	Increased expression of *P5CS*, *P5CR* (proline synthesis genes), *Cadhn*, *sHSP* (drought-sensitive gene), *bZIP1* (ABA-related genes), *LOX, COI1* (JA-related genes), POX, glutathione, but decreased CAT and SOD	Kim et al. (2022)

2021; Luo et al., 2022). *Bacillus amyloliquefaciens* (SN13) is a bio-protective agent against *Rhizoctonia solani* (causative agent of sheath blight disease), which enhances defense response in the rice plants. The colonized plants showed alteration in phytohormone content (increased level of SA, ABA and GA), and MAPKinases (increased level of phospholipase D and serine-threonine protein kinases) signaling pathways that helped in controlling disease proliferation. Apart from these, SN13 treatment also modulated the production of secondary metabolites (quinazoline) and ROS regulators (arabitol, proline and mannitol, sugars like β-D-glucopyranose, fructopyranose, and myoinositol, ferric reducatse glutathione *S*-transferase and peroxidase precursor) (Srivastava et al., 2016). Moreover, cotton (*Gossypium hirsutum*) plants treated with blend of *Bacillus* spp. enhanced secretion of gossypol (allelochemical) and JA (defense related phytohormone) which in turn reduced larval feeding of *Spodoptera exigua* (beet armyworm). In addition, treated cotton plants exhibited increased expression of genes involved in synthesis of allelochemicals i.e. *(+)-δ-cadinene synthase* (*CAD1* gene family, including *Cad1- C1, Cad1-A, Cad1-C14 and Cdn-C3*) and jasmonates *(allene oxide synthase*) (*GhAOS), 13-lipoxygenase (GhLOX1)* and *12-oxo-phytodienoicacid reductase 3* (*GhOPR3* [Figure 1A; (Zebelo et al., 2016)]. Another rhizobacterium strain *Bacillus amyloliquefaciens* SQRT3 strongly inhibited tomato bacterial wilt disease (caused by *Ralstonia solanacearum*). The application of SQRT3 increased the expression of POD, PPO, stress marker genes like *proteinase inhibitor 2* (*PIN2*) (related to JA pathway) and *pathogenesis related protein-1a (PR-1a*) (related to SA pathway) and *Omsotin-like* (related to ET pathway) (Chunyu et al., 2017). Likewise, *Bacillus velezensis* enhanced resistance of pepper plants against *Botrytis cinerea* BC1301 (causative agent of gray mold disease) by triggering antioxidants SOD, CAT, POD and SA- mediated defense signaling genes namely *non-expressor of pathogenesis-related genes 1* (*NPR1*), *pathogenesis related protein-1 (PR1)* and *peroxidase*. However, no effect was observed in the expression of *proteinase inhibitor 2 (PIN2)* and (*TIN1)* genes (Jiang et al., 2018). In another study, anti-pathogenic role of *Bacillus amyloliquefaciens* Ba13 against tomato yellow leaf curl virus (TYLCV) was observed. It was found that inoculation of this beneficial microbe improved tomato growth by elevating the expression of systemic resistance related genes including *pathogenesis related protein-1, 2, 3 (PR1*, *PR2* and *PR3*), chitinase, PAL, POD, PPO, and β-1,3-glucanase (Guo et al., 2019). Furthermore, an interesting work underline the effectiveness of *Bacillus amyloliquefaciens* YN201732 against tobacco powdery mildew disease (caused by *Erysiphe cichoracearum*). Results highlight that bacterial treatment inhibited pathogenic fungi growth over tobacco cultivar by increasing the expression of disease-related genes like *non-expressor of pathogenesis-related genes 1 (NPR1), plant defensin 1.2 (PDF1.2), chitinase (chit)* and PPO, whereas, no significant change was observed in POD and PAL activity (Jiao et al., 2020). Also, *Enterobacter asburiae* BQ9 imparted tolerance against tomato yellow leaf curl virus by enhancing the expression of antioxidant enzymes such as POD, CAT, PAL and SOD; defense-related genes i.e. *pathogenesis related protein-1a, 1b* (*PR1a* and *PR1b*) (Li et al., 2016). These findings illustrate that treatment with PGPR may activate biochemical and molecular changes to restrict pathogenic invasions in plants. Additionally, in *Nicotiana tabacum* cv. plants *Peanibacillus lentimorbus* B-30488 inoculation reduced cucumber mosaic virus (CMV) RNA accumulation by ~12 fold (91%). This ISR was linked with an increase in expression of stress related genes *Brassinosteroid signaling kinase 1* (*BR-SK1*), *RNA dependent RNA polymerase 2* (*RdRP2*), *zinc finger – homeodomain (ZF-HD), pathogenesis related protein 1(PR1), β-1,3-glucanase* (*Gluc), asparagine synthetase (AsSyn), tetrahydrocannabinolic acid synthase* (*TCAS*) and antioxidant enzyme (APX, GPX, SOD and CAT) (Kumar et al., 2016). Similarly, *Peanibacillus lentimorbus* B-30488 also provides resistance against southern blight (caused by *Scelerotium rolfsii)* disease in tomato. The treated plants showed alteration of the ET pathway by significantly suppressing *1-aminocyclopropane-1-carboxylate synthase (ACC synthase)* and *oxidase (ACO)* enzymes, and antioxidant enzyme activities (APX, GPX and SOD); whereas systemic tolerance was associated with expression of *pathogenesis – related protein -1, 2A, 4, 7* (*PR1, PR2A, PR4* and *PR7)*, CAT, *chitinase (CHI3* and *CHI9), β-1, 3- glucanase* (*GLU*), calmodulin and PPO (Dixit et al., 2016). Another report elucidates the role of *Pseudomonas aeruginosa* in controlling fungus (*Botrytis cinerea*) infection in *Brassica napus* by inducing the expression of transcription factor (*BnWRKY33),mitogen-activated protein kinase 3, 4 (BnMPK3* and *BnMPK4*) and *pathogenesis related protein-1 and 4 (BnPR1* and *BnPR4*) (Monnier et al., 2018).

Apart from this, some PGPRs also impart resistance against pathogens by secreting anti-microbial compounds (Hamid et al., 2021; Ji et al., 2021). For example, different *Bacillus* strains such as

Bacillus velezensis QST713, *Bacillus velezensis* (C2), *Bacillus velezensis* OEE1, *Bacillus subtilis* release antifungal compounds such as lipopeptides (fengycin, bacillomycin and surfactin), polyketides (bacillaene, macrolactin and difficidin), dipeptide bacilysin, antifungal VOCs (phenylethyl alcohol, benzeneacetic acid, benzaldehyde, 1-decene, tetradecane) and lytic enzymes (chitinase, protease and β-glucanase), 3-indolylacetonitrile and suppresses green mold (caused by *Trichoderma aggressivum* f. *europaeum*), Verticillium wilt disease (caused by *Verticillium dahliae*), Septoria tritici blotch (caused by *Zymoseptoria tritici*), in button mushroom, tomato, olive, wheat (Mejri et al., 2018; Pandin et al., 2018; Dhouib et al., 2019; Azabou et al., 2020). Moreover, a research illustrated that application of *Herbaspirillum seropedicae* (BAC) suppressed *Xanthomonas euvesicatoria* (Xe), a causative agent of bacterial spot disease in 'Micro-Tom' *Solanum lycopersicum* L by downscaling the concentration of various organic acids such as oxalic acid, succinic acid, citric acid that enhances pathogens virulence (da Silva et al., 2021). The compiled studies (Table 2) clearly illustrate that PGPRs help in imparting tolerance against pathogen by activating pathway(s) that leads to production of array of defense related metabolites in plants.

Developing stress resilient crops

To full fill the goal of providing nutritious food for an increasing population, it is critical to develop resilient crops which can withstand the pressure of climate change. Scientists are strategizing, investigating, and discovering key genes which can aid in developing new transgenic crops tolerant to biotic and abiotic stresses without compromising on productivity. Wheat and rice are major staple crops grown across different regions of the world. Hence a lot of efforts are being made to develop better varieties through the classical breeding approach and newer biotechnological tools. The transgenic approach is one of the common techniques for inserting a gene of interest to achieve the desired trait. Among the transgenic approach, one of the studies illustrated that mutated transcription factor (*HaHB4*) from sunflower belonging to homeodomain-leucine zipper family (*HD-Zip*) improved water use efficiency and productivity of wheat (González et al., 2019). In another study introduction of beta gene-encoding choline dehydrogenase enhanced the glycine betaine content making the transformed wheat tolerant to drought (He et al., 2011). Similarly, in rice introduction of transcription factor *PeSNAC-1* lead to increased production of proline, thereby making the plant tolerant to salinity and drought (Hou et al., 2020). Also, insertion of gene *MYB49* lead to increased POD, SOD activity, chlorophyll content offering better resistance to drought, salinity and pathogen *Phytophthora infestans* in tomato (Cui et al., 2018). The transcription factor, *SlDREB3* increased membrane stability and prevented ROS from imparting tolerance to chilling (Wang et al., 2019). The above studies clearly suggest that modifications at the genetic level either by inserting specific genes; altering transcription factor families like *WRKY, DREB, MYB, NAC* and *ERF* and modifying signal transduction genes significantly enhances the tolerance of plants to various stresses (Khan et al., 2019d). Similar physiological changes can be introduced in target plants by applying appropriate PGPRs (Tables 1, 2 and Figure 2). In wheat, over expression of *TaWRKY2* (drought stress tolerance gene) improved drought tolerance by withholding water for 8-10 days before re-watering and enhancing proline content compared to wild type plant (Gao et al., 2018). Similarly, application of *Azospirillum lipoferum* significantly augmented the proline content in wheat seedling resulting in higher drought tolerance by withholding water for 10 days before watering (Kanwal et al., 2017). Engineering of *E. coli cold shock protein* (*CspA* and *CspB)* genes to convert it into plant-preferred codon namely *SeCspA* and *SeCspB* resulted in better stress tolerance potential by lowering MDA content, preventing water loss, reduced Na^+ level and higher levels of chlorophyll, proline content under drought and salt stresses (200 mM NaCl) compared to the control wheat plants (Yu et al., 2017), similar response was evoked by endophytic strain *Bacillus subtilis* in wheat wherein lower MDA level was observed in plants grown under (340 mM NaCl) compared to control counter parts (Lastochkina et al., 2017). Cloning of *Arabidopsis WRKY30 (AtWRKY30)* transcription factor followed by its over-expression in wheat seedling subjected to drought stress by with-holding water for 12 days exhibited enhanced activities of antioxidant enzymes CAT, SOD, POX and APX (El-Esawi et al., 2019). Similar observation was made by (Akhtar et al., 2021) wherein antioxidant enzyme namely SOD, CAT and POX increased upon treating wheat with *Bacillus* sp., *Azospirillum lipoferum* and *Azospirillum brasilense* and subjected to drought stress by withholding water to 40% field capacity. Overexpression of *TaFER-5B* improved multiple stress including heat stress tolerance in wheat induced by keeping 10 days old seedling under 40°C, the protective mechanism was attributed to ROS scavenging activity (Zang et al., 2017). Similarly, modulation in expression of heat shock proteins (HSPs) was noticed in wheat seeds primed with *Bacillus safensis*. The treated plants tolerated heat shock (40°C) without generation of excessive ROS (Sarkar et al., 2021). Introduction of *HVA1* (ABA-responsive barley gene) in wheat improved water use efficiency subjected to drought stress in wheat (Sivamani et al., 2000). Similarly, application of bacteria based bioformulation improved relative water content and range of antioxidants of wheat seedlings experiencing drought stress (40% the field capacity) (Akhtar et al., 2021). Several scientific studies conclusively indicate that applications of PGPRs could lead to development of resilient plants tolerant to sudden fluctuations in the weather, rise in temperature, salinity, drought, disease and insect attack. The association between plant and rhizospheric soil microbes is a functionally dynamic association and changes in the environment are perceived even at miniscule level triggering a cascade of appropriate stress-related responses making the plant tolerant to perceived challenges. In spite of beneficial attribute of transgenic crops, their acceptability for human consumption is still an issue of concern. Hence, knowledge-based application of the right PGPR could be a sustainable and ecofriendly approach to develop stress resilient crops.

Conclusion

A sustainable agriculture system strives to protect the environment, without compromising crop yield to provide sufficient

TABLE 2 PGPRs along with their mode of action in combating biotic stress.

PGPR Strain	Host Plant	Pathogen	Disease	Mechanism of action	Benefits	References
Bacillus amyloliquefaciens (SN13)	Rice	*Rhizoctonia solani*	Sheath blight	Target phytohormones (SA, ABA, GA), MAPK, ROS signaling. Increased secondary metabolite production	Reduces fungal dry mass, number, size, length and diameter of spot lesions	Srivastava et al. (2016)
Bacillus sp.	Cotton	*Spodoptera exigua*	–	Increased gossypol and JA production	Significant reduction in larvae mortality rate	Zebelo et al. (2016)
Enterobacter asburiae BQ9	Tomato	*Tomato yellow leaf curl virus*	–	Enhanced expression of *PR1a, PR1b*, POD, CAT, POL and SOD	Milder disease symptoms (stunting, yellowing, curling of leaves)	Li et al. (2016)
Peanibacillus lentimorbus B-30488	Tobacco cv.	*Cucumber mosaic virus*	–	Targets PR genes and antioxidant enzymes	Reduces CMV RNA accumulation with 20-75% viral elimination rate	Kumar et al. (2016)
Peanibacillus lentimorbus B-30488	Tomato	*Scelerotium rolfsii*	Southern blight	Increased expression of PR genes but down-regulation of antioxidant enzymes and ethylene signaling	Reduces fungal dry biomass, mycelia growth	Dixit et al. (2016)
Bacillus amyloliquefaciens SQRT3	Tomato	*Ralstonia solanacearum*	Bacterial wilt	Modulates JA/ET/SA hormonal signaling and POD, PPO expression	Suppresses disease incidence with 84.1% biocontrol efficacy	Chunyu et al. (2017)
Bacillus subtilis	Wheat	*Zymoseptoria tritici*	Septoria tritici blotch	Produces lipopeptides such as mycosubtilin, surfactin, fengycin	Reduces mycelia growth	Mejri et al. (2018)
Bacillus velezensis	Pepper	*Botrytis cinerea*	Gray mold	Stimulates SA signaling genes and antioxidants (SOD, CAT and POD)	Suppresses sporulation, mycelia growth	Jiang et al. (2018)
Pseudomonas aeruginosa	Rapeseed	*Botrytis cinerea*	Gray mold	Activates MAPK and PR genes	Reduces pathogenic lesions size, mycelium development	Monnier et al. (2018)
Bacillus velezensis QST713	Button mushroom	*Trichoderma aggressivum* f. *europaeum*	Green mold	Produces antifungal secondary metabolites (macrolactin, bacillaene, bacillomycin D, fengycin, surfactin,bacilysin, subtilin- like/ericin, difficidin and bacillibactin (siderophore)	Inhibits fungus sporulation, mycelium growth	Pandin et al. (2018)
Bacillus amyloliquefaciens Ba13	Tomato	*Tomato yellow leaf curl virus*	–	Induces expression of resistance related genes and defense enzymes	Milder disease symptoms (stunting, yellowing, curling of leaves) with decrease in virus load in leaves	Guo et al. (2019)
Bacillus velezensis OEE1	Olive	*Verticillium dahliae*	Verticillium wilt	Produces antifungal secondary metabolites/ lipopeptides (surfactin A, iturin C and D, bacillomycin C, fengycin A, B and D, plipastatin, macrolactin, bacillaene, difficidin and bacilysin)	Inhibits conidia, microsclerotia germination (92% inhibition)	Azabou et al. (2020)
Bacillus velezensis (C2)	Tomato	*Verticillium dahliae*	Verticillium wilt	Production of metabolites, lipopeptides and lytic enzymes	Significant reduction in disease incidence (70.43 ± 7.08%)	Dhouib et al. (2019)
Bacillus amyloliquefaciens YN201732	Tobacco	*Erysiphe cichoracearum*	Tobacco powdery mildew	Promotes the expression of PPO and chitinases, also triggers JA/ET signaling	Inhibits conidia germination (86.11%)	Jiao et al. (2020)
Herbaspirillum seropedicae	Micro-Tom Tomato	*Xanthomonas euvesicatoria*	Bacterial spot	Reduces the concentration of organic acids	50% reduction in disease severity	da Silva et al. (2021)

food for the growing population. In order to provide adequate food for all, approaches like conventional breeding and genetic engineering have been extensively used for crop improvement. But these processes are costly, tedious, labor intensive and raise safety issues. Further, to improve crop productivity, heavy doses of chemical fertilizers, pesticides, and fungicides have been rampantly used which lead to large scale deterioration of the environment and soil health ultimately impacting human health. Hence, ecofriendly sustainable means are being explored to increase plant productivity and in this respect application of microbial based biostimulants in agriculture is one of the promising means. PGPR modulates physiological responses in crops and equips them to survive under abiotic and biotic stresses. The majority of these responses are common to both biotic and abiotic challenges implying that the impact of PGPR in plant systems

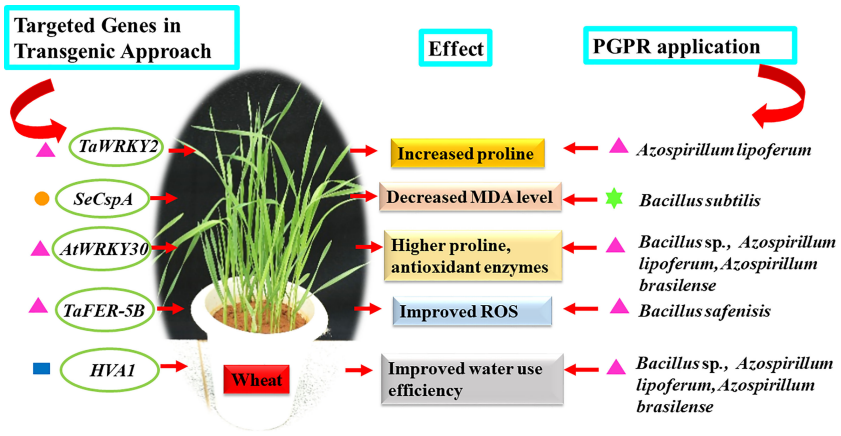


FIGURE 2
Model representing common physiological responses observed in wheat crop *via* transgenic approach and PGPRs application. Different colored symbols in the figure indicates the conditions of the experiment (Pots, Filter Paper, Hydroponics, Greenhouse potting mix).

is broad-based, interaction is multifarious and beneficial in more than one way. Further studies are necessary to unravel the underlying mechanism of plant-microbe interactions and to understand the signal transduction network in an integrated perspective. Holistic information about plant-PGPR functionality will pave the way for developing novel microbial based biostimulants for boosting crop yield for future generations in a sustainable manner.

Author contributions

PK designed, drafted, revised, finalized the original manuscript and prepared the figures; NA drafted and revised the manuscript. Structuring, reviewing, and editing were done by SS. Conceptualization and input in hormonal interaction component was done by PP. Conceptualization, reviewing and editing was done by AP. All authors contributed to the article and approved the submitted version.

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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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Micropropagation and elicited production of triterpenoid saponin glycosides and stigmasterol *via* precursor and elicitor feeding in *Bacopa floribunda* (R.Br.) Wettst.—A potential nootropic herb

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Bacopa floribunda (Family: Plantaginaceae) is used in folklore medicines for the management of cognitive dysfunction. It has nootropic, antiaging, anti-inflammatory, anti-cholinesterase, and antioxidant properties. We developed an efficient and reproducible protocol for *in vitro* propagation of *B. floribunda* using the nodal explants. We assessed the effects of Murashige and Skoog (MS) medium fortified with various plant growth regulatory substances (PGRs), a precursor, and elicitors and their optimal combinations on regeneration and production of total saponins, triterpenoid saponin glycosides (bacoside A3, bacopaside X, bacopaside II, and bacosaponin C), and stigmasterol content in *in vitro* grown biomass of *B. floribunda*. The growth of the shoots and roots was stimulated by MS + 2.0 mg/l BAP + 2.0 mg/l KIN and MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA, respectively. After 10 weeks of acclimatization, plantlets of *B. floribunda* had a survival rate of 95%. The highest total saponin content (35.95 ± 0.022 mg DE/g DW) was noted in the treatment of MS + 2.0 mg/l BAP + 1.5 µM SQ. Similarly, total triterpenoid saponin glycosides and stigmasterol were found maximum in biomass derived from MS + 2.0 mg/l BAP + 1.5 µM SQ and MS + 2.0 mg/l BAP, respectively. At the same treatments, bacoside A3 (1.01 ± 0.195 mg/g DW), bacopaside II (43.62 ± 0.657 mg/g DW), bacopaside X (1.23 ± 0.570 mg/g DW), bacosaponin C (0.19 ± 0.195 mg/g DW), and stigmasterol (7.69 ± 0.102 mg/g DW) were reported. The present findings will help to highlight *B. floribunda* as a potent memory-enhancing herb, and in future also, it could be a potential substitute to *B. monnieri*. The current work is the first to describe the micropropagation and the elicited production of bioactive metabolites from the *in vitro* grown biomass of *B. floribunda*. In addition, further research is needed on production of bioactives, their pharmacological effects, and the elicited production using callus, cell suspension, and hairy root cultures.

KEYWORDS

plant growth regulators (PGRs), *Bacopa*, triterpenoid saponin glycosides, HPLC, nootropic, elicitation, methyl jasmonate (MeJA)

Introduction

Bacopa floribunda (R.Br.) Wettest. (Family: Plantaginaceae) is indigenous to Madagascar, Africa, and Asia. In Ayurveda, it has been utilized as a brain tonic and antiaging and also to treat psychological disorders. It is an excellent source of natural medicine due to its cholinergic enzyme-inhibiting, anti-inflammatory, and antioxidant properties. Because of its potent nootropic effects, which decrease acetylcholinesterase (AChE) inhibition, it is used in traditional remedies to improve memory (Olatunji et al., 2019; Adetuyi et al., 2022). Triterpenoid saponin glycosides are said to be the active chemical components in the genus *Bacopa* and categorized as pseudojujubogenin (bacoside II and bacosaponin C) and jujubogenin glycosides (bacoside A3 and bacopaside X) and to exhibit memory-improving effects (Mathur et al., 2016; Nuengchamnong et al., 2016; Watcharatanon et al., 2019; Bhandari et al., 2020). Triterpenoid saponin glycosides from *B. monnieri* (Brahmi) are the well-known marker compounds used to improve nerve impulse transmission. Triterpenoid saponin glycosides help injured neurons' recovery by promoting kinase activity, neuronal synthesis, and the restoration of synaptic function. It ultimately results in restoration of nerve impulse transmission and an increase in *de novo* synthesis of proteins in the brain. Nerve impulse transmission plays a crucial role in promoting healthy cognitive functions such as attention span, focus, concentration, learning, and memory (Mathur et al., 2016; Singh and Dhawan 1997).

Due to its usage in the production of pharmaceuticals and herbal extracts, *B. monnieri* (Brahmi) is in very high demand in India, which promotes massive collection from their natural habitats and the extinction of the species. The greatest obstacle to the use of Brahmi in several industrial applications is the high variability in the levels of bioactive principles (Christopher et al., 2017). Since ancient times, *B. monnieri* (Brahmi) has been used as a brain tonic or memory booster. *Bacopa* formulations have been employed for therapeutic purposes by numerous academics and pharmaceutical firms worldwide, including those in India, New Zealand, the United States, and Australia. It has been estimated that approximately 10,000 t of fresh plant material was harvested from natural habitats each year that caused a significant decline in wild populations. Due to the presence of bioactive compounds, Brahmi has unique medicinal utility. Low yields, geographical and environmental variability, and other factors are linked to the synthesis and accumulation of secondary metabolites in the field-grown plants of Brahmi (Christopher et al., 2017; Watcharatanon et al., 2019; Asadollahei et al., 2022). Considering the problems associated with the growth and importance of the species, it is necessary to find out alternative sources and their mass multiplication, conservation, and production of bioactive metabolites. *In vitro* propagation makes it possible for cells, tissues, and organs to grow aseptically without the presence of microbes, which is crucial for the development of therapeutic plants (Ali et al., 2021; Bhattacharyya et al., 2022). The demand of providing high-quality-stock plants for conservation and pharmaceutical needs through micropropagation has remarkably increased (Komakech et al., 2020). *In vitro* produced cells, tissues, and organ cultures offered a good source of homogenous, sterile, and suitable materials for the conservation of plant species, their biochemical characterization, and the identification of bioactive constituents (Patil et al., 2013). *In vitro* propagation techniques have been used earlier to conserve several commercially important, ornamental, medicinal, and RET species (Nikam et al., 2009; Gupta et al., 2016; Chandra et al., 2020).

Recent studies have shown numerous methods for large-scale production; the synthesis of secondary metabolites includes strain improvement, optimization, elicitations, medium and culture environment manipulation, and nutrient and precursor feeding (Murthy et al., 2014). Precursors and elicitors can trigger morphological and physiological alterations that result in the accumulation of phytoalexins. Elicitation with abiotic and biotic treatments can also boost the production of bioactive metabolites (Watcharatanon et al., 2019). Elicitors have the ability to activate a cell immune system and produce secondary metabolites as a protection mechanism (Asadollahei et al., 2022). It is believed that adding precursors into the plant biosynthesis pathway will facilitate the production of secondary metabolites (Watcharatanon et al., 2019). The use of elicitors like methyl jasmonate (MJ), salicylic acid (SA), and precursors like squalene (SQ) for production of secondary metabolites in *in vitro* culture systems has been reported earlier (Largia et al., 2015; Baek et al., 2019;). Several reports are available on micropropagation and enhanced metabolite production in *B. monnieri* (Banerjee and Shrivastava, 2008; Parale and Nikam, 2009; Parale et al., 2010; Largia et al., 2015; Sharma et al., 2015; Muzynska et al., 2016; Watcharatanon et al., 2019).

Although *B. floribunda* is a versatile medicinal herb, there are no reports available on its *in vitro* propagation and elicited production of bioactive compounds. Taking all of this into consideration, the objectives of the current study were to develop a micropropagation protocol and production of secondary metabolites. To achieve these goals, MS media supplemented with different plant growth regulatory substances (PGRs), precursors, and elicitors and their optimal concentrations were studied for the production of total saponins, four triterpenoid saponin glycosides, and stigmasterol.

Materials and methods

Standards and reagents

Standard triterpenoid saponin glycosides (bacoside A3, bacoside II, bacopaside X, and bacosaponin C) and stigmasterol of HPLC grade were purchased from Sigma-Aldrich and TCI Chemicals. Methanol, water, and acetonitrile (ACN) used in the analysis were of HPLC grade. All the marker compounds used in the study had a purity of above 95%. All the chemicals, nutrient media, gelling agents, PGRs, precursors, and elicitors were procured from Sigma-Aldrich (USA), TCI Chemicals (Tokyo, Japan), Sisco Research Laboratory (SRL), and HiMedia (Mumbai, India).

Collection of plant materials and establishment of cultures

Plants of *Bacopa floribunda* (R.Br.) Wettest. were collected from the rice fields of Rajapur, Maharashtra, India, during March 2021.

Abbreviations: AChE, acetylcholinesterase; IAA, indole acetic acid; IBA, indole,3-butyric acid; BAP, 6-benzylaminopurine; DW, dry weight; FW, fresh weight; HPLC, high performance liquid chromatography; KIN, kinetin; KCl, potassium chloride; MJ, methyl jasmonate; $MgSO_4$, magnesium sulfate; $HgCl_2$, mercuric chloride; MS, Murashige and Skoog; NAA, naphthalene acetic acid; PCA, principle component analysis; PGRs, plant growth regulatory substances; SA, salicylic acid; SQ, squalene.

The sample specimen was deposited in an herbarium (SUK) of the Department of Botany, Shivaji University, Kolhapur, Maharashtra (Voucher no. SSO 032). The plants were brought to the laboratory, carefully washed under running water, then treated with 0.1% (v/v) Tween-20 for 5 min and rinsed five times with sterile distilled water. These explants (nodal segments) were further surface sterilized by first rinsing them with aqueous $HgCl_2$ (0.1% w/v) for 4 min and then washed five times with sterile distilled water to remove the traces of $HgCl_2$. Initially, cultures were established on MS medium (1962) devoid of any PGRs (Murashige and Skoog, 1962). In further investigations, different PGRs including auxins (IAA, IBA, and NAA), cytokinins (BAP, and KIN), a precursor (squalene, SQ), and elicitors [methyl jasmonate (MJ), salicylic acid (SA), potassium chloride (KCl), and magnesium sulfate ($MgSO_4$)] were added to MS medium. All the surface-sterilized nodal explants (1.0 cm) were excised and then transferred to a semisolid nutrient medium under aseptic conditions. All nutrient media used in the study contained vitamins and also supplemented with 3% sucrose, with a pH level maintained at 5.8 ± 0.5, solidified with 8 g/l agar, and autoclaved at 121°C for 15 min. All cultures in this experiment were maintained under controlled conditions (25 ± 2°C temperature, 80 ± 10% relative humidity, 8-h photoperiod, and 30–40 µmol m^{-2} S^{-1} light intensity).

Effects of different PGRs on shoot and root regeneration

Initially, cultures were established on MS medium devoid of any PGRs. Further, healthy nodal explants (1.0 cm) were carefully excised and inoculated on MS medium (50 ml) fortified with different concentrations (0.5, 1.0, 1.5, and 2.0 mg/l) of cytokinins (BAP, KIN). Likewise, varied levels of auxins (IAA, IBA, and NAA; 0.5, 1.0, 1.5, and 2.0 mg/l) were used.

Rooting of shoots and acclimatization of plantlets

MS medium enriched with the optimal concentrations of auxins was used, and rooting of shoots was studied after 3 weeks of culture. Shoots with multiple roots from the abovesaid medium were acclimatized under field conditions. Well-grown rooted shoots were carefully removed from the culture bottles, and surplus culture media were removed carefully. They were also delicately moved into the plastic pots containing soil and coco pit mixture (1:1). To maintain humidity (80–90%), all the plantlets were covered with transparent polythene bags. These pots were kept inside a greenhouse for 2 weeks. Controlled conditions were maintained as 50 µmol m^{-2} S^{-1} light intensity, 25 ± 5°C temperature, and 80 ± 10% relative humidity. Initially, half-strength MS was given to the plantlets for a period of 2 weeks and then watered regularly. After 3 weeks, polythene bags were removed and plantlets were exposed to natural conditions. After 10 weeks, the survival rate was calculated and expressed as percentage.

Effects of PGRs, precursors, and elicitors on bioactive metabolites (total triterpenoid saponin glycosides and stigmasterol)

In the study, different elicitors, *viz*., methyl jasmonate (MJ; 30, 60, 80, 100 µM), salicylic acid (SA; 30, 60, 80, 100 µM), potassium chloride (KCl; 30, 60, 80, 100 mM), and magnesium sulfate ($MgSO_4$; 30, 60, 80, 100 mM) were added separately to the nutrient medium. Likewise, squalene alone (SQ; 0.5, 1.0, 1.5, 2.0 µM) was used as a precursor. Further, the influence of optimal concentrations of auxins and cytokinins in combination with the optimal concentration of the precursor was used and the contents of triterpenoid saponin glycosides and stigmasterol were studied. Details of the treatments used were as follows: MS + 2.0 mg/l BAP + 2.0 mg/l KIN (OCC*), MS + 0.5 mg/IAA + 0.5 mg/IBA + 1.0 mg/l NAA (OCA*), MS + 2.0 mg/ BAP + 2.0 mg/l KIN + 1.5 µM SQ (OCC*P°), MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA + 1.5 µM SQ (OCA*P°), MS + 2.0 BAP mg/ l + 1.5 µM SQ (OCC*P1°), MS + 2.0 mg/l KIN + 1.5 µM SQ (OCC*P2°),MS + 1.0 mg/l NAA + 2.0 mg/l KIN + 1.5 µM SQ (OCAC*P°), and MS + 1.0 mg/l NAA + 2.0 mg/l KIN (OCAC*).

In the present study, nodal explants (four explants per culture bottle and 10 bottles for each treatment) initially grown on plane MS medium were used. All cultures in this experiment were maintained as per the abovementioned conditions. After a culture period of 21 days, several parameters such as average shoot length, number of leaves per shoot, fresh and dry weights, number of roots, average root length, and percent regeneration were determined.

Analysis of total saponins, triterpenoid saponin glycosides, and stigmasterol contents

Preparation of samples and standard solutions

An ultrasonicator (Rivotek, India) was used to extract the saponins and stigmasterol. *In vitro* grown and hardened plant biomass (0.5 g) were mixed with 10 ml of methanol and subjected to ultrasonic assisted extraction for a period of 10 min. The homogenate was then centrifuged at 10,000 rpm (Emtek Instruments, India). Further, the supernatant was collected and condensed and the volume adjusted to 1 ml using the same solvent. Standard compounds including diosgenin, triterpenoid saponin glycosides, and stigmasterol were carefully weighed (1 mg) and dissolved separately in HPLC-grade methanol (1 ml) to obtain a standard stock solution (mg/ml) (Careri et al., 2001; Bhandari et al., 2006). The preparation of working and stock solutions included dilution of the stock solution with the appropriate solvent to provide six distinct concentrations. All of the extracts and working and stock solutions were stored at 4°C until further use. Prior to HPLC analysis, all the standards and extracts were filtered using 0.22-µm nylon filters (HiMedia, India). A standard curve with different concentrations (20–100 µg/ml) was prepared, and results were expressed as mg/g DW.

Estimation of total saponin content

Total saponin content (TSC) was evaluated by using the protocol of Uysal et al. (2017) with slight modifications. A plant extract (200 µl) in mg/ml was mixed with 200 µl of 8% vanillin, and 1,000 µl of 72% H_2SO_4 acid was added to it; the reaction mixture was further incubated for 10 min in a water bath at 60°C (Equitron, India). After complete incubation, reaction mixtures were cooled for 15 min and absorbance was recorded at 544 nm using a UV-vis spectrophotometer (Jasco V-730, Japan). Diosgenin was used as a standard, and results were represented as mg diosgenin equivalent (DE)/g DW.

Estimation of triterpenoid saponin glycosides using HPLC

The Jasco LC-2000 Plus chromatographic system, which has a binary pump, an autosampler, and a UV detector (UV-2070), was used for the detection of triterpenoid saponin glycosides. Utilizing a Hiber C18 column (5 µM, 250 X 4.6 mm), separation of bacoside A3, bacoside II, bacopaside X, and bacosaponin C was carried out. Built-in ChromNAV software was utilized for data processing. A flow rate of 0.8 ml/min, an injection volume of 20 µl, and a mobile phase consisting of water:acetonitrile (70:30 v/v) was used (Bhandari et al., 2006). The peaks were observed at 205 nm with a run time of 20 min. The amount of respective saponins was calculated by comparing the chromatogram with standard and expressed as milligrams per gram of dry weight (mg/g DW).

HPLC analysis of stigmasterol

Detection of stigmasterol was carried out using the same instrumentation system as specified above. An isocratic mobile phase consisting acetonitrile:water (70:30 v/v) was used with a 1-ml/min flow rate, a 20-µl injection volume, and a 20-min run period. The peaks from standard and samples were monitored at 210 nm (Careri et al., 2001). By comparing their retention time with those of the standard, stigmasterol from the extracts was identified. The amount of stigmasterol was calculated and expressed as milligrams per gram of dry weight (mg/g DW).

By spiking with known concentrations of the respective standards, triterpenoid saponin glycosides and stigmasterol were verified. Samples and standards were analyzed in triplicates to improve the quality of the results.

Statistical analysis

All the analyses were performed in triplicate, and values were represented as mean ± standard error. The data were subjected to one-way analysis of variance (ANOVA), and significant differences between mean values were determined by Duncan's multiple-range test ($P < 0.05$) using SPSS software ver. 16. Principal component analysis (PCA) was used to analyze the data produced from the investigated morphological responses and bioactive compounds from various concentrations of PGRs, precursors, and elicitors using Minitab software (trial ver. 19).

Results

In vitro micropropagation of *B. floribunda*

In the study, the influence of different cytokinins (BAP and KIN) on various parameters was tested and results are depicted in Table 1 and Figure 1. A reliable protocol for direct *in vitro* propagation using fresh nodal segments of *B. floribunda* was standardized (Figure 1A). After a period of 21 days, cytokinins (BAP, KIN) at all concentrations (0.5, 1.0, 1.5, and 2.0 mg/l) denoted the improved shoot growth. Nodal explants of *B. floribunda* cultured on the higher concentrations of BAP and KIN (2.0 mg/l) revealed 100% regeneration. Among all the different PGRs tested, the highest number of leaves per shoot (15.60 ± 0.40) with a maximum shoot length (6.4 ± 0.40 cm) was obtained in MS medium supplemented with 2.0 mg/l KIN (Table 1, Figure 1B). However, the shoot growth depended on the type and concentration of cytokinin. The statistical analysis revealed that among all the cytokinin concentrations, MS + 2.0 mg/l KIN resulted in a considerably higher shoot length (6.4 ± 0.40 cm). MS + 2.0 mg/l BAP also demonstrated the second highest enhancement in shoot length (5.4 ± 0.40 cm). However, nodal explants treated with MS + 1.0 mg/l BAP had the shortest shoot length (3.95 ± 0.22 cm). Similarly, MS + 2.0 mg/l KIN revealed the highest number of leaves per shoot (15.60 ± 0.40), followed by MS + 2.0 mg/l BAP with 15.2 ± 0.49 leaves per shoot. In contrast, MS + 1.0 mg/l BAP exhibited the lowest number of leaves per shoot (9.0 ± 0.58). The maximum fresh (2.93 ± 0.5 g) and dry (0.150 ± 0.02 g) weights were found in the cultures grown on MS + 2.0 mg/l BAP (Table 1). The highest concentrations of both the cytokinins revealed superior responses in terms of the axillary shoot initiation, number of leaves, and fresh and dry weights in the cultures of *B. floribunda*. These cultures were further maintained on the medium supplemented with cytokinins for a period of 2 months by repeated subculturing, and multiple shoots with the roots were observed (Figure 1C). These well-developed shoots maintained on the same medium resulted into multiple-shoot formation (Figure 1D). Shoots grown on basal MS medium were further subjected to different concentrations of IAA, IBA, and NAA (0.5–2.0 mg/l), and results are presented in Table 2 and Figure 1E. The tested concentrations of auxins promoted the root growth after 21 days of incubation. The root initiation and growth depend upon the auxin type and its concentration. Results showed that among the auxins used at different concentrations, MS + 1.0 mg/l NAA produced significantly higher root numbers (32 ± 1.35) and also showed the highest average root length (2.6 ± 0.16 cm). In contrast, levels of NAA (0.5 and 2.0 mg/l) showed the lowest number of roots per shoot (4.0 ± 0.10) with an average root length of 1.9 ± 0.11 cm. The roots that were initiated using MS + 1.0 mg/l NAA were more robust and tightly connected to the base of the well-grown shoots. Levels of other auxins (0.5 and 1.5 mg/l IAA, 0.5 and 2.0 mg/l IBA, and 1.0 and 1.5 mg/l NAA) failed to affect the root length in a significant way. Incorporation of auxins in the nutrient medium significantly affected the fresh weight (FW) that ranged between 1.3 and 2.75 g wherein MS + 1.0 mg/l NAA indicated the maximum fresh weight. Similarly, maximum dry weight (DW) was reported when the MS medium was enriched with 0.5 mg/l IAA (0.130 ± 0.02 g), followed by 1.0 mg/l NAA and 0.5 mg/l IBA. Our results

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TABLE 1 Effect of cytokinins (BAP and KIN) on regeneration of *Bacopa floribunda*.

Cytokinins (mg/l)		Average shoot length (cm)	No. of leaves per shoot	No. of roots per shoot	Average root length (cm)	FW (g)	DW (g)	Response (%)
BAP	0.5	5.13 ± 0.97^{abc}	11.33 ± 0.67^{bc}	15.67 ± 4.70^{a}	3.23 ± 0.33^{a}	1.74 ± 0.04^{ab}	0.275 ± 0.07^{a}	90^{c}
	1.0	3.95 ± 0.22^{c}	9.00 ± 0.58^{c}	10.00 ± 0.58^{b}	2.90 ± 0.20^{a}	0.75 ± 0.03^{b}	0.108 ± 0.01^{b}	95^{b}
	1.5	4.20 ± 0.20^{c}	10.40 ± 0.40^{bc}	6.40 ± 0.40^{c}	3.20 ± 0.20^{a}	2.44 ± 0.43^{a}	0.114 ± 0.04^{b}	100^{b}
	2.0	5.40 ± 0.40^{ab}	15.20 ± 0.49^{a}	9.20 ± 0.49^{bc}	3.40 ± 0.24^{a}	2.93 ± 0.50^{a}	0.150 ± 0.02^{b}	100^{a}
KIN	0.5	5.12 ± 0.71^{abc}	12.00 ± 1.15^{b}	13.20 ± 0.75^{ab}	3.40 ± 0.28^{a}	1.52 ± 0.40^{ab}	0.109 ± 0.01^{b}	95^{b}
	1.0	5.38 ± 0.21^{abc}	12.40 ± 0.81^{ab}	13.40 ± 0.87^{ab}	3.50 ± 0.26^{a}	1.35 ± 0.30^{ab}	0.114 ± 0.01^{b}	100^{a}
	1.5	4.60 ± 0.24^{abc}	12.80 ± 0.49^{ab}	13.20 ± 0.49^{ab}	3.40 ± 0.24^{a}	1.90 ± 0.10^{ab}	0.140 ± 0.02^{b}	100^{a}
	2.0	6.40 ± 0.40^{a}	15.60 ± 0.40^{a}	14.40 ± 1.17^{a}	3.60 ± 0.24^{a}	2.20 ± 0.32^{ab}	0.133 ± 0.01^{b}	100^{a}

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

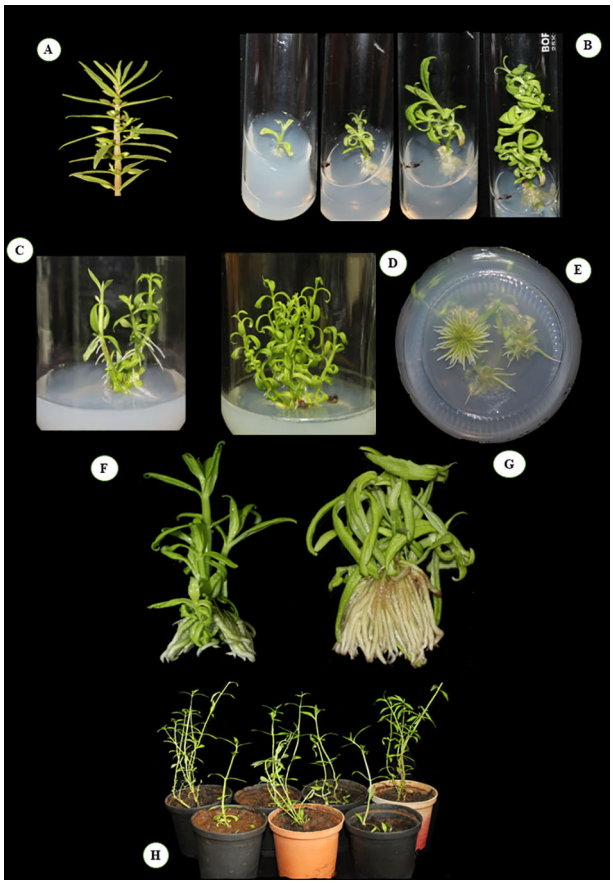


FIGURE 1
Different stages of micropropagation in *Bacopa floribunda*. **(A)** Mother plant; **(B)** various stages of caulogenesis. **(C, D)** Development of multiple shoots on cytokinin containing media. **(E)** Rooting of shoots. **(F)** Initiations of roots in optimum combination auxins. **(G)** Formation of multiple roots using optimum auxins. **(H)** Hardened plants (10 weeks old).

TABLE 2 Effect of auxins (IAA, IBA, and NAA) on regeneration of *Bacopa floribunda*.

Auxins (mg/l)		Average shoot length (cm)	No. of leaves per shoot	No. of roots per shoot	Average root length (cm)	FW (g)	DW (g)	Response (%)
IAA	0.5	5.1 ± 071^{bc}	10 ± 0.63^{ab}	19.4 ± 2.25^{a}	2.6 ± 0.15^{a}	2.28 ± 0.1^{ab}	0.130 ± 0.002^{a}	100^{a}
	1.0	4.3 ± 0.17^{bc}	8.4 ± 0.40^{ab}	18.4 ± 0.87^{bc}	2.2 ± 0.07 ^{ab}	1.46 ± 0.30^{ab}	0.114 ± 0.005^{a}	100^{a}
	1.5	3.0 ± 0.10^{c}	8.4 ± 0.40^{ab}	11.8 ± 0.4^{d}	2.6 ± 0.20^{a}	1.80 ± 0.17^{ab}	0.121 ± 0.006^{a}	100^{a}
	2.0	3.0 ± 0.10^{c}	8.5 ± 0.5^{ab}	11.75 ± 0.5^{d}	2.25 ± 0.3 ^{ab}	1.71 ± 0.2^{ab}	0.126 ± 0.005^{a}	100^{a}
IBA	0.5	6.9 ± 0.71^{a}	12.8 ± 0.49^{a}	18.6 ± 1.25^{bc}	2.6 ± 0.18^{a}	1.80 ± 0.2^{ab}	0.122 ± 0.007^{a}	100^{a}
	1.0	6.8 ± 0.53^{a}	10.4 ± 0.75^{ab}	13.4 ± 0.75^{b}	2.1 ± 0.11^{ab}	1.70 ± 0.11^{ab}	0.116 ± 0.001^{a}	100^{a}
	1.5	3.0 ± 0.10^{c}	12.6 ± 0.40^{a}	16.6 ± 0.67^{c}	2.4 ± 0.058 ^{ab}	1.30 ± 0.28^{b}	0.118 ± 0.001^{a}	90^{b}
	2.0	3.0 ± 0.10^{c}	12.4 ± 0.49^{a}	13.2 ± 0.49^{d}	2.6 ± 0.11 ^{a}	1.76 ± 0.02^{ab}	0.118 ± 0.001^{a}	100^{a}
NAA	0.5	5.5 ± 0.76^{b}	11.0 ± 0.52^{a}	20.0 ± 2.03^{b}	1.9 ± 0.11 ^{ab}	1.42 ± 024^{ab}	0.117 ± 0.001^{a}	90^{b}
	1.0	6.8 ± 0.72^{a}	9.5 ± 2.50^{ab}	32.0 ± 1.35^{a}	2.6 ± 0.16 ^{a}	2.75 ± 0.45^{a}	0.124 ± 0.002^{a}	100^{a}
	1.5	3.0 ± 0.10^{c}	7.2 ± 0.49^{b}	4.0 ± 0.10^{c}	2.6 ± 0.24 ^{a}	2.04 ± 0.20^{ab}	0.113 ± 0.001^{a}	100^{a}
	2.0	3.0 ± 0.10^{c}	7.6 ± 0.89^{b}	4.0 ± 0.10^{c}	2.2 ± 0.45^{ab}	1.62 ± 0.20^{ab}	0.107 ± 0.001^{a}	100^{a}

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

highlighted that MS fortified with 1.0 mg/l NAA showed the best initiation and growth of roots (Table 2, Figure 1E). In the case of cytokinins, response of KIN was comparatively higher than that of BAP. Similarly, among all the auxins used, NAA was found to be the most responsive followed by IAA and IBA (Table 2).

Regeneration responses of *B. floribunda* using precursors and elicitors

The precursor (squalene) and elicitors (methyl jasmonate, salicylic acid, potassium chloride, magnesium sulfate) were added to MS, and growth responses were studied (Table 3). Incorporation of the precursor and elicitors showed the variations in the studied responses (average shoot length, number of leaves per shoot, number of roots per shoot, average root length, FW, DW, and percent response). Our results highlighted that within the studied levels of precursor and elicitors, the MS medium enriched with 1.5 µM SQ, 30 µM SA, 80 µM MJ, 30 mM $MgSO_4$, and 60 mM KCl showed the highest responses. The average shoot length of 3 to 5 cm was reported at all the concentrations of precursor and elicitors used. The highest shoot length (5.2 ± 0.22 cm) and maximum number of leaves (18 ± 0.71 cm) were recorded in MS + 1.5 µM SQ, whereas addition of 30 mM KCl to the medium revealed the lowest responses (3.0 ± 0.24 cm and 8.4 ± 0.40, respectively). In contrast, the highest number of roots (18.0 ± 0.75) with maximum root length (3 ± 0.10 cm) was observed from the squalene-enriched medium (MS + 1.5 µM SQ). In the study, MS + 100 mM KCl denoted the minimum root number (4.6 ± 1.0) and root length (2.0 ± 0.10 cm). Incorporation of MJ, SA, and $MgSO_4$ in the nutrient medium showed intermediate responses (Table 3). Moreover, the highest FW and DW were noted from the biomass raised on MS medium amended with 1.5 µM SQ. After analyzing the current findings, it was noted that squalene at a concentration of 1.5 µM promoted the *in vitro* growth of *B. floribunda* (Table 3).

Combined effects of optimal cytokinins, auxins, and precursors on *in vitro* regeneration

Based on the findings from Tables 1–3, the optimal concentrations of auxins, cytokinins, and squalene were used in combinations, and results are presented in Table 4. It was revealed that combinations of cytokinins, auxins, and precursors together altered the regeneration responses in *B. floribunda*. According to the statistical study, MS + 2.0 mg/l BAP + 1.5 µM SQ produced longer shoots (5.12 ± 0.71 cm) than the rest of the combinations. Likewise, MS + 2.0 mg/l BAP + 2.0 mg/l KIN exhibited the highest leaves per

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TABLE 3 Effect of precursor (squalene) and elicitors (methyl jasmonate, salicylic acid, potassium chloride, and magnesium sulfate) on regeneration of *Bacopa floribunda*.

Precursors and elicitors		Average shoot length (cm)	No. of leaves per shoot	No. of roots per shoot	Average root length (cm)	FW (g)	DW (g)	Response (%)
SQ (µM)	0.5	5.0 ± 0.12^{ab}	15.3 ± 0.67^{bc}	15.3 ± 0.64^{abc}	2.6 ± 0.33^{b}	1.40 ± 0.12^{bcd}	0.120 ± 0.01^{a}	90^{c}
	1.0	4.8 ± 0.49^{abc}	17.6 ± 0.75^{ab}	15.6 ± 0.71^{ab}	2.6 ± 0.24^{b}	1.35 ± 0.31^{bcd}	0.119 ± 0.02^{a}	100^{a}
	1.5	5.2 ± 0.22^{a}	18.0 ± 0.70^{a}	18.0 ± 0.75^{a}	3.0 ± 0.10^{a}	2.10 ± 0.06^{a}	0.126 ± 0.01^{a}	100^{a}
	2.0	3.0 ± 0.10^{e}	9.6 ± 0.40^{fghi}	8.0 ± 0.10^{efg}	2.4 ± 0.24^{b}	1.45 ± 0.10^{bcd}	0.120 ± 0.04^{a}	100^{a}
MJ (µM)	30	3.0 ± 1.3^{e}	12.4 ± 5.50^{cdef}	7.6 ± 3.4^{fgh}	3.0 ± 1.30^{b}	1.08 ± 0.12^{cdefg}	0.110 ± 0.01^{a}	100^{a}
	60	3.8 ± 0.20^{de}	13.6 ± 075^{cdef}	11.6 ± 0.40^{def}	3.2 ± 0.10^{a}	1.16 ± 0.10^{bcdef}	0.120 ± 0.01^{a}	100^{a}
	80	4.4 ± 0.24^{abc}	17.6 ± 0.75^{ab}	14.6 ± 0.75^{abcd}	3.2 ± 1.3^{a}	1.54 ± 0.11^{b}	0.140 ± 0.03^{a}	100^{a}
	100	4.0 ± 0.10^{cde}	14.0 ± 0.50^{bcd}	11.6 ± 1.6^{def}	3.0 ± 0.10^{b}	0.768 ± 0.18^{fghi}	0.108 ± 0.04^{a}	100^{a}
SA (µM)	30	5.0 ± 0.10^{ab}	17.6 ± 1.17^{ab}	13.2 ± 0.49^{cde}	3.0 ± 0.10^{b}	0.806 ± 0.37^{efghi}	0.102 ± 0.02^{a}	100^{a}
	60	3.0 ± 0.10^{e}	12 ± 0.63^{cdefg}	8.8 ± 0.49^{ef}	2.0 ± 0.10^{b}	0.680 ± 0.033^{ghi}	0.100 ± 0.02^{a}	100^{a}
	80	3.8 ± 0.10^{de}	12.4 ± 0.40^{cdefg}	8.8 ± 0.49^{ef}	2.0 ± 0.10^{b}	0.790 ± 0.07^{fghi}	0.100 ± 0.03^{a}	100^{a}
	100	3.0 ± 0.10^{e}	11.6 ± 0.40^{defgh}	4.8 ± 0.49^{i}	2.2 ± 0.10^{b}	0.420 ± 0.00^{i}	0.100 ± 0.02^{a}	100^{a}
KCl (mM)	30	3.0 ± 0.24^{e}	8.4 ± 0.40^{hi}	5.4 ± 0.49^{hi}	2.0 ± 0.10^{b}	0.940 ± 0.07^{defgh}	0.100 ± 0.02^{a}	100^{a}
	60	3.4 ± 0.20^{e}	12.8 ± 0.49^{cdef}	10.8 ± 0.40^{cde}	2.4 ± 0.24^{b}	1.40 ± 0.01^{bcd}	0.129 ± 0.01^{a}	100^{a}
	80	3.2 ± 0.20^{e}	12.4 ± 0.40^{cdef}	10.4 ± 0.24^{cde}	2.0 ± 0.10^{b}	0.720 ± 0.04^{fghi}	0.100 ± 0.02^{a}	100^{a}
	100	3.2 ± 0.4^{e}	10 ± 0.60^{efghi}	4.6 ± 1.0^{hi}	2.0 ± 0.10^{b}	0.900 ± 0.01^{dfghi}	0.100 ± 0.01^{a}	100^{a}
$MgSO_4$ (mM)	30	4.2 ± 0.4^{cde}	16.0 ± 1.40^{bc}	15 ± 1.0^{ab}	3.0 ± 0.10^{b}	1.360 ± 0.26^{bcd}	0.136 ± 0.01^{a}	100^{b}
	60	4.0 ± 0.01^{cde}	13.0 ± 0.58^{cdef}	11.5 ± 0.5^{cdef}	2.5 ± 0.29^{b}	1.024 ± 0.12^{bcde}	0.100 ± 0.06^{a}	100^{b}
	80	3.25 ± 0.30^{e}	11.5 ± 0.50^{defgh}	8.5 ± 0.5^{ef}	3.0 ± 0.10^{b}	1.00 ± 0.06^{cdefgh}	0.110 ± 0.02^{a}	90^{a}
	100	3.0 ± 0.10^{e}	10.5 ± 0.50^{defgh}	7.5 ± 0.5^{fgh}	2.75 ± 0.25^{b}	0.775 ± 0.14^{fghi}	0.120 ± 0.02^{a}	80^{a}

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

shoot (22.33 ± 1.45), followed by MS + 2.0 mg/l BAP + 2.0 mg/l KIN + 1.5 µM SQ, which had the second highest number of leaves (21.6 ± 1.17). However, the type and concentration of auxin determined the initiation and further elongation of roots. Findings revealed that MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA produced considerably maximum number of roots (36.0 ± 0.89) with average root length (3.5 ± 0.04 cm). The roots that were grown in the same medium were stronger and closely affixed to the base of the plantlets. Referring to the above findings, it was found that the optimal combination of auxins (MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA) was

TABLE 4 Effect of optimal concentrations and combinations of PGRs and precursor on regeneration of *Bacopa floribunda*.

PGRs and pre-cursor (mg/l–µM)	Average shoot length (cm)	No. of leaves per shoot	No. of roots per shoot	Average root length (cm)	FW (g)	DW (g)	Response (%)
OCC*	2.33 ± 0.33^{de}	22.33 ± 1.45^{a}	3.67 ± 0.4^{c}	1.0 ± 0.10^{c}	4.33 ± 0.75^{a}	0.191 ± 0.08^{a}	100^{a}
OCA*	3.00 ± 0.10^{cd}	15.75 ± 0.58^{b}	36 ± 0.89^{a}	3.5 ± 0.04^{a}	3.09 ± 0.44^{ab}	0.114 ± 0.01^{ab}	100^{a}
OCC*P°	4.0 ± 0.10^{ab}	21.6 ± 1.17^{a}	10 ± 0.63^{bc}	2.16 ± 0.10^{b}	1.79 ± 0.13^{b}c	0.105 ± 0.01^{b}	80^{b}
OCA*P°	1.9 ± 0.10^{e}	11.2 ± 1.02^{cd}	12.4 ± 0.75^{b}	2.2 ± 0.20^{b}	1.44 ± 0.4^{bc}	0.112 ± 0.01^{b}	100^{a}
OCC*P1°	5.12 ± 0.71^{a}	12.0 ± 1.15^{bc}	4.8 ± 1.22^{c}	2.2 ± 0.20^{b}	2.88 ± 0.19^{ab}	0.111 ± 0.01^{b}	100^{a}
OCC*P2°	3.2 ± 0.20^{bc}	12.4 ± 0.81^{bc}	13.4 ± 0.87^{b}	2.64 ± 0.14^{ab}	1.85 ± 0.45^{bc}	0.113 ± 0.01^{b}	80^{b}
OCAC*P°	2 ± 0.10^{e}	7.6 ± 0.49^{e}	13.2 ± 0.49^{b}	2.6 ± 0.24^{ab}	0.470 ± 0.08^{c}	0.048 ± 0.01^{b}	100^{a}
OCAC*	2.6 ± 0.40^{de}	15.6 ± 0.40^{bc}	4.2 ± 0.66^{c}	2.52 ± 0.18^{ab}	1.44 ± 0.01b^{c}	0.077 ± 0.02^{b}	100^{a}

*(mg/l), °(µM)

OCC* - MS + 2.0 mg/l BAP + 2.0 mg/l KIN, OCA* - MS + 0.5 mg/IAA + 0.5 mg/IBA + 1.0 mg/l NAA, OCC*P° - MS + 2.0 mg/BAP + 2.0 mg/l KIN + 1.5 µM SQ, OCA*P° - MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA + 1.5 µM SQ, OCC*P1°- MS + 2.0 BAP mg/l + 1.5 µM SQ, OCC*P2°- MS + 2.0 mg/l KIN + 1.5 µM SQ, OCAC*P° - MS + 1.0 mg/l NAA + 2.0 mg/l KIN + 1.5 µM SQ, OCAC* - MS + 1.0 mg/l NAA + 2.0 mg/l KIN.

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

found to be the most ideal for root initiation (Figure 1G). In comparison with other treatments, the optimal combination of cytokinins (MS + 2.0 mg/l BAP + 2.0 mg/l KIN) had the highest FW (4.33 ± 0.75 g) and DW (0.191 ± 0.08 g), followed by MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA (3.09 ± 0.44 and 0.114 ± 0.01 g, respectively). Development of shoots, number of leaves, FW, DW, maximum root number, root length, and root development was observed in the cultures when the optimum combinations of cytokinins (MS + 2.0 mg/l BAP + 2.0 mg/l KIN) and auxins (MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA) were used. From the above results, it was observed that growth parameters (number of roots per shoot and leaves per shoot) in *B. floribunda* were significantly enhanced by using optimum combinations of auxins and cytokinins (Table 4, Figures 1F, G).

Acclimatization of plantlets

Nutrient medium amended with the optimal auxin combinations, i.e., MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA, showed the best rooting responses. After 3 weeks of cultures, *in vitro* grown rooted shoots were taken out from the culture vessels and used for acclimatization. After 10 weeks of acclimatization, *in vitro* regenerated plantlets of *B. floribunda* had a survival rate of 95%. The acclimatized plants grew well and were morphologically identical to the mother plants (Figure 1H). The total height of hardened plantlets (10 weeks old) was approximately 8 to 10 cm with 13–18 leaves per plant. The acclimatized *in vitro* plantlets showed no evident variation in morphology or growth characteristics (Figure 1H).

Estimation of total saponins, triterpenoid saponin glycosides, and stigmasterol contents from *in vitro* grown biomass

In vitro biomass derived from the experiments (auxins, cytokinins, precursor, and elicitors and their combinations) were subjected to estimation of various bioactive compounds, particularly total saponins (TSC), triterpenoid saponin glycosides, and stigmasterol using spectrophotometry and HPLC (Figure 2).

