MAXIMIZING NITROGEN FIXATION IN LEGUMES AS A TOOL FOR SUSTAINABLE AGRICULTURE INTENSIFICATION

EDITED BY: Pilar Irisarri, Juan Imperial, Fernando Alfredo Lattanzi, Jorge Monza, Jose Palacios, Juan Sanjuan and Julie Grossman

PUBLISHED IN: Frontiers in Agronomy and Frontiers in Plant Science

frontiers Research Topics

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-88974-044-4
DOI 10.3389/978-2-88974-044-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

MAXIMIZING NITROGEN FIXATION IN LEGUMES AS A TOOL FOR SUSTAINABLE AGRICULTURE INTENSIFICATION

Topic Editors:
Pilar Irisarri, Universidad de la República, Uruguay
Juan Imperial, Spanish National Research Council (CSIC), Spain
Fernando Alfredo Lattanzi, National Institute for Agricultural Research (INIA), Uruguay
Jorge Monza, Universidad de la República, Uruguay
Jose Palacios, Polytechnic University of Madrid, Spain
Juan Sanjuan, Experimental Station of Zaidín, Spanish National Research Council (CSIC), Spain
Julie Grossman, University of Minnesota Twin Cities, United States

Citation: Irisarri, P., Imperial, J., Lattanzi, F. A., Monza, J., Palacios, J., Sanjuan, J., Grossman, J., eds. (2022). Maximizing Nitrogen Fixation in Legumes as a Tool for Sustainable Agriculture Intensification. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-044-4

Table of Contents

05 ***Editorial: Maximizing Nitrogen Fixation in Legumes as a Tool for Sustainable Agriculture Intensification***

Pilar Irisarri, Juan Imperial, Fernando A. Lattanzi, Jorge Monza, Jose Palacios, Juan Sanjuan and Julie Grossman

08 ***The Rhizosphere Talk Show: The Rhizobia on Stage***

Alice Checcucci and Marta Marchetti

15 ***Will Phosphate Bio-Solubilization Stimulate Biological Nitrogen Fixation in Grain Legumes?***

Walid Janati, Bouchra Benmrid, Wissal Elhaissoufi, Youssef Zeroual, Joshua Nasielski and Adnane Bargaz

27 ***Applying Agronomic Principles of Rhizobial Inoculation to the Conservation of a Keystone Legume Species in a High Mountain Ecosystem on an Oceanic Island***

Laura Pulido-Suárez, Águeda M. González-Rodríguez, Jonay Cubas, Marcelino del Arco-Aguilar, José L. Martín-Esquivel and Milagros León-Barrios

39 ***Non-specific Lipid Transfer Proteins in Legumes and Their Participation During Root-Nodule Symbiosis***

Citlali Fonseca-García, Jorge Solis-Miranda, Ronal Pacheco and Carmen Quinto

56 ***Characterization of* Bradyrhizobium *spp. Nodulating* Lupinus cosentinii *and* L. luteus *Microsymbionts in Morocco***

Mustapha Missbah El Idrissi, Omar Bouhnik, Salma ElFaik, Soufiane Alami, Hanane Lamin, Eulogio J. Bedmar and Hanaa Abdelmoumen

68 ***Soybean Nodulation Response to Cropping Interval and Inoculation in European Cropping Systems***

Mosab Halwani, Moritz Reckling, Dilfuza Egamberdieva, Richard Ansong Omari, Sonoko D. Bellingrath-Kimura, Johann Bachinger and Ralf Bloch

77 ***Control of the Rhizobia Nitrogen-Fixing Symbiosis by Common Bean MADS-Domain/AGL Transcription Factors***

Litzy Ayra, María del Rocio Reyero-Saavedra, Mariel C. Isidra-Arellano, Luis Lozano, Mario Ramírez, Alfonso Leija, Sara-Isabel Fuentes, Lourdes Girard, Oswaldo Valdés-López and Georgina Hernández

94 ***Why Should Nodule Cysteine-Rich (NCR) Peptides Be Absent From Nodules of Some Groups of Legumes but Essential for Symbiotic N-Fixation in Others?***

J. Allan Downie and Eva Kondorosi

101 ***Introduction of H_2-Uptake Hydrogenase Genes Into Rhizobial Strains Improves Symbiotic Nitrogen Fixation in* Vicia sativa *and* Lotus corniculatus *Forage Legumes***

Mariana Sotelo, Ana Claudia Ureta, Socorro Muñoz, Juan Sanjuán, Jorge Monza and Jose Palacios

108 ***Rhizobial Exopolysaccharides and Type VI Secretion Systems: A Promising Way to Improve Nitrogen Acquisition by Legumes***
Bruna Fernanda Silva De Sousa, Tereza Cristina Luque Castellane, Lilia Tighilt, Eliana Gertrudes de Macedo Lemos and Luis Rey

118 ***Efficient CRISPR/Cas9 Genome Editing in Alfalfa Using a Public Germplasm***
Emilia Bottero, Gabriela Massa, Matías González, Margarita Stritzler, Hiromi Tajima, Cristina Gómez, Romina Frare, Sergio Feingold, Eduardo Blumwald, Nicolás Ayub and Gabriela Soto

126 ***Analysis of Nitrogenase Fe Protein Activity in Transplastomic Tobacco***
Jose A. Aznar-Moreno, Xi Jiang, Stefan Burén and Luis M. Rubio

137 ***Black Seedcoat Pigmentation Is a Marker for Enhanced Nodulation and N_2 Fixation in Bambara Groundnut (*****Vigna Subterranea** ***L. Verdc.) Landraces***
Doris K. Puozaa, Sanjay K. Jaiswal and Felix D. Dakora

152 ***Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the*** **Rhizobium*****-Legume Symbioses***
Marcela Mendoza-Suárez, Stig U. Andersen, Philip S. Poole and Carmen Sánchez-Cañizares

178 ***Genomic Diversity of Pigeon Pea (*****Cajanus cajan** ***L. Millsp.) Endosymbionts in India and Selection of Potential Strains for Use as Agricultural Inoculants***
Beatriz Jorrin, Marta Maluk, Nagvanti Atoliya, Shiv Charan Kumar, Danteswari Chalasani, Andrzej Tkacz, Prachi Singh, Anirban Basu, Sarma VSRN Pullabhotla, Murugan Kumar, Santosh Ranjan Mohanty, Alison K. East, Vinoy K. Ramachandran, Euan K. James, Appa Rao Podile, Anil Kumar Saxena, DLN Rao and Philip S. Poole

197 ***Inhibition of N_2 Fixation by N Fertilization of Common Bean (*****Phaseolus vulgaris** ***L.) Plants Grown on Fields of Farmers in the Eastern Cape of South Africa, Measured Using ^{15}N Natural Abundance and Tissue Ureide Analysis***
Simon J. Habinshuti, Sipho T. Maseko and Felix D. Dakora

frontiers in Agronomy

EDITORIAL
published: 23 November 2021
doi: 10.3389/fagro.2021.796717

Editorial: Maximizing Nitrogen Fixation in Legumes as a Tool for Sustainable Agriculture Intensification

Pilar Irisarri[1]*, Juan Imperial[2], Fernando A. Lattanzi[3], Jorge Monza[1], Jose Palacios[4], Juan Sanjuan[5] and Julie Grossman[6]

[1] Department of Biología Vegetal, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay, [2] Consejo Superior de Investigaciones Científicas, Madrid, Spain, [3] Programa Pasturas y Forrajes, Estación Experimental INIA La Estanzuela, Instituto Nacional de Investigación Agropecuaria (INIA), Colonia, Uruguay, [4] Department Biotechnology-Plant Biology, CBGP UPM-IINIA, ETSIAAB, Universidad Politécnica de Madrid, Madrid, Spain, [5] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental de Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain, [6] Department of Horticultural Science, University of Minnesota Twin Cities, St. Paul, MN, United States

Keywords: biological nitrogen fixation, inoculants, symbiosis, rhizobia, legumes

OPEN ACCESS

Edited and reviewed by:
Davey Jones,
Bangor University, United Kingdom

****Correspondence:***
Pilar Irisarri
irisarri@fagro.edu.uy

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *17 October 2021*
Accepted: *26 October 2021*
Published: *23 November 2021*

Citation:
Irisarri P, Imperial J, Lattanzi FA, Monza J, Palacios J, Sanjuan J and Grossman J (2021) Editorial: Maximizing Nitrogen Fixation in Legumes as a Tool for Sustainable Agriculture Intensification. Front. Agron. 3:796717. doi: 10.3389/fagro.2021.796717

Editorial on the Research Topic

Maximizing Nitrogen Fixation in Legumes as a Tool for Sustainable Agriculture Intensification

Today's agricultural systems are challenged by providing sufficient food for a growing world population while improving soil and water quality, maintaining farmer profitability (Stephens et al., 2018), and contributing to climate change mitigation (Niles et al., 2018). Nitrogen is an essential nutrient for plant growth and development, and intensification of agricultural systems typically encourages greater use of nitrogen to increase yields. Unfortunately, nitrogen is also directly linked to negative environmental impact *via* direct (N_2O) and indirect (NH_3) greenhouse gas emissions, ozone depletion (NO), and water pollution (NO_{3^-}). Thus, nitrogen must play a central role in the sustainable transformation that global food production faces (Ying et al., 2017).

Most, if not all, agricultural production systems are limited by nitrogen availability, hence the widespread -and increasing- use of fertilizers (Rütting et al., 2018). Fertilizers are a source of nitrogen that is costly, of only moderate efficiency and with a relatively large CO_2 footprint, due to the energy intensity of the synthesis of chemical fertilizers by the Haber-Bosch process. Globally, the second largest source of agroecosystem nitrogen input is atmospheric N_2 fixation driven by a symbiotic relationship between legumes and soil bacteria collectively known as rhizobia. In this process, known as biological nitrogen fixation (BNF), rhizobia use plant assimilates to reduce N_2 to a plant-usable form through their nitrogenase enzyme. Compared to chemical fertilizers, BNF is cheaper and may result in a lower carbon footprint. Unsurprisingly, improving the effectiveness of BNF has been the focus of much research, and has risen in importance due to its critical role in sustainable agricultural systems. Improvement of the BNF process will improve our capacity to design sustainable diversified agroecosystems through the inclusion of legumes. This special issue addresses different approaches directed toward the exploitation of biological nitrogen fixation as a tool to develop more sustainable agricultural systems.

Rhizobia as soil inhabitants are important players in the legume rhizosphere microbiome, as supported by the minireview of Checcucci and Marchetti in this Research Topic. The signals involved in the rhizobium-legume interactions are shaped by plant exudates and rhizosphere microbiome composition. From the plant partner symbiotic perspective, Downie and Kondorosi discuss why different types of legume nodules, including both terminally differentiated rhizobia or

not, depend on plant-encoded defense-like small peptides which include Nodule-specific Cysteine-Rich (NCR) peptides. Their minireview explores why NCR genes are absent from many legumes, and regulate bacteroid development and rhizobial strain discrimination in others.

Improving the biological nitrogen fixation (BNF) process has reinforced the attention to the rhizobia inoculation of legumes. Mendoza-Suárez et al. review the implications of rhizobial competitiveness for the establishment of a successful symbiosis. They describe ongoing approaches for inoculant development based on native strains to ensure optimal performance of both symbiotic partners.

While characterizing rhizobia partners for grain legumes, Missbah El Idrissi et al. report that the selection of lupine microsymbionts utilized as inoculants in the south Mediterranean should depend on soil type and pH. Some *Bradyrhizobium* spp. strains isolated from *Lupinus luteus* and *L. cosentinii* may constitute new genospecies while *nodA* and *nodC* nodulation genes correspond to members of the genistearum symbiovar.

Pigeon pea (*Cajanus cajan*) is a legume well-adapted to dry land areas and India is its major producer. Jorrin et al. performed the first genomic study of pigeon pea endosymbionts in India. The major species nodulating pigeon pea was *Bradyrhizobium yuanmingense*, whereas nodulation-related features showed similarity to *B. cajani* and to *B. zhanjiangense*. They also recommend candidates for inoculant formulation in different agro-climatic regions.

Previous evidence supports the presence of indigenous soybean-nodulating bradyrhizobia in central Europe soils under cold-growing conditions, with Halwani et al. advancing our understanding of these strains by showing they remained viable in soil and were still symbiotically competent for up to 4 years after soybean cultivation. However, findings that their symbiotic performance was not sufficient in all cases makes inoculation with commercial products still necessary.

The symbiotic process is highly dependent on the dynamic exchange of signals and molecular nutrients between partners. The mini-review of Rey et al. highlights the importance of exopolysaccharide production and presence and possible function of type VI secretion systems, two key determinants in successful symbiotic interaction between rhizobia and pulse crops. Both exopolysaccharide composition and quantity play important roles in plant signaling and in bacterial protection to stressful conditions but type VI secretion systems are only recently studied in rhizobia.

Among other factors affecting N_2 fixation, Habinshuti et al. confirmed inhibition by N fertilization of common bean despite tissue N derived from BNF being high for bean plants receiving a double-dose of N fertilizer. In contrast, Bargaz et al. review how P bio-solubilization bacteria may enhance N_2 fixation in grain legumes. Rhizobial inoculation promotes growth of *Spartocytisus supranubius*, a keystone native legume species in a high mountain ecosystem on an oceanic island (Pulido-Suárez et al.). This article shows the importance of inoculation also for conservation purposes.

Nitrogenase activity results in the evolution of hydrogen but some rhizobia induce an uptake hydrogenase to recycle this hydrogen, improving the efficiency of the process. Sotelo et al. report the generation and symbiotic behavior of hydrogenase-positive *Rhizobium leguminosarum* and *Mesorhizobium loti* strains effective in vetch and birdfoot trefoil. The inoculation of these forage crops with the engineered strains leads to increases in the levels of nitrogen incorporated and indicates that hydrogen recycling has the potential to improve symbiotic nitrogen fixation in forage plants.

On the side of plant improvement, Puozaa et al. report that black seedcoat pigmentation in Bambara groundnut, the most important food legume in Africa, is a biomarker for increased nodulation and N_2 fixation that can be used in breeding programs.

Plants MADS-domain/AGL proteins constitute a large transcription factor family that control root development among others, but their role in legumes was almost unexplored. Ayra et al. study the involvement of AGL from common bean as regulator of different stages of the rhizobia-legume symbiosis. They used composite plants with transgenic roots/nodules overexpressing or silencing AGL gene expression. The silencing plants were affected in the generation and growth of transgenic roots, decreased rhizobial infection with lower expression level of early symbiotic genes, and increased number of small ineffective nodules. This alteration in the autoregulation of nodulation symbiotic process is proposed to be related with the interplay with NIN, the master symbiotic regulator.

Non-specific lipid transfer proteins (LTPs) constitute a large protein family in plants while their role in mutualistic interactions is still unknown. Fonseca-García et al. find that genes of different classes of LTPs were expressed in roots inoculated with rhizobia and nodules of legumes. Specifically, common bean LTP genes are differentially expressed during the early and late stages of nodulation, and they might be regulated by ROS production.

Bottero et al. reported the high-efficiency of alfalfa mutagenesis by using public and regenerative alfalfa clone C23 and the CRISP/Cas9 system. This advance in the efficiency of CRISP/Cas9 genome editing suggests that this approach can be used to reduce the cost of production of edited cultivars for this important forage legume.

Finally, the integration of prokaryotic nitrogen fixation (*nif*) genes into the plastid genome for expression of functional nitrogenase components could render plants capable of assimilating atmospheric N_2. However, N_2 fixation is a complex trait involving among others Nif proteins that are very sensitive to O_2 exposure. Aznar-Moreno et al. show that NifH protein expressed in transplastomic tobacco plants is functional when isolated from leaves collected at the end of the dark period. Non-unwanted negative effects from expression of functional NifH and NifM in plastids were observed, allowing to continue with attempts to engineer nitrogenase in crops.

DEDICATION

This Topic is dedicated to the memory of Tomás Ruiz-Argüeso in recognition for a lifetime of research on the improvement of symbiotic biological nitrogen fixation in legumes.

OBITUARY

Tomas Ruiz Argüeso (Villamol 1943–Madrid 2020) was Full Professor of Microbiology and, until his death, Emeritus Professor at the Universidad Politécnica de Madrid. After completing his Doctoral Thesis, focused on the study of the microbiology of honey, he became interested in the symbiotic nitrogen fixation process, which he would pursue throughout his long research career. He initially worked on the isolation and characterization of endosymbionts from soybean, a crop that was being introduced in Spain at that time and contributed to the development of the first rhizobial inoculants in the country. He later made postdoctoral stays at the laboratories of Harold Evans' lab at Oregon State University and at Donald Helinski's lab in La Jolla (UCSD). He devoted a long and fruitful research work to the study of the hydrogen recycling system of rhizobia, a subject on which he published over 50 research papers that covered from basic, mechanistic aspects of this metalloenzyme to its role in improving nitrogen fixation by legumes. In recent years he became interested in the characterization of new symbiotic systems, thus expanding our understanding on the high diversity of soil bacteria capable of establishing diazotrophic symbiosis. He published more than 80 articles in international journals, led over 30 research projects, presented almost 300 contributions at national and international conferences, and directed 16 Doctoral Theses. In parallel, he had a long teaching career in Microbiology, and a very notable task of service in different national and European research organizations and entities. And beyond his many achievements as a scientist and professor, Tomás always kept open the door of scientific curiosity and drawing pleasure from doing science, along with a friendly and close character, always ready to listen and help others. Those of us who have been fortunate and privileged to share these years with such an extraordinary researcher, teacher and friend will miss him. We are left with his many teachings and his memory. May he rest in peace.

AUTHOR CONTRIBUTIONS

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

FUNDING

FL was financially supported by PROCISUR and FONTAGRO through the project Uso de leguminosas en sistemas ganaderos sudamericanos. RTI2018-94985-B-100 (to JP). JS was supported by Project BIO2017-83533-P and funded by MCIN/AEI/10.13039/501100011033/FEDER A way to make Europe.

REFERENCES

Niles, M., Ahuja, R., Barker, T., Esquivel, J., Gutterman, S., Heller, M., et al. (2018). Climate change mitigation beyond agriculture: a review of food system opportunities and implications. *Renew. Agric. Food Syst.* 33, 297–308. doi: 10.1017/S1742170518000029

Rütting, T., Aronsson, H., and Delin, S. (2018). Efficient use of nitrogen in agriculture. *Nutr. Cycl Agroecosyst.* 110, 1–5. doi: 10.1007/s10705-017-9900-8

Stephens, E. C., Jones, A. D., and Parsons, D. (2018). Agricultural systems research and global food security in the 21st century: an overview and roadmap for future opportunities. *Agric. Syst.* 163, 1–6. doi: 10.1016/j.agsy.2017.01.011

Ying, H., Ye, Y., Cui, Z., and Chen, X. (2017). Managing nitrogen for sustainable wheat production. *J. Clean. Prod.* 162, 1308–1316. doi: 10.1016/j.jclepro.2017.05.196

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Irisarri, Imperial, Lattanzi, Monza, Palacios, Sanjuan and Grossman. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

MINI REVIEW
published: 02 December 2020
doi: 10.3389/fagro.2020.591494

The Rhizosphere Talk Show: The Rhizobia on Stage

Alice Checcucci[1] and Marta Marchetti[2,3]*

[1] Department of Agricultural and Food Science, University of Bologna, Bologna, Italy, [2] INRA, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France, [3] CNRS, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2594, Castanet-Tolosan, France

From bacterial quorum sensing to the signals of bees, communication is the basis of biotic interactions. Frequently, more than two organisms can take part in the speeches, resulting in a complex network of cross-talks. Recent advances in plant-microbe interactions research have shown that communication, both inter-kingdom and intra-kingdom, is shaped by a broad spectrum of factors. In this context, the rhizosphere (i.e., the soil close to the root surface) provides a specific microhabitat where complex interactions occur. The complex environment that makes up the rhizosphere can select for certain microbial populations, which are adapted to this unique niche. Among them, rhizobia have emerged as an important component of the rhizospheric microbiome. The aim of this review is to explore the components of such a rhizospheric Talk Show in the frame of the rhizobium-legume interactions. This symbiosis is a complex process that involves several signals that can be shaped by plant rhizospheric exudates and microbiome composition. The relationship established by rhizobia with other rhizospheric organisms, together with the influence of the environmental factors, results in their beneficial role on host plant health. Here, we resume research accounting strategies, molecules, and organisms that influence the place of rhizobia in the rhizosphere. The focus is on the most recent approaches for the study and subsequent exploitation of the diversity of the organisms. Indeed, the study of plant-microbes communication and evolution is fundamental to develop highly efficient inoculants able to reduce the use of fertilizers in agriculture.

Keywords: rhizosphere, rhizobia, communication, microbial communities, plant-microorganisms interaction

OPEN ACCESS

Edited by:
Pilar Irisarri,
Universidad de la República, Uruguay

Reviewed by:
Ivan John Oresnik,
University of Manitoba, Canada
Luciano Kayser Vargas,
State Secretariat of Agriculture, Livestock and Irrigation, Brazil

***Correspondence:**
Alice Checcucci
alice.checcucci2@unibo.it

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *04 August 2020*
Accepted: *16 November 2020*
Published: *02 December 2020*

Citation:
Checcucci A and Marchetti M (2020) The Rhizosphere Talk Show: The Rhizobia on Stage. Front. Agron. 2:591494. doi: 10.3389/fagro.2020.591494

THE TALK SHOW IN THE RHIZOSPHERE

Prokaryotes and eukaryotes have interacted for millions of years, evolving and refining over time their communication systems. As proposed by Hauser in 1996 (Hauser, 1996), biological signals and the exchange of information are part of the definition of communication, while the signals themselves are considered as "every structure able to shape the behavior of the organisms" (Smith and Harper, 2003; Schott-Phillips, 2008). Consequently, the signals can evolve and persist thanks to the interaction between signals producers and receivers. Then, cooperation and fitness improvement are the basis of biological communication (Zahavi, 2008).

In a particular environment, individuals can communicate and interact with multiple partners, and the nature of interaction can determine variable costs and benefits to the partner, as a biological market (Werner et al., 2015). One of the most fascinating environments

where complex biological interactions occur is the rhizosphere. Indeed, a large number of signals can be exchanged involving the plant itself, insects, fungi and microbes. This all take place in a high-density environmental niche. Usually, communication is the result of chemical responses of cells to signatory molecules coming from other cells. These signals affect both the metabolism and transcription of genes activating several regulatory mechanisms.

Frequently, in the rhizosphere more than two organisms (and species, across the tree of life) can take part in the communication, resulting in a complex network of interactions and cross-talks which can influence the fitness of all participating partners. Thus, this environment can be considered a hot spot for numerous inter-kingdom signals exchange, which involves plant-associated microbial communities (rhizobiome). The microbial community's composition is mainly shaped and recruited by hundreds of metabolites released in the soil by plant roots, which normally facilitate the interactions with the biotic and abiotic environment. Often the plant can modulate their diversity based on the benefits in terms of growth and health (Plant Growth Promoting, PGP) (Hartmann et al., 2009). Nevertheless, a large number of nutrients issued by the plant can be of interest for the pathogenic organisms, which can take advantage of plant products for their survival in the rhizosphere (Rasmann and Turlings, 2016).

It stands to reason that the plants play a fundamental role in the rhizosphere *scene* (Bending, 2017) (**Figure 1**). Indeed, because of the chemical signals conveyed by nutrient-rich exudates released by the plant roots, a large variety of microbes can first colonize the rhizosphere and then gradually penetrate the root and the overall plant tissue (endophytes) (Hardoim et al., 2008). Otherwise, they can colonize the host plant establishing a lasting and beneficial symbiotic relationship (Chi et al., 2005). To date, numerous investigations on root exudates composition have been performed (Hartmann et al., 2009; Bulgarelli et al., 2013; Venturi and Keel, 2016).

The most known plant-microbe *dialogue* on the rhizosphere *scene*, which determine direct and indirect advantages to the

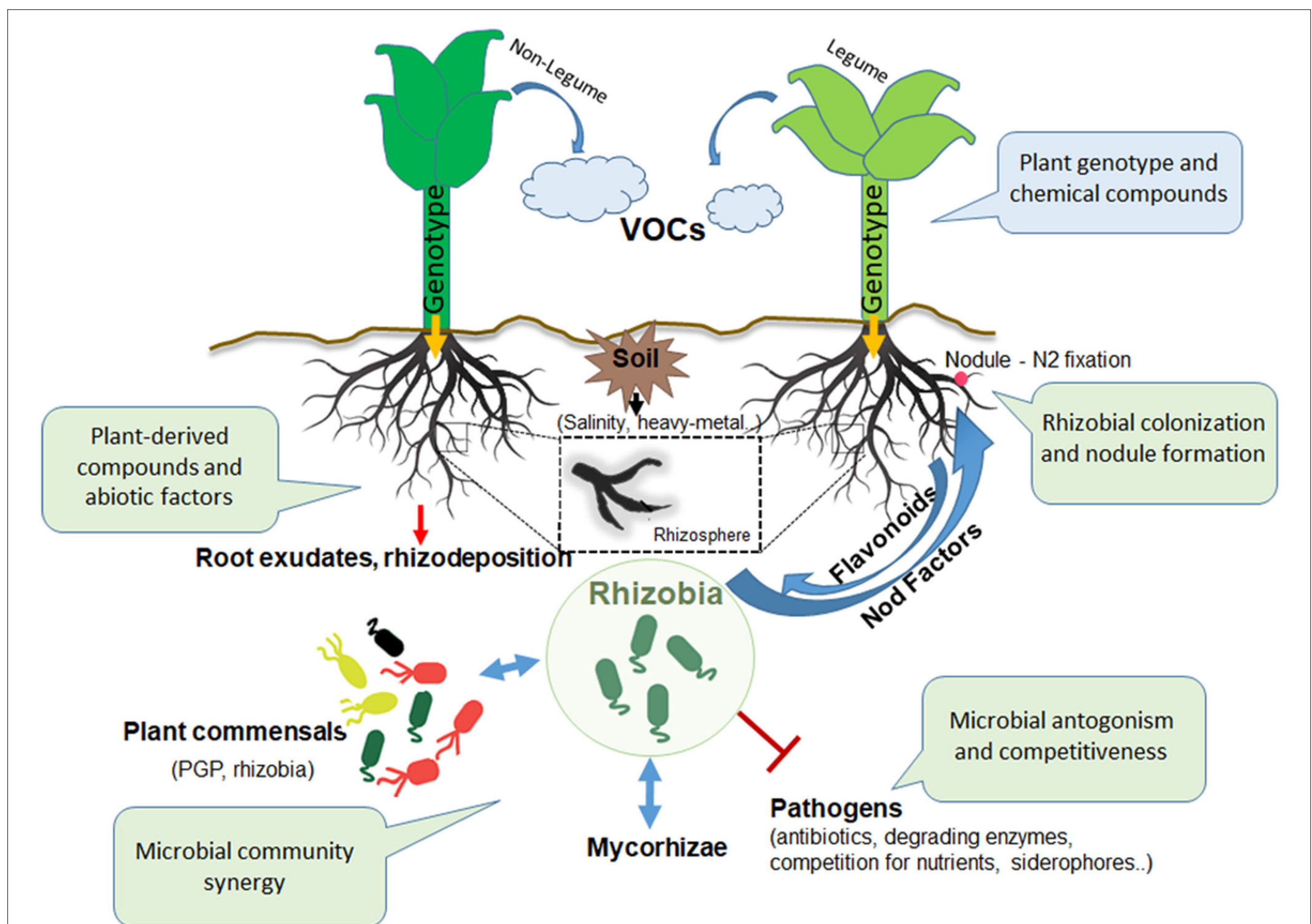


FIGURE 1 | Actors and interactions in the rhizosphere. Inter-kingdom and intra-kingdom communication involving plants and microbes in the rhizosphere: the consistent role of rhizobia. VOCs, volatile organic compounds; PGP, plant growth promoting; AMF, arbuscular mycorrhizal fungi.

partners, was properly addressed as early as 1904 when Hiltner described the symbiotic interaction among legumes and rhizobia (Hartmann et al., 2008). This symbiosis is a highly specific process in which the genetic and chemical communication signals are strictly plant-bacterium specific. In this mutualistic interaction, rhizobia positively influence the host's growth thanks to the nitrogen fixation process and at the same time can benefit from the nutrients provided by the plant.

This symbiosis has been extensively studied over the last few decades, and many studies on the communication and the signaling between the two partners at different steps of the symbiosis (from root infection to nodules development) have been elucidated (Oldroyd et al., 2011; Oldroyd, 2013). However, the knowledge about the earlier steps of rhizosphere colonization, namely the opening line at the root surface, remains poorly characterized. Nonetheless, increasing data have shown the importance of intraspecies and multispecies communications among rhizospheric biotic components for the improvement of rhizobia–legumes interaction. In addition, it has been shown that rhizobia are part of the rhizosphere of a wide variety of non-legume plants where they can play a role as PGP components, recovering a central role in plant core microbiome (Yeoh et al., 2016).

This review provides an outline of the most studied intra- and inter-kingdom communication strategies in the rhizobium-legume cross talk, defining the most relevant mechanisms of rhizospheric communication. We conceived the exchange of signals between plants and microorganisms as an intricate Talk Show *discussion*, in which a large variety of *dialogues* can take place, regulated and coordinated by a defined line-up, but at the same time, free to influence each other.

THE PLACE OF RHIZOBIA: SETTING UP THE *SCENE*

Rhizosphere complexity makes it difficult to identify and analyze the microbiome components and in particular, the laws governing the dynamics and the maintenance of plant associated microbial communities. Among factors able to influence those microbial communities, nutrients availability and soil composition have a strong influence (Naylor and Coleman-Derr, 2018). At this level, the root soil interface is rich in exudates that can differently alter gas and soil composition, thus selecting for certain microbial populations adapted to this unique niche. Root exudates contain a large variety of secondary metabolites, such as organic acids, amino acids and sugars, which are mainly used to attract microbes (PGP or pathogenic microorganisms) through chemotaxis process, and phenolic compounds such as flavonoids, fundamental for the signaling mechanism that allowst the symbiotic interaction with rhizobia and mycorrhizal fungi to start. Interestingly, several phytormones and antimicrobial compounds (phenolics and terpenoids) are secreted by roots and used by plant as defense weapons against soil-born pathogens, to select among beneficial microbes (Baetz and Martinoia, 2014). Additionally, roots are able to produce volatile organic compounds (VOCs), which fullfill the role of rhizosphere microbial growth regulator (as antimicrobial compounds) or as significant carbon source (Peñuelas et al., 2014). Therefore, the rhizodeposition makes microbial composition of the rhizophere significantly different from that found in bulk soil (de Oliveira et al., 2017).

The plant acts as a *mediator* in the rhizospheric stage, consistently contributing to the balance of soil microbiome composition (Hartmann et al., 2009). On the other hand, the soil microbial community actively participates in the composition of the rhizospheric environment, taking part in degradation and production of organic compounds, for its own benefit (Paterson et al., 2007).

Among bacteria living in the rhizosphere and in association with plants, rhizobia can impact on surrounding microbial population by affecting legumes rhizosphere's composition and structure of the soil microbiome (Nwoke et al., 2008; Fan et al., 2018). The role of rhizobia as the main actor is mediated by a plethora of chemical and molecular signals that regulate bacterial invasion and intracellular colonization, modulate host plant defense response and nutrient exchange (Gibson et al., 2008).

Furthermore, water deficiency, salinity, heavy metals, acidity, as well as low nutrients levels are all abiotic factor that frequently can interfere with the role of rhizobia in the rhizosphere *scene* (Fagorzi et al., 2018; Bellabarba et al., 2019).

In addition, recent data have revealed that under identical soil conditions, the plant genotype, through its phenotypic features, can filter and modulate the microbial community structure and function, as well as the diversity of root associated bacteria (el Zahar Haichar et al., 2008; Berg and Smalla, 2009) carrying out a partner choice in rhizobia mutualism (Simms and Taylor, 2002).

Increasing field studies including large-scale rhizosphere samplings are now emerging with the aim to better understand the rhizospheric microbiome rules, focusing on the structure, the preservation and on the importance of communication occurring among the plants. In every case, the abiotic environmental factors (positive or negative), together with the communication among plants and rhizobial/non-rhizobial species are able to influence the rhizosphere Talk Show, giving order to the existing messy *scenario*.

SYNERGIC EFFECTS OF INTRA-KINGDOM BACTERIAL INTERACTION

The communication of microorganisms in the rhizosphere is mediated by a large diversity of microbial metabolites and physical signals, as happens in a noisy Talk Show, in which every *dialogue* can influence the success of the performance. The soil bacteria attracted by root exudates (**Figure 1**), root cell lysates and mucilages, profusely colonize the rhizosphere, thus starting the rhizosphere *dialogue*. Quorum sensing (QS), the bacterial population-dependent regulatory mechanism, is involved in bacterial cell aggregation and in the first phases of rhizosphere colonization through biofilm formation (Ng and Bassler, 2009). Indeed, rhizobia typically produce and release into the surrounding environment diffusible N-acyl homoserine lactone (AHL) molecules, which are used as signals for the control of plant- microbe interaction. The recognition of AHL molecules by bacterial cells starts and controls several processes

as the biofilm formation, Extracellular Polymeric Substances (EPS) production, bacterial motility and expression of bacterial genes relevant for symbiosis and nitrogen fixation (Loh et al., 2002; Sanchez-Contreras et al., 2007; Yang et al., 2009).

The rhizobial QS is based on the LuxR – LuxI type regulatory system. It is dependent on the threshold level of AHL which allows to induce expression of specific target genes (Veliz-Vallejos et al., 2020).

Actually, at least three LuxRI-type QS regulatory systems have been identified (Zheng et al., 2015b), and their role in interspecies communication also in co-inoculation experiments has been well demonstrated (Miao et al., 2018). Since several decades, extensive investigations of the role of the QS in rhizobial biofilms all along the root surface and inside the plants (i.e., endophytes) have been performed. Thus, strains with an effective roots colonizing ability through biofilm formation (Velmourougane et al., 2017) and flagellar-dependent chemotaxis ability (Zheng et al., 2015a) are always more advantaged than those strains without biofilm formation capabilities. Furthermore, a large variety of strategies are important for rhizobial roots colonization, such as the secretion of antibiotics and/or cell-wall degrading enzymes, which have been reported to be important for the protection against root phytopathogens (Siddiqui et al., 2000; Chandra et al., 2007) (**Figure 1**).

A wide spectrum of other physiological traits plays an important role in the rhizosphere by contributing to the complex phenomenon of nodulation competitiveness (Triplett and Sadowsky, 1992). These traits include swarming motility, type III secretion system, plasmid transfer, cell division, metabolism and transport (Calatrava-Morales et al., 2018). It is of note that their occurrence varies among species.

Microbial communities in the rhizosphere can compete for nutrients and for the plant roots colonization. Several rhizobia are able to interfere with the infecting capability of other stains through the production of bacteriocins, antimicrobial compounds that act on relative close phylogenetic species (Oresnik et al., 1999; Venter et al., 2001). Sinorhizobium meliloti, one of the most studied symbiotic species, is able to produce high-affinity siderophores which deprive antagonists of the available iron, thus limiting their plant growth (Arora et al., 2001).

Contrarily, in some cases, soil microbial communities can improve the infection capabilities of particular rhizobia and their communication with the host plant (Mehboob et al., 2013). The recent work by Miao et al. (2018) highlighted that strains of Rhizobium fabae are able to improve Rhizobium etli nodulation capabilities through a particular intra-species QS mechanism.

In this context, rhizosphere *actors* can participate in setting up the Talk Show by improving their performance. Synergic effects of specific co-inocula formulations should include a fair combination of PGP bacteria and rhizobia (Remans et al., 2008). Indeed, a range of PGP microbes can be used with rhizobia for the improvement of legumes growth and rhizobial mutalistic efficiency. The free - living diazotrophs *Azospirillum, Azotobacter, Bacillus, Pseudomonas, Serratia*, and *Enterobacter* are some of the genera that were successfully used with rhizobia as co-inoculants, potentiating growth and yield of several leguminous crops through different strategies, such as the production of phytohormones or biocontrol agents against rhizosphere phytopathogens (Tchebotar et al., 1998; Tilak et al., 2006; Remans et al., 2008). Presence of *Azospirillum* species can increase the size of the rhizobial infection site, providing space for infection and facilitating the nodulation ability of rhizobia (Tchebotar et al., 1998). Although there are many combinations of bacteria co-inoculation that have been explored for the improvement of rhizobia-legumes symbiosis, there is still the need for advanced comprehensive research in their communication systems. Indeed, a selection of the most appropriate and performing panels of PGP strains, might be used in the near future as inoculants replacing fertilizers by a more environmentally friendly agricultural practice.

THE MOST *ACCLAIMED* RHIZOSPHERIC INTER-KINGDOM COMMUNICATIONS

A large part of the rhizobia-host communication strategies in the rhizosphere influences the highly regulated and ongoing rhizobial interactions in the root tissue. In compatible legume cultivars, after the establishment in the rhizosphere, rhizobia detect plant-derived flavonoids inducing *nod* genes, which are involved in Nod factors (lipochitooligosaccharides) production (Poole et al., 2018).

Then, rhizobia gradually adhere to roots surfaces, penetrate the plant root tissues and address their lifestyle inside the newly formed root organ, the nodule. Here, rhizobia differentiate into bacteroids, the N_2 fixing form of rhizobia, thus reducing atmospheric nitrogen to ammonia benefiting, in return, of protection and availability of nutrients from the plant (Kereszt et al., 2011). In this scenario, the accessibility to the symbiotic niche is naturally regulated and limited by the host plant to respond to nitrogen needs and by competition among rhizobial strains present in the neighboring soil (Triplett and Sadowsky, 1992).

The bi-directional inter-kingdom interaction between rhizobia and the compatible legume host involves a large panel of molecular signals as well as the exchange of metabolic resources, largely explored by scientist for decades (Oldroyd, 2013; Udvardi and Poole, 2013; Poole et al., 2018). The inter-kingdom molecular communication of these social interactions includes the volatile organic compounds (VOCs) and flavonoids issued by the plant and the bacterial Nod factors responsible for the nodulation signaling pathway. Bacteria cheaters among rhizospheric populations can profit of the plant permission state to escape plant sanctions and infect the plant tissue (Kiers et al., 2003; Sachs et al., 2010; Checcucci et al., 2016; diCenzo et al., 2018).

Representing more than 70% of all lands plants, legumes are able to interact with arbuscular mycorrhizal fungi (AMF) through the recognition of the mycorrhizal oligosaccharides' factors (Myc factors). Plant-fungi association allows the fungal elongation into the root cortex, where AMF can benefit the host mediating the nutrient uptake. It is largely demonstrated that in legumes, the Talk Show *dialogue* between fungi and rhizobia can take place at the root level (Barea and Pozo, 2005; Meghvansi et al., 2008; Kaschuk et al., 2010).

To our knowledge, Crush was the first researcher who observed that the presence of mycorrhizas stimulated nodulation and consequently plant growth (Crush, 1974). Then, many others reported additional effects produced by the tripartite interaction of Arbuscular Mycorrhizal Fungi – Rhizobia - Plant (ARP): it enhances symbiotic nitrogen fixation and effectiveness in drought soils (Hao et al., 2019; Laouane et al., 2019), increases number and dry weight of nodules (Antunes and Goss, 2005) and root soluble sugar contents (Hao et al., 2019; Tang et al., 2019). For more literature on the arguments, we refers to Anandakumar et al. (2019), Sakamoto et al. (2019), Sui et al. (2019) and Zhang et al. (2020).

Besides positive effects, fungi can act as antagonistic plant pathogens, which rhizobia can counteract as biocontrol agents for multiple plant species (Deshwal et al., 2013). Furthermore, it was demonstrated that the multiple inoculations of fungi, PGP bacteria can improve symbiotic behavior and plant nutrients availability, as was shown in semi-arid and alkaline soils (Requena et al., 1997; Abd-Alla et al., 2014).

It seems that communication which rhizobia can establish with the other components of the stage, can be a potential reserve for the improvement of rhizosphere interactions, and primarily those that contribute to host plant growth (Artursson et al., 2006). Recently, transcriptomic analysis in the presence of fungal exudates has shown their positive role in the transcription of rhizobial genes associated with the chemosensory (Zhang et al., 2020). In the future, further research should be addressed to the development of new technologies and applications to enable the optimization and the subsequent exploitation of ARP tripartite interactions. In particular, the monitoring of expression profiles of genes associated with communication mechanisms, the development of microcosm systems which simulate the natural condition, the analysis of the metabolic potential of microbial (and fungal) consortia in association with host plants, might be good starting points to better understand the multi-organism's rhizosphere communication. The most recent metagenomic approaches for the study of rhizobial microbial communities, as well as the determination of the role of each actor in the rhizosphere scene, will be essential for the understanding and the subsequent exploitation of organism diversity for sustainable agriculture (Faure et al., 2009; Arora et al., 2020).

CONCLUDING REMARKS

The current understanding of the rhizosphere is highlighting the complexity of the communication strategies taking place in this model environmental *scenario*. Increasing evidence is pointing out how in this scenario rhizobia occupy an important place that extends to non-legume plants. This niche is a consequence of the influence of several factors (biotic and abiotic), at different levels (intra- and inter-kingdom) and different degrees (competition and cooperation).

Every interaction and exchange of signals taking place in one of the most known complex environments cannot be conceived as uni or bidirectional reactions, but it can be imagined as a complex network of *dialogues*, a model of noisy *discussion* while remaining inexplicably tidy and regulated by the partners themselves. Therefore, the recent studies, focused on microbial community networks models (Melke et al., 2010; Barto et al., 2012; Succurro and Ebenhöh, 2018; Mai et al., 2019), have to be adapted to the complexity of the rhizosphere. The dissection of such rhizospheric communication is essential for the improvement of the benefic aspects of such communication (Mueller and Sachs, 2015; Checcucci et al., 2017; Mueller et al., 2019) with a view to agricultural applications.

The rhizosphere *scene* can be considered as a highly suitable model for the application of a system biology study approach, including the large number of plants and microorganisms sequenced genomes, the studies on partners metabolic functionalities (Korenblum et al., 2020) and on the transcriptomic changes related to different partners interaction (Pathan et al., 2020). It is expected that future studies will continue to explore the selective forces that shape rhizosphere microbiome further elucidate the potential of the communication among the different rhizospheric partners.

AUTHOR CONTRIBUTIONS

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

FUNDING

This review was performed at the LIPM belonging to the Laboratoire d'Excellence (LABEX) entitled TULIP (ANR-10-LABX-41) and in the Laboratory of Microbiology, Department of Agricultural and Food Science, University of Bologna.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Lenie Dijkshoorn for the critical review and the English grammar editing of the manuscript.

REFERENCES

Abd-Alla, M. H., El-Enany, A.-W. E., Nafady, N. A., Khalaf, D. M., and Morsy, F. M. (2014). Synergistic interaction of *Rhizobium leguminosarum* bv. viciae and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (*Vicia faba* L.) in alkaline soil. *Microbiol. Res.* 169, 49–58. doi: 10.1016/j.micres.2013.07.007

Anandakumar, S., Kalaiselvi, T., and Sivakumar, U. (2019). Arbuscular mycorrhizal fungi (*Glomus intraradices*) and diazotrophic bacterium (Rhizobium BMBS) primed defense in blackgram against herbivorous insect (*Spodoptera litura*) infestation. *Microbiol. Res.* 231:126355. doi: 10.1016/j.micres.2019.126355

Antunes, P. M., and Goss, M. J. (2005). Communication in the tripartite symbiosis formed by arbuscular mycorrhizal fungi, rhizobia, and legume plants: a review. *Agronomy* 48:199. doi: 10.2134/agronmonogr48.c11

Arora, N. K., Fatima, T., Mishra, I., and Verma, S. (2020). "Microbe-based inoculants: role in next green revolution," in: *Environmental Concerns and Sustainable Development*, eds V. Shukla and N. Kumar (Singapore: Springer), 191–246.

Arora, N. K., Kang, S. C., and Maheshwari, D. K. (2001). Isolation of siderophore-producing strains of Rhizobium meliloti and their biocontrol potential against Macrophomina phaseolina that causes charcoal rot of groundnut. *Curr. Sci.* 81, 673–677.

Artursson, V., Finlay, R. D., and Jansson, J. K. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environ. Microbiol.* 8, 1–10. doi: 10.1111/j.1462-2920.2005.00942.x

Baetz, U., and Martinoia, E. (2014). Root exudates: the hidden part of plant defense. *Trends Plant Sci.* 19, 90–98. doi: 10.1016/j.tplants.2013.11.006

Barea, J. M., and Pozo, M. J. (2005). Microbial co-operation in the rhizosphere. *J. Exp. Bot.* 56:e1778. doi: 10.1093/jxb/eri197

Barto, E. K., Weidenhamer, J. D., Cipollini, D., and Rillig, M. C. (2012). Fungal superhighways: do common mycorrhizal networks enhance below ground communication? *Trends Plant Sci.* 17, 633–637. doi: 10.1016/j.tplants.2012.06.007

Bellabarba, A., Fagorzi, C., diCenzo, G. C., Pini, F., Viti, C., and Checcucci, A. (2019). Deciphering the symbiotic plant microbiome: translating the most recent discoveries on rhizobia for the improvement of agricultural practices in metal-contaminated and high saline lands. *Agronomy* 9:529. doi: 10.3390/agronomy9090529

Bending, G. D. (2017). "The rhizopshere and its microorganisms," in *Encyclopedia of Applied Plant Sciences, 2nd edn*, eds B. Thomas, B. G. Murray, and D. J. Murphy (Academic Press), 347–351. doi: 10.1016/B978-0-12-394807-6.00165-9

Berg, G., and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13. doi: 10.1111/j.1574-6941.2009.00654.x

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E. V. L., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Calatrava-Morales, N., McIntosh, M., and Soto, M. (2018). Regulation mediated by N-Acyl homoserine lactone quorum sensing signals in the rhizobium-legume symbiosis. *Genes* 9:263. doi: 10.3390/genes9050263

Chandra, S., Choure, K., Dubey, R. C., and Maheshwari, D. K. (2007). Rhizosphere competent Mesorhizobiumloti MP6 induces root hair curling, inhibits *Sclerotinia sclerotiorum* and enhances growth of Indian mustard (*Brassica campestris*). *Braz. J. Microbiol.* 38, 124–130. doi: 10.1590/S1517-83822007000100026

Checcucci, A., Azzarello, E., Bazzicalupo, M., Galardini, M., Lagomarsino, A., Mancuso, S., et al. (2016). Mixed nodule infection in *Sinorhizobium meliloti*–*Medicago sativa* symbiosis suggest the presence of cheating behavior. *Front. Plant Sci.* 7:835. doi: 10.3389/fpls.2016.00835

Checcucci, A., DiCenzo, G. C., Bazzicalupo, M., and Mengoni, A. (2017). Trade, diplomacy, and warfare: the quest for elite rhizobia inoculant strains. *Front. Microbiol.* 8:2207. doi: 10.3389/fmicb.2017.02207

Chi, F., Shen, S.-H., Cheng, H.-P., Jing, Y.-X., Yanni, Y. G., and Dazzo, F. B. (2005). Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl. Env. Microbiol.* 71, 7271–7278. doi: 10.1128/AEM.71.11.7271-7278.2005

Crush, J. R. (1974). Plant growth responses to vesicular-arbuscular mycorrhiza VII. Growth and modulation of some herbage legumes. *New Phytol.* 73, 743–749. doi: 10.1111/j.1469-8137.1974.tb01302.x

de Oliveira, M. C. F., Mafezoli, J., and Gunatilaka, A. A. L. (2017). Natural products of the rhizosphere and its microorganisms. *Chem. Biol. Nat. Prod.* 29, 285-331. doi: 10.1201/9781315117089-10

Deshwal, V. K., Singh, S. B., Kumar, P., and Chubey, A. (2013). Rhizobia unique plant growth promoting rhizobacteria: a review. *Int. J. Life Sci.* 2, 74–86.

diCenzo, G. C., Debiec, K., Krzysztoforski, J., Uhrynowski, W., Mengoni, A., Fagorzi, C., et al. (2018). Genomic and biotechnological characterization of the heavy-metal resistant, arsenic-oxidizing bacterium Ensifer sp. M14. *Genes* 9:379. doi: 10.3390/genes9080379

el Zahar Haichar, F., Marol, C., Berge, O., Rangel-Castro, J. I., Prosser, J. I., Balesdent, J., et al. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *ISME J.* 2:1221. doi: 10.1038/ismej.2008.80

Fagorzi, C., Checcucci, A., diCenzo, G., Debiec-Andrzejewska, K., Dziewit, L., Pini, F., et al. (2018). Harnessing rhizobia to improve heavy-metal phytoremediation by legumes. *Genes* 9:542. doi: 10.3390/genes9110542

Fan, M., Xiao, X., Guo, Y., Zhang, J., Wang, E., Chen, W., et al. (2018). Enhanced phytoremdiation of Robinia pseudoacacia in heavy metal-contaminated soils with rhizobia and the associated bacterial community structure and function. *Chemosphere* 197, 729–740. doi: 10.1016/j.chemosphere.2018.01.102

Faure, D., Vereecke, D., and Leveau, J. H. J. (2009). Molecular communication in the rhizosphere. *Plant Soil* 321, 279–303. doi: 10.1007/s11104-008-9839-2

Gibson, K. E., Kobayashi, H., and Walker, G. C. (2008). Molecular determinants of a symbiotic chronic infection. *Annu. Rev. Genet.* 42, 413–441. doi: 10.1146/annurev.genet.42.110807.091427

Hao, Z., Xie, W., Jiang, X., Wu, Z., Zhang, X., and Chen, B. (2019). Arbuscular mycorrhizal fungus improves Rhizobium–Glycyrrhiza seedling symbiosis under drought stress. *Agronomy* 9:572. doi: 10.3390/agronomy9100572

Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* 16, 463–471. doi: 10.1016/j.tim.2008.07.008

Hartmann, A., Rothballer, M., and Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant Soil* 312, 7–14. doi: 10.1007/s11104-007-9514-z

Hartmann, A., Schmid, M., van Tuinen, D., and Berg, G. (2009). Plant-driven selection of microbes. *Plant Soil.* 321, 235–257. doi: 10.1007/s11104-008-9814-y

Hauser, M. D. (1996). *The Evolution of Communication*. Cambridge, MA: MIT Press.

Kaschuk, G., Leffelaar, P. A., Giller, K. E., Alberton, O., Hungria, M., and Kuyper, T. W. (2010). Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: a meta-analysis of potential photosynthate limitation of symbioses. *Soil Biol. Biochem.* 42, 125–127. doi: 10.1016/j.soilbio.2009.10.017

Kereszt, A., Mergaert, P., and Kondorosi, E. (2011). Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Mol. Plant. Microbe Interact.* 24, 1300–1309. doi: 10.1094/MPMI-06-11-0152

Kiers, E. T., Rousseau, R. A., West, S. A., and Denison, R. F. (2003). Host sanctions and the legume–rhizobium mutualism. *Nature* 425:78. doi: 10.1038/nature01931

Korenblum, E., Dong, Y., Szymanski, J., Panda, S., Jozwiak, A., Massalha, H., et al. (2020). Rhizosphere microbiome mediates systemic root metabolite exudation by root-to-root signaling. *Proc. Natl. Acad. Sci.* 117, 3874–3883. doi: 10.1073/pnas.1912130117

Laouane, R., Ben, M.eddich, A., Bechtaoui, N., and Oufdou, K., Wahbi, S. (2019). Effects of arbuscular mycorrhizal fungi and rhizobia symbiosis on the tolerance of *Medicago sativa* to salt stress. *Gesunde Pflanz.* 71, 135–146. doi: 10.1007/s10343-019-00461-x

Loh, J., Pierson, E. A., Pierson, I. I. I., L. S., Stacey, G., and Chatterjee, A. (2002). Quorum sensing in plant-associated bacteria. *Curr. Opin. Plant Biol.* 5, 285–290. doi: 10.1016/S1369-5266(02)00274-1

Mai, T. H., Schnepf, A., Vereecken, H., and Vanderborght, J. (2019). Continuum multiscale model of root water and nutrient uptake from soil with explicit consideration of the 3D root architecture and the rhizosphere gradients. *Plant Soil* 439, 273–292. doi: 10.1007/s11104-018-3890-4

Meghvansi, M. K., Prasad, K., Harwani, D., and Mahna, S. K. (2008). Response of soybean cultivars toward inoculation with three arbuscular mycorrhizal fungi and Bradyrhizobium japonicum in the alluvial soil. *Eur. J. Soil Biol.* 44, 316–323. doi: 10.1016/j.ejsobi.2008.03.003

Mehboob, I., Naveed, M., Zahir, Z. A., and Sessitsch, A. (2013). "Potential of rhizosphere bacteria for improving Rhizobium-legume symbiosis," in: *Plant Microbe Symbiosis: Fundamentals and Advances*, ed N. K. Arora (New Delhi: Springer), 305–349.

Melke, P., Sahlin, P., Levchenko, A., and Jönsson, H. (2010). A cell-based model for quorum sensing in heterogeneous bacterial colonies. *PLoS Comput. Biol.* 6:e1000819. doi: 10.1371/journal.pcbi.1000819

Miao, J., Zhang, N., Liu, H., Wang, H., Zhong, Z., and Zhu, J. (2018). Soil commensal rhizobia promote *Rhizobium etli* nodulation efficiency through CinR-mediated quorum sensing. *Arch. Microbiol.* 200, 685–694. doi: 10.1007/s00203-018-1478-2

Mueller, C. W., Carminati, A., Kaiser, C., Subke, J.-A., and Gutjahr, C. (2019). Rhizosphere functioning and structural development as complex interplay between plants, microorganisms and soil minerals. *Front. Environ. Sci*:7, 130. doi: 10.3389/fenvs.2019.00130

Mueller, U. G., and Sachs, J. L. (2015). Engineering microbiomes to improve plant and animal health. *Trends Microbiol.* 23, 606–617. doi: 10.1016/j.tim.2015.07.009

Naylor, D., and Coleman-Derr, D. (2018). Drought stress and root-associated bacterial communities. *Front. Plant Sci.* 8:2223. doi: 10.3389/fpls.2017.02223

Ng, W.-L., and Bassler, B. L. (2009). Bacterial quorum-sensing network architectures. *Annu. Rev. Genet*. 43, 197–222. doi: 10.1146/annurev-genet-102108-134304

Nwoke, O. C., Diels, J., Abaidoo, R., Nziguheba, G., and Merckx, R. (2008). Organic acids in the rhizosphere and root characteristics of soybean (Glycine max) and cowpea (*Vigna unguiculata*) in relation to phosphorus uptake in poor savanna soils. *Afr. J. Biotechnol*. 7, 3620–3627. doi: 10.5897/AJB08.438

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol*. 11:252. doi: 10.1038/nrmicro2990

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., and Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet*. 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Oresnik, I. J., Twelker, S., and Hynes, M. F. (1999). Cloning and characterization of a *Rhizobium leguminosarum* gene encoding a bacteriocin with similarities to RTX toxins. *Appl. Environ. Microbiol*. 65, 2833–2840. doi: 10.1128/AEM.65.7.2833-2840.1999

Paterson, E., Gebbing, T., Abel, C., Sim, A., and Telfer, G. (2007). Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytol*. 173, 600–610. doi: 10.1111/j.1469-8137.2006.01931.x

Pathan, S. I., Ceccherini, M. T., Sunseri, F., and Lupini, A. (2020). "Rhizosphere as hotspot for plant-soil-microbe interaction," in: *Carbon and Nitrogen Cycling in Soil*, eds R. Datta, R. S. Meena, S. I. Pathan, and M. T. Ceccherini (Singapore: Springer), 17–43.

Peñuelas, J., Asensio, D., Tholl, D., Wenke, K., Rosenkranz, M., Piechulla, B., et al. (2014). Biogenic volatile emissions from the soil. *Plant Cell Environ*. 37, 1866–1891. doi: 10.1111/pce.12340

Poole, P., Ramachandran, V., and Terpolilli, J. (2018). Rhizobia: from saprophytes to endosymbionts. *Nat. Rev. Microbiol*. 16:291. doi: 10.1038/nrmicro.2017.171

Rasmann, S., and Turlings, T. C. J. (2016). Root signals that mediate mutualistic interactions in the rhizosphere. *Curr. Opin. Plant Biol*. 32, 62–68. doi: 10.1016/j.pbi.2016.06.017

Remans, R., Ramaekers, L., Schelkens, S., Hernandez, G., Garcia, A., Reyes, J. L., et al. (2008). Effect of Rhizobium–Azospirillum coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. *Plant Soil* 312, 25–37. doi: 10.1007/s11104-008-9606-4

Requena, N., Jimenez, I., Toro, M., and Barea, J. M. (1997). Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and Rhizobium spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. *New Phytol*. 136, 667–677. doi: 10.1046/j.1469-8137.1997.00786.x

Sachs, J. L., Ehinger, M. O., and Simms, E. L. (2010). Origins of cheating and loss of symbiosis in wild Bradyrhizobium. *J. Evol. Biol*. 23, 1075–1089. doi: 10.1111/j.1420-9101.2010.01980.x

Sakamoto, K., Ogiwara, N., Kaji, T., Sugimoto, Y., Ueno, M., Sonoda, M., et al. (2019). Transcriptome analysis of soybean (Glycine max) root genes differentially expressed in rhizobial, arbuscular mycorrhizal, and dual symbiosis. *J. Plant Res*. 132, 541–568. doi: 10.1007/s10265-019-01117-7

Sanchez-Contreras, M., Bauer, W. D., Gao, M., Robinson, J. B., and Allan Downie, J. (2007). Quorum-sensing regulation in rhizobia and its role in symbiotic interactions with legumes. *Philos. Trans. R. Soc. B Biol. Sci.* 362, 1149–1163.

Schott-Phillips, T. C. (2008). Defining biological communication. *J. Evol. Biol*. 21, 387–395. doi: 10.1111/j.1420-9101.2007.01497.x

Siddiqui, I. A., Ehteshamul-Haque, S., Zaki, M. J., and Abdul, G. (2000). Effect of urea on the efficacy of Bradyrhizobium Sp. and *Trichoderma harzianum* in the control of root infecting fungi in mungbean and sunflower. *Sarhad J. Agric*. 16, 403–406.

Simms, E. L., and Taylor, D. L. (2002). Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integr. Comp. Biol*. 42, 369–380. doi: 10.1093/icb/42.2.369

Smith, J. M., and Harper, D. (2003). *Animal Signals*. Oxford: Oxford University Press.

Succurro, A., and Ebenhöh, O. (2018). Review and perspective on mathematical modeling of microbial ecosystems. *Biochem. Soc. Trans*. 46, 403–412. doi: 10.1042/BST20170265

Sui, X., Zhang, T., Tian, Y., Xue, R., and Li, A. (2019). A neglected alliance in battles against parasitic plants: arbuscular mycorrhizal and rhizobial symbioses alleviate damage to a legume host by root hemiparasitic Pedicularis species. *New Phytol*. 221, 470–481. doi: 10.1111/nph.15379

Tang, T.-T., Xie, M.-M., Chen, S.-M., Zhang, S.-M., and Wu, Q.-S. (2019). Effects of arbuscular mycorrhizal fungi and rhizobia on physiological activities in white clover (*Trifolium repens*). *Biotechnology* 18, 49–54. doi: 10.3923/biotech.2019.49.54

Tchebotar, V. K., Kang, U. G., Asis Jr, C. A., and Akao, S. (1998). The use of GUS-reporter gene to study the effect of Azospirillum-Rhizobium coinoculation on nodulation of white clover. *Biol. Fertil. Soils* 27, 349–352. doi: 10.1007/s003740050442

Tilak, K., Ranganayaki, N., and Manoharachari, C. (2006). Synergistic effects of plant-growth promoting rhizobacteria and Rhizobium on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*). *Eur. J. Soil Sci*. 57, 67–71. doi: 10.1111/j.1365-2389.2006.00771.x

Triplett, E. W., and Sadowsky, M. J. (1992). Genetics of competition for nodulation of legumes. *Annu. Rev. Microbiol*. 46, 399–422. doi: 10.1146/annurev.mi.46.100192.002151

Udvardi, M., and Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annu. Rev. Plant Biol*. 64, 781–805. doi: 10.1146/annurev-arplant-050312-120235

Veliz-Vallejos, D. F., Kawasaki, A., and Mathesius, U. (2020). The presence of plant-associated bacteria alters responses to N-acyl homoserine lactone quorum sensing signals that modulate nodulation in *Medicago truncatula*. *Plants* 9:777. doi: 10.3390/plants9060777

Velmourougane, K., Prasanna, R., and Saxena, A. K. (2017). Agriculturally important microbial biofilms: present status and future prospects. *J. Basic Microbiol*. 57, 548–573. doi: 10.1002/jobm.201700046

Venter, A. P., Twelker, S., Oresnik, I. J., and Hynes, M. F. (2001). Analysis of the genetic region encoding a novel rhizobiocin from *Rhizobium leguminosarum* bv. viciae strain 306. *Can. J. Microbiol*. 47, 495–502. doi: 10.1139/w01-043

Venturi, V., and Keel, C. (2016). Signaling in the rhizosphere. *Trends Plant Sci*. 21, 187–198. doi: 10.1016/j.tplants.2016.01.005

Werner, G. D. A., Cornwell, W. K., Cornelissen, J. H. C., and Kiers, E. T. (2015). Evolutionary signals of symbiotic persistence in the legume–rhizobia mutualism. *Proc. Natl. Acad. Sci*. 112, 10262–10269. doi: 10.1073/pnas.1424030112

Yang, M., Sun, K., Zhou, L., Yang, R., Zhong, Z., and Zhu, J. (2009). Functional analysis of three AHL autoinducer synthase genes in *Mesorhizobium loti* reveals the important role of quorum sensing in symbiotic nodulation. *Can. J. Microbiol*. 55, 210–214. doi: 10.1139/W08-128

Yeoh, Y. K., Paungfoo-Lonhienne, C., Dennis, P. G., Robinson, N., Ragan, M. A., Schmidt, S., et al. (2016). The core root microbiome of sugarcanes cultivated under varying nitrogen fertilizer application. *Environ. Microbiol*. 18, 1338–1351. doi: 10.1111/1462-2920.12925

Zahavi, A. (2008). "The handicap principle and signalling in collaborative systems," in: *Sociobiology of Communication*, eds P. d'Ettorre and D. P. Hughes (Oxford; New York, NY: Oxford University Press), 1–10.

Zhang, W., Li, X. G., Sun, K., Tang, M. J., Xu, F. J., Zhang, M., et al. (2020). Mycelial network-mediated rhizobial dispersal enhances legume nodulation. *ISME J*. 14, 1015–1029. doi: 10.1038/s41396-020-0587-5

Zheng, H., Mao, Y., Teng, J., Zhu, Q., Ling, J., and Zhong, Z. (2015a). Flagellar-dependent motility in *Mesorhizobium tianshanense* is involved in the early stage of plant host interaction: study of an flgE mutant. *Curr. Microbiol*. 70, 219–227. doi: 10.1007/s00284-014-0701-x

Zheng, H., Mao, Y., Zhu, Q., Ling, J., Zhang, N., Naseer, N., et al. (2015b). The quorum sensing regulator CinR hierarchically regulates two other quorum sensing pathways in ligand-dependent and-independent fashions in *Rhizobium etli*. *J. Bacteriol*. 197, 1573–1581. doi: 10.1128/JB.00003-15

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

Copyright © 2020 Checcucci and Marchetti. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

REVIEW
published: 11 March 2021
doi: 10.3389/fagro.2021.637196

Will Phosphate Bio-Solubilization Stimulate Biological Nitrogen Fixation in Grain Legumes?

*Walid Janati[1,2], Bouchra Benmrid[1], Wissal Elhaissoufi[1], Youssef Zeroual[3], Joshua Nasielski[4] and Adnane Bargaz[1]**

[1] AgroBioSciences, Laboratory of Plant-Microbe Interactions, Mohammed VI Polytechnic University, Ben Guerir, Morocco, [2] Department of Life Sciences, Laboratory of Functional Ecology and Environmental Engineering, Faculty of Sciences and Techniques, Sidi Mohamed Ben Abdellah University, Fez, Morocco, [3] Situation Innovation, OCP Group, El Jadida, Morocco, [4] Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada

Biological nitrogen fixation (BNF) refers to a bacterially mediated process by which atmospheric N_2 is reduced, either symbiotically or non-symbiotically, into ammonia (NH_3) in the presence of the enzyme complex nitrogenase. In N_2-fixing grain legumes, BNF is often hampered under low phosphorus (P) availability. The P status of legumes, particularly nodules, as well as P availability in the rhizosphere, play a vital role in regulating BNF. Aside from increasing P availability via fertilization, other plant traits (i.e., extensive rooting system and their spatial distribution, hyper-nodulation, root exudates, rhizosphere acidification, and heterogeneity) contribute to greater P uptake and hence more effective BNF. The positive interaction between P availability and BNF can be exploited through beneficial soil P solubilizing microorganisms (PSM). These microorganisms can increase plant-available P by modifying either rhizosphere soil processes or promoting plant traits, which lead to increased P uptake by the production of plant growth-promoting substances, both of which could indirectly influence the efficiency of BNF in legumes. In this review, we report on the importance of microbial P bio-solubilization as a pathway for improving BNF in grain legumes via PSM and P solubilizing bacteria (PSB). Because BNF in legumes is a P-requiring agro-ecological process, the ability of soil PSB to synergize with the rhizobial strains is likely a key belowground process worth investigating for advanced research aiming to improve rhizosphere biological functions necessary for sustainable legume-based cropping systems.

Keywords: crops, inoculant, legumes, nodules, nitrogen fixation, rhizobia, rhizosphere, sustainability

OPEN ACCESS

Edited by:
Jorge Monza,
Universidad de la República, Uruguay

Reviewed by:
Mahaveer P. Sharma,
ICAR Indian Institute of Soybean Research, India
Bahar S. Razavi,
University of Kiel, Germany

Correspondence:
Adnane Bargaz
adnane.bargaz@um6p.ma

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *02 December 2020*
Accepted: *17 February 2021*
Published: *11 March 2021*

Citation:
Janati W, Benmrid B, Elhaissoufi W, Zeroual Y, Nasielski J and Bargaz A (2021) Will Phosphate Bio-Solubilization Stimulate Biological Nitrogen Fixation in Grain Legumes? Front. Agron. 3:637196. doi: 10.3389/fagro.2021.637196

INTRODUCTION

Legume crops play key roles both in food security and agro-ecosystems sustainability by providing several services at the food- and production-system levels (Stagnari et al., 2017). For instance, different legume crops varieties constitute a major source of plant-based food and feed and these crops can provide many other beneficial services (i.e., biological nitrogen (N_2) fixation, mitigation of greenhouse emission, suppression of weeds and plant diseases, etc.) (Sanginga et al., 2003; Stagnari et al., 2017; Mitran et al., 2018). In fact, legume crops play a key role in integrated soil fertility management owing to their capacity to fix atmospheric N_2 in association with rhizobia, which can lead to increased soil fertility and decreased dependence on N-fertilizers in low-input farming systems (Atienza and Rubiales, 2017; Vanlauwe et al., 2019).

Traditionally, the application of organic and inorganic fertilizers to farm fields has been used to correct nutrient deficiencies and maintain nutrient balances (Li et al., 2020; Tewari et al., 2020), but negative environmental impacts could occur as a result of excessive and irrational nutrients application in agro-ecosystems that has received justified attention from stakeholders along the entire food value chain (Choudhury and Kennedy, 2005). There is increasing attention paid to the ability of purposefully selected soil microorganisms to enhance soil fertility through biological methods. The role of soil microorganisms in the cycling of N and P are well-known, particularly the bacteria involved in BNF. But there is a growing understanding of the importance of other bacteria and fungi, either in the soil or in the plant (endophytes), for grain legume performance. Indeed, the exploitation and use of these soil microorganisms is a viable ecological strategy to reduce farmer dependence on inorganic fertilizers and enhance soil health (Zaidi et al., 2010). Enhancing biological nitrogen fixation (BNF) in grain legumes *via* soil microorganisms not directly involved in BNF could be a propitious option for increasing the supply of affordable and eco-friendly protein for human consumption while also improving the overall sustainability of cropping systems (Meena and Lal, 2018).

Some microorganisms at the soil root interface have the ability to establish a N_2-fixing symbiosis with legumes based on nodule formation on roots or stems (Skorupska et al., 2010). Rhizobial symbiosis induces the formation of nodules in host plants' roots and fixes N_2 to provide plants with ammonium-N (Ahmad et al., 2014). In addition, rhizobia can also promote legume growth by other direct or indirect mechanisms (Zaidi et al., 2010). For instance, by improving water and nutrient uptake *via* tripartite symbiosis established by association with arbuscular mycorrhizal fungi (AMF) (Püschel et al., 2017). Another group of soil microorganisms namely P solubilizing microorganisms (PSM) can efficiently solubilize inorganic P forms, by transforming insoluble P into soluble and plant-available forms. These microbial activities can enhance legume production while reducing reliance on fertilizer inputs (mainly N and P) (Ahmad et al., 2014). Therefore, for sustainable legume-based cropping systems, biological fertilization *via* the application of specific microbes should be promising for both crop productivity and economic-environmental sustainability.

The BNF is an energetically costly bioprocess that requires a significant investment in carbon and P (Püschel et al., 2017). Indeed the efficiency of BNF in grain legumes can be limited by P (Khan et al., 2007; Nasto et al., 2014; Aziz et al., 2016). According to Mitran et al. (2018), a number of studies reported a significant correlation between nodular P content and N_2 fixation. In line with this, Attar et al. (2012) unveiled that P fertilization at 90 kg ha^{-1} enhanced the ability of common bean (*Phaseolus vulgaris*) to fix N_2. In addition, it increased the release of hydrogen by roots which increased the plant-available form of P in the rhizosphere; which is the zone of soil surrounding the roots, rich in nutrients and contains a wide variety of microorganisms able to solubilize P (Vacheron et al., 2013); eventually leading to increased P uptake (Attar et al., 2012). In fact, adequate supply of P to plants has been found to regulate several metabolic processes related to BNF, such as ammonium assimilation into amino acids and ureides (Mitran et al., 2018), as well as the synthesis of mitochondrial and symbiosome membranes for functional N_2-fixing nodules (Bargaz et al., 2018; Si et al., 2019). Moreover, several authors claimed that the high demand of P during this biological process is also due to N_2-fixing bacteria, whose P requirement was found to be higher compared to the non-N_2-fixing bacteria (Nasto et al., 2017; Van Gerven et al., 2019; Fernández-Juárez et al., 2020). Altogether, nodule formation, nodule functioning, and the energy costs of BNF in legumes strongly depend on the P status in plants and nodule tissues, and inadequate P status will reduce potential BNF contribution to N uptake (Bargaz et al., 2018). Given all of the knowledge regarding the key role of P in enhancing plant growth either by boosting the size of the root system (i.e., root hair density, lateral root growth, root surface area, and nodulation) or by stimulating the BNF process, soil PSM represents a promising strategy that could be adopted to increase plant-accessible forms of P to maintain soil health, fertility and agricultural sustainability (Wani et al., 2007; Qureshi et al., 2011; Bargaz et al., 2018; Elhaissoufi et al., 2020).

In the context of enhancing BNF in legumes, the present review summarizes available knowledge on the importance of PSM, with a focus on PSB, on BNF in grain legumes. We begin with an overview of the role of P in BNF. We then focus on recent research examining the ability of P bio-solubilization to enhance BNF. In addition, we hypothesized that rates of BNF could be sustainably enhanced in grain legumes via farmer use of PSM inoculation, given the importance of PSM as a biological component.

IMPORTANCE OF P IN LEGUME BNF

Biological N_2 fixation is a symbiotic process (Lindström and Mousavi, 2020), during which plants provide shelter and carbon as an energy source to the symbiotic microorganisms in exchange for bacterially reduced nitrogenous compounds that can be readily assimilated by the host plant (Skorupska et al., 2010; Lazali et al., 2013; Lindström and Mousavi, 2020). Indeed, several published literatures reported that BNF supplies legume plants with an average of 80% of N needs (Skorupska et al., 2010; Santachiara et al., 2017), thus reducing the amount of mineral N needed especially in low inputs legume-based cropping systems (Hardarson and Atkins, 2003).

However, the establishment of this symbiotic relationship is shown to be affected by various biotic and abiotic factors among which is P deficiency (Mitran et al., 2018). It is of note that P is the second major macronutrient required for plant growth after N, and it plays essential roles during all plant growth stages (Malhotra et al., 2018). Phosphorus is involved in the biosynthesis of major cellular constituents (i.e., nucleic acids, enzymes, phospholipids, ATP, and nucleotides), as well as in the control of vital processes such as photosynthesis, respiration, and energy generation (Balemi and Negisho, 2012; Sanz-Saez et al., 2017; Malhotra et al., 2018). While P deficiency reduces overall plant function and growth, its impact on bacterial BNF is one of the indirect functions played by P in legumes. Additionally, P

controls the survival of soil microorganisms such as diazotrophic bacteria, as they require P for their growth; low levels of this nutrient may influence early-stage colonization processes and later nodules formation in legumes (Malhotra et al., 2018).

In fact, BNF as one of the main rhizosphere biological processes, is correlated with P availability wherein P deficiency strongly limits the activity of diazotrophic (i.e., N_2-fixing bacteria), reduces the symbiotic partnership between plant host and rhizobia, as well as the N_2-fixation process itself (Aziz et al., 2016; Martins et al., 2017; Sanz-Saez et al., 2017; Li et al., 2020; Tewari et al., 2020). Tewari et al. (2020) further highlighted the role of P as a structural element in controlling the BNF process. As an example, Tindwa et al. (2019) found that increased soil P availability in the presence of liming led to a subsequent increase in nodulation parameters such as, nodule number, nodule weight and volume, which in turn increased soybean (*Glycine max*) growth and the amount of fixed N_2. Generally, P deficiency reduces nodule formation, development and function, reducing the supply of energy allocated to support nodule metabolic activity (Zaidi et al., 2010). Additionally, it was found that the P requirement for N_2-fixing plants is higher than that of non-N_2-fixing plants (Sanz-Saez et al., 2017). An increased P requirement of grain legumes is mainly due to the high P demand for BNF processes (Sanz-Saez et al., 2017; Mitran et al., 2018). In contrast, other authors claimed that the high demand of P during this biological process is due to the needs of N_2-fixing bacteria, whose P requirement were found to be higher compared to non-N_2_fixers (Nasto et al., 2017; Van Gerven et al., 2019; Fernández-Juárez et al., 2020). Interestingly, the authors reported that the availability of various phospholipids (such as phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine) might play a role in nodulation and maintenance of legume-rhizobia symbiosis (Tewari et al., 2020). These compounds are essential for the establishment of a proper rhizobial membrane architecture that allows effective participation in BNF. In addition to its important role in BNF that takes place in the bacteroids, Mitran et al. (2018) reported a significant correlation between P concentration in the plant cell fraction of nodules and N_2-fixation. This is because several metabolic processes that occur in plant cells notably, ammonium assimilation into amino acids and ureides were also found to be critically affected by P concentration (Batterman et al., 2013; Singh et al., 2013; Mitran et al., 2018). Li et al. (2020) found that increasing P content in soil and wheat (*Triticum aestivum*) plant tissues also enhanced total N and nitrogenase activity in plant shoots and roots along with *nifH* gene expression, supporting our understanding of the role of P in the enhancement of BNF (Wani et al., 2007).

The importance of adequate P availability to support BNF of grain legumes is receiving more attention (Olivera et al., 2004). A study by Taliman et al. (2019) found that N_2 fixation and nodule biomass of soybean increased in response to high P fertilization. Co-application of biochar and P fertilizer to cowpea (*Vigna unguiculata*) improved soil properties encompassing cation exchange capacity, soil organic carbon, and total N, which correlated positively with nodules formation and subsequently N_2 fixation capacity (Phares et al., 2020). Kyei-Boahen et al. (2017) found that cowpea treated with rhizobia and P fertilizer showed greater N_2 fixation as a result to increased number and biomass of nodules, ultimately leading to increased yield. Moreover, Sanz-Saez et al. (2017) reported that nodule and energy-generating metabolism depend strongly on P availability, as P deficiency reduces P-nodules and Pi-adenylates levels, resulting in decreased nitrogenase activity. Ultimately, this reduces the proportion of plant N derived from BNF (Sanz-Saez et al., 2017). To minimize the effects of P deficiency on BNF, nodules develop mechanisms to recycle and conserve internal P instead of acquiring external P (Vardien et al., 2014; Sanz-Saez et al., 2017). Indeed, leguminous plants tend to reallocate P from both leaves and roots to their nodules, which in turn reduced aboveground processes such as photosynthesis, disturbing plant growth directly or indirectly (Sanz-Saez et al., 2017; Stevens et al., 2019).

PHOSPHATE SOLUBILISING MICROORGANISMS STIMULATE BNF IN GRAIN LEGUMES

The role of PSM such as bacteria and fungi in increasing soil P available fraction has been widely investigated by many researchers (Manzoor et al., 2017; Adhikari and Pandey, 2019; Faried et al., 2019). Phosphate solubilizing bacteria are the most known and studied microorganisms for their potential to solubilize P. These microbes participate in bio-geochemical P cycling mainly through solubilization of inorganic forms of P and mineralization of organic ones (Manzoor et al., 2017; Abbasi and Manzoor, 2018). The microbial solubilization of insoluble P has been often attributed to organic acid excretion (i.e., lactic acid, succinic acid, acetic acid, fumaric acid, etc.) (Bhowmik and Das, 2018). In addition to their P bio-solubilization ability, PSM enhance plant growth through various mechanisms such as the release of important growth-promoting substances, the increase in soil availability of other micronutrients as well as the enhancement of N_2 fixation efficiency (Zaidi et al., 2009; Rahman et al., 2017). Therefore, the application of these microbes to nutrient-deficient soils, where large amounts of nutrients are fixed in unavailable forms, could help farmers increase crop yield and financial returns *via* more rational use of P fertilizers inputs and an increase in BNF (Püschel et al., 2017). This fact was confirmed by Belimov et al. (1995) whose study consisted of using the ^{15}N isotope dilution technique, and which revealed that a combination of N_2-fixing (*Azospirillum lipoferum*) and P-solubilizing (*Agrobacterium radiobacter*) bacteria significantly increased N accumulation in barley (*Hordeum vulgare*). This corroborates with findings by Wani et al. (2007), who showed that triple inoculation of chickpea (*Cicer arietinum*) with a PSB (from genus *Bacillus* or *Pseudomonas*) and two N_2 fixing Bacteria (*Mesorhizobium cicero* and *Azotobacter chroococcum*) increased soil fertility in terms of P and N, enhanced nodulation, and stimulated chickpea growth compared to single inoculation. These positive effects were attributed to the synergies between PSM (particularly PSB) and N_2 fixing bacteria, which resulted in enhanced BNF and increased nutrient use efficiency (Wani

et al., 2007). Likewise, the combination of *Rhizobium* and a PSB strain (*Bacillus megatherium*), increased yield and seed quality of faba bean (*Vicia faba*) (Rugheim and Abdelgani, 2012) as well as yield, nodule biomass, shoot biomass and the N and P content of lablab bean (*Lablab purpureus*) shoots (Hassan and Abdelgan, 2009). These positive effects were concomitant with increased soil nutrients availability (N and P) and uptake in unfertilized soils.

Therefore, the synergy between soil PSM and rhizobial strains nodulating leguminous plants represent a key rhizosphere process that deserves to be thoroughly investigated to improve rhizosphere biological functions in grain legumes and enhance the sustainability of legume-based cropping systems. In line with this, several studies reported that soil application of PSM, including PSB, individually or in a consortium, increased plant growth through solubilization of insoluble P and subsequent increase in BNF (Batterman et al., 2013; Singh et al., 2013). In this context, Zaidi et al. (2010) found that PSB (genus *Serratia*) in association with N_2-fixers (*Mesorhizobium ciceri*) and AMF (*Glomus fasciculatum*) could increase grain legume growth through a diverse range of mechanisms (i.e., enhanced BNF along with increased soil N and P availability), hence providing a promising solution to sustain low-input agricultural cropping systems. This finding was further confirmed by Ahmad et al. (2019) who showed that increased growth and nutritional status of mung bean (*Vigna radiata*) plants following their inoculation with PSB strains (*Bacillus aryabhattai* S10 and *Bacillus subtilis* ZM63) were concomitant with enhanced nodulation, measured in terms of nodules number and weight. A recent study by Li et al. (2020) showed that wheat co-inoculation with a diazotrophic bacterium (*Paenibacillus beijingensis* BJ-18) and a PSB (*Paenibacillus sp.* B1) significantly increased plant growth as well as P and N content of both the plant (roots and shoots) and the soil. Elkoca et al. (2008) indicated a significant increase in nodulation, plant N content, yield, and seed protein content in chickpea in response to a mixed inoculation with *Rhizobium*, N_2 fixing (*Bacillus subtilis* OSU-142) and PSB (*Bacillus megaterium*M-3). Furthermore, Qureshi et al. (2011) reported an increase of mung bean root parameters (root mass, root length, nodule number, and nodule mass) in response to their inoculation with *Rhizobium phaseoli* and *Bacillus megaterium*, which subsequently increased uptake and use efficiency of N and P. Likewise, when chickpea was inoculated with N_2-fixing bacteria (*Mesorhizobium ciceri*), a phosphate solubilizing fungus (*Penicillium* WF6) and/or a PSB (*Serratia* T1), it resulted in an increase of P availability and uptake and also positively impacted BNF (Zaidi and Khan, 2007).

The synergies between legumes root, rhizobia, and AMF was also confirmed by Tajini et al. (2012) who carried out an experiment in which common bean plants treated with rhizobia (*Rhizobium tropici*) and inoculated with AMF (*Glomus intraradices*) showed enhanced BNF under low P conditions. This effect was likely mediated by improved P acquisition during legume-rhizobia-AMF symbiosis. According to Sanz-Saez et al. (2017), the increase in P uptake can be explained by the fact that AMF develops an important fungal hyphae network, which increases the root absorptive area and capacity. Results from these cited studies provide strong evidence regarding the synergy between BNF and the use of PSB and PSM, either applied solely or in combined treatment. Adding PSB or PSM appears to increase BNF as well as P uptake, resulting in overall greater grain legume productivity. Optimizing PSM/PSB use in legume-based cropping systems may not only increase soil P availability, which would itself stimulate BNF, but may also enhance crop growth and yield through various mechanisms (i.e., production of plant growth regulators, siderophores, hydrogen cyanide, suppression of phytopathogens, etc.) that indirectly increase BNF as well. In light of this, **Table 1** represents several published works of literature that have highlighted the role of microorganisms (including PSB/PSM) in maximizing BNF and increasing plant growth. However, further research investigations are needed in order to decipher the mechanisms by which these microbes communicate with their host plants, at the molecular and cellular levels so as to better understand the plant-microbe and microbe-microbe interactions.

PHOSPHATE-SOLUBILISING BACTERIAL MECHANISMS FOR IMPROVED NODULATION AND BIOLOGICAL NITROGEN FIXATION

Several researchers have advocated for direct farmer use of soil microbes, particularly PSM as bio-inoculants to make available nutrients previously fixed in unavailable forms, enhance BNF and overall soil health and fertility (**Table 1**) (Ahmad et al., 2014; Kumar, 2016). Phosphate solubilizing microorganisms applied to agricultural soils supply plants with P by converting insoluble forms of P into soluble ones in an environmental-friendly manner (Elkoca et al., 2008). The most studied PSM are PSB, among which pseudomonads, rhizobia, *Azotobacter*, bacilli were qualified as the most potent bacteria in terms of P bio-solubilization. Phosphate solubilizing bacteria may not only solubilize soil P, but also increase the uptake and use efficiency of inorganic fertilizers and enhance crop production in different agro-ecological niches (Ahmad et al., 2014). Farmer use of PSB requires successful introduction into the microbial community of the rhizosphere, as it forms the symbiotic relationship with plant roots (Lindström and Mousavi, 2020). Indeed, microbes such as PSB possess multifarious mechanisms by which they contribute to plant growth promotion in general, and BNF in particular (**Figure 1**).

Indirect Stimulation of BNF via Bacterially Produced Hormones

One of the important mechanisms exerted by PSB is the production of plant growth regulators such as auxins, cytokinins, gibberellins, and ethylene (Anand et al., 2016). These molecules are known to promote root growth and make available previously inaccessible pools of P and this, in turn, contributes to fulfilling indirectly the energy costs of the BNF process (Zaidi et al., 2010). Indeed, several researchers found high concentrations

TABLE 1 | Effects of phosphate solubilizing microorganisms on grain legumes growth, biological nitrogen fixation and nutrient uptake.

	Bacterial species	Growth conditions	Effects on plants	References
Legume species				
Chickpea	*Rhizobium* + *P. striata* or *B. Polymyxa*	Greenhouse	Increased the yield, nutrient uptake, and nodulation also nitrogenase activity.	Algawadi and Gaur, 1988
Chickpea	*M. ciceri* C-2/2 alone or with *P.* + *jessenii* PS06	Field conditions	Enhanced symbiosis and produced higher nodule mass, nodule number and shoot N content.	Valverde et al., 2007
Chickpea	*M. ciceri* RC4 +*A. chroococuum*A10 + *Bacillus*PSB9	Experimental Field	Increased N and P concentration and uptake also nodule number and biomass.	Wani et al., 2007
Chickpea	*Rhizobium*, N_2 fixing *Bacillus subtilis (OSU-142)*, + PS*B. megaterium (M-3)*	Controlled conditions and field conditions	Increased plant height, shoot, root, and nodule dry weight, N%, chlorophyll content, pod number, seed yield, total biomass yield, and seed protein content.	Elkoca et al., 2008
Chickpea	*P. fluorescens* BHUPSB06 + *Mesorhizobium sp*. BHURC02	Glasshouse conditions	Increase in nodule number, dry matter, and nutrient content.	Verma et al., 2012
Chickpea	*Mesorhizobium sp.* BHURC03 and *P. aeruginosa* BHUPSB02	Control at Field experiment	Enhanced the nodulation and N_2fixation	Verma et al., 2013
Common Bean	*Paenibacillus polymyxa* (*DSM 36*) + *R. tropici* (*CIAT899*)	Greenhouse	Increase leghemoglobin concentrations, nitrogenase activity and N_2 fixation efficiency.	Figueiredo et al., 2008
Common Bean	*Azospirillum* and *Rhizobium*	Greenhouse and farm	Increased the amount of fixed nitrogen and the yield.	Roseline et al., 2008
Pigeon pea	*Bacillus sp.* and *Azospirillum sp* + *Rhizobium*	Controlled conditions	Enhance nodulation and N_2 fixation.	Rajendran et al., 2008
Pea	*B. simplex* 30N-5 or *B. subtilis* 30VD-1 +*R. leguminosarum bv. viciae* 128C53	Greenhouse	Nodules were larger, clustered, and developed more highly branched vascular bundles, supporting nitrogen fixation.	Schwartz et al., 2013
Pea	*Rhizobium leguminosarum*	Controlled conditions	Enhanced N_2-fixing activity.	Downey and van Kessel, 1990
Soybean	*Bacillus thuringiensis* NEB17 +*Bradyrhizobium japonicum*	Greenhouse and Field conditions	Increases nodule number and weight, total biomass, total nitrogen and grain yield.	Bai et al., 2003
Soybean	*B. megaterium* and *Bradyrhizobium japonicum (MN110)*	Greenhouse	Improved plant N and nodule leghemoglobin.	Subramanian et al., 2015
Non-legume species				
Sugarcane	*Azospirillum*	Greenhouse	Increased N content of leaves.	Muthukumrasamy et al., 1999
Wheat	*Azospirillum brasilense* and *Azospirillum lipoferum* with *Zoogloea Ky1*	Controlled conditions	Contributed 7–12 % of plant N by BNF.	Malik et al., 2002
Maize	*Klebsiella sp*. Br1, *K. pneumoniae* Fr1, and*B. pumilus* S1r1	Greenhouse	Ability to BNF and increased the total N content.	Kuan et al., 2016

of auxin in root nodules, which confirms the vital role of this hormone in nodule formation and functioning (Mathesius et al., 1998; Van Noorden et al., 2006). Rao (2014) reported that co-inoculation of AMF (*Glomus fasciculatum*) along with rhizobia significantly improved nodulation, BNF, shoot N and P content and yield of soybean. Likewise, Qureshi et al. (2012) reported that co-inoculation of mash bean (*Vigna mungo* L.) with *Rhizobium* and a P solubilizing strain (*Bacillus* sp.) increased plant growth not only due to P solubilizing and N_2-fixation abilities but also due to their capacity to produce auxin. These authors reported that microbial auxin altered endogenous hormonal level, which in turn increased root length, root biomass, number of nodule and number of infection sites, which further resulted in increased plant growth. This corroborates with findings by Ahmad et al. (2019) who showed that co-inoculation of mung bean plants with *Bacillus aryabhattai* S10 and *Bacillus subtilis* ZM63 increased nodulation, plant growth, and the nutritional status

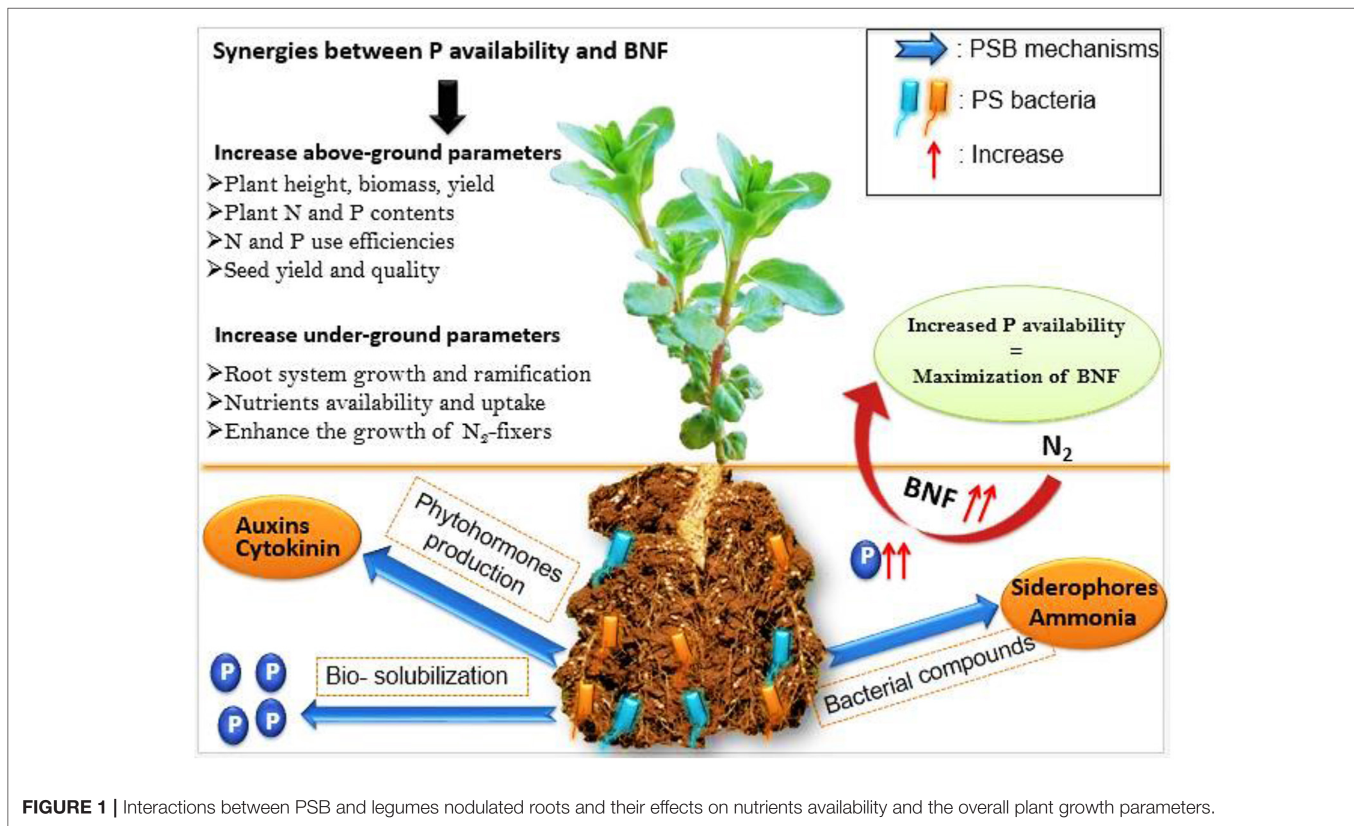


FIGURE 1 | Interactions between PSB and legumes nodulated roots and their effects on nutrients availability and the overall plant growth parameters.

of the tested plants. The increment of nodulation parameters was likely attributed to the improved formation of nodules through the increased surface area of rhizobial infection during nodulation. Mixed-inoculation of soybean plants with *Rhizobium*, *Enterobacter* and PSM (not identified) increased plant biomass, weight and length of pods as well as root nodulation parameters. The latter being a consequence of increased production of auxin and gibberellin by the combined action of the tested microbes, subsequently leading to increased cell division and elongation. Eventually, increasing the overall plant growth, yield and productivity (Yasmeen and Bano, 2014).

Similarly, Figueiredo et al. (2008) stated that cytokinin produced by a PSB strain (*Paenibacillus polymyxa* DSM 36) could influence BNF in common bean plants, most likely through the stimulation of rhizobial growth, nodule development and the number of infections in roots (**Figure 1**). This corroborates with findings by Elkoca et al. (2008) who revealed that production of plant growth regulators by *Bacillus subtilis* (OSU-142) and *Bacillus megaterium* (M-3) increased root system growth which in turn increased the number of infection sites. Moreover, Kannapiran and Ramkumar (2011) reported that black gram (*Phaseolus mungo*) N status was enhanced following inoculation with several bacteria amongst two PSB strains (*Pseudomonas aeruginosa* and *Bacillus sp*). In fact, these positive effects were attributed to several metabolites produced by the tested bacteria, such as siderophores, HCN, auxin and ammonia.

Phosphorus Bio-Availability as a Key Mechanism for Enhanced BNF

According to several studies, P solubilization may be one of the main mechanisms by which PSB enhances the BNF (Wani et al., 2007; Püschel et al., 2017; Bargaz et al., 2018; Bhowmik and Das, 2018). Common and soybean plants treated with biofertilizer (a consortium of three PSB: *Pseudomonas* sp., *Burkholderia* sp., and *Enterobacter* sp.) combined with rock phosphate in soil with low fertility found to increase the soil and plants P content, providing favourable conditions to plant nodulation which in turn increased the efficiency of BNF (Fankem et al., 2015). Stajković et al. (2011) shown that bioavailable P has a positive influence on the legume-rhizobia symbiosis. These authors observed positive effects of bio-available P on nodulation and nitrogenase activity in common bean plants following their inoculation with *Rhizobium phaseoli* and *Pseudomonas* sp. (a P solubilizing bacteria). Additionally, Korir et al. (2017) revealed that mixed-inoculation of common bean plants with *Rhizobium*, *Bacillus megaterium* and *Paenibacillus polymyxa* enhanced shoot and root weight of the plants as well as nodule number and biomass. These authors stated that nodulation parameters were enhanced as a result of increased P availability, which is known to stimulate growth and expansion of roots, thus offering to the rhizobial strains more active sites for nodulation. In addition, a field experiment conducted by Dutta and Bandyopadhyay (2009), showed that co-inoculation of chickpea plants with P fertilizer at 26.2 kg/ha along with P

bio-fertilizers [*Rhizobium* and phosphobacterin (*Pseudomonas striata*)] enhanced symbiotic properties measured in terms of number and weight of nodules, leghemoglobin content, and nitrogenase activity, which ultimately enhanced plant growth and yield. In fact, these beneficial effects were particularly attributed to the positive effects of increased P availability on the bacteria rather than the host plant. According to Jat and Ahlawat (2004), P availability controls the motility and migration of the bacterial cells, which is a prerequisite for the host infection and thus for the nodules development and the overall BNF process.

Additionally, it is reported that the PSB when applied with other plant growth-promoting rhizobacteria (PGPR) could reduce P fertilizer application by 50% without any significant reduction in crop yields or BNF, allowing more rational use of Mahanta et al. (2018). Therefore, the exploitation of PSM as bio-fertilizers holds great promise for sustaining crop production. Ahmad et al. (2014) suggest that PSB can interact positively with other microorganisms such as N_2-fixers and AMF, eventually leading to increased legume growth through a range of mechanisms. In fact, the beneficial influence of AMF has been attributed to the increase in auxin and abscisic acid contents in roots, shoots, and nodules, which increased the size and activity of nodules, enhanced micronutrient uptake from the soil, and increasing protection against biotic and abiotic stresses (Ahmad et al., 2014; Rao, 2014).

Although there are numerous reports highlighting interactions between PSB, N_2-fixing bacteria, and AMF, the underlying mechanisms behind these associations are not yet fully understood.

Thus, further studies are needed to deepen our understanding about these microorganisms' beneficial interactions. Additionally, based on the above described knowledge regarding the beneficial effect of PSB in fostering below-ground bio-processes, these microbial strains should be evaluated in extensive field trials to evaluate their potential to be used as bio-fertilizers. However, based on the cited studies, the mechanisms by which the bioavailable P affects nodulation and BNF is not yet deciphered. Therefore, further studied aiming to understand profoundly, at the molecular and cellular scale, the effects of the bio-available P on BNF are needed.

NITROGEN-FIXING BACTERIA AND PHOSPHATE SOLUBILIZING MICROORGANISMS: KEY AGRO-ECOLOGICAL ENGINEERING DRIVERS GEARING TOWARD SUSTAINABLE AGRICULTURE

Ecological engineering is a system-based approach aims to restore, optimize or even enhance the normal functioning of an ecosystem (Mitsch, 2012). This approach was first adopted in China in 1978 (Mitsch et al., 1993) and has been applied to address sustainability concerns in many other ecosystems, including agro-ecosystems (Mitsch and Jørgensen, 2003; Power, 2010). This strategy relies on the use of internal ecological processes, not only to restore the normal function of a given ecosystem but also to prevent further disruption of other ecosystems (Hengsdijk and Van Ittersum, 2003). Soil supports the growth of different microbial populations, many of which are involved in providing different categories of soil ecosystem services, directly or indirectly, such as nutrient cycling, organic matter degradation, contaminants removal, supporting soil fertility, resistance to different stresses, production of bioactive compounds that stimulate plants growth and other beneficial effects (Barrios, 2007; Wagg et al., 2014; Saccá et al., 2017). These functions are mostly the consequence of mutualistic associations established between plants and root-associated microorganisms (Martin et al., 2017). Given recent scientific advances regarding the beneficial effects provided by these microbes, it is evident that N_2-fixing bacteria and PSB/PSM play a key role in providing agro-ecosystem services, which ultimately lead to maintain, sustain, and enhance agro-ecosystems services (Bender et al., 2016).

Phosphorus and N play an important role in agro-ecosystems nutrient balances, as they are often limiting factors for soil fertility in low-input systems (Sharma et al., 2013). Soil microorganisms such as N_2-fixing symbionts and PSB/PSM contribute directly to the restoration of soil fertility by cycling nutrients such as N and P, ultimately leading to enhanced soil fertility and the maintenance of adequate plant growth (Bender et al., 2016). This process involves both biotic and abiotic factors with PSM considered among the key biotic factors that influence the cycling of P (i.e., sorption-desorption, dissolution-precipitation, and mineralization-immobilization) (Sharma et al., 2013) and N (i.e., N_2-fixation into plant-available ammonium; nitrification of ammonium into nitrogen oxides; or denitrification of NO_3 into N_2O and N_2) within the soil (Bender et al., 2016).

As described previously, PSB releases insoluble P (Rfaki et al., 2020), whereas N_2-fixing symbionts reduce N_2 into ammonia either symbiotically or non-symbiotically (Bhattacharjee et al., 2008). Therefore, these microbes could act as promising ecological engineers contributing to more sustainable agriculture. N_2-fixing bacteria (symbiotic and non-symbiotic) have been widely studied in the last decade because of their role in supplying plants with N (Oberson et al., 2013). Kuan et al. (2016) noted that almost 80% of the N available to plants comes from the symbiotic group, including *Rhizobium* which infects plants to form nodules. Whereas the non-symbiotic group consists of free-living microorganisms, such as, *Bacillus*, *Azotobacter*, *Azospirillum*, and *Herbaspirillum* (Arfarita et al., 2019), which contributes to approximately 5–10% of the biological N inputs into the soil N cycle (Son, 2001). In a two-year field study, Amirnia et al. (2019) demonstrated that N_2-fixing bacterial strain belonging to the genus of *Azotobacter* in combination with AMF (*Glomus intraradices*) enhanced soil fertility and plant growth under rainfed conditions, as application resulted in higher biomass yield, seed yield and seed protein of lentil (*Lens culinaris* Medik), thus the authors suggested that this bio-fertilizer has great potential in an arid and semi-arid area, in order to fulfill plant nitrogenous requirements and productivity under rainfed conditions. Moreover, Raklami et al. (2019) stated that co-inoculation of faba bean plant with a consortium of PGPR

(*Acinetobacter* sp. and *Rahnella aquatilis*) rhizobia (*Ensifer meliloti* (RhOF4) and *Ensifer meliloti* (RhOF155) and AMF (*Glomus* sp. *Sclerocystis* sp. *Acaulospora* sp.), PGPR-Rhizobia-AMF increased soil fertility in terms of N and P, subsequently leading to increased plant growth.

As discussed above, AMF were shown to be involved in managing agro-ecosystems functioning. These fungi form a tripartite symbiosis with plants and N_2-fixing bacteria. Musyoka et al. (2020) revealed that tripartite symbiosis involving mung bean (*Vigna radiata* L.) plants, *Bradyrhizobium*, and an AMF-consortium (*Rhizophagus irregularis, Funneliformis mosseae, Glomus etunicatum, Glomus aggregatum*) enhanced N_2 fixation, nutrients acquisition, and overall plant growth. Intercropping systems based on mixing legumes and non-legumes could also take advantage of this type of symbiosis. For instance, a study by Meng et al. (2015) highlighted the contribution of AMF and rhizobium in improving yield advantages of maize/soybean intercropping systems. In this experiment, the bacteria increased nodulation and N_2-fixation, while the AMF, through its hyphae, increased the degree of contact between soybean and maize which resulted in more N transfer to non-legume crops (Meng et al., 2015).

Besides their capacity in increasing, restoring, and/or maintaining soil fertility (Xavier and Germida, 2003), these microbes contribute to providing various ecosystem services in order to enhance and maintain agro-ecosystems productivity, stability, and sustainability (Paudyal et al., 2007; Ismaiel et al., 2014; Ullah et al., 2017). Disease suppression is considered as one of the microbial potentials to be involved indirectly in plant protection. This effect was illustrated by Negi et al. (2019) who showed that bio-priming of French bean (*Phaseolus vulgaris* cv. Contender) seed with *Rhizobium* (B1), *Trichoderma viride* and a PGPR strain enhanced plant growth, pod yield, seed yield, seed quality, seed vigour and reduced incidence of diseases such as root rot and angular leaf spot under field conditions. Moreover, growth of auxin plant under water stress conditions was found to be improved as a response to *Bradyrhizobium*, and AMF-consortium (*Rhizophagus irregularis, Funneliformis mosseae, Glomus etunicatum, and Glomus aggregatum*) inoculation (Musyoka et al., 2020).

Faba bean growth in alkaline conditions was found to be increased following their inoculation with *Rhizobium Legumiosarum* and AMF (*Acaulospora laevis, Glomus geosporum, Glomus mosseae,* and *Scutellospora armeniaca*) (Abd-Alla et al., 2014). Plant growth increase was likely attributed to the increased number and biomass of nodules, nitrogenase activity, leghemoglobin, and mycorrhizal colonization. In fact, BNF is enhanced due to AMF-mediated increases in plant uptake of nutrients such as P, Fe, and K (among others) (Abd-Alla et al., 2014). Under saline conditions, inoculation of chickpea plant with *Rhizobium*, AMF and *Stenotrophomonas maltophilia* resulted in enhanced plant growth, which is most likely due to increased nutrient availability, including P (Abd-Alla et al., 2019).

Additionally, soil contaminants (organic waste, xenobiotics) are one of the most common problems that threaten soil health and plant growth (Saccá et al., 2017). However, Ismaiel et al. (2014) revealed that plants inoculation with N_2-fixing *Rhizobium leguminosarum* bv. viceae and AMF (*Glomus mosseae*, *Gigaspora* sp., and *Acaulospora* sp) alleviated heavy metals toxicity and enhanced plant growth simultaneously. Taken all together, these microorganisms either used solely or in mixed inoculation showed promising results both on plant nutrient uptake, growth and protection. The use of PSM in legume-based cropping systems in an integrated manner, along with other sources of nutrients such as manure and inorganic fertilizers, show promise as a cost-effective strategy to increase nutrient use efficiency, BNF and overall yield and grain quality, particularly in low-input systems. However, the full contribution of soil biota including N_2-fixing bacteria, PSB, and PSM to agro-ecosystem functioning in a range of climates, soil types, and cropping systems has yet to be deciphered. Additional research addressing the functional capacity of PSM, including their ability to synergize BNF in the field is required to unlock the potential. Agricultural field- experiments are needed to further understand the agronomic relevance of PSM, as well as the functional mechanisms underlying the plant-microbe and microbe-microbe interactions.

CONCLUSION

Nitrogen and P are two essential macronutrients required for plant growth and development. While many farmers rely on inorganic sources of P to avoid nutrient deficiencies, non-trivial amounts of these fertilizers are fixed in the soil or lost through different mechanisms, thus remain unavailable for plant uptake. In legume-based cropping systems, P deficiencies can also lead to N deficiency and reduced crop yields. Based on the studies cited in this review, bio-fertilization using rhizosphere microorganisms such as PSM/PSB and/or N_2-fixing bacteria showed promising effects on BNF in particular and overall plant growth in general. Targeted application of specific soil microorganisms thus presents a propitious cost-effective management strategy that could increase optimization of N and P fertilizer eco-efficiency in an environmentally-friendly manner. However, there is still a need to conduct further research investigations in order to improve PSM responses under variable agro-climatic conditions. Field trials conducted under agriculturally relevant conditions, measuring crop-level responses to PSM, are required for validation. Additionally, the mechanisms underlying the rhizobial-PSM-plant interactions as well as the microbe-microbe interaction are not yet fully deciphered. Therefore, further research should focus on improving knowledge regarding the functional mechanisms behind these microbial interactions, taking into account those interactions with crop species, so that compatible organisms can be determined and used as effective bio-fertilizers in sustainable crop production systems.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

This review is carried out within the framework of MSc theses of both WJ and BB hosted at the Plant-Microbe Interactions Laboratory at Mohammed VI Polytechnic University. This work is part of a research project (AS17 2019-2022) financially supported by OCP Group at Mohammed VI Polytechnic University.

REFERENCES

Abbasi, K. M., and Manzoor, M. (2018). Biosolubilization of phosphorus from rock phosphate and other P fertilizers in response to phosphate solubilizing bacteria and poultry manure in a silt loam calcareous soil. *J. Plant Nutr. Soil Sci*. 181, 345–356. doi: 10.1002/jpln.201800012

Abd-Alla, M. H., El-Enany, A. W. E., Nafady, N. A., Khalaf, D. M., and Morsy, F. M. (2014). Synergistic interaction of *Rhizobium leguminosarum* bv. viciae and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (Vicia faba L.) in alkaline soil. *Microbiol. Res*. 169, 49–58. doi: 10.1016/j.micres.2013.07.007

Abd-Alla, M. H., Nafady, N. A., Bashandy, S. R., and Hassan, A. A. (2019). Mitigation of effect of salt stress on the nodulation, nitrogen fixation and growth of chickpea (*Cicer arietinum* L.) by triple microbial inoculation. *Rhizosphere* 10:100148. doi: 10.1016/j.rhisph.2019.100148

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

Ahmad, E., Zaidi, A., and Khan, M. S. (2014). "Response of PSM inoculation to certain legumes and cereal crops," in *Phosphate Solubilizing Microorganisms*, ed. M. S. Khan, A. Zaidi and J. Musarrat (Cham: Springer), 175–205. doi: 10.1007/978-3-319-08216-5_8

Ahmad, M., Adil, Z., Hussain, A., Mumtaz, M. Z., Nafees, M., Ahmad, I., et al. (2019). Potential of phosphate solubilizing bacillus strains for improving growth and nutrient uptake in mungbean and maize crops. *Pak. J. Agri. Sci*. 56, 283–289. doi: 10.21162/PAKJAS/19.7285

Algawadi, A. R., and Gaur, A. C. (1988). Associative effect of Rhizobium and phosphate-solubilizing bacteria on the yield and nutrient uptake uptake of chickpea. *Plant Soil* 105, 241–246. doi: 10.1007/BF02376788

Amirnia, R., Ghiyasi, M., Moghaddam, S. S., Rahimi, A., Damalas, C. A., and Heydarzadeh, S. (2019). Nitrogen-fixing soil bacteria plus mycorrhizal fungi improve seed yield and quality traits of lentil (Lens culinaris Medic). *J. Soil Sci. Plant Nutr*. 19, 592–602. doi: 10.1007/s42729-019-00058-3

Anand, K., Kumari, B., and Mallick, M. A. (2016). Phosphate solubilizing microbes: an effective and alternative approach as biofertilizers. *Int. J. Pharm. Pharm. Sci*. 8, 37–40.

Arfarita, N., Muhibuddin, A., and Imai, T. (2019). Exploration of indigenous free nitrogen-fixing bacteria from rhizosphere of *Vigna radiata* for agricultural land treatment. *J. Degrade. Min. Land Manage*. 6:1617. doi: 10.15243/jdmlm.2019.062.1617

Atienza, S. G., and Rubiales, D. (2017). Legumes in sustainable agriculture. *Crop Past. Sci.* 68, i-ii. doi: 10.1071/CPv68n11_FO

Attar, H. A., Blavet, D., Selim, E. M., Abdelhamid, M. T., and Drevon, J. J. (2012). Relationship between phosphorus status and nitrogen fixation by common beans (*Phaseolus vulgaris* L.) under drip irrigation. *Int. J. Environ. Sci. Technol*. 9, 1–13. doi: 10.1007/s13762-011-0001-y

Aziz, A., Ahiabor, B., Opoku, A., and Abaidoo, R. (2016). Contributions of rhizobium inoculants and phosphorus fertilizer to biological nitrogen fixation, growth and grain yield of three soybean varieties on a fluvic luvisol. *Am. J. Exp. Agric*. 10, 1–11. doi: 10.9734/AJEA/2016/20072

Bai, Y. M., Zhou, X. M., and Smith, D. L. (2003). Enhanced soybean plant growth resulting from coinoculation of Bacillus strains with *Bradyrhizobium japonicum*. *Crop Sci.* 43, 1774–1781. doi: 10.2135/cropsci2003.1774

Balemi, T., and Negisho, K. (2012). Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop production: a review. *J. Soil Sci. Plant Nutr*. 12, 547–561. doi: 10.4067/S0718-95162012005000015

Bargaz, A., Lyamlouli, K., Chtouki, M., Zeroual, Y., and Dhiba, D. (2018). Soil microbial resources for improving fertilizers efficiency in an integrated plant nutrient management system. *Front. Microbiol*. 9:1606. doi: 10.3389/fmicb.2018.01606

Barrios, E. (2007). Soil biota, ecosystem services and land productivity. *Ecol. Econ*. 64, 269–285. doi: 10.1016/j.ecolecon.2007.03.004

Batterman, S. A., Wurzburger, N., and Hedin, L. O. (2013). Nitrogen and phosphorus interact to control tropical symbiotic N2 fixation: a test in Inga punctate. *J. Ecol*. 101, 1400–1408. doi: 10.1111/1365-2745.12138

Belimov, A. A., Kojemiakov, A. P., and Chuvarliyeva, C. V. (1995). Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. *Plant Soil*. 173, 29–37. doi: 10.1007/BF00155515

Bender, S. F., Wagg, C., and van der Heijden, M. G. A. (2016). An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol. Evol*. 31, 440–452. doi: 10.1016/j.tree.2016.02.016

Bhattacharjee, R. B., Singh, A., and Mukhopadhyay, S. N. (2008). Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. *Appl. Microbiol. Biotechnol*. 80, 199–209. doi: 10.1007/s00253-008-1567-2

Bhowmik, S. N., and Das, A. (2018). "Biofertilizers: a sustainable approach for pulse production," in *Legumes for Soil Health and Sustainable Management*, eds R. S. Meena, A. Das, G. Singh and Y. Rattan Lal (Singapore: Springer), 445–485. doi: 10.1007/978-981-13-0253-4_14

Choudhury, A. T. M. A., and Kennedy, I. R. (2005). Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. *Commun. Soil Sci. Plant Anal*. 36, 1625–1639. doi: 10.1081/CSS-200059104

Downey, J., and van Kessel, C. (1990). Dual inoculation of *Pisum sativum* with *Rhizobium leguminosarum* and *Penicillium bilaji*. *Biol. Fertil. Soils* 10, 194–196. doi: 10.1007/BF00336135

Dutta, D., and Bandyopadhyay, P. (2009). Performance of chickpea (*Cicer arietinum* L.) to application of phosphorus and bio-fertilizer in laterite soil. *Arch. Agron. Soil Sci*. 55, 147–155. doi: 10.1080/03650340802398864

Elhaissoufi, W., Khourchi, S., Ibnyasser, A., Ghoulam, C., Rchiad, Z., Zeroual, Y., et al. (2020). Phosphate solubilizing rhizobacteria could have a stronger influence on wheat root traits and aboveground physiology than Rhizosphere P solubilization. *Front. Plant Sci*. 11, 1–15. doi: 10.3389/fpls.2020.00979

Elkoca, E., Kantar, F., and Sahin, F. (2008). Influence of nitrogen fixing and phosphorus solubilizing bacteria on the nodulation, plant growth, and yield of chickpea. *J. Plant Nutr*. 31, 157–171. doi: 10.1080/01904160701742097

Fankem, H., Tchuisseu Tchakounte, G. V., Ngo Nkot, L., Mafokoua, H. L., Tchouomo, D. D., Simo, C., et al. (2015). Common bean (*Phaseolus vulgaris* L.) and soya bean (Glycine max) growth and nodulation as influenced by rock phosphate solubilising bacteria under pot grown conditions. *Int. J. Agric. Policy Res*. 3, 242–250. doi: 10.15739/IJAPR.046

Faried, A. S. M., Mohamed, H. M., El-Dsouky, M. M., and El-Rewainy, H. M. (2019). Isolation and characterization of phosphate solubilizing actinomycetes from rhizosphere soil. *AJAS* 49, 125–137. doi: 10.21608/ajas.2018.28381

Fernández-Juárez, V., Bennasar-Figueras, A., Sureda-Gomila, A., Ramis-Munar, G., and Agawin, N. S. R. (2020). Differential effects of varying concentrations of phosphorus, iron, and nitrogen in N_2-fixing cyanobacteria. *Front. Microbiol*. 11, 1–19. doi: 10.3389/fmicb.2020.541558

Figueiredo, M. V. B., Martinez, C. R., Burity, H. A., and Chanway, C. P. (2008). Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (*Phaseolus vulgaris* L.). *World J. Microbiol. Biotechnol*. 24, 1187–1193. doi: 10.1007/s11274-007-9591-4

Hardarson, G., and Atkins, C. (2003). Optimising biological N_2 fixation by legumes in farming systems. *Plant Soil*. 252, 41–54. doi: 10.1023/A:1024103818971

Hassan, M. A., and Abdelgan, M. E. (2009). Effect of microbial biofertilization on nodulation, nitrogen and phosphorus content and forage yield of lablab bean (*Lablab purpureus* L.). *Am.-Eurasian J. Sustain. Agric.* 3, 829–835.

Hengsdijk, H., and Van Ittersum, M. K. (2003). Formalizing agro-ecological engineering for future-oriented land use studies. *Eur. J. Agron*. 19, 549–562. doi: 10.1016/S1161-0301(03)00002-9

Ismaiel, A. A., Hegazy, H. S., and Azb, M. A. (2014). Physiological response of *Vicia faba* L. to inoculation with Rhizobium and arbuscular mycorrhizal fungi: comparative study for irrigation with Nile water and wastewater. *Aust. J. Crop Sci*. 8, 781–790.

Jat, R. S., and Ahlawat, I. P. S. (2004). Effect of vermicompost, biofertilizer and phosphorus on growth, yield and nutrient uptake by gram (*Cicer arietinum*) and their residual effect on fodder maize (Zea mays). *Indian J. Agric. Sci*. 74, 359–361.

Kannapiran, E., and Ramkumar, V. S. (2011). Inoculation effect of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth of black gram (Phaseolus mungo Roxb ; Eng). *Ann. Biological Res*. 2, 615–621.

Khan, M. S., Zaidi, A., and Wani, P. A. (2007). Role of phosphate-solubilizing microorganisms in sustainable agriculture —a review. *Agron. Sustain. Dev*. 27, 29–43. doi: 10.1051/agro:2006011

Korir, H., Mungai, N. W., Thuita, M., Hamba, Y., and Masso, C. (2017). Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Front. Plant Sci*. 8, 141. doi: 10.3389/fpls.2017.00141

Kuan, K. B., Othman, R., Rahim, K. A., and Shamsuddin, Z. H. (2016). Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. *PLoS ONE* 11, 1–19. doi: 10.1371/journal.pone.0152478

Kumar, A. (2016). Phosphate solubilizing bacteria in agriculture biotechnology: diversity, mechanism and their role in plant growth and crop yield. *Int. J. Adv. Res*. 4, 116–124. doi: 10.21474/IJAR01/111

Kyei-Boahen, S., Savala, C. E. N., Chikoye, D., and Abaidoo, R. (2017). Growth and yield responses of cowpea to inoculation and phosphorus fertilization in different environments. *Front. Plant Sci*. 8, 1–13. doi: 10.3389/fpls.2017.00646

Lazali, M., Zaman-Allah, M., Amenc, L., Ounane, G., Abadie, J., and Drevon, J. J. (2013). A phytase gene is overexpressed in root nodules cortex of Phaseolus vulgaris–rhizobia symbiosis under phosphorus deficiency. *Planta* 238, 317–324. doi: 10.1007/s00425-013-1893-1

Li, Y., Li, Q., Guan, G., and Chen, S. (2020). Phosphate solubilizing bacteria stimulate wheat rhizosphere and endosphere biological nitrogen fixation by improving phosphorus content. *PeerJ*. 8:e9062. doi: 10.7717/peerj.9062

Lindström, K., and Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol*. 13, 1314–1335. doi: 10.1111/1751-7915.13517

Mahanta, D., Rai, R. K., Dhar, S., Varghese, E., Raja, A., and Purakayastha, T. J. (2018). Modification of root properties with phosphate solubilizing bacteria and arbuscular mycorrhiza to reduce rock phosphate application in soybean-wheat cropping system. *Ecol. Eng*. 111, 31–43. doi: 10.1016/j.ecoleng.2017.11.008

Malhotra, H., Vandana, S. S., and Pande, R. (2018). "Phosphorus nutrition: plant growth in response to deficiency and excess," in *Plant Nutrients and Abiotic Stress Tolerance*, eds. M. Hasanuzzaman, M. Fujita, H. Nahar, K. Oku and B. Hawrylak-Nowak (Singapore: Springer) 171–190. doi: 10.1007/978-981-10-9044-8_7

Malik, K. A., Mirza, M. S., Hassan, U., Mehnaz, S., Rasul, G., Haurat, J., et al. (2002). "The role of plant associated beneficial bacteria in rice-wheat cropping system,"in *Biofertilisers in Action. Rural Industries Research and Development Corporation,* eds I. R. Kennedy A. T. M. A. Chaudhry (Canberra), 73–83.

Manzoor, M., Abbasi, M. K., and Sultan, T. (2017). Isolation of phosphate solubilizing bacteria from maize rhizosphere and their potential for rock phosphate solubilization–mineralization and plant growth promotion. *Geomicrobiol. J*. 34, 81–95. doi: 10.1080/01490451.2016.1146373

Martin, F. M., Uroz, S., and Barker, D. G. (2017). Ancestral alliances: plant mutualistic symbioses with fungi and bacteria. *Science* 356:6340. doi: 10.1126/science.aad4501

Martins, D., Macovei, A., Leonetti, P., Balestrazzi, A., and Araújo, S. (2017). "The infuence of phosphate defciency on legume symbiotic N2 fixation," in *Legume Nitrogen Fixation in Soils with Low Phosphorus Availability*, eds S. Sulieman and L. S. P. Tran (Cham: Springer), 41–76. doi: 10.1007/978-3-319-55729-8_3

Mathesius, U., Schlaman, H. R. M., Spaink, H. P., Sautter, C., Rolfe, B. G., and Djordjevic, M. A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant J*. 14, 23–34 doi: 10.1046/j.1365-313X.1998.00090.x

Meena, R. S., and Lal, R. (2018). "Legumes and sustainable use of soils," in *Legumes for Soil Health and Sustainable Management*, eds R. S. Meena, A. Das, G. S. Yadav and R. Lal (Singapore: Springer), 1–31. doi: 10.1007/978-981-13-0253-4_1

Meng, L., Zhang, A., Wang, F., Han, X., Wang, D., and Li, S. (2015). Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci*. 6, 1–10. doi: 10.3389/fpls.2015.00339

Mitran, T., Meena, R. S., Lal, R., Layek, J., Kumar, S., and Datta, R. (2018). Role of soil phosphorus on legume production. *Legum. Soil Heal. Sustain. Manag.* 487–510. doi: 10.1007/978-981-13-0253-4_15

Mitsch, W. J. (2012). What is ecological engineering? *Ecol. Eng*. 45, 5–12. doi: 10.1016/j.ecoleng.2012.04.013

Mitsch, W. J., and Jørgensen, S. E. (2003). Ecological engineering: a field whose time has come. *Ecol. Eng*. 20, 363–377. doi: 10.1016/j.ecoleng.2003.05.001

Mitsch, W. J., Yan, J., and Cronk, J. K. (1993). Ecological engineering—contrasting experiences in China with the West. *Ecol. Eng*. 2, 177–191. doi: 10.1016/0925-8574(93)90013-6

Musyoka, D. M., Njeru, E. M., Nyamwange, M. M., and Maingi, J. M. (2020). Arbuscular mycorrhizal fungi and Bradyrhizobium co-inoculation enhances nitrogen fixation and growth of green grams (*Vigna radiata* L.) under water stress. *J. Plant Nutr*. 43, 1036–1047. doi: 10.1080/01904167.2020.1711940

Muthukumrasamy, R., Revathi, G., and Lakshminarasimhan, C. (1999). Diazotrophic association in sugar can cultivation in South India. *Trop Agri*. 76, 171–178.

Nasto, M. K., Alvarez-Clare, S., Lekberg, Y., Sullivan, B. W., Townsend, A. R., and Cleveland, C. C. (2014). Interactions among nitrogen fixation and soil phosphorus acquisition strategies in lowland tropical rain forests. *Ecol. Lett*. 17, 1282–1289. doi: 10.1111/ele.12335

Nasto, M. K., Osborne, B. B., Lekberg, Y., Asner, G. P., Balzotti, C. S., Porder, S., et al. (2017). Nutrient acquisition, soil phosphorus partitioning andcompetition among trees in a lowland tropical rain forest. *New Phytol*. 214, 1506–1517. doi: 10.1111/nph.14494

Negi, S., Bharat, N. K., and Kumar, M. (2019). Effect of seed biopriming with indigenous PGPR, Rhizobia and Trichoderma sp. on growth, seed yield and incidence of diseases in French bean (Phaseolus vulgaris L.). *Legum. Res*. 47, 288–295. doi: 10.18805/LR-4135

Oberson, A., Frossard, E., Bühlmann, C., Mayer, J., Mäder, P., and Lüscher, A. (2013). Nitrogen fixation and transfer in grass-clover leys under organic and conventional cropping systems. *Plant Soil* 371, 237–255. doi: 10.1007/s11104-013-1666-4

Olivera, M., Tejera, N., Iribarne, C., Ocana, A., and Lluch, C. (2004). Growth, nitrogen fixation and ammonium assimilation in common bean (*Phaseolus vulgaris*): effect of phosphorus. *Physiol. Plant* 121, 498–505. doi: 10.1111/j.0031-9317.2004.00355.x

Paudyal, S., Aryal, R. R., Chauhan, S., and Maheshwari, D. (2007). Effect of heavy metals on growth of rhizobium strains and symbiotic efficiency of two species of tropical legumes. *Sci. World* 5, 27–32. doi: 10.3126/sw.v5i5.2652

Phares, C. A., Atiah, K., Frimpong, K. A., Danquah, A., Asare, A. T., and Aggor-Woananu, S. (2020). Application of biochar and inorganic phosphorus fertilizer influenced rhizosphere soil characteristics, nodule formation and phytoconstituents of cowpea grown on tropical soil. *Heliyon. Heliyon*. 6:e05255. doi: 10.1016/j.heliyon.2020.e05255

Power, A. G. (2010). Ecosystem services and agriculture: tradeoffs and synergies. *Philos. Trans. R Soc. B Biol. Sci*. 365, 2959–2971. doi: 10.1098/rstb.2010.0143

Püschel, D., Janoušková, M., Voríšková, A., Gryndlerová, H., Vosátka, M., and Jansa, J. (2017). Arbuscular mycorrhiza stimulates biological nitrogen fixation in two Medicago spp. through improved phosphorus acquisition. *Front. Plant Sci*. 8, 1–12. doi: 10.3389/fpls.2017.00390

Qureshi, M. A., Iqbal, A., Akhtar, N., Shakir, M. A., and Khan, A. (2012). Co-inoculation of phosphate solubilizing bacteria and rhizobia in the presence of L-tryptophan for the promotion of mash bean (*Vigna mungo* L.). *Soil Environ*. 31, 47–54.

Qureshi, M. A., Shakir, M. A., Iqbal, A., Akhtar, N., and Khan, A. (2011). Co-inoculation of phosphate solubilizing bacteria and rhizobia for improving growth and yield of mungbean (*Vigna radiata* L.). *J. Anim. Plant Sci*. 21, 491–497.

Rahman, C. H., Ahcene, B., Miloud, B., and Rachid, D. (2017). Screening and characterization of plant growth promoting traits of phosphate. *Malays. J. Microbiol*. 13, 124–131.

Rajendran, G., Sing, F., Desai, A. J., and Archana, G. (2008). Enhanced growth and nodulation of pigeon pea by co-inoculation of Bacillus strains with Rhizobium spp. *Bioresour. Technol.* 99, 544–550. doi: 10.1016/j.biortech.2007.06.057

Raklami, A., Bechtaoui, N., Tahiri, A. I., Anli, M., Meddich, A., and Oufdou, K. (2019). Use of rhizobacteria and mycorrhizae consortium in the open field as a strategy for improving crop nutrition, productivity and soil fertility. *Front. Microbiol.* 10:1106. doi: 10.3389/fmicb.2019.01106

Rao, D. L. N. (2014). Recent advances in biological nitrogen fixation in agricultural systems. *Proc. Indian Natl. Sci. Acad*. 80, 359–378. doi: 10.16943/ptinsa/2014/v80i2/55114

Rfaki, A., Zennouhi, O., Aliyat, F. Z., Nassiri, L., and Ibijbijen, J. (2020). Isolation, selection and characterization of root-associated rock phosphate solubilizing bacteria in Moroccan Wheat (*Triticum aestivum* L.). *Geomicrobiol. J.* 37, 230–241. doi: 10.1080/01490451.2019.1694106

Roseline, R., Lara, R., Sarah, S., German, H., Aurelio, G., Jorge, R., et al. (2008). Effect of Rhizobium-Azospirillum coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. *Plant Soil.* 302, 149–161. doi: 10.1007/s11104-008-9606-4

Rugheim, A. M. E., and Abdelgani, M. E. (2012). Effects of microbial and chemical fertilization on yield and seed quality of faba bean (*Vicia faba*). *Int. Food Res. J.* 19, 417–422.

Saccá, M. L., Caracciolo, A. B., and Lenola, M. D. (2017). "Ecosystem services provided by soil microorganisms", in *Soil Biological Communities and Ecosystem Resilience*, eds. M. Lukac, P. Grenni and M. Gamboni (Cham: Springer), 9–24. doi: 10.1007/978-3-319-63336-7_2

Sanginga, N., Dashiell, K. E., Diels, J., Vanlauwe, B., Lyasse, O., Carsky, R. J., et al. (2003). Sustainable resource management coupled to resilient germplasm to provide new intensive cereal-grain-legume-livestock systems in the dry savanna. *Agric. Ecosyst. Environ*. 100, 305–314. doi: 10.1016/S0167-8809(03)00188-9

Santachiara, G., Borrás, L., Salvagiotti, F., Gerde, J. A., and Rotundo, J. L. (2017). Relative importance of biological nitrogen fixation and mineral uptake in high yielding soybean cultivars. *Plant Soil.* 418, 191–203. doi: 10.1007/s11104-017-3279-9

Sanz-Saez, A., Morales, F., Arrese-Igor, C., and Aranjuelo, I. (2017). "P Defciency: A Major Limiting Factor for Rhizobial Symbiosis," in *Legume Nitrogen Fixation in Soils with Low Phosphorus Availability*, eds S. Sulieman and L. S. P. Tran (Cham: Springer), 21–40. doi: 10.1007/978-3-319-55729-8_2

Schwartz, A. R., Ortiz, I., Maymon, M., Herbold, C. W., Fujishige, N. A., Vijanderan, J. A., et al. (2013). Bacillus simplex—a little known PGPB with anti-fungal activity—alters pea legume root architecture and nodule morphology when co-inoculated with Rhizobium leguminosarum Bv. Viciae. *Agrononmy* 3, 595–620. doi: 10.3390/agronomy3040595

Sharma, S. B., Sayyed R. Z., Trivedi, M. H., and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *Springerplus*. 2:587. doi: 10.1186/2193-1801-2-587

Si, Z., Yang, Q., Liang, R., Chen, L., Chen, D., and Li, Y. (2019). Digalactosyldiacylglycerol synthase gene MTDGD1 plays an essential role in nodule development and nitrogen fixation. *Mol. Plant-Microbe Interact*. 32, 1196–1209. doi: 10.1094/MPMI-11-18-0322-R

Singh, O., Gupta, M., Mittal, V., Kiran, S., Nayyar, H., Gulati, A., et al. (2013). Novel phosphate solubilizing bacteria 'Pantoea cypripedii PS1' along with Enterobacter aerogenes PS16 and Rhizobium Cicero enhance the growth of chickpea (*Cicer arietinum* L.). *Plant Growth Regul.* 73, 79–89. doi: 10.1007/s10725-013-9869-5

Skorupska, A., Wielbo, J., Kidaj, D., and Marek-Kozaczuk, M. (2010). "Enhancing rhizobium–legume symbiosis using signaling factors", in *Microbes for Legume Improvement,* eds Khan, A. Zaidi and J. Musarrat (Vienna: Springer), 27–54. doi: 10.1007/978-3-211-99753-6_2

Son, Y. (2001). Non-symbiotic nitrogen fixation in forest ecosystems. *Ecol. Res*. 16, 183–196. doi: 10.1046/j.1440-1703.2001.00385.x

Stagnari, F., Maggio, A., Galieni, A., and Pisante, M. (2017). Multiple benefits of legumes for agriculture sustainability: an overview. *Chem. Biol. Technol. Agric*. 4, 1–13. doi: 10.1186/s40538-016-0085-1

Stajković, O., Delic, D., Josic, D., Kuzmanovic, D., Rasulic, N., and Knezevic-Vukcevic, J. (2011). Improvement of common bean growth by co-inoculation with Rhizobium and plant growth-promoting bacteria. *Rom. Biotechnol. Lett*. 16, 5919–5926.

Stevens, G. G., Pérez-Fernández, M. A., Morcillo, R. J. L., Kleinert, A., Hills, P., Brand, D. J., et al. (2019). Roots and nodules response differently to p starvation in the mediterranean-type legume virgilia divaricata. *Front. Plant Sci*. 10:73. doi: 10.3389/fpls.2019.00073

Subramanian, P., Kim, K., Krishnamoorthy, R., Sundaram, S., and Tongmin, S. (2015). Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with Bradyrhizobium japonicum MN110. *Plant Growth Regul.* 76, 327–332. doi: 10.1007/s10725-014-9993-x

Tajini, F., Trabelsi, M., and Drevon, J. J. (2012). Combined inoculation with Glomus intraradices and Rhizobium tropici CIAT899 increases phosphorus use efficiency for symbiotic nitrogen fixation in common bean *(Phaseolus vulgaris* L.). *Saudi J. Biol. Sci.* 19, 157–163. doi: 10.1016/j.sjbs.2011.11.003

Taliman, N. A., Dong, Q., Echigo, K., Raboy, V., and Saneoka, H. (2019). Plants effect of phosphorus fertilization on the growth, photosynthesis, nitrogen fixation, mineral accumulation, seed yield, and seed quality of a soybean low-phytate line. *Plants* 8:119. doi: 10.3390/plants8050119

Tewari, S., Pooniya, V., and Sharma, S. (2020). Next generation bioformulation prepared by amalgamating Bradyrhizobium, cell free culture supernatant, and exopolysaccharides enhances the indigenous rhizospheric rhizobial population, nodulation, and productivity of pigeon pea. *Appl. Soil Ecol*. 147:103363. doi: 10.1016/j.apsoil.2019.103363

Tindwa, H. J., Kachiguma, A., and Mrema, J. P. (2019). Incubation of soil with agricultural lime and phosphorus enhances biological nitrogen fixation and yield of soybean (Glycine max (L.) merrill) in an ultisol. *J. Cent. Eur. Agric*. 20, 938–952. doi: 10.5513/JCEA01/20.3.2118

Ullah, S., Khan, M. Y., Asghar, H. N., Akhtar, M. J., and Zahir, Z. A. (2017). Differential response of single and co-inoculation of *Rhizobium leguminosarum* and *Mesorhizobium ciceri* for inducing water deficit stress tolerance in wheat. *Ann. Microbiol*. 67, 739–749. doi: 10.1007/s13213-017-1302-2

Vacheron, J., Desbrosses, G., Bouffaud, M. L., Touraine, B., Moënne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci*. 4:356. doi: 10.3389/fpls.2013.00356

Valverde, A., Burgos, A., Fiscella, T., Rivas, R., Velazquez, E., Rodriguez-Barrueco, C., et al. (2007). "Differential effects of coinoculations with Pseudomonas jessenii PS06 (a phosphate-solubilizing bacterium) and Mesorhizobium ciceri C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions," in *First International Meeting on Microbial Phosphate Solubilization. Developments in Plant and Soil Sciences*, *Vol. 102,* eds E. Velázquez and C. Rodríguez-Barrueco (Dordrecht: Springer), 111–115.

Van Gerven, L. P. A., Kuiper, J. J., Mooij, W. M., Janse, J. H., Paerl, H. W., and De Klein, J. J. M. (2019). Nitrogen fixation does not axiomatically lead to phosphorus limitation in aquatic ecosystems. *Oikos* 128, 563–570. doi: 10.1111/oik.05246

Van Noorden, G. E., Ross, J. J., Reid, J. B., Rolfe, B. G., and Mathesius, U. (2006). Defective long-distance auxin transport regulation in the Medicago truncatula super numeric nodules mutant 1[W]. *Plant Physiol*. 140, 1494–1506. doi: 10.1104/pp.105.075879

Vanlauwe, B., Hungria, M., Kanampiu, F., and Giller, K. E. (2019). The role of legumes in the sustainable intensification of African smallholder agriculture: Lessons learnt and challenges for the future. *Agric. Ecosyst. Environ*. 284:106583. doi: 10.1016/j.agee.2019.106583

Vardien, W., Valentine, A. J., Mesjasz-Przybyłowicz, J., Przybyłowicz, W. J., Wang, Y., and Steenkamp, E. T. (2014). Nodules from Fynbos legume *Virgilia divaricata* have high functional plasticity under variable P supply levels. *J. Plant Physiol*. 171, 1732–1739. doi: 10.1016/j.jplph.2014.08.005

Verma, J. P., Yadav, J., and Tiwari, K. N. (2012). Enhancement of nodulation and yield of Chickpea by co-inoculation of indigenous Mesorhizobium spp. and plant growth–promoting Rhizobacteria in Eastern Uttar Pradesh. *Commun. Soil Sci. Plant Anal.* 43:3, 605–621, doi: 10.1080/00103624.2012.639110

Verma, J. P., Yadav, J., Tiwari, K. N., and Kumar, A. (2013). Effect of indigenous Mesorhizobium spp. and plant growth promotingrhizobacteria on yields and

nutrients uptake of chickpea (Cicer arietinum L.) under sustainable agriculture. *Ecol. Eng.*. 51, 282–286. doi: 10.1016/j.ecoleng.2012.12.022

Wagg, C., Bender, S. F., Widmer, F., and Van Der Heijden, M. G. A. (2014). Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5266–5270. doi: 10.1073/pnas.1320054111

Wani, P. A., Khan, M. S., and Zaidi, A. (2007). Synergistic effects of the inoculation with nitrogen-fixing and phosphate-solubilizing rhizobacteria on the performance of field-grown chickpea. *J. Plant Nutr. Soil Sci.* 170, 283–287. doi: 10.1002/jpln.200620602

Xavier, L. J. C., and Germida, J. J. (2003). Selective interactions between arbuscular mycorrhizal fungi and Rhizobium leguminosarum bv. viceae enhance pea yield and nutrition. *Biol. Fertil. Soils* 37, 261–267. doi: 10.1007/s00374-003-0605-6

Yasmeen, S., and Bano, A. (2014). Combined effect of phosphate-solubilizing microorganisms, Rhizobium and Enterobacter on root nodulation and physiology of soybean (Glycine max L.). *Commun. Soil Sci. Plant Anal.* 45, 2373–2384. doi: 10.1080/00103624.2014.939192

Zaidi, A., Ahemad, M., Oves,., M., Ahmad, E., and Saghir, K. M. (2010). "Role of phosphate-solubilizing bacteria in legume improvement," in *Microbes for Legume Improvemen*t, eds. Khan, A. Zaidi and J. Musarrat (Vienna: Springer) 273–292. doi: 10.1007/978-3-211-99753-6_11

Zaidi, A., and Khan, M. S. (2007). Stimulatory effects of dual inoculation with phosphate solubilising microorganisms and arbuscular mycorrhizal fungus on chickpea. *Aust. J. Exp. Agric.* 47, 1016–1022. doi: 10.1071/EA06046

Zaidi, A., Khan, M. S., Ahemad, M., Oves, M., and Wani, P. A. (2009). Recent advances in plant growth promotionby phosphate-solubilizing microbes. *Microbial. Strat. Crop Improv*. 23–50. doi: 10.1007/978-3-642-01979-1_2

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.

Copyright © 2021 Janati, Benmrid, Elhaissoufi, Zeroual, Nasielski and Bargaz. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

ORIGINAL RESEARCH
published: 15 April 2021
doi: 10.3389/fagro.2021.660574

Applying Agronomic Principles of Rhizobial Inoculation to the Conservation of a Keystone Legume Species in a High Mountain Ecosystem on an Oceanic Island

Laura Pulido-Suárez[1], *Águeda M. González-Rodríguez*[2], *Jonay Cubas*[2], *Marcelino del Arco-Aguilar*[2], *José L. Martín-Esquivel*[3] and *Milagros León-Barrios*[1]*

[1] Departamento de Bioquímica, Microbiología, Biología Celular y Genética, área Microbiología, Universidad de La Laguna, La Laguna, Spain, [2] Departamento de Botánica, Ecología y Fisiología vegetal, Universidad de La Laguna, La Laguna, Spain, [3] Teide National Park, La Orotava, Spain

OPEN ACCESS

Edited by:
Juan Sanjuan,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Adriana Fabra,
National University of Río Cuarto, Argentina
Lourdes Girard,
National Autonomous University of Mexico, Mexico
Fatima Maria De Souza Moreira,
Universidade Federal de Lavras, Brazil

***Correspondence:**
Milagros León-Barrios
mileonba@ull.edu.es

In Memoriam:
This paper is dedicated to the memory of Prof. Tomás Ruiz-Argüeso.

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: 29 January 2021
Accepted: 19 March 2021
Published: 15 April 2021

Citation:
Pulido-Suárez L, González-Rodríguez ÁM, Cubas J, del Arco-Aguilar M, Martín-Esquivel JL and León-Barrios M (2021) Applying Agronomic Principles of Rhizobial Inoculation to the Conservation of a Keystone Legume Species in a High Mountain Ecosystem on an Oceanic Island. Front. Agron. 3:660574. doi: 10.3389/fagro.2021.660574

The Teide broom, *Spartocytisus supranubius*, is an endemism of the Canary Islands (Spain) and the dominant legume of the Tenerife high-mountain ecosystem in Teide National Park (N.P.). Biotic and abiotic stresses are causing a progressive deterioration and decline of the population of this keystone legume. Since its symbiosis with rhizobia is the main nitrogen (N) input into these soils, diminishing the biological nitrogen fixation could compromise the maintenance of this alpine ecosystem. Symbiotically efficient nitrogen-fixing rhizobia have been widely and successfully used as inoculants for agronomic purposes. However, only rarely has rhizobial inoculation been used for legume species conservation in natural ecosystems. In this study, we assessed three *Bradyrhizobium* sp. strains as inoculants for *S. supranubius* on seedlings grown in a greenhouse experiment and on juvenile individuals (2-years-old) transplanted on a field trial in the N.P. Plant growth as well as symbiotic and plant physiological parameters were measured to evaluate the effect of rhizobia inoculation. Our results show that broom plants responded positively to the inoculation both in the greenhouse and field trials. The SSUT18 inoculated plants had significantly higher number and weight of nodules, greater sizes (biovolume) and biomass and also showed the highest N which, being not significant in our experimental conditions, it still contributed to more N per planted hectare than control plants, which could be important for the ecosystem maintenance in these N-poor soils. Positive effects of inoculation were also detected on the plant survival rate and water content. The bradyrhizobial inoculation, by accelerating the plant growth can shorten the greenhouse period and by producing more robust juvenile plants, they could help them to cope better with stresses in its natural habitat. Therefore, inoculation with selected rhizobia is a successful strategy to be integrated into conservation campaigns for this threatened legume species.

Keywords: nitrogen-fixation, bradyrhizobia, *Spartocytisus supranubius*, alpine ecosystem, Teide National Park, Canary Islands

INTRODUCTION

The Teide broom, *Spartocytisus supranubius* (L.f.) Christ ex G. Kunkel, is a species endemic to the Canary Islands. It is the dominant legume and the most characteristic species of the summit shrubland vegetation of the Tenerife high mountain ecosystem. In the past, intensive grazing and its use as forage (Pérez de Paz et al., 1986; Rodríguez-Delgado and Elena-Roselló, 2006) were the main threats to this highly palatable legume, from which it was protected after the establishment of Teide National Park (N.P.) in 1954 (del Arco-Aguilar and Rodríguez-Delgado, 2018). However, these were far from being its only problem, as in this ecosystem it also has to cope with several other biotic and abiotic stresses. In the decade 1980–1990, the favorable trend due to N.P. protection reversed and *S. supranubius* populations started to recede (Cubas et al., 2018; Ibarrola-Ulzurrun et al., 2019; Bello-Rodríguez et al., 2020).

In recent decades, regeneration of *S. supranubius* populations has been compromised by introduced herbivores, mainly rabbits (Cubas et al., 2018). Although present in the N.P. since their introduction in the sixteenth century, larger populations of rabbits have been favored by the milder climatic conditions in the last few decades (Bello-Rodríguez et al., 2020). On the other hand, these broom populations thrive between 2,000 and 3,100 m a.s.l. (Kyncl et al., 2006), where conditions are highly demanding for plants. The Mediterranean bioclimate is diverse throughout the Teide N.P. varying from dry Supramediterranean in the southern part to dry-subhumid Supramediterranean in the north, and semiarid or arid Oromediterranean at the summit of Teide peak (del Arco-Aguilar et al., 2006; del Arco-Aguilar and Rodríguez-Delgado, 2018). Broom plants have to face intense sunlight exposure and a high daily and annual temperature variation with means ranging from below 0°C in winter to over 25°C in summer. The annual precipitation is 330–450 mm, mostly occurring in winter (when some snow and frost episodes are possible) and the summer is extremely dry, with a drought period from May to September (del Arco-Aguilar et al., 2006). Morphological adaptations of *S. supranubius* include deep roots, small ephemeral deciduous leaves and multibranched photosynthetic stems, which branch almost from the base (**Supplementary Figure 1**). These features mold this species well to its natural environment (Wheeler and Dickson, 1990), where specimens up to 4 m high and 10 m diameter can be found (Olano et al., 2017). In addition to the harsh climatic conditions of the alpine ecosystem, prolonged drought in recent decades and an overall temperature increase due to global warming are causing a progressive deterioration of *S. supranubius* adult individuals (González-Rodríguez et al., 2017; Olano et al., 2017; Martín-Esquivel et al., 2020).

Nitrogen (N) availability can be another stress factor for broom plants in soils of Teide N.P. As a legume, *S. supranubius* can establish a symbiosis with N-fixing rhizobia. This is fundamental, since broom plants grow on poorly developed soils, where N content is very low and limits vegetation establishment (Arbelo et al., 2009; Díaz et al., 2011; Rodríguez et al., 2014). Therefore, biological nitrogen fixation (BNF) seems to be the key input of this element into the soil of this ecosystem (Wheeler and Dickson, 1990; Pulido-Suárez et al., 2021), highlighting the need for the maintenance of the Teide broom population. Thus, given the ecological importance of this legume in the N cycle, its decline could compromise the maintenance of the ecosystem and affect its total productivity.

Symbiotically-efficient-rhizobia inoculants have been widely and successfully applied in legume crop agriculture for about a century. Moreover, in the last decades the use of rhizobia inoculants has also been tested with other, different purposes. Chaer et al. (2011) describe several examples of successful inoculation of leguminous trees in the reclamation of degraded areas, resulting in soil recovery and the restoration of the ecosystem biodiversity. In addition, several studies have characterized the rhizobia nodulating different threatened legume species and have identified efficient N-fixers (Donate-Correa et al., 2007; Lorite et al., 2010; Fonseca et al., 2012; Martínez-Hidalgo et al., 2016; Safronova et al., 2017). Even so, rhizobial inoculation is not a common practice in natural ecosystems for legume species conservation and only rarely have they been used for this purpose. In recent years, Navarro et al. (2014) carried out a good example, applying rhizobial inoculation in a field trial to conserve the endangered legume *Lupinus mariae-josephae*. They achieved a successful plant reproductive cycle dependent on seedling inoculation with effective bradyrhizobia, reporting higher seed production and yield (Navarro et al., 2014).

In a previous study we described that strains of genus *Bradyrhizobium* are the main microsymbionts of *S. supranubius* (Pulido-Suárez et al., 2021) and showed, coinciding with Wheeler and Dickson (1990), the positive influence of this symbiosis in the ecosystem, since the soils around this legume have higher N content. However, the symbiotic effectiveness of the *S. supranubius* bradyrhizobia has not yet been evaluated nor how these endosymbiotic bacteria promote growth of the broom plants in their natural habitat. Hence, in the present study our aim was to evaluate the effect of rhizobial inoculation on *S. supranubius* when growing under the environmental conditions of its natural habitat of Teide N.P. We hypothesized that inoculation of broom plants with selected rhizobial strains before transplanting them on the field would produce more robust plants, better prepared to cope with the local external stresses.

MATERIALS AND METHODS

Rhizobial Strains Used in This Study

The three rhizobial strains used in this study, *Bradyrhizobium* sp. SSUT18, *Bradyrhizobium* sp. SSUT74 and *Bradyrhizobium* sp. SSUT109, were isolated from root nodules of *S. supranubius* trap-plants grown in soils from three areas of Teide N.P., Tenerife (Fasnia volcanoes, Llano de Maja plain, and Chiquero Mountain). These strains were selected because in a previous infectivity test on the original host plant (performed in greenhouses at the University of La Laguna) they showed a relative symbiotic efficiency of about 60%, calculated as: (Inoculated plant Dry Weight/N-fertilized plant Dry Weight) x 100. Strain SSUT18 had been characterized in a previous study (Pulido-Suárez et al., 2021) and the other two are characterized here by

sequencing the genes *glnII* (glutamine synthetase II) and *nodC* (nodulation protein C). Genomic DNA was extracted, quantified and normalized as described in Pulido-Suárez et al. (2021). The *glnII* genes were amplified using primers glnII12F and glnII689R (Vinuesa et al., 2005b). The *nodC* was amplified with primers nodCF and nodCI (Laguerre et al., 2001) for SSUT18 and SSUT109. For SSUT74, *nodC* gene amplification was attempted by combining the nodCI reverse primer with primers nodCF, nodCF4 and nodCFu (Laguerre et al., 2001). Purified fragments were sequenced using Macrogen Inc., Spain. The *S. supranubius* inoculation experiments with these three strains were performed at Teide N.P., either in the greenhouse or in the field. Our procedures for plant manipulation in both experiments strictly followed those protocols used by the Teide N.P. managers, with the exception of the rhizobia inoculation, as explained below.

Nursery Experiments: The Greenhouse and Acclimatization Stages

The growth of Teide Brooms in the plant nursery has two phases. First, a greenhouse stage where seeds are germinated, and seedlings are grown in trays. Second, an acclimatization period in which seedlings are transplanted to pots and grown outside the greenhouse. For the greenhouse experiment, the National Park provided broom seedlings which had been previously germinated following the usual protocols of the park managers. In brief, seeds are germinated in plastic trays with a non-sterilized substrate consisting of a mix of commercial peat, local volcanic lapilli and sieved local soil. A month after their germination, the seedlings were inoculated with the bradyrhizobia and transplanted to 5 × 5 × 15 cm plastic conical-pot trays filled with the same substrate and inoculated with the bradyrhizobia. The inoculants were prepared by growing the bacteria on YMB (Yeast-Mannitol Broth), at 28°C for 5 days. Well-grown cultures were then centrifuged and the pellet was suspended in sterile saline solution to adjust cell density to 2U McFarland. Five milliliter of the bacterial suspension were used to inoculate each broom plant. Plants were divided into four treatments. Treatment 1 consisted of control plants (uninoculated, watered with 5 ml irrigation water) and the other three treatments consisted of plants inoculated with one of the three selected strains: *Bradyrhizobium* sp. SSUT18, *Bradyrhizobium* sp. SSUT74 or *Bradyrhizobium* sp. SSUT109. A total of 224 seedlings, 56 for each treatment, were used. The effect of a possible positional effect was corrected by a random distribution of the trays. Plants were grown in the greenhouse at El Portillo (Teide N.P., Tenerife) from June 2019 to November 2019, and sprinkler watered daily (following the usual pattern at the park greenhouse). Five-month-old plants were collected and analyzed. Number of nodules, ramifications, survival, whole fresh plant (FW) and dry weight (DW) were determined. The Absolute Water Content (AWC) was calculated using the following formula: AWC = [(FW-DW)/FW] × 100 (Macar and Ekmekçi, 2008; Kang et al., 2012). Survival was evaluated at the end of the experiment.

Field Trial Experiment

Site Description

The study site, called Llano de Maja, is a flat area at 2,283 m a.s.l. located in the NE of Teide N.P., an area within a dry-Supramediterranean bioclimatic belt (del Arco-Aguilar et al., 2006). At Maja, the average annual temperature is 10°C (annual maximum 15.7°C and annual minimum 4.4°C in 2019) (personal data from N.P. managers). The Maja plain has soils rich in clay, sand and small pyroclasts. These soils have been described as slightly acidic (average pH 6.44) with an average N of 1.5 $g.kg^{-1}$ (Arbelo et al., 2009; Rodríguez et al., 2014). Specifically, the plot selected for field trial experiments was within an herbivore exclosure area situated on that plain (UTM 28R348679E/3130629N) (**Supplementary Figure 2**). The fence covers a total area of 30 ha and was built in 2015 to protect seedlings from grazing by introduced herbivores.