Total saponin content

TSC from the *in vitro* grown biomass was determined using a spectrophotometer, and results are represented in Figure 2. Fortification of PGRs, precursor, and elicitors in MS showed reliable TSC which was found in the range of 17 to 32 mg/g DW. Among all the levels of cytokinins, MS + 1.5 mg/l KIN showed the least TSC (20.92 ± 4.8 mg DE/g DW), whereas MS + 1.0 mg/l BAP exhibited the highest content (31.79 ± 0.011 mg DE/g DW) followed by MS + 0.5 mg/l BAP and MS + 1.0 KIN mg/l (Figure 2A). In the case of auxins, MS + 0.5 mg/l IBA was found to be the best medium for total saponin content (32.0 ± 0.001 mg DE/g DW), whereas the lowest content (17.78 ± 2.1 mg DE/g DW) was found in NAA (0.5 mg/l) containing medium (Figure 2A). Additionally, when compared with other concentrations of the studied precursor and elicitors, MS + 1.5 µM SQ was found to be the best treatment that offered the highest TSC (31.7 ± 0.04 mg DE/g DW). Furthermore, in the elicitors tested, addition of KCl in the nutrient medium showed the highest TSC, followed by SA, $MgSO_4$, and MJ (Figure 2B). MS modified with 80

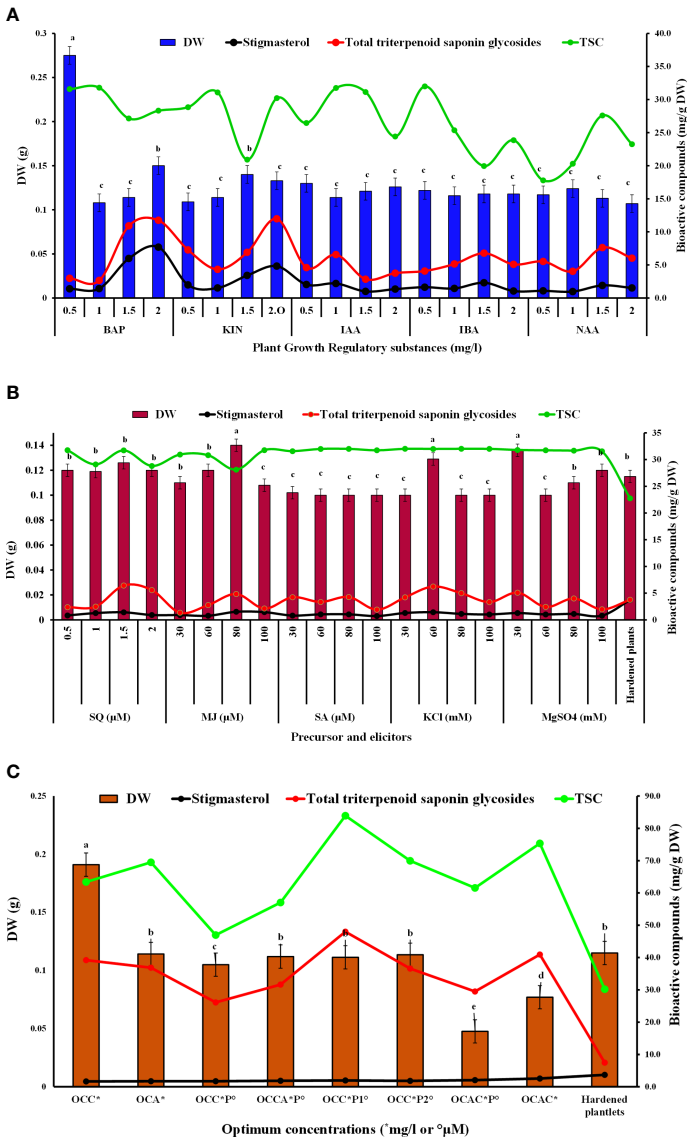


FIGURE 2
Effects of different PGRs **(A)**, precursor and elicitors **(B)**, and their optimal combinations **(C)** on dry weight, total saponins, total triterpenoid saponin glycosides, and stigmasterol contents from *in vitro* grown biomass of *B. floribunda*.

mM KCl showed the highest TSC (32.01 ± 0.10 mg DE/g DW), whereas the least response (28.10 ± 0.079 mg DE/g DW) was observed when 80 mM MJ was added in the medium. Other treatments including SA, $MgSO_4$, and MJ showed intermediate contents of total saponins. Based on the findings from Figures 2A, B, optimal concentrations of auxins and cytokinins along with the optimal concentration of squalene were used in combinations and results are presented in Figure 2C. It was revealed that the combination of cytokinins, auxins, and precursors together enhanced the total saponin content in cultures of *B. floribunda*. At the same combinations studied, TSC was found in the range of 20 to 36 mg/g DW wherein MS + 2.0 mg/l BAP + 1.5 µM SQ revealed the highest content of saponins (35.95 ± 0.022 mg DE/g DW) (Figure 2C). Furthermore, 10-week-old hardened plants of *B. floribunda* had comparatively lower TSC (27.71 ± 0.045 mg DE/g DW). From the findings, it was clearly shown that elicitor and precursor feeding increased the TSC amount in the *in vitro* grown biomass of *B. floribunda*. These treatments showed the reliable amount of TSC from the *in vitro* grown biomass of *B. floribunda* and also indicated that it could be a good source of saponins (Figures 2A–C).

Estimation of triterpenoid saponin glycosides using HPLC

In the current study, an HPLC analysis of triterpenoid saponin glycosides (bacoside A3, bacoside II, bacopaside X, and bacosaponin C) from the *in vitro* grown biomass and hardened plants of *B. floribunda* was carried out. The combination of jujubogenin (bacoside A3 and bacopaside X) and pseudojujubogenin (bacopaside II and bacosaponin C) glycosides was considered as total triterpenoid saponin glycosides. MS media fortified with various concentrations of PGRs, precursor, and elicitors and combinations of their optimal concentrations were examined.

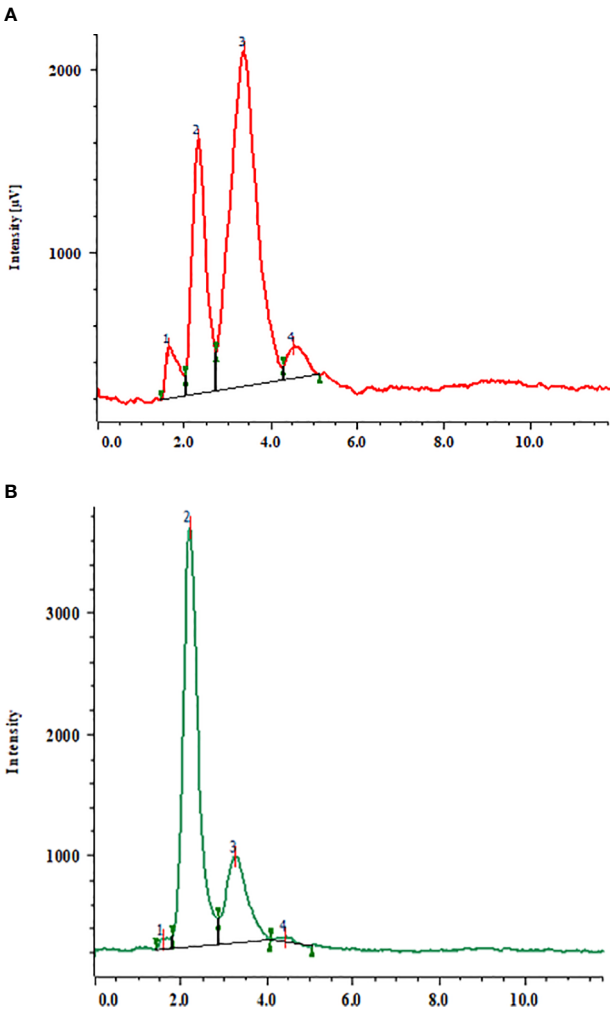


FIGURE 3
HPLC chromatogram of a mixture of triterpenoid saponin glycosides, **(A)** (1) bacoside A3; (2) bacopaside II; 3) bacopaside X; 4) bacopasaponin C and **(B)**, triterpenoid saponin glycosides from *in vitro* grown biomass (MS + 2.0 mg/l BAP + 1.5 µM SQ).

Additionally, a total of four triterpenoid saponin glycoside compounds were separated successfully and results are presented in Figure 3A. Extracts of *in vitro* grown biomass raised on MS medium fortified with KIN denoted a significant amount of total triterpenoid saponin glycosides (2 to 12 mg/g DW), followed by BA, NAA, IBA, and IAA. MS + 2.0 mg/l KIN revealed the highest (11.98 ± 0.10 mg/g DW) content of saponins followed by MS + 2.0 mg/l BAP (11.74 ± 0.10 mg/g DW). Likewise in the case of auxins studied, MS + 1.5 mg/l NAA showed the highest saponin content (7.61 ± 0.10 mg/g DW). In contrast, the least content (2.84 ± 0.10 mg/g DW) was observed when cultures were grown on MS + 1.5 mg/l IAA (Figure 2A). Furthermore, among all the tested concentrations of precursor and elicitors, MS + 1.5 µM SQ was identified as the best source of total triterpenoid saponin glycosides (6.42 ± 0.10 mg/g DW) as compared with its remaining concentrations wherein the least content (2.36± 0.10 mg/g DW) was denoted from the lowest level (0.5 µM) of squalene (Figure 2B). In elicitors, MS + 60 mM KCl indicated the highest saponin content (6.23 ± 0.10 mg/g DW), whereas incorporation of 30 µM MJ to the medium denoted the lowest amount (1.34 ± 0.10 mg/g DW) (Figure 2B). The triterpenoid saponin glycosides are said to be the active chemical constituents reported from the genus *Bacopa* and mainly categorized as pseudojujubogenin (bacoside II and bacosaponin C) and jujubogenin glycosides (bacoside A3 and bacopaside X). The effects of different PGRs on production of triterpenoid saponin glycosides (jujubogenin glycoside and pseudojujubogenin glycoside) from *in vitro* grown biomass of *Bacopa floribunda* were investigated, and results are depicted in Table 5. In the present investigation, different treatments of PGRs showed variable responses wherein total jujubogenin and pseudojujubogenin glycoside contents were found in the range of 0.62 to 2.20 and 1.91 to 9.99 mg/g DW, respectively. Moreover, the highest total jujubogenin glycoside content (2.20 ± 0.10 mg/g DW) was observed from the biomass derived from MS + 2.0 mg/l BAP. Similar to this, maximum bacopaside X (2.150 ± 0.069 mg/g DW) and bacoside A3 (0.081 ± 0.019 mg/g DW) were reported from the auxins containing media (MS + 2.0 mg/l BAP and MS + 1.0 mg/l BAP, respectively). Further, the highest total pseudojujubogenin glycosides (9.99 ± 0.10 mg/g DW) were reported from the cultures derived from MS + 2.0 mg/l KIN. At the same time, maximum bacopaside II (9.93 ± 0.27 mg/g DW) and bacosaponin C (0.142 ± 0.090 mg/g DW) were reported from MS medium enriched with 2 mg/l KIN and 1.5 mg/l BAP, respectively. The addition of 1.5 mg/l IBA in the nutrient medium produced the highest amount of total jujubogenin glycoside content (1.429 ± 0.010 mg/g DW) wherein a maximum content of bacopaside X (1.425 ± 0.018 mg/g DW) was noted. In a similar way, MS + 0.5 mg/l IAA revealed the best results in terms of bacoside A3 content (0.206 ± 0.001 mg/g DW). The highest total pseudojujubogenin glycoside content (6.20 ± 0.10 mg/g DW) was obtained from the biomass derived from MS + 1.5 mg/l NAA (Table 5**).** In the current findings, we noted that bacopaside X and bacopaside II were the main ingredients found responsible for enhancing total jujubogenin and pseudojujubogenin glycosides in *B. floribunda*. Like PGRs, the influence of precursor and elicitors on production of bioactive compounds was also studied (Table 6). In the case of precursor studies, MS + 1.5 µM SQ denoted the highest total jujubogenin (0.936 ± 0.10 mg/g DW) and total pseudojujubogenin glycoside (5.49 ± 0.10 mg/g DW) contents. Among the studied elicitors, all the concentrations denoted a reliable amount of total jujubogenin and pseudojujubogenin glycosides, where MS + 80 µM MJ displayed the highest total jujubogenin glycoside content (0.878 ± 0.10 mg/g DW). Total pseudojujubogenin glycoside content was comparatively found to be higher (5.419 ± 0.10 mg/g DW) when 60 mM KCl was added to the medium. In the same way, MS + 80 mM KCl exhibited the highest bacoside A3 (0.020 ± 0.017 mg/g DW) content; however, bacopaside X content was maximum when the shoots were treated with 80 µM MJ (0.873 ± 0.049 mg/g DW). Similarly, the highest content of bacopaside II (4.242 ± 0.145 mg/g DW) was reported from the culture derived from the medium supplemented with 30 mM $MgSO_4$ (Table 6**).**

Furthermore, optimal concentrations of auxins and cytokinins along with squalene were used in combinations to boost the total amount of triterpenoid saponin glycosides. Among all the optimum tested concentrations, MS + 2.0 mg/l BAP + 1.5 µM SQ was found to be the best with the highest content of total triterpenoid saponin glycosides (46.04 ± 0.10 mg/g DW) (Figure 3B). Similarly, other remaining optimal combinations revealed total saponins in the range

TABLE 5 Effects of different PGRs on total triterpenoid saponin glycosides (jujubogenin glycoside and pseudojujubogenin glycoside) from *in vitro* grown biomass of *Bacopa floribunda*.

PGRs (mg/l)		Total triterpenoid saponin glycosides (mg/g DW)						
		Jujubogenin glycoside		Total Jujubogenin glycoside	Pseudojujubogenin glycoside		Total Pseudojujubogenin glycosides	Total jujubogenin and pseudojujubogenin glycosides
		Bacoside A3	Bacopaside X		Bacopaside II	Bacosaponin C		
BAP	0.5	0.032 ± 0.01^{c}	0.588 ± 0.12^{e}	0.62 ± 0.10^{e}	2.320 ± 0.02^{l}	0.034 ± 0.002^{b}	2.35 ± 0.10^{o}	2.97 ± 0.10^{f}
	1.0	0.081 ± 0.019^{b}	0.663 ± 0.120^{de}	0.74 ± 0.10^{e}	1.862 ± 0.098^{m}	0.048 ± 0.001^{b}	1.91 ± 0.10^{q}	2.65 ± 0.10^{f}
	1.5	0.041 ± 0.019^{c}	1.913 ± 0.059^{a}	1.95 ± 0.10^{a}	8.814 ± 0.27^{c}	0.142 ± 0.090^{a}	8.96 ± 0.10^{b}	10.91 ± 0.10^{b}
	2.0	0.050 ± 0.002^{b}	2.150 ± 0.069^{a}	2.20 ± 0.10^{a}	9.504 ± 0.270^{b}	0.038 ± 0.014^{b}	9.54 ± 0.10^{b}	11.74 ± 0.10^{a}
KIN	0.5	0.023 ± 0.01^{d}	1.014 ± 0.042^{c}	1.04 ± 0.10^{c}	6.169 ± 0.155^{d}	0.060 ± 0.024^{b}	6.23 ± 0.10^{c}	7.27 ± 0.10^{c}
	1.0	0.025 ± 0.001^{d}	0.909 ± 0.140^{cd}	0.93 ± 0.10^{e}	3.337 ± 0.085^{i}	0.061 ± 0.028^{b}	3.40 ± 0.10^{k}	4.33 ± 0.10^{d}
	1.5	0.026 ± 0.001^{d}	1.309 ± 0.127^{b}	1.33 ± 0.010^{c}	5.498 ± 0.003^{e}	0.065 ± 0.040^{b}	5.56 ± 0.10^{d}	6.90 ± 0.10^{c}
	2.0	0.013 ± 0.001^{d}	1.972 ± 0.101^{a}	1.99 ± 0.10^{a}	9.934 ± 0.276^{a}	0.060 ± 0.038^{b}	9.99 ± 0.10^{a}	11.98 ± 0.10^{a}
IAA	0.5	0.206 ± 0.001^{a}	0.542 ± 0.380^{e}	0.749 ± 0.10^{d}	3.825 ± 0.115^{h}	0.020 ± 0.009^{b}	3.84 ± 0.10^{i}	4.59 ± 0.10^{d}
	1.0	0.035 ± 0.034^{c}	0.761 ± 0.319^{cde}	0.796 ± 0.10^{d}	5.738 ± 0.192^{e}	0.023 ± 0.001^{b}	5.76 ± 0.10^{c}	6.55 ± 0.10^{c}
	1.5	0.059 ± 0.054^{b}	0.574 ± 0.152^{e}	0.633 ± 0.10^{d}	2.178 ± 0.125^{l}	0.029 ± 0.004^{b}	2.20 ± 0.10^{p}	2.84 ± 0.10^{f}
	2.0	0.044 ± 0.039^{c}	0.786 ± 0.097^{cde}	0.830 ± 0.10^{d}	2.870 ± 0.112^{k}	0.059 ± 0.001^{b}	2.92 ± 0.10^{n}	3.75 ± 0.10^{e}
IBA	0.5	0.018 ± 0.015^{d}	0.971 ± 0.083^{c}	0.989 ± 0.10^{c}	3.039 ± 0.079^{jk}	0.055 ± 0.018^{b}	3.09 ± 0.10^{m}	4.08 ± 0.10^{c}
	1.0	0.002 ± 0.001^{e}	0.972 ± 0.084^{c}	0.974 ± 0.010^{c}	4.111 ± 0.139^{g}	0.055 ± 0.019^{b}	4.16 ± 0.10^{h}	5.14 ± 0.10^{d}
	1.5	0.004 ± 0.002^{e}	1.425 ± 0.018^{b}	1.429 ± 0.010^{b}	5.290 ± 0.094^{e}	0.053 ± 0.015^{b}	5.34 ± 0.10^{e}	6.77 ± 0.10^{c}
	2.0	0.005 ± 0.001^{e}	1.263 ± 0.237^{b}	1.268 ± 0.10^{b}	3.753 ± 0.018^{h}	0.060 ± 0.023^{b}	3.81 ± 0.10	5.08 ± 0.10^{d}
NAA	0.5	0.003 ± 0.002^{e}	0.894 ± 0.045^{cd}	0.897 ± 0.10^{c}	4.578 ± 0.025^{f}	0.046 ± 0.007^{b}	4.62 ± 0.10^{g}	5.52 ± 0.10^{d}
	1.0	0.004 ± 0.001^{e}	0.819 ± 0.025^{cde}	0.823 ± 0.10^{c}	3.162 ± 0.066^{ij}	0.044 ± 0.014^{b}	3.20 ± 0.10^{l}	4.02 ± 0.10^{c}
	1.5	0.004 ± 0.001^{e}	1.404 ± 0.044^{b}	1.407 ± 0.10^{b}	6.165 ± 0.056^{d}	0.043 ± 0.001^{b}	6.20 ± 0.10^{c}	7.61 ± 0.10^{c}
	2.0	0.004 ± 0.002^{e}	1.298 ± 0.024^{b}	1.302 ± 0.10^{b}	4.667 ± 0.328^{f}	0.046 ± 0.005^{b}	4.71 ± 0.10^{f}	6.01 ± 0.10^{c}

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

of 24 to 46 mg/g DW, wherein the total jujubogenin glycoside content was 5.69 ± 0.10 mg/g DW from MS + 2.0 mg/l BAP + 2.0 mg/l KIN. Similarly, MS + 2.0 mg/l BAP + 1.5 µM SQ was identified as the best treatment and offered the highest total pseudojujubogenin glycosides (43.80 ± 0.10 mg/g DW). Among all the triterpenoid saponin glycosides, the highest bacoside A3 content (1.23 ± 0.064 mg/g DW) was obtained from the biomass grown on MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 NAA mg/l + 1.5 µM SQ (Table 7, Figure 2C).

TABLE 6 Effects of precursor and elicitors on total triterpenoid saponin glycosides (jujubogenin glycoside and pseudojujubogenin glycoside) from *in vitro* grown biomass of *Bacopa floribunda*.

Precursor/ elicitors		Total triterpenoid saponin glycosides (mg/g DW)						
		Jujubogenin glycoside		Total jujubogenin glycoside	Pseudojujubogenin glycosides		Total pseudojujubogenin glycoside	Total amount of jujubogenin and pseudojujubogenin glycosides
		Bacoside A3	Bacopaside X		Bacopaside II	Bacosaponin C		
SQ (μM)	0.5	0.011 ± 0.007^{b}	0.476 ± 0.177^{ijkl}	0.487 ± 0.10e	1.835 ± 0.412^{jkl}	0.044 ± 0.029^{b}	1.879 ± 0.10^{d}	2.366 ± 0.10^{d}
	1.0	0.014 ± 0.006^{b}	0.519 ± 0.082^{hijk}	0.533 ± 0.10^{d}	1.902 ± 0.086^{hij}	0.037 ± 0.022^{c}	1.939 ± 0.10^{d}	2.472 ± 0.10^{d}
	1.5	0.005 ± 0.003^{b}	0.930 ± 0.088^{a}	0.936 ± 0.10^{a}	5.463 ± 0.211^{a}	0.031 ± 0.003^{c}	5.493 ± 0.10^{a}	6.429 ± 0.10^{a}
	2.0	0.045 ± 0.041^{a}	0.833 ± 0.028^{ab}	0.878 ± 0.10^{b}	4.673 ± 0.119^{bc}	0.020 ± 0.001^{c}	4.693 ± 0.10^{b}	5.571 ± 0.10^{b}
MJ (μM)	30	0.011 ± 0.006^{b}	0.418 ± 0.046^{kl}	0.429 ± 0.10^{e}	0.872 ± 0.045^{m}	0.043 ± 0.016^{b}	0.915 ± 0.10^{f}	1.344 ± 0.10^{f}
	60	0.006 ± 0.004^{b}	0.581 ± 0.098^{efgh}	0.587 ± 0.10^{d}	2.125 ± 0.078^{ghi}	0.052 ± 0.024^{b}	2.177 ± 0.10^{d}	2.764 ± 0.10^{d}
	80	0.005 ± 0.001^{b}	0.873 ± 0.049^{ab}	0.878 ± 0.10^{a}	3.880 ± 0.020^{d}	0.079 ± 0.042^{a}	3.959 ± 0.10^{c}	4.837 ± 0.10^{c}
	100	0.008 ± 0.001^{b}	0.497 ± 0.007^{kl}	0.505 ± 0.10^{d}	1.551 ± 0.037^{l}	0.067 ± 0.022^{a}	1.618 ± 0.10^{e}	2.123 ± 0.10^{d}
SA (μM)	30	0.003 ± 0.002^{b}	0.611 ± 0.038^{efg}	0.614 ± 0.10^{c}	3.585 ± 0.027^{e}	0.027 ± 0.001^{c}	3.612 ± 0.10^{c}	4.226 ± 0.10^{c}
	60	0.005 ± 0.001^{b}	0.518 ± 0.017^{hijk}	0.523 ± 0.10^{d}	2.810 ± 0.058^{f}	0.016 ± 0.004^{d}	2.826 ± 0.10^{d}	3.349 ± 0.10^{d}
	80	0.009 ± 0.003^{b}	0.644 ± 0.031^{efg}	0.653 ± 0.10^{c}	3.605 ± 0.024^{e}	0.014 ± 0.008^{d}	3.619 ± 0.10^{d}	4.273 ± 0.10^{c}
	100	0.011 ± 0.002^{b}	0.397 ± 0.021^{l}	0.408 ± 0.10^{e}	1.485 ± 0.039^{l}	0.023 ± 0.005^{c}	1.507 ± 0.10^{e}	1.915 ± 0.10^{e}
KCl (mM)	30	0.005 ± 0.001^{b}	0.683 ± 0.076^{def}	0.688 ± 0.10^{c}	3.498 ± 0.098^{e}	0.022 ± 0.006^{c}	3.521 ± 0.10^{c}	4.208 ± 0.10^{c}
	60	0.004 ± 0.001^{b}	0.816 ± 0.061^{bc}	0.820 ± 0.10^{a}	5.406 ± 0.041^{a}	0.013 ± 0.001^{d}	5.419 ± 0.10^{a}	6.239 ± 0.10^{a}
	80	0.020 ± 0.017^{b}	0.738 ± 0.028^{cde}	0.759 ± 0.10^{b}	4.212 ± 0.125^{c}	0.019 ± 0.005^{d}	4.231 ± 0.10^{b}	4.989 ± 0.10^{c}
	100	0.005 ± 0.002^{b}	0.552 ± 0.001^{ghi}	0.557 ± 0.10^{d}	2.756 ± 0.103^{f}	0.018 ± 0.004^{d}	2.774 ± 0.10^{e}	3.331 ± 0.10^{d}
$MgSO_4$ (mM)	30	0.009 ± 0.001^{b}	0.773 ± 0.110^{bcd}	0.782 ± 0.10^{b}	4.242 ± 0.145^{c}	0.058 ± 0.044^{b}	4.301 ± 0.10^{b}	5.082 ± 0.10^{a}
	60	0.006 ± 0.001^{b}	0.432 ± 0.009^{jkl}	0.438 ± 0.10^{e}	1.957 ± 0.167^{hij}	0.019 ± 0.003^{d}	1.976 ± 0.10^{e}	2.414 ± 0.10^{d}
	80	0.007 ± 0.001^{b}	0.575 ± 0.03^{defgh}	0.582 ± 0.10^{d}	3.387 ± 0.550^{e}	0.029 ± 0.010^{c}	3.416 ± 0.10^{c}	3.998 ± 0.10^{c}
	100	0.011 ± 0.003^{b}	0.409 ± 0.01^{ghijkl}	0.419 ± 0.10^{e}	1.534 ± 0.086^{kl}	0.017 ± 0.005^{d}	1.551 ± 0.10^{e}	1.970 ± 0.10^{e}
Hardened plantlet		0.001 ± 0.001^{c}	1.48 ± 0.001^{a}	1.485 ± 0.01^{a}	1.59 ± 0.001^{kl}	0.67 ± 0.001^{a}	2.27 ± 0.001^{e}	3.754 ± 0.001^{c}

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

TABLE 7 Effects of optimal concentrations of PGRs and precursor combinations on total triterpenoid saponin glycosides (jujubogenin glycoside and pseudojujubogenin glycoside) from *in vitro* grown biomass of *Bacopa floribunda*.

PGRs and precursor (mg/l–µM)	Total triterpenoid saponin glycosides (mg/g DW)						
	Jujubogenin glycoside		Total jujubogenin glycoside	Pseudojujubogenin glycoside		Total Pseudojujubogenin glycosides	Total amount of jujubogenin and pseudojujubogenin glycosides
	Bacoside A3	Bacopaside X		Bacopaside II	Bacosaponin C		
OCC*	0.74± 0.066^{b}	4.95 ± 0.587^{a}	5.69 ± 0.10^{a}	31.57 ± 0.966^{c}	0.29 ± 0.148^{a}	31.86 ± 0.10^{c}	37.55 ± 0.10^{c}
OCA*	0.98 ± 0.120^{a}	2.84 ± 0.103^{b}	3.81 ± 0.10^{d}	31.11 ± 1.19^{c}	0.22 ± 0.117^{a}	31.33 ± 0.10^{c}	35.15 ± 0.10^{d}
OCC*P°	1.16± 0.058^{a}	2.93 ± 0.754^{b}	4.08 ± 0.10^{b}	20.17 ± 1.2^{e}	0.15 ± 0.117^{a}	20.31 ± 0.10^{e}	24.39 ± 0.10^{h}
OCA*P°	1.23 ± 0.064^{a}	2.51 ± 0.419^{bc}	3.75 ± 0.10^{e}	25.77 ± 2.2^{d}	0.27 ± 0.07^{a}	26.04 ± 0.10^{d}	29.79 ± 0.10^{f}
OCC*P1°	1.01 ± 0.195^{a}	1.23 ± 0.570^{d}	2.24 ± 0.10^{h}	43.62 ± 0.657^{a}	0.19 ± 0.195^{a}	43.80 ± 0.10^{a}	46.04 ± 0.10^{a}
OCC*P2°	1.09 ± 0.195^{a}	2.76 ± 0.43^{bc}	3.84 ± 0.10^{c}	30.77 ± 2.00^{c}	0.17 ± 0.053^{a}	30.94 ± 0.10^{c}	34.79 ± 0.10^{e}
OCAC*P°	0.99 ± 0.157^{a}	1.68 ± 1.08^{cd}	2.67 ± 0.10^{g}	24.64 ± 1.59^{d}	0.13 ± 0.040^{a}	24.78 ± 0.10^{d}	27.45 ± 0.10^{g}
OCAC*	1.11 ± 0.127^{a}	2.16 ± 0.37^{bc}	3.27 ± 0.10^{f}	34.96 ± 2.7^{b}	0.13 ± 0.040^{a}	35.09 ± 0.10^{b}	38.36 ± 0.10^{b}

(mg/l), °(µM), OCC - MS + 2.0 mg/l BAP + 2.0 mg/l KIN, OCA* - MS + 0.5 mg/IAA + 0.5 mg/IBA + 1.0 mg/l NAA, OCC*P° - MS + 2.0 mg/BAP + 2.0 mg/l KIN + 1.5 µM SQ, OCA*P° - MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA + 1.5 µM SQ, OCC*P1°- MS + 2.0 BAP mg/l + 1.5 µM SQ, OCC*P2°- MS + 2.0 mg/l KIN + 1.5 µM SQ, OCAC*P° - MS + 1.0 mg/l NAA + 2.0 mg/l KIN + 1.5 µM SQ, OCAC* - MS + 1.0 mg/l NAA + 2.0 mg/l KIN.

Values represent the averages and standard errors of three independent measurements. According to Duncan's multiple-range test, mean values in the same column with various alphabets exhibited statistically significant differences.

Among the triterpene saponins studied, maximum bacopaside X (4.95 ± 0.58 mg/g DW) was found when the nutrient medium was enriched with BAP and KIN (MS + 2.0 mg/l BAP + 2.0 mg/l KIN). Similarly, higher levels of bacopaside II (34.96 ± 2.7 mg/g DW) and bacosaponin C (0.29 ± 0.148 mg/g DW) were reported from the *in vitro* biomass grown on combinations of auxins and cytokinins (MS + 1.0 mg/l NAA + 2.0 mg/l KIN and MS + 2.0 mg/l BAP + 2.0 mg/l KIN, respectively). In the same way, 10-week-old hardened plants of *B. floribunda* also highlighted the appreciable amount of saponins (3.75 ± 0.001 mg/g DW) with higher levels of bacopaside X and bacopaside II (1.48 ± 0.001 and 1.59 ± 0.001 mg/g DW, respectively). These results revealed that the combinations of PGRs and the precursor (SQ) at its optimum concentrations exhibited the highest amount of triterpenoid saponin glycosides. According to the data, it was established that *B. floribunda* could be a reliable source of triterpenoid saponin glycosides and may serve as an alternative and potential candidate to the commonly used *B. monnieri* (Tables 5–7; Figures 2A–C).

Estimation of stigmasterol content

Similar to total triterpenoid saponin glycosides, stigmasterol content from the *in vitro* grown biomass of *Bacopa floribunda* was studied using HPLC (Figures 2 and 4). Separation of compound was carried out using acetonitrile and water (70:30 v/v), and the compound was detected at 3.01 ± 0.1 min (Figure 4A). In the cytokinin study, biomass obtained from MS nutrient medium containing 2.0 mg/l BAP had the highest stigmasterol content (7.69 ± 0.102 mg/g DW), whereas the least was found in the case of MS + 0.5 mg/l BAP (1.39 ± 0.31 mg/g DW). Similar to cytokinins, addition of 1.5 mg/l IBA to the MS medium denoted the maximum stigmasterol content (2.31 ± 0.66 mg/g DW) and the lowest in MS + 1.0 mg/l NAA (0.98 ± 0.17 mg/g DW) (Figure 2A). In precursor studies, incorporation of 1.5 µM SQ offered the best response (1.14 ± 0.13 mg/g DW) followed by MS + 1.0 µM SQ (1.27 ± 0.043 mg/g DW), 2.0 µM SQ (0.91 ± 0.069 mg/g DW), and 0.5 µM SQ (0.82 ± 0.034 mg/g DW), respectively (Figure 2B). Among all the elicitors studied, the content of stigmasterol was noted in the range of 0.72 to 1.51 mg/g DW. Utilization of 80 µM MJ in the medium revealed the highest stigmasterol content (1.51 ± 0.10 mg/g DW), whereas the minimum was found when biomass was grown on MS + 100 mM SA. An intermediate content of stigmasterol (0.72 to 1.43 mg/g DW) was observed when the cultures were raised using remaining elicitors such as SA, KCl, and $MgSO_4$. Further, combinations of optimum concentrations were studied and all the tested treatments showed comparatively less amount (1.6 to 2.6 mg/g DW) of stigmasterol (Figure 2C). A similar trend was noted in the case of 10-week-old hardened plants (3.71 ± 0.106 mg/g DW). From the above results, it can be concluded that MS along with 2.0 mg/l BAP showed an elevated level of stigmasterol as compared with the other treatments (Figures 2A–C and 4B). These findings indicated that all the PGRs, precursor, and elicitors and the combinations of their optimal concentrations displayed a significant amount of stigmasterol and also highlighted that *B. floribunda* could be a reliable source of phytosterol (Figure 2).

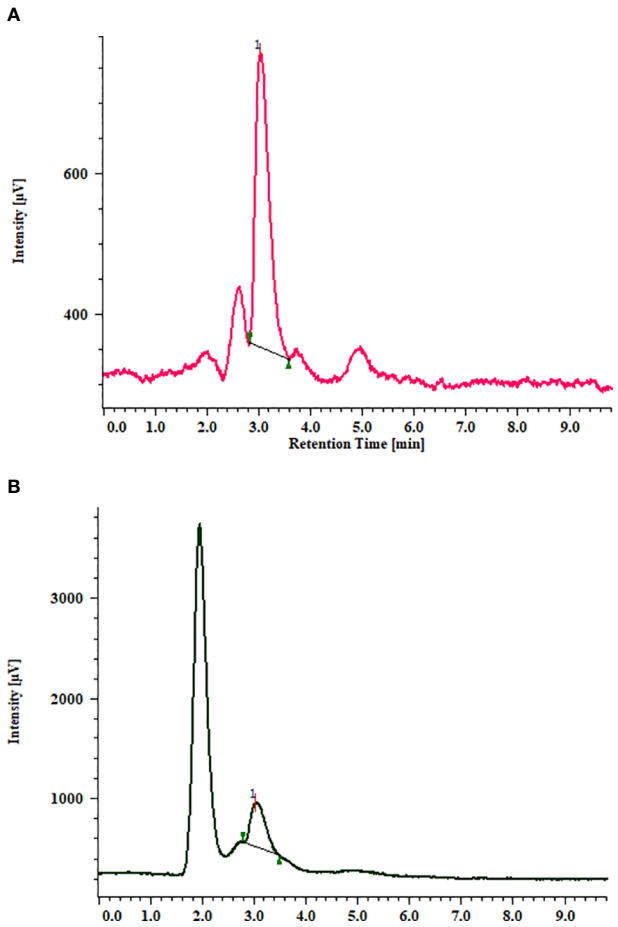


FIGURE 4
HPLC chromatogram of standard stigmasterol **(A)**. Stigmasterol from *in vitro* grown biomass of MS + 2.0 mg/l BAP **(B)**.

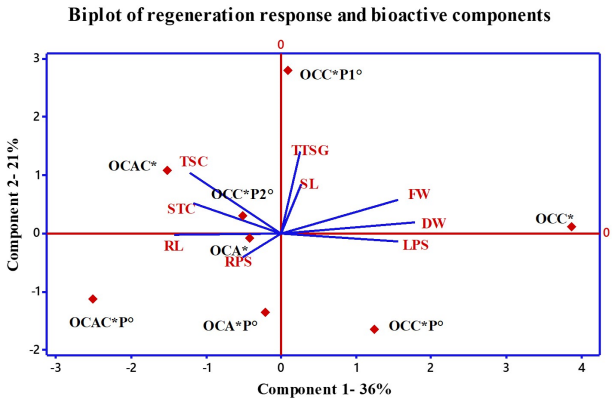


FIGURE 5
Principal component analysis (scores and loading plots, biplot) of optimal combinations of PGRs with precursors on growth and production of bioactive compounds from *B. floribunda*. *mg/l; °µM. SL, shoot length; LPS, leaves per shoot; RL, root length; RPS, roots per shoot; FW, fresh weight; DW, dry weight; TSC, total saponin content; STC, stigmasterol content; TTSG, total triterpenoid saponin glycosides; OCC, optimal combination of cytokinins; OCCP1, optimal combination of cytokinin with precursor; OCCP2, optimal combination of cytokinin with precursor; OCA, optimal combination of auxins; OCAP, optimal combination of auxin with precursor; OCACP, optimal combination of auxin, cytokinin, and precursor; OCAC, optimum combination of auxins with cytokinin; OCCP, optimum combination of cytokinin with precursor.

Chemometric analysis

PCA was performed to understand the relationship between the studied morphological responses along with detected bioactive compounds (total saponins, stigmasterol, and triterpenoid saponin glycosides). In the analysis, responses from various optimal concentrations of PGRs and precursors and their combinations were used (Figure 5). Multivariate analysis showed that components 1 and 2 highlighted 36 and 21% variability, respectively. Figure 5 also shows that the positive planes of both components were occupied by TBC (0.07), FW (0.42), DW (0.49), and SL (0.07). Optimal combination of cytokinins (OCC) and optimal combination of cytokinin and precursor (OCCP1) enjoyed the positive plane of both the components. Only parameter LPS having a loading value of 0.42 occupied the positive plane of PC1 and the negative plane of PC2. Similarly, component 2 was mainly dominated by TTSG and STC with 0.48 and 0.23 loading values, respectively. OCCP2 and OCAC enjoyed the positive plane of component 2. From Figure 5, it was clearly shown that OCA along with RL and RPS occupied the negative planes of PC1 and PC2. Treatments like OCACP, OCAP, and OCCP were found less effective for the regeneration responses. However, results also highlighted that treatments like OCAC, OCCP2, and OCA contributed toward the higher contents of total saponins, total triterpenoid saponin glycosides, and stigmasterol (Figure 5).

Discussion

Bacopa species are mainly found in wetland areas of tropical and subtropical countries, and its growth under natural conditions is limited. In order to increase mass multiplication of *B. floribunda* and secondary metabolites including triterpenoid saponin glycosides and sterols, it is necessary to develop an efficient *in vitro* micropropagation protocol. In order to accomplish this goal, we focused on micropropagation and augmented production of stigmasterol and triterpenoid saponin glycosides from the unexplored plant species known as *B. floribunda*. Owing to their enormous potential in medicinal purposes, most of *B. monnieri* have been collected extensively from their native habitat, resulting in the species' extinction and threat. Search of alternate herbs and their mass multiplication is also needed to reduce the overexploitation of *B. monnieri*. To achieve this goal, we aimed at micropropagation and elicited production of triterpenoid saponin glycosides as well as stigmasterol in *B. floribunda*. Seeds of *B. monnieri* had poor germination, low viability, and frequent two-leaf seedling deaths, but it can be propagated efficiently using stem cuttings (Parale and Nikam, 2009; Parale et al., 2010). In the study, fresh nodal segments were used as explants and cultures were established using MS medium supplemented with varied concentrations of auxins and cytokinins. The present investigation revealed that MS supplemented with PGRs (BAP, KIN, IAA, IBA, and NAA) alone and in combination revealed the formation of multiple shoots and roots in *B. floribunda*. Our study revealed optimal regeneration responses when the MS medium was supplemented with cytokinins such as KIN and BAP. These findings are in accordance with earlier reports where researchers suggested that the highest concentrations of cytokinins promoted the regeneration of *B. monnieri* through axillary nodes and internodes

(Banerjee and Shrivastava, 2008). In addition, several researchers also reported that BAP was found to be the most effective in cytokinin, which played an important role in induction of multiple shoots in *B. monnieri* (Tiwari et al., 2001; Ceasar et al., 2010). According to Banerjee and Shrivastava (2008), internode explants of *B. monnieri* revealed a significantly higher number of shoots when inoculated into MS media supplemented with 1.0 mg/l BAP and 0.5 mg/l KIN. Our findings are in good agreement with Parale and Nikam (2009) who observed that BA and KIN at concentrations of 1 to 10 µM improved the shoot regeneration in *B. monnieri*. In all the auxin treatments, incorporation of 1.0 mg/l NAA in nutrient medium showed optimum root regeneration (Banerjee and Shrivastava, 2008). Researchers reported that roots of *B. monnieri* showed optimum growth when MS medium was supplemented with 1.5 mg/l NAA. It is a fact that PGRs such as cytokinins (BAP and KIN) stimulate the initiation and further elongation of shoots whereas auxins (IAA, IBA, and NAA) promoted the root initiation, growth, and development. In addition, the composition of nutrient media used in *in vitro* propagation also affected the plant growth and development (Komakech et al., 2020). Plant cell culture growth is mainly affected by nutrient concentration, stress variables, photoperiod, light quality, genotype, and concentration as well as combinations of plant growth regulators (Chavan et al., 2010).

The present study reported that among the precursor and elicitors used, utilization of 1.5 µM SQ in MS medium showed optimum regeneration responses. In accordance with our studies, Baek et al. (2019) also reported that squalene at different concentrations (up to 10 mM) increased the growth index of hairy roots of *Centella asiatica*. Likewise, elicitors at various concentrations altered the growth performance and the best response was observed when 80 µM MJ was added to the nutrient medium. Furthermore, cultures of *B. floribunda* grown on MS nutrient medium with methyl jasmonate, salicylic acid, potassium chloride, and magnesium chloride also flourished well. In the same way, Sharma et al. (2015) reported that abiotic elicitors (jasmonic acid, copper sulfate, and salicylic acid) stimulated the biomass production in *in vitro* shoot cultures of *B. monnieri*. Moreover, Largia et al. (2015) studied the influence of elicitors (methyl jasmonate, salicylic acid) on growth, biomass, and morphology of *B. monnieri*. Patil et al. (2013) also found that elicitation feeding improved the *in vitro* growth of *Digitalis purpurea*. Elicitors are compounds well known to increase the metabolic activities of phytochemicals and further improve plant performance. The plant physiology and biochemistry of the tissues are also affected by the type and/or concentration of the elicitor. Responses of the species also depend on the elicitor and its concentration added in the medium. In accordance with our findings, in plant cell cultures, the use of elicitors has been found to be beneficial (Guru et al., 2022). Additionally, a number of precursors and elicitors have been used earlier to induce morphological and physiological alterations in *in vitro* cultures (Patil et al., 2013; Watcharatanon et al., 2019). Optimal concentrations of PGRs, precursor, and elicitors alone and in combination were used to study the growth responses in *B. floribunda*. The goal of this experiment was to increase the production of *in vitro* biomass of *B. floribunda*. From the present investigations, we found that optimum cytokinin concentrations, i.e., MS + 2.0 mg/l KIN + 2.0 mg/l BAP, demonstrated the highest growth responses (average shoot length, number of leaves per shoot, FW, and DW). Martins et al. (2022) found that the combination of two cytokinins can be more advantageous than using only one, which is consistent with our findings. In our studies, also the combination of auxins (MS + 0.5 IAA mg/l + 0.5 IBA mg/l + 1.0 mg/l NAA) promoted the root initiation, elongation, and number of roots per shoot. According to earlier reports, rooting of the *in vitro* produced shoots of *B. monnieri* was higher when auxin-rich MS medium was used (Banerjee and Shrivastava, 2008; Sudheer et al., 2022). Selection of PGRs plays a crucial role in *in vitro* propagation of the plant. Cytokinins and auxins were commonly used in culture media to influence physiological reactions that led to promotion of the growth of shoots, roots, and whole plant. A number of researchers have investigated the impact of auxins (NAA, IAA) and cytokinins (BAP, KIN) on *in vitro* growth and secondary metabolites from the plant (Amoo and Staden, 2012; Martins et al., 2022). The highest concentrations of cytokinins offered the best outcomes, which may be due to the ability of BAP to promote cell proliferation and lateral bud development. It also plays a key role in altering the axillary bud dormancy. BAP exhibits a more sustained stimulation of cell division due to its stable nature and resistance to oxidation characteristics. In addition, it cannot easily broken down by plants (Komakech et al., 2020; Okello et al., 2021; Martins et al., 2022).

Well-developed plantlets were successfully acclimatized to the field conditions with a good survival rate of 95%, which was in agreement with earlier reports (Tiwari et al., 2001; Sharma et al., 2016;). These plantlets were morphologically similar to the field-grown plants. Acclimatized plants may have aberrant anatomy, physiology, or morphology, which takes longer to adapt and lowers growth. From various tested concentrations of PGRs, precursor, and elicitors, a reliable amount of total saponin content was noted. In cytokinins, MS + 1.0 mg/l BAP showed the highest TSC (31.79 ± 0.011 mg DE/g DW), whereas, among the tested auxins, incorporation of IBA in the medium MS + 0.5 mg/l IBA proved to be the best (32.0 ± 0.001 mg DE/g DW). In our studies, addition of precursor SQ (1.5 µM) revealed the highest TSC. Likewise, among all the elicitors tested, incorporation of KCl (80 mM) to the nutrient medium offered the highest content of total saponins. Additionally, combination of cytokinins and squalene (MS + 2.0 mg/l BAP + 1.5 µM SQ) demonstrated the best results as compared with other treatments. Our findings are in good agreement with Phrompittayarat et al. (2011). Triterpenoid saponin glycosides of *B. monnieri* were earlier reported from the aerial parts as well as shoots. The saponin content may vary depending on several variables, including the plant components, environment, season, and harvesting time. Watcharatanon et al. (2019) reported improvement in total triterpenoid saponin glycosides when *in vitro* cultures of *B. monnieri* were fed with the precursors and exposed to light-emitting diode (LED). The study suggested that saponin content may be enhanced as a result of the feeding of precursors and LED light exposure to *in vitro* cultures of *B. monnieri*.

HPLC analysis confirmed the presence of four triterpenoid saponin glycosides (bacoside A3, bacopaside X, bacopaside II, and bacosaponin C) from the *in vitro* grown biomass treated with various PGRs, precursor, and elicitors as well as acclimatized plantlets. On the other hand, several researchers identified and quantified bacoside

saponins from *B. monnieri* and also reported potent biological activities like anti-inflammatory, antioxidant, anti-hepatotoxic, and analgesic and superoxide inhibition (Bhandari et al., 2006; Sudheer et al., 2022). It is a classified and well-known drug used in the improvement of memory or mental power (Ganzera et al., 2004; Deepak et al., 2005; Bhandari et al., 2006; Naik et al., 2012; Saini et al., 2012; Bansal et al., 2014; Christopher et al., 2017; Bhandari et al., 2020).

Bacosides are the mixture of four saponin glycosides, *viz*., bacoside A3, bacopaside II, bacosaponin C, and bacopaside X, generally referred to as bacoside A (Nuengchamnong et al., 2016). Additionally, LC-ESI-QTOF-MS-based screening of the mixture of the authenticated saponin (bacoside A) also revealed that each of the individual compounds was identified individually by its mass as bacoside A3, bacopaside II, bacopaside X, and bacosaponin C. Additionally, total ion chromatogram (TIC) of the authenticated compounds proved that the bacoside mixture includes bacoside A3, bacopaside II, bacopaside X, and bacosaponin C (Nuengchamnong et al., 2016).

In the present findings, HPLC analysis confirmed the presence of four triterpenoid saponin glycosides in the *in vitro* grown biomass and acclimatized plantlets of *B. floribunda*. MS + 2.0 mg/l KIN alone highlighted the highest triterpenoid saponin glycosides, in which bacoside A3 (0.013 ± 0.001 mg/g DW), bacopaside II (9.934 ± 0.276 mg/g DW), bacopaside X (1.972 ± 0.101 mg/g DW), and bacosaponin C (0.060 ± 0.038 mg/g DW) contents were recorded. Similar to cytokinins tested, MS + 1.5 mg/l NAA revealed the highest contents of bacoside A3 (0.004 ± 0.001 mg/g DW), bacopaside II (6.16 ± 0.05 mg/g DW), bacopaside X (1.40 ± 0.044 mg/g DW), and bacosaponin C (0.043 ± 0.001 mg/g DW). Our results clarified that the higher concentrations of cytokinins (2 mg/l) proved to be the best and offered the highest triterpenoid saponin glycoside content. Our results are in agreement with reported results of Watcharatanon et al. (2019) who reported the highest triterpenoid saponin glycosides from *in vitro* grown cultures of *B. monnieri* (17.35 ± 0.13 mg/g DW). Likewise, Muzynska et al. (2016) also reported that shoots grown on MS medium supplemented with 1.0 mg/l BAP and 0.2 mg/l NAA revealed the highest bacoside A3 (2.18 ± 0.15 mg/g DW), bacopaside II (19.19 ± 1.1 mg/g DW), bacopaside X (0.67 ± 0.05 mg/g DW), and bacosaponin C (4.75 ± 0.01 mg/g DW) contents. Kamonwannasit et al. (2008) reported that shoots produced from internodal explants using MS + 0.5 mg/l TDZ showed remarkable pseudojujubogenin glycoside content (30.62 ± 1.29 mg/g DW). Among the different concentrations of the precursor used, MS medium enriched with 1.5 µM SQ revealed the highest total bacoside content (6.4 ± 0.10 mg/g DW). In accordance with our studies, Baek et al. (2019) also reported the enhancement of targeted triterpenoids in *Centella asiatica* hairy root cultures fed with 2.5 mM SQ. Dasari et al. (2020) also revealed that productivity of bioactive compounds depends on the concentration of chemicals used in the nutrient medium and also the exposure time. In *in vitro* grown biomass of *B. floribunda*, elicitors such as MJ, SA, KCl, and $MgSO_4$ were found to be the most effective for the production of triterpenoid saponin glycosides. In line with our findings, elicitors like methyl jasmonate and salicylic acid enhanced the bacoside production in shoot cultures of *B. monnieri* (Largia et al., 2015). Sharma et al. (2015) noted enhancement of triterpenoid saponins from *B. monnieri* by utilizing methyl jasmonate as the key elicitor. Researchers also studied the influence of different elicitors (jasmonic acid, copper sulfate, and salicylic acid) on stimulation of bacoside production in shoot cultures of *B. monnieri*. Biotic (chitosan) and abiotic (salicylic acid, methyl jasmonate) elicitors directly affected the metabolite(s) production in medicinal plants (Kaur et al., 2022). Among all various studied elicitors, MJ and SA have been well documented for the increased production of triterpenoid saponins in plant cell cultures (Largia et al., 2015). As compared with previous reported results, we found satisfactory results from *in vitro* grown biomass of *B. floribunda* treated with MJ and SA. Elicitation is a technique of inducing or increasing the production of secondary metabolites in plant tissue culture (Kaur et al., 2022). According to the studies, elicitors may serve as signaling molecules in proper quantities, and when perceived by a plasma membrane receptor, they trigger a complex signal transduction network that regulates gene expression and leads to the production of the targeted compound(s). The complexity of elicitor signal transduction, the diversity and specificity of connections between elicitor signals and plant cell receptors, and subsequent plant cell defense responses may play a role in the variation in yield of targeted compounds (Chavan et al., 2010). As most of the elicitors are easily available in the market or can be produced and given to cell cultures in the laboratory, they can be used to produce secondary metabolites on a large scale (Chavan et al., 2010). Based on the aforesaid findings, an optimal level of the precursor was combined with the optimal concentrations of PGRs to get the maximum amount of metabolite accumulation. Surprisingly, we discovered that the combination of SQ and PGRs used in the medium promoted the bacoside content (46.04 ± 0.10 mg/g DW). When cytokinin (BAP) and precursor (SQ) were used in combination, it significantly enhanced total *Bacopa* saponins. Additionally, among all tested concentrations of elicitors and precursors, squalene was discovered to be the best. As SQ was found in the production route of saponins, it considerably improved the triterpenoid saponin glycosides. The mevalonate pathway is a crucial metabolic pathway with end products such as 3,3-dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) that play a key mediating role in the formation of various secondary metabolites in plants. In order to produce different glycosylated triterpenoids, several enzymes participate in the structural alteration of these intermediates by substitution, oxidation, and glycosylation. Farnesyl pyrophosphate (FPP) molecules are converted to squalene by the enzyme squalene synthase, which is a key regulator in the biosynthesis of sterols and triterpenoids (Jeena et al., 2017; Murthy, 2022). According to earlier reports, *B. monnieri* was found to be an excellent source of bacosides. The results of our investigations showed that *in vitro* grown biomass of *B. floribunda* may serve as an alternate source of bacosides.

Additionally, stigmasterol is a subclass of phytosterols and has been shown to be preventative against cancer, diabetes, and hepatic and cardiovascular disorders. These sterols can reduce cancer risk by 20%, if included into the diet (Shahzad et al., 2017). Phytosterols have attracted the attention of researchers due to its diverse medicinal properties. In the current studies, cytokinin alone (2.0 mg/l BAP) showed the highest stigmasterol (7.69 ± 0.102 mg/g DW) content as compared with other levels of cytokinins. Similarly, MS supplemented

with IBA alone (1.5 mg/l) showed the highest stigmasterol content (2.31 ± 0.665 mg/g DW). In precursor and elicitor studies, incorporation of squalene (1.5 µM) and methyl jasmonate (80 µM) in the nutrient medium revealed the maximum stigmasterol content (1.41 ± 0.13 and 1.51 ± 0.10 mg/g DW, respectively). In optimal concentrations of PGRs, elicitors, precursor, and their combinations (MS + 1.0 mg/l NAA + 2.0 mg/l KIN) denoted the maximum (2.56 ± 0.03 mg/g DW) stigmasterol content. These findings are in good agreement with Ghosh et al. (2011). The authors reported stigmasterol from the aerial portions of *B. monnieri*. It has been demonstrated that phytosterols have the ability to decrease cholesterol and also possess anticancer properties (Ghosh et al., 2011). Numerous secondary metabolites including stigmasterol also demonstrated potential biological functions and play a significant role in the food and pharmaceutical industries. Different plant parts have medicinal potential due to the presence of secondary metabolites such as phenolics, flavonoids, alkaloids, terpenoids, and sterols (Otari et al., 2022; Patel et al., 2022; Ghane et al., 2018; Lekhak et al., 2021). Many bioactive substances appear to have a variety of health benefits; thus, the demand for them in the food and pharmaceutical industries is high (Bhattacharyya et al., 2022; Otari et al., 2022). *B. floribunda* was also identified as a potential candidate with an additional source of bioactive constituents including triterpenoid saponin glycosides and stigmasterol.

In the present investigation, the correlation among studied optimal mixed concentrations with their respective regeneration responses along with bioactive metabolites (total saponins, triterpenoid saponin glycosides, and stigmasterol) was studied using a data reduction tool, i.e., PCA. The optimum concentration of cytokinin (MS + 2.0 mg/l BAP + 2.0 mg/l KIN) was identified as the best treatment that showed the highest shoot length, higher number of leaves per shoot, FW, and DW. Similarly, optimum mixed combinations of cytokinin with the precursor (MS + 2.0 mg/l BAP + 1.5 µM SQ) contributed toward the triterpenoid saponin glycosides. Similarly, the combination of auxins (MS + 0.5 mg/IAA + 0.5 mg/IBA + 1.0 mg/l NAA) revealed the highest root length and number of roots per shoot. According to the above findings, it can be concluded that cytokinins (BAP and KIN) were responsible for shoot growth whereas root initiation and multiple roots per shoot were mainly controlled by the combined treatment of optimal auxins (NAA, IBA, and IAA). Significant amounts of bioactive compounds were found in the optimal mixed combinations of cytokinin and precursor (MS + 2.0 mg/l BAP + 1.5 µM SQ). Jauhari et al. (2019) reported a strong correlation between total bacosides and phytochemical content due to incorporation of elicitors in the nutrient medium. It was noted that precursors play a vital role in boosting the bioactive potential in plants. Previous reports stated that PCA is the most powerful statistical method for evaluating the relationship between a large number of variables and condensing the data into small elements (Otari et al., 2022; Patel and Ghane, 2021; Ghane et al., 2018).

A thorough analysis confirmed the remarkable amount of the desired metabolites (total saponins, triterpenoid saponin glycosides, and stigmasterol) in *in vitro* grown biomass of *B. floribunda*. The favorable outcomes also underlined the goal of improving the production of secondary metabolites in the *in vitro* cultures of *B. floribunda* by using precursor and elicitor feeding. The current research work provides an appropriate approach for mass multiplication and also validates the biosynthesis and accumulation of secondary metabolites in *in vitro* grown biomass of *B. floribunda*. It forms the first report on *in vitro* propagation and elicited production of total saponins, triterpenoid saponin glycosides, and stigmasterol contents from *B. floribunda*. It could be a reliable source of nootropic drugs as well as an important substitute to *B. monnieri*.

Conclusion

From the studies, we conclude that MS medium fortified with 2.0 mg/l BAP + 2.0 mg/l KIN was found to be the best for shoot development. Similarly, MS + 0.5 mg/l IAA + 0.5 mg/l IBA + 1.0 mg/l NAA promoted the root initiation and number of roots per shoots. The *in vitro* regenerated *B. floribunda* plants showed a survival rate of up to 95% after acclimatization and was found to be identical to the mother plant. The presence of triterpenoid saponin glycosides and stigmasterol in *in vitro* grown biomass was confirmed by HPLC, wherein MS + 2.0 mg/l BAP + 1.5 µM SQ offered the highest content of total pseudojujubogenin glycosides. Similarly, biomass obtained from MS medium fortified with 2.0 mg/l BAP exhibited the highest content of stigmasterol. The species must be conserved in order to produce valuable pharmaceuticals commercially (such as bacosides and stigmasterol), and its sustainable utilization is required. It may serve as a potential candidate and alternate substitute to the Indian Pennywort. Further, studies on detailed phytochemical diversity, pharmacological activities, and elicited production of bioactives using biotechnological tools are needed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SGG designed the experiment. The experiment was carried out by SSO, SPD, and SBP performed all the experiments. SO drafted the manuscript and had it critically reviewed by SGG. SSO, SBP and SGG took part in the statistical analysis and assisted in refining the manuscript. All authors contributed to the article and approved the submitted version.

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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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Inductive role of the brown alga *Sargassum polycystum* on growth and biosynthesis of imperative metabolites and antioxidants of two crop plants

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The potential of macroalgae as biostimulants in agriculture was proved worthy. *Vicia faba* and *Helianthus annuus* are socioeconomic crops owing to their increasing demand worldwide. In this work, we investigated the energetic role of seed presoaking and irrigation by the brown seaweed, *Sargassum polycystum* aqueous extract (SAE) on certain germination and growth traits, photosynthetic pigments, carbohydrates, phenolics, flavonoids, and the total antioxidant activity. Compared to the control plants, our consequences revealed that seeds that received the SAE improved all the germination and growth criteria for both crop plants. Furthermore, the SAE significantly increased the carotenoids, total photosynthetic pigments, and total carbohydrates by (14%, 7%, and 41%) for *V. faba* and (17%, 17%, and 38%) for *H. annuus*, respectively. Phenolics and flavonoids were significantly induced in *Vicia* but slightly promoted in *Helianthu* plants, whereas the total antioxidant activity in both crops non significantly elevated. Even though The NPK contents were significantly stimulated by the SAE in *Vicia* plants, the effect was different in *Helianthus*, where only nitrogen content was significantly enhanced, whereas phosphorus and potassium showed little enhancement. Thus, the SAE treatment is one of the superlative sustainable strategies for food, feed, and as excellent plant conditioner.

KEYWORDS

biostimulation, seaweeds, polysaccharides, antioxidants, animal nutrition, soil health

Introduction

Nowadays, fertilizer improvement has become an urgent global concern because of economic needs (Agathokleous et al., 2022). There is anxiety about environmental pollution from the application of chemical fertilizers, which cause long-term, extensive adverse effects on soil fertility, environmental dysfunction, greater soil salinity and degeneration, the introduction of cadmium into the crops *via* the application of P fertilizers, and higher production costs (Pahalvi et al., 2021).

Broad bean (*Vicia faba.* L., Leguminosae) crop is among the most important legume crops because of its high nutritional value that is cultivated for its green pods. It is considered a valuable and cheap protein source instead of animal protein. Also, it increases soil fertility nitrogen fixation, and the beans, husks, and hay are used as animal fodder (Hassan and Ghani, 2001).

On the other hand, sunflower (*Helianthus annuus* L., Asteraceae) is the world's fourth largest oil crop containing high-quality edible oil. It has excellent nutritional properties and is easily cultivated and grown in different conditions and soils. The phytoremediation properties of *Helianthus* are widely established. Also, it could be composted and returned to the soil as fertilizer (Fozia et al., 2008)

Hence, organic fertilizers are strongly recommended for more agricultural safety. The term "bio-fertilization" refers to a sustainable agriculture method that involves the use of bio-fertilizers to improve soil nutritional quality, resulting in greater yields. Macroalgal extracts have really been applied as agricultural biostimulants (Abs) to enhance plant growth and development (Ammar et al., 2022).

Seaweeds include a wide variety of antioxidants, plant growth promoters, minerals, and other unique biomolecules. In addition, stimulation of plant defense mechanisms *via* polysaccharides and oligosaccharides derived from seaweeds is a potential protective strategy (Benhamou and Rey, 2012). Importantly, marine macroalgae are renewable, biodegradable, eco-friendly, and cost-effective sources of biofertilizer (Ammar et al., 2022). Natural seaweed as a biofertilizer can substitute the synthetic fertilizer that has reduced soil fertility, turning it more acidic and unfavorable for growing crops (Pahalvi et al., 2021).

Recently, several studies reported the stimulatory effect of seaweeds extracts on different legumes and Asteraceae crop plants. A strong response of the three peanut varieties was caused by applying the *Sargassum vulgare* extract, which led to higher antioxidant activity (Ben Ghozlen et al., 2023). Seaweed organic fertilizer combined with standard NPK dosage can increase vitamin C and fiber content in bush beans (Rahayu et al., 2021). In two different chickpea genotypes, several agronomical parameters recorded maximum values in treatment with the *Ascophyllum nodosum* extract (Kurakula and Rai, 2021). On the other hand, soil application of mineral fertilizer combined with foliar spraying of seaweed extracts was most beneficial for the yield and quality parameters of globe artichoke plants (Elsharkawy et al., 2021; Petropoulos et al., 2022). In addition, a foliar fertilizer extract from seaweed achieved better yield and quality for lettuce crop (Hoa et al., 2022).

The goal of our effort is to appraise the impact of seed presoaking and irrigation by the *Sargassum polycystum* aqueous extract (SAE) on germination, plant vegetative growth and different crucial metabolic contents of two socioeconomic crops in Egypt, *Vicia faba* and *Helianthus annuus*. We tried to clarify the vital role of the SAE secondary metabolites that greatly affect the nutritional quality of the two tested crops. Moreover, we shed the light of the co-benefits of using the algal extracts of brown seaweed; as a biostimulant in different commercially important applications such as human consumption, animal nutrition, and improving the soil health for facing the incident climatic changes and the food crises that strike our planet nowadays.

Materials and methods

Seaweeds collection and preparation

The samples of the seaweed, *Sargassum polycystum*, were taken in July 2020 from the coast-line region near the industrial area in Jizan city, which is located on the Red Sea in the Kingdom of Saudi Arabia (about 16°49'20.8" North, 42°37'17.0" East). On-site seaweeds were rinsed with seawater, tap water three times, and bottled drinking water and morphologically recognized on genus level (Guiry, 2010). Shade-dried seaweeds were 40°C for 7 days. Dry algal biomass was chopped, processed, and sieved using 2 mm screen for future use.

Algal extract preparation

In a 250 ml flask, 5 g of dried and grinded algal biomass were added to 100 ml of distilled water. The mixture was kept in a water bath at 40 ° for 12 h, then filtered using Whatman number 1 filter paper to get a pure seaweed aqueous extract. After that, the prepared extract was preserved at 4° for further work.

Plant materials

Healthy-looking and uniform-sized seeds of *Vicia faba L.*, Leguminosae and *Helianthus annuus* L., Asteraceae were obtained from the Agriculture Research Centre (ARC), Giza, Egypt.