Rhizobia Inoculation

For the field trial experiment, 2-year old broom plants (provided by the N.P.) were sown following the N.P. procedures, in which plants are transplanted to the field after 2 years of acclimatization. During that stage, plants were grown in 8 x 8 x 17 cm plastic conical pots on tables outside the N.P. greenhouses (El Portillo). In our experiment, we inoculated the legume roots with the bradyrhizobia during the last months of the acclimatization period. Inocula were prepared as previously explained in section Nursery Experiments: The Greenhouse and Acclimatization Stages, except that the 2-year old plants were inoculated with 50 ml twice, in summer (July 2019) and fall (November 2019). In December 2019, a selected a set of 20 homogeneous broom plants per treatment were transplanted out to Llano de Maja. A simple random design with 3 m intervals between plants was used, occupying a total area of 1,092 m^2 (42 × 26 m).

Assessment of Inoculation: Effect on Nodulation and Plant Growth

The inoculated 2-year old broom plants were transplanted to the field on December 17, 2019. We evaluated the effect of rhizobial inoculation at the beginning of summer (July 9, 2020) and in mid-fall (November 13, 2020), 29 and 74 weeks after the transplantation, respectively. Direct measurements (destructive method) of plant growth were carried out from randomly selected plants, in a sample of $n = 5$ plants/treatment in the summer and for $n = 10$ plants/treatment in the fall.

For the summer sampling (July 2020), fresh weights of shoots (SFW) and roots (RFW) were measured at the field. Shoot and root dry weights (SDW and RDW) were determined from plants dried at 70°C for 72 h. Total fresh (TFW) and dry weights (TDW) were calculated by adding SFW+RFW or SDW+RDW, respectively. Plant fresh and dry weights were also used to calculate the AWC, as previously explained in section Nursery Experiments: The Greenhouse and Acclimatization Stages.

Nitrogen content was also determined from the dried material and analyzed using the Kjeldahl method (at the Institute of Natural Products and Agrobiology, CSIC, La Laguna, Tenerife). The number of nodules (NN), nodules fresh (NFW) and dry weights (NDW) were also determined. The parameter NN/RDW

was calculated for each plant to determine the production of nodules per g of root dry weight. Plant growth was also estimated in July 2020 from non-destructive measurements using all the plant specimens in the field. The number, average height, and diameter of total branches were obtained from the main branches (MB, those which directly sprouted from the stem) and the secondary branches (SB, those ramified from MB). We also determined the plant biovolume. Usually, to calculate it, a geometrical criterion is followed (Castro et al., 1996). That is, adjusting its general shape to a given solid body. In our case, an approximation to the plant biovolume was calculated using a cylindrical model (Nilsen et al., 1993), given the cylindrical shape of the branches of *S. supranubius* (**Figure 1C**). The volume of a single branch was estimated using the cylinder volume formula ($V_{branch} = \pi hr^2$) and subsequently multiplied by the total number of branches ($V_{Plant} = V_{branch} \times$ number of branches).

By the fall sampling (November 2020), the root system had widely developed, thus making a total uprooting impossible. Consequently, only shoot parameters (SFW and SDW) were taken into account. Plant height (cm) and widest width (cm) were also considered.

Plant survival was evaluated at the end of the acclimatization stage 6 months after the rhizobial inoculation, just before transplantation to the field (December 2019).

Estimation of the Physiological State of Inoculated Plants

The maximum photochemical efficiency (Fv/Fm) of photosystem 2 (PSII) is a parameter commonly used as an indirect indicator of the physiological condition of a plant (Paknejad et al., 2007; Ashraf and Harris, 2013). It was measured with a portable fluorimeter (Handy PEA, Plant Efficiency Analyzer, Hansatech, UK). Prior to the measurement, the photosynthetic stems were adapted to darkness for a minimum of 30 min in order to determine basal fluorescence (Fo). After a saturating red light pulse (650 nm, 3,000 μmol photons m^{-2} s^{-1}) flashed by an array of ultra-bright red light emitting diodes, the maximum fluorescence (Fm) was determined. From these parameters, the maximum photochemical efficiency (Fv/Fm) was calculated as the ratio (Fm-Fo)/Fm according to Maxwell and Johnson (2000). As we aimed to evaluate the adaptation of the plants to the field, measurements were taken in winter (January 2020) shortly after transplantation and in early summer (June 2020) on eight randomly selected plants/treatment.

Data Analysis

The measured plant parameters were analyzed with IMB SPSS Statistics V21.0. We assessed the normal distribution of the data (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). Differences between the treatments were studied using a one-way ANOVA and a *post-hoc* LSD or T2 Tamhane test, according to the result of the Levene test. When the data did not fit a normal distribution, a non-parametric test (Kruskal-Wallis) was used. We set the significance level for all tests to $p \leq 0.05$. Boxplot graphs were plotted with function "boxplot" and function "ggplot" using the statistical platform (R Core Team, 2017) and package "vegan" (Oksanen et al., 2016).

The phylogenetic analyses were conducted with MEGA version X (Kumar et al., 2018) and the tree was constructed using neighbor-joining (NJ) with Kimura's 2-parameter model. The robustness of the tree topology was calculated from bootstrap analysis with 1,000 replications. The accession numbers of the sequences generated in this study are specified in **Supplementary Figures 3**, **4**.

RESULTS

Rhizobia Strains

Given that the highly conserved sequences of the 16S rRNA genes of the *S. supranubius* bradyrhizobia produced a poor resolution to the species level (Pulido-Suárez et al., 2021), the taxonomic characterization of the three selected strains used in this study was based on the *glnII* housekeeping gene sequences (**Supplementary Figure 3**). In this phylogeny the strain SSUT18 is classified as a close relative of the species *Bradyrhizobium canariense*, while strains SSUT74 and SSUT109 clustered close to species *B. rifense*. The nodulation *nodC* gene sequences included strains SSUT18 and SSUT109 within symbiovar genistearum (**Supplementary Figure 4**), the common symbiovar found in rhizobia nodulating the Genistea (Vinuesa et al., 2005a; Stepkowski et al., 2018). However, the *nodC* gene failed to amplify for strain SSTU74, despite using several primer pairs and temperature profiles.

Rhizobial Inoculation on Broom Plants Under Greenhouse Conditions

Table 1 and **Supplementary Figure 5** show the results of the inoculation on plant growth, nodule number and water content of broom seedlings after 5 months growing under greenhouse conditions in Teide N.P. The use of non-sterilized soils explains the nodulation of the uninoculated plants. However, these control plants had a significantly lower nodule number than the inoculated plants ($p < 0.0001$). The SSUT109 and SSUT18 inoculated seedlings had significantly higher plant weights than uninoculated control plants ($p < 0.001$ for FW and $p < 0.001$ for DW. The number of branches on plants inoculated with SSUT18 was also statistically significant ($p < 0.0001$), a key parameter for broom plants taking into account that their physiology greatly depends on multibranched photosynthetic stems. The worst result was obtained for SSUT74 inoculated plants, which had lower values for FW, DW and branches (**Table 1**), rather similar to the control plants. The AWC for each plant/treatment was obtained from the plant's FW and DW, showing that all inoculated plants had statistically significant higher values than controls ($p < 0.0001$) (**Table 1**).

Rhizobial Inoculation of Broom Plants in the Field Trial

Broom plants were transplanted to the field (**Supplementary Figure 6**) a month after their second inoculation, in mid-December. The effect of inoculation on plants in the field was evaluated in two seasons of 2020, in summer (July 2020) and in fall (November 2020), respectively seven and eleven months after transplanting. In the summer

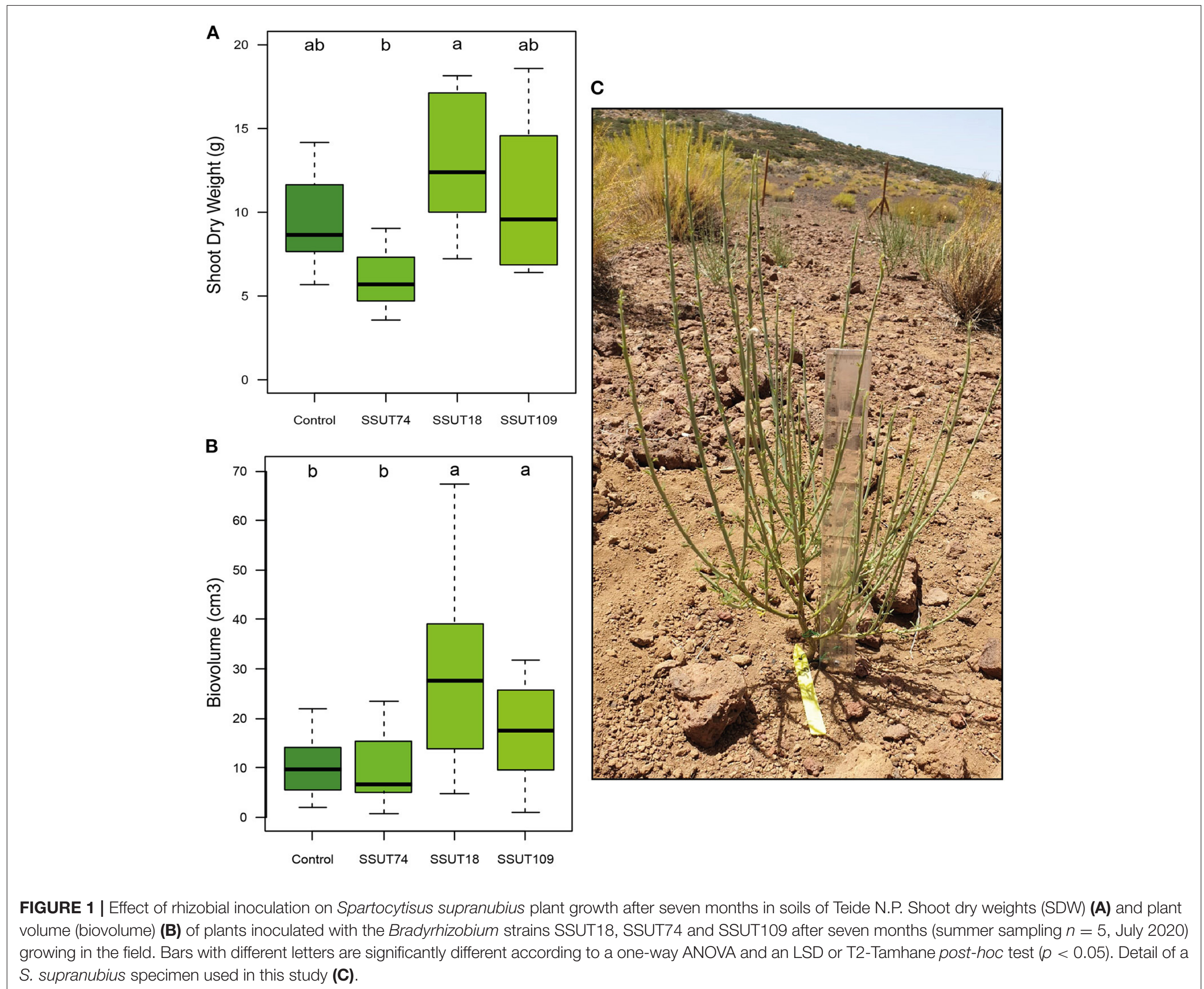


FIGURE 1 | Effect of rhizobial inoculation on *Spartocytisus supranubius* plant growth after seven months in soils of Teide N.P. Shoot dry weights (SDW) **(A)** and plant volume (biovolume) **(B)** of plants inoculated with the *Bradyrhizobium* strains SSUT18, SSUT74 and SSUT109 after seven months (summer sampling $n = 5$, July 2020) growing in the field. Bars with different letters are significantly different according to a one-way ANOVA and an LSD or T2-Tamhane *post-hoc* test ($p < 0.05$). Detail of a *S. supranubius* specimen used in this study **(C)**.

sampling (**Table 2** and **Figure 1**) the SSUT18 inoculated plants had significantly higher heights ($p < 0.01$), and increased by 1.5 times the SFW and SDW of control plants (**Table 2** and **Figure 1A**). Plants produced 12–51 nodules (**Figure 2A**) depending on the inoculated strain. The number of nodules (NN) was statistically higher ($p < 0.05$) in plants inoculated with SSUT18. This SSUT18 strain, in addition with SSUT109, showed the highest nodule weights ($p < 0.05$) (**Figure 2B**) and visually their nodules were bigger, redder and more branched than those of SSUT74 and control plants (nodulated by other native rhizobia in the field). The number of nodules was also expressed as the number per gram of root DW (ratio NN/RDW), showing that the SSUT18 inoculated plants also had the highest NN/RDW values as well as the best N content per plant. N contents were statistically not significant between treatments, which can be partially explained because the small deciduous leaves of *S. supranubius* had already fallen by the time of the sampling and therefore the N content values correspond just to the stems.

Since only a small number of plants ($n = 5$/treatment) were used for direct measurements in summer, in this first sampling we also estimated the plant growth for all specimens (80 plants) using non-destructive methods (**Supplementary Table 1** and **Figure 1B**). Estimated plant volume was significantly higher ($p < 0.0001$) in broom plants inoculated with strains SSUT18 and SSUT109, whereas SSUT74 inoculated plants were similar to controls (**Figure 1B**). Correlation between SDW (biomass) and the estimated biovolume was good ($r^2 = 0.847$, $p < 0.0001$), in agreement with the observed tendency in the direct measurements. It is worth mentioning that inoculated plants with SSUT18 and SSUT109 had on average 10 branches more per plant that controls (**Supplementary Table 1**), which may of relevance for this species because photosynthesis is mainly carried out by its photosynthetic stems (Kyncl et al., 2006).

TABLE 1 | Effect of the inoculation with three *Bradyrhizobium* sp. strains on the nodule number, plant (fresh and dry) weight, number of branches and water content of *Spartocytisus supranubius* seedlings grown 5 months under greenhouse conditions at Teide National Park.

Treatment	N° Nodules	PFW (g)	PDW(g)	Branches	AWC
SSUT18	7.3 ± 1.0 a	1.0 ± 0.1 ab	0.3 ± 0.04 a	1-5 a	65.5 ± 0.7 b
SSUT74	6.4 ± 1.0 a	0.6 ± 0.1 bc	0.2 ± 0.03 b	1-3 b	67.7 ± 0.8 ab
SSUT109	9.7 ± 1.4 a	1.3 ± 0.2 a	0.4 ± 0.05 a	1-4 ab	68.9 ± 0.5 a
Control	2.4 ± 0.6 b	0.5 ± 0.1 c	0.2 ± 0.02 b	1 b	62.1 ± 1.0 c

Average number of nodules per plant (N° Nodules), Plant (shoot + root) Fresh Weight (PFW) and Dry Weight (PDW), number of branches (branches) and Absolute Water Content (AWC). Mean ± standard error. Values with different letters in the same column are significantly different according to a Kruskal-Wallis or a one-way ANOVA and a LSD or T2 Tamhane post-hoc test ($p < 0.05$).

TABLE 2 | Assessment of the response of *Spartocytisus supranubius* plants to the inoculation with three *Bradyrhizobium* sp. strains after 7 months (sampling July 2020) growing in natural soils of Teide National Park.

Treatment	Height (cm)	SFW (g/plant)	SDW (g/plant)	RFW (g/plant)	RDW (g/plant)	NFW (g/plant)	NN/RDW	N(g/plant)	AWC (%)
SSUT18	27.15 ± 1.12 a	29.0 ± 4.7a	9.3 ± 1.7a	10.6 ± 1.6a	3.7 ± 0.6a	0.4 ± 0.07ab	19.6 ± 4.2a	0.20 ± 0.03a	67.4 ± 1.1a
SSUT74	23.05 ± 4.45 ab	13.4 ± 2.6b	4.2 ± 0.7b	4.1 ± 0.3a	1.9 ± 0.3b	0.1 ± 0.03c	8.5 ± 1.1ab	0.10 ± 0.01a	68.5 ± 0.9a
SSUT109	25.74 ± 1.09 ab	24.2 ± 5.7ab	8.0 ± 1.9ab	9.6 ± 1.5a	3.2 ± 0.5ab	0.5 ± 0.08a	11.8 ± 3.2ab	0.18 ± 0.04a	66.8 ± 1.0a
Control	21.60 ± 1.10 b	18.4 ± 4.5ab	6.7 ± 1.0ab	8.6 ± 2.6a	3.1 ± 1.7ab	0.2 ± 0.02b	4.2 ± 1.0b	0.15 ± 0.02a	65.7 ± 0.3a

Averages for Plant height (cm), Shoot Fresh (SFW) and Dry weight (SDW), Root Fresh (RFW) and Dry weight (RDW), nodule fresh weight (NFW), ratio number of nodules/root dry weight (NN/RDW), Shoot Nitrogen content (g/plant) and Absolute Water Content (AWC) for a n = 5, sampling of July 2020. Mean ± standard error. Values with different letters in the same column are significantly different according to one-way ANOVA and a LSD or T2-Tamhane post-hoc test ($p < 0.05$).

Contrary to the seedlings at the greenhouse, the AWC for this summer sampling (**Table 2**) produced very similar values in all treatments. These ranged from 65 to 68%, which were not statistically significant ($p > 0.05$), although the plants inoculated with the three bradyrhizobia showed slightly higher averages.

During the fall sampling in November, we only made direct (destructive) measurements of shoots, as explained in section Assessment of Inoculation: Effect on Nodulation and Plant Growth. The results showed (**Figure 3** and **Table 3**) that broom plants inoculated with strains SSUT18 and SSUT109 showed significantly higher SFW ($p < 0.05$) (**Table 3**) and SDW for SSUT18-inoculated plants (**Figure 3A**) ($p < 0.05$). In this second sampling, all plants almost doubled the height measured in July, although no significant differences were observed between treatments. Furthermore, plants inoculated with strains SSUT18 and SSUT109 were visually more voluminous and branched (**Figure 3B**) and significantly wider (**Table 3**) than control plants or those with SSUT74. Height and width are two important parameters when comparing the broom plant growth, taking into account the special morphology of this legume (**Figure 1C**). Nodulation data were not recorded in November due to difficulty in pulling up the whole root system.

Plant Survival

The survival rate for seedlings grown in the greenhouse was higher in inoculated plants, being over 57% and 52.90% in plants inoculated with strains SSUT18 and SSUT109, respectively, whereas control plants presented a higher mortality rate, as only 35.7% of the transplanted seedlings survived (**Supplementary Table 2**). Survival of juvenile plants in acclimatization (field trial experiment), scored 6 months after their inoculation, was much higher in all cases (**Supplementary Table 2**). However, these results coincided with the greenhouse experiment in showing a higher mortality in uninoculated broom plants (16%), more than double the rate of inoculated plants, whose mortality was 6.67% (SSUT74), 3.33% (SSUT18) and 0% (SSUT109).

Photosynthetic Parameters

The ratio Fv/Fm is a parameter commonly used as an indirect measurement of plant stress, with values below 0.75 being considered a stress indicator (Paknejad et al., 2007, Ashraf and Harris, 2013). In January, a month after being transplanted to the field, plants from all treatments had values below this threshold (**Supplementary Figure 7**), which indicates stress and therefore a low photosynthetic yield (Paknejad et al., 2007). Six months later (in June) the values suggest that plants were acclimated in the field, as reflected in the higher FvFm values (**Supplementary Figure 7**).

DISCUSSION

Spartocytisus supranubius, the dominant species in the Teide N.P. is currently experiencing a decrease in population (Cubas et al., 2018; Martín-Esquivel et al., 2020). In order to slow down this decline, periodic transplanting of juvenile individuals is a frequent strategy in Teide N.P. About 10,000 broom plants are produced each year, a fraction of which is planted in the wild for ecological restoration purposes. The usual protocol followed by the environmental managers at the Teide N.P. includes a 2–3 years acclimatization period outside the greenhouses before transplanting them at

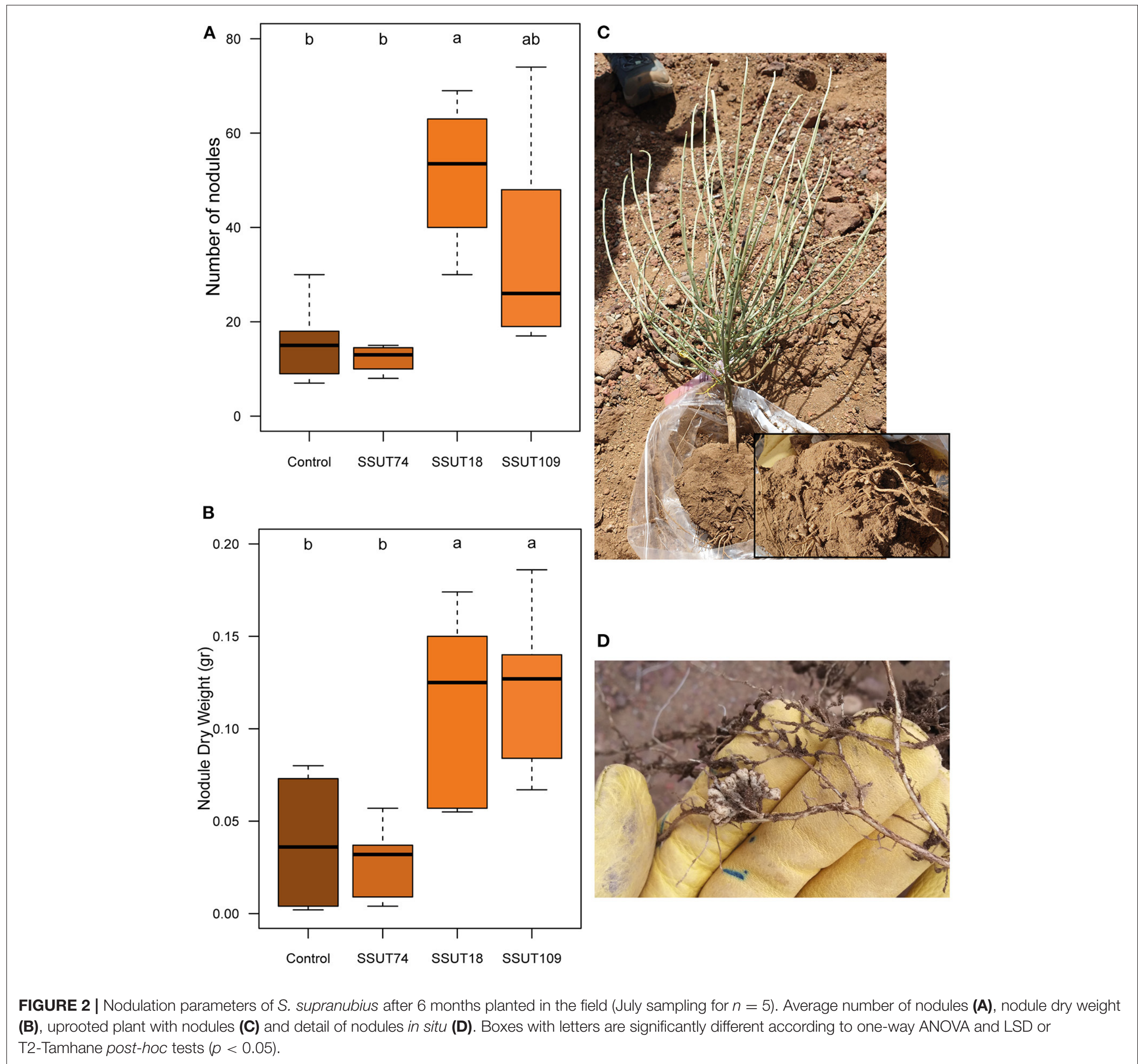


FIGURE 2 | Nodulation parameters of *S. supranubius* after 6 months planted in the field (July sampling for $n = 5$). Average number of nodules **(A)**, nodule dry weight **(B)**, uprooted plant with nodules **(C)** and detail of nodules *in situ* **(D)**. Boxes with letters are significantly different according to one-way ANOVA and LSD or T2-Tamhane *post-hoc* tests ($p < 0.05$).

the field. However, high mortality rates among transplanted individuals is observed, especially in the southern area of the park, where it is two or three times higher than in the northern area. Despite the relevance of N-fixation associated with this legume has already been evidenced (Wheeler and Dickson, 1990; Pulido-Suárez et al., 2021), the inoculation with rhizobia has never been incorporated into the legume species conservation strategies. In the present study we have assessed the response of *S. supranubius* to the rhizobial inoculation at the three transplanting stages followed by technicians at the Teide NP: growing seedlings at the greenhouses, during the acclimatization period, and in a field trial.

Growing legumes under greenhouse conditions is a very common practice to assess the symbiotic efficiency of the rhizobial inoculation. However, it is worth mentioning that here we evaluate the response of *S. supranubius* to the rhizobial inoculation in greenhouses located in Teide N.P., at 2050 m a.s.l., therefore in conditions similar to its natural habitat in the high mountain ecosystem of Tenerife. Although under greenhouse conditions plants are protected from some stresses (such as solar irradiation and winds), inside this greenhouse temperatures are similar to the outside and frozen plants are not rare on the coldest days of the year. In addition, the substrate used in the N.P. to grow seedlings in the greenhouse stage is not sterile, containing local soils and fertilized peat, so the presence of native rhizobia

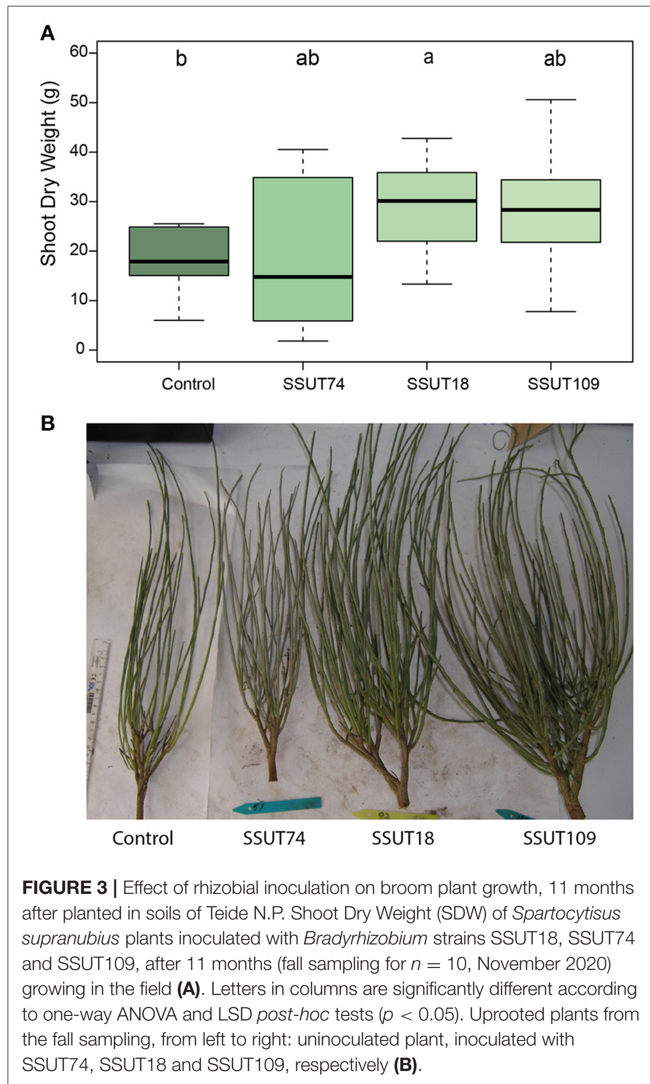


FIGURE 3 | Effect of rhizobial inoculation on broom plant growth, 11 months after planted in soils of Teide N.P. Shoot Dry Weight (SDW) of *Spartocytisus supranubius* plants inoculated with *Bradyrhizobium* strains SSUT18, SSUT74 and SSUT109, after 11 months (fall sampling for $n = 10$, November 2020) growing in the field **(A)**. Letters in columns are significantly different according to one-way ANOVA and LSD *post-hoc* tests ($p < 0.05$). Uprooted plants from the fall sampling, from left to right: uninoculated plant, inoculated with SSUT74, SSUT18 and SSUT109, respectively **(B)**.

TABLE 3 | Effect of the inoculation with three *Bradyrhizobium* sp. strains on the plant growth of *Spartocytisus supranubius* grown for 11 months in natural soils of the Teide National Park (sampling November 2020).

Treatment	SFW (g)	SDW (g)	Plant height (cm)	Plant width (cm)
SSUT18	63.3 ± 6.9 a	28.6 ± 3.1 a	50.0 ± 2.5 a	17.8 ± 1.1 ab
SSUT74	39.6 ± 9.8 bc	18.5 ± 4.6 ab	44.2 ± 3.7 a	13.2 ± 1.7 c
SSUT109	60.4 ± 8.5 ab	27.5 ± 4.0 ab	47.8 ± 3.8 a	18.3 ± 1.6 a
Control	37.0 ± 4.8 c	17.4 ± 2.2 b	49.0 ± 2.7 a	13.8 ± 1.4 bc

Average Shoot Fresh Weight (SFW), Shoot Dry Weight (SDW), Plant height and Plant width for a $n = 10$, sampling of November 2020. Mean ± standard error. Values with different letters in the same column are significantly different according to one-way ANOVA and a LSD or T2-Tamhane post-hoc test ($p < 0.05$).

and some N input is possible. Despite of that, our results showed that broom seedlings responded positively to the inoculation with selected rhizobia, enhancing the natural nodulation and promoting plant growth, pointing to the effectiveness of these "extra" nodules compared to the more weakly nodulated control plants. The results for those inoculated with strains SSUT18 and SSUT109 were particularly positive as broom seedlings with these two strains doubled the biomass (FW and DW) of control plants and showed more branches, a key factor for this plant since photosynthesis mainly occurs in its photosynthetic stems (Kyncl et al., 2006). However, strain SSUT74 did not perform with the expected effectiveness, neither in the greenhouse nor in the field trial experiment. An important difference was that the substrate used to grow plants in the previous screening experiment was sterile vermiculite, while the experiments of this study were performed in non-sterile soils. The results obtained with SSUT74 inoculated plants might reflect poor root colonization and/or its inability to compete with other less effective, more competitive native strains in the soils, which could have occupied the nodules instead. In fact, an ERIC-PCR fingerprint from nodules recovered in the summer sampling (**Supplementary Figure 8**) seem to support this. Whereas, ERIC profiles confirmed nodule occupancy for SSUT18 and SSUT109, for SSUT74 it was observed that at least some nodules contained a different strain. This result was unexpected, because this strain had been isolated from local soils, so other unknown factors could be involved.

In addition to promoting growth, broom-seedling inoculation also had positive effects on AWC (**Table 1**) and plant survival (**Supplementary Table 2**). AWC in inoculated seedlings was statistically higher than controls. Interestingly, this effect seems unrelated with effectiveness, as this positive response to inoculation included strain SSUT74, which could be classified as symbiotically inefficient. Many studies have shown the sensitivity of the rhizobium-legume symbiosis to water deficit (Zahran, 1999; Marino et al., 2007; Marinković et al., 2019). However, few studies deal with the benefits of rhizobial inoculation of legumes under drought stress. An interesting example is the study by Agele et al. (2017), who observed positive effects in four legume species under several water stress levels (statistically significant for the less severe watering regime). Also, a significant delay in drought-induced leaf senescence was found in nodulated *Medicago truncatula* in comparison to non-nodulated plants (Staudinger et al., 2016). In turn, Defez et al. (2019), showed that overproduction of rhizobial indole-acetic acid inside root nodules positively affected drought stress response in nodulated alfalfa plants. Our preliminary results point to a positive effect of rhizobial inoculation on water content, at least during the early development stages of broom plants, and it will require further studies to confirm this tendency and investigate the mechanisms behind it.

Seedling mortality (above 60%) in the park greenhouse was considerably high during the first months. At this early stage, seedlings benefited from rhizobial inoculation, showing a survival rate about 20% higher when inoculated with strains SSUT18 and SSUT109. These results were similar to those by Navarro et al. (2014) who reported survival rates 25% higher for the rhizobia-inoculated pre-germinated seeds of *Lupinus mariae-josephae*, compared to uninoculated controls, after six months in the field. It has been shown that the first months of growth are critical for plant survival in alpine ecosystems (Forbis, 2003). Therefore, by promoting growth and decreasing mortality

in this delicate early stage of seedling development, rhizobial inoculation can contribute to the success of the early steps of replanting campaigns.

Field experiments to study the effect of rhizobial inoculation on legumes are often complicated or difficult. Many failures occur because many adverse environmental conditions influence symbiosis under field conditions (Bordeleau and Prévost, 1994; Zahran, 1999) and/or due to a lack of competitiveness of the introduced rhizobia inoculants are easily supplanted by more competitive, less effective native rhizobia (diCenzo et al., 2019; Irisarri et al., 2019).

Seasonal variations are of great relevance to plant growth, especially in alpine ecosystems. Our first sampling in the field was in July. Although for other ecosystems the summer could already be an unfavorable time, in the alpine ecosystem of Teide N.P. spring comes late and early summer is still beneficial to plants in the park, so this sampling can be considered representative of a favorable period. The second sampling was 4 months later, in mid-November (fall). Early fall in the park is usually unfavorable after the extremely dry summers. However, the first rains had already fallen before the November sampling, which made this sampling more positive than originally expected.

The results obtained for the field experiment for both seasons were good, showing a significant benefit of inoculation for most parameters. The lack of statistical significance in some of the studied parameters could be explained by the natural high morphological variability of *S. supranubius*.

Plants inoculated with the SSUT18 strain had significantly higher number and weight of nodules, greater sizes (biovolume) and biomass. This strain also had the highest N content per plant that, being not statistically significant in our experimental conditions, it still contributed to more N per planted hectare than control plants, a fact that deserves to be taken into consideration. Six-months transplanted juvenile-brooms inoculated with this strain produced 0.2 g N per plant. This means that in the small field trial experiment we performed with 20 plant/treatment, SSUT18 would have produced 4 g N. Total nitrogen contents in soils in the majority of the areas of the park are below 3 g.kg-1 and mainly contained as organic matter (Arbelo et al., 2009). Some calculations using the same transplanting experimental design of one plant every three meters would give N values for one planted hectare after 6 months in the field of 135 g·ha-1 for the control plants (spontaneously nodulated by rhizobia present in the local soils), but of 180 g·ha-1 for those plant inoculated with strain SSUT18, which is about 25% more N in a planted ha. Therefore, taking this into account, even this moderate N content detected in this field trial still could be important for the ecosystem maintenance in these N-poor soils.

Destructive methods, albeit providing accurate measurements of biomass, obviously have the disadvantage of eliminating the samples (Catchpole and Wheelert, 1985), which in case of threatened species is a serious drawback. Therefore, for *S. supranubius* in the first July sampling, together with the destructive method we also performed a non-destructive approach and calculated the apparent biovolume. Following the description of Gough (2010), who describes young *S. supranubius* as cone shaped, the biovolume was first estimated using the cone volume formula. However, when establishing a correlation between the biovolume and biomass (SDW), the regression coefficient was low. It has been pointed out that the apparent biovolume is sometimes difficult to estimate accurately given the spatial arrangements of organs (such as branches), and because it is a combination of plant tissue and void (Pottier and Jabot, 2017). Consequently, to obtain a more accurate biovolume, we considered individual Teide broom branches as cylinders (**Figure 1C**). The regression coefficient obtained using a cylindric approach is considered a good fit for an allometric equation, and is similar to other results in previous studies (Pottier and Jabot, 2017). This good correlation indicates that a non-destructive estimation of biovolume is a simple method that could be used as a tool to indirectly determine plant growth when plant destruction is not possible, desired or permitted, thus monitoring the effect of inoculation on growth several times during longer periods.

Height was also greater in inoculated broom plants in the two samplings (especially in July). This could be advantageous for the plants' establishment, since plant height has been associated with competitive vigor (Cornelissen et al., 2003). Furthermore, although more speculative, faster growth of inoculated plants could also have another positive effect, indirectly contributing to reduce the negative impact of rabbits. Cubas et al. (2018) considered that individuals 70–100 cm high are tall enough to escape rabbit grazing. Kyncl et al. (2006) established a lower escape height from rabbit herbivory, at 55 cm. Our inoculated brooms reached about 50 cm height, and hence by introducing rhizobial inoculation in the greenhouse and during the acclimatization stage, it is expected that in a shorter period of time the inoculated broom plants can reach this key rabbit grazing-resistant threshold, thus ensuring survival outside the exclosure plots. Furthermore, SSUT18- and SSUT109-inoculated broom plants had on average 10 branches more than uninoculated controls, a finding that deserves to be highlighted because the number of branches is important for this species for two reasons. One, most of the photosynthesis is carried out by its stems, due to its small, deciduous leaves (Wheeler and Dickson, 1990). Two, the broom also presents clonal growth, that is, its branches have the capacity to root and form new individuals (Kyncl et al., 2006). This strategy has proven to be an important propagation method for this species (Kyncl et al., 2006).

We could not however evaluate survival in the field trial experiment, since all planted individuals survived. In order to explain this apparent 100% survival rate, it must be taken into account that the field experiment started with a set of 30 juvenile broom plants (2 years old), which were inoculated twice several months before transplanting them into the field. For transplantation, we selected only 20 healthy-looking individuals per treatment, with well-established nodulation. In fact, of the original 30 plants, some did die during acclimatization (**Supplementary Table 2**). However, survival was still much higher (above 90%) than scored in seedlings.

In the field trial, the effect of the rhizobia on plant water content was not clear. Contrasting with results for inoculated seedlings, only slightly higher, statistically non-significant AWC values were registered (**Supplementary Table 1**). Whether the

fact that inoculated plants were already more than 2 years old when inoculated or some environmental conditions in the study area might have had an influence is not clear. Different sensitivity to water deficit has been reported among rhizobial strains (Mahler and Wollum, 1980; Fuhrmann et al., 1986; Busse and Bottomley, 1989) and so, isolation of water-stress adapted rhizobia strains may well be possible. The results for water content of inoculated seedlings encourage us to further study the effect of inoculation with selected bradyrhizobial strains, so as to alleviate drought stress in broom plants. Given the long drought episodes in the N.P. (Olano et al., 2017, del Arco-Aguilar and Rodríguez-Delgado, 2018), it would be extraordinarily desirable to demonstrate positive effects of rhizobial inoculation.

CONCLUSION

Inoculation with *Bradyrhizobium* strains SSUT18 and SSUT109 significantly enhanced biomass and biovolume and improved survival of *S. supranubius* seedlings and juvenile individuals. These were tested after growing in their natural ecosystem in the Teide N.P., under greenhouse and field conditions. By producing larger, more robust plants in a shorter time compared to uninoculated broom plants, bradyrhizobia inoculation reduces the greenhouse period and juvenile plants better cope with and survive stresses in field trials. Therefore, inoculation with selected rhizobia is a successful strategy to be integrated into conservation campaigns for threatened legume species. It could become a key tool in replanting and reforestation campaigns, aiding the maintenance of this fundamental key species in the delicate high mountain ecosystem of Tenerife.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT210556; https://www.ncbi.nlm.nih.gov/genbank/, MW439206; https://www.ncbi.nlm.nih.gov/genbank/, MW439207; https://www.ncbi.nlm.nih.gov/genbank/, MT321501; https://www.ncbi.nlm.nih.gov/genbank/, MW439203.

AUTHOR CONTRIBUTIONS

ML-B conceived the research and funding acquisition. ML-B and ÁG-R designed methodology. ML-B, LP-S, MA-A, and JM-E planned and set up the greenhouse and the field experiments. ÁG-R and LP-S took measurements of plant physiological parameters. ML-B, MA-A, and LP-S collected and took measures of the plant samples. LP-S and ML-B analyzed the data. JC and LP-S performed non-destructive methods and edited figures. ML-B and LP-S wrote the manuscript. All authors revised and made significant contributions to the write-up and agreed to the published version of the manuscript.

FUNDING

This work was supported by Fundación CajaCanarias-Caixa (grant 2017REC13). LP-S received a researcher-contract from grant 2017REC13 and a FPU fellowship from the Spanish Ministerio de Ciencia, Innovación y Universidades.

ACKNOWLEDGMENTS

The authors thank the local environmental authorities responsible for Teide National Park (Área de Sostenibilidad, Medio Ambiente, Aguas y Seguridad, Parque Nacional del Teide, Cabildo de Tenerife) for providing seeds, juvenile plants and permits and the technical personal for their help in the field work. We are especially grateful to Omar Trujillo for his enthusiasm with our work and for being always willing to lend a hand with the work in the greenhouse and the field.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.660574/full#supplementary-material

REFERENCES

Agele, S., Ajayi, A., and Olawanle, F. (2017). Effects of watering regime and *rhizobium* inoculation on the growth, functional and yield traits of four legume species. *Int. J. Plant Soil Sci.* 17, 1–15. doi: 10.9734/IJPSS/2017/32891

Arbelo, C. D., Sánchez, J., Notario, J., and Mora, J. (2009). "Parque Nacional del Teide. Dinámica de nutrientes y carbono en los suelos" in *Proyectos de Investigación en Parques Nacionales: 2005-2008*, eds L. Ramírez, and B. Asensio (Spain: Organismo Autónomo de Parques Nacionales), 43–71.

Ashraf, M., and Harris, P. J. C. (2013). Photosynthesis under stressful environments: an overview. *Photosynthetica* 51, 163–190. doi: 10.1007/s11099-013-0021-6

Bello-Rodríguez, V., Mateo, R. G., Pellissier, L., Cubas, J., Cooke, B., and González-Mancebo, J. M. (2020). Forecast increase in invasive rabbit spread into ecosystems of an oceanic island (Tenerife) under climate change. *Ecol. Appl.* 31:e02206. doi: 10.1002/eap.2206

Bordeleau, L. M., and Prévost, D. (1994). Nodulation and nitrogen fixation in extreme environments. *Plant Soil.* 161, 115–125. doi: 10.1007/BF02183092

Busse, M. D., and Bottomley, P. J. (1989). Growth and nodulation responses of *rhizobium meliloti* to water stress induced by permeating and nonpermeating solutes. *Appl. Environ. Microbiol.* 55, 2431–2436. doi: 10.1128/AEM.55.10.2431-2436.1989

Castro, I., Casado, M. Á., Ramirez-Sanz, L., de Miguel, J. M., Costa, M., and Díaz-Pineda, F. (1996). Funciones de estimación de la biomasa aérea de varias especies del matorral mediterráneo del centro de la península Ibérica. *Orsis Org. Sist.* 11, 107–116.

Catchpole, W. R., and Wheelert, C. J. (1985). Estimating plant biomass: a review of techniques. *Austral Ecol.* 17,121–131. doi: 10.1111/j.1442-9993.1992.tb00790.x

Chaer, G. M., Resende, A. S., Campello, E. F. C., de Faria, S. M., and Boddey, R.B. (2011). Nitrogen-fixing legume tree species for the reclamation of severely degraded lands in Brazil. *Tree Physiol.* 31, 139–49. doi: 10.1093/treephys/tpq116

Cornelissen, J. H. C., Lavorel, S., Garnier, E., Díaz, S., Buchmann, N., Gurvich, D. E., et al. (2003). A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. *Aust. J. Bot.* 51, 335–380. doi: 10.1071/BT02124

Cubas, J., Martín-Esquivel, J. L., Nogales, M., Irl, S. D. H., Hernández-Hernández, R., López-Darias, M., et al. (2018). Contrasting effects of invasive rabbits on endemic plants driving vegetation change in a subtropical alpine insular environment. *Biol. Invasions* 20, 793–807. doi: 10.1007/s10530-017-1576-0

Defez, R., Andreozzi, A., Romano, S., Pocsfalvi, G., Fiume, I., Esposito, R., et al. (2019). Bacterial IAA-delivery into medicago root nodules triggers a balanced stimulation of C and N metabolism leading to a biomass increase. *Microorganisms* 7:403. doi: 10.3390/microorganisms7100403

del Arco-Aguilar, M., Pérez-De-Paz, P. L., Acebes, J. R., González-Mancebo, J. M., Reyes-Betancort, J. A., Bermejo, J. A., et al. (2006). Bioclimatology and climatophilous vegetation of the Island of Tenerife (Canary Islands). *Ann. Bot. Fenn.* 43, 167–192.

del Arco-Aguilar, M., and Rodríguez-Delgado, O. (2018). *Vegetation of the Canary Islands*. Cham: Springer. doi: 10.1007/978-3-319-77255-4

Díaz, F. J., Tejedor, M., Jiménez, C., and Dahlgren, R. A. (2011). Soil fertility dynamics in runoff-capture agriculture, Canary Islands, Spain. *Agric. Ecosyst. Environ.* 144, 253–261. doi: 10.1016/j.agee.2011.08.021

diCenzo, G. C., Zamani, M., Checcucci, A., Fondi, M., Griffitts, J. S., Finan, T. M., et al. (2019). Multidisciplinary approaches for studying rhizobium–legume symbioses. *Can. J. Microbiol.* 65, 1–33. doi: 10.1139/cjm-2018-0377

Donate-Correa, J., Leon-Barrios, M., Perez-Galdona, R., and del Arco-Aguilar, M. (2007). Different *Mesorhizobium* species sharing the same symbiotic genes nodulate the shrub legume *Anagyris latifolia*. *Syst. Appl. Microbiol.* 30, 615–623. doi: 10.1016/j.syapm.2007.07.002

Fonseca, M. B., Peix, A., de Faria, S. M., Mateos, P. F., Rivera, L. P., Simões-Araujo, J. L., et al. (2012). Nodulation in *Dimorphandra wilsonii* Rizz. *(Caesalpinioideae*), a Threatened Species Native to the Brazilian Cerrado. *PLoS ONE* 7:e49520. doi: 10.1371/journal.pone.0049520

Forbis, T. A. (2003). Seedling demography in an alpine ecosystem. *Am. J. Bot.* 90, 1197–1206. doi: 10.3732/ajb.90.8.1197

Fuhrmann, J., Davey, C. B., and Wollum, A. G. (1986). Desiccation tolerance of clover rhizobia in sterile soils. *Soil Sci. Soc. Am. J.* 50, 639–644. doi: 10.2136/sssaj1986.03615995005000030019x

González-Rodríguez, Á. M., Brito, P., Lorenzo, J. R., Gruber, A., Oberhuber, W., and Wieser, G. (2017). Seasonal cycles of sap flow and stem radius variation of *Spartocytisus supranubius* in the alpine zone of Tenerife, Canary Islands. *Alp. Bot.* 127, 97–108. doi: 10.1007/s00035-017-0189-7

Gough, L. (2010). *The spatial ecology of an endemic desert shrub* (PhD thesis). Nottingham: University of Nottingham.

Ibarrola-Ulzurrun, E., Marcello, J., Gonzalo-Martín, C., and Martín-Esquivel, J. L. (2019). Temporal dynamic analysis of a mountain ecosystem based on multi-source and multi-scale remote sensing data. *Ecosphere* 10, 1–17. doi: 10.1002/ecs2.2708

Irisarri, P., Cardozo, G., Tartaglia, C., Reyno, R., Gutiérrez, P., Lattanzi, F. A., et al. (2019). Selection of competitive and efficient rhizobia strains for white clover. *Front. Microbiol.* 10:768. doi: 10.3389/fmicb.2019.00768

Kang, G., Li, G., Xu, W., Peng, X., Han, Q., Zhu, Y., et al. (2012). Proteomics reveals the effects of salicylic acid on growth and tolerance to subsequent drought stress in wheat. *J. Proteome Res.* 11, 6066–6079. doi: 10.1021/pr300728y

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Kyncl, T., Suda, J., Wild, J., Wildova, R., and Herben, T. (2006). Population dynamics and clonal growth of *Spartocytisus supranubius* (Fabaceae), a dominant shrub in the alpine zone of Tenerife, Canary islands. *Plant Ecol.* 186, 97–108. doi: 10.1007/s11258-006-9115-6

Laguerre, G., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P., and Amarger, N. (2001). Classification of rhizobia based on nodC and nifH gene analysis reveals a close phylogenetic relationship among Phaseolus vulgaris symbionts. *Microbiology* 147, 981–993. doi: 10.1099/00221287-147-4-981

Lorite, M. J., Donate-Correa, J., del Arco-Aguilar, M., Pérez Galdona, R., Sanjuán, J., and León-Barrios, M. (2010). *Lotus* endemic to the Canary Islands are nodulated by diverse and novel rhizobial species and symbiotypes. *Syst. Appl. Microbiol.* 33, 282–290. doi: 10.1016/j.syapm.2010.03.006

Macar, T. K., and Ekmekçi, Y. (2008). PSII photochemistry and antioxidant responses of a chickpea variety exposed to drought. *Zeitschrift Naturforsch.* 63, 583–594. doi: 10.1515/znc-2008-7-820

Mahler, R. L., and Wollum, A. G. (1980). Influence of water potential on the survival of rhizobia in a goldsboro loamy sand. *Soil Sci. Soc. Am. J.* 44, 988–992. doi: 10.2136/sssaj1980.03615995004400050023x

Marinković, J., Bjelić, D., Dordević, V., Balešević-Tubić, S., Jošić, D., and Vucelić-Radović, B. (2019). Performance of different *Bradyrhizobium* strains in root nodule symbiosis under drought stress. *Acta Physiol. Plant.* 41:37. doi: 10.1007/s11738-019-2826-9

Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., et al. (2007). Nitrogen fixation control under drought stress. Localized or systemic? *Plant Physiol.* 143, 1968–1974. doi: 10.1104/pp.107.097139

Martín-Esquivel, J. L., Marrero-Gómez, M., Cubas, J., González-Mancebo, J. M., Olano, J. M., and del Arco-Aguilar, M. (2020). Climate warming and introduced herbivores disrupt alpine plant community of an oceanic island (Tenerife, Canary Islands). *Plant Ecol.* 221, 1117–1131. doi: 10.1007/s11258-020-01066-5

Martínez-Hidalgo, P., Pérez-Yépez, J., Velázquez, E., Pérez-Galdona, R., Martínez-Molina, E., and León-Barrios, M. (2016). Symbiovar loti genes are widely spread among *Cicer canariense* mesorhizobia, resulting in symbiotically effective strains. *Plant Soil* 398, 25–33. doi: 10.1007/s11104-015-2614-2

Maxwell, K., and Johnson, G. (2000). Chlorophyll fluorescence-a practical guide. *J. Exp. Bot.* 51, 659–668. doi: 10.1093/jexbot/51.345.659

Navarro, A., Fos, S., Laguna, E., Durán, D., Rey, L., Rubio-Sanz, L., et al. (2014). Conservation of endangered *Lupinus mariae-josephae* in its natural habitat by inoculation with selected, native *Bradyrhizobium* strains. *PLoS ONE* 9:e102205. doi: 10.1371/journal.pone.0102205

Nilsen, E. T., Karpa, D., Mooney, H. A., and Field, C. (1993). Patterns of stem photosynthesis in two invasive legumes (*Spartium junceum, Cytisus scoparius*) of the California coastal region. *Am. J. Bot.* 80, 1126–1136. doi: 10.1002/j.1537-2197.1993.tb15344.x

Oksanen, A. J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2016). *Vegan: Community Ecology Package. Ordination Methods, Diversity Analysis and Other Functions for Community and Vegetation Ecologists*. Available online at: https://CRAN.R-project.org/package=vegan (accessed January 12, 2021).

Olano, J. M., Brito, P., González-Rodríguez, Á. M., Martín-Esquivel, J. L., García-Hidalgo, M., and Rozas, V. (2017). Thirsty peaks: drought events drive keystone shrub decline in an oceanic island mountain. *Biol. Conserv.* 215, 99–106. doi: 10.1016/j.biocon.2017.09.008

Paknejad, F., Nasri, M., Moghadam, H. R. T., Zahedi, H., and Alahmad, M. A. (2007). Effects of drought stress on chlorophyll fluoresence parameters, chlorophyll content and grain yield of wheat cultivars. *J. Biol. Sci.* 7, 841–847. doi: 10.3923/jbs.2007.841.847

Pérez de Paz, P., Del Arco, M., Acebes, J., and Wilpret, W. (1986). *Leguminosas Forrajeras de CANARIAS.* Santa Cruz de Tenerife: Museo Insular de Ciencias Naturales.

Pottier, J., and Jabot, F. (2017). Non-destructive biomass estimation of herbaceous plant individuals: a transferable method between contrasted environments. *Ecol. Indic.* 72, 769–776. doi: 10.1016/j.ecolind.2016.08.030

Pulido-Suárez, L., Diaz-Peña, F., Notario del Pino, J. S., Cabrera-Medina, A., and Leon-Barrios, M. (2021). Alteration of soil rhizobial populations by rabbit latrines could impair symbiotic nitrogen fixation in the insular alpine ecosystem of Teide National Park. *Agric. Ecosyst. Environ. Appl. Soil Ecol.* 160:103850. doi: 10.1016/j.apsoil.2020.103850

R Core Team (2017). *R: A Language and Environment for Statistical Computing*. Available online at: https://www.r-project.org/

Rodríguez, N., Notario, J., Arbelo, C. D., Rodríguez-Rodríguez, A., and Guerra, J. A. (2014). Spatial variability of soil properties and soils classification in Teide National Park (Tenerife, Canary Islands). *GlobalSoilMap* 191–196. doi: 10.1201/b16500-37

Rodríguez-Delgado, O., and Elena-Roselló, R. (2006). *Evolución del Paisaje Vegetal del Parque Nacional del Teide.* Madrid: Ministerio de Medio Ambiente.

Safronova, V., Belimov, A., Andronov, E., Popova, J., Tikhomirova, N., Orlova, O., et al. (2017). Method for obtaining root nodules of the Baikal relict legumes in laboratory pot experiments. *Int. J. Environ. Stud.* 74, 700–705. doi: 10.1080/00207233.2017.1283948

Staudinger, C., Mehmeti-Tershani, V., Gil-Quintana, E., Gonzalez, E. M., Hofhansl, F., Bachmann, G., et al. (2016). Evidence for a rhizobia-induced drought stress response strategy in *Medicago truncatula*. *J. Proteomics* 136, 202–213. doi: 10.1016/j.jprot.2016.01.006

Stepkowski, T., Banasiewicz, J., Granada, C. E., Andrews, M., and Passaglia, L. M. P. (2018). Phylogeny and phylogeography of rhizobial symbionts nodulating legumes of the tribe genisteae. *Genes* 9:163. doi: 10.3390/genes9030163

Vinuesa, P., León-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Pérez-Galdona, R., et al. (2005a). *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. genistearum, Bradyrhizobium genospecies alpha and *Bradyrhizobium* genospecies beta. *Int. J. Syst. Evol. Microbiol.* 55, 569–575. doi: 10.1099/ijs.0.63292-0

Vinuesa, P., Silva, C., Werner, D., and Martínez-Romero, E. (2005b). Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Mol. Phylogenet. Evol.* 34, 29–54. doi: 10.1016/j.ympev.2004.08.020

Wheeler, C., and Dickson, J. (1990). Symbiotic nitrogen fixation and distribution of *Spartocytisus supranubius* on Las Cañadas, Tenerife. *Vieraea* 19, 309–314.

Zahran, H. H. (1999). Rhizobium -legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63, 968–989. doi: 10.1128/MMBR.63.4.968-989.1999

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.

Copyright © 2021 Pulido-Suárez, González-Rodríguez, Cubas, del Arco-Aguilar, Martín-Esquivel and León-Barrios. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

ORIGINAL RESEARCH
published: 22 April 2021
doi: 10.3389/fagro.2021.660100

Non-specific Lipid Transfer Proteins in Legumes and Their Participation During Root-Nodule Symbiosis

*Citlali Fonseca-García, Jorge Solis-Miranda, Ronal Pacheco and Carmen Quinto**

Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

OPEN ACCESS

Edited by:
Jose Palacios, Polytechnic University of Madrid, Spain

Reviewed by:
Estibaliz Larrainzar, Public University of Navarre, Spain
Kanika Khanna, Guru Nanak Dev University, India

***Correspondence:**
Carmen Quinto
carmen.quinto@ibt.unam.mx

In Memoriam:
This paper is dedicated to the memory of Dr. Tomás Ruiz-Argüeso.

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *28 January 2021*
Accepted: *22 March 2021*
Published: *22 April 2021*

Citation:
Fonseca-García C, Solis-Miranda J, Pacheco R and Quinto C (2021) Non-specific Lipid Transfer Proteins in Legumes and Their Participation During Root-Nodule Symbiosis. Front. Agron. 3:660100. doi: 10.3389/fagro.2021.660100

Non-specific lipid transfer proteins (LTPs) constitute a large protein family in plants characterized by having a tunnel-like hydrophobic cavity, which allows them to transfer different lipid molecules. LTPs have been studied in various model plants including those of agronomic interest. Recent studies have demonstrated that LTPs play key functions in both biotic and abiotic stress. In plants-pathogen interaction, they act as either positive or negative regulators of defense responses. However, little is known about the roles of LTPs in symbiotic interactions, especially in root nodule symbiosis. Here, we performed a broad genome analysis of LTP family members in legumes and other important model plants, focusing on their possible roles in legume-rhizobium symbiosis. *In silico* analysis showed that legumes contain large LTP families, with at least 70 LTP members clustered into four clades. Although the structures of LTP genes and proteins are conserved among species, differences were observed between clades from different species. LTPs are widely expressed in different plant tissues. In general, genes of the *LTP1* and *LTP2* classes are highly expressed in shoot and reproductive tissues in all analyzed species. Furthermore, genes of the different classes are also expressed in roots inoculated with rhizobia and nodules of legumes. RT-qPCR expression profile analysis of seven *PvLTP* genes in common bean (*Phaseolus vulgaris*) revealed that these genes are differentially expressed during the early and late stages of nodulation and they are genetically regulated by *PvRbohA*. These findings provide insight into the putative roles of LTP family members in legume-rhizobium symbiosis and their possible interactions with RBOH-dependent ROS production.

Keywords: expression profile, legumes, lipid transfer proteins, nodule symbiosis, phylogenetic analysis

INTRODUCTION

Lipid transfer proteins (LTPs) constitute a large family of plant proteins that bind to different lipids in a non-specific manner. As LTPs can transfer a wide range of lipid molecules, such as phospholipids, galactolipids, and free fatty acids, they are also known as non-specific LTPs (nsLTPs) (Wei and Zhong, 2014; Edqvist et al., 2018). LTPs have a low molecular weight and contain four or five α-helices connected by four disulfide bonds. These boundaries are formed by the conserved eight-cysteine motif (8CM) with the general structure C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C, where X represents any amino acid (Shin et al., 1995; Boutrot et al., 2008; Lascombe et al., 2008). LTPs are also characterized by the presence of a hydrophobic cavity that allows them to bind to lipid molecules (Lascombe et al., 2008).

LTP family members have been identified in various angiosperms, including rice (*Oryza sativa L.*), *Arabidopsis thaliana* (L.) Heynh, wheat (*Triticum aestivum* L.) (Boutrot et al., 2008), barley (*Hordeum vulgare* L.) (Zhang et al., 2019), maize (*Zea mays* L.) (Wei and Zhong, 2014), and potato (*Solanum tuberosum* L.) (Li et al., 2019). Also in non-vascular plants, such as *Marchantia polymorpha* L., *Physcomitrium* (*Physcomitrella*) *patens* (Hedw.) Bruch and Schimp., *Selaginella moellendorffii* Hieron, the fern *Adiantum capillus-veneris* L., and the conifer *inus taeda* L. (Edstam et al., 2011).

Plant LTPs are classified into 2 subgroups based on their structural organization: LTP1s, with a molecular weight of 9–10 kDa and ∼90–95 amino acids; and LTP2s, with a molecular weight of 6–7 kDa and 65–70 amino acids (Finkina et al., 2016). LTPs have also been characterized based on the presence and positions of introns, the presence of a glycosylphosphatidylinositol (GPI) modification site, the Cys spacing in the 8CM, and the similarity of their amino acid sequences. Based on these features, LTPs are grouped into 10 subtypes: LTP1, LTP2, LTPc, LTPd, LTPe, LTPf, LTPg, LTPh, LTPj, and LTPk (Edstam et al., 2011).

LTPs participate in lipid barriers synthesis, facilitating the transportation of lipid polymers in the apoplastic space, as well as in the loosening of the cell wall (Edqvist et al., 2018). Under *in vitro* conditions, *As*E246, an LTP from Chinese milk vetch (*Astralagus sinicus* L.), was shown to bind to different fatty acids, mainly those with chains 16–18 carbons in length. Under the same conditions, *As*E246 also binds to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), monogalactosyldiacylglycerol, (MGDG), and to digalactosyldiacylglycerol (DGDG) (Lei et al., 2014).

Genetic approaches have reveled LTPs functions in plant responses to pathogens. For instance, the Arabidopsis *LTP3* gene transcript accumulated in response to the bacterial pathogen *Pseudomonas syringae pv. tomato (Pst)* DC3000 and its overexpression enhanced the susceptibility to this pathogen (Gao et al., 2016). Transcript accumulation of the LTP4 from *Nicotiana tabacum L.* (*NtLTP*$_4$) also increased in response to wounding and infection with *Ralstonia solanacearum* (Xu et al., 2018). Moreover, salicylic acid (SA), a hormone that induces the plant systemic acquired resistance (SAR) of plants against biotrophic bacterial pathogens (Zhao, 2015), enhanced *NtLTP*$_4$ transcription (Xu et al., 2018). Xu et al. (2018) also found that jasmonic acid (JA), a hormonal regulator of defense responses to wounding and pathogen attack (Halim et al., 2006), increased *NtLTP*$_4$ transcription.

The Arabidopsis nsLPT-related gene *DRN1* (disease related nonspecific lipid transfer protein) is suppressed in response to *Pst* DC3000 and *Pseudomonas syringae pv. maculicola* ES4326 (*Psm* ES4326). Interestingly, when plants were challenged with bacteria with a defective type three secretion system (TTSS), *DRN1* was not suppressed. Additionally, *drn1* mutants showed less resistance to both bacterial (*Pst* DC3000 and *Psm* ES4326) and fungal pathogens (*Botrytis cinerea*). Since *DRN1* expression was also abrogated in SA, JA, and ethylene (ET)-mediated resistance, it is probably regulated by a pathway independent of these hormones. In this context, it was shown that *DRN1* expression is apparently regulated by superoxide radicals (Dhar et al., 2020).

In root nodule symbiosis, LTP functions have also been reported. For instance, *As*E246 was shown to bind DGDG from Chinese milk vetch nodule extract; interestingly, *As*E246 knockdown reduced lipid abundance (PC, PE, PI, and DGDG) in the nodules. *As*E246 knockdown also reduced number of nodules and infection threads (IT) and impaired the development of symbiosomes. Conversely, *As*E246 overexpression increased the number of nodules and IT (Lei et al., 2014). Recently, *As*E246 was observed to interact with the high temperature protein G (HtpG) of *Mesorhizobium huakuii* in infected nodule cells of *A. sinicus* and in symbiosome membranes (Zhou et al., 2019). Furthermore, downregulation of the LTP nodulin 5 from *Medicago truncatula* Gaertn. (MtN5) caused an increase in the curling of the root hair, but a reduction of invaded nodule primordia. On the contrary, MtN5 overexpression favored both root hair curling and invaded nodule primordia, also, the overexpression of MtN5 increased the content of lipids, MGDG, DGDG, PE and glucosyl ceramides, in infected roots (Santi et al., 2017).

Since LTPs bind to and transfer a variety of lipids, they are thought to play important roles in membrane remodeling. Both membrane remodeling and the formation of new membranes are key processes for the development of nodules during legume-rhizobium symbiosis. During this symbiotic interaction, rhizobium (soil bacteria capable of fixing atmospheric nitrogen) are internalized into nodule cells via a mechanism similar to endocytosis that encapsulates the bacteria within the plant membrane (Oldroyd and Downie, 2008; Roy et al., 2020). Inside the nodules, bacteria differentiate into bacteroids, which are surrounded by a membrane of plant origin. In these compartments, known as symbiosomes, bacteroids fix atmospheric nitrogen, and the plant-derived membrane acts as a symbiotic interface, allowing nutrient exchange (Oldroyd and Downie, 2008; Bapaume and Reinhardt, 2012).

Some legume species are widely exploited as crops, serving as a source of nutrients for human and animal consumption. The ability of legumes to develop symbiosis with nitrogen-fixing bacteria makes them suitable crops for the development of sustainable agriculture. However, little is known about the roles of LTPs in legume-rhizobium symbiosis. Therefore, in the current study, we examined the possible roles of these proteins in this process. We identified LTP family members in several model plant species, including seven species of legumes, using the criteria proposed by Edstam et al. (2011). To expand our knowledge of the evolutionary relationships of the LTPs, we constructed a phylogenetic tree based on the amino acid sequences of LTPs from legumes, cereals, and non-seed plants. Analysis of the gene structures of LTPs and their expression profiles in different organs of legumes and other species was performed based on several databases. Several LTPs are expressed in the roots and nodules of legumes. RT-qPCR assays showed that seven LTPs genes of common bean (*Phaseolus vulgaris L.*) were differential expressed in roots at different stages of nodule development and that some of these genes were regulated by downregulation of the NADPH oxidase *PvRbohA*. Therefore, we hypothesize that LTPs could regulate legume-rhizobium

symbiosis under the regulation of ROS production, dependent on RBOHs.

MATERIALS AND METHODS

Identification of LTP Family Members

To identify *LTP* genes in various plants (15 species, **Supplementary Table 1**), several databases were searched. LTPs from 12 species were identified in the *Phytozome 12.1.6* database (https://phytozome.jgi.doe.gov) (Goodstein et al., 2012). LTPs from *Lotus japonicus* L. were identified by searching *LotusBase* (https://lotus.au.dk) (Mun et al., 2016). The *LIS: Legume Information System* database (https://legumeinfo.org) (Dash et al., 2016) was used to identify *LTPs* in the genomes of *Pisum sativum L.* and *Lupinus albus*. *A. thaliana* LTP amino acid sequences were used as queries in all BLASTP searches.

Gene/Protein Sequence Analysis, Chromosomal Localization, and Synteny Analysis of LTPs in Legume Species and *A. thaliana*

Exon-intron gene structure information was obtained from the corresponding databases (**Supplementary Table 2**). Gene structure mapping was carried out using Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/Gsds_about.php). The conserved motifs and domains in the full-length amino acid sequences of all LTPs of legume species and *A. thaliana* were identified using the Multiple Expression motifs for Motif Elicitation version 5.2.0 tool (MEME) (Bailey et al., 2009). Chromosome localization was carried out with PhenoGram Plot (http://visualization.ritchielab.org/phenograms/plot) (Wolfe et al., 2013).

Synteny analysis was performed using the amino acid sequences of LTPs of *L. japonicus*, *Glycine max L.*, *P. vulgaris*, *M. truncatula*, and *A. thaliana*. In all cases, a BLASTP file in m8 format and a simplified gff file were used as inputs for the MCScanx Collinearity Scanner Toolkit (http://chibba.pgml.uga.edu/mcscan2/) (Wang et al., 2012).

In silico Analysis of LTP Expression in *P. vulgaris*, *G. max*, *M. truncatula*, *L. japonicus*, and *A. thaliana*

The expression profiles of *P. vulgaris LTPs* were obtained from the Common Bean Gene Expression Atlas, PvGEA (https://plantgrn.noble.org/PvGEA/) (O'Rourke et al., 2014). The *LTP* expression profiles of *G. max*, *A. thaliana*, *S. moellendorffii*, *Sorghum bicolor (L.) Moench*, *O. sativa*, and *Z. mays* were retrieved from Bio-Analytic Resource for Plant Biology (BAR) (Schmid et al., 2005; Libault et al., 2010). The expression profiles of *M. truncatula LTPs* were downloaded from the *M. truncatula* Gene Expression Atlas (MtGEA) (Benedito et al., 2008; He et al., 2009). The expression profiles of *L. japonicus LTPs* were retrieved from LotusBASE (https://lotus.au.dk/) (Mun et al., 2016), and the expression profiles of *P. patens LTPs* were retrieved from PEATmoss (https://peatmoss.online.uni-marburg.de/) (Fernandez-Pozo et al., 2020). Heat maps of the transcript levels of the genes were constructed using the heatmap.2 function of the gplot package (Warnes et al., 2015) with R Project.

Phylogenetic Analysis of LTPs

All protein sequences were aligned with the MUSCLE algorithm and manually edited using MEGA version X (Kumar et al., 2018) to remove misaligned sequences. The phylogenetic tree was constructed with the IQ-TREE algorithm 1.6.12 (Nguyen et al., 2014) using the maximum-likelihood method based on a VT+R9 substitution model with 1,000 bootstraps.

Wild-Type Plant Growth and Generation of Composite Plants

Common been seeds (*P. vulgaris* cv. Negro Jamapa) were surface sterilized as described by Estrada-Navarrete et al. (2007) and incubated in a germination chamber for 2 days at 28°C in the dark. At 2 days postgermination (dpg), the seedlings were placed in pots with sterile vermiculite and inoculated with *Rhizobium tropici* (CIAT 899) at an OD_{600} of 0.05 or they were not inoculated (control plants). All plants were watered three times per week with Fahraeus medium; control plants were treated with KNO_3 (10 mM) to avoid rhizobial contamination. At 7, 14, 21, and 30 days post-inoculation (dpi), the roots were harvested, frozen in liquid nitrogen, and stored at −75°C until RNA extraction.

To generate common bean composite plants (wild-type plants with transgenic hairy roots), 2 dpg seedlings were infected with *Agrobacterium rhizogenes* K599 strains carrying the *PvRbohA-RNAi* (Arthikala et al., 2017) and *PvRbohB-RNAi* (Montiel et al., 2012) constructs. As a control, composite plants carrying an inert RNAi vector were used (see details in Montiel et al., 2012); these plants are referred to as pTdT-SAC hereafter. All composite plants were generated as described by Estrada-Navarrete et al. (2007) with some modifications (**Supplementary Material**: protocol 1). Three to five days after removing the primary root, hairy roots of composite plants were observed under an epifluorescence microscope to confirm the presence of the reporter gene (*RFP*) and to remove non-transformed roots. Composite plants were potted in sterile vermiculite and inoculated with *R. tropici* (CIAT 899) as previously described. Hairy roots were harvested at 24 h after inoculation, frozen in liquid nitrogen, and stored at −75°C until RNA extraction.

RNA Extraction and qPCR Assays

Total RNA was extracted from frozen root tissue using TRIzol Reagent (Ambion®, Life Technologies™, USA) following the manufacturer's instructions. RNA integrity and concentration were verified by agarose gel electrophoresis (1%) and spectrometry (NanoDrop 2000/200c, Thermo Scientific, Waltham, MA, USA), respectively. DNA contamination was removed by incubation with RNase-free DNase (10 U/µl, Roche) at 37°C for 30 min. Complementary DNA (cDNA) was synthesized from 200 ng/µl of DNA-free RNA using RevertAid Reverse Transcriptase (200 U/µl, Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) assays were performed

TABLE 1 | Number of *LTP* genes identified in the analyzed species.

Species	LTP identified	LTP classes					
		LTP1	*LTP2*	*LTPc*	*LTPd*	*LTPe*	*LTPg*
C. reinhardtii	1	–	–	–	1	–	–
M. polymorpha	13	–	–	–	13	–	–
P. patens	28	–	–	–	20	–	8
S. moellendorffii	23	–	–	–	13	–	10
Z. mays	68	10	9	2	17	–	30
S. bicolor	63	10	13	1	17	–	22
O. sativa	73	19	13	3	12	–	26
A. thaliana	82	14	17	3	15	2	30
P. vulgaris	77	37	5	1	10	1	23
G. max	120	29	8	2	31	2	48
M. truncatula	95	45	6	1	15	1	27
T. pratense	85	39	5	–	18	1	22
L. japonicus	72	16	3	4	16	–	33
L. albus	87	36	9	1	11	2	28
P. sativum	73	32	3	–	16	1	21
	960						

using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA) on a RT-qPCR system (QuantStudio 5; Applied Biosystems, Waltham, MA, USA) as follows: 95°C for 10 min, 30 cycles of 95°C for 15 s, and 60°C for 60 s. The primer sequences used in the RT-qPCR assays are listed in **Supplementary Table 1**.

Relative transcript levels were calculated by the $2^{-\Delta\Delta CT}$ method using the transcript profile of the *elongation factor 1α* (*EF1α*) gene for normalization. The samples for the RT-qPCR assays were derived from three independent plants, with three biological replicates and three technical repeats performed per sample.

Statistical Analysis

The non-parametric Mann-Whitney test was performed using the *wilcox.test* function from the statistics package in R Project.

RESULTS

Phylogenetic Analysis Indicates That LTPs Are an Ancient Protein Family

Previous studies aimed at identifying the members of the LTP family have focused on only a few model species, such as *A. thaliana* and a few cereal crops (Edstam et al., 2011; Salminen et al., 2016). To analyze the potential roles of LTP subfamily members in legume-rhizobium symbiosis, we identified these proteins in the most recent versions of the genomes of 14 plants and a green alga from various databases (**Supplementary Table 1**). We searched for LTP homologs in each species, followed by domain analysis of homologous proteins to verify the presence of the characteristic lipid transfer protein domain of the LTP family. We identified 960 LTPs in the 15 species analyzed (**Table 1**, **Supplementary Table 3**). Remarkably, the *Chlamydomonas reinhardtii P. A. Dang.* genome had one significant hit related to the LTP family, making it the first reported LTP in a chlorophyte species. In addition, we identified at least 13 LTPs encoded in each terrestrial plant genome, with fewer homologous LTPs present in the analyzed marchantiophyta, bryophyte, and lycophyte species (**Supplementary Table 3**).

A robust phylogenetic analysis of the amino acid sequences of these 960 LTPs divided them into 4 major clades (**Figure 1**, **Supplementary Figure 1**). Following the guidelines proposed by Edstam et al. (2011), the clades were named according to the content of the characteristic 8CM motif C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C (**Figure 1**, **Supplementary Table 3**). One of these clades, consisting exclusively of the LTP1 class, was identified in all angiosperm species analyzed in this study (**Figure 1**, **Supplementary Figure 1**, light blue clade). Additionally, another clade consisting exclusively of the LTPg class was identified in all bryophyte and angiosperm species studied (**Figure 1**, **Supplementary Figure 1**, red clade). By contrast, two clades contained a mixture of classes, including one with classes LTP2, LTPc, and LTPd (**Figure 1**, **Supplementary Figure 1**, green clade) and the other with classes *LTPd* and *LTP*e (**Figure 1**, **Supplementary Figure 1**, lilac clade). At least one member of the *LTPd* class was present in all species analyzed. A few previously classified LTPs were reassigned based on a phylogenetic analysis and on the arrangement of 8CM motifs determined using the latest available versions of their genomes. These results suggest that LTPs are an ancient protein family that appeared before the first terrestrial plant; it was represented in the chlorophyte species by the LTPd class.

Features of LTPs Are Variable Among Plant Species

Our phylogenetic analysis showed that LTPs are a large family of conserved proteins in the phylum Plantae. However, some differences were detected in the physicochemical characteristics of LTPs in legumes vs. the other species. The angiosperm species contained more LTPs than the ancient lineages, suggesting that a major duplication event occurred during evolution. Although the total number of LTPs identified in legumes was similar to that in *A. thaliana* and cereals, the number of members in different classes varied. Our sequence analysis showed that LTPs in the legumes studied had a reduced number of members of the LTP2 class, with an average of 6 members, whereas *A. thaliana* and the cereal species had an average of 13 members (**Table 1**). By contrast, the LTP1 class was larger in legumes than in the other species, with an average of 33 members in the legume species studied and only an average of 13 members in the other angiosperm species (**Table 1**). These differential characteristics in the legumes could be related to their specific functions in this plant family, likely in root nodule symbiosis.

The 960 LTPs have variable amino acid length and molecular weight (MW), ranging from 55 to 793 amino acids and 8.8–20.8 kDa (**Supplementary Table 4**), respectively. In all species studied, the MW was closely related to the LTP class, with the

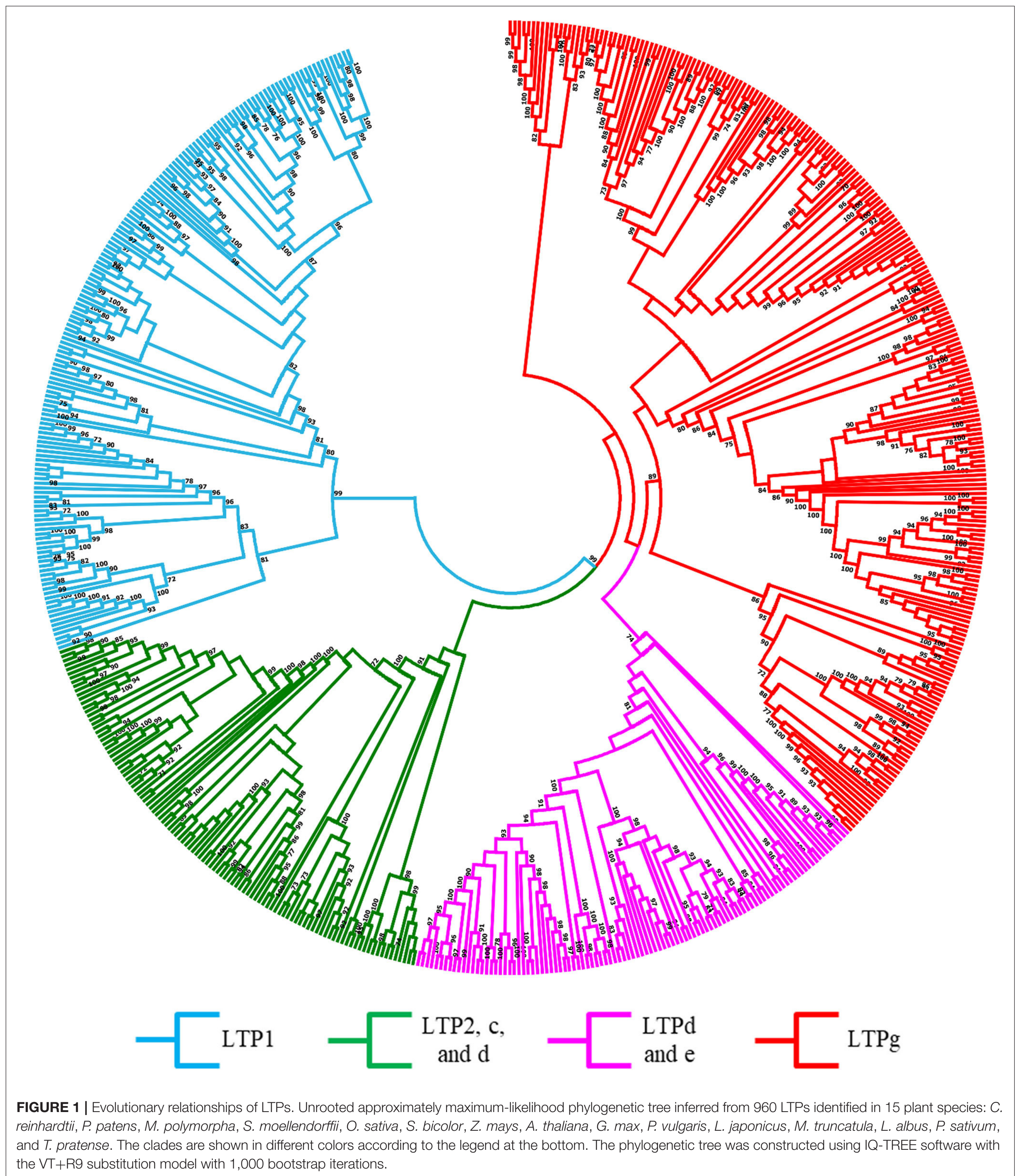


FIGURE 1 | Evolutionary relationships of LTPs. Unrooted approximately maximum-likelihood phylogenetic tree inferred from 960 LTPs identified in 15 plant species: *C. reinhardtii*, *P. patens*, *M. polymorpha*, *S. moellendorffii*, *O. sativa*, *S. bicolor*, *Z. mays*, *A. thaliana*, *G. max*, *P. vulgaris*, *L. japonicus*, *M. truncatula*, *L. albus*, *P. sativum*, and *T. pratense*. The clades are shown in different colors according to the legend at the bottom. The phylogenetic tree was constructed using IQ-TREE software with the VT+R9 substitution model with 1,000 bootstrap iterations.

highest MW in the LTPg class and the lowest in the LTPc class (**Supplementary Figure 2A**). Similarly, the isoelectric point (IP) showed some differences between LTP classes, ranging from 3.7 to 11.1 (**Supplementary Table 4**, **Supplementary Figure 1B**). The LTPc class showed more acidic IP values, while the remaining LTP classes tended to show basic IP values. As expected, the LTPd

identified in the alga *C. reinhardtii* showed a slightly basic IP of 7.56, confirming this categorization of IP values by LTP class and the trend toward a basic IP for the LTPd class.

LTPs are characterized by the presence of a lipid transfer domain and an 8CM arrangement along the sequence (**Figure 2B**). To characterize these conserved motifs and examine their relationship to legume nodule symbiosis, we analyzed the LTP sequences of the legumes and *A. thaliana* using MEME software (**Figure 2**). In the 691 sequences examined, 15 different motifs with diverse cysteine arrangements were identified throughout the sequences (**Figures 2A,C**). The motifs were mostly class specific for all species analyzed, showing clear differential patterns between the LTP1 and LTP2 classes and the remaining classes. Classes LTP1 and LTPg showed five specific motifs, while classes LTPc and LTPd shared two or three motifs in some genes. The motif located in the LTP2 class (motif number two) was the longest and one of the most conserved motifs. Surprisingly, we identified a specific motif in the LTP1 class in legumes that form indeterminate nodules, suggesting it might play a specific role in this type of symbiotic nodule (**Figures 2A,C**). Our findings indicate that the physicochemical characteristics of LTPs in plants vary between classes, but also between species, suggesting specific functions for each species.

Synteny of *LTP* Genes in Legumes and *A. thaliana* Showed a Duplicated Gene Family

To analyze the distribution of *LTP* genes in the genomes of the legumes and *A. thaliana*, we performed *in silico* mapping of the gene loci using PhenoGram Plot. The *LTP* gene family is distributed among all *A. thaliana* and legume chromosomes (**Supplementary Figure 2**). The *LTP* loci are clustered in a particular manner in some legume chromosomes by LTP class, which was not observed in *A. thaliana*. In *P. vulgaris*, 51% of the *LTP1* genes are located on chromosome 9, while 40% of *LTP1* genes are located on chromosome 7 in *M. truncatula*. Similarly, 50% of the *LTPd* genes are located on chromosome 18 in *G. max*, and 50% of *LTPg* genes are clustered on chromosomes 1 and 5 in *L. japonicus*.

We explored the evolutionary pathways of the *LTP* genes in the four legumes and *A. thaliana* individually and between species using MCScanx software. Individual syntenic analysis showed that *A. thaliana*, *P. vulgaris*, *L. japonicus*, and *M. truncatula* have a low percentage of *LTP* collinear genes in their genomes (10–17%), whereas *G. max* contains 52% of these genes (**Supplementary Table 4**, **Supplementary Figure 3**). These collinear genes represent less than half of the percentage of genetic synteny in the overall genomes of *A. thaliana* and *P. vulgaris*, which range from 27 to 29%. By contrast, in *L. japonicus* and *M. truncatula*, these collinear genes represent 1- or 2-fold that of their overall genomes, i.e., 5–13% (**Supplementary Figure 4**).

Syntenic analysis between species revealed 112 collinear *LTP* genes between *P. vulgaris* and *G. max*; 46 between *P. vulgaris* and *L. japonicus*; 54 between *P. vulgaris* and *M. truncatula*; 87 between *L. japonicus* and *G. max*; 93 between *G. max* and *M. truncatula*; and 49 between *L. japonicus* and *M. truncatula* (**Figures 3A–C,E–G**, **Supplementary Table 4**). Within these collinear genes, the *LTPg* class represents the highest percentage of syntenic genes (58%), followed by the *LTP1* and *LTPd* classes (17 and 14%, respectively). Analysis of the collinearity between the *LTPs* of these four legumes against *A. thaliana* revealed 124 legume genes that are syntenic to those of the Brassicaceae. Of these 124 syntenic genes, 29 belong to *P. vulgaris*, 21 to *L. japonicus*, 52 to *G. max*, and 22 to *M. truncatula* (**Figure 3D**, **Supplementary Table 5**). *G. max* has the highest number of syntenic genes, and *M. truncatula* has the lowest number compared with *A. thaliana*, which are mainly represented by the *LTPg* class in all instances. These results indicate that *LTP* genes were subjected to various duplication events throughout the evolution of legumes.

Conserved Exon-Intron Arrangement of *LTP* Genes in Legumes and *A. thaliana*

To analyze the functional roles of *LTP* genes, we assessed the exon-intron organization of the *LTP* genes in *P. vulgaris*, *G. max*, *L japonicus*, *M. truncatula*, *T. pratense*, and *A. thaliana* using Gene Structure Display Server 2.0 software. The positions of the introns are variable between all species analyzed and the *LTP* classes, but the number of introns showed little variation. In *A. thaliana*, four *LTP* genes lack introns (*AtLTP2.8*, *AtLTP2.11*, *AtLTPe.2*, and *AtLTPg.18*); the remaining genes of the *LTP1*, *LTPc*, and *LTPd* classes have one to three introns, with more genes containing three introns; genes of the *LTP2* class have one or two introns; *AtLTPe.1* has one intron, while *LTPd* genes have two to four introns, most with four introns (**Figure 4A**).

Among legumes, *M. truncatula* and *P. vulgaris* have the highest number of *LTP* genes without introns (20 and 11, respectively), followed by *G. max* and *L. japonicus* (6 and 1, respectively); all these genes are distributed among *LTP1*, *LTPc*, *LTPd*, *LTPe*, and *LTPg* (**Figures 4B–F**). Legumes that form determinate nodules have one to three introns in the *LTP1* class (**Figures 4B–D**), while genes belonging to this class from legumes that form indeterminate nodules have up four introns (**Figures 4E,F**). Additionally, the classes *LTP2*, *LTPd*, and *LTPg* exhibit a homogeneous intron distribution, with two, one to three, and one to five introns, respectively, in almost all legumes. Finally, genes in the *LTPc* and *LTPe* classes contain one to three introns, depending on the legume species. Although some conservation of introns was observed among the six plants analyzed, more introns are present in *LTP1* and *LTPg* genes in the legumes than in *A. thaliana*. However, more similarities between this non-legume dicot and legumes that form determinate vs. indeterminate nodules were observed.

Analysis of the Expression Profiles of *LTP* Genes

To analyze the expression profiles of the *LTP* genes in various plant organs, we retrieved expression data for ancient species, cereals, legumes, and *A. thaliana*, available in different gene expression atlases. Comparison of expression data between plant species and between *LTP* classes was performed. The results are presented in the following sections.

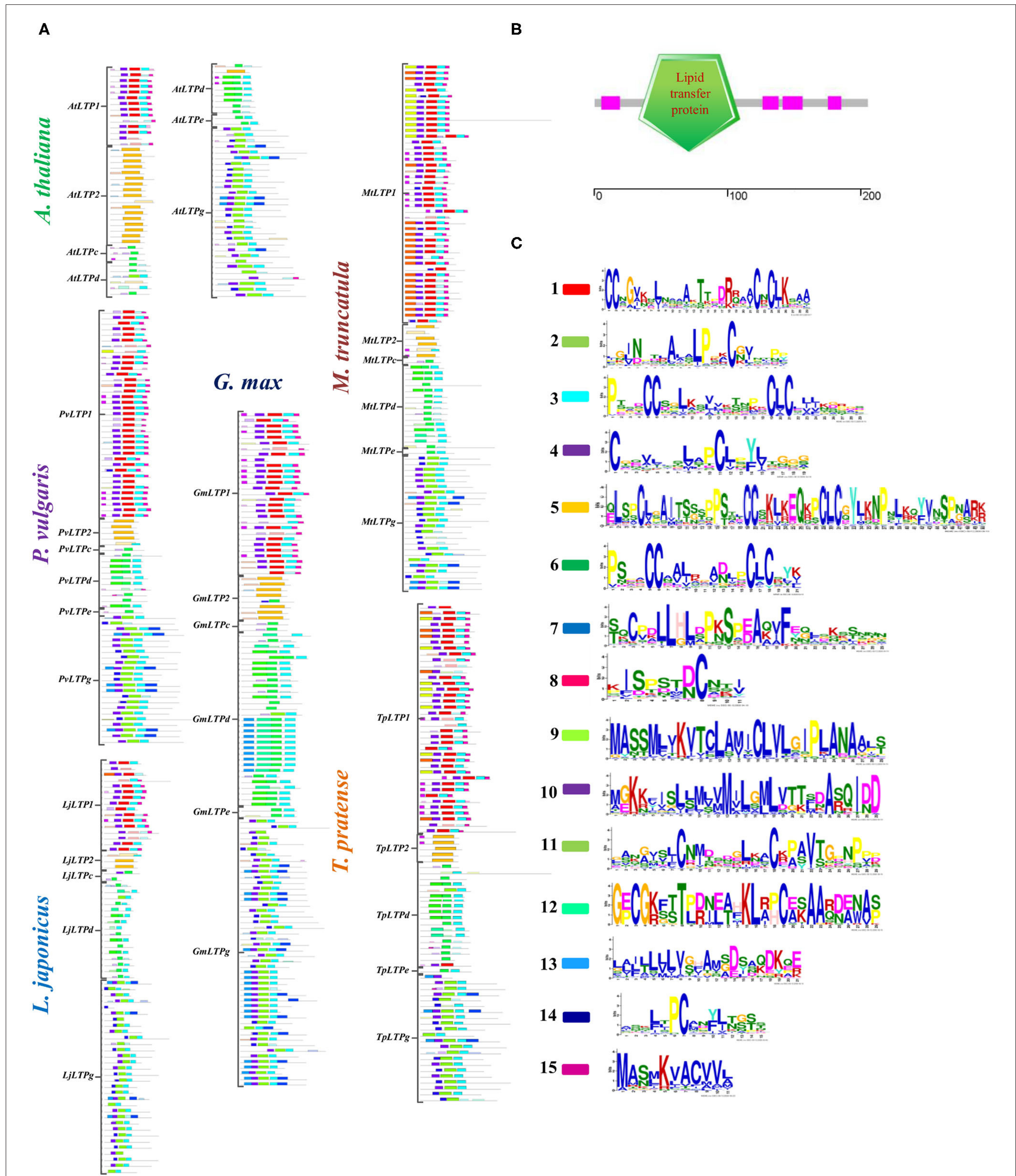


FIGURE 2 | Conserved functional motifs in LTPs. **(A)** Schematic representation of the motifs identified in LTP amino acid sequences from *P. vulgaris*, *G. max*, *L. japonicus*, *M. truncatula*, *T. pratense*, and *A. thaliana*. Significantly overrepresented motifs are shown by bars in their predicted positions. **(B)** Characteristic lipid transfer domains in LTP sequences. **(C)** Logo of the identified overrepresented motifs; the color code is the same as in **(A)**. The *LTP* classes on the left indicate the corresponding sequences for each class and species. MEME software was used to identify significantly overrepresented motifs.

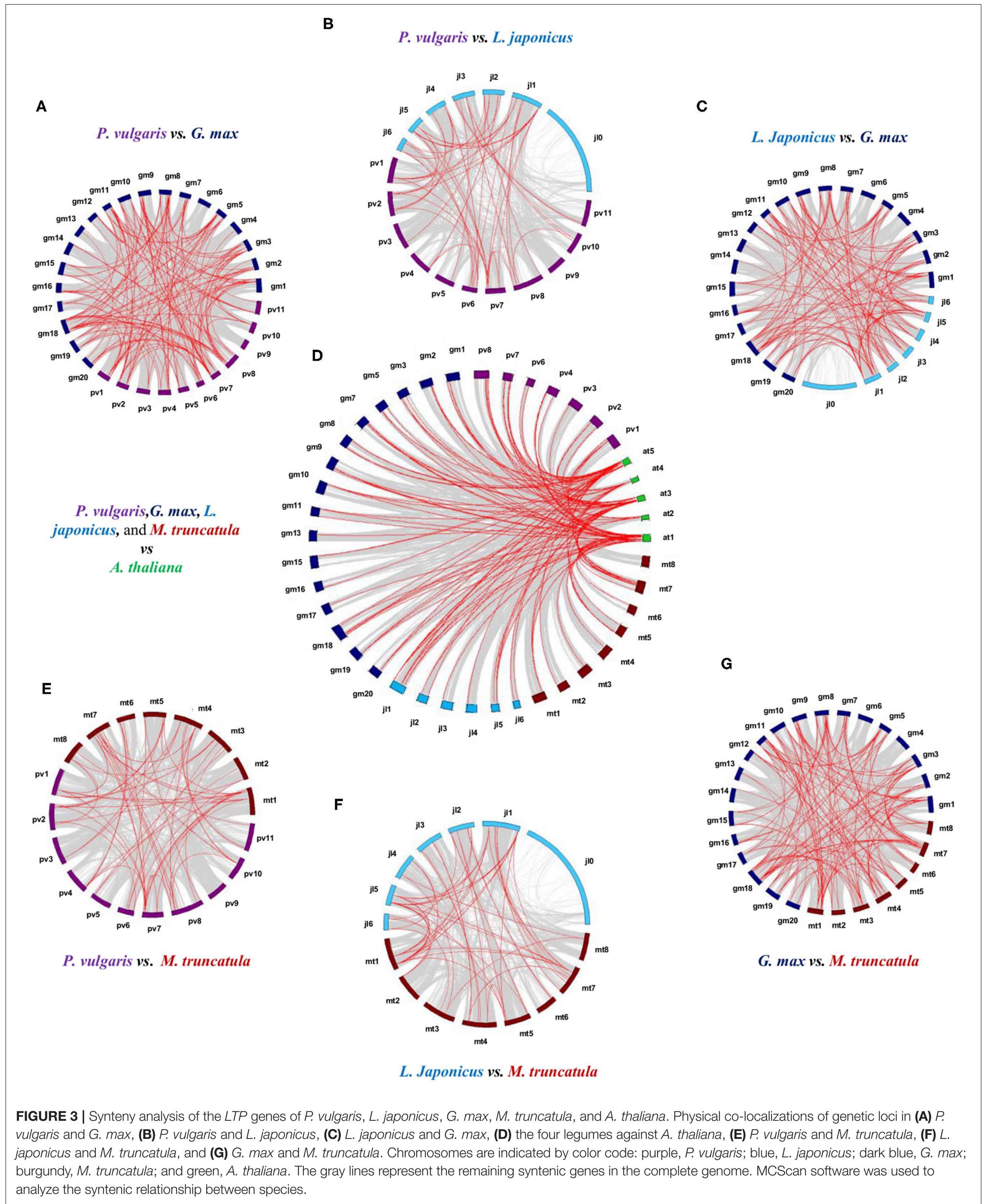


FIGURE 3 | Synteny analysis of the *LTP* genes of *P. vulgaris*, *L. japonicus*, *G. max*, *M. truncatula*, and *A. thaliana*. Physical co-localizations of genetic loci in **(A)** *P. vulgaris* and *G. max*, **(B)** *P. vulgaris* and *L. japonicus*, **(C)** *L. japonicus* and *G. max*, **(D)** the four legumes against *A. thaliana*, **(E)** *P. vulgaris* and *M. truncatula*, **(F)** *L. japonicus* and *M. truncatula*, and **(G)** *G. max* and *M. truncatula*. Chromosomes are indicated by color code: purple, *P. vulgaris*; blue, *L. japonicus*; dark blue, *G. max*; burgundy, *M. truncatula*; and green, *A. thaliana*. The gray lines represent the remaining syntenic genes in the complete genome. MCScan software was used to analyze the syntenic relationship between species.

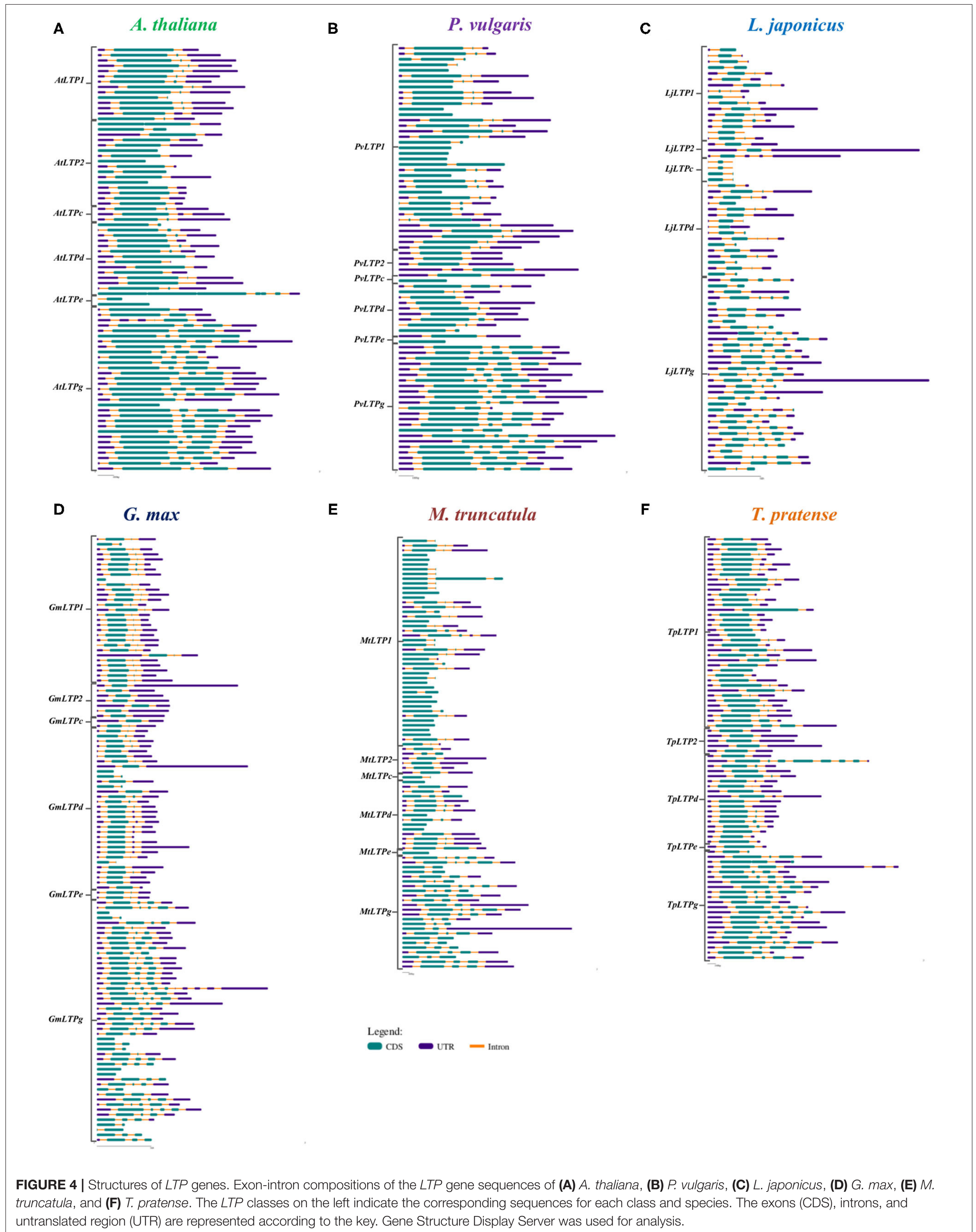


FIGURE 4 | Structures of *LTP* genes. Exon-intron compositions of the *LTP* gene sequences of **(A)** *A. thaliana*, **(B)** *P. vulgaris*, **(C)** *L. japonicus*, **(D)** *G. max*, **(E)** *M. truncatula*, and **(F)** *T. pratense*. The *LTP* classes on the left indicate the corresponding sequences for each class and species. The exons (CDS), introns, and untranslated region (UTR) are represented according to the key. Gene Structure Display Server was used for analysis.

Ancient Plants

Expression data for *P. patens* (a bryophyte) and *S. moellendorffii* (a lycophyte) were retrieved from the BAR for Plant Biology database. These species only possess the *LTPd* and *LTPg* classes, which exhibit differential expression patterns between species (**Supplementary Figures 5A,B**). In *P. patens*, the *LTPd* class (*LTPd15*, *LTPd17*, and *LTPd.18*) is highly expressed in whole gametophores, gametophores without rhizoids, and green sporophytes (**Supplementary Figure 5A**). Genes in the *LTPg* class are expressed 90% lower than *LTPd* genes, but are expressed in germinating spores and protonema. In *S. moellendorffii*, the *LTPd* class (*LTPd.8* and *LTPd.9*) is mainly expressed in roots and rhizophores, while the *LTPg* class (*LTPg.9*) is 99% higher expressed in strobili than in other tissues (**Supplementary Figure 5B**). These data suggest that the *LTPd* class is more strongly associated with aboveground tissue in bryophytes and belowground tissue in lycophytes, but members of the *LTPg* class are more closely involved in reproductive organ activity in both species. However, additional comparative analyses with ancient species are needed to test this hypothesis.

Monocotyledons

To analyze the expression patterns of *LTP* family genes in monocot species, we also retrieved expression data for the cereal species, *S. bicolor*, *O. sativa*, and *Z. mays* from the BAR for Plant Biology database. The *LTP* genes are mainly expressed in the shoot systems (basically leaves, stems, flowers, and seeds) of the three monocot species, with *LTP1* genes being the most highly represented class (**Supplementary Figures 5C–E**). In *S. bicolor*, the most highly expressed gene is *SbLTP1.9* in the vegetative meristem, followed by *SbLTP1.3* in shoots (**Supplementary Figure 5C**). In *O. sativa*, the most highly expressed gene is *OsLTP1.4* in stems, followed by *OsLTP2.4* in stems (**Supplementary Figure 5D**). The most highly expressed gene in *Z. mays* is *ZmLTP1.8* in young seeds (**Supplementary Figure 5E**). In these cereal species, the *LTP* genes were expressed 70% lower in belowground vs. aboveground tissue, where genes in classes *LTP1* and *LTPd* are mainly expressed (**Supplementary Figures 5C–E**). *SbLTPd.10* and *ZmLTPd.10* show the highest expression levels in *S. bicolor* and *Z. mays*, respectively, whereas 11 *OsLTP1* genes and *OsLTP2.3* show the highest expression levels in *O. sativa*. These data suggest that genes belonging to the *LTP1* class could play various roles in shoot development and reproduction in cereals.

Non-legume Dicotyledons

LTP family proteins have been studied more extensively in *A. thaliana* than in any other species. In the present study, *A. thaliana* was included as a model non-legume dicotyledon to compare the expression profiles of LTPs in legumes against dicotyledons. We retrieved expression data for *A. thaliana* from the BAR for Plant Biology database. In this dicot species, *AtLTP* genes are expressed at their highest levels in siliques and flowers, followed by leaves and seeds (**Figure 5A**). The *AtLTP1*, *AtLTP2*, and *AtLTPd* classes are the most highly represented classes in these organs, whereas genes in the *LTPg* class are expressed 67% less. Despite the generally lower expression levels of *AtLTP* genes in roots compared to siliques and flowers, *AtLTP2.5*, *AtLTP2.11*, *AtLTPd.4*, and *AtLTPg.19* are more highly expressed in roots than in these organs. These results suggest that members of the LTP family could play an important role in the reproductive process of this dicot species, as observed in the monocot species.

Legumes

To analyze the possible roles of LTP family members in root nodule symbiosis, we evaluated the expression patterns of these genes in the following legumes: *P. vulgaris*, *G. max*, *L. japonicus*, and *M. truncatula*. We observed both similarities and differences in the expression patterns of *LTP* genes in these legumes compared to non-legumes species. Genes in classes *LTP1* and *LTP2* are highly expressed in leaves, flowers, and seeds at different stages of development (**Figures 5B–E**). Although some *LTP* genes are highly expressed in flowers and seeds in all four legumes, the different classes of *LTPs* are also highly expressed in roots and nodules of the legumes (**Figures 5B–E**).

In *P. vulgaris*, *PvLTPg.11* is the most highly expressed gene in roots inoculated with rhizobia and in young nodules, followed by *PvLTPd.4*, *PvLTPd.6*, and *PvLTPd.10* (**Figure 5B**), whereas in *G. max*, *GmLTPd.1* and *GmLTP1.1* are the most expressed genes in roots (**Figure 5C**). *LjLTPd.1*, *LjLTPd.2*, *LjLTPd.3*, and *LjLTPc.1* are primarily expressed in roots and mature nodules of *L. japonicus* (**Figure 5D**). In *M. truncatula*, which forms indeterminate nodules, *MtLTPd.1*, *MtLTPd.2*, and *MtLTP2.1* show high expression levels in young and mature nodules and roots (**Figure 5E**). Among legumes, the *LTP1* and *LTPd* classes are the most commonly represented classes in roots and nodules, showing a differential expression pattern during early and late stages of nodulation.

These data suggest that some *LTP* classes are active in legume roots, roots inoculated with rhizobia, and nodules. To validate the expression patterns of *LTP* genes in the legumes analyzed *in silico*, we evaluated the expression profiles of seven *P. vulgaris* genes during the early and late stages of nodulation by RT-qPCR. We selected *LTP* genes that are potentially expressed under nodulation conditions in this legume that might also be expressed in the three other legumes examined. Also, we analyzed the expression profile of these genes in a home transcriptomic data of *P. vulgaris* roots inoculated and non-inoculated at 7 dpi with *R. tropici* (Fonseca-García et al., 2019), here they showed the highest fold changes with the highest read counts of the gene family in this legume (**Supplementary Table 6**). These seven genes (*PvLTP1.6*, *PvLTP2.3*, *PvLTP2.5*, *PvLTPd.4*, *PvLTPd.6*, *PvLTPd.10*, and *PvLTPg20*) presented two different expression profiles during nodulation in *P. vulgaris* (**Figure 6**).

Two genes were downregulated during the late stages of nodulation: *PvLTP1.6* was significantly suppressed at 14, 21, and 30 dpi, while *PvLTP2.3* was clearly downregulated at 14 and 21 dpi (**Figures 6A–C**). By contrast, 4 genes were upregulated between the early and late stages of nodulation: *PvLTPd.6*, *PvLTPd.10*, and *PvLTPg20* were upregulated at 7, 14, and 21 dpi, and *PvLTPg20* was also upregulated at 30 dpi (**Figures 6E–G**). Similarly, *PvLTPd.4* was upregulated at 7 and 14 dpi, but, intriguingly, it was downregulated at 30 dpi (**Figure 6D**). These results indicate that these seven *LTP* genes are differentially

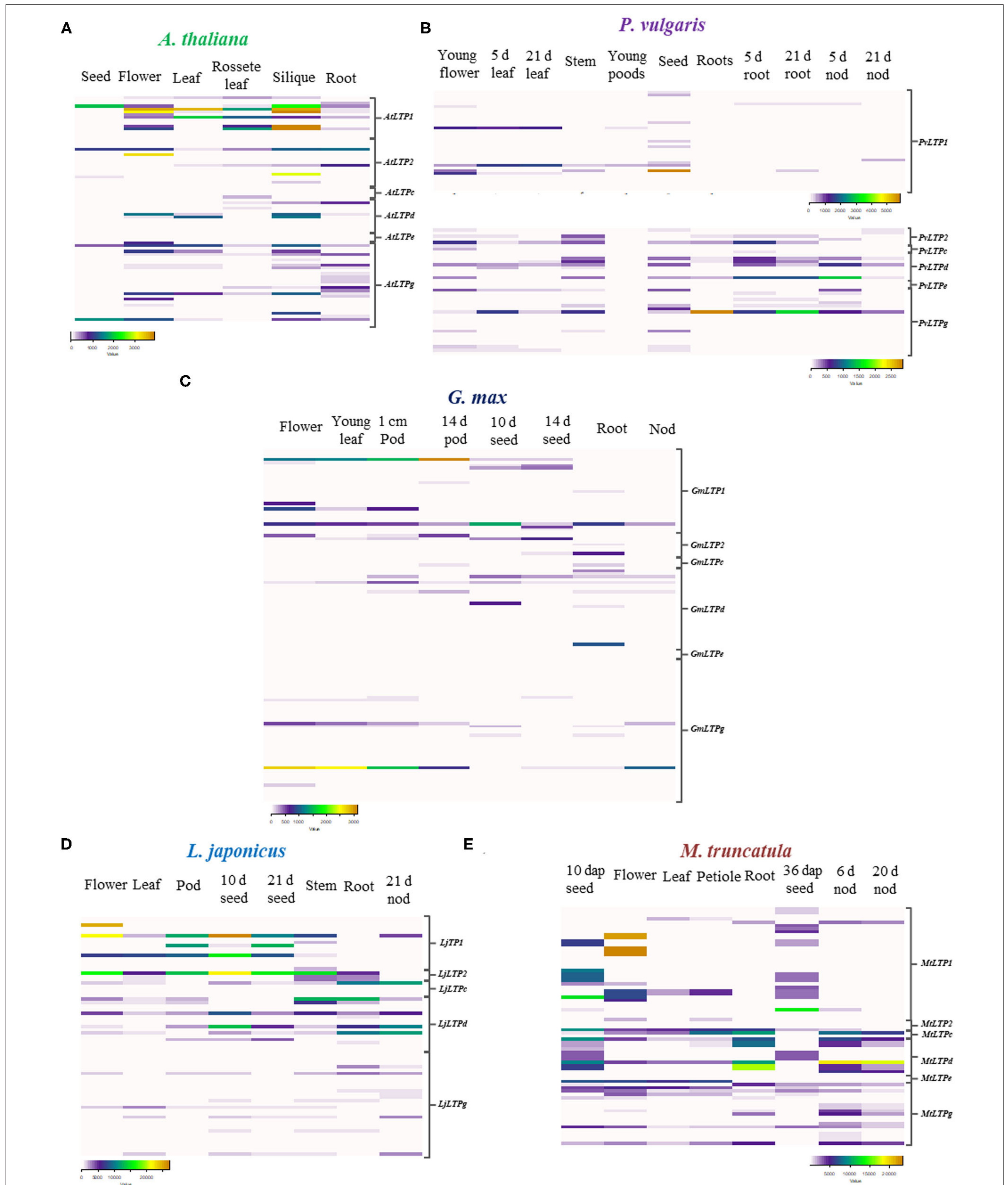


FIGURE 5 | Expression patterns of *LTP* genes. Heat maps of *LTP* gene expression values in **(A)** *A. thaliana*, **(B)** *P. vulgaris*, **(C)** *G. max*, **(D)** *L. japonicus*, and **(E)** *M. truncatula*. Expression data were retrieved from the PvGEA, LotusBASE, MtGEA, and BAR databases. RPKM values are represented as color codes below each heat map. The *LTP* classes on the left indicate the corresponding sequences for each class and species. dap, days after pollination; d, days; nod, nodules.

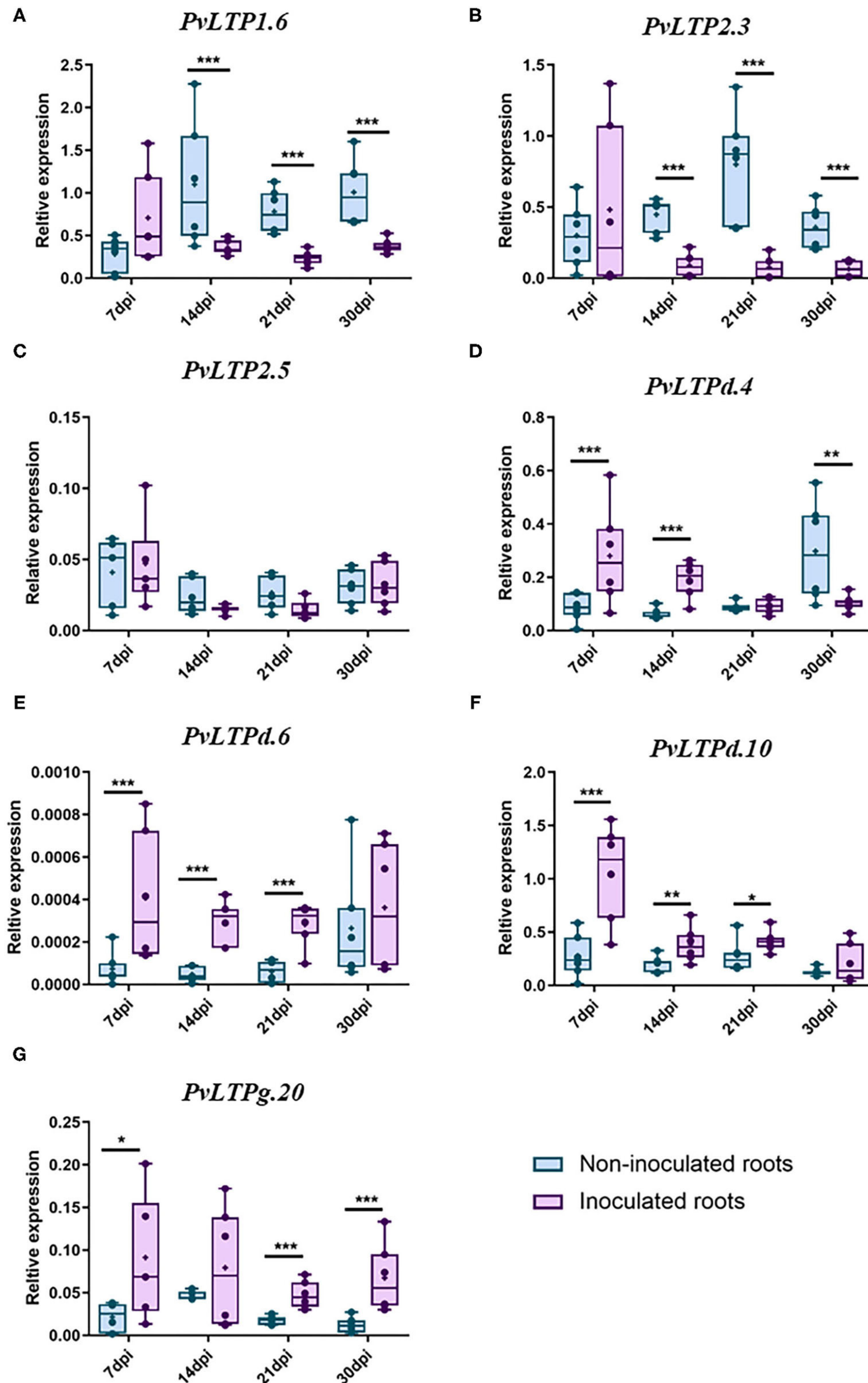


FIGURE 6 | RT-qPCR analysis of selected *P. vulgaris LTP* genes. The relative expression profiles of seven *LTP* genes from wild-type *P. vulgaris* roots inoculated or non-inoculated with *R. tropici*: **(A)** *PvLTP1.6*, **(B)** *PvLTP2.3*, **(C)** *PvLTP2.5*, **(D)** *PvLTPd.4*, **(E)** *PvLTPd.6*, **(F)** *PvLTPd.10*, and **(G)** *PvLTPg.20.* The transcript abundance of the selected genes was evaluated by RT-qPCR and normalized according to the expression of the *elongation factor 1α* (*EF1α*) gene. The blue bars represent non-inoculated roots, and the purple bars represent roots inoculated with *R. tropici*. The borders of the boxes represent the first quartile to the third quartile, the horizontal line within the box represents the median, and the whiskers represent the smallest and the largest outlier point of the data set ($n = 6$). A non-parametric Mann-Whitney test was performed to evaluate significant differences, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

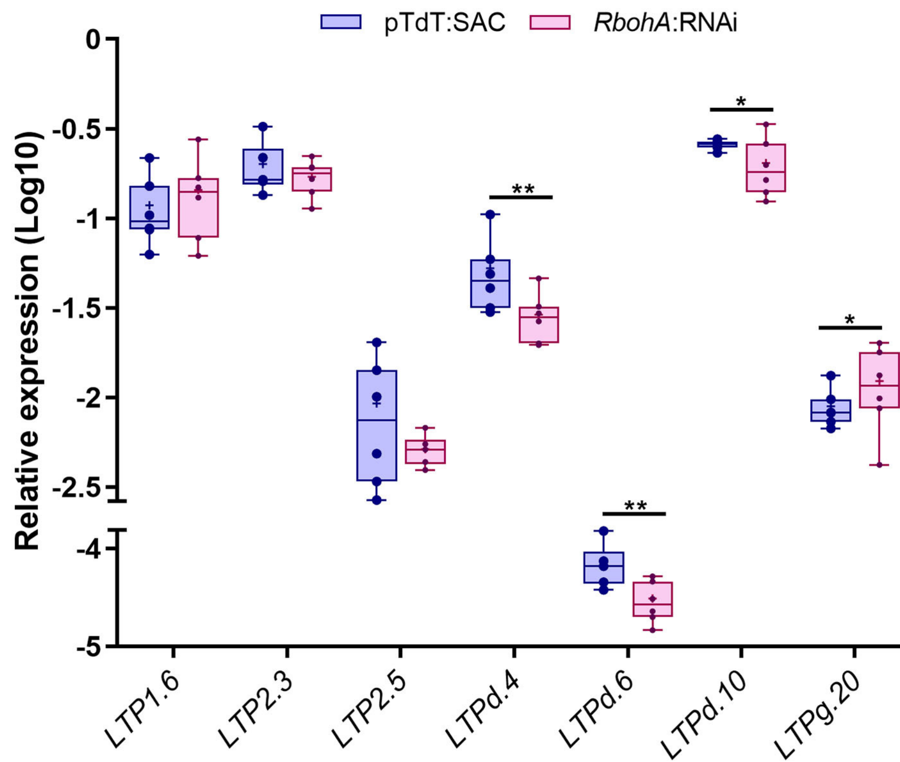


FIGURE 7 | RT-qPCR analysis of selected *P. vulgaris LTP* genes in *RbohA*:RNAi transgenic roots. Relative expression profiles of the seven *LTP* genes in control (pTdT-SAC) and *RbohA*:RNAi transgenic roots of *P. vulgaris* inoculated with *R. tropici.* The transcript levels were normalized according to the expression of the *elongation factor 1α* (*EF1α*) gene. The blue bars represent control (pTdT-SAC) transgenic roots, and the purple bars represent *RbohA*:RNAi transgenic roots inoculated with *R. tropici*. The borders of the boxes represent the first quartile to the third quartile, the horizontal line within the box represents the median, and the whiskers represent the smallest and the largest outlier point of the data set ($n = 6$). A non-parametric Mann-Whitney test was performed to evaluate significant differences, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

expressed during different stages of nodulation in common bean, suggesting that they are fine-tuning regulated in root nodule symbiosis.

Previously, our group demonstrated that ROS play an important role in common bean nodulation through RBOHs activity (Cárdenas et al., 2008; Montiel et al., 2012; Arthikala et al., 2017). To investigate the possible roles of these seven *PvLTP* genes in nodulation in more detail, we evaluated their transcript accumulation in transgenic roots expressing an RNA interference construct targeting *PvRbohA* following inoculation with *R. tropici*. *PvRbohA* encodes a NADPH oxidase required for the early stages of root nodule development in common bean (Arthikala et al., 2017). All three *LTPd* genes were downregulated with 23–54% less in the inoculated roots carrying the *RbohA*:RNAi construct at 1 dpi than in control roots, whereas *LTPg.20* was upregulated with 38% more under the same conditions (**Figure 7**). Furthermore, the *LTP2.5* gene presented a downregulation tendency with 44% less in the *RbohA*:RNAi roots than control, but *LTP1.6* and *LTP2.3* were expressed at similar levels in silenced and control roots. These results indicate that five of the seven genes analyzed could be regulated by ROS, produced by *RbohA* during the early stages of rhizobial symbiosis.

DISCUSSION

Legumes are valuable crops that serve as sources of food and animal feed throughout the world. These plants interact with soil bacteria called rhizobia, which through a molecular dialog and a coordinated cellular reorganogenesis in the plant cortex, form a new organ, the nodule. In the nodules, rhizobia differentiate into bacteroids and are enclosed to form symbiosomes, in which atmospheric dinitrogen is transformed into ammonia, a source of nitrogen that is assimilable by the plant (Roy et al., 2020). In plants, *LTP* genes comprise an abundant family of proteins and perform various functions in their life cycle (Edqvist et al., 2018). This gene family has been identified in some important plant species such as, *A. thaliana*, rice, wheat (Boutrot et al., 2008), maize (Wei and Zhong, 2014), barley (Zhang et al., 2019) and potato (Li et al., 2019). However, little is known about *LTPs* in legumes and their role in the root-nodule symbiosis. In this study, we performed a genome identification of LPTs family in various legumes species and other important plant model, moreover, we analyzed the putative roles of *LTPs* in rhizobial symbiosis.

Through a robust phylogenetic analysis, we identified 960 *LTP* genes in 15 different plant species, which were grouped into 6 classes (*LTP1*, *LTP2*, *LTPc*, *LTPd*, *LTPe*, and *LTPg*), according to

their phylogenetic features and the characteristic motif 8CM. We used the latest versions of the genomes of all species studied, allowing us to use the complete sequences of the putative *LTP* genes instead of the ESTs used in previous studies (Edstam et al., 2011); consequently, some previously reported *LTP* genes were reassigned in the current study. In addition, 30 genes showed incomplete 8CMs with at least 1 missing C; however, these sequences were considered to be *LTPs* because they all possess characteristic lipid transfer domains. This modification occurred in the 15 species studied and in the 6 classes of *LTPs*, suggesting that this feature was conserved during evolution. Notably, we identified the first reported *LTP* gene (*LTPd*) in an algae species, *C. reinhardtii*, indicating that *LTPs* are an ancient family of genes that has been present since the division of Chlorophyta, before the first land plant appeared.

Our phylogenetic analysis clustered the 960 LTPs into 4 clades (**Figure 1**, **Supplementary Figure 1**). Ancient species here studied, *P. patens* (Rensing et al., 2020) and *S. moellendorffii* (Ferrari et al., 2020), only contained classes LTPd and LTPg, suggesting that these two classes are the oldest in this gene family. On the contrary, the LTPe class was present only in dicot species, indicating that the class is specific to these angiosperms and that these genes could be the youngest members of the LTP family (**Table 1**). Additionally, we detected notable differences in the number of *LTP1* and *LTP2* genes among monocotyledonous and dicotyledonous species. In particular, legumes had fewer *LTP2* genes and more *LTP1* genes than the other species (**Table 1**), which contained a specific motif in legumes that form indeterminate nodules (**Figures 2A,C**). These results suggest that these classes of genes exhibit specific gene duplication, as well as structural modifications in legumes that could have specific functions in these plants, which has also been suggested in other plant systems (Moore and Purugganan, 2005; Conant and Wolfe, 2008; Flagel and Wendel, 2009).

Genome duplication is a primary source of genetic novelty, providing increased functionality in angiosperms and has played a fundamental role in their evolution (Davies et al., 2004). Many reports have documented duplication events of *LTP* genes in various species, such as *A. thaliana*, rice, sorghum (Salminen et al., 2016), potato (Li et al., 2019), maize (Wei and Zhong, 2014), and wheat (Kouidri et al., 2018; Odintsova et al., 2019). Gene duplication and functional redundancy allow *LTP* gene sequences to gather mutations, that result in increased divergence and thus the expansion and evolution of the gene family (Conant and Wolfe, 2008). Our synteny analysis of *LTP* genes revealed a conserved homology between some genes in the four legumes and *A. thaliana*. We observed a low percentage of collinear *LTP* genes in the individual genomes of *A. thaliana*, *P. vulgaris*, *L. japonicus*, and *M. truncatula*, while more than half of the *LTP* genes in *G. max* showed collinearity (**Supplementary Table 4**, **Supplementary Figure 3**). This could be due to *G. max* is an allotetraploid species that originated from hybridization events involving two different diploid progenitors (Cannon and Shoemaker, 2012). Also, we observed a higher percentage of collinear genes between *G. max* and the other legumes than within *A. thaliana*, *P. vulgaris*, *L. japonicus*, and *M. truncatula*. The LTP class with the most syntenic genes was *LTPg*, which was generally observed in all species tested with an extended number of members., It could be because *LTPg* class regulate the export of cuticular wax and suberin (Kim et al., 2012; Lee and Suh, 2018), important processes for plant development.

Gene function has been associated with different structural characteristics such as exon-intron composition, which bear the marks of the evolution of a gene family (Moore and Purugganan, 2005; Flagel and Wendel, 2009). According to our data, the LTP family formed before the differentiation of terrestrial plants and generated new classes during their evolution. The dicotyledonous species analyzed have a variable arrangement of exon-introns among the *LTP* classes, and legume genes have more introns than *A. thaliana* genes (**Figure 4**). Also, legumes have more genes without introns in all *LTP* classes compared to *A. thaliana*. *M. truncatula* has the highest number of genes without introns (22, including 17 in the *LTP1* class), followed by *P. vulgaris* (10 genes) and *A. thaliana* (only 5 genes). A compact genetic structure could be advantageous to plants responding to stimuli by allowing rapid gene expression (Jeffares et al., 2008). However, the expression patterns of intron less *LTP* genes in legumes were similar to those in *A. thaliana* (**Figure 5**).

Even though many potential *LTP* genes have been identified in some plant species using bioinformatics approaches, the functions of only a few *LTPs* have been revealed. However, some reports suggest that the a mechanism of action of LTPs is related to the transfer and deposition of the monomers required for the assembly of the waterproof lipid barriers (Charvolin et al., 1999; Han et al., 2001; Cheng et al., 2004). Root nodule symbiosis involve two complex processes that are simultaneously coordinated, rhizobial infection and nodule development, where the number of dividing cells increases dramatically, requiring membrane biosynthesis by the plant (Roy et al., 2020). Also, for the formation of symbiosomes the synthesis of the peribacteroidal membrane to enclose the bacteroids is required (Verma, 1992). The mentioned ability of LTPs to bind to and transfer a wide variety of lipids suggests that these proteins participate in membrane remodeling during root nodule symbiosis. In fact, the LTP *As*E246 from Chinese milk vetch bids DGDG from nodule extract and its knockdown impairs symbiosomes development (Lei et al., 2014). In addition, *in silico* analysis of the expression profiles showed that many *LTP* genes, especially *LTPd* genes, are active in legume roots and nodules (**Figure 5**).

RT-qPCR assays confirmed that genes belonging to the *PvLTPd* and *PvLTPg* classes are highly expressed in *P. vulgaris* roots inoculated with rhizobia during the early and late stages of nodulation (**Figures 6D–G**). In addition, genes of the *PvLTP1* and *PvLTP2* classes are repressed during late stages of nodulation in this legume (**Figures 6A–C**). Indeed, a novel *LTP* gene in Chinese milk vetch might be involved in the establishment of rhizobial infection and the transport of plant-synthesized lipids to the symbiosome membrane (Lei et al., 2014). Another report suggests that an *AtLTP1* gene is involved in regulating the ethylene response and signaling in *A. thaliana* (Wang et al., 2016), importantly, this phytohormone is a well-known negative regulator of nodulation. Our results suggest that genes of the *PvLTPd* and *PvLTPg* classes could

mediate positively root nodule symbiosis, transporting plant-synthesized lipids. Whereas, members of the *PvLTP1* and *PvLTP2* classes might inhibit this process interacting with negatives regulators.

ROS function as early signals in root hairs during the establishment of root nodule symbiosis (Montiel et al., 2016). In root hair cells of *P. vulgaris*, a rapid, transient ROS burst was detected 15–30 s after treatment with Nod factors (Cárdenas et al., 2008). In plants, ROS are mainly produced by the activity of the NADPH oxidase enzymes RBOHs, which have been implicated in effective rhizobial infection and in promoting nodule organogenesis in *P. vulgaris* (Montiel et al., 2012; Arthikala et al., 2014) and *M. truncatula* (Marino et al., 2011). In the current study, we provided evidence that the expression of *LTPd* and *LTPg* class genes is regulated by the downregulation of a *Pv*RBOH (*Pv*RBOHA) in *P. vulgaris* roots during the early stages of nodulation. Our findings strongly suggest that *LTP* genes could play different roles during nodulation in *P. vulgaris*, and possibly interacting with RBOH-dependent ROS production since the early stages. ROS as early signals in this symbiotic process could activate the synthesis of lipids in the plasma membrane necessary for rhizobia infection and nodule organogenesis, however further functional analysis is needed to confirm this interaction.

CONCLUSIONS

In this study, we performed a comprehensive genome analysis of the LTP family in legumes and other important model plants. We identified 960 LTPs in 14 plant species and 1 green alga, which were grouped into 4 clades according to the LTP class. We report the first *LTP* gene identified in a chlorophyte species, *C. reinhardtii*, indicating that the LTP family appeared before the first terrestrial plant. Sequence analysis showed that the gene and protein structures of the LTP family members are highly conserved among species, but clear differences were observed between the LTP classes. Expression profiling analyses revealed that *PvLTP* genes are differentially expressed during the early and late stages of nodulation and that they might be regulated by RBOH-dependent ROS production. These findings shed light on the possible roles of LTP family members in the legume-rhizobium symbiosis, but functional analyses are necessary to increase our knowledge and, as a good start, *PvLTP* genes could be good candidates.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Phytozome 12.1.6 database https://phytozome.jgi.doe.gov; LotusBase https://lotus.au.dk; LIS: Legume Information System database https://legumeinfo.org.

AUTHOR CONTRIBUTIONS

CQ and CF-G conceived the study. CF-G and JS-M analyzed the data. RP and CF-G collected some of the plant tissues and performed RNA extraction and purification, cDNA synthesis, and RT-qPCR assays. CQ, CF-G, and RP drafted, edited, and revised the manuscript, which was approved by all authors. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Consejo Nacional de Ciencia y Tecnología in México (CONACyT) under Fronteras de la ciencia (FC) grants: FC-2016/1503 to CQ and CV200519 to Luis Cárdenas, with a postdoctoral fellowship to CF-G (268158) and doctoral fellowships to JS-M (463767) and RP (823333).

ACKNOWLEDGMENTS

We are grateful to Noreide Nava for her technical support in the collection of plant tissues. In addition, we thank Dr. Paul Gaytan and MsC Jorge Arturo Yañez from Unidad de Síntesis y Secuenciación at Instituto de Biotecnología, UNAM for their technical contributions. We thank to Dr. Luis Cárdenas for providing the QuantStudio 5 equipment for the qPCR assays.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.660100/full#supplementary-material

REFERENCES

Arthikala, M. K., Montiel, J., Sánchez-López, R., Nava, N., Cárdenas, L., and Quinto, C. (2017). Respiratory burst oxidase homolog gene a is crucial for rhizobium infection and nodule maturation and function in common bean. *Front. Plant Sci.* 8, 1–15. doi: 10.3389/fpls.2017.02003

Arthikala, M. K., Sánchez-López, R., Nava, N., Santana, O., Cárdenas, L., and Quinto, C. (2014). RbohB, a Phaseolus vulgaris NADPH oxidase gene, enhances symbiosome number, bacteroid size, and nitrogen fixation in nodules and impairs mycorrhizal colonization. *New Phytol.* 202, 886–900. doi: 10.1111/nph.12714

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., et al. (2009). MEME Suite: Tools for motif discovery and searching. *Nucleic Acids Res.* 37, 202–208. doi: 10.1093/nar/gkp335

Bapaume, L., and Reinhardt, D. (2012). How membranes shape plant symbioses: signaling and transport in nodulation and arbuscular mycorrhiza. *Front. Plant Sci.* 3:223. doi: 10.3389/fpls.2012.00223

Benedito, V. A., Torres-Jerez, I., Murray, J. D., Andriankaja, A., Allen, S., Kakar, K., et al. (2008). A gene expression atlas of the model legume *Medicago truncatula*. *Plant J.* 55, 504–513. doi: 10.1111/j.1365-313X.2008.03519.x

Boutrot, F., Chantret, N., and Gautier, M. F. (2008). Genome-wide analysis of the rice and arabidopsis non-specific lipid transfer protein (nsLtp) gene families and identification of wheat nsLtp genes by EST data mining. *BMC Genom.* 9, 1–19. doi: 10.1186/1471-2164-9-86

Cannon, S. B., and Shoemaker, R. C. (2012). Evolutionary and comparative analyses of the soybean genome. *Breed. Sci.* 61, 437–444. doi: 10.1270/jsbbs.61.437

Cárdenas, L., Martínez, A., Sánchez, F., and Quinto, C. (2008). Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). *Plant J*. 56, 802–813. doi: 10.1111/j.1365-313X.2008.03644.x

Charvolin, D., Douliez, J. P., Marion, D., Cohen-Addad, C., and Pebay-Peyroula, E. (1999). The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 A resolution. *Eur. J. Biochem*. 264, 562–568. doi: 10.1046/j.1432-1327.1999.00667.x

Cheng, H.-C., Cheng, P.-T., Peng, P., Lyu, P.-C., and Sun, Y.-J. (2004). Lipid binding in rice nonspecific lipid transfer protein-1 complexes from *Oryza sativa*. *Protein Sci*. 13, 2304–2315. doi: 10.1110/ps.04799704

Conant, G. C., and Wolfe, K. H. (2008). Turning a hobby into a job: how duplicated genes find new functions. *Nat. Rev. Genet*. 9, 938–950. doi: 10.1038/nrg2482

Dash, S., Campbell, J. D., Cannon, E. K. S., Cleary, A. M., Huang, W., Kalberer, S. R., et al. (2016). Legume information system (LegumeInfo.org): a key component of a set of federated data resources for the legume family. *Nucleic Acids Res*. 44, D1181–D1188. doi: 10.1093/nar/gkv1159

Davies, T. J., Barraclough, T. G., Chase, M. W., Soltis, P. S., Soltis, D. E., and Savolainen, V. (2004). Darwin's abominable mystery: insights from a supertree of the angiosperms. *Proc. Natl. Acad. Sci. U. S. A*. 101, 1904–1909. doi: 10.1073/pnas.0308127100

Dhar, N., Caruana, J., Erdem, I., and Raina, R. (2020). An arabidopsis DISEASE RELATED NONSPECIFIC LIPID TRANSFER PROTEIN 1 is required for resistance against various phytopathogens and tolerance to salt stress. *Gene* 753, 1–13. doi: 10.1016/j.gene.2020.144802

Edqvist, J., Blomqvist, K., Nieuwland, J., and Salminen, T. A. (2018). Plant lipid transfer proteins: are we finally closing in on the roles of these enigmatic proteins? *J. Lipid Res*. 59, 1374–1382. doi: 10.1194/jlr.R083139

Edstam, M. M., Viitanen, L., Salminen, T. A., and Edqvist, J. (2011). Evolutionary history of the non-specific lipid transfer proteins. *Mol. Plant* 4, 947–964. doi: 10.1093/mp/ssr019

Estrada-Navarrete, G., Alvarado-Affantranger, X., Olivares, J. E., Guillén, G., Díaz-Camino, C., Campos, F., et al. (2007). Fast, efficient and reproducible genetic transformation of Phaseolus spp. by *Agrobacterium rhizogenes. Nat. Protoc*. 2, 1819–1824. doi: 10.1038/nprot.2007.259

Fernandez-Pozo, N., Haas, F. B., Meyberg, R., Ullrich, K. K., Hiss, M., Perroud, P. F., et al. (2020). PEATmoss (Physcomitrella Expression Atlas Tool): a unified gene expression atlas for the model plant Physcomitrella patens. *Plant J*. 102, 165–177. doi: 10.1111/tpj.14607

Ferrari, C., Shivhare, D., Hansen, B. O., Pasha, A., Esteban, E., Provart, N. J., et al. (2020). Expression atlas of *Selaginella moellendorffii* provides insights into the evolution of vasculature, secondary metabolism, and roots. *Plant Cell* 32, 853–870. doi: 10.1105/tpc.19.00780

Finkina, E. I., Melnikova, D. N., Bogdanov, I. V., and Ovchinnikova, T. V. (2016). Lipid transfer proteins as components of the plant innate immune system: structure, functions, and applications. *Acta Nat*. 8, 47–61. doi: 10.32607/20758251-2016-8-2-47-61

Flagel, L. E., and Wendel, J. F. (2009). Gene duplication and evolutionary novelty in plants. *New Phytol*. 183, 557–564. doi: 10.1111/j.1469-8137.2009.02923.x

Fonseca-García, C., Zayas, A. E., Montiel, J., Nava, N., Sánchez, F., and Quinto, C. (2019). Transcriptome analysis of the differential effect of the NADPH oxidase gene RbohB in *Phaseolus vulgaris* roots following *Rhizobium tropici* and *Rhizophagus irregularis* inoculation. *BMC Genom*. 20:800. doi: 10.1186/s12864-019-6162-7

Gao, S., Guo, W., Feng, W., Liu, L., Song, X., Chen, J., et al. (2016). LTP3 contributes to disease susceptibility in Arabidopsis by enhancing abscisic acid (ABA) biosynthesis. *Mol. Plant Pathol*. 17, 412–426. doi: 10.1111/mpp.12290

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., et al. (2012). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res*. 40, D1178–D1186. doi: 10.1093/nar/gkr944

Halim, V. A., Vess, A., Scheel, D., and Rosahl, S. (2006). The role of salicylic acid and jasmonic acid in pathogen defence. *Plant Biol*. 8, 307–313. doi: 10.1055/s-2006-924025

Han, G. W., Lee, J. Y., Song, H. K., Chang, C., Min, K., Moon, J., et al. (2001). Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray crystallography. *J. Mol. Biol*. 308, 263–278. doi: 10.1006/jmbi.2001.4559

He, J., Benedito, V. A., Wang, M., Murray, J. D., Zhao, P. X., Tang, Y., et al. (2009). The *Medicago truncatula* gene expression atlas web server. *BMC Bioinform*. 10:a441. doi: 10.1186/1471-2105-10-441

Jeffares, D. C., Penkett, C. J., and Bähler, J. (2008). Rapidly regulated genes are intron poor. *Trends Genet*. 24, 375–378. doi: 10.1016/j.tig.2008.05.006

Kim, H., Lee, S. B., Kim, H. J., Min, M. K., Hwang, I., and Suh, M. C. (2012). Characterization of glycosylphosphatidylinositol-anchored lipid transfer protein 2 (LTPG2) and overlapping function between LTPG/LTPG1 and LTPG2 in cuticular wax export or accumulation in *Arabidopsis thaliana*. *Plant Cell Physiol*. 53, 1391–1403. doi: 10.1093/pcp/pcs083

Kouidri, A., Whitford, R., Suchecki, R., Kalashyan, E., and Baumann, U. (2018). Genome-wide identification and analysis of non-specific lipid transfer proteins in hexaploid wheat. *Sci. Rep*. 8:17087. doi: 10.1038/s41598-018-35375-7

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*. 35, 1547–1549. doi: 10.1093/molbev/msy096

Lascombe, M.-B., Bakan, B., Buhot, N., Marion, D., Blein, J.-P., Larue, V., et al. (2008). The structure of "defective in induced resistance" protein of *Arabidopsis thaliana*, DIR1, reveals a new type of lipid transfer protein. *Protein Sci*. 17, 1522–1530. doi: 10.1110/ps.035972.108

Lee, S. B., and Suh, M. C. (2018). Disruption of glycosylphosphatidylinositol-anchored lipid transfer protein 15 affects seed coat permeability in Arabidopsis. *Plant J*. 96, 1206–1217. doi: 10.1111/tpj.14101

Lei, L., Chen, L., Shi, X., Li, Y., Wang, J., Chen, D., et al. (2014). A nodule-specific lipid transfer protein AsE246 participates in transport of plant-synthesized lipids to symbiosome membrane and is essential for nodule organogenesis in Chinese milk vetch. *Plant Physiol*. 164, 1045–1058. doi: 10.1104/pp.113.232637

Li, G., Hou, M., Liu, Y., Pei, Y., Ye, M., Zhou, Y., et al. (2019). Genome-wide identification, characterization and expression analysis of the non-specific lipid transfer proteins in potato. *BMC Genom*. 20:375. doi: 10.1186/s12864-019-5698-x

Libault, M., Farmer, A., Joshi, T., Takahashi, K., Langley, R. J., Franklin, L. D., et al. (2010). An integrated transcriptome atlas of the crop model *Glycine max*, and its use in comparative analyses in plants. *Plant J*. 63, 86–99. doi: 10.1111/j.1365-313X.2010.04222.x

Marino, D., Andrio, E., Danchin, E. G. J., Oger, E., Gucciardo, S., Lambert, A., et al. (2011). A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning. *New Phytol*. 189, 580–592. doi: 10.1111/j.1469-8137.2010.03509.x

Montiel, J., Arthikala, M. K., Cárdenas, L., and Quinto, C. (2016). Legume NADPH oxidases have crucial roles at different stages of nodulation. *Int. J. Mol. Sci*. 17:680. doi: 10.3390/ijms17050680

Montiel, J., Nava, N., Cárdenas, L., Sánchez-López, R., Arthikala, M. K., Santana, O., et al. (2012). A Phaseolus vulgaris NADPH oxidase gene is required for root infection by Rhizobia. *Plant Cell Physiol*. 53, 1751–1767. doi: 10.1093/pcp/pcs120

Moore, R. C., and Purugganan, M. D. (2005). The evolutionary dynamics of plant duplicate genes. *Curr. Opin. Plant Biol*. 8, 122–128. doi: 10.1016/j.pbi.2004.12.001

Mun, T., Bachmann, A., Gupta, V., Stougaard, J., and Andersen, S. U. (2016). Lotus base: an integrated information portal for the model legume *Lotus japonicus*. *Sci. Rep*. 6, 1–18. doi: 10.1038/srep39447

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., and Minh, B. Q. (2014). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol*. 32, 268–274. doi: 10.1093/molbev/msu300

Odintsova, T. I., Slezina, M. P., Istomina, E. A., Korostyleva, T. V., Kovtun, A. S., Kasianov, A. S., et al. (2019). Non-specific lipid transfer proteins in Triticum kiharae dorof. Et migush.: Identification, characterization and expression profiling in response to pathogens and resistance inducers. *Pathogens* 8:221. doi: 10.3390/pathogens8040221

Oldroyd, G. E. D., and Downie, J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. *Ann. Rev. Plant Biol*. 59, 519–546. doi: 10.1146/annurev.arplant.59.032607.092839

O'Rourke, J. A., Iniguez, L. P., Fu, F., Bucciarelli, B., Miller, S. S., Jackson, S. A., et al. (2014). An RNA-Seq based gene expression atlas of the common bean. *BMC Genom*. 15, 1–16. doi: 10.1186/1471-2164-15-866

Rensing, S. A., Goffinet, B., Meyberg, R., Wu, S.-Z., and Bezanilla, M. (2020). The Moss Physcomitrium (Physcomitrella) patens: a model organism for non-seed plants. *Plant Cell* 32, 1361–1376. doi: 10.1105/tpc.19.00828

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., et al. (2020). Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell* 32, 15–41. doi: 10.1105/tpc.19.00279

Salminen, T. A., Blomqvist, K., and Edqvist, J. (2016). Lipid transfer proteins: classification, nomenclature, structure, and function. *Planta* 244, 971–997. doi: 10.1007/s00425-016-2585-4

Santi, C., Molesini, B., Guzzo, F., Pii, Y., Vitulo, N., and Pandolfini, T. (2017). Genome-wide transcriptional changes and lipid profile modifications induced by medicago truncatula N5 overexpression at an early stage of the symbiotic interaction with sinorhizobium meliloti. *Genes* 8:396. doi: 10.3390/genes8120396

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., et al. (2005). A gene expression map of *Arabidopsis thaliana* development. *Nat. Genet*. 37, 501–506. doi: 10.1038/ng1543

Shin, D. H., Lee, J. Y., Hwang, K. Y., Kyu Kim, K., and Suh, S. W. (1995). High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* 3, 189–199. doi: 10.1016/S0969-2126(01)00149-6

Verma, D. P. S. (1992). Signals in root nodule organogenesis and endocytosis of Rhizobium. *Plant Cell* 4, 373–382. doi: 10.2307/3869439

Wang, H., Sun, Y., Chang, J., Zheng, F., Pei, H., Yi, Y., et al. (2016). Regulatory function of Arabidopsis lipid transfer protein 1 (LTP1) in ethylene response and signaling. *Plant Mol. Biol*. 91, 471–484. doi: 10.1007/s11103-016-0482-7

Wang, Y., Tang, H., Debarry, J. D., Tan, X., Li, J., Wang, X., et al. (2012). MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res*. 40, 1–14. doi: 10.1093/nar/gkr1293

Warnes, G. R., Bolker, B., Huber, W., Lumley, T., Maechler, M., Magnusson, A., et al. (2015). *gplots: Various R Programming Tools for Plotting Data*, p. 70. Available online at: https://cran.r-project.org/web/packages/gplots/index.html

Wei, K., and Zhong, X. (2014). Non-specific lipid transfer proteins in maize. *BMC Plant Biol*. 14, 1–18. doi: 10.1186/s12870-014-0281-8

Wolfe, D., Dudek, S., Ritchie, M. D., and Pendergrass, S. A. (2013). Visualizing genomic information across chromosomes with PhenoGram. *BioData Mining* 6, 1–12. doi: 10.1186/1756-0381-6-18

Xu, Y., Zheng, X., Song, Y., Zhu, L., Yu, Z., Gan, L., et al. (2018). NtLTP4, a lipid transfer protein that enhances salt and drought stresses tolerance in *Nicotiana tabacum*. *Sci. Rep*. 8, 1–14. doi: 10.1038/s41598-018-27274-8

Zhang, M., Kim, Y., Zong, J., Lin, H., Dievart, A., Li, H., et al. (2019). Genome-wide analysis of the barley non-specific lipid transfer protein gene family. *Crop J*. 7, 65–76. doi: 10.1016/j.cj.2018.07.009

Zhao, J. (2015). Phospholipase D and phosphatidic acid in plant defence response: from protein-protein and lipid-protein interactions to hormone signalling. *J. Exp. Bot*. 66, 1721–1736. doi: 10.1093/jxb/eru540

Zhou, D., Li, Y., Wang, X., Xie, F., Chen, D., Ma, B., et al. (2019). *Mesorhizobium huakuii* HtpG interaction with nsLTP AsE246 is required for symbiotic nitrogen fixation. *Plant Physiol*. 180, 509–528. doi: 10.1104/pp.18.00336

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.

Copyright © 2021 Fonseca-García, Solis-Miranda, Pacheco and Quinto. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

ORIGINAL RESEARCH
published: 11 May 2021
doi: 10.3389/fagro.2021.661295

Characterization of *Bradyrhizobium* spp. Nodulating *Lupinus cosentinii* and *L. luteus* Microsymbionts in Morocco

Mustapha Missbah El Idrissi[1], Omar Bouhnik[1], Salma ElFaik[1], Soufiane Alami[1], Hanane Lamin[1], Eulogio J. Bedmar[2] and Hanaa Abdelmoumen[1]*

[1] Faculty of Sciences, Centre de Biotechnologies Végétale et Microbienne, Biodiversité et Environnement, Mohammed V University, Rabat, Morocco, [2] Departamento de Microbiología del Suelo y Sistemas Simbióticos Estación Experimental del Zaidín, Granada, Spain

OPEN ACCESS

Edited by:
*Jose Palacios,
Polytechnic University of Madrid, Spain*

Reviewed by:
*Luis Rey,
Polytechnic University of Madrid, Spain
Durgesh K. Jaiswal,
Banaras Hindu University, India*

***Correspondence:**
*Mustapha Missbah El Idrissi
missbah49@yahoo.fr*

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *30 January 2021*
Accepted: *22 March 2021*
Published: *11 May 2021*

Citation:
Missbah El Idrissi M, Bouhnik O, ElFaik S, Alami S, Lamin H, Bedmar EJ and Abdelmoumen H (2021) Characterization of Bradyrhizobium spp. Nodulating Lupinus cosentinii and L. luteus Microsymbionts in Morocco. Front. Agron. 3:661295. doi: 10.3389/fagro.2021.661295

In this work, we analyzed the diversity of the nodule-forming bacteria associated with *Lupinus luteus* and *Lupinus cosentinii* grown in the Maamora Cork oak forest acidic soils in Morocco. The phenotypic analysis showed the high diversity of the strains nodulating the two lupine's species. The strains were not tolerant to acidity or high alkalinity. They do not tolerate salinity or high temperatures either. The strains isolated from *L. luteus* were more tolerant to antibiotics and salinity than those isolated from *L. cosentinii*. The plant growth promoting (PGP) activities of our strains are modest, as among the 28 tested isolates, only six produced auxins, six produced siderophores, whereas three solubilized phosphates. Only two strains possess the three activities. The rrs gene sequences from eight representative strains selected following ARDRA and REP-PCR results revealed that they were members of the genus *Bradyrhizobium. Six* strains were then retained for further molecular analysis. The *glnII*, *recA*, *gyrB*, *dnaK*, and *rpoB* housekeeping gene sequence phylogeny showed that some strains were close to *B. lupini* LMG28514^T whereas others may constitute new genospecies in the genus *Bradyrhizobium*. The strains were unable to nodulate *Glycine max* and *Phaseolus vulgaris* and effectively nodulated *L. luteus, L. cosentinii, L. angustifolius, Chamaecytisus albidus*, and *Retama monosperma*. The *nodC* and *nodA* symbiotic gene phylogenies showed that the strains are members of the genistearum symbiovar.

Keywords: ***Lupinus, Bradyrhizobium*, symbiosis, nodulation, diversity, MLSA**

INTRODUCTION

Legumes (*Fabaceae*) are of great importance in different aspects, whether in agriculture, medicine, or ecology. Some species of this plant family can establish a specific symbiosis with rhizobia. The latter are soil bacteria normally found in the rhizosphere and in large numbers on the root surface, because of the nutrients secreted by the plant as root exudates (Sessitsch et al., 2002). In the rhizobia–legume symbiosis, bacteria provide plants with nitrogen compounds and plants provide them with carbon sources. A successful symbiotic interaction is based on a molecular dialogue between the two partners and generally involves rhizobial lipo-chitooligosaccharide signals called Nod factors (NF) (Wang et al., 2018; Lindström and Mousavi, 2020). These nodulation factors

secreted by the rhizobia and perceived by the root cell receptors will initiate molecular and physiological responses in the plant, leading to the formation of the nodule, which is the site of fixation of the nitrogen (Clúa et al., 2018). Nitrogen fixation and uptake contributes to the high seed protein content and also provides residual N for the following crops, particularly cereals (Kermah et al., 2018).

Rhizobia are also collectively called root or shoot nodule bacteria, comprise more than 238 species in 18 genera regrouped in two clades; however, the description of rhizobial species included only 23% of the 19,000 legume species that exist throughout the world (Shamseldin et al., 2017).

Lupinus is one of the most important genera in *Fabaceae* with a rich diversity of species divided into Mediterranean "Old World" species and American "New World" species. Wild lupines are distributed over a wide range of climates around the world, from the coldest to the warmest and from the driest to the most humid (Wolko et al., 2011). On the other hand, the centers of diversity of *Lupinus* are the Americas, the Mediterranean region, and the regions of North and East Africa (Susek et al., 2016).

Wild lupines are toxic to humans and unpalatable to animals because their seeds contain alkaloids. Lupine cultivation improved in the 1930's, when breeders discovered sweet varieties of white lupine. Thus, its culture was stimulated in different European countries (Kohajdova et al., 2011). Nowadays, lupines are mainly consumed by ruminants. They are used in human food for the manufacture of ingredients used in the bakery and pastry industries in Europe and Australia (Hall et al., 2005; Smith et al., 2006). The current cultivated varieties of lupines, known as sweet varieties, are devoid of bitterness such as white and yellow lupines, which are used for human consumption. The white lupine is exploited generally as flour, recommended in low glycemic index protein, and gluten-free diets (Kohajdova et al., 2011; Yorgancilar and Bilgiçli, 2014).

The extensive and ongoing development of molecular biology tools over the last 20 years has facilitated the identification of new nodulating bacteria and has resulted in significant changes in the classification and proposition of new and different species. Lupines were first thought to be mainly nodulated by slow-growing rhizobia of the genus *Bradyrhizobium*, although fast-growing strains associated with lupines have been identified since 1988 (Miller and Pepper, 1988). *Lupinus* is currently reported as a promiscuous host that can be nodulated by different symbiotic bacteria belonging to the genera *Bradyrhizobium*, *Ochrobactrum*, *Microvirga*, *Phyllobacterium*, *Neorhizobium*, and *Rhizobium* (Jarabo-Lorenzo et al., 2003; Trujillo et al., 2005; Velázquez et al., 2010; Ardley et al., 2012; Bourebaba et al., 2016; Msaddak et al., 2018; Tounsi-Hammami et al., 2019; Missbah El Idrissi et al., 2020).

There has probably been a horizontal transfer of symbiotic genes between different *Bradyrhizobium* spp. associated with *Lupinus* spp. and other legumes of the *Genisteae* tribe, and it is very likely that the *Microvirga* and *Ochrobactrum* species isolated from different lupins have obtained their symbiotic genes from other more common rhizobial genera (Andrews et al., 2018).

Lateral transfer of specific symbiosis genes into rhizobial genera is an important mechanism for legumes to form symbiosis with selected rhizobia more adapted to particular soils and a legume-specific rhizobium strain symbiosis can develop in specific habitats.

In this work, we isolated 36 bacteria from the root nodules of two Lupine species, *Lupinus cosentinii* and *Lupinus luteus*, grown in soils of the Maamora forest, one of the most important Cork Oak Forest in the world (Aafi et al., 2005). The bacteria were first screened for their nodulation gene possession as a marker for their belonging to symbiotic microsymbionts and then characterized by molecular and phenotypic analysis.

MATERIALS AND METHODS

Bacterial Strains

All the 36 strains were isolated from different soils in Maamora forest in the region of Rabat known by its mild climate (Aafi et al., 2005) (**Supplementary Table 1**). Twenty isolates were isolated from *L. cosentinii* and 16 were isolated from *L. luteus* plants root nodules, according to the method of Howieson and Dilworth (2016). The root segments bearing nodules were collected, washed under running tap water and surface sterilized by immersion in 5% sodium hypochlorite for 3 min, and finally washed seven times with sterile distilled water. Each nodule was then crushed, and the extract was streaked onto plates of YMA. Plates were incubated for 15 days at 28°C, and then the single colonies obtained were checked for purity by repeated streaking on YMA medium, supplemented with Congo red dye. The incubation period of 2 weeks was sufficient to check for the growth of any slow-growing symbiotic bacteria on YEM medium. Pure isolates were maintained at −20°C in 50% (m/v) glycerol and at 4°C in YMA that was routinely used for rhizobial culture.

Nodulation and Host Range Experiments

The seeds of the two lupines were surface disinfected with 70% ethanol for 1 min and later sterilized with 5% sodium hypochlorite for 15 min. The seeds were scarified with concentrated sulfuric acid for 10 min and germinated in water agar (0.6% w/v) containing Petri plates. The seedlings were then transferred to Gibson tubes (Howieson and Dilworth, 2016) and inoculated with 1 ml per plant of a suspension containing $\sim 10^8$ isolates CFU ml^{-1}. The plants were cultivated at 26°C for 60 days under a 16.0/8.0 h light/dark photoperiod. Eight weeks after inoculation, the plants were checked for nodules appearance and plant aspect. Indirect effectiveness of the nodules for N_2 fixation was estimated by visual assay of red leghemoglobin presence in cross-sections and by the dark green intensity of the leaves compared to uninoculated control plants.

The selected strains Lcos6, Lcos7.2.1, Lcos8.1, Lcos10.2, Llut4, Llut5, Llut6, and Llut8 were also tested for nodulation on *Lupinus angustifolius, Chamaecytisus albidus*, *Retama monosperma*, *Cytisus monspesulanus, Glycine max*, and *Phaseolus vulgaris* in the same conditions.

DNA Extraction and REP-PCR Fingerprinting

Bacteria were grown on solid Tryptone-Yeast Extract (TY) slants (Beck et al., 1993) for 96 h at 28°C, and colonies were

suspended in 2 ml of distilled water. The suspensions were then centrifuged twice at 3,000 r/min for 15 min. The resulting pellet was treated with proteinase K (20 mg/ml), and total DNA was extracted as previously described by Guerrouj et al. (2013). Rep-PCR (Repetitive Extragenic Palindromic Polymerase Chain Reaction) using ERIC1R and ERIC2 primers (De Bruijn, 1992) was used for amplification of the DNA. The DNA template was denatured for 5 min at 95°C, and PCR was carried out for 35 cycles (94°C for 30 s, 52°C for 1 min, and 72°C for 1 min), with an elongation step at 72°C for 7 min. PCR products were analyzed by horizontal electrophoresis in 2% agarose gels in TAE buffer at 55 V for 4 h. Gels were stained with ethidium bromide, visualized under UV radiation, and photographed with a digital camera. Cluster analysis of the fingerprints obtained was performed with Gelcompar II program (version 2.15) (**Supplementary Figure 1**).

PCR Amplification and Genes Sequencing

PCR amplification was performed as described by Lamrabet et al. (2020) in 25-μl reaction mixtures containing 2.5 μ DNA extract, 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 0.001% (w/v) gelatin, 1 U fastTaq DNA polymerase (Applied Biosystems), 200 mM of each deoxyribonucleotide, and 50 ng of each primer in Applied Biosystems 2720 Thermal Cycler.

All the amplifications consisted in an initial 3-min denaturation at 94°C and 35 cycles of 30-s denaturation at 94°C, 30 s of annealing depending on the primers used, and an extension at 72°C for 45 s. The final extension is performed at 72°C for 3 min. PCR amplification of 16S rRNA gene fragments was done using the two opposing primers fD1 and rD1 and the primer pairs nodCFn–nodCI and nodA1f–nodAb1r were used for amplification of the *nodC* and *nodA* gene (Chaintreuil et al., 2001; Laguerre et al., 2001).

Primers *dnaK* 1466F/1777R, *glnII* 12F/689R, *gyrB* 343F/1034R, *TSrecA* F/R, and *rpoB* 83F/1061R were used to amplify the chaperone protein DnaK (dnaK), the glutamine synthase (*glnII*), DNA gyrase subunit B (*gyrB*) genes, RecA (*recA*), and RNA polymerase β subunit gene (*rpoB*), using the annealing temperatures indicated in **Supplementary Table 2**. The PCR products and their concentration were assessed by electrophoresis of 6 μl of product on a 1% agarose gel and staining with ethidium bromide. A molecular mass marker (Promega 1-kb ladder) was included to estimate the length of the amplicons.

The amplification products were purified using a Qiaquick PCR purification kit (Qiagen) and sequenced using the same primers as for PCR amplification using the dideoxynucleotide chain-termination method with fluorescent ddNTPs (Applied Biosystems), with ABI Prism Dye Chemistry, and analyzed with a 3130xl automatic sequencer at the sequencing facilities of UATRS (Unités d'Appui Technique à la Recherche Scientifique, CNRST, Rabat, Morocco) and the CSIC sequencing facilities in Zaidin experimental station, CSIC, Granada, Spain. The sequences obtained were compared with those from GenBank using the BLASTN program (Altschul et al., 1990).

Sequences Analysis and Phylogeny

Sequences were aligned using MEGA 7 software (Kumar et al., 2016), and the distances calculated according to Kimura's two-parameter model (Kimura, 1980) were used to infer phylogenetic trees with the neighbor-joining analysis (Saitou and Nei, 1987) with MEGA 7 software (Kumar et al., 2016). Phylogenetic trees were subjected to 1000 bootstrap replications, and preferred topologies were plotted.

Virtual DNA Ribotyping

An *in silico* ARDRA of the 16S rDNA gene using 30 restriction enzymes (*Acc*II, *Acu*I, *Acv*I, *Age*I, *Alo*I, *Asp*LEI, *Bcc*I, *Bpm*I, *Bsa*XI, *Bsl*I, *Bsp*143I, *Bst*6I, *Bst*C8I, *Bst*F5I, *Cac*8I, *Cfr*9I, *Eco*91I, *Faq*I, *Fok*I, *Hae*III, *Hga*I, *Hha*I, *Hinf*I, *Lwe*I, *Mly*I, *Msp*I, *Psp*N4I, *Rsa*I, *Sfa*NI, and *Taq*I) was performed using the Silent Mutator program (Cermak, 2018b), which scans through the sequences and recognizes sites for each restriction enzyme. The enzymes were selected following the results obtained with the Restriction Comparator program (Cermak, 2018a). Finally, a dendrogram of similarities based on the concatenated sequences database was generated using Statistica software version 7.0 (StatSoft Inc, 2004).

Phenotypic Characterization

The analysis was pursued by determining some phenotypic properties of the isolated strains. Tests were performed in broth tubes or agar plates inoculated with an exponentially growing liquid culture. The tolerance of the rhizobial isolates to high temperatures was tested on TY broth medium (Beringer, 1974) at 30, 35, 40, and 45°C. The ability of isolates to grow in acidic or basic media was determined on YMA Petri dishes for which the pH has been adjusted and buffered to 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, 9.5, or 10.0, as described by Zerhari et al. (2000). The salt tolerance of the isolates was tested at 0, 86, 171, 342, 510, and 685 mM.

Utilization of 12 amino acids as sole nitrogen source was investigated on a modified YMB medium in which the yeast extract was replaced by the amino acid to test. The amino acids were sterilized separately by filtration and added to the medium at a concentration of at 1% (w/v). Carbohydrate assimilation was carried out on solid YEM containing 0.005% YEM and 0.4% (w/v) of glucose, fructose, sucrose, maltose, lactose or starch. The carbohydrates were sterilized separately by filtration.

The antibiotics resistance of the isolates was determined on solid YEM supplemented with the following antibiotics (μg/ml): kanamycin (20), ampicillin (20), bacitracin (20), tetracycline (10), vancomycin (20), tetracycline (10), streptomycin (25), trimethoprim (10), nalidixic acid (20), gentamicin (20), and chloramphenicol (20).

The intrinsic heavy metal resistance of the isolates was determined on TY agar plates containing the following heavy metals (μg/ml): $CuSO_4$, $5H_2O$ (500), $AlCl_3$ (100), $AlCl_3$ (450), $HgCl_2$ (5), $CdSO_4$ (50), $ZnSO_4$ (250), Pb-acetate (1000), $MnCl_2$, $4H_2O$ (500), $MnSO_4$ (500), $MgCl_2$ (1000), $MgSO_4$ (1000), $BaCl_2$, $2H_2O$ (1000), $CoSO_4$ (150), $FeCl_3$ (500), and $NiCl_2$ (500).

To evaluate oxidase activity, a loopful of bacterial mass from the colonies of the isolates was spread out over cellulose

paper soaked in a solution containing 1% N,N-dimethyl-p-phenylenediamine oxalate 98%. The appearance of blue color is indicative of oxidase activity.

PGPR Characteristics

We used the PVK solid medium containing 2.5 g L^{-1} of $(Ca_3PO_4)_2$ to determine the ability of the isolates to solubilize phosphates. To this purpose, 5 µl of the bacterial suspension was plated onto PVK medium in Petri dishes and incubated at 28°C for 10 days. The size of the halos that appeared around the colonies and the size of the colonies were measured to estimate the ability of the isolates to solubilize phosphate. Siderophore-producing isolates were identified by the development of orange halos around the colonies according to Lakshmanan et al. (2015). To assess the ability of the isolates to produce indole acetic acid (IAA) and IAA-related compounds, a volume of 5 µl of the bacterial suspension was placed on the solid YEM medium supplemented with 0.5 g L^{-1} tryptophan, and the plates were then incubated at 28°C for 10 days. The production of IAA was detected using the Salkowski reagent followed by the appearance of a pink halo around the colonies after incubation for 30 to 60 min at 28°C.

RESULTS

Strain Isolation, REP-PCR, and ARDRA Analysis

Thirty-six bacterial cells were isolated from root nodules of *L. cosentinii* (20) and *L. luteus* (16) grown in Maamora cork oak forest soils. Among the strains, 28 were able to re-nodulate their original host under axenic conditions using Gibson's tubes. REP-PCR using primers REP1R-I and REP2-I grouped the strains into six clusters and some single strains (**Supplementary Figure 1**). The REP PCR technique is employed for analysis of the genetic diversity of rhizobia (Laguerre et al., 1997; Menna et al., 2009).

Eight strains representing the different groups were randomly selected and used for *in vitro* ARDRA, based on 1,450 nucleotides of rrs gene, with the restriction enzymes *cfo*I, *Hae*III, and *Msp*I, which distributed the strains in three ribotypes (**Supplementary Table 3**). Furthermore, the *in silico* ARDRA of the eight representative strains, together with other 45 bradyrhizobial species and the outgroup *R. gallicum* R602, using 30 restriction enzymes, including *Msp*I, *Hae*III, and *Rsa*I, showed that the strains from the two lupines clustered in three main groups, with *B. lupini*–*B. canariense* and *B. cytisi* (**Supplementary Figure 2**).

rrs Sequences Analysis

The *rrs* gene sequences of strains Llut4 (MT468660), Llut5 (MT468656), Llut6 (MT468653), Llut8 (MT468657), Lcos6 (MT468659), Lcos7.2 (MW494674), Lcos8.1 (MW494669), and Lcos102 (MT468658) revealed they were members of the genus *Bradyrhizobium* of the Alphaproteobacteria and share 99 to 99.93% of similitude. The strains Llu4, Lut6, Lcos6, Lcos7.2, and Lcos102 have 99.43–100% of similitude with *B. lupini* USDA 3051^T and share similarities from 99.55 to 100% with strains nodulating *L. angustifolius*, *L micranthus*, and *L. luteus* in Algeria and Tunisia (Msaddak et al., 2017; Mellal et al., 2019).

The strains Lcos102 and Llut5 have similarity values of 99.79 and 99.86% with *B. canariense* BTA-1^T. They have similarities of 99.08–99.85% with the North African strains tested (**Supplementary Table 4**). The strains Lcos8.1 and Llut8 share similarities of 99.64 and 99.79% with *B. cytisi* $CTAW11^T$ and share also similarities of 98.93–99.85% with the strains isolated from the Algerian and Tunisian lupines.

The phylogenetic tree based on the 16S rRNA sequences showed that the strains Lut6, Lcos6, and Lcos7.2 clustered together with different strains isolated from North African lupines, in Algeria and Tunisia in group I, along with *B. lupini* USDA 3051^T. The strains Lcos102 and Llut5 clustered in group II with *B. canariense* LMG 22265^T, whereas strains Lcos8.1 and Llut8 clustered in group III with *B. cytisi* $CTAW^T$ and some strains isolated from Algerian and Tunisian lupines. The strain Llut4 grouped further into the cluster composed of groups 1 and 2 (**Figure 1**). The *rrs* sequences of strains Lcos6, Lcos7.2, and Llut6 are 100% similar, and thus, we selected one strain (Llcos6) to represent the three. The strains retained as representatives for further molecular analyses are Lcos6, Llut4, Llut5, Lcos102, Lcos8.1, and Llut8.

Multilocus Sequence Analysis

Five housekeeping genes, *dnaK, glnII, gyrB, recA*, and *rpoB* of the six representative strains were analyzed in this study. The *recA and glnII* sequences were analyzed and compared with other strains isolated from North African lupines root nodules. All the sequence accession numbers are reported on the different figures. We could not compare our sequences with the *dnaK, gyrB*, and *rpoB* sequences of Algerian and Tunisian strains since they are not published.

The sequence analyses showed that the *recA* genes of strains Llut5 (MK346962) and Lcos6 (MK346959) are 100% similar and have 99.80% of similarity with *B. lupini* 3051^T. The two strains share 99.58–100% similarity with strain LaO10, LaO17, and Lat46 isolated from *L. angustifolius* in Algeria (Mellal et al., 2019), and 99.59% with strain LmiT3 isolated from the root nodules of *L. micranthus* in Tunisia (Msaddak et al., 2017). The strains Lcos8.1 (MW527003), Llut8 (MK346964), and Lcos102 (MK346960) share 99.23 to 99.60% and have similarity percentages of 94.43 to 95.23% with Llut4 (MK346961). They have percentages of similarities ranging from 93.51 to 94.30 with the different Algerian and Tunisian strains. The phylogenetic tree based on the *recA* sequences (**Supplementary Figure 3**) shows that strains Lcos6 and Llut5 clustered with strains nodulating *L. micranthus* and *L. angustifolius* in Algeria all together with the type strain *B. lupini* USDA3051. The strain Llut4 was closer to *B. cytisi* $LMG25866^T$ with which it constituted a group containing *B. japonicum* $LMG6138^T$ and *B. diazoefficiens* $USDA110^T$. The strains Llut8, Lcos8.1, and Lcos102 were regrouped in a different cluster with *B. ganzhouense* $RITF806^T$.

The *glnII* sequences of strains Lcos6 (MK346977) and Llut5 (MW565871) share 98.98% similarity and have similarity percentages of 98.81 and 99.84 with *B. lupini* 3051^T, respectively.

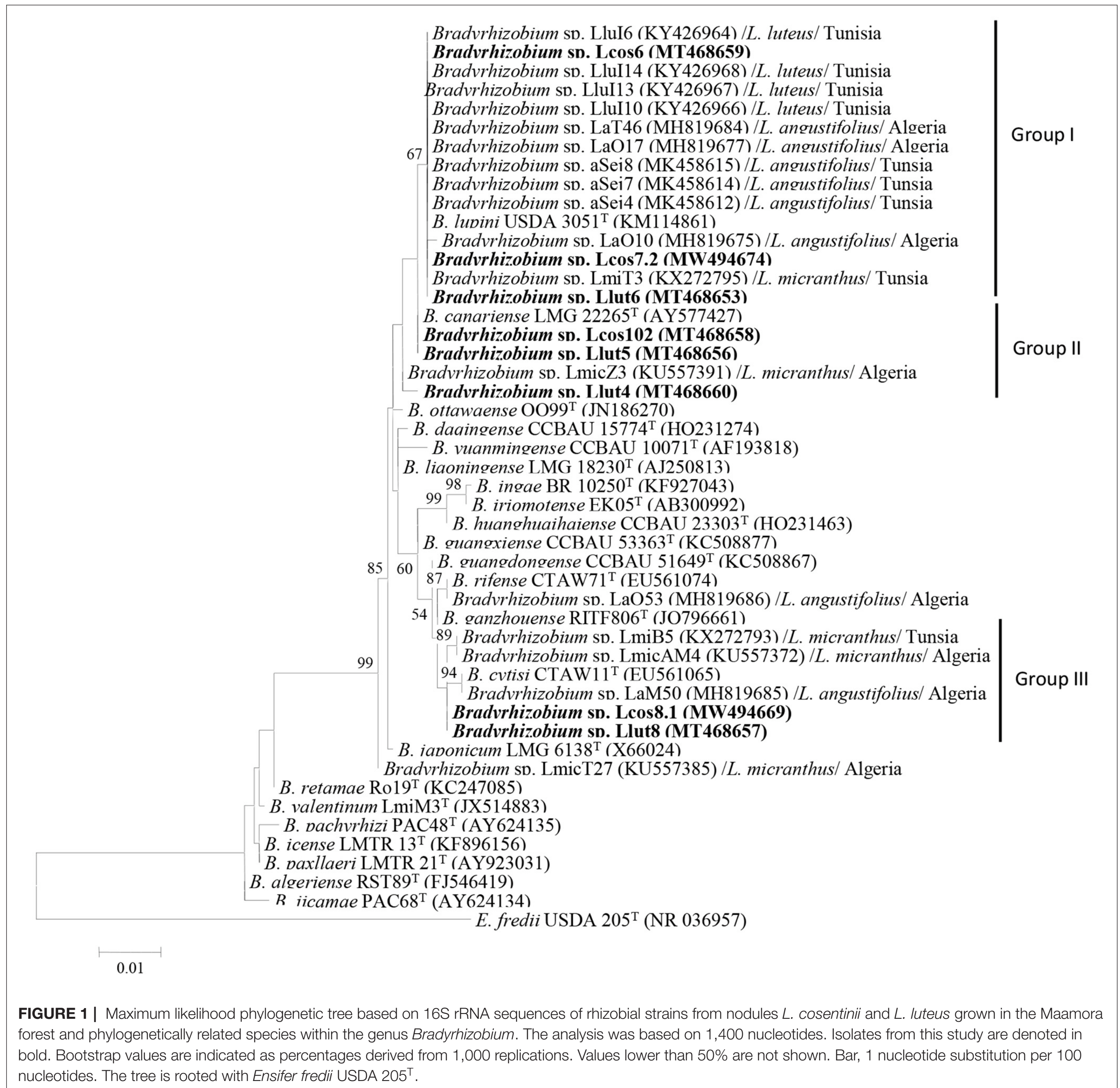


FIGURE 1 | Maximum likelihood phylogenetic tree based on 16S rRNA sequences of rhizobial strains from nodules *L. cosentinii* and *L. luteus* grown in the Maamora forest and phylogenetically related species within the genus *Bradyrhizobium*. The analysis was based on 1,400 nucleotides. Isolates from this study are denoted in bold. Bootstrap values are indicated as percentages derived from 1,000 replications. Values lower than 50% are not shown. Bar, 1 nucleotide substitution per 100 nucleotides. The tree is rooted with *Ensifer fredii* USDA 205^T.

They have also percentages of similarity ranging between 98.45 and 99.83 with strains LaO10, LaO17, LaT46 (Mellal et al., 2019), and LmiT3 (Msaddak et al., 2017).

The *glnII* neighbor-joining phylogenetic tree (**Supplementary Figure 3**) shows that strains Lcos6 and Llut5 are close to *B. lupini* 3051^T with which they were regrouped along with strains LaO10, LaO17, and LaT46 isolated from *L. angustifolius* in Algeria and strain LmiT3 isolated *from L. micranthus* in Tunisia.

Similar results were obtained with the *gyrB, dnaK*, and *rpoB* sequences (**Supplementary Figures 4–8**). However, we could not compare our sequences with those of other North African lupines' microsymbionts.

The concatenation of the *glnII, gyrB*, and *recA* genes showed that the strains Llut8, Llcos81, and Llcos102 have identity percentages of 95.8, 95.73, and 95.59% with *B. ganzhouense* RITF806 ^{T} and share similarities of 99.43–99.57%. The strains Lcos6 and Llut5 share 99.70 similarities and have identity percentages of 99.43 and 99.50% with *B. lupini* LMG28514^T, respectively. The strain Llut4 has low similarities with all the strains tested and shares a maximum identity of 94% with *B. cytisi* LMG25866 ^{T} (**Supplementary Table 4**). The phylogenetic

tree based on the concatenation of the three genes (**Figure 2**) revealed also that *B. cytisi* LMG25866^T is the closest parent to strain Llut4, whereas the strains Lut5 and Lcos6 clustered with *B. lupini* LMG28514^T, and strains Llut8, Llcos81, and Llcos102 were regrouped with *B. ganzhouense* RITF806^T.

nodA and *nodC* Symbiotic Genes Sequences Analysis

The *nodC* sequences of the strains Lut5, Llut8, and Lcos6 share 99.9–100% similarity. The *nodC*-based phylogenetic tree (**Figure 3**) showed that they were regrouped in a same cluster with strain LAM15, isolated from the nodules of *L. angustifolius* in Algeria (Mellal et al., 2019), with which they have a similarity percentage of 92.1%, within the genistearum symbiovar. The strain Llut4 has a 95.9% percentage similarity with the three other representatives and was regrouped with *B. cytisi* LMG25866T with which it shares 97% similarity.

The analysis of the *nodA* gene sequences showed also that our strains were very close and share 99.6 to 100% similarity. The phylogenetic tree based on the *nodA* sequences (**Supplementary Figure 9**) showed also that they formed a single sub-cluster in the symbiovar genistearum. The phylogenetic analysis of the two symbiotic genes' concatenated sequences also produced similar results (**Supplementary Figure 10**).

The Moroccan as well as the Algerian and the Tunisian strains were clustered together in the same clade with *B. lupini*, *B. canariense, B. cytisi*, and *B. rifense*, members of the genistearum symbiovar.

All the strains selected for the host range experiments were able to nodulate *L. angustifolius, L. cosentinii, L. luteus, C. monspessilanus, C. albidus*, and *R. monosperma*, but not *L. albus*, *P. vulgaris*, or *G. max* (**Supplementary Table 5**).

Phenotypic Tests

All the isolated strains were slow growing, as their colonies appeared on YEM solid medium after 7 days incubation time at 28°C. The phenotypic results reported in **Supplementary Table 6** show that the strains grow in media with different pH values ranging from 6 to 8. All strains nodulating *L. cosentinii* use trehalose as sole carbon source; 93% assimilate fructose and myoinositol, and 64% grew in the presence of arabinose, while only 7% can assimilate maltose. The latter cannot be assimilated by strains nodulating *L. luteus*. The results also showed that all strains of *L. luteus* assimilate arabinose and fructose, while 79% of these strains use myo-inositol and 36% develop in the presence of trehalose. None of the two lupine species microsymbionts is capable to use lactose, starch, or carboxy-methyl-cellulose (CMC).

The strains nodulating lupines in the Maâmora forest are able to use a wide range of amino acids as sole nitrogen source. All the strains nodulating *L. luteus* assimilate 10 amino acids, arginine, tyrosine, proline, asparagine, histidine, valine, serine, tryptophan, leucine, and phenylalanine, while 93% of these strains use methionine, and 64% use alanine, whereas only 14% are able to use glycine, lysine, and aspartic acid. On the other hand, all strains that nodulate *L. cosentinii* are able to use tyrosine, serine, and phenylalanine as the sole source of nitrogen, while 93% assimilate arginine, proline, valine, tryptophan, and leucine, whereas 86% can assimilate asparagine and histidine while 57% are able to assimilate methionine. Glycine, lysine, and aspartic acid are used by only 21% of these strains.

We also tested some enzymatic activities in the strains studied. Thus, 18% of strains nodulating *L. cosentinii* and 9% among strains of *L. luteus* produce gelatinase, and all the strains isolated from the two lupine species produce catalase.

Tolerance to NaCl varies considerably between strains nodulating the two species. The results show that all strains tested can grow in the presence of 85 mM NaCl, while 21% of *L. cosentinii* strains and 14% of *L. luteus* strains develop in salt concentrations between 170 mM and 680 mM, while only 7% of the strains nodulating *L. cosentinii* and 7% of those of *L. luteus* continue to develop at 854 mM NaCl.

Strains react differently when grown in YEM media with different pH values. All the strains nodulating the two species of lupine have the ability to grow in a slightly acidic to neutral pH (6 to 7). All the strains nodulating *L. luteus* grow in a slightly alkaline pH (pH 7.5) and 93% grow at pH 8. They are sensitive to more alkaline pH, with only 50% of the strains able to grow at pH 8.5 and 14% at pH 9. The sensitivity of these strains was also noted with respect to acidic pH; thus, no strain tolerated pH 5; however, 14% of these strains were able to grow at pH 5.5. The results also show that the majority of strains nodulating *L. cosentinii* (86%) can develop at pH 7.5 and that with the increase in pH, the number of strains that manage to grow there decreases, 36% grow at pH 8, 29% at pH 8.5, and only 21% at pH 9. In acidic environments, 21% of the strains manage to develop at pH 5.5 and 7% at pH 5.

Among all the heavy metals tested, barium, manganese chloride, manganese sulfate, aluminum, and cadmium (at 100 μg/ml) were the least harmful for the growth of the strains nodulating *L. luteus* with 79–93% that resist. On the other hand, the presence of cobalt and copper inhibited the growth of half of the strains. The heavy metals most toxic to strains of *L. luteus* are mercury, magnesium, zinc, and lead, and only 21% could grow in their presence. The results showed also that 50% of strains nodulating *L. cosentinii* develop in the presence of manganese chloride and 43% are resistant to aluminum. On the other hand, in presence of barium, cobalt, and manganese sulfate, 36% of the strains were able to resist, and 28% of the strains were able to develop in the presence of copper. Mercury, magnesium, zinc at 500 μg/ml, and lead at 1,000 μg/ml were found to be more toxic with only 21% of strains that were able to tolerate their presence.

The tolerance to antibiotic is one of the defining criteria in the analysis of rhizobial biodiversity. In this work, we tested 11 antibiotics on the different strains isolated from the two lupines and the results showed that there is variability in tolerance. Thus, all the strains nodulating *L. luteus* were resistant to several families of antibiotics: beta-lactams (ampicillin and penicillin), quinolones (nalidixic acid), glucopeptides (vancomycin), aminoglycosides (spectinomycin), gramicidins (bacitracin), and trimethoprims. They grew also in presence of tetracyclines and aminoglycosides (gentamycins and kanamycin) with high percentages between 70 and 86%. The chloramphenicol affects the growth of these bacteria since only 36% were

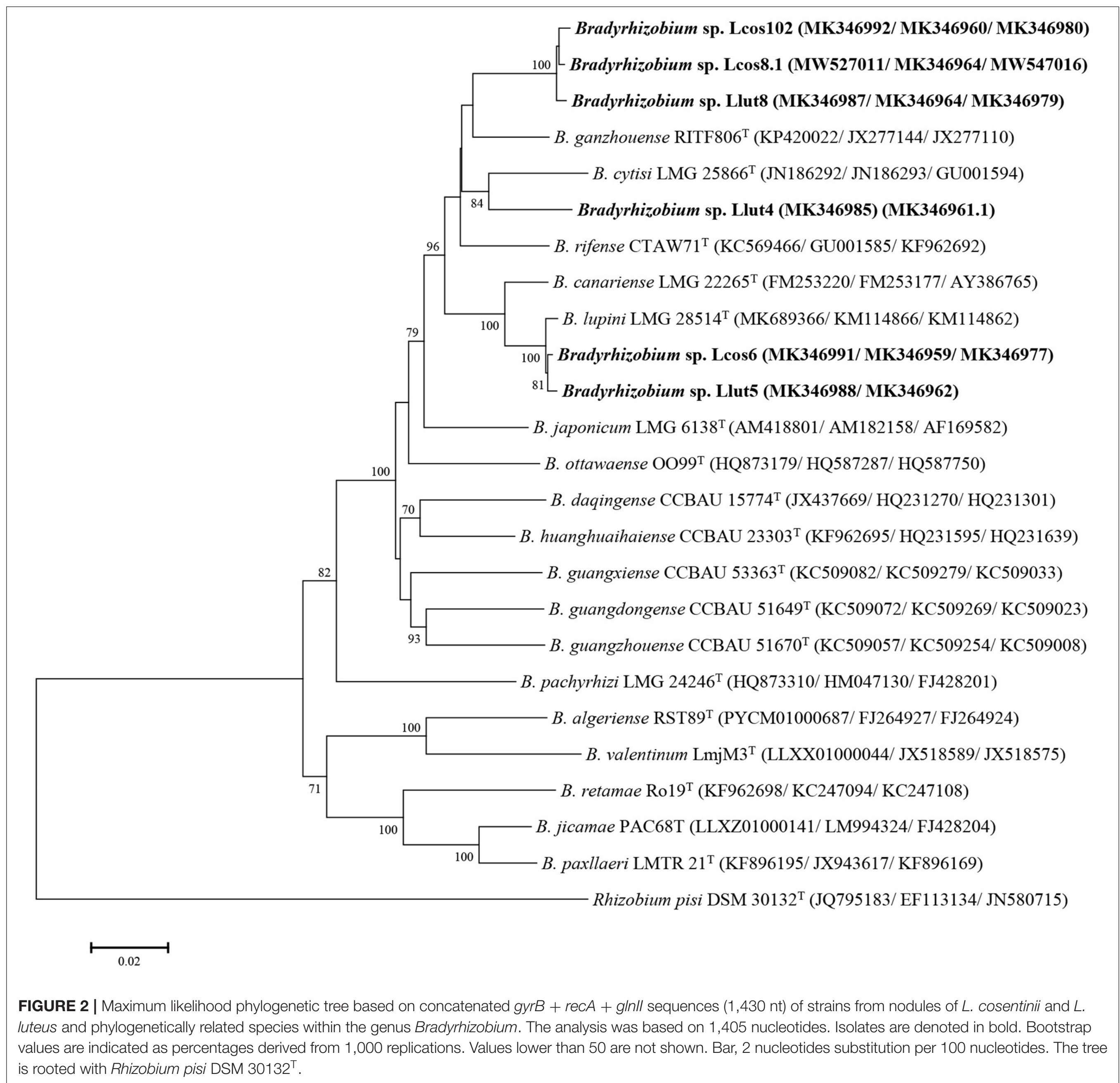


FIGURE 2 | Maximum likelihood phylogenetic tree based on concatenated *gyrB* + *recA* + *glnII* sequences (1,430 nt) of strains from nodules of *L. cosentinii* and *L. luteus* and phylogenetically related species within the genus *Bradyrhizobium*. The analysis was based on 1,405 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1,000 replications. Values lower than 50 are not shown. Bar, 2 nucleotides substitution per 100 nucleotides. The tree is rooted with *Rhizobium pisi* DSM 30132^T.

able to grow in its presence, at the concentrations used. The results also showed that 43% of the strains nodulating *L. cosentinii* tolerated the majority of antibiotics: nalidixic acid, vancomycin, spectinomycin, bacitracin, trimethoprim, and beta-lactams, and 36% developed in the presence of chloramphenicol. These strains are sensitive to aminoglycosides, since only 21% are resistant to kanamycin and 14% are resistant to gentamycin.

Temperature also has a differential effect on strains' growth. Thus, all the strains grew at 30°C, whereas at 35°C, the strains showed a fluctuation in their responses, with 79% of the strains nodulating *L. cosentinii*, and 71% for the strains nodulating *L. luteus*, which were able to grow at this temperature. No strain can grow above 40°C.

PGPR Growth Promoting Activities Test Results

Out of the 28 strains tested, six strains produced the IAA, four strains from *L. cosentinii* and two from *L. luteus*. Only three strains were able to solubilize the inorganic phosphates, two strains from *L. cosentinii* and one strain among *L. luteus* microsymbionts. Two strains were hence very effective with a solubilization index of

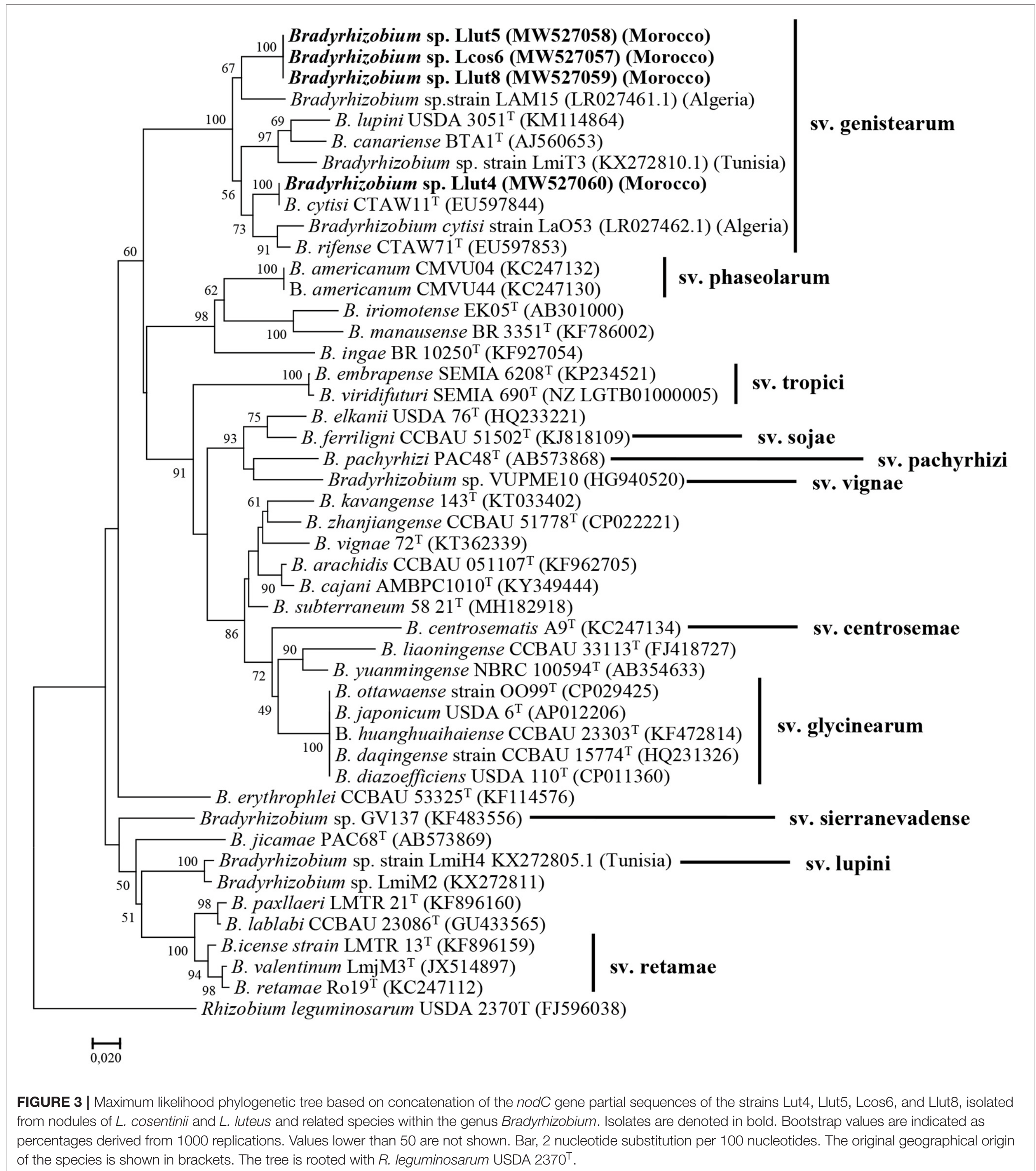


FIGURE 3 | Maximum likelihood phylogenetic tree based on concatenation of the *nodC* gene partial sequences of the strains Lut4, Llut5, Lcos6, and Llut8, isolated from nodules of *L. cosentinii* and *L. luteus* and related species within the genus *Bradyrhizobium*. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 50 are not shown. Bar, 2 nucleotide substitution per 100 nucleotides. The original geographical origin of the species is shown in brackets. The tree is rooted with *R. leguminosarum* USDA 2370^T.

275% for lut5 and 375% for cos10.2. Six strains produced siderophores, four strains from *L. cosentinii* and two from *L. luteus*. The two strains Lcos10.2 and Llut5 have the capacity to solubilize phosphates and produce siderophores and IAA.

DISCUSSION

In this work, we aimed to characterize and identify some bacterial strains isolated from the root nodules of two lupines grown in different soils of the Maamora forest, in the vicinity of Rabat.

We started the molecular characterization by the analysis of the eight representative strains' *rrs* gene sequences, and their phylogeny revealed that they are more related to *B. cytisi* and *B. lupini*. The *rrs* gene was considered as a preliminary genetic marker to situate a strain taxonomic position at the genus level, although it does not provide clear phylogenetic diversity for species of the *Bradyrhizobium* genus (Martinez-Romero and Ormeño-Orrillo, 2019). To this purpose, an MLSA of different housekeeping genes as additional phylogenetic markers is frequently used for accurate identification of *Bradyrhizobium* species and strains (Tampakaki et al., 2017; Wójcik and Kalita, 2019).

Six strains were then selected from the eight representatives, as the *rrs* sequences of strains Llut6 and Lcos7.2 were identical (100%) to strain Lcos6 *rrs* sequence. The phylogenies of the three individual genes *glnII*, *gyrB*, and *recA* showed that strains Lcos6 and Llut5 were closely related to *B. lupini* LMG 28514^T. In fact, we sequenced also the *dnaK* and *rpoB* genes of most strains, but we failed to amplify them in some. Furthermore, data concerning these two genes in *B. lupini* are not available in the databases, and the *dnaK* (JACJNR010000003.1) and *rpoB* (JACJNR010000001.1) sequences of the strain *B. lupini* DSM 30140 extracted from the whole genome sequence available in the NCBI database are more related to *B. japonicum* LMG6138^T (**Supplementary Figures 5**, **6**). Consequently, we preferred to continue with only three genes.

The concatenated housekeeping genes' sequences phylogeny confirmed that the closest parent of the strains Lcos6 and Llut5 is *B. lupini* LMG 28514^T, whereas strain Llut4 was more related to *B. cytisi* LMG 25866^T. The three strains Lcos8.1, Lcos102 and Llut8 were regrouped in a single cluster more related to *B. ganzhouense* RITF806^T, isolated from the rot nodules of *Acacia melanoxylon* in China (Lu et al., 2014). The two strains Lcos8.1 and Lcos102 were isolated from *L. cosentinii*, whereas strain Llut8 was isolated from *L. luteus.* None of the three strains grew in YEMA medium in the presence of 1% NaCl. The strain Lcos6 grows between pH 6 and 7.5, while cos8.1 grows between pH 6 and 8, and the Lut8 strain grows in pH 6–8.5, but do not grow at 40°C. They use glucose, fructose, mannose, and sucrose as sole carbon sources. Inversely, the type strain of *B. ganzhouense* does not assimilate sucrose; it grows at pH 5–12 and develops in the presence of 3% NaCl (Lu et al., 2014). Their low percentages of similarity with their closest parent suggest that they may constitute a putative new genospecies in the genus *Bradyrhizobium*.

Plant species of the genus *Lupinus* were considered as nodulated exclusively by *Bradyrhizobium* spp. for a long time until Ardley et al. (2012) reported the isolation and identification of three symbiotic *Microvirga* species from *L. texensis* and *Listia angolensis* root nodules. More recently, lupines were found to be nodulated by *Microvirga* strains in Tunisian, Moroccan, and American soils (Beligala et al., 2017; Msaddak et al., 2017, 2019; Rejili et al., 2019; Missbah El Idrissi et al., 2020). Msaddak et al. (2017) suggested that lupines would prefer *Bradyrhizobium* spp. strains as microsymbionts in acidic soils and *Microvirga* spp. in the alkaline soils. Lamrabet et al. (2020) also reported that in alkaline soils, some Genistae such as *Retama* spp. would prefer members of *Microvirga* as microsymbiont instead of *Bradyrhizobium*, although Tounsi-Hammami et al. (2019) found that strains isolated from *L. albus* in alkaline soils were affiliated to *Rhizobium* and *Neorhizobium*. Furthermore, Durán et al. (2014) reported the nodulation of the endemic *Lupinus mariae-josephae* by *Bradyrhizobium valentinum* in alkaline-limed soils in Eastern Spain.

The phenotypic analysis showed that all the strains assimilate different carbon sources including glucose, mannose, galactose, and sucrose, while no strain used starch or lactose; this result agrees with previously reported results (Rome et al., 1996; Missbah El Idrissi et al., 2020). *Bradyrhizobium canariense* BTA-1T does not use sucrose or lactose either (Vinuesa et al., 2005).

There was diversity in the utilization of amino acids, but all the strains were able to assimilate tyrosine and phenylalanine as sole nitrogen source, which contradicts results of Miller and Pepper (1988) who reported that the asparagine was the most preferred amino acid for the growth of rhizobia isolated from lupines in the desert of Mexico. Vinuesa et al. (2005) reported also that *B. canariense* and *B. yuanmingense* do not use glycine. Some strains possess the gelatinase activity, an enzyme that hydrolyzes collagen into amino acids or peptides, which allows them to persist in in the rhizosphere while waiting for the plant partner.

We analyzed the effect of environmental factors including salinity, drought, acidity, and alkalinity as well as the behavior of strains toward heavy metals and antibiotics, factors that compromise survival, growth, and nitrogen fixing ability of rhizobia strains (Zahran et al., 1994). We noticed a low tolerance to salinity in the majority of the strains as reported by Vinuesa et al. (2005), but some strains tolerated salt concentrations as high as 854 mM. Many studies have reported very interesting resistance profiles in strains isolated from lupines in Egypt that can tolerate up to 1,700 mM NaCl (Zahran et al., 1994). However, the limits of salinity tolerance between rhizobia can vary considerably from one species to another (Elsheikh and Wood, 1989), and even between strains of the same species (Boukhatem et al., 2012).

Peix et al. (2015) found that among 11 type strains of *Bradyrhizobium* species tested, only *B. betae*, *B. japonicum*, and *B. ganzhouense* grew at 171 mM of NaCl. In fact, in saline environments, the symbiosis between rhizobia and lupines depends not only on the bacteria but on the host plant also. Some strains were able to grow at pH 9, while few strains tolerated pH 5. Our strains tolerate more alkaline than acidic pHs. The type strains of *B. cytisi* and *B. rifense* are reported to grow at pH 4.5 (Peix et al., 2015). All the isolates grow up to 35°C, while no strain grew at 40°C, which is very common in bradyrhizobia (Peix et al., 2015). *Bradyrhizobium* sp. strains isolated from *L. luteus* in Tunisia were not able to grow at 37°C, pH 12, and 1% NaCl (Msaddak et al., 2017). Other studies have shown that certain rhizobial strains can tolerate higher temperatures, but it has been suggested that rhizobia may be protected against different soil constraints by living in particular niches in the rhizosphere and inside nodules (Boukhatem et al., 2012).

L. luteus nodulating strains are more resistant to antibiotics (ATB) and heavy metals than strains isolated from *L. cosentinii*, among which only 50% are able to resist to some antibiotics. The resistance level varies between strains, depending on the type of

ATB and its mechanism of action. Similar results were obtained by (Msaddak et al., 2017).

The majority of our strains showed modest activities of phosphate solubilization and production of siderophores or auxins. Sarkar and Laha (2013) reported that 80% of rhizobia have the capacity to produce IAA and IAA-related compounds. However; siderophore production is usually low and phosphate solubilization is variable in rhizobia (Lamrabet et al., 2020). Few rhizobia may possess all the plant growth promoting (PGP) activities and the plants need hence to interact with different beneficial strains in their rhizosphere. To sustain their development, the most healthy plants attract the best PGPRs (Backer et al., 2018; Gouda et al., 2018).

It is evident that environmental factors such as salinity, drought, acidity, alkalinity, heavy metals, and the presence of antibiotics compromise the survival, growth, and the ability to fix nitrogen of strains of rhizobia (De la Peña and Pueyo, 2012), which shows the interest of knowing their phenotypic and biochemical characteristics in advance before being introduced and inoculated to the plants in the field, to ensure their survival, their adaptability, and their competitiveness (Boukhatem et al., 2012).

The *nodC* symbiotic gene (encoding the N-acetylglucosaminyl transferase protein) is essential for nodulation of compatible host legumes (Laguerre et al., 2001). However, although its phylogeny is not congruent with the core genes' phylogeny (Andrews and Andrews, 2017), it is used to characterize the new isolates at the symbiovar level (Rogel et al., 2011).

Analysis of the individual and concatenated *nodA* and *nodC* gene sequences and their phylogeny showed that the strains Llut5, Lcos6, Llut4, and Llut8 are members of the genistearum symbiovar.

All the strains are able to nodulate *R. monosperma*, *L. luteus, L. albus, L. cosentinii, C. albidus*, and *C. monspessulanus* but not *G. max* or *Phaseolus vulgaris*. It is known that the Genisteae microsymbionts do not nodulate soybean (*G. max* or G. *soya*: Phaseoleae tribe). However, they are able to nodulate different genera and species of the tribe Genisteae (Vinuesa et al., 2005). The fact that the strains are able to nodulate and fix nitrogen with different Genisteae is an advantage to members of this tribe. It is important to note that we observed no significant visible differences between the two lupine plants inoculated with the Lcos strains (isolated from *L. cosentinii*) or the Llut strains (isolated from *L. luteus*). All the plants were in good health as shown by the leaves' green color after 3 months. This represents an advantage for these legume plants, because they can establish a nitrogen fixing symbiosis with different strains with different PGP potentialities, which contributes to their resilience in the case of any edaphoclimatic changes.

CONCLUSION

In this work, we report on the characterization of some strains isolated from the root nodules of two lupines grown in the acidic soils of the Maamora cork forest, in Morocco.

We found that *L. cosentinii* is nodulated by members of the genus *Bradyrhizobum*, whereas the species has been reported as nodulated by *Microvirga* sp. in alkaline soils of the same forest. As already reported, the selection of lupine microsymbionts in the south Mediterranean would depend on the type and pH of the soil. Some *Bradyrhizobium* spp. strains newly isolated from *L. luteus* and *L. cosentinii* may constitute a new genospecies, and this needs confirmation by more sophisticated research such as whole genome sequencing or DNA–DNA hybridization.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GenBank [accession: MW526996-MW527060, MW494669-MW494676, MW547016, and MW565871-MW565872].

AUTHOR CONTRIBUTIONS

MM participated in the conception and design of the experimentations and discussion of the results. OB participated in the analysis of genomic data and phylogenies. SE, HL, and SA participated in the isolation and characterization of isolates. EB participated in the sequencing experiments. HA participated in the conception of the project. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.661295/full#supplementary-material

REFERENCES

Aafi, A., El Kadmiri, A. A., Benabid, A., and Rochdi, M. (2005). Richesse et diversité floristique de la suberaie de la Mamora (Maroc). *Acta Bot. Malacit.* 30, 127–138. doi: 10.24310/abm.v30i0.7187

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Andrews, M., and Andrews, M. E. (2017). Specificity in legume-rhizobia symbioses. *Int. J. Mol. Sci.* 18, 705. doi: 10.3390/ijms18040705

Andrews, M., De Meyer, S., James, E. K., Stepkowski, T., Hodge, S., Simon, M. F., et al. (2018). Horizontal transfer of symbiosis genes within and between rhizobial genera: occurrence and importance. *Genes.* 9:321. doi: 10.3390/genes9070321

Ardley, J. K., Parker, M. A., De Meyer, S. E., Trengove, R. D., O'Hara, G. W., Reeve, W. G., et al. (2012). Microvirga lupini sp. *nov., Microvirga lotononidis* sp. nov. and *Microvirga zambiensis* sp. nov. are alphaproteobacterial root-nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. *Int. J. Syst. Evol. Microbiol.* 62, 2579–2588. doi: 10.1099/ijs.0.035097-0

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1473. doi: 10.3389/fpls.2018.01473

Beck, D. P., Materon, L. A., and Afandi, F. (1993). Practical rhizobium-legume technology manual. *Technical Manual*. ICARDA. 19.

Beligala, D. H., Michaels, H. J., Devries, M., and Phuntumart, V. (2017). Multilocus sequence analysis of root nodule bacteria associated with *Lupinus* spp. and glycine max. *Adv. Microbiol.* 7, 790–812. doi: 10.4236/aim.2017.711063

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Microbiology* 84, 188–198. doi: 10.1099/00221287-84-1-188

Boukhatem, Z. F., Domergue, O., Bekki, A., Merabet, C., Sekkour, S., Bouazza, F., et al. (2012). Symbiotic characterization and diversity of rhizobia associated with native and introduced acacias in arid and semi-arid regions in Algeria. *FEMS Microbiol. Ecol.* 80, 534–547. doi: 10.1111/j.1574-6941.2012.01315.x

Bourebaba, Y., Durán, D., Boulila, F., Ahnia, H., Boulila, A., Temprano, F., et al. (2016). Diversity of *Bradyrhizobium* strains nodulating *Lupinus micranthus* on both sides of the Western Mediterranean: Algeria and Spain. *Syst. Appl. Microbiol.* 39, 266–274. doi: 10.1016/j.syapm.2016.04.006

Cermak, V. (2018a). *Restriction Comparator*. Available online at: http://www.molbiotools.com/restrictioncomparator.html (accessed November, 2010).

Cermak, V. (2018b). *Silent Mutator*. Available online at: http://www.molbiotools.com/silentmutator.html

Chaintreuil, C., Boivin, C., Dreyfus, B., and Giraud, E. (2001). Characterization of the common nodulation genes of the photosynthetic *Bradyrhizobium* sp. ORS285 reveals the presence of a new insertion sequence upstream of *nodA*. *FEMS Microbiol. Lett.* 194, 83–86. doi: 10.1111/j.1574-6968.2001.tb09450.x

Clúa, J., Roda, C., Zanetti, M. E., and Blanco, F. A. (2018). Compatibility between legumes and rhizobia for the establishment of a successful nitrogen-fixing symbiosis. *Genes.* 9:125. doi: 10.3390/genes9030125

De Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* 58, 2180–2187. doi: 10.1128/AEM.58.7.2180-2187.1992

De la Peña, T. C., and Pueyo, J. J. (2012). Legumes in the reclamation of marginal soils, from cultivar and inoculant selection to transgenic approaches. *Agron. Sustain. Dev.* 32, 65–91. doi: 10.1007/s13593-011-0024-2

Durán, D., Rey, L., Navarro, A., Busquets, A., Imperial, J., and Ruiz-Argüeso, T. (2014). Bradyrhizobium valentinum sp. *nov*., isolated from effective nodules of *Lupinus mariae-josephae,* a lupine endemic of basic-lime soils in Eastern Spain. *Syst. Appl. Microbiol*. 37, 336–341. doi: 10.1016/j.syapm.2014.05.002

Elsheikh, E. A. E., and Wood, M. (1989). Response of chickpea and soybean rhizobia to salt: osmotic and specific ion effects of salts. *Soil Biol. Biochem.* 21, 889–895. doi: 10.1016/0038-0717(89)90077-1

Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H.-S., and Patra, J. K. (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. *Microbiol. Res.* 206, 131–140. doi: 10.1016/j.micres.2017.08.016

Guerrouj, K., Pérez-Valera, E., Chahboune, R., Abdelmoumen, H., Bedmar, E. J., and El Idrissi, M. M. (2013). Identification of the rhizobial symbiont of *Astragalus glombiformis* in Eastern Morocco as *Mesorhizobium camelthorni*. *Antonie Van Leeuwenhoek* 104, 187–198. doi: 10.1007/s10482-013-9936-y

Hall, R. S., Thomas, S. J., and Johnson, S. K. (2005). Australian sweet lupin flour addition reduces the glycaemic index of a white bread breakfast without affecting palatability in healthy human volunteers. *Asia Pac. J. Clin. Nutr.* 14:91.

Howieson, J. G., and Dilworth, M. J. (Eds.). (2016). *Working with Rhizobia. ACIAR Monograph No. 173*. Canberra: Australian Centre for International Agricultural Research (ACIAR). p. 312.

Jarabo-Lorenzo, A., Pérez-Galdona, R., Donate-Correa, J., Rivas, R., Velázquez, E., Hernández, M., et al. (2003). Genetic diversity of bradyrhizobial populations from diverse geographic origins that nodulate *Lupinus* spp. *and Ornithopus* spp. *Syst. Appl. Microbiol.* 26, 611–623. doi: 10.1078/072320203770865927

Kermah, M., Franke, A. C., Adjei-Nsiah, S., Ahiabor, B. D. K., Abaidoo, R. C., and Giller, K. E. (2018). N2-fixation and N contribution by grain legumes under different soil fertility status and cropping systems in the Guinea savanna of northern Ghana. *Agric. Ecosyst. Environ.* 261, 201–210. doi: 10.1016/j.agee.2017.08.028

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120. doi: 10.1007/BF01731581

Kohajdova, Z., KaroVičoVá, J., and Schmidt, Š. (2011). Lupin composition and possible use in bakery-a review. *Czech J. Food Sci.* 29, 203–211. doi: 10.17221/252/2009-CJFS

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Laguerre, G., Nour, S. M., Valérie, M., Sanjuan, J., Drouin, P., and Amarger, N. (2001). Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among Phaseolus vulgaris symbionts. *Microbiology* 147, 981–993. doi: 10.1099/00221287-147-4-981

Laguerre, G., van Berkum, P., Amarger, N., and Prévost, D. (1997). Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus*, *Oxytropis*, and *Onobrychis*. *Appl. Environ. Microbiol.* 63, 4748–4758. doi: 10.1128/AEM.63.12.4748-4758.1997

Lakshmanan, V., Shantharaj, D., Li, G., Seyfferth, A. L., Janine Sherrier, D., and Bais, H. P. (2015). A natural rice rhizospheric bacterium abates arsenic accumulation in rice (*Oryza sativa* L.). *Planta* 242, 1037–1050. doi: 10.1007/s00425-015-2340-2

Lamrabet, M., Lamin, H., Bouhnik, O., Bennis, M., Abdelmoumen, H., Eulogio, J. B., et al. (2020). Nodulation of Retama species by members of the genus Microvirga in Morocco. *Symbiosis*. 82, 249–258. doi: 10.1007/s13199-020-00725-5

Lindström, K., and Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol.* 13, 1314–1335. doi: 10.1111/1751-7915.13517

Lu, J. K., Dou, Y. J., Zhu, Y. J., Wang, S. K., Sui, X. H., and Kang, L. H. (2014). Bradyrhizobium ganzhouense sp. nov., an effective symbiotic bacterium isolated from Acacia melanoxylon R. Br. nodules. *Int. J. Syst. Evol. Microbiol.* 64:1900. doi: 10.1099/ijs.0.056564-0

Martinez-Romero, E., and Ormeño-Orrillo, E. (2019). A genomotaxonomy view of the *Bradyrhizobium* genus. *Front. Microbiol.* 10:1334. doi: 10.3389/fmicb.2019.01334

Mellal, H., Yacine, B., Boukaous, L., Khouni, S., Benguedouar, A., Castellano-Hinojosa, A., et al. (2019). Phylogenetic diversity of *Bradyrhizobium* strains isolated from root nodules of *Lupinus angustifolius* grown wild in the North East of Algeria. *Syst. Appl. Microbiol.* 42, 397–402. doi: 10.1016/j.syapm.2019.01.003

Menna, P., Pereira, A. A., Bangel, E. V., and Hungria, M. (2009). Rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool. *Symbiosis* 48, 120–130. doi: 10.1007/BF03179991

Miller, M. S., and Pepper, I. L. (1988). Physiological and biochemical characteristics of a fast-growing strain of lupin rhizobia isolated from the Sonoran Desert. *Soil Biol. Biochem.* 20, 319–322. doi: 10.1016/0038-0717(88)90011-9

Missbah El Idrissi, M., Lamin, H., ElFaik, S., Tortosa, G., Peix, A., Bedmar, E. J., et al. (2020). *Microvirga* sp. symbiovar mediterranense nodulates *Lupinus cosentinii* grown wild in Morocco. *J. Appl. Microbiol.* 128, 1109–1118. doi: 10.1111/jam.14526

Msaddak, A., Rejili, M., Durán, D., Mars, M., Palacios, J. M., Ruiz-Argüeso, T., et al. (2019). Microvirga tunisiensis sp. nov., a root nodule symbiotic bacterium isolated from *Lupinus micranthus* and *L. luteus* grown in Northern Tunisia. *Syst. Appl. Microbiol.* 42:126015. doi: 10.1016/j.syapm.2019.126015

Msaddak, A., Rejili, M., Durán, D., Rey, L., Imperial, J., Palacios, J. M., et al. (2017). Members of *Microvirga* and *Bradyrhizobium* genera are native endosymbiotic bacteria nodulating *Lupinus luteus* in Northern Tunisian soils. *FEMS Microbiol. Ecol.* 93, 1–13. doi: 10.1093/femsec/fix068

Msaddak, A., Rejili, M., Durán, D., Rey, L., Palacios, J. M., Imperial, J., et al. (2018). Definition of two new symbiovars, sv. lupini and sv. mediterranense, within the genera Bradyrhizobium and Phyllobacterium efficiently nodulating Lupinus micranthus in Tunisia. *Syst. Appl. Microbiol.* 41, 487–493. doi: 10.1016/j.syapm.2018.04.004

Peix, A., Ramírez-Bahena, M. H., Flores-Félix, J. D., Alonso de la Vega, P., Rivas, R., Mateos, P. F., et al. (2015). Revision of the taxonomic status of the species *Rhizobium lupini* and reclassification as *Bradyrhizobium lupin*i comb. nov. *Int. J. Syst. Evol. Microbiol.* 65, 1213–1219. doi: 10.1099/ijs.0.000082

Rejili, M., Msaddak, A., Filali, I., Benabderrahim, M. A., Mars, M., and Marín, M. (2019). New chromosomal lineages within *Microvirga* and *Bradyrhizobium* genera nodulate *Lupinus angustifolius* growing on different Tunisian soils. *FEMS Microbiol. Ecol.* 95:fiz118. doi: 10.1093/femsec/fiz118

Rogel, M. A., Ormeño-Orrillo, E., and Martinez Romero, E. (2011). Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Syst. Appl. Microbiol.* 34, 96–104. doi: 10.1016/j.syapm.2010.11.015

Rome, S., Fernandez, M. P., Brunel, B., Normand, P., and Cleyet-Marel, J.-C. (1996). *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int. J. Syst. Evol. Microbiol.* 46, 972–980. doi: 10.1099/00207713-46-4-972

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sarkar, D., and Laha, S. (2013). Production of phytohormone auxin (IAA) from soil born *Rhizobium* sp, isolated from different leguminous plant. *Int. J. Appl. Environ. Sci.* 8, 521–528.

Sessitsch, A., Howieson, J. G., Perret, X., Antoun, H., and Martinez-Romero, E. (2002). Advances in rhizobium research. *CRC Crit. Rev. Plant Sci.* 21, 323–378. doi: 10.1080/0735-260291044278

Shamseldin, A., Abdelkhalek, A., and Sadowsky, M. J. (2017). Recent changes to the classification of symbiotic, nitrogen-fixing, legume-associating bacteria: a review. *Symbiosis* 71, 91–109. doi: 10.1007/s13199-016-0462-3

Smith, S. C., Choy, R., Johnson, S. K., Hall, R. S., Wildeboer-Veloo, A. C. M., and Welling, G. W. (2006). Lupin kernel fiber consumption modifies fecal microbiota in healthy men as determined by rRNA gene fluorescent *in situ* hybridization. *Eur. J. Nutr.* 45, 335–341. doi: 10.1007/s00394-006-0603-1

StatSoft Inc (2004). *STATISTICA (Data Analysis Software System), Version 7*. Available online at: www.statsoft.com (accessed November, 2010).

Susek, K., Bielski, W. K., Hasterok, R., Naganowska, B., and Wolko, B. (2016). A first glimpse of wild lupin karyotype variation as revealed by comparative cytogenetic mapping. *Front. Plant Sci.* 7:1152. doi: 10.3389/fpls.2016.01152

Tampakaki, A. P., Fotiadis, C. T., Ntatsi, G., and Savvas, D. (2017). Phylogenetic multilocus sequence analysis of indigenous slow-growing rhizobia nodulating cowpea (*Vigna unguiculata* L.) in Greece. *Syst. Appl. Microbiol.* 40, 179–189. doi: 10.1016/j.syapm.2017.01.001

Tounsi-Hammami, S., Le Roux, C., Dhane-Fitouri, S., De Lajudie, P., Duponnois, R., and Jeddi, F., Ben (2019). Genetic diversity of rhizobia associated with root nodules of white lupin (*Lupinus albus* L.) in Tunisian calcareous soils. *Syst. Appl. Microbiol.* 42, 448–456. doi: 10.1016/j.syapm.2019.04.002

Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A.-M., Rivas, R., Ludena, D., et al. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl. Environ. Microbiol.* 71, 1318–1327. doi: 10.1128/AEM.71.3.1318-1327.2005

Velázquez, E., Valverde, A., Rivas, R., Gomis, V., Peix, Á., Gantois, I., et al. (2010). Strains nodulating *Lupinus albus* on different continents belong to several new chromosomal and symbiotic lineages within *Bradyrhizobium*. *Antonie Van Leeuwenhoek* 97, 363–376. doi: 10.1007/s10482-010-9415-7

Vinuesa, P., Silva, C., Werner, D., and Martínez-Romero, E. (2005). Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Mol. Phylogenet. Evol.* 34, 29–54. doi: 10.1016/j.ympev.2004.08.020

Wang, J., Andersen, S. U., and Ratet, P. (2018). Molecular and cellular mechanisms of the legume-rhizobia symbiosis. *Front. Plant Sci.* 9:1839. doi: 10.3389/fpls.2018.01839

Wójcik, M., and Kalita, M. (2019). Numerical analysis of phenotypic properties, genomic fingerprinting, and multilocus sequence analysis of *Bradyrhizobium* strains isolated from root nodules of *Lembotropis nigricans* of the tribe Genisteae. *Ann. Microbiol.* 69, 1123–1134. doi: 10.1007/s13213-019-01491-6

Wolko, B., Clements, J. C., Naganowska, B., and Nelson, M. N. (2011). "Lupinus," in *Wild Crop Relatives: Genomic And Breeding Resources*, Ed C. Kole (Verlag Berlin Heidelberg: Springer), 153–206. doi: 10.1007/978-3-642-14387-8_9

Yorgancilar, M., and Bilgiçli, N. (2014). Chemical and nutritional changes in bitter and sweet lupin seeds (*Lupinus albus* L.) during bulgur production. *J. Food Sci. Technol.* 51, 1384–1389. doi: 10.1007/s13197-012-0640-0

Zahran, H. H., Räsänen, L. A., Karsisto, M., and Lindström, K. (1994). Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. *World J. Microbiol. Biotechnol.* 10, 100–105. doi: 10.1007/BF00357572

Zerhari, K., Aurag, J., Khbaya, B., Kharchaf, D., and Filali-Maltouf, A. (2000). Phenotypic characteristics of rhizobia isolates nodulating *Acacia* species in the arid and Saharan regions of Morocco. *Lett. Appl. Microbiol.* 30, 351–357. doi: 10.1046/j.1472-765x.2000.00730.x

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.

Copyright © 2021 Missbah El Idrissi, Bouhnik, ElFaik, Alami, Lamin, Bedmar and Abdelmoumen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 June 2021
doi: 10.3389/fpls.2021.638452

Soybean Nodulation Response to Cropping Interval and Inoculation in European Cropping Systems

Mosab Halwani[1], Moritz Reckling[1,2], Dilfuza Egamberdieva[1,3], Richard Ansong Omari[1,4], Sonoko D. Bellingrath-Kimura[1,4], Johann Bachinger[1] and Ralf Bloch[5]*

[1]Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany, [2]Department of Crop Production Ecology, Swedish University of Agricultural Sciences, Uppsala, Sweden, [3]Faculty of Biology, National University of Uzbekistan, Tashkent, Uzbekistan, [4]Faculty of Life Sciences, Humboldt-University of Berlin, Berlin, Germany, [5]Faculty of Landscape Management and Nature Conservation, Eberswalde University for Sustainable Development, Eberswalde, Germany

OPEN ACCESS

Edited by:
*Juan Imperial,
Consejo Superior de Investigaciones Científicas, Spanish National Research Council (CSIC), Spain*

Reviewed by:
*Li Youguo,
Huazhong Agricultural University, China
Claire Prigent-Combaret,
UMR5557 Ecologie Microbienne (LEM), France*

***Correspondence:**
*Mosab Halwani
mosab.halwani@zalf.de;
mosab.halwani@student.hu-berlin.de*

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

Received: *06 December 2020*
Accepted: *06 May 2021*
Published: *04 June 2021*

Citation:
Halwani M, Reckling M, Egamberdieva D, Omari RA, Bellingrath-Kimura SD, Bachinger J and Bloch R (2021) Soybean Nodulation Response to Cropping Interval and Inoculation in European Cropping Systems. Front. Plant Sci. 12:638452. doi: 10.3389/fpls.2021.638452

To support the adaption of soybean [*Glycine max* (L) Merrill] cultivation across Central Europe, the availability of compatible soybean nodulating Bradyrhizobia (SNB) is essential. Little is known about the symbiotic potential of indigenous SNB in Central Europe and the interaction with an SNB inoculum from commercial products. The objective of this study was to quantify the capacity of indigenous and inoculated SNB strains on the symbiotic performance of soybean in a pot experiment, using soils with and without soybean history. Under controlled conditions in a growth chamber, the study focused on two main factors: a soybean cropping interval (time since the last soybean cultivation; SCI) and inoculation with commercial Bradyrhizobia strains. Comparing the two types of soil, without soybean history and with 1–4 years SCI, we found out that plants grown in soil with soybean history and without inoculation had significantly more root nodules and higher nitrogen content in the plant tissue. These parameters, along with the leghemoglobin content, were found to be a variable among soils with 1–4 years SCI and did not show a trend over the years. Inoculation in soil without soybean history showed a significant increase in a nodulation rate, leghemoglobin content, and soybean tissue nitrogen concentration. The study found that response to inoculation varied significantly as per locations in soil with previous soybean cultivation history. An inoculated soybean grown on loamy sandy soils from the location Müncheberg had significantly more nodules as well as higher green tissue nitrogen concentration compared with non-inoculated plants. No significant improvement in a nodulation rate and tissue nitrogen concentration was observed for an inoculated soybean grown on loamy sandy soils from the location Fehrow. These results suggest that introduced SNB strains remained viable in the soil and were still symbiotically competent for up to 4 years after soybean cultivation. However, the symbiotic performance of the SNB remaining in the soils was not sufficient in all cases and makes inoculation with commercial products necessary. The SNB strains found in the soil of Central Europe could also be promising candidates for the development of inoculants and already represent a contribution to the successful cultivation of soybeans in Central Europe.

Keywords: soybean, Bradyrhizobia, cropping interval, inoculation, nodulation, Central Europe

INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) is one of the most extensively cultivated crops worldwide, representing approximately 57 and 79% of the global pulse area and production in 2018, respectively (FAO, 2020). In Europe-28, however, areas under soybean cultivation are still below 1 million ha, and the region depends largely on imports from North and South America to meet its protein demand (Watson et al., 2017). The demand for soybeans from Europe is increasing, and there is a growing interest by farmers to experiment with the crop (Reckling et al., 2020) and to diversify their cropping systems that are dominated by cereals (Hufnagel et al., 2020). There are studies to identify potent indigenous and commercial Bradyrhizobia inoculants suitable for European conditions (Zimmer et al., 2016; Yuan et al., 2020) and attempt to incorporate soybeans in the no-till cover-crop system (Halwani et al., 2019). However, very little is known about the potential of soybeans in higher latitudes (Lamichhane et al., 2020; Schoving et al., 2020). Soybean seeds contain high nutrients for feed and food use, and the crop establishes nitrogen-fixing symbiosis with Bradyrhizobia, fixing 68% and 119 kg ha^{-1} of nitrogen aboveground from the atmosphere (Peoples et al., 2009). The Bradyrhizobia are either indigenous and present in the soils along with the appropriate hosts or are introduced with a new host by seed inoculation (Corman et al., 1987). In areas where soybeans were domesticated centuries ago, soybean-nodulating Bradyrhizobia (SNB) usually survive and are present in soils in high diversity, making the utilization of commercial inoculants less important (Zhang et al., 2011). In contrast, a soybean is a novel crop in Central Europe (Shurtleff and Aoyagi, 2016), and it has been shown that such soils lack native SNB (Crozat et al., 1987; Madrzak et al., 1995; Narożna et al., 2015). Thus, enhancing soybean expansion and productivity in Europe will also depend on effective inoculation, and farmers currently cultivate soybeans, using commercially available Bradyrhizobium inoculants (Zhang et al., 2003; Zimmer et al., 2016; Reckling et al., 2020).

Previous research in Central Europe has been mainly focused on the selection of the most efficient Bradyrhizobia strains from different inoculants under cold growing conditions (Kadiata et al., 2012) and their interaction with different early maturing soybean varieties (Zimmer et al., 2016; Kühling et al., 2018). In these studies, soybean inoculation with Bradyrhizobia increased the grain yield by up to 56%, protein content by 26% (Zimmer et al., 2016), chlorophyll content by 120% (Kühling et al., 2018); and improved nodulation and N_2-fixation (Kadiata et al., 2012). These experiments tested the symbiotic performance of soybeans with inoculants in soils with no history of soybean cultivation. On the other hand, previously introduced strains, subsequently, might remain genetically isolated in soil or may undergo genetic changes to fit the local conditions (Perrineau et al., 2014). Indeed, inoculants are often applied to soils with soybean cultivation history and established SNB (Obaton et al., 2002; Narożna et al., 2015; Yuan et al., 2020). Little is known about the possible influences of the previously introduced SNB on the symbiotic performance of commercial inoculants. Research in the North and South America showed that the response of inoculation with commercial Bradyrhizobia inoculants differed in soils when soybean was included in previous rotations. This introduced SNB in the soil can either enhance, stay neutral or compete with the elite strain in the commercial inoculants (Thies et al., 1991; Piccinetti et al., 2013; Iturralde et al., 2019).

There is sufficient evidence regarding the presence of SNB in European soils at high latitudes with cold winters. The presence of indigenous bacteria can be seen as a chance for the SNB to adapt to European conditions. It may also cause a problem by escalating the competition between indigenous and inoculated strains. However, no information is available concerning the symbiotic performance of soybeans with the present SNB in the soil of Central Europe and their competition with commercial inoculants. The objectives of this study were to (i) investigate the nodulation and symbiotic efficiency of indigenous SNB in soils from different soybean cropping intervals, and (ii) assess the response of a soybean to commercial inoculants in the presence of indigenous SNB in a pot experiment.

MATERIALS AND METHODS

A pot experiment was conducted in growth chambers at the Leibniz Centre for Agricultural Landscape Research (ZALF), located in Müncheberg, Germany. Plants were grown from January to February 2018 for 40 days in tall and narrow 2.5-L plastic pots filled with 2.3-kg soil.

Soil Sampling

Eleven soil samples with different soybean cultivation histories (**Table 1**) were collected from two locations in the northeast of Germany, Fehrow (N 51° 51′ 9.915″ E 14° 16′ 42.874″) and Müncheberg (N 52° 30′ 56.16″ E 14° 7′ 38.639″) in November 2017. At each location, soil samples were collected from five random spots at a depth of 0 to 20 cm. The main agronomic practices at both locations: The maize is amended with cattle manure in early spring; manure is immediately incorporated, using a chisel plow. Grass-clover mix is mowed 2–3 times per year, and the biomass is raked and baled. Weeds are managed using rotary hoeing and row cultivation until crop canopy closure.

Soybean cropping interval (SCI) refers to the year(s) since the last soybean cultivation. In the sampling sites with zero SCI, there is no history of soybean cultivation. In the other sampling sites, soybean cultivar "Merlin" had been cultivated, and seeds were inoculated with HiStick®, a commercial SNB strain from BASF. Similar agronomic practices were used in all sampled sites.

Physical and Chemical Characteristics of Soil

Müncheberg soils consist of 65–77% sand, 17–27% silt, and 5–7% clay. Their particle size distribution categorizes the soil as slightly loamy sand. The field capacity lies between 9.5 and 12%. The soil in Fehrow is medium loamy sand with 8–12% sand, 10–40% silt, and 48–82% clay. Compared with Müncheberg soils, soils in Fehrow have a higher field capacity in the range of 10.5–21%. Soil pH in water ranged from neutral (pH 7.0) to slightly acidic (pH 6.0) in soil samples at both locations.

TABLE 1 | Crop sequence history in the sampling sites.

Location	SCI[1]	Year of soil sampling	Pre-crops		
		2017	2016	2015	2014
Fehrow	1	Soybean	Winter wheat	Grass-clover mix	Winter wheat
	2	Winter wheat	Soybean	Winter wheat	Grass-clover mix
	3	Grass-clover mix	Winter wheat	Soybean	Maize
	4	Maize	Grass-clover mix	Winter wheat	Soybean
	0	Grass-clover mix	Grass-clover mix	Grass-clover mix	Grass-clover mix
Müncheberg	1	Soybean	Winter wheat	Winter oilseed rape	Winter rye
	2	Oat	Soybean	Winter barley	Winter wheat
	3	Grass-alfalfa mix	Spring oat	Soybean	Maize
	4	Grass-clover mix	Grass-clover mix	Spring oat	Soybean
	0	Maize	Alfalfa-grass mix	Winter rye	Winter rye
	0*	Grass	Grass	Grass	Grass

[1]*Soybean cropping interval (year/s since the last soybean cultivation).*
**No legumehistory at all.*

The carbon content in the soils of Müncheberg and Fehrow was 1–2% and <0.8%, respectively; while nitrogen contents were 0.1–0.2% and 0.05–0.08, respectively. At both locations, the contents of double lactate extractable phosphorus, exchangeable potassium, and magnesium ranged from 1.7–9, 2.9–12, and 0.9–1.8 g kg^{-1}, respectively.

Experimental Conditions

The seeds of early maturing soybean cultivar "Merlin" (maturity group 000) were surface sterilized by immersion in 10% v/v bleach NaOCl solution for 45 s and then in 70% ethanol for 45 s before being rinsed five times, using sterile water. Surface-sterilized, bold, and healthy seeds were sown two seeds per pot at a depth of 3 cm. There were two treatments: non-inoculation and inoculation with a commercial inoculant. The commercial soybean inoculant, HiStick®, obtained from BASF, North Carolina, NC, USA, containing *Bradyrhizobium japonicum* (4×10^9 viable cells $gram^{-1}$), was used for inoculation of soybean seeds. Each treatment was replicated six times.

A trap host approach, described by Howieson et al. (2016), was used to sow the surface-sterilized soybean seeds directly into the soil. The conditions in the growth chambers were a 16/8 h light regime and at a temperature range of 22/15°C of day/night, respectively (Martyniuk et al., 2016). The soil temperatures in the pot were measured, using a soil thermometer. The Soil–Plant Analysis Development (SPAD) was measured weekly, using chlorophyll meter SPAD 502 Plus (Konica Minolta Optics, Inc., Osaka, Japan).

Plant Harvest for Assessing Nodulation and Plant Growth Parameters

After 40 days, the aboveground plant growth parameters, such as the height and dry weight of the plant shoots, were determined. Afterward, roots were washed carefully with water over a metal sieve to ensure minimum nodule loss and to reduce possible shedding and rupturing of nodules. The number of nodules and average weight of nodules per plant were determined. Nitrogen concentrations in the plant green tissues were measured according to the Kjeldahl method. Olympus AT200 auto analyzer was used to measure the nitrogen contents in shoots.

Leghemoglobin Test

Leghemoglobin in the soybean nodules was determined by using the modified method of Wilson and Reisenauer (1963). The leghemoglobin was extracted with Drabkin's solution and measured colorimetrically as the CMLHb complex after centrifuging. The leghemoglobin content (mg g^{-1}) of each sample was determined directly from the calibration curve.

Root Architecture

Each plant root system was washed and cleansed of soil particles. Harvested roots were scanned in water with a flatbed scanner (Epson Expression 10,000 xL, SEIKO Epson CORPORATION, Japan, resolution 2,400 dpi). Root volume, root length, a root surface area, a root diameter, and root tips were measured from the scanned images using the commercial root-scanning system WinRhizo™ 2007 (Régent Instruments Inc., Canada).

Statistical Analysis

To evaluate the response of nodulation and symbiotic performance of SNB to a soybean cropping interval, separate data of each location from non-inoculated treatments were subjected to the variance analysis. The differences between the means were tested by applying Tukey's test. To evaluate the response of nodulation and symbiotic performance to inoculation, data collected from each location were divided into sites with and without soybean history. After satisfying the assumptions for normality and homogeneity of variance, the software SAS® 9.2 (SAS Institute Inc., Cary, NC, United States) was employed, using the PROC MIXED procedure for data evaluation. The Pearson's correlation coefficients among the nodule, plant growth parameters, and root architecture traits were calculated, using the JMP Pro® (version14.3) software for multivariate data analysis (SAS Institute Inc., Cary, NC, United States). The results were expressed at the $p < 0.05$ (*) and $p < 0.01$ (**) levels of probability, respectively.

RESULTS

Plant Growth Response to Soybean Cropping Interval Without Inoculation

The analysis of variance showed that the plant height, dry weight of shoot, and SPAD-value were not significantly ($p \leq 0.05$) affected by SCI at both locations. The plant heights ranged from 22- to 27-cm $plant^{-1}$ and from 25- to 27-cm $plant^{-1}$,

while the weights ranged from 1.2- to 1.6-g plant^{-1} and from 1.3- to 1.4-g plant^{-1}, and its SPAD-values ranged from 35 to 36 and from 29 to 32 in Fehrow and Müncheberg, respectively. Meanwhile, the root system architecture traits divided the soil samples in Fehrow into two groups. The first group consists of soils with 2-year SCI and soils with 4-year SCI. The second group consists of soils with no soybean history, soils with 1-year SCI, and soils with 3-year SCI, which were statistically the same in all measured root system architecture traits. Root length ranged from 17×10^6 cm to 20×10^6 cm and from 22×10^6 cm to 25×10^6 cm, a surface area ranged from 27×10^5 cm^2 to 34×10^5 cm^2 and 36–45 $\times 10^5$ cm^2, a root volume ranged from 33×10^2 cm^3 to 44×10^2 cm^3 and from 47×10^2 cm^3 to 66×10^2 cm^3, a root diameter ranged from 0.50 to 51 mm and from 0.52 to 0.58 mm, and root tips ranged from 19×10^2 to 25×10^2 and from 33×10^2 to 40×10^2 in the first and second groups, respectively. Plants grown in Müncheberg soils with no soybean or legume history had root length of 27–29 $\times 10^6$ cm, a root surface area of 46–62 $\times 10^5$ cm^2, a root volume of 64–106 $\times 10^2$ cm^3, a root diameter of 0.6–0.68 mm, and root tips of 35–53 $\times 10^2$. These root system architecture traits were significantly lower in all soils with 1–4 SCI and ranged as follows: root length from 20×10^6 to 24×10^6 cm, a root surface area from 37×10^5 cm^2 to 42×10^5 cm^2, root volume from 54×10^2 cm^3 to 60×10^2 cm^3, a root diameter from 0.57 to 0.59 mm, and root tips from 27×10^2 to 34×10^2.

Nodulation Response to a Soybean Cropping Interval Without Inoculation

Results of the nodulation rate, the average weight of nodules, leghemoglobin content, and nitrogen concentration in the green tissue were significantly different between locations and were analyzed separately.

Nodulation Response at the Location Fehrow

The nodule number formed on the roots of soybean plants was significantly affected ($p = 0.0036$) by the SCI at the location Fehrow (**Table 2**). The nodule number formed on plants grown in soils with 1- and 2-year SCI was not significantly different from soils with 3- and 4-year SCI, and soils with no soybean history. However, plants grown in soils with 3-year SCI formed significantly more nodules than that with 4-year SCI and no soybean history. The average weight of nodules was significantly ($p = 0.0095$) higher in plants grown in all soils with no soybean history compared with plants grown in soil with 1–4 SCI. The average weight of nodules was 13.1 mg for soil samples with no soybean history, while, in soils with 1–4 SCI, the average weight of nodules ranged from 3.9 to 5.1 mg (**Table 2**).

Among the soil samples with 1–4-year SCI, the leghemoglobin contents of nodules were affected significantly ($p = 0.0003$) by SCI. However, the leghemoglobin content of nodules in soil with no soybean history was 18.6 mg g^{-1} and was similar to values obtained in sites with 1–4 SCI. Nitrogen concentration in the plant tissue was significantly ($p < 0.0001$) higher in plants grown in all soils with 1–4 SCI than the plants grown in soil with no soybean history (**Table 2**).

Nodulation Response at the Location Müncheberg

The plants grown in Müncheberg soil with no soybean or no legume history formed significantly ($p = 0.001$) the lowest number of nodules of 7 and 3 nodule plants^{-1}, respectively (**Table 3**). The number of nodules did not differ among soils with no soybean history, 1-year SCI and 3-year SCI. Plants grown in soil with 2-year SCI formed the highest number of nodules of 41 plants^{-1} and were similar to plants grown in soil with 4-year SCI (**Table 3**). The average weight of nodules was significantly ($p = 0.045$) higher in plants grown in all soils with no soybean history compared with the plants grown in soil with 1–4 SCI. The average weight of nodules ranged from 44.4 to 50 mg for soil samples with no soybean history, while it ranged from 9.1 to 24.6 mg in the soils with 1–4 SCI (**Table 3**). The highest leghemoglobin content of 46 mg g^{-1} of fresh nodule compared with all other soils. Among the soils with soybean history, the leghemoglobin contents ranged from 16 to 30 mg g^{-1} with no statistical differences between soils with 1, 2, and 4 SCI.

Nitrogen concentration in the plant tissue was significantly ($p = 0.0004$) higher in soybean plants grown in soil with 1–4 year SCI compared with that grown in soil with no legume history (**Table 3**). Tissue nitrogen content in soil with no legume history was 2.1%, while it ranged from 2.4 to 3.2% for soils with 1–4 SCI.

Nodulation and Plant Growth Response to Inoculation and Soybean Cropping Intervals

Inoculation showed a significant influence on the nodulation rate in the soil with no soybean history. Relative to non-inoculation, the nodulation rate increased by 75.8% ($p = 0.017$) and 360%

TABLE 2 | Influence of soybean cropping interval on nodulation rate, average weight of nodules, content of leghemoglobin in nodules as well as nitrogen concentration in plant shoot at Fehrow.

SCI[1]	Nodulation rate (nodule plant^{-1})	Average weight of nodule (mg)	Leghemoglobin content (mg g^{-1})	Nitrogen concentration in plant (%)
1	63^{ab}	5.1^{b}	24.2^{a}	3.2^{c}
2	46^{ab}	3.9^{b}	15.6^{bc}	3.2^{d}
3	83.7^{a}	4.4^{b}	23.4^{ab}	3.7^{a}
4	41.7^{b}	4.2^{b}	10.3^{c}	3.5^{b}
0	40^{b}	13.1^{a}	18.6^{ab}	3.1^{e}

Data was collected from non-inoculated plants. Means followed by a common letter are not significantly different by the Tukey's test at the 5% level of significance.

[1]Soybean cropping interval (year/s since the last soybean cultivation).

TABLE 3 | Influence of soybean cropping interval on nodulation rate, average weight of nodules, content of leghemoglobin in nodules as well as nitrogen concentration in plant shoot at Müncheberg.

SCI[1]	Nodulation rate (nodule plant^{-1})	Average weight of nodule (mg)	Leghemoglobin content (mg g^{-1})	Nitrogen concentration in plant (%)
1	10^{cd}	24.6^{b}	28.7^{bc}	2.4^{e}
2	40.7^{a}	13.2^{b}	30.2^{b}	3.1^{b}
3	22.7^{bc}	9.1^{b}	15.7^{c}	2.9^{c}
4	28^{ab}	14.5^{b}	17.6^{bc}	3.2^{a}
0	7.3^{cd}	44.4^{a}	29^{bc}	2.7^{d}
0*	2.7^{d}	50^{a}	45.9^{a}	2.1^{f}

Data was collected from non-inoculated plants. Means followed by a common letter are not significantly different by the Tukey's test at the 5% level of significance.
[1]Soybean cropping interval (year/s since the last soybean cultivation).
**No legume history at all.*

(p = 0.0041) with commercial inoculant in Fehrow and Müncheberg soils with no soybean history, respectively (**Figure 1**). In the soils with soybean history, the response to inoculation differed between the locations. While no significant (p = 0.82) differences were found between inoculated and non-inoculated plants grown in Fehrow soils, there was a significant increase (p = 0.049) in the nodulation rate by an average of 57.9% in Müncheberg soils with soybean history (**Figure 1**).

Inoculation showed a significant (p < 0.0001) influence on the average weight of nodules in the soil with no soybean history at both locations. In Fehrow, the average weight of nodules reduced from 13.1 to 7.2 mg, following inoculation. Also, the average weight of nodules reduced from 47.2 to 10.5 mg with inoculation in Müncheberg. In contrast, the average weight of nodules did not differ significantly (p = 0.34 and p = 0.133) with inoculation in the soil samples with soybean history in Fehrow and Müncheberg, respectively.

The leghemoglobin contents of soybean nodules did not increase significantly by inoculation. Instead, leghemoglobin content decreased significantly (p = 0.046) by 31% with inoculation at Müncheberg soil with no soybean history (**Figure 1**).

The shoot nitrogen concentration showed a positive response to the commercial inoculant tested in the soils with no soybean history at both locations, i.e., Fehrow (p = 0.004) and Müncheberg (p = 0.0034). Soybean tissue nitrogen concentration increased in inoculated soils with no soybean history by 7.1% at Fehrow and 27.6% at Müncheberg. In soils previously cultivated with soybeans, the inoculated plants had a significantly (p = 0.0004) higher tissue nitrogen concentration of 15.6% compared with the non-inoculated plant at Müncheberg (**Figure 1**). However, the plant tissue nitrogen concentration did not increase significantly (p = 0.8185) with inoculation in the soils with soybean history at Fehrow. The plant height, dry weight of shoot, and SPAD value as well as all root architecture traits did not show a positive response to the commercial inoculant tested in this experiment (data not presented).

The correlation matrix of the studied parameters revealed positive correlations between the nodulation rate and chlorophyll content measured by SPAD (r^2 = 0.69; p < 0.05) and nitrogen concentration in the plant tissue (r^2 = 0.90; p < 0.001). Furthermore, the correlation analysis also showed a significant (r^2 = 0.84; p < 0.01) positive relationship between SPAD values measured and nitrogen concentration in the green tissue. In contrast, the dry weight of the shoot showed a significantly positive correlation with all root architecture traits (r^2 from 0.46 to 0.52; p < 001). A significant positive relationship existed among the root architecture traits: root length, a surface area, volume, a diameter, and tips (r^2 ranged from 0.69 to 0.96; p < 0.01). No correlations were found for the nodulation rate and the following parameters: leghemoglobin content in the nodules, plant height, dry weight of shoot, and all root architecture traits.

DISCUSSION

This study confirmed the presence of indigenous SNB on the soybean plant grown at both locations (Fehrow and Müncheberg) with no soybean history, as well as with no legume history in Müncheberg. The observed nodules had a very high leghemoglobin content (**Tables 2** and **3**), which suggests evidence of symbiotic nitrogen fixation (Ott et al., 2005). A minimal number of nodules observed even in the first soybean cultivation at non-inoculated conditions were already reported in Germany (Kühling et al., 2017). It might be related to the ability of soybeans to establish symbiosis with other rhizobia, which has a broad range of host plants, confirming the results obtained in northeastern China (Yan et al., 2014). Yan et al. (2014) isolated a high diversity of soybean Bradyrhizobia from grassland, which has been cultivated for 28 years with a mixture of legume and non-legume wild species. Yuan et al. (2020) selected one symbiotic isolate, GMM49, which is closely related to *R. tropici* and *R. lusitanum*, from the soil with no legume history in Germany. This isolate is capable of forming nodules with soybeans more effectively than the commercial isolates. Another possibility might be that the soil was contaminated by Bradyrhizobia through wind and/ or water erosion as well as through farming equipment used over the years (Larson, 2013; Mason et al., 2016). Vargas et al. (1994), in Brazil, found a strain of *B. japonicum* in soil samples, although the sampled site was thousands of kilometers away from the area where it had been introduced as an inoculant. However, at both locations in the present study, the nodulation rate of plants grown in soils with no soybean or legume history was lower than plants grown in soils with 1–4 years SCI and was accompanied by a high average nodule weight per plant and low shoot nitrogen concentration. These observations support the assertion that SNB can be present in soils at low population densities (Thies et al., 1991).

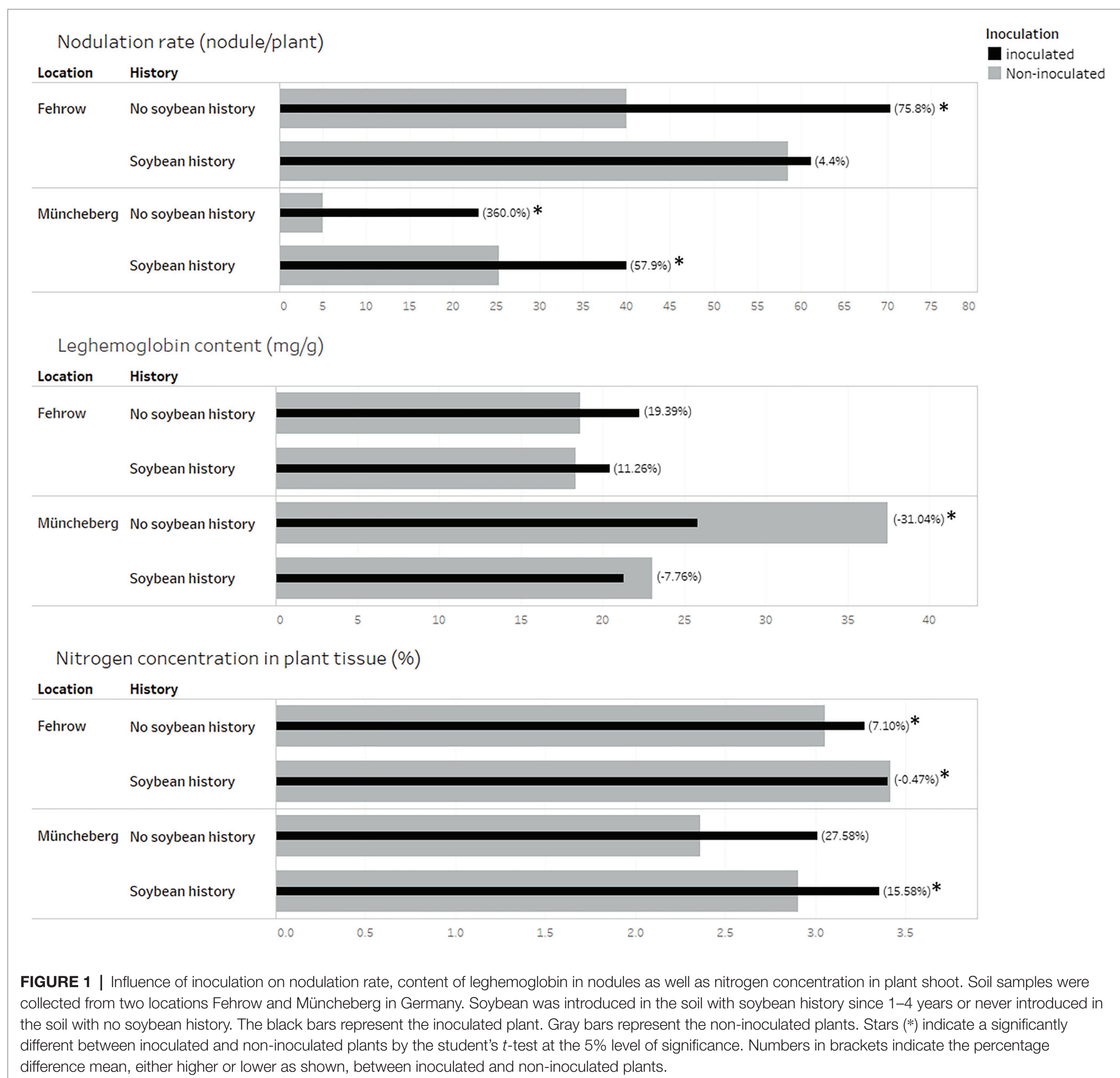


FIGURE 1 | Influence of inoculation on nodulation rate, content of leghemoglobin in nodules as well as nitrogen concentration in plant shoot. Soil samples were collected from two locations Fehrow and Müncheberg in Germany. Soybean was introduced in the soil with soybean history since 1–4 years or never introduced in the soil with no soybean history. The black bars represent the inoculated plant. Gray bars represent the non-inoculated plants. Stars (*) indicate a significantly different between inoculated and non-inoculated plants by the student's *t*-test at the 5% level of significance. Numbers in brackets indicate the percentage difference mean, either higher or lower as shown, between inoculated and non-inoculated plants.

The nodulation rate was high when the inoculated soybean seeds were grown in the soils from the sites with no soybean history, which was initially devoid of SNB or had it in low populations (**Figure 1**). The positive response to inoculation is consistent with the other experiments conducted under controlled conditions in growth chambers (Kadiata et al., 2012) and natural soil conditions (Piccinetti et al., 2013; Zimmer et al., 2016; Kühling et al., 2017; Reckling et al., 2020). The higher nodulation rate was associated with a higher nitrogen concentration in the plant shoots (**Figure 1**), which was also found in other studies (Koutroubas et al., 1998; Sogut, 2006; Kadiata et al., 2012; Kühling et al., 2018). These observations indicate that the native or nonspecific soybean strains present in the soil are neutral and did not compete with the commercial inoculants (Sinclair and Nogueira, 2018). However, the leghemoglobin content of the nodules formed from these native or nonspecific soybean strains was as high as in the elite strains applied (**Figure 1**), which indicates good coordination between SNB and a plant host (Ott et al., 2005) and implies that their establishment in the soil at low population density was the main reason for their low nodulation rates (Thies et al., 1991). Increasing the nodulation rate by inoculation corresponded with decreasing an average nodule weight per plant and leghemoglobin content as well as increasing nitrogen concentration in plant tissues. This observation reflects a high nitrogenase enzyme activity in the small nodules despite the

low leghemoglobin contents. Singh and Varma (2017) indicated that the presence of leghemoglobin is essential for the nitrogenase enzyme, but vice versa is true, and the relationship between leghemoglobin content and nitrogenase activity varies, depending on the strain, plant host, nodule size, and nodule age.

The non-inoculated soybean grown in soils with 1–4-year SCI formed more nodules than those grown in soil with no soybean history (**Tables 2** and **3**). These established SNBs were active and had sufficient leghemoglobin content that played an essential role in the N_2 fixation of soybean nodules by facilitating O_2 supply to the Bradyrhizobia for respiration (Ohyama et al., 2011) and, at the same time, protected the nitrogenase enzyme from oxygen denaturation (Singh and Varma, 2017). As a result, the shoot nitrogen concentration of soybean plants grown in soils with 1–4-year SCI was higher than those grown in soils with no soybean history. This finding suggests that the inoculated strains had been originally established in these soils or had changed genetically to fit the local conditions (Melchiorre et al., 2011; Perrineau et al., 2014). According to Nazir et al. (2013), introduced SNB strains could either become well adapted to the soil environment, displace a native population, and might occupy its niche (Nazir et al., 2013). This confirms the results of Yuan et al. (2020), who showed the close genetic relationship between some Bradyrhizobium isolates from soils in Germany and elite strains from commercial inoculants.

The nodulation rate and leghemoglobin content in the nodules, as well as plant tissue nitrogen concentration response to varying SCI, were significantly different between non-inoculated plants grown in soils with 1–4 year SCI history at both locations. However, these parameters did not show a consistent trend among the different SCI (**Tables 2** and **3**). This variation in symbiotic performance reflects the low effects of the introduced SNB strains in the soil or its nodulation activities by SCI. Factors such as soil management, crop rotation, organic fertilizer use (Triplett and Sadowsky, 1992) and land use patterns (Yan et al., 2014), as well as soil characteristics (Revellin et al., 1996), are likely to contribute to the variability in the response.

We observed significant variability between the locations in response to inoculation in the soils from sites with soybean history but were not able to identify the reasons for these differences (soils, climate, and management were different). A comparative analysis of the soybean rhizobia symbiotic genes in Germany showed no significant differences in *nodD* and *nifH* genes, which indicates that the soybean rhizobia symbiotic genes in Germany belong to only one type (Yuan et al., 2020). Moreover, in multilocus sequence analysis (MLSA), the majority of isolates were identified as *Bradyrhizobium*, and some isolates were shown to belong to the genus *Rhizobium*. The isolates identified as *Rhizobium* did not have the ability to form nodules on soybeans, depending on the phylogenetic analysis of symbiotic genes for *nodD* and *nifH* (Yuan et al., 2020).

In Fehrow, the nodulation rate, leghemoglobin, content and shoot nitrogen concentration in soil with soybean history were similar for non-inoculated and inoculated soybean seeds, showing no response to the commercial inoculation (**Figure 1**). This finding is in consonance with several previous studies in France (Revellin et al., 1996; Obaton et al., 2002) and at 73 environments in the USA (De Bruin et al., 2010; Mason et al., 2016). It is, however, in contrast to a common perception that the nodulation rate and symbiotic performance improve by using the inoculants containing elite strains of Bradyrhizobia (Mendes et al., 2004; Argaw, 2014). With respect to the results of Thies et al. (1991) and Mendes et al. (2004), who indicated that the response of legumes to rhizobial inoculants was inversely related to the population of present rhizobia in the soil, the modest response to commercial inoculant refers to the large populations of introduced SNB present in the soil and gives rise to two assumptions. Similar observations were reported for soils in Poland, where Bradyrhizobia strains were re-isolated 17 years later and compared with the same strains from the original inoculum strains. The strains remained viable, symbiotically capable, and had equal numbers, although a soybean was never grown in the field (Narożna et al., 2015).

On the other hand, there was a positive response of inoculation on a nodulation rate and shoot nitrogen concentration in soils from sites with soybean history in Müncheberg (**Figure 1**), representing a reduction in the introduced SNB abundance in the soil (Sinclair and Nogueira, 2018). The neutral response of leghemoglobin to inoculation refers to the high ability of established SNB to form a symbiotic association with a plant host (Ott et al., 2005). The decline in numbers of established SNB is likely to be a response to the direct and indirect harsh environmental conditions attributable to the sandy soil and drought conditions in the sampled area (Sinclair and Nogueira, 2018). Dudeja and Khurana (1989) reported degrees of the abundance of the Bradyrhizobia sp. (Cujanur) CC 1021 strain after 90 days of inoculation in the sandy loam soil for 2 years.

Inoculation with commercial inoculant should enhance growth parameters (García et al., 2004) as well as root system architecture traits (Yang et al., 2017). Nonetheless, our results showed no increase and even a slight decrease in SPAD value, plant height, and root architecture traits with inoculation (data not presented). This result is possibly due to the substantial demand for carbohydrates and nutrients for symbiotic microorganisms like Bradyrhizobia. The photosynthetically derived carbon from the legume host plants during the symbiosis process may constitute a possible competitive effect during the nodulation and nitrogen fixation (Thies et al., 1991; Reich et al., 2006; Aleman et al., 2010; Li et al., 2016). Takahashi et al. (1995) and Guo et al. (2011) indicated that soybean roots were shorter, and the root surface was limited by inoculation of the seed with symbiotic rhizobia.

CONCLUSION

Nodulation and shoot nitrogen concentration differed among the varying SCIs in non-inoculated soils, but there was no consistency in the trend in both locations. A significant variability between the locations in response to inoculation with commercial inoculants was observed in the soils with soybean history. While a nodulation rate, leghemoglobin content, and shoot

nitrogen concentration were similar to non-inoculated and inoculated soybean plants at the location Fehrow, a positive response to inoculation was observed at the location Müncheberg. Furthermore, the results of this study emphasize the presence of viable and symbiotically competent SNB strains in soils previously cultivated with soybeans. These previously introduced and adapted SNB whose presence in soil influenced the symbiotic performance of the commercial inoculants are promising candidates for ensuring effective inoculation in cold conditions of Central Europe. Further investigations, especially on the adapted SNB strains that fit the environments in Central Europe, are urgently 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

MH, RB, and DE conceived and designed the experiment. MH performed the agronomic analysis and wrote the manuscript. MR, JB, DE, RO, and SB-K provided many helpful conceptual discussions. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Innovation Network to Improve Soybean Production under the Global Change (INNISOY) through the European Interest Group CONCERT-Japan (01DR17011A), the SusCrop-ERA-NET project LegumeGap (031B0807A), and the web-based nutrient management in organic farming (Web-Man)-2818OE051. MR was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 420661662.

ACKNOWLEDGMENTS

The authors acknowledge the support of the German Research Foundation (DFG) and the Open Access Publication Fund of Humboldt-Universität zu Berlin. We would like to cordially thank Gerlinde Stange, Gunhild Rosner and the staff of the field research station in Müncheberg for their excellent technical support, as well as Georg Ludwig for providing the soil samples from Fehrow.

REFERENCES

Aleman, L., Ortega, J. L., Martinez-Grimes, M., Seger, M., Holguin, F. O., Uribe, D. J., et al. (2010). Nodule-enhanced expression of a sucrose phosphate synthase gene member (MsSPSA) has a role in carbon and nitrogen metabolism in the nodules of alfalfa (*Medicago sativa* L.). *Planta* 231, 233–244. doi: 10.1007/s00425-009-1043-y

Argaw, A. (2014). Symbiotic effectiveness of inoculation with *Bradyrhizobium* isolates on soybean [*Glycine max* (L.) Merrill] genotypes with different maturities. *SpringerPlus* 3:753. doi: 10.1186/2193-1801-3-753

Corman, A., Crozat, Y., and Cleyet-Marel, J. C. (1987). Modelling of survival kinetics of some *Bradyrhizobium japonicum* strains in soils. *Biol. Fertil. Soils* 4, 79–84.

Crozat, Y., Cleyet-Marel, J., and Corman, A. (1987). Use of the fluorescent antibody technique to characterize equilibrium survival concentrations of *Bradyrhizobium japonicum* strains in soil. *Biol. Fertil. Soils* 4, 85–90.

De Bruin, J., Pedersen, P., Conley, S., Gaska, J., Naeve, S., Kurle, J. E., et al. (2010). Probability of yield response to inoculants in fields with a history of soybean. *Crop Sci.* 50, 265–272. doi: 10.2135/cropsci2009.04.0185

Dudeja, S., and Khurana, A. (1989). Persistence of *Bradyrhizobium* sp.(Cajanus) in a sandy loam. *Soil Biol. Biochem.* 21, 709–713. doi: 10.1016/0038-0717(89)90068-0

FAO (2020). FAOSTAT Statistical Database. Food and Agriculture Organization of the United Nations.

García, J. L., Probanza, A., Ramos, B., Barriuso, J., and Manero, F. G. (2004). Effects of inoculation with plant growth promoting rhizobacteria (PGPRs) and Sinorhizobium fredii on biological nitrogen fixation, nodulation and growth of *Glycine max* cv. Osumi. *Plant Soil* 267, 143–153. doi: 10.1007/s11104-005-4885-5

Guo, W., Zhao, J., Li, X., Qin, L., Yan, X., and Liao, H. (2011). A soybean β-expansin gene GmEXPB2 intrinsically involved in root system architecture responses to abiotic stresses. *Plant J.* 66, 541–552. doi: 10.1111/j.1365-313X.2011.04511.x

Halwani, M., Reckling, M., Schuler, J., Bloch, R., and Bachinger, J. (2019). Soybean in no-till cover-crop systems. *Agronomy* 9:883. doi: 10.3390/agronomy9120883

Howieson, J., Yates, R., Bala, A., and Hungria, M. (2016). "Collecting nodules for isolation of rhizobia," in *The Working With Rhizobia*. eds. J. G. Howieson and M. J. Dilworth (Canberra: Australian Centre for International Agricultural Research), 25–37.

Hufnagel, J., Reckling, M., and Ewert, F. (2020). Diverse approaches to crop diversification in agricultural research. A review. *Agron. Sustain. Dev.* 40:14. doi: 10.1007/s13593-020-00617-4

Iturralde, E. T., Covelli, J. M., Alvarez, F., Pérez-Giménez, J., Arrese-Igor, C., and Lodeiro, A. R. (2019). Soybean-nodulating strains with low intrinsic competitiveness for nodulation, good symbiotic performance, and stress-tolerance isolated from soybean-cropped soils in Argentina. *Front. Microbiol.* 10:1061. doi: 10.3389/fmicb.2019.01061

Kadiata, B. D., Schubert, S., and Yan, F. (2012). Assessment of different inoculants of *Bradyrhizobium japonicum* on nodulation, potential N2 fixation and yield performance of soybean (*Glycine max* L.). *J. Anim. Plant Sci.* 13, 1704–1713.

Koutroubas, S., Papakosta, D., and Gagianas, A. (1998). The importance of early dry matter and nitrogen accumulation in soybean yield. *Eur. J. Agron.* 9, 1–10. doi: 10.1016/S1161-0301(97)00067-1

Kühling, I., Hüsing, B., Bome, N., and Trautz, D. (2017). Effekte der Saatgutimpfung im ökologischen Soja-Anbau unter den Umweltbedingungen nördlicher Breiten. A public lecture at: 14. Wissenschaftstagung Ökologischer Landbau, Campus Weihenstephan, Freising-Weihenstephan. March 07–10, 2017.

Kühling, I., Hüsing, B., Bome, N., and Trautz, D. (2018). Soybeans in high latitudes: effects of Bradyrhizobium inoculation in Northwest Germany and southern West Siberia. *Org. Agric.* 8, 159–171. doi: 10.1007/s13165-017-0181-y

Lamichhane, J. R., Constantin, J., Schoving, C., Maury, P., Debaeke, P., Aubertot, J.-N., et al. (2020). Analysis of soybean germination, emergence, and prediction of a possible northward establishment of the crop under climate change. *Eur. J. Agron.* 113:125972. doi: 10.1016/j.eja.2019.125972

Larson, K. (2013). Evaluation of soybean inoculant products and techniques to address soybean nodulation problems in Kansas. master's thesis. Kansas: Kansas State University

Li, X., Zeng, R., and Liao, H. (2016). Improving crop nutrient efficiency through root architecture modifications. *J. Integr. Plant Biol.* 58, 193–202. doi: 10.1111/jipb.12434

Madrzak, C. J., Golinska, B., Kroliczak, J., Pudelko, K., Lazewska, D., Lampka, B., et al. (1995). Diversity among field populations of *Bradyrhizobium japonicum* in

Poland. *Appl. Environ. Microbiol.* 61, 1194–1200. doi: 10.1128/AEM.61.4.1194-1200.1995

Martyniuk, S., Kozieł, M., and Gałązka, A. (2016). Survival of rhizobia on seeds, nodulation and growth of soybean as influenced by synthetic and natural seed-applied fungicides. *Polish J. Agron.* 27, 96–99.

Mason, S., Galusha, T., and Kmail, Z. (2016). Soybean yield and nodulation response to crop history and inoculation. *Agron. J.* 108, 309–312. doi: 10.2134/agronj2015.0245

Melchiorre, M., De Luca, M. J., Anta, G. G., Suarez, P., Lopez, C., Lascano, R., et al. (2011). Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants. *Biol. Fertil. Soils* 47, 81–89. doi: 10.1007/s00374-010-0503-7

Mendes, I. C., Hungria, M., and Vargas, M. A. (2004). Establishment of *Bradyrhizobium japonicum* and *B. elkanii* strains in a Brazilian Cerrado oxisol. *Biol. Fertil. Soils* 40, 28–35. doi: 10.1007/s00374-004-0739-1

Narożna, D., Pudełko, K., Króliczak, J., Golińska, B., Sugawara, M., Mądrzak, C. J., et al. (2015). Survival and competitiveness of *Bradyrhizobium japonicum* strains 20 years after introduction into field locations in Poland. *Appl. Environ. Microbiol.* 81, 5552–5559. doi: 10.1128/AEM.01399-15

Nazir, R., Semenov, A. V., Sarigul, N., and van Elsas, J. D. (2013). Bacterial community establishment in native and non-native soils and the effect of fungal colonization. *Microbiol. Discov.* 2, 1–10. doi: 10.7243/2052-6180-1-8

Obaton, M., Bouniols, A., Piva, G., and Vadez, V. (2002). Are *Bradyrhizobium japonicum* stable during a long stay in soil? *Plant Soil* 245, 315–326. doi: 10.1023/A:1020447928911

Ohyama, T., Fujikake, H., Yashima, H., Tanabata, S., Ishikawa, S., Sato, T., et al. (2011). "Effect of nitrate on nodulation and nitrogen fixation of soybean," in *The Soybean: Physiology and Biochemistry*. ed. H. El-Shemy (Croatia: InTech).

Ott, T., van Dongen, J. T., Gu, C., Krusell, L., Desbrosses, G., Vigeolas, H., et al. (2005). Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Curr. Biol.* 15, 531–535. doi: 10.1016/j.cub.2005.01.042

Peoples, M. B., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., et al. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* 48, 1–17. doi: 10.1007/BF03179980

Perrineau, M. M., Le Roux, C., Galiana, A., Faye, A., Duponnois, R., Goh, D., et al. (2014). Differing courses of genetic evolution of Bradyrhizobium inoculants as revealed by long-term molecular tracing in *Acacia mangium* plantations. *Appl. Environ. Microbiol.* 80, 5709–5716. doi: 10.1128/AEM.02007-14

Piccinetti, C., Arias, N., Ventimiglia, L., Díaz-Zorita, M., Murua, L., Sanchez, H., et al. (2013). "Efectos positivos de la inoculación de soja sobre la nodulación, la FBN y en los parámetros de producción del cultivo," in *Microbiología Agrícola: Un Aporte de la Investigación en Argentina. 2nd Edn.* ed. A. S. Albanesi (Tucumán: Magna Publicaciones), 283–297.

Reckling, M., Bergkvist, G., Watson, C. A., Stoddard, F. L., and Bachinger, J. (2020). Re-designing organic grain legume cropping systems using systems agronomy. *Eur. J. Agron.* 112:125951. doi: 10.1016/j.eja.2019.125951

Reich, P. B., Hungate, B. A., and Luo, Y. (2006). Carbon-nitrogen interactions in terrestrial ecosystems in response to rising atmospheric carbon dioxide. *Annu. Rev. Ecol. Evol. Syst.* 37, 611–636. doi: 10.1146/annurev.ecolsys.37.091305.110039

Revellin, C., Pinochet, X., Beauclair, P., and Catroux, G. (1996). Influence of soil properties and soya bean cropping history on the *Bradyrhizobium japonicum* population in some French soils. *Eur. J. Soil Sci.* 47, 505–510. doi: 10.1111/j.1365-2389.1996.tb01850.x

Schoving, C., Stöckle, C. O., Colombet, C., Champolivier, L., Debaeke, P., and Maury, P. (2020). Combining simple Phenotyping and Photothermal algorithm for the prediction of soybean phenology: application to a range of common cultivars grown in Europe. *Front. Plant Sci.* 10:1755. doi: 10.3389/fpls.2019.01755

Shurtleff, W., and Aoyagi, A. (2016). *History of Soybeans and Soyfoods in Germany (1712–2016): Extensively Annotated Bibliography and Sourcebook*. Lafayette: Soyinfo Center.

Sinclair, T. R., and Nogueira, M. A. (2018). The next step to increase grain legume N_2 fixation activity: selection of host-plant genotype. *J. Exp. Bot.* 69, 3523–3530. doi: 10.1093/jxb/ery115

Singh, S., and Varma, A. (2017). "Structure, function, and estimation of leghemoglobin," in *Rhizobium Biology and Biotechnology.* Cham: Springer, 309–330.

Sogut, T. (2006). Rhizobium inoculation improves yield and nitrogen accumulation in soybean (*Glycine max*) cultivars better than fertiliser. *N. Z. J. Crop. Hortic. Sci.* 34, 115–120. doi: 10.1080/01140671.2006.9514395

Takahashi, M., Kokubun, M., and Akao, S. (1995). Characterization of nitrogen assimilation in a supernodulating soybean mutant En6500. *Soil Sci. Plant Nutr.* 41, 567–575. doi: 10.1080/00380768.1995.10419618

Thies, J. E., Singleton, P. W., and Bohlool, B. B. (1991). Modeling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil. *Appl. Environ. Microbiol.* 57, 29–37. doi: 10.1128/AEM.57.1.29-37.1991

Triplett, E. W., and Sadowsky, M. J. (1992). Genetics of competition for nodulation of legumes. *Annu. Rev. Microbiol.* 46, 399–422. doi: 10.1146/annurev.mi.46.100192.002151

Vargas, M. A., Mendes, I. C., Suhet, A. R., and Peres, J. R. R. (1994). Inoculation of soybean in Cerrado soils with established populations of *Bradyrhizobium japonicum*. *Rev. Microbiol.* 25, 245–250.

Watson, C., Reckling, M., Preissel, S., Bachinger, J., Bergkvist, G., Kuhlman, T., et al. (2017). Grain legume production and use in European agricultural systems. *Adv. Agron.* 144, 235–303. doi: 10.1016/bs.agron.2017.03.003

Wilson, D., and Reisenauer, H. (1963). Determination of leghemoglobin in legume nodules. *Anal. Biochem.* 6, 27–30. doi: 10.1016/0003-2697(63)90004-6

Yan, J., Han, X. Z., Ji, Z. J., Li, Y., Wang, E. T., Xie, Z. H., et al. (2014). Abundance and diversity of soybean-nodulating rhizobia in black soil are impacted by land use and crop management. *Appl. Environ. Microbiol.* 80, 5394–5402. doi: 10.1128/AEM.01135-14

Yang, Y., Zhao, Q., Li, X., Ai, W., Liu, D., Qi, W., et al. (2017). Characterization of genetic basis on synergistic interactions between root architecture and biological nitrogen fixation in soybean. *Front. Plant Sci.* 8:1466. doi: 10.3389/fpls.2017.01466

Yuan, K., Reckling, M., Ramirez, M. D. A., Djedidi, S., Fukuhara, I., Ohyama, T., et al. (2020). Characterization of rhizobia for the improvement of soybean cultivation at cold conditions in Central Europe. *Microbes Environ.* 35, 1–13. doi: 10.1264/jsme2.ME19124

Zhang, Y. M., Li, Y., Chen, W. F., Wang, E. T., Tian, C. F., Li, Q. Q., et al. (2011). Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China plain. *Appl. Environ. Microbiol.* 77, 6331–6342. doi: 10.1128/AEM.00542-11

Zhang, H., Prithiviraj, B., Charles, T., Driscoll, B., and Smith, D. (2003). Low temperature tolerant *Bradyrhizobium japonicum* strains allowing improved nodulation and nitrogen fixation of soybean in a short season (cool spring) area. *Eur. J. Agron.* 19, 205–213. doi: 10.1016/S1161-0301(02)00038-2

Zimmer, S., Messmer, M., Haase, T., Piepho, H.-P., Mindermann, A., Schulz, H., et al. (2016). Effects of soybean variety and Bradyrhizobium strains on yield, protein content and biological nitrogen fixation under cool growing conditions in Germany. *Eur. J. Agron.* 72, 38–46. doi: 10.1016/j.eja.2015.09.008

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.

Copyright © 2021 Halwani, Reckling, Egamberdieva, Omari, Bellingrath-Kimura, Bachinger and Bloch. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 June 2021
doi: 10.3389/fpls.2021.679463

Control of the Rhizobia Nitrogen-Fixing Symbiosis by Common Bean MADS-Domain/AGL Transcription Factors

*Litzy Ayra[1], María del Rocio Reyero-Saavedra[2], Mariel C. Isidra-Arellano[2], Luis Lozano[3], Mario Ramírez[1], Alfonso Leija[1], Sara-Isabel Fuentes[1], Lourdes Girard[4], Oswaldo Valdés-López[2] and Georgina Hernández[1]**

[1] Programa de Genómica Funcional de Eukaryotes, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Mexico, [2] Laboratorio de Genómica Funcional de Leguminosas, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla de Baz, Mexico, [3] Unidad de Análisis Bioinformáticos, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Mexico, [4] Programa de Biología de Sistemas y Biología Sintética, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

OPEN ACCESS

Edited by:
*Juan Imperial,
Consejo Superior de Investigaciones Científicas (CSIC), Spain*

Reviewed by:
*Elena A. Dolgikh,
All-Russian Research Institute of Agricultural Microbiology of the Russian Academy of Agricultural Sciences, Russia
Jesus Montiel,
Aarhus University, Denmark*

***Correspondence:**
*Georgina Hernández
gina@ccg.unam.mx*

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

Received: *11 March 2021*
Accepted: *10 May 2021*
Published: *07 June 2021*

Citation:
Ayra L, Reyero-Saavedra MdR, Isidra-Arellano MC, Lozano L, Ramírez M, Leija A, Fuentes S-I, Girard L, Valdés-López O and Hernández G (2021) Control of the Rhizobia Nitrogen-Fixing Symbiosis by Common Bean MADS-Domain/AGL Transcription Factors. Front. Plant Sci. 12:679463. doi: 10.3389/fpls.2021.679463

Plants MADS-domain/AGL proteins constitute a large transcription factor (TF) family that controls the development of almost every plant organ. We performed a phylogeny of (*ca.* 500) MADS-domain proteins from Arabidopsis and four legume species. We identified clades with Arabidopsis MADS-domain proteins known to participate in root development that grouped legume MADS-proteins with similar high expression in roots and nodules. In this work, we analyzed the role of AGL transcription factors in the common bean (*Phaseolus vulgaris*) – *Rhizobium etli* N-fixing symbiosis. Sixteen *P. vulgaris AGL* genes (*PvAGL*), out of 93 family members, are expressed – at different levels – in roots and nodules. From there, we selected the *PvAGL* gene denominated *PvFUL*-like for overexpression or silencing in composite plants, with transgenic roots and nodules, that were used for phenotypic analysis upon inoculation with *Rhizobium etli*. Because of sequence identity in the DNA sequence used for RNAi-*FUL*-like construct, roots, and nodules expressing this construct -referred to as RNAi_*AGL*- showed lower expression of other five *PvAGL* genes highly expressed in roots/nodules. Contrasting with *PvFUL*-like overexpressing plants, rhizobia-inoculated plants expressing the RNAi_*AGL* silencing construct presented affection in the generation and growth of transgenic roots from composite plants, both under non-inoculated or rhizobia-inoculated condition. Furthermore, the rhizobia-inoculated plants showed decreased rhizobial infection concomitant with the lower expression level of early symbiotic genes and increased number of small, ineffective nodules that indicate an alteration in the autoregulation of the nodulation symbiotic process. We propose that the positive effects of PvAGL TF in the rhizobia symbiotic processes result from its potential interplay with NIN, the master symbiotic TF regulator, that showed a CArG-box consensus DNA sequence recognized for DNA binding of AGL TF and presented an increased

or decreased expression level in roots from non-inoculated plants transformed with OE_*FUL* or RNAi_*AGL* construct, respectively. Our work contributes to defining novel transcriptional regulators for the common bean – rhizobia N-fixing symbiosis, a relevant process for sustainable agriculture.

Keywords: transcription factors, MADS, AGL, common bean, rhizobia, symbiotic nitrogen fixation, nodule

INTRODUCTION

Transcription factors (TFs) are master control proteins in all living cells, regulating gene expression in response to different stimuli. These exhibit one or more sequence-specific DNA-binding domains that bind to the promoter and/or enhancer regions of multiple target genes to activate or repress transcription. In this way, TF regulates essential biological processes such as development, growth, metabolism, cell cycle progression, and responses to the environment (Czechowski et al., 2004; Libault et al., 2009). An average of 5.7% plant genes code for TF has been distributed among 62 gene families (Libault et al., 2009).

The MADS-domain proteins constitute a eukaryotic family of TFs involved in various relevant functions. Its name derived from the initials of the four founding members of this family *MINICHROMOSOME MAINTENANCE 1* (*MCM1*) from *Saccharomyces cerevisiae*, which regulates mating type-specific genes, *AGAMOUS* (*AG*) from *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) and *DEFICIENS* (*DEF*) form *Antirrhinum majus*, which act as homeotic factors that control flower development, and *SERUM RESPONSE FACTOR* (*SRF*) from *Homo sapiens* which controls serum inducible and muscle-specific gene expression (Schwarz-Sommer et al., 1990; Yanofsky et al., 1990; Pollock and Treisman, 1991; Mead et al., 2002). MADS TF recognizes a consensus DNA sequence called the CArG-box [CC(A/T)$_6$GG] (Riechmann et al., 1996).

In plants, the MADS-domain TFs family has expanded largely with 109 members in Arabidopsis, compared with just a few in mammals, *Drosophila melanogaster*, *Saccharomyces cerevisiae,* and *Caenorhabditis elegans* (Olson et al., 1995; Parenicova et al., 2003). This family of TFs can be divided into two main lineages, type I and type II, based on their protein structure. The best-studied MADS TFs from Arabidopsis and other plant species belong to the type II lineage. Plants type II MADS proteins have a modular domain structure, referred to as the MIKC structure, that contains the N-terminal located ($\sim$ 60 amino acids) conserved DNA-binding domain (M), followed by moderately conserved intervening (I) and keratin-like (K) domains, and a variable C-terminal domain (C) that, together with K, have roles for protein complex formation (Alvarez-Buylla et al., 2000b; Smaczniak et al., 2012). These MADS proteins include the regulators of floral transition and floral organ identity determination that, after over 20 years of intensive study, led to the establishment of a general model for flower organ identity in higher plants, the so-called ABCDE model in which floral whorl-specific combinations of class A, B, C, D, or E genes specify floral organ identity (Alvarez-Buylla et al., 2000b; Murai, 2013).

Although initially, *MADS*-box genes were relevant for floral organ speciation, more recent studies have revealed that these genes are relevant for the morphogenesis of almost all plant organs. The role of MADS TF in root development has received relatively less attention, but recently it was comprehensively reviewed by Alvarez-Buylla et al. (2019). About 41 Arabidopsis *MADS*-box genes that show high and intermediate expression levels in different root tissues and zones have been identified (Alvarez-Buylla et al., 2019). Arabidopsis research using genetic, molecular, biochemical approaches has contributed to understanding the role of root-expressed *MADS*-box genes, from different clades, in primary or lateral root development. The latter include *XAL1* (*XAANTAL1*), *XAL2* (*XAANTAL2*), *ANR1* (*Arabidopsis NITRATE REGULATED1*), and *AGL21* (*AGAMOUS-Like21*) (Gan et al., 2005; Garay-Arroyo et al., 2013; Garcia-Cruz et al., 2016). In addition, orthologs of Arabidopsis root *MADS*-box genes from different plant species show similar expression patterns and potential functional conservation. These include *MADS*-box genes from rice (*Oryza sativa*), sweet potato (*Ipomoea batatas*), and *Chrysanthemum morifolium* (Ku et al., 2008; Puig et al., 2013; Sun et al., 2018; Alvarez-Buylla et al., 2019). Studies regarding the expression/function of *MADS*-box genes in legume species are yet scant. *XAL-1* and *XAL-2* genes have been identified from soybean (*Glycine max*) and alfalfa (*Medicago sativa*). These show a high level of expression in the root in soybean, but expression data from alfalfa genes are not available (Alvarez-Buylla et al., 2019). Also, high expression in roots and root hairs has been documented for *AGL21* soybean ortholog (Alvarez-Buylla et al., 2019). Previous work from our group identified orthologs of Arabidopsis root *MADS*-box genes from common bean (*Phaseolus vulgaris*) and soybean that are expressed in root tissues (Íñiguez et al., 2015).

Legumes can establish two types of endosymbiotic associations, with arbuscular mycorrhiza fungi and nitrogen-fixing bacteria, collectively called rhizobia (Olah et al., 2005; Oldroyd, 2013). These associations facilitate the plant acquisition of nutrients such as phosphorus and nitrogen (Oldroyd, 2013; Venkateshwaran et al., 2013).

An efficient symbiotic association with rhizobia results in the formation of root nodules where rhizobia are allocated and fix atmospheric nitrogen (N_2) informs that the legume host can assimilate in exchange for a carbon source. Symbiotic nitrogen fixation (SNF) is ecologically and economically important for sustainable agriculture because it reduces synthetic fertilizers and the cost of legume cultivation (Venkateshwaran et al., 2013; Castro-Guerrero et al., 2016). The infection of rhizobia to the root tissue is a complex process that involves communication between rhizobia and legumes through molecular signals, such

as flavonoids released by the plant root to the rhizosphere that is sensed by compatible rhizobia that, in turn, produce the lipochitooligosaccharides, known as nodulation factors (NF). NF perception by the plant triggers a series of molecular responses essential for rhizobial infection and nodule development (Roy et al., 2020). These responses include the rhizobia-induced root hair deformations or curling required to entrap the rhizobia into an infection chamber and initiate the formation of an infection thread -an invasive invagination of the plant cells- and the infection of the cortical root cells (Suzaki et al., 2015). After rhizobia infection, legumes activate intricate signaling pathways that include finely regulated nodulin genes to develop mature nodules with functionally differentiated bacteroids. Research from the last 20 years has identified a suite of *ca*. 200 legume genes relevant for establishing an effective symbiosis with rhizobia (Roy et al., 2020). The NODULE INCEPTION (NIN) TF gene is a master regulator for the rhizobia symbiosis. It plays an indispensable role in the rhizobial infection and nodule organogenesis symbiotic processes (Schauser et al., 1999; Marsh et al., 2007; Liu and Bisseling, 2020). Recently, it was shown that NIN also plays a major role in the transition from infection into functional nodules, the development of symbiosomes, and nodule senescence (Liu et al., 2021).

Because of the high carbon demand, SNF is an energy-demanding process for the legume host; therefore, the number of infections and the number of nodules per plant is tightly regulated by a systemic feedback regulatory mechanism termed autoregulation of nodulation (AON, Reid et al., 2011; Ferguson et al., 2019). Upon infection, rhizobia induce the root production of CLAVATA3/endosperm surrounding region-related (CLE) peptides, encoded by the *RIC1* and *RIC2* genes in soybean and common bean (Reid et al., 2011; Ferguson et al., 2014). These CLE peptides are transported from the root to the shoot, where they are detected by the nodule autoregulation receptor kinase (NARK) to activate the generation of a shoot-derived signal (i.e., miR2111 and cytokinin) (Tsikou and Yan, 2018; Ferguson et al., 2019). The shoot signals are transported to the root to inhibit further nodule development through the participation of the negative regulator TML (Too Much Love), a kelch repeat-containing F-box protein (Takahara et al., 2013). By interplay with other regulators, the NIN TF plays a relevant role in the AON process through the transcriptional activation of the CLE peptides (Soyano et al., 2014; Roy et al., 2020).

The information about legume *MADS*-box genes as regulators of the legume -rhizobia symbiosis is scarce. Three *MADS*-box genes from alfalfa (*Medicago sativa*) from the *AGL17* subfamily with high expression in nodules were cloned (*nmh7*, *nmhC5*, *ngl9*) (Heard et al., 1997; Zucchero et al., 2001). Expression analysis of the alfalfa *NMH7* gene revealed its expression in flowers and nodules and the parenchyma or the root tip and the root elongation zone from seedlings (Páez-Valencia et al., 2008). The soybean *GmNMHC5* gene is highly expressed in roots and nodules, and its overexpression promotes lateral root development and nodulation (Liu et al., 2015). Contrastingly, the *GmNMH7*, *MADS-box* gene, highly expressed in roots and nodules, exerts negative control to nodulation, probably through regulating the content of gibberellin (Ma et al., 2019).

Common bean (*Phaseolus vulgaris*) is the most important crop legume for human consumption and the principal source of non-animal protein for human consumption in the developing world (Broughton et al., 2003). Research from our group focused on the common bean – rhizobia symbiosis has contributed to identifying microRNAs/TF target gene nodes that play a relevant role in the regulation of the SNF, such as miR172c/AP2-1 and miR319d/TCP10 (Nova-Franco et al., 2015; Martin-Rodriguez et al., 2018). We have also identified common bean and soybean *MADS*-box genes expressed in roots, similar to their Arabidopsis orthologs (Íñiguez et al., 2015). This work aimed to analyze the role of *MADS*-box genes from *P. vulgaris*, hereafter denominated *PvAGL* genes, in the common bean – rhizobia symbiosis that, to our knowledge, have not been studied in this legume. We performed a phylogenetic analysis of *MADS*-box genes for Arabidopsis and four legume species that identified clades, including root-expressed MADS-box genes from Arabidopsis and legumes. Expression analysis revealed that *PvAGL* genes are highly expressed in both rhizobia-inoculated roots and nodules. The functional analysis of composite *Rhizobium etli*-inoculated common bean plants with a modulated expression of *PvAGL* genes revealed the participation of TF from this gene family in rhizobial infection, expression of early symbiotic genes, root architecture, nodulation, nitrogenase activity, and the AON symbiotic process. Our work contributes to the knowledge of the participation of members from the *MADS*-box/*AGL* TF family in the legume – rhizobia SNF, a relevant process for sustainable agriculture.

MATERIALS AND METHODS

Phylogenetic Analysis

Gene sequences and the RNA-seq gene expression data for the *MADS*-box TFs genes for each plant species were retrieved from the following databases. For Arabidopsis, The Arabidopsis Information Resource TAIR (https://www.arabidopsis.org); for *P. vulgaris* the Gene Expression Atlas (GEA), *Pv* GEA (http://plantgrn.noble.org/PvGEA/) (O'Rourke et al., 2014); for *G. max* the ePlant BAR page (http://bar.utoronto.ca/eplant_soybean/) (Waese et al., 2017), for *Medicago truncatula* the MtGEA (https://mtgea.noble.org/v3/) and *Lotus japonicus* the Lotus Base (https://lotus.au.dk/).

The translated protein sequences from the *MADS*-box genes were analyzed in Interpro (http://www.ebi.ac.uk/interpro/) to confirm the presence of the characteristic domain IPR002100 from this gene family. Next, the protein sequences were analyzed with blast v2.6.0 and aligned with clustalw v2.1 (Thompson et al., 1994) to confirm that proteins share a stereotypical MIKC structure with four domains characteristic of type II *MADS*-box genes of the plant (Alvarez-Buylla et al., 2000b; Smaczniak et al., 2012). Finally, the proteins with MIKC structure were re-analyzed by multiple alignments to verify the integrity of the MADS domain (∼60 amino acidic region). Some protein sequences from each species were excluded for phylogenetic analysis because these did not have a complete MADS domain sequence. The whole set of 511 MADS protein sequences from

Arabidopsis and four legume species used for phylogenetic analysis are listed in the **Supplementary Table 1**.

The best evolutionary model (JTT+G) was determined with Prottest3 v3.4.2, and a maximum likelihood phylogeny was made with PhyML v3.0 using approximate likelihood-ratio test SH-like as a statistical test for branch support. Finally, the Interactive Tree of Life (iTOL5) (Letunic and Bork, 2019) was used for displaying the phylogenetic tree.

Plant Material and Growth Conditions

The common bean (*P. vulgaris*) Mesoamerican cv BAT93 was used in this work (Vlasova et al., 2016). Seeds were surface sterilized in 10% (V/V) commercial sodium hypochlorite for 10 min and finally rinsed three to four times in sterile distilled water. Then seeds were germinated on sterile moistened filter paper at 30°C in darkness for 2 days. Germinated seedlings were planted in pots with wet sterile vermiculite. For SNF conditions, plantlets were inoculated with 1 ml saturated liquid culture of the *Rhizobium etli* CE3 strain per plant. Plants were grown in growth chambers under controlled environmental conditions (25–28°C, 16 h photoperiod) and were watered every 3 days with N-free B&D nutrient solution (Broughton and Dilworth, 1971). For non-inoculated conditions, a full nutrient B&D solution (5 mM N-content) was used to water the plants. Common bean composite plants with transgenic roots were generated as described below and grown in sterile pots with perlite to prevent harming the transgenic root/nodules when taking these out of the pot at harvest time. The growth conditions of composite plants were similar to those described for wild-type plants.

RNA Isolation and Quantitative RT-PCR Analysis

Total RNA was isolated from frozen tissues collected directly into liquid nitrogen and stored at −80°C. The wet weight used for RNA isolation from each tissue was: 100 mg for nodules detached from roots, 250 mg for roots, and 200 mg for leaves. Trizol™ Reagent (Thermo Fischer Scientific, Inc., Waltham, MA, USA) was used, following the manufacturer's instructions. Total RNA was quantified using the NanoDrop spectrophotometer (Thermo Fischer Scientific, Inc.). For quantification of transcripts levels, total RNA (2 μg) was treated with DNaseI RNase-free (Thermo Fischer Scientific, Inc., Waltham, MA, USA) to remove genomic DNA. First, strand cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The resulting cDNAs were then diluted and used to perform qRT-PCR assays using SYBR Green PCR Master Mix (Thermo Fischer Scientific, Inc.), following the manufacturer's instructions. The sequences of oligonucleotide primers used for qRT-PCR of each gene are provided (**Supplementary Table 2**). Assays were run in 96-well plates using the 7300 Real-Time PCR System and 7300 System Software (Applied Biosystems, Foster City, CA, USA) with settings of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 57°C for 60 s. Relative expression for each sample was calculated using the "comparative Ct method" and normalized with the geometrical mean of three housekeeping genes (HSP, MDH, and UBQ9) (Vandesompele et al., 2002). Student's t-test was performed to evaluate the significance of the differential expression using the mean values from three biological replicates for each condition, using the GraphPad Prism v8.0 for Windows (GraphPad Software, San Diego, CA, USA).

Plasmid Construction, Plant Transformation, and Generation of Composite Plants

For overexpression of the Phvul.008G027800.1 *P. vulgaris MADS*-box gene, hereafter denominated *PvFUL*-like (Phvul.008G027800) based on its ortholog Arabidospis *FUL* (*FRUITFULL*), was PCR-amplified using as template cDNA from common bean roots and the specific primers Up_*FUL* 5′-CCCT CGAGCTTTTCCACAATTGCC-3′ and Lw_*FUL* 5′-GCCC GGATCCTAACTAGTAAGTAG-3′. The purified PCR product (767 bp) was cloned into the pTOPO2.1 intermediate vector and confirmed by sequencing. To construct the OE_*FUL* plasmid, the *PvFUL*-like DNA region was excised using XhoI/BamHI sites and cloned into the pTDTO plasmid (Aparicio-Fabre et al., 2013). This expression plasmid contains the 35S cauliflower mosaic virus (35SCaMV) promoter and the tdTomato (red fluorescent protein) gene as a visible reporter gene. For silencing *PvAGL* genes, by RNAi strategy, specific primers flanking a DNA region coding for the MADS-domain from the *PvFUL*-like gene (Up_RNAi 5′-TCAGCTCAAGCGGATCG-3′ and Lw_RNAi 5′-CACCACGTTCCAAGACATCTT-3′) were used to amplify a 194 bp fragment using as template cDNA from common bean roots. This DNA fragment, that share homology among *PvAGL* genes highly expressed in roots/nodules, was cloned by the Gateway system into the intermediate vector pENTR and finally in the pTDT-DC-RNAi plasmid (Valdes-Lopez et al., 2008), which also contains the 35S cauliflower mosaic virus (35SCaMV) promoter and the tdT (tandem double Tomato, red fluorescent protein) gene as a visible reporter gene.

The empty vectors pTDTO and pTDT-DC-RNAi, hereafter denominated EV, and the resulting plasmids OE_*FUL* and RNAi_*AGL* were introduced by electroporation into *Agrobacterium rhizogenes* K599, which was then used for plant transformation as described (Estrada-Navarrete et al., 2007) with minor modifications (Aparicio-Fabre et al., 2013). In addition, the presence of red fluorescence from the tdTomato reporter gene was routinely checked in the putative transgenic roots/nodules using a fluorescence stereomicroscope.

Identification of Putative *Cis*-Regulatory Elements in *P. vulgaris* Genes

DNA sequences from the region upstream of the initiation codon were retrieved from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html). The length of the intergenic 5′-upstream sequences analyzed was 20 kbp or shorter depending on the position of the next contiguous gene. For *PvFUL*-like gene 7.3-kb sequence was analyzed using the New PLACE tool (A Database of Plant *Cis*-acting Regulatory Elements; https://www.dna.affrc.go.jp/PLACE/?action=newplace) (Higo et al., 1999) to identify putative *cis*-regulatory elements related to nodulation or root development; these are shown in **Supplementary Figure 2**.

Upstream sequences of *P. vulgaris* early symbiotic genes were analyzed using SnapGene Viewer (GSL Biotech LLC, Chicago, IL, USA; available at snapgene.com) to identify the consensus CArG-box sequence that is recognized by AGL TF (Riechmann et al., 1996).

Root Hair Deformation Analysis

Common bean composite plants, expressing the empty vectors (EV) or OE_*FUL*/RNAi_*AGL* plasmids and growing in 25 cm × 25 cm Petri dishes containing nitrogen-free Fahräeus medium (Catoira et al., 2000), were inoculated with 1 mL of saturated *Rhizobium etli* CE3 culture ($OD_{600} = 1$). Five days after inoculation, tdTomato-positive transgenic roots were collected. The root-susceptible zone for rhizobial infection covered around 2 cm was stained with methylene blue for 1 h to maximize contrast and washed three times with double-distilled water. The quantification of the number of rhizobia-induced root hair deformations was determined from 1 cm root segments from the susceptible zone. Deformation events were observed with a bright-field microscope equipped with an 18 MP Digital Camera with Aptina CMOS Sensor (Cientifica Vela Quin, Iztapalapa, Mexico city, Mexico). A total of 20 independent biological replicates were generated, each one including 10 plants.

Phenotypic Analysis and Nodule Histology

For root length, root area, and nodule perimeter evaluations, pictures from root or nodule tissues of composite plants (18 or 28 dpi) were processed with SmartRoot software (Lobet et al., 2011).

Determination of the Nase-enzyme activity was determined in nodulated roots (28 dpi) by acetylene reduction assay (ARA) described by Hardy et al. (1968). Specific activity is expressed as nmol ethylene h^{-1}/plant.

For histological analysis, nodules (28 dpi) were collected from composite plants transformed with EV or OE_*FUL*/RNAi_*AGL* plasmids and were treated with the procedure described by Reyero-Saavedra et al. (2017). In addition, representative images from safranine-stained sections (25 μm) were taken with a NIKON camera coupled to a bright-field microscope.

Statistical Analyses

The graphs and statistical analysis were made with the R software 3.0.1 or the GraphPad Prism v8.0. The specific statistical tests performed are indicated in the legend of the corresponding figures.

RESULTS

Phylogeny of the MADS-Domain TF From Arabidopsis and Legume Plants

In plants, the *MADS*-box is a large gene family of TF. The number of family members varies in different plant species. In this work, we identified *MADS*-box genes for four legume species. For *P. vulgaris* (common bean), we identified 93 *AGL* genes, for *G. max* (soybean) 183, for *M. truncatula* (Medicago) 143, and *L. japonicus* (Lotus) 79. For the phylogenetic analysis of MADS TF from Arabidopsis and four legume species, those MADS protein sequences that did not show a complete MADS domain sequence were excluded. Thus, a 511 MADS-domain protein sequences set (**Supplementary Table 1**) was used for the maximum likelihood phylogenetic tree shown in **Figure 1**.

The majority of clades from the phylogenetic tree (**Figure 1**) group MADS from Arabidopsis and the four legumes species analyzed, suggesting that MADS TF from the same clade share their tissue expression and their participation in regulatory networks from a certain vegetative tissue. In **Figure 1**, we indicated seven Arabidopsis root-expressed *MADS*-box genes from five different clades involved in regulating root development (Alvarez-Buylla et al., 2019). We assessed if the legume *MADS*-box genes grouped in these monophyletic clades were also expressed in root and if, additionally, these were expressed in root-nodules elicited by rhizobia. The clade from **Figure 2A** includes *ANR1 (ARABIDOPSIS NITRATE REGULATED 1*; (Gan et al., 2005) and six legume *MADS*-box genes with high expression in root tissues, and five of these also showed expression in nodules. The Arabidopsis *XAL1* (Garcia-Cruz et al., 2016) clade (**Figure 2B**) groups five legume genes that showed enhanced expression in roots, and three of these also showed expression in nodules. The clade depicted in **Figure 2C** includes *XAL2* and *AGL19* (Alvarez-Buylla et al., 2000a, 2019; Garay-Arroyo et al., 2013) as well as five legume *MADS*-box genes that were expressed in root and nodules. **Figure 2D** shows the clade with *AGL17/21* genes (Burgeff et al., 2002; Alvarez-Buylla et al., 2019) grouped with five legume genes that showed expression in roots, and four of these were also expressed in nodules. The clade from **Figure 2E** includes the *AGL16* (Nawy et al., 2005) and five legume genes whose expression in roots or nodules was >50%, compared with the expression level from other organs.

Expression Analysis of *PvAGL* Genes in Root/Nodule

Previous work from our group identified the *MADS*-box genes from common bean and soybean, an ortholog of root-expressed Arabidopsis *MADS*-box genes that are expressed in roots and nodules (Íñiguez et al., 2015). Our previous report (Íñiguez et al., 2015) and data presented in **Figures 1**, **2** prompted us to propose that *P. vulgaris AGL* genes highly expressed in roots/nodules participate as regulators of the common bean - rhizobia symbiosis. In this work, we updated the analysis of *PvAGL* genes based on the recent annotation of the *P. vulgaris* genome sequence (www.phytozome.net/commonbean.php) and the identification of the total number of *PvAGL* genes from this work (**Figure 1**). RNA-seq data analysis revealed 16 *PvAGL* genes, out of 93 gene family members, with expression in root and nodule tissues compared with their expression in the stem, leaf, pod, and seed tissues (**Supplementary Figure 1**). To assess the reliability of the RNA-seq data, we performed qRT-PCR gene expression analysis from root, nodules, and leaves tissues and confirmed that these *PvAGL* genes are expressed in root/nodules, albeit at different levels (**Supplementary Figure 1**).

We pursued the expression analysis of six *PvAGL* genes that showed the highest expression in root/nodule tissues (**Supplementary Figure 1**) by assaying their transcript levels

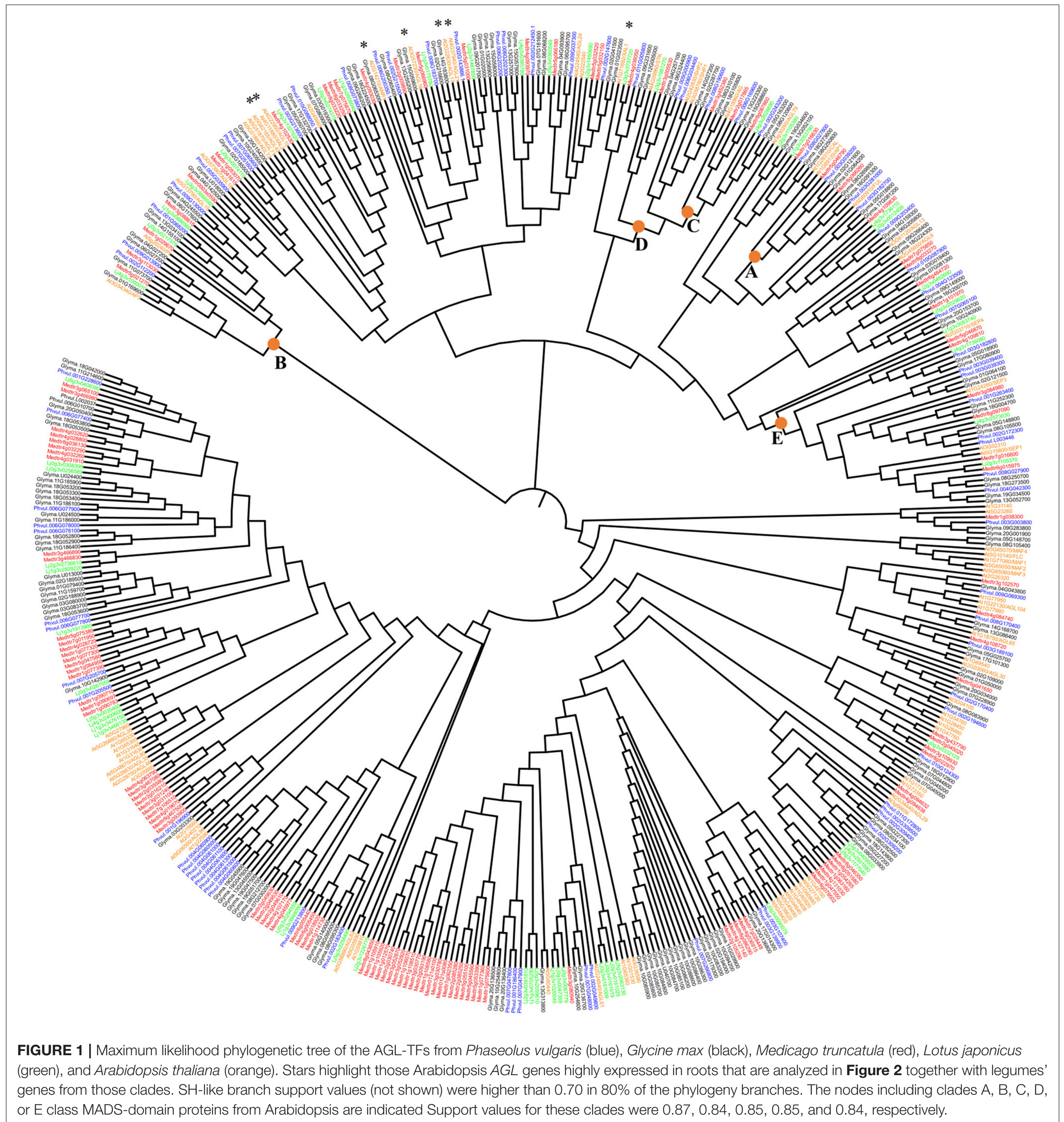


FIGURE 1 | Maximum likelihood phylogenetic tree of the AGL-TFs from *Phaseolus vulgaris* (blue), *Glycine max* (black), *Medicago truncatula* (red), *Lotus japonicus* (green), and *Arabidopsis thaliana* (orange). Stars highlight those Arabidopsis *AGL* genes highly expressed in roots that are analyzed in **Figure 2** together with legumes' genes from those clades. SH-like branch support values (not shown) were higher than 0.70 in 80% of the phylogeny branches. The nodes including clades A, B, C, D, or E class MADS-domain proteins from Arabidopsis are indicated Support values for these clades were 0.87, 0.84, 0.85, 0.85, and 0.84, respectively.

from these tissues of *R. elti*-inoculated plants as well as from root of non-inoculated plants at different developmental stages. According to our phylogenetic analysis, the name we assigned to each PvAGL gene corresponds to its Arabidopsis ortholog (**Figure 1**). **Figure 3** shows expression level values for *PvAGL16*-like, *PvSVP*-like (*SHORT VEGETATIVE PHASE*), *PvXAL1*-like, *PvSOC1*-like *(SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1)*, *PvAGL24*-like, and *PvFUL*-like (*FRUITFULL*). The six genes analyzed showed significantly lower expression in young roots compared with increased levels in roots from later developmental stages, except for *PvXAL1*-like that showed high expression in 12-h roots (**Figure 3**). These genes were also expressed in immature (10–15 dpi), mature (22 dpi), and senescent (35 dpi) nodules, albeit at a lower level than

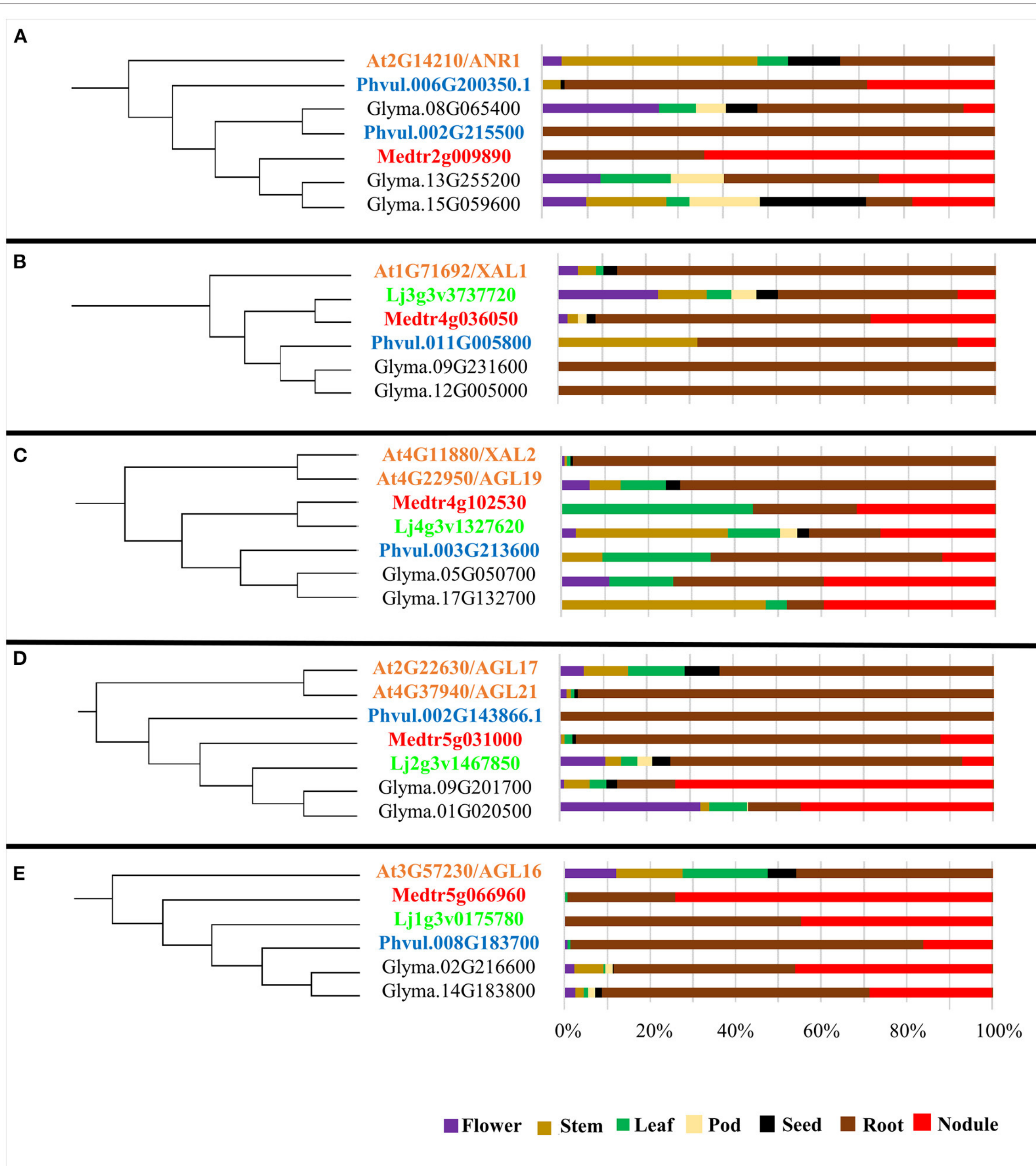


FIGURE 2 | Expression profile by tissue of *AGL* genes from different plant species grouped in clades including Arabidopsis *AGL*s highly expressed in roots. **(A–E)** The left side of each panel shows one selected clade (highlighted in **Figure 1**); the color code used for genes from each species is the same as in **Figure 1**. The right side of each panel shows the % expression level in each tissue (flower, purple; stem, ocher; leaf, green; pod, beige; seed, black; root, brown and nodule, red) of the corresponding *AGL* gene. As indicated in the Material and methods section, expression level values were extracted from the Gene Expression Atlas for each species.

in non-inoculated or inoculated roots, except for *PvFUL*-like (**Figure 3**).