Bioassay for the effect of algal aqueous extract

Twenty seeds from each of the two tested plants were surface sterilized using 0.01% $HgCl_2$ for one minute and rinsed gently with sterilized distilled water. Twenty seeds from each tested plant were presoaked in the SAE. Simultaneously, another 20 seeds of each crop were treated with distilled water only as control samples, then kept on filter paper (Whatman No. 1) inside sterilized Petri dishes (9 cm) at room temperature (28°C ± 1). For both control and treatment seeds, each filter paper was kept moist *via* regular tap water addition.

Germination parameters

Germination percentage (GP), the seedling Vigor index (SVI), as well as seedling growth characteristics, such as radicle length (RL; cm), plumule length (PL; cm), seedling height (SH; cm), seedling fresh weight (FW; g seedlings^{-1}), and seedling dry weight (DW; g seedlings^{-1}), were also determined 10 days after sowing (10 DAS).

Germination (%): the number of normal seedlings was counted according to the following formula:

$$\text{Germination}(\%) = \frac{\text{No . of germinated seeds}}{\text{Total No . of tested seeds}} \text{x}100$$

Seedling Vigor index (SVI) was calculated according to Abdul-Baki and Anderson (1973) using the next formula:

SVI = Germination (%) * Total seedling length (cm)

Treatments pattern and experimental design

The experiment was performed with 40 plastic pots containing soil mixture (sand: clay, 1:2 V/V) and watered with water holding capacity using a completely randomized block design (CRD). Fertilizers such as superphosphate and urea were put in all pots. The 40 pots were then divided into four groups (treatments). Each group has ten pots, one for each treatment's replication. The sterilized seeds of *Vicia faba* L. and *Helianthus annuus* L. were separately soaked in deionized water (50 seeds of each strain for control). Before sowing, the other 50 seeds of each strain were individually soaked in *Sargassum* aqueous extract (SAE). The control and treated seeds were irrigated by tap water except for irrigation practice on the 8th day after sowing, where the treated plants were irrigated by the mixture of algal extract and water (1:3 V/V), while the control plant was normally irrigated by tap water. Plants were picked 45 days after sowing (DAS) to evaluate growth biomarkers and physiological characteristics.

Growth biomarkers

After eliminating soil particles from the roots and washing them with distilled water, the shoot length was measured on a meter scale, and the fresh biomass of the shoot was measured using a weighing balance. All samples were kept in the oven for 48 h at 70 °. After allowing the dried plants to cool at room temperature, they were weighed again to record the shoot dry weight. The plant moisture content was calculated on the basis of wet weight according to by means of the following formula:

$$\text{Moisture content}\ (\%) = \frac{\text{W2} - \text{W3 x 100}}{\text{W2} - \text{W1}}$$

W1 = weight of the empty container with lid

W2 = weight of the container with lid and sample before drying

W3 = weight of the container with lid and sample after drying.

The number of leaves per plant was counted. The leaf area (LA) per plant was determined using the squared papers method and using the equation,

M2 = M1 x W2/W1, (Haroun, 1985)

Where M1 and W1 are the area and weight of the square paper and M2 and W2 are the area and weight of the plant leaf, respectively.

Leaf area index (LAI) was calculated according to Amanuallah et al. (2007) using following formula,

Leaf Area Index = Leaf area per plant (m^2) x No. of plants per m^2

Physiological measurements

Extraction and estimation of photosynthetic pigments

For the determination of different pigments, we applied the methods authorized by Arnon (1949) and Horvath et al. (1972) and modified by Kissimon (1999). Briefly, the plant leaves were carefully ground with 80% acetone for 5 minutes. After 3 minutes of centrifugation at 1000 rpm, the supernatant was measured at 480, 644, and 663 nm wavelengths.

Estimation of carbohydrates content

Soluble sugar extraction from pre-dried samples of plant shoots was performed using 80% ethanol determined according to the anthrone sulfuric acid procedure (Whistler et al., 1962). Polysaccharides concentrations were estimated in plant residue left after the extraction of soluble sugars. Finally, the total content of carbohydrates was determined by the summation of the total contents of polysaccharides and soluble sugars for each sample. All results were recorded as mg 100 g^{-1} DW of shoots.

Assay of total phenolics

The Folin-Ciocalteu reagent was used in accordance with Singleton and Rossi's (1965) methodology to determine the total phenolic content of the plant. At a wavelength of 725 nm, the absorbance was measured. Total phenolics were determined using a standard curve, and the results were expressed as gallic acid equivalents (GAE) in milligrams per one hundred grams of dried material.

Assay of total flavonoids

The Aluminum Chloride Calorimetric Assay was used to derive an estimate of the total flavonoid content (Zhuang et al., 1992). The total flavonoid was determined using the standard plot, and the results were presented in the form of mg catechin equivalent per 100g of dried sample.

Assay of total antioxidant capacity

The phosphomolybdenum method measured the plant methanolic extract's antioxidant capability (Prieto et al., 1999). Ascorbic acid equivalent mg/100g dry sample represents the antioxidant activity.

Quantification of total nitrogen

The total plant nitrogen was evaluated by the micro- Kjeldahl method (Pregi, 1945). Titration against a standard sulphuric acid

(0.0143 N) was used to determine each sample, with bromocresol green and methyl red (3:2 v/v) as indicators until the end point (a faint red color) was reached. The titration figures were converted into mg nitrogen using:

1 ml of 0.0143 NH_2SO_4 = 0.28 mg Nitrogen

Estimation of potassium and phosphorus

Based on the wet ashing method, the dried plant matter was digested according to Chapman and Pratt (1962). Potassium was determined by the flame emission technique as adopted by Ranganna (1977). Phosphorus was estimated simultaneously by inductively coupled plasma optical emission (ICP) Spectrometry using the method of Soltanapour (1985). Data were calculated as ppm.

Qualitative analysis of phytochemical substances in algal extracts

The *S. polycystum* extract was phytochemically screened using Harborne's method (1998). The alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins, quinones, and glycosides were identified *via* phytochemical screening. The general responses that took place throughout these studies revealed whether or not the algal extracts that were analyzed contained these chemicals.

Test for Alkaloids: Alkaloid identification required 2 mL of strong hydrochloric acid and 2 mL algal extract. Mayer's reagent was added. Alkaloids appear green or white.

Test for Terpenoids: Terpenoids were identified by adding 2 milliliter chloroform and concentrated sulphuric acid to 0.5 ml algal extract. A reddish brown interface shows terpenoids.

Test for Steroids: 2 mL chloroform and 1 mL sulphuric acid were added to 0.5 mL algal extract to identify steroids. Steroids cause reddish brown interface rings.

Test for Tannins: The algal extract was mixed with 1 mL of 5% ferric chloride to identify tannins. Tannins cause dark blue or greenish-black.

Test for Saponins: 2 mL distilled water and 2 mL algal extract were agitated in a graduated cylinder for 15 min to identify saponins. Saponins cause 1 cm foam.

Test for Flavonoids: 2 mL algal extract was mixed with 1 mL 2N sodium hydroxide to identify flavonoids. Yellow denotes flavonoids.

Test for Phenols: Adding 2 mL of distilled water and a few drops of 10% ferric chloride to 1 mL of algal extract identified phenols. phenols cause blue/green color.

Test for Coumarins: In order to identify the coumarins, 1 milliliter of algal extract was mixed with 1 milliliter of 10% sodium hydroxide. The appearance of a yellow color is a clear signal that coumarins are present.

Test for Quinones: Algal extract was mixed with 1 mL concentrated sulphuric acid to identify Quinone. Red color denotes quinones.

Test for Glycosides: Glycosides were identified by adding 3 mL chloroform and 10% ammonium solution to 2 mL algal extract. Pink denotes glycosides.

Statistical analysis

All experiments were conducted in three replicates, and the results are recorded as the mean ± standard deviation. For statistical purposes, one-way ANOVA was applied to test the significant differences between treatments using the statistical software SPSS (IBM, v. 25) treatments followed by *post hoc* Duncan's test to determine the difference in growth and metabolic parameters between the target treatment group and the control group at a probability level (P) ≤ 0.05.

Results

Effect of *Sargassum polycystum* aqueous extract on the germination traits

The data documented in Table 1 and illustrated by Figure 1 revealed that SAE does not actually affect the germination percentage as it recorded 100% for both control and treated *V. faba* and *H. annus* plants. At the same time, the seedling vigor index (SVI) improved by (24.09%) and (22.73%) in *V. faba* and *H. annuus*, respectively, over their controls.

Although the SAE treatment non-significantly enhanced the length of plumule and seedling dry weight of *Vicia* plants (30.25% and 8.05%) over the control plants, all the other estimated germination traits were significantly increased (17.76%, *Helianthus* plumule length), and (24.09% *Vicia* radicle) and (20.16% *Helianthus* radicle), (41.22% *Vicia* seedling height) and (22.73% *Helianthus* seedling height). The fresh weights were significantly induced up to 18.15% and 31.62% in *Vicia* and *Helianthus*, respectively, whereas *Helianthus* dry weight raised by 7.14% over the control plants.

Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on growth biomarkers

Shoot growth criteria

Biostimulants are natural organic compounds that, in low concentrations, can improve plant growth, nutrient absorption, stress tolerance, and crop productivity. Except for *Helianthus* dry weight, data itemized in Table 2 showed that the SAE exhibited a significant effect on all estimated morphological traits. However, *faba* plants were more affected by the SAE, as it achieved a higher increment in shoot length, shoot fresh and dry weights, and shoot water content. These boosts were evaluated by 49, 32, 19, and 1.9%, respectively, compared to the *faba* control. The corresponding improvements for the same growth criteria were estimated by 15, 18, 33, and 1.05% one-to-one in *Helianthus* shoots.

Leaf growth parameters

Data which were enumerated in Table 2, exposed that the SAE significantly increased leaves number/plant (19 and 33%), leaf area

TABLE 1 Effect of presoaking in *Sargassum polycystum* aqueous extract on germination parameters of *Vicia faba* and *Helianthus annuus* plants.

		Plumule Length (cm)	Radicle Length (cm)	Seedling total height (cm)	Seedling fresh weight $(g)^{-1}$	Seedling dry weight $(g)^{-1}$	Seedling Water content	Seedling vigor index
V.faba	Control	2.856^{a} ± 0.573	4.730^{b} ± 0.839	7.156^{b} ± 1.131	2.865^{b} ± 0.403	0.696^{b} ± 0.002	75.266^{a} ± 3.615	715.56^{b} ± 113.148
	Treated	3.720^{a} ± 0.783	5.570^{a} ± 1.462	8.880^{a} ± 2.371	3.385^{a} ± 0.497	0.752^{a} ± 0.002	77.265^{a} ± 4.011	888.00^{a} ± 237.056
H.annuus	Control	6.579^{b} ± 1.218	2.926^{b} ± 0.738	9.715^{b} ± 1.271	1.145^{b} ± 0.115	0.070^{b} ± 0.002	93.864^{b} ± 0.657	971.48^{b} ± 127.144
	Treated	7.935^{a} ± 1.061	4.132^{a} ± 4.132	11.923^{a} ± 2.193	1.507^{a} ± 0.218	0.075^{a} ± 0.001	94.923^{a} ± 0.587	1193.16^{a} ± 219.340

^{a}, ^{b}significantly different according to DMRTs at 0.05 level.

(19.51 and 9.16%), and leaf area index (17.65 and 11.11%) one-to-one in *V. faba* and *H. annuus* respectively.

Flowering and nodulation

Table 2 exposed that the algal treatment produced more than two times (55.6%) root nodules in *Vicia* roots as compared to their control and enhanced nodules size remarkably (Figure 2). With respect to flower formation, the employed SAE treatment significantly amplified the flower number in *V.faba* only (Table 2), while the *H. annuus* did not bloom till the end of the experiment schedule (45 DAS). In addition, the SAE significantly enhanced flowering in faba plants (48.3%).

Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on the photosynthetic pigments

Although, the SAE treatment significantly increased the carotenoids and total photosynthetic pigments by 14.34% and 17.09% and 7% and 17% over the control plants of *Vicia* and *Helianthus* in that order (Figure 3). Chlorophylls a and b were not significantly affected in *Vicia* but significantly affected in *Helianthus* plants.

Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on the carbohydrate content

Complementarily with our previous results, the SAE treatment significantly improved carbohydrate content in both crop plants. Although soluble sugars moderately enhanced (15% and 16%), polysaccharides highly improved by 47% and 48% for both plants. Consequently, total plant carbohydrate content increased by 41% and 38% for *Vicia* and *Helianthus*, respectively Figure 4

Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on some non-enzymatic antioxidants component; phenolics and flavonoids

According to the data presented in Figure 5, phenolic and flavonoid contents were significantly induced in *Vicia* but not significantly promoted in *Helianthus* plants. Phenolic content was enhanced by 18% and 43%. Whereas flavonoids improved by 28% and 18% in *Vicia* and *Helianthus* shoots, respectively. This enrichment nominates the treated plants as preferable fodder for animal nutrition.



FIGURE 1
Effect of presoaking in *Sargassum polycystum* aqueous extract (SAE) on the germination of *Vicia faba* and *Helianthus annuus*, 10 days after sowing **(A)** Control and treated *Vicia* plants, **(B)** Control and treated *Helianthus* plants.

TABLE 2 Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on the growth biomarkers of *V. faba* and *H.annuus* plants.

		Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Water content %	Leaf number per plant	Leaf area (mm^2)/ plant	Leaf area index	Number of root nodules	Number of Flowers
V.faba	Control	13.64^{b} ± 1.05	2.62^{b} ± 0.11	0.35^{b} ± 0.01	86.49^{b} ± 0.39	10.4^{b} ± 0.89	46.49^{b} ± 2.56	.0017^{b} ± 0.0	1.6^{b} ± 0.548	5.8^{b} ± 1.304
	Treated	20.32^{a} ± 1.86	3.45^{a} ± 0.53	0.41^{a} ± 0.05	88.13^{a} ± 0.63	12.4^{a} ± 0.55	55.56^{a} ± 3.95	.0020^{a} ± 0.0	2.8^{a} ± 0.837	8.6^{a} ± 1.140
H. annuus	Control	19.04^{b} ± 1.27	2.32^{b} ± 0.23	0.17^{a} ± 0.02	92.39^{b} ± 0.24	4.8^{b} ± 1.1	24.56^{b} ± 2.51	.0009^{b} ± 0.0	–	–
	Treated	21.82^{a} ± 0.4	2.74^{a} ± 0.14	0.18^{a} ± 0.02	93.36^{a} ± 0.44	6.4^{a} ± 0.89	26.81^{a} ± 0.99	.0010^{a} ± 0.0	–	–

$^{a, b}$significantly different according to DMRTs at 0.05 level.

Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on the total antioxidant capacity

Referring to Figure 5, The total antioxidant activity was not significantly induced by 8.5% and 8.4% for *Vicia* and *Helianthus*, respectively.

Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on NPK content

The data in Figure 6 exposed that the algal extract application significantly promoted the production of the total -N content by 7% and 5% in *Vicia* and *Helianthus* compared to untreated plants. Although The phosphorus and potassium contents were significantly stimulated by the SAE in *Vicia* plants (10% and 26%), The situation was different in *Helianthus*, where phosphorus and potassium non significantly raised (12% and 4%) in *Helianthus* shoots.

Discussion

Among important sources of biostimulants are humic acid, chitosan, fungi, beneficial bacteria, and seaweed extracts (El-Beltagi et al., 2019). Seaweeds show an array of physiological and biochemical characteristics. Macroalgal extracts are rich sources of plants growth regulators (auxins, gibberellins, cytokinins and abscisic acid) in addition to the macronutrients, and micronutrients as well as amino acids and vitamins (Issa et al., 2019) and polysaccharide and oligosaccharide contents (Benhamou and Rey, 2012).

Our findings in Table 1 are consistent with the results of El-Sheekh and El-Saied (2000), who documented that the crude extract of the green seaweed *Corallina mediterranea* enhanced seed germination, root and shoot length and lateral roots number of

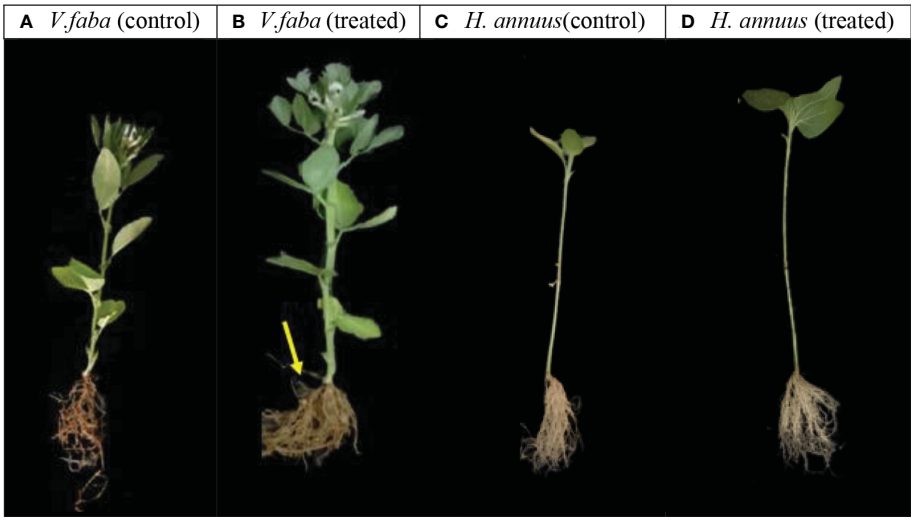


FIGURE 2
Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract on shoots and Roots morphology of treated *V. faba* **(A, B)** and *H. annuus* **(C, D)** 45 days of planting.(Yellow arrow shows nodule size in the treated *Vica* plants).

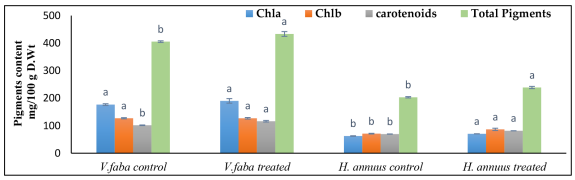


FIGURE 3
Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract (SAE) on the photosynthetic pigments of *Vicia* and *Helianthus* plants 45 days after sowing. Bars with different letters are significantly different according to DMRTs at 0.05 level.

Vicia faba. In harmony, Baroud et al. (2019) documented that *Fucus spiralis* extract improved the germination percentage, radicle and hypocotyls length, total seedling length, dry weights and seedling biomass of pepper (*Capsicum annuum*).

Raj et al. (2015), reported the existence of steroids in the methanol extract of *Sargassum tenerrimum*. Sterols are cell membrane structural components that modulate membrane permeability and fluidity. Sterols, as a component of the algal extract, have been proven to have a beneficial influence on seed germination. For example, seeds of transgenic *Brassica juncea* overexpressing the HMG-COA SYNTHASE gene resulted in greater sterol centration and speeder germination than the wild type (Wang et al., 2012). Moreover, Vriet et al. (2012), stated that BRs and sterols are involved in germinating seeds under regular and stressed conditions. The incident growth improvement that happened in the *vicia* and *Helianthus* plumule, radical, shoot and root criteria (Tables 1, 2) may be attributed to the existence of the plant growth hormone as a component of the seaweed extract as recorded by the speculation of Crouch and Van Staden (1993) when cited that, the existence of a broad spectrum of plant growth-promoters is related to the wide variety of growth responses elicited by seaweed extracts. This interpretation has strongly supported the role of auxins and cytokinins, which regulate shoot and root development (Kurepa and Smalle, 2022).

In the same connection, Baroud et al. (2019) reported that plants treated with (2%) of the Bifurcaria bifurcate and *Fucus spiralis*, aqueous extract showed higher t growth biomarkers (root and shoot length, total plant height, and dry weight). Leaf length did not differ between cultivars, but leaf area index (LAI) and leaf number may differ between them throughout the growing season (Castro-Nava et al., 2016). The leaf area index (LAI) is a non-dimensional variable used to evaluate different agronomic traits such as canopy, photosynthesis and evapotranspiration, which play a vital function in the transformation of energy and mass between the atmosphere and plant canopy in an ecosystem (Law et al., 2001).

Leaf area index (LAI) is related to liquid photosynthesis performed by crop plants (Wiedenfeld and Enciso, 2008). This association exists because the LAI is closely related to the amount of light absorbed and, as a consequence, to the photosynthetic activity conducted by plants (Scarpari and Beauclair, 2009). The recorded boosting LAI (Table 2) as a response to the SAE treatment is probably due to the increment in leaf area and number per plant. This finding may also contribute to a considerable relation between total photosynthetically active surface and crop productivity, as documented by Sandhu et al. (2012).

The attained enhancement in *Vicia* flower numbers (Table 2) a result of the SAE treatment may be explicated by the existence of the phytohormone cytokinin as a constituent of seaweed extract (Issa et al., 2019). Cytokinins play a role in different aspects of plant development and growth and physiological evidence suggests that they also are involved in floral transition. D'Aloia et al. (2011) elucidated the putative roles of cytokinins during the floral transition in Arabidopsis.

Seaweeds' hormonal profiles are very similar to those of higher plants. Many plant hormones have been discovered, such as biologically active forms of auxins, ABA, gibberellins, and cytokinins (Issa et al., 2019). Salicylic acid, ethylene, brassinosteroids, strigolactones, and jasmonates, were also detected in macroalgal extracts (Stirk and Van Staden, 2014).

In accordance, flowering enhancement may also be interpreted by the incidence of poly- and oligosaccharides in the SAE (Benhamou and Rey, 2012). Sugars, in low concentrations, control different phases of the cell life cycle, including cell differentiation, vegetative and organ development, flower and

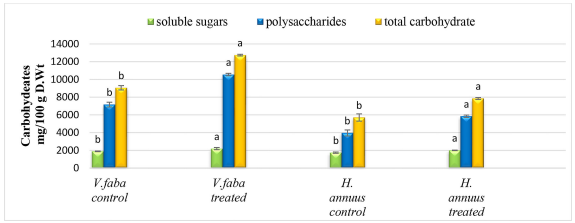


FIGURE 4
Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract (SAE) on the carbohydrates content of *Vicia* and *Helianthus* plants 45 days after sowing. Bars with different letters are significantly different according to DMRTs at 0.05 level.

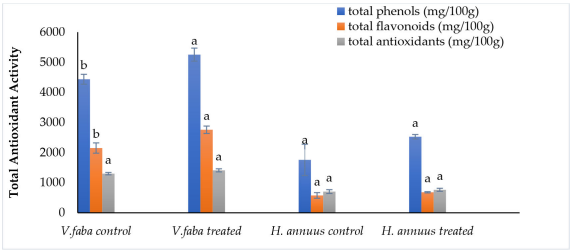


FIGURE 5
Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract (SAE) on the phenolics, flavonoids content and total antioxidant activity of *Vicia* and *Helianthus* plants 45 days after sowing. Bars with different letters are significantly different according to DMRTs at 0.05 level.

fruit production, as well as defense reactions and maturity (Ciereszko, 2018).

The intensification in nodules number in Vicia roots (Table 1) may reflect the enhancement in total nitrogen production in the same treated plants (Figure 6). In a recent study, Darwish et al. (2022) registered an improved nodulation both in nodules numbers and fresh weight in parallel with the enhancement of root weight and length by enhancing strigolactone biosynthesis in soybean plants. Strigolactones were also detected in macroalgal extracts (Stirk and Van Staden, 2014).

In coordination with our outcomes, Abd El-Gawad et al. (2015) indicated that the algal extract positively affected nodulation in *V.faba* as it enhanced both Nodule dry weight and nitrogen content. Recently Darwish et al. (2022) registered a raised nodule number, fresh nodule weight, both root length and weight, by enhancing strigolactone biosynthesis in soybean plants.

The synchronized changes in leaf area (Table 2) and pigment contents (Figure 3) faced in our investigation are powerfully supported by the postulation of Beckett et al. (1994), who assumed that seaweed extract acts as some kind of bio-stimulant which increases yield at least in two ways. First, it might have increased the source capacity of the leaves, thus increasing the available assimilation supply by increasing leaf area and photosynthetic rates. Second, the algal extract might increase seed weight *via* enhancing the sink potential of the fruit for assimilates, thus increasing cotyledon cell number and final seed mass.

In support of the former attitude, Salah El Din et al. (2008) and Scarpari and Beauclair (2009) interrelated the variation of plant photosynthesis rate to the leaf size. They suggested that, a larger leaf size has a larger surface area with more chloroplasts, receives more sunlight, and has more stomata on the surface of the leaves, which play a vital role in gas exchange during the photosynthesis process. The enhanced leaf area and photosynthetic pigments recorded by Table 1 and Figure 3 routinely led to elevated sugar contents (Figure 4) in both tested plant strains, *Vicia* and *Helianthus*, especially the levels of the polysaccharides which exceeded too much those of the soluble sugars. The last speculation may be correlated to the fact that the seaweed extracts contain polysaccharide and oligosaccharide contents (Benhamou and Rey, 2012).

In the same concern, El-Sheekh and El-Saied (2000) reported that the crude extracts of different seaweeds increased chlorophyll content and total soluble sugars in *V. faba*. The stimulated growth of seedlings was a response to the employed algal extract but to different degrees. Likewise, Baroud et al. (2019) registered a high enhancement in the total sugar content of pepper plants as an outcome of treating the plant seeds with aqueous extracts of brown algae.

In synchronization, Abbas (2013) demonstrated that algae extract positively influences various metabolic functions such as respiration, photosynthetic activity, ion uptake, as well as leaf pigmentation. Generally, the influence of SAE on chlorophyll levels may be correlated to the probable existence of betaines (Baroud et al., 2019). On the other hand, Abdel Hadi et al. (1993) attributed the high increments in growth criteria and chlorophyll contents of a soybean plant to algalization.

This elevation of sugar content (Figure 4) as a consequence of SAE induces appropriate cellular reactions and affects some gene

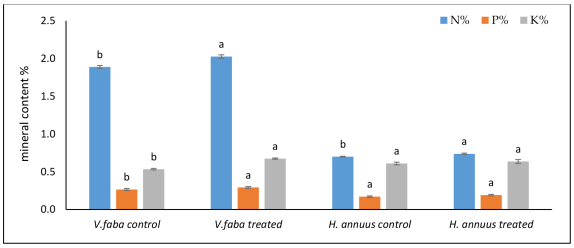


FIGURE 6
Effect of presoaking and irrigation by *Sargassum polycystum* aqueous extract (SAE) on NPK content of *Vicia* and *Helianthus* plants 45 days after sowing. Bars with different letters are significantly different according to DMRTs at 0.05 level.

expressions and metabolic processes. Sugars serve as structural and storage compounds, respiratory substrates, and intermediate metabolites in various metabolic activities. They can also serve as signaling molecules and be transported over long distances (Ciereszko, 2018).

An urgent challenge faced the animal scientists is the augmented demand for safe animal food from natural feed additives. Animal health is impacted or even regulated by antioxidant supplementation in animal feed (Christaki et al., 2020). On the other hand, agricultural waste and by-products are rich sources of valuable compounds, such as phenols and antioxidants, which could be exploited as functional elements in animal feeds (Fontana et al., 2013).

The presence of simple phenols was reported in green seaweed. Furthermore, brown macroalgae show higher contents of phenolics than other types of seaweeds. Phenolics and flavonoid compounds which has been demonstrated to exist in the tested SAE (Table 3), have a fundamental role in different biological activities in plants. In addition, they are believed to be secondary ROS-scavenging systems that guard the plant against diverse environmental stresses (Fini et al., 2011).

As presented in Table 3, *S. polycystum* extract contains several chemical components that may be responsible for the stimulatory effect on both tested plants. In addition, phenols were markedly presented in the extract, which may explain the increased phenolic content in the treated plants compared to the control.

From this point of view, our outcomes illustrated in Figure 5 revealed that the SAE treatment improved both crops' phenolic and flavonoid content. These outcomes are not only of great significance for plant growth but also to introduce these crops by products as nontoxic animal fodder (Hassan and Ghani, 2001) and hence a human health preservation target.

Adding phenolics to animal diets may have positive impacts on animal gut health, involving anti-inflammatory and antibacterial activities, saving action on vitamin antioxidants, and oxidative stability of food originating from farm animals (Starčević et al., 2015). Thus, phenolics have a known impact on meat quality directly or indirectly.

This obtained enhancement in phenolics and flavonoids contents (Figure 5) may be owed to the richness of seaweed aqueous and methanolic extracts by numerous metabolites such as steroids, terpenoids, phenolics, flavonoids, carbohydrates, and xanthoproteins (Raj et al., 2015; Ammar et al., 2022).

Cheynier et al. (2013) interpreted the improvement of flavonoids content to the implementation of allelochemicals which cause various alterations in secondary metabolism, especially in the synthesis of flavonoids by increasing the activity of biosynthetic enzymes such as phenylalanine ammonia-lyase (PAL), implying a shift away from sucrose production and toward repair and repair defense processes.

Similarly, the elevation of phenolics content as a result of the SAE treatment may be attributed to increased production of growth hormones and nutritional uptake in the plant roots, as well as improved polyphenol oxidase activity, which enhances phenolic buildup and thus plants antioxidant activity (Chrysargyris et al., 2018).

Marine macroalgae are subjected to both oxygen and light, resulting in the production of free radicals and different highly oxidizing factors. Yet, lacking oxidative damage in macroalgae structural elements (i.e., polyunsaturated fatty acids) and their resistance to oxidation during storage indicate that their structure has protective antioxidant defense systems. Like vascular plants, algae have protection enzymes (peroxidase, superoxide dismutase, catalase and glutathione reductase) and antioxidative molecules such as phlorotannins, tocopherols, carotenoids, ascorbic acid, phospholipids, bromophenols, and catechins (El-Beltagi et al., 2019).

Higher contents of secondary compounds such as polyphenol, flavonoid and β-carotene, provide the plant with a greater antioxidant capacity. Several latest studies have proven the close relationship between plant phenols, flavonoids, carotenoid content and antioxidant activity (Sarker et al., 2022; Gorni et al., 2022).

The antioxidant activities of phlorotannins isolated from *Sargassum pallidum* and *Fucus vesiculosus* have been demonstrated. Furthermore, Sulfated polysaccharides such as fucoidan, laminarin, and alginic acid from *Turbinaria* have demonstrated antioxidant activity. Many other sulfated polysaccharides extracted from seaweeds, such as sulfated galactans, galactans, sulfated glycosaminoglycan, and porphyrin, have also shown highly radical scavenging properties (El-Beltagi et al., 2019).

Seaweed extracts serve as chelators, improving the plant's mineral and nutrient uptake characteristics while also improving the soil's structure and aeration, promoting root growth. Seaweed extract gained this feature as it contains mineral elements, vitamins, fatty acids, and amino acids, besides the unique composition of growth regulators, which cannot normally be found in higher plants (Issa et al., 2019).

In a similar recent study, total phenols and flavonoids content and DPPH radical scavenging activity for three peanut varieties were determined after treatment with *Sargassum vulgare* extract. The data illustrate a strong response from the three peanut varieties tested. Application of algal extract promoted the antioxidant potential, corresponding to the accumulation of total phenolics and induced the accumulation of resveratrol and its derivatives leading to the highest antioxidant activity (Ben Ghozlen et al., 2023).

TABLE 3 Qualitative analyses of phytochemical composition of algal aqueous extracts.

Alkaloids	Terpenoids	Steroids	Tannins	Saponins	Flavonoids	Phenols	Coumarins	Quinones	Glycosides
+	+	+	+	++	+	++	-	-	+

++: intensely present, +: Present, -: Absent.

Saponins are structurally complex amphiphatic glycosides of steroids and triterpenoids that are widely produced by plants and also by certain marine organisms and play a role in plant development. Besides, Confalonieri et al. (2009), reported the involvement of b-amyrin and derived saponins in the regulation of root nodulation.

In a study on fenugreek seeds (*Trigonella foenum-graecum*) it was found that the diffusible saponin substances located both in the endosperm and perisperm inhibited the production of a-galactosidase activity, which is a step needed for seed germination (Zambou et al., 1993). Another role of saponins is the induction of callose synthesis in carrot cells by a spirostanol saponin (Messiaen et al., 1995). A c-pyronyl triterpenesoid saponin termed chromosaponin 1 (CSI) was reported to stimulate root growth and regulate gravitropic response by inhibiting or stimulating the uptake of endogenous auxin in root cells (Rahman et al., 2001). Furthermore, saponin affects cell elongation by inhibiting ethylene signaling (Rahman et al., 2000).

Petropoulos et al. (2022) suggested that the positive effects of applying seaweed extract on artichoke plants may be attributed to the improved nutrient uptake due to the hormone-like and chelating properties of the applied seaweed extract.

The application of seaweeds as biofertilizers in sufficient quantities improved soil conditions and crop growth parameters in different field crops. The use of seaweed biofertilizer compensated for the deficiency of N, P, and K and other minerals necessary for plant growth (Singh et al., 2016). The addition of seaweed species; *Sargassum* sp. and *Gracilaria verrucosa;* caused chemical changes in clay and sandy soils as a soil fertility indicator, improved organic content, and lowered C/N ratio in both clay and sandy soil (Ammar et al., 2022).

In accordance, Xu and Leskovar (2015) reported an increase in *Eruca vesicaria* L. antioxidant activity caused by seaweed extract treatment. In a different study, Alhasan et al. (2021), recorded a direct correlation between antioxidant activity and phenolic content. Hence, the knowledge of the nutritional contents of diverse plant residues, including their application in plant nutrition during a crop cycle, is critical, especially in sustainable agriculture (Torma et al., 2018).

Our study found that the SAE treatment improved the nitrogen and potassium content in both plant shoots and increased plant growth and final dry biomass (Table 2 and Figure 6). According to Torma et al. (2018), the quantity of nutrients (particularly nitrogen and potassium) left in the soil after the crop harvest is considerable; at least 50% of the residual plant nutrients are available for the following crop cultivated without further fertilization.

Vicia Faba is well known for its great prominence in improving soil fertility through nitrogen fixation by root nodules, improving soil's natural properties (Hassan and Ghani, 2001). Nitrogen-fixing bacteria (Rhizobia) coexist with legume roots and convert atmospheric N_2 to NH_3 by nitrogenase enzyme in plant root nodules. Furthermore, nitrogen in residual biomass of harvested crops is leached to a smaller extent than nitrogen in inorganic fertilizers, which improves groundwater quality (Aulakh et al., 2000).

On the other hand, because *Helianthus* stems contain phosphate and potassium, they can be composted and put back into the soil as fertilizer (Fozia et al., 2008). Figure 6 shows the positive impact of the SAE in inducing a higher level of phosphorus content in both crops. Mamun et al. (2020) strengthened our findings; they reported that, instead of total nitrogen, total phosphate is the most important regulatory element for algal growth. The phosphate function in respiration is to transfer high-energy molecules such as adenosine triphosphate (ATP). Accordingly, this accessible phosphate inspires more seed respiration during *Vicia* and *Helianthus* germination, which may justify the amplified seedling growth vigor recoded by Table 1.

Conclusion

Sargassum polycystum could be viewed as a sustainable, eco-friendly bio-based stimulant for green agriculture, as a trial to reduce the addition of harmful chemical fertilizers and face the offensive climatic changes. The qualitative analysis of the SAE opened a pandora box of varied bioactive secondary metabolites such as saponins, phenolics, flavonoids as well as the terpenes and steroids which are suggested to reinforce the vegetative growth, primary and secondary metabolites of both *Vicia faba* and *Helianthus annuus*. In addition, seaweed extract enhanced the antioxidant activity of both tested plants. Importantly, there is also a positive indication of the ability of dual utilization of *Vicia faba* and *Helianthus annuus* residual biomass in animal nutrition, as well as extraction of phytochemicals and further conversion into value-added products and utilization as safe biostimulants.

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 authors.

Author contributions

Conceptualization: ME-S, SM, SH, and AN. Methodology: ME-S, SM, SH, MA-H, AE, AN. Validation: ME-S, SM, SH, MA-H, AE, and AN. Formal analysis: ME-S, SM, SH, MA-H, AE, and AN. Investigation: ME-S, AE, and AN. Resources: ME-S, SM, SH, MA-H, AE, and AN. Data curation: ME-S, SM, SH, MA-H, AE, and AN. Writing—original draft preparation: SM, SH, and AN. Writing—review and editing: ME-S, SM, SH, MA-H, AE, and AN. Visualization: SM, SH, AE, and AN. Supervision: ME-S, AE, and AN. Project administration: ME-S, MA, and AE. Funding acquisition: ME-S, MA-H, and AE. All authors contributed to the article and approved the submitted version.

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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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Temperature-induced modulation of stress-tolerant PGP genes bioprospected from *Bacillus* sp. IHBT-705 associated with saffron (*Crocus sativus)* rhizosphere: A natural -treasure trove of microbial biostimulants

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There is a renewed interest in sustainable agriculture wherein novel plant growth-promoting rhizobacteria (PGPR) are being explored for developing efficient biostimulants. The key requirement of a microbe to qualify as a good candidate for developing a biostimulant is its intrinsic plant growth-promoting (PGP) characteristics. Though numerous studies have been conducted to assess the beneficial effects of PGPRs on plant growth under normal and stressed conditions but not much information is available on the characterization of intrinsic traits of PGPR under stress. Here, we focused on understanding how temperature stress impacts the functionality of key stress tolerant and PGP genes of *Bacillus* sp. IHBT-705 isolated from the rhizosphere of saffron (*Crocus sativus*). To undertake the study, *Bacillus* sp. IHBT-705 was grown under varied temperature regimes, their PGP traits were assessed from very low to very high-temperature range and the expression trend of targeted stress tolerant and PGP genes were analyzed. The results illustrated that *Bacillus* sp. IHBT-705 is a stress-tolerant PGPR as it survived and multiplied in temperatures ranging from 4°C-50°C, tolerated a wide pH range (5-11), withstood high salinity (8%) and osmolarity (10% PEG). The PGP traits varied under different temperature regimes indicating that temperature influences the functionality of PGP genes. This was further ascertained through whole genome sequencing followed by gene expression analyses wherein certain genes like *cspB, cspD, hslO, grpE*, *rimM, trpA, trpC, trpE, fhuC, fhuD*, *acrB5* were found to be temperature sensitive while, cold tolerant (*nhaX* and *cspC)*, heat tolerant *(htpX)* phosphate solubilization (*pstB1)*, siderophore production (*fhuB* and *fhuG*), and root colonization (*xerC1* and *xerC2*) were found to be highly versatile as they could express well both under low and high temperatures. Further, the biostimulant potential was checked through a pot study on rice (*Oryza sativa*), wherein the application of

Bacillus sp. IHBT-705 improved the length of shoots, roots, and number of roots over control. Based on the genetic makeup, stress tolerance potential, retention of PGP traits under stress, and growth-promoting potential, *Bacillus* sp. IHBT-705 could be considered a good candidate for developing biostimulants.

KEYWORDS

saffron rhizosphere, stress tolerant PGP potential, long-read sequencing, gene expression, biostimulants for soil and plant health

Introduction

PGPRs are now widely recognized for promoting sustainable agriculture (Gouda et al., 2018) for their ability to enhance plant productivity in multiple ways (Raza et al., 2016). PGPRs enable higher nutrient acquisition through the solubilization of phosphate (Ahemad and Khan, 2012), the production of siderophore for better uptake of iron (Beneduzi et al., 2012), the synthesis of plant growth regulators like indole acetic acid, the release of volatile organic compounds, the production of antifungal enzymes like chitinase, glucanase, and ACC-deaminase (1-Aminocyclopropane-1-carboxylate) and inducing resistance against pathogens (Choudhary et al., 2011; García-Fraile et al., 2015). Numerous studies have illustrated the beneficial application of PGPR under stress (Sapre et al., 2018; Ali and Khan, 2021). PGPRs improved yield, and tolerance against salinity stress in wheat (Lastochkina et al., 2017; Ansari et al., 2019), enhanced tolerance to drought in chickpeas (Vurukonda et al., 2016; Khan et al., 2019), and enhanced the growth of the tomato under cold stress (Subramanian et al., 2016). Under heat stress, thermotolerant PGPRs stimulated heat-responsive genes (*GmHSP*, *GmLAX3*, and *GmAKT2*) in soybean (Khan et al., 2020). Despite the multitude of positive traits, the commercial application of PGPR as a biostimulant experiences certain limitations, the most common being the variability of performance under field conditions (Timmusk et al., 2017; Glick, 2018). This is primarily due to the mode of selection process wherein PGP traits of microbes are assessed at a laboratory scale under highly controlled conditions, while field conditions are markedly different (Vasseur-Coronado et al., 2021). In fields, multiple factors interact, hence the PGP potentials of microbes are often lost or masked leading to underperformance (Backer et al., 2018; Mellidou and Karamanoli, 2022). Hence, the selection of the right microbe which does not lose its PGP traits under field conditions and various stresses is crucial (Amaya-Gómez et al., 2020). Unraveling the genetic makeup of PGPR can help in understanding its stress tolerance potential and predict its functionality as a biostimulant. The expression trend of genes will have a direct effect on their PGP potential and field performance (Bruto et al., 2014). Hence in this study, we analyzed the PGP traits of *Bacillus* sp. IHBT-705 under stressed conditions and carried out gene expression studies of key PGP genes over a wide range of temperatures to understand how temperature impacts their functionality.

Bacillus sp. IHBT-705 was isolated from the rhizosphere of saffron (*Crocus sativus*) cultivated in Patti village, Kangra District, Himachal Pradesh India since 2018. Among several PGPRs isolated from the region, *Bacillus* sp. IHBT-705 was found to highly stress tolerant and interestingly its key PGP traits like phosphate and potassium solubilizing potential, siderophore IAA and ACC deaminase, were evident over varied temperature regimes. Hence, the present study focused on assessing the stress tolerance capacity and analyzing the PGP trait of the bacteria, *Bacillus* sp. IHBT-705. We also wanted to understand the underlining functioning of key PGP genes in response to different temperature regimes i.e., very low (4°C) to very high (50°C). To elucidate the functional characteristics of PGP and stress-related genes, whole genome sequencing of *Bacillus* sp. IHBT-705 was carried out using a long-read sequencing system, PacBio RS II (Pacific Bioscience, USA). Genome sequencing followed by data mining lead to the identification of several key genes associated with stress tolerance and PGP traits. Further, the expression analyses of key PGP genes were studied in detail under varied temperature regimes. This study provided new insight into understanding the functionality of PGP-related genes of *Bacillus* sp. IHBT- 705 over a wide range of temperatures. For the first time, the study illustrated that certain PGP genes are highly sensitive to temperature while some genes are versatile and can function well over wide temperature regimes.

Materials and methods

Sampling and physiochemical properties of soil

Soil samples were collected from the rhizosphere of saffron (*Crocus sativus*) grown at Patti village, Palampur (HP), India (32.0708° North & 76.5415° East) (Supplementary Figure S1) from a depth of 8- 15cm. Five independent soil samples were collected from five different places in the field, wherein each sample consisted of four subsamples pooled together from the four sides of the saffron (*Crocus sativus*) rhizosphere from the same site. Physiochemical properties of soil i.e pH, electrical conductivity (EC), Distribution of Nitrogen (N), Phosphorus (P), Organic carbon (OC), Potassium (K), Iron (Fe), Manganese (Mn), Zinc (Zn), Calcium (Ca) and Copper (Cu) were analyzed. The pH of the

soil and electrical conductivity were measured by immersing the electrodes into the soil: water-1:2.5 (w:v) extraction by using a calibrated pH meter (Thermo Scientific Eutech PC450, USA) and conductivity meter (Thermo Scientific Eutech Cyber Scan CON45, USA). Total N in the soil was determined by the micro-Kjeldahl method after digestion in concentrated sulfuric acid. The total P was determined by Bray's method (Bray and Kurtz, 1945), whereas the OC was estimated as per the standard dichromate oxidation method (Nelson and Sommers, 1983). Available K, Fe, Mn, Zn, Ca, and Cu was determined by using a flame photometer (model BWB XP, BWB Technologies UK Ltd., UK) after Mehlich -3 extraction (Mehlich, 1984).

Isolation of PGP bacteria

Soil samples were serially diluted (10^{-8}), spread on Tryptone Soya Agar (TSA) media plates (Himedia, India) incubated in an incubator shaker (Innova 12444, New Brunswick Scientific, USA) at 28 ± 0.1°C for 48 hrs for the isolation of PGP bacteria. The morphology of isolated colonies was observed for shape, texture, color, margin, opacity, and elevation. Gram's reaction was performed as per the method optimized by Vincent (Vincent, 1970). Pure colonies were preserved in glycerol stock (−80°C) till further use.

Stress tolerance potential of *Bacillus* sp. IHBT-705

To check the temperature tolerance, *Bacillus* sp. IHBT-705 was grown in Tryptone Soya Broth (TSB) media (Himedia, India) at 180 rpm for 48 hrs in an incubator shaker at ambient temperature (28° C). After 48 hrs, the optical density of bacterial suspension was observed at 600nm and found to be 1 (OD-1) and 100 µl of this bacterial suspension was inoculated in 100 ml of TSB media and incubated at targeted temperatures i.e very low (4°C and15°C), low (20°C), optimum (28°C), high (42°C) and very high (50°C) in an incubator shaker. For pH tolerance, pH (4, 5, 6, 7, 8, 9, 10, 11, and 12) was adjusted in TSB media by 1N NaOH and 1M HCl. Thereafter 100 µl of 48 hrs old *Bacillus* sp. IHBT-705 suspension (OD-1) was inoculated in 100 ml of selected pH media and incubated at targeted temperatures in an incubator shaker (Getahun et al., 2020). For salinity tolerance, concentrations of sodium chloride (0%, 2%, 4%, 6%, 8%, 10%, 12%, and 15%) were varied in TSB media and 100 µl of 48 hrs old *Bacillus* sp. IHBT-705 suspension (OD-1) was inoculated in 100 ml of selected NaCl TSB media and incubated at targeted temperatures in an incubator shaker (Ramadoss et al., 2013). For osmotic tolerance, concentrations of polyethylene glycol (PEG-10,000MW) (0%, 5%, 10%, 15%, 20%, 25%, and 30%) were varied in TSB media. Thereafter, 100 µl of 48 hrs old *Bacillus* sp. IHBT-705 suspension (OD-1) was inoculated in 100 ml of selected PEG TSB media and incubated at targeted temperatures in an incubator shaker (Niu et al., 2018). The optical density of bacterial growth was measured with a microplate reader (Synergy™ LX Multi-Mode BioTek) and incubated in an incubator shaker (Innova 12444, New Brunswick Scientific, USA) at 180 for 48 hrs for all the abiotic stresses mentioned above at targeted all temperatures.

PGP potential of *Bacillus* sp. IHBT-705

The initial screening and qualitative estimation of inorganic phosphate (P) solubilization of *Bacillus* sp. IHBT-705 was done on Pikovskaya's (PVK) agar plates (Nautiyal, 1999). Qualitative estimation of potassium (K) solubilization assay was done on Aleksandrow agar media (Dhaked et al., 2017). The siderophore production was checked by Chrome Azurol S assay (CAS) (Schwyn and Neilands, 1987). The IAA production ability was evaluated by a colorimetric detection test (Loper, 1986). ACC deaminase assay was done according to the method proposed by Dworkin and Foster (Dworkin and Foster, 1958). All PGPR attributes were done at all targeted temperatures. The qualitative estimation of P, K solubilization and siderophore production was calculated by solubilization index (SI) =colony diameter + halo zone diameter/ colony diameter (Paul and Sinha, 2013).

Molecular identification based on 16S rRNA gene sequencing

For molecular identification, the genomic DNA of *Bacillus* sp. IHBT-705 was extracted by using the GenElute bacterial genomic DNA isolation kit (Sigma-Aldrich, USA) and the quality and quantity of genomic DNA were determined by gel electrophoresis (Bio-Rad, USA) and NanoDrop 2000 (Thermo Scientific, USA) and Qubit version 2.0 fluorometer (Invitrogen, USA) respectively. The 16S rRNA amplification was performed by polymerase chain reaction (PCR) using a genomic DNA template and universal primers 27F (5'AGA-GTT-TGA-TCC-TGG-CTC-AG3') and 1492R (5'TAC-GGT-TAC-CTT-GTT-ACG-ACT3') were used to a near-full length, approximately 1500 bp PCR product (Weisburg et al., 1991). For PCR amplification, 25µl standard reaction mixture containing: GoTaq Green Master Mix 1x, primer (forward and reverse primer,1µM each), and DNA template was prepared and cycled at 94°C for 2min for initial denaturation, followed by 35 cycles consisting of denaturation at 94°C for 30sec, annealing at 55° C for 30sec, and elongation at 72°C for 2min; a final 7min elongation step at 72°C. The amplified product was visualized on a 1% agarose gel under UV light for a positive amplification signal, and the PCR product was purified Exosap-IT solution (Applied Biosystem, USA) according to the manufacturer's instructions. DNA ladder (1Kb, Qiagen, Netherlands) was used for size identification. Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, USA) was used according to the manufacturer's instructions and its PCR product was purified by Montage sequencing reaction kit (Millipore USA). The purified PCR product was injected into a Genetic analyzer (Applied Biosystems, USA) for Sanger sequencing technology. Sequences obtained were submitted as a query to the BLASTn (https://www.ncbi.nlm.nih.gov/) search algorithm. Strain identification

was done at the species level using EzTaxon (https://www.ezbiocloud.net/) and sequences of homologous strains were aligned using CLUSTALW (Larkin et al., 2007), and a phylogenetic tree was constructed with the MEGA version 11 (Tamura et al., 2021) (https://www.megasoftware.net/) using the neighbor-joining method with 1,000 bootstrap replicates (Saitou and Nei, 1987).

Pot experiment

Inoculation of *Bacillus* sp. IHBT-705 on rice (*Oryza sativa*)

To carry out the germination assay, seeds of Rice (HPR 2880 B/S) procured from CSKHPKV university Palampur Himachal Pradesh India, were surface sterilized (Nezarat and Gholami, 2009) and the experiment was set up in five replicates with two treatments i.e., treated (*Bacillus* sp. IHBT-705) and control (without *Bacillus* sp. IHBT-705). For the treated set, *Bacillus* sp. IHBT-705 was grown in TSB (Himedia, India) and incubated in an incubator shaker (Innova 12444, New Brunswick Scientific, USA) at 180 rpm for 24 hrs at 28°C, thereafter, centrifuged at 8000 rpm for 10 min. The centrifuged pellet was resuspended in autoclaved water for making OD-1 and used for further experiments. The effect of *Bacillus* sp. IHBT-705 on the growth of rice was studied by soaking rice seeds in the *Bacillus* sp. IHBT-705 suspension (OD-1) for 30 min. The seeds of control set were soaked in sterile distilled water. Each pot was filled with 2 kg autoclaved soil and fifty soaked seeds were planted, thereafter10 ml of *Bacillus* sp. IHBT-705 suspension was added in treated set. Seeds treated with sterile distilled water without bacterial suspension were taken as control. The treatments were arranged in a completely randomized design (CRD) with five replicates and placed in a glass house. After 60 days of sowing, ten plants were randomly uprooted and washed under running water, and root/shoot lengths and numbers of roots were measured.

Complete genome sequencing and annotation of *Bacillus* sp. IHBT-705

Complete genome sequencing was carried out to understand the genomic complexity of *Bacillus* sp. IHBT-705. The genomic DNA of *Bacillus* sp. IHBT-705 was isolated as mentioned above and ~10µg of high-quality intact genomic DNA was used for long reads library preparation using a PacBio SMRTbell template preparation kit with 20Kb insert size (Pacific Biosciences, USA) version 1.0 as per manufacturer's instructions. Briefly, intact gDNA was fragmented using g-tubes (Covaris, Inc. USA) and the quality of the sheared DNA was checked using gel electrophoresis (Supplementary Figure S2A). Followed by end-repairing, adaptor ligation, and purification of library molecules using Bluepippin size-selection system (Sage Science, USA) called SMRTbell template (Supplementary Figure S2B). The QC of the library was checked with Bioanalyzer DNA 12000 chip (Agilent Technologies, USA) and Qubit Fluorometer v3.0 (Invitrogen, USA) as shown in (Supplementary Figure S2C). Further, genome sequencing of *Bacillus* sp. IHBT-705 was performed on the PacBio RS II system with polymerase P6-C4 sequencing chemistry. The annotation was performed using Rapid Annotation Using Subsystems Technology (RAST) (Aziz et al., 2008). Denovo assembly of sequenced sub-reads was carried out using HGAP (Hierarchical Genome Assembly Process) (Supplementary Figure S3) protocol version 3.0 in SMRT analysis version 2.2.0 (Pacific Biosciences, USA) and produced a complete circular genome sequence with high coverage.

Whole-genome alignment with closest strain

For comparative genome analysis reference genome sequence of *Bacillus altitudinis* ASM97268 (Top hit in 16S rRNA Sequencing) was downloaded from the NCBI (Accession number NZ_CP011150.1), and imported into the CLCBio genomics workbench (Qiagen, Netherlands). The complete genome sequence of *Bacillus* sp. IHBT-705 (CP074101) was used against *Bacillus altitudinis* (ASM97268) for whole-genome alignment. The alignment was performed using CLC Genomics Workbench (v21.0.3) whole-genome alignment tool with default parameters (minimum initial seed length 15bp and minimum alignment block length (100bp). This tool primarily works by identifying seeds i.e., short stretches of nucleotide sequence that are shared between multiple genomes but not present multiple times in the same genome. These seeds are extended using the HOXD scoring matrix (Chiaromonte et al., 2002) until the local alignment score drops below a fixed threshold.

Genome-based average nucleotide identification with closest strain

The genomic similarity between the *Bacillus* sp. IHBT-705 and the closest strain *B. altitudinis* ASM97268 from the NCBI database were calculated using the Orthologous Average Nucleotide Identity algorithm (OrthoANIu) (https://www.ezbiocloud.net).

Expression analysis of stress-tolerant and PGP genes

Genes responsible for stress (cold and heat), pH tolerance, and PGP attributes such as Phosphate solubilization, IAA production, siderophore production, ACC deaminase activity, and root colonization were selected based on the available literature (Table 1) and screened from the whole genome annotation of selected PGP and stress-tolerant bacteria by BLAST to GO (Conesa et al., 2005) for expression studies. Primers for stress-tolerant and PGP genes were designed by using the Primer Express software version 3 (Applied Biosystem USA) as shown in (Supplementary Table S1).

TABLE 1 List of stress-tolerant and PGP genes selected from the whole genome of *Bacillus* sp. IHBT-705 for expression studies.

Genes	Reported Attributes	Ref.
nhaX *cspD* *cspB* *cspC*	Cold Shock Proteins	(Gupta et al., 2014; Kishor PB, 2019; Lalaouna et al., 2019)
dnaJ *dnaK* *hslO* *htpX* *grpE*	Heat Shock Genes	(Gupta et al., 2014; Suarez et al., 2019)
trpA *trpB* *trpD* *trpC* *trpE*	IAA Biosynthesis.	(Gupta et al., 2014; Dahmani et al., 2020; Zaid et al., 2022).
fhuC *fhuD* *fhuB* *fhuG* *acrB5*	Siderophore Production	(Gupta et al., 2014; Suarez et al., 2019)
nhaK	pH tolerance	(Noori et al., 2021)
pstB1	Phosphate Solubilization	(Suarez et al., 2019)
rimM	ACC Deaminase Activity	(Gupta et al., 2014)
xerC1 *xerC2*	Rhizosphere Colonizer	(Shen et al., 2013; Eida et al., 2020)

RNA extraction, cDNA synthesis, and qRT-PCR analyses

Bacillus sp. IHBT-705 was grown in TSB media (Himedia India) at all targeted temperatures at 180 rpm for 24 hrs in an incubator shaker and after 24 hrs total RNA was isolated separately from *Bacillus* sp. IHBT-705 suspension (OD-1) by using RNeasy Mini Kit (Qiagen, Netherlands). The quality of RNA was checked by using RNA nanochip on a bioanalyzer (Agilent Technologies, USA). Further, the RNA quality and quantity were verified by agarose gel electrophoresis (Bio-Rad, USA) followed by ND-1000 NanoDrop (Thermo Scientific, USA) respectively. Total RNA was reverse transcribed into cDNA using a verso cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Briefly, the reaction was prepared in 20µl volume with random hexamer primer conducted at 42˚C for 60min and 95˚C for 2min for heat denaturation of enzymes. Prepared cDNA was confirmed by PCR amplification using 16S primer pair followed by agarose gel electrophoresis (Bio-Rad, USA) (Cárdenas Espinosa et al., 2021). Real-time qRT-PCR was performed with Syber Green PCR Master Mix (Thermo Fisher Scientific, USA) and conducted on Step One Plus 2.0 Real-Time PCR System (Applied Biosystems, USA). The reaction mixture included 2µl of diluted cDNA (1:10), 0.5µl each primer, and SYBR green 5µl. The final reaction volume of 10µl was adjusted with nuclease-free water. The following PCR condition was used: 95˚C for 7min, 40 cycles of 95˚C for 15sec, and 60˚C for 30sec. The melting temperature-determining dissociation step was run at 95˚C for 15sec and 60˚C for 1min and 95˚C for 15sec at the end of the amplification. All the reactions were carried out in triplicate for each cDNA sample (Jian et al., 2020).

Statistical analysis

Relative gene expression calculations of stress-tolerant and PGP genes were calculated according to the comparative critical threshold (2ΔΔCT) method (Livak and Schmittgen, 2001). The expression of two housekeeping genes (*16S*, *secA*) was analyzed for stress-tolerant and PGP gene primers. At all targeted temperatures, the expression of the *16S* gene was highly stable, and similar results were obtained by using it as a normalization gene at 28˚C (optimum temperature for gene expression) and comparison of Ct-values further revealed that expression of *16S* reference gene was stably expressed with constant Ct values (Supplementary Figure S4A, B). Reference gene *secA* was not stably expressed in the targeted temperatures. Hence, only the 16S reference gene was considered as the internal reference. All experiments were performed in triplicates for gene expression and in five replicates for pot experiments. All data (Significance in fold-change and Pot experiment) was analyzed by using one-way ANOVA followed by Duncan multiple range test (DMRT) (p-value = 0.05) (https://ccari.icar.gov.in/wasp/index.php).

Results

Physicochemical properties of soil

We wanted to understand the nature of soil being the habitat of *Bacillus* sp. IHBT-705, hence the soil characteristics (pH, EC, available N2, P, OC, K, Fe Mn, Zn, Ca, and Cu) were analyzed (Table 2).

Morphological characterization of *Bacillus* sp. IHBT-705

Colonies of *Bacillus* sp. IHBT-705 were off-white, translucent, elevated, and round with a regular margin. Gram staining illustrated it to be rod-shaped gram-positive bacteria.

TABLE 2 Physiochemical properties of rhizosphere soil. Values represented are mean ± standard error of three replicates.

pH	EC	N_2	P	K	OC	Zn	Fe	Ca	Cu	Mn
	(dS m^{-1})	Kg/ha			%	ppm				
4.8 ± 0.02	131 ± 0.04	294.57 ± 0.02	19.29 ± 0.25	22.2 ± 0.02	2.81 ± 0.02	15 ± 0.02	453.23 ± 0.07	5.26 ± 0.24	12.73 ± 0.02	8.4 ± 0.03

Stress tolerance potential of *Bacillus* sp. IHBT-705

Bacillus sp. IHBT-705 exhibited growth on temperatures ranging from 4°C- 50°C on TSA as well as in TSB medium. However, 28°C was found to be the optimum temperature for growth and multiplication (Figure 1A). We wanted to understand the nature of *Bacillus* sp. IHBT-705 particularly under a stressed environment, so we subjected it to different stresses like temperature, pH, salinity, and osmoticum. *Bacillus* sp. IHBT-705 exhibited thermotolerance on temperatures ranging from 4°C- 50°C (Figure 1A) and could multiply at pH ranging from 5 to 11 at all the targeted temperatures indicating that it can tolerate and propagate both in acidic as well as the alkaline environment (Figure 1B). The salinity (NaCl) tolerance level of *Bacillus* sp. IHBT-705 was up to 6% at all the targeted temperatures and increased up to 8% at 28°C and 42°C (Figure 1C). *Bacillus* sp. IHBT-705 exhibited relatively slow growth in medium supplemented with PEG-10,000MW (10% onwards) from 15°C-42°C and almost no growth at 50°C (Figure 1D). All these stress assessment results indicate *Bacillus* sp. IHBT-705 to be a stress-tolerant PGP.

PGP potential of *Bacillus* sp. IHBT-705

Though *Bacillus* sp. IHBT-705 was found to be a stress-tolerant PGPR, but it was essential to check if it retained its growth-promoting attributes under stress or not so that it can serve as an effective biostimulant. Hence, we checked the key PGP traits under all the targeted temperatures. The P solubilization and siderophore production of *Bacillus* sp. IHBT-705 was confirmed by a clear zone around the colonies on PKV and blue CAS agar media at all targeted temperatures however, the potential varied. K solubilization by *Bacillus* sp. IHBT-705 was confirmed by a clear zone around the colonies on Aleksandrow medium at all targeted temperatures except 4°C, IAA production by the pink color formation in the salkowaski reagent at all targeted temperatures except 4°C and ACC deaminase activity was confirmed by the growth of *Bacillus* sp. IHBT-705 on DF salt minimal medium at all targeted temperatures except 4°C (Table 3).

Molecular identification based on 16S rRNA gene sequencing

Phylogenetic analysis of *Bacillus* sp. IHBT-705 based on 16S rRNA sequence showed a close evolutionary relationship of 99.46% similarity with *Bacillus altitudinis* (ASJC01000029) as per the closest neighbor-joining method (Figure 2). The16S rRNA nucleotide sequence of *Bacillus* sp. IHBT-705 has been submitted to the NCBI gen bank under accession number MW959130 and its pure culture was deposited under the accession number MTCC25416 at the Microbial Culture Collection (MCC) at IMTECH, Chandigarh, India.

Effect of *Bacillus* sp. IHBT-705 on rice

Through *in vitro* assay the *Bacillus* sp. IHBT-705 was found to exhibit promising plant growth potential this was further investigated through a pot study. *Bacillus* sp. IHBT-705 inoculation positively affected the growth of rice seedlings (Figure 3A). The treatment of rice with *Bacillus* sp. IHBT-705 significantly increased the length of shoots (42 cm ±0.1) as compared to the control treatment (28 cm ±0.3). Similarly, the length of roots was higher in *Bacillus* sp. IHBT-705 treated plants (13.08cm ±0.20) as compared to the control treatment of (8.48 ± 0.40; Figure 3B). Importantly, the numbers of total roots were higher in treated plants (85.58 ± 0.91) as compared to the control treatment (65.2 ± 1.78).

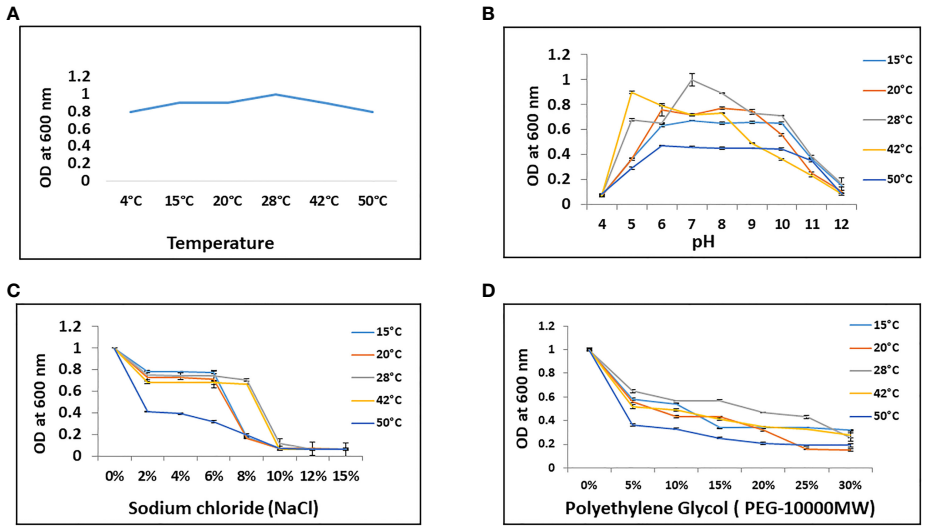


FIGURE 1
Stress tolerance potential of *Bacillus* sp. IHBT-705. **(A)** Thermotolerance potential. **(B)** pH tolerance. **(C)** Salt (NaCl) tolerance. **(D)** Osmotic (PEG 10,000MW) tolerance. All the experiments were done on targeted temperatures and Error bars represent the standard error of three replicates.

TABLE 3 PGP Potential of *Bacillus* sp. IHBT-705. Values represented are the mean of three replicates.

PGPR Strain	Targeted temperatures	Phosphate Solubilization (Zone in mm)	Potassium Solubilization (Zone in mm)	Siderophore production (Zone in mm)	IAA Synthesis	ACC Deaminase Activity
***Bacillus* sp. IHBT-705**	4°C	7	–	8	–	–
	15°C	10	7	12	+	+
	20°C	10	7	12	+	+
	28°C	12	10	15	++	+
	42°C	12	8	15	++	+
	50°C	10	6	12	+	+

+ indicates moderate activity, ++ indicates high activity, - indicates absence of activity.

Complete genome sequencing of *Bacillus* sp. IHBT-705

The complete genome and plasmid sequences of *Bacillus* sp. IHBT-705 genome sequences were deposited at DDBJ/EMBL/NCBI GenBank under the accession numbers CP074101 under BioProject PRJNA725988 and BioSample SAMN18917214. Obtained a total of 312,490,430 bases and 39,800 reads (N50 Read length 10,827 bases and mean read length 7,851 bases). Out of which 36,038 reads (91%) were mapped and generated a complete assembly of 3,773,439 bases with 67.91X coverage and 41.3% GC content. Rapid Annotations using the Subsystems Technology (RAST) server predicted 4,015 genes for protein-coding (CDSs), 106 genes for RNAs, and 327 RAST subsystem categories were functionally assigned through the predicted genes as shown (Figure 4A). Out of 1682 features, 319 were found in amino acids and derivatives, 238 in carbohydrates and 206 in protein metabolism. The comparison of the *Bacillus* sp. IHBT-705 genome sequence with *Bacillus altitudinis* showed a close alignment using the whole-genome alignment tool as CLCBio Genomics Workbench (v21.0.3) with default parameters (minimum initial seed length 15bp and minimum alignment block length 100bp). The alignment was found to be matched at 5403 blocks (Figure 4B).

Genome-based average nucleotide identification of *Bacillus* sp. IHBT-705 with closest strain *B. altitudinis* ASM97268

The draft genome length of *B. altitudinis* ASM 97268 was ~3.74 Mbp, while the genome size of *Bacillus* sp. IHBT-705 was ~3.77 Mbp. Close to 98.57% of total orthologous clusters were shared among two genomes.

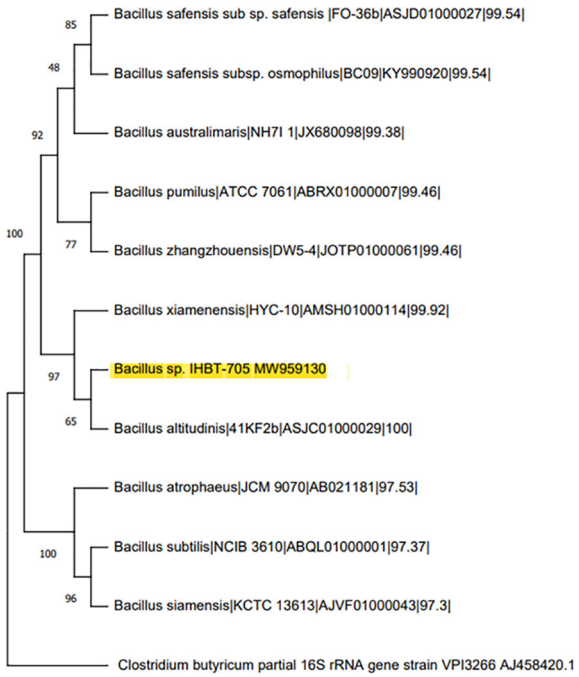


FIGURE 2
Phylogenetic relationships of 16S sequence of *Bacillus* sp. IHBT-705 and its homologs to the other species within the genus *Bacillus* and *Clostridium butyricum* as an outgroup.

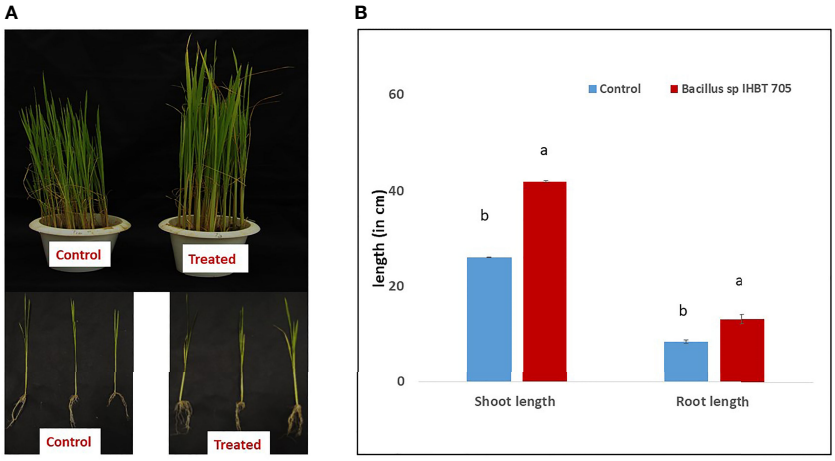


FIGURE 3
Effect of *Bacillus* sp. IHBT-705 on Rice **(A)** growth of rice seedlings under-treated and control conditions **(B)** shoot and root length. Different letters indicate significant differences among the treated and control treatments at p = 0.05. Data (mean ± SE) are averages of five replicates (n=5).

Expression analysis of stress-tolerant and PGP genes of *Bacillus* sp. IHBT-705 under selected temperatures

Having observed that *Bacillus* sp. IHBT-705 retains its major PGP traits under temperature stress, we wanted to understand the functionality of genes responsible for these traits. Not much information is available on the response of key PGP genes under varying temperature regimes, hence we carried out expression analysis of targeted genes to observe their modulation with the rise and fall of temperature. The observation of the study can pave the way to predict the efficiency of *Bacillus* sp. IHBT-705 as a biostimulant for plants growing in areas exposed to low and high temperatures. A total of twenty-four genes, governing stress (cold and heat), pH tolerance, and PGP attributes (IAA production, siderophore production, ACC deaminase activity, phosphate solubilization, and root colonization) were targeted based on the information available through literature (Table 1) for expression analysis studies.

Four genes *nhaX, cspB, cspC*, and *cspD* were targeted to unravel the cold tolerance capability of *Bacillus* sp. IHBT-705. Out of four

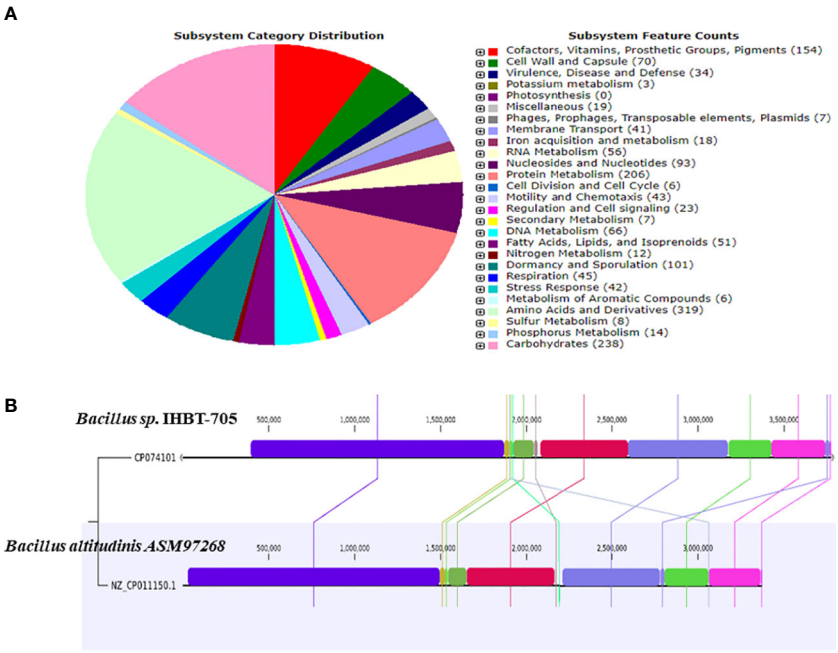


FIGURE 4
Genome annotation of *Bacillus* sp. IHBT-705. **(A)** Genome annotation of *Bacillus* sp. IHBT-705 using RAST (Rapid Annotations using Subsystems Technology). **(B)** Whole genome alignment of *Bacillus* sp. IHBT-705 (CP074101.1) with closest species reference genome *B. altitudinis* (NZ_CP011150.1).

cold tolerant genes, *cspC* was found to be versatile as it upregulated at 15°C, 20°C, and 42°C. Gene *nhaX* gene upregulated at 20°C and 42°C. Gene *cspB* and *cspD* both upregulated only at 20°C. Gene *cspB* downregulated at 4°C,15°C, and 42°C while gene *cspD* downregulated at 4°C and 42°C. All these four cold tolerant genes downregulated at 50°C (Figure 5A). Out of the five heat-tolerant genes (*dnaJ, dnaK, hslO, htpX*, and *grpE*), gene *dnaJ* did not upregulat at any targeted temperature while it stably expressed at 20°C and downregulated at all the other targeted temperatures. Both genes *dnaK*, and *hslO* upregulated at 20°C, stably expressed at 42°C and downregulated at 4°C, 15°C, and 50°C. While gene *htpX* upregulated at 15°C, 20°C, and 42°C, stably expressed at 4°C, and 50°C. Gene *grpE* upregulated only at 20°C and downregulated at all the other temperatures (Figure 5B). Out of five IAA synthesis genes *trpA, trpB, trpD, trpC*, and *trpE*, gene *trpA* upregulated at 42°C, stably expressed at 15°C and 20°C and downregulated at 4°C, and 50°C. Gene *trpB* stably expressed at 4°C, 15°C, and 42°C and downregulated at 20°C and 50°C. Gene *trpD* stably expressed at 20°C and 42°C and downregulated at 4°C, 15°C, and 50°C. Gene *trpC*, and *trpE* both upregulated at 20°C. Gene *trpC* expressed stably at 4°C, 15°C, 42°C and downregulated at 50°C. Gene *trpE* downregulated at all the targeted temperature except 20°C (Figure 5C). Genes *fhuB, fhuC, fhuD, fhuG*, and *acrB5* targeted for the siderophore production potential and out of these five genes, Genes *fhuC* and *fhuD* upregulated at 42°C, *fhuC* expressed stably at 20°C and *fhuD* at 4°C, both these genes downregulated on remaining other targeted temperatures. Gene *fhuB* upregulated at 4°C, 20°C 42°C and 50°C and downregulated at 15°C, while *fhuG* upregulated at 20°C and 42°C and stably expressed at 4°C and 15°C and downregulated at 50°C. Gene *acrB5* upregulated at 20°C and stably expressed at 4°C, 15°C, 42°C, and 50°C indicating retention of siderophore-producing activity at different temperature ranges (Figure 5D). Gene *nhaK* was targeted for pH tolerance and it stably expressed only at 20°C and downregulated at all the other targeted temperatures. Gene *rimM* gene targeted for ACC deaminase activity and it upregulated at only 20°C, stably expressed at 42°C and downregulated at all the other targeted temperatures. Gene *pstB1* targeted for the phosphate solubilizing potential and it upregulated at 15°C, 20°C, and 42°C and downregulated at 4°C and 50°C. Both the genes for root colonizations *xerC1* and *xerC2* upregulated at 20° C and 42°C, *xerC1* expressed stably and *xerC2* were downregulated at 4°C, 15°C and 50°C (Figure 5E).

Discussion

Several species of *Bacillus* are well known for their plant growth-promoting attributes and during the present study plant growth-promoting potentials of *Bacillus* sp. IHBT-705 was unraveled through *in vitro* analyses, pot study, and gene expression studies. The bacteria exhibited thermo-tolerance, could multiply in the acidic and alkaline range and could tolerate salt and high PEG concentration indicating that it is a good stress-tolerant PGPR.

Upon whole genome analysis, several key genes like *cspB, cspC, cspD, nhaX*, and *htpX* were identified. These genes are known to confer cold stress tolerance in microbes (Kishor PB, 2019; Lalaouna et al., 2019; Suarez et al., 2019), hence it is likely that these genes

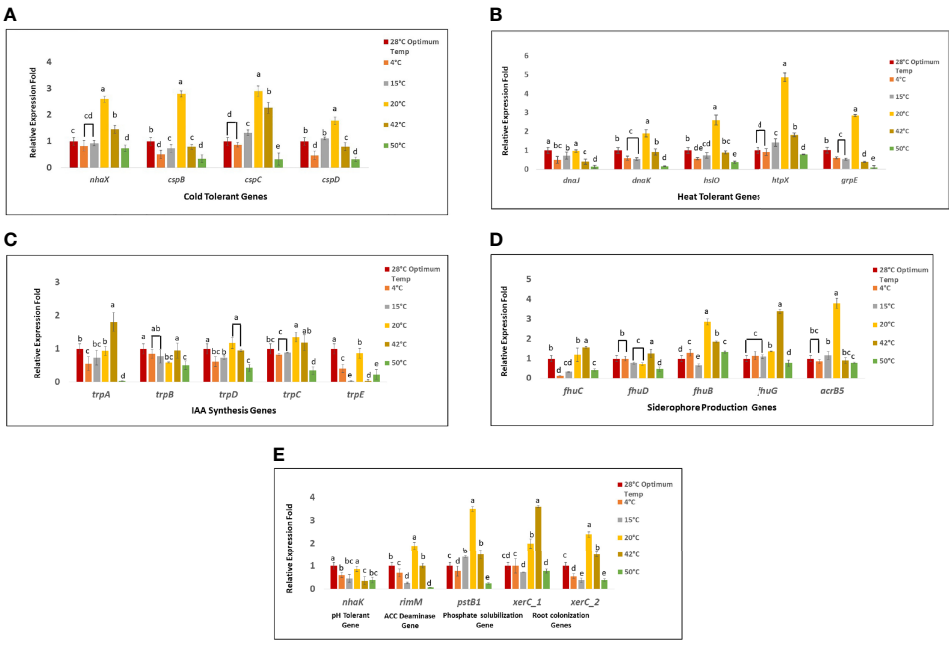


FIGURE 5
The relative expression of stress-related and PGP genes **(A)** Cold tolerant genes **(B)** Heat tolerant genes **(C)** Genes involved in IAA biosynthesis **(D)** Genes related to Siderophore-production **(E)** pH tolerant gene, ACC deaminase gene, phosphate solubilization and genes for root colonization. The results were represented as the mean of three replicates (n=3). Different letters indicate significant differences in gene expression among all targeted temperatures (P = 0.05).

could have conferred stress tolerance capability to *Bacillus* sp. IHBT-705. This gene expression study revealed that *nhaX* upregulated at low as well as high temperatures indicating that it could be responsible for imparting heat and cold tolerance and makes the bacterial strain tolerant to both cold and heat stress. Cold shock proteins (*csp*) family are known for their stress tolerance potential and found to be constitutively expressed during stress conditions in bacteria and even transgenic plants overexpressing bacterial *csp* exhibited tolerance to cold, salt, and drought stresses (Gupta et al., 2014; Kishor PB, 2019). In our study gene *cspC* overexpressed at high as well as low temperature suggesting that it may also impart heat and cold tolerance. Hence, presence of *cspC* gene makes *Bacillus* sp. IHBT-705 a valuable microbe from stress tolerance perspective. However, *cspB* and *cspD* exhibited a narrow range of functionality as both these genes upregulated only at low temperature.

Literature suggests that genes *dnaJ, dnaK, HslO, htpX*, and *grpE* are responsible for heat tolerance (Gupta et al., 2014; Suarez et al., 2019). However, we observed that genes *dnaK grpE, hslO* upregulated only at low temperature but not at high temperature, hence these genes were found to exhibit a narrow range of functionality. The gene *htpX* upregulated at low as well as high temperatures indicating its broad range of functionality and could have imparted stress tolerance ability, hence this gene appears to be crucial in conferring thermo-tolerance to *Bacillus* sp. IHBT-705. As the bacteria could tolerate up to 50°C, but none of the targeted gene upregulated at very high temperature further investigations by targeting other heat-tolerant genes are required to understand its extreme temperature tolerance capability.

The colorimetric tests illustrated that *Bacillus* sp. IHBT-705 efficiently produces IAA both at low as well as at high temperatures. Earlier studies indicate that *trp* genes (*trpA, trpB, trpD*, *trpC, trpE*) are involved in multiple biological processes including IAA biosynthesis (Gupta et al., 2014; Dahmani et al., 2020; Zaid et al., 2022). Interestingly, the present gene expression study revealed that specific temperature triggers the upregulation of specific sets of IAA-producing genes. Gene *trpA* was upregulated at high while *trpC* and *trpE* were upregulated at low temperature. The expression of *trpB* largely remained unaltered at both low and high temperatures, hence it could be considered a versatile gene with the ability to function over a broad temperature range with optimum efficacy. The role of microbial IAA in shaping root architecture ultimately benefitting plant productivity has been experimentally proven (Kazan, 2013; Sukumar et al., 2013; Grover et al., 2021), hence IAA is considered an important plant growth-promoting attribute, and in this respect, the complementary functioning of IAA-producing genes at both low and high temperature possibly enable retention of IAA producing capability of *Bacillus* sp. IHBT-705 under temperature stress which is a beneficial attribute.

Siderophore-mediated iron uptake in plants is a major benefit imparted by PGPRs (Braud et al., 2006; Mandal and Kotasthane, 2014). Siderophores are grouped into three principal categories based on their functional groups which are hydroxamates, catecholate, and carboxylates (Suarez et al., 2019; Kramer et al., 2020). Earlier studies revealed that the iron (III)-hydroxamate ABC transporter cluster *fhuCDB* and the ferric hydroxamate uptake gene *fhuA* are responsible for the transport of ferrichrome and other Fe3 + hydroxamate compounds (Gupta et al., 2014; Paul and Lade, 2014), hence in our study *fhuCDBG* and *acrB5* genes associated with the production of siderophore were targeted for expression analysis studies. Among these targeted genes, *fhuB* was found to be highly versatile as it upregulated from very low to very high temperatures. Similarly, *fhuG* upregulated from low to high temperature while *acrB5* upregulated at very low temperature, hence functionality of gene *fhuB* is a positive attribute of *Bacillus* sp. IHBT-705. Compared to IAA-producing genes, siderophore-related genes exhibited better expression at both low and high temperatures. The colorimetric study also illustrated that siderophore producing potential of *Bacillus* sp. IHBT-705 was active from very low to high temperature which is highly desirable trait from a biostimulant perspective.

The gene *nhaK* is known for imparting tolerance to pH (Noori et al., 2021), in this study gene *nhaK* was downregulated at all targeted temperatures thereby indicating a narrow range of functionality with respect to temperature. PGPRs produce extracellular polymeric substances and synthesize 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme or its homologue D-cysteine desulfhydrase, these enzymes lower ethylene accumulation in stressed plants (Sashidhar and Podile, 2010; Wagh et al., 2014) and enable plants to become stress tolerant (Paul and Lade, 2014). Gene *rimM* responsible for ACC deaminase activity (Gupta et al., 2014), *Bacillus* sp. IHBT-705 is an ACC-deaminase-producing strain consisting gene *rimM* and thus can act as a stress buster also. The expression analysis of *rimM* exhibited upregulation at low as well as a high temperature suggesting that *Bacillus* sp. IHBT-705 can function efficiently under temperature stress.

Qualitative assay phosphate solubilization potential of *Bacillus* sp. IHBT-705 was observed from very low to very high temperature which were further illustrated through gene expression study. The literature (Hudek et al., 2016; Suarez et al., 2019) suggests that *pstB1* plays a functional role in phosphate uptake. In our study gene *pstB1* upregulated from very low to high temperature, indicating that this gene is versatile and remains active both under low and high temperatures. This attribute is significant from a biostimulant perspective.

The ability to colonize the rhizosphere is an important attribute of PGPRs and genes *xerC1* and *xerC2* have been found to code site-specific recombinase which facilitates efficient colonization (Shen et al., 2013; Eida et al., 2020), hence these two genes were targeted. The expression analysis revealed overexpression of *xerC1* and *xerC2* at low and high temperature indicating that *Bacillus* sp. IHBT-705 could be a good colonizer both low and high temperatures. Apart from 28°C which is the optimum temperature for the growth of *Bacillus* sp. IHBT-705, among the targeted genes, the maximum number of genes were upregulated at 20°C and 42°C indicating its PGP potential at both low and high-temperature (Figure 6). Our study suggests that owing to its high-stress tolerance capability, *Bacillus* sp. IHBT-705 could be used as a potential biostimulant for crops growing in cooler mountainous regions as well as in warmer plains.

Conclusion

Recent years have witnessed an upsurge in microbial-based biostimulant. Several studies on PGP traits illustrate that the intrinsic characteristics of microbes are critical to their biostimulant potential. In this regard, physiological, molecular, and gene expression study of *Bacillus* sp. IHBT-705 revealed that it could survive both under very low to very high temperatures, low and high pH, and withstand salinity and osmolarity, suggesting that it is a highly stress-tolerant PGPR. Whole genome sequencing revealed the presence of several genes responsible for imparting stress tolerance and multiple genes for a single PGP trait. Furthermore, expression analysis of key PGP genes revealed that temperature is a critical factor governing the upregulation/downregulation of specific PGP genes hence temperature strongly regulates the functionality of PGP traits. As a PGPR, *Bacillus* sp. IHBT-705 has unique genetic makeup with multiple genes for a specific PGP trait wherein the downregulation of one gene is compensated by the upregulation of complementary genes, making *Bacillus* sp. IHBT-705 a good candidate for developing biostimulant(s) for agriculture in hilly terrain, warmer plains, and land affected by sodic soils. The genes *nhaX, cspC, htpX, fhuB, fhuG, pstB1, xerC1, and xerC2* were found to be versatile genes owing to their expression both under low and high temperatures while *cspB, cspD, hslO, grpE, rimM, trpA, trpC, trpE, fhuC, fhuD, acrB5* were found to be temperature sensitive. The presence of stress-tolerant genes may serve as marker genes for selecting other PGPRs as stress tolerant PGPRs for developing biostimulants (s) suitable for agriculture in different terrains.

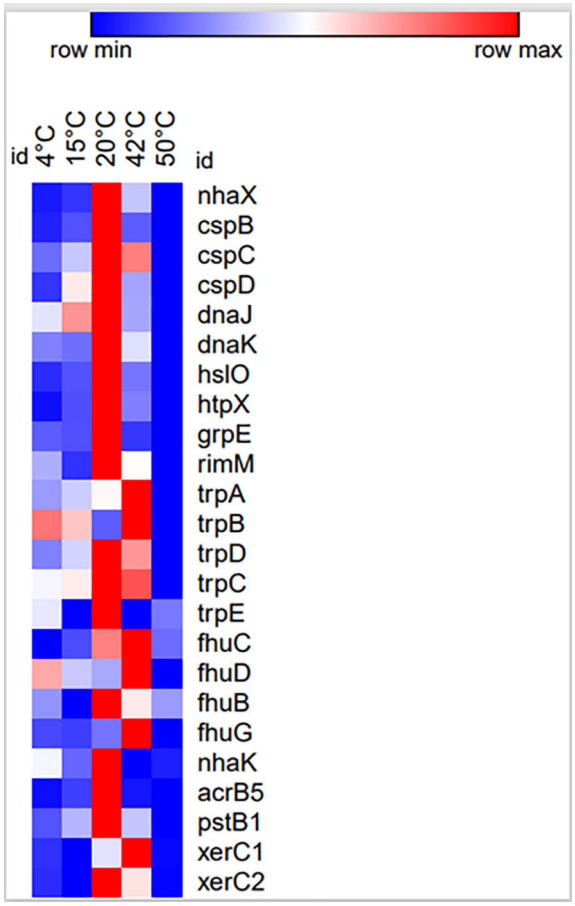


FIGURE 6
Heat map of twenty-four stress tolerant and PGP genes expression on targeted temperatures.

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

NA performed the experimentation, data analysis, and drafted the manuscript. MS contributed to experimentation and data analysis. RV helped in experimentation. PK edited and proofread the manuscript. AP conceptualized, guided, and structured the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1141538/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Map of soil sampling site for isolation of PGPR.

SUPPLEMENTARY FIGURE 2
Library validation of gDNA for whole genome sequencing of *Bacillus* sp. IHBT-705. **(A)** Gel profile of isolated high-quality gDNA of *Bacillus* sp. IHBT-705 with 1Kb ladder. **(B)** Bioanalyzer profile of *Bacillus* sp. IHBT-705 SMRTbell templates for sequencing with ladder. **(C)** Bioanalyzer profile of prepared library size (~9.8 Kb) of *Bacillus* sp. IHBT-705.

SUPPLEMENTARY FIGURE 3
Genome assembly features of PacBio RS II data using HGAP v3.0. **(A)** Polymerase read length distribution of post-filter reads. **(B)** Polymerase read quality distribution of post-filter reads. **(C)** Subread filtering of the whole genome of *Bacillus* sp. IHBT-705 sequencing reads. **(D)** Coverage across a reference of single contig assembly (with an average coverage of 69X).

SUPPLEMENTARY FIGURE 4
Expressions analysis of targeted stress tolerant and PGP genes by qRT-PCR. **(A)** Amplification plots **(B)** Melt curves of targeted genes.

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Hormones regulate the flowering process in saffron differently depending on the developmental stage

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Flowering in saffron is a highly complex process regulated by the synchronized action of environmental cues and endogenous signals. Hormonal regulation of flowering is a very important process controlling flowering in several plants, but it has not been studied in saffron. Flowering in saffron is a continual process completed in months with distinct developmental phases, mainly divided into flowering induction and flower organogenesis/formation. In the present study, we investigated how phytohormones affect the flowering process at different developmental stages. The results suggest that different hormones differentially affect flower induction and formation in saffron. The exogenous treatment of flowering competent corms with abscisic acid (ABA) suppressed both floral induction and flower formation, whereas some other hormones, like auxins (indole acetic acid, IAA) and gibberellic acid (GA), behaved contrarily at different developmental stages. IAA promoted flower induction, while GA suppressed it; however, GA promoted flower formation, whereas IAA suppressed it. Cytokinin (kinetin) treatment suggested its positive involvement in flower induction and flower formation. The expression analysis of floral integrator and homeotic genes suggests that ABA might suppress floral induction by suppressing the expression of the floral promoter (LFY, FT3) and promoting the expression of the floral repressor (*SVP*) gene. Additionally, ABA treatment also suppressed the expression of the floral homeotic genes responsible for flower formation. GA reduces the expression of flowering induction gene *LFY*, while IAA treatment upregulated its expression. In addition to these genes, a flowering repressor gene, *TFL1-2*, was also found to be downregulated in IAA treatment. Cytokinin promotes flowering induction by increasing the expression levels of the *LFY* gene and decreasing the *TFL1-2* gene expression. Moreover, it improved flower organogenesis by increasing the expression of floral homeotic genes. Overall, the results suggest that hormones differently regulate flowering in saffron *via* regulating floral integrator and homeotic gene expression.

KEYWORDS

flowering (evocation), phytohormones, homeotic genes, floral integrators, floral induction

Introduction

Saffron (*Crocus sativus* L.) is a monocot that belongs to the plant family Iridaceae and grows as a perennial stemless herb in Iran, Spain, Greece, India, Italy, and Nepal. Because of its triploid chromosome makeup, this plant reproduces asexually by corm feeding and produces sterile offspring (Esmaeili et al., 2011). The most valuable component of saffron is the flower, which has six tepals, three stamens, and three stigmas. Stigma is used as a spice, coloring, and flavoring agent in agro-food and cosmetic industries (Gohari et al., 2013). Flowering in saffron is highly complex and regulated by various external and internal factors. Flowering is the most important stage in saffron plant development, which is directly related to crop yield and productivity. External factors, mainly temperature, have been suggested as a major regulator controlling flowering in saffron. Despite the temperature, other regulatory networks also control flowering, including phytohormones. Plant hormones are an important factor that helps transmit signals from inside or outside the plant and contributes to the flowering process (Conti, 2017). Considering the importance of flowering in saffron production, several studies have been conducted to understand the process, with the majority of them directed towards understanding the influence of temperature on saffron flowering but only a few on the effect of hormones (Molina et al., 2005a; Molina et al., 2005b; Renau-Morata et al., 2021; Javid et al., 2022). The influence of hormones on the flowering process in saffron is partly explored. Therefore, unravelling the hormonal network in saffron flower induction and formation is of great interest concerning its utilization for maximized flowering and yield.

Plant hormones, gibberellic acid (GA), abscisic acid (ABA), cytokinin, ethylene, and auxin, are known to regulate a series of developmental processes such as seed germination, plant growth, senescence, flowering, *etc*. There has always been a dilemma on the plant hormones' exact role in regulating the flowering process—for example, GA has been positively correlated with flowering in several plant species like *Arabidopsis*, tobacco, radish, *etc*. (Gallego-Giraldo et al., 2007; Porri et al., 2012; Jung et al., 2020; Fukazawa et al., 2021), and contrary to many perennial fruit species, it inhibits flowering in apples, grapes, and citrus (Boss and Thomas, 2002; Garmendia et al., 2019; Zhang et al., 2019). GA has been shown to affect the different phases of flowering, from flowering induction to flower development (Mutasa-Göttgens and Hedden, 2009). Besides the model plants, the roles of GA in flowering have also been studied in some geophytes such as in *Zantedeschia*, lily, *Tulip*, *Allium sativum*, *Anemone*, *Paeonia*, *Lilium*, *Hyacinthus*, saffron, *etc*. ((Rudnicki et al., 1976; Evans et al., 1990; Ramzan et al., 2014; Hu et al., 2020; Renau-Morata et al., 2021; Yari et al., 2021). However, detail of the said mechanism is still required. In *Zantedeschia* GA promotes flower initiation and development processes, and the number of inflorescences is determined by the doses and duration of GA treatment (Brooking and Jamieson, 2002). In general, GA treatment has been shown to overcome and compensate for the low temperature requirement for flowering in the model plant *Arabidopsis* (Moon et al., 2003; King et al., 2008), including *Tulips* (Kurtar and Ayan, 2005). The contradictory role of GA in regulating flowering has also been suggested in different studies carried out on saffron (Renau-Morata et al., 2021). Their research correlating hormone signaling during flower induction and development suggested that flower initiation might be suppressed by gibberellins, while another study by Hu et al. (2020) suggested higher accumulations of GA and downregulation of GA2ox, an inhibitor of GA pathway, suggesting a positive role of GA in promoting flowering. Thus, more studies are required to suggest the exact role of GA in flowering regulation in saffron. ABA is another important plant hormone with a suggested role in flowering. Various studies using ABA mutants suggest a negative effect on flowering in *Arabidopsis* (Wang et al., 2013; Shu et al., 2016). Although the exact mode of action of ABA in regulating flowering is not known, ABA controls *Flowering Locus T* (*FT*) gene transcription *via GIGANTEA* (*GI*), *CONSTANS* (*CO*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) expression (Riboni et al., 2016; Martignago et al., 2020). A negative role of ABA on flowering induction has been suggested in saffron (Hu et al., 2020; Renau-Morata et al., 2021).

Auxins are considered as a key regulator for flower development and floral organ patterning (Nemhauser et al., 1998; Aloni et al., 2006; Cheng and Zhao, 2007). Auxins can affect floral initiation in multiple ways by either promoting floral organ primordia genes or by inhibiting the pluripotency of stem cells. During floral initiation, auxins are shown to regulate the expression of *LEAFY* (*LFY*) and *APETALA 1* (*AP1*) genes in *Arabidopsis* (Yamaguchi et al., 2013; Yamaguchi et al., 2016), while at a later stage during flower organ development auxin (ARF3) was found to regulate the expression of *APETALA2* and *AGAMOUS* genes (Liu et al., 2014). In addition to auxins, cytokinins have been suggested to have a role in promoting flowering induction in many plant species—such as in *Arabidopsis*, BAP promotes flowering *via* the activation of FT paralog TSF (TWIN SISTER OF FT) (D'Aloia et al., 2011; Han et al., 2014); in rose, cytokinin promotes flowering production (Zieslin et al., 1985); in strawberry, cytokinin is also involved in flowering (Eshghi and Tafazoli, 2007); and in *Iris*, zeatin and isopentenyl-adenine play an important role in flower bud blasting (Vonk et al., 1986). The cytokinin pathway also mediates *APETALA1* function in the establishment of floral meristems in *Arabidopsis* (Han et al., 2014). Different auxin and cytokinin ratios are responsible for stamen and ovule development in *Hyacinthus* from perianth explant (Lu et al., 1988). Plant hormones altogether have been suggested to have a species-specific and distinct role during different flower developmental stages.

The flowering process in saffron, similar to other plant species, can be divided into two main stages, *i.e*., (1) floral induction/initiation and (2) floral organ development. These two distinct developmental stages have different sets of genes involved and categorized as floral integrators and floral homeotic genes (Figure 1). Floral integrator genes comprising *FT, LFY, SVP* and *TFL* genes. *FT* and *LFY* regulate positively while *TFL* and *SVP* negatively regulate the flowering induction. (Tsaftaris et al., 2007; Kalivas et al., 2007a; Kalivas et al., 2007b; Tsaftaris et al., 2010; Tsaftaris et al., 2011; Tsaftaris et al., 2012; Tsaftaris et al., 2013; Kalia et al., 2022), while floral homeotic genes consist of ABCE model genes such as *APETALA*, *PISTILLATA*, *SEPALLATA*, and *DROOPING LEAF*-like genes that are involved in floral organ development and patterning (Renau-Morata et al., 2021). The role of a few of these genes has been studied—for example, a lot still remain undeciphered concerning flowering regulation in saffron. By analyzing *PEBP* genes, floral integrator genes in saffron have been

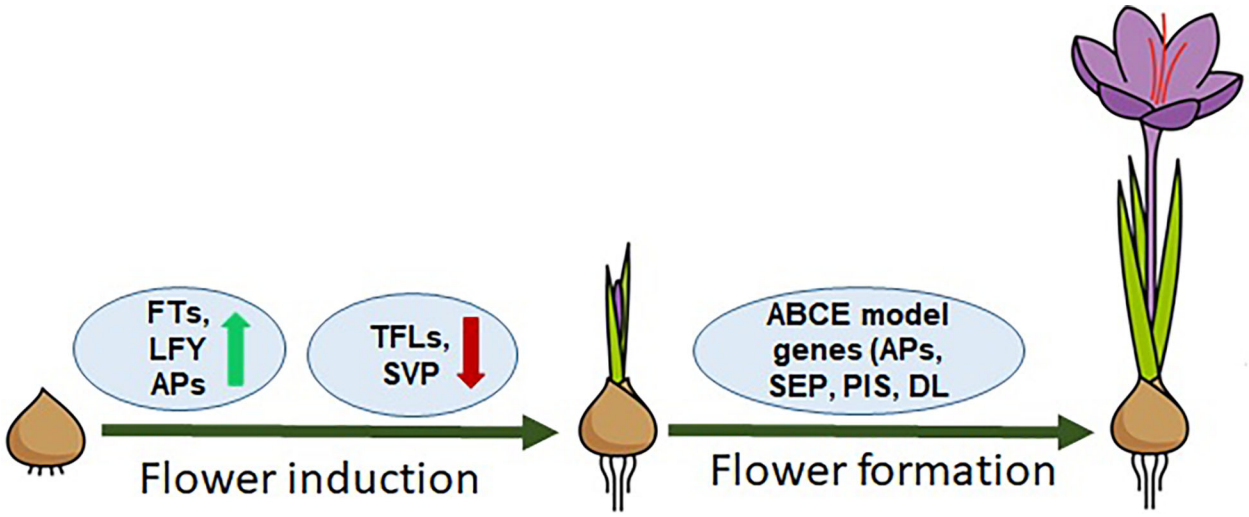


FIGURE 1
Model representing the flowering regulation in saffron. FT, LFY genes are flower inducers; TFLs, SVP are flowering inhibitors; ABCE model genes: APs, SEPs, PISs, and DLs are homeotic genes/flower organogenesis.

identified, but their hormonal regulation remain unexplored (Tsaftaris et al., 2013; Kalia et al., 2022). The spatiotemporal expression profiling of FT genes identified from saffron has suggested different roles during flowering and vegetative growth. *CsatFT3* gene has been suggested in temperature and sugar mediated regulation of flowering in saffron (Tsaftaris et al., 2013; Kalia et al., 2022; Jose-Santhi et al., 2023). A *SVP* homolog gene, which is a negative regulator of flowering, was shown to be expressed only in the vegetative buds of small-sized corms, which are not competent to flower (Haghighi et al., 2020). This finding indicates that, similar with other plants, SVP-like genes might be involved in regulating *FT* gene expression in saffron. However, more research is required to provide a detailed analysis. Similarly, floral homeotic genes have been identified in saffron, but studies are confined only to cloning and limited expression analysis. The plausible roles of these genes were based on sequence similarities and expression analysis (Tsaftaris et al., 2007; Kalivas et al., 2007a; Tsaftaris et al., 2011). Additionally, the major limitation in studying the hormonal regulation of flowering in saffron is that, when applied exogenously, their effect does not last longer and the whole flowering process can take months and is ineffective for observing long-term effects. In that case, it is very difficult to predict that it is the cause or the consequence that is seen after a long interval of hormone application.

Thus, in the present study, to define specific function of a hormone in regulating a distinct developmental stage, we have used two stages for hormone application. The first exogenous hormone application was done at the dormant stage, and it was followed for their ability to induce the process. Secondly, to understand their role in flower formation i.e. development of flowers, corms with pre induced flowering process were used. After hormone treatment, the morphological, histological, and transcriptional changes during different developmental stages were studied. The study suggests that hormones differentially affect flower induction and formation in saffron *via* regulation of floral integrator and Homeotic genes expression.

Materials and methods

Plant material

Saffron (*Crocus sativus* L.) corms grown in the field of CSIR-IHBT, Palampur, Himachal Pradesh, India, were used for the study. The corms were planted in October 2020 and harvested in April 2021. After harvesting, the corms were air-dried and sorted according to weight for the experiments. Corms with a weight range of 10–12 g that are considered competent to flower were used for the experimentation. In total 20 corms per treatment were used for each developmental stages. Different tissues of the saffron plant—namely, leaves, roots, corms, and flower tissues (stigma, stamens, and tepals)—were also collected separately for tissue-specific expression of genes.

Hormonal treatment

To understand the role of hormones involved in flowering induction, corms 10–12 g in size were treated with different hormones. Briefly, a hormonal solution of IAA (0.5 mg/l or 2.85 µm), ABA (0.5 mg/l or 1.89 µm), GA (0.5 mg/l or 1.44 µm), and kinetin (0.5 mg/l or 2.32 µm) was prepared in distilled water, and the corms were dipped in solution and then infiltrated for 2 h under vacuum. The control sample was dipped only with distilled water for the same span of time. After treatment, the corms were wiped with tissue, dried, and then stored at 25°C for floral induction in the dark for 90 days. Samples were collected for morphological and gene expression analysis from apical buds after 45 days (stage 1) and 90 days (stage 2) of treatment. Three biological replicates for each treatment were used for the study. The samples were harvested, frozen in liquid nitrogen, and stored at -80°C until further use.

To study the role of hormones in flower formation, flowering competent corms (10–12 g) that were stored at 25°C for 3 months in a growth chamber (June–August) and which already have floral initiation in them were used for the experiment. In early September, after exogenous hormone treatments (IAA, ABA, GA, and kinetin), corms were stored at 15/20°C during the night and day, respectively, with 8/16-h light/dark cycle for flower emergence in the growth chamber. Samples were collected at 45 days' interval in mid-October (stage III) and at the end of November (stage IV) when flowers emerged and were visible.

Histochemical and morphological analysis

Apical bud tissue at 45 days of the developmental stage of large corms were collected and fixed quickly in 4% paraformaldehyde solution. The fixed samples were dehydrated with ethanol series (10%-100%). Samples were stained with 0.05% eosin and replaced by Histo-Clear gradually. Then, the samples were embedded in paraffin and sliced to a thickness of 7–10 µm with Thermo Scientific HM 355S automatic rotary microtome. The sliced samples were photographed under Leica DM2000 optical microscope (Javelle et al., 2011).

Gene selection for the study

The genomic resource (genome sequences) of saffron is not yet available, making it difficult to mine genes involved in the process. In order to study the genes involved in flowering regulation, we selected genes which were previously reported either in saffron or other plants. Briefly, floral integrator genes that comprise *FTs*, *TFL1s*, *LFY*, and floral repressor *SVP* along with floral homeotic genes (*APATELLA*, *PISTILLATA*, *SEPATELLA*, *DROOPING LEAF*, *etc.*) were obtained from in-house and online database and used for the study. The list of genes studied, their source, and the references used in the present work have been listed in Supplementary Table S1.

RNA extraction and cDNA preparation

Total RNA was extracted from apical tissues using Spectrum Plant Total RNA kit (Sigma-Aldrich) according to manufacturer's instructions. Portions of RNA at 10 µg were treated with RNase-free DNase I (Thermo Fisher), and portions 2 µg were then utilized in cDNA preparation. The cDNA was prepared using a verso cDNA synthesis kit (Thermo Fisher) as per the manufacturer's instruction. Expression analysis was done by using RT-PCR.

RT-PCR and amplification conditions

Real-time PCR (RT-PCR) was done to quantify the expression of genes using gene-specific primers (Supplementary Table S2). The reactions were performed using three biological replicates and three technical replicates on Applied Biosystems real-time PCR machine. The analyzed real-time reaction data was the mean of biological and technical triplicates. The PCR conditions were as follows: 2 min at 50°C, 95°C for 3 min, followed by 40 cycles of 15 s at 95°C, 15 s at 55°C, and 45 s at 72°C. Tubulin was used as the internal control.

Statistical analysis

Tukey–Kramer multiple-comparison test by GraphPad software (significance level of $P < 0.05$; GraphPad URL: https://www.graphpad.com) and one-way ANOVA test (significance level of $P < 0.05$) were used to analyze the real-time PCR and corm morphological data, respectively.

Results

Morphological changes in the apical bud during flowering

To identify the morphological changes and development of different organs during the flowering process, the apical buds were monitored for 6 months (May–November), and apical bud samples were collected and analyzed after every 45 days from the start of the experiment. The morphological studies suggest that flower induction occurred in the first 45 days (stage 1), whereas the stamen and stigma start to develop between 45 and 90 days (stage 2). Stage 3 is marked with elongation in the stamen and stigma, and tepals development was observed between stage 3 and stage 4. A complete flower with all the organs developed was seen at stage 4 (Supplementary Figure S1).

Morphological changes in response to different hormones during flower induction

To study the effect of hormones on flower induction in saffron, dormant corms were treated with different hormones (GA, ABA, IAA, and kinetin) and were analyzed for floral induction process. After 45 days (stage 1), the apical meristem was fixed and used for microscopic analysis after sectioning. The morphological studies suggest that GA and ABA inhibited flower induction as seen in the longitudinal cross-section of the apical bud (small and semi-conical in shape, undifferentiated apical bud meristem tissues) (Figure 2A). Corroborating with the results seen after 45 days in ABA and GA treated samples, no floral organ formation can be seen in them after 90 days (Figure 2A). However, in the control, IAA- and kinetin-treated apical bud floral induction was prominently visible (differentiated apical bud meristem tissues), which is further confirmed by apical bud images at 90 days (stage 2) after the respective treatment that shows the development of the stamen and stigma (Figure 2A). The effect of different hormone treatments was also visible on apical and axillary bud length. IAA and kinetin promoted apical bud growth, which is reflected as increased apical and axillary bud length, whereas a significant reduction in apical and axillary bud length is observed in ABA and GA treated samples (Figures 2D, E).

Expression analysis of floral integrator and homeotic genes during flowering in saffron

The expressions of floral integrator genes (*FTs*, *TFL1s*, *LFY*, and *SVP*) were checked during the different stages of the flowering process. The results show that *CsatFT1* and *CsatFT2* gene expression gradually increased from stage 0 to stage 4 by up to three- and fivefold,

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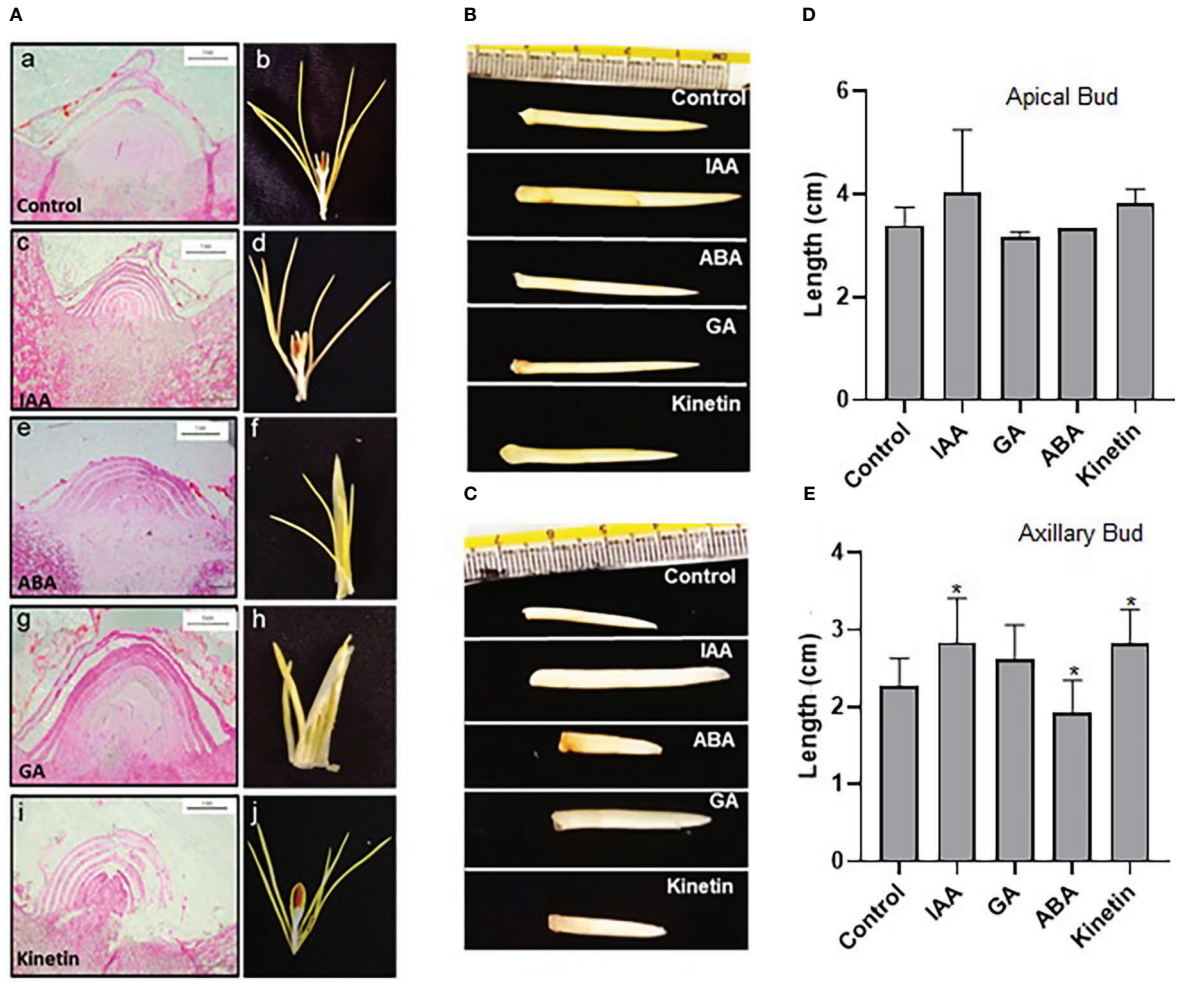


FIGURE 2
Morphological characteristics of saffron apical bud during flower induction stages after the hormonal treatments. **(A)** Cross-section images of the apical bud after 45 and 90 days of hormonal treatments, representing stage I and stage II, respectively: a, b—control (mock treatment); c, d—IAA-treated; e, f—ABA-treated; g, h—GA-treated; and i, j—kinetin-treated in stage I and stage II, respectively. **(B, C)** Representative pictures of the apical bud and axillary bud, respectively, in stage II of treatments. **(D)** Graphical representation of apical bud length. **(E)** Graphical representation of axillary bud length in stage II. The data presented is the mean of three biological replicates. Error bars represent standard error. *$P < 0.05$, with respect to control (Student's *t*-test).

respectively (Figure 3). The increase was observed from stage 1 and 2 in *CsatFT1* and *CsatFT2* genes, respectively, and no significant increase in expression was seen at stage 0 (flowering induction stage). However, *CsatFT3* comparatively showed a sixfold increase in stage 1, and then its expression declined in further stages. The *CsatFT4* gene transcript levels remained low throughout the stages and rapidly increased by up to ~15 folds in stage 4. The *CsatTFL1-1* and *CsatTFL1-2* genes showed a similar expression pattern (Figure 3). The transcript of TFLs was higher at stage 0 but reduced rapidly in stage 1 and remained low throughout all the stages except at stage 4, where their expression increased again by more than approximately twofold. *CsatLFY* gene expression was observed from stage 0, and it gradually increased at further stages. The expression of *CsatSVP* gene was not found to be significant at the early developmental stages but declined slightly at stage 3 and stage 4 (Figure 3).

The homeotic genes showed a different expression in the developmental stages of saffron, such as the *CsatAP3* expression which started to increase from stage 2, had its highest expression by up to approximately fivefold in stage 3, and further showed a small reduction of its transcript levels in stage 4. *CsatPISTILLATA-1* and *CsatPISTILLATA-2* showed the same expression patterns (Figure 3). The *CsatPISTILLATA-1* and *CsatPISTILLATA-2* transcripts started to appear from stage 2 and stayed highest by approximately 60- and 30-fold, respectively, in stage 4. The *CsatSEP3A* and *CsatSEP3B* genes had different expression patterns. The *CsatSEP3A* gene expression was highest in stage 2 (approximately 60-fold), and the transcript levels were rapidly decreased by 60%–70% in stage 3 and stage 4. The *CsatSEP3B* transcripts were also accumulated from stage 2 and went to its highest by ~30-fold in stage 4 (Figure 3). Another gene, *DL*, showed a gradual increase in expression pattern from stage 1 to stage 3 (approximately sevenfold), while the transcript levels were decreased by 40%–50% in stage 4. The *AP1* gene also started to accumulate from stage 1 and declined slightly in stage 3 and stage 4 (Figure 3).

Tissue-specific expression profiling of floral integrator and homeotic genes

Floral integrator and homeotic genes show tissue-specific expression in plants, suggesting their role in different plant and organ developmental processes. Our previous studies have provided the tissue-specific expression of *FT* and *TFL* genes in saffron (Kalia et al., 2022), but it

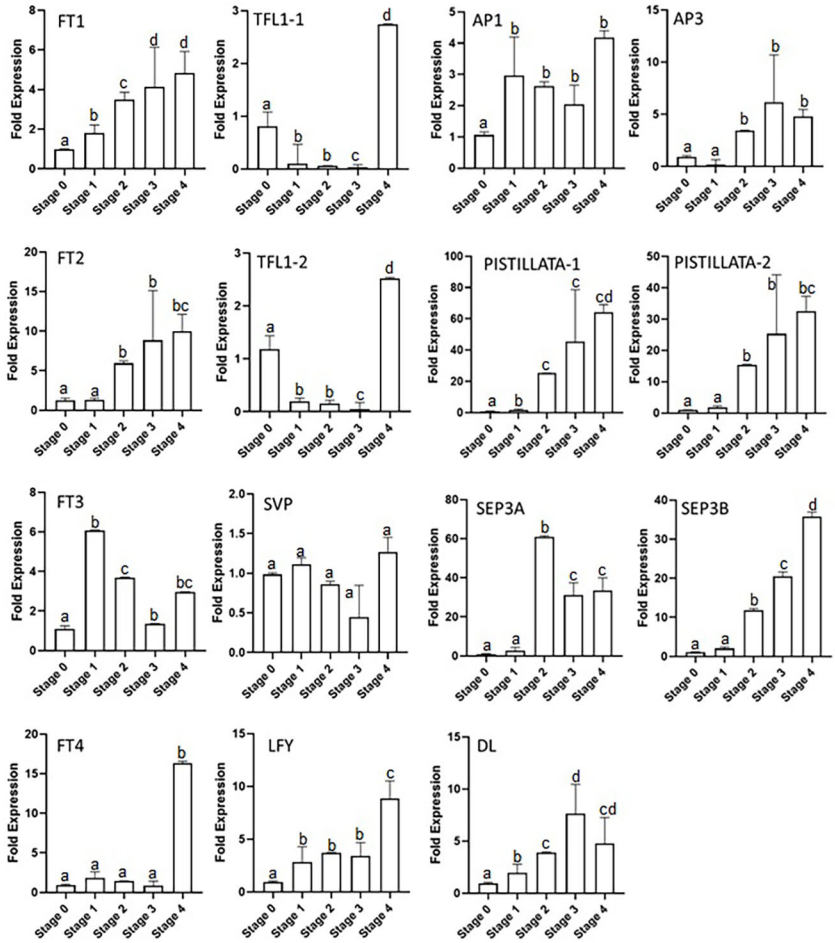


FIGURE 3
Expression profiling of floral integrator and homeotic genes at different developmental stages of saffron flowering. Stages 0–4 represent different developmental stages during saffron flowering. Stage 0, dormant corms; stage 1, flower induction stage; stage 2, stamen and stigma formation; stage 3, stamen and stigma development and elongation; and stage 4, tepal development. Error bars represent ± SD of three biological replicates. Stage I morphologically represents the flower induction stage, and stage II represents the stamen and stigma formation stage. Letters (a–d) over the bars indicate significant differences at $P < 0.05$ (means followed by the same letter are not significantly different at $P = 0.05$).

remains unknown for floral homeotic genes and other genes such as *SVP* and *LFY*. Thus, we examined the expression of these genes in different tissues. The expression patterns found that *CsatDL* (dropping leaf) gene was highly and specifically expressed in the stigma tissue (Supplementary Figure S2). The *CsatPISTILLATA-2*, *CsatSEP3A*, *CsatSEP3B*, and *CsatAP3* genes were expressed in stamen, stigma, and tepals tissues, whereas *CsatPISTILLATA-1* expression was confined to tepals and stigma tissues (Supplementary Figure S3). However, *SVP* expressions were only observed in leaf and corm tissues of saffron. The *CsatLFY* gene was expressed in all the tissues studied, with a higher expression in leaf and corm tissues. Similar to *CsatLFY*, *CsatAP1* expression was also found in most of the tissues except in the roots.

Effect of hormone treatment on the expression of floral integrator genes during floral induction

Morphological studies identified stage 1 as the flower induction stage in saffron flowering. Hormone treatments also differently affected them. We next examined the expression of previously identified/suggested floral integrator genes to establish a correlation between the effect of hormone on flower induction and organ formation *via* their transcriptional regulation. *FT* and *LFY* genes are positive regulators of floral induction. In our study ABA and GA treatments negatively co-related with flowering induction (Figure 2A). ABA and GA significantly reduced the expression of *LFY* gene in stage 2 (Figure 4). Moreover, ABA reduced the the expression of *FT3* gene in stage 1 (Figure 4). No significant effect of ABA and GA was observed on *CsatFT1*, *CsatFT2*, and *CsatFT4* expression compared with the control. Moreover, ABA significantly induced the expression of *CsatTFL1-1* and *CsatSVP* genes that are considered as floral repressors at floral induction stage 1 (Figure 4). Contrary to this, IAA and kinetin, which promoted flower induction, also promoted the expression of *CsatFT3* and *CsatLFY* genes that are known to be involved in floral induction. At stage 2, most of the positive floral integrator genes were downregulated significantly, while floral repressors were upregulated during this stage in comparison with the control (Figure 4).

Effect of different hormones on the expression of floral homeotic genes at the early stage of flower organ differentiation

Stage 2 was marked as the stamen and stigma differentiation stage (Figure 2). To identify the role of conserved floral homeotic genes, also known as ABCE model genes, and their regulation *via* different hormones, we studied the expression of these genes during stage 1 and stage 2 of flowering. The expression of the studied genes was not significantly changed during stage 1, and majority of these genes were expressed at low levels except for the *CsatAP1* and *CsatAP3* genes (Figure 5). The AP3 gene was significantly downregulated in GA treated samples. A significant reduction in the gene expression of *CsatPISTILLATA-1* and *CsatPISTILLATA*-2 (involved in stamen and tepals formation) was observed in ABA and GA treated samples, whereas upregulation was seen in IAA and kinetin treated samples, respectively, at stage 2 (Figure 5). Similarly, members of the *CsatSEPTELLA* (*SEP3A* and *SEPB*) genes were also downregulated in ABA and GA treated samples and upregulated in IAA and kinetin treated samples at stage 2. The *dropping leaf* (*DL*)-like gene also showed a significant downregulation in expression compared with the control in ABA and GA treated samples (Figure 5). Overall, the results

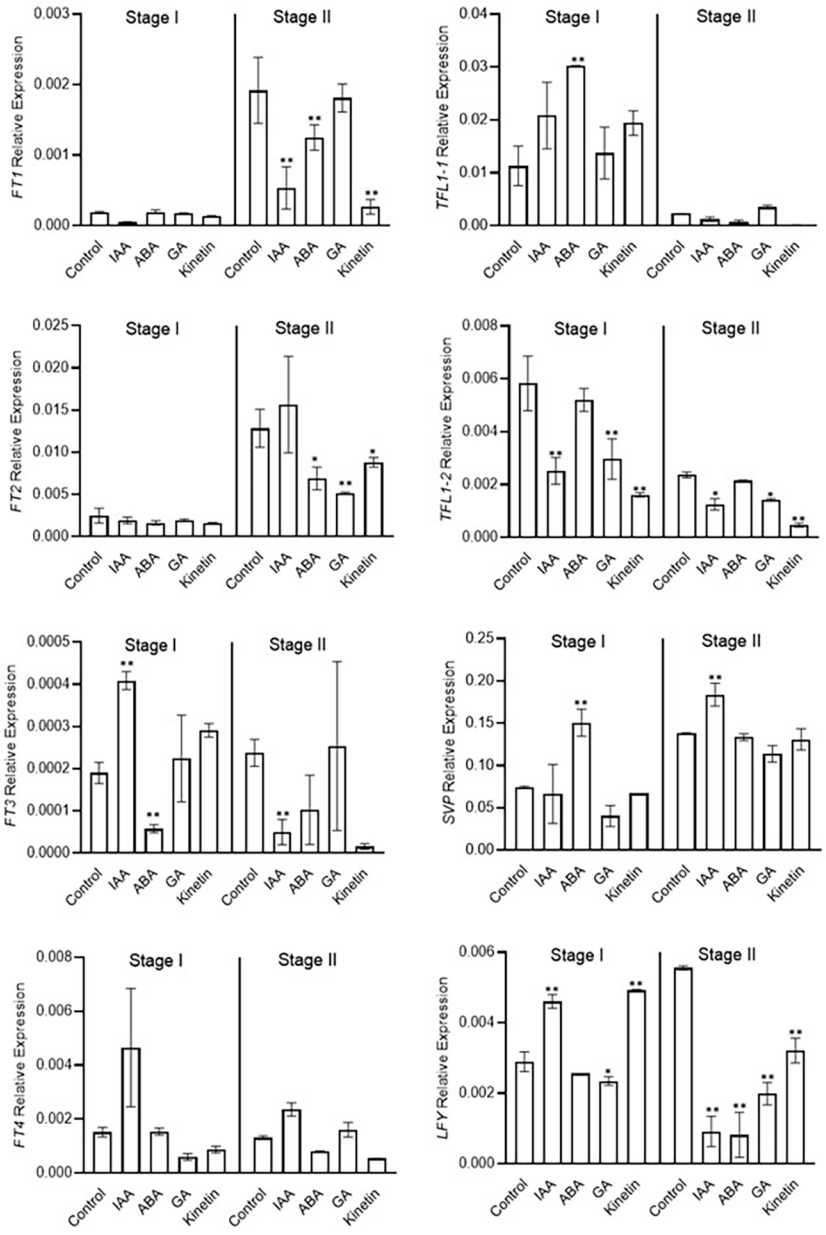


FIGURE 4
Expression profiling of genes involved in flowering induction quantified by real-time PCR in response to different hormones. Total RNA was isolated from the apical bud of corms treated with different hormones at 45 days (stage I) and 90 days (stage II) of treatments. Reactions from three separate pools of apical bud RNA samples were run in triplicates with tubulin as the internal control for normalization. Error bars represent ± SD of three biological replicates. Stage I morphologically represents the flower induction stage, and stage II represents the stamen and stigma formation stage. Data represented is mean of three biological replicates. Error bars represent standard error (SE), * indicates P <0.05, ** indicates P <0.01, with respect to control (Student's t-test).

suggest that hormones that negatively affect flower induction and differentiation suppressed the expression of floral homeotic genes involved in stamen and stigma development at the early stages of flower organ differentiation.