Interestingly, the transcript level of the *PvFUL*-like gene in rhizobia-inoculated plants was similar in roots compared with immature nodules and was higher in mature nodules compared with roots. In contrast, at nodule senescence, the expression in roots was highly increased (**Figure 3**). Furthermore, the *cis*-elements *in silico* analysis of *PvFUL*-like gene promoter sequence (**Supplementary Figure 2**) revealed the presence of the organ-specific elements OSE1ROOTNODULE (Vieweg et al., 2004) and OSE2ROOTNODULE (Fehlberg et al., 2005) with characteristic consensus sequences motifs of the promoters activated in infected cells of root nodules. These were repeated 9 and 18 times, respectively, among the analyzed sequence (**Supplementary Figure 2**). In addition, one *cis*-element related

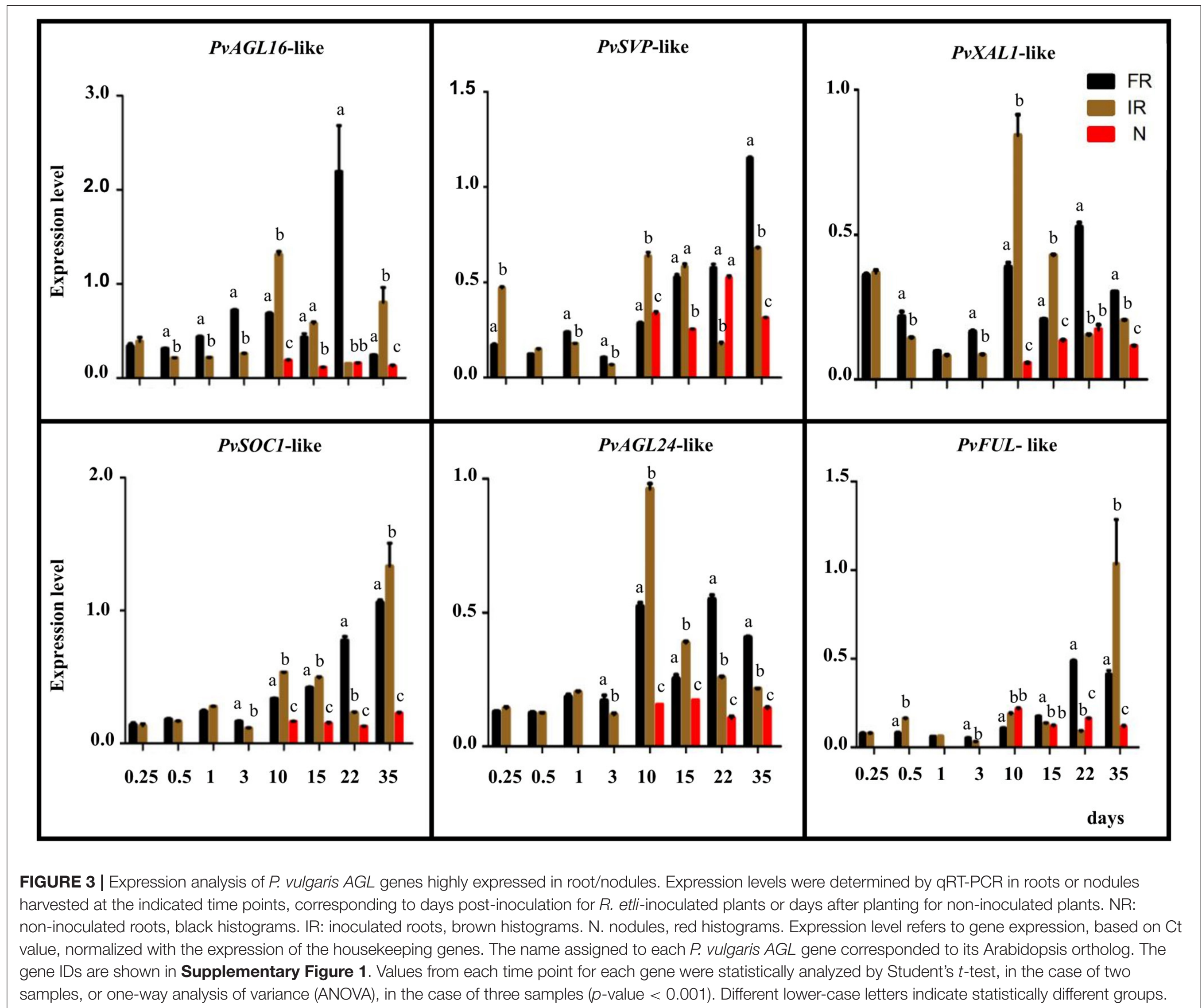


FIGURE 3 | Expression analysis of *P. vulgaris AGL* genes highly expressed in root/nodules. Expression levels were determined by qRT-PCR in roots or nodules harvested at the indicated time points, corresponding to days post-inoculation for *R. etli*-inoculated plants or days after planting for non-inoculated plants. NR: non-inoculated roots, black histograms. IR: inoculated roots, brown histograms. N. nodules, red histograms. Expression level refers to gene expression, based on Ct value, normalized with the expression of the housekeeping genes. The name assigned to each *P. vulgaris AGL* gene corresponded to its Arabidopsis ortholog. The gene IDs are shown in **Supplementary Figure 1**. Values from each time point for each gene were statistically analyzed by Student's *t*-test, in the case of two samples, or one-way analysis of variance (ANOVA), in the case of three samples (p-value < 0.001). Different lower-case letters indicate statistically different groups.

to root development, repeated 39 times, was also identified (**Supplementary Figure 2**).

Effect of PvAGL TF in Rhizobia-Infection of *R. etli*-inoculated Common Bean Plants

To further investigate the regulatory role of root/nodule-expressed *PvAGL* TF genes in the common bean-rhizobia N-fixing symbiosis, we aimed to modulate their expression in common bean composite plants – with transgenic root/nodules and untransformed aerial organs – generated through *A. rhizogenes*-mediated transformation (Estrada-Navarrete et al., 2007).

The *PvFUL*-like gene was selected for the over-expression construct (OE_*FUL*) and the silencing construct (RNAi_*AGL*) driven by the 35SCaMV promotor. These constructs and the empty control vector (EV) contained the tdTomato (red fluorescent protein) reporter gene (Valdes-Lopez et al., 2008; Aparicio-Fabre et al., 2013). As expected, a very high *PvFUL*-like transcript level was expressed in transgenic roots and nodules from composite plants transformed with OE_*FUL* (**Supplementary Figure 3**). The (194 bp) cDNA fragment from the *PvFUL*-like gene used for the RNAi construct (**Supplementary Figure 4**) codes for the *MDS*-box sequence. Multiple sequence alignment analysis of this *PvFUL*-like gene sequence with a corresponding sequence of *PvAGL* genes showed similar sequence identity, ranging from *ca.* 50–70% (**Supplementary Figure 4**). Gene expression level from the six *PvAGL* genes highly expressed in root/nodules (**Figure 3**) was determined from transgenic roots and nodules expressing the EV or the RNAi constructs (**Supplementary Figure 5A**). Compared with control EV tissues, the roots and nodules expressing the silencing construct have a significantly decreased expression level of PvFUL-like gene and *PvXAL1*-like, *PvSVP*-like, *PvSOC1*-like and *PvAGL16*-like, and *PvAGL24*-like. Gene silencing

in nodules was high, showing a very low expression level; generally, the silencing effect in roots was lower than in nodules (**Supplementary Figure 5A**). In agreement with published GEA data (O'Rourke et al., 2014; Íñiguez et al., 2015) and data from **Supplementary Figure 1**, we confirmed very low, negligible, expression of other 10 *PvAGL* genes in EV and RNAi_*AGL* roots/nodules (**Supplementary Figure 5B**). The simultaneous silencing of several *PvAGL* genes in transgenic roots/nodules interpreted as a result of the homology among *MADS*-box cDNA sequence cloned for the RNAi silencing strategy would avoid functional redundancy among these genes. It may result in highly altered phenotypes of RNAi_*AGL* plants (see below).

Common bean plantlets transformed with *A. rhizogenes* bearing the RNAi_*AGL* developed fewer and shorter hairy roots, emerging from the infection site, instead of those transformed with OE_*FUL* or EV that showed numerous and longer hairy roots (**Figure 4A**). In addition, the RNAi-*AGL* composite plants grown under non-inoculated conditions showed reduced root growth (**Figure 4D**).

To assess if the negative effect of *PvAGL* silencing in hairy root formation/growth also affects the common bean symbiosis with rhizobia, we first analyzed rhizobial infection to young transgenic roots inoculated with *R. etli* CE3. No significant differences in root hair development nor root hair density were observed in control (EV) plants than roots with modulation of *PvAGL*-gene expression (**Supplementary Figure 6**). Next, we quantified the root hair deformation upon rhizobia infection (**Figure 5**). Notably, inoculated roots expressing the RNAi_*AGL* construct showed significantly fewer effective deformed or curled roots hairs (**Figures 5A,B**) that form an infection chamber, where rhizobia are trapped would allow the formation of the infection thread (Fournier et al., 2015). In addition, *PvAGL*-silenced roots showed an increased number of non-effective or spatula-like (Reyero-Saavedra et al., 2017) root hair deformations (**Figures 5C,D**). The inoculated roots overexpressing the *PvFUL*-like gene showed a similar number of curled root hair than control EV inoculated roots (**Figure 5B**).

The fact that the silencing of Pv*AGL* genes negatively affected the rhizobia infection process led us to hypothesize that the Pv*AGL* TFs regulate, either directly or indirectly, the expression of key symbiosis-related genes. To test this hypothesis, we evaluated the expression level of *PvENOD93 (EARLY NODULIN 93)*, *PvENOD40 (EARLY NODULIN 40)*, *PvNIN*, *PvNSP2 (NODULATION SIGNALING PATHWAY 2)*, *PvFNSII (FLAVONE SYNTHASE II)*, and *PvFLOT2 (FLOTILLIN 2)* genes from RNAi_*AGL*, OE_*FUL* and EV roots inoculated with *R. etli* for 2 days. The data presented in **Figure 6** showed that, compared with gene expression level in EV control roots, every gene evaluated showed reduced transcript level in RNAi-*AGL* roots but an increased transcript level in OE_*FUL* roots. Thus, there was a correlation between altered root hair deformation and the expression of early symbiotic genes essential for rhizobial infection (**Figures 5**, **6**). Furthermore, to infer if these *P. vulgaris* early symbiotic genes could be direct targets of PvAGL transcriptional regulation, we searched for the consensus DNA sequence recognized by AGL TFs, the so-called CArG-box, in their promoter region. As a result, we identified a CArG-box in the promoter region of *PvNIN* and *PvNSP2* (**Table 1**). We then determined the expression level of these two genes in 2-day-old non-inoculated transgenic roots. Our data indicated that the basal expression level of *PvNSP*2 was similar in the control (EV) and modulated (RNAi_*AGL* or OE_*FUL* roots). However, the gene expression level of *PvNIN* in non-inoculated control EV roots (0.023 ± 0.003) was significantly decreased in RNAi_*AGL* roots (0.001 + 7 – 0.0001) while it significantly increased in OE-*FUL* roots (0.052 ± 0.003). This data supported our hypothesis proposing that PvAGL TF might be implicated in *PvNIN* regulation.

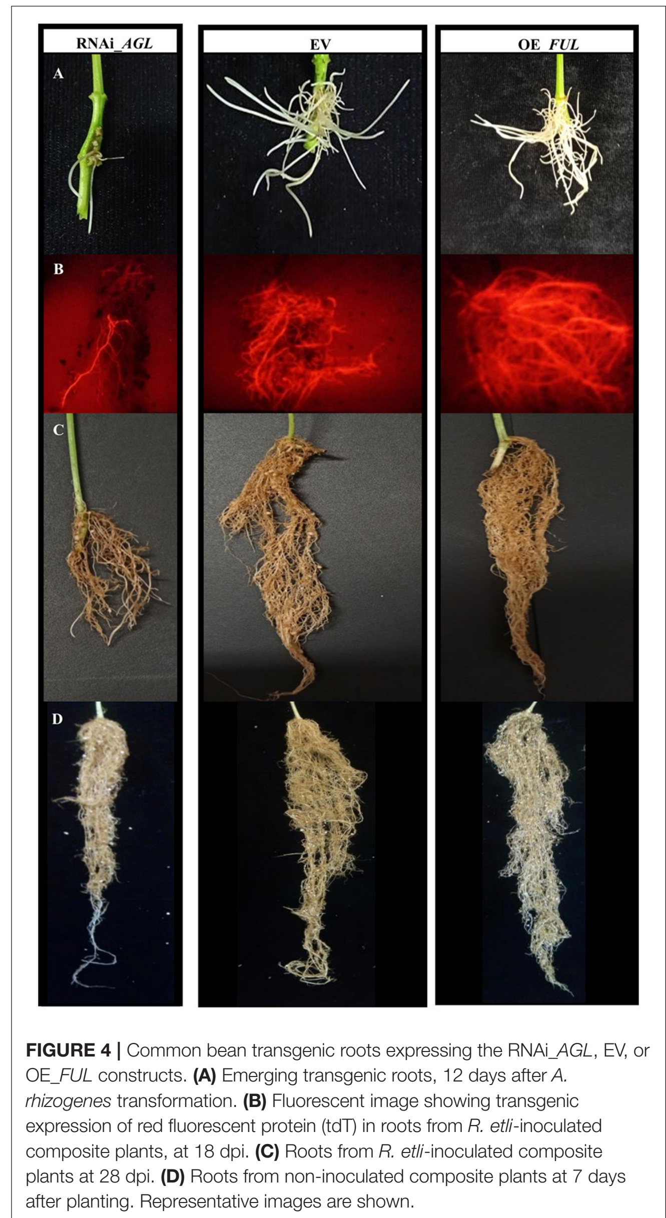


FIGURE 4 | Common bean transgenic roots expressing the RNAi_*AGL*, EV, or OE_*FUL* constructs. **(A)** Emerging transgenic roots, 12 days after *A. rhizogenes* transformation. **(B)** Fluorescent image showing transgenic expression of red fluorescent protein (tdT) in roots from *R. etli*-inoculated composite plants, at 18 dpi. **(C)** Roots from *R. etli*-inoculated composite plants at 28 dpi. **(D)** Roots from non-inoculated composite plants at 7 days after planting. Representative images are shown.

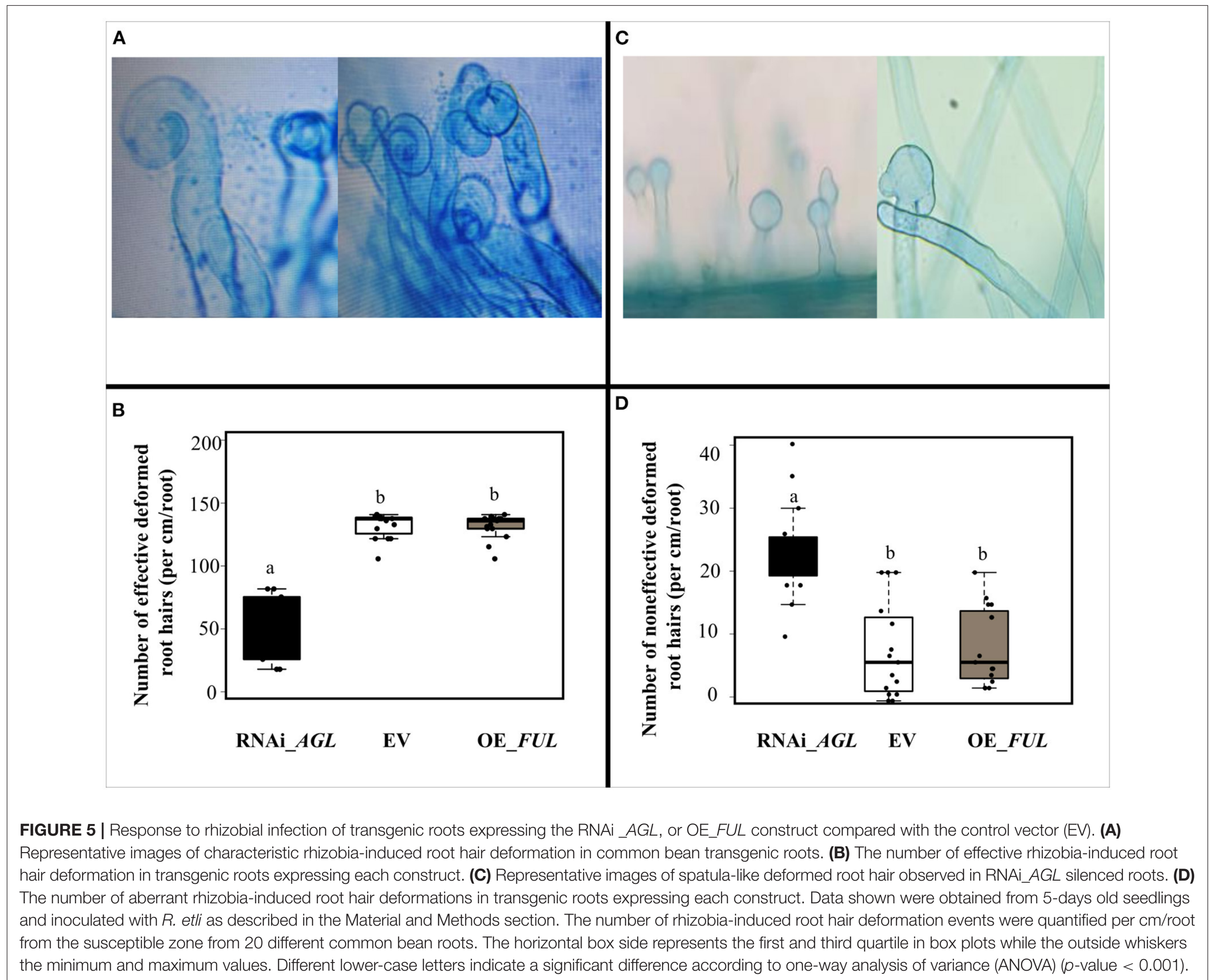


FIGURE 5 | Response to rhizobial infection of transgenic roots expressing the RNAi _*AGL*, or OE_*FUL* construct compared with the control vector (EV). **(A)** Representative images of characteristic rhizobia-induced root hair deformation in common bean transgenic roots. **(B)** The number of effective rhizobia-induced root hair deformation in transgenic roots expressing each construct. **(C)** Representative images of spatula-like deformed root hair observed in RNAi_*AGL* silenced roots. **(D)** The number of aberrant rhizobia-induced root hair deformations in transgenic roots expressing each construct. Data shown were obtained from 5-days old seedlings and inoculated with *R. etli* as described in the Material and Methods section. The number of rhizobia-induced root hair deformation events were quantified per cm/root from the susceptible zone from 20 different common bean roots. The horizontal box side represents the first and third quartile in box plots while the outside whiskers the minimum and maximum values. Different lower-case letters indicate a significant difference according to one-way analysis of variance (ANOVA) (p-value < 0.001).

Effect of PvAGL TF in Root and Nodule Development of Common Bean in Symbiosis With *R. etli*

We assessed if the affection of rhizobia-infection and early symbiotic gene expression in composite plants with modulation in *PvAGL*-gene expression results in altered root and nodulation phenotype.

An evident decrease in the transgenic roots formed, and the root growth was observed in *PvAGL*-silenced composite plants (**Figures 4B,C**). Analysis of root architecture revealed decreased root length, area, and biomass of RNAi_*AGL* roots compared with control (EV) roots, both at 18 and 28 dpi (**Figures 7A–C**). In agreement, the foliage/root ratio was increased in RNAi_*AGL* composite plants (**Figure 7D**). Contrastingly, plants transformed with OE_*FUL* showed an adequate formation and root architecture of transgenic roots (**Figures 4B,C**). In addition, the roots overexpressing *PvFUL*-like showed increased root length, area, and biomass compared with EV roots at 28 dpi (**Figures 7B,C**).

Regarding the nodule phenotype, we observed that compared with EV plants, RNAi_*AGL* composite plants formed fewer nodules at 18 dpi, but at a later stage (28 dpi), their nodule number increased (**Figure 8A**). A high proportion of small nodules were observed in RNAi_*AGL* transgenic roots at 28 dpi (**Figures 8B,C**). However, each plant's nodule dry weight per root system was similar among the RNAi_*AGL*, OE_*FUL*, and EV plants. Microscopic image of RNAi_*AGL* nodule sections revealed defective, small nodules with decreased number of infected cells (**Figure 9**). By contrast, compared with EV plants, OE_*FUL* forms fewer nodules but with increased size (**Figures 8A,B**). A high proportion of medium and large size nodules was observed in OE_*FUL* plants (**Figures 8C**, **9**). The OE_*FUL* healthy nodules showed a full infected zone (**Figure 9**).

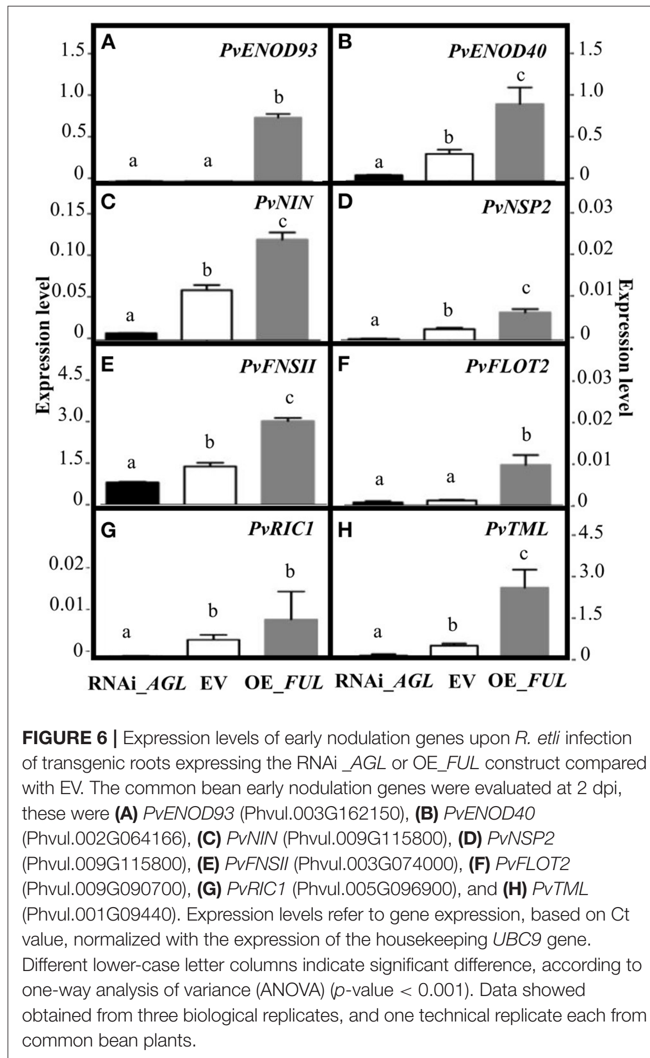


FIGURE 6 | Expression levels of early nodulation genes upon *R. etli* infection of transgenic roots expressing the RNAi _*AGL* or OE_*FUL* construct compared with EV. The common bean early nodulation genes were evaluated at 2 dpi, these were **(A)** *PvENOD93* (Phvul.003G162150), **(B)** *PvENOD40* (Phvul.002G064166), **(C)** *PvNIN* (Phvul.009G115800), **(D)** *PvNSP2* (Phvul.009G115800), **(E)** *PvFNSII* (Phvul.003G074000), **(F)** *PvFLOT2* (Phvul.009G090700), **(G)** *PvRIC1* (Phvul.005G096900), and **(H)** *PvTML* (Phvul.001G09440). Expression levels refer to gene expression, based on Ct value, normalized with the expression of the housekeeping *UBC9* gene. Different lower-case letter columns indicate significant difference, according to one-way analysis of variance (ANOVA) (p-value < 0.001). Data showed obtained from three biological replicates, and one technical replicate each from common bean plants.

Nodule function was then evaluated by determining nitrogenase activity through ARA. In agreement with their smaller size and fewer infected cells (**Figures 8**, **9**), RNAi_*AGL* nodules displayed a significantly lower nitrogenase activity compared with that from EV or OE_*FUL* nodules (**Figure 8D**).

Effect of PvAGL TF in the AON of the Common Bean – *R. etli* Symbiosis

Knock-out mutants from different legume species, defective in the shoot nodule autoregulation receptor kinase (*NARK*, in common bean and soybean) present a characteristic hypernodulation or supernodulation phenotype; but the nodules formed, in higher number, are ineffective, white and small (Reid et al., 2011). This phenotype resembles the one observed in composite common bean plants silenced in *PvAGL* genes (**Figures 8**, **9**). Thus, we analyzed if RNAi_*AGL* composite plants are altered in the root components of the AON. As shown in **Figure 6G**, the expression level of the *PvRIC1* gene was very low in *R. etli*-inoculated roots expressing the RNAi_*AGL* construct. This correlates with the low expression of *PvNIN* (**Figure 6C**), known to be the transcriptional regulator of *RIC* genes (Soyano et al., 2019; Roy et al., 2020). In addition, the *TML* gene, encoding an AON negative regulator of further nodule formation, also shows diminished expression in RNAi_*AGL* transgenic roots (**Figure 6H**). By contrast, the roots overexpressing *PvFUL*-like formed a lower number of medium or large nodules compared with EV (control) plants (**Figures 8**, **9**) and showed higher expression of *PvNIN* and *PvTML* (**Figures 6C,H**). Furthermore, we identified CArG-box sequences repeated three times in the *PvTML* promoter region (**Table 1**); however, the expression level of *PvTML* in non-inoculated transgenic roots was similar to EV silenced and overexpressing roots. Taken together, these results indicate a positive effect of *PvAGL* genes in the AON process of the rhizobia N-fixing symbiosis, possibly exerted through the interaction with *PvNIN*, relevant for the regulation of this process.

TABLE 1 | Identification of CArG-box sequences in *P. vulgaris* early symbiotic genes.

Gene name	Gene ID	CArG sequence	Position relative to the initiation codon
PvNIN	Phvul.009G115800	CCTTTATAGG	−9166/−9157
PvNSP2	Phvul.009G122700	CCATATATGG	−3736/−3727
PvRIC1	Phvul.005G096901	CCAAAAAAGG	−16998/−16989
PvTML	Phvul.001G094400	CCATTTATGG	−718/−709
		CCAAATTTGG	−7715/−7706
		CCATTAAAGG	−8099/−8090

DISCUSSION

In plants, the *MADS*-box/*AGL* genes constitute a large TF family with diverse and essential biological functions to regulate the development of almost every plant organ. Phylogenies of plant *MADS*-box genes have provided information for studies of evolution and developmental genetic pathways (Alvarez-Buylla et al., 2000a; Liljegren et al., 2000). In this work, we present a phylogeny of *MADS*-box genes from Arabidopsis and the legumes: common bean, soybean, Medicago, and Lotus. Initial research of Arabidopsis *MADS*-box genes focused on floral development, and it gave rise to the so-called ABCDE model of floral organ specification (Smaczniak et al., 2012). The phylogenetic tree (**Figure 1**) indicates the clades of Arabidopsis homeotic genes from A, B, C, D, or E classes that group *MADS*-box genes from the four legumes analyzed. Data from legume GEAs generally indicated a high expression of such legume genes in flowers. For example, the clade, including the class A *AP1* gene from Arabidopsis, involved in specifying sepals and petals (Mandel et al., 1992), also includes genes from the four legume species highly expressed in flowers. However, its expression in specific flower tissues, i.e., sepals and petals, has not been documented. This agrees with previous knowledge indicating

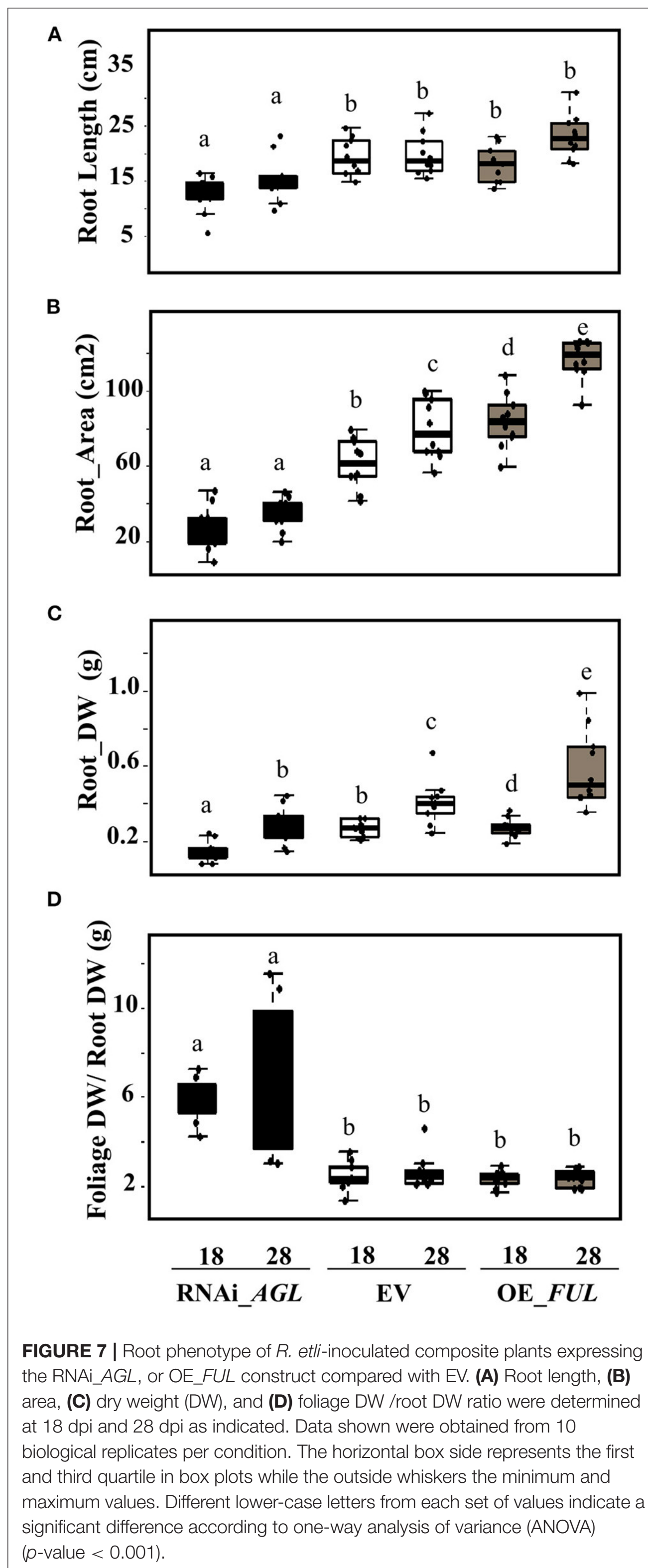


FIGURE 7 | Root phenotype of *R. etli*-inoculated composite plants expressing the RNAi_*AGL*, or OE_*FUL* construct compared with EV. **(A)** Root length, **(B)** area, **(C)** dry weight (DW), and **(D)** foliage DW /root DW ratio were determined at 18 dpi and 28 dpi as indicated. Data shown were obtained from 10 biological replicates per condition. The horizontal box side represents the first and third quartile in box plots while the outside whiskers the minimum and maximum values. Different lower-case letters from each set of values indicate a significant difference according to one-way analysis of variance (ANOVA) (p-value < 0.001).

that Arabidopsis *MADS*-box genes that form a monophyletic clade share similar tissue expression and likely share regulatory function (Alvarez-Buylla et al., 2000a).

On the other hand, the phylogenetic analysis suggested that the evolution of the MADS-domain family has involved a simultaneous functional diversification in vegetative and reproductive structures (Alvarez-Buylla et al., 2000a; Liljegren et al., 2000). Examples of such diversification were found in the class B genes represented by the *AP3* and *PISTILLATA* (*PI*) gene clades (Jack et al., 1992; Goto and Meyerowitz, 1994). The legume genes grouped in the *AP3* clade include the soybean genes Glyma04G245500 that, according to GEA data, is expressed in flowers and root; and Glyma.06G027200 (*NMH7*) gene that is highly expressed in roots and nodules (Ma et al., 2019). According to the GEA data, the PI clade includes the Medicago Medtr1g029670 gene that, in addition to flower, is highly expressed in nodules.

Based on data from Arabidopsis and legumes gene expression atlas, we analyzed the expression pattern of seven genes from five clades that include seven Arabidopsis *MADS*-box genes known to be important regulators of root development (Alvarez-Buylla et al., 2019; **Figure 1**). These genes are: *ANR1* that is a key determinant for developmental root plasticity and has a regulatory role in nutrient response through controlling lateral root elongation in response to nitrate (Gan et al., 2005); *XAL1* that plays a principal role during root development, possibly regulating the expression of genes that are components of the cell cycle (Garcia-Cruz et al., 2016); *XAL2*, another relevant gene for Arabidopsis root development, that controls auxin transport via *PIN* (Garay-Arroyo et al., 2013; Alvarez-Buylla et al., 2019); *AGL19* that is expressed in the columella, lateral root cap and epidermal cells of the meristematic region of the primary and lateral root tips (Alvarez-Buylla et al., 2000a); *AGL17* gene that seems to be a lateral root cap marker in the root tip (Burgeff et al., 2002); *AGL21*, which is highly expressed in lateral root primordia and it has a punctual expression in the primary root meristem (Alvarez-Buylla et al., 2019) and *AGL16* gene that is expressed at relatively high levels in the Arabidopsis root quiescent center (Nawy et al., 2005) and shows an intermediate expression level in phloem, xylem and procambium o the root mature zone (Alvarez-Buylla et al., 2019). Evident variations in the expression pattern from seven plant organs were observed among Arabidopsis and the legume *MADS*-box genes grouped in each clade (**Figure 2**). Such variations may be related to the different ages/conditions of the different organs from the consulted database from each plant species. For example, data for *AGL* genes expression in leaf were from 21-day-old common bean leaves, Gm, 28 days old for Medicago, 42 days old for Lotus, and young leaves for Arabidopsis consulted gene expression atlas. However, all the legume *MADS*-box genes from analyzed clades showed expression in roots, ranging from 9 to 100% compared with the level of expression in other organs. Notably, all the legume genes, except *P. vulgaris* (Phvul.002G143866, **Figure 2D**), were also expressed in legume nodules (7 to 74%), developed from roots inoculated with rhizobia.

Recent research in Lotus and Medicago has demonstrated that legumes co-opted a lateral root developmental program, from Arabidopsis, to control nodule organogenesis (Schiessl et al., 2019; Soyano et al., 2019). The case of study in these reports are the legume orthologs of the Arabidopsis *ASYMMETRIC*

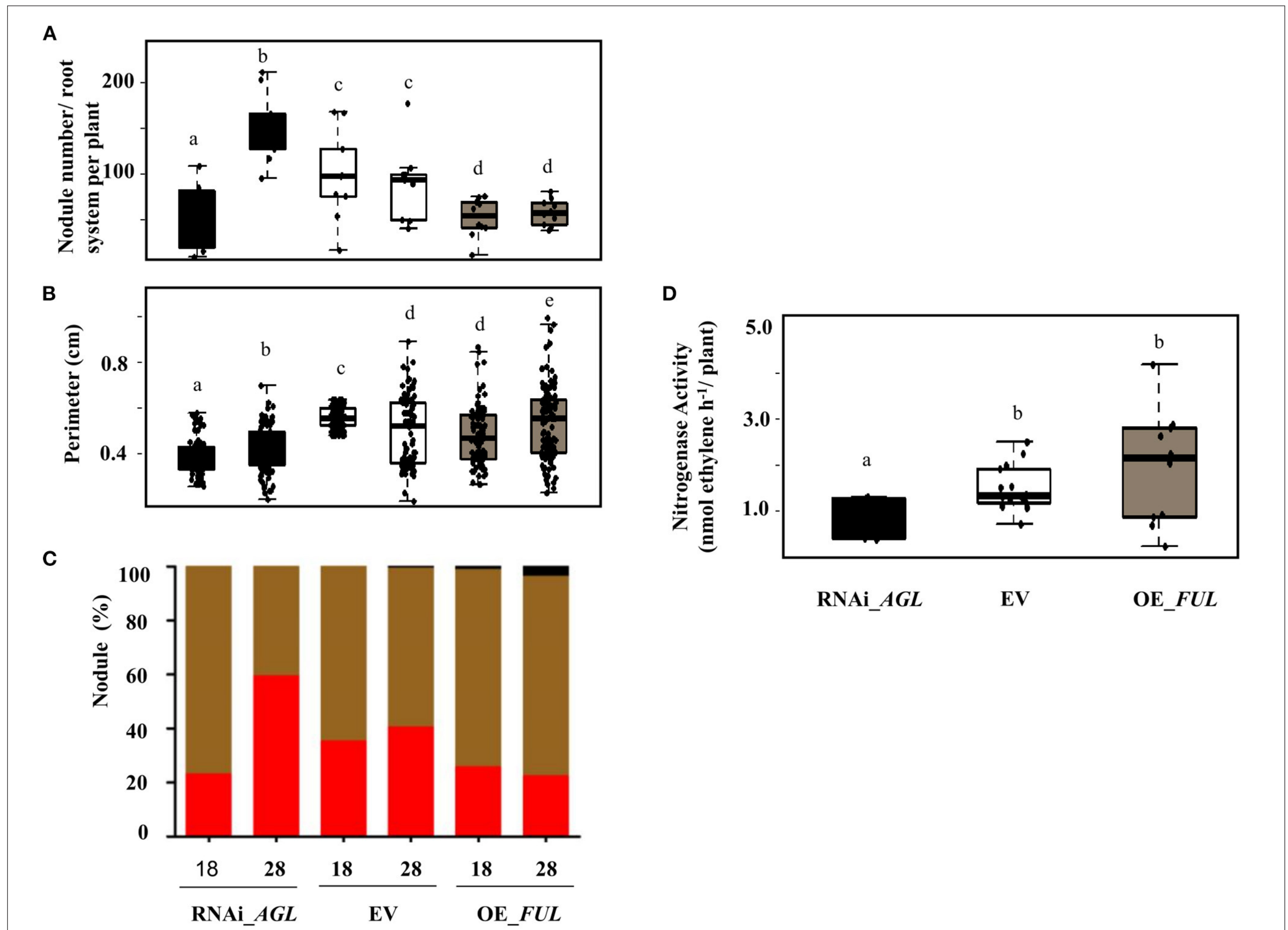


FIGURE 8 | Nodulation phenotype of *R. etli*-inoculated composite plants expressing the RNAi_*AGL*, or OE_*FUL* construct compared with EV. Nodule parameters were determined at the indicated dpi. **(A)** Nodule number from the whole root system per plant was obtained from 10 biological replicates per condition. **(B)** Nodule perimeter was calculated using the ImageJ program from 50 different nodules obtained from three to five plants per condition. **(C)** Based on their perimeter values, nodules were classified as small (0.2–0.5 cm; red bar), medium (0.51–0.75 cm; brown bar), or large (>0.76; black bar), the percentage of nodules from each category is shown for each condition. **(D)** Nitrogenase activity was determined at 28 dpi using the acetylene reduction assay (ARA). Data were obtained from 10 biological replicates per condition. Different lower-case letter columns indicate a significant difference according to one-way analysis of variance (ANOVA) (p-value < 0.001).

LEAVES 2-LIKE 18/LATERAL ORGAN BOUNDARIES DOMAIN 16a (*ASL18/LDB16a*) gene. In Arabidopsis, the ASL18/LBD16a TF is involved in establishing the asymmetry required for divisions of the pericycle founder cells that produce lateral root primordia (Goh et al., 2012). In legumes, the nodules are lateral root organs that initiate from the inner root layers in response to rhizobial perception. In contrast, lateral roots emerge from predefined founder cells as an adaptive response to external stimuli. Thus, despite differential induction, lateral roots and nodules share developmental programs through *ASL18/LBD16a* (Schiessl et al., 2019; Soyano et al., 2019; Shahan and Benfey, 2020). Thus, the master regulator for legume symbioses, the NIN TF, is essential for recruiting the lateral root and nodule organogenesis developmental programs. In Medicago, cytokinin-induction of *NIN* allows induction of this program during nodulation through activation of *ASL18/LBD16a* that promotes induction of auxin-responsive gene and auxin biosynthesis (Schiessl et al., 2019). In Lotus, *NIN* regulates the *ASL18/LBD16a* and *NUCLEAR FACTOR-Y* (*NF-Y*) genes that genetically interact during nodule development (Soyano et al., 2019). On this basis, research presented in this work about root/nodules *PvAGL* TF genes (namely, *PvFUL*-like, *PvXAL1*-like, *PvAGL24*-like, *PvSOC1*-like, *PvSVP*-like, and *PvAGL16*-like) led us to propose that Arabidopsis *AGL* TF that regulate root development have been co-opted by legumes to control, both, root and nodule development. However, further experimentation is needed to confirm this hypothesis.

The negative effect of *PvAGL* gene silencing in root development was evident in non-inoculated and *R. etli* inoculated plants. These roots showed delayed transgenic roots after *A. rhizogenes* infection and reduced root growth (**Figure 4**). Furthermore, the inoculated RNAi_*AGL* plants showed altered

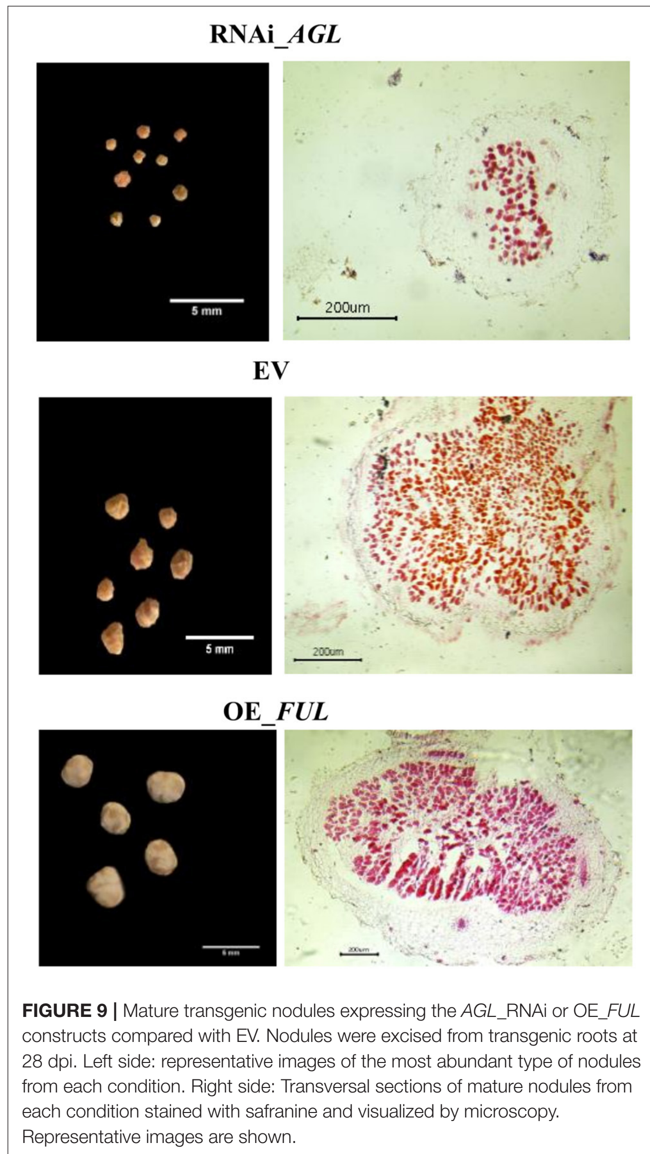


FIGURE 9 | Mature transgenic nodules expressing the *AGL*_RNAi or OE_*FUL* constructs compared with EV. Nodules were excised from transgenic roots at 28 dpi. Left side: representative images of the most abundant type of nodules from each condition. Right side: Transversal sections of mature nodules from each condition stained with safranine and visualized by microscopy. Representative images are shown.

root architecture evidenced by diminished root length, area, and biomass (**Figures 4**, **7**). Thus, orthologs of Arabidopsis *AGL* genes that regulate root development have a similar function in common beans.

The gene silencing of root/nodule *PvAGL* genes did not affect the root hair development or density (**Supplementary Figure 6**). However, it negatively affected rhizobial infection of common bean roots, perhaps because due to ineffective chemical communication between the symbionts, something that was evidenced by a significant reduction in the rhizobia-induced effective deformed root hairs that was concomitant with a significant decrease in the expression level of early symbiotic genes essential for rhizobial infection (**Figures 5**, **6**). The genes analyzed include *PvFNSII* involved in the synthesis of flavones, the chemical signal of the plant sensed by compatible rhizobia, and genes involved in the infection thread initiation and progression (*PvFLOT2*, *PvENOD40*, and *PvENOD93*) that act downstream of the TF NSP2 and NIN (Roy et al., 2020). Thus, the effect of PvAGL TF in the transcriptional regulation of early symbiotic genes (**Figure 6**) could be direct or indirect. In addition, several other genes are known to be involved in the rhizobial infection process, and infection thread formation and progression (Roy et al., 2020) could be down-regulated in RNAi_*AGL* roots and would negatively affect initial symbiotic stages. Future experiments based on transcriptomic approaches would provide evidence about this proposition.

Research in different legumes indicates that a delay in the rhizobial infection process results in a defective activation of the nodule organogenesis program (Oldroyd, 2013; Roy et al., 2020). In agreement, a defective nodulation phenotype (**Figures 8**, **9**) was evident in *PvAGL*-silenced plants affected by a rhizobial infection. The nodule-specific NIN TF is an essential symbiosis regulator expressed in the epidermis and controls rhizobial infection (Schauser et al., 1999; Liu and Bisseling, 2020). NIN is also expressed in the pericycle and is essential for regulating nodule primordia formation in Medicago (Liu et al., 2019). The complex pattern of spatiotemporal regulation exerted by NIN in Medicago requires different upstream *cis*-regulatory sequences. These include a remote (-18 kb) *cis*-element with cytokinin-response elements essential for nodule organogenesis (Liu et al., 2019). In addition, recently, it was shown that *NIN* is expressed in the proximal part of the infection zone in nodules and plays an essential role in the transition from infection to fixation zones for establishing a functional symbiosis (Liu et al., 2021). Our *in silico* analysis of the *PvNIN* promoter region revealed a CArG-box *cis*-element at−9166 bp upstream of the initiation codon (**Table 1**). It is noteworthy that *PvNIN* expression was reduced in *PvAGL* silenced root as opposite to *PvFUL*-like overexpressing roots both from inoculated and non-inoculated plants, something that supports the hypothesis of its possible direct transcriptional regulation by AGL TF acting as an essential regulator of rhizobial infection and nodule organogenesis in common bean. However, further work is required to identify the *P. vulgaris* gene targets of *PvAGL* transcription regulation in common bean roots and nodules. Experimental approaches demonstrate their specific binding to CArG-box present in gene promoter regions.

The observed increased nodule number with altered nodule morphology and function in RNAi_*AGL* plants (**Figures 8**, **9**) led us to propose that the downregulation of *PvAGL* genes results in an alteration of the AON symbiotic process. Recently it was shown that gibberellins signaling is a key regulator of the AON process in Lotus (Akamatsu et al., 2020). The endogenous gibberellins from Lotus nodules induce *NIN* expression via its gibberellin-responsive *cis*-acting region. NIN directly induces *CLE-RS* genes (*RIC* genes in the common bean) to activate the AON process (Soyano et al., 2014; Akamatsu et al., 2020). We showed a significantly low expression of *PvRIC1* and *PvTML* (**Figure 6**), a negative regulator of AON, in *PvAGL*-silenced roots/nodules. In addition, we showed that the expression level of *PvNIN*, with CArG-box *cis*-elements in its promoter region (**Table 1**), is up- or down-regulated both in inoculated as well as in non-inoculated roots, with increased or silenced *PvAGL* expression, respectively. Thus, we propose that PvAGL TF

positively regulates *PvNIN* that, in turn, positively regulates *PvRIC1* via gibberellins. The AON is a complex process that includes almost 20 different genes (Roy et al., 2020). Therefore, the down-regulation of the expression of other genes relevant to regulating the AON process in RNAi_*AGL* roots/nodules could not be excluded. Taken together, our results indicate that the downregulation of *PvAGL*s in roots/nodules negatively affects the AON resulting in a higher number of small, ineffective nodules formed.

The possible contribution of legume AGL TF as regulators of the rhizobia symbiosis is scarcely documented. In alfalfa, three *MADS*-box genes expressed in roots and nodules have been reported, though their functional analysis is lacking (Heard et al., 1997; Zucchero et al., 2001; Páez-Valencia et al., 2008). In soybean, one report characterizes the *GmNMHC5, MADS*-box gene that acts as a positive regulator of root development and nodulation, while the study of *GmNMH7* revealed its negative regulation of nodulation (Liu et al., 2015; Ma et al., 2019). Future in-depth studies are needed to find the commonalities and differences among MADS/AGL TF roles from different legumes as regulators of the N-fixing symbiosis.

CONCLUSIONS

This is the first report about the participation of PvAGL TF as positive regulators of the common bean – rhizobia symbiosis. The data presented in this study attest to the relevance of PvAGL TF as positive regulators of several processes of the common bean – rhizobia symbioses such as root development, rhizobial infection, nodule organogenesis/function, and the autoregulation of nodulation. Furthermore, we propose that *PvAGL* control be exerted via interplay with *PvNIN*, the master symbiosis regulator. Certainly, the knowledge of AGL TF as regulators of N-fixing symbiosis in different legume species would be relevant for a future improvement of this relevant process for ecological and economic reasons.

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

GH and LA conceived and designed the study. LA, MI-A, MR-S, MR, AL, and S-IF performed the experiments. LA and LL performed *in silico* analyses. LA, OV-L, LG, and GH adviced experiments and analyzed data. LA and GH wrote the manuscript. All authors contributed to the critical revision of the manuscript, read, and approved the submitted version.

FUNDING

Partial financial support for this work was provided by grants A1-S-22570 to GH and A1-S-9454 to OV-L from CONACyT; IN201320 to OV-L, IN204320 to LG, and IN203819 to GH from DGAPA, UNAM. LA is doctoral student from the Programa de Doctorado en Ciencias Biomédicas - UNAM and she received the Fellowship 582722 form Consejo Nacional de Ciencia y Tecnología and from DGAPA, UNAM (IN203819).

ACKNOWLEDGMENTS

We are grateful to Ma. de la Paz Eelizabeth Salas, Víctor Bustos, and Joel Gómez Espíndola for technical assistance and to Damien Formey and Luis P. Íñiguez for advice in data bases and bioinformatic analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.679463/full#supplementary-material

REFERENCES

Akamatsu, A., Nagae, M., Nishimura, Y., Romero Montero, D., Ninomiya, S., Kojima, M., et al. (2020). Endogenous gibberellins affect root nodule symbiosis via transcriptional regulation of NODULE INCEPTION in Lotus japonicus. *Plant J.* 105, 1507–1520. doi: 10.1111/tpj.15128

Alvarez-Buylla, E. R., Garcia-Ponce, B., Sanchez, M. P., Espinosa-Soto, C., Garcia-Gomez, M. L., Pineyro-Nelson, A., et al. (2019). MADS-box genes underground becoming mainstream: plant root developmental mechanisms. *New Phytol.* 223, 1143–1158. doi: 10.1111/nph.15793

Alvarez-Buylla, E. R., Liljegren, S. J., Pelaz, S., Gold, S. E., Burgeff, C., Ditta, G. S., et al. (2000a). MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. *Plant J.* 24, 457–466. doi: 10.1046/j.1365-313x.2000.00891.x

Alvarez-Buylla, E. R., Pelaz, S., Liljegren, S. J., Gold, S. E., Burgeff, C., Ditta, G. S., et al. (2000b). An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5328–5333. doi: 10.1073/pnas.97.10.5328

Aparicio-Fabre, R., Guillen, G., Loredo, M., Arellano, J., Valdes-Lopez, O., Ramirez, M., et al. (2013). Common bean (Phaseolus vulgaris L.) PvTIFY orchestrates global changes in transcript profile response to jasmonate and phosphorus deficiency. *BMC Plant Biol.* 13:26. doi: 10.1186/1471-2229-13-26

Broughton, W. J., and Dilworth, M. J. (1971). Control of leghaemoglobin synthesis in snake beans. *Biochem. J.* 125, 1075–1080. doi: 10.1042/bj1251075

Broughton, W. J., Hernández, G., Blair, M., Beebe, S., Gepts, P., and Vanderleyden, J. (2003). Beans (Phaseolus spp.) – model food legumes. *Plant Soil* 252, 55–128. doi: 10.1023/A:1024146710611

Burgeff, C., Liljegren, S. J., Tapia-Lopez, R., Yanofsky, M. F., and Alvarez-Buylla, E. R. (2002). MADS-box gene expression in lateral primordia, meristems and differentiated tissues of Arabidopsis thaliana roots. *Planta* 214, 365–372. doi: 10.1007/s004250100637

Castro-Guerrero, N. A., Isidra-Arellano, M. C., Mendoza-Cozatl, D. G., and Valdés-López, O. (2016). Common bean: a legume model on the rise for unraveling responses and adaptations to iron, zinc, and phosphate deficiencies. *Front. Plant Sci.* 7:600. doi: 10.3389/fpls.2016.00600

Catoira, R., Galera, C., de Billy, F., Penmetsa, R. V., Journet, E. P., Maillet, F., et al. (2000). Four genes of Medicago truncatula controlling components of a nod factor transduction pathway. *Plant Cell* 12, 1647–1666. doi: 10.1105/tpc.12.9.1647

Czechowski, T., Bari, R. P., Stitt, M., Scheible, W. R., and Udvardi, M. K. (2004). Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant J*. 38, 366–379. doi: 10.1111/j.1365-313X.2004.02051.x

Estrada-Navarrete, G., Alvarado-Affantranger, X., Olivares, J. E., Guillen, G., Diaz-Camino, C., Campos, F., et al. (2007). Fast, efficient and reproducible genetic transformation of Phaseolus spp. by *Agrobacterium rhizogenes*. *Nat. Protoc*. 2, 1819–1824. doi: 10.1038/nprot.2007.259

Fehlberg, V., Vieweg, M. F., Dohmann, E. M., Hohnjec, N., Puhler, A., Perlick, A. M., et al. (2005). The promoter of the leghaemoglobin gene VfLb29: functional analysis and identification of modules necessary for its activation in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots. *J. Exp. Bot*. 56, 799–806. doi: 10.1093/jxb/eri074

Ferguson, B. J., Li, D., Hastwell, A. H., Reid, D. E., Li, Y., Jackson, S. A., et al. (2014). The soybean (Glycine max) nodulation-suppressive CLE peptide, GmRIC1, functions interspecifically in common white bean (*Phaseolus vulgaris*), but not in a supernodulating line mutated in the receptor PvNARK. *Plant Biotechnol. J*. 12, 1085–1097. doi: 10.1111/pbi.12216

Ferguson, B. J., Mens, C., Hastwell, A. H., Zhang, M., Su, H., Jones, C. H., et al. (2019). Legume nodulation: the host controls the party. *Plant Cell Environ*. 42, 41–51. doi: 10.1111/pce.13348

Fournier, J., Teillet, A., Chabaud, M., Ivanov, S., Genre, A., Limpens, E., et al. (2015). Remodeling of the infection chamber before infection thread formation reveals a two-step mechanism for rhizobial entry into the host legume root hair. *Plant Physiol*. 167, 1233–1242. doi: 10.1104/pp.114.253302

Gan, Y., Filleur, S., Rahman, A., Gotensparre, S., and Forde, B. G. (2005). Nutritional regulation of ANR1 and other root-expressed MADS-box genes in Arabidopsis thaliana. *Planta* 222, 730–742. doi: 10.1007/s00425-005-0020-3

Garay-Arroyo, A., Ortiz-Moreno, E., de la Paz Sanchez, M., Murphy, A. S., Garcia-Ponce, B., Marsch-Martinez, N., et al. (2013). The MADS transcription factor XAL2/AGL14 modulates auxin transport during Arabidopsis root development by regulating PIN expression. *EMBO J*. 32, 2884–2895. doi: 10.1038/emboj.2013.216

Garcia-Cruz, K. V., Garcia-Ponce, B., Garay-Arroyo, A., Sanchez, M. P., Ugartechea-Chirino, Y., Desvoyes, B., et al. (2016). The MADS-box XAANTAL1 increases proliferation at the Arabidopsis root stem-cell niche and participates in transition to differentiation by regulating cell-cycle components. *Ann. Bot*. 118, 787–796. doi: 10.1093/aob/mcw126

Goh, T., Joi, S., Mimura, T., and Fukaki, H. (2012). The establishment of asymmetry in Arabidopsis lateral root founder cells is regulated by LBD16/ASL18 and related LBD/ASL proteins. *Development* 139, 883–893. doi: 10.1242/dev.071928

Goto, K., and Meyerowitz, E. M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes Dev*. 8, 1548–1560. doi: 10.1101/gad.8.13.1548

Hardy, R. W., Holsten, R. D., Jackson, E. K., and Burns, R. C. (1968). The acetylene-ethylene assay for n(2) fixation: laboratory and field evaluation. *Plant Physiol*. 43, 1185–1207. doi: 10.1104/pp.43.8.1185

Heard, J., Caspi, M., and Dunn, K. (1997). Evolutionary diversity of symbiotically induced nodule MADS box genes: characterization of nmhC5, a member of a novel subfamily. *Mol. Plant Microbe Interact*. 10, 665–676. doi: 10.1094/MPMI.1997.10.5.665

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res*. 27, 297–300. doi: 10.1093/nar/27.1.297

Íñiguez, L. P., Nova-Franco, B., and Hernandez, G. (2015). Novel players in the AP2-miR172 regulatory network for common bean nodulation. *Plant Signal Behav*. 10:e1062957. doi: 10.1080/15592324.2015.1062957

Jack, T., Brockman, L. L., and Meyerowitz, E. M. (1992). The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell* 68, 683–697. doi: 10.1016/0092-8674(92)90144-2

Ku, A. T., Huang, Y. S., Wang, Y. S., Ma, D., and Yeh, K. W. (2008). IbMADS1 (Ipomoea batatas MADS-box 1 gene) is involved in tuberous root initiation in sweet potato (Ipomoea batatas). *Ann. Bot*. 102, 57–67. doi: 10.1093/aob/mcn067

Letunic, I., and Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res*. 47, W256–W259. doi: 10.1093/nar/gkz239

Libault, M., Joshi, T., Benedito, V. A., Xu, D., Udvardi, M. K., and Stacey, G. (2009). Legume transcription factor genes: what makes legumes so special? *Plant Physiol*. 151, 991–1001. doi: 10.1104/pp.109.144105

Liljegren, S. J., Ditta, G. S., Eshed, Y., Savidge, B., Bowman, J. L., and Yanofsky, M. F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* 404, 766–770. doi: 10.1038/35008089

Liu, J., and Bisseling, T. (2020). Evolution of NIN and NIN-like genes in relation to nodule symbiosis. *Genes* 11:777. doi: 10.3390/genes11070777

Liu, J., Rasing, M., Zeng, T., Klein, J., Kulikova, O., and Bisseling, T. (2021). NIN is essential for development of symbiosomes, suppression of defence and premature senescence in Medicago truncatula nodules. *New Phytol*. 230, 290–303. doi: 10.1111/nph.17215

Liu, J., Rutten, L., Limpens, E., van der Molen, T., van Velzen, R., Chen, R., et al. (2019). A remote cis-regulatory region is required for NIN expression in the pericycle to initiate nodule primordium formation in medicago truncatula. *Plant Cell* 31, 68–83. doi: 10.1105/tpc.18.00478

Liu, W., Han, X., Zhan, G., Zhao, Z., Feng, Y., and Wu, C. (2015). A novel sucrose-regulatory MADS-box transcription factor GmNMHC5 promotes root development and nodulation in soybean (Glycine max [L.] Merr.). *Int. J. Mol. Sci*. 16, 20657–20673. doi: 10.3390/ijms160920657

Lobet, G., Pages, L., and Draye, X. (2011). A novel image-analysis toolbox enabling quantitative analysis of root system architecture. *Plant Physiol*. 157, 29–39. doi: 10.1104/pp.111.179895

Ma, W.-y., Liu, W., Hou, W.-s., Sun, S., Jiang, B.-j., Han, T.-f., et al. (2019). GmNMH7, a MADS-box transcription factor, inhibits root development and nodulation of soybean (Glycine max [L.] Merr.). *J. Integr. Agric*. 18, 553–562. doi: 10.1016/S2095-3119(18)61992-6

Mandel, M. A., Gustafson-Brown, C., Savidge, B., and Yanofsky, M. F. (1992). Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. *Nature* 360, 273–277. doi: 10.1038/360273a0

Marsh, J. F., Rakocevic, A., Mitra, R. M., Brocard, L., Sun, J., Eschstruth, A., et al. (2007). Medicago truncatula NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol*. 144, 324–335. doi: 10.1104/pp.106.093021

Martin-Rodriguez, J. A., Leija, A., Formey, D., and Hernandez, G. (2018). The MicroRNA319d/TCP10 node regulates the common bean - rhizobia nitrogen-fixing symbiosis. *Front. Plant Sci*. 9:1175. doi: 10.3389/fpls.2018.01175

Mead, J., Bruning, A. R., Gill, M. K., Steiner, A. M., Acton, T. B., and Vershon, A. K. (2002). Interactions of the Mcm1 MADS box protein with cofactors that regulate mating in yeast. *Mol. Cell Biol*. 22, 4607–4621. doi: 10.1128/MCB.22.13.4607-4621.2002

Murai, K. (2013). Homeotic genes and the ABCDE model for floral organ formation in wheat. *Plants* 2, 379–395. doi: 10.3390/plants2030379

Nawy, T., Lee, J. Y., Colinas, J., Wang, J. Y., Thongrod, S. C., Malamy, J. E., et al. (2005). Transcriptional profile of the Arabidopsis root quiescent center. *Plant Cell* 17, 1908–1925. doi: 10.1105/tpc.105.031724

Nova-Franco, B., Iniguez, L. P., Valdes-Lopez, O., Alvarado-Affantranger, X., Leija, A., Fuentes, S. I., et al. (2015). The micro-RNA72c-APETALA2-1 node as a key regulator of the common bean-Rhizobium etli nitrogen fixation symbiosis. *Plant Physiol*. 168, 273–291. doi: 10.1104/pp.114.255547

Olah, B., Briere, C., Becard, G., Denarie, J., and Gough, C. (2005). Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in Medicago truncatula via the DMI1/DMI2 signalling pathway. *Plant J*. 44, 195–207. doi: 10.1111/j.1365-313X.2005.02522.x

Oldroyd, G. E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol*. 11, 252–263. doi: 10.1038/nrmicro2990

Olson, E. N., Perry, M., and Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev. Biol*. 172, 2–14. doi: 10.1006/dbio.1995.0002

O'Rourke, J. A., Iniguez, L. P., Fu, F., Bucciarelli, B., Miller, S. S., Jackson, S. A., et al. (2014). An RNA-Seq based gene expression atlas of the common bean. *BMC Genomics* 15:866. doi: 10.1186/1471-2164-15-866

Páez-Valencia, J., Sánchez-Gómez, C., Valencia-Mayoral, P., Contreras-Ramos, A., Hernández-Lucas, I., Orozco-Segovia, A., et al. (2008). Localization of the MADS domain transcriptional factor NMH7 during seed, seedling and nodule development of *Medicago sativa*. *Plant Sci*. 175, 596–603. doi: 10.1016/j.plantsci.2008.06.008

Parenicova, L., de Folter, S., Kieffer, M., Horner, D. S., Favalli, C., Busscher, J., et al. (2003). Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: new openings to the MADS world. *Plant Cell* 15, 1538–1551. doi: 10.1105/tpc.011544

Pollock, R., and Treisman, R. (1991). Human SRF-related proteins: DNA-binding properties and potential regulatory targets. *Genes Dev*. 5, 2327–2341. doi: 10.1101/gad.5.12a.2327

Puig, J., Meynard, D., Khong, G. N., Pauluzzi, G., Guiderdoni, E., and Gantet, P. (2013). Analysis of the expression of the AGL17-like clade of MADS-box transcription factors in rice. *Gene Expr. Patterns* 13, 160–170. doi: 10.1016/j.gep.2013.02.004

Reid, D. E., Ferguson, B. J., Hayashi, S., Lin, Y. H., and Gresshoff, P. M. (2011). Molecular mechanisms controlling legume autoregulation of nodulation. *Ann. Bot*. 108, 789–795. doi: 10.1093/aob/mcr205

Reyero-Saavedra, M. D. R., Qiao, Z., Sanchez-Correa, M. D. S., Diaz-Pineda, M. E., Reyes, J. L., Covarrubias, A. A., et al. (2017). Gene silencing of Argonaute5 negatively affects the establishment of the legume-rhizobia symbiosis. *Genes* 8:352. doi: 10.3390/genes8120352

Riechmann, J. L., Wang, M., and Meyerowitz, E. M. (1996). DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. *Nucleic Acids Res*. 24, 3134–3141. doi: 10.1093/nar/24.16.3134

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., et al. (2020). Celebrating 20 Years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell* 32, 15–41. doi: 10.1105/tpc.19.00279

Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. *Nature* 402, 191–195. doi: 10.1038/46058

Schiessl, K., Lilley, J. L. S., Lee, T., Tamvakis, I., Kohlen, W., Bailey, P. C., et al. (2019). NODULE INCEPTION recruits the lateral root developmental program for symbiotic nodule organogenesis in *Medicago truncatula*. *Curr. Biol.* 29, 3657–3668. doi: 10.1016/j.cub.2019.09.005

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H., and Sommer, H. (1990). Genetic control of flower development by homeotic genes in *Antirrhinum majus*. *Science* 250, 931–936. doi: 10.1126/science.250.4983.931

Shahan, R., and Benfey, P. N. (2020). A co-opted regulator of lateral root development controls nodule organogenesis in lotus. *Dev. Cell* 52, 6–7. doi: 10.1016/j.devcel.2019.12.009

Smaczniak, C., Immink, R. G., Angenent, G. C., and Kaufmann, K. (2012). Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. *Development* 139, 3081–3098. doi: 10.1242/dev.074674

Soyano, T., Hirakawa, H., Sato, S., Hayashi, M., and Kawaguchi, M. (2014). Nodule Inception creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. *Proc. Natl. Acad. Sci. U. S. A*. 111, 14607–14612. doi: 10.1073/pnas.1412716111

Soyano, T., Shimoda, Y., Kawaguchi, M., and Hayashi, M. (2019). A shared gene drives lateral root development and root nodule symbiosis pathways in Lotus. *Science* 366, 1021–1023. doi: 10.1126/science.aax2153

Sun, C. H., Yu, J. Q., Duan, X., Wang, J. H., Zhang, Q. Y., Gu, K. D., et al. (2018). The MADS transcription factor CmANR1 positively modulates root system development by directly regulating CmPIN2 in chrysanthemum. *Hortic. Res*. 5:52. doi: 10.1038/s41438-018-0061-y

Suzaki, T., Yoro, E., and Kawaguchi, M. (2015). Leguminous plants: inventors of root nodules to accommodate symbiotic bacteria. *Int. Rev. Cell Mol. Biol*. 316, 111–158. doi: 10.1016/bs.ircmb.2015.01.004

Takahara, M., Magori, S., Soyano, T., Okamoto, S., Yoshida, C., Yano, K., et al. (2013). Too much love, a novel Kelch repeat-containing F-box protein, functions in the long-distance regulation of the legume-Rhizobium symbiosis. *Plant Cell Physiol*. 54, 433–447. doi: 10.1093/pcp/pct022

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Tsikou, D., and Yan, Z. (2018). Systemic control of legume susceptibility to rhizobial infection by a mobile microRNA. *Science* 362, 233–236. doi: 10.1126/science.aat6907

Valdes-Lopez, O., Arenas-Huertero, C., Ramirez, M., Girard, L., Sanchez, F., Vance, C. P., et al. (2008). Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots. *Plant Cell Environ.* 31, 1834–1843. doi: 10.1111/j.1365-3040.2008.01883.x

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034

Venkateshwaran, M., Volkening, J. D., Sussman, M. R., and Ane, J. M. (2013). Symbiosis and the social network of higher plants. *Curr. Opin. Plant Biol*. 16, 118–127. doi: 10.1016/j.pbi.2012.11.007

Vieweg, M. F., Frühling, M., Quandt, H. J., Heim, U., Bäumlein, H., Pühler, A., et al. (2004). The promoter of the Vicia faba L. leghemoglobin gene VfLb29 is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants. *Mol. Plant Microbe Interact*. 17, 62–69. doi: 10.1094/MPMI.2004.17.1.62

Vlasova, A., Capella-Gutierrez, S., Rendon-Anaya, M., Hernandez-Onate, M., Minoche, A. E., Erb, I., et al. (2016). Genome and transcriptome analysis of the Mesoamerican common bean and the role of gene duplications in establishing tissue and temporal specialization of genes. *Genome Biol*. 17:32. doi: 10.1186/s13059-016-0883-6

Waese, J., Fan, J., Pasha, A., Yu, H., Fucile, G., Shi, R., et al. (2017). ePlant: visualizing and exploring multiple levels of data for hypothesis generation in plant biology. *Plant Cell* 29, 1806–1821. doi: 10.1105/tpc.17.00073

Yanofsky, M. F., Ma, H., Bowman, J. L., Drews, G. N., Feldmann, K. A., and Meyerowitz, E. M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. *Nature* 346, 35–39. doi: 10.1038/346035a0

Zucchero, J. C., Caspi, M., and Dunn, K. (2001). ngl9: a third MADS box gene expressed in alfalfa root nodules. *Mol. Plant Microbe Interact*. 14, 1463–1467. doi: 10.1094/MPMI.2001.14.12.1463

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.

Copyright © 2021 Ayra, Reyero-Saavedra, Isidra-Arellano, Lozano, Ramírez, Leija, Fuentes, Girard, Valdés-López and Hernández. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

MINI REVIEW
published: 22 June 2021
doi: 10.3389/fagro.2021.654576

Why Should Nodule Cysteine-Rich (NCR) Peptides Be Absent From Nodules of Some Groups of Legumes but Essential for Symbiotic N-Fixation in Others?

J. Allan Downie[1]* *and Eva Kondorosi*[2]

[1] Department of Molecular Microbiology, John Innes Centre, Norwich, United Kingdom, [2] Institute of Plant Biology, Biological Research Center of the Eötvös Loránd Research Network, Szeged, Hungary

In nitrogen-fixing nodules of legumes such as pea *(Pisum)* and *Medicago* spp. the plant induces terminal differentiation in the rhizobial endosymbionts by targeting nodule-specific cysteine-rich defensin-like peptides into the bacteria. However, in nodules of other legumes such as soybean and *Lotus* spp. terminal bacterial differentiation does not occur; these legumes lack genes encoding equivalent peptides controlling rhizobial development. Here, we review the effects of some of these peptides on rhizobia and address the question as to how and why such peptides may have evolved to enslave rhizobia and become essential for nitrogen fixation in some clades of legumes but not in others.

Keywords: cysteine-rich peptides, nitrogen fixation, NCR, *Rhizobium*, *Sinorhizobium*, *Medicago*, *Lotus*

OPEN ACCESS

Edited by:
Juan Imperial,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Zhenhua Zhang,
Hunan Agricultural University, China
Esther Menendez,
University of Evora, Portugal

***Correspondence:**
J. Allan Downie
allan.downie@jic.ac.uk

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *16 January 2021*
Accepted: *25 May 2021*
Published: *22 June 2021*

Citation:
Downie JA and Kondorosi E (2021) Why Should Nodule Cysteine-Rich (NCR) Peptides Be Absent From Nodules of Some Groups of Legumes but Essential for Symbiotic N-Fixation in Others? Front. Agron. 3:654576. doi: 10.3389/fagro.2021.654576

INTRODUCTION

About 90% of 19,000 species of legumes have nitrogen-fixing nodules, which can have diverse structures in different legume genera (Sprent, 2001). There is a profound difference in the fate of nitrogen-fixing rhizobia in different types of legume nodules because in some, the rhizobia become terminally differentiated and cannot be cultured, whereas in others, the nitrogen-fixing rhizobia can return to the free-living state. These differences are caused by plant-encoded small peptides that can induce terminal bacterial differentiation.

Symbiotic nitrogen fixation has been most studied in legumes producing root nodules with either "determinate" or "indeterminate" meristems, that are usually infected by rhizobia entering roots *via* plant-made infection threads formed after rhizobial-legume signaling (Oldroyd et al., 2011). The infection threads are tunnel-like structures that allow the rhizobia to grow into the region of the root in which cell proliferation has initiated nodule organogenesis (Oldroyd et al., 2011).

"Indeterminate" nodules are cylindrical with a continuously active meristem, a bit like very stubby lateral roots. Such nodules, which e.g., are formed on *Pisum, Medicago* spp., have an age gradient along their length with a growing meristem at the tip, an infection zone, a nitrogen-fixation zone and a so-called interzone between the infection and nitrogen-fixation zones (**Figure 1**). Rapid bacterial differentiation occurs in the interzone and is initiated in the proximal infection zone after the bacteria are released from infection threads and are endocytosed into the cytoplasm surrounded by a plant-made membrane. Many of the legumes forming indeterminate nodules lack an ancient

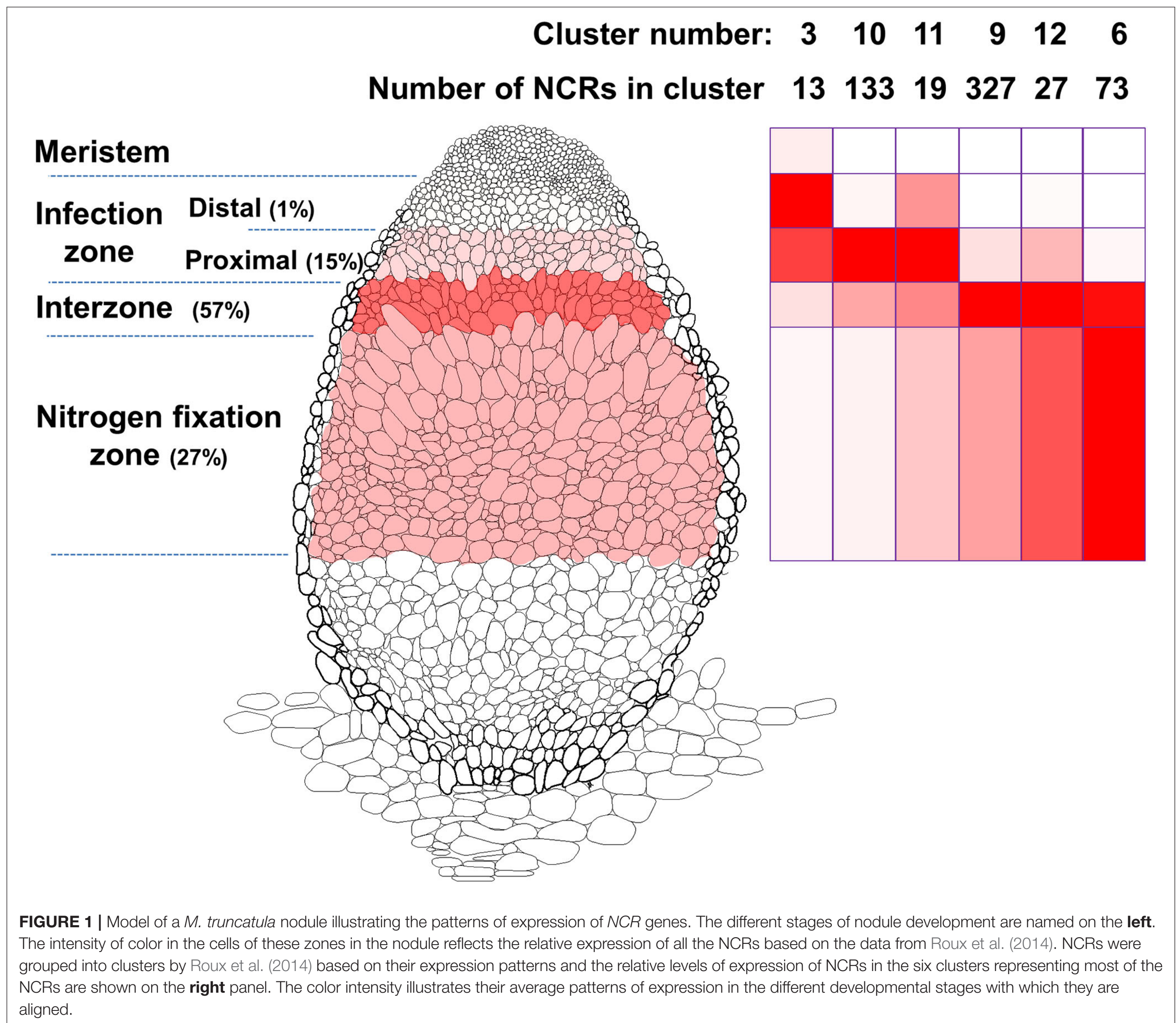


FIGURE 1 | Model of a *M. truncatula* nodule illustrating the patterns of expression of *NCR* genes. The different stages of nodule development are named on the **left**. The intensity of color in the cells of these zones in the nodule reflects the relative expression of all the NCRs based on the data from Roux et al. (2014). NCRs were grouped into clusters by Roux et al. (2014) based on their expression patterns and the relative levels of expression of NCRs in the six clusters representing most of the NCRs are shown on the **right** panel. The color intensity illustrates their average patterns of expression in the different developmental stages with which they are aligned.

inverted repeat region indicating a deep phylogenetic split with other legumes (Wojciechowski et al., 2004); consequently, they are referred to as the "Inverted-Repeat-Lacking Clade" (IRLC) of legumes.

"Determinate" nodules such as those formed on soybean, *Phaseolus* bean and *Lotus* spp. are spherical and develop as a consequence of transient cell proliferation; so all infected cells in mature nodules are essentially at the same developmental stage.

NCR PEPTIDES

Peptides Control Rhizobial Development in Some but Not Other Legumes

In the nodules of IRLC clade legumes such as pea and *Medicago* spp., nitrogen-fixing bacteria (bacteroids) become terminally differentiated and cannot be cultured (Mergaert et al., 2006), whereas with legumes like soybean and *L. japonicus*, nitrogen-fixing rhizobia do not terminally differentiate and can regrow (Gresshoff and Rolfe, 1978). Terminally differentiated nitrogen-fixing bacteroids show chromosomal endoreduplication, arrest of cell division, cell enlargement and changes in cell walls, membrane permeability, and patterns of gene expression (Batut et al., 2011; Kereszt et al., 2011; Haag et al., 2013; Kondorosi et al., 2013; Maroti and Kondorosi, 2014; Alunni and Gourion, 2016). Many of these changes are caused by plant-made small defensin-like peptides. This has been best characterized in *M. truncatula* in which there are two gene families, one (of about 650) encoding Nodule-specific Cysteine-Rich (NCR) peptides and one (of 24) encoding Glycine-Rich Peptides (GRPs). Similar genes have been identified in other members of the IRLC legumes (Kevei et al.,

2002; Graham et al., 2004; Alunni et al., 2007; Montiel et al., 2017) but neither NCR nor GRP-encoding genes could be identified in the sequenced genomes of *L. japonicus* and soybean (Alunni et al., 2007). The NCRs are delivered through the plant-made membrane surrounding the bacteroids *via* a plant-determined secretion complex that recognizes the highly conserved N-terminal secretion signal on NCR peptides (Wang et al., 2010; Stonoha-Arther and Wang, 2018). The mature peptides (usually 35–55 residues) then enter the bacteroids, inducing the changes associated with terminal differentiation (Mergaert et al., 2006; Van de Velde et al., 2010; Tiricz et al., 2013; Farkas et al., 2014; Penterman et al., 2014). The effects of NCR peptides have been reviewed recently (Mergaert et al., 2020; Roy et al., 2020).

NCR Peptides: Activity and Rhizobial Protection Mechanisms

Most eukaryotes produce anti-microbial defensin peptides and these fall into different groups including cysteine-rich peptides that bind to microbial proteins. In such defensins, cysteine crosslinks hold together α-helical and β sheet regions such that highly variable "interactive" domains can bind efficiently to proteins (Shafee et al., 2017). NCRs are similar to, but different from most cysteine–rich defensins, usually having 4 or 6 cysteine residues rather than 8 or 10 seen in true defensins (Maroti et al., 2015). The sequences of NCR peptides are highly diverse and fall into cationic, anionic, and neutral groupings. The NMR-derived structure of NCR044 produced in *Pichia pastoris* revealed that it was mostly disordered, highly dynamic and internally cross-linked via *two* disulfide bonds that linked two antiparallel beta strands and linked one of these to a very short potential alpha helix. NCR044 entered cells of the fungal pathogen *Botrytis cinerea* via discrete membrane foci causing a loss of turgor and the production of reactive oxygen species (Velivelli et al., 2020). Another peptide (NCR247) can bind to multiple proteins in bacteroids (Farkas et al., 2014) and this is associated with inhibition of transcription, translation and cell division (Farkas et al., 2014; Penterman et al., 2014; Shabab et al., 2016). Cysteine crosslinking within NCR247 was essential for effects on transcription, was important but not essential for inhibition of translation and was not required for inhibition of cell division (Haag et al., 2011, 2012; Shabab et al., 2016). Rhizobia require some degree of protection against such potentially widespread disruptive effects. Some *S. meliloti* strains have a plasmid-encoded peptidase (HrrP), that suppresses nitrogen fixation on some *Medicago* species but not on others (Crook et al., 2012; Price et al., 2015). HrrP can degrade several NCR peptides resulting in premature nodule senescence and a defective symbiosis, similar to the phenotypes caused by mutations in *NCR* genes (see below). Another mechanism conferring protection against NCRs is mediated via rhizobial *bacA* (or *bclA*) genes that are essential for terminal bacteroid development in IRLC legumes but not in legumes in which nitrogen-fixing rhizobia can regrow (Glazebrook et al., 1993; Karunakaran et al., 2010; Maunoury et al., 2010; Guefrachi et al., 2015; Barriere et al., 2017). BacA may play a role in the transport of peptides, and the *bacA* mutation increased sensitivity to the stable folded form of NCR247 (Haag et al., 2011). BacA and BclA have been implied to act either by importing NCR peptides to remove them from the bacterial cell surface (a likely site of activity), or by exporting them to reduce their cytoplasmic toxicity (Haag et al., 2011, 2013; Barriere et al., 2017). Mutations affecting rhizobial cell envelope polysaccharides, inner and outer membrane proteins and transcriptional regulators affect the sensitivity to NCR247 (Arnold et al., 2017, 2018).

Different Legumes Have Widely Differing Numbers of NCR Peptides

*M. truncat*ula has >650 NCR peptides predicted from genome sequencing (Young et al., 2011) of which about 600 were identified in RNA sequencing of nodule tissue (Roux et al., 2014). The expression of NCR peptides in nodules was reduced by added nitrate (Liese et al., 2017; Schulze et al., 2020) in parallel with nitrate-induced decreases in other nodule proteins. Mutations in specific NCR genes (Horvath et al., 2015; Kim et al., 2015), or affecting the secretion complex that delivers the peptides to bacteroids, abolish symbiotic nitrogen fixation (Van de Velde et al., 2010; Wang et al., 2010).

IRLC legumes express different numbers of NCRs; 7 were identified in *Glycyrrhiza uralensis* (Chinese licorice), 63 in *Cicer arietinum* (chickpea), 353 in *Pisum sativum* (pea), and 469 in *M. sativa* (alfalfa). The degree of bacteroid differentiation in the tested legumes correlated with the number and composition of NCR peptides (Montiel et al., 2016, 2017). The ability of the legumes to induce bacteroid swelling (an aspect of differentiation) was predicted to be acquired independently in at least five independent lineages and bacteroid morphotypes even within a single legume can vary (Oono et al., 2010). Such differentiation is not necessarily imposed on all rhizobia nodulating legumes in the IRLC clade. For example, in *G. uralensis* nodules *Sinorhizobium fredii* strain HH103 did not show the signs of bacteroid differentiation (Crespo-Rivas et al., 2016) that were seen with *Mesorhizobium tianenshenense* isolated from *G. uralensis* nodules (Montiel et al., 2016, 2017). This fits with the observation that *S. fredii* HH103 showed little *in-vitro* sensitivity to NCR peptides (Crespo-Rivas et al., 2016), that are toxic to other rhizobia (Tiricz et al., 2013). However, only cationic NCR peptides with a pI > 9.5 have so far been confirmed to have antimicrobial activity (Van de Velde et al., 2010; Ordogh et al., 2014) and *G. uralensis* lacks such NCRs.

Phylogenetic analyses indicated there were many legume-species-specific NCRs (Montiel et al., 2017). Such independent evolution of NCR peptides is consistent with the conclusion that there has been convergent evolution of endosymbiont differentiation driven by NCR-like peptides in the Dalbergoid clade of legumes that evolved separately from the IRLC clade (Czernic et al., 2015). However, the diversity in number and sequences of NCR peptides also point toward rapid evolution and diversification of this group of genes; the chromosomal clustering of groups of NCR genes and pseudogenes with related sequences in *M. truncatula* (Young et al., 2011) indicates that this diversification occurs *via* gene duplication. A comparison of 26 mature NCR sequences from different accessions of *M.*

truncatula revealed a relatively high pattern of diversifying selection consistent with recent and rapid evolution leading to new functions of NCRs (Nallu et al., 2013). A comparison of NCR peptides in pea and lentil also indicated parallel evolution of NCR peptides and *NCR* genes arising from gene duplication (Duran et al., 2021). These observations imply that a single rhizobial genotype can encounter different NCR peptides in nodules within the same cross-inoculation group of legumes.

NCR Genes Regulate Bacteroid Development and Rhizobial Strain Discrimination

The rapid evolution and selection of NCRs implies that they confer a strong selective advantage. However, the absence of NCR peptides in several nitrogen-fixing legumes shows that NCR peptides are not required *per se* for symbiotic nitrogen fixation. Therefore, IRLC legumes probably use NCRs to manipulate rhizobia to optimize nitrogen fixation. The distribution of NCRs and the effects of mutations in individual NCR genes raise a few paradoxes. Firstly, NCRs have antimicrobial activity *in vitro* and yet the loss of individual NCR genes causes rapid senescence of bacteroids (Horvath et al., 2015; Kim et al., 2015). Secondly, although mutations in individual NCRs such as NCR169 and NCR211 cause rapid bacteroid senescence, these NCRs are not required for nitrogen fixation in those legumes lacking all NCRs. Thirdly, why should some legumes such as *G. uralensis* manage with few (Montiel et al., 2017) when *M. truncatula* has >600 nodule-expressed NCR genes?

One reason for having different NCR genes could relate to their expression pattern. **Figure 1** was made based on RNA sequencing and identification of gene expression clusters of *NCR* genes (Roux et al., 2014). It shows the pattern of expression of about 600 NCRs in different tissues of *M. truncatula* nodules; analysis of these data reveals that cationic NCR peptides with antimicrobial activity are restricted to the interzone and nitrogen fixing zone. Few (if any) *NCR* genes are expressed in the nodule meristem. Some *NCRs* are expressed in the distal part of the infection zone, in which many rhizobia remain in infection threads or some have recently been endocytosed into plant cells. Most rhizobia are released from infection threads in the proximal infection zone, where about 15% of the nodule-expressed *NCR* genes are induced. Most (56%) *NCR* genes are expressed in the interzone, which corresponds to the region of the nodule in which *S. meliloti* bacteroids are differentiating. Thus, over 70% of the *NCR* genes are induced before the onset of nitrogen fixation and many of these genes are switched off in the nitrogen fixation zone. The transient pattern of transcription of many *NCR* genes is correlated with the increasing ploidy levels of both the differentiating rhizobia (Mergaert et al., 2006) and the plant cells as they differentiate (Nagymihaly et al., 2017).

Different NCRs may enable legumes to discriminate against some rhizobia. Rhizobial-specific nitrogen fixation appears to be common among different accessions of *M. truncatula* (Liu et al., 2014) and discrimination between some rhizobial strains can be attributed directly to the presence of specific *NCR* genes (Wang et al., 2017, 2018; Yang et al., 2017). As observed in alfalfa and *M. truncatula*, different NCRs can be induced by different rhizobial strains (Burghardt et al., 2017; Kang et al., 2020). Mutation of some *NCR* genes conferred nitrogen fixation on a strain that was unable to fix nitrogen in plants carrying the *NCR* gene. One of these *NCR* genes was expressed in the proximal infection and transitional zones (Yang et al., 2017). This is an odd stage to apply sanctions to some rhizobia, because it follows the commitment to nodule development, but precedes an assessment of effectiveness of nitrogen fixation. Perhaps the effects of some NCRs could be an undesirable consequence of NCR function. A focus of future research will be to identify the modes of action of different NCR peptides.

DISCUSSION

Why Are There So Many NCRs in Some Legumes?

To address this, we should consider the potential roles of NCR peptides. Some cationic peptides cause membrane depolarization and disintegration *in vitro* (Tiricz et al., 2013). This may be due to the relatively high concentrations used; at low concentrations labeled NCR247 could enter cells without causing membrane damage but at higher concentrations increased membrane permeability. It seems unlikely that *M. truncatula* would produce >600 proteins primarily causing membrane permeabilization and so these effects are probably not the primary role of most NCR peptides. Tagged NCR247 (Farkas et al., 2014) pulled down proteins associated with at least nine separate complexes (Ribosomal proteins, FtsZ, GroEL, Pyruvate dehydrogenase, transaldolase, RNA polymerase, Elongation factors, a Maf-like protein, and nitrogenase). Thus, some NCRs, like antimicrobial peptides, are probably "sticky" and can bind to many other proteins, possibly inhibiting or modulating their function. If even only 10% of the 600 or so NCRs expressed in *M. truncatula* nodules are "sticky," then these NCRs have the potential to modulate activity of over a 100 rhizobial proteins. Why should some legumes evolve so many NCR peptides and to what end? Three reasons for having NCRs could be: (1) The NCRs may constrain rhizobial growth in nodules. (2) The NCRs may play a role in selection against cheaters (non-nitrogen fixing bacteria that get into nodules) or to select against rhizobia with poor nitrogen fixation. (3) Plants may use NCRs to take control of bacteroid development (and possibly aspects of metabolism) to optimize nitrogen fixation. Since each would induce positive selection, it is probable that some legumes combine aspects of each.

How might this have evolved? How can we explain why the loss of a single NCR can block nitrogen fixation, whereas other legumes fix nitrogen without that specific NCR? Let us assume that an early role of NCRs was to suppress growth in nodules of cheaters (or rhizobia that are poor at N-fixation). A nodule-expressed NCR may have evolved from a defensin to suppress growth of a cheater by targeting a protein that is absent from (or sufficiently different from that of) the rhizobial endosymbiont. This could confer symbiotic benefit and

subsequent gene duplication and diversification could allow the acquisition of several related NCRs targeting different cheaters. However, the observation that a single NCR can bind to several targets implies that some side effects might negatively affect the symbiosis, for example by causing accumulation of some intermediate that limits symbiotic nitrogen fixation. If this did occur, the plant could resolve the problem by losing the NCR peptide. Alternatively, it could in theory, suppress the negative side effects by acquiring another NCR peptide that could down-regulate more of the pathway thereby decreasing accumulation of the problematic intermediate. It is possible that the acquisition of several NCRs could enable the plant to control different aspects of rhizobial development (and possibly metabolism) such that there would be selection against cheaters and optimization of nitrogen fixation. However, once such a control network had developed it could easily be associated with an interdependence of NCRs, such that e.g., if the first NCR was lost (e.g., by mutation) then an imbalance in the control network due to its loss, could result in a poor or ineffective symbiosis as seen with loss of NCR 169 or NCR211. Acquisition of NCRs that benefit some rhizobial strains could be detrimental to the symbiosis established with others. Therefore, there is the potential for both positive and negative selection, which could explain the observed diversifying selection (Nallu et al., 2013).

Why Are NCR Genes Absent From Many Legumes?

If the acquisition of NCRs is of some benefit to legumes in the IRLC species, why are they not present in legumes such as soybean and *Lotus* spp? One possibility is that there may be a disadvantage for legumes to impose terminal differentiation on bacteroids in legumes like soybean, that lack persistent infection threads or other means of protecting some of the rhizobia from NCRs. Indeterminate nodules such as those in *Medicago* spp. have many persistent infection threads from which rhizobia are released, and these released rhizobia then enlarge mostly without undergoing cell division. Rhizobia within infection threads in *M. truncatula* do not differentiate, implying that these rhizobia are not exposed to NCRs (Mergaert et al., 2006). Therefore, the bacteria cultured from such nodules are probably those from within the infection threads, which are seen in sections of almost all infected cells.

In contrast, rhizobia released into cells of determinate nodules as in soybean do divide (Goodchild and Bergersen, 1966) and so infection threads can be much reduced and shorter compared with the complex architecture of infection threads seen e.g., in *M. trunctula* (Gage, 2004; Monahan-Giovanelli et al., 2006). Therefore, if a legume with determinate nodules acquired NCRs that induce terminal bacteroid differentiation, relatively few bacteria would be protected within infection threads. A successful symbiosis requires mutual benefit to both partners; if the legume were to impose constraints on bacteroid survival, such that there were insufficient rhizobia that could escape from senescing nodules, then a key aspect of the symbiosis, namely rhizobial benefit, would be lost. Thus, any advantage of controlling bacterial growth by inducing terminal differentiation could be outweighed by the disadvantage of having two few rhizobia surviving the symbiosis. It remains to be established whether, in diverse legumes, there is a correlation between protection of rhizobia within infection threads and acquisition of terminal differentiation of bacteroids induced by NCR peptides.

Future Perspectives

Key questions remain with regard to understanding the functions of NCR peptides acting alone and/or in combination. One problem is that they can bind many proteins (including other NCRs), but several of the observed interactions may be spurious and have no effect in nodules. Another issue is that the targets of NCRs may be expressed only in nitrogen-fixing bacteroids, making it difficult to use rhizobial genetics to identify their targets. So how can the analysis of the physiological functions of NCRs be addressed? One approach could be to analyze NCR function in IRLC legumes that express few NCRs, but getting the molecular genetics systems established for such legumes could be difficult. Another approach as suggested (Van de Velde et al., 2010) could be to introduce NCRs from IRLC legumes into a transformable non-IRLC legume such as *L. japonicus* or a promiscuous legume such as *Phaseolus vulgaris*. Although such a transgenic legume may be compromised for rhizobial survival in nodules, this would not be a problem with laboratory-maintained plants. Such an approach could be feasible using NCR genes from an IRLC legume that has relatively few NCRs that can induce terminal bacteroid differentiation. The technology is now in place to allow legume transformation using single constructs carrying multiple genes, so it should be possible to introduce multiple NCR genes into a transformable IRLC legume such as *L. japonicus* and determine effects on bacteroid differentiation and symbiotic nitrogen fixation.

AUTHOR CONTRIBUTIONS

JAD and EK discussed the ideas and edited the manuscript prior to submission. JAD drafted the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

JAD was a recipient of a grant from the Hungarian Academy of Sciences to facilitate a working visit to the laboratory of EK. EK is a recipient of the NKFIH Frontline Research project KKP129924 and the Balzan research grant.

ACKNOWLEDGMENTS

We thank Jeremy Murray for help in preparation of the figure and the colleagues of EK for helpful discussions. This article is dedicated to the memory of our friend and colleague Tomás Ruiz Argüeso.

REFERENCES

Alunni, B., and Gourion, B. (2016). Terminal bacteroid differentiation in the legume-rhizobium symbiosis: nodule-specific cysteine-rich peptides and beyond. *N. Phytolol*. 211, 411–417. doi: 10.1111/nph.14025

Alunni, B., Kevei, Z., Redondo-Nieto, M., Kondorosi, A., Mergaert, P., and Kondorosi, E. (2007). Genomic organization and evolutionary insights on GRP and NCR genes, two large nodule-specific gene families in *Medicago truncatula*. *Mol. Plant Microbe Interact*. 20, 1138–1148. doi: 10.1094/MPMI-20-9-1138

Arnold, M. F. F., Penterman, J., Shabab, M., Chen, E. J., and Walker, G. C. (2018). Important late-stage symbiotic role of the *Sinorhizobium meliloti* exopolysaccharide succinoglycan. *J. Bacteriol*. 200, e00665–e00617. doi: 10.1128/JB.00665-17

Arnold, M. F. F., Shabab, M., Penterman, J., Boehme, K. L., Griffitts, J. S., and Walker, G. C. (2017). Genome-wide sensitivity analysis of the microsymbiont *Sinorhizobium meliloti* to symbiotically important, defensin-like host peptides. *mBio* 8, e01060-17. doi: 10.1128/mBio.01060-17

Barriere, Q., Guefrachi, I., Gully, D., Lamouche, F., Pierre, O., Fardoux, J., et al. (2017). Integrated roles of BclA and DD-carboxypeptidase 1 in Bradyrhizobium differentiation within NCR-producing and NCR-lacking root nodules. *Sci. Rep*. 7:9063. doi: 10.1038/s41598-017-08830-0

Batut, J., Mergaert, P., and Masson-Boivin, C. (2011). Peptide signalling in the Rhizobium-legume symbiosis. *Curr. Opin. Microbiol*. 14, 181–187. doi: 10.1016/j.mib.2010.12.010

Burghardt, L. T., Guhlin, J., Chun, C. L., Liu, J. Q., Sadowsky, M. J., Stupar, R. M., et al. (2017). Transcriptomic basis of genome by genome variation in a legume-rhizobia mutualism. *Mol. Ecol*. 26, 6122–6135. doi: 10.1111/mec.14285

Crespo-Rivas, J. C., Guefrachi, I., Mok, K. C., Villaecija-Aguilar, J. A., Acosta-Jurado, S., Pierre, O., et al. (2016). Sinorhizobium fredii HH103 bacteroids are not terminally differentiated and show altered O-antigen in nodules of the Inverted Repeat-Lacking Clade legume Glycyrrhiza uralensis. *Environ. Microbiol*. 18, 2392–2404. doi: 10.1111/1462-2920.13101

Crook, M. B., Lindsay, D. P., Biggs, M. B., Bentley, J. S., Price, J. C., Clement, S. C., et al. (2012). Rhizobial plasmids that cause impaired symbiotic nitrogen fixation and enhanced host invasion. *Mol. Plant Microbe Interact*. 25, 1026–1033. doi: 10.1094/MPMI-02-12-0052-R

Czernic, P., Gully, D., Cartieaux, F., Moulin, L., Guefrachi, I., Patrel, D., et al. (2015). Convergent evolution of endosymbiont differentiation in Dalbergioid and Inverted Repeat-Lacking Clade legumes mediated by nodule-specific cysteine-rich peptides. *Plant Physiol*. 169, 1254–1265. doi: 10.1104/pp.15.00584

Duran, D., Albareda, M., Garcia, C., Marina, A.-I., Ruiz-Argueso, T., and Palacios, J.-M. (2021). Proteome analysis reveals a significant host-specific response in *Rhizobium leguminosarum* bv. *viciae* endosymbiotic cells. *Mol. Cell Proteomics* 20:100009. doi: 10.1074/mcp.RA120.002276

Farkas, A., Maroti, G., Durgo, H., Gyorgypal, Z., Lima, R. M., Medzihradszky, K. F., et al. (2014). *Medicago truncatula* symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5183–5188. doi: 10.1073/pnas.1404169111

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev*. 68, 280–300. doi: 10.1128/MMBR.68.2.280-300.2004

Glazebrook, J., Ichige, A., and Walker, G. C. (1993). A Rhizobium meliloti homolog of the *Escherichia coli* peptide-antibiotic transport protein SbmA is essential for bacteroid development. *Genes Dev*. 7, 1485–1497. doi: 10.1101/gad.7.8.1485

Goodchild, D. J., and Bergersen, F. J. (1966). Electron microscopy of the infection and subsequent development of soybean nodule cells. *J. Bacteriol*. 92, 204–213. doi: 10.1128/JB.92.1.204-213.1966

Graham, M. A., Silverstein, K. A., Cannon, S. B., and VandenBosch, K. A. (2004). Computational identification and characterization of novel genes from legumes. *Plant Physiol*. 135, 1179–1197. doi: 10.1104/pp.104.037531

Gresshoff, P. M., and Rolfe, B. G. (1978). Viability of Rhizobium bacteroids isolated from soybean nodule protoplasts. *Planta* 142, 329–333. doi: 10.1007/BF00385085

Guefrachi, I., Pierre, O., Timchenko, T., Alunni, B., Barriere, Q., Czernic, P., et al. (2015). Bradyrhizobium BclA is a peptide transporter required for bacterial differentiation in symbiosis with Aeschynomene legumes. *Mol. Plant Microbe Interact*. 28, 1155–1166. doi: 10.1094/MPMI-04-15-0094-R

Haag, A. F., Arnold, M. F., Myka, K. K., Kerscher, B., Dall'Angelo, S., Zanda, M., et al. (2013). Molecular insights into bacteroid development during Rhizobium-legume symbiosis. *FEMS Microbiol. Rev*. 37, 364–383. doi: 10.1111/1574-6976.12003

Haag, A. F., Baloban, M., Sani, M., Kerscher, B., Pierre, O., Farkas, A., et al. (2011). Protection of Sinorhizobium against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biol*. 9:e1001169. doi: 10.1371/journal.pbio.1001169

Haag, A. F., Kerscher, B., Dall'Angelo, S., Sani, M., Longhi, R., Baloban, M., et al. (2012). Role of cysteine residues and disulfide bonds in the activity of a legume root nodule-specific, cysteine-rich peptide. *J. Biol. Chem*. 287, 10791–10798. doi: 10.1074/jbc.M111.311316

Horvath, B., Domonkos, A., Kereszt, A., Szucs, A., Abraham, E., Ayaydin, F., et al. (2015). Loss of the nodule-specific cysteine rich peptide, NCR169, abolishes symbiotic nitrogen fixation in the *Medicago truncatula* dnf7 mutant. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15232–15237. doi: 10.1073/pnas.1500777112

Kang, W. J., Jiang, Z. H., Chen, Y. G., Wu, F., Liu, C., Wang, H. F., et al. (2020). Plant transcriptome analysis reveals specific molecular interactions between alfalfa and its rhizobial symbionts below the species level. *BMC Plant Biol*. 20:293. doi: 10.1186/s12870-020-02503-3

Karunakaran, R., Haag, A. F., East, A. K., Ramachandran, V. K., Prell, J., James, E. K., et al. (2010). BacA is essential for bacteroid development in nodules of galegoid, but not phaseoloid, legumes. *J. Bacteriol*. 192, 2920–2928. doi: 10.1128/JB.00020-10

Kereszt, A., Mergaert, P., and Kondorosi, E. (2011). Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Mol. Plant Microbe Interact*. 24, 1300–1309. doi: 10.1094/MPMI-06-11-0152

Kevei, Z., Vinardell, J. M., Kiss, G. B., Kondorosi, A., and Kondorosi, E. (2002). Glycine-rich proteins encoded by a nodule-specific gene family are implicated in different stages of symbiotic nodule development in *Medicago* spp. *Mol. Plant Microbe Interact*. 15, 922–931. doi: 10.1094/MPMI.2002.15.9.922

Kim, M., Chen, Y. H., Xi, J. J., Waters, C., Chen, R. J., and Wang, D. (2015). An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15238–15243. doi: 10.1073/pnas.1500123112

Kondorosi, E., Mergaert, P., and Kereszt, A. (2013). A paradigm for endosymbiotic life: cell differentiation of Rhizobium bacteria provoked by host plant factors. *Annu. Rev. Microbiol*. 67, 611–628. doi: 10.1146/annurev-micro-092412-155630

Liese, R., Schulze, J., and Cabeza, R. A. (2017). Nitrate application or P deficiency induce a decline in *Medicago truncatula* N2-fixation by similar changes in the nodule transcriptome. *Sci. Rep*. 7:46264. doi: 10.1038/srep46264

Liu, J. G., Yang, S. M., Zheng, Q. L., and Zhu, H. Y. (2014). Identification of a dominant gene in *Medicago truncatula* that restricts nodulation by Sinorhizobium memiloti strain Rm41. *BMC Plant Biol*. 14:167. doi: 10.1186/1471-2229-14-167

Maroti, G., Downie, J. A., and Kondorosi, E. (2015). Plant cysteine-rich peptides that inhibit pathogen growth and control rhizobial differentiation in legume nodules. *Curr. Opin. Plant Biol*. 26, 57–63. doi: 10.1016/j.pbi.2015.05.031

Maroti, G., and Kondorosi, E. (2014). Nitrogen-fixing Rhizobium-legume symbiosis: are polyploidy and host peptide-governed symbiont differentiation general principles of endosymbiosis? *Front. Microbiol*. 5:326. doi: 10.3389/fmicb.2014.00326

Maunoury, N., Redondo-Nieto, M., Bourcy, M., Van de Velde, W., Alunni, B., Laporte, P., et al. (2010). Differentiation of symbiotic cells and endosymbionts in *Medicago truncatula* nodulation are coupled to two transcriptome-switches. *PLoS ONE* 5:e9519. doi: 10.1371/journal.pone.0009519

Mergaert, P., Kereszt, A., and Kondorosi, E. (2020). Gene expression in nitrogen-fixing symbiotic nodule cells in *Medicago truncatula* and other nodulating plants. *Plant Cell* 32, 42–68. doi: 10.1105/tpc.19.00494

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., et al. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5230–5235. doi: 10.1073/pnas.0600912103

Monahan-Giovanelli, H., Pinedo, C. A., and Gage, D. J. (2006). Architecture of infection thread networks in developing root nodules induced by the symbiotic bacterium *Sinorhizobium meliloti* on *Medicago truncatula*. *Plant Physiol*. 140, 661–670. doi: 10.1104/pp.105.072876

Montiel, J., Downie, J. A., Farkas, A., Bihari, P., Herczeg, R., Balint, B., et al. (2017). Morphotype of bacteroids in different legumes correlates with the number and type of symbiotic NCR peptides. *Proc. Natl. Acad. Sci. U.S.A.* 114, 5041–5046. doi: 10.1073/pnas.1704217114

Montiel, J., Szucs, A., Boboescu, I. Z., Gherman, V. D., Kondorosi, E., and Kereszt, A. (2016). Terminal bacteroid differentiation is associated with variable morphological changes in legume species belonging to the inverted repeat-lacking clade. *Mol. Plant Microbe Interact*. 29, 210–219. doi: 10.1094/MPMI-09-15-0213-R

Nagymihaly, M., Veluchamy, A., Gyorgypal, Z., Ariel, F., Jegu, T., Benhamed, M., et al. (2017). Ploidy-dependent changes in the epigenome of symbiotic cells correlate with specific patterns of gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 114, 4543–4548. doi: 10.1073/pnas.1704211114

Nallu, S., Silverstein, K. A. T., Samac, D. A., Bucciarelli, B., Vance, C. P., and VandenBosch, K. A. (2013). Regulatory patterns of a large family of defensin-like genes expressed in nodules of *Medicago truncatula*. *PLoS ONE* 8:e60355. doi: 10.1371/journal.pone.0060355

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., and Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet*. 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Oono, R., Schmitt, I., Sprent, J. I., and Denison, R. F. (2010). Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. *N. Phytol*. 187, 508–520. doi: 10.1111/j.1469-8137.2010.03261.x

Ordogh, L., Voros, A., Nagy, I., Kondorosi, E., and Kereszt, A. (2014). Symbiotic plant peptides eliminate *Candida albicans* both *in vitro* and in an epithelial infection model and inhibit the proliferation of immortalized human cells. *Biomed. Res. Int*. 2014, 320796. doi: 10.1155/2014/320796

Penterman, J., Abo, R. P., De Nisco, N. J., Arnold, M. F. F., Longhi, R., Zanda, M., et al. (2014). Host plant peptides elicit a transcriptional response to control the *Sinorhizobium meliloti* cell cycle during symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 111, 3561–3566. doi: 10.1073/pnas.1400450111

Price, P. A., Tanner, H. R., Dillon, B. A., Shabab, M., Walker, G. C., and Griffitts, J. S. (2015). Rhizobial peptidase HrrP cleaves host-encoded signaling peptides and mediates symbiotic compatibility. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15244–15249. doi: 10.1073/pnas.1417797112

Roux, B., Rodde, N., Jardinaud, M. F., Timmers, T., Sauviac, L., Cottret, L., et al. (2014). An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J*. 77, 817–837. doi: 10.1111/tpj.12442

Roy, P., Achom, M., Wilkinson, H., Lagunas, B., and Gifford, M. L. (2020). Symbiotic outcome modified by the diversification from 7 to over 700 nodule-specific cysteine-rich peptides. *Genes* 11:348. doi: 10.3390/genes11040348

Schulze, J., Liese, R., Ballesteros, G., Casieri, L., Salinas, G., and Cabeza, R. A. (2020). Ammonium acts systemically while nitrate exerts an additional local effect on *Medicago truncatula* nodules. *Plant Sci*. 292:110383. doi: 10.1016/j.plantsci.2019.110383

Shabab, M., Arnold, M. F., Penterman, J., Wommack, A. J., Bocker, H. T., Price, P. A., et al. (2016). Disulfide cross-linking influences symbiotic activities of nodule peptide NCR247. *Proc. Natl. Acad. Sci. U.S.A.* 113, 10157–10162. doi: 10.1073/pnas.1610724113

Shafee, T. M. A., Lay, F. T., Phan, T. K., Anderson, M. A., and Hulett, M. D. (2017). Convergent evolution of defensin sequence, structure and function. *Cell. Mol. Life Sci*. 74, 663–682. doi: 10.1007/s00018-016-2344-5

Sprent, J. I. (2001). *Nodulation in Legumes*. London: Royal Botanic Gardens, Kew.

Stonoha-Arther, C., and Wang, D. (2018). Tough love: accommodating intracellular bacteria through directed secretion of antimicrobial peptides during the nitrogen-fixing symbiosis. *Curr. Opin. Plant Biol*. 44, 155–163. doi: 10.1016/j.pbi.2018.04.017

Tiricz, H., Szucs, A., Farkas, A., Pap, B., Lima, R. M., Maroti, G., et al. (2013). Antimicrobial nodule-specific cysteine-rich peptides induce membrane depolarization-associated changes in the transcriptome of *Sinorhizobium meliloti*. *Appl. Env. Microbiol*. 79, 6737–6746. doi: 10.1128/AEM.01791-13

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., et al. (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* 327, 1122–1126. doi: 10.1126/science.1184057

Velivelli, S. L. S., Czymmek, K. J., Li, H., Shaw, J. B., Buchko, G. W., and Shah, D. M. (2020). Antifungal symbiotic peptide NCR044 exhibits unique structure and multifaceted mechanisms of action that confer plant protection. *Proc. Natl. Acad. Sci. U.S.A.* 117, 16043–16054. doi: 10.1073/pnas.2003526117

Wang, D., Griffitts, J., Starker, C., Fedorova, E., Limpens, E., Ivanov, S., et al. (2010). A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis. *Science* 327, 1126–1129. doi: 10.1126/science.1184096

Wang, Q., Liu, J. G., Li, H., Yang, S. M., Kormoczi, P., Kereszt, A., et al. (2018). Nodule-specific cysteine-rich peptides negatively regulate nitrogen-fixing symbiosis in a strain-specific manner in *Medicago truncatula*. *Mol. Plant Microbe Interact*. 31, 240–248. doi: 10.1094/MPMI-08-17-0207-R

Wang, Q., Yang, S. M., Liu, J. G., Terecskei, K., Abraham, E., Gombar, A., et al. (2017). Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. *Proc. Natl. Acad. Sci. U.S.A.* 114, 6854–6859. doi: 10.1073/pnas.1700715114

Wojciechowski, M. F., Lavin, M., and Sanderson, M. J. (2004). A phylogeny of legumes (Leguminosae) based on analysis of the plastid matK gene resolves many well-supported subclades within the family. *Am. J. Bot*. 91, 1846–1862. doi: 10.3732/ajb.91.11.1846

Yang, S. M., Wang, Q., Fedorova, E., Liu, J. G., Qin, Q. L., Zheng, Q. L., et al. (2017). Microsymbiont discrimination mediated by a host-secreted peptide in *Medicago truncatula*. *Proc. Natl. Acad. Sci. U.S.A.* 114, 6848–6853. doi: 10.1073/pnas.1700460114

Young, N. D., Debelle, F., Oldroyd, G. E. D., Geurts, R., Cannon, S. B., Udvardi, M. K., et al. (2011). The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* 480, 520–524. doi: 10.1038/nature10625

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.

Copyright © 2021 Downie and Kondorosi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

BRIEF RESEARCH REPORT
published: 29 June 2021
doi: 10.3389/fagro.2021.661534

Introduction of H_2-Uptake Hydrogenase Genes Into Rhizobial Strains Improves Symbiotic Nitrogen Fixation in *Vicia sativa* and *Lotus corniculatus* Forage Legumes

Mariana Sotelo[1†], *Ana Claudia Ureta*[2†‡], *Socorro Muñoz*[3†], *Juan Sanjuán*[3], *Jorge Monza*[1] *and Jose Palacios*[2*]

[1] Departamento de Biología Vegetal, Laboratorio de Bioquímica, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay, [2] Centro de Biotecnología y Genómica de Plantas UPM-INIA, Campus de Montegancedo, and Departamento de Biotecnología-Biología vegetal, ETSIAAB, Universidad Politécnica de Madrid, Madrid, Spain, [3] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Granada, Spain

OPEN ACCESS

Edited by:
Bahar S. Razavi,
University of Kiel, Germany

Reviewed by:
Mika Tapio Tarkka,
Helmholtz Center for Environmental Research (UFZ), Germany
Vinay Kumar,
Central University of Punjab, India

***Correspondence:**
Jose Palacios
jose.palacios@upm.es

† These authors have contributed equally to this work

‡Present address:
Ana Claudia Ureta,
División de Inocuidad y Calidad de Alimentos, Dirección General de Servicios Agrícolas, Ministerio de Ganadería, Agricultura y Pesca, Montevideo, Uruguay

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: 30 January 2021
Accepted: 01 June 2021
Published: 29 June 2021

Citation:
Sotelo M, Ureta AC, Muñoz S, Sanjuán J, Monza J and Palacios J (2021) Introduction of H_2-Uptake Hydrogenase Genes Into Rhizobial Strains Improves Symbiotic Nitrogen Fixation in Vicia sativa and Lotus corniculatus Forage Legumes. Front. Agron. 3:661534. doi: 10.3389/fagro.2021.661534

Biological nitrogen fixation by the *Rhizobium*-legume symbiosis allows the conversion of atmospheric nitrogen into ammonia within root nodules mediated by the nitrogenase enzyme. Nitrogenase activity results in the evolution of hydrogen as a result of a side reaction intrinsic to the activity of this enzyme. Some rhizobia, and also other nitrogen fixers, induce a NiFe uptake hydrogenase (Hup) to recycle hydrogen produced by nitrogenase, thus improving the efficiency of the nitrogen fixation process. In this work we report the generation and symbiotic behavior of hydrogenase-positive *Rhizobium leguminosarum* and *Mesorhizobium loti* strains effective in vetch (*Vicia sativa)* and birsfoot trefoil (*Lotus corniculatus*) forage crops, respectively. The ability of hydrogen recycling was transferred to these strains through the incorporation of *hup* minitransposon Tn*HB100*, thus leading to full recycling of hydrogen in nodules. Inoculation of *Vicia* and *Lotus* plants with these engineered strains led to significant increases in the levels of nitrogen incorporated into the host legumes. The level of improvement of symbiotic performance was dependent on the recipient strain and also on the legume host. These results indicate that hydrogen recycling has the potential to improve symbiotic nitrogen fixation in forage plants.

Keywords: hup genes, *Rhizobium leguminosarum*, *Mesorhizobium loti*, legume, inoculant

INTRODUCTION

Symbiotic nitrogen fixation, carried out by rhizobia expressing nitrogenase in association with legume plants, allows reducing the use of N fertilizers, thus positioning legume crops as key players in sustainable agriculture (Laranjo et al., 2014). Nitrogen fertilizers used in agriculture are incorporated into plants with low efficiency (40–50%) resulting in the release of an enormous amount of reactive nitrogen into the biosphere (Zhang et al., 2020). Forage legumes (alfalfa, clover, vetch, trefoil, etc.) can be used as cut fodder (either directly or after fermentation) or as grazed pasture, and are also relevant at a global scale for ruminant's feeding.

Common vetch (*Vicia sativa*) is a multi-purpose, cool season annual legume widely used in low- to medium rainfall areas as source of both forage and grain for animal feeding, due to its high protein content (Huang et al., 2017). *V. sativa* has a high potential of nitrogen fixation through its association with endosymbiotic bacteria. *V. rhizobia* were traditionally placed within *Rhizobium leguminosarum* species, but systematic analysis of isolates from different regions has revealed that other rhizobial species such as *R. sophorae* and *R. laguerreae*, are also endosymbionts of this legume (Zhang et al., 2019).

Lotus is a widely spread legume genus comprising 100–130 species originally native from Europe, Asia, Africa and Australia. Its current worldwide distribution is a consequence of human-driven introduction into non-native areas due to its interest as forage legume and its adaptability to different environmental stresses, which make it a relevant alternative in ecological restoration programs (Escaray et al., 2012). Just a few species, including *Lotus corniculatus, L. subbiflorus,* and *L. tenuis*, have been domesticated and subject to plant breeding programs for its use as forage for livestock. Among them, *L. corniculatus* is a major component of pastures in South America (Díaz et al., 2005). *Lotus* rhizobia are widely diverse, with over 20 species dispersed amongst several genera (*Mesorhizobium, Bradyrhizobium Rhizobium, Ensifer* and *Aminobacter;* Lorite et al., 2018). The study of the *Lotus* symbiosis has been greatly advanced by the establishment of *L. japonicus* as a model system for genetic and molecular studies (Tabata and Stougaard, 2014).

Growth of pastures based in forage legumes is closely associated with the rate of biological N fixation, since nitrogen is often the main limiting nutrient for herbage production (Phelan et al., 2015). Nitrogenase activity results in the evolution of hydrogen as a result of a side reaction of the enzyme, thus accounting for at least 25% of electrons channeled into the enzyme. As a consequence, nodules in root legumes evolve large amounts of hydrogen, which diffuses into the soil, constituting a source of inefficiency of the system; some rhizobial strains induce a hydrogenase system able to recycle the hydrogen produced by nitrogenase, thus improving the efficiency of the nitrogen fixation process (Ruiz-Argüeso et al., 2000; Palacios et al., 2005). This hydrogenase is a metalloenzyme with a complex NiFe heterobimetallic cofactor. As with other metalloenzymes such as nitrogenase, the synthesis of the NiFe hydrogenase is a complex process that, in the case of *R. leguminosarum* bv. viciae, requires 18 proteins whose genetic determinants (*hupSLCDEFGHIJKhypABFCDEX*) are clustered in the symbiotic plasmid of this bacterium (Palacios et al., 2005). Synthesis of hydrogenase is co-regulated with nitrogenase though the action of NifA, the master regulator of nitrogen fixation genes (Brito et al., 1997).

The cloning of the whole cluster of hydrogenase genes a cosmid (Leyva et al., 1987) allowed the introduction of hydrogen-uptake (Hup) activity into hydrogenase-deficient strains of different rhizobial species, including *R. leguminosarum, R. etli, and Mesorhizobium loti* (Brito et al., 2000). In order to avoid unstability of the introduced genes derived from its presence as an extrachromosomal entity, a mini-transposon (Tn*HB100*) including the whole set of *hup*/*hyp* genes along with a spectinomycin resistance marker gene was generated (Bascones et al., 2000). The use of this transposon allowed the generation of transposants inducing high levels of symbiotic hydrogenase in Hup^{-} recipient strains from *B. japonicum*, *R. etli*, and *R. leguminosarum* bv viciae, leading to nodules that evolved little or no hydrogen at all. In contrast, transposants obtained from *Sinorhizobium meliloti* and *M. cicer* carrying the same transposon expressed very low levels of hydrogenase activity, resulting in nodules evolving high levels of hydrogen (Bascones et al., 2000).

The evaluation of the effect of hydrogen recycling has been carried out in several systems, and positive effects on the level of nitrogen fixation had been shown in different legumes such as cowpea, and common beans (Baginski et al., 2005; Torres et al., 2020). These experiments had been carried out with grain legumes, and there are scarce data available on the effect of hydrogen recycling on forage legumes. Rhizobial strains effective in alfalfa and in clover lack hydrogenase activity. Strategies for heterologous expression of *hup* genes, either through plasmid transfer or Tn*HB100* transposon insertion, have been attempted without much success. The model *S. meliloti* / alfalfa system does not express hydrogenase under symbiotic conditions, even though vegetative cells of the same *Sinorhizobium* strain can express high levels of hydrogenase activity (Brito et al., 2002), so it was important to elucidate whether the same system can also improve the growth of forage legumes in which hydrogenase is expressed. In this work we have incorporated the *R. leguminosarum* hydrogenase genes into rhizobial strains effective on two forage legumes permissive for hydrogenase expression (*Lotus* and *Vicia*), and the effect of hydrogenase activity on symbiotic performance of the new strains has been determined.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

R. leguminosarum Rlv13 and Rlv21 were isolated from *V. sativa* root nodules at Zaidín Estacion Experimental (Granada, Spain). *M. loti* U261 (=NZP2037, Irisarri et al., 1996) was obtained from the Department of Scientific and Industrial Research, Palmerston South, New Zealand. *Rhizobium* and *Mesorhizobium* strains were grown at 28°C in tryptone-yeast extract (TY), yeast-manitol broth (YMB), or *Rhizobium* minimal media (Rmin) (Bascones et al., 2000). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium supplemented with the corresponding antibiotics. Minitransposon Tn*HB100*, carrying the entire *R. leguminosarum hup* cluster was transferred into (*Meso*)*Rhizobium* strains by conjugation as previously described (Bascones et al., 2000). For bacterial conjugations, fresh cell cultures of *E. coli* S17.1 λ*pir*(pTn*HB100*) donor (Simon et al., 1983) and rhizobial recipient strains were mixed on TY plates and incubated overnight at 28°C. Transconjugants were selected on Rmin plates supplemented with 200 μg/ml of spectinomycin.

Plant Material and Growth Conditions

Seeds of *L. corniculatus cv. La Estanzuela*, *L. tenuis*, *L. subbiflorus*, and *L. uliginosus* were surface sterilized with 10%

sodium hypochlorite and pregerminated at 28°C for 2 days in the dark. *V. sativa* cv. Filón seeds were sterilized with ethanol/sodium hypochlorite treatment and pregerminated as previously described (Brito et al., 2000). Three-day old seedlings were sown in pots containing vermiculite supplied with a nitrogen-free nutrient solution (Handberg and Stougaard, 1992; Brito et al., 1994) and inoculated with liquid cultures of rhizobial strains. Plants were maintained in a growth chamber under controlled conditions (16/8 h light/dark; Monza et al., 1992; Brito et al., 2000). Bacteroid supensions were prepared from freshly detached nodules as previously described (Brito et al., 2008).

Recombinant DNA Techniques

Plasmid DNA preparations, restriction enzyme digestions, DNA cloning, transformation of DNA into *E. coli*, agarose gel electrophoresis, Southern hybridizations, and PCR amplifications were performed by standard methods (Sambrook and Russell, 2002). Total DNA from rhizobial strains was isolated as described (Bascones et al., 2000). For the determination of nucleotide sequence flanking the Tn*HB100* insertions, the region was first cloned as a *Pst*I restriction fragment in a pBluescript vector plasmid (Agilent Technologies, La Jolla, CA), and the DNA sequence was determined using a primer complementary to the 3′ end of the spectinomycin resistance cassette (5′-GCTGGCTTTTTCTTGTTATCG-3′). Transposon insertions in Rlv13H9 and Rlv21H19 were located in genes encoding potential galactonate dehydratase/epimerases highly similar to protein RLV_6446 from *R. leguminosarum* bv viciae (Sanchez-Canizares et al., 2018). These proteins are moderately expressed in pea and lentil bacteroids induced by Rlv UPM791 (Duran et al., 2021), and might function in carbon metabolism pathway, so a potential effect of the gene inactivation on bacterial performance cannot be excluded. In contrast, insertion in Rlv13H6 was located in an intergenic region, with no similarity to described genes, whereas Rlv21H10 carried Tn*HB100* insertion in a hypothetical protein not expressed in bacteroids as deduced from a recent proteomic analysis (Duran et al., 2021). In the case of Ml U261H4, the site of insertion was located in an intergenic region downstream of a potassium uptake gene (*kup*) located at positions 2366770-2368683 of the genome (Kelly et al., 2014).

The removal of spectinomycin cassette from Rlv13 and Rlv21 Tn*HB100*-containing derivatives was carried out as described (Bascones et al., 2000). Briefly, DNA regions flanking the resistance marker on each strain were obtained and fused to *hupS* upstream region by restriction cloning in pK18mobSac suicide vector (Schäfer et al., 1994). Then, the plasmid was conjugated into the strains carrying the Tn*HB100* insertion, and a double recombination event excising Spc^R marker was obtained based on the selection provided by *sacB* gene.

Determination of Nitrogenase and Hydrogenase Activities and Plant Tests

Nitrogenase activity was determined by the acetylene reduction assay, and hydrogenase activity was estimated by measuring bacteroid hydrogen uptake amperometrically, or hydrogen evolution from nodules by gas chromatography (Brito et al., 2000, 2008) using detopped nodulated roots from 35 day-old plants. Plant dry weight was measured after maintaining shoots in an oven at 80°C for 48 h. Nitrogen content of *Lotus* plants was determined from detached leaves collected after 40, 60 and 70 days growing period and analyzed for total nitrogen with a mass spectrometer (IRMS Micromass Isochrom) at SIDI- Laboratorio de Isótopos Estables (Montevideo, Uruguay). Nitrogen content was calculated according with IAEA (IAEA, 2001). Nitrogen content of *Vicia* shoots was determined by a combustion method using a nitrogen analyzer (FP-528-LECO). Experiments were established in a completely random design with at least three replicates. Data were analyzed using ANOVA and means were compared using Fisher's least significant difference (LSD) test at 5% probability level.

RESULTS

Generation of Hydrogen-Recycling Strains

In order to analyze the potential effect of hydrogen recycling in symbiotic performance of forage legumes, the *R. leguminosarum* hydrogenase gene cluster was introduced into strains effective in *Vicia sativa* (*R. leguminosarum* bv viciae Rlv13 and Rlv21) and in *L. corniculatus* (*Mesorhizobium loti* U261, a broad-range *Lotus* inoculant strain). Stable insertion of *hup* gene package into the recipient strains was carried using transposon Tn*HB100*. Following conjugation with *E. coli* S17.1λ*pir*(pTn*HB100*), spectinomycin-resistant derivatives arose at frequencies of *ca.* 10^{-4}/recipient (Rlv strains) or 10^{-8} /recipient (Ml U261). The presence of *hup* genes in spectinomycin-resistant transconjugants was confirmed through identification of *hupS*-hybridizing bands in Southern experiments (**Supplementary Figure 1**) or through PCR amplification of specific bands corresponding to *hypB* gene using adequate primers (data not shown).

The ability of transposants to effectively recycle hydrogen evolved by nitrogenase was assessed in several derivatives per each recipient strain. In the case of strains effective in *V. sativa*, four derivatives obtained from each of Rlv13 and Rlv21 strains were analyzed for hydrogenase and nitrogenase activities in vetch nodules (**Table 1**). With one exception, all transconjugants from *Rlv* strains showed high levels of nitrogenase activity. The exception was the Rlv13 derivative Rlv13H24, which showed a poor symbiotic performance, attributed to a potential deleterious effect of the insertion. Although there were differences in the levels of hydrogenase activity induced in each case, in both sets of strains we found derivatives with levels of hydrogenase activity high enough as to fully recycle the hydrogen evolved by nitrogenase, thus resulting in nodules with no detectable hydrogen evolution: Rlv13H6 and H9, and Rlv21H10 and H19 (**Table 1**). Insertion sites were determined for these four derivatives (see Materials and Methods), and derivatives Rlv 13H6 and Rlv21H10, bearing insertions with no predicted effect on bacteroid physiology were selected for the analysis of the effect of hydrogen recycling on vetch productivity.

Transposon Tn*HB100* carries a Spc^R determinant whose presence in bacteria designed to be used as inoculant in the field is not desirable. In order to generate marker-free derivatives more acceptable for field release, strains Rlv13H6 and Rlv21H10 were

TABLE 1 | Nitrogenase and hydrogenase activities of wild-type and engineered strains incorporating hydrogenase genes from *R. leguminosarum* UPM791.

Strain	Nitrogenase activity[a]	Hydrogenase activity[b]	H_2 evolution[a]
R. leguminosarum/Vicia sativa			
Rlv13 (WT)	51.6 ± 2.3	< 50	14.1 ± 0.7
Rlv13H6	45.2 ± 5.5	1,187 ± 321	< 0.05
Rlv13H9	60.2 ± 0.2	1,272 ± 90	< 0.05
Rlv13H21	30.5± 1.3	609 ±198	1.2 ± 0.1
Rlv13H24	< 10	< 50	< 0.05
Rlv21 (WT)	37.9 ± 2.3	< 50	8.7 ± 0.2
Rlv21H10	26.8 ± 0.5	1,117 ± 546	< 0.05
Rlv21H16	44.1 ±0.2	1,044 ± 506	2.5 ± 0.7
Rlv21H19	41.4 ± 1.1	1,043 ± 473	< 0.05
Rlv21H22	35.4 ± 0.3	198 ±18	8.1 ± 0.8
Mesorhizobium loti/Lotus corniculatus			
Ml U261 (WT)	2.4 ± 0.8	n.d.	1.07 ± 0.4
Ml U261H4	0.6 ± 0.2	n.d.	< 0.05
Ml U261H6	1.4 ± 0.3	n.d.	0.6 ± 0.1
Ml U261H15	1.5 ± 0.1	n.d.	0.5 ± 0.3
Ml U261H16	1.2 ± 0.1	n.d.	0.8 ± 0.1
Ml U261H23	1.0 ± 0.1	n.d.	0.6 ± 0.3
Ml U261H 25	2.1 ± 0.7	n.d.	0.3 ± 0.1

[a] *Values are expressed in μmoles.h^{-1}. (g nodule fresh weight)$^{-1}$, and represent the mean of at least three determinations ± S.D.*
[b] *Values are expressed in nmoles of H_2.h^{-1}. (mg protein)$^{-1}$.*
n.d., not determined.

TABLE 2 | Effect of the introduction of *hup* genes on symbiotic performance of *Rlv* strains in association with *V. sativa* plants.

Strain[a]	N content (mg/plant)	% increase	SDW (mg/plant)
Rlv13	15.6 ± 0.8a		338 ± 71a
Rlv13H6	18.6 ± 1.0b	19	420 ± 44a
Rlv13H6DS	17.8 ± 1.0b	14	411 ± 51a
Rlv21	16.1 ± 1.2a		348 ± 40a
Rlv21H10	16.0 ± 0.7a		343 ± 30a
Rlv21H10DS	16.9 ± 1.1a		360 ± 50a

Values are the mean of 4 replicates ± S.E. Values followed by a different letter indicate significant differences with the corresponding wild-type strain according to Fisher's L.S.D test.

further engineered for the removal of Spc^R determinant by a double recombination event carried out as described in Materials and Methods. The absence of the spectinomycin resistance cassette in the resulting strains was confirmed by Southern hybridization of genomic DNA using a probe internal to the Spc^R gene (**Supplementary Figure 1**). Both strains, designated as Rlv13H6DS and Rlv21H10DS, induced nodules with levels of nitrogenase activity similar to those in the strains bearing the Spc^R determinant. Bacteroids from these strains showed high levels of hydrogenase activity that fully recycled the nitrogenase-derived hydrogen (data not shown).

In the case of *M. loti* U261 derivatives, 6 transconjugants were selected to assess the expression of the introduced *hup* genes in symbiosis with *L. corniculatus* as the legume host. Each of the transconjugants was used as inocula for *L. corniculatus* plants, and the levels of nitrogenase activity and hydrogen evolution from nodules were determined (**Table 1**). Levels of nitrogenase activity in the transconjugant strains did not show significant differences with that from the wild type. Nodules induced by the *hup*-containing derivatives showed significant reductions of the level of hydrogen evolution as compared to nodules induced by the wild-type strain (**Table 1**). The complete suppression of hydrogen evolution, indicating full recycling of nitrogenase-produced hydrogen, was detected in one of the transconjugants (*Ml* U261H4). Inspection of the corresponding genomic region (see Materials and Methods section) revealed that this insertion is likely in an intergenic region not affecting the expression of any other gene.

Analysis of the Effect of Hydrogen Recycling on Plant Productivity

In order to determine the effect of the hydrogen recycling on symbiotic performance of the vetch/*R. leguminosarum* bv viciae system, *V. sativa* plants were inoculated with the wild type strains Rlv13 and Rlv21 and with the *hup*-engineered derivatives obtained from them and described above. Inoculated plants were maintained in a growth chamber, and shoot dry matter and nitrogen accumulation were determined 30 days after inoculation. In this analysis, hydrogen-recycling strains derived from Rlv13 showed significant increases ($p < 0.05$) in N fixed per plant (**Table 2**). Values of shoot dry weight also showed marked increases (24.1% and 21.7 %, respectively) although in this case the higher variability of the data led to a lower significance of the differences ($p < 0.1$). In the case of strain Rlv21, derivatives incorporating the ability of full hydrogen recycling did not show significant variations of any of the two productivity parameters analyzed. These data suggest that the rhizobial background is relevant for the expression of the benefits associated to hydrogen recycling.

Symbiotic performance of *M. loti* U261 and its derivative strain U261H4 were compared in *L. corniculatus* plants grown under controlled conditions. Plant shoot dry weight, and N content of the shoots were determined at 40, 60, and 70 days after inoculation. 60- and 70-day old plants inoculated with the engineered strain showed levels of nitrogen accumulation significantly higher than those inoculated with the wild-type strain. This result was consistently observed in three independent experiments carried out under controlled chamber conditions. The increase in nitrogen was accompanied with an increase in dry weight at 70 days (**Table 3**). At 40 days plants showed higher values of N accumulation, but the variability of the data made these differences not statistically significant.

Since, *M. loti* U261 is effective in other *Lotus* species of agronomical interest (Gonnet and Díaz, 2000), we also estimated the potential benefits of hydrogen recycling carried out by *M. loti* U261H4 when in symbiosis with other 3 hosts of the same genus, namely *L. tenuis*, *L. subbiflorus*, and *L. uliginosus*. Similarly to what was observed in *L. corniculatus*, nodules induced by *M. loti* U261H4 engineered strain showed undetectable levels of hydrogen evolution in symbiosis with

TABLE 3 | Effect of the introduction of *hup* genes on symbiotic performance of *M. loti* U261 in association with *L. corniculatus* plants.

Strain	40 days		60 days		70 days	
	N content	DW	*N* content	DW	*N* content	DW
U261	114 ± 15a	32 ± 8a	552 ± 25a	138 ± 33a	253 ± 22a	100 ± 24a
U261H4	146 ± 20a	41 ± 10a	627 ± 32b	128 ± 31a	527 ± 26b	161 ± 39a

N content (mg/plant) was determined by mass spectrometry in detached leaves. Dry weight (DW) corresponds to mg leaves/plant. Parameters were measured at the indicated time points after inoculation. Values are the mean of 3 replicates ± S.D. Values following by different letters indicate significant differences (p < 0.05) according to a Fisher's L.S.D. test.

TABLE 4 | Effect of the introduction of *hup* genes on symbiotic performance of *M. loti* U261 strain in association with the indicated host legumes.

Strain	*Lotus tenuis*		*Lotus subbiflorus*		*Lotus uliginosus*	
	N content	DW	*N* content	DW	*N* content	DW
U261	604 ± 180a	155 ± 96a	533 ± 160a	116 ± 59a	504 ± 45a	126 ± 26a
U261H4	864 ± 210a	201 ± 56a	766 ± 180a	187 ± 21a	457 ± 60a	127± 19a

N content (mg/plant) was determined by mass spectrometry in detached leaves. Dry weight (DW) corresponds to mg leaves/plant. Parameters were measured 60 days after inoculation. Values are the mean of 3 replicates ± S.D. Values following by different letters indicate significant differences (p < 0.05) according to a Fisher's L.S.D. test.

these 3 hosts, whereas nodules induced by the wild type strain *Ml* U261 evolved large amounts of hydrogen in all cases (data not shown). These results indicate that *R. leguminosarum hup* system is expressed under symbiotic conditions in all host partners at levels high enough as to fully recycle hydrogen evolved by nitrogenase. Despite full hydrogen recycling, there were no significant differences between the wild type and U261H4 engineered strain neither in shoot dry weight nor in N content in symbiosis with *L. tenuis*, *L. subbiflorus*, *L. uliginosus* at 60 days of cultivation (**Table 4**), indicating a relevant role for the host on the effect of hydrogen-recycling on nitrogen fixation.

DISCUSSION

Hydrogenase Activity and Forage Legume Productivity

Forage legumes constitute a relevant input of protein for the sustainable feeding of livestock, a crucial issue in a context of increased demand of livestock products (FAO, 2011; Michalk et al., 2019). The productivity of pastures is dependent on an adequate supply of nitrogen (Phelan et al., 2015), therefore ensuring optimal functioning of symbiotic nitrogen fixation is a relevant aspect for pasture management. This optimization includes the use of highly efficient rhizobial strains. Even the most efficient rhizobial strains still dedicate a minimum of 25% of electrons going through nitrogenase to reduce protons to hydrogen. In this work we have generated rhizobial strains effective in two forage legumes (*Lotus* and *Vicia*) and able to fully recycle the hydrogen evolved from nitrogenase activity in the nodules. Engineered strains were associated to levels of nitrogen fixation higher than the respective wild-type strains under controlled conditions. It has to be noted, however, that for *L. tenuis* and *L. subbiflorus* the data showed a higher level of variability which, along with the limited number of replicates, did not allow to adscribe statistical significance to the observed variation of the mean values.

Previous evidence had shown that *R. leguminosarum* hydrogenase system is expressed in nodules when the hydrogenase gene cluster is introduced into strains effective on *Vicia* and *Lotus* (Monza et al., 1997; Brito et al., 2000). In contrast, introduction of the same system into *Sinorhizobium meliloti* strains resulted in only residual levels of hydrogenase activity in alfalfa nodules. It was concluded that several factors, including nickel provision and effectiveness of heterologous regulators, limit the expression in different backgrounds (Brito et al., 2000). The original transposon contains a spectinomycin resistance cassette to help on the selection of transposants (Bascones et al., 2000). We have also generated derivative strains in which such marker has been deleted, thus resulting in engineered strains in which only rhizobial DNA was present. As expected, removal of Spc^R tag did not change the symbiotic performance of the strains. These strains should be more amenable to its use as inoculants.

The results presented in this work indicate that the introduced ability of hydrogen recycling has the potential to increase nitrogen fixation in forage legumes, confirming the notion of hydrogen evolution as a source of inefficiency of nitrogen fixation by the *Rhizobium*-legume symbiosis (Evans et al., 1988). There was scarce previous information on the effect of hydrogen recycling on forage legumes. The presence of hydrogenase genes in rhizobia is highly variable depending of the system (Ruiz-Argüeso et al., 2000), and in fact there are no reports on the presence of functional *hup* gene clusters in rhizobia isolated from forage legumes such as clover or alfalfa. Even in *R. leguminosarum* bv viciae the presence of hydrogenase system is highly uncommon (Fernandez et al., 2005), and so the actual evolutionary advantage of possessing such system is a matter of controversy. Now we demonstrate for the first time that the advantages of hydrogen recycling can be exploited also in forage legumes, at least under controlled conditions. The results

obtained also indicate that both the host species and the rhizobial strain condition the existence of significant increases of fixed nitrogen associated to hydrogenase activity. Factors proposed to account for the beneficial effect of hydrogen recycling are the protection of nitrogenase against oxygen and against the detrimental effect of hydrogen, and the availability of additional energy for nitrogen fixation (Palacios et al., 2005). Additional work is required to elucidate the reasons for the observed effect of both the rhizobial background and host legume. It has to be noted that nickel provision and effectiveness of heterologous regulators for expression of hydrogenase genes have been cited before as potential limiting factors (Brito et al., 2000).

Hydrogen and Soil Fertility

The role of hydrogen evolution and hydrogen recycling in plant productivity is not a simple question. Results presented here refer to the levels of nitrogen fixed by the inoculated legume, and have been obtained in an artificial substrate without the soil microbiome. From a more integrated point of view, the positive role of hydrogen recycling in the productivity of legumes must be considered along with the potential effect of hydrogen evolved from nodules in the fertility of surrounding soil. It has been shown that the presence of hydrogen evolving nodules in legume plants inoculated with hydrogenase-negative rhizobia results in an increase of the ratio of H_2-oxidizing bacteria in soil surrounding nodules, as could be expected from the presence of an additional energy substrate in the environment. In fact, a "hydrogen fertilization effect" has been proposed as a factor contributing to the observed benefit of legumes on the following crop in rotation practices (Golding and Dong, 2010). The direct addition of hydrogen to soils resulted in increases in tiller/plant and plant dry weight in barley (Dong et al., 2003). There is also one report indicating that barley crop following soybean inoculated with Hup^-, hydrogen evolving *B. japonicum* strain resulted in higher grain yield than in the case of isogenic, hydrogen recycling strain (Dean et al., 2006). In that work an increase of hydrogen oxidation activity in soils surrounding Hup^- nodules was also detected. Changes in soil microbiome around nodules producing hydrogen are expected, and it has been recently shown that exposure of soil to hydrogen levels similar to those on the soil-nodule interface indeed causes changes in soil microbiome structure and function, including the stimulation of carbon turnover (Khdhiri et al., 2017). All these data indicate that the potential role of hydrogen metabolism in plant productivity should be analyzed from an integrated perspective. The hydrogen-recycling strains effective in forage legumes developed in this work can be used to further study the effect of hydrogen metabolism on mixed grass-legume pastures in which the potential role of hydrogen in stimulating other crops can be analyzed in the same season as legume symbiosis occurs, so a more integrated study of the effect of nitrogen fixation on mixed pasture production can be carried out.

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

MS, AU, and SM performed experiments and analyzed data. JM and JS designed experiments, validated data and review the manuscript. JP designed experiments, validated data, wrote draft and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are extremely grateful to Drs. José Olivares and Tomás Ruiz-Argüeso for providing resources and intellectual support to this work for so many years in the context of EU's IMPACT and other projects. JP is funded by grant from Spain's Ministerio de Ciencia, Innovación y Universidades (RTI2018-094985-B-100). JM obtained funds from Programa de Desarrollo de Ciencias Básicas and INIA-Uruguay (Project 4-componente IV), and by Sistema Nacional de Investigadores. This paper is dedicated to the memory of Tomás Ruiz-Argüeso, who passed away in March 2020 due to Covid19.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.661534/full#supplementary-material

REFERENCES

Baginski, C., Brito, B., Imperial, J., Ruiz-Argüeso, T., and Palacios, J. M. (2005). Symbiotic hydrogenase activity in *Bradyrhizobium* sp (*Vigna*) increases nitrogen content in *Vigna unguiculata* plants. *Appl. Environ. Microb.* 71, 7536–7538. doi: 10.1128/AEM.71.11.7536-7538.2005

Bascones, E., Imperial, J., Ruiz-Argueso, T., and Palacios, J. M. (2000). Generation of new hydrogen-recycling *Rhizobiaceae* strains by introduction of a novel *hup* minitransposon. *Appl. Environ. Microb* 66, 4292–4299. doi: 10.1128/AEM.66.10.4292-4299.2000

Brito, B., Martínez, M., Fernández, D., Rey, L., Cabrera, E., Palacios, J. M., et al. (1997). Hydrogenase genes from *Rhizobium leguminosarum* bv. viciae are controlled by the nitrogen fixation regulatory protein NifA. *Proc. Natl. Acad. Sci. U.S.A.* 94, 6019–6024. doi: 10.1073/pnas.94.12.6019

Brito, B., Monza, J., Imperial, J., Ruiz-Argüeso, T., and Palacios, J. M. (2000). Nickel availability and *hupSL* activation by heterologous regulators limit symbiotic expression of the *Rhizobium leguminosarum* bv. viciae hydrogenase system in *Hup- rhizobia. Appl. Environ. Microb.* 66, 937–942. doi: 10.1128/AEM.66.3.937-942.2000

Brito, B., Palacios, J. M., Hidalgo, E., Imperial, J., and Ruiz-Argüeso, T. (1994). Nickel availability to pea (*Pisum sativum* L.) plants limits hydrogenase activity of *Rhizobium leguminosarum* bv. viciae bacteroids by affecting the processing of the hydrogenase structural subunits. *J. Bacteriol.* 176, 5297–5303. doi: 10.1128/jb.176.17.5297-5303.1994

Brito, B., Palacios, J. M., Imperial, J., and Ruiz-Argüeso, T. (2002). Engineering the *Rhizobium leguminosarum* bv. viciae hydrogenase system for expression in free-living microaerobic cells and increased symbiotic hydrogenase activity. *Appl. Environ. Microbiol.* 68, 2461–2467. doi: 10.1128/AEM.68.5.2461-2467.2002

Brito, B., Toffanin, A., Prieto, R. I., Imperial, J., Ruiz-Argüeso, T., and Palacios, J. M. (2008). Host-dependent expression of *Rhizobium leguminosarum* bv. viciae hydrogenase is controlled at transcriptional and post-transcriptional levels in legume nodules. *Mol. Plant Microbe Interact.* 21, 597–604. doi: 10.1094/MPMI-21-5-0597

Dean, C. A., Sun, W. C., Dong, Z. M., and Caldwell, C. D. (2006). Soybean nodule hydrogen metabolism affects soil hydrogen uptake and growth of rotation crops. *Can. J. Plant Sci.* 86, 1355–1359. doi: 10.4141/P06-082

Díaz, P., Borsani, O., and Monza, J. (2005). *Lotus-Related Species and Their Agronomic Importance. Lotus Japonicus Handbook*. Dordrecht, Netherlands: Springer. 25–37. doi: 10.1007/1-4020-3735-X_2

Dong, Z., W, L., Kettlewell, B., Caldwell, C. D., and Layzell, D. B. (2003). Hydrogen fertilization of soils - is this a benefit of legumes in rotation? *Plant Cell Environ.* 26, 1875–1879. doi: 10.1046/j.1365-3040.2003.01103.x

Duran, D., Albareda, M., Garcia, C., Marina, A. I., Ruiz-Argüeso, T., and Palacios, J. (2021). Proteome analysis reveals a significant host-specific response in *Rhizobium leguminosarum* bv viciae endosymbiotic cells. *Mol. Cell. Proteomics* 20: 100009. doi: 10.1074/mcp.RA120.002276

Escaray, F. J., Menendez, A. B., Garriz, A., Pieckenstain, F. L., Estrella, M. J., Castagno, L. N., et al. (2012). Ecological and agronomic importance of the plant genus *Lotus*. Its application in grassland sustainability and the amelioration of constrained and contaminated soils. *Plant Sci.* 182, 121–133. doi: 10.1016/j.plantsci.2011.03.016

Evans, H. J., Russell, S. A., Hanus, F. J., and Ruiz-Argüeso, T. (1988). *The Importance of Hydrogen Recycling in Nitrogen Fixation by Legumes. World crops: Cool Season Food Legumes*. Boston: Kluwer Academic Publ. 777–791. doi: 10.1007/978-94-009-2764-3_62

FAO (2011). Mapping supply and demand for animal-source foods to 2030. Available at: http://www.fao.org/3/i2425e/i2425e00.htm (accessed December 2020).

Fernandez, D., Toffanin, A., Palacios, J. M., Ruiz-Argüeso, T., and Imperial, J. (2005). Hydrogenase genes are uncommon and highly conserved in *Rhizobium leguminosarum* bv. viciae. *FEMS Microbiol. Lett.* 253, 83–88. doi: 10.1016/j.femsle.2005.09.022

Golding, A. L., and Dong, Z. M. (2010). Hydrogen production by nitrogenase as a potential crop rotation benefit. *Environ. Chem. Lett.* 8, 101–121. doi: 10.1007/s10311-010-0278-y

Gonnet, S., and Díaz, P. (2000). Glutamine synthetase and glutamate synthase activities in relation to nitrogen fixation in Lotus spp. *Braz. J. Plant Physiol.* 12, 195–202. doi: 10.1590/S0103-31312000000300003

Handberg, K., and Stougaard, J. (1992). *Lotus-japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant J.* 2, 487–496. doi: 10.1111/j.1365-313X.1992.00487.x

Huang, Y. F., Gao, X. L., Nan, Z. B., and Zhang, Z. X. (2017). Potential value of the common vetch (*Vicia sativa* L.) as an animal feedstuff: a review. *J. Anim. Physiol. Anim. Nutr.* 101, 807–823. doi: 10.1111/jpn.12617

IAEA (2001). Use of isotope and radiation methods in soil and water managment and crop nutrition. IAEA- TCS-14, Vienna. Vienna: IAEA TCS-14.

Irisarri, P., Milnitsky, F., Monza, J., and Bedmar, E. J. (1996). Characterization of rhizobia nodulating *Lotus subbiflorus* from Uruguayan soils. *Plant Soil* 180, 39–47. doi: 10.1007/BF00015409

Kelly, S., Sullivan, J., Ronson, C., Tian, R., Brau, L., Davenport, K., et al. (2014). Genome sequence of the *Lotus* spp. microsymbiont *Mesorhizobium loti* strain NZP2037. *Front Stand. Genimic Sci.* 9:7. doi: 10.1186/1944-3277-9-7

Khdhiri, M., Piche-Choquette, S., Trembla, J., Tringe, S. G., and Constant, P. (2017). The Tale of a neglected energy source: elevated hydrogen exposure affects both microbial diversity and function in soil. *Appl. Environ. Microb.* 83,e00275–e00217. doi: 10.1128/AEM.00275-17

Laranjo, M., Alexandre, A., and Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiol. Res.* 169, 2–17. doi: 10.1016/j.micres.2013.09.012

Leyva, A., Palacios, J. M., Mozo, T., and Ruiz-Argüeso, T. (1987). Cloning and characterization of hydrogen uptake genes from *Rhizobium leguminosarum. J. Bacteriol.* 169, 4929–4934. doi: 10.1128/JB.169.11.4929-4934.1987

Lorite, M. J., Estrella, M. J., Escaray, F. J., Sannazzaro, A., Castro, I. M. V. E., Monza, J., et al. (2018). The rhizobia-lotus symbioses: deeply specific and widely diverse. *Front. Microbiol.* 9:2055. doi: 10.3389/fmicb.2018.02055

Michalk, D. L., Kemp, D. R., Badgery, W. B., Wu, J. P., Zhang, Y. J., and Thomassin, P. J. (2019). Sustainability and future food security-a global perspective for livestock production. *Land. Degrad. Dev.* 30, 561–573. doi: 10.1002/ldr.3217

Monza, J., Díaz, P., Borsani, O., Ruiz-Argüeso, T., and Palacios, J. M. (1997). Evaluation and improvement of the energy efficiency of nitrogen fixation in *Lotus corniculatus* nodules induced by *Rhizobium loti* strains indigenous to Uruguay. *World J. Microbiol. Biotech.* 13, 565–571. doi: 10.1023/A:1018573527503

Monza, J., Fabiano, E., and Arias, A. (1992). Characterization of an indigenous population of rhizobia nodulating *Lotus corniculatus*. *Soil Biol. Biochem.* 24, 241–247. doi: 10.1016/0038-0717(92)90225-M

Palacios, J. M., Manyani, H., Martinez, M., Ureta, A. C., Brito, B., Bascones, E., et al. (2005). Genetics and biotechnology of the H(2)-uptake [NiFe] hydrogenase from *Rhizobium leguminosarum* bv. viciae, a legume endosymbiotic bacterium. *Biochem. Soc. Trans.* 33, 94–96. doi: 10.1042/BST0330094

Phelan, P., Moloney, A. P., McGeough, E. J., Humphreys, J., Bertilsson, J., O'Riordan, E. G., et al. (2015). Forage legumes for grazing and conserving in ruminant production systems. *Crit. Rev. Plant Sci.* 34, 281–326. doi: 10.1080/07352689.2014.898455

Ruiz-Argüeso, T., Imperial, J., and Palacios, J. M. (2000). *Uptake Hydrogenases in Root Nodule Bacteria. Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process*. Wymondham, UK: Horizon Scientific Press. 489–507.

Sambrook, J., and Russell, D. W. (2002). *Molecular Cloning: A Laboratory Manual*. New Yok, NY: Cold Spring Harbor.

Sanchez-Canizares, C., Jorrin, B., Duran, D., Nadendla, S., Albareda, M., Rubio-Sanz, L., et al. (2018). Genomic diversity in the endosymbiotic bacterium *Rhizobium leguminosarum*. *Genes* 9:60. doi: 10.3390/genes9020060

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145, 69–73. doi: 10.1016/0378-1119(94)90324-7

Simon, R., Priefer, U., and Pühler, A. (1983). *Vector Plasmids for in-vivo and in-vitro Manipulations of Gram-Negative Bacteria. Molecular Genetics of the Bacteria-Plant Interactions*. Berlin, Springer-Verlag. 98–106. doi: 10.1007/978-3-642-69338-0_11

Tabata, S., and Stougaard, J. (2014). *The Lotus Japonicus Genome Preface to the Volume. Compend Pl Genome: Xi-Xi*. doi: 10.1007/978-3-662-44270-8

Torres, A. R., Brito, B., Imperial, J., Palacios, J. M., Ciampitti, I. A., Ruiz-Argueso, T., et al. (2020). Hydrogen-uptake genes improve symbiotic efficiency in common beans (*Phaseolus vulgaris* L.). *Antonie van Leeuwenhoek* 113, 687–696. doi: 10.1007/s10482-019-01381-6

Zhang, J. J., Shang, Y. M., Peng, S. S., Chen, W. F., Wang, E. T., de Lajudie, P., et al. (2019). *Rhizobium sophorae*, *Rhizobium laguerreae*, and two novel *Rhizobium* genospecies associated with *Vicia sativa* L. in Northwest China. *Plant Soil* 442, 113–126. doi: 10.1007/s11104-019-04168-w

Zhang, X. N., Ward, B. B., and Sigman, D. M. (2020). Global nitrogen cycle: critical enzymes, organisms, and processes for nitrogen budgets and dynamics. *Chem. Rev.* 120, 5308–5351. doi: 10.1021/acs.chemrev.9b00613

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.

Copyright © 2021 Sotelo, Ureta, Muñoz, Sanjuán, Monza and Palacios. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

MINI REVIEW
published: 05 July 2021
doi: 10.3389/fagro.2021.661468

Rhizobial Exopolysaccharides and Type VI Secretion Systems: A Promising Way to Improve Nitrogen Acquisition by Legumes

Bruna Fernanda Silva De Sousa[1,2†], *Tereza Cristina Luque Castellane*[3†], *Lilia Tighilt*[1,4], *Eliana Gertrudes de Macedo Lemos*[3*] *and Luis Rey*[1,2*]

[1] Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM)-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Campus de Montegancedo, Madrid, Spain, [2] Departamento de Biotecnología y Biología Vegetal, ETSI Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Madrid, Spain, [3] Laboratório de Bioquímica de Microrganismos e Plantas, Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal, Universidade Estadual Paulista Júlio de Mesquita Filho, São Paulo, Brazil, [4] Laboratoire d'Ecologie Microbienne, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia, Algeria

OPEN ACCESS

Edited by:
Juan Imperial, Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Xiancan Zhu, Anhui Normal University, China
Jose Enrique Ruiz-Sainz, Sevilla University, Spain
Francisco Pérez Montaño, Sevilla University, Spain

***Correspondence:**
Luis Rey
luis.rey@upm.es
Eliana Gertrudes de Macedo Lemos
elianag.lemos@gmail.com

[†] *These authors share first authorship*

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: 30 January 2021
Accepted: 04 June 2021
Published: 05 July 2021

Citation:
De Sousa BFS, Castellane TCL, Tighilt L, Lemos EGM and Rey L (2021) Rhizobial Exopolysaccharides and Type VI Secretion Systems: A Promising Way to Improve Nitrogen Acquisition by Legumes. Front. Agron. 3:661468. doi: 10.3389/fagro.2021.661468

At present, there are numerous examples in which symbiotic nitrogen fixation by rhizobia can totally replace the use of nitrogen fertilizers in legume crops. Over the years, there has been a great effort by research institutions to develop and select rhizobial inoculants adapted for these crops. The symbiotic process is highly dependent on the dynamic exchange of signals and molecular nutrients between partners. Our focus in this review was to discuss the two key determinants in successful symbiotic interactions of rhizobia to nodulate pulses. One of them is the production of exopolysaccharides (EPS) and the other the presence of the type VI secretion system (T6SS). EPS are extracellular polymers weakly associated with the bacterial surface and are abundantly released into acid soils facilitating, among other functions, an adaptation of rhizobia to this environment. On the other hand, different protein secretion systems, involved in symbiosis, have been described in rhizobia. This is not the case with the T6SS. The current availability of various rhizobial genomes offers the possibility of discussing its role in symbiosis. The study of these determinants will be of great utility for the selection of effective inoculants for legumes, a promising way to improve nitrogen acquisition by legumes.

Keywords: *Rhizobium*, root nodulating bacteria, effectors, type VI secretion systems, inoculant, exopolysaccharide, symbiosis

RHIZOBIA FOR PULSES

Legumes are of capital importance for human and animal feed and for most ecosystems. This is due, among other factors, to the fact that most species are capable of biologically fixing nitrogen (BNF) by association with soil diazotrophic bacteria called rhizobia. Most legume crops can self-supply the nitrogen they need, this being a fact of great relevance to mitigate the negative effects of nitrogen fertilizer leaching in the environment. All this has led to great interest in the selection of effective inoculants. Brazil is considered a global leader in the use of inoculants and one of the

largest producers of pulses worldwide (Santos et al., 2019). A reference is made in the following section to the most commonly selected strains (*Bradyrhizobium* and *Rhizobium*), as well as the beneficial role that rhizobial exopolysaccharides (EPS) and T6SS can have in an effective symbiosis and therefore in BNF.

BRADYRHIZOBIUM SPECIES IN BRAZILIAN SOILS

Reports on legume nodulation by *Bradyrhizobium* strains in Brazil are quite abundant, mainly in terms of legumes and bradyrhizobial isolates diversity. However, there are still a large number of rhizobial isolates waiting to be characterized. In this section, we summarize data on the history and evolution of studies on *Bradyrhizobium* in soybean (*Glycine max*).

The SEMIA Rhizobia Collection of Brazil originated in 1950 through the isolation and selection of efficient native strains for legumes of economic importance by professionals from the Agricultural Microbiology Section (SEMIA) of the Agronomic Research Institute (IPAGRO today FEPAGRO). Brazilian soybean crops boosted the beginning of inoculant production in Brazil in the 1960s due to the lack of native bacteria that were able to efficiently nodulate soybean, therefore, supplied mainly with North American strains. The first record of the SEMIA Collection was in 1973. In the 1980s, studies showed enormous genetic and physiological variability between the strains of *Bradyrhizobium japonicum*, suggesting the subdivision of *Bradyrhizobium* into two species, *B. japonicum* and *B. elkanii* (Kuykendall et al., 1992).

Argentina, Brazil, and the United States are the most important soybean producers. *Bradyrhizobium* is the most prominent and widely studied in terms of nodulation and nitrogen fixation in soybeans. The Ministry of Agriculture, Livestock and Food (MAPA) of Brazil emitted official reports about the quality of soybean inoculants (number of viable cells, strain identification, and presence of contaminants). In Brazil and South America, today, MAPA authorizes the use of different *Bradyrhizobium* strains in the formulation of commercial inoculants (**Supplementary Table 1**), consequently settling these populations in most of the soybean Brazilian soils (Hungria et al., 2001; Delamuta et al., 2013). Since the beginning of inoculant production in the 1980s, seven species that were able to efficiently nodulate soybean have been described, *B. japonicum, B. diazoefficiens, B. elkanii,* and *B. liaoningense* (Willems, 2006); and *Mesorhizobium tianshanense* (Chen et al., 1995); *Sinorhizobium fredii* and *S. xinjiangense* (Chen et al., 1988). The number of validated *Bradyrhizobium* species published has greatly increased in recent years, and ∼35% are from South America and another 35% from other regions mainly from China.

The soybean BNF is consolidated though many farmers use synthetic fertilizers. *Bradyrhizobium* inoculants are alternatives to reduce water pollution and nitrous oxide (N_2O) emission (Akiyama et al., 2016). However, Obando et al. (2019) showed the first report that pointed out the incomplete denitrification pathway in five of the most used strains for soybean.

Göttfert et al. (2001) described symbiosis-specific genes in a 410-kilobase DNA region of the *B. diazoefficiens* USDA110 chromosome and in 2002, the complete genomic sequence was described (Kaneko et al., 2002). The pioneering study reporting the high diversity of *Bradyrhizobium* genospecies was through a DNA–DNA hybridization conducted by Willems et al. (2001). *Bradyrhizobium*'s genomes are larger than those of other genera of rhizobia, reflecting their vast metabolic, and different life cycles. In addition, high phenotypic and/or genotypic diversity, both intra- and interspecific, between the strains of *Bradyrhizobium* was observed (Guimarães et al., 2012, 2015; Rufini et al., 2014). Despite this diversity, *Bradyrhizobium* species have a monophyletic character with few exceptions, particularly the photosynthetic bradyrhizobia (Moulin et al., 2004). Iida et al. (2015) reported the presence of some islands of symbiosis shuffled with abundant insertion sequences in the genomes of extra-slow-growing *Bradyrhizobium* strains. In the symbiotic islands, there are insertion sequences and groups of symbiotic genes, such as *nod*, *nif*, *fix*, and *rhc*. *nifH* and *nod* Genes are the most used for phylogenetic reconstructions of symbiosis, both transferred vertical and horizontally between different chromosomal backgrounds (Menna and Hungria, 2011). Generally, the transfer of specific symbiotic genes is considered a fundamental mechanism that allows legumes to form symbioses with rhizobia adapted to private soils (Andrews et al., 2017).

RHIZOBIUM TROPICI, A SPECIES RESISTANT TO ACIDIC GROWTH CONDITIONS

Rhizobium species are widely used as inoculants. *R. tropici* CIAT899 establishes symbiosis with different legume species, including common beans (*Phaseolus vulgaris*) (Martínez-Romero et al., 1991). However, the success of this process can be limited by different environmental conditions, such as high temperature, low pH, presence of host legumes, and soil (Martínez-Romero et al., 1991; Hungria et al., 2001; Vinuesa et al., 2003; Puozaa et al., 2017).

When pH is reduced by plant exudates containing protons and organic acids, it provides a limitation for the survival of microorganisms and nodulation, and BNF can be severely affected. Finding acid-tolerant plants and compatible rhizobia are of remarkable agronomic and ecological relevance. *R. tropici* CIAT899 is more resistant to acidic conditions than most rhizobia. This resistance is related to several factors, such as hydroxylated ornitine lipids, which make the membrane less fluid and less permeable to protons (Vinuesa et al., 2003; Vences-Guzmán et al., 2011). Guerrero-Castro et al. (2018) identified 26 genes in *R. tropici* CIAT899 involved in the pH stress response, and transcriptomic analysis from cells grown under different pH conditions allowed the identification of 383 genes that are differentially expressed. The genes included response regulators and membrane transporters, enzymes involved in the metabolism of amino acids and carbohydrates, and proton extrusion.

Biosynthesis of EPS encoded in the *R. tropici* CIAT899 genome has been associated with acid tolerance, and implication of EPS in symbiosis is discussed in the next section.

IMPORTANCE OF EXOPOLYSACCHARIDES PRODUCTION IN SYMBIOSIS

Rhizobial EPS are biopolymers of high–and low–molecular weight, secreted in the environment both in free living and in symbiosis (Skorupska et al., 2006). EPS contribute to tolerance of rhizobia against unfavorable conditions: reactive oxygen species, detergents, salt, acidic pH, drought, antimicrobial agents, etc. (Staehelin et al., 2006; Geddes et al., 2014; Naseem et al., 2018; Sun et al., 2020). Also, they participate in biofilm formation and attachment to abiotic or plant surfaces (Russo et al., 2006; Schäper et al., 2019). In the *Rhizobium*–legume interactions, EPS affect pre-infection events with the host, such as root hair curvature (Downie, 2010; Janczarek, 2011) and suppress the defense responses of the host plant (Jones et al., 2007). EPS can be perceived by plant LysM kinase receptors impairing or facilitating symbiosis with rhizobia depending on the polysaccharide composition, in *L. japonicus*, EPR3 receptor recognizes EPS controlling bacterial infection (Kawaharada et al., 2017; Kelly et al., 2017; Wong et al., 2020). EPS can have a negative effect on symbiosis as seen in *S. fredii* HH103/*Glycine max*, where flavonoids activate transcription of nodulation genes but repress EPS production (Acosta-Jurado et al., 2016).

EPS biosynthesis is unknown in most of the inoculants authorized in Brazil (**Supplementary Table 1**), particularly in *Bradyrhizobium* strains, however, it has been well-studied in *Sinorhizobium meliloti* and *Rhizobium leguminosarum* and required *exo, exs, exp,* and *muc* genes, which are mainly grouped and conserved in different strains (Janczarek et al., 2015). They are distributed on the chromosome or in symbiotic plasmids (Ivashina and Ksenzenko, 2012; Ormeño-Orrillo et al., 2012). EPS contain mostly D-glucose and D-galactose (Castellane et al., 2014, 2017, 2018, 2019), uronic acids and non-sugar substitutions can also be very important (López-Baena et al., 2016). Enzymes such as glycosyltransferase (PssA) and galactosyltransferase (PssJ) are responsible for assembling EPS sub-units in *R. leguminosarum* (Marczak et al., 2020).

Rhizobia are capable of producing two EPS forms, type I (succinoglycans) involved in *S. meliloti* nodule development (Reuber et al., 1991; Becker et al., 2002; Skorupska et al., 2006) and type II (galactoglycans) required for root hair attachment and biofilm formation (Rüberg et al., 1999; Becker et al., 2002; Skorupska et al., 2006; Sorroche et al., 2010). Also, *R. leguminosarum* EPS protect against zinc stress in the symbiosis with *Trifolium* (Kopycińska et al., 2018). In the symbiosis of *S. fredii* HH103/*Glycyrrhiza uralensis,* EPS were not strictly necessary (Margaret-Oliver et al., 2012) but *Mesorhizobium tianshanense* non-mucoid mutants were defective in nodulation with the same legume (Wang et al., 2008). *R. tropici* CIAT899 EPS are not required for bean nodulation but it is involved in competitiveness (Milner et al., 1992). A *R. leguminosarum pssZ* mutant showed reduced EPS production and nodule formation and affected bacteroids development (Lipa et al., 2018). Different amounts of EPS do not seem to interfere with the host, but with the ability to survive in the rhizosphere (Donati et al., 2013). *S. meliloti* Rm8530 produced three times more EPS than an *expR* mutant (Primo et al., 2020), and both had the same efficiency in the symbiosis with *Medicago sativa* (Pellock et al., 2002). Despite all these studies, the role of EPS in symbiosis remains unclear (Skorupska et al., 2006; Marczak et al., 2017).

In acidic soils, rhizobia from different hosts, such as *Cicer, Phaseolus, Lens,* and *Leucaena* can produce a considerable quantity of EPS (Cunningham and Munns, 1984). Secreted EPS are increased under abiotic stress (Hirsch, 1996; Sorroche et al., 2018; Primo et al., 2020). However, at acid pH *R. tropici* CIAT899 showed a lower production of biopolymers (Avelar Ferreira et al., 2012), whereas EPS production was not affected in *R. favelukesii* LPU83 (Nilsson et al., 2021).

The EPS in *P. vulgaris* induce genes involved in carbon metabolism, transcriptional regulation, circadian cycle, and phytohormone production (Via et al., 2015).

TYPE VI SECRETION SYSTEM (T6SS) IN RHIZOBIA

The T6SS secretion system has been hardly related to symbiosis. It was described in 2006 in *Vibrio cholerae* and *Pseudomonas aeruginosa* (Mougous et al., 2006; Pukatzki et al., 2006). Since then it has been characterized in numerous bacteria, mainly Proteobacteria (Boyer et al., 2009). The T6SS is a nano-syringe that enables bacteria to inject proteins called effectors into both eukaryotic and prokaryotic cells (Basler and Mekalanos, 2012).

T6SS biosynthesis usually requires 13 core structural proteins encoded by *tss* genes and accessories named *tag* (type six accessory gene) (Shalom et al., 2007; Silverman et al., 2012).

Its structure contains three elements: a trans-membrane complex (TssMLJ), a cytoplasmic baseplate (TssEFGK), and a double tube, the outer one formed by a contractile sheath (TssBC) and the inner one by Hcp (TssD) ending with the puncturing device VgrG (TssI)-PAAR (Wang et al., 2019) (**Figure 1**). The PAAR domain (proline–alanine–alanine–arginine) is a sharp conical extension on the VgrG spike, is similar to DUF4150 domain, and facilitates secretion of a broad range of toxins (Shneider et al., 2013). The assembly of the inner tube and the contractile sheath requires TssA. After contraction, the T6SS tube is recycled by TssH (ClpV), an ATPase that disassembles the structure (Wang et al., 2019).

Many of the known effectors targeting bacteria have a toxic function (lipases, DNases, and peptidoglycanases) killing potential competitors (Coulthurst, 2019). Toxic effector genes are adjacent to cognate immunity genes that prevent self-toxicity or toxicity of sibling cells, and they are known as effector–immunity pairs (E/I pairs) (Yang et al., 2018). It is relevant that the presence of genes coding for possible effectors not accompanied by their cognate immunity gene and called orphan genes could encode for effectors whose target was eukaryotic

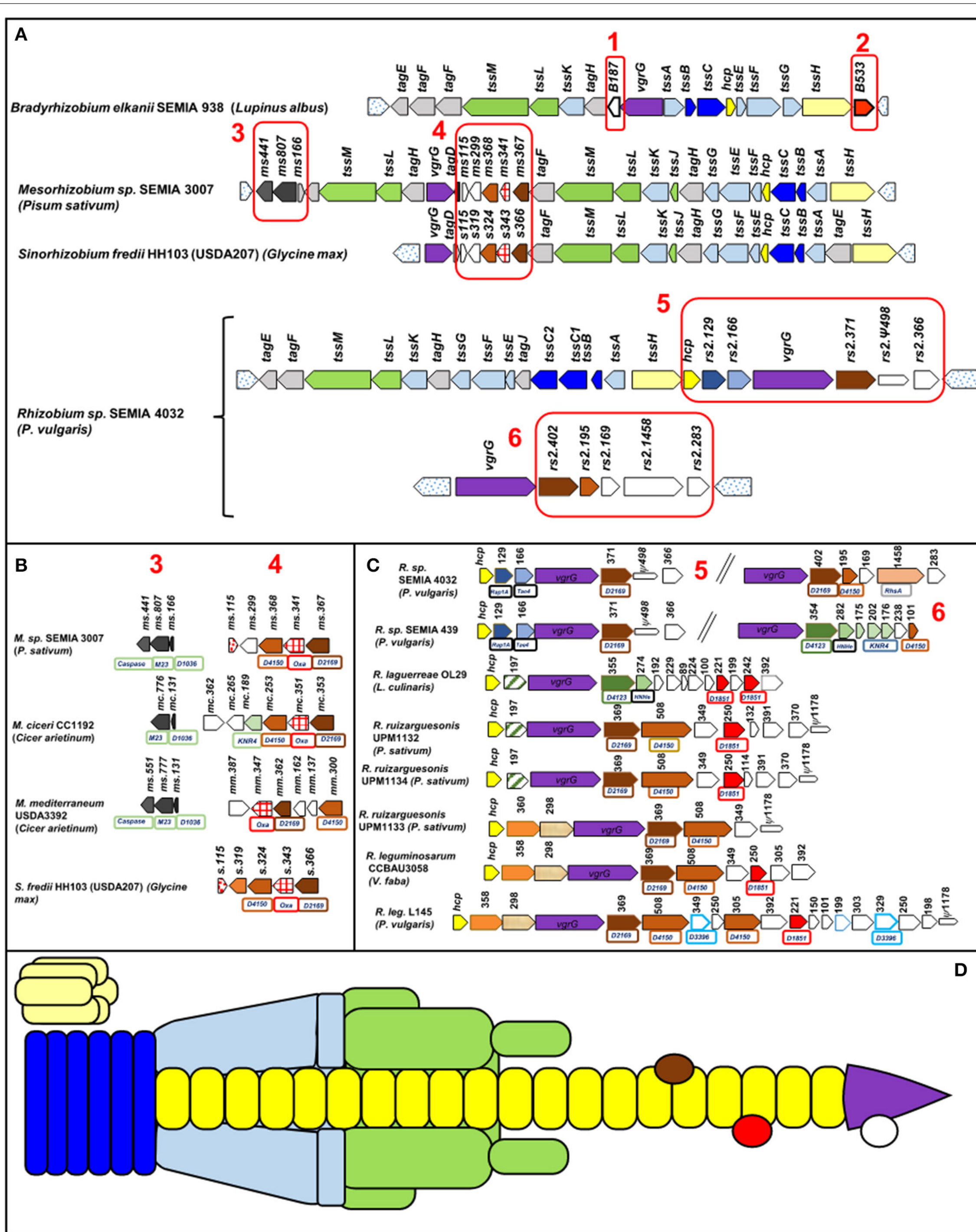


FIGURE 1 | T6SS gene cluster organization of different rhizobia. In parentheses is the host legume. Orthologous genes show the same color and pattern. The numbered Boxes 1–6 contain mainly not *tss* or *tag* genes. Box 6 corresponds to strains harboring a second cluster with the *vgrG* gene. Uncolored genes encode proteins with no known domains. Pseudogenes are indicated by thinner arrows. Dotted arrows have not been considered part of the T6SS. The numbers above the genes indicate the number of amino acids of the corresponding protein and boxes under the genes indicate identified domains (**D**: DUF, Oxa: oxoacyl-ACP synthases. **(A)** General organization in four rhizobia genera, **(B)** detailed gene organization of Boxes 3,4 of *Mesorhizobium* and *Sinorhizobium* strains, **(C)** detailed gene organization of Boxes 5, 6 of *Rhizobium* strains, and **(D)** structure of a T6SS. The colors are the same as those of the genes that encode these units in **(A–C)**. The effectors are symbolized by ovoid shapes.

TABLE 1 | Proteins encoded by different rhizobial T6SS genes (no *tss* or *tag* genes).

Host	Strains	Origin	Number of aa of proteins from not *tss* nor *tag* genes	References and NCBI sequence
Arachis hypogaea	*B. nanningense* CCBAU 53390	China	187, 528 (**Figure 1A,** Boxes 1, 2)	DS[a] NZ_LBJC01000000
Lupinus albus	*B.elkanii* SEMIA 938	Brazil	187, 533 (**Figure 1A,** Boxes 1, 2)	Hungria et al., 2019, NZ_SZZP01000000
Vigna unguiculata	*B. pachyrhizi* BR3262	Brazil	187, 528 (**Figure 1A,** Boxes 1, 2)	Simões-Araújo et al., 2016 LJYE00000000
Glycine max	*B. elkanii* SEMIA 587	Brazil	187, 528 (**Figure 1A,** Boxes 1, 2)	De Souza et al., 2012 NZ_SWAO01000000
	B. elkanii SEMIA 5019	Brazil	187, 533 (**Figure 1A,** Boxes 1, 2)	DS[a] NZ_SWAN01000000
	B. japonicum SEMIA 5079	Brazil	116, 533 (**Figure 1A,** Boxes 1, 2)	Siqueira et al., 2014 NZ_CP007569.1
	B. diazoefficiens SEMIA 5080	Brazil	187, 533 (**Figure 1A,** Boxes 1, 2)	Siqueira et al., 2014 ADOU00000000
	S. fredii HH103 (USDA207)	China	115, 319, 324, 343, 366 (**Figures 1A,B,** Box 4)	Sugawara et al., 2013 NZ_WITA01000128
Cicer arietinum	*M. ciceri* CC1192	Israel	776, 131 361, 265, 189, 253, 351, 353 (**Figures 1A,B,** Boxes 3, 4)	Haskett et al., 2016 NZ_CP015063
	M. mediterraneum USDA3392	France	551, 777, 131 387, 347, 362. 162, 137, 300 (**Figures 1A,B,** Boxes 3, 4)	DS[a] NZ_NPKI01000026
Pisum sativum	*Mesorhizobium* SEMIA 3007	Mexico	441, 807, 166, 115, 299, 368, 341, 367 (**Figures 1A,B,** Boxes 3, 4)	DS[a] NZ_MDLH01000000
	R. ruizarguesonis UPM1132	Italy	197; 369, 508, 349, 250, 132, 391, 370 (**Figures 1A,C,** Box 5)	Jorrín et al., 2020 NZ_PQIH01000000
	R. ruizarguesonis UPM1133	Italy	360, 298, 365, 508, 349 (**Figures 1A,C,** Box 5)	Jorrín et al., 2020 NZ_PQIG01000000
	R. ruizarguesonis UPM1134	Italy	197; 369, 508, 349, 250, 114, 391, 370 (**Figures 1A,C,** Box 5)	Jorrín et al., 2020 NZ_PQIF01000000
Lens culinaris	*R. laguerreae* OL29	Algeria	355, 274, 192, 229, 89, 224, 100, 221, 199, 242, 392 (**Figures 1A,C,** Box 5)	DS[a] NZ_WIFJ01000000
	R. leguminosarum L145	France	358, 298, 369, 499, 330, 128, 500, 370, 221, 150, 101, 199, 303, 329, 250, 198 (**Figures 1A,C,** Box 5)	DS[a] NZ_WIED00000000
Phaseolus vulgaris	*Rhizobium* sp. SEMIA 4032	Brazil	129, 166, 371, 366, 402, 195, 169, 1,458, 283 (**Figures 1A,C,** Boxes 5, 6)	DS[a] NZ_QERH01000000
	Rhizobium sp. SEMIA 439	Argentina	129, 166,371, 366, 101 354, 282, 175, 202, 176, 238 (**Figure 1C,** Boxes 5, 6)	DS[a] NZ_QERG01000000
Vicia faba	*R. leguminosarum* CCBAU 03058	China	358, 298, 250, 392, 369, 508, 305, 349 (**Figures 1A,C,** Box 5)	DS[a] NZ_WIEO01000000

[a]*DS, Direct submission.*

cells (Trunk et al., 2018). Sometimes there is a third gene that codes a chaperone or adaptor for loading the effector onto the cognate VgrG. Adaptors contain conserved domains as DUF2169 or DUF4123 (Lien and Lai, 2017). Genes encoding effectors are often close to *hcp* and *vgrG* genes (Ma et al., 2014; Bernal et al., 2017).

The relationship between T6SS and rhizobial symbiosis was studied before the description of the independent role of T6SS. In 2003, a group of genes impairing pea nodulation of *Rhizobium leguminosarum* RBL5523 were identified (Bladergroen et al., 2003). These genes corresponded to what we already know as *tss*. Since then, a few researchers have studied the role of T6SS in symbiosis beyond mentioning its presence. In two such studies, it was found that mutations in T6SS genes do not affect symbiotic efficiency but symbiotic competitiveness (De Campos et al., 2017; Lin et al., 2018), and in another study, a positive effect on symbiosis was demonstrated (Salinero-Lanzarote et al., 2019).

So far the symbiotic importance of any T6SS of recommended inoculants such as those included in **Supplementary Table 1** is not known. **Figure 1** shows the organization of 19 T6SSs of strains representing the main genera of rhizobia that nodulate pulses. When possible the strains were chosen from **Supplementary Table 1**.

GENOMIC ORGANIZATION OF RHIZOBIA T6SS CLUSTERS

Organization and arrangement of T6SS genes with structural, regulatory, or accessory functions called *tss* or *tag* genes are totally conserved in all the species of each genus included in **Table 1** and **Figure 1**. Genes not identified as *tss* or *tag*, match mostly to hypothetical proteins and among them are potential effectors as discussed below. These genes have been

marked in **Figure 1** by boxes numbered 1–6 and they are not as conserved as the previous ones, especially when different genera are compared. The T6SSs of all the *Bradyrhizobium* strains in **Table 1** have identical gene organization (**Figure 1A**). The three *Mesorhizobium* T6SSs presented slight differences among them as shown in Boxes 3, 4 of **Figure 1**. *Sinorhizobium* T6SS is similar to that of *Mesorhizobium*. The T6SSs of the *Rhizobium* strains show the greatest diversity in genes of Boxes 5, 6. Genomes of *R.* SEMIA 439 and *R.* SEMIA 4032 have an extra copy of gene *vgrG* (**Figure 1C**, Box 6).

PUTATIVE EFFECTORS IN RHIZOBIA T6SS

Numerous effectors are found in the vicinity of the structural genes *vgrG* and *hcp,* and it is common to find three genes in tandem so that the first corresponds to an adaptor with DUF2169 or DUF 4123 domains, the second to a toxic effector, and the third to an immunity protein. Other possibilities are E/I pair or orphan effectors (Ma et al., 2014; Bondage et al., 2016; Trunk et al., 2018; Yang et al., 2018). Many effectors have an N-terminal DUF4150 or PAAR domain followed by a C-terminal region of variable function and some immunity proteins have a similar structure to KNR4 proteins or contain DUF1851 domains (Zhang et al., 2011; Ma et al., 2014). Possible effectors are discussed below based on their location in T6SS or on domains noted in Pfam protein families database (El-Gebali et al., 2019) (**Supplementary Table 2**).

Two conserved genes in *Bradyrhizobium* T6SS could encode orphan effectors (**Figure 1**, Boxes 1, 2). Proteins from these genes were designated B.187 and B.528. Their number of amino acids depends on the strain (**Table 1**). No functional domain has been identified in B.187. The other, B.528, has a methyltransferase domain. The role of these proteins is unknown. Other orphan effector (197 aa) could be encoded by the gene between *hcp* and *vrgG* in *R. laguerrae* and *R. ruizarguesonis* UPM1132 and UPM 1134 (**Figure 1C**).

Box 3 in **Figures 1A,B** contains genes for three types of *Mesorhizobium* proteins, Ms166 has the DUF1036 domain mainly present in membrane proteins of alphaproteobacteria. Ms808 is homologous to M23 metallopeptidases that lyse bacterial cell wall peptidoglycans (Lewis et al., 2019). And the third, Ms441, not present in *M. ciceri*, presents a caspase domain. Caspases (cysteine–aspartic proteases) participate in programmed cell death in animal tissues; however, the function of most bacterial caspase homologs are unknown (Asplund-Samuelsson, 2015). Box 4 of **Figures 1A,B** includes genes encoding proteins with DUF2169, DUF4150, or oxoacyl-ACP synthases domains, but their roles are unknown.

Pairs E/I are present in *R.* sp. SEMIA 4032 and SEMIA 439 as Rap1/Tae4 pair (**Figure 1C**). Rap1 is homologous to an immunity protein that neutralizes the amidase Tae4 able to cleave muropeptides of peptidoglycans (English et al., 2012; Srikannathasan et al., 2013; Zhang et al., 2013). Other E/I pair could correspond to the genes between *hcp* and *vgrG* in the *Rhizobium* strains UPM1133, CCBAU3058, and L145, although they do not have similarity with any E/I pair described.

Proteins with N-terminal PAAR-like/DUF4150 domain followed by a C-terminal region that contains a putative effector domain (Bondage et al., 2016) are present in Boxes 4–6 of **Figure 1**. No function for these proteins has been identified with the exception of *M. ciceri* Mc253 that contains a DNase_NucA_NucB domain (**Figure 1B,** Box 4). Rse.1458 from *R.* sp. SEMIA4032, **Figure 1** Box 6, has two toxic RhsA domains often present in effectors secreted by T6SS (Pei et al., 2020).

CONCLUSIONS

It is desirable that collections of rhizobia, such as the Brazilian SEMIA, have and provide well-characterized rhizobia capable of maximizing nitrogen fixation in legumes.

Rhizobial EPS role in plant signaling and in bacterial protection against different stresses should be elucidated because they play a crucial role in symbiosis. EPS can promote competitiveness, the development of nodules, and therefore influence the effectiveness of inoculants. The connection between different symbiotic signals and regulation of EPS and other relevant surface polysaccharide expression in different rhizobia should also be considered.

The presence of T6SS in inoculants with high nitrogen-fixing capacity can lead to (i) better competitiveness against native soil endosymbionts able to nodulate the same host legume; (ii) biocontrol against pathogens/microorganisms in the rhizosphere as demonstrated by *Pseudomonas* preventing phytopathogenic bacteria (Bernal et al., 2017, 2018) and by the antifungal activity of *Serratia marcescens* (Trunk et al., 2018); and (iii) targeting effectors to host legumes as revealed by some nodulation external proteins (Nops). Nops are type III secretion system–dependent effectors with a positive, negative, or neutral effect on symbiosis (Deakin and Broughton, 2009; Miwa and Okazaki, 2017; Kusakabe et al., 2020). It has been identified that T6SS anti-eukaryotic effectors enable infectious bacteria to survive against the immune response of their hosts (Monjarás- Feria and Valvano, 2020).

AUTHOR CONTRIBUTIONS

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

FUNDING

LR was supported by the Ministerio de Ciencia, Innovación y Universidades, Spain (RTI2018-094985-B-100). TC and EL were supported by the National Council for Scientic and Technological Development (CNPq) (Proceeding No. 401886/2016-1) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Proceeding No. 19/13493-1). BD was supported by a scholarship from CNPq-GDE-204842/2018-2. LT was supported by scholarships from the National Exceptional Program (PNE), Ministry of Higher Education and Scientific Research of Algeria, and from the Erasmus+ Programme project 2019-1-ES01-KA107-063778.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of Prof. Tomás Ruiz-Argüeso (1943–2020) whose teachings and friendship will always be with us.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.661468/full#supplementary-material

REFERENCES

Acosta-Jurado, S., Alias-Villegas, C., Navarro-Gómez, P., Zehner, S., Murdoch, P. D., RodrÃíguez-Carvajal, M. A., et al. (2016). The *Sinorhizobium fredii* HH103 MucR1 global regulator is connected with the nod regulon and is required for efficient symbiosis with *Lotus burttii* and *Glycine max* cv. *Williams. Mol. Plant Microbe Interact*. 29, 700–712. doi: 10.1094/mpmi-06-16-0116-r

Akiyama, H., Hoshino, Y. T., Itakura, M., and Shimomura, Y. (2016). Mitigation of soil N_2O emission by inoculation with a mixed culture of indigenous *Bradyrhizobium diazoefficiens*. *Nat. Publ. Gr*. 6:32869. doi: 10.1038/srep32869

Andrews, M., and Andrews, M., E. (2017). Specificity in legume-rhizobia symbioses. *Int. J. Mol. Sci*. 18:705. doi: 10.3390/ijms18040705

Asplund-Samuelsson, J. (2015). The art of destruction: revealing the proteolytic capacity of bacterial caspase homologs. *Mol. Microbiol*. 98, 1–6. doi: 10.1111/mmi.13111

Avelar Ferreira, P. A., Bomfeti, C. A., Lima Soares, B., and Moreira, F. M. (2012). Efficient nitrogen-fixing *Rhizobium* strains isolated from amazonian soils are highly tolerant to acidity and aluminium. *World J. Microbiol. Biotechnol*. 28, 1947–1959. doi: 10.1007/s11274-011-0997-7

Basler, M., and Mekalanos, J. (2012). Type 6 secretion dynamics within and between bacterial cells. *Science* 337, 815–815. doi: 10.1126/science.1222901

Becker, A., Ruberg, S., Baum Garth, B., Bertram-Drogatz, P. A., Quester, I., and Pühler, A. (2002). Regulation of succinoglycan and galactoglucan biosynthesis in *Sinorhizobium meliloti*. *J. Mol. Microbiol. Biotechnol*. 4,187–190.

Bernal, P., Allsopp, L. P., Filloux, A., and Llamas, M. A. (2017). *The Pseudomonas putida* T6SS is a plant warden against phytopathogens. *ISME J*. 11, 972–987. doi: 10.1038/ismej.2016.169

Bernal, P., Llamas, M. A., and Filloux, A. (2018). Type VI secretion systems in plant-associated bacteria. *Environ. Microbiol*. 20, 1–15. doi: 10.1111/1462-2920.13956

Bladergroen, M. R., Badelt, K., and Spaink, H. P. (2003). Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. *Mol. Plant Microbe Interact*. 16, 53–64. doi: 10.1094/MPMI.2003.16.1.53

Bondage, D. D., Lin, J. S., Ma, L. S., Kuo, C. H., and Lai, E. M. (2016).VgrG C terminus confers the type VI effector transport specificity and is required for binding with PAAR and adaptor-effector complex. *Proc. Natl. Acad. Sci. U.S.A*. 113, E3931–E3940. doi: 10.1073/pnas.1600428113

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC Genomics*. 10:104. doi: 10.1186/1471-2164-10-104

Castellane, T. C. L., Campanharo, J. C., Colnago, L. A., Coutinho, I. D., Lopes, É. M., Lemos, M. V. F., et al. (2017). Characterization of new exopolysaccharide production by *Rhizobium tropici* during growth on hydrocarbon substrate. *Int. J. Biol. Macromol*. 96, 361–369. doi: 10.1016/j.ijbiomac.2016.11.123

Castellane, T. C. L., de Sousa, B. F. S., and Lemos, E. G. D. M. (2019). "Exopolysaccharide Produced from *Rhizobium spp*. An interesting product for industry and environment". *Curr. Appl. Polym. Sci*. 3:157. doi: 10.2174/2452271603666191016143811

Castellane, T. C. L., Lemos, M. V. F., and Lemos, E. G. D. M. (2014). Evaluation of the biotechnological potential of *Rhizobium tropici* strains for exopolysaccharide production. *Carbohydr. Polym*. 111, 191–197. doi: 10.1016/j.carbpol.2014.04.066

Castellane, T. C. L., Lemos, M. V. F., and Lemosa, E. G. M. (2018). Exploring and utilization of some bacterial exopolysaccharide. *Biopolym. Res*. 2:1000106.

Chen, W., Wang, E., Wang, S., Li, Y., Chen, X., and Li, Y. (1995). Characteristics of Rhizobium tianshanense sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *Int. J. Syst. Evol. Microbiol*. 45, 153–159. doi: 10.1099/00207713-45-1-153

Chen, W. X., Yan, G. H., and Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium gen*. nov. *Int. J. Syst. Evol. Microbiol*. 38, 392–397. doi: 10.1099/00207713-38-4-392

Coulthurst, S. (2019). The Type VI secretion system: a versatile bacterial weapon. *Microbiology* 165, 503–515. doi: 10.1099/mic.0.000789

Cunningham, S. D., and Munns, D. N. (1984). The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. *Soil Sci. Soc. Am. J*. 48:1273. doi: 10.2136/sssaj1984.03615995004800060001

De Campos, S. B., Lardi, M., Gandolfi, A., Eberl, L., and Pessi, G. (2017). Mutations in two *Paraburkholderia phymatum* type VI secretion systems cause reduced fitness in interbacterial competition. *Front. Microbiol*. 8, 2473. doi: 10.3389/fmicb.2017.02473

De Souza, J. A., Tieppo, E., Magnani Gde, S., Alves, L. M., Cardoso, R. L., Cruz, L. M., et al. (2012). Draft genome sequence of the nitrogen-fixing symbiotic bacterium *Bradyrhizobium elkanii 587*. *J. Bacteriol*. 194, 3547–3548. doi: 10.1128/JB.00563-12

Deakin, W. J., and Broughton, W. J. (2009). Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nat. Rev. Microbiol*. 7, 312–320. doi: 10.1038/nrmicro2091

Delamuta, J. R. M., Ribeiro, R. A., Ormeno-Orrillo, E., Melo, I. S., Martínez-Romero, E., and Hungria, M. (2013). Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens sp. nov*. *Int. J. Syst. Evol. Microbiol*.63, 3342–3351. doi: 10.1099/ijs.0.049130-0

Donati, A. J., Lee, H. I., Leveau, J. H., and Chang, W. S. (2013). Effects of indole-3-acetic acid on the transcriptional activities and stress tolerance of *Bradyrhizobium japonicum*. *PLoS ONE* 8:e76559. doi: 10.1371/journal.pone.0076559

Downie, J. A. (2010). The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. *FEMS Microbiol. Rev*. 34, 150–170. doi: 10.1111/j.1574-6976.2009.00205.x

El-Gebali, S., Mistry, J., Bateman, A., Eddy, S. R., Luciani, A., Potter, S. C., et al. (2019). The Pfam protein families database in 2019. *Nucleic Acids Res*. 47, D427–D432. doi: 10.1093/nar/gky995

English, G., Trunk, K., Rao, V. A., Srikannathasan, V., Hunter, W. N., and Coulthurst, S. J. (2012). New secreted toxins and immunity proteins encoded within the Type VI secretion system gene cluster of *Serratia marcescens*. *Mol. Microbiol*. 86, 921–936. doi: 10.1111/mmi.12028

Geddes, B. A., González, J. E., and Oresnik, I. J. (2014). Exopolysaccharide production in response to medium acidification is correlated with an increase in competition for nodule occupancy. *Mol. Plant Microbe Interact*. 27, 1307–1317. doi: 10.1094/MPMI-06-14-0168-R

Göttfert, M., Röthlisberger, S., Kündig, C., Beck, C., Marty, R., and Hennecke, H. (2001). Potential symbiosis-specific genes uncovered by sequencing a 410-kilobase DNA region of the *Bradyrhizobium japonicum* chromosome. *J. Bacteriol*. 183 1405–1412. doi: 10.1128/JB.183.4.1405-1412.2001

Guerrero-Castro, J., Lozano, L., and Sohlenkamp, C. (2018). Dissecting the acid stress response of *Rhizobium tropici CIAT 899*. *Front. Microbiol*. 9:846. doi: 10.3389/fmicb.2018.00846

Guimarães, A. A., Florentino, L. A., Almeida, K. A., Lebbe, L., Silva, K. B., Willems, A., et al. (2015). High diversity of *Bradyrhizobium* strains isolated from several legume species and land uses in Brazilian tropical ecosystems. *Syst. Appl. Microbiol*. 38, 433–441. doi: 10.1016/j.syapm.2015.06.006

Guimarães, A. A., Jaramillo, P. M. D., Nóbrega, R. S. A., Florentino, L. A., Silva, K. B., and de Souza Moreira, F. M. (2012). Genetic and symbiotic diversity of nitrogen-fixing bacteria isolated from agricultural soils in the western Amazon by using cowpea as the trap plant. *Appl. Environ. Microbiol.* 78, 6726–6733. doi: 10.1128 /AEM.01303-12

Haskett, T., Wang, P., Ramsay, J., O'Hara, G., Reeve, W., Howieson, J., et al. (2016). Complete genome sequence of *Mesorhizobium ciceri* strain CC1192, an efficient nitrogen-fixing microsymbiont of *Cicer arietinum*. *Genome Announc.* 4:16. doi: 10.1128/genomeA.00516-16

Hirsch, P. R. (1996). Population dynamics of indigenous and genetically modified rhizobia in the field. *New Phytol.* 133, 159–171. doi: 10.1111/j.1469-8137.1996.tb04351.x

Hungria, M., Campo, R. J., and Mendes, I. D. C. (2001). *Fixação Biológica Do Nitrogênio Na Cultura Da Soja*. Londrina: Embrapa Soja-Circular Técnica (INFOTECA-E).

Hungria, M., Delamuta, J. R. M., Ribeiro, R. A., and Nogueira, M. A. (2019). Draft genome sequence of *Bradyrhizobium elkanii* Strain SEMIA 938, used in commercial inoculants for *Lupinus spp*. *in Brazil. Microbiol. Resour. Announc.* 8, e00546–e00519. doi: 10.1128 / MRA.00546-19

Iida, T., Itakura, M., Anda, M., Sugawara, M., Isawa, T., Okubo, T., et al. (2015). Symbiosis island shuffling with abundant insertion sequences in the genomes of extra-slow-growing strains of soybean bradyrhizobia. *Appl. Environ. Microbiol.* 81, 4143–4154. doi: 10.1128/AEM.00741-15

Ivashina, T. V., and Ksenzenko, V. N. (2012). "Exopolysaccharide biosynthesis in *Rhizobium leguminosarum*: from genes to functions," in *The Complex World of Polysaccharides, Desiree Nedra Karunaratne* (IntechOpen), 99–127. doi: 10.5772/51202

Janczarek, M. (2011). Environmental signals and regulatory pathways that influence exopolysaccharide production in rhizobia. *Int. J. Mol. Sci.* 12, 7898–7933. doi: 10.3390/ijms12117898

Janczarek, M., Rachwał, K., and Kopcińska, J. (2015). Genetic characterization of the Pss region and the role of PssS in exopolysaccharide production and symbiosis of *Rhizobium leguminosarum bv.* trifolii with clover. *Plant Soil* 396, 257–275. doi: 10.1007/s11104-015-2567-5

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat. Rev. Microbiol.* 5, 619–633. doi: 10.1038/nrmicro1705

Jorrín, B., Palacios, J. M., Peix, A., and Imperial, J. (2020). *Rhizobium ruizarguesonis sp. nov.,* isolated from nodules of *Pisum sativum L. Syst. Appl. Microbiol.* 43:126090. doi: 10.1016/j.syapm.2020.126090

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., et al. (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res.* 9, 189–197. doi: 10.1093/ dnares/9.6.189

Kawaharada, Y., Nielsen, M., Kelly, S., et al. (2017). Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. *Nat. Commun.* 8:14534. doi: 10.1038/ncomms 14534

Kelly, S., Radutoiu, S., and Stougaard, J. (2017). Legume LysM receptors mediate symbiotic and pathogenic signalling. *Curr. Opin. Plant Biol.* 39, 152–158. doi: 10.1016/j.pbi.2017.06.013

Kopycińska, M., Lipa, P., Cieśla, J., Kozie,ł, M., and Janczarek, M. (2018). Extracellular polysaccharide protects *Rhizobium leguminosarum* cells against zinc stress *in vitro* and during symbiosis with clover. *Environ. Microbiol. Rep.* 10, 355–368. doi: 10.1111/1758-2229.12646

Kusakabe, S., Higasitani, N., Kaneko, T., Yasuda, M., Miwa, H., Okazaki, S., et al. (2020). Lotus accessions possess multiple checkpoints triggered by different type III secretion system effectors of the wide-host-range symbiont *Bradyrhizobium elkanii* USDA61. *Microbes Environ.* 35:ME19141. doi: 10.1264/jsme2.ME19141

Kuykendall, L. D., Saxena, B., Devine, T. E., and Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can. J. Microbiol.* 38, 501–505. doi: 10.1139/m92-082

Lewis, J. M., Deveson Lucas, D., Harper, M., and Boyce, J. D. (2019). Systematic identification and analysis of *Acinetobacter baumannii* type VI secretion system effector and immunity components. *Front. Microbiol.* 10:2440. doi: 10.3389/fmicb.2019.02440

Lien, Y. W., and Lai, E. M. (2017). Type VI secretion effectors: methodologies and biology. *Front. Cell. Infect. Microbiol.* 7:254. doi: 10.3389/fcimb.2017.00254

Lin, H. H., Huang, H. M., Yu, M., Lai, E. M., Chien, H. L., and Liu, C. T. (2018). Functional exploration of the bacterial type VI secretion system in mutualism: *Azorhizobium caulinodans ORS571–Sesbania rostrata* as a research model. *Mol. Plant Microbe Interact.* 31, 856–867. doi: 10.1094/MPMI-01-18-0026-R

Lipa, P., Vinardell, J. M., Kopcińska, J., Zdybicka-Barabas, A., and Janczarek, M. (2018). Mutation in the *pssZ* gene negatively impacts exopolysaccharide synthesis, surface properties, and symbiosis of *Rhizobium leguminosarum bv. trifolii* with clover. *Gene* 97:369. doi: 10.3390/genes9070369

López-Baena, F. J., Ruiz-Sainz, J. E., Rodríguez-Carvajal, M. A., and Vinardell, J. M. (2016). Bacterial molecular signals in the *Sinorhizobium fredii*-soybean symbiosis. *Int. J. Mol. Sci.* 17:755. doi: 10.3390/ijms17050755

Ma, L. S., Hachani, A., Lin, J. S., Filloux, A., and Lai, E. M. (2014). *Agrobacterium tumefaciens* deploys a superfamily of type VI secretion DNase effectors as weapons for interbacterial competition in planta. *Cell Host Microbe* 16, 94–104. doi: 10.1016/j.chom.2014.06.002

Marczak, M., Mazur, A., Koper, P., Zebracki, K., and Skorupska, A. (2017). Synthesis of rhizobial exopolysaccharides and their importance for symbiosis with legume plants. *Genes* 8:360. doi: 10.3390/genes8120360

Marczak, M., Wójcik, M., Zebracki, K., Turska-Szewczuk, A., and Talarek, K., Nowak, D., et al. (2020). PssJ is a terminal galactosyltransferase involved in the assembly of the exopolysaccharide subunit in *Rhizobium leguminosarum bv. trifolii. Int. J. Mol. Sci.* 21:7764. doi: 10.3390/ijms21207764

Margaret-Oliver, I., Lei, W., Parada, M., Rodríguez-Carvajal, M. A., Crespo-Rivas, J. C., Hidalgo, Á., et al. (2012). *Sinorhizobium fredii HH103* does not strictly require KPS and/or EPS to nodulate *Glycyrrhiza uralensis*, an indeterminate nodule-forming legume. *Arch. Microbiol.* 194, 87–102. doi: 10.1007/s00203-011-0729-2

Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., and Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris L. beans and Leucaena sp.* trees. *Int. J. Syst. Evol. Microbiol.* 41, 417–426. doi: 10.1099/00207713-41-3-417

Menna, P., and Hungria, M. (2011). Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. *Int. J. Syst. Evol. Microbiol.* 61, 3052–3067. doi: 10.1099/ijs.0.02 8803-0

Milner, J. L., Araujo, R. S., and Handelsman, J. (1992). Molecular and symbiotic characterization of exopolysaccharide-deficient mutants of *Rhizobium tropici* strain CIAT899. *Mol. Microbiol.* 6, 3137–3147. doi: 10.1111/j.1365-2958. 1992.tb01770.x

Miwa, H., and Okazaki, S. (2017). How effectors promote beneficial interactions. *Curr. Opin. Plant Biol.* 38, 148–154. doi: 10.1016/j.pbi.2017.05.011

Monjarás- Feria, J., and Valvano, M. A. (2020). An overview of anti-eukaryotic T6SS effectors. *Front. Cell. Infect. Microbiol.* 10:617. doi: 10.3389/fcimb.2020.584751

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., et al. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312, 1526–1530. doi: 10.1126/science.1128393

Moulin, L., Béna, G., Boivin-Masson, C., and Stepkowski, T. (2004). Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium genus*. *Mol. Phylogenet. Evol.* 30, 720–732. doi: 10.1016/S1055-7903(03)00255-0

Naseem, H., Ahsan, M., Shahid, M. A., and Khan, N. (2018). Exopolysaccharides producing rhizobacteria and their role in plant growth and drought tolerance. *J. Basic Microbiol.* 58, 1009–1022. doi: 10.1002/jobm.201800309

Nilsson, J. F., Castellani, L. G., Draghi, W. O., Mogro, E. G., Wibberg, D., Winkler, A., et al. (2021). Global transcriptome analysis of *Rhizobium favelukesii* LPU83 in response to acid stress. *FEMS Microbiol. Ecol.* 97:235. doi: 10.1093/femsec/fiaa235

Obando, M., Correa-Galeote, D., Castellano-Hinojosa, A., Gualpa, J., Hidalgo, A., Alché, J. D. D., et al. (2019). Analysis of the denitrification pathway and greenhouse gases emissions in *Bradyrhizobium sp.* strains used as biofertilizers in South America. *J. Appl. Microbiol.* 127, 739–749. doi: 10.1111/jam.14233

Ormeño-Orrillo, E., Menna, P., Almeida, L. G., Ollero, F. J., Nicolás, M. F., Pains Rodrigues, E., et al. (2012). Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium sp.*

PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris L.*). *BMC Genom*. 13:735. doi: 10.1186/1471-2164-13-735

Pei, T. T., Li, H., Liang, X., Wang, Z. H., Liu, G., Wu, L. L., et al. (2020). Intramolecular chaperone-mediated secretion of an Rhs effector toxin by a type VI secretion system. *Nat. Commun.* 11, 1–13. doi: 10.1038/s41467-020-15774-z

Pellock, B. J., Teplitski, M., Boinay, R. P., Bauer, W. D., and Walker, G. C. (2002). A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J. Bacteriol.* 184, 5067–5076. doi: 10.1128/jb.184.18.5067-5076.2002

Primo, E. D., Cossovich, S., Nievas, F., Bogino, P., Humm, E. A., Hirsch, A. M., et al. (2020). Exopolysaccharide production in *Ensifer meliloti* laboratory and native strains and their effects on alfalfa inoculation. *Arch. Microbiol.* 202, 391–398. doi: 10.1007/s00203-019-01756-3

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., et al. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1528–1533. doi: 10.1073/pnas.0510322103

Puozaa, D. K., Jaiswal, S. K., and Dakora, F. D. (2017). African origin of *Bradyrhizobium* populations nodulating Bambara groundnut (*Vigna subterranea L.* Vedic) in Ghanaian and South African soils. *PLoS ONE*. 12:e0184943. doi: 10.1371/journal.pone.0184943

Reuber, T. L., Reed, J., Glazebrook, J., Glucksmann, M. A., Ahmann, D., Marra, A., et al. (1991). *Rhizobium meliloti* exopolysaccharides: genetic analyses and symbiotic importance. *Biochem. Soc. Trans.* 19, 636–644. doi: 10.1042/bst0190636

Rüberg, S., Pühler, A., and Becker, A. (1999). Biosynthesis of the exopolysaccharide galactoglucan in *Sinorhizobium meliloti* is subject to a complex control by the phosphate-dependent regulator PhoB and the proteins ExpG and MucR. *Microbiology*. 145, 603–611. doi: 10.1099/13500872-145-3-603

Rufini, M., Da Silva, M. A. P., Ferreira, P. A. A., de Souza Cassetari, A., Soares, B. L., De Andrade, M. J. B., et al. (2014). Symbiotic efficiency and identification of rhizobia that nodulate cowpea in a Rhodic Eutrudox. *Biol. Fert. Soils*. 50, 115–122. doi: 10.1007/s00374-013-0832-4

Russo, D. M., Williams, A., Edwards, A., Posadas, D. M., Finnie, C., Dankert, M., et al. (2006). Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by *Rhizobium leguminosarum*. *J. Bacteriol.* 188, 4474–4486. doi: 10.1128/JB.00 246-06

Salinero-Lanzarote, A., Pacheco-Moreno, A., Domingo-Serrano, L., Durán, D., Ormeño-Orrillo, E., Martínez-Romero, E., et al. (2019). The Type VI secretion system of *Rhizobium etli Mim1* has a positive effect in symbiosis. *FEMS Microbiol. Ecol.* 95:54. doi: 10.1093/femsec/fiz054

Santos, M. S., Nogueira, M. A., and Hungria, M. (2019). Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. *AMB Express*. 9, 1–22. doi: 10.1186/s13568-019-0932-0

Schäper, S., Wendt, H., Bamberger, J., Sieber, V., Schmid, J., and Becker, A. (2019). A bifunctional UDP-sugar 4-epimerase supports biosynthesis of multiple cell surface polysaccharides in *Sinorhizobium meliloti*. *J. Bacteriol.* 201, e00801–e00818. doi: 10.1128/JB.00801-18

Shalom, G., Shaw, J. G., and Thomas, M. S. (2007). In vivo expression technology identifies a type VI secretion system locus in *Burkholderia pseudomallei* that is induced upon invasion of macrophages. *Microbiology* 153, 2689–2699. doi: 10.1099/mic.0.2007/006585-0

Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., and Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* 15, 350–353. doi: 10.1038/nature 12453

Silverman, J. M., Brunet, Y. R., Cascales, E., and Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annu. Rev. Microbiol.* 66, 453–472. doi: 10.1146/annurev-micro-121809-151619

Simões-Araújo, J. L., Leite, J., Marie Rouws, L. F., Passos, S. R., Xavier, G. R., Rumjanek, N. G., et al. (2016). Draft genome sequence of *Bradyrhizobium sp.* strain BR 3262, an effective microsymbiont recommended for cowpea inoculation in Brazil. *Braz. J. Microbiol.* 47, 783–784. doi: 10.1016/j.bjm.2016.03.001

Siqueira, A. F., Ormeno-Orrillo, E., Souza, R. C., Rodrigues, E. P., Almeida, L. G. P., Barcellos, F. G., et al. (2014). Comparative genomics of *Bradyrhizobium japonicum CPAC 15* and *Bradyrhizobium diazoefficiens CPAC 7*: elite model strains for understanding symbiotic performance with soybean. *BMC Genom.* 15:420. doi: 10.1186/1471-2164-15-420

Skorupska, A., Janczarek, M., Marczak, M., Mazur, A., and Król, J. (2006). Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microb. Cell Fact.* 5, 1–19. doi: 10.1186/1475-2859-5-7

Sorroche, F., Bogino, P., Russo, D. M., Zorreguieta, A., Nievas, F., Morales, G. M., et al. (2018). Cell autoaggregation, biofilm formation, and plant attachment in a *Sinorhizobium meliloti* lpsB Mutant. *Mol. Plant Microbe. Interact.* 31, 1075–1082. doi: 10.1094/MPMI-01-18-0004-R

Sorroche, F. G., Rinaudi, L. V., Zorreguieta, Á., and Giordano, W. (2010). EPS II-dependent auto aggregation of *Sinorhizobium meliloti* planktonic cells. *Curr. Microbiol.* 61, 465–470. doi: 10.1007/s00284-010-9639-9

Srikannathasan, V., English, G., Bui, N. K., Trunk, K., O'Rourke, P. E., Rao, V. A., et al. (2013). Structural basis for type VI secreted peptidoglycan DL-endopeptidase function, specificity and neutralization in *Serratia marcescens*. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 69, 2468–2482. doi: 10.1107/S0907444913022725

Staehelin, C., Forsberg, L. S., D'Haeze, W., Gao, M. Y., Carlson, R. W., Xie, Z. P., et al. (2006). Exo-oligosaccharides of *Rhizobium sp.* strain *NGR234* are required for symbiosis with various legumes. *J. Bacteriol.* 188, 6168–6178. doi: 10.1128/JB.00365-06

Sugawara, M., Epstein, B., Badgley, B. D., Unno, T., Xu, L., Reese, J., et al. (2013). Comparative genomics of the core and accessory genomes of 48 *Sinorhizobium* strains comprising five genospecies. *Genome Biol.* 14:R17. doi: 10.1186/gb-2013-14-2-r17

Sun, Y., Liu, Y., Liu, X., Dang, X., Dong, X., Xie, Z., et al. (2020). *Azorhizobium caulinodans* c-di-GMP phosphodiesterase Chp1 involved in motility, EPS production, and nodulation of the host plant. *Appl. Microbiol. Biotechnol.* 104, 2715–2729. doi: 10.1007/s00253-020-10404-6

Trunk, K., Peltier, J., Liu, Y. C., Dill, B. D., Walker, L., Gow, N. A., et al. (2018). The type VI secretion system deploys antifungal effectors against microbial competitors. *Nat. Microbiol.* 3, 920–931. doi: 10.1038/s41564-018-0191-x

Vences-Guzmán, M. A., Guan, Z., Ormeno-Orrillo, E., González-Silva, N., López-Lara, I. M., Martínez-Romero, E., et al. (2011). Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici CIAT899*. *Mol. Microbiol.* 79, 1496–1514. doi: 10.1111/j.1365-2958.2011.07535.x

Via, V. D., Narduzzi, C., Aguilar, O. M., Zanetti, M. E., and Blanco, F. A. (2015). Changes in the common bean transcriptome in response to secreted and surface signal molecules of *Rhizobium etli*. *Plant Physiol.* 169, 1356–1370. doi: 10.1104/pp.15.00508

Vinuesa, P., Neumann-Silkow, F., Pacios-Bras, C., Spaink, H. P., Martínez-Romero, E., and Werner, D. (2003). Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. *Mol. Plant-Microbe Interact.* 16, 159–168. doi: 10.1094/MPMI.2003.16.2.159

Wang, J., Brodmann, M., and Basler, M. (2019). Assembly and subcellular localization of bacterial type VI secretion systems. *Annu. Rev. Microbiol.* 73, 621–638. doi: 10.1146/annurev-micro-020518-115420

Wang, P., Zhong, Z., Zhou, J., Cai, T., and Zhu, J. (2008). Exopolysaccharide biosynthesis is important for *Mesorhizobium tianshanense*: plant host interaction. *Arch. Microbiol.* 189, 525–530. doi: 10.1007/s00203-007-0345-3

Willems, A. (2006). The taxonomy of rhizobia: an overview. *Plant Soil.* 287, 3–14. doi: 10.1007/s11104-006-9058-7

Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P., et al. (2001). DNA-DNA hybridization study of *Bradyrhizobium* strains. *Int. J. Syst. Evol. Microbiol.* 51, 1315–1322. doi: 10.1099/00207713-51-4-1315

Wong, J. E. M. M., Gysel, K., Birkefeldt, T. G., Vinther, M., Muszyński, A., Azadi, P., et al. (2020). Structural signatures in EPR3 define a unique class of plant carbohydrate receptors. *Nat. Commun.* 11:3797. doi: 10.1038/s41467-020-17568-9

Yang, X., Long, M., and Shen, X. (2018). Effector–immunity pairs provide the T6SS nanomachine its offensive and defensive

capabilities. *Molecules* 23:1009. doi: 10.3390/molecules23051009

Zhang, D., Iyer, L. M., and Aravind, L. (2011). A novel immunity system for bacterial nucleic acid degrading toxins and its recruitment in various eukaryotic and DNA viral systems. *Nucleic Acids Res.* 39, 4532–4552. doi: 10.1093/nar/gkr036

Zhang, H., Gao, Z. Q., Wang, W. J., Liu, G. F., Xu, J. H., Su, X. D., et al. (2013). Structure of the type VI effector-immunity complex (Tae4-Tai4) provides novel insights into the inhibition mechanism of the effector by its immunity protein. *J. Biol. Chem*. 288, 5928–5939. doi: 10.1074/jbc.M112.434357

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.

Copyright © 2021 De Sousa, Castellane, Tighilt, Lemos and Rey. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

BRIEF RESEARCH REPORT
published: 16 July 2021
doi: 10.3389/fagro.2021.661526

Efficient CRISPR/Cas9 Genome Editing in Alfalfa Using a Public Germplasm

*Emilia Bottero[1,2], Gabriela Massa[3,4], Matías González[3], Margarita Stritzler[1,2], Hiromi Tajima[5], Cristina Gómez[1,2], Romina Frare[1,2], Sergio Feingold[3], Eduardo Blumwald[5], Nicolás Ayub[1,2] and Gabriela Soto[1,2]**

[1] Instituto de Agrobiotecnología y Biología Molecular, CONICET - INTA, Buenos Aires, Argentina, [2] Instituto de Genética "Edwald Alfredo Favret", INTA, Buenos Aires, Argentina, [3] Laboratorio de Agrobiotecnología, IPADS, CONICET - INTA, Balcarce, Argentina, [4] Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Balcarce, Argentina, [5] Department of Plant Sciences, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Juan Sanjuan,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Jun Shan,
Institute of Soil Science (CAS), China
Longlong Wang,
Huazhong Agricultural University, China

***Correspondence:**
Gabriela Soto
soto.gabrielacinthia@inta.gob.ar
orcid.org/0000-0002-3492-1268

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: 30 January 2021
Accepted: 17 June 2021
Published: 16 July 2021

Citation:
Bottero E, Massa G, González M, Stritzler M, Tajima H, Gómez C, Frare R, Feingold S, Blumwald E, Ayub N and Soto G (2021) Efficient CRISPR/Cas9 Genome Editing in Alfalfa Using a Public Germplasm. Front. Agron. 3:661526. doi: 10.3389/fagro.2021.661526

Because its ability to acquire large amounts of nitrogen by symbiosis, tetraploid alfalfa is the main source of vegetable proteins in meat and milk production systems in temperate regions. Alfalfa cultivation also adds fixed nitrogen to the soil, improving the production of non-legumes in crop rotation and reducing the use of nitrogen fertilizers derived from fossil fuel. Despite its economic and ecological relevance, alfalfa genetics remains poorly understood, limiting the development of public elite germplasm. In this brief article, we reported the high-efficiency of alfalfa mutagenesis by using the public clone C23 and the CRISPR/Cas9 system. Around half of the *GUS* overexpressing plants (35S-*GUS* under C23 genomic background) transformed with an editing plasmid containing two sgRNAs against the *GUS* gene and the Cas9 nuclease exhibited absence of *GUS* activity. Nucleotide analysis showed that the inactivation of GUS in CRISPR/Cas9-editing events were produced via different modifications in the GUS gene, including frameshift and non-sense mutations. Using the CRISPR/Cas9 system and two sgRNAs, we have also edited the alfalfa gene *NOD26*, generating plants with different doses of alleles at this locus, including complete gene knockout at high efficiency (11%). Finally, we discuss the potential applications of genome-editing technologies to polyploid research and to alfalfa improvement public programs.

Keywords: alfalfa, genome editing, CRISPR/Cas9, mutants, transformation

INTRODUCTION

The production of mutant alleles has been essential for plant functional genetics. Efficient generation of stable plant mutant lines has been achieved using different methods, including ethyl methanesulfonate, ionizing radiation, RNA interference, artificial microRNAs, and the insertion of T-DNA and retrotransposons. During the last years, there has been an explosion in the production of knockout plant lines through the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 genome-editing system of *Streptococcus pyogenes*, which requires the canonical PAM motif NGG (N, any nucleotide; G, guanine) in dsDNA-bound states for DNA targeting. This system contains two components: one encoding the Cas9 endonuclease and the other containing a synthetic single guide RNA (sgRNA), with the latter typically under the control of the U6 small nuclear RNA gene promoter. Cas9 is normally efficient at inducing double-strand DNA breaks in

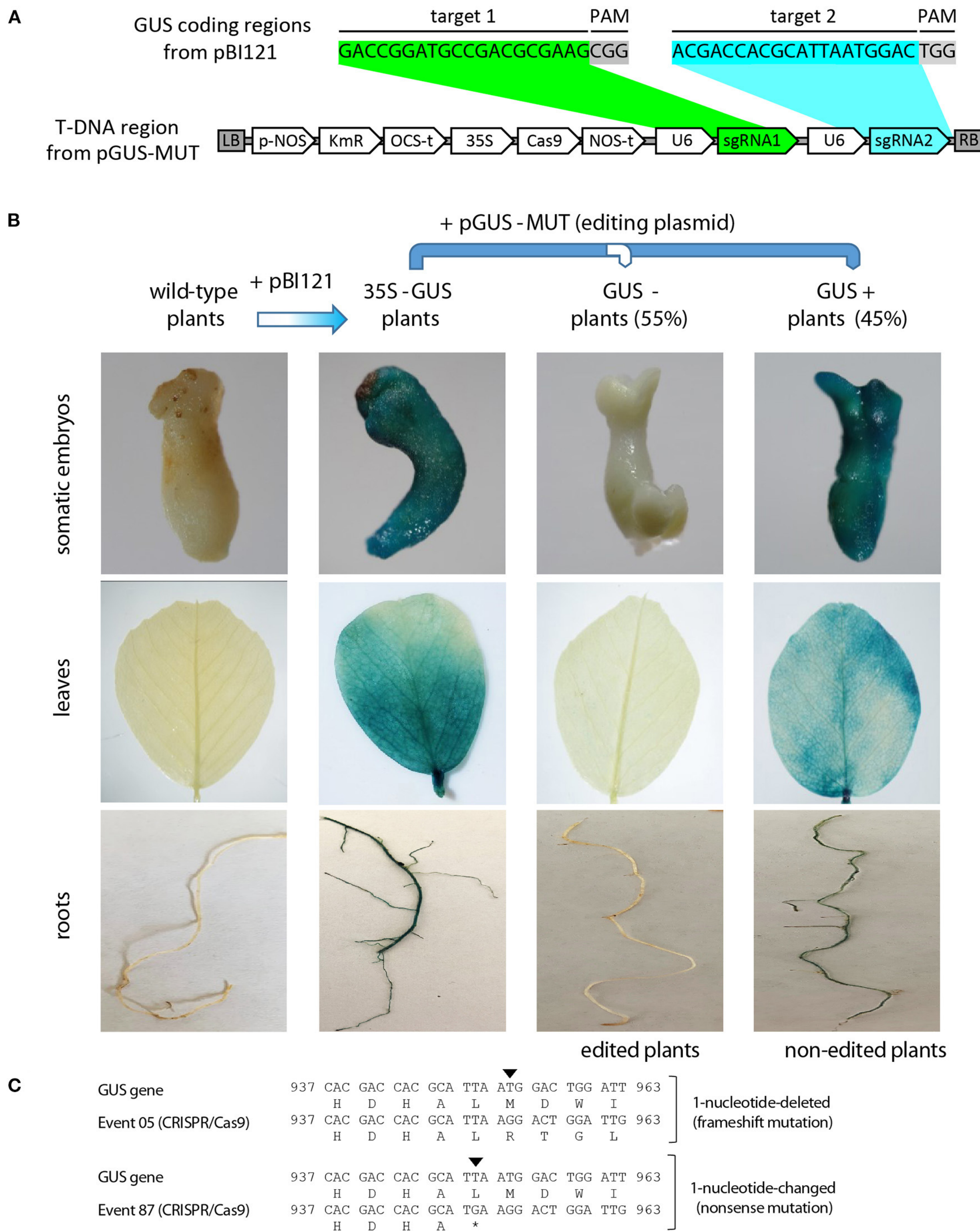


FIGURE 1 | High-efficiency alfalfa mutagenesis by using regenerative alfalfa clone C23 and the CRISPR/Cas9 system. **(A)** Above: Schematic representation of a fragment of the *GUS* coding sequence from the binary vector pBI121 used to produce transgenic alfalfa with high *GUS* activities, specifically showing the sequence targeted by the sgRNA (target 1 and target 2) and the sequence recognized by the Cas9 nuclease (PAM sequences). Below: Schematic representation of the T-DNA region from the binary vector p*GUS*-MUT used for the CRISPR/Cas9-mediated knockout of *GUS* reporter. In the last vector, the nopaline synthase (pNOS), the *Arabidopsis thaliana* U6 (U6), and the CaMV 35S promoters were used to drive the constitutive expression of the NPTII gene for kanamycin selection *in planta* (KmR), a plant codon-optimized Cas9 gene for genome editing in dicots (Cas9) and two sgRNA (sgRNA1 and sgRNA2) that targeted in two regions (target 1 and target 2) of the *GUS* gene. The octopine synthase (OCS-t) and nopaline synthase (NOS-t) terminators within the T-DNA region are shown. **(B)** Representative images of *GUS* activity in somatic embryos and leaves of alfalfa clone C23 without synthetic elements (wild-type plants), alfalfa clone C23 containing the *GUS* reporter from pBI121 (35S-*GUS* plants), and importantly, *GUS*-positive (*GUS*+) and *GUS*-negative (*GUS*-) plants derived from 35S-*GUS* plants transformed with the CRISPR/Cas9 system for *GUS* edition from p*GUS*-MUT. As described in the text and summarized in this picture, around half of the 35S-*GUS* plants transformed with the Cas9 nuclease

(Continued)

FIGURE 1 | exhibited absence of *GUS* activity (*GUS*- plants), showing that this genome editing system (CRISPR/Cas9) is an excellent tool for the mutagenesis of this genomic background (alfalfa clone C23). **(C)** Sequence analysis showed different mutations within the *GUS* genes from edited alfalfa events (CRISPR/Cas9) that generate mutant versions of *GUS* proteins in these plants, such as frameshift and non-sense mutations within target 2 in CRISPR/Cas9-editing events 5 and 87.

both model plants and important crops. When repaired by the error-prone non-homologous end-joining pathway, these DNA breaks eventually result in the emergence of short deletions and/or insertions (indels) that give rise to stop codons or non-sense and frameshift mutations (Bortesi and Fischer, 2015).

Alfalfa (*Medicago sativa* L.), commonly known as the "Queen of Forages," is the main source of plant protein for the meat and milk global production. Because of its ability to establish effective symbiosis with nitrogen-fixing bacteria, the production of animal protein based on alfalfa feeds does not require the use of nitrogen fertilizers derived from fossil fuel, reducing our dependence on non-renewable energy sources and mitigating the emission of greenhouse gases. Although during the last years the production of stable mutant libraries has been extended from model plants (e.g., Arabidopsis thaliana, Medicago truncatula, and Brachypodium distachyon) to main crops (e.g., potato, soybean, and sorghum), public projects have not produced mutant libraries of alfalfa, limiting functional genomics studies from this important legume crop. Consequently, there is a continuing and increasing interest in the development of alternative methods for the editing of alfalfa genome, which partially depends on the regenerative genotype used during alfalfa transformation (Gao et al., 2018; Chen et al., 2020; Wolabu et al., 2020). Previously, we developed a highly effective procedure for the transformation of the public alfalfa clone C23 via *Agrobacterium tumefaciens* (Garcia et al., 2014; Jozefkowicz et al., 2016, 2018; Stritzler et al., 2018; Pascuan et al., 2020). In this brief research report, we used this framework for the production of CRISPR/Cas9 gene-edited alfalfa from clone C23.

MATERIALS AND METHODS

For the construction of the p*GUS*-MUT plasmid, two gRNAs were produced using the gh51 plasmid (Narimatsu et al., 2018) as template and specific primers designed against the *GUS* gene (**Supplementary Figure 1**). The binary vector p*GUS*-MUT was constructed in two steps by using the Golden Gate cloning system (Weber et al., 2011), and BsaI and BbSI restriction enzymes supplemented with T4 ligase for Level 1 and Level 2 vectors, respectively. First, the Level 1 vectors pICH47751 and pICH47761 (Weber et al., 2011) were used in combination with pICSL01009::AtU6p (Nekrasov et al., 2013), to obtain sgRNA*GUS*Mse and sgRNA*GUS* under the control of the *A. thaliana* U6 promoter. Next, Level 1 constructs (pICH47751::AtU6p::sgRNA*GUS*Mse and pICH47761::AtU6p::sgRNA*GUS*), pICH47732::NOSp-NPTII-OCST (Castel et al., 2019), pICH47742::35S-Cas9-NOST (Belhaj et al., 2013) and the linker pICH41780 (Weber et al., 2011) were assembled into the Level 2 vector pAGM4723 (Weber et al., 2011). For molecular and histochemical analyses of transgenic and edited plants, genomic DNA of alfalfa plants was isolated from leaf tissue by using the DNeasy plant mini kit (Cat.#69104, Qiagen). The presence and integrity of the *GUS* reporter in alfalfa plants were analyzed by PCR studies against the *GUS* reporter and Sanger sequencing. In PCR analysis, a 1,098-bp fragment derived from the *GUS* reporter was amplified by using primers *GUS*-UP and *GUS*-Low (**Supplementary Figure 1**), and the following amplification conditions: 1 cycle at 94°C for 5 min, 34 cycles of 94°C for 1 min, 60°C for 1 min 30 s and 72°C for 1 min, and a final extension of 72°C for 8 min. We verified that the *GUS* gene was edited in a subset of 25 plants via Sanger sequencing. Histochemical detection of *GUS* activity in alfalfa plants was analyzed as previously (Kelemen et al., 2002).

For the insertion and mutation of the *GUS* gene in the alfalfa genome, two transgenic alfalfa events, called here 3-1 and 5-1, which show high and constitutive expression of the *GUS* reporter, were generated by transforming the regenerative alfalfa clone C2-3 with the binary vector pBI121 (Accession Number: AF485783.1) as described previously (Garcia et al., 2014; Jozefkowicz et al., 2018). To determine the number of T-DNA insertions in 3-1 and 5-1, these 35S-*GUS*-transformed alfalfa events were individually crossed manually with CW1010 wild-type alfalfa by using transgenic parental plants as pollen donors. The progenies of 3-1 and 5-1 were analyzed by histochemical detection of *GUS* activity, showing in both cases a Mendelian inheritance pattern of a single gene. For the editing of the *GUS* reporter in 3-1 and 5-1 via CRISPR/Cas9, the binary vector p*GUS*-MUT was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation, as described by Shen and Forde (1989). Petioles of 3-1 and 5-1 were transformed with p*GUS*-MUT via *A. tumefaciens* and cultured *in vitro* as described previously (Garcia et al., 2014), with some modifications to minimize escapes from the kanamycin selection and maximize the efficiency of genome editing in alfalfa. Petiole tissues were decontaminated by dipping in 70% ethyl alcohol for 1 min and 2% sodium hypochlorite for 20 min. The petioles were washed five times in sterile bidistilled water. Explants earlier damaged with a scalpel were inoculated with an *A. tumefaciens* culture for 3 min ($OD_{600nm} = 0.6$), and then dried in Whatman filter paper and transferred to SHK medium (Garcia et al., 2014) supplemented with 100 μM acetosyringone and 100 mg/l cefotaxime, and incubated for 3 days at 25°C in the dark. The alfalfa explants were then washed five times with 400 mg/l cefotaxime and transferred to SHK medium containing 400 mg/l cefotaxime and 25 mg/l kanamycin, and then, routinely transferred to fresh medium every 1 week, at 25°C and 16 h light (150 μmoles/m^2s). Somatic embryos were transferred to MS rooting medium, composed of MS medium (Jozefkowicz et al., 2016) diluted 1:2 with water. Around 6 months after callus formation, regenerated plantlets were transferred to

growth chambers programmed for 16 h light at 23°C and 8 h dark at 20°C. CRISPR/Cas9-mediated gene-editing events were propagated by cuttings to increase the plant material available for histochemical and molecular studies. Kanamycin-tolerant plants derived from different calluses were considered independent gene-editing events.

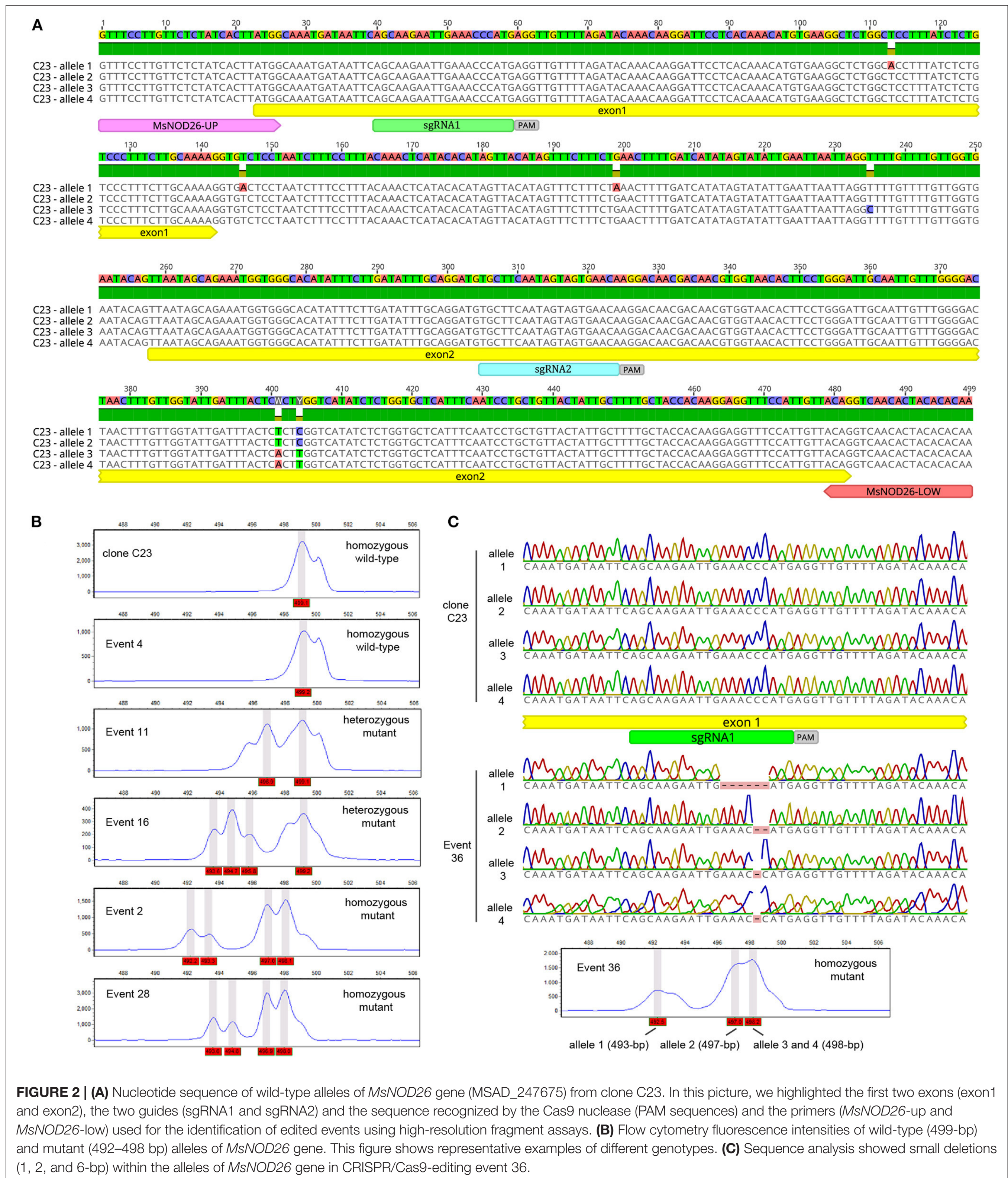


FIGURE 2 | (A) Nucleotide sequence of wild-type alleles of *MsNOD26* gene (MSAD_247675) from clone C23. In this picture, we highlighted the first two exons (exon1 and exon2), the two guides (sgRNA1 and sgRNA2) and the sequence recognized by the Cas9 nuclease (PAM sequences) and the primers (*MsNOD26*-up and *MsNOD26*-low) used for the identification of edited events using high-resolution fragment assays. **(B)** Flow cytometry fluorescence intensities of wild-type (499-bp) and mutant (492–498 bp) alleles of *MsNOD26* gene. This figure shows representative examples of different genotypes. **(C)** Sequence analysis showed small deletions (1, 2, and 6-bp) within the alleles of *MsNOD26* gene in CRISPR/Cas9-editing event 36.

The p*NOD26*-MUT plasmid was produced using the modular system and the Webtools for the Voytas Lab Plant Genome Engineering Toolkit previously described by the Voytas group (Cermak et al., 2017). To corroborate the complete conservation of the nucleotide sequence to edit in the genome of the C23 clone, the two first exons and the first intron of the *NOD26* gene from *Medicago sativa* (MSAD_247675) was cloned into a pGEM-T easy vector (Biodynamics, Argentina) and sequenced. Two sgRNAs were designed for each target, specifically exon 1 and exon 2 of *MsNOD26* (**Supplementary Figure 1**). PCR products containing BsaI restriction sites were digested and ligated through a Golden Gate reaction, resulting in one polycistronic element containing the CmYLCV promoter, sgRNAs, Csy4 sites and the CaMV terminator. The subsequent fragment was digested with SapI enzyme and cloned into pMOD_B2103. The pMODB vector harboring the polycistronic construct and three other modular vectors (pMOD_A0501, pMOD_C3003, and pTRANS_220d) were assembled together with AarII digestion and ligation, resulting in the binary vector p*NOD26*-MUT. The transformation of the C23 clone with the p*NOD26*-MUT vector via Agrobacterium and the selection of transgenic plants using kanamycin were performed as described above. The presence of the Cas9 gene in regenerated plants was confirmed by PCR studies. In this analysis, a 353-bp fragment derived from the Cas9 gene was amplified by using specific primers (**Supplementary Figure 1**), and the following amplification conditions: 1 cycle at 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension of 72°C for 5 min. For the analysis of *NOD26*-edited alleles in transgenic plants, genomic DNA was extracted from young leaves as described above. DNA fragments derived from the target region was amplified by using specific primers (**Supplementary Figure 1**) in a final volume of 20 L in the presence of 75 ng DNA, 1 U of Taq DNA polymerase (Platinum Taq DNA Polymerase, Invitrogen), 2.5 mM MgCl2, 0.2 mM of each dNTP, 2 L of 10 X PCR Buffer (Invitrogen) and 0.5 mM of each primer. The forward primer was labeled with 5'6-FAM (Thermo Fisher Scientific, Argentina). The PCR protocol was: 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 55 °C for 30 s, and 72°C for 40 s, and a final extension of 10 min at 72°C. The amplified fragments were detected by a Genetic Analyzer GA3500XL (Applied Biosystems), as previously (Cuyeu et al., 2013, 2014). Each PCR was performed six times.

RESULTS AND DISCUSSION

To evaluate the rate of induced mutation frequency via CRISPR/Cas9 in alfalfa by using the C23 genotype, we produced two transgenic alfalfa events (3-1 and 5-1) with the pBI121 binary vector containing the *GUS* gene (**Figure 1A**) and expressing high *GUS* activities in both somatic embryos and leaves (**Figure 1B**), which were re-transformed with the p*GUS*-MUT plasmid containing two sgRNAs against the *GUS* gene under the control of the U6 promoter for the editing of this reporter via CRISPR/Cas9 (**Figure 1A**). As expected, no *GUS* inactivation was observed in 97 regenerated plants derived from 3-1 to 5-1, confirming the absence of spontaneous *GUS* inactivation under our specific regeneration condition (data not shown). Contrarily, *GUS* inactivation was observed in plants derived from 3-1 to 5-1 transformed with the p*GUS*-MUT plasmid (**Figure 1B**), where the frequency of *GUS* inactivation was 48% (37 *GUS*-negative and 40 *GUS*-positive plants) and 62% (58 *GUS*-negative and 35 *GUS*-positive plants), respectively, showing the high efficiency (an average of 55%) of CRISPR/Cas9 genome editing system in C23 genomic background. As expected, nucleotide analysis showed that *GUS* inactivation in CRISPR/Cas9-editing events (CRISPR/Cas9) were produced via different alterations in the *GUS* gene, including frameshift and non-sense mutations (**Figure 1C**).

Due to the polyploidy nature of cultivated alfalfa (*Medicago sativa*), it is important to analyze the efficiency of CRISPR/Cas9 to mutagenize the four alleles of an endogenous gene of the C23 genotype. In this context, we produced 35 transgenic events with the p*NOD26*-MUT plasmid containing CRISPR/Cas9 and two sgRNAs against the *NOD26* gene (Frare et al., 2018) under the CmYLCV promoter. Specifically, these sgRNAs target to the first two exons of the *MsNOD26* gene (**Figure 2A**). Due to the high conservation of the alleles of the *MsNOD26* gene in the region selected to edit, the 499-bp amplified fragment derived from wild-type alleles can be rapidly detected by flow cytometry (**Figure 2B**). This amplification showed 31 alleles, with band sizes of 492–499 bp and multiple products per events (ranging from 1 to 4), at an average of 1.7 alleles per locus in the 36 transgenic events (**Supplementary Table 1**), supporting the high efficiency of the CRISPR/Cas9 genome editing system in the C23 genomic background (**Supplementary Table 1**). This approach preferentially induces small indels in edited alleles (**Supplementary Table 1**), and consequently, these directed mutations mimic spontaneous mutations. The occurrence of this particular type of genetic modifications improves the commercial perspectives of the edited alfalfa germplasm in major alfalfa-producing countries (i.e., USA, Canada, Australia and Argentina), where there are particular legislations regarding new plant-breeding technologies (Menz et al., 2020). Interestingly, it is possible to generate different genotypes for this simple locus (*MsNOD26* gene) with a relative small set of transgenic events. These genotypes include non-edited (wild-type) plants and homozygous and heterozygous mutants for the *MsNOD26* gene (**Figures 2B,C**). Therefore, the mutagenesis of alfalfa clone C23 via the CRISPR/Cas9 system may help to explore the effect of different doses of alleles at an individual locus, expanding our understanding of its complex genetics. Additionally, the CRISPR/Cas9 system delivers gene knockout at high efficiencies (11%) in the C23 genomic background (**Supplementary Table 1**), a common rate-limiting step to understand the role of a single gene in this important legume crop.

The alfalfa-related species *Medicago truncatula* has been consolidated as the model plant for legume genetics and genomics, due to several important features, including its diploid genome, short generation time, small genome size, capacity to reproduce by self- and out-crossing, and the availability of

its complete genome sequence and vast mutant libraries. In addition, there are clear examples that elegantly illustrate how knowledge gained from this model species can be used to improve alfalfa (Yang et al., 2008; Gou et al., 2018). However, as normally observed for any pair of related plant species, *Medicago truncatula* and alfalfa have numerous differences that limit the application of this model in alfalfa breeding. Since cultivated alfalfa is a tetraploid, allogamous, perennial species, species-specific factors influence the survival and high production of an alfalfa stand (e.g., plant regrowth), which depends on complex allelic variants. Besides, although alfalfa exhibits high levels of self-incompatibility, the homozygosis in an individual locus can be rapidly and inexpensively generated via alfalfa selfing by using non-emasculated hand crosses under greenhouse conditions (Jozefkowicz et al., 2018). Consequently, full allelic knock-out of an individual alfalfa gene could be obtained by autofecundation of edited plants, independently of the efficiency of the CRISPR/Cas9 system to simultaneously target multiple alleles in this crop. In genetically heterozygous tetraploid potato, high-efficacy full-allelic CRISPR/Cas9 gene editing was obtained by replacing the Arabidopsis U6 promoter (the same used in this work to control of expression of *sgRNA* against the *GUS* reporter), driving expression of the CRISPR factors, with endogenous potato U6 promoters (Johansen et al., 2019). In this line, we recently generated native-optimized vectors (e.g., binary vectors containing alfalfa *H3.2* promoter)

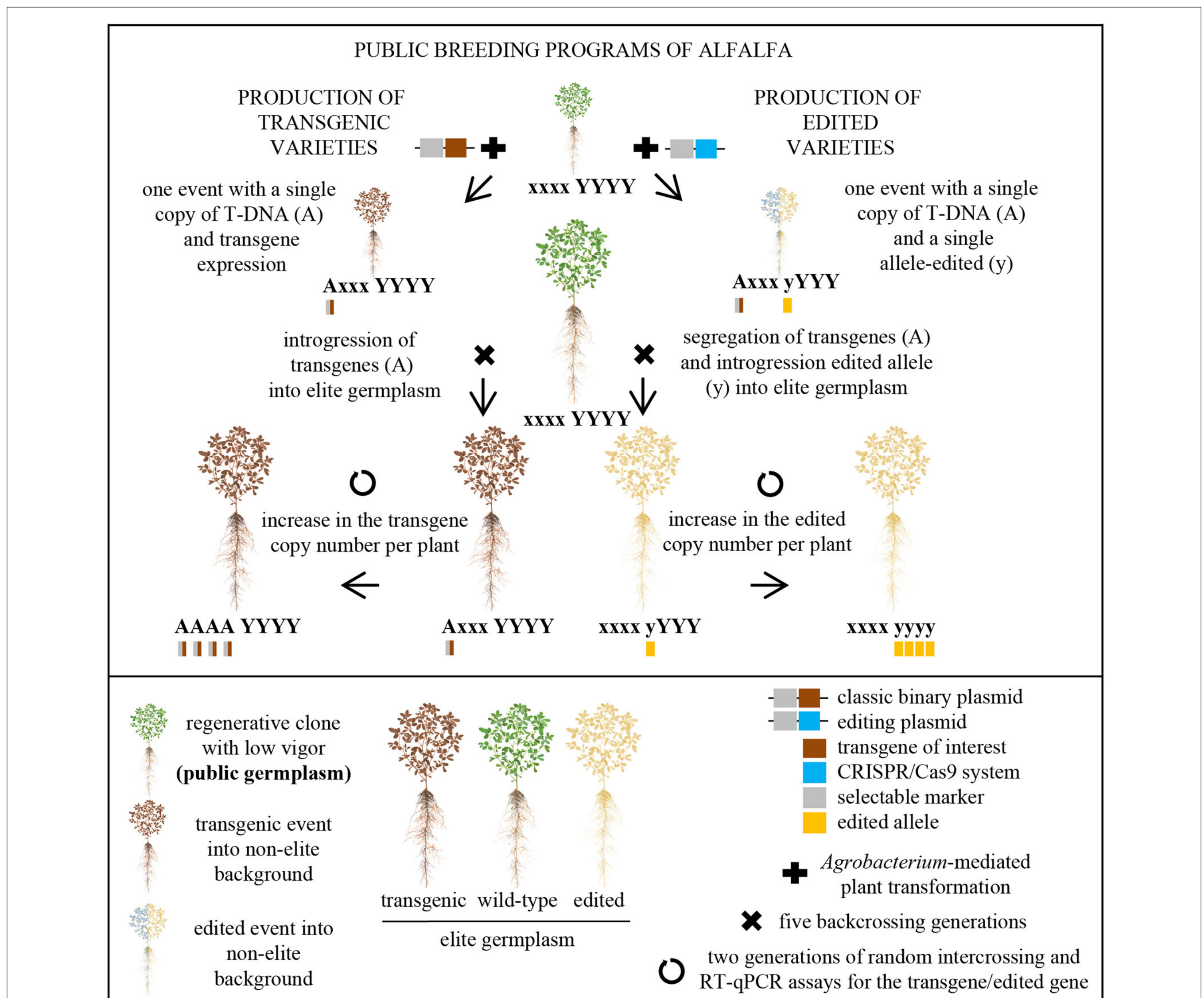


FIGURE 3 | Schematic representation of the similarities between the production of public commercial alfalfa varieties containing only one transgenic (left) or edited (right) trait. These approaches contain the same three critical steps: the transformation of a public alfalfa germplasm (e.g., clone C23) with a binary plasmid via *Agrobacterium tumefaciens*, the introgression of the transgene or the edited allele into elite germplasm, and the increase in the transgenic or the edited copy number per plant.

to improve the CRISPR/Cas9 system in alfalfa (Pascuan et al., 2020). Moreover, the recent release of the genome sequence of alfalfa (Chen et al., 2020) may help to improve the design of sgRNA guides for editing alfalfa genes in the next years.

While there is a legal structure for the deregulation of transgenic crops and there are several commercial transgenic cultivars in the world, practically no country envisages the release of genetically modified microbes (e.g., recombinant nitrogen-fixing rhizobia) into agroecosystems. Consequently, symbiotic nitrogen fixation improvement in legume crops (e.g., Soybean and Alfalfa) via genetic manipulation requires the engineering of the host genome, at least in the next years or few decades. Recently, we described a robust platform for the incorporation of transgenic traits into elite germplasm (Jozefkowicz et al., 2018), which can be easily adapted to the production of edited varieties (**Figure 3**). In this context, we propose the use of the public clone C23 and CRISPR/Cas9 system for the rapid incorporation of alfalfa mutants in public breeding programs, and the application of the genome-editing technology to guide the engineering of the nitrogen fixation system in this major legume crop.

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

GS designed and funded the experiments. EBo, MS, RF, GM, MG, HT, and CG performed the experiments. EBo, MS, RF, GM, MG, HT, CG, SF, NA, EBl, and GS helped with the experiments and data analysis. GS, NA, SF, and EBl wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Grant PICT 2017-0484 to GS and by INTA PE I115. EBo, MG, and MS were supported by fellowships from CONICET and IUBMB.

ACKNOWLEDGMENTS

We thank Carmen Soria for her technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.661526/full#supplementary-material

REFERENCES

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., and Nekrasov, V. (2013). Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods* 9:39. doi: 10.1186/1746-4811-9-39

Bortesi, L., and Fischer, R. (2015). The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.* 33, 41–52. doi: 10.1016/j.biotechadv.2014.12.006

Castel, B., Tomlinson, L., Locci, F., Yang, Y., and Jones, J. D. G. (2019). Optimization of T-DNA architecture for Cas9-mediated mutagenesis in Arabidopsis. *PLoS ONE* 14:e0204778. doi: 10.1371/journal.pone.0204778

Cermak, T., Curtin, S. J., Gil-Humanes, J., Cegan, R., Kono, T. J. Y., Konecna, E., et al. (2017). A multipurpose toolkit to enable advanced genome engineering in plants. *Plant Cell* 29, 1196–1217. doi: 10.1105/tpc.16.00922

Chen, H., Zeng, Y., Yang, Y., Huang, L., Tang, B., Zhang, H., et al. (2020). Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. *Nat. Commun.* 11:2494. doi: 10.1038/s41467-020-16338-x

Cuyeu, R., Pagano, E., Rosso, B., Soto, G., and Ayub, N. D. (2014). The genetic diversity of wild rescuegrass is associated with precipitation levels. *J. Genet.* 93, e29–34. doi: 10.1007/s12041-014-0349-0

Cuyeu, R., Rosso, B., Pagano, E., Soto, G., Fox, R., and Ayub, N. D. (2013). Genetic diversity in a world germplasm collection of tall fescue. *Genet. Mol. Biol.* 36, 237–242. doi: 10.1590/S1415-47572013005000021

Frare, R., Ayub, N., Alleva, K., and Soto, G. (2018). The ammonium channel NOD26 is the evolutionary innovation that drives the emergence, consolidation, and dissemination of nitrogen-fixing symbiosis in angiosperms. *J. Mol. Evol.* 86, 554–565. doi: 10.1007/s00239-018-9867-3

Gao, R., Feyissa, B. A., Croft, M., and Hannoufa, A. (2018). Gene editing by CRISPR/Cas9 in the obligatory outcrossing *Medicago sativa*. *Planta* 247, 1043–1050. doi: 10.1007/s00425-018-2866-1

Garcia, A. N., Ayub, N. D., Fox, A. R., Gomez, M. C., Dieguez, M. J., Pagano, E. M., et al. (2014). Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. *BMC Plant Biol.* 14:248. doi: 10.1186/s12870-014-0248-9

Gou, J., Debnath, S., Sun, L., Flanagan, A., Tang, Y., Jiang, Q., et al. (2018). From model to crop: functional characterization of SPL8 in *M. truncatula* led to genetic improvement of biomass yield and abiotic stress tolerance in alfalfa. *Plant Biotechnol. J.* 16, 951–962. doi: 10.1111/pbi.12841

Johansen, I. E., Liu, Y., Jørgensen, B., Bennett, E. P., Andreasson, E., Nielsen, K. L., et al. (2019). High efficacy full allelic CRISPR/Cas9 gene editing in tetraploid potato. *Sci. Rep.* 9:17715. doi: 10.1038/s41598-019-54126-w

Jozefkowicz, C., Bottero, E., Pascuan, C., Pagano, E., Ayub, N. D., and Soto, G. (2016). Minimizing the time and cost of production of transgenic alfalfa libraries using the highly efficient completely sequenced vector pPZP200BAR. *Plant Cell Rep.* 35, 1987–1990. doi: 10.1007/s00299-016-2026-7

Jozefkowicz, C., Frare, R., Fox, R., Odorizzi, A., Arolfo, V., Pagano, E., et al. (2018). Maximizing the expression of transgenic traits into elite alfalfa germplasm using a supertransgene configuration in heterozygous conditions. *Theor. Appl. Genet.* 131, 1111–1123. doi: 10.1007/s00122-018-3062-1

Kelemen, Z., Mai, A., Kapros, T., Feher, A., Gyorgyey, J., Waterborg, J. H., et al. (2002). Transformation vector based on promoter and intron sequences of a replacement histone H3 gene. A tool for high, constitutive gene expression in plants. *Transgenic Res* 11, 69–72. doi: 10.1023/A:1013923826979

Menz, J., Modrzejewski, D., Hartung, F., Wilhelm, R., and Sprink, T. (2020). Genome edited crops touch the market: a view on the global development and regulatory environment. *Front. Plant Sci.* 11:586027. doi: 10.3389/fpls.2020.586027

Narimatsu, Y., Joshi, H. J., Yang, Z., Gomes, C., Chen, Y. H., Lorenzetti, F. C., et al. (2018). A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome. *Glycobiology* 28, 295–305. doi: 10.1093/glycob/cwx101

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., and Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 691–693. doi: 10.1038/nbt.2655

Pascuan, C., Bottero, E., Kapros, T., Ayub, N., and Soto, G. (2020). pBAR-H3.2, a native-optimized binary vector to bypass transgene silencing in alfalfa. *Plant Cell Rep.* 39, 683–685. doi: 10.1007/s00299-020-02521-3

Shen, W. J., and Forde, B. G. (1989). Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Res.* 17:8385. doi: 10.1093/nar/17.20.8385

Stritzler, M., Elba, P., Berini, C., Gomez, C., Ayub, N., and Soto, G. (2018). High-quality forage production under salinity by using a salt-tolerant AtNXH1-expressing transgenic alfalfa combined with a natural stress-resistant nitrogen-fixing bacterium. *J. Biotechnol.* 276–277, 42–45. doi: 10.1016/j.jbiotec.2018.04.013

Weber, E., Engler, C., Gruetzner, R., Werner, S., and Marillonnet, S. (2011). A modular cloning system for standardized assembly of multigene constructs. *PLoS ONE* 6:e16765. doi: 10.1371/journal.pone.0016765

Wolabu, T. W., Cong, L., Park, J. J., Bao, Q., Chen, M., Sun, J., et al. (2020). Development of a highly efficient multiplex genome editing system in outcrossing tetraploid alfalfa (*Medicago sativa*). *Front. Plant Sci.* 11:1063. doi: 10.3389/fpls.2020.01063

Yang, S., Gao, M., Xu, C., Gao, J., Deshpande, S., Lin, S., et al. (2008). Alfalfa benefits from medicago truncatula: The RCT1 gene from *M. truncatula* confers broad-spectrum resistance to anthracnose in alfalfa. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12164–12169. doi: 10.1073/pnas.080251 8105

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.

Copyright © 2021 Bottero, Massa, González, Stritzler, Tajima, Gómez, Frare, Feingold, Blumwald, Ayub and Soto. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

ORIGINAL RESEARCH
published: 21 July 2021
doi: 10.3389/fagro.2021.657227

Analysis of Nitrogenase Fe Protein Activity in Transplastomic Tobacco

Jose A. Aznar-Moreno[1†], *Xi Jiang*[1,2†], *Stefan Burén*[1†] and *Luis M. Rubio*[1,2*†]

[1] *Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM), Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain,* [2] *Departamento de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaría y de Biosistemas, UPM, Madrid, Spain*

Integration of prokaryotic nitrogen fixation (*nif*) genes into the plastid genome for expression of functional nitrogenase components could render plants capable of assimilating atmospheric N_2 making their crops less dependent of nitrogen fertilizers. The nitrogenase Fe protein component (NifH) has been used as proxy for expression and targeting of Nif proteins within plant and yeast cells. Here we use tobacco plants with the *Azotobacter vinelandii nifH* and *nifM* genes integrated into the plastid genome. NifH and its maturase NifM were constitutively produced in leaves, but not roots, during light and dark periods. Nif protein expression in transplastomic plants was stable throughout development. Chloroplast NifH was soluble, but it only showed *in vitro* activity when isolated from leaves collected at the end of the dark period. Exposing the plant extracts to elevated temperatures precipitated NifM and apo-NifH protein devoid of $[Fe_4S_4]$ clusters, dramatically increasing the specific activity of remaining NifH protein. Our data indicate that the chloroplast endogenous [Fe-S] cluster biosynthesis was insufficient for complete NifH maturation, albeit a negative effect on NifH maturation due to excess NifM in the chloroplast cannot be excluded. NifH and NifM constitutive expression in transplastomic plants did not affect any of the following traits: seed size, germination time, germination ratio, seedling growth, emergence of the cotyledon and first leaves, chlorophyll content and plant height throughout development.

Keywords: nifH, chloroplast, nitrogen fixation, crop improvement, transplastomic plants, fertilizer, biotechnology

OPEN ACCESS

Edited by:
Juan Imperial,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Zhenhua ZHANG,
Hunan Agricultural University, China
Ray Dixon,
John Innes Centre, United Kingdom

Correspondence:
Luis M. Rubio
lm.rubio@upm.es

†ORCID:
Jose A. Aznar-Moreno
orcid.org/0000-0002-4474-8710
Xi Jiang
orcid.org/0000-0002-1819-9041
Stefan Burén
orcid.org/0000-0002-8487-2732
Luis M. Rubio
orcid.org/0000-0003-1596-2475

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *22 January 2021*
Accepted: *28 June 2021*
Published: *21 July 2021*

Citation:
Aznar-Moreno JA, Jiang X, Burén S and Rubio LM (2021) Analysis of Nitrogenase Fe Protein Activity in Transplastomic Tobacco. Front. Agron. 3:657227. doi: 10.3389/fagro.2021.657227

INTRODUCTION

Under optimal climate and water availability, crop yields correlate directly with chemical fertilizer inputs. Among these, nitrogen (N) fertilizers are critical to maximize commercial crop yields (Mueller et al., 2012). However, production and application of N fertilizers are major sources of pollution (Erisman et al., 2015). First, conversion of dinitrogen gas (N_2) into ammonia (NH_3) by the Haber-Bosh process is energetically costly and uses fossil fuels. Then, once applied in the field, NH_3 is converted to nitrate (NO_3^-) by nitrification in the soil. This causes N runoff into ground waters and aquatic systems and is often followed by toxic algal and cyanobacterial blooms. Finally, some of the NO_3^- is denitrified into the potent greenhouse gas nitrous oxide (N_2O). As the world's population is expected to reach 9.7 billion by 2050 and as the agricultural yield overall should increase with 60% from 2007 levels to meet coming food demand (Alexandratos and Bruinsma, 2012), new technologies could be important tools to reduce future agricultural impact on the environment.

Many of the effects from intense N fertilization could be avoided if crops could directly utilize atmospheric N_2 as N source by using biological N_2 fixation (BNF) (Curatti and Rubio, 2014; Oldroyd and Dixon, 2014), a trait only performed by a small (albeit phylogenetically diverse) group of prokaryotes collectively called diazotrophs. These select bacteria and archaea reduce N_2 to NH_3 using nitrogenase, a metalloenzyme that in its most prevalent form is composed of the Fe protein (encoded by *nifH*) and the MoFe protein (encoded by *nifD* and *nifK*) [for recent reviews see (Burén et al., 2020; Seefeldt et al., 2020)]. Progress has already been achieved in the path to engineer N_2 fixation by transfer of *nif* genes to unicellular eukaryotes (Cheng et al., 2005; López-Torrejón et al., 2016; Burén et al., 2017, 2019; Burén and Rubio, 2018) and to higher plants (Ivleva et al., 2016; Allen et al., 2020; Eseverri et al., 2020; Jiang et al., 2021).

Genetically modified plants are most often created by gene insertion into the nuclear genome (nuclear transformation) or the plastid genome (transplastomic, plastid transformation) (Bock, 2013). Currently, there is no standard methodology to insert exogenous DNA into the genome of plant mitochondria, although recent work in this direction is encouraging (Kazama et al., 2019). While nuclear transformation is relatively straightforward and can be achieved either by *A. tumefaciens*-mediated transformation or by particle bombardment, manipulation of chloroplast genome is only possible through bombardment (Ruf et al., 2019). Plastid genome transformation offers some important advantages over nuclear transformation, e.g. "in house" expression of proteins destined for the plastid, the possibility to organize genes into operons and to obtain high transgene expression, and it is devoid of gene silencing (Adem et al., 2017).

Importantly, many Nif proteins, including the nitrogenase structural components, carry metal clusters that are extremely sensitive to O_2. The NifH protein in particular is rapidly and irreversibly inactivated by O_2 (Shah and Brill, 1973). Due to this extreme sensitivity, and because NifH function involves fewer ancillary proteins than NifDK, the former has been used as proxy for nitrogenase engineering in eukaryotic cells. Proof of concept was obtained when NifH and NifM (a putative peptidyl-prolyl cis-trans isomerase involved in NifH maturation) were targeted to the mitochondria of aerobically cultured yeast cells (López-Torrejón et al., 2016). Active NifH has also been isolated from *Agrobacterium tumefaciens*-infiltrated tobacco leaves expressing chloroplast or mitochondria targeted NifH (Eseverri et al., 2020; Jiang et al., 2021). In these studies, NifU and NifS where included, in addition to NifM, as they are involved in the assembly of [Fe-S] clusters destined for Nif proteins (Burén et al., 2020).

Previously, Ivleva and colleagues generated transplastomic tobacco plants for the expression of *Azotobacter vinelandii* NifH and NifM, and demonstrated NifH protein activity in extracts from plants incubated at 10% O_2 (Ivleva et al., 2016). Although NifH protein activity was very low, these results were encouraging and deserved further investigation. In the present work we have studied these transplastomic tobacco plants in more detail. We show that NifH and NifM proteins were constitutively expressed in leaves, but not roots, and accumulated in both light and darkness throughout the plant development. Importantly, we show that NifH protein was active when isolated from plants grown under standard air atmosphere if they were collected at the end of the dark period. Exposing the plant extracts to elevated temperatures precipitated NifM and apo-NifH protein devoid of $[Fe_4S_4]$ clusters, which significantly increased the specific activity of remaining NifH protein. Finally, constitutive expression of NifH and NifM in the chloroplast did not cause pleiotropic effects that could otherwise impact the agronomic relevance of the attempts to engineer nitrogenase in crops.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transplastomic tobacco plants (*Nicotiana tabacum*) with *A. vinelandii nifH* and *nifM* genes integrated in the chloroplast genome downstream of *rbcL* using plasmid pMON261406 (carrying the *aadA* gene cassette) were kindly provided by Bayer Cropscience. In short, spectinomycin resistant transplastomic lines were generated by biolistic bombardment of tobacco leaves with pMON261406 and recovered in tissue culture after plant regeneration steps. Presence of the desired DNA insert was verified by Southern blot analysis (Ivleva et al., 2016). The plants were designed to constitutively express NifH and NifM proteins from a chimeric two-gene operon controlled by the ribosomal RNA promoter *Prrn*. Two transplastomic lines were recovered that carried *nifH* and *nifM* integrated in the same location of the chloroplast genome and expressed identical amounts of NifM and NifH polypeptides. One line was used for the present study.

Seeds were sown in soil inside a growth chamber set at 25°C (day, 17 h) and 23°C (night, 7 h), and 55% humidity. Four-week-old plants were irrigated with Sequestrene G100 (1 $g \cdot L^{-1}$) plus $FeSO_4$ (1 $g \cdot L^{-1}$) starting one week before leaves harvesting. Leaves were harvested either at the end of the dark period, or at least after five hours into the light period.

Preparation of NifH-Enriched Samples From Tobacco Leaves

About 100 g of leaves from four-week-old tobacco plants were used for each preparation. Harvested leaves were immediately snap-frozen and maintained in liquid N_2. The material was ground into powder using a mortar and pestle in liquid N_2, and then transferred into an anaerobic glovebox to avoid O_2 exposure during protein extraction.

The leaf powder was resuspended in anaerobic lysis buffer containing 50 mM Tris-HCl (pH 7.6), 2 mM sodium dithionite (DTH), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $\mu g \cdot ml^{-1}$ leupeptin, and 5 $\mu g \cdot ml^{-1}$ DNase I, at a 1:2 (leaf weight to buffer volume) ratio. For heat treatment, the total extract was incubated at 50°C in a closed bottle submerged in a water bath with stirring for 5 min, filtered through Miracloth (Merck) and centrifuged at 4°C and 58,000×g for 1 h. The resulting soluble cell free extract (CFE) was passed through a Minisart 0.45 μm CA filter (Sartorius AG) and loaded into a 5 ml Q-Sepharose column (GE healthcare) at 1.5 $ml \cdot min^{-1}$. The column was washed with five column volumes of buffer A (50 mM Tris-HCl, pH 7.6, 2 mM DTH) at the same flow rate before applying a linear gradient

spanning 0–500 mM NaCl (gradient volume of 100 ml mixing buffer A and B (50 mM Tris, pH 7.6, 500 mM NaCl, 2 mM DTH)). The flow rate during the gradient was set at 2 ml·min^{-1} and fractions were collected with 2.5 ml intervals. Attempts to reduce contamination of Rubisco with NifH included: extended washing with five column volumes of 100 or 150 mM NaCl before starting the linear gradient, NifH elution using stepwise gradients (five column volumes each of 100, 200, 300, 400 and 500 mM NaCl), and changes in the chromatography flow rates. However, we were not able to remove Rubisco from NifH-enriched fractions.

The NifH elution profile was determined by immunoblot analysis. Fractions containing NifH were pooled and concentrated using Amicon Ultra 30K centrifugal filters (Merck), snap-frozen and then kept in liquid N_2 until further analysis.

Immunoblots

Samples were mixed with Laemmli buffer, heated for 5 min at 90°C, and centrifuged at 10,000×*g* for 30 s to remove cell debris. Protein samples (final volume 20 µl) were resolved by SDS-PAGE at 200 V for 40 min. Gels were either stained directly with Coomassie or transferred to nitrocellulose membranes using a Trans-Blot Semi-Dry system (BioRad) and then stained with Ponceau. Membranes were blocked in phosphate buffer saline (PBS) containing 5% skimmed milk at room temperature for 1 h, washed 3 times for 15 min with PBS, and then incubated overnight at 4°C in an orbital shaker with polyclonal antibodies (1:5,000 dilution) detecting either *A. vinelandii* NifH (López-Torrejón et al., 2016) or *A. vinelandii* NifM (Burén and Rubio, 2018). Membranes were washed 3 times for 15 min with PBS before addition of secondary antibody (A0545-1ML, Sigma-Aldrich) at 1:50,000 dilution in PBS and incubated for 1 h at room temperature. Finally, membranes were washed 3 times for 15 min in PBS, and then 1 ml of ECL reagent were added to the membrane. Signals were recorded using an iBright FL1000 Imaging System (Thermo Fisher Scientific).

Quantification of NifH levels in the final samples was performed as previously described (Poza-Carrión et al., 2014). Membranes from gels loaded with increasing amounts of sample were probed with NifH-targeting antibodies, scanned at high resolution, and analyzed using the ImageJ software. NifH band intensities were compared to membranes (processed in parallel) originating from gels loaded with known amounts of NifH purified from *A. vinelandii*.

Heat Treatment Analysis

Two types of samples were subjected to heat treatment: (i) tobacco NifH-enriched fractions (containing NifH and NifM) and (ii) purified *A. vinelandii* NifH or apo-NifH. Protein samples were incubated either at 35°C or 55°C for 5 min and then centrifuged at 12,000×*g* for 10 min. Soluble (corresponding to the supernatant remaining after centrifugation) and pellet (corresponding to the pellet after resuspension in 1 ml lysis buffer) fractions were analyzed by immunoblotting. For heat treatment experiments using purified *A. vinelandii* NifH or apo-NifH, 1 ml of tobacco WT total protein extract from the corresponding dark or light periods were added to 12 µg apo-NifH or holo-NifH before the treatment.

Nitrogenase Activity

In vitro NifH protein activity was assayed in 9 ml serum vials sealed with serum stoppers according to Shah and Brill with minor modifications (Shah and Brill, 1973). Nitrogenase activity was measured by the acetylene reduction assay (ARA) using a NifH:NifDK protein molar ratio of 9:1. For this, 8.2 µg of NifH was mixed with 3 µg of NifDK and an ATP-regenerating mixture (1.23 mM ATP, 18 mM phosphocreatine, 2.2 mM $MgCl_2$, 3 mM DTH and 40 µg of creatine phosphokinase). Reactions (in a total volume of 0.8 ml) were incubated at 30°C for 30 min in a rotary shaker, and then stopped by addition of 0.1 ml of 8 M NaOH. The produced ethylene was measured by injecting 500 µl of the head space gas into a gas chromatograph (GC2014, Shimadzu) equipped with a Porapak N 80/100 column. Positive control reactions were performed with NifH and NifDK proteins purified from *A. vinelandii* (Curatti et al., 2007). Negative control reactions were performed using enriched samples from WT plants, processed and collected identically to those from the transplastomic plants. For each experiment, nitrogenase activities were measured in at least three independent biological replicates.

Apo-NifH Generation and Reconstitution

Apo-NifH was prepared *in vitro* according to Rangaraj and colleagues (Rangaraj et al., 1997) with slight modifications. To remove the [Fe_4S_4] cluster from the NifH dimers, 50 µM of NifH purified from *A. vinelandii* was incubated with 2.5 mM MgATP, 2 mM DTH, and 40 mM 2,2′-bipyridyl in 22 mM Tris-HCl (pH 7.4) for 30 min at 25°C. The protein was desalted twice using Sephadex G-25 PD-10 columns (Amersham Biosciences) and collected in buffer containing 200 mM NaCl, 10% glycerol, 2 mM DTH and 100 mM Tris-HCl (pH 8.0). Finally, the generated apo-NifH was concentrated using Amicon Ultra 10K centrifugal filters (Merck) and quantified using the BCA protein assay (Pierce).

Reconstitution assays using tobacco extracts were carried out with holo-NifH purified from *A. vinelandii* or with apo-NifH prepared as described above. Total protein extracts from WT tobacco leaves harvested at the end of the dark period were separately mixed with 12.33 µg NifH (holo-NifH or apo-NifH) in a final volume of 200 µl, and then either directly used for ARA or first heat-treated at 55°C for 5 min and then used for ARA. In parallel and following the same procedure, apo-NifH activation was performed using WT tobacco total extracts supplemented with NifU that had been previously loaded with [Fe-S] clusters as described in Dos Santos et al. (2004) and López-Torrejón et al. (2016). Apo-NifH reconstitution was estimated by ARA following the addition of 5 µg of NifDK protein and ATP-regenerating mixture as described above.

NifH Specific Activity Determination After Reconstruction of Co-existence of Apo- and Holo-NifH in Tobacco Extracts

A mixture of NifH polypeptides (12.33 µg total at a 1:6 ratio of holo-NifH to apo-NifH) were mixed with 80 µl of tobacco cell-free extract. Four hundred eighty µl of this mix was prepared and split into two tubes of 240 µl each. Samples were

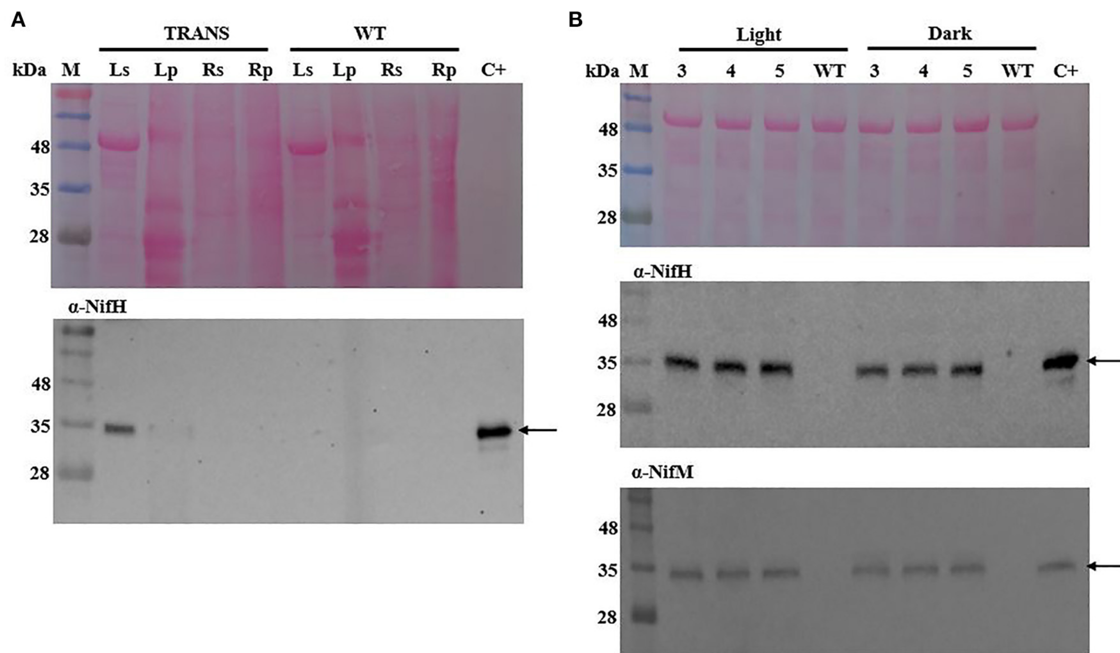


FIGURE 1 | Immunoblot analysis of NifH and NifM polypeptide accumulation in transplastomic tobacco. **(A)** NifH accumulation in soluble (s) or membrane-associated pellet (p) fractions from leaf (L) and root (R) tissues of transplastomic (TRANS) or wild-type (WT) tobacco. **(B)** Accumulation of soluble NifH and NifM in leaves of transplastomic tobacco harvested during light or dark periods at three (3), four (4) and five (5) weeks after sowing. Arrows point to polypeptides detected with antibodies against the *A. vinelandii* NifH and NifM proteins. C+, pure *A. vinelandii* NifH and NifM proteins; M, molecular mass markers (kDa); WT, total protein extract from 4-week-old WT tobacco leaves. Ponceau staining of membranes are shown as control of protein loading and transfer.

heated either at 35°C or 55°C for 5 min and centrifuged at 12,000× *g* for 5 min to separate soluble and precipitated protein. Eighty μl of each supernatant was mixed with ATP-regeneration mixture containing 5 μg of purified NifDK, and ARA was performed as described above. The remaining supernatant solution and the pellets left after centrifugation from each heat treatment condition were used for protein quantification based on immunoblotting assays.

Plant Development and Seed Size Analyses

Three independent experiments were performed to determine emergence of the cotyledon and the first pair of leaves. For each experiment at least 60 WT or transplastomic seeds were sown in soil and daily monitored. Plant height and chlorophyll content (CCM-200plus, OPTI-SCIENCES) were measured from 3 to 6 weeks after sowing seeds.

Seed sizes were analyzed following a previously established protocol (Herridge et al., 2011). Approximately 200 randomly selected seeds from 4 different WT or transplastomic plants were photographed using a Leica M165FC microscope, and then measured using the ImageJ software (version 1.48v).

RESULTS

Accumulation of *A. vinelandii* NifH and NifM Polypeptides in Transplastomic Tobacco

Accumulation of the nitrogenase NifH protein polypeptide was analyzed in leaf (aerial) and root (lower oxygen concentration below soil) tissues of transplastomic tobacco plants. The protein migrated similarly to native *A. vinelandii* NifH in SDS-PAGE gels and was almost exclusively detected in the soluble fraction of leaf extracts, with only a faint band being associated to the membrane fraction after centrifugation (**Figure 1A**). That NifH accumulated as soluble protein was indicative of successful maturation by NifM, as previous reports indicate that absence of NifM renders insoluble NifH polypeptides (Roberts et al., 1978; Howard et al., 1986; Eseverri et al., 2020). No NifH was detected in root tissue of transplastomic plants.

To analyze the accumulation of NifH and NifM proteins during the development of the transplastomic plants, protein extracts were prepared from leaves harvested in both light and darkness at three, four, and five weeks after sowing. The levels of NifH and NifM in soluble extracts were analyzed by immunoblotting and were found to be constant over time which indicated stable and consistent expression (**Figure 1B**).

Effect of Light and Darkness on Function of Plastid-Expressed NifH Protein

The metabolic status of plants varies during light and darkness (Ort and Oxborough, 1992; Dodd et al., 2015). The circadian cycle affects both physical and biochemical factors that are critical to NifH protein maturation, stability and activity, e.g., O_2 levels, availability of ATP and reducing power (Bulen and LeComte, 1966; Shah and Brill, 1973). Ivleva and colleagues previously demonstrated low NifH protein activity in chloroplasts enriched from transplastomic tobacco (Ivleva et al., 2016). However, these plants were incubated under atmosphere with 10%

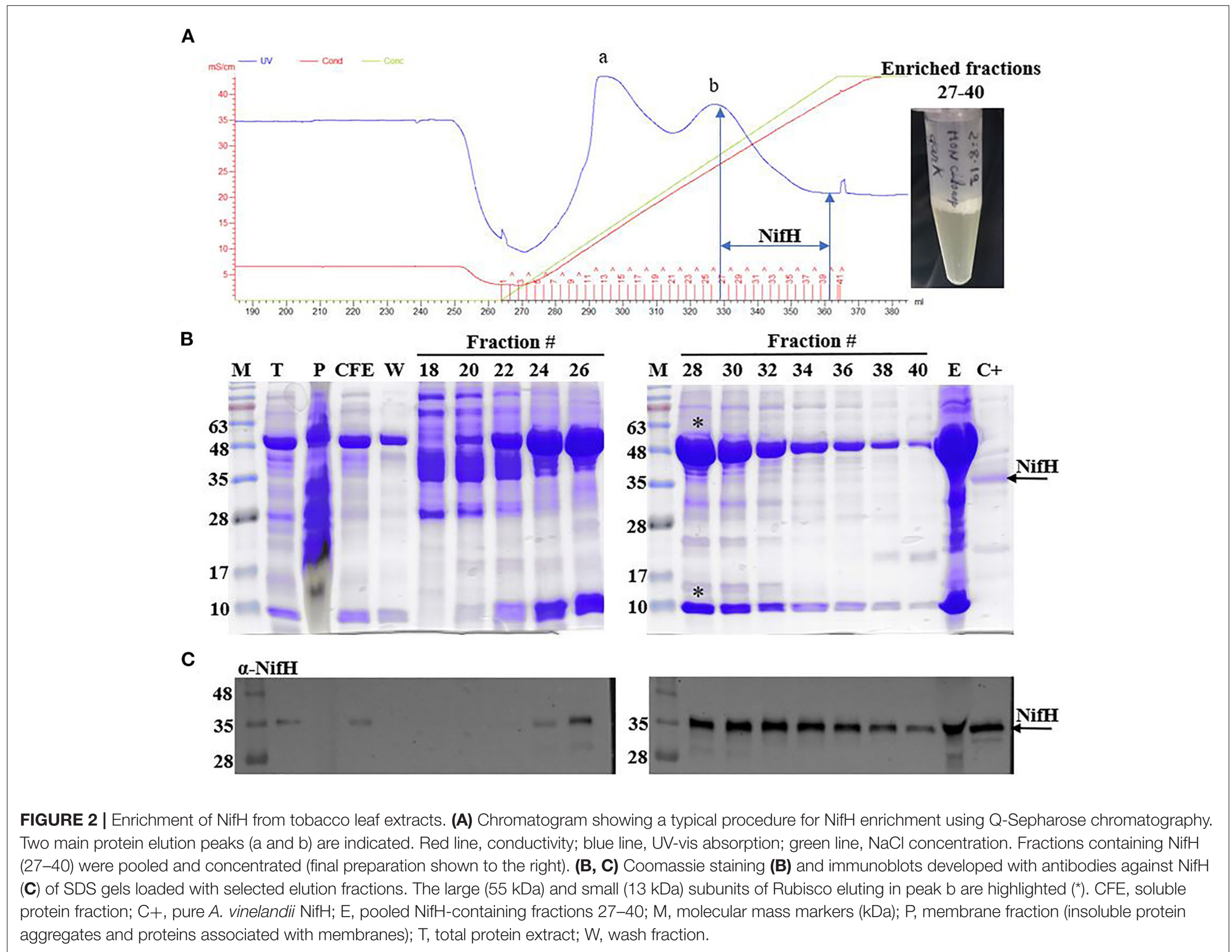


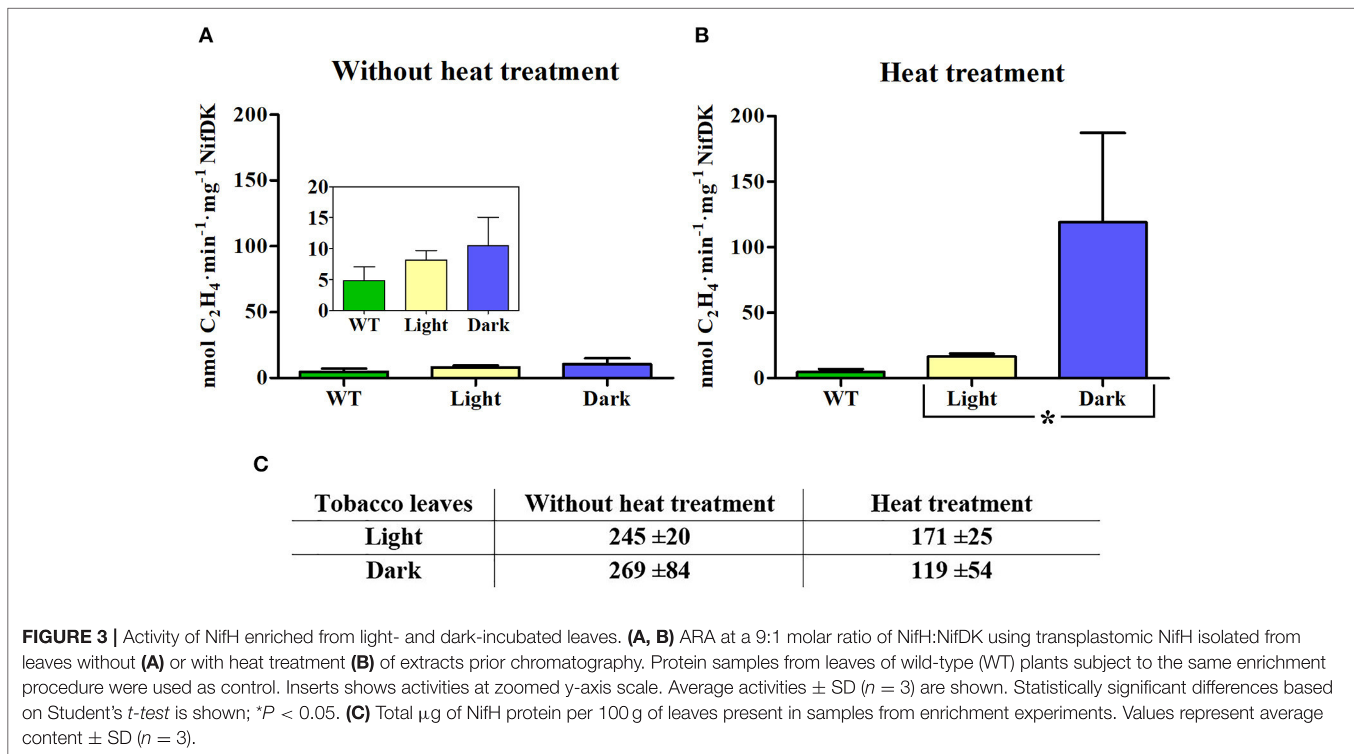
FIGURE 2 | Enrichment of NifH from tobacco leaf extracts. **(A)** Chromatogram showing a typical procedure for NifH enrichment using Q-Sepharose chromatography. Two main protein elution peaks (a and b) are indicated. Red line, conductivity; blue line, UV-vis absorption; green line, NaCl concentration. Fractions containing NifH (27–40) were pooled and concentrated (final preparation shown to the right). **(B, C)** Coomassie staining **(B)** and immunoblots developed with antibodies against NifH (**C**) of SDS gels loaded with selected elution fractions. The large (55 kDa) and small (13 kDa) subunits of Rubisco eluting in peak b are highlighted (*). CFE, soluble protein fraction; C+, pure *A. vinelandii* NifH; E, pooled NifH-containing fractions 27–40; M, molecular mass markers (kDa); P, membrane fraction (insoluble protein aggregates and proteins associated with membranes); T, total protein extract; W, wash fraction.

O_2. Understanding how plant metabolic and physiological conditions, such as those imposed by light and darkness, affects NifH function is therefore of major importance to engineer nitrogen fixation *in planta*.

For this analysis, anion exchange chromatography was employed to enrich NifH from tobacco soluble protein extracts prepared from leaves harvested either in the light or at the end of the dark period (**Figure 2A**). Two main protein peaks were observed in the chromatogram, where the second peak was dominated by the large (55 kDa) and small (13 kDa) subunits of Rubisco (**Figure 2B**). Immunoblot analysis using antibodies recognizing NifH was required to unambiguously identify which fractions contained the NifH protein. NifH was normally found in fractions 27–40 of the linear NaCl gradient, which corresponded to 325–475 mM NaCl (25–39 mS cm^{-1}) (**Figure 2C**).

To reduce protein contaminants in fractions containing NifH, transplastomic leaf extracts were heat-treated for 5 mins and centrifuged prior to chromatography. Heat treatment can denature proteins and this method has been described to remove approximately 60% of the soluble proteins in *A. vinelandii* extracts at the cost of only minor NifH loss (Burgess et al., 1980). We therefore employed this protocol expecting that this procedure would reduce the amounts of Rubisco in leaf extracts. However, Rubisco was still prominent in NifH enriched fractions under all conditions tested (see Material and Methods), which impaired isolating pure NifH leading us to determine NifH concentration in the enriched samples using immunoblotting (**Supplementary Figure 1**).

The Fe protein activity of NifH enriched preparations was determined by the ARA, in which NifDK reduces acetylene to ethylene using electrons donated by NifH. NifDK for ARA was purified from *A. vinelandii*, and so was the NifH protein used in positive control reactions (**Supplementary Figure 2**). Since the amount of NifH protein in the enriched fractions was a limiting factor, activity assays were carried out at 1:9 (NifDK:NifH) ratio. When NifH was isolated from leaves collected during the light period the measured NifH protein activity was low (8.2 nmol $C_2H_4 \cdot min^{-1} \cdot mg$ $NifDK^{-1}$), which corresponded to about twice the amount of ethylene measured using samples



Tobacco leaves	Without heat treatment	Heat treatment
Light	245 ±20	171 ±25
Dark	269 ±84	119 ±54

FIGURE 3 | Activity of NifH enriched from light- and dark-incubated leaves. **(A, B)** ARA at a 9:1 molar ratio of NifH:NifDK using transplastomic NifH isolated from leaves without **(A)** or with heat treatment **(B)** of extracts prior chromatography. Protein samples from leaves of wild-type (WT) plants subject to the same enrichment procedure were used as control. Inserts shows activities at zoomed y-axis scale. Average activities ± SD ($n = 3$) are shown. Statistically significant differences based on Student's *t-test* is shown; $^*P < 0.05$. **(C)** Total µg of NifH protein per 100 g of leaves present in samples from enrichment experiments. Values represent average content ± SD ($n = 3$).

isolated from WT tobacco leaves (**Figure 3A**, inset). A further two-fold increase in ethylene production was observed when NifH from heat-treated leaves (also harvested in light) was used (**Figure 3B**). Similarly, NifH samples obtained from leaves collected at the end of the dark period showed low NifH protein activity (9.2 nmol C_2H_4·min^{-1}·mg NifDK^{-1}) (**Figure 3A**), but heat-treatment increased NifH protein specific activity 13-fold. Importantly, heat treatment was more effective in increasing NifH activity in samples from dark-collected leaves than those from light-exposed leaves (**Figure 3B**).

As previously reported for *A. vinelandii* extracts (Burgess et al., 1980), heat treatment reduced the amount of soluble NifH in transplastomic leaf extracts (**Figure 3C**; **Supplementary Figures 1** and **3**). By comparing the specific activity of NifH protein enriched from chloroplasts to that of NifH purified from *A. vinelandii* (**Supplementary Figure 2**), it was estimated that about half of the heat resistant NifH isolated from transplastomic leaves at the end of the dark period was active. Although this result does not necessarily encompass all active NifH present in transplastomic leaves (some active holo-NifH protein could have precipitated), it strongly suggests that darkness improved the cellular environment for NifH protein maturation and/or stability.

Heat-Precipitation of NifM Does Not Increase NifH Protein Activity

NifH is highly abundant in N_2-fixing *A. vinelandii* and *Klebsiella oxytoca* cells, about 50-fold higher than NifM (Poza-Carrión et al., 2014; Yang et al., 2018). One limitation with the transplastomic plants used is the expected equimolar stoichiometry of NifH and NifM, which could impair NifH activity. Incorrect Nif protein stoichiometry has been shown to affect nitrogenase maturation *in vitro* (Curatti et al., 2007; Hernandez et al., 2011) and *in vivo* (Temme et al., 2012). Analysis of the NifH-enriched samples showed that NifM was absent when the leaf extracts had been heated prior to chromatography (**Figure 4A**; **Supplementary Figure 3**). Data in **Figure 4A** shows that while some NifH was resistant to the elevated temperature all NifM precipitated to the pellet. We therefore speculated that NifH function was impaired by NifM present in non-heated samples. To test this possibility, ARA were performed in which increasing amounts of NifM were added to the reaction mixtures. No inhibitory effect from NifM but rather a slight increase in nitrogenase activity was observed in the ARA (**Figure 4B**). This result argued against inhibition of NifH activity by NifM in non-heated samples. Thus, heat mediated NifM precipitation did not explain the increase in NifH activity.

Apo-NifH Removal by Heat Treatment Correlates With Increased NifH Protein Activity

The NifH activity increase upon heat treatment could be explained if [Fe_4S_4] clusters were liberated from (other) denatured plant metalloproteins and incorporated into cluster-deficient apo-NifH. To test this hypothesis, purified *A. vinelandii* apo-NifH was added to WT tobacco leaf extracts and then subject to the same heat treatment. No apo-NifH activation was observed indicating lack of [Fe_4S_4] cluster transfer (**Figure 5A**). A similar experiment was performed in the presence of pure

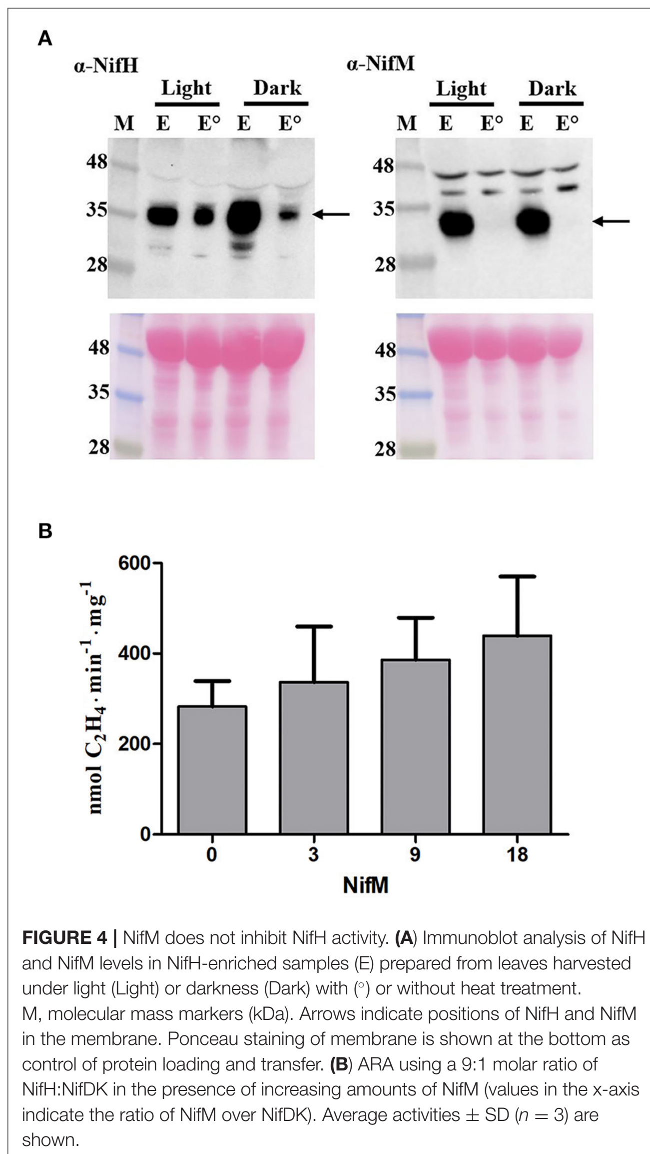


FIGURE 4 | NifM does not inhibit NifH activity. **(A)** Immunoblot analysis of NifH and NifM levels in NifH-enriched samples (E) prepared from leaves harvested under light (Light) or darkness (Dark) with (°) or without heat treatment. M, molecular mass markers (kDa). Arrows indicate positions of NifH and NifM in the membrane. Ponceau staining of membrane is shown at the bottom as control of protein loading and transfer. **(B)** ARA using a 9:1 molar ratio of NifH:NifDK in the presence of increasing amounts of NifM (values in the x-axis indicate the ratio of NifM over NifDK). Average activities ± SD ($n = 3$) are shown.

NifU, a physiological donor of [Fe-S] clusters to nitrogenase proteins that had been previously purified from *E. coli* and loaded with [Fe_4S_4] clusters *in vitro*. NifU-mediated reconstitution of apo-NifH has been previously demonstrated (Dos Santos et al., 2004; López-Torrejón et al., 2016). Consistently, NifU was able to activate apo-NifH presumably by transferring [Fe_4S_4] clusters (**Figure 5B**). In all experiments, the heat treatment decreased the level of apo-NifH activation in the extract, likely due to partial NifH precipitation (**Figure 5**). Thus, no direct positive effect on NifH activity was obtained by heating the extract. We note that the NifU-reconstituted apo-NifH appeared to be more sensitive to heat than the holo-NifH protein.

We also tested whether native tobacco proteins present in the extracts were inhibiting NifH activity, whose disappearance upon heating could explain improved NifH functionality. However, pure holo-NifH mixed with WT tobacco protein extract showed the same activity as control reactions lacking the tobacco extracts (**Figure 5A**).

The yield of NifH in enriched fractions was reduced when tobacco protein extracts had been previously heated, although the specific activity was higher (**Figure 3**). This inverse correlation between yield and activity suggested that extract-heating positively selected for functional NifH protein. We therefore tested whether holo-NifH was more heat-resistant than apo-NifH. While about half of the holo-NifH remained in solution upon heating at 55°C for 5 min (**Figure 6A**) most apo-NifH precipitated. We also tested whether the presence of apo-NifH inhibited holo-NifH activity. When half of holo-NifH was replaced with apo-NifH nitrogenase activity was also reduced by half (**Figure 6B**), indicating that apo-NifH did not compete with holo-NifH for NifDK (and inhibit NifDK) during ARA under the tested conditions. Finally, a reconstruction experiment was conducted in which known mixtures of apo-NifH and holo-NifH (6:1 ratio) were added to tobacco extracts, incubated at either 35°C or 55°C, and analyzed for NifH precipitation and activity (**Supplementary Figure 4**). Again, a correlation between NifH precipitation and NifH specific activity was observed with activities of samples incubated at 55°C being significantly higher than those incubated at 35°C. Taken together, these results suggest that the NifH protein enriched from heated tobacco extracts was more active due to precipitation of non-functional and inactive apo-NifH.

Agronomic Traits Are Not Affected by Constitutive Expression of NifH and NifM in Transplastomic Plants

Plastids possesses an impressive capacity to synthesize and accumulate proteins of industrial and pharmaceutical importance (Oey et al., 2009; Scotti et al., 2009; Petersen and Bock, 2011). However, transplastomic plants can show pleiotropic effects that reduce crop yield (Scotti and Cardi, 2014). We therefore examined some physiological parameters of transplastomic tobacco plants expressing NifH and NifM. Transplastomic seed germination time and ratio were not affected compared to WT plants. This was expected as NifH and NifM expression is driven by the ribosomal Prrn promoter which is not active in plastids of non-green tissues (Zhang et al., 2012). NifH and NifM expression did neither affect seedling growth nor emergence of the cotyledon and first leaves (**Figure 7A**), suggesting that plant development was not impaired. Since chlorosis has been described in transplastomic plants (Oey et al., 2009; Scotti et al., 2009; Petersen and Bock, 2011), the chlorophyll content was quantified in leaves from plants three to six weeks after sowing (**Table 1**). It is well established that photosynthetic efficiency directly correlates with crop yield (Long et al., 2015; Wu et al., 2019). No effect on chlorophyll content was observed suggesting that constitutive expression of NifH and NifM did not affect photosynthesis. Other traits such as plant height and seed size were also determined. Growth of transplastomic plants expressing NifH and NifM was similar to that of WT plants (**Table 2**) and no morphological differences were observed. Moreover, the seed size was not affected (**Figure 7B**). Altogether,

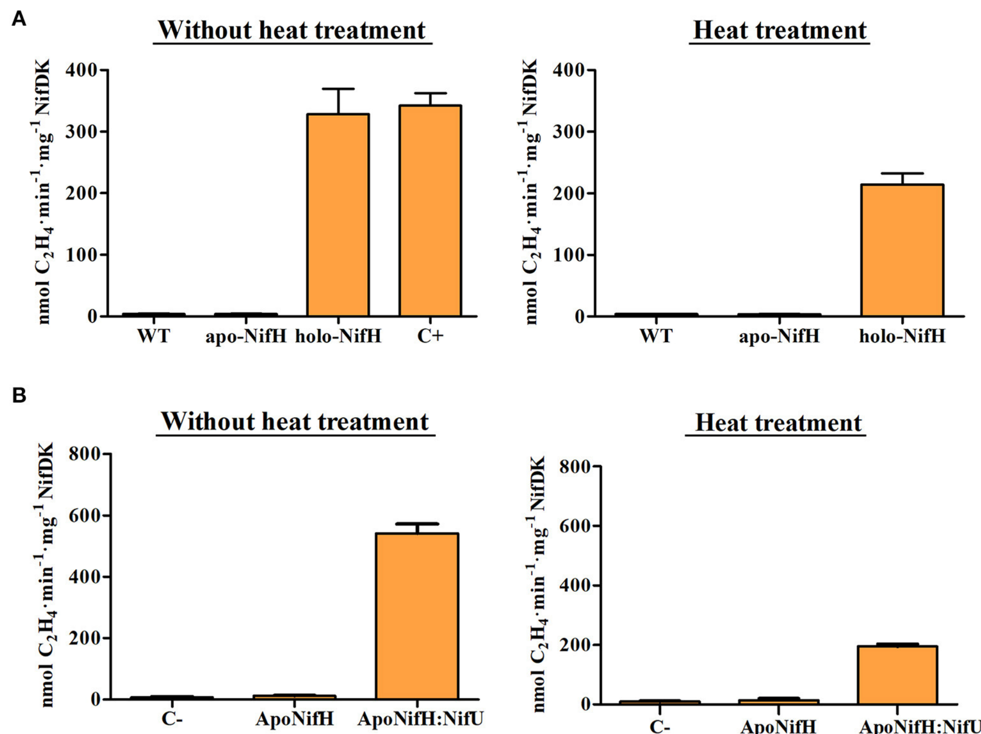


FIGURE 5 | Reconstitution of apo-NifH added to tobacco protein extracts. **(A)** Pure apo-NifH and NifH (holo-NifH) isolated from *A. vinelandii* were mixed with tobacco WT tobacco protein extracts without a heat treatment (left) or subjected to heat treatment (right). NifH activity was determined by ARA using 9:1 molar ratio of NifH:NifDK. WT, control reactions containing only WT tobacco leaf extract. C+, control reactions including the same amount of holo-NifH but lacking WT tobacco protein extracts. Average activities ± SD ($n = 4$) are shown. **(B)** Reconstitution of apo-NifH by NifU added to WT tobacco total protein extracts without (left) and with (right) heat treatment. Pure *A. vinelandii* apo-NifH was mixed with WT tobacco leaf extracts and then, [Fe-S] cluster-loaded NifU was added to the mixtures. After 10 min incubation at room temperature, pure NifDK was added and samples were analyzed for ARA. C-, WT tobacco leaf extracts without added NifH. Average activities ± SD ($n = 4$) are shown.

these results indicate that relevant agronomical traits were not affected by constitutive expression of NifH and NifM in the chloroplast.

DISCUSSION

In this work, we have characterized in more detail a previously generated transplastomic tobacco line that expresses the *A. vinelandii nifH* and *nifM* genes in the chloroplast. These plants showed very low NifH protein activity in chloroplast-enriched samples when plants were transiently incubated at low O_2 concentrations (Ivleva et al., 2016). In contrast, here we analyzed NifH protein activity from these plants grown at standard atmosphere. We have used anion exchange chromatography, which is a well-established method to isolate NifH, that takes advantage of the relative strong interaction between the NifH polypeptide and this type of resins. Unfortunately, the presence of Rubisco -the most abundant protein in the green tissues of plants- in NifH-enriched fractions could not be avoided (**Figure 2B**). This contaminant precluded direct NifH polypeptide quantitation and other biochemical analyses such as spectroscopy and metal content determination.