Hormonal effect on flower formation

To further investigate the roles of hormones in the flower formation of saffron, hormone treatments were performed in corms that have already induced flowering. After treatment, the corms were observed for flower development mainly flower formation. The results suggest that ABA just like in floral induction negatively affected flower formation. The corms that were treated with ABA showed flower atrophy, and the already initiated flower did not develop further, but the corm produced healthy leaves although with damage to the corms (Figure 6). Similar results were observed in IAA treated corms (flower atrophy with normal leaf development). On the other hand, GA and kinetin treatment accelerated flower formation compared with the control (Figure 6). The flowers in GA and kinetin treated corms showed no major difference in flower formation compared with the control. Interestingly, IAA, which

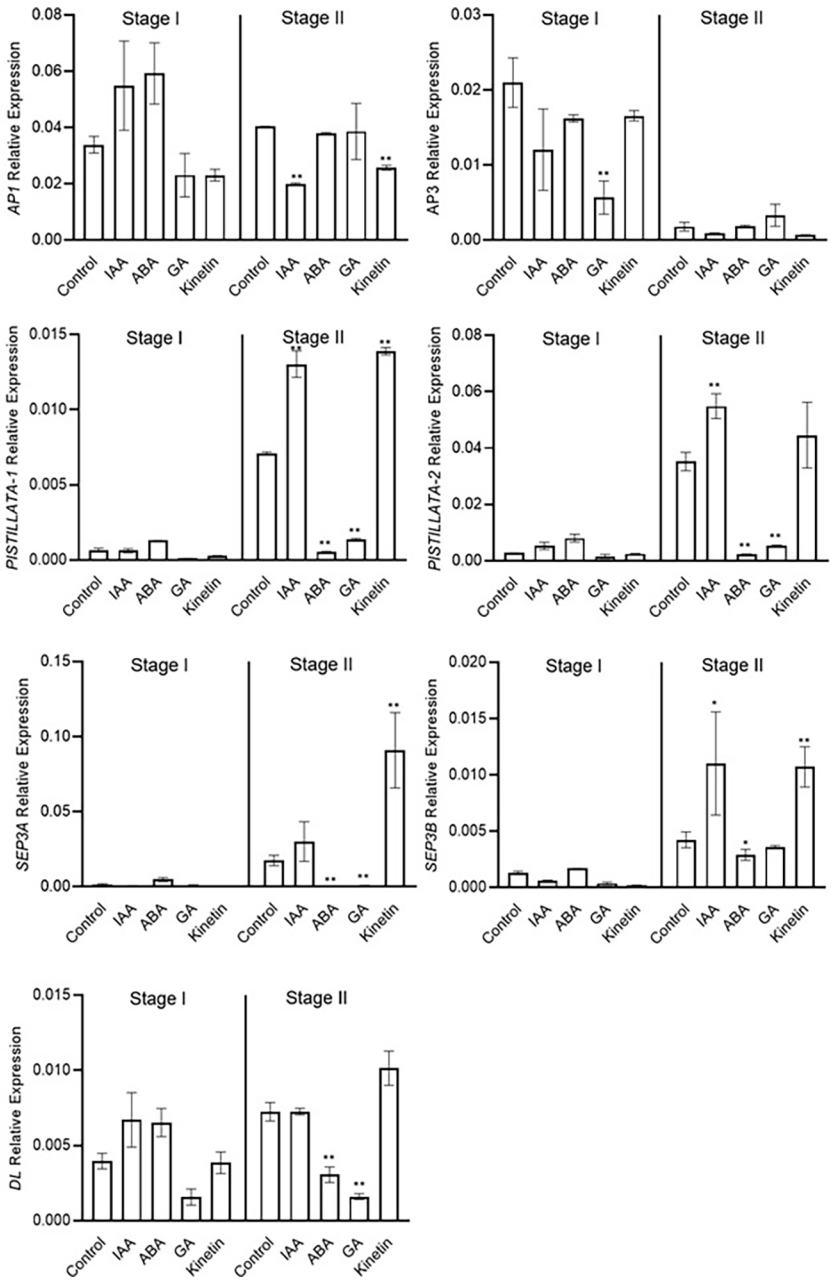


FIGURE 5
Expression profiling of genes involved in flower formation quantified by real-time PCR in response to different hormones. Total RNA was isolated from the apical bud of corms treated with different hormones at 45 days (stage I) and 90 days (stage II) of treatments. Reactions from three separate pools of root RNA samples were run in triplicates with tubulin as the internal control for normalization. Error bars represent ± SD of three biological replicates. Stage I morphologically represents the flower induction stage, and stage II represents the stamen and stigma formation stage. Data represented is mean of three biological replicates. Error bars represent standard error (SE), * indicates P <0.05, ** indicates P < 0.01, with respect to control (Student's t-test).

promoted flower induction, suppressed flower formation, whereas GA that inhibited flower induction showed no significant effect on flower formation (Figure 6).

Differential regulation of flower development genes (ABCE model) in response to different hormones

The expression analysis of flower formation genes was carried out at stage 3 and stage 4 of saffron flowering after different hormone treatments. ABA and IAA negatively affected flower formation and also downregulated the expression of ABCE model genes (Figure 7). The expression of *CsatAP3*, *CsatPISTILATTA-1*, *CsatPISTILATTA*-2, *CsatSEP3A*, *CsatSEP3B*, and *CsatDL*-like genes were significantly downregulated in stage 3 and stage 4 in ABA and IAA treated corms (Figure 7). The results correlated with the flower atrophy phenotype observed in corms treated with these hormones. There was no significant upregulation of any of the genes studied in GA and kinetin treated corms in comparison with the control. Moreover, the expression of most of these genes also decreased in GA and kinetin treated corms such as *CsatPISTILLATA2*, *CsatAP3*, and *CsatDL* (Figure 7). The expression of floral homeotic genes suggests that hormones regulate their expression in determining floral organ formation.

Discussion

Flowering is an important development process which determines plant productivity. The flowering process in plants is controlled by various environmental factors such as light, photoperiod, vernalization, and hormones (Freytes et al., 2021). Among them, plant hormones are one of the important factors that transmit signals from inside or outside the plant and play crucial roles in regulating the flowering process (Khodorova and Boitel-Conti, 2013; Conti, 2017). Hormones are present in minute quantities, and determining their endogenous content and its correlation in regulating a process is difficult. Hence, exogenous treatments are commonly used to study their effect on several plant development processes. Different hormones have been suggested to play a role during the process, but their distinct role in regulating the different developmental stages of flowering in saffron has not been elucidated. As the saffron flower develops, it goes through two distinct stages: flower induction and flower development or organogenesis. This study found that, in addition to temperature, these developmental stages are differentially regulated by hormones.

The spatiotemporal expression profiling of floral integrator genes has been previously described in saffron (Kalia et al., 2022). Tissue- and time-specific regulation of floral homeotic gene expression ensures proper and precise floral organ development. The studied floral homeotic genes showed tissue- and stage-specific expression patterns, suggesting that they are involved in specific organ development processes similar to other

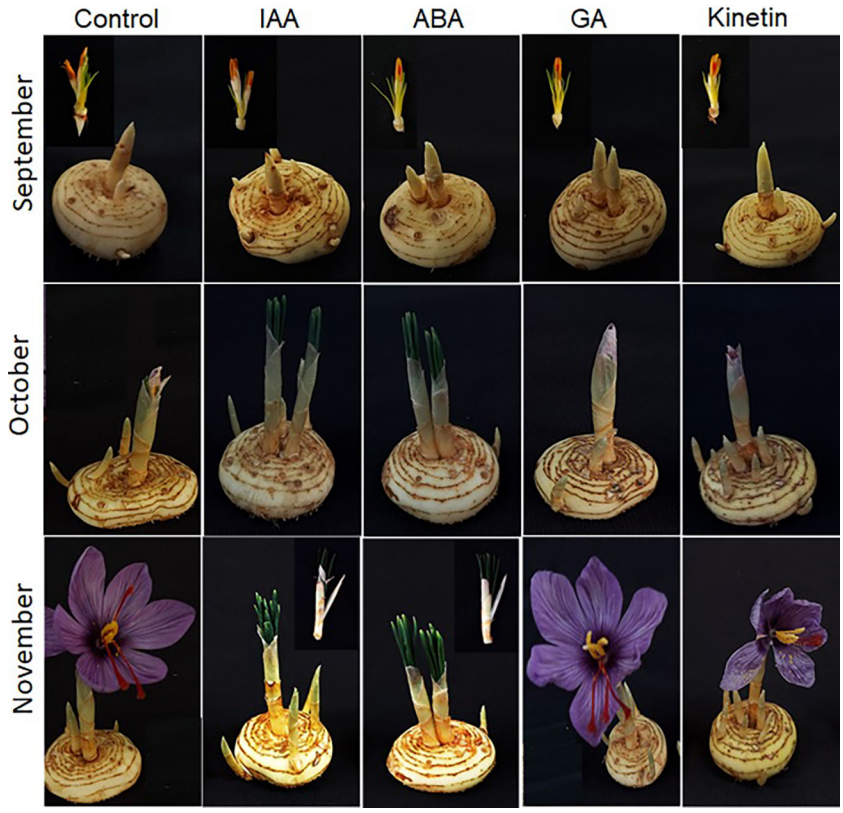


FIGURE 6
Morphological changes during flower formation in saffron after hormonal treatments. Corms already floral-initiated in early September were treated with different hormones and sampled after 45 days (mid-October) and 90 days (end of November).

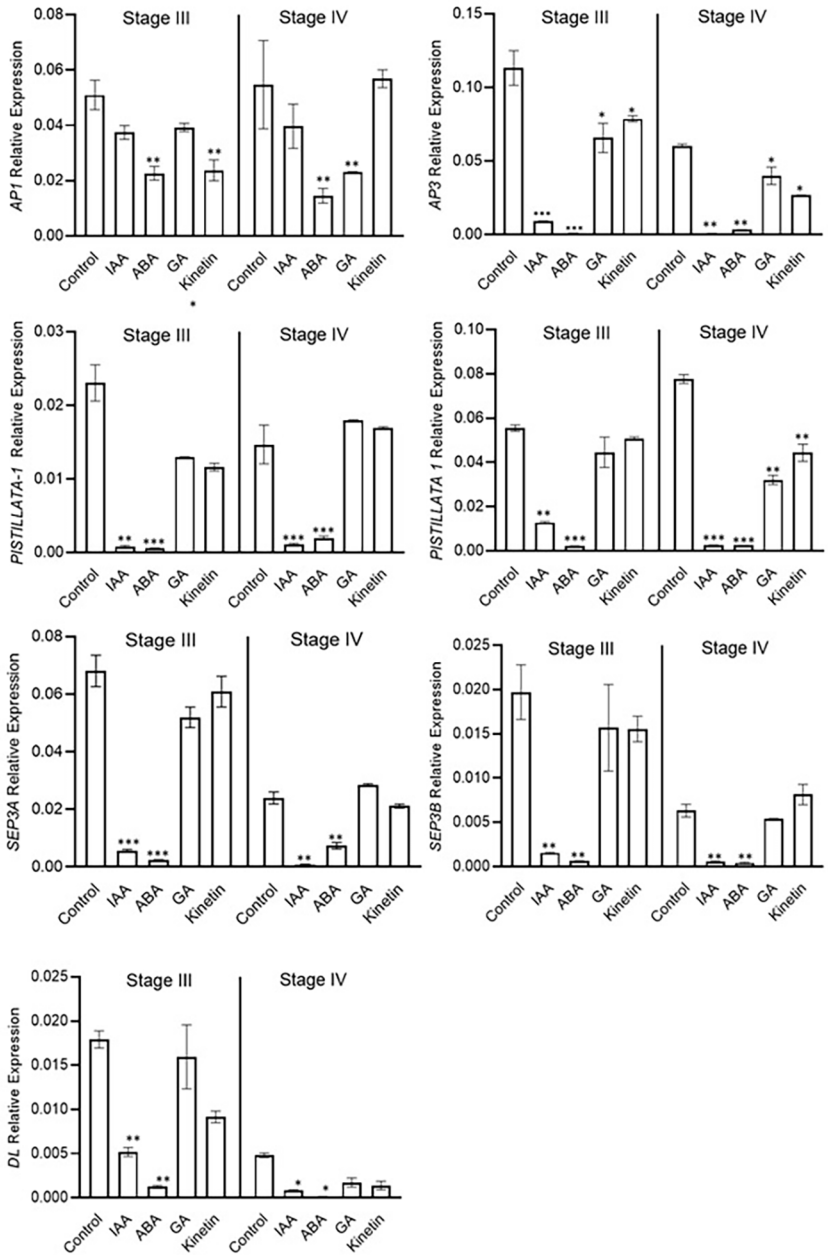


FIGURE 7
Expression profiling of genes involved in flower formation after the hormone treatment. Corms already initiated flowering were used for the treatment. The apical bud samples were collected after 45 and 90 days after the hormonal treatment. Error bars represent ± SD of three biological replicates. Stage III morphologically represents the stamen and stigma elongation stage, and stage IV represents the tepal formation stage. Data represented is mean of three biological replicates. Error bars represent standard error (SE), * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ with respect to control (Student's t-test).

plants (Liu and Mara, 2010). Similarly in other plants and in saffron, the *PISTILLATA* and *SEPATALLA* genes showed stamen-, stigma-, and tepal-specific expression, suggesting that they are involved in saffron flower organ development (Kalivas et al., 2007b; Tsaftaris et al., 2011). The expression of these genes correlated with specific stages when the stigma, stamen, and tepals are formed. Recently, a study carried out by Renau-Morata et al. (2021) reported for the first time *DL*-like genes and their involvement in flower formation in saffron but which lacked tissue-specific expression. Our results showed stigma-specific expression of *DL* and its expression during carpel (stigma, style, and ovule) development and suggest its involvement in the process. Our results are in accordance with the partial results shown by Kalivas et al. (2007a); Tsaftaris et al. (2011); Renau-Morata et al. (2021), and Tsaftaris et al. (2010).

Gibberellic acid has a distinct role during flowering

In our study, we found that hormones differentially affect the induction and formation of flowers in saffron. GA is the class of hormones which is best documented in the flowering process of *Arabidopsis*. It enhances the flowering process in *Arabidopsis* plant

(Mutasa-Göttgens and Hedden, 2009), but, on the other hand, restricts flowering in several perennial species (Boss and Thomas, 2002; Garmendia et al., 2019). GA regulates flowering in *Arabidopsis* by promoting the *LFY* and *FT* genes expression (Moon et al., 2003; King et al., 2008). This study has found that GA regulates the flower induction and flower development processes of saffron in distinct ways. GA represses the flower initiation process and promoted the flower development process in saffron. Our results are in accordance with the findings of Renau-Morata et al. (2021), which also predicted by internal GA quantification that GA might inhibit floral induction in saffron. Contrary to this though, the study by Hu et al. (2020) suggested a positive role of GA in promoting floral induction in saffron. The results presented by Hu et al. were based only on transcript analysis, and the contradiction may be due to the different stages of floral induction. It is possible that GA might naturally inhibit floral induction, as in other perennials, and promote flower organ formation. More support to our observation in the role of GA is provided by the transcriptional analysis of floral integrator genes. We observed that GA treatment significantly downregulated the expression of *CsatLFY* that is a floral integrator gene. In addition to this, GA is known to overcome the chilling requirement that is a must for many plant species to flower (Heggie and Halliday, 2004), but in the case of saffron, ambient high temperatures are required for flowering, and low temperature treatment suppresses flowering (Molina et al., 2005b). This specific temperature requirement for flower induction might be the reason for the divergent role of GA in saffron flowering compared with other flowering plants.

ABA inhibits flower induction and flower formation

ABA acts antagonistically to GA in various developmental processes, including flowering (Vanstraelen and Benková, 2012; Liu and Hou, 2018). It has a negative effect on flower initiation in *Arabidopsis* (Wang et al., 2013). However, ABA promotes flowering in *Litchi chinensis* (Cui et al., 2013). The suppression of flowering by ABA is shown to be mediated by effecting the expression of *FT*, *SVP*, and *FLC*-like genes (Martignago et al., 2020). The effect of ABA on geophytes, including saffron, has not been studied; rather, most of the studies are on leaf senescence. As flowering in saffron is accompanied with sprouting and dormancy release, it is very interesting to see its effects on saffron flowering. Our results also show the inhibitory role of ABA in the flower induction process in saffron and the repression of *FT3* and *LFY* gene expression. In addition to this, ABA treatment also increased the expression of flowering repressor genes *CsatTFL1-1* and *CsatSVP* in stage I of the flower initiation process. *TFL* and *SVP* genes have roles in dormancy establishment and release (Singh et al., 2018) other than flowering regulation. All homeotic genes except *CsatAP3* also showed downregulation in the initiation process. A negative role of ABA in the flower induction process in saffron is also suggested by Renau-Morata et al. (2021) based on internal hormonal content and the genes involved in ABA signaling. There are not many studies on the hormonal regulation of flowering in saffron, including ABA, although the role of ABA in regulating saffron corm dormancy has been studied (Rubio-Moraga et al., 2014).

Cytokinin (kinetin) is a positive regulator of floral induction and formation in saffron

Cytokinins have been studied in many plants in connection with flowering with contrasting roles. Cytokinin application in rice reduced the expression of *FT1* gene and delayed the flowering time (Cho et al., 2022), whereas in *Arabidopsis* it promotes flowering *via* the transcriptional activation of the *FT* paralog TSF (D'Aloia et al., 2011). Cytokinins are also important for ovule development in *Arabidopsis* (Higuchi et al., 2004; Bencivenga et al., 2012; Galbiati et al., 2013). However, this study found that cytokinin promotes both flower initiation and the developmental process of saffron by downregulating the flowering repressor gene *CsatTFL1-2* and upregulation of flower developmental genes *CsatPISTILLATA-1*, *CsatSEP3A*, and *CsatSEP3B*. Cytokinins have been implicated in dormancy release (Subbaraj et al., 2010; Wu et al., 2019) in several geophytes by the regulation of cell proliferation and division *via* cell cyclin genes. In calla lilies, a cross-talk between cytokinin and GA regulates dormancy and flowering (Subbaraj et al., 2010). Cytokinin is also essential for *in vitro* flower development in *Panax ginseng* (Soon Lee et al., 1991).

Auxins differently affect floral induction and formation

Auxins (IAA) are another group of well-known phytohormones which regulate various aspects of plant growth and development (William et al., 2006; Spaepen et al., 2007; Spaepen and Vanderleyden, 2011). It has been found that IAA plays a crucial role in gynoecium development of *Arabidopsis* (Nemhauser et al., 2000). Our study shows that IAA is able to initiate flowering through activating flowering induction genes *FT3* and *LFY* and suppressing the flower suppressor genes *TFL1-2*. Auxin also induces the expression of homeotic genes such as *SEP3B*, *PISTILLATA-1*, and *PISTILLATA-2*. In *Arabidopsis*, auxins regulate *LFY* expression in promoting flowering (Yamaguchi et al., 2013; Yamaguchi et al., 2016). In tulips, auxin has been identified as the main hormone involved in floral induction (Rietveld et al., 2000). However, unlike kinetin, IAA has a negative impact on the development of flower formation in saffron. Our results are in corroboration with the findings of Renau-Morata et al. (2021) where they also suggested promotion of flowering induction by auxins.

Conclusion

The findings of the study have been summarized in Figure 8. In conclusion, ABA negatively and cytokinin positively regulate both flowering induction and flower formation, whereas GA and IAA have an inverse effect on the different developmental stages. These hormones regulate the expression of genes, mainly floral integrator (*FT* and *LFY*) and repressor (*SVP* and *TFL1-2*) genes, during flower induction. Furthermore, during flower formation, they regulate the expression of floral homeotic genes (*PISTILLATA*, *SEPETALLA*, and *DL*). The findings of this study provide molecular insights into the hormonal regulation of flowering in saffron that can be utilized to

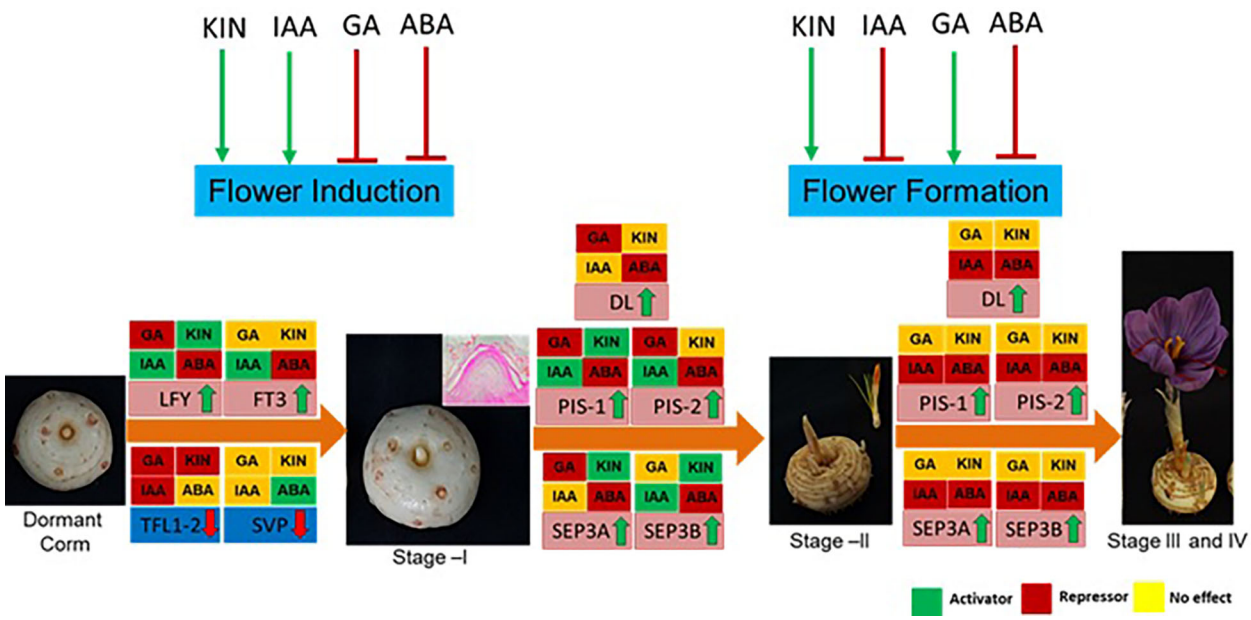


FIGURE 8
Summary of the effect of hormones on flowering process, gene regulation, and their effect at different developmental stages of saffron flowering. Briefly, ABA negatively regulates both flower induction and formation, whereas kinetin promotes both. Indole acetic acid (IAA) promotes flower induction, while gibberellic acid (GA) suppresses it. IAA inhibits flower formation, and GA promotes it. Green arrows show positive regulation, and red shows negative regulation. Hormonal effect on gene expression at different stages is marked by green, red, and yellow colors. The green color indicates induced expression levels, the red color indicates reduced expression levels, and the yellow color shows no significant changes in effect on the expression levels of the genes.

alter flowering in saffron as per requirement. Additionally, the results can be utilized to induce *in vitro* flowering in saffron. Moreover, how these hormone cross-talk during the process is an area to explore that will deepen the insights of flowering regulation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Author contributions

RS conceived the experiment idea. DS, SS, JJ-S, and DK performed the experiments and data analysis. All authors contributed to writing and reviewing the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1107172/full#supplementary-material

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Selected indigenous drought tolerant rhizobium strains as promising biostimulants for common bean in Northern Spain

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Drought is the most detrimental abiotic stress in agriculture, limiting crop growth and yield and, currently, its risk is increasing due to climate change. Thereby, ensuring food security will be one of the greatest challenges of the agriculture in the nearest future, accordingly it is essential to look for sustainable strategies to cope the negative impact of drought on crops. Inoculation of pulses with biostimulants such as rhizobium strains with high nitrogen fixation efficiency and drought-tolerance, has emerged as a promising and sustainable production strategy. However, some commercial inoculums are not effective under field conditions due to its lower effectiveness against indigenous rhizobium strains in the establishment of the symbiosis. Thus, in the present study, we evaluated the ability to improve drought tolerance in common bean plants of different indigenous rhizobia strains isolated from nearby crop fields in the Basque Country either affected by drought or salinity. The plants in this trial were grown in a climatic chamber under controlled conditions and exposed to values of 30% relative soil water content at the time of harvest, which is considered a severe drought. From the nine bacteria strains evaluated, three were found to be highly efficient under drought (namely 353, A12 and A13). These strains sustained high infectiveness (nodulation capacity) and effectiveness (shoot biomass production) under drought, even surpassing the plants inoculated with the CIAT899 reference strain, as well as the chemically N-fertilized plants. The tolerance mechanisms developed by plants inoculated with 353, A12 and A13 strains were a better adjustment of the cell wall elasticity that prevents mechanical damages in the plasma membrane, a higher WUE and an avoidance of the phenological delay caused by drought, developing a greater number of flowers. These results provide the basis for the development of efficient common bean inoculants able to increase the yield of this crop under drought conditions in the Northern Spain and, thus, to be used as biostimulants. In addition, the use of these efficient nitrogen fixation bacteria strains is a sustainable alternative to chemical fertilization, reducing cost and minimizing its negative impact on environment.

KEYWORDS

growth, inoculation, native strains, nodulation, *Phaseolus vulgaris*, phenology alteration, symbiosis, water relations

1 Introduction

Drought is the most severe abiotic stress in agriculture, limiting crop growth and yield (Soureshjani et al., 2019) and due to climate change its duration and severity is expected to increase in upcoming years, especially in southern Europe (IPCC, 2021). Common bean (*Phaseolus vulgaris* L.) is the grain legume for direct human consumption of greatest production worldwide (FAO, 2021) and one of the crops with the highest nutritional value due to its high content of protein, fibers, vitamins, minerals and bioactive compounds (FAO, 2021). Unfortunately, drought is, among abiotic stresses, the most detrimental constraint of grain yield for common bean, affecting about 60% of its production areas (Polania et al., 2017; Soureshjani et al., 2019; Rai et al., 2020). Its yield is reduced between 10 and 90% depending on the frequency, duration and intensity of the drought (Soureshjani et al., 2019; Rai et al., 2020).Thereby, is an urgent need to increase common bean yield under drought conditions in order to fulfill food demands of the continuously growing global population (FAO, 1992). Consequently, maintaining food security will be one of the greatest challenges of the agriculture in the nearest future.

Soil and plant roots are colonized by a great variety of microorganisms many of which are beneficial to plants (Singh et al., 2017; Khan et al., 2020). Among them are the so-called plant growth-promoting rhizobacteria (PGPR) that inhabit rhizosphere and endo-rhizosphere of plants promoting plant growth through multiple mechanisms, such as nitrogen fixation (Verma et al., 2010; Goyal et al., 2021). Rhizobia, bacteria that perform symbiosis with legume plants, are included as PGPR mainly for their ability to fix atmospheric nitrogen through the biological nitrogen fixation (BNF) (Lindström & Mousavi, 2020). Thanks to the establishment of efficient symbiotic relationships, the legume crops productivity is increased, as well as the N content of soils, without synthetic N-fertilizers application (Weese et al., 2015; Abebe & Deressa, 2017; Torabian et al., 2019; Goyal et al., 2021), avoiding their economic and environmental costs, such as degradation of the quality of limited and scarce resources (water and soil) and increase in agricultural GHG emissions, among others (FAO, 1990; Szparaga et al., 2019; Hossain & Bakhsh, 2020). In fact, agricultural techniques that provide the necessary fertilizers from natural sources, as occurs with the establishment of legume efficient symbiotic relationships, are particularly relevant today, as a result of the large price increases experienced in recent years due to the energy crisis (Horoias et al., 2022; Sargentis et al., 2022; Statista, 2022). Common bean is considered a poor nitrogen fixer crop since it tends to establish inefficient symbiotic relationships with rhizobia species (Michiels et al., 1998). Therefore, finding rhizobium strains that are able to promote high nitrogen fixation should be a priority in order to increase yields without chemical fertilizers.

In legume plants, drought is also one of the main factors that affect more negatively the rhizobial-plant interactions and therefore the biological nitrogen fixation. Water stress affects adversely all the aspects of the legume-rhizobium symbiosis, being one of the mayor problems the rhizobia survival and persistence in the soils (Prudent et al., 2019; Santillana Villanueva, 2021; Sharaf et al., 2019; Sindhu et al., 2020). Drought also inhibits nodulation and nitrogen fixation). Few strains of rhizobia show high tolerance to water stress (Sharaf et al., 2019).

In order to maintain legume yield under water stress conditions, inoculation of pulses with highly efficient and drought-tolerant nitrogen fixing rhizobium strains has emerged as a promising sustainable production strategy. In this way, promising results have been obtained by inoculating drought-tolerant rhizobia in chickpea (Aulakh et al., 2020; Laranjeira et al., 2021), lentil (Sijilmassi et al., 2020), soybean (Igiehon et al., 2021; Omari et al., 2022), mungbean (Sapna & Sharma, 2020; Nawaz et al., 2021), pea (Prudent et al., 2019) and common bean (Figueiredo et al., 2008; Sijilmassi et al., 2020; Rodiño et al., 2021), leading to an improvement of legume drought tolerance and increasing yield under water stress. In addition, some osmotolerant rhizobial strains are also capable of surviving and nodulating efficiently under drought conditions in the soil (Fernandez-Aunión et al., 2010; Mhadhbi et al., 2011; Viti et al., 2021), and its inoculation increases the drought tolerance in legumes (Kibido et al., 2019). Mhadhbi et al. (2011) observed how the drought tolerance of common bean improved greatly after inoculating the plants with the osmotolerant strain *Ensifer meliloti* 4H41, isolated from common bean nodules from a Tunisian oasis (Mnasri et al., 2007).

The most widely used commercial rhizobium inoculum for the improvement of common bean production in the world is the Brazilian strain *Rhizobium tropici* CIAT 899 (Hungria et al., 2000). This strain is also able to nodulate a variety of other legumes and has been shown to have high tolerance to salt and drought stress (Mhadhbi et al., 2011; Sijilmassi et al., 2020) and to high temperatures (Hungria et al., 2000). Due to these characteristics, it has been used as a control or reference strain in countless common bean and soybean research (Mhadhbi et al., 2011; Cardoso et al., 2017).

However, commercial or reference inoculum may vary in effectiveness with soil type or legume genotypes. Firstly, rhizobia strains need to survive for long time in the soil in the absence of the legume (Sindhu & Dadarwal, 2000; Ruiz-Díez et al., 2012) meaning that different geographical areas with different edaphoclimatic characteristics may not be favourable for the survival of the commercial inoculum or for the establishment of the symbiosis (Sindhu et al., 2020; Omari et al., 2022). Secondly some commercial inoculum lost effectiveness when translated from greenhouse to field due to the competition with indigenous rhizobia strains that are more effective in the establishment of the symbiosis (Kumar &

Abbreviations: A, Assimilation rate; BNF, Biological nitrogen fixation; D, Drought treatment; DH, Dehydration; DS, Developmental stage; DW, Dry weight; EC, Electrolytic conductivity; EL, Electrolyte leakage; FEL, Full expanded leaf; Fv/Fm, Photochemical efficiency of PSII in dark-adapted leaves; Fv′/Fm′, Photochemical efficiency of PSII in light-adapted leaves; FW, Fresh weight; Gs, Stomatal conductance; OA, Osmotic adjustment; PGPR, Plant growth promoting rhizobacteria; RLWC, Relative leaf water content; RSWC, Relative soil water content; T, Whole plant transpiration; TLA, Total leaf area; TW, Turgid weight; WA, Water availability; WUE, Water use efficiency; WW, Well-watered treatment; E, Volumetric elastic modulus; Ψo, Osmotic potential; Ψt, Turgor pressure or cell wall potential; Ψw, Water potential at midday; $\Delta^{13}C$, Carbon isotope discrimination; $\partial^{15}N$, Natural isotopic ratio of 15N.

Verna, 2018; Sindhu et al., 2020). Thus, usually, indigenous rhizobia are better adapted to local edaphoclimatic conditions and are more competitive with the microbiota of the specific area being more successful in nodulating the genotypes cultivated in that region (Koskey et al., 2017; Omari et al., 2022). For these reasons, we selected 8 rhizobium strains isolated from nodules of common bean cultivars grown under drought and saline field conditions in Northern Spain, with the objective of testing the effect of inoculation on different plant physiological variables related with drought tolerance, such as osmotic adjustment, cell wall elasticity adjustment, early flowering or increase in water use efficiency, among others.

Thus, the present work was based on the hypothesis that the inoculation of common bean plants with different indigenous rhizobia strains isolated under drought and saline conditions would also enhance common bean tolerance to water stress. With this aim, we studied the different drought tolerance mechanisms developed by plants after the inoculation with indigenous rhizobia isolated from local soils exposed to water and salinity stress conditions, and we selected the most efficient ones, expecting that in the future, they could be used as biostimulants to improve common bean yield under water stress in the Northern Spain.

2 Materials and methods

2.1 Biological material and growth conditions

The bacterial strains tested in this research belong to a collection of potentially drought-tolerant indigenous rhizobia cultures and to a collection of osmotolerant indigenous rhizobia cultures of the Department of Plant Biology and Ecology of the University of the Basque Country (UPV/EHU), preserved at -80°C in 50% glycerol TY medium (Table 1). Two rhizobium species, *Rhizobium giardinii* (343) and *Rhizobium gallicum* (353), were isolated from common bean plants grown under drought conditions in agricultural soils located in Álava (Basque Country, Spain), and six more species were isolated from common beans grown in saline soils located in Las Bardenas Reales (Navarra, Spain): *Rhizobium giardinii* (A5), *Rhizobium phaseoli* (A9), and five cultures identified as *Rhizobium etli* (A10, A11, A12 and A13) (del-Canto, 2022). All of them isolated from ecologically managed soils, with a high microbial diversity. In addition to these strains, the *Rhizobium tropici* CIAT899 strain was also tested as a reference strain of proven efficiency under drought conditions (Mhadhbi et al., 2011; Sijilmassi et al., 2020). This strain was provided by Dr. Juan Sanjuan from the Plant-Bacteria Interactions Laboratory of the Zaidín Experimental Station, CSIC (Granada, Spain).

TABLE 1 Bacterial strains selected for the study.

Code	Specie	Strain colection
343	*Rhizobium giardinii*	Potentially drought tolerant
353	*Rhizobium gallicum*	Potentially drought tolerant
A5	*Rhizobium giardinii*	Osmotolerant strains
A9	*Rhizobium phaseoli*	Osmotolerant strains
A10	*Rhizobium etli*	Osmotolerant strains
A11	*Rhizobium etli*	Osmotolerant strains
A12	*Rhizobium etli*	Osmotolerant strains
A13	*Rhizobium etli*	Osmotolerant strains

The common bean genotype selected for the assay was Arrocina de Álava, a local genotype of great economic interest, widely cultivated in Álava, Spain, and certified with the seal of quality from the Basque Country (Eusko Label, https://euskolabel.hazi.eus/es/producto-eusko-label/legumbres-del-pais-vasco/), but not studied to date.

2.2 Experimental design

The symbiotic performance and the drought tolerance conferred by their inoculation were tested in a pot experiment in a controlled environment grow chamber (ASL Ibercex S.A., Alcalá de Henares). Seeds were surface-sterilized by immersion in NaClO 1% (v/v) for 10 min and thoroughly washed with sterilized distilled water. The seeds were sown in 3L capacity pots, suitable for plant growth evaluations in rhizobia experiments for common beans (Obede da et al., 2020), filled with sterile perlite: vermiculite (2:1 v/v). To reduce the possibility of cross-contamination, the pots were placed in individual plastic plates and covered with thick plastic bags, all previously autoclaved (121°C, 30 min). Three seeds per pot were sown and once the seedlings emerged, only one was left, ensuring that all had a homogeneous size and vigor. Plants were grown under 14 h light photoperiod and 500 µmol photon·m^{-2} s^{-1} of light intensity with a day/night temperature of 23/18°C and 65/70% relative humidity, respectively.

The water and inoculation treatments were assigned following a completely randomized design with 10 inoculum treatments and 2 water availability treatments and replicated 8 times. The inoculation treatments were: non-inoculated plants and N-fertilized; inoculated with CIAT899 reference strain (*Rhizobium tropici*); strain 343 (*Rhizobium giardinii*); strain 353 (*Rhizobium gallicum*); strain A5 (*Rhizobium giardinii*); strain A9 (*Rhizobium phaseoli*); strain A10 (*Rhizobium etli*); strain A11 (*Rhizobium etli*); strain A12 (*Rhizobium etli*) and strain A13 (*Rhizobium etli*). The 160 pots were randomly distributed and rotated periodically on the growth chamber tables to avoid intra-chamber variability.

Throughout the plant development, the whole plant transpiration data, phenological stage, and the number of flower and leaves were recorded. The day before harvest, photosynthesis, fluorescence, SPAD and water potential data were taken from the central leaflet of full-expanded leaf. All plants were harvested at 40 days after emergence, after 25 days of drought treatment.

2.3 Inoculation and water stress treatment

Once sterilized, the seeds were inoculated before sowing with the nine selected bacteria according to a modified version from

Sanz-Sáez et al. (2015). Summarizing, 20 mL of 10^9cell·mL^{-1} TY medium of each strain was taken, centrifuged at 5,100 *g* for 10 min and re-suspended in 200 µL of sterile distilled water with 2% PVPP. These 200 µL water rhizobial cell suspension of 10^{11} cell·mL^{-1} concentration was added to another 50 mL of sterile water and pour it over the approximately 700 seeds and let for 24 hours, until the evaporation of water. Uninoculated seeds were also included in the experiment as a sterility control treatment. The seeds were treated as described previously for inoculated seeds but with TY medium without inoculum. In addition, the rhizosphere of each seedling was inoculated three times at 3, 7 and 11 days after emergence with 3 mL of a water rhizobial cell suspension of 10^8 cell mL^{-1} per plant in the inoculated treatments, and with sterilized water in non-inoculated treatment (Sanz-Sáez et al., 2015).

The plants were watered at field capacity (100% relative soil water content) until the establishment of the drought. Once the drought treatment began (15 days after emergence), the well-watered plants were irrigated at field capacity, while the stressed plants were subjected to drought by completely withholding watering until reaching 30% relative soil water content. From this moment, only the transpired water was replaced to maintain the plants at this relative soil water content. The plants were irrigated three times a week, alternating water and nutritive solution to avoid salt accumulation. The inoculated plants were watered with Evans N free solution (Evans, 1974), while the uninoculated plants were watered with Evans's solution with 10 mM KNO_3. Thus, in the inoculated plants, as they were grown on an inert substrate watered with N free nutrient solution, all the nitrogen presented by plants came from the seed reservoir and from the plant's nitrogen fixation (Díaz de Alcántara, 2010).

2.4 Phenological and growth parameters

The phenological stage, number of leaves and flowers of all plants were recorded three times a week, the watering days, from sowing to harvest. The phenological stage of all plants was determined based on the scale established by CIAT (Fernández et al., 1986).

At harvest time, the plants were separated into aerial and root biomass and different growth parameters analyzed. The area of the youngest full-expanded leaf (cm^2) and the plant total leaf area (m^2) were determined using a Licor 3100 area meter (LI-COR Biosciences). Leaves, stems and the full-expanded leaf were weighed separately to obtain their fresh weight (g). The dry weight (g) was obtained after drying in an oven at 80°C, a minimum of 48 hours. From these data leaf biomass (g), stem biomass (g) and shoot biomass (g), as the sum of leaf and stem biomass, were obtained.

The root systems were washed, and nodules were detached and weighed to determine the nodule fresh weights per plant (g). Then, the root dry weight (g) was obtained as explained before. Total biomass (g) was calculated adding the root biomass and shoot biomass.

2.5 Water relations

The whole plant transpiration (T, g H_2O·plant^{-1}) was calculated by gravimetric method by weighing each pot before and after watering and calculating the evapotranspirated water from the previous irrigation. Thus, adding all the obtained values per day, the accumulated evapotranspiration per plant was calculated. The whole plant water use efficiency (WUE, g DW·Kg^{-1} H_2O) was calculated as the relation between the total dry matter produced and the total water transpired (Medrano et al., 2007).

The day before harvest, the leaf water potential at midday (Ψw, MPa) was measured on the full-expanded leaf´s terminal leaflet, using a pressure chamber (Skye model SKPM1400) according to the method described by Scholander et al. (1965) and modified by Mena-Petite et al. (2001). The relative soil water content (%) was determined by the gravimetric method described by Epron (1997) and modified by Miranda-Apodaca et al. (2015). Immediately after harvesting the plants, about 100 mL of humid substrate from central zone of the roots was weighed obtaining the fresh weight (g), dried at 80°C for a minimum of 48 hours and weighed again to obtain the dry weight (g). With these data, the relative soil water content was calculated as follows:

$$\text{RSWC}(\%) = \frac{\text{FW} - \text{DW}}{(\text{DW}(\text{FWi}/\text{DWi})) - \text{DW}} * 100$$

Where FWi was the fresh weight of each pot kept at field capacity and DWi was the dry weight of each pot kept at field capacity (droughted) of the well-watered plants.

The relative leaf water content was calculated using the gravimetric method according to Pérez-López et al. (2009a). After obtaining the fresh weight (FW, g) of three discs of 0,8 mm of diameter of the central portion of the right lateral leaflet of the full-expanded leaf, they were introduced into a Petri dish with distilled water and kept during 24 h at 4°C and in the dark (to avoid processes of respiration and photosynthesis, and thus changes of dry matter). After that, they were weighed to obtain the turgid weight (TW, g) and then dried in oven for a minimum of 8 h at 80° C to quantify the dry weight (DW, g). The relative leaf water content (RLWC) was then calculated using the following equation:

$$\text{RLWC}(\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} * 100$$

The osmotic potential, (Ψo, MPa) and osmotic potential at maximum turgor, Ψo^{100} (MPa) were obtained from fresh and turgid plant material, respectively, of central zone of the full-expanded leaf´s central leaflet as described Pérez-López et al. (2009a). To obtain the turgid plant material, the leaflet was rehydrated in a Petri dish with distilled water for 24 h at 4°C in the dark. After that, in both cases, the plant material was cut in small pieces and introduced into a 0.5 mL Eppendorf tube perforated at the base that was introduced in another 1.5 mL Eppendorf tube and frozen in liquid nitrogen to achieve the rupture of the cell membranes. Then, the samples were stored at -80°C. After a few days, the tubes were defrosted and centrifuged at

13,200 *g* for approximately 5 minutes. This technique allows to separate cellular sap, which remains in the larger Eppendorf, from the solid fraction, which remains in the small one. 25 µl of cellular sap were taken and kept in a bath at 25°C, for 10 minutes moment at which the osmolarity (osmol·Kg^{-1}) was measured by an osmometer (Osmomat 3000 basic, Gonotec, Germany).

The osmotic potential was calculated using the Varit Hoff's Law according to Wyn jones & Gorham (1983):

$$\Psi o(MPa) = -n * R * T$$

Where *n* is the osmolarity provided by the osmometer (osmol·Kg^{-1}), *R* corresponds to the universal gas constant (0.0083 Kg·MPa·mol^{-1}·K^{-1}) and *T* is the absolute temperature of the sample (K°). The osmotic adjustment (MPa), was calculated as the difference between the mean value of Ψo^{100} of the control plants (WW) and the mean value of Ψo^{100} of plants subjected to water stress (D). The osmotic adjustment (OA) of the irrigated control plants is assumed to be zero.

$$OA = \Psi o^{100}ww - \Psi o^{100}D$$

The dehydration (DH, MPa) of the leaves (full-expanded leaf) from six plant per treatment was estimated as the difference between the solute potential values of fresh material and the material at full turgor using following formula:

$$DH = \Psi o - \Psi o^{100}$$

The turgor pressure or cell wall potential (Ψt, MPa), was calculated as the difference between the leaf water potential (Ψw) and osmotic water potential (Ψo).

$$\Psi t\,(MPa) = \Psi w - \Psi o$$

The wall cell elastic modulus (ϵ, MPa), was estimated according to Rivelli et al. (2002), with the following formula:

$$\epsilon\,(MPa) = \frac{\Delta\Psi t}{AV/V}$$

where ΔΨt is the difference of turgor pressure (MPa) between full turgor conditions ($\Psi t^{100} = \Psi o^{100}$) and growth conditions (Ψt):

$$\Delta\Psi t = (\Psi o^{100} = \Psi t^{100}) - \Psi t$$

and AV/V is the difference between the relative leaf water content in growth conditions (RLWC) and the the relative leaf water content of the fully hydrated tissue ($RLWC^{100}$),

$$AV/V = (RLWC^{100} - RLWC)/100$$

$$RLWC^{100} - RLWC)/100$$

The leaf electrolyte leakage (%) was determined as described by Mena-Petite et al. (2001). Three discs of 0.8 cm diameter from the full-expanded leaf´s left lateral leaflet were introduced into plastic vials, (previously washed with abundant deionized water) containing 16 mL of deionized water of known electrical conductivity (EC_0). Electrolytic conductivity was measured using a digital conductivity meter (EC meter 19101-10 Cole-Parmer Intrument Company Vernon Hills, USA). The vial was closed and shaken to ensure that the plant material was in contact with the water. After 24 h at room temperature, a new electrical conductivity measurement was taken (EC_{24}). Then, the samples were autoclaved (10 min 110°C), to disrupt the cell membranes and release all electrolytes from the cell and cooled at room temperature to measure the total electrical conductivity (EC_T). The electrolyte leakage (EL, %), was calculated according the following equation:

$$EL\,(\%) = 100\left(\frac{EC_{24} - EC_0}{EC_T - EC_0}\right)$$

2.6 Photosynthetic parameters

The determination of gas exchange parameters such as assimilation rate (µmol CO_2·m^{-2}·s^{-1}) and stomatal conductance (gs) was performed the day before harvest using an infrared gas analyzer system (IRGA, CIRAS-2, PP Systems, Hitchin, UK) at a photon flux density of 700 µmol m^{-2} s^{-1}. Measurements were taken in the terminal leaflet of the full-expanded leaf, 2 h after the lights of the chamber were switch on, with a cuvette at a stable temperature of 24°C, relative humidity of 60%, and the CO_2 concentration at 410 ppm.

The photochemical efficiency of PSII in dark-adapted leaves, or also called maximum efficiency of PSII photochemistry, Fv/Fm (Genty et al., 1989), was measured in the central zone of the terminal leaflet of the full-expanded leaf the day before harvesting with a portable fluorometer (FluorPen FP 100, Photon Systems Instruments, spol. s.r.o., Drasov, Czech Republic). The Fv/Fm data in the plant adapted to dark conditions were taken in predawn conditions, while the Fv'/Fm' data, were taken two hours after sunrise, once the plants were adapted to light conditions.

The determination of chlorophyll content was carried out by a portable chlorophyll meter (SPAD-502 plus, Konica Minolta Sensing, Inc), by measuring the terminal leaflet of the full-expanded leaf, the day before harvest (Adamsen et al., 1999).

2.7 ^{13}C and ^{15}N stable isotope analysis and C and N content

^{13}C and ^{15}N stable isotope analysis were performed as an estimation of WUE and biological nitrogen fixation, respectively. The determination of the isotopic ratio of ^{15}N ($\partial^{15}N$) and the carbon isotope discrimination ($\Delta^{13}C$) was carried out from a pool of dry foliar material of each plant at the Research Support Service (SAI) of the Elvina Campus (A Coruña). Samples were ground and weighed into tin capsules using a microbalance UMX-2 balance (Mettler, Toledo). The combustion was performed on an elemental analyzer (Flash EA 1112, Thermo Finnigan) coupled *via* a ConfloII interface to an isotope ratio mass spectrometer (DeltaV Advantage, Thermo Scientific). The results for leaf C and N content were expressed in %, and those for $\partial^{13}C$ and $\partial^{15}N$ were expressed in ‰ relative to VPDB standards (Vienna Pee Dee Belemmite) and

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atmospheric air, respectively. The ratio (R) of $^{13}C/^{12}C$ was showed as $\delta^{13}C$ (‰) indicating the C isotope composition:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}}\right) - 1$$

where, R_{sample} and $R_{standard}$ are the isotope ratios ($^{13}C/^{12}C$) of the sample and international secondary standards of Known $^{13}C/^{12}C$ ratios (USGS 40, USGS41a, IAEA-N-1, IAEA-N-2 y USGS-25), respectively.

The natural ^{15}N isotopic ratio ($\delta^{15}N$) was calculated using the formula described by Shearer et al. (1982):

$$\delta^{15}N = \frac{R_{sample}}{(R_{air} - 1)} * 1,000$$

where, R_{sample} and R_{air} are the isotope ratios ($^{15}N/^{14}N$) of the sample and air, respectively.

The analysis of carbon isotope discrimination, $\Delta^{13}C$ (‰) was calculated from the $\partial^{13}C$ (‰) values, according to, Polania et al. (2016),

$$\Delta^{13}C = \left(\frac{C_{atm} - \partial^{13}C_{sample}}{1}\right) + \frac{\partial^{13}_{sample}}{1,000}$$

where $\partial^{13}C_{atm}$ is the carbon isotope composition of atmospheric CO_2 (−8‰) (Farquhar et al., 1989) and $\partial^{13}C_{sample}$ is the C isotope composition in seed samples.

From the values of leaf nitrogen content (LNC, %), the parameters of leaf nitrogen total content per plant (LNTC, g) was calculated.

$$LNTC = LNC * \text{leaf DW}$$

where, DW was the total foliar biomass (g).

2.8 Soluble proteins

The extract used for the quantification of soluble proteins was carried out as Pérez-López et al. (2009b). The extracts were obtained using 0.15 g of fresh material from the central area of the full-expanded leaf collected at harvest and ground with liquid nitrogen on a previously cooled mortar. Eight plants per treatment were analyzed. The extract was homogenized with 3 mL of extraction buffer (50 mM phosphate buffer, pH 7.8; 0.1 mM EDTA; 5 mM Cysteine; 2 mM Asa), filtered through two layers of muslin and centrifuged at 4°C for 25 minutes at 16,100 *g*.

Five µl of supernatant were diluted in 595 µl of commercial Bradford reagent (BIO-RAD protein Assay) diluted in 1:4 MilliQ water. The reaction was maintained for 10 minutes in the darkness and the absorbance was determined at 595 nm (Bradford, 1976). A calibration curve was made with bovine serum albumin (BSA) (Abs_{595} = 0.34 mg prot · mL^{-1} + 0.0326; R^2 = 0.990; using 8 calibration points, from 0 to 0.7 mg·mL^{-1} BSA to further determine the leaf protein concentration (LPC, mg prot g^{-1} DW). The leaf protein total content (LPTC, mg) was calculated from the LPC values, considering the foliar biomass (DW, g).

$$LPTC = LPC * \text{leaf DW}$$

2.9 Statistical analysis

The data were analyzed using the statistical package SPSS Statistics 24.0 (IBM Corporation, Armonk, NY, USA). The normality of the non-standardized residuals of the data was studied using the Shapiro-Wilk test (considering normal data if p >0.05) and the homoscedasticity of the variance was studied with the Levenne test (considering homoscedasticity of the variance if p > 0.05). A two-way analysis of variance (ANOVA) was performed with water stress treatment and inoculation treatment as main factors and replicates as random effect. When the interaction between water treatment and inoculation treatment was significant, least square means *post-hoc* test was performed to compare means (Tukey or Kruskal Wallis, for parametric and non-parametric data, respectively).

To study the effect of the inoculation without presence of interaction, the multiple comparisons abovementioned were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions). The effect of water availability without presence of interaction was studied for each inoculation treatment separately performing a 1-factor ANOVA test, in the case of parametric data, or the U Mann-Withney test for two independent samples for non-parametric data indicating with asterisks the existence of significant differences.

For the correlations, the Pearson's Coefficient for parametric data and Spearman's for non-parametric data has been shown, also indicating the significance of these correlations by asterisks (* p< 0.05; ** p<0.01 and *** p<0.001).

In addition, a comparision between inoculated and non-inoculated plants was performed to evaluate the differences between plant with a non-inoculated treatment watered with mineral N-fertilization and plant inoculated with potentially drought tolerant rhizobia. Thus, data were analyzed using a two-way analysis of variance (ANOVA), with water stress treatment and "nitrogen source" category as main factor and replicates as random effect.

3 Results

3.1 Phenology and growth parameters

Harvesting was done at flowering (R6) and all the studied phenological and growth parameters were affected both by the availability of water and by the inoculated strain (Figures 1–3). In general, drought delayed the development process by, at least, one stage (R5, Figure 1 and Supplementary Material Table S.1) except in plants inoculated with the strains 353, A12 and A13 that showed the same developmental stage (R6) than well-watered plants at harvest (Figure 1). Plants inoculated with 353, A12 and A13 showed better response than the plants inoculated with the CIAT899 and especially those not inoculated (N-fertilized) that showed a significant phenological delay at harvest (vegetative state V4).

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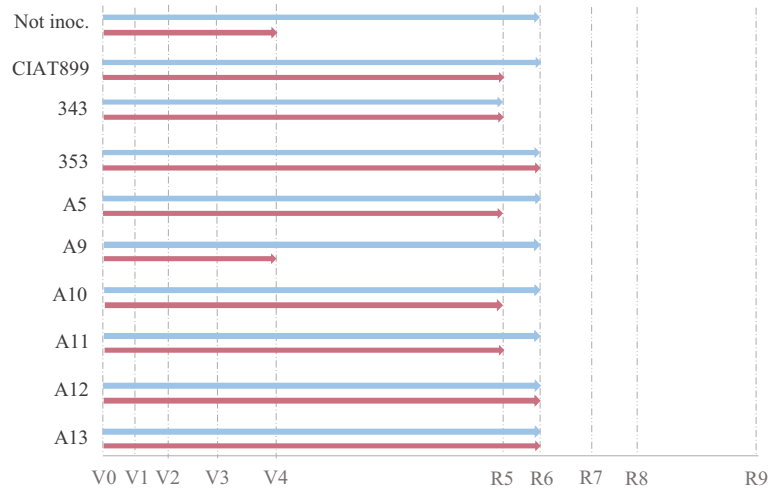


FIGURE 1
Phenological state of non-inoculated common bean (Not inoc.) and inoculated with different strains of rhizobium (CIAT899, 343, 353, A5, A9, A10, A11, A12 and A13), under different water availability conditions: well-watered (blue) and drought (red).

The number of flowers per plant (Figure 2C) was reduced from around 18 flowers in the well-watered conditions to 6 flowers under drought, a reduction of 65.95%. In well-watered conditions, no significant differences in the number of flowers were observed among the majority of inoculation treatments while, under drought, all inoculation treatments reduced significantly the number of flowers. However, with 353, A12 and A13 the reduction in flower number was lower than observed in the rest

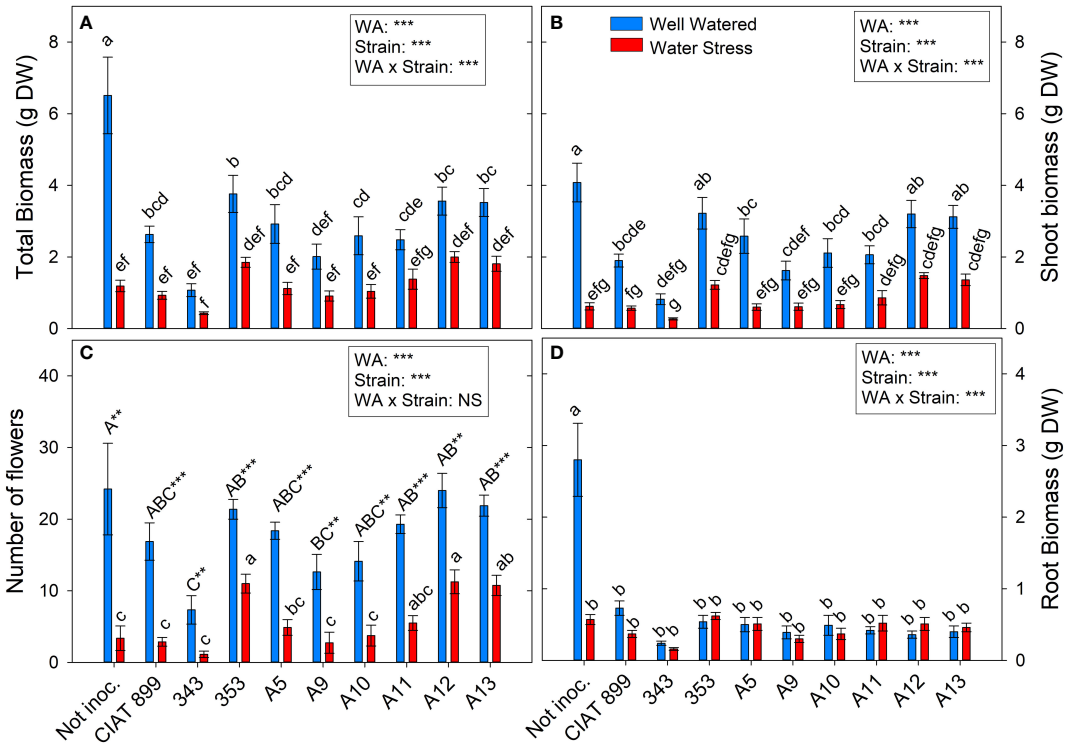


FIGURE 2
Phenological and biomass parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): ** p<0.01 and *** p<0.001). **(A)** Total biomass, DW g; **(B)** Shoot biomass, DW g; **(C)** Number of flowers; and **(D)** Root biomass, DW (g) DW, dry weight.

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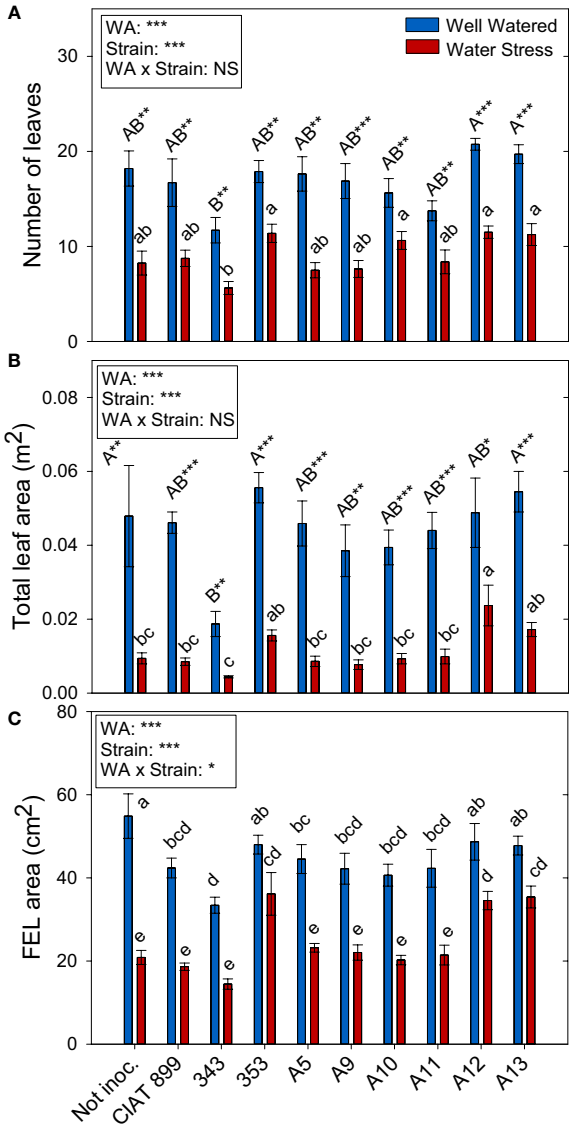


FIGURE 3
Leaf growth parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** Number of leaves; **(B)** Total leaf area, m2; and **(C)** Full-expanded leaf (FEL) area, cm2.

of treatments. Drought reduced on average for all treatment shoot biomass by 66.5% and total biomass by 59%. Non-inoculated plants (N-fertilized) (Figure 2A) produced the highest total biomass in well-watered conditions, especially in root biomass (Figure 2D), but were the treatment most negatively affected by drought. In inoculated plants, drought reduced all biomass parameters except for the root biomass (Figure 2D), and no differences among strains were observed, although the plants inoculated with the strain 353, A12 and A13 reached the highest values of leaf biomass under water deficit conditions, surpassing N-fertilized and plants inoculated with strain CIAT 899 (Supplementary Figure 1).

Total leaf area (Figure 3B) was more negatively affected by drought than leaf biomass (Supplementary Figure 1). This fall in total leaf area was due to a reduction in the number of leaves per plant (46%, Figure 3A) and a reduction of the size of the leaves (full-expanded leaf, 43.9%, Figure 3C). In general, in well-watered conditions, plants not inoculated and those inoculated with the 353, A12 and A13 strains showed the highest values in biomass parameters whereas, under drought, these strains responded better than the nitrogen fertilized plants and the plants inoculated with CIAT 899 (Figure 3). In the other hand, the strain 343 showed the lowest value both in control and drought conditions in all biomass parameters evaluated.

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3.2 Water relations

At harvest, control plants were at 100% of field capacity while in drought stressed plants the relative water content of the soil was close to 30% (20% in plants inoculated with strain A13, Figure 4A), confirming the drought level at which different treatments were exposed. The drought treatment significantly decreased leaf water potential (Ψw), from an average value of -0.46 MPa in the well-watered control treatment, to an average value of -4.46 MPa under drought (Figure 4B). The inoculation treatment showed no differences in Ψw in within the well-watered plants although under drought, the plants inoculated with the strain A11 reached the lowest water potential values (-5.28 MPa) and those inoculated with A5 the highest one (-3.47 MPa). This drop in Ψw was mainly due to the loss of cellular turgor, due to a decrease in cell wall pressure potential (Ψt, Figure 4C) showing a very similar behaviour in most inoculated plants. In the other hand, in the non-inoculated plants and in the inoculated plants with CIAT899, 343, A9 and A11, the drop in Ψw was also due to a significant drop in osmotic potential (Ψo, Figure 4D). This drop in Ψo could be due to a passive loss of water, dehydration (Figure 5D), as observed in non-inoculated and in plants inoculated with 343, A9 and A11 strains that showed also a reduction in the relative water content of the leaf (Figure 5A). This decrease could also be due to an active process of solutes accumulation (osmotic adjustment, AO Figure 5C) being the plants inoculated with reference strain CIAT899 and A11 the ones that showed the highest AO values while the inoculated with 343 showed the lowest.

All the plants showed a significant increase in the elasticity of the cell wall (ϵ) with drought stress (Figure 5B) being significantly lower in plants inoculated with bacteria, especially in plants inoculated with A9, A12, CIAT899, A13 and 353 strains. Drought also caused a degradation of the membranes as indicated by the increase in electrolyte leakage (Figure 6A), being the plants inoculated with A12 and the CIAT899 those least affected by membrane leakage; whereas the highest values were observed in not inoculated plants and those inoculated with strains 343 and A10, the most drought sensitive inoculation treatments.

Concerning the total transpiration accumulated during the whole experiment, non-inoculated plants were the ones that showed highest values under well-watered conditions and especially under drought (Figure 6B), therefore, they showed a large drop in the total water use efficiency (WUE) under drought (76.9%, Figure 6C). Among inoculated plants, drought reduced total transpiration and got similar WUE than control plants. The lowest WUE was observed in plants inoculated with 343 strains whereas plants inoculated with strains 353, A12 and A13 showed higher WUE values and lower carbon isotope discrimination ($\Delta^{13}C$) values (Figure 6D) under drought.

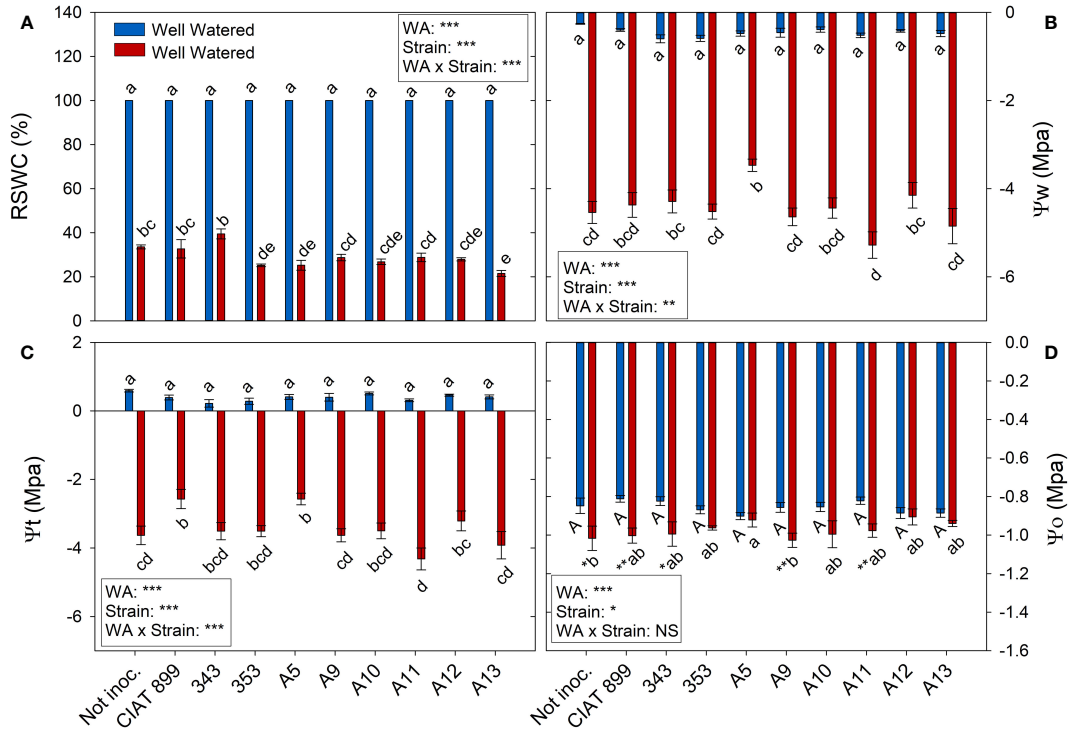


FIGURE 4
Relative soil water content (RSWC) and water potential parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** Relative soil water content, RSWC %; **(B)** Water potential, Ψw MPa); **(C)** Cell wall pressure potential, Ψt MPa); and **(D)** Osmotic potential, Ψo MPa.

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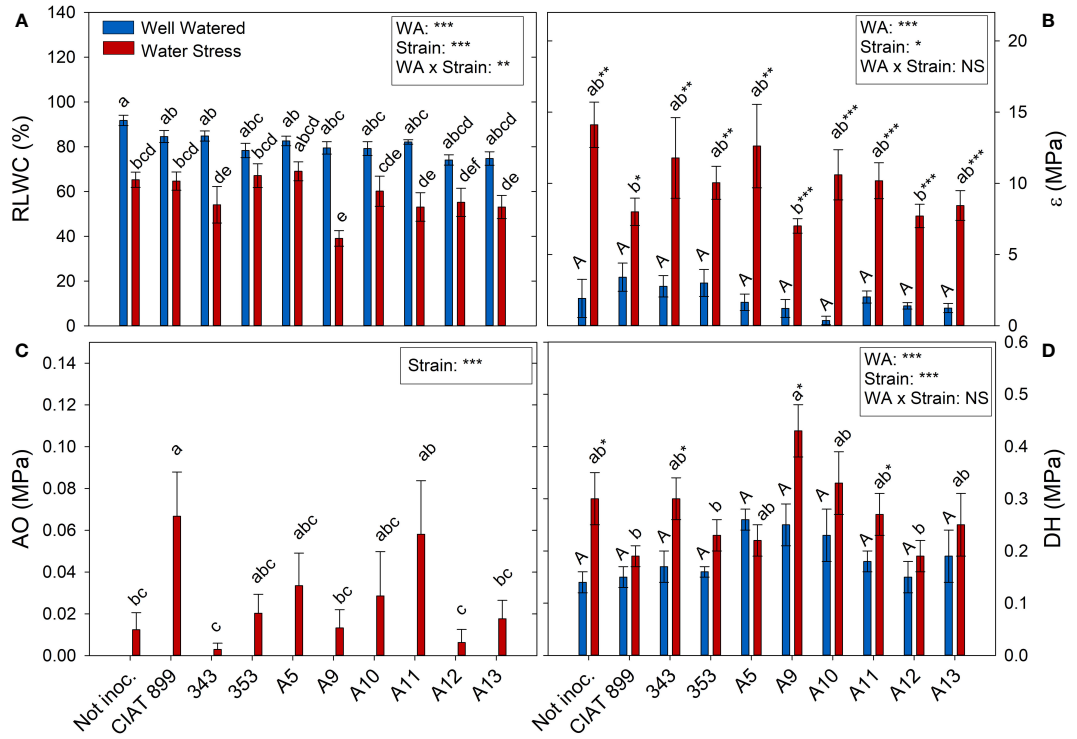


FIGURE 5
Relative leaf water content, **(A)** RLWC %; **(B)** Cell elastic modulus, ϵ MPa; **(C)** Osmotic adjustment, OA MPa; and **(D)** Dehydration, DH MPa, of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant).

3.3 Photosynthetic parameters

Drought and the different strains had a direct effect on photosynthesis (A) and stomatal conductance (gs) but no interaction between these factors two factors was found (Figure 7). Drought reduced on average for all treatments photosynthesis by 32%, and stomatal conductance by 30%. However, photochemical efficiency of PSII in dark adapted plants (Fv/Fm) were not affected by drought and all values were close to 0.8 (data not shown). Similar response was detected in light-adapted leaves (Fv′/Fm′) that were not affected neither by drought nor by inoculation treatments. Similarly, no significant differences were observed in the chlorophyll content among treatments, except for plants inoculated with the 343 strain under drought conditions (Figure 7C).

The leaf C% was affected by water deficit in the plants inoculated with 343, A9 and A11 strains (supplementary material Supplementary Figure 2A), while the plants inoculated with the strains CIAT899 and A5 showed the highest percentage under both water availability treatments. The total leaf carbon content (mg) was higher in plants inoculated with the strains 353, A12 and A13 both in well-watered conditions and under drought, whereas the lowest values were obtained in the plants inoculated with the strain 343 (supplementary material Supplementary Figure 2B).

3.4 Nitrogen fixation and content

The highest nodule production was observed in plants inoculated with 353 and A12 strains in well-watered conditions (1.63 and 1.56 g per plant, respectively) and were also the ones that showed the highest nodule biomass (0.447 and 0.371 g respectively) under drought conditions although the stress reduced by more than 70% the nodule biomass (Figure 8A). The strain with the lowest $\delta^{15}N$ values, which represents higher biological nitrogen fixation, were CIAT899, 353, A12 and A13 in both water availably treatments while 343 presented the lowest, especially under drought (Figure 8B).

Drought reduced the overall N% content of leaves by 26.99%, being the decrease significant in the plants inoculated with the strains CIAT899, A11, A13 and especially in 343 (Supplementary Figure 3A). The higher leaf total N content under well-watered conditions was found in plants fertilized with N and those inoculated with the strain 353, A12 and A13 (Figure 8C). Drought reduced this value in all inoculation treatments being the plants inoculated with A12 the ones that showed lower reduction.

3.5 Soluble protein

The foliar concentration of soluble proteins (Figure 8D) and total leaf protein content (supplementary material Supplementary

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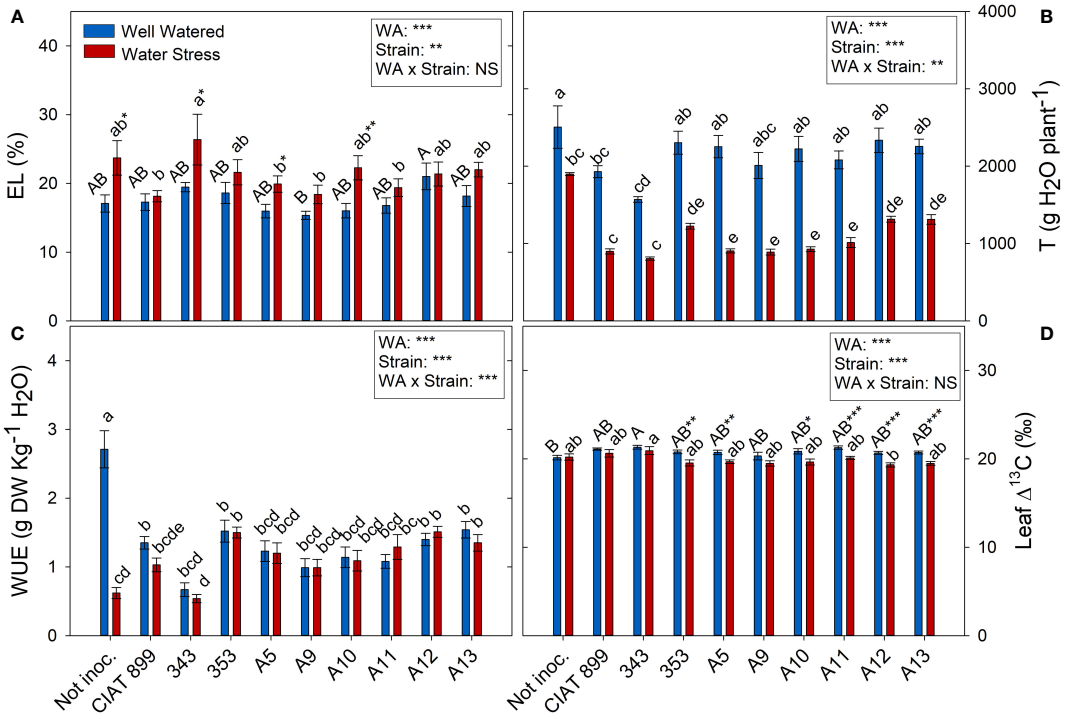


FIGURE 6
Electrolyte leakage and transpiration related parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** Electrolyte leakage, EL %; **(B)** Accumulated transpiration, T g H2O·plant-1; **(C)** Water use efficiency, WUE g DW·Kg-1 H2O; and **(D)** Leaf Δ13C, ‰. DW, dry weight.

Figure 3B) were affected by water supply and inoculation treatments. Under irrigation conditions, non-inoculated plants showed the lowest leaf protein concentration in comparison with rhizobia inoculation treatments, reaching the maximum value in plants inoculated with strain A9 and A13. Protein content decreased an average of 40.87% under drought conditions, however, no significant differences were observed among inoculation treatments. The total leaf protein content showed a similar behaviour to the observed in the total N content of the foliar biomass (Figure 8B).

3.6 Correlations between studied parameters

A strong positive correlation was detected between the developmental stage and the number of flowers per plant at harvest time (R=0.848***, Table 2); as well as between the number of flowers and both shoot and foliar biomass (R=0.833*** and R=0.825*** respectively), and between the number of flowers and nodule fresh weight (R=0.704***). A weaker correlation, although significant, was observed between the developmental stage and shoot biomass (R=0.647***) and between the number of flowers and the assimilation rate (R=0.638***, Table 2). The correlations between number of flowers and developmental stage with the total biomass was also significant, R=0.791*** and R=0.582***, respectively (Table 2).

Among the water relation parameters, the accumulated transpiration depends on the shoot biomass and total leaf area of the plants as shown by the strong correlation observed between both parameters, R=0.855*** and R=0.794***, respectively (Table 2). A strong correlation was also obtained between Ψw and Ψt (R=0.987***), compared to the weaker correlation between Ψw and Ψo (R=0.440***, Table 2). A positive correlation was also detected between the Ψt and the shoot biomass (R=0.630***, Table 2). The correlations obtained between Ψo and osmotic adjustment (OA) and dehydration (DH) confirms that in the case of CIAT899 the drop in osmotic potential was associated with the osmotic adjustment (Table 3). In the case of plants inoculated with the strains 353 and A11, the drop in Ψo was correlated both to OA and to dehydration. By the contrary, in non-inoculated plants and plants inoculated with 343, A9 and A10 strains, the drop in Ψo was correlated to dehydration. A positive correlation was also detected between ϵ values and electrolyte leakage (R=0.237**).

The relationship of CO_2 assimilation rate (A) and biomass production occurred mainly between shoot biomass and assimilation rate, rather than between total biomass and A, as shown by the correlation coefficients obtained (R=0.610 ** and R=0.558 ** respectively). A correlation between total leaf area and assimilation rate (R=0.679 ***, Table 2), and between the relative

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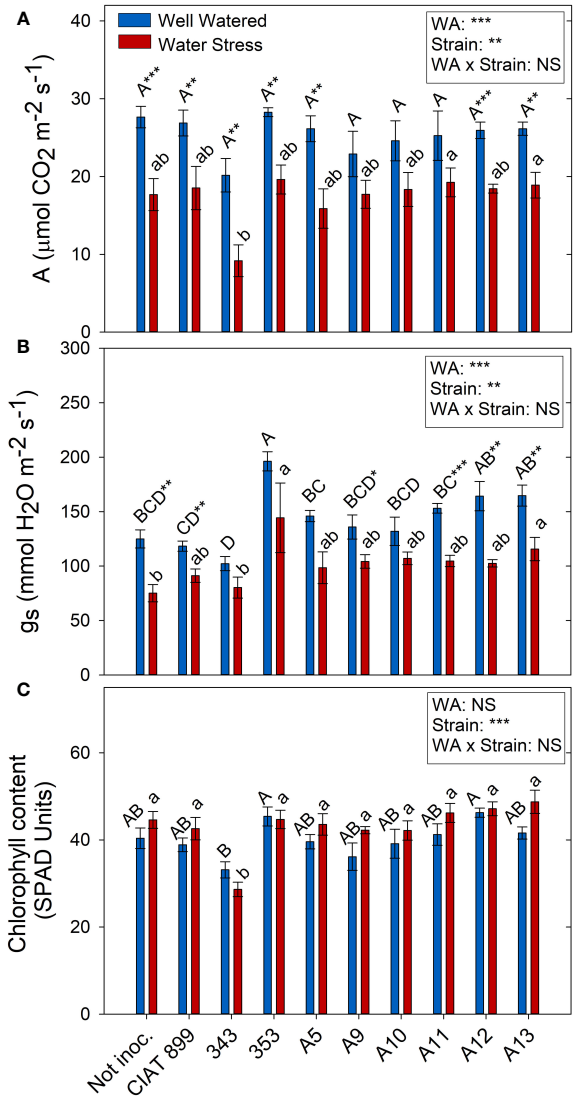


FIGURE 7
Photosynthetic parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** Assimilation rate, A µmol CO2·m-2·s-1); **(B)** Stomatal conductance, mmol H2O·m-2·s-1; **(C)** Chlorophyll content, SPAD units.

leaf water content and assimilation rate (R=0.313***, Table 2) were also detected

A positive correlation was observed between nodule fresh weight per plant and shoot biomass production (R=0.647 ***), and negative between nodule fresh weight and isotopic analysis of ^{15}N (R=-0.366 **, Table 2).

4 Discussion

4.1 Water relations

Common bean plants are considered to be water stressed when soil reaches 50% relative water content (Sánchez-Reinoso et al., 2019; Nawaz et al., 2021). The plants in this trial were exposed to values of 30% relative soil water content at the time of harvest, which is considered a severe drought (Xu and Zhou, 2006). This affected considerably the growth and development of the plants with a reduction of 66.5% in shoot biomass, 74.1% in total leaf area, a decrease in number and size of leaves and in number of flowers. This soil water content, caused a large drop in the water potential, reaching an average value of -4.46 MPa, very low values compared to the reached in others water stress studies (-1.5 MPa) with common bean (Hageman et al., 2020). The drop in Ψw was mainly due to the drop in the pressure potential (Ψt), especially in the non-inoculated plants and in those inoculated with CIAT899, 343, A9 and A11 strains, a drop in osmotic potential was also significant.

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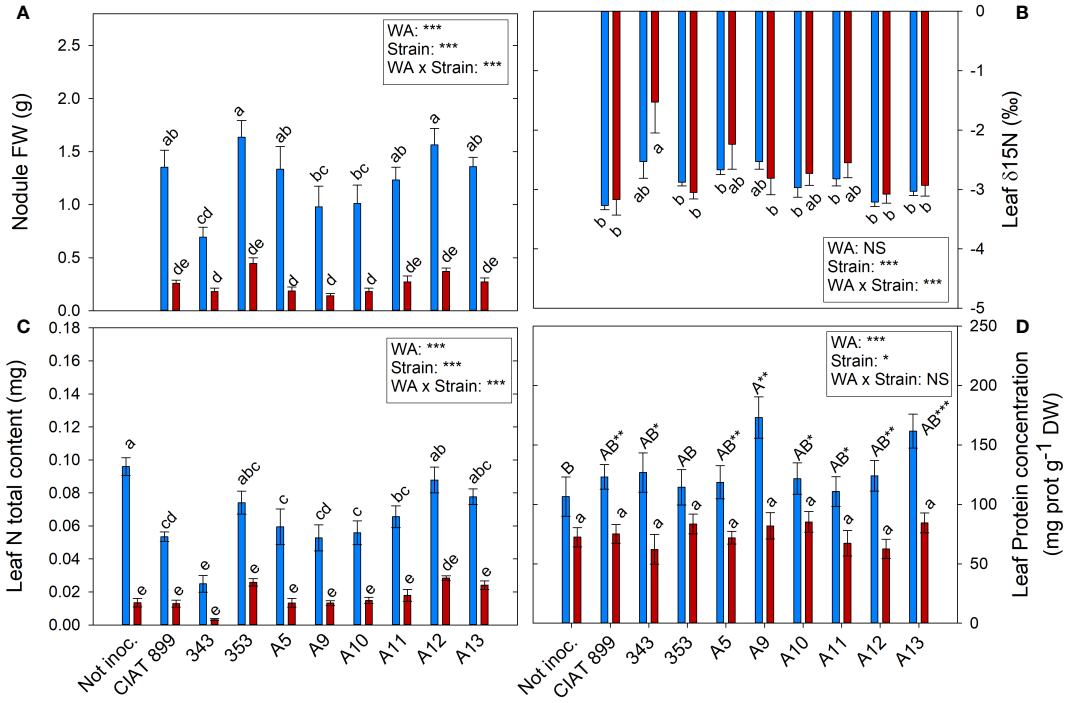


FIGURE 8
Nitrogen fixation related parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** Nodule fresh weigh, g; **(B)** leaf δ15N, ‰; **(C)** leaf N total content, mg; **(D)** leaf protein concentration, %.

Although a slight osmotic adjustment was detected mainly in plants inoculated with the reference strain CIAT 899, in the non-inoculated plants and in some of the more drought sensitive strains (especially 343); the drop in osmotic potential was due to dehydration. In addition, these plants suffered a great increase in the elasticity of the cell wall and a significant reduction in the relative water content of the leaves, as has also been reported in other not very successful bacterial inoculation tests under drought (Curá et al., 2017; Sapna and Sharma, 2020; Steiner et al., 2020). The cell wall elasticity adjustment is considered to be one of the most important physiological mechanisms of tolerance to water stress in several species (Marshall & Dumbroff, 1999; De Diego et al., 2013). This helps to maintain cellular turgor and volume and prevents mechanical damages in the plasma membrane, (Mena-Petite et al., 2001; De Diego et al., 2013) as indicated by low values of electrolyte leakage of the plants inoculated with CIAT899, A12, A13 and 353 strains. These plants also showed the lowest increase of ϵ values under drought, maintaining a better adjustment of the cell wall elasticity and higher leaf water content, as confirm the detected correlation between ϵ values and electrolyte leakage (R=0.237**). These results agree with many other studies that shown that inoculation with efficient strains reduces membrane damage in severe stress situations in different legumes such as soybean (Abbasi et al., 2013; Igiehon & Babalola, 2017; Kibido et al., 2019) or common bean (Steiner et al., 2020). This could explain the higher drought tolerance of CIAT899, 353, A12 and A13 strains which showed lower dehydration, higher leaf water content and, in the case of CIAT899, a better osmotic adjustment. In this sense, the accumulation of osmolytes such as trehalose and proline in the nodules has been related to the tolerance to drought of some plant genotypes (Goyal et al., 2021; Santillana Villanueva, 2021).

4.2 Photosynthetic parameters

Drought reduced the photosynthetic assimilation in most inoculation treatments due in part to stomatal limitations of photosynthesis (Figure 6) (Robredo et al., 2007) and did not produced any photochemical limitations since there were no significant changes in Fv/Fm nor in Fv'/Fm' values. These results indicate the absence of damage at the PSII level and that the capture and transduction of energy from the antenna complex to the PSII was not affected (Mathobo et al., 2017; Calzone et al., 2020). In addition, similarly to the observations of Boydston et al. (2018), plants subjected to water deficit did not show significant differences in their chlorophyll content (SPAD values). Among all the plants, those inoculated with 353, A12 and A13 indigenous strains together with the reference strain CIAT899 showed the highest photosynthesis and the highest percentage of leaf C concentration under drought, contrary to the plants inoculated with the 343 strain. In addition, similarly to Sapna & Sharma (2020), a positive strong correlation was detected between photosynthetic rate and relative

TABLE 2 Pearson's correlation indices (R) between the analyzed parameters: Transpiration (T, g H_2O·plant-1), water use efficiency (WUE, g DW·Kg-1H_2O), relative leaf water content (RLWC, %), water potential (Ψw, MPa), osmotic potential (Ψo, MPa), cell wall pressure potential (Ψt, MPa), dehydration (DH, MPA), osmotic adjustment (OA, MPa), electrolyte leakage (EL, %), nodule fresh weigh (NFW, g), flower number (FN), developmental stage (DS), total leaf area (TLA, m2), root biomass (RB, g), shoot biomass (SB, g), total biomass (TB, g), isotopic ratio of ^{15}N ($\delta^{15}N$, ‰), leaf N concentration (N, %), assimilation rate (A, µmol CO2·m-2·s-1) and cell elastic modulus (ε, MPa) (indicating the significance of these correlations by asterisks (* p < 0.05 and ** p <0.01).

	T	WUE	RLWC	Ψw	Ψo	Ψt	DH	OA	EL	NFW	FN	DS	TLA	RB	SB	TB	δN^{15}	N%	A	ϵ
T	1	,448**	,508**	,757**	,226**	,752**	-,252**	-,357**	-,263**	,731**	,770**	,616**	,794**	,442**	,855**	,797**	0,096	,347**	,642**	-,604**
WUE	,448**	1	0,079	,228**	0,023	,240**	-,168*	-0,054	-0,084	,189*	,596**	,481**	,487**	,779**	,751**	,838**	,200*	-0,067	,378**	-,286**
RLWC	,508**	0,079	1	,655**	,320**	,663**	-,286**	-,278**	-,341**	,411**	,402**	,300**	,445**	0,139	,351**	,313**	,191*	,548**	,313**	-,243**
Ψw	,757**	,228**	,655**	1	,440**	,987**	-,309**	-,387**	-,398**	,690**	,667**	,570**	,722**	,191*	,623**	,539**	0,055	,611**	,550**	-,728**
Ψo	,226**	0,023	,320**	,440**	1	,408**	-,489**	-,217**	-,184*	,248**	,201*	,232**	,306**	0,054	,159*	0,140	0,119	,309**	,171*	-,353**
Ψt	,752**	,240**	,663**	,987**	,408**	1	-,315**	-,350**	-,403**	,696**	,658**	,555**	,722**	,196*	,630**	,547**	0,040	,613**	,584**	-,741**
DH	-,252**	-,168*	-,286**	-,309**	-,489**	-,315**	1	-0,132	,209*	-,246**	-,227**	-,218*	-,294**	-0,165	-,273**	-,262**	-0,103	-,241**	-,199*	,172*
OA	-,357**	-0,054	-,278**	-,387**	-,217**	-,350**	-0,132	1	-0,058	-,263**	-,307**	-,262**	-,291**	-0,057	-,265**	-,221**	-0,056	-0,155	-,168*	,302**
EL	-,263**	-0,084	-,341**	-,398**	-,184*	-,403**	,209*	-0,058	1	-,239**	-,172*	-0,122	-,327**	-0,076	-,218**	-,187*	0,035	-,442**	-,177*	,237**
NFW	,731**	,189*	,411**	,690**	,248**	,696**	-,246**	-,263**	-,239**	1	,704**	,596**	,676**	-0,058	,647**	,472**	-,366**	,333**	,553**	-,576**
FN	,770**	,596**	,402**	,667**	,201*	,658**	-,227**	-,307**	-,172*	,704**	1	,848**	,775**	,373**	,833**	,791**	-0,077	,292**	,638**	-,580**
DS	,616**	,481**	,300**	,570**	,232**	,555**	-,218*	-,262**	-0,122	,596**	,848**	1	,632**	,254**	,647**	,582**	-,184*	,227**	,534**	-,539**
TLA	,794**	,487**	,445**	,722**	,306**	,722**	-,294**	-,291**	-,327**	,676**	,775**	,632**	1	,366**	,784**	,724**	0,004	,349**	,679**	-,590**
RB	,442**	,779**	0,139	,191*	0,054	,196*	-0,165	-0,057	-0,076	-0,058	,373**	,254**	,366**	1	,580**	,782**	,523**	-0,044	,270**	-,172*
SB	,855**	,751**	,351**	,623**	,159*	,630**	-,273**	-,265**	-,218**	,647**	,833**	,647**	,784**	,580**	1	,961**	0,114	0,140	,610**	-,541**
TB	,797**	,838**	,313**	,539**	0,140	,547**	-,262**	-,221**	-,187*	,472**	,791**	,582**	,724**	,782**	,961**	1	,258**	0,092	,558**	-,469**
δN^{15}	0,096	,200*	,191*	0,055	0,119	0,040	-0,103	-0,056	0,035	-,366**	-0,077	-,184*	0,004	,523**	0,114	,258**	1	-0,014	-0,073	0,118
N%	,347**	-0,067	,548**	,611**	,309**	,613**	-,241**	-0,155	-,442**	,333**	,292**	,227**	,349**	-0,044	0,140	0,092	-0,014	1	,298**	-,427**
A	,642**	,378**	,313**	,550**	,171*	,584**	-,199*	-,168*	-,177*	,553**	,638**	,534**	,679**	,270**	,610**	,558**	-0,073	,298**	1	-,508**
ϵ	-,604**	-,286**	-,243**	-,728**	-,353**	-,741**	,172*	,302**	,237**	-,576**	-,580**	-,539**	-,590**	-,172*	-,541**	-,469**	0,118	-,427**	-,508**	1

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leaf water content and, according to their observations, both parameters are related to grain yield in Mungbean plants inoculated with drought efficient rhizobia strains, confirming the efficiency of the aforementioned strains (CIA899, 353, A12 and A13).

4.3 Water use efficiency

As consequence of stomatal closure and the reduction of number and biomass of leaves under drought, the whole plant transpiration was reduced to cope with lack of water, maintaining the whole plant water use efficiency similar to the control plants (WUE), that decreased only in non-inoculated plants. Although under drought conditions, plants generally tend to increase WUE (Polania et al., 2016; Sanz-Saez et al., 2019) under severe stomatal closure, the efficiency of water use decreases again (Salazar-Tortosa et al., 2018). Thus, no differences were found in the WUE among the inoculation treatments. However, when considering the carbon isotope discrimination ($\Delta^{13}C$), another way of measuring the water use efficiency of plants since both parameters are negatively correlated (Farquhar et al., 1989), bigger differences were observed. Thus, strains 353, A12 and A13, showed higher water use eficiency (lower $\Delta^{13}C$) under drought, while the plants inoculated with CIAT899 did not showed any change. This confirmed the efficiency of these three strains (353, A12 and A13), since WUE or "more crop per drop" is considered as an important component of drought tolerance in different crops (Blum, 2009; Polania et al., 2016).

4.4 Nitrogen fixation and content

In inoculated plants the only source of nitrogen was the atmospheric N_2, accordingly, all the N content in these plants came from the biological nitrogen fixation (BFN); therefore, the total N content in the tissues is an estimate of the efficiency of the symbiosis. In addition, a positive correlation between the N% and nodule fresh weight was detected (R=0.333***). There are many studies using nodule weight as an indicator of a strain´s infectiveness and many others report that drought reduces nodulation (Kibido et al., 2019; Aulakh et al., 2020; Santillana Villanueva, 2021). It has also been stated that the more drought tolerant a strain is, the greater nodulation capacity it has under drought conditions (Aulakh et al., 2020; Steiner et al., 2020; Omari et al., 2022). The negative correlation between nodules fresh weight and $\partial^{15}N$ (R=-0.366**) would confirm that the greater the biomass of nodules, the greater the capacity for nitrogen fixation. From all the strains tested, plants inoculated with 353, A12, A13 and CIAT899 bacteria showed the highest infectiveness (higher nodule weight) and the lowest $\delta^{15}N$ values (higher biological nitrogen fixation) in both water availably treatments. The most efficient strains allow plants to develop a greater number of nodules and fix more nitrogen under stress conditions, resulting in a greater accumulation of N in their tissues higher leaf chlorophyll content and more shoot biomass (Steiner et al., 2020; Rodiño et al., 2021; Omari et al., 2022), in a similar way to what was observed in plants inoculated with the strain 353, A12 and A13, confirming these strains as the ones with the highest nitrogen fixation, highlighting the strain A12. On the contrary, the strain 343 showed the worst behaviour with the lowest values in all parameters tested.

TABLE 3 Pearson's correlation indices (R) between Ψo and the parameters of osmotic adjustment (OA) and dehydration (DH), in common bean plants without microsymbiont (not inoc.) and inoculated with different strains of rhizobium: CIAT 899, A5, A9, A10, A11, A12 and A13 (* p< 0.05; ** and p<0.01).

	DH	OA
Not Inoc.	-0.696**	-0.198
CIAT899	-0.469	-0.692*
343	-0.681*	0.12
353	-0.757*	-0.657*
A5	-0.309	-0.036
A9	-0.75**	-0.186
A10	-0.603*	0.134
A11	-0.677**	-0.577*
A12	-0.365	-0.208
A13	-0.205	-0.065

4.5 Phenology and growth parameters

Biomass production is also usually used as strain effectiveness indicator (Kibido et al., 2019; Aserse et al., 2020). In this study, there was a high correlation (R=0.647 ***) between nodule fresh weight and shoot biomass. Thus, the strains with the highest nodulation capacity under drought (353, A12, A13 and CIAT899) were those with the highest biomass production, except in the case of CIAT899, which did not develop much shoot biomass. On the contrary, the plants inoculated with 343 rhizobia, presented the lowest shoot dry weight in both water treatments, confirming the low efficiency of this strain. Furthermore, shoot dry weight has also been related to grain yield in several crop species such as common bean (Beebe et al., 2013; Rai et al., 2020). Therefore, the strains capable of increasing the shoot biomass are expected to achieve higher yields as confirmed in many studies (Igiehon et al., 2021), which is why it is considered a criterion for the selection of inoculants in addition to an indicator of strains effectiveness (Aserse et al., 2020; Aulakh et al., 2020). In our study this is further confirmed by the strong positive correlation between nodule fresh weight and number of flowers (R=0.704 ***). In fact, Omari et al. (2022) found that the number of nodules was positively correlated with soybean yield and protein content, confirming many previous reports that concluded that nodulation enhancement is essential for increasing soybean grain yield (Thilakarathna and Raizada, 2017; Moretti et al., 2018). Therefore, the strain 353, A12 and A13 were the most efficient strains under drought stress, for the studied genotype, surpassing even the reference strain CIAT899. These strains showed as well the

highest total leaves area in both water availability conditions, keeping higher photosynthetic capacity (higher total content of C in leaves) per plant.

Drought delayed the phenological development of the plants by one stage (from R6 in well-watered conditions to R5) and reduced the number of flowers by 65.9% since drought stress affects flower initiation (Nuñez Barrios et al., 2005), as well as flower abortion (Monteiro-Galvao et al., 2019) affecting yield (Nuñez Barrios et al., 2005; Steiner et al., 2020). However, inoculation of Arrocina de Álava plants with efficient strains (353, A12 and A13) avoided this phenological delay keeping them in R6 like the well-watered plants and also developed a greater number of flowers, even improving the effects of the inoculation with CIAT899 reference strain. On the contrary, the nitrate fertilized plants showed the greatest delay in development under drought remaining in a vegetative state (V4). Thus, the inoculation of efficient strains increased the plant tolerance by accelerating flowering, in the studied genotype, which is considered a common strategy for "evasion" of water stress, since it allows plants to escape the drought by completing their life cycle before the onset of severe stress conditions (Polania et al., 2016; Sedlar et al., 2020).

4.6 Selection of tested drought tolerant indigenous rhizobia

The results of this work showed that from the nine strains of potentially drought-tolerant bacteria evaluated, three were found to be efficient strains under drought for the studied genotype. One isolated from common bean productive plants under drought conditions, 353 (*Rhizobium gallicum*) and two strains of *Rhizobium etli* isolated from saline soils: A12 and A13. These 3 strains showed high infectiveness (nodule fresh weight) and effectiveness (shoot dry weight) and nitrogen accumulation, even surpassing the plants inoculated with the CIAT899 reference strains in many of the analyzed parameters. In contrast, the strain 343 (*Rhizobium giardinii*) was identified as a sensitive strain, showing the worst results in both water regimes. These results are consistent with the fact that *R. etli* and *R. gallicum* have been previously characterized as microsymbiont that form effective symbiosis with common bean, while *R. giardinii* include both effective and ineffective strains (Ribeiro Torres et al., 2009).

The selected strains (353, A12 and A13) did not present significant differences with the plants chemically fertilized with nitrate neither in the aerial biomass nor in the number of flowers in well-watered conditions and even showed a higher foliar protein concentration. On the other hand, drought affected the N-fertilized plants more intensely, suffering a greater delay in phenological development, and a greater reduction in shoot biomass and number of flowers. Considering that both, the shoot biomass and the number of flowers are parameters closely related to yield (Beebe et al., 2013), the results of the present study showed how through inoculation of plants of Arrocina de Álava with efficient rhizobia strains, yields similar to those obtained with nitrogen fertilization could be obtained in well-watered conditions and surpass them under drought conditions.

The tolerance mechanisms developed by plants of the studied genotype with the inoculation with 353, A12 and A13 strains were a better adjustment of the cell wall elasticity that helps to maintain cellular turgor and volume, prevention of mechanical damages in the plasma membrane, to maintain higher WUE values and to avoid the phenological delay caused by drought developing a greater number of flowers. In further studies we will characterize the selected bacterial strains and delve into the drought tolerance mechanism that underlies this response by evaluating some metabolites related to water stress such as hormone levels, and proline and trehalose content among others. The results obtained provide the basis for the development of "elite" inoculants to increase the yield of an economically interesting genotype common bean under intense drought field conditions in the Northern also covering the need to expand the pool of inoculum resources in Europe (Omari et al., 2022). Therefore, to evaluate the efficiency of these selected rhizobia (353, A12 and A13), a field trial will be carried out under drought conditions. Although the results obtained in the field often differ from those obtained under controlled conditions, very favourable results have been achieved, even with the inoculation of strains with little history of edaphoclimatic adaptation (Sindhu & Dadarwal, 2000; Omari et al., 2022). Thus, taking into account the long tradition of bean cultivation in the Basque Country (Eustat, 2019), that the selected strains were isolated from stressed soils and that common bean is a highly promiscuous species (Shamseldin & Velázquez, 2020), we expect to obtain promising results under field conditions, for Arrocina de Álava genotype, of great economic interest, such as those obtained by other authors with common bean, in other edaphoclimatic zones (Aserse et al., 2020; Rodiño et al., 2021). In future research, we also hope to conduct similar studies with other common bean genotypes, since different genotypes respond differently to the same inoculum (Goyal et al., 2021; Omari et al., 2022).

5 Conclusion

From the analysed bacteria, three were found to be highly efficient strains under drought (353, A12 and A13) showing high nodulation capacity, high biological nitrogen fixation and shoot biomass production, even surpassing the plants inoculated with the CIAT899 reference strain, as well as the chemically N-fertilized plants. The tolerance mechanisms developed by inoculated with 353, A12 and A13 strains were, a better adjustment of the cell wall elasticity that prevents mechanical damages in the plasma membrane, higher WUE and avoidance of the phenological delay caused by drought, developing a greater number of flowers. The results confirm that these indigenous strains could have an agronomical application in common bean crops in Northern Spain and be used as biostimulants to reduce negative drought effects related to climate change in a sustainably way, as well as

being an alternative to chemical fertilization. Inoculation with these strains could contribute to avoid the environmental N-pollution and costs associated with the production of synthetic fertilizers of great importance nowadays.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AD-C, AS-S and ML conceived the experiment. AD-C, AS-M, EM, ML conducted the experiment. AD-C, AS-S and ML analyzed data and contributed to the drafting of the manuscript. AD-C wrote the manuscript. AS-S and ML supervised the last version of the manuscript. ML supervised and funded the whole project. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1046397/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Leaf biomass (DW, g) of common bean plants nitrate-fertilized (not inoc.) and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13) under different water availability (WA) conditions: well-watered (blue) and drought (red). When there is no interaction of factors, the multiple comparisons were made comparing all the inoculation treatments with each other, but separating the data according to the water regime (capital letters for control conditions and lower case for drought conditions), and the effect of water availability was studied for each inoculation treatment separately (using asterisks to show the effect): * p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant).

SUPPLEMENTARY FIGURE 2
C parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). (* p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** leaf C concentration, %; **(B)** leaf C total content (mg).

SUPPLEMENTARY FIGURE 3
Nitrogen content related parameters of common bean plants without inoculum and nitrate-fertilized (not inoc.), and inoculated with different strains of rhizobium (CIAT899, A5, A9, A10, A11, A12 and A13), under different water availability (WA) conditions: well-watered (blue) and drought (red). (* p< 0.05; ** p<0.01 and *** p<0.001; NS, non-significant). **(A)** leaf N concentration, %; **(B)** leaf protein total content (mg).

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Microalgae as next generation plant growth additives: Functions, applications, challenges and circular bioeconomy based solutions

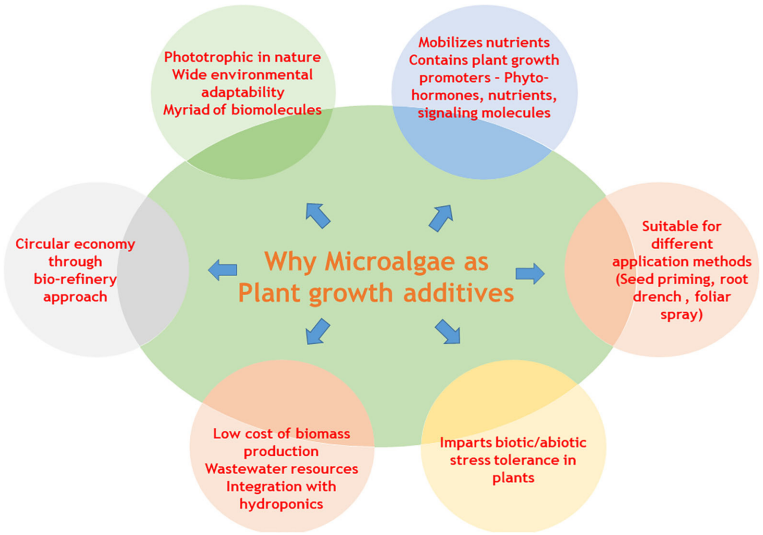
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Sustainable agriculture practices involve the application of environment-friendly plant growth promoters and additives that do not negatively impact the health of the ecosystem. Stringent regulatory frameworks restricting the use of synthetic agrochemicals and the increase in demand for organically grown crops have paved the way for the development of novel bio-based plant growth promoters. In this context, microalgae biomass and derived agrochemicals offer novel sources of plant growth promotors that enhance crop productivity and impart disease resistance. These beneficial effects could be attributed to the presence of wide range of biomolecules such as soluble amino acid (AA), micronutrients, polysaccharides, phytohormones and other signaling molecules in microalgae biomass. In addition, their phototrophic nature, high photosynthetic efficiency, and wide environmental adaptability make them an attractive source of biostimulants, biofertilizers and biopesticides. The present review aims to describe the various plant growth promoting metabolites produced by microalgae and their effects on plant growth and productivity. Further, the effects elicited by microalgae biostimulants with respect to different modes of applications such as seed treatments, foliar spray and soil/root drenching is reviewed in detail. In addition, the ability of microalgae metabolites to impart tolerance against various abiotic and biotic stressors along with the mechanism of action is discussed in this paper. Although the use of microalgae based biofertilizers and biostimulants is gaining popularity, the high nutrient and water requirements and energy intensive downstream processes makes microalgae based technology commercially unsustainable. Addressing this challenge, we propose a circular economy model of microalgae mediated bioremediation coupled with biorefinery approaches of generating high value metabolites along with biofertilizer applications. We discuss and review new trends in enhancing the sustainability of microalgae biomass production by co-cultivation of algae with hydroponics and utilization of agriculture effluents.

KEYWORDS

biostimulant, biofertilizers, biopesticides, biorefinery, circular economy, stress tolerance, bioremediation



GRAPHICAL ABSTRACT

1 Introduction

The global population is projected to reach 9.6 billion by 2050 and the demand for food production is continuously increasing (Wu et al., 2014). However, the arable land available for crop production is limited and is expected to grow at a very negligible rate of 0.10% from 1592 million ha in 2005-07 estimate to a projected 1661 million ha in 2050 (Alexandratos and Bruinsma, 2012). To meet the global food requirements, intensive agricultural practices have been followed such as the use of chemical fertilizers, pesticides, and growth enhancers for maximizing crop productivity. Continuous use of these chemicals has deteriorated soil health mainly the physicochemical profile and soil micro-flora reducing the crop yield (Abinandan et al., 2019). This has led to a range of environmental issues such as nutrient leaching, contamination of surface and groundwater, eutrophication, greenhouse gas emissions, loss of aquatic biodiversity, and xenobiotics-induced human diseases (Mahapatra et al., 2022). In addition to the environmental concerns, the depletion of fossil fuels and non-renewable resources makes synthetic/chemical-based agriculture expensive (Woods et al., 2010). Further, the increasing consumer demand for organically grown crops and pesticide-free agriculture commodities necessitates the identification of safe, biologically derived, and sustainable alternatives for agricultural applications.

According to the European Biostimulant Industry Council (EBIC, 2022, https://biostimulants.eu/highlights/economic-overview-of-the-european-biostimulants-market/), a plant biostimulant refers to a material or a formulation which contains substance(s) and/or microorganisms whose function, when applied to plants or the rhizosphere is to stimulate natural processes to benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independently of its nutrient content (Ricci et al., 2019). In this context, microalgae and cyanobacteria have the potential to act as environmental friendly biostimulants/biofertilizers that improve crop quality and yield (Gonçalves, 2021). Microalgae are unicellular, mostly phototrophic organisms with wide environmental adaptability (Barsanti and Gualtieri, 2018). The ability of microalgae biomass to elicit a positive impact on plant growth and soil health could be attributed to the presence of a wide range of biomolecules such as N-fixing enzymes, soluble AAs, bio-mineral conjugates, polysaccharides and phytohormones (Kapoore et al., 2021; Lee and Ryu, 2021). Microalgae have been projected as a potential industrial feedstock owing to their high photosynthetic efficiency, their ability to grow in non-potable waters such as industrial effluents, and their ability to modulate metabolite biosynthetic pathways in response to varying environment (Ahmed et al., 2022; Chen et al., 2022; Zhao et al., 2022). Some of the industrially important microalgae species such as *Arthrospira platensis* (*Spirulina* spp.), *Chlorella* spp., *Heamatococcus pluvialis, Dunaliella salina, Nostoc* spp., *Anabaena* spp., *Scenedesmus* spp., *Nannochlorpsis* spp., *Phaeodactylum tricornutum*, etc. have been used as a renewable source of food, nutraceuticals, animal feed, agrochemicals (Cordeiro et al., 2022). Although the use of seaweed

Abbreviations: AA, Amino acid; ABA, Abscisic acid; APX, Ascorbate peroxidase; BOD, Biological oxygen demand; BSC, Biological soil crust; Ca, Calcium; CAT, Catalase; CEC, Cation exchange capacities; COD, Chemical oxygen demand; CPC, C-Phycocyanin; DW, Dry weight; EPS, Extracellular polysaccharide; Fe, Iron; FS, Foliar spray; GA, Gibberellic acid; GAE, Gallic acid equivalent; GRX, Glutathione reductase; IAA, Indole-3-acetic acid; JA, Jasmonic acid; K, Potassium; LCA, Life cycle analysis; LOX, Lipoxygenase; N, Nitrogen; ORP, Open raceways pond; P, phosphorus; PAL, Phenylalanine ammonia lyase; PBR, Photo bioreactor; POD, Peroxidase; PSM, Phosphate solubilizing microbe; QE, Quercetin equivalent; ROS, Reactive oxygen species; SC, Salicylic acid; SOD, Superoxide dismutase; UV, Ultra Violet.

extracts and cyanobacteria in agriculture has been traditionally practiced, the newer developments such as the *omics* approach in microalgae biotechnology and biorefinery approaches in algal biomass utilization have reinforced the applications of microalgae and cyanobacteria in agriculture (Behera et al., 2021).

Microalgal metabolites have been reported to improve soil fertility, impart resistance to plants against abiotic stress, stimulate defense response against pathogens and infection, and improve nutrient uptake from soil such as phosphorus (P), potassium (K), N, and minerals (Berthon et al., 2021; Gonçalves, 2021). Several reports on the use of microalgae for the improvement of crop quality and productivity in various Agri-Horti crops have been published in recent years (Abinandan et al., 2019; Gonçalves, 2021; Kapoore et al., 2021; Lee and Ryu, 2021). Although the different classes of microalgae metabolites with biostimulant and biofertilizer properties have been identified and compiled earlier (Kapoore et al., 2021); however, their mechanisms of action, and impact on plant physiology have not been clearly understood. Further, the effect of a different mode of biostimulant applications on plants, concerning microalgae has been seldom discussed in earlier reports.

A Large quantity of microalgae biomass is the foremost requirement for agriculture applications, especially as plant growth promoters and fertilizers. Despite enormous research on the microalgae biomass production, sustainably achieving high biomass productivity is still far from reality (Calijuri et al., 2022). Commercial production of microalgae throughout the year have been possible only in few tropical and sub-tropical regions offering high light and conducive temperature without affecting conventional agriculture productivity (Correa et al., 2019; Casanova et al., 2022; Rasheed et al., 2022). Use of artificial lighting and controlled photobioreactors (PBRs) for continuous biomass production have been demonstrated (Peter et al., 2022). However, the process is economically unviable owing to the high capital and energy inputs reducing sustainability. Apart from the light requirements, some of the other critical challenges involved in microalgae biomass production are high water footprint, high nutrient costs, and energy-intensive downstream processing (Maiolo et al., 2020; Ighalo et al., 2022). These challenges offset the benefits imparted by the microalgae biomass for agriculture applications necessitating sustainable microalgae bioprocesses. Integration of wastewater bioremediation and flue gas utilization for microalgae cultivation and deployment of biorefinery strategies for biomass utilization have been identified to be sustainable choices (Carraro et al., 2022; Zafar et al., 2022). However, a detailed review of the feasibility of the above processes and the risks involved are not available.

Thus in the present review, we summarize the different types of plant growth-promoting activity viz., biofertilizers, biostimulants, and biopesticides, exhibited by microalgae. In addition, we describe the mechanism of biostimulant action of various microalgae metabolites along with their effects on plants under different modes of application namely seed treatments, foliar spray and soil drenching. Although few reviews and status papers on this topic are available, they seldom discuss the functionalities of various class of biostimulants present in microalgae (Chiaiese et al., 2018; Arahou et al., 2022; González-Pérez et al., 2022). Another important highlight of this review is the description of mechanisms involved in the abiotic and biotic stress tolerances imparted by microalgae on crops along with case studies; which have not been reviewed critically in earlier reports such as (Kapoore et al., 2021; González-Pérez et al., 2022). Further, we address the various challenges involved in the microalgae bioprocesses and describe different strategies such as integration of bioremediation with biomass production and biorefinery approaches to improve the sustainability quotient. We emphasize the need for a closed-loop circular economy model for sustainable agriculture with the case study of integrating microalgae cultivation with modern agriculture technologies such as hydroponics. The main aim of this review is to popularize the commercial use of microalgae-based plant growth additives and highlight the various strategies to combat the challenges involved thereof.

2 Growth-promoting properties of microalgae

2.1 Microalgae as biofertilizers

Microalgae impart growth-promoting properties in three different modes, namely as biofertilizers, biostimulants, and biopesticides. These properties could be attributed to the presence of a variety of biomolecules such as soluble AAs, phenolic compounds, phytohormone-like compounds, terpenoids, and polysaccharides (Lee and Ryu, 2021). The most common mode of utilization of microalgae biomass is biofertilizers. Biofertilizers are live microorganisms or compounds derived from microbes that enhance or augment plant nutrition by mobilizing or enhancing the nutrient availability in soils by colonizing the rhizosphere, rhizoplane, or root interiors (Mitter et al., 2021). Based on the characteristic functions, biofertilizers can be mainly categorized into a) N-fixing fertilizers b) potassium solubilizing fertilizers c) potassium mobilizing fertilizers, d) phosphate mobilizing and solubilizing fertilizers (Win et al., 2018; Gonçalves, 2021).

Microalgae and cyanobacteria along with fungi and bacteria constitute the uppermost layers of soil collectively called biological soil crust (BSC) which plays a critical role in enhancing soil fertility and crop productivity (Abinandan et al., 2019). Several reports have been published on the presence of microalgae and cyanobacteria in the formation of BSC, especially in a variety of soil types ranging from clay loams, desert soils, semi-arid, silt loam to sandstone and granite (Acea et al., 2003; Malam Issa et al., 2007; Nisha et al., 2007; Wang et al., 2009; Lichner et al., 2013; Manjunath et al., 2016; Renuka et al., 2016; Dineshkumar et al., 2018). These soils are characterized by poor organic (carbon (C) and N and micronutrient content owing to higher surface temperature, Ultra-voilet (UV) irradiation, and elevated carbon dioxide (CO_2) levels. Despite such harsh environmental conditions and lack of moisture on the soil surface, microalgae and cyanobacteria initiate BSC formation and survive these harsh conditions through adaptive mechanisms such as the formation of heterocysts, secretion of hydrophobic AAs, and extracellular polysaccharides (EPS) and specialty molecules known as phytochelatins that prevent desiccation of intracellular contents, protection from UV-B radiations and degradation of nucleic acids (Garcia-Pichel et al., 2001; Bhargava et al., 2005). A survey of various agroecosystems and soil microbial communities revealed a significant presence of cyanobacteria groups such as Oscillatoriales, Nostocales,

Chroococcales, Synechococcales, Chroococcidiopsidales, Pleurocapsales, Microcoleaceae, Chlorellales (Trivedi et al., 2016). Experimental inoculation of microalgae/cyanobacteria in different soil types improved soil nutrients (organic C, N, P, and other minerals) concentration, soil stability, soil moisture content, and water penetration to soil (Acea et al., 2003; Malam Issa et al., 2007; Nisha et al., 2007; Wang et al., 2009; Lichner et al., 2013).

2.1.1 N fixation by cyanobacteria and microalgae

The foremost function exhibited by microalgae, specifically cyanobacteria, as biofertilizer is atmospheric N fixation and enhancement of the soil N content. The most abundant source of N (N_2) in the earth is atmospheric N, however, they are inert and require high energy for its reduction to ammonia for further uptake by plants. Atmospheric N_2 enters the biological N cycle in three main ways, viz., through biological fixation (prokaryotic conversion of N_2 to ammonia); by atmospheric fixation (lightning and photochemical conversion of N_2 to nitrate); and by the Haber–Bosch industrial process where ammonia is produced from N_2 (Kraiser et al., 2011). N supplementation in intensive agricultural practice is through the application of N-rich fertilizers such as urea and ammonium sulfate to the soil. However, the major drawback is that only 50% of the N is taken up by the plant while the remaining is lost to the environment due to ammonia volatilization, nitrification, leaching, and surface runoff. This deficit of soil N content can be corrected by the fixation of abundant atmospheric N (Bouwman et al., 2009).

Cyanobacteria have special mechanisms to fix inert atmospheric N. They are diazotrophs and utilize nitrogenase enzyme complex to convert atmospheric N to ammonia at the expense of 16 ATP molecules, a highly energy-intensive process under anoxic (oxygen-free) conditions (Stal, 2015). High light and oxygen exposure inhibits N fixation in cyanobacteria, and they have adopted several strategies for simultaneous photosynthetic and N fixation processes (Gallon, 1992). Among these, the spatial separation of photosynthesis and anaerobic N fixation process between vegetative cells and heterocysts in heterocystous cyanobacteria is one such mechanism (Maldener and Muro-Pastor, 2010). The second mechanism is by temporal separation of photosynthesis and N_2 fixation process under light and dark regimes in non-heterocystous cyanobacteria exemplified by *Lyngbya* spp., and *Cyanothece* spp., (Misra, 1999). The third mechanism is by the combination of spatial and temporal separation exemplified by *Gleothece* spp., (Compaoré and Stal, 2010*)* and lastly by genome reduction and loss of oxygenic photosynthesis as observed in *Trichodesmium* spp., (Bergman et al., 2013).

Among the various types of N_2 fixing mechanisms, the most predominant and commonly observed in terrestrial ecosystems is heterocyst based N_2 fixation. This process happens through a symbiotic relationship with the host plant where cyanobacterial species colonize the leaf and roots of the host plant (Krings et al., 2009). Cyanobacteria enter the leaf tissue through the stomata and colonize the intercellular spaces by forming a cyanobacterial loop while in the case of roots, they form loose colonies on the root hair and tight colonies on the root surfaces (Lee and Ryu, 2021). Some examples include the colonization of roots of wheat and cotton by *Anabaena* spp., and *Tolypothrix* spp., and rice by *Nostoc* spp., (Babu et al., 2015). The colonization of roots and further process by cyanobacteria is called 'Gland formation' and the process has been elucidated and the mechanism is similar to the nodule formation by *Rhizobium* spp. or gall formation by *Agrobacterium tumefaciens*. The process involves cell penetration, intracellular colonization, hormogonium formation, and gland development with host specificity (Santi et al., 2013). Microalgae as a source of N_2 is applied to the soil as live culture in case of cyanobacteria or as dried biomass or suspension in the case of green algae (Alvarez et al., 2021). The major advantage of the use of microalgae in soil as a source of N is that there is lesser chance of leaching or loss as run offs unlike chemical based N fertilizers since less than 5% of N content in microalgae biomass is in the mineralized form (Mulbry et al., 2005). Further, the issue of NH_3 volatilization is negligible with dried algae biomass application unlike urea or other manures (de Siqueira Castro et al., 2017).

2.1.2 Phosphorus solubilization

Apart from N, P exists as the second most limiting nutrient for plant growth. P exist in the soil in the form of inorganic phosphates or in complex organic forms making them unavailable to plants, necessitating the use of P-rich fertilizers (Elser, 2012). Despite the enormous use of P fertilizer in agro-production only a portion of the P is available to plants as the significant amount is lost due to erosion and leaching leading to the contamination of groundwater and water eutrophication (Cordell et al., 2009). The safe and effective alternative that can cut down the overuse of P fertilizers in crop production is use of phosphate solubilizing microbes (PSM) and biofertilizers that augment P uptake from soil (Gyaneshwar et al., 2002). Among the PSM, cyanobacteria and microalgae play a critical role in phosphate solubilization to plants. Depending on the soil pH, the P is bound to calcium (Ca) or aluminium in soil. Cyanobacteria solubilize bound P in two ways either by releasing chelators that bind Ca ions or by releasing organic acids that promote solubilization (Alvarez et al., 2021). Experimental observations revealed that cyanobacterial species such as *Anabaena variabilis* and W*estelliopsis* spp., secrete pthalic acid for P solubilization from phosphate rock and tricalcium phosphate (Yandigeri et al., 2011). In addition to solubilization, cyanobacteria and microalgae mineralize P from organic P sources. The most commonly occurring organic P sources are phytates and phosphoesters and microalgae produce P hydrolyzing enzymes such as alkaline phosphatases, phosphodiesteratses, 5'nucleotidases and phytases that release bound P from organic molecules (Markou et al., 2014).

Further, microalgae and cyanobacteria demonstrate a luxury uptake mechanism where they accumulate intracellular P reserves as polyphosphate granules. This P reserve is utilized by the algae when the P levels in the surrounding medium is depleted (Powell et al., 2009). This luxury uptake mechanism is further supported by additional physiological processes such as membrane lipid remodeling to reallocate P based on its availability from surrounding medium. This is achieved through modulation of lipid composition, where P starvation induces remodeling of membrane lipid moieties such as phosphatidylethanolamine, phosphatidylcholine, and phosphatidylglycerol (phospholipids) to non-P containing glycolipids or betaine class of lipids such as sulfoquinovosyldiacylglycerol or diacylglyceroltrimethylhomoserine leading to P reallocation (Çakirsoy et al., 2022). Such physiological

mechanisms offer microalgae to accommodate additional P from surrounding medium or reallocate P during P deficiency by way of polar lipid remodeling as observed in few fast growing and high P uptake microalgae species such as *Nanochloropsis oceania, Nannochloropsis gaditana, Tetraselmis suecia* (Cañavate et al., 2017).This phenomenon of luxury uptake of P by microalgae could be utilized for delivering soluble P to plants.

2.1.3 Enhancing micronutrient bioavailability

In addition to aforesaid macronutrients, minerals such as iron (Fe) play a crucial role in the growth of plants. The typical Fe requirement of plants is in the order of 10^{-6} moles (M). Although Fe is abundant in soil, their bioavailability to plants is negligible owing to physico-chemical properties of soil. Under aerated conditions and soil pH > 7.00, the inorganic Fe becomes poorly soluble and bioavailable Fe concentration is in the range of 10^{-10} M leading to Fe scarcity (Colombo et al., 2014). To counter this problem, soil and rhizosphere bacteria release low molecular weight, organic molecules called siderophores. Siderophores are nitrogenous compounds with strong affinity for Fe^{3+} ions and contribute to the solubilization and mobilization of Fe into plants (Chakraborty et al., 2019). Similar to bacteria, microalgae and cyanobacteria secrete siderophores (Årstøl and Hohmann-Marriott, 2019).

Siderophores form a strong hexadentate, octahedral complex with Fe^{3+} ion. Based on the primary oxygen donating ligands, siderophores are primarily classified into hydroxamates, catecholates, and carboxylates (Hider and Kong, 2010). The predominant class of siderophores observed in cyanobacteria are hydroxamates. The two hydroxamate siderophores whose structure has been determined are schizokinen and synechobactin in *Anabaena* spp., and *Synechococcus* spp., respectively (Simpson and Neilands, 1976). The structure of the cyanobacterial siderophore is similar to the bacterial siderophore rhizobactin and aerobactin (Årstøl and Hohmann-Marriott, 2019). Additionally, a catecholates group of siderophores named anachelin has been detected in *Anabaena cylindrica* which chelates Fe^{3+} ions through a catechol moiety (Beiderbeck et al., 2000). The presence of siderophore not only helps in Fe binding and mobilization but also in preventing heavy metal toxicity to the algae. In *Anabaena* spp., PCC 7120, under high copper conditions (copper toxicity) chelation of Cu ions by siderophores was observed. Cellular recycling of siderophores results in exclusion of Cu ions reducing the toxicity of Cu ions to cyanobacterial cells (Clarke et al., 1987). This mechanism can be exploited in heavy metal sequestration in contaminated soils. Few observations on the ability of cyanobacterial hydroxamates to sequester heavy metals have been reported such as such as uranium sequestration by *Synechococcus elongates* BDU 130911 and cadmium chelation by *Anabaena oryzae* under Fe replete conditions (Rashmi et al., 2013; Singh et al., 2016). Thus cyanobacteria have wide applications as natural biofertilizers in replenishing of soil nutrients and their mobilization to plants.

2.2 Microalgae as biostimulants

Biostimulants are compounds other than fertilizers that enhance the crop productivity by acting directly on the plants regardless of its nutrient content (Du Jardin, 2015). These are group of organic compounds that enhance the crop productivity by increasing the nutrient uptake in plants, imparting resistance to various biotic and abiotic stresses, improve soil water use efficiency, reinforcement of root system, and maintenance of physiological processes such as respiration, photosynthetic activity, Fe uptake and nucleic acid synthesis (Du Jardin, 2015; Lee and Ryu, 2021; Kumar et al., 2022d). Microalgae have been identified with several biostimulatory compounds such as phenolics, phytohormones mimicking compounds, terpenoids, polysaccharides and AAs (Ronga et al., 2019; Gonçalves, 2021). Extracts and metabolites obtained from microalgae species such as *Chlorella* spp., *Spirulina platensis*, *Acutodesmus* spp., *Scenedesmus* spp., *Dunaliella* spp., *Calothrix elenkini* etc. are commonly used as biostimulants (Ronga et al., 2019; Colla and Rouphael, 2020).

2.2.1 Phytohormones mimicking compounds from microalgae

Phytohormones are a low molecular weight, structurally unrelated signaling molecules that occur naturally in plants and provide stimulatory effects at very low concentrations in the various plant development processes such as root and shoot formation, tissue differentiation, fertilization and plant senescence, defense and tolerance to various biotic and abiotic stresses (Gray, 2004; Santner et al., 2009; Fahad et al., 2015). Based on their functions, the phytohormones are classified into auxins, cytokinins, gibberellic, abscisic acid (ABA) and ethylene.

2.2.1.1 Auxins

Auxins are tryptophan derived plant hormones involved in regulation of key physiological processes such as cell division and elongation, vascular tissue differentiation, tropism apical dominance and stress response (Wang et al., 2001; Eyidogan et al., 2012). They are the first identified class of phytohormones, consisting compounds like indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid, indole-3-butyric acid (IBA), indol-3-acetamide (IAM), and 2-phenylacetic acid (Górka et al., 2015). Microalgae such as *Chlorella* spp., *Coenochloris* spp., *Acutodesmus* spp., and *Scenedesmus* spp., *Chlorococcum* spp., were found to contain auxins in the concentration ranging between 0.18 to 99.83 nmol g^{-1} dry weight (DW) biomass consisting two major compounds viz., IAA and IAM. However, IAA was the predominant auxin detected in almost 24 microalgae species (Stirk et al., 2013b; Kapoore et al., 2021). The direct role of IAA derived from microalgae extracts in root formation and elongation was observed with *Petunia x hybrida* plants. Foliar spray (FS) of extracts obtained from *Scenedesmus* spp., on *Petunia* plants resulted in increased dry root weight of plants. Analysis of the extracts indicated the presence of IAA at a concentration of 5965 ng g^{-1} (Plaza et al., 2018). The auxins, specifically IAA, have been attributed with the role as signaling molecule during plant cyanobacteria interactions especially for root colonization (Lee and Ryu, 2021). The IAA produced in cyanobacterial species such as *Nostoc* spp., *Synechocystis* spp., *Leptolyngbya* spp., have been found to improve the growth in rice and wheat plants and were found in highest concentrations during colonization of plant roots (Hussain et al., 2013; Hussain et al., 2015). In addition to this, auxins (IAA) and soluble AAs secreted by cyanobacteria have been identified to enhance soil microbial content and micro biome quality (Karthikeyan et al., 2009; Lee and Ryu, 2021).

2.2.1.2 Cytokinins

Cytokinins are N6-substituted adenine derivatives containing either aromatic or isoprenoid side chains (Santner et al., 2009). They play a significant role in plant developmental processes such as shoot differentiation, cell division, nutrient mobilization, photomorphogenic development, chloroplast biogenesis, apical dominance and vascular differentiation (Fahad et al., 2015). The most predominant cytokinins observed in microalgae are zeatin, zeatin riboside, kinetin, isopentenyladenosine (Górka et al., 2015). The cytokinins content ranged between 0.29 nmol g^{-1} DW and 21.40 nmol g^{-1} DW with maximum concentration observed in *Stigeoclonium nanum*. *cis*-Zeatin and isopentenyl adenine were the predominant cytokinins while trans-zeatin and dihydrozeatin were found in low concentrations in addition to free bases, ribosides (Stirk et al., 2013b). Apart from physiological and developmental functions, microalgae derived cytokinins have been reported to impart abiotic stress tolerance in host plants. For example, use of cytokinin containing extracts of *Nannochloropsis* spp., alleviated water and N stress in tomato plants (Oancea et al., 2013; Lu et al., 2014). The putative mechanism behind the stress tolerance in plants induced by microalgae cytokinins could be attributed to the free radical scavenging properties of cytokinins (Fahad et al., 2015).

2.2.1.3 Gibberellic acids

Gibberellic acids (GA) are specific class of phytohormones with main functions in abiotic stress tolerance in plants (Fahad et al., 2015). GAs modulate photosynthetic efficiency of plants and promote the redistribution of photosynthesis, thus balancing source – sink relationship during abiotic stress (Iqbal et al., 2011). About 19 different types of GAs have been identified in microalgae with main functions of stem elongation, initiation of seed germination *via* enzyme activation (alpha-amylase), initiation of flowering, floral organ development and influencing protein biosynthesis (Kapoore et al., 2021). The total concentration of GAs in microalgae range between 3 pg mg^{-1} biomass for GA7 in *Gyoerffyana humicola* to 3452.9 pg mg^{-1} for GA15 in *Scotiellopsis terrestris* (Stirk et al., 2013a). It has been observed that extracts containing GA3 derived from *Chlorella vulgaris* reduced the adverse effects caused by heavy metal stress and impart defense against lead and Cadmium (Han et al., 2018).

2.2.1.4 Ethylene

Ethylene is a gaseous hormone that regulates developmental processes such as senescence, fruit ripening, cell division and elongation, and tolerance to biotic and biotic stresses (Han et al., 2018). Microalgae species belonging to the genus *Chlamydomonas*, *Chlorella*, *Scenedesmus* and cyanobacteria such as *Synechococcus* spp., *Anabaena* spp., *Nostoc* spp., *Calothrix* spp., *Scytonema* spp., and *Cylindrospermum* spp., have been reported to synthesize ethylene (Lu and Xu, 2015). Plaza et al. (2018) reported ethylene content of 341 ng g^{-1} DW and 546 ng g^{-1} DW in *Scenedemsus* spp., and *Spirulina platensis* respectively.

2.2.1.5 Abscisic acid

Abscisic acid is a C_{15} sesquiterpenoid hormone which play an important role in the adaptive responses of plants to various biotic and abiotic stresses. They function by modulating stomatal closure, biosynthesis of proteins and compatible solutes/osmolytes, and maintenance of water status in plants enabling tolerance in plants to stressors (Eyidogan et al., 2012). In general, they are general inhibitors of growth and metabolic functions and functions in conjunction with other phytohormones or signaling molecules such as auxins, cytokinins, ethylene and brassinosteroids (Fahad et al., 2015).

2.2.2 Other hormone like signaling molecules

In addition to phytohormones, microalgae and cyanobacteria accumulate low molecular weight signaling molecules such as brassinosteroids, polyamines and jasmonic and salicylic acids (Kapoore et al., 2021; Lee and Ryu, 2021). Brassinosteroids are steroidal plant hormones that either exist freely or are conjugated to sugars or fatty acids with primary function in seed germination, vascular differentiation, leaf bending and pollen tube elongation (Fahad et al., 2015). They have been associated with the stress response mechanisms of plants, especially in the tolerance to salt stress and enhance the enzymatic and non-enzymatic defence response in stressed plants (Sharma et al., 2013). Microalgae have been identified with two types of brassinosteroids, viz., brassinolide and catasterone. The brassinosteroid contents ranged from 117.3 pg g^{-1} DW in *Raphidocelis subcapitata* MACC 317 to 977.8 pg g^{-1} DW in *Klebsormidium flaccidum* MACC 692 (Stirk et al., 2013b).

Jasmonic (JA) and Salicylic acids (SA) are ubiquitous messenger/signaling molecules in plant defence systems with a significant role in biotic stress response. They play a critical role in seed germination, glycolysis, flowering, upregulation of antioxidant genes, ion uptake and transport, photosynthetic rate, stomatal conductance, transpiration, thermo-tolerance, senescence and nodulation (Fahad et al., 2015). The SA and JA signaling pathway are interconnected with phytohormones signaling and they act antagonistically to auxins, cytokinins and GA responses while acting synergistically with ethylene and ABA (Kapoore et al., 2021). JA levels increase in response to various inductive signals such as mechanical wound, herbivory and abiotic stresses while SA levels increase with infection of the host plants by a broad range of pathogens (Santner et al., 2009). Jasmonic and salicylic acids have been detected in most of the microalgae species, and in significant quantities in *Scenedesmus* spp., 75.13 ng g^{-1} and 156714 ng g^{-1} for JA and SA respectively (Plaza et al., 2018; Kapoore et al., 2021).

Polyamines are low molecular weight poly-cations that play a prominent role in plant growth and development processes and stress responses (Kusano et al., 2008; Chen et al., 2019). The most common forms of polyamines are putrescine, spermidine and spermine (Mustafavi et al., 2018). Among the various microalgae species, *Spirulina platensis* contained significant quantities of polyamines *viz*., 0.76 µg of putrescine, 3.31 µg of spermine and 0.67 µg of spermidine per gram dry biomass (Tarakhovskaya et al., 2007). The polyamines of *Spirulina* have been reported to enhance the growth of lettuce seedlings, exhibiting a biostimulant behavior (Mógor et al., 2018a).

2.2.3 Microalgal polysaccharides as bio stimulatory compounds

Polysaccharides are complex macromolecular polymers of neutral sugars with diverse compositions with varied degree of

polymerization, chemical substitution and biological activity (Chanda et al., 2019). Polysaccharides stimulate plant growth and metabolism by modulating physiological and biochemical processes. Some of the stimulatory activities exhibited by polysaccharides are enhancement of root growth, nutrient availability and mobilization through chelation of minerals, and enhancement of photosynthesis through increased synthesis of Rubisco and tolerance to biotic and abiotic stress and act as signaling molecules (Moreira et al., 2022).

The typical mechanism by which microalgal polysaccharides exhibit stimulatory properties is through microbial associated molecular patterns dependent signaling pathways (Chanda et al., 2019). The mechanism has been elucidated in seaweed polysaccharide extracts (Mukherjee and Patel, 2020) which can be well extrapolated to microalgal polysaccharides. Briefly, the complex polysaccharides are hydrolyzed by soil enzymes such as beta glucanase, chitinase secreted by microorganisms and these neutral sugars from polysaccharides could be recognized by the receptors on plant membranes as microbial derived compounds that induce signaling cascades by (i) activation of Ca2+ influx, (ii) stimulation of octadecanoid and phenylpropanoid pathways leading through the enzymes lipoxygenase (LOX) and phenylalanine ammonia lyase (PAL), (iii) activation of SA and JA signaling pathway, (iv) reactive oxygen species (ROS) scavenging enzymes such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) and synthesis of phenolic and secondary metabolites that act as defense molecules (Chanda et al., 2019; Farid et al., 2019). Among the various microalgae species, cyanobacteria such as *Spirulina platensis, Nostoc* spp., *Phormidium* spp., *Calothrix* spp., *Plectonema* spp., are known to produce polysaccharides that are secreted to the surrounding medium and termed as exopolysaccharides (EPS) (Parwani et al., 2021). Similarly, eukaryotic microalgae such as *Chlorella vulgaris, Chlorella stigmatophora, Porphyridium cruentum, Tetraselmis* spp., *Dunaliella salina* produce polysaccharides that have biostimulatory potential (Chanda et al., 2019).

The EPS secreted by cyanobacteria have various benefits in terms of enhancing crop productivity. The foremost of them is the bioadhesive property of EPS that results in the formation of microbial mats and biofilms promoting BSC formation (Rossi and De Philippis, 2015; Abinandan et al., 2019). Further, cyanobacterial EPS have soil conditioning properties where they promote the formation of micro-aggregates of soil leading to moisture retention, nutrient accumulation and proliferation of soil microflora (Rossi and De Philippis, 2015). EPS-secreting cyanobacterial biofilms are concentrated source of nutrients and offer benefits such as mineralization of complex soil nutrients, C and N fixation in soil, protection against drought and desiccation and heavy metal sequestration (Garlapati et al., 2019; Parwani et al., 2021; Moreira et al., 2022).

Field applications of cyanobacteria and microalgae polysaccharides have shown beneficial effects in host plants. Foliar application of polysaccharide-rich extracts from *Spirulina platensis* resulted in enhanced growth of tomato and pepper plants (Elarroussia et al., 2016). Similarly, polysaccharide extracts of *Chlorella vulgaris, Chlorella sorokiniana*, *Chlamydomonas reinhardtii*, *Dunaliella salina* had shown bio stimulatory properties when injected into tomato plant seedlings (Farid et al., 2019). The group observed that polysaccharide extracts of above mentioned green microalgae enhanced the activity of defence enzymes such as LOX, PAL, and ROS scavenging antioxidant enzymes such as CAT, POD and ascorbate peroxidase (APX). In addition, they enhanced very long chain fatty acids in the leaves of tomato plant which constitute the cuticular wax composition indicating activation of plant defence mechanisms against external stressful stimuli. In another study, El Arroussi et al. (2018) reported enhanced stress tolerance to salinity in tomato plants when applied with EPS obtained from *Dunaliella salina*. The literature amply demonstrates that polysaccharides from microalgae and cyanobacteria have plant growth promotion and biostimulatory properties that can be exploited for enhanced crop productivity.

2.2.4 Other microalgae metabolites in crop productivity enhancement

Similar to polysaccharides, protein hydrolysates, peptides and free AAs obtained from microalgae have been reported to enhance crop productivity (Kapoore et al., 2021). The primary function of these hydrolysates and AAs are nutrient mobilization into plants through complexation and chelation of essential minerals (Du Jardin, 2015). Additionally, these AAs play a critical role in abiotic stress mitigation by acting as osmoprotectants and antioxidants such as glycine, betaine and proline against environmental stress such as heavy metals and salinity (Bulgari et al., 2015). Further, application of AAs and protein hydrolysates enhanced the plant growth promoting bacteria by acting as a source of reduced N to the microflora thus promoting the soil microbiome (Colla et al., 2017; Lee and Ryu, 2021). The protein content of microalgae and cyanobacteria range up to 63% with AAs contents constituting between 40% and 48% of total proteins (Hempel et al., 2012; Kumar et al., 2022c). High amounts of certain AAs like arginine and tryptophan in species such as *Spirulina platensis* make them an attractive option for biostimulant application as they act as precursors for polyamines and auxins respectively (Bulgari et al., 2019). Foliar application of protein rich extracts of *Spirulina platensis* on red beet increased the hypocotyl growth, chlorophyll and nutrient composition (Mógor et al., 2018b). while in case of *Petunia x hybrida* the number of flowers, flower fresh and DW was enhanced (Plaza et al., 2018). In another study, application of AA rich extracts of green microalgae enhanced the solids content, total organic and capsaicinoids content in three varieties of hot pepper (*Capsicum* spp.). The study suggested variety specific outcome with respect to biostimulatory treatments (Zamljen et al., 2021).

Antioxidants and micronutrients are another important group of nutrients offered by microalgae and cyanobacteria towards enhancing crop productivity. Some of the important class of micronutrients are vitamins, specifically ascorbic acid that impart tolerance to both biotic and abiotic stresses (Kapoore et al., 2021). Among the antioxidants like phenols, terpenoids and carotenoids, the foremost group is carotenoids that play a central role in photosynthesis and photoprotection. In addition, carotenoids contribute to the pigmentation of seeds, fruits, and flowers and act as precursors for plant signaling hormones such as ABA and strigolactone (Sun et al., 2022). The prominent group of antioxidants found in microalgae are carotenoids such as alpha and beta carotene in *Dunaliella salina*, *Chlorella vulgaris*, fucoxanthin in *Phaeodactylum tricornutum* and *Isochrysis cabana*. The carotenoid composition of microalgae and

cyanobacteria have been well reviewed by several authors previously (Vidyashankar et al., 2017; Cezare-Gomes et al., 2019). Although the role of carotenoids in plant growth and development is characterized, the mechanisms of carotenoid conversion and uptake from microalgae biomass is not elucidated requiring further detailed studies.

2.2.5 Microalgae phenolics as biostimulants

Phenolics are another important group of metabolites that play a critical role in stress signaling and defence responses to infection and injury (Mandal et al., 2010). Phenolic compounds have been attributed to various defence mechanisms observed in plants in response to abiotic stresses. In addition, phenolics increase nutrient absorption by chelating ions and mobilize the uptake of nutrients such as Ca, zinc, and Fe leading to enhanced porosity of soils (Perera and Tirimanne, 2021). Phenolic acids and flavonoids act as sunscreens protecting plants from UV-B radiation, specifically kaempferol and derivatives (Tamrat Alemu and Roro, 2020). Further, they are involved in drought, salinity and heavy metal stress tolerance with a primary role of scavenging ROS generated during oxidative stress. These abiotic stresses generate hydrogen peroxides, hydroxyl and superoxide ions that act as free radicals (Dehghanian et al., 2022). In addition to abiotic stress, phenolic acids are generated as a defence response to insects, phytopathogens and herbivory (Dehghanian et al., 2022). Phenolic acids and derivatives such as hydroxycinnamate conjugates and hydroxycoumarins are produced during infections from phytopathogens (Kumar et al., 2020). Microalgal polyphenols and flavonoids are less explored compared to the other metabolites in them. The total polyphenol content of microalgae ranged between 0.16 mg gallic acid equivalent (GAE) g^{-1} in *Neochloris* spp., to 60 mg GAE g^{-1} in *Nostoc*. spp., while flavonoids ranged between 0.84 mg Quercetin equivalent (QE) g^{-1} in *Phaeodactylum* to 4.03 mg QE g^{-1} in *Desmodesmus* spp., (Del Mondo et al., 2021). Gallic, ferulic, caffeic, chlorogenic, synapic and coumaric acids and hydroxybenzoates are commonly found phenolic while in the case of flavonoids, rutin, quercetin, kaempferol are predominantly observed among different microalgae species (Del Mondo et al., 2021).

2.2.6 C-phycocyanin as biostimulant

C-phycocyanin (CPC) is a water-soluble phycobiliprotein (protein-pigment complex) majorly distributed in cyanobacteria with light-harvesting functions. Cyanobacteria such as *Spirulina platensis*, *Phormidium* spp., *Nostoc* spp., *Anabaena fertilissima* PUPCCC 410.5, *Synechocystis* spp., and *Galdieria sulphuraria* are known to contain significant content of CPC (Kaur et al., 2019; Avci and Haznedaroglu, 2022). Commercially, CPC is produced from *Spirulina platensis* towards food (as a natural blue colourant) and therapeutic applications (as an antioxidant) (Patel et al., 2022). Apart from the nutraceutical applications, CPC has been recently identified with plant biostimulant properties (Varia et al., 2022). Treatment of tomato seeds with CPC enhanced the germination index, shoot and radicle length along with the modulation of chemical composition, specifically secondary metabolites such as phenolics and flavonoids (Metwally et al., 2022). Application of CPC in vertical hydroponic systems promoted early maturity in lettuce along with enhanced biomass productivity, leaf diameter and total flavonoids (quercetin and luteolin) content (Varia et al., 2022). In addition to these, CPC modulated microbial diversity and abundance in the hydroponic growth medium promoting actinobacteria and firmicutes suggesting possible plant prebiotic properties of CPC by its ability to stabilize plant growth promoting bacteria and enhancing plant growth (Varia et al., 2022). The major advantage of use of CPC as biostimulants is that the molecule is well characterized along with its downstream processes such as extraction and purification well standardized and commercialized (Arahou et al., 2022). Further, CPC is water soluble in nature allowing its scalability as bio stimulants. The major bottleneck in the use of CPC in open conditions is its light-sensitive nature and poor half-life at high light intensities (Adjali et al., 2021). However, under controlled atmospheric growth conditions such as hydroponics and other vertical farming systems, CPC can be well exploited as biostimulants. A summary of various bio stimulatory compounds present in microalgae and cyanobacteria and their functions are presented in Figure 1.

2.2.7 Micronutrients from microalgae biomass

Microalgae are rich source of micronutrients like K, Ca, P and trace elements such as, iron, zinc, copper and manganese. The total P content of the biomass ranged between 0.73% to 1.46% w/w with highest content observed in marine microalgae *Tetraselmis chuii* and freshwater oleaginous microalga *Botryococcus braunii* (1.45 - 1.46% w/w) (Tibbetts et al., 2015). The Ca content ranged between 0.1% to 2.9% w/w with highest content observed in *Tetraselmis chuii* (2.99% w/w) and *Phaeodactylum tricornutum* (2.91% w/w) followed by *Porphyridium cruentum* (2.06% w/w) (Tibbetts et al., 2015; Di Lena et al., 2020). The K content was highest in *Phaeodactylum tricornutum* (2.4% w/w) and other microalgae species containing K ranging from 0.7% w/w to 1.8% w/w (Tibbetts et al., 2015; Di Lena et al., 2020). Among the most commonly cultivated microalgae viz., *Spirulina platensis* and *Chlorella vulgaris*, the P content was twofold higher in *Chlorella* in comparison with *Spirulina* while in case of K, the trend was vice *versa* with *Spirulina* containing thirty-fold higher K (1.3% w/w - 1.5% w/w) in comparison to *Chlorella* (0.049% w/w) and Ca content ranging between 0.59% and 0.89% w/w (Tokuşoglu and Üunal, 2003). Among the trace elements, Fe was highest in *Porphyridium aerugineum* (1110 mg $100g^{-1}$) followed by *Botryococcus braunii* (620.31 mg $100g^{-1}$) (Tibbetts et al., 2015). Among freshwater microalgae, *Chlorella* spp., (259.1 mg $100g^{-1}$) contained two-fold higher Fe content compared to *Spirulina* (103.6 mg $100g^{-1}$) (Tokuşoglu and Üunal, 2003). Other trace elements such as Zn, Mn and Cu ranged between 1.5 mg to 13 mg $100g^{-1}$ (Tokuşoglu and Üunal, 2003; Tibbetts et al., 2015; Di Lena et al., 2020). As discussed in previous section, microalgae accumulate P and Fe using special mechanisms and store as polyphosphate granules and Fe reservoirs respectively (Powell et al., 2009). The mechanism of luxury uptake of minerals observed in microalgae could be exploited for agriculture applications. Microalgae passively bio-absorb nutrients especially, trace metals like Zn, molybdenum (Mo), selenium (Se), Cu, etc. The surface composition of microalgae cell contains charged polysaccharide molecules with charged moieties like carboxyl, sulfonic acids, hydroxyl ions that in turn bind to metal ions and form complexes. Microalgae absorb these

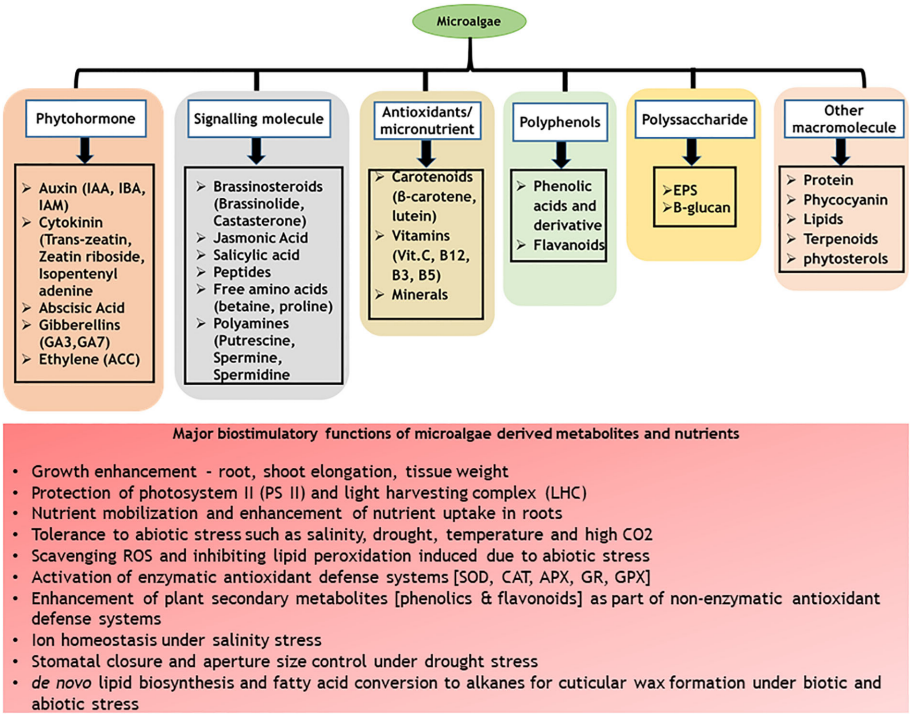


FIGURE 1
A summary of various bio stimulatory compounds present in microalgae and cyanobacteria.

metals/charged ions through ion exchange and complexation mechanisms (Michalak and Chojnacka, 2015).

Apart from minerals, microalgae synthesize vitamins that act as growth promoting factors for plants. Among the various water soluble vitamins evaluated, vitamin C was the most abundant, ranging between 100.2 mg kg^{-1} in *Chlorella stigmatophora* and 191 mg kg^{-1} in *Tetraselmis suecia* compared to other B complex vitamins in marine microalgae biomass (Fabregas and Herrero, 1990). Among the vitamin B complex, nicotinic acid was highest ranging between 77.7 mg kg^{-1} in *Isochrysis galbana* to 89.3 mg kg^{-1} in *Tetraselmis suecia* (Fabregas and Herrero, 1990). In case of freshwater microalgae, riboflavin, niacin, folic acid and cyanocobalamin were predominant vitamins present in microalgae such as *Spirulina platensis*, *Chlorella* spp. (Edelmann et al., 2019). The riboflavin content varied between 21 and 41 µg g^{-1} while niacin ranged from 0.13 to 0.28 mg g^{-1} DW respectively, in the aforesaid microalgae species. The folic acid content was six times higher in *Chlorella* spp., 19.7 µg g^{-1} compared to *Spirulina platensis* 3.5 µg g^{-1} (Edelmann et al., 2019). Watanabe et al. (2013) reported presence of true form of vitamin B_{12} in *Chlorella vulgaris* while *Spirulina platensis* and other cyanobacteria such as *Nostoc commune*, *Nostoc flagelliforme* and *Nostochopsis* spp., contained a corrinoid compound or pseudo form of vitamin B_{12} [adeninyl cobalamin] that are not biologically active in mammals. However, these compounds could be potentially bioactive for plant health and plants may utilize these corrinoid compounds as precursors or signaling molecules. Application of vitamin B complex rich microalgae biomass to the soil enhance the vitamin contents of the plant tissues as plants have known to absorb vitamin B12 and other B complex vitamins through roots as evidenced by use of bio fertilizers in cultivation of soybean, spinach and barley (Mozafar, 1994). Apart from micronutrients, microalgae produce metabolites such as terpenoids, humic substances, betains, and peptide (cyanotoins) that act as messenger/signaling molecules, bio stimulants or as allelochemicals that inhibit growth of weeds and microbes and function as bio pesticides (Kapoore et al., 2021).

3 Methods of application of microalgae-based bio fertilizer and biostimulants

Cyanobacteria and microalgae, either in the form of live biomass or dried biomass have been applied as biofertilizers while their cellular extracts and hydrolysates as biostimulants in enhancing plant growth and crop yield. Admixtures of soil with live or dry algae biomass, dipping of seeds with cell extracts of microalgae (seed priming), and root drenching are some of the common methods of biostimulant/ biofertilizer application (Lee and Ryu, 2021). The mode of application of biostimulants is dependent upon the particular need of the crops such as nutrient supplementation or for micronutrient enrichment or disease suppression. A further type of crop greatly influences the mode of application as whether they are direct seed sown or nursery grown and transplanted in the field (Renuka et al., 2018).

3.1 Seed treatments

Seed treatment with biostimulants generally involves three different processes, namely seed priming, seed coating and seed dipping (Gupta et al., 2022). Seed priming is a pre-sowing treatment where the seeds are

hydrated in a controlled manner such that the radicle does not protrude. This technique enhances seed germination rate and enhances root formation in plants (Sharma et al., 2014). In seed coating, the seeds are sprayed or coated with biostimulants to form a uniform layer while in seed dipping or soaking treatments, the seeds are soaked for a definite time (18-24 h) prior to sowing. The major advantage with these methods is that it gives a head start in germination and consequently enhances germination index, seedling vigor, increased shoot and radicle length and reduction in harmful seed micro flora (Rocha et al., 2019).

The use of microalgae extracts and live cell suspension in seed treatments resulted in enhanced growth of plants in a variety of crops such as cereals, vegetables and spices. In a recent study, priming of spinach seeds with whole cell extracts and cell lysates of green microalgae *Chlorococcum* spp., *Micractinium* spp., *Scenedesmus* spp., and *Chlorella* spp., resulted in enhanced seed germination, faster cotyledon emergence and seedlings weight (Rupawalla et al., 2022). Similarly, priming of seeds of vegetable crops such as tomato, lettuce and cucumber with cellular extracts of *Spirulina* spp., *Chlorella* spp., *Chlorella vulgaris, Scendesmus* spp., *Synechocystis* spp., and *Acutodesmus obliquus* resulted in higher germination rate compared to untreated seeds (Garcia-Gonzalez and Sommerfeld, 2016; Bumandalai and Tserennadmid, 2019; Supraja et al., 2020a). In addition to enhancing the germination rate and seedling vigour, hydration of seeds with extracts obtained from *Chlorella vulgaris* and *Scenedesmus quadricauda* resulted in increased root length, root diameter and root surface area and the number of root tips in sugar beet (Puglisi et al., 2022). Similar observations were made with cereal crops namely maize and wheat when extracts and cell-free supernatants of cyanobacteria such as *Anabaena* sp. PCC 7120, *Calothrix* spp., *Hapalosiphon* spp., *Nostoc* spp., and *Westiellopsis* spp were applied to the seeds (Karthikeyan et al., 2009; Grzesik and Romanowska-Duda, 2014). Apart from the application of microalgae extracts, inoculation of live cyanobacteria suspensions like *Anabaena laxa* and *Calothrix elenkinii* with the seeds of spice crops such as pepper, coriander, fennel and cumin promoted germination and enhanced root and shoot formation (Guzmán-Murillo et al., 2013; Kumar et al., 2013). The ability of microalgae cell suspensions and cell-free supernatants in enhancing the growth of seedlings, germination rate and root formation could be attributed to the presence of EPS in the supernatant while that of microalgal extracts could be attributed to the presence of phytohormones such as cytokinins (trans-Zeatin, dihydrozeatin, isopentyladenine and kinetin), gibberellins (GA1, GA3, GA4, GA20 and GA29), auxin (IAA) and ABA (Rupawalla et al., 2022).

3.2 Foliar spray

Foliar spray of biostimulants is a commonly employed method for enhancing crop productivity in several crops owing to the faster response of plants to nutrients supplemented compared to other treatments (Arahou et al., 2022). FS of biostimulants enhanced the water use efficiency and stomatal functioning of the plants (Renuka et al., 2018). Although widely used method, the mechanism of uptake of biostimulants and nutrients through FS is still not clearly understood. Four major pathways of entry have been hypothesized namely through cuticle cracks, stomata, aqueous and ectodesmatal pores (Ishfaq et al., 2022). The generally accepted phenomenon is the cuticular route of nutrient entry (Oosterhuis, 2009). A typical nutrient absorption pathway through foliage involves foliar adsorption, followed by cuticular penetration, uptake and absorption into cellular compartments of leaf, followed by translocation and utilization. Another possible mechanism is through stomatal penetration through the process of diffusion along the stomatal pores (Fernández et al., 2013).

The efficacy of nutrient and biostimulants uptake in plants depends on the physicochemical properties of spray formulation such as pH, the surface tension of the spray liquids, and retention of spray fluid on the leaf surface. Additionally, the inherent nature of the spray formulation such as the molecular size of the nutrients or stimulatory compounds, ionic charge and solubility determines the success of penetration into the leaf (Pandey et al., 2013). FS is generally applied either through fertigation (addition of stimulants/ nutrients in irrigation systems) process or through aerial sprays which are effective in improving nutrient use efficiency (Renuka et al., 2018). Foliar application of microalgae extracts has been proven successful in enhancing the growth of several crop plants. Cellular extracts of *Chlorella vulgaris*, *Spirulina platensis*, *Scenedesmus* spp., *Nostoc* spp., *Anabaena* spp., *Dunaliella salina* have been reported to enhance the growth, biomass yield (shoot weight), fruit yield, leaf pigment content, tolerance to abiotic stress such as drought, salinity and temperature stress in horticultural crops such as tomato (Gitau et al., 2022), lettuce (La Bella et al., 2021; Puglisi et al., 2022), capsicum (Elarroussia et al., 2016), onion (Gemin et al., 2022) and beans (Li et al., 2014; Elarroussia et al., 2016). In addition to the horticulture crops, FS of cellular extracts of *Spirulina platensis* and *Scenedesmus* spp., resulted in enhanced root weight, plant growth, number of flowers per plant, earliness of flowering and flower diameter in *Petunia* x hybrid plant (Plaza et al., 2018). Evaluation of the composition of microalgae cell hydrolysates revealed the presence of plant growth-promoting substances such as phytohormones (auxins and cytokinins), signaling molecules such as betaines, AAs, vitamins, polyamines, (spermine and spermidine), and polysaccharides mainly beta-glucan apart from micronutrients (González-Pérez et al., 2022). Among the various microalgae species, extracts and hydrolysates derived from *Spirulina platensis*, *Chlorella vulgaris* and *Scendesmus* spp., are most utilized for FS application (Arahou et al., 2022; González-Pérez et al., 2022).

3.3 Soil and root drench

Roots are the interface between soil and plant that sustain plant growth mainly by mobilizing nutrients, conducting external stimuli and initiating plant defence response to stressors while the soil is a finite nonrenewable resource that forms the basis of agriculture (Ma et al., 2022). Maintaining the health of roots and soil is essential for sustainable agriculture and this is achieved through the application of soil conditioners and fertilizers that replenish soil health and provide nutrients to roots. However, in intensive agricultural practices, the use of chemical fertilizers and conditioners has resulted in soil compaction, acidification, decreased fertility and imbalance of soil microflora aggravating soil diseases (Ye et al., 2020). This necessitates

the use of biodegradable, less harmful soil conditioners and fertilizers. Soil drenching involves proportionate mixing of biofertilizers or stimulants during sowing that enhances plant growth. The mechanism of action of biostimulants/biofertilizers by soil drenching method is by agglomeration of soil particles with organic molecules such as polysaccharides, promotion of biological mineralization of complex nutrients and restoration of soil microflora (Karthikeyan et al., 2009; Alvarez et al., 2021).

Microalgae-based soil drenching applications involve the addition of live microalgae suspensions or dried biomass with suitable carrier materials for the application. Soil is the most economically viable carrier for agriculture applications. However, aerial contamination is the major issue with soil, necessitating alternative carrier materials. Agricultural and agro-industrial wastes such as bagasse, peat, wheat straw, vermiculite and animal manure are effective carriers in the application of cyanobacteria and microalgae in soil drenching applications (Renuka et al., 2018). Application of algae as biofertilizer in soil drenching is done as consortia of cyanobacteria/microalgae or as a consortium consisting of a combination of cyanobacteria and rhizobial bacterial species in a suitable carrier (Alvarez et al., 2021). As discussed earlier, the main application of these consortia is for soil amendment and crop productivity enhancement. The most commonly used cyanobacterial combination is *Anabaena* spp., and *Nostoc* spp., finding application in a variety of crops such as corn, rice, wheat, cotton and chickpea. The use of cyanobacterial consortium resulted in enhanced availability of soil N and P, increased soil enzyme activity such as nitrogenase, dehydrogenases, and proliferation of soil microflora leading to enhanced biomass and crop productivity (Karthikeyan et al., 2009; Prasanna et al., 2015a; Prasanna et al., 2015b). Further, a synergistic effect of cyanobacteria with rhizobial bacteria such as *Brevundimonas diminuta*, *Mesorhizobium ciceri*, *Azotobacter* spp., *Pseudomonas putida* etc., resulted in enhanced N_2 fixation, P solubilization, soil micronutrient content and microbial activity (Alvarez et al., 2021). Several authors reported that the use of synergistic bacterial and cyanobacterial consortium in pot or field experiments resulted in the enhancement of macro and micronutrients in cereal crops such as rice (Rana et al., 2015). Similarly, there was an significant increase in the increased grain yield and leghaemoglobin content in nodules of chickpea (Prasanna et al., 2017). The beneficial effects were observed in flower crops such as chrysanthemum with increased flower diameter (Kanchan et al., 2019).

The major benefit of inoculating cyanobacteria was a reduction in the requirement for chemical NPK fertilizers. Most of the above-mentioned reports utilized live cyanobacteria or consortia partially replacing the chemical NPK fertilizers. The available reports uniformly suggest that the use of cyanobacterial consortia resulted in grain yield in crops such as corn and rice similar to chemical fertilizers albeit with a savings of N fertilizer to the tune of 50% (Prasanna et al., 2015a; Prasanna et al., 2015b; Prasanna et al., 2015c). In the case of green microalgae, the application of *Chlorella vulgaris* in synergy with *Pseudomonas putida* resulted in enhanced soil P mobilization to rice plants and prevented arsenic translocation in plant tissues suggesting the dual role of microalgae as biofertilizer and heavy metal sequestration (Srivastava et al., 2018). In the case of rice, treatment with cyanobacteria species such as *Anabaena variablis* and *Nostoc* spp., by root drench method enhanced the plant height, leaf length and grain yield compared to the inorganic fertilizers (Singh and Datta, 2007; Innok et al., 2009). Similarly, the use of eukaryotic microalgae biomass, specifically green algae *Chlorella* spp., enhanced the growth and yield of vegetable plants. For example, the application of *Chlorella pyrenoidosa* by root drench method enhanced the grain yield and shoot weight in soybean (Dubey and Dubey, 2010). Extracts of *Chlorella vulgaris* enhanced the growth of wheat, maize and tomato (Shaaban, 2001b; Coppens et al., 2016; Barone et al., 2018). The other microalgae species that have been reported to possess biofertilizer properties are *Scenedesmus* spp., *Dunaliella* spp., *Spirulina* spp., *Scenedesmus quadricauda*, and *Nannochloropsis* spp. (Ammar et al., 2022). A summary of different modes of application of biostimulants derived from microalgae is presented in Table 1.

3.4 Effect of biostimulants and their method of applications on plant metabolism

As discussed earlier, the method of bio stimulant application is mainly dependent on the type of crop and the need of the crop, i.e., whether for nutrient enhancement or for tolerance against biotic and abiotic stresses. The method of biostimulant application and timing of application significantly affect the response of plants to stimulants. For example, in a study with lettuce, the response to *Chlorella vulgaris* extracts was different between root drenching and FS applications (Puglisi et al., 2022). In the case of root drenching, the microalgae extract exerted significant influence on carbon metabolism compared to a foliar application as observed with enhanced activities of malate dehydrogenase and citrate synthase, key enzymes involved in carbon fixation (Kreb's cycle) supported with enhanced carbon content in the biomass and root and shoot weight (Puglisi et al., 2022). However, the N metabolism was influenced both by root drenching and FS as evidenced by increased activity of enzymes such as glutamate synthase and glutamine synthetase involved in N metabolism with both the treatments (Puglisi et al., 2022). This was morphologically validated with enhanced protein content in the shoot, increased leaf pigments and biomass weight. Similar observations of enhanced N metabolism and increased shoot and root N and biomass protein contents were observed with foliar application of *Scendesmus quadricauda* extracts in lettuce, commercial algae-based biostimulants on spinach (Fan et al., 2013; Ronga et al., 2019; Puglisi et al., 2020).

In addition to supporting plant growth, bio stimulant application promotes stress tolerance in plants by means of activation of secondary metabolism. Several reports have been published with enhanced abiotic/biotic stress tolerance (Table 1). This observation can be attributed to the increased activity of the enzyme PAL involved in phenylpropanoid pathway involved in the synthesis of phenolics and flavonoids which function as plant defense molecules (Dehghanian et al., 2022). Microalgae extracts derived from *Chlorella vulgaris*, *Scenedesmus quadricauda* significantly enhanced the PAL activity in crops such as lettuce and sugar beet (Barone et al., 2018; Puglisi et al., 2020; Puglisi et al., 2022). Among the different methods of application, FS acted instantly in increasing the enzymatic activity compared to soil drenching. This was evidenced by time dependent expression of key enzymes (Puglisi et al., 2022). The PAL

TABLE 1 Methods of application of microalgae biostimulants and their effect on plants.

Microalgae/extracts	Crop evaluated	Outcome(s)					References
		Enhanced germination rate & seedling vigor	Improved root parameters	Increased biomass yield	Enhanced nutritional content of seeds	Biotic/ Abiotic stress tolerance	
Seed treatments							
Spirulina platensis (seed coating of Spirulina platensis extracts)	*Raphanus sativus*	+				+	(Godlewska et al., 2019)
Spirulina extract	• *Triticum aestivum* • *Hordeum vulgare*	+		+			(Akgül, 2019)
Spirulina platensis extract	*Calotropis procera* Ait	+	+			+	(Bahmani Jafarlou et al., 2021)
Spirulina platensis extract	*Vigna mungo* L.	+	+	+	+	+	(Thinh, 2021)
Phycocyanin extract [*Spirulina platensis*]	*Solenum lycopersicum* L.	+		+	+		(Metwally et al., 2022)
Chlorella spp. cell suspension	• *Triticum aestivum* • *Hordeum vulgare*	+	+	+			(Odgerel and Tserendulam, 2016)
Chlorella vulgaris	• *Solanum lycopersicum* L., • *Cucumus sativus*		+	+			(Bumandalai and Tserennadmid, 2019)
Acutodesmus dimorphus Live culture and extracts	*Solanum lycopersicum* var. *Roma*	+					(Garcia-Gonzalez and Sommerfeld, 2016)
Nostoc commune aqueous extracts	*Oryza sativa* L.	+	+	+			(Abedi Firoozjaei et al., 2021)
• *Dunaliella salina* extract • *Phaeodactylum tricornutum* extract	*Capsicum annuum* L.	+	+			+	. (Guzmán-Murillo et al., 2013)
Consortia of • *Microcystis aeruginosa* MKR 0105 • *Anabaena* spp. PCC 7120 • *Chlorella* spp.	*Zea mays* L.	+	+	+			(Grzesik and Romanowska-Duda, 2014)
• *Chlorella vulgaris* extract • *Scenedesmus quadricauda* extract	*Beta vulgaris*	+	+				(Puglisi et al., 2020)
Extracts of consortium • *Chlorella* spp., • *Scenedesmus* spp., • *Spirulina* spp., • *Synechocystis* spp.	*Solanum lycopersicum* L.	+	+	+			(Supraja et al., 2020a)
• *Chlorella vulgaris* • *Scenedesmus quadricauda*	*Beta vulgaris* L.		+				(Barone et al., 2018)
Consortia of • *Chlorococcum* spp. • *Micractinium* spp. • *Scenedesmus* spp. • *Chlorella* spp.	*Spinacia oleraceae*	+		+	+		(Rupawalla et al., 2022)

(Continued)

TABLE 1 Continued

Soil/Root drenching							
Microalgae/ extracts	Crop evaluated	Improved root parameters	Enhanced biomass/crop yield	Enhanced leaf phytochemical content	Improved soil quality & soil enzyme activity	Biotic/ Abiotic stress tolerance	References
Spirulina platensis	*Zea mays* L.		+		+		(Tuhy et al., 2015)
Spirulina platensis suspension	*Vicia faba* var. Giza 843	+	+			+	(Osman et al., 2016)
Scenedesmus subspicatus	*Allium cepa L*	+					(Gemin et al., 2022)
Navicula Spp.	• *Solanum lycopersicum* L., • *Capsicum annuum* L., • *Solanum melongena*		+		+		(Alshehrei et al., 2021)
Extract of *Spirulina maxima* and *Chlorella ellipsoida*	*Triticum aestivum*			+	+	+	(Abd El-Baky et al., 2010)
Nostoc, Anabaena, Westiellopsis, Aulosira, and Scytonema	*Oryza sativa* L.		+		+		(Paudel et al., 2012)
Extract of *Chlorella vulgaris* CCAP 211/11C	*Lactuca sativa* L.	+	+	+	+		(Puglisi et al., 2022)
Polysaccharide extracts of Spirulina platensis, Dunaliella salina, porphyridium spp.	*Solanum lycopersicum* L. var. Jana F1		+	+	+	+	(Rachidi et al., 2020)
Scenedesmus obliquus Chlorella vulgaris and Anabaena oryzae biomass	*Musa* spp.	+		+	+	+	(Hamouda and El-Ansary, 2017)
Chlorella vulgaris biomass with cow dung	*Solanum lycopersicum* L.	+		+	+	+	(Suchithra et al., 2022)
• Nannochloropsis • Consortia of *Ulothrix* spp. and *Klebsormidium* spp.	• *Solanum lycopersicon* cv., • *Solanum lycopersicum* var. Maxifort		+	+			(Coppens et al., 2016)
Foliar spray							
Microalgae/ extracts	Crop evaluated	Enhanced biomass/crop yield	Early flowering and increased enzymatic activity	Enhanced leaf pigment content & number of fruits/ flowers	Improved nutritional quality of plants	Biotic/ Abiotic stress tolerance	References
Arthrospira spp	*Beta vulgaris* L.	+			+		(de Oliveira et al., 2013)
Polysaccharides extract of *Spirulina platensis*	• *Solanum lycopersicum* L. • *Capsicum annuum* L.,	+			+		(Elarroussia et al., 2016)
Spirulina extract	*Cynara cadunculus* L.	+		+	+		(Amer et al., 2019)
Spirulina extract	*Zea mays* L.			+	+		(Kiran et al., 2020)
Arthrospira fusiformis	*Allium sativum*				+		(Shalaby and El-Ramady, 2014)
Cell extract of *Chlorella vulgaris*	*Triticum aestivum* L. var. Giza 69	+			+		(Shaaban, 2001a)

(Continued)

TABLE 1 Continued

Foliar spray							
Microalgae/ extracts	Crop evaluated	Enhanced biomass/crop yield	Early flowering and increased enzymatic activity	Enhanced leaf pigment content & number of fruits/ flowers	Improved nutritional quality of plants	Biotic/ Abiotic stress tolerance	References
Chlorella vulgaris cell suspension	*Vicia faba*					+	(Li et al., 2014)
Chlorella fusca	• *Cucumis sativus* • *Arabidopsis thaliana*					+	(Kim et al., 2018; Lee et al., 2020)
Chlorella vulgaris	*Cyamopsis tetragonoloba* (L.) Taub.		+		+	+	(Kusvuran and Can, 2020)
Chlorella vulgaris extract	*Lactuca sativa*	+		+	+		(La Bella et al., 2021)
Chlorella vulgaris	*Brassica oleracea* var. italica		+	+		+	(Kusvuran, 2021)
Chlorella vulgaris extract	Lactuca sativa L.		+		+		(Puglisi et al., 2022)
Scenedesmus spp. extract	*Triticum aestivum* L. var. Gemmiza 9	+			+		(Shaaban et al., 2010)
Dunaliella salina exopolysaccharides	*Solanum lycopersicum* L.	+	+	+		+	(El Arroussi et al., 2018)
Scenedesmus subspicatus	*Allium cepa L*	+			+		(Gemin et al., 2022)
• *Acutodesmus dimorphus* extract	*Solanum lycopersicum* var. *Roma*	+	+		+		(Garcia-Gonzalez and Sommerfeld, 2016)
• *Scenedesmus* spp. extract • *Arthrospira platensis* cell hydrolysate	*Petunia x hybrida*	+	+		+		(Plaza et al., 2018)
• *Nostoc muscorum SOS14* extract • *Anabaena oryzae SOS13* extract	*Lactuca sativa* L.			+	+		(Mohsen, 2016)
Extracts of microalgae consortium • *Chlorella* spp., • *Scenedesmus* spp., • *Spirulina* spp., • *Synechocystis* spp	*Solanum lycopersicum* L.	+		+	+		(Supraja et al., 2020a)
Cell lysates of • *Chlamydomonas reinhardtii* CC124 • *Chlorella* sp.MACC360	*Solanum lycopersicum L.*	+	+	+			(Gitau et al., 2022)
Polysaccharides Extract of • *Dunaliella salina* MS002 and MS067 • *Phaeodactylum tricornotum* MS023, • *Porphyridium* spp. MS081, *Desmodesmus* spp. • *Spirulina platensis* MS001	*Solanum lycopersicum* L.				+	+	(Rachidi et al., 2021)
Polysaccharide extracts of • *Chlorella vulgaris* • *Chlorella Sorokiniana*	*Solanum lycopersicum* L.		+			+	(Farid et al., 2019)

+ indicated positive effects.

enzyme showed immediate increase in its activity after FS while the root drenched samples showed delayed expression of the enzymes at 4th day indicating influence of application method on the time of physiological response (Puglisi et al., 2022). The immediate physiological response to FS as compared to root drenching applications could be attributed to the faster rate of absorption through stomata compared to root cells (Hong et al., 2021; Arahou et al., 2022). This has been earlier validated with nano-fertilizer applications of micronutrients where foliar application of minerals modulated plant growth better and faster compared to soil application (Alshaal and El-Ramady, 2017). For commercial and large scale applications, root drench and foliar applications seem to be best options of bio stimulant application. However, for faster physiological response in plants, FS of biostimulants is generally preferred. It is reiterated again that the mode of bio stimulant application is case dependent and varies with respect to the crops requirement. A summary of functions attributed with different methods of bio stimulant application is presented in Figure 2.

4 Microalgae in amelioration of abiotic stress in plants

Increasing human population and climate change exacerbated by anthropogenic activities has resulted in severe stress on the global crop production (Pereira, 2016). One of the significant impact of climate change is global warming resulting in a 0.3°C per decade mean increase in the global temperatures and a corresponding decrease in global crop productivity by -5% per °C (Lobell and Gourdji, 2012). Climate change and environmental conditions such as prolonged heat waves (higher atmospheric temperature), varying intensity of rainfall, failure of monsoons, and high CO_2 concentration negatively affect plant physiology and consequently affect crop productivity (Ferguson, 2019; Chaudhry and Sidhu, 2021). This can be attributed to the altered photosynthetic and carbon assimilation mechanisms in plants in response to climate change (Reddy et al., 2010). Some of the major impacts of climate change on plant physiology, growth and development are (i) lower germination rate, (ii) decreased photosynthetic rate, (iii) reduction in shoot biomass and root/shoot length, (iv) root/shoot ratio imbalance, (v) poor stomatal conductance, (vi) reduced number of leaves, (vii) declined chlorophyll content, (viii) lower protein content, (ix) poor membrane stability (Chaudhry and Sidhu, 2021). The common underlying mechanism behind most of the negative impacts of climate change induced abiotic stress is through generation of ROS that alter protein synthesis, induce lipid peroxidation, alter membrane permeability, inactivate enzymes and degrade nucleic acids leading to cell death (Singh et al., 2019).

Plants have developed important adaptive strategies to counter climate change-induced abiotic stress such as *de novo* synthesis and regulation of phytohormones, accumulation of osmolytes, *de novo* synthesis of heat shock proteins, and enhanced activity of enzymatic antioxidants (Chaudhry and Sidhu, 2021). Phytohormones and signaling molecules play an important role in the upregulation of the abiotic stress defence mechanisms exhibited by plants. For example, drought stress in plants leads to enhancement in ABA levels, which in turn induces stomatal closure and regulates water balance in plants (Sah et al., 2016). Another mechanism to counter drought stress and salt stress is by an accumulation of osmolytes such as proline, trehalose, sucrose and glycine betaine. Accumulation of these molecules creates a negative osmotic potential inside the cell which leads to the entry of water into cells to regulate turgidity and maintain water balance and cellular osmolarity (Sharma et al., 2019). Similarly, under high temperature and salinity stress, JA and SA promote the expression of antioxidant enzymes like superoxide dismutase, APX, and CAT that scavenge ROS (Wang et al., 2020b).

Use of microalgae and derived extracts as bio stimulant, especially towards mitigating abiotic stress has been in an upward trend in recent years. As described in earlier section, microalgae derived metabolites have wide range of functions such as signaling molecules, growth enhancers, antioxidant, natural mineral chelators that promote plant growth (Lee and Ryu, 2021). Among the various metabolites, EPS derived from both cyanobacteria and microalgae have shown plant immuno stimulatory properties along with other growth promoting activities in the rhizosphere regions such as mineral complexation, water retention etc. (Chanda et al., 2019). Application of sulfated polysaccharide extracts derived from *Dunaliella salina* enhanced the salinity tolerance in tomato and pepper plants by enhancing the antioxidant enzyme (POD, SOD, APX, CAT) activities (El Arroussi et al., 2018). In another study, polysaccharide extracts derived from *Spirulina platensis*, *Dunaliella salina*, *Porphyridium* spp., and *Phaeodacylum tricorntum* showed bio stimulatory effects when applied on tomato plants by enhancing PAL and chitinase enzyme, total polyphenol content, ROS scavenging activity and biosynthesis of very long chain fatty acids that constitute cuticular wax of leaves (Rachidi et al., 2020). Mutale-Joan et al. (2020) reported that injection of polysaccharides enriched liquid extracts of several microalgae species into tomato plants stimulated *de novo* lipid biosynthesis especially, palmitic and stearic acid involved in cuticular wax formation and linoleic acid involved in jasmonate pathway. The group further reported enhanced shoot, root and chlorophyll accumulation and correlated these observations to higher uptake of N and K in roots. Metabolomics' analysis revealed that microalgal polysaccharides triggered accumulation of pyridine-3-carboxamide, (active amide form of vitamin B3) that promotes growth in plants. In another study by the group, a combined extract of microalgae and cyanobacteria such as *Dunaliella salina, Chlorella ellipsoidea, Aphanothece* spp., *and Spirulina maxima* offered defense against salinity stress up to 150 mM NaCl in tomato plants by enhancing the activity of SOD and CAT, restoration of ion homeostasis by enhanced K^+ uptake over Na^+ ions and triggering of fatty acid degradation and conversion to alkane biosynthesis that constitute cuticular wax towards maintenance of water levels in hydric stressed plants. Further, it was observed that, application of combined microalgae-cyanobacteria extracts enhanced osmolytes accumulation, in this case proline (Mutale-Joan et al., 2020). A report by Kusvuran (2021) demonstrated that foliar application of *Chlorella vulgaris* extracts at 5% v/v strength alleviated drought stress induced oxidative stress in broccoli plants by enhancing the activities of CAT, SOD, APX, glutathione reductase and inhibiting lipid peroxidation. Further, *C. vulagris* extracts enhanced total chlorophyll, carotenoid, polyphenols and flavonoid content in plant tissues thereby promoting growth and secondary metabolite (non-enzymatic antioxidant defense response). The various abiotic stress

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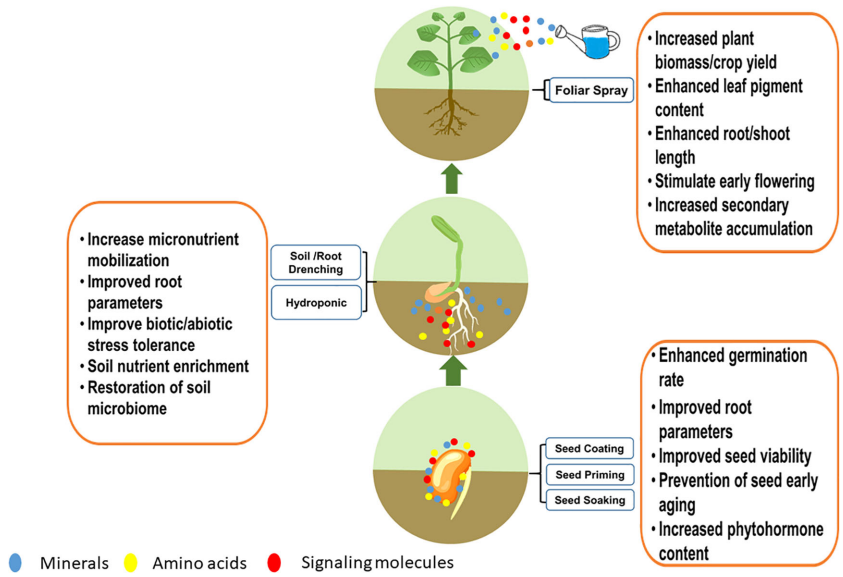


FIGURE 2
Methods of microalgae biostimulant application and their effects in plants.

tolerance imparted by microalgae derived extracts and bio stimulatory compounds are listed in Table 2 and Figure 3.

5 Microalgae as biopesticides

Biopesticides are naturally occurring organisms or biologically derived compounds that suppress the growth and proliferation of plant pathogens ranging from bacteria, fungi, insects and nematodes (Fenibo et al., 2021). Bio pesticides are broadly two types viz., microbial and biochemical; and microalgae derived bio pesticides fall under both these categories. Microalgal biopesticides act by inhibiting the growth, development and reproduction of plant pathogens or by competitively inhibiting the nutrients to the pathogen restricting their growth (Costa et al., 2019). Among the various species of algae, cyanobacteria have found significant applications as biopesticides. The most commonly used cyanobacteria are *Anabeana* spp., *Nostoc* spp., *Spirulina platensis,*

TABLE 2 Microalgae mediated abiotic stress tolerance in plants.

Microalgae/ consortia	Plant species	Abiotic factor	Observations	Mode of action	References
Dunaliella salina Phaeodactylum tricornutim	*Capsicum annuum* L.	Salinity stress [Up to 50mM NaCl]	• Increase in germination rate • Increase in shoot/root fresh weight • Resistance to salinity induced oxidative stress	• Scavenging superoxide free radicals • Inhibition of lipid peroxidation • Increased CAT and SOD activity • Ion balance and enhanced water uptake in roots	(Guzmán-Murillo et al., 2013)
Dunaliella salina Chlorella ellipsoidea Aphanothece spp., *Arthrospira maxima*	*Solanum lycopersicum* L. *Var. Jana F1* [tomato]	Salinity stress [Up to 150mM NaCl]	• Improved photosynthetic activity • Enhanced nutrient absorption • Increased shoot and root weight • Enhanced root volume • Larger leaf area • Improved osmotic adjustment	• Promotes osmolyte [proline] accumulation • Scavenging of ROS through increased CAT and SOD activity • Enhanced K^+ uptake over Na^+ • Restoration of ion homeostasis • Promoting fatty acid transformation to alkanes and cuticular wax biosynthesis	(Mutale-Joan et al., 2020)
Dunliella salina exopolysaccharides	*Solanum lycopersicum* L. *Var. Jana F1* [tomato]	Salinity stress [up to 6 gL^{-1}]	• Increase in root and shoot length • Increase in total weight of the plant • Increased water holding capacity of roots	• Enhanced protein biosynthesis • Restoration of ion homeostasis • Enhanced K^+ uptake over Na^+ ions • Enhanced osmolyte accumulations [proline, glycine-betaine] • Enhanced alkane content in leaves with	(El Arroussi et al., 2018)

(Continued)

TABLE 2 Continued

Microalgae/ consortia	Plant species	Abiotic factor	Observations	Mode of action	References
				concomitant decrease in very long chain fatty acids	
Nannochloropsis salina *Chlorella vulgaris*	*Moringa oleifera* LAM	Salinity stress [up to $6gL^{-1}$]	• Enhancement of leaf area and leaf number • Improved photosynthetic activity • Enhanced stem and root DW • Enhanced polyphenol content [rutin and gallic acid] in leaves	• Enhanced K^+ uptake over Na^+ ions • Increase in non-enzymatic antioxidant defense systems [polyphenols]	(Al Dayel and El Sherif, 2021)
Chlorella vulgaris - [applied as live culture suspensions]	*Vicia faba* L. cv. Da qing pi	Drought stress	• Improved water use efficiency • Reduction in leaf transpiration rate	• Induction of stomatal closure • Reduction in stomatal aperture through ROS mediated signaling pathway • Promotes CAT activity and scavenges H_2O_2	(Li et al., 2014)
Chlorella vulgaris [applied as foliar spray]	*Brassica oleracea* var. *italica 'Barokka'*	Drought stress	• Enhanced growth of the plants • Enhanced photosynthetic pigment content • Increased nutrient uptake	• Inhibition of lipid peroxidation • Mitigation of oxidative stress through ➢ Increased secondary metabolites like phenolic acids and flavonoids [non-enzymatic defense response] ➢ Increased antioxidant enzymes activity such as SOD, CAT, APX, GR	(Kusvuran, 2021)
Oscillatoria agardhii [Applied as nano silicon product]	Triticum spp., [Wheat]	Drought stress	• Increase in grain yield • Grain flour protein content	• Increase in the activity of hydrolytic and defense enzymes (CAT, SOD, APX)	(Haggag et al., 2018)
General biostimulant properties exhibited by microalgae					
Chlorella vulgaris *Scenedesmus quadricauda* extracts	*Beta vulgaris* L.	Biostimulant effect	Enhanced root growth characterized by • Increased root length • Increased number of root tips • Increased fine root length • Increased root surface area	• Upregulation of genes involved in biosynthetic pathway of primary and secondary metabolism • Upregulation of intracellular transport • Upregulation of nutrient acquisition genes such as inorganic phosphate transporter	(Barone et al., 2018)
Arthrospira platensis, *Dunaliella salina*, *Porphyridium* spp. [Polysaccharides extract]	*Solanum lycopersicum* L. *Var. Jana F1*	Biostimulant and growth enhancement effect	• Increased shoot length, weight and shoot nodes number • Increased photosynthetic pigments content	• Increased activity of N assimilatory enzymes - nitrate reductase & NAD-GDH activities • Enhancement of sterols/steroidal glycol-alkaloids in plants • Increased alkanes content in leaves suggesting cuticle wax formation • Enhanced protein biosynthesis	(Rachidi et al., 2020)
Chlorella vulgaris [Applied as FS]	*Vigna mungo* L.	Biostimulant and growth enhancement effect	• Increase number of root nodules • Increased root and shoot length • Increased root and shoot weight • Increased photosynthetic pigment content in plants • Increased number of pods, seeds and weight of seeds	Enhanced nutrient uptake through mineral solubilzation and mobilization into roots	(Dineshkumar et al., 2019)
Cyanobacteria - *Anabaena oryzae* *Anabaena doliolum* *Phormidium fragile* *Caothrixx geitonos*	*Oryza sativa Var UPR 1823*	Plant growth promotion and stress tolerance in rice plants	• Increased root and shoot length • Increased plant fresh weight • Increased photosynthetic	• Root colonization and N fixation • Increased antioxidant enzymes activity - peroxidase and PAL • Increased phytohormone – IAA & IBA	(Singh et al., 2011)

(Continued)

TABLE 2 Continued

Microalgae/ consortia	Plant species	Abiotic factor	Observations	Mode of action	References
Hapalosiphon intricatus *Aulosira fertilissima* *Tolypothrix tenuis* *Oscillatoria acuta* *Plectonema boryanum* [Applied as Live cultures]			pigment content • Increased metabolite content in leaves [phenolics]	concentration in leaves promoting plant growth	

ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase; APX, Ascorbate Peroxidase; CAT, Catalase; GR, Glutathione Reductase; NAD-GDH, nicotinamide adenine dinucleotide glutamate dehydrogenase; PAL, Phenylalanine Ammonia Lyase; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid.

Oscillatoria spp., *and Tolypothrix* spp., (Costa et al., 2019; Hernández-Fernández et al., 2021). Along with these cyanobacteria, chlorophycean microalgae such as *Chlorella vulgaris*, *Chlorella fusca, Scendesmus* spp., have been attributed with biopesticide activity (Bileva, 2013).

5.1 Anti-microbial properties of cyanobacteria and microalgae

Cyanobacteria are known to secrete EPS and anti-microbial compounds that inhibit the growth of plant pathogens. *Nostoc* spp., *Nodularia harveyana*, produce cyclic compounds such as 4,4' –dihydroxybiphenyl, norharmane and diterpenoids that show anti-bacterial activity (Volk and Furkert, 2006). Norharmane is indole alkaloids mostly secreted by cyanobacterial species belonging to the order Nostacales that have mutagenic properties and inhibit key metabolic enzymes such as monoamine oxidase, indoleamine 2,3-dioxygenase, nitric oxide synthase (Volk, 2008). These antimicrobials were effective against several fungal pathogens. For example, Abdel-Hafez et al. (2015) reported the fungicidal activity of extracts obtained from *Nostoc muscorum* and *Oscillatoria* against *Alternaria porri* that causes a purple blotch of onion. The group analyzed the fungicidal extracts revealing the presence of inhibitory compounds such as β-ionone, norharmane, and α-isomethyl ionone. Along with these, a variety of antimicrobial compounds such as nostocyclyne A, nostocin A, ambigol A and B, hapalindoles and scytophycins, tjipanazoles, fischerellin-A have been identified and applied as biocontrol agents obtained from cyanobacteria such as *Nostoc* spp., *Scytonema* spp., *Tolypothrix* spp., *Fischerella* spp. respectively (Singh et al., 2016). These antimicrobials inhibit protein synthesis, enzymatic functions and cell division in pathogens. In another mechanism, some anti-microbial compounds especially siderophore type molecules competitively bind to nutrients such as Fe, Cu and deprive the nutrients to pathogens as observed in the case of biocidal activities of *Pseudomonas* spp., (Lee and Ryu, 2021). Further, cyanobacteria such as *Anabaena laxa*, *Anabaena variabilis*, *Nostoc* spp., *Calothrix elenkinii* produce hydrolytic enzymes, mainly cell wall-degrading enzymes such as chitosanase, β-1,4-glucanase, and β-1,3-glucanase that hydrolyse the cell wall of pathogens such as *Pythium*

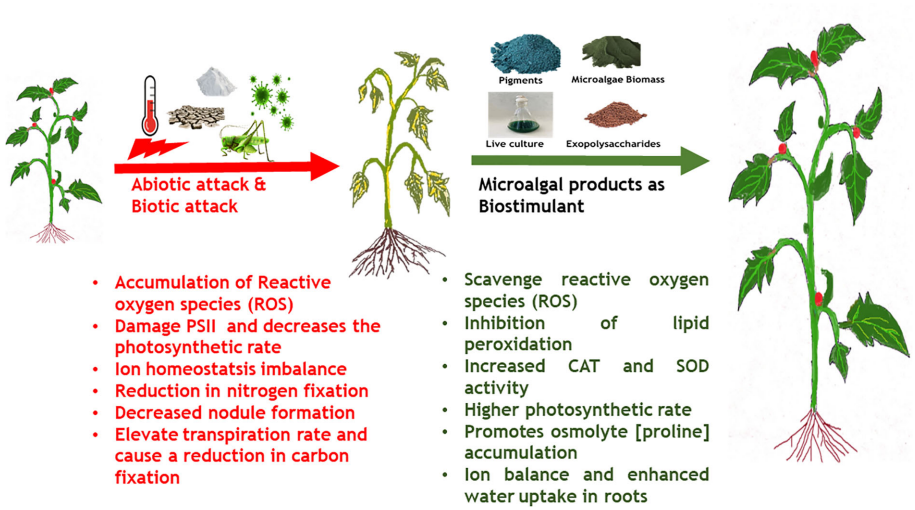


FIGURE 3
Effects of microalgae bio stimulants in biotic and abiotic stress tolerance in plants.

aphanidermatum (Prasanna et al., 2008; Gupta et al., 2011; Natarajan et al., 2013). Apart from directly acting on fungal pathogens, microalgal and cyanobacterial extracts enhance plant defence mechanisms by enhancing the activity of hydrolytic enzymes (endochitinases such as β-N-acetylhexosamindase, chitin-1,4-β-chitobiosidase, Endoglucanase – β-1,3 glucanase), polyphenol oxidase and POD (Roberti et al., 2015). Further, cyanobacterial extracts enhance the activity of PAL enzyme that catalyzes phenylpropanoid pathway involved in biosynthesis of SA attributed to systemic resistance to infections in plants and phenolics and phytoalexins (Singh et al., 2011; Babu et al., 2015). Application of cyanobacterial biomass and cell-free extracts to the soil resulted in reduction in disease symptoms. For example, Chaudhary et al. (2012) and Prasanna et al. (2013) reported that biopesticide formulation of *Anabaena* spp., led to a 10% to 15% reduction in damping-off disease in tomato seedlings and significant increase in plant growth when challenged with pathogenic fungi. Similarly, the application of *Spirulina platensis* resulted in a reduction of root rot, root wilt and damping off symptoms in Moringa plant (Imara et al., 2021).

5.2 Insecticidal properties of cyanobacteria and microalgae

In addition to the antimicrobial properties, microalgae possess insecticidal properties, specifically nematicidal properties owing to the secretion of neurotoxins such as anatoxin-A, microcystin, nodularins inhibiting the growth of nematode pests. Anatoxin-A, mimics neurotransmitter acetylcholine and bind irreversibly to acetylcholine receptors leading to continuous muscle contraction in nematode pests leading to their immobility (Mankiewicz et al., 2003). Number of cyanobacterial species such as *Oscillatoria chlorina*, *Aulosira fertilissima, Spirulina platensis, Amphora cofeaeformis* have been reported to exhibit nematicidal properties against soil borne nematodes such as root knot nematode (*Meloidogyne arenaria, Meloidogyne incognita), Xiphinema index* that affect the root formation (Khan et al., 2007; Chandel, 2009; Bileva, 2013; El-Eslamboly et al., 2019). Root knot nematodes forms gall like structure in the roots leading to hardening of root surface ultimately affecting nutrient uptake in plants. Soil application of cyanobacteria and microalgae such as *Chlorella vulgaris* inhibited the nematode growth and reduced the gall formation in roots and promoted root and shoot growth in plants (Khan et al., 2007). Microalgae derived secondary metabolites such as hexamethyl phenol, methoxyphenyl and flavonoids were shown to inhibit nematode growth and reduction of gall formation in roots of *Cucumis sativus* plants and concomitant enhancement in fruit yield and quality (El-Eslamboly et al., 2019). Additionally, cyanobacteria such as *Anabaena* spp., and *Scytonema* spp., produce peptide toxins that act as repellents to insect pests. (Sathiyamoorthy and Shanmugasundaram, 1996) reported presence of low molecular weight (<12 kDa) peptides in *Scytonema* spp., that have strong odour repelling cotton leaf chewing insects such as *Helicoverpa armigera* and *Stylepta derogate*. Similarly, Abdel-Rahim and Hamed (2013) reported insecticidal properties of extracts obtained from *Anabaena flos aquae* against *Spodoptera littoralis* larvae (Cotton leaf worm) that destroys cotton crops. The authors reported that the cyanobacterial extracts suppressed the oviposition in the insects and caused sterility in insects. A list of bio pesticide and crop protection properties of cyanobacteria and microalgae is presented in Table 3 and the mode of action is presented in Figure 3.

Review of literature clearly indicates that microalgae and cyanobacteria have excellent plant growth promoting properties. However, their utilization in large scale has not seen the commercial light. Some of the important challenges associated with commercial utilization of microalgae in agro-chemicals sector are high cost of cultivation, varying biomass productivities, high cost and energy intensive downstream processes in obtaining purified metabolites demonstrating plant growth promoting applications (Sharma et al., 2013; Smetana et al., 2017). This warrants identification of processes and technologies that reduce the cost of biomass production and improve energy efficiency in downstream processes. Following section describes the various steps involved in microalgae biomass generation and discuss strategies for low cost biomass production and multi product utilization to achieve environmental sustainability along with economic viability.

6 Production of microalgae based bio fertilizers and biostimulants

6.1 Microalgae biomass production

Microalgae have been considered as a potential industrial source of food, chemicals and bio-products owing to their higher photosynthetic efficiency and high unit dry matter per yield/land area in comparison to land plants (Chen et al., 2022). The presence of myriad of chemical compounds comes from their ability to adapt to wide environmental conditions and adopt different modes of nutrition such as autotrophic (completely photosynthetic), mixotrophic (ability to utilize both reduced organic carbon and light sources) and heterotrophic (fermentative approach with utilization of organic carbon source) (Udayan et al., 2022). Microalgae biomass is the functional material and the primary basis for biofertilizer or biostimulants production. Systematic evaluation of biomass production processes and understanding their suitability is very critical in mass production of biofertilizers and agro-chemicals. A typical microalgae biomass production or derived products involves three major steps, *viz*., (i) cultivation, (ii) harvesting and (iii) downstream processing consisting dewatering, extraction, and purification or formulation. The production strategies vary with respect to intended application such as for food, feed, fuel or agriculture applications. Microalgae are generally mass cultivated either in open raceway ponds (ORP) or closed PBRs utilizing the phototrophic mode of nutrition exploiting solar energy, carbon dioxide and inorganic minerals. Amongst the two systems, ORP are commercially preferred over PBRs owing to their low capital and operational costs while PBRs are preferred for accurate control of culture parameters and production of high value products such as proteins, pigments (carotenoids and xanthophylls), and

TABLE 3 Biotic stress tolerances imparted by microalgae.

Microalgae/consortia	Host plant species	Biopesticide activity	Mode of action	References
Anti-fungal activity				
Nostoc strain ATCC 53789	*Solanum lycopersicum* L.	• Fungicidal activity against *Sclerotinia sclerotiorum*, • Concentration dependent fungicidal activity • Enhanced root and shoot length	High adventitious root formation increasing nutrient uptake in the plants	(Biondi et al., 2004)
Anabaena, Oscillatoria, Nostoc, Nodularia and *Calothrix* species	NR	Fungicidal activity against *Alternaria alternate, Botrysis cinerea, Rhizopus stolonifer* *Phytopthora capsici*, fusarium oxysporium, *Colletotrichum gleosporoides*	• Reduced fungal colonization in plant tissues • Enhancement of plant defense mechanisms mainly activity of hydrolytic enzymes - PPO, chitinase, and PAL • Contains depsipeptide named cryptophycin 1 which promotes antiproliferative and anti-mitotic activity • Glycosylated lipopeptides – hassallidins	(Kim et al., 2006; Kim and Kim, 2008; Vestola et al., 2013)
Nostoc commune FA-103	*Solanum lycopersicum* L.	• Fungicidal activity against f. sp. *Lycopersici* • Minimum inhibitory concentration – 150 µg per seed • Reduction in number of infected seedlings • Increase in root, stem length • Increase in number of hairy roots		
Anabaena variabilis RPAN59 and *Anabaena oscillarioides* RPAN69	*Solanum lycopersicum* L	• Fungicidal activity against *Pythium debaryanum, Fusarium oxysporum lycopersici, Fusarium moniliforme and Rhizoctonia solani* • Increase in the number of leaves • Enhancement of plant height and weight • Reduction in disease severity	Fungicidal activity due to presence of hydrolytic activity of cyanobacterial extracts	(Chaudhary et al., 2012)
Anabaena laxa and *Calothrix elenkinii*	*Coriandrum sativum* L. *Var*. NRCSS-ACr1, *Cuminum cyminum* L. Var. RZ-209, *Foeniculum vulgare* Mill. *Var*. NRCSS-AF-1	• Fungicidal activity against *Fusarium oxysporum* ITCC 95 and *Macrophomina phaseolina* ITCC 5141 • Enhancement in germination rate and soil chlorophyll • Increase in root and shoot length • Improvement in the vigour index of seed	• Enhancement of plant defense mechanisms mainly activity of hydrolytic enzymes - PPO, chitinase, • Increased activity of endogluconase and peroxidase in plants	(Kumar et al., 2013)
Nostoc muscorum and *Oscillatoria* spp.	*Allium cepa* L.	• Fungicidal activity against *Alternaria porri* that causes Purple blotch of Onion • Disease reduction by 55.1% – 66.5%	• Phenolic acids, alkaloids derived from cyanobacterial extracellular filtrates induce fungicidal activity • Major fungicidal compounds include - β-ionone, norharmane, and α-isomethyl ionone	(Abdel-Hafez et al., 2015)
Consortia of cyanobacteria [*Calothrix elenkinii* and *Anabaena laxa* RPAN8] and *Trichoderma* spp.,	*Gossypium hirsutum* F1861 and Gossypium arboreum CISA 310	• Fungicidal activity against Rhizoctonia spp. • Root colonization by cyanobacteria • Increased N fixation • Enhancement of soil N and P • Increased fresh and DW of plant	• Enhanced defense enzyme activity such as of β-1,3 endoglucanase, β-1,4 endoglucanase and chitosanase in plants • Enhanced activity of stress defense enzymes such as peroxidase and PAL	(Babu et al., 2015)
Anabaena spp., BEA0300B strain	*Cucurbita pepo* [Zucchini plants cv. Consul]	Fungicidal activity against *Podosphaera xanthii* (Castagne) U. Braun and Shishkoff 25.4% reduction of the infected leaf area	Fungicidal activity attributed to increased activity of ➢ Endochitinases such as β-N acetylhexosamindase, chitin-1,4-β-chitobiosidase, ➢ Endoglucanase – β-1,3 glucanase and peroxidase	(Roberti et al., 2015)
Phycobiliproteins from *Spirulina platensis* and *Hydropuntia cornea*	*Lycopersicum esculentum* L	• Fungicidal activity against *Botrytis cinerea* • Inhibited mycelial growth and spore germination • Reduced fruit disease incidence	• Antioxidant property of phycobilins attributed to fungicidal activity	(Righini et al., 2020)

(Continued)

TABLE 3 Continued

Microalgae/consortia	Host plant species	Biopesticide activity	Mode of action	References
Spirulina platensis	*Moringa oleifera* LAM	• Fungicidal activity against *Sclerotium rolfsii*, *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani* • Controlling Damping-off, Root Rot and Wilt Diseases • Increased shoot weight and growth of plant • Enriched nutrients of Moringa leaf	NR	(Imara et al., 2021)
Insecticidal activity				
Anabaena cyliindrica, Anabaena oyzae, Nostoc muscorum, Tolypothrix tenuis	*Oryzae sativa* var. Indica IR 28 *[short duration]*	• Insecticidal activity against *Chilo agamemnon* [stem borer] and *Hydrellia prosternalis* [leaf miner] • Increased grain yield and weight • Reduction in use of chemical N fertilizer	NR	(Yanni and Abdallah, 1990)
Anabaena flos aqua	*In vitro* studies	• Insecticidal activity against *Spodoptera littoralis* (Boisd.) larvae [Cotton leaf worm] • Reduction in inset pupation • Reduction in fertility of eggs	• Cyanobacterial extracts suppressed oviposition of the adults • Induced sterility and reduced fecundity in insects	(Abdel-Rahim and Hamed, 2013)
Oscillatoria chlorina [Cyanobacterial biomass applied to the soil]	*Lycopersicum esculentum* L	• Nematicidal activity against *Meloidogyne arenaria* [root knot nematode] • Reducing the number of galls in the root • Increased vegetative growth and root mass production	• Potential nematicidal activity due to presence of cyanobacterial neurotoxins – Anatoxin-a • Additionally, secondary metabolites such as polyketides, cyclic peptides, lipopeptides induce toxicity in nematodes	(Khan et al., 2007)
Chlorella vulgaris	*Vitis vinifera* grapevine cv. Palieri	Nematicidal activity against root ectoparasite *Xiphinemia index* Increase in root and shoot length Enhancement of fresh and dry weight of plant	NR	(Bileva, 2013)
Spirulina platensis *Amphora cofeaeformis*	*Cucumis sativus* L, *Hesham hybrid F1*	• Nematicidal activity against *Meloidogyne incognita* [root knot nematode] • Reduction in the egg production • Reducing the number of galls in the root • Increased host plant vegetative growth, fruit quality and yield	Microalgae derived secondary metabolites such as asetamide and phenolic compounds such as hexamethyl phenol, methoxyphenyl phenolic acids, flavonoids act as nematicides	(El-Eslamboly et al., 2019)
Aulosira fertilissima	*In vitro* studies	• Nematicidal activity against Meloidogyne triticoryzae and Meloidogyne incognita • Inhibition of the hatching of root knot nematode	Extracellular secretions such as polysaccharides induce nematicidal activity	(Chandel, 2009)

NR, Not reported; PPO, Polyphenol Oxidase; PAL, Phenylalanine Ammonia Lyase.

polyunsaturated fatty acids (Udayan et al., 2022). The choice of cultivation depends on the species/strains and further downstream processes selected. Although ORP is widely preferred, major disadvantages include contamination of culture, low biomass productivity and susceptibility to predators and environmental conditions (Tan et al., 2020). Photo bioreactors offer better control the over the cultures with high biomass productivity, reduced contamination risks and higher metabolite yield; however, the major disadvantages are high capital investment, higher energy requirements in circulating culture and maintenance of temperature and risk of bio fouling and increased dissolved oxygen in cultures leading to cell death (Tan et al., 2020; Udayan et al., 2021; Udayan et al., 2022).

The second important step in the biomass production process is harvesting, which involves the separation of microalgae cells from the growth medium and the recycling of the spent medium. A typical microalgae culture has asolids content of less than 1%, (approximately 0.6 to 1 g biomass per litre culture) and needs to be concentrated between 15% and 25% solids content for further downstream processing (Milledge and Heaven, 2013). Several processes such as chemical -induced flocculation, bio-flocculation (coagulation induced by bacterial/fungal species), electrocoagulation, flotation, and membrane filtration have been developed and evaluated for harvesting microalgae biomass (Milledge and Heaven, 2013; Vandamme et al., 2013; Barros et al., 2015; Udayan et al., 2022). Despite the availability of enormous literature on the harvesting of

microalgae, it is a general consensus that harvesting is the major bottleneck in the commercial production of microalgae biomass. This could be attributed to the wide range of cellular properties such as size, shape, the surface charge of microalgae cells necessitating customized solutions limiting commercial scalability (Kumar et al., 2022a). However, commercially a combination of flocculation followed by pressure filtration or centrifugation is considered economically viable with lower energy inputs (less than 0.1 $kWh{\cdot}kg^{-1}$ algae) (Fasaei et al., 2018). The success of a harvesting process is entirely dependent on the cultivation step where the cell density of the culture determines the energy expenditure incurred during harvesting. Cultivation in ORP is generally performed at low cell densities to avoid the shading effect and consequent dark fermentation at the bottom of the ponds (Shekh et al., 2022). This necessitates the deployment of multiple harvesting steps such as two-stage process of initial concentration to 2% to 3% solids content followed by dewatering to 15% solids content (Milledge and Heaven, 2013). However, this problem can be avoided with microalgae cultivation in flat plate PBRs at higher cell density reducing the energy and cost requirements during the harvesting stage (Milledge and Heaven, 2013).

The final step involved in typical microalgae biomass production is dewatering and downstream processing for further production of commercial products. Dehydration is the most energy-intensive process and has been a major area of concern and a limiting factor in the commercialization of microalgae products. The choice of dehydration is dependent on the end product application and earlier reports indicate that convective air drying of harvested biomass paste is the most economical option for large-scale production (Sirohi et al., 2022). However, it has been observed that heating of biomass beyond 70°C resulted in the loss of heat-labile compounds such as phycobiliproteins, antioxidants and secondary metabolites (Phong et al., 2017). For the biofertilizer application, mainly as a source of macro and micronutrients, the dewatering process does not influence biomass stability. However, for use as biostimulants such as phytohormone-rich extracts or pesticidal extracts, dehydration plays a crucial role as many of these compounds are heat sensitive necessitating optimization of dewatering and further downstream processes.

6.2 Life cycle analysis and sustainability of microalgae biomass production

In order to select a particular cultivation, harvesting and downstream strategy, a thorough evaluation of sustainability aspects involved in various stages of biomass production is required. For commodity products such as food, animal feed, biofuel or biofertilizers the choice of cultivation method must be cheaper with minimal energy expenditure. However, the choice of process varies between species/strains necessitating a detailed sustainability analysis. This study is known as life cycle analysis (LCA). A typical LCA involves assessment of process against six parameters namely global warming potential expressed as g CO_2 eq. per kg biomass, acidification potential expressed as g SO_2 eq. per kg biomass, eutrophication potential expressed as g PO_4^- eq. per kg biomass, cumulative energy demand expressed as MJ per kg biomass, land use expressed as m^2 per kg biomass and water usage expressed as L per kg biomass. These parameters could also be tested against other functional units that are potentially generated from microalgae biomass such as energy produced (in Kcal) in case of biofuels, or proteins/polysaccharides/pigments/or metabolite of interest (expressed in grams/kg biomass) (Kumar et al., 2022a).

Life cycle analysis of microalgae based products have been studied for various scenarios such as biofuels (Huang et al., 2022; Kim et al., 2022), food and nutraceuticals (Schade and Meier, 2020; Schade et al., 2020), multiproduct refineries (Arashiro et al., 2022; Ubando et al., 2022) biofertilizers (de Siqueira Castro et al., 2020; Arashiro et al., 2022). The major consensus among most of these studies is that the cultivation of microalgae in ORP is cheaper and less energy intensive compared to PBRs. However, the biomass productivity is roughly 12 times higher with PBRs (1.5 kg dry biomass m^{-3} day^{-1}) compared to ORP (0.117 kg dry biomass m^{-3} day^{-1}) (Silva et al., 2015). The lower sustainability quotient with respect to PBRs based cultivation could be attributed to the high capital costs involved in construction of PBRs which contribute to almost 85% of the total energy consumed in the process (Silva et al., 2015). Calculation of net energy returns, a simple ratio of energy produced over energy consumed for various cultivation steps indicated that biomass production using horizontal tubular PBRs had NER <1 while biomass produced with ORP or flat plate PBRs had NER >1, suggesting commercial feasibility of ORP based biomass production (Jorquera et al., 2010).

Energy required for heating cultures in ORP and the energy required for injecting CO_2 and maintaining the flow of cultures in PBRs and supply of reduced organic carbon sources in mixotrophic or heterotrophic cultivation are some critical factors that negatively influence microalgae based processes (Ighalo et al., 2022). Microalgae based processes had relatively lower land and water use compared to aquaculture processes, however, in comparison to poultry and insect farming the water and nutrient requirements were relatively higher for microalgae biomass production (Maiolo et al., 2020; Schade et al., 2020). The overall energy requirements for production of one metric ton (1 MT) of microalgae biomass was estimated between 12000 and 22000 kWh, which was 4 to 7 folds higher compared to poultry or insect meal based protein production (Maiolo et al., 2020). The capital and operational inputs contributing to the energy consumption as part of the life cycle assessment during microalgae cultivation and biomass processing is presented in Figure 4.

Water and nutrient requirements are the two critical parameters that determine the economics of microalgae biomass production. For example, the estimated nutrient requirement (inorganic salts) for the production of 1 MT of fresh biomass of microalga *Tetraselmis suecia* was 90 kg and the water requirement was approximately 15000 m^3 per m^2 per month, which is 10 times higher compared to other poultry and insect meal production (Maiolo et al., 2020). This high water requirement makes microalgae biomass-based products and processes unsustainable, necessitating the identification of cheaper sources of water and nutrients. Wastewater obtained from industrial processes could be a potential source of nutrient-rich water for the cultivation of microalgae (Dagnaisser et al., 2022). The concept of utilization of industrial wastewater for the cultivation of microalgae has been practised for quite some time now with several success stories and enormous literature (Carraro et al., 2022; Nagarajan et al., 2022; Satya et al., 2022; Yan et al., 2022). Microalgae have the unique ability to grow

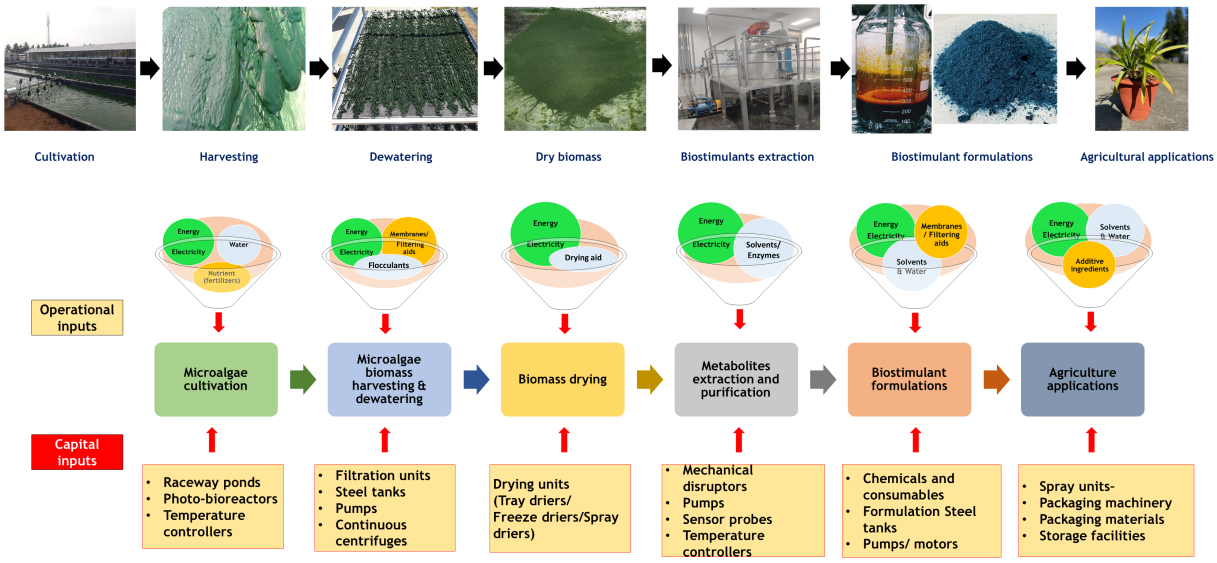


FIGURE 4
Energy inputs involved in microalgae biomass production towards biostimulants application.

in non-potable waters and have excellent bioremediation potential with high removal efficiencies against nitrates, phosphates, and ammonia in waste streams and reduce the chemical (COD) and biological oxygen demand (BOD) of wastewaters (Ahmed et al., 2022; Díaz et al., 2022). Further, the ability to use industrial flue gas as a source of CO_2 and the presence of carbon concentration mechanism in microalgae make them an attractive resource for dual applications of bioremediation and metabolite/biomass production (Çakirsoy et al., 2022; Ighalo et al., 2022; Ma et al., 2022).

6.3 Dual application of bioremediation and biofertilizer application

The dual application of bioremediation and microalgae biomass production has been successfully exploited previously for biofuel production (Ali et al., 2022; Das et al., 2022). The concept has helped to improve the economics of biomass production with microalgae having the ability to grow in different types of industrial wastewater streams such as that of steel slag, cement, food processing, distillery, aquaculture, leather tannery and anaerobic digestates (You et al., 2021; Rambabu et al., 2022; Venugopal, 2022). As discussed earlier, microalgae consistently showed a nutrient removal efficiency against nitrates, phosphates, organic carbon, ammonia and other nutrients above 70%, and in a few cases above 90% from the initial levels (Abdelfattah et al., 2022; Nagarajan et al., 2022). The ability of microalgae to absorb nutrients in abundance could be attributed to the special physiological mechanisms such as carbon concentration mechanism, lipid remodeling and P allocation in fast-growing microalgae as described earlier (Çakirsoy et al., 2022). Microalgae prefer ammonia over inorganic N sources as their assimilation is less energy intensive and can be directly incorporated into AAs and other N moieties (Sarma et al., 2021). This provides an opportunity for ammonia-rich wastewaters such as agriculture run- offs, agro-industrial residues and anaerobic digestates to act as an excellent N sources in microalgae cultivation (Wang et al., 2018).

A typical microalgae biomass contains relatively higher nutrient content compared to other organic fertilizers, especially N and P. The average N content ranges between 4.9% and 7.1% N while the P content ranges from 1.5% to 2.1% (Khan et al., 2019). Further with the presence of a luxury uptake mechanism, microalgae accumulate nutrients from the surrounding medium and act as a concentrated source of nutrients for plant growth. The use of wastewater-derived microalgae biomass as biofertilizers and source of biostimulants has been a rising trend with several case studies being published in recent years (Álvarez-González et al., 2022; Arashiro et al., 2022; Dagnaisser et al., 2022). Table 4 lists various case studies available on the applications of wastewater-generated biomass as biofertilizer. Domestic and municipal wastewater has been predominantly used as a source of nutrients for microalgae biomass production (Table 4). However, the major disadvantage of this is the varying levels of nutrients as frequent nutrient composition changes affect biomass productivity. Among the various wastewater resources evaluated, food industry effluents are promising for the recovery of various algae-derived bioproducts owing to their better quality compared to other effluents (Arashiro et al., 2022). Further, the group reported that microalgae biomass production utilizing wastewater significantly reduced the energy expenditure and environmental impact thus enhancing the sustainability of the whole process. Further integration of industrial gases, specifically CO_2-rich flue gases along with wastewater utilization enhances biomass productivity and bioproducts recovery (Yadav et al., 2019; Carraro et al., 2022).

6.4 Challenges associated with wastewater algae as biofertilizers

Although microalgae show prolific growth and efficiently remediate nutrients in waste effluents, the process of utilizing

wastewater treatment derived biomass for agriculture applications has certain inherent challenges. These include scalability of biomass production, presence of xenobiotic residues and heavy metals in biomass and contamination of cultures with bacteria, fungi and virus limiting their widespread application (Abdelfattah et al., 2022; Amin et al., 2022). The first critical challenge in wastewater mediated biomass production is meeting the light requirement for cultures. The industrial effluents derived from food, livestock, aquaculture processing and sewage have high suspended solids, reducing the light penetration into the growth medium consequently affecting photosynthetic rate and biomass productivity (Abdelfattah et al., 2022). This light penetration issue is a major challenge in scalability of wastewater mediated large scale biomass production. The average biomass yield obtained utilizing wastewater ranged between 0.7 g L^{-1} to 1.4 g L^{-1} with microalgae such as *Scendesmsus* spp., *Chlorella* spp., *Desmodesmus* spp., *Tetradesmus* spp., *Selenastrum* spp., predominantly effective in effluent treatment (Rani et al., 2021; Amin et al., 2022). Further the biomass productivity significantly varies with respect to the temperature, pH, effluent nature and nutrient content (Rani et al., 2021). A "one shoe size fits all" approach cannot be applied with respect to biomass production utilizing industrial effluents, thus requiring a case specific standardization and cultivation strategy. The second critical issue is harvesting and dewatering of microalgae from effluent treatment ponds and reactors. As discussed earlier the biomass concentration is very low in large scale algae cultures requiring energy intensive dewatering steps.

Apart from scalability, microalgae cultivated utilizing dye and tannery effluents, and pharmaceutical wastes pose the challenge of presence of xenobiotic residues and heavy metals in the biomass. Microalgae have the ability to degrade organic hydrocarbons such as phenols, azo dyes, and benzene compounds such as naphthalene, antibiotics and hormones residues present in wastewater (Xiong et al., 2021; Touliabah et al., 2022). The mechanisms behind the degradation of organic compounds by microalgae include (i) secretion of extracellular polymeric substances such as polysaccharides, proteins and humic acid that degrade organic molecules, (ii) bioaccumulation of organic compounds followed by intracellular biotransformation through redox reactions, hydrolysis and conjugation with macromolecules and (iii) photolysis (Xiong et al., 2021). A range of antibiotics belonging to classes such as tetracyclines, cephalosporins, macrolides, penicillins, and beta-lactams have been degraded using microalgae with varied efficiency (Chandel et al., 2022; Wang et al., 2022). Similarly, microalgae remove heavy metals from effluents through the phenomenon of bio adsorption and bioaccumulation followed by chelation and vacuolar sequestration or biotransformation utilizing special intracellular chelating agents called 'phytochelatins' (Chugh et al., 2022). Although the ability of microalgae to remediate xenobiotics, heavy metals have been demonstrated, there are no pilot scale studies involved on the additive toxicity of microalgae biomass enriched with a heavy metal or a bio transformed molecules. On the other hand, high concentrations of heavy metals or xenobiotics, colored dyes have shown to induce oxidative stress in microalgae causing cell death (Bhatt et al., 2022). This defeats the whole purpose of microalgae mediated bioremediation. Further the coexistence of fungi, bacteria and viruses in the waste effluents pose another challenge in utilization of microalgae biomass. Microalgae and other microbes have been known to interact both mutually and competitively in a polluted environment such as industry effluents and sewage. The interactions have been supported through nutritional reciprocity, chemotaxis, communication through volatile organic compounds and signal transduction between microalgae and microbiome present in the wastewater environment (Ashraf et al., 2022; Astafyeva et al., 2022; Chegukrishnamurthi et al., 2022).

In addition to heavy metals and xenobiotics, co-occurrence of virulent microbes along with microalgae biomass and their consequent application as biofertilizers poses the risk of introduction of virulence into the ecosystem. Further occurrence of horizontal gene transfers between micro biome and microalgae in polluted waters have been demonstrated offering special adaptive mechanisms to microalgae such as tolerance to heavy metals and impart extremophilic properties (You et al., 2021). Though HGTs offer niche adaptive benefits to algae, it poses the risk of transfer of antibiotic resistance genes, disrupting existing transcription mechanisms of biotransformation thereby affecting the bioremediation process and consequently the biomass application as biofertilizer. The major drawback in deployment of microalgae biomass produced from wastewaters and effluents for biofertilizer application is the lack of pilot scale/industrial scale trials and understand the real time effect of environment on microalgae biomass production and its impact on the biomass productivity. Most of the published literature are based on lab scale trials that do not truly reflect real time scenarios necessitating systematic studies. Nevertheless, the concept of biomass generation through bioremediation of wastewater and industrial effluents offers an exciting opportunity in increasing the sustainability of microalgae biomass production; since microalgae biomass is the primary basis of supplying nutrients and stimulants for plant growth.

7 Microalgae based bio refineries and circular economy for sustainable agriculture

It has been advocated recently that the sustainability and commercial feasibility of the microalgae-mediated technologies come only from multiproduct utilization, *i.e*, through a biorefinery approach (Mehariya et al., 2021). Although means for producing microalgae biomass at low costs, utilizing wastewater and CO_2-rich flue gas have been worked out, the high cost of downstream processing creates a net negative energy balance making the process unfeasible for commodity applications such as that of animal feed, agrochemicals and industrial chemicals (fuels) (Subhash et al., 2022). With newer findings on the potential of microalgae biomass and its metabolites for agricultural applications, it is imperative to identify sustainable biomass utilization routes for commercial feasibility. In this context, a biorefinery approach of complete valorization of microalgae biomass coupled with a circular economy route of reuse, recycling and refurbishing with minimal energy footprint could be effective in achieving the commercial feasibility of microalgae-derived agrochemicals (Geissdoerfer et al., 2017; Behera et al., 2021). In the case of microalgae-based circular economy, a typical model would be integrating wastewater treatment coupled with biomass production followed by downstream processing of the biomass for value-added products and utilizing the residual biomass obtained thereafter for food, feed, agriculture or bioenergy applications (Kholssi et al., 2021).

TABLE 4 Wastewater mediated microalgae biomass production towards biofertilizer and biostimulant applications.

Wastewater source	Microalgae used	Nutrient removal efficiency	Method of Application	Crop(s) evaluated	Observation	References
Domestic waste water	*Chlorella* spp.	Selenium (Sodium selenite) - 44% TSS - 86% COD – 70% TC - 67% TP - 77% NH_4^+ - 93%	• Foliar spray • Soil drenching • Seed treatment	*Phaseolus vulgaris*	• Enhanced germination rate • Selenium enrichment in seeds and leaves [bio-fortification]	(Li et al., 2021)
Domestic waste water	Consortia of *Chlorella* spp., *and Scenedesmus* spp.	Nitrates - 96% NH_4^+ - 98% $PO4^{3-}$ – 95% COD- 83% TOC - 86% TN - 94%	Deoiled algal biomass as biofertlizer	*Solanum lycopersicum*	• Enhanced shoot and root weight • Enhanced macro [N, P, K] and micronutrients [Ca, Mg, Fe] in biomass • Increased tomato yields	(Silambarasan et al., 2021a)
Aquaculture wastewater	*Spirulina platensis*	Nitrates – 50% NH_4^+ - 95%	• Soil drenching [admixtures of soil and dry biomass] • Seed soaking	• *Eruca sativa* • *Ameranthus gangeticus*, • *Brassica rapa ssp. Chinensis*	• Enhanced germination rate • Increased seedling vigor • Enhanced plant height and root length • Increased biomass yield • Increased chlorophyll content	(Wuang et al., 2016)
Domestic waste water	*Scenedesmus* spp.	COD- 69% TIN - 91% TP - 81% NH_4^+ - 95%	Soil drenching [admixtures of microalga biomass paste and substrate]	*Ocimum basilicum L.*	• Enhanced leaf weight • Increased magnesium content in leaves • Overall performance similar to inorganic fertilizers	(Álvarez-González et al., 2022)
Sewage waste water	*Chlorella minutissima*	TDS - 96% TP - 70% K - 45% NH_4^+ - 90% BOD - >90% COD – 80% Nitrates – 89%	Soil drenching [admixtures of dry microalga biomass and soil]	• *Spinacia oleraceae* • *Zea mays*	• Enhanced available N and organic carbon in soil • Enhanced biomass and root yield • Increased soil enzymes activity [urease, nitrate reductase & dehydrogenase]	(Sharma et al., 2021)
Brewery effluents	*Scenedesmus obliquus*	COD - 71% TN - 88% TP - 30% NH_4^+ - 81%	• Algal cell suspension • Deoiled biomass fertilizers	• *Triticum aestivum* • *Hordeum vulgare*	Increased the germination and sprouting	(Ferreira et al., 2019)
Piggery waste water	• *Tetradesmus obliquus* • *Chlorella protothecoides* • *Chlorella vulgaris* • *Synechocystis* spp., • *Neochloris oleoabundans* • *Nostoc* spp.,	COD - 62-79% NH_4^+ - 79-92% PO_4^{3-} - 90 -98%	Seed treatments with algae biomass	• *Solanum lycopersicum* • *Cucumis sativus* • *Hordeum vulgare* • *Glycine max* • *Nasturium officinale* • *Triticum aestivum*	• Increased germination index • Increased root length in wheat and cucumber • Biopesticidal activity against *Fusarium oxysporum*	(Ferreira et al., 2021)
Dairy effluent	*Chlorella pyrenoidosa*	BOD - 88% COD - 85% $NH4^+$ - 99% $PO4^{3-}$ – - 97%	Encapsulated algal biomass as biofertilizers	*Oryza sativa*	• Enhanced the length of root and shoot in rice seedlings	(Yadavalli and Heggers, 2013)
Paddy soaked rice mill waste water	*Chlorella pyrenoidosa*	$NH4^+$ - 69.4% $PO4^{3-}$ – 64.7%	• Seed treatment with microalgae cell suspensions • Soil drenching with live algae biomass	*Abelmoschus angulosus*	• Increased germination percentage • Increase number of leaves • Increased plant height	(Umamaheswari and Shanthakumar, 2021)

TSS, Total Suspended Solids; TDS, Total Dissolved Solids; TOC, Total Organic Carbon; TC, Total Carbon; BOD, Biological Oxygen Demand; COD, Chemical Oxygen Demand; TN, Total nitrogen; TP, Total phosphorous; TIN, Total Inorganic nitrogen.

7.1 Microalgae bio refineries

Microalgae bio refineries involve complete fractionation and utilization of biomass analogous to petrochemical refinery (Vermuë et al., 2018). As discussed earlier, a typical microalgae based ingredient [food/feed/fuel] production would involve cultivation, harvesting, dewatering, extraction and purification steps involving multiple unit processes that are energy and cost intensive. To achieve commercial feasibility, prudent selection of processes followed by generation of high value ingredients is essential. Among the various commodities that can be produced from microalgae, biofuels are supposed to be cheapest. However, with the high process costs, the commercial feasibility of microalgae derived biofuels is questionable. To offset this uncertainty, several scenarios of multiproduct generation and biomass valorization strategies are being suggested such as (i) production of animal feed after biofuel extraction, (ii) gasification of deoiled biomass for generation of syngas, (iii) liquefaction of deoiled biomass for production of bio crude/bio char, (iv) anaerobic digestion of biomass to produce methane, (v) fermentation of deoiled microalgae biomass for bioethanol, (vi) application of deoiled biomass as biofertilizers and soil conditioners (Katiyar et al., 2021). For further economic feasibility and reduced energy footprints, integration of bio refinery with bioremediation of wastewaters and CO_2 could enhance the commercial prospects along with environmental benefits. A typical microalgae biorefinery involving multiproduct utilization is presented in Figure 5.

Experimental trials have indicated positive outcomes from integrated biorefinery based routes towards biofertilizers application. Nayak et al. (2019) reported the use of deoiled *Scenedesmus* spp., biomass cultivated using domestic wastewater and coal-fired flue gas [2.5% v/v] as biofertilizers in the cultivation of rice. Deoiled *Scenedesmus* spp., biomass contained an entire spectrum of AAs and carbohydrates that promoted plant growth and reduced the requirements for chemical fertilizers. Similarly, *Chlorella minutissima* cultivated using sewage water was used as biofertilizer in field experiments for the cultivation of spinach and baby corn (Sharma et al., 2021). Phycoremediation with *Chlorella minutissima* resulted in a significant reduction (up to 80%) of both BOD and COD, and >50% of ammonia, nitrate and phosphate levels in the sewage water. The NPK content of microalgae biomass was higher compared to other natural manures such as vermicomposting, oil cake and biogas slurry. Application of microalgae biomass resulted in enhanced soil microbial activity, total organic carbon, available N and P as compared to soils enriched with chemical fertilizers (Sharma et al., 2021). Microalgae biomass obtained from external CO_2 supplementation from flue gas showed enhanced nutrient content and exhibited biofertilizer potential. Sinha et al. (2021) reported that deoiled biomass of CO_2 tolerant strain *Tetradesmus obliquus* CT02 exhibited biofertilizer properties when evaluated upon tomato plants. The deoiled biomass enhanced the germination percentage and index of tomato seedlings in comparison with commercial NPK fertilizers. In similar lines, cellular extracts of deoiled biomass of *Nostoc* spp. cultivated using municipal wastewater showed biostimulatory effects and improved the growth of lettuce plants. Foliar application of extracts derived from deoiled *Nostoc* spp., LS04 resulted in enhanced shoot and root length, fresh weight, DW, leaf chlorophyll and nutrient content compared to control groups (Silambarasan et al., 2021b).

7.2 Microalgae biochar for agricultural applications

In addition to the direct utilization of microalgae deoiled biomass and its extracts, bio char obtained through thermochemical conversion processes (hydrothermal carbonization, pyrolysis and Torre faction) of deoiled biomass could be used for agriculture applications (Mona et al., 2021). This integrated bio refinery route typically involves four steps *viz*., (i) cultivation of microalgae using wastewater; (ii) harvesting and downstream processing of biomass for lipid/hydrocarbon extraction; (iii) thermochemical conversion of deoiled biomass for bio char production and (iv) biofertilizer application of microalgae based bio char. Previous reports on use of bio char obtained from various agro-residues and biomass like straw, coffee husk, bamboo and variety of wood have been reviewed and reported to possess biofertilizer properties (Wang et al., 2020a). Application of bio char obtained from these biomasses in agro-forestry systems have resulted in enhanced soil fertility, improved soil texture especially in sandy and loamy soils through enhanced water retention, improvement in soil cation exchange capacities (CEC) leading to increased soil pH, and nutrient availability for plants in the rhizosphere region (Wang et al., 2020a). Thermochemical conversion of biomass results in oxidation of aromatic carbon groups and other complex organic molecules resulting in availability of carboxyl, carbonyl and other negatively charged functional groups that form complexes with cations thus enhancing the CEC of soil leading to nutrient retention (Laird et al., 2010). Further the porous microstructure and higher surface area of bio char causes slow release of nutrients to the plants thereby acting as slow release fertilizers reducing the problem of leaching and surface runoff (Gwenzi et al., 2015; Yu et al., 2019; Wang et al., 2020a). Thermochemical conversion of deoiled microalgae biomass could yield up to 90% bio char with higher nutritive value compared to deoiled microalgae biomass. Ultimate analysis of bio char and deoiled biomass revealed that thermochemical conversion enhances the net N, carbon, C/N ratio in bio char over the raw biomass (Phusunti et al., 2017). However, this depends on the thermos-conversion process parameters such as pyrolysis temperature and residence time during the process (Gan et al., 2018). The ultimate elemental analysis of biochar obtained from *Chlorella* spp. and *Nannochloropsis* spp., consisted 33% -50% w/w carbon, 33% - 57% w/w oxygen, 1% -5% w/w hydrogen and 4%-12% w/w N along with minerals such as Ca, Fe, Mg and K. The chemical composition of biochar, especially the mineralized N content indicated their suitability as biofertilizers especially for nutrient enrichment of soil (Borges et al., 2014; Chang et al., 2015). Biochar obtained from *Scenedesmus abundans* biomass cultivated using domestic wastewater had bio fertilization properties. The biochar contained 54.23% w/w carbon, 9.8% w/w hydrogen, 4.2% w/w ammoniacal N, 2.7% w/w sulphur and 28% w/w oxygen (Arun et al., 2020). The authors reported that biochar obtained from wastewater grown *Scendemsus abundans* biomass enhanced the growth, leaf and shoot weight and leaf chlorophyll content in tomato plants compared to chemical fertilizers. Apart from enhancing and mobilizing nutrient content in soil, microalgae derived biochar have been found to improve soil enzyme activity and promote soil microflora and soil microbial carbon content (Palanisamy et al., 2017). In another case study, use of algal biochar promoted defense response to drought stress in maize plants under deficit irrigation conditions. It was observed that algal biochar in

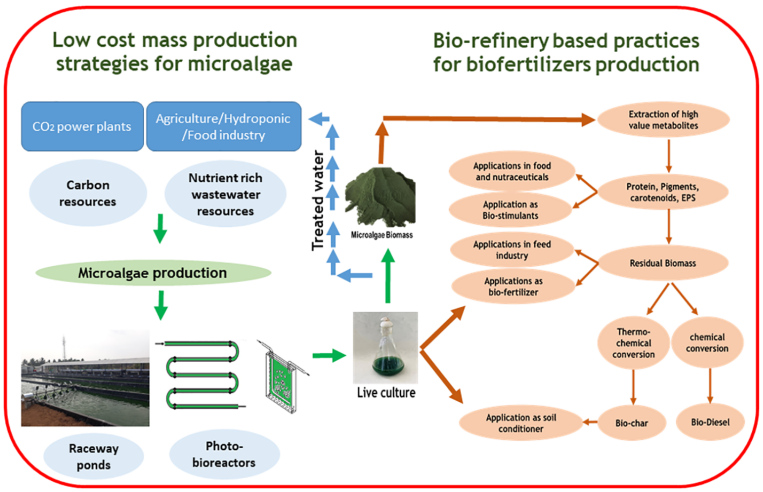


FIGURE 5
A typical microalgae biorefinery scheme.

consortia with plant growth promoting rhizobacteria *Serratia odorifera* accelerated plant growth by enhancing the nutrient availability in soil and enhancing photosynthetic activity (Ullah et al., 2020).

7.3 Integration of hydroponics and microalgae cultivation towards circular economy

Along with the use of municipal and sewage wastewaters, microalgae biomass obtained from wastewater generated from hi-tech agriculture systems such as hydroponics could be utilized used for biofertilizer application in a closed-loop model. Soilless cultivation such as hydroponics hasgained significant attention in recent years as the system can achieve maximum yield under a shorter time duration with excellent quality (Richa et al., 2020). Hydroponics utilizes nutrient solutions consisting of fertilizer salts in a definite ratio as per plant's growth requirements. Typically, the nutrient solutions used for the cultivation of vegetables and other horticulture plants have electrical conductivity in the range between 0.8 and 2.5 dS m^{-1} and pH between 5 and 7 and are generally prepared using salts that have high solubility in water (Hosseinzadeh et al., 2017). The physicochemical properties of nutrient solutions used in hydroponic systems meet the essential nutrient requirements of microalgae suggesting its potential in microalgae cultivation (Zhang et al., 2017). Even the wastewater (hydroponic system drainage) solution obtained after a plant cultivation cycle contains high concentrations of residual N (150–600 mg L^{-1}) and P (30–100 mg L^{-1}) along with other nutrients such as sulphates, K, Ca, trace minerals and organic substances like humic acids and root exudates (Saxena and Bassi, 2013). This wastewater solution that when released into the environment results in eutrophication and imbalance in the aquatic ecosystem (Richa et al., 2020). Nutrient recycling of hydroponics wastewater using membrane filtration technologies [reverse osmosis, ultrafiltration], sand filtration, UV treatment and denitrification using biological filters, and chemical treatment has been generally practised (Patel et al., 2020; Rufí-Salís et al., 2020). However, challenges exist in wastewater recycling treatments as organic residues and root exudates present in wastewater require multiple strategies as they promote microbial growth (Richa et al., 2020).

Hydroponics wastewater treatment and nutrient recycling could be effectively achieved by microalgae co-cultivation strategies. Co-cultivation of microalgae such as *Chlorella vulgaris* and *Scenedesmus quadricauda* with tomato plants in hydroponic system resulted in enhanced growth of both the plant and algae with biostimulatory properties exhibited by microalgae. This could be attributed to the mutual benefits exchanged by plant and algae where the roots exudates and inorganic nutrients promote algae growth and algal exudates induce stimulatory effects on to the plants (Barone et al., 2019). Zhang et al. (2017) reported that co-cultivation of microalgae *Chlorella infusionum* and tomato plants in hydroponic systems resulted in higher microalgae and crop biomass productivities when compared to individual monocultures. The enhancement in biomass productivity could be attributed to the aeration induced by microalgal photosynthesis and simultaneous utilization of CO_2 from crop root respiration. Further co-cultivation reduces nutrient load during the drainage of the hydroponic wastewater. Supraja et al. (2020b) observed the above-mentioned phenomena of enhanced plant and microalgae biomass productivities and reduced the nutrient load in drain water during co-cultivation of microalgae consortia consisting *Chlorella* spp., *Scenedesmus* spp., *Synechocystis* spp., and *Spirulina* spp., with tomato plants. Co-cultivation resulted in the utilization > 80% of initial N, P and K in the nutrient media. All the aforesaid studies suggest that microalgae co-cultivation in hydroponics is a cost-effective solution for simultaneous microalgae biomass production and wastewater treatment. A typical closed loop circular economy model of microalgae integrated biorefinery is presented in Figure 6.

8 Microalgae based agro-chemicals: Market trends and way forward

Use of microalgae biomass and derived extracts as bio-fertilizers and growth stimulants have gained significant global attention in the

recent past. Although, role of cyanobacteria and few microalgae species in N fixation and plant growth promotion have been known for many decades; the discovery of new applications of microalgae metabolites in agriculture and need for biorefinery approach in microalgae biomass utilization have renewed the interests of microalgae as alternative to synthetic agrochemicals (Kapoore et al., 2021; Lee and Ryu, 2021; Sharma et al., 2021). Analysis of the patent landscape of microalgae utilization in agriculture showed specific applications in plant growth promotion, plant protection (disease control), weed management and post-harvest quality improvement (Murata et al., 2021). The current global market for plant growth promoters and bio-stimulants is valued at USD 3.2 billion and forecasted to reach USD 5.6 billion by the year 2026 (https://www.marketsandmarkets.com/Market-Reports/biostimulant-market-1081.html). The demand for microalgae based fertilizers and plant growth stimulants was valued at USD 9479 thousand in 2021 with a compounded annual growth of 7.7% and is expected to grow to 8.7% by 2031. The market survey indicated that *Spirulina platensis*, *Chlorella vulgaris* and *Dunaliella salina* cumulatively contributed to 27.5% of microalgae based offerings to the fertilizers sector. [https://www.futuremarketinsights.com/reports/microalgae-fertilizers-sector]. As discussed in previous sections, microalgae biomass and extracts rich in polysaccharides, AAs have been commercially used for biofertilizer/bio stimulant applications. The bio-stimulant activity from microalgae have been demonstrated through different modes of applications such as (i) amendment of soil with algal formulations with suitable carriers, (ii) amendment of soil with algal dry biomass or suspended liquid culture, (iii) foliar spray of microalgae extracts and cell free supernatant, (iv) substrate or soil drench with alga culture (Chiaiese et al., 2018). Among the various application methods, FS was found to be the most effective (González-Pérez et al., 2022).

Despite the availability of enormous literature, commercial realization of microalgal technologies for agrochemical sector is still far, with several challenges to be overcome which are discussed below. Most critical challenges are survival, colonization and interaction of microalgae/cyanobacteria with the host plant. Challenges of lab to field transitions are critical as many good strains/consortia of strains with excellent performance *in vitro* perform very poorly under field conditions (Mitter et al., 2021). Some of the challenges involved in field trials are edaphic factors such as negative biotic interactions with resident microbiomes of host plant leading to competition and antagonism and high variability in soil physico-chemical properties like pH, moisture, temperature and salinity (de Siqueira Castro et al., 2017; Schütz et al., 2018). Apart from these, host plant acceptance and variability in plant growth [plant age] and its physiological status affect the functionality of biofertilizer or bio stimulant (Dennis et al., 2010). Thirdly, viability and stability of microbial formulations, in our case microalgal/cyanobacteria, under field conditions, persistence to varying temperatures and salinity are some critical parameters that affect the widespread use of biofertilizers (Kaminsky et al., 2019). Finally, practical challenges such as cost effectiveness, scalability and farmer's acceptance determine the success of the technology.

Some potential solutions to overcome the aforesaid challenges are (i) developing a consortia of biofertilizers with wide ecological adaptations with broad pH, thermo, psychro and osmotic tolerances, (ii) isolation of strains from the host rhizosphere and native soil, (iii) personalized biofertilizers specific to soil or a crop, (iv) biofilm forming organisms for enhanced stability, (v) formulations and carriers that are stable to environmental fluctuations and use of technology such as encapsulation and milder dehydration processes that retain the bioactivity of the formulations (Mitter et al., 2021). Apart from plant growth enhancement, human biosafety of cyanobacterial and microalgal biofertilzers are essential as many cyanobacterial strains secrete toxins such as microcystins, nodularin's and analchalins which affect neurological functions in humans and pose a significant risk (Kubickova et al., 2019). Currently, majority of commercial biofertilizers formulations consist of N_2-fixing organisms such as *Actinorhizobium* spp., *Azospirillum* spp., *Azotobacter* spp., and *Rhizobium* spp. that have low health risk and has history of safe applications (Sessitsch et al., 2019). Therefore, it becomes imperative to evaluate the toxicology aspects of novel algae based biofertilizers in experimental animal models before its widespread use. A list of commercially marketed microalgae derived biostimulants and plant growth promoters are listed in Table 5.

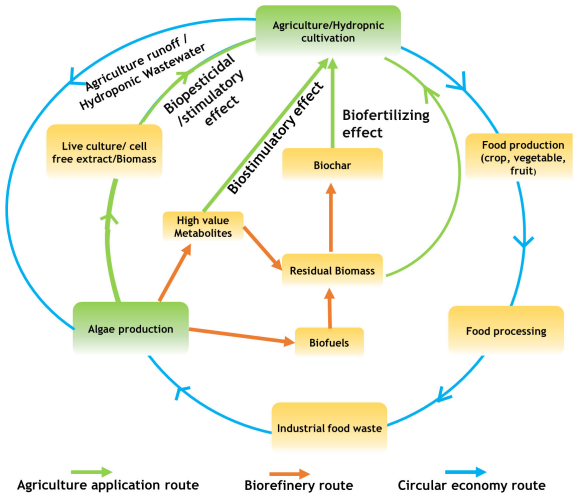


FIGURE 6
A typical closed loop circular economy model of microalgae integrated biorefinery coupled with biofertilizer and biostimulatory applications.

9 Concluding remarks

The Use of biofertilizers and natural biostimulants has been on the upward trend in recent years to overcome the dependency on chemical fertilizers and non-renewable resources. Microalgae and cyanobacteria have been proven to enhance and stimulate plant growth and offer systemic immune resistance to various biotic and abiotic stresses. However, the success of microalgae-based biofertilizers and agro-chemical depends on the low cost of biomass production, and lower energy footprints in biomass production technologies. This necessitates the integration of bioremediation and biorefinery models to improve the commercial feasibility of microalgae-based agro technologies. Co-cultivation of microalgae and cyanobacteria in closed-loop hydroponic systems could be an effective system in contributing to global food security. Some key research and developmental directions that are required for the success of microalgae-based technologies for crop productivity enhancement are

TABLE 5 List of commercially marketed microalgae based bio-stimulants and plant growth promoters.

Marketed product	Company	Microalgae species	Claim Application	References
Algafert	Biorizon Biotech	Spirulina	• Growth and fattening of fruits. • Faster crop rooting • Natural color stimulation in fruits. • Size and homogeneous ripening. • Improving resistance against pests, thermal stress and disease.	https://www.biorizon.es/bioenhancers/hydrolyzed/algafert/?lang=en
Agrialgae organic orginal	AlgaEnergy	Microalgae	• High quality agricultural bio-stimulant based on an optimized combination of microalgae • High assimilation by the plant, promoting both the root and the foliage development of the plant • Increases its photosynthetic capacity and promotes the regeneration of damaged tissues • provides the crops with the tools to reinforce its immune system, making it more resistant to or boosting the recovery from stress episodes	https://www.agrialgae.es/tienda-online-bioestimulantes/agrialgae-organic-en/agrialgae-organic-original-2/?lang=en
Agrialgae premium			• AgriAlgae® Premium includes biostimulants specifically designed to provide your crop with what it needs in each of the phenological phases of development. • Provides solutions for different stages like flowering, fruit setting, ripening and fattening, rooting, sprouting and stress	https://www.agrialgae.es/bioestimulantes-agricolas/agrialgae-premium-en/?lang=en
Blue Green Algae Biofertilizer	AlgEnerg Pvt Ltd	Blue Green Algae	• 10-14% increase in crop yield • Highly effective and environment friendly	https://www.algenerg.co.in/blue- green-algae-biofertilizer.htm
TrueSolum	True Algae	*Chlorella*	• Enhanced soil microbial diversity. • More vigorous root systems. • Increased yield. • Better crop quality. • Extended shelf life of their crops	https://truealgae.com/
BYAS-A601	Back of the yard algae sciences	Microalgae	• Potent microalgae based bio-stimulant • Proven natural accelerator of plant growth and phytochemical production. • Improves taste, yield, shelf life • Can be used in controlled environment agriculture and indoor/outdoor fruit and vegetable farming	https://www.algaesciences.com/

Adapted from (Kumar et al., 2022b).

- Bio prospection of microalgae and cyanobacterial strains with plant growth promotion properties
- Identification of microalgae strains with wide environmental adaptability such as tolerances to desiccation, wide pH range, temperatures and osmolarity
- Studies evaluating the host plant interaction with microalgae/cyanobacteria strains
- Chemical fingerprinting and establishment of a database on the distribution of plant growth-promoting metabolites such as phytohormones, phenolics and signaling molecules present in microalgae/cyanobacteria strains
- A large number of field trials validating *in vitro* results on fertilizing or stimulatory or pesticidal activity of microalgae-derived extracts
- Development of circular economy loop/routes utilizing agriculture wastewaters and industrial wastes for the cultivation of microalgae biomass and utilizing them in agri-applications
- Safety and risk assessment of biomass produced utilizing wastewaters for heavy metals, xenobiotic residues, horizontal gene transfers
- Development of cost-effective harvesting and dewatering processes for mass production of microalgae biomass
- Education and involvement of farmers on microalgae applications in largescale field trials for product acceptance and widespread use

Author contributions

VS conceptualized the review. PP, RK and YN created the tables and figures. All the authors contributed in writing the manuscript. VS

reviewed the manuscript. All the authors contributed to the article and approved the submitted version.

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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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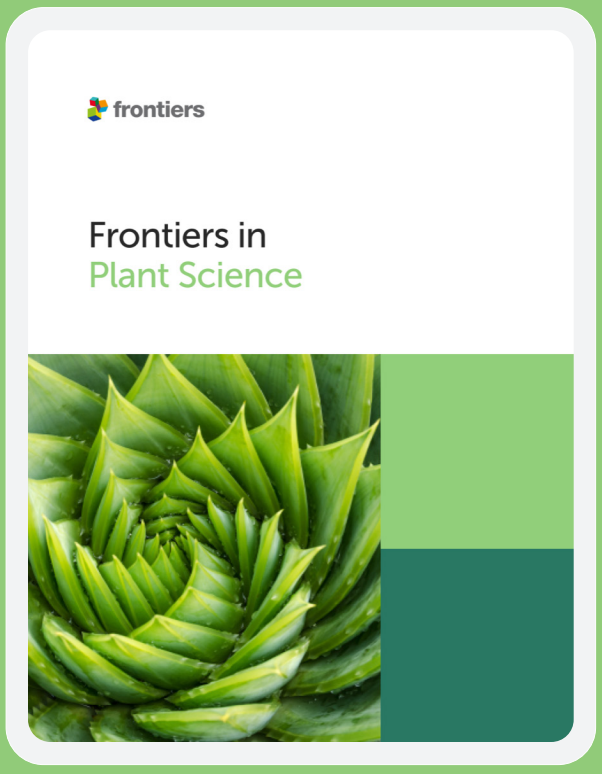
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