The O_2 levels at which the plants were grown were not manipulated in this study, as the atmosphere cannot be manipulated in field conditions. Instead, we investigated the effect of oxygenic photosynthesis on NifH accumulation and activity. NifH and NifM accumulation in chloroplasts was similar during light and darkness indicating stability of NifH polypeptides. No Nif protein expression was detected in plastids of non-green root tissue (**Figure 1**), consistent with other studies using the Prrn promoter (Zhang et al., 2012).

Significant NifH activity was only obtained using extracts from leaves harvested at the end of the dark period and required heating of the leaf protein extract prior to enrichment by chromatography. The data suggest that the heat treatment had two immediate consequences over NifH. First, NifM and non-functional apo-NifH precipitated out of the soluble extract. Second, while apo-NifH removal reduced the total NifH yield it increased the apparent specific activity because NifH preparations were more enriched in holo-NifH after the treatment. Assuming that NifH folding was not affected by light, the most plausible explanation for higher NifH activity observed in leaves harvested in darkness is $[Fe_4S_4]$ cluster loading or stability. Lower internal O_2 levels at night (Hitoshi and Hideo, 1985) is likely to increase NifH $[Fe_4S_4]$ cluster stability.

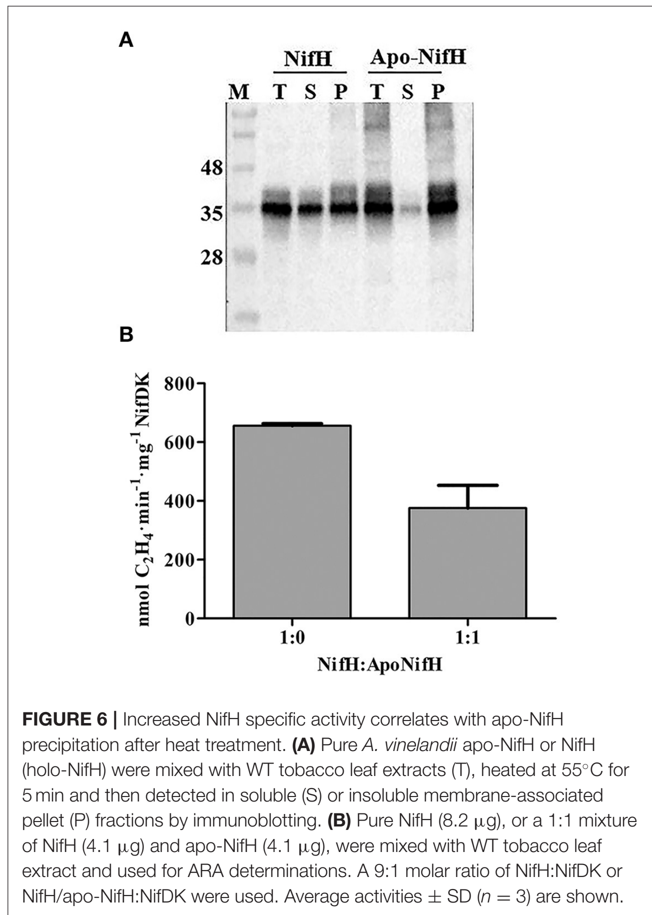


FIGURE 6 | Increased NifH specific activity correlates with apo-NifH precipitation after heat treatment. **(A)** Pure *A. vinelandii* apo-NifH or NifH (holo-NifH) were mixed with WT tobacco leaf extracts (T), heated at 55°C for 5 min and then detected in soluble (S) or insoluble membrane-associated pellet (P) fractions by immunoblotting. **(B)** Pure NifH (8.2 μg), or a 1:1 mixture of NifH (4.1 μg) and apo-NifH (4.1 μg), were mixed with WT tobacco leaf extract and used for ARA determinations. A 9:1 molar ratio of NifH:NifDK or NifH/apo-NifH:NifDK were used. Average activities ± SD ($n = 3$) are shown.

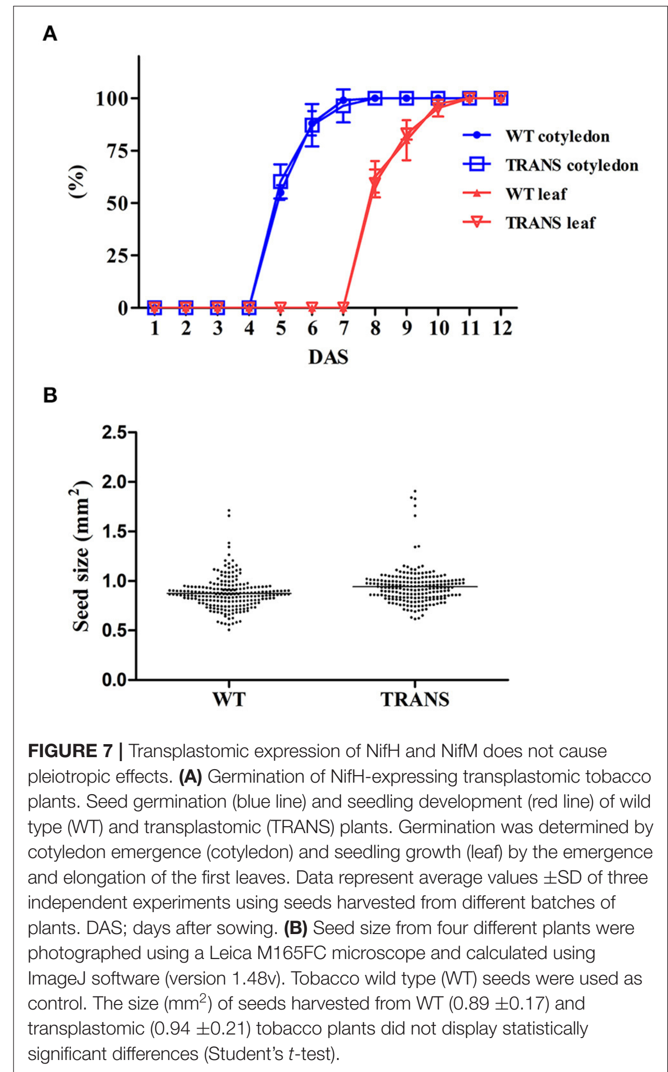


FIGURE 7 | Transplastomic expression of NifH and NifM does not cause pleiotropic effects. **(A)** Germination of NifH-expressing transplastomic tobacco plants. Seed germination (blue line) and seedling development (red line) of wild type (WT) and transplastomic (TRANS) plants. Germination was determined by cotyledon emergence (cotyledon) and seedling growth (leaf) by the emergence and elongation of the first leaves. Data represent average values ±SD of three independent experiments using seeds harvested from different batches of plants. DAS; days after sowing. **(B)** Seed size from four different plants were photographed using a Leica M165FC microscope and calculated using ImageJ software (version 1.48v). Tobacco wild type (WT) seeds were used as control. The size (mm^2) of seeds harvested from WT (0.89 ±0.17) and transplastomic (0.94 ±0.21) tobacco plants did not display statistically significant differences (Student's *t*-test).

Moreover, NifH has shown functional during darkness for chlorophyll biosynthesis in the unicellular algae *Chlamydomonas reinhardtii* (Cheng et al., 2005). Activity of chloroplast targeted NifH has also been observed in leaves harvested at the end of the dark period (Eseverri et al., 2020).

If [Fe_4S_4] cluster occupancy was the limiting factor for NifH maturation it would be interesting to co-express NifU and NifS together with NifH and NifM in the transplastomic tobacco line. NifU and NifS are proteins involved in assembly of [Fe-S] clusters destined for Nif proteins. Although NifU and NifS were not required for NifH maturation in mitochondria of *Saccharomyces cerevisiae* (López-Torrejón et al., 2016), they were required to produce active yeast NifB (Burén et al., 2019). In a recent study from our laboratory, we also showed that tobacco plants transiently expressing high levels of chloroplast-targeted NifH and NifM required NifU and NifS (Eseverri et al., 2020). There are some important differences in the experimental approach between the two studies that might explain this contradiction. First, transient expression from the strong 35S promoter results in a sudden burst of protein production. Although the Prrn promoter used in the transplastomic plants also results in high protein expression, endogenous chloroplast iron-sulfur cluster biosynthesis could perhaps deal better with constitutive and stable rather than transient expression. Second, the transplastomic tobacco plants were grown in soil supplemented with $FeSO_4$, which could alleviate the requirement for NifU and NifS. While it is difficult to directly compare NifH specific activities from the two studies, it would be interesting to determine the specific activity of heat-treated NifH when expressed together with NifU and NifS, as there is a good chance that the activities in the previous study (Eseverri et al., 2020) were underestimated because of presence of apo-NifH.

Only one transplastomic line was used in this study. Although results must be interpreted with care, a number of conclusions can be drawn. This study shows that functional nitrogenase NifH protein can be expressed in tobacco chloroplasts during the dark period, even if plants are grown at standard atmospheric O_2 levels. In contrast to mitochondria-targeted *K. oxytoca* NifH, which is insoluble even when expressed together with NifM (Okada et al., 2020), the *A. vinelandii* NifH expressed with NifM inside chloroplasts was soluble overcoming a major obstacle for nitrogenase engineering in plants. Chloroplast metabolism in darkness appears to permit NifH protein maturation, which

TABLE 1 | Chlorophyll content in transplastomic (TRANS) and wild type (WT) tobacco leaves from 3, 4, 5 and 6 weeks after seed sowing.

	3rd week		4th week		5th week		6th week	
	Average (cci)	**±SD**	**Average (cci)**	**±SD**	**Average (cci)**	**±SD**	**Average (cci)**	**±SD**
TRANS	5.8	0.43	7.0	0.88	8.9	1.30	10.3	2.03
WT	5.8	0.47	6.9	0.63	8.8	1.25	10.5	1.87

At least two individual leaves from sixty plants were assayed. Cci; chlorophyll content index (a ratio between transmission of radiation at 931 nm divided by transmission of radiation at 653 nm). Not statistically significant differences were detected between transplastomic and WT leaves.

TABLE 2 | Height of transplastomic (TRANS) and wild type (WT) tobacco plants at 3, 4, 5 and 6 weeks after seed sowing.

	3rd week		4th week		5th week		6th week	
	Average (cm)	**±SD**	**Average (cm)**	**±SD**	**Average (cm)**	**±SD**	**Average (cm)**	**±SD**
TRANS	1.0	0.72	1.9	0.38	6.3	0.95	10.1	1.89
WT	1.0	0.32	2.0	0.38	6.4	0.68	10.6	1.82

Sixty plants of each genotype were measured. Not statistically significant differences were detected between transplastomic and control plants.

opens new avenues for controlling Nif protein expression by the circadian cycle or for using promoters for expression in root plastids. Although no positive effects on yield are expected from NifH expression alone (as nitrogenase is a two-component enzyme), we show that NifH and NifM expression did not induce any apparent pleiotropic effects demonstrating that accumulation of nitrogenase components in the chloroplast does not necessarily cause unwanted secondary effects.

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

JAAM, XJ, and LMR designed experiments. XJ purified and reconstituted NifU, performed apo-NifH reconstitutions. JAAM performed all other experiments. JAAM, XJ, SB, and LMR analyzed data and wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

Funding for this research was provided by the Bill & Melinda Gates Foundation (BMGF) Grant OPP1143172 (to LMR). JAAM is supported by Juan de la Cierva fellowship (IJCI-2017-31937) from the Ministerio de Economía, Industria y Competitividad. XJ is supported by a doctoral fellowship from the Universidad Politécnica de Madrid.

ACKNOWLEDGMENTS

We thank Bayer Cropscience for sharing the transplastomic tobacco plant used in this study under the terms of BMGF Global Access policy. We thank Carlos Echavarri-Erasun for assistance in protein purifications and critically reading the article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.657227/full#supplementary-material

REFERENCES

Adem, M., Beyene, D., and Feyissa, T. (2017). Recent achievements obtained by chloroplast transformation. *Plant Methods* 13, 30–30. doi: 10.1186/s13007-017-0179-1

Alexandratos, N., and Bruinsma, J. (2012). World agriculture towards 2030/2050: the 2012 revision. ESA Working Papers 12–03.

Allen, R. S., Gregg, C. M., Okada, S., Menon, A., Hussain, D., Gillespie, V., et al. (2020). Plant expression of NifD protein variants resistant to mitochondrial degradation. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23165–23173. doi: 10.1073/pnas.2002365117

Bock, R. (2013). Strategies for metabolic pathway engineering with multiple transgenes. *Plant Mol. Biol.* 83, 21–31. doi: 10.1007/s11103-013-0045-0

Bulen, W., and LeComte, J. (1966). The nitrogenase system from Azotobacter: two-enzyme requirement for N2 reduction, ATP-dependent H2 evolution, and ATP hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* 56:979. doi: 10.1073/pnas.56.3.979

Burén, S., Jiménez-Vicente, E., Echavarri-Erasun, C., and Rubio, L. M. (2020). Biosynthesis of nitrogenase cofactors. *Chem. Rev.* 120, 4921–4968. doi: 10.1021/acs.chemrev.9b00489

Burén, S., Pratt, K., Jiang, X., Guo, Y., Jimenez-Vicente, E., Echavarri-Erasun, C., et al. (2019). Biosynthesis of the nitrogenase active-site cofactor precursor NifB-co in andlt;emandgt;Saccharomyces cerevisiaeandlt;/emandgt. *Proc. Nat. Acad. Sci.* 116:25078. doi: 10.1073/pnas.1904903116

Burén, S., and Rubio, L. M. (2018). State of the art in eukaryotic nitrogenase engineering. *FEMS Microbiol. Lett.* 365:fnx274. doi: 10.1093/femsle/fnx274

Burén, S., Young, E. M., Sweeny, E. A., Lopez-Torrejón, G., Veldhuizen, M., Voigt, C. A., et al. (2017). Formation of nitrogenase NifDK tetramers in the mitochondria of *Saccharomyces cerevisiae*. *ACS Synth. Biol.* 6, 1043–1055. doi: 10.1021/acssynbio.6b00371

Burgess, B. K., Jacobs, D. B., and Stiefel, E. I. (1980). Large-scale purification of high activity *Azotobacter vinelandii* nitrogenase. *Biochim. Biophys. Acta (BBA) Enzymol* 614, 196–209. doi: 10.1016/0005-2744(80)90180-1

Cheng, Q., Day, A., Dowson-Day, M., Shen, G.-F., and Dixon, R. (2005). The *Klebsiella pneumoniae* nitrogenase Fe protein gene (nifH) functionally substitutes for the chlL gene in *Chlamydomonas reinhardtii*. *Biochem. Biophys. Res. Commun.* 329, 966–975. doi: 10.1016/j.bbrc.2005.02.064

Curatti, L., Hernandez, J. A., Igarashi, R. Y., Soboh, B., Zhao, D., and Rubio, L. M. (2007). In vitro synthesis of the iron-molybdenum cofactor of nitrogenase from iron, sulfur, molybdenum, and homocitrate using purified proteins. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17626–17631. doi: 10.1073/pnas.0703050104

Curatti, L., and Rubio, L. M. (2014). Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. *Plant Sci.* 225, 130–137. doi: 10.1016/j.plantsci.2014.06.003

Dodd, A. N., Belbin, F. E., Frank, A., and Webb, A. A. R. (2015). Interactions between circadian clocks and photosynthesis for the temporal and spatial coordination of metabolism. *Front. Plant Sci.* 6, 245–245. doi: 10.3389/fpls.2015.00245

Dos Santos, P. C., Smith, A. D., Frazzon, J., Cash, V. L., Johnson, M. K., and Dean, D. R. (2004). Iron–sulfur cluster assembly: NifU-directed activation of the nitrogenase Fe protein. *J. Biol. Chem.* 279, 19705–19711. doi: 10.1074/jbc.M400278200

Erisman, J. W., Galloway, J. N., Dise, N. B., Sutton, M. A., Bleeker, A., Grizzetti, B., et al. (2015). *Nitrogen: Too Much of a Vital Resource: Science Brief*. Zeist: WWF Netherlands.

Eseverri, Á., López-Torrejón, G., Jiang, X., Burén, S., Rubio, L. M., and Caro, E. (2020). Use of synthetic biology tools to optimize the production of active nitrogenase Fe protein in chloroplasts of tobacco leaf cells. *Plant Biotechnol. J.* 18, 1882–1896. doi: 10.1111/pbi.13347

Hernandez, J. A., Phillips, A. H., Erbil, W. K., Zhao, D., Demuez, M., Zeymer, C., et al. (2011). A Sterile α-Motif domain in NafY targets apo-NifDK for iron-molybdenum cofactor delivery via a tethered domain. *J. Biol. Chem.* 286, 6321–6328. doi: 10.1074/jbc.M110.168732

Herridge, R. P., Day, R. C., Baldwin, S., and Macknight, R. C. (2011). Rapid analysis of seed size in Arabidopsis for mutant and QTL discovery. *Plant Methods* 7:3. doi: 10.1186/1746-4811-7-3

Hitoshi, S., and Hideo, C. (1985). Photosynthesis measurement by oxygen electrode as a simple bioassay method. *JARQ* 18, 252–259.

Howard, K. S., McLean, P. A., Hansen, F. B., Lemley, P. V., Koblan, K. S., and Orme-Johnson, W. H. (1986). *Klebsiella pneumoniae* nifM gene product is required for stabilization and activation of nitrogenase iron protein in *Escherichia coli*. *J. Biol. Chem.* 261, 772–778. doi: 10.1016/S0021-9258(17)36161-6

Ivleva, N. B., Groat, J., Staub, J. M., and Stephens, M. (2016). Expression of active subunit of nitrogenase via integration into plant organelle genome. *PLoS ONE* 11:e0160951. doi: 10.1371/journal.pone.0160951

Jiang, X., Payá-Tormo, L., Coroian, D., García-Rubio, I., Castellanos-Rueda, R., Eseverri, Á., et al. (2021). Exploiting genetic diversity and gene synthesis to identify superior nitrogenase NifH protein variants to engineer N2-fixation in plants. *Commun. Biol.* 4:4. doi: 10.1038/s42003-020-01536-6

Kazama, T., Okuno, M., Watari, Y., Yanase, S., Koizuka, C., Tsuruta, Y., et al. (2019). Curing cytoplasmic male sterility via TALEN-mediated mitochondrial genome editing. *Nat. Plants* 5, 722–730. doi: 10.1038/s41477-019-0459-z

Long, S. P., Marshall-Colon, A., and Zhu, X. G. (2015). Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. *Cell* 161, 56–66. doi: 10.1016/j.cell.2015.03.019

López-Torrejón, G., Jiménez-Vicente, E., Buesa, J. M., Hernandez, J. A., Verma, H. K., and Rubio, L. M. (2016). Expression of a functional oxygen-labile nitrogenase component in the mitochondrial matrix of aerobically grown yeast. *Nat. Commun.* 7:11426. doi: 10.1038/ncomms11426

Mueller, N. D., Gerber, J. S., Johnston, M., Ray, D. K., Ramankutty, N., and Foley, J. A. (2012). Closing yield gaps through nutrient and water management. *Nature* 490, 254–257. doi: 10.1038/nature11420

Oey, M., Lohse, M., Kreikemeyer, B., and Bock, R. (2009). Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J.* 57, 436–445. doi: 10.1111/j.1365-313X.2008.03702.x

Okada, S., Gregg, C. M., Allen, R. S., Menon, A., Hussain, D., Gillespie, V., et al. (2020). A synthetic biology workflow reveals variation in processing and solubility of nitrogenase proteins targeted to plant mitochondria, and differing tolerance of targeting sequences in a bacterial nitrogenase assay. *Front. Plant Sci.* 11:1388. doi: 10.3389/fpls.2020.552160

Oldroyd, G. E., and Dixon, R. (2014). Biotechnological solutions to the nitrogen problem. *Curr. Opin. Biotechnol.* 26, 19–24. doi: 10.1016/j.copbio.2013.08.006

Ort, D. R., and Oxborough, K. (1992). In situ regulation of chloroplast coupling factor activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43, 269–291. doi: 10.1146/annurev.pp.43.060192.001413

Petersen, K., and Bock, R. (2011). High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. *Plant Mol. Biol.* 76, 311–321. doi: 10.1007/s11103-011-9742-8

Poza-Carrión, C., Jiménez-Vicente, E., Navarro-Rodríguez, M., Echavarri-Erasun, C., and Rubio, L. M. (2014). Kinetics of andlt;emandgt;nifandlt;/emandgt; gene expression in a nitrogen-fixing bacterium. *J. Bacteriol.* 196:595. doi: 10.1128/JB.00942-13

Rangaraj, P., Shah, V. K., and Ludden, P. W. (1997). ApoNifH functions in iron-molybdenum cofactor synthesis and apodinitrogenase maturation. *Proc. Natl. Acad. Sci. U.S.A.* 94, 11250–11255. doi: 10.1073/pnas.94.21.11250

Roberts, G. P., MacNeil, T., MacNeil, D., and Brill, W. J. (1978). Regulation and characterization of protein products coded by the nif (nitrogen fixation) genes of *Klebsiella pneumoniae*. *J. Bacteriol.* 136, 267–279. doi: 10.1128/jb.136.1.267-279.1978

Ruf, S., Forner, J., Hasse, C., Kroop, X., Seeger, S., Schollbach, L., et al. (2019). High-efficiency generation of fertile transplastomic Arabidopsis plants. *Nat. Plants* 5, 282–289. doi: 10.1038/s41477-019-0359-2

Scotti, N., Alagna, F., Ferraiolo, E., Formisano, G., Sannino, L., Buonaguro, L., et al. (2009). High-level expression of the HIV-1 Pr55 gag polyprotein in transgenic tobacco chloroplasts. *Planta* 229, 1109–1122. doi: 10.1007/s00425-009-0898-2

Scotti, N., and Cardi, T. (2014). Transgene-induced pleiotropic effects in transplastomic plants. *Biotechnol. Lett.* 36, 229–239. doi: 10.1007/s10529-013-1356-6

Seefeldt, L. C., Yang, Z.-Y., Lukoyanov, D. A., Harris, D. F., Dean, D. R., Raugei, S., et al. (2020). Reduction of substrates by nitrogenases. *Chem. Rev.* 120, 5082–5106. doi: 10.1021/acs.chemrev.9b00556

Shah, V. K., and Brill, W. J. (1973). Nitrogenase IV. Simple method of purification to homogeneity of nitrogenase components from Azotobacter vinelandii. *Biochim. Biophys. Acta Bioenerg.* 305, 445–454. doi: 10.1016/0005-2728(73)90190-4

Temme, K., Zhao, D., and Voigt, C. A. (2012). Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7085–7090. doi: 10.1073/pnas.1120788109

Wu, A., Hammer, G. L., Doherty, A., von Caemmerer, S., and Farquhar, G. D. (2019). Quantifying impacts of enhancing photosynthesis on crop yield. *Nat. Plants* 5, 380–388. doi: 10.1038/s41477-019-0398-8

Yang, J., Xie, X., Xiang, N., Tian, Z.-X., Dixon, R., and Wang, Y.-P. (2018). Polyprotein strategy for stoichiometric assembly of nitrogen fixation components for synthetic biology. *Proc. Nat. Acad. Sci. U.S.A.* 115:E8509. doi: 10.1073/pnas.1804992115

Zhang, J., Ruf, S., Hasse, C., Childs, L., Scharff, L. B., and Bock, R. (2012). Identification of cis-elements conferring high levels of gene expression in non-green plastids. *Plant J.* 72, 115–128. doi: 10.1111/j.1365-313X.2012.05065.x

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.

Copyright © 2021 Aznar-Moreno, Jiang, Burén and Rubio. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Agronomy

ORIGINAL RESEARCH
published: 13 August 2021
doi: 10.3389/fagro.2021.692238

Black Seedcoat Pigmentation Is a Marker for Enhanced Nodulation and N_2 Fixation in Bambara Groundnut (*Vigna Subterranea* L. Verdc.) Landraces

Doris K. Puozaa[1,2], *Sanjay K. Jaiswal*[3] *and Felix D. Dakora*[3]*

[1] Department of Crop Sciences, Tshwane University of Technology, Pretoria, South Africa, [2] CSIR-Savanna Agricultural Research Institute, Tamale, Ghana, [3] Chemistry Department, Arcadia Campus, Tshwane University of Technology, Pretoria, South Africa

Field studies on the effect of differing seedcoat colour on plant growth and symbiotic performance of three Bambara groundnut landraces using the ^{15}N natural abundance technique and ureide analysis in the xylem stream and petiole extracts revealed a consistent pattern in nodule function between ureide levels and shoot $\delta^{15}N$ values, as well as between ureide levels and percent N derived from fixation at Gbalahi, Kpalisogu, and Manga. At those three sites, where shoot $\delta^{15}N$ was low and percent N derived from fixation high, ureide concentrations in the xylem sap and petiole extracts were also high, indicating that the two techniques were comparatively quite robust in estimating N_2 fixation at a higher precision in the test Bambara groundnut landraces. A comparison of the effect of differing seedcoat pigmentation on the concentration of phenolics released by imbibed seeds showed that the Black landrace contained higher levels of flavonoids and anthocyanins, followed by the Red, and lowest in the Cream landrace. The Black landrace also recorded much higher nodule number and nodule fresh weight per plant, greater shoot biomass, lower shoot $\delta^{15}N$ values, greater N derived from fixation, higher symbiotic N contribution, and increased water-use efficiency across all the five study sites, followed by the Red landrace, which produced the highest grain yield. Black seedcoat pigmentation in Bambara groundnut is therefore a marker for increased nodulation, N_2 fixation, and plant growth that can be tapped in breeding programs for developing high N_2-fixing, water-use efficient grain legumes for use by farmers in Africa.

Keywords: ^{15}N natural abundance, shoot δ ^{15}N, ureides, N-fixed, nodulation, phenolics, flavonoids and anthocyanins

OPEN ACCESS

Edited by:
Juan Imperial, Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Vinay Kumar, Central University of Punjab, India
Dulce Nombre Rodriguez-Navarro, IFAPA Centro Las Torres Tomejil, Spain

***Correspondence:**
Felix D. Dakora
dakorafd@tut.ac.za

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: *07 April 2021*
Accepted: *16 July 2021*
Published: *13 August 2021*

Citation:
Puozaa DK, Jaiswal SK and Dakora FD (2021) Black Seedcoat Pigmentation Is a Marker for Enhanced Nodulation and N_2 Fixation in Bambara Groundnut (Vigna Subterranea L. Verdc.) Landraces. Front. Agron. 3:692238. doi: 10.3389/fagro.2021.692238

INTRODUCTION

Bambara groundnut (*Vigna subterranea* L. Verdc.) is the third most important food legume in Africa after groundnut and cowpea, both in consumption and land area under cultivation. The crop is high in protein (20.6%) and carbohydrate (56.5%), and has 6.6% fat as well as 6.3% fibre, thus making it a complete meal (Mohale et al., 2013). It is cultivated by smallholder farmers either as a monoculture, in rotation with cereals, or mixed cultures with cereals, root, and tuber crops.

Despite its importance, Bambara groundnut yields have remained low due to lack of improvement. Plant growth, symbiotic N_2 fixation and grain yield of Bambara groundnut have been found to vary considerably across Africa (Nyemba and Dakora, 2005; Mabhaudhi et al., 2013; Mohale et al., 2013) due to environmental factors and the symbiotic efficacy of native rhizobia nodulating Bambara groundnut. Grain yield of Bambara groundnut has been reported to double with rhizobial inoculation (Gueye and Bordeleau, 1988), indicating that native soil rhizobia are sometimes inefficient in meeting the N demand of the crop. Applying adequate numbers of competitive and efficient rhizobia on legume seed at planting can be an insurance for enhanced nodulation and N_2 fixation for increased legume crop yields.

A few field studies have evaluated N_2 fixation in Bambara groundnut using the 15N natural abundance method (Nyemba and Dakora, 2005; Mohale et al., 2013). The 15N isotopic technique has been used to estimate N_2 fixation in grain legumes, pasture species, and tree/shrub legumes (Unkovich et al., 2008; Pule-Meulenberg and Dakora, 2009; Belane and Dakora, 2010; Mohale et al., 2013; Muhaba and Dakora, 2020).

As the reproductive unit, seeds carry the genetic material of crop species over time and space, while the seedcoat plays a vital role in protecting the embryo, as well as determining seed dormancy and germination (Moise et al., 2005). The seedcoat also contains a host of novel compounds that are released during germination when the seed imbibes water. These molecules include flavonoids, proteins, peptides, amino acids, alkaloids, and terpenoids (Ndakidemi and Dakora, 2003) and play an important role in plant development, including plant defence against insect pests and pathogens, as well as induction of nodulation genes during symbiotic establishment in N_2-fixing legumes (Ndakidemi and Dakora, 2003). The importance of these seedcoat compounds in legume nodulation has been demonstrated; Hungria and Phillips, 1993; Subramanian et al., 2007), and variation in seedcoat colour may be responsible for the differences in N_2 fixation observed under field conditions among legume genotypes.

The seedcoat colour of Bambara groundnut landraces can vary distinctly due to the presence of different phenolic compounds (Ndakidemi and Dakora, 2003) and are often used as descriptors for landraces (Heller et al., 1997). Legume accessions are sometimes given local names based on the colour of the testa or seedcoat (Wang et al., 2008). Seedcoat compounds are also known to play a role in the nodulation of symbiotic legumes. In studies by Hungria et al. (1991) and Hungria and Phillips (1993), seedcoat pigmentation was found to strongly influence the nodulation of common bean. An isogenic line of common bean with black seedcoat colour formed more nodules than its cream mutant counterpart, due to differences in seedcoat flavonoid concentration and profile. In a recent study, Bambara groundnut landraces with different seedcoat colours also exhibited differences in grain yield and water-use efficiency under field conditions (Mabhaudhi et al., 2013). So far, however, the link between seedcoat colour and symbiotic performance of legumes has not been established under field conditions. Yet, seedcoat pigmentation could be a useful marker in breeding programs for selecting Bambara groundnut landraces for superior performance. The aim of this study was to assess plant growth, grain yield, N_2 fixation, and water-use efficiency in field plants of three Bambara groundnut landraces with different seedcoat colours, using the ^{15}N natural abundance technique.

MATERIALS AND METHODS

Seed Source and Characteristics

Three Bambara groundnut landraces with different seedcoat colours (Black, Red, and Cream) were used in this study. Seed material of the Black and Red landraces were obtained from farmers at Tiza in the Upper West Region, while the Cream landrace was purchased from Tamale market in the Northern Region, Ghana.

The Bambara groundnut genotypes used in this study differed in seedcoat pigmentation. Otherwise, they exhibited similar growth habit, and phenology, recorded 50% flowering between 52 and 56 days after planting, and matured in 110–120 days.

Description of Field Study Sites

The field trials were set up in July 2013 at three locations within two contrasting agro-ecologies of Ghana. Three field experiments were sited in the Guinea savanna of Northern Region, at Savelugu (latitude 9.624722, longitude 0.827778), Gbalahi (latitude 9.433333, longitude 0.766667) and Kpalisogu (latitude 9.405066, longitude 1.002990), and two at Googo (latitude 10.7545041, longitude 0.4879915) and Manga (latitude 11.017331, longitude 0.264352) in the Sudano-sahelian savanna of the Upper East Region. The two regions have a unimodal rainfall. The annual rainfall in 2013 was 1,047.8 mm in Northern Region and 870 mm in Upper East Region (**Table 1**). Average daily maximum temperature was 30 to 35°C.

Experimental Design and Planting

In June 2013, Bambara groundnut landraces with black, red, and cream seedcoat pigmentation were evaluated for growth and symbiotic performance in five districts of the Northern and Upper East Regions of Ghana. Each field experiment was laid using a randomised complete block design with four replicate plots. Three seeds were planted per hole and latter thinned out to two seedlings per stand. To ensure optimal nodulation at each site, seeds of the three Bambara groundnut landraces were inoculated with *Bradyrhizobium* strain CB756 and planted at 50 cm between rows and 20 cm within rows. There were 12 plants per row and six rows per plot. A distance of 1.5 m was maintained between plots and 2 m between blocks. In June 2013, Bambara groundnut landraces with black, red and cream seedcoat pigmentation were evaluated for growth and symbiotic performance in five farming districts of the Northern and Upper East Regions of Ghana. Each field experiment was laid using a randomised complete block design with four replicate plots. Three seeds were planted per hole and latter thinned out to two seedlings per stand. To endure optimal nodulation at each site, seeds of the three Bambara groundnut landraces were inoculated with *Bradyrhizobium* strain CB756 and planted at 50 cm between rows and 20 cm within rows. There were 12 plants per row and

TABLE 1 | Physical and chemical properties of bulk soil collected from each site prior to planting.

Location	Texture	pH (KCl)	Org C%	Total N	Org matter	Ca	Mg	K	Na	CEC	P	Zn	Cu	Mn	Fe
							$mg.kg^{-1}$								
Kpalisogu	Sandy loam	4.7	0.51	0.05	0.88	320	96	59	9	484	8	10.8	3.9	32	1.5
Gbalahi	Sandy loam	5.1	0.63	0.06	1.09	640	224	47	7	918	7	20.8	3.4	25	2.4
Manga	Loamy sandy	4.7	0.48	0.05	0.83	214	32	98	12	355	23	12.8	4.6	18	1.4
Googo	Sandy loam	4.6	0.9	0.08	1.38	534	168	140	16	851	15	9.9	8.2	70	2
Savelugu	Sandy loam	4.9	0.39	0.04	0.67	320	64	55	7	445	8	1.8	6.3	30	1

six rows per plot. A distance of 1.5 m was maintained between plots in a row and 2 m between blocks.

Seed Inoculation

Seeds were inoculated with a peat-based inoculant of *Bradyrhizobium* strain CB756 at a rate of 10 g inoculant per kg seed of 10^8 rhizobial cells.gpeat^{-1}. Inoculated seeds were allowed to dry under shade for a few minutes before planting. The uninoculated control seeds were planted first, followed by the inoculated treatment to avoid contamination. The plots were kept weed-free by hand hoeing, when necessary.

Soil Sampling and Analysis

Following land preparation but prior to planting at Savelugu, Gbalahi, Kpalisogu, Googo, and Manga, 20 soil samples were randomly collected from 0 to 20 cm depth from across each experimental plot using an augur. The soil samples were pooled per plot and thoroughly mixed. The composite soil sample was air-dried in the laboratory, and sieved (2.0 mm) for analysis of texture, organic matter, organic C, pH, CEC, P, Ca, K, Mg, Na, Fe, Zn, Cu, and Mn (ammonium acetate method), as well as total N using the Kjeldahl digestion method (Muhaba and Dakora, 2020).

Collection of Xylem Sap and Ureide Analysis

At flowering at 56 days after planting (DAP), the shoots of 10 to 15 plants per plot were deccapitated at the crown level, and the root bleeding sap collected into Eppindorf tubes using Pasteur pipets. The Eppindorf tubes containing xylem sap were stored in ice, transported to the laboratory, where they were analysed for ureides, as described by Unkovich et al. (2008).

Plant Sampling and Processing

The decapitated shoots of 10 to 15 plants per plot were placed in labelled paper bags. The roots were dug up and root nodules separated, countered, and weighed. The shoots of individual plants were oven-dried (70°C) for 48 h, weighed, and finely ground (0.85 mm) for ^{15}N and ^{13}C isotopic analysis. Shoots of non-legume plant species growing within the experimental plots were concurrently collected, oven-dried (70°C for 48 h) and similarly processed for ^{15}N isotopic analysis in order to estimate soil N uptake by the legume.

$^{15}N/^{14}N$ Isotopic Analysis, Percent N Derived From Fixation, and Amount of N-fixed

About 2.0–2.5 mg of ground sample of legume and reference plant shoots were fed into a mass spectrometer [Carlo Erba NA1500 elemental analyzer (Fisons Instruments SpA, Strada, Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer (Fisons Instrument SpA, Strada, Rivoltana, Italy) *via* conflo II open-split device] to measure $^{15}N/^{14}N$ isotopic composition. The $\delta^{15}N$ of the shoot samples was computed as (Muhaba and Dakora, 2020):

$$\delta^{15}N(^o/_{oo}) = \frac{[^{15}N/^{14}N]_{sample} - [^{15}N/^{14}N]_{atm}}{[^{15}N/^{14}N]_{atm}} \times 1000 \quad (1)$$

where the $^{15}N/^{14}N_{sample}$ is the abundance ratio of ^{15}N and ^{14}N in the sample and $^{15}N/^{14}N_{atm}$ is the abundance ratio of ^{15}N and ^{14}N in the atmosphere. The %N and %C of shoot samples were obtained directly from the mass spectrometer, and shoot C/N ratio computed as the ratio of C to N.

The N content of shoot samples was calculated as the product of shoot DM and %N. The %Ndfa of the test legumes was calculated as (Muhaba and Dakora, 2020):

$$\%Ndfa = \frac{\delta^{15}N_{ref} - \delta^{15}N_{leg}}{\delta^{15}N_{ref} - B} \times 100 \quad (2)$$

where $\delta^{15}N_{ref}$ is the ^{15}N natural abundance of reference plant, $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of legume plant, and the *B value* is the ^{15}N natural abundance of Bambara groundnut plants completely dependent on atmospheric N_2 fixation for their N nutrition. The mean $\delta^{15}N$ of all reference plants sampled from each study site was used to estimate the %Ndfa of landraces from that site.

The amount of N-fixed in Bambara groundnut plants was calculated as:

N-fixed = %Ndfa × shoot N content. The soil N uptake by Bambara groundnut was calculated as the difference between total shoot N content and amount of N-fixed.

$^{13}C/^{12}C$ Isotopic Analysis

Percent C and ^{13}C isotopic analysis of soybean plants was done using a mass spectrometer as done for ^{15}N. To determine $^{13}C/^{12}C$ ratio, 2–3 mg plant sample was weighed into Al capsules, and fed into a Carlo Erba NA1500 elemental analyzer (Fisons Instruments SpA, Strada, Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer *via* conflo II open-split device. The ratio of $^{13}C/^{12}C$ in each shoot sample was used to calculate the ^{13}C natural abundance (or $\delta^{13}C$) as (Muhaba and Dakora, 2020):

$$\delta^{13}C(^{o}/_{oo}) = \left[\frac{(13_C/12_C)_{sample}}{(13_C/12_C)_{standard}} - 1 \right] \times 1000 \quad (3)$$

Where $(^{13}C/^{12}C)_{sample}$ is the isotopic ratio of soybean shoot and $(^{13}C/^{12}C)_{standard}$ is the isotopic ratio of Pee Dee Belemite (PDB) limestone formation, a universally accepted standard from IAEA (Muhaba and Dakora, 2020).

Determination of Flavonoids and Anthocyanins in Seed Extracts

The concentration of flavonoids and anthocyanin in the seeds of Bambara groundnut landraces was measured, as described by Makoi et al. (2010). Seeds of each landrace were weighed (50 g/landrace), counted to record seed number, and finely ground (0.85 mm sieve).Flavonoids and anthocyanins were extracted using 10 g powder in 50 ml of acidified methanol (79:20:1 MeOH H_2O HCl). The mixture was incubated on a shaker for 48 h in darkness, filtered, and absorbance of the filtrate measured spectrometrically (UV-VIS) at 300, 530, and 657 nm using acidified methanol as control. The concentration of flavonoid was measured at 300 nm and expressed as Abs $g.DM^{-1}$ or Abs. $seed^{-1}$, while the anthocyanin was measured as Abs530 −1/3Abs657 and expressed as Abs $g.DM^{-1}$ or Abs. $seed^{-1}$.

Statistical Analysis

The data collected from individual sites were subjected to a one-way analysis of variance (ANOVA), while a 2-factorial arrangement comprising seedcoat colour of genotype and environment was used to analyse the data from across the five experimental sites. Where means were significantly different, they were separated using the Duncan multiple range test. Pearson's correlation analysis was carried out to determine the relationship between plant growth and N nutrition parameters as well as N nutrition and shoot $\delta^{13}C$. The analyses were carried out using STATISTIX version 10 software (StaSoft Inc., Tulsa, OK, USA) package.

RESULTS

Soil Analysis

As shown in **Table 1**, the soils from the five sites were similar in texture (i.e., sandy loam) and pH, which ranged from pH 4.6 to 5.1. Gbalahi and Googo recorded the highest level of organic matter (1.09 and 1.38%, respectively) and %C (0.63 and 0.90%, respectively). As a result, Gbalahi and Googo had the highest CEC (918 and 851 mg kg^{-1}, respectively), Ca (640 and 534 mg kg^{-1}, respectively), Mg (224 and 168 mg kg^{-1}, respectively), N (0.06 and 0.08 mg kg^{-1}, respectively), Zn (20.8 and 9.9 mg kg^{-1}, respectively) and Fe (2.4 and 2.0 mg kg^{-1}, respectively).

Flavonoid and Anthocyanin Concentrations in Seed Extracts of Bambara Groundnut Landraces

The concentrations of flavonoids and anthocyanin released by imbibed seeds of the Bambara groundnut landraces in acidified methanol were higher in the landrace with black seedcoat (Black), followed by red seedcoat (Red) and least in the cream seedcoat (Cream), whether measured as absorbance per gramme of seed dry matter or on the basis of absorbance per single seed (**Table 2**). As would be expected, all the three test genotypes had more flavonoids than anthocyanins in the crude seed extract (**Table 2**). There was also a highly positive significant correlation (r = 0.85, p = 0) between flavonoid and anthocyanin levels in the test Bambara groundnut landraces (data not shown) implying that anthocyanin makes up a great proportion of the total seed flavonoid content.

$\delta^{15}N$ of Reference Plants

At Gbalahi, five reference plant species were analysed, and the $\delta^{15}N$ values ranged from +2.76 to +5.19‰, with a combined mean $\delta^{15}N$ value of +3.75‰ (**Table 3**). The $\delta^{15}N$ of the three reference plants sampled at Googo ranged from +4.97‰ to +5.39‰, with a mean value of +5.23‰. Four reference plant species were collected from each site at Kpalisogu and Manga, and their mean $\delta^{15}N$ values were +3.95 and +4.30‰, respectively (**Table 3**). At Savelugu, the minimum and maximum $\delta^{15}N$ values were +2.47‰ and +2.71‰, with a combined mean $\delta^{15}N$ value of +2.61‰ for the six reference plant species (**Table 3**).

TABLE 2 | Concentration of flavonoids and anthocyanin released by imbibing seeds of Bambara groundnut landraces in acidified MeOH.

Seedcoat colour	Flavonoids	Anthocyanin	Flavonoids	Anthocyanin
	Abs.g DM^{-1}		Abs.seed^{-1}	
Black	1.57 ± 0.08a	1.001 ± 0.0591a	1.31 ± 0.06a	0.8344 ± 0.0359a
Red	1.25 ± 0.02b	0.024 ± 0.001b	1.00 ± 0.07b	0.0192 ± 0.0014b
Cream	1.02 ± 0.02c	0.008 ± 0.0003c	0.74 ± 0.02c	0.0059 ± 0.0002c
F-statistics	31.86***	277.86***	9.50**	174.84***

*Values (Mean ± SE) followed by dissimilar letters in a column are significantly different at **P ≤ 0.01, ***P < 0.001.*

TABLE 3 | Shoot δ^{15} N values of reference plants used for estimating soil N uptake by Bambara groundnut landraces.

Location	Reference plant	δ^{15} N (‰)
Gbalahi	*Oryza sativa*	+2.76
	Cyperus sp.	+5.18
	Zea mays	+5.19
	Commelina sp.	+2.80
	Mean	**+3.75**
Googo	*Zea mays*	+4.97
	Corchorus sp.	+5.33
	Sorghum bicolor	+5.39
	Mean	**+5.23**
Kpalisogu	*Pennisetum glaucum*	+4.15
	Zea mays	+3.19
	Ageratum sp.	+4.97
	Sorghum bicolor	+3.49
	Mean	**+3.95**
Manga	*Cyperus* sp.	+3.48
	Commelina sp.	+3.38
	Corchorus olitorius	+4.77
	Hibiscus sabdariffa	+5.58
	Mean	**+4.30**
Savelugu	*Ageratum* sp.	+2.57
	Commelina sp.	+2.64
	Zea mays	+2.65
	Sorghum bicolor	+2.65
	Corchorus olitorius	+2.47
	Cassia sp.	+2.71
	Mean	**+2.61**

Plant Growth, Symbiotic Performance, Water-Use Efficiency and Grain Yield of Bambara Landraces Planted at Different Experimental Sites

At Gbalahi, the Bambara groundnut landrace Black formed more nodules, with higher nodule fresh weight per plant compared to the Red and Cream landraces (**Table 4**). Plant growth (measured as shoot dry matter) and δ^{13}C (water-use efficiency) were however similar for all three landraces. The Red landrace produced the highest grain yield relative to Black and Cream (**Table 4**).

The Black and Red landraces showed significantly increased shoot N concentration (%N) but not N content. Shoot δ^{15}N was lowest in the Black landrace, and resulted in greater %Ndfa when compared to the Red and Cream landraces (**Table 5**). The Black landrace also showed the highest ureide concentration in the xylem stream.

Although seedcoat colour had no effect on nodule number per plant at Googo, nodule fresh weight was greater in the Black landrace, followed by Red and least in the Cream landrace (**Table 4**). The three Bambara groundnut landraces revealed similar shoot DM and water-use efficiency (δ^{13}C) at Googo. However, they differed in grain yield, with the Red landrace producing higher grain yield than the Cream and Black landraces (**Table 4**).

The shoot %N was highest in the Red landrace, which when combined with its slightly higher shoot DM resulted in much greater N content relative to the Black and Cream landraces (**Table 5**). The three landraces were however similar shoot δ^{15}N, %Ndfa and N-fixed. Although soil N uptake was similar for the three landraces, the Red Banbara groundnut showed much higher ureide concentration compared to the others (**Table 5**).

At Kpalisogu, the Black landrace produced significantly higher nodule number and nodule fresh weight per plant, followed by the Red landrace and Cream (**Table 4**). Shoot dry matter was also markedly higher in the Black landrace, followed by Red, and lowest in the Cream landrace. Shoot δ^{13}C values were however similar for all three landraces at the Kpalisogu site (**Table 4**). The Black landrace also recorded much higher shoot biomass and greater grain yield, which almost doubled the yield of the Cream landrace (**Table 4**).

Shoot N concentration and N content at Kpalisogu were markedly higher in the Black and Red landraces than the Cream (**Table 5**). The Black landrace also showed much lower shoot δ^{15}N, followed by the Red. This was in contrast to the Cream landrace which recorded a much greater shoot δ^{15}N (**Table 5**). As a result, the percent N derived from fixation and amounts of N-fixed were higher in the Black and Red compared to the Cream landrace (**Table 5**). Ureides in the xylem stream and petiole extracts was also much greater in the Black and Red than the Cream landrace, though not statistically significant (**Table 5**).

At Manga, nodule number and fresh weight per plant were both greater in the Black landrace, followed by Red, and least in the Cream landrace (**Table 4**). The Black landrace also recorded much higher shoot DM compared to the other two landraces, but shoot δ^{13}C and grain yield were similar (**Table 4**).

Although shoot N concentration was similar for all three landraces, the N content was significantly higher in the Black

TABLE 4 | Nodulation, shoot dry matter (DM), $\delta^{13}C$ values and grain yield of Bambara groundnut landraces with different seedcoat colours sampled from five sites in Ghana.

Seedcoat colour	Nodule no.	Nodule Fwt	Shoot DM	$\delta^{13}C$	Grain yield
	.plant^{-1}	mg.plant^{-1}	g.plant^{-1}	‰	kg.ha^{-1}
Gbalahi					
Black	27 ± 3a	446.9 ± 50.3a	17.0 ± 0.9a	−28.95 ± 0.07a	284.2 ± 32.5c
Red	20 ± 2b	258.2 ± 16.4b	13.3 ± 0.8a	−29.16 ± 0.05a	499.6 ± 28.9a
Cream	20 ± 2b	259.8 ± 28.6b	14.9 ± 1.6a	−28.95 ± 0.11a	378.2 ± 27.2b
F-statistic	3.82*	9.77**	2.63	2.29	13.30***
Googo					
Black	7 ± 1a	168.1 ± 5.5a	23.7 ± 1.5a	−27.87 ± 0.14a	697.2 ± 61.3c
Red	7 ± 1a	71.3 ± 6.3b	24.2 ± 1.8a	−27.99 ± 0.09a	2028.7 ± 146.5a
Cream	7 ± 1a	59.5 ± 3.5c	20.6 ± 1.1a	−28.16 ± 0.14a	1368.4 ± 128.8b
F-statistic	0.04	130.37***	1.79	1.34	72.55***
Kpalisogu					
Black	33 ± 4a	286.7 ± 6.0a	25.1 ± 0.6a	−28.66 ± 0.18a	1566.3 ± 119.4a
Red	17 ± 2b	83.8 ± 5.2b	21.8 ± 1.3b	−28.87 ± 0.05a	1067.9 ± 102.7b
Cream	9 ± 1c	49.5 ± 3.3c	19.4 ± 1.2c	−28.53 ± 0.04a	830.9 ± 55.5c
F-statistic	20.43***	664.16***	6.91**	2.51	15.16***
Manga					
Black	13 ± 2a	328.1 ± 17.1a	18.8 ± 0.4a	−27.59 ± 0.10a	443.5 ± 41.3a
Red	7 ± 1b	220.9 ± 17.3b	11.6 ± 0.9b	−28.03 ± 0.07a	497.2 ± 47.8a
Cream	5 ± 1b	142.3 ± 11.0c	10.6 ± 1.0b	−27.54 ± 0.26a	384.5 ± 23.8a
F-statistic	25.02***	36.74***	29.73	2.67	2.09
Savelugu					
Black	27 ± 2a	484.2 ± 15.6a	21.58 ± 0.96a	−29.01 ± 0.09a	907.8 ± 64.2b
Red	19 ± 1b	365.8 ± 30.2b	16.11 ± 1.33b	−29.25 ± 0.06a	1331.3 ± 76.4a
Cream	15 ± 1c	299.1 ± .37.8b	17.65 ± 1.39b	−29.15 ± 0.05a	1217.2 ± 81.5a
F-statistic	16.74**	14.64***	5.16**	3.07	8.68***

Values (Mean ± SE) followed by dissimilar letters in a column are significantly different at $^*p \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P < 0.001$.

landrace due its greater shoot biomass. Shoot $\delta^{15}N$ values were much lower in the Black and Red landraces. As a result, the percent N derived from fixation and amount of N-fixed by Black and Red landraces were higher than the Cream. Ureides in the xylem sap of Red landrace was also much higher than the Cream. Soil N uptake by the Red landrace was less than the other two landraces (**Table 5**).

Nodule number and nodule fresh weight per plant were both greater in the Black landrace at the Savelugu, followed by Red, and much lower in the Cream landrace (**Table 4**). The Black landrace also produced significantly higher shoot DM but recorded much lower grain yield than the other two landraces.

The shoot N concentration and N content were similar for the three landraces at Savelugu. However, the shoot $\delta^{15}N$ was lowest in the Red Bambara groundnut landrace, and resulted in significantly higher %Ndfa than the Black and Cream landraces (**Table 5**). The amount of N-fixed in the Black landrace was greater in magnitude, but not statistically. Soil N uptake was lower in the Red landrace relative to Black and Cream. Ureide-N in the xylem sap was however similar for all three landraces. As found at the other study sites, shoot $\delta^{13}C$ was unaffected by seedcoat colour at Savelugu.

Effects of the Seedcoat Colour × Environment Interactions on Plant Growth, Nodulation, Grain Yield and Water-Use in Bambara Groundnut Landraces

A 2-way analysis of variance involving seedcoat colour and environment revealed marked differences in plant growth, $\delta^{13}C$ and grain yield among the Bambara groundnut landraces in the different environments. Nodule number and nodule fresh weight were relatively higher at Gbalahi and Savelugu and lowest at Googo (**Table 6**). Shoot biomass was much greater at Googo and Kpalisogu, followed by Savelugu, and lowest at Manga and Gbalahi. Grain yield was also markedly higher at Googo, followed by Kpalisogu and lowest at Gbalahi and Manga (**Table 6**). Shoot $\delta^{13}C$ was however greater at Manga in the Upper East Region, where rainfall was lower (867.6 mm), while Savelugu with high rainfall (1047.8 mm) recorded the lowest $\delta^{13}C$ values.

The Black landrace produced significantly higher nodule number and nodule fresh weight per plant across all locations, followed by the Red and Cream landraces (which was the lowest). Grain yield was however higher in the Red landrace, but similar for the Black and Cream landraces (**Table 6**). Shoot $\delta^{13}C$ was greater in the Black than the Red and Cream landraces.

TABLE 5 | Shoot N concentration, N-fixed, ureide concentration and soil N uptake of Bambara groundnut landraces with different seedcoat colours sampled at flowering from five sites in Ghana.

Seedcoat colour	Shoot N	N content	$\delta^{15}N$	Ndfa	N-fixed	Soil N uptake	Ureide-N μg.ml^{-1}
	%	g.plant^{-1}	‰	%	kg.ha^{-1}	kg.ha^{-1}	
Gbalahi							
Black	2.41 ± 0.05a	0.41 ± 0.02a	−0.47 ± 0.04b	81.9 ± 4.8a	67.2 ± 2.9a	14.4 ± 1.2b	201.87 ± 5.88a
Red	2.59 ± 0.07a	0.35 ± 0.03a	0.29 ± 0.03a	67.1 ± 3.6c	46.5 ± 3.5a	22.9 ± 2.1a	143.03 ± 8.32c
Cream	1.96 ± 0.16b	0.30 ± 0.05a	−0.12 ± 0.01b	75.1 ± 2.3b	45.1 ± 9.3a	16.0 ± 1.6b	167.75 ± 15.08b
F-statistic	9.86***	2.13	148.42***	148.42***	1.72	15.63**	7.90**
***Googo* μg.g DM$^{-1\dagger}$**							
Black	2.76 ± 0.17b	0.66 ± 0.07b	3.34 ± 0.23a	28.5 ± 3.5a	49.61 ± 3.9a	92.0 ± 7.1a	0.35 ± 0.04b
Red	3.35 ± 0.11a	0.80 ± 0.04a	3.05 ± 0.14a	33.0 ± 2.1a	53.92 ± 6.0a	106.8 ± 3.6a	0.47 ± 0.05a
Cream	2.71 ± 0.22b	0.56 ± 0.06c	2.78 ± 0.21a	37.0 ± 3.1a	42.36 ± 6.8a	69.6 ± 6.4b	0.22 ± 0.01c
F-statistic	4.34**	4.72*	2.03	2.03	1.05	10.17***	11.50**
***Kpalisogu* μg.ml$^{-1}\ell$**							
Black	3.27 ± 0.16a	0.82 ± 0.05a	1.04 ± 0.03b	54.4 ± 3.5a	89.5 ± 5.6a	74.8 ± 4.5a	91.24 ± 6.42a
Red	3.37 ± 0.14a	0.73 ± 0.04a	1.15 ± 0.05b	52.4 ± 2.9a	75.8 ± 2.9b	69.6 ± 5.3a	102.78 ± 8.80a
Cream	2.55 ± 0.20b	0.51 ± 0.07b	1.52 ± 0.14a	45.5 ± 2.5b	45.5 ± 6.3c	55.6 ± 8.0a	77.04 ± 7.31a
F-statistic	7.14**	9.45***	8.56**	8.56**	19.21***	2.65	2.90
***Manga* μg.g DM$^{-1\dagger}$**							
Black	2.30 ± 0.13a	0.43 ± 0.03a	−0.56 ± 0.05b	85.2 ± 1.9a	73.8 ± 4.8a	12.6 ± 1.5a	0.54 ± 0.05b
Red	2.50 ± 0.11a	0.29 ± 0.02b	−0.64 ± 0.05c	86.7 ± 1.8a	50.3 ± 4.0b	7.6 ± 1.0b	1.09 ± 0.21a
Cream	2.70 ± 0.10a	0.29 ± 0.04b	−0.46 ± 0.04a	83.5 ± 1.7b	48.3 ± 5.7b	9.9 ± 1.6ab	0.53 ± 0.07b
F-statistic	3.10	8.23**	4.25*	4.25*	8.49**	5.39**	6.47**
***Savelugu* μg.ml$^{-1}\ell$**							
Black	2.62 ± 0.14a	0.57 ± 0.05a	−0.30 ± 0.02b	72.59 ± 4.9b	82.7 ± 7.0a	31.6 ± 3.1a	213.40 ± 15.87a
Red	2.65 ± 0.08a	0.43 ± 0.04a	−0.92 ± 0.06c	88.1 ± 1.5a	74.8 ± 6.3a	10.6 ± 1.9b	205.85 ± 1.92a
Cream	2.90 ± 0.11a	0.51 ± 0.05a	−0.26 ± 0.01a	71.6 ± 2.7b	73.3 ± 6.8a	29.2 ± 2.8a	250.30 ± 18.86a
F-statistic	1.87	2.57	101.16***	101.16***	0.57	19.74**	2.78

*Values (Mean ± SE) followed by dissimilar letters in a column are significantly different at *$p \leq 0.05$, **$P \leq 0.01$, ***$P < 0.001$. †petiole extract, $^{\ell}$xylem sap.*

TABLE 6 | Effects of environment and seedcoat colour (SCC) on nodulation, shoot dry matter (DM), $\delta^{13}C$ values and grain yield of Bambara groundnut landraces.

Treatment	Nodule no.	Nodule Fwt	Shoot DM	$\delta^{13}C$	Grain yield
	.plant^{-1}	mg.plant^{-1}	g.plant^{-1}	‰	kg.ha^{-1}
Location					
Gbalahi	23 ± 1a	321.6 ± 26.6b	15.0 ± 0.7c	−29.02 ± 0.05d	387.4 ± 24.6c
Googo	7 ± 1c	99.7 ± 10.5e	22.9 ± 0.9a	−28.01 ± 0.07b	1364.8 ± 130.7a
Kpalisogu	20 ± 3b	140.0 ± 22.0d	22.1 ± 0.8a	−28.69 ± 0.07c	1155.1 ± 83.1b
Manga	8 ± 1c	230.4 ± 18.0c	13.7 ± 0.9c	−27.72 ± 0.10a	441.7 ± 23.6c
Savelugu	20 ± 1b	383.0 ± 20.9a	18.4 ± 0.8b	−29.14 ± 0.04d	1152.1 ± 55.5b
Seedcoat colour (SCC)					
Black	21 ± 2a	342.8 ± 21.1a	21.2 ± 0.6a	−28.42 ± 0.11a	779.8 ± 77.6b
Red	14 ± 1b	200.0 ± 19.2b	17.4 ± 0.9b	−28.66 ± 0.09c	1085.0 ± 99.4a
Cream	11 ± 1c	162.0 ± 18.0c	16.6 ± 0.8b	−28.47 ± 0.11bc	835.8 ± 72.9b
F-statistics					
Location	53.86***	101.72***	37.10***	87.97***	98.43***
SCC	44.44***	108.47***	22.25***	6.36**	21.24 ***
SCC*Location	6.72***	2.28*	2.39*	1.31	20.72***

*Values (Mean ± SE) followed by dissimilar letters in a column are significantly different at *$p \leq 0.05$, **$P \leq 0.01$, ***$P < 0.001$.*

Seedcoat colour × location interaction was significant for nodule number, nodule fresh weight, shoot biomass and grain yield, but not shoot $\delta^{13}C$ (**Table 6**). Comparing the performance of each landrace across all five study sites revealed significant location effects. For example, with the Black landrace, nodule number was higher at Kpalisogu, followed by Gbalahi, and lowest at Googo (**Figure 1A**). With the Red and Cream landraces, nodule number was highest at Gbalahi, followed by Kpalisogu (**Figure 1A**). All three landraces recorded their highest nodule fresh weights at Savelugu, followed by Gbalahi, and their lowest at Googo and Kpalisogu (**Figure 1B**).

For all three landraces, shoot biomass was much higher at Googo and Kpalisogu, and the lowest for the Red and Cream at Manga (**Figure 1C**). Across all five study sites, grain yield of the Black landrace was highest at Kpalisogu, and lowest at Gbalahi (**Figure 1D**). With the Red and Cream landraces, grain yield was greater at Googo, followed by Savelugu, and lowest at Manga and Gbalahi (**Figure 1D**).

Effect of Seedcoat Colour × Environment Interaction on Symbiotic N Nutrition in Bambara Groundnut

The seedcoat colour × location interaction was significant for shoot N concentration and N content, $\delta^{15}N$, percent N derived from fixation, amount of N-fixed and soil N uptake (**Table 7**). A comparison of symbiotic performance of each landrace across the five study sites revealed significant location effects. For example, shoot %N of the Black and Red landraces was higher at Googo and Kpalisogu than the other three sites, while the Cream landrace showed higher shoot %N at Savelugu, and lowest at Gbalahi (**Figure 2A**). Shoot N content of the Black and Red landraces was similarly greater Googo and Kpalisogu, and much lower at Gbalahi and Manga, while the Cream landrace showed similar and higher N content at Googo, Kpalisogu, and Savelugu, and lower amounts at Gbalahi and Manga (**Figure 2B**). Shoot $\delta^{15}N$ was highest at Googo, followed by Kpalisogu, and lowest at Manga and Savelugu for all the three landraces (**Figure 2C**).

The percent N derived from fixation was highest for the Black landrace at Manga, followed by Gbalahi and Savelugu, and lowest at Googo (**Figure 2D**). In contrast, the Red landrace showed much higher percent N derived from fixation at Savelugu, followed by Manga, and lowest at Googo. In contrast was much , shoot percent N obtained from fixation by the Cream landrace was much greater at Gbalahi and Manga, followed by Savelugu, and lowest at Googo and Kpalisogu (**Figure 2D**). Across all study sites, the amounts of N-fixed were generally higher for the Black landrace, followed by Red and then the Cream landrace (**Figure 2E**). However, for site-to- site comparison, the Black

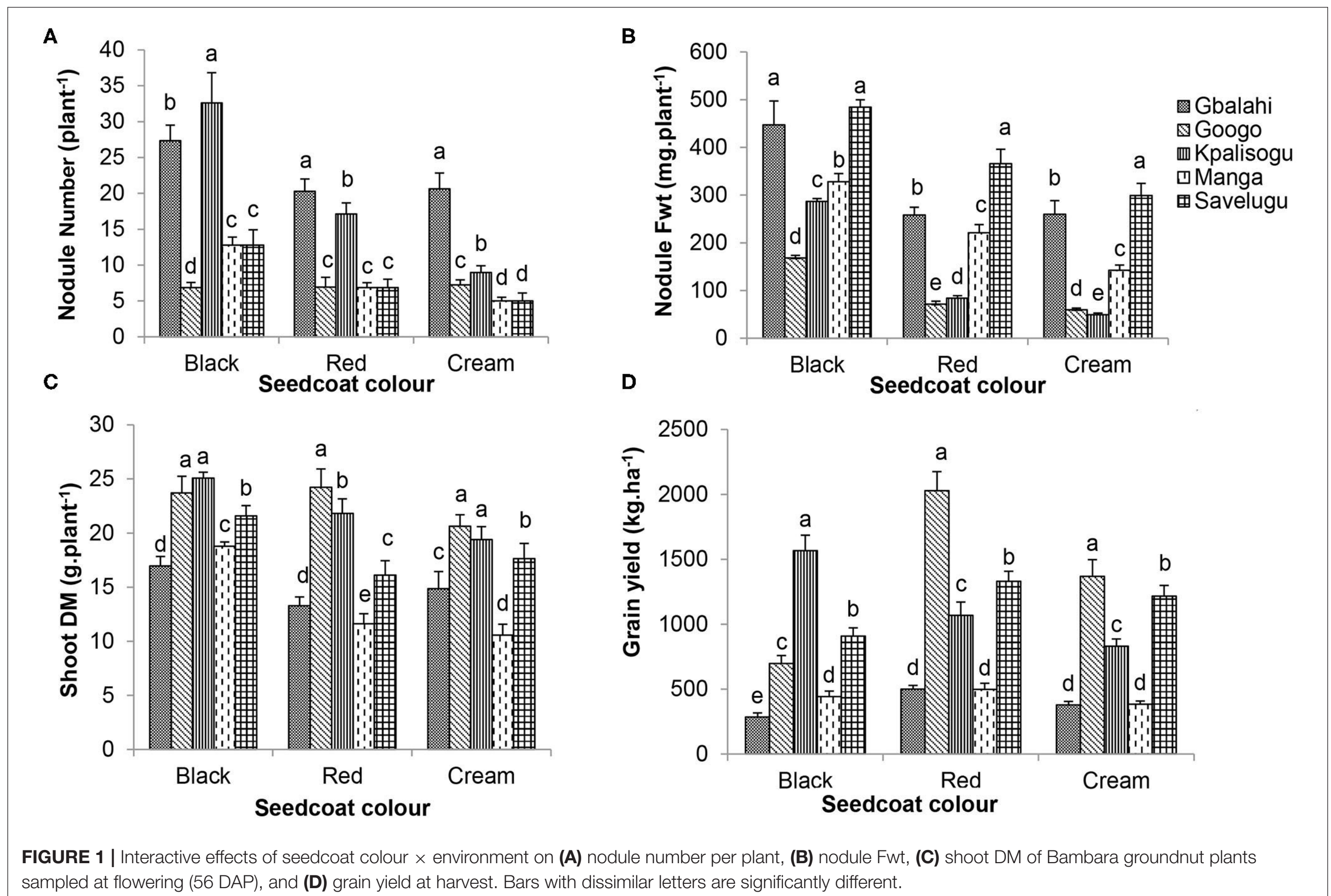


FIGURE 1 | Interactive effects of seedcoat colour × environment on **(A)** nodule number per plant, **(B)** nodule Fwt, **(C)** shoot DM of Bambara groundnut plants sampled at flowering (56 DAP), and **(D)** grain yield at harvest. Bars with dissimilar letters are significantly different.

TABLE 7 | Effects of seedcoat colour (SCC) and environment on the N nutrition of Bambara groundnut landrace sampled at flowering (56 DAP).

Treatment	Shoot N	N content	$\delta^{15}N$	Ndfa	N-fixed	Soil N uptake
	%	g.plant^{-1}	‰	%	kg.ha^{-1}	kg.ha^{-1}
Location						
Gbalahi	2.32 ± 0.08d	0.35 ± 0.02c	−0.10 ± 0.07c	74.7 ± 1.3c	52.5 ± 3.6b	18.0 ± 1.4c
Googo	2.94 ± 0.11ab	0.67 ± 0.04a	3.06 ± 0.12a	32.8 ± 1.8e	48.6 ± 3.3b	89.5 ± 4.6a
Kpalisogu	3.06 ± 0.12a	0.68 ± 0.04a	1.23 ± 0.06b	50.8 ± 1.2d	70.3 ± 4.8a	66.7 ± 3.8b
Manga	2.50 ± 0.07cd	0.34 ± 0.02c	−0.55 ± 0.03d	85.1 ± 0.5a	57.5 ± 3.6b	10.1 ± 0.7d
Savelugu	2.72 ± 0.07bc	0.50 ± 0.03b	−0.49 ± 0.07d	77.4 ± 1.7b	76.9 ± 3.8a	23.8 ± 2.4c
Seedcoat colour (SCC)						
Black	2.67 ± 0.08b	0.58 ± 0.03a	0.68 ± 0.23a	63.1 ± 3.3a	71.4 ± 3.1a	46.3 ± 5.3a
Red	2.89 ± 0.08a	0.52 ± 0.04a	0.58 ± 0.23a	65.4 ± 3.4a	60.2 ± 2.8b	43.5 ± 6.3a
Cream	2.56 ± 0.09c	0.43 ± 0.03b	0.62 ± 0.22a	63.9 ± 3.2a	51.8 ± 3.5c	35.0 ± 4.4b
F-statistics						
Location	14.68***	41.06***	701.66***	536.36***	13.26***	217.91***
SCC	7.52***	12.91***	1.20	2.62	14.78***	10.56***
SCC*Location	4.76***	3.65***	10.47***	16.73***	2.65**	6.95***

*Values (Mean ± SE) followed by dissimilar letters in a column are significantly different at *p ≤ 0.05, **P ≤ 0.01, ***P < 0.001.*

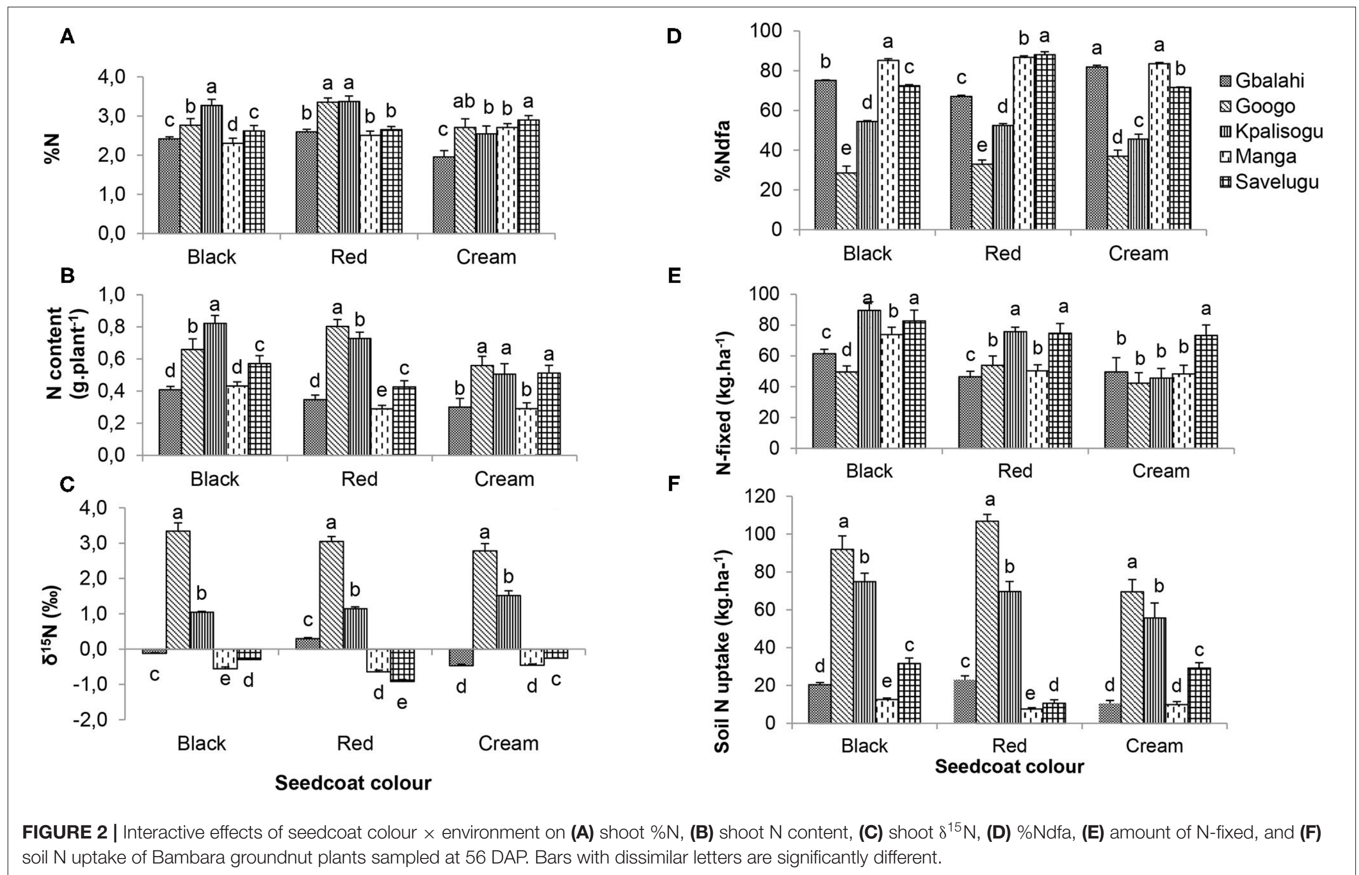


FIGURE 2 | Interactive effects of seedcoat colour × environment on **(A)** shoot %N, **(B)** shoot N content, **(C)** shoot $\delta^{15}N$, **(D)** %Ndfa, **(E)** amount of N-fixed, and **(F)** soil N uptake of Bambara groundnut plants sampled at 56 DAP. Bars with dissimilar letters are significantly different.

landrace recorded greater amounts of N-fixed at Kpalisogu and Savelugu, followed by Manga, and lowest at Googo and Gbalahi, which recorded much higher endogenous soil N concentrations that probably inhibited N_2 fixation (Mbah and Dakora, 2018). The Red landrace similarly exhibited higher amounts of N-fixed at Kpalisogu and Savelugu, and the lowest at Gbalahi. In contrast, the amount of N-fixed by the Cream landrace was much greater at Savelugu than the other four sites, which showed

similar N contribution (**Figure 2E**). Soil N uptake was highest at Googo (which had the highest soil N concentration), followed by Kpalisogu, and lowest at Manga for all three landraces (**Figure 2F**).

Correlation Analysis

There was an inverse relationship between nodule number and $\delta^{15}N$, between nodule fresh weight and $\delta^{15}N$, between nodule number and soil N uptake, as well as nodule fresh weight and soil N uptake (**Figures 3A–D**, respectively). Similarly, there was a significantly negative correlation between $\delta^{15}N$ and %Ndfa, as well as %Ndfa and soil N uptake (**Figures 4A,B**). However, shoot $\delta^{13}C$ (or water-use-efficiency) was positively correlated with shoot N content ($p = 0.824$), and also with soil N uptake ($p = 0.013$) (**Figures 4C,D**).

DISCUSSION

The Black Seedcoat of Grain Legumes Is a Natural Source of Nutraceuticals and Anthocyanins for Useful Exploitation

In this study, the three Bambara groundnut landraces tested differed significantly in their levels of flavonoids and anthocyanins in the grain, a finding consistent with the report by Wang et al. (2008) who also found variations in the concentration of phenolic compounds in the seeds of the same legume species. The phenolic concentrations were much greater in the seeds of Black and Red landraces than the lighter Cream (**Table 2**); these results were confirmed using UPLC-qTOF-MS analysis (Tsamo et al., 2018). Earlier studies also found higher levels of phenolic compounds in the darker-coloured pea (Troszyńska and Ciska, 2002), azuki bean and soybean with black seedcoat (Lin and Lai, 2006). Ndakidemi and Dakora (2003) similarly reported higher concentrations of anthocyanins and flavonoids in a black-seeded Bambara groundnut accession relative to its cream counterpart. Seedcoat pigmentation is reported to be a major determinant of the profile and concentrations of flavonoids, anthocyanins and anthocyanidins in cowpea, Bambara groundnut and Kersting's groundnut seeds (Tsamo et al., 2018, 2019, 2021). Based on metabolite profiling of cowpea seed coats, as well as Bambara groundnut and Kersting's bean seeds, Tsamo et al. (2020) found black seeds of legumes to be a natural source of nutraceuticals for human consumption, and a reservoir of anthocyanins that can be exploited for developing cosmetic, food and pharmaceutical products.

However, consumer preference for cowpea and Bambara groundnut grain consumption across West Africa (where the most cowpea is produced in Africa) is guided by seed coat pigmentation. The majority of consumers in West Africa prefer cowpea and Bambara groundnut with cream or white seedcoat colour (Ira et al., 2019), which is in contrast to Brazil where "black beans" are most preferred by consumers possibly because they contain the high levels of total phenolics, flavonoids, anthocyanins, anthocyanidins, and antioxidant activity when compared to their counterparts with cream or white seedcoat colour (Hungria et al., 1991; Tsamo et al., 2018, 2019, 2021). The consumption of these antioxidant compounds have implications for human nutrition/health (Jiang et al., 2016; Panche et al., 2016).

Effect of Seedcoat Colour on Plant Growth, Water-Use Efficiency and Grain Yield of Bambara Groundnut Landraces

Relative to the Red and Cream landraces, the Black Bambara groundnut landrace showed greater plant growth (measured as shoot dry matter) at all five study sites, though this was significant at only three locations. The variation in plant growth among the landraces could be linked to varying efficiencies in intercepting solar radiation for conversion to biomass (Toure et al., 2012), inherent genetic differences in photosynthate production, and/or greater resistance to environmental stress factors that limit crop productivity. Improved seedling vigour is also important for early crop establishment, and might be linked to seedcoat colour, as Mabhaudhi et al. (2013) found differences in seedling vigour among Bambara groundnut landraces with varying seedcoat pigmentation. In this study, site-specific comparison showed that the Red landrace consistently produced higher grain yield than the Black and Cream landraces. In Cameroon and Tanzania, red seedcoat pigmentation among Bambara groundnut landraces was associated with increased grain yield (Collinson et al., 2000; Wamba et al., 2012).

Bambara groundnut is generally regarded as a drought-tolerant legume species. Although, in this study, the ^{13}C isotopic analysis was used to measure water-use efficiency in the Bambara groundnut landraces (Mohale et al., 2013; Muhaba and Dakora, 2020), there was no direct relationship between seedcoat colour and water-use efficiency at individual experimental sites (**Table 4**). However, there were marked differences in shoot $\delta^{13}C$ or water-use efficiency among the test Bambara groundnut landraces across the study sites (**Table 6**). The Black landrace, for example, showed much greater $\delta^{13}C$ and therefore higher water-use efficiency than the Red and Cream landraces (**Table 6**). High tissue flavonoid concentration is known to protect the photosynthetic apparatus in plants under drought conditions (Nogués et al., 1998) and this probably explains the slightly higher shoot $\delta^{13}C$ values of the Black landrace. However, enhanced N nutrition also seems to induce greater water-use efficiency (Cabrera-Bosquet et al., 2007). Thus, the significant correlation found between shoot $\delta^{13}C$ and shoot N content, as well as shoot $\delta^{13}C$ and soil N uptake (**Figures 2C,D**) in this study suggest greater water-use efficiency in the Black landrace, which was superior in N_2 fixation relative to the Red and Cream landraces.

Effect of Seedcoat Pigmentation on Nodulation and Symbiotic N Nutrition of Bambara Groundnut Landraces

In this study, the Black Bambara groundnut landrace consistently showed greater nodule number and nodule fresh weight per plant at each site and across all sites, followed by the Red landrace (**Tables 4**, **6**). The Black and Red landraces also generally recorded lower shoot $\delta^{15}N$ values, derived a higher proportion of their N from fixation, and produced more

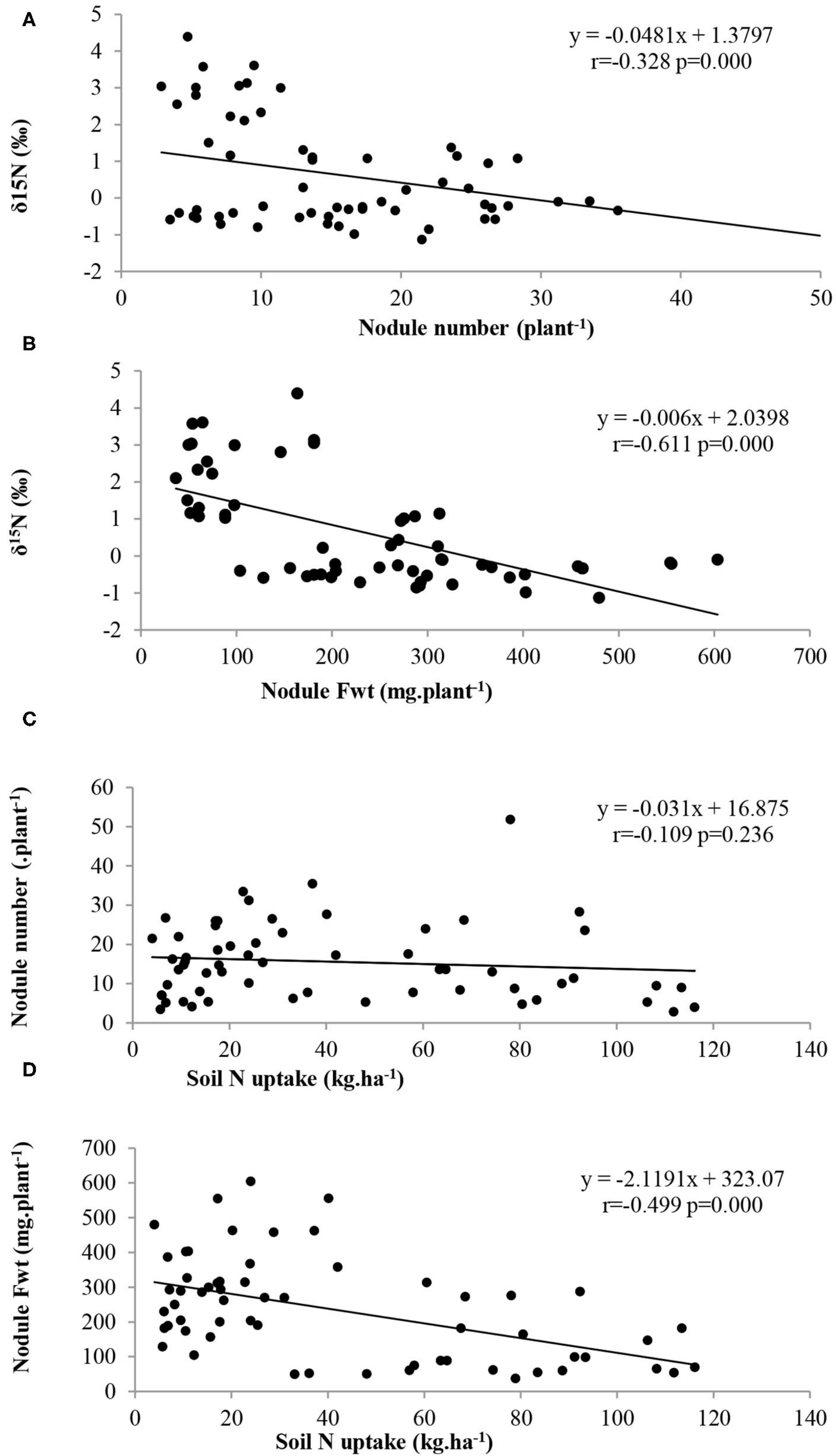


FIGURE 3 | Relationships between **(A)** δ^{15}N and nodule number, **(B)** δ^{15}N and nodule Fwt, **(C)** nodule number and soil N uptake, and **(D)** nodule fresh weight and soil N uptake of Bambara groundnut landraces sampled at flowering at 65 DAP.

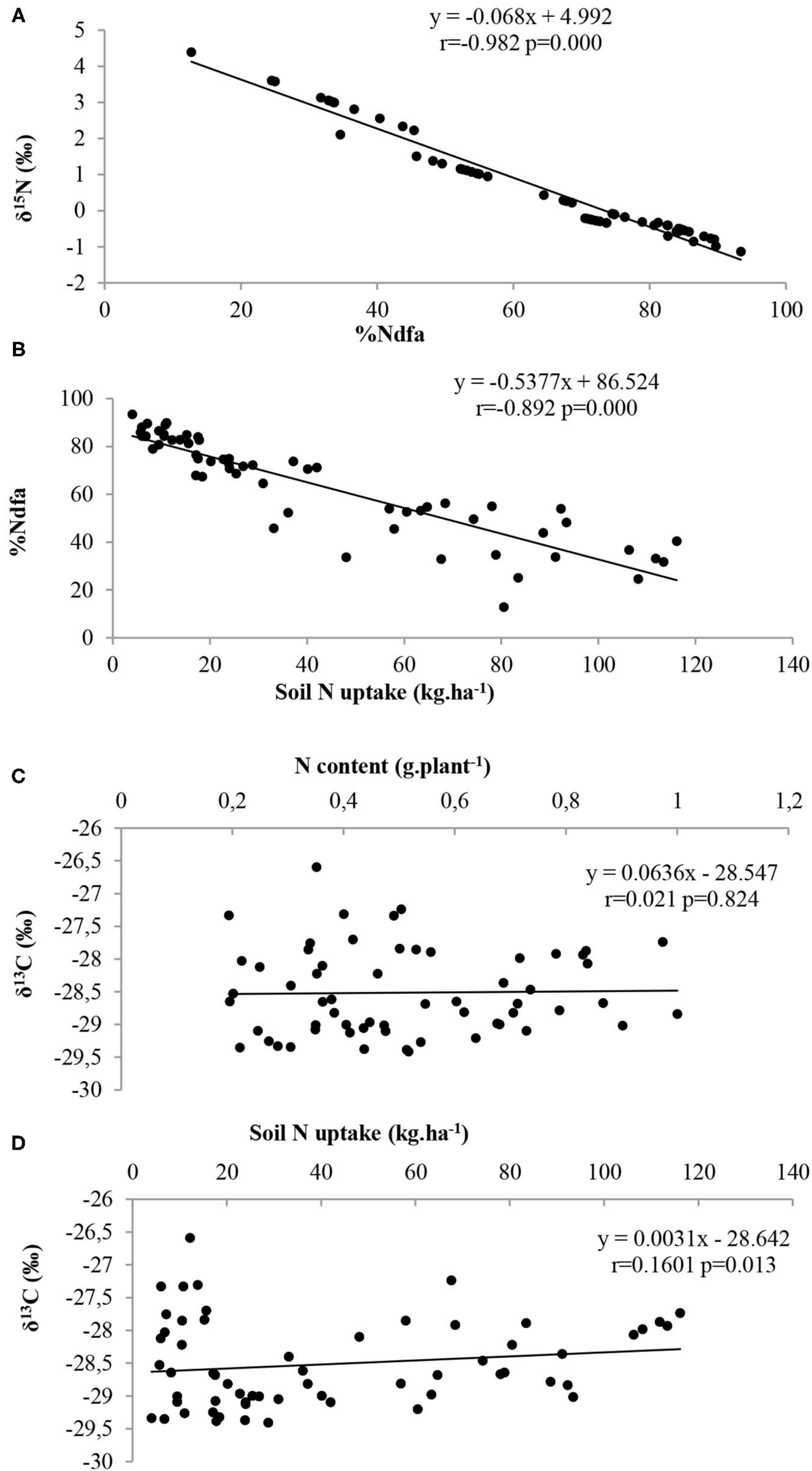


FIGURE 4 | Relationships between **(A)** %Ndfa from ureide-N and $\delta^{15}N$, **(B)** %Ndfa and soil N uptake, **(C)** $\delta^{13}C$ and shoot N content, and **(D)** $\delta^{13}C$ and soil N uptake **(D)** of Bambara groundnut landrace at flowering at 56 DAP.

symbiotic N. Anthocyanidins and their anthocyanin aglycones from black-seeded common bean have been reported to be potent inducers of nodulation genes in symbiotic rhizobia (Hungria et al., 1991). We therefore attribute the superior symbiotic performance of the Black Bambara groundnut landrace to its ability to produce and release higher concentrations of nod-gene-inducing anthocyanins and flavonoids than its Red and Cream counterparts (**Table 2**; Tsamo et al., 2018, 2019, 2020). This argument is supported by the findings of Hungria and Phillips (1993) who also showed that a black-seeded common bean genotype that released higher concentrations of *nod*-gene-inducing flavonoids elicited greater nodulation than its isogenic cream genotype with reduced biosynthesis of the flavonoid *nod*-gene inducers. Therefore, the markedly higher concentrations of anthocyanins and flavonoids found in the seedcoats of black cowpea and Bambara groundnut landraces (Tsamo et al., 2019, 2020) probably induce greater *nod*-gene expression in the microsymbionts of these species, leading to increased nodulation and N_2 fixation, as found in this study (**Tables 4**, **5**).

Although it can be argued that, besides seedcoat pigmentation, the observed differences in symbiotic functioning between the test Bambara groundnut landraces were probably due to their genetic makeup, the three landraces used in this study were similar in many traits except seedcoat colour. For example, they exhibited similar growth habit and phenology, recorded 50% flowering between 52 and 56 days after planting, and matured in 110 to 120 days. The seed colour trait, which differentiates them, must therefore be the dominant factor influencing gene expression in relation to N_2 fixation.

There were marked differences in nodulation and N_2 fixation of the test Bambara groundnut landraces within each location and across study sites. Variations in nodule formation and N_2 fixation have been reported for Bambara groundnut in a number of studies (Egbe et al., 2013; Mohale et al., 2013). Possibly, due to the high levels of organic matter, soil %C, Ca, Mg, Zn, and Fe, and the relatively high pH (which all play a role in the nodulation process), the plants sampled from the Gbalahi site in this study had the highest nodule number per plant, followed by Savelugu, and lowest at Manga and Googo. However, nodule mass was much greater at Savelugu when compared to the other sites (**Table 6**). Furthermore, relative to the other sites, the plants from Manga formed fewer nodules, but derived over 80% of their N nutrition from symbiosis, an indication that though fewer in population, those nodules were highly efficient in N_2 fixation due likely to the high concentration of plant-available P in the soil at Manga ($23\,mg.kg^{-1}$), which supported an increase in N_2 fixation.

Although the Bambara groundnut symbiosis with *Bradyrhizobium* strain CB 756 has been reported to be tolerant of mineral N in the rooting medium (Dakora, 1998), the relatively high endogenous soil N at Googo (0.08%) appears to have suppressed nodulation and nodule functioning in Bambara groundnut plants at that site (Saito et al., 2014; Mbah and Dakora, 2018), as evidenced by the low N derived from fixation. Even then, there was greater nodule number and nodule fresh weight per plant of the Black landrace than its Red and Cream counterparts at all the study sites. Despite its greater shoot biomass and superior symbiotic performance, the Black landrace produced the least grain yield, in contrast to other studies which found a significantly strong correlation between N_2 fixation and shoot biomass and/or grain yield (Belane et al., 2011; Mokgehle et al., 2014).

Because of the very high percent N derived from fixation at Manga, soil N uptake by all three landraces was lowest at that site, but much higher at Googo and Kpalisogu where N_2 fixation levels were relatively lower. Although the soil population of microsymbionts nodulating Bambara groundnut was small at Manga due to the fewer nodules produced, they were highly efficient in N_2 fixation, as evidenced by the lower shoot $\delta^{15}N$ values and the higher percent N derived from fixation.

In this study, N_2 fixation was measured using the ^{15}N natural abundance technique and analysis of ureides (allanton and allantoic acid) in the xylem stream and petiole extracts. As found with the ^{15}N methodology where shoot $\delta^{15}N$, percent N derived from fixation, and amounts of N-fixed differed significantly among the landraces, there were similarly marked variations in the ureide concentration of xylem sap and petiole extracts of the Bambara groundnut landraces (**Table 5**). A close scrutiny of the results revealed a consistent pattern in nodule function between ureide levels and shoot $\delta^{15}N$, as well as ureide levels and percent N derived from fixation at Gbalahi, Kpalisogu and Manga (**Table 5**). Where shoot $\delta^{15}N$ values were low and percent N from fixation was high, as found at Gbalahi, Kpalisogu, and Manga, the ureide concentrations in xylem sap and petiole extract were also high (**Table 5**). This clearly shows that the two techniques were quite robust in estimating N_2 fixation at a higher precision in the Bambara groundnut landraces (Unkovich et al., 2008). Where the trends among the landraces differed for the two techniques, this could possibly be attributed to the fact that, contrary to the ^{15}N isotopic discrimination method which is cumulative over the life span of the plant, the ureide technique is an instantaneous measure that is easily influenced by environmental factors such as temperature and water, which could have affected transpiration rates at the time of sampling (Unkovich et al., 2008).

In conclusion, the darker seeds of Bambara groundnut landrace was found to contain higher levels of flavonoids, anthocyanin, anthocyanidins, and anti-oxidant activity relative to the Cream landrace. Seedlings from the black-seeded Bambara groundnut landrace also exhibited better plant growth, increased water-use efficiency, higher root nodulation and greater N contribution under field conditions. Black seedcoat pigmentation in Bambara groundnut is therefore a biomarker for increased nodulation and N_2 fixation that can be tapped in breeding programs for developing high N_2-fixing, water-use efficient grain legumes for use by farmers in Africa.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DP collected samples and analysed the data. DP and SJ drafted the manuscript. FD conceived the idea, edited, and approved the final version of the paper. All authors contributed to the article and approved the submitted version.

FUNDING

Bill and Melinda Gates Foundation Project (OPP1028057) on Capacity Building in legume Sciences in Africa.

ACKNOWLEDGMENTS

This work was supported with grants from the Bill and Melinda Gates Foundation Project on Capacity Building in Legume Sciences in Africa, the South African Department of Science and Technology, the Tshwane University of Technology, the National Research Foundation in Pretoria, and the South African Research Chair in Agrochemurgy and Plant Symbioses. DP is grateful for a competitive doctoral fellowship from the Bill and Melinda Gates Foundation Project on Capacity Building in Legume Sciences in Africa.

REFERENCES

Belane, A. K., Asiwe, J. A. N., and Dakora, F. D. (2011). Assessment of N_2 fixation in 32nodulated field-grown cowpea (*Vigna unguiculata* L. Walp) genotypes at Taung in South Africa, using ^{15}N natural abundance. *Afr. J. Biotech.* 10, 11450–11458. doi: 10.5897/AJB11.674

Belane, A. K., and Dakora, F. D. (2010). Symbiotic N_2 fixation in 30 field-grown cowpea (*Vigna unguiculata* L. Walp.) genotypes in the Upper West Region of Ghana, measured using ^{15}N natural abundance. *Biol. Fertil. Soil* 46, 191–198. doi: 10.1007/s00374-009-0415-6

Cabrera-Bosquet, L., Molero, G., Bort, J., Nogués, S., and Araus, J. L. (2007). The combined effect of constant water deficit and nitrogen supply on WUE, NUE and Δ ^{13}C in durum wheat potted plants. *Ann. Appl. Biol.* 151, 277–289. doi: 10.1111/j.1744-7348.2007.00195.x

Collinson, S. T., Sibuga, K. P., Tarimo, A. J. P., and Azam-Ali, S. N. (2000). Influence of sowing date on the growth and yield of Bambara groundnut landraces in Tanzania. *Exp. Agric.* 36, 1–13. doi: 10.1017/S0014479700361038

Dakora, F. D. (1998). Nodule function in symbiotic Bambara groundnut (*Vigna subterranea* L) and Kerstings bean (*Macrotyloma geocarpum* L.) is tolerant of nitrate in the root medium. *Ann. Bot.* 82, 687–690. doi: 10.1006/anbo.1998.0720

Egbe, M. O., Alhassan, G. A., and Ijoyah, M. (2013). Nodulation, nitrogen yield and fixation by Bambara groundnut (Vigna subterranea L. *Verdc.)* landraces intercropped with cowpea and maize in Southern Guinea savanna of Nigeria. *Agric. Sci.* 1, 15–28. doi: 10.12735/as.v1i4p15

Gueye, M., and Bordeleau, L. M. (1988). Nitrogen fixation in Bambara groundnut (*Voandzea subterranea* L..Thouars). *Mircen. J.* 4, 365–375. doi: 10.1007/BF01096142

Heller, J., Begemann, F., and Mushonga, J. (1997). Bambara groundnut, *Vigna subterranea* (L.) Verdc. *Prom. Conserv. Underutiliz. Neglect.* 9:166. ISBN: 978-92-9043-299-9, ISBN: 92-9043-299-3

Hungria, M, Joseph, C. M., and Phillips, D. A. (1991). Anthocyanindins and flavonols, major *nod*-gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). *Plant Physiol.* 97, 751–758. doi: 10.1104/pp.97.2.751

Hungria, M., and Phillips, D. (1993). Effects of a seed colour mutation on rhizobial *nod*-gene-inducing flavonoids and nodulation in common bean. *Mol. Plant-Microbe Interact.* 6, 418–422. doi: 10.1094/MPMI-6-418

Ira, A., Zhenyu, J., and Francis, K. (2019). Market preferences for cowpea (*Vigna unguiculata* L. *Walp.)* dry grain in Ghana. *Afr. J. Agric. Res.* 14, 928–934. doi: 10.5897/AJAR2019.13997

Jiang, N., Doseff, A. I., and Grotewold, E. (2016). Flavones: from biosybnthesis to health benefits. *Plants* 5:27. 5020027doi: 10.3390/plants5020027

Lin, P. Y., and Lai, H. M. (2006). Bioactive compounds in legumes and their germinated products. *J. Agric. Food Chem.* 54, 3807–3814. doi: 10.1021/jf060002o

Mabhaudhi, M., Modi, A. T., and Beletse, Y. G. (2013). Growth, phenological nd yield responses of a Bambara groundnut (*Vigna subterranea* L. Verdc.) landrace to imposed water stress: II: Rainfall shelter conditions. *Water SA* 39:22. Doi.org/10.4314/wsa.v39i2.2. doi: 10.4314/wsa.v39i2.2

Makoi, J. H. J. R., Belane, A. K., Chimphango, S. B. M., and Dakora, F. D. (2010). Seed flavonoids and anthocyanins as markers of enhanced plant defence in nodulated cowpea (*Vigna unguiculata* L. *Walp.) Field Crop. Res.* 118, 21–27. doi: 10.1016/j.fcr.2010.03.012

Mbah, G. C., and Dakora, F. D. (2018). Nitrate inhibition of N_2 fixation and its effect on micronutrient accumulation in shoots of soybean (*Glycine max* L. *Merr.),* Bambara groundnut *(Vigna subterranea L. Vedc.)* and Kersting's groundnut *(Macrotyloma geocarpum Harms.). Symbiosis* 75, 205–216. doi: 10.1007/s13199-017-0531-2

Mohale, K. C., Belane, A. K., and Dakora, F. D. (2013). Symbiotic N nutrition, C assimilation, and plant water-use efficiency in Bambara groundnut (*Vigna subterranea* L. Verdc) grown in farmers' fields in South Africa, measured using ^{15}N and ^{13}C natural abundance. *Biol. Fertil. Soil* 50, 307–319. doi: 10.1007/s00374-013-0841-3

Moise, A. J., Han, S., Loreta Gudynaite-Savitch, L., Johnson, D. A., and Miki, B. L. A. (2005). Seed coats: structure, development, composition, and biotechnology. *In Vitro Cell. Develop. Biol. Plant* 41, 620–644. doi: 10.1079/IVP2005686

Mokgehle, S. N., Dakora, F. D., and Mathews, C. (2014). Variation in N_2 fixation and N contribution by 25 groundnut (*Arachis hypogaea* L.) varieties grown in different agro-ecologies, measured using ^{15}N natural abundance. *Agric. Ecosys. Environ.* 195, 161–172. doi: 10.1016/j.agee.2014.05.014

Muhaba, S. K., and Dakora, F. D. (2020). Symbiotic performance, shoot biomass and water-use efficiency of three groundnut (*Arachis hypogea* L.) genotypes in response to phosphorus supply under field conditions in Ethiopia. *Front. Agric. Sci. Eng.* 7, 455–466. doi: 10.15302/J-FASE-2020354

Ndakidemi, P. A., and Dakora, F. D. (2003). Legume seed flavonoids and nitrogenous metabolites as signals and protectants in early seedling development. *Funct. Plant Biol.* 30, 729–745. doi: 10.1071/FP03042

Nogués, S., Allen, D. J., Morison, I. L., and Baker, N. R. (1998). Ultraviolet-B radiation effects on water relations, leaf development, and photosynthesis in droughted pea plants. *Plant. Physiol.* 117, 173–81. doi: 10.1104/pp.117.1.173

Nyemba, R. C., and Dakora, F. D. (2005). Identifying suitable arable weeds as reference plants for measuring N_2 fixation in grain legumes in Zambia using ^{15}N natural abundance. *Symbiosis* 40, 79–86.

Panche, A. N., Diwan, A. D., and Chandra, S. R. (2016). Flavonoids: an overview. *J. Nutr. Sci.* 5, 1–15. doi: 10.1017/jns.2016.41

Pule-Meulenberg, F., and Dakora, F. D. (2009). Assessing the symbiotic dependency of grain and tree legumes on N_2 fixation for their N nutrition in five agro-ecological zones of Botswana. *Symbiosis* 48, 68–77. doi: 10.1007/BF03179986

Saito, A., Tanabata, S., Tanabata, T., Tajima, S., Ueno, M., Ishikawa, S., et al. (2014). Effect of nitrate on nodule and root growth of soybean (*Glycine max* (L.) Merr.). *Internat. J. Mol. Sci.* 15, 4464–4480. doi: 10.3390/ijms15034464

Subramanian, S., Stacey, G., and Yu, O. (2007). Distinct crucial roles of flavonoids during legume nodulation. *Trend Plant Sci.* 12, 282–285. doi: 10.1016/j.tplants.2007.06.006

Toure, Y., Kone, M., Tanoh, H. K., and Kone, D. (2012). Agromorphological and phenological variability of 10 Bambara groundnut [*Vigna subterranea* L. Verdc. (Fabaceae)] landraces cultivated in the Ivory Coast. *Tropicultura* 30, 216–221.

Troszyńska, A., and Ciska, E. (2002). Phenolic compounds of seedcoats of white and coloured varieties of pea (*Pisum sativum* L.) and their total antioxidant activity. *Czech J. Food Sci.* 20, 15–22. doi: 10.17221/3504-CJFS

Tsamo, A. T., Mohammed, H., Mohammed, M., Ndibewu, P. P., and Dakora, F. D. (2019). Seed coat metabolite profiling of cowpea (*Vigna unguiculata* L. *Walp.)* accessions from Ghana using UPLC-PDA-QTOF-MS and chemometrics. *Nat. Prod. Res.* 28, 808–811. doi: 10.1080/14786419.2018.1548463

Tsamo, A. T., Mohammed, M., and Dakora, F. D. (2020). Metabolite fingerprinting of Kersting's groundnut [*Macrotyloma geocarpum* (Harms) Maréchal and Baudet] seeds using UPLC-qTOF-MS reveals the nutraceutical and antioxidant potentials of the orphan legume. *Front. Nutr.* 7:306. doi: 10.3389/fnut.2020.593436

Tsamo, A. T., Mohammed, M., and Ndibewu P. P., and Dakora, F. D. (Jaiswal SK and Dakora FD (2021)). Identification and quantification of anthocyanins in seeds of Kersting's groundnut [*Macrotyloma geocarpum* (Harms) Marechal and Baudet] landraces of varying seed coat pigmentation. *J. Food Measur. Charact.* 13, 2310–2317. doi: 10.1007/s11694-019-00150-3

Tsamo, A. T., Ndibewu, P. P., and Dakora, F. D. (2018). Phytochemical profile of seeds from 21 Bambara groundnut landraces *via* UPLC-qTOF-MS. *Food Res. Internat.* 112, 160–168. doi: 10.1016/j.foodres.2018.06.028

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, R., Giller, K., et al. (2008). *Measuring plant-associated nitrogen fixation in agricultural systems (ACIAR Mono)*. Canberra: Australian Centre for International Agricultural Research (ACIAR).

Wamba, O., Taffouo, V., Youmbi, E., Ngwene, B., and Amougou, A. (2012). Effects of organic and inorganic nutrient sources on growth, total chlorophyll and yield of three Bambara groundnut landraces in the Coastal Region of Cameroom. *J. Agron.*, 11, 31–42. doi: 10.3923/ja.2012.31.42

Wang, M. L., Gillaspie, A. G., Morris, J. B., Pittman, R. N., Davis, J., and Pederson, G. A. (2008). Flavonoid content in different legume germplasm seeds quantified by HPLC. *Plant Gen Resourc* 6: 62–69. doi: 10.1017/S1479262108923807

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Puozaa, Jaiswal and Dakora. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Plant Science

REVIEW
published: 19 August 2021
doi: 10.3389/fpls.2021.690567

Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the *Rhizobium*-Legume Symbioses

Marcela Mendoza-Suárez[1†], Stig U. Andersen[1†], Philip S. Poole[2†] and Carmen Sánchez-Cañizares[2†]*

[1] Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark, [2] Department of Plant Sciences, University of Oxford, Oxford, United Kingdom

OPEN ACCESS

Edited by:
Juan Sanjuan,
Estación Experimental de Zaidín,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain

Reviewed by:
Monika Janczarek,
Marie Curie-Skłodowska University,
Poland
Antonio Lagares,
Universidad Nacional de La Plata,
Argentina
Anibal Roberto Lodeiro,
CONICET Instituto de Biotecnologia y
Biologia Molecular (IBBM), Argentina

***Correspondence:**
Carmen Sánchez-Cañizares
carmen.sanchez-canizares@plants.ox.ac.uk

†ORCID:
Marcela Mendoza-Suárez
orcid.org/0000-0003-3419-1362
Stig U. Andersen
orcid.org/0000-0002-1096-1468
Philip S. Poole
orcid.org/0000-0001-5087-6455
Carmen Sánchez-Cañizares
orcid.org/0000-0003-4620-5274

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

Received: 03 April 2021
Accepted: 19 July 2021
Published: 19 August 2021

Citation:
Mendoza-Suárez M,
Andersen SU, Poole PS and
Sánchez-Cañizares C (2021)
Competition, Nodule Occupancy,
and Persistence of Inoculant Strains:
Key Factors in the Rhizobium-Legume
Symbioses.
Front. Plant Sci. 12:690567.
doi: 10.3389/fpls.2021.690567

Biological nitrogen fixation by *Rhizobium*-legume symbioses represents an environmentally friendly and inexpensive alternative to the use of chemical nitrogen fertilizers in legume crops. Rhizobial inoculants, applied frequently as biofertilizers, play an important role in sustainable agriculture. However, inoculants often fail to compete for nodule occupancy against native rhizobia with inferior nitrogen-fixing abilities, resulting in low yields. Strains with excellent performance under controlled conditions are typically selected as inoculants, but the rates of nodule occupancy compared to native strains are rarely investigated. Lack of persistence in the field after agricultural cycles, usually due to the transfer of symbiotic genes from the inoculant strain to naturalized populations, also limits the suitability of commercial inoculants. When rhizobial inoculants are based on native strains with a high nitrogen fixation ability, they often have superior performance in the field due to their genetic adaptations to the local environment. Therefore, knowledge from laboratory studies assessing competition and understanding how diverse strains of rhizobia behave, together with assays done under field conditions, may allow us to exploit the effectiveness of native populations selected as elite strains and to breed specific host cultivar-rhizobial strain combinations. Here, we review current knowledge at the molecular level on competition for nodulation and the advances in molecular tools for assessing competitiveness. We then describe ongoing approaches for inoculant development based on native strains and emphasize future perspectives and applications using a multidisciplinary approach to ensure optimal performance of both symbiotic partners.

Keywords: bioinoculants, biofertilizers, competition, rhizobia, sustainable agriculture, symbiosis, biological nitrogen fixation, legume

INTRODUCTION

Biological nitrogen fixation (BNF) is an important source of nitrogen, and the various legume crops and pasture species often fix as much as 200 to 300 kg of nitrogen per hectare per year (Peoples et al., 1995). Altogether, the legume-*Rhizobium* symbioses contribute the equivalent of approximately a quarter of the nitrogen applied to arable land annually as chemical fertilizers

(Herridge et al., 2008). The use of legumes in rotations also offers control of crop diseases and pests (Graham and Vance, 2000). The benefits of the symbioses between legumes and nitrogen-fixing bacteria are crucial in farming systems worldwide. Rhizobia are ubiquitous in soil but show great variation in their number and composition of natural populations depending on properties of the soils (Brockwell et al., 1995; Hirsch, 1996; Vuong et al., 2017). Their ability to form nodules in the presence of other strains determines their nodulation competitiveness (referred to here as rhizobial competitiveness; Yates et al., 2011; Onishchuk et al., 2017). While nitrogen fixation in an inoculated pasture is assumed to be due to the strain used in the inoculant, the identity of the strains occupying the nodules is generally unknown (Irisarri et al., 2019). Several rhizobia strains can inhabit nodules within the same host plant, even co-inhabiting the same nodule (Mendoza-Suárez et al., 2020), and compete for host resources with non-fixing ("cheating") strains (Checcucci et al., 2017). However, it has been shown that plants can sanction nodules that are inefficient at fixing nitrogen (Kiers et al., 2003; Regus et al., 2017; Westhoek et al., 2021). Moreover, legumes are able to control the number of nodules formed through an "autoregulation" process in which a shoot-derived signal limits infection (Kinkema et al., 2006; Ferguson et al., 2010; Reid et al., 2011; Mortier et al., 2012). The phytohormone ethylene also has an inhibitory effect on nodulation in most legumes (Penmetsa and Cook, 1997; Penmetsa et al., 2003; Lin et al., 2020), although rhizobia can influence these mechanisms by altering ethylene levels via the production of a rhizobiotoxine which inhibits ethylene biosynthesis of host roots (Ma et al., 2002; Sugawara et al., 2006).

Rhizobial competitiveness has important practical implications for agriculture, as differences in nitrogen fixation efficiency between strains can be large (Slattery and Pearce, 2002; Irisarri et al., 2019). Elite rhizobial inoculants must be highly effective in providing the plant with fixed nitrogen (N_2-effectiveness) and, at the same time, be highly competitive for nodule occupancy (competitiveness) in a background of native rhizobia that may show high competitiveness combined with low N_2-effectiveness (Checcucci et al., 2017; Onishchuk et al., 2017).

Microbial interactions in agriculture are part of a multicomponent equation that includes (i) plant genotype, (ii) environment, and (iii) plant and soil microbiomes. These factors should be taken into account when evaluating the success of beneficial microbes (Sessitsch et al., 2002; Busby et al., 2017; Onishchuk et al., 2017; diCenzo et al., 2019). Nodule formation, and therefore rhizobial competitiveness, is affected by soil type and its physicochemical properties (i.e., temperature, pH, and moisture; Hungria and Franco, 1993; Frey and Blum, 1994; Anyango et al., 1995; Montañez et al., 1995; Zahran, 1999; Rao et al., 2002; Rathi et al., 2018), nutrient availability and the ability of microbes to use them (Rynne et al., 1994; Kyei-Boahen et al., 2017; Kasper et al., 2019), the population of native rhizobia and the remaining soil microbiome (Meade et al., 1985; Siefert et al., 2018; Han et al., 2020), previous inoculation history (Laguerre et al., 2003; Batista et al., 2015), and/or the choice of inoculation method (Danso and Bowen, 1989; López-García et al., 2009). The degree to which the rhizobial strains adapt to the local soil conditions will strongly influence the competition between strains.

But how is this endosymbiotic relationship with legumes established? Nitrogen-fixing rhizobia have complex life cycles (Poole et al., 2018). Rhizobia are found as free-living bacteria in soil and in the rhizosphere, which are highly heterogeneous environments in both space and time. In brief, the symbiosis is initiated in the rhizosphere following an exchange of signals between both partners (Oldroyd and Downie, 2004; Oldroyd et al., 2011; Udvardi and Poole, 2013; **Figure 1A**). Root exudates released into the rhizosphere are also part of this chemical dialogue (Badri and Vivanco, 2009), being specific for the plant genotype (Monchgesang et al., 2016) and changing during the life cycle of the plant, the root zone, the rhizosphere microbial community and with time (Zhalnina et al., 2018; Canarini et al., 2019; Korenblum et al., 2020). In nature, host legumes are surrounded by other plants, and other roots or mesofauna may also exude chemoattractants, adding noise to the host root exudate (De-la-Peña et al., 2008). Therefore, bacterial attraction and initial root colonization occurs at multiple stages that are defined by spatial and temporal separation (Massalha et al., 2017; Poole, 2017). Indeed, a microfluidics-based approach combined with advanced microscopy showed that the first step in colonization seems to involve newly divided and undifferentiated cells in the nutrient-rich microenvironment of the root elongation zone (Massalha et al., 2017). Bacterial accumulation at this site occurs prior to the attachment to differentiated root-hair cells (Massalha et al., 2017; Poole, 2017). They usually adhere to roots using adsorption, root attachment being affected by soil and root physicochemical properties, such as pH, Ca^{2+} and Mg^{2+} concentrations and water availability (Rodríguez-Navarro et al., 2007).

The infection process starts when rhizobia enter root systems through natural cracks between epidermal cells at the base of emerging lateral roots (termed "crack entry"), or, more commonly, when compatible rhizobia induce curling and deformation of growing root hairs around the bacterial cells that subsequently enter through an intracellular infection thread (IT, **Figure 1A**; Rae et al., 2021). Crack entry infection is considered more primitive than ITs because the host does not experience sophisticated cellular differentiation of root hairs (Sprent, 2008; Gage, 2019), although some species of plants (e.g., *Lotus japonicus*) can alter their mode of infection depending on the site of infection (Montiel et al., 2021). Only the emerging root hairs are infectable, with polar root hair growth required to achieve the necessary root hair deformation and cell wall invagination to form an IT (Turgeon and Bauer, 1985; Esseling et al., 2003). Hence, this process is a transient phenomenon, as root hairs remain infectable for only a few hours in a given root region. Whereas the transiency of root hair infectability highlights the relevance of the reversible and generally polar attachment to emerging root hairs, the irreversible attachment and biofilm formation seems relevant for rhizosphere and soil colonization, although not for infection and nodulation, as it would be completed only after the root hairs are no longer infectable. IT initiation and elongation continues through cortical cell layers until rhizobia are released into dividing

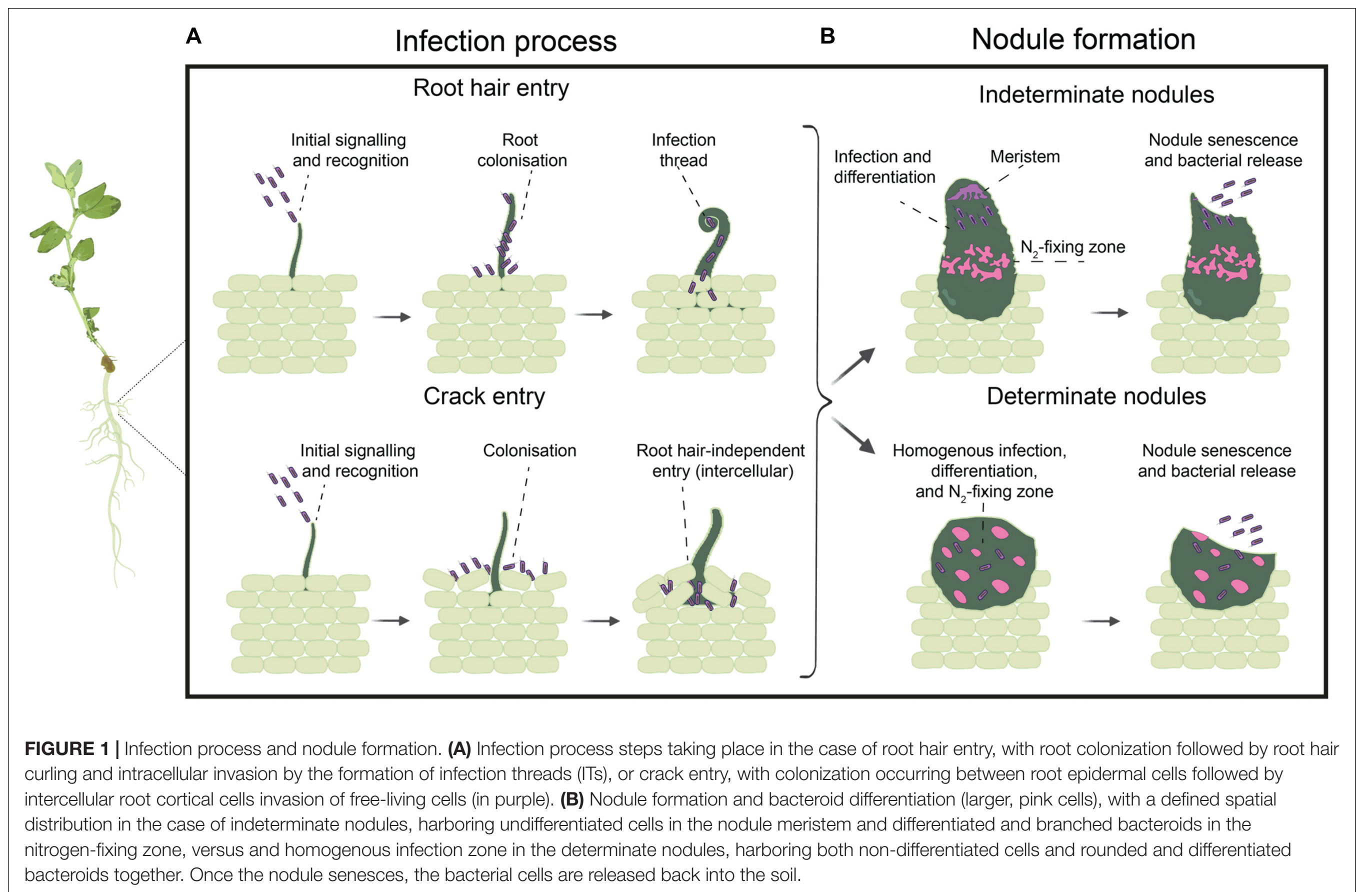


FIGURE 1 | Infection process and nodule formation. **(A)** Infection process steps taking place in the case of root hair entry, with root colonization followed by root hair curling and intracellular invasion by the formation of infection threads (ITs), or crack entry, with colonization occurring between root epidermal cells followed by intercellular root cortical cells invasion of free-living cells (in purple). **(B)** Nodule formation and bacteroid differentiation (larger, pink cells), with a defined spatial distribution in the case of indeterminate nodules, harboring undifferentiated cells in the nodule meristem and differentiated and branched bacteroids in the nitrogen-fixing zone, versus and homogenous infection zone in the determinate nodules, harboring both non-differentiated cells and rounded and differentiated bacteroids together. Once the nodule senesces, the bacterial cells are released back into the soil.

cortical cells. They are taken up into the plant cytosol and surrounded by a plant-derived membrane (**Figure 1B**). The rhizobial cells with their plant surrounding membrane are known as symbiosomes, which are temporary plant organelles where nitrogen fixation takes place (Gage, 2019; Pérez-Giménez et al., 2021). In the symbiosomes, rhizobia adjust their metabolism in response to the stress conditions, differentiating into bacteroids, after which the nitrogenase complex is expressed, leading to active N_2-fixation (Poole et al., 2018; Ledermann et al., 2021). There is no general consensus on the morphological types of legume nodules and their internal structure. Although some authors use a classification system with up to five nodule types – aeschynomenoid, desmodioid, indeterminate, lupinoid, and primitive (Sprent, 2008) – the major types of nodule shape and structure are determinate and indeterminate nodules (**Figure 1B**). Determinate nodules are spherical, lacking a persistent meristem and therefore cease growing at some point, whereas indeterminate nodules are elongated, with a persistent meristem, resulting in indefinite growth and branching in irregular shapes (Ferguson et al., 2010). These two patterns of nodule development differ also in bacteroid differentiation, with determinate nodules showing a more synchronic, time-dependent and homogeneous maturation of most rhizobia within the nodules, in contrast to indeterminate nodules, in which rhizobia at different stages of differentiation are observed within the same nodule, with bacteroid maturation depending on the different nodule spatial zones (Ferguson et al., 2010; Poole et al., 2018). Bacteroids in determinate nodules are viable and retain their morphology and their capacity to divide and revert to free-living cells, whereas in indeterminate nodules, only a small fraction of rhizobial cells in the saprophytic zone of nodules remains in a viable and vegetative form, being able to multiply in the infection threads and regrow outside the nodule, increasing the bacterial population in the soil after nodule senescence (Mergaert et al., 2006; Montiel et al., 2017). When the nodule senesces, the saprophytic zone that is formed is where rhizobia are nourished by the products of organic breakdown and decay and multiply massively (Timmers et al., 2000; Wielbo et al., 2010). What we do not yet understand is which phases in the nodulation process are exposed to competition.

The improvement of symbiotic nitrogen fixation is one of the main challenges facing agricultural research. It is therefore necessary to evaluate nodule occupancy in order to assess the competitiveness of a strain. Although competition assays must include field assessments that confirm the results obtained from experiments under controlled conditions, there are limitations when they are performed at a large scale. Here, we will review the molecular factors that can influence rhizobial competitiveness and how the development of molecular tools is simplifying this task and we will discuss the implications of rhizobial competitiveness for the establishment of a successful symbiosis and for the search for elite rhizobial strains. We will also

emphasize the importance of performing N_2-effectiveness and competitiveness assays using relevant field soils and plant genotypes. Although crop management and environmental factors (for example: soil pH, soil temperature, salinity, moisture, soil texture, among others) are not discussed in this review, these factors can also influence rhizobial competitiveness and, therefore, future inoculant performance (reviewed in detail by Vlassak and Vanderleyden, 1997; Saad et al., 2020).

TYPES OF COMPETITION AND GENETIC FEATURES INFLUENCING COMPETITIVENESS

Competition for nodulation is a key adaptive feature of rhizobia that is of great importance in the practical application of inoculants. However, the genetic basis of competitiveness for nodule formation is not fully understood yet. What we know is that competitiveness, similarly to nitrogen fixation, is controlled by specific genes that are expressed during different time points in nodule development (Ampe et al., 2003; Barnett et al., 2004). In addition, rhizobia are part of the plant microbiome community and, as such, they have to interact with the non-rhizobial accompanying members of this dynamic community in order to undertake a range of beneficial functions including growth promotion, nutrient acquisition, pathogen resistance or stress tolerance, thus, exploiting the versatile benefits offered by plant-microbe interactions (as reviewed by Compant et al., 2019; Trivedi et al., 2020). The assemblage of the plant microbiome community is currently the focus of active investigation, as different components have been shown to have a crucial impact in the assembly process, such as the soil and seed-born initial microbial repertoires, both accounting for key microbe-microbe interactions within this microbial consortium (Philippot et al., 2013; Turner et al., 2013; Zgadzaj et al., 2016; Nelson, 2018). Indeed, Plant Growth-Promoting Rhizobacteria (PGPR) have been found to be enriched in the soybean rhizosphere, reinforcing the idea that rhizosphere recruitment is an important first step in symbiotic interactions (Liu et al., 2019). Hence, elite inoculant behaviors appear to be a consequence of gene assortment, with genes involved in an efficient symbiosis with their plant hosts, PGPR traits, or secretion systems (Pastor-Bueis et al., 2019). Onishchuk et al. (2017) identified two types of competition influenced by different factors: exploitative (indirect) competition, involving a more effective use of a common limiting nutrient, or interference (direct) competition, whereby other cells are prevented from growing and surviving in the environment. Hence, to be both competitive and N_2-effective, an elite strain should have genetic components that enable it to (i) successfully colonize the rhizosphere and benefit from available nutrients in an effective way, (ii) prevent the growth of other bacterial cells, (iii) establish an efficient symbiosis, and (iv) promote plant growth. Here, we will review some of the components that are relevant to the four points mentioned above and summarized in **Table 1**. The genetic knowledge on the pre-infection, infection and nodulation stages has mainly been gained from experiments with defective mutants and, more recently, with high-throughput technologies. Hence, although these data come from laboratory experiments, such traits may also be present in the soil rhizobia populations. Manipulating the expression of these traits, either genetically or through culture conditions, may lead to improvements in the ability of rhizobial strains to compete against endogenous soil populations.

Genetic Components for Rhizosphere Colonization

When it comes to exploitative competition, where bacteria compete for the same common resources without directly interacting, bacterial chemotaxis toward exuded compounds is an important trait for root colonization and plant-driven selection of microorganisms (Bais et al., 2006; Raina et al., 2019). Motility and chemotaxis are factors affecting nodulation efficiency and competitiveness (Mellor et al., 1987; Caetano-Anollés et al., 1988; Bauer and Caetano-Anollés, 1990). Disruption of the flagellum hook gene *flgE* in *Mesorhizobium tianshanense* caused a flagellar-less phenotype, leading to the complete loss of swimming ability, a heavier biofilm formation and decreased bacterial attachment on the root hair. These *in vitro* assays suggest that flagella are involved in the early stage of the symbiosis process (Zheng et al., 2015). The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae,* Che1, has been shown to be essential for competitive nodulation (Miller et al., 2007; Wheatley et al., 2020). It has to be noted that these observations have been mainly made from laboratory experiments carried out either in liquid media or in flooded substrates and cannot be extrapolated to soil conditions (Iturralde et al., 2019). Motility of inoculated rhizobia in soils at field-capacity is generally scarce; therefore, the distribution of the rhizobia in the soil profile is important, as it facilitates entry of growing roots in contact with the static rhizobia (López-García et al., 2002, 2009). Motility and chemotaxis might only have an effect during the short periods of rainfall or watering, during which the soil pores are water-saturated. Consequently, inoculant competitiveness may be improved through the application of liquid in-furrow inoculants, contributing to rhizobial dispersion, instead of seed-coated dried inoculation.

In the case of root colonization patterns, PGPR share common mechanisms with rhizobia for colonizing roots (Drogue et al., 2012). Attachment of bacteria to root surfaces is a multi-step process. An initial reversible attachment is followed by an irreversible attachment that occurs several hours after initial attachment, and, finally, biofilms can form over a few days (Dazzo et al., 1984; Smit et al., 1992). Two adhesion mechanisms have been described in *R. leguminosarum* to mediate the reversible and polar attachment to root hairs depending on the soil pH: in acidic conditions, rhizobial surface polysaccharide glucomannan binds to plant lectin, expressed on root-hair tips (Laus et al., 2006; Williams et al., 2008). Whereas under neutral or alkaline conditions, root lectins are solubilised and rhicadhesin, a hitherto unidentified calcium-binding protein, was proposed long ago to facilitate attachment to root hairs (Smit et al., 1989), although the gene encoding it

TABLE 1 | Examples of some of the genes reported to be involved in competition.

Category	Gene function	Gene name	Rhizobial strain object of the study	Publication
(i) Genetic Components for Rhizosphere Colonization	Motility	*motA, motB* *flg* and *fli* genes	*E. meliloti* L5-30	Bauer and Caetano-Anollés, 1988
	Chemotaxis	*cheAWRBYD* (Che1 cluster)	*R. leguminosarum* bv. *viciae* 3841	Miller et al., 2007; Wheatley et al., 2020*
	ABC transporters	*teuBAC1C2 (root exudates)* *aapJQMP (branched-chain amino acids)* *livM (branched-chain amino acids)*	*R. tropici CFN299* *R. leguminosarum* *E. meliloti* 2011	Rosenblueth et al., 1998 Green et al., 2019 Pobigaylo et al., 2008*
	EPS biosynthesis	*dgoK* *pssA*	*E. meliloti* *R. leguminosarum* bv. *trifolii*	Geddes et al., 2014 Janczarek et al., 2009
		pssD	*R. leguminosarum* bv. *viciae* 3841	Wheatley et al., 2020*
		exoY	*S. meliloti* 1021	Jones, 2012
	Lypopolysaccharide biosynthesis	*lpsB* *acpXL*	*E. meliloti* 2011/1021	Niehaus et al., 1998; Lagares et al., 1992; Campbell et al., 2002; Sharypova et al., 2003
	Attachment	*praR*	*R. leguminosarum* bv. *viciae* 3841	Frederix et al., 2014
	Rhizopine biosynthesis and catabolism	*mosABC* *mocCABRDEF*	*E. meliloti* L5-30	Gordon et al., 1996; Murphy et al., 1987
	PHB synthesis and degradation	*phaC* *bdhA*	*E. meliloti*	Aneja et al., 2005
	Homoserine catabolism	pRL80079-pRL80088	*R. leguminosarum* bv. *viciae* 3841	Vanderlinde et al., 2014
	Proline catabolism	*proDH*	*E. meliloti* GRM8	Jimenez-Zurdo et al., 1995
	Rhamnose catabolism	*rhaRSTPQUK, rhaDI*	*R. leguminosarum* bv. *trifolii* Rlt100	Oresnik et al., 1998
	Myo-inositol catabolism	*iolDEB* *iolA, iolRCDEB*	*R. leguminosarum* *E. meliloti* 2011	Fry et al., 2001 Pobigaylo et al., 2008*; Kohler et al., 2010
	Glycerol catabolism	*glpDSTPQUVK*	*R. leguminosarum* bv. *viciae* VF39	Ding et al., 2012
	Transcriptional regulation	*rsh* *rosR*	*B. diazoefficiens* USDA 110 *R. etli* CE3 *R. leguminosarum bv. trifolii* 24.2	Pérez-Giménez et al., 2021 Bittinger et al., 1997 Janczarek et al., 2010; Rachwał et al., 2016
(ii) Genetic components to prevent the growth of other bacterial cells	Bacteroicin production	*cinRIS* (small bacteriocin) *tfxABCDEFG* (trifolitoxin)	*R. leguminosarum* *Rhizobium trifolii* T24	Schripsema et al., 1996 Schwinghamer and Belkengren, 1968; Triplett and Barta, 1987
	T1SSd (biofilm production)	*prsD* and *prsE*	*R. leguminosarum* bv. *viciae*	Russo et al., 2006
	Type III secretion system (T3SS)	*rhcJ, rhcLNQRSTU, hrpW, rhcVD, nops* genes *nopP*	*S. meliloti* *Rhizobium* sp. NGR234	Jimenez-Zurdo et al., 1995 Marie et al., 2003 Ausmees et al., 2004
	Type IV secretion system – pilus (T4SS)	*virB1-virB11*	*S. meliloti*	Nelson et al., 2017
	Type VI secretion system (T6SS)	*tssHD* *tssABC1C2* *tagH* *tssEFGGHKLMFE*	*R. etli* Mim1	Salinero-Lanzarote et al., 2019
(iii) Genetic components to establish an efficient symbiosis	Nodulation	*nodD*	*R. tropici* strain CIAT 899 *R. leguminosarum* bv. trifolii	del Cerro et al., 2015 Ferguson et al., 2020
(iv) Genetic components to promote plant growth	Tryptophan biosynthesis (precursor of IAA and auxin)	*trpEF* *trpC trpF*	 *E. meliloti* 2011	Barsomian et al., 1992 Pobigaylo et al., 2008*
		trpB	*R. etli CE3*	Taté et al., 1999
	Phosphate solubilization	*phoR, phoUB* *ptsSCAB*	*E. meliloti* 2011	Pobigaylo et al., 2008*
	Siderophore production (Rhizobactin 1021)	*rhbABCDEF* *rhtA, rhrA*	 *E. meliloti* 1021	 Lynch et al., 2001

**Studies with several other genes reported to affect competition, but not all included in this table.*

is still a mystery. When competing with the wild type during nodule infection, the glucomannan mutant (*gmsA*) was strongly outcompeted (Williams et al., 2008). There are also other plant components influencing the attachment of *R. leguminosarum* to surfaces such as an arabinogalactan protein (Xie et al., 2012). Weak adherence and reversible attachment are mediated mainly by proteins and anchoring, while stronger adherence and irreversible attachment is mediated by polysaccharides (reviewed by Wheatley and Poole, 2018). Rhizobial adhering proteins (Raps) promote attachment and aggregation by rhizobia (Ausmees et al., 2001). Mutation of the transcriptional regulator *praR*, modulating the expression of the genes encoding Raps, results in enhanced *in vitro* biofilm formation, attachment to root hairs and increased nodulation competitiveness primarily due to the enhanced expression of Rap proteins (Frederix et al., 2014). Cyclic glucans (CG), exopolysaccharides (EPS), lipopolysaccharides (LPS), and capsular polysaccharides (KPS) are the main rhizobial surface polymers required for successful nodulation (Margaret et al., 2011). EPS is an extracellular carbon polymer weakly associated with the bacterial surface and thus abundantly released into the surrounding environment (Marczak et al., 2017). The recognition of EPS by specific LysM receptors modulates plant-bacteria recognition and potentially competition for nodulation (Geddes et al., 2014; Kawaharada et al., 2015). The two basic nodule morphologies appear to exhibit different rhizobial exopolysaccharide requirements. In rhizobia inducing determinate nodules, EPS mutants still induce effective nodules, with EPS playing a signaling role at the late stages of both infection thread initiation and bacterial release (Kelly et al., 2013). However, in symbioses forming indeterminate nodules, EPS is absolutely essential for a successful interaction (reviewed by Fraysse et al., 2003; Acosta-Jurado et al., 2021). EPS-altered mutants (exhibiting conserved LPS) generated in an isogenic strain, *R. loti* PN184, able to nodulate *Lotus pedunculatus* (determinate nodules) and *Leucaena leucocephala* (indeterminate nodules) showed that they were fully effective on a determinate-nodulating host but ineffective on the indeterminate one (Hotter and Scott, 1991). Working on *L. japonicus*, Kawarada and co-workers showed that perception of EPS synthesized by *M. loti* is important for maintaining an intracellular infection mode, with the plant LysM receptor protein EPR3 acting in the root cortex and nodule primordia to support and sustain the containment of rhizobia and to facilitate an efficient infection process. They observed a reduced and delayed nodulation either in the plant *epr3* mutants or in *M. loti* mutants affected in EPS biosynthesis due to a reduction of the normal intracellular infection thread mode and increased intercellular infection (Kawaharada et al., 2017). Interestingly, ITs of determinate nodules are narrower than those from indeterminate ones, where EPS is a critical component of the internal matrix and relevant for the cortex rhizobial invasion (Stacey et al., 1991). In indeterminate nodules, bacteria have to spread out by means of continuous IT penetration in the new cortex cells, whereas in the determinate nodules they spread by division of already infected cells (Fraysse et al., 2003). Consequently, EPS plays an important role in determining symbiotic competence; indeed, *R. leguminosarum* bv. *trifolii* strains overexpressing the biosynthesis gene *pssA* overproduced EPS and showed enhanced competitiveness, nodule occupancy, and symbiotic effectiveness with *Trifolium pratense* (red clover) in relation to their wild type strains (Janczarek et al., 2009). Likewise, *S. meliloti* 1021 mutant overexpressing the *exoY* gene, which encodes the enzyme responsible for the first step in succinoglycan (EPSI) biosynthesis, resulted in an increased production of this surface polymer and, as a consequence, enhanced symbiotic effectiveness with *Medicago truncatula* plants (Jones, 2012). Succinoglycan is essential for infection thread formation on plant hosts and has been shown to be more important than Nod factors for bacterial survival inside nodules (Maillet et al., 2020). Among other surface polysaccharides, EPS also seems to play an essential role also in protection against host plant defense during early and late symbiotic stages of rhizobial growth, when rhizobia are subjected to a prolonged oxidative burst from their plant hosts (Santos et al., 2001; D'Haeze and Holsters, 2004; Davies and Walker, 2007). Succinoglycan also protects *S. meliloti* against the antimicrobial activity of plant-derived nodule-specific cysteine-rich peptides in nodule occupancy and bacteroid differentiation and potentially against pH stress inside symbiosomes (Montiel et al., 2017; Arnold et al., 2018). Regarding LPS, contrary to what happens with EPS, an intact polymer seems to be needed for the formation of narrow ITs from determinate nodules (Stacey et al., 1991). A *B. japonicum* mutant defective in LPS synthesis was able to attach and induce root hair curling but failed to penetrate the root and induce ITs and nodules in soybean. However, in indeterminate nodules, mutants with alterations in this surface polymer formed ITs, although they led to an altered symbiotic phenotype in *Ensifer meliloti* during the association with alfalfa that affects the timing of nodule emergence, the infection development, and the strain competitiveness for nodulation (Lagares et al., 1992; Campbell et al., 2002; Sharypova et al., 2003). *lpsB* is a nice example of a surface mutation which does not affect the rate of infection initiation (i.e., no change in root nodule distribution) but the rate of nodule development and emergence. Such post-infection delay strongly reduces nodulation competitiveness by a factor of nearly 100. Beside this, the *lpsB* mutation shows how surface changes may also modify the host range for N2-fixation (Niehaus et al., 1998).

A common characteristic found in rhizobiales and other soil bacteria is the large number of ATP-binding cassette (ABC) transporters and methyl-accepting chemiotaxis proteins (M), which allows them to thrive in such a heterogenous and changing environment as soil, and to detect a high number of metabolites and influence their motility (Mauchline et al., 2006). ABC transporters, such as the genes *teuBAC1C2*, required for the utilization of root exudates (Rosenblueth et al., 1998) may confer competitive ability (Oger et al., 1997). The genes encoding the amino acid transporter AapJQMP have been shown to be up-regulated in bean and pea bacteroids, indicating the importance of the plant supply for branched-chain amino acids isoleucine and valine (Green et al., 2019). The assimilation of different nutrients and energy also influences competitiveness at different stages, such as during multiplication and survival in bulk soil (Sessitsch et al., 2002) or multiplication from the nodule environment after root senescence thanks to the ability

of catabolizing rhizopines, a compound made by bacteroids and subsequently catabolized by free-living cells of the producing strain (Murphy et al., 1987; Gordon et al., 1996). Recent work on genetically engineered *M. truncatula* and alfalfa plants that produce and exude rhizopine into the rhizosphere opened the door for the regulation of the root microbiome (Geddes et al., 2019b). Plant-supplied carbon can be diverted into storage molecules, such as polyhydroxybutyrate (PHB), increasing future survival and reproduction during free-living life-history stages once released into the soil after nodule senescence (Aneja et al., 2005; Ratcliff et al., 2008). Indeed, model prediction shows that the amount of PHB stored per cell could support the survival of active cells for a few days, or over a century for sufficiently dormant cells (Muller and Denison, 2018). Their PHB quantification experiments with *Bradyrhizobium* field isolates done in starvation conditions suggest that PHB is partitioned asymmetrically in dividing cells and that high-PHB isolates used more PHB over the first month, still retaining sufficient PHB for potential long-term survival in a dormant state. Homoserine is another amino acid shown to confer an advantage on those *R. leguminosarum* bv. *viciae* strains whose Sym plasmid harbors the genetic determinants for the catabolism of this plant-associated compound in the rhizosphere of pea roots (Hynes and O'Connell, 1990). The catabolism of other compounds such as rhamnose, proline, myo-inositol, and glycerol has also been reported to influence competitiveness (Jimenez-Zurdo et al., 1995; Oresnik et al., 1998; Fry et al., 2001; Kohler et al., 2010; Ding et al., 2012). Transcriptional regulators involved in metabolism are also important. A *Bradyrhizobium diazoefficiens* mutant with an impaired *rsh* gene (from *relA*-*spoT* homologous), responsible for a pleiotropic adaptation under stressful and starving conditions known as the stringent response, was less competitive than the wild type in occupying soybean nodules (Pérez-Giménez et al., 2021). A functional *rosR* is also important for competitive nodulation. This regulator has pleiotropic effects leading to defective attachment, infection thread formation, and bacteroid differentiation and senescence (Bittinger et al., 1997; Janczarek et al., 2010; Rachwał et al., 2016). As *pssA*, in terms of competitiveness, an increase in the quantity of nodules formed by strains carrying multiple copies of *rosR* was observed in comparison to *R. leguminosarum* bv. *trifolii* wild type strains (Janczarek et al., 2009).

Genetic Components to Prevent the Growth of Other Bacterial Cells

In terms of interference competition, one strategy is the production of antibacterial compounds, such as bacteriocins, which may play a role in competition for rhizosphere and root colonization (Hirsch et al., 1980; Oresnik et al., 1999; Venter et al., 2001). The quorum sensing system *cinRIS* is responsible for the production of the small bacteriocin, produced by strains of all three biovars of *R. leguminosarum* and inhibiting the growth of several strains of this species (Schripsema et al., 1996). The antibiotic trifolitoxin also improves rhizosphere colonization and increases competitiveness for nodule occupancy of clover (Schwinghamer and Belkengren, 1968; Triplett and Barta, 1987; Robleto et al., 1998). Members of the nodule microbiome of *Medicago sativa* also produce britacidins and tyrocidines (Hansen et al., 2020), supporting the idea that, in addition to nitrogen fixation, legume root nodules are sites of active antimicrobial production, presumably to provide protection from pathogens that might infect these organs and ensure that nitrogen-fixation activity is preserved. The presence of secretion systems in PGPRs and rhizobial strains may play a role in their plant growth-promoting (PGP) functions and also provides means of interference competition against other strains in the rhizosphere (Gupta et al., 2014). For instance, the T1SSd proteins orthologous to the PrsD and PrsE proteins are required for biofilm formation (Russo et al., 2006). The T3SS, T4SS, and T6SS are generally used to inject effector proteins, such as nodulation outer proteins (Nops), directly into eukaryotic host cells or into other bacteria, which can mediate compatibility with the host in rhizobia, modulating partner choice (Jimenez-Zurdo et al., 1995; Marie et al., 2003; Ausmees et al., 2004; Nelson and Sadowsky, 2015). The secretion of Nops through T3SS in the presence of flavonoids is able to induce the transcription of nodulation genes (Jiménez-Guerrero et al., 2017) and might modulate the plant defense response upon infection (Pérez-Giménez et al., 2021). While T3SS orthologs are present in *R. etli* CFN42, *S. fredii* HH103, *B. japonicum* USDA110, and *Rhizobium* sp. NGR234, they are not common in *R. leguminosarum* or *Sinorhizobium* species (Black et al., 2012). The T4SS, encoded by *traGDCAFBHMR,* is involved in conjugal transfer, whereas the T4SS-pili (*virB1*-*virB11*) system is involved in the colonization of surfaces in gram-negative bacteria (Juhas et al., 2008). *S. meliloti* with a truncated T4SS was less competitive for nodule formation compared to wild type (Nelson et al., 2017). T6SS, encoded by the *imp* (*tss*) and *hcp* clusters, was first identified in the α-rhizobial strain *R. leguminosarum* (Bladergroen et al., 2003) and shown to be important for nodulation of pea plants. Impaired T6SS mutants in *R. etli* Mim1 have been shown to generate small, white nodules in *Phaseolus vulgaris*, although with similar activity (Salinero-Lanzarote et al., 2019). The authors suggested a positive role for T6SS in competition with other soil bacteria, as it was active at a high cell density and in the presence of plant exudates. Many gram-negative bacteria use this secretion system for killing competitors (Schwarz et al., 2010; de Campos et al., 2017; Allsopp et al., 2020) and presumably even as an inter-bacterial communication system mediated by the T6SS–quorum sensing cross-talk (Gallique et al., 2017). These transport secretion systems are often found in the accessory genome of rhizobial strains.

Genetic Components for Establishing an Efficient Symbiosis

Rhizobial genomes are extremely variable (MacLean et al., 2007), with the secondary replicons generally more genetically diverse between strains than is the primary chromosome (Galardini et al., 2013). Chromids – large replicons carrying essential genes and adopting plasmid-type maintenance and a *repABC* replication system – appear to contain genus-specific genes in *Rhizobium*, *Ensifer,* and *Agrobacterium* (Harrison et al., 2010).

When an organism arrives in a new niche, it needs to adapt to the new environment, undergoing genome expansions due to the duplication of existing genes or the acquisition of new ones through horizontal gene transfer in order to acquire new functions that improve the fitness of these strains in the new environment (Martins Dos Santos et al., 2004; Aguilar et al., 2018). This is the case, for example, with *nodD*. NodD regulates the expression of the *nodABCFE* cluster and is therefore involved in Nod factor production. Nod factor decorations are critical for host specificity and their levels are tightly regulated during infection (Krönauer and Radutoiu, 2021). Five *nodD* reiterations were found in *R. tropici* CIAT899, necessary to engage the symbiont in nodulation with different legume plants (del Cerro et al., 2015). They were also present in different nitrogen-fixing rhizobial strains from *P. vulgaris,* suggesting a potential role in host range (Peralta et al., 2016). In *R. leguminosarum* bv. *trifolii*, a second copy, NodD2, enhanced nodule colonization and competitiveness in symbiosis with clover (Ferguson et al., 2020). These accessory *nod* genes may reflect the variations within the interactions among rhizobial and plant species. Indeed, *nodD* has been extensively used for the identification and classification of rhizobial isolates (Boivin et al., 2020; Fields et al., 2021). Sequence heterogeneity within symbiotic plasmids also shows extensive genomic rearrangements, recombination rates, lateral transfer events, and relaxation or intensification of selective pressures (González et al., 2003). Indeed, the diversity of associations between genomic backgrounds and Sym genotypes may be greater in bulk soils than in nodules, as the first symbiotic organs are formed by the most competitive strains' genotypes for a given host plant (Louvrier et al., 1996; Laguerre et al., 2003) triggering autoregulation of nodulation which start to inhibit further nodulation (Mortier et al., 2012). Legume hosts seem to select differentially within the same soil populations. As an example, pea and faba bean have been reported to select A1 and B1 *nod* groups of *R. leguminosarum* bv. *viciae* as their symbiotic partner to form nodules (Laguerre et al., 2003; Mutch and Young, 2004; Jorrin and Imperial, 2015). When *R. etli nodC* variants from the centers of bean genetic diversification were inoculated on wild and cultivated *P. vulgaris* (common bean), yield was best when plant and bacteria were from the same geographic origin, suggesting mutual symbiotic selectivity and coevolution (Aguilar et al., 2004). During the growth cycle of beans, more competitive rhizobia will return to the soil after nodule senescence and therefore contribute to an increase in their representation in soil, explaining this synergism (Aguilar et al., 2004). The minimal symbiotic genome has been defined recently in *E. meliloti,* allowing gain-of-function approaches that can be used to elucidate genes in the Sym plasmid, as well as those genes from other highly competitive rhizobia that contribute to nodulation competitiveness (Geddes et al., 2021).

Genetic Components to Promote Plant Growth

The term biofertilizer comprises formulations of different living microbial cells, either a single strain or multiple strains, that promote plant growth by increasing nutrient availability and acquisition (Riaz et al., 2020). Thus, elite rhizobial inoculants, besides the traits involved in competition, should also combine a subset of other important traits, with PGP abilities among them (Vessey, 2003; Lugtenberg and Kamilova, 2009; Jaiswal et al., 2021). These traits enhance yields and cause positive changes in soil structure and microbial community. The production of phytohormones is a major property that has evolved in plant-associated bacteria. It leads to an increased size of plant root system and, subsequently, the exploitation of a larger soil volume, thus improving the mineral and aqueous nutrition available to the plant (Cai et al., 2018). In most bacterial pathways, auxin biosynthesis mainly relies on tryptophan, which acts as a biosynthetic precursor of indole acetic acid (IAA), a common auxin produced by rhizobacteria, and as a signal inducing *ipdC/ppdC* transcription. *E. meliloti trpE* mutants, blocked at the first step in tryptophan biosynthesis, form N_2-ineffective symbionts (Barsomian et al., 1992). Similarly, Tn5-induced *B. japonicum* tryptophan synthetase mutants lacked the ability to fix nitrogen and were symbiotically defective (Kummer and David Kuykendall, 1989). This deficiency in N_2-fixation seems to be most likely due to the pleitropic effects of the amino acid auxotrophy, with the host plant unable to supply the nutrients needed for the endosymbiont to establish an effective symbiosis. Interestingly, the tryptophan auxotroph mutant *trpB* of *R. etli* CE3 was unable to produce Nod factors in free-living conditions unless tryptophan was added to the growth medium, rendering nodules ineffective in its symbiotic partner *P. vulgaris.* This indicated that even though this *trpB* mutant was able to induce the development of a nodule primordium, a shortage of aromatic amino acids during nodule invasion strongly altered its ability to subsequently invade the nodule and differentiate into bacteroids (Taté et al., 1999). Some PGPR of the genera *Bacillus, Enterobacter*, or *Pseudomonas* promote plant growth by solubilizing phosphate from the soil. Genes implicated in phosphate solubilization are the sensor kinase *phoR*, and the genes involved in regulation and transport, *phoUB* and *pstSCAB* (Chhabra et al., 2013). Intriguingly, bean nodule bacteroids seem to be phosphate-limited (Green et al., 2019). The secretion of siderophores also promotes plant growth by enhancing iron uptake and utilization (Liu et al., 2017). These molecules have a high affinity for Fe^{3+}, scavenging iron from environmental stocks where soil iron is low and reducing it to Fe^{2+} for uptake and utilization (Kramer et al., 2020). As an example, Rhizobactin 1021 is a hydroxymate siderophore produced by the soil bacterium *E. meliloti* 2011 which appears to contribute to the competitive ability of free-living *E. meliloti* in iron-depleted soils (Persmark et al., 1993; Barton et al., 1996; Lynch et al., 2001).

Future Understanding of Rhizobial Genomes

Understanding the mechanisms behind competitiveness for nodulation may lead to improved inoculation strategies (Bourion et al., 2018). A plethora of molecular mechanisms play a role in competition, concerning both microbe-microbe, and plant-microbe interactions. Sequencing efforts have improved our understanding of rhizobial genomes, with sequence analysis of

whole genomes enabling in-depth studies and comparisons of genome structures (Burghardt et al., 2018; Epstein et al., 2018; Cavassim et al., 2020). The high-throughput identification of genes relevant to competitiveness was first approached with signature-tagged mutagenesis (STM) *in planta*, which allowed screening of hundreds of mutants in one passage through the host (Pobigaylo et al., 2008). This study identified 38 mutations in genes that were not previously known to be involved in competitiveness or symbiosis in *E. meliloti*, confirming 23 with attenuated competitiveness phenotypes when they were tested for competition against the wild type. Among them are some of the genetic determinants already mentioned, such as: *trpC* and *trpF*, involved in the conversion of tryptophan to IAA; *pstA* and *pstC*, encoding components of the high-affinity phosphate transporter system PstSCAB; or *iolA*, encoding a methylmalonate-semialdehyde dehydrogenase involved in myo-inositol catabolism (Pobigaylo et al., 2008). STM also allowed the identification of more than a hundred genes in *E. meliloti* associated with rhizosphere colonization of a host (alfalfa) and a non-host plant (pea; Salas et al., 2017), supporting the ancient character of those genes. The high-throughput identification of essential genes in specific conditions is today performed by transposon-insertion sequencing (Tn-Seq or INSeq), a saturation insertion mutagenesis performed in a pool of colonies in a given environment. Each colony contains a single insertion that can be mapped across an entire genome by next-generation sequencing, allowing the determination of gene fitness at genome-scale (Goodman et al., 2011; Perry and Yost, 2014; Chao et al., 2016). This method has been applied in rhizobia, leading to the elucidation of genes involved in competition, such as the ORF SMc00911, conserved and highly expressed in the nodule. This insertion mutant strain strongly outcompeted the *E. meliloti* 1021 wild type strain (Queiroux et al., 2012). More recently, this method has allowed the identification at a genome-wide scale of genes involved in various stages of the rhizobial lifestyle, including those genes required when in competition with other bacteria, which were traditionally missed in the artificial environment of inoculation with a single strain (Wheatley et al., 2020). Among the mutants assesed in this study for their ability to compete against wildtype to form nodules were the genes encoding the chemotaxis protein CheA, glutamine synthetase II (*glnII*), or the polysaccharide biosynthesis protein PssD. A more powerful method moving this field forward are unique but random DNA barcodes used in insertional mutagenesis (Bar-Seq). The abundance of transposon insertions can be followed with a single PCR step to amplify the barcodes followed by NGS, allowing the study of gene fitness across multiple growth conditions (Robinson et al., 2014; Wetmore et al., 2015). Multi-strain barcoding can be used for analyzing how bacteria interact with the plant and one another during competitive root colonization competition coupled to sequencing (Cole et al., 2017; Knights et al., 2021).

Active competition between rhizobial strains might take place throughout the entire course of the symbiosis, from the recruitment of the endosymbionts in the rhizosphere to nodule decay (Wielbo et al., 2010). These methods will improve our understanding of which phases in the nodulation process are exposed to competition. Indeed, although much less explored relative to competition in the rhizosphere, we have previously seen that competition also takes place inside plant nodules, highlighting the complexity of the interactions between the plant host and rhizobia. Studies on rhizobial competitiveness have revealed that competition between strains also extends to the process of infection thread initiation and the growth of rhizobia in the infection threads (Stuurman et al., 2000; Duodu et al., 2009). The INSeq work carried out in *R. leguminosarum* bv. *viciae* has shown that the chemotaxis cluster Che2 is needed in the infection thread (Wheatley et al., 2020). Numerous molecular techniques are available for the evaluation of competitive abilities of rhizobia in the rhizosphere, whereas the formation of infection threads remains poorly understood. New methods based on Periodic Acid-Schiff to visualize the three-dimensional structure of infection threads in sufficient detail using novel and traditional cell wall fluorescent labels combined with laser confocal scanning microscopy presents an exciting opportunity for research in this area, including competition (Rae et al., 2021).

METHODS TO ASSESS RHIZOBIAL COMPETITIVENESS FOR NODULATION

Experiments involving single- and multi-inoculation with pea plants have shown that the nodulation ability of a strain does not predict its competitiveness for nodulation and is not correlated with its N_2-effectiveness (Bourion et al., 2018). The high competitiveness of a given strain does not ensure high nitrogen-fixing efficiency or high biomass production for the plant, indicating that competitiveness for nodulation is controlled by multiple genetic factors from both the host and the rhizobia strain. This study highlighted that competitiveness for nodulation and nitrogen fixation efficiency must both be considered as selection criteria for improving pea crop production. Although many unknows remain regarding the molecular and genetic mechanisms driving competition for nodulation, it has become clear that, in the development of elite rhizobial inoculants, it is necessary to consider colonization and competition for nodulation separately from symbiotic nitrogen fixation abilities (Checcucci et al., 2017). Therefore, one of the main challenges is still the selection of elite rhizobial strains based on their high performance in the field due to their symbiotic performance, combined with relevant genetic features (Checcucci et al., 2017; Aguilar et al., 2018).

Despite one of the first co-inoculation assays being presented in 1930 by Löhnis (1930), follow by Dunham and Baldwin (1931), there has been limited progress in this area due to the complexity of directly observing and identifying interactions between bacteria and plants. The methods to assess competitiveness have traditionally been time-consuming and restricted to a small sample size. However, several techniques are now emerging, based on advances in imaging (Jeckel and Drescher, 2020) and sequencing technologies, which allow a high-throughput approach, as summarized in **Table 2**.

Competition assays were initially performed using morphological, serological, or antibiotic markers that allowed

TABLE 2 | The most frequently used methods to assess rhizobial competitiveness for nodulation.

Method	Principle	Pros	Cons	Example of latest studies using this method to assess competitiveness
Antibiotic markers Graham, 1969; Josey et al., 1979	Scoring rhizobial infection by plating nodule samples on suitable selected media	• No need for sophisticated equipment; • No need to genetically modify the strains; • Competitiveness of strain not affected.	• Limited number of strains can be evaluated; • Mixed nodules are often missed; • Relies on strain viability and culturability in different antibiotics which becomes very labor intensive.	Bogino et al., 2011; Laguerre et al., 2012; Bourion et al., 2018
Strain-specific fingerprints De Bruijn, 1992; Laguerre et al., 2003	Targeting specific plasmid profiles or genes; afterwards patterns of the resulting PCR products are analyzed	• Suitable as a first step to classify closely related strains in large collections.	• Requires strict standardization of reaction parameters; • Complex comparative analysis of banding patterns; • Does not allow the identification of mixed nodules.	Lardi et al., 2017; Irisarri et al., 2019; Pastor-Bueis et al., 2019
Sequential double staining to detect *gusA* and *celB* Sessitsch et al., 1996	Scoring of nodule infection by color detection after enzymatic reactions	• Allows efficient scoring of single or double nodule infections without requiring sophisticated equipment; • Stable insertion of marker genes, ideal for ecological experiments; • Can be used in large-scale assays and in the presence of an unmarked background population.	• Only possible to score two tagged strains simultaneously; • Toxic buffers are needed for the enzymatic reaction to distinguish nodule occupancy; • Not possible to recover viable rhizobia from stained nodules.	Sánchez-Cañizares and Palacios, 2013; Ferguson et al., 2020; Mendoza-Suárez et al., 2020 Westhoek et al., 2021
Fluorescent proteins Stuurman et al., 2000	Detection of dual fluorescence by microscopy	• High resolution even at single cell level; • Viable rhizobia can be recovered from nodules.	• Only a few nodules per plant or a small plant sample size can be assessed due to microscopy complexity.	Checcucci et al., 2016; Regus et al., 2017; Bellabarba et al., 2020
NGS of full genome, core genes or accessory genes	Analysis of partial or full genome to identify individual strains	• Large numbers of rhizobial strains can be assessed simultaneously; • Measures relative strain diversity; • Tracks dynamic changes in strain populations.	• DNA samples are pooled, loosing information of individual strain-nodule relation; • Pre-sequencing of the genomes from the strains is needed; • Complex sequencing data analysis.	Ji et al., 2017; Burghardt et al., 2018; Boivin et al., 2020; Moeskjær et al., 2020
NGS of synthetic DNA fragments Mendoza-Suárez et al., 2020	Introduced unique barcodes (IDs) are detected by NGS to score bacterial populations in individual nodules	• Large numbers of rhizobial strains can be assessed simultaneously; • Strains not previously isolated and genome-sequenced can be identified; • Information at a nodule level; • Easy identification of mixed nodules; • Simultaneous assessment of competitiveness and effectiveness.	• High-throughput cloning methods are needed and bacteria library preparation; • A blue-light transilluminator is needed to detect GFP nodules from tagged strains vs native strains.	Mendoza-Suárez et al., 2020

for strain discrimination (Löhnis, 1930; Holland, 1966; Graham, 1969; Josey et al., 1979), followed by strain-specific genomic fingerprints (De Bruijn, 1992; Laguerre et al., 2003; **Figure 2A**). These methods are labor-intensive, as they involve isolating strains from nodules and plating them in selective media or complex and sometimes barely-reproducible PCR profile analyses. Marker genes such as *lacZ* (Drahos et al., 1986) and *luxAB* (O'Kane et al., 1988) facilitated observation of the bacteria-plant interaction, although the downsides were that ß-galactosidase activity from *lacZ* had a high background in both rhizobia and the plant host, and the luciferase assays with *luxAB* needed sophisticated equipment to be detected.

A tool that simplified these assays was the use of marker genes and chemical staining to detect *gusA* (Streit et al., 1992), and the simultaneous detection of *gusA* and *celB* (Sessitsch et al., 1996) by enzymatic activities rendering colored products (**Figure 2B**), or by fluorescent proteins (Stuurman et al., 2000; **Figures 2C,D**). However, these assays are still restricted to inoculum mixes of only two strains. More novel approaches today allow high-throughput assays by using either NGS of full genomes, core genes, or accessory genes, or NGS of synthetic DNA fragments (Mendoza-Suárez et al., 2020). It is worth noting the promoter driving the expression of the reporter genes. While constitutive promoters have been standard, Wilson et al. (1995b) and Sessitsch et al. (1996) used promoters that express only in symbiosis and nitrogen-fixing conditions and are a more specific alternative, such as the *nifH* promoter. A broader, more novel approach is the use of a universal *nifH* synthetic promoter, based on a consensus sequence adapted to different rhizobia by Mendoza-Suárez et al. (2020). These reporters can be either replicated in stable low-copy plasmids (Pini et al., 2017; Geddes et al., 2019a), through a quick conjugation step with the strains of interest, or can be integrated in the genome by stable mini-Tn7 vectors (Romero-Jiménez et al., 2015). The transformational breakthrough in Mendoza-Suárez et al. (2020) is the ability to simultaneously assess competitiveness and estimate rates of nitrogen fixation in individual nodules of pea plants. This is achieved thanks to the combination of a module for evaluating

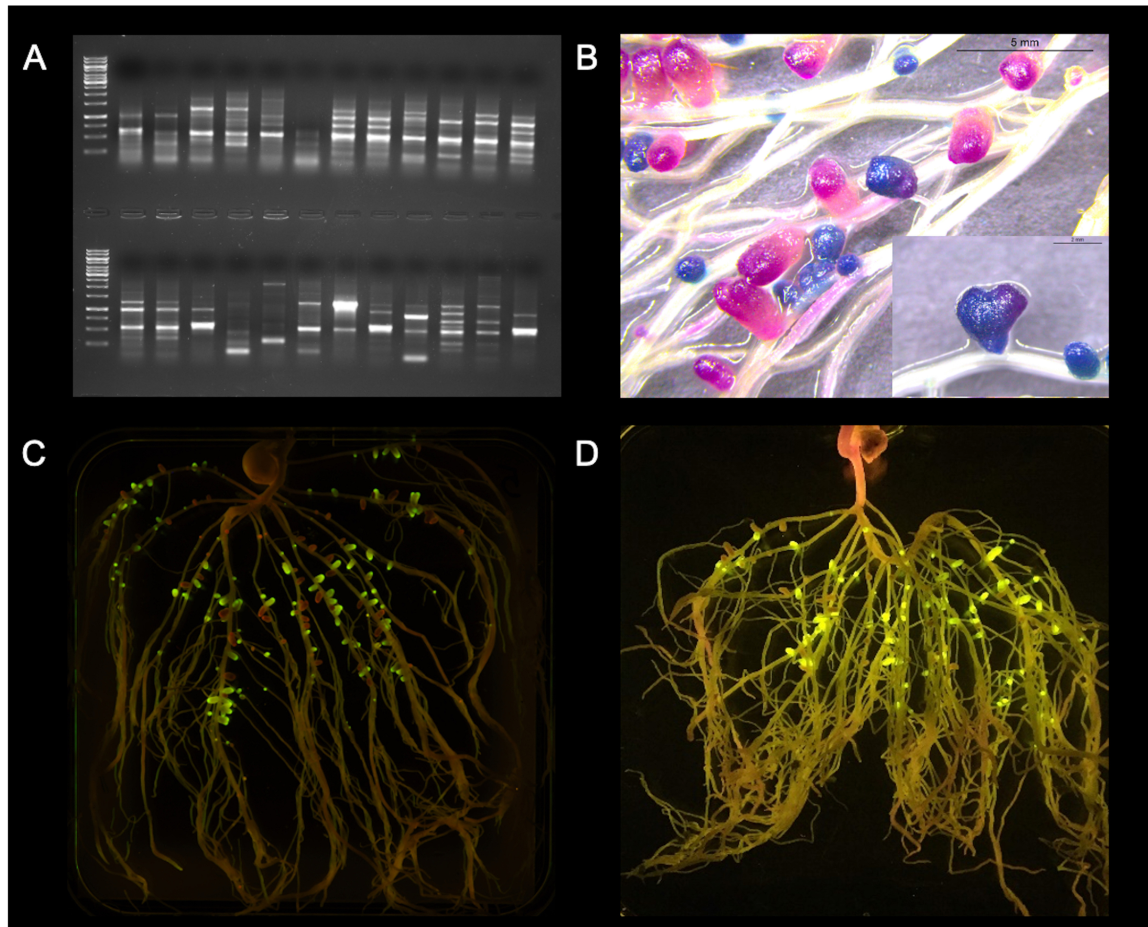


FIGURE 2 | Visualization of some methods for assessing rhizobial competitiveness for nodulation. **(A)** Strain-specific genomic fingerprints: ERIC-PCR from same plant nodule isolates from a trapping assay using faba bean as a host; **(B)** Sequential double staining to detect *gusA* and *celB*: Pea roots were sequentially double-stained with Magenta-GlcA and X-Gal after thermal treatment, resulting in pink nodules formed by UPM791gusA (*gusA* constitutively expressed) and blue nodules formed by Rlv3841celB (*celB* constitutively expressed); **(C)** Fluorescent proteins: Rlv3841 labeled with mini-Tn7 J23104 GFP or mCherry, respectively; and **(D)** NGS of synthetic DNA fragments: Example of pea roots grown in non-sterile soil and exposed to a blue-light transilluminator. Tagged rhizobia, expressing GFP under Ps*nifH* control, lead to fluorescent nodules, while indigenous rhizobia do not. Photo credit: **(A,B,D)** Marcela Mendoza-Suárez; **(C)** Laura Clark.

N_2-effectiveness that included a consensus *nifH* (nitrogenase) promoter driving nodule-specific expression of green fluorescent protein as reporter gene, together with a second module consisting of a unique synthetic nucleotide sequence as a barcode strain identifier (ID; **Figure 2D**), allowing the screening of large libraries of bacterial strains.

We must not forget that competitiveness of a *Rhizobium* strain and how efficiently it fixes nitrogen is determined by its genetics and physiology, in interaction with the legume host genotype (Laguerre et al., 2003; Burghardt et al., 2018; Boivin et al., 2020) and the inoculation context, determined by soil influence (Batista et al., 2015) and climatic conditions (Frey and Blum, 1994; Vuong et al., 2017). The population of rhizobia in different soils is heterogeneous and varies quantitatively and qualitatively, responding to different abiotic and biotic factors (Graham, 2008; Kasper et al., 2019). A given elite strain may thrive in one climate or soil type but fail in a different environment to which it is poorly adapted. Therefore, when elite strains are found, these are for a particular soil and plant genotype, and may well not perform as well in a different plant genotype or different environment (**Figure 3**). It is of utmost importance that future competition assays are performed in non-sterile conditions; ultimately, rhizobial strains are not alone in the rhizosphere. It is therefore essential to apply and improve existing techniques, such as those of Burghardt et al. (2018) and Mendoza-Suárez et al. (2020), to other *Rhizobium* species. Like this, more studies could combine the assessment of a large number of strains for nodule occupancy using different host genotypes with the assessment of symbiotic N_2-fixation in agricultural soils, whilst minimizing financial and time costs through increased testing efficiency.

HOW MANY STRAINS ARE THERE IN A MIXED NODULE?

Nodules containing more than one rhizobial strain, called "mixed nodules," were identified several decades ago (Johnston and Beringer, 1976). Experimental results have shown

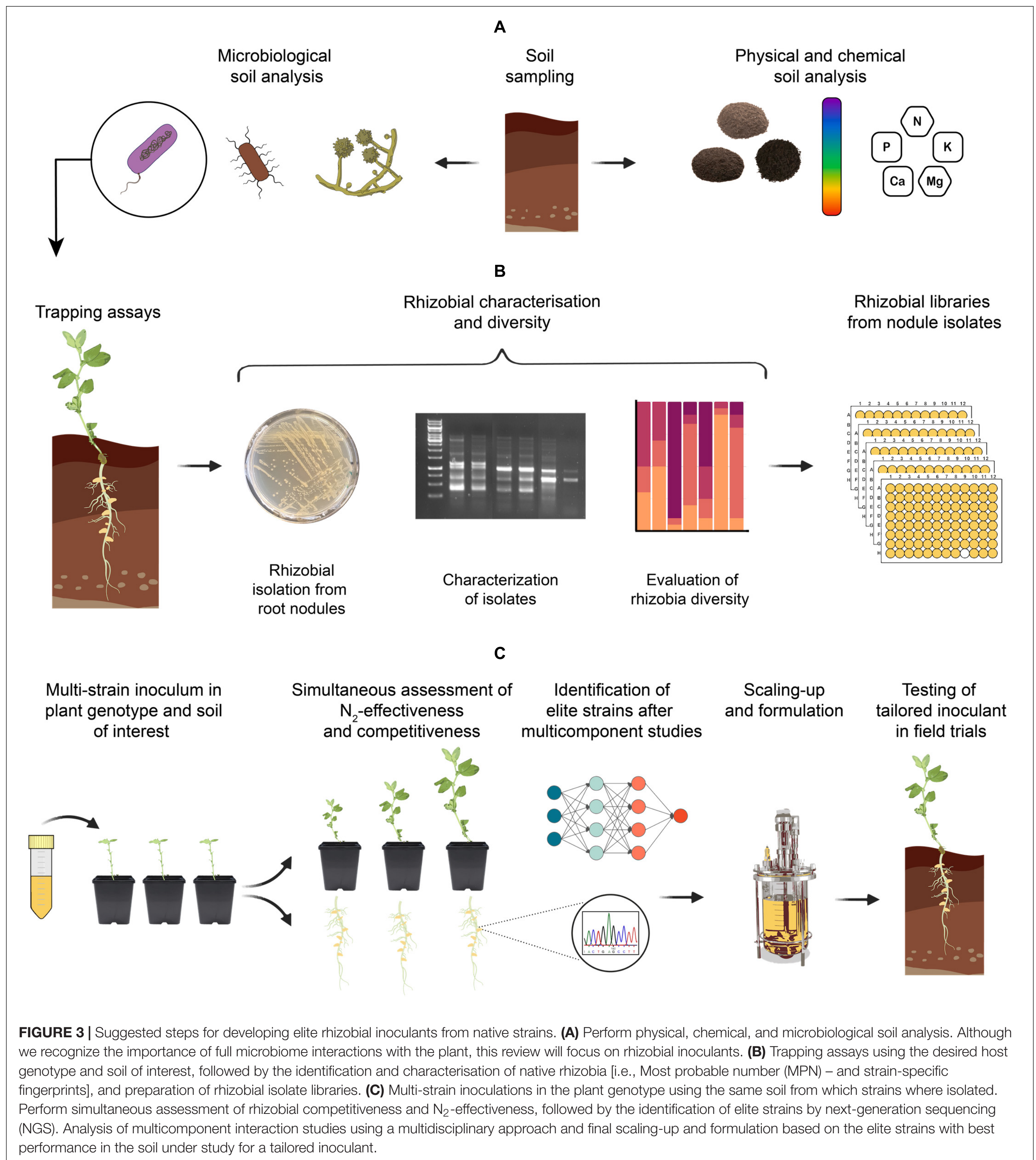


FIGURE 3 | Suggested steps for developing elite rhizobial inoculants from native strains. **(A)** Perform physical, chemical, and microbiological soil analysis. Although we recognize the importance of full microbiome interactions with the plant, this review will focus on rhizobial inoculants. **(B)** Trapping assays using the desired host genotype and soil of interest, followed by the identification and characterisation of native rhizobia [i.e., Most probable number (MPN) – and strain-specific fingerprints], and preparation of rhizobial isolate libraries. **(C)** Multi-strain inoculations in the plant genotype using the same soil from which strains where isolated. Perform simultaneous assessment of rhizobial competitiveness and N_2-effectiveness, followed by the identification of elite strains by next-generation sequencing (NGS). Analysis of multicomponent interaction studies using a multidisciplinary approach and final scaling-up and formulation based on the elite strains with best performance in the soil under study for a tailored inoculant.

that the percentage of mixed nodules is sufficiently high that it should be carefully considered in rhizobial studies, particularly in competition assays (May and Bohlool, 1983; Moawad and Schmidt, 1987). However, these competition experiments were traditionally conducted with two-strain mixtures. Thanks to the development of new methods to assess rhizobial competitiveness, advances in microscopy, and the continued decrease in price of NGS, it has now been possible to assess more simultaneously-competing strains, which has also made the easy identification of mixed nodules possible. These new studies suggest that not

only are mixed nodules more common than thought previously, but they can also be occupied by more than two different strains. In fact, Mendoza-Suárez et al. (2020) found up to six different strains in a single nodule using unique barcode identifiers (ID). This also reinforces the hypothesis that there may be cooperative partnerships between strains.

CHEATERS AND PLANT SANCTIONING BEHAVIORS

Since multiple rhizobial strains may occupy the same nodule (Checcucci et al., 2016; Mendoza-Suárez et al., 2020), cheating behaviors have emerged (Sachs et al., 2010; Checcucci et al., 2016; Regus et al., 2017). Experiments with near-isogenic mutants demonstrated that a N_2-ineffective mutant had a similar level of competitiveness to its parent (Amarger, 1981; Daubech et al., 2017), supporting the idea that plants do not select rhizobial strains by their fixing abilities at root entry level. Indeed, N_2-ineffective rhizobial strains with superior competitiveness often gain advantage over N_2-effective strains, despite offering suboptimal growth to their plant host (Sachs et al., 2010). Perennial legumes, where nodulation is a continuous and sequential process, establish symbiosis with less competitive rhizobial strains when they have no other choice. A study performed in *Caragana microphylla* with different *Mesorhizobium* strains demonstrated how the plant host could be infected with a more favorable rhizobial strain when the nodules were first occupied by less competitive rhizobial strains (Ji et al., 2017). However, if a legume had nodules occupied by its most favorable rhizobial strain first, it was unlikely that other rhizobial strains in the rhizosphere would be able to form new nodules on the roots, even if the density of the other strains was higher. This dynamism over time and life phase of the host plant has been reported in the nodule microbiome of *M. sativa,* indicating that its members strongly interact through cooperation and competition (Hansen et al., 2020). In fact, pea plants have been shown to tolerate intermediate fixers only when a better strain was not available (Westhoek et al., 2021). Post-infection plant control over bacteroid metabolism is essential due to the high fitness cost of nodule formation and bacteroid maintenance. The plant host must therefore monitor symbiotic performance and respond accordingly (Ledermann et al., 2021). However, plants cannot select effective nitrogen-fixing rhizobia from a mixture of effective and ineffective strains in the soil in the early stages of the symbiotic interaction (Westhoek et al., 2021). Instead, to avoid cheaters displacing effective symbionts once the infection has occurred, legumes limit cheating through host sanctions, which reduce the fitness of cheaters, and partner choice, where each partner can identify and reject forming relationships with cheaters (West et al., 2002; Kiers et al., 2003; Kiers and Denison, 2008; Sachs et al., 2010; Oono et al., 2011; Daubech et al., 2017; Westhoek et al., 2021). Sanctioning of ineffective nodules occurs by reducing the number of viable cells present in nodules and reducing their reproductive success (Kiers et al., 2003), by preferentially promoting nodule development in number and size with most BNF-efficient rhizobia due to the stimulation of plant cell multiplication and bacteroid differentiation (Laguerre et al., 2012), or presumably by reducing resource allocation to a nodule and shutting it down (Kiers et al., 2003; Westhoek et al., 2021). As seen in mixed nodules, where nodules are co-occupied by different strains, the host plant appears to spatially structure symbionts, separating in individual plant cells effective from ineffective partners. Therefore, when nodules are co-inhabited with a fixing strain, the inefficient strain is sanctioned rapidly in a cell-autonomous way (Regus et al., 2017). This means that plant sanctions are targeted specifically to those individual host cells housing ineffective partners, being an effective host strategy to sanction poorly performing strains. Even in mixed nodule infections, each individual plant cell appears to only have one strain. Plant nodules have been described as autonomous compartments where the host is able to actively rewire investment away from symbiont reproduction, and toward nitrogen fixation (Chomicki et al., 2020). In evolutionary terms, compartmentalization of host-microbe interactions helps to stabilize cooperation by allowing hosts to: (i) isolate symbionts and control their reproduction; (ii) reward cooperative symbionts and punish or stop interactions with non-cooperative symbionts; and (iii) reduce direct conflict among different symbiont strains in a single host (Chomicki et al., 2020). Hosts with symbiotic promiscuity (analyzed in detail in Perret et al., 2000) could face a stronger selection pressure to evolve effective post-infection discrimination, although the mechanisms by which the host monitors individual nodules, once formed, are still largely unknown (Chomicki et al., 2020).

CURRENT PRODUCTION OF INOCULANTS

The use of inoculants is well stablished in agricultural systems. Based on *Bradyrhizobium* strains, soybean is currently the crop that consumes the most inoculant production worldwide (Santos et al., 2019). Rhizobial strains have traditionally been isolated either from bulk soil (**Figure 3A**) in a semi-selective medium or with trapping assays designed for a soil and a host of interest (**Figure 3B**; Laguerre et al., 2003). For a rhizobial strain to be considered as a potential inoculant, the easiest approach is to first characterize the isolate in the laboratory and show its effectiveness at nitrogen fixation, its ability to successfully fight for rhizosphere colonization, and its relative competitiveness against native rhizobia for nodule occupancy. Screening for the relative ability of rhizobia to compete for the rhizosphere appears a quite good predictor of nodule occupancy as indicated by the strikingly linear impact that access to the rhizosphere has been shown to have on nodule occupancy (Salas et al., 2017). Once these characteristics have been determined, it is crucial to test rhizobial strains in greenhouse and field trials to analyze the effects of the inoculant treatment on agronomic traits, such as: nodulation, plant biomass, shoot N content, grain yield, and/or grain N content (Werner and Newton, 2005; Lupwayi et al., 2006). Once the screen for a set of elite rhizobia performing well in the rhizosphere has been made, these wild type strains can then be incorporated into more complex assays,

such as Tn-Seq/INSeq or BarSeq, where the mutagenesis can be done in soil conditions where a natural microbiome will be present, or genome comparisons between an elite strain versus non-competitive strains to identify the genetic determinants involved in competition.

Those strains selected as inoculants are typically grown at a fermenter scale and used to coat seeds of compatible legume hosts to introduce them into the soil (**Figure 3C**; diCenzo et al., 2019). However, this inoculation method often results in a high density of bacteria near the seed, with nodulation restricted to the upper tap root but reduced in the more distal part of the tap root and the lateral roots due to the low density of the inoculant strain in the bulk soil (Vlassak and Vanderleyden, 1997). Alternatively, in-furrow inoculation – placing the rhizobial strain in the seed bed – is an approach that enhances rhizobial motility and consequently, increases nodule occupancy (López-García et al., 2009). It also works well for legumes infected by crack entry, such as peanut and bradyrhizobia, as the chances of encountering breaks in the secondary root epidermis and establishing symbiosis are increased (Bogino et al., 2011). Therefore, to maximize rhizobial inoculant efficacy, research must focus on the following key aspects: the intrinsic characteristics of the rhizobial strain; the delivery system into the soil, known as inoculant formulation; the optimization of the production process by industrial fermentation, and the compatibility with the farmer's practices (Catroux et al., 2001; Temprano et al., 2002; Herridge et al., 2008; Checcucci et al., 2017; Pastor-Bueis et al., 2019).

Although an elite strain is an essential prerequisite in the development of successful inoculants, there are several factors to consider when working in laboratory conditions. Firstly, nodule-dominant strain genotypes from soil populations do not necessarily show superior competitiveness for nodulation compared to minor occupants when evaluated under non-soil conditions (Laguerre et al., 2003). Secondly, legumes grown in sterile conditions under nitrogen starvation regimes can show significant growth differences between the inoculated and non-inoculated treatments. However, real agricultural conditions are never entirely devoid of nitrogen, and populations of native rhizobia are usually present (Wilson et al., 1995a). As the sole inoculant applied to legumes under laboratory or controlled conditions, specific strains of rhizobia may increase nitrogen fixation (Wielbo et al., 2010); however, once these strains are applied to legumes under real agricultural conditions, they often fail in the competition with native strains for nodulation due to soil conditions, rooting depth, humidity, temperature, and the inoculant formulation (López-García et al., 2002; Naeem et al., 2004; Yates et al., 2011; Drew et al., 2012). Finally, another important trait in an inoculant is the survival rate; both in the carrier – a wide range of carriers can be used (peat, compost, vermiculite, perlite, and sand) – and in the soil. The strain used as the inoculant needs to be either robust or well protected in order to survive under harsh conditions (Parnell et al., 2016). Rhizobia should survive and grow in the soil in the absence of the host plant and be able to colonize the host plant rhizosphere prior to competition for root infection and nodule formation (Laguerre et al., 2003). The use of coated seeds is the most convenient delivery system, but while rhizobia survive well in inoculant formulations, some die rapidly after seed-coating owing to osmotic and desiccation stress (O'Callaghan, 2016; Atieno et al., 2018). Formulation of inoculants is a crucial issue, but little research has been conducted on this subject. It can improve field performance, shelf life, and stability while reducing variability (Parnell et al., 2016; Santos et al., 2019). Both liquid and solid formulations are widely used. Currently, the most widespread dry formulation consists of peat as a carrier, plus other additives such as bacterial protectors and adhesives (Bashan et al., 2014; Atieno et al., 2018; Santos et al., 2019). Drying of microorganisms has been recognized as an efficient way of long-term preserving; however, desiccation is a physiologically challenging process, so protectants are added externally to the bacterial cells prior to drying (Berninger et al., 2017). Among other mineral or organic carriers, compost and biochar—the solid, carbon-rich product of heating biomass with the exclusion of air, with high porosity, large specific surface area, adsorption ability, and high cation exchange capacity—have been proposed as carriers with outstanding properties (Albareda et al., 2008; Arif et al., 2017; Egamberdieva et al., 2017; Song et al., 2020). It has also been shown that the combination of an inoculant strain together with a carbon source, such as glycerol in microgranules, confers a competitive advantage to the inoculant bacterium (Duquenne et al., 1999; Sessitsch et al., 2002). Biomaterials adopted from the field of drug delivery have been proposed as a technological opportunity for developing an advanced seed-coating (Zvinavashe et al., 2019). These authors have developed a seed-coating based on silk fibronin and trehalose that stabilizes and preserves rhizobia in saline and, possibly, arid environments. However, the non-biological components of the formulations remain key bottlenecks in the commercial development of inoculants (Bashan et al., 2014; Pastor-Bueis et al., 2019). For all these reasons, experiments in soil conditions are crucial, with the ultimate goal being the design of a successful inoculant based on an elite native strain with an adequate formulation which can result in greater grain yields than without the use of rhizobial inoculants. Or, in some cases, grain yields can even be similar or higher compared to chemical fertilization practices in inoculated fields with native strains (Mulas et al., 2015). Of course, the latest results have to be considered in an arid soil context, where the loss of total nitrogen input is leached at rates of up to 58% (Hu et al., 2010). In seeking elite strains for several pulse legumes in areas of difficult soils placing substantial stress on inoculant survival, Howieson et al., 2000, successfully developed an inoculant program based on cross-row experiments in successive years. The authors screened for nitrogen fixation, edaphic adaptation and performance *in situ* of rhizobial strains originally collected from the Mediterranean region, which reflects the edaphic characteristics of the target soils in Southern Australia. Inoculation with these better adapted strains, selected for their superior N_2-effectiveness, increased yield and nodulation in legume crops in infertile soils combining acidity and desiccation. Moreover, despite the competition for nodulation by background rhizobia at the site, assessment of nodule occupancy by the inoculant strains revealed all were present in >90% of nodules, securing the establishment of

pulse crops in difficult soils. Below we explain why rhizobial inoculant strains that are applied to legumes in field conditions often fail to nodulate legumes in competition with native strains (López-García et al., 2002; Naeem et al., 2004; Yates et al., 2011; Drew et al., 2012).

GENETIC STABILITY OF INOCULANT STRAINS

The genetic instability of inoculant strains and the exchange of symbiotic plasmids contribute to the diversity of naturalized populations and the lack of inoculant persistence (Ronson and Lowther, 1995). Several reports based on the phylogeny of *nod* genes—located in the symbiotic plasmid or symbiotic islands—have shown that the Sym plasmid is not strictly associated with the chromosomal background in natural populations of rhizobia (Schofield et al., 1987; Young and Wexler, 1988; Laguerre et al., 1992; Louvrier et al., 1996; Andrews et al., 2018). Since lateral gene and plasmid transfers are the major drivers of symbiotic phenotype evolution, bacterial genospecies are not ecologically relevant for symbiotic traits (Kumar et al., 2015; Andrews et al., 2018; Boivin et al., 2020). Symbiovars reflect the symbiosis plasmid rather than chromosome diversity (Kumar et al., 2015).

While the transfer of symbiosis genes to bacteria adapted to local soil conditions can allow them to become symbionts of previously incompatible legumes growing in those soils (Sullivan et al., 1995; Barcellos et al., 2007; Rivas et al., 2007; Andrews et al., 2018), in the case of inoculant strains this common and widespread phenomenon in fact results in the opposite outcome. Natural transfer of symbiotic islands by mobile integrative and conjugative elements has been demonstrated in field trials with mesorhizobia (Sullivan et al., 1995; Sullivan and Ronson, 1998; Nandasena et al., 2006, 2007; Hill et al., 2021), where resident non-nodulating bacteria accepted symbiotic genes from the inoculant *Mesorhizobium* strain, often resulting in highly competitive strains with poor nitrogen fixation capabilities that outcompete the original inoculant, potentially rendering it ineffective (Sullivan et al., 1995; Nandasena et al., 2007; Sotelo et al., 2011). Self-transmissible plasmids may be maintained within field isolates because they confer selective advantages on host strains (Meade et al., 1985). Genes required for the catabolism of plant exudates or the utilization of a range of carbon sources are often located on plasmids. For example, the genes involved in the degradation of rhamnose were reported to be induced by root extracts from the host plant, playing a role in competition of *R. leguminosarum* bv. *trifolii* in the early stages of the symbiotic interaction (Oresnik et al., 1998). Mutants unable to utilize this carbon source had impaired competitive abilities. The same applies to homoserine, an amino acid abundantly exuded by pea roots. *R. leguminosarum* bv. *viciae* strains able to use homoserine as a carbon and nitrogen source were found to be prevalent in pea nodules (Hynes and O'Connell, 1990; Vanderlinde et al., 2014). However, this does not exclude the possibility that some genes present on the chromosome may also contribute to the symbiotic phenotype and its variation (Boivin et al., 2020; Ferguson et al., 2020).

ERRATIC PERFORMANCE OF INOCULANTS

In many locations, native rhizobial populations are either not effective, or do not occur in sufficient number to meet the nitrogen demand of promiscuous cultivars, leading to a safer inoculation approach with exotic elite rhizobial strains instead of relying on resident strains of unknown potential (Chibeba et al., 2017). Moreover, although rhizobia are ubiquitous in the soil, the introduction of new plant species in a different location usually results in a lack of co-evolved rhizobial strains in soils abroad (Mpepereki et al., 2000; Giller, 2001; Simonsen et al., 2017; Bouznif et al., 2019). Successful introduction of crops into new regions is, therefore, dependent on inoculation with exotic rhizobia. Many examples in the Southern hemisphere illustrate this phenomenon, such as African soils and soybean cultivars (Okogun and Sanginga, 2003; Abaidoo et al., 2007; Klogo et al., 2015), forage and grain legumes in Australian and New Zealand soils (Ronson and Lowther, 1995; Hill et al., 2021), soybean in Argentina (Iturralde et al., 2019), common bean in Brazilian soils (Hungria et al., 2003), or forage production of *Lotus corniculatus* and clover in Uruguay (Sotelo et al., 2011, Irisarri et al., 2019). However, this leads to another problem with promiscuous cultivars: commercial inoculants based on exotic rhizobial strains selected for their efficiency in nitrogen fixation fail to establish a successful symbiosis by not being competitive against inefficient native rhizobia (Martínez-Romero, 2003; Pastor-Bueis et al., 2019; Shamseldin and Velázquez, 2020). Indeed, the absence of the inoculated strain in the nodules leads to low productivity, attributed to poor performance of the inoculant, and lack of consistency in field performance (Sotelo et al., 2011; Irisarri et al., 2019). The infection of legume crops by native populations of ineffective but competitive local rhizobia is known as "the competition problem" and causes yield decreases of legume crops (Triplett and Sadowsky, 1992; Friesen, 2012). Due to these inoculation problems, farmers prefer to use chemical fertilizers rather than rely on the capacity of the inoculants to carry out an efficient BNF (Pastor-Bueis et al., 2019). Therefore, the design of tailored inoculants by selecting naturally-evolved and locally-sourced rhizobia with outstanding symbiotic performance (effectiveness) that are already adapted to the agroclimatic conditions of a particular region (competitiveness) is of fundamental importance for agricultural lands worldwide in order to tackle this competition problem and increase crop yields without the use of nitrogen fertilizers (Chibeba et al., 2017; Pastor-Bueis et al., 2019). Indeed, local adaptation is a more important evolutionary force shaping microbial cooperation than is partner choice (Batstone et al., 2020). External factors are major determinants of the high competitiveness of soil native populations, such as the physiological condition and the growth age of the rhizobial culture, the distribution of the bacterial cells into the soil, the presence of salt and the soil pH of the sampling location, the soil surface coverage and soil temperatures during the crop season, or the resistance to the herbicides applied to the soil as part of the agricultural practice (López-García et al., 2002; Albareda et al., 2006; Iturralde et al., 2019). Therefore, different field scenarios need to be investigated. Methods to conduct field

trials with those elite strains are being developed to ascertain their superiority in fixing nitrogen in the presence of native and/or commercial strains (Mendoza-Suárez et al., 2020).

MOVING TOWARD TAILORED INOCULANTS

The addition of chemical fertilizers has a negative impact on the soil microbiome, as plants no longer need to interact with beneficial bacteria to access the nutrients that are being externally supplied and, therefore, the diversity of the microbial community in the root environment is reduced (Zhu et al., 2016; Kavamura et al., 2018). A study performed in wheat has shown that chemical fertilizers reduce the number of bacteria associated with the roots that solubilise nutrients such as nitrogen, potassium, phosphorous, iron, and zinc (Reid et al., 2021). Strikingly, the number of growth-promoting bacteria living on the roots fell from 91% of total bacteria for unfertilized plants, to just 19% for those that received the fertilizer dose. Chemical nitrogen inputs for a period of 22 years have guided the evolution of less-mutualistic rhizobia which produce 17–30% less biomass on *Trifolium* species compared to plants inoculated with rhizobia from non-fertilized control plots (Weese et al., 2015), which clearly shows the great negative environmental consequences of long-term input of chemical fertilizers in agricultural systems. Therefore, optimizing microbiome function is essential for sustainable agriculture (Hartmann et al., 2015).

On the other hand, Batstone et al. (2020) have found that after five plant generations, *M. truncatula* select for a more efficient *E. meliloti* symbiont. The superior host benefits were observed when the plant-microbe partnership shared an evolutionary history. Incorporating rhizobial strains with closely-related hosts is more likely to be effective, but it is necessary to give the new microbe sufficient time to adapt to their new environment. Schlaeppi and Bulgarelli (2015) were the first authors to foresee next-generation agriculture that aims to customize practices and tools, such as microbial inoculants, inspired by the concept of personalized diagnosis in medicine. Bell et al. (2019) proposed a customizable field-scale microbial inoculant that could have long-lasting effects with appropriate implementation. These innovative biofertilizer technologies based on tailored design and the implementation of effective agricultural microbiome manipulations and management strategies will benefit both consumers and producers of worldwide food supply (Busby et al., 2017; Mitter et al., 2021). Particularly in marginal soils of arid and semi-arid regions, inoculation is a crucial agricultural practice, with BNF being the major way to introduce nitrogen (Zahran, 1999). Not only that, but rhizobial inoculants also have other important and promising ecological applications, such as the restoration of habitats or the conservation of endangered plant species (Rodríguez-Echeverría et al., 2003; Thrall et al., 2005; Beyhaut et al., 2014; Navarro et al., 2014).

Although, as we have seen, it is well known that taking into account *Rhizobium* genotype (Gr) interactions together with root and soil microbiota ecology (M), plant host genotype (Gp), and/or environment (E), is important for evaluating whether selected strains are going to be suitable as inoculants (**Figure 3C**; Roskothen, 1989; Hungria and Vargas, 2000; Sessitsch et al., 2002; Busby et al., 2017; diCenzo et al., 2019)—note that authors have used different abbreviations—much research on competition has nevertheless focussed on changing only one or a few variables at a time. This is due to the complexity of distinguishing specific strains of interest, the limitations of running scaled-up plant experiments, and a lack of knowledge in understanding the ecological component of heterogenous communities and complex environments (including field trials). Of course, the big challenge is which one of these variables is most important to study or control? Although multicomponent interaction studies can be perceived as an overwhelming and titanic task, we can rely on a multidisciplinary approach to tackle the problem. Indeed, more and more multicomponent interaction studies are being conducted (Vuong et al., 2017; Burghardt et al., 2018; Gunnabo et al., 2019; Batstone et al., 2020; Mendoza-Suárez et al., 2020; Fagorzi et al., 2021), and they are already generating important knowledge to help understand some of these interactions and give more weight to the performance of rhizobial inoculants. Additionally, the rapid progress in NGS with open-source laboratory equipment automation (**Figure 3C**; Wong et al., 2018; Faiña et al., 2020), and the application of machine learning in big data analysis of microbiome studies (Cammarota et al., 2020; Ghannam and Techtmann, 2021) and in biological-image analysis (Berg et al., 2019; Chung et al., 2020; Tausen et al., 2020), will make multicomponent interactions studies more achievable, providing the opportunity to identify a larger number of elite strains in less time. The introduction of a wider number of variables in the experimental assays conducted to identify elite strains will increase the probability of designing rhizobial inoculants with better performance under field conditions. Once elite strains are identified in greenhouse experiments exploring multicomponent interactions, strict testing needs to be conducted in field trials before their commercialisation (Soumare et al., 2020; **Figure 3C**). Due to the complexity and genetic diversity within the soil and plant microbiome, it is unlikely that one formulation will be effective for all fields (Mitter et al., 2021). Yet, it might be unrealistic to design tailored inoculants for each individual field (Parnell et al., 2016). However, it may be possible to first carry out broader and cheaper assays to assess soil factors such as pH, nutrient and organic material content, and then correlate the physicochemical properties of the field with an already established collection of strains that perform well in those field conditions. Tailored inoculants may also become cheaper with increased production, which gives an opportunity to learn how to improve the production process and begin a positive-feedback cycle of increasing demand and falling prices (Schmidt et al., 2017; Lafond et al., 2020). For this reason, we suggest that efforts should be made to develop a multidisciplinary validation system that targets optimal performance for a given set of components. Thanks to progress in multicomponent interactions and multidisciplinary studies, it could become possible to offer farmers tailored rhizobial inoculants in the near future by conducting full physicochemical and microbiological soil analysis, in a similar way to fertilizer consultants offering

an "inoculant recommendation" for specific legume crops to grow in the next agricultural cycle (**Figure 3**). This inoculant advice would work in the same way that chemical fertilizer recommendations based on soil testing are currently offered and would reinforce and complement the farmer's current agricultural practice.

CONCLUSION

Ensuring the sustainability of agriculture becomes more important in light of future challenges such as climate change or the rapid growth of the human population (Schlaeppi and Bulgarelli, 2015). Next-generation agriculture will greatly benefit from the development of rhizobial bioinoculants based on elite strains that combine effectiveness and competitiveness under field conditions. The aforementioned areas of research in competition are shedding light on many of the processes that affect the performance of the inoculant and should be taken into account. The advances in imaging and sequencing technologies are improving our knowledge about all the stages in the symbiotic process, deciphering the mechanisms of cheating and cooperative bacterial behaviors, uncovering the genetic features behind competition and allowing high-throughput approaches to assess this trait, also under field conditions. Moreover, machine learning will allow us to automate assays and data analysis. In the longer term, these advances will contribute to solving the competition problem by allowing cost-efficient design and production of site-specific inoculants.

AUTHOR CONTRIBUTIONS

MM-S and CS-C wrote the manuscript. SA and PP provided critical feedback. All authors conceived and approved the final version of the manuscript.

FUNDING

This work was supported by The Leverhulme Trust (grant RPG-2019-246), the Biotechnology and Biological Sciences Research Council (grant number BB/N003608/1), and the ERA-NET Cofound SusCrop (grant N°771134), being part of the Joint Programming Initiative on Agriculture, Food Security and Climate Change (FACCE-JPI; SA).

ACKNOWLEDGMENTS

This article is dedicated to Tomas Ruíz-Argüeso, who was a warm, inspiring, and incredibly supportive mentor of many researchers working in the field of nitrogen fixation. We thank Leila Walker for editing the manuscript and Laura Clark for providing the competition assay photo using fluorescent proteins. We apologize to any researchers whose work was omitted without intention.

REFERENCES

Abaidoo, R. C., Keyser, H. H., Singleton, P. W., Dashiell, K. E., and Sanginga, N. (2007). Population size, distribution, and symbiotic characteristics of indigenous *Bradyrhizobium* spp. that nodulate TGx soybean genotypes in Africa. *Appl. Soil Ecol.* 35, 57–67. doi: 10.1016/j.apsoil.2006.05.006

Acosta-Jurado, S., Fuentes-romero, F., Ruiz-sainz, J. E., Janczarek, M., and Vinardell, J. M. (2021). Rhizobial exopolysaccharides: genetic regulation of their synthesis and relevance in symbiosis with legumes. *Int. J. Mol. Sci.* 22, 1–27. doi: 10.3390/ijms22126233

Aguilar, A., Mora, Y., Dávalos, A., Girard, L., Mora, J., and Peralta, H. (2018). Analysis of genome sequence and symbiotic ability of rhizobial strains isolated from seeds of common bean (*Phaseolus vulgaris*). *BMC Genomics* 19:645. doi: 10.1186/s12864-018-5023-0

Aguilar, O. M., Riva, O., and Peltzer, E. (2004). Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports coevolution in centers of host diversification. *Proc. Natl. Acad. Sci. U.S.A.* 101, 13548–13553. doi: 10.1073/PNAS.0405321101

Albareda, M., Dardanelli, M. S., Sousa, C., Megías, M., Temprano, F., and Rodríguez-Navarro, D. N. (2006). Factors affecting the attachment of rhizospheric bacteria to bean and soybean roots. *FEMS Microbiol. Lett.* 259, 67–73. doi: 10.1111/j.1574-6968.2006.00244.x

Albareda, M., Rodríguez-Navarro, D. N., Camacho, M., and Temprano, F. J. (2008). Alternatives to peat as a carrier for rhizobia inoculants: solid and liquid formulations. *Soil Biol. Biochem.* 40, 2771–2779. doi: 10.1016/j.soilbio.2008.07.021

Allsopp, L. P., Bernal, P., Nolan, L. M., and Filloux, A. (2020). Causalities of war: the connection between type VI secretion system and microbiota. *Cell. Microbiol.* 22:e13153. doi: 10.1111/cmi.13153

Amarger, N. (1981). Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti*. *Soil Biol. Biochem.* 13, 475–480. doi: 10.1016/0038-0717(81)90037-7

Ampe, F., Kiss, E., Sabourdy, F., and Batut, J. (2003). Transcriptome analysis of *Sinorhizobium meliloti* during symbiosis. *Genome Biol.* 4:R15. doi: 10.1186/gb-2003-4-2-r15

Andrews, M., De Meyer, S., James, E. K., Stępkowski, T., Hodge, S., Simon, M. F., et al. (2018). Horizontal transfer of symbiosis genes within and between rhizobial genera: occurrence and importance. *Genes* 9:321. doi: 10.3390/genes9070321

Aneja, P., Zachertowska, A., and Charles, T. C. (2005). Comparison of the symbiotic and competition phenotypes of *Sinorhizobium meliloti* PHB synthesis and degradation pathway mutants. *Can. J. Microbiol.* 51, 599–604. doi: 10.1139/w05-042

Anyango, B., Wilson, K. J., Beynon, J. L., and Giller, K. E. (1995). Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Appl. Environ. Microbiol.* 61, 4016–4021. doi: 10.1128/aem.61.11.4016-4021.1995

Arif, M. S., Shahzad, S. M., Riaz, M., Yasmeen, T., Shahzad, T., Akhtar, M. J., et al. (2017). Nitrogen-enriched compost application combined with plant growth-promoting rhizobacteria (PGPR) improves seed quality and nutrient use efficiency of sunflower. *J. Plant Nutr. Soil Sci.* 180, 464–473. doi: 10.1002/jpln.201600615

Arnold, M. F. F., Penterman, J., Shabab, M., Chen, E. J., and Walker, G. C. (2018). Important late-stage symbiotic role of the *Sinorhizobium meliloti* Exopolysaccharide Succinoglycan. *J. Bacteriol.* 200:e00665-17. doi: 10.1128/JB.00665-17

Atieno, M., Wilson, N., Casteriano, A., Crossett, B., Lesueur, D., and Deaker, R. (2018). Aqueous peat extract exposes rhizobia to sub-lethal stress which may prime cells for improved desiccation tolerance. *Appl. Microbiol. Biotechnol.* 102, 7521–7539. doi: 10.1007/s00253-018-9086-2

Ausmees, N., Jacobsson, K., and Lindberg, M. (2001). A unipolarly located, cell-surface-associated agglutinin, RapA, belongs to a family of Rhizobium-adhering proteins (Rap) in *Rhizobium leguminosarum* bv. *trifolii*. *Microbiology* 147, 549–559.

Ausmees, N., Kobayashi, H., Deakin, W. J., Marie, C., Krishnan, H. B., Broughton, W. J., et al. (2004). Characterization of NopP, a type III secreted effector of *Rhizobium* sp. strain NGR234. *J. Bacteriol.* 186, 4774–4780. doi: 10.1128/JB.186.14.4774-4780.2004

Badri, D. V., and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant. Cell Environ.* 32, 666–681. doi: 10.1111/j.1365-3040.2009.01926.x

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Barcellos, F. G., Menna, P., Batista, J. S. D. S., and Hungria, M. (2007). Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium* (Ensifer) *fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil. *Appl. Environ. Microbiol.* 73, 2635–2643. doi: 10.1128/AEM.01823-06

Barnett, M. J., Toman, C. J., Fisher, R. F., and Long, S. R. (2004). A dual-genome Symbiosis Chip for coordinate study of signal exchange and development in a prokaryote-host interaction. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16636–16641. doi: 10.1073/pnas.0407269101

Barsomian, G. D., Urzainqui, A., Lohman, K., and Walker, G. C. (1992). *Rhizobium meliloti* mutants unable to synthesize anthranilate display a novel symbiotic phenotype. *J. Bacteriol.* 174, 4416–4426. doi: 10.1128/jb.174.13.4416-4426.1992

Barton, L. L., Johnson, G. V., Schitoskey, K., and Wertz, M. (1996). Siderophore-mediated iron metabolism in growth and nitrogen fixation by alfalfa nodulated with *Rhizobium meliloti*. *J. Plant Nutr.* 19, 1201–1210. doi: 10.1080/01904169609365191

Bashan, Y., De-Bashan, L. E., Prabhu, S. R., and Hernandez, J. P. (2014). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998-2013). *Plant Soil* 378, 1–33. doi: 10.1007/s11104-013-1956-x

Batista, L., Irisarri, P., Rebuffo, M., Cuitiño, M. J., Sanjuán, J., and Monza, J. (2015). Nodulation competitiveness as a requisite for improved rhizobial inoculants of *Trifolium pratense*. *Biol. Fertil. Soils* 51, 11–20. doi: 10.1007/s00374-014-0946-3

Batstone, R. T., O'Brien, A. M., Harrison, T. L., and Frederickson, M. E. (2020). Experimental evolution makes microbes more cooperative with their local host genotype. *Science* 370, 23–26. doi: 10.1126/science.abb7222

Bauer, W. D., and Caetano-Anollés, G. (1990). Chemotaxis, induced gene expression and competitiveness in the rhizosphere. *Plant Soil* 129, 45–52. doi: 10.1007/BF00011690

Bell, T. H., Kaminsky, L. M., Gugino, B. K., Carlson, J. E., Malik, R. J., Hockett, K. L., et al. (2019). Factoring ecological, societal, and economic considerations into inoculant development. *Trends Biotechnol.* 37, 572–573. doi: 10.1016/j.tibtech.2019.02.009

Bellabarba, A., Bacci, G., Decorosi, F., Aun, E., Azzarello, E., Remm, M., et al. (2020). Competitiveness prediction for nodule colonization in *Sinorhizobium meliloti* through combined in vitro tagged strain characterization and genome-wide association analysis. *bioRxiv* [Preprint]. doi: 10.1101/2020.09.15.298034

Berg, S., Kutra, D., Kroeger, T., Straehle, C. N., Kausler, B. X., Haubold, C., et al. (2019). ilastik: interactive machine learning for (bio)image analysis. *Nat. Methods* 16, 1226–1232. doi: 10.1038/s41592-019-0582-9

Berninger, T., González López, Ó, Bejarano, A., Preininger, C., and Sessitsch, A. (2017). Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microb. Biotechnol.* 11, 277–301. doi: 10.1111/1751-7915.12880

Beyhaut, E., Larson, D. L., Allan, D. L., and Graham, P. H. (2014). Legumes in prairie restoration: evidence for wide cross-nodulation and improved inoculant delivery. *Plant Soil* 377, 245–258. doi: 10.1007/s11104-013-1999-z

Bittinger, M. A., Milner, J. L., Saville, B. J., and Handelsman, J. (1997). rosR, A determinant of nodulation competitiveness in *Rhizobium etli*. *Mol. Plant Microbe Interact.* 10, 180–186. doi: 10.1094/MPMI.1997.10.2.180

Black, M., Moolhuijzen, P., Chapman, B., Barrero, R., Howieson, J., Hungria, M., et al. (2012). The genetics of symbiotic nitrogen fixation: comparative genomics of 14 rhizobia strains by resolution of protein clusters. *Genes* 3, 138–166. doi: 10.3390/genes3010138

Bladergroen, M. R., Badelt, K., and Spaink, H. P. (2003). Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. *Mol. Plant Microbe Interact.* 16, 53–64. doi: 10.1094/MPMI.2003.16.1.53

Bogino, P., Nievas, F., Banchio, E., and Giordano, W. (2011). Increased competitiveness and efficiency of biological nitrogen fixation in peanut via in-furrow inoculation of rhizobia. *Eur. J. Soil Biol.* 47, 188–193. doi: 10.1016/j.ejsobi.2011.01.005

Boivin, S., Ait Lahmidi, N., Sherlock, D., Bonhomme, M., Dijon, D., Heulin-Gotty, K., et al. (2020). Host-specific competitiveness to form nodules in *Rhizobium leguminosarum* symbiovar viciae. *New Phytol.* 226, 555–568. doi: 10.1111/nph.16392

Bourion, V., Heulin-Gotty, K., Aubert, V., Tisseyre, P., Chabert-Martinello, M., Pervent, M., et al. (2018). Co-inoculation of a Pea core-collection with diverse Rhizobial strains shows competitiveness for nodulation and efficiency of nitrogen fixation are distinct traits in the interaction. *Front. Plant Sci.* 8:2249. doi: 10.3389/fpls.2017.02249

Bouznif, B., Guefrachi, I., Rodríguez de la Vega, R. C., Hungria, M., Mars, M., Alunni, B., et al. (2019). Phylogeography of the *Bradyrhizobium* spp. Associated With Peanut, Arachis hypogaea: Fellow Travelers or New Associations?. *Front. Microbiol.* 10:2041. doi: 10.3389/fmicb.2019.02041

Brockwell, J., Bottomley, P. J., and Thies, J. E. (1995). Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant Soil* 174, 143–180. doi: 10.1007/BF00032245

Burghardt, L. T., Epstein, B., Guhlin, J., Nelson, M. S., Taylor, M. R., Young, N. D., et al. (2018). Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. *Proc. Natl. Acad. Sci. U.S.A.* 115, 2425–2430. doi: 10.1073/PNAS.1714246115

Busby, P. E., Soman, C., Wagner, M. R., Friesen, M. L., Kremer, J., Bennett, A., et al. (2017). Research priorities for harnessing plant microbiomes in sustainable agriculture. *PLoS Biol.* 15:e2001793. doi: 10.1371/journal.pbio.2001793

Caetano-Anollés, G., Wall, L. G., De Micheli, A. T., Macchi, E. M., Bauer, W. D., and Favelukes, G. (1988). Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti*. *Plant Physiol.* 86, 1228–1235. doi: 10.1104/pp.86.4.1228

Cai, H., Bai, Y., and Guo, C. (2018). Comparative genomics of 151 plant-associated bacteria reveal putative mechanisms underlying specific interactions between bacteria and plant hosts. *Genes Genomics* 40, 857–864. doi: 10.1007/s13258-018-0693-1

Cammarota, G., Ianiro, G., Ahern, A., Carbone, C., Temko, A., Claesson, M. J., et al. (2020). Gut microbiome, big data and machine learning to promote precision medicine for cancer. *Nat. Rev. Gastroenterol. Hepatol.* 17, 635–648. doi: 10.1038/s41575-020-0327-3

Campbell, G. R. O., Reuhs, B. L., and Walker, G. C. (2002). Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3938–3943. doi: 10.1073/pnas.062425699

Canarini, A., Kaiser, C., Merchant, A., Richter, A., and Wanek, W. (2019). Root exudation of primary metabolites: mechanisms and their roles in plant responses to environmental stimuli. *Front. Plant Sci.* 10:157. doi: 10.3389/fpls.2019.00157

Catroux, G., Hartmann, A., and Revellin, C. (2001). Trends in rhizobial inoculant production and use. *Plant Soil* 230, 21–30. doi: 10.1023/A:1004777115628

Cavassim, M. I. A., Moeskjær, S., Moslemi, C., Fields, B., Bachmann, A., Vilhjálmsson, B. J., et al. (2020). Symbiosis genes show a unique pattern of introgression and selection within a *Rhizobium leguminosarum* species complex. *Microb. Genomics* 6:e000351. doi: 10.1099/mgen.0.000351

Chao, M. C., Abel, S., Davis, B. M., and Waldor, M. K. (2016). The design and analysis of transposon insertion sequencing experiments. *Nat. Rev. Microbiol.* 14, 119–128. doi: 10.1038/nrmicro.2015.7

Checcucci, A., Azzarello, E., Bazzicalupo, M., Galardini, M., Lagomarsino, A., Mancuso, S., et al. (2016). Mixed nodule infection in *Sinorhizobium meliloti-Medicago sativa* symbiosis suggest the presence of cheating behavior. *Front. Plant Sci.* 7:835. doi: 10.3389/fpls.2016.00835

Checcucci, A., diCenzo, G. C., Bazzicalupo, M., and Mengoni, A. (2017). Trade, diplomacy, and warfare: the Quest for elite rhizobia inoculant strains. *Front. Microbiol.* 8:2207. doi: 10.3389/fmicb.2017.02207

Chhabra, S., Brazil, D., Morrissey, J., Burke, J. I., O'Gara, F., and Dowling, D. N. (2013). Characterization of mineral phosphate solubilization traits from a barley rhizosphere soil functional metagenome. *Microbiologyopen* 2, 717–724. doi: 10.1002/mbo3.110

Chibeba, A. M., Kyei-Boahen, S., Guimarães, M., de, F., Nogueira, M. A., and Hungria, M. (2017). Isolation, characterization and selection of indigenous *Bradyrhizobium* strains with outstanding symbiotic performance to increase soybean yields in Mozambique. *Agric. Ecosyst. Environ.* 246, 291–305. doi: 10.1016/j.agee.2017.06.017

Chomicki, G., Werner, G. D. A., West, S. A., and Kiers, E. T. (2020). Compartmentalization drives the evolution of symbiotic cooperation: compartmentalisation drives symbiosis. *Philos. Trans. R. Soc. B Biol. Sci.* 375:20160083.

Chung, Y. S., Lee, U., Heo, S., Silva, R. R., Na, C. I., and Kim, Y. (2020). Image-based machine learning characterizes root nodule in soybean exposed to silicon. *Front. Plant Sci.* 11:520161. doi: 10.3389/fpls.2020.520161

Cole, B. J., Feltcher, M. E., Waters, R. J., Wetmore, K. M., Mucyn, T. S., Ryan, E. M., et al. (2017). Genome-wide identification of bacterial plant colonization genes. *PLoS Biol.* 15:1–24. doi: 10.1371/journal.pbio.2002860

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

D'Haeze, W., and Holsters, M. (2004). Surface polysaccharides enable bacteria to evade plant immunity. *Trends Microbiol.* 12, 555–561. doi: 10.1016/j.tim.2004.10.009

Danso, S. K. A., and Bowen, G. D. (1989). Methods of inoculation and how they influence nodulation pattern and nitrogen fixation using two contrasting strains of *Bradyrhizobium japonicum*. *Soil Biol. Biochem.* 21, 1053–1058. doi: 10.1016/0038-0717(89)90043-6

Daubech, B., Remigi, P., de Moura, G. D., Marchetti, M., Pouzet, C. C., Auriac, M. C., et al. (2017). Spatio-temporal control of mutualism in legumes helps spread symbiotic nitrogen fixation. *eLife* 6:e28683. doi: 10.7554/eLife.28683

Davies, B. W., and Walker, G. C. (2007). Identification of novel *Sinorhizobium meliloti* mutants compromised for oxidative stress protection and symbiosis. *J. Bacteriol.* 189, 2110–2113. doi: 10.1128/JB.01802-06

Dazzo, F. B., Truchet, G. L., Sherwood, J. E., Hrabak, E. M., Abe, M., and Pankratz, S. H. (1984). Specific phases of root hair attachment in the *Rhizobium trifolii*-clover symbiosis. *Appl. Environ. Microbiol.* 48, 1140–1150. doi: 10.1128/aem.48.6.1140-1150.1984

De Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* 58, 2180–2187.

de Campos, S. B., Lardi, M., Gandolfi, A., Eberl, L., and Pessi, G. (2017). Mutations in Two *Paraburkholderia phymatum* Type VI secretion systems cause reduced fitness in interbacterial competition. *Front. Microbiol.* 8:2473. doi: 10.3389/fmicb.2017.02473

del Cerro, P., Rolla-Santos, A. A., Gomes, D. F., Marks, B. B., del Rosario Espuny, M., Rodríguez-Carvajal, M. Á., et al. (2015). Opening the "black box" of nodD3, nodD4 and nodD5 genes of *Rhizobium tropici* strain CIAT 899. *BMC Genomics* 16:864. doi: 10.1186/s12864-015-2033-z

De-la-Peña, C., Lei, Z., Watson, B. S., Sumner, L. W., and Vivanco, J. M. (2008). Root-microbe communication through protein secretion. *J. Biol. Chem.* 283, 25247–25255. doi: 10.1074/jbc.M801967200

diCenzo, G. C., Zamani, M., Checcucci, A., Fondi, M., Griffitts, J. S., Finan, T. M., et al. (2019). Multidisciplinary approaches for studying rhizobium–legume symbioses. *Can. J. Microbiol.* 65, 1–33. doi: 10.1139/cjm-2018-0377

Ding, H., Yip, C. B., Geddes, B. A., Oresnik, I. J., and Hynes, M. F. (2012). Glycerol utilization by *Rhizobium leguminosarum* requires an ABC transporter and affects competition for nodulation. *Microbiology* 158, 1369–1378. doi: 10.1099/mic.0.057281-0

Drahos, D. J., Hemming, B. G., and McPherson, S. (1986). Tracking recombinant organisms in the environment: β-gaiactosidase as a selectable non-antibiotic marker for fluorescent *Pseudomonas*. *Biotechnology* 4, 439–444. doi: 10.1038/nbt0586-439

Drew, E., Herridge, D., Ballard, R., Hara, G. O., Deaker, R., Denton, M., et al. (2012). *Inoculating Legumes: A Practical Guide*. Canberra: Grains Research and Development Corporation.

Drogue, B., Doré, H., Borland, S., Wisniewski-Dyé, F., and Prigent-Combaret, C. (2012). Which specificity in cooperation between phytostimulating rhizobacteria and plants? *Res. Microbiol.* 163, 500–510. doi: 10.1016/j.resmic.2012.08.006

Dunham, D. H., and Baldwin, I. L. (1931). Double infection of leguminous plants with good and poor strains of rhizobia. *Soil Sci.* 32, 235–249. doi: 10.1097/00010694-193109000-00007

Duodu, S., Brophy, C., Connolly, J., and Svenning, M. M. (2009). Competitiveness of a native *Rhizobium leguminosarum* biovar trifolii strain for nodule occupancy is manifested during infection. *Plant Soil* 318, 117–126.

Duquenne, P., Chenu, C., Richard, G., and Catroux, G. (1999). Effect of carbon source supply and its location on competition between inoculated and established bacterial strains in sterile soil microcosm. *FEMS Microbiol. Ecol.* 29, 331–339. doi: 10.1111/j.1574-6941.1999.tb00624.x

Egamberdieva, D., Reckling, M., and Wirth, S. (2017). Biochar-based *Bradyrhizobium inoculum* improves growth of lupin (*Lupinus angustifolius* L.) under drought stress. *Eur. J. Soil Biol.* 78, 38–42. doi: 10.1016/j.ejsobi.2016.11.007

Epstein, B., Abou-Shanab, R. A. I., Shamseldin, A., Taylor, M. R., Guhlin, J., Burghardt, L. T., et al. (2018). Genome-wide association analyses in the model Rhizobium *Ensifer meliloti*. *mSphere* 3:e00386-18. doi: 10.1128/msphere.00386-18

Esseling, J. J., Lhuissier, F. G. P., and Emons, A. M. C. (2003). Nod factor-induced root hair curling: continuous polar growth towards the point of Nod factor application. *Plant Physiol.* 132, 1982–1988. doi: 10.1104/pp.103.021634

Fagorzi, C., Bacci, G., Huang, R., Cangioli, L., Checcucci, A., Fini, M., et al. (2021). Nonadditive Transcriptomic Signatures of Genotype-by-genotype interactions during the initiation of plant-Rhizobium Symbiosis. *mSystems* 6:e00974-20. doi: 10.1101/2020.06.15.152710

Faiña, A., Nejati, B., and Stoy, K. (2020). Evobot: an open-source, modular, liquid handling robot for scientific experiments. *Appl. Sci.* 10:814. doi: 10.3390/app10030814

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M. H., Lin, Y. H., Reid, D. E., et al. (2010). Molecular analysis of legume nodule development and autoregulation. *J. Integr. Plant Biol.* 52, 61–76. doi: 10.1111/j.1744-7909.2010.00899.x

Ferguson, S., Major, A. S., Sullivan, J. T., Bourke, S. D., Kelly, S. J., Perry, B. J., et al. (2020). *Rhizobium leguminosarum* bv. *trifolii NodD*2 enhances competitive nodule colonization in the clover-rhizobium symbiosis. *Appl Environ Microbiol.* 86:e01268-20. doi: 10.1128/AEM.01268-20

Fields, B., Moeskjær, S., Friman, V. P., Andersen, S. U., and Young, J. P. W. (2021). MAUI-seq: metabarcoding using amplicons with unique molecular identifiers to improve error correction. *Mol. Ecol. Resour.* 21, 703–720. doi: 10.1111/1755-0998.13294

Fraysse, N., Couderc, F., and Poinsot, V. (2003). Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. *Eur. J. Biochem.* 270, 1365–1380. doi: 10.1046/j.1432-1033.2003.03492.x

Frederix, M., Edwards, A., Swiderska, A., Stanger, A., Karunakaran, R., Williams, A., et al. (2014). Mutation of praR in *Rhizobium leguminosarum* enhances root biofilms, improving nodulation competitiveness by increased expression of attachment proteins. *Mol. Microbiol.* 93, 464–478. doi: 10.1111/mmi.12670

Frey, S. D., and Blum, L. K. (1994). Effect of pH on competition for nodule occupancy by type I and type II strains of *Rhizobium leguminosarum* bv. *phaseoli*. *Plant Soil* 163, 157–164. doi: 10.1007/BF00007964

Friesen, M. L. (2012). Widespread fitness alignment in the legume-rhizobium symbiosis. *New Phytol.* 194, 1096–1111. doi: 10.1111/j.1469-8137.2012.04099.x

Fry, J., Wood, M., and Poole, P. S. (2001). Investigation of myo-inositol catabolism in *Rhizobium leguminosarum* bv. *viciae* and its effect on nodulation competitiveness. *Mol. Plant Microbe Interact.* 14, 1016–1025. doi: 10.1094/MPMI.2001.14.8.1016

Gage, D. J. (2019). "Infection thread development in model legumes," in *The Model Legume Medicago truncatula*, ed. F. de Bruijn (New York, NY: Taylor and Francis), 579–588. doi: 10.1002/9781119409144.ch72

Galardini, M., Pini, F., Bazzicalupo, M., Biondi, E. G., and Mengoni, A. (2013). Replicon-dependent bacterial genome evolution: the case of *Sinorhizobium meliloti*. *Genome Biol. Evol.* 5, 542–558. doi: 10.1093/gbe/evt027

Gallique, M., Bouteiller, M., and Merieau, A. (2017). The Type VI secretion system: A dynamic system for bacterial communication? *Front. Microbiol.* 8:1454. doi: 10.3389/fmicb.2017.01454

Geddes, B. A., González, J. E., and Oresnik, I. J. (2014). Exopolysaccharide production in response to medium acidification is correlated with an increase in

competition for nodule occupancy. *Mol. Plant Microbe Interact.* 27, 1307–1317. doi: 10.1094/MPMI-06-14-0168-R

Geddes, B. A., Kearsley, J. V. S., Huang, J., Zamani, M., Muhammed, Z., Sather, L., et al. (2021). Minimal gene set from *Sinorhizobium* (Ensifer) *meliloti* pSymA required for efficient symbiosis with Medicago. *Proc. Natl. Acad. Sci. U.S.A.* 118, e2018015118. doi: 10.1073/pnas.2018015118

Geddes, B. A., Mendoza-Suárez, M. A., and Poole, P. S. (2019a). A Bacterial Expression Vector Archive (BEVA) for flexible modular assembly of golden gate-compatible vectors. *Front. Microbiol.* 9:3345. doi: 10.3389/fmicb.2018.03345

Geddes, B. A., Paramasivan, P., Joffrin, A., Thompson, A. L., Christensen, K., Jorrin, B., et al. (2019b). Engineering transkingdom signalling in plants to control gene expression in rhizosphere bacteria. *Nat. Commun.* 10:3430. doi: 10.1038/s41467-019-10882-x

Ghannam, R. B., and Techtmann, S. M. (2021). Machine learning applications in microbial ecology, human microbiome studies, and environmental monitoring. *Comput. Struct. Biotechnol. J.* 19, 1092–1107. doi: 10.1016/j.csbj.2021.01.028

Giller, K. E. (2001). *Nitrogen Fixation in Tropical Cropping Systems*, 2nd Edn. Wallingford: CABI. doi: 10.1079/9780851994178.0000

González, V., Bustos, P., Ramírez-Romero, M. A., Medrano-Soto, A., Salgado, H., Hernández-González, I., et al. (2003). The mosaic structure of the symbiotic plasmid of *Rhizobium etli* CFN42 and its relation to other symbiotic genome compartments. *Genome Biol.* 4:R36. doi: 10.1186/gb-2003-4-6-r36

Goodman, A. L., Wu, M., and Gordon, J. I. (2011). Identifying microbial fitness determinants by insertion sequencing using genome-wide transposon mutant libraries. *Nat. Protoc.* 6, 1969–1980. doi: 10.1038/nprot.2011.417

Gordon, D. M., Ryder, M. H., Heinrich, K., and Murphy, P. J. (1996). An experimental test of the rhizopine concept in *Rhizobium meliloti*. *Appl. Environ. Microbiol.* 62, 3991–3996. doi: 10.1128/aem.62.11.3991-3996.1996

Graham, P. H. (2008). "Ecology of the root-nodule bacteria of legumes," in *Nitrogen-Fixing Leguminous Symbioses. Nitrogen Fixation: Origins, Applications, and Research Progress*, eds M. J. Dilworth, E. K. James, and J. I. Sprent (Berlin: Springer), 22–58. doi: 10.1007/978-1-4020-3548-7_2

Graham, P. H. (1969). Selective medium for growth of Rhizobium. *Appl. Microbiol.* 17, 769–770. doi: 10.1128/aem.17.5.769-770.1969

Graham, P. H., and Vance, C. P. (2000). Nitrogen fixation in perspective: an overview of research and extension needs. *Field Crops Res.* 65, 93–106. doi: 10.1016/S0378-4290(99)00080-5

Green, R. T., East, A. K., Karunakaran, R., Downie, J. A., and Poole, P. S. (2019). Transcriptomic analysis of *Rhizobium leguminosarum* bacteroids in determinate and indeterminate nodules. *Microb. Genomics* 5:e000254. doi: 10.1099/mgen.0.000254

Gunnabo, A. H., Geurts, R., Wolde-meskel, E., Degefu, T., Giller, K. E., and van Heerwaarden, J. (2019). Genetic interaction studies reveal superior performance of *Rhizobium tropici* CIAT899 on a range of diverse east african common bean (*Phaseolus vulgaris* L.) genotypes. *Appl. Environ. Microbiol.* 85:e01763-19. doi: 10.1128/AEM.01763-19

Gupta, A., Gopal, M., Thomas, G. V., Manikandan, V., Gajewski, J., Thomas, G. V., et al. (2014). Whole genome sequencing and analysis of plant growth promoting bacteria isolated from the rhizosphere of plantation crops coconut, cocoa and arecanut. *PLoS One* 9:e104259. doi: 10.1371/journal.pone.0104259

Han, Q., Ma, Q., Chen, Y., Tian, B., Xu, L., Bai, Y., et al. (2020). Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. *ISME J.* 14, 1915–1928. doi: 10.1038/s41396-020-0648-9

Hansen, B. L., Pessotti, R., de, C., Fischer, M. S., Collins, A., El-Hifnawi, L., et al. (2020). Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *mBio* 11:e01917-20. doi: 10.1128/mBio.01917-20

Harrison, P. W., Lower, R. P. J., Kim, N. K. D., and Young, J. P. W. (2010). Introducing the bacterial "chromid": not a chromosome, not a plasmid. *Trends Microbiol.* 18, 141–148. doi: 10.1016/j.tim.2009.12.010

Hartmann, M., Frey, B., Mayer, J., Mäder, P., and Widmer, F. (2015). Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J.* 9, 1177–1194. doi: 10.1038/ismej.2014.210

Herridge, D. F., Peoples, M. B., and Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311, 1–18. doi: 10.1007/s11104-008-9668-3

Hill, Y., Colombi, E., Bonello, E., Haskett, T., Ramsay, J., O'Hara, G., et al. (2021). Evolution of diverse effective N2-fixing microsymbionts of Cicer arietinum following horizontal transfer of the *Mesorhizobium ciceri* CC1192 symbiosis integrative and conjugative element. *Appl. Environ. Microbiol.* 87:e02558-20. doi: 10.1128/aem.02558-20

Hirsch, P. R. (1996). Population dynamics of indigenous and genetically modified rhizobia in the field. *New Phytol.* 133, 159–171. doi: 10.1111/j.1469-8137.1996.tb04351.x

Hirsch, P. R., Van Montagu, M., and Johnston, A. W. B. (1980). Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 120, 403–412. doi: 10.1099/00221287-120-2-403

Holland, A. A. (1966). Serologic characteristics of certain root-nodule bacteria of legumes. *Antonie Van Leeuwenhoek* 32, 410–418. doi: 10.1007/BF02097492

Hotter, G. S., and Scott, D. B. (1991). Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. *J. Bacteriol.* 173, 851–859. doi: 10.1128/jb.173.2.851-859.1991

Howieson, J. G., Malden, J., Yates, R. J., and O'Hara, G. W. (2000). Techniques for the selection and development of elite inoculant strains of *Rhizobium leguminosarum* in southern Australia. *Symbiosis* 28, 33–48.

Hu, K., Li, Y., Chen, W., Chen, D., Wei, Y., Edis, R., et al. (2010). Modeling nitrate leaching and optimizing water and nitrogen management under irrigated maize in desert oases in Northwestern China. *J. Environ. Qual.* 39, 667–677. doi: 10.2134/jeq2009.0204

Hungria, M., and Franco, A. A. (1993). Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L. *Plant Soil* 149, 95–102. doi: 10.1007/BF00010766

Hungria, M., and Vargas, M. A. T. (2000). Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Res.* 65, 151–164. doi: 10.1016/S0378-4290(99)00084-2

Hungria, M., Campo, R. J., and Mendes, I. C. (2003). Benefits of inoculation of the common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains. *Biol. Fertil. Soils* 39, 88–93. doi: 10.1007/s00374-003-0682-6

Hynes, M. F., and O'Connell, M. P. (1990). Host plant effect on competition among strains of *Rhizobium leguminosarum*. *Can. J. Microbiol.* 36, 864–869. doi: 10.1139/m90-150

Irisarri, P., Cardozo, G., Tartaglia, C., Reyno, R., Gutiérrez, P., Lattanzi, F. A., et al. (2019). Selection of competitive and efficient Rhizobia Strains for White Clover. *Front. Microbiol.* 10:768. doi: 10.3389/fmicb.2019.00768

Iturralde, E. T., Covelli, J. M., Alvarez, F., Pérez-Giménez, J., Arrese-Igor, C., and Lodeiro, A. R. (2019). Soybean-nodulating strains with low intrinsic competitiveness for nodulation, good symbiotic performance, and stress-tolerance isolated from soybean-cropped soils in Argentina. *Front. Microbiol.* 10:1061. doi: 10.3389/fmicb.2019.01061

Jaiswal, S. K., Mohammed, M., Ibny, F. Y. I., and Dakora, F. D. (2021). Rhizobia as a source of plant growth-promoting molecules: potential applications and possible operational mechanisms. *Front. Sustain. Food Syst.* 4:619676. doi: 10.3389/fsufs.2020.619676

Janczarek, M., Jaroszuk-Ściseł, J., and Skorupska, A. (2009). Multiple copies of rosR and pssA genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in *Rhizobium leguminosarum* bv. *trifolii*. *Antonie Van Leeuwenhoek* 96, 471–486. doi: 10.1007/s10482-009-9362-3

Janczarek, M., Kutkowska, J., Piersiak, T., and Skorupska, A. (2010). *Rhizobium leguminosarum* bv. *trifolii* rosR is required for interaction with clover, biofilm formation and adaptation to the environment. *BMC Microbiol.* 10:284. doi: 10.1186/1471-2180-10-284

Jeckel, H., and Drescher, K. (2020). Advances and opportunities in image analysis of bacterial cells and communities. *FEMS Microbiol. Rev.* 062, 1–14. doi: 10.1093/femsre/fuaa062

Ji, Z. J., Yan, H., Cui, Q. G., Wang, E. T., Chen, W. F., and Chen, W. X. (2017). Competition between rhizobia under different environmental conditions affects the nodulation of a legume. *Syst. Appl. Microbiol.* 40, 114–119. doi: 10.1016/j.syapm.2016.12.003

Jimenez-Zurdo, J. I., Van Dillewijn, P., Soto, M. J., De Felipe, M. R., Olivares, J., and Toro, N. (1995). Characterization of a *Rhizobium meliloti* proline

dehydrogenase mutant altered in nodulation efficiency and competitiveness on alfalfa roots. *Mol. Plant Microbe Interact.* 8, 492–498. doi: 10.1094/MPMI-8-0492

Jiménez-Guerrero, I., Pérez-Montaño, F., Medina, C., Ollero, F. J., and López-Baena, F. J. (2017). The *Sinorhizobium (Ensifer) fredii* HH103 nodulation outer protein NopI is a determinant for efficient nodulation of soybean and cowpea plants. *Appl. Environ. Microbiol.* 83:16. doi: 10.1128/AEM.02770-16

Johnston, A. W. B., and Beringer, J. E. (1976). Pea root nodules containing more than one *Rhizobium* species. *Nature* 263, 502–504. doi: 10.1038/263502a0

Jones, K. M. (2012). Increased production of the exopolysaccharide succinoglycan enhances *Sinorhizobium meliloti* 1021 symbiosis with the host plant *Medicago truncatula*. *J. Bacteriol.* 194, 4322–4331. doi: 10.1128/JB.00751-12

Jorrin, B., and Imperial, J. (2015). Population genomics analysis of legume host preference for specific rhizobial genotypes in the *Rhizobium leguminosarum* bv. *viciae symbioses*. *Mol. Plant Microbe Interact.* 28, 310–318. doi: 10.1094/MPMI-09-14-0296-FI

Josey, D. P., Beynon, J. L., Johnston, A. W. B., and Beringer, J. E. (1979). Strain identification in Rhizobium using intrinsic antibiotic resistance. *J. Appl. Bacteriol.* 46, 343–350. doi: 10.1111/j.1365-2672.1979.tb00830.x

Juhas, M., Crook, D. W., and Hood, D. W. (2008). Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. *Cell. Microbiol.* 10, 2377–2386. doi: 10.1111/j.1462-5822.2008.01187.x

Kasper, S., Christoffersen, B., Soti, P., and Racelis, A. (2019). Abiotic and biotic limitations to nodulation by leguminous cover crops in south Texas. *Agriculture* 9:209. doi: 10.3390/agriculture9100209

Kavamura, V. N., Hayat, R., Clark, I. M., Rossmann, M., Mendes, R., Hirsch, P. R., et al. (2018). Inorganic nitrogen application affects both taxonomical and predicted functional structure of wheat rhizosphere bacterial communities. *Front. Microbiol.* 9:1074. doi: 10.3389/fmicb.2018.01074

Kawaharada, Y., Kelly, S., Nielsen, M. W., Hjuler, C. T., Gysel, K., Muszyński, A., et al. (2015). Receptor-mediated exopolysaccharide perception controls bacterial infection. *Nature* 523, 308–312. doi: 10.1038/nature14611

Kawaharada, Y., Nielsen, M. W., Kelly, S., James, E. K., Andersen, K. R., Rasmussen, S. R., et al. (2017). Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. *Nat. Commun.* 8:14534. doi: 10.1038/ncomms14534

Kelly, S. J., Muszyński, A., Kawaharada, Y., Hubber, A. M., Sullivan, J. T., Sandal, N., et al. (2013). Conditional requirement for exopolysaccharide in the Mesorhizobium-Lotus symbiosis. *Mol. Plant Microbe Interact.* 26, 319–329. doi: 10.1094/MPMI-09-12-0227-R

Kiers, E. T., and Denison, R. F. (2008). Sanctions, cooperation, and the stability of plant-Rhizosphere Mutualisms. *Annu. Rev. Ecol. Evol. Syst.* 39, 215–236. doi: 10.1146/annurev.ecolsys.39.110707.173423

Kiers, E. T., Rousseau, R. A., West, S. A., and Denlson, R. F. (2003). Host sanctions and the legume-rhizobium mutualism. *Nature* 425, 78–81. doi: 10.1038/nature01931

Kinkema, M., Scott, P. T., and Gresshoff, P. M. (2006). Legume nodulation: successful symbiosis through short- and long-distance signalling. *Funct. Plant Biol.* 33, 707–721. doi: 10.1071/FP06056

Klogo, P. Y., Ofori, J., and Amaglo, H. (2015). Soybean (*Glycine Max* (L) Merill) promiscuity reaction to indigenous Bradyrhizobia inoculation In Some Ghanaian Soils. *Int. J. Sci. Technol. Res.* 4, 306–313.

Knights, H. E., Jorrin, B., Haskett, T. L., and Poole, P. S. (2021). Deciphering bacterial mechanisms of root colonisation. *Environ. Microbiol. Rep.* 13, 428–444. doi: 10.1111/1758-2229.12934

Kohler, P. R. A., Zheng, J. Y., Schoffers, E., and Rossbach, S. (2010). Inositol catabolism, a key pathway in *Sinorhizobium meliloti* for competitive host nodulation. *Appl. Environ. Microbiol.* 76, 7972–7980. doi: 10.1128/AEM.01972-10

Korenblum, E., Dong, Y., Szymanski, J., Panda, S., Jozwiak, A., Massalha, H., et al. (2020). Rhizosphere microbiome mediates systemic root metabolite exudation by root-to-root signaling. *Proc. Natl. Acad. Sci. U.S.A.* 117, 3874–3883. doi: 10.1073/PNAS.1912130117

Kramer, J., Özkaya, Ö., and Kümmerli, R. (2020). Bacterial siderophores in community and host interactions. *Nat. Rev. Microbiol.* 18, 152–163. doi: 10.1038/s41579-019-0284-4

Krönauer, C., and Radutoiu, S. (2021). Understanding Nod factor signalling paves the way for targeted engineering in legumes and non-legumes. *Curr. Opin. Plant Biol.* 62:102026. doi: 10.1016/j.pbi.2021.102026

Kumar, N., Lad, G., Giuntini, E., Kaye, M. E., Udomwong, P., Shamsani, N. J., et al. (2015). Bacterial genospecies that are not ecologically coherent: population genomics of *Rhizobium leguminosarum*. *Open Biol.* 5:140133. doi: 10.1098/rsob.140133

Kummer, R. M., and David Kuykendall, L. (1989). Symbiotic properties of amino acid auxotrophs of *Bradyrhizobium japonicum*. *Soil Biol. Biochem.* 21, 779–782. doi: 10.1016/0038-0717(89)90170-3

Kyei-Boahen, S., Savala, C. E. N., Chikoye, D., and Abaidoo, R. (2017). Growth and yield responses of cowpea to inoculation and phosphorus fertilization in different environments. *Front. Plant Sci.* 8:646. doi: 10.3389/fpls.2017.00646

Lafond, F., Greenwald, D. S., and Farmer, J. D. (2020). Can stimulating demand drive costs down? World war II as a natural experiment. *Soc. Sci. Res. Netw.* 1–61. doi: 10.2139/ssrn.3519913

Lagares, A., Caetano-Anolles, G., Niehaus, K., Lorenzen, J., Ljunggren, H. D., Puhler, A., et al. (1992). A *Rhizobium meliloti* lipopolysaccharide mutant altered in competitiveness for nodulation of alfalfa. *J. Bacteriol.* 174, 5941–5952. doi: 10.1128/jb.174.18.5941-5952.1992

Laguerre, G., Heulin-Gotty, K., Brunel, B., Klonowska, A., Le Quéré, A., Tillard, P., et al. (2012). Local and systemic N signaling are involved in *Medicago truncatula* preference for the most efficient Sinorhizobium symbiotic partners. *New Phytol.* 195, 437–449. doi: 10.1111/j.1469-8137.2012.04159.x

Laguerre, G., Louvrier, P., Allard, M. R., and Amarger, N. (2003). Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar viciae for nodulation of host legumes. *Appl. Environ. Microbiol.* 69, 2276–2283. doi: 10.1128/AEM.69.4.2276-2283.2003

Laguerre, G., Mazurier, S. I., and Amarger, N. (1992). Plasmid profiles and restriction fragment length polymorphism of *Rhizobium leguminosarum* bv. *viciae* in field populations. *FEMS Microbiol. Ecol.* 101, 17–26. doi: 10.1111/j.1574-6941.1992.tb01644.x

Lardi, M., de Campos, S. B., Purtschert, G., Eberl, L., and Pessi, G. (2017). Competition experiments for legume infection identify *Burkholderia phymatum* as a highly competitive β-rhizobium. *Front. Microbiol.* 8:1527. doi: 10.3389/fmicb.2017.01527

Laus, M. C., Logman, T. J., Lamers, G. E., Van Brussel, A. A. N., Carlson, R. W., and Kijne, J. W. (2006). A novel polar surface polysaccharide from *Rhizobium leguminosarum* binds host plant lectin. *Mol. Microbiol.* 59, 1704–1713. doi: 10.1111/j.1365-2958.2006.05057.x

Ledermann, R., Schulte, C. C. M. M., and Poole, P. S. (2021). How rhizobia adapt to the nodule environment. *J. Bacteriol.* 203:e0539-20. doi: 10.1128/jb.00539-20

Lin, J., Frank, M., and Reid, D. (2020). No home without hormones: How plant hormones control legume nodule organogenesis. *Plant Commun.* 1:100104. doi: 10.1016/j.xplc.2020.100104

Liu, D., Yang, Q., Ge, K., Hu, X., Qi, G., Du, B., et al. (2017). Promotion of iron nutrition and growth on peanut by *Paenibacillus illinoisensis* and *Bacillus* sp. strains in calcareous soil. *Braz. J. Microbiol.* 48, 656–670. doi: 10.1016/j.bjm.2017.02.006

Liu, F., Hewezi, T., Lebeis, S. L., Pantalone, V., Grewal, P. S., and Staton, M. E. (2019). Soil indigenous microbiome and plant genotypes cooperatively modify soybean rhizosphere microbiome assembly. *BMC Microbiol.* 19:201. doi: 10.1186/s12866-019-1572-x

Löhnis, M. P. (1930). Can *Bacterium radicicola* assimilate nitrogen in the absence of the host plant? *Soil Sci.* 29, 37–57.

López-García, S. L., Perticari, A., Piccinetti, C., Ventimiglia, L., Arias, N., De Battista, J. J., et al. (2009). In-furrow inoculation and selection for higher motility enhances the efficacy of *Bradyrhizobium japonicum* nodulation. *Agron. J.* 101, 357–363. doi: 10.2134/agronj2008.0155x

López-García, S. L., Vázquez, T. E. E. E., Favelukes, G., and Lodeiro, A. R. (2002). Rhizobial position as a main determinant in the problem of competition for nodulation in soybean. *Environ. Microbiol.* 4, 216–224. doi: 10.1046/j.1462-2920.2002.00287.x

Louvrier, P., Laguerre, G., and Amarger, N. (1996). Distribution of symbiotic genotypes in *Rhizobium leguminosarum* biovar viciae populations isolated directly from soils. *Appl. Environ. Microbiol.* 62, 4202–4205. doi: 10.1128/aem.62.11.4202-4205.1996

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

Lupwayi, N. Z., Clayton, G. W., and Rice, W. A. (2006). Rhizobial inoculants for legume crops. *J. Crop Improv.* 15, 97–125. doi: 10.1300/J411v15n02

Lynch, D., O'Brien, J., Welch, T., Clarke, P., Cuív, P. O., Crosa, J. H., et al. (2001). Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti*. *J. Bacteriol.* 183, 2576–2585. doi: 10.1128/JB.183.8.2576-2585.2001

Ma, W., Penrose, D. M., and Glick, B. R. (2002). Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. *Can. J. Microbiol.* 48, 947–954. doi: 10.1139/w02-100

MacLean, A. M., Finan, T. M., and Sadowsky, M. J. (2007). Genomes of the symbiotic nitrogen-fixing bacteria of legumes. *Plant Physiol.* 144, 615–622. doi: 10.1104/pp.107.101634

Maillet, F., Fournier, J., Mendis, H. C., Tadege, M., Wen, J., Ratet, P., et al. (2020). *Sinorhizobium meliloti* succinylated high-molecular-weight succinoglycan and the *Medicago truncatula* LysM receptor-like kinase MtLYK10 participate independently in symbiotic infection. *Plant J.* 102, 311–326. doi: 10.1111/tpj.14625

Marczak, M., Mazur, A., Koper, P., Żebracki, K., and Skorupska, A. (2017). Synthesis of rhizobial exopolysaccharides and their importance for symbiosis with legume plants. *Genes* 8:360. doi: 10.3390/genes8120360

Margaret, I., Becker, A., Blom, J., Bonilla, I., Goesmann, A., Göttfert, M., et al. (2011). Symbiotic properties and first analyses of the genomic sequence of the fast growing model strain *Sinorhizobium fredii* HH103 nodulating soybean. *J. Biotechnol.* 155, 11–19. doi: 10.1016/j.jbiotec.2011.03.016

Mauchline, T. H., Fowler, J. E., East, A. K., Sartor, A. L., Zaheer, R., Hosie, A. H. F., et al. (2006). Mapping the *Sinorhizobium meliloti* 1021 solute-binding protein-dependent transportome. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17933–17938. doi: 10.1073/pnas.0606673103

Marie, C., Deakin, W. J., Viprey, V., Kopciñska, J., Golinowski, W., Krishnan, H. B., et al. (2003). Characterization of Nops, nodulation outer proteins, secreted via the type III secretion system of NGR234. *Mol. Plant Microbe Interact.* 16, 743–751. doi: 10.1094/MPMI.2003.16.9.743

Martínez-Romero, E. (2003). Diversity of Rhizobium-*Phaseolus vulgaris* symbiosis: overview and perspectives. *Plant Soil* 252, 11–23. doi: 10.1023/A:1024199013926

Martins Dos Santos, V. A. P., Heim, S., Moore, E. R. B., Strätz, M., and Timmis, K. N. (2004). Insights into the genomic basis of niche specificity of *Pseudomonas* putida KT2440. *Environ. Microbiol.* 6, 1264–1286. doi: 10.1111/j.1462-2920.2004.00734.x

Massalha, H., Korenblum, E., Malitsky, S., Shapiro, O. H., and Aharoni, A. (2017). Live imaging of root-bacteria interactions in a microfluidics setup. *Proc. Natl. Acad. Sci. U.S.A.* 114, 4549–4554. doi: 10.1073/pnas.1618584114

May, S. N., and Bohlool, B. B. (1983). Competition Among *Rhizobium leguminosarum* Strains for Nodulation of Lentils (Lens esculenta). *Appl. Environ. Microbiol.* 45, 960–965.

Meade, J., Higgins, P., and O'Gara, F. (1985). Studies on the Inoculation and Competitiveness of a *Rhizobium leguminosarum* Strain in Soils Containing Indigenous Rhizobia. *Appl. Environ. Microbiol.* 49, 899–903. doi: 10.1128/aem.49.4.899-903.1985

Mellor, H. Y., Glenn, A. R., Arwas, R., and Dilworth, M. J. (1987). Symbiotic and competitive properties of motility mutants of Rhizobium trifolii TA1. *Arch. Microbiol.* 148, 34–39. doi: 10.1007/BF00429644

Mendoza-Suárez, M. A., Geddes, B. A., Sánchez-Cañizares, C., Ramírez-González, R. H., Kirchhelle, C., Jorrin, B., et al. (2020). Optimizing Rhizobium- legume symbioses by simultaneous measurement of rhizobial competitiveness and N2 fixation in nodules. *Proc. Natl. Acad. Sci. U.S.A.* 117, 9822–9831. doi: 10.1073/pnas.1921225117

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., et al. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5230–5235. doi: 10.1073/pnas.0600912103

Miller, L. D., Yost, C. K., Hynes, M. F., and Alexandre, G. (2007). The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol. Microbiol.* 63, 348–362. doi: 10.1111/j.1365-2958.2006.05515.x

Mitter, E. K., Tosi, M., Obregón, D., Dunfield, K. E., and Germida, J. J. (2021). Rethinking crop nutrition in times of modern microbiology: innovative biofertilizer technologies. *Front. Sustain. Food Syst.* 5:606815. doi: 10.3389/fsufs.2021.606815

Moawad, M., and Schmidt, E. L. (1987). Occurrence and nature of mixed infections in nodules of field-grown soybeans (*Glycine max*). *Biol. Fertil. Soils* 5, 112–114. doi: 10.1007/BF00257644

Moeskjær, S., Tausen, M., Andersen, S. U., and Young, J. P. W. (2020). Amplicons and isolates: Rhizobium diversity in fields under conventional and organic management. *bioRxiv* [Preprint]. doi: 10.1101/2020.09.22.307934

Monchgesang, S., Strehmel, N., Schmidt, S., Westphal, L., Taruttis, F., Muller, E., et al. (2016). Natural variation of root exudates in *Arabidopsis thaliana*-linking metabolomic and genomic data. *Sci. Rep.* 6:29033. doi: 10.1038/srep29033

Montañez, A., Danso, S. K. A., and Hardarson, G. (1995). The effect of temperature on nodulation and nitrogen fixation by five *Bradyrhizobium japonicum* strains. *Appl. Soil Ecol.* 2, 165–174. doi: 10.1016/0929-1393(95)00052-M

Montiel, J., Downie, J. A., Farkas, A., Bihari, P., Herczeg, R., Bálint, B., et al. (2017). Morphotype of bacteroids in different legumes correlates with the number and type of symbiotic NCR peptides. *Proc. Natl. Acad. Sci. U.S.A.* 114, 5041–5046. doi: 10.1073/pnas.1704217114

Montiel, J., Reid, D., Gronbaek, T. H., Benfeldt, C. M., James, E. K., Ott, T., et al. (2021). Distinct signaling routes mediate intercellular and intracellular rhizobial infection in Lotus japonicus. *Plant Physiol.* 185, 1131–1147. doi: 10.1093/PLPHYS/KIAA049

Mortier, V., Holsters, M., and Goormachtig, S. (2012). Never too many? How legumes control nodule numbers. *Plant Cell Environ.* 35, 245–258. doi: 10.1111/j.1365-3040.2011.02406.x

Mpepereki, S., Javaheri, F., Davis, P., and Giller, K. E. (2000). Soyabeans and sustainable agriculture Promiscuous soyabeans in southern Africa. *Field Crops Res.* 65, 137–149. doi: 10.1016/S0378-4290(99)00083-0

Mulas, D., Seco, V., Casquero, P. A., Velázquez, E., and González-Andrés, F. (2015). Inoculation with indigenous rhizobium strains increases yields of common bean (*Phaseolus vulgaris* L.) in northern Spain, although its efficiency is affected by the tillage system. *Symbiosis* 67, 113–124. doi: 10.1007/s13199-015-0359-6

Muller, K. E., and Denison, R. F. (2018). Resource acquisition and allocation traits in symbiotic rhizobia with implications for life-history outside of legume hosts. *R. Soc. Open Sci.* 5:124. doi: 10.1098/rsos.181124

Murphy, P. J., Heycke, N., and Banfalvi, Z. (1987). Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. *Proc. Natl. Acad. Sci. U.S.A.* 84, 493–497. doi: 10.1073/pnas.84.2.493

Mutch, L. A., and Young, J. P. W. (2004). Diversity and specificity of *Rhizobium leguminosarum* biovar viciae on wild and cultivated legumes. *Mol. Ecol.* 13, 2435–2444. doi: 10.1111/j.1365-294X.2004.02259.x

Naeem, F. I., Ashraf, M. M., Malik, K. A., and Hafeez, F. Y. (2004). Competitiveness of introduced Rhizobium strains for nodulation in fodder legumes. *Pak. J. Bot.* 36, 159–166.

Nandasena, K. G., O'Hara, G. W., Tiwari, R. P., and Howieson, J. G. (2006). Rapid in situ evolution of nodulating strains for *Biserrula pelecinus* L. through lateral transfer of a symbiosis island from the original mesorhizobial inoculant. *Appl. Environ. Microbiol.* 72, 7365–7367. doi: 10.1128/AEM.00889-06

Nandasena, K. G., O'Hara, G. W., Tiwari, R. P., Sezmiş, E., and Howieson, J. G. (2007). In situ lateral transfer of symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia suboptimal in symbiotic nitrogen fixation on the pasture legume *Biserrula pelecinus* L. *Environ. Microbiol.* 9, 2496–2511. doi: 10.1111/j.1462-2920.2007.01368.x

Navarro, A., Fos, S., Laguna, E., Durán, D., Rey, L., Rubio-Sanz, L., et al. (2014). Conservation of Endangered *Lupinus mariae-josephae* in Its Natural Habitat by Inoculation with Selected, Native Bradyrhizobium Strains. *PLoS One* 9:e102205. doi: 10.1371/journal.pone.0102205

Nelson, E. B. (2018). The seed microbiome: origins, interactions, and impacts. *Plant Soil* 422, 7–34. doi: 10.1007/s11104-017-3289-7

Nelson, M. S., and Sadowsky, M. J. (2015). Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. *Front. Plant Sci.* 6:491. doi: 10.3389/fpls.2015.00491

Nelson, M. S., Chun, C. L., and Sadowsky, M. J. (2017). Type IV effector proteins involved in the *Medicago-sinorhizobium* symbiosis. *Mol. Plant Microbe Interact.* 30, 28–34. doi: 10.1094/MPMI-10-16-0211-R

Niehaus, K., Lagares, A., and Pühler, A. (1998). A *Sinorhizobium meliloti* lipopolysaccharide mutant induces effective nodules on the host plant *Medicago sativa* (Alfalfa) but fails to establish a symbiosis with *Medicago truncatula*. *Mol. Plant Microbe Interact.* 11, 906–914. doi: 10.1094/MPMI.1998.11.9.906

O'Callaghan, M. (2016). Microbial inoculation of seed for improved crop performance: issues and opportunities. *Appl. Microbiol. Biotechnol.* 100, 5729–5746. doi: 10.1007/s00253-016-7590-9

O'Kane, D. J., Lingle, W. L., Wampler, J. E., Legocki, M., Legocki, R. P., and Szalay, A. A. (1988). Visualization of bioluminescence as a marker of gene expression in rhizobium-infected soybean root nodules. *Plant Mol. Biol.* 10, 387–399. doi: 10.1007/BF00014945

Oger, P., Petit, A., and Dessaux, Y. (1997). Genetically engineered plants producing opines alter their biological environment. *Nat. Biotechnol.* 15, 369–372. doi: 10.1038/nbt0497-369

Okogun, J. A., and Sanginga, N. (2003). Can introduced and indigenous rhizobial strains compete for nodule formation by promiscuous soybean in the moist savanna agroecological zone of Nigeria? *Biol. Fertil. Soils* 38, 26–31. doi: 10.1007/s00374-003-0611-8

Oldroyd, G. E. D. D., Murray, J. D., Poole, P. S., and Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Oldroyd, G. E. D., and Downie, J. A. (2004). Calcium, kinases and nodulation signalling in legumes. *Nat. Rev. Mol. Cell Biol.* 5, 566–576. doi: 10.1038/nrm1424

Onishchuk, O. P., Vorobyov, N. I., and Provorov, N. A. (2017). Nodulation competitiveness of nodule bacteria: genetic control and adaptive significance: review. *Appl. Biochem. Microbiol.* 53, 131–139. doi: 10.1134/S0003683817020132

Oono, R., Anderson, C. G., and Denison, R. F. (2011). Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. *Proc. R. Soc. B Biol. Sci.* 278, 2698–2703. doi: 10.1098/rspb.2010.2193

Oresnik, I. J., Pacarynuk, L. A., O'Brien, S. A. P., Yost, C. K., and Hynes, M. F. (1998). Plasmid-encoded catabolic genes in *Rhizobium leguminosarum* bv. *trifolii:* evidence for a plant-inducible rhamnose locus involved in competition for nodulation. *Mol. Plant Microbe Interact.* 11, 1175–1185. doi: 10.1094/MPMI.1998.11.12.1175

Oresnik, I. J., Twelker, S., and Hynes, M. F. (1999). Cloning and characterization of a *Rhizobium leguminosarum* gene encoding a bacteriocin with similarities to RTX toxins. *Appl. Environ. Microbiol.* 65, 2833–2840. doi: 10.1128/aem.65.7.2833-2840.1999

Parnell, J. J., Berka, R., Young, H. A., Sturino, J. M., Kang, Y., Barnhart, D. M., et al. (2016). From the lab to the farm: an industrial perspective of plant beneficial microorganisms. *Front. Plant Sci* 7:1110. doi: 10.3389/fpls.2016.01110

Pastor-Bueis, R., Sánchez-Cañizares, C., James, E. K., and González-Andrés, F. (2019). Formulation of a highly effective inoculant for common bean based on an autochthonous elite strain of *Rhizobium leguminosarum* bv. *phaseoli*, and Genomic-Based Insights Into Its Agronomic Performance. *Front. Microbiol.* 10:2724. doi: 10.3389/fmicb.2019.02724

Penmetsa, R. V., and Cook, D. R. (1997). A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* 275, 527–530. doi: 10.1126/science.275.5299.527

Penmetsa, R. V., Frugoli, J. A., Smith, L. S., Long, S. R., and Cook, D. R. (2003). Dual genetic pathways controlling nodule number in *Medicago truncatula*. *Plant Physiol.* 131, 998–1008. doi: 10.1104/pp.015677

Peoples, M. B., Herridge, D. F., and Ladha, J. K. (1995). Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? *Plant Soil* 174, 3–28. doi: 10.1007/BF00032239

Peralta, H., Aguilar, A., Díaz, R., Mora, Y., Martínez-Batallar, G., Salazar, E., et al. (2016). Genomic studies of nitrogen-fixing rhizobial strains from *Phaseolus vulgaris* seeds and nodules. *BMC Genomics* 17:711. doi: 10.1186/s12864-016-3053-z

Pérez-Giménez, J., Iturralde, E. T., Tejerizo, G. T., Quelas, J. I., Krol, E., Borassi, C., et al. (2021). A stringent-response-defective *Bradyrhizobium diazoefficiens* Strain Does Not Activate the Type 3 Secretion System, Elicits an Early Plant Defense Response, and Circumvents NH4NO3- Induced Inhibition of Nodulation. *Appl. Environ. Microbiol.* 87:e02989-20. doi: 10.1128/AEM.02989-20

Perret, X., Staehelin, C., and Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64, 180–201. doi: 10.1128/MMBR.64.1.180-201.2000

Perry, B. J., and Yost, C. K. (2014). Construction of a mariner-based transposon vector for use in insertion sequence mutagenesis in selected members of the Rhizobiaceae. *BMC Microbiol.* 14:298. doi: 10.1186/s12866-014-0298-z

Persmark, M., Pittman, P., Buyer, J. S., Schwyn, B., Gill, P. R., and Neilands, J. B. (1993). Isolation and Structure of Rhizobactin 1021, a Siderophore from the Alfalfa Symbiont *Rhizobium meliloti* 1021. *J. Am. Chem. Soc.* 115, 3950–3956. doi: 10.1021/ja00063a014

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Van Der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Pini, F., East, A. K., Appia-Ayme, C., Tomek, J., Karunakaran, R., Mendoza-Suárez, M., et al. (2017). Bacterial biosensors for *in vivo* spatiotemporal mapping of root secretion. *Plant Physiol.* 174, 1289–1306. doi: 10.1104/pp.16.01302

Pobigaylo, N., Szymczak, S., Nattkemper, T. W., and Becker, A. (2008). Identification of genes relevant to symbiosis and competitiveness in *Sinorhizobium meliloti* using signature-tagged mutants. *Mol. Plant Microbe Interact.* 21, 219–231. doi: 10.1094/MPMI-21-2-0219

Poole, P. (2017). Shining a light on the dark world of plant root-microbe interactions. *Proc. Natl. Acad. Sci. U.S.A.* 114, 4281–4283. doi: 10.1073/pnas.1703800114

Poole, P., Ramachandran, V., and Terpolilli, J. (2018). Rhizobia: from saprophytes to endosymbionts. *Nat. Rev. Microbiol.* 16, 291–303. doi: 10.1038/nrmicro.2017.171

Queiroux, C., Washburn, B. K., Davis, O. M., Stewart, J., Brewer, T. E., Lyons, M. R., et al. (2012). A comparative genomics screen identifies a *Sinorhizobium meliloti* 1021 sodM-like gene strongly expressed within host plant nodules. *BMC Microbiol.* 12:74. doi: 10.1186/1471-2180-12-74

Rachwał, K., Boguszewska, A., Kopcińska, J., Karaś, M., Tchórzewski, M., and Janczarek, M. (2016). The Regulatory Protein RosR Affects *Rhizobium leguminosarum* bv. *trifolii* Protein Profiles, Cell Surface Properties, and Symbiosis with Clover. *Front. Microbiol.* 7:1302. doi: 10.3389/fmicb.2016.01302

Rae, A. E., Rolland, V., White, R. G., and Mathesius, U. (2021). New methods for confocal imaging of infection threads in crop and model legumes. *Plant Methods* 17:24. doi: 10.1186/s13007-021-00725-6

Raina, J. B., Fernandez, V., Lambert, B., Stocker, R., and Seymour, J. R. (2019). The role of microbial motility and chemotaxis in symbiosis. *Nat. Rev. Microbiol.* 17, 284–294. doi: 10.1038/s41579-019-0182-9

Rao, D. L. N., Giller, K. E., Yeo, A. R., and Flowers, T. J. (2002). The effects of salinity and sodicity upon nodulation and nitrogen fixation in chickpea (*Cicer arietinum*). *Ann. Bot.* 89, 563–570. doi: 10.1093/aob/mcf097

Ratcliff, W. C., Kadam, S. V., and Denison, R. F. (2008). Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiol. Ecol.* 65, 391–399. doi: 10.1111/j.1574-6941.2008.00544.x

Rathi, S., Tak, N., Bissa, G., Chouhan, B., Ojha, A., Adhikari, D., et al. (2018). Selection of Bradyrhizobium or Ensifer symbionts by the native Indian caesalpinioid legume *Chamaecrista pumila* depends on soil pH and other edaphic and climatic factors. *FEMS Microbiol. Ecol.* 94:fiy180.

Regus, J. U., Quides, K. W., O'Neill, M. R., Suzuki, R., Savory, E. A., Chang, J. H., et al. (2017). Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *Am. J. Bot.* 104, 1299–1312. doi: 10.3732/ajb.1700165

Reid, D. E., Ferguson, B. J., Hayashi, S., Lin, Y. H., and Gresshoff, P. M. (2011). Molecular mechanisms controlling legume autoregulation of nodulation. *Ann. Bot.* 108, 789–795. doi: 10.1093/aob/mcr205

Reid, T. E., Kavamura, V. N., Abadie, M., Torres-Ballesteros, A., Pawlett, M., Clark, I. M., et al. (2021). Inorganic chemical fertilizer application to wheat reduces the abundance of putative plant growth-promoting Rhizobacteria. *Front. Microbiol.* 12:642587. doi: 10.3389/fmicb.2021.642587

Riaz, U., Mehdi, S. M., Iqbal, S., Khalid, H. I., Qadir, A. A., Anum, W., et al. (2020). "Bio-fertilizers: eco-friendly approach for plant and soil environment," in *Bioremediation and Biotechnology: Sustainable Approaches to Pollution Degradation*, eds K. Hakeem, R. Bhat, and H. Qadri (Berlin: Springer), 189–213. doi: 10.1007/978-3-030-35691-0_9

Rivas, R., Laranjo, M., Mateos, P. F., Oliveira, S., Martínez-Molina, E., and Velázquez, E. (2007). Strains of *Mesorhizobium amorphae* and *Mesorhizobium*

tianshanense, carrying symbiotic genes of common chickpea endosymbiotic species, constitute a novel biovar (ciceri) capable of nodulating *Cicer arietinum*. *Lett. Appl. Microbiol.* 44, 412–418. doi: 10.1111/j.1472-765X.2006.02086.x

Robinson, D. G., Chen, W., Storey, J. D., and Gresham, D. (2014). Design and analysis of bar-seq experiments. *G3* 4, 11–18. doi: 10.1534/g3.113.008565

Robleto, E. A., Kmiecik, K., Oplinger, E. S., Nienhuis, J., and Triplett, E. W. (1998). Trifolitoxin production increases nodulation competitiveness of *Rhizobium etli* CE3 under agricultural conditions. *Appl. Environ. Microbiol.* 64:2630. doi: 10.1128/aem.64.7.2630-2633.1998

Rodríguez-Echeverría, S., Pérez-Fernández, M. A., Vlaar, S., and Finnan, T. (2003). Analysis of the legume-rhizobia symbiosis in shrubs from central western Spain. *J. Appl. Microbiol.* 95, 1367–1374. doi: 10.1046/j.1365-2672.2003.02118.x

Rodríguez-Navarro, D. N., Dardanelli, M. S., and Ruíz-Saínz, J. E. (2007). Attachment of bacteria to the roots of higher plants. *FEMS Microbiol. Lett.* 272, 127–136. doi: 10.1111/j.1574-6968.2007.00761.x

Romero-Jiménez, L., Rodríguez-Carbonell, D., Gallegos, M. T., Sanjuán, J., and Pérez-Mendoza, D. (2015). Mini-Tn7 vectors for stable expression of diguanylate cyclase PleD* in Gram-negative bacteria. *BMC Microbiol.* 15:190. doi: 10.1186/s12866-015-0521-6

Ronson, C. W., and Lowther, W. L. (1995). Issues affecting the competitiveness of white clover rhizobia in New Zealand pastures. *Agron. Soc. New Zeal.* 5, 87–90.

Rosenblueth, M., Hynes, M. F., and Martínez-Romero, E. (1998). Rhizobium tropici teu genes involved in specific uptake of *Phaseolus vulgaris* bean-exudate compounds. *Mol. Gen. Genet.* 258, 587–598. doi: 10.1007/s004380050772

Roskothen, P. (1989). Genetic effects on Host x Strain Interaction in the Symbiosis of *Vicia faba* and *Rhizobium leguminosarum*. *Plant Breed.* 102, 122–132. doi: 10.1111/j.1439-0523.1989.tb00325.x

Russo, D. M., Williams, A., Edwards, A., Posadas, D. M., Finnie, C., Dankert, M., et al. (2006). Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by Rhizobium leguminosarum. *J. Bacteriol.* 188, 4474–4486. doi: 10.1128/JB.00246-06

Rynne, F. G., Glenn, A. R., and Dilworth, M. J. (1994). Effect of mutations in aromatic catabolism on the persistence and competitiveness of *Rhizobium leguminosarum* bv. *trifolii*. *Soil Biol. Biochem.* 26, 703–710. doi: 10.1016/0038-0717(94)90262-3

Saad, M. M., Eida, A. A., Hirt, H., and Doerner, P. (2020). Tailoring plant-associated microbial inoculants in agriculture: a roadmap for successful application. *J. Exp. Bot.* 71, 3878–3901. doi: 10.1093/jxb/eraa111

Sachs, J. L., Ehinger, M. O., and Simms, E. L. (2010). Origins of cheating and loss of symbiosis in wild *Bradyrhizobium*. *J. Evol. Biol.* 23, 1075–1089. doi: 10.1111/j.1420-9101.2010.01980.x

Salas, M. E., Lozano, M. J., López, J. L., Draghi, W. O., Serrania, J., Torres Tejerizo, G. A., et al. (2017). Specificity traits consistent with legume-rhizobia coevolution displayed by Ensifer meliloti rhizosphere colonization. *Environ. Microbiol.* 19, 3423–3438. doi: 10.1111/1462-2920.13820

Salinero-Lanzarote, A., Pacheco-Moreno, A., Domingo-Serrano, L., Durán, D., Ormeño-Orrillo, E., Martínez-Romero, E., et al. (2019). The Type VI secretion system of *Rhizobium etli* Mim1 has a positive effect in symbiosis. *FEMS Microbiol. Ecol.* 95:fiz054. doi: 10.1093/femsec/fiz054

Sánchez-Cañizares, C., and Palacios, J. (2013). Construction of a marker system for the evaluation of competitiveness for legume nodulation in *Rhizobium* strains. *J. Microbiol. Methods* 92, 246–249. doi: 10.1016/j.mimet.2012.12.022

Santos, M. S., Nogueira, M. A., and Hungria, M. (2019). Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. *AMB Express* 9:205. doi: 10.1186/s13568-019-0932-0

Santos, R., Hérouart, D., Sigaud, S., Touati, D., and Puppo, A. (2001). Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Mol. Plant Microbe Interact.* 14, 86–89. doi: 10.1094/MPMI.2001.14.1.86

Schlaeppi, K., and Bulgarelli, D. (2015). The plant microbiome at work. *Mol. Plant Microbe Interact.* 28, 212–217. doi: 10.1094/MPMI-10-14-0334-FI

Schmidt, O., Hawkes, A., Gambhir, A., and Staffell, I. (2017). The future cost of electrical energy storage based on experience rates. *Nat. Energy* 2:17110. doi: 10.1038/nenergy.2017.110

Schofield, P. R., Gibson, A. H., Dudman, W. F., and Watson, J. M. (1987). Evidence for genetic exchange and recombination of *Rhizobium* Symbiotic plasmids in a soil population. *Appl. Environ. Microbiol.* 53, 2942–2947. doi: 10.1128/aem.53.12.2942-2947.1987

Schripsema, J., de Rudder, K. E., van Vliet, T. B., Lankhorst, P. P., de Vroom, E., Kijne, J. W., et al. (1996). Bacteriocin small of *Rhizobium leguminosarum* belongs to the class of N-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. *J. Bacteriol.* 178, 366–371. doi: 10.1128/JB.178.2.366-371.1996

Schwarz, S., Hood, R. D., and Mougous, J. D. (2010). What is type VI secretion doing in all those bugs? *Trends Microbiol.* 18, 531–537. doi: 10.1016/j.tim.2010.09.001

Schwinghamer, E. A., and Belkengren, R. P. (1968). Inhibition of rhizobia by a strain of *Rhizobium trifolii*: some properties of the antibiotic and of the strain. *Arch. Mikrobiol.* 64, 130–145. doi: 10.1007/BF00406972

Sessitsch, A., Howieson, J. G., Perret, X., Antoun, H., and Martínez-Romero, E. (2002). Advances in *Rhizobium* research. *CRC Crit. Rev. Plant Sci.* 21, 323–378. doi: 10.1080/0735-260291044278

Sessitsch, A., Wilson, K., Akkermans, A., and de Vos, W. (1996). Simultaneous detection of different *Rhizobium* strains marked with either the *Escherichia coli gusA* gene or the *Pyrococcus furiosus celB* gene. *Appl. Environ. Microbiol.* 62, 4191–4194.

Shamseldin, A., and Velázquez, E. (2020). The promiscuity of *Phaseolus vulgaris* L. (common bean) for nodulation with rhizobia: a review. *World J. Microbiol. Biotechnol.* 36:63. doi: 10.1007/s11274-020-02839-w

Sharypova, L. A., Niehaus, K., Scheidle, H., Holst, O., and Becker, A. (2003). *Sinorhizobium meliloti acpXL* mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does not express a slow migrating form of lipopolysaccharide. *J. Biol. Chem.* 278, 12946–12954. doi: 10.1074/jbc.M209389200

Siefert, A., Zillig, K. W., Friesen, M. L., and Strauss, S. Y. (2018). Soil microbial communities alter conspecific and congeneric competition consistent with patterns of field coexistence in three *Trifolium* congeners. *J. Ecol.* 106, 1876–1891. doi: 10.1111/1365-2745.13042

Simonsen, A. K., DInnage, R., Barrett, L. G., Prober, S. M., and Thrall, P. H. (2017). Symbiosis limits establishment of legumes outside their native range at a global scale. *Nat. Commun.* 8:14790. doi: 10.1038/ncomms14790

Slattery, J., and Pearce, D. (2002). "Development of Elite Inoculant Rhizobium Strains in Southeastern Australia," in *Inoculants and Nitrogen Fixation of Legumes in Vietnam*, ed. D. Herridge (Jakarta: ACIAR), 86–94.

Smit, G., Logman, T. J., Boerrigter, M. E., Kijne, J. W., and Lugtenberg, B. J. (1989). Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* Ca^{2+}-dependent adhesin, which mediates the first step in attachment of cells of the family *Rhizobiaceae* to plant root hair tips. *J. Bacteriol.* 171, 4054–4062. doi: 10.1128/jb.171.7.4054-4062.1989

Smit, G., Swart, S., Lugtenberg, B. J., and Kijne, J. W. (1992). Molecular mechanisms of attachment of *Rhizobium* bacteria to plant roots. *Mol. Microbiol.* 6, 2897–2903.

Song, L., Hou, L., Zhang, Y., Li, Z., Wang, W., and Sun, Q. (2020). Regular biochar and bacteria-inoculated biochar alter the composition of the microbial community in the soil of a Chinese fir plantation. *Forests* 11:951. doi: 10.3390/f11090951

Sotelo, M., Irisarri, P., Lorite, M. J., Casaretto, E., Rebuffo, M., Sanjuán, J., et al. (2011). Diversity of rhizobia nodulating *Lotus corniculatus* grown in northern and southern regions of Uruguay. *Appl. Soil Ecol.* 49, 197–207. doi: 10.1016/j.apsoil.2011.05.005

Soumare, A., Diedhiou, A. G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., et al. (2020). Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. *Plants* 9:1011. doi: 10.3390/plants9081011

Sprent, J. I. (2008). "Evolution and diversity of legume symbiosis," in *Nitrogen-fixing Leguminous Symbioses Nitrogen Fixation: Origins, Applications, and Research Progress*, eds M. Dilworth, E. James, J. Sprent, and W. Newton (Berlin: Springer), 1–21. doi: 10.1007/978-1-4020-3548-7_1

Stacey, G., So, J. S., Roth, L. E., Lakshmi, B. S. K., and Carlson, R. W. (1991). A lipopolysaccharide mutant of *Bradyrhizobium japonicum* that uncouples plant from bacterial differentiation. *Mol. Plant Microbe Interact.* 4, 332–340. doi: 10.1094/MPMI-4-332

Streit, W., Kosch, K., and Werner, D. (1992). Nodulation competitiveness of *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* strains measured by glucuronidase (gus) gene fusion. *Biol. Fertil. Soils* 14, 140–144. doi: 10.1007/BF00336264

Stuurman, N., Pacios Bras, C., Schlaman, H. R. M., Wijfjes, A. H. M., Bloemberg, G., and Spaink, H. P. (2000). Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. *Mol. Plant Microbe Interact.* 13, 1163–1169. doi: 10.1094/MPMI.2000.13.11.1163

Sugawara, M., Okazaki, S., Nukui, N., Ezura, H., Mitsui, H., and Minamisawa, K. (2006). Rhizobitoxine modulates plant-microbe interactions by ethylene inhibition. *Biotechnol. Adv.* 24, 382–388. doi: 10.1016/j.biotechadv.2006.01.004

Sullivan, J. T., and Ronson, C. W. (1998). Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5145–5149. doi: 10.1073/pnas.95.9.5145

Sullivan, J. T., Patrick, H. N., Lowther, W. L., Scott, D. B., and Ronson, C. W. (1995). Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc. Natl. Acad. Sci. U.S.A.* 92, 8985–8989. doi: 10.1073/pnas.92.19.8985

Taté, R., Riccio, A., Caputo, E., Cermola, M., Favre, R., and Patriarca, E. J. (1999). The *Rhizobium etli trpB* gene is essential for an effective symbiotic interaction with *Phaseolus vulgaris*. *Mol. Plant Microbe Interact.* 12, 926–933. doi: 10.1094/MPMI.1999.12.10.926

Tausen, M., Clausen, M., Moeskjær, S., Shihavuddin, A. S. M., Dahl, A. B., Janss, L., et al. (2020). Greenotyper: image-based plant phenotyping using distributed computing and deep learning. *Front. Plant Sci.* 11:1181. doi: 10.3389/fpls.2020.01181

Temprano, F. J., Albareda, M., Camacho, M., Daza, A., Santamaría, C., and Rodríguez-Navarro, D. N. (2002). Survival of several *Rhizobium/Bradyrhizobium* strains on different inoculant formulations and inoculated seeds. *Int. Microbiol.* 5, 81–86. doi: 10.1007/s10123-002-0067-y

Thrall, P. H., Millsom, D. A., Jeavons, A. C., Waayers, M., Harvey, G. R., Bagnall, D. J., et al. (2005). Seed inoculation with effective root-nodule bacteria enhances revegetation success. *J. Appl. Ecol.* 42, 740–751. doi: 10.1111/j.1365-2664.2005.01058.x

Timmers, A. C. J. J., Soupène, E., Auriac, M.-C. C., de Billy, F., Vasse, J., Boistard, P., et al. (2000). Saprophytic intracellular Rhizobia in Alfalfa Nodules. *Mol. Plant Microbe Interact.* 13, 1204–1213. doi: 10.1094/MPMI.2000.13.11.1204

Triplett, E. W., and Barta, T. M. (1987). Trifolitoxin Production and Nodulation Are Necessary for the Expression of Superior Nodulation Competitiveness by *Rhizobium leguminosarum* bv. *trifolii* Strain T24 on Clover. *Plant Physiol.* 85, 335–342. doi: 10.1104/pp.85.2.335

Triplett, E. W., and Sadowsky, M. J. (1992). Genetics of competition for nodulation of legumes. *Annu. Rev. Microbiol.* 46, 399–428. doi: 10.1146/annurev.micro.46.1.399

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

Turgeon, B. G., and Bauer, W. D. (1985). Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. *Planta* 163, 328–349. doi: 10.1007/BF00395142

Turner, T. R., James, E. K., and Poole, P. S. (2013). The plant microbiome. *Genome Biol.* 14:209. doi: 10.1186/gb-2013-14-6-209

Udvardi, M., and Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annu. Rev. Plant Biol.* 64, 781–805. doi: 10.1146/annurev-arplant-050312-120235

Vanderlinde, E. M., Hynes, M. F., and Yost, C. K. (2014). Homoserine catabolism by *Rhizobium leguminosarum* bv. *viciae* 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation. *Environ. Microbiol.* 16, 205–217. doi: 10.1111/1462-2920.12196

Venter, A. P., Twelker, S., Oresnik, I. J., and Hynes, M. F. (2001). Analysis of the genetic region encoding a novel rhizobiocin from *Rhizobium leguminosarum* bv. *viciae strain* 306. *Can. J. Microbiol.* 47, 495–502. doi: 10.1139/cjm-47-6-495

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* 255, 571–586. doi: 10.1023/A:1026037216893

Vlassak, K. M., and Vanderleyden, J. (1997). Factors influencing nodule occupancy by inoculant Rhizobia. *CRC Crit. Rev. Plant Sci.* 16, 163–229. doi: 10.1080/07352689709701948

Vuong, H. B., Thrall, P. H., and Barrett, L. G. (2017). Host species and environmental variation can influence rhizobial community composition. *J. Ecol.* 105, 540–548. doi: 10.1111/1365-2745.12687

Weese, D. J., Heath, K. D., Dentinger, B. T. M., and Lau, J. A. (2015). Long-term nitrogen addition causes the evolution of less-cooperative mutualists. *Evolution* 69, 631–642. doi: 10.1111/evo.12594

Werner, D., and Newton, W. E. (2005). *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment*, eds D. Werner and W. E. Newton (Berlin: Springer). doi: 10.1007/1-4020-3544-6

West, S. A., Kiers, E. T., Simms, E. L., and Denison, R. F. (2002). Sanctions and mutualism stability: Why do rhizobia fix nitrogen? *Proc. R. Soc. B Biol. Sci.* 269, 685–694. doi: 10.1098/rspb.2001.1878

Westhoek, A., Clark, L. J., Culbert, M., Dalchau, N., Griffiths, M., Jorrin, B., et al. (2021). Conditional sanctioning in a legume-*Rhizobium* mutualism. *Proc. Natl. Acad. Sci. U.S.A.* 118:e2025760118. doi: 10.1073/pnas.2025760118

Wetmore, K. M., Price, M. N., Waters, R. J., Lamson, J. S., He, J., Hoover, C. A., et al. (2015). Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons. *MBio* 6:e00306-15. doi: 10.1128/mBio.00306-15

Wheatley, R. M., and Poole, P. S. (2018). Mechanisms of bacterial attachment to roots. *FEMS Microbiol. Rev.* 42, 448–461. doi: 10.1093/femsre/fuy014

Wheatley, R. M., Ford, B. L., Li, L., Aroney, S. T. N., Knights, H. E., Ledermann, R., et al. (2020). Lifestyle adaptations of *Rhizobium* from rhizosphere to symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23823–23834. doi: 10.1073/pnas.2009094117

Wielbo, J., Kuske, J., Marek-Kozaczuk, M., and Skorupska, A. (2010). The competition between *Rhizobium leguminosarum* bv. *viciae* strains progresses until late stages of symbiosis. *Plant Soil* 337, 125–135. doi: 10.1007/s11104-010-0510-3

Williams, A., Wilkinson, A., Krehenbrink, M., Russo, D. M., Zorreguieta, A., and Downie, J. A. (2008). Glucomannan-mediated attachment of *Rhizobium leguminosarum* to pea root hairs is required for competitive nodule infection. *J. Bacteriol.* 190, 4706–4715. doi: 10.1128/JB.01694-07

Wilson, K. J., Peoples, M. B., and Jefferson, R. A. (1995a). New techniques for studying competition by Rhizobia and for assessing nitrogen fixation in the field. *Plant Soil* 174, 241–253. doi: 10.1007/BF00032250

Wilson, K. J., Sessitsch, A., Corbo, J. C., Giller, K. E., Akkermans, A. D. L., and Jefferson, R. A. (1995b). β-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. *Microbiology* 141, 1691–1705. doi: 10.1099/13500872-141-7-1691

Wong, B. G., Mancuso, C. P., Kiriakov, S., Bashor, C. J., and Khalil, A. S. (2018). Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER. *Nat. Biotechnol.* 36, 614–623. doi: 10.1038/nbt.4151

Xie, F., Williams, A., Edwards, A., and Downie, J. A. (2012). A plant arabinogalactan-like glycoprotein promotes a novel type of polar surface attachment by *Rhizobium leguminosarum*. *Mol. Plant Microbe Interact.* 25, 250–258. doi: 10.1094/MPMI-08-11-0211

Yates, R. J., Howieson, J. G., Reeve, W. G., and O'Hara, G. W. (2011). A re-appraisal of the biology and terminology describing rhizobial strain success in nodule occupancy of legumes in agriculture. *Plant Soil* 348, 255–267. doi: 10.1007/s11104-011-0971-z

Young, J. P. W., and Wexler, M. (1988). Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. *Microbiology* 134, 2731–2739. doi: 10.1099/00221287-134-10-2731

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63, 968–989.

Zgadzaj, R., Garrido-Oter, R., Jensen, D. B., Koprivova, A., Schulze-Lefert, P., and Radutoiu, S. (2016). Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 113, E7996–E8005. doi: 10.1073/pnas.1616564113

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., da Rocha, U. N., Shi, S., et al. (2018). Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat. Microbiol.* 34, 470–480. doi: 10.1038/s41564-018-0129-3

Zheng, H., Mao, Y., Teng, J., Zhu, Q., Ling, J., and Zhong, Z. (2015). Flagellar-dependent motility in *Mesorhizobium tianshanense* is involved in the early stage of plant host interaction: study of an *flgE* mutant. *Curr. Microbiol.* 70, 219–227. doi: 10.1007/s00284-014-0701-x

Zhu, S., Vivanco, J. M., and Manter, D. K. (2016). Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. *Appl. Soil Ecol.* 107, 324–333. doi: 10.1016/j.apsoil.2016.07.009

Zvinavashe, A. T., Lim, E., Sun, H., and Marelli, B. (2019). A bioinspired approach to engineer seed microenvironment to boost germination and mitigate soil salinity. *Proc. Natl. Acad. Sci. U.S.A.* 116, 25555–25561. doi: 10.1073/pnas.1915902116

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Mendoza-Suárez, Andersen, Poole and Sánchez-Cañizares. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 September 2021
doi: 10.3389/fpls.2021.680981

Genomic Diversity of Pigeon Pea (*Cajanus cajan* L. Millsp.) Endosymbionts in India and Selection of Potential Strains for Use as Agricultural Inoculants

Beatriz Jorrin [1*]***, Marta Maluk*** [2]***, Nagvanti Atoliya*** [3]***, Shiv Charan Kumar*** [4]***, Danteswari Chalasani*** [5]***, Andrzej Tkacz*** [1]***, Prachi Singh*** [4]***, Anirban Basu*** [5]***, Sarma VSRN Pullabhotla*** [5]***, Murugan Kumar*** [4]***, Santosh Ranjan Mohanty*** [3]***, Alison K. East*** [1]***, Vinoy K. Ramachandran*** [1]***, Euan K. James*** [2]***, Appa Rao Podile*** [5*]***, Anil Kumar Saxena*** [4*]***, DLN Rao*** [3*] ***and Philip S. Poole*** [1*]

[1] Department of Plant Sciences, University of Oxford, Oxford, United Kingdom, [2] The James Hutton Institute, Dundee, United Kingdom, [3] ICAR-Indian Institute of Soil Science, Bhopal, India, [4] ICAR-National Bureau of Agriculturally Important Microorganisms, Mau, India, [5] Department of Plant Sciences, School of Life Science, University of Hyderabad, Hyderabad, India

Pigeon pea (*Cajanus cajan* L. Millsp.) is a legume crop resilient to climate change due to its tolerance to drought. It is grown by millions of resource-poor farmers in semiarid and tropical subregions of Asia and Africa and is a major contributor to their nutritional food security. Pigeon pea is the sixth most important legume in the world, with India contributing more than 70% of the total production and harbouring a wide variety of cultivars. Nevertheless, the low yield of pigeon pea grown under dry land conditions and its yield instability need to be improved. This may be done by enhancing crop nodulation and, hence, biological nitrogen fixation (BNF) by supplying effective symbiotic rhizobia through the application of "elite" inoculants. Therefore, the main aim in this study was the isolation and genomic analysis of effective rhizobial strains potentially adapted to drought conditions. Accordingly, pigeon pea endosymbionts were isolated from different soil types in Southern, Central, and Northern India. After functional characterisation of the isolated strains in terms of their ability to nodulate and promote the growth of pigeon pea, 19 were selected for full genome sequencing, along with eight commercial inoculant strains obtained from the ICRISAT culture collection. The phylogenomic analysis [Average nucleotide identity MUMmer (ANIm)] revealed that the pigeon pea endosymbionts were members of the genera *Bradyrhizobium* and *Ensifer*. Based on *nodC* phylogeny and *nod* cluster synteny, *Bradyrhizobium yuanmingense* was revealed as the most common endosymbiont, harbouring *nod* genes similar to those of *Bradyrhizobium cajani* and *Bradyrhizobium zhanjiangense*. This symbiont type (e.g., strain BRP05 from Madhya Pradesh) also outperformed all other strains tested on pigeon pea, with the notable

OPEN ACCESS

Edited by:
Julie Grossman, University of Minnesota Twin Cities, United States

Reviewed by:
Elisa Pellegrino, Sant'Anna School of Advanced Studies, Italy
Attila Kereszt, Eötvös Loránd Research Network, Hungary

****Correspondence:***
Beatriz Jorrin
beatriz.jorrin@plants.ox.ac.uk
Philip S. Poole
philip.poole@plants.ox.ac.uk
DLN Rao
dlnrao.icar@gmail.com
Anil Kumar Saxena
saxena461@yahoo.com
Appa Rao Podile
podilerao@gmail.com

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

Received: *15 March 2021*
Accepted: *06 August 2021*
Published: *07 September 2021*

Citation:
Jorrin B, Maluk M, Atoliya N, Kumar SC, Chalasani D, Tkacz A, Singh P, Basu A, Pullabhotla S, Kumar M, Mohanty SR, East AK, Ramachandran VK, James EK, Podile AR, Saxena AK, Rao D and Poole PS (2021) Genomic Diversity of Pigeon Pea (Cajanus cajan L. Millsp.) Endosymbionts in India and Selection of Potential Strains for Use as Agricultural Inoculants. Front. Plant Sci. 12:680981. doi: 10.3389/fpls.2021.680981

exception of an *Ensifer alkalisoli* strain from North India (NBAIM29). The results provide the basis for the development of pigeon pea inoculants to increase the yield of this legume through the use of effective nitrogen-fixing rhizobia, tailored for the different agroclimatic regions of India.

Keywords: *Bradyrhizobium*, *Ensifer (Sinorhizobium)*, pigeon pea *(Cajanus cajan)*, *nod* cluster, nodulation outer proteins (Nop), comparative genomics, India

INTRODUCTION

Pigeon pea (*Cajanus cajan* L. Millsp.) is grown by millions of resource-poor farmers in semiarid and tropical subregions of Asia and Africa as a major contributor to their food security (Mula and Saxena, 2010; Varshney et al., 2010). The initial domestication of pigeon pea was started in central India over 3,500 years ago, from its wild progenitor *Cajanus cajanifolius* (Vavilov, 1951; Saxena et al., 2014). Pigeon pea is the sixth most important legume in the world, representing 5% of the total pulse production (4.92 M ha), with India contributing more than 70% of the total (3.6 M ha) and harbouring a wide variety of cultivars (218 making up 73% of the total) (Saxena, 2006). It was estimated by the Food and Agriculture Organisation (FAO) that the worldwide annual production of pigeon pea in 2019 was 5.6 Mt, of which ∼59% was produced by India alone [FAO statistics (www.fao.org/faostat)].

Pigeon pea is a perennial shrub normally cultivated as an annual crop and, in India, can be used in rotation and intercrop systems with different cereal crops. Moreover, pigeon pea develops a deep root system, making it drought tolerant. These traits encourage cultivation in rain-fed drylands, although the poor growth conditions (e.g., aridity, nutrient-poor soils) mean that yields remain low. Effective symbiosis may improve nitrogen (N) content in this pulse legume and, hence, seed quality and quantity. However, legume-rhizobium symbioses are sensitive to drought, and, therefore, N fixation can be inefficient (Serraj et al., 1999; Mula and Saxena, 2010; Varshney et al., 2012). Selecting from among the diversity of pigeon pea cultivars sown in India may lead to improved symbiotic partners, as in the case for other legumes like soybean (Yang et al., 2010). To increase pigeon pea yields, it is important to select superior rhizobial strains that perform well under a wide variety of various stresses. Such bacteria can be developed into pigeon pea inoculants, tailored to perform well under different agroclimatic conditions.

However, until now, genomic diversity studies have only been performed in countries in the American and African continents, showing that the preferred endosymbionts are *Bradyrhizobium* spp. In Trinidad and Tobago, the main symbiont was *Bradyrhizobium elkanii* (Ramsubhag et al., 2002), whereas, in the Dominican Republic, *Bradyrhizobium yuanmingense* dominated. In the Ivory Coast, two different clades can nodulate pigeon pea, one associated with the *B. elkanii* group and a second one later assigned as the new species *B. ivorense* (Fossou et al., 2016, 2020). Additionally, another new species isolated from pigeon pea in the Dominican Republic has been defined as *Bradyrhizobium cajani* (Araújo et al., 2017), illustrating the great diversity present within pigeon pea endosymbionts across the world. *Ensifer* (syn. *Sinorhizobium*) has been reported as a symbiont only rarely, but strains were isolated using pigeon pea as a trap plant in soybean fields in Brazil (Coutinho et al., 1999; Stepkowski et al., 2003). Diverse pigeon pea rhizobia have been reported in Indian soils and have a long history of usage as inoculants; nevertheless, rigorous diversity studies have not been performed on these endosymbionts. In this study, we applied a mechanistic-holistic approach to study the diversity of pigeon pea native endosymbionts across India.

The aim in this study was to characterise the pigeon pea endosymbiont population isolated from a diversity of soil types in South (Alfisols), Central (Vertisols), and North India (Inceptisols). To achieve this, we isolated representative Indian pigeon pea rhizobia, sequenced representative strains, assessed their ability to nodulate pigeon pea and promote its growth, and analysed their genetic and genomic features. We uncovered the diversity of this population and the relationship between pigeon pea and members of the genera *Bradyrhizobium* and *Ensifer*. Comparisons of symbiotic-related features and the putative proteomes of these strains reveal the preferred pigeon pea endosymbionts in India.

MATERIALS AND METHODS

Strain Isolation From Nodules

Pigeon pea nodules were collected from three different regions in India: South India representing Alfisols (Telangana/Andhra Pradesh, Hyderabad University, HU strains), Central India representing Vertisols (Madhya Pradesh, Bhopal Rhizobia Pigeon pea, BRP strains), and North India, representing Inceptisols (Uttar Pradesh/Haryana/Punjab, National Bureau of Agricultural Important Microorganisms, NBAIM strains) (**Supplementary Table S1**). Nodules were surface sterilised by washing with ethanol (70%) for 1 min, followed by 2% sodium hypochlorite for 5 min, and finally washing with sterile-distilled water. The nodules were homogenised in 0.9% NaCl and directly streaked on Yeast Mannitol media (YM), supplemented with Congo red (0.0025%) (CRYEMA) for visual screening (Vincent, 1970; Somasegaran and Hoben, 1994). Plates were incubated at 28°C for up to 3–5 days. Selected colonies were streaked onto fresh CRYEMA plates to obtain pure cultures.

Eight pigeon pea inoculant strains were obtained from the Microbial Germplasm collection of the International Crops Research Institute for the Semiarid Tropics (ICRISAT), Hyderabad, India, were also included as reference strains (**Supplementary Table S1**).

Assessment of Bacterial Diversity by BOX-PCR

DNA extraction was achieved by alkaline lysis (0.05-M NaOH, 0.25% SDS) (Rivas et al., 2001). Isolated DNA was used as a template to generate BOX-PCR fingerprints, using the specific BOXA1R primer (CTACGGCAAGGCGACGCTGACG) (Versalovic et al., 1994). Amplification was carried out in a 25-μl PCR reaction containing 5–10 ng of isolated DNA and 1 U of OneTaq polymerase (NEB). BOX-PCR products were visualised on 2% agarose gels at 100 V until clear band separation. Gel images of the BOX-PCR fingerprint of each strain in the IU population were compared to find those that were the same and those that were different from each other.

Nodulation Test

Seeds of *C. cajan* cv. Asha (ICPL 87119) were surface sterilised with sodium hypochlorite (3% active chlorine) and 0.1% (v/v) Tween 20 for 6 min and rinsed three times with sterile-distilled water. Surface-sterilised seeds were germinated on 0.5% distilled water agar in petri plates at 28°C in the dark. Germinated seedlings were transferred to sterile test tubes containing 30 ml of vermiculite: perlite mixture (1:1) and 30 ml of B&D nutrient solution. The tubes were transferred to a growth chamber at a temperature of 28°C, 16/8-h day/night light regime, a 70% moisture level, and 100 μmol m^{-2} s^{-1} irradiances. Each tube was inoculated with 1 ml of bacterial liquid culture (10^8 CFU). Negative control tubes were left uninoculated. Five test tubes for each isolate were completely randomised in the growth chamber. Plants were harvested and scored for nodulation after 8 weeks of growth.

Assessment of Plant Growth Promotion

A representative strain from each BOX-PCR pattern was used as an inoculant with pigeon pea to assess its plant growth-promoting potential, using a temperature of 28°C, 16/8-h day/night light regime, a 70% moisture level, and 100 μmol m^{-2} s^{-1} irradiances. The experiment was run as a completely randomised design with five replications. Sterile 1 L pots were filled with a 1:1 mixture of vermiculite: perlite and 400 ml B&D nutrient solution (Broughton and Dilworth, 1971). Seeds were surface sterilised and germinated as described above. Seedlings were transferred to 1-L pots and inoculated with 1 ml of bacterial liquid culture (10^8 CFU). To prevent cross-contamination during watering, the pots were covered with plastic film with a hole for the shoot. Plants were fed weekly with a B&D nutrient solution and watered daily, or as required. The plants were harvested 8 weeks after inoculation, and shoot and root biomass obtained from five replicates was quantified after drying in an oven at 70°C for 5 days. The pigeon pea endosymbiont reference strains, IC3195, IC3342, IC4059, IC4060, and IC4062 were included as positive controls.

Genome Sequencing, Annotation and Analysis

Culture samples were outsourced to Microbes NG, Birmingham, United Kingdom for Illumina sequencing (MiSeq v2, PE 2 × 250 bp). The closest available reference genome for each sample was identified with Kraken v2 (Wood and Salzberg, 2014), and reads were mapped to the reference genome using bwa-mem v0.7.17 (Li and Durbin, 2009) to assess the quality of the data. *De novo* assembly was performed with SPAdes v3.14.1 (Bankevich et al., 2012). Automated annotation was made using Prokka v1.12 (Seemann, 2014). Geneious R10 (v10.2.6) was used to investigate genome annotation. The rRNA copy number was estimated by calculating the relative coverage of 16S rRNA vs. that of *rpoB*, a single-copy gene. All genomes were uploaded to GenBank (BioProject PRJNA679722). BioSample IDs are given in **Supplementary Table S2**.

Phylogenetic and Phylogenomic Analysis

nodC sequences from strains were extracted from annotated genomes or obtained from GenBank (**Supplementary Table S3**). Alignment was performed using MUSCLE software (Edgar, 2004). Distances were calculated according to the two-parameter model of Kimura (1980). Phylogenies of *nodC* were inferred using the neighbour-joining (NJ) method. All analyses were performed using MEGA X software (Kumar et al., 2018). All nodes with a bootstrap value lower than 70% were removed. The similarity of draught genome sequences of India-UK (IU) strains and ICRISAT (IC) strains (**Table 1**), together with genome sequences from closely related species considered as references, was analysed by calculating pairwise average nucleotide identity (ANI) values (Konstantinidis and Tiedje, 2005; Goris et al., 2007). ANI was performed using the Nucmer algorithm [Average nucleotide identity MUMmer (ANIm)] (Kurtz et al., 2004) as implemented in the JSpecies software v.1.2.1. Pairwise similarity percentage was transformed into a dissimilarity distance matrix and plotted as an NJ cladogram (Saitou and Nei, 1987) on MEGA X (Kumar et al., 2018). BioSample codes for each genome used can be found in **Supplementary Table S3**.

Cluster Synteny

nod cluster regions were extracted from GenBank files using Geneious R10 (v10.2.6). Synteny analysis was performed in CloVR-Comparative (Angiuoli et al., 2011; Agrawal et al., 2017) and visualised with Sybyl in this platform (Riley et al., 2011). Sybyl defines an orthologue when a protein sequence has an identity >70%, a coverage cutoff of 80%, and an e-value $>1^{e-5}$.

Nodulation Outer Protein (Nop) Analysis

A local blast database was constructed with IU and IC draught proteomes. Well-characterised genes associated with Type 3 Secretion System (T3SS) machinery (*rhcQ*, *rhcU, ttsI*, *nolU,* and *nolV*) and its putative effectors (Nop: *nopA*, *nopB*, *nopC*, *nopD*, *nopE*, *nopF*, *nopJ*, *nopL*, *nopM*, *nopP*, *nopT*, *nopX, nopAA*, *nopAC*, and *nopAR*) were obtained from UniProt and NCBI databases (as shown in **Supplementary Table S4**), and blastp was performed. A blastp hit of at least 50% identity, 50% coverage, and e-value $>1^{e-5}$ in protein sequence was considered an orthologue (as shown in **Supplementary Table S4** for locus tags). Clustered heatmaps were generated using the pheatmap R package (Kolde, 2019).

TABLE 1 | Strains sequenced and used for genomic comparison.

Species	Strain	Host	Location
Bradyrhizobium yuanmingense	BRP05	*Cajanus cajan* cv Asha	Madhya Pradesh
Ensifer sp.	BRP08	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium yuanmingense	BRP09	*Cajanus cajan* cv Asha	Madhya Pradesh
Ensifer aridi	BRP14	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium yuanmingense	BRP19	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium yuanmingense	BRP20	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium sp.	BRP22	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium yuanmingense	BRP23	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium brasilense	BRP56	*Cajanus cajan* cv Asha	Madhya Pradesh
Bradyrhizobium yuanmingense	NBAIM01	*Cajanus cajan* cv Asha	Uttar Pradesh
Bradyrhizobium yuanmingense	NBAIM02	*Cajanus cajan* cv Asha	Punjab
Bradyrhizobium yuanmingense	NBAIM03	*Cajanus cajan* cv Asha	Punjab
Bradyrhizobium yuanmingense	NBAIM08	*Cajanus cajan* cv Asha	Uttar Pradesh
Bradyrhizobium yuanmingense	NBAIM14	*Cajanus cajan* cv Asha	Punjab
Bradyrhizobium yuanmingense	NBAIM16	*Cajanus cajan* cv Asha	Punjab
Bradyrhizobium yuanmingense	NBAIM18	*Cajanus cajan* cv Asha	Uttar Pradesh
Bradyrhizobium yuanmingense	NBAIM20	*Cajanus cajan* cv Asha	Uttar Pradesh
Ensifer alkalisoli	NBAIM29	*Cajanus cajan* cv Asha	Punjab
Bradyrhizobium yuanmingense	NBAIM32	*Cajanus cajan* cv Asha	Uttar Pradesh
Bradyrhizobium yuanmingense	IC4061	*Pongamia pinnata*	Uttar Pradesh
Bradyrhizobium yuanmingense	IC4060	*Pongamia pinnata*	Haryana
Bradyrhizobium yuanmingense	IC3069	*Indigofera glandulosa*	Telangana
Bradyrhizobium yuanmingense	IC4059	*Pongamia pinnata*	Tamil Nadu
Bradyrhizobium yuanmingense	IC3195	*Macroptilium atropurpureum*	Telangana
Bradyrhizobium yuanmingense	IC3123	*Aarachis hypogaea*	Maharashtra
Ensifer sp.	IC3342	*Macroptilium atropurpureum*	Telangana
Ensifer sp.	IC4062	unknown	Maharashtra

Genetic Features Analysis

CMG-Biotools were used to infer core genomes and pangenomes of IU and IC strains using for orthologue analysis protein files (Vesth et al., 2013). Protein files were uploaded to OrthoVenn2 running locally. Orthovenn2 uses a cutoff $1e^{-5}$ to define paralogues (within genomes) and orthologues (between genomes).

Statistical Analyses

For PcoA plots construction, data were analysed in PRIMER 6 (PRIMER-E). Data were normalised and a similarity matrix was calculated using Euclidian distance. Strain samples that lacked a value in any tested variable were removed from the analysis. Permutational multivariate analysis of variance (PERMANOVA) was run in PRIMER 6 (PRIMER-E) using 9,999 unrestricted permutations of raw data. PERMANOVA produces pseudo-F values as a proxy for the difference between beta-diversity and alpha-diversity using a given factor. Statistical analyses were performed on PRISM 9 v9.0.2.

RESULTS

Bacterial Diversity Revealed by BOX-PCR

A total of 111 strains [termed collectively India-UK (IU) strains] were isolated from *C. cajan* root nodules in three different regions of India with different soils: 32 from South India (Alfisols; Telangana/Andhra Pradesh, HU strains), 47 from Central India (Vertisols; Madhya Pradesh, BRP strains), and 32 from North India (Inceptisols; Uttar Pradesh/Haryana/Punjab, NBAIM strains) (**Supplementary Table S1**, **Figure 1A**). BOX-PCR showed a total of 59 different profiles (a to bg) (**Supplementary Table S5**). A single representative strain from each region was selected from each BOX-PCR profile group, resulting in a total of 65: 20 HU strains, 27 BRP strains, and 18 NBAIM strains (**Supplementary Table S5**).

Ability of Isolates to Nodulate and Influence Plant Growth

The 65 IU strains selected were used to investigate their effect on the growth of pigeon pea plants under controlled conditions (**Supplementary Table S6**). Some of these IC strains were included as positive controls since some of these are used as pigeon pea inoculants in India. In addition, *B. cajani* AMBPC1010^{T} and *B. cajani* AMBPC1011, isolated from *C. cajan* in the Dominican Republic (Araújo et al., 2017), were included as controls. Nineteen of the tested strains produced nodules on pigeon pea: 9 (BRP) and 10 (NBAIM) (**Supplementary Table S6**). None of the strains selected from the Telangana/Andhra Pradesh region (HU) formed nodules on *C. cajan* under these test conditions. Results varied from the increased dry weight of both root and shoot to a detrimental effect when inoculated with

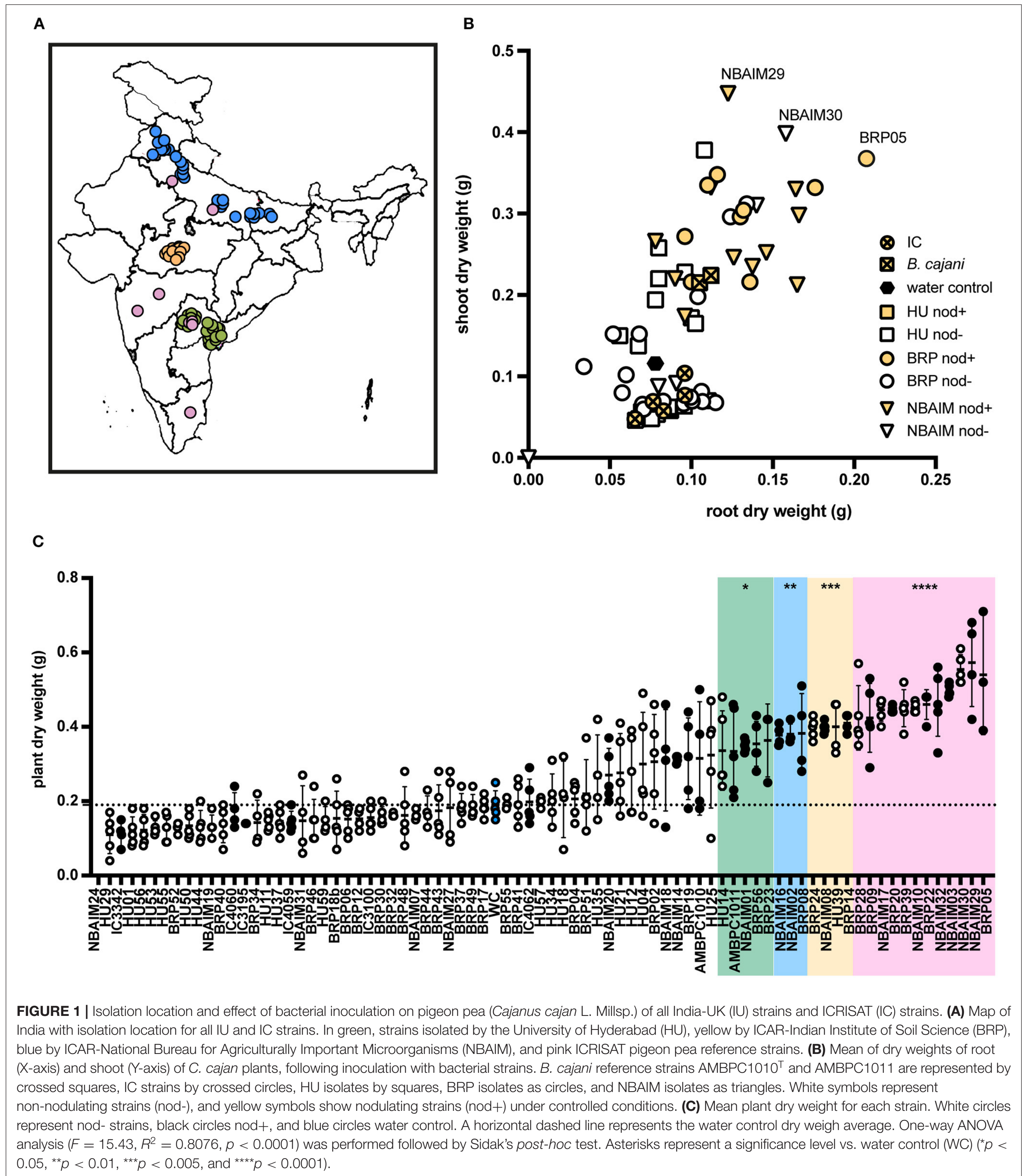


FIGURE 1 | Isolation location and effect of bacterial inoculation on pigeon pea (*Cajanus cajan* L. Millsp.) of all India-UK (IU) strains and ICRISAT (IC) strains. **(A)** Map of India with isolation location for all IU and IC strains. In green, strains isolated by the University of Hyderabad (HU), yellow by ICAR-Indian Institute of Soil Science (BRP), blue by ICAR-National Bureau for Agriculturally Important Microorganisms (NBAIM), and pink ICRISAT pigeon pea reference strains. **(B)** Mean of dry weights of root (X-axis) and shoot (Y-axis) of *C. cajan* plants, following inoculation with bacterial strains. *B. cajani* reference strains AMBPC1010^{T} and AMBPC1011 are represented by crossed squares, IC strains by crossed circles, HU isolates by squares, BRP isolates as circles, and NBAIM isolates as triangles. White symbols represent non-nodulating strains (nod-), and yellow symbols show nodulating strains (nod+) under controlled conditions. **(C)** Mean plant dry weight for each strain. White circles represent nod- strains, black circles nod+, and blue circles water control. A horizontal dashed line represents the water control dry weigh average. One-way ANOVA analysis ($F = 15.43$, $R^2 = 0.8076$, $p < 0.0001$) was performed followed by Sidak's *post-hoc* test. Asterisks represent a significance level vs. water control (WC) (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.0001$).

NBAIM24 where plants were dead (**Figures 1B,C**). It is clear that IC strains have a less beneficial effect on the growth of these plants than any of the IU-nodulating strains isolated in this study. Even though IC strains are used as pigeon pea inoculants across India, none were originally isolated from pigeon pea plants (**Table 1**) (Rupela et al., 1991). The strains that show the most significant increases in plant dry weight compared to uninoculated water controls are NBAIM29 (nod+) and BRP05 (nod+) (**Figure 1C**).

It is important to mention that there are non-nodulating strains, which under these conditions promote the growth of pigeon pea by an unknown mechanism. The nod^{-} strain NBAIM30 has the best performance of a strain that does not form any nodules, with a positive effect on both shoot and root, outcompeting many nodulating strains (**Figure 1C**).

Phylogenomic Diversity

We sequenced 27 genomes, 19 IU strains, which formed nodules under the test conditions, together with 8 IC strains (**Table 1**), for comparison with reference strains and to decipher the taxonomic diversity among them. Based on ANIm phylogeny, IU and IC strains were associated either with *Bradyrhizobium* (22 strains) or *Ensifer* (5 strains) (**Figure 2**). Twenty strains are related to *B. yuanmingense* CCBAU 10071^{T}, showing ANIm similarity values 96.8–98.3%. They all show lower ANIm values (82.3–90.7%) with the next most similar type strains: *Bradyrhizobium forestalis* INPA54B^{T}, *Bradyrhizobium liaoningense* CCBAU 83689^{T}, *B. cajani* AMBPC1010^{T}, and *Bradyrhizobium japonicum* USDA 6^{T} (**Supplementary Table S7**). We can consider that these twenty strains (BRP05, BRP09, BRP19, BRP20, BRP23, NBAIM01, NBAIM02, NBAIM03, NBAIM08, NBAIM14, NBAIM16, NBAIM18, NBAIM20, NBAIM32, IC3069, IC3965, IC3123, IC4059, IC4060, and IC4061) belong to *B. yuanmingense*; henceforth, they are defined as such in subsequent figures. Two strains cluster within *Bradyrhizobium* superclade II (Ormeño-Orrillo and Martínez-Romero, 2019). BRP56 has an ANIm of 96.5% with *Bradyrhizobium brasilense* UFLA03-321^{T}, 95.5% with *B. elkanii* USDA 76^{T}, and 95.4% with *Bradyrhizobium pachyrhizi* PAC 48^{T}; henceforth, it is referred to as *B. brasilense* BRP56 in subsequent figures. Within the same superclade, BRP22 shows an ANIm similarity lower than 85.9% to all closely related type strains: *Bradyrhizobium macuxiense* BR 10303^{T}, *B. ivorense* CI-1B^{T}, *Bradyrhizobium tropiciagri* SEMIA 6148^{T}, *B. elkanii* USDA 76^{T}, *B. brasilense* UFLA 03-321^{T}, and *B. pachyrhizi* PAC 48^{T}. Strain BRP22 could represent a new species due to its ANIm similarity value lower than 96%, although new species descriptions based on a single strain are discouraged, given the requirement to demonstrate intraspecific diversity (De Lajudie et al., 2019). Therefore, we cannot, as yet, assign BRP22 to any given species, so it will subsequently be referred to as *Bradyrhizobium* sp. BRP22. For the five strains in the *Ensifer* group, NBAIM29 showed 98.9% similarity with *Ensifer alkalisoli* YIC4027^{T} (*E. alkalisoli* NBAIM29), BRP14 showed 95.8% similarity with *Ensifer aridi* LMR002^{T} (*E. aridi* BRP14), and BRP08, IC3342, and IC4062 showed just 91.2, 91.1, and 90.9% similarity, respectively, with the closest type strain, *Ensifer terangae* USDA 4894^{T}, meaning that we cannot assign these latter three strains to any known species, i.e., *Ensifer* sp.BRP08, *Ensifer* sp.IC3342, and *Ensifer* sp.IC4062, respectively.

Genome Characteristics

The IU and IC *Bradyrhizobium* strains have a genome of 7.5–9.2 Mb, a GC-content >62.7%, absence of replication plasmid genes (*repABC*), and 20 out of 22 have a single estimated copy of 16S rRNA (**Supplementary Table S2**). Most *Bradyrhizobium* genomes range between 7 and 10 Mb with an average of 8.6 Mb (Ormeño-Orrillo and Martínez-Romero, 2019). However, *B. brasilense* BRP56 presents a remarkably larger genome among the IU and IC strains at 9.2 Mb (**Supplementary Table S2**). This strain is phylogenetically related to *B. elkanii*, which characteristically contains genomes larger than 9 Mb (Reeve et al., 2017). Since there is an inherent difficulty in resolving repetitive regions with short reads by assemblers (Waters et al., 2018), we estimate the rRNA copy number as the coverage ratio between 16S rRNA and the single-copy housekeeping gene *rpoB*. Most *Bradyrhizobium* IU and IC strains showed a single predicted copy, except for *B. yuanmingense* IC3069 and *B. yuanmingense* NBAIM32. Even though it is uncommon within the genus *Bradyrhizobium*, strains with closed genomes like *B. japonicum* USDA 6^{T} or *Bradyrhizobium* sp. BTAi1 have two copies of the rRNA cluster (Cytryn et al., 2008; Kaneko et al., 2011). There is a direct correlation between rRNA copy number and the time taken for a soil bacterium to respond to nutrient availability (Klappenbach et al., 2000), which could be translated into an adaptive advantage in a rhizosphere environment. In fact, *B. yuanmingense* NBAIM32 showed a significantly improved performance in plant growth experiments compared with other members of the By group (**Figure 3**, **Supplementary Table S6**). None of the IU and IC strains revealed the presence of plasmid-like replication genes (**Supplementary Table S2**). Although infrequent, plasmid presence was confirmed in *Bradyrhizobium* sp. BTAi1 and in *Bradyrhizobium* sp. DOA9 (Cytryn et al., 2008; Okazaki et al., 2015).

The IU and IC *Ensifer* strains showed a genome size of 6.5-7.4 Mb, 61-62 GC%, 3-6 estimated rRNA copies, and 2-3 plasmids (**Supplementary Table S2**). Most *Ensifer* spp. genomes have three copies of rRNA, as in *Ensifer fredii* NGR234 (Viprey et al., 2000), with the exception of *Ensifer* sp. IC4062, which shows six copies of rRNA. *Ensifer* IC strains have two copies of *repABC*, whereas IU strains have three, reflecting a different genomic organisation (**Supplementary Table S2**). These replicon numbers are in agreement with the work of Sugawara et al. (2013) with 48 different *Ensifer* spp., which showed 2–5 plasmids in Eckhart gels.

Phylogeny-Based on *nodC*

The *nodC* phylogenetic tree (**Figure 4**) shows that the twenty-seven sequenced strains fall into five main groups: *B. yuanmingense* (By), 8 strains; *B. cajani* (Bc), 12 strains; *B. elkanii* (Be), 1 strain; *B. icense* (Bi), 1 strain; and *E. fredii* (Ef), 5 strains. The IU and IC strains assigned as *B. yuanmingense* belong to either the By or Bc group. In the By group, *B. yuanmingense* NBAIM32, NBAIM18, NBAIM20, BRP09, IC4060, IC4061, IC3069, and IC4059 show 95% *nodC* nucleotide identity with *B. yuanmingense* reference strains, which were isolated in China from *Glycine max* (soybean) or *Lespedeza cuneata,* among which is *B. yuanmingense* CCBAU 10071^{T}. These IU and IC By strains share 89–90% *nodC* identity with the closed group formed by *B. diazoefficiens*-related strains (**Supplementary Table S8**). The second main group, Bc, comprises *B. yuanmingense* strains IC3195, IC3123, BRP19, BRP20, NBAIM14, NBAIM03, BRP05, BRP23, NBAIM02, NBAIM08, and NBAIM16, which showed more than 96.8% *nodC* nucleotide identity with *Bradyrhizobium*

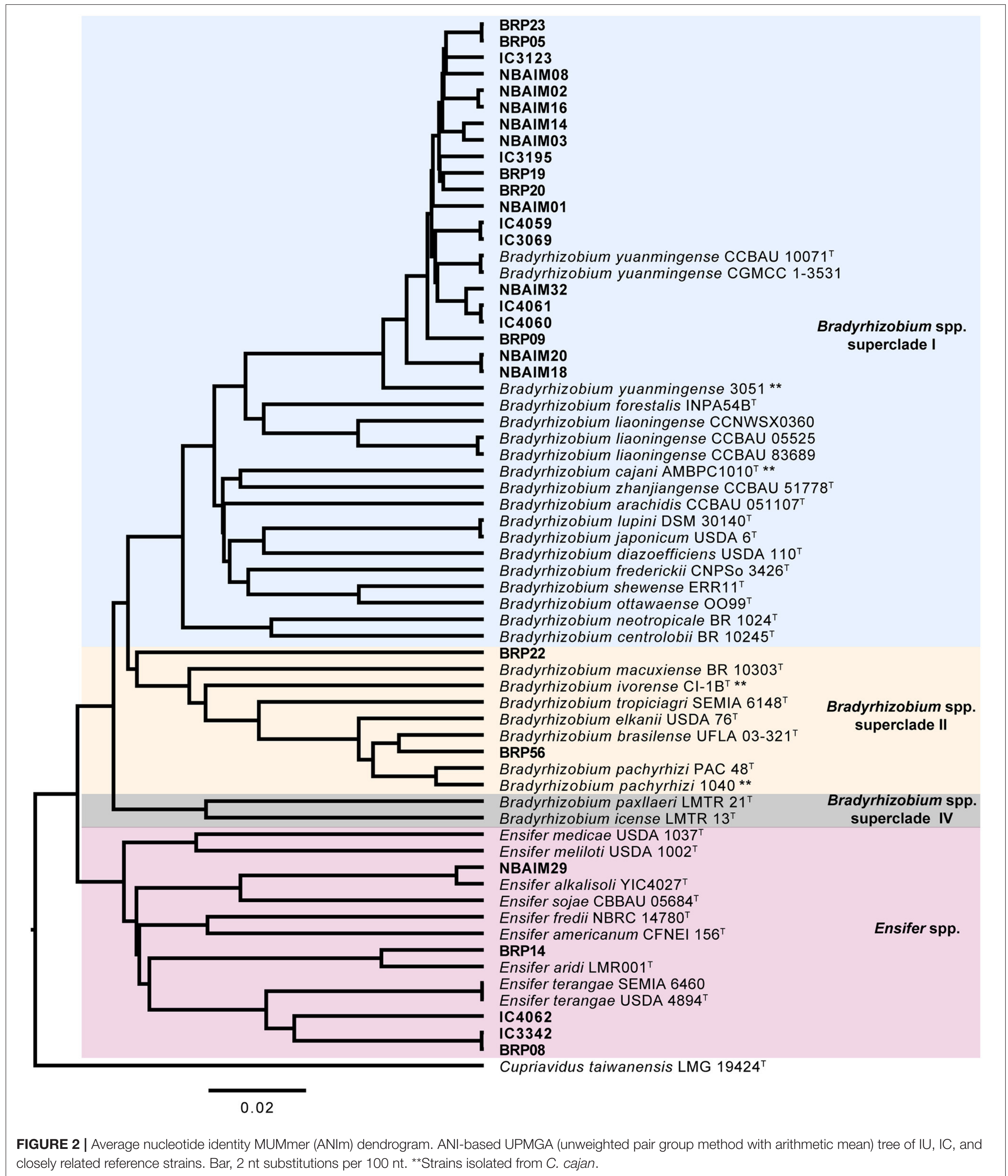


FIGURE 2 | Average nucleotide identity MUMmer (ANIm) dendrogram. ANI-based UPMGA (unweighted pair group method with arithmetic mean) tree of IU, IC, and closely related reference strains. Bar, 2 nt substitutions per 100 nt. **Strains isolated from *C. cajan*.

zhanjiangense CCBAU 51778^T and more than 92% with *B. cajani* AMBPC1010^T (**Figure 4**). *B. yuanmingense* NBAIM01 is more distant and shares 88.8–89.3% identity with other Bc, IU, and IC strains, and 87–87.7% with the aforementioned reference strains (**Supplementary Table S8**). The closest *nodC* sequence to that of *B. yuanmingense* NBAIM01 is from *Bradyrhizobium*

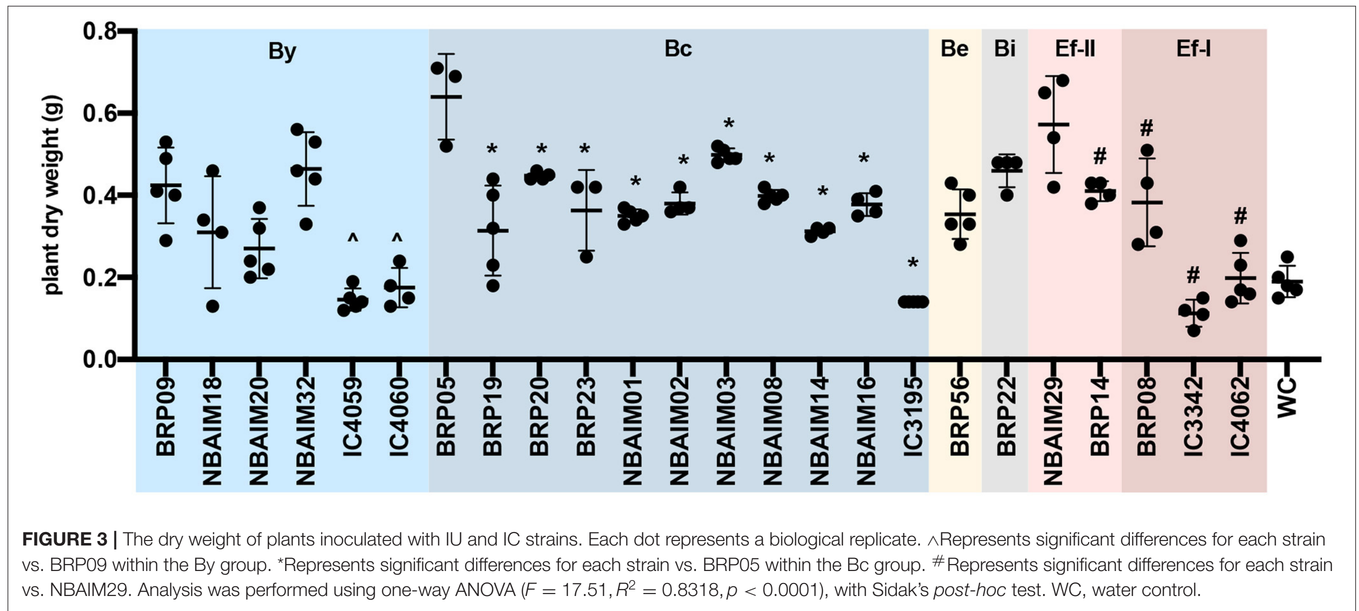


FIGURE 3 | The dry weight of plants inoculated with IU and IC strains. Each dot represents a biological replicate. ∧Represents significant differences for each strain vs. BRP09 within the By group. *Represents significant differences for each strain vs. BRP05 within the Bc group. #Represents significant differences for each strain vs. NBAIM29. Analysis was performed using one-way ANOVA ($F = 17.51, R^2 = 0.8318, p < 0.0001$), with Sidak's *post-hoc* test. WC, water control.

sp. LCT2 (91.23%). It is within this Bc group that 44% of the sequenced strains clade together, showing that this is the most common *nodC* type found in Indian *C. cajan* endosymbionts. *B. brasilense* BRP56 (Be group) has a *nodC* very similar to that of *B. elkanii* strains (99.7% identity) and 91.3% with that of *B. ivorense* CI-1B^T, a pigeon pea endosymbiont isolated in the Ivory Coast (Fossou et al., 2020). *Bradyrhizobium* sp. BRP22 is found in group Bi, with its *nodC* sequence, showing 83.2% and 81.9% similarity, respectively, to *B. icense* LMTR 13^T and *Bradyrhizobium paxllaeri* LMTR 21^T (**Figure 4**).

The IU and IC *Ensifer* strains have a *nodC* similar to that of *E. fredii* (Ef group), which clade in two subgroups, Ef-I (*Ensifer* sp. BRP08, IC3342, and IC4062) and Ef-II (*E. aridi* BRP14 and *E. alkalisoli* NBAIM29). Within Ef-I, the *nodC* similarity is 99.3–99.9% and <94% with *nodC* from Ef-II. The Ef-II IU strains share 98.4% *nodC* identity, and *circa*, 96%, with *E. fredii* and *E. sojae* reference strains in the same Ef-II group (**Figure 4**).

Nod Cluster Synteny

Nod gene cluster synteny analysis was performed for strains in each *nodC* group: By, Bc, Be, Bi, and Ef (**Figure 4**). All IU and IC strains have *nodABCIJ* as a core cluster, which is present in all symbiotic nod factor (NF)-dependent rhizobia.

Bradyrhizobium

All newly sequenced strains in the By group show the presence of the same nodulation-related genes, *nolY*-[]-*nolA*-[]-*D2*-[]-*D1YABCSUIJ*-*nolN*-*nodZ* (**Supplementary Figure S1**). Representative strains *B. yuanmingense* BRP09, *B. yuanmingense* IC4060, and *B. yuanmingense* NBAIM32 were selected to further investigate their synteny with *B. yuanmingense* CCBAU 10071^T and *B. diazoefficiens* USDA 110^T (**Figure 5A**). *B. diazoefficiens* USDA 110^T has three extra nodulation-related genes, *nolZ*, *nolM*, and *nolO*, not present in any of the newly sequenced strains. The By *nod* cluster is highly conserved, albeit with evidence of different insertion events. We can conclude that the By strains *B. yuanmingense* NBAIM18, NBAIM20, NBAIM32, BRP09, IC3069, IC4059, IC4060, and IC4061 have the same *nod* cluster as *B. yuanmingense* CCBAU 10071^T. A highly conserved *nod* cluster, *nolA*-[]-*nodD2D1YABCSUIJ*-*nolO*-*nodZ*, is present in the newly sequenced strains belonging to the Bc group (**Supplementary Figure S2**). *B. yuanmingense* NBAIM08 and IC3195 were selected as representative and aligned with *B. cajani* AMBPC1010^T and *B. zhanjiangense* CCBAU 51778^T (**Figure 5B**). Notwithstanding transposase-related genes in *B. yuanmingense* IC3195 (which are not present in either of the type strains), we can conclude that the *nod* cluster and its genomic context are the same as that of *B. cajani* AMBPC1010^T and *B. zhanjiangense* CCBAU 51778^T. The *nodC* phylogeny shows that *B. brasilense* BRP56 belongs to the Be group, together with *B. ivorense* CI-1B^T and *B. elkanii* USDA76^T (**Figure 4**). The observed *nod* cluster is *nolY*-[]-*nolA*-[]-*nodD2D1*-[]-*ABCSUIJ*-*nolO*-*nodZ* (**Figure 5C**). *B. brasilense* BRP56 has a *nodK* gene among *nodD1* and *nodA,* which is not annotated in CI-1B^T, and neither is it in *B. elkanii* USDA 76^T. Both reference strains have an open reading frame (ORF) in this region, which, in USDA 76^T, has an amino acid (aa) identity of 97.7% to *nodK* of *B. brasilense* BRP56, whereas, in CI-1B^T, it is just 59.4%. Furthermore, both *B. brasilense* BRP56 and *B. elkanii* USDA 76^T have *nopM* downstream of *nifA* (**Figure 5C**). Overall, we suggest that *B. brasilense* BRP56 has a typical *B. elkanii*-type *nod* cluster. *Bradyrhizobium* sp. BRP22, together with *B. icense* LMTR 13^T and *B. paxllaeri* LMTR 21^T, belongs to the Bi *nodC* group (**Figure 4**). Analysis of their *nod* cluster synteny shows that all strains have *nolA*-[]-*nodD2D1*-[]-*ABCSUIJ*-*nolO*-*nodZ*-[]-*noeE* (**Figure 5D**). In addition, *Bradyrhizobium* sp. BRP22 and *B. icense* LMTR 13^T have *nopM* downstream of *noeE,* whereas *B. paxllaeri* LMTR 21^T shows a pseudogene (pg) with 50–54% aa identity to the N-terminal part of *nopM*. We conclude that *Bradyrhizobium* sp. BRP22 has a *B. icense*-type *nod* cluster.

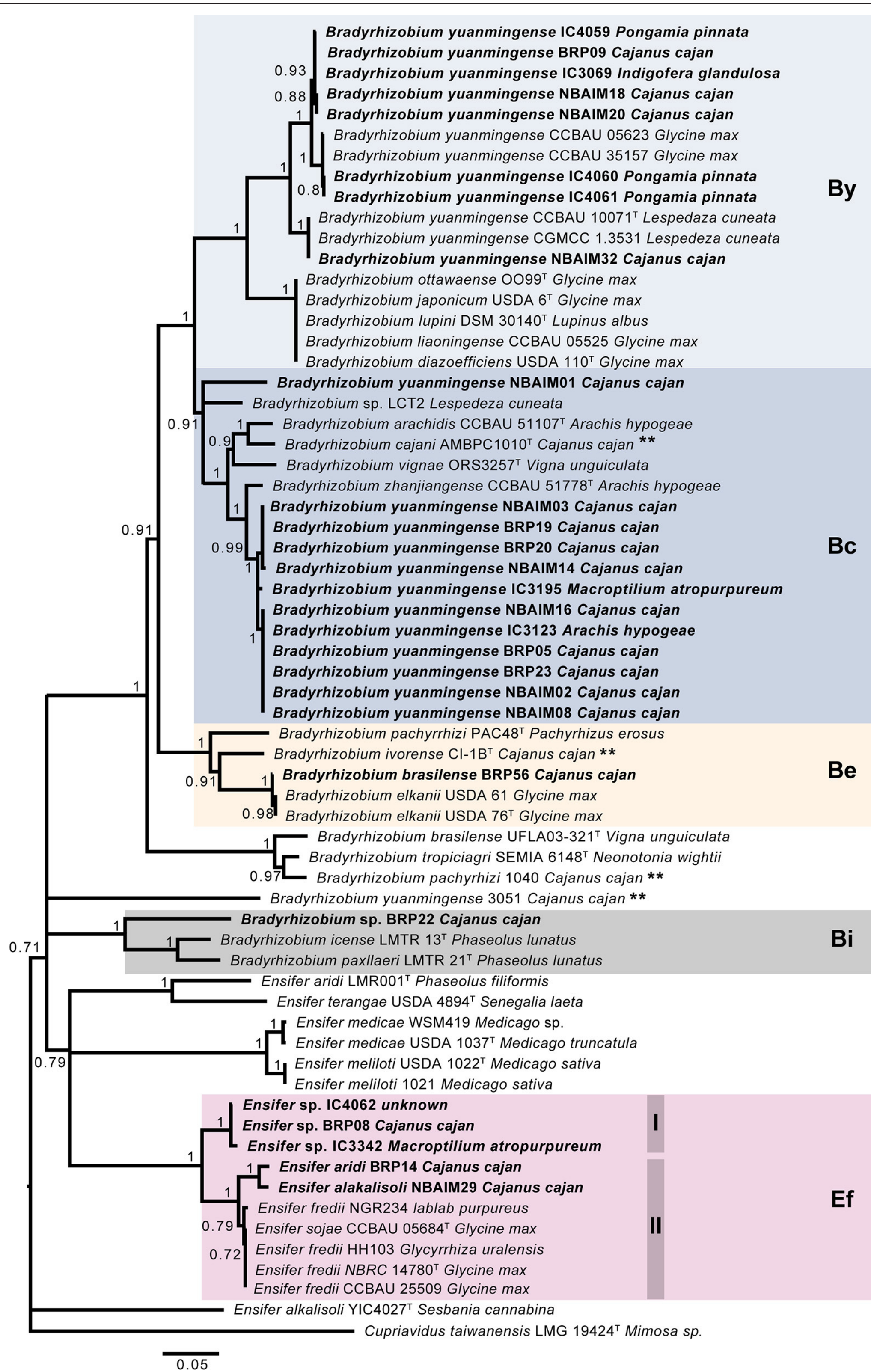


FIGURE 4 | Tree-based on *nodC* phylogeny. Neighbour-joining (NJ) phylogenetic tree based on *nodC* sequence (1,458 nt) of IU and IC strains with closely related species. Each is shown together with the plant from which it was isolated. Bootstrap values (only values > 70%, expressed as a percentage of 1,000 replications) are shown at the branching points. Bar, 5 nt substitutions per 100 nt. **Strains isolated from *C. cajan*. By (*B. yuanmingense*), Bc (*B. cajani*), Be (*B. elkanii*), Bi (*B. icense*), and Ef (*E. fredii*).

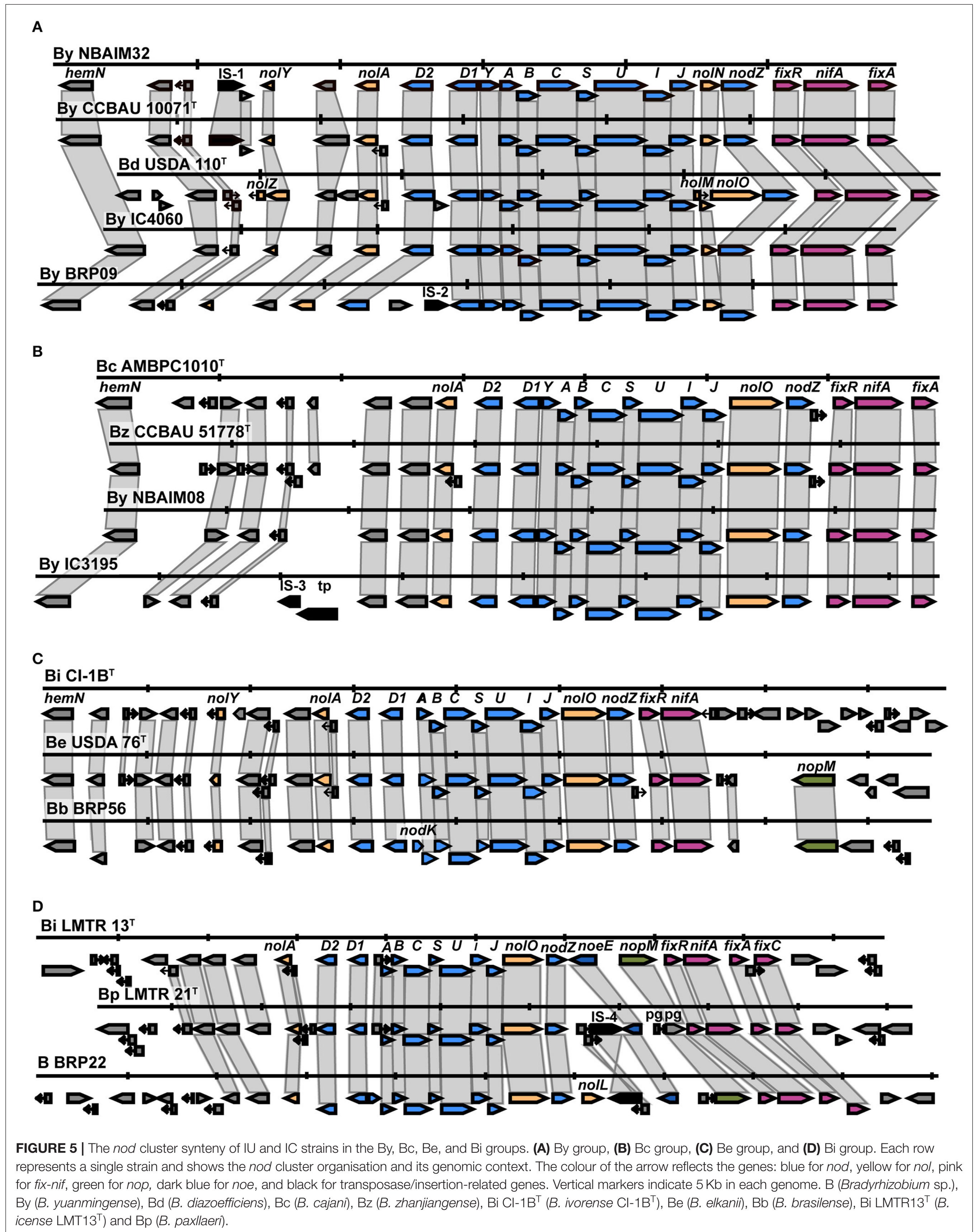


FIGURE 5 | The *nod* cluster synteny of IU and IC strains in the By, Bc, Be, and Bi groups. **(A)** By group, **(B)** Bc group, **(C)** Be group, and **(D)** Bi group. Each row represents a single strain and shows the *nod* cluster organisation and its genomic context. The colour of the arrow reflects the genes: blue for *nod*, yellow for *nol*, pink for *fix-nif*, green for *nop,* dark blue for *noe*, and black for transposase/insertion-related genes. Vertical markers indicate 5 Kb in each genome. B (*Bradyrhizobium* sp.), By (*B. yuanmingense*), Bd (*B. diazoefficiens*), Bc (*B. cajani*), Bz (*B. zhanjiangense*), Bi CI-1B^{T} (*B. ivorense* CI-1B^{T}), Be (*B. elkanii*), Bb (*B. brasilense*), Bi LMTR13^{T} (*B. icense* LMT13^{T}) and Bp (*B. paxllaeri*).

In selected representative strains from each *Bradyrhizobium nodC* group (**Figures 4**, **5**), this region of DNA was aligned (**Supplementary Figure S3**). As all strains have *nolA*-[]-*nodD2*-[]-*nodD1*-[]-*nodABCSUIJ*-[]-*nodZ*, this could be considered the minimum *nod* cluster necessary to nodulate pigeon pea. Other nodulation-related genes (*nolY*, *nodK*, *nodY*, *nolO*, *nolN*, *nolL*, or *noeE*) may be associated with host specificity and, therefore, play a part in the symbiotic performance.

Ensifer

The *nod* cluster regions for the five newly sequenced *Ensifer* strains were aligned and, together with reference strains in the same *nodC* phylogenetic clade, reveal a high degree of synteny within the group (**Figure 6**). The observed canonical *nod* cluster is *nodABCIJ-nolO-noeI*-[]-*noeE*. There are two genomic contexts for each *nod*C group (Ef-I and Ef-II), which suggests that they could have a different origin (**Figure 6**). Despite this, the IU and IC strains show great conservation of the *nod* cluster, except for the absence of *nolL* from *E. alkalisoli* NBAIM29. Instead, it has *cysNC* (adenylyl-sulphate kinase, A0A4S5J185). The lack of *nolL* could give *E. alkalisoli* NBAIM29 an advantage in plant recognition, which would explain its enhanced growth promotion phenotype observed *in planta* (**Figure 3**), although, as it is based only on a single strain, this is highly speculative.

Presence of Gene-Encoding Nops

There are multiple pieces of evidence supporting a T3SS and Nops and their key roles in the establishment of symbiosis and host specificity in certain rhizobia-legume interactions (Pueppke and Broughton, 1999; López-Baena et al., 2016). We have confirmed the presence of T3SS machinery by finding orthologues (>50% aa identity and coverage) for *rhcQ, rhcU, ttsI, nolV*, and *nolU* from the well-characterised T3SS of *B. vignae* ORS3257 (Teulet et al., 2019) and *E. fredii* NGR234 (Freiberg et al., 1997) in all the newly sequenced strains presented in this study (**Supplementary Table S4**). To determine the putative range of T3SS effectors, we have based analysis on the Nops and used Nop sequences from well-characterised *Ensifer* and *Bradyrhizobium* spp. to find homologues in the IU and IC genomes (as shown in **Supplementary Table S4**).

Within the *Bradyrhizobium* strains, all IU and IC strains have orthologues for *nopT*, *nopP2*, *nopM2*, and *nopM3*. These T3SS effectors could be needed for establishing symbiosis between *Bradyrhizobium* spp. and pigeon pea (**Figure 7A**). The groups formed based on the presence and absence of Nop orthologues in *Bradyrhizobium* spp. (**Figure 7A**) are highly correlated with those observed in the *nodC* phylogeny and *nod* cluster synteny (**Figures 4**, **5**). Cluster I (By) and cluster II (Bc) are distinguished from each other by the presence or absence of two groups of *nop* genes: group A (*nopC*, *nopAA*, *nopM1*, and *nopX*), group B (*nopD*, *nopAR*, *nopL*, and *nopE*), where cluster I (By) has group A genes but not those of group B and cluster II (Bc), vice versa (**Figure 7A**). There are a few orphan strains that present a different presence/absence pattern, e.g., *B. yuanmingense* BRP09 has all the Nop genes present in the other By strains (cluster I), plus *nopAA* (**Figure 7A**). However, we have not observed differences in plant dry weight between *B. yuanmingense* BRP09 and other IU strains of the By group (**Figure 3**), suggesting that the presence of *nopAA* is uncorrelated with plant performance. *Bradyrhizobium yuanmingense* NBAIM01 belongs to the Bc *nod* group (cluster II), although it shows a very different set of Nop homologues. Its lack of *nopX* and *nopC* (*nolJ*) could be counterbalanced by the presence of *nopB*, nopL, and/or *nopE* effectors since the plant performance of *B. yuanmingense* NBAIM01 is similar to that of other Bc members (**Figure 3**). Finally, *B. yuanmingense* BRP05 displays the common Nop genes for Bc strains (group II), plus *nopA* (**Figure 7A**). This strain promotes plant growth significantly more than any other Bc member or, indeed, any other *Bradyrhizobium* strains tested (**Figure 3**), which could be a result in part of the presence of this T3SS effector, although, without further strains showing similar characteristics, it is impossible to draw firm conclusions at this stage.

In comparison to *Bradyrhizobium,* a total of only seven *nop* genes are present in *Ensifer* (**Figure 7B**). All *Ensifer* IU and IC strains show orthologues for *nopA*, *nopB*, *nopL*, *nopM*, and *nopX*. Nevertheless, only *E. alkalisoli* NBAIM29 has *nopC*, *nopP*, and *nopT*, and, together with the lack of *nolL*, could equip it for improved performance on pigeon pea (**Figure 3**). However, further strains showing the same characteristics are required to test this speculation.

Genetic Features

Putative proteomes of *Bradyrhizobium* and *Ensifer* IU and IC strains were analysed to infer their core genome and pangenomes (**Figures 8A,D**). Both groups of strains showed an open pangenome of 17,596 and 10,458, respectively, and a core genome of *circa* 3,500 genes for both. Non-core genes present in each group of strains could play a role in soil endurance, competition for root colonisation, host specificity, or symbiosis establishment and, therefore, may help explain the differences observed in plant growth promotion (**Figure 3**).

Bradyrhizobium Orthologues

To reduce the complexity and computational time, we chose representative strains (shown in bold) between those sharing ANIm values greater than 99% similarity: *B. yuanmingense* **BRP05**-BRP23 (99.91%), **IC3069**-IC4069 (99.86%), **NBAIM03**-NBAIM14 (99.18%), NBAIM32-IC4060-**IC4061** (99.27-99.28%), and NBAIM18-**NBAIM20** (99.89%). All IU and IC strains share a total of 3,879 orthologue clusters with enrichment of DNA-related functions (GO:0006412, GO:0006313, and GO:0003700), transmembrane transport (GO0055085 and GO:0008643), and cell shape regulation (GO:0008360) (**Figure 8B**). Thirteen out of 16 analysed strains show exclusive orthologue clusters, which are not present in any other strain. *Bradyrhizobium* sp. BRP22 and *B. brasilense* BRP56 show the greatest number of exclusive groups of orthologues, 98 and 93, respectively (**Figure 8B**), which could be the result of the phylogenomic differences with other *B. yuanmingense* strains (**Figures 2**, **4**, **5**).

Since the most distinct feature observed among IU and IC strains is the nodulation-related gene groups (**Figure 5**, **Supplementary Figure S3**), we have compared the orthologue

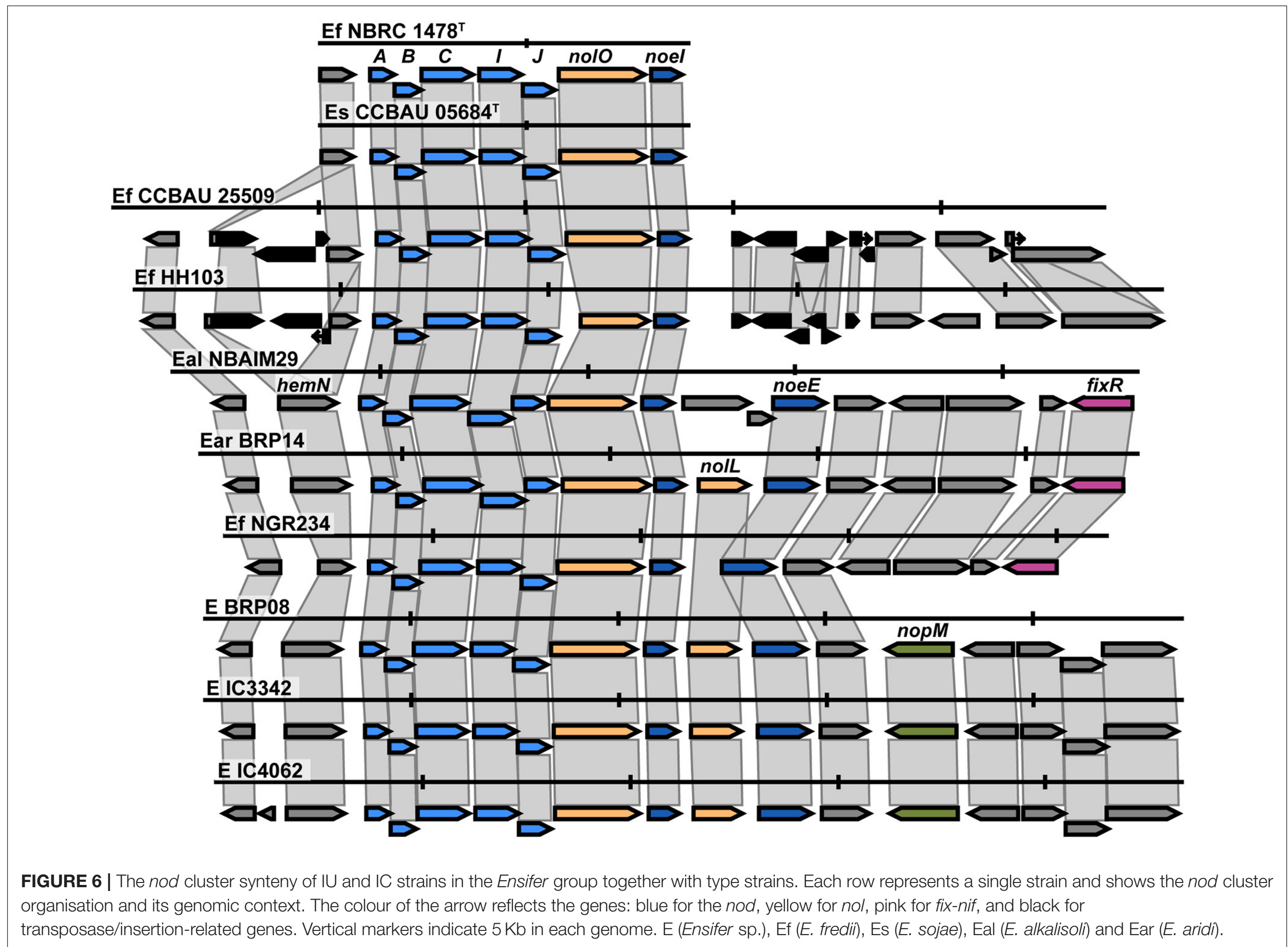


FIGURE 6 | The *nod* cluster synteny of IU and IC strains in the *Ensifer* group together with type strains. Each row represents a single strain and shows the *nod* cluster organisation and its genomic context. The colour of the arrow reflects the genes: blue for the *nod*, yellow for *nol*, pink for *fix-nif*, and black for transposase/insertion-related genes. Vertical markers indicate 5 Kb in each genome. E (*Ensifer* sp.), Ef (*E. fredii*), Es (*E. sojae*), Eal (*E. alkalisoli*) and Ear (*E. aridi*).

clusters shared among By, Bc, Be, and Bi (**Figure 8C**). The exclusive genes for each group represent orthologue clusters that are present in all strains within that specific group. By is the only group that exclusively shows enrichment in GO functions for carbohydrate transport (GO: 0008643 and GO: 0015407). Among the 1,117 orthologue groups shared between By and Bc strains there is enrichment in clusters associated with chemoreceptors (GO: 0007165), permeases (GO: 005585), and flagellum-dependent cell motility (GO: 0071973), which are not present in *Bradyrhizobium* sp. BRP22 and *B. brasilense* BRP56. Nevertheless, these strains could partially compensate for this absence through the catabolism of aromatic compounds (GO: 0019439), which are a component of pigeon pea root exudates (Ae et al., 1990).

Ensifer Orthologues

We compared the orthologues groups of IU and IC *Ensifer* strains, which all share 3,730 orthologue clusters (**Figure 8E**). In this core set, there is enrichment in different DNA-related biological processes (GO: 0006412, GO: 0006313, and GO: 0035556) and transmembrane transport (GO: 0055085). The comparison between IU and IC strains shows enrichment for IC strains in an orthologue cluster annotated as putative adenylate cyclase 3 (*cya3*, GO: 0035556), which could be the reason for the significantly different plant performance between IC and IU strains (**Figure 3**). *Ensifer* sp. IC3342, *Ensifer* sp. IC4062, *Ensifer* sp. BRP08, and *E. aridi* BRP14 strains shared 626 orthologues families that are not present in *E. alkalisoli* NBAIM29 (**Figure 8E**). Within this group of orthologues, there is enrichment in the biosynthesis pathway of rhizobactin 1021 (GO: 0019289). Since *E. alkalisoli* NBAIM29 has a significantly better plant performance (**Figure 3**), we hypothesise that synthesis of rhizobactin 1021 might be a cost, which *E. alkalisoli* NBAIM29 would not sustain.

Genotype-Metadata Correlation

We have analysed the pigeon pea population structure based on GC%, genome length, number of tRNAs, rRNA clusters, *repABC*, *nod* type, and presence/absence of *nod* and *nop* genes (**Figure 9**). The population separates based on the *nod* group each strain belongs to (**Figure 9A**). In addition, we have run PERMANOVA using bacterial species (*B. yuanmingense*, *Bradyrhizobium* sp., *Ensifer* sp., *E. alkalisoli*, and *E. aridi*), *nod* type (By, Bc, Be, Bi, Ef-I, and Ef-II), *nop* profile (B-*nop*-I, B-*nop*-II, B-*nop*-II,

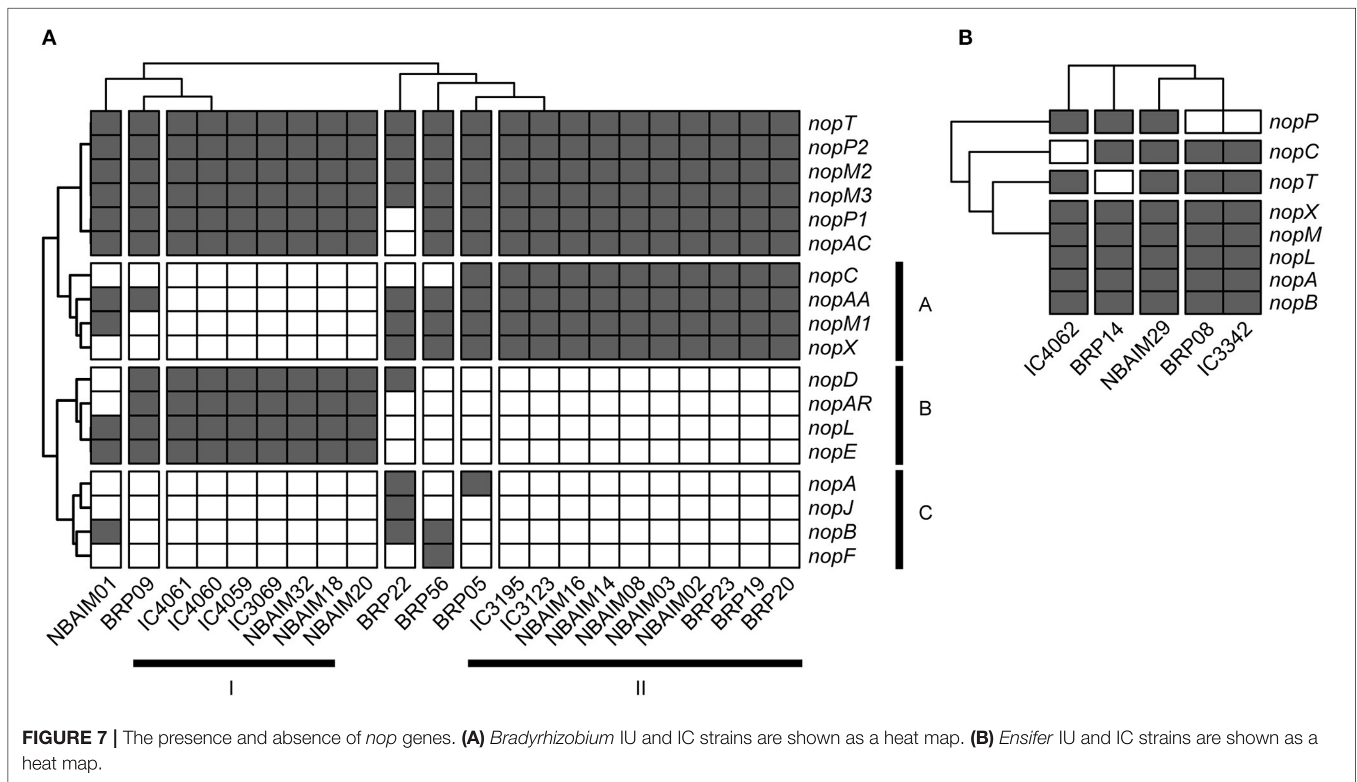


FIGURE 7 | The presence and absence of *nop* genes. **(A)** *Bradyrhizobium* IU and IC strains are shown as a heat map. **(B)** *Ensifer* IU and IC strains are shown as a heat map.

B-*nop*-IV, B-*nop*-V, B-*nop*-VI, B-*nop*-VII, E-*nop*-I, E-*nop*-II, E-*nop*-III, and E-*nop*-IV), location of isolation (Madhya Pradesh, Uttar Pradesh, Punjab, Haryana, Tamil Nadu, and Maharashtra), the plant host from which the strain was originally isolated (*C. cajan* cv. Asha, *C. cajan* cv. Bahar, *Indigofera glandulosa*, *Arachis hypogaea*, *Macroptilium atropurpureum*, and *Pongamia pinnata*), and the culture collection (or origin) (BRP, IC, and NBAIM) (**Figure 9B**). The main factor controlling the assembly of the pigeon pea endosymbiont population is the type of *nod* genes, followed by the species the strain belongs, to and finally, the *nop* gene set that each strain contains. The factors that had no significance were the location of isolation, the plant host from which the strain was originally isolated, and the culture collection from which the strain came (origin).

DISCUSSION

Among the BOX-PCR-reduced population of 65 strains, only 19 were able to establish symbiosis with pigeon pea under laboratory conditions. The isolation of non-symbiotic bacteria from nodule samples has previously been reported (Wu et al., 2011), as well as opportunistic infection (Zgadzaj et al., 2015). Moreover, Fossou et al. (2016) in their sampling of nodule-isolated strains from pigeon pea in Ivory Coast showed that 22% of the population did not display any rhizobia-like features. In addition, they were unable to amplify nitrogenase-encoding sequences (*nifH*) from 5% of the selected strains.

In our pigeon pea endosymbiont population of 27 IU and IC strains, the diversity is moderately driven by the species the strain belongs to (**Figure 9**). The main species-nodulating pigeon pea in India is *B. yuanmingense* (20 out of 27, **Figure 2**). In their study of pigeon pea endosymbionts in the Dominican Republic, Araújo et al. (2015) found that all strains investigated had a 99.8% identity to *B. yuanmingense* CCBAU 10071^{T}. Moreover, one of the sequenced pigeon pea endosymbionts from this study, *B. yuanmingense* 3051 (ALSPC3051), shows a high ANIm similarity (95.7–96%) to *B. yuanmingense* IU and IC strains (**Figure 2**, **Supplementary Table S7**). It is interesting to note that *B. yuanmingense* may be a predominant symbiont in India since it has been isolated from several legumes throughout the country (Ojha et al., 2017; Rathi et al., 2018). *B. brasilense* BRP56 and *Bradyrhizobium* sp. BRP22 are rare strains in this population; however, within this superclade II exist other pigeon pea-nodulating species isolated in the Dominican Republic, Brazil, and Ethiopia, such as *B. ivorense* and *B. elkanii* (Stepkowski et al., 2003; Wolde-Meskel et al., 2005; Fossou et al., 2016, 2020).

Nodulation-related genes are the main diversity driver in the pigeon pea endosymbiont population (**Figure 9**). The *nodC* sequences defined clear groups, with Bc the most common (12 out of 27). This sequence is similar to the reference strains, including *B. zhanjiangense* CCBAU 51778^{T}, a Chinese strain isolated from *A. hypogaea* (Li et al., 2019), and *B. cajani* AMBPC1010^{T}, a *C. cajan* strain isolated in the Dominican Republic (Araújo et al., 2017). Despite differences observed in *nodC*, all *Bradyrhizobium* spp. share a common *nod* cluster *nolA*-[]-*nodD2*-[]-*nodD1*-[]-*nodABCSUIJ*-[]-*nodZ* (**Supplementary Figure S3**). However, different presence/absence patterns were observed for genes

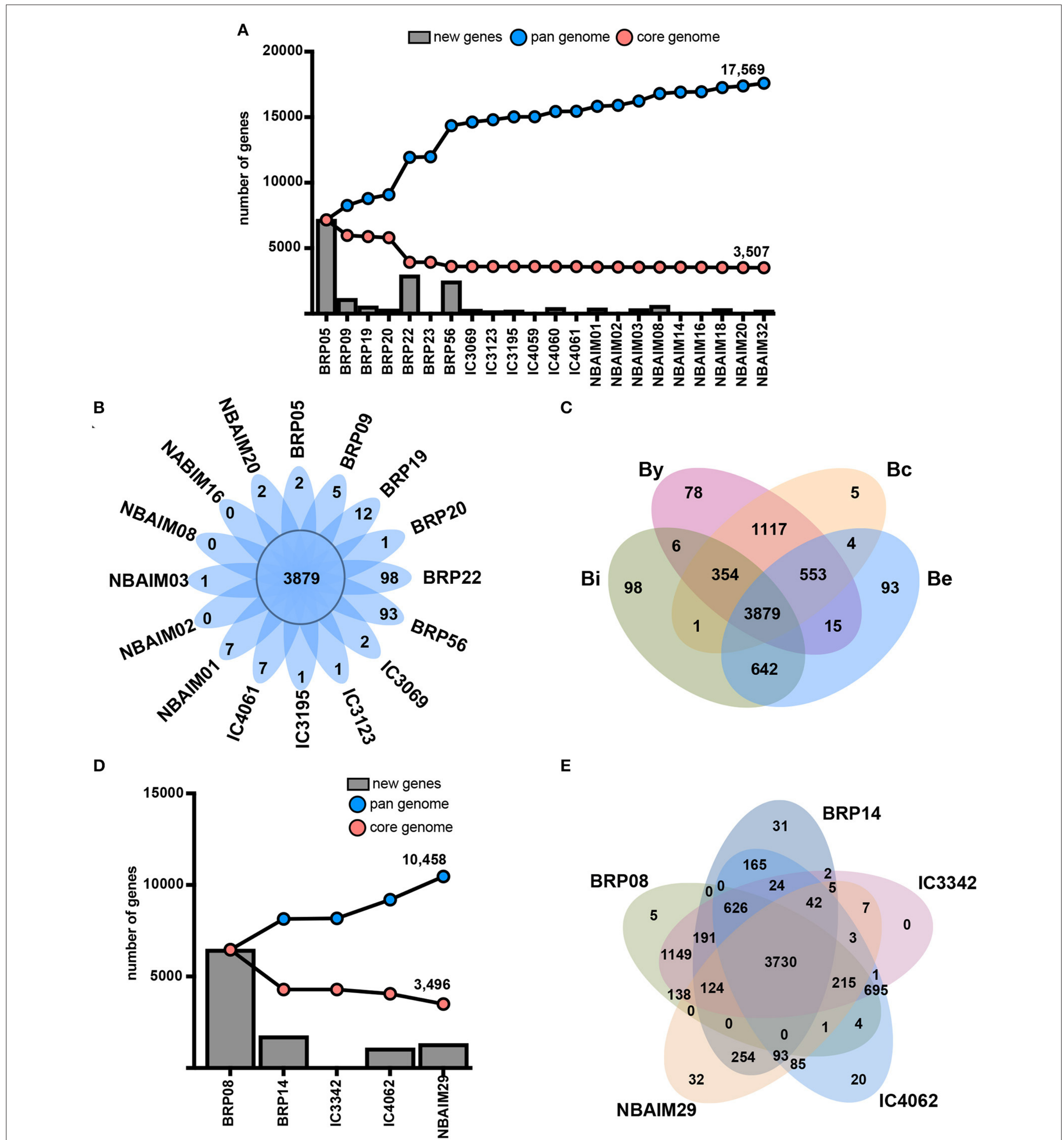


FIGURE 8 | Genetic features. **(A)** Core genome and pangenome of *Bradyrhizobium* where X-axis shows strains and the Y-axis, the number of genes. Blue line: pangenome. Red line: core genome. New genes: bars. **(B)** Core and exclusive orthologue clusters for each *Bradyrhizobium* strain. **(C)** Venn diagram showing shared and exclusive orthologue families among nodulation-type groups. By: the *B. yuanmingensenod*-type group. Bc: the *B. cajaninod*-type group. Be: the *B. elkaniinod*-type group. Bi: the *B. icensenod*-type group. **(D)** Coregenome and pangenome of *Ensifer* where X-axis shows strains and the Y-axis, the number of genes. Blue line: pangenome. Red line: core genome. New genes: bars. **(E)** Core and exclusive orthologue clusters for each *Ensifer* strain.

related to NF modifications, *nolY*, *nodK*, *nodY*, *nolO*, *nolN*, *nolL*, or *noeE* for each *Bradyrhizobium nodC* type group, By, Bc, Be, and Bi (**Figures 4**, **5**). The IU and IC *Bradyrhizobium* strains either have *nodY* or *nodK* between nodD1 and *nodA*

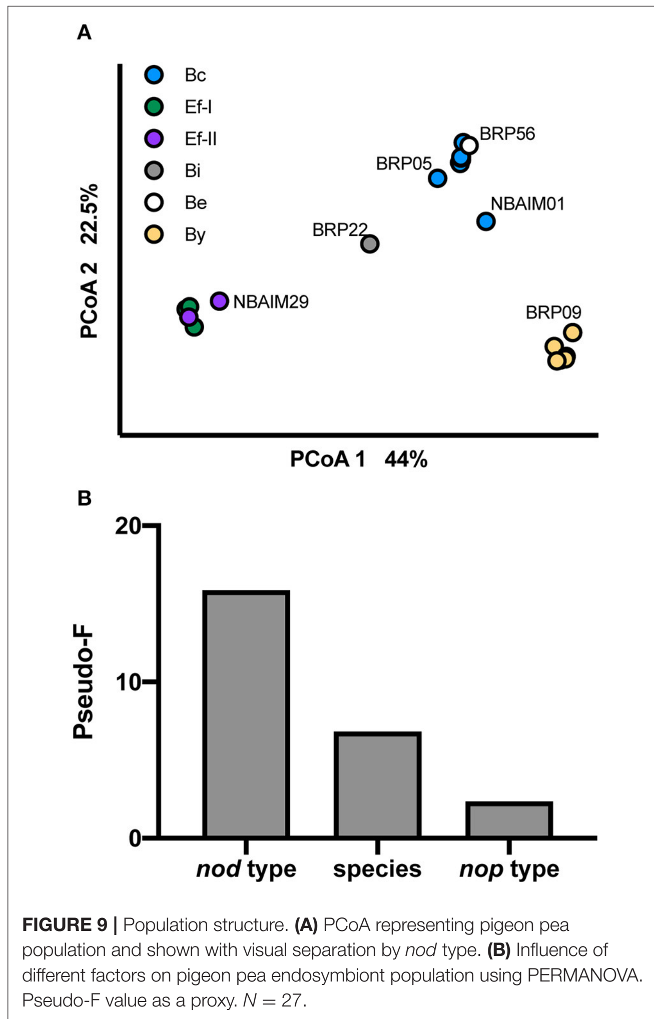


FIGURE 9 | Population structure. **(A)** PCoA representing pigeon pea population and shown with visual separation by *nod* type. **(B)** Influence of different factors on pigeon pea endosymbiont population using PERMANOVA. Pseudo-F value as a proxy. $N = 27$.

(**Supplementary Figure S3**), whose functions have not yet been elucidated (Menna and Hungria, 2011). The IU and IC strains from the Bc and Be groups have a *nolY* homologue upstream from *nolA* (**Figures 5B,C**). In *B. diazoefficiens* USDA 110^T, a *nolY* mutant showed a significant disadvantage in nodule kinetics on *Vigna radiata* (mung bean), but this detrimental effect was not so strong in soybean (Dockendorff et al., 1994), suggesting that the presence or absence of *nolY* in *Bradyrhizobium* spp. could be related to their host range. *Bradyrhizobium* sp. BRP22 is the only strain with *nolL* and *noeE* homologues, which seem to modify the NF playing a role in host specificity (**Figure 5D**) (Corvera et al., 1999; Wei et al., 2008). Moreover, differences were observed in the presence and absence of the annotated carbamoyltransferases *nolN* and *nolO* (**Supplementary Figure S3**). Bc, Be, and Bi strains have the *nolO* homologue between *nodJ* and *nodZ*, whereas By strains have *nolN.* In *B. diazoefficiens* USDA 110^T *nolMNO* is part of the *nod* operon, and *nolNO,* together with *nodZ,* acts in the NF 2-O-methylfucosylation. However, mutants in *nolO* or *nolNO* in this strain showed the same phenotype: delayed nodule formation and a reduced percentage of nodules per plant in legumes like soybean or mung bean (Luka et al., 1993). Since both genes encode carbamoyltransferases, which probably undertake the same function, it is possible that NF 2-O-methylfucosilation is essential to establish symbiosis in pigeon pea. Since differences in plant performance were not observed (**Supplementary Figure S4B**), we conclude that the common functional *nod* cluster for IU and IC *Bradyrhizobium* spp. is *nolA-nodD2D1-nodY/K*-nodABCSUIJ-*nolO/nolN*. Genes like *nodY*, *nodK*, *noeE*, and *nolL* only reflect phylogenetic diversity among these strains. The absence of *nolY* could have been positively selected, since the major group, Bc, does not show a *nolY* homologue. However, this selection has no impact on plant performance (**Supplementary Figure S4A**).

Based on the orthologue analysis, each *Bradyrhizobium* group could have developed a different strategy to endure in the pigeon pea rhizosphere (**Figure 8C**). Bc is the most common pigeon pea symbiont group in our population, and we hypothesise that it may be better adapted to the pigeon pea root environment. Together with By, both have homologues of chemoreceptors and flagella, which Be and Bi do not possess. These groups of genes are essential in rhizobium-legume symbioses (Jiang et al., 2016; Wheatley et al., 2020). However, *Bradyrhizobium* sp. BRP22 and *B. brasilense* BRP56 could partially compensate for this absence through the catabolism of aromatic compounds, which are present in pigeon pea root exudates (Ae et al., 1990). The By group shows enrichment in carbohydrate transporters. Since sugars are one of the main components of root exudates (Lynch and Whipps, 1990), it is possible that having a greater pool of carbohydrate transporters could give the By group an adaptative advantage in the pigeon pea rhizosphere, resulting in their increased prevalence in nodules.

Among IU and IC *Bradyrhizobium* spp., the Bc strain *B. yuanmingense* BRP05 promotes plant growth more significantly than any other strain tested (**Figure 3**), which could be, in part, a result of the presence of T3SS pili structures like *nopA* (**Figure 7A**). NopA is part of the external T3SS apparatus, and its deletion completely abolishes the secretion of other Nops, since it is a major component of the T3SS pili (Krishnan et al., 2003). However, in *E. fredii* USDA 257, the absence of *nopA* extends the host range to other soybean varieties, whereas, in cowpea, it has a slightly deleterious effect (Kim and Krishnan, 2014). Nevertheless, it is impossible to draw firmer conclusions without further strains showing similar characteristics to *B. yuanmingense* BRP05.

Ensifer spp. is an infrequent pigeon pea endosymbiont in the population since only five (of 22) IU and IC strains were assigned to this genus. There are a few records of *Ensifer* strains,-nodulating pigeon pea, including strains isolated in Cerrado soil in Brazil and India (Coutinho et al., 1999; Stepkowski et al., 2003). Their rarity is probably related to pigeon pea specificity rather than low *Ensifer* spp. numbers in soil, since, in India, *Ensifer* spp. are common endosymbionts of native legumes growing in alkaline soils (Gehlot et al., 2013; Tak et al., 2016; Sankhla et al., 2017; Rathi et al., 2018; Choudhary et al., 2020). Regarding nodulation genes, the most relevant feature is *nolL*, where its absence correlates with a significantly improved plant performance in *E. alkalisoli* NBAIM29 (**Figures 3**, **6**). *nolL*

determines 4-O-acetylation of the fucosyl residue in NF, and its deletion has been shown to have a negative effect on *R. etli* CE3 nodule kinetics in some *Phaseolus vulgaris* cultivars and in *V. umbellata* (Corvera et al., 1999). Furthermore, the heterologous expression of *nolL* in *E. fredii* USDA 257 extends its host range to other legumes like *Leucaena leucocephala* and *L. halophilus* (Berck et al., 1999). NolL plays a role in both host specificity and host range. Therefore, we hypothesise that the lack of the NF fucosyl acetylation might give *E. alkalisoli* NBAIM29 an advantage in plant recognition, explaining the phenotype observed *in planta* (**Figure 3**). However, with only one strain, this is highly speculative. Moreover, *E. alkalisoli* NBAIM29 is the only IU and IC *Ensifer* spp. strain that has *nopC*, *nopP*, and *nopT*; all of them are well-characterised T3SS effectors with functions related to host-range and interaction with the plant immune system. The deletion of any of these *nop* genes results in a reduction of nodules in the symbiosis between *E. fredii* and different legumes (soybean and *P. vulgaris*) (Boundy-Mills et al., 1994; Skorpil et al., 2005; Dai et al., 2008; López-Baena et al., 2009).

The comparison of orthologue groups between the IU and IC *Ensifer* spp. strains showed an exclusive group in IC annotated as an adenylate cyclase 3 (cya3), which modulate the extent of epidermal infection during nodulation (Tian et al., 2012). Indeed, a mutation in *cya3* (*cya5*) in *E. meliloti* CXM1-105 significantly increased alfalfa shoot dry weight (Sharypova et al., 1999), which could be reflected in the different plant performances between IC and IU strains (**Figure 3**).

Remarkably, *E. alkalisoli* NBAIM29 lacks an orthologue family related to the synthesis of rhizobactin 1021 (*rhbBCDEF*), a siderophore that chelates iron (Fe) (Lynch et al., 2001). We hypothesise that the biosynthesis of a siderophore might be redundant in the pigeon pea rhizosphere since it exudes piscidic acid, an aromatic compound that solubilises phosphorous (P) by chelating Fe from P-Fe compounds (Ae et al., 1990). Siderophore biosynthesis would represent a metabolic cost to the other *Ensifer* strains, *Ensifer* sp. IC3342, *Ensifer* sp. IC4062, *Ensifer* sp. BRP08, and *E. aridi* BRP14, and could explain their significantly lower plant performance compared to that of *E. alkalisoli* NBAIM29 (**Figure 3**). It is possible that the presence of these Nop proteins, together with the lack of *nolL* and the rhizobactin 1021 biosynthesis pathways, endows *E. alkalisoli* NBAIM29 with improved plant recognition machinery that could translate into better performance with pigeon pea, but without further strains showing the same characteristics, it is impossible to tell at this stage.

Due to its intrinsic capacity to tolerate drought (grown on drylands), pigeon pea is a promising candidate for resilience to climate change; however, its yield remains low. The use of symbionts well-adapted to the growth conditions of pigeon pea could increase its productivity (Pellegrino et al., 2011; Pellegrino and Bedini, 2014; Pastor-Bueis et al., 2019). Our findings demonstrate that the most common pigeon pea endosymbiont in India is a *B. yuanmingense* strain with a *B. cajani-B. zhanjiangens* (Bc) *nod* type, defined mainly by the absence of *nolY* and the presence of *nolO*. Since we have not observed location to be a driving factor in population diversity, our findings may apply to much, if not all, of India. Due to its intrinsic capabilities for persisting and establishing symbiosis, in addition to its genetic and genomic features, we suggest that *B. yuanmingense* BRP05 could be a good candidate for inclusion in inoculum formulations for pigeon pea in India. However, testing a range of IU strains for symbiotic performance in field trials is essential to assess their real-world performance. Moreover, the less common *Ensifer* strains, like *E. alkalisoli* NBAIM29, may be better for alkaline conditions, where members of this genus often perform well (Gehlot et al., 2013; Tak et al., 2016; Sankhla et al., 2017; Rathi et al., 2018; Choudhary et al., 2020).

This study presents a first step in defining and collecting strains that can nodulate pigeon pea in Indian soils. Their ability to influence plant performance has been investigated in glasshouse experiments under sterile conditions. Therefore, extensive trialling in the field in India, using a range of different varieties of pigeon pea, is now suggested to evaluate their performance under these agronomic conditions. We are confident that such studies will lead to the selection of a group of highly effective strains for use in inoculant technology, improving the symbiotic performance of this essential legume in India.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in GenBank repository, BioProject PRJNA679722.

AUTHOR CONTRIBUTIONS

BJ, PP, DR, AS, AP, and VR conceived the study and designed the manuscript. BJ, MM, AB, DC, SP, NA, SM, SK, PS, and MK performed the experiments. BJ, MM, and AT analysed the data. BJ prepared the manuscript. BJ, AT, AE, VR, EJ, PP, MM, DR, AP, AS, and AB critically reviewed the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the study, and approved it for publication.

FUNDING

This study was supported by the Biotechnology and Biological Sciences Research Council [Grant No. BB/N013387/1], granted to PP; and by the Department of Biotechnology, Government of India [Grant No. BT/IN/UK-VNC/41/DLN/2015-16], granted to DR, AS, and AP.

ACKNOWLEDGMENTS

We thank the Indian Council of Agricultural Research, New Delhi, and the Director IISS, Bhopal, India, for administrative support. We thank International Crops Research Institute for the Semiarid Tropics (ICRISAT), Hyderabad, India, for supplying pigeon pea seeds and rhizobia strains (IC). We

would like to thank Prof. Fernando González-Andrés from Universidad de León, Spain, for sharing *B. cajani* AMBPC 1010^{T} and *B. cajani* AMPBC 1011 strains. We would like to thank Lockdown for giving us the opportunity to put this study together for publishing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.680981/full#supplementary-material

REFERENCES

Ae, N., Arihara, J., Okada, K., Yoshihara, T., and Johansen, C. (1990). Phosphorus uptake by pigeon pea and its role in cropping systems of the Indian subcontinent. *Science* 248, 477–480. doi: 10.1126/science.248.4954.477

Agrawal, S., Arze, C., Adkins, R. S., Crabtree, J., Riley, D., Vangala, M., et al. (2017). CloVR-Comparative: automated, cloud-enabled comparative microbial genome sequence analysis pipeline. *BMC Genomics* 18:332. doi: 10.1186/s12864-017-3717-3

Angiuoli, S. V., Matalka, M., Gussman, A., Galens, K., Vangala, M., Riley, D. R., et al. (2011). CloVR: a virtual machine for automated and portable sequence analysis from the desktop using cloud computing. *BMC Bioinformatics* 12:356. doi: 10.1186/1471-2105-12-356

Araújo, J., Díaz-Alcántara, C.-A., Velázquez, E., Urbano, B., and González-Andrés, F. (2015). *Bradyrhizobium yuanmingense* related strains form nitrogen-fixing symbiosis with *Cajanus cajan* L. *in* dominican republic and are efficient biofertilizers to replace N fertilization. *Sci. Hortic.* 192, 421–428. doi: 10.1016/j.scienta.2015.06.009

Araújo, J., Flores-Félix, J. D., Igual, J. M., Peix, A., González-Andrés, F., Díaz-Alcántara, C. A., et al. (2017). *Bradyrhizobium cajani* sp. nov. isolated from nodules of *Cajanus cajan. Int. J. Syst. Evol. Microbiol.* 67, 2236–2241. doi: 10.1099/ijsem.0.001932

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Berck, S., Perret, X., Quesada-Vincens, D., Promé, J. C., Broughton, W. J., and Jabbouri, S. (1999). NolL of *Rhizobium* sp. strain NGR234 is required for *O*-acetyltransferase activity. *J. Bacteriol.* 181, 957–964. doi: 10.1128/JB.181.3.957-964.1999

Boundy-Mills, K., Kosslak, R., Tully, R., Pueppke, S., and Lohrke, S. (1994). Induction of the *Rhizobium fredii* nod box-independent nodulation gene *nolJ* requires a functional *nodD1* gene. *MPMI* 7, 305–308. doi: 10.1094/MPMI-7-0305

Broughton, W. J., and Dilworth, M. J. (1971). Control of leghaemoglobin synthesis in snake beans. *Biochem. J.* 125, 1075–1080. doi: 10.1042/bj1251075

Choudhary, S., Tak, N., Bissa, G., Chouhan, B., Choudhary, P., Sprent, J. I., et al. (2020). The widely distributed legume tree *Vachellia* (Acacia) *nilotica* subsp. indica is nodulated by genetically diverse Ensifer strains in India. *Symbiosis* 80, 15–31. doi: 10.1007/s13199-019-00658-8

Corvera, A., Promé, D., Promé, J. C., Martínez-Romero, E., and Romero, D. (1999). The *nolL* gene from *Rhizobium etli* determines nodulation efficiency by mediating the acetylation of the fucosyl residue in the nodulation factor. *MPMI* 12, 236–246. doi: 10.1094/MPMI.1999.12.3.236

Coutinho, H. L. C., Oliveira, V. M., Lovato, A., Maia, A. H. N., and Manfio, G. P. (1999). Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans. *Appl. Soil Ecol.* 13, 159–167. doi: 10.1016/S0929-1393(99)00031-1

Cytryn, E. J., Jitacksorn, S., Giraud, E., and Sadowsky, M. J. (2008). Insights learned from pBTAi1, a 229-kb accessory plasmid from *Bradyrhizobium* sp. strain BTAi1 and prevalence of accessory plasmids in other *Bradyrhizobium* sp. strains. *ISME J.* 2, 158–170. doi: 10.1038/ismej.2007.105

Dai, W. J., Zeng, Y., Xie, Z. P., and Staehelin, C. (2008). Symbiosis-promoting and deleterious effects of NopT, a novel type 3 effector of *Rhizobium* sp. strain NGR234. *J. Bacteriol.* 190, 5101–5110. doi: 10.1128/JB.00306-08

De Lajudie, P. M., Andrews, M., Ardley, J., Eardly, B., Jumas-Bilak, E., Kuzmanović, N., et al. (2019). Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *Int. J. Syst. Evol. Microbiol.* 69, 1852–1863. doi: 10.1099/ijsem.0.003426

Dockendorff, T. C., Sharma, A. J., and Stacey, G. (1994). Identification and characterization of the *nolYZ* genes of Bradyrhizobium japonicum. *MPMI* 7, 173–180. doi: 10.1094/MPMI-7-0173

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Fossou, R. K., Pothier, J. F., Zéz,é, A., and Perret, X. (2020). Bradyrhizobium ivorense sp. nov. as a potential local bioinoculant for *Cajanus cajan* cultures in Côte d'Ivoire. *Int. J. Syst. Evol. Microbiol.* 70, 1421–1430. doi: 10.1099/ijsem.0.003931

Fossou, R. K., Ziegler, D., Zézé, A., Barja, F., and Perret, X. (2016). Two major clades of Bradyrhizobia dominate symbiotic interactions with pigeonpea in fields of Côte d'Ivoire. *Front. Microbiol.* 7:1793. doi: 10.3389/fmicb.2016.01793

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., and Perret, X. (1997). Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* 387, 394–401. doi: 10.1038/387394a0

Gehlot, H. S., Tak, N., Kaushik, M., Mitra, S., Chen, W. M., Poweleit, N., et al. (2013). An invasive *Mimosa* in India does not adopt the symbionts of its native relatives. *Ann. Bot.* 112, 179–196. doi: 10.1093/aob/mct112

Goris, J., Konstantinidis, K. T., Klappenbach, J. A., Coenye, T., Vandamme, P., and Tiedje, J. M. (2007). DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *Int. J. Syst. Evol. Microbiol.* 57, 81–91. doi: 10.1099/ijs.0.64483-0

Jiang, N., Liu, W., Li, Y., Wu, H., Zhang, Z., Alexandre, G., et al. (2016). A chemotaxis receptor modulates nodulation during the *Azorhizobium caulinodans-Sesbania rostrata* symbiosis. *Appl. Environ. Microbiol.* 82, 3174–3184. doi: 10.1128/AEM.00230-16

Kaneko, T., Maita, H., Hirakawa, H., Uchiike, N., Minamisawa, K., Watanabe, A., et al. (2011). Complete genome sequence of the soybean symbiont *Bradyrhizobium japonicum* Strain USDA6T. *Genes* 2, 763–787. doi: 10.3390/genes2040763

Kim, W. S., and Krishnan, H. B. (2014). A *nopA* deletion mutant of *Sinorhizobium fredii* USDA257, a soybean symbiont, is impaired in nodulation. *Curr. Microbiol.* 68, 239–246. doi: 10.1007/s00284-013-0469-4

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120. doi: 10.1007/BF01731581

Klappenbach, J. A., Dunbar, J. M., and Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. *Appl. Environ. Microbiol.* 66, 1328–1333. doi: 10.1128/AEM.66.4.1328-1333.2000

Kolde, R. (2019). "*Pheatmap: Pretty Heatmaps*". R package version 1.0.12. Available online at: https://cran.r-project.org/web/packages/pheatmap/index.html

Konstantinidis, K. T., and Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2567–2572. doi: 10.1073/pnas.0409727102

Krishnan, H. B., Lorio, J., Kim, W. S., Jiang, G., Kim, K. Y., Deboer, M., et al. (2003). Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type III protein secretion system in *Sinorhizobium fredii* USDA257. *MPMI* 16, 617–625. doi: 10.1094/MPMI.2003.16.7.617

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Kurtz, S., Phillippy, A., Delcher, A., Smoot, M., Shumway, M., Antonescu, C., et al. (2004). Versatile and open software for

comparing large genomes. *Genome Biol*. 5:R12. doi: 10.1186/gb-2004-5-2-r12

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, Y. H., Wang, R., Sui, X. H., Wang, E. T., Zhang, X. X., Tian, C. F., et al. (2019). Bradyrhizobium nanningense sp. *nov., Bradyrhizobium guangzhouense sp. nov. and Bradyrhizobium zhanjiangense sp. nov*., isolated from effective nodules of peanut in Southeast China. *Syst. Appl. Microbiol*. 42:126002. doi: 10.1016/j.syapm.2019.126002

López-Baena, F. J., Monreal, J. A., Pérez-Montaño, F., Guasch-Vidal, B., Bellogín, R. A., Vinardell, J. M., et al. (2009). The absence of Nops secretion in *Sinorhizobium fredii* HH103 increases GmPR1 expression in Williams soybean. *MPMI* 22, 1445–1454. doi: 10.1094/MPMI-22-11-1445

López-Baena, F. J., Ruiz-Sainz, J. E., Rodríguez-Carvajal, M. A., and Vinardell, J. M. (2016). Bacterial molecular signals in the *Sinorhizobium fredii*-soybean symbiosis. *Int. J. Mol. Sci.* 17:755. doi: 10.3390/ijms17050755

Luka, S., Sanjuan, J., Carlson, R. W., and Stacey, G. (1993). *nolMNO* genes of *Bradyrhizobium japonicum* are co-transcribed with *nodYABCSUIJ*, and *nolO* is involved in the synthesis of the lipo-oligosaccharide nodulation signals. *J. Biol. Chem.* 268, 27053–27059. doi: 10.1016/S0021-9258(19)74217-3

Lynch, D., O'brien, J., Welch, T., Clarke, P., ÓcuiV., P., Crosa, J. H., et al. (2001). Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti*. *J. Bacteriol.* 183, 2576–2585. doi: 10.1128/JB.183.8.2576-2585.2001

Lynch, J. M., and Whipps, J. M. (1990). Substrate flow in the rhizosphere. *Plant Soil.* 129, 1–10. doi: 10.1007/BF00011685

Menna, P., and Hungria, M. (2011). Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. *Int. J. Syst. Evol. Microbiol.* 61, 3052–3067. doi: 10.1099/ijs.0.028803-0

Mula, M. G., and Saxena, K. B. (2010). *Lifting the Level of Awareness on Pigeonpea-a Global Perspective.* Patancheru: International Crops Research Institute for the Semi-Arid Tropics.

Ojha, A., Tak, N., Rathi, S., Chouhan, B., Rao, S. R., Barik, S. K., et al. (2017). Molecular characterization of novel *Bradyrhizobium* strains nodulating *Eriosema chinense* and *Flemingia vestita*, important unexplored native legumes of the sub-Himalayan region (Meghalaya) of India. *Syst. Appl. Microbiol.* 40, 334–344. doi: 10.1016/j.syapm.2017.06.003

Okazaki, S., Noisangiam, R., Okubo, T., Kaneko, T., Oshima, K., Hattori, M., et al. (2015). Genome analysis of a novel *Bradyrhizobium* sp. DOA9 carrying a symbiotic plasmid. *PLoS ONE* 10:e0117392. doi: 10.1371/journal.pone.0117392

Ormeño-Orrillo, E., and Martínez-Romero, E. (2019). A genomotaxonomy view of the *Bradyrhizobium* genus. *Front. Microbiol.* 10:1334. doi: 10.3389/fmicb.2019.01334

Pastor-Bueis, R., Sánchez-Cañizares, C., James, E. K., and González-Andrés, F. (2019). Formulation of a highly effective inoculant for common bean based on an autochthonous elite strain of *Rhizobium leguminosarum* bv. phaseoli, and genomic-based insights into its agronomic performance. *Front. Microbiol.* 10:2724. doi: 10.3389/fmicb.2019.02724

Pellegrino, E., and Bedini, S. (2014). Enhancing ecosystem services in sustainable agriculture: biofertilization and biofortification of chickpea (Cicer arietinum L.) by arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* 68, 429–439. doi: 10.1016/j.soilbio.2013.09.030

Pellegrino, E., Bedini, S., Avio, L., Bonari, E., and Giovannetti, M. (2011). Field inoculation effectiveness of native and exotic arbuscular mycorrhizal fungi in a Mediterranean agricultural soil. *Soil Biol. Biochem.* 43, 367–376. doi: 10.1016/j.soilbio.2010.11.002

Pueppke, S. G., and Broughton, W. J. (1999). *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *MPMI* 12, 293–318. doi: 10.1094/MPMI.1999.12.4.293

Ramsubhag, A., Umaharan, P., and Donawa, A. (2002). Partial 16S rRNA gene sequence diversity and numerical taxonomy of slow growing pigeonpea (*Cajanus cajan* L. *Millsp) nodulating rhizobia. FEMS Microbiol. Lett.* 216, 139–144. doi: 10.1111/j.1574-6968.2002.tb11427.x

Rathi, S., Tak, N., Bissa, G., Chouhan, B., Ojha, A., Adhikari, D., et al. (2018). Selection of *Bradyrhizobium* or *Ensifer* symbionts by the native Indian caesalpinioid legume *Chamaecrista pumila* depends on soil pH and other edaphic and climatic factors. *FEMS Microbiol. Ecol.* 94:fiy180. doi: 10.1093/femsec/fiy180

Reeve, W., Van Berkum, P., Ardley, J., Tian, R., Gollagher, M., Marinova, D., et al. (2017). High-quality permanent draft genome sequence of the *Bradyrhizobium elkanii* type strain USDA 76T, isolated from *Glycine max* (L.) Merr. *Stand. Genomic. Sci.* 12, 26–26. doi: 10.1186/s40793-017-0238-2

Riley, D. R., Angiuoli, S. V., Crabtree, J., Dunning Hotopp, J. C., and Tettelin, H. (2011). Using Sybil for interactive comparative genomics of microbes on the web. *Bioinformatics* 28, 160–166. doi: 10.1093/bioinformatics/btr652

Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F., and Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis* 22, 1086–1089. doi: 10.1002/1522-2683()22:6<1086::AID-ELPS1086>3.0.CO;2-6

Rupela, O. P., Kumar Rao, J. V. D. K., Sudarshana, M. R., Usha Kiran, M., and Anjaiah, V. (1991). *Rhizobium germplasm Resources at ICRISAT Center.* Patancheru: International Crops Research Institute for the Semi-Arid Tropics.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sankhla, I. S., Tak, N., Meghwal, R. R., Choudhary, S., Tak, A., Rathi, S., et al. (2017). Molecular characterization of nitrogen fixing microsymbionts from root nodules of *Vachellia* (Acacia) *jacquemontii*, a native legume from the Thar Desert of India. *Plant Soil.* 410, 21–40. doi: 10.1007/s11104-016-2838-9

Saxena, K. B. (2006). *Seed Production Systems in Pigeonpea ICRISAT.* Patancheru: International Crops Research Institute for the Semi-Arid Tropics.

Saxena, R. K., Von Wettberg, E., Upadhyaya, H. D., Sanchez, V., Songok, S., Saxena, K., et al. (2014). Genetic diversity and demographic history of *Cajanus* spp. illustrated from genome-wide SNPs. *PLoS ONE* 9:e88568. doi: 10.1371/journal.pone.0088568

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Serraj, R., Sinclair, T. R., and Purcell, L. C. (1999). Symbiotic N2 fixation response to drought. *J. Exp. Bot.* 50, 143–155. doi: 10.1093/jxb/50.331.143

Sharypova, L. A., Yurgel, S. N., Keller, M., Simarov, B. V., Pühler, A., and Becker, A. (1999). The *eff*-482 locus of *Sinorhizobium meliloti* CXM1-105 that influences symbiotic effectiveness consists of three genes encoding an endoglycanase, a transcriptional regulator and an adenylate cyclase. *Mol. Gen. Genet.* 261, 1032–1044. doi: 10.1007/s004380051052

Skorpil, P., Saad, M. M., Boukli, N. M., Kobayashi, H., Ares-Orpel, F., Broughton, W. J., et al. (2005). NopP, a phosphorylated effector of *Rhizobium* sp. strain NGR234, is a major determinant of nodulation of the tropical legumes *Flemingia congesta* and *Tephrosia vogelii*. *Mol. Microbiol.* 57, 1304–1317. doi: 10.1111/j.1365-2958.2005.04768.x

Somasegaran, P., and Hoben, H. J. (1994). *Handbook for Rhizobia : Methods in Legume-Rhizobium Technology.* New York, NY: Springer-Verlag. doi: 10.1007/978-1-4613-8375-8

Stepkowski, T., Czaplińska, M., Miedzinska, K., and Moulin, L. (2003). The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha proteobacteria. *Syst. Appl. Microbiol.* 26, 483–494. doi: 10.1078/072320203770865765

Sugawara, M., Epstein, B., Badgley, B. D., Unno, T., Xu, L., Reese, J., et al. (2013). Comparative genomics of the core and accessory genomes of 48 *Sinorhizobium* strains comprising five genospecies. *Genome Biol.* 14:R17. doi: 10.1186/gb-2013-14-2-r17

Tak, N., Awasthi, E., Bissa, G., Meghwal, R. R., James, E. K., Sprent, J. S., et al. (2016). Multi locus sequence analysis and symbiotic characterization of novel *Ensifer* strains nodulating *Tephrosia* spp. *in the Indian Thar Desert. Syst. Appl. Microbiol.* 39, 534–545. doi: 10.1016/j.syapm.2016.08.002

Teulet, A., Busset, N., Fardoux, J., Gully, D., Chaintreuil, C., Cartieaux, F., et al. (2019). The rhizobial type III effector ErnA confers the ability to form nodules in legumes. *Proc. Natl. Acad. Sci. U.S.A.* 116, 21758–21768. doi: 10.1073/pnas.1904456116

Tian, C. F., Garnerone, A.-M., Mathieu-Demazière, C., Masson-Boivin, C., and Batut, J. (2012). Plant-activated bacterial receptor adenylate cyclases modulate epidermal infection in the *Sinorhizobium meliloti–Medicago* symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6751–6756. doi: 10.1073/pnas.1120260109

Varshney, R. K., Chen, W., Li, Y., Bharti, A. K., Saxena, R. K., Schlueter, J. A., et al. (2012). Draft genome sequence of pigeonpea (*Cajanus cajan*), an

orphan legume crop of resource-poor farmers. *Nat. Biotechnol.* 30, 83–89. doi: 10.1038/nbt.2022

Varshney, R. K., Penmetsa, R. V., Dutta, S., Kulwal, P. L., Saxena, R. K., Datta, S., et al. (2010). Pigeonpea genomics initiative (PGI): an international effort to improve crop productivity of pigeonpea (*Cajanus cajan* L.). *Mol. Breed.* 26, 393–408. doi: 10.1007/s11032-009-9327-2

Vavilov, N. I. (1951). *The Origin, Variation, Immunity and Breeding of Cultivated Plants: Selected Writings*. Waltham: Chronica Botanica Company.

Versalovic, J., Schneider, M., De Bruijn, F., and Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Biol.* 5, 25–40.

Vesth, T., Lagesen, K., Acar, Ö., and Ussery, D. (2013). CMG-biotools, a free workbench for basic comparative microbial genomics. *PLoS ONE* 8:e60120. doi: 10.1371/journal.pone.0060120

Vincent, J. M. (1970). *A Manual for the Practical Study of Root-Nodule Bacteria.* Oxford: International Biological Programme, Blackwell Scientific.

Viprey, V., Rosenthal, A., Broughton, W. J., and Perret, X. (2000). Genetic snapshots of the *Rhizobium* species NGR234 genome. *Genome Biol.* 1:research0014.0011. doi: 10.1186/gb-2000-1-6-research0014

Waters, N. R., Abram, F., Brennan, F., Holmes, A., and Pritchard, L. (2018). riboSeed: leveraging prokaryotic genomic architecture to assemble across ribosomal regions. *Nucleic Acids Res.* 46, e68–e68. doi: 10.1093/nar/gky212

Wei, M., Yokoyama, T., Minamisawa, K., Mitsui, H., Itakura, M., Kaneko, T., et al. (2008). Soybean seed extracts preferentially express genomic loci of *Bradyrhizobium japonicum* in the initial interaction with soybean, *Glycine max* (L.) Merr. *DNA Res.* 15, 201–214. doi: 10.1093/dnares/dsn012

Wheatley, R. M., Ford, B. L., Li, L., Aroney, S. T. N., Knights, H. E., Ledermann, R., et al. (2020). Lifestyle adaptations of *Rhizobium* from rhizosphere to symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23823–23834. doi: 10.1073/pnas.2009094117

Wolde-Meskel, E., Terefework, Z., Frostegård, Å., and Lindström, K. (2005). Genetic diversity and phylogeny of rhizobia isolated from agroforestry legume species in southern Ethiopia. *Int. J. Syst. Evol. Microbiol.* 55, 1439–1452. doi: 10.1099/ijs.0.63534-0

Wood, D. E., and Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15:R46. doi: 10.1186/gb-2014-15-3-r46

Wu, L. J., Wang, H. Q., Wang, E. T., Chen, W. X., and Tian, C. F. (2011). Genetic diversity of nodulating and non-nodulating rhizobia associated with wild soybean (*Glycine soja* Sieb. *& Zucc.)* in different ecoregions of China. *FEMS Microbiol. Ecol.* 76, 439–450. doi: 10.1111/j.1574-6941.2011.01064.x

Yang, S., Tang, F., Gao, M., Krishnan, H. B., and Zhu, H. (2010). *R* gene-controlled host specificity in the legume–rhizobia symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 107:18735. doi: 10.1073/pnas.1011957107

Zgadzaj, R., James, E. K., Kelly, S., Kawaharada, Y., De Jonge, N., Jensen, D. B., et al. (2015). A legume genetic framework controls infection of nodules by symbiotic and endophytic bacteria. *PLoS Genet.* 11:e1005280. doi: 10.1371/journal.pgen.1005280

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Jorrin, Maluk, Atoliya, Kumar, Chalasani, Tkacz, Singh, Basu, Pullabhotla, Kumar, Mohanty, East, Ramachandran, James, Podile, Saxena, Rao and Poole. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

ORIGINAL RESEARCH
published: 08 September 2021
doi: 10.3389/fagro.2021.692933

Inhibition of N_2 Fixation by N Fertilization of Common Bean (*Phaseolus vulgaris* L.) Plants Grown on Fields of Farmers in the Eastern Cape of South Africa, Measured Using ^{15}N Natural Abundance and Tissue Ureide Analysis

Simon J. Habinshuti[1,2], Sipho T. Maseko[2] and Felix D. Dakora[3]*

[1] Department of Agricultural Production, School of Agricultural Sciences, College of Agricultural and Environmental Sciences, Makerere University, Kampala, Uganda, [2] Department of Crop Sciences, Tshwane University of Technology, Pretoria, South Africa, [3] Chemistry Department, Tshwane University of Technology, Pretoria, South Africa

OPEN ACCESS

Edited by:
Jose Palacios,
Polytechnic University of Madrid, Spain

Reviewed by:
Mahaveer P. Sharma,
ICAR Indian Institute of Soybean Research, India
Glaciela Kaschuk,
Federal University of Paraná, Brazil

***Correspondence:**
Felix D. Dakora
DakoraFD@tut.ac.za

Specialty section:
This article was submitted to Plant-Soil Interactions, a section of the journal Frontiers in Agronomy

Received: 09 April 2021
Accepted: 21 June 2021
Published: 08 September 2021

Citation:
Habinshuti SJ, Maseko ST and Dakora FD (2021) Inhibition of N_2 Fixation by N Fertilization of Common Bean (Phaseolus vulgaris L.) Plants Grown on Fields of Farmers in the Eastern Cape of South Africa, Measured Using ^{15}N Natural Abundance and Tissue Ureide Analysis. Front. Agron. 3:692933. doi: 10.3389/fagro.2021.692933

Inhibition of N_2 fixation in N-fertilized common bean (*Phaseolus vulgaris* L.) plants growing on the fields of farmers in the Eastern Cape of South Africa was measured using ^{15}N natural abundance and tissue ureide analysis. The N-fertilized bean plants revealed greater soil N uptake, higher concentrations of nitrate in organs, low tissue ureide levels, and much lower percent relative ureide-N abundance when compared with unfertilized plants. In contrast, the unfertilized plants showed greater nodule fresh weight, higher N derived from fixation (e.g., 84.6, 90.4, and 97.1% at Lujecweni fields 2, 3, and 4, respectively), increased amount of N-fixed (e.g., 163.3, 161.3, and 140.3 kg ha^{-1} at Lujecweni fields 2, 3, and 4, respectively), greater ureide concentration in stems and petioles, higher % relative ureide-N abundance, and low soil N uptake. We also found that the percent N derived from fixation (%Ndfa) was very high for some bean plants receiving a double dose of N fertilizer [e.g., Lujecweni field 1 (51.8%) and Tikitiki field 1 (53.3%], and quite high for others receiving a single dose of N fertilizer [e.g., Tikitiki field 2 (50.1%), Mfabantu fields 1 and 2 (45.5 and 79.9%, respectively), and St. Luthberts field 1 (58.9%)]. Though not assessed in this study, it is likely that the rhizobia that effectively nodulated the N-fertilized bean plants and fixed considerable amounts of symbiotic N had constitutive and/or inducible nitrate reductase genes for reducing nitrate in nodules and bacteroids, hence their ability to form root nodules and derived high %Ndfa in bean with added N. While single- and double-dose N fertilizer applications increased plant growth and grain yield compared to unfertilized bean plants, the single-dose N fertilizer application produced much greater grain yield than the double dose. This indicates that farmers should stop using a double dose of N fertilizers on bean production, as it decreases yields and can potentially pollute the environment. This study has however shown that government supply of free N fertilizers to resource-poor farmers in South Africa increased bean yields for food/nutritional security.

Keywords: symbiosis, soil N uptake, N-fixed, %Ndfa, ureide, percent relative ureide abundance

INTRODUCTION

Legume N_2 fixation is important for sustainable cropping systems in both tropical and subtropical environments, where the soils are naturally deficient in N (Graham and Vance, 2003). The legume-rhizobia symbiosis can provide symbiotic N for meeting the N demand of legumes and, in so doing, spare endogenous soil N for use by cereal crops, in addition to increasing soil N supply through decomposition of legume residues and improving soil structure and organic matter content (Maingi et al., 2000).

Several studies have assessed N nutrition in common bean (Farid and Navabi, 2015; Farid et al., 2016; Jiang et al., 2020), and symbiotic N_2 fixation has generally been found to be lower in common bean when compared to other legumes (Farid et al., 2016). However, applying *Rhizobium* strain HB-429 to common bean crop increased plant growth, percent N derived from fixation (%Ndfa), the amount of N-fixed, and grain yield by 19, 17, 54, and 48%, respectively, over the uninoculated control in Ethiopia (Samago et al., 2018). These findings show that supplying rhizobial inoculants can replace chemical N fertilizer use and increase bean yields in Ethiopia. An additional benefit following grain harvest in legumes is the release of fixed-N by the legume residues into the soil, which is generally regarded as slow and can match plant uptake from the soil with symbiotic N supply by residues (Chikanai et al., 2018), thus reducing leaching, runoff, and environmental pollution, which are common with added N fertilizer.

In an attempt to increase grain yield and improve household food security, the South African government embarked on a program of providing free inputs, such as improved seeds and the fertilizers NPK, urea, and limestone ammonium nitrate (LAN), for use by resource-poor farmers instead of rhizobial inoculants. Although N fertilizers can be used to overcome the inherently low nutrient levels in African soils and increase crop yields, they are expensive and inaccessible to resource-poor farmers, hence the free N supply to poor farmers by the South African government. Although government supply of fertilizers has increased the production of maize and common bean, in particular, the two major staple foods in South Africa, no study has evaluated the effect of frequent N fertilizer use on plant growth and N_2 fixation of common bean on the fields of farmers. However, the suppressive effects of N fertilizers on legume N_2 fixation is known. For example, high concentrations of NH_3 can inhibit *nod*-gene expression, nodule formation, and nitrogenase activity in legumes (Vieira et al., 1998; Li et al., 2009), thus reducing their symbiotic N yield. Nitrate is also known to reduce the oxygen diffusion barrier and inhibit nodule functioning *via* the formation of nitrosylleghemoglobin, which reduces oxygen supply to respiring bacteroids for ATP formation (Dakora and Atkins, 1989).

Several techniques are available for estimating legume N_2 fixation in the field, which include the ^{15}N natural abundance technique and the ureide assay. Although the ^{15}N natural abundance technique is robust in quantifying N_2 fixation in legumes (Unkovich et al., 1994; Mohale et al., 2013), it assumes plant uptake of only two sources of N (soil N and atmospheric N_2), which therefore limits its application in situations where external N is added to the soil (Unkovch et al., 2008). Even if there are non-fixing, non-legume plant species growing on the field and sourcing N from the soil and added fertilizer, the ^{15}N natural abundance technique can still be used to estimate N_2 fixation in the legume species, such as the N-fertilized common bean plants on the fields of farmers. Additionally, tropical legumes belonging to the tribe Phaseoleae (e.g., common bean) export their fixed-N from nodules to shoots in the form of ureides (allantoin and allantoic acid) *via* the xylem stream (Herridge and People, 1990; Dakora et al., 1992). The concentration of ureides relative to other nitrogenous solutes (nitrate and amino acids) in the xylem sap, stems, or petioles of the tribe Phaseoleae is a reliable measure of N_2 fixation (Herridge and People, 1990; Unkovich et al., 2008) that is not limited by external N inputs.

The application of N fertilizer to symbiotic legumes can decrease nodulation, N_2 fixation, and even grain yield. We hypothesize that the program of providing free N fertilizers (NPK, urea, and LAN) by the South African government to resource-poor farmers could increase grain yield but decrease nodulation and N_2 fixation in beans. This study aimed to assess the effect of N fertilization on plant growth, N_2 fixation, and grain yield of common bean planted without rhizobial inoculation on the fields of farmers in the Eastern Cape Province of South Africa, using the ^{15}N natural abundance technique and ureide assay.

MATERIALS AND METHODS

Soil Sampling and Analysis

In each field, bulk soil was sampled (0–20 cm depth) from empty spaces in between plants using an augur. The soil samples from each farm were pooled, taken to the laboratory, air-dried, and sieved (2 mm). Subsamples were taken and analyzed for pH (KCl), %C (Walkley and Black, 1934), P (Bray and Kurtz, 1945), CEC, Ca, K, Mg, Fe, Mn, and B (ammonium acetate method), and total N using the Kjeldahl digestion.

Plant Sampling and Processing

In this study, plants were sampled from the fields of 14 farmers located in six villages [namely, Lujecweni (31.6103°S; 28.8619°E), Ngcolosi (31.4000°S; 28.7000°E), Tikitiki (31.4000°S; 28.7000°E), Mfabantu (31.5706°S; 29.0014°E), Nomhala (31.1523°S; 28.3714°E), and St. Luthberts] in the Eastern Cape Province of South Africa. The number of farmer fields sampled varied from village to village depending on the willingness of farmers to allow plants to be collected from their fields. A paired legume-reference plant sampling procedure was used, as described by Unkovich et al. (1994). Ten plants were randomly selected from each farm at the pod-filling stage, dug up, and separated into nodulated roots and shoots plus pods. The shoots of non-legume plant species growing among the common bean plants in the field of each farmer (and which received the same blanket N fertilizer treatment applied to the field before planting the beans) were also harvested as reference plants for the estimation of soil N uptake by legumes. All bean plant materials (i.e., shoots plus pods) and shoots only of reference plants were oven-dried separately at 60°C for 48 h, weighed,

and milled to a fine powder (0.85 mm sieve) for ^{15}N and ^{13}C isotopic analysis.

Common Bean Stem and Petiole Collection, and Processing for Ureide and Nitrate Analysis

As the 10 bean plants were sampled from the field of each farmer for measuring N_2 fixation using the ^{15}N natural abundance method, the stems and petioles of each plant were removed, oven-dried separately at 60°C for 48 h, weighed, and milled to a fine powder (0.85 mm sieve) for ureide and nitrate analysis, after warm water extraction (Unkovich et al., 2008).

Ureide and Nitrate Assay

Ureides and nitrate in finely ground powder of stems and petioles were extracted using hot water, as described by Unkovch et al. (2008). The ureide concentration in plant extracts was analyzed using the Rimini–Schryver reaction as described by Young and Conway (1942), whereas nitrate was analyzed using the salicylic acid method (Cataldo et al., 1975), as outlined by Unkovich et al. (2008). The concentrations of nitrate and ureides in stems and petioles were expressed on per gram oven-dried organ basis, and total nitrate-N and ureide-N were estimated for each sample (Unkovich et al., 2008). The percent relative ureide abundance in stems and petioles was calculated as described by Herridge (1982a,b):

$$\text{Relative ureide abundance(\%)} = \left(\frac{400\ ureide - N}{4\ ureide - N + nitrate - N} \right) \times 100$$

Measurement of N_2 Fixation

$^{15}N/^{14}N$ Isotopic Analysis

Finely ground plant material was weighed into Al tin capsules (1–2 mg/legume sample and 2–3 mg/reference plant) and analyzed for %Ndfa and $^{15}N/^{14}N$ ratio using a Carlo Erba NA1500 elemental analyzer (Fisons Instruments SpA, Strada, Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer *via* Conflo II open-split device. The ^{15}N natural abundance was expressed as δ (delta) notation and is per mille deviation of the ^{15}N natural abundance of the sample from atmospheric (atm) N_2 (0.36637 atom %^{15}N). The isotopic composition ($\delta^{15}N$) was measured as described by Unkovich et al. (2008):

$$\delta^{15}N = \frac{\left(^{15}N/_{14}N \right)_{sample} - \left(^{15}N/_{14}N \right)_{atm}}{\left(^{15}N/_{14}N \right)_{atm}} \times 1000$$

where $^{15}N/^{14}N_{sample}$ is the abundance ratio of ^{15}N and ^{14}N in the common bean sample, and $^{15}N/^{14}N_{atm}$ is the abundance ratio of ^{15}N and ^{14}N in the atmosphere.

Shoot N Content

The N content of common bean shoots was determined as the product of %Ndfa and shoot dry weight (Pausch et al., 1996; Peoples et al., 2009; Angus and Peoples, 2012).

Percent N Derived From Fixation

The percent N derived from the symbiotic fixation of atmospheric N_2 was estimated using the equation (Shearer and Kohl, 1986; Unkovich et al., 2008):

$$\%Ndfa = \frac{\left(\delta^{15}N_{ref} - \delta^{15}N_{leg}\right)}{\left(\delta^{15}N_{ref} - B_{value}\right)} \times 100$$

where the $\delta^{15}N_{ref}$ is the ^{15}N natural abundance of reference plants, $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of legume, and the B-value is the ^{15}N natural abundance of the test legume (common bean) wholly dependent on N_2 fixation for its N nutrition. The *B*-value replaces the value of atmospheric N_2 as it incorporates the isotopic fractionation associated with N_2 fixation (Unkovich et al., 2008). In this study, the B-value used (−2.16‰) was obtained from Unkovich et al. (2008).

Amount of N-fixed

The amount of N-fixed was calculated as described by Maskey et al. (2001):

$$N - fixed = amount\ \ of\ \ shoot\ \ N \times \%Ndfa\ \ of\ \ shoots$$

N-fixed per hectare was estimated as the product of N-fixed in shoots per plant and plant density per hectare. The plant density in each field was measured by counting the number of common bean plants in a 3-m^2 quadrant, which was then used to estimate plant population per hectare.

Statistical Analysis

Statistical analyses were carried out using the STATISTICA package (StatSoft Inc., Tulsa, OK, USA). Data were subjected to a normal distribution test, followed by a one-way ANOVA. Where there were significant differences, treatment means were separated using the Duncan multiple range test at $p \leq 0.05$.

RESULTS

Soil Chemical Properties

All the sites used in this study were characterized by acidic soils with pH ranging from pH 4.1 at Tikitiki fields 1 and 2, and Lujecweni field 4 to pH 5.3 at Mfabantu field 2. Generally, the soils appeared to have low levels of N and intermediate to high levels of %C, Ca, Mg, P, Mn, B, and Fe. Nevertheless, plant-available P was lowest in the soil from Nomhala field 2, followed by Tikitiki field 1, Ngcolosi field 1, and St. Luthberts field 1 (**Table 1**).

$\delta^{15}N$ of Reference Plants

At least five non-legume plant species were collected from the field of each farmer for $^{15}N/^{14}N$ isotopic analysis. Across the fields of farmers, the $\delta^{15}N$ of all reference plants ranged from +0.02 to +9.37‰, whereas the combined $\delta^{15}N$ of the reference plant species sampled from the different fields ranged from +3.03 to +6.87‰ (**Table 2**).

TABLE 1 | Chemical properties of bulk soil (0–20 cm depth) sampled from the fields of farmers.

Location	Farm	Fertilizer used	pH (KCl)	N	C	CEC	P_{Avail}	K	Ca	B	Fe	Mn
				%		Cmol(+)/kg						
Lujecweni	1	NPK, Urea	4.3	0.026	0.65	5.6	25	124	282	0.16	135	213
	2	None	4.2	0.030	0.57	5.9	25	171	282	0.17	171	211
	3	None	4.5	0.030	0.61	3.4	12	150	284	0.16	131	162
	4	None	4.1	0.033	0.55	6.3	17	106	248	0.17	168	242
	5	NPK, Urea	4.2	0.035	0.61	7.8	14	136	258	0.17	119	166
Ngcolosi	1	NPK,LAN	4.5	0.030	0.66	5.7	9	84	320	0.13	141	377
Tikitiki	1	NPK, LAN	4.1	0.035	1.04	5.9	7	64	290	0.18	145	161
	2	NPK	4.1	0.082	1.46	14.1	13	64	416	0.22	120	128
Mfabantu	1	NPK	5.2	0.052	0.98	13.3	27	98	884	0.16	146	226
	2	NPK	5.3	0.042	0.83	12.1	34	94	762	0.16	128	210
Nomhala	1	NPK	4.5	0.031	0.78	5.0	12	71	352	0.13	151	187
	2	NPK	4.4	0.025	0.68	6.6	5	86	368	0.14	155	188
	3	NPK	4.3	0.024	0.57	5.8	12	84	252	0.12	131	212
St. Luthberts	1	NPK	4.2	0.075	1.56	9.5	10	68	488	0.24	132	111

Each value is the average of a duplicate soil analysis.

TABLE 2 | Shoot $\delta^{15}N$ values of reference plants sampled from the fields of farmers in the Eastern Cape and Limpopo Provinces for estimating percent N derived from fixation (%Ndfa) and soil N uptake by common bean.

Location	Field no.	No. of reference plants per field	$\delta^{15}N$ values (‰)		
			Min	Max	Mean ± SE
Lujecweni	1	5	+2.24	+4.40	+3.04 ± 0.37
	2	5	+0.37	+5.02	+3.11 ± 0.09
	3	6	+2.11	+4.61	+3.52 ± 0.46
	4	5	+1.30	+4.17	+3.05 ± 0.68
	5	5	+3.49	+5.64	+4.75 ± 0.40
Ngcolosi	1	5	+4.51	+5.95	+4.92 ± 0.26
Tikitiki	1	5	+1.01	+6.62	+4.19 ± 0.91
	2	5	+3.07	+5.64	+4.33 ± 0.57
Mfabantu	1	5	+0.02	+9.37	+3.03 ± 0.64
	2	5	+0.83	+9.35	+4.08 ± 0.25
Nomhala	1	5	+5.12	+8.28	+6.85 ± 0.56
	2	5	+5.54	+8.26	+6.87 ± 0.44
	3	5	+3.91	+7.51	+6.41 ± 0.64
St. Luthberts	1	5	+2.90	+4.18	+3.77 ± 0.23

Plant Growth

Plant growth (measured as shoot dry matter) of common bean variety PAN128 was significantly ($p \leq 0.05$) greater at Nomhala fields 1 (156.92 g plant^{-1}) and 2 (134.44 g plant^{-1}), followed by those grown at Mfabantu fields 2 (107.86 g plant^{-1}) and 1 (106.96 g plant^{-1}) (**Figure 1C**). On the other hand, the common bean plants from Nomhala field 2 ranked least in nodule number and nodule fresh weight, despite recording high plant growth (**Figures 1A–C**). However, the plants sampled from Mfabantu field 2 ranked highest in nodule number, nodule fresh weight, and plant growth. Plant growth was lowest at Tikitiki field 1 (25.72 g plant^{-1}), Lujecweni fields 1 (51.32 g plant^{-1}) and 3 (53.30 g plant^{-1}), Ngcolosi field 1 (54.76 g plant^{-1}), and Lujecweni field 4 (57.54 g plant^{-1}). The least nodulation and plant growth were recorded in the common beans that were sampled from Lujecweni field 1 and Ngcolosi field 1 (**Figures 1A–C**).

In general, the common bean plants grown without fertilizer application (e.g., Lujecweni fields 2, 3, and 4) recorded much higher nodulation but lower shoot DM when compared to their

counterparts supplied with N fertilizers by farmers in the Eastern Cape (**Figures 1A–C**).

Grain Yield

There were marked ($p \leq 0.05$) differences in the grain yield of the common bean plants from the fields of various farmers (**Figure 1D**). PAN128, the test bean variety, recorded the highest grain yield at Mfabantu field 1 (1,065 kg ha^{-1}) and Tikitiki field 2 (1,060 kg ha^{-1}), followed by Lujecweni field 1 (875 kg ha^{-1}), Mfabantu field 2 (797 kg ha^{-1}), and Tikitiki 1 (787 kg ha^{-1}). The fields that showed the highest yields (Mfabantu field 1 and Tikitiki field 2) each received a single dose of mineral N fertilizer, whereas intermediate yields were recorded at Lujecweni field 5 (630 kg ha^{-1}), Nomhala field 1 (715 kg ha^{-1}), St. Luthberts field 1 (710 kg ha^{-1}), and Ngcolosi field 1 (750 kg ha^{-1}). Most of the fields producing intermediate yields received a double dose of mineral N. In contrast, the lowest grain yield was recorded at Lujecweni fields 4 (400 kg ha^{-1}), 2 (503 kg ha^{-1}), and 3 (570 kg ha^{-1}), which were all unfertilized (**Figure 1D**).

Nodulation

Nodulation (measured as nodule number and nodule fresh weight per plant) varied significantly ($p \leq 0.05$) among common bean plants collected from the fields of 14 farmers in the Eastern Cape Province (**Figures 1A,B**). The bean plants grown at Nomhala field 2 (1.6 nodules per plant) had the least number of nodules, followed by the plants grown at Tikitiki field 2 (6.4 nodules per plant), Ngcolosi field 1 (6.7 nodules per plant), and then Lujecweni field 1 (7.6 nodules per plant). The plants from Mfabantu field 2 recorded the highest nodulation (53.2 nodules per plant), followed by Lujecweni field 5 (35.4 nodules per plant), Lujecweni field 3 (21.8 nodules per plant), Lujecweni field 2 (15.2 nodules per plant), Lujecweni field 4 (12.3 nodules per plant), Mfabantu field 1 (11.9 nodules per plant), and then St. Luthberts field 1 (11.2 nodules per plant). The fresh weight of nodules was markedly greater at Lujecweni field 2 (338.41 mg $plant^{-1}$), Mfabantu field 2 (233.56 mg $plant^{-1}$), Lujecweni field 4 (189.43 mg $plant^{-1}$), Lujecweni field 3 (153.86 mg $plant^{-1}$), Lujecweni field 5 (147.23 mg $plant^{-1}$), Tikitiki field 1 (104.88 mg $plant^{-1}$), and St. Luthberts field 1 (103.06 mg $plant^{-1}$). In contrast, the common bean plants from Nomhala field 2, Lujecweni field 1, Ngcolosi field 1, and Tikitiki field 2 recorded much lower nodule fresh weights of 6.5, 28.1, 29.11, and 34.83 mg $plant^{-1}$, respectively (**Figures 1A,B**). There was a strong correlation between nodule number and nodule fresh weight ($r = 0.82^{***}$) of the common bean plants sampled from the unfertilized fields. In contrast, there was no significant correlation between nodule number and nodule fresh weight of plants sampled from the N-fertilized fields.

Ureides, Nitrate, and Percent Relative Ureide-N Abundance in Plant Organs

Warm water extracts of ground common bean stems and petioles were analyzed for ureides and nitrate. The sum of the two nitrogenous solutes from both organs was used to estimate nitrate-N, ureide-N, and percent relative ureide-N abundance (**Table 3**). The common bean plants grown without N fertilization at Lujecweni fields 2, 3, and 4 in the Eastern Cape exhibited greater nodulation but low shoot biomass and also recorded the lowest nitrate concentration in their stems and petioles (**Table 3**). Total nitrate-N of plants from Lujecweni field 4 was 0.11 mg g^{-1}, whereas those of Lujecweni fields 2 and 3 were 0.15 and 0.16 mg g^{-1}, respectively. The common bean plants that exhibited intermediate levels of nitrate in their stems and petioles, and intermediate total nitrate in the two organs were sampled from Nomhala fields 1 (3.83 mg g^{-1}) and 2 (4.53 mg g^{-1}) and also from Lujecweni field 1 (4.80 mg g^{-1}). As noted before, plants sampled from Nomhala fields 1 and 2 showed better growth, despite low nodulation in field 2. It is interesting to note that, although plants from Lujecweni field 5 showed intermediate shoot growth (80.50 g $plant^{-1}$), those from Ngcolosi field1 had much lower shoot DM (54.76 g $plant^{-1}$), and they both recorded much higher nitrate in stems and petioles (**Table 3**).

Ureide analysis of tissue extracts revealed significant ($p \leq 0.05$) differences in the concentration of ureide-N per organ (stem and petiole) of the bean plants (**Table 3**). Common bean grown at St. Luthberts showed significantly higher concentration of ureides in stems (1.49 mg g^{-1}), petioles (1.46 mg g^{-1}), and total ureide-N in the two organs (2.95 mg g^{-1}) (**Table 3**). The next higher ureide concentrations were recorded in the tissues of plants grown at Mfabantu field 2 (2.10 mg g^{-1}) and Lujecweni field 2 (1.99 mg g^{-1}). Intermediate concentrations of total ureide-N were recorded for the stems and petioles of common bean plants sampled from Mfabantu field 1 (1.87 mg g^{-1}), Tikitiki field 2 (1.60 mg g^{-1}), and Lujecweni field 3 (1.57 mg g^{-1}). In contrast, plants from Nomhala fields 1, 2, and 3 showed the lowest concentrations of ureide-N in organs (0.55 mg g^{-1} in field 3, 0.82 mg g^{-1} in field 1, and 0.95 mg g^{-1} in field 2; see **Table 3**).

The percent relative ureide-N abundance varied markedly ($p \leq 0.05$) between and among plants sampled from the fields of different farmers (**Table 3**). Common bean plants grown without N fertilizer application at Lujecweni fields 2, 3, and 4 recorded much higher relative ureide-N abundance, in the order of 92.3, 91.6, and 90.2%, respectively. Mfabantu fields 1 (49.15%) and 2 (60.53%), St. Luthberts field 1 (51.57%), and Tikitiki field 2 (50.15%), which received a single dose of N fertilizer at the planting stage, produced plants with intermediate relative ureide-N abundance (**Table 3**). In contrast, the plants grown at Ngcolosi fields 1 (8.17%), Nomhala field 3 (9.20%), and Lujecweni field 5 (10.97%), which were supplied with N fertilizer both at planting and flowering, revealed the lowest relative ureide-N abundance (**Table 3**).

Shoot N, $\delta^{15}N$, %Ndfa, and Amount of N-fixed

All the bean fields surveyed in the Eastern Cape received mineral N at the planting stage and/or the pod-filling stage, except for Lujecweni fields 2, 3, and 4. That notwithstanding, the ^{15}N natural abundance technique was applied to estimate N_2 fixation in the N-fertilized bean plants using reference plants sampled from among the bean plants in each field. This was based on the understanding that, like the bean plants, the reference plants also took up the added mineral N.

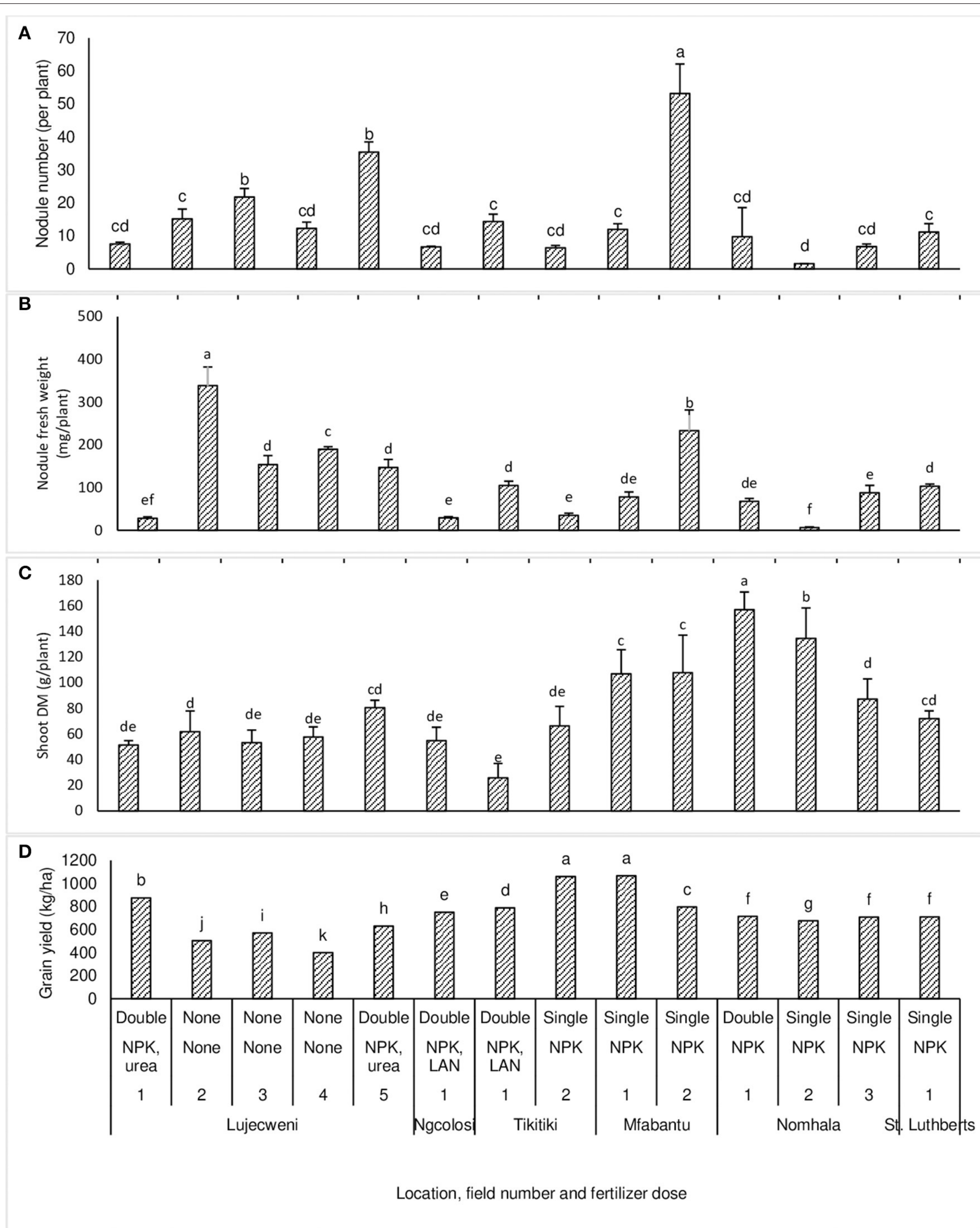


FIGURE 1 | Nodulation **(A,B)**, plant biomass **(C)**, and grain yield **(D)** of common bean variety PAN128 sampled from the fields of 14 farmers in Eastern Cape Province, in the 2014 cropping season. NPK (2:3:2), limestone ammonium nitrate (LAN, 28% N), and urea were each used at rates of 100 kg ha^{-1}. For each location on the *x*-axis, the numbers 1, 2, 3, … represent different fields within that location. Bars with dissimilar letters are significantly different at $p \leq 0.05$.

Shoot N concentration in bean plants ranged from 1.61 to 2.77%, and N content from 0.61 to 3.59 g $plant^{-1}$ (**Table 4**). Shoot $\delta^{15}N$ values were much lower in the unfertilized bean plants at Lujecweni fields 2, 3, and 4, and much higher in plants from Nomhala fields 1, 2, and 3 (**Table 4**). As a result, the %Ndfa was higher at Lujecweni fields 2, 3, and 4 (85, 90, and 97%, respectively) and much lower at Nomhala fields 1 and 2 (12 and 4%, respectively). Symbiotic N contribution by common bean

TABLE 3 | Symbiotic performance of common bean variety PAN128 sampled from the fields of 14 farmers.

Location	Field	N supply (dosage)	Nitrate-N			Ureide-N			Relative ureide-N
			Stems mg g^{-1}	Petioles mg g^{-1}	Total nitrate-N (stems + petioles) mg g^{-1}	Stems mg g^{-1}	Petioles mg g^{-1}	Total ureide-N (stems + petioles) mg g^{-1}	%
Lujecweni	1	Double	2.72 ± 0.37cd	2.09 ± 0.46cd	4.80 ± 0.81cd	0.78 ± 0.14bc	0.50 ± 0.14bc	1.28 ± 0.20cd	23.17 ± 6.01d
	2	None	0.13 ± 0.04g	0.02 ± 0.01g	0.15 ± 0.05g	1.50 ± 0.20a	0.49 ± 0.08bc	1.99 ± 0.22b	92.30 ± 2.86a
	3	None	0.11 ± 0.03g	0.05 ± 0.01g	0.16 ± 0.04g	0.95 ± 0.19b	0.61 ± 0.10bc	1.57 ± 0.18bcd	91.58 ± 1.52a
	4	None	0.09 ± 0.01g	0.02 ± 0.01g	0.11 ± 0.00g	0.61 ± 0.05bc	0.43 ± 0.04bc	1.05 ± 0.08cd	90.19 ± 0.43a
	5	Double	5.56 ± 0.03b	4.87 ± 0.29b	10.43 ± 0.28b	0.75 ± 0.14bc	0.56 ± 0.09bc	1.31 ± 0.23c	10.97 ± 1.62e
Ngcolosi	1	Double	7.48 ± 0.4a	6.77 ± 0.41a	14.25 ± 0.80a	0.70 ± 0.06bc	0.55 ± 0.07bc	1.24 ± 0.11cd	8.17 ± 0.92e
Tikitiki	1	Double	1.14 ± 0.11cde	0.11 ± 0.06g	1.52 ± 0.10f	0.84 ± 0.21b	0.30 ± 0.24b	1.14 ± 0.19cd	42.86 ± 3.08c
	2	Single	1.51 ± 0.49de	0.08 ± 0.39cde	1.59 ± 0.84de	1.08 ± 0.43a	0.52 ± 0.28a	1.60 ± 0.41bcd	50.15 ± 8.50bc
Mfabantu	1	Single	1.53 ± 0.25ef	0.53 ± 0.20fg	2.06 ± 0.38f	1.35 ± 0.15a	0.52 ± 0.05bc	1.87 ± 0.16bc	49.15 ± 4.05bc
	2	Single	1.16 ± 0.40f	0.30 ± 0.13g	1.46 ± 0.37fg	1.62 ± 0.26a	0.47 ± 0.06bc	2.10 ± 0.30b	60.53 ± 7.35b
Nomhala	1	Double	2.19 ± 0.41cde	1.64 ± 0.34de	3.83 ± 0.73de	0.55 ± 0.12cd	0.27 ± 0.03c	0.82 ± 0.14fg	19.44 ± 5.19de
	2	Single	2.83 ± 0.52cd	1.69 ± 0.50cde	4.53 ± 0.69cd	0.54 ± 0.06cd	0.41 ± 0.07bc	0.95 ± 0.07fg	18.60 ± 3.01de
	3	Single	3.02 ± 0.25c	2.49 ± 0.19c	5.50 ± 0.39c	0.34 ± 0.08d	0.22 ± 0.03c	0.55 ± 0.11g	9.20 ± 1.77e
St. Luthberts	1	Single	1.57 ± 0.21ef	1.17 ± 0.31ef	2.73 ± 0.17ef	1.49 ± 0.13a	1.46 ± 0.31a	2.95 ± 0.30a	51.57 ± 2.56c
F-Statistics			**44.99*** **	**46.95*** **	**63.02*** **	**5.89*** **	**10.17*** **	**14.17*** **	**53.79*** **

Values (mean ± SE) with dissimilar letters in the same column are significantly different at $^{***}p \le 0.001$.

plants ranged from 0.14 to 2.27 g plant^{-1} or 16 to 163 kg ha^{-1} (**Table 4**). Soil N uptake also varied from 0.05 to 3.14 g plant^{-1} or 6 to 337 kg ha^{-1} (**Table 4**).

Correlation Analysis

Correlation analysis revealed a significantly positive relationship between % relative ureide-N abundance and nodule fresh weight ($r = 0.50^{***}$) and also between % relative ureide-N abundance and %Ndfa ($r = 0.75^{***}$) for common bean plants sampled from the N-fertilized fields. In contrast, the % relative ureide-N abundance was negatively correlated with shoot DM ($r = -0.31^{*}$), whereas relative ureide-N abundance and %Ndfa from ^{15}N natural abundance were both negatively correlated with tissue nitrate concentration ($r = -0.78^{***}$ and $r = -0.32^{***}$, respectively) for N-fertilized bean plants. With the unfertilized common bean plants, the correlation analysis showed a significantly positive relationship between nodule number and nodule fresh weight ($r = 0.82^{***}$) and between %Ndfa measured from ^{15}N natural abundance and % relative ureide-N abundance ($r = 0.96^{***}$). But %Ndfa was negatively correlated with tissue nitrate. Furthermore, % relative ureide-N was positively correlated with nodule number ($r = 0.56^{*}$) and nodule fresh weight ($r = 0.82^{***}$). In contrast, there was a significantly negative correlation between relative ureide-N abundance and shoot DM ($r = -0.61^{**}$), and tissue NO_3^- concentration ($r = -0.84^{***}$).

DISCUSSION

The South African government policy of providing free N fertilizers to resource-poor farmers has had a positive impact on food security, especially among rural households. Both maize and common bean yields rose with the supply of free fertilizer inputs to emerging small-scale farmers (**Figure 1D**). In this study, the low endogenous soil N concentrations (0.030–0.033%) found in the unfertilized fields of the Eastern Cape do justify the program of supplying free fertilizer by the government to resource-poor farmers. However, %Ndfa of 84.6, 90.4, and 97.1%, and symbiotic N contribution of 163.3, 161.3, and 140.3 kg ha^{-1} by unfertilized common bean plants at Lujecweni fields 2, 3, and 4, respectively, when compared to their N-fertilized counterparts, suggest that the use of chemical N fertilizers in common bean production in South Africa is not sustainable due to (i) the high symbiotic performance of unfertilized bean plants relative to their fertilized counterparts, (ii) the high cost of N fertilizers, and (iii) the negative impact of N fertilizers on the environment with frequent use. Exploiting symbiotic N for increased dry bean production in South Africa is therefore a better and sustainable alternative to the use of chemical N fertilizers for increased food and nutritional security.

Additionally, in this study, N-fertilization had a directly negative effect on the symbiotic process of common bean plants. For example, with the unfertilized bean plants, there was a significantly positive correlation between nodule number and nodule fresh weight ($r = 0.82^{***}$) and between %Ndfa measured from ^{15}N natural abundance and % relative ureide-N

TABLE 4 | Growth and symbiotic performance of common bean variety PAN128 plants sampled from the fields of farmers.

Location	Field	N supply	Plant density	Shoot DM	N conc'n	N content	$\delta^{15}N$	Ndfa	N-fixed	N-fixed	Soil N uptake	Soil N uptake
		(Dosage)	**ha^{-1}**	**g plant^{-1}**	**%**	**g plant^{-1}**	‰	**%**	**g plant^{-1}**	**kg ha^{-1}**	**g plant^{-1}**	**kg ha^{-1}**
Lujecweni	1	Double	130 000	51.3 ± 3.5de	2.21 ± 0.17cde	1.19 ± 0.22de	1.25 ± 0.47cd	51.8 ± 6.1bc	0.6 ± 0.1bcdef	52.3 ± 11.7cde	0.58 ± 0.16cde	48.9 ± 4.9bcd
	2	None	105 000	61.8 ± 16.2d	2.38 ± 0.36bcd	1.43 ± 0.33de	−1.35 ± 0.43f	84.6 ± 8.2a	1.2 ± 0.3bc	163.3 ± 10.3a	0.22 ± 0.09cde	29.7 ± 6.9bcd
	3	None	102 000	53.3 ± 9.9de	2.77 ± 0.25a	1.45 ± 0.16de	−1.66 ± 0.48f	90.4 ± 9.2a	1.3 ± 0.2b	161.3 ± 13.2a	0.13 ± 0.04de	16.1 ± 3.9cd
	4	None	95 000	57.5 ± 7.9de	2.00 ± 0.10def	1.17 ± 0.19de	−2.01 ± 0.37f	97.1 ± 7.0a	1.1 ± 0.2bcd	140.3 ± 16.2ab	0.05 ± 0.01e	6.4 ± 3.2d
	5	Double	84 200	80.5 ± 5.8cd	2.11 ± 0.15cde	1.71 ± 0.19cde	2.02 ± 0.30c	39.2 ± 4.3bcd	0.7 ± 0.1bcdef	29.0 ± 3.5de	1.05 ± 0.16bc	46.4 ± 6.8bcd
Ngcolosi	1	Double	85 000	54.8 ± 10.5de	1.91 ± 0.07ef	0.99 ± 0.09de	1.43 ± 0.38cd	31.1 ± 4.4de	0.3 ± 0.1ef	39.9 ± 3.8cde	0.68 ± 0.10cde	88.5 ± 12.4b
Tikitiki	1	Double	103 000	25.7 ± 11.3e	2.48 ± 0.17abc	0.61 ± 0.26e	0.81 ± 0.35cd	53.3 ± 5.4bc	0.7 ± 0.1bcdef	29.2 ± 9.2de	0.33 ± 0.07cde	33.7 ± 5.8bcd
	2	Single	74 200	66.4 ± 15.1de	2.58 ± 0.22abc	1.66 ± 0.39cde	1.08 ± 0.53cd	50.1 ± 8.2bcd	0.9 ± 0.0bcdef	68.1 ± 5.2cde	0.74 ± 0.12cde	54.9 ± 8.8bcd
Mfabantu	1	Single	103 000	105.1 ± 18.7c	2.62 ± 0.09ab	2.74 ± 0.88abc	0.67 ± 0.57d	45.5 ± 5.9bcd	1.0 ± 0.4bcdef	22.7 ± 2.5e	1.75 ± 0.08b	40.2 ± 3.3bcd
	2	Single	137 000	107.9 ± 29.2c	2.62 ± 0.18ab	2.72 ± 0.60abc	−0.90 ± 0.43ef	79.9 ± 6.9a	2.3 ± 0.7a	84.1 ± 6.2bcd	0.44 ± 0.14cde	16.4 ± 2.4cd
Nomhala	1	Double	102 000	156.9 ± 13.8a	2.3 ± 0.1abcd	3.59 ± 0.33a	5.77 ± 0.69a	11.9 ± 2.7ef	0.5 ± 0.3cdef	46.1 ± 6.1cde	3.14 ± 0.39a	320.3 ± 12.3a
	2	Single	120 000	134.4 ± 23.8b	2.29 ± 0.08cde	2.95 ± 0.50ab	6.47 ± 0.16a	4.4 ± 1.75f	0.1 ± 0.0f	16.4 ± 2.4e	2.81 ± 0.48a	337.1 ± 10.3a
	3	Single	54 200	87.2 ± 15.8d	1.61 ± 0.12f	1.41 ± 0.29de	3.50 ± 0.52b	34.0 ± 6.1cd	0.41 ± 0.0def	22.3 ± 2.6e	1.00 ± 0.32bcd	54.0 ± 17.1
St. Luthberts	1	Single	89 200	72.0 ± 6.0cd	2.71 ± 0.18a	1.95 ± 0.20bcd	0.28 ± 0.38de	58.9 ± 6.4b	1.1 ± 0.1bcd	99.4 ± 7.5bc	0.84 ± 0.19cde	74.7 ± 4.6ab
F-Statistics				**4.44*****	**3.71*****	**4.88*****	**32.39*****	**15.24*****	**4.06*****	**6.15*****	**9.19*****	**22.04*****

Values (mean ± SE) with dissimilar letters in the same column are significantly different at $^{***}p \leq 0.001$.

abundance ($r = 0.96^{***}$). Furthermore, the % relative ureide-N was positively correlated with nodule number ($r = 0.56^{*}$) and nodule fresh weight ($r = 0.82^{***}$), a finding consistent with an uninhibited functional symbiosis. The significantly positive relationship between % relative ureide-N abundance and nodule fresh weight ($r = 0.50^{***}$) and between % relative ureide-N abundance and %Ndfa ($r = 0.75^{***}$) in N-fertilized common bean plants suggests that the N-fertilized common bean plants were still able to fix N_2 for their growth as they could achieve up to 60% relative ureide-N abundance with a single N-dose application. Tsai et al. (1993) and Tahir et al. (2009) reported that the use of some starter N at planting was beneficial in achieving a synergistic effect on N_2 fixation in common bean by stimulating nodule formation, nitrogenase activity, and plant growth, especially in infertile soils. However, in this study, the % relative ureide-N abundance was negatively correlated with shoot DM ($r = -0.31^{*}$), an indication of inadequate symbiotic N supply to shoots. Several studies have shown that plant growth and grain yield of legumes in Africa are directly correlated with symbiotic functioning, and that grain legumes with high levels of N_2 fixation generally elicit greater biomass accumulation, leading to higher grain yield, and *vice versa* (Belane and Dakora, 2009, 2010; Peoples et al., 2009; Mohale et al., 2013).

The low relative ureide-N (9.2%) observed at Nomhala field 3 that received a double *N*-dose application (**Table 3**) shows that, in this study, farmer application of N fertilizers suppressed N_2 fixation in common bean (Vargas et al., 2000; Salvagiotti et al., 2008; Li et al., 2009; Reinprecht et al., 2020); as a result, those bean plants had to complement symbiotic N supply with greater soil N uptake in order to meet their N demand (Van Vessel and Hartley, 2000; Adu-gyamfi et al., 2007). This argument is reinforced by the high nitrate concentration found in stems and petioles of the common bean plants receiving a single and a double dose of N-fertilizer. In fact, Unkovich et al. (2008) have shown that the N solutes in the xylem stream and aqueous stem extracts of members of the tribe Phaseoleae can change from one dominated by ureides in N_2-dependent plants to a system dominated by nitrate and amino acids in plants utilizing soil N. This could therefore also explain the strong inverse relationship found between relative ureide-N abundance and tissue NO_3^- concentrations of the bean plants in this study. Given that the ureide technique provides an instantaneous measure of legume N_2 fixation at the time of sampling (Alves et al., 2000; Unkovich et al., 2008), the higher ureide-N concentration in stems than petioles could suggest that the stem extract contained xylem ureides that were in transit from root nodules to the leaves, the major sink for fixed-N assimilation.

In this study, although N fertilization markedly suppressed nodulation and nodule symbiotic functioning in bean plants (**Tables 3**, **4**), its application as a single dose generally increased grain yield over either double-dose application or zero-N fertilization (**Figure 1D**). The fact that double-dose application increased shoot growth at the expense of grain yield is consistent with a recent report by Boddey et al. (2017), who observed higher vegetative growth and lower grain yields in cowpea receiving N fertilizer. The findings of this study therefore suggest that, if N must be applied to bean plants, a single dose supply could help to increase yields while reducing government expenditure to resource-poor farmers. However, the fact that symbiotic dependence of bean plants was markedly decreased by a single dose of N fertilizer applied suggests the need to explore the possibility of achieving greater symbiotic N nutrition and increased grain yield at lower starter N doses in low N soils.

A comparison of the data obtained for symbiotic performance from measuring N_2 fixation using the ^{15}N natural abundance technique and ureide assay revealed similar trends for plants sampled from the fields of the same farmer, an indication of their precision in measuring N_2 fixation in bean plants on the fields of farmers. For example, %Ndfa by unfertilized bean plants at Lujecweni fields 2, 3, and 4 were 84.6, 90.4, and 97.1%, respectively, using the ^{15}N natural abundance method, values comparable to the 92.3, 91.6, and 90.2% relative ureide abundance estimated using the ureide technique. Similarly, symbiotic N contribution by unfertilized common bean plants at Lujecweni fields 2, 3, and 4 using the ^{15}N method was 163.3, 161.3, and 140.3 kg ha^{-1} respectively, with values similar in magnitude to the total ureides in stems and petioles of 2.0, 1.6, and 1.1 mg g^{-1} (**Tables 3**, **4**). These trends indicate that the two techniques were similar in their precision and robustness in estimating N_2 fixation in the bean plants on the fields of farmers.

In this study, %Ndfa and ureide levels in stems/petioles were also comparatively similar in trend when the two techniques were used on N-fertilized bean plants from Lujecweni field 1, Mfabantu fields 1 and 2, St. Luthberts field 1, and, to some extent, Tikitiki fields 1 and 2 (**Tables 3**, **4**), and therefore this further confirmed the robustness of the two methods used. However, we also found that the values of %Ndfa at Lujecweni field 1 (51.8%) and Tikitiki field 1 (53.3%) were very high for plants receiving a double dose of N fertilizer and quite high for Tikitiki field 2 (50.1%), Mfabantu fields 1 and 2 (45.5 and 79.9%), and St. Luthberts field 1 (58.9%), which received a single dose of N fertilizer. Some legume symbioses are tolerant of nitrate (Dakora 1998). The only possible explanation for this observation would be that the rhizobia, and therefore bacteroids in root nodules of these N-fertilized bean plants, had a constitutive and/or inducible nitrate reductase activity. Rhizobia with high expression of constitutive nitrate reductase or inducible nitrate reductase genes can form root nodules in the presence of nitrate, but nitrate reductase-*minus* strains cannot (Serrano and Chamber, 1990). Similarly, bacteroids with a high expression of constitutive nitrate reductase and inducible nitrate reductase genes can fix N_2 in the presence of nitrate, but nitrate reductase-*minus* strains cannot (Serrano and Chamber, 1990). Therefore, the observed differences in N_2 fixation of bean plants supplied with high doses of mineral N on the fields of farmers could be attributed to the presence of native soil rhizobia with the ability to reduce mineral N and convert N_2 into NH_3 in root nodules, following differentiation into bacteroids.

CONCLUSION

In conclusion, this study has shown that government supply of free N fertilizers to resource-poor farmers in South Africa increased bean yields for food/nutritional security. Although single- and double-dose N fertilizer application increased plant growth and grain yield compared to unfertilized bean plants, single-dose N fertilizer application produced a much greater grain yield than double dose. This suggests that farmers should stop using a double dose of N fertilizers on bean production, as it decreases yields and can potentially pollute the environment. However, relative to the N-fertilized bean plants, their unfertilized counterparts produced greater nodule fresh weight, higher N derived from fixation, increased amount of symbiotic N, higher percent relative ureide-N abundance, and greater ureide concentration in stems and petioles. Furthermore, contrary to the hypothesis of reduced N_2 fixation with N supply, %Ndfa was very high for some bean plants receiving a double dose of N fertilizer [e.g., Lujecweni field 1 (51.8%) and Tikitiki field 1 (53.3%)] and high for those receiving a single dose of N fertilizer [e.g., Tikitiki field 2 (50.1%), Mfabantu fields 1 and 2 (45.5 and 79.9%), and St. Luthberts field 1 (58.9%)]. Although this was not assessed in our study, it is likely that the rhizobia that effectively nodulated the N-fertilized bean plants and fixed considerable amounts of symbiotic N had constitutive and/or inducible nitrate reductase genes for reducing nitrate in nodules and bacteroids, hence their ability to form root nodules and derived high percent N from fixation in bean with added N.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SH and SM collected the samples. SH analyzed data and drafted the manuscript. FD conceived the idea, edited and approved the final version of the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported with grants from the Bill and Melinda Gates Foundation Project on Capacity Building in Legume Sciences in Africa, the South African Department of Science and Technology, the Tshwane University of Technology, the National Research Foundation in Pretoria, and the South African Research Chair in Agrochemurgy and Plant Symbioses. SH is grateful for a competitive masters fellowship from the Bill and Melinda Gates Foundation Project on Capacity Building in Legume Sciences in Africa.

REFERENCES

Adu-gyamfi, J. J., Myaka, F. A., Sakala, W. D., Odgaard, R., Vesterager, J. M., and Høgh-Jensen, H. (2007). Biological nitrogen fixation and nitrogen and phosphorus budgets in farmer-managed intercrops of maize-pigeon pea in semi-arid Southern and Eastern Africa. *Plant Soil.* 295, 127–136. doi: 10.1007/s11104-007-9270-0

Alves, B., Resende, A., Urquiaga, S., and Boddey, R. (2000). Biological nitrogen fixation by two tropical forage legumes assessed from the relative ureide abundance of stem solutes: 15N calibration of the technique in sand culture. *Nutr. Cycling Agroecosyst.* doi: 10.1023/A:1009865816182

Angus, J. F., and Peoples, M. B. (2012). Nitrogen from Australian dryland pastures. *Crop Pasture Sci.* doi: 10.1071/CP12161

Belane, A. K., and Dakora, F. D. (2009). Measurement of N2 fixation in 30 cowpea (*Vigna unguiculata* L. Walp.) genotypes under field conditions in Ghana, using the 15N natural abundance technique. *Symbiosis* 48, 47–56. doi: 10.1007/BF03179984

Belane, A. K., and Dakora, F. D. (2010). Symbiotic N2 fixation in 30 field-grown cowpea (*Vigna unguiculata* L. Walp.) genotypes in the Upper West Region of Ghana measured using 15N natural abundance. *Biol. Fertil. Soils* 46, 191–198. doi: 10.1007/s00374-009-0415-6

Boddey, R. M., Fosu, M., Atakora, W. K., Miranda, C. H., Boddey, L. H., Guimaraes, A. P., et al. (2017). Cowpea (Vigna unguiculata) crops in Africa can respond to inoculation with rhizobium. *Exp. Agric.* 53, 578–587. doi: 10.1017/S0014479716000594

Bray, R. H., and Kurtz, L. T. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Sci.* 59, 39–46.

Cataldo, D., Maroon, M., Schrader, L., and Youngs, V. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* doi: 10.1080/00103627509366547

Chikanai, V., Chikowo, R., and Vanlauwe, B. (2018). Response of common bean (*Phaseolus vulgaris L.*) to nitrogen, phosphorus and rhizobia inoculation across variable soils in Zimbabwe. *Agric. Ecosyst. Environ.* doi: 10.1016/j.agee.2018.08.010

Dakora, F. D., and Atkins, C. A. (1989). Diffusion of oxygen in relation to structure and function in legume root nodules. *Funct. Plant Biol.* 16, 131–140. doi: 10.1071/PP9890131

Dakora, F. D., Atkins, C. A., and Pate, J. S. (1992). Effect of NO3 on N2 fixation and nitrogenous solutes of xylem in two nodulated West African geocarpic legumes, Kersting's bean (*Macrotyloma geocarpum* L.) and Bambara groundnut (*Vigna subterranea* L.). *Plant Soil.* 140, 255–262. doi: 10.1007/BF00010602

Farid, M., Earl, H. J., and Navabi, A. (2016). Yield stability of dry bean genotypes across nitrogen-fixation-dependent and fertilizer-dependent management systems. *Crop Sci.* 56, 173–182. doi: 10.2135/cropsci2015.06.0343

Farid, M., and Navabi, A. (2015). N_2 fixation ability of different dry bean genotypes. *Can. J. Plant Sci.* 95, 1243–1257. doi: 10.4141/cjps-2015-084

Graham, P. H., and Vance, C. P. (2003). Legumes: importance and constraints to greater use. *Plant Physiol.* doi: 10.1104/pp.017004

Herridge, D. F. (1982a). Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. *Plant Physiol.* doi: 10.1104/pp.70.1.1

Herridge, D. F. (1982b). Use of the ureide technique to describe the nitrogen economy of field-grown Soybeans. *Plant Physiol.* doi: 10.1104/pp.70.1.7

Herridge, D. F., and People, M. B. (1990). Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. *Plant Physiol.* doi: 10.1104/pp.93.2.495

Jiang, Y., MacLean, D. E., Perry, G. E., Marsolais, F., Hill, B., and Pauls, K. P. (2020). Evaluation of beneficial and inhibitory effects of nitrate on nodulation and nitrogen fixation in common bean (*Phaseolus vulgaris*). *Legume Sci.* 2:e45. doi: 10.1002/leg3.45

Li, Y., Yu, C., Cheng, X., Li, C., Sun, J., Zhang, F., et al. (2009). Intercropping alleviates the inhibitory effect of N fertilisation on nodulation and symbiotic N_2 fixation of faba bean. *Plant and Soil.* doi: 10.1007/s11104-009-9938-8

Maingi, J. M., Shisanya, C. A., Nkanata, M. G., and Hornetz, B. (2000). Nitrogen fixation by common bean (*Phaseolus vulgaris* L.) in pure and mixed stands

in semi-arid south-east Kenya. *Eur. J. Agron.* doi: 10.1016/S1161-0301(00)00080-0

Maskey, S. L., Bhattarai, S., Peoples, M. B., and Herridge, D. F. (2001). On-farm measurements of nitrogen fixation by winter and summer legumes in the Hill and Terai regions of Nepal. *Field Crops Res.* doi: 10.1016/S0378-4290(01)00140-X

Mohale, K. C., Belane, A. K., and Dakora, F. D. (2013). Symbiotic N nutrition, C assimilation, and plant water use efficiency in Bambara groundnut (*Vigna subterranea* L. Verdc) grown in farmers' fields in South Africa, measured using ^{15}N and ^{13}C natural abundance. *Bio. Fertil. Soils* doi: 10.1007/s00374-013-0841-3

Pausch, R. C., Charles, L., Mulchi, C. L., Lee, E. H., and Meisinger, J. J. (1996). "Use of ^{13}C and ^{15}N isotopes to investigate O_3effects on C and N metabolism in soybeans," in *Part II. Nitrogen uptake, fixation, and partitioning. Agriculture Ecosystem and Environment.* Available online at: 10.1016/S0167-8809(96)01062-6

Peoples, M. B., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., et al. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis.* doi: 10.1007/BF03179980

Reinprecht, L., Vidholdová, Z., and Iždinský, J. (2020). Bacterial and mold resistance of selected tropical wood species. *BioResources* 15, 5198–5209.

Salvagiotti, F., Cassman, K. G., Specht, J. E., Walters, D. T., Weiss, A., and Dobermann, A. (2008). Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. *Field Crops Res.* doi: 10.1016/j.fcr.2008.03.001

Samago, T. Y., Endalkachew, W. A., and Dakora, F. D. (2018). Grain yield of common bean (*Phaseolus vulgaris* L.) varieties is markedly increased by rhizobial inoculation and phosphorus application in Ethiopia. *Symbiosis* 75, 245–255. doi: 10.1007/s13199-017-0529-9

Serrano, A., and Chamber, M. (1990). Nitrate reduction in *Bradyrhizobium* sp. *(Lupinus)* strains and its effects on their symbiosis with *Lupinus luteus. J. Plant Physiol.* 136, 240–246. doi: 10.1016/S0176-1617(11)81673-1

Shearer, G., and Kohl, D. H. (1986). N_2 fixation in field settings: Estimation based on natural ^{15}N abundance. *Aus. J. Plant Physiol.* doi: 10.1071/PP9860699

Tahir, M. M., Abbasi, M. K., Rahim, N., Khaliq, A., and Kazmi, M. H. (2009). Effect of Rhizobium inoculation and NP fertilisation on growth, yield and nodulation of soybean *(Glycine max L.)* in the sub-humid hilly region of Rawalakot Azad Jammu and Kashmir, Pakistan. *Afr. J. Biotech.* 8, 6191–6200. doi: 10.5897/AJB09.1039

Tsai, S. M., Bonetti, R., Agbala, S. M., and Rossetto, R. (1993). "Minimizing the effect of mineral nitrogen on biological nitrogen fixation in common bean by increasing nutrient levels," in *Enhancement of Biological Nitrogen Fixation of Common Bean in Latin America. Developments in Plant and Soil Sciences,*eds F. A. Bliss, G. Hardarson (Dordrecht: Springer). Available online at: doi: 10.1007/978-94-011-2100-2_14

Unkovch, M. J., Herridge, D. F., Peoples, M. B., Cadisch, G., Boddey, R. M., and Giller, K. E. (2008). *Measuring Plant-Associated Nitrogen Fixation in Agricultural Systems*. Canberra: ACIAR.

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., et al. (2008). *Measuring Plant-Associated Nitrogen Fixation in Agricultural Systems.* Canberra, ACT: Australian Centre for International Agricultural Research.

Unkovich, M. J., Pate, J. S., Sanford, P., and Amstrong, E. L. (1994). Potential precision of the $\delta^{15}N$ natural abundance method in field estimation of nitrogen fixation by crop and pasture legumes in South-west Australia. *Aust. J. Agric. Res.* doi: 10.1071/AR9940119

Van Vessel, C., and Hartley, C. (2000). Agricultural management of grain legumes: has it led to an increase in nitrogen fixation? *Field Crops Res.* doi: 10.1016/S0378-4290(99)00085-4

Vargas, M. A., Mendes, I. C., and Hungria, M. (2000). Response of field-grown bean (Phaseolus vulgaris L.) to Rhizobium inoculation and nitrogen fertilization in two Cerrados soils. *Biol. Fertil. Soils* 32, 228–233. doi: 10.1007/s003740000240

Vieira, R. F., Cardoso, E. J. B. N., Vieira, C., and Cassini, S. J. A. (1998). Foliar application of molybdenum in common beans: Nitrogenase and reductase activities in a soil of high fertility. *J. Plant Nutri.* doi: 10.1080/01904169809365391

Walkley, A., and Black, I. A. (1934). An examination of the degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Sci.* 37, 29–38. doi: 10.1097/00010694-193401000-00003

Young, E. G., and Conway, C. F. (1942). On the estimation of allantoin by the Rimini-Schryver reaction. *J. Bio. Chem.* 142, 839–853. doi: 10.1016/S0021-9258(18)45082-X

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Habinshuti, Maseko and Dakora. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership