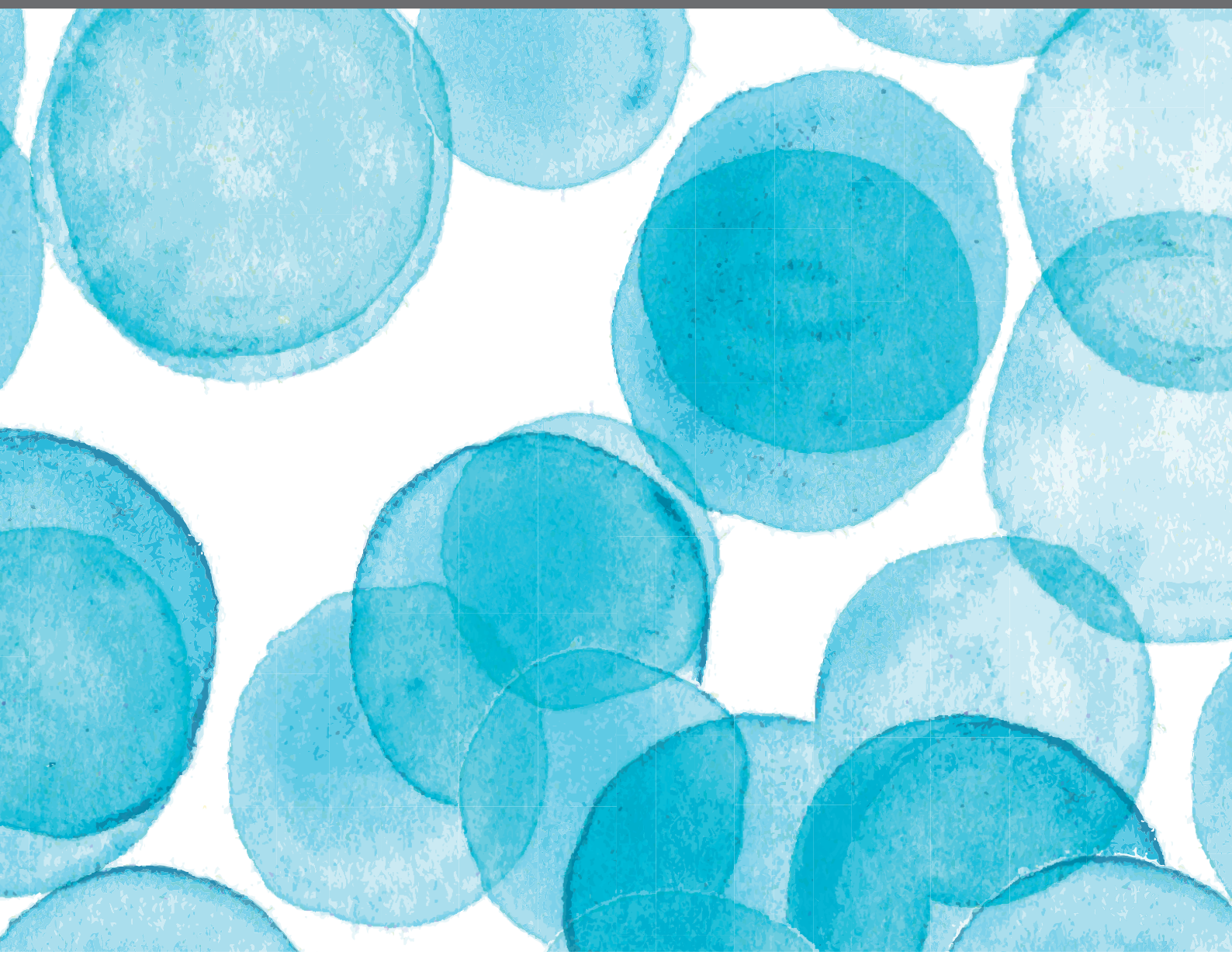
PLANT MICROBIOME: DIVERSITY, FUNCTIONS, AND APPLICATIONS

EDITED BY: Khondoker M. G. Dastogeer, Jenny Kao-Kniffin and Shin Okazaki
PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-441-3
DOI 10.3389/978-2-83250-441-3

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

PLANT MICROBIOME: DIVERSITY, FUNCTIONS, AND APPLICATIONS

Topic Editors:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh
Jenny Kao-Kniffin, Cornell University, United States
Shin Okazaki, Tokyo University of Agriculture and Technology, Japan

Citation: Dastogeer, K. M. G., Kao-Kniffin, J., Okazaki, S., eds. (2022). Plant Microbiome: Diversity, Functions, and Applications. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-441-3

Table of Contents

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 26 September 2022
DOI 10.3389/fmicb.2022.1039212

OPEN ACCESS

EDITED AND REVIEWED BY
Trevor Carlos Charles,
University of Waterloo, Canada

*CORRESPONDENCE
Khondoker M. G. Dastogeer
dastogeer.ppath@bau.edu.bd

SPECIALTY SECTION
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

RECEIVED 07 September 2022
ACCEPTED 13 September 2022
PUBLISHED 26 September 2022

CITATION
Dastogeer KMG, Kao-Kniffin J and Okazaki S (2022) Editorial: Plant microbiome: Diversity, functions, and applications.
Front. Microbiol. 13:1039212.
doi: 10.3389/fmicb.2022.1039212

Editorial: Plant microbiome: Diversity, functions, and applications

Khondoker M. G. Dastogeer[1,2]*, Jenny Kao-Kniffin[3] and Shin Okazaki[4]

[1]Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh, [2]Department of Environmental Science, Policy, and Management, University of California, Berkeley, Berkeley, CA, United States, [3]Horticulture Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, United States, [4]Plant Microbiology Laboratory, Tokyo University of Agriculture and Technology, Tokyo, Japan

KEYWORDS

endophytes, metabolites, microbiome diversity, plant health, plant microbiome

Editorial on the Research Topic
Plant microbiome: Diversity, functions, and applications

Considerable attention on plant microbiomes has been growing over the past decade featuring model plants, natural, and agricultural crop systems (Berg et al., 2020; Chialva et al., 2022). Studies are being carried out to understand microbial community composition, assembly process and underlying factors, evolution and ecology, and functional roles (Compant et al., 2019; Dastogeer et al., 2020; Xu et al., 2021; Trivedi et al., 2022). The power of advanced omics and multi-omics techniques, computational power, biostatistics, and remodeling of biological and evolutionary theories allows for a deeper understanding of plant—-microbiome interactions (Subramanian et al., 2020; Xu et al., 2021). The study of how the microbiome interacts with other elements of the environment is gaining momentum, and efforts are being made to understand the functional roles of the microbiome in translating microbiome potentials into sustainable plant production and protection. Currently, the engineering of microbial flora and the development and application of the synthetic community (SynComs) approach have revealed an essential role of microorganisms in plant adaptability and productivity (Liu et al., 2019; Nerva et al., 2022).

This Research Topic hosts eleven original research articles and a review article. The original research articles span experimental findings of various plants with their associated microbes, such as apple, aspen, avocado, chrysanthemum, eucalyptus, licorice, morning glories, tea, tobacco, and yellow witchweed.

Crop management modulates microbiome diversity and composition

It is increasingly evident that crop management practices, including plant protection measures, influence the microbiome of plants and soil (Jing et al., 2022). The study by

Wang et al. substantiates that monoculture practice significantly modulates microbiome compositions. They reported that continuous monocropping changed the profiles of the bacterial and fungal communities in cut chrysanthemum. The longer the monocropping time, the greater the reduction of biocontrol bacteria but the augmentation of pathogenic microbes in the rhizosphere of plants. These changes in microbial composition were also correlated with concomitant changes in soil properties in the monoculture practice.

Glycyrrhiza uralensis known as licorice is one of the most commonly used bulk medicinal materials in traditional Chinese medicine (Jiang et al., 2020). The quality of the desired product depends on many factors, including the techniques of licorice processing. Li et al. studied the association of endophytic microorganisms and the accumulation of metabolites in different licorice drying methods. Natural drying impacted the microbial diversity of licorice and Nectriaceae, with Enterobacteriaceae becoming more abundant under these conditions. They reported a correlation between dominant endophyte taxon and the metabolite types present in fresh vs. dry processing, suggesting that fresh-processing is an effective drying method to ensure the quality of licorice.

Association of the microbiome with plant health status

Understanding the role of the microbiome in plant health is essential in pinpointing the etiology of disease and devising appropriate management solutions (Dastogeer et al., 2022; Trivedi et al., 2022). Mahnkopp-Dirks et al. conducted experiments with apple replant disease (ARD) to understand whether it influences structuring plant microbiome composition and diversity. This disease has worldwide occurrence in many tree nurseries, but there still remains a lack of knowledge about the cause and management of this complex disease (Mazzola and Manici, 2012; Winkelmann et al., 2019). Mahnkopp-Dirks et al. used culture-independent and culture-dependent methods to compare the endophytic root microbiome of apple plants grown in ARD-affected soil vs. those in control soil. They reported that several *Streptomyces* increased over time, particularly in virgin soil, and based on this, they speculated that Streptomyces might be associated with ARD etiology. Also, ARD-affected soil had a reduced abundance of *Pseudomonas* roots endophytes, indicating its importance for a balanced microbiome in healthy soils. Culturing bacteria in media also showed a high diversity of *Pseudomonas,* supporting the view of their microbiome study. Ahmed et al. employed similar methods to gain a microbiome perspective of bacterial wilt-infected Flue-Cured Tobacco (*Nicotiana tabacum*) plants. They reported that the healthy plants exhibited higher microbial diversity than diseased plants with increased abundances of several bacteria such as *Bacillus*, *Bradyrhizobium*, *Ensifer*, *Neorhizobium*, and *Lysobacter,* which have been variously reported to be plant growth promoters and disease suppressor. There were increased abundances of bacterial wilt pathogen *Ralstonia solanacearum* rhizosphere soil and root of diseased plants. In addition, several fungi commonly identified as plant pathogens, *Plectosphaerella cucumerina, Alternaria alternata, Thanatephorus cucumeris*, and *Fusarium* sp., were encountered more frequently in the rhizosphere soil and root. They hypothesized that higher abundances of beneficial bacteria in the healthy plants and pathogenic fungi in diseased plants may be associated with the bacterial wilt disease in tobacco and that the complexity of bacterial and fungal communities possibly interact with each other (microbe–microbe) and host (host-microbe) to influence growth and induce resistance against bacterial wilt disease.

Cao et al. showed that infection of root-knot nematode (RKN) causes significant alteration in the diversity and composition of root microorganisms in tobacco. While the diversity of rhizosphere bacteria was reduced, the diversity of rhizosphere fungi increased upon infection by RNK. In particular, the abundance of *Rhizobiaceae* and *Chryseobacterium* increased after RKN infection. Nematode infection altered the overall composition of root microbiome but only at specific growth stages of plants, indicating variable nematode-microbe-plant interactions at different development stages of plants. Farooq et al. conducted microbial profiling of rhizosphere soil and root endosphere of avocado plants infested or not infested with *Phytophthora cinnamomi*. They compared the changes in microbial diversity and community composition of infested and non-infested plants grown with various soil additives or by spraying plants with phosphite. The results revealed phosphite treatment or soil application of mineral mulch applied to the soil reduced *Phytophthora* infestation. Bacterial abundance and diversity were reduced in infested rhizospheres and root endospheres. The mineral mulch application significantly changed diversity and rhizosphere community composition more than any other treatment. Some rhizosphere bacterial groups, especially Actinobacteria and Proteobacteria, had significantly higher relative abundance in the presence of *Phytophthora*. The bacterial communities of root endospheres were lower in abundance than rhizosphere communities and not affected by soil treatments or phosphite but increased in abundance after infection with *P. cinnamomi*. These findings suggested that adding silicate-based mineral mulch protects against *Phytophthora* root rot, which changes in rhizosphere bacterial community composition may partly mediate. However, the changes to the microbiome induced by spraying plants with phosphite are different from those resulting from applying mineral mulch to the soil. Plant fungal communities are diverse and influenced by many biotic and abiotic factors. The influence of biotic factors, however, is still poorly understood. Messal et al. investigated how insect infestation and gall formation on *Eucalyptus* foliage influence the diverse community composition of foliar fungi in *Eucalyptus*

grandis trees. The infestation of the wasp *Leptocybe invasa* (Eulophidae: Hymenoptera) indeed brought about significant changes in the composition and diversity of fungal communities and was correlated with the severity of infestations. Their network analysis indicated that the co-occurrence of potential pathogens differed significantly between no to mild and medium to heavy infestation. This study increased our microbial interactions, especially the role of pathogens which may help design management approaches for pests employing beneficial host-associated microbial communities.

Endophytes: Diversity, composition, evolution, and sources of novel metabolites

All plants in natural ecosystems appear to be symbiotic with fungal and bacterial endophytes, and their diversity is very high (Rodriguez et al., 2009; Afzal et al., 2019). They play important roles in helping plant growth and development. Tea is an economically important crop worldwide. The information about the distribution pattern and potential functions of endophytic communities in tea trees has been limited. Lin et al. investigated the diversity and community composition of endophytic bacteria and fungi of tea plants using amplicon sequencing. Ecological niches shape the diversity of endophytic microorganisms. They found that bacterial diversity was highest in root tissues followed by the stem, old leaves, and new leaves, and fungal diversity was highest in the old leaves followed by new leaves, roots, and stems. The cooperative relationship between endophytic bacteria and fungi in the new leaves was more substantial than that in the old leaves, which can better participate in the metabolism of tea material. Microorganisms, specifically the endophytes of medicinal plants, are recognized as valuable sources of novel secondary metabolites. Maela et al. isolated and identified five bacterial endophytes, *Alectra sessiliflora*, an important medicinal plant. *Bacillus* was the most dominant bacterial endophyte with two other genera: *Lysinibacillus* and *Peribacillus*. The crude extracts of three bacterial strains showed more than 90% growth inhibition against all the cancer cell lines at a concentration of 1,000 μg/ml. They also performed untargeted secondary metabolite profiling and reported the presence of compounds known to have biological activity. In addition to various abiotic and biotic factors, plant-fungal communities are also shaped by management factors. Witzell et al. hypothesized that nitrogen fertilization (N) modulates the quality of aspen (*Populus tremula*) leaves and thus alters the internal "chemical landscape" for the fungi. The study suggested that nitrogen treatment reduced foliar concentrations of CT precursor catechin but not that of condensed tannins (CTs). The N application also boosted the level of the amino acids and lowered the level of certain sugars. When a beetle herbivore was used as a treatment, no significant alteration in chemical composition or endophyte diversity was noted. A few rare fungi were associated with and potentially vectored by the beetle herbivores. They inferred that under controlled conditions and in a short period, the growth conditions, not the internal chemical quality, are the moderators of the fungal diversity in aspen leaves. The production of bioactive metabolites by endosymbiotic fungi is particularly important to the fitness of the fungus and the host species. The benefits and costs of symbiosis are not well-understood in many cases. The fungal endosymbiont of several plants, locoweeds (Fabaceae), morning glories (Convolvulaceae), and two species of Malvaceae are known to produce swainsonine. Swainsonine has toxic effects on livestock that feed on these plants and has potential pharmaceutical applications. Quach et al. evaluated 244 morning glory species for the presence of swainsonine and built a phylogeny based on available internal transcribed spacer (ITS) sequences. Only a single morning glory clade has swainsonine, and this symbiosis developed ∼5 million years. Various orders of swainsonine-producing fungal endosymbionts in different plant families are genetically diverse. Further research would be exciting to know whether different symbioses have similar effects on host plants from other families and fungal-host specificity.

Gruet et al. provide an excellent review that sheds light on our current understanding of the microbiome of wheat root and rhizosphere based on pre-and post-domestication wheat history. Wheat, one of the major crops in the world, has had a complex history that includes genomic hybridizations between *Triticum* and *Aegilops* species and several domestication events. The author discusses differences between wild and domesticated wheat, ancient and modern types of cultivars, as well as individual cultivars within a given wheat species. Their analyses pointed out two significant trends. The majority of the investigations were carried out toward taxonomic diversity rather than the microbial functioning microbiota and more for bacteria and mycorrhizal fungi. Second, studies primarily compared wheat genotypes of *T. aestivum* cultivars, sometimes with little consideration for their particular genetic and physiological traits. The author hoped that the employment of current state-of-the-art sequencing technologies would enable revisiting the wheat microbiome's diversity to understand the wheat evolutionary history's significance better. This would provide the baseline information needed to develop microbiome-based breeding strategies for sustainable wheat farming.

The twelve articles comprising this special topic on *Plant Microbiome: Diversity, Functions, and Applications* highlight the advances and progress in research on a great diversity of plant species globally. The rapid developments in omics technologies and bioinformatics analysis are continuing to evolve the microbiome science field forward. We can expect many more novel discoveries in the coming years featuring innovative experimentation and predictive analyses.

Author contributions

KD: conceptualization and draft preparation. JK-K and SO: revision and modification. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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.

References

Afzal, I., Shinwari, Z. K., Sikandar, S., and Shahzad, S. (2019). Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. *Microbiol. Res.* 221, 36–49. doi: 10.1016/j.micres.2019.02.001

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., et al. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8, 1–22. doi: 10.1186/s40168-020-00875-0

Chialva, M., Lanfranco, L., and Bonfante, P. (2022). The plant microbiota: composition, functions, and engineering. *Curr. Opin. Biotechnol.* 73, 135–142. doi: 10.1016/j.copbio.2021.07.003

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

Dastogeer, K. M., Tumpa, F. H., Sultana, A., Akter, M. A., and Chakraborty, A. (2020). Plant microbiome–an account of the factors that shape community composition and diversity. *Curr. Plant Biol.* 2020:100161. doi: 10.1016/j.cpb.2020.100161

Dastogeer, K. M., Yasuda, M., and Okazaki, S. (2022). Microbiome and pathobiome analyses reveal changes in community structure by foliar pathogen infection in rice. *Front. Microbiol.* 2918. doi: 10.3389/fmicb.2022.949152

Jiang, M., Zhao, S., Yang, S., Lin, X., He, X., Wei, X., et al. (2020). An "essential herbal medicine"—Licorice: A review of phytochemicals and its effects in combination preparations. *J. Ethnopharmacol.* 249, 112439. doi: 10.1016/j.jep.2019.112439

Jing, J., Cong, W.-F., and Bezemer, T. M. (2022). Legacies at work: plant–soil–microbiome interactions underpinning agricultural sustainability. *Trends Plant Sci.* 27, 781–792. doi: 10.1016/j.tplants.2022.05.007

Liu, Y.-X., Qin, Y., and Bai, Y. (2019). Reductionist synthetic community approaches in root microbiome research. *Curr. Opin. Microbiol.* 49, 97–102. doi: 10.1016/j.mib.2019.10.010

Mazzola, M., and Manici, L. M. (2012). Apple replant disease: role of microbial ecology in cause and control. *Annu. Rev. Phytopathol.* 50, 45–65. doi: 10.1146/annurev-phyto-081211-173005

Nerva, L., Sandrini, M., Moffa, L., Velasco, R., Balestrini, R., and Chitarra, W. (2022). Breeding toward improved ecological plant–microbiome interactions. *Trends Plant Sci.* (in press). doi: 10.1016/j.tplants.2022.06.004

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

Subramanian, I., Verma, S., Kumar, S., Jere, A., and Anamika, K. (2020). Multi-omics data integration, interpretation, and its application. *Bioinform. Biol. Insights* 14:1177932219899051. doi: 10.1177/1177932219899051

Trivedi, P., Batista, B. D., Bazany, K. E., and Singh, B. K. (2022). Plant–microbiome interactions under a changing world: Responses, consequences, and perspectives. *New Phytol.* 234, 1951–1959. doi: 10.1111/nph.18016

Winkelmann, T., Smalla, K., Amelung, W., Baab, G., Grunewaldt-Stöcker, G., Kanfra, X., et al. (2019). Apple replant disease: causes and mitigation strategies. *Curr. Issues Mol. Biol.* 30, 89–106. doi: 10.21775/cimb.030.089

Xu, L., Pierroz, G., Wipf, H. M.-L., Gao, C., Taylor, J. W., Lemaux, P. G., et al. (2021). Holo-omics for deciphering plant-microbiome interactions. *Microbiome* 9, 1–11. doi: 10.1186/s40168-021-01014-z

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 October 2021
doi: 10.3389/fmicb.2021.740721

Dynamic Changes in Endophytic Microorganisms and Metabolites During Natural Drying of Licorice

Ting Li, Guangxi Ren, Dan Jiang and Chunsheng Liu**

School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing, China

The method of drying licorice is an important factor affecting the quality of the final product. To determine the best processing method of licorice postharvest, we investigated the interaction of increasing aridity between the endophytic microorganisms and the accumulation of metabolites. Samples from the roots of licorice growing along an aridity gradient during the natural drying process were collected, and the metabolic components, the content of the main active substances and the dynamic changes of the endophytic microbial community were assessed. The glycyrrhizic acid and liquiritin contents decreased slightly or remained flat during natural drying, whereas those of liquiritigenin and isoliquiritigenin increased slightly. Moreover, the Shannon index of endophytic microbial diversity of licorice was the highest in the fresh period and showed a downward trend during the drying process. When the licorice were fresh, Cladosporiaceae and Burkholderiaceae were the dominant family present, but after drying, Nectriaceae and Enterobacteriaceae were the dominant families. A similar trend was also found in which the differential metabolites of licorice were reduced during natural drying. Furthermore, correlation analysis between dominant families and differential metabolites showed that there was a correlation between the two. Therefore, fresh processing is an effective drying method to ensure the quality of licorice. This study revealed the relationship of endophytic microbiota and changes in the licorice metabolites during different stages of drying, which provided a scientific basis for the drying method of licorice.

Keywords: natural drying, water change, metabolism, endophytic microorganisms, licorice

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Vineet Kumar Mishra, Indian Institute of Chemical Biology (CSIR), India
Dilfuza Egamberdieva, Leibniz Center for Agricultural Landscape Research (ZALF), Germany

****Correspondence:***
Chunsheng Liu
max_liucs@263.net
Dan Jiang
jiangdan1027@163.com

Specialty section:
This article was submitted to Microbe and Virus Interactions With Plants, a section of the journal Frontiers in Microbiology

Received: *13 July 2021*
Accepted: *23 September 2021*
Published: *14 October 2021*

Citation:
Li T, Ren G, Jiang D and Liu C (2021) Dynamic Changes in Endophytic Microorganisms and Metabolites During Natural Drying of Licorice. Front. Microbiol. 12:740721. doi: 10.3389/fmicb.2021.740721

INTRODUCTION

Glycyrrhiza uralensis Fisch. (Licorice) is the most commonly used bulk medicinal material in traditional Chinese medicine. Licorice and its extracts have anti-inflammatory and antiviral properties (Chen et al., 2021; van de Sand et al., 2021). It is also an important additive in cosmetics, healthcare products, tobacco and other industries, and its annual demand is huge (Hayashi and Sudo, 2009; Shakeri et al., 2018; Yin et al., 2020). The licorice plant needs to be processed in a series of steps to become a medicinal material. Drying licorice is the most critical step in this process, ensuring the licorice does not rot due to high water content (Icier et al., 2021). Recent studies have focused on the microwave drying and vacuum freeze drying of licorice (Balbay and Sahin, 2012; Li et al., 2017). However, in general, sun-drying is the

method primarily used in cultivation areas; that is, licorice is dug out once it has grown for approximately 3 years, gathered into small bundles in the cultivation field, dried in the sun, remoisturized with water and finally cut into decoction pieces. However, this particular method of drying licorice in the sun takes a significant amount of time, which can negatively affect the quality and effectiveness of the final medicinal materials (Icier et al., 2021). Therefore, the analysis of the content of the effective components of licorice in the sun-drying process would be helpful in characterizing the best drying and processing technology of licorice production areas, helping to improve the quality of licorice.

Endophytic microorganisms live in various tissues and organs of healthy host plants without negatively affecting the plant's health or functions (Wu et al., 2021). Endophytic microorganisms have important effects on plant growth, development and the accumulation of metabolites (Lugtenberg et al., 2016; Zhou et al., 2020; Liu and Wei, 2021; Santos et al., 2021; Singh et al., 2021). It has been reported that Bacillus pumilus can alleviate the drought stress of licorice and increase the accumulation of licorice metabolites (Xie et al., 2019). Endophytic microorganisms are affected by a variety of external factors, including changes in the water content (Huang et al., 2021; Tian et al., 2021). As an important part of licorice, the microbial community composition and function will also change as the water content changes during the drying process. However, the effect of licorice on the functional microbial community during the natural drying process is still largely unknown. Therefore, a clearer understanding of the composition and function of the microbial community during the drying process provides a scientific basis for perfecting licorice production, which is conducive to perfecting the production area and processing of licorice, and provides a scientific basis for obtaining high-quality licorice.

In recent years, researchers have begun using metabolomics to study the types and quantities of plant endogenous metabolites and their changes under the action of internal and external factors. Metabolomics has unique advantages in uncovering these patterns, such as high throughput sequencing, rapid separation, high sensitivity, and widespread use (Jiang et al., 2021; Li et al., 2021). The metabolic components of traditional Chinese medicines are relatively complicated, and the existing detection and analysis methods have been mostly used to focus on one or several known components, making it difficult to achieve a comprehensive quality analysis. Therefore, metabolomics technology can provide an effective method for the qualitative and quantitative analysis of traditional Chinese medicine. At present, metabolomics technology has begun to be used to study the synthesis and transformation of metabolites during the growth and storage of Chinese medicinal materials (Ma et al., 2021). For this reason, metabolomics was used to analyze the dynamic changes in the drying process of licorice in this study.

Here, we analyzed the changes in the content of the effective components of licorice during the drying process and used the Illumina MiSeq high-throughput sequencing platform to analyze the structure and function of endophytic microorganisms in the licorice during the drying process. At the same time, nontargeted metabolomic sequencing was performed on licorice with different water contents, and the relationship between the changes in metabolites and endophytic microorganisms was studied. In summary, our research provides the diversity and composition of the endophytic microbial community of licorice and the simultaneous changes in licorice metabolites during the natural drying process. In addition, it provides a favorable scientific basis for choosing the best licorice processing method.

MATERIALS AND METHODS

Materials

Wild licorice (all *G. uralensis* Fisch.) was collected from an arid hillside in Suide County, Shaanxi Province, transported on ice and stored at −80°C. It was identified by Professor Chunsheng Liu of Beijing University of Chinese Medicine. Numbers W-1, W-2, and W-3 were three groups of licorice with a thickness of 0.8, 0.9, and 1.1 cm, and each group was composed of five licorice roots of the same thickness. The samples were all mixed with five licorice roots to form one sample. The reference substances liquiritin (Lot number: Z13J11X108109), glycyrrhizic acid (Lot number: P24J10F91300), isoliquiritigenin (Lot number: C03A8Q41092), liquiritigenin (Lot number: Z20J8X40265), and glycyrrhetinic acid (Lot number: T27J7X18416) were purchased from Shanghai Yuanye Bio Technology Co., Ltd. (Shanghai, China).

Drying Method

The fresh licorice root was dried in a simulated production area (natural drying). During the process, the water content was measured every 6 days (0 day: fresh, 6 days: early stage of drying, 12 days: middle stage of drying, 18 days: completely dried). Then, the previous operation was repeated until the water content of licorice was below 10%, when the medicinal material was considered to have reached the dry standard. At the same time, the licorice root was sampled every 6 days (The samples were mixed with five licorice roots to form one sample). The metabolites of the samples were analyzed and determined by HPLC–MS. The licorice was remoisturized and sliced within half a year after it was completely dried, which was also in line with the processing method of licorice. After that, the content of the active ingredients of licorice was tested.

Extraction and UPLC Analysis of the Effective Components of Licorice

The licorice powder was extracted with 70% methanol and detected by UPLC. Liquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizic acid, and glycyrrhetinic acid were taken in appropriate amounts to prepare mother liquor with a liquiritin concentration of 0.139 mg/ml, liquiritigenin concentration of 0.051 mg/ml, isoliquiritigenin concentration of 0.065 mg/ml, glycyrrhizic acid concentration of 0.24 mg/ml and glycyrrhetinic acid concentration of 0.099 mg/ml. Then, 0.5, 1, 1.5, 2, and

2.5 ml was taken and diluted to 10 ml with methanol to a series of concentrations. The chromatographic conditions were as follows: BEH C18 column (100 mm × 2.1 mm, 1.7 μm, Waters, United States) acetonitrile (A)-0.1% phosphoric acid water (B); gradient elution: 1 min, 10% A; 7 min, 14% A; 14 min, 21% A; 21 min, 30% A; 25 min, 45% A; 28 min, 70% A; 34 min, 10% A; 35 min, 10% A. The flow rate was 0.4 ml/min, and the injection volume was 1 μl. Each experiment was repeated five times.

Extraction of Metabolites and HPLC–MS Analysis Conditions

One hundred milligram of licorice tissue was added to 800 μl of 80% cold methanol solution, which was then crushed by a high-throughput tissue breaker under low temperature conditions. After being mixed, it was placed on ice and ultrasonically extracted for 10 min, repeated three times. The sample was placed at −20°C for 30 min and centrifuged at 13,000 *g* at 4°C for 15 min. After centrifugation, the supernatant was collected for detection by LC–MS. The chromatographic conditions were as follows: the column was a BEHC18 column (100 mm × 2.1 mm, 1.7 μm, Waters, United States). The mobile phase was acetonitrile (A)-0.1% phosphoric acid water (B); gradient elution: 0 min, 95% B; 3 min, 80% B; 9 min, 5% B; 13.0 min, 5% B; 13.1 min, 95% B; 16 min, 95% B. The flow rate was 0.4 ml/min, the column temperature was 40°C, and the injection volume was 10 μl. Using positive and negative ion scanning modes, the positive electrode ionization voltage was 3.5 kV, the negative electrode ionization voltage was 2.8 kV, the ion source heating temperature was 400°C, the sheath gas flow rate was 40 psi and the aus gas flow rate was 10 psi. Each experiment was repeated six times.

DNA Extraction and Diversity Sequencing of Endophytic Microorganisms

The licorice root was sampled every 6 days for sequencing after sterilization. The sterilization method was as follows: rinsing with purified water for 30 min, rinsing with sterile water 2–3 times, disinfecting with 75% ethanol for 2 min, rinsing with sterile water 2–3 times, sterilizing with 0.1% mercury for 5 min, and rinsing with sterile water 2–3 times. The last rinse was used to verify that it was cleaned. Total DNA was extracted from the samples (licorice roots at 0, 6, 12, and 18 days of drying) with a DNA kit (Fast DNA Soil, MP, United Sates) according to the instructions. Primers were designed according to the 16S rRNA and ITS conserved regions. The sequences were as follows: 799F: AACMG GATTAGATACCCKG; 1193R: ACGTCATCCCCACCTTCC; ITS1F: CTTGGTCATTTAGAGGAAGTAA; ITS2R: GCTGC GTTCTTCATCGATGC. The DNA was amplified by PCR, purified and quantified, after which the sequencing library was constructed. Sequencing was performed using Illumina HiSeq 2500. The PE reads obtained by MiSeq sequencing were first spliced according to the overlap relationship, and the sequence quality was controlled and filtered at the same time. OTU clustering was performed after distinguishing the samples. Species annotation and abundance analysis were carried out to reveal the composition of the samples. Alpha and β diversity of species was analyzed. In addition, significant species difference analysis was used to explore the differences of samples under different drying times. Picrust2 was used to predict the function of endophytic microorganisms.

Data Analysis

The content of effective components is expressed as mean ± SD. Statistical significance was evaluated using a one-way ANOVA for multiple comparisons (SAS 9.4 Software). A value of $p < 0.05$ was considered statistically significant. The raw metabolic data were imported into the metabolomics processing Progenesis QI (Waters Corporation, Milford, United States) for baseline filtering, peak identification, integration, retention time correction, peak alignment, and finally a data matrix of retention time, mass-to-charge ratio and peak area was obtained. Then, the data were preprocessed, and the MS and MS/MS mass spectra information were matched with the metabolic database. The main database used were http://www.hmdb.ca; http://metlin.scripps.edu and other public databases. Correlations between the microbial community and metabolites were carried out based on Spearman correlation coefficients. All sequences produced from Illumina sequencing were uploaded to the sequence read archive (SRA) of the NCBI database. The accession number of all samples is PRJNA748524.

RESULTS

Dynamic Changes in Effective Components in Licorice During Drying

Because the age of wild licorice could not be ascertained, the licorice was divided into three groups according to root thickness, and each group was composed of five licorice roots of the same thickness to eliminate the risk of error caused by unequal root thickness. Five index components were selected for the determination: liquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizic acid, and glycyrrhetinic acid. However, it may have been impossible to detect glycyrrhetinic acid in some samples due to the extremely small concentration in licorice roots, so we did not analyze the glycyrrhetinic acid content of the samples. Compared with fresh licorice, the contents of liquiritin and glycyrrhizic acid in the three groups of samples decreased slightly or remained flat with increasing drying time (**Figures 1A,D**), while the contents of liquiritigenin and isoliquiritigenin increased slightly (**Figures 1B,C**). It was speculated that the aglycone was removed from glucose and converted into glycosides or other compounds during the natural drying of licorice. Except for the difference between the content of isoliquiritigenin and that of completely dried isoliquiritigenin in the W-3 group, the contents of liquiritin, liquiritigenin, isoliquiritigenin and glycyrrhizic acid in the other groups were significantly different between fresh and completely dried samples. In addition, after a long period of drying and then moistening with water, the content of all active ingredients

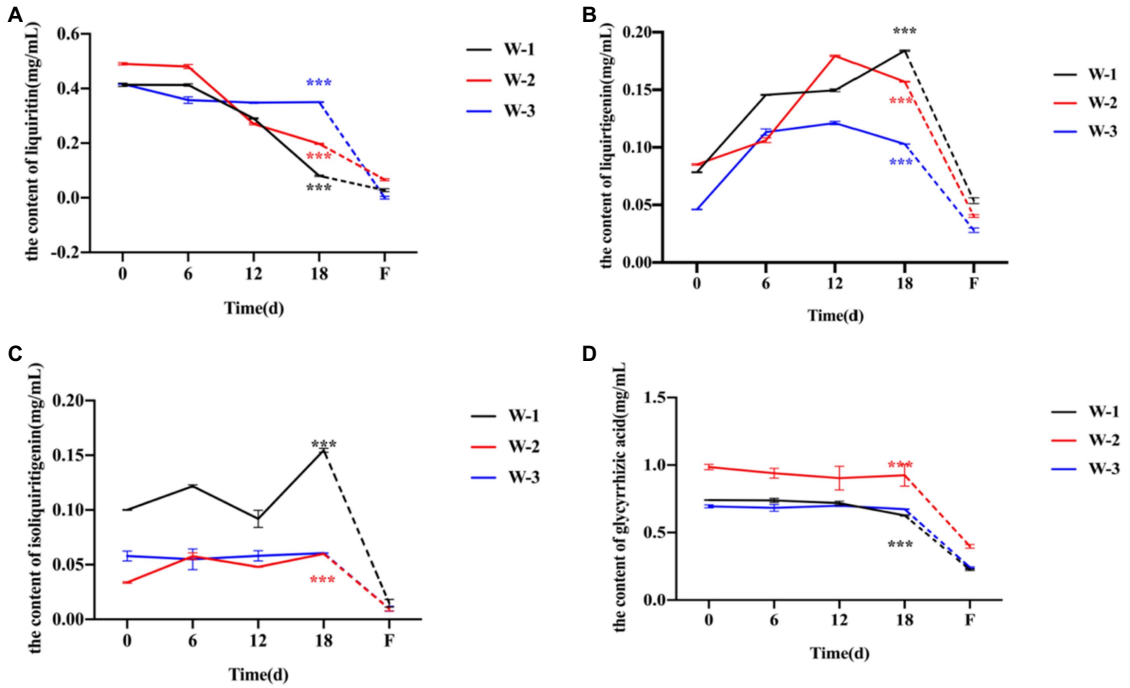


FIGURE 1 | Dynamic changes in the active ingredient content of licorice every 6 days during the natural drying process. **(A)** Dynamic changes in liquiritin content during different drying periods. **(B)** Dynamic changes in liquiritigenin content during different drying periods. **(C)** Dynamic changes in isoliquiritigenin content during different drying periods. **(D)** Dynamic changes in glycyrrhizic acid content during different drying periods. The dotted line indicates that the licorice is moistened again after drying for a long time, and *** of different colors indicates a significant difference between fresh and completely dried components of different groups, $p < 0.01$. Each sample was made by mixing five licorice roots, and the measurement was repeated five times.

in licorice was extremely reduced. Therefore, it was speculated that a large portion of the effective ingredients of licorice will be lost after traditional processing technology (after remoistening and cutting).

Dynamic Changes in Endophytic Microorganisms During Drying

Structural Characteristics of the Endophytic Microorganism Community

To better understand changes in microbial communities during drying, 16S rRNA and ITS gene amplification sequences were used to investigate the microbial community within licorice. The analysis results showed that the sequence length of the endophytic fungi was between 180 and 320 bp (**Figure 2A**), belonging to 128 OTUs (indicating 128 different endophytic fungi), and 66 OTUs were distributed when licorice was fresh, 42 of which were unique. There were 44 OTUs at the beginning of drying, 14 of which were unique. There were 44 OTUs in the mid-drying period, 12 of which were unique. There were 47 OTUs when the licorice was completely dried, 17 of which were unique (**Figure 2B**). Each experimental group had a different proportion of endophytic fungi. The number first declined rapidly and then remained almost unchanged. At the family level of fungi, unclassified fungi (average abundance of 31.07%), Cladosporiaceae (average abundance of 14.37%), Helotiales-fam-incertae-sedis (average abundance of 10.4%) and Aspergillaceae (average abundance of 9.59%) composed the entire fresh licorice bacterial community. However, Nectriaceae emerged as the dominant family, and the abundance increased dramatically in the slow drying (**Figure 2C**). The Shannon index was used to estimate the diversity of the microbial community. The Shannon index was 2.39 when the licorice was fresh, and the diversity of endophytic fungi was the highest at this time. In the subsequent drying process, the diversity of endophytic fungi showed a decreasing trend (**Figure 2D**).

The sequences of endophytic bacteria ranged from 360 to 400 bp (**Figure 2E**), belonging to 350 OTUs. When licorice was fresh, there were 343 OTUs and 315 unique OTUs (**Figure 2F**). There were 18 OTUs and 17 OTUs in the early and mid-drying stages, respectively, and there were no unique OTUs. When licorice was completely dried, there were 30 OTUs and four of which were unique. At the family level (**Figure 2G**), Burkholderiaceae (average abundance of 62.25%) and Nocardiaceae (average abundance of 16.19%) composed the entire fresh licorice bacterial community. In the early stage of drying, Enterobacteriaceae (average abundance of 72.52%) and Erwiniaceae (average abundance of 21.83%) were the main dominant bacteria. After the mid-drying period, Yersiniaceae was the main dominant bacteria, with an average abundance of 90.63%. When the licorice was completely dried, Enterobacteriaceae (average abundance of 67.85%) and Erwiniaceae (average abundance of 29.25%) were the main dominant bacteria. When the licorice was fresh, its Shannon index was the highest, indicating that the microorganisms were

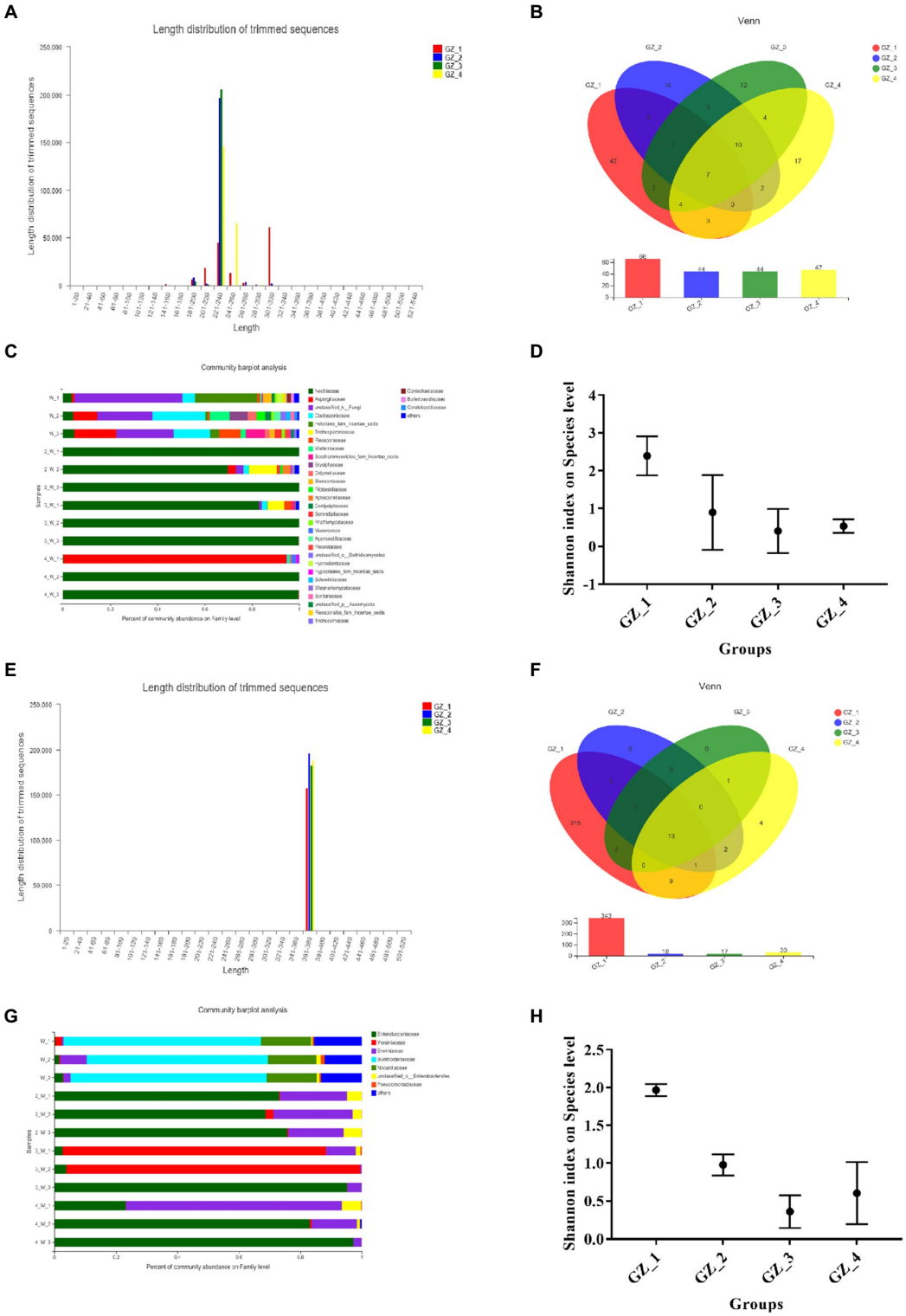


FIGURE 2 | Structural characteristics of the endophytic microbial community of licorice during the natural drying process (GZ_1: fresh licorice; GZ_2: early drying: 6 days after drying; GZ_3: mid-drying: 12 days after drying; and GZ_4: complete drying: 18 days after drying. Each sample was made by mixing five licorice roots). **(A)** The distribution range of the sequence length of endophytic fungi. **(B)** Venn diagram of endophytic fungi in different groups. **(C)** Endophytic fungal community composition. **(D)** Shannon index at the level of endophytic fungal species in different groups. **(E)** The distribution range of the length of the endogenetic bacteria sequencing sequence. **(F)** Venn diagram of endophytic bacteria in different groups. **(G)** Endophytic bacterial community composition. **(H)** Shannon index at the level of different groups of endophytic bacteria.

diverse and abundant at this time (**Figure 2H**). In short, endophytic fungi or bacteria had the highest diversity in fresh licorice.

Analysis of the Microbial Function of Licorice During Slow Drying

Based on ITS and 16S rRNA sequences, PICRUSt2 was used to predict and analyze the function of fungal and bacterial communities in licorice during the drying process. The main types and gene functions of the obtained samples were analyzed to predict their molecular functions to further elucidate the metabolic activity of endophytic bacteria during the drying process (Tian et al., 2021). The MetaCyc pathway results of endophytic fungi showed that the main function of endophytic fungi with the highest abundance was aerobic respiration I and II, palmitate biosynthesis I, glyoxylate cycle, fatty acid and beta, oxidation and GDP-mannose biosynthesis (**Supplementary Material 1**). Among them, glucose and glucose-1-phosphate degradation also had certain functions. Surprisingly, we unearthed the squalene synthase and glucose transferase (**Supplementary Material 2**) that encode the triterpene biosynthetic pathway in the KEGG library of endophytic fungi (Kaewkla and Franco, 2021).

The MetaCyc pathway results of endophytic bacteria showed that the main function with the highest abundance was pyruvate fermentation into isobutanol (engineering), pentose phosphate pathway (nonoxidative branch), fatty acid β-oxidation I, D-fructose ester degradation, glycine biosynthesis (anaerobic), L-alanine biosynthesis super pathway and the fatty acid biosynthesis initiation super pathway (*Escherichia coli*, etc.). COG functional classification (**Figure 3**) showed that in addition to the relatively high abundance of unknown functions, cell cycle control, cell division, chromosome partitioning, energy production and conversion, carbohydrate transport and metabolism and organic ion transport and metabolism were still the most important functions.

Dynamic Changes of Metabolites During Drying

The positive and negative ion patterns of the samples were identified, and the structure of the metabolites in licorice was determined by matching the retention time, molecular mass, secondary fragmentation mass spectrum, collision energy and other information. A total of 328 compounds were identified in positive ion mode, and a total of 288 compounds were identified in negative ion mode. Principal component analysis (PCA) was used to comprehensively analyze the clustering trend. The results showed that the metabolite expression patterns of licorice samples were similar during the drying process, and only fresh licorice metabolites were significantly different from other groups. To verify the reliability of the data, QC samples were selected to verify the chromatography and quality inspection system. The PCA score chart showed that the QC samples were close to the origin of the meta-axis and tightly clustered, which showed the stability and reproducibility of the method (**Figure 4A**). To better reflect the differences in samples during the drying process, a Venn diagram was established for the detected cations. The results showed that the samples contained 14 unique metabolites when fresh. However, licorice contained 4, 5, and 4 unique compounds in the early, middle and complete drying stages, respectively (**Figure 4B**).

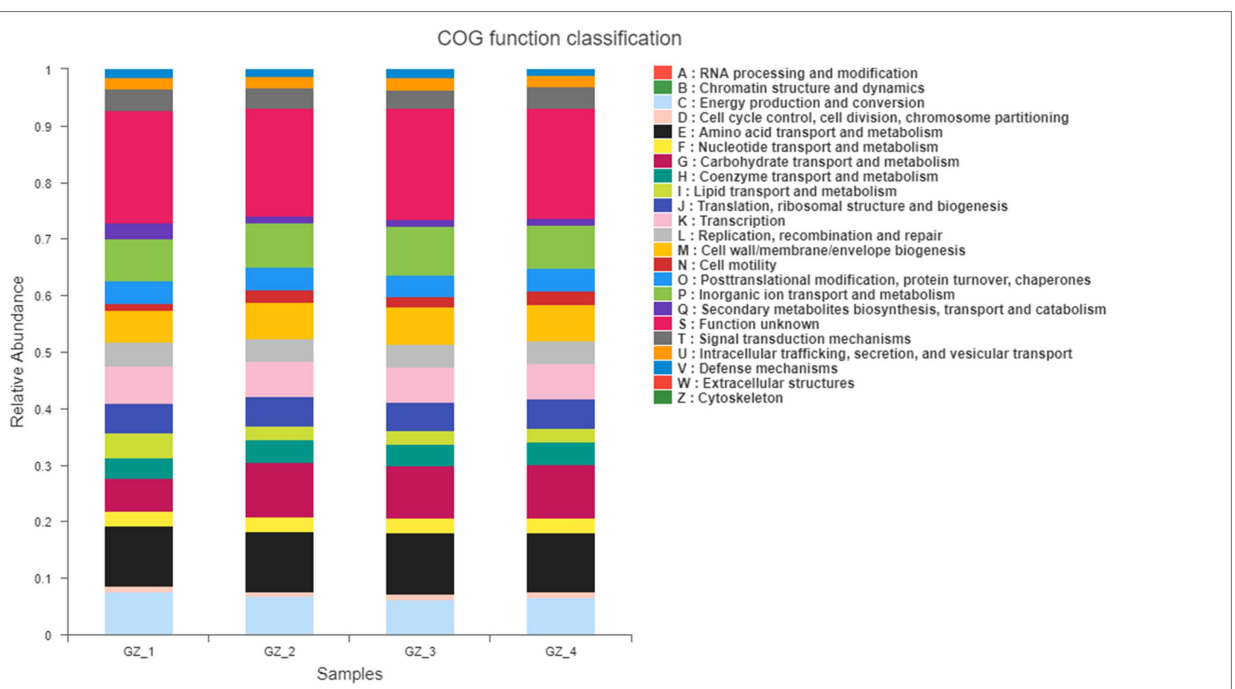


FIGURE 3 | COG function classification statistical histogram (The *x*-axis is different groups. GZ_1: fresh licorice; GZ_2: early drying-6 days after drying; GZ_3: mid-drying-12 days after drying; and GZ_4: completely drying-after 18 days. The *y*-axis is the relative abundance of function).

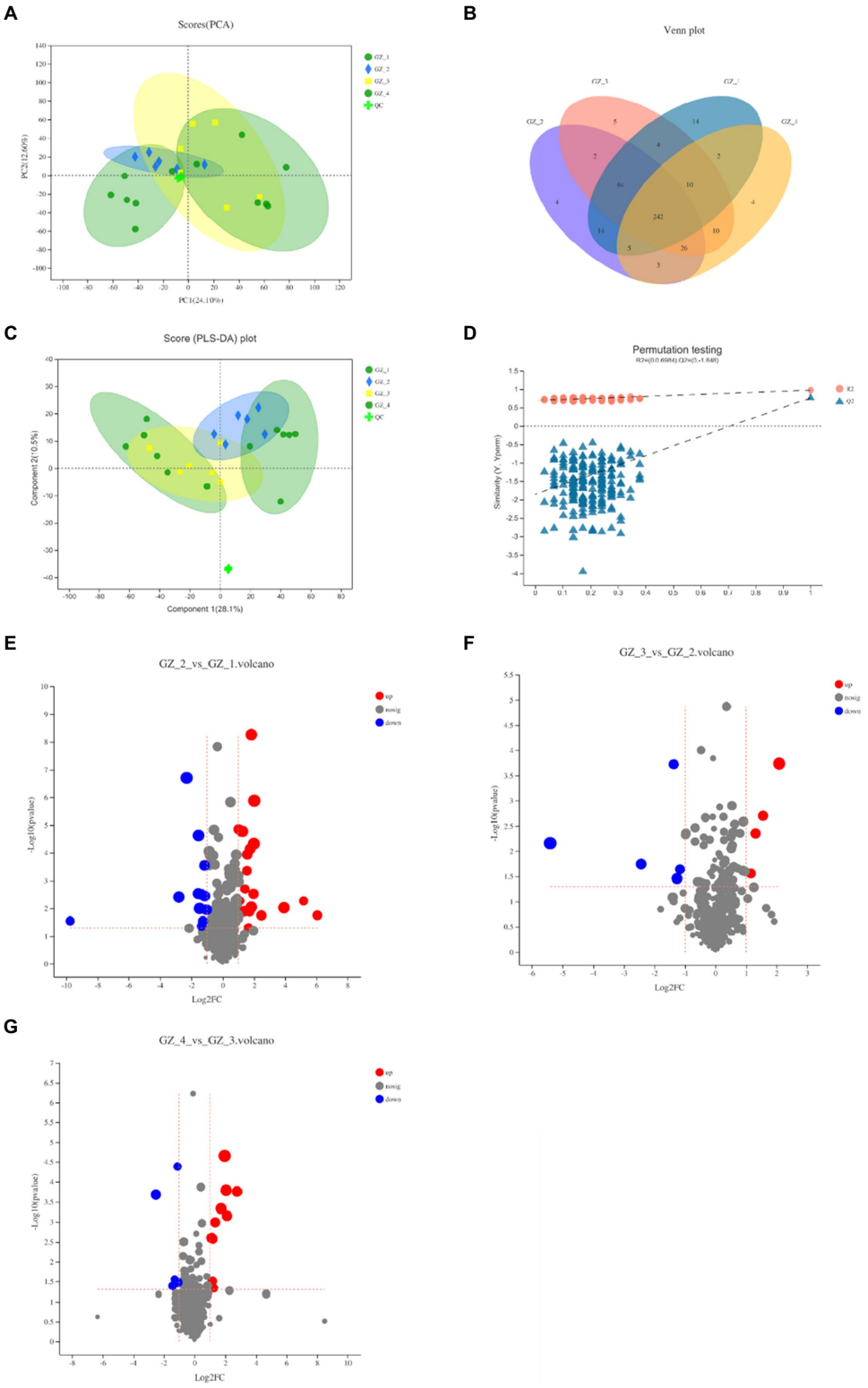


FIGURE 4 | Changes of metabolites of licorice during the four periods of natural drying process (GZ_1: fresh licorice; GZ_2: early drying-6 days after drying; GZ_3: mid-drying-12 days after drying; and GZ_4: completely dried-18 days after drying. Each sample was made by mixing five licorice roots, and the measurement was repeated six times). **(A)** Different periods of cationic PCA scores. **(B)** Venn diagram of metabolites in different periods. **(C)** PLS-DA score chart of different periods. **(D)** PLS-DA model verification. **(E)** Fresh licorice and early drying licorice metabolites' volcano graph. **(F)** Volcano map of different metabolites of licorice in the early and mid-drying period. **(G)** Volcano map of the different metabolites of licorice in the mid-drying period and completely dry.

In addition, the PLS-DA analysis was established, and the cross-validation of the 200 displacement tests showed that the intercepts R2 and Q2 were 0.6984 and −1.848, respectively (**Figure 4D**), indicating that the PLS-DA model was reliable. Fresh licorice still had a good degree of separation from the other groups, and the other groups were relatively close, showing no significant difference (**Figure 4C**).

To further screen the marker compounds related to each group, a volcano map was drawn according to the factor of difference (FC) and value of *p* to analyze the differences between the fresh licorice and the early drying period, the early drying period and the mid-drying period, and the mid-drying period and the complete drying. When fresh licorice was slowly dried for 6 days, a total of 23 compounds were upregulated, among which glycyrrhizin C2 was upregulated, while glycyrrhizin G2, apiosylglucosyl 4-hydroxybenzoate and 12 other compounds were downregulated (**Figure 4E**). In the mid-dry stage, there were a total of nine different compounds, which were significantly reduced compared to the previous stage, with five upregulating compounds and four downregulating compounds in epidermin, diacetone alcohol, and dihydronaringenin-O-sulphate (**Figure 4F**). From the mid-drying stage to the complete drying stage, there were a total of 15 different compounds, of which 10 were upregulated compounds and five were downregulated compounds (**Figure 4G**). The types of compounds changed greatly in the fresh period, and **Figure 4E** clearly shows that the content was relatively high. Related to the endobiotic species we obtained in the previous period, the types of compounds and endophytic microorganisms are the highest in the fresh period of licorice.

Correlation Analysis Between Microorganisms and Host Metabolites During Drying

Correlation analysis was carried out between the proportion of the dominant family of endophytic microorganisms and the differential metabolites of licorice during slow drying. The results showed that the endophytic fungi Cladosporiaceae and Helotiales_fam_Incertae_sedis and the endophytic bacteria Erwiniaceae and Enterobacteriaceae were significantly correlated with the metabolites diacetone alcohol and dihydronaringenin-O-sulphate (**Figure 5**). Yersiniaceae and Nectriaceae had a significant correlation with the metabolite menthoside (isoflavone glycoside).

DISCUSSION

Fresh Processing Can Improve the Content of Active Ingredients in Licorice

The postharvest processing of Chinese medicinal materials is the most critical factor affecting their quality (Liu et al., 2019; Su et al., 2019). The biologically active ingredients of licorice dynamically changed during natural slow drying. We found that fresh licorice had a high content of liquiritin and glycyrrhizic acid, and the content of these components showed a downward trend with slow drying (**Figure 1**). This was consistent with most existing reports that the extension of the drying process time will adversely affect the content of active ingredients (Yabar et al., 2011). Therefore, fresh processing methods after harvesting licorice can reduce the loss of internal components and drying time, thus increasing drying efficiency. In addition, during the determination of the content of active ingredients, it was found that liquiritin, glycyrrhizic acid and other substances in the form of glycosides will slowly lose glucose molecules during the drying process to form aglycones. At present, many medicinal plants are processed when they are harvested fresh (Luz et al., 2009). With the advantage of fresh processing, an increasing number of producers will likely choose this method of fresh processing for medicinal material in the future.

Diversity and Function of the Endophytic Microbial Community

According to previous studies, plants that can survive in extreme environments usually have a unique microbial community. These microbes play a very important role in improving host resistance and productivity (Habibzadeh et al., 2013; Coleman-Derr et al., 2016; Dai et al., 2020). We found that the families Nectriaceae and Enterobacteriaceae in completely dried licorice were the main families that existed after licorice drying, proving that they have a certain "drought resistance" property. These "drought-resistant" microorganisms may have a correlation with the content of active ingredients. For example, the content of isoliquiritigenin in this study had a very significant correlation with the abundance ratio of Cladosporiaceae, Helotiales-fam-incertae-sedis and Enterobacteriaceae (**Supplementary Material 3**). In addition, some studies have reported that the isolation of microorganisms from plants growing in arid environments can promote plant growth and drought resistance (Marasco et al., 2012; Fan et al., 2020; Dubey et al., 2021). It is of great significance to isolate and culture these "drought resistant" microorganisms to determine whether they can play a role in the host's tolerance to natural drought stress and to further clarify whether they have an impact on the metabolic pathway of licorice. Therefore, we will continue to isolate and culture these "drought resistant" microorganisms in the future.

Endophytes may be involved in these pathways and affect the changes in the effective components of the host. For example, it has been reported that endophytic microorganisms are involved in the metabolic pathways of amino acids, causing differences in the metabolic profile of the host (Llorens et al., 2019). Some functional prediction analyses have shown that microorganisms have related enzymes and genes in the synthesis pathway of plant secondary metabolism. It has been speculated that these microorganisms have the ability to synthesize or transform secondary metabolites. In addition, we also found some beneficial enzymes in the prediction of microbial function, such as amylase and chitinase, which play an important role in degrading plant residues and plant polysaccharides (Kaewkla and Franco, 2021). The interaction between microorganisms and plants was also further demonstrated to some extent. PICRUSt2 can enrich our understanding of the diversity of internal microbial functions, as well as the proportion of these functions in the flora, and further provide support for the

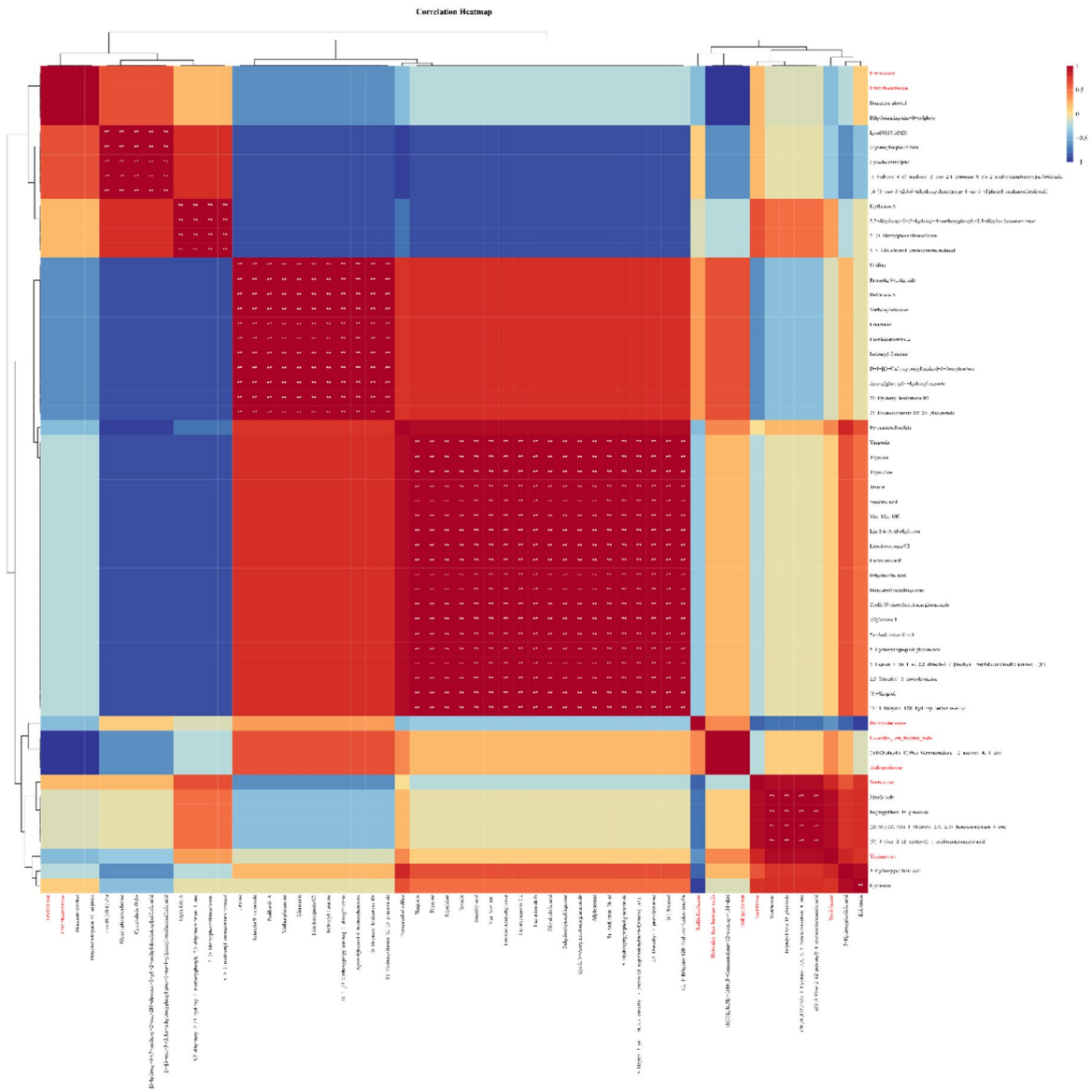


FIGURE 5 | The correlation between the main metabolites and the main endophytic microorganisms of licorice (red font is the family of the microorganism, black font is the identified metabolite, the red square indicates positive correlation, and the blue square indicates negative correlation, ** is $p < 0.01$).

development and utilization of microorganisms. However, in view of the limitations of PICRUSt2, it will be further determined by combining metagenomics and transcriptomics. In addition, these endophytes can be further isolated and cultured to verify their functions.

Endophytic Microorganisms May Cause Differences in Host Metabolites

We simulated the natural drying process of licorice and performed LC–MS metabolome analysis on licorice in the fresh, early and late drying stages. Licorice metabolites were quite different between the drying stages. The variety of different metabolites was the most abundant in the fresh period. At this time, the diversity of endophytic microorganisms in licorice was also the highest. It is speculated that the enzymes of the microorganisms in the fresh period of licorice have better activity and participate more in the metabolic pathway of the host. After drying for a period of time, the enzymes of the microorganisms or the host's enzymes are gradually inactivated, which may be due to changes in various biochemical processes, including the participation of endophytic microorganisms in the formation of host metabolites and the action of enzymes or the activity of the host's own enzymes. For example, it has been reported in the literature that *Salvia miltiorrhiza* has a rich microbial community, which helps the host plant's genome encode metabolic processes, thus affecting the tanshinone synthesis pathway (Chen et al., 2018). This result further shows that endophytic microorganisms can indeed affect the metabolic

pathway of the host, and the specific mechanism by which they interact needs further study. Subsequently, the correlation between the differential metabolites and the main endophytic microorganisms was analyzed, and the results showed that the endophytic microbial community has a certain correlation with the metabolites. Therefore, it can be inferred that host metabolites were also closely related to endophytic microorganisms. This may be caused by changes in host metabolites caused by endophytic microorganism metabolites or by more complex microbial-host interactions. However, how endogenous microorganisms affect host metabolites should continue to be studied further in the future.

CONCLUSION

This study explored the dynamic changes in endophytic microorganisms, the content of active ingredients, and metabolites of licorice during natural drying. During the drying process, the decrease in water content caused the contents of glycyrrhizic acid and liquiritin to decrease, while the liquiritigenin and isoliquiritigenin contents increased. The microbial diversity in the roots of licorice also showed a downward trend of decreasing content. The endophytic fungus Cladosporiaceae and endophytic bacteria Burkholderiaceae were the dominant family when fresh, but Nectriaceae and Enterobacteriaceae were the dominant families after drying. In addition, the metabolomics results showed that the differential metabolites of licorice were reduced during the natural drying process. Correlation analysis between dominant families and differential metabolites showed that there was a certain correlation between the two. Based on these results, we suggest that fresh processing is better after harvest.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA748524.

AUTHOR CONTRIBUTIONS

TL, DJ, and CL designed the study and prepared the manuscript. GR participated in the experiments and data analysis. TL conducted the experiments and data analysis. DJ and CL revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work gratefully acknowledges the financial support provided by the National Natural Science Foundation of China (81773838 and 81703645), 2019–2020 National Medical Products Administration Project Funding Project, and the Fundamental Research Funds for the Central Universities (Beijing University of Chinese Medicine, no. 2020-JYB-ZDGG-037).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.740721/full#supplementary-material

REFERENCES

Balbay, A., and Sahin, O. (2012). Microwave drying kinetics of a thin-layer liquorice root. *Dry. Technol.* 30, 859–864. doi: 10.1080/07373937.2012.670682

Chen, J., Li, L. F., Hu, X. R., Wei, F., and Ma, S. C. (2021). Network pharmacology-based strategy for elucidating the molecular basis Forthe pharmacologic effects of Licorice (Glycyrrhiza spp.). *Front. Pharmacol.* 12:590477. doi: 10.3389/fphar.2021.590477

Chen, H. M., Wu, H. X., Yan, B., Zhao, H. G., Liu, F. H., Zhang, H. H., et al. (2018). Core microbiome of medicinal plant *Salvia miltiorrhiza* seed: a rich reservoir of beneficial microbes for secondary metabolism? *Int. J. Mol. Sci.* 19:672. doi: 10.3390/ijms19030672

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., et al. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native agave species. *New Phytol.* 209, 798–811. doi: 10.1111/nph.13697

Dai, Y., Li, X. Y., Wang, Y., Li, C. X., He, Y., Lin, H. H., et al. (2020). The differences and overlaps in the seed-resident microbiome of four leguminous and three Gramineous forages. *Microb. Biotechnol.* 13, 1461–1476. doi: 10.1111/1751-7915.13618

Dubey, A., Saiyam, D., Kumar, A., Hashem, A., Abd-Allah, E. F., and Khan, M. L. (2021). Bacterial root Endophytes: characterization of their competence and plant growth promotion in soybean (Glycine max (L.) Merr.) under drought stress. *Int. J. Environ. Res. Public Health* 18:931. doi: 10.3390/ijerph18030931

Fan, D., Subramanian, S., and Smith, D. L. (2020). Plant endophytes promote growth and alleviate salt stress in *Arabidopsis thaliana*. *Sci. Rep.* 10:12740. doi: 10.1038/s41598-020-69713-5

Habibzadeh, Y., Pirzad, A., Zardashti, M. R., Jalilian, J., and Eini, O. (2013). Effects of Arbuscular Mycorrhizal fungi on seed and protein yield under water-deficit stress in Mung bean. *Agron. J.* 105, 79–84. doi: 10.2134/agronj2012.0069

Hayashi, H., and Sudo, H. (2009). Economic importance of licorice. *Plant Biotechnol.* 26, 101–104. doi: 10.5511/plantbiotechnology.26.101

Huang, Q., Jiao, F., Huang, Y. M., Li, N., Wang, B. R., Gao, H., et al. (2021). Response of soil fungal community composition and functions on the alteration of precipitation in the grassland of loess plateau. *Sci. Total Environ.* 751:142273. doi: 10.1016/j.scitotenv.2020.142273

Icier, F., Ozmen, D., Cevik, M., and Cokgezme, O. F. (2021). Drying of licorice root by novel radiative methods. *J. Food Process. Preserv.* 45:13. doi: 10.1111/jfpp.15214

Jiang, L., Akram, W., Luo, B., Hu, S., Faruque, M. O., Ahmad, S., et al. (2021). Metabolomic and pharmacologic insights of aerial and underground parts of *Glycyrrhiza uralensis* Fisch. ex DC. for maximum utilization of medicinal resources. *Front. Pharmacol.* 12:658670. doi: 10.3389/fphar.2021.658670

Kaewkla, O., and Franco, C. M. M. (2021). Genome mining and description of Streptomyces albidus sp. nov., an endophytic actinobacterium with antibacterial potential. *Antonie Van Leeuwenhoek* 114, 539–551. doi: 10.1007/s10482-021-01539-1

Li, H. H., Feng, T., Wen, Y. L., Li, L., Liu, Y. N., Ren, X. L., et al. (2021). Comparative investigation for raw and processed products of euodiae fructus based on high-performance liquid chromatography fingerprints and chemical pattern recognition. *Chem. Biodivers.* 18:e2100281. doi: 10.1002/cbdv.202100281

Li, Y. H., Li, Y. N., Li, H. T., Qi, Y. R., Wu, Z. F., and Yang, M. (2017). Comparative study of microwave-vacuum and vacuum drying on the physicochemical properties and antioxidant capacity of licorice extract powder. *Powder Technol.* 320, 540–545. doi: 10.1016/j.powtec.2017.07.076

Liu, Y., Chen, J.-F., Xu, N., Lin, W.-G., Liu, Y.-M., Chen, M.-L., et al. (2019). Effects of fresh-cut on drying rate and quality of Panax notoginseng. *Zhongguo Zhong Yao Za Zhi* 44, 1381–1391. doi: 10.19540/j.cnki.cjcmm.20181221.002

Liu, Y., and Wei, X. L. (2021). Dark septate endophyte improves the drought-stress resistance of Ormosia hosiei seedlings by altering leaf morphology and photosynthetic characteristics. *Plant Ecol.* 222, 761–771. doi: 10.1007/s11258-021-01135-3

Llorens, E., Sharon, O., Camanes, G., Garcia-Agustin, P., and Sharon, A. (2019). Endophytes from wild cereals protect wheat plants from drought by alteration of physiological responses of the plants to water stress. *Environ. Microbiol.* 21, 3299–3312. doi: 10.1111/1462-2920.14530

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

Luz, J. M. Q., Ehlert, P. A. D., and Innecco, R. (2009). Harvest moment and drying period of *Ocimum gratissimum* L. *Hortic. Bras.* 27, 539–542. doi: 10.1590/S0102-05362009000400022

Ma, Y., Yao, Y., Wang, Q., Gu, Z., Wang, P., Han, Y., et al. (2021). Mechanism of nitric oxide enhancing NaCl tolerance of barley seedlings based on physiol-biochemical analysis and LC-MS metabolomics. *Environ. Exp. Bot.* 189:104533. doi: 10.1016/j.envexpbot.2021.104533

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., et al. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. *PLoS One* 7:e48479. doi: 10.1371/journal.pone.0048479

Santos, S. S., Rask, K. A., Vestergard, M., Johansen, J. L., Prieme, A., Froslev, T. G., et al. (2021). Specialized microbiomes facilitate natural rhizosphere microbiome interactions counteracting high salinity stress in plants. *Environ. Exp. Bot.* 186:104430. doi: 10.1016/j.envexpbot.2021.104430

Shakeri, A., Masullo, M., D'Urso, G., Iranshahi, M., Montoro, P., Pizza, C., et al. (2018). In depth chemical investigation of Glycyrrhiza triphylla Fisch roots guided by a preliminary HPLC-ESIMSn profiling. *Food Chem.* 248, 128–136. doi: 10.1016/j.foodchem.2017.12.031

Singh, S., Pandey, S. S., Tiwari, R., Pandey, A., Shanker, K., and Kalra, A. (2021). Endophytic consortium with growth-promoting and alkaloid enhancing capabilities enhance key terpenoid indole alkaloids of Catharanthus roseus in the winter and summer seasons. *Ind. Crop. Prod.* 166:113437. doi: 10.1016/j.indcrop.2021.113437

Su, X. Y., Wu, Y. J., Li, Y., Huang, Y. F., Liu, Y., Luo, P., et al. (2019). Effect of different post-harvest processing methods on the chemical constituents of Notopterygium franchetii by an UHPLC-QTOF-MS-MS metabolomics approach. *Molecules* 24:3188. doi: 10.3390/molecules24173188

Tian, G. P., Shen, Y., Hu, X. R., Zhang, T., Zhang, L. M., and Bian, B. (2021). The change of water content and role of microbe in the sludge drying process. *J. Environ. Manag.* 286:112254. doi: 10.1016/j.jenvman.2021.112254

van de Sand, L., Bormann, M., Alt, M., Schipper, L., Heilingloh, C. S., Steinmann, E., et al. (2021). Glycyrrhizin effectively inhibits SARS-CoV-2 replication by inhibiting the viral Main protease. *Viruses* 13:609. doi: 10.3390/v13040609

Wu, W., Chen, W. H., Liu, S. Y., Wu, J. J., Zhu, Y. T., Qin, L. P., et al. (2021). Beneficial relationships Between Endophytic bacteria and medicinal plants. *Front. Plant Sci.* 12:646146. doi: 10.3389/fpls.2021.646146

Xie, Z. C., Chu, Y. K., Zhang, W. J., Lang, D. Y., and Zhang, X. H. (2019). *Bacillus pumilus* alleviates drought stress and increases metabolite accumulation in *Glycyrrhiza uralensis* Fisch. *Environ. Exp. Bot.* 158, 99–106. doi: 10.1016/j.envexpbot.2018.11.021

Yabar, E., Pedreschi, R., Chirinos, R., and Campos, D. (2011). Glucosinolate content and myrosinase activity evolution in three maca (Lepidium meyenii Walp.) ecotypes during preharvest, harvest and postharvest drying. *Food Chem.* 127, 1576–1583. doi: 10.1016/j.foodchem.2011.02.021

Yin, Y., Li, Y. P., Jiang, D., Zhang, X. N., Gao, W., and Liu, C. S. (2020). De novo biosynthesis of liquiritin in Saccharomyces cerevisiae. *Acta Pharm. Sin. B* 10, 711–721. doi: 10.1016/j.apsb.2019.07.005

Zhou, J. Y., Liu, Z. L., Wang, S. F., Li, J., Li, Y. K., Chen, W. K., et al. (2020). Fungal endophytes promote the accumulation of Amaryllidaceae alkaloids in Lycoris radiata. *Environ. Microbiol.* 22, 1421–1434. doi: 10.1111/1462-2920.14958

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.

frontiers in Microbiology

REVIEW
published: 04 January 2022
doi: 10.3389/fmicb.2021.782135

Significance of the Diversification of Wheat Species for the Assembly and Functioning of the Root-Associated Microbiome

*Cécile Gruet, Daniel Muller and Yvan Moënne-Loccoz**

Univ Lyon, Université Claude Bernard Lyon 1, Centre National de la Recherche Scientifique (CNRS), Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), VetAgro Sup, UMR 5557 Ecologie Microbienne, Villeurbanne, France

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Gilles Bena, Institut de Recherche Pour le Développement (IRD), France
Marco Nuti, Sant'Anna School of Advanced Studies, Italy

***Correspondence:**
Yvan Moënne-Loccoz
yvan.moenne-loccoz@univ-lyon1.fr

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *23 September 2021*
Accepted: *30 November 2021*
Published: *04 January 2022*

Citation:
Gruet C, Muller D and Moënne-Loccoz Y (2022) Significance of the Diversification of Wheat Species for the Assembly and Functioning of the Root-Associated Microbiome. Front. Microbiol. 12:782135. doi: 10.3389/fmicb.2021.782135

Wheat, one of the major crops in the world, has had a complex history that includes genomic hybridizations between *Triticum* and *Aegilops* species and several domestication events, which resulted in various wild and domesticated species (especially *Triticum aestivum* and *Triticum durum*), many of them still existing today. The large body of information available on wheat-microbe interactions, however, was mostly obtained without considering the importance of wheat evolutionary history and its consequences for wheat microbial ecology. This review addresses our current understanding of the microbiome of wheat root and rhizosphere in light of the information available on pre- and post-domestication wheat history, including differences between wild and domesticated wheats, ancient and modern types of cultivars as well as individual cultivars within a given wheat species. This analysis highlighted two major trends. First, most data deal with the taxonomic diversity rather than the microbial functioning of root-associated wheat microbiota, with so far a bias toward bacteria and mycorrhizal fungi that will progressively attenuate thanks to the inclusion of markers encompassing other micro-eukaryotes and archaea. Second, the comparison of wheat genotypes has mostly focused on the comparison of *T. aestivum* cultivars, sometimes with little consideration for their particular genetic and physiological traits. It is expected that the development of current sequencing technologies will enable to revisit the diversity of the wheat microbiome. This will provide a renewed opportunity to better understand the significance of wheat evolutionary history, and also to obtain the baseline information needed to develop microbiome-based breeding strategies for sustainable wheat farming.

Keywords: wheat, domestication, rhizosphere, root microbiome, microbial interactions, symbiosis

Abbreviations: BP, Before Present; DAPG, 2,4-Diacetylphloroglucinol; AM, Arbuscular Mycorrhizal; ISR, Induced Systemic Resistance; MIR, Mycorrhiza-Induced Resistance; SAR, Systemic Acquired Resistance; QTL, Quantitative Trait Loci; IAA, Indole-3-Acetic Acid; ACC, 1-Aminocyclopropane-1-Carboxylate; PSB, Phosphate-Solubilizing Bacteria.

INTRODUCTION

Plants interact with a myriad of microorganisms, and plant-microbe interactions are now considered a key facet of plant evolution, adaptation and ecology (Simon et al., 2019), both for wild and domesticated plants (Hassani et al., 2018). Hence, the plant needs to be seen as a holobiont (i.e., macro-organism and its associated microbiota), which requires a more integrated perspective on the significance of their microbial partners and the extended plant phenotypes they confer (Haichar et al., 2008; Vandenkoornhuyse et al., 2015).

The vast majority of plant microorganisms are in interaction with roots (Moënne-Loccoz et al., 2015). There are three distinct root-associated compartments for microorganisms, which are (i) the root endosphere (i.e., root internal tissues), (ii) the rhizoplane (i.e., the interface between the root surface and soil), and (iii) the rhizosphere (i.e., soil in the immediate vicinity of the root) (**Figure 1A**). Endophytic microorganisms inhabit the endosphere, where probably they have direct access to certain plant metabolites (Reinhold-Hurek and Hurek, 2011). They are often transmitted horizontally (Edwards et al., 2015), but some of them may be transmitted vertically (Liu et al., 2012; Hodgson et al., 2014; Truyens et al., 2015). Many of them if not most are thought to benefit their plant host (Schulz and Boyle, 2006; Reinhold-Hurek et al., 2015). In the rhizosphere, where soil is under the direct influence of the root (Hiltner, 1904), microorganisms from the surrounding soil are attracted by and benefit from rhizodeposits including root exudates (Zhalnina et al., 2018), leading to microbial proliferation and enhanced activity, i.e., the rhizosphere effect (Buée et al., 2009). Plant genotype influences the rhizosphere microbiota (Badri and Vivanco, 2009; Berg and Smalla, 2009; Micallef et al., 2009; Bouffaud et al., 2014), because different plant genotypes display different root properties and lead to different rhizosphere conditions for microbial partners. In turn, rhizosphere microorganisms can be either beneficial, pathogenic or have no effect on the plant (Vacheron et al., 2013; Nowell et al., 2016; Parnell et al., 2016). These plant-microbe interactions are essential for the ecological functioning of soil ecosystems (Lu et al., 2018).

Wheat, of the *Poaceae* family, is one of the major crops in the world with rice and maize. The crop provides 20% of calories in the human diet (Gill et al., 2004). Durum wheat is of significance as a food crop to make for example pasta, couscous, burghul, and bread wheat is used to prepare bread, pastries, etc. The Food and Agricultural Organization of the United Nations predicts a production of 776 million tons of wheat in 2022, an increase of 118 million compared to 2012. Demand for wheat is increasing with the change of diet in several large countries, such as China or India (Brisson et al., 2010). This increase in production needs to be achieved despite the growing number of challenges facing the crop, including climatic change, diminishing water resources, restrictions in the use of fertilizers and pesticides, and the risk caused by new and more aggressive pests (Tian et al., 2021). Intensive cereal systems for increasing yields are environmentally deleterious in the long-term (Lobell et al., 2009), and developing sustainable crops based on ecological intensification is essential. Exploiting the potential of wheat interactions with soil microorganisms that can enhance plant productivity, by contributing to plant nutrition and health (Bhattacharyya and Jha, 2012; Vacheron et al., 2013) is a promising strategy to reach this goal. This will require a better, more comprehensive understanding of the microbial community associated with wheat, and to identify new avenues to exploit them for sustainable wheat farming.

Recent methodology improvements, especially in sequencing technologies, have enabled to revisit our knowledge of the interactions between wheat and root-associated microbial community. For instance, the wheat microbiome has been recently described, with a focus on environmental factors driving microbiome assembly and identifying beneficial microorganisms important for sustainable wheat farming (Kavamura et al., 2021). This review aims at putting into perspective the growing knowledge on wheat-microbe interactions, by considering the evolutionary history of wheats and then its implications for the wheat microbiome. The particular patterns of microbial selection in the different root compartments (rhizosphere, rhizoplane, and endosphere) are described, ranging from bacteria and archaea to fungi and other microeukaryotes. Finally, we focus on the functional diversity of the wheat root microbiome and its implication for wheat growth and health.

WHEAT PARTICULARITIES OF RELEVANCE FOR PLANT-MICROBE INTERACTIONS

Hybridization, Polyploidy, and Domestication History

The *Triticum* and *Aegilops* ancestors of bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) underwent hybridization, as well as polyploidization events (Haberer et al., 2016) involving genomes A, S, B and D (**Figure 2A**). The A and S genomes arose by divergence from a common ancestor circa 7 million years Before Present (BP) (Pont et al., 2019). D genome might have originated from homoploid hybrid speciation of A and S genomes, 5–6 million years BP (Glémin et al., 2019). Two wild diploid wheats ($2n = 14$), i.e., *Triticum urartu* (AA genome) and a close descendant of *Aegilops speltoides* (BB genome) (Pont et al., 2019), hybridized about 500,000 years BP and gave a tetraploid wild wheat ($2n = 28$) termed *Triticum dicoccoides* (wild emmer wheat; AABB genome) (Pont et al., 2019). A second hybridization took place about 10,000 years BP, between domesticated emmer and a direct ascendant of the current diploid species *Aegilops tauschii* (DD genome), giving rise to a wild hexaploid wheat ($2n = 42$; AABBDD genome) at the origin of domesticated *T. aestivum*. Hexaploid wheat might have arisen from more than one crossing event (Dvorak et al., 1998). In both hybridization events, the seven chromosomes of each genome (A, B, or D) could not pair for subsequent mitosis, which resulted in chromosome doubling and thus allopolyploidy (Glover, 2016). On one hand, hybridization can lead to a loss

of genetic diversity, since only a limited number of individuals of each species is involved in the crossing. On the other hand, polyploidy may lead to particular gene expression patterns, and probably also to particular properties in terms of root exudation, root uptake, etc. (Saia et al., 2019; Iannucci et al., 2021), which can be expected to impact on microorganisms.

Wheat has undergone several domestication events. The wild einkorn *Triticum monococcum* subsp. *beoticum* (genomically close to *T. urartu*; Fricano et al., 2014) was domesticated and gave *T. monococcum* subsp. *monococcum,* a crop seldom cultivated nowadays (Salamini et al., 2002) (and therefore is not portrayed in **Figure 2A**). The wild emmer wheat *T. dicoccoides* (AABB genome) gave rise to the domesticated emmer wheat *T. dicoccon* (perhaps on several independent occasions (Özkan et al., 2011)), which later evolved into durum wheat *T. durum* (**Figure 2A**). The cross between wild emmer and the ascendant of *A. tauschii* (DD genome) either (i) resulted in an unknown hexaploid wild wheat, from which derived the domesticated wheat *T. aestivum* (Salamini et al., 2002), or (ii) was concomitant with the domestication event itself. Agronomic traits of wheat changed gradually upon domestication. As for other *Poaceae*, domesticated wheat presents bigger grains and higher seed number per spike (Salamini et al., 2002). Wheat domestication resulted also in lower root biomass (Waines and Ehdaie, 2007), with more fine roots and a shallower root system (Roucou et al., 2018), and with more seminal roots (Golan et al., 2018), but these traits display heterogeneity at inter and infra-species levels. Domestication represents a genetic bottleneck, with an estimated 50–60% reduction of wheat genetic diversity (Bonnin et al., 2014).

Wheat Geography

Nowadays, wild wheat habitats are still located in the area that was 10,000 years ago the Fertile Crescent (**Supplementary Figure 1**), and where *T. urartu*, *T. beoticum*, *T. dicoccoides*, *A. speltoides*, and *A. tauschii* can be found. Beyond the Fertile Crescent, wild wheats grow mainly in temperate climates between latitudes 30°N and 40°N, but they may occur also within the Arctic Circle and to higher elevations near the equator (Haas et al., 2019). Therefore, wild wheats grow under a wide range of pedoclimatic conditions, which means they may encounter different types of soil microbial communities.

Whereas wild wheats are mostly winter type, domesticated wheat can be either winter or spring type (i.e., does not need vernalization), which enables to find domesticated wheat in a larger range of climatic zones (and soil conditions). In comparison with spring wheat, winter wheat is sown in the autumn, which means the root system develops and interact with soil microorganisms over a much longer duration in the year. Domesticated wheats (both durum and bread wheats) are found in a broad range of areas and climates, and are present on all continents (Di Paola et al., 2018; Mideksa et al., 2018; Dong et al., 2019; Tidiane Sall et al., 2019). They are therefore likely to be included in a diversity of cropping systems and farming practices (i.e., regarding tillage, fertilizers, etc.) in contrasted conditions of soil and climate, which means exposure to very different types of soil microbial communities (Chaudhary et al., 2017; Dong et al., 2017; Gajda et al., 2017; Somenahally et al., 2018; Wang et al., 2018).

Selection and Modern Breeding

Growth of domesticated wheats in diverse environments and climatic conditions required local adaptations (Dwivedi et al., 2016). Farmers, through mass selection, led to the creation of particular, still genetically heterogeneous (Bonjean, 2001) wheat genotypes (called landraces) well-adapted to local environments (Kiszonas and Morris, 2018) and to specific stresses (Feldman and Kislev, 2007). Thus, the growth of landraces results in stands consisting of mixtures of many different closely related genotypes. Considering features important for plant-microbe interactions, this means an expected heterogeneity in terms of root system traits, plant physiology, rhizodeposition patterns and rhizosphere chemistry within a given plot.

Modern breeding aimed at higher yield. Genealogical selection (Gayon and Zallen, 1998) resulted into (i) limited plant-to-plant genetic heterogeneity within these cultivars, (ii) preferential allocation of N and C compounds to shoots rather than roots, probably leading to reduced rhizodeposition for microorganisms (Lindig-Cisneros et al., 1997), (iii) enhanced mineral uptake (Zhang et al., 2020; Cantarel et al., 2021), and (iv) particularities in root functioning and rhizosphere chemistry (George et al., 2014).

During the Green Revolution (from 1950 to late 1960s), crosses with semi-dwarf varieties were implemented (Brancourt-Hulmel et al., 2003). Hybrids were produced (Šramková et al., 2009). Chromosome engineering methodologies have been employed to transfer specific disease genes from other members of the tribe Triticeae into wheat, conferring new immune system defenses against phytopathogens (Rong et al., 2000; Niu et al., 2011). More recently, molecular markers and quantitative trait loci (QTLs) (Peng et al., 2003; Pestsova et al., 2005; Peleg et al., 2011) have been used successfully to facilitate breeding, whereas CRISPR-Cas9 (Kiszonas and Morris, 2018) and genome sequencing (Trebbi et al., 2011; Jia et al., 2013; Ling et al., 2013; Maccaferri et al., 2014; Soriano et al., 2016; International Wheat Genome Sequencing Consortium [IWGSC], Appels et al., 2018) open new perspectives, with potentially an impact on wheat-microbe interactions.

TAXONOMIC DIVERSITY OF MICROORGANISMS IN THE RHIZOSPHERE AND ROOTS OF WHEAT

Importance and Analysis of Root-Associated Wheat Microbiome

Soil type (Donn et al., 2015; Simonin et al., 2020) as well as cultivation history (Hilton et al., 2018) and practices (e.g., tillage, soil amendments) are the main factors shaping wheat root microbiota (Ahlawat et al., 2018; Kavamura et al., 2018). The second most important factor is the wheat genotype, both at the species and intra-species (varieties) levels (Mahoney et al., 2017; Stromberger et al., 2017; Ellouze et al., 2018; Naz et al., 2018;

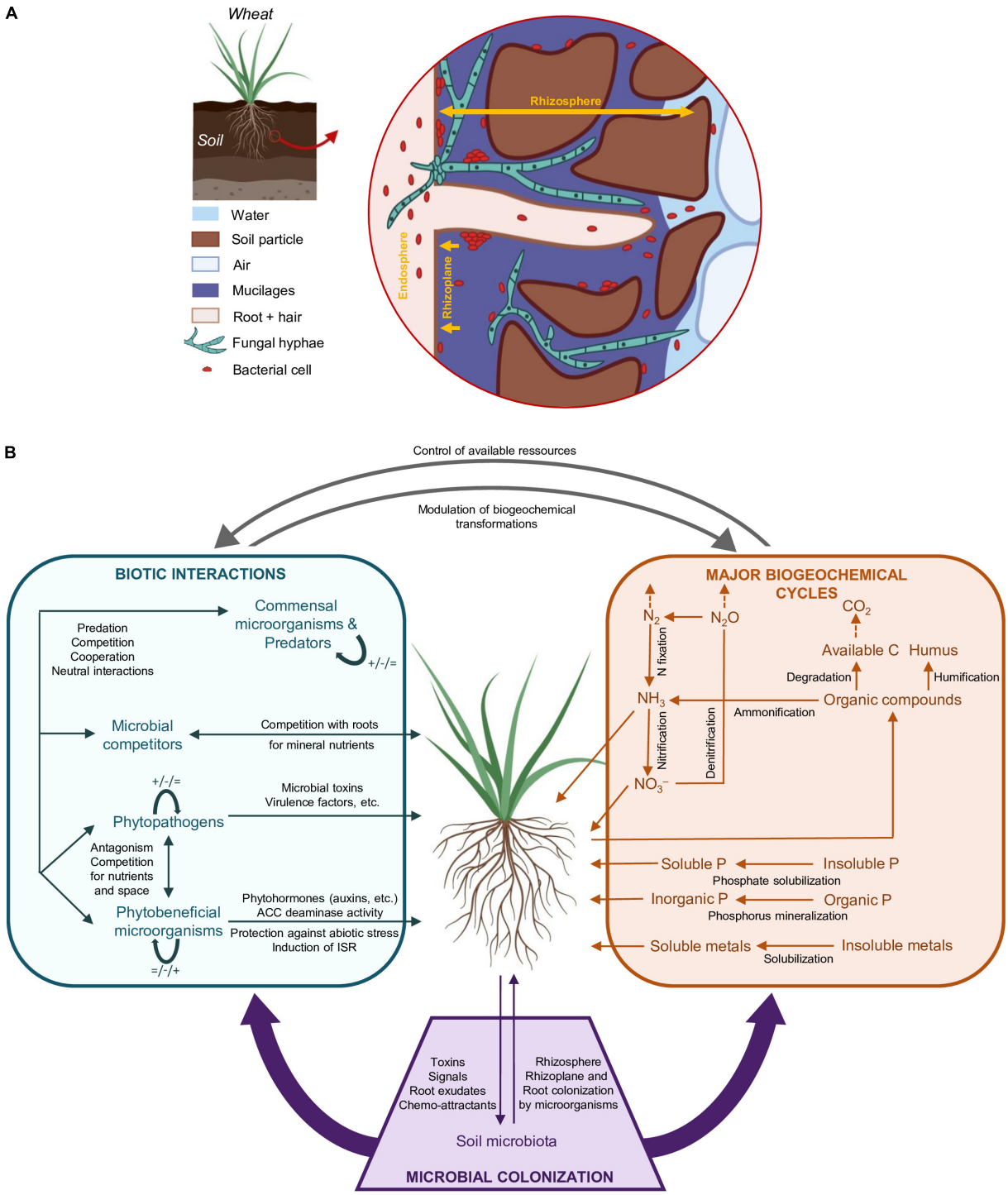


FIGURE 1 | Relationship between wheat roots and soil/microbial components. **(A)** Structure of the rhizosphere, rhizoplane, and endosphere (not to scale). The rhizosphere is the soil in the immediate vicinity of the root, where the root has a major direct impact on soil organization and microbial functioning. The rhizoplane is the interface between the root surface and the soil. The endosphere corresponds to root internal tissues. Adapted from York et al. (2016) and Ding et al. (2019). **(B)** Major root-level microbial contributions to biotic interactions and biogeochemical cycles linked to plant growth and health. Root colonization by microorganisms is mediated by plant signals and exudates, which attract or repel soil microorganisms. Biotic interactions in the rhizosphere include plant-microorganism interactions and microorganism/microorganism interactions, with beneficial (+), deleterious (-) or neutral effects (=). Major microbial transformations are indicated for C, N, and P biogeochemical cycles. Metal biotransformations are not reviewed. A particular microbial taxon may be involved in several different biotic interactions (left box) and biotransformations (right box). ISR, Induced Systemic Resistance; ACC, 1-AminoCyclopropane-1-Carboxylate. Dashed arrows are used for abiotic volatilization phenomena.

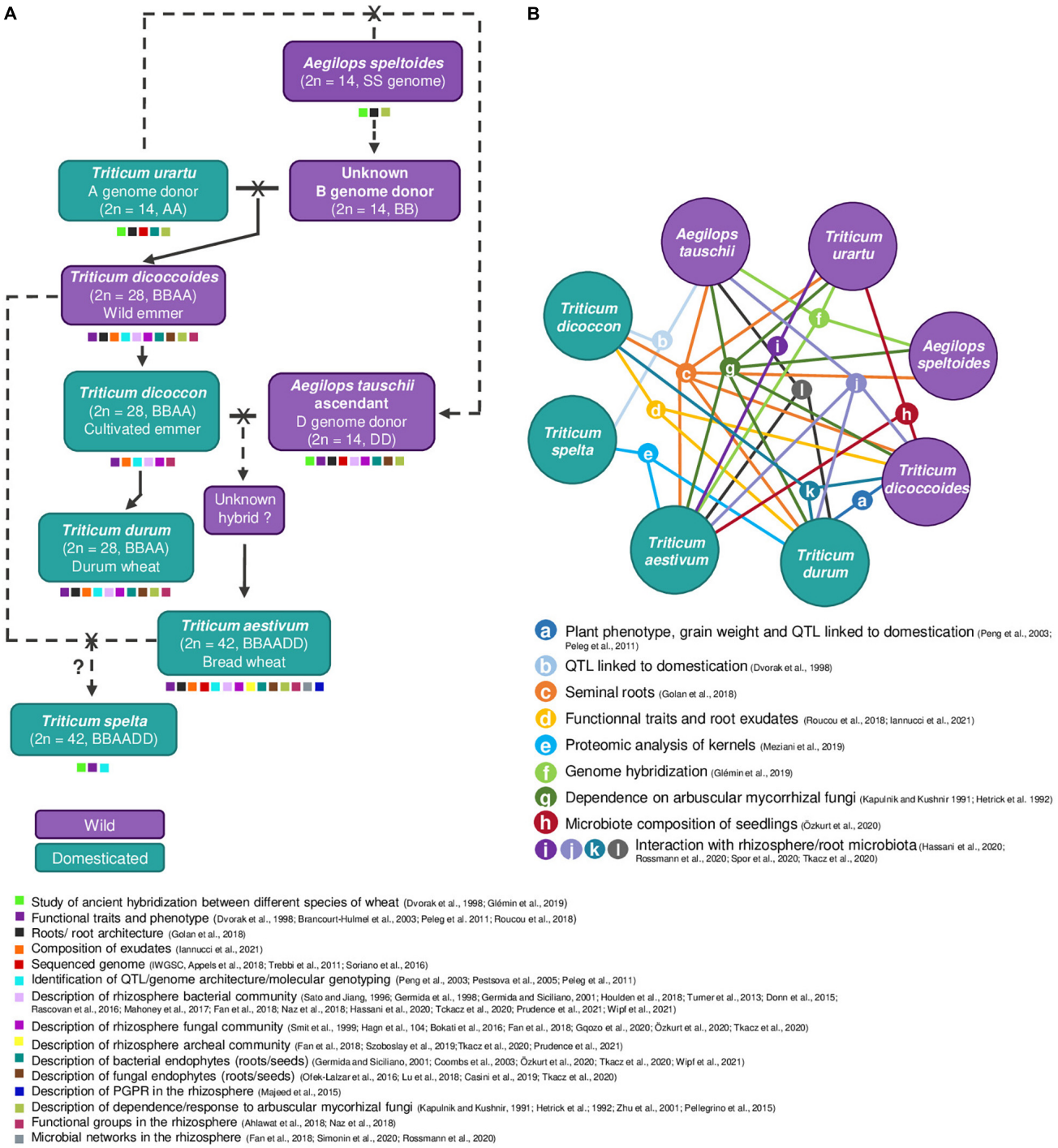


FIGURE 2 | The origin of durum and bread wheat, and literature comparisons. Wild wheats are represented in purple, while domesticated wheats are in turquoise. **(A)** Wild and domesticated species involved in wheat evolution and leading to pasta (*T. durum*) and bread (*T. aestivum*) wheats are indicated (adapted from Mujeeb-Kazi, 2006), as well as examples of key scientific issues investigated with them (shown with small squares with the color code indicated below the panel). The ancestors of pasta and bread wheats underwent hybridization and polyploidization events involving genomes A, S, B, and D. A simplified version of wheat evolutionary history is depicted. The A and S genomes arose by divergence from a common ancestor (not shown) circa 7 million years Before Present (BP) (Pont et al., 2019). The B genome probably descends from the S genome and is therefore a close relative of *Aegilops speltoides* (SS) (Fricano et al., 2014). A first hybridization event is speculated to have taken place between A (*T. urartu*) and S (*A. speltoides/A. mutica*) genomes, 5–6 million years BP (Glémin et al., 2019), leading to the D genome upon homoploid hybrid speciation. A second hybridization event took place about 500,000 years BP between this B genome donor

(Continued)

FIGURE 2 | and *T. urartu* (A genome), leading to the wild tetraploid *T. dicoccoides*, and later to the domesticated emmer *T. dicoccon*. A third hybridization event (10,000 years BP) involved *T. dicoccon* and an ascendant of current *A. tauschii* (D genome), leading to the hexaploid wheat *T. aestivum*. It is unclear whether the latter hybridization and domestication events took place at the same time or not, and the wild form of the hexaploid hybrid remains unknown. A fourth cross, between *T. aestivum* and *T. dicoccon*, is probably at the origin of the hexaploid wheat *Triticum spelta* (Fricano et al., 2014). Wheat genomes are composed of 14 (AA, BB or DD), 28 (AABB), or 42 chromosomes (AABBDD). Dashed arrows are used for uncertain events. In the history of Triticeae, other domestication events also occurred but without leading to species extensively cultivated nowadays, as for example the wild einkorn *Triticum monococcum* subsp. *beoticum* (A genome, genomically close to but not interfertile with *T. urartu*; Fricano et al., 2014) was domesticated to become *Triticum monococcum* subsp. *monococcum* (not shown). **(B)** Key literature comparisons between individual wheat species are indicated using colored lines connecting the corresponding species included; the type of comparison is shown using letters a-l, and is specified in the legend, along with the corresponding reference(s). The figure points to an unbalance in the consideration of wheat species, as previous investigation have studied *T. durum*, *T. aestivum*, and *T. dicoccoides* extensively, *T. urartu*, *T. dicoccon*, and *A. tauschii* to a lesser extent, but the other species have been seldom considered. We identified eight studies comparing wheat genomic and phenotypic properties and seven others comparing the microbiota associated to different wheat species, which shows that plant properties and microbiota properties are described to the same extent. Multiple comparisons between *T. durum*, *T. dicoccon*, and *T. dicoccoides* were made (five studies), probably because this represents a good model for domestication studies, but only one considered the microbiota (h). Only 3 of 15 studies, with a focus on seminal roots (c) or arbuscular mycorrhizal fungi (g), covered all main events of wheat history.

Kinnunen-Grubb et al., 2020; Iannucci et al., 2021). Growth stage and plant physiology matter less (Houlden et al., 2008; Donn et al., 2015), despite significant shifts after tillering (Wang J. et al., 2016) and when heading starts (Hilton et al., 2018).

In the case of wheats (Triticeae tribe), most work on rhizosphere and root microbiomes has focused on bread wheat *T. aestivum*, whereas durum wheat *T. durum* has received little attention (**Figure 2B**). Therefore, knowledge on other *Triticum* species and *Aegilops* species is very incomplete (Özkurt et al., 2020; Tkacz et al., 2020).

Other species of the Triticeae tribe have been considered mostly for comparison with *T. aestivum* and *T. durum*, to decipher the impact of domestication and selection on the wheat microbiome (Golan et al., 2018; Roucou et al., 2018; Meziani et al., 2019) (**Figure 2B**). For instance, the interactions with *Glomeromycota* fungi have been compared between wild (*T. urartu, A. speltoides*, etc.) and domesticated wheats (*T. aestivum,* etc.) (Kapulnik and Kushnir, 1991; Hetrick et al., 1992).

Microorganisms are subjected to stronger plant selection in the root endosphere than the rhizosphere (Compant et al., 2010; Fitzpatrick et al., 2018), including in the case of wheat. This materializes by greater dominance effects in the wheat endosphere, i.e., with fewer taxa but in greater relative abundance, both for bacteria and fungi (Lu et al., 2018; Özkurt et al., 2020; Tkacz et al., 2020; Prudence et al., 2021). While the rhizosphere was extensively studied, fewer studies have focused on the root endosphere of wheats (Germida and Siciliano, 2001; Bokati et al., 2016; Özkurt et al., 2020; Tkacz et al., 2020). In addition, the bacterial community has been more investigated than the archaeal and fungal communities. Information about the archaeal community of wheat rhizosphere and root endosphere exists almost only for *T. aestivum*, and this community has been documented by culture-independent methods only (Fan et al., 2018; Szoboszlay et al., 2019; Tkacz et al., 2020; Prudence et al., 2021), archaea being difficult to isolate with methods routinely used in rhizosphere ecology. Very few studies have focused on fungi in the root endosphere, whether with culture-dependent (Bokati et al., 2016) or culture-independent methods (Bokati et al., 2016; Ofek-Lalzar et al., 2016; Özkurt et al., 2020). It must be kept in mind that the various root-associated compartments, i.e., root endosphere, rhizoplane and rhizosphere, are not always straightforward to distinguish from one another from an experimental point of view, which complicates comparisons between studies.

Rhizosphere, Rhizoplane, and Root Endosphere Microbiomes of Bread Wheat

Rhizosphere Microbiome

The rhizosphere bacterial community of *T. aestivum* is dominated at almost 40% by *Proteobacteria*, as indicated by culture-independent methods (**Table 1**). The other dominant phyla (10–15%) are *Acidobacteria*, *Actinobacteria*, and *Bacteroidetes*, whereas the remaining phyla represent <5% each (Turner et al., 2013; Donn et al., 2015; Rascovan et al., 2016; Mahoney et al., 2017; Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021). Culture-dependent methods point to *Proteobacteria* and *Actinobacteria* (each representing about 25%) and then *Firmicutes* (10%) as main phyla in the *T. aestivum* rhizosphere (**Table 2**; Juhnke et al., 1987; Sato and Jiang, 1996a,b; Germida et al., 1998; Germida and Siciliano, 2001). Within the *Proteobacteria*, the *Gammaproteobacteria* are the most abundant, with especially the families *Pseudomonadaceae* and *Xanthomonadaceae* (Donn et al., 2015).

In the *archaea,* the *Thaumarchaeota* represent more than two thirds of the rhizosphere community of *T. aestivum*, the *Euryarchaeota* <10%, and a range of unidentified phyla a total of about 20% (**Table 1**; Fan et al., 2018; Szoboszlay et al., 2019; Tkacz et al., 2020; Prudence et al., 2021). In the studies cited in **Table 1** and **Figure 3B**, the *Crenarchaeota* were not detected in *T. aestivum* rhizosphere. One investigation also considered lower taxonomic levels, showing that the *Nitrosphaeraceae* (*Thaumarchaeota*) was the most abundant family in the rhizosphere (Prudence et al., 2021).

The rhizosphere fungal community of *T. aestivum* is dominated by *Ascomycota*, which represent 40–50% of the total community with culture-independent (**Table 1**; Fan et al., 2018; Lu et al., 2018; Gqozo et al., 2020; Tkacz et al., 2020) and culture-dependent methods (**Table 3**; Smit et al., 1999; Hagn et al., 2003). The other dominant phyla are *Basidiomycota* and *Chytridiomycota* (5–15% each; **Table 1**). At genus level, *Mortiella* (phylum *Mucoromycota*), *Verticillum* (*Ascomycota*),

TABLE 1 | Occurrence of phyla in the rhizosphere of *T. aestivum*, as documented by culture-independent methods.

Phyla	Occurrence (%)[a]			Number (and list[b]) of *T. aestivum* genotypes	Number of countries	References
	Min	Max	Mean			
Bacteria						
Proteobacteria	16.5 (Bawburgh, UK; a)	52.0 (Villa Saboya, Argentina; l)	38.6	14 (a, b, c, d, e, f, g, h, i, j, k, l, NS)	5	Turner et al., 2013; Donn et al., 2015; Rascovan et al., 2016; Mahoney et al., 2017; Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021
Acidobacteria	0.5 (Gundibinyal, Australia; k)	23.4 (Bawburgh, UK; a)	11.5	14 (a, b, c, d, e, f, g, h, i, j, k, l, NS)	5	Turner et al., 2013; Donn et al., 2015; Rascovan et al., 2016; Mahoney et al., 2017; Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021
Actinobacteria	1.0 (Villa Saboya, Argentina; l)	26.0 (Gundibinyal, Australia; k)	12.9	14 (a, b, c, d, e, f, g, h, i, j, k, l, NS)	5	Turner et al., 2013; Donn et al., 2015; Rascovan et al., 2016; Mahoney et al., 2017; Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021
Bacteroidetes	0.9 (Villa Saboya, Argentina; l)	28.0 (Pullman, WA; g)	14.6	14 (a, b, c, d, e, f, g, h, i, j, k, l, NS)	5	Turner et al., 2013; Donn et al., 2015; Rascovan et al., 2016; Mahoney et al., 2017; Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021
Chloroflexi	0.5 (Northern China; NS)	6.3 (Bawburgh, UK; a)	0.9	7 (a, k, l, NS)	4	Donn et al., 2015; Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021
Cyanobacteria	0.5 (Norwich, UK; a)	3.0 (Bawburgh, UK; a)	0.2	1 (a)	2	Turner et al., 2013; Tkacz et al., 2020
Firmicutes	9.6 (Norwich, UK; a)	20.9 (Bawburgh, UK; a)	2.9	2 (a, NS)	3	Turner et al., 2013; Rascovan et al., 2016; Prudence et al., 2021
Armatimonadetes	1.0 (Pullman, WA; d)	4.6 (Pullman, WA; e)	1.2	9 (b, c, d, e, f, g, h, i, j)	1	Mahoney et al., 2017
Planctomycetes	0.2 (Northern China; NS)	8.9 (Villa Saboya, Argentina; l)	1.0	4 (a, h, l, NS)	3	Turner et al., 2013; Rascovan et al., 2016; Mahoney et al., 2017; Fan et al., 2018
Saccharibacteria	2.0 (Pullman, WA; i)	4.0 (Pullman, WA; b)	2.0	9 (b, c, d, e, f, g, h, i, j)	1	Mahoney et al., 2017
Gemmatimonadetes	0.5 (Northern China; NS)	7.0 (Pullman, WA; b)	2.3	10 (b, c, d, e, f, g, h, i, j, NS)	2	Fan et al., 2018; Naz et al., 2018
Verrucomicrobia	0.7 (Northern China; NS)	10.2 (Villa Saboya, Argentina; l)	2.7	12 (a, b, c, d, e, f, g, h, i, j, l, NS)	4	Turner et al., 2013; Rascovan et al., 2016; Mahoney et al., 2017; Tkacz et al., 2020; Prudence et al., 2021
Other taxa	2.0 (Villa Saboya, Argentina; l)	23.5 (Bawburgh, UK; a)	2.2	2 (a, l)	3	Turner et al., 2013; Rascovan et al., 2016; Prudence et al., 2021
Unidentified taxa	1.0 (Pullman, WA; e)	71.6 (Northern China; NS)	7.12	11 (b, c, d, e, f, g, h, i, j, k)	2	Donn et al., 2015; Mahoney et al., 2017
Archaea						
Euryarchaeota	24.5 (Bawburgh, UK; a)	24.5 (Bawburgh, UK; a)	6.1	1 (a)	1	Prudence et al., 2021
Thaumarchaeota	22.2 (Northern China; NS)	100.0 (Bawburgh, UK; a)	74.3	3 (a, NS)	3	Fan et al., 2018; Szoboszlay et al., 2019; Tkacz et al., 2020; Prudence et al., 2021
Other taxa	0.2 (Bawburgh, UK; a)	0.2 (Bawburgh, UK; a)	0.05	1 (a)	1	Prudence et al., 2021
Unidentified taxa	0.4 (Bawburgh, UK; a)	77.8 (Northern China; NS)	19.6	2 (a, NS)	2	Fan et al., 2018; Prudence et al., 2021
Fungi						
Zygomycota	16.0 (Bawburgh, UK; a)	16.0 (Bawburgh, UK; a)	2.7	1 (a)	1	Tkacz et al., 2020
Glomeromycota	0.5 (Bethlehem, South Africa; o)	1.5 (Bawburgh, UK; a)	0.8	4 (a, m, n, o)	2	Gqozo et al., 2020; Tkacz et al., 2020
Chytridiomycota	0.9 (Bawburgh, UK; a)	22.3 (Pretoria, South Africa; n)	5.4	4 (m, n, o, p)	2	Gqozo et al., 2020; Tkacz et al., 2020
Basidiomycota	0.5 (Hangzhou, China; NS)	24.3 (Bethlehem, South Africa; o)	12.6	5 (a, m, n, o, NS)	3	Smit et al., 1999; Lu et al., 2018; Tkacz et al., 2020
Ascomycota	28.3 (Bawburgh, UK; a)	60.6 (Napier, South Africa; m)	45.4	6 (a, m, n, o, NS)	3	Fan et al., 2018; Lu et al., 2018; Gqozo et al., 2020; Tkacz et al., 2020
Other taxa	12.6 (Bethlehem, South Africa; o)	39.5 (Bawburgh, UK; a)	14.2	4 (a, m, n, NS)	2	Gqozo et al., 2020; Tkacz et al., 2020
Unidentified taxa	51.0 (Northern China; NS)	63.5 (Hangzhou, China; NS)	19.1	2 (NS)	1	Lu et al., 2018

Min and Max correspond to minimum and maximum occurrences observed for each phylum in the specified references, after combining data from all replicate plants of one genotype (indicated between parentheses), at one growth stage, for one treatment in one soil of one geographic location (indicated between parentheses). For each phylum, the mean is calculated from all the values obtained from the different geographic locations and T. aestivum genotypes.

[a] *Abbrevations are used to designate United Kingdom (UK) and Washington State (WA).*

[b] *Genotypes were cultivars Paragon (a), Madsen (b), PI561725 (c), Eltan (d), Finch (e), Hill81 (f), Lewjain (g), PI561722 (h), PI561726 (i), PI561725 (j), Janz (k), Cadenza (l), SST88 (m), Kariega (n), Eland (o) or were not specified (NS).*

TABLE 2 | Occurrence of phyla in the rhizosphere of *T. aestivum*, as documented by culture-dependent methods.

Phyla	Occurrence (%)			Number (and list[a]) of *T. aestivum* genotypes	Number of countries	References
	Min	Max	Mean			
Bacteria						
Proteobacteria	9.4 (Watrous, Canada; NS)	77.0 (Kawatabi, Japan; d)	23.7	6 (a, b, c, d, f, NS)	3	Sato and Jiang, 1996b; Germida et al., 1998; Germida and Siciliano, 2001; Rascovan et al., 2016
Actinobacteria	5.0 (Watrous, Canada; NS)	88.9 (Kawatabi, Japan; d)	24.0	6 (a, b, c, d, f, NS)	3	Juhnke et al., 1987; Sato and Jiang, 1996a; Germida et al., 1998; Germida and Siciliano, 2001
Bacteroidetes	1.9 (Watrous, Canada; NS)	23.0 (Kawatabi, Japan; d)	5.9	5 (a, b, c, d, NS)	2	Sato and Jiang, 1996b; Germida et al., 1998; Germida and Siciliano, 2001
Firmicutes	3.4 (Saskatoon, Canada; b)	28.3 (Watrous, Canada; NS)	10.0	5 (a, b, c, d, NS)	2	Sato and Jiang, 1996a,b; Germida et al., 1998; Germida and Siciliano, 2001
Other taxa	55.4 (Watrous, Canada; NS)	55.4 (Watrous, Canada; NS)	7.9	1 (NS)	1	Germida et al., 1998
Unidentified taxa	60.6 (Saskatoon, Canada; a)	70.0 (Saskatoon, Canada; c)	28.5	3 (a, b, c)	1	Germida and Siciliano, 2001
Fungi						
Zygomycota	5.9 (Utrecht, The Netherlands; NS)	5.9 (Utrecht, The Netherlands; NS)	5.9	1 (NS)	1	Smit et al., 1999
Chytridiomycota	3.7 (Utrecht, The Netherlands; NS)	6.7 (Utrecht, The Netherlands; NS)	3.7	1 (NS)	1	Smit et al., 1999
Basidiomycota	32.8 (Utrecht, The Netherlands; NS)	32.8 (Utrecht, The Netherlands; NS)	32.8	1 (NS)	1	Smit et al., 1999
Ascomycota	54.6 (Utrecht, The Netherlands; NS)	54.6 (Utrecht, The Netherlands; NS)	54.6	1 (NS)	1	Smit et al., 1999
Other taxa	3.7 (Utrecht, The Netherlands; NS)	6.7 (Utrecht, The Netherlands; NS)	3.7	1 (NS)	1	Smit et al., 1999

Min and Max correspond to minimum and maximum occurrences observed for each phylum in the specified references, after combining data from all replicate plants of one genotype (indicated between parentheses), at one growth stage, for one treatment in one soil of one geographic location (indicated between parentheses). For each phylum, the mean is calculated from all the values obtained from the different geographic locations and T. aestivum genotypes.
[a]*Genotypes were cultivars PI167549 (a), Red Fife (b), CDC Teal (c), Aoba (d), GSTR 11562 (e), Pondera (f), or not specified (NS).*

and *Cryptococcus* (*Basidiomycota*) are enriched in the rhizosphere (Tkacz et al., 2020).

Rhizoplane Microbiome

The rhizoplane is very poorly documented with sequencing methods, which is surprising considering the importance of bread wheat as a crop. This situation probably results from sampling limitations for the root-sol interface and an increased focus on the root endosphere in recent years. In comparison with the rhizosphere, the rhizoplane displays a bacterial community that changes with wheat growth to a larger extent, with a decrease in *Proteobacteria* and an increase in *Actinobacteria* between the vegetative and ripening stages, as well as a decrease in *Bacteroidetes* associated with senescing roots compared to ripening stage (Donn et al., 2015). A lower abundance of *Acidobacteria* is observed at the rhizoplane in comparison with the rhizosphere (Donn et al., 2015).

Root Endosphere Microbiome

The bacterial community of the root endosphere of *T. aestivum,* in contrast with that of the rhizosphere, is dominated by *Actinobacteria* (Tkacz et al., 2020; Prudence et al., 2021). They represent about 40% of the total community based on culture-independent methods (**Table 3**), but <10% with culture-dependent methods (Germida and Siciliano, 2001; Bokati et al., 2016). They are followed by *Proteobacteria* (about 30%), and then *Bacteroidetes, Acidobacteria* and *Verrucomicrobia* (each at 5–10%). At family level, the *Streptomycetaceae* (*Actinobacteria*) dominates the endophytic community, followed by *Chitinophagaceae* (*Bacteroidetes*) and *Polyangiaceae* (*Proteobacteria*) (Prudence et al., 2021), whereas at genus level *Streptomyces, Microbispora*, *Micromonospora*, and *Nocardioides* (all in the *Actinobacteria* phylum) are prevalent (Coombs and Franco, 2003).

Root archaeal endophytes consist mainly of *Thaumarchaeota* (about 60% of the community) and *Euryarchaeota* (about 30%) (**Table 3**; Tkacz et al., 2020). It is a situation reminiscent of the one in the rhizosphere, but the abundance of *Thaumarchaeota* is lower in the root endosphere compared with the rhizosphere (Tkacz et al., 2020). The family *Nitrosphaeraceae* (*Thaumarchaeota*) also dominates in the root endosphere (about 75% of the community; Prudence et al., 2021). *Methanobacteriaceae* and *Methanocellaceae* are also present (about 10% of the community; Prudence et al., 2021).

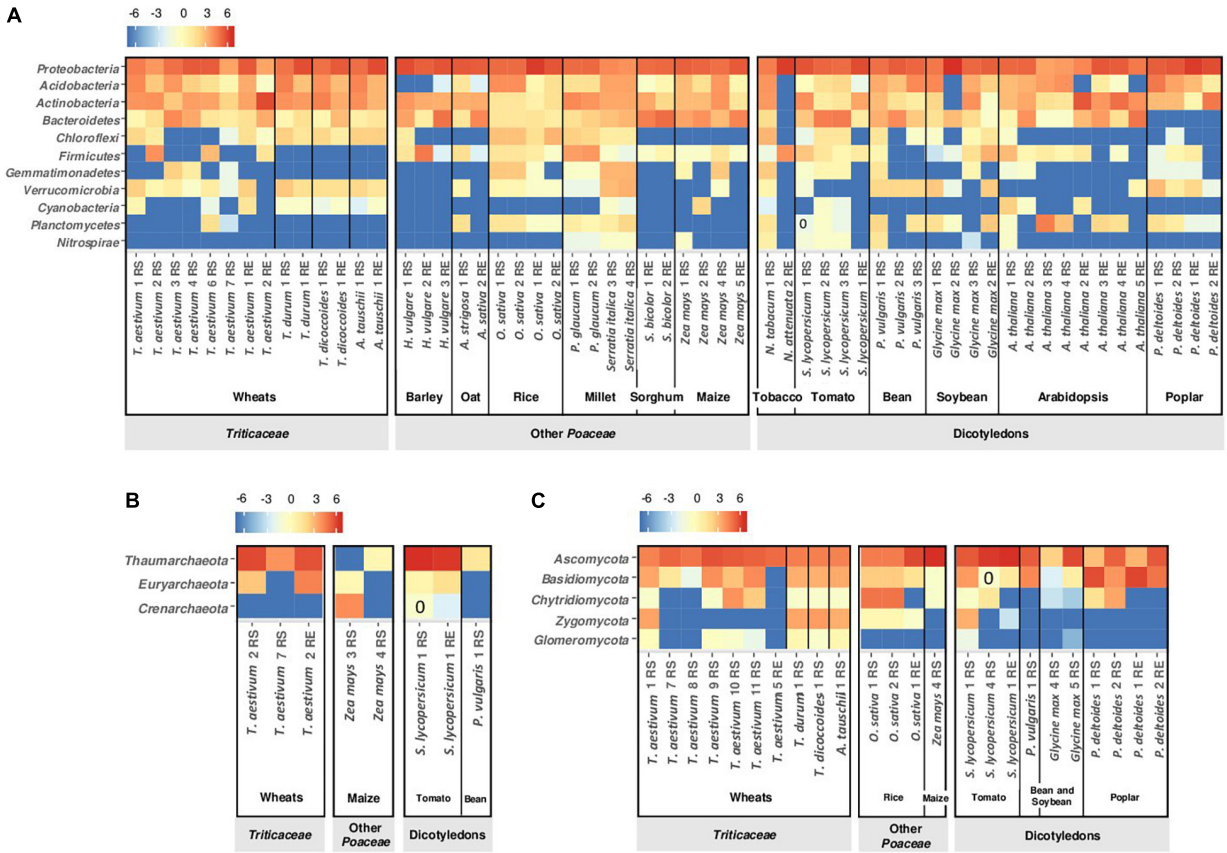


FIGURE 3 | Heatmap of major phyla affiliated with **(A)** bacteria, **(B)** archaea, and **(C)** fungi in rhizosphere soil (RS) and root/endosphere (RE) of wheats and non-wheat plants based on results from selected studies. Only phyla with relative abundance >0.5% in at least one study are shown. The color intensity in each cell denotes the transformed relative abundance [$\log_2((100x)+0.02)$] of a phylum in each study for each plant type. For details on individual conditions, see **Supplementary Table 1**.

For fungi, the predominance of *Ascomycota* in *T. aestivum* root endosphere (about 80%; **Table 3**) is documented with culture-independent and culture-dependent methods (Bokati et al., 2016; Özkurt et al., 2020). Basidiomycota represent <1% (Özkurt et al., 2020).

Evolutionary History of Wheats and Microbiome Effects

Hybridization and Domestication

Inter-generic hybridizations and domestication events led to a range of wheat species with phenotypic differences between one another and with their wild progenitors (see above section). Wheat polyploidy is a trait thought to lead to slightly different bacterial community diversity in root and rhizosphere. Wipf and Coleman-Derr (2021) documented a higher abundance of *Actinobacteria* in roots of tetraploid and hexaploid species and a higher α-diversity in the rhizosphere, in comparison with diploids. Several studies investigated the impact of domestication on the microbial community of rhizosphere and root endosphere of wheat (**Figure 2B**; Kapulnik and Kushnir, 1991; Hetrick et al., 1992; Hassani et al., 2018; Özkurt et al., 2020; Tkacz et al., 2020; Wipf and Coleman-Derr, 2021). Comparison of *T. durum* and *T. aestivum* with *T. dicoccoides* and *A. tauschii* (Tkacz et al., 2020) evidenced the same bacterial phyla but not in the same proportions, depending on the wheat species and root compartment. In the rhizosphere, the abundance of *Verrucomicrobia* is lower and the abundance of *Actinobacteria* is higher for *T. dicoccoides* than the other wheats (**Figure 3A**; Tkacz et al., 2020). Wild varieties displayed higher bacterial α-diversity than domesticated ones when comparing diploid wheats (Wipf and Coleman-Derr, 2021). In the root endosphere, a higher proportion at the heading/flowering stage is found for *Bacteroidetes* in *T. dicoccoides*, *Chloroflexi* in *A. tauschii* and *Cyanobacteria* in *T. aestivum* compared with the other wheat species (**Figure 3A**; Tkacz et al., 2020). The root endosphere of seedlings displays a higher abundance of *Proteobacteria* and a lower abundance of *Firmicutes* for *T. dicoccoides* than for *T. aestivum* (Özkurt et al., 2020). Results with root samples pointed to higher α-diversity for wild polyploid wheats than domesticated polyploids, but shifts were of small magnitude (Wipf and Coleman-Derr, 2021). Higher stochasticity (e.g., priority effects) was found in *T. aestivum* than the wild species *T. dicoccoides* (Tkacz et al., 2020).

TABLE 3 | Occurrence of phyla in the root endosphere of *T. aestivum*, as documented by culture-independent and culture-dependent methods.

Phyla	Occurrence (%)[a]			Number (and list[b]) of *T. aestivum* genotypes	Number of countries	References
	Min	Max	Mean			
Culture-independent methods						
Bacteria						
Proteobacteria	16.1 (Bawburgh, UK; a)	51.1 (Bawburgh, UK; a)	33.6	1 (a)	1	Tkacz et al., 2020; Prudence et al., 2021
Acidobacteria	1.2 (Bawburgh, UK; a)	12.4 (Bawburgh, UK; a)	6.8	1 (a)	1	Tkacz et al., 2020; Prudence et al., 2021
Actinobacteria	14.9 (Bawburgh, UK; a)	60.1 (Bawburgh, UK; a)	37.5	1 (a)	1	Tkacz et al., 2020; Prudence et al., 2021
Bacteroidetes	5.1 (Bawburgh, UK; a)	11.1 (Bawburgh, UK; a)	8.1	1 (a)	1	Tkacz et al., 2020; Prudence et al., 2021
Chloroflexi	1.0 (Bawburgh, UK; a)	5.2 (Bawburgh, UK; a)	3.1	1 (a)	1	Tkacz et al., 2020; Prudence et al., 2021
Cyanobacteria	4.3 (Bawburgh, UK; a)	4.3 (Bawburgh, UK; a)	2.2	1 (a)	1	Tkacz et al., 2020
Firmicutes	3.5 (Bawburgh, UK; a)	3.5 (Bawburgh, UK; a)	1.6	1 (a)	1	Prudence et al., 2021
Verrucomicrobia	7.17 (Bawburgh, UK; a)	7.17 (Bawburgh, UK; a)	3.6	1 (a)	1	Tkacz et al., 2020
Other taxa	7.0 (Bawburgh, UK; a)	7.0 (Bawburgh, UK; a)	3.5	1 (a)	1	Prudence et al., 2021
Archaea						
Euryarchaeota	28.0 (Bawburgh, UK; a)	28.0 (Bawburgh, UK; a)	28.0	1 (a)	1	Prudence et al., 2021
Thaumarchaeota	62.0 (Bawburgh, UK; a)	62.0 (Bawburgh, UK; a)	62.0	1 (a)	1	Prudence et al., 2021
Other taxa	7.9 (Bawburgh, UK; a)	7.9 (Bawburgh, UK; a)	7.9	1 (a)	1	Prudence et al., 2021
Unidentified taxa	2.1 (Bawburgh, UK; a)	2.1 (Bawburgh, UK; a)	2.1	1 (a)	1	Prudence et al., 2021
Fungi						
Ascomycota	66.0 (Kirksville, MO; b)	99.3 (Northern Germany; NS)	82.7	1 (b, NS)	1	Bokati et al., 2016
Basidiomycota	0.7 (Northern Germany; NS)	0.7 (Northern Germany; NS)	0.7	1 (NS)	1	Özkurt et al., 2020
Other taxa	34.0 (Kirksville, MO; b)	34.0 (Kirksville, MO; b)	17.0	1 (b)	1	Bokati et al., 2016
Culture-dependent methods						
Bacteria						
Proteobacteria	17.8 (Saskatoon, Canada; c)	24.3 (Saskatoon, Canada; e)	21.0	3 (c, d, e)	1	Germida and Siciliano, 2001
Actinobacteria	4.7 (Saskatoon, Canada; c)	11.8 (Saskatoon, Canada; e)	8.0	3 (c, d, e)	1	Germida and Siciliano, 2001
Bacteroidetes	1.0 (Saskatoon, Canada; d)	4.1 (Saskatoon, Canada; c)	2.5	3 (c, d, e)	1	Germida and Siciliano, 2001
Firmicutes	0.8 (Saskatoon, Canada; e)	1.6 (Saskatoon, Canada; c)	1.1	3 (c, d, e)	1	Germida and Siciliano, 2001
Unidentified taxa	59.0 (Saskatoon, Canada; e)	73.6 (Saskatoon, Canada; c)	67.3	3 (c, d, e)	1	Germida and Siciliano, 2001
Fungi						
Ascomycota	75.0 (Kirksville, MO; b)	75.0 (Kirksville, MO; b)	75.0	1 (b)	1	Bokati et al., 2016
Unidentified taxa	15.0 (Kirksville, MO; b)	15.0 (Kirsville, MO; b)	15.0	1 (b)	1	Bokati et al., 2016

Min and Max correspond to minimum and maximum occurrences observed for each phylum in the specified references, after combining data from all replicate plants of one genotype (indicated between parentheses), at one growth stage, for one treatment in one soil of one geographic location (indicated between parentheses). For each phylum, the mean is calculated from all the values obtained from the different geographic locations and T. aestivum genotypes.

[a] *Abbrevations are used to designate Missouri (MO) and United Kingdom (UK).*

[b] *Genotypes were cultivars Paragon (a), GSTR 11562 (b), PI167549 (c), Red Fife (d), CDC Teal (e) or not specified (NS).*

Archaea were studied (Tkacz et al., 2020), but sequencing targeted bacteria and archaea together, yielding limited numbers of sequences for archaea (<5%). This approach gave similar levels of *Thaumarcheota*, in the rhizosphere and the root endosphere, for *A. tauschii, T. dicoccoides, T. durum*, and *T. aestivum*.

For fungi, *A. tauschii* presented fewer *Zygomycota* in its rhizosphere than the other wheats did (**Figure 3C**; Tkacz et al., 2020). At genus level, fewer *Mortierella* (*Mucoromycota*) were found in *A. tauschii* and more *Verticillum* (*Ascomycota*) in *A. tauschii* and *T. dicoccoides* in comparison with *T. aestivum* (Tkacz et al., 2020). The less abundant fungal phylum in *T. aestivum* rhizosphere corresponded to the *Glomeromycota* (about 1%, **Table 1**), which were more abundant in the rhizosphere of *A. tauschii* (Tkacz et al., 2020). The selection of *Glomeromycota* and the plant response to these fungi are controlled by the D genome of *A. tauschii*, in comparison

TABLE 4 | Literature comparisons of root-associated microbial functional groups considering (i) wheat of different species, wild or domesticated, (ii) landraces, ancient, or modern varieties within wheat species, and (iii) different modern cultivars within wheat species.

Microbial function	Analysis of individual microorganisms				Analysis of functional groups			
	Microorganism studied	**(i) Wheat evolution/ domestication**	**(ii) Genotype categories within wheat species**	**(iii) Wheat cultivars**	**Methodology used**	**(i) Wheat evolution/ domestication**	**(ii) Genotype categories within wheat species**	**(iii) Wheat cultivars**
Biotic interactions								
DAPG synthesis	*Pseudomonas brassicacearum* Q8r1-96			Okubara and Bonsall, 2008; Yang et al., 2018				
	Pseudomonas fluorescens Q2-87							
	Pseudomonas ogarae F113		Valente et al., 2020	Valente et al., 2020				
					PCR-RFLP and sequence analysis of *Pseudomonas* isolates			Mazzola et al., 2004
Phenazine synthesis	*Pseudomonas chlororaphis*			Mahmoudi et al., 2019				
Synthesis of antimicrobial compound(s)					*in silico* prediction from *rrs* metabarcodes			Mahoney et al., 2017
Fungal inhibition					PCR-RFLP of *Pseudomonas* isolates			Gu and Mazzola, 2003
Rhizoctonia inhibition					Agar plate assays of *Pseudomonas* isolates			Mazzola and Gu, 2002
Induction of root defense	*Pseudomonas brassicacearum* Q8r1-96			Maketon et al., 2012				
	Pseudomonas putida			Pérez-de-Luque et al., 2017				
	Rhizophagus irregularis							
	Pseudomonas brassicacearum Q8r1-96			Okubara et al., 2010				
IAA synthesis	*Azotobacter chroococcum*			Narula et al., 2000				
					Salkowski method and sequence analysis			Venieraki et al., 2011
ACC deaminase activity					Absorbance quantification of α-ketobutyrate product			Stromberger et al., 2017
Yield promotion	*Azospirillum brasilense* Cd	Pagnani et al., 2020						
	Gluconacetobacter diazotrophicus Pal5							
	Herbaspirillum seropedicae Z67							

(Continued)

TABLE 4 | (Continued)

Microbial function	Analysis of individual microorganisms				Analysis of functional groups			
	Microorganism studied	**(i) Wheat evolution/ domestication**	**(ii) Genotype categories within wheat species**	**(iii) Wheat cultivars**	**Methodology used**	**(i) Wheat evolution/ domestication**	**(ii) Genotype categories within wheat species**	**(iii) Wheat cultivars**
Biogeochemical cycles								
Malate production					*in silico* prediction from *rrs* metabarcodes			Mahoney et al., 2017
Degradation of organic compound					Biolog^{TM} plate assays		Siciliano et al., 1998	
Cellulose decomposition					Counts on cellulose Congo Red medium			Zuo et al., 2014
Urease, catalase, sucrose, and dehydrogenase synthesis					Colorimetric assays of potential enzymatic activities			Zuo et al., 2014
Nitrogen metabolism					Metabarcoding (*rrs*) predicted gene functioning			Mahoney et al., 2017
N_2 fixation					Counts on N-free Ashby's medium	Zuo et al., 2014		
					Acetylene reduction assays and *nifH* sequence analysis			Venieraki et al., 2011
					qPCR (*nifH*)	Spor et al., 2020		Rilling et al., 2018
Nitrification					Counts on improved Stephenson's medium			Zuo et al., 2014
					qPCR (*amoA*)	Spor et al., 2020		
Denitrification					Measurement of nitrate reductase potential activity			Gill et al., 2006
					qPCR (*nirK/nirS, nosZI/nosZII*)	Spor et al., 2020		
Sulfur metabolism					*in silico* prediction from *rrs* metabarcodes			Mahoney et al., 2017
Phosphorus metabolism					*in silico* prediction from *rrs* metabarcodes			Mahoney et al., 2017
Phosphate solubilization	*Azotobacter chroococcum*			Narula et al., 2000				

These comparisons were carried out at the level of individual microorganisms (whereby one or several microorganisms was/were inoculated on wheat) or entire functional groups (i.e., taking into account most or all microorganisms potentially contributing to a given microbial function). RFLP, Restriction Fragment Length Polymorphism. List of references is available in ***Supplementary Material 1****.*

with genomes A (*T. urartu*) and B (*A. speltoides*) (Kapulnik and Kushnir, 1991; Hetrick et al., 1992; Zhu et al., 2001). The analysis of 32 *T. durum* genotypes indicated that *Glomeromycota* composition depended on plant genotype, and that certain *T. durum* genotypes associated strongly with *Paraglomus* and *Dominikia*, which were undetected in other genotypes (Ellouze et al., 2018). In the root endosphere, differences between *T. aestivum* and *T. dicoccoides* seedlings were evidenced, with a

higher abundance (73 vs. 47%) of *Pleosporales* (*Ascomycota*) in *T. aestivum* than *T. dicoccoides* roots (Özkurt et al., 2020).

Post-domestication Selection

The analysis of bacterial rhizosphere isolates showed a higher diversity with a landrace compared with two cultivars of *T. aestivum*, with the genera *Aureobacter* and *Salmonella* found only in the landrace (Germida and Siciliano, 2001). With culture-independent methods, landraces presented in the rhizosphere a higher abundance of *Bacteroidetes* and a lower abundance of *Actinobacteria* in comparison with modern *T. aestivum* cultivars (Rossmann et al., 2020). Landraces also displayed a core microbiome with more bacterial genera that were specific, i.e., found only with landraces. In the rhizosphere, the fungal genus *Dominikia* (*Glomeromycota*) was detected with 80% of *T. durum* landraces but only 60% of modern genotypes (Ellouze et al., 2018). The endophytic fungal community of *T. durum* landrace Perciasacchi (winter type) is dominated by *Ascomycota* (with *Alternaria* and *Gibberella*) and that of *T. durum* landrace Tumminia (spring type) by *Basidiomycota* (and particularly *Sporobolomyces* and *Puccinia*) (Casini et al., 2019), but these landraces were not compared with durum wheat cultivars. When comparing root and rhizosphere microorganisms associated with landraces and modern cultivars of *T. aestivum*, old accessions were enriched in *Acidobacteria* and *Actinobacteria*, and modern cultivars in *Verrumicrobia* and *Firmicutes* (Kinnunen-Grubb et al., 2020). Landraces are genetically more heterogeneous than cultivars, including for traits that influence root-microorganism interactions, e.g., root system size and root exudation (Waines and Ehdaie, 2007; Haichar et al., 2008; Matthews et al., 2019; Iannucci et al., 2021), and thus at the scale of a field they are likely to select a wider range of soil microorganisms overall. At the scale of individual plants, however, the microbiota of both old and ancient bread wheats followed a neutral assembly model, and the root microbiome displayed higher stochasticity (i.e., less deterministic selection) with modern *T. aestivum* cultivars than with landraces (Kinnunen-Grubb et al., 2020).

The Green Revolution resulted in the selection of semi-dwarf cultivars. In comparison with older, tall cultivars, the rhizosphere bacterial community of semi-dwarf bread wheat cultivars displays lower levels of *Actinobacteria* (1.4 vs. 13%), *Bacteroidetes* (5.0 vs. 16%) and *Proteobacteria* (8.6 vs. 29%) and higher levels of *Verrucomicrobia* (7.9 vs. 2.9%), *Planctomycetes* (2.1 vs. 0.7%) and *Acidobacteria* (2.9 vs. 1.4%) (Kavamura et al., 2020). The latter phyla are typically well present in bulk soil, which suggests a lower selection intensity by semi-dwarf than tall cultivars (Kavamura et al., 2020). When considering rhizosphere selection of individual strains, the comparison of 192 *T. aestivum* varieties evidenced that old varieties had a higher ability than modern cultivars to recruit the bacterium *Pseudomonas ogarae* (ex-*fluorescens* ex-*kilonensis*) F113 (Valente et al., 2020). Root systems of mid and later-generation semi-dwarf wheats are smaller than those of early Green-Revolution wheats (Waines and Ehdaie, 2007), whereas the amount of simple sugars released by roots of modern wheats is higher (Shaposhnikov et al., 2016), probably due to less stringent control of sugar exudation (Pérez-Jaramillo et al., 2018). Much remains to be done to understand differences in microbial community between old and modern cultivars, notably for archaea and fungi (not considered so far).

Among modern bread wheat cultivars, differences in bacterial selection can be significant, for certain phyla (**Figure 3A**; Mahoney et al., 2017). The abundance of *Actinobacteria, Firmicutes* and *Cyanobacteria* in the rhizosphere of cultivar Madsen is lower than for cultivar Paragon (8 vs. 19%, 0.2 vs. 20%, and 0.5 vs. 2.8%, respectively; **Figure 3A**). Such differences can also be evidenced at lower taxonomic levels, and root colonization levels by *P. ogarae* F113 varied between modern *T. aestivum* cultivars (Valente et al., 2020). Different *T. aestivum* cultivars can present dissimilar abundance levels of *Thaumarchaeota* and *Euryarchaeota* (**Figure 3B**), but data scarcity does not enable to conclude on archaeal ecology (Fan et al., 2018; Tkacz et al., 2020; Prudence et al., 2021). With fungi, higher rhizosphere levels were found for *Basidiomycota* (>15% vs. <10%) in cultivars Paragon, SST88 and Eland (Gqozo et al., 2020; Tkacz et al., 2020) and for *Chytridiomycota* (about 20% vs. <10%) in cultivar Kariega (Gqozo et al., 2020; **Figure 3C**). Among 94 *T. aestivum* genotypes, variations in mycorrhizal colonization were observed following inoculation with *Rhizophagus* and *Claroideoglomus* species, which could vary depending on old vs. recent cultivars (Lehnert et al., 2017). The abundance of *Paraglomus* (*Glomeromycota*) depends on the modern *T. durum* cultivar in the rhizosphere but not in the root endosphere (Ellouze et al., 2018).

Microbiome of Wheats vs. Other *Poaceae* and Non-*Poaceae*

Wheats vs. Other *Poaceae*

Compared with other *Poaceae*, Triticea members show some specificity in the level of certain phyla in the rhizosphere or root endosphere. Using selected publications (**Supplementary Table 1**), we found trends for (i) a higher abundance of *Cyanobacteria* and *Glomeromycota* and a lower abundance of *Firmicutes* in the rhizosphere, and (ii) a higher abundance of *Chloroflexi* in the root endosphere (**Figures 3A,C**). Differences between rhizobacterial communities increase with the phylogenetic distance between *Poaceae* (Bouffaud et al., 2016), but this is not apparent when considering phyla abundance. For example, *Verrucomicrobia* are found at the same level of magnitude in the rhizospheres of millet, rice and wheat (**Figure 3A**; Shi et al., 2019), even though the former two are distant from the Triticeae tribe. Moreover, rice (the closest to wheats in **Figure 3C**) displays a higher abundance of *Chytridiomycota* in the rhizosphere than Triticeae, whereas maize (although more distant) exhibits rhizosphere levels of *Chytridiomycota* closer to those of the Triticeae. This is also the case for the root endosphere, as the abundance of *Acidobacteria* in Triticeae is higher than in barley and oat but similar to levels in rice, sorghum and maize, which are comparatively more distant from wheats (**Figure 3A**). Nevertheless, such a variability between wheats and *Poaceae* needs to be considered in light of the high variability that exists between the different Triticeae species, and even between different *T. aestivum* cultivars.

Wheats vs. Non-*Poaceae*

Differences in bacterial community composition at phylum level can be found between Triticeae and non-*Poaceae*. This includes a lower abundance in rhizosphere and root endosphere of *Bacteroidetes* (for poplar) and *Chloroflexi* (for poplar and arabidopsis) compared with the Triticeae. A higher abundance of *Thaumarchaeota* (in rhizosphere and root endosphere) and *Nitrospirae* (in rhizosphere) is observed for tomato compared with *T. aestivum* (**Figure 3B**). In the rhizosphere, *Glomeromycota* are more abundant with Triticeae members than with tomato, bean, soybean and poplar (**Figure 3C**). However, significant microbiota similarities may also be observed when considering Triticeae and *non-Poaceae* plants mentioned in **Figure 3**. For instance, *Proteobacteria* and *Ascomycota* dominate the rhizobacterial community of both wheats and dicotyledons except for two varieties of *Arabidopsis thaliana* (Bulgarelli et al., 2012).

The comparison between Triticeae and non-*Poaceae* also reveals unexpected features, as certain differences do not coincide with the divide between these two groups. Barley (the closest to wheat in **Figure 3A**), displays surprisingly a low abundance of *Acidobacteria* in comparison with the Triticeae, the other *Poaceae* and also the non-*Poaceae*. The *Thaumarchaeota* dominate the rhizospheres of wheat and tomato, but not maize (**Figure 3B**). The rhizosphere and root endosphere of dicotyledons and certain *T. aestivum* are poorly colonized by *Zygomycota*, unlike for rice, other *Triticum* species and *A. tauschii*. In fact, extensive variability exists within the Triticeae, including between *T. aestivum* cultivars, and it is not necessarily lower than the variability between Triticeae and *non-Poaceae* observed in **Figure 3**.

FUNCTIONAL DIVERSITY OF THE WHEAT MICROBIOME

Functional Network of the Wheat Root Microbiome

Microorganisms play key roles in the biogeochemical cycles of carbon, nitrogen, sulfur, etc. (Philippot et al., 2013; Louca et al., 2016; **Figure 1B**). In root environments, the plant is the major provider of organic C and stimulate microorganisms, leading to the synthesis of various microbial metabolites, many of them with feed-back effects on the plant (Raaijmakers et al., 2009; Compant et al., 2010; Vacheron et al., 2013). The range of possible interactions between microorganisms and plant host is very broad (**Figure 1B**), from parasitism and competition to commensalism and mutualism (Lambers et al., 2009; Newton et al., 2010). Root-associated microorganisms also interact with one another, which modulates their own interactions with the plant (Raaijmakers et al., 2009; Huang et al., 2012). Due to the importance of root metabolism and rhizodeposition, it can be expected that exudate differences between wheat genotypes have the potential to materialize in significant differences in the implementation of biogeochemical cycles and biotic interactions in the root zone, but this possibility remains poorly documented.

Microbial functioning involves a complex network of elementary transformations (e.g., the conversion of N_2 into NH_3) or interactions (e.g., the inhibition mediated by a given antibiotic), each corresponding to a particular function. Each individual microorganism is endowed with many of these functions, and typically each function is common to different strains and species, leading to functional redundancy in the microbial community (Louca et al., 2016). All microorganisms participating to the same function form a functional group, and therefore each organism is likely to belong to several functional groups (Louca et al., 2016). For root-associated microorganisms, the context of the holobiont adds a supplementary dimension when considering microbial functioning (Vandenkoornhuyse et al., 2015; Lemanceau et al., 2017; Hassani et al., 2020). Whereas taxonomic variation within individual functional groups does not seem to fluctuate extensively with soil and other environmental conditions, the functional potential of the microbiota is thought to be strongly linked to environmental conditions (soil physico-chemistry, plant genotype, and growth stage) (Louca et al., 2016; Lemanceau et al., 2017; Guo et al., 2018). Accordingly, similar environments should promote similar microbial functional communities, while allowing for taxonomic variation inside an individual functional group.

Global Functioning of the Wheat Microbiota

The emergence of metagenomics has made it possible to glimpse the global functional and metabolic capacities of a microbiota. The assessment of the rhizosphere metagenome of *T. durum* showed an overrepresentation of two categories of microbial functions (Ofek-Lalzar et al., 2016; Ofaim et al., 2017). A first category corresponded to basic metabolism, important for root colonization (Santi et al., 2013; Vacheron et al., 2013), such as chemotaxis, lipopolysaccharide metabolism, nitrogen metabolism, pentose and glucoronate interconversions, starch, and sucrose metabolism. The second category was related to secondary metabolism, e.g., anthocyanin production or xenobiotic metabolism (Ofaim et al., 2017; Lu et al., 2018). Whether metagenome differences occur between wheat species or lines of individual wheat species remains to be determined. Despite methodological limitations, functional metagenome predictions from metabarcoding-based OTU datasets did suggest microbial differences between wheat lines (Mahoney et al., 2017). These predictions differed also according to *T. aestivum* growth stage (Kavamura et al., 2018). Energy metabolism dominates in the rhizosphere microbiota during early wheat development, vs. degradation of complex organic compounds with older, photosynthetically active plants. A similar rhizosphere acclimatization was found with oat (Nuccio et al., 2020). Metatranscriptomic analysis of the rhizosphere of one *T. aestivum* genotype revealed metabolic capabilities for rhizosphere colonization, including cellulose degradation and methylotrophy (Turner et al., 2013).

Metagenomic or metatranscriptomic studies have been useful to describe global metabolic activity in root environments, but they have not been implemented yet to compare different wheat

species or lines. Therefore, it remains difficult to assess the impact of intra-species variation, domestication and hybridization of wheats on global microbial functioning in the root zone, and this is a topic in strong need of research attention.

Microbial Interactions in the Wheat Root and Rhizosphere

Rhizosphere microorganisms develop deleterious, beneficial or neutral interactions with one another (**Figure 1B**). The extent of these interactions, and the density and complexity of the resulting interaction network depend on taxa richness, microbial abundance and activity levels (Finlay et al., 1997; Torsvik and Øvreås, 2002; Fuhrman, 2009). Wheat root system architecture and rhizodeposition traits determine the root surface that can be colonized and the amount of root exudates. Since these characteristics were affected by domestication and subsequent crop selection (Waines and Ehdaie, 2007; Shaposhnikov et al., 2016; Iannucci et al., 2021), wild wheats, landraces and modern cultivars probably display different patterns of microbial colonization and of microbial interactions in their roots and rhizosphere. Microbial interactions and competition can also be modulated by predators (nematodes and protozoa), which thrive to different extents in the rhizospheres of oat, pea and wheat (Turner et al., 2013), and perhaps also in the rhizosphere of different wheat genotypes. Microbiota network analysis of *T. aestivum* rhizosphere revealed the co-occurrences of cercozoa (protozoa), bacterial and fungal taxa, reminiscent of a predator-prey system (Rossmann et al., 2020). Co-occurrence levels were higher and trophic networks more entangled with landraces than modern cultivars, which suggests a higher level of microbial interactions in the rhizosphere of landraces.

Pathogens infecting wheat roots cause significant damage, including the take-all fungus *Gaeumannomyces graminis* var. *tritici* (Wilkinson et al., 1985), *Rhizoctonia* spp. causing root rot or damping-off (Ingram and Cook, 1990), and *Pythium* species leading to root rot (Wiese, 1987; Ingram and Cook, 1990). Differences in sensitivity to root pathogens exist according to wheat genotype. *A. speltoides* and *T. durum* are more sensitive than *T. monococcum* to *G. graminis* var. *tritici* (McMillan et al., 2014), whereas inter-cultivar variability of resistance to this pathogen is high within *T. aestivum* (Golizadeh et al., 2017). Susceptibility to *Rhizoctonia* was similar for *T. monococcum* and *T. durum*, as well as certain *T. aestivum* cultivars, whereas other cultivars of *T. aestivum* were more sensitive (Oros et al., 2013). Differences in tolerance to *G. graminis* (Golizadeh et al., 2017), root-infecting *Fusarium graminearum* (Wang et al., 2018), foot, crown and root rot-causing *Fusarium culmorum* (Erginbaş Orakcı et al., 2018) and *Pythium* (Higginbotham et al., 2004) occurred among *T. aestivum* cultivars. Wheat is also affected by parasitic nematodes, with cultivar-level differences in susceptibility of *T. aestivum* to cereal cyst nematodes *Heterodera* spp. (Cui et al., 2016) and root-lesion nematode *Pratylenchus curvicauda* (Begum et al., 2020). Apart from differences in disease sensitivity, modern *T. aestivum* cultivars tend to be more colonized by *Fusarium*, *Neoascochyta* and *Microdochium* root pathogens compared with landraces (Kinnunen-Grubb et al., 2020).

Wheat cultivars might rely on specific microbial populations for phytoprotection, which may entail pathogen inhibition (*via* competition or antagonism) or systemic induction of plant defense pathways. Fluorescent *Pseudomonas* inhibit *Pythium, Rhizoctonia* and *G. graminis* (Weller and Cook, 1986; Mavrodi et al., 2013), using 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), or phenazines (Keel et al., 1992; Kwak and Weller, 2013; Mavrodi et al., 2013; Imperiali et al., 2017). The diversity of bacterial populations producing these compounds and rhizosphere expression of the corresponding genes depend on plant host genotype (Latz et al., 2015). DAPG and HCN-producing microorganisms can be studied *via* the marker genes *phlD a*nd *hcnABC*, respectively (**Supplementary Table 2**), and DAPG-producing microorganisms associated with different wheat genotypes have been well-studied in comparison with the case of other antagonistic compounds (**Table 4**). Modern cultivars of *T. aestivum* differentially select for and benefit from DAPG-producing *Pseudomonas* species in resident soil populations (Berg et al., 2002; Mazzola et al., 2004; Meyer et al., 2010). Cultivar differences were evidenced in the interaction with DAPG-producing *P. brassicacearum* in soil suppressive to take-all (Yang et al., 2018). Suppression of *Rhizoctonia* root rot and take-all is cultivar-dependent, through enhanced recruitment of specific *Pseudomonas* populations by cultivars less affected by disease (Mazzola et al., 2004; Yang et al., 2018). Phytopathogens may also be inhibited by other saprophytic microorganisms, including bacteria and fungi (Barnett et al., 2017), and for the latter the ability to colonize wheat roots can depend on the cultivar (Osborne et al., 2018). In addition, Arbuscular Mycorrhizal (AM) fungi (division *Glomeromycota*) also inhibit wheat pathogens *via* competition (Ganugi et al., 2019) or production of cellulases and chitinases, which may affect pathogen cell wall and provide wheat protection (Pérez-de-Luque et al., 2017), but the significance of wheat genotypes is not documented. Induced resistance is poorly documented in monocots, including wheat (Balmer et al., 2013). Induced Systemic Resistance (ISR), which involves jasmonate and ethylene signaling, is triggered in wheat by certain *Pseudomonas* strains (Balmer et al., 2013). Similarly, saprophytic fungi from *Aspergillus*, *Penicillium* and *Trichoderma* genera and protecting against Rhizoctonia wilt trigger ISR in wheat (El-Maraghy et al., 2020). Mycorrhizae also induce systemic plant resistance, termed Mycorrhiza-Induced Resistance (MIR) (Mustafa et al., 2016), which is reminiscent of ISR (Balmer et al., 2013) but displays also features of Systemic Acquired Resistance (SAR), especially the priming of salicylic acid-dependent genes (Pérez-de-Luque et al., 2017). Thus, MIR by the AM fungus *Funneliformis mosseae* upregulated several defense genes in wheat, and protected wheat from the powdery mildew pathogen *Blumeria graminis* f. sp. *tritici* (Mustafa et al., 2016). In addition, *Bacillus velezensis* CC09 stimulated SAR pathways, inducing *PR1* genes and enhancing lignin accumulation, and protected wheat from take-all (Kang et al., 2018). Little has been done to compare induced resistance in different wheat genotypes (**Table 4**). The two cultivars studied in Pérez-de-Luque et al. (2017) displayed different levels of systemic priming for chitosan-induced callose after co-inoculation with *Pseudomonas putida* and *Rhizophagus irregularis*. One of the two cultivars showed higher level of callose deposition after

co-inoculation than inoculation of *P. putida* or *R. irregularis* alone, suggesting additive or synergistic effects in some but not all genotypes, probably linked to cultivar differences in the signaling pathway leading to systemic immune priming (Haichar et al., 2016). Root expression of defense gene homologs induced by *P. brassicacearum* Q8r1-96 differed between the three wheat cultivars analyzed (Okubara et al., 2010). Since wheat species and varieties differ in their abilities to recruit microbial taxa containing strains with induced resistance potential, especially AM fungi (**Figure 2A**), it raises the possibility that some varieties are more prone to be protected by ISR. If so, this might explain some of the differences in sensitivity to diseases observed among cultivars.

Apart from plant protection, several microbial functional groups present in the rhizosphere are beneficial, by ensuring better plant growth (Vacheron et al., 2013; Lemanceau et al., 2017). However, information about their abundance and activity according to wheat genotype is scarce, even for the best described functional groups. Mineral nutrition of wheat can be promoted by different functional groups directly affecting nutrient bioavailability or stimulating plant development through microbial modulation of its hormonal balance (Finkel et al., 2017). Wheat growth can be promoted by microorganisms that produce phytohormones, such as Indole-3-Acetic Acid (IAA) (Spaepen et al., 2007), cytokinins and gibberellin, or secondary metabolites interfering with auxin, such as DAPG (Landa et al., 2003) and nitric oxide (NO) (Molina-Favero et al., 2008). Inoculation with different P-solubilizing, IAA-producing strains of *Azotobacter chroococcum* carried out on three cultivars of *T. aestivum* showing contrasted P responses resulted in enhanced N, P, K uptakes, probably as a consequence of phytohormone effects, but without difference between wheat genotypes (Narula et al., 2000). In contrast, cultivar differences were found following *Azospirillum* inoculation, when considering phenotypic traits such as root system architecture or plant height, especially during early growth phases, but without an effect on grain yield (Kazi et al., 2016). Using the Opata × synthetic mapping population, one QTL region on chromosome 1A was identified for *Azospirillum* adhesion to wheat roots (Díaz De León et al., 2015). DAPG, at low concentration, induces the plant's auxinic pathways, which stimulates root exudation and branching (Brazelton et al., 2008; Combes-Meynet et al., 2010). Application of wheat root exudates to a soil modified the composition of the DAPG-producing community in a cultivar-specific manner (Gu and Mazzola, 2003). The activity of DAPG-producing *Pseudomonas* in the rhizosphere and DAPG accumulation on the rhizoplane of *T. aestivum* is influenced by specific cultivar-bacterial strain associations (Bergsma-Vlami et al., 2005; Okubara et al., 2010). Variability is also observed between old and modern cultivars, as colonization by *P. ogarae* F113 and expression of *phl* genes (coding for DAPG production) are higher for ancient genotypes of *T. aestivum* (Valente et al., 2020). Not much data is available on the effect of wheat genotype on the abundance or activity of other phytohormone-producing microorganisms (**Table 4**). 1-aminocyclopropane-1-carboxylate (ACC) is the precursor of ethylene in plants and more ACC is produced in case of stress, which may have deleterious effects on plant growth (Glick, 2014). ACC deaminase activity, which catalyzes the cleavage of ACC into ammonium and alpha-ketobutyrate (Glick, 2005, 2014), is found in many microorganisms living in the rhizosphere (Bouffaud et al., 2018). This activity can improve the growth and yield of *T. aestivum* under salt stress and drought conditions, acting as an ACC sink that lowers ethylene level without stopping stress-induced reactions (Zahir et al., 2009; Shakir et al., 2012; Hassan et al., 2014). The abundance of microorganisms with ACC deaminase activity in the rhizosphere varies with plant genotype (Bouffaud et al., 2018), and the abundance, composition and activity of the corresponding functional group depends also on *T. aestivum* cultivar (Stromberger et al., 2017). Under drought or well-watered conditions, the response of *T. aestivum* to inoculation with ACC deaminase-producing bacteria is genotype dependent, as some cultivars showed higher root length while others had increased above-ground biomass (Salem et al., 2018). The ability of these bacteria to promote drought resistance may depend on wheat genotype (Stromberger et al., 2017). The significance of ACC deaminase activity has not been studied for wheat species other than *T. aestivum* (**Figure 2A**). *Pseudomonas* producing phenazine (usually studied for its antimicrobial properties) are thought to be involved in drought resistance as well. Indeed, when inoculated, their presence on roots (at population levels that are wheat cultivar dependent; Mahmoudi et al., 2019) leads to added protection of seedlings against drought in cultivars that are genetically drought resistant (Mahmoudi et al., 2019). Moreover, *Pseudomonas* producing phenazine were shown to be abundant in non-irrigated soil (Mavrodi et al., 2012, 2013). Furthermore, resistance to drought (along with other abiotic stress like salinity or metals; Seguel et al., 2016; Aguilera et al., 2018; Ganugi et al., 2019) may be conferred by AM fungi following the induction of particular metabolomic responses in wheat roots (Bernardo et al., 2019). This protection varies with *T. aestivum* cultivars and QTLs have been identified, especially on chromosomes 3D and 7D (Lehnert et al., 2017). More generally, genome-wide association studies for the establishment of AM symbiosis have highlighted QTL regions on chromosomes 3A, 4A, and 7A in *T. aestivum* inoculated with *Rhizophagus intraradices*, *Claroideoglomus claroideum* and *Claroideoglomus etunicatum* (Lehnert et al., 2017) and on chromosomes 1A, 2B, 5A, 6A, 7A, and 7B for *T. durum* when inoculated individually with *Funneliformis mosseae* or *Rhizoglomus irregulare* (De Vita et al., 2018). Depending on the species (*T. aestivum* or *T. durum*), changes in rhizosphere microbiota traits due to drought will differ, suggesting that different wheat genotypes recruit their own specific microbiota to help alleviate abiotic stresses (Azarbad et al., 2018, 2020).

Biogeochemical Cycles in the Wheat Root and Rhizosphere

The rhizosphere is characterized by the release of organic exudates and other rhizodeposits, and the uptake of mineral nutrients by roots (**Figure 1B**). The biogeochemical cycles of carbon, nitrogen and phosphorus are well-understood, and several primers are available to target microbial markers

associated with these cycles (**Supplementary Table 2**). However, microbial activities involved in other important cycles such as those of potassium, sulfur or iron are less described in the wheat root and rhizosphere (Narula et al., 2000; Sheng and He, 2011; Jacoby et al., 2017; Kumar et al., 2018).

Carbon

Carbon efflux from the root is important (Kuzyakov and Cheng, 2001) and wheat root-derived CO_2 represents between 25 and 50% of the total CO_2 efflux from soil (Kuzyakov and Cheng, 2001). Most microbial functions related to carbon in the rhizosphere are linked to degradation of organic exudates (Derrien et al., 2004; Haichar et al., 2016). The rhizosphere priming effect (i.e., the increase of microbial activity following exudation) intensifies decomposition and mineralization (Cheng et al., 2003), and is an important cause of carbon loss from the rhizosphere.

Bacteria and fungi are primary decomposers, releasing extracellular hydrolytic enzymes to catalyze decomposition of organic matter (Berg and McClaugherty, 2008). Actinobacteria, found in the rhizosphere of *T. aestivum* (**Table 1**) include different species involved in decomposition and humus formation (Wang C. et al., 2016; Bao et al., 2021). In the rhizosphere, the potential activity of microbial enzymes (mostly cellulases) associated with carbohydrate degradation differed according to *T. aestivum* cultivar (**Table 4**; Zuo et al., 2014). Moreover, endophytic microorganisms isolated from roots of ancient *T. durum* cultivars differed in their ability to degrade organic compounds compared with those from more recent cultivars (Siciliano et al., 1998). Indeed, microorganisms isolated from recent cultivar CDC Teal degraded carboxylic acids at a higher rate, whereas polymers and amino acids were degraded at a higher rate by microorganisms isolated from ancient cultivars Red Fife and PI 167549.

Nitrogen

Diazotrophs are well-represented in the plant rhizosphere, where they find sufficient energy resources for the costly functioning of the dinitrogenase that fixes N_2 into assimilable NH_3 (Herridge et al., 2008). N_2 fixation is the best studied activity when comparing different wheat genotypes (**Table 4**). The ability of N_2-fixing *Azospirillum* to colonize roots depends on *T. aestivum* cultivar, as the bacteria were detected either in the root tissues and intercellular spaces or only at the root surface (Schloter and Hartmann, 1998). *Cyanobacteria* of the genera *Nostoc* (Gantar et al., 1993) and *Azospirillum brasilense* FP2 fix N_2 when colonizing *T. aestivum* roots (Van Dommelen et al., 2009; Camilios-Neto et al., 2014). Free-living nitrogen-fixing prokaryotes contribute to nitrogen requirement of wheat (Dellagi et al., 2020), up to 76 and 32% for shoots and roots, respectively (Majeed et al., 2015). In addition, higher yields were observed for *T. aestivum* inoculated with engineered strains able to fix nitrogen constitutively (Fox et al., 2016). The diazotroph community varies in size and activity with plant species (Perin et al., 2006; Mao et al., 2013; Bouffaud et al., 2016) and cultivars of *T. aestivum*, with a higher number of rhizosphere diazotrophs for Xiaoyan than for other *T. aestivum* cultivars (Mahoney et al., 2017).

Even though N is often limiting for plant growth, wheat roots may release nitrogen in the form of NH_3/NH_4^+ in the rhizosphere (26 mg for the entire growing season, 18% of the total N yield of the plant; Janzen, 1990). Within the wheat rhizosphere, NH_3 is oxidized into NO_2^- and NO_3^- by aerobic nitrifiers. Ammonium oxidizers include ammonium-oxidizing bacteria (AOB) and ammonium-oxidizing archaea (AOA; Chen et al., 2011). Wheat domestication and selection had an effect on the interaction with nitrifiers, as they are less abundant in the rhizosphere of modern *T. durum* cultivars compared with *T. dicoccoides* and *T. dicoccon* (Spor et al., 2020). Differences in abundance of nitrifying bacteria also exist between *T. aestivum* cultivars, with higher rhizosphere numbers for cultivar Xiaoyan than the others (Mahoney et al., 2017). In addition, some *T. aestivum* landraces can inhibit nitrification in their rhizosphere (O'Sullivan et al., 2016).

Denitrifying bacteria harboring NO_2^- reductase genes *nirK*/*nirS* (Chen et al., 2011) reduce NO_2^- into NO. In the *T. aestivum* rhizosphere, denitrification is stimulated by root exudates (as denitrifiers are heterotrophs) (Wollersheim et al., 1987) and soil waterlogging (as it results in anoxia) (Hamonts et al., 2013). Thus, *T. aestivum* modulates denitrification activity and influences the composition of the denitrifying community (Achouak et al., 2019). Among modern *T. aestivum* cultivars, differences in rhizosphere denitrification activity and N_2O emissions are significant (Hayashi et al., 2015), including for cultivars that can even inhibit nitrate reductase activity (Gill et al., 2006).

Phosphorus

Phosphorus is mostly present as insoluble phosphate or organic forms in the soils. Microorganisms can degrade P-containing organic matter *via* phosphatases, thereby mineralizing phosphorus and making it potentially available for plants. Some bacteria also act as Phosphate-Solubilizing Bacteria (PSB), thanks to the production of organic acids, protons, IAA (Gyaneshwar et al., 2002). PSB have been isolated from the rhizosphere of *T. aestivum*, e.g., *Streptomyces* spp. (Jog et al., 2014), *Pseudomonas* sp. BR2 (Babana et al., 2013) and *Bacillus* sp. (Majeed et al., 2015), and the rhizosphere of *T. durum* (Cherchali et al., 2019; Di Benedetto et al., 2019). The number of culturable PSB varies with plant species (Kundu et al., 2009). Since PSB are heterotrophic, their abundance in the rhizosphere of *T. aestivum* is influenced by organic matter content (Abderrazak et al., 2017), and differences between wheat cultivars might be expected since each may exude differently (Waines and Ehdaie, 2007; Iannucci et al., 2021). Wheat cultivars differed in the abundance of microbiota sequences linked to phosphate metabolism (Mahoney et al., 2017). Inoculation with a PSB from *Azotobacter chroococcum* in the rhizosphere of three cultivars of *T. aestivum* increased the number of grains per spike, straw yield, and root biomass, but without significant difference between cultivars (Narula et al., 2000). This is the only study dealing with P solubilization activity in different wheat cultivars (**Table 4**).

In addition to bacteria, AM fungi associated to wheat can mineralize organic phosphorus. The symbiotic network formed by mycorrhizal fungi with plant roots increases the volume of soil exploited for nutrients, providing the plant with P sources

while the fungus acquires organic carbon from the plant (Hamel et al., 2004; Li et al., 2006; Pellegrino et al., 2015). The importance of AM fungi in wheat phosphorus feeding depends on the combination of plant and fungal genotypes (Hamel et al., 2004; Pérez-de-Luque et al., 2017). Indeed, the abundance of AM fungi differs between *T. durum* varieties, with a higher abundance associated with landraces than with modern cultivars (Ellouze et al., 2018). Similarly, wild wheats of genome A (*T. urartu*) and B (*A. speltoides*) showed more mycorrhizal dependence (i.e., the degree of plant growth and nutrition obtained with the help of AM fungi) than wild wheat of the D genome (*A. tauschii*) (Hetrick et al., 1992). Thus, the response to AM fungi in hexaploid wheat is probably controlled by the D genome (Kapulnik and Kushnir, 1991; Hetrick et al., 1992). Mycorrhizal dependence was lower in modern cultivars in comparison to old cultivars of *T. aestivum* (Zhu et al., 2001). Modern wheat crops seem to select their partner less (Hetrick et al., 1992; Zhu et al., 2001) and mycorrhizal dependence has been reported to decrease with wheat domestication (domesticated *T. durum* vs. wild *T. dicoccoides*; Martín-Robles et al., 2018). Indeed, in agroecosystems where different fertilization regimes are applied, modern cultivars probably rely less on microorganisms for their nutrition since they are well-provided with fertilizers (Duhamel and Vandenkoornhuyse, 2013).

CONCLUSION

Wheat, one of the three most important crops in the world, has undergone a particularly complex evolutionary history involving several inter-genus crosses, genomic hybridizations and domestication events, which resulted in the formation of several wheat species able to grow in contrasted climates and cultivated (mostly *T. aestivum* and *T. durum*) for various feed and food purposes. The implications of this very particular evolutionary history on the recruitment and functioning of root and rhizosphere microbiomes are poorly understood.

Most studies have focused on the taxonomic features of these microbiomes, describing the abundance and diversity of microorganisms associated with wheat species. The most dominant bacterial phylum corresponds to *Proteobacteria* in the rhizosphere (as for other plant taxa) and *Actinobacteria* in the root endosphere (but *Proteobacteria* for other *Poaceae* and for non-*Poaceae* taxa). In both compartments, the archaeal and fungal communities are dominated by, respectively, *Thaumarchaeota* (as for other plant taxa) and *Ascomycota* (as for the other plant taxa investigated except poplar, where *Basidiomycota* dominate).

Domestication, selection and modern breeding created wheats selecting different root and rhizosphere communities in comparison with those of their wild or ancient relatives. Evidence for the importance of genomic hybridizations relates especially to the case of *Glomeromycota*, whose mycorrhizal association is controlled by D genome factors. Otherwise, the main differences in root and rhizosphere microorganisms seem to stem from post-domestication selection, based on the information available so far. Indeed, landraces are associated with a larger microbial diversity, which probably results from their higher plant-to-plant genetic heterogeneity, and their core microbiome presents certain bacterial families not found in modern cultivars. However, at the level of individual plants, strong microbial selection takes place in the rhizosphere of landraces, with higher taxa co-occurrence levels. This is attributed to a stronger selective pressure in the rhizosphere of pre-Green Revolution wheat compared to semi-dwarf varieties and modern cultivars of *T. aestivum*. Overall, the vast majority of analyses considering wheat genetic diversity have been restricted to the comparison of different cultivars of *T. aestivum*, showing microbial variability between them, to an extent not necessarily lower than that found between Triticeae and other *Poaceae* or *non-Poaceae*.

At the functional level, much less is documented on root-associated microorganisms in comparison with taxonomic data. Information is scarce on their functional traits, both for metagenomic and metatranscriptomic investigations targeting the entire microbial community and studies dealing with particular microbial functional groups. Differences in recruitment of disease-suppressive microorganisms are seen between cultivars of *T. aestivum*, contributing to differences in wheat health. The best-documented impact of domestication and post-domestication selection concerns the ability to interact and benefit from AM fungi, which decreases along the domestication/selection gradient.

Overall, it appears that wheat evolution has resulted into crop varieties with particular microbiome profiles, which probably rely less on their underground microbial partners for provision of growth resources and protection against diseases, and thus they are more dependent on human management. The development of omics tools targeting microbial functions in the rhizosphere is expected to provide new insights into the significance of wheat domestication and diversification for wheat-microorganisms interactions. It should also facilitate the design of novel breeding strategies integrating the contribution of root symbiotic partners for sustainable wheat farming.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this review article and approved the submitted version.

FUNDING

CG was funded by a grant from the Ministry of Research, Paris, France. Support from the Breakthrough project "Phytobiome" (IDEX Lyon) is acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.782135/full#supplementary-material

REFERENCES

Abderrazak, R., Laila, N., and Jamal, I. (2017). Occurrence of phosphate solubilizing bacteria in the rhizosphere of *Triticum aestivum* L. from Meknes, Morocco. *Am. J. Microbiol. Biotechnol.* 4, 1–7.

Achouak, W., Abrouk, D., Guyonnet, J., Barakat, M., Ortet, P., Simon, L., et al. (2019). Plant hosts control microbial denitrification activity. *FEMS Microbiol. Ecol.* 95:fiz021. doi: 10.1093/femsec/fiz021

Aguilera, P., Larsen, J., Borie, F., Berríos, D., Tapia, C., and Cornejo, P. (2018). New evidences on the contribution of arbuscular mycorrhizal fungi inducing Al tolerance in wheat. *Rhizosphere* 5, 43–50. doi: 10.1002/jsfa.10088

Ahlawat, O. P., Tiwari, R., and Singh, G. P. (2018). Metagenomics of wheat rhizosphere for abiotic stress management. *Wheat Barley Res.* 10, 64–77.

Azarbad, H., Constant, P., Giard-Laliberté, C., Bainard, L. D., and Yergeau, E. (2018). Water stress history and wheat genotype modulate rhizosphere microbial response to drought. *Soil Biol. Biochem.* 126, 228–236. doi: 10.1016/j.soilbio.2018.08.017

Azarbad, H., Tremblay, J., Giard-Laliberté, C., Bainard, L. D., and Yergeau, E. (2020). Four decades of soil water stress history together with host genotype constrain the response of the wheat microbiome to soil moisture. *FEMS Microbiol. Ecol.* 96:fiaa098. doi: 10.1093/femsec/fiaa098

Babana, A. H., Dicko, A. H., Maïga, K., and Traoré, D. (2013). Characterization of rock phosphate-solubilizing microorganisms isolated from wheat (*Triticum aestivum* L.) rhizosphere in Mali. *J. Microbiol. Microbial Res.* 1, 1–6.

Badri, D. V., and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant Cell Environ.* 32, 666–681. doi: 10.1111/j.1365-3040.2008.01926.x

Balmer, D., Planchamp, C., and Mauch-Mani, B. (2013). On the move: induced resistance in monocots. *J. Exp. Bot.* 64, 1249–1261. doi: 10.1093/jxb/ers248

Bao, Y., Dolfing, J., Guo, Z., Chen, R., Wu, M., Li, Z., et al. (2021). Important ecophysiological roles of non-dominant Actinobacteria in plant residue decomposition, especially in less fertile soils. *Microbiome* 9: 84. doi: 10.1186/s40168-021-01032-x

Barnett, S., Zhao, S., Ballard, R., and Franco, C. (2017). Selection of microbes for control of Rhizoctonia root rot on wheat using a high throughput pathosystem. *BiolControl* 113, 45–57.

Begum, F., Jones, M. G. K., and Fosu-Nyarko, J. (2020). Assessment of the pest status of *Pratylenchus curvicauda* and ultrastructural changes in roots of infected wheat and barley. *Plant Pathol* 69, 1574–1588. doi: 10.1111/ppa.13232

Berg, B., and McClaugherty, C. (2008). "Decomposition of fine root and woody litter," in *Plant Litter: Decomposition, Humus Formation, Carbon Sequestration*, eds B. Berg and C. McClaugherty (Berlin: Springer), 193–209. doi: 10.1007/978-3-540-74923-3_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

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., and Smalla, K. (2002). Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. *Appl. Environ. Microbiol.* 68, 3328–3338. doi: 10.1128/AEM.68.7.3328-3338.2002

Bergsma-Vlami, M., Prins, M. E., and Raaijmakers, J. M. (2005). Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* spp. *FEMS Microbiol. Ecol.* 52, 59–69. doi: 10.1016/j.femsec.2004.10.007

Bernardo, L., Carletti, P., Badeck, F. W., Rizza, F., Morcia, C., Ghizzoni, R., et al. (2019). Metabolomic responses triggered by arbuscular mycorrhiza enhance tolerance to water stress in wheat cultivars. *Plant Physiol. Biochem.* 137, 203–212. doi: 10.1016/j.plaphy.2019.02.007

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9

Bokati, D., Herrera, J., and Poudel, R. (2016). Soil influences colonization of root-associated fungal endophyte communities of maize, wheat, and their progenitors. *J. Mycol.* 2016:8062073.

Bonjean, A. (2001). Histoire de la culture des céréales et en particulier de celle du blé tendre (*Triticum aestivum* L.). *Doss. Environ. INRA* 21, 29–37.

Bonnin, I., Bonneuil, C., Goffaux, R., Montalent, P., and Goldringer, I. (2014). Explaining the decrease in the genetic diversity of wheat in France over the 20th century. *Agric. Ecosyst. Environ.* 195, 183–192. doi: 10.1007/s00122-005-2014-8

Bouffaud, M. L., Renoud, S., Dubost, A., Moënne-Loccoz, Y., and Muller, D. (2018). 1-Aminocyclopropane-1-carboxylate deaminase producers associated to maize and other *Poaceae* species. *Microbiome* 6:114. doi: 10.1186/s40168-018-0503-7

Bouffaud, M. L., Renoud, S., Moënne-Loccoz, Y., and Muller, D. (2016). Is plant evolutionary history impacting recruitment of diazotrophs and *nifH* expression in the rhizosphere? *Sci. Rep.* 6:21690. doi: 10.1038/srep21690

Bouffaud, M., Poirier, M., Muller, D., and Moënne-Loccoz, Y. (2014). Root microbiome relates to plant host evolution in maize and other Poaceae. *Environ. Microbiol.* 16, 2804–2814. doi: 10.1111/1462-2920.12442

Brancourt-Hulmel, M., Doussinault, G., and Lecomte, C. (2003). Genetic improvement of agronomic traits of winter wheat cultivars released in France from 1946 to 1992. *Crop Sci.* 43, 37–45. doi: 10.2135/cropsci2003.0037

Brazelton, J. N., Pfeufer, E. E., Sweat, T. A., McSpadden Gardener, B. B., and Coenen, C. (2008). 2,4-Diacetylphloroglucinol alters plant root development. *Mol. Plant-Microbe Interact.* 21, 1349–1358. doi: 10.1094/MPMI-21-10-1349

Brisson, N., Gate, P., Gouache, D., Charmet, G., Oury, F. X., and Huard, F. (2010). Why are wheat yields stagnating in Europe? A comprehensive data analysis for France. *Field Crops Res.* 119, 201–212.

Buée, M., De Boer, W., Martin, F., van Overbeek, L., and Jurkevitch, E. (2009). The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil* 321, 189–212. doi: 10.1007/s11104-009-9991-3

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Camilios-Neto, D., Bonato, P., Wassem, R., Tadra-Sfeir, M. Z., Brusamarello-Santos, L. C., Valdameri, G., et al. (2014). Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. *BMC Genomics* 15:378. doi: 10.1186/1471-2164-15-378

Cantarel, A. A. M., Allard, V., Andrieu, B., Barot, S., Enjalbert, J., Gervaix, J., et al. (2021). Plant functional trait variability and trait syndromes among wheat varieties: the footprint of artificial selection. *J. Exp. Bot.* 72, 1166–1180. doi: 10.1093/jxb/eraa491

Casini, G., Yaseen, T., Abdelfattah, A., Santoro, F., Varvaro, L., Drago, S., et al. (2019). Endophytic fungal communities of ancient wheat varieties. *Phytopathol. Mediter.* 58, 151–162.

Chaudhary, S., Dheri, G. S., and Brar, B. S. (2017). Long-term effects of NPK fertilizers and organic manures on carbon stabilization and management index under rice-wheat cropping system. *Soil Tillage Res.* 166, 59–66. doi: 10.1016/j.still.2016.10.005

Chen, X., Zhang, L. M., Shen, J. P., Wei, W. X., and He, J. Z. (2011). Abundance and community structure of ammonia-oxidizing archaea and bacteria in an acid paddy soil. *Biol. Fertil. Soils* 47, 323–331.

Cheng, W., Johnson, D. W., and Fu, S. (2003). Rhizosphere effects on decomposition. *Soil Sci. Soc. Am. J.* 67, 1418–1427. doi: 10.2136/sssaj2003.1418

Cherchali, A., Boukhelata, N., Kaci, Y., Abrous-Belbachir, O., and Djebbar, R. (2019). Isolation and identification of a phosphate-solubilizing *Paenibacillus polymyxa* strain GOL 0202 from durum wheat (*Triticum durum* desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. *Biocatal. Agric. Biotechnol.* 19:101.

Combes-Meynet, E., Pothier, J. F., Moënne-Loccoz, Y., and Prigent-Combaret, C. (2010). The *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol is a signal inducing rhizoplane expression of *Azospirillum* genes involved in plant-growth promotion. *Mol. Plant-Microbe Interact.* 24, 271–284. doi: 10.1094/MPMI-07-10-0148

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42, 669–678. doi: 10.1016/j.soilbio.2009.11.024

Coombs, J. T., and Franco, C. M. M. (2003). Isolation and Identification of *Actinobacteria* from surface-sterilized wheat roots. *Appl. Environ. Microbiol.* 69, 5603–5605. doi: 10.1128/aem.69.9.5603-5608.2003

Cui, L., Sun, L., Gao, X., Song, W., Wang, X. M., Li, H. L., et al. (2016). The impact of resistant and susceptible wheat cultivars on the multiplication of *Heterodera filipjevi* and *H. avenae* in parasite-infested soil. *Plant Pathol.* 65, 1192–1199. doi: 10.1111/ppa.12495

De Vita, P., Avio, L., Sbrana, C., Laidò, G., Marone, D., Mastrangelo, A. M., et al. (2018). Genetic markers associated to arbuscular mycorrhizal colonization in durum wheat. *Sci. Rep.* 8:10. doi: 10.1038/s41598-018-29020-6

Dellagi, A., Quillere, I., and Hirel, B. (2020). Beneficial soil-borne bacteria and fungi: a promising way to improve plant nitrogen acquisition. *J. Exp. Bot.* 71, 4469–4479. doi: 10.1093/jxb/eraa112

Derrien, D., Marol, C., and Balesdent, J. (2004). The dynamics of neutral sugars in the rhizosphere of wheat: an approach by 13C pulse-labelling and GC/C/IRMS. *Plant Soil* 267:243.

Di Benedetto, N. A., Campaniello, D., Bevilacqua, A., Cataldi, M. P., Sinigaglia, M., Flagella, Z., et al. (2019). Isolation, screening, and characterization of plant-growth-promoting bacteria from durum wheat rhizosphere to improve N and P nutrient use efficiency. *Microorganisms* 7:541. doi: 10.3390/microorganisms7110541

Di Paola, A., Caporaso, L., Di Paola, F., Bombelli, A., Vasenev, I., Nesterova, O. V., et al. (2018). The expansion of wheat thermal suitability of Russia in response to climate change. *Land Use Policy* 78, 70–77.

Díaz De León, J. L., Castellanos, T., Ling, J., Rojas-Hernández, A., and Röder, M. S. (2015). Quantitative trait loci underlying the adhesion of *Azospirillum brasilense* cells to wheat roots. *Euphytica* 204, 81–90. doi: 10.1007/s10681-014-1334-7

Ding, L. J., Cui, H. L., Nie, S. A., Long, X. E., Duan, G. L., and Zhu, Y. G. (2019). Microbiomes inhabiting rice roots and rhizosphere. *FEMS Microbiol. Ecol.* 95:fiz040. doi: 10.1093/femsec/fiz040

Dong, G., Wei, M., Yang, Y., Liu, R., Wang, J., Chen, L., et al. (2019). A brief history of wheat utilization in China. *Front Agr. Sci. Eng.* 6, 288–295. doi: 10.15302/J-FASE-2019266

Dong, W., Liu, E., Yan, C., Tian, J., Zhang, H., and Zhang, Y. (2017). Impact of no tillage vs. conventional tillage on the soil bacterial community structure in a winter wheat cropping succession in northern China. *Eur. J. Soil Biol.* 80, 35–42.

Donn, S., Kirkegaard, J. A., Perera, G., Richardson, A. E., and Watt, M. (2015). Evolution of bacterial communities in the wheat crop rhizosphere. *Environ. Microbiol.* 17, 610–621. doi: 10.1111/1462-2920.12452

Duhamel, M., and Vandenkoornhuyse, P. (2013). Sustainable agriculture: possible trajectories from mutualistic symbiosis and plant neodomestication. *Trends Plant Sci.* 18, 597–600. doi: 10.1016/j.tplants.2013.08.010

Dvorak, J., Luo, M. C., Yang, Z. L., and Zhang, H. B. (1998). The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. *Theor. Appl. Genet.* 97, 657–670. doi: 10.1016/j.compbiolchem.2019.107144

Dwivedi, S. L., Ceccarelli, S., Blair, M. W., Upadhyaya, H. D., Are, A. K., and Ortiz, R. (2016). Landrace germplasm for improving yield and abiotic stress adaptation. *Trends Plant Sci.* 21, 31–42. doi: 10.1016/j.tplants.2015.10.012

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Ellouze, W., Hamel, C., Singh, A. K., Mishra, V., DePauw, R. M., and Knox, R. E. (2018). Abundance of the arbuscular mycorrhizal fungal taxa associated with the roots and rhizosphere soil of different durum wheat cultivars in the Canadian prairies. *Can. J. Microbiol.* 64, 527–536. doi: 10.1139/cjm-2017-0637

El-Maraghy, S. S., Tohamy, T. A., and Hussein, K. A. (2020). Role of plant-growth promoting fungi (PGPF) in defensive genes expression of *Triticum aestivum* against wilt disease. *Rhizosphere* 15: 100.

Erginbaş Orakcı, G., Morgounov, A., and Dababat, A. A. (2018). Determination of resistance in winter wheat genotypes to the dryland root rots caused by *Fusarium culmorum* in Turkey. *Uluslar Tarım Yaban Hayatı Bilimleri Dergisi* 4, 193–202. doi: 10.24180/ijaws.414501

Fan, K., Weisenhorn, P., Gilbert, J. A., and Chu, H. (2018). Wheat rhizosphere harbors a less complex and more stable microbial co-occurrence pattern than bulk soil. *Soil Biol. Biochem.* 125, 251–260. doi: 10.1016/j.soilbio.2018.07.022

Feldman, M., and Kislev, M. E. (2007). Domestication of emmer wheat and evolution of free-threshing tetraploid wheat. *Isr. J. Plant Sci.* 55, 207–221.

Finkel, O. M., Castrillo, G., Herrera Paredes, S., Salas González, I., and Dangl, J. L. (2017). Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant Biol.* 38, 155–163. doi: 10.1016/j.pbi.2017.04.018

Finlay, B. J., Maberly, S. C., and Cooper, J. I. (1997). Microbial diversity and ecosystem function. *Oikos* 80, 209–213.

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., and Johnson, M. T. J. (2018). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proc. Natl. Acad. Sci. U.S.A.* 115, E1157–E1165. doi: 10.1073/pnas.1717617115

Fox, A. R., Soto, G., Valverde, C., Russo, D., Lagares, A., Zorreguieta, Á, et al. (2016). Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X. *Environ. Microbiol* 18, 3522–3534. doi: 10.1111/1462-2920.13376

Fricano, A., Brandolini, A., Rossini, L., Sourdille, P., Wunder, J., Effgen, S., et al. (2014). Crossability of *Triticum urartu* and *Triticum monococcum* wheats, homoeologous recombination, and description of a panel of interspecific introgression lines. *G3* 4, 1931–1941. doi: 10.1534/g3.114.013623

Fuhrman, J. A. (2009). Microbial community structure and its functional implications. *Nature* 459, 193–199. doi: 10.1038/nature08058

Gajda, A. M., Czyż, E. A., Stanek-Tarkowska, J., Furtak, K. M., and Grządziel, J. (2017). Effects of long-term tillage practices on the quality of soil under winter wheat. *Plant Soil Environ.* 63, 236–242. doi: 10.17221/223/2017-pse

Gantar, M., Kerby, N. W., and Rowell, P. (1993). Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria: III. The role of a hormogonia-promoting factor. *New Phytol.* 124, 505–513. doi: 10.1111/j.1469-8137.1993.tb03842.x

Ganugi, P., Masoni, A., Pietramellara, G., and Benedettelli, S. (2019). A review of studies from the last twenty years on plant–arbuscular mycorrhizal associations and their uses for wheat crops. *Agronomy* 9: 112.

Gayon, J., and Zallen, D. T. (1998). The role of the Vilmorin Company in the promotion and diffusion of the experimental science of heredity in France, 1840-1J. *Hist. Biol.* 31, 241–262. doi: 10.1023/a:1004335619901

George, T. S., French, A. S., Brown, L. K., Karley, A. J., White, P. J., Ramsay, L., et al. (2014). Genotypic variation in the ability of landraces and commercial cereal varieties to avoid manganese deficiency in soils with limited manganese availability: is there a role for root-exuded phytases? *Physiol. Plant.* 151, 243–256. doi: 10.1111/ppl.12151

Germida, J. J., Siciliano, S. D., Renato de Freitas, J., and Seib, A. M. (1998). Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiol. Ecol.* 26, 43–50. doi: 10.1111/j.1574-6941.1998.tb01560.x

Germida, J., and Siciliano, S. (2001). Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. *Biol. Fertil. Soil.* 33, 410–415. doi: 10.1007/s003740100343

Gill, B. S., Appels, R., Botha-Oberholster, A. M., Buell, C. R., Bennetzen, J. L., Chalhoub, B., et al. (2004). A workshop report on wheat genome sequencing: international genome research on wheat consortium. *Genetics* 168, 1087–1096. doi: 10.1534/genetics.104.034769

Gill, S., Abid, M., and Azam, F. (2006). Root-induced changes in potential nitrification and nitrate reductase activity of the rhizospheric soil of wheat (*Triticum aestivum* l.) and chickpea (*Cicer arietinum* l.). *Pak. J. Bot.* 38:991.

Glémin, S., Scornavacca, C., Dainat, J., Burgarella, C., Viader, V., Ardisson, M., et al. (2019). Pervasive hybridizations in the history of wheat relatives. *Sci. Adv.* 5:eaav9188. doi: 10.1126/sciadv.aav9188

Glick, B. R. (2005). Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol. Lett.* 251, 1–7. doi: 10.1016/j.femsle.2005.07.030

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

Glover, N. M. (2016). Homoeologs: what are they and how do we infer them? *Trends Plant Sci.* 21, 609–621. doi: 10.1016/j.tplants.2016.02.005

Golan, G., Hendel, E., Espitia, G. E. M., Schwartz, N., and Peleg, Z. (2018). Activation of seminal root primordia during wheat domestication reveals underlying mechanisms of plant resilience. *Plant Cell Environ.* 41, 755–766. doi: 10.1111/pce.13138

Golizadeh, V. M., Dashti, H., Riseh, R. S., and Bihamta, M. R. (2017). Screening bread wheat germplasm for resistance to take-all disease (*Gaeumannomyces graminis* var. tritici) in greenhouse conditions. *J. Agric. Sci. Tech.* 19, 1173–1184.

Gqozo, M. P., Bill, M., Siyoum, N., Labuschagne, N., and Korsten, L. (2020). Fungal diversity and community composition of wheat rhizosphere and non-rhizosphere soils from three different agricultural production regions of South Africa. *Appl. Soil Ecol.* 151: 103543. doi: 10.1016/j.apsoil.2020.103543

Gu, Y. H., and Mazzola, M. (2003). Modification of fluorescent pseudomonad community and control of apple replant disease induced in a wheat cultivar-specific manner. *Appl. Soil Ecol.* 24, 57–72. doi: 10.1016/s0929-1393(03)00066-0

Guo, X., Zhou, X., Hale, L., Yuan, M., Feng, J., Ning, D., et al. (2018). Taxonomic and functional responses of soil microbial communities to annual removal of aboveground plant biomass. *Front. Microbiol.* 9:954. doi: 10.3389/fmicb.2018.00954

Gyaneshwar, P., Naresh Kumar, G., Parekh, L. J., and Poole, P. S. (2002). Role of soil microorganisms in improving P nutrition of plants. *Plant Soil* 245, 83–93.

Haas, M., Schreiber, M., and Mascher, M. (2019). Domestication and crop evolution of wheat and barley: genes, genomics, and future directions. *J. Integr. Plant Biol.* 61, 204–225. doi: 10.1111/jipb.12737

Haberer, G., Mayer, K. F., and Spannagl, M. (2016). The big five of the monocot genomes. *Curr. Opin. Plant Biol.* 30, 33–40. doi: 10.1016/j.pbi.2016.01.004

Hagn, A., Pritsch, K., Schloter, M., and Munch, J. C. (2003). Fungal diversity in agricultural soil under different farming management systems, with special reference to biocontrol strains of *Trichoderma* spp. *Biol. Fertil. Soil.* 38, 236–244.

Haichar, F. Z., Heulin, T., Guyonnet, J. P., and Achouak, W. (2016). Stable isotope probing of carbon flow in the plant holobiont. *Curr. Opin. Biotechnol* 41, 9–13. doi: 10.1016/j.copbio.2016.02.023

Haichar, F. Z., 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, 1221–1230. doi: 10.1038/ismej.2008.80

Hamel, P., Saint-Georges, Y., Pinto, B. D., Lachacinski, N., Altamura, N., and Dujardin, G. (2004). Redundancy in the function of mitochondrial phosphate transport in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. *Mol. Microbiol* 51, 307–317. doi: 10.1046/j.1365-2958.2003.03810.x

Hamonts, K., Clough, T. J., Stewart, A., Clinton, P. W., Richardson, A. E., Wakelin, S. A., et al. (2013). Effect of nitrogen and waterlogging on denitrifier gene abundance, community structure and activity in the rhizosphere of wheat. *FEMS Microbiol. Ecol.* 83, 568–584. doi: 10.1111/1574-6941.12015

Hassan, W., Bano, R., Bashir, F., and David, J. (2014). Comparative effectiveness of ACC-deaminase and/or nitrogen-fixing rhizobacteria in promotion of maize (*Zea mays* L.) growth under lead pollution. *Environ. Sci. Pollut. Res.* 21, 10983–10996.

Hassani, M. A., Duran, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6:58. doi: 10.1186/s40168-018-0445-0

Hassani, M. A., Özkurt, E., Franzenburg, S., and Stukenbrock, E. (2020). Ecological assembly processes of the bacterial and fungal microbiota of wild and domesticated wheat species. *Phytobiomes J.* 4, 217–224. doi: 10.1094/pbiomes-01-20-0001-sc

Hayashi, K., Tokida, T., Kajiura, M., Yanai, Y., and Yano, M. (2015). Cropland soil–plant systems control production and consumption of methane and nitrous oxide and their emissions to the atmosphere. *Soil Sci. Plant Nutr.* 61, 2–33.

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

Hetrick, B. A. D., Wilson, G. W. T., and Cox, T. S. (1992). Mycorrhizal dependence of modern wheat varieties, landraces, and ancestors. *Can. J. Bot.* 70, 2032–2518. doi: 10.1139/b92-253

Higginbotham, R. W., Paulitz, T. C., Campbell, K. G., and Kidwell, K. K. (2004). Evaluation of adapted wheat cultivars for tolerance to Pythium root rot. *Plant Dis.* 88, 1027–1032. doi: 10.1094/PDIS.2004.88.9.1027

Hiltner, L. (1904). Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung und Brache. *Arb DLG* 98, 59–78.

Hilton, S., Bennett, A. J., Chandler, D., Mills, P., and Bending, G. D. (2018). Preceding crop and seasonal effects influence fungal, bacterial and nematode diversity in wheat and oilseed rape rhizosphere and soil. *Appl. Soil Ecol.* 126, 34–46. doi: 10.1016/j.apsoil.2018.02.007

Hodgson, S., de Cates, C., Hodgson, J., Morley, N. J., Sutton, B. C., and Gange, A. C. (2014). Vertical transmission of fungal endophytes is widespread in forbs. *Ecol. Evol.* 4, 1199–1208. doi: 10.1002/ece3.953

Houlden, A., Timms-Wilson, T. M., Day, M. J., and Bailey, M. J. (2008). Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops. *FEMS Microbiol. Ecol.* 65, 193–201. doi: 10.1111/j.1574-6941.2008.00535.x

Huang, M., Sanchez-Moreiras, A. M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., et al. (2012). The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (E)−β−caryophyllene, is a defense against a bacterial pathogen. *New Phytol.* 193, 997–1008. doi: 10.1111/j.1469-8137.2011.04001.x

Iannucci, A., Canfora, L., Nigro, F., De Vita, P., and Beleggia, R. (2021). Relationships between root morphology, root exudate compounds and rhizosphere microbial community in durum wheat. *Appl. Soil Ecol.* 158, 103781. doi: 10.1016/j.apsoil.2020.103781

Imperiali, N., Chiriboga, X., Schlaeppi, K., Fesselet, M., Villacrés, D., Jaffuel, G., et al. (2017). Combined field inoculations of *Pseudomonas* bacteria, arbuscular mycorrhizal fungi, and entomopathogenic nematodes and their effects on wheat performance. *Front. Plant Sci.* 8:1809. doi: 10.3389/fpls.2017.01809

Ingram, D. M., and Cook, R. J. (1990). Pathogenicity of four *Pythium* species to wheat, barley, peas and lentils. *Plant Pathol.* 39, 110–117. doi: 10.1111/j.1365-3059.1990.tb02481.x

International Wheat Genome Sequencing Consortium [IWGSC], Appels, R., Eversole, K., Stein, N., Feuillet, C., Keller, B., et al. (2018). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science* 361:eaar7191. doi: 10.1126/science.aar7191

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., and Kopriva, S. (2017). The role of soil microorganisms in plant mineral nutrition—Current knowledge and future directions. *Front. Plant Sci.* 8:1617. doi: 10.3389/fpls.2017.01617

Janzen, H. H. (1990). Deposition of nitrogen into the rhizosphere by wheat roots. *Soil Biol. Biochem.* 22, 1155–1160. doi: 10.1111/nph.13966

Jia, J., Zhao, S., Kong, X., Li, Y., Zhao, G., He, W., et al. (2013). *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. *Nature* 496, 91–95. doi: 10.1038/nature12028

Jog, R., Pandya, M., Nareshkumar, G., and Rajkumar, S. (2014). Mechanism of phosphate solubilization and antifungal activity of *Streptomyces* spp. isolated from wheat roots and rhizosphere and their application in improving plant growth. *Microbiology* 160, 778–788. doi: 10.1099/mic.0.074146-0

Juhnke, M. E., Mathre, D. E., and Sands, D. C. (1987). Identification and characterization of rhizosphere-competent bacteria of wheat. *Appl. Environ. Microbiol.* 53, 2793–2799. doi: 10.1128/aem.53.12.2793-2799.1987

Kang, X., Zhang, W., Cai, X., Zhu, T., Xue, Y., and Liu, C. (2018). *Bacillus velezensis* CC09: a potential 'vaccine' for controlling wheat diseases. *Mol. Plant-Microbe Interact.* 31, 623–632. doi: 10.1094/MPMI-09-17-0227-R

Kapulnik, Y., and Kushnir, U. (1991). Growth dependency of wild, primitive and modern cultivated wheat lines on vesicular-arbuscular mycorrhiza fungi. *Euphytica* 56, 27–36. doi: 10.1007/bf00041740

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

Kavamura, V. N., Mendes, R., Bargaz, A., and Mauchline, T. H. (2021). Defining the wheat microbiome: Towards microbiome-facilitated crop production. *Comput. Struct. Biotechnol. J.* 19, 1200–1213. doi: 10.1016/j.csbj.2021.01.045

Kavamura, V. N., Robinson, R. J., Hughes, D., Clark, I., Rossmann, M., Melo, I. S., et al. (2020). Wheat dwarfing influences selection of the rhizosphere microbiome. *Sci. Rep.* 10:1452. doi: 10.1038/s41598-020-58402-y

Kazi, N., Deaker, R., Wilson, N., Muhammad, K., and Trethowan, R. (2016). The response of wheat genotypes to inoculation with *Azospirillum brasilense* in the field. *Field Crop Res.* 196, 368–378.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., et al. (1992). Suppression of root diseases by *Pseudomonas* fluorescens CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol. Plant-Microbe Interact.* 5, 4–13. doi: 10.1094/mpmi-5-004

Kinnunen-Grubb, M., Sapkota, R., Vignola, M., Nunes, I. M., and Nicolaisen, M. (2020). Breeding selection imposed a differential selective pressure on the wheat

root-associated microbiome. *FEMS Microbiol. Ecol.* 96:fiaa196. doi: 10.1093/femsec/fiaa196

Kiszonas, A. M., and Morris, C. F. (2018). Wheat breeding for quality: a historical review. *Cereal Chem.* 95, 17–34.

Kumar, P., Thakur, S., Dhingra, G. K., Singh, A., Pal, M. K., Harshvardhan, K., et al. (2018). Inoculation of siderophore producing rhizobacteria and their consortium for growth enhancement of wheat plant. *Biocatal. Agric. Biotechnol.* 15, 264–269. doi: 10.1016/j.bcab.2018.06.019

Kundu, B. S., Nehra, K., Yadav, R., and Tomar, M. (2009). Biodiversity of phosphate solubilizing bacteria in rhizosphere of chickpea, mustard and wheat grown in different regions of Haryana. *Indian J. Microbiol.* 49, 120–127. doi: 10.1007/s12088-009-0016-y

Kuzyakov, Y., and Cheng, W. (2001). Photosynthesis controls of rhizosphere respiration and organic matter decomposition. *Soil Biol. Biochem.* 33, 1915–1925. doi: 10.1016/s0038-0717(01)00117-1

Kwak, Y. S., and Weller, D. M. (2013). Take-all of wheat and natural disease suppression: a review. *Plant Pathol. J.* 29, 125–135. doi: 10.5423/PPJ.SI.07.2012.0112

Lambers, H., Mougel, C., Jaillard, B., and Hinsinger, P. (2009). Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. *Plant Soil* 321, 83–115. doi: 10.3389/fpls.2021.636709

Landa, B. B., Mavrodi, D. M., Thomashow, L. S., and Weller, D. M. (2003). Interactions between strains of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* in the rhizosphere of wheat. *Phytopathology* 93, 982–994. doi: 10.1094/phyto.2003.93.8.982

Latz, E., Eisenhauer, N., Scheu, S., and Jousset, A. (2015). Plant identity drives the expression of biocontrol factors in a rhizosphere bacterium across a plant diversity gradient. *Funct. Ecol.* 29, 1225–1234.

Lehnert, H., Serfling, A., Enders, M., Friedt, W., and Ordon, F. (2017). Genetics of mycorrhizal symbiosis in winter wheat (*Triticum aestivum*). *New Phytol.* 215, 779–791. doi: 10.1111/nph.14595

Lemanceau, P., Blouin, M., Muller, D., and Moënne-Loccoz, Y. (2017). Let the core microbiota be functional. *Trends Plant Sci.* 22, 583–595. doi: 10.1016/j.tplants.2017.04.008

Li, C., Zhou, A., and Sang, T. (2006). Genetic analysis of rice domestication syndrome with the wild annual species *Oryza nivara*. *New Phytol.* 170, 185–193. doi: 10.1111/j.1469-8137.2005.01647.x

Lindig-Cisneros, R., Benrey, B., and Espinosa-García, F. (1997). Phytoalexins, resistance traits, and domestication status in *Phaseolus coccineus* and *Phaseolus lunatus*. *J. Chem. Ecol.* 23, 1997–2011.

Ling, H. Q., Zhao, S., Liu, D., Wang, J., Sun, H., Zhang, C., et al. (2013). Draft genome of the wheat A-genome progenitor *Triticum urartu*. *Nature* 496, 87–90. doi: 10.1038/nature11997

Liu, Y., Zuo, S., Xu, L., Zou, Y., and Song, W. (2012). Study on diversity of endophytic bacterial communities in seeds of hybrid maize and their parental lines. *Arch. Microbiol.* 194, 1001–1012. doi: 10.1007/s00203-012-0836-8

Lobell, D. B., Cassman, K. G., and Field, C. B. (2009). Crop yield gaps: their importance, magnitudes, and causes. *Annu. Rev. Environ. Resour.* 34, 179–204. doi: 10.1111/gcb.13617

Louca, S., Parfrey, L. W., and Doebeli, M. (2016). Decoupling function and taxonomy in the global ocean microbiome. *Science* 353, 1272–1277. doi: 10.1126/science.aaf4507

Lu, T., Ke, M., Peijnenburg, W. J. G. M., Zhu, Y., Zhang, M., Sun, L., et al. (2018). Investigation of rhizospheric microbial communities in wheat, barley, and two rice varieties at the seedling stage. *J. Agric. Food Chem.* 66, 2645–2653. doi: 10.1021/acs.jafc.7b06155

Maccaferri, M., Cane', M., Sanguineti, M. C., Salvi, S., Colalongo, M. C., Massi, A., et al. (2014). A consensus framework map of durum wheat (*Triticum durum* desf.) suitable for linkage disequilibrium analysis and genome-wide association mapping. *BMC Genomics* 15:873. doi: 10.1186/1471-2164-15-873

Mahmoudi, T. R., Yu, J. M., Liu, S., Pierson, L. S., and Pierson, E. A. (2019). Drought-stress tolerance in wheat seedlings conferred by phenazine-producing rhizobacteria. *Front. Microbiol.* 10:1590. doi: 10.3389/fmicb.2019.01590

Mahoney, A. K., Yin, C., and Hulbert, S. H. (2017). Community structure, species variation, and potential functions of rhizosphere-associated bacteria of different winter wheat (*Triticum aestivum*) cultivars. *Front. Plant Sci.* 8:132. doi: 10.3389/fpls.2017.00132

Majeed, A., Abbasi, M. K., Hameed, S., Imran, A., and Rahim, N. (2015). Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. *Front. Microbiol.* 6:198. doi: 10.3389/fmicb.2015.00198

Maketon, C., Fortuna, A.-M., and Okubara, P. A. (2012). Cultivar-dependent transcript accumulation in wheat roots colonized by *Pseudomonas* fluorescens Q8r1-96 wild type and mutant strains. *Biol. Control* 60, 216–224. doi: 10.1016/j.biocontrol.2011.11.002

Mao, Y., Yannarell, A. C., Davis, S. C., and Mackie, R. I. (2013). Impact of different bioenergy crops on N-cycling bacterial and archaeal communities in soil. *Environ. Microbiol.* 15, 928–942.

Martín-Robles, N., Lehmann, A., Seco, E., Aroca, R., Rillig, M. C., and Milla, R. (2018). Impacts of domestication on the arbuscular mycorrhizal symbiosis of 27 crop species. *New Phytol* 218, 322–334. doi: 10.1111/nph.14962

Matthews, A., Pierce, S., Hipperson, H., and Raymond, B. (2019). Rhizobacterial community assembly patterns vary between crop species. *Front. Microbiol.* 10:581. doi: 10.3389/fmicb.2019.00581

Mavrodi, D. V., Parejko, J. A., Mavrodi, O. V., Kwak, Y. S., Weller, D. M., Blankenfeldt, W., et al. (2013). Recent insights into the diversity, frequency and ecological roles of phenazines in fluorescent *Pseudomonas* spp. *Environ Microbiol.* 15, 675–686. doi: 10.1111/j.1462-2920.2012.02846.x

Mavrodi, O. V., Walter, N., Elateek, S., Taylor, C. G., and Okubara, P. A. (2012). Suppression of Rhizoctonia and Pythium root rot of wheat by new strains of *Pseudomonas*. *BioControl* 62, 93–102. doi: 10.1016/j.biocontrol.2012.03.013

Mazzola, M., Funnell, D. L., and Raaijmakers, J. M. (2004). Wheat cultivar-specific selection of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* species from resident soil populations. *Microb. Ecol.* 48, 338–348. doi: 10.1007/s00248-003-1067-y

Mazzola, M., and Gu, Y.-H. (2002). Wheat genotype-specific induction of soil microbial communities suppressive to disease incited by *Rhizoctonia solani* anastomosis group (AG)-5 and AG-8. *Phytopathology* 92, 1300–1307. doi: 10.1094/PHYTO.2002.92.12.1300

McMillan, V. E., Gutteridge, R. J., and Hammond-Kosack, K. E. (2014). Identifying variation in resistance to the take-all fungus, *Gaeumannomyces graminis var. tritici*, between different ancestral and modern wheat species. *BMC Plant Biol.* 14:212. doi: 10.1186/s12870-014-0212-8

Meyer, J. B., Lutz, M. P., Frapolli, M., Péchy-Tarr, M., Rochat, L., Keel, C., et al. (2010). Interplay between wheat cultivars, biocontrol pseudomonads, and soil. *Appl. Environ. Microbiol.* 76, 6196–6204. doi: 10.1128/AEM.00752-10

Meziani, S., Nadaud, I., Gaillard-Martinie, B., Chambon, C., Benali, M., and Branlard, G. (2019). Proteomic analysis of mature kernel aleurone layer of *Triticum spelta* and three wheat related species. *Nutr. Santé* 8, 27–35.

Micallef, S. A., Shiaris, M. P., and Colón-Carmona, A. (2009). Influence of accessions on rhizobacterial communities and natural variation in root exudates. *J. Exp. Bot.* 60, 1729–1742. doi: 10.1093/jxb/erp053

Mideksa, T., Letta, T., Bayisa, T., Abinasa, M., Tilahun, A., Hundie, B., et al. (2018). Bread wheat varietal development and release in southeastern highlands of Ethiopia. *Am. J. Biol. Environ. Stat.* 4, 15–19. doi: 10.11648/j.ajbes.20180401.13

Moënne-Loccoz, Y., Mavingui, P., Combes, C., Normand, P., and Steinberg, C. (2015). "Microorganisms and biotic interactions," in *Environmental Microbiology: Fundamentals and Applications*, eds J. C. Bertrand, P. Caumette, P. Lebaron, R. Matheron, P. Normand, and T. Sime-Ngando (Dordrecht: Springer), 395–444. doi: 10.1007/978-94-017-9118-2_11

Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L. (2008). Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato. *Mol. Plant-Microbe Interact.* 21, 1001–1009. doi: 10.1094/MPMI-21-7-1001

Mujeeb-Kazi, A. (2006). "Utilization of genetic resources for bread wheat improvement," in *Genetic Resources, Chromosome Engineering, and Crop Improvement*, eds R. J. Singh and P. P. Jauhar (Boca Raton, FL: CRC Series), 61–97. doi: 10.1201/9780203489260.ch3

Mustafa, G., Randoux, B., Tisserant, B., Fontaine, J., Magnin-Robert, M., Lounès-Hadj Sahraoui, A., et al. (2016). Phosphorus supply, arbuscular mycorrhizal fungal species, and plant genotype impact on the protective efficacy of mycorrhizal inoculation against wheat powdery mildew. *Mycorrhiza* 26, 685–697. doi: 10.1007/s00572-016-0698-z

Narula, N., Kumar, V., Behl, R. K., Deubel, A., Gransee, A., and Merbach, W. (2000). Effect of P-solubilizing *Azotobacter chroococcum* on N, P, K uptake in

P-responsive wheat genotypes grown under greenhouse conditions. *J. Plant Nutr. Soil Sci.* 163, 393–398.

Naz, I., Mirza, M. S., and Bano, A. (2018). Molecular characterization of rhizosphere bacterial communities associated with wheat (*Triticum aestivum* l.) cultivars at flowering stage. *J. Anim. Plant Sci.* 24, 1123–1134.

Newton, A. C., Fitt, B. D. L., Atkins, S. D., Walters, D. R., and Daniell, T. J. (2010). Pathogenesis, parasitism and mutualism in the trophic space of microbe–plant interactions. *Trends Microbiol.* 18, 365–373. doi: 10.1016/j.tim.2010.06.002

Niu, Z., Klindworth, D. L., Friesen, T. L., Chao, S., Jin, Y., Cai, X., et al. (2011). Targeted introgression of a wheat stem rust resistance gene by DNA marker-assisted chromosome engineering. *Genetics* 187, 1011–1021. doi: 10.1534/genetics.110.123588

Nowell, R. W., Laue, B. E., Sharp, P. M., and Green, S. (2016). Comparative genomics reveals genes significantly associated with woody hosts in the plant pathogen *Pseudomonas syringae*: adaptation to woody hosts in *Pseudomonas syringae*. *Mol. Plant Pathol.* 17, 1409–1424. doi: 10.1111/mpp.12423

Nuccio, E. E., Starr, E., Karaoz, U., Brodie, E. L., Zhou, J., Tringe, S. G., et al. (2020). Niche differentiation is spatially and temporally regulated in the rhizosphere. *ISME J.* 14, 999–1014. doi: 10.1038/s41396-019-0582-x

O'Sullivan, C. A., Fillery, I. R. P., Roper, M. M., and Richards, R. A. (2016). Identification of several wheat landraces with biological nitrification inhibition capacity. *Plant Soil* 404, 61–74. doi: 10.1007/s11104-016-2822-4

Ofaim, S., Ofek-Lalzar, M., Sela, N., Jinag, J., Kashi, Y., Minz, D., et al. (2017). Analysis of microbial functions in the rhizosphere using a metabolic-network based framework for metagenomics interpretation. *Front. Microbiol.* 8:1606. doi: 10.3389/fmicb.2017.01606

Ofek-Lalzar, M., Gur, Y., Ben-Moshe, S., Sharon, O., Kosman, E., Mochli, E., et al. (2016). Diversity of fungal endophytes in recent and ancient wheat ancestors *Triticum dicoccoides* and *Aegilops sharonensis*. *FEMS Microbiol. Ecol.* 92, fiw152. doi: 10.1093/femsec/fiw152

Okubara, P. A., and Bonsall, R. F. (2008). Accumulation of *Pseudomonas*-derived 2,4-diacetylphloroglucinol on wheat seedling roots is influenced by host cultivar. *Biol. Control* 46, 322–331. doi: 10.1016/j.biocontrol.2008.03.013

Okubara, P. A., Call, D. R., Kwak, Y., and Skinner, D. Z. (2010). Induction of defense gene homologues in wheat roots during interactions with *Pseudomonas fluorescens*. *BioControl* 55, 118–125.

Oros, G., Naár, Z., and Magyar, D. (2013). Susceptibility of wheat varieties to soil-borne *Rhizoctonia* infection. *Am. J. Plant Sci.* 4:2.

Osborne, S. J., McMillan, V. E., White, R., and Hammond-Kosack, K. E. (2018). Elite UK winter wheat cultivars differ in their ability to support the colonization of beneficial root-infecting fungi. *J. Exp. Bot.* 69, 3103–3115. doi: 10.1093/jxb/ery136

Özkan, H., Willcox, G., Graner, A., Salamini, F., and Kilian, B. (2011). Geographic distribution and domestication of wild emmer wheat (*Triticum dicoccoides*). *Genet. Resour. Crop Evol.* 58, 11–53. doi: 10.1007/s10722-010-9581-5

Özkurt, E., Hassani, M. A., Sesiz, U., Künzel, S., Dagan, T., Özkan, H., et al. (2020). Seed-derived microbial colonization of wild emmer and domesticated bread wheat (*Triticum dicoccoides* and *Triticum aestivum*) seedlings shows pronounced differences in overall diversity and composition. *mBio* 11:e02637–20. doi: 10.1128/mBio.02637-20

Pagnani, G., Galieni, A., Stagnari, F., Pellegrini, M., Del Gallo, M., and Pisante, M. (2020). Open field inoculation with PGPR as a strategy to manage fertilization of ancient *Triticum* genotypes. *Biol. Fertil. Soils* 56, 111–124. doi: 10.1007/s00374-019-01407-1

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.* 7:1. doi: 10.3389/fpls.2016.01110

Peleg, Z., Fahima, T., Korol, A. B., Abbo, S., and Saranga, Y. (2011). Genetic analysis of wheat domestication and evolution under domestication. *J. Exp. Bot.* 62, 5051–5061. doi: 10.1093/jxb/err206

Pellegrino, E., Öpik, M., Bonari, E., and Ercoli, L. (2015). Responses of wheat to arbuscular mycorrhizal fungi: a meta-analysis of field studies from 1975 to 2013. *Soil Biol. Biochem.* 84, 210–217.

Peng, J., Ronin, Y., Fahima, T., Roder, M. S., Li, Y., Nevo, E., et al. (2003). Domestication quantitative trait loci in *Triticum dicoccoides*, the progenitor of wheat. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2489–2494. doi: 10.1073/pnas.252763199

Pérez-de-Luque, A., Tille, S., Johnson, I., Pascual-Pardo, D., Ton, J., and Cameron, D. D. (2017). The interactive effects of arbuscular mycorrhiza and plant growth-promoting rhizobacteria synergistically enhance host plant defences against pathogens. *Sci. Rep.* 7:16409. doi: 10.1038/s41598-017-16697-4

Pérez-Jaramillo, J. E., Carrión, V. J., de Hollander, M., and Raaijmakers, J. M. (2018). The wild side of plant microbiomes. *Microbiome* 6:143. doi: 10.1186/s40168-018-0519-z

Perin, L., Martínez-Aguilar, L., Castro-González, R., Santos, P. E., Cabellos-Avelar, T., Guedes, H. V., et al. (2006). Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. *Appl. Environ. Microbiol.* 72, 3103–3110. doi: 10.1128/AEM.72.5.3103-3110.2006

Pestsova, E. G., Börner, A., and Röder, M. S. (2005). Development and QTL assessment of *Triticum aestivum–Aegilops tauschii* introgression lines. *Theor. Appl. Genet.* 112:634. doi: 10.1007/s00122-005-0166-1

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

Pont, C., Leroy, T., Seidel, M., Tondelli, A., Duchemin, W., Armisen, D., et al. (2019). Tracing the ancestry of modern bread wheats. *Nat. Genet.* 51, 905–911. doi: 10.1038/s41588-019-0393-z

Prudence, S. M. M., Newitt, J. T., Worsley, S. F., Macey, M. C., Murrell, J. C., Lehtovirta-Morley, L. E., et al. (2021). Soil, senescence and exudate utilisation: characterisation of the Paragon var. spring bread wheat root microbiome. *Environ. Microbiome* 16:12. doi: 10.1186/s40793-021-00381-2

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361. doi: 10.1007/s11104-008-9568-6

Rascovan, N., Carbonetto, B., Perrig, D., Díaz, M., Canciani, W., Abalo, M., et al. (2016). Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. *Sci. Rep.* 6, 1–12. doi: 10.1038/srep28084

Reinhold-Hurek, B., and Hurek, T. (2011). Living inside plants: bacterial endophytes. *Curr. Opin. Plant Biol.* 14, 435–443. doi: 10.1016/j.pbi.2011.04.004

Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., and Hurek, T. (2015). Roots shaping their microbiome: global hotspots for microbial activity. *Annu. Rev. Phytopathol.* 53, 403–424. doi: 10.1146/annurev-phyto-082712-102342

Rilling, J. I., Acuña, J. J., Sadowsky, M. J., and Jorquera, M. A. (2018). Putative nitrogen-fixing bacteria associated with the rhizosphere and root endosphere of wheat plants grown in an andisol from southern Chile. *Front. Microbiol.* 9:2710. doi: 10.3389/fmicb.2018.02710

Rong, J. K., Millet, E., Manisterski, J., and Feldman, M. (2000). A new powdery mildew resistance gene: Introgression from wild emmer into common wheat and RFLP-based mapping. *Euphytica* 115, 121–126.

Rossmann, M., Chiaramonte, B., Dumack, K., Fiore-Donno, A. M., Mendes, L. W., Raaijmakers, J. M., et al. (2020). Multitrophic interactions in the rhizosphere microbiome of wheat: from bacteria and fungi to protists. *FEMS Microbiol. Ecol.* 96:fiaa032. doi: 10.1093/femsec/fiaa032

Roucou, A., Violle, C., Fort, F., Roumet, P., Ecarnot, M., and Vile, D. (2018). Shifts in plant functional strategies over the course of wheat domestication. *J. Appl. Ecol.* 55, 25–37. doi: 10.1111/1365-2664.13029

Saia, S., Fragasso, M., De Vita, P., and Beleggia, R. (2019). Metabolomics provides valuable insight for the study of durum wheat: a review. *J. Agric. Food Chem.* 67, 3069–3085. doi: 10.1021/acs.jafc.8b07097

Salamini, F., Özkan, H., Brandolini, A., Schäfer-Pregl, R., and Martin, W. (2002). Genetics and geography of wild cereal domestication in the near east. *Nat. Rev. Genet.* 3, 429–441. doi: 10.1038/nrg817

Salem, G., Stromberger, M. E., Byrne, P. F., Manter, D. K., El-Feki, W., and Weir, T. L. (2018). Genotype-specific response of winter wheat (*Triticum aestivum* L.) to irrigation and inoculation with ACC deaminase bacteria. *Rhizosphere* 8, 1–7. doi: 10.1016/j.rhisph.2018.08.001

Santi, C., Bogusz, D., and Franche, C. (2013). Biological nitrogen fixation in non-legume plants. *Ann. Bot.* 111, 743–767. doi: 10.1093/aob/mct048

Sato, K., and Jiang, H. Y. (1996a). Gram-positive bacterial flora on the root surface of wheat (*Triticum aestivum* L.) grown under different soil conditions. *Biol. Fertil. Soils* 23, 121–125. doi: 10.1007/s003740050148

Sato, K., and Jiang, H. Y. (1996b). Gram-negative bacterial flora on the root surface of wheat (*Triticum aestivum*) grown under different soil conditions. *Biol. Fertil. Soils* 23, 273–281. doi: 10.1007/bf00335955

Schloter, M., and Hartmann, A. (1998). Endophytic and surface colonization of wheat roots (*Triticum aestivum*) by different *Azospirillum brasilense* strains studied with strain-specific monoclonal antibodies. *Symbiosis* 25, 159–179.

Schulz, B., and Boyle, C. (2006). "What are endophytes?," in *Microbial Root Endophytes*, eds B. J. E. Schulz, C. J. C. Boyle, and T. N. Sieber (Berlin: Springer), 1–13. doi: 10.1007/3-540-33526-9_1

Seguel, A., Castillo, C. G., Morales, A., Campos, P., Cornejo, P., and Borie, F. (2016). Arbuscular mycorrhizal symbiosis in four Al-tolerant wheat genotypes grown in an acidic Andisol. *J. Soil Sci. Plant Nutr.* 16, 164–173.

Shakir, M. A., Bano, A., and Arshad, M. (2012). Rhizosphere bacteria containing ACC-deaminase conferred drought tolerance in wheat grown under semi-arid climate. *Soil Environ.* 31, 108–112.

Shaposhnikov, A. I., Morgounov, A. I., Akin, B., Makarova, N. M., Belimov, A. A., and Tikhonovich, I. A. (2016). Comparative characteristics of root systems and root exudation of synthetic, landrace and modern wheat varieties. *Agric. Biol.* 51, 68–78.

Sheng, X. F., and He, L. Y. (2011). Solubilization of potassium-bearing minerals by a wild-type strain of *Bacillus edaphicus* and its mutants and increased potassium uptake by wheat. *Can. J. Microbiol.* 52, 56–72. doi: 10.1139/w05-117

Shi, S., Chang, J., Tian, L., Nasir, F., Ji, L., Li, X., et al. (2019). Comparative analysis of the rhizomicrobiome of the wild versus cultivated crop: insights from rice and soybean. *Arch. Microbiol.* 201, 879–888. doi: 10.1007/s00203-019-01638-8

Siciliano, S. D., Theoret, C. M., de Freitas, J. R., Hucl, P. J., and Germida, J. J. (1998). Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. *Can. J. Microbiol.* 44, 844–851. doi: 10.1139/w98-075

Simon, J. C., Marchesi, J. R., Mougel, C., and Selosse, M.-A. (2019). Host-microbiota interactions: from holobiont theory to analysis. *Microbiome* 7: 5. doi: 10.1186/s40168-019-0619-4

Simonin, M., Dasilva, C., Terzi, V., Ngonkeu, E. L. M., Diouf, D., Kane, A., et al. (2020). Influence of plant genotype and soil on the wheat rhizosphere microbiome: evidences for a core microbiome across eight African and European soils. *FEMS Microbiol. Ecol.* 96:fiaa067. doi: 10.1093/femsec/fiaa067

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J. D., and Wernars, K. (1999). Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* 65, 2614–2621. doi: 10.1128/AEM.65.6.2614-2621.1999

Somenahally, A., DuPont, J. I., Brady, J., McLawrence, J., Northup, B., and Gowda, P. (2018). Microbial communities in soil profile are more responsive to legacy effects of wheat-cover crop rotations than tillage systems. *Soil Biol. Biochem.* 123, 126–135. doi: 10.1016/j.soilbio.2018.04.025

Soriano, J. M., Villegas, D., Aranzana, M. J., del Moral, L. F. G., and Royo, C. (2016). Genetic structure of modern durum wheat cultivars and mediterranean landraces matches with their agronomic performance. *PLoS One* 11:e0160983. doi: 10.1371/journal.pone.0160983

Spaepen, S., Vanderleyden, J., and Remans, R. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* 31, 425–448. doi: 10.1111/j.1574-6976.2007.00072.x

Spor, A., Roucou, A., Mounier, A., Bru, D., Breuil, M.-C., Fort, F., et al. (2020). Domestication-driven changes in plant traits associated with changes in the assembly of the rhizosphere microbiota in tetraploid wheat. *Sci. Rep.* 10:12234. doi: 10.1038/s41598-020-69175-9

Šramková, Z., Gregová, E., and Šturdík, E. (2009). Chemical composition and nutritional quality of wheat grain. *Acta Chimi. Slov.* 2, 115–138.

Stromberger, M. E., Abduelafez, I., Byrne, P., Canela, M. M., Elamari, A. A., Manter, D. K., et al. (2017). Genotype-specific enrichment of 1-aminocyclopropane-1-carboxylic acid deaminase-positive bacteria in winter wheat rhizospheres. *Soil Sci. Soc. Am. J.* 81, 796–805. doi: 10.2136/sssaj2016.12.0437

Szoboszlay, M., Näther, A., Liu, B., Carrillo, A., Castellanos, T., Smalla, K., et al. (2019). Contrasting microbial community responses to salinization and straw amendment in a semiarid bare soil and its wheat rhizosphere. *Sci. Rep.* 9:9795. doi: 10.1038/s41598-019-46070-6

Tian, Z., Wang, J. W., Li, J., and Han, B. (2021). Designing future crops: challenges and strategies for sustainable agriculture. *Plant J.* 105, 1165–1178. doi: 10.1111/tpj.15107

Tidiane Sall, A., Chiari, T., Legesse, W., Seid-Ahmed, K., Ortiz, R., van Ginkel, M., et al. (2019). Durum wheat (*Triticum durum* desf.): origin, cultivation and potential expansion in Sub-Saharan Africa. *Agronomy* 9:263.

Tkacz, A., Pini, F., Turner, T. R., Bestion, E., Simmonds, J., Howell, P., et al. (2020). Agricultural selection of wheat has been shaped by plant-microbe interactions. *Front. Microbiol.* 11:132. doi: 10.3389/fmicb.2020.00132

Torsvik, V., and Øvreås, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5, 240–245. doi: 10.1016/s1369-5274(02)00324-7

Trebbi, D., Maccaferri, M., de Heer, P., Sørensen, A., Giuliani, S., Salvi, S., et al. (2011). High-throughput SNP discovery and genotyping in durum wheat (*Triticum durum* desf.). *Theor. Appl. Genet.* 123, 555–569. doi: 10.1007/s00122-011-1607-7

Truyens, S., Weyens, N., Cuypers, A., and Vangronsveld, J. (2015). Bacterial seed endophytes: genera, vertical transmission and interaction with plants: bacterial seed endophytes. *Environ. Microbiol. Rep.* 7, 40–50. doi: 10.3389/fmicb.2019.02659

Turner, T. R., Ramakrishnan, K., Walshaw, J., Heavens, D., Alston, M., Swarbreck, D., et al. (2013). Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. *ISME J.* 7, 2248–2258. doi: 10.1038/ismej.2013.119

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

Valente, J., Gerin, F., Le Gouis, J., Moënne-Loccoz, Y., and Prigent–Combaret, C. (2020). Ancient wheat varieties have a higher ability to interact with plant growth-promoting rhizobacteria. *Plant Cell Environ* 43, 246–260. doi: 10.1111/pce.13652

Van Dommelen, A., Croonenborghs, A., Spaepen, S., and Vanderleyden, J. (2009). Wheat growth promotion through inoculation with an ammonium-excreting mutant of *Azospirillum brasilense*. *Biol. Fertil. Soils* 45, 549–553. doi: 10.1007/s00374-009-0357-z

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Van, A. L., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Venieraki, A., Dimou, M., Pergalis, P., Kefalogianni, I., Chatzipavlidis, I., and Katinakis, P. (2011). The genetic diversity of culturable nitrogen-fixing bacteria in the rhizosphere of wheat. *Microb. Ecol.* 61, 277–285. doi: 10.1007/s00248-010-9747-x

Waines, J. G., and Ehdaie, B. (2007). Domestication and crop physiology: roots of green-revolution wheat. *Ann. Bot.* 100, 991–998. doi: 10.1093/aob/mcm180

Wang, C., Dong, D., Wang, H., Müller, K., Qin, Y., Wang, H., et al. (2016). Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of *Actinobacteria* in lignocellulose decomposition. *Biotechnol. Biofuel* 9: 22. doi: 10.1186/s13068-016-0440-2

Wang, J., Zhang, D., Zhang, L., Li, J., Raza, W., Huang, Q., et al. (2016). Temporal variation of diazotrophic community abundance and structure in surface and subsoil under four fertilization regimes during a wheat growing season. *Agric. Ecosyst. Environ.* 216, 116–124. doi: 10.1016/j.agee.2015.09.039

Wang, Y., Zhao, X., Guo, Z., Jia, Z., Wang, S., and Ding, K. (2018). Response of soil microbes to a reduction in phosphorus fertilizer in rice-wheat rotation paddy soils with varying soil P levels. *Soil Tillage Res.* 181, 127–135. doi: 10.1016/j.still.2018.04.005

Weller, D. M., and Cook, R. J. (1986). "Suppression of root diseases of wheat by fluorescent pseudomonads and mechanisms of action," in *Iron, Siderophores, and Plant Diseases*, ed. T. R. Swinburne (Boston, MA: Springer), 99–107. doi: 10.1007/978-1-4615-9480-2_12

Wiese, M. V. (1987). *Compendium of Wheat Diseases*, 2nd Edn. St Paul, MN: American Phytopathological Society.

Wilkinson, H. T., Cook, R. J., and Alldredge, J. R. (1985). Relation of inoculum size and concentration to infection of wheat roots by *Gaeumannomyces graminis* var. tritici. *Phytopathology* 75, 98–103.

Wipf, H. M. L., and Coleman-Derr, D. (2021). Evaluating domestication and ploidy effects on the assembly of the wheat bacterial microbiome. *PLoS One* 16:e0248. doi: 10.1371/journal.pone.0248030

Wollersheim, R., Trolldenier, G., and Beringer, H. (1987). Effect of bulk density and soil water tension on denitrification in the rhizosphere of spring wheat (*Triticum vulgare*). *Biol. Fertil. Soils* 5, 181–187.

Yang, M., Mavrodi, D. V., Thomashow, L. S., and Weller, D. M. (2018). Differential response of wheat cultivars to *Pseudomonas brassicacearum* and take-all decline soil. *Phytopathology* 108, 1363–1372. doi: 10.1094/PHYTO-01-18-0024-R

York, L. M., Carminati, A., Mooney, S. J., Ritz, K., and Bennett, M. J. (2016). The holistic rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. *J. Exp. Bot.* 67, 3629–3643. doi: 10.1093/jxb/erw108

Zahir, Z. A., Ghani, U., Naveed, M., Nadeem, S. M., and Asghar, H. N. (2009). Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. *Arch. Microbiol.* 191, 415–424. doi: 10.1007/s00203-009-0466-y

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.* 3, 470–480. doi: 10.1038/s41564-018-0129-3

Zhang, L., Du, Y. L., and Li, X. G. (2020). Modern wheat cultivars have greater root nitrogen uptake efficiency than old cultivars. *J. Plant Nutr. Soil Sci.* 183, 192–199. doi: 10.1002/jpln.201900353

Zhu, Y. G., Smith, S. E., Barritt, A. R., and Smith, F. A. (2001). Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. *Plant Soil* 237, 249–255.

Zuo, S., Li, X., Ma, Y., and Yang, S. (2014). Soil microbes are linked to the allelopathic potential of different wheat genotypes. *Plant Soil* 378, 49–58.

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 February 2022
doi: 10.3389/fmicb.2022.807057

Tobacco Root Microbial Community Composition Significantly Associated With Root-Knot Nematode Infections: Dynamic Changes in Microbiota and Growth Stage

Yi Cao, Zhi-Xiao Yang, Dong-Mei Yang, Ning Lu, Shi-Zhou Yu, Jian-Yu Meng and Xing-Jiang Chen*

Guizhou Academy of Tobacco Science, Guiyang, China

OPEN ACCESS

Edited by:
Jenny Kao-Kniffin, Cornell University, United States

Reviewed by:
Manoj Kumar Solanki, University of Silesia in Katowice, Poland
Laith Khalil Tawfeeq Al-Ani, Universiti Sains Malaysia, Malaysia

***Correspondence:**
Yi Cao
yicao1001@163.com

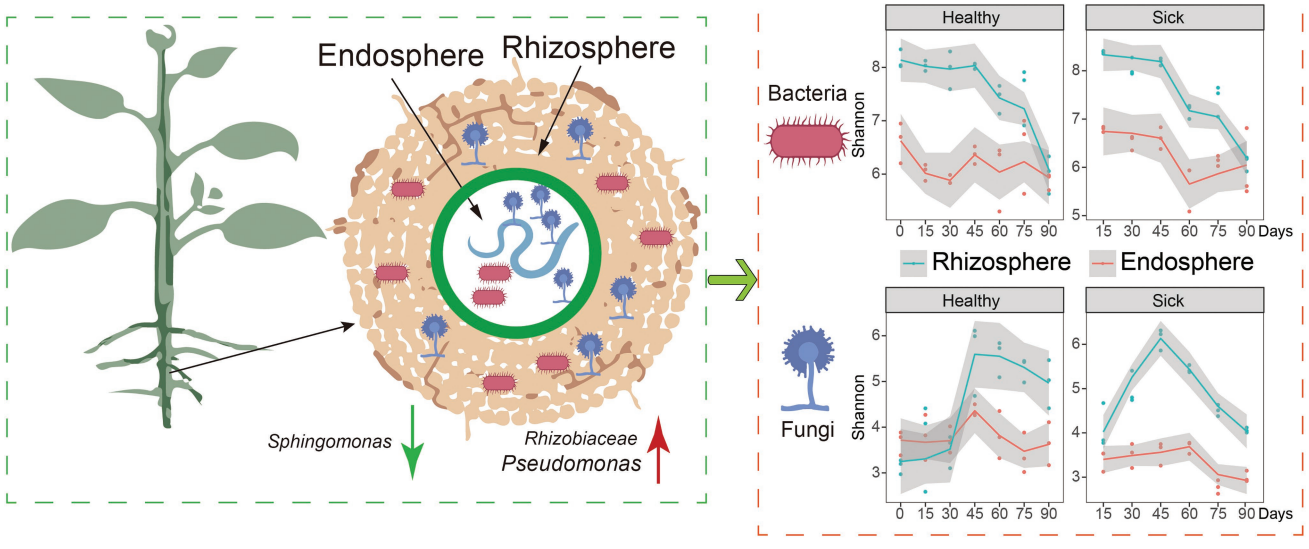
Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *01 November 2021*
Accepted: *07 January 2022*
Published: *09 February 2022*

Citation:
Cao Y, Yang Z-X, Yang D-M, Lu N, Yu S-Z, Meng J-Y and Chen X-J (2022) Tobacco Root Microbial Community Composition Significantly Associated With Root-Knot Nematode Infections: Dynamic Changes in Microbiota and Growth Stage. Front. Microbiol. 13:807057. doi: 10.3389/fmicb.2022.807057

The root-knot nematode (RKN) is an important pathogen that affects the growth of many crops. Exploring the interaction of biocontrol bacteria-pathogens-host root microbes is the theoretical basis for improving colonization and controlling the effect of biocontrol bacteria in the rhizosphere. Therefore, 16S and 18S rRNA sequencing technology was used to explore the microbial composition and diversity of tobacco roots (rhizosphere and endophytic) at different growth stages in typical tobacco RKN-infected areas for 2 consecutive years. We observed that RKN infection changed the α-diversity and microbial composition of root microorganisms and drove the transformation of microorganisms from bacteria to fungi. The abundance of *Sphingomonas* decreased significantly from 18% to less than 3%, while the abundance of *Rhizobiaceae* increased from 4 to 15% at the early growth stage during the first planting year, and it promoted the proliferation of *Chryseobacterium* at the late growth stage in rhizosphere microorganisms with the highest abundance of 17%. The overall trend of rhizosphere microorganisms changed in the early growth stage with increasing growth time. The specific results were as follows: (1) *Rhizobiaceae* and *Chryseobacterium* increased rapidly after 75 days, became the main abundant bacteria in the rhizosphere microorganisms. (2) The dominant flora in fungi were *Fusarium* and *Setophoma*. (3) Comparing the root microbes in 2017 and 2018, RKN infection significantly promoted the proliferation of *Pseudomonas* and *Setophoma* in both the rhizosphere and endophytes during the second year of continuous tobacco planting, increasing the relative abundance of *Pseudomonas* from 2 to 25%. *Pseudomonas* was determined to play an important role in plant pest control. Finally, a total of 32 strains of growth-promoting bacteria were screened from tobacco rhizosphere bacteria infected with RKN through a combination of 16S rRNA sequencing and life-promoting tests. The results of this research are helpful for analyzing the relationship between RKNs and bacteria in plants, providing reference data for elucidating the pathogenesis of RKNs and new ideas for the biological control of RKNs.

Keywords: root-knot nematode, microbiota, microbial community, root, time dynamic



GRAPHICAL ABSTRACT |

INTRODUCTION

Root-knot nematode (RKN) (*Meloidogyne* spp.) disease is an important crop disease that has become one of the main factors limiting sustainable crop production (Gorny et al., 2019; Hemmati and Saeedizadeh, 2020). RKN infects plant roots and forms nodules, which hinder the absorption of nutrients and water by the roots, leading to wilting and withering; in addition, RKN infection reduces plant resistance, causes wounds, is conducive to infection by other pathogens, and often causes complex diseases (EFSA Panel on Plant Health et al., 2018; Khan and Ahamad, 2020), such as Fusarium wilt, root rot, and bacterial diseases (Khan and Ahamad, 2020; Leonetti and Molinari, 2020). With the prohibition of methyl bromide and the appearance of chemical nematicides with high toxicity and high residue, such as imidacloprid, aldicarb, and carbofuran, there are few nematicides that both have the control effect and are safe for use in agriculture (Huang et al., 2016). Therefore, due to the lack of effective resistant varieties, the high multiple cropping index of crops, the limitation of the use of chemical nematicides with high toxicity and high residue, and the increase in drug resistance, it is particularly important to develop biological nematicides and biological control strategies to control RKN that are environmentally friendly and safe for humans and animals. Owing to the high toxicity of chemicals toward humans and the environment, the use of biocontrol bacteria, a promising method for controlling RKNs, has gained attention. In the prevention and control of plant RKN diseases, biocontrol agents have the advantages of high safety, environmental friendliness, strong specificity, and long control periods (Huang et al., 2015; Liang et al., 2019). At present, our understanding of the interactions between ecosystems, plants, RKNs, and rhizosphere microorganisms at the cellular and molecular levels is limited. Elucidating the molecular mechanism of the interactions between key microorganisms and RKN is of great significance for developing efficient biocontrol agents against plant nematodes and providing new biocontrol strategies for the future control of plant nematodes. Although many nematicidal metabolites from some microorganisms have been identified and some biocontrol bacteria have been developed, there are still many unknown mechanisms involved in biocontrol activity. Therefore, a better understanding of the molecular mechanism of the microbe nematode interaction will provide more effective strategies for further research (Ma et al., 2013; Li et al., 2015). One important aspect is to clarify the "biocontrol bacteria-pathogens-host root microorganisms" interaction, as the relationship is the theoretical basis for improving the colonization and control effect of biocontrol bacteria in the rhizosphere.

Soil is home to the most diverse and abundant microorganisms in the world (Marupakula et al., 2020). Root exudates can "shape" the unique rhizosphere soil bacterial population of plants and have a significant impact on the composition of rhizosphere microorganisms (Zhang et al., 2020a; Liang et al., 2021). Understanding the mutual equilibrium relationship between soil microorganisms and the balance of soil ecosystems is key to preventing the disease from continuing to occur and maintaining the healthy growth of crops (Liu et al., 2016). Microbial imbalances in the "plant–soil–microorganism" ecosystem play an important role in the occurrence of diseases (Shan et al., 2019).

At present, a large number of studies show that the occurrence of plant diseases is closely related to the changes of microbial composition and diversity (Li et al., 2015). The composition and interaction of plant rhizosphere microorganisms and pathogen-associated microorganisms may largely determine the occurrence, development, and spreading of plant diseases and then affect the implementation and effect of biological control. At the same time, the root exudates of different plants at different growth stages are different, which affects the species,

quantity, and distribution characteristics of rhizosphere microorganisms. Therefore, this project aims to examine the relationship between tobacco RKNs and rhizosphere microbial populations, analyze the dynamic changes of tobacco rhizosphere bacterial communities and RKNs over various periods of time.

MATERIALS AND METHODS

Sample Collection

Yunyan 97 was used as the experimental flue-cured tobacco variety, and tobacco seedling raising and field management were carried out according to conventional cultivation and management measures (no application of chemicals containing RKNs); the susceptible samples were from the perennial disease site of southern RKNs (2 years in the same plot), and the healthy samples were from a healthy growing tobacco field in the same region (2 years in the same plot). The varieties and cultivation and management measures of the two groups of samples were the same.

BS represents the abbreviation of the soil before transplanting plants, AS represents the soil after tobacco planting and harvesting, and HR0 represents the root of the tobacco seedlings to be transplanted. The flue-cured tobacco seedlings were intensively raised, and the seedlings of the two areas were all raised with the same batch of materials and seeds and in the same period. Therefore, unified sampling was adopted for the tobacco seedlings in the transplanting period. Three seedling trays were randomly selected from the seedling pool, and three seedlings were taken from each tray, which were mixed to form a duplicate sample.

After transplanting, the samples were collected according to different growth stages (15, 30, 45, 60, 75, and 90 days). The field sample collection was performed according to the method described by Peiffer et al. (2013). The roots of three random plants were sampled from the middle of each plot. For each plant, a root segment 5 cm in length and 0.5–3 mm in diameter was collected near the base of the plant, along with any adherent soil particles. The preparation of the collected samples was performed according to the literature of Lundberg et al. (2012). One portion was frozen in a −80°C refrigerator, and the other was used for soil total DNA extraction.

DNA Extraction, PCR Amplification, and Sequencing

For all soil, rhizosphere and endophytic microbial DNA extractions were performed with the MP Bio Fast DNA Spin Kit for soil (SDS/mechanical lysis; Lundberg et al., 2012). Primers were designed as follows: 515 forward (5′-GTGCCAGCMGCCGCGG-3′) and 806 reverse (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3-V4 region of the 16S rDNA gene, and SSU0817 forward (5′-TTAGCATGGAATAATRRAATAGGA-3′) and 1,196 reverse (5′-TCTGGACCTGGTGAGTTTCC-3) were used to amplify the V5-V7 region of the 18S rDNA gene. PCR was carried out with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 μM forward and reverse primers, and approximately 10 ng of template DNA. The thermal cycling process consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and finally, the sample was held at 72°C for 5 min. The library was sequenced on an Illumina NovaSeq platform.

Bioinformatics and Statistical Analyses

Sequence analysis was performed using Uparse software (Uparse v7.0.1001). Sequences with ≥97% similarity were assigned to the same OTUs. To study the phylogenetic relationships of different OTUs and the differences between the dominant species in different groups, multiple sequence alignment was conducted using MUSCLE software (version 3.8.31). Alpha diversity was applied to analyze the complexity of the species diversity for a sample through three indices, including Chao1, Shannon, and good coverage. All these indices in our samples were calculated with QIIME (version 1.7.0) and visualized with R software (version 2.15.3). The beta diversity based on weighted UniFrac was calculated using QIIME software (version 1.9.1). Random forest analysis evaluates the importance of each predictor by determining how much the mean square error (MSE) increases. The variables were selected when the predictor variables were randomly replaced and the other variables remained unchanged. These analyses were performed using the "RandomForest" package in R. The "Hmisc" package was used to calculate the correlation, the "igraph" package was used to analyses the co-occurrence network (Csardi and Nepusz, 2006), and Gephi 0.9.2 software was used to draw the network map. The statistical analyses using the R software and GraphPad Prism.

Isolation of Rhizosphere Bacteria

The rhizosphere soil samples of tobacco infected with RKN 15 and 75 days after transplanting were cleaned with sterilized 10 mM $MgCl_2$. The cleaned samples were placed into a 2 ml round bottom centrifuge tube, an appropriate number of sterilized metal grinding balls were added, and the sample was ground twice (30 s each time) with a sample grinder. The milled sample was precipitated for 15 min at room temperature; after 5 min, the supernatant was taken for gradient dilution (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}), and the diluted sample was transferred into a 96-well plate with liquid medium (repeated three times for each concentration). After 5 days at a constant temperature of 28°C, the culture medium was drawn and coated in the corresponding solid medium. After culturing at 28°C, plates with fewer than 30 colonies within 10 days were selected, and then, the colonies were marked and purified at −80°C until use.

Identification of the Isolated Rhizosphere Bacteria at the Molecular Level

A 16S rRNA amplification sequence was used to identify the isolated tobacco rhizosphere bacteria. A single bacterial isolate was cultured in LB medium shaken at 30°C (180 rpm), and the total genomic DNA was extracted from the overnight culture of a single bacterial isolate using a DNeasy® UltraClean® Microbial Kit (QIAGEN GmbH) according to the kit's instructions. The 16S rRNA gene was amplified by PCR using the universal primers 27F (5′-agagtgtgatcmtggctcag-3′) and

1492r (5′-tacggytaccttgttacgactt-3′). The polymerase chain reaction (50 μl) consisted of 1 μl of bacterial DNA template, 25 μl of 2× Taq PCR master mix, 1 μl each of forward and reverse primers, and 22 μl of ddH_2O. The PCR conditions were as follows: denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and 30 s, a total of 35 cycles, and extension at 72°C for 10 min. The sequencing results were submitted to the NCBI GenBank database for homology sequence alignment.

Determination of the Growth-Promoting Activity of the Strains

Screening of phosphate and potassium-dissolving bacteria: the screened strains were inoculated using high-pressure sterilized toothpicks into plates of organic phosphorus, inorganic phosphorus, and a silicate bacteria culture medium and then cultured in a 28°C constant temperature incubator for 7 days to observe whether there were clear and transparent circles and oil drop-like colonies on the plates.

Screening of nitrogen-fixing bacteria: the screened strains were inoculated with disposable inoculation rings on Azotobacter culture medium and observed continuously for 7 days to select the strains with good growth.

Screening of the indole acetic acid (IAA)-producing ability of strains: the classic Salkowski method was used. The activated strains were inoculated in King's medium containing L-tryptophan and shaken at 30°C and 180 R/min for 2 days. One hundred microliters of bacterial suspension was placed into the wells of white porcelain plates, and then, 100 μl of Salkowski colorimetric solution was added. At the same time, 100 μl of culture medium inoculated with sterile water was added to a Salkowski colorimetric solution as the control. After the white porcelain plate was placed in the dark for 15 min, the control culture medium had no color change, while the culture medium in the hole of white porcelain plate turned red, indicating that the tested strain had the ability to secrete IAA.

Screening of the ability of a strain to produce an iron carrier: the screened strain was inoculated with a sterilized toothpick on the CAS plate, with three points on a plate and three replicates for each strain, and incubated at 30°C for 48 h. An obvious orange halo indicative of an iron carrier appeared around colonies of bacteria secreting an iron carrier. Generally, the larger the circle, the darker the color, and the greater the ability to produce iron carriers.

RESULTS

The Diversity of Tobacco Rhizosphere Bacteria Was Reduced by Infection With RKN

For the alpha diversity analysis, Chao1 estimators and Shannon indices were used to assess the community richness and diversity, respectively. We first analyzed the effect of RKN infection on bacteria. The results indicate that the richness of rhizosphere bacteria infected with RKNs was significantly higher than that of healthy tobacco at the beginning of the first year of the test (**Figure 1A**). When the planting reached 45 days, the richness of the rhizosphere bacteria of both healthy and diseased plants was significantly reduced, indicating that when the growth process reached 45 days, the abundance of rhizosphere bacteria changed significantly. At the same time, we observed the change trend of Chao1 in the second year of planting, and we found that the rhizosphere microbial abundance of the RKN-infected plants was significantly lower than that of healthy plants at this stage. There was no significant difference between the two at 45 days of planting. However, when the growth cycle reached 45 days, the microbial abundance of healthy and RKN-infected rhizospheres also decreased significantly, indicating that tobacco growth at 45 days may be an important period for the change in rhizosphere microbial abundance (**Figure 1A**). In general, the abundance of RKN-infected and healthy plants was significantly different at the initial stage, and as the plants grew and developed, the difference between the two would decrease. By 45 days, the microbes in the rhizosphere and endophytes were significantly reduced. Meanwhile, the abundance of rhizosphere bacteria was significantly higher than that of endophytic bacteria.

We also analyzed the Shannon index of each group to explore the trend and law of its diversity. At the beginning of the second year, the diversity index of rhizosphere bacteria in the sick group was significantly lower than that of the healthy group, which indicated that the rhizosphere bacterial diversity was significantly reduced by infection with RKN at this stage, and the overall diversity showed a gradual decreasing trend with time (**Figure 1B**). The diversity of endophytic bacteria infected with RKN was significantly higher than that of healthy tobacco at the beginning of the first year of planting, and there was no significant decreasing or increasing trend in the overall endophytic bacteria diversity with increasing time. In the second year of continuous planting, when the growth cycle reached 45 days, the diversity of endophytic bacteria decreased significantly. In general, in the first year of planting, the diversity of rhizosphere bacteria in RKN-infected tobacco was not significant, similar to that of healthy tobacco, but the diversity of endophytic bacteria significantly increased. In the second year of infection, the diversity of infected strains was significantly lower than that of healthy plants, and the overall diversity showed a downward trend.

The Diversity of Tobacco Rhizosphere Fungi Was Increased by Infection With RKN

We analyzed the abundance of rhizosphere and endophytic fungi. The abundance of rhizosphere fungi in the healthy tobacco group did not change significantly in the first year of planting. The abundance of rhizosphere fungi in the RKN-infected group increased gradually within 0–45 days ($p < 0.01$) and began to decrease gradually after 45 days ($p < 0.01$). In the second year of continuous planting, the abundance of rhizosphere fungi in the RKN-infected tobacco group showed no significant change. Similar to the first year, the abundance of rhizosphere fungi was highest at 45 days and then decreased significantly ($p < 0.05$). We further analyzed

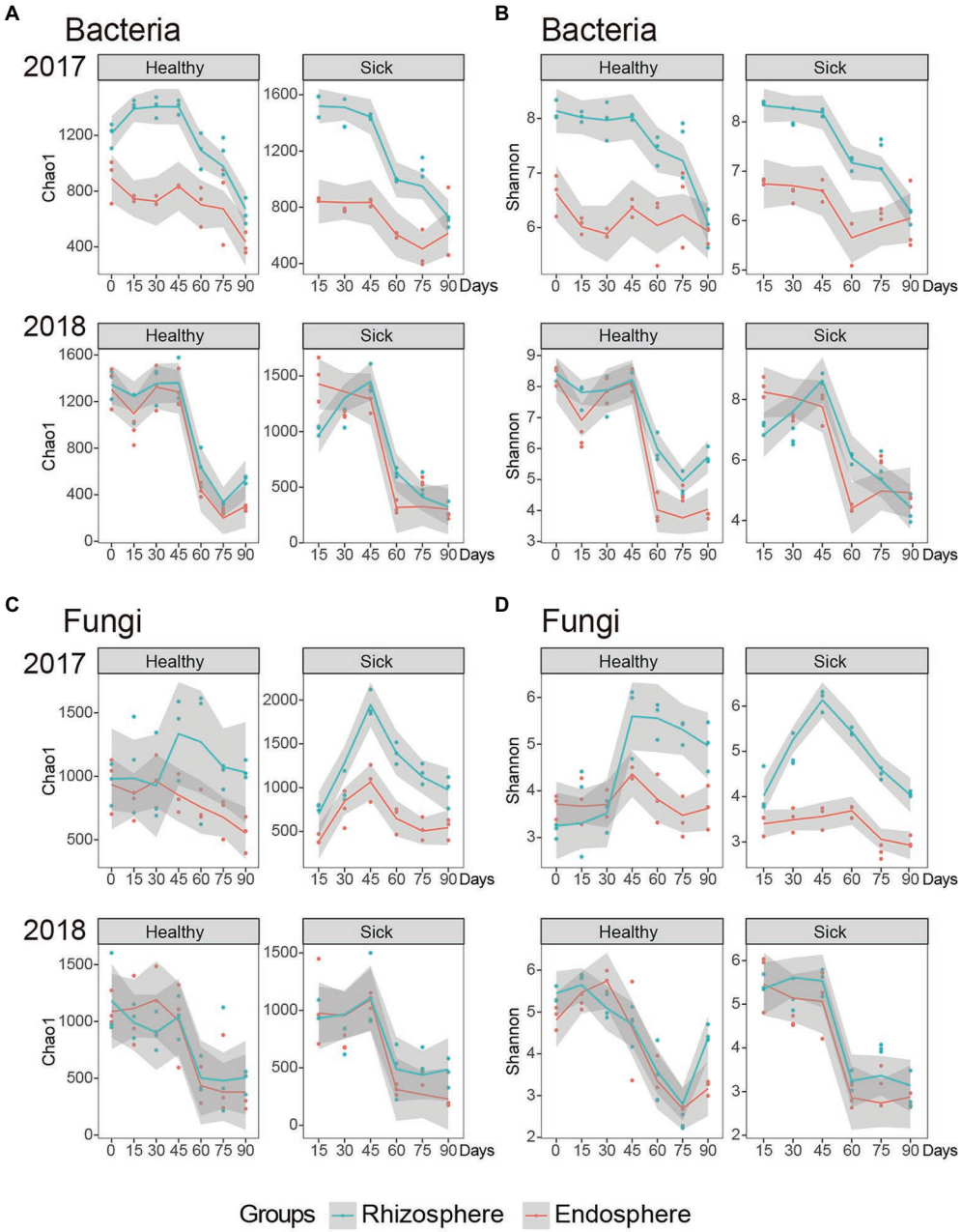


FIGURE 1 | Abundance and diversity of root microorganisms in RKN-infected and healthy tobacco. **(A)** The Chao1 index of rhizosphere and endophytic bacteria. **(B)** The Shannon index of rhizosphere and endophytic bacteria. **(C)** The Chao1 index of rhizosphere and endophytic fungi. **(D)** The Shannon index of rhizosphere and endophytic fungi.

the abundance of endophytic fungi and found that began to decrease in both the healthy and infected groups after 45 days of the growth cycle in the first year of planting and the second year of continuous planting ($p < 0.01$; **Figure 1C**). In general, RKN infection significantly increased the abundance of rhizosphere fungi in the early growth stage, which was significantly higher than that in the healthy group, and significantly decreased the fungal abundance in the endophytes at 45 days of growth.

By analyzing the diversity of rhizosphere and endophytic fungi, we found that the diversity of rhizosphere fungi in the RKN-infected group was significantly higher than that in the healthy group in the first year of planting and showed a significant increasing trend in the growth cycle of 0–45 days ($p < 0.01$). At 45 days, the maximum value gradually decreased ($p < 0.01$). In the second year of continuous planting, the diversity of rhizosphere fungi did not change significantly during Days 0–45 but decreased significantly after 45 days ($p < 0.01$). The diversity of endophytic

fungi in the RKN-infected group was significantly lower than that in the healthy group after 75 days of the growth cycle ($p < 0.05$) and decreased significantly from 45 days in the second year of continuous planting ($p < 0.01$; **Figure 1D**).

Two-way ANOVA was conducted to determine the effects of treatment (healthy and sick) and the time period and their interactions on Shannon and Chao1 index (**Supplementary Table S1**). Through the results, we found that the Shannon and Chao1 index of the bacteria and fungi communities remained relatively stable under treatment in all periods.

The Effects of RKN Infection on Tobacco Root Microorganisms

To measure the extent of the similarity between microbial communities, we performed PCoA analysis on rhizosphere microbes and endophytic microbes for each group of samples based on the weighted UniFrac algorithm. We found that whether rhizosphere bacteria or endophytic bacteria (**Figure 2A**) were present, there were significant differences in the total microbial composition among the groups. Among the PCoA analyses, the PC1 distribution with the largest contribution rate revealed changes of 22.04 and 28.72%, and the PC2 distribution revealed changes of 56.96 and 38.68%, respectively. This result indicates that different growth and development times could significantly change and affect the composition of the tobacco rhizosphere and endophytic bacteria. At the same time, the microbial compositions of the infected and healthy tobacco plants were similar at some time points, and some were significantly different, which indicated that the effects of RKN infection on specific healthy tobacco root microorganisms were different, which may be related to the time of growth and development and the mechanism of infection. The PCoA analysis to compare the similarity of fungal microorganisms in different treatment groups found that the results were similar to those of bacteria (**Figure 2B**). In summary, the above results showed that RKN infection changed the overall composition of bacteria and fungi in the root system and changed the composition and distribution of root microorganisms in different growth stages of healthy tobacco with increasing time.

RKN Infection Caused Changes in Bacterial Community Structure at Different Times

To explore the specific distributions in different growth periods and the change rule after RKN infection, we further analyzed the composition of microorganisms at the phylum and genus

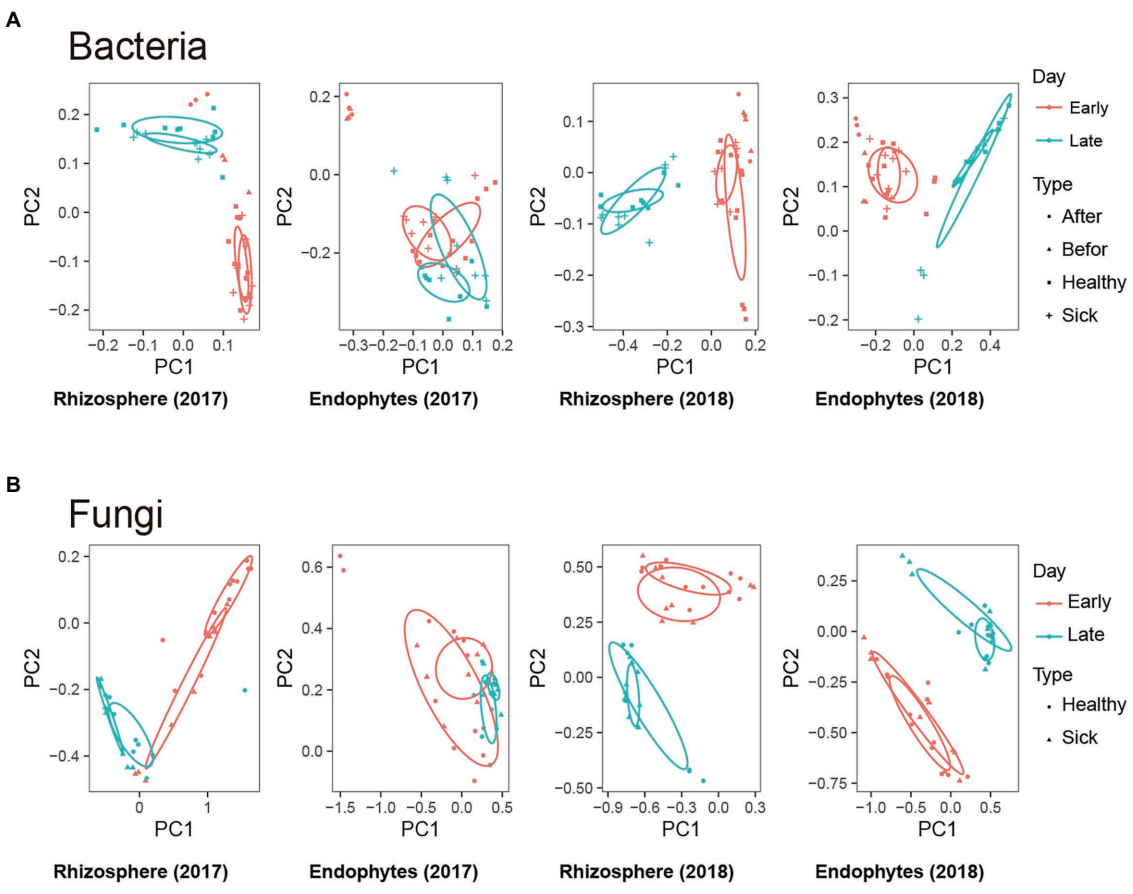


FIGURE 2 | Effects of root-knot nematode (RKN) infection on tobacco root microorganisms. **(A,B)** Representative tobacco rhizosphere bacteria, endophytic bacteria, rhizosphere fungi and endophytic fungi based on a weighted UniFrac algorithm of PCoA analysis. Early: 0–45 days, Late: 45–90 days.

levels on rhizosphere and endophytic bacteria. We first observed and analyzed the bacteria at the phylum level (**Figure 3A**). The rhizosphere bacteria were mainly composed of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Acidobacteria*. There was no significant difference between the rhizosphere bacteria of the plants infected with RKN and those of healthy tobacco plants at the phylum level, and there was no significant change trend with time. The composition of soil microorganisms was different from that of rhizosphere microorganisms, especially *Gemmatimonadetes*. It was found that the structure of endophytic bacteria was similar to that of the rhizosphere. The results showed that RKN infection did not significantly change the root microbial composition at the phylum level.

We further analyzed the composition of bacteria at the genus level in tobacco and found that they were mainly composed of *Pseudomonas*, *Streptomyces*, *Chryseobacterium*, *Rhizobiaceae*, and *Sphingomonas* (**Figure 3B**). The relative abundance of *Pseudomonas* increased significantly in the second year compared with the first year. The abundance of *Sphingomonas* in the rhizosphere bacteria was significantly reduced in the early growth period and the abundance of *Rhizobiaceae* increased after RKN infection. RKN infection significantly promoted the proliferation of *Chryseobacterium* when growth reached 3 months. In the first year of planting, the relative abundance of *Streptomyces* of endophytic bacteria decreased significantly in the late growth period, while the relative abundance of *Pseudomonas* increased. The results showed that infection with RKN can change the root microorganisms of healthy tobacco.

RKN Infection Caused Changes in Fungal Community Structure at Different Times

We analyzed the specific composition of fungal microorganisms in different treatment groups at the phylum level and found that both rhizosphere and intraroot fungi were mainly composed of *Ascomycota* and *Chytridiomycota* (**Figure 3C**). In the first year of planting, *Chytridiomycota* was the most abundant and dominant microorganism in the rhizosphere and endomycota. With the increase in the growth cycle, the relative abundance of *Chytridiomycota* decreased significantly and that of *Ascomycota* increased, which gradually became the dominant flora. As the growth period increased, the fungal composition was stable, and *Ascomycota* became the main dominant fungus. In the

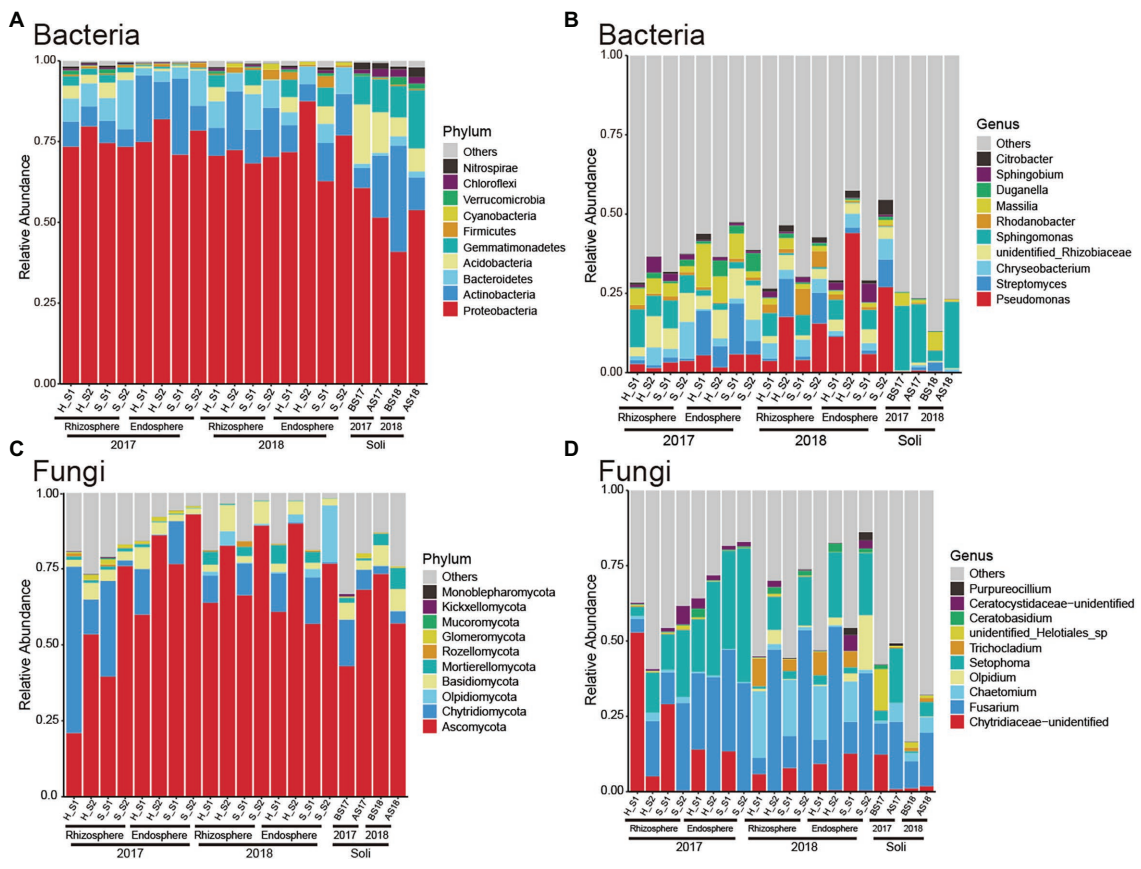


FIGURE 3 | Composition of microorganisms at the phylum and genus levels in RKN-infected and healthy tobacco at different times. **(A)** The relative contributions of the top 10 phyla of bacteria at the phylum level at different times in RKN-infected and healthy tobacco. **(B)** The relative contributions of the top 10 genera of bacteria. **(C)** The relative contributions of the top 10 fungi at the phylum level at different times. **(D)** The relative contributions of the top 10 genera of fungi at the genus level at different times in tobacco roots. S1: 0–45 days, S2: 45–90 days.

second year of continuous planting, *Ascomycota* and *Chytridiomycota* together formed different groups of fungi in the early growth cycle.

We analyzed the distribution and composition of fungal microorganisms at the genus level (**Figure 3D**) and found that in the first year of planting, during the early growth cycle, the fungi were mainly composed of *Chytridiaceae*, *Fusarium*, and *Setophoma*, the relative abundance of *Chytridiaceae* gradually decreased, the *Fusarium* abundance gradually increased, and the relative abundance of *Setophoma* also gradually increased, accounting for approximately 10%. At this time, we found that the abundance of *Fusarium* in endophytes was greater than that in the rhizosphere. At the late growth cycle, the abundance of *Chytridiaceae* decreased to a very low level, *Fusarium* and *Setophoma* became the dominant fungi, and the abundance of *Setophoma* increased from 10 to 50%. The fungal compositions in the rhizosphere and endophytes were similar and were composed of *Fusarium* and *Setophoma*, and there was no significant difference in their relative abundance.

Major Differential Microbial Species in RKN-Infected and Healthy Tobacco Root Microorganisms in Different Growth Stages

To verify and further determine the microbial OTUs that could be used to discriminate the time of RKN infection, random forest was performed to identify the most important microbial taxa (% Increase MSE) in predicting soil microbes. We selected 10 of the most important time-discriminant OTUs of bacteria and fungi (**Figures 4A,B**). The results indicated that the most important bacterial phyla taxa were classified *Proteobacteria* and *Armatimonadetes*, and the most important fungi phyla were classified *Chytridiomycota*, *Ascomycota*, *Basidiomycota*, and *Mortierellomycota*. The relative abundances of most of them were low, and they were relatively rare bacterial taxa. At the same time, the abundance of these microorganisms will also change with planting time. For example, the relative abundances of *Sphingomonas*, *Xanthomonadales*, and *Micropepsaceae* were high in the early growth stage of tobacco planting. However, *Burkholderiaceae* was the key group in the late stage of infection (**Figure 4C**). *Fusarium* and *Chytridiaceae* were the two most abundant fungi. *Chytridiaceae* was the key fungus

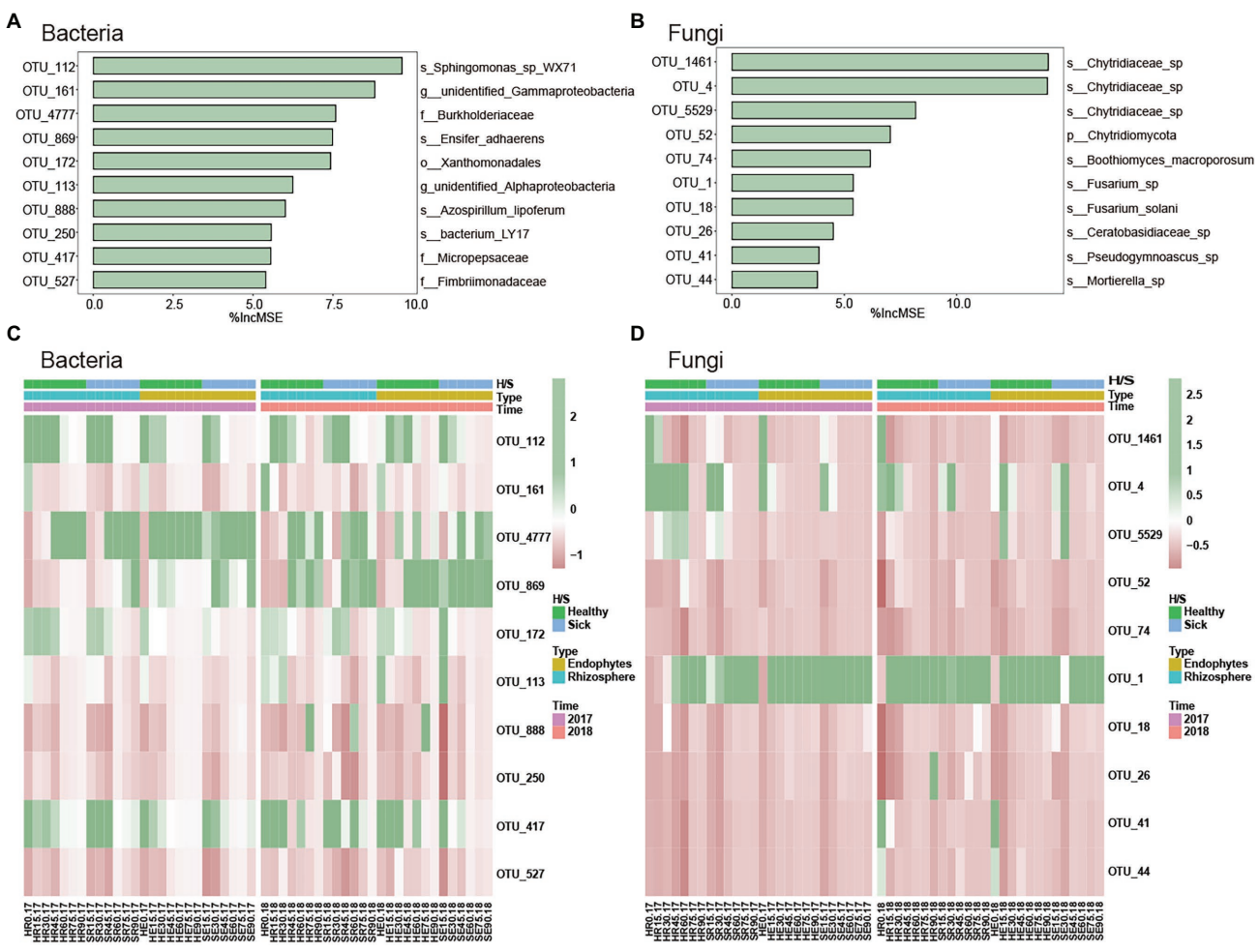


FIGURE 4 | Major differential microbial species in RKN-infected and healthy tobacco root microorganisms in different growth stages. **(A,B)** The top 10 OTUs selected according to %IncMSE through random forest. **(C,D)** Heatmap of the relative abundances of important biomarker microorganisms.

in the early stage, and the relative abundance of *Fusarium* was high except in the early stages (**Figure 4D**).

Comparison of Root Microbial Composition After RKN Infection in Different Planting Years

In addition to the analysis of the dynamic changes of root microorganism in different growth periods (0, 15, 30, 45, 60, 75, and 90 days), we also compared and analyzed the root microbial composition in 2017 and 2018 to explore the effect of RKN infection on root microorganisms in different planting years. We found that the root microbes in 2017 were significantly different from those in 2018 (**Figure 5A**; **Supplementary Figures S1A–C**). At the same time, we analyzed the rhizosphere bacteria in the second year of continuous planting and found that the relative abundance of *Pseudomonas* in SR60.18 was significantly higher than that in HR60.18 from the 60-day growth cycle ($p < 0.05$, **Figure 5B**), while the relative abundance of *Streptomyces* was significantly lower than that in HR60.18 ($p < 0.01$, **Figure 5C**). *Pseudomonas* plays an important role in plant pest control, which also shows that RKN infection from the second year of continuous planting will significantly change the composition of rhizosphere bacteria, especially the proliferation of *Pseudomonas* and the reduction of *Streptomyces*.

Network Chart of Microbial Flora Based on Co-occurrence

Based on the Spearman correlation analysis of relative abundances at the genus level, we analyzed the symbiotic network relationship between the healthy group and the sick group, respectively (**Figure 6**). The results showed that with the change of planting time from 2017 to 2018, the symbiotic dominant flora in the healthy group changed from *Arsenicitalea*, *Pedobacter*, *Arenimonas*, *Gemmatimonas*, *Bauldia*, etc. to *Neurospora*, *Thermomyces*, *Gemmatimonadacea*, *Alphaproteobacteria*, etc., which as the new dominant flora. It promoted the symbiotic relationship dominated by bacteria to gradually transform into fungi. We also analyzed the symbiotic relationship in the infection group and found that it changed with the planting time from 2017 to 2018, the dominant symbiotic flora after RKN infection gradually changed from *Arsenicitalea*, *Rhodobacter*, etc. to *Sphingomonas*, *Arenimonas*, *Kineosporia*, etc. The above results not only show that RKN infection will change the core flora of the dominant symbiotic relationship, but also show that fungi gradually occupy part of the symbiotic relationship with the increase of planting time.

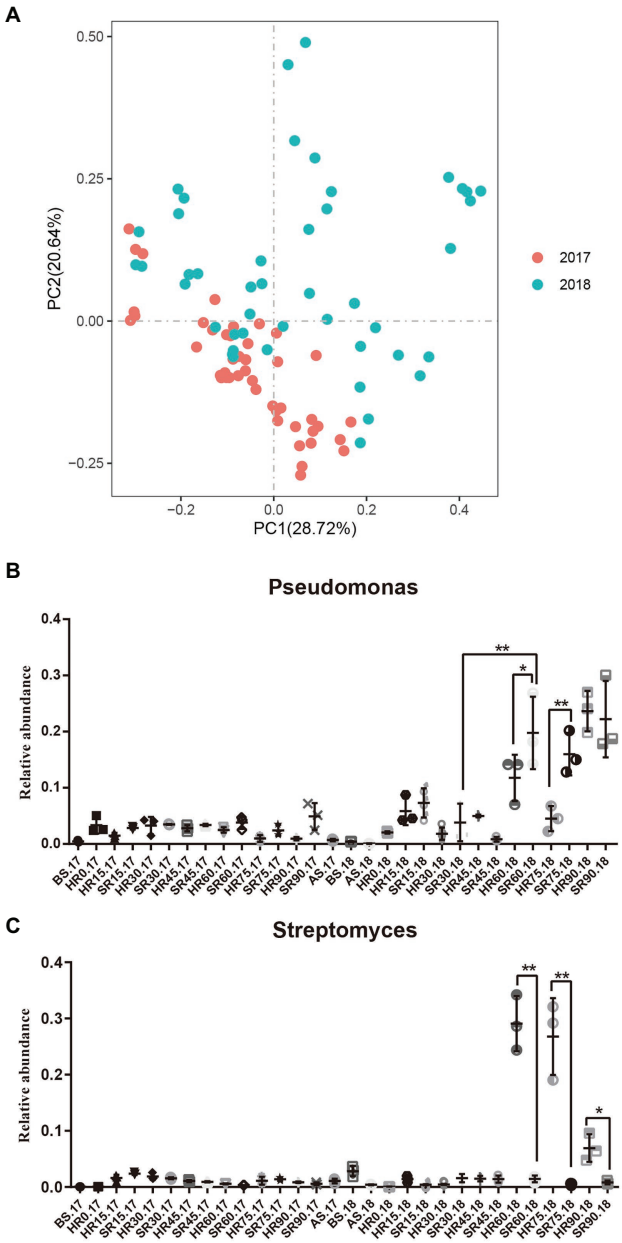


FIGURE 5 | Comparison of root microbial composition after RKN infection in different planting years. **(A)** The distribution and composition of rhizosphere bacteria in 2017 and 2018 analyzed by PCoA based on a weighted UniFrac algorithm. **(B,C)** The relative abundances of *Pseudomonas* and *Streptomyces* in rhizosphere bacteria. Data were analyzed by ANOVA (*$p < 0.05$ and **$p < 0.01$).

Analysis of the Growth-Promoting Characteristics of Strains

According to the abovementioned 16S rRNA sequencing results, 45 different representative strains were selected based on the analysis of the different microorganisms between the RKN-infected group and the healthy group. A total of 32 strains of growth-promoting bacteria were screened from tobacco rhizosphere bacteria infected with RKN (**Table 1**). Three strains of phosphorite-dissolving bacteria, seven strains of phosphate-solubilizing strains, two potassium-dissolving strains, three nitrogen-fixing strains, 17 IAA-producing strains, and 13 siderophore-producing strains were used. Among them, *Agrobacterium fabrum* YC2096 had the ability to dissolve potassium, IAA, and siderophores; *Pseudomonas* YC2103 had the ability to dissolve phosphorus and iron carriers; *Bacillus* YC2128 had the ability to dissolve phosphorus and produce IAA; and *Enterobacter* YC2132 and *Pantoea agglomerans* YC2174 both had the ability to dissolve phosphorus and produce IAA and siderophores.

In recent years, it has been found that the bacteria and metabolic pathways producing IAA and iron carriers are significantly

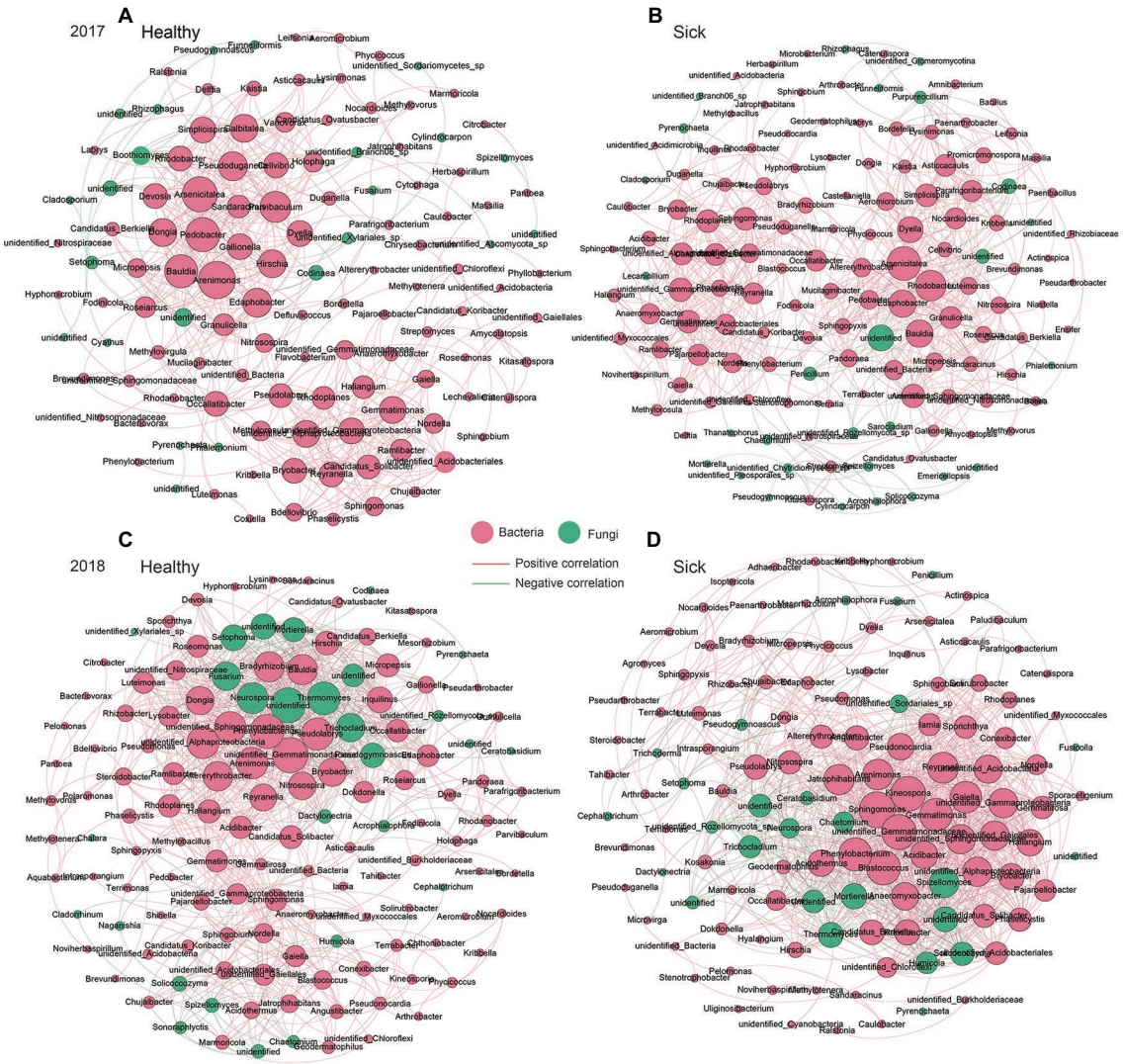


FIGURE 6 | Co-occurrence network relationship between the healthy group and the sick group. **(A)** Healthy tobacco in 2017. **(B)** Sick tobacco in 2017. **(C)** Healthy tobacco in 2018. **(D)** Sick tobacco in 2018. Screen the correlation greater than 0.8, $p < 0.05$. The red edges represent bacteria, and the green edges represent fungi, the red lines between the nodes indicate a positive correlation, and the green lines indicate negative correlation.

related to nematode infection in plants. Studies on the microbiome and biocontrol have found that strains and mixtures with these functions have more research and application prospects in biocontrol. Therefore, a combination of 16S rRNA sequencing and life-promoting tests was used to screen a variety of strains with high biological activity, which has important application value in controlling RKN infection and promoting plant growth and development and provides strain resources and a new reference basis for the development of biologically controlled bacteria.

DISCUSSION

At present, biocontrol microorganisms are an important resource for the development of nematode biocontrol preparations (Dong et al., 2018). Nematophagous fungi kill nematodes through predation, parasitism, and toxin production. They are an important group of microorganisms that control nematode populations in nature; nematophagous bacteria are another important resource for the biocontrol of nematodes. The bionematocides developed using nematode-preying fungi and bacteria have been registered and used to control nematode disease in plants. Therefore, the development of biologically controlled bacteria has important significance for and effects on the treatment of RKN. This experiment explored the effects of different growth periods (0, 15, 30, 45, 60, 75, and 90 days) and continuous planting on the root system microorganisms (rhizosphere and endophytic) of healthy tobacco after infection with RKN through 16S and 18S rRNA technology. We found that in the first year of planting, the diversity of rhizosphere bacteria in RKN-infected tobacco was not significant, similar to that of healthy tobacco, but it significantly increased the diversity of endophytic bacteria. In

TABLE 1 | Test results of the growth-promoting activity of the strain.

Serial number	Strain number	Bacteria	Phosphorus dissolving (3)	Phosphorus solution (7)	Nitrogen fixation (3)	Potassium solution (2)	IAA (17)	Iron carried (13)
1	YC2076	*Bacillus oleronius*	–	–	–	–	–	–
2	YC2077	*Devosia riboflavina*	–	–	–	+	–	+
3	YC2083	*Herbaspirillum seropedicae*	–	–	–	–	+	–
4	YC2096	*Agrobacterium fabrum*	–	–	+	–	+	+
5	YC2097	*Rathayibacter*	–	–	–	–	+	+
6	YC2098	*Stenotrophomonas rhizophila*	–	–	–	–	+	–
7	YC2099	*Sphingomonas*	–	–	–	–	–	–
8	YC2100	*Arthrobacter*	–	–	–	–	+	–
9	YC2101	*Bacillus oleronius*	–	–	–	–	–	–
10	YC2102	*Burkholderia paludis*	–	–	–	–	–	–
11	YC2103	*Pseudomonas*	+	+	–	–	–	+
12	YC2109	*Chryseobacterium*	–	–	–	–	+	–
13	YC2111	*Shinella*	–	–	–	–	+	+
14	YC2114	*Agrobacterium*	–	+	–	–	–	+
15	YC2115	*Advenella incenata*	–	–	–	–	–	+
16	YC2116	*Brachybacterium*	–	–	–	–	–	+
17	YC2117	*Ensifer*	–	–	+	–	+	–
18	YC2120	*Stenotrophomonas rhizophila*	–	–	–	–	–	–
19	YC2122	*Microbacterium*	–	–	–	–	+	–
20	YC2124	*Lysobacter capsici*	+	–	–	–	–	–
21	YC2126	*Pedobacter soli*	–	–	–	–	+	+
22	YC2128	*Bacillus*	+	+	–	–	+	–
23	YC2130	*Pectobacterium*	–	–	–	–	+	–
24	YC2132	*Enterobacte*	–	+	–	–	+	+
25	YC2134	*Pectobacterium*	–	+	–	–	+	–
26	YC2136	*Delftia*	–	–	–	–	–	+
27	YC2137	*Alcaligenaceae bacterium*	–	–	–	–	–	+
28	YC2138	*Delftia*	–	–	–	–	–	+
29	YC2139	*Enterobacter*	–	–	–	–	–	–
30	YC2140	*Pseudomonas*	–	–	–	–	–	+
31	YC2141	*Agrobacterium*	–	–	+	–	+	+
32	YC2143	*Stenotrophomonas rhizophila*	–	–	–	–	–	+
33	YC2145	*Pseudomonas punonensis*	–	–	–	–	–	–
34	YC2147	*Erwinia*	–	–	–	–	–	–
35	YC2149	*Chryseobacterium*	–	–	–	–	+	–
36	YC2151	*Variovorax paradoxus*	–	–	–	–	–	–
37	YC2153	*Arthrobacter nitrophenolicus*	–	–	–	+	–	+
38	YC2155	*Pseudomonas putida*	–	–	–	–	–	–
39	YC2157	*Alcaligenaceae bacterium*	–	–	–	–	–	+
40	YC2159	*Alcaligenes*	–	–	–	–	–	+
41	YC2165	*Rothia marina*	–	–	–	–	–	–
42	YC2167	*Janibacter melonis*	–	–	–	–	–	–
43	YC2169	*Bacterium*	–	–	–	–	–	–
44	YC2171	*Exiguobacterium acetylicum*	–	+	–	–	–	+
45	YC2174	*Pantoea agglomerans*	–	+	–	–	+	+

the second year of infection, the diversity of infected strains was significantly lower than that of healthy plants, and the overall diversity showed a downward trend. Therefore, by comparing the composition and diversity of rhizosphere bacteria between infected and healthy tobacco, we found that there were some differences in the α-diversity and distribution of rhizosphere bacteria between diseased and healthy tobacco, indicating that RKN infection affected the rhizosphere bacterial flora of host plants to a certain extent.

We found that RKN infection significantly changed and affected the composition and abundance of rhizosphere and endophytic bacteria in tobacco. At the same time, the composition of root microorganisms changed significantly with the time of different growth stages. RKNs significantly reduced the abundance of *Sphingomonas*, increased the abundance of *Rhizobiaceae* in the early growth stage of the first year of planting, and promoted the proliferation of *Chryseobacterium* during the late growth stage. From 60 days after the second year of planting, the RKNs significantly promoted the proliferation of *Pseudomonas* and reduced the abundance of *Streptomyces*. In the first year of planting of the endophytic bacteria, the relative abundance of *Streptomyces* decreased significantly, while the relative abundance of *Pseudomonas* increased. In the second year of planting, the abundance of *Pseudomonas* increased from 60 days of growth. Therefore, we found that both rhizosphere and endophytic bacteria showed significant regular change trends with different growth stages, and the

microorganisms compositions were different. Infection with RKN can partially change the root microorganisms of healthy tobacco. On the 90th day of the second year of continuous planting, the relative abundance of *Bacillaceae* in the RKN group was significantly higher than that in the healthy group. In the "plant-pathogen-beneficial bacteria" interaction system, the plant root exudes nutrients and exogenous signaling substances, thereby affecting the behavior of rhizosphere bacteria, and selecting beneficial microbial populations to resist pathogen infection has been confirmed and widely accepted (Buchan et al., 2010). RKNs infect plants, leading to host physiological changes. Nutrients and metabolites are released through root cells fed by nematodes through the symplast, which changes the composition of root exudates, such as water-soluble carbon and metal ions (Tian et al., 2015), and then affects the composition of rhizosphere bacteria. Rhizosphere bacteria play key roles in plant growth, health, and development and are mainly determined by the soil community. To some extent, they are regulated by the biological clock, related to plant metabolism, and affect carbon metabolism and the exchange of plant microorganisms (Staley et al., 2017). Mavrodi et al. (2012) found that the population of *Pseudomonas* spp. producing DAPG was higher in the rhizosphere soil of wheat cultivated in paddy fields dominated by *Gaeumannomyces graminis*, while in the same region, the number of *Pseudomonas* spp. producing the resistance metabolite phenazine was higher in the rhizosphere soil of early wheat with sheath blight. The results showed that there was no significant difference in the soil microbial population structure between inoculated and uninoculated barley, but the overall decline phenomenon only occurred in the soil inoculated with the pathogen (Schreiner et al., 2010). This conclusion was consistent with our findings. We found that the relative quantities of *Bacillaceae* and *Pseudomonas* increased significantly after RKN infection with increasing growth time, and RKN infection caused a large number of proliferations of *Pseudomonas* in the second year of continuous tobacco planting. *Pseudomonas* bacteria have been widely reported to inhibit the activity of RKN by various mechanisms, which is beneficial for inhibiting soil-borne diseases and providing a healthy soil environment to promote root growth (Li et al., 2015; Nikolić et al., 2019). Tao et al. (2020) conducted a high-throughput screening of 886 rhizosphere samples of 886 plants in China's three major cotton-producing areas and found that two *Pseudomonas* strains showed significant inhibitory effects on Vibrio dahlia. Through comparative genomic and phenotypic analyses, it was shown that *P. protegens* XY2F4 and *P. donghuensis* 22G5 were the most effective strains to protect cotton plants against verticillium wilt because they produced specific biological control products. In addition, they found and confirmed that the natural tropolone compound 7-hydroxytropolone (7-HT) had a significant effect on the general public. Research has revealed that *Pseudomonas* bacteria have a specific gene cluster that can produce effective antipathogenic metabolites, which can now be used as new drugs for the biological control of Verticillium wilt. Continuous cropping leads to obstacles in crop productivity by the accumulation of p-hydroxybenzoic acid (PHBA) and ferulic acid (FA). Zhang et al. (2020b) used transcriptomics to explore the mechanism by which a strain of *Pseudomonas* CFA has higher PHBA and FA degradation abilities in soil, proposed a complete pathway for the conversion of PHBA and FA to acetyl-CoA, and discovered that 4-hydroxybenzoate 3-monooxygenase and vanillate O-demethylase were rate-limiting enzymes by gene overexpression. A previous study showed that *Pseudomonas* CFA has the potential to alleviate the PHBA and FA stress of cucumber and alleviate continuous cropping obstacles. The above results all indicate that plant growth-promoting rhizobacteria are efficient candidates for application in agricultural fields to enhance crop yield and suppress plant diseases. However, a wide variety of root exudates enter the rhizosphere soil after RKN infection. Some of these root exudates can directly antagonize diseases and insect pests, while others act as signal substances to gather and select microbial flora conducive to plant growth under the action of microbial chemotaxis (Bücking et al., 2008). The above results showed that beneficial microorganisms could be selected specifically to resist pathogen infection. That is, RKN infection could affect the behavior of rhizosphere bacteria and select some beneficial microbial populations to resist pathogen infection. The above results indicate the mechanism by which *Pseudomonas* regulates and inhibits pests and diseases, suggesting that in this experiment, tobacco infected with RKN can promote the proliferation of Pseudomonas and play a biological control function.

In addition, comparing the results of root microbes in 2017 and 2018, we found that in the second year of continuous tobacco planting, RKN infection significantly reduced the abundance of *Streptomyces* in both the rhizosphere and endophytes. *Streptomyces* is a main plant rhizosphere growth-promoting bacterium and an important nematode biocontrol bacterium. The bioactive substances produced by *Streptomyces*, such as antibiotics, enzymes, and inhibitors, have good inhibitory effects on RKNs and pathogens. In this study, in the second year of planting, the relative abundance of *Streptomyces* in rhizosphere soil infected by RKN and that in healthy tobacco were significantly different. The abundance of *Streptomyces* in healthy tobacco was significantly higher than that in the rhizosphere after RKN infection, indicating that the composition and abundance of *Streptomyces* may be related to RKN infection. The above results not only reveal that the root microorganisms after 2 consecutive years of planting have significantly different trends but also suggest that *Streptomyces* may be closely related to RKN infection, and subsequent studies can be conducted to define the mechanism of interaction between *Pseudomonas* and *Streptococcus* and RKN.

In this study, 18S rRNA sequencing technology was also used to study the composition of fungal microorganisms. Rhizosphere and endophytic fungi are widely distributed in almost all plant groups and are important microbial plant symbionts. They grow in plant cells or within cells, obtain nutrition from host plants and survive for long periods of time. Their main functions are to promote plant growth, improve the ecological adaptability of host plants, dilute active secondary metabolites, and promote plant restoration. The mechanism by which endophytic fungi promote plant growth is multifaceted. They can assist host plants in absorbing iron ions from soil through biological nitrogen fixation, synthesize, or promote plants to synthesize a variety of plant growth hormones, promote the growth of host roots and the absorption of a variety of inorganic ions, and synthesize

some small molecular substances or enzymes to improve the host plants' resistance to frost and other harmful environmental conditions and the sensitivity of harmful pathogens to promote plant growth. In addition, endophytic fungi can also improve host resistance to fungal diseases. Some studies have also found that endophytic fungal infection can affect the soil microbial community. We found that *Fusarium* and *Setophoma* were the main dominant flora of RKN-infected and healthy tobacco plants in the second year of continuous planting, and the RKN infection group significantly increased the relative abundance of *Setophoma*. The results of this study can provide some theoretical and technical means for the biological control of knot nematode disease.

In this experiment, a combination of 16S rRNA sequencing and a life-promoting test was used to comprehensively screen out strains with a variety of high biological activities. Thirty-two strains of growth-promoting bacteria were screened from tobacco rhizosphere bacteria. At present, *Rhizobia*, *Pseudomonas fluorescens*, and *Bacillus* have been found to have the potential for disease prevention and growth promotion. The growth-promoting mechanism of the abovementioned rhizosphere bacteria for plants mainly includes the synthesis of substances that directly promote the growth and development of plants, such as IAA, which change the form of elements to promote the absorption of nutrients (such as nitrogen fixation and phosphorus solubilization). However, most rhizosphere growth-promoting bacteria have a single function in overcoming nutrient and disease adversity. Therefore, it is very important to screen growth-promoting strains with complex abilities in this experiment to improve rhizosphere nutrition and regulate the soil microecological environment. For example, the *Pseudomonas* found in this study has the compound functions of phosphate solubilization and iron carriage. Iron is one of the essential micronutrients in plants and an indispensable element in life activities. Studies have shown that some biocontrol bacteria can form a competitive iron ion relationship with pathogenic bacteria by producing iron carriers to achieve an inhibitory effect on pathogenic bacteria (Saha et al., 2016). It can also reduce trivalent iron ions, which are not easily absorbed by plants, to bivalent iron ions, which are easily absorbed by plant cells by secreting and producing iron carriers, to improve the iron absorption efficiency of plants and promote the growth of plants. Manwar et al. (2004) found that *Pseudomonas aeruginosa* could increase the germination rate and chlorophyll content of peanut plants and promote root bifurcation and nodule formation. This study provides strain resources and a reference basis for the development of biocontrol and growth-promoting bacteria.

CONCLUSION

This study used 16S and 18S rRNA sequencing technology to explore the microbial composition and diversity of tobacco roots at different growth stages in typical tobacco RKN-infected areas for 2 consecutive years to analyze the dynamic changes in the rhizosphere and endophytic microbial communities in tobacco and significantly associated the microbiota of RKN on a time scale. We discovered the overall change trend of tobacco rhizosphere and root endophytic microorganisms with different microorganism growth periods and found that in the "Biocontrol bacteria-pathogens-host root microorganisms" interaction system, infection with RKN after continuous planting will significantly promote the proliferation of *Pseudomonas*, affect the composition of rhizosphere microorganisms, and make the rhizosphere select beneficial microbial populations to resist pathogen infection. This research provides reference data for elucidating the pathogenesis of RKNs and provides new ideas for the biological control of RKNs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA694781.

AUTHOR CONTRIBUTIONS

YC contributed to the conception of the study and drafted the manuscript. Z-XY, D-MY, and NL performed the experiment and manuscript preparation. S-ZY performed the data analyses. J-YM and X-JC helped perform the analysis with constructive discussions and revisions of text passages. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by the National Natural Science Foundation of China (No. 31660544), the Science and Technology Program of Guizhou Tobacco Company (2021XM12), the Major Science and Technology Program of China Tobacco Corporation [110202101055(LS-15)], the Guizhou Academician Workstation of Microbe and Health [Talent Platform (2020)4004], and the Guizhou Science and Technology Project [(2020)1Y106].

ACKNOWLEDGMENTS

The authors thank Jing Xiong and Jianguang Ma for help with fieldwork.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.807057/full#supplementary-material

REFERENCES

Buchan, A., Crombie, B., and Alexandre, G. M. (2010). Temporal dynamics and genetic diversity of chemotactic-competent microbial populations in the rhizosphere. *Environ. Microbiol.* 12, 3171–3184. doi: 10.1111/j.1462-2920.2010.02290.x

Bücking, H., Abubaker, J., Govindarajulu, M., Tala, M., Pfeffer, P. E., Nagahashi, G., et al. (2008). Root exudates stimulate the uptake and metabolism of organic carbon in germinating spores of Glomus intraradices. *New Phytol.* 180, 684–695. doi: 10.1111/j.1469-8137.2008.02590.x

Csardi, G., and Nepusz, T. (2006). The igraph software package for complex network research. *Int. J. Complex Syst.* 1695, 1–9.

Dong, L., Xu, J., Zhang, L., Cheng, R., Wei, G., Su, H., et al. (2018). Rhizospheric microbial communities are driven by Panax ginseng at different growth stages and biocontrol bacteria alleviates replanting mortality. *Acta Pharm. Sin. B* 8, 272–282. doi: 10.1016/j.apsb.2017.12.011

EFSA Panel on Plant HealthJeger, M., Bragard, C., Caffier, D., Candresse, T., Chatzivassiliou, E., et al. (2018). Pest categorisation of *Nacobbus aberrans*. *EFSA J.* 16:e05249. doi: 10.2903/j.efsa.2018.5249

Gorny, A. M., Wang, X., Hay, F. S., and Pethybridge, S. J. (2019). Development of a species-specific PCR for detection and quantification of *Meloidogyne hapla* in soil using the 16D10 root-knot nematode effector gene. *Plant Dis.* 103, 1902–1909. doi: 10.1094/PDIS-09-18-1539-RE

Hemmati, S., and Saeedizadeh, A. (2020). Root-knot nematode, *Meloidogyne javanica*, in response to soil fertilization. *Braz. J. Biol.* 80, 621–630. doi: 10.1590/1519-6984.218195

Huang, Y., Ma, L., Fang, D. H., Xi, J. Q., Zhu, M. L., Mo, M. H., et al. (2015). Isolation and characterisation of rhizosphere bacteria active against *Meloidogyne incognita*, *Phytophthora nicotianae* and the root knot-black shank complex in tobacco. *Pest Manag. Sci.* 71, 415–422. doi: 10.1002/ps.3820

Huang, W. K., Wu, Q. S., Peng, H., Kong, L. A., Liu, S. M., Yin, H. Q., et al. (2016). Mutations in Acetylcholinesterase2 (ace2) increase the insensitivity of acetylcholinesterase to fosthiazate in the root-knot nematode *Meloidogyne incognita*. *Sci. Rep.* 6:38102. doi: 10.1038/srep38102

Khan, M. R., and Ahamad, F. (2020). Incidence of root-knot nematode (*Meloidogyne graminicola*) and resulting crop losses in paddy rice in northern India. *Plant Dis.* 104, 186–193. doi: 10.1094/PDIS-12-18-2154-RE

Leonetti, P., and Molinari, S. (2020). Epigenetic and metabolic changes in root-knot nematode-plant interactions. *Int. J. Mol. Sci.* 21:7759. doi: 10.3390/ijms21207759

Li, J., Zou, C., Xu, J., Ji, X., Niu, X., Yang, J., et al. (2015). Molecular mechanisms of nematode-nematophagous microbe interactions: basis for biological control of plant-parasitic nematodes. *Annu. Rev. Phytopathol.* 53, 67–95. doi: 10.1146/annurev-phyto-080614-120336

Liang, J. F., Li, Q. W., Gao, J. Q., Feng, J. G., Zhang, X. Y., Wu, Y. Q., et al. (2021). Biochar rhizosphere addition promoted *Phragmites australis* growth and changed soil properties in the Yellow River Delta. *Sci. Total Environ.* 761:143291. doi: 10.1016/j.scitotenv.2020.143291

Liang, L. M., Zou, C. G., Xu, J., and Zhang, K. Q. (2019). Signal pathways involved in microbe-nematode interactions provide new insights into the biocontrol of plant-parasitic nematodes. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 374:20180317. doi: 10.1098/rstb.2018.0317

Liu, X., Zhang, S., Jiang, Q., Bai, Y., Shen, G., Li, S., et al. (2016). Using community analysis to explore bacterial indicators for disease suppression of tobacco bacterial wilt. *Sci. Rep.* 6:36773. doi: 10.1038/srep36773

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Ma, L., Cao, Y. H., Cheng, M. H., Huang, Y., Mo, M. H., Wang, Y., et al. (2013). Phylogenetic diversity of bacterial endophytes of *Panax notoginseng* with antagonistic characteristics towards pathogens of root-rot disease complex. *Antonie Van Leeuwenhoek* 103, 299–312. doi: 10.1007/s10482-012-9810-3

Manwar, A. V., Khandelwal, S. R., Chaudhari, B. L., Meyer, J. M., and Chincholkar, S. B. (2004). Siderophore production by a marine *Pseudomonas aeruginosa* and its antagonistic action against phytopathogenic fungi. *Appl. Biochem. Biotechnol.* 118, 243–252. doi: 10.1385/abab:118:1-3:243

Marupakula, S., Mahmood, S., Clemmensen, K. E., Jacobson, S., Hogbom, L., and Finlay, R. D. (2020). Root associated fungi respond more strongly than rhizosphere soil fungi to N fertilization in a boreal forest. *Sci. Total Environ.* 766:142597. doi: 10.1016/j.scitotenv.2020.142597

Mavrodi, O. V., Mavrodi, D. V., Parejko, J. A., Thomashow, L. S., and Weller, D. M. (2012). Irrigation differentially impacts populations of indigenous antibiotic-producing *Pseudomonas* spp. in the rhizosphere of wheat. *Appl. Environ. Microbiol.* 78, 3214–3220. doi: 10.1128/AEM.07968-11

Nikolić, I., Berić, T., Dimkić, I., Popović, T., Lozo, J., Fira, D., et al. (2019). Biological control of *Pseudomonas* syringae pv. aptata on sugar beet with *Bacillus pumilus* SS-10.7 and *Bacillus amyloliquefaciens* (SS-12.6 and SS-38.4) strains. *J. Appl. Microbiol.* 126, 165–176. doi: 10.1111/jam.14070

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U. S. A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S., and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environ. Sci. Pollut. Res. Int.* 23, 3984–3999. doi: 10.1007/s11356-015-4294-0

Schreiner, K., Hagn, A., Kyselková, M., Moënne-Loccoz, Y., Welzl, G., Munch, J. C., et al. (2010). Comparison of barley succession and take-all disease as environmental factors shaping the rhizobacterial community during take-all decline. *Appl. Environ. Microbiol.* 76, 4703–4712. doi: 10.1128/AEM.00481-10

Shan, S., Wang, W., Song, C., Wang, M., Sun, B., Li, Y., et al. (2019). The symbiotic bacteria *Alcaligenes faecalis* of the entomopathogenic nematodes *Oscheius* spp. exhibit potential biocontrol of plant- and entomopathogenic fungi. *Microb. Biotechnol.* 12, 459–471. doi: 10.1111/1751-7915.13365

Staley, C., Ferrieri, A. P., Tfaily, M. M., Cui, Y., Chu, R. K., Wang, P., et al. (2017). Diurnal cycling of rhizosphere bacterial communities is associated with shifts in carbon metabolism. *Microbiome* 5:65. doi: 10.1186/s40168-017-0287-1

Tao, X., Zhang, H., Gao, M., Li, M., Zhao, T., and Guan, X. (2020). *Pseudomonas* species isolated via high-throughput screening significantly protect cotton plants against verticillium wilt. *AMB Express* 10:193. doi: 10.1186/s13568-020-01132-1

Tian, B. Y., Cao, Y., and Zhang, K. Q. (2015). Metagenomic insights into communities, functions of endophytes, and their associates with infection by root-knot nematode, *Meloidogyne incognita*, in tomato roots. *Sci. Rep.* 5:17087. doi: 10.1038/srep17087

Zhang, Y., Chen, C. X., Feng, H. P., Wang, X. J., Roessner, U., Walker, R., et al. (2020b). Transcriptome profiling combined with activities of antioxidant and soil enzymes reveals an ability of *Pseudomonas* sp. CFA to mitigate p-hydroxybenzoic and ferulic acid stresses in cucumber. *Front. Microbiol.* 11:522986. doi: 10.3389/fmicb.2020.522986

Zhang, Y., Tian, C., Xiao, J., Wei, L., Tian, Y., and Liang, Z. (2020a). Soil inoculation of *Trichoderma asperellum* M45a regulates rhizosphere microbes and triggers watermelon resistance to Fusarium wilt. *AMB Express* 10:189. doi: 10.1186/s13568-020-01126-z

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

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/fmicb.2022.801546

Rhizosphere Microbial Community Diversity and Function Analysis of Cut Chrysanthemum During Continuous Monocropping

Tan Wang[1,2†], Kexin Yang[3†], Qingyun Ma[1,2], Xu Jiang[1], Yiqing Zhou[1], Delong Kong[1], Zhiye Wang[4], Rebecca E. Parales[5], Lin Li[2], Xin Zhao[3*] and Zhiyong Ruan[1,6,7*]*

[1] CAAS-CIAT Joint Laboratory in Advanced Technologies for Sustainable Agriculture, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, China, [2] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China, [3] Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China, [4] Key Laboratory of Microbial Resources Exploitation and Application of Gansu Province, Institute of Biology, Gansu Academy of Sciences, Lanzhou, China, [5] Department of Microbiology and Molecular Genetics, College of Biological Sciences, University of California, Davis, Davis, CA, United States, [6] College of Resources and Environment, Tibet Agricultural and Animal Husbandry University, Linzhi, China, [7] College of Life Sciences, Yantai University, Yantai, China

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Deep Chandra Suyal, Eternal University, India
Makoto Kanasugi, Nicolaus Copernicus University in Toruń, Poland

***Correspondence:**
Lin Li
lilin@mail.hzau.edu.cn
Xin Zhao
zhaoxin01@caas.cn
Zhiyong Ruan
ruanzhiyong@caas.cn

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: 25 October 2021
Accepted: 19 January 2022
Published: 16 March 2022

Citation:
Wang T, Yang K, Ma Q, Jiang X, Zhou Y, Kong D, Wang Z, Parales RE, Li L, Zhao X and Ruan Z (2022) Rhizosphere Microbial Community Diversity and Function Analysis of Cut Chrysanthemum During Continuous Monocropping. Front. Microbiol. 13:801546. doi: 10.3389/fmicb.2022.801546

As an ornamental flower crop, the long-term continuous monocropping of cut chrysanthemum causes frequent occurrence of diseases, seriously affecting the quality of cut chrysanthemum. The rhizosphere microbial community plays an important role in maintaining the healthy growth of plants, whereas the composition and dynamics of rhizosphere microbial community under continuous monocropping of cut chrysanthemum have not been fully revealed. In this study, the Illumina MiSeq high-throughput sequencing platform was used to monitor the dynamic changes of rhizosphere microbial communities in four varieties of cut chrysanthemum during 0–3 years of monocropping, and the soil physicochemical properties were also determined. Results showed that continuous monocropping significantly increased the fungal community richness and altered the profiles of the bacterial and fungal communities, leading to variation of community beta-diversity. With the increase of continuous cropping time, biocontrol bacteria decreased, while some plant pathogenic fungi were enriched in the rhizosphere of cut chrysanthemum. FAPROTAX-based functional prediction showed that the abundance of gene related to nitrogen and sulfur metabolism and chitin lysis was reduced in the rhizosphere of cut chrysanthemum. FUNGuild-based fungal function prediction showed that plant pathogenic fungal taxa were increasing in the rhizosphere of cut chrysanthemum, mainly *Acremonium*, *Plectosphaerellaceae*, *Fusarium*, and *Cladosporium*. Continuous cropping also reduced the content of ammonium nitrogen and increased soil salinity, resulting in deterioration of soil physical and chemical properties, which, together with the transformation of rhizosphere microbial community, became part of the reasons for the continuous cropping obstacle of cut chrysanthemum.

Keywords: cut chrysanthemum, rhizosphere, community structure, soil physical and chemical property decline, continuous cropping barrier

INTRODUCTION

Cut chrysanthemum is a popular flower crop because of its easy breeding, long bottle life, and storage transport. It is also known as a major cut flower worldwide, and its main production areas cover Netherlands, China, Japan, and Korea (Li et al., 2016). In China, the planting area of cut chrysanthemum exceeds 7,000 hectares, and the annual sales reach 2.65 billion sprays with a sales volume of 1.52 billion yuan (China Ministry of Agriculture, 2016). Cut chrysanthemum is generally grown in greenhouse sheds, which can be harvested up to five times a year, to obtain great economic benefits. However, similar to other crops, such as soybean, tomato, watermelon, and vanilla, long-term continuous monocropping will generate replanting problems. The replanting problem of cut chrysanthemum will induce adverse symptoms such as short stature, yellowing, and increased infection rate (Li et al., 2017). In commercial production, the replanting problem has led to a significant decline in the yield and quality of cut chrysanthemum, resulting in great economic losses for growers.

At present, a number of measures to address the replanting problem have been developed. Rotation can reduce weed and pest occurrence, facilitate soil nutrient utilization (Nevens and Reheul, 2001), and improve crop yield and quality (Zhao et al., 2020). Organic fertilizer contains a variety of organic acids (OA), peptides, and rich nutrient elements such as nitrogen, phosphorus, and potassium, which can not only provide comprehensive nutriment for crops but also increase and renew soil organic matter, promote microbial reproduction, and improve soil physical and chemical properties and biological activity (Chen et al., 2018). Long-term continuous monocropping will cause the accumulation of pathogenic microorganisms, and the application of soil sterilization (including high-temperature sterilization and reagent fumigation) can effectively reduce the number of pathogenic microorganisms (Li et al., 2019). However, soil sterilization may damage the soil microbial ecology, which is contrary to the objective of sustainable agriculture. Rhizosphere microbial communities are closely related to plant health (Raaijmakers et al., 2009). The root exudates from plants consist of phenolic acids (PA), OA, sugars, amino acids, fatty acids, alkaloids, terpenoids, enzymes, flavonoids, and proteins (De Weert et al., 2002; Bais et al., 2006). A variety of carbon and nitrogen sources obtain abundant microorganisms. Exploiting microorganisms isolated from the environment as antagonists or growth-promoting rhizobacteria can effectively reduce the occurrence of diseases and promote plant growth (Verbon and Liberman, 2016; Morales-Cedeño et al., 2021).

Nevertheless, the aforementioned method always transforms the soil microbiome to a healthier direction. As the second genome of plants, plant-associated microbiome plays an important role in plant growth. With regard to nutrient utilization, the symbiotic interactions between plants and arbuscular mycorrhizal fungi and *Rhizobium* bacteria lead to nutrient acquisition (Richardson and Simpson, 2011; Corné et al., 2014; Trivedi et al., 2016; Rachel et al., 2018). Non-symbiotic plant-growth-promoting bacteria can improve not only the bioavailability of insoluble minerals but also the root system architecture of host plants, thereby increasing the exploratory capacity of the root for water and minerals (Richardson and Simpson, 2011; Trivedi et al., 2016). A recent study suggested that the differences in nitrogen use efficiency in rice varieties are due to a high proportion of nitrogen-cycling-related bacterial supplementation, resulting in a more efficient transformation of nitrogen in the root environment in indica rice than in japonica rice varieties (Zhang et al., 2019). Rhizosphere microorganisms extend the bioavailability of nitrogen by nitrification, delay flowering time by converting tryptophan to the plant hormone indoleacetic acid, and stimulate plant growth by downregulating flowering genes (Lu et al., 2018). Plant-related microbial groups can mobilize nutrients that are not easily available to plants, such as inorganic phosphate and iron, through dissolution, mineralization, or excretion through iron-chelated siderophores. Moreover, plant-related microbiome helps plants fight diseases. The effect of plant defense based on natural microorganisms on plant health has been clearly demonstrated in suppressive soils. Plant root exudates stimulate, enrich, and support soil microorganisms as the first line of defense against soil-borne pathogens (Mendes et al., 2011). Community-based analyses of suppressive soils have demonstrated that no single phylum is uniquely associated with disease suppression (Mendes et al., 2011; Penton et al., 2014; Santhanam et al., 2015; Cha et al., 2016; Trivedi et al., 2017). A recent study has modeled general disease suppression of *Fusarium oxysporum* and identified bacterial abundance in *Actinomycetes* and *Firmicutes* as predictive markers for continent-scale soil disease suppression. Under abiotic or biological stress conditions, plants usually select a microbial community that promotes stress resistance (Wagner et al., 2014; Eida et al., 2018; Naylor and Coleman-Derr, 2018; Naylor et al., 2018; Timm et al., 2018; Fitzpatrick et al., 2019), which modifies plant evolutionary responses to environmental stress by altering the fitness of individual plant genotypes, the expression of plant traits related to fitness, and the strength or direction of natural selection occurring within populations that experience environmental stress through the effects of microorganisms on reproductive fitness (Lau and Lennon, 2012; Eida et al., 2018; Fitzpatrick et al., 2018, 2019). In the effect of fungal endophytes on plant growth performance under water stress, traits related to resource utilization and stress tolerance account for 26–53% (Kudjordjie et al., 2019). In addition, the complex microbial community can help plants deal with other adverse effects, such as heavy metals (Yang et al., 2020), herbicides (Fawzy et al., 2015), and pesticides (Li et al., 2020). The composition and structure of the microbiome must remain stable to continuously exert the functional potential of the plant-related microbiome, and the loss of its steady state may cause negative effects such as imbalance in soil nutrient conversion and the proliferation of pathogenic bacteria. Therefore, determining the dynamic changes of the rhizosphere microbial community under different continuous cropping periods is necessary to explore the mechanism of continuous cropping obstacles of cut chrysanthemum.

Based on previous studies, the rhizosphere microbial community of cut chrysanthemum is based on denaturing gradient gel electrophoresis (Duineveld et al., 2001; Song et al., 2013), which is not sufficient to reveal the overall dynamic changes of bacteria and fungi during continuous cropping of cut chrysanthemum. With the wide application of high-throughput sequencing technology, the microbial community landscape in

the environment is gradually revealed. Here, we used Illumina MiSeq sequencing technology to detect the dynamic changes of microbial communities in the rhizosphere soil of four varieties of cut chrysanthemum during 0–3 years of monocropping period and measured the physical and chemical properties, nutrients, and other indicators of soil to evaluate the health status of the soil during continuous cropping. We hypothesized that (i) during continuous monocropping for 0–3 years, harmful microorganisms would accumulate in the rhizosphere. (ii) Differences in rhizosphere exudates of different varieties of cut chrysanthemum could affect the rhizosphere microbial community. (iii) With the increase of continuous monocropping time, soil physicochemical properties decline, and nutrient conversion slows down.

MATERIALS AND METHODS

Study Site and Experimental Design

The experimental site was located in Nankou experimental base of the Chinese Academy of Agricultural Sciences, Beijing (116.57033, 40.237361), China. Cut chrysanthemums with different monocropping times were planted in different greenhouses at a distance of 8 m from each other. Each greenhouse is 80 m in length. In the greenhouse, each variety of cut chrysanthemum was randomly planted in three plots, separated by other cut chrysanthemum varieties. All plots adopted the same water and fertilizer management practices. During sampling, each plot randomly took soil samples of three chrysanthemum plants and mixed them into one replicate. The monoculture time selected in the experiment was 0 years (CK, before planting cut chrysanthemum), 0.5 years (H, planting for one crop), and 3 years (T, planting for six crops). The sampling period was the mature period. A total of four varieties were selected, namely, *Glitter Orange* (variety 1), *Gem Yellow* (variety 2), *Cloud Red* (variety 3), and *Mona Lisa Pink* (variety 4). Thus, the sample names were CK (0 years); H1, H2, H3, and H4 (0.5 years); and T1, T2, T3, and T4 (3 years). CK had four replicates; the others had three replicates. In obtaining rhizosphere soil, the fibrous roots and main roots of the plant were cut off with sterile scissors, and then the roots were placed into a sterilized 50 ml centrifuge tube. Subsequently, the root tissues were added with 0.2 × PBS buffer solution and swirled for 1 min. Afterward, the root tissues were removed and centrifuged at 8,000 rpm for 5 min. The supernate was discarded, and the residual part was the rhizosphere soil. The rhizosphere soil samples were placed at –80°C for subsequent DNA extraction.

Soil Physicochemical Properties

The planted soil of cut chrysanthemum (bulk soil) was collected to determine the physical and chemical properties. The sampling method of bulk soil referred to that of Edwards et al. (2014). The collected soil was air-dried on a kraft paper and screened by a 1 mm sieve, and the resulting soil was determined as dried soil. Soil pH was measured using a glass conductance meter in a 1:2.5 soil/water (W/V) suspension (Qiu et al., 2012). The content of soil total carbon, total nitrogen, and organic carbon was determined by using an elemental analyzer (Elementar Analysensysteme GmbH, Germany). Soil urease (Kandeler and Gerber, 1988), catalase (Johansson, 1988), sucrase (Gao et al., 2013), and alkaline phosphatase (Scandinavica et al., 2014) activities were determined using the soil enzyme activity assay kit from Solarbio Science & Technology Co. (Beijing, China). Soil available P was extracted using sodium bicarbonate and measured with the molybdenum blue method (Olsen, 1954). Soil available K was extracted using ammonium acetate and determined with flame photometry (nova 300, Analytic Jena, Germany) (Frank et al., 1998). Soil ammonium nitrogen and nitrate nitrogen were extracted with 1 M KCl and the indophenol-blue colorimetric and double wavelength (220 and 275 nm) methods, respectively, and their concentrations were measured using a spectrophotometer (UV-6000, China) (Zhao et al., 2017). Soil salinity was determined by an oven-drying method following the NY/T 1121.16-2006 standard water-soluble salt analysis of soil.

Soil DNA Extraction and High-Throughput Amplicon Sequencing

Total genome DNA from 0.5 g of fresh soil samples was extracted using a E.Z.N.A^{TM} Mag-Bind Soil DNA Kit (OMEGA, Norcross, United States) following the manufacturer's instructions. DNA integrity was detected on 1% agarose gel, and DNA concentration was quantitatively detected by Qubit. The V3–V4 region of the 16S rRNA gene was amplified using the PCR primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) for bacterial community analysis, and the ITS1 region of the transcribed spacer was amplified using the PCR primers ITS1F (5′-CTTGGTCATTTA GAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′). 16S rRNA genes were amplified using a specific primer with a barcode. The first round of PCRs was performed in 30 μl of reactions with 15 μl of 2 × Hieff Robust PCR Master Mix, 1 μl of Bar-PCR primer F and 1 μl of Primer R, and 10–20 ng of template DNA. PCR amplification cycles consisted of an initial denaturation at 94°C for 3 min, followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 20 s, and elongation at 65°C for 30 s; then 20 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and elongation at 72°C for 30 s; and finally elongation at 72°C for 5 min after cycles.

Illumina bridge PCR-compatible primers were introduced in the second round of amplification. The reaction system was identical to that of the first round; however, the primers were replaced by Primer F and Index-PCR Primer R. Library quality was assessed using a Qubit@ 3.0 fluorometer (Thermo Scientific, Waltham, MA, United States). Finally, the library was sequenced on an Illumina MiSeq platform, and 250 bp paired-end reads were generated.

Community Diversity and Function Analysis

USearch was used to cluster the sequences in the libraries. Operational taxonomic unit (OTU) clustering was performed on non-repeated sequences (excluding single sequences) based

on 97% similarity, and the representative sequence of OTU was obtained by removing chimeras during clustering. Then, an RDP classifier was used to annotate taxonomic information for each representative sequence. In calculating alpha diversity, we refined the OTU table and calculated two indicators, namely, ACE index and Shannon even index, to estimate the species richness and species evenness of the community, respectively.

The 10 most abundant phyla were selected from all the samples included in the phylum-level species annotation and abundance information, and a histogram was drawn for each sample with regard to relative abundance. In addition, 30 genera with the highest abundance were selected to draw a heatmap. LEfSe (Segata et al., 2011) was used to compare data among samples and select biomarkers for each sample, and 2.0 was set as the threshold for the logarithmic LDA score for discriminating features. Principal co-ordinate analysis (PCoA) was performed to compare the community profiles of different samples in R[1] by using the amplicon and phyloseq packages. Redundancy analysis (RDA) was performed to evaluate the relationship between microbial community profiles and soil physical and chemical properties by using the ggvegan package. Linear fitting analysis was used to evaluate the correlation between soil enzyme activity and soil nutrient element content and between soil enzyme activity and microbial community richness index. To further explore the impact of microbial community change, functional prediction with PICRUSt (Langille et al., 2013), FAPROTAX (Louca et al., 2016), and FUNGuild (Nguyen et al., 2015) annotation tools was performed. The predicted results were visualized by a principal component analysis (PCA) plot and boxplot. The figures were adjusted, combined, and modified by Adobe Illustrator 2021.

RESULTS

Changes of Soil Physicochemical Properties of Cut Chrysanthemum During Continuous Monoculture

During continuous monocropping for 0–3 years, the contents of soil total carbon, total nitrogen, and organic carbon increased significantly in soil planted with "variety 1" and "variety 4," whereas no evident change was observed in soil pH of all varieties (**Table 1**). Ammonium nitrogen content showed a decreasing trend in bulk soil of all varieties. In general, the content of nitrate nitrogen initially increased and then decreased during continuous monocropping, but it increased remarkably in soil planted with "variety 1" in the third year of monocropping. The content of available phosphorus and potassium also showed an increasing trend in replanted soil, and the increment in bulk soil planted with "variety 1" and "variety 4" was high. Likewise, the total salt content in soil increased in the third year of monocropping, and the maximum increment was observed in soil planted with "variety 1."

During monocropping of cut chrysanthemum, the activities of four soil enzymes showed an upward trend in general. Urease activity of soil planted with "variety 1," "variety 3," and "variety 4" significantly increased in the third year, and a high increment was observed in bulk soil planted with "variety 1" (**Figure 1**). The activity of soil catalase also showed an increasing trend with the increase of monocropping time. The activity of sucrase was almost zero from 0 to 0.5 years, but it increased greatly in the third year (T1). Meanwhile, alkaline phosphatase activity increased from 0.5 to 3 years.

Diversity Changes of Rhizosphere Microbial Community With Continuous Monocropping Period

During the continuous monocropping period of 0–3 years, the richness and evenness of the rhizosphere bacterial community of the four varieties of cut chrysanthemum did not change significantly (**Figures 2A,B**). The richness of the fungal community increased remarkably in the third year, whereas the evenness remained constant (**Figures 2C,D**). During the monoculture period, the structure and composition of the rhizosphere microbial community changed dramatically. At the phylum level of bacteria (**Figure 3A**), *Proteobacteria* with the highest relative abundance maintained stable abundance with continuous monocropping. In another dominant phylum, *Actinobacteria*, its abundance decreased significantly in the third year of continuous monocropping, particularly in the rhizosphere of "variety 2." On the contrary, the abundance of *Bacteroidetes* (Top4 phylum) showed a significant increase in the third year. Furthermore, the abundance of other dominant phyla, such as *Verrucomicrobia*, *Gemmatimonadetes*, and *Chloroflexi*, remained relatively stable in the rhizosphere. In the fungal community (**Figure 3B**), *Ascomycota* was the most abundant phylum. In year 0.5, the relative abundance of *Ascomycota* varied greatly in the rhizosphere of the four varieties of cut chrysanthemum, but it remained stable in the rhizosphere of the four varieties during the follow-up period. *Basidiomycota* (Top3 phylum) gradually enriched around the root with continuous monocropping, and it showed preference to "variety 1." The relative abundance of *Mortierellomycota* (Top4 phylum) in CK soil was higher than that in the rhizosphere, and it occupied great proportion in the rhizosphere with continuous monocropping. At the genus level, *Arthrobacter* (Top1 genus) was largely enriched in the rhizosphere of 0.5 year samples, but it declined in the third year (**Figure 3C**). The relative abundance of *Pseudomonas* (Top4 genus) and *Lysobacter* (Top24 genus) also decreased in the third year of monoculture. *Sphingobacterium* (Top7 genus) was significantly enriched in the rhizosphere of all varieties. Some taxa, such as *Gp6* (Top5 genus), *Streptophyta* (Top9 genus), and *Ohtaekwangia* (Top18 genus), only showed differences in some samples. In the fungal community (**Figure 3D**), *Cladosporium* (Top1 genus) was greatly enriched in the rhizosphere of cut chrysanthemum with continuous monoculture. *Acremonium* (Top2 genus) and *Thyronectria* (Top14 genus) showed the same trend. An unclassified genus (Top29), and *Botryotrichum* (Top30 genus), showed high abundance in the rhizosphere of cut chrysanthemum in 0.5 years. However, by the third year of monoculture, their abundance dropped dramatically.

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

TABLE 1 | A summary of soil physicochemical properties in bulk soil of cut chrysanthemum.

Treatments	Total carbon (g/kg)	Total nitrogen (g/kg)	Organic carbon (g/kg)	Soil pH	Ammonium nitrogen (mg/kg)	Nitrate nitrogen (mg/kg)	Available phosphorus (mg/kg)	Available potassium (mg/kg)	Salt content (g/kg)
CK	8.84 ± 2.49^{d}	0.59 ± 0.14^{b}	5.00 ± 0.93^{b}	8.63 ± 0.19^{a}	1.61 ± 0.25^{a}	16.15 ± 4.46^{b}	27.77 ± 6.15^{ef}	64.80 ± 8.14^{f}	0.95 ± 0.09^{bc}
H1	8.28 ± 0.16^{d}	0.59 ± 0.05^{b}	3.84 ± 0.19^{b}	8.43 ± 0.16^{a}	1.20 ± 0.42^{ab}	21.75 ± 7.62^{b}	29.84 ± 2.32^{de}	87.47 ± 2.47^{ef}	0.53 ± 0.09^{de}
H2	10.87 ± 0.14^{bcd}	0.55 ± 0.07^{b}	4.19 ± 0.14^{b}	8.53 ± 0.24^{a}	1.03 ± 0.34^{ab}	19.63 ± 6.50^{b}	29.30 ± 3.34^{de}	96.13 ± 5.40^{ef}	0.64 ± 0.05^{cde}
H3	10.18 ± 0.16^{cd}	0.56 ± 0.03^{b}	4.26 ± 0.24^{b}	8.51 ± 0.19^{a}	1.07 ± 0.38^{ab}	19.45 ± 6.77^{b}	34.91 ± 3.71^{de}	110.87 ± 0.58^{de}	0.87 ± 0.07^{bcd}
H4	13.31 ± 0.72^{ab}	0.51 ± 0.07^{b}	5.06 ± 1.14^{b}	8.41 ± 0.05^{a}	0.95 ± 0.32^{ab}	28.95 ± 4.24^{b}	18.17 ± 2.93^{f}	76.07 ± 11.5^{ef}	1.20 ± 0.06^{b}
T1	15.07 ± 0.52^{a}	1.19 ± 0.05^{a}	10.70 ± 1.09^{a}	8.22 ± 0.10^{a}	0.54 ± 0.15^{b}	65.98 ± 25.27^{a}	137.01 ± 3.32^{b}	215.17 ± 17.42^{b}	1.73 ± 0.05^{a}
T2	10.34 ± 0.29^{cd}	0.55 ± 0.04^{b}	4.77 ± 0.87^{b}	8.59 ± 0.25^{a}	0.58 ± 0.20^{b}	13.26 ± 2.60^{b}	39.56 ± 0.92^{d}	133.37 ± 0.46^{cd}	1.13 ± 0.08^{bc}
T3	10.21 ± 0.14^{cd}	0.64 ± 0.06^{b}	5.13 ± 0.27^{b}	8.53 ± 0.07^{a}	0.49 ± 0.05^{b}	13.66 ± 1.68^{b}	58.69 ± 5.41^{c}	165.40 ± 13.11^{c}	1.04 ± 0.13^{bc}
T4	12.62 ± 0.45^{abc}	1.17 ± 0.05^{a}	10.37 ± 0.53^{a}	8.27 ± 0.22^{a}	0.81 ± 0.20^{ab}	24.37 ± 5.36^{b}	150.35 ± 0.97^{a}	488.73 ± 21.44^{a}	1.05 ± 0.15^{bc}

Statistical significance was set at a level of $p < 0.05$ using Tukey's HSD tests. The same letter in the table represents no significant difference.

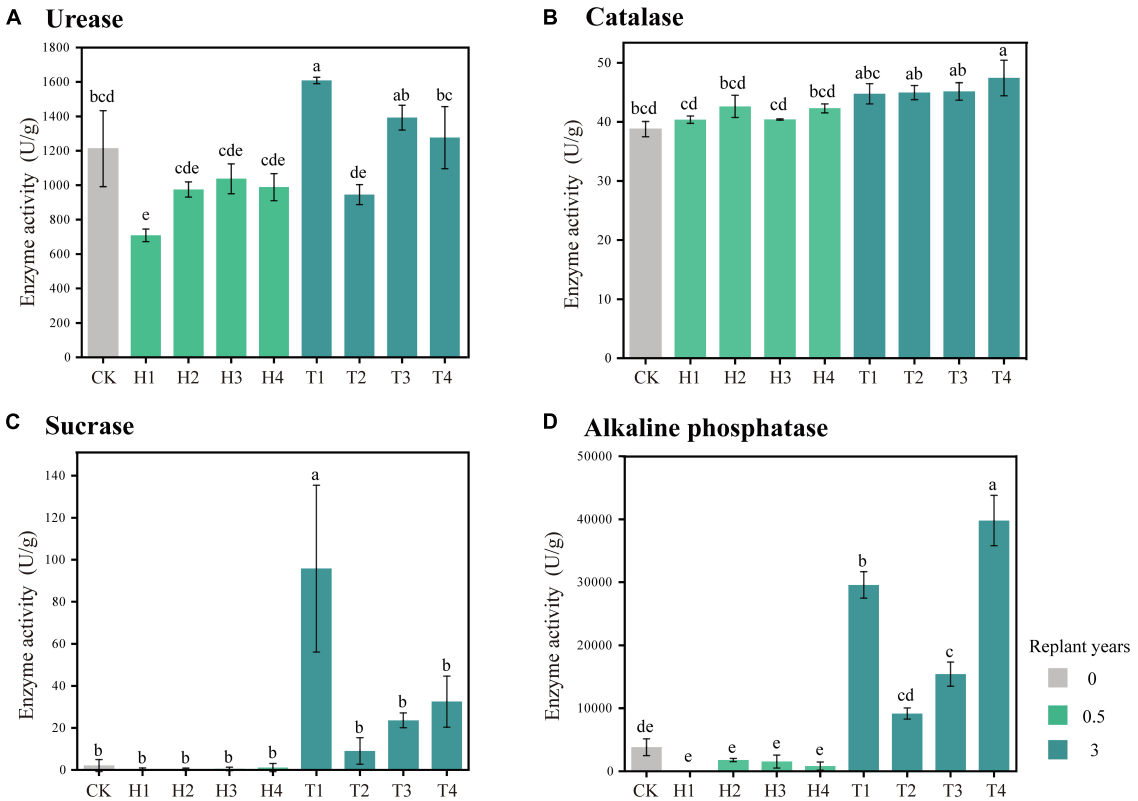


FIGURE 1 | Changes of the activities of four pivotal soil enzymes with the continuous monoculture period (Tukey's HSD test). **(A)** Urease. **(B)** Catalase. **(C)** Sucrase. **(D)** Alkaline phosphatase.

LEfSe identified the taxa that were significantly different among samples, which were defined as biomarkers (**Figure 4**). In the bacterial community, the abovementioned bacteria changed significantly in the third year of monoculture, which primarily belonged to *Bacteroidetes* and *Acidobacteria* (**Figure 4A**). At a low classification level, the families *Sphingobacteriaceae* (Top5 family), *norank_Acidobacteria_Gp4* (Top7 family), *Chitinophagaceae* (Top4 family), *Flavobacteriaceae* (Top25 family), *Planctomycetaceae* (Top9 family), and *Enterobacteriaceae* (Top63 family) and the genera *Sphingobacterium* (Top6 genus), *Gp6* (Top3 genus), *Chryseobacterium* (Top37 genus), *Chryseolinea* (Top29 genus), *Gp7* (Top18 genus), *Sphingopyxis* (Top31 genus), *Pelagibacterium* (Top46 genus), *Rhizobium* (Top40 genus), *Variovorax* (Top33 genus), *Flavobacterium* (Top61 genus), *Olivibacter* (Top63 genus), *Halomonas* (Top51 genus), *Stenotrophomonas* (Top82 genus), *Buttiauxella* (Top106 genus), and *Gp10* (Top35 genus) contributed to the occurrence of differences in the phylum level. For the fungal community, the difference in the rhizosphere community in the third year of monoculturing primarily occurred on *Ascomycota* and *Rozellomycota* (**Figure 4B**).

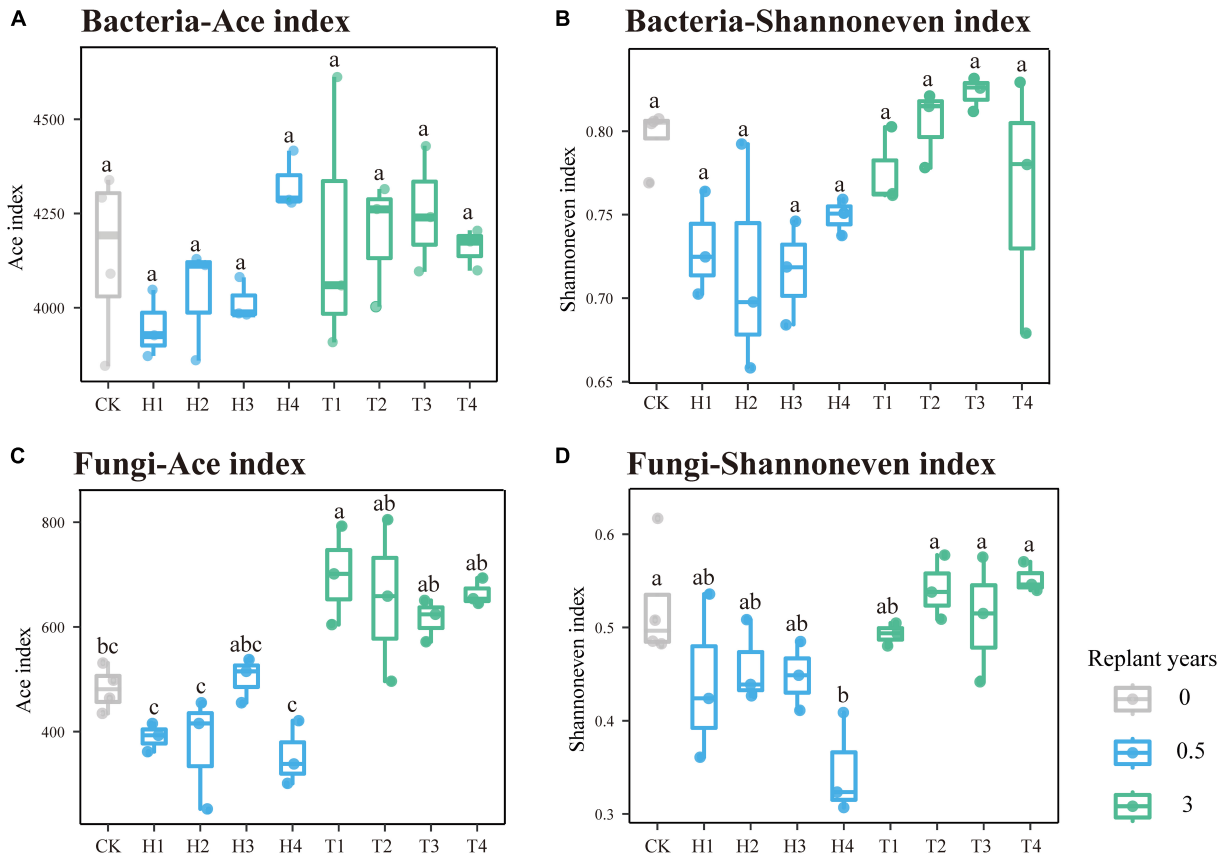


FIGURE 2 | Variations of the rhizosphere microbial community richness and evenness in different monoculture years. The ACE index indicates the richness of community, where the Shannon even index represents the evenness of the community. Box colors show the length of continuous monoculture time. **(A,B)** Prokaryotic community. **(C,D)** Fungal community.

At the species level, *Cladosporium_sphaerospermum* (Top1 species), *unclassified_Acremonium* (Top3 species), *unclassified_Gibellulopsis* (Top5 species), *Acrostalagmus_luteoalbus* (Top4 species), *Thyronectria rhodochlora* (Top7 species), and *Podospora pyriformis* (Top10 species) had high LDA scores, which partly resulted in the difference at the phylum level.

Principal co-ordinate analysis based on the Bray–Curtis distance showed that bacterial or fungal communities of different monocropping periods were separated on the first axis, and the differences accounted for 31% (**Figure 5A**) and 37% (**Figure 5B**), respectively. The PERMANOVA test showed significant differences among the three-period samples ($P < 0.01$). RDA of the rhizosphere microbial community and soil physicochemical properties during monocropping showed that (**Figures 6A–C**) the total contribution rates of soil total carbon (C), total nitrogen (N), organic carbon (OC), ammonium nitrogen (AN), nitrate nitrogen (NN), available phosphorus (AP), available potassium (AK), and soil salinity (SC) in shaping bacterial and fungal communities were 44.9 and 46.9%, respectively, of which the content of ammonium nitrogen had the highest contribution rate (13.0% for bacteria and 14.0% for fungi). On the first axis, the microbial community was primarily separated by AN, C, SC, and NN, whereas on the second axis, the microbial community was separated by OC, N, AP, and AK.

Functional Prediction of Rhizosphere Microbial Community in Cut Chrysanthemum During Continuous Monocropping

Principal component analysis was performed with the results of COG and KEGG function annotations (**Figure 7**), and the results showed that the functional composition of rhizosphere microorganisms of cut chrysanthemum has changed significantly from 0.5 to 3 years (PERMANOVA, COG: $P = 0.007$, KO: $P = 0.007$). The five COG classifiers with the highest contribution rates that cause differences between samples were COG0583, COG1629, COG1595, COG2204, and COG0515. According to the distribution of sample points of different monocropping years on the PCA plot, it showed that COG1629 was the most influential COG classifier that caused differences of functional profiles. COG1629 was annotated as outer membrane receptor proteins, mostly referring to Fe transport. In the KEGG annotated results, the five most volatile KEGG classifications were K02026, K02004, K03088, K02027, and K02025. The K03088, belonging to

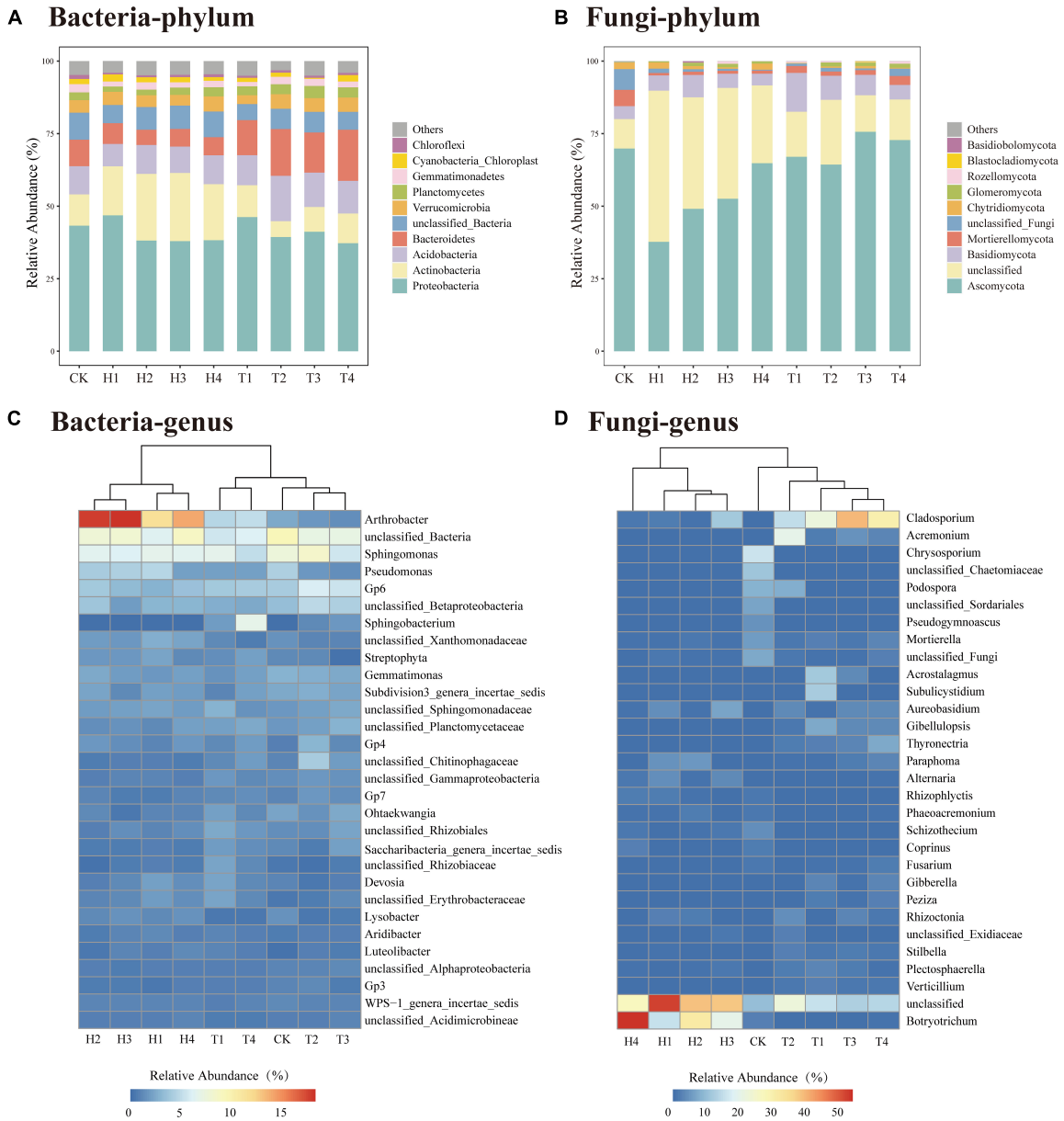


FIGURE 3 | The dynamics of the rhizosphere microbial community composition on phylum and genus levels during the continuous monoculture time series. **(A)** Bacterial community on phylum level. **(B)** Fungal community on phylum level. **(C)** Bacterial community on genus level. **(D)** Fungal community on genus level.

RNA polymerase sigma-70 factor, ECF subfamily, had the greatest contribution to differences between groups (0.5 vs. 3 years).

Based on the results of FAPROTAX and FUNGuild, significant changes in the abundance of some metabolic pathways of bacterial community (**Figure 8A**) and the plant pathogen of fungal community (**Figure 8B**) had occurred. Among these pathways, most of them involved nitrogen and sulfur metabolism, and one was associated with chitin lysis. Continuous monocropping obviously reduced the abundance of these metabolic genes. For the fungal community, all species were matched with different functional groups (Guild). Guilds which were part of plant pathogens had been observed in the rhizosphere, and some of them were massively enriched with continuous monocropping.

DISCUSSION

Continuous Monocropping Resulted in Soil Degradation of Cut Chrysanthemum

Here, we monitored the changes of soil-related physical and chemical properties of four varieties of cut chrysanthemum after continuous monocropping for 3 years, and the results showed that the content of soil carbon, nitrogen, available phosphorus,

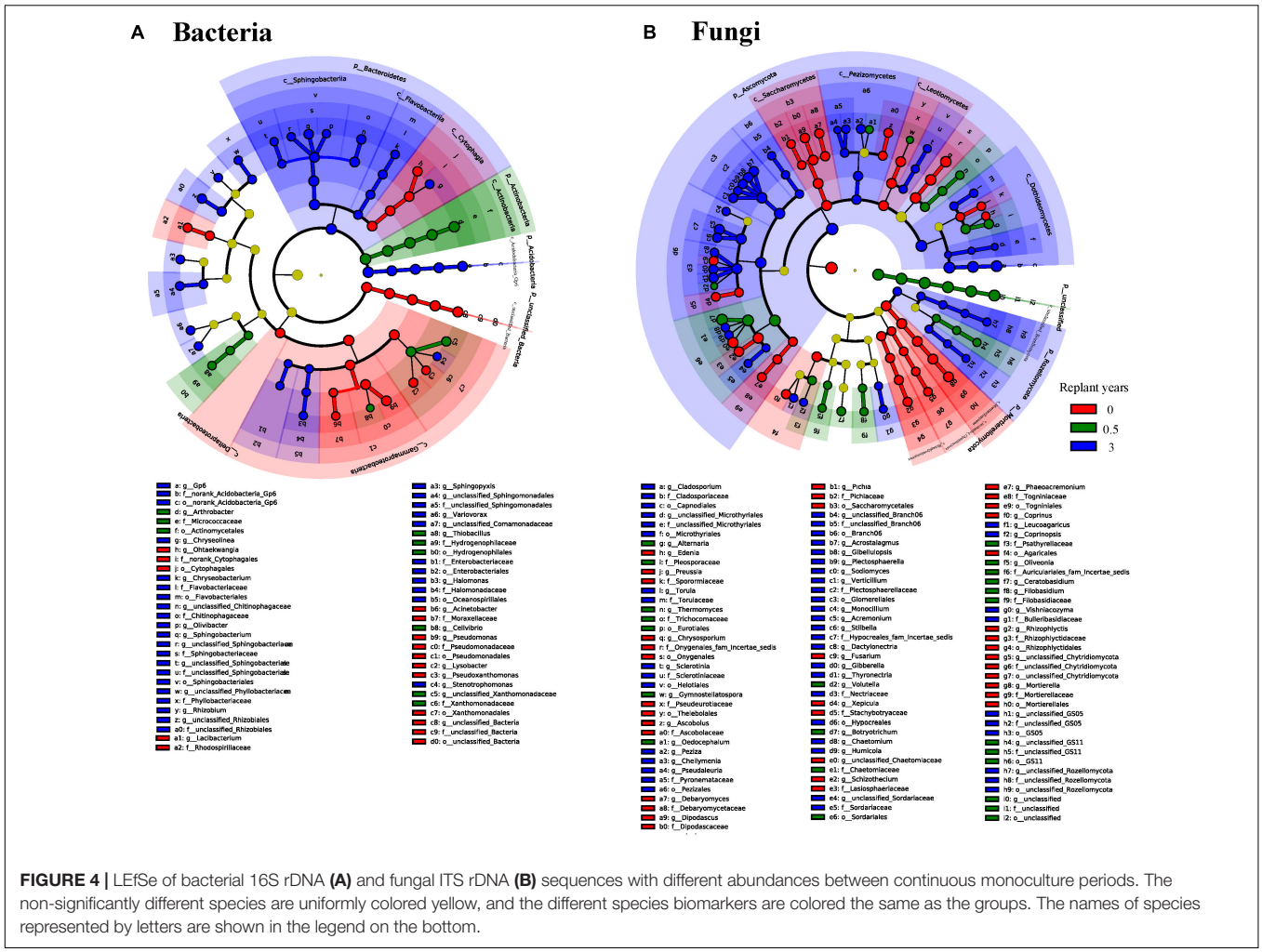
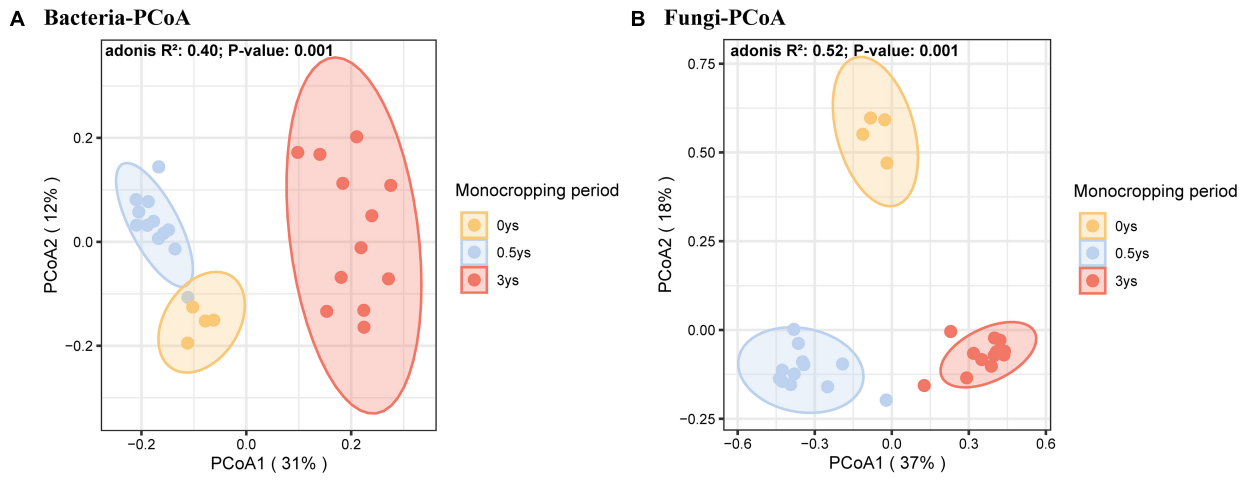


FIGURE 4 | LEfSe of bacterial 16S rDNA **(A)** and fungal ITS rDNA **(B)** sequences with different abundances between continuous monoculture periods. The non-significantly different species are uniformly colored yellow, and the different species biomarkers are colored the same as the groups. The names of species represented by letters are shown in the legend on the bottom.

and available potassium showed different degrees of increment in the third year of monocropping (**Table 1**). The soil pH did not change dramatically, but a slight reduction occurred in the 3 year soil planted with "variety 1" and "variety 4." Notably, the content of available nitrogen (including ammonium nitrogen and nitrate nitrogen) in the soil basically decreased with continuous cropping, which may cause nutrient imbalance and hinder the growth of cut chrysanthemum. In this study, the salt content in the soil showed an increasing trend with continuous monocropping. Soil salinity is also closely related to the growth of plants. Higher soil salinity produces higher osmotic pressure, which leads to the inability of crops to effectively absorb water and nutrients in the soil (Kluitenberg and Biggar, 1992; Tian et al., 2020), resulting in vegetable deficiency symptoms and decreasing crop quality and yield (Riaz et al., 2018). Soil enzyme, which is produced by plants, animals, and microorganisms, participates in the synthesis and oxygenolysis of various organic and inorganic substances, and it is an indicator for evaluating soil fertility (Dick and Kandeler, 2004; Wang et al., 2019). Soil urease (S-UE) is an enzyme that hydrolyzes amide organic nitrides into inorganic nitrides that can be directly utilized by plants (Singh and Ghoshal, 2013; Cai et al., 2015), while high urease activity rapidly hydrolyzes applied urea to ammonia which contributes to soil nitrogen losses and reduces N use efficiency of crop plants (Rana et al., 2021). Furthermore, ammonium accumulation in soil, particularly at high pH, may hinder the nitrification process at the midway and result in the accumulation of toxic levels of nitrites (Breuillin-sessoms et al., 2017). Soil catalase (S-CAT) is an important oxidoreductase, which is closely related to the activity of oxygen-consuming microbes. It can promote the decomposition of hydrogen peroxide—a metabolic intermediate—and alleviate its toxicity (Sun et al., 2013). The enzymatic activity of catalase can characterize the strength of soil humus and the conversion rate of organic matter (Lee et al., 2009). The soil sucrase activity (S-SC) reflects the maturation degree and fertility level of soil and is an important index for evaluating soil fertility (Stępniewska et al., 2009). Further, sucrase activity is directly related to the growth of crops and closely related to the transformation of organic matter and respiratory intensity (Gianfreda et al., 2005; Tripathi et al., 2007). Alkaline phosphatase enzymes (S-ALP) are believed to be mainly released by microorganisms (Nannipieri et al., 2011) and to be affected by plant species, microbial community composition, and rhizodeposition (Badri and Vivanco, 2009; Wasaki et al., 2018;



pairs	R²	p.value	p.adjusted
0.5ys vs 3ys	0.3515764	0.001	0.0015
0.5ys vs 0ys	0.3079967	0.001	0.0015
3ys vs 0ys	0.2647027	0.002	0.0020

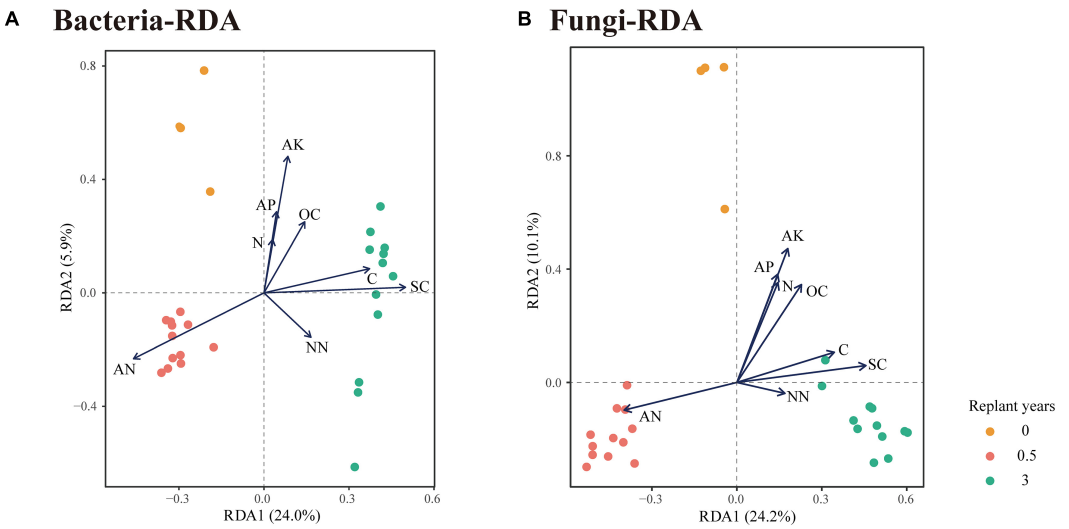
pairs	R²	p.value	p.adjusted
0.5ys vs 3ys	0.4678740	0.001	0.0015
0.5ys vs 0ys	0.4136082	0.001	0.0015
3ys vs 0ys	0.4035999	0.005	0.0050

FIGURE 5 | Difference of the rhizosphere microbial community profiles of various monoculture years. Points' color represents different monoculture periods. The points are covered by a 95% confidence ellipse. Pairwise PERMANOVA is shown below the PCoA plots. **(A)** PCoA of prokaryotic communities. **(B)** PCoA of fungal communities.

Teixeira et al., 2019). Moreover, alkaline phosphatase enzymes are ubiquitous in soils, but their activity is somehow influenced by the quantity and quality of rhizodeposits (Ragot, 2016; Wasaki et al., 2018; Wu et al., 2018; Wei et al., 2019). In the current research, the activities of four soil enzymes all increased with continuous monocropping (**Figure 1**). Part of possible reasons were revealed by performing the linear fitting analysis of enzyme activity and soil physicochemical properties (**Figure 9A**) and ACE diversity index of microbial community (**Figure 9B**). Strong correlations were observed in S-UE and N ($R^2 = 0.36$, $P < 0.001$), S-CAT and OC ($R^2 = 0.24$, $P = 0.008$), S-SC and OC ($R^2 = 0.27$, $P = 0.005$), and S-ALP and AP ($R^2 = 0.37$, $P < 0.001$). The residual amount of nutrient elements differentiated due to the different utilization capacity of each cut chrysanthemum, and higher residual amounts always corresponded to higher enzyme activity. Still, the ACE index of fungal community increased with the increase of enzyme activity (**Figures 9Be–h**), which may indicate that cut chrysanthemum recruited more fungal taxa to increase enzyme production. The continuous increase of urease activity would be one of the reasons for the occurrence of replanting problems.

Continuous Monocropping Caused Changes in the Bacterial Community Composition and a Decrease in the Abundance of Nutrient Element Conversion Genes

The richness and evenness of bacterial community did not change significantly during continuous monocropping of the four varieties (**Figures 2A,B**). However, variations in the abundance of some taxa occurred in the rhizosphere of cut chrysanthemum (**Figures 3A,C**, **4A**). *Proteobacteria* is the dominant taxon in different geographical regions and soil types (Janssen, 2006), and members of this phylum play important roles in phylogenetic, ecological, and pathological values and energy metabolism (Bryant and Frigaard, 2006; Mukhopadhya et al., 2012). Root exudates and plant species play a key role in shaping the rhizosphere microbial community, which leads to genotype-specific communities of plants within the same soil type (Grayston et al., 1998; Marschner et al., 2004; Chaparro et al., 2014). The differences in the abundance of *Proteobacteria* may be primarily attributed to the varieties of cut chrysanthemum (**Figure 3A**). Based on previous studies, *Actinobacteria* is associated with pathogen antagonism (Mendes et al., 2011; Xiong et al., 2017), which is enriched in large amounts in suppressive soil and significantly decreased with the increase of monocropping time (Xiong et al., 2015). In our study, the relative abundance of *Actinobacteria* reduced significantly in the third year of monoculture. *Acidobacteria* is also a member of a relatively rich and prevalent soil bacterial community (Janssen, 2006), and it plays an important role in organic matter degradation and nutrient cycling (Eichorst et al., 2018). Fierer et al. (2007) showed that *Acidobacteria* had the highest abundance in soil with low resource availability. In general, continuous monocropping reduces soil nutrients and soil pH, which may explain the high abundance of *Acidobacteria* in replanted soil of cut chrysanthemum. In addition, *Bacteroidetes* participates in carbon and nitrogen metabolism, such as organic matter degradation and nitrite oxidation (Clemens, 2017; Han et al., 2017; Eichorst et al., 2018; Fang et al., 2018), and it has higher abundance in environments with high carbon content.

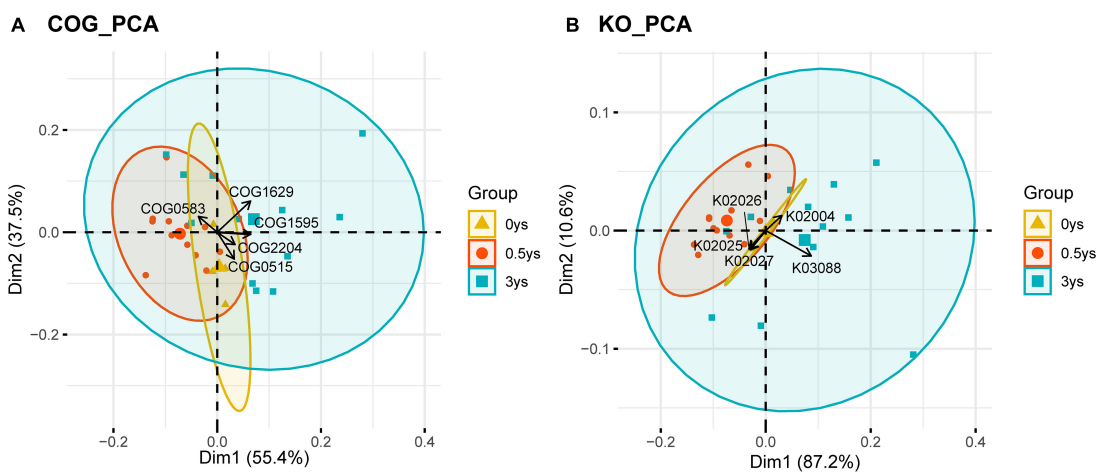


C

		Bacteria R² (%)	F	Pr(>F)		Fungi R² (%)	F	Pr(>F)
	C	3.6%	1.24	0.215		3.3	1.24	0.223
	N	2.7%	0.93	0.435		3.3	1.24	0.219
	OC	5.0%	1.72	0.071.		5.2	1.93	0.053.
Bacteria	AN	13.0%	4.46	0.002**	Fungi	14.0	5.24	0.001***
	NN	4.7%	1.61	0.080.		5.1	1.91	0.056.
	AP	5.2%	1.80	0.083.		3.9	2.35	0.014*
	AK	4.5%	1.54	0.112		5.5	2.06	0.026*
	SC	6.2%	2.14	0.028*		6.6	2.48	0.020*

Signif. codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1

FIGURE 6 | Correlations between soil physicochemical properties and rhizosphere microbial species. **(A,B)** RDA of bacterial and fungal community profiles correlated to soil properties. **(C)** Explanation rate of soil physicochemical properties to the differences of community profiles (conditional effect).



adonis R²: 0.24; P-value: 0.011

pairs	R²	p.value	p.adjusted
0.5ys vs 3ys	0.24123754	0.007	0.021
0.5ys vs 0ys	0.25368606	0.030	0.045
3ys vs 0ys	0.03709727	0.605	0.605

adonis R²: 0.23; P-value: 0.02

pairs	R²	p.value	p.adjusted
0.5ys vs 3ys	0.21706740	0.007	0.0210
0.5ys vs 0ys	0.24583898	0.021	0.0315
3ys vs 0ys	0.03505417	0.595	0.5950

FIGURE 7 | Principal component analysis based on COG **(A)** and KEGG **(B)** functional annotations. The arrows represent the most volatile COG and KEGG classifiers in the functional prediction results. PERMANOVA was used to test the significance of the differences.

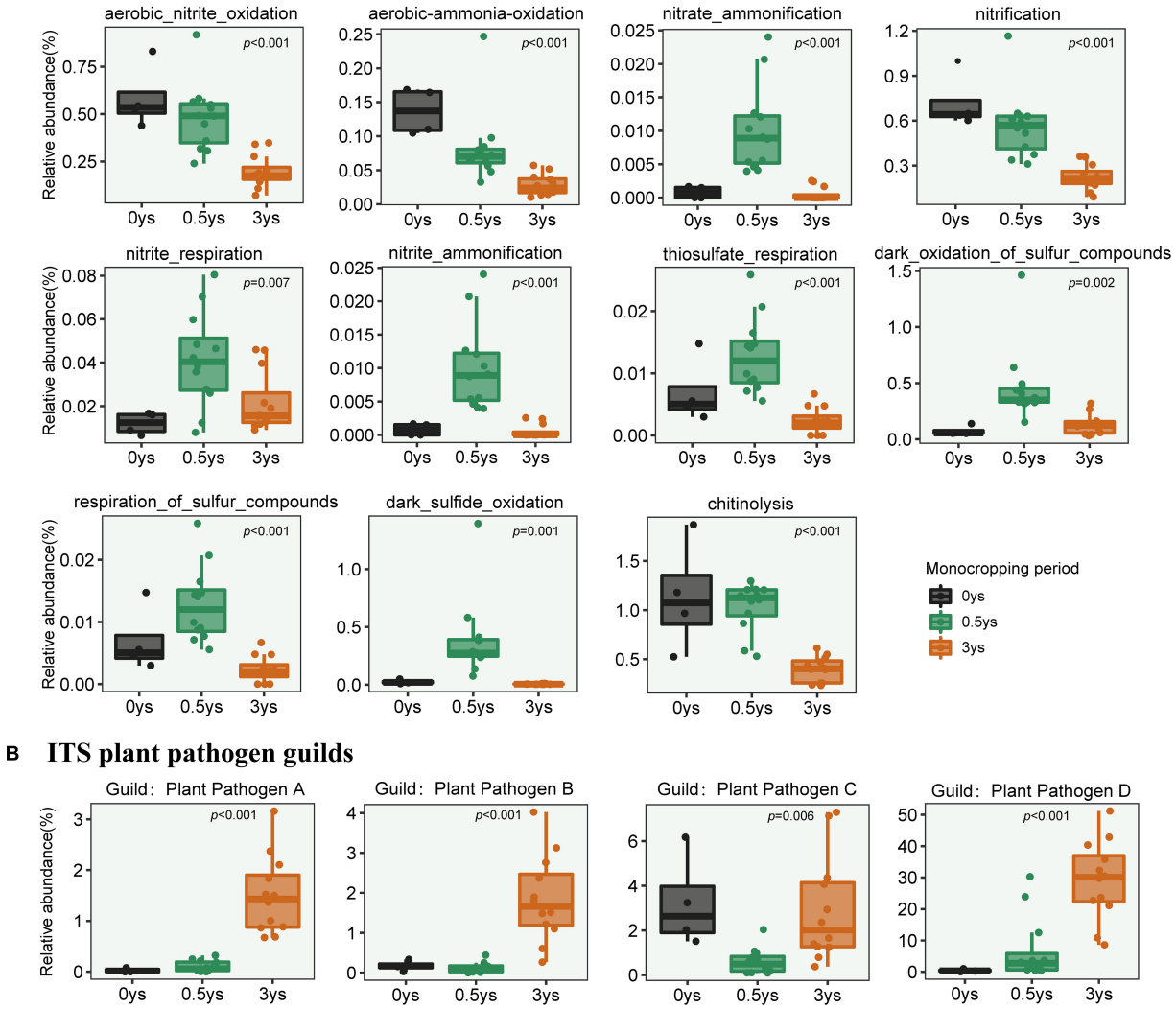


FIGURE 8 | Changes in relative abundance of metabolic pathways in prokaryotic communities based on FAPROTAX **(A)** and variation of abundance of plant pathogen guilds based on FUNGuild **(B)**. ANOVA was used to test the significance of differences.

The proportion of *Bacteroidetes* in replanted soil increased probably because more debris were produced in the roots of cut chrysanthemum. Other dominant phyla, *Acidobacteria* and *Chloroflexi*, also play an important role in organic matter degradation and nutrient cycling (Eichorst et al., 2018; Fang et al., 2018). For low classification levels, some species of *Pseudomonas* are involved in plant pathogen inhibition in many soils, and it serves as PGPR to reduce plant diseases (Liu et al., 1995; Kinkel et al., 2012). In the rhizosphere of cut chrysanthemum, the abundance of *Pseudomonas* decreased remarkably in the third year of continuous monocropping. Many *Bacillus* species, such as *Bacillus drentensis*, *Bacillus simplex*, and *Bacillus aryabhattai*, secrete fengycin, bacillaene, difficidin, and iturins by nutritional competition to antagonize pathogens such as *Rhizoctonia solani* and *Botrytis cinerea* (Ongena and Jacques, 2008; Jeong et al., 2012). The relative abundance of *Bacillus* did not change significantly in our study. *Rhizobium*, as a nitrogen fixer, was largely enriched in the rhizosphere of cut chrysanthemum, which may be attributed to the consumption of ammonium nitrogen in bulk soil. However, it should not be ignored that the species of *Pseudomonas* are not all beneficial; some are even plant pathogenic bacteria. In order to further improve the recognition rate of species and strains, the non-clustering algorithm of DADA3 may be better than that of UPARSE.

Changes in the composition of the bacterial community led to variations in metabolic genes, which were predicted by FAPROTAX based on 16S rRNA sequences. Continuous monocropping significantly reduced the abundance of genes related to nitrogen and sulfur metabolism in the rhizosphere of cut chrysanthemum (**Figure 8A**). Nitrogen is an important component of plant cell proteins, nucleic acids, chlorophyll,

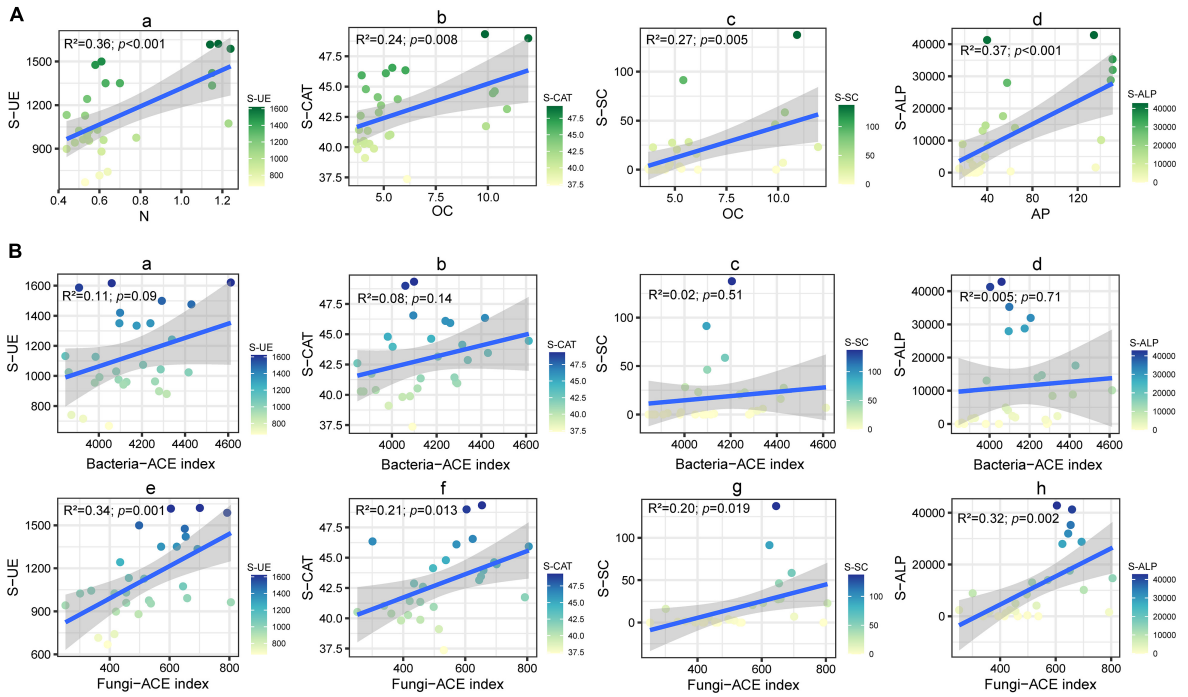


FIGURE 9 | Linear fitting analysis of soil enzyme activity and soil physical and chemical properties **(Aa–Ad)** and microbial community richness index **(Ba–Bh)**. R^2 represented the magnitude of the correlation coefficient, and the *p*-value was generated by a *t*-test.

enzymes, etc., which can improve photosynthetic efficiency and increase crop yield and quality. As an element equally important to plants as NPK, sulfur is essential for the function of plant proteins, enzymes, and other physiologically active substances. The lack of sulfur element may lead to smaller plants and yellow leaves, which are symptoms of nitrogen deficiency, and ultimately result in the loss of crop yield and quality. With the increase of continuous cropping years, the reduction of the related metabolic genes will reduce the conversion rate of the rhizosphere nutrient of cut chrysanthemum and gradually cause a state of "element deficiency." This state may contribute to the occurrence of reduced buds, smaller flowers, and increased diseases in cut chrysanthemum.

Continuous Monocropping Significantly Increased the Number of Phytopathogenic Fungi in the Rhizosphere of Cut Chrysanthemum

During continuous monocropping, the richness of the rhizosphere fungal community significantly increased (**Figure 2C**). LEfSe showed significant changes in the abundance of multiple fungal taxa (**Figure 4B**). *Ascomycota* is the most dominant phylum in the rhizosphere of cut chrysanthemum. It contains many plant pathogens and is the main source of toxins that cause replanting diseases (Ecker et al., 2005; Li et al., 2014). It was significantly enriched in the rhizosphere of cut chrysanthemum with the increase of monocropping time. The third dominant phylum, *Basidiomycota*, has some species that are saprophytic, and many of its large saprophytic fungi cause wood rot. Nevertheless, other species can also promote the growth of plants by forming mycorrhizas with plants. In this study, *Basidiomycota* was significantly enriched in the rhizosphere of "variety 1." At the genus level, *Mortierella* was involved in plant pathogen inhibition, which can inhibit the occurrence of the Chinese cabbage bulb disease and protect bananas against *Fusarium wilt* and root rot (Narisawa et al., 1998). It has higher abundance in vanilla suppressive soil, while it gradually accumulated in the rhizosphere of cut chrysanthemum with increasing planting time. *Humicola* also served as a biocontrol fungus (Sun et al., 2014), whose relative abundance increased significantly in the rhizosphere of cut chrysanthemum. Pathogenic fungi of some plant diseases, such as *Alternaria*, which can cause black spot in cabbage and *Alternaria Panax* in ginseng (Zhao et al., 1993; Ko et al., 2011), and *Fusarium*, which can cause wilt in chrysanthemums (Li et al., 2017), were enriched in the rhizosphere of cut chrysanthemum. *Rhizoctonia*, a pathogen that can cause blight in chrysanthemums, watermelons, etc. (Crater, 1992; Fehér, 1993), did not change significantly in our study.

To further reveal the changes of fungal community function, the FUNGuild method was used to divide fungal species into multiple guilds. A guild represents a more subdivided nutrition mode based on pathotroph, saprotroph, and symbiotroph, one of which is a plant pathogen. Guilds with significant changes were detected based on ANOVA. We found that some of the guilds belonging to plant pathogens were significantly enriched

in the rhizosphere of cut chrysanthemum (**Figure 8B**). According to FUNGuild results, these guilds mostly belong to *Acremonium* (Hou et al., 2019), *Plectosphaerellaceae* (Giraldo and Crous, 2019), *Fusarium* (Ma et al., 2013), and *Cladosporium* (Carolina et al., 2021), which have been reported to be pathogens of certain crops. The pathogenic genus *Acremonium* (corresponding to Guild: Plant Pathogen D) has a relative abundance that even reached 50% in the rhizosphere of cut chrysanthemum (Top2 genus). Therefore, we speculated that the increased incidence of diseases during continuous monocropping of cut chrysanthemum was caused by not only *Fusarium* but also the joint action of multiple pathogens.

In the current research, four cultivars of cut chrysanthemum were selected to investigate the changes of the rhizosphere microbial community and soil physicochemical properties under different continuous cropping times. Continuous monoculture had less effect on the bacterial community, but it significantly reduced the number of bacteria that metabolized nitrogen and sulfur. Continuous cropping significantly increased the richness of rhizosphere fungi and decreased the abundance of chitin-lysis-related genes, prompting the rhizosphere of cut chrysanthemum to become a fungal community. The taxa largely enriched in rhizosphere belonged to phytopathogenic fungi, which may combine to disrupt the otherwise healthy microbial community and increase diseases in cut chrysanthemum. Continuous cropping did not always increase the number of pathogenic bacteria; some beneficial microorganisms were also enriched and promoted the increase of soil enzyme activity. But they seem unable to resist the trend of community degradation. In this study, ammonium nitrogen (AN) was one of the most important factors shaping microbial communities, and the variation of its content caused significant changes in bacterial and fungal communities. However, the total contribution rate of determined soil physicochemical properties to the community transformation was less than 50%, which suggested that there were other factors involved in shaping the rhizosphere microbial community. Allelochemicals are produced by leaves, roots, stems, etc., which have beneficial or adverse effects on other plants (Rice, 1984). Allelochemicals consist of OA, straight-chain alcohols, aldehydes or ketones, unsaturated lactones, fatty acids, etc. (Gross and Parthier, 1994; Einhellig, 1995; Rice, 1995; Seigler, 1996). If the donor and the recipient belong to the same species, this leads to intraspecific allelopathy, and the term used is autotoxicity (Singh et al., 2010). Autotoxicity is a major obstacle in continuous cropping under protected cultivation (Zhou et al., 2009), and it is also found in the cultivation of cut chrysanthemum. A variety of allelochemicals participate in the transformation of the composition and structure of the rhizosphere microbial community of cut chrysanthemum, and together with plant pathogens, they contribute to the replanting problem of cut chrysanthemum.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ZR, XZ, and LL conceived and designed the experiments. TW, KY, and QM performed most of the experiments. TW and QM analyzed the data. TW wrote the manuscript. XJ and YZ helped to handle the plants. DK, ZW, and RP helped perform the analysis with constructive discussion. All authors read and approved the submitted version.

FUNDING

This research was partially supported by the National Natural Science Foundation of China (32070004 and 31670006). This study was also supported by the Fundamental Research Funds for Central Non-profit Scientific Institution (1610132020009), Central Public-interest Scientific Institution Basal Research Fund (Y2021GH18), Key Laboratory of Microbial Resources Exploitation and Application of Gansu Province (2019cx-04), and the Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Ministry of Agriculture and Rural Affairs, China.

ACKNOWLEDGMENTS

We are grateful to the Enpapers company for their editing services for the article and to our colleagues who helped us in the experiment.

REFERENCES

Badri, D. V., and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant Cell Environ*. 32, 666–681. doi: 10.1111/j.1365-3040.2008.01926.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

Breuillin-sessoms, F., Venterea, R. T., Sadowsky, M. J., Coulter, J. A., Clough, T. J., and Wang, P. (2017). Soil Biology & Biochemistry Nitri fi cation gene ratio and free ammonia explain nitrite and nitrous oxide production in urea-amended soils. *Soil Biol. Biochem*. 111, 143–153. doi: 10.1016/j.soilbio.2017.04.007

Bryant, D. A., and Frigaard, N. U. (2006). Prokaryotic photosynthesis and phototrophy illuminated. *Trends Microbiol.* 14, 488–496. doi: 10.1016/j.tim.2006.09.001

Cai, Z., Li, S., Zhang, W., and Ma, J. (2015). Effects of the novel pyrimidynyloxybenzoic herbicide ZJ0273 on enzyme activities, microorganisms and its degradation in Chinese soils. *Environ. Sci. Pollut. Res. Int.* 22, 4425–4433. doi: 10.1007/s11356-014-3674-1

Carolina, T., Javier, A., Adrián, C., and Graciela, P. (2021). Cladosporium species causing "' Cladosporium rot "' on "' Bosc "' pear fruit in Argentina. *Rev. Argent. Microbiol.* 53, 75–77. doi: 10.1016/j.ram.2019.11.006

Cha, J. Y., Han, S., Hong, H. J., Cho, H., Kim, D., Kwon, Y., et al. (2016). Microbial and biochemical basis of a Fusarium wilt-suppressive soil. *ISME J.* 10, 119–129. doi: 10.1038/ismej.2015.95

Chaparro, J. M., Badri, D. V., and Vivanco, J. M. (2014). Rhizosphere microbiome assemblage is affected by plant development. *ISME J.* 8, 790–803. doi: 10.1038/ismej.2013.196

Chen, W., Teng, Y., Li, Z., Liu, W., Ren, W., Luo, Y., et al. (2018). Mechanisms by which organic fertilizer and effective microbes mitigate peanut continuous cropping yield constraints in a red soil of south China. *Appl. Soil Ecol.* 128, 23–34. doi: 10.1016/j.apsoil.2018.03.018

China Ministry of Agriculture (2016). Available online at: http://zdscxx.moa.gov.cn:8080/nyb/pc/search.jsp

Clemens, S. (2017). The Environmental Detection of Comammox Nitrospira. Vienna: University of Vienna. doi: 10.25365/thesis.45220

Corné, M. J., Pieterse, Christos Zamioudis, Roeland, L., Berendsen, David, M., et al. (2014). Induced systemic resistance by beneficial microbes. *Ann. Rev. Phytopathol*. 52, 347–375.

Crater, G. D. (1992). "Potted Chrysanthemums," in *Introduction to Floriculture*, (Berlin: Elsevier), 249–287. doi: 10.1016/b978-0-12-437651-9.50015-4

De Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., et al. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas* fluorescens. *Mol. Plant-Microbe Interact.* 15, 1173–1180. doi: 10.1094/MPMI.2002.15.11.1173

Dick, R. P., and Kandeler, E. (2004). "Enzymes in Soils," in *Encyclopedia of Soils in the Environment*, (Netherland: Elsevier Inc.), 448–456. doi: 10.1016/B0-12-348530-4/00146-6

Duineveld, B. M., Kowalchuk, G. A., Keijzer, A., Van Elsas, J. D., and Van Veen, J. A. (2001). Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* 67, 172–178. doi: 10.1128/AEM.67.1.172-178.2001

Ecker, D. J., Sampath, R., Willett, P., Wyatt, J. R., Samant, V., Massire, C., et al. (2005). The Microbial Rosetta Stone Database: A compilation of global and emerging infectious microorganisms and bioterrorist threat agents. *BMC Microbiol.* 5:19. doi: 10.1186/1471-2180-5-19

Edwards, J., Johnson, C., Santos-medellín, C., Lurie, E., and Kumar, N. (2014). Structure, variation, and assembly of the root-associated microbiomes of rice. *PNAS* 112, E911–E920. doi: 10.1073/pnas.1414592112

Eichorst, S. A., Trojan, D., Roux, S., Herbold, C., Rattei, T., and Woebken, D. (2018). Genomic insights into the Acidobacteria reveal strategies for their success in terrestrial environments. *Environ. Microbiol.* 20, 1041–1063. doi: 10.1111/1462-2920.14043

Eida, A. A., Ziegler, M., Lafi, F. F., Michell, C. T., Voolstra, C. R., Hirt, H., et al. (2018). Desert plant bacteria reveal host influence and beneficial plant growth properties. *PLoS One*. 13:e0208223. doi: 10.1371/journal.pone.0208223

Einhellig, F. A. (1995). "Allelopathy: current status and future goals," in *Allelopathy: Organisms, Processes and Applications*, eds K. M. M. Inderjit Dakshini and F. A. Einhellig (Washington, DC: American Chemical Society), 1–24.

Fang, D., Zhao, G., Xu, X., Zhang, Q., Shen, Q., Fang, Z., et al. (2018). Microbial community structures and functions of wastewater treatment systems in plateau and cold regions. *Bioresour. Technol.* 249, 684–693. doi: 10.1016/j.biortech.2017.10.063

Fawzy, O., Maillard, E., Vuilleumier, S., Millet, M., and Imfeld, G. (2015). Science of the Total Environment Degradation of chloroacetanilide herbicides and bacterial community composition in lab-scale wetlands. *Sci. Total Environ.* 520, 222–231. doi: 10.1016/j.scitotenv.2015.03.061

Fehér, T. (1993). "Watermelon," in *Genetic Improvement of Vegetable Crops*, (Netherland: Elsevier), 295–311. doi: 10.1016/b978-0-08-040826-2.50025-4

Fierer, N., Bradford, M. A., and Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology* 88, 1354–1364. doi: 10.1890/05-1839

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., and Johnson, M. T. J. (2018). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proc. Natl. Acad. Sci. U.S.A.* 115, E1157–E1165. doi: 10.1073/pnas.1717617115

Fitzpatrick, C. R., Mustafa, Z., and Viliunas, J. (2019). Soil microbes alter plant fitness under competition and drought. *J. Evol. Biol.* 32, 438–450. doi: 10.1111/jeb.13426

Frank, K., Beegle, D., and Denning, J. (1998). *Recommended Chemical Soil test Procedures for the North Central Region*. Columbia: University of Missour.

Gao, M., Song, W., Zhou, Q., Ma, X., and Chen, X. (2013). Interactive effect of oxytetracycline and lead on soil enzymatic activity and microbial biomass. *Environ. Toxicol. Pharmacol.* 36, 667–674. doi: 10.1016/j.etap.2013.07.003

Gianfreda, L., Antonietta, M., Piotrowska, A., Palumbo, G., and Colombo, C. (2005). Soil enzyme activities as affected by anthropogenic alterations?: intensive agricultural practices and organic pollution. *Sci. Total Environ.* 341, 265–279. doi: 10.1016/j.scitotenv.2004.10.005

Giraldo, A., and Crous, P. W. (2019). Studies in Mycology. *Stud. Mycol.* 92, 227–286. doi: 10.1016/j.simyco.2018.10.005

Grayston, S. J., Wang, S., Campbell, C. D., and Edwards, A. C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol. Biochem.* 30, 369–378. doi: 10.1016/S0038-0717(97)00124-7

Gross, D., and Parthier, B. (1994). Novel natural substances acting in plant growth regulation. *J. Plant Growth Regul.* 13, 93–114. doi: 10.1007/bf00210953

Han, S., Luo, X., Liao, H., Nie, H., Chen, W., and Huang, Q. (2017). Nitrospira are more sensitive than Nitrobacter to land management in acid, fertilized soils of a rapeseed-rice rotation field trial. *Sci. Total Environ.* 599–600, 135–144. doi: 10.1016/j.scitotenv.2017.04.086

Hou, Y. M., Zhang, X., Zhang, N. N., Naklumpa, W., Zhao, W. Y., Liang, X. F., et al. (2019). Genera Acremonium and Sarocladium Cause Brown Spot on Bagged Apple Fruit in China. *Plant Dis.* 103, 1889–1901. doi: 10.1094/PDIS-10-18-1794-RE

Janssen, P. H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72, 1719–1728. doi: 10.1128/AEM.72.3.1719

Jeong, H., Jeong, D. E., Kim, S. H., Song, G. C., Park, S. Y., Ryu, C. M., et al. (2012). Draft genome sequence of the plant growth-promoting bacterium Bacillus siamensis KCTC 13613T. *J. Bacteriol.* 194, 4148–4149. doi: 10.1128/JB.00805-12

Johansson, L. H. (1988). A spectrophotometric method for determination of catalase activity in small tissue samples. *Anal. Biochem.* 174, 331–336. doi: 10.1016/0003-2697(88)90554-4

Kandeler, E., and Gerber, H. (1988). Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol. Fert Soils* 6, 68–72.

Kinkel, L. L., Schlatter, D. C., Bakker, M. G., and Arenz, B. E. (2012). Streptomyces competition and co-evolution in relation to plant disease suppression. *Res. Microbiol.* 163, 490–499. doi: 10.1016/j.resmic.2012.07.005

Kluitenberg, G. J., and Biggar, J. W. (1992). Canopy temperature as a measure of salinity stress on sorghum. *Irrig. Sci.* 13, 115–121. doi: 10.1007/BF00191053

Ko, W. H., Yang, C. H., Lin, M. J., Chen, C. Y., and Tsou, Y. J. (2011). Humicola phialophoroides sp. nov. from soil with potential for biological control of plant diseases. *Bot. Stud.* 52, 197–202.

Kudjordjie, E. N., Sapkota, R., Steffensen, S. K., Fomsgaard, I. S., and Nicolaisen, M. (2019). Maize synthesized benzoxazinoids affect the host associated microbiome. *Microbiome* 7:59. doi: 10.1186/s40168-019-0677-7

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lau, J. A., and Lennon, J. T. (2012). Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14058–14062. doi: 10.1073/pnas.1202319109

Lee, S., Lee, J., Jeong, Y., and Kim, J. (2009). Chemosphere In situ stabilization of cadmium-, lead-, and zinc-contaminated soil using various amendments. *Chemosphere* 77, 1069–1075. doi: 10.1016/j.chemosphere.2009.08.056

Li, H., Qiu, Y., Yao, T., Ma, Y., Zhang, H., Yang, X., et al. (2020). Evaluation of seven chemical pesticides by mixed microbial culture (PCS-1): degradation ability, microbial community, and *Medicago sativa* phytotoxicity. *J. Hazard. Mater.* 389:121834. doi: 10.1016/j.jhazmat.2019.121834

Li, K., DiLegge, M. J., Minas, I. S., Hamm, A., Manter, D., and Vivanco, J. M. (2019). Soil sterilization leads to re-colonization of a healthier rhizosphere microbiome. *Rhizosphere* 12:100176. doi: 10.1016/j.rhisph.2019.100176

Li, P., Chen, J., Li, Y., Zhang, K., and Wang, H. (2017). Possible mechanisms of control of Fusarium wilt of cut chrysanthemum by Phanerochaete chrysosporium in continuous cropping fields: A case study. *Sci. Rep.* 7:15994. doi: 10.1038/s41598-017-16125-7

Li, P., Zhang, F., Chen, S., Jiang, J., Wang, H., Su, J., et al. (2016). Genetic diversity, population structure and association analysis in cut chrysanthemum

(Chrysanthemum morifolium Ramat.). *Mol. Genet. Genomics* 291, 1117–1125. doi: 10.1007/s00438-016-1166-3

Li, X. G., Ding, C. F., Zhang, T. L., and Wang, X. X. (2014). Fungal pathogen accumulation at the expense of plant-beneficial fungi as a consequence of consecutive peanut monoculturing. *Soil Biol. Biochem.* 72, 11–18. doi: 10.1016/j.soilbio.2014.01.019

Liu, L., Kloepper, J. W., and Tuzun, S. (1995). Induction of systemic resistance in cucumber by plant growth-promoting rhizobacteria: duration of protection and effect of host resistance on protection and root colonization. *Phytopathology* 85, 1064–1068. doi: 10.1094/phyto-85-1064

Louca, S., Parfrey, L. W., and Doebeli, M. (2016). Decoupling function and taxonomy in the global ocean microbiome. *Science* 353, 1272–1277. doi: 10.1126/science.aaf4507

Lu, T., Ke, M., Lavoie, M., Jin, Y., Fan, X., Zhang, Z., et al. (2018). Rhizosphere microorganisms can influence the timing of plant flowering. *Microbiome* 6:231. doi: 10.1186/s40168-018-0615-0

Ma, L., Geiser, D. M., Proctor, R. H., Rooney, A. P., Donnell, K. O., Trail, F., et al. (2013). Fusarium Pathogenomics. *Annu. Rev. Microbiol.* 67, 399–416. doi: 10.1146/annurev-micro-092412-155650

Marschner, P., Crowley, D., and Yang, C. H. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* 261, 199–208. doi: 10.1023/B:PLSO.0000035569.80747.c5

Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., Van Der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Morales-Cedeño, L. R., Orozco-Mosqueda, M., del, C., Loeza-Lara, P. D., Parra-Cota, F. I, de los Santos-Villalobos, S., et al. (2021). Plant growth-promoting bacterial endophytes as biocontrol agents of pre- and post-harvest diseases: Fundamentals, methods of application and future perspectives. *Microbiol. Res.* 242:126612. doi: 10.1016/j.micres.2020.126612

Mukhopadhya, I., Hansen, R., El-Omar, E. M., and Hold, G. L. (2012). IBD-what role do Proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* 9, 219–230. doi: 10.1038/nrgastro.2012.14

Nannipieri, P., Giagnoni, L., Landi, L., and Renella, G. (2011). "Role of phosphatase enzymes in soil," in *Phosphorus in Action*, Vol. 26, eds E. Bünemann, A. Oberson, and E. Frossard (Berlin: Springer), 215–243.

Narisawa, K., Tokumasu, S., and Hashiba, T. (1998). Suppression of clubroot formation in Chinese cabbage by the root endophytic fungus, Heteroconium chaetospira. *Plant Pathol.* 47:206. doi: 10.1046/j.1365-3059.1998.00225.x

Naylor, D., and Coleman-Derr, D. (2018). Drought stress and root-associated bacterial communities. *Front. Plant Sci.* 8:223. doi: 10.3389/fpls.2017.02223

Naylor, D., Dong, Z., Simmons, T., Hixson, K. K., Kim, Y., Zink, E. M., et al. (2018). Erratum: Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria (Proceedings of the National Academy of Sciences of the United States of America (2018) 115 (E4284-E4293) *Proc. Natl. Acad. Sci. U.S.A.* 115:E4952. doi: 10.1073/pnas.1807275115

Nevens, F., and Reheul, D. (2001). Crop rotation versus monoculture; yield, N yield and ear fraction of silage maize at different levels of mineral N fertilization. *Netherlands J. Agric. Sci.* 49, 405–425. doi: 10.1016/S1573-5214(01)80026-9

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., et al. (2015). FUNGuild?: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* 20, 241–248. doi: 10.1016/j.funeco.2015.06.006

Olsen, S. R. (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate. *Miscell. Paper Instit. Agricult. Res. Samaru*. 418, 18–19.

Ongena, M., and Jacques, P. (2008). Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16, 115–125. doi: 10.1016/j.tim.2007.12.009

Penton, C. R., Gupta, V. V. S. R., Tiedje, J. M., Neate, S. M., Ophel-Keller, K., Gillings, M., et al. (2014). Fungal community structure in disease suppressive soils assessed by 28S LSU gene sequencing. *PLoS One* 9:e93893. doi: 10.1371/journal.pone.0093893

Qiu, M., Zhang, R., Xue, C., Zhang, S., Li, S., Zhang, N., et al. (2012). Application of bio-organic fertilizer can control Fusarium wilt of cucumber plants by regulating microbial community of rhizosphere soil. *Biol. Fertil. Soils* 48, 807–816. doi: 10.1007/s00374-012-0675-4

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361. doi: 10.1007/s11104-008-9568-6

Rachel, Backer, J., Stefan, Rokem, Gayathri, 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

Ragot, S. (2016). Soil phoD and phoX alkaline phosphatase gene diversity responds to multiple environmental factors. *FEMS Microbiol. Ecol.* 93:fiw212. doi: 10.1093/femsec/fiw212

Rana, M. A., Mahmood, R., and Ali, S. (2021). Soil urease inhibition by various plant extracts. *PLoS One* 16:e0258568. doi: 10.1371/journal.pone.0258568

Riaz, M., Arif, M. S., Ashraf, M. A., Mahmood, R., Yasmeen, T., Shakoor, M. B., et al. (2018). "A comprehensive review on rice responses and tolerance to salt stress," in *Advances in Rice Research for Abiotic Stress Tolerance*, (Berlin: Elsevier), 133–158. doi: 10.1016/B978-0-12-814332-2.00007-1

Rice, E. L. (1984). *Allelopathy*, 2nd Edn. New York, NY: Academic Press.

Rice, E. L. (1995). *Biological Control of Weeds and Plant Diseases*. Norman, USA: University of Oklahomka Press.

Richardson, A. E., and Simpson, R. J. (2011). Soil microorganisms mediating phosphorus availability update on microbial phosphorus. *Plant Physiol.* 156, 989–996. doi: 10.1104/pp.111.175448

Santhanam, R., Weinhold, A., Goldberg, J., Oh, Y., and Baldwin, I. T. (2015). Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. *Proc. Natl. Acad. Sci. U.S.A.* 112, E5013–E5020. doi: 10.1073/pnas.1505765112

Scandinavica, A. A., Soil, S. B., Tan, X., Kong, L., Yan, H., Wang, Z., et al. (2014). Influence of soil factors on the soil enzyme inhibition by Cd. *Acta Agric. Scand. Sect. B* 64, 666–674. doi: 10.1080/09064710.2014.953985

Segata, N., Izard, J., Walron, L., Gevers, D., Miropolsky, L., Garrett, W., et al. (2011). Metagenomic Biomarker Discovery and Explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Seigler, D. S. (1996). Chemistry and Mechanisms of Allelopathic Interactions. *A. J.* 88, 876–885.

Singh, A., and Ghoshal, N. (2013). European Journal of Soil Biology Impact of herbicide and various soil amendments on soil enzymes activities in a tropical rainfed agroecosystem. *Eur. J. Soil Biol.* 54, 56–62. doi: 10.1016/j.ejsobi.2012.10.003

Singh, H. P., Batish, D. R., Kohli, R. K., Singh, H. P., Batish, D. R., and Kohli, R. K. (2010). Autotoxicity: Concept, Organisms, and Ecological Significance. *Crit. Rev. Plant Sci. Autot.* 2689, 757–772. doi: 10.1080/07352689991309478

Song, A., Zhao, S., Chen, S., Jiang, J., Chen, S., Li, H., et al. (2013). The abundance and diversity of soil fungi in continuously monocropped chrysanthemum. *Sci. World J.* 2013:632920. doi: 10.1155/2013/632920

Stępniewska, Z., Wolińska, A., and Ziomek, J. (2009). Response of soil catalase activity to chromium contamination. *J. Environ. Sci.* 21, 1142–1147. doi: 10.1016/s1001-0742(08)62394-3

Sun, Y., Sun, G., Xu, Y., Wang, L., Liang, X., and Lin, D. (2013). Geoderma Assessment of sepiolite for immobilization of cadmium-contaminated soils. *Geoderma* 19, 149–155. doi: 10.1016/j.geoderma.2012.07.012

Sun, Z., Yang, L. M., Ma, X. J., and Zhang, Y. M. (2014). Screening and identification of antagonistic bacteria on alternaria panax Whetz. *J. Jilin Agric. Univ*. 36, 276–281.

Teixeira, P. L., Id, S. S., Law, T. F., and Jose, P. (2019). The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response. *PLoS Biol.* 17:e3000534. doi: 10.1371/journal.pbio.3000534

Tian, F., Hou, M., Qiu, Y., Zhang, T., and Yuan, Y. (2020). Salinity stress effects on transpiration and plant growth under different salinity soil levels based on thermal infrared remote (TIR) technique. *Geoderma* 357:113961. doi: 10.1016/j.geoderma.2019.113961

Timm, C. M., Carter, K. R., Aa Carrell, Jun, S. R., and Weston, D. J. (2018). Abiotic stresses shift belowground populus -associated bacteria toward a core stress microbiome. *mSystems* 3, e70–e17. doi: 10.1128/mSystems.00070-17

Tripathi, S., Chakraborty, A., and Chakrabarti, K. (2007). Enzyme activities and microbial biomass in coastal soils of India. *Soil Biol. Biochem.* 39, 2840–2848. doi: 10.1016/j.soilbio.2007.05.027

Trivedi, P., Delgado-Baquerizo, M., Trivedi, C., Hamonts, K., Anderson, I. C., and Singh, B. K. (2017). Keystone microbial taxa regulate the invasion of a fungal

pathogen in agro-ecosystems. *Soil Biol. Biochem.* 111, 10–14. doi: 10.1016/j.soilbio.2017.03.013

Trivedi, P., Trivedi, C., Grinyer, J., Anderson, I. C., and Singh, B. K. (2016). Harnessing host-vector microbiome for sustainable plant disease management of phloem-limited bacteria. *Front. Plant Sci.* 7:1423. doi: 10.3389/fpls.2016.01423

Verbon, E. H., and Liberman, L. M. (2016). Beneficial Microbes Affect Endogenous Mechanisms Controlling Root Development. *Trends Plant Sci.* 21, 218–229. doi: 10.1016/j.tplants.2016.01.013

Wagner, M. R., Lundberg, D. S., Coleman-Derr, D., Tringe, S. G., Dangl, J. L., and Mitchell-Olds, T. (2014). Natural soil microbes alter flowering phenology. *Ecol. Lett*. 17, 717–726. doi: 10.1111/ele.12276.Natural

Wang, X., Fan, J., Xing, Y., Xu, G., Wang, H., Deng, J., et al. (2019). "The Effects of Mulch and Nitrogen Fertilizer on the Soil Environment of Crop Plants," in *Advances in Agronomy*, (New York, NY: Academic Press Inc), 121–173. doi: 10.1016/bs.agron.2018.08.003

Wasaki, J., Sakaguchi, J., Yamamura, T., Ito, S., Osaki, M., and Kandeler, E. (2018). Soil Science and Plant Nutrition P and N deficiency change the relative abundance and function of rhizosphere microorganisms during cluster root development of white lupin (Lupinus albus L.). *Soil Sci. Plant Nutr.* 00, 1–11. doi: 10.1080/00380768.2018.1536847

Wei, X., Hu, Y., Razavi, B. S., Zhou, J., Shen, J., Nannipieri, P., et al. (2019). Rare taxa of alkaline phosphomonoesterase-harboring microorganisms mediate soil phosphorus mineralization. *Soil Biol. Biochem*. 131, 62–70. doi: 10.1016/j.soilbio.2018.12.025

Wu, L., Kobayashi, Y., Wasaki, J., and Koyama, H. (2018). Soil Science and Plant Nutrition Organic acid excretion from roots?: a plant mechanism for enhancing phosphorus acquisition, enhancing aluminum tolerance, and recruiting beneficial rhizobacteria. *Soil Sci. Plant Nutr.* 00, 1–8. doi: 10.1080/00380768.2018.1537093

Xiong, W., Li, R., Ren, Y., Liu, C., Zhao, Q., Wu, H., et al. (2017). Distinct roles for soil fungal and bacterial communities associated with the suppression of vanilla Fusarium wilt disease. *Soil Biol. Biochem.* 107, 198–207. doi: 10.1016/j.soilbio.2017.01.010

Xiong, W., Zhao, Q., Zhao, J., Xun, W., Li, R., Zhang, R., et al. (2015). Different Continuous Cropping Spans Significantly Affect Microbial Community Membership and Structure in a Vanilla-Grown Soil as Revealed by Deep Pyrosequencing. *Microb. Ecol.* 70, 209–218. doi: 10.1007/s00248-014-0516-0

Yang, Y., Ding, J., Chi, Y., and Yuan, J. (2020). Characterization of bacterial communities associated with the exotic and heavy metal tolerant wetland plant Spartina alterniflora. *Sci. Rep*. 10:17985. doi: 10.1038/s41598-020-75041-5

Zhang, J., Liu, Y. X., Zhang, N., Hu, B., Jin, T., Xu, H., et al. (2019). Nrt1.1b is associated with root microbiota composition and nitrogen use in field-grown rice. *Nat. Biotechnol.* 37, 676–684. doi: 10.1038/s41587-019-0104-4

Zhao, J., Yang, Y., Zhang, K., Jeong, J., Zeng, Z., and Zang, H. (2020). Does crop rotation yield more in China? A meta-analysis. *F. Crop. Res.* 245:107659. doi: 10.1016/j.fcr.2019.107659

Zhao, Q., Jian, S., Nunan, N., Maestre, F. T., Tedersoo, L., He, J., et al. (2017). Altered precipitation seasonality impacts the dominant fungal but rare bacterial taxa in subtropical forest soils. *Biol. Fertil. Soils* 53, 231–245. doi: 10.1007/s00374-016-1171-z

Zhao, Y. F., Wu, L. J., Yang, Y. J., and Li, X. H. (1993). Study on the pathogon of ginseng phytophthoya blight. *Acta Phytopathol. Sin*. 3, 19–20.

Zhou, K., Guo, W. M., Wang, Z. F., and Hao, F. G. (2009). Autotoxicity of aquatic extracts from chrysanthemum and rhizosphere soil on photosynthesis in the same plant species. *Chin. J. Eco Agricult.* 17, 318–322. doi: 10.3724/sp.j.1011.2009.00318

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 March 2022
doi: 10.3389/fmicb.2022.846208

Aspen Leaves as a "Chemical Landscape" for Fungal Endophyte Diversity—Effects of Nitrogen Addition

*Johanna Witzell[1,2], Vicki Huizu Guo Decker[3], Marta Agostinelli[2], Carmen Romeralo[2,4], Michelle Cleary[2] and Benedicte Riber Albrectsen[3]**

[1] Forestry and Wood Technology, Linnaeus University, Växjö, Sweden, [2] Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, Alnarp, Sweden, [3] Department of Plant Physiology, Umeå Plant Science Center, Umeå University, Umeå, Sweden, [4] Forest Research Centre (INIA, CSIC), Madrid, Spain

Abiotic and biotic factors may shape the mycobiome communities in plants directly but also indirectly by modifying the quality of host plants as a substrate. We hypothesized that nitrogen fertilization (N) would determine the quality of aspen (*Populus tremula*) leaves as a substrate for the endophytic fungi, and that by subjecting the plants to N, we could manipulate the concentrations of positive (nutritious) and negative (antifungal) chemicals in leaves, thus changing the internal "chemical landscape" for the fungi. We expected that this would lead to changes in the fungal community composition, in line with the predictions of heterogeneity–diversity relationship and resource availability hypotheses. To test this, we conducted a greenhouse study where aspen plants were subjected to N treatment. The chemical status of the leaves was confirmed using GC/MS (114 metabolites, including amino acids and sugars), LC/MS (11 phenolics), and UV-spectrometry (antifungal condensed tannins, CTs), and the endophytic communities were characterized using culture-dependent sequencing. We found that N treatment reduced foliar concentrations of CT precursor catechin but not that of CTs. Nitrogen treatment also increased the concentrations of the amino acids and reduced the concentration of some sugars. We introduced beetle herbivores (H) as a second treatment but found no rapid changes in chemical traits nor strong effect on the diversity of endophytes induced by herbivores. A few rare fungi were associated with and potentially vectored by the beetle herbivores. Our findings indicate that in a controlled environment, the externally induced changes did not strongly alter endophyte diversity in aspen leaves.

Keywords: ***Populus tremula*, phenolics, condensed tannins, fungal endophytes, *Chrysomela tremula*, heterogeneity-diversity relationship hypothesis**

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Richard Lindroth, University of Wisconsin-Madison, United States
Ahmed M. Abdel-Azeem, Suez Canal University, Egypt

****Correspondence:***
Benedicte Riber Albrectsen
benedicte.albrectsen@umu.se

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *30 December 2021*
Accepted: *19 January 2022*
Published: *21 March 2022*

Citation:
Witzell J, Decker VHG, Agostinelli M, Romeralo C, Cleary M and Albrectsen BR (2022) Aspen Leaves as a "Chemical Landscape" for Fungal Endophyte Diversity—Effects of Nitrogen Addition. Front. Microbiol. 13:846208. doi: 10.3389/fmicb.2022.846208

IMPORTANCE

Fungal endophytes that colonize plants without causing symptoms may influence the growth and resistance traits of plants. To learn how fungal mycobiomes may potentially support sustainable plant growth, it is important to understand how endophyte communities are regulated by environmental vs. plant inherent factors. In a greenhouse study, we found that the endophyte

communities in the leaves of young aspen trees remained rather stable regardless of the subtle changes in chemical quality that were caused by nitrogen fertilization (reduction of potentially antifungal catechins and decrease in sugars and increase in amino acids). A short-term exposure to insect feeding did not cause marked changes to plant chemistry or fungal communities, although some rare fungi occurred only in connection to insect feeding. Thus, in controlled conditions and over a short period of time, the growth environment rather than the internal chemical quality seemed to be a stronger determinant of the fungal diversity in aspen leaves.

INTRODUCTION

Wherever plants grow, in natural ecosystems or cultivated environments, fungal endophytes form a hidden stratum of biodiversity, imbedded inside the plant tissues (Wilson and Lindow, 1994; Bailey et al., 2005; Albrectsen et al., 2010). Currently, studies on such endophyte communities are in the frontline of plant ecology (e.g., Hardoim et al., 2015), and interest in these communities is also boosted by the prospects of utilizing the biosynthetic capacities of the endophytes in plant protection and plant growth promotion in agriculture and forestry (Busby et al., 2016; Witzell and Martín, 2018; Terhonen et al., 2019; Blacutt et al., 2020), and in medical or industrial applications (Nisa et al., 2015; Gouda et al., 2016). Advancement of molecular tools has made it possible to obtain more detailed information about the taxonomic structure of these hidden communities (Jumpponen and Jones, 2009; Albrectsen and Witzell, 2012; Unterseher et al., 2016), but investigations are still challenged by their temporal and spatial dynamics, the complexity of fungal taxonomy, and the lack of knowledge about the roles of fungal species and their inter- and intraspecies interactions inside the plants (Witzell et al., 2014). Especially in the case of large-sized and long-lived forest trees, the mechanisms determining endophyte community composition have remained puzzling (Ragazzi et al., 2003; Albrectsen and Witzell, 2012; Witzell and Martín, 2018).

The ecological and evolutionary processes that shape terrestrial communities include dispersal (movement of organisms across space), drift (stochastic changes in species abundance), speciation (creation of new species), and selection (which involves biotic and abiotic interactions including intra- and interspecific interactions) (Vellend, 2010); moreover, at the landscape level, environmental heterogeneity promotes species richness, as it increases opportunities for niche partitioning (Ben-Hur and Kadmon, 2020). Nutrient availability theory also suggests that plant defenses change character along nutrient gradients, for example, by emphasizing constitutive defenses in nutrient poor soils, to change to investment in both growth and defense in nutrient-richer environments (López-Goldar et al., 2020). In aspen, environmental effects are documented to shape both arthropod community structure (Robinson et al., 2016) and pathogen susceptibility (Bandau et al., 2021).

The impact of soil nutrients on fungal endophyte communities in aspen leaves has received less attention. Trees receive their endophytes mainly from the surrounding environment (horizontal spreading; Clay, 1988; Petrini et al., 1993; Clay and Holah, 1999; Siddique et al., 2022), and the influence of season and climatic conditions on the community structure has been found to be high (Gomes et al., 2018). Edaphoclimatic factors can strongly determine the dispersal and establishment of endophytes in trees (Carroll, 1995; Sieber, 2007), either directly by influencing the quality and quantity of the available inoculum, or indirectly, through effects on the host plant as a substrate for the fungi. Although landing of viable fungal spores on potential host plants is a stochastic event, the chemical environment on and inside the plant may act as selective factor, supporting the germination and growth of some fungi and suppressing others. For instance, several studies have found a connection between endophyte infections and phenolic plant metabolites in trees (Bailey et al., 2005; Albrectsen et al., 2010; Martín et al., 2013; Albrectsen et al., 2018). These ubiquitous plant metabolites are potentially antifungal or fungistatic (Witzell and Martín, 2008), but some fungi may also be able to use them as a carbon source (Blumenstein et al., 2015). Soil fertility is known to influence production of phenolics in plants, and especially the availability of soil nitrogen tends to be negatively correlated with phenolic concentrations in plant tissues (Bandau et al., 2015). Phenolic metabolism is known to readily increase in response to stress, and several phenolics have antioxidant, antimicrobial, or antiherbivore properties (Witzell and Martín, 2008; Gourlay and Constabel, 2019). Because phoretic associations between fungi and insects are likely to be common (Lewinsohn et al., 1994; Rice et al., 2007), insect herbivores may influence the fungal community both directly and indirectly by induction of plant responses. On the other hand, endophytic fungi may also influence the herbivores (Coblentz and Van Bael, 2013), further complicating the interactions.

The goal of our study was to increase the current understanding of how foliar endophyte assemblages in woody plants vary in response to multiple, interacting factors. Our basic hypothesis was that the intrinsic status of phenolic metabolites, dominant defensive chemicals in most boreal and temperate zone trees species, would strongly influence the quality of plants as a substrate for endophytic fungi and thus shape their community structure (Agostinelli et al., 2018; Albrectsen et al., 2018). Moreover, we anticipated that external factors could modify the phenolic status and result in changes in the fungal community. To test this hypothesis, we isolated endophytic fungi from leaves of vegetatively propagated plants of 12 aspen (*Populus tremula* L.) genotypes that were subjected to nitrogen amendment and leaf beetle herbivory, alone and in combination. The genotypes represented a similar chemotype in terms of their content of salicinoid phenolic glycosides (SPGs, low molecular weight phenolics that are characteristic for Salicaceae plants; Keefover-Ring et al., 2014), but differed with regard to concentration of high-molecular weight phenolics, condensed tannins (CTs; Robinson et al., 2012). The plant material thus allowed us to specifically focus on the importance of the latter compounds, which have earlier been identified as potentially antifungal compounds in aspen (Ullah et al., 2017).

We expected that fertilization would generally reduce the concentrations of phenolic metabolites (Witzell and Shevtsova, 2004; Bandau et al., 2015; Decker et al., 2017) in aspen leaves. Furthermore, we expected that it would also reduce the within-group variation in phenolic concentrations, i.e., the phenolic concentration would vary less between N treated replicate plants as compared to the control plant group (Edenius et al., 2012; Albrectsen et al., 2018). We expected that this quantitative response could lead to a more homogenous nutritional niche that would support a less diverse endophytic community than what is found in control plants. In contrast, we hypothesized that herbivory could lead to locally induced accumulation of constitutive or induced phenolics (Sampedro et al., 2011; Massad et al., 2014) or oxidation of phenolics due to tissue damage (Constabel et al., 2000; Salminen and Karonen, 2011). We expected that this would result in a more variable (multidirectional) and compartmentalized chemical environment in leaf endosphere, leading to higher diversity of nutritional niches and thus more diverse fungal communities, as predicted by the heterogeneity–diversity relationship hypothesis (Ben-Hur and Kadmon, 2020). The possible phoretic interactions could add to the diversity of fungal communities in H-treated plants. We used isolation method in order to capture the fast-growing fraction of the total endophyte community because we expected that it would most rapidly respond to the treatment effects in our short-term experiment. To study the metabolic status of the plants, we conducted a global metabolite analysis using GC/MS and completed it with a targeted LC/MS analysis of low-molecular weight phenolics. We also analyzed the concentrations of condensed tannins. The results are discussed within the framework of the heterogeneity–diversity relationship hypothesis (Ben-Hur and Kadmon, 2020).

MATERIALS AND METHODS

Plant Material

Plants belonging to 12 aspen (*P. tremula*) genotypes were chosen from the SwAsp collection (genotypes number 4, 6, 7, 26, 41, 51, 69, 72, 79, 92, 98, and 100; Robinson et al., 2012). The selected clones represented the tremuloides-like chemotype, with four dominating SPGs (salicin, tremuloidin, salicortin, and tremulacin) (Robinson et al., 2012; Keefover-Ring et al., 2014). Aspen plants were produced from *in vitro* tissue cultures (Umeå Plant Science Centre, Umeå, Sweden) and planted into 5-L pots on May 23, 2014. The pots were placed in the greenhouse 20°C, 60% R.H., and a 16:8 h D/N cycle. Lateral branches were removed within the first 4 weeks after planting to promote apical growth.

Experimental Setup

Two individual plants from each of the 12 genotypes were randomly assigned to one of four treatments: control (C), nitrogen fertilization (N), herbivory (H), and their combination (NH) (n = 24 plants per treatment). The whole experiment thus comprised a total of 96 plants. For N treatment, a Weibulls Rika-S® solution (containing 84 gr/liter nitrogen, NH_4NO_3) was applied weekly starting from June 17th for 4 weeks. This resulted in a final N input that corresponds to the level of industrial forest fertilization in Sweden, 150 kg N ha^{-1} y^{-1} (Nohrstedt, 2001; Decker et al., 2017). The first fully expanded leaf on each plant was marked on June 17th, and this leaf was later used to assess baseline leaf chemistry (see below).

After 4 weeks of fertilization treatment, a mousseline fabric net was placed over the first three fully expanded leaves that had developed above the marked leaf after the first fertilization event. Five adult aspen leaf beetles (*Chrysomela tremula* Fabricius) were placed into the nets after the last fertilization event and allowed to feed for 5 days (treatments H and NH). The beetles had been reared in the laboratory at Umeå Plant Science Centre since the beginning of May and belonged to the second generation of a culture captured close to Ekebo, in southern Sweden. Empty nets were placed in similar position also on plants in C and N treatments to control for the effect of the mousseline bag.

Sampling and Growth Measurement

After 5 days of herbivory treatment, the experiment was ended. Leaves were collected from each plant for phytochemical analysis (the leaf below the netted leaves) and for fungal endophyte analysis (the netted leaves). Leaves for the chemical analyses were flash-frozen in liquid nitrogen, freeze-dried, and stored at −20°C until they were ground to fine powder using a bead mill (MM 301 Vibration Mill, Retsch GmbH and Co., KG, Haan, Germany) at 25 Hz for 3 min. The fine powder was stored in vials at −20°C until chemical analyses. Leaves designated for endophyte analysis were collected and stored at 4°C for a maximum of 3 days prior to surface sterilization and endophyte isolation. In case the petiole of a netted leaf was chewed by the beetle (causing wilting or the death of the leaf), those leaves were discarded from further analyses. A total of 90 plants were included in the endophyte analysis. The growth of plants and insects was recorded in the end of the experiment (**Supplementary Table 1**).

Phytochemical Analysis

For the global GC/MS analysis, 6.00 (±1.00) mg of leaf powder was extracted in 1 ml methanol:chloroform:water (v:v:v) at 4°C. Deuterated salicylic acid [$^2H_6$] (Isotec, Miamisburg, OH, United States) was used as an internal standard in all samples. Samples were centrifuged at 4°C, and 100 μl of the resulting supernatant was evaporated in vacuum. The residues were resolved in 30 μl of methoxyamine (15 μg/μl in pyridine) and 30 μl of MSTFA. Methyl stearate (30 μl of 15 ng/μl in heptane) was added before analysis, and 1 μl of each aliquot was injected by a CTC Combi Pal autosampler (CTC Analytics AG, Switzerland) into an Agilent 6890 gas chromatograph. The chromatograph was equipped with a 10 m × 0.18 mm fused silica capillary column with a chemically bonded 0.18-μm DB 5-MS UI stationary phase (J&W Scientific, Corston, Bath, United Kingdom). The compound detection was performed in a Pegasus III time-of-flight mass spectrometer, GC/TOFMS (Leco Corp., St Joseph, MI, United States). MATLAB™ R2011b (Mathworks, Natick, MA, United States) was used to quantify the mass by means of integrated peak areas. All pretreatment data procedures, such as baseline correction, chromatogram alignment, data compression, and hierarchical

multivariate curve resolution (H-MCR), were performed using custom scripts according to Jonsson et al. (2005). The extracted mass spectra were identified by comparisons of their retention index and mass spectra with libraries of retention time indices and mass spectra (Schauer et al., 2005). Identification of compounds was based on comparison with mass spectra libraries (in-house database) as well as the Kovats retention index. The identified chemicals were quantified by peak area and assigned to metabolite classes (phenolics, amino acids, fatty acids).

For the targeted LC/MS analysis, samples were prepared as above, but the residue was suspended in 25 μl of methanol and 25 μl of 0.1% v/v aqueous formic acid. A 2.0-μl aliquot was injected onto a C18 UPLC^{TM} column (2.1 × 100 mm, 1.7 μm), and chromatographic separation was performed on a LCT Premier TOF/MS in negative mode (Waters, Milford, MA, United States), following the method by Abreu et al. (2011). The standard compounds used in this analysis were from our in-house library (the Swedish Metabolomics Centre: SMC, Umeå, Sweden) including salicinoid standards (salicin, tremulacin, salicortin, and tremuloidin). MassHunter^{TM} Qualitative Analysis software package (version B06.00, Agilent Technologies Inc., Santa Clara, CA, United States) was used to acquire the mass feature extraction (MFE), and extracted features were aligned and matched between samples using Mass Profiler Professional^{TM} 12.5 (Agilent Technologies Inc., Santa Clara, CA, United States). The concentrations of salicinoids were quantified according to the peak area of each compound using linear standard curves. Masses of either or both of the deprotonated ion ([M−H]−) and the formate adduct ([M−H + FA]−) were assessed based on molecular weights according to Abreu et al. (2011) and Keefover-Ring et al. (2014) and guided by retention times where available.

Condensed tannin concentrations were assessed based on a modified Porter's assay (acid: butanol method) (Porter et al., 1985; Bandau et al., 2015): 10 ± 1 mg fine powder of freeze-dried leaf sample was added to 800 μl of an acetone/ascorbic acid solution (70% acetone, 30% Milli-Q water, with 10 mM ascorbic acid) then incubated with an iron and acid-butanol reagent in boiling water for 1 h. Absorbance was measured on a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, United States) at A 550 nm. Procyanidin B2 ($C_{30}H_{26}O_{12}$, Sigma- Aldrich®, St. Louis, MO, United States) was used as the tannin standard.

Fungal Endophyte Isolation and Identification

Endophytic fungi were isolated following the method described by Albrectsen et al. (2010). From each leaf, 10 segments (about cm^2) were cut using a sterilized scalpel. The segments were surface-sterilized using 95% ethanol and 2% sodium hypochlorite, and rinsed in sterile water before placing them individually on potato dextrose agar (PDA, Merck 70139-2.5KG) in petri dishes (12 × 12 cm) that had been autoclaved and received added streptomycin to avoid bacterial growth. The leaf segments on the plates were checked for emerging fungal growth every second day, and emerging colonies transferred to new PDA-dishes. Three leaves were harvested from every C and N treated aspen plant; and one to three leaves were harvested from the plants that had been exposed to herbivores. The total number of leaves (and petri dishes) was 228.

The recovered fungal cultures (n = 1,642) were classified into 30 distinct morphotypes (MTs) based on their visual morphological traits and growth rate estimated on a four-step scale (fast, medium, slow, very slow). In order to obtain information about the taxonomy of the fungal community, a total of 59 cultures representing the 30 MTs were selected for sequencing. From each culture, plugs of PDA containing growing mycelium were transferred to malt extract broth. After an incubation period of 2 weeks (room temperature, darkness), mycelia were filtered, placed in 50-ml Falcon tubes, and lyophilized for 48 h. Freeze-dried samples were then pulverized in a FastPrep®-24 homogenizer (MP biomedicals, Santa Ana, CA, United States). Total genomic DNA was extracted using the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, United States). The extracted nuclear DNA was measured using NanoDrop® ND-1000 (Wilmington, United States). PCR amplification of the internal transcribed spacer (ITS) region of the rDNA was performed with the primers ITS1 and ITS4 (White et al., 1990). Each 50-μl PCR reaction mixture contained 5 μl of 10x PCR buffer, 0.4 μM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1U Taq polymerase, and 10 ng fungal DNA. The PCR program consisted of 94°C for 5 min, 33 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 10 min. PCR samples were kept at 4°C before analyzing *via* gel electrophoresis on 1.5% Agarose (BIO-RAD) gel and visualized under UV light with 0.1% GelRed (GelRed^{TM} Nucleic Acid Gel Stain, 10,000X in DMSO). PCR products were purified with the HT ExoSAP-IT High-throughput PCR Product Cleanup (Affymetrix, Santa Clara, CA, United States) following the manufacturer's instruction. After quantification of DNA concentrations with Qubit fluorometer 3.0 (Life Technologies, Carlsbad, CA, United States), samples were sent to the National Genomics Infrastructure (NGI) at Science for Life Laboratory (SciLifeLab, Uppsala, Sweden). Visualization of raw sequence data was done with the software Chromas (version 2.4.4, Technelysium, South Brisbane, QLD, Australia). All sequences were aligned and manually edited using BioEdit (Ibis Biosciences, Carlsbad, CA, United States). Sequencing data were blasted against best matches in the reference database at NCBI.[1] The ITS sequence homology for delimiting fungal taxa was set to >98.5% for presumed species (**Supplementary Table 2**).

Data Analysis

Kruskal–Wallis non-parametric tests (**Table 1**) were used to assess the significance of effects on specific chemicals and the summary of the statistical analyses conducted for those relationships that are presented in **Figure 2**. PCA plots were performed for 114 targeted and non-targeted metabolites from LC/MS and GC/MS and for 11 targeted phenolics (mainly

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

TABLE 1 | Summary of Kruskal–Wallis statistics on treatment effects as presented in **Figure 2**.

Chemical	Chi-square, df = 3	*p*-value	Significance
Condensed tannins	5.73	0.1255	n.s.
Catechin	25.46	1.239e-05	***
Phenylalanine	31.45	6.81e-07	***
Tryptophan	42.04	3.928e-09	***
Glucose	4.86	0.1817	n.s.
Maltose	21.30	9.122e-05	***

*The non-parametric test was chosen for all comparisons to standardize between compounds with and without normally distributed residuals. The individual chemicals were chosen from the metabolomics data to provide examples of chemical responses to treatment that generally agreed with the compound group: phenolic compounds (as exemplified in condensed tannins and their precursor catechin), the amino acids (phenylalanine and tryptophan), and the sugars (glucose and maltose, with maltose responses varying when compared to the other sugars). ***$P < 0.0001$.*

SPGs from LC/MS), respectively, using R version 3.6.1 (2019-07-05) packages: ggplot2,[2] FactorMineR[3] (Lê et al., 2008), and factoextra.[4]

Fungal diversity (richness, S; Whittaker, 1972) was tracked as the number of distinct morphotypes (MTs) found in each plant, and the MT abundance (frequency) was recorded as the number of isolates of a given MT per sampled plant (n = 24 plants for C and N, and 21 plants for H and NH treatments). Venn diagram was constructed to illustrate the number of shared and unique MTs per treatment (Shade and Handelsman, 2012). Relative abundance was determined for each MT as the number of isolates of the given morphotype divided by the total number of isolates emerged from all samples (n = 1642). To examine the possible shifts in the community composition among the different treatments (C, N, H, and NH), a permutational multivariate analysis of variance (Permanova) of relative abundances was performed with the adonis function in vegan package[5] using 999 permutations, followed by a pairwise comparison with pairwise.adonis (Martinez Arbizu, 2019).

Morphotype richness values were used to create sample-size-based rarefaction (interpolation) and extrapolation (prediction) curves (Colwell et al., 2012; Chao et al., 2014; Hsieh et al., 2016) with an endpoint of 42 individuals and 100 bootstrap repetitions. The curves were generated with the iNEXT (iNterpolation and EXTrapolation) R package[6] and visualized with ggiNEXT, the ggplot2 extension for iNEXT. To compare the complexity of communities, Simpson's (D) and Shannon (H') diversity indices and Pielou's index for evenness (J') were calculated using the Vegan package (Oksanen et al., 2016; **Supplementary Table 3**). The effect of the treatments (C, N, H, NH) on the ecological diversity indexes and on the fungal richness (S) was assessed with an analysis of variance followed by a multiple comparison test (residuals were checked for normality, homoscedasticity, and linearity). When the data did not meet these requirements, a robust ANOVA based on trimmed means was performed with the WRS2 package.[7] Before all the analyses were performed, data were balanced using a decision tree to generate new samples from the minority classes (H and NH treatments) with an oversampling algorithm (random walk oversampling) from the imbalance package in R[8] (R Development Core Team, 2016).

[2]https://cran.r-project.org/web/packages/ggplot2/

[3]https://cran.r-project.org/web/packages/FactoMineR/index.html

[4]https://cran.r-project.org/web/packages/factoextra/index.html

[5]https://cran.r-project.org/web/packages/vegan/index.html

[6]https://cran.r-project.org/web/packages/iNEXT/index.html

RESULTS

Treatment Effects on Aspen Chemistry and Growth

The score plot (PCA) of global metabolite analysis (data included 114 metabolites) showed a weak separation between fertilized and non-fertilized treatments (**Figure 1A**). The plot of targeted metabolites (11 phenolics, including catechin but excluding condensed tannins) did not show any clear distinction between fertilized and non-fertilized plants (**Figure 1B**).

Nitrogen treatment reduced the concentration of the CT precursor, catechin, in aspen leaves, and a decreasing (non-significant) trend was observed also in the CT concentration (**Table 1** and **Figures 2A,B**). The lowest within-treatment variation in CTs was found in N-treated plants (**Table 1** and **Figure 2E**), despite the varied genetic background of the trees (12 clones). Overall, the effect of N on chemical characters appeared stronger compared to the effect of H (**Table 1** and **Figures 2A–F**), and there were no significant N × H interactions.

The concentrations of amino acids increased in plants receiving N treatment (**Table 1** and **Figures 2A,B**). Most sugars were somewhat reduced by H and increased by N treatment (data for glucose are shown as an example; **Table 1** and **Figure 2C**). A deviating pattern was found only for maltose, which was reduced by treatments, especially by N, alone and in combination with H (**Table 1** and **Figure 2D**).

Nitrogen treatment had a strong effect on both the above-ground biomass and height of the trees [**Supplementary Table 1**; ANOVA: $F_{(df\ =\ 3)} = 44.07$; $p < 0.0001$, $F_{(df\ =\ 3)} = 15.96$; $p < 0.0001$, respectively], and there was no effect of herbivory ($p > 0.9$).

Endophyte Diversity in Aspen Leaves

Among the 30 MTs, 18 were identified to class (2 MTs), genus (10 MTs), or species (6 MTs) level (**Table 1** and **Supplementary Table 2**). Of the identified isolates, 17 belonged to *Ascomycota* (class *Dothideomycetes*, *Eurotiomycetes*, *Hypocreales*, and *Sordariomycetes*) and one to *Basidiomycota* (MT25: *Rhodotorula* sp.). The three most common morphotypes across the treatments, *Ramularia* sp. (MT1), and the unidentified morphotypes MT2 and MT3 had 576 isolates (35% of all isolates), 336 (20% of all isolates), and 329 (20% of all isolates), respectively. A total of 18 MTs were captured as a maximum of three isolates (<0.2% relative abundance) and were thus considered as rare MTs.

[7]https://cran.r-project.org/web/packages/WRS2/index.html

[8]https://cran.r-project.org/web/packages/imbalance/index.html

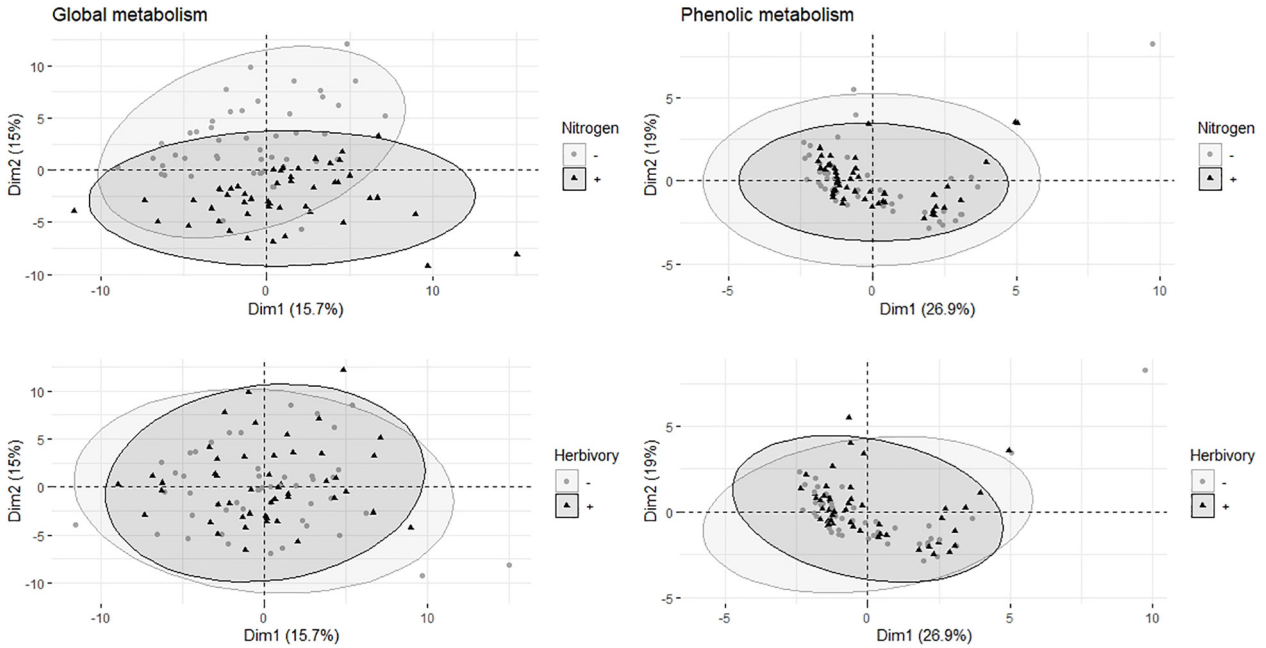


FIGURE 1 | Multivariate analyses of metabolites in the leaves of aspen (*Populus tremula*) plants exposed to environmental endophytic inoculum in controlled, greenhouse conditions: untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment). Shown are the principal component analysis score plots of 114 targeted and non-targeted foliar metabolites (global analysis, data acquired using gas chromatography/mass spectrometry) and 11 targeted metabolites (the main salicinoid phenolic glycosides and catechin; data acquired using liquid chromatography).

The sample size-based rarefaction and extrapolation curves (**Figure 3A**) are predicted to accurately estimate the number of MTs per sampling unit (tree) within the double number of species when compared to the reference sample size (Chao et al., 2014). At least two fungal isolates were recovered from each plant, and the highest increase in diversity was obtained when increasing the number of sampled trees from 1 to 5 within all the treatments. The non-asymptotic rarefaction curves suggest that additional sampling efforts would yield more MTs, especially in H treatment. However, the coverage-based rarefaction and extrapolation curves (**Figure 3B**) suggested that the sample size (24 trees for C and N each, and 21 for H and NH) allowed high completeness with the method that was used: the sample coverage values were 0.95, 0.97, 0.91, and 0.93 for C, N, H, and NH, respectively.

Treatment Effects on Endophytes

The treatments caused significant differences in endophyte richness (robust ANOVA, F = 3.70, p = 0.02) and relative abundance (Permanova, F = 1.67, p = 0.04). These differences were located between N and H treatments (p = 0.01 for richness; and F = 2.92, p = 0.02, for abundance), while the rest of combinations did not result in significant differences. Together, the isolates belonging to the three dominating MTs made up 81% of all isolates in C plants, but their proportion tended to decrease by treatments: in N and H treatments, they made up 73% of all isolates, and in NH 71%. At the same time, the relative abundance of the other (less frequent, and rare) morphotypes increased correspondingly (**Table 1**).

The Shannon index (H') did not differ among the treatments (ANOVA, F = 1.54, p = 0.21), with mean (±se) and SE values 1.35 (±0.07) for C; 1.50 (±0.06) for N, 1.24 (±0.10) for H, and 1.36 (±0.08) for NH. Likewise, the Simpson index (D) values did not vary among treatments (0.68 ± 0.02 for C; 0.72 ± 0.02 for N; 0.62 ± 0.04 for H; and 0.68 ± 0.03 for NH; robust ANOVA, F = 0.41, p = 0.74) nor did the values of Pielou's measure of evenness (J) (0.85 ± 0.02 for C; 0.84 ± 0.02 for N, 0.82 ± 0.03 for H and 0.86 ± 0.02 for NH; robust ANOVA, F = 1.03, p = 0.39).

In total, 11 MTs were shared among all four treatments (**Figure 4**), of which six morphotypes were identified at least to the genus level (*Ramularia* sp., *Cladosporium* sp., *Penicillium olssonii*, *Arthrinium* sp. and 2, *Penicillium* sp.) and five remained unknown (**Table 2**). Three rare MTs were recovered only from C plants, including a Dothideomycetes and a *Coleosporium* species, and three others from N-treated plants (a Dothideomycetes, *Physalospora scripi*, and an unknown species). Three rare MTs, including the identified *Aureobasidium* species (*A. microstictum* and *A. pullulans*), were found only in H treatments (**Table 2**).

DISCUSSION

Nitrogen treatment induced some of the expected changes in the quality of aspen leaves: in particular, the global metabolite analysis and catechin levels highlighted the difference in the

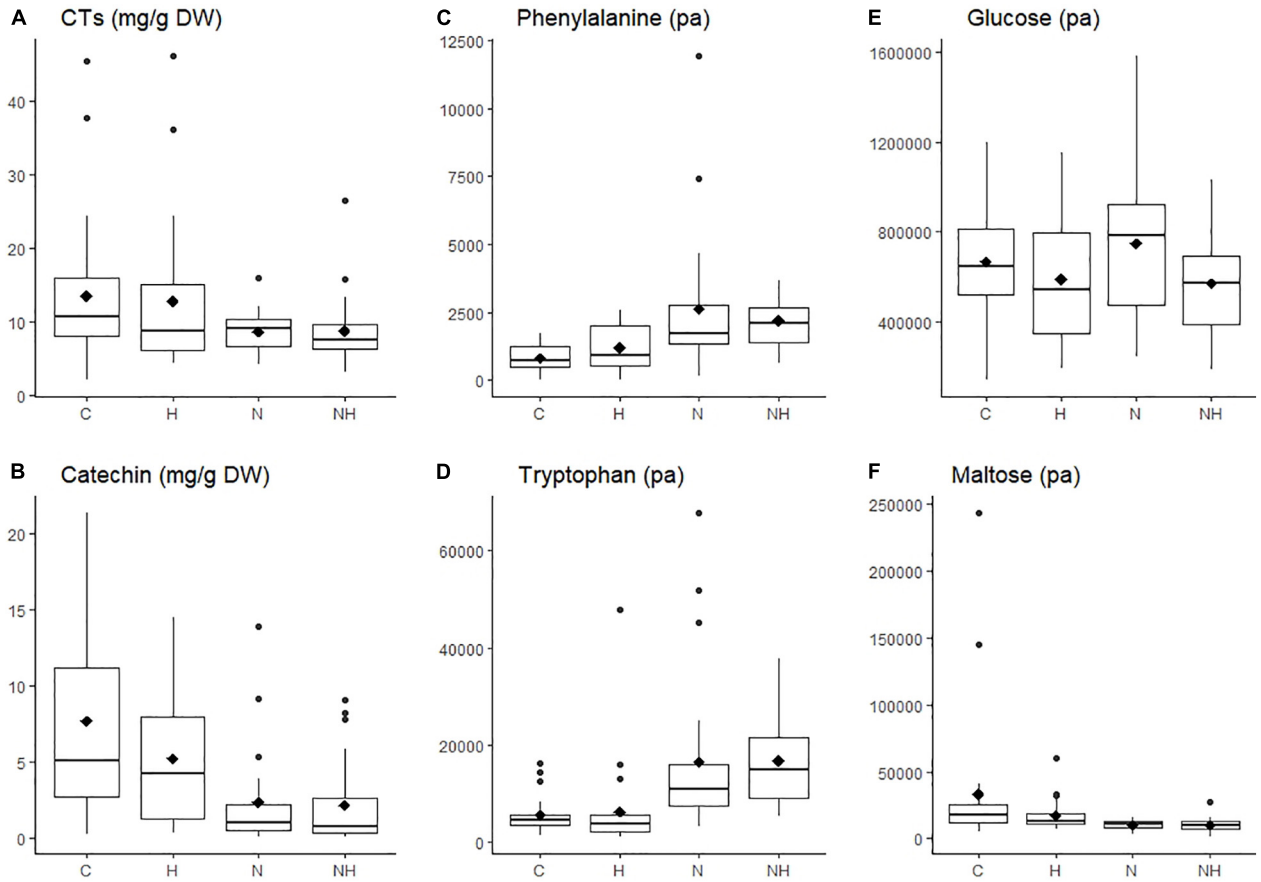


FIGURE 2 | Concentration (mg g^{-1} DW) or level (measured as normalized peak area, pa) of individual chemical compounds in the leaves of aspen (*Populus tremula*) plants exposed to environmental endophytic inoculum in controlled, greenhouse conditions: untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment). Shown are selected examples for phenolics (**A,B**: concentrations of condensed tannins, CTs, and their precursor catechin), amino acids (**C,D**: phenylalanine and tryptophan), and sugars (**E,F**: glucose, maltose). The data were acquired using liquid chromatography, except for condensed tannins, which we analyzed using a spectroscopy.

chemical profile between fertilized and non-fertilized aspen plants. A characteristic change in the global metabolite pool seemed to be due to increased concentrations of amino acids and a decrease in some sugars, which could reflect the expected positive effect of N on growth and associated metabolic changes in the plants (**Supplementary Table 1**, growth data). While the N effect on CTs was not significant, we found lower concentration of CTs in the leaves of fertilized plants, and the N treatment also tended to reduce the variation in CT concentration among the studied trees. Thus, nitrogen fertilization seemed to render the leaves to a more nutritious and less toxic environment, assuming bioactive effects of catechins (Gaur et al., under review), but also a more homogenous substrate for the fungi.

In contrast to our initial expectation, however, the detected quantitative changes were not accompanied by a more uniform fungal community in aspen leaves when compared with the control plants. Thus, our findings did not support for the classic heterogeneity–diversity relationship hypothesis among the endophyte community in aspen leaves (i.e., the apparently more homogenous chemical environment, especially in terms of condensed tannins, was not accompanied by a lower endophyte diversity). However, recent studies suggest that heterogeneity–diversity relationships may be non-linear and more complex than expected from the niche-based perspective (Ben-Hur and Kadmon, 2020). Resource availability may further alter the relative investment in different defense mechanisms in plants (López-Goldar et al., 2020), and earlier studies have shown that growing environment also determines aspens' investment in CTs (Decker et al., 2017). In our greenhouse study, the CT level was generally low as compared to other studies where the detection has been done against the same commercial standard (e.g., Bandau et al., 2016), but it is unclear if the low CT level had consequences for the responses of the fungal community in our study. A deeper taxonomic analysis, using culture-independent approaches, may be needed to reveal these relationships. Clearly, more detailed information about nutritional niches and functional dynamics of different endophytic fungi (Blumenstein et al., 2015; Paungfoo-Lonhienne et al., 2015) would facilitate the analyses of the niche-dependent mechanisms behind the observed patterns in fungal diversity.

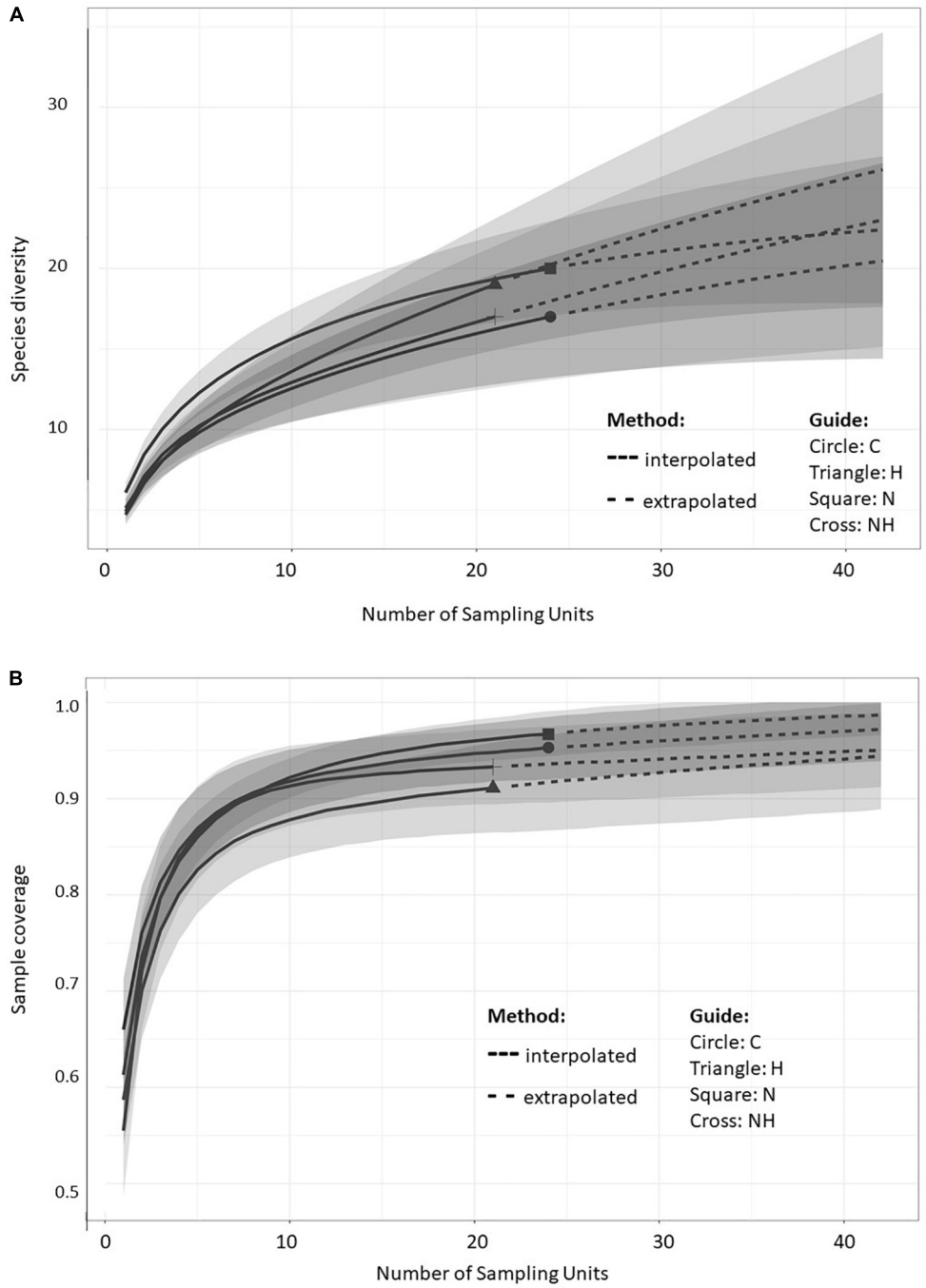


FIGURE 3 | Sample-size-based **(A)** and coverage-based **(B)** rarefaction (solid line) and extrapolation (dotted line) curves comparing fungal morphotype richness in the leaves of greenhouse-cultivated aspen (*Populus tremula*) plants (n = 96): untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment). The shaded areas represent the 95% confidence intervals. The different symbols represent the reference samples.

We expected that leaf herbivory would increase both the compositional (magnitude) and configurational (spatial) chemical heterogeneity in the leaf tissues by inducing changes in phenolic metabolism, and that this change would lead to a more complex community structure among the relatively fast growing, culturable fraction of endophyte communities. However, we did not find evidence for the expected changes in CTs or other phenolics in response to herbivory. Possibly, the impact of herbivory could have been stronger in younger leaves (a systemic induction) or after a longer feeding period. Moreover, the MT

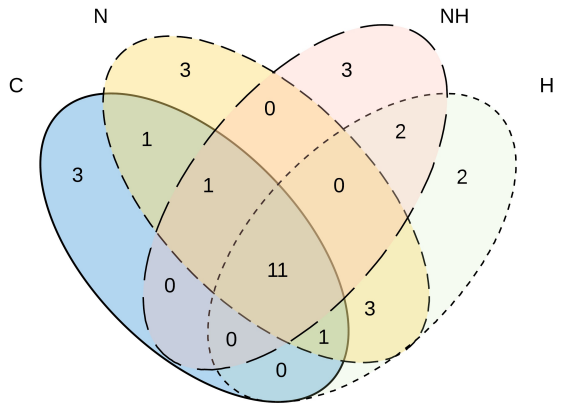


FIGURE 4 | Venn diagram showing the number of unique and shared morphotypes of fungal endophytes in the leaves of greenhouse-cultivated aspen (*Populus tremula*) plants untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment).

TABLE 2 | An overview of morphotype identity and abundance (calculated as the number of isolates of a morphotype per plant normalized after the total number isolates) from leaves of aspen clones belonging to the Swedish Aspen collection (Luquez et al., 2008) and divided after treatment (C, control; N, nitrogen fertilization; and subsequent herbivory H of controls and nitrogen treated plants, H and NH, respectively).

Morphotype	Identity	Treatment			
		C	N	H	NH
Total nr of isolates		543	520	268	311
MT1	*Ramularia* sp.	8.83	6.88	5.14	4.33
MT2		4.71	5.13	1.48	3.29
MT5		4.83	3.92	2.71	2.95
MT13	*Cladosporium* sp. 1	1.08	1.29	0.71	0.76
MT18		0.88	0.83	0.38	0.43
MT12	*Penicillium olssonii*	0.42	0.67	0.57	0.81
MT22	*Arthrinium* sp. 1	0.54	0.75	0.52	0.52
MT15	*Penicillium* 1	0.54	0.54	0.10	0.52
MT26		0.29	0.29	0.43	0.71
MT17	*Penicillium brevicompactum*	0.00	0.46	0.19	0.00
MT23	*Arthrinium* sp. 2	0.08	0.25	0.05	0.05
MT3	*Fusarium oxysporum*	0.04	0.04	0.00	0.05
MT4		0.00	0.04	0.05	0.00
MT6		0.17	0.08	0.05	0.05
MT7		0.04	0.00	0.00	0.00
MT8	*Dothideomycetes* 1	0.00	0.08	0.00	0.00
MT9	*Dothideomycetes* 2	0.04	0.00	0.00	0.00
MT10		0.00	0.00	0.05	0.05
MT11		0.00	0.00	0.05	0.00
MT14	*Cladosporium* sp. 2	0.04	0.00	0.00	0.00
MT16		0.00	0.04	0.00	0.00
MT19		0.04	0.04	0.05	0.00
MT20	*Penicillium* sp. 2	0.00	0.00	0.00	0.05
MT21	*Penicilium* sp. 3	0.04	0.08	0.00	0.00
MT24	*Arthrinium* sp. 3	0.00	0.00	0.05	0.00
MT25	*Rhodotorula* sp.	0.00	0.21	0.14	0.00
MT27	*Aureobasidium microstictum*	0.00	0.00	0.00	0.14
MT28	*Aureobasidium pullulans*	0.00	0.00	0.05	0.05
MT29		0.00	0.00	0.00	0.05
MT30	*Physalospora scirpi*	0.00	0.04	0.00	0.00

richness in H-treated plants did not increase as compared to C plants. Interestingly, we found that three of the MTs were found only in connection to herbivory, supporting the view that insects may influence the fungal diversity by carrying specific fungi to the plants (Albrectsen et al., 2018). Wounding by herbivores may also open up more entry points for the ubiquitous fungi such as *Aureobasidium pullulans,* which was identified among the three H-treatment-specific MTs and is commonly found as an epiphyte and endophyte in different environments (Martín et al., 2013; Albrectsen et al., 2018).

The combined effect of N and H on the chemical traits or fungal diversity seemed to be dominated by the N effect. The longer impact time of N was likely to accentuate its effect as a stronger bottom-up force and allowed it to shape the quality of leaves as a substrate for fungi more than the short-term herbivory. Fungi transmitted to plants by the feeding beetles would probably need a longer incubation time than what was possible in our study before they can be captured using the culturing approach. It should also be noted that in our study, the insects were reared in laboratory conditions and thus exposed to only a limited environmental inoculum. Therefore, the results of this study may underestimate the importance of insect-mediated facilitation of infections that occurs in natural environments.

In accordance with the universal pattern of species abundance distribution (McGill et al., 2007), we found that the culturable endophyte community in aspen leaves was composed of a few common species (the main community member *Ramularia* spp. and two unknown ones) but many rare species. The identified taxa represented *Ascomycota* commonly found as endophytes in trees (Arnold et al., 2007; Rodriguez et al., 2009; Unterseher, 2011). The only *Basidiomycota* species identified, *Rhodotorula* sp., has been reported as a common endophyte in trees in previous studies (Jumpponen and Jones, 2010; Unterseher, 2011; Rajala et al., 2014), and it has also been linked to herbivory treatment (Albrectsen et al., 2018). In our study, however, only weak link was found between *Rhodotorula* sp. and herbivory. Intriguing was also that some common tree endophytes, such as *Alternaria* and *Phomopsis* (Albrectsen et al., 2010; Crous and Groenewald, 2013; Martín et al., 2013), were not detected, possibly because the greenhouse acted as a filter for the environmental inocula and reduced the frequency of otherwise common endophytes. Moreover, the time point of sampling in early summer may be reflected in the community composition: the species diversity is likely to increase during the season as the infections accumulate. Among the other genera identified, *Cladosporium*, *Ramularia* (anamorph *Mycosphaerella*), and *Physalospora* have been previously described as plant pathogens (Thomma et al., 2005; Tadych et al., 2012; Videira et al., 2016), but no signs of pathogen attacks were found on the leaves, suggesting

that the conditions in our experiment supported endophytic lifestyle of these and other fungi.

In conclusion, our results indicate that the culturable fraction of fungal endophyte community in aspen leaves is rather stable against the directional alterations induced in the chemical "landscape" within the leaves by nitrogen fertilization or disturbances due to herbivory. This is in line with the results of the study by Christian et al. (2016) who reported high resistance among endophyte communities against biotic and anthropogenic perturbations. However, more research is needed to better comprehend the nutritional requirements of endophytes and the signals influencing the dynamics of the microbial communities in the endosphere. This is particularly important in future conditions where climate change may directly or indirectly cause major alterations in nutrient availability for plants (Kreuzwieser and Gessler, 2010). While our results did not agree with the expectations we made based on the heterogeneity–diversity relationship hypothesis, we propose that by implementing theoretical frameworks of community ecology, it is possible to gain new insights into the processes and traits driving the structure and functions of endophyte communities in perennial plants, such as forest trees.

DATA AVAILABILITY STATEMENT

The data are available at doi: 10.5061/dryad.3j9kd51kp after publishing (https://datadryad.org/stash/share/USovDaEtgAaireb D6HyROVNXuDkv0V8PsQjtp-0-AFY).

AUTHOR CONTRIBUTIONS

VD and MA planned the research supervised by BA and JW. VD performed the experiments and performed the chemical analyses. MA, CR, BA, and MC performed data analyses. VD and MA wrote the first draft. JW and BA wrote the manuscript. All authors contributed to the final version of the manuscript.

FUNDING

This research was supported by FORMAS, a Swedish Research Council for Sustainable Development (2012-01358 and 2016-00907) to JW and MA, The Royal Swedish Academy of Agriculture and Forestry to MA, MSCA-IF from the EU's Horizon 2020 Research and Innovation Program (grant agreement No. 845419) to CR, and Swedish Strategic Research to BA. The work was further supported by the Swedish Metabolomics Centre and by the Berzelii Center at UPSC, Umeå.

ACKNOWLEDGMENTS

We thank Sylvia S. Chen for assistance in the experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.846208/full#supplementary-material

REFERENCES

Abreu, I. N., Ahnlund, M., Moritz, T., and Albrectsen, B. R. (2011). UHPLC-ESI/TOFMS determination of salicylate-like phenolic gycosides in *Populus tremula* leaves. *J. Chem. Ecol.* 37, 857–870. doi: 10.1007/s10886-011-9991-7

Agostinelli, M., Cleary, M., Martín, J. A., Albrectsen, B. R., and Witzell, J. (2018). Pedunculate oaks (*Quercus robur* L.) differing in vitality as reservoirs for fungal biodiversity. *Front. Microbiol.* 9:1758. doi: 10.3389/fmicb.2018.01758

Albrectsen, B. R., and Witzell, J. (2012). "Disentangling functions of fungal endophytes in forest trees," in *Fungi: Types, Environmental Impact And Role In Disease*, eds A. Paz Silva and M. Sol (Huntington, NY: Nova Science Publishers), 235–246.

Albrectsen, B. R., Björkén, L., Varad, A., Hagner, Å, Wedin, M., Karlsson, J., et al. (2010). Endophytic fungi in European aspen (*Populus tremula*) leaves - diversity, detection, and a suggested correlation with herbivory resistance. *Fungal Div.* 41, 17–28. doi: 10.1007/s13225-009-0011-y

Albrectsen, B. R., Siddique, A. B., Decker, V. H. G., Unterseher, M., and Robinson, K. M. (2018). Both plant genotype and herbivory shape aspen endophyte communities. *Oecologia* 187, 535–545. doi: 10.1007/s00442-018-4097-3

Arnold, A. E., Henk, D. A., Eells, R. L., Lutzoni, F., and Vilgalys, R. (2007). Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* 99, 185–206. doi: 10.3852/mycologia.99.2.185

Bailey, J. K., Deckert, R., Schweitzer, J. A., Rehill, B. J., Lindroth, R. L., Gehring, C., et al. (2005). Host plant genetics affect hidden ecological players: links among *Populus*, condensed tannins, and fungal endophyte infection. *Can. J. Bot.* 83, 356–361. doi: 10.1139/b05-008

Bandau, F., Albrectsen, B. R., Julkunen-Tiitto, R., and Gundale, M. (2016). Genotypic variability in *Populus tremula* L. affects how anthropogenic nitrogen enrichment influences litter decomposition. *Plant Soil* 310:467. doi: 10.1007/s11104-016-3033-8

Bandau, F., Decker, V. H. G., Gundale, M. J., and Albrectsen, B. R. (2015). Genotypic tannin levels in *Populus tremula* impact the way nitrogen enrichment affects growth and allocation responses for some traits and not for others. *PLoS One* 10:e0140971. doi: 10.1371/journal.pone.0140971

Bandau, F., Robinson, K., Albrectsen, B. R., and Gundale, M. (2021). Populus genotypes with high compared to low constitutive tannin defenses grow taller in response to anthropogenic nitrogen enrichment. *For. Ecol. Manage.* 487:118985. doi: 10.1016/j.foreco.2021.118985

Ben-Hur, E., and Kadmon, R. (2020). Heterogeneity-diversity relationships in sessile organisms: a unified framework. *Ecol. Lett.* 23, 193–207. doi: 10.1111/ele.13418

Blacutt, A., Ginnan, N., Dang, T., Bodaghi, S., Vidalakis, G., Ruegger, P., et al. (2020). An in vitro pipeline for screening and selection of citrus-associated microbiota with potential anti-"*Candidatus Liberibacter asiaticus*" properties. *Appl. Environ. Microbiol.* 86, e2883–e2889. doi: 10.1128/AEM.02883-19

Blumenstein, K., Albrectsen, B. R., Martín, J. A., Hultberg, M., Sieber, T. N., Helander, M., et al. (2015). Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree disease. *Biocontrol* 60, 655–667. doi: 10.1007/s10526-015-9668-1

Busby, P. E., Peay, K. G., and Newcombe, G. (2016). Common foliar fungi of *Populus trichocarpa* modify *Melampsora* rust disease severity. *New Phytol.* 209, 1681–1692. doi: 10.1111/nph.13742

Carroll, G. (1995). Forest endophytes: pattern and process. *Can. J. Bot.* 73, 1316–1324. doi: 10.1139/b95-393

Chao, A., Gotelli, N. J., Hsieh, T. C., Sander, E. L., Ma, K. H., Colwell, R. K., et al. (2014). Rarefaction and extrapolation with Hill numbers: a framework for

sampling and estimation in species diversity studies. *Ecol. Monogr.* 84, 45–67. doi: 10.1890/13-0133.1

Christian, N., Sullivan, C., Visser, N. D., and Clay, K. (2016). Plant host and geographic location drive endophyte community composition in the face of perturbation. *Microb Ecol.* 72, 621–632. doi: 10.1007/s00248-016-0804-y

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* 69, 10–16.

Clay, K., and Holah, J. (1999). Fungal endophyte symbiosis and plant diversity in successional fields. *Science* 285, 1742–1745. doi: 10.1126/science.285.5434.1742

Coblentz, K. E., and Van Bael, S. A. (2013). Field colonies of leaf-cutting ants select plant materials containing low abundances of endophytic fungi. *Ecosphere* 4, 1–10. doi: 10.1890/ES13-00012.1

Colwell, R. K., Chao, A., Gotelli, N. J., Lin, S.-Y., Mao, C. X., Chazdon, R. L., et al. (2012). Models and estimators linking individual-based and sample-based rarefaction, extrapolation and comparison of assemblages. *J. Plant Ecol.* 5, 3–21.

Constabel, C. P., Yip, L., Patton, J. J., and Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.* 124, 285–295. doi: 10.1104/pp.124.1.285

Crous, P. W., and Groenewald, J. Z. (2013). A phylogenetic re-evaluation of *Arthrinium*. *IMA Fungus* 4, 133–154. doi: 10.5598/imafungus.2013.04.01.13

Decker, V. H. G., Bandau, F., Gundale, M. J., Cole, C. T., and Albrectsen, B. R. (2017). Aspen phenylpropanoid genes' expression levels correlate with genets' tannin richness and vary both in responses to soil nitrogen and associations with phenolic profiles. *Tree Physiol.* 37, 270–279. doi: 10.1093/treephys/tpw118

Edenius, L., Mikusinski, G., Witzell, J., and Bergh, J. (2012). Effects of repeated fertilization of young Norway spruce on phenolics and arthropods: implications for insectivorous birds' food resources. *For. Ecol. Manage.* 277, 38–45. doi: 10.1016/j.foreco.2012.04.021

Gomes, T., Pereira, J. A., Benhadi, J., Lino-Neto, T., and Baptista, P. (2018). Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem. *Microb. Ecol.* 76, 668–679. doi: 10.1007/s00248-018-1161-9

Gouda, S., Das, G., Sen, S. K., Shin, H.-S., and Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. *Front. Microbiol.* 7, 1–8. doi: 10.3389/fmicb.2016.01538

Gourlay, G., and Constabel, P. (2019). Condensed tannins are inducible antioxidants and protect hybrid poplar against oxidative stress. *Tree Physiol.* 39, 345–355. doi: 10.1093/treephys/tpy143

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

Hsieh, T. C., Ma, K. H., and Chao, A. (2016). INEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Meth. Ecol. Evol.* 7, 1451–1456. doi: 10.1111/2041-210X.12613

Jonsson, P., Johansson, A. I., Gullberg, J., Trygg, J., Grung, B., Marklund, S., et al. (2005). High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. *Anal. Chem* 77, 5635–5642. doi: 10.1021/ac050601e

Jumpponen, A., and Jones, K. L. (2009). Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytol.* 184, 438–448. doi: 10.1111/j.1469-8137.2009.02990.x

Jumpponen, A., and Jones, K. L. (2010). Seasonally dynamic fungal communities in the *Quercus macrocarpa* phyllosphere differ between urban and nonurban environments. *New Phytol.* 186, 496–513. doi: 10.1111/j.1469-8137.2010.03197.x

Keefover-Ring, K., Ahnlund, M., Abreu, I. N., Jansson, S., Moritz, T., and Albrectsen, B. R. (2014). No evidence of geographical structure of salicinoid chemotypes within *Populus tremula*. *PLoS One* 9:e107189. doi: 10.1371/journal.pone.0107189

Kreuzwieser, J., and Gessler, A. (2010). Global climate change and tree nutrition: influence of water availability. *Tree Physiol.* 30, 1221–1234. doi: 10.1093/treephys/tpq055

Lê, S., Josse, J., and Husson, F. (2008). FactoMineR: an r package for multivariate analysis. *J. Stat. Soft.* 25, 1–18. doi: 10.18637/jss.v025.i01

Lewinsohn, D., Lewinsohn, E., Bertagnolli, C. L., and Patridge, A. D. (1994). Blue-stain fungi and their transport structures on the Douglas-fir beetle. *Can. J. For. Res.* 24, 2275–2283. doi: 10.1139/x94-292

López-Goldar, X., Zas, R., and Sampedro, L. (2020). Resource availability drives microevolutionary patterns of plant defences. *Funct. Ecol.* 34, 1640–1652. doi: 10.1111/1365-2435.13610

Luquez, V., Hall, D., Albrectsen, B. R., Karlsson, J., Ingvarsson, P., and Jansson, S. (2008). Natural phenological variation in aspen (*Populus tremula*): the SwAsp Collection. *Tree Genet. Genomes* 4, 279–292. doi: 10.1007/s11295-007-0108-y

Martín, J. A., Witzell, J., Blumenstein, K., Rozpedowska, E., Helander, M., Sieber, T. N., et al. (2013). Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms (*Ulmus* spp.). *PLoS One* 8:e56987. doi: 10.1371/journal.pone.0056987

Martinez Arbizu, P. (2019). *Pairwiseadonis: Pairwise Multilevel Comparison Using Adonis*. R Package Version 0.3.

Massad, T. J., Trumbore, S. E., Ganbat, G., Reichelt, M., Unsicker, S., Boeckler, A., et al. (2014). An optimal defense strategy for phenolic glycoside production in *Populus trichocarpa* – isotope labeling demonstrates secondary metabolite production in growing leaves. *New Phytol.* 203, 607–619. doi: 10.1111/nph.12811

McGill, B. J., Etienne, R. S., Gray, J. S., Alonso, D., Anderson, M. J., Benecha, H. K., et al. (2007). Species abundance distributions: moving beyond single prediction theories to integration within an ecological framework. *Ecol. Lett.* 10, 995–1015. doi: 10.1111/j.1461-0248.2007.01094.x

Nisa, H., Kamili, A. N., Nawchoo, I. A., Shafi, S., Shameem, N., and Bandh, S. A. (2015). Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: a review. *Microb. Pathog.* 82, 50–59. doi: 10.1016/j.micpath.2015.04.001

Nohrstedt, H. -Ö (2001). Response of coniferous forest ecosystems on mineral soils to nutrient additions: a review of Swedish experiences. *Scand. J. For. Res.* 16, 555–573. doi: 10.1080/02827580152699385

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., et al. (2016). *Vegan: Community Ecology Package*. R Package Version 2.4-0.

Paungfoo-Lonhienne, C., Yeoh, Y. K., Kasinadhuni, N. R. P., Lonhienne, T. G. A., Robinson, N., Hugenholtz, P., et al. (2015). Nitrogen fertilizer dose alters fungal communities in sugarcane soil and rhizosphere. *Sci. Rep.* 5:8678. doi: 10.1038/srep08678

Petrini, O., Sieber, T. N., Toti, L., and Viret, O. (1993). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Nat. Toxins* 1, 185–196. doi: 10.1002/nt.2620010306

Porter, J. K., Bacon, C. W., Cutler, H. G., Arrendale, R. F., and Robbins, J. D. (1985). In vitro auxin production by *Balansia epichloë*. *Phytochemistry* 24, 1429–1431. doi: 10.1016/S0031-9422(00)81037-7

R Development Core Team (2016). R: A Language And Environment For Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Available online at: https://www.r-project.org/ (accessed June 21, 2020).

Ragazzi, A., Moricca, S., Capretti, P., Dellavalle, I., and Turco, E. (2003). Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining *Quercus* species in Italy. *For. Pathol.* 33, 31–38. doi: 10.1046/j.1439-0329.2003.3062003.x

Rajala, T., Velmala, S. M., Vesala, R., Smolander, A., and Pennanen, T. (2014). The community of needle endophytes reflects the current physiological state of Norway spruce. *Fungal Biol.* 118, 309–315. doi: 10.1016/j.funbio.2014.01.002

Rice, A. V., Thormann, M. N., and Langor, D. W. (2007). Mountain pine beetle associated blue-stain fungi cause lesions on jack pine, lodgepole pine, and lodgepole × jack pine hybrids in Alberta. *Can. J. Bot.* 85, 307–315. doi: 10.1139/B07-014

Robinson, K. M., Hauzy, C., Loeuille, N., and Albrectsen, B. R. (2016). Relative impacts of environmental variation and evolutionary history on the nestedness and modularity of tree–herbivore networks. *Ecol. Evol.* 5, 2898–2915. doi: 10.1002/ece3.1559

Robinson, K. M., Ingvarsson, P. K., Jansson, S., and Albrectsen, B. R. (2012). Genetic variation in functional traits influences arthropod community composition in *Aspen (Populus tremula L)*. *PLoS One* 7:e37679. doi: 10.1371/journal.pone.0037679

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

Salminen, J. P., and Karonen, M. (2011). Chemical ecology of tannins and other phenolics: we need a change in approach. *Funct. Ecol.* 25, 325–338. doi: 10.1111/j.1365-2435.2010.01826.x

Sampedro, L., Moreira, X., and Zas, R. (2011). Costs of constitutive and herbivore- induced chemical defences in pine trees emerge only under low nutrient availability. *J. Ecol.* 99, 818–827. doi: 10.1111/j.1365-2745.2011.01814.x

Schauer, N., Steinhauser, D., Strelkov, S., Schomburg, D., Allison, G., Moritz, T., et al. (2005). GC–MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Letters* 579, 1332–1337. doi: 10.1016/j.febslet.2005.01.029

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi: 10.1111/j.1462-2920.2011.02585.x

Sieber, T. N. (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biol. Rev.* 21, 75–89. doi: 10.1016/j.fbr.2007.05.004

Siddique, A. B., Albrectsen, B. R., Ilbi, H., and Siddique, A. B. (2022). Optimization of protocol for construction of fungal ITS amplicon library for high-throughput illumina sequencing to study the mycobiome of aspen leaves. *Appl. Sci.* 12:1136. doi: 10.3390/app12031136

Tadych, M., Bergen, M. S., Johnson-Cicalese, J., Polashock, J. J., Vorsa, N., and White, J. F. (2012). Endophytic and pathogenic fungi of developing cranberry ovaries from flower to mature fruit: diversity and succession. *Fungal Div.* 54, 101–116. doi: 10.1007/s13225-012-0160-2

Terhonen, E., Blumenstein, K., Kovalchuk, A., and Asiegbu, F. (2019). Forest tree microbiomes and associated fungal endophytes: functional roles and impact on forest health. *Forests* 10:42. doi: 10.3390/f10010042

Thomma, B. P. H. J., Van Esse, H. P., Crous, P. W., and De Wit, P. J. G. M. (2005). *Cladosporium fulvum (syn. Passalora fulva),* a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Mol. Plant Pathol.* 6, 379–393. doi: 10.1111/j.1364-3703.2005.00292.x

Ullah, C., Unsicker, S. B., Fellenberg, C., Constabel, P., Schmidt, A., Gershenzon, J., et al. (2017). Flavan-3-ols are an effective chemical defense against rust infection. *Plant Physiol.* 175, 1560–1578. doi: 10.1104/pp.17.00842

Unterseher, M. (2011). "Diversity of fungal endophytes in temperate forest trees," in *Endophytes of Forest Trees: Biology and Applications*, ed. A. C. Frank (Dordrecht: Springer), 31–46. doi: 10.1007/978-94-007-1599-8_2

Unterseher, M., Siddique, A. B., Brachmann, A., and Peršoh, D. (2016). Diversity and composition of the leaf mycobiome of beech (*Fagus sylvatica*) are affected by local habitat conditions and leaf biochemistry. *PLoS One* 11:e0152878. doi: 10.1371/journal.pone.0152878

Vellend, M. (2010). Conceptual synthesis in community ecology. *Quart. Rev. Biol.* 85, 183–206. doi: 10.1086/652373

Videira, S. I. R., Groenewald, J. Z., Braun, U., Shin, H. D., and Crous, P. W. (2016). All that glitters is not *Ramularia*. *Stud. Mycol.* 83, 49–163. doi: 10.1016/J.SIMYCO.2016.06.001

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomial RNA genes for phyologenetics," in *PCR Protocols: A Guide To Methods And Applicaitions*, eds T. J. Innis, D. H. Gelfand, and J. J. Sninsky (New York, NY: Academic Press Inc.,), 315–322.

Whittaker, R. H. (1972). Evolution and measurement of species diversity. *Taxon* 21, 213–251.

Wilson, M., and Lindow, S. E. (1994). Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Appl. Environ. Microbiol.* 60, 4468–4477.

Witzell, J., and Martín, J. A. (2008). Phenolic metabolites in the resistance of northern forest trees to pathogens — past experiences and future prospects. *Can. J. For. Res.* 38, 2711–2727. doi: 10.1139/X08-112

Witzell, J., and Martín, J. A. (2018). "Endophytes and forest health," in *Endophytes Of Forest Trees*, ed. C. A. Frank (New York, NY: Springer International Publishing), 261–282. doi: 10.1007/978-3-319-89833-9_12

Witzell, J., and Shevtsova, A. (2004). Nitrogen-induced changes in phenolics of *Vaccinium myrtillus* - implications for interaction with a parasitic fungus. *J. Chem. Ecol.* 10, 1919–1938. doi: 10.1023/b:joec.0000045587.75128.a4

Witzell, J., Martín, J. A., and Blumenstein, K. (2014). "Ecological aspects of endophyte-based biocontrol of forest diseases," in *Advances in Endophytic Research*, eds V. C. Verma and A. C. Gange (New Delhi: Springer India), 321–333. doi: 10.1007/978-81-322-1575-2_17

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 March 2022
doi: 10.3389/fmicb.2022.841558

Dynamics of Bacterial Root Endophytes of *Malus domestica* Plants Grown in Field Soils Affected by Apple Replant Disease

*Felix Mahnkopp-Dirks[1], Viviane Radl[2], Susanne Kublik[2], Silvia Gschwendtner[2], Michael Schloter[2] and Traud Winkelmann[1]**

[1] Section Woody Plant and Propagation Physiology, Institute of Horticultural Production Systems, Leibniz Universität Hannover, Hanover, Germany, [2] Research Unit for Comparative Microbiome Analysis, Helmholtz Zentrum München, Munich, Germany

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Murali Gopal, Central Plantation Crops Research Institute (ICAR), India
Marco Nuti, Sant'Anna School of Advanced Studies, Italy
Pankaj Prakash Verma, Central Potato Research Institute (ICAR), India

***Correspondence:**
Traud Winkelmann traud.winkelmann@zier.uni-hannover.de

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *22 December 2021*
Accepted: *14 February 2022*
Published: *25 March 2022*

Citation:
Mahnkopp-Dirks F, Radl V, Kublik S, Gschwendtner S, Schloter M and Winkelmann T (2022) Dynamics of Bacterial Root Endophytes of Malus domestica Plants Grown in Field Soils Affected by Apple Replant Disease. Front. Microbiol. 13:841558. doi: 10.3389/fmicb.2022.841558

Apple replant disease (ARD) is a worldwide problem for tree nurseries and orchards leading to reduced plant growth and fruit quality. The etiology of this complex phenomenon is poorly understood, but shifts of the bulk soil and rhizosphere microbiome seem to play an important role. Since roots are colonized by microbes from the rhizosphere, studies of the endophytic microbiome in relation to ARD are meaningful. In this study, culture-independent and culture-dependent approaches were used in order to unravel the endophytic root microbiome of apple plants 3, 7, and 12 months after planting in ARD-affected soil and ARD-unaffected control soil at two different field sites. Next to a high diversity of *Pseudomonas* in roots from all soils, molecular barcoding approaches revealed an increase in relative abundance of endophytic Actinobacteria over time in plants grown in ARD and control plots. Furthermore, several amplicon sequence variants (ASVs) linked to *Streptomyces*, which had been shown in a previous greenhouse ARD biotest to be negatively correlated to shoot length and fresh mass, were also detected in roots from both field sites. Especially in roots of apple plants from control soil, these *Streptomyces* ASVs increased in their relative abundance over time. The isolation of 150 bacterial strains in the culture-dependent approach revealed a high diversity of members of the genus *Pseudomonas,* confirming the data of the molecular barcoding approach. However, only partial overlaps were found between the two approaches, underlining the importance of combining these methods in order to better understand this complex disease and develop possible countermeasures. Overall, this study suggests a key role of *Streptomyces* in the etiology of ARD in the field.

Keywords: endophyte, apple replant disease (ARD), *Malus domestica*, *Streptomyces*, microbiome, *Pseudomonas*

INTRODUCTION

Apple replant disease (ARD) is a worldwide complex problem, which affects apple tree nurseries and orchards, causing reductions in tree growth, fruit yield, and quality (Mazzola and Manici, 2012; Manici et al., 2013; Winkelmann et al., 2019). It occurs when apple is repeatedly planted at the same site and is defined as a "harmfully disturbed physiological and morphological reaction of apple plants to soils that faced alterations in their (micro-)biome due to previous apple cultures"

(Winkelmann et al., 2019). The exact etiology of ARD is still not known, but there is increasing evidence that, next to changes in the abundance of specific pathogens, shifts of the bulk soil and rhizosphere microbiome are an important driver of ARD (Winkelmann et al., 2019; Balbín-Suárez et al., 2021). With the rise of next-generation sequencing, several studies revealed shifts of the rhizosphere microbiome community structure (Sun et al., 2014; Franke-Whittle et al., 2015; Yim et al., 2015; Nicola et al., 2018; Tilston et al., 2018; Balbín-Suárez et al., 2021) and showed an enrichment of potential ARD fungal pathogens [*Acremonium*, *Fusarium*, *Cylindrocarpon* (Franke-Whittle et al., 2015), *Pythium* (Tilston et al., 2018), *Ilyonectria*, and *Nectria* sp. (Balbín-Suárez et al., 2021)] and several bacteria associated with ARD [*Lysobacter*, *Pseudomonas* (Sun et al., 2014), *Chitinophaga*, *Hyphomicrobium* (Franke-Whittle et al., 2015), *Streptomyces*, and *Variovorax* (Balbín-Suárez et al., 2021)].

The plant endobiome is strongly influenced by colonization from the rhizosphere microbiome and, thus, is very likely subjected to changes in ARD-affected soil. Moreover, beneficial endophytes can support plants in coping with abiotic and biotic stresses. Therefore, analyses of the root endobiome of apple in replant-affected soils is of interest to understand the disease etiology, but also to develop mitigation strategies. However, studies of the endophytic microbiome and its role in ARD are rare and were mostly focused on fungal pathogens in apple roots. Kelderer et al. (2012) identified *Cylindrocarpon* spp. and *Rhizoctonia* sp. as pathogenic root endophytes in row (ARD affected) and inter-row (control) planted apple trees. *Fusarium oxysporum* and *Fusarium solani* were most abundant in roots in this study, but not considered as pathogens. Root endophytic *Cylindrocarpon*-like fungi (*Thelonectria* sp. and *Ilyonectria* spp.) were also shown by Manici et al. (2013) next to *Pythium* spp. to be correlated to the growth reduction in the rootstock M9 growing in ARD-affected soil. Different species of Nectriaceae were also found in ARD-affected cortex cells applying laser microdissection (Popp et al., 2020). Several fungal endophytes from ARD-affected apple roots were isolated and re-inoculated in a soil free biotest by Popp et al. (2019). Negative effects on plant health were reported for *Cadophora*, *Calonectria*, *Dactylonectria*, *Ilyonectria*, and *Leptosphaeria*.

For bacterial root endophytes, even less data are available. Only two studies were conducted, which investigated the role of bacterial endophytes to the context of ARD (Tewoldemedhin et al., 2011; Van Horn et al., 2021). Tewoldemedhin et al. (2011) used a cultivation-dependent approach to investigate the biocontrol properties of Actinobacteria isolates (92 isolates belonging to the genus *Streptomyces* and four to *Nocardiopsis*) from ARD-affected roots but inoculation of selected isolates resulted in no effects on plant growth. Amplicon sequencing was used by Van Horn et al. (2021) to characterize the endophytic community structure of rootstock genotypes reported to be susceptible (M26 and M9) and tolerant (G210, G41, G890, and G935) to ARD. The strongest community differences were found between tolerant and susceptible genotypes and the overall most abundant endophytes were members belonging to the genera *Arthrobacter*, *Burkholderia*, *Halospirulina*, and *Streptomyces*. In a previous study (Mahnkopp-Dirks et al., 2021), we conducted a biotest with apple plants of the ARD-sensitive rootstock genotype M26 grown in three soils differing in soil properties and compared for each soil the bacterial root endophytic community of plantlets grown in ARD-affected soil or control (grass) soil using a molecular barcoding approach. Results showed several amplicon sequence variants (ASVs) linked to *Streptomyces*, which were exclusively found in plants grown in ARD soil. Moreover, these ASVs were negatively correlated to shoot length and shoot fresh mass. These results were achieved for young plantlets (8 weeks of age) under controlled greenhouse conditions using *in vitro* propagated plant material, which did not represent field conditions. To validate the relevance of the observations of our greenhouse biotest under field conditions and to assess seasonal dynamics, we performed a field experiment at the two sites, from which the soils for the biotests were obtained. The bacterial root endophytic community structure in plants grown in ARD-affected or grass control soil using seed-propagated rootstocks (cv. 'Bittenfelder Saemling', hereafter referred to as Bittenfelder) was characterized. Samples were taken over 1 year to cover a complete vegetation cycle. We postulated that overall diversity and seasonal dynamics of bacterial root endophytes are less pronounced in plantlets grown in ARD-affected soils, as in these soils single bacterial endophytes dominate the microbiome of root endophytes, which negatively impact plant growth and overall plant performance. Moreover, we intended to obtain isolates to complement the culture-independent data and to serve as potential inoculants. Thus, we also used a culture-dependent approach in order to isolate a broad spectrum of bacterial root endophytes, established pure cultures, and identified them based on their 16S rRNA gene sequence. This will enable us to study their effects on apple plants and potentially to help and overcome the complex ARD phenomenon.

MATERIALS AND METHODS

Field Sites and Sampling

The field experiments were carried out at two different sites in northern Germany: Heidgraben (x-coordinate 53.699199; y-coordinate 9.683171; WGS 84, Schleswig-Holstein) and Ellerhoop (x-coordinate 53.71435; y-coordinate 9.770143 WGS 84, Schleswig-Holstein), which differed in their soil properties (Mahnkopp et al., 2018). Based on World Reference Base for soil resources, the textures of the top soil (0–20 cm) of the two sites were classified as sand (Heidgraben) and loamy sand (Ellerhoop) (Mahnkopp et al., 2018). In 2017, the annual mean temperature for both sides was 10°C, and the annual precipitation reached 1,142 mm.

At both sites, two different variants were established, each in four replicates (plots): (i) ARD plots, where ARD was successfully induced by repeatedly replanting Bittenfelder apple seedling rootstocks since 2009 in a 2-year cycle, and (ii) control plots, which were covered with grass since then. In spring 2016, at Heidgraben and spring 2017 at Ellerhoop, one-third of these grass control plots were planted with Bittenfelder plants representing the first apple planting generation (hereafter referred to as grass plots). ARD plots at Ellerhoop were replanted for the last time in spring 2017 and at Heidgraben in spring 2016 representing the fifth replant generation at the time of sampling (**Table 1**). Both

sites were managed as closely as possible to nursery practice and were treated in a similar way. However, since the apple plants were grown not only at the two different sites but also in two different years, the management was not completely the same. Per year, each site was fertilized with 54 kg of nitrogen per hectare.

The planting material for this study was obtained from a specialized nursery (Stahl Baumschulen GmbH, Tornesch, Germany) as 1-year-old Bittenfelder seedlings. These were sown in spring 2015 and 2016, respectively, on a loamy sandy soil (fertilized with 95 kg nitrogen per hectare), uprooted in the following autumn, and stored over winter in a cooling chamber. At Heidgraben, plants were planted on April 5 and 6, 2016 for both plots; at Ellerhoop, April 10 and 11, 2017. At both sites, plants were sampled at three time points after planting: 3 months after planting (summer), 7 months after planting (late autumn), and 1 year after planting (spring). At Heidgraben, sampling was performed on July 27, 2016 (summer), November 16, 2016 (autumn), and April 25, 2017 (spring); at Ellerhoop, on July 25, 2017 (summer), November 13, 2017 (autumn), and April 23, 2018 (spring). Three plants were sampled per plot and sampling time point (in total 24 per site and season).

To determine the root fresh weight of plants taken after 12 months (spring), adhering soil was removed by carefully washing the roots under running tap water until no soil was visible any more followed by briefly drying the roots using paper towels. Since fresh roots were analyzed further by project partners, determining the root dry weight was not possible. However, in previous studies with apple roots (Mahnkopp et al., 2018), we observed a highly significant Pearson correlation [$r(214) = 0.97$, $p < 0.05$] between root fresh mass and root dry mass. In addition, samples of the planting material (plants taken before the transfer into the soil) were taken at Ellerhoop in spring 2017 and served as "time point zero" (T0) plants.

Root Surface Disinfection

The following surface disinfection was performed as described in Mahnkopp-Dirks et al. (2021): To get rid of the adhering soil, roots were washed carefully. Afterward, they were rinsed for 30 s in EtOH (70%), followed by stirring in 2% NaOCl for 7.5 min and finally washing five times in sterile deionized water. The final washing water was plated on 523 medium (Viss et al., 1991) and R2A Agar (Reasoner and Geldreich, 1985) and incubated for 1 week at room temperature. Plating resulted in <10 CFU per plate in all cases. Roots were stored in sterile 2-ml Eppendorf tubes at −80°C until DNA extraction for amplicon sequencing.

Molecular Barcoding of Root Endophytic Bacteria

For Illumina sequencing, DNA was extracted as mentioned in Mahnkopp-Dirks et al. (2021) using the Invisorb Spin Plant Mini Kit (Stratec, Berlin, Germany) according to the manufacturer's instructions. The extracted DNA of three biological replicates was pooled. For each sampling time point per site, four replicates (pooled DNA) were used for sequencing. For seven samples, no PCR product could be amplified, resulting in reduced replicate number (**Table 2**).

Amplicon sequencing was done using the primer combination 335F (CADACTCCTACGGGAGGC)/769R (ATCCTGTTTGMTMCCCVCRC) (Dorn-In et al., 2015) to amplify the V3–V4 region of the 16S rRNA gene. Amplicon library preparation and bioinformatics analysis were described in detail in Mahnkopp-Dirks et al. (2021). Briefly, PCR was performed using 2x Phusion High-Fidelity Master Mix (Thermo Fisher Scientific, Waltham, MA, United States), 10 pmol of each primer, and 5 ng of DNA template in a final volume of 10 µl with PCR conditions: 98°C for 10 s, 30 cycles of 98°C for 1 s–59°C

TABLE 1 | Experimental setting and sampling time points at the two sites "Heidgraben" and "Ellerhoop" (1 = sampling in July, 2 = sampling in November, and 3 = sampling in April); $n = 4$ plots with 3 plants each.

Year	Plot	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Heid-graben	ARD	1. apple gen.	2. apple gen.		3. apple gen.		4. apple gen.		5. apple gen. 1 2 3		6. apple gen.
	Con-trol	Grass							1 2 3 1. apple gen.		
Eller-hoop	ARD	1. apple gen.		2. apple gen.		3. apple gen.		4. apple gen.		5. apple gen. 1 2 3	
	Con-trol	Grass								1 2 3 1. apple gen.	

TABLE 2 | Richness and diversity of endophytic bacterial communities based on amplicon sequence variants (ASVs) in roots from Bittenfelder plants grown in apple replant disease (ARD) plots or grass plots at the sites Heidgraben and Ellerhoop.

Site	Soil	*n*	Observed ASVs	Chao1	Shannon	Simpson
Ellerhoop	T0	3	244 ± 26	253 ± 30	4.6 ± 0.12	0.98 ± 0.00
Heidgraben (Summer 16)	ARD	3	260 ± 39	266 ± 41	5.0 ± 0.19	**0.99 ± 0.00**
	Grass	4	210 ± 78	222 ± 85	4.19 ± 0.47	**0.96 ± 0.02***
Heidgraben (Autumn 16)	ARD	4	291 ± 96	301 ± 104	4.82 ± 0.43	0.98 ± 0.01
	Grass	4	240 ± 95	248 ± 100	4.48 ± 0.59	0.97 ± 0.02
Heidgraben (Spring 17)	ARD	4	251 ± 41	264 ± 39	4.85 ± 0.31	0.99 ± 0.01
	Grass	4	236 ± 21	243 ± 19	4.76 ± 0.24	0.98 ± 0.01
Ellerhoop (Summer 17)	ARD	4	236 ± 72	245 ± 77	4.42 ± 0.61	0.97 ± 0.02
	Grass	3	166 ± 43	175 ± 43	3.89 ± 0.49	0.96 ± 0.02
Ellerhoop (Autumn 17)	ARD	2	160 ± 21	162 ± 22	4.05 ± 0.01[a]	0.96 ± 0.01
	Grass	3	171 ± 41	176 ± 37	4.04 ± 0.96	0.94 ± 0.06
Ellerhoop (Spring 18)	ARD	3	253 ± 77	263 ± 83	4.72 ± 0.17[b]	0.98 ± 0.00
	Grass	4	159 ± 28	161 ± 29	4.06 ± 0.73	0.92 ± 0.10

Additionally, T0 plants before planting at Ellerhoop are shown. Different letters indicate significant differences within the sites between the sampling times (Tukey's test at $p \leq 0.05$). No letters indicate no significant differences. Significant differences between ARD and grass are shown in bold (t-test at $^*p \leq 0.05$). Given are mean ± standard deviation of n replicates.

for 5 s–72°C for 45 s, 72°C for 1 min. After purification with Agencourt AMPure XP kit (Beckman Coulter, United States) indexing PCR (98°C for 30 s, 8 cycles of 98°C for 10 s–55°C for 30 s–72°C for 30 s, 72°C for 10 min) was performed using Nextera XT Index Kit v2 (Illumina, United States). Purified samples were equimolarly pooled to 4 nM and sequenced on Illumina Miseq platform. FASTQ files were trimmed using AdapterRemoval (Schubert et al., 2016) and analyzed using the QIIME 2 software package release 2017.11 (Caporaso et al., 2010) with default parameters. Quality control was performed *via* QIIME 2 plugin DADA2 (Callahan et al., 2016), with removal of 10 bp n-terminally, length truncation at position 300 (forward) and 260 (reverse), and an expected error of 2. Taxonomic assignment of the resulting ASVs was performed using primer-specific pre-trained Naive Bayes classifiers of the SILVA_132_QIIME release 99% and the q2-feature-classifier plugin. Raw sequence data were deposited in GenBank[1] under the BioProject accession number PRJNA795995.

PCR-negative control showed no ASVs; thus, contamination during sample processing could be excluded. For further data analysis, unassigned reads, singletons, plastid sequences, and sequences assigned to archaea and eukaryotes were removed (in sum 37% of all reads), resulting in total in 4,694 different ASVs, of which 4,422 (94.2%) were covered after rarefying at 4,213 reads (**Supplementary Figure 2**). The relative abundance was calculated by dividing the number of reads per ASV in the samples by the sum of total reads per sample and finally multiplied by 100. To calculate the overall relative abundance of the corresponding phylum/genus, ASVs belonging to the same phylum/genus were merged.

Determination of species diversity (Shannon and Simpson) and richness (Chao1) indices of the amplicon data was done using the "Phyloseq" (McMurdie and Holmes, 2013) and "Vegan" (Oksanen et al., 2019) packages of R v3.6.1 (R Development Core Team, 2019).[2] Normal distribution based on Shapiro-Wilk test (Shapiro and Wilk, 1965) and homogeneity of variance based on Levene's test (Levene et al., 1960) were tested using the program PAST3 v. 3.20 (Hammer et al., 2001). If the null hypotheses of normal distribution and equal variances were rejected, the Tukey test based on Herberich et al. (2010) was used at $p < 0.05$ to determine significant differences of the diversity and richness scores. In order to compare the relative abundance of different phyla in different seasons and ARD variants to grass variants, a DESeq2 analysis using generalized linear models and pairwise comparisons ($p < 0.05$) was performed [DESeq2, Love et al. (2014)]. Non-metric multidimensional scaling (NMDS) was performed with the program PAST3 v. 3.20 (Hammer et al., 2001) using the Bray–Curtis similarity index and analysis of similarity (ANOSIM) in order to visualize the community composition of the different samples. Vectors showing the correlation between the corresponding genus and the NMDS score were added to indicate the influence of the corresponding genera.

Isolation of Bacterial Root Endophytes

In order to isolate bacterial endophytes, four random 1-cm pieces of surface disinfected fine roots (Ø < 2 mm) of each plant were placed per Petri dish containing 523 (Viss et al., 1991) and R2A medium (Reasoner and Geldreich, 1985). For each plant, three Petri dishes per medium were prepared as replicates. After

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

[2]http://www.R-project.org

approximately 7 days at room temperature, colonies were picked based on different morphology and purified by dilution plating using the same media.

To avoid slow-growing colonies to be overgrown by fast-growing bacteria, additionally 100 mg of surface-disinfected roots were cut into small pieces and transferred into a 50-ml centrifuge tube containing 10 ml of saline (0.85% NaCl). Samples were shaken at 150 rpm and 4°C for 22 h. One hundred microliters of the solution as well as dilutions up to $1:10^5$ were plated onto three Petri dishes containing 523 medium and R2A medium, respectively, and evenly distributed. After 7–28 days, colonies were picked and streaked out. Selection of different colonies was based on different appearance and morphology with the aim to obtain a broad spectrum of different bacterial root endophytes.

Single colonies were transferred to liquid medium 523 and incubated for 1–7 days at room temperature on a shaker at 150 rpm until growth was visible. One milliliter of this suspension was used for DNA extraction based on the protocol of Quambusch et al. (2014).

Partial sequences of the 16S rRNA gene of 140 isolates were obtained using the primers 27f (AGAGTTTGATCCT GGCTCAG) and 1492r (GGYTACCTTGTTACGACTT) (Weisburg et al., 1991). Each PCR reaction (25 µl) contained 10 ng of DNA, 1 × Williams Buffer (100 mM Tris–HCl, pH 8.3 at 25°C; 500 mM KCl; 20 mM $MgCl_2$; 0.01% gelatin), 200 µM dNTPs, 10 pmol of each primer, and 1 U Biotaq DNA polymerase (Bioline, London, United Kingdom). The thermal cycler protocol started with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing of the primers at 52°C for 40 s, and elongation at 72°C for 60 s, and ended with a final elongation at 72°C for 5 min.

Fragments were separated *via* gel electrophoresis [1 × Tris–acetate-EDTA (TAE) buffer, Aaij and Borst, 1972; Hayward and Smith, 1972] and the PCR products of about 1,500 bp were excised from 1% agarose gels and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey and Nagel, Düren, Germany). The 16S rRNA gene fragments were sequenced with the Sanger method (Sanger et al., 1977) by Microsynth Seqlab (Göttingen, Germany) using the same primers as described above.

For phylogenetic analysis, an alignment of the nucleotide sequences of 150 isolates was done using BioEdit (version 7.2.5, Hall, 1999). Therefore, all sequences were cut at 1,320 bp before ClustalW multiple alignment (Thompson et al., 1994) was done with the number of bootstraps set to 1,000, resulting in a total of 62 different sequences that were deposited in GenBank (see footnote text 1) under the accession MW580614:MW580673[accn]. This alignment was used for phylogenetic tree construction with the program MEGA X (Kumar et al., 2018) using the Maximum Likelihood method and Tamura–Nei model (Tamura and Nei, 1993). Identities and origins of the different isolates can be seen in **Supplementary Table 1**.

To link the culture-independent approach and the culture-dependent approach, a local Blastn of the 16S rRNA sequences of the isolates against all ASVs obtained from amplicon sequencing was done using BioEdit (version 7.2.5, Hall, 1999).

TABLE 3 | Fresh weight of roots after 12 months of growth in ARD or grass plots at Heidgraben and Ellerhoop.

	ARD	Grass
Heidgraben	36.88 ± 15.65	52.61 ± 25.34
Ellerhoop	21.64 ± 10.91[a]	78.21 ± 33.49[b]

Different letters indicate significant differences between ARD and grass variants within a site (Welch Two Sample t-test $p \leq 0.05$, $n = 12$).

RESULTS

After 12 months of growing in ARD or grass plots, roots of apple plantlets showed clear differences (**Table 3** and **Supplementary Figure 1**). Roots of plants from Ellerhoop growing in ARD soil had significantly lower mass (21.64 ± 10.91 g) compared to roots from those in the grass soil (78.21 ± 33.49 g). At Heidgraben, roots from ARD soil had a lower mass (36.88 ± 15.65) than roots grown in grass soil (52.61 ± 25.34 g), but the differences were not significant due to high plant-to-plant variation.

Molecular Barcoding of Bacterial Root Endophytes

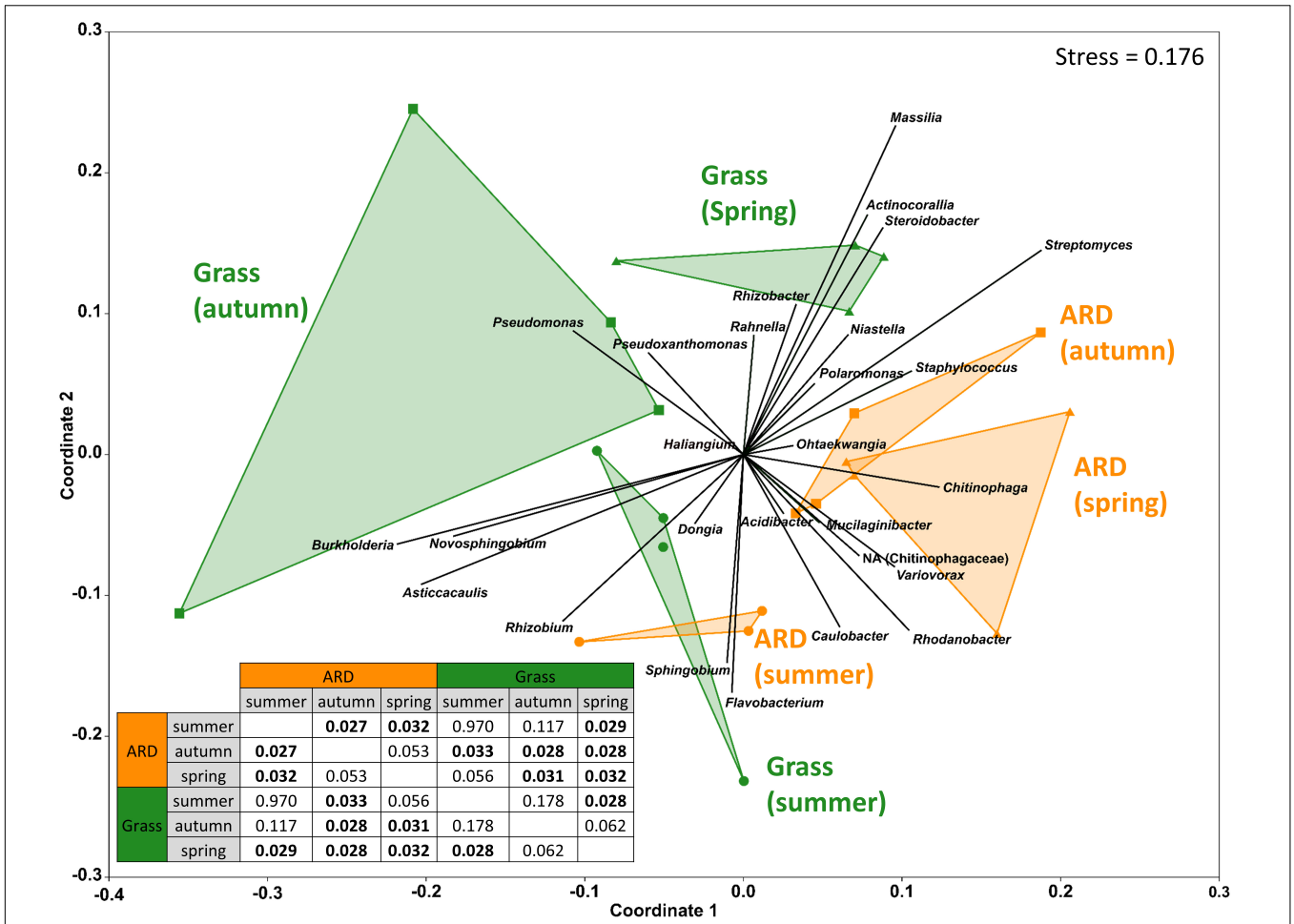
In order to compare the bacterial diversity of endophytes of roots growing in ARD-affected and non-affected soils, a metabarcoding approach using extracted DNA from the roots after surface disinfection and 16S rRNA gene amplification was performed.

Overall, the richness (observed ASVs, Chao1) of bacterial endophytes was higher in roots grown in soil at Heidgraben compared to Ellerhoop. Seasonal fluctuations at both sites differed: Whereas the highest numbers of observed ASVs were found in autumn (2017) at Heidgraben, at Ellerhoop, at the same time point of the experiment (autumn 2018), the numbers of observed ASVs at Ellerhoop were the lowest (**Table 2**). In five out of six variants (site + season), richness of bacterial ASVs in roots grown in ARD soil was higher compared to roots from plants grown in grass soil, but the differences did not reach the level of significance due to high variations between replicate samples. Evenness (Simpson index) of root endophytes was not affected by the sampling site or by season and treatment.

The large plant-to-plant variation is also depicted in the results of β diversity analyses. At both sites, roots from grass plots clustered separately from those grown in ARD plots, except for the first sampling 3 months after planting (summer, **Figure 1** and **Supplementary Figure 3**). For the separation of samples from grass and ARD plots, several bacterial genera had an effect, such as *Pseudomonas, Burkholderia, Chitinophaga*, or *Streptomyces* at Heidgraben (**Figure 1**) and *Pseudomonas*, *Halomonas*, *Streptomyces*, or *Sphingobium* at Ellerhoop (**Supplementary Figure 3**). For samples from Ellerhoop, endophytic bacterial communities of the planting material (T0) were clearly different from those of plants grown for 3–12 months in the different field plots (**Supplementary Figure 3**).

Proteobacteria

Proteobacteria were the dominant bacterial phylum detected as root endophytes with a mean relative abundance of 77.3% in



		ARD			Grass		
		summer	autumn	spring	summer	autumn	spring
ARD	summer		**0.027**	**0.032**	0.970	0.117	**0.029**
	autumn	**0.027**		0.053	**0.033**	**0.028**	**0.028**
	spring	**0.032**	0.053		0.056	**0.031**	**0.032**
Grass	summer	0.970	**0.033**	0.056		0.178	**0.028**
	autumn	0.117	**0.028**	**0.031**	0.178		0.062
	spring	**0.029**	**0.028**	**0.032**	**0.028**	0.062	

FIGURE 1 | Three-dimensional non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarity of roots taken in summer and autumn after planting or the following spring at Heidgraben. Vectors represent the correlation coefficient between the corresponding genus and the NMDS score. Relative lengths and the directions of the vectors indicate the influence of the respective genera (RA > 0.5%). The third axis is not shown. Results of the one-way analysis of similarities are shown in the lower left corner; significant differences are highlighted in bold ($p \leq 0.05$).

all samples (**Figure 2**). Significant differences in the relative abundance of Proteobacteria between roots from plants grown in ARD and control soil were only detected for Ellerhoop (autumn) with a reduced relative abundance in roots grown in ARD-affected soil. The most abundant proteobacterial genus was *Pseudomonas* (mean of relative abundance over all samples 20.1%).

At Ellerhoop, *Pseudomonas* showed a different development in roots grown in grass soil compared to ARD soil over time. In grass soil, *Pseudomonas* was constantly the dominating genus at all sampling times (sum of RA of all *Pseudomonas* ASVs in summer = 35.6%, autumn = 34%, spring = 31.1%), whereas in ARD soil, the relative abundance decreased over time (sum of RA in summer = 20%, autumn = 1.4%, spring = 2.3%; **Figure 3**).

At Heidgraben, the overall relative abundance of *Pseudomonas* showed a similar pattern between roots grown in ARD and grass soil. In both soils, roots had the highest relative abundance of *Pseudomonas* in autumn (ARD = 20%, Grass = 23.5%) and decreased over time to the lowest abundance in spring (ARD = 8.6%, Grass = 11%).

Amplicon sequence variants linked to *Pseudomonas* were not only most abundant in the analyzed root samples, but also highly diverse. Overall, 34 ASVs could be detected (based on RA > 0.5), with a slightly higher number of differing ASVs obtained from Ellerhoop compared to Heidgraben (26 and 23 for Ellerhoop and Heidgraben, respectively). Most of these ASVs were classified as *P. corrugata* and *P. turukhanskensis* (14.7% each). While the number of ASVs linked to *Pseudomonas* at both sites in roots grown in grass soil slightly decreased after summer (Heidgraben summer = 22, autumn = 18, spring = 19; Ellerhoop summer = 26, autumn = 16, spring = 18), the number continuously decreased even more in roots grown in ARD sites at Heidgraben (summer = 20, autumn = 16, spring = 14) and especially after summer at Ellerhoop (Ellerhoop summer = 22, autumn = 6, spring = 8).

Twenty-seven ASVs linked to *Pseudomonas* were present in roots from T0 plants. Most of them disappeared over time in roots grown in ARD soil. After 1 year (spring), only 7 out of 27 ASVs were still present in roots grown in ARD soil. However, in roots grown in grass soil, 16 ASVs were still detected, most of them being highly abundant.

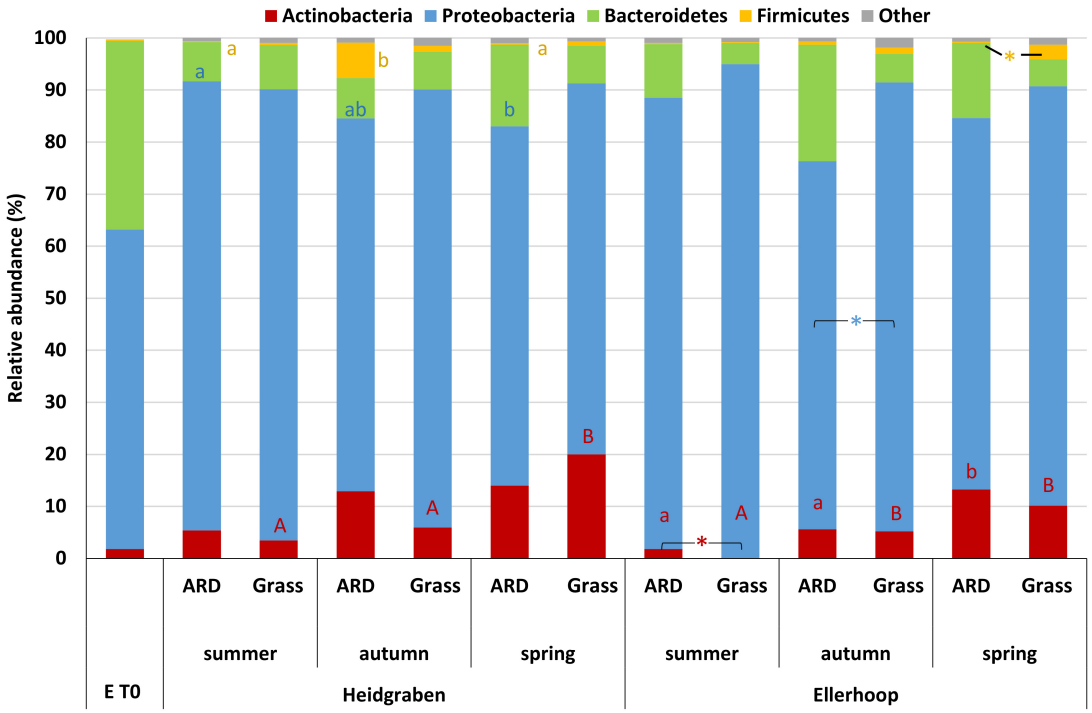


FIGURE 2 | Relative abundance of dominant phyla in roots of Bittenfelder plants grown in apple replant disease (ARD) plots or grass plots at Heidgraben and Ellerhoop taken in summer and autumn after planting or the following spring. Different letters indicate statistically significant differences within one site in ARD plots (lower case) or grass plots (upper case) between the seasons (DESeq2 analysis using a generalized linear model and multiple comparisons with $p \leq 0.05$). Significant differences between ARD and grass within one season are indicated by an asterisk (DESeq2 analysis using a generalized linear model and pairwise comparisons with $p \leq 0.05$). Different colored letters belong to the respective phyla. *N* numbers are shown in **Table 2**.

Interestingly, 15 ASVs related to *Pseudomonas* were present at both sites at Ellerhoop and Heidgraben. Whereas these ASVs showed different development at ARD plots at both sites in response to sampling time and treatment, 7 ASVs linked to *Pseudomonas* showed a similar development at grass plots and were mainly decreasing in abundance over time (**Figure 3**, ASV8873, ASV8914, ASV8938, ASV8953, ASV8994, and ASV9009).

The second most abundant proteobacterial genus was *Rhizobium* (mean of relative abundance over all samples 4.5%). While, in both sites, the overall relative abundance of *Rhizobium* showed the same pattern over time in roots grown in ARD soil (Heidgraben sum of RA in summer = 4.3%, autumn = 2.7%, spring = 4.3%; Ellerhoop in summer = 2.9%, autumn = 1.4%, spring = 2%), a different pattern was observed in roots grown in grass soil. At Heidgraben, the overall relative abundance decreased (sum of RA in summer = 15.3%, autumn = 5.7%, spring = 1%), while it increased at Ellerhoop over time (sum of RA in summer = 0.3%, autumn = 1.9%, spring = 2.3%). In total, seven different ASVs linked to *Rhizobium* were found in roots from Heidgraben and 5 ASVs were found in roots from Ellerhoop (which were all shared between both sites). From a total of 7 ASVs, three were classified as *R. etli* and three as *R. alamii*. No clear patterns in number of ASVs over time were observed.

Amplicon sequence variants linked to the proteobacterial genus *Rhanella* were only found at Ellerhoop in autumn but with high abundance in roots grown in both soils (ARD = 20%; grass = 19.9%).

Bacteroidetes

The second most abundant phylum was Bacteroidetes (average abundance 12.8% across all samples). Here, no significant differences in overall relative abundance between plants grown in control (grass) soils and ARD-affected soils were detected. The most abundant genera in the group of Bacteroidetes were *Flavobacterium* (2.99%), *Mucilaginibacter* (1.3%), and *Chitinophaga* (1.16%, average over all samples). However, *Flavobacterium* was only present at Ellerhoop. Interestingly, 13 ASV linked to *Flavobacterium* were present in T0 plants, which was clearly the dominating genus with 23.9%. However, the number of ASVs linked to *Flavobacterium* decreased after planting in ARD soil (summer = 5, autumn = 0, spring = 1), and after initially decreasing over time, it slightly increased again in grass soil (summer = 8, autumn = 1, spring = 6).

In total, 7 ASVs linked to *Chitinophaga* were found (5 at Ellerhoop and 2 at Heidgraben). Only 1 ASV was found in both sites and classified as *C. ginsengisoli*. Except for ASV859 (classified as *C. oryziterraeno*), ASVs linked to *Chitinophaga* were not present in roots grown in grass soil. At Ellerhoop, the overall relative abundance of *Chitinophaga* peaked in autumn (sum of RA in summer = 0.66%, autumn = 12.01%, spring = 0.02%).

Actinobacteria

Actinobacteria had a mean relative abundance of 7.5% in average, with higher abundance observed for Heidgraben (**Figure 2**).

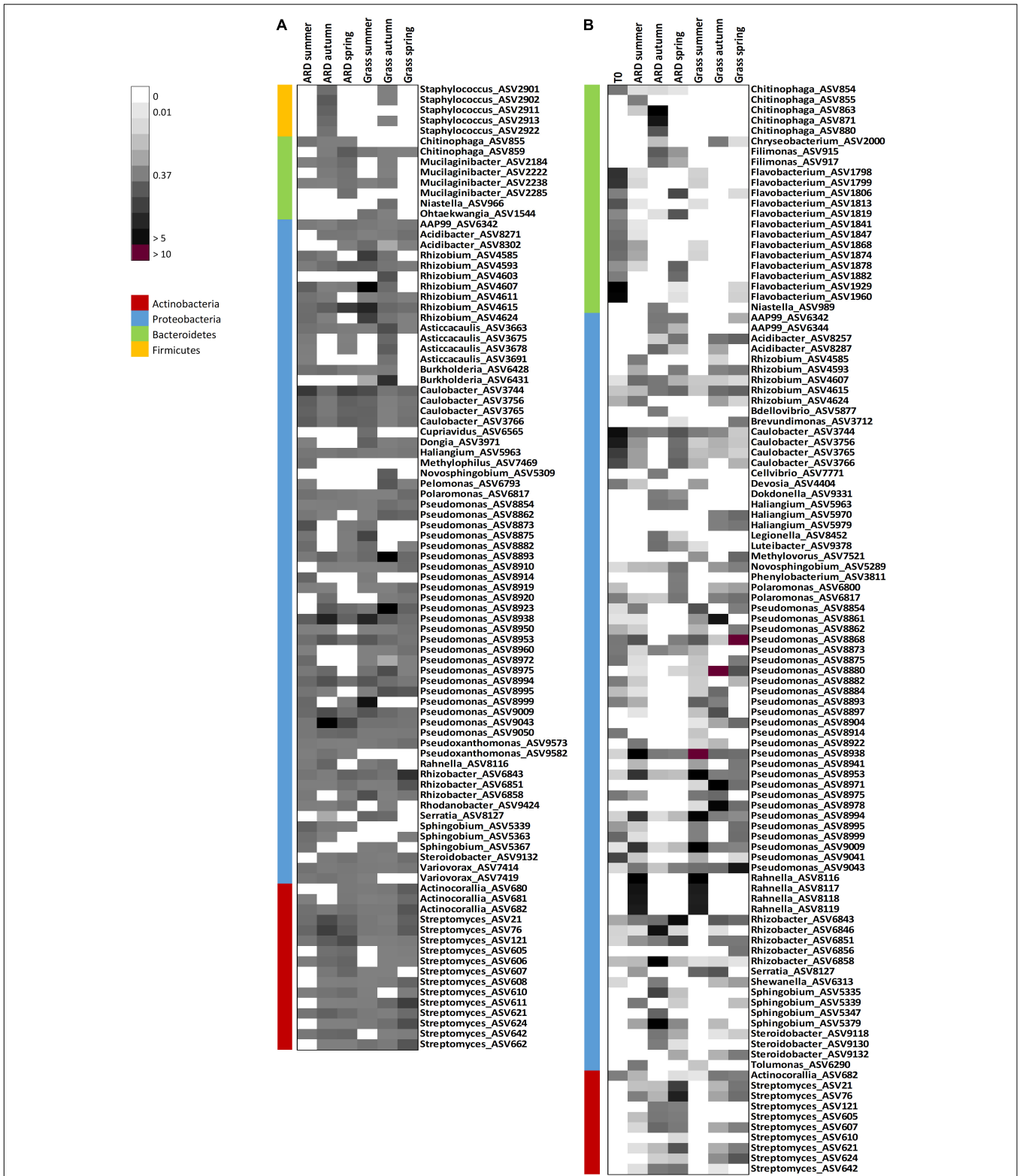
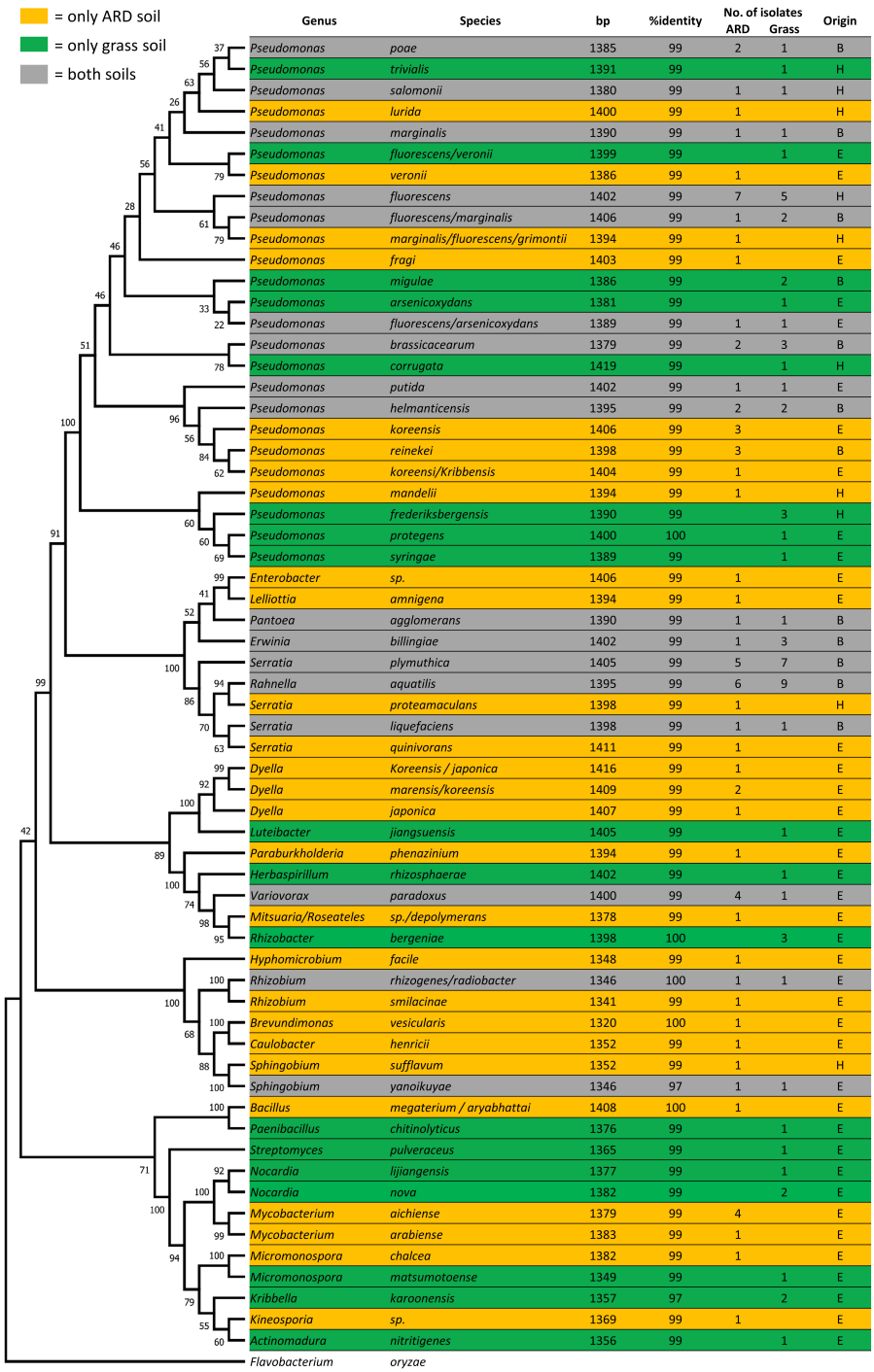


FIGURE 3 | Heatmap showing the abundance of different amplicon sequence variants (ASVs) (RA > 0.5%) in roots of Bittenfelder plants from ARD and grass plots taken 3 months (summer), 7 months (autumn), and 12 months (spring) after planting at Heidgraben **(A)** in 2016/17 and Ellerhoop **(B)** in 2017/18. For each sampling time per site and soil, only ASVs with an abundance greater than 0.5% were selected, and their relative abundance was compared with all other variants. The color code indicates the range from low relative abundance (light gray, 0.01%), medium abundance [gray, 0.37% (median)], to high abundance (black > 5%, purple > 10%). Different colors on the left side indicate the corresponding phylum of the ASVs.



Genus	Species	bp	%identity	No. of isolates ARD	No. of isolates Grass	Origin
Pseudomonas	*poae*	1385	99	2	1	B
Pseudomonas	*trivialis*	1391	99		1	H
Pseudomonas	*salomonii*	1380	99	1	1	H
Pseudomonas	*lurida*	1400	99	1		H
Pseudomonas	*marginalis*	1390	99	1	1	B
Pseudomonas	*fluorescens/veronii*	1399	99		1	E
Pseudomonas	*veronii*	1386	99	1		E
Pseudomonas	*fluorescens*	1402	99	7	5	H
Pseudomonas	*fluorescens/marginalis*	1406	99	1	2	B
Pseudomonas	*marginalis/fluorescens/grimontii*	1394	99	1		H
Pseudomonas	*fragi*	1403	99	1		E
Pseudomonas	*migulae*	1386	99		2	B
Pseudomonas	*arsenicoxydans*	1381	99		1	E
Pseudomonas	*fluorescens/arsenicoxydans*	1389	99	1	1	E
Pseudomonas	*brassicacearum*	1379	99	2	3	B
Pseudomonas	*corrugata*	1419	99		1	H
Pseudomonas	*putida*	1402	99	1	1	E
Pseudomonas	*helmanticensis*	1395	99	2	2	B
Pseudomonas	*koreensis*	1406	99	3		E
Pseudomonas	*reinekei*	1398	99	3		B
Pseudomonas	*koreensi/Kribbensis*	1404	99	1		E
Pseudomonas	*mandelii*	1394	99	1		H
Pseudomonas	*frederiksbergensis*	1390	99		3	H
Pseudomonas	*protegens*	1400	100		1	E
Pseudomonas	*syringae*	1389	99		1	E
Enterobacter	*sp.*	1406	99	1		E
Lelliottia	*amnigena*	1394	99	1		E
Pantoea	*agglomerans*	1390	99	1	1	B
Erwinia	*billingiae*	1402	99	1	3	B
Serratia	*plymuthica*	1405	99	5	7	B
Rahnella	*aquatilis*	1395	99	6	9	B
Serratia	*proteamaculans*	1398	99	1		H
Serratia	*liquefaciens*	1398	99	1	1	B
Serratia	*quinivorans*	1411	99	1		E
Dyella	*Koreensis / japonica*	1416	99	1		E
Dyella	*marensis/koreensis*	1409	99	2		E
Dyella	*japonica*	1407	99	1		E
Luteibacter	*jiangsuensis*	1405	99		1	E
Paraburkholderia	*phenazinium*	1394	99	1		E
Herbaspirillum	*rhizosphaerae*	1402	99		1	E
Variovorax	*paradoxus*	1400	99	4	1	E
Mitsuaria/Roseateles	*sp./depolymerans*	1378	99	1		E
Rhizobacter	*bergeniae*	1398	100		3	E
Hyphomicrobium	*facile*	1348	99	1		E
Rhizobium	*rhizogenes/radiobacter*	1346	100	1	1	E
Rhizobium	*smilacinae*	1341	99	1		E
Brevundimonas	*vesicularis*	1320	100	1		E
Caulobacter	*henricii*	1352	99	1		E
Sphingobium	*sufflavum*	1352	99	1		H
Sphingobium	*yanoikuyae*	1346	97	1	1	E
Bacillus	*megaterium / aryabhattai*	1408	100	1		E
Paenibacillus	*chitinolyticus*	1376	99		1	E
Streptomyces	*pulveraceus*	1365	99		1	E
Nocardia	*lijiangensis*	1377	99		1	E
Nocardia	*nova*	1382	99		2	E
Mycobacterium	*aichiense*	1379	99	4		E
Mycobacterium	*arabiense*	1383	99	1		E
Micromonospora	*chalcea*	1382	99	1		E
Micromonospora	*matsumotoense*	1349	99		1	E
Kribbella	*karoonensis*	1357	97		2	E
Kineosporia	*sp.*	1369	99	1		E
Actinomadura	*nitritigenes*	1356	99		1	E
Flavobacterium	*oryzae*					

FIGURE 4 | Phylogenetic tree based on 16S rRNA gene sequences of all different endophytic isolates using the Maximum Likelihood method and Tamura–Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test [1,000 replicates (Felsenstein, 1985)] are shown next to the branches. The closest hit with species level and corresponding identity using the NCBI database is shown. Only isolates with more than 1,300 bp were selected for alignment. *Flavobacterium oryzae* was used as an outgroup to root the tree. Isolates were obtained from roots grown only in ARD plots (yellow), grass plots (green), or both plots (gray) from the sites Heidgraben (H), Ellerhoop (E), or both (B) sites.

Interestingly, the relative abundance for this phylum increased over time in ARD and grass plots at both sites. Significant differences between ARD-affected and grass plots were observed for Actinobacteria when root samples from Ellerhoop (summer) were compared, with higher numbers in roots from plants obtained from ARD-affected plots.

The most abundant actinobacterial genus was *Streptomyces*. Their relative abundance increased over time in roots grown in ARD-affected soil at Heidgraben (sum of RA in summer = 3.36%, autumn = 10.93%, spring = 10.38%) and Ellerhoop (sum of RA in summer = 0.84%, autumn = 3.81%, spring = 11.65%), making *Streptomyces* the most abundant genus in these variants. An increase in relative abundance over time was also observed in roots grown in grass soil at Heidgraben (sum of RA in summer = 1.93%, autumn = 2.78%, spring = 11.65%) and Ellerhoop (sum of RA in summer = 0.00%, autumn = 1.20%, spring = 4.65%). A comparable pattern could be found for the diversity. In total, 13 different ASVs linked to *Streptomyces* were observed from which nine were shared between the sites. At both sites, the number of ASVs increased over time in roots grown in ARD-affected soil (Heidgraben: summer = 7, autumn = 13, spring = 13; Ellerhoop: summer = 6, autumn = 8, spring = 9) as well as in grass soil (Heidgraben: summer = 10, autumn = 12, spring = 12; Ellerhoop: summer = 0, autumn = 6, spring = 5). At both sites, most ASVs linked to *Streptomyces* increased in their relative abundance over time in roots grown in ARD-affected soil but especially in grass soil. Three out of 13 ASVs were classified as *S. camponoti*. No ASVs linked to *Streptomyces* were found in T0 plants.

Firmicutes

Firmicutes was a phylum with a low relative abundance (1.3%) and only found in roots from Heidgraben. This phylum was represented by five different ASVs linked to the genus *Staphylococcus*. In roots grown in ARD soil and grass soil, these ASVs were only present in autumn (ARD = 5 ASVs, Grass = 3 ASVs). Their relative abundance was higher in ARD (sum of RA = 4.9%) compared to grass soil (0.11%).

Culture-Dependent Approach

Next to molecular barcoding, a culture-dependent approach was performed in order to obtain a wide range of different endophytic bacterial isolates. In total, 150 isolates were obtained from both sites and sampling times (**Figure 4**), belonging to 69 different bacterial species and 29 genera. Thirty-one species were only found in roots grown in ARD soil, 19 only in grass soil, and 19 in both soils. Most species (25 out of 69) were classified as *Pseudomonas*, confirming the molecular data; 62.2% of all isolates obtained from Heidgraben and 31.9% from Ellerhoop were classified as *Pseudomonas*. At both sites, their distribution showed only slight differences between the percentage of isolates obtained from ARD or grass plots (Heidgraben: ARD = 59.1%, grass = 65.2%; Ellerhoop: ARD = 33.3%, grass = 30.2%).

Only one isolate classified as *Streptomyces* was found. Species that were isolated most frequently were *Rhanella aquatilis* (15), *Pseudomonas fluorescens* (12), and *Serratia plymuthica* (12).

To link the isolates obtained from the culture-dependent approach to the ASVs of the culture-independent approach, their 16S rRNA gene sequences were blasted against the sequences obtained from amplicon sequencing using a local Blastn. Nearly all isolates showed a very high similarity to one or more of the ASVs (**Supplementary Table 2**). However, only 20 Isolates out of 62 (>1,300 bp) showed a 100% identity to ASVs. The isolate *Kribella karoonensis* showed with 89.3% [to NA_ASV568 (Nocardioidaceae)] the lowest identity to the amplicon data followed by *Actinomadura nitritigenes* with 93.2% to NA_ASV677 (Non-omuraea). The isolates that were obtained frequently, e.g., *Pseudomonas fluorescens* (12x) or *Rhanella aquatilis* (15x), were only found in low abundance in the amplicon data. The five most abundant ASVs linked to *Pseudomonas* were classified as *P. brassicacearum* (ASV8938 and ASV9009), *P. corrugata* (ASV8893 and ASV8880), and *P. frederiksbergensis* (ASV9043), which were also identified in the culture-dependent approach (**Supplementary Table 2**).

DISCUSSION

Community Structure and Relative Abundance Over Time

In most studies, in which rhizosphere or bulk soils of ARD-affected sites were analyzed (Sun et al., 2014; Franke-Whittle et al., 2015; Yim et al., 2015; Peruzzi et al., 2017; Tilston et al., 2018), Proteobacteria were the dominant phylum with a mean relative abundance of 35% (Nicola et al., 2018). However, a clearly higher relative abundance of Proteobacteria was observed, when the root endobiome was investigated in greenhouse-grown plants in the frame of an ARD biotest (Mahnkopp-Dirks et al., 2021) as root endophytes. These results were confirmed in the present study for roots grown in the field at Heidgraben and Ellerhoop. At both sites, Proteobacteria showed in roots grown in ARD plots an average relative abundance of 76%, respectively (**Figure 2**). However, in roots grown in grass soil, the relative abundance was even higher with an average of 84%. Due to their high metabolic activity and fast growth, members of this phylum are known to usually dominate the endosphere (Lundberg et al., 2012; Reinhold-Hurek et al., 2015).

During the first year of growth after planting, the community structure changed over time. This was also observed by Rumberger et al. (2007) for the bacterial rhizosphere community of apple trees grown in ARD-affected sites using terminal restriction fragment length polymorphism (T-RFLP) analyses. In the present study, the relative abundances of Actinobacteria increased over time in roots grown in ARD and grass plots at both sites (**Figure 2**). At the Heidgraben site, 12 months after planting (spring), the relative abundance was even higher in roots grown in grass plots than in ARD plots. Microscopic analysis revealed Actinobacteria to be more often found in roots grown in ARD-affected soil than in unaffected soil (Grunewaldt-Stöcker et al., 2019). Also, in-depth investigations of typical symptomatic root segments revealed a high frequency of Actinobacteria on the root surface and in the cortex (Grunewaldt-Stöcker et al., 2021). Actinobacteria were also observed in higher abundance in the previous greenhouse biotest in roots grown in untreated ARD soil in comparison to controls (Mahnkopp-Dirks et al., 2021). An increase of Actinomycetes (Actinobacteria) in the rhizosphere was also observed by Čatská et al. (1982) with increasing age of apple trees grown in ARD-affected soils.

Čatská et al. (1982) also reported a decline in "fluorescent pseudomonads" in apple trees within 30 months after planting in ARD-affected soil but not in control soil. A reduction of *Pseudomonas* in the rhizosphere over years after replanting confirmed by others (Rumberger et al., 2007; Jiang et al., 2017) was also observed for the endophytic root microbiome in the present study, especially at Ellerhoop in roots grown in ARD soil. Here, the total abundance of *Pseudomonas* ASVs was reduced 10-fold after summer but stayed nearly on the same level in roots grown in grass soil (**Figure 3**). This also points to the link between the rhizosphere and endosphere community since the main way of entering the root interior is through natural cracks during lateral root emergence and root tips (Hardoim et al., 2008; Bulgarelli et al., 2013). Members of *Pseudomonas* are known for their good rhizosphere competence and fast growth (Haas and Keel, 2003; Santoyo et al., 2012), and numerous strains are reported as plant growth promoting, e.g., by producing iron-chelating siderophores (Santoyo et al., 2012; David et al., 2018). These siderophores can prevent potential phytopathogens from acquiring (enough) soluble iron, thus inhibiting their growth and proliferation (Kloepper et al., 1980; Loper and Henkels, 1999; David et al., 2018). Furthermore, this trait of *Pseudomonas* was associated with disease-suppressive soils, among others (Kloepper et al., 1980). Moreover, several members, which are also associated with plants, are able to produce antibiotics (Rosales et al., 1995; Raaijmakers et al., 1997; Haas and Keel, 2003; Paulsen et al., 2005). Mazzola and Gu (2000) could show that a suppression of potential ARD causing pathogenic fungi was attributed to a transformation in composition of the fluorescent pseudomonad community in the apple rhizosphere with an increase in proportion of *Pseudomonas putida* in the population and a decrease in recovery of *P. syringae* and *P. fluorescens*. Therefore, it is possible that a decrease of the abundance of members of plant growth-promoting *Pseudomonas* could play a role in the establishment of ARD.

Overall, we could not verify a lower diversity of bacterial root endophytes in plantlets grown in ARD-affected soils. However, the dominance of single bacterial ASVs (e.g., from the group of *Streptomyces*) may lead to an outcompetition of other bacterial species with plant growth-promoting properties in the subsequent years, which may have a strong effect on plant performance.

Twenty months after planting, the apple rootstocks were uprooted, because both sites belong to a central experimental area on which ARD was induced and is now maintained by biannual replanting. Also in tree nurseries, rootstocks are cultivated only for one or two vegetation periods before being used for grafting. Thus, an observation of the bacterial endobiome over a longer time, which might have revealed even more pronounced changes, was not possible in this study.

Apple Roots Grown in Field Soils Seem to Attract *Streptomyces*

The majority of Actinobacteria reads belonged to the genus *Streptomyces*, which had previously been suggested to play a role in ARD: In our greenhouse biotest (Mahnkopp-Dirks et al., 2021), we could show that the relative abundance of several unique ASVs linked to *Streptomyces* in roots from ARD soil from three different sites (including Heidgraben and Ellerhoop) was negatively correlated to shoot fresh mass and shoot length. Of these unique ASVs, 6 (Streptomyces_ASV76, 607, 611, 21, 121, and 621) and 5 (Streptomyces_ASV76, 607, 21, 621, and 121) were now detected in field-grown roots of the ARD plots at Heidgraben and Ellerhoop, respectively. Even though we observed a higher ARD severity [difference between fresh mass of roots growing in ARD and grass soil (**Table 3**)] in Ellerhoop, the overall relative abundance of *Streptomyces* was similar in both sites after 1 year (Heidgraben = 10.93%, Ellerhoop = 11.65%). However, two ASVs that were the most abundant in Ellerhoop (ASV21 and ASV76) were comparatively less abundant in Heidgraben, indicating the importance of the different ASVs. One of the most abundant unique *Streptomyces* ASVs in the greenhouse biotest, ASV76, which was present in 2016 at Heidgraben and Ellerhoop and 2017 at Heidgraben, was in total also the most abundant one in roots grown in ARD soil in the field sites Heidgraben and Ellerhoop. Overall, most ASVs linked to *Streptomyces* increased over time. Especially in roots grown in grass soil at Ellerhoop, where 3 months after planting in summer none of these *Streptomyces* ASVs were present, in the following spring, *Streptomyces* represented the second most abundant genus. With increasing root biomass over time, the total amount of root exudates is also increasing. It was shown that *Streptomyces* is more abundant in the rhizosphere of *Arabidopsis thaliana* (Badri et al., 2013; Lebeis et al., 2015) and their root colonization rate increased (Chewning et al., 2019) when plant exudates were present in comparison to when they were absent. Their accumulation could also lead to the assumption of pathogenicity of *Streptomyces*. After planting, their abundance is increasing over time. Even after removing the plant and planting non-Rosaceae for several years, *Streptomyces* could remain in high amount in the soil due to their ability to form spores, which can persist for years even under harsh conditions (Bobek et al., 2017). This would correlate with ARD, which is known to persist for decades after removing apple plants (Savory, 1966). After replanting apple, these highly abundant spores could germinate, triggered by plant material/exudates and therefore be a causative part of ARD. The question whether *Streptomyces* is phytopathogenic and could be a key player in ARD is discussed in detail in Mahnkopp-Dirks (2021) and Mahnkopp-Dirks et al. (2021). However, the accumulation of *Streptomyces* ASVs over time in roots grown in grass soil, which do not cause ARD symptoms, speaks against this hypothesis. However, *Streptomyces* is known to be able to suppress the plant defense response (Lehr et al., 2007; Tarkka et al., 2008; Vurukonda et al., 2018) by reducing the peroxidase activity and pathogenesis-related peroxidase gene (Spi2) expression and promote fungal root infections, which was shown in *Picea abies* (Lehr et al., 2007). This could mean that they enable easier colonization for potential fungal ARD pathogens. It was shown by Busnena et al. (2021) and Rohr et al. (2021) that some biphenyl and dibenzofuran compounds are present even under non-ARD conditions. Already at the first

plant generation (here grass soil), *Streptomyces*, triggered and attracted by these phytoalexins, will accumulate over time. If apple is replanted, these plants face an already highly abundant *Streptomyces* population, which might reduce the plant defense response and enable easier colonization for potential fungal ARD pathogens.

In summary, in this study, we could show that the same *Streptomyces* ASVs identified previously in the biotest, which were negatively correlated to shoot length and shoot fresh mass, were also present in the field at Heidgraben and Ellerhoop during the season. Furthermore, in comparison to the biotest, Bittenfelder seedlings were used instead of the genotype M26. Thus, these ASVs linked to *Streptomyces* were associated with ARD independent of the genotype (Bittenfelder seedlings or M26), field or greenhouse, at two different sites and independent of seasons or years.

Comparison of Culture-Dependent and -Independent Approach

Functional analyses of effects of certain endophytes often rely on inoculation experiments. Therefore, we conducted an additional culture-dependent isolation approach, which resulted in a collection of 150 bacterial isolates. To compare culture-dependent and culture-independent approaches, Liu et al. (2017) summarized the proportion of different endophytic bacterial phyla in different plants based on 25 different references. They found that root endophytic bacterial communities are typically dominated by Proteobacteria (≈50% in relative abundance), Actinobacteria (≈10%), Firmicutes (≈10%), and Bacteroidetes (≈10%). By 16S amplicon sequencing of xylem tissue from different apple genotype shoots, Liu et al. (2018) found the same four dominant different phyla, despite in slightly different relative abundance [Proteobacteria (58.4%), Firmicutes (23.8%), Actinobacteria (7.7%), and Bacteroidetes (2%)]. In the present study, the culture-independent 16S amplicon sequencing also revealed a root endophytic bacterial community dominated by Proteobacteria (80%), Bacteroidetes (9.7%), Actinobacteria (8.2%), and Firmicutes (1.2%) (**Figure 2**, mean of all plots and time points). The 150 isolates obtained in the present study by the culture-dependent approach were comparably dominated by Proteobacteria (85.3%), Actinobacteria (10%), and Firmicutes (2%). However, despite the so far similar phyla abundances between the culture-independent and -dependent approach, Bacteroidetes were not isolated.

In the culture-independent approach, 4,422 ASVs were found in total. They represent different sequences with at least 1 nucleotide difference, hence do not represent species level, which is often considered at a threshold of 97% sequence identity. Since the sequences of the 150 isolates all have at least one nucleotide difference, they would represent 3.4% of the total amount of ASVs found in the independent approach {sequencing errors cannot be excluded [Taq error rate ranges from 1.1×10^{-4} errors/bp (Barnes, 1992) to 8.9×10^{-5} errors/bp (Cariello et al., 1991)]}. The ASVs were linked to 473 different known genera. In the culture-dependent approach, isolates belonging to 29 different genera were obtained, which represent 6.13%. It is thought that only 0.1–10% of the total diversity of an environment is culturable (Handelsman and Smalla, 2003). Other studies indicate that more than 99% of all microorganisms are unculturable (Schloss and Handelsman, 2005; Vartoukian et al., 2010; Pham and Kim, 2012). Based on these numbers, the proportion of culturable bacteria in this study seems to be high. However, the total amount of 4,422 ASVs did not fully represent the total bacterial endophytic root community. Several biases in amplicon sequencing have an influence on the total bacterial endophytic root community (reviewed by Pollock et al., 2018). For instance, the universal primer pair used in our study for amplicon sequencing were chosen because of minimal non-target DNA amplification like mitochondrial or chloroplast DNA (Dorn-In et al., 2015). However, despite being "universal," comparing the primer sequences to the 16S rRNA sequence collection of the Ribosomal Database Project (RDP, Cole et al., 2014) using "probe match" resulted in 1,122,475 hits out of 3,482,181 (32%) sequences in the domain Bacteria (when using 0 mismatches; 1 mismatch = 1,596,717; 2 mismatches = 1,910,059). Next to the primer used, the DNA extraction protocol has a strong influence on the bacterial community composition (Carrigg et al., 2007; Pollock et al., 2018).

Even though the two different culture media used resulted in several different cultured isolates, the number of potentially culturable bacterial endophytes will definitely increase with the use of more different media and physiological conditions. To also isolate obligate endophytes, the addition of plant extract to the medium might increase the number of different isolates (Eevers et al., 2015).

The most diverse genus in the culture-independent approach was *Pseudomonas*, with 138 ASVs linked to it. Likewise, isolates obtained from the culture-dependent approach belonging to the genus *Pseudomonas* were with 25 different species also the most diverse group. However, ASVs linked to the genus *Streptomyces* belonged to the most abundant ones, especially in roots grown in ARD soils, whereas in the culture-dependent approach, only one isolate could be obtained. One reason for this could be that the growth of *Streptomyces* was rather slow on the media used compared to other isolates, which might have outcompeted them. Another reason is that the outgrowth of isolates took place at room temperature. The optimal growth temperature for *Streptomyces* species is described as 28°C (Shepherd et al., 2010). Tewoldemedhin et al. (2011) were able to isolate 92 *Streptomyces* strains from surface-disinfected roots from six ARD-affected sites in South Africa using Casein-Starch medium and water agar supplemented with cycloheximide at 27°C for 4 weeks.

There were also some discrepancies in the abundance of some isolates compared to their corresponding ASVs (**Supplementary Table 2**). Several isolates were isolated frequently from roots, like *Rhanella aquatilis* or *Pseudomonas fluorescens*, but their corresponding ASVs were not found in high abundance in the amplicon sequencing. Reasons mentioned above like primer selection or DNA extraction methods could select against these bacteria in the culture-independent approach. Since both of these isolates were found to be fast growing on the media used, it is likely that the culture-dependent approach selected

for them. Both genera were also isolated in high abundance from apple roots and rhizosphere soil by Dos Passos et al. (2014). With *Kribella karoonensis*, there was also one isolate, whose genus was not found in the culture-independent approach. The reason for this is probably that the primer 769R does not have any coverage in this genus based on 0 mismatches in the SILVA database. Several other isolates, like *Enterobacter*, *Lelliotti*, *Erwinia*, or *Rhanella*, were not found directly in the independent approach because the corresponding ASV sequences had several hits of different genera with the same score (resulting in NA), which means that the amplified sequence might not be long enough to discriminate between these genera. Discrepancies between culture-dependent and independent approaches were also observed in the phyllosphere of apple, where Actinomycetales were found only among isolates (Yashiro et al., 2011). In the present study, the culture-dependent approach was rather used as a qualitative method rather than a quantitative one to enable upcoming inoculation experiments.

CONCLUSION

In this study, we provide evidence that the same six *Streptomyces* ASVs, which were found to be negatively correlated to shoot growth and fresh mass in a previous greenhouse biotest, were also found in high abundance in roots of a different rootstock cultivar grown in the field at two sites. Interestingly, most of these ASVs were increasing over time especially in newly planted apple plants in grass (virgin) soil leading to the assumption that the accumulation of these ASVs could play a role in ARD etiology. Furthermore, in this study, we could observe a decrease of the total abundance of *Pseudomonas* in the endophytic microbiome in roots grown in ARD soil, which may indicate that the presence of *Pseudomonas* is of importance for a balanced microbiome in healthy soils that is disturbed in ARD soils (dysbiosis). Next to the culture-independent approach, the isolation of 69 different bacterial strains showed on the one hand a comparable community structure with *Pseudomonas* being the most diverse genus. However, there is a need for further isolation efforts including different bacterial culture media and conditions in order to complement the collection of isolates, especially with regard to *Streptomyces*. On the other hand, the discrepancies between these two approaches underline the importance of combining different methods.

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/, MW580614:MW580673[accn]; https://www.ncbi.nlm.nih.gov/genbank/, PRJNA795995.

AUTHOR CONTRIBUTIONS

TW conceived and designed the experiments. FM-D performed the experiments. FM-D, SG, SK, and VR analyzed the data. TW and MS contributed the reagents, materials, and analysis tools. FM-D, SG, SK, VR, TW, and MS contributed to writing the manuscript and approved the submitted version.

FUNDING

The German Federal Ministry of Research and Education funded the project BonaRes ORDIAmur (FKZ 031B0512) within the framework of the BonaRes program.

ACKNOWLEDGMENTS

We are thankful for the help of several colleagues and students at Leibniz Universität Hannover, especially Ewa Schneider, Carolin Popp, and Alicia Balbín-Suárez from JKI Braunschweig for providing root fresh weight data and photos. We would also like to thank Bärbel Fösel from Helmholtz Center Munich for providing microbiome-associated NGS service. Parts of this study were published in a Ph.D. thesis by Mahnkopp-Dirks (2021).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.841558/full#supplementary-material

Supplementary Figure 1 | Apple roots in spring after 12 months of growth in grass soil (left) or ARD soil (right) at Ellerhoop (photo: Alicia Balbín-Suárez).

Supplementary Figure 2 | Rarefaction curves showing the number of observed ASVs in all samples of Bittenfelder roots grown in ARD or grass soil at Heidgraben **(A)** and Ellerhoop **(B)** and taken in 3 months (summer), 7 months (autumn) or 12 months after planting (spring). Each line represents one sample of Bittenfelder roots taken in summer, autumn or spring. All samples were rarefied at 4,213 reads.

Supplementary Figure 3 | Three-dimensional non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity of roots taken in summer and autumn after planting or the following spring at Ellerhoop. Vectors represent the correlation coefficient between the corresponding genus and the NMDS score. Relative lengths and the directions of the vectors indicate the influence of the respective genera (RA > 0.5%). The third axis is not shown. Results of the one-way analysis of similarities are shown at the table above, significant differences are highlighted in bold ($p \leq 0.05$).

Supplementary Table 1 | Origins of the different isolates. Isolates were obtained from roots grown only in ARD plots (yellow), grass plots (green) or both plots (gray). Isolates were identified using Sanger sequencing and blasting against the NCBI database. The closest hit at species level is shown.

Supplementary Table 2 | Isolates obtained in the culture independent approach blasted against ASVs from amplicon sequencing (culture independent approach). Shown is the isolate identified by Sanger sequencing (closest species hit at NCBI database) and the corresponding ASV, which showed the highest Blast score in a local Blastn. The identity shows the matching bp of the isolate (query) divided by the bp of the matching ASV (sbjct) in percentage. The mean relative abundance (average over the three sampling times) of these ASVs in roots grown in ARD and grass soils from Heidgraben (H) and Ellerhoop (E) is shown.

REFERENCES

Aaij, C., and Borst, P. (1972). The gel electrophoresis of DNA. biochimica et biophysica acta (BBA). *Nucleic Acids Prot. Synth.* 269, 192–200. doi: 10.1016/0005-2787(72)90426-1

Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., and Vivanco, J. M. (2013). Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J. Biol. Chem.* 288, 4502–4512. doi: 10.1074/jbc.M112.433300

Balbín-Suárez, A., Jacquiod, S., Rohr, A. D., Liu, B., Flachowsky, H., Winkelmann, T., et al. (2021). Root exposure to apple replant disease soil triggers local defense response and rhizoplane microbiota dysbiosis. *FEMS Microbiol. Ecol.* 97:31. doi: 10.1093/femsec/fiab031

Barnes, W. M. (1992). The fidelity of Taq polymerase catalyzing PCR is improved by an N-terminal deletion. *Gene* 112, 29–35. doi: 10.1016/0378-1119(92)90299-5

Bobek, J., Šmídová, K., and Čihák, M. (2017). A waking review: old and novel insights into the spore germination in *streptomyces*. *Front. Microbiol.* 2017:5. doi: 10.3389/fmicb.2017.02205

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

Busnena, B. A., Beuerle, T., Mahnkopp-Dirks, F., Winkelmann, T., Beerhues, L., and Liu, B. (2021). Formation and exudation of biphenyl and dibenzofuran phytoalexins by roots of the apple rootstock M26 grown in apple replant disease soil. *Phytochemistry* 192:112972. doi: 10.1016/j.phytochem.2021.112972

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Cariello, N. F., Swenberg, J. A., and Skopek, T. R. (1991). Fidelity of *Thermococcus litoralis* DNA polymerase (Vent) in PCR determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* 19, 4193–4198. doi: 10.1093/nar/19.15.4193

Carrigg, C., Rice, O., Kavanagh, S., Collins, G., and O'Flaherty, V. (2007). DNA extraction method affects microbial community profiles from soils and sediment. *Appl. Microbiol. Biotechnol.* 77, 955–964. doi: 10.1007/s00253-007-1219-y

Čatská, V., Vančura, V., Hudská, G., and Pøikryl, Z. (1982). Rhizosphere microorganisms in relation to the apple replant problem. *Plant Soil* 69, 187–197. doi: 10.1007/BF02374514

Chewning, S., Grant, D., O'Banion, B., Gates, A., Kennedy, B., Campagna, S., et al. (2019). Root-associated *streptomyces* isolates harboring melc genes demonstrate enhanced plant colonization. *Phytob. J.* 3:5. doi: 10.1094/PBIOMES-01-19-0005-R

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. doi: 10.1093/nar/gkt1244

David, B. V., Chandrasehar, G., and Selvam, P. N. (2018). *Pseudomonas fluorescens*: A Plant-Growth-Promoting Rhizobacterium (PGPR) with potential role in biocontrol of pests of crops. *Crop Imp. Through Microbial. Biotechnol.* 2018, 221–243. doi: 10.1016/b978-0-444-63987-5.00010-4

Dorn-In, S., Bassitta, R., Schwaiger, K., Bauer, J., and Hölzel, C. S. (2015). Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. *Microbiol. Methods.* 113, 50–56. doi: 10.1016/j.mimet.2015.04.001

Dos Passos, J. F., da Costa, P. B., Costa, M. D., Zaffari, G. R., Nava, G., Boneti, J. I., et al. (2014). Cultivable bacteria isolated from apple trees cultivated under different crop systems: Diversity and antagonistic activity against Colletotrichum gloeosporioides. *Genet. Mol. Biol.* 37, 560–572. doi: 10.1590/s1415-47572014000400013

Eevers, N., Gielen, M., Sánchez-López, A., Jaspers, S., White, J. C., Vangronsveld, J., et al. (2015). Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. *Microbial. Biotechnol.* 8, 707–715. doi: 10.1111/1751-7915.12291

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evol. Int. J. Organ. Evol.* 39, 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Franke-Whittle, I. H., Manici, L. M., Insam, H., and Stres, B. (2015). Rhizospherebacteria and fungi associated with plant growth in soils of three replanted apple orchards. *Plant Soil* 395, 317–333. doi: 10.1007/s11104-015-2562-x

Grunewaldt-Stöcker, G., Mahnkopp, F., Popp, C., Maiss, E., and Winkelmann, T. (2019). Diagnosis of apple replant disease (ARD): Microscopic evidence of early symptoms in fine roots of different apple rootstock genotypes. *Sci. Horticult.* 243, 583–594. doi: 10.1016/j.scienta.2018.09.014

Grunewaldt-Stöcker, G., Popp, C., Wamhoff, D., Maiss, E., and Winkelmann, T. (2021). Microscopic evidence of Nectriaceae and other microbes in infected fine root tissue of replant diseased apple plants. *EuroJ. Hort. Sci.* 86, 29–40 doi: 10.17660/ejhs.2021/86.1.4

Haas, D., and Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp: and relevance for biological control of plant disease. *Ann. Rev. Phytopathol.* 41, 117–153. doi: 10.1146/annurev.phyto.41.052002.095656

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41, 95–98.

Hammer, O., Harper, D. A. T., and Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontol. Electron* 4:9.

Handelsman, J., and Smalla, K. (2003). Techniques: conversations with the silent majority. *Curr. Opin. Microbiol.* 6, 271–273. doi: 10.1016/S1369-5274(03)00062-6

Hardoim, P. R., Van Overbeek, L. S., and 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

Hayward, G. S., and Smith, M. G. (1972). The chromosome of bacteriophage T5. II. Arrangement of the single-stranded DNA fragments in the T5 + and T5st(O) chromosomes. *J. Mol. Biol.* 63, 397–407. doi: 10.1016/0022-2836(72)90436-6

Herberich, E., Sikorski, J., and Hothorn, T. (2010). A robust procedure for comparing multiple means under heteroscedasticity in unbalanced designs. *PLoS One* 5:3. doi: 10.1371/journal.pone.0009788

Jiang, J., Song, Z., Yang, X., Mao, Z., Nie, X., Guo, H., et al. (2017). Microbial community analysis of apple rhizosphere around Bohai Gulf. *Sci. Rep.* 7:8918. doi: 10.1038/s41598-017-08398-9

Kelderer, M., Manici, L. M., Caputo, F., and Thalheimer, M. (2012). Planting in the 'inter-row' to overcome replant disease in apple orchards: a study on the effectiveness of the practice based on microbial indicators. *Plant Soil* 357, 381–393. doi: 10.1007/s11104-012-1172-0

Kloepper, J. W., Leong, J., and Teintze, M. (1980). *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. *Curr. Microbiol.* 4, 317–320. doi: 10.1007/BF02602840

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

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., and McDonald, M. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–864. doi: 10.1126/science.aaa8764

Lehr, N. A., Schrey, S. D., Bauer, R., Hampp, R., and Tarkka, M. T. (2007). Suppression of plant defence response by a mycorrhiza helper bacterium. *New Phytol.* 2007, 892–903. doi: 10.1111/j.1469-8137.2007.02021.x

Levene, H., Olkin, I., and Hotelling, H. (1960). *Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling, Robust tests for equality of variances*. Redwood City: Stanford University Press, 278–292.

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

Liu, J., Abdelfattah, A., Norelli, J., Burchard, E., Schena, L., Droby, S., et al. (2018). Apple endophytic microbiota of different rootstock/scion combinations

suggests a genotype-specific influence. *Microbiome* 6:18. doi: 10.1186/s40168-018-0403-x

Loper, J. E., and Henkels, M. D. (1999). Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Appl. Environ. Microbiol.* 65, 5357–5363. doi: 10.1128/AEM.65.12.5357-5363.1999

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Mahnkopp, F., Simon, M., Lehndorff, E., Patzold, S., Wrede, A., and Winkelmann, T. (2018). Induction and diagnosis of apple replant disease (ARD): a matter of heterogeneous soil properties? *Sci. Horticult.* 241, 167–177. doi: 10.1016/j.scienta.2018.06.076

Mahnkopp-Dirks, F. (2021). Bacterial endophytes in apple (Malus domestica) rootstocks grown in apple replant disease affected and non-affected soils. *Hannover: Gottfried Wilhelm Leibniz Universität, Diss.* 132:11138. doi: 10.15488/11138

Mahnkopp-Dirks, F., Radl, V., Kublik, S., Gschwendtner, S., Schloter, M., and Winkelmann, T. (2021). Molecular barcoding reveals the genus *Streptomyces* as associated root endophytes of apple (Malus domestica) plants grown in soils affected by apple replant disease. *Phytob. J.* 2, 177–189. doi: 10.1094/PBIOMES-07-20-0053-R

Manici, L. M., Kelderer, M., Franke-Whittle, I. H., Rühmer, T., Baab, G., Nicoletti, F., et al. (2013). Relationship between root-endophytic microbial communities and replant disease in specialized apple growing areas in Europe. *Appl. Soil Ecol.* 72, 207–214. doi: 10.1016/j.apsoil.2013.07.011

Mazzola, M., and Gu, Y.-H. (2000). Impact of wheat cultivation on microbial communities from replant soils and apple growth in greenhouse trials. *Phytopathology* 90, 114–119. doi: 10.1094/PHYTO.2000.90.2.114

Mazzola, M., and Manici, L. M. (2012). Apple replant disease: role of microbial ecology in cause and control. *Annu. Rev. Phytopathol.* 50, 45–65. doi: 10.1146/annurev-phyto-081211-173005

McMurdie, P. J., and Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Nicola, L., Insam, H., Pertot, I., and Stres, B. (2018). Reanalysis of microbiomes in soils affected by apple replant disease (ARD): Old foes and novel suspects lead to the proposal of extended model of disease development. *Appl. Soil Ecol.* 129, 24–33. doi: 10.1016/j.apsoil.2018.04.010

Oksanen, J., Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: Community Ecology Package. R package version 2.5-6.*

Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S., Mavrodi, D. V., et al. (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat. Biotechnol.* 23, 873–878. doi: 10.1038/nbt1110

Peruzzi, E., Franke-Whittle, I. H., Kelderer, M., Ciavatta, C., and Insam, H. (2017). Microbial indication of soil health in apple orchards affected by replant disease. *Appl. Soil Ecol.* 119, 115–127. doi: 10.1016/j.apsoil.2017.06.003

Pham, V. H., and Kim, J. (2012). Cultivation of unculturable soil bacteria. *Trends Biotechnol.* 30, 475–484. doi: 10.1016/j.tibtech.2012.05.007

Pollock, J., Glendinning, L., Wisedchanwet, T., and Watson, M. (2018). The madness of microbiome: attempting to find consensus "best practice" for 16S microbiome studies. *Appl. Environ. Microbiol.* 84, e2627–e2617. doi: 10.1128/AEM.02627-17

Popp, C., Grunewaldt-Stöcker, G., and Maiss, E. (2019). A soil-free method for assessing pathogenicity of fungal isolates from apple roots. *J. Plant Dis. Prot.* 126, 329–341. doi: 10.1007/s41348-019-00236-6

Popp, C., Wamhoff, D., Winkelmann, T., Maiss, E., and Grunewaldt-Stocker, G. (2020). Molecular identification of Nectriaceae in infections of apple replant disease affected roots collected by Harris Uni-Core punching or laser microdissection. *J. Plant Dis. Prot.* 127, 571–582. doi: 10.1007/s41348-020-00333-x

Quambusch, M., Pirttilä, A. M., Tejesvi, M. V., Winkelmann, T., and Bartsch, M. (2014). Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes. *Tree Physiol.* 34, 524–533. doi: 10.1093/treephys/tpu027

R Development Core Team (2019). R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.

Raaijmakers, J. M., Weller, D. M., and Thomashow, L. S. (1997). Frequency of antibiotic-producing *pseudomonas* spp. in natural environments. *Appl. Environ. Microbiol.* 63, 881–887. doi: 10.1128/AEM.63.3.881-887.1997

Reasoner, D. J., and Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 49, 1–7. doi: 10.1128/aem.49.1.1-7.1985

Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., and Hurek, T. (2015). Roots shaping their microbiome: global hotspots for microbial activity. *Ann. Rev. Phytopathol.* 53, 403–424. doi: 10.1146/annurev-phyto-082712-102342

Rohr, A. D., Staudt, J., Cziborra, K., Fritz, A., Schmitz, M., and Winkelmann, T. (2021). Split-root approach reveals localized root responses towards apple replant disease (ARD) in terms of ARD biomarker gene expression and content of phenolic compounds. *Sci. Horticult.* 286:110117. doi: 10.1016/j.scienta.2021.110117

Rosales, A. M., Thomashow, L., Cook, R. J., and Mew, T. W. (1995). Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. *Phytopathology* 85, 1028–1032. doi: 10.1094/phyto-85-1028

Rumberger, A., Merwin, I. A., and Thies, J. E. (2007). Microbial community development in the rhizosphere of apple trees at a replant disease site. *Soil Biol. Biochem.* 39, 1645–1654. doi: 10.1016/j.soilbio.2007.01.023

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci. USA* 74, 5463–5467. doi: 10.1073/pnas.74.12.5463

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

Savory, B. M. (1966). *Specific replant diseases causing root necrosis and growth depression in perennial fruit and plantation crops. Research Review No. 1. Commonwealth Bureau of Horticulture and Plantation Crops, East Malling.* Maidstone: Commonwealth Agricultural Bureaux

Schloss, P. D., and Handelsman, J. (2005). Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. *Genome Biol.* 6:229. doi: 10.1186/gb-2005-6-8-229

Schubert, M., Lindgreen, S., and Orlando, L. (2016). AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC Res. Notes* 9:88–88. doi: 10.1186/s13104-016-1900-2

Shapiro, S. S., and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika* 52, 591–611. doi: 10.1093/biomet/52.3-4.591

Shepherd, M. D., Kharel, M. K., Bosserman, M. A., and Rohr, J. (2010). Laboratory maintenance of *Streptomyces* species. *Curr. Protoc. Microbiol.* 10:18. doi: 10.1002/9780471729259.mc10e01s18

Sun, J., Zhang, Q., Zhou, J., and Wei, Q. (2014). Illumina Amplicon Sequencing of 16S rRNA Tag reveals bacterial community development in the rhizosphere of apple nurseries at a replant disease site and a new planting site. *PLoS One* 9:e111744. doi: 10.1371/journal.pone.0111744

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.ution* 10, 512–526. doi: 10.1093/oxfordjournals.molbev.a040023

Tarkka, M. T., Lehr, N. A., Hampp, R., and Schrey, S. D. (2008). Plant behavior upon contact with Streptomycetes. *Plant Signal. Behav.* 3, 917–919. doi: 10.4161/psb.5996

Tewoldemedhin, Y. T., Mazzola, M., Labuschagne, I., and McLeod, A. (2011). A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. *Soil Biol. Biochem.* 43, 1917–1927. doi: 10.1016/j.soilbio.2011.05.014

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

Tilston, E. L., Deakin, G., Bennett, J., Passey, T., Harrison, N., O'Brien, F., et al. (2018). Candidate causal organisms for apple replant disease in the UK. *Phytob. J.* 2, 261–274. doi: 10.1094/PBIOMES-11-18-0050-R

Van Horn, C., Somera, T. S., and Mazzola, M. (2021). Comparative analysis of the rhizosphere and endophytic microbiomes across apple rootstock genotypes in replant orchard soils. *Phytob. J.* 5, 231–243. doi: 10.1094/PBIOMES-08-20-0058-R

Vartoukian, S. R., Palmer, R. M., and Wade, W. G. (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol. Lett.* 309, 1–7. doi: 10.1111/j.1574-6968.2010.02000.x

Viss, P., Brooks, E., and Driver, J. (1991). A simplified method for the control of bacterial contamination in woody plant tissue culture. *Vitro Cell Dev. Biol.* 27:42. doi: 10.4103/0973-1296.96547

Vurukonda, S., Giovanardi, D., and Stefani, E. (2018). Plant Growth Promoting and Biocontrol Activity of *Streptomyces* spp. as Endophytes. *Int. J. Mol. Sci.* 19:952. doi: 10.3390/ijms19040952

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. doi: 10.1128/jb.173.2.697-703.1991

Winkelmann, T., Smalla, K., Amelung, W., Baab, G., Grunewaldt-Stocker, G., Kanfra, X., et al. (2019). Apple replant disease: causes and mitigation strategies. *Curr. Issues Mol. Biol* 30, 89–106. doi: 10.21775/cimb.030.089

Yashiro, E., Spear, R. N., and McManus, P. S. (2011). Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *J. Appl. Microbiol.* 110, 1284–1296. doi: 10.1111/j.1365-2672.2011.04975.x

Yim, B., Winkelmann, T., Ding, G. C., and Smalla, K. (2015). Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis – contribution to improved aboveground apple plant growth? *Front. Microbiol.* 6:1224. doi: 10.3389/fmicb.2015.01224

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 March 2022
doi: 10.3389/fmicb.2022.841621

Fungal Communities of *Eucalyptus grandis* Leaves Are Influenced by the Insect Pest *Leptocybe invasa*

Mandy Messal[1], María Vivas[1,2], Martin Kemler[3], Dominik Begerow[3], Andreas Brachmann[4], Frederick Witfeld[3], Sanushka Naidoo[1] and Bernard Slippers[1]**

[1]*Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa,* [2]*Institute for Dehesa Research (INDEHESA), University of Extremadura, Plasencia, Spain,* [3]*Evolution of Plants and Fungi, Ruhr University Bochum, Bochum, Germany,* [4]*Faculty of Biology, Ludwig-Maximilians-Universität München, München, Germany*

OPEN ACCESS

Edited by:
Khondoker M.G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Ari Mikko Hietala, Norwegian Institute of Bioeconomy Research (NIBIO), Norway
Bamisope Steve Bamisile, South China Agricultural University, China

***Correspondence:**
Mandy Messal
mandy.messal@fabi.up.ac.za
Bernard Slippers
bernard.slippers@fabi.up.ac.za

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *22 December 2021*
Accepted: *08 March 2022*
Published: *31 March 2022*

Citation:
Messal M, Vivas M, Kemler M, Begerow D, Brachmann A, Witfeld F, Naidoo S and Slippers B (2022) Fungal Communities of Eucalyptus grandis Leaves Are Influenced by the Insect Pest Leptocybe invasa. Front. Microbiol. 13:841621. doi: 10.3389/fmicb.2022.841621

Fungal communities in above-ground tree tissues are hyperdiverse and are influenced by biotic interactions with other organisms living in or on these tissues. These biotic interactions are, however, still poorly understood. In this study, we aimed to understand how insect-associated gall formation on *Eucalyptus* foliage correlates with the diversity of foliar fungal communities in surrounding healthy leaf tissue, as well as the co-occurrence patterns among the members of the fungal community. We used ITS metabarcoding to characterise the foliar fungal communities of 179 individual *E. grandis* trees. These trees were assigned to infestation levels of the wasp *Leptocybe invasa* (Eulophidae: Hymenoptera), which causes gall formation on shoot tips and leaves of its host. Fungal community networks were calculated using a Pearson correlation coefficient. The composition and diversity of fungal communities were influenced by the severity of *L. invasa* infestations. We identified potential *Eucalyptus* pathogens with high sequence abundance at all disease severity levels, but network analysis indicated that the co-occurrence of potential pathogens between no to mild and medium to heavy infestation differed significantly. A better understanding of microbial interactions, especially the role of pathogens, can be useful for controlling disease- and beneficial host-associated microbial communities.

Keywords: *Eucalyptus* microbiome, microbial networks, phyllosphere fungal community, biotic plant stress, fungal-plant interaction, plantation trees, amplicon sequencing

INTRODUCTION

Plant galls are the growth of abnormal plant tissue induced by other organisms (e.g. viruses, bacteria, fungi, nematodes or insects) and are found on many plant species. Insect-induced plant galls impact plant development by triggering morphological and physiological changes in the host plant tissues. Oviposition in leaf tissues initiates these cellular modifications through changes in plant development pathways, nutrient concentrations, the disruption of plant defence, selection for gall induction traits and the advent of insect-derived effectors (Giron et al., 2016; Oates et al., 2021). Plant tissues stressed in such a way, potentially also change the conditions for the colonisation or proliferation of co-existing organisms, including foliar fungi.

It is well known that fungal community diversity and the colonisation of plant host niches are influenced by abiotic, microbial and host factors (e.g. Bálint et al., 2013; Kemen, 2014; Vivas et al., 2017; Gomes et al., 2018). A clear difference between the fungal communities of healthy and yellowing *Citrus limon* leaves, where yellowing leaves had the least species diversity, exemplifies the influence of plant physiology on microbial community patterns (Douanla-Meli et al., 2013). It must therefore be assumed that galls, which function as metabolic sinks (Allison and Schultz, 2005; Dardeau et al., 2014), also influence fungal community diversity. Some studies indeed show that fungal communities in galls are different than in the surrounding leaf tissue. Cultivated fungal communities associated with aphid-induced galls in cottonwood (*Populus deltoides*) for instance exhibit distinct fungal richness and diversity in galls compared to the surrounding tissues (Lawson et al., 2014). A metabarcoding study of fungal communities showed differences in richness, diversity and composition between galls induced by *Dryocosmus kuriphilus* and surrounding chestnut leaf tissues (Fernandez-Conradi et al., 2019). Whether different levels of severity of gall formation affect the fungal community in surrounding healthy tissue is, however, not known.

Eucalyptus plantations are of high economic value globally (Wingfield et al., 2015). In recent years, their yield has been jeopardised by the gall-forming wasp *Leptocybe invasa* Fisher and LaSalle (Hymenoptera: Eulophidae), which deposits eggs into new growth of *Eucalyptus* trees (Naidoo et al., 2011; Dittrich-Schröder et al., 2018; Mhoswa et al., 2020). A high density of *L. invasa* can cause heavy galling, malformation, stunted growth and in extreme cases, tree death (Mendel et al., 2004; Zheng et al., 2014; Csóka et al., 2017). The egg oviposition into the *Eucalyptus* spp. leaf tissues initiates the expression of pathogen-related genes by the host and localised cell death causing desiccation, detachment or is directly ovicidal (Geuss et al., 2017; Griese et al., 2017). Within 24 h after oviposition, *Eucalyptus* spp. tissues accumulate reactive oxygen species and phenolics, as well as phytohormones (especially jasmonic acid, salicylic acid and ethylene) as a defence against biotic stress (Berens et al., 2017). The egg and oviposition fluid may redirect the hosts' responses towards gall development (e.g. cell division) and is thus responsible for initiating galling (Oates et al., 2021). Gall-forming insects are also known to modify the availability of sugars, lipids and proteins in the nutritive tissue of the gall chamber (Huang et al., 2014; Ferreira et al., 2015). A study on the influence of *L. invasa* gall development on frost resistance in eucalypts found that the physiological changes on the plant foliage increased plant defence mechanisms against cold. The toll of galling by herbivores may thus have a positive indirect effect on the host plant (Rocha et al., 2013).

Fungal diversity associated with healthy and diseased *Eucalyptus* spp. has been explored for several decades (e.g. Bird et al., 1974; Bettucci and Alonso, 1997; Barbed et al., 2003; Roux et al., 2003; Hunter et al., 2011; Márquez et al., 2011; Jimu et al., 2015). The recent application of high-throughput sequencing of fungal-specific PCR amplicons has revealed enormous species diversity and richness in *Eucalyptus* spp. (Kemler et al., 2013). Such studies have identified potential pathogens existing in the fungal community of trees without visible symptoms of disease or decay. Additionally, community patterns have been shown to be highly dependent on environmental factors, as well as maternal effects that could influence the formation of fungal communities in seedlings (Vivas et al., 2017). With increasing *L. invasa* infestations in plantations (Hurley et al., 2016; Dittrich-Schröder et al., 2018), it is important to understand its influence on the associated fungal community and whether the added stress increases the occurrence of potentially pathogenic fungi.

In this study, we analysed fungal community diversity, composition and co-occurrence network structures in *E. grandis* trees with different levels of *L. invasa* infestation. We aimed to answer three questions: (i) Do *L. invasa* infestation levels correlate with fungal community diversity and composition in surrounding healthy leaf tissue?; (ii) Do co-occurrence patterns of fungal taxa correlate with *L. invasa* infestation?; and (iii) Do increased *L. invasa* infestation levels lead to an increased abundance of potential *Eucalyptus* pathogens?

MATERIALS AND METHODS

Plant Material

A *Eucalyptus grandis* half-sib population, situated at a non-irrigated coastal site (Siya Qubeka) in KwaZulu-Natal, South Africa, was selected. The 14-month-old *Eucalyptus* trees were scored for *L. invasa* infestation symptoms. Tree infestation was characterised by one of the following categories: 0 - no infestation, 1 - infestation with evidence of oviposition, but no gall development, 2 - infestation with galls on leaves, mid-ribs or petioles and 3 - infestation with stunted overall growth and lethal gall formation. The foliar fungal communities of 179 individual trees (0, $n = 49$; 1, $n = 50$; 2, $n = 56$; 3, $n = 24$) were analysed. The *Eucalyptus* population was analysed and genotyped in previous studies (Naidoo et al., 2018; Mhoswa et al., 2020) and DNA extracts from those studies were used. Importantly, DNA was extracted from non-surface sterilised leaf punches of non-symptomatic leaf tissues and care was taken to avoid the galled areas of the leaves; thus, non-symptomatic tissue was used for all DNA samples.

Molecular Methods and Sequencing

Polymerase Chain Reactions (PCR) were performed on the 179 *E. grandis* leaf DNA samples and PCR products were quantified through gel electrophoresis and ImageJ (Schneider et al., 2012). The primers ITS1-F (5′-CTTGGTCATTTAGA GGAAGTAA, Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC, White et al., 1990) were used to amplify the fungal Internal Transcribed Spacer (ITS) rDNA gene region. The amplicon library for sequencing was prepared in two consecutive PCR steps using GoTaq G2 Hot Start polymerase (Promega, Mannheim, Germany). The first PCR amplified the fungal ITS region with specific ITS1-F and ITS4 primers including a tag sequence (**Supplementary Table 1**). The PCR products were purified using ExoSAP-Clean Up (New England BioLabs®) following the manufactures' instructions.

The second PCR was conducted with primers containing the Illumina adaptor sequences to receive a unique tag-index combination (**Supplementary Table 2**). The products of the second PCR were quantified using 1% agarose gel electrophoresis and residual reaction chemicals were removed using the CleanPCR kit (CleanNA). Subsequently, equimolar sample pools were generated in repetitive steps. Equimolar pooled sequencing libraries (2 × 250 bp paired-end) were sequenced on an Illumina MiSeq (Illumina Inc., San Diego, CA, United States) using the MiSeq® Reagent Kit v3 Chemistry at the Genomics Service Unit (LMU Biocenter, Planegg-Martinsried, Germany).

Illumina MiSeq Sequence Processing

Sequence information was obtained as fastq files for the forward and reverse sequence reads, respectively. Samples were demultiplexed in QIIME v1.9.1 (Caporaso et al., 2010) based on forward and reverse reads. However, only the forward read was used for downstream analyses, as read lengths of 250 bp can prevent merging forward and reverse reads, thereby excluding diversity from the analyses. Subsequent sequence quality control, OTU clustering and taxonomic assignments were performed in QIIME v1.9.1 and programmes implemented therein. During quality control, reads smaller than 200 bp, containing homopolymers of a length more than 6 and a Phred score below 30, were filtered out. Chimeric sequences were removed de-novo using usearch61 (Edgar, 2010). OTUs were clustered at a 97% sequence similarity using uclust v1.2.22q (Edgar, 2010). For the taxonomic assignment, representative sequences for each OTU were queried against the UNITE database (v7_99_s_28.06.2017; Kõljalg et al., 2013) using BLAST (Altschul et al., 1990) at an e-value of $1e^{-30}$. For subsequent analyses, OTUs with less than ten sequences, as well as OTUs with no blast hits and non-fungal hits, were removed from the data set.

Fungal Community Diversity and Composition

To analyse the fungal diversity among *E. grandis* trees with different *L. invasa* infestation levels, Shannon, Simpson and Invsimpson diversity indices were calculated for each tree. The effect of the different *L. invasa* infestation levels on each diversity index was analysed using a one-way ANOVA followed by a Tukey's Honest Significant Difference (HSD) *post-hoc* test to do pairwise comparisons of the means. In all cases, model validity was checked and the *agricolae* package of the R software was used to analyse fungal diversity (R Core Team, 2018; de Mendiburu and Yaseen, 2021).

The fungal taxonomic composition of *E. grandis* leaves was summarised in a taxonomic tree using the *MetacodeR* package of the R software (Foster et al., 2017; R Core Team, 2018). Differences among community composition of *E. grandis* trees with different *L. invasa* infestation levels were visualised using a Principal Coordinate Analysis (PCoA). PCoA was performed on an abundance matrix applying Bray-Curtis dissimilarity. To assess whether community composition statistically varied among different *L. invasa* infestation levels, we employed a permutational multivariate analysis of variance (PERMANOVA). The *vegan* package of the R software (Oksanen et al., 2018; R Core Team, 2018) was used to analyse the community composition.

The community composition analysis was paired with a differential heat tree illustrating the differences in the fungal abundance of each taxon for the order Capnodiales among the *L. invasa* infestation levels using *MetacodeR*. The order of Capnodiales was selected due to their apparent role as foliar pathogens of *Eucalyptus* spp. A Wilcoxon Rank Sum test was applied, and the resulting *p*-values were corrected for multiple comparisons using the false discovery rate (FDR).

Fungal Community Networks

Networks were calculated using the Calypso web-server (Zakrzewski et al., 2016). For this analysis, only OTUs that were assigned at least to the genus level were used. During upload, taxa that had less than 0.01% relative abundance were removed from the OTU table. Total sum normalisation and square-root transformation were performed on the dataset. Networks were calculated using a Pearson correlation coefficient and only edges that correlated significantly at $p < 0.05$ were connected. The resulting network was imported into Cytoscape v3.7.2 (Shannon et al., 2003) for depiction. To analyse the number of edges between the subnetworks, we used a one-way ANOVA followed by a Tukey's Honest Significant Difference (HSD) *post-hoc* test to do pairwise comparisons of the means. Model validity was checked and the *agricolae* package of the R software was used to analyse fungal diversity (R Core Team, 2018; de Mendiburu and Yaseen, 2021). In order to understand possible differences in the ecology of the fungi in the network, the feeding modes of fungal genera were analysed using FungalTraits (Põlme et al., 2020).

RESULTS

Sequencing

A total of 1,104,065 filtered and non-chimeric fungal ITS1 sequences were used for community analyses. Sequencing of leaves with *L. invasa* infestation level 0 resulted in 250,261 reads, *L. invasa* infestation level 1 in 269,086 reads, *L. invasa* infestation level 2 in 377,422 reads and *L. invasa* infestation level 3 in 207,296 reads. We assigned 171 individual OTUs to 77 different fungal genera (**Figure 1**) and 91 different fungal species (**Supplementary Tables 3–10**). Out of the 171 detected OTUs, 140 OTUs were shared between the fungal communities of the four *L. invasa* infestation levels (**Figure 2**). We found 155 OTUs in *L. invasa* infestation level 0, 160 OTUs in *L. invasa* infestation level 1, 159 OTUs in *L. invasa* infestation level 2 and 154 OTUs in *L. invasa* infestation level 3, respectively.

Fungal Community Diversity and Composition

Fungal community diversity was different between the four *L. invasa* infestation levels. Shannon, Simpson and Invsimpson indexes were higher in trees with low *L. invasa* infestation than in trees with a higher infestation ($p < 0.001$; **Figure 3**).

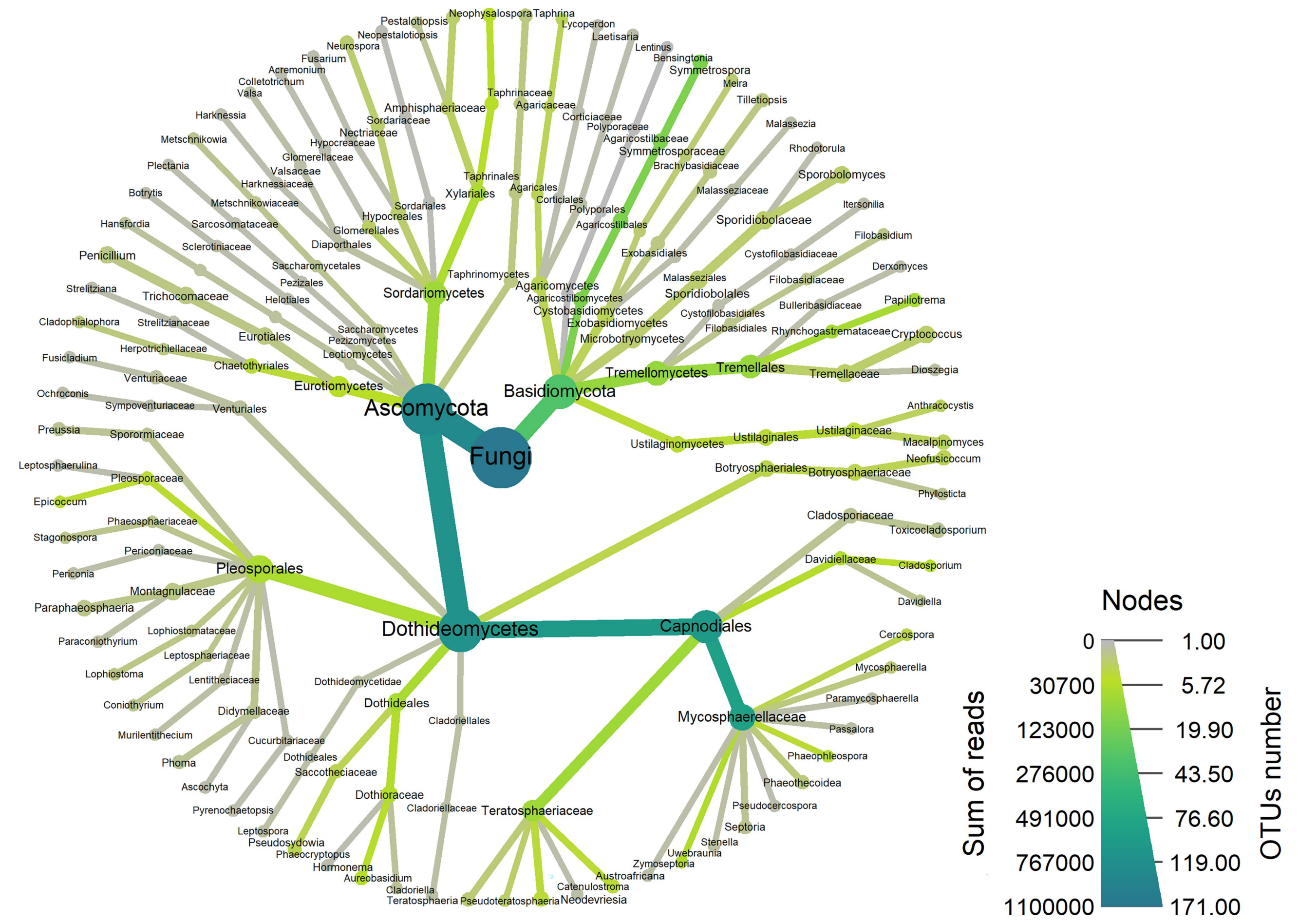


FIGURE 1 | Taxonomic composition of the fungi associated with *Eucalyptus grandis* leaves. The heat tree represents the fungal community structure as a taxonomic hierarchy up to the genus level. Node and edge sizes are proportional to the number of OTUs within each taxon and colours represent absolute taxon abundance (sum of reads number).

The PCoA plot showed that the fungal community composition differed between the *L. invasa* infestations levels 0 and 1 (healthy to mild infestation) and levels 2 and 3 (medium to high infestation), respectively (**Figure 4**). PERMANOVA confirmed that the *L. invasa* infestation levels significantly explained the variation in fungal community composition ($F_{1,3} = 37.82$, $r^2 = 0.393$, $p < 0.001$).

Out of all OTUs, 67% belonged to Ascomycota, 25% to Basidiomycota and 9% could not be identified beyond Fungi. The total numbers of reads assigned to the order Capnodiales were 37% at *L. invasa* infestation level 0, 53% at level 1, 68% at level 2 and 72% at level 3. Of the total number of reads, 28% were assigned to the family Mycosphaerellaceae at *L. invasa* infestation level 0, 40% at level 1, 63% at level 2 and 69% at level 3.

In the differential heat tree of Capnodiales, *Eucalyptus* leaves with higher *L. invasa* infestation levels showed a higher abundance of taxa in the Mycosphaerellaceae in comparison with leaves with a lower infestation level (**Figure 5**). Most of the OTUs within the Capnodiales (22 out of 37 OTUs) were assigned to the family Mycosphaerellaceae.

Fungal Community Network Results

We recovered two subnetworks that did not show any statistically significant co-occurrence among each other and 16 genera that did not show any significant co-occurrence patterns (**Figure 6**). Subnetwork 1 represents genera, which were largely retrieved from samples with *L. invasa* infestation levels 0 or 1 (red and blue in **Figure 6**) and subnetwork 2 represents genera, which were largely retrieved from samples with *L. invasa* infestation levels 2 or 3 (yellow and mauve in **Figure 6**). Fungal genera occurring in subnetwork1 have significant fewer edges (61) than fungal taxa in subnetwork 2 (98; $p < 0.001$, **Table 1**; **Supplementary Figure 1**; **Supplementary Table 11**), while the number of nodes is comparable (subnetwork 1 = 30, subnetwork 2 = 26). In our network analysis, a node represents a taxon or OTU grouped at a specific level, e.g. genus level, while edges are lines connecting nodes and represent significant correlations between these nodes.

The fungal feeding modes were analysed using FungalTraits (Põlme et al., 2020). No difference could be observed between feeding types of the fungal genera of subnetwork 1 (*L. invasa* infestation level 0 and 1) compared to subnetwork 2 (*L. invasa*

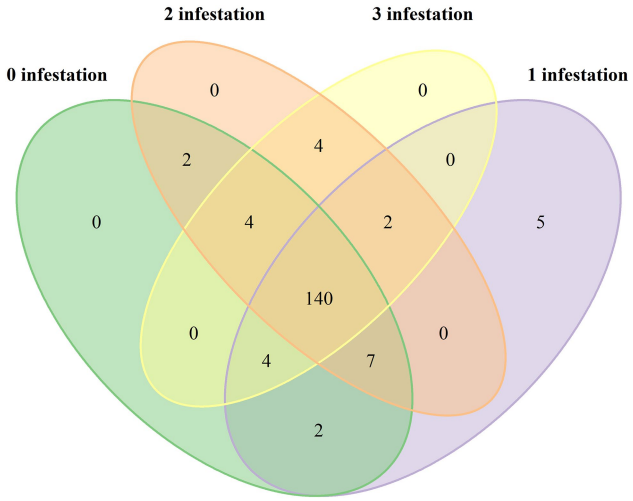


FIGURE 2 | Venn diagram showing the abundance of fungal species associated with *Eucalyptus grandis* leaves among four infestation levels of the wasp *Leptocybe invasa* (0—no infestation, 1—infestation with evidence of oviposition, but no gall development, 2—infestation with galls on leaves, mid-ribs or petioles and 3—infestation with stunted overall growth and lethal gall formation).

infestation level 0 and 1). In both subnetworks, the majority of genera were classified as plant pathogens (**Supplementary Table 10**; **Supplementary Figure 2**).

DISCUSSION

In this study, we show that the fungal community structure of *E. grandis* leaves is influenced by the insect pest *L. invasa*. We characterised a high fungal diversity, with 171 fungal taxa identified from these leaf tissues in one site. The fungal communities were clearly structured by the level of *L. invasa* infestation. Potential phytopathogenic taxa were present in all four levels of infested leaves. This is the first demonstration of such a clear relationship between the level of *L. invasa* infestation in *E. grandis* leaves and fungal community structure.

Leaves with no or mild *L. invasa* infestation (*L. invasa* infestation levels 0 and 1) had a significantly higher fungal community diversity than leaves showing medium and high infestation (*L. invasa* infestation levels 2 and 3). It has been shown before that a plant's health status influences its associated fungal diversity. For example, gall tissue induced by the gall wasp *Dryocosmus kuriphilus* in chestnut leaves harboured a significantly lower fungal community diversity compared to the surrounding plant tissue (Fernandez-Conradi et al., 2019) and increasing levels of powdery mildew infection in pumpkin leaves correlated negatively with fungal community diversity (Zhang et al., 2018). The correlations between increased infection levels and fungal community diversity are, however, controversial. While *Fusarium* head blight infections increase *Fusarium* spp. in wheat spikelets and kernels, overall fungal community diversity only reduced significantly in kernels and not in spikelets (Rojas et al., 2020).

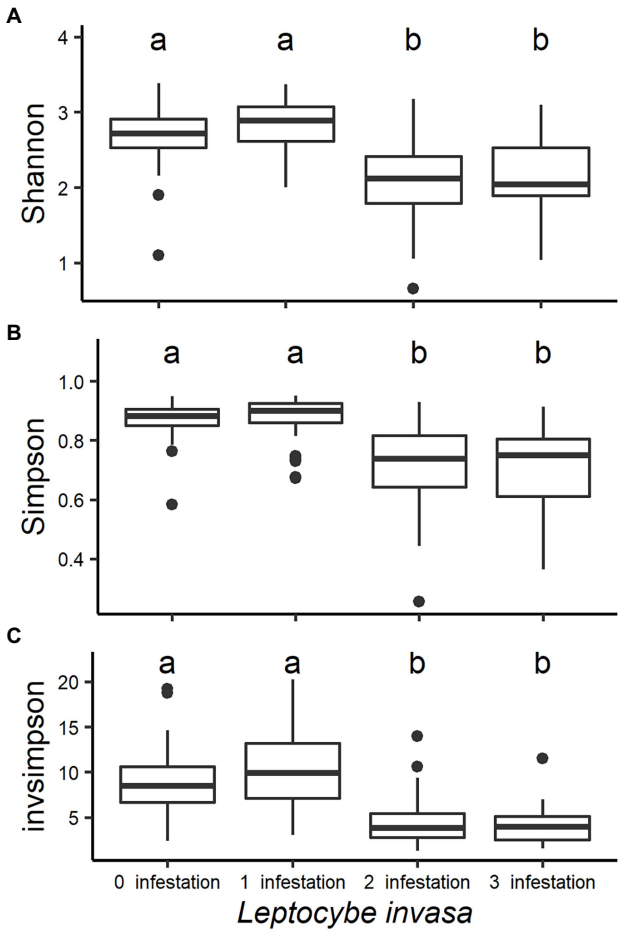


FIGURE 3 | Box plots analysing the fungal diversity indices (A-Shannon, B-Simpson and C-Invsimpson) of *Eucalyptus grandis* leaves among four infestation levels of the wasp *Leptocybe invasa* (0—no infestation, 1—infestation with evidence of oviposition, but no gall development, 2—infestation with galls on leaves, mid-ribs or petioles and 3—infestation with stunted overall growth and lethal gall formation). Bars show standard errors and different letters indicate significant differences ($p < 0.05$).

In this study, we sampled healthy tissues that were adjacent to the affected tissues. This indicated that galling has indirect effects on fungal species diversity in non-symptomatic tissues. Changes in fungal community diversity have been attributed to factors including changes in plant nutrients, secondary metabolites, competition, plant defence reactions, as well as phytohormone regulation (Cameron et al., 2013; Pieterse et al., 2014; Abdelfattah et al., 2016; Bennett and Cahill, 2016; Shen et al., 2018; Gluck-Thaler et al., 2020). These changes can also be seen in the surrounding gall tissues, hence affecting the host's mycobiome (Lawson et al., 2014; Fernandez-Conradi et al., 2019).

The *L. invasa* infestation did not only lower fungal diversity within respective leaves but also led to a deterministic pattern of fungal community composition between leaves of different infestation levels. The communities associated with no or mild *L. invasa* infestation and those associated with a medium or heavy infestation were clearly separated on PCo 1. Interestingly,

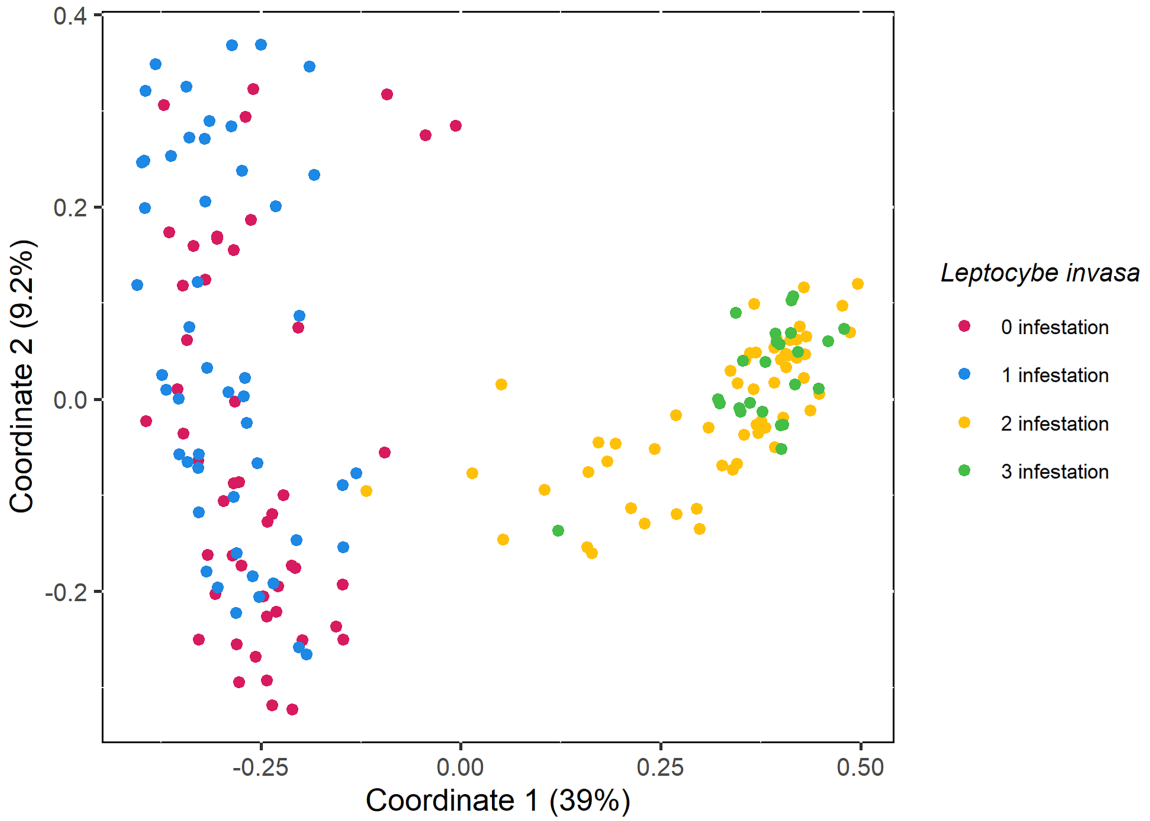


FIGURE 4 | Principal coordinates analysis (PCoA) of fungal community composition associated with *Eucalyptus grandis* leaves among four infestation levels of the wasp *Leptocybe invasa* (0—no infestation, 1—infestation with evidence of oviposition, but no gall development, 2—infestation with galls on leaves, mid-ribs or petioles and 3—infestation with stunted overall growth and lethal gall formation.). The variance explained by each dimension is shown between brackets.

there was no gradual transition between infestation levels, but two separate clusters were formed of leaves with no/mild infestation and leaves with medium/heavy infestation. This could indicate a limitation in our scoring system, as we might have been unable to determine subtle infections in what were assumed to be healthy leaves. However, the clear separation between two clusters might also be consistent with an interpretation that the *Leptocybe-Eucalyptus* interaction causes a threshold, where the associated fungal community switches in composition. Such tipping points have been observed in other host-associated microbiomes (Tytgat et al., 2019) and raise interesting questions about the conditions at which such bifurcations occur in the tripartite interaction of host, *Leptocybe* and mycobiome for further investigation.

Fungal communities on healthy or mildly infested leaves (*L. invasa* infestation levels 0 and 1) showed more dispersed fungal community composition than heavily and medium infested leaves (*L. invasa* infestation levels 2 and 3). Such differences have been observed in disease systems before, except that it was mostly the sick individuals that showed a more dispersed diversity (Turnbaugh et al., 2009; Hong et al., 2015). This so-called Anna Karenina principle has been attributed to a more stochastic distribution of factors affecting the structure of microbial communities in non-healthy individuals and predicts that all healthy microbiomes are alike, and each disease-associated microbiome is 'sick' in its own way (Zaneveld et al., 2017). The reason for the less dispersed composition in sick individuals, and how common this might be in plant gall associated microbial communities, is not known.

A sudden shift in *Eucalyptus* leaf physiology due to galling could explain our observed shift in fungal diversity. Putative physiological changes due to gall development and *Eucalyptus* defence mechanisms have been observed, including cell wall reinforcement, protease inhibitors, cell cycle suppression and regulatory hormone signalling pathways (Oates et al., 2015). It is well known that fungi take advantage of the physiological state of their host. For example, when comparing the fungal communities of healthy and yellowing *C. limon* leaves, a result of either nutrient deficiency or drought stress, the potential phytopathogen *Colletotrichum gloeosporioides* increased in abundance, whereas most other fungal species decreased (Douanla-Meli et al., 2013). These fungal species may have advantageous traits for coping with physiological disturbance, whereas the other fungal endophytes might rely on nutrition and protection from their healthy plant host.

Oviposition initiates physiological changes in leaf tissues, including cellular modifications, changes in plant development pathways and nutrient concentrations, the disruption of plant defence, selection for gall induction traits and the advent of insect-derived effectors (Giron et al., 2016). The cynipid gall wasp *Andricus petiolicolus*, for example elicits galls on chestnut oak leaves (*Quercus prinus*) that significantly differ from the

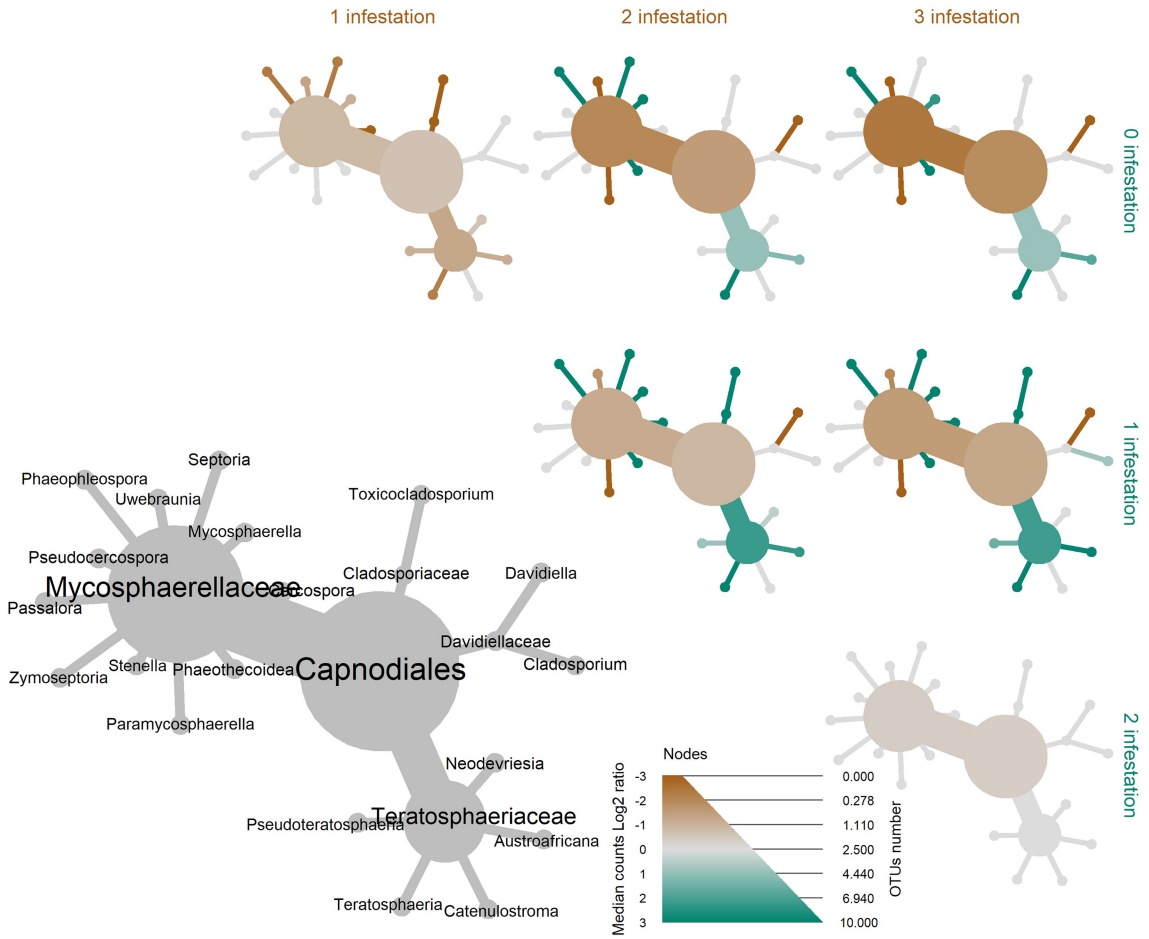


FIGURE 5 | Differential heat tree up to the order level illustrating the effect of four *Leptocybe invasa* infestation levels (0—no infestation, 1—infestation with evidence of oviposition, but no gall development, 2—infestation with galls on leaves, mid-ribs or petioles and 3—infestation with stunted overall growth and lethal gall formation) on the abundance of leaf-associated fungi in *Eucalyptus grandis*. Node and edge sizes are proportional to the different number of OTUs between the groups for each taxon and colours represent the log of the ratio of median abundance between the groups for each taxon. When a taxon has more counts in samples from groups on the right side of the graph, it is coloured green. And, when a taxon has more counts in samples from groups on the upper side of the graph, it is coloured brown.

leaf tissue from which they are formed (Allison and Schultz, 2005). The gall cortex and epidermis exhibit higher peroxidase and invertase activities and greater condensed tannin concentrations than the nutritive tissues or leaves. The woolly poplar aphid *Phloeomyzus passerinii* can induce a pseudogall within the bark on trunks of poplars, where the gall acts as a sink accumulating nutrients, like amino acids, soluble sugars or starch drawn from the surrounding tissues (Dardeau et al., 2014). A study on the transcriptome and terpene profiles of *E. grandis* challenged with *L. invasa* shows that indeed changes are induced by oviposition, including mono- and sesquiterpene profiles, phytohormone responses and lignification locally at the site of oviposition (Oates et al., 2015). A joint transcriptomics study (Dual-RNA) analysing plant and fungal expressed genes associated with insect gall development will increase the understanding of these biotic interactions.

Distinct fungal community subnetworks could be observed between healthy/mildly infested and medium/heavily infested *E. grandis* leaf samples. All but 16 genera showed significant co-occurrence within their subnetworks, i.e. nodes are connected by edges. Even though co-occurrence is not evidence of ecological interactions (Blanchet et al., 2020), we observe a non-random co-occurrence pattern, possibly caused by a biological driver. The higher number of edges in subnetwork 2 could be explained by the physiological changes of *L. invasa* gall development on the host plant, which outweigh the impact of general environmental factors. These findings might also be influenced by biotic factors, e.g. microbial interactions. Subnetwork 2 also has a higher average number of neighbours than subnetwork 1, which agrees with the fungal community composition pattern shown in our PCoA and PERMANOVA analyses.

Trait analysis suggested that potential phytopathogens occur in the fungal community of both, *L. invasa* infested tissues, as well as healthy/mildly infested plants. Fungi that are reputedly 'phytopathogenic' were also recovered from healthy plant tissues in multiple previous studies (Rodriguez et al., 2009; Hardoim

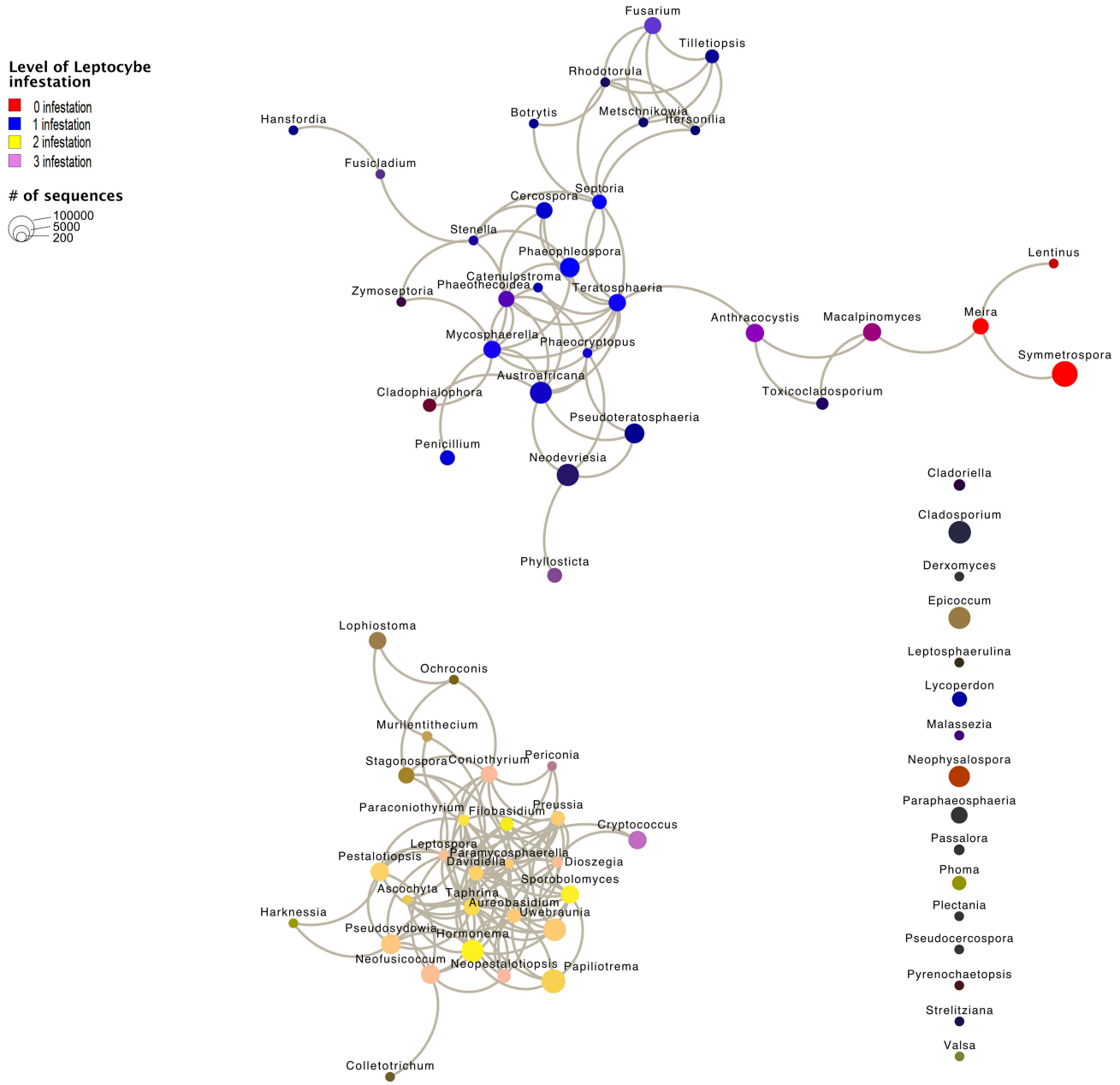


FIGURE 6 | Network graph calculated using Calypso. Node size represents the abundance of the genus. Edges are significant correlations between the nodes. Node colour is made up of four infestation levels of the wasp *Leptocybe invasa* (0—[red] no infestation, 1—[blue] infestation with evidence of oviposition, but no gall development, 2—[yellow] infestation with galls on leaves, mid-ribs or petioles and 3—[mauve] infestation with stunted overall growth and lethal gall formation). Node colours are merged based on the correlation to the *Leptocybe invasa* infestation level, e.g. *Fusarium* (purple) appears mainly in *Leptocybe invasa* infestation levels 0 and 1 samples. 16 fungal genera did not show significant correlations.

et al., 2015; Almario et al., 2017). It has been proposed that stress (e.g. pathogen attack or drought) impacts a plant's microbiome, likely *via* alterations in physiology, which could result in favourable conditions for pathogens to cause disease (Slippers and Wingfield, 2007; Sherwood et al., 2015; Oliva et al., 2021). Such stress is, however, not a prerequisite for infection by pathogens, who then remain dormant until conditions are conducive to their further development.

We found an increase in abundance of several Mycosphaerellaceae taxa with increasing *L. invasa* infestation level, indicating that some Mycosphaerellaceae taxa are favoured by the morphological and physiological changes in the leaf *E. grandis* environment after *L. invasa* oviposition. Our results support a newly proposed micro-evolutionary approach of fungal ecological niches (Selosse et al., 2018). We have at least two different micro-environments in our study, namely, the healthy and the gall surrounding tissues.

TABLE 1 | Summary network statistics calculated in Cytoscape.

	Subnetwork 1	Subnetwork 2
Number of nodes	30	26
Number of edges	61	98
Average number of neighbours	4.067	7.538

The subnetworks refer to **Figure 6** *where subnetwork 1 is made up of fungal genera with high abundances in samples with Leptocybe invasa infestation levels 0 and 1 and subnetwork 2 is made up of fungal genera with high abundances in samples with Leptocybe invasa infestation levels 2 and 3.*

Growing evidence that many fungi have more complex niche adaptations than previously imagined has been reported (Selosse et al., 2018). Although the number of pathogenic taxa in our study is similar between the micro-environments, some pathogenic taxa (i.e. Mycosphaerellaceae) have more counts (higher abundance) in gall surrounding tissues compared to healthy leaf tissues. Our study raises the questions, why potentially pathogenic taxa abundances change and what the relevance of microbial interactions following physiological changes in the host is Carrión et al. (2019).

In conclusion, we were able to show that the fungal communities surrounding *L. invasa* oviposition sites change with an increasing infestation level. This knowledge helps to further understand the outcomes of the *E. grandis-L. invasa* interaction, as well as factors that influence fungal community composition in general. Future work could use functional genetic studies to untangle the intricacies of biotic interactions in this system by using combined microscopic, transcriptomic, proteomic and metabolomic approaches.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at https://www.ncbi.nlm.nih.gov/, PRJNA791551.

AUTHOR CONTRIBUTIONS

BS, MK and SN conceived the study. FW and MM prepared the sequence library. AB performed Illumina sequencing. MM, MV, and MK performed the data analysis, visualisation and interpretation. MM drafted the initial manuscript. BS, SN and DB obtained funding to support the research. All authors contributed to the article and approved the submitted version.

FUNDING

Funding for this work was provided by members of the Tree Protection Co-operative Programme (TPCP) and the DST/NRF Centre of Excellence in Plant Health Biotechnology (CPHB). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscripts.

ACKNOWLEDGMENTS

We thank Lorraine Mhoswa and the Eucalyptus and Pine Pathogen Interaction (EPPI) research group for helping with the data and sample collections. We thank the Department of Evolution of Plants and Fungi for the help during sequence library preparation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fmicb.2022.841621/full#supplementary-material

REFERENCES

Abdelfattah, A., Wisniewski, M., Nicosia, M. G. L. D., Cacciola, S. O., and Schena, L. (2016). Metagenomic analysis of fungal diversity on strawberry plants and the effect of management practices on the fungal community structure of aerial organs. *PLoS One* 11:e0160470. doi: 10.1371/journal.pone.0160470

Allison, S. D., and Schultz, J. C. (2005). Biochemical responses of chestnut oak to a galling cynipid. *J. Chem. Ecol.* 31, 151–166. doi: 10.1007/s10886-005-0981-5

Almario, J., Jeena, G., Wunder, J., Langen, G., Zuccaro, A., Coupland, G., et al. (2017). Root-associated fungal microbiota of nonmycorrhizal *Arabis alpina* and its contribution to plant phosphorus nutrition. *Proc. Natl. Acad. Sci.* 114, E9403–E9412. doi: 10.1073/pnas.1710455114

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

Bálint, M., Tiffin, P., Hallström, B., O'Hara, R. B., Olson, M. S., Fankhauser, J. D., et al. (2013). Host genotype shapes the foliar fungal microbiome of balsam poplar (*Populus balsamifera*). *PLoS One* 8:e53987. doi: 10.1371/journal.pone.0053987

Barbed, P. A., Smith, I. W., and Keane, P. J. (2003). Foliar diseases of *eucalyptus* spp. grown for ornamental cut foliage. *Australas. Plant Pathol.* 32, 109–111. doi: 10.1071/AP02055

Bennett, J. A., and Cahill, J. F. (2016). Fungal effects on plant–plant interactions contribute to grassland plant abundances: evidence from the field. *J. Ecol.* 104, 755–764. doi: 10.1111/1365-2745.12558

Berens, M. L., Berry, H. M., Mine, A., Argueso, C. T., and Tsuda, K. (2017). Evolution of hormone signaling networks in plant defense. *Annu. Rev. Phytopathol.* 55, 401–425. doi: 10.1146/annurev-phyto-080516-035544

Bettucci, L., and Alonso, R. (1997). A comparative study of fungal populations in healthy and symptomatic twigs of *Eucalyptus grandis* in Uruguay. *Mycol. Res.* 101, 1060–1064. doi: 10.1017/S0953756297003717

Bird, T., Kile, G. A., and Podger, F. D. (1974). The eucalypt crown diebacks—A growing problem for forest managers. *Aust. For.* 37, 173–187. doi: 10.1080/00049158.1974.10675609

Blanchet, F. G., Cazelles, K., and Gravel, D. (2020). Co-occurrence is not evidence of ecological interactions. *Ecol. Lett.* 23, 1050–1063. doi: 10.1111/ele.13525

Cameron, D. D., Neal, A. L., van Wees, S. C. M., and Ton, J. (2013). Mycorrhiza-induced resistance: more than the sum of its parts? *Trends Plant Sci.* 18, 539–545. doi: 10.1016/j.tplants.2013.06.004

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., et al. (2019). Pathogen-induced activation of disease-suppressive

functions in the endophytic root microbiome. *Science* 366, 606–612. doi: 10.1126/science.aaw9285

Csóka, G., Stone, G. N., and Melika, G. (2017). Non-native gall-inducing insects on forest trees: a global review. *Biol. Invasions* 19, 3161–3181. doi: 10.1007/s10530-017-1466-5

Dardeau, F., Deprost, E., Laurans, F., Lainé, V., Lieutier, F., and Sallé, A. (2014). Resistant poplar genotypes inhibit pseudogall formation by the wooly poplar aphid, *Phloeomyzus passerinii* sign. *Trees* 28, 1007–1019. doi: 10.1007/s00468-014-1014-1

de Mendiburu, F., and Yaseen, M. (2021). Agricolae: statistical procedures for agricultural research. R package version 1.4.0. Available at: https://cran.r-project.org/web/packages/agricolae/agricolae.pdf (Accessed November 24, 2021).

Dittrich-Schröder, G., Hoareau, T. B., Hurley, B. P., Wingfield, M. J., Lawson, S., Nahrung, H. F., et al. (2018). Population genetic analyses of complex global insect invasions in managed landscapes: a *Leptocybe invasa* (hymenoptera) case study. *Biol. Invasions* 20, 2395–2420. doi: 10.1007/s10530-018-1709-0

Douanla-Meli, C., Langer, E., and Talontsi Mouafo, F. (2013). Fungal endophyte diversity and community patterns in healthy and yellowing leaves of *Citrus Limon*. *Fungal Ecol.* 6, 212–222. doi: 10.1016/j.funeco.2013.01.004

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fernandez-Conradi, P., Fort, T., Castagneyrol, B., Jactel, H., and Robin, C. (2019). Fungal endophyte communities differ between chestnut galls and surrounding foliar tissues. *Fungal Ecol.* 42:100876. doi: 10.1016/j.funeco.2019.100876

Ferreira, B. G., Carneiro, R. G. S., and Isaias, R. M. S. (2015). Multivesicular bodies differentiate exclusively in nutritive fast-dividing cells in *Marcetia taxifolia* galls. *Protoplasma* 252, 1275–1283. doi: 10.1007/s00709-015-0759-8

Foster, Z. S. L., Sharpton, T. J., and Grünwald, N. J. (2017). Metacoder: An R package for visualization and manipulation of community taxonomic diversity data. *PLoS Comput. Biol.* 13:e1005404. doi: 10.1371/journal.pcbi.1005404

Gardes, M., and Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113–118. doi: 10.1111/j.1365-294X.1993.tb00005.x

Geuss, D., Stelzer, S., Lortzing, T., and Steppuhn, A. (2017). *Solanum dulcamara*'s response to eggs of an insect herbivore comprises ovicidal hydrogen peroxide production. *Plant Cell Environ.* 40, 2663–2677. doi: 10.1111/pce.13015

Giron, D., Huguet, E., Stone, G. N., and Body, M. (2016). Insect-induced effects on plants and possible effectors used by galling and leaf-mining insects to manipulate their host-plant. *J. Insect Physiol.* 84, 70–89. doi: 10.1016/j.jinsphys.2015.12.009

Gluck-Thaler, E., Haridas, S., Binder, M., Grigoriev, I. V., Crous, P. W., Spatafora, J. W., et al. (2020). The architecture of metabolism maximizes biosynthetic diversity in the largest class of fungi. *Mol. Biol. Evol.* 37, 2838–2856. doi: 10.1093/molbev/msaa122

Gomes, E. A., Lana, U. G. P., Quensen, J. F., de Sousa, S. M., Oliveira, C. A., Guo, J., et al. (2018). Root-associated microbiome of maize genotypes with contrasting phosphorus use efficiency. *Phytobiomes J.* 2, 129–137. doi: 10.1094/PBIOMES-03-18-0012-R

Griese, E., Dicke, M., Hilker, M., and Fatouros, N. E. (2017). Plant response to butterfly eggs: inducibility, severity and success of egg-killing leaf necrosis depends on plant genotype and egg clustering. *Sci. Rep.* 7:7316. doi: 10.1038/s41598-017-06704-z

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

Hong, B.-Y., Araujo, M. V. F., Strausbaugh, L. D., Terzi, E., Ioannidou, E., and Diaz, P. I. (2015). Microbiome profiles in priodontitis in relation to host and disease characteristics. *PLoS One* 10:e0127077. doi: 10.1371/journal.pone.0127077

Huang, M.-Y., Huang, W.-D., Chou, H.-M., Lin, K.-H., Chen, C.-C., Chen, P.-J., et al. (2014). Leaf-derived cecidomyiid galls are sinks in *Machilus thunbergii* (Lauraceae) leaves. *Physiol. Plant.* 152, 475–485. doi: 10.1111/ppl.12186

Hunter, G. C., Crous, P. W., Carnegie, A. J., Burgess, T. I., and Wingfield, M. J. (2011). Mycosphaerella and Teratosphaeria diseases of *eucalyptus*; easily confused and with serious consequences. *Fungal Divers.* 50, 145–166. doi: 10.1007/s13225-011-0131-z

Hurley, B. P., Garnas, J., Wingfield, M. J., Branco, M., Richardson, D. M., and Slippers, B. (2016). Increasing numbers and intercontinental spread of invasive insects on eucalypts. *Biol. Invasions* 18, 921–933. doi: 10.1007/s10530-016-1081-x

Jimu, L., Wingfield, M. J., Mwenje, E., and Roux, J. (2015). Diseases on *eucalyptus* species in Zimbabwean plantations and woodlots. *South. For. J. For. Sci.* 77, 221–230. doi: 10.2989/20702620.2014.1001682

Kemen, E. (2014). Microbe–microbe interactions determine oomycete and fungal host colonization. *Curr. Opin. Plant Biol.* 20, 75–81. doi: 10.1016/j.pbi.2014.04.005

Kemler, M., Garnas, J., Wingfield, M. J., Gryzenhout, M., Pillay, K.-A., and Slippers, B. (2013). Ion torrent PGM as tool for fungal community analysis: a case study of endophytes in *Eucalyptus grandis* reveals high taxonomic diversity. *PLoS One* 8:e81718. doi: 10.1371/journal.pone.0081718

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Lawson, S. P., Christian, N., and Abbot, P. (2014). Comparative analysis of the biodiversity of fungal endophytes in insect-induced galls and surrounding foliar tissue. *Fungal Divers.* 66, 89–97. doi: 10.1007/s13225-013-0268-z

Márquez, S. S., Bills, G. F., and Zabalgogeazcoa, I. (2011). Fungal species diversity in juvenile and adult leaves of *Eucalyptus globulus* from plantations affected by Mycosphaerella leaf disease. *Ann. Appl. Biol.* 158, 177–187. doi: 10.1111/j.1744-7348.2010.00449.x

Mendel, Z., Protasov, A., Fisher, N., and La Salle, J. (2004). Taxonomy and biology of *Leptocybe invasa* gen. & sp. n. (hymenoptera: Eulophidae), an invasive gall inducer on *eucalyptus*. *Aust. J. Entomol.* 43, 101–113. doi: 10.1111/j.1440-6055.2003.00393.x

Mhoswa, L., O'Neill, M. M., Mphahlele, M. M., Oates, C. N., Payn, K. G., Slippers, B., et al. (2020). A genome-wide association study for resistance to the insect pest *Leptocybe invasa* in *Eucalyptus grandis* reveals genomic regions and positional candidate defense genes. *Plant Cell Physiol.* 61, 1285–1296. doi: 10.1093/pcp/pcaa057

Naidoo, S., Christie, N., Acosta, J. J., Mphahlele, M. M., Payn, K. G., Myburg, A. A., et al. (2018). Terpenes associated with resistance against the gall wasp, *Leptocybe invasa*, in *Eucalyptus grandis*. *Plant Cell Environ.* 41, 1840–1851. doi: 10.1111/pce.13323

Naidoo, S., Naidoo, R., Oates, C., Wilken, F., and Myburg, A. (2011). Investigating *eucalyptus* – pathogen and pest interactions to dissect broad spectrum defense mechanisms. *BMC Proc.* 5:P97. doi: 10.1186/1753-6561-5-S7-P97

Oates, C. N., Denby, K. J., Myburg, A. A., Slippers, B., and Naidoo, S. (2021). Insect egg-induced physiological changes and transcriptional reprogramming leading to gall formation. *Plant Cell Environ.* 44, 535–547. doi: 10.1111/pce.13930

Oates, C. N., Külheim, C., Myburg, A. A., Slippers, B., and Naidoo, S. (2015). The transcriptome and terpene profile of *Eucalyptus grandis* reveals mechanisms of defence against the insect pest, *Leptocybe invasa*. *Plant Cell Physiol.* 56, 1418–1428. doi: 10.1093/pcp/pcv064

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2018). Vegan: community ecology package. R Package Version, 2.5-2.

Oliva, J., Ridley, M., Redondo, M. A., and Caballol, M. (2021). Competitive exclusion amongst endophytes determines shoot blight severity on pine. *Funct. Ecol.* 35, 239–254. doi: 10.1111/1365-2435.13692

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

Põlme, S., Abarenkov, K., Henrik Nilsson, R., Lindahl, B. D., Clemmensen, K. E., Kauserud, H., et al. (2020). Fungal traits: a user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Divers.* 105, 1–16. doi: 10.1007/s13225-020-00466-2

R Core Team (2018). R: A Language and Environment for Statistical Computing. R Found. Stat. Comput.

Rocha, S., Branco, M., Boas, L. V., Almeida, M. H., Protasov, A., and Mendel, Z. (2013). Gall induction may benefit host plant: a case of a gall wasp and eucalyptus tree. *Tree Physiol.* 33, 388–397. doi: 10.1093/treephys/tpt009

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

Rojas, E. C., Sapkota, R., Jensen, B., Jørgensen, H. J. L., Henriksson, T., Jørgensen, L. N., et al. (2020). Fusarium head blight modifies fungal endophytic communities during infection of wheat spikes. *Microb. Ecol.* 79, 397–408. doi: 10.1007/s00248-019-01426-3

Roux, J., Myburg, H., Wingfield, B. D., and Wingfield, M. J. (2003). Biological and phylogenetic analyses suggest that two *Cryphonectria* spp. cause cankers of *eucalyptus* in Africa. *Plant Dis.* 87, 1329–1332. doi: 10.1094/PDIS.2003.87.11.1329

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Selosse, M.-A., Schneider-Maunoury, L., and Martos, F. (2018). Time to re-think fungal ecology? Fungal ecological niches are often prejudged. *New Phytol.* 217, 968–972. doi: 10.1111/nph.14983

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shen, Q., Liu, Y., and Naqvi, N. I. (2018). Fungal effectors at the crossroads of phytohormone signaling. *Curr. Opin. Microbiol.* 46, 1–6. doi: 10.1016/j.mib.2018.01.006

Sherwood, P., Villari, C., Capretti, P., and Bonello, P. (2015). Mechanisms of induced susceptibility to *Diplodia* tip blight in drought-stressed Austrian pine. *Tree Physiol.* 35, 549–562. doi: 10.1093/treephys/tpv026

Slippers, B., and Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biol. Rev.* 21, 90–106. doi: 10.1016/j.fbr.2007.06.002

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540

Tytgat, H. L. P., Nobrega, F. L., van der Oost, J., and de Vos, W. M. (2019). Bowel biofilms: tipping points between a healthy and compromised gut? *Trends Microbiol.* 27, 17–25. doi: 10.1016/j.tim.2018.08.009

Vivas, M., Kemler, M., Mphahlele, M. M., Wingfield, M. J., and Slippers, B. (2017). Maternal effects on phenotype, resistance and the structuring of fungal communities in *Eucalyptus grandis*. *Environ. Exp. Bot.* 140, 120–127. doi: 10.1016/j.envexpbot.2017.06.002

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc. Guide Methods Appl.* 18, 315–322. doi: 10.1016/B978-0-12-372180-8.50042-1

Wingfield, M. J., Brockerhoff, E. G., Wingfield, B. D., and Slippers, B. (2015). Planted forest health: The need for a global strategy. *Science* 349, 832–836. doi: 10.1126/science.aac6674

Zakrzewski, M., Proietti, C., Ellis, J. J., Hasan, S., Brion, M.-J., Berger, B., et al. (2016). Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. *Bioinformatics* 33, btw725–btw783. doi: 10.1093/bioinformatics/btw725

Zaneveld, J. R., McMinds, R., and Vega Thurber, R. (2017). Stress and stability: applying the Anna Karenina principle to animal microbiomes. *Nat. Microbiol.* 2:17121. doi: 10.1038/nmicrobiol.2017.121

Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., et al. (2018). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res.* 46, W95–W101. doi: 10.1093/nar/gky418

Zheng, X. L., Li, J., Yang, Z. D., Xian, Z. H., Wei, J. G., Lei, C. L., et al. (2014). A review of invasive biology, prevalence and management of *Leptocybe invasa* Fisher & La Salle (hymenoptera: Eulophidae: Tetrastichinae). *Afr. Entomol.* 22, 68–79. doi: 10.4001/003.022.0133

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.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 April 2022
doi: 10.3389/fmicb.2022.845310

Microbial Cross-Talk: Dissecting the Core Microbiota Associated With Flue-Cured Tobacco (*Nicotiana tabacum*) Plants Under Healthy and Diseased State

Waqar Ahmed[1,2,3], Zhenlin Dai[2,3], Qi Liu[2,3], Shahzad Munir[2], Jun Yang[2,3,4], Samantha C. Karunarathna[5], Shichen Li[6], Jinhao Zhang[2,3], Guanghai Ji[2,3] and Zhengxiong Zhao[1]**

[1] College of Resources and Environment, Yunnan Agricultural University, Kunming, China, [2] State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan Agricultural University, Kunming, China, [3] Key Laboratory of Agro-Biodiversity and Pest Management of Ministry of Education, Yunnan Agricultural University, Kunming, China, [4] College of Resources, Environment, and Chemistry, Chuxiong Normal University, Chuxiong, China, [5] Biological Resources Protection and Utilization, College of Biological Resources and Food Engineering, Qujing Normal University, Qujing, China, [6] College of Agronomy and Biotechnology, Yunnan Agricultural University, Kunming, China

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer,
Bangladesh Agricultural University,
Bangladesh

Reviewed by:
Muhammad Aamer,
Jiangxi Agricultural University, China
Chunhao Jiang,
Nanjing Agricultural University, China

***Correspondence:**
Zhengxiong Zhao
zhaozx0801@163.com
Guanghai Ji
jghai001@163.com

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: 29 December 2021
Accepted: 28 January 2022
Published: 14 April 2022

Citation:
Ahmed W, Dai Z, Liu Q, Munir S, Yang J, Karunarathna SC, Li S, Zhang J, Ji G and Zhao Z (2022) Microbial Cross-Talk: Dissecting the Core Microbiota Associated With Flue-Cured Tobacco (Nicotiana tabacum) Plants Under Healthy and Diseased State. Front. Microbiol. 13:845310. doi: 10.3389/fmicb.2022.845310

Bacterial wilt caused by *Ralstonia solanacearum* is a devastating disease of flue-cured tobacco production which poses significant yield losses all around the world. In this study, we evaluated the rhizosphere microbiome of healthy and bacterial wilt-infected (diseased) flue-cured tobacco plants through amplification of V3-V4 and ITS1-5f variable regions of 16S and internal transcribed spacer (ITS) rRNA. The study was based on the location (Qujing, Shilin, and Wenshan), plant components (rhizosphere soil and roots), and sample types (healthy and diseased) to assess the diversity of bacterial and fungal communities. Bacterial and fungal communities present in roots primarily emanated from rhizosphere soil. Healthy flue-cured tobacco plants exhibit high microbial diversity compared to diseased plants. Among three variables, plant components significantly influence the diversity of microbial communities, whereas rhizosphere soil harbors higher microbial diversity than roots. Bacterial phyla Cyanobacteria and Proteobacteria were found in high relative abundance in roots and rhizosphere soil samples, respectively. As far as fungi is concerned, a high relative abundance of Ascomycota and Basidiomycota was found in both rhizosphere soil and root. Bacterial genera such as *Bacillus*, *Bradyrhizobium*, *Ensifer*, *Neorhizobium*, and *Lysobacter* related to plant growth promotion and disease suppressing abilities were dominant than fungal genera. Analysis of relative abundance at specie-level revealed that most fungal species are pathogenic to flue-cured tobacco and could provide a conducive environment for wilt infection. In conclusion, *R. solanacearum* significantly influences the microbial diversity of flue-cured tobacco plants and negatively affects the bacterial community composition. Altogether, our study demonstrates the complexity of bacterial and fungal communities

that possibly interact with each other (microbe–microbe) and host (host–microbe). This cross-talk could be helpful for healthy flue-cured tobacco plant growth and to induce resistance against bacterial wilt disease.

Keywords: flue-cured tobacco, disease resistance, locations, microbial diversity, plant components, *Ralstonia solanacearum*

INTRODUCTION

Bacterial wilt disease caused by soilborne pathogenic bacterium *Ralstonia solanacearum* is a serious threat to flue-cured tobacco (*Nicotiana tabacum* L.) production worldwide, including China (Cai et al., 2021). *Ralstonia solanacearum* is widely distributed in tropical and subtropical regions of the world and has a broad host range that infects more than 250 plant species (Paudel et al., 2020). It is a serious threat to important field crops of the *Solanaceae* family including tomato, potato, tobacco, ginger, eggplant, and pepper, with average yield losses that range from 10 to 55% (Kim et al., 2016).

Ralstonia solanacearum infects all tobacco plant parts (roots, stalk, and leaves) and generally produces symptoms of yellowing and wilting of leaves, discoloration of xylem vessels, and black necrotic spots on the stem. It multiplies systemically in the xylem vessels followed by death of whole plant (Cai et al., 2021). Infected soil acts as a primary source of inoculum, and the pathogen oozes out in the rhizosphere soil of diseased plants from the roots upon completion of life cycle (Wu et al., 2020). The bacterium survives in soil, water, and plant residues for a longer period as saprophytism in the absence of a specific host plant (Li et al., 2014). It spreads from diseased to healthy plants through rain splashes, irrigation water, and mechanical operations (Qi et al., 2020).

As far as losses caused by this disease are concerned, in China, incidence and yield losses vary from region to region, host to host, climatic condition, and pathogenic strain (Jiang et al., 2017). However, incidence and yield losses are recorded between 15 to 35% but can reach up to 75% and 50–60%, respectively, when disease is present with root rot pathogen *Phytophtora nicotianae* (Jiang et al., 2017). In high humidity and mono-cropping regions, it occurs in epidemic form, and yield losses reach up to 100%. Soil physicochemical properties, climatic conditions, locations, and rhizosphere microbial diversity play an important role on the occurrence of soilborne diseases (Cai et al., 2021).

The plant rhizosphere is considered as one of the most complex ecosystems on earth and hot spot habitat for diverse microbes (Raaijmakers, 2015). Most disease-resistant and developmental mechanisms in plants are directly related to the diversity of rhizosphere microbes (Mendes et al., 2013; Dong et al., 2019). Plant genotype and soil type are the two main factors that are responsible for assembling a healthy rhizosphere microbiome (Dong et al., 2019). Advancements in science and modern sequencing tools made the study of host–microbe interaction easier (Govindasamy et al., 2014; Mhlongo et al., 2018). However, knowledge gaps are still present between host–microbe interactions and their underlying mechanism, which need to be filled (Bulgarelli et al., 2013).

Nowadays, biological control *via* disease suppressive specific endophytes and rhizobacteria such as *Bacillus*, *Lysobacter*, *Streptomyces*, and *Pseudomonas* is considered as practical approach to suppress the incidence of many soilborne diseases including bacterial wilt, *Fusarium* wilt, and clubroot by the mechanism of direct antagonism, reshaping the rhizospheric microbial diversity, and the production of metabolites (Ma et al., 2018; Wu et al., 2020; Zhang et al., 2020a; Wei et al., 2021). The application of bioorganic fertilizer and biochar along with biocontrol agents significantly suppresses the incidence of tobacco bacterial wilt disease (Liu et al., 2013; Zhang et al., 2017; Li et al., 2022). Because of the broad host range, species complexity, wide geographical distribution, and persistent nature, no effective control exists to date against this devastating disease, and it is difficult to completely control the incidence of tobacco bacterial wilt disease (Li et al., 2014; Shen et al., 2018).

Flue-cured tobacco is a major cash crop in Yunnan Province, China. Yunnan is well-known for its unique environment and climatic conditions. It produces high-quality flue-cured tobacco famous for its pure taste, fragrant aroma, and golden color (Li et al., 2019). Yunnan produces about 50% of China's total tobacco leaf yield, with an annual production of around 750,000 tons and 320,000 ha of agricultural land under tobacco cultivation (Tang et al., 2020). However, *R. solanacearum* poses significant yield losses every year. Thus, to successfully mitigate the bacterial wilt pathogen, it is necessary to understand the population dynamics and distribution of the microbiome in tobacco plants. Therefore, the present study aims to explore the core microbiota (bacteria and fungi) associated with different locations (Qujing, Shilin, and Wenshan), plant components (rhizosphere soil and roots), and nature of plant (healthy and diseased). We hypothesized that this study helps us to establish a model for studying the naturally occurring flue-cured tobacco microbiome to mitigate the incidence of tobacco bacterial wilt disease.

MATERIALS AND METHODS

Sample Collection

Rhizosphere soil and root samples were collected from healthy and diseased tobacco plants from three locations; Qujing (25.4900°N, 103.7962°E), Shilin (25.0950°N, 121.5246°E), and Wenshan (23.3863°N, 104.2325°E) in Yunnan Province, China, in September 2020 (**Figure 1**). Tobacco has been continuously grown in those fields for the past 10 years. For sample collection, upper 2–3 cm layer of soil was removed, and tobacco plants were uprooted (three plants per field from three different fields for both healthy and diseased plants). Bulk soil was removed by

shaking the roots, and the tiny soil particles attached to roots were collected as rhizosphere soil samples and fibrous roots as root samples. A total of 12 composite samples (three replicates per sample) were collected from three different locations in Yunnan (**Supplementary Figure 1**). Samples were put in polythene bags and placed in an icebox until delivered to the laboratory and stored at −80°C for further study.

DNA Extraction and Polymerase Chain Reaction Amplification

Total genomic DNA was extracted from 0.5 g of soil per sample to 1 g of roots per sample using the Soil and Plant DNA Extraction Kit (Zymo Research Corp., Irvine, CA, United States), respectively, following the manufacturer's instructions. The quality of extracted DNA was quantified at $OD_{260/280\ nm}$ 1.7-1.9 using a NanoDrop spectrophotometer (ND2000, Thermo Scientific, Madison, WI, United States), and extracted DNA was stored at -20°C for future use. The V3-V4 and ITS1-5F variable regions of 16S and internal transcribed spacer (ITS) rRNA genes of bacteria and fungi were amplified using two pairs of universal primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), and 1743F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and 2043R (5′-GCTGCGTTCTTCATCGATGC-3′) for bacterial and fungal diversity analysis, respectively (Zhang et al., 2020a).

Library Preparation and Sequencing

Amplicon library was prepared by the Nextera XT Index Kit (Illumina Inc. Madison, WI, United States) as per 16S and ITS Metagenomic Sequencing Library preparation protocols. Amplicon quality was visualized using gel electrophoresis, and 1X AMPure XP beads were used for amplicons library purification, checked on Agilent DNA1000 chip with Bioanalyzer2100, and quantified by Qubit Fluorometer 2.0 using a Qubit dsDNA assay kit (Life Technologies Cat. No. Q328520) (Gao et al., 2018). The same numbers of purified amplicons were pooled for subsequent sequencing analysis and sequenced on an Illumina MiSeq platform at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China).

Quality Control

Raw data were collected in FASTQ format from DNA sequencing, and Trimmomatic software was used for the cutoff of low-quality reads (score < 20) and preparation of paired-end reads (Bolger et al., 2014). FLASH software was used to assemble paired-end reads with 10 bp/200 bp and 20% minimum/maximum overlapping and maximum mismatch rate, respectively. UCHIME software was used for chimeras removal and production of clean reads (Edgar et al., 2011).

Data Processing

Clean reads were processed with the UPARSE pipeline to generate operational taxonomic units (OTUs) at ≥97% similarity level (Edgar, 2013). For taxonomic information, species annotation was performed for all representative read and OTUs using ribosomal database project (RDP) classifier in SILVA database for bacteria (at 70% confidence threshold) and UNITE database for fungi (Quast et al., 2012; Kõljalg et al., 2013). OTUs were analyzed for relative abundance at genus and phylum levels, and alpha and beta diversity indices were calculated to obtain species richness and uniformity information. A Venn diagram was used for common and unique OTUs among different variables such as sample types, plant components, and locations.

Statistical Analysis

Data were statistically analyzed using a *t*-test ($P < 0.05$). All statistical analyses were performed using IBM SPSS Verison 20.0 (SPSS Inc., Chicago, IL, United States). QIIME software (Version 1.9.1) was used to calculate the observed OTUs, Chao1, Shannon, and abundance-based coverage estimator (ACE) indices. The Bray–Curtis dissimilarity was calculated for beta diversity analysis of bacterial and fungal communities and used for principal coordinate analysis (PCoA) with QIIME. The relative abundance bar plots at the phylum level, relative abundance heatmaps at the genera level, and relative abundance bar plots at the species level were generated using R scripts in R software (version 2.15.3) (Dong et al., 2018). Co-occurrence network analysis was conducted using sparcc in R for OTUs at the phylum level ($P < 0.05$ and correlation coefficient > 0.3). The network properties were calculated and visualized in Gephi 0.9.2. All figures were processed and illustrated using Adobe Illustrator CC 2019 (Adobe Systems Inc., San Francisco, CA, United States).

RESULTS

General Characteristics of Tobacco Microbiome

We explored the bacterial and fungal communities associated with different parts of the tobacco plant (rhizosphere soil and roots), different geographic locations (Qujing, Shilin, and Wenshan), and different sample types (healthy and diseased). Data related to raw reads (#), clean reads (#), and quality control (Q20% and Q30%) through amplification of 16S (V3-V4)/ITS (1-5f) rRNA of bacteria/fungi, respectively, are shown in **Supplementary Table 1**. After quality control and chimeras filtering, an average of 80,477 bacterial and 92,685 fungal clean reads per sample were obtained with an average length of 412 bps/244 bps per sample by Illumina sequencing (**Supplementary Table 1**). Rarefaction curves generated from the OTUs demonstrated that high sampling coverage was achieved in all samples for both fungal and bacterial communities (**Supplementary Figure 2**).

Effects of Different Locations, Plant Components, and Sample Types on Beta Diversity

The effect of variables such as locations (Qujing, Shilin, and Wenshan), plant components (rhizosphere soil and roots), and sample types (healthy and diseased) on bacterial and fungal community composition was analyzed. Bray–Curtis dissimilarity was used to determine the beta diversity (variation in bacterial and fungal communities structure) for all 12 composite samples (**Figure 2**). Among the three variables, it was observed that

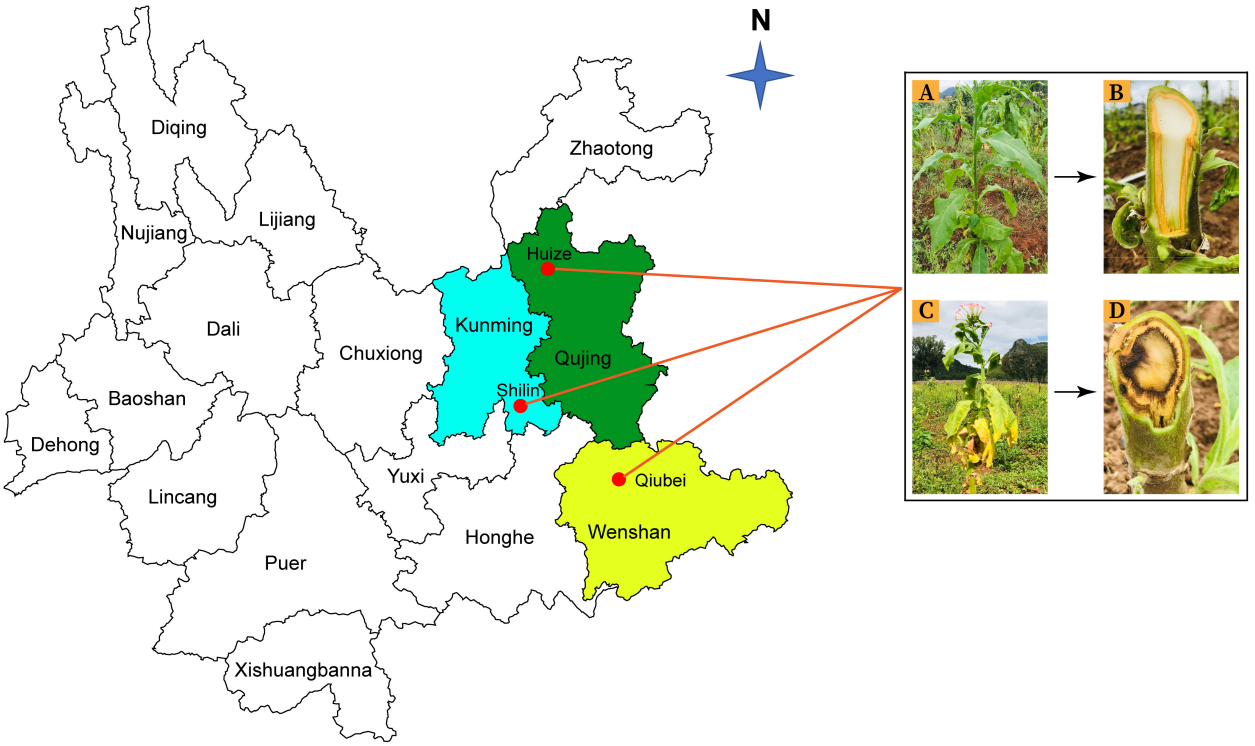


FIGURE 1 | Rhizosphere soil and root samples were collected from three different locations in Yunnan Province: Kunming (Shilin), Qujing (Huize), and Wenshan (Qiubei), from healthy and bacterial wilt infected flue-cured tobacco plants. Healthy flue-cured tobacco plant with green leaves and white-colored xylem vessels **(A,B)**. Disease flue-cured tobacco plant with typical bacterial wilt symptoms (yellowing, wilting of leaves, necrotic lesions on stem, and discoloration of xylem vessels) **(C,D)**.

plant components (rhizosphere soil and roots) significantly influenced bacterial and fungal community composition. The separation of samples at the one axis for bacterial community composition indicates that the influence of this variable on bacterial community structure was more significant than on fungal community structure. Sample types and locations have little impact on bacterial and fungal communities. A different pattern was observed using PCoA, with a difference of 37.17% and 18.67% in bacterial and fungal community composition, respectively. Distance heatmap graphs based on Weighted UniFrac (based on abundances of taxa) and Unweighted UniFrac (sensitive to rare taxa) of all 12 samples were generated to estimate the beta diversity of bacterial and fungal communities (**Supplementary Figure 3**).

Effects of Different Locations, Plant Components, and Sample Types on Alpha Diversity

The observed species, Shannon, Chao 1, and ACE at cutoff levels of 3% are shown in **Figure 3**. The locations have little impact on alpha diversity indices of bacterial communities compared with fungal communities (**Supplementary Table 2**). In plant components (rhizosphere soil and roots), alpha diversity indices of observed species, Shannon, Chao 1, and ACE for rhizosphere soil were found higher than those of roots for both bacterial and fungal communities. It indicates that the number of bacteria and fungi in the rhizosphere was higher than in roots. Among the sample types (healthy and diseased), alpha diversity indices of bacterial and fungal communities were more elevated in healthy samples than in diseased samples.

Analysis of Operational Taxonomy Units

Operational taxonomic units count was recorded maximum in rhizosphere soil samples than in root samples. For bacterial and fungal communities, the rhizosphere soil exhibited a high diversity and richness in OTUs compared with roots, and high diversity was found in healthy plant samples than in diseased plant samples (**Figure 4**). Analysis of OTUs revealed that a total of 4,233 and 3,014 specific OTUs were recovered for both bacterial and fungal communities from different locations (Qujing, Shilin, and Wenshan), respectively, and 1,849 (bacterial) and 949 (fungal) OTUs were found as common OTUs. Plant components (rhizosphere soil and roots) significantly impact the bacterial communities than fungal communities, and fungal OTU count in rhizosphere soil and roots was almost the same. In plant components, the specific bacterial OTUs in rhizosphere soil (1,520) were significantly higher than the specific OTUs (231) in the root tissue, and 2,482 OTUs were common. The specific fungal OTUs in root tissues (680) were slightly higher than specific OTUs (618) in rhizosphere soil, and 1,716 common OTUs were found in rhizosphere soil and roots. Further analysis of OTUs among sample types (healthy

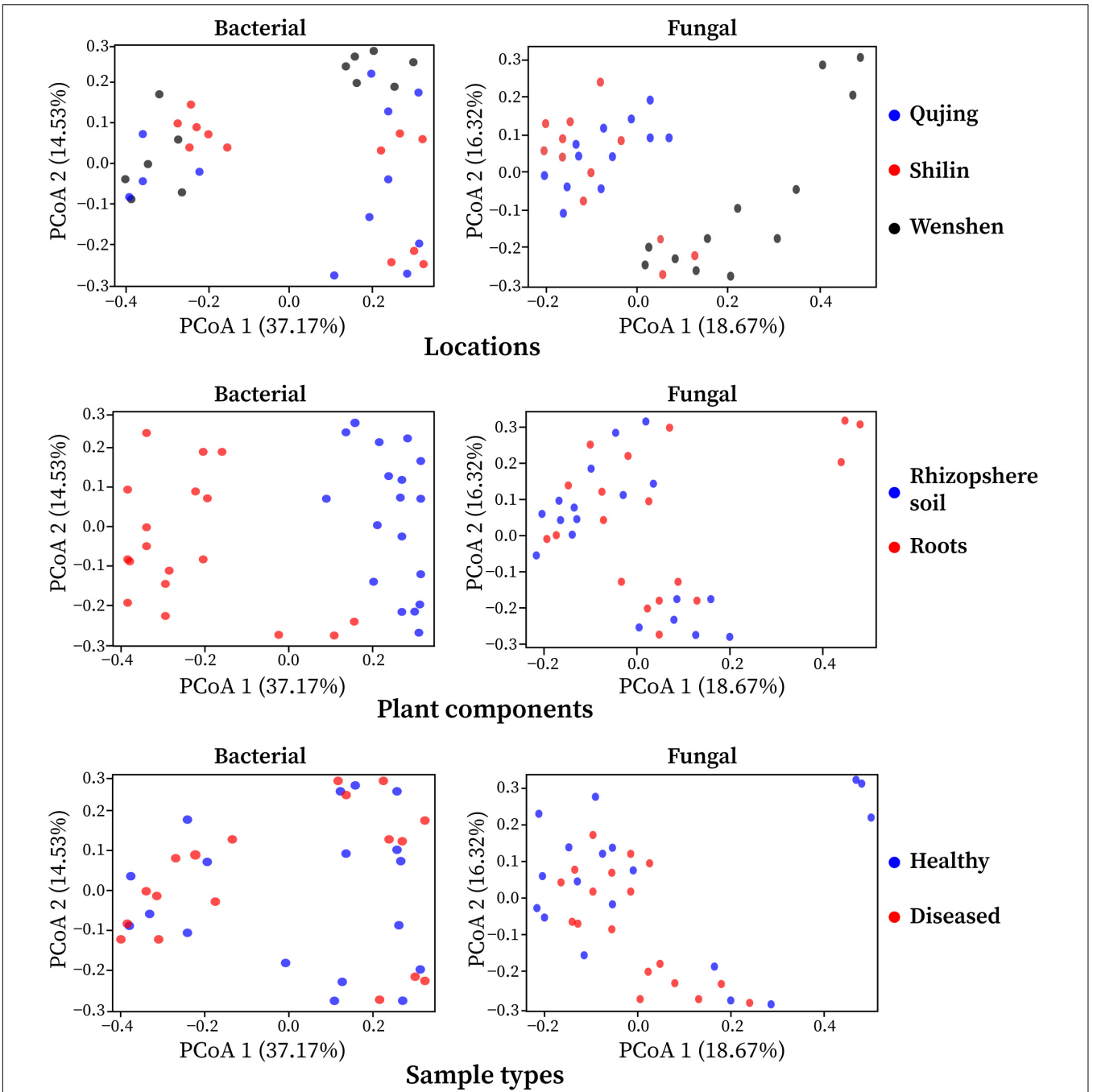


FIGURE 2 | Principal coordinate analysis (PCoA) was based on the Bray–Curtis dissimilarity metrics showing the beta diversity analysis for all 12 composite samples (three replicates per sample) of flue-cured tobacco plants under three variables.

and diseased) showed that a total of 703 and 407 unique bacterial OTUs and 3,123 common bacterial OTUs were found among healthy and diseased plants, respectively. The unique fungal OTUs in healthy samples (724) were significantly higher than diseased samples (391), and 1,899 OTUs were found as common. The results indicate that bacterial wilt pathogen had a more significant impact on bacterial communities than fungal communities.

Bacterial and Fungal Community Composition at Phylum Level

The top 10 bacterial and fungal phyla with relative abundance greater than 1% are shown in **Figure 5** and **Supplementary Table 3**. The dominant bacterial phyla in all rhizosphere soil and root samples with a relative abundance greater than 1% are Acidobacteriota,

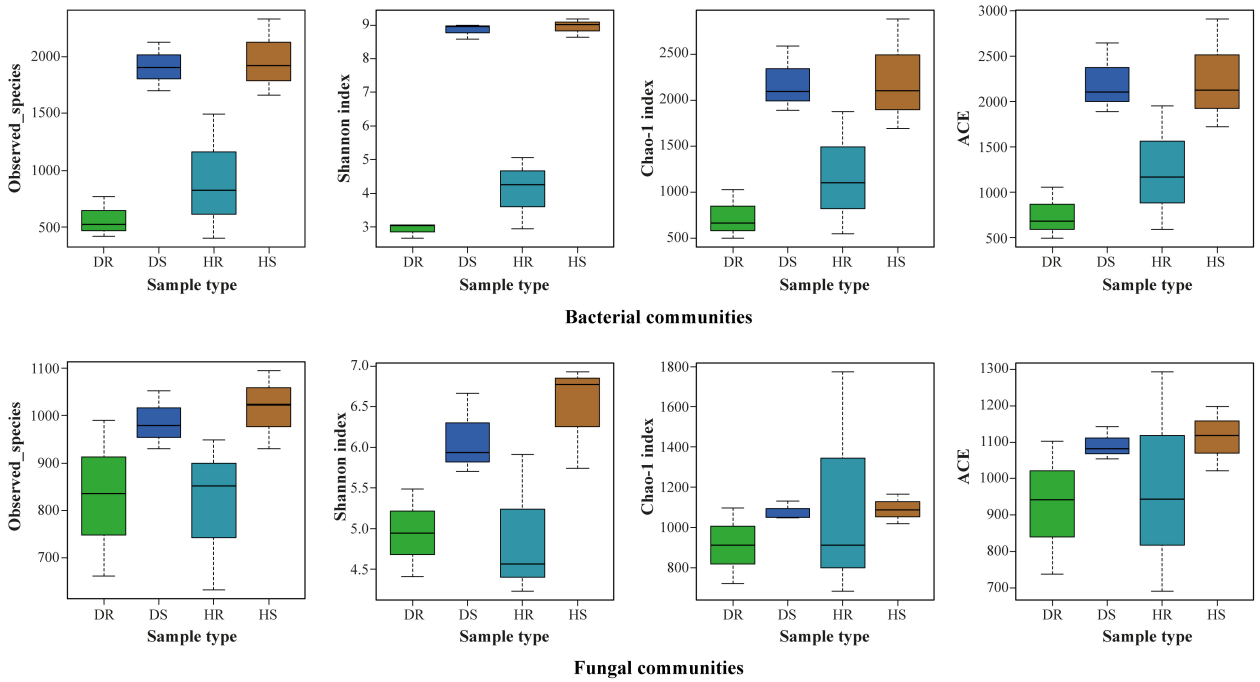


FIGURE 3 | Boxplot of bacterial **(top)** and fungal **(bottom)** showing alpha diversity indexes of flue-cured tobacco plants under three variables. HS, healthy rhizosphere soil; DS, diseased rhizosphere soil; HR, healthy roots; DR, diseased roots.

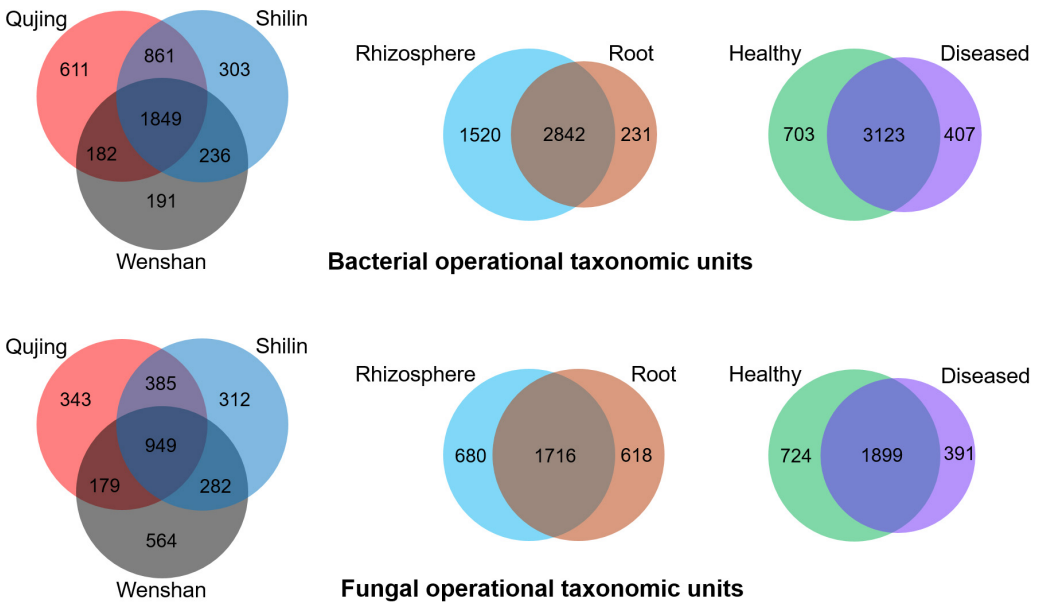


FIGURE 4 | Distribution of bacterial **(top)** and fungal **(bottom)** operational taxonomic units in three variables, i.e., locations (Qujing, Shilin, and Wenshan), plant components (rhizosphere soil and roots), and sample types (healthy and diseased).

Actinobacteriota, Bacteroidota, Cyanobacteria, Crenarchaeota, Chloroflexi, Gemmatimonadetes, Firmicutes, Proteobacteria, Unidentified_Bacteria, and others (**Figure 5A**). The dominant fungal phyla in all rhizosphere soil and root samples with a relative abundance greater than 1% are Ascomycota, Basidiomycota, Blastocladiomycota, Basidiobolomycota, Chytridiomycota, Glomeromycota, Mortierellomycota, Mucoromycota, Rozellomycota, Olpidiomycota, and others (**Figure 5B**). Among the three variables (locations, plant components, and sample types), plant components (rhizosphere

soil and roots) significantly influence the bacterial community composition. High relative abundance of phyla Cyanobacteria (average of 59.61%) and Proteobacteria (average of 21.44%) was found in roots; however, phyla Proteobacteria (average of 29.20%) and Actinobacteria (average of 16.46%) were found in high relative abundance in rhizosphere soil (**Figure 5A**). No significant impact was observed of these three variables on fungal community composition. Fungal phyla such as Ascomycota (average of 65 and 20%) and Basidiomycota (average of 62 and 10%) were present in high relative abundance both in root and rhizosphere soil samples, respectively. However, phylum Basidiomycota was found in low relative abundance in rhizosphere soil (average 10%) than in roots (average 20%) (**Figure 5B**).

Relative Abundance of Bacterial and Fungal Community Composition at Genera Level

We determined the relative abundance of the top 35 bacterial and fungal genera in the above screened top 10 bacterial and fungal phyla. On the basis of the species relative abundance in all samples, the top 35 bacterial and fungal genera were selected to create a heatmap to determine which genera present in high or low abundance in respective sample type (healthy and diseased). The relative abundance heatmaps of the top 35 bacterial and fungal genera in group-wise comparison under three variables (locations, plant components, and sample types) are shown in **Figure 6**. Bacterial genera such as *Streptomyces*, *Amycolatopsis*, and *Ensifer*; *Chitinophaga*, *Dyella*, Dongia, *Neorhizobium*, *Pelomonas*, *Pseudonocardia*, and *Sphingopyxis*; *Esherichia-Shigella*, *Sphingomonas*, *Ramlibacter*, *Flavisobater*, *Gemmatimonas*, and *Sphingobium*; and *Lysobacter*, *Arthrobacter*, *Bryobacter*, *Bacillus*, *MND1*, *Gaiella*, and *Pontibacter* were found in high relative abundance in diseased roots (DR), healthy roots (HR), diseased rhizosphere soil (DS), and healthy rhizosphere soil (HS), respectively. However, a high abundance of genus *Ralstonia* was present in diseased rhizosphere soil and root samples (**Figure 6A**). Similarly, fungal genera such as *Chaetomium*, *Gibberella*, *Myceliophthora*, and *Alternaria*; *Ophiocordyceps*, *Conocybe*, *Cercophora*, *Purpureocillium*, *Humicola*, and *Mortierella*; *Codinaea*, *Rhizophlycits*, *Ceratobasidium*, *Entoloma*, *Paramyrothecium*, *Setophoma*, and *Psathyrella*; and *Marasmius*, *Meyerozyma*, *Sampaiozyma*, and Unidentified_Ascomycota sp. were found in high relative abundance in DS, HS, DR, and HR, respectively. Whereas genus *Thanatephorus* was highly abundant with diseased rhizosphere soil and root samples (**Figure 6B**).

Relative Abundance of Bacterial and Fungal Communities at the Species Level

We assessed the relative abundance of the top 10 bacteria and fungi in the top 35 bacterial and fungal genera. The relative abundance bar plots for the top 10 bacteria and fungi in group-wise comparison under three variables (locations, plant components, and sample types) are shown in **Figure 7**. A high abundance of bacterial wilt pathogen *Ralstonia solanacearum* was found in diseased roots and rhizosphere soil. *Neorhizobium galegae* (well-known nitrogen fixer), *Ensifer adhaerens* (a bacterial predator of bacteria in the soil), and *Lysobacter dokdonensis* were found in high abundance in HR, DR, and HS, respectively (**Figure 7A**). Fungal species such as *Alternaria alternata* and *Thanatephorus cucumeris*, disease causing agents of tobacco brown spot and leaf spot disease, respectively, were found in high abundance with diseased rhizosphere soil and root samples. Moreover, higher abundance of *Fusarium* sp. was found in DR and DS (**Figure 7B**).

Characteristics of Co-occurrence Network

A microbial co-occurrence network was constructed for bacterial and fungal OTUs at the phylum level for each healthy and diseased sample associated with different plant components (rhizosphere soil and roots) (**Figure 8**). It was observed that, for bacterial communities, the average degree, number of nodes, and number of edges were found higher in healthy samples compared with diseased samples. In the fungal communities, the average degree, number of nodes, and number of edges were found higher in diseased samples than healthy samples while have no effect on the rhizosphere network. It indicates that the connection degree between members of the bacterial microbial network increased in healthy samples and members interact, whereas the fungal microbial network showed an opposite trend.

DISCUSSION

Ralstonia solanacearum is a serious threat to flue-cured tobacco production worldwide, including China. It is estimated that approximately 47,133 hm^{-2} lands are affected by this pathogen, resulting in millions dollars losses every year (Cai et al., 2021). Many integrated disease management strategies are adopted to control the incidence of bacterial wilt pathogen (Jiang et al., 2017). In recent years, biological control *via* disease suppressive biocontrol agents is considered a promising approach to reduce the incidence of many field crop diseases (Munir et al., 2021; Ahmed et al., 2022). Studies have proven that soil health and rhizosphere microbial diversity are the key factors responsible for soilborne disease occurrence and plant health (Köberl et al., 2013; Dong et al., 2018; Hao and Ashley, 2021).

Soil sickness affects both crop quality and yields when the same crop or its relative species are continuously grown in the same soil under a monocropping system (Zhang et al., 2020b). The rhizosphere microbiome acts as the first line of defense against soilborne pathogen infection and abiotic stress (Bulgarelli et al., 2013; Mendes et al., 2013). In previous studies, flue-cured tobacco bacterial wilt diseases associated with bacterial and fungal communities were studied by direct isolation and high-throughput sequencing technique. However, most studies performed on rhizosphere microbial diversity aimed at bacterial communities with little knowledge about fungal communities of flue-cured tobacco grown on a specific location in pots and field experiments after

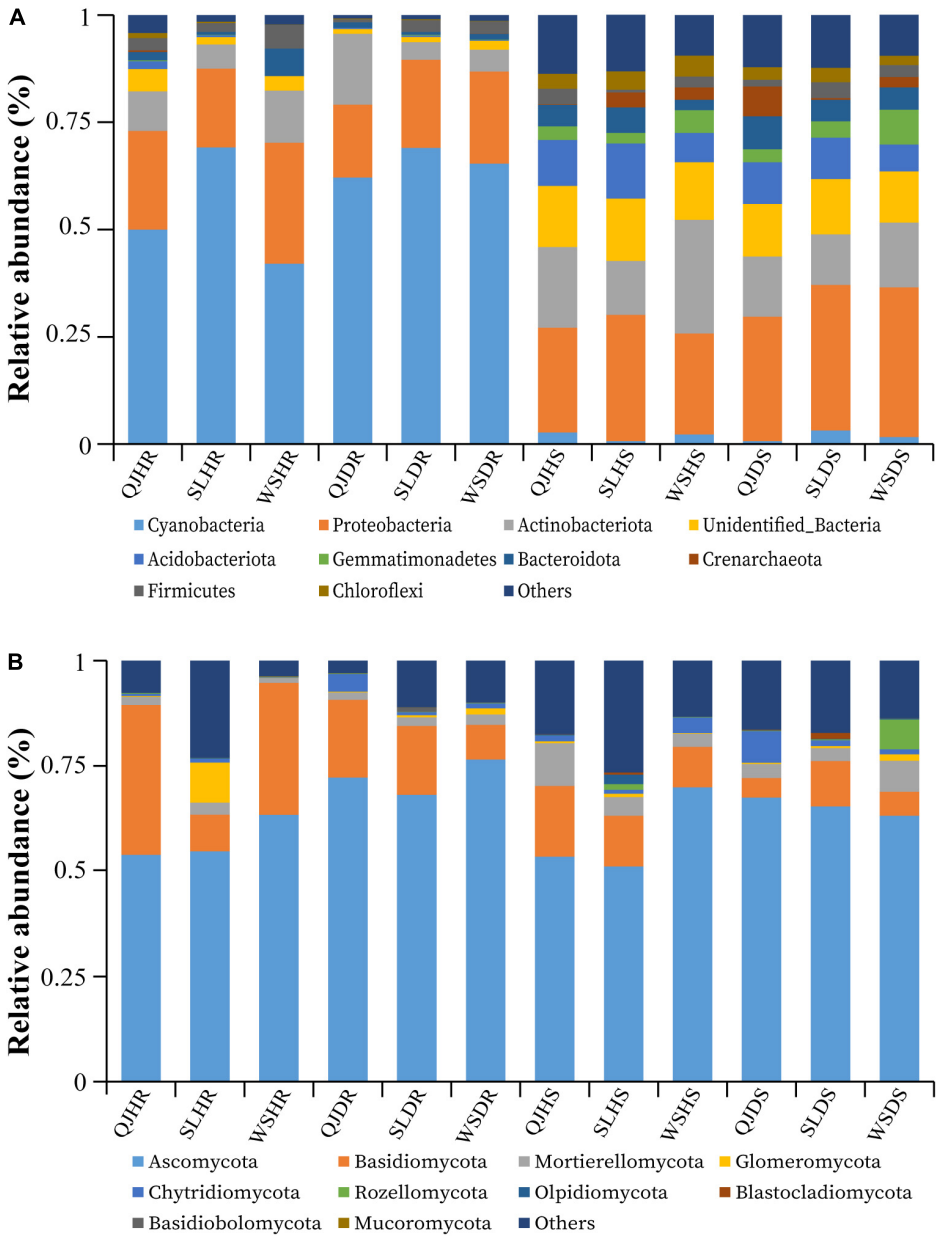


FIGURE 5 | Relative abundance bar plots at phylum level based on the species annotation results in 12 composite samples (average of three replicates per sample) of flue-cured tobacco plants under three variables. **(A)** Relative abundance at the phylum level in bacterial communities and **(B)** relative abundance at the phylum level in fungal communities. QJ, Qujing; SL, Shilin; WS, Wenshan; HS, healthy rhizosphere soil; DS, diseased rhizosphere soil; HR, healthy roots; DR, diseased roots.

artificial inoculation of *R. solanacearum* combined with other biocontrol agents.

In this study, we provided a comprehensive view of the naturally occurring core microbiome of healthy and bacterial wilt–infected (diseased) flue-cured tobacco plants. We presented an in-depth appraisal of bacterial and fungal communities associated with different locations, sample types, and plant components through 16S and ITS1-5f high-throughput sequencing analysis. Our results support the concept that distinct plant components have a significant impact on bacterial and fungal communities, irrespective of sample types and location.

Different plant components have been observed to study the impact of rhizospheric and endophytic bacterial communities on the growth and health of plants with no specific assemblage pattern (Compant et al., 2008; Lundberg et al., 2012; Lebeis et al., 2015). The plant's rhizosphere acts as the primary host, hot spot habitat, and passage for colonization of microbes into different plant components through roots, especially for bacterial communities (Bulgarelli et al., 2013;

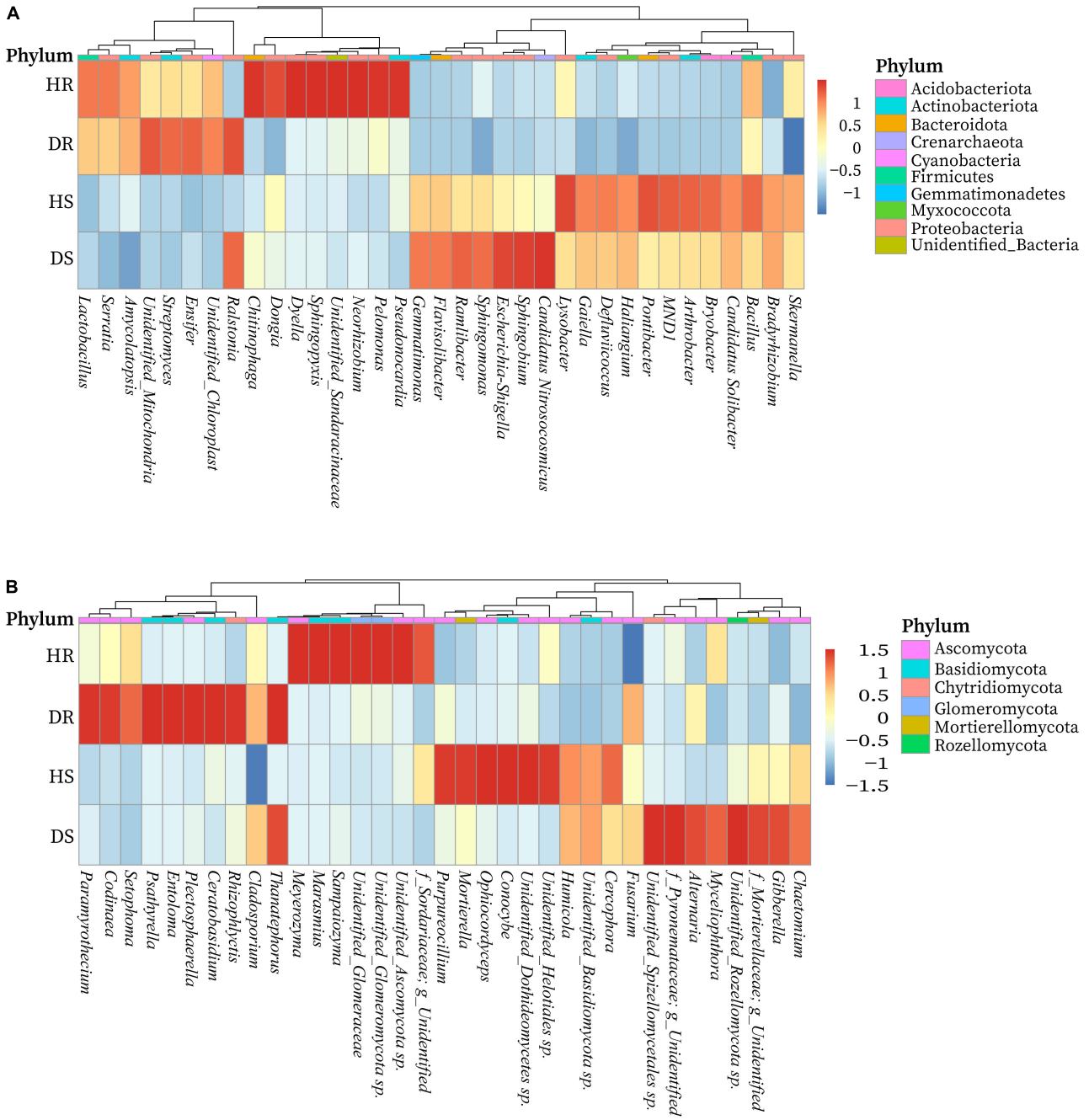


FIGURE 6 | Relative abundance heatmaps at the genus level for top 35 bacterial **(A)** and fungal **(B)** genera in group-wise comparison under three variables. HS, healthy rhizosphere soil; DS, diseased rhizosphere soil; HR, healthy roots; DS, diseased roots.

Kaushal et al., 2020). In our study, samples were collected from fields where flue-cured tobacco has been continuously grown for the past 10 years as an annual crop for commercial production. Therefore, infected soil and tools used for mechanical operations are primary carriers of bacterial wilt pathogen from one field to another.

Our study demonstrated that rhizosphere soil greatly influences microbial diversity and host more bacterial and fungal communities than roots. Most of the communities present in roots are also present in rhizosphere soil in all locations. The results suggest the microbial communities present in roots were transferred and colonized from the rhizosphere soil. Studies have proven that host plants selectively promote colonization of specific bacterial and fungal communities in roots from rhizosphere soil (Turnbaugh et al., 2007; Lundberg et al., 2012). It was found that rhizosphere soil showed a high microbial

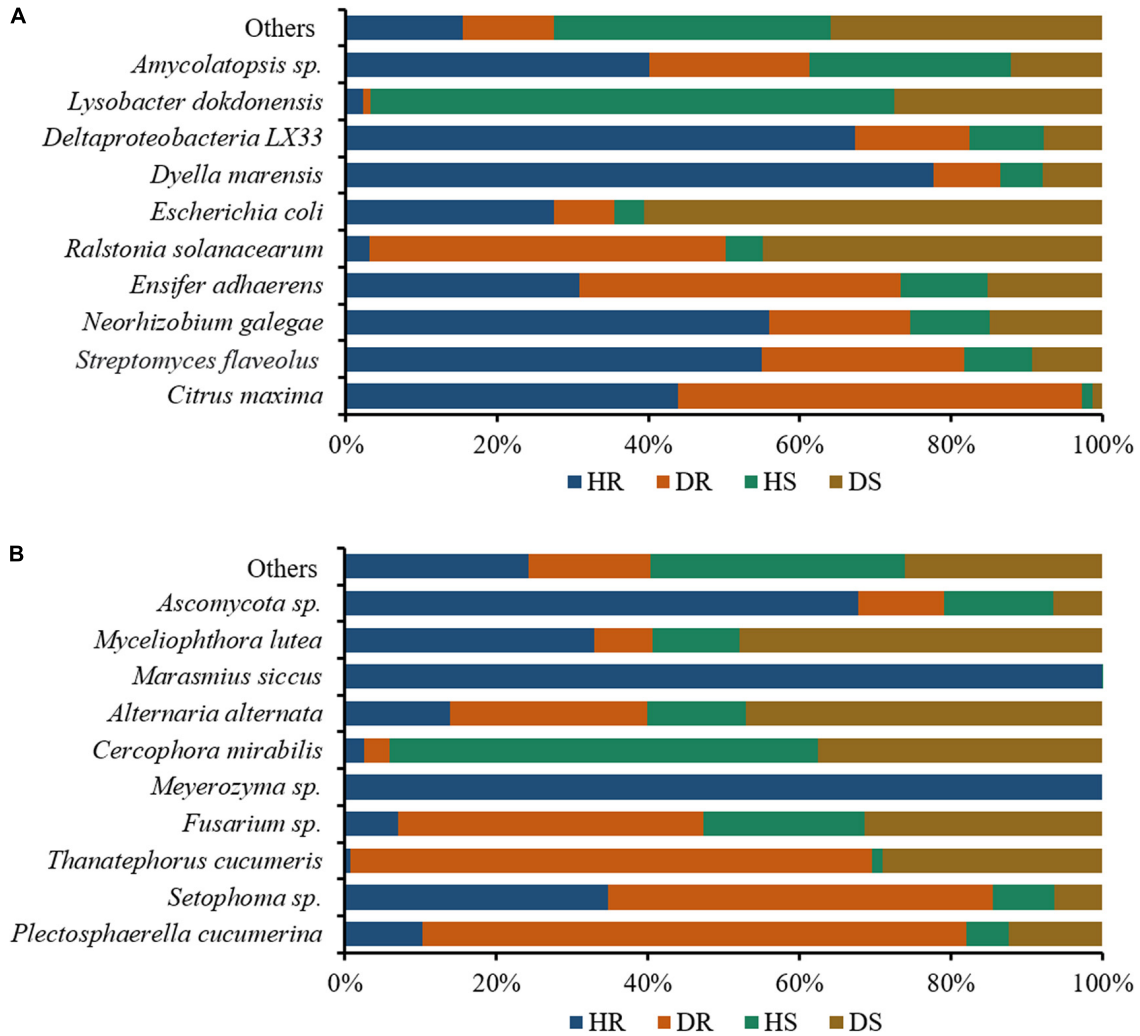


FIGURE 7 | Relative abundance bar plots for top 10 bacterial **(A)** and fungal **(B)** species in group-wise comparison under three variables. HS, healthy rhizosphere soil; DS, diseased rhizosphere soil; HR, healthy roots; DS, diseased roots.

diversity than roots. However, healthy tobacco plant rhizosphere soil and root samples host a high diversity of bacterial and fungal communities than bacterial wilt–infected tobacco plant samples. This may be due to disease stress, which is directly linked to the decreased value of available carbon for microbes in the rhizosphere and regulating specific microbe's growth (Kaushal et al., 2020). Flue-cured tobacco plant resistance and severity of *R. solanacearum* in different plant components also contribute to the prevalence of microbial communities. Bacterial and fungal OTU count was recorded maximum in all locations and plant components in rhizosphere soil samples than in the root samples.

Host plant environment is known to support or suppress the colonization of certain bacterial and fungal genera with different plant components. Thus, bacterial and fungal communities in these specific plant components are either boosted or exhausted (Kaushal et al., 2020). We observed the core stable bacterial and fungal communities in all sample types that were colonized in different plant components with respect to OTUs distribution. In all rhizosphere soil and root samples for bacterial community composition phylum, Proteobacteria and Cyanobacteria were found in high relative abundance, respectively. Whereas for fungal community composition, phyla Ascomycota and Basidiomycota were found in high relative abundance in both rhizosphere soil and root samples. Results showed that bacterial wilt pathogen has a great impact and significantly influences the bacterial communities compared with fungal communities.

Most of the diverse bacteria and fungi can enhance plant growth, suppress disease incidence, and confer other biological functions that benefit the plants (Santoyo et al., 2016; Gouda et al., 2018). A fraction of core bacterial communities identified in this study are known as plant growth promoters, enhance resistance in plants, and suppress the incidence of disease *via* production

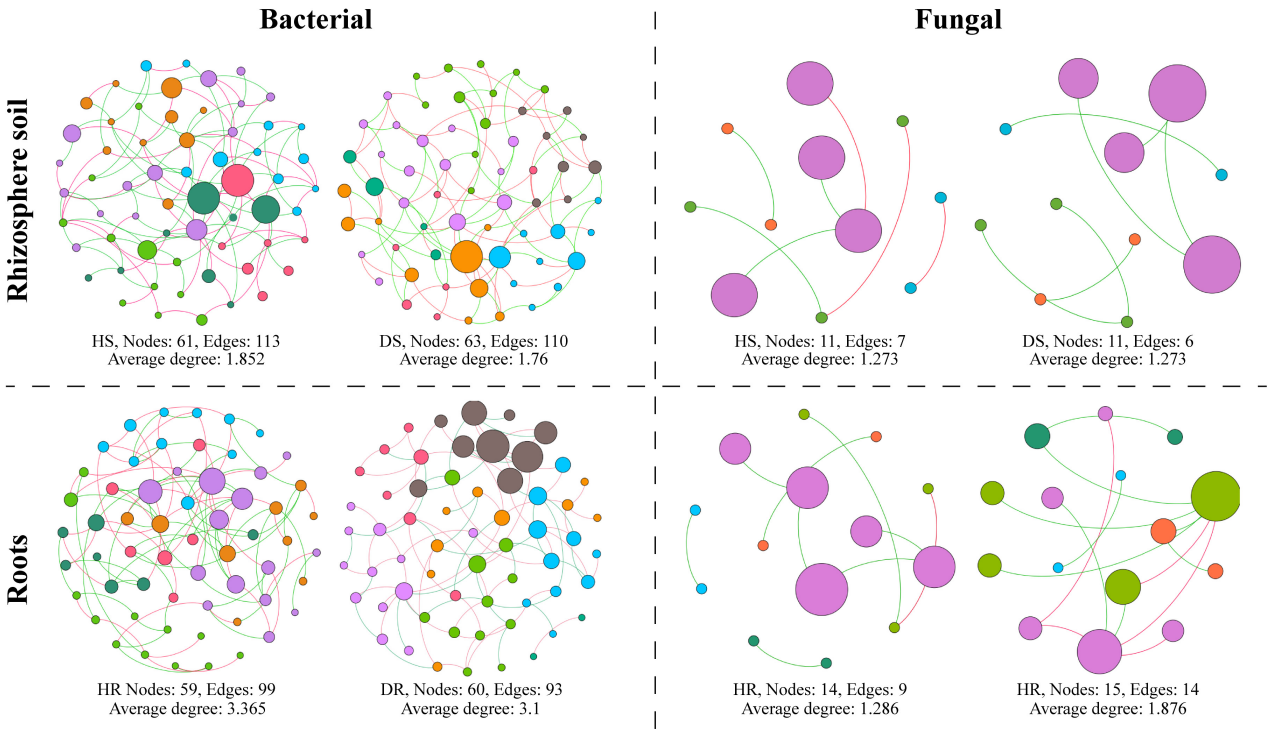


FIGURE 8 | Co-occurrence network analysis of bacterial and fungal communities associated with healthy and diseased samples collected from different plant components (rhizosphere soil and roots). HS, healthy soil; DS, diseased soil; HR, healthy roots; DR, diseased roots.

of a specific antibiotic, volatile organic compounds, secondary metabolites, and nitrogen fixation in many fields crops. Bacterial genera such as *Amycolatopsis*, *Bacillus*, *Streptomyces*, *Ensifer*, *Sphingopyxis*, *Neorhizobium*, *Pseudonocardia*, *Sphingomonas*, *Haliangium*, *Bradyrhizobium*, *Arthrobacter*, *Bryobacter*, and *Lysobacter* were found in high relative abundance, which contribute to plant growth promotion, nutrition acquisition, suppression of soilborne diseases and other plant pathogens. However, a minor fraction of fungal communities such as *Gibberella* and *Purpureocillium* was found as plant growth promoters and biocontrol agents, whereas others are saprophytic and pathogenic and cause disease in many important field crops.

Bacterial wilt pathogen *R. solanacearum* was highly abundant in diseased rhizosphere soil and root. A high abundance of the bacterium was found with nitrogen-fixing ability, producing secondary metabolites, and bacterial predator of bacteria in the soil such as *Neorhizobium galegae*, *Lysobacter dokdonensis*, and *Ensifer adhaerens*. Plants pathogenic fungi such as *Plectosphaerella cucumerina*, *Alternaria alternata*, *Thanatephorus cucumeris*, and *Fusarium* sp. were found in high relative abundance diseased rhizosphere soil and root. Thus, we conclude that these beneficial bacterial communities are positively related to flue-cured tobacco plant health, whereas the pathogenic fungal communities enhance the population of *R. solanacearum* and the incidence of bacterial wilt disease. Our results are in accordance with the study of Jiang et al. (2017), who reported that the incidence of tobacco bacterial wilt disease increases when it prevails with other root rot pathogen *P. nicotiana*. Analysis of co-occurrence networks showed that *R. solanacearum* has a significant impact on the bacterial community structure compared with fungal communities. Fewer negative correlations were found between bacterial communities in healthy plants compared to diseased plants.

CONCLUSION

In this study, we conclude that flue-cured tobacco plants host a range of bacterial and fungal communities. Plant components significantly impact bacterial and fungal communities. Rhizosphere soil is enriched with bacterial and fungal communities compared to roots, whereas high diversity was found in healthy plants rather than diseased plants. By comparing the microbial communities with other crops, we suggest that several bacterial communities discovered in the flue-cured tobacco microbiome aid in plant growth promotion and disease suppression. A fraction of fungal communities pathogenic to flue-cured tobacco was found in diseased plants, which correlates with the occurrence of tobacco bacterial wilt disease. However, future work should focus on studying the microbiome of flue-cured tobacco cultivars with resistance to bacterial wilt pathogen both as naturally occurring and artificial inoculation. This will provide experimental and theoretical information, that microbiome of flue-cured tobacco can be engineered to manage the bacterial wilt disease for better yield and quality production.

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

ZZ and GJ designed the experiment. WA, QL, SL, and JZ visited the tobacco-growing areas and collected samples. WA, ZD, and JY analyzed data and drew figures. WA, SM, and SK wrote the initial draft of manuscript. WA, SM, GJ, and ZZ wrote, reviewed, and edited final draft of the manuscript. All authors contributed to the final draft of the manuscript and agreed to the published version of the manuscript.

FUNDING

This study was financially supported by the Yunnan Agricultural University Scientific Research Foundation (KX900187), the Science and Technology Platform Plan of Yunnan Province (2019IC005), the National Key R&D Program of China (2019YFD1002000), the Yunnan Ten Thousand Talents Plan Leading Talents of Industrial Technology Project of China (YNWR-CYJS-2019-046), and National Natural Science Foundation of China (No. 32060601).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.845310/full#supplementary-material

REFERENCES

Ahmed, W., Yang, J., Tan, Y., Munir, S., Liu, Q., Zhang, J., et al. (2022). *Ralstonia solanacearum*, a deadly pathogen: revisiting the bacterial wilt biocontrol practices in tobacco and other Solanaceae. *Rhizosphere* 21:100479. doi: 10.1016/j.rhisph.2022.100479

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

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

Cai, Q., Zhou, G., Ahmed, W., Cao, Y., Zhao, M., Li, Z., et al. (2021). Study on the relationship between bacterial wilt and rhizospheric microbial diversity of flue-cured tobacco cultivars. *Eur. J. Plant Pathol.* 160, 265–276. doi: 10.1007/s10658-021-02237-4

Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., Ait Barka, E., and Clément, C. (2008). Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. *FEMS Microbiol. Ecol.* 63, 84–93. doi: 10.1111/j.1574-6941.2007.00410.x

Dong, C.-J., Wang, L.-L., Li, Q., and Shang, Q.-M. (2019). Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. *PLoS One* 14:e0223847. doi: 10.1371/journal.pone.0223847

Dong, L., Xu, J., Zhang, L., Cheng, R., Wei, G., Su, H., et al. (2018). Rhizospheric microbial communities are driven by Panax ginseng at different growth stages and biocontrol bacteria alleviates replanting mortality. *Acta Pharmac. Sin. B* 8, 272–282. doi: 10.1016/j.apsb.2017.12.011

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Gao, H., Hua, C., and Tong, M. (2018). Impact of Dinophysis acuminata feeding Mesodinium rubrum on nutrient dynamics and bacterial composition in a microcosm. *Toxins* 10:443. doi: 10.3390/toxins10110443

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

Govindasamy, V., Franco, C. M., and Gupta, V. V. (2014). "Endophytic actinobacteria: diversity and ecology," in *Advances In Endophytic Research*, eds V. C. Verma and A. C. Gange (New Delhi: Springer), 27–59.

Hao, J., and Ashley, K. (2021). Irreplaceable role of amendment-based strategies to enhance soil health and disease suppression in potato production. *Microorganisms* 9:1660. doi: 10.3390/microorganisms9081660

Jiang, G., Wei, Z., Xu, J., Chen, H., Zhang, Y., She, X., et al. (2017). Bacterial wilt in China: history, current status, and future perspectives. *Front. Plant Sci.* 8:1549. doi: 10.3389/fpls.2017.01549

Kaushal, M., Swennen, R., and Mahuku, G. (2020). Unlocking the microbiome communities of banana (*Musa* spp.) under disease stressed (*Fusarium wilt*) and non-stressed conditions. *Microorganisms* 8:443. doi: 10.3390/microorganisms8030443

Kim, B.-S., French, E., Caldwell, D., Harrington, E. J., and Iyer-Pascuzzi, A. S. (2016). Bacterial wilt disease: host resistance and pathogen virulence mechanisms. *Physiol. Mol. Plant Pathol.* 95, 37–43. doi: 10.1016/j.pmpp.2016.02.007

Köberl, M., Schmidt, R., Ramadan, E. M., Bauer, R., and Berg, G. (2013). The microbiome of medicinal plants: diversity and importance for plant growth, quality and health. *Front. Microbiol.* 4:400. doi: 10.3389/fmicb.2013.00400

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., McDonald, M., et al. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–864. doi: 10.1126/science.aaa8764

Li, C., Ahmed, W., Li, D., Yu, L., Xu, L., Xu, T., et al. (2022). Biochar suppresses bacterial wilt disease of flue-cured tobacco by improving soil health and functional diversity of rhizosphere microorganisms. *Appl. Soil Ecol.* 171:104314. doi: 10.1016/j.apsoil.2021.104314

Li, L., Feng, X., Tang, M., Hao, W., Han, Y., Zhang, G., et al. (2014). Antibacterial activity of *Lansiumamide* B to tobacco bacterial wilt (*Ralstonia solanacearum*). *Microbiol. Res.* 169, 522–526. doi: 10.1016/j.micres.2013.12.003

Li, Y., Ren, K., Zou, C., Xie, J., He, X., Chen, Y., et al. (2019). Effects of ferrous iron toxicity on agronomic, physiological, and quality indices of flue-cured tobacco. *Agron. J.* 111, 2193–2206. doi: 10.2134/agronj2018.12.0786

Liu, Y., Shi, J., Feng, Y., Yang, X., Li, X., and Shen, Q. (2013). Tobacco bacterial wilt can be biologically controlled by the application of antagonistic strains in combination with organic fertilizer. *Biol. Fertil. Soils* 49, 447–464. doi: 10.1007/s00374-012-0740-z

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Ma, L., Zhang, H.-Y., Zhou, X.-K., Yang, C.-G., Zheng, S.-C., Duo, J.-L., et al. (2018). Biological control tobacco bacterial wilt and black shank and root colonization by bio-organic fertilizer containing bacterium *Pseudomonas aeruginosa* NXHG29. *Appl. Soil Ecol.* 129, 136–144. doi: 10.1016/j.apsoil.2018.05.011

Mendes, R., Garbeva, P., and Raaijmakers, J. M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* 37, 634–663. doi: 10.1111/1574-6976.12028

Mhlongo, M. I., Piater, L. A., Madala, N. E., Labuschagne, N., and Dubery, I. A. (2018). The chemistry of plant–microbe interactions in the rhizosphere and the potential for metabolomics to reveal signaling related to defense priming and induced systemic resistance. *Front. Plant Sci.* 9:112. doi: 10.3389/fpls.2018.00112

Munir, S., Ahmed, A., Li, Y., He, P., Singh, B. K., He, P., et al. (2021). The hidden treasures of citrus: finding Huanglongbing cure where it was lost. *Crit. Rev. Biotechnol.* 1–16. doi: 10.1080/07388551.2021.1942780

Paudel, S., Dobhal, S., Alvarez, A. M., and Arif, M. (2020). Taxonomy and phylogenetic research on ralstonia solanacearum species complex: a complex pathogen with extraordinary economic consequences. *Pathogens* 9:886. doi: 10.3390/pathogens9110886

Qi, G., Chen, S., Ke, L., Ma, G., and Zhao, X. (2020). Cover crops restore declining soil properties and suppress bacterial wilt by regulating rhizosphere bacterial communities and improving soil nutrient contents. *Microbiol. Res.* 238:126505. doi: 10.1016/j.micres.2020.126505

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Raaijmakers, J. M. (2015). "The minimal rhizosphere microbiome," in *Principles Of Plant-Microbe Interactions*, ed. B. Lugtenberg (Heidelberg: Springer), 411–417.

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M., and Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiol. Res.* 183, 92–99. doi: 10.1016/j.micres.2015.11.008

Shen, G., Zhang, S., Liu, X., Jiang, Q., and Ding, W. (2018). Soil acidification amendments change the rhizosphere bacterial community of tobacco in a bacterial wilt affected field. *Appl. Microbiol. Biotechnol.* 102, 9781–9791. doi: 10.1007/s00253-018-9347-0

Tang, Z., Chen, L., Chen, Z., Fu, Y., Sun, X., Wang, B., et al. (2020). Climatic factors determine the yield and quality of Honghe flue-cured tobacco. *Sci. Rep.* 10, 1–12. doi: 10.1038/s41598-020-76919-0

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., and Gordon, J. I. (2007). The human microbiome project. *Nature* 449, 804–810. doi: 10.1038/nature06244

Wei, L., Yang, J., Ahmed, W., Xiong, X., Liu, Q., Huang, Q., et al. (2021). Unraveling the association between metabolic changes in inter-genus and intra-genus bacteria to mitigate clubroot disease of chinese cabbage. *Agronomy* 11:2424.

Wu, X., Li, H., Wang, Y., and Zhang, X. (2020). Effects of bio-organic fertiliser forti?ed by Bacillus cereus QJ-1 on tobacco bacterial wilt control and soil quality improvement. *Biocontrol Sci. Technol.* 30, 351–369. doi: 10.1080/09583157.2020.1711870

Zhang, C., Lin, Y., Tian, X., Xu, Q., Chen, Z., and Lin, W. (2017). Tobacco bacterial wilt suppression with biochar soil addition associates to improved soil physiochemical properties and increased rhizosphere bacteria abundance. *Appl. Soil Ecol.* 112, 90–96. doi: 10.1016/j.apsoil.2016.12.005

Zhang, J., Wei, L., Yang, J., Ahmed, W., Wang, Y., Fu, L., et al. (2020a). Probiotic consortia: Reshaping the rhizospheric microbiome and its role in suppressing root-rot disease of *Panax notoginseng*. *Front. Microbiol.* 11:701. doi: 10.3389/fmicb.2020.00701

Zhang, J., Fan, S., Qin, J., Dai, J., Zhao, F., Gao, L., et al. (2020b). Changes in the microbiome in the soil of an American ginseng continuous plantation. *Front. Plant Sci.* 11:572199. doi: 10.3389/fpls.2020.572199

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.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 April 2022
doi: 10.3389/fmicb.2022.870900

Changes to the Bacterial Microbiome in the Rhizosphere and Root Endosphere of *Persea americana* (Avocado) Treated With Organic Mulch and a Silicate-Based Mulch or Phosphite, and Infested With *Phytophthora cinnamomi*

Qurrat Ul Ain Farooq[1,2]*, *Giles Edward St. John Hardy*[1,3], *Jen A. McComb*[1], *Peter Campbell Thomson*[4] *and Treena Isobel Burgess*[1]

[1] *Phytophthora Science and Management, Harry Butler Institute, Murdoch University, Perth, WA, Australia,* [2] *Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan,* [3] *ArborCarbon, ROTA Compound Murdoch University, Murdoch, WA, Australia,* [4] *Sydney School of Veterinary Science, The University of Sydney, Camden, NSW, Australia*

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Laith Khalil Tawfeeq Al-Ani, Universiti Sains Malaysia, Malaysia
Manoj Kumar Solanki, University of Silesia in Katowice, Poland

***Correspondence:**
Qurrat Ul Ain Farooq
anniepk_elegant@yahoo.com

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *07 February 2022*
Accepted: *11 March 2022*
Published: *28 April 2022*

Citation:
Farooq QUA, Hardy GESJ, McComb JA, Thomson PC and Burgess TI (2022) Changes to the Bacterial Microbiome in the Rhizosphere and Root Endosphere of Persea americana (Avocado) Treated With Organic Mulch and a Silicate-Based Mulch or Phosphite, and Infested With Phytophthora cinnamomi. Front. Microbiol. 13:870900. doi: 10.3389/fmicb.2022.870900

Plant growth and responses of the microbial profile of the rhizosphere soil and root endosphere were investigated for avocado plants infested or not infested with *Phytophthora cinnamomi* and the changes were compared in plants grown with various soil additives or by spraying plants with phosphite. Soil treatments were organic mulches or silica-based mineral mulch. Reduction of root growth and visible root damage was least in the infested plants treated with phosphite or mineral mulch applied to the soil. Rhizosphere soils and root endospheres were analyzed for bacterial communities using metabarcoding. Bacterial abundance and diversity were reduced in infested rhizospheres and root endospheres. The presence or absence of mineral mulch resulted in greater diversity and larger differences in rhizosphere community composition between infested and non-infested pots than any other treatment. Some rhizosphere bacterial groups, especially Actinobacteria and Proteobacteria, had significantly higher relative abundance in the presence of *Phytophthora*. The bacterial communities of root endospheres were lower in abundance than rhizosphere communities and not affected by soil treatments or phosphite but increased in abundance after infection with *P. cinnamomi*. These findings suggested that the addition of silicate-based mineral mulch protects against *Phytophthora* root rot, which may be partly mediated through changes in rhizosphere bacterial community composition. However, the changes to the microbiome induced by spraying plants with phosphite are different from those resulting from the application of mineral mulch to the soil.

Keywords: soil amendments, silicate-based mulch, microbial profile, rhizosphere soil, phosphite

INTRODUCTION

Most soil-borne disease-causing agents are acclimatized to live in bulk soil and cause plant diseases when changes in physical and biotic soil conditions allow them to colonize the rhizosphere and damage plants (Avis et al., 2008). Changes in cultural practices or the application of soil additives resulting in improved disease suppression is thought to function by altering the soil microbiota. However, it is not usually known which soil microbes are impacted, and which changes lead to the desired outcome (Bakker et al., 2015). As high throughput sequencing techniques allow the analysis of the composition of the entire soil microbial population, they will provide information on how microbial populations change in the presence of a pathogen, and in response to soil additives will provide a better understanding of which microbes enhance or reduce disease expression. The molecular techniques mean that this analysis is no longer restricted to microbes easy to isolate and culture. In the longer term, this knowledge may allow more efficient exploitation of living microorganisms to suppress disease using eco-friendly, non-chemical methods.

Avocadoes are cultivated in at least 59 countries in tropical and subtropical regions. *Phytophthora* root rot or avocado wilt complex caused by the oomycete *Phytophthora cinnamomi* Rands (Ramirez-Gil et al., 2017, 2018; Hardham and Blackman, 2018) is one of the worst diseases for avocados worldwide, especially in Australia. *Phytophthora* root rot can cause losses from 45 to 90% in avocado (Molano, 2007; Perez-Jimenez, 2008) and may increase to 100% if appropriate controls are not adopted. Management of *Phytophthora* root rot is usually achieved by using chemical sprays such as phosphite and metalaxyl (Pegg et al., 1987; Dobrowolski et al., 2008; Ramirez-Gil et al., 2017; Belisle et al., 2019), but most growers adopt an integrated management strategy. This includes site management to reduce waterlogging and minimize disease spread by personnel or machinery, as well as the application of inorganic fertilizers, such as calcium, phosphorus, magnesium, and silicon, which are shown to reduce the impact of the disease (Dann and Le, 2017; Ramirez-Gil et al., 2017). The addition of organic matter as mulches, manures, and composts is a major part of an integrated strategy (Bulluck and Ristaino, 2002; Bonanomi et al., 2007; Klein et al., 2011; Ando et al., 2014; Gilardi et al., 2016; van Bruggen et al., 2016).

Organic soil additives add nutrients and improve water holding capacity (Bhadha et al., 2017; Oldfield et al., 2018). They may also reduce plant disease either directly by reducing the population of pathogenic bacteria and fungi in the soil, or indirectly by increasing the abundance of bacteria that induce systemic disease resistance in the host plant (Zhang et al., 1998; Hoitink and Boehm, 1999; Aviles et al., 2011). For example, compost enhances the density of soil bacteria that have antibiotic activity against *Fusarium oxysporum*, *F. solani,* and *Rhizoctonia solani* (Jambhulkar et al., 2015) through enhancement of populations of strains of *Pseudomonas, Streptomyces,* or *Bacillus* spp. Of particular interest here are reports of some Proteobacteria or Actinobacteria suppressing *Phytophthora* root rot in avocado (You et al., 1996; Yin et al., 2004; Cazorla et al., 2007; Guevara-Avendano et al., 2018). In some cases, commercial preparations of beneficial bacteria have been effective for disease control in crops such as solanaceous vegetables, fruit crops, other vegetables, and ornamentals (Junaid et al., 2013; Hu et al., 2016; Mukta et al., 2017), but in a pot trial of avocado, probiotics containing *Bacillus* spp. did not reduce root damage from *P. cinnamomi* (Farooq et al., 2022).

Junaid et al. (2013) reviewed direct and indirect mechanisms by which soil microbes might suppress a pathogen. The direct mechanisms include hyperparasitism, nutrient competition (Pal and Gardener, 2006; Jambhulkar et al., 2015), commensalism (Yoon et al., 1977; Chisholm et al., 2006), mutualism (Bronstein, 1994; Chisholm et al., 2006), and the production of antibiotic compounds (Garbeva et al., 2006, 2011; Postma et al., 2008). Indirect mechanisms include stimulation of plant growth through the production of plant growth-promoting hormones and siderophores (Bhattacharjee and Dey, 2014), and disease protection through the induction of systemic host resistance (Nakkeeran et al., 2004; Bent, 2006).

Mineral soil conditioners, particularly silicate-based ones, have shown encouraging outcomes for plant disease control and crop growth (Pozza et al., 2015; Tubana et al., 2016). Silica (Si) is not an essential element for plant growth but has been categorized as "quasi-essential." A recent review by Rajput et al. (2021) shows there is a wide array of positive effects of silica on plants and soil microbiota, and that application of silicate-based nanoparticles may be more efficacious than conventional application. Conventional silica application has been effective for the control of anthracnose (*Colletotrichum lindemuthianum;* Moraes et al., 2009) and powdery mildew (*Sphaerotheca fuliginea*) (Menzies et al., 1992; Belanger et al., 2003). Although most of the research has been on herbaceous crops, Dann and Le (2017) showed a silica soil amendment improved root biomass, enhanced new root growth, and reduced root necrosis in avocado seedlings infected with *P. cinnamomi* or *Calonectria ilicicola*. It appears silica has its effect both through changes to the plant metabolism and the soil microbiome. Silica accumulation in the plant results in a physical barrier in the cell wall (Samuels et al., 1991; Fawe et al., 2001) as well as the stimulation of plant defense enzymes such as lipoxygenase polyphenol oxidase, peroxidase, and phenylalanine ammonia lyase (Fauteux et al., 2005; Shetty et al., 2011; Prabhu et al., 2012). It alters soil microbial diversity and richness in rhizosphere soil, but its impact varies with the season (Gao et al., 2022). It may promote beneficial bacteria (Li et al., 2019), but one study showed it could also decrease populations of beneficial bacteria such as *Rhizobacteria*. However, the application of silicate-based nanoparticles did not have this detrimental effect (Rajput et al., 2021).

Treatment of plants with pesticides can have an impact on the soil microbiome, which can be beneficial or detrimental (Lo, 2010). Phosphite is widely used to control *P. cinnamomi* damage in avocado orchards, and there is little data on how this might affect the soil microbiome. In tomato crops, phosphite has a beneficial effect; it suppresses *Ralstonia solanacearum*, and, when applied in conjunction with the biocontrol agent *Bacillus*

amyloliquefaciens, it enhances its antagonistic activity (Su et al., 2021b).

In non-infested avocado plants, the addition of organic mulch, a silicate-based mineral mulch, and organic mulch, or spraying plants with phosphite increased total root growth and fine root growth (Farooq et al., 2022) (**Supplementary Table S1**). When *P. cinnamomi* was present, mineral mulch or phosphite treatments increased fine root weight and reduced root damage (Farooq et al., 2022). The silicate-based mineral mulch resulted in almost the same level of protection against *P. cinnamomi* as phosphite (Farooq et al., 2022). The current study focuses on the changes in microbial consortia in root endospheres and rhizospheres of avocado infested with *P. cinnamomi*, and how these changes are modulated by the application of organic mulches (chicken manure, jarrah (*Eucalyptus marginata*) wood mulch, and avocado mulch), a silicate-based mineral mulch in addition to the organic ones, or spraying the plants with phosphite. The rhizosphere and root microbial populations were analyzed using amplicon 16S primers.

Firstly, we hypothesized that the abundance and diversity of bacteria in the microbiome would increase in response to the addition of organic mulch, and there would be further changes with the addition of mineral mulch or spraying plants with phosphite. Secondly, the presence of *P. cinnamomi* would change the microbial profiles of the soils in similar ways, regardless of the soil additives. Thirdly, the abundance and diversity of the bacteria in the microbiome would be greatest in the treatments that suppressed *Phytophthora* root damage. Fourthly, since avocado root damage from *phytophthora* is reduced to a similar extent by the silicate-based mineral mulch or by spraying plants with phosphite, these two treatments will induce similar changes in the bacteria in the rhizosphere and root endosphere.

MATERIALS AND METHODS

Glasshouse Experiment

Rhizosphere soil and avocado root tips were collected from plants grown in polybags in the glasshouse under conditions that simulated the integrated control measures for *P. cinnamomi* used by many avocado growers. Details are given in Farooq et al. (2022). Briefly, 5-month-old plants were transplanted into 150 × 380 mm (7 L) free-draining polybags (Garden City Plastics, Forrestfield, Western Australia) containing a well-drained clay loam soil with good porosity and water holding capacity from an avocado growing area in Carabooda, Western Australia and mixed 1:1 with river sand. There were 20 plants in each treatment, and 38 days after transplanting, the soil of ten of these were inoculated with *P. cinnamomi* (isolate MP 94-48 from the *Phytophthora* Science and Management culture collection, Genbank Accession number for ITS gene region is JX113294). Plants were harvested 12 weeks after inoculation and root damage and growth parameters were assessed (Farooq et al., 2022). These data showed that applying organic mulches, one of the two silicate-based mineral mulches tested or spraying plants with phosphite improved plant growth and reduced root damage to a level comparable with phosphite (**Supplementary Table S1**). Plants from these treatments and the control pots with no soil additives were selected to analyze the soil microbiota (**Table 1**). Details of these treatments are Treatment 1, "No mulch": pots with no additives; Treatment 2, "Organic mulches": pots with well-composted chicken manure fertilizer, jarrah wood mulch for moisture retention, and mulch from an avocado orchard to simulate orchard conditions; Treatment 3, "Mineral mulch" organic mulches with the addition of a silicate-based mineral mulch (which also contained some calcium and trace elements such zinc, copper, boron) (https://mineralmulch.com/), and Treatment 4, "Phosphite" organic mulches and plants were sprayed with phosphite (Agri-Fos 600) (Nufarm Australia) to run off (**Table 1**).

At harvest, bulk soil was shaken gently from the roots, and then ∼10 ml of the adhering rhizosphere soil was gently brushed into 15-ml freezing tubes (ThermoFisher scientific). Healthy white root tips (∼1 ml) were randomly collected from each plant during harvesting and placed in a 1.5-ml Eppendorf tube. The rhizosphere soil samples and the root tips were immediately immersed in liquid nitrogen and stored at −20°C before DNA extraction.

Analysis of the Soil and Plant Microbial Profile (Metabarcoding)

A total of 250 mg of rhizosphere soil and 50 mg of roots were used for the extraction of DNA using DNeasy® PowerSoil® Pro Kit (QAIGEN group) and DNeasy Plant Pro and Plant Kits (QAIGEN group), respectively following the manufacturer's instructions. One sample was analyzed from each rhizosphere and soil.

For taxonomic profiling, 16S primers (Klindworth et al., 2013) were used in combination for the amplification of the hypervariable V3 and V4 regions of the bacterial 16S rRNA gene following the procedures of 16 S RNA gene amplicon suggested for Illumina Miseq systems (Illumina documents 2019). For each sample, PCR amplicons were generated from three technical replicates using 25 μl mixtures containing 2.5 μl microbial genomic DNA, 1 μl of Illumina 16S forward and reverse primers each, 8 μl PCR grade water, and 12.5 μl of GoTraq® green master mix per sample. This PCR was carried out on a thermal cycler and had a first denaturation cycle at 95°C for 3 min, 25 cycles of the second denaturation at 95°C for 30 s followed by primer annealing at 55°C for 30 s with further extension at 72°C for 30 s, at the end with a final step of heating at 72°C for 5 min. To reduce the variation in each PCR, amplicons of the technical replicates were pooled and cleaned up using AMPure XP beads (Beckman Coulter, USA) according to the manufacturer's instructions. After clean-up, PCR Illumina sequencing adapters were attached by an index PCR step using the Nextra XT Index kit (Illumina Inc., San Diego, CA USA). The mixture consisted of 5 μl DNA, 5 μl Nextra XP index Primers 1 and 2 each, 25 μl of 2× KAPA HiFi Hotstart ReadyMix, and 10 μl PCR grade water. The PCR program was run on a thermal cycler using an initial denaturation step at 95°C for 3 min, followed by 8 cycles of the second denaturation at 95°C for 30 s, then primer annealing at 55°C for 30 s and further extension at 72°C for 30 s, and finally a heating cycle at 72°C for 5 min. Then the amplicons

TABLE 1 | Experimental treatments.

Treatment code	Chicken manure	Mulch			Chemical spray
	50 g per pot applied monthly	Jarrah wood 75 g per pot at the time of transplantation	Avocado 75 g per pot at the time of transplantation	Mineral 100 g/pot at the time of transplantation	0.5% phosphite with 133 μl L^{-1} penetrant (BS-1000) applied to foliage 28 and 38 days after transplanting
No mulch	-	-	-	-	-
Organic mulches	+	+	+	-	-
Mineral mulch	+	+	+	+	-
Phosphite	+	+	+	-	+

were again cleaned up following the same procedure described previously. The quantification of amplicon libraries was done using Qubit (Invitrogen, CA, USA), and all the samples were combined in equimolar amounts (4 mM each).

Library preparation was performed per the Illumina Guide for 16S Metagenomic Sequencing Library Preparation (Illumina). Indexed amplicon pools were sequenced on the Illumina MiSeq platform using 2 x 300 bp paired-end chemistry. Using the FASTA manifest protocol, de-multiplexed paired-end reads were imported into the Quantitative Insights into the Microbial Ecology platform (QIIME 2). Read trimming, primer removal, denoising, read merging, and amplicon sequence variant (ASV) clustering were performed using the DADA2 plugin pipeline. Taxonomy was assigned using the QIIME 2 feature classifier with the Greengenes v13.8 99% OTU 16s rRNA genes after removing chloroplast and mitochondrial operational taxonomic units (OTUs) from the dataset.

Statistical Analysis

Statistical analysis was computed in R software (Version 4.1.1). Alpha diversity indices (Shannon indices) were calculated with the vegan package in R from the OTU table normalized by rarefaction to reduce the impact of sequencing depth on results. The alpha diversity indices were subjected to ANOVA to assess the difference between treatments and infection status, with statistical significance calculated from a permutation test. Linear-mixed effects model was used to calculate the species richness. Beta diversity (Bray-Curtis metric) indices were calculated through the vegan package. The effects of treatments and infection status on beta diversity indices were assessed with permutational multivariate ANOVA using distance matrices (*via* the "adonis" function through the vegan package). Non-metric multidimensional scaling (NMDS) based on UniFrac weighted and unweighted distances was performed using the vegan package, and plant root assessment factors were added to NMDS plots through the "envfit" function in the R vegan package. Data on total root weight, fine root weight, and root damage were from Farooq et al. (2022) (**Supplementary Table S1**).

A permutational multivariate ANOVA was used to compare the structure of bacterial communities of treatments with infection status using the vegan package unifrac weighted and unweighted distance matrix with 999 permutations. Significant differences in bacterial taxa relative abundance between treatments and infection status were calculated through the Kruskal–Wallis tests. The difference of relative abundance between infested and non-infested plants for each treatment and between treatments for each organism within a particular classification was compared by Mann-Whitney (Wilcoxon) tests. Tree-view of differences in the bacterial abundance was generated through the metacoder package in R. A cluster analysis to compare similarities of bacterial communities across the samples was undertaken based on the relative abundance data. Multivariate distances between samples were calculated using the vegan package in R and the cluster analysis with associated dendrogram and heatmap were generated from these distance data using the heatmap.2 function in the gplots package of R. Venn diagrams were generated to observe the shared and unique OTUs among the groups based on the prevalence of OTUs in sample groups, regardless of their relative abundance by using the VennDiagram package. A significance value of $p < 0.05$ was used where statistical testing was performed.

RESULTS

Bacterial Diversity and Species Richness

In the rhizosphere, there were significant differences in the Shannon (alpha) diversity measure for bacterial communities between the four treatments ($p = 0.03$; **Figure 1A**), but there were no significant differences in alpha diversity indices for the root endospheres (**Figure 1B**). Adding mineral mulch or spraying plants with phosphite resulted in greater bacterial diversity in rhizospheres with greater consistency between pots than in the rhizosphere of plants from other treatments (**Figure 1A**).

The linear mixed-effects model suggested that rarefied species richness in the rhizosphere of plants treated with mineral mulch or phosphite was significantly higher than those from organic mulch for non-infested plants, and significantly higher than those from no mulch or organic mulch for infested plants (**Figure 2A**). No significant differences in species richness in root endospheres were observed between treatments or between non-infested and infested (**Figure 2B**).

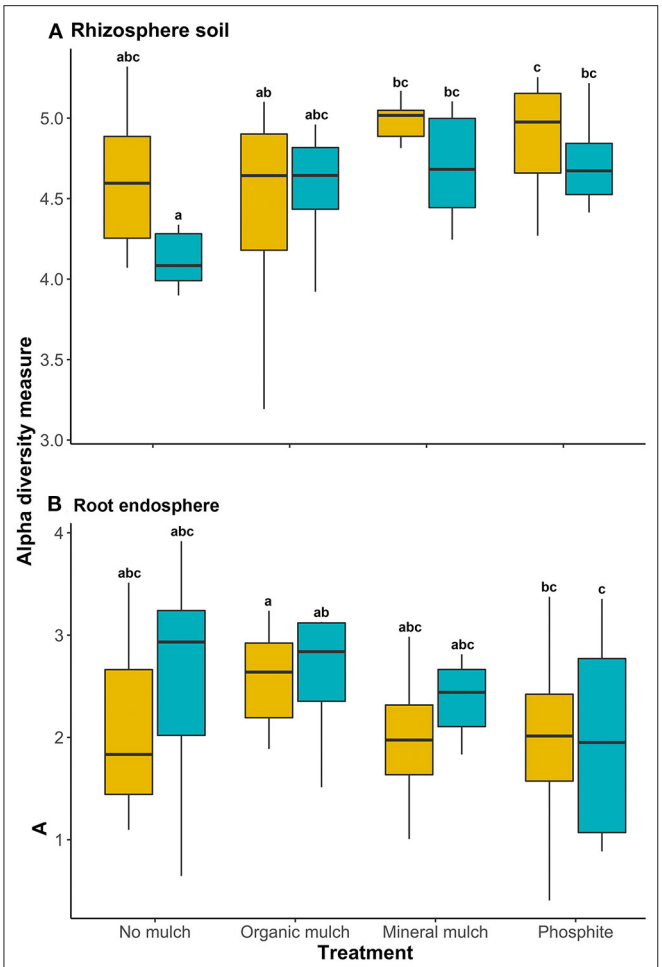


FIGURE 1 | Alpha diversity of the microbial biome of **(A)** rhizosphere soil and **(B)** root endosphere of avocado plants given four soil treatments. Yellow boxplots show alpha diversity, measured by the Shannon diversity index for non-infested avocado, blue boxes represent plants infested with *Phytophthora cinnamomi*. Different lowercase letters indicate a significant ($P < 0.05$) difference.

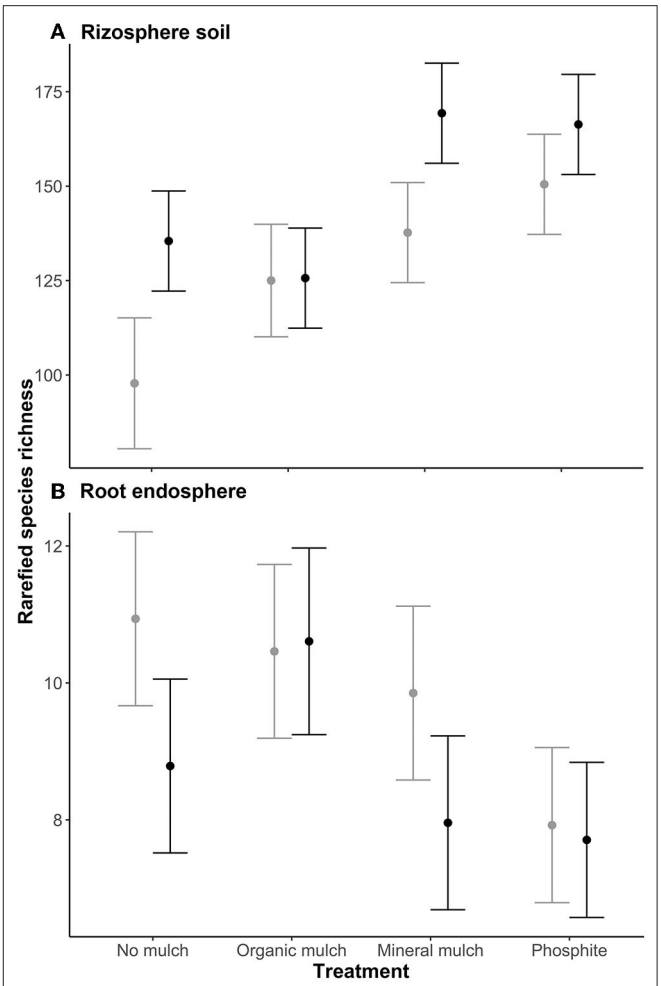


FIGURE 2 | Mean rarefied species richness of the microbial biome of **(A)** rhizosphere soil, and **(B)** root endosphere of avocado plants given four soil treatments. Values are estimated marginal means ± standard error. Gray lines indicate values for non-infested plants, black lines plants infested with *P. cinnamomi*.

Beta Diversity

Beta-diversity (Bray-Curtis metrics) indices of the microbial populations showed the greatest difference between non-infested and infested rhizosphere soil from plants with the mineral mulch treatment, with the first dimension (NMDS1) separating the infested from non-infested soil samples (**Figure 3A**). The NMDS plots showed low variability in bacterial communities between infested and non-infested plants in all other treatments and minimal separation of bacterial communities between treatments regardless of infection status.

There were significant differences among treatments regarding infection status in the beta analysis shown by Adonis analysis of the rhizosphere soil. For bacterial communities, the main effect of treatments and infection status and their interaction on beta-diversity were highly significant (Adonis) for rhizosphere soil. For bacteria, treatments, infection status and their interactions accounted for 7, 2, and 5% of the total variability, respectively. The interaction was primarily because the difference between infested and non-infested samples was greatest for mineral mulch.

Explanatory Variables Significantly Related With NMDS Axis

To visualize the relationship between the variability in the community composition among the treatments with regard to infection status, the root damage fine root and total root dry weights were plotted in the NMDS ordination as fitted explanatory variables. The variation in community composition of rhizosphere soil was correlated significantly with root damage ($p = 0.01$, $R^2 = 0.02$, F.mod = 1.61) and total root dry weight ($p = 0.04$, $R^2 = 0.01$, F.mod = 1.39; **Figure 3A**). For root samples, the variation in community composition was significantly correlated to fine root dry weight ($p = 0.01$, $R^2 = 0.02$, F.mod = 1.90) and total root dry weight ($p = 0.03$, $R^2 = 0.02$, F.mod= 1.71; **Figure 3B**).

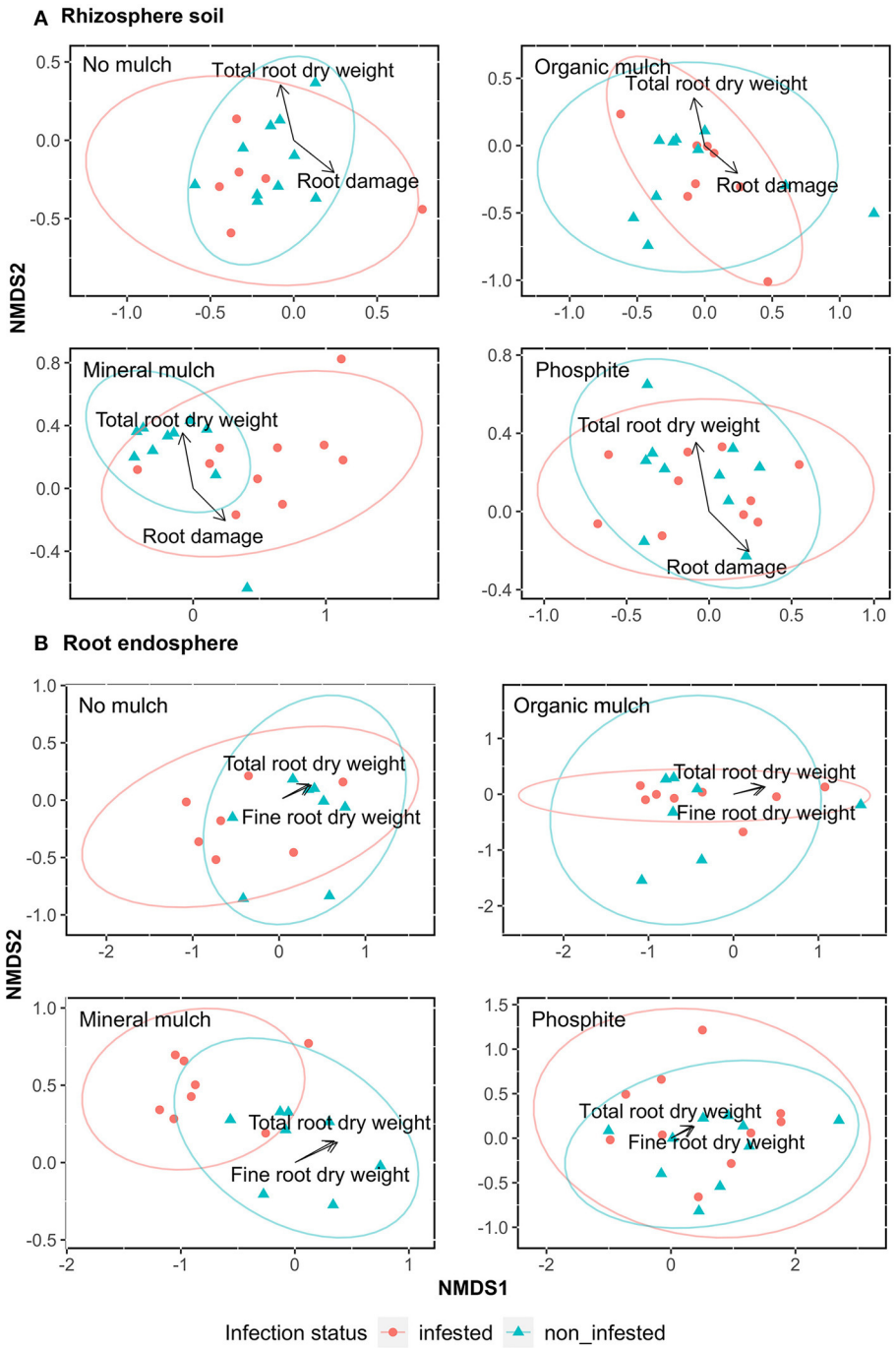


FIGURE 3 | Association of bacterial communities with parameters of root health (total root dry weight, fine root dry weight and root damage) of avocado plants given four soil treatments. Non-metric multidimensional scaling (NMDS) showing the bacterial taxonomic clustering of microbes in **(A)** rhizosphere soil, and **(B)** root endosphere from the four treatments. Blue triangles indicate samples from non-infested and red dots treatments infested with *P. cinnamomi*. The analysis was based on the Bray-Curtis dissimilarity matrix using relative abundance data obtained from a Hellinger-transformation of the number of microbe reads. Data for root health was selected through Adonis PERMANOVA and were from Farooq et al. (2022) **Supplementary Table S1**.

Relative Abundance

In rhizosphere soil, significant differences in relative abundance were observed for the Actinobacteria and Proteobacteria across the four treatments for non-infested plants, with the relative abundance of Proteobacteria being lower, and Actinobacteria higher than in the other treatments (**Figure 4A**, **Table 2**).

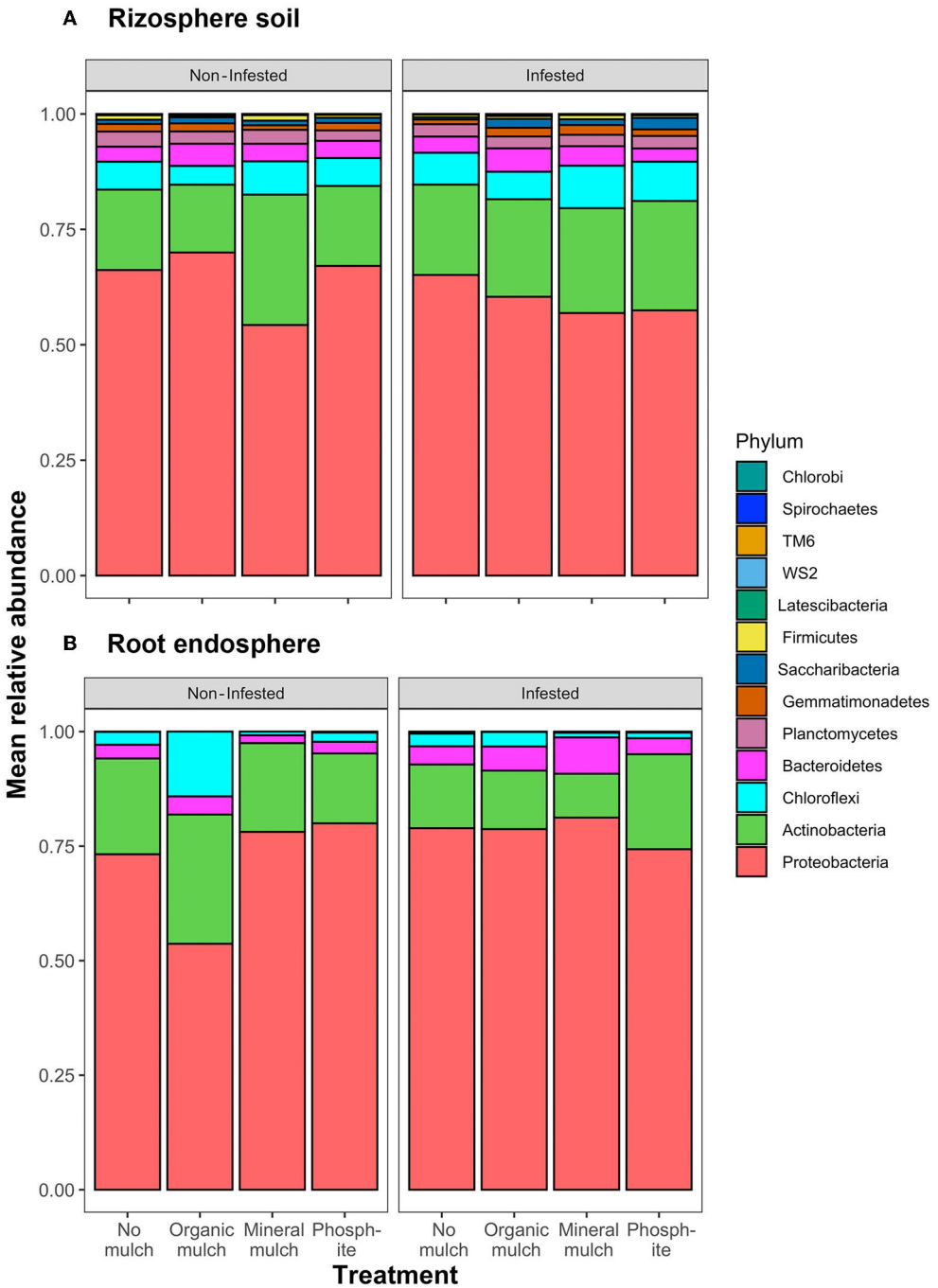


FIGURE 4 | Relative abundance of phyla across the four treatments and comparing infested and non-infested plants in **(A)** rhizosphere soil and **(B)** root endosphere.

Comparing relative abundance in infested and non-infested rhizospheres from the same soil treatment indicated little difference in the treatment without mulch. In the mineral mulch and phosphite treatments, Actinobacteria increased (28%) in abundance, while they decreased (15%) in the organic mulch treatment. There was also a significant increase in Spirochaetes in infested plants from treatments with organic mulch, mineral mulch, or phosphite compared with no mulch (**Figure 4A, Supplementary Figure S1A**, **Table 2**). In root endospheres, there were significantly fewer phyla present. In the non-infested root endosphere from the organic mulch treatment, the proportion of Chloroflexi was high (7%), but the increase was not statistically significant, whereas in infested root endosphere, the relative abundance of Bacteroidetes was

TABLE 2 | Comparison of relative abundance of bacterial communities between treatments within each infested/non-infested group tested by Kruskal-Wallis test.

Phylum	Infection status	Rhizosphere soil	Root endosphere
Actinobacteria	non-infested	0.0003	
Proteobacteria	non-infested	0.0001	
Spirochaetes	infested	0.0120	
Bacteroidetes	non-infested		0.0080
Chloroflexi	non-infested		0.0018

Only $p < 0.05$ for significant differences between treatments are displayed.

significantly higher in the mineral mulch treatment (7.9%) than in other treatments (ranging from 2 to 4%). A major difference between non-infested and infested root endosphere was seen between those from the mineral mulch treatment in which non-infested endophytic roots had fewer Actinobacteria (36%) and Bacteroidetes (93%) than the infested root endosphere (**Figure 4B**, **Supplementary Figure S1B**, **Table 2**).

Analysis of relative abundance of the bacteria at the genus level (**Supplementary Data Sheets S1A,B**) illustrates the significant difference in the bacterial profiles resulting from mineral mulch or phosphite treatment. In non-infested rhizospheres, species of 11 genera significantly increased in relative abundance in response to both mineral mulch or phosphite treatments, 2 in phosphite but not mineral mulch, and 17 in mineral mulch but not phosphite. When the changes after infestation are examined further, there is a large difference between the groups that increase in relative abundance: in mineral mulch, three Actinobacteria, two Chloroflexi, two Gamma Proteobacteria, and six Proteobacteria increased relative to the levels in non-infested pots; whereas in phosphite-treated soils, only one Actinobacteria and one Proteobacteria genus increased in relative abundance. No distinctive clustering of bacterial communities was observed in heatmaps of rhizosphere soil samples for relative abundance among treatments and infection status groups (**Supplementary Figure S4A**). A more "continuous" variation in the biological samples of soils was found as indicated by the more uniform color of the heatmap and the structure of the accompanying dendrogram, as expressed by their relative abundance. Nonetheless, a small level of clustering of bacterial communities was present in mineral mulch compared with phosphite (**Supplementary Figure S4A**).

In contrast, there was a very distinctive clustering of bacterial communities in the heat maps of the endophytic root bacteria, indicating a similarity of groups of samples as expressed by their relative abundances. However, despite this, clustering was not related to treatments or infection status in a pairwise comparison of treatments (**Supplementary Figure S4B**).

Total Abundance

In the rhizospheres of plants in non-infested soil, the addition of organic mulch did not increase the bacterial abundance; however, the addition of mineral mulch or phosphite treatment did cause a rise, with a marked increase in the number of Actinobacteria in the mineral mulch treatment and Proteobacteria in both treatments (**Supplementary Figures S1A, S2**). Bacteria were less abundant in all treatments in infested soils, but there was an abundant increase with the application of organic mulch, and there were additional rises with mineral mulch and phosphite treatments.

In the root endosphere, bacterial abundance was eight times lower than in the rhizosphere, and fewer phyla were represented. In the roots from non-infested soil, organic mulch resulted in a doubling of the endophytic bacterial population with a marked increase in the abundance of Actinobacteria and Chloroflexi, but the other treatments were similar to the no mulch treatment. There were more bacteria present in the root endosphere from infested soils with the mineral mulch treatment having high levels of Proteobacteria and Bacteroidetes (**Supplementary Figures S1B, S3**).

Comparison of the OTUs Present in Different Treatments

Differences in the composition of the bacterial microbiome and identification of the OTUs unique to a soil treatment or common across treatments are presented in the Venn diagrams (**Figure 5A** I, II, III). In non-infested rhizosphere soil, total OTUs were the least in treatments with no mulch or avocado mulch, and highest in the treatments with mineral mulch (65% of the total OTUs) or phosphite (60% of the total OTUs), which also had much higher numbers of unique OTUs (**Figure 5A** I). In infested soil, there was a reduction (10%) in the total number of OTUs in all treatments, but again, the highest total number and number of unique OTUs were in treatments with mineral mulch (54% of total OTUs) or phosphite (57% of total OTUs) (**Figure 5A** II). The number of OTUs common to all treatments in non-infested soil was 167 (17% of total OTUs), dropping to 90 (10% of total OTUs) in infested soils. The eight phyla that occurred in all treatments were Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria, and TM7 (Saccharibacteria). When the data from treatments were bulked, the number of OTUs common to infested and non-infested treatments were higher than the number unique to either treatment (**Figure 5A** III).

Overall, endophytic roots had many fewer OTUs than the rhizosphere. Some of the main phyla found in rhizosphere soil such as TM6, Latescibacteria, Spirochaetes Planctomycetes, Firmicutes and Chlorobi were absent from roots samples. No mulch and mineral mulch treatments had the highest numbers, and the proportion of OTUs common to non-infested and infested treatments was higher than in the rhizosphere (**Figure 5B** I, II, III). The four phyla that occurred in all treatments were Actinobacteria, Bacteroidetes, Chloroflexi, and Proteobacteria. The number of OTUs unique to root endospheres was 8% of the total OTUs found in soil and root combined.

DISCUSSION

The Effect of Additives on Bacterial Diversity and Abundance

The abundance and diversity of the soil microbiome were greatest in treatments in which *Phytophthora* root damage was suppressed, that is, soils from plants supplied with mineral mulch

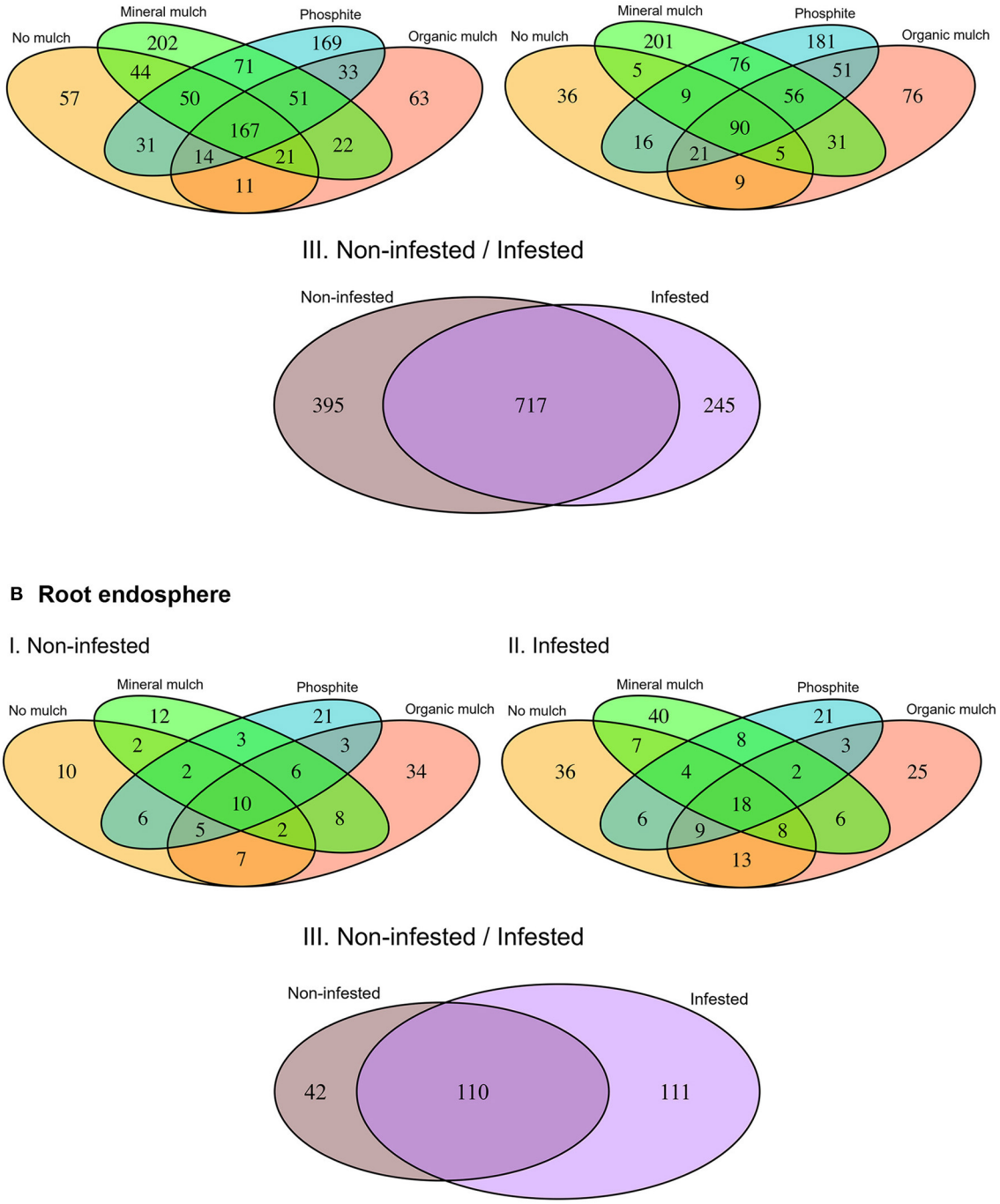


FIGURE 5 | Venn diagrams representing the operational taxonomic units (OTUs) in common and different in the microbiome of **(A)** rhizosphere soil and **(B)** root endosphere among treatments.

or in which plants were sprayed with phosphite. The NMDS plots showed the strongest link between root health and the variability of the rhizosphere microbes was in the treatment with mineral mulch and this microbiome showed more difference between the non-infested and infested pots than any other treatment. The total number of OTUs was highest in treatment with mineral

mulch or phosphite, although infection *with P. cinnamomi* reduced the total number of OTUs detected, and in both non-infested and infested treatments the highest numbers of OTUs were in the mineral mulch and phosphite treatments. Of note, a strong clustering of bacterial communities was seen in the root samples but not in the rhizosphere, as shown from the heatmaps. This is in contrast to most of the other findings in this study where differences in diversity, species richness, and abundances were found in root samples but not in the rhizosphere.

Several studies have reported a decrease in microbial abundance and diversity in the root endosphere or the rhizosphere with infection. Yang and Ancona (2021) found a decrease in abundance and diversity of microbial communities in the root endosphere after infection with *P. nicotianae* in *Citrus*. Liu et al. (2020) observed a reduction in microbial diversity in the root endosphere after infection with *P. nicotianae* in *Nicotiana tabacum,* but the opposite occurred in the soil. The decrease in bacterial composition in soil was also observed by Byers et al. (2020) in kauri forest soils in which *P. agathidicida* was present, and Wei et al. (2021) found that diseased cotton plants infested with *Verticillium* wilt had a higher total microbial biomass than healthy plants but less bacterial diversity.

Changes in the Microbiome Due to the Additives

The total number of OTUs detected in the various treatments indicated that the diversity was not markedly increased by the addition of organic mulch, but was significantly increased when mineral mulch or phosphite treatments were added. Mean relative abundance of different phyla in the rhizosphere was not markedly affected by the addition of organic mulch or spraying the plants with phosphite. However, mineral mulch caused a significant drop in the proportion of Protobacteria and an increase in Actinobacteria in non-infested treatments. When *P. cinnamomi* was present, the rhizospheres from soils with mineral mulch or phosphite, both had less Proteobacteria but more Actinobacteria and Chloroflexi than the treatments with no mulch or organic mulch treatments.

The abundance and diversity of the bacterial microbiomes in the root endospheres were much lower than in the rhizosphere in all treatments. There was a marked increase in Chloroflexi in non-infested pots with organic mulch, but this was not seen in the corresponding infested treatment. In the infested roots, the proportions of endophytic Actinobacteria and Bacteroidetes were different in the two treatments most suppressive of *Phytophthora*, namely the mineral mulch and phosphite treatments.

Actinobacteria, a phylum whose relative abundance increases in effective treatments, such as mineral mulch and phosphite, includes taxa such as *Streptomyces* known to produce antibiotic compounds effective against *Phytophthora* (Koberl et al., 2013). Isolates have also been shown to control *P. drechsleri* damping-off in cucumber (Sadeghi et al., 2017). However, *Streptomyces* species did not increase with the treatments applied in the current experiments.

Other genera that include species known to suppress fungal pathogens and *Phytophthora* by producing a wide range of antibiotic or volatile compounds, siderophores, and growth-promoting compounds are *Pseudomonas* and *Burkholderia* in the Proteobacteria (Inderbitzin et al., 2018; Youseif, 2018). In the current study, the proportion of this group was reduced by the treatments most effective against *Phytophthora*. In many other studies, *Pseudomonas* spp. have been shown to be effective for the control of *Phytophthora* (Broadbent et al., 1971; Broadbent and Baker, 1974; Duvenhage et al., 1991; You et al., 1996; Yang et al., 2001; Hunziker et al., 2015; Martins et al., 2021). In the current study, *Pseudomonas* spp. were not more abundant in the effective treatments (mineral mulch and phosphite) than in organic mulch alone before infection; however after infection, *Pseudomonas* spp. were more abundant in mineral mulch treatment. *Bukholdaria* was more abundant in phosphite but not mineral mulch. Thus, the changes in the microbial profiles following treatment with mineral mulch or phosphite were very different.

Impact of *P. cinnamomi* on the Bacterial Microbiome

Infestation of the soil with *P. cinnamomi* resulted in a decrease in OTUs in all treatments. However, the decrease was accompanied by different alterations in the relative abundance of the phyla in the various treatments. Many more genera changed in abundance at the genus level in response to infection in the mineral mulch treatment compared with phosphite. There was no significant change in the abundance of *Streptomyces*, *Pseudomonas*, or *Burkholdaria* after infestation in the treatments with no mulch or phosphite.

It was hypothesized that as avocado root damage from *Phytophthora* is reduced to a similar extent by silicate-based mineral mulch or spraying plants with phosphite, then both treatments would induce similar changes in the soil microbiome. However, the relative abundance of the various phyla in these two treatments was very different. In non-infested rhizospheres, the proportion of OTUs in common between these two treatments was similar to that of ineffective treatments. The dissimilarity of genera increase in abundance after adding mineral mulch or phosphite, and the changes following infection indicate that each treatment affects different bacterial taxa. Taxa shown to be important for disease control in other studies did not appear in this study and it appears there are many genera worthy of further study concerning whether they protect plants from *Phytophthora* root rot.

Many studies have shown that the addition of organic matter improves the physical and chemical properties of soil (Duffy et al., 1997; Tenuta and Lazarovits, 2002), change bacterial diversity, composition, and overall activity (Van and Van Ginkel, 2001; Bulluck and Ristaino, 2002; Cohen et al., 2005; Perez-Piqueres et al., 2006), as well as increasing the disease resistance of plants (Bonanomi et al., 2010; Aviles et al., 2011). However, in the current experiment, the addition of organic mulch (the chicken manure, jarrah mulch, and avocado mulch) increased the total abundance and changed the relative abundance of bacterial phyla present in the rhizosphere but did not result in significantly less

damage from *P. cinnamomi*. Although the potting medium was designed to replicate field conditions as far as possible in the field, greater fluctuations in temperature and moisture may alter the responses of both the microbiome and the plants.

Possible Modes of Action of the Suppressive Treatment–Mineral Mulch and Phosphite

The mineral mulch contained silicon and calcium, compounds known to improve bacterial diversity and richness significantly and in particular, to increase the relative abundance of Proteobacteria and Actinobacteria (Samaddar et al., 2019; Chen et al., 2021). An increase in Actinobacteria was also observed in the current study, but there was a decrease in the relative abundance of Proteobacteria. Silicon may also improve the production of plant metabolites that are associated with plant defense mechanisms against pathogens (Cherif et al., 1994; Rahman et al., 2015). When plant defensive mechanisms are activated, root exudates change, which impacts the soil microbiome (Ansari, 2018; Hu et al., 2018). Silicon may also decrease the disease severity by creating a physical barrier between the cell wall and cuticle (Song et al., 2021). Silicon is an environmentally friendly treatment and can be used in organic farming (Artyszak, 2018).

Phosphite, like silica, activates plant defense mechanisms (Ramezani et al., 2018; Mohammadi et al., 2020) and also has a direct effect on the pathogen. The changes in microbiome seen after phosphite spraying on the leaves are likely to be mediated through changes in root exudates (Eshraghi et al., 2014; Achary et al., 2017; Gill et al., 2018). The addition of phosphite increased the abundance of soil bacteria, but the relative abundance of all the phyla was very similar across the treatments. Su et al. (2021a) examined the effects of phosphite on the microbiome of tomatoes and found it enhanced the abundance of Proteobacteria and Actinobacteria.

Endophytic Bacteria in Roots Compared to the Rhizosphere

The rhizosphere soil had more taxa and thus greater bacterial diversity and richness than the root endosphere (as shown by Venn diagrams and alpha diversity). In a previous study by Cordero et al. (2020), infection with *P. cinnamomi* resulted in an increase in the number of endophytic microbes in the roots and in the number of unique OTUs in many crop species. However, in the current study, although the number of microbes was increased after infection with *P. cinnamomi,* the total number of OTUs in the roots endosphere remained much lower than the rhizosphere soil.

As the roots were not surface-sterilized before analysis, the bacteria detected included some from the rhizosphere as well as endophytic organisms. Of the total root OTUs 30–50% were not detected from the comparable rhizosphere, suggesting that up to half of the microbes have a preference for, or are only found as endophytes in roots. These include the Actinobacteria (e.g., *Williamsia serinedens, Streptomyces reticuliscabie,* and *Goprdonia* sp.), Bacteroidetes (e.g., *Flavobacterium succinicans*, *Mucilaginibacter* sp., and *Sphingobacteriia* sp.), and Proteobacteria (e.g., *Sphiingomonas wittichii*, *Rhizobium giardinii,* and *Asticcacaulis* sp.). Several phyla that occur in the rhizosphere (Chlorobi, Firmicutes, TM6, Planctomycetes, Latescibacteria, and Spirochaetes) were absent from the roots. Yang and Ancona (2021) examined the endophytic bacteria in *Citrus* roots from healthy trees and those infested with *P. nicotianae*. They also recorded a decrease in the bacterial abundance and diversity resulting from infection. However, in their case, the relative abundance of Bacteroidetes increased and that of Proteobacteria decreased, whereas the opposite was the case in the avocado roots studied here. In roots of species in each of the unrelated plant genera *Persea, Citrus,* and *Arabis,* the most abundant root bacteria are from the phyla Proteobacteria, Actinobacteria, and Bacteroidetes but they differ in that in *Arabis*, Firmicutes, and in *Citrus,* Acidobacteria are also common phyla (Dombrowski et al., 2017; Zhang et al., 2017).

CONCLUSION

The observed similarity in the reduction of *Phytophthora* root damage through the application of mineral mulch or phosphite was not linked to parallel changes in the rhizosphere or root microbiome. It is not possible to determine the relative importance of changes to the soil microbiome and changes to plant metabolism induced by the application of silica or phosphite. Changes to the soil microbiome are important, and they are different after the application of silica or spraying with phosphite. Analysis of the whole microbiome suggested that a wide range of bacteria may be involved in suppressing *Phytophthora* root rot, but the major genera are likely to come from the phyla Actinobacteria, Firmicutes, and Proteobacteria. Further insight into the crucial groups involved will be obtained from RNA sequencing.

DATA AVAILABILITY STATEMENT

The data sets related to this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://data.mendeley.com/drafts/h7jxb8frm2, 10.17632/h7jxb8frm2.1.

AUTHOR CONTRIBUTIONS

QF, JM, GH, and TB designed the experiments. QF carried out the experiments. Data were analyzed by QF and PT. All authors contributed equally to the manuscript and agreed to the submitted version.

FUNDING

This research was supported by Murdoch University Scholarship and HIA (Horticulture Innovation Australia) project AV10067.

ACKNOWLEDGMENTS

We thank Russell Delroy from Delroy Orchards for providing mulch from his orchard, Ziad Mekkawi, Rajah Behlaj, and Bill Dunstan for assistance in the glasshouse, Diane White for laboratory work, Sam Abraham and Mark O'Dea for conducting the metabarcoding in their laboratory, Emma Steel for providing help for statistical analysis, and Kmaran Mujahid for figure editing. We also thank the company Mineral Mulch for providing the commercial soil additive.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.870900/full#supplementary-material

Supplementary Figure S1 | Total abundance of phyla across the four treatments and comparing infested and non-infested plants in a) rhizosphere soil and b) root endosphere.

Supplementary Figure S2 | Difference between proportions of reads observed across the four treatments and comparing infested and non-infested plants in rhizosphere soil. The gray taxa on the lower left side represents a key for unlabelled trees. Each mini tree represents a comparison across the treatments in the rows and columns. Taxa colored in green are more abundant in treatments of the row and taxa in tan color are more abundant in treatments in columns. For figure a) and b) the color of each taxon represents the log-2 ratio of median proportion of reads observed in each treatment. Taxa colored in blue are abundant in the treatment mineral mulch whereas taxa colored in tan are more abundant in no mulch. Taxa in gray showed no difference across treatments.

Supplementary Figure S3 | Difference between proportions of reads observed across the four treatments and comparing infested and non-infested plants in root endosphere. The gray taxa on the lower left side represents a key for unlabelled trees. Each mini tree represents a comparison across the treatments in the rows and columns. Taxa colored in green are more abundant in treatments of the row and taxa in tan color are more abundant in treatments in columns. For figure a) and b) the color of each taxon represents the log-2 ratio of median proportion of reads observed in each treatment. Taxa colored in blue are abundant in the treatment mineral mulch whereas taxa colored in tan are more abundant in no mulch. Taxa in gray showed no difference across treatments.

Supplementary Figure S4 | Cluster analysis of **(A)** rhizosphere soil and **(B)** root endosphere bacterial samples across the four treatments. Samples with +Pc indicates those infested with *Phytophthora cinnamomi* and -Pc indicates non-infested.

Supplementary Table S1 | The effect of four soil treatments on shoot dry weight, total root dry weight, fine root dry weight and root damage of non-infested avocado plants or those infested with *Phytophthora cinnamomi*. Significant differences between means for each growth parameter are indicated by different superscript letters. For each parameter, means for non-infested and infested plants in the same treatment that are significantly ($P < 0.05$) different are shown in bold. Root damage scores are 1 (least damaged) to 5 (most damaged). Data from Farooq et al. (2022).

Supplementary Data Sheet S1 | (A) A comparison of the bacterial communities present (*OTU*s) and relative abundance within a particular classification (up to genus level) in non-infested and infested rhizospheres (for each of the four treatments) for which the abundance changed significantly following infection. Significance of differences were assessed by a Mann-Whitney (Wilcoxon) test with significant differences having $P < 0.05$. "-" indicates that the relative abundance at infested rhizosphere had a significantly lower relative abundance than in non-infested rhizosphere, when collapsed to the particular classification level, while a "+" indicates a significantly higher abundance at non-infested rhizosphere, respectively. The columns "n-" ad "n+" refer to the number of OTUs within a genus where an infested rhizosphere had a significantly lower (-) or higher (+) relative abundance, respectively. **(B)** A comparison of the relative abundance of bacterial communities within a particular classification present in the rhizosphere of non-infested pots with only organic mulch and those with, in addition, mineral mulch or in which plants were treated with phosphite. The columns "n-" and "n+" refer to the number of OTUs within a genus where organic mulch treatment had a significantly lower (-) or higher (+) relative abundance, than the mineral mulch or phosphite treatments, respectively.

REFERENCES

Achary, V. M. M., Ram, B., Manna, M., Datta, D., Bhatt, A., Reddy, M. K., et al. (2017). Phosphite: a novel P fertilizer for weed management and pathogen control. *Plant Biotechnol. J.* 15, 1493–1508. doi: 10.1111/pbi.12803

Ando, S., Ito, T., Kanno, T., Kobayashi, T., Morikawa, T., and Honda, K.-,i., et al. (2014). Impact of organic crop management on suppression of bacterial seedling diseases in rice. *Organic Agri.* 4, 187–196. doi: 10.1007/s13165-014-0067-1

Ansari, M. I. (2018). Plant microbiome and its functional mechanism in response to environmental stress. *Int. J. Green Pharm.* 12, S81. https://doi.org/10.22377/ijgp.v12i01.1603

Artyszak, A. (2018). Effect of silicon fertilization on crop yield quantity and quality—A literature review in Europe. *Plants.* 7, 54. doi: 10.3390/plants7030054

Aviles, M., Borrero, C., and Trillas, M. (2011). Review on compost as an inducer of disease suppression in plants grown in soilless culture. *Dyn Soil Dyn Plant.* 5, 1–11. Available online at: http://www.globalsciencebooks.info/Journals/DSDP.html

Avis, T. J., Gravel, V., Antoun, H., and Tweddell, R. J. (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. *Soil Biol. Biochem.* 40, 1733–1740. doi: 10.1016/j.soilbio.2008.02.013

Bakker, M. G., Chaparro, J. M., Manter, D. K., and Vivanco, J. M. (2015). Impacts of bulk soil microbial community structure on rhizosphere microbiomes of *Zea mays*. *Plant Soil.* 392, 115–126. doi: 10.1007/s11104-015-2446-0

Belanger, R. R., Benhamou, N., and Menzies, J. G. (2003). Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f. sp. tritici). *Phytopathology.* 93, 402–412. doi: 10.1094/PHYTO.2003.93.4.402

Belisle, R. J., Hao, W., McKee, B., Arpaia, M. L., Manosalva, P., and Adaskaveg, J. E. (2019). New oomycota fungicides with activity against *Phytophthora cinnamomi* and their potential use for managing avocado root rot in California. *Plant Dis.* 103, 2024–2032. doi: 10.1094/PDIS-09-18-1698-RE

Bent, E. (2006). "Induced systemic resistance mediated by plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF)," in *Multigenic and Induced Systemic Resistance in Plants,* Tuzun, S., and Bent, E. (eds) Boston, MA: Springer. p. 225–258. doi: 10.1007/0-387-23266-4_10

Bhadha, J. H., Capasso, J. M., Khatiwada, R., Swanson, S., and LaBorde, C. (2017). "Raising soil organic matter content to improve water holding capacity," in *SL447. Series UF/IFAS extension University of Florida* (Florida, FL: UF/IFAS Extension, University of Florida), 1–5.

Bhattacharjee, R., and Dey, U. (2014). An overview of fungal and bacterial biopesticides to control plant pathogens/diseases. *Afr. J. Microbiol. Res.* 8, 1749–1762. doi: 10.5897/AJMR2013.6356

Bonanomi, G., Antignani, V., Capodilupo, M., and Scala, F. (2010). Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. *Soil Biol. Biochem.* 42, 136–144. doi: 10.1016/j.soilbio.2009.10.012

Bonanomi, G., Antignani, V., Pane, C., and Scala, F. (2007). Suppression of soilborne fungal diseases with organic amendments. *J. Plant Pathol.* 311–324. Available online at: https://www.jstor.org/stable/41998410?seq=1

Broadbent, P., Baker, K., and Waterworth, Y. (1971). Bacteria and Actinomycetes antogonistic to fungal root pathogens in Australian soils. *Aust. J. Biol. Sci.* 24, 925–944. doi: 10.1071/BI9710925

Broadbent, P., and Baker, K. F. (1974). Behaviour of *Phytophthora cinnamomi* in soils suppressive and conducive to root rot. *Aust. J. Agric. Res.* 25, 121–137. doi: 10.1071/AR9740121

Bronstein, J. L. (1994). Our current understanding of mutualism. *Q. Rev. Biol.* 69, 31–51. doi: 10.1086/418432

Bulluck, L. R., and Ristaino, J. B. (2002). Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. *Phytopathology.* 92, 181–189. doi: 10.1094/PHYTO.2002.92.2.181

Byers, A.-K., Condron, L., O'callaghan, M., Waipara, N., and Black, A. (2020). Soil microbial community restructuring and functional changes in ancient kauri (*Agathis australis*) forests impacted by the invasive pathogen *Phytophthora agathidicida*. *Soil Biol. Biochem.* 150, 108016. doi: 10.1016/j.soilbio.2020.108016

Cazorla, F. M., Romero, D., Perez-Garcia, A., Lugtenberg, B., Vicente, A., d., et al. (2007). Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *J. Appl. Microbiol.* 103, 1950–1959. doi: 10.1111/j.1365-2672.2007.03433.x

Chen, H., Zhao, J., Jiang, J., Zhao, Z., Guan, Z., Chen, S., et al. (2021). Effects of inorganic, organic and bio-organic fertilizer on growth, rhizosphere soil microflora and soil function sustainability in chrysanthemum monoculture. *Agriculture.* 11, 1214. doi: 10.3390/agriculture11121214

Cherif, M., Asselin, A., and Belanger, R. R. (1994). Defense responses induced by soluble silicon in cucumber roots infected by *Pythium* spp. *Phytopathology.* 84, 236–242. doi: 10.1094/Phyto-84-236

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell.* 124, 803–814. doi: 10.1016/j.cell.2006.02.008

Cohen, M. F., Yamasaki, H., and Mazzola, M. (2005). *Brassica napus* seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. *Soil Biol. Biochem.* 37, 1215–1227. doi: 10.1016/j.soilbio.2004.11.027

Cordero, J., de Freitas, J. R., and Germida, J. J. (2020). Bacterial microbiome associated with the rhizosphere and root interior of crops in Saskatchewan, Canada. *Can. J. Microbiol.* 66, 71–85. doi: 10.1139/cjm-2019-0330

Dann, E. K., and Le, D. P. (2017). Effects of silicon amendment on soilborne and fruit diseases of avocado. *Plants (Basel).* 6, 51–65. doi: 10.3390/plants6040051

Dobrowolski, M. P., Shearer, B. L., Colquhoun, I. J., O'brien, P. A., and Hardy, G. E. S. J. (2008). Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide. *Plant Pathol.* 57, 928–936. doi: 10.1111/j.1365-3059.2008.01883.x

Dombrowski, N., Schlaeppi, K., Agler, M. T., Hacquard, S., Kemen, E., Garrido-Oter, R., et al. (2017). Root microbiota dynamics of perennial *Arabis alpina* are dependent on soil residence time but independent of flowering time. *ISME J.* 11, 43–55. doi: 10.1038/ismej.2016.109

Duffy, B. K., Ownley, B. H., and Weller, D. M. (1997). Soil chemical and physical properties associated with suppression of take-all of wheat by *Trichoderma koningii*. *Phytopathology.* 87, 1118–1124. doi: 10.1094/PHYTO.1997.87.11.1118

Duvenhage, J., Kotz,é, J., and Maas, E. M. (1991). Suppressive soils and biological control of *Phytophthora* root rot. *South African Avocado Growers Association Yearbook.* 14, 06–11.

Eshraghi, L., Anderson, J. P., Aryamanesh, N., McComb, J. A., Shearer, B., and Hardy, G. E. S. J. (2014). Defence signalling pathways involved in plant resistance and phosphite-mediated control of *Phytophthora cinnamomi*. *Plant Mol. Biol. Rep.* 32, 342–356. doi: 10.1007/s11105-013-0645-5

Farooq, Q., McComb, J., Hardy, G. S. J., and Burgess, T. I. (2022). Soil amendments and suppresssion of *Phytophthora* root rot in avocado (*Persea indica*). *BIORXIV,* 478582. doi: 10.1101/2022.01.31.478582

Fauteux, F., Remus-Borel, W., Menzies, J. G., and Belanger, R. R. (2005). Silicon and plant disease resistance against pathogenic fungi. *FEMS Microbiol. Lett.* 249, 1–6. doi: 10.1016/j.femsle.2005.06.034

Fawe, A., Menzies, J. G., Cherif, M., and Belanger, R. R. (2001). "Silicon and disease resistance in dicotyledons," in *Studies in plant science,* Datnoff, L. E., Snyder, G. H., and Korndorfer, G. H. (eds). Amsterdam: Elsevier. *159*—169. doi: 10.1016/S0928-3420(01)80013-6

Gao, Z., Jiang, Y., Yin, C., Zheng, W., Nikolic, N., Nikolic, M., et al. (2022). Silicon fertilization influences microbial assemblages in rice roots and decreases arsenic concentration in grain: A five-season in-situ remediation field study. *J. Hazard. Mater.* 423, 127180. doi: 10.1016/j.jhazmat.2021.127180

Garbeva, P., Postma, J., Van Veen, J., and Van Elsas, J. (2006). Effect of above-ground plant species on soil microbial community structure and its impact on suppression of *Rhizoctonia solani* AG3. *Environ. Microbiol.* 8, 233–246. doi: 10.1111/j.1462-2920.2005.00888.x

Garbeva, P., Silby, M. W., Raaijmakers, J. M., Levy, S. B., and De Boer, W. (2011). Transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors. *ISME J.* 5, 973. doi: 10.1038/ismej.2010.196

Gilardi, G., Pugliese, M., Gullino, M. L., and Garibaldi, A. (2016). Effect of different organic amendments on lettuce fusarium wilt and on selected soilborne microorganisms. *Plant Pathol.* 65, 704–712. doi: 10.1111/ppa.12460

Gill, U. S., Sun, L., Rustgi, S., Tang, Y., von Wettstein, D., and Mysore, K. S. (2018). Transcriptome-based analyses of phosphite-mediated suppression of rust pathogens *Puccinia emaculata* and *Phakopsora pachyrhizi* and functional characterization of selected fungal target genes. *Plant J.* 93, 894–904. doi: 10.1111/tpj.13817

Guevara-Avendano, E., Carrillo, J. D., Ndinga-Muniania, C., Moreno, K., Mendez-Bravo, A., Guerrero-Analco, J. A., et al. (2018). Antifungal activity of avocado rhizobacteria against *Fusarium euwallaceae* and *Graphium* spp., associated with *Euwallacea* spp. nr. fornicatus, and Phytophthora cinnamomi. *Antonie Van Leeuwenhoek* 111, 563–572. doi: 10.1007/s10482-017-0977-5

Hardham, A. R., and Blackman, L. M. (2018). *Phytophthora cinnamomi*. *Mol. Plant Pathol.* 19, 260–285. doi: 10.1111/mpp.12568

Hoitink, H. A. J., and Boehm, M. J. (1999). Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. *Annu. Rev. Phytopathol.* 37, 427–446. doi: 10.1146/annurev.phyto.37.1.427

Hu, J., Wei, Z., Friman, V.-P., Gu, S.-,h., Wang, X.-,f., Eisenhauer, N., et al. (2016). Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. *MBio.* 7, e01790–e01716. doi: 10.1128/mBio.01790-16

Hu, L., Robert, C. A., Cadot, S., Zhang, X., Ye, M., Li, B., et al. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nat. Commun.* 9, 1–13. doi: 10.1038/s41467-018-05122-7

Hunziker, L., Bönisch, D., Groenhagen, U., Bailly, A., Schulz, S., and Weisskopf, L. (2015). *Pseudomonas* strains naturally associated with potato plants produce volatiles with high potential for inhibition of *Phytophthora infestans*. *Appl. Environ. Microbiol.* 81, 821–830. doi: 10.1128/AEM.02999-14

Inderbitzin, P., Ward, J., Barbella, A., Solares, N., Izyumin, D., Burman, P., et al. (2018). Soil microbiomes associated with Verticillium wilt-suppressive broccoli and chitin amendments are enriched with potential biocontrol agents. *Phytopathology.* 108, 31–43. doi: 10.1094/PHYTO-07-17-0242-R

Jambhulkar, P. P., Sharma, M., Lakshman, D., and Sharma, P. (2015). "Natural mechanisms of soil suppressiveness against diseases caused by *Fusarium, Rhizoctonia, Pythium,* and *Phytophthora*," in *Organic Amendments and Soil Suppressiveness in Plant Disease Management,* Meghvansi, M. K., and Varma, A. (eds). Cham: Springer. 95–123. doi: 10.1007/978-3-319-23075-7_5

Junaid, J. M., Dar, N. A., Bhat, T. A., Bhat, A. H., and Bhat, M. A. (2013). Commercial biocontrol agents and their mechanism of action in the management of plant pathogens. *Int. J. Mod. Plant and Ani. Sci.* 1, 39–57. Available online at: https://www.ModernScientificPress.com/Journals/IJPlant.aspx

Klein, E., Katan, J., and Gamliel, A. (2011). Soil suppressiveness to Fusarium disease following organic amendments and solarization. *Plant Dis.* 95, 1116–1123. doi: 10.1094/PDIS-01-11-0065

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, e1–10.1093. doi: 10.1093/nar/gks808

Koberl, M., Ramadan, E. M., Adam, M., Cardinale, M., Hallmann, J., Heuer, H., et al. (2013). *Bacillus* and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt. *FEMS Microbiol. Lett.* 342, 168–178. doi: 10.1111/1574-6968.12089

Li, M., Wang, Q., Liu, Z., Pan, X., and Zhang, Y. (2019). Silicon application and related changes in soil bacterial community dynamics reduced ginseng black spot incidence in Panax ginseng in a short-term study. *BMC Microbiol.* 19, 1–12. doi: 10.1186/s12866-019-1627-z

Liu, T., Xiao, Y., Yin, J., Yi, T., Zhou, Z., Hsiang, T., et al. (2020). Effects of cultured root and soil microbial communities on the disease of Nicotiana tabacum caused by *Phytophthora nicotianae*. *Front. Microbiol.* 11, 929. doi: 10.3389/fmicb.2020.00929

Lo, C. C. (2010). Effect of pesticides on soil microbial community. *J. Environ. Sci. Health B.* 45, 348–359. doi: 10.1080/03601231003799804

Martins, J., Ares, A., Casais, V., Costa, J., and Canhoto, J. (2021). Identification and characterization of *Arbutus unedo* L. Endophytic bacteria isolated from wild and cultivated trees for the biological control of *Phytophthora cinnamomi*. *Plants* 10, 1569. doi: 10.3390/plants10081569

Menzies, J., Bowen, P., Ehret, D., and Glass, A. D. (1992). Foliar applications of potassium silicate reduce severity of powdery mildew on cucumber, muskmelon, and zucchini squash. *J. Am. Soc. Hort. Sci.* 117, 902–905. doi: 10.21273/JASHS.117.6.902

Mohammadi, M. A., Han, X., Zhang, Z., Xi, Y., Boorboori, M., and Wang-Pruski, G. (2020). Phosphite application alleviates *Pythophthora infestans* by modulation of photosynthetic and physio-biochemical metabolites in potato leaves. *Pathogens.* 9, 170. doi: 10.3390/pathogens9030170

Molano, P. J. T. (2007). Enfermedades del aguacate. *Revista politécnica.* 3, 51–70. Available online at: https://revistapolitecnica.epn.edu.ec/ojs2/index.php/revista_politecnica2

Moraes, S. R. G., Pozza, E. A., Pozza, A. A. A., de Carvalho, J. G., and de Souza, P. E. (2009). Nutrition in bean plants and anthracnose intensity in function of silicon and copper application. *Acta Sci. Agron.* 31, 283–291.

Mukta, J. A., Rahman, M., Sabir, A. A., Gupta, D. R., Surovy, M. Z., Rahman, M., et al. (2017). Chitosan and plant probiotics application enhance growth and yield of strawberry. *Biocatal. Agric. Biotechnol.* 11, 9–18. doi: 10.1016/j.bcab.2017.05.005

Nakkeeran, S., Kavitha, K., Mathiyazhagan, S., Fernando, W., Chandrasekar, G., and Renukadevi, P. (2004). Induced systemic resistance and plant growth promotion by *Pseudomonas chlororaphis* strain PA-23 and *Bacillus subtilis* strain CBE4 against rhizome rot of turmeric (*Curcuma longa* L.). *Can. J. Plant Pathol.* 26, 417–418.

Oldfield, E. E., Wood, S. A., and Bradford, M. A. (2018). Direct effects of soil organic matter on productivity mirror those observed with organic amendments. *Plant Soil.* 423, 363–373. doi: 10.1007/s11104-017-3513-5

Pal, K. K., and Gardener, B. M. (2006). Biological control of plant pathogens. *Plant Health Instr.* 1–25. doi: 10.1094/PHI-A-2006-1117-02

Pegg, K. G., Whiley, A. W., Langdon, P. W., and Saranah, J. B. (1987). Comparison of phosetyl-Al, phosphorous acid and metalaxyl for the long-term control of *Phytophthora* root rot of avocado. *Aust. J. Exp. Agric.* 27, 471–474. doi: 10.1071/EA9870471

Perez-Jimenez, R. M. (2008). Significant avocado diseases caused by fungi and oomycetes. *Eur. J. Plant Sci. Biotech.* 2, 1–24. Available online at: http://www.globalsciencebooks.info/Journals/EJPSB.html

Perez-Piqueres, A., Edel-Hermann, V., Alabouvette, C., and Steinberg, C. (2006). Response of soil microbial communities to compost amendments. *Soil Biol. Biochem.* 38, 460–470. doi: 10.1016/j.soilbio.2005.05.025

Postma, J., Schilder, M. T., Bloem, J., and van Leeuwen-Haagsma, W. K. (2008). Soil suppressiveness and functional diversity of the soil microflora in organic farming systems. *Soil Biol. Biochem.* 40, 2394–2406. doi: 10.1016/j.soilbio.2008.05.023

Pozza, E. A., Pozza, A. A. A., and Botelho, D. M. D. S. (2015). Silicon in plant disease control. *Rev. Ceres.* 62, 323–331. doi: 10.1590/0034-737X201562030013

Prabhu, A. S., Barbosa Filho, M. P., Datnoff, L. E., Snyder, G. H., Berni, R. F., Rodrigues, F. A., et al. (2012). Silicon reduces brown spot severity and grain discoloration on several rice genotypes. *Trop. Plant Pathol.* 37, 409–414. doi: 10.1590/S1982-56762012000600005

Rahman, A., Wallis, C. M., and Uddin, W. (2015). Silicon-induced systemic defense responses in perennial ryegrass against infection by *Magnaporthe oryzae*. *Phytopathology.* 105, 748–757. doi: 10.1094/PHYTO-12-14-0378-R

Rajput, V. D., Minkina, T., Feizi, M., Kumari, A., Khan, M., Mandzhieva, S., et al. (2021). Effects of silicon and silicon-based nanoparticles on rhizosphere microbiome, plant stress and growth. *Biology.* 10, 791. doi: 10.3390/biology10080791

Ramezani, M., Ramezani, F., Rahmani, F., and Dehestani, A. (2018). Exogenous potassium phosphite application improved PR-protein expression and associated physio-biochemical events in cucumber challenged by *Pseudoperonospora cubensis*. *Sci. Hortic.* 234, 335–343. doi: 10.1016/j.scienta.2018.02.042

Ramirez-Gil, J. G., Castañeda-Sánchez, D. A., and Morales-Osorio, J. G. (2017). Production of avocado trees infected with *Phytophthora cinnamomi* under different management regimes. *Plant Pathol.* 66, 623–632. doi: 10.1111/ppa.12620

Ramirez-Gil, J. G., Morales, J. G., and Peterson, A. T. (2018). Potential geography and productivity of "Hass" avocado crops in Colombia estimated by ecological niche modeling. *Sci. Hortic.* 237, 287–295. doi: 10.1016/j.scienta.2018.04.021

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

Samaddar, S., Truu, J., Chatterjee, P., Truu, M., Kim, K., Kim, S., et al. (2019). Long-term silicate fertilization increases the abundance of Actinobacterial population in paddy soils. *Biol. Fertility Soils.* 55, 109–120. doi: 10.1007/s00374-018-01335-6

Samuels, A. L., Glass, A. D. M., Ehret, D. L., and Menzies, J. G. (1991). Mobility and deposition of silicon in cucumber plants. *Plant, Cell Environ.* 14, 485–492. doi: 10.1111/j.1365-3040.1991.tb01518.x

Shetty, R., Frette, X., Jensen, B., Shetty, N. P., Jensen, J. D., Jorgensen, H. J. L., et al. (2011). Silicon-induced changes in antifungal phenolic acids, flavonoids, and key phenylpropanoid pathway genes during the interaction between miniature roses and the biotrophic pathogen *Podosphaera pannosa*. *Plant Physiol.* 157, 2194–2205. doi: 10.1104/pp.111.185215

Song, X.-P., Verma, K. K., Tian, D.-D., Zhang, X.-Q., Liang, Y.-J., Huang, X., et al. (2021). Exploration of silicon functions to integrate with biotic stress tolerance and crop improvement. *Biol. Res.* 54, 19. doi: 10.1186/s40659-021-00344-4

Su, L., Mo, X., Sun, J., Qiu, P., Zhang, R., Kuramae, E. E., et al. (2021a). Potassium Phosphite Modulated the Soil Microbiome and Enriched the Antagonistic Bacteria *Streptomyces Coelicoflavus* and *Paenibacillus Favisporus* to Inhibit the Tomato Pathogen *Ralstonia Solanacearum* Synergistically. *Res. Squ*. doi: 10.21203/rs.3.rs-143372/v1

Su, L., Qiu, P., Fang, Z., Mo, X., Sun, J., Liu, Y., et al. (2021b). Potassium phosphite enhances the antagonistic capability of *Bacillus amyloliquefaciens* to manage tomato bacterial wilt. *Plant Dis*. doi: 10.1094/PDIS-08-21-1601-RE

Tenuta, M., and Lazarovits, G. (2002). Ammonia and nitrous acid from nitrogenous amendments kill the microsclerotia of *Verticillium dahliae*. *Phytopathology.* 92, 255–264. doi: 10.1094/PHYTO.2002.92.3.255

Tubana, B. S., Babu, T., and Datnoff, L. E. (2016). A review of silicon in soils and plants and its role in US agriculture: history and future perspectives. *Soil Sci.* 181, 393–411. doi: 10.1097/SS.0000000000000179

van Bruggen, A. H., Gamliel, A., and Finckh, M. R. (2016). Plant disease management in organic farming systems. *Pest Manage. Sci.* 72, 30–44. doi: 10.1002/ps.4145

Van, O. S. G. J., and Van Ginkel, J.H. (2001). Suppression of *Pythium* root rot in bulbous Iris in relation to biomass and activity of the soil microflora. *Soil Biol. Biochem.* 33, 1447–1454. doi: 10.1016/S0038-0717(01)00053-0

Wei, F., Feng, H., Zhang, D., Feng, Z., Zhao, L., Zhang, Y., et al. (2021). Composition of rhizosphere microbial communities associated with healthy and Verticillium wilt diseased cotton plants. *Front. Microbiol.* 12, 618169. doi: 10.3389/fmicb.2021.618169

Yang, C., and Ancona, V. (2021). Metagenomic analysis reveals reduced beneficial microorganism associations in roots of foot-rot-affected citrus trees. *Phytobiomes J.* 5, 305–315. doi: 10.1094/PBIOMES-07-20-0049-R

Yang, C. H., Crowley, D. E., and Menge, J. A. (2001). 16S rDNA fingerprinting of rhizosphere bacterial communities associated with healthy and *Phytophthora* infected avocado roots. *FEMS Microbiol. Ecol.* 35, 129–136. doi: 10.1111/j.1574-6941.2001.tb00796.x

Yin, B., Scupham, A. J., Menge, J. A., and Borneman, J. (2004). Identifying microorganisms which fill a niche similar to that of the pathogen: a new

investigative approach for discovering biological control organisms. *Plant Soil.* 259, 19–27. doi: 10.1023/B:PLSO.0000020944.45798.56

Yoon, H., Klinzing, G., and Blanch, H. (1977). Competition for mixed substrates by microbial populations. *Biotechnol. Bioeng.* 19, 1193–1210. doi: 10.1002/bit.260190809

You, M. P., Sivasithamparam, K., and Kurtboke, D. I. (1996). Actinomycetes in organic mulch used in avocado plantations and their ability to suppress *Phytophthora cinnamomi*. *Biol. Fertility Soils.* 22, 237–242. doi: 10.1007/BF00382518

Youseif, S. H. (2018). Genetic diversity of plant growth promoting rhizobacteria and their effects on the growth of maize plants under greenhouse conditions. *Ann. Agric. Sci.* 63, 25–35. doi: 10.1016/j.aoas.2018.04.002

Zhang, W., Han, D. Y., Dick, W. A., Davis, K. R., and Hoitink, H. A. J. (1998). Compost and compost water extract-induced systemic acquired resistance in cucumber and *Arabidopsis*. *Phytopathology.* 88, 450–455. doi: 10.1094/PHYTO.1998.88.5.450

Zhang, Y., Xu, J., Riera, N., Jin, T., Li, J., and Wang, N. (2017). Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. *Microbiome.* 5, 1–17. doi: 10.1186/s40168-017-0304-4

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.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 May 2022
doi: 10.3389/fmicb.2022.871148

Phylogenetic Patterns of Swainsonine Presence in Morning Glories

*Quynh N. Quach[1], Dale R. Gardner[2], Keith Clay[1] and Daniel Cook[2]**

[1] Department of Ecology and Evolutionary Biology, Tulane University, New Orleans, LA, United States, [2] United States Department of Agriculture – Agricultural Research Service, Poisonous Plant Research Laboratory, Logan, UT, United States

Endosymbionts play important roles in the life cycles of many macro-organisms. The indolizidine alkaloid swainsonine is produced by heritable fungi that occurs in diverse plant families, such as locoweeds (Fabaceae) and morning glories (Convolvulaceae) plus two species of Malvaceae. Swainsonine is known for its toxic effects on livestock following the ingestion of locoweeds and the potential for pharmaceutical applications. We sampled and tested herbarium seed samples (n = 983) from 244 morning glory species for the presence of swainsonine and built a phylogeny based on available internal transcribed spacer (ITS) sequences of the sampled species. We show that swainsonine occurs only in a single morning glory clade and host species are established on multiple continents. Our results further indicate that this symbiosis developed ∼5 mya and that swainsonine-positive species have larger seeds than their uninfected conspecifics.

Keywords: swainsonine, fungal symbiosis, morning glory, heritable symbiosis, *Ipomoea*

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Hao Lu, Northwest A&F University, China
Rufin Toghueo, Institute of Natural Resources and Agrobiology of Salamanca (CSIC), Spain
Weilan Gomes Paixão Melo, University of São Paulo, Brazil

***Correspondence:**
Daniel Cook
daniel.cook@usda.gov

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *07 February 2022*
Accepted: *07 March 2022*
Published: *03 May 2022*

Citation:
Quach QN, Gardner DR, Clay K and Cook D (2022) Phylogenetic Patterns of Swainsonine Presence in Morning Glories. Front. Microbiol. 13:871148. doi: 10.3389/fmicb.2022.871148

INTRODUCTION

Heritable micro-organisms, passed down from mother to offspring through seeds or eggs, play critical roles in the life cycles of many organisms but their prevalence and functional roles are unknown for most plants. This stands in contrast with the well-documented ancient endosymbiotic origins of chloroplasts and mitochondria. Differing from the diverse endophytic fungal communities in plants that result from the environmental transmission and form highly localized infections within single leaves or other tissues (Christian et al., 2015), heritable micro-organisms, most often fungal endosymbionts, are associated with only a few selected major plant families. When a vertically transmitted symbiont benefits the host, it has an indirect positive effect on its own fitness as hereditary symbionts are completely dependent on the host for their own propagation and reproductive fitness and so should not reduce host fitness (Ewald, 1987; Lipsitch et al., 1996). Understanding the origin, distribution, and function of heritable fungi provide insights into the evolution and ecology of major plant groups.

Fungi in general produce a wide array of secondary metabolites. The production of bioactive metabolites by endosymbiotic fungi that is observed in multiple hereditary plant-endosymbiotic fungal associations might have particular importance to the fitness of both the fungus and the

Abbreviations: Swainsonine positive (S+); swainsonine negative (S−).

host species. The benefits and costs of the symbiosis are not well understood in these systems except for the fact that many of these metabolites have toxic effects upon ingestion of the host plant. The indolizidine alkaloid swainsonine (**Figure 1**) is the toxic principle in a number of plant species worldwide and causes severe toxicosis in livestock grazing these plants (Colegate et al., 1979; Molyneux and James, 1982; Molyneux et al., 1995; de Balogh et al., 1999; Colodel et al., 2002; Dantas et al., 2007). Swainsonine is an alpha-mannosidase and mannosidase II inhibitor that alters glycoprotein processing and causes lysosomal storage disease (Colegate et al., 1979; Dorling et al., 1980; Tulsiani et al., 1988). Consumption of swainsonine-containing plants by grazing animals leads to a chronic disease characterized by weight loss, depression, altered behavior, decreased libido, infertility, and death, which are estimated to cause tens of millions of dollars in livestock losses annually (Panter et al., 1999). Swainsonine occurs sporadically in three diverse plant families: Fabaceae (Fabales), Malvaceae (Malvales), and the morning glory family Convolvulaceae (Solanales) (Cook et al., 2014).

Many of the plant species identified to date that contain swainsonine primarily result from episodes of poisoning as the clinical signs and associated pathology are similar in poisoned livestock (Cook et al., 2014). These cases of livestock poisoning include more than 25 swainsonine-containing legumes commonly referred to as locoweeds (Fabaceae), such as *Astragalus*, *Oxytropis*, and *Swainsona* species. Toxic species within these genera have been identified in Australia, China, North America, and South America. Likewise, seven morning glory species belonging to the genus *Ipomoea* have been reported to contain swainsonine due to episodes of livestock poisoning in Africa, Australia, and South America (Cook et al., 2014). Lastly, two swainsonine-containing *Sida* species (Malvaceae) have been identified in South America due to poisoning of livestock (Colodel et al., 2002; Micheloud et al., 2017). More recently, preserved herbarium specimens have been used to test and identify several *Astragalus*, *Oxytropis*, and *Swainsona* species that contain swainsonine (Cook et al., 2016, 2017b,c,d). Herbarium specimens provide an excellent resource to investigate the phytochemical composition of plants and their distribution among species (Cook et al., 2021).

Prior research has demonstrated that all swainsonine-containing plant taxa investigated to date are associated with systemic, heritable, seed-transmitted fungal symbionts that produce swainsonine. Swainsonine-containing legumes (*Astragalus, Oxytropis*, and *Swainsona* spp.) are associated with a fungal symbiont from *Alternaria* section *Undifilum* (Pleosporales) (Braun et al., 2003; Yu et al., 2010; Baucom et al., 2012; Grum et al., 2013). By contrast, the swainsonine-containing convolvulaceous taxon, *Ipomoea carnea* is associated with a distinct fungal symbiont belonging to the order Chaetothyriales (Cook et al., 2013 and **Supplementary Figure 1**). Other phylogenetically disjunct groups of swainsonine-producing fungi are found to associate with distantly related plant families. *Metarhizium* sp. (Hypocreales) are both insect pathogens and plant symbionts while *Slafractonia leguminicola* (Pleosporales), a pathogen of red clover (*Trifolium pratense*), also produce swainsonine (Gough and Elliott, 1956; Cook et al., 2014). Swainsonine-producing fungi that are not associated with plants include several species in the Arthrodermataceae (Onygenales), a family of fungi that causes athlete's foot and ringworm diseases in humans and other mammals (Cook et al., 2017a). Recently, molecular genetic studies have identified and characterized the orthologous swainsonine biosynthetic genes in the above four orders of fungi (Cook et al., 2017a).

It is notable that other species of morning glories (Convolvulaceae) are associated with a distinct group of hereditary fungi classified in the genus *Periglandula* (Clavicipitaceae), that produce the ergot and indole diterpene alkaloids similar to those found in grasses infected with fungal endophytes from the same fungal family (Steiner et al., 2011; Schardl et al., 2013). A recent study found that *Periglandula* fungi are associated with particular clades of morning glories, where they produce distinct mixtures of ergot alkaloids and where symbiotic hosts have significantly larger seed sizes than non-symbiotic species (Beaulieu et al., 2021).

To further our understanding of the morning glory/Chaetothyriaceae/swainsonine association and to test the hypothesis that the presence of swainsonine, used here to indicate the presence of the heritable symbiont, is found in particular phylogenetic groups, we (1) tested for the presence of swainsonine in seeds of 244 morning glory species from diverse, worldwide herbarium collections in order to determine the global distribution and diversity of Chaetothyriaceae symbiosis, (2) obtained phylogenetically informative internal transcribed spacer (ITS) sequences from GenBank and determined the distribution of swainsonine-positive (S+) and S − species across the morning glory host phylogeny to elucidate the evolutionary history of the symbiosis, and (3) evaluated whether swainsonine and Chaetothyriaceae symbiosis vary with a key plant life-history trait to understand potential selective benefits of this symbiosis for the host. Our results provide new insights into the evolution of heritable fungal symbiosis and the diversification of swainsonine-producing fungi in morning glories.

MATERIALS AND METHODS

Herbarium Survey

We obtained permissions to sample mature seeds and/or vegetative parts from herbarium specimens from the Missouri Botanical Garden (MOBOT), Vadense Herbarium (WAG, Netherlands), Western Australian Herbarium (PERTH), and the Australian National University Herbarium (ANU), in addition to seeds from personal collections of Convolvulaceae researchers. We sampled mature seeds and/or vegetative parts from a total of 244 morning glory species from 983 herbarium sheets, where species identity, mean swainsonine content, mean seed mass, and mean collection latitude are reported by species for each herbarium specimen where available (**Supplementary Data 1**). The sampled species include representatives from all continents except Europe (where we had only one sample; **Figure 2**) and Antarctica. Some species have localized distributions, while others have regional or pantropical distributions (**Supplementary Data 1**). Our samples represented

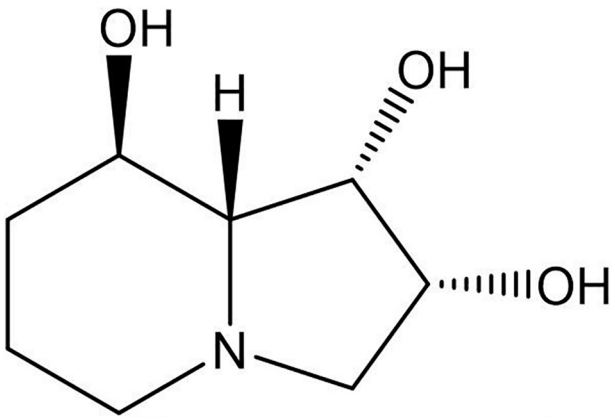


FIGURE 1 | Structure of the indolizidine alkaloid swainsonine.

a subset of all potential host species because most herbarium specimens examined did not have mature seeds. We relied on the records of herbarium specimen labels but realize that misidentifications are possible and have updated any naming conventions to the best of our abilities.

Swainsonine Analysis

Swainsonine was extracted using a modification of a procedure described by Gardner and Cook (2011). A measured quantity of plant material was extracted in a volume of 2% acetic acid for 18 h with agitation. After extraction, samples were centrifuged and an aliquot from the extraction was diluted into 20 mM ammonium acetate in a 1 ml auto-sampler vial. Samples were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) to detect swainsonine as previously described (Gardner et al., 2001). Presence/absence data were recorded primarily with concentrations being recorded when the sample permitted.

Species that tested positive for swainsonine by the LC-MS/MS method were subsequently verified to contain swainsonine by gas chromatography-mass spectrometry (GC-MS) as a secondary screen. Sample preparation was similar to those described by Cook et al. (2016). All samples were analyzed by GC-MS for swainsonine (trimethylsilyl [TMS] derivative) using the GC-MS conditions previously described (Gardner et al., 2001).

Estimating Morning Glory Phylogeny With ITS Sequence Data

We downloaded the ITS sequence entries from GenBank available in 2021 for 201 species (**Supplementary Data 2**) across multiple genera (*Argyreia, Distimake, Ipomoea, Jacquemontia, Stictocardia*, and *Merremia*) that we sampled for swainsonine. For each species, we obtained the complete ITS1 + 5.8S rRNA + ITS2 sequence where possible. In some cases, this was obtained by combining partial sequences from multiple accessions of the same species. In total, we obtained 194 complete ITS1 + 5.8S + ITS2 sequences, three sequences from combining partial sequences and four were partial ITS1 +5.8S + ITS2 sequences. These sequences were aligned and trimmed using MAFFT v7.489 (Katoh et al., 2019) using the L-INS-i alignment strategy with a 1.7 gap penalty. We used jModelTest2 (Darriba et al., 2012) on CIPRES Science Gateway (Miller et al., 2010) to determine the best substitution model, which was GTR+G+I based on Akaike Information Criteria (AIC). A maximum-likelihood phylogeny was then estimated using RAxML-HPC2 on XSEDE on CIPRES Science Gateway (Miller et al., 2010) using the GTR+G model, as the developers of RAxML do not recommend using the proportion of invariable sites estimation, with 1,000 bootstrap replicates. We also estimated a Bayesian tree using MrBayes v3.2.7a on XSEDE on CIPRES Science Gateway (Miller et al., 2010) with two runs and four independent chains using the GTR+G+I model for 10,000,000 generations, sampled every 1,000 generations, with the first 25% as burn in. We used Tracer v1.7.2 (Rambaut et al., 2018) to check for convergence. For both methods, we designated *Merremia sibirica* as the outgroup species based on previous Convolvulaceae phylogenetic work (Simoes et al., 2015).

We recognize the limitations of inferring a phylogeny based on a single gene, but most of our sampled species had no other gene sequences available. Our results are in general agreement with published smaller, multi-gene phylogenies, and the ITS sequences used here have been used to construct phylogenies in other published studies (Muñoz-Rodríguez et al., 2019; Beaulieu et al., 2021).

Testing Phylogenetic Signal for Swainsonine and Seed Mass

i) Swainsonine presence: We tested for phylogenetic signal in the presence of swainsonine to determine whether S+ species across the Ipomoeeae tribe are closely related species. We followed the analyses methods described by Beaulieu et al. (2021). In short, we used the function "fitDiscrete" from the R package "geiger" (Pennell et al., 2014) to fit different models of character evolution (FitzJohn et al., 2009). We used the all-rates-different (ARD) model based on the lowest AIC score. We then used Pagel's λ (Pagel, 1999) to assess the phylogenetic signal where there is a strong phylogenetic signal if λ is equal to or near 1. We transformed our phylogeny to one with no phylogenetic signal ($\lambda = 0$) and compared its likelihood scores with our observed phylogeny and computed the *p*-value to determine if our observed phylogeny significantly differed from the no-signal phylogeny.

We also computed Fritz and Purvis's D (FPD) (Fritz and Purvis, 2010) as an alternative measure of the phylogenetic signal using the function "phylo.d" from R package "caper" (Orme et al., 2013). Where D is a measure of phylogenetic dispersion and can be less than 1 (non-random distribution) or equal to 1 (random distribution).

ii) Seed mass: All average seed masses were log10-transformed to normalize the data then phylogenetic signal testing for seed mass was conducted as was done for the presence of swainsonine, except the "fitContinuous" function from the R package "geiger" (Pennell et al., 2014) was used since the data are continuous. The

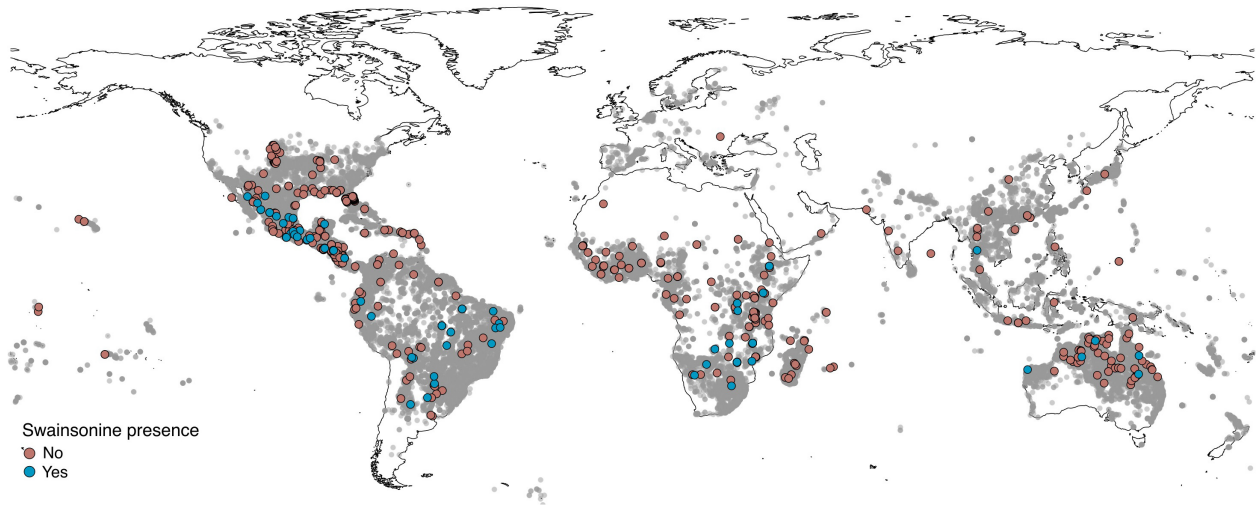


FIGURE 2 | Distribution of samples. Global distribution of samples with geographic information ($n = 666$) tested for swainsonine. Gray circles represent herbarium specimens from *Argyreia, Ipomoea, Stictocardia,* and *Turbina* (now *Ipomoea*) species from the Global Biodiversity Information Facility (GBIF.org, 2021a,b,c,d) to illustrate the geographical distribution of the tribe Ipomoeeae with higher concentrations of sample in darker areas.

FPD statistic can only be applied to binary traits so we did not apply it to seed mass.

Ancestral Character-State Reconstruction

We followed the analyses methods described by Beaulieu et al. (2021). In short, we reconstructed ancestral states for the presence of swainsonine (yes, no) and seed mass (large, small) using the R packages "ape" (Paradis and Schliep, 2019) and "phytools" (Revell, 2012). We used "ace" from R package "ape" to fit the best model of trait evolution for our data by comparing AIC scores, which show that the ARD model was better than other models (equal-rates and symmetric). This produces a likelihood at each internal node of being S+ or S −, or large or small seeds, character states. Ancestors were designated as being one state or another if the likelihood of it being that state was greater than 75%.

Correlated Evolution in Swainsonine Presence, Concentration, and Seed Mass

We followed the analyses methods described by Beaulieu et al. (2021). In short, we used two methods to test for correlated evolution between swainsonine and seed mass. First, using log10-transformed seed mass data, a phylogenetic logistic regression was performed using the function "phyloglm" from the R package "phylolm" (Ho and Ané, 2014). Second, the function "fitPagel" from the R package "phytools" (Revell, 2012) was used to perform Pagel's test of correlation (Pagel, 1994). Pagel's correlation assesses correlated evolution between two binary characters, so we transformed seed mass into a binary trait. The average seed mass of all surveyed species was log10-transformed and was used to separate the large (> 1.615, 41.23 mg) from the small (< 1.615, 41.23 mg) group.

In addition to a linear regression, we use the function "phylolm" from the R package "phylolm" (Ho and Ané, 2014) to perform a phylogenetic linear regression to test for a correlation between seed mass and swainsonine concentration in S+ species ($n = 21$) where both values were log10-transformed.

RESULTS

Distribution of Swainsonine in Morning Glory Species

In total, 32 of 244 (13%) morning glory species that we evaluated from herbarium specimens (**Supplementary Data 1**) contained swainsonine and are therefore symbiotic (**Figure 2**). In total, 24 of the 32 species were not previously known to be S+, and S+ species were distributed across multiple continents (**Table 1**). *Ipomoea sericosepala* was previously reported to be S+ (Mendonça et al., 2018) but we did not detect swainsonine in our samples of the species. Given that there are more than 800 morning glory species, the total number of S+ species will certainly become larger with further sampling.

Swainsonine Distribution in Host Phylogeny

To evaluate the occurrence of swainsonine in relation to morning glory host phylogeny, we obtained published ITS sequences for 201 of our 244 surveyed species (**Supplementary Data 2**). In both our maximum likelihood and Bayesian inferred phylogenies (**Figures 3**, **4** and **Supplementary Figure 2**), we found that all S+ species occurred within a single clade of closely related species and that swainsonine was absent from the rest of the morning

TABLE 1 | Swainsonine positive species.

Swainsonine positive	Location	References	Average seed mass (mg)	Swainsonine concentration (%)
Ipomoea albivenia	Africa		71.5	1.07
Ipomoea arborescens	Mexico		52.5	0.23
Ipomoea brasiliana[1]	S. America	Dantas et al., 2007; Mendonça et al., 2012, 2018	108	0.5
Ipomoea calobra[1]	Australia	Molyneux et al., 1995	N/A	0.11
Ipomoea carnea[1]	Pantropical	de Balogh et al., 1999; Haraguchi et al., 2003	66.15	0.07
Ipomoea cavalcantei	S. America		20	0.06
Ipomoea chilopsidis	Mexico		N/A	N/A
Ipomoea costata[1]	Australia	Cook et al., 2019	114	0.09
Ipomoea cuneifolia	S. America		10	0.12
Ipomoea descolei	S. America		58	0.4
Ipomoea haenkeana	S. America		8	0.07
Ipomoea hieronymi[1]	S. America	Cholich et al., 2021	33	0.25
Ipomoea intrapilosa	Mexico		79	0.04
Ipomoea lapidosa	Africa		123	0.79
Ipomoea longifolia	C. America		117.5	0.1
Ipomoea malvaeoides	S. America		30	0.08
Ipomoea marcellia	S. America		N/A	N/A
Ipomoea marmorata	Africa		213	0.45
Ipomoea megapotamica[1]	S. America	Barbosa et al., 2006	N/A	N/A
Ipomoea murucoides	C. America		154.29	0.14
Ipomoea opulifolia	S. America		56	N/A
Ipomoea pauciflora	C. & S. America		73.6	0.14
Ipomoea polpha[1]	Australia	Molyneux et al., 1995	99	N/A
Ipomoea populina	C. America		65	N/A
Ipomoea prismatosyphon	Africa		71	N/A
Ipomoea pterocaulis[1]	S. America	Barbosa et al., 2006	20	0.74
Ipomoea rosea[3]	S. America	Mendonça et al., 2018	N/A	N/A
Ipomoea saopaulista	S. America		4.25	0.03
Ipomoea sericosepala[2]	S. America	Mendonça et al., 2018	44.33	N/A
Ipomoea sumatrana	Asia		N/A	N/A
Ipomoea valenzuelensis	S. America		N/A	N/A
Ipomoea verbascoidea	Africa		56.17	0.67
Ipomoea wolcottiana	C. & S. America		34.5	0.32
Ipomoea yardiensis	Australia		N/A	N/A
Jacquemontia corymbulosa[3]	S. America	Mendonça et al., 2018	N/A	N/A

Bolded species were previously reported to contain swainsonine; non-bolded reported here for the first time. All species were analyzed in this study unless noted otherwise. N = North, C = Central, S = South.

[1] *Species confirmed to contain swainsonine in this survey.*

[2] *Species was confirmed to be negative through this survey.*

[3] *This species was not investigated in this survey.*

glory phylogeny. We found a significant phylogenetic signal for the presence of swainsonine (Pagel's $\lambda = 0.9$, $D = -0.67$, $p < 0.001$), suggesting that species with swainsonine are more closely related than expected by chance (**Figures 3**, **4**). Our results suggest that the oldest common ancestor observed was S − and that this symbiosis has arisen relatively recently within the morning glory phylogeny given that the S+ clade was the last to split from the lineage (**Figures 3**, **4**). This pattern also suggests that the symbiosis is strictly heritable and vertically transmitted from a single S+ ancestor, especially given the wide geographic distribution of S+ species. However, several S − species occur within this primarily S+ clade, suggesting that the symbiosis can be lost.

Seed Mass and Swainsonine

Species with larger seeds are often larger as adults, longer-lived, and have lower growth rates than species with smaller seeds (Silvertown, 1981; Moles et al., 2005). Therefore, we measured seed mass as a proxy for plant life history strategy. We found a significant phylogenetic signal in seed mass ($\lambda = 0.89$, $p < 0.001$), suggesting that closely related species have more similar seed mass. Furthermore, seeds of S+ species were approximately

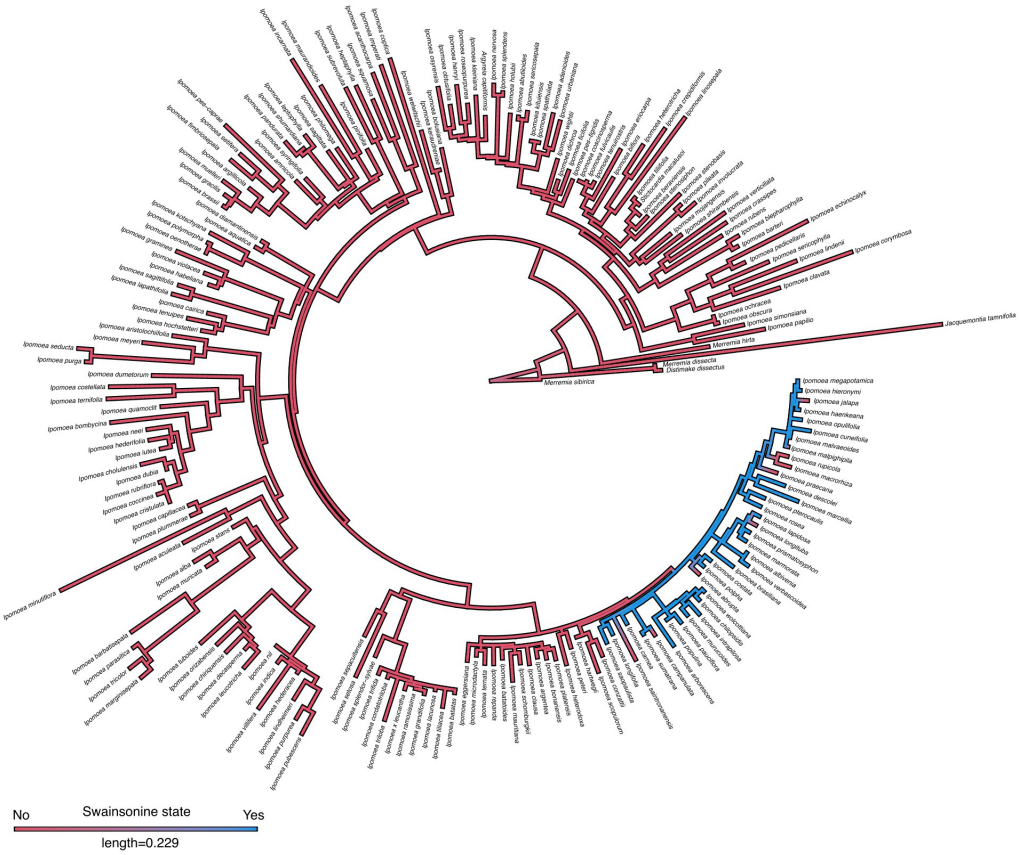


FIGURE 3 | Internal transcribed spacer (ITS) phylogeny of morning glories. Maximum likelihood ITS phylogeny of morning glories ($n = 201$) with ancestral state reconstruction of swainsonine presence by density map based on 1,000 stochastic character maps. Branch color represents the probability of the character state. The length of the legend equals units of substitution per site.

two times larger than seeds of S − species (69.5 vs. 37.3 mg, t(32.6) =, $p < 0.005$, **Figure 5A**). When we transformed seed mass to a binary state, we found a significantly higher proportion of species with large seeds containing swainsonine than species with small seeds ($X^2 = 18.4$, $p < 0.001$, **Figure 5B**, **Supplementary Figure 3**). We then performed Pagel's test for correlated evolution between two discrete characters after accounting for host phylogeny (Pagel, 1994) and found a significant positive correlation between large seed mass and swainsonine ($p = 0.047$, **Figure 4**). However, our phylogenetic logistic regression analysis (Ives and Garland, 2010), which tests for a correlation between discrete and continuous characters based on a phylogeny, showed that seed mass as a continuous variable is not a direct predictor of swainsonine presence. Finally, phylogenetic regression (Ho and Ané, 2014) did not show a significant correlation between seed mass and swainsonine concentration in S+ species (**Supplementary Figure 4**).

DISCUSSION

The results reported here show that all S+ *Ipomoea* species occur in just a single clade while the rest of the phylogeny is S −. Many of the S+ *Ipomoea* species reported here are part of Clade A1 described in Wood et al.'s (2020) monograph of *Ipomoea* species from the Americas. In fact, 22 of 26 species in clade A1 from the Americas surveyed here contained swainsonine. Species in this clade are perennial, generally have tuberous roots, and have distinctive pollen and floral traits (Wood et al., 2020). Within Clade A1, there are additional groups, for example, the Arborescens clade, which is composed of small trees, large shrubs, or lianas, where many are S+ that includes *I. pauciflora*, *I. arborescens*, *I. chilopsidis*, *I. intrapilosa*, *I. populina*, *I. wolcottiana*, and *I. murucoides*. These common morphological features within this clade might have played a role in the evolution of the swainsonine symbiosis.

This pattern of closely related S+ species observed here is similar to observations in the legume genus *Astragalus*, which like *Ipomoea* is species rich and morphologically and phylogenetically complex (Barneby, 1964; Wojciechowski et al., 1999). Unlike the phylogeny of *Ipomoea* reported by Muñoz-Rodríguez et al. (2019) and herein, the phylogeny of *Astragalus* generally has poor resolution with only a few well-defined clades due to the recency of the radiation (Wojciechowski et al., 1999). One exception is a phylogenetic clade composed of six North American and eight South American *Astragalus* species (Wojciechowski et al., 1999;

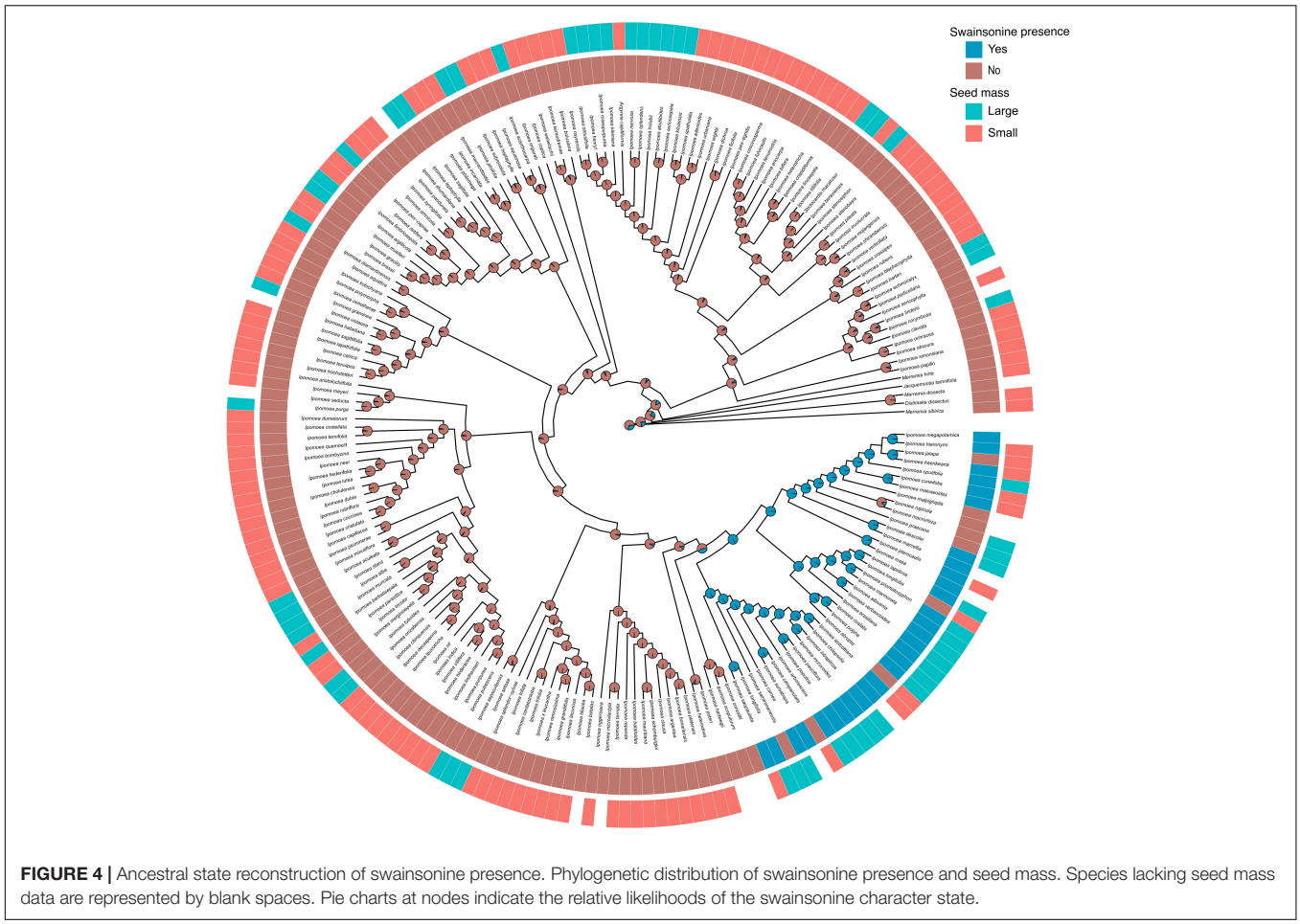


FIGURE 4 | Ancestral state reconstruction of swainsonine presence. Phylogenetic distribution of swainsonine presence and seed mass. Species lacking seed mass data are represented by blank spaces. Pie charts at nodes indicate the relative likelihoods of the swainsonine character state.

Scherson et al., 2008). Surveys for S+ species of *Astragalus* identified swainsonine in 13 of these 14 species in this clade that share a common phylogenetic origin (Cook et al., 2017b,c). Among the more than 50 species of *Astragalus* that were reported to contain swainsonine in North and South America that includes the 13 mentioned above (Cook et al., 2017b,c), many share common morphological features, such as an inflated pod, as they co-occur in morphologically related taxonomic sections (Barneby, 1964).

Documentation of swainsonine-containing morning glories is relatively recent as compared to other swainsonine-containing legumes (Colegate et al., 1979; de Balogh et al., 1999). Our inferred phylogeny also suggests that the swainsonine symbiosis is a relatively recent plant-fungal association in the Convolvulaceae. We estimate the divergence age of the S+ clade to be approximately 5 mya based on the conclusions of a recently published time-calibrated nuclear phylogeny study of the family (Muñoz-Rodríguez et al., 2019). Further, all S+ species are closely related, which strongly suggest that the fungal endosymbiont is strictly transmitted vertically, consistent with observations on the transmission of the fungal endosymbiont of *I. carnea* (Cook et al., 2013). Furthermore, this pattern suggests that the symbiosis is transmitted vertically during the speciation process. Though S+ species that belong to the same morning glory clade, they are widely distributed geographically across Australia, Africa, Americas, and Southeast Asia, suggesting that these species originated from a common S+ ancestor. The occurrence of occasional S − species within the same clade suggests that the symbiosis has been occasionally lost in the lineage. These S − species also occur over multiple continents. It is highly likely that there are many other *Ipomoea* species that contain swainsonine given that our samples represent 26 of 127 species (20%) in Clade A1 described by Wood et al. (2020). Based upon our survey reported here, the frequency of S+ species in the clade is greater than 85% or over 100 species.

The Chaetothyriales symbiont associated with *I. carnea* has been shown to grow as an epibiont on the adaxial leaf surface associated with oil glands (Cook et al., 2013), similar to *Periglandula* on the adaxial surface of *Ipomoea asarifolia* (Steiner et al., 2011). Plants that lack swainsonine lack the corresponding epibiont on the leaf surface (Cook et al., 2013). The epibiont has only been detected growing on the leaf surface and has not been observed growing between cells in different plant tissues. Notably, there is no evidence of horizontal transmission as plants containing the epibiont and plants lacking the epibiont grown in proximity to each other in the greenhouse maintain their original chemical phenotype (Cook et al., 2013; Noor et al., 2021). It is not known whether the Chaetothyriales symbiont

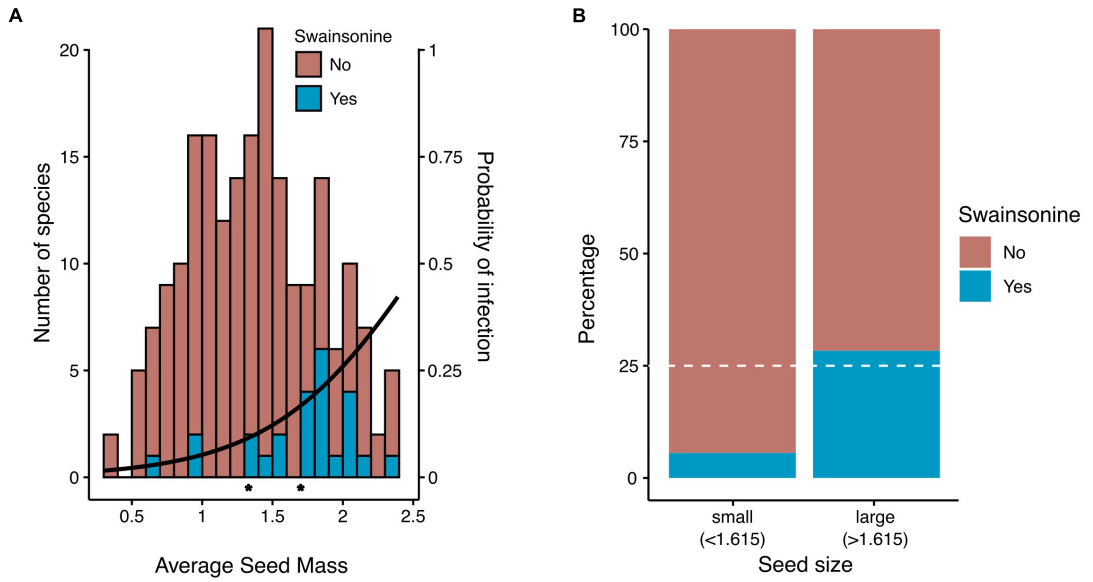


FIGURE 5 | Relationship of seed mass and swainsonine. Seed mass is log10-transformed. **(A)** The number of species with ($n = 34$) or without ($n = 210$) swainsonine by mean seed mass. Stars along the x-axis indicate the mean seed mass of each group. The binomial regression line shows the probability of infection based on mean seed mass. **(B)** Proportion of small and large-seeded species with or without swainsonine. Dashed lines represent the expected number of swainsonine species if the proportion of S+ species is equivalent across seed mass.

grows as an epibiont in other swainsonine-containing *Ipomoea* species, but should be examined. In *I. asarifolia*, *Periglandula* is associated with and is thought to feed on the oils secreted from the glandular trichomes. Potentially, an analogous type of association may be occurring with the Chaetothyriales symbiont wherein it benefits from the plant through its association with the glandular trichomes thus facilitating the plant host relationship. It is less clear what potential benefits the host plant obtains but theory suggests that there should be a benefit to the host plant.

Interestingly, S − species in the rest of the phylogeny include those that have been shown to contain ergot alkaloids produced by another heritable fungal endosymbiont (*Periglandula* sp.) found in morning glories, where 25% of species examined contained ergot alkaloids (Beaulieu et al., 2021). Thus, about 40% of all morning glory species examined thus far are symbiotic with one heritable fungus or the other. Swainsonine might affect plant growth in ways that were not captured in our study and this non-overlapping host species distribution of endosymbionts potentially reflects symbiont exclusion to reduce physiological costs to the host or redundancy in functions for the host. A previous study has shown that *Periglandula*-infected *I. tricolor* plants have reduced biomass when grown in isolation, but performed better when grown in the presence of root-knot nematodes as compared to non-infected conspecifics (Durden et al., 2019).

Similar to the *Periglandula* sp. symbiosis in the morning glory family (Beaulieu et al., 2021), S+ species have larger seeds than their S − congeners in our survey. Although the toxic properties of swainsonine can lead to poisoning of animals when ingested, a previous study did not find any evidence that animals avoid S+ plants (*Oxytropis* and *Ipomoea*) (Pfister et al., 2020). However, larger seeds represent a larger energy investment by the maternal plant, and swainsonine found in seeds might serve as a deterrent for other potential seed predators, such as birds or insects. For example, *Metarhizium* sp., which produces toxic metabolites, such as swainsonine, is widely used as an organic plant insecticide (Zimmermann, 2007; St Leger and Wang, 2010). Swainsonine may also increase the fitness of the seed by affecting other fungi associated with the seed or potential seed pathogens. Recent studies have shown that the presence of the heritable symbiont *Alternaria fulva* and associated swainsonine in the spotted locoweed, *Astragalus lentiginosus* (Fabaceae), alters the richness and diversity of other fungi associated with the plant (Harrison et al., 2018).

Greater seed mass has also been shown to be characteristic of long-lived plants (Silvertown, 1981) and many of the S+ species here are woody, perennial species (Wood et al., 2020). Larger seeds can increase seedling survival that would be required for the longer time that larger plants take to reach maturity (Moles and Westoby, 2004). Seed mass also correlates with the net primary productivity of the plant and some environmental conditions, such as precipitation (Moles et al., 2005), so it is possible that the correlation of swainsonine present with larger seed mass may reflect other factors than plant life history.

Swainsonine concentrations have been shown to vary between plant tissues, as a function of phenology, and due to endophyte genotype in the legume *Oxytropis sericea*. In *O. sericea*, little or no swainsonine was detected in the root or crown of the plant with greater concentrations found in above-ground parts (Cook et al., 2009). Concentrations have been also shown to vary in above-ground parts as a function of developmental age with the greatest concentrations as the plant reaches

maturity (Cook et al., 2012). Swainsonine concentrations also vary by endophyte genotype within and between host species, which influences the concentrations found in plant tissues (Braun et al., 2003; Cook et al., 2013). The variation of swainsonine concentrations among different plant tissues and across developmental stages should be examined in different S+ *Ipomoea* species. Although we did not find a significant correlation between seed mass and swainsonine concentration in S+ species, there is a positive trend that might become stronger with increased sampling ($p = 0.075$, **Supplementary Figure 4**). Another interesting pattern is that African S+ species have higher concentrations than S+ species from other continents (**Supplementary Figure 4**). This group of African species forms a subclade within the S+ clade and their high swainsonine concentrations could suggest a difference in endosymbiont genotypes, consistent with observations in *Astragalus* and *Oxytropis* species (Braun et al., 2003; Cook et al., 2013).

Although our study comprised only a fraction (30%) of all described *Ipomoea* species, it demonstrates the distribution and heritability of swainsonine-producing fungi in morning glory species. The closely related group of S+ morning glories presents a promising future model system for studying heritable fungal symbiosis and its evolution. One important question is whether the diversity and density of other horizontally acquired fungal endophytes in the host microbiome vary with the presence of the heritable symbiont and its swainsonine chemistry as had been shown in other host species (Harrison et al., 2018). In addition, experimental performance and functional studies of S+ vs. S − morning glory species would provide further insights into the costs and benefits of this symbiosis. The genetic diversity and relatedness of the various orders of swainsonine-producing fungal endosymbionts in different plant families also pose interesting directions for studying the origins of these chemically related symbioses, whether these distinct symbioses have similar effects on host plants from different families and fungal-host specificity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: doi: 10.6084/m9.figshare.19010885.

AUTHOR CONTRIBUTIONS

DC, QQ, and KC conceptualized the study. DC, DG, and KC collected and generated data. QQ performed statistical analyses with input from KC. All authors contributed to writing, reviewing, and editing the final manuscript.

FUNDING

This study was provided by grant 429440 from the Simons Foundation to the Smithsonian Tropical Research Institute to KC (W. Wcislo, PI).

ACKNOWLEDGMENTS

We thank Eric Knox (Indiana U.), James Solomon, Charlotte Taylor, and George Yatskievtch (Missouri Botanical Garden), Brendan Lepschi and Kirsten Cowley (Australian National Herbarium), and Jan Wieringa (Vadense Herbarium, Wageningen U.) for help in obtaining seeds from herbarium specimens. We thank Margaret S. Devall (USDA Center for Bottomlands Hardwood Research) and Kathleen Keeler (U. Nebraska-Lincoln) for seeds from their own collections. We also thank Richard E. Miller (U. Colorado) for his previous research on morning glory biology, valuable input on interpreting morning glory phylogeny and recommendations on our phylogenetic analyses. We thank Pablo Muñoz-Rodríguez and Robert Scotland (University of Oxford) for their extensive sequence database for *Ipomoea*, which made our phylogenetic analyses possible. We also thank John Wood (University of Oxford) for his help in the identification of some specimens. Special thanks to Wesley T. Beaulieu (Jaeb Center for Health Research) who helped make the morning glory seed collections as part of his Ph.D. dissertation at Indiana University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.871148/full#supplementary-material

REFERENCES

Barbosa, R. C., Riet-Correa, F., Medeiros, R. M. T., Lima, E. F., Barros, S. S., Gimeno, E. J., et al. (2006). Intoxication by *Ipomoea sericophylla* and *Ipomoea riedelii* in goats in the state of Paraíba, Northeastern Brazil. *Toxicon* 47, 371–379.

Barneby, R. C. (1964). *Atlas of North American Astragalus*. New York, NY: Botanical Garden.

Baucom, D. L., Romero, M., Belfon, R., and Creamer, R. (2012). Two new species of *Undifilum*, fungal endophytes of *Astragalus* (locoweeds) in the United States. *Botany* 90, 866–875. doi: 10.1139/b2012-056

Beaulieu, W. T., Panaccione, D. G., Quach, Q. N., Smoot, K. L., and Clay, K. (2021). Diversification of ergot alkaloids and heritable fungal symbionts in morning glories. *Commun. Biol.* 4:1362. doi: 10.1038/s42003-021-02870-z

Braun, K., Romero, J., Liddell, C., and Creamer, R. (2003). Production of swainsonine by fungal endophytes of locoweed. *Mycol. Res.* 107, 980–988. doi: 10.1017/s095375620300813x

Cholich, L. A., Martinez, A., Micheloud, J. F., Pistán, M. E., García, E. N., Robles, C. A., et al. (2021). Alpha-mannosidosis caused by toxic plants in ruminants of Argentina. *An. Acad. Bras. Cienc.* 93:e20191496. doi: 10.1590/0001-3765202120191496

Christian, N., Whitaker, B. K., and Clay, K. (2015). Microbiomes: unifying animal and plant systems through the lens of community ecology theory. *Front. Microbiol.* 6:869. doi: 10.3389/fmicb.2015.00869

Colegate, S. M., Dorling, P. R., and Huxtable, C. R. (1979). A spectroscopic investigation of swainsonine: an α-mannosidase inhibitor isolated from *Swainsona canescens*. *Aust. J. Chem.* 32, 2257–2264.

Colodel, E. M., Gardner, D. R., Zlotowski, P., and Driemeier, D. (2002). Identification of swainsonine as a glycoside inhibitor responsible for *Sida carpinifolia* poisoning. *Vet. Hum. Toxicol.* 44, 177–178.

Cook, D., Beaulieu, W. T., Mott, I. W., Riet-Correa, F., Gardner, D. R., Grum, D., et al. (2013). Production of the alkaloid swainsonine by a fungal endosymbiont of the Ascomycete order Chaetothyriales in the host *Ipomoea carnea*. *J. Agric. Food Chem.* 61, 3797–3803. doi: 10.1021/jf4008423

Cook, D., Gardner, D. R., Lee, S. T., Pfister, J. A., Stonecipher, C. A., and Welsh, S. L. (2016). A swainsonine survey of North American *Astragalus* and *Oxytropis* taxa implicated as locoweeds. *Toxicon* 118, 104–111. doi: 10.1016/j.toxicon.2016.04.033

Cook, D., Gardner, D. R., Martinez, A., Robles, C. A., and Pfister, J. A. (2017b). Screening for swainsonine among South American *Astragalus* species. *Toxicon* 139, 54–57. doi: 10.1016/j.toxicon.2017.09.014

Cook, D., Gardner, D. R., and Pfister, J. A. (2014). Swainsonine-containing plants and their relationship to endophytic fungi. *J. Agric. Food Chem.* 62, 7326–7334. doi: 10.1021/jf501674r

Cook, D., Gardner, D. R., Pfister, J. A., Lee, S. T., Welch, K. D., and Welsh, S. L. (2017c). A screen for swainsonine in select North American *Astragalus* species. *Chem. Biodivers.* 14:e1600364. doi: 10.1002/cbdv.201600364

Cook, D., Gardner, D. R., Ralphs, M. H., Pfister, J. A., Welch, K. D., and Green, B. T. (2009). Swainsoninine concentrations and endophyte amounts of *Undifilum oxytropis* in different plant parts of *Oxytropis sericea*. *J. Chem. Ecol.* 35, 1272–1278. doi: 10.1007/s10886-009-9710-9

Cook, D., Gardner, D. R., Welch, K. D., and Allen, J. G. (2017d). A survey of swainsonine content in *Swainsona* species. *Rangeland J.* 39, 213–218.

Cook, D., Donzelli, B. G. G., Creamer, R., Baucom, D. L., Gardner, D. R., Pan, J., et al. (2017a). Swainsonine biosynthesis genes in diverse symbiotic and pathogenic fungi. *G3* 7, 1791–1797. doi: 10.1534/g3.117.041384

Cook, D., Lee, S. T., Gardner, D. R., Molyneux, R. J., Johnson, R. L., and Taylor, C. M. (2021). Use of herbarium voucher specimens to investigate phytochemical composition in poisonous plant research. *J. Agric. Food Chem.* 69, 4037–4047. doi: 10.1021/acs.jafc.1c00708

Cook, D., Lee, S. T., Panaccione, D. G., Leadmon, C. E., Clay, K., and Gardner, D. R. (2019). Biodiversity of Convolvulaceous species that contain ergot alkaloids, indole diterpene alkaloids, and swainsonine. *Biochem. Syst. Ecol.* 86:103921. doi: 10.1016/j.bse.2019.103921

Cook, D., Shi, L., Gardner, D. R., Pfister, J. A., Grum, D., Welch, K. D., et al. (2012). Influence of phenological stage on swainsonine and endophyte concentrations in *Oxytropis sericea*. *J. Chem. Ecol.* 38, 195–203. doi: 10.1007/s10886-012-0067-0

Dantas, A. F. M., Riet-Correa, F., Gardner, D. R., Medeiros, R. M. T., Barros, S. S., Anjos, B. L., et al. (2007). Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of *Turbina cordata* in Northeastern Brazil. *Toxicon* 49, 111–116. doi: 10.1016/j.toxicon.2006.08.012

Darriba, D., Taboada, G. L., Doallo, R., and Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9:772. doi: 10.1038/nmeth.2109

de Balogh, K. K., Dimande, A. P., van der Lugt, J. J., Molyneux, R. J., Naudé, T. W., and Welman, W. G. (1999). A lysosomal storage disease induced by *Ipomoea carnea* in goats in Mozambique. *J. Vet. Diagn. Invest.* 11, 266–273. doi: 10.1177/104063879901100310

Dorling, P. R., Huxtable, C. R., and Colegate, S. M. (1980). Inhibition of lysosomal alpha-mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*. *Biochem. J* 191, 649–651. doi: 10.1042/bj1910649

Durden, L., Wang, D., Panaccione, D., and Clay, K. (2019). Decreased root-knot nematode gall formation in roots of the morning glory *Ipomoea tricolor* symbiotic with ergot alkaloid-producing fungal *Periglandula* sp. *J. Chem. Ecol.* 45, 879–887. doi: 10.1007/s10886-019-01109-w

Ewald, P. W. (1987). Transmission modes and evolution of the parasitism-mutualism continuum. *Ann. N. Y. Acad. Sci.* 503, 295–306. doi: 10.1111/j.1749-6632.1987.tb40616.x

FitzJohn, R. G., Maddison, W. P., and Otto, S. P. (2009). Estimating trait-dependent speciation and extinction rates from incompletely resolved phylogenies. *Syst. Biol.* 58, 595–611. doi: 10.1093/sysbio/syp067

Fritz, S. A., and Purvis, A. (2010). Selectivity in mammalian extinction risk and threat types: a new measure of phylogenetic signal strength in binary traits. *Conserv. Biol.* 24, 1042–1051. doi: 10.1111/j.1523-1739.2010.01455.x

Gardner, D. R., and Cook, D. (2011). A comparison of alternative sample preparation procedures for the analysis of swainsonine using LC-MS/MS. *Phytochem. Anal.* 22, 124–127. doi: 10.1002/pca.1257

Gardner, D. R., Molyneux, R. J., and Ralphs, M. H. (2001). Analysis of swainsonine: extraction methods, detection, and measurement in populations of locoweeds (*Oxytropis* spp.). *J. Agric. Food Chem.* 49, 4573–4580. doi: 10.1021/jf010596p

GBIF.org (2021a). *GBIF Occurrence Download*. Copenhagen: GBIF, doi: 10.15468/dl.fbx35m

GBIF.org (2021b). *GBIF Occurrence Download*. Copenhagen: GBIF, doi: 10.15468/dl.mpcfzz

GBIF.org (2021c). *GBIF Occurrence Download*. Copenhagen: GBIF, doi: 10.15468/dl.qqz5r5

GBIF.org (2021d). *GBIF Occurrence Download*. Copenhagen: GBIF, doi: 10.15468/dl.z94jjv

Gough, F. J., and Elliott, E. S. (1956). *Blackpatch of Red Clover and Other Legumes Caused by Rhizoctonia leguminicola* sp. nov. *West Virginia Agricultural and Forestry Experiment Station Bulletins* 387T. Rome: FAO.

Grum, D. S., Cook, D., Baucom, D., Mott, I. W., Gardner, D. R., Creamer, R., et al. (2013). Production of the alkaloid swainsonine by a fungal endophyte in the host *Swainsona canescens*. *J. Nat. Prod.* 76, 1984–1988. doi: 10.1021/np400274n

Haraguchi, M., Gorniak, S. L., Ikeda, K., Minami, Y., Kato, A., Watson, A. A., et al. (2003). Alkaloidal components in the poisonous plant, *Ipomoea carnea* (Convolvulaceae). *J. Agric. Food Chem.* 51, 4995–5000. doi: 10.1021/jf0341722

Harrison, J. G., Parchman, T. L., Cook, D., Gardner, D. R., and Forister, M. L. (2018). A heritable symbiont and host-associated factors shape fungal endophyte communities across spatial scales. *J. Ecol.* 106, 2274–2286.

Ho, Ls, and Ané, C. (2014). A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. *Syst. Biol.* 63, 397–408. doi: 10.1093/sysbio/syu005

Ives, A. R., and Garland, T. Jr. (2010). Phylogenetic logistic regression for binary dependent variables. *Syst. Biol.* 59, 9–26. doi: 10.1093/sysbio/syp074

Katoh, K., Rozewicki, J., and Yamada, K. D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* 20, 1160–1166. doi: 10.1093/bib/bbx108

Lipsitch, M., Siller, S., and Nowak, M. A. (1996). The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution* 50, 1729–1741. doi: 10.1111/j.1558-5646.1996.tb03560.x

Mendonça, F. S., Albuquerque, R. F., Evêncio-Neto, J., Freitas, S. H., Dória, R. G. S., Boabaid, F. M., et al. (2012). Alpha-mannosidosis in goats caused by the swainsonine-containing plant *Ipomoea verbascoidea*. *J. Vet. Diagn. Invest.* 24, 90–95. doi: 10.1177/1040638711425948

Mendonça, F. S., Silva Filho, G. B., Chaves, H. A. S., Aires, L. D. A., Braga, T. C., Gardner, D. R., et al. (2018). Detection of swainsonine and calystegines in *Convolvulaceae* species from the semiarid region of Pernambuco. *Pesqui. Vet. Bras.* 38, 2044–2051.

Micheloud, J. F., Marin, R., Colque-Caro, L. A., Martínez, O. G., Gardner, D., and Gimeno, E. J. (2017). Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of *Sida rodrigoi* Monteiro in North-western Argentina. *Toxicon* 128, 1–4. doi: 10.1016/j.toxicon.2016.12.011

Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). "Creating the CIPRES Science Gateway for inference of large phylogenetic trees," in *Proceedings of the Gateway Computing Environments Workshop*, (New Orleans, LA), 1–8.

Moles, A. T., Ackerly, D. D., Webb, C. O., Tweddle, J. C., Dickie, J. B., Pitman, A. J., et al. (2005). Factors that shape seed mass evolution. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10540–10544. doi: 10.1073/pnas.0501473102

Moles, A. T., and Westoby, M. (2004). Seedling survival and seed size: a synthesis of the literature. *J. Ecol.* 92, 372–383. doi: 10.1111/j.0022-0477.2004.00884.x

Molyneux, R. J., and James, L. F. (1982). Loco intoxication: indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). *Science* 216, 190–191. doi: 10.1126/science.6801763

Molyneux, R. J., McKenzie, R. A., O'Sullivan, B. M., and Elbein, A. D. (1995). Identification of the glycosidase inhibitors swainsonine and calystegine B2 in weir vine (*Ipomoea* sp. Q6 aff. *calobra*) and correlation with toxicity. *J. Nat. Prod.* 58, 878–886. doi: 10.1021/np50120a009

Muñoz-Rodríguez, P., Carruthers, T., Wood, J. R. I., Williams, B. R. M., Weitemier, K., Kronmiller, B., et al. (2019). A taxonomic monograph of *Ipomoea* integrated across phylogenetic scales. *Nat Plants* 5, 1136–1144. doi: 10.1038/s41477-019-0535-4

Noor, A. I., Nava, A., Neyaz, M., Cooke, P., Creamer, R., and Cook, D. (2021). Ectopic growth of the *Chaetothyriales* fungal symbiont on *Ipomoea carnea*. *Botany* 99, 619–627. doi: 10.1139/cjb-2021-0037

Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., et al. (2013). *The Caper Package: Comparative Analysis of Phylogenetics and Evolution in R*. Available online at: http://caper.r-forge.r-project.org (accessed February 1, 2022).

Pagel, M. (1994). Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 255, 37–45.

Pagel, M. (1999). The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. *Syst. Biol.* 48, 612–622. doi: 10.1080/106351599260184

Panter, K. E., James, L. F., Stegelmeier, B. L., Ralphs, M. H., and Pfister, J. A. (1999). Locoweeds: effects on reproduction in livestock. *J. Nat. Toxins* 8, 53–62.

Paradis, E., and Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35, 526–528. doi: 10.1093/bioinformatics/bty633

Pennell, M. W., Eastman, J. M., Slater, G. J., Brown, J. W., Uyeda, J. C., FitzJohn, R. G., et al. (2014). geiger v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics* 30, 2216–2218. doi: 10.1093/bioinformatics/btu181

Pfister, J., Cook, D., Lee, S. T., Gardner, D. R., and Riet-Correa, F. (2020). Livestock preference for endophyte-infected or endophyte-free *Oxytropis sericea*, *Ipomoea carnea*, and *Ipomoea asarifolia*. *Poisonous Plant Res.* 3, 58–73.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., and Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst. Biol.* 67, 901–904. doi: 10.1093/sysbio/syy032

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210x.2011.00169.x

Schardl, C. L., Young, C. A., Hesse, U., Amyotte, S. G., Andreeva, K., Calie, P. J., et al. (2013). Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. *PLoS Genet.* 9:e1003323. doi: 10.1371/journal.pgen.1003323

Scherson, R. A., Vidal, R., and Sanderson, M. J. (2008). Phylogeny, biogeography, and rates of diversification of New World *Astragalus* (Leguminosae) with an emphasis on South American radiations. *Am. J. Bot.* 95, 1030–1039. doi: 10.3732/ajb.0800017

Silvertown, J. W. (1981). Seed size, life span, and germination date as coadapted features of plant life history. *Am. Nat.* 118, 860–864.

Simoes, A. R., Culham, A., and Carine, M. (2015). Resolving the unresolved tribe: a molecular phylogenetic framework for the *Merremieae* (Convolvulaceae). *Bot. J. Linn. Soc.* 179, 374–387.

Steiner, U., Leibner, S., Schardl, C. L., Leuchtmann, A., and Leistner, E. (2011). *Periglandula*, a new fungal genus within the Clavicipitaceae and its association with Convolvulaceae. *Mycologia* 103, 1133–1145. doi: 10.3852/11-031

St Leger, R. J., and Wang, C. (2010). Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests. *Appl. Microbiol. Biotechnol.* 85, 901–907. doi: 10.1007/s00253-009-2306-z

Tulsiani, D. R., Broquist, H. P., James, L. F., and Touster, O. (1988). Production of hybrid glycoproteins and accumulation of oligosaccharides in the brain of sheep and pigs administered swainsonine or locoweed. *Arch. Biochem. Biophys.* 264, 607–617. doi: 10.1016/0003-9861(88)90327-x

Wojciechowski, M. F., Sanderson, M. J., and Hu, J.-M. (1999). Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and chloroplast DNA trnL intron data. *Syst. Bot.* 24, 409–437.

Wood, J. R. I., Muñoz-Rodríguez, P., Williams, B. R. M., and Scotland, R. W. (2020). A foundation monograph of *Ipomoea* (Convolvulaceae) in the New World. *PhytoKeys* 143, 1–823. doi: 10.3897/phytokeys.143.32821

Yu, Y., Zhao, Q., Wang, J., Wang, J., Wang, Y., Song, Y., et al. (2010). Swainsonine-producing fungal endophytes from major locoweed species in China. *Toxicon* 56, 330–338. doi: 10.1016/j.toxicon.2010.03.020

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Technol.* 17, 879–920.

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.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 July 2022
doi: 10.3389/fmicb.2022.870821

The Antibacterial, Antitumor Activities, and Bioactive Constituents' Identification of *Alectra sessiliflora* Bacterial Endophytes

*Mehabo Penistacia Maela[1], Hendriëtte van der Walt[2] and Mahloro Hope Serepa-Dlamini[1]**

[1] Department of Biotechnology and Food Technology, Faculty of Science, University of Johannesburg, Doornfontein Campus, Johannesburg, South Africa, [2] Advanced Materials Division, Nanotechnology, Mintek, Randburg, South Africa

OPEN ACCESS

Edited by:
Khondoker M. G. Dastogeer, Bangladesh Agricultural University, Bangladesh

Reviewed by:
Weaam Ebrahim, Mansoura University, Egypt
Vijay K. Sharma, Agricultural Research Organization (ARO), Israel
Vipin Kumar Singh, Banaras Hindu University, India

***Correspondence:**
Mahloro Hope Serepa-Dlamini
hopes@uj.ac.za

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *07 February 2022*
Accepted: *02 June 2022*
Published: *05 July 2022*

Citation:
Maela MP, van der Walt H and Serepa-Dlamini MH (2022) The Antibacterial, Antitumor Activities, and Bioactive Constituents' Identification of Alectra sessiliflora Bacterial Endophytes. Front. Microbiol. 13:870821. doi: 10.3389/fmicb.2022.870821

Due to increased antimicrobial resistance against current drugs, new alternatives are sought. Endophytic bacteria associated with medicinal plants are recognized as valuable sources of novel secondary metabolites possessing antimicrobial, antitumor, insecticidal, and antiviral activities. In this study, five bacterial endophytes were isolated and identified from the medicinal plant, *Alectra sessiliflora,* and their antibacterial and antitumor activities were investigated. In addition, the crude extracts of the endophytes were analyzed using gas chromatography (GC) coupled with time-of-flight mass spectrometry (TOF-MS). The identified bacterial endophytes belong to three genera *viz Lysinibacillus, Peribacillus,* and *Bacillus,* with the latter as the dominant genus with three species. Ethyl acetate extracts from the endophytes were used for antimicrobial activity against eleven pathogenic strains through minimum inhibitory concentration (MIC). The antitumor activity against the Hela cervical, Hek 293 kidney, and A549 lung carcinoma cells was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. *Lysinibacillus* sp. strain AS_1 exhibited broad antibacterial activity against the pathogenic strains with MIC values ranging from 4 to 8 mg/ml, while *Bacillus* sp. strain AS_3 displayed MIC of 0.25 mg/ml. Crude extracts of *Lysinibacillus* sp. strain AS_1, *Peribacillus* sp. strain AS_2, and *Bacillus* sp. strain AS_3 showed growth inhibition of more than 90% against all the cancer cell lines at a concentration of 1,000 μg/ml. Untargeted secondary metabolite profiling of the crude extracts revealed the presence of compounds with reported biological activity, such as antimicrobial, antioxidant, anti-inflammatory, antitumor, and antidiabetic properties. This study reported for the first time, bacterial endophytes associated with *A. sessiliflora* with antibacterial and antitumor activities.

Keywords: *Alectra sessiliflora*, antibacterial activity, bioactive compounds, bacterial endophytes, antitumor activity

INTRODUCTION

The frequency of infections caused by pathogenic bacteria has increased exponentially in the previous decades (Roca et al., 2015; Attia et al., 2020). In addition, the abuse and misuse of antimicrobial drugs, some of which are available over the counter without a prescription, has turned into a global health concern (Ayukekbong et al., 2017). All these compounded by the lack of new effective antimicrobial agents are contributing to the rise in antimicrobial resistance, and bacteria and fungi have developed resistance through a variety of mechanisms, including enzyme activation, altered target sites, decreased cell permeability, and increased efflux due to over-expression, among others (Baptista et al., 2018). This has resulted in a continual decrease in the development of new antimicrobial drugs; it is, therefore, necessary to discover and develop novel antimicrobial drugs from natural products (Sciarretta et al., 2016; Farhat et al., 2019).

To tackle antimicrobial resistance, recent breakthroughs in microbial ecology have led researchers to focus on studying ground-breaking and promising antimicrobial compounds from natural sources, such as medicinal plants. Medicinal plants have long been used to cure a variety of ailments, including skin conditions, coughs, microbiological infections, diabetes, colds, urinary issues, and inflammations (Aswani et al., 2020; Alotaibi et al., 2021). Medicinal plants have been recognized as good sources of bioactive substances that are vital for good health and are reservoirs for various microorganisms categorized as endophytes, such as bacteria, fungi, and actinomycetes (Petrini et al., 1993; Duhan et al., 2020).

Endophytes are microorganisms like fungi, bacteria, and actinomycetes, which have a mutual relationship with the host plant and inhabit the host tissues without causing detrimental symptoms (Gunatilaka, 2006). Bacterial endophytes have been identified as the prospective source of natural metabolites such as alkaloids, steroids, phenols, terpenoids, flavonoids, isocoumarins, and quinones, which have agricultural, industrial (Zinniel et al., 2002), and pharmaceutical applications (Palanichamy et al., 2018; Attia et al., 2020). Bacterial endophytes benefit the host plants by helping them survive abiotic and biotic conditions, solubilize minerals, nutrient acquisition, and protection against pathogens and parasitic nematodes (Dutta et al., 2014). Bacterial endophytes are diverse and range from Gram-positive to Gram-negative species of various genera such as *Pantoea, Achromobacter, Acinetobacter, Xanthomonas, Bacillus, Agrobacterium,* etc. (Sun et al., 2013). Bioactive compounds produced by various bacterial endophytes have antimicrobial and anticancer compounds that may be used for various diseases (Gouda et al., 2016). Furthermore, bioactive compounds which have been extracted from endophytic microorganisms exhibit antidiabetic, antifungal, immunosuppressant, and anti-inflammatory properties, thus they have received attention in drug discovery research (Egamberdieva et al., 2017; Panigrahi and Rath, 2021; Singh et al., 2021).

Research on medicinal plants and their associated endophytes, and their potential to synthesize distinct bioactive compounds, have opened the possibility of looking into more medicinal plants as well to explore their diverse endophytic bacteria (Duhan et al., 2020). *Alectra sessiliflora* is a medicinal plant that grows throughout Sub-Saharan Africa, China, India, and the Philippines (Morawetz and Wolfe, 2011; Gasa, 2015; Katembo et al., 2021). The eastern and southwestern provinces of South Africa, which include the Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North-West, and Western Cape provinces, are home to *A. sessiliflora* (Morawetz and Wolfe, 2011). *A. sessiliflora* has been used in traditional medicine to treat toothaches, diarrhea, scabies, gastrointestinal illnesses, and oral thrush (Gasa, 2015; Katembo et al., 2021). It is used to treat tuberculosis in several African countries, such as Nigeria, and its leaves are used as a galactogen by pregnant women in Central Africa (Ogbole and Ajaiyeoba, 2010; Oosthuizen et al., 2019). The phytochemistry of *A. sessiliflora* has received significant attention, however, there is no information on its endophytes (Ogbole and Ajaiyeoba, 2010; Oosthuizen et al., 2019). The goal of this study was to isolate and identify bacterial endophytes from *A. sessiliflora* collected in Limpopo province, South Africa. In addition, the antibacterial and antitumor activities of the endophyte's secondary metabolite crude extracts were investigated and further identified using gas chromatography high-resolution time-of-flight mass spectrometry (GC-TOF-MS).

MATERIALS AND METHODS

Collection and Identification of the Plant Material

The whole plant with a height up to 25 cm from the ground was collected from Eisleben, Botlokwa (23°31′49.5″S 29°49′27.1″E) in Limpopo province, South Africa. The whole plant including roots was placed in sterile polyethylene bags and transported to the laboratory at 4°C. The plant material was collected in March 2017 from a site with grassland. The identification of the plant material was carried out at the University of Johannesburg Herbarium (JRAU). A sample specimen of the plant material was deposited in the University of Johannesburg Herbarium (JRAU) with voucher specimen number Serepa-Dlamini 205 and species name *A. sessiliflora*. The remaining plant material was immediately processed in the laboratory.

Isolation and Culturing of Bacterial Endophytes

The bacterial endophytes were isolated from fresh leaves (approximately 10–15 leaves) of 1 whole plant following the method by Ding and Melcher (2016). Following isolation of pure colonies of the bacterial endophytes, 35% glycerol (glycerol diluted in sterile distilled water) stock cultures were prepared and stored at −80°C for future use. Stock cultures of five bacterial isolates were retrieved from long-term storage and sub-cultured on fresh nutrient agar (NA) media followed by incubation for 2–7 days at 30°C. Sub-culturing of each bacterial isolate was repeated several times until pure colonies were obtained.

Genomic DNA Extraction of Bacterial Endophytes

The bacterial strains were grown on NA for 24 h at 30°C and genomic DNA was extracted using the Zymo Research Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, United States) as per the manufacturer's protocol. The concentration of each endophyte DNA was quantified using the Nanodrop Spectrophotometer (Thermo Fisher Scientific, United States).

Polymerase Chain Reaction Amplification and Sequencing of the 16S rRNA Gene

The 16S rRNA genes of each bacterial strain were amplified by polymerase chain reaction (PCR) using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′CGGTTA CCTTGTTACGACTT-3′) primers (Yeates et al., 1997). The 25 μl PCR reactions contained 12.5 μl 2X PCR Master mix with standard buffer (20 mM Tris-HCl, 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCl, 0.2 mM dNTPs, 5% glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20, 25 units/ml One Taq® DNA polymerase), 2.5 μl of each primer (10 μM), 2.5 μl nuclease-free water, and 5 μl of each DNA (>50 ng/μl) template. A negative control containing all the PCR mix without any DNA was included in the PCR experiment. The amplification was carried out on a MyCycler™ Thermal Cycler (Bio-Rad, United States). The PCR reaction conditions were initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, and a final extension of 72°C for 10 min. The amplicons were purified with ExoSAP-it™ (Thermo Fisher Scientific, United States) after which they were sent to a commercial service provider, Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa for sequencing.

Phylogenetic Analysis

Raw sequence data of the 16S rRNA genes for each endophytic bacteria were used to create consensus sequences using the BioEdit Sequence Alignment Editor v.7.2.6 (Hall, 1999). The consensus sequences were subjected to BLAST analysis at the National Center for Biotechnology Information (NCBI) against the prokaryotic rRNA sequence database (Bacteria and Archaea), from which closely related bacterial species were identified (Altschul et al., 1990), and the type strains from the EzBioCloud database[1] were also included. All phylogenetic analyses post-BLAST were performed using molecular evolutionary genetics analysis version (MEGA) v.7.27 software (Kumar et al., 2016). The sequences were aligned using multiple sequence comparison by log-expectation (MUSCLE) with default settings (Edgar, 2004). Phylogenetic trees were constructed using maximum likelihood (ML) following the Jukes-Cantor model (Jukes and Cantor, 1969). A total of 1,000 replications were used for the statistical confidence of the nodes. For Bayesian inference, a Markov Chain Monte Carlo (MCMC) method was used to reconstruct the phylogenetic trees using BEAST v.1.10.4 (Suchard et al., 2018). The resulting trees were visualized in FigTree v.1.4.4 (Rambaut, 2018).

[1]https://www.ezbiocloud.net/

Biological Activity Assays

Extraction of Secondary Metabolites From Endophytes

Secondary metabolites of each bacterial isolate were extracted using the method described by Maloney et al. (2009) with slight modifications. Briefly, the endophytic bacteria isolated from *A. sessiliflora* were cultured in 1 L Luria Bertani (LB) broth and agitated at 200 rpm at 28°C for 7 days. After cultivation, 20 g/L of the Amberlite® XAD7HP 20–60 mesh (Sigma-Aldrich, Darmstadt, Germany) was added to each flask to absorb the secondary metabolites and was further agitated at 180 rpm for 2 h. A cheesecloth was used to filter the resin after which it was washed three times with 200 ml acetone. The acetone was concentrated using a rotary vapor (Lab Tech, Nantong, Jiangsu, China) at 5°C until a dark brown viscous extract was obtained. The residual water containing the crude extracts was transferred into a measuring cylinder and an equal volume of ethyl acetate (1:1 [v/v]) was added. The mixture was agitated vigorously for 5–10 min after which it was separated using a funnel. This process was repeated three times, and subsequently the ethyl acetate fraction was evaporated using a rotary vapor. The crude extracts were transferred into sterile beakers and covered with foil, then left at room temperature to dry.

Antibacterial Activities of Endophyte's Crude Extracts

In this study, the minimum inhibitory concentration (MIC) method described by Andrews (2001), was used to determine the antibacterial activities of the crude extracts from the bacterial endophytes with slight modifications. The test bacterial species included human clinical pathogens, and a number of the strains have previously exhibited antibiotic resistance to various antibiotics such as penicillin, ampicillin, quinolone, carbenicillin, cefalotin, cefotaxime, trimethoprim-sulfamethoxazole, clindamycin, dicloxacillin, and cetyltrimethylammonium bromide (Wagenlehner et al., 2003; Zhang et al., 2016; Hernández et al., 2021). Care was taken to include methicillin-resistant *Staphylococcus saprophyticus* (Higashide et al., 2008), members of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp.) (Flores-Paredes et al., 2021), with the exception of *Acinetobacter baumannii* and *Enterobacter* spp.; and methicillin-susceptible *S. aureus* (MSSA) (Ham et al., 2010): The test strains included, *Bacillus cereus* (ATCC 10876), *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Klebsiella oxytoca* (ATCC 13182), *Mycobacterium smegmatis* (ATCC 21293), *Pseudomonas aeruginosa* (NCTC 10662), *Staphylococcus aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305), *Staphylococcus epidermidis* (ATCC 14990), *Veillonella parvula* (ATCC 10790), and *Enterococcus faecium* (ATCC 13048). Briefly, stock solutions of the crude endophyte extracts were prepared by dissolving 0.19 g in 1 ml dimethyl sulfoxide (DMSO) to make a stock solution of 32 mg/ml. This was then serially diluted to concentrations of 16 mg/L down

to 0.25 mg/ml using Mueller-Hinton broth (MHB). Using McFarland 0.5 standard, 10 μl of each pathogenic strain was inoculated in 20 ml MHB and incubated at 30°C for 24 h. Using sterile 96 well microtiter plates, 100 μl of each pathogenic strain was added horizontally while 100 μl of the diluted crude extracts were added vertically starting from 16 mg/ml down to 0.25 mg/ml. The antibiotic Streptomycin with a concentration of 1 mg/ml (Sigma-Aldrich, Switzerland) was used as positive control while DMSO was used as a negative control. The MIC was conducted in triplicates. The plates were incubated at 37°C for 24 h after which 10 μl resazurin salt solution [0.02% (w/v)] was added to the wells as an indicator of microbial growth and incubated for an additional 2 h. The color change from blue to pink to clear indicated reduction had taken place as oxygen becomes limited within the medium, indicating that metabolism has taken place. The wells in which no color change occurred indicated no bacterial growth while the wells with a pink or clear color indicated bacterial growth. The MIC with the lowest concentration was visually inspected for color change.

Antitumor Activity of Endophyte's Crude Extracts

The effect of bacterial endophyte's crude extracts on the survival and growth of human cancer cell lines A549 lung carcinoma, Hek 293 kidney adenocarcinoma, and HeLa cervical adenocarcinoma cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) *in vitro* cytotoxic assay. The crude extracts were prepared as described above and different concentrations of each extract (31.30, 62.60, 125, 250, 500, and 1,000 μg/ml) were prepared. Briefly, the A549, HeLa, and Hek 293 cells were grown using normal tissue culture techniques with the addition of 10% fetal bovine serum (FBS). The cells (1×10^6 cells/ml) were incubated in 96 well microtiter plates at 37°C for 24 h. Following incubation, the media was removed and 100 μl of fresh media was added to all the wells along with 100 μl of the diluted extracts from high (1,000 μg/ml) to low (31.3 μg/ml) concentrations. The cells were incubated for 72 h, after which 5 μl [3- (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) was added to the cells. The absorbance values were measured at 490 nm at 0, 1, 2, 3, and 4 h incubation periods using the Molecular Devices SpectraMax ABS Plus, and data were acquired with SoftMax Pro 7.1 Data Acquisition and Analysis Software. Auranofin was used as a positive control and DMSO was used as a negative control. In the MTT assay, the MTS compound is metabolized by viable cells from yellow to purple formazan by the mitochondria of viable cells which is detected at 490 nm. The cytotoxicity tests of the crude extracts were analyzed in duplicates across three plates (n = 6) and the absorbance value was reported. The results were expressed as growth inhibition and IC_{50} values were determined using the AAT Bioquest IC_{50} calculator [AAT Bioquest (2022), Sunnyvale, CA, United States] available at https://www.aatbio.com/tools/ic50-calculator. The IC_{50} is the half-maximal inhibitory concentration, which measures the effectiveness of a crude extract in inhibiting a given biological sample or process by half, in this study the inhibition of human cancer cell lines.

Gas Chromatography-Mass Spectrophotometry Analysis

Metabolite profiling of the endophyte extracts was carried out on a GC-TOF-MS system (LECO Corporation St. Joseph, MI, United States) using the following conditions: primary column and a Rxi-5Sil MS (30 m, 250 μm i.d., 0.25 μm d_f) (Restek, Pennsylvania, United States) and a Rxi-17Sil MS (2 m, 250 μm i.d., 0.25 μm d_f) (Restek, Bellefonte, PA, United States) secondary column. In brief, samples were first prepared by adding 1 ml HPLC grade methanol (Sigma-Aldrich, Aston Manor, South Africa) to the extracts, 1 μl of each sample was injected, and Helium was used as a carrier gas with a flow rate of 1 ml/min. The oven temperature was maintained at 60°C for 1 min and then programmed at 10°C/min increment to 330°C, then 5°C/min to 280°C. The inlet temperature was 250°C. Mass spectra (MS) were optimized at an electron energy of −70 eV with an ion source at 250°C. The mass fragments used were from 40–660 m/z with an acquisition rate of 10 spectra/second. The interpretation of GC-MS mass-spectra was analyzed using the ChromaTOF software (LECO Corporation, St. Joseph, MI, United States). The functional groups and biological activities of the compounds were analyzed using the NCBI PubChem and PASS online databases available at https://pubchem.ncbi.nlm.nih.gov and http://www.way2drug.com/passonline, respectively.

Statistical Analysis

Unless otherwise stated all experiments were carried out in triplicates. The mean values were calculated using the Microsoft Excel program version 2010. The t-test was performed to determine the significance of the difference between the mean values. One-way ANOVA was performed at $p \leq 0.05$ significant levels to determine the variance.

RESULTS

Molecular Identification of Bacterial Endophytes Associated With *Alectra sessiliflora*

In this study, a total of five bacterial endophytes all belonging to the Firmicute phylum were isolated and identified through 16S rRNA gene sequencing as shown in **Table 1**. The 16S rRNA sequences were deposited in GenBank with accession numbers from MZ976846—MZ976850. The 16S rRNA gene sequences of each strain were compared with other bacterial species available on the GenBank-NCBI database. The NCBI database confirmed the identity of the bacterial endophytes belonging to three genera *Lysinibacillus, Peribacillus,* and *Bacillus* with three isolates as shown in **Table 1**. All the isolates showed 94–99% similarities with other closely related strains retrieved from the NCBI database as indicated in **Supplementary Table 1**.

Phylogenetic Analysis

The evolutionary relationships between all the endophytic bacteria isolated from *A. sessiliflora* with other closely related species were constructed using the ML and Bayesian MCMC

TABLE 1 | Morphological characteristics of bacterial endophytes isolated from *Alectra sessiliflora*.

Bacterial sample code	Assigned isolate name	Assigned accession number	Phylum	Gram stain reaction	Cell shape
AS_1	*Lysinibacillus* sp. strain AS_1	MZ976846	Firmicutes	+ve	Rods
AS_2	*Peribacillus* sp. strain AS_2	MZ976847	Firmicutes	+ve	Rods
AS_3	*Bacillus* sp. strain AS_3	MZ976848	Firmicutes	+ve	Rods
AS_4	*Bacillus* sp. strain AS_4	MZ976849	Firmicutes	+ve	Rods
AS_5	*Bacillus* sp. strain AS_5	MZ976850	Firmicutes	+ve	Rods

+ve positive: Gram-positive.

methods. Each species was delineated with closely related species in separate phylogenetic trees (**Figures 1**–**3**). *Lysinibacillus* sp. Strain AS_1 formed a polytomy clade with other *Lysinibacillus macroides* and two *L. fusiformis* strains, supported by a 95% bootstrap value (**Figure 1**). In the Bayesian phylogenetic tree (**Supplementary Figure 1**), *Lysinibacillus* sp. Strain AS_1 formed a paraphyletic group with *L. fusiformis* and *L. endophyticus*.

Peribacillus strain AS_2 showed a polytomy relationship with *Peribacillus simplex* and *P. muralis* supported by an 83% bootstrap value (**Figure 2**). In the Bayesian phylogenetic tree (**Supplementary Figure 2**) *Peribacillus* strain AS_2 formed a sister clade with *Peribacillus soganii*. *Bacillus* sp. Strain AS_3 had a polytomy clade with *Bacillus luti* and *B. proteolyticus* with a 23% bootstrap value (**Figure 3**). Strain AS_4 had a sister clade with *B. cereus*, and strain AS_5 did not cluster with any of the species (**Figure 3**). In the Bayesian phylogenetic tree (**Supplementary Figure 3**), strains AS_3 and AS_5 had sister clades with *B. albus* and *B. pacificus*, respectively. Strain AS_4 did not cluster with any species, although there was no congruency between the two methods, both indicate that strains in this study belong to *Lysinibacillus, Peribacillus,* and *Bacillus* genera. Strains AS_4 and AS_5 could represent new species and further studies are recommended.

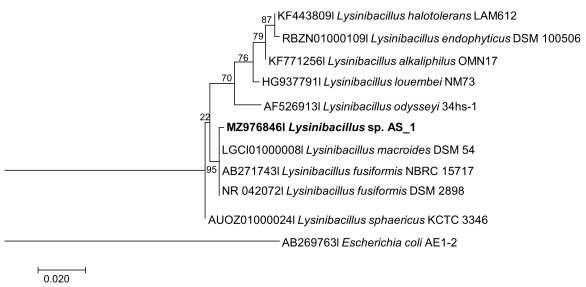


FIGURE 1 | Maximum likelihood phylogenetic tree based on analysis of partial 16S rDNA nucleotide sequence of *Lysinibacillus* sp. Strain AS_1 with related strains from the *Lysinibacillus* genus. Numbers above or below the nodes indicate bootstrap values generated after 1,000 replications. *Escherichia coli* AE-1 (AB269763) was used as an outgroup.

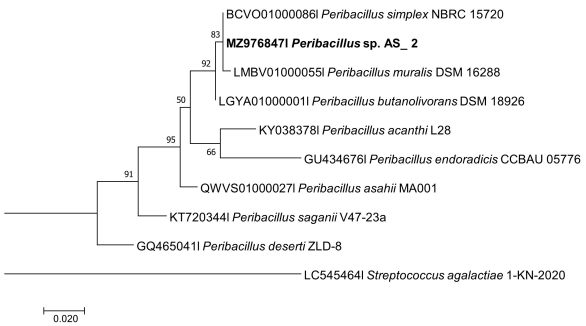


FIGURE 2 | Maximum likelihood phylogenetic tree based on analysis of partial 16S rDNA nucleotide sequence of *Peribacillus* sp. Strain AS_2 with related strains from the *Peribacillus* genus. Numbers above or below the nodes indicate bootstrap values generated after 1,000 replications. *Streptococcus agalactiae* AE-1 (LC545464) was used as an outgroup.

Antibacterial Activity of Endophytic Bacteria

The five isolated endophytic bacteria from medicinal plant *A. sessiliflora* were tested against 11 pathogenic strains for antibacterial activity as shown in **Table 2**. The minimum inhibitory concentration of extracted secondary metabolites ranged from 8 to 0.25 mg/ml. The crude extracts of *Bacillus* sp. strain AS_3 and *Bacillus* sp. strain AS_5 showed no inhibition against all the indicator strains. The lowest MIC value

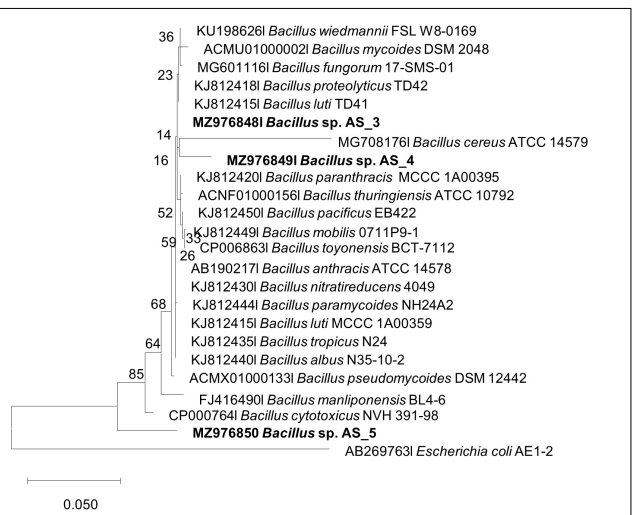


FIGURE 3 | Maximum likelihood phylogenetic tree based on analysis of partial 16S rDNA nucleotide sequences of *Bacillus* sp. strain AS_3, *Bacillus* sp. AS_4 and *Bacillus* sp. AS_5 with related strains from the *Bacillus* genus. Numbers above or below the nodes indicate bootstrap values generated after 1,000 replications. *Escherichia coli* AE1-2 was used as an outgroup.

TABLE 2 | Minimum inhibitory concentrations of crude extracts of bacterial endophytes associated with *Alectra sessiliflora*.

Test strain	Crude extracts mg/mL					Positive control
	AS_1	AS_2	AS_3	AS_4	AS_5	Streptomycin 1 mg/mL
B. cereus	8	2	–	–	–	0.25
E. coli	8	–	–	–	–	0.25
E. faecium	8	–	–	–	–	0.25
K. oxytoca	4	–	–	–	–	0.25
K. pneumoniae	8	–	–	0.25	–	0.125
M. smegmatis	8	–	–	–	–	0.25
S. aureus	8	16	–	–	–	0.25
S. epidermidis	4	–	–	–	–	0.25
S. saprophyticus	8	2	–	–	–	0.25
P. aeruginosa	–	–	–	–	–	0.25
V. parvula	8	–	–	–	–	0.25

–, No inhibition; AS_1, Lysinibacillus sp. strain AS_1; AS_2, Peribacillus sp. AS_2; AS_3, Bacillus sp. strain AS_3; AS_4, Bacillus sp. strain AS_4; AS_5, Bacillus sp. strain AS_5.

was recorded against *K. pneumoniae* (0.25 mg/ml), *B. cereus* (2 mg/ml), and *S. saprophyticus* (2 mg/ml) from strains AS_2 and AS_4. The highest concentration was recorded against *M. smegmatis*, *E. coli*, and *V. parvula* with MIC values ranging from 8 to 16 mg/ml from strains AS_1 and AS_2. Statistical analysis showed that only two of the endophytes' extracts, strains AS_2 and AS_4 had significant ($p < 0.05$) inhibition values with the lowest MIC values of 0.25 and 2 mg/ml.

Antitumor Activity of Bacterial Endophytes Crude Extracts Against Cancer Cells

Antitumor Activity of Crude Extracts Against Hela Cervical Adenocarcinoma Cells

Different concentrations of crude ethyl acetate extracts of *Lysinibacillus* sp. strain AS_1, *Peribacillus* sp. strain AS_2, *Bacillus* sp. strain AS_3, *Bacillus* sp. strain AS_4, and *Bacillus* sp. strain AS_5 were used to determine antitumor activity against three cancer cell lines (A549, Hela and Hek 293). Dimethyl sulfoxide was used as a negative control, while auranofin was used as a positive control as it is known to kill most cancer cells by inhibiting thioredoxin reductase and the ubiquitin-proteasome system (McCauley et al., 2013; Li et al., 2016). Endophytic crude extracts showed varying activities against Hela cervical carcinoma cells with AS_1 and AS_2 showing 99% reduction at a concentration of 1,000–500 μg/ml (**Figure 4**). A cell reduction of 61% was observed for AS_3 at a concentration of 1,000 μg/ml. An increase in cell viability > 100% was noted for AS_4 and AS_5. Overall, only three strains AS_1, AS_2, and AS_3 had significant inhibition values ($p < 0.05$) except for strains AS_4 and AS_5 for Hela and Hek 293 cancer cell lines. Only strain AS_2 showed the most significant inhibition at higher concentrations (500 and 1,000 μg/ml) for A549 adenocarcinoma cells. The cell line viability was in the following order: Hela > Hek 293 > A459.

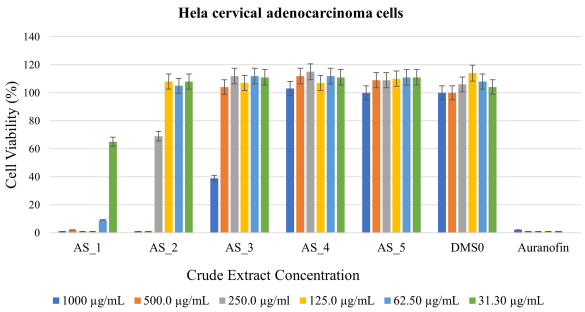


FIGURE 4 | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxic assay of endophyte-derived secondary metabolites on Hela cervical adenocarcinoma cells tested at different concentrations ranging from 1,000 to 31.30 μg/ml. Auranofin was used as a positive control. AS_1, *Lysinibacillus* sp. strain AS_1; AS_2, *Peribacillus* sp. AS_2; AS_3, *Bacillus* sp. strain AS_3; AS_4, *Bacillus* sp. strain AS_4; AS_5, *Bacillus* sp. strain AS_5.

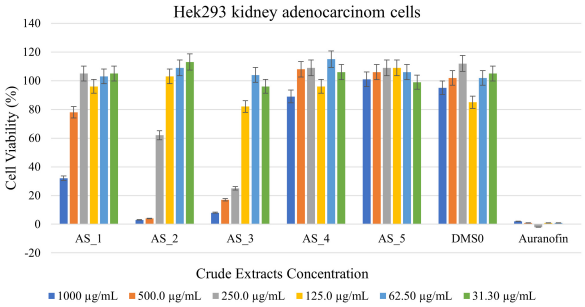


FIGURE 5 | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxic assay of endophyte-derived secondary metabolites on Hek293 kidney adenocarcinoma cells tested at different concentrations ranging from 1,000 to 31.3 500 μg/ml. Auranofin was used as a positive control. AS_1, *Lysinibacillus* sp. strain AS_1; AS_2, *Peribacillus* sp. AS_2; AS_3, *Bacillus* sp. strain AS_3; AS_4, *Bacillus* sp. strain AS_4; AS_5, *Bacillus* sp. strain AS_5.

Antitumor Activity of Crude Bacterial Endophyte Extracts Against Hek 293 Kidney Adenocarcinoma Cells

Strain AS_2 showed the highest cell reduction of 96% against Hek 293 kidney cells at a concentration of 500 μg/ml (**Figure 5**) and strain AS_3 showed a cell reduction of 92% (1,000 μg/ml), 83% (500 μg/ml), and 75% (250 μg/ml). A reduction of 62% was noted for strain AS_1 at a concentration of 1,000 μg/ml. No notable reduction was observed for strains AS_4 and AS_5 there was an increase in cell viability.

Antitumor Activity of Crude Bacterial Endophyte Extracts Against A549 Lung Adenocarcinoma Cells

Bacillus sp. strain AS_3 crude extracts were able to kill all the A549 lung cells at a concentration of 1,000 μg/ml having cell viability of 0% (**Figure 6**) and strain AS_2 extracts showed a cell reduction of 99% at concentrations of 1,000–500 μg/ml respectively. Strain AS_1 showed a cell reduction of less than 50% at a concentration of 125 and 62.5 μg/ml respectively. An increase in cell viability was observed for strains AS_4 and AS_5.

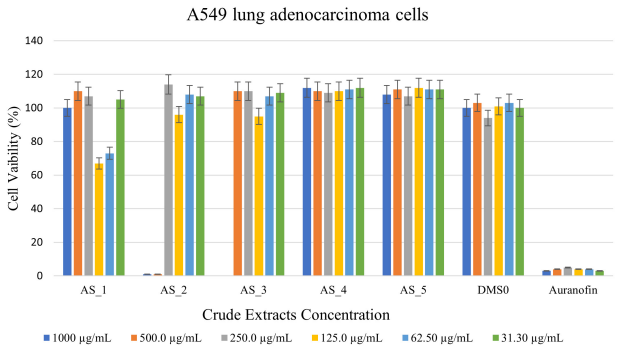


FIGURE 6 | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxic assay of endophyte-derived secondary metabolites on A549 lung adenocarcinoma cells tested at different concentrations ranging from 1,000 to 31.3 500 µg/ml. Auranofin was used as a positive control. AS_1, *Lysinibacillus* sp. strain AS_1; AS_2, *Peribacillus* sp. AS_2; AS_3, *Bacillus* sp. strain AS_3; AS_4, *Bacillus* sp. strain AS_4; AS_5, *Bacillus* sp. strain AS_5.

TABLE 3 | IC_{50} values of crude extracts from *Alectra sessiliflora* against different cancer cells.

IC_{50} (µg/mL)			
Crude extract	**Hela**	**Hek293**	**A549**
AS_1	52.8	523.8	190.9
AS_2	262	262.2	380.6
AS_3	700.7	169.4	753.3
AS_4	>1,000	>1,000	>1,000
AS_5	>1,000	18.3	165.4
Auranofin	>1,000	500.06	>1,000

AS_1, Lysinibacillus sp. strain AS_1; AS_2, Peribacillus sp. AS_2; AS_3, Bacillus sp. strain AS_3; AS_4, Bacillus sp. strain AS_4; AS_5, Bacillus sp. strain AS_5.

The IC_{50} values were determined on all three cancer cell lines. From **Table 3** it can be observed that the IC_{50} for Hela was 52.78, 262, and 700.7 µg/ml for the Hela adenocarcinoma cells for strains AS_1, AS_2, and AS_3, respectively. For Hek 293 adenocarcinoma cells, 50% inhibition was observed at 523.8 µg/ml (strain AS_1), 262.2 µg/ml (strain AS_2), 169.4 µg/ml (strain AS_3), and 18.31 µg/ml (strain AS_5). For A549 cells, 50% inhibition was observed at concentrations ranging from 190.9, 380.6, 753.3, and 165.4 µg/ml for strains AS_1, AS_2, AS_3, and AS_5, respectively. No notable inhibition was observed for strain AS_4 for all the cancer cell lines and AS_5 for Hela cells.

Gas Chromatography-Mass Spectrophotometry Analysis

Metabolite profiling of the endophyte's crude extracts from *A. sessiliflora* was subjected to GC-MS analysis. The bioactive compounds were identified and tabulated (**Table 4**). **Table 4** shows the metabolite profiles for the ethyl acetate extracts. Only compounds having a retention time (RT) of ≥3 min were recorded. The gas chromatography results of the bacterial crude extracts identified a total of 80 secondary metabolites (**Table 4**, **Supplementary Table 2**, and **Supplementary Figures 4–8**), with AS_1 (**Supplementary Figure 4**) having the most identified compounds. The compounds prevalent in all the extracts were tridecane (C13H28), hexadecane (C16H34), tetracosane (C24H50), and ergotaman-3′,6′,18-trione,9,10-dihydro-12′-hydroxy-2′-methyl-5′-(phenylmethyl)-, (5′a,10a) (C33H37N5O5). Other interesting metabolites included benzyl benzoate (C9H16N2O2), benzene-acetamide (C8H9NO), 2-coumaranone (C8H6O2), and octacosane (C28H58).

DISCUSSION

Endophytic bacteria isolated from medicinal plants have gained much interest from researchers as they have been shown to possess antibacterial and antifungal activities (Duhan et al., 2020). They are also known to produce a wide range of secondary metabolites with various biological activities including antioxidant, antimalarial, antidiabetic, antimicrobial, anti-inflammatory, and cytotoxic (Abdalla et al., 2020). Due to the increasing number of deaths from infections caused by drug-resistant bacteria and cancer, there is an urgent need to search for new sources of drugs (Kusari et al., 2013; Prakash et al., 2020). *Alectra sessiliflora* is a medicinal plant with a limited history in ethnobotanical applications, but studies on the endophytic bacteria associated with it are scarce.

Based on the NCBI-BLAST database, the isolated strains had a 93–99% similarity to *Lysinibacillus fusiformis* strain POB29, *Peribacillus simplex* strain TP 141-1, *Bacillus cereus* strain NCIM 2158, *Bacillus proteolyticus* strain KLR12, and *Bacillus safensis* strain MF-86-1 (**Supplementary Table 1**). Although five bacterial endophytes were isolated in this study, the data adds to the minimally reported phyllosphere bacterial endophytes (Sivakumar et al., 2020). Li et al. (2021), reported a minimal number of bacterial endophytes from halophytes, with five bacterial endophytes isolated each from *Reaumuria soongorica* (PalL Maxim.) and *Peganum harmala* L., and three bacterial endophytes each from *Artemisia carvifolia* (Buch-Ham. ex Roxb. Hort. Beng.) and *Suaeda dendroides* (C. A. Mey. Moq.). The current study is the first to isolate and identify bacterial endophytes from *A. sessiliflora*, we strongly believe that more bacterial endophytes are associated with this plant host, thus necessitating further identification of its endophytes. The phyllosphere bacterial endophyte community is known to be affected by plant genotype, immune system and species, soil type, climatic conditions, and geographic location (Copeland et al., 2015). This could explain the low number of endophytes isolated in the current study. Similarly, previous studies reported the occurrence of *Bacillus* endophyte species within the phyllosphere of lettuce (Rastogi et al., 2012), and grapevine (Kembel et al., 2014). *Peribacillus* spp. and *Lysinibacillus* spp. have not been reported as phyllosphere endophytes, nonetheless, *Lysinibacillus* spp. have previously been isolated from tomato roots (Zhu et al., 2021), rice roots (Khaskheli et al., 2020; Shabanamol et al., 2021); and *Peribacillus* spp. was previously isolated from *P. harmala* (Li et al., 2021) and canola crop roots (Martínez-Hidalgo et al., 2021), making our study one of

TABLE 4 | GC-HRTOFMS analysis of bacterial endophyte's crude extracts associated with *Alectra sessiliflora*.

Compound	Molecular formula	RT (min)	Area%	ion m/z	Biological activity	Bacterial endophyte	References
1-Undecanol	$C_{11}H_{24}O$	11,62	0,03	155.0726	Antimicrobial	AS_1	Mukherjee et al., 2013
Tridecane	$C_{13}H_{28}$	7,25	0,07	127.0543	Antibacterial, antioxidant and anti-inflammatory activity	AS_1, AS_2, AS_3, AS_5	Barupal et al., 2019
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	12,31	0,02	206,116	Antioxidant and anti-inflammatory activity	AS_1, AS_3, AS_5	Vahdati et al., 2022
Undecanoic acid	$C_{11}H_{22}O_2$	13	0,04	169,0761	Insecticidal and antioxidant activity	AS_1, AS_3	Lodyato et al., 2003; Farag et al., 2021
7-Hexadecene, (Z)-	$C_{16}H_{32}$	13,55	0,06	153,127	Insecticidal activity	AS_1, AS_3, AS_5	Ekalu, 2021
2-Dodecanone	$C_{12}H_{24}O$	13,62	0,04	134,14	Antimicrobial	AS_1, AS_3, AS_4, AS_5	Mukherjee et al., 2013
Hexadecane	$C_{16}H_{34}$	12,04	0,06	113.1323	Insecticidal activity	AS_1, AS_2, AS_3, AS_4, AS_5	Ekalu, 2021
4-Mercaptophenol	C_6H_6OS	16,25	0,02	126,0423	Anticancer, antibacterial, and antiseptic activity	AS_1	Kumar and Mishra, 2018
Pentadecane	$C_{15}H_{32}$	16,31	0,025	204,2481	Antibacterial, antioxidant and anti-inflammatory activity	AS_1	Barupal et al., 2019
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	16,73	2,03	197,127	Antibiotic, antifungal drugs, cholesterol, and antitumor agents	AS_1, AS_3, AS_4, AS_5	Rao and Lakshmi, 2018
Eicosane	$C_{20}H_{42}$	16,25	1,28	254,965	Antibacterial, antioxidant and anti-inflammatory activity	AS_1, AS_3, AS_4, AS_5	Barupal et al., 2019
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	18,14	0,06	213.1846	Antifungal and antibacterial activity	AS_1, AS_2	Hsou et al., 2011
Tetracosane	$C_{24}H_50$	20,52	0,43	225,2578	Antibacterial, antioxidant and anti-inflammatory activity	AS_1, AS_2, AS_3, AS_4, AS_5	Barupal et al., 2019
2-Coumaranone	$C_8H_6O_2$	7,87	0,06	134.0360	Anticancer activity and Anti-HIV agents	AS_2	Cole et al., 2016; Ma et al., 2016
Phenol, 2,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	12,3	0,03	206,1662	Anticancer, antibacterial and antiseptic activity	AS_2, AS_4	Kumar and Mishra, 2018
Octacosane	$C_{28}H_{58}$	22,15	0,14	196,1204	Insecticidal activity	AS_2, AS_3, AS_4, AS_5	Ponsankar et al., 2016
1,2-Benzenedicarboxylic acid, dipropyl ester	$C_{14}H_{18}O_4$	18,205	0,03	150,0265	Antifungal and antibacterial activity	AS_3, AS_4, AS_5	Hsou et al., 2011
Tetracosanol-1	$C_{24}H_{50}O$	19,35	0,04	139,8193	Antimutagenic activity	AS_5	Makhafola et al., 2017

RT (m), retention time (minutes); m/z, mass-to-charge ratio; AS_1, Lysinibacillus sp. strain AS_1; AS_2, Peribacillus sp. AS_2; AS_3, Bacillus sp. strain AS_3; AS_4, Bacillus sp. strain AS_4; AS_5, Bacillus sp. strain AS_5.

the few to isolate and report *Peribacillus* sp. as an endophyte. The 16S rRNA approach remains the gold standard for the initial identification of bacterial species, however, this approach cannot differentiate closely related bacterial species as is indicated by the formation of polytomy relationships in **Figures 1–3** (**Supplementary Figures 1–3**; Kitahara and Miyazaki, 2013). The 16S rRNA gene has identified the endophytes to genus level, further studies like whole genome sequencing and multilocus sequence analysis (MLSA) are required for species delineation.

New and effective therapeutic drugs are required to combat microbial drug resistance (World Health Organization [WHO], 2021), and increasing incidence of cancers some of which have drug resistance (Housman et al., 2014). The use of medicinal plants as a source of bioactive compounds has paved the way for the discovery of novel drugs against microbial and cancer infections (Gagana et al., 2020). However, one setback of using medicinal plants is that several factors including the chemical composition of the plant, season and geographical specificity, cultivation requirements, and random use of the plant may limit their potential use (Katiyar et al., 2012). Moreover, their overuse can ultimately lead to plant extinction. Several studies suggest that endophytic bacteria isolated from medicinal plants can produce the same or similar bioactive compounds as their host plant including novel compounds (Mehanni and Safwat, 2009; Alvin et al., 2014; Gouda et al., 2016). These findings have attracted the interest of researchers as this indicates that endophytes can act as substitutes for plants when searching for novel bioactive compounds without causing major impacts on the environment.

A previous study has shown that plant extracts of *A. sessiliflora* exhibited antibacterial activity against selected pathogens including *S. aureus*, *P. aeruginosa*, *E. coli, B. pumilus,* and *Shigella dysenteria*e at MIC values ranging from 3.13–25 mg/ml (Mariita et al., 2010). In the current study, the antimicrobial activity of bacterial endophytes associated with *A. sessiliflora* was investigated against 11 pathogenic strains. A plant extract with a MIC value of $\leq$8 mg/ml is considered to possess some antimicrobial activity while those with a MIC value of $\leq$1 mg/ml are considered to have significant antibacterial activity (Fabry et al., 1998; Van Vuuren, 2008). Uche-Okereafor et al. (2019), reported antibacterial activities of bacterial endophytes isolated from *Solanum mauritianum* against pathogenic bacteria such as *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*, and the results indicated antimicrobial activity with MIC concentrations

ranging from 0.0625 to 8 mg/ml. In a similar study by Tapfuma et al. (2020) bacterial endophytes isolated from *Celtis africana* had an antibacterial activity with MIC concentrations ranging from 4 to 8 mg/ml against *B. cereus, E. coli,* and *S. aureus*. Among the five bacterial endophytes, *Lysinibacillus* strain AS_1 extract had antibacterial activity against 10 test strains with antibacterial activity ranging from 4 and 8 mg/ml (**Table 2**), with the exception of *P. aeruginosa*. The most significant MIC value of 4 mg/ml was noted on the *K. oxytoca* and *S. epidermidis*. The previous studies demonstrated that the genus *Lysinibacillus* produced secondary metabolites such as antibiotics, hydrolytic enzymes, and bacteriocins with strong antibacterial activity against selected pathogens such as *K. pneumoniae*, *S. aureus,* and *P. aeruginosa* (Naureen et al., 2017). *Peribacillus* sp. strain AS_2 extracts had an MIC of 2 and 16 mg/ml, with *S. saprophyticus* and *B. cereus* being the most susceptible with an MIC value of 2 mg/ml except for *S. aureus* which had a higher MIC value of 16 mg/ml. All the test bacterial species were resistant to the *Peribacillus* crude extracts. To the best of our knowledge, this is the study on the antibacterial activity of bioactive compounds from *Peribacillus* sp. *Bacillus* sp. strain AS_4 had an MIC of 0.25 mg/ml against *K. pneumoniae*, which was the lowest MIC recorded in the study. A study by Akpor et al. (2021), determined the antibacterial potential of metabolites produced by *B. proteolyticus, B. thuringiensis, B.* cereus, and *B. subtilis,* and all the extracts showed antimicrobial activity against test pathogens at an MIC of 200 mg/ml. In a similar study by Makuwa and Serepa-Dlamini (2021), bioactive metabolites of *Bacillus* species isolated from a medicinal plant, *Dicoma anomala* were found to be effective against selected pathogens such as *E. coli*, *K. oxytoca*, and *S. aureus* with MIC values ranging from 0.625 to 10 mg/ml. According to Borriss et al. (2019), *Bacillus* species produce antibacterial agents such as surfactin and bacteriocins which may be responsible for their antibacterial activities (Huo et al., 2019). Interestingly, no antibacterial activities were reported for the crude extracts of *Bacillus* sp. strain AS_3 and *Bacillus* sp. strain AS_5 for all the pathogenic strains (**Table 2**). Strain AS_1 had MIC activities against most test strains, and we thus recommend its test against multi-drug resistant bacteria.

The antitumor activity of the bacterial endophyte's crude extracts from *A. sessiliflora* was evaluated against three human cancer cells, Hela cervical, Hek 293 kidney, and A549 lung adenocarcinoma cells. Oosthuizen et al. (2019), conducted a study to determine the cytotoxic effects of *A. sessiliflora* plant extracts against U937 human macrophage cells, and the results showed an increase in cell viability of the U937 cells. In this study, crude secondary metabolites from *A. sessiliflora* bacterial endophytes showed the best antitumor activities showing cytotoxic effects against the three cancer cell lines. To the best of our knowledge, this is the first report on the antitumor cytotoxic activity of bacterial endophyte's crude extracts from *Lysinibacillus* sp. strain AS_1, *Peribacillus* sp. strain AS_2, *Bacillus* sp. stain AS_3; *Bacillus* sp. strain AS_4, and *Bacillus* sp. strain AS_5, all isolated from *A. sessiliflora*.

Lysinibacillus sp. strain AS_1 crude extract showed antitumor activity against Hela cervical cells, with growth inhibition of more than 90% at concentrations ranging from 1,000 to 62.50 μg/ml (**Figure 4**). The minimum concentration of 31.3 μg/ml showed growth inhibition of 35%. For Hek 293 kidney cells (**Figure 5**), cell growth inhibition reduction of 68% was observed at a concentration of 1,000 μg/ml, while at concentrations of 500 and 125 a growth inhibition of less than 50% was recorded. Similar results were observed for A549 lung cells as no growth inhibition of more than 50% was noted (**Figure 6**). However, there was an increase in cell growth in the A549 lung cells. *Peribacillus* sp. strain AS_2 crude endophyte extract showed to have antitumor activity against all the cancer cell lines, with growth inhibition of more than 95% with concentrations ranging from 1,000 to 500 μg/ml. Growth inhibitions of 31 and 38% were observed at a concentration of 250 μg/ml for Hela cervical and Hek293 kidney cells, respectively. An increase in cell growth was observed at concentrations ranging from 125 to 31.3 μg/ml for Hela and Hek293, respectively, with the exception of A549 cells which showed less activity with a 4% reduction.

For *Bacillus* species, only *Bacillus* sp. strain AS_3 showed notable antitumor activity against all the cancer cells at a concentration of 1,000 μg/ml. Growth inhibition of 61% was observed for Hela cells at a concentration of 1,000 μg/ml. Growth inhibition of 92.83 and 75% was observed for Hek 293 kidney cells with a concentration ranging from 1,000 to 250 μg/ml, respectively (**Figures 4–6**). A 100% growth inhibition was achieved for A459 lung cells at a concentration of 1,000 μg/ml. No notable activity was observed for strains AS_4 and AS_5 for all the cancer cells, instead, there was an increase in cell growth. Overall, strains AS_1, AS_2, and AS_3, showed a significant effect on the growth inhibition of cells by decreasing the three cancer cells as compared to strains AS_4 and AS_5. *Bacillus* species are known to produce bioactive metabolites with antitumor and antibacterial species (Shao et al., 2021). Sebola et al. (2020), conducted a study to evaluate the antitumor activity of crude extracts from the medicinal plant *Crinum macowanii* Baker, and the results showed that the crude extracts of *B. safensis* had growth inhibition of 50% against A549 cells at a concentration of 100 μg/ml. Ramasubburayan et al. (2015), conducted a similar study in which the anticancer activity of the crude extract of *B. subtilis* subsp. *subtilis* RG was tested against MCF-7 human breast adenocarcinoma cells, and the results indicated growth of 37% at a concentration of 25 μg/ml.

The IC_{50} values were further determined to show the concentration at which 50% inhibition of the tumor or cancerous cells occurred. It has been proposed that extracts with IC_{50} values <20 μg/ml are significant when tested against cancer cell lines, whereas IC_{50} values <50 μg/ml are moderate, low when IC_{50} values are <200 μg/ml and non-toxic when $IC_{50} > 200$ μg/ml (Kuete and Efferth, 2015). For Hela cervical adenocarcinoma cells, strain AS_1 showed a low IC_{50} of 52.78 μg/ml. Strains AS_4 and AS_5 were found to be non-toxic ($IC_{50} > 200$ μg/ml) to the Hela cancer cells. For Hek 293 kidney adenocarcinoma cells, stain AS_5 showed a significant IC_{50} of 18.31 μg/ml, whereas strain AS_3 showed a low IC_{50} value of 169.4 μg/ml. For A549 lung cells, strain AS_5 showed low IC_{50} at a concentration of 165.4 μg/ml. Strains AS_1, AS_2, and AS_3 showed strong cytotoxic activities while the other extracts had poor activity. Bostanci et al. (2022), investigated the anticancer activity of the

Salvia marashica plant in two cancer cell lines, breast cancer cells (MF-7) and healthy endothelial cell line (HUVEC), and the plant showed IC_{50} values at concentrations of 125 and 1,650 μg/ml, respectively. A similar study by Abdel-Fatah et al. (2021), showed that extracts isolated from *Gink biloba* showed anticancer activity of IC_{50} values of 4.06 and 6.07 μM for cancer cell lines HEPG2 (liver) and MCF7 (breast) cells, respectively.

The chemical composition of *Alectra sessiliflora* bacterial endophyte's crude extracts was further analyzed using gas chromatography. The analysis identified 80 compounds (**Table 4**, **Supplementary Table 2**, and **Supplementary Figures 4–8**) belonging to different chemical groups of acids, alcohols, amino acids, aldehydes, amines, amides, ethers, esters, hydrocarbons, ketones, and carbohydrates. Several major compounds were identified in all the extracts including pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), tridecane, eicosane, tetracosane, and hexadecane as shown in **Table 4**. Some alkane compounds or derivatives such as octacosane, tetracosane, and eicosane secreted by *Bacillus* sp. AS_3 have been reported to be a potential inhibitor against different cancer cells including cervical carcinoma, breast carcinoma, and, human embryonic lung cells W1-38 (Strobykina et al., 2019).

Phenol and phenol derivatives were identified in some bacterial extracts such as 4-mercaptophenol (AS_1), phenol,2,2-bis(1,1dimethyl) (AS_2 and AS_4), and 2,4-di-tertbutylphenol (AS_1, AS_3 and AS_5). These compounds are well-known to possess vital therapeutic properties including anticancer, antibacterial, antiseptic, and anti-inflammatory (Kumar and Mishra, 2018). The compound *n*-tetracosanol was isolated from leave extracts of *Combretum microphyllum* and it was found to have antimutagenic activity against *Salmonella typhimurium* TA98 at a low concentration of 5 μg/ml, and because mutations play a role in the pathogenesis and development of cancerous cells, it thus prevents the pathological process of cancer which can be caused by mutations (Makhafola et al., 2017). Vergara et al. (2015) conducted a study on the antiproliferative evaluation of tetracosanol over Chinese hamster ovary cells K1 (CHO-K1) and human melanoma cells and it was found that tetracosanol had no cytotoxic effect on the growth of CHO-K1 cells whereas, for human melanoma cells, it affected the cell density and inhibited growth by 58%. In this study, only *Bacillus* sp. strain AS_5 secreted this metabolite. Most of the compounds identified in this study have antibacterial and antitumor activity, which explains the results obtained from the crude extracts, especially for strains AS_1 and AS_2. The use of organic solvents such as ethyl acetate to extract bioactive metabolites from medicinal plants has been reported to yield a high number of metabolites with higher purity compared to water-based methods (Jose et al., 2002; Pintać et al., 2018). The culturing of bacterial endophytes for 7 days was sufficient to yield the expected metabolites. In a similar study by El-Naggar et al. (2017), 22 antimicrobial metabolites were achieved using the solvent ethyl acetate. Chakraborty et al. (2021), conducted a study in which 42 compounds from *Streptomyces levis* strain KS46 were found to possess antibacterial, antioxidant, antifungal, and antiproliferative activities. In this study, *Lysinibacillu*s sp. strain AS_1 crude extract exhibited a high number of bioactive compounds which explains the significant antibacterial and antitumor activities as compared to the other bacterial endophyte extracts. In general, the presence of bioactive compounds in all the crude extracts of bacterial endophytes especially those with antibacterial and antitumor activities should be investigated further as they have shown potential to inhibit pathogenic bacteria and cancer cells and this further necessitates their investigation and use for drug development. To our knowledge, this is the first study to report on the bacterial endophytes associated with *A. sessiliflora*, with antibacterial and antitumor activity against bacterial pathogens and cancer cells, respectively.

CONCLUSION

With more studies conducted on plant-associated bacterial endophytes, more evidence has demonstrated that endophytes provide significant benefits to various sectors, such as pharmaceuticals, industry, and agriculture. The current results in this study showed that *A. sessiliflora* does harbor bacterial endophytes, some with notable antibacterial and antitumor activities, which are attributed to their bioactive constituents, we thus recommend further studies to be conducted for the isolation of more diverse endophytes from *A. sessiliflora*. The antibacterial and antitumor activities of the crude extracts show the potential use of endophytic bacteria and thus should be considered as a novel source for the isolation and production of pure bioactive compounds. Moreover, further research needs to be conducted on the specific compounds responsible for the antibacterial and antitumor activities of the endophytes as this would be useful in developing new antimicrobial drugs and understanding the mechanism of action of these compounds on the studied cancer cells.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene sequences of this study are available from the corresponding author upon request. The isolated bacterial endophytes sequences in this study have been deposited in GenBank with the following accession numbers: MZ976846–MZ976850.

AUTHOR CONTRIBUTIONS

MM contributed to the experimental work. HW contributed to materials used for the antitumor studies. MS-D conceptualized the study, provided the materials used for the isolation, identification, and antimicrobial studies, and was the main supervisor of the project. All authors were involved in the writing of this manuscript and approved the submitted version.

FUNDING

This research was funded by the South African National Research Foundation Thuthuka grant (TTK210216586709). MM received the UJ Faculty of Science Bursary.

ACKNOWLEDGMENTS

We would like to acknowledge the South African National Research Foundation (NRF), Thuthuka, for the financial support for this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.870821/full#supplementary-material

REFERENCES

AAT Bioquest (2022). *Quest Graph*^{TM} *IC50 Calculator*. Sunnyvale, CA: AAT Bioquest.

Abdalla, M. A., Aro, A. O., Gado, D., Passari, A. K., Mishra, V. K., Singh, B. P., et al. (2020). Isolation of endophytic fungi from South African plants, and screening for their antimicrobial and extracellular enzymatic activities and presence of type I polyketide synthases. *S. Afr. J. Bot.* 134, 336–342. doi: 10.1016/j.sajb.2020.03.021

Abdel-Fatah, S. S., El-Batal, A. I., El-Sherbiny, G. M., Khalaf, M. A., and El-Sayed, A. S. (2021). Production, bioprocess optimization and γ-irradiation of *Penicillium polonicum*, as a new taxol producing endophyte from *Ginko biloba*. *Biotechnol. Rep.* 30:e00623. doi: 10.1016/j.btre.2021.e00623

Akpor, O. B., Akinwusi, O. D., and Ogunnusi, T. A. (2021). Production, characterization and pesticidal potential of *Bacillus* species metabolites against sugar ant (*Camponotus consobrinus*). *Heliyon* 7:08447. doi: 10.1016/j.heliyon.2021.e08447

Alotaibi, S. S., Alshoaibi, D., Alamari, H., Albogami, S., Khan, E., Alshanbari, A., et al. (2021). Potential significance of medicinal plants in forensic analysis: a review. *Saudi J Biol Sci.* 28, 3929–3935. doi: 10.1016/j.sjbs.2021.03.071

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

Alvin, A., Miller, K. I., and Neilan, B. A. (2014). Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiol. Res.* 169, 483–495. doi: 10.1016/j.micres.2013.12.009

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* 48, 5–16. doi: 10.1093/jac/48.suppl_1.5

Aswani, R., Jishma, P., and Radhakrishnan, E. K. (2020). "Endophytic bacteria from the medicinal plants and their potential applications," in *Microbial Endophytes*, eds A. Kumar and V. K. Singh (Sawston: Woodhead Publishing), 15–36. doi: 10.1016/B978-0-12-818734-0.00002-4

Attia, E. Z., Farouk, H. M., Abdelmohsen, U. R., and Mo'men, H. (2020). Antimicrobial and extracellular oxidative enzyme activities of endophytic fungi isolated from alfalfa (*Medicago sativa*) assisted by metabolic profiling. *S. Afr. J. Bot.* 134, 156–162. doi: 10.1016/j.sajb.2019.12.003

Ayukekbong, J. A., Ntemgwa, M., and Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries causes and control strategies. *Antimicrob. Resist. Infect. Control.* 6, 1–8. doi: 10.1186/s13756-017-0208-x

Baptista, P. V., McCusker, M. P., Carvalho, A., Ferreira, D. A., Mohan, N. M., Martins, M., et al. (2018). Nano-strategies to fight multidrug resistant bacteria "A Battle of the Titans". *Front. Microbiol.* 9:1441. doi: 10.3389/fmicb.2018.01441

Barupal, T., Meena, M., and Sharma, K. (2019). Inhibitory effects of leaf extract of *Lawsonia inermis* on *Curvularia lunata* and characterization of novel inhibitory compounds by GC–MS analysis. *Biotechnol. Rep.* 23:e00335. doi: 10.1016/j.btre.2019.e00335

Borriss, R., Wu, H., and Gao, X. (2019). "Secondary metabolites of the plant growth promoting model Rhizobacterium *Bacillus velezensis* FZB42 are involved in direct suppression of plant pathogens and in stimulation of plant-induced systemic resistance," in *Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms*, eds H. B. Singh, C. Keswani, M. S. Reddy, E. S. Royano, and C. García-Estrada (Singapore: Springer), 147–168.

Bostanci, M. T., Bulbul, A. S., Celik, I. S., Kocabas, Y. Z., Burhan, H., Bayat, R., et al. (2022). Investigation of antibacterial, antifungal, antibiofilm, antioxidant and anticancer properties of methanol extracts of *Salvia marashica* İlçim, Celep and Doğan and *Salvia caespitosa* Montbret and Aucher ex Benth plants with medicinal importance. *Chemosphere* 288:132602. doi: 10.1016/j.chemosphere.2021.132602

Chakraborty, B., Kumar, R. S., Almansour, A. I., Gunasekaran, P., and Nayaka, S. (2021). Bioprospection and secondary metabolites profiling of marine *Streptomyces levis* strain KS46. *Saudi. J. Biol. Sci.* 29, 667–679. doi: 10.1016/j.sjbs.2021.11.055

Cole, A. L., Hossain, S., Cole, A. M., and Phanstiel, O. IV (2016). Synthesis and bioevaluation of substituted chalcones, coumaranones and other flavonoids as anti-HIV agents. *Bioorganic Med. Chem.* 24, 2768–2776. doi: 10.1016/j.bmc.2016.04.045

Copeland, J. K., Yuan, L., Layeghifard, M., Wang, P. W., and Guttman, D. S. (2015). Seasonal community succession of the phyllosphere microbiome. *Mol. Plant Microbe Interact.* 28, 274–285. doi: 10.1094/MPMI-10-14-0331-FI

Ding, T., and Melcher, U. (2016). Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. *PLoS Pathog.* 11:e0150895. doi: 10.1371/journal.pone.0150895

Duhan, P., Bansal, P., and Rani, S. (2020). Isolation, identification and characterization of endophytic bacteria from medicinal plant *Tinospora cordifolia*. *S. Afr. J. Bot.* 134, 43–49. doi: 10.1016/j.sajb.2020.01.047

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

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform.* 5:113. doi: 10.1186/1471-2105-5-113

Egamberdieva, D., Wirth, S., Behrendt, U., Ahmad, P., and Berg, G. (2017). Antimicrobial activity of medicinal plants correlates with the proportion of antagonistic endophytes. *Front. Microbiol.* 8:199. doi: 10.3389/fmicb.2017.00199

Ekalu, A. (2021). Medicinal uses, phytochemistry, and pharmacological activities of *Mitracarpus* species (Rubiaceae): a review. *Sci. Afr.* 11:e00692. doi: 10.1016/j.sciaf.2020.e00692

El-Naggar, N. E. A., El-Bindary, A. A. A., Abdel-Mogib, M., and Nour, N. S. (2017). *In vitro* activity, extraction, separation and structure elucidation of antibiotic produced by *Streptomyces anulatus* NEAE-94 active against multidrug-resistant *Staphylococcus aureus*. *Biotechnol. Equip.* 31, 418–430. doi: 10.1080/13102818.2016.1276412

Fabry, W., Okemo, P. O., and Ansorg, R. (1998). Antibacterial activity of East African medicinal plants. *J. Ethnopharmacol.* 60, 79–84. doi: 10.1016/S0378-8741(97)00128-1

Farag, S. M., Essa, E. E., Alharbi, S. A., Alfarraj, S., and El-Hassan, G. M. A. (2021). Agro-waste derived compounds (flax and black seed peels): toxicological effect against the West Nile virus vector, *Culex pipiens* L. with special reference to GC-MS analysis. *Saudi. J. Biol. Sci.* 28, 5261–5267. doi: 10.1016/j.sjbs.2021.05.038

Farhat, H., Urooj, F., Tariq, A., Sultana, V., Ansari, M., Ahmad, V. U., et al. (2019). Evaluation of antimicrobial potential of endophytic fungi associated with healthy plants and characterization of compounds produced by endophytic *Cephalosporium* and *Fusarium solani*. *Biocatal. Agric. Biotechnol.* 18:101043. doi: 10.1016/j.bcab.2019.101043

Flores-Paredes, W., Luque, N., Albornoz, R., Rojas, N., Espinoza, M., Pons, M. J., et al. (2021). Evolution of antimicrobial resistance levels of eskape microorganisms in a peruvian iv-level hospital. *J. Infect. Chemother.* 53:449. doi: 10.3947/ic.2021.0015

Gagana, S. L., Kumaraswamy, B. E., and Shivanna, M. B. (2020). Diversity, antibacterial and antioxidant activities of the fungal endophytes associated with *Schleichera oleosa* (Lour.) Merr. *S. Afr. J. Bot.* 134, 369–381. doi: 10.1016/j.sajb.2020.06.012

Gasa, N. (2015). *Antibiofilm Activity of South African plant Extracts Against Mycobacterium spp. and their Mechanism of action Using Mycothiol reductase*. Pretoria: University of Pretoria.

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

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

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *In Nucleic Acids Symp. Ser.* 41, 95–98.

Ham, J. S., Lee, S. G., Jeong, S. G., Oh, M. H., Kim, D. H., Lee, T., et al. (2010). Powerful usage of phylogenetically diverse *Staphylococcus aureus* control strains for detecting multidrug resistance genes in transcriptomics studies. *Mol. Cells.* 30, 71–76. doi: 10.1007/s10059-010-0090-3

Hernández, A. G. C., Ortiz, V. G., Gómez, J. L. A., López, M. ÁR., Morales, J. A. R., Macías, A. F., et al. (2021). Detection of Bacillus cereus sensu lato isolates posing potential health risks in Mexican chili powder. *Microorganisms* 9:2226. doi: 10.3390/microorganisms9112226

Higashide, M., Kuroda, M., Omura, C. T. N., Kumano, M., Ohkawa, S., Ichimura, S., et al. (2008). Methicillin-resistant *Staphylococcus saprophyticus* isolates carrying staphylococcal cassette chromosome mec have emerged in urogenital tract infections. *Antimicrob. Agents Chemother.* 52, 2061–2068. doi: 10.1128/AAC.01150-07

Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., et al. (2014). Drug resistance in cancer: an overview. *Cancers* 6, 1769–1792. doi: 10.3390/cancers6031769

Hsou, A. B., Trigui, M., Mansour, R. B., Jarraya, R. M., Damak, M., and Jaoua, S. (2011). Chemical composition, cytotoxicity effect and antimicrobial activity of *Ceratonia siliqua* essential oil with preservative effects against listeria inoculated in minced beef meat. *Int. J. Food Microbiol.* 148, 66–72. doi: 10.1016/j.ijfoodmicro.2011.04.028

Huo, L., Hug, J. J., Fu, C., Bian, X., Zhang, Y., and Müller, R. (2019). Heterologous expression of bacterial natural product biosynthetic pathways. *Nat. Prod. Rep.* 36, 1412–1436. doi: 10.1039/c8np00091c

Jose, V. M. L. F., Ana, F. F. U., Sissi, M. F., and Vania, M. M. M. (2002). Antibacterial activity of extracts of six macroalgae from the northeastern brazilian coast. *Braz. J. Microbiol.* 33, 311–313. doi: 10.1590/S1517-83822002000400006

Jukes, T. H., and Cantor, C. R. (1969). "Evolution of protein molecules," in *Mammalian Protein Metabolism*, ed. H. N. Munro (New York, NY: Academic Press), 21–132. doi: 10.1016/B978-1-4832-3211-9.50009-7

Katembo, S. P., Mukatakamba, G. K., Charles, V., and Ngulusansi, A. (2021). Clinical trial of *Alectra sessiliflora* (VAHL.) Kunze powder in the treatment of sheep's foot rot in Lubero territory (North-Kivu/DR Congo). *J. Anim. Plant. Sci.* 48, 8722–8728. doi: 10.35759/JAnmPlSci.v48-3.3

Katiyar, C., Gupta, A., Kanjilal, S., and Katiyar, S. (2012). Drug discovery from plant sources: an integrated approach. *Ayu* 33:10. doi: 10.4103/0974-8520.100295

Kembel, S. W., O'Connor, T. K., Arnold, H. K., Hubbell, S. P., Wright, S. J., and Green, J. L. (2014). Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *Proc. Natl. Acad. Sci.* 111, 13715–13720. doi: 10.1073/pnas.1216057111

Khaskheli, M. A., Wu, L., Chen, G., Chen, L., Hussain, S., Song, D., et al. (2020). Isolation and characterization of root-associated bacterial endophytes and their biocontrol potential against major fungal phytopathogens of rice (*Oryza sativa* L.). *Pathogens* 9:172. doi: 10.3390/pathogens9030172

Kitahara, K., and Miyazaki, K. (2013). Revisiting bacterial phylogeny: natural and experimental evidence for horizontal gene transfer of 16S rRNA. *Mob. Gent. Elements* 3:e24210. doi: 10.4161/mge.24210

Kuete, V., and Efferth, T. (2015). African flora has the potential to fight multidrug resistance of cancer. *Biomed. Res. Int.* 2015:914813. doi: 10.1155/2015/914813

Kumar, A., and Mishra, A. K. (2018). Biological importance of phenol derivatives as potent bioactive compound: a review. *Lett. Org. Chem.* 15, 251–264. doi: 10.2174/1570178614666171130155539

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

Kusari, S., Pandey, S. P., and Spiteller, M. (2013). Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. *Phytochemistry* 91, 81–87. doi: 10.1016/j.phytochem.2012.07.021

Li, H., Hu, J., Wu, S., Wang, L., Cao, X., Zhang, X., et al. (2016). Auranofin-mediated inhibition of PI3K /AKT/mTOR axis and anticancer activity in non-small cell lung cancer cells. *Oncotarget* 7:3548. doi: 10.18632/oncotarget.6516

Li, L., Gao, L., Liu, Y., Fang, B., Huang, Y., Mohamad, O. A., et al. (2021). Diversity of cultivable endophytic bacteria associated with halophytes in Xinjiang of China and their plant beneficial traits. *J. Arid Land.* 13, 790–800. doi: 10.1007/s40333-021-0016-2

Lodyato, V. I., Yurkova, I. L., Sorokin, V. L., Shadyro, O. I., Dolgopalets, V. I., and Kisel, M. A. (2003). Synthesis and properties of 11-(3, 5-Di-tert-butyl-2-hydroxyphenylcarbamoyl) undecanoic acid, a new amphiphilic antioxidant. *Bioorg. Med. Chem. Lett.* 13, 1179–1182. doi: 10.1016/S0960-894X(03)00041-6

Ma, Q., Liu, Y., Zhan, R., and Chen, Y. (2016). A new isoflavanone from the trunk of *Horsfieldia pandurifolia*. *Nat. Prod. Res.* 30, 131–137. doi: 10.1080/14786419.2015.1043554

Makhafola, T. J., Elgorashi, E. E., McGaw, L. J., Awouafack, M. D., Verschaeve, L., and Eloff, J. N. (2017). Isolation and characterization of the compounds responsible for the antimutagenic activity of *Combretum microphyllum* (Combretaceae) leaf extracts. *BMC Complement. Altern. Med.* 17:446. doi: 10.1186/s12906-017-1935-5

Makuwa, S. C., and Serepa-Dlamini, M. H. (2021). The antibacterial activity of crude extracts of secondary metabolites from bacterial endophytes associated with *Dicoma anomala*. *Int. J. Microbiol.* 2021:8812043. doi: 10.1155/2021/8812043

Maloney, K. N., MacMillan, J. B., Kauffman, C. A., Jensen, P. R., DiPasquale, A. G., Rheingold, A. L., et al. (2009). Lodopyridone, a structurally unprecedented alkaloid from a marine actinomycete. *Org. Lett.* 11, 5422–5424. doi: 10.1021/ol901997k

Mariita, R., Ogol, C. K. P. O., Oguge, N., and Okemo, P. (2010). Antitubercular and phytochemical investigation of methanol extracts of medicinal plants used by the samburu community in Kenya. *Trop. J. Pharm. Res.* 9, 379–385. doi: 10.4314/tjpr.v9i4.58935

Martínez-Hidalgo, P., Flores-Félix, J. D., Sánchez-Juanes, F., Rivas, R., Mateos, P. F., Santa Regina, I., et al. (2021). Identification of canola roots endophytic bacteria and analysis of their potential as biofertilizers for canola crops with special emphasis on sporulating bacteria. *J. Agron.* 11:1796. doi: 10.3390/agronomy11091796

McCauley, J., Zivanovic, A., and Skropeta, D. (2013). "Bioassays for anticancer activities," in *Metabolomics Tools for Natural Product Discovery*, eds U. Roessner and D. Dias (Totowa, NJ: Humana Press), 191–205.

Mehanni, M. M., and Safwat, M. S. A. (2009). Endophytes of medicinal plants. *Acta Hortic.* 854, 31–39. doi: 10.17660/ActaHortic.2010.854.3

Morawetz, J. J., and Wolfe, A. D. (2011). Taxonomic revision of the *Alectra sessiliflora* complex (Orobanchaceae). *Syst. Bot.* 36, 141–152. doi: 10.1600/036364411X553234

Mukherjee, K., Tribedi, P., Mukhopadhyay, B., and Sil, A. K. (2013). Antibacterial activity of long-chain fatty alcohols against mycobacteria. *FEMS Microbiol. Lett.* 338, 177–183. doi: 10.1111/1574-6968.12043

Naureen, Z., Rehman, N. U., Hussain, H., Hussain, J., Gilani, S. A., Al Housni, S. K., et al. (2017). Exploring the potentials of *Lysinibacillus sphaericus* ZA9 for plant growth promotion and biocontrol activities against phytopathogenic fungi. *Front. Microbiol.* 8:1477. doi: 10.3389/fmicb.2017.01477

Ogbole, O., and Ajaiyeoba, E. (2010). Traditional management of tuberculosis in Ogun State of Nigeria: the practice and ethnobotanical survey. *Afr. J. Tradit. Complement. Altern. Med.* 7, 79–84. doi: 10.4314/ajtcam.v7i1.57270

Oosthuizen, C. B., Gasa, N., Hamilton, C. J., and Lall, N. (2019). Inhibition of mycothione disulphide reductase and mycobacterial biofilm by selected South African plants. *S. Afr. J. Bot.* 120, 291–297. doi: 10.1016/j.sajb.2018.09.015

Palanichamy, P., Krishnamoorthy, G., Kannan, S., and Marudhamuthu, M. (2018). Bioactive potential of secondary metabolites derived from medicinal plant endophytes. *Egypt. J. Basic. Appl. Sci.* 5, 303–312. doi: 10.1016/j.ejbas.2018.07.002

Panigrahi, S., and Rath, C. C. (2021). *In vitro* characterization of antimicrobial activity of an endophytic bacterium *Enterobacter cloaca* (MG001451) isolated from *Ocimum sanctum*. *S. Afr. J. Bot.* 143, 90–96. doi: 10.1016/j.sajb.2021.07.044

Petrini, O., Sieber, T. N., Toti, L., and Viret, O. (1993). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Nat. Toxins.* 1, 185–196. doi: 10.1002/nt.2620010306

Pintać, D., Majkić, T., Torović, L., Orčić, D., Beara, I., Simin, N., et al. (2018). Solvent selection for efficient extraction of bioactive compounds from grape pomace. *Ind. Crops. Prod.* 111, 379–390. doi: 10.1016/j.indcrop.2017.10.038

Ponsankar, A., Vasantha-Srinivasan, P., Senthil-Nathan, S., Thanigaivel, A., Edwin, E. S., Selin-Rani, S., et al. (2016). Target and non-target toxicity of botanical insecticide derived from *Couroupita guianensis* L. flower against generalist herbivore, *Spodoptera litura* Fab. and an earthworm, *Eisenia foetida* Savigny. *Ecotoxicol. Environ. Saf.* 133, 260–270. doi: 10.1016/j.ecoenv.2016.06.043

Prakash, S., Elavarasan, N., Subashini, K., Kanaga, S., Dhandapani, R., Sivanandam, M., et al. (2020). Isolation of hesperetin-A flavonoid from *Cordia sebestena* flower extract through antioxidant assay guided method and its antibacterial, anticancer effect on cervical cancer *via in vitro* and *in silico* molecular docking studies. *J. Mol. Struct.* 1207:127751. doi: 10.1016/j.molstruc.2020.127751

Ramasubburayan, R., Sumathi, S., Bercy, D. M., Immanuel, G., and Palavesam, A. (2015). Antimicrobial, antioxidant and anticancer activities of mangrove associated bacterium *Bacillus subtilis* subsp. subtilis RG. *Biocatal. Agric. Biotechnol.* 4, 158–165. doi: 10.1016/j.bcab.2015.01.004

Rambaut, A. (2018). *FigTree v.1.4.4*. Available online at: https://github.com/rambaut/figtree/releases (accessed March 18, 2022).

Rao, M. R. K., and Lakshmi, N. V. (2018). Preliminary phytochemical and GC MS analysis of different extracts of *Sphaeranthus indicus* leaves. *Indo Am. J. Pharm.* 5, 1511–1520. doi: 10.5281/zenodo.1204485.svg

Rastogi, G., Sbodio, A., Tech, J. J., Suslow, T. V., Coaker, G. L., and Leveau, J. H. (2012). Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J.* 6, 1812–1822. doi: 10.1038/ismej.2012.32

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., et al. (2015). The global threat of antimicrobial resistance: science for intervention. *New. Microbes. New. Infect.* 6, 22–29. doi: 10.1016/j.nmni.2015.02.007

Sciarretta, K., Røttingen, J. A., Opalska, A., Van Hengel, A. J., and Larsen, J. (2016). Economic incentives for antibacterial drug development: literature review and considerations from the transatlantic task force on antimicrobial resistance. *Clin. Infect. Dis.* 63, 1470–1474. doi: 10.1093/cid/ciw593

Sebola, T. E., Uche-Okereafor, N. C., Mekuto, L., Makatini, M. M., Green, E., and Mavumengwana, V. (2020). Antibacterial and anticancer activity and untargeted secondary metabolite profiling of crude bacterial endophyte extracts from *Crinum macowanii* baker leaves. *Int. J. Microbiol.* 2020:8839490. doi: 10.1155/2020/8839490

Shabanamol, S., Thampi, M., Sajana, P., Varghese, S., Karthika, S., George, T. K., et al. (2021). Characterization of the major antifungal extrolite from rice endophyte *Lysinibacillus sphaericus* against *Rhizoctonia solani*. *Arch. Microbiol.* 203, 2605–2613. doi: 10.1007/s00203-021-02229-2

Shao, Y., Wang, X. Y., Qiu, X., Niu, L. L., and Ma, Z. L. (2021). Isolation and purification of a new *Bacillus subtilis* Strain from deer dung with anti-microbial and anti-cancer activities. *Curr. Med. Sci.* 41, 832–840. doi: 10.1007/s11596-021-2383-5

Singh, A., Kumar, J., Sharma, V. K., Singh, D. K., Kumari, P., Nishad, J. H., et al. (2021). Phytochemical analysis and antimicrobial activity of an endophytic *Fusarium proliferatum* (ACQR8), isolated from a folk medicinal plant *Cissus quadrangularis* L. *S. Afr. J. Bot.* 140, 87–94. doi: 10.1016/j.sajb.2021.03.004

Strobykina, I. Y., Nemtarev, A. V., Garifullin, B. F., Voloshina, A. D., Sapunova, A. S., and Kataev, V. E. (2019). Synthesis and biological activity of alkane-1, 1-diylbis (phosphonates) of diterpenoid isosteviol. *Russ. J. Org. Chem.* 55, 17–24. doi: 10.1134/S1070428019010044

Sivakumar, N., Sathishkumar, R., Selvakumar, G., Shyamkumar, R., and Arjunekumar, K. (2020). "Phyllospheric microbiomes: diversity, ecological significance, and biotechnological applications," in *Plant Microbiomes for Sustainable Agriculture*, eds A. Yadav, J. Singh, A. Rastegari, and N. Yadav (Cham: Springer), 113–172. doi: 10.1007/978-3-030-38453-1_5

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., and Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol.* 4:vey016. doi: 10.1093/ve/vey016

Sun, H., He, Y., Xiao, Q., Ye, R., and Tian, Y. (2013). Isolation, characterization, and antimicrobial activity of endophytic bacteria from *Polygonum cuspidatum*. *Afr. J. Microbiol. Res.* 7, 1496–1504. doi: 10.5897/AJMR12.899

Tapfuma, K. I., Nchabeleng, E. K., Adebo, O. A., Hussan, R., Williams, R. D., Ravuluvulu, A. B., et al. (2020). Antibacterial activity and gas chromatography mass spectrometry (GC–MS)-based metabolite profiles of *Celtis africana* and its endophytic extracts. *Ind. Crops. Prod.* 157:112933. doi: 10.1016/j.indcrop.2020.112933

Uche-Okereafor, N., Sebola, T., Tapfuma, K., Mekuto, L., Green, E., and Mavumengwana, V. (2019). Antibacterial activities of crude secondary metabolite extracts from *Pantoea* species obtained from the stem of *Solanum mauritianum* and their effects on two cancer cell lines. *Int. J. Environ. Res. Public Health* 16:602. doi: 10.3390/ijerph16040602

Vahdati, S. N., Lashkari, A., Navasatli, S. A., Ardestani, S. K., and Safavi, M. (2022). Butylated hydroxyl-toluene, 2, 4-Di-tert-butylphenol, and phytol of Chlorella sp. protect the PC12 cell line against H2O2-induced neurotoxicity. *Biomed. Pharmacother.* 145:112415. doi: 10.1016/j.biopha.2021.112415

Van Vuuren, S. F. (2008). Antimicrobial activity of South African medicinal plants. *J. Ethnopharmacol.* 119, 462–472. doi: 10.1016/j.jep.2008.05.038

Vergara, M., Olivares, A., and Altamirano, C. (2015). Antiproliferative evaluation of tall-oil docosanol and tetracosanol over CHO-K1 and human melanoma cells. *Electron. J. Biotechnol.* 18, 291–294. doi: 10.1016/j.ejbt.2015.05.004

Wagenlehner, F. M. E., Heisig, P., Irtenkauf, C., Notka, F., Decker, J., Lehn, N., et al. (2003). Clinically significant borderline resistance of sequential clinical isolates of *Klebsiella pneumoniae*. *Int. J. Antimicrob. Agents* 22, 367–373. doi: 10.1016/S0924-8579(03)00126-2

World Health Organization [WHO] (2021). *Antimicrobial Resistance*. Geneva: World Health Organization.

Yeates, C., Gillings, M. R., Davison, A. D., Altavilla, N., and Veal, D. A. (1997). PCR amplification of crude microbial DNA extracted from soil. *Lett. Appl. Microbiol.* 25, 303–307. doi: 10.1046/j.1472-765X.1997.00232.x

Zhang, A., He, X., Meng, Y., Guo, L., Long, M., Yu, H., et al. (2016). Antibiotic and disinfectant resistance of *Escherichia coli* isolated from retail meats in Sichuan, China. *Microb. Drug Resist.* 22, 80–87. doi: 10.1089/mdr.2015.0061

Zhu, L., Guo, J., Sun, Y., Wang, S., and Zhou, C. (2021). Acetic acid-producing endophyte *Lysinibacillus fusiformis* orchestrates jasmonic acid signaling and contributes to repression of cadmium uptake in tomato plants. *Front. Plant Sci.* 12:1041. doi: 10.3389/fpls.2021.670216

Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczmarski, D., Higley, P., et al. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.* 68, 2198–2208. doi: 10.1128/AEM.68.5.2198-2208.2002

Conflict of Interest: HW was employed by Mintek.

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

Publisher's Note: 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.

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 23 September 2022
DOI 10.3389/fmicb.2022.872034

OPEN ACCESS

EDITED BY
Khondoker M. G. Dastogeer,
Bangladesh Agricultural University,
Bangladesh

REVIEWED BY
Laith Khalil Tawfeeq Al-Ani,
Universiti Sains Malaysia,
Malaysia
Yinwu Shi,
Xinjiang Academy of Agricultural Sciences,
China
Bartholomew Saanu Adeleke,
Ondo State University of Science and Technology, Nigeria

*CORRESPONDENCE
Zhonghua Liu
larkin-liu@163.com

SPECIALTY SECTION
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

RECEIVED 09 February 2022
ACCEPTED 07 July 2022
PUBLISHED 23 September 2022

CITATION
Lin H, Liu C, Peng Z, Tan B, Wang K and Liu Z (2022) Distribution pattern of endophytic bacteria and fungi in tea plants. *Front. Microbiol.* 13:872034. doi: 10.3389/fmicb.2022.872034

Distribution pattern of endophytic bacteria and fungi in tea plants

Haiyan Lin[1,2], Changwei Liu[1,2], Zhong Peng[1,2], Bin Tan[1,2], Kunbo Wang[1,2,3] and Zhonghua Liu[1,2,3]*

[1]Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha, China, [2]National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha, China, [3]Co-Innovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha, China

Endophytes are critical for plant growth and health. Tea is an economically important crop in China. However, little is known about the distribution pattern and potential functions of endophytic communities in tea trees. In this study, two genotypes (BXZ and MF) cultivated under the same conditions were selected, and endophytic bacteria and fungi were analyzed through 16S rRNA and ITS high-throughput sequencing technologies, respectively. For endophytic bacteria, root tissues harbored the most diverse endophytes, followed by stems and old leaves, and new leaves possessed the lowest diversity. In contrast, old leave tissues harbored more diverse endophytic fungi than did root and stem tissues. Most of the dominant endophytes showed obvious cultivar and tissue preferences. Tissue type played a more important role in shaping community structure than did cultivar. Nevertheless, some endophytic bacterial groups, which mainly affiliated to *Chryseobacterium, Sphingomonas, Rhizobium, Morganella, Methylobacterium* and *Comamonadaceae*, could parasitize different tissues, and the average relative abundance of endophytic bacteria was as high as 72.57%. Some endophytic fungal populations, such as *Colletotrichum, Uwebraunia, Cladosporium, and Devriesia,* could also parasitize tea, and the relative abundance accounted for approximately 25.70–97.26%. The cooperative relationship between endophytic bacteria and fungi in the new leaves was stronger than that in the old leaves, which can better participate in the metabolism of tea material.

KEYWORDS

tea, leaf, endophytes, bacteria, fungi

Introduction

Endophytes are able to live inside plant tissues without inducing any apparent symptoms in their hosts (Borer et al., 2013). Numerous studies indicated that endophytes play important roles in plant disease control, secondary metabolites synthesis, plant growth regulation, and environmental resistance (Oukala et al., 2021). For this reason, endophytes have been receiving increasing attention from scientists since the latter part of the twentieth century. Fungi and bacteria are the most common microbes that exist as endophytes. It is

reported that more than half of the isolated endophytes had different kinds of plant growth-promoting traits (Spaepen et al., 2009; Bulgarelli et al., 2012). These endophytes can promote plant growth by accelerating the availability of mineral nutrients, helping in the production of phytohormones, siderophores, and enzymes, and by activating systemic resistance against insect pests and pathogens in plants (Santoyo et al., 2016; Kushwaha et al., 2020). They can also regulate plant growth through improving nitrogen fixation, phosphate solubilization, siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and indole-3-acetic acid (IAA) synthesis Santoyo. Meanwhile, endophytes can induce plant defenses through jasmonic acid, salicylic acid and ethylene pathways (Santoyo et al., 2016). Furthermore, it was also revealed that bacterial endophytes can improve salt tolerance by adjusting osmotic balance, ion homeostasis, phytohormone production, extracellular molecules and exopolysaccharides, which can be a more environmentally friendly and cost-effective solution to curtail the harmful effect of salinity on crop growth and yield (Ali et al., 2014; Singh et al., 2017). Likewise, some fungal endophytes, such as *Leptontidium*, *Phialocephala* and *Beauveria*, also affect plant growth and plant responses to pathogens, herbivores, and environmental changes (Hong et al., 2005; Porras-Alfaro and Bayman, 2011; Huang et al., 2020). All these studies suggested that endophytes would play important roles in helping plant growth and alleviating environmental stress on plant, but the distribution and interaction patterns between many host plants and endophytes remains unknown.

Tea (*Camellia sinensis*) is a perennial woody plant and an economically important crop in China (Li et al., 2022). The secondary metabolites of tea, such as tea polyphenols, theophylline, organic acids, can excite and relieve fatigue, detoxify and quench thirst, diuresis and improve eyesight, supplement nutrition, etc. (Bhuyan et al., 2013; Shan et al., 2018; Rothenberg and Zhang, 2019). Recently, a few researches has shown that there were also some endophytes colonized in tea plant, and these endophytes may also play important roles in helping plant growth and improving tea quality. For example, Yan et al. (2018) isolated 274 bacterial isolates from two tea cultivars, these endophytic bacterial mainly affiliated to Proteobacteria, Firmicutes and Bacteroidetes. Some of the endophytic bacteria appeared plant-growth promoting (PGP) traits, such as nitrogen fixation, P-solubilization, siderophore, IAA production or ACC deaminase. Sun et al. (2019) isolated an endophytic bacterial strain (identified as *Luteibacter* spp.) with strong biocatalytic activity for converting both glutamine and ethylamine to theanine. Likewise, endophytic fungus *Colletotrichum gloeosporioides* isolated from healthy tea plant tissues also showed a strong inhibitory activity on tea plant pathogens of *Pestalotiopsis theae* and *Colletotrichum camelliae*, and the inhibitory mechanism may attribute to the fungus' high efficient chitinase and protease (Rabha et al., 2014). However, these few studies mainly focused on the functions of endophytic strains based on traditional isolation, which would extremely limit our comprehensive understanding of the distribution pattern of endophytes in tea plant.

In this study, two tea plant varieties under the same environmental conditions and planting management measures were selected as materials, and the characteristics of endophytic populations in roots, stems, old leaves and new leaves were systematically compared and analyzed through 16S and high-throughput sequencing technology. The purpose is to explore the population distribution characteristics of the quality endophytes, the functional activity of dominant populations and their interaction with environmental microorganisms and to clarify the characteristics of the distribution of the endophytic bacterial population in tea plants. Therefore, exploring the population distribution characteristics and functions of tea tree endophytes can lay a foundation for the evaluation of the influence of endophytes on the formation of tea quality and provide a basis for further research and development of tea tree endophytic resources.

Materials and methods

Sampling of tea plant tissue

The experiment was carried out in the Chang'an station of Hunan Agricultural University. The type of soil was red soil. The physical and chemical properties of the soil were as follows: organic carbon 0.95 g·kg^{-1}, total nitrogen 0.97 g·kg^{-1}, and total phosphorus 0.35 g·kg^{-1}. Total potassium 1.52 g kg^{-1}, available potassium 92 mg kg^{-1}, available phosphorus 26.6 mg kg^{-1}, alkaline hydrolysis nitrogen 112 mg kg^{-1}, available iron 138 mg kg^{-1}, available manganese 15.4 mg kg^{-1}, available copper 1.14 mg kg^{-1}, available zinc 1.57 mg kg^{-1}, pH 3.87. Two typical cultivars of tea plants (BXZ and MF) with 5 years planting history under the same management practices (such as fertilization) were selected in 2017. For each cultivar., 15 healthy tea plants with the uniform growth were selected, and for each plant, the third and sixth leaves of each plant were sampled as new and old leaves, respectively. Roots and stems were simultaneously collected and washed off soil particles with water. All the tissues were surface-sterilized by successively submersing in 75% ethyl alcohol for 3 min, 1.2% sodium hypochlorite for 3 min, and then 75% ethyl alcohol for 1 min, followed by five rinses with sterilized deionized water. The final rinse water was used to verify the sterility of root surfaces by both PCR amplification of 16S rRNA gene and plate cultivation method (Seghers et al., 2004). The verified tissue samples were immediately frozen in liquid nitrogen and stored at-80°C for molecular analysis.

DNA extraction and endophytic bacterial sequencing

A Fast DNA Spin Kit (Fast DNA Spin Kit for Soil, MP) was used for DNA extraction from tea plants. The concentration and purity of DNA were determined using a NanodropND-1,000 (NanoDrop Technologies, Delaware, United States). After DNA concentration was diluted to 30 ng uL-1 with nuclease-free water,

all the samples were performed first PCR to amplify bacterial 16S rRNA (V5-V7) with 799F/1492R primers (Bulgarelli et al., 2012). The reaction solution consisted 1 μl (10 μmol L − 1) of each forward and reverse primer, 2 μl of template DNA, 25 μl 2 × Power Taq Master Mix (Tiangen, Beijing, China), and 21 μLnuclease-free water. PCR reaction was performed with a Mastercycler pro gradient PCR Cycler (Eppendorf AG, Hamburg, German) as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s,72°C for 45 s, and with a final extension of 72°C for 6 min. The amplification of genes was checked using gel electrophoresis (1.5% agarose) and the band of approximately 700 bp was excised and purified using an AxyPrep DNA kit (Axygen, California, United States). The purified PCR product from the first PCR reaction was used as templates for the second PCR amplification (using same conditions) with the primer set 799F/1193R (Lundberg et al., 2012) that comprised an eight-base barcoded at the 5′ end. For the endophytic fungi, primer set of ITS1F/ITS2R was used to amplify ITS genes according to Arnold et al. (2003). The PCR amplicons were gel purified as described above and quantified using a NanodropND-1,000 spectrophotometer. These purified products were pooled in equimolar aliquots and sent for paired-end sequencing on an Illumina Miseq PE300 platform (Shanghai MajorbioBioPharm Technology Co., Ltd., Shanghai, China).

Bioinformatics and statistical analysis

The obtained raw 16S rRNA gene sequences were quality filtered, assembled, de-multiplexed and assigned to individual samples using Quantitative Insights into Microbial Ecology (QIIME) pipeline (version 1.9.0). Briefly, sequences were discarded if they contained any ambiguous base, had more than two mismatches to the primers, one mismatch to the barcode sequence, or a minimum sequence length of 200 bp or average quality score of 20. After filtering and chimera removal, the operational taxonomic units (OTUs) picking was performed using USEARCH at 97% sequence identity. The singletons were filtered, and subsequently, taxonomic annotation of the representative 16S rRNA and ITS sequences was assigned based on the Greengenes database (version 13.5) and unite database (version 7.0) using RDP classifier Bayes algorithm with a confidence threshold of 70% (Goyer et al., 2022). After removing the sequences related to chloroplast or mitochondria, number of OTUs, Shannon, and Chao1 indexes were calculated based on the OTU table that was unified to 9,146 and 36,037 sequences (the minimum number of sample sequences) per sample for 16S rRNA and ITS genes, respectively. Hierarchical cluster diagram and principal coordinates analysis (PCoA) were performed to display the similarities or differences in the community compositions between samples. In order to further identify endophytic bacterial/fungal taxa detected in tea trees that are omnipresent across all cultivar and tissue types or locally resident in specific cultivar or tissue types, a Venn diagram was used to split the overall community into two general categories: "common" - OTUs detected in all samples, "specific"—OTUs found only in the specific tissue samples from a specific cultivar. One-way analysis of variance (ANOVA) was used to detect the significant difference between the properties of soil and plant with SPSS software (SPSS Inc., Chicago, United States). The Duncan's multiple range was used for detecting significant difference among treatments, and the statistical significance was determined at $p < 0.05$ and highly significant difference at $p < 0.01$.

The correlation between the endophyte populations in the sample was analyzed on the online tool of Majorbio Cloud Platform with default parameters.[1]

Results

Community diversity of endophytic bacteria and fungi in tea trees

After quality control and chloroplast or mitochondrial sequences removal, a total of 530,608 bacterial sequences and 2,442,189 fungal sequences were obtained from 24 samples. These sequences were assigned to 636 bacterial OTUs and 718 fungal OTUs, respectively. In comparison, the OTU numbers and Chao index of endophytic bacteria in roots and stems were significantly higher than that in leaves for both BXZ and MF ($p < 0.05$), while no significant differences ($p > 0.05$) were detected between roots and stems, and old and new leaves. But for the shannon index, although similar pattern were observed, only MF-root and BXZ-stem samples appeared significantly higher values than new leaves, while other tissues showed no significant differences (Figure 1A).However, no significant differences in bacterial diversity were detected between two cultivars for specific tissue (Figure 1A). Unlike bacteria, for the endophytic fungi, it was detected that the new leaves generally showed the lowest OTU numbers and Shannon index for both BXZ and MF, while similar fungal diversity was detected in root, stem and old leaves, except for the old leaves showed higher OTU numbers (Figure 1B). Furthermore, significant differences in fungal diversity were also detected between two cultivars for specific tissues, e.g., OTU numbers in stem, Chao1 in root ($p < 0.05$).

Community structures of endophytic bacteria and fungi in tea trees

From the PCoA plots (Figure 2), it was detected that although cultivar also induced some changes in their community structure for specific tissue types, all the samples separately clustered according to tissue types, suggesting that the tissue type may play a more important role in shaping endophytic bacterial community structure than did cultivar (Figure 2A). Among the four tissue types, the bacterial community in root and stem samples clustered closer while that in old and new leaves was

1 https://cloud.majorbio.com/page/tools/

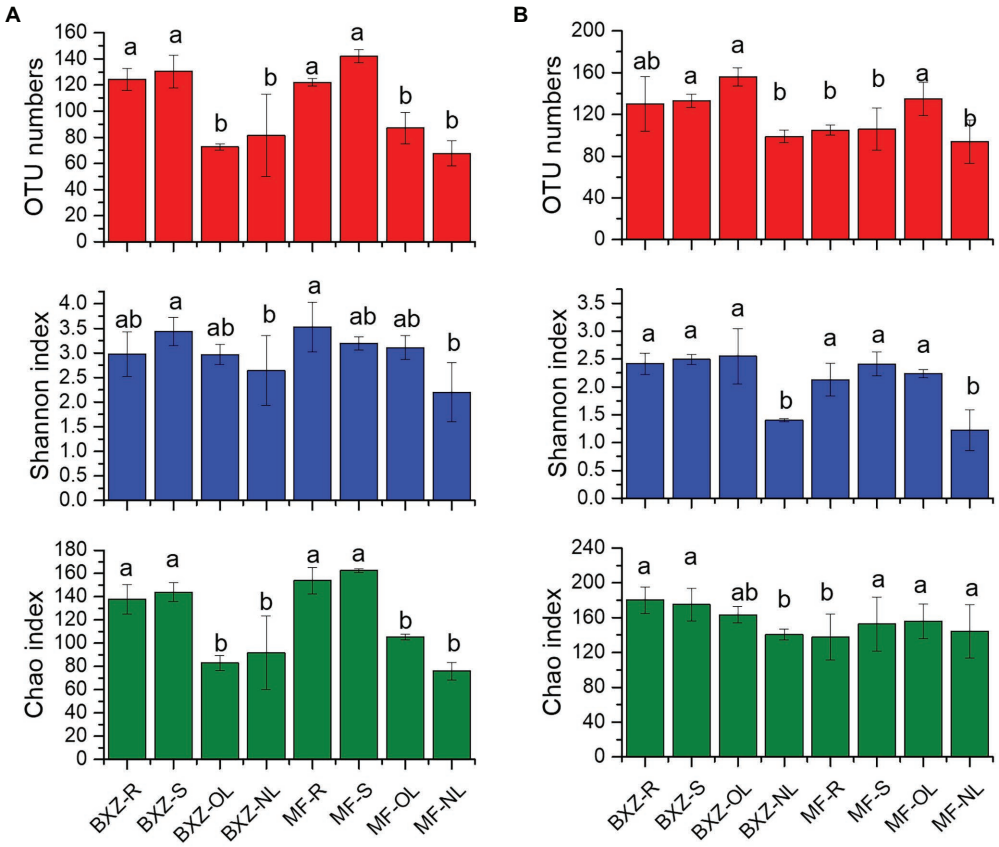


FIGURE 1
Diversity of endophytic bacteria **(A)** and fungi **(B)** in tea trees. BXZ_R, BXZ_S, BXZ_OL 556 and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar, while MF_R, 557 MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar. Different letter indicates (a) BXZ tea tree α diversity and (b) MF tea tree α diversity.

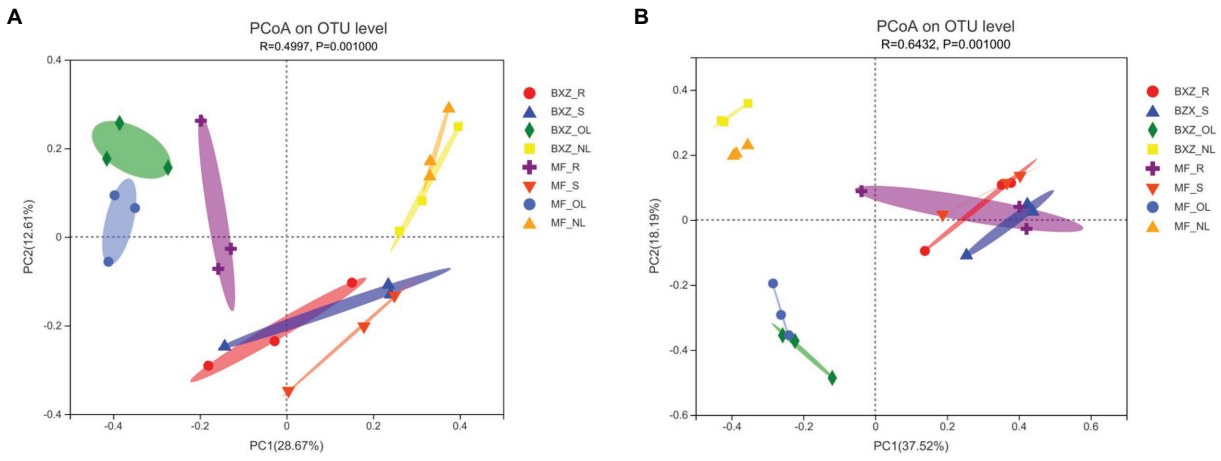


FIGURE 2
PCoA analysis of community structures of endophytic bacteria **(A)** and fungi **(B)** in tea trees. BXZ_R, BXZ_S, BXZ_OL and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar., while MF_R, MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar.

clearly apart. These results suggested that the root and stem may share relative similar community structure of endophytic bacteria and fungi, while old or new leaves may harbor distinct endophytic community structures ($p < 0.01$; Figure 2A). Generally, the distribution pattern of the fungal community in tea trees were quite similar with that of bacteria (Figure 2B), which were also

characterized by separately clustered according to tissue types with some modification induced by cultivar.

The taxonomic analysis further revealed that the endophytic bacteria and fungi differed markedly among the tissue types and cultivars (Figure 3). It was observed that, on average, more than 90% of observed endophytic bacteria were affiliated to six bacterial classes, including Alpha-, Beta-and Gamma-proteobacteria, Actinobacteria, Flavobacteriia and Bacilli (Figure 3A). However, the relative abundance of these taxa varied among different tissue types as well as cultivars (Figure 3A). For example, new leaves in both BXZ and MF were predominated by Alphaproteobacteria and Flavobacteriia, which totally account for 84.8 and 79.2% of the total endophytic bacterial population, respectively. However, the relative abundances of these two dominant bacterial groups were decreased to 10.2 and 9.8% in old leaves of BXZ and MF, respectively. Instead, the minor groups Actinobacteria and Gammaproteobacteria in new leaves (average total proportion 3.2–3.6%), were significantly increased to be predominant endophytic bacterial groups (averagely 24.7–32.0%) in old leaves for both BXZ and MF. The root and stem samples generally shared similar community structure of endophytic bacteria for both cultivars, and the relative abundance of the dominant groups described above were generally in between new and old leaves. Finer taxonomic division at genus level (Supplementary Figure S1A) showed that members of *Morganella, Acidovorax,* and *Rhizobium* were dominant populations in roots and stems. But in new leaves, approximately 70% of the bacteria belonged to the four major bacterial genera *Chryseobacterium, Sphingomonas, Rhizobium* and *Methylobacterium*. The population composition of old leaves was more complex than that of new leaves. *Arsenophonus, Corynebacterium, Actinomyces, Pseudomonas* and other taxa were dominant populations in old leaves, but the amounts in other tissues were relatively low or even undetectable.

Likewise, most of the dominant endophytic fungal taxa also appeared tissue preference(Figure 3B). For example, the roots and stems were dominated by fungal classes of Dothideomycetes, Sordariomycetes and Leotiomycetes, which totally took account 87.0–93.5% of the overall community of endophytic fungi. Dothideomycetes and Sordariomycetes were still dominant fungal group in old leaves, but the relative abundance of Leotiomycetes were significantly decreased by 26.7–34.8%, which only represented 0.3 and 0.5% of the total fungal population in the old leaves of BXZ and MF, respectively. With regards to new leaves, it was interestingly found that approximately 76.0 and 81.6% of the endophytic fungal community belonged to Dothideomycetes, while other taxa only took account minor proportion. When analysis at genus level (Supplementary Figure S1B), it was found that approximately 38.0–90.3% were unclassified taxa. Of the classified taxa, *Colletotrichum* (4.8–9.7%), *Scleropezicula* (2.2–17.2%) and *Devriesia* (1.1–2.7%) were dominant fungal groups in roots and stems across two cultivars with proportion > 1%. With respected to old leaves, the proportion of *Colletotrichum*, *Cladosporium* and *Aspergillus* were significantly increased to 36.2–41.4%, 4.6–6.0% and 1.9–2.1%, respectively. Instead, the dominant group in root and stems, such as *Scleropezicula* and *Devriesia*, were decreased to be minor groups with relative abundance less than 0.1%. Compared to old leaves, the proportion of *Colletotrichum* was sharply decreased to 0.05–0.16% in new leaves, while *Uwebraunia* remarkably increased to be the predominant taxa with relative abundance 8.2–49.6%.

Partitioning of endophytic bacterial and fungal community

The distribution of endophytic bacterial and fungal OTUs among samples could be visualized in the Venn diagrams (Figure 4). It was detected that 45 bacterial OTUs were shared by all tissue samples across two cultivars; these OTUs formed 25.0% on average (12.9–40.5%) of the total OTU numbers from a given tissue type. By comparison, the factions of common OTUs in leaves were higher than that in roots and stems across two

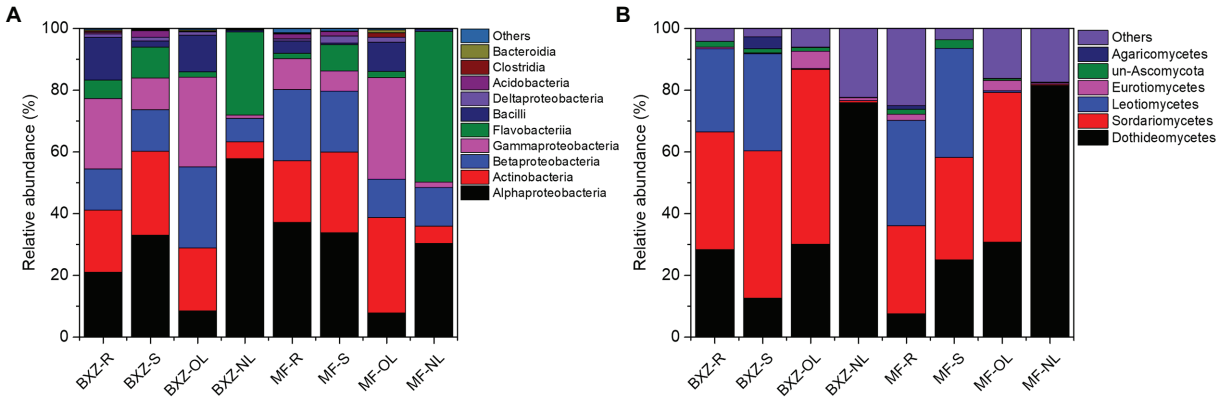


FIGURE 3
Taxonomic analysis of the endophytic bacterial **(A)** and fungal **(B)** communities at class level. BXZ_R, BXZ_S, BXZ_OL and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar., while MF_R, MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar.

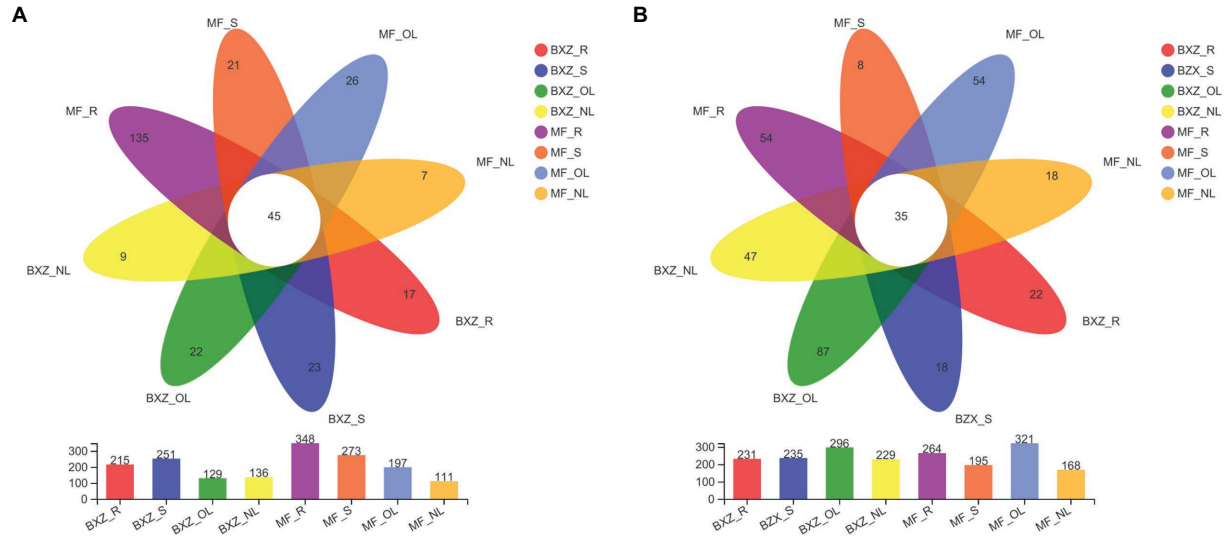


FIGURE 4
Common, tissue/cultivar-specific bacterial **(A)** and fungal **(B)** OTUs in tea tree. BXZ_R, BXZ_S, BXZ_OL and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar., while MF_R, MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar.

cultivars (Figure 4A). However, when examining the relative abundance of the common OTUs in a specific tissue sample, they represented, on average, 72.6% of the total bacterial sequence reads, with the highest proportion of 95.9% in new leaves, followed by stems (81.2%), and the lower in old leaves and root samples at 56.0 and 54.3%, respectively (Table 1).

For the endophytic fungal communities, it was detected that 35 OTUs were assigned as common community (Figure 4B). On average, these common OTUs represented 15.0% (10.9–20.8%) of the total OTU numbers in fungal community in a specific tissue sample, with the highest proportion of 15.3 and 20.8% in new leaves of BXZ and MF, respectively, and the lowest in old leaves at 11.8 and 10.9%, respectively. However, the relative abundance of these common communities in the total fungal population of a specific sample varied among tissue types. The average relative abundance of common groups was approximately 94.1–97.3% in new leaves, and 84.4–85.1% in old leaves, which were significantly higher than that in root and stem samples (32.8 and 31.9% on average).

When concerning the bacterial and fungal OTUs occurred in only specific tissue and cultivar., it was revealed that the proportions of the specific bacterial OTUs were generally lower than the common communities, which took account 13.3% of the total OTU numbers in individual sample, on average. The lowest number of OTUs assigned to the specific category was observed for new leaves in both BXZ and MF, representing 6.62 and 6.31% of their total OTU numbers, respectively. However, their proportions in old leaves were significantly increased to 17.05 and 13.20%, respectively. The proportion of specific OTUs in roots varied significantly with cultivar., with 7.91% in BXZ but 38.79% in MF. Unlike endophytic bacteria, the lowest specific fungal OTU number proportion were observed in stems, the percentage in BXZ and MF were 7.66 and 4.10%, respectively. The highest proportion in BXZ occurred in old leaves (29.39%), but in MF, it was changed to roots (20.45%). However, although the specific bacterial or fungal endophytic taxa averagely represented 13.34–14.90% in the total OTU pools, their relative abundance within the endophytic population were very low, on average, only 1.96 and 1.37% of the total bacterial and fungal reads in a specific sample, respectively.

Taxonomy analysis of common and tissue/cultivar specific community of endophytic bacteria and fungi

Taxonomy analysis showed that the common bacterial OTUs were mainly affiliated to 7 bacterial classes of Alpha-, Beta-and Gamma-proteobacteria, Actinobacteria, Flavobacteriia, Bacilli (Figure 5A), and 31 bacterial genera, such as *Chryseobacterium, Sphingomonas, Rhizobium, Morganella, Methylobacterium* and *Comamonadaceae* (Figure 5A). The common fungal taxa were mainly distributed in 4 fungal classes (*Dothideomycetes, Eurotiomycetes, Sordariomycetes, Agaricomycetes,* etc.), 12 fungal orders (*Capnodiales, Pleosporales, Eurotiales,* etc.), and 15 fungal genera (*Colletotrichum, Uwebraunia, Cladosporium,* etc.; Figure 5B). However, although these common taxa diversely distributed in various tissue types across cultivar., their relative abundance varied significantly among samples. For example, new leaves harbored the highest proportion of common communities affiliated to bacterial genera of *Chryseobacterium* and *Devosia*, followed by stems and roots, and the old leaves possessed the

TABLE 1 Proportions of common and specific taxa in total OTU numbers and population sizes of endophytic bacteria and fungi in tea trees.

	Proportion of OTU numbers (%)				Proportion of sequence reads (%)			
	Bacteria		Fungi		Bacteria		Fungi	
	Common	Specific	Common	Specific	Common	Specific	Common	Specific
BXZ-R	20.93	7.91	15.15	9.52	72.64	0.75	39.92	0.07
BXZ-S	17.93	9.16	14.89	7.66	82.29	0.68	29.07	0.05
BXZ-OL	34.88	17.05	11.82	29.39	57.58	4.45	84.37	2.16
BXZ-NL	33.09	6.62	15.28	20.52	94.05	0.30	94.14	0.22
MF-R	12.93	38.79	13.26	20.45	35.90	6.83	25.70	7.49
MF-S	16.48	7.69	17.95	4.10	80.07	0.46	34.65	0.01
MF-OL	22.84	13.20	10.90	16.82	60.38	1.96	85.14	0.88
MF-NL	40.54	6.31	20.83	10.71	97.68	0.24	97.26	0.07

BXZ_R, BXZ_S, BXZ_OL and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar. while MF_R, MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar.

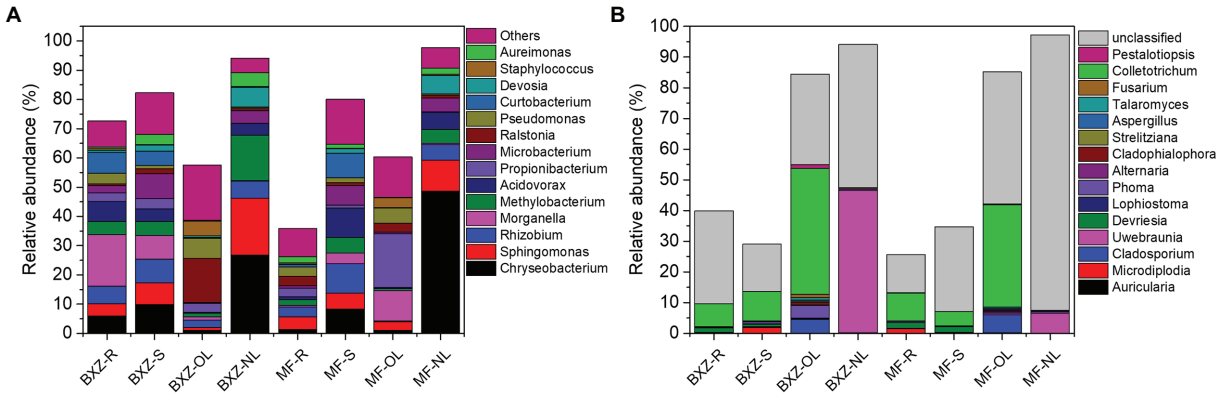


FIGURE 5
Common bacterial **(A)** and fungal **(B)** endophytic communities that shared by all tissues across two cultivars (genus level). BXZ_R, BXZ_S, BXZ_OL and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar., while MF_R, MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar.

lowest proportion. The relative abundance of *Curtobacterium* in root and stem samples were significantly higher than that in old and new leaves ($p < 0.05$; Figure 5A). For the fungal community, old leaves had between 2.7–6.0 times higher proportion of *Colletotrichum* than root and stem samples, and the proportion of this taxa sharply decreased from 33.3–41.0% in old leaves to 0.05–0.13% in new leaves. In contrast, the new leaves possessed the highest proportion of *Uwebraunia* (46.3% in BXZ and 6.5% in MF), followed by old leaves (0.3 and 0.5%), and the proportion in roots and stems was very low (less than 0.02%; Figure 5B).

In comparison with the common community, the specific communities of endophytic bacteria and fungi were diversely distributed in 31 bacterial and 11 fungal classes, and 96 bacterial and 81 fungal genera, respectively. Furthermore, the taxonomy categories of both bacterial and fungal OTUs identified as specific were also closely related to tissue and cultivar types (Figure 6). For example, of the total specific bacterial OTUs identified in old leaves of BXZ, about 61.09% of the community related to the class Bacilli (e.g., genera *Alicyclobacillus*) and other specific OTUs belonging to Alpha-and Gamma-proteobacteria (*Cardiobacterium, Nevskia*), Flavobacteriia (*Flavobacterium, Candidatus_Uzinura*), were observed in lower proportions (> 0.2%). In old leaves of MF, bacterial classes of Alpha-and Beta-proteobacteria, Clostridia, and Bacilli, and bacterial genera of Prevotella, Paenibacillus, Eubacterium, Corynebacterium, and Alloprevotella were the abundant specific groups with relative abundance ranged from 0.14 to 0.51%. Whereas in the root samples of MF, Alpha-proteobacteria (e.g., *Roseomonas, Sphingobium, Inquilinus*), Actinobacteria (*Catenulispora, Actinospica, Acidothermus, Nocardia*, etc.), and Acidobacteria (*Bryobacter, Candidatus_Solibacter*) were predominant groups with relative abundance >0.5%, which totally accounted 75.72% of the total specific community. For the specific fungal community (Figure 6B), it was detected that the old leaves in BXZ were dominated by fungal classes of Sordariomycetes (45.86% of the total specific fungal community), Eurotiomycetes (29.97%), and fungal genera of *Acremonium*, *Talaromyces*, *Rachicladosporium, Knufia*, and *Verticillium*. While approximately 86.71% of the

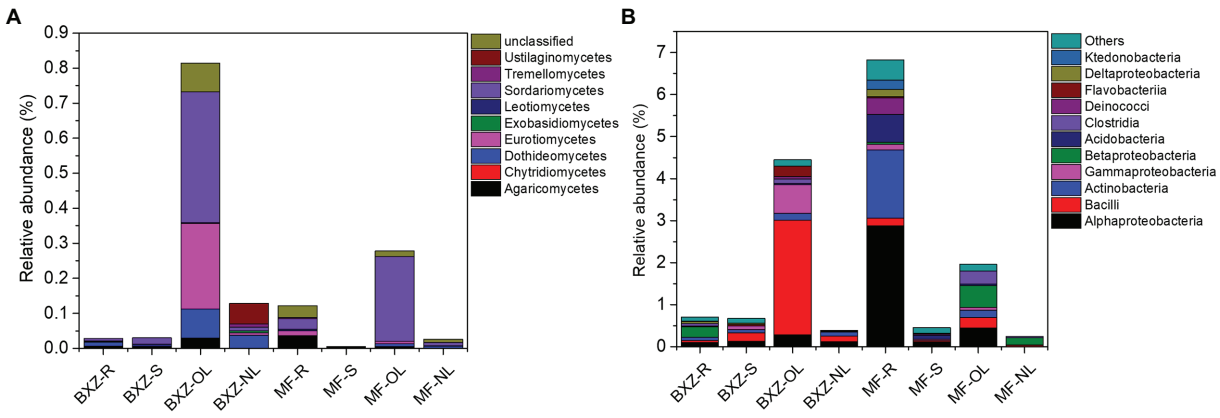


FIGURE 6
Specific bacterial **(A)** and fungal **(B)** endophytic communities that only occurred in specific tissue/cultivar (class level). BXZ_R, BXZ_S, BXZ_OL and BXZ_NL represented root, stem, old leave and new leave samples of BXZ cultivar. while MF_R, MF_S, MF_OL and MF_NL represented root, stem, old leave and new leave samples for MF cultivar.

specific fungal community in MF old leaves were affiliated to *Sordariomycetes*, including fungal genera of *Lecanicillium, Myrmecridium*, and *Rosellinia*.

Analysis of the interaction between endophytic bacterial and fungal populations

The relationship between the endophytic bacteria and fungi in the different tissues of BXZ and MF was analyzed through the species correlation network.

There were significant interactions between different species of endophytic bacteria and fungi among different tissues. The degree of correlation was significantly different, which was represented by root > stem > old leaves > new leaves (Figure 7). The number of bacterial and fungal populations (a positive correlation) in the new leaves was much higher than that in the old leaves. Compared with new leaves, the number of populations with a negative correlation in old leaves increased significantly. The correlation between bacterial populations in roots and stems was more complex, mainly manifested in the existence of positive and negative correlations between species.

Discussion

In this study, 16S rRNA and ITS high-throughput sequencing analysis were performed on the endophytes of BXZ and MF under the same cultivation management, and the difference and contrast analysis of the endophyte population composition and diversity in different tissues were carried out. These findings indicate that the endophytes in different tissues of different varieties are significantly different and are represented by roots > stems > old leaves > new leaves.

The endophyte community structure within the plant is dynamic and is influenced by abiotic and biotic factors such as soil conditions, biogeography, plant species, microbe-microbe interactions and plant-microbe interactions, both at local and larger scales (García-Guzmán and Heil, 2014). There is a long-term coevolution relationship between endophytes and tea. We found 636 species of endophytic bacteria of tea plants in this study, belonging to 20 bacterial phyla, 35 bacterial classes, 81 bacterial orders, 169 bacterial families, and 308 bacterial genera.

Endophytes are rich in plant tissues and play important roles in plant-microbial interactions and plant-growth regulation (Hardoim et al., 2008; Hacquard and Schadt, 2015; Wani et al., 2015). The bacterial groups of different tea plant species under the same cultivation conditions may be determined by different root exudates, different signaling molecules, etc. (Shyam et al., 2010; Frank et al., 2017). The root system was in direct contact with the soil, and the bacteria in the soil had more opportunities to infect the root system. Therefore, the diversity of endophytes in the roots was much higher than that in other tissues. Generally, host plants and rhizosphere endophytes are mostly symbiotic relationships. Previous studies have found that *Azoarcus, Burkholderia, Gluconobacter, Herbaspirillum, Klebsiella, Plantoea and Rahnella* and other bacterial genera can be detected in many plants (Dudeja et al., 2021). These bacteria can help host plants relieve nutrient stress by fixing nitrogen and secreting growth hormone under relatively infertile conditions (Dudeja et al., 2021). Some endophytic bacterial populations in plants, such as *Bacillus, Enterobacter, Pseudomonas, Streptomyces,* etc., can help host plants resist pathogenic bacteria and drought, salinity and other biological and abiotic stresses (Ali et al., 2014; Mukherjee, 2014; Naveed et al., 2014). We found that some bacterial genera, such as *Chryseobacterium, Sphingomonas, Rhizobium, Morganella, Methylobacterium Propionibacterium,* etc. were dominant bacterial populations coexisting in different tissues of BXZ and MF. It has been proven that bacterial populations such as *Chryseobacterium* have strong environmental tolerance and can

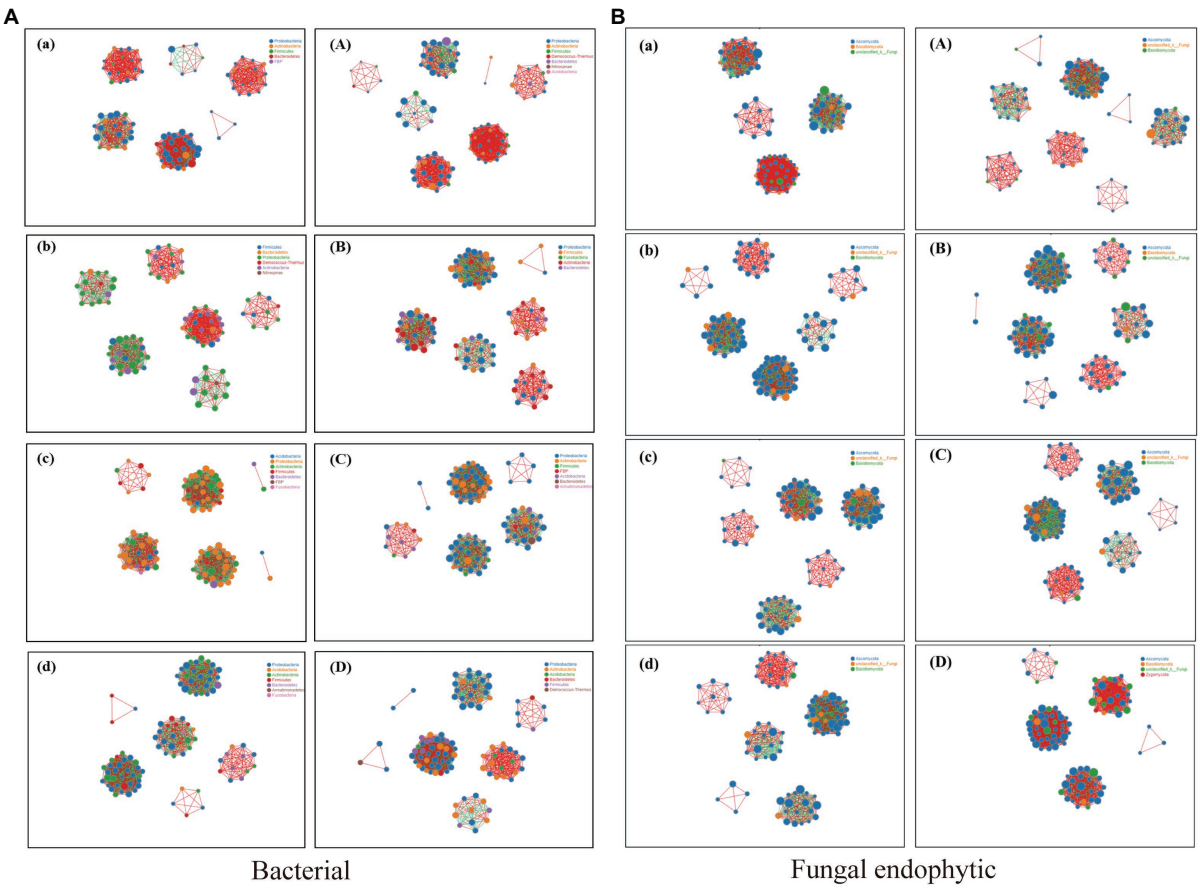


FIGURE 7
Network analysis showing the correlations between bacterial and fungal endophytic communities. (The figure shows the species with Spearman coefficient>0.8 and p<0.01 by default; the size of the node in the figure indicates the species abundance, and different colors indicate different species; the color of the line indicates a positive and negative correlation, and red indicates a positive correlation. Green indicates a negative correlation; the thickness of the line indicates the size of the Pearson correlation coefficient. The thicker the line, the higher the correlation between species; the more lines, the closer the relationship between the species and other species.) **(A)** Network analysis of correlation between BXZ and endophytic bacteria. **(B)** Network analysis of correlation between MF and endophytic bacteria. (a) BXZ new leaf; (b) BXZ old leaf; (c) BXZ stem; (d) BXZ root system; (A) MF new leaf; (B) MF old leaves; (C) MF stem; (D) MF root system.

adapt to oligotrophic and hypoxic environments (Kampfer et al., 2003; Liu et al., 2010).

The distribution of endophytic bacteria in tea trees showed an obvious alternating pattern during the maturation of tea leaves (Arnold et al., 2003; Suryanarayanan and Thennarasan, 2004). The types and numbers of endophytic bacteria gradually increased as the leaves matured. For example, the contents of *Arsenophonus, Corynebacterium, Actinomyces, Pseudomonas*, etc. in old leaves had significantly increased when compared with new leaves. Is the genetic background or growth characteristics of the tea plant itself or the external environmental conditions responsible for the differences in the distribution of endophytic bacteria populations? The answers to these questions need to be further explored and studied. The endophytic bacteria in the new leaves had a positive correlation, and they participated in the formation of tea metabolites together and jointly promoted the growth of the new leaves. However, the endophytic bacteria in the old leaves mainly showed a competitive relationship. To satisfy their own growth, the endophytic bacterial populations had a competitive relationship for nutrients.

Some endophytic bacterial populations could be parasitic in different tissues or varieties (Wei et al., 2018). Our results indicated that these endophytic bacterial populations, including *Chryseobacterium, Sphingomonas, Rhizobium, Morganella, Methylobacterium and Comamonadaceae*, that exist in different varieties and under different cultivation conditions may be common endophytic bacterial populations in tea varieties. The abundance, distribution and diversity of endophytes may be influenced by the genotypes and tissue types of tea plants and the length of plant growth. In this study, among the 636 different species of bacteria obtained from BXZ and MF planted in the same cultivation environment, only 45 species were shared. The microenvironment in different species or tissues affects endophytic bacterial survival. Some endophytic bacteria preferred the microenvironment in the leaf, and the abundance in the leaf was higher than that in other tissues. Previous studies have shown that environmental requirements, such as nutrient status, pH, oxygen concentration, etc. had a great influence on the growth of tea microorganisms. The difference in habitat conditions can

directly affect their living conditions and functional activity (Horner-Devine et al., 2007; Fierer and Ladau, 2012; Hazard et al., 2013). Factors such as genetic background and growth characteristics will lead to certain differences in the physical structure, chemical composition and nutritional components of different tissues, which will cause changes in the composition and structure of the tea plant bacterial population. The differences in the endophytic bacterial population structure of BXZ and MF may be related to the genetic characteristics of tea plants.

There was also a long-term coevolutionary relationship between endophytic fungi and plants. Some endophytic fungi have developed into an inseparable part of the plant (Osono and Mori, 2003). The distribution of endophytic fungi in tea plants had obvious specificity among different tissues. We found an obvious trend of old leaves > new leaves > roots > stems according to the types and quantity of endophytic fungi. Different tissues had different chances of contacting fungal spores floating in the air and fungi in the soil during the growth process. These endophytic fungi could produce metabolites with the same or similar chemical structure as the host (Zeng et al., 2000). Tea plants might recruit and colonize specific endophytic fungal groups based on their genetic and biochemical characteristics to maintain their own healthy growth (Su et al., 2010). However, which endophytic fungal groups are closely related to the genetics of tea trees and whether these endophytic fungal groups have a certain internal connection between the healthy growth of tea trees and tea quality are still unclear.

In addition to a large number of bacterial populations, there are also a large number of fungi in the soil, which greatly enriches the diversity of endophytic fungi in the root system. Hu et al. (2013) isolated 7 genera and 12 species of endophytic fungi from the tea roots of 10-year-old "Longjing long leaves." The 35 strains of endophytic fungi isolated by Agusta et al. (2005) belonged to *Fusarium, Penicillium, Schizophyllum and Diaporthe*. Studies have also found that the infection of tea plant endophytic fungi may be related to the number of stomata on the leaves. As the leaves mature, the number of stomata gradually increases (Xie et al., 2006; Xie, 2007). The composition of endophytic fungi in leaves at different developmental stages may be related to the internal and external structure and chemical factors of the leaves. How tea biochemistry affects the succession of endophytic fungi and whether endophytes have an impact on tea biochemistry require further research.

Endophytic fungi can survive in different endophytic microenvironments of tea plants. Taxonomic analysis found that most of the dominant fungal populations coexisting in different parts of different tea plant species belong to the genera of *Colletotrichum, Uwebraunia, Cladosporium*, and *Devriesia*. These ubiquitous endophytic fungi could live widely in plants; they changed in the microenvironment and affected their survival status. Most fungi had specific habitat requirements, including nutrient status, pH, oxygen concentration, etc. The differences in habitat conditions could directly affect their survival status and functional activity. The different varieties of tea plants used in this study had certain differences in the physical structure, chemical composition, and nutrient conditions of their roots, stems and leaves due to the genetic background and growth characteristics. The composition and structure of the fungal population in the organ would be different.

The endophytic fungal populations had a similar bacterial trend, the new leaves were more in the form of a positive correlation, and they participated in the material metabolism of tea through mutual help. The nutrients in the old leaves were reduced, and the endophytic fungal populations competed for nutrients to satisfy self-growth, which led to a significant reduction in the cooperative relationship between the endophytic fungal populations.

Conclusion

Tea plant harbored high diversity of endophytic bacteria and fungi. Tissue type played a more important role in shaping community structure and diversity of endophytic bacteria and fungi than did cultivar. Roots harbored the most diverse endophytic bacteria while the new leaves possessed the lowest diversity. Whereas for endophytic fungi, old leave displayed higher diversity than did root and stem tissues. Most of the dominant endophytes showed obvious cultivar and tissue preferences. Nevertheless, some core endophytic groups with high abundance, such as bacterial general of *Chryseobacterium, Sphingomonas, Rhizobium, Morganella, Methylobacterium* and *Comamonadaceae,* and fungal groups of *Colletotrichum, Uwebraunia, Cladosporium*, and *Devriesia*, could parasitize different tissues. At the same time, the distinctive characters of individual tissue/cultivar resulted in highly diverse specific communities that appear to be restricted to specific environments.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA861887.

Author contributions

HL, KW, and ZL conceived and designed research. HL, CL, and ZP conducted the experiments. HL, ZP, and BT analyzed the data. HL wrote the manuscript. KW and ZL revised and perfected the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the National Key R&D Program of China (Grant no. 2018YFC1604403). Open research project of Hunan Key Laboratory for crop germplasm innovation and resource utilization Key scientific research project of the Department of education 21A0130.

Acknowledgments

The authors wish to thank the Key Laboratory of Tea Science of Ministry of Education, China, the National Research Center of Engineering Technology for Utilization of Functional Ingredients from Botanicals in China, and the Co-Innovation Center of Education Ministry for Utilization of Botanical Functional Ingredients in China for their support.

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.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.872034/full#supplementary-material

References

Agusta, A., Maehara, S., Ohashi, K., et al. (2005). Stereoselective oxidation at C-4 of Flavans by the Endophytic fungus Diaporthe sp. *Isolated from a Tea Plant.* 37, 1565–1569.

Ali, S., Charles, T. C., and Glick, B. R. (2014). Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. *Plant Physiology & Biochemistry.* 80, 160–167. doi: 10.1016/j.plaphy.2014.04.003

Arnold, A. E., Mejia, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., et al. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci. U. S. A.* 100, 15649–15654. doi: 10.1073/pnas.2533483100

Bhuyan, L. P., Sabhapondit, S., Baruah, B. D., Bordoloi, C., Gogoi, R., Bhattacharyya, P.. (2013). Polyphenolic compounds and antioxidant activity of CTC black tea of north-East India[J]. *Food Chem.* 141: 3744–3751. doi: 10.1016/j.foodchem.2013.06.086

Borer, E. T., Kinkel, L. L., May, G., and Seabloom, E. W. (2013). The world within: quantifying the determinants and outcomes of a host's microbiome. *Basic and Applied Ecology.* 14, 533–539. doi: 10.1016/j.baae.2013.08.009

Bulgarelli, D., Rott, M., Schlaeppi, K., Loren, Ver., van Themaat, E., Ahmadinejad, N., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95.

Dudeja, S. S., Suneja-Madan, P., Paul, M., Maheswari, R., and Kothe, E. (2021). Bacterial endophytes: Molecular interactions with their hosts. *J. Basic Microbiol.* 61, 475–505.

Fierer, N., and Ladau, J. J. N. M. (2012). Predicting microbial distributions in space and time. *Nat. Methods* 9, 549–551. doi: 10.1038/nmeth.2041

Frank, A. C., Guzman, J. P. S., and Shay, J. E. (2017). Transmission of Bacterial Endophytes. *Microorganisms* 5:70. doi: 10.3390/microorganisms5040070

García-Guzmán, G., and Heil, M. (2014). Life histories of hosts and pathogens predict patterns in tropical fungal plant diseases. *New Phytologist* 201, 1106–1120.

Goyer, C., Neupane, S., Zebarth, B. J., Burton, D. L., Wilson, C., and Sennett, L. (2022). Diverse compost products influence soil bacterial and fungal community diversity in a potato crop production system. *Applied Soil Ecology* 169:104247

Hacquard, S., and Schadt, C. W. (2015). Towards a holistic understanding of the beneficial interactions across the Populus microbiome. *New Phytol.* 205, 1424–1430. doi: 10.1111/nph.13133

Hardoim, P. R., LSV, O., and JDV, E. (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

Hazard, C., Gosling, P., Van, D. G., Christopher, J., et al. (2013). The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale. *ISME J.* 7, 498–508. doi: 10.1038/ismej.2012.127

Hong, Y. C., Xin, W., Lai, Y. B., et al. (2005). Isolation of endophytic antifungal and pesticide degrading bacteria from tea plant. *J. Tea Sci.* 3, 183–188.

Horner-Devine, C., Silver, J. M., Leibold, M. A., Bohannan, B. J. M., et al. (2007). A comparison of taxon co-occurrence patterns for macro-and microorganisms. *Ecology* 88, 1345–1353. doi: 10.1890/06-0286

Hu, Y. F., Zhang, Y., Zhang, C. L., et al. (2013). Diversity analysis of the endophytic fungi in root and fungi in rhizosphere soil of tea plant in different seasons. *Journal of Nanjing Agricultural University* 36, 41–46.

Huang, X., Ren, J., Li, P., Feng, S., Dong, P., and Ren, M. (2020). Potential of microbial endophytes to enhance the resistance to postharvest diseases of fruit and vegetables. *J. Sci. Food Agric.* 101, 1744–1757. doi: 10.1002/jsfa.10829

Kampfer, P., Dreyer, U., Neef, A., Dott, W., and Busse, H. J. (2003). *Chryseobacterium defluvii* sp. nov. isolated from wastewater. *Int. J. Syst. Evol. Microbiol.* 53, 93–97. doi: 10.1099/ijs.0.02073-0

Kushwaha, P., Kashyap, P. L., Bhardwaj, A. K., Kuppusamy, P., Srivastava, A. K., and Tiwari, R. K. (2020). Bacterial endophyte mediated plant tolerance to salinity: growth responses and mechanisms of action. *World Journal of Microbiology & Biotechnology.* 36:26. doi: 10.1007/s11274-020-2804-9

Li, J., Xiao, Y., Zhou, X., Liao, Y., Wu, S., Chen, J., et al (2022). Characterizing the cultivar-specific mechanisms underlying the accumulation of quality-related metabolites in specific Chinese tea (Camellia sinensis) germplasms to diversify tea products. *Food Res. Int.* 111824.

Liu, W. J., Wang, K., Li, B. Z., Yuan, H. L., and Yang, J. S. (2010). Production and characterization of an intracellular bioflocculant by chryseobacterium daeguense w6 cultured in low nutrition medium. *Bioresour. Technol.* 101, 1044–1048. doi: 10.1016/j.biortech.2009.08.108

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., and Malfatti, S. (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature*, 488, 86–90.

Mukherjee. (2014). Biotechnological applications of bacterial endophytes. *Current Biotechnol.* 3, 1616–8542. doi: 10.1007/978-3-662-45097-0

Naveed, M., Mitter, B., Reichenauer, T. G., Wieczorek, K., and Sessitsch, A. (2014). Increased drought stress resilience of maize through endophytic colonization by *burkholderia phytofirmans* psjn and enterobacter sp. fd17. *Environmental & Experimental Botany* 97, 30–39. doi: 10.1016/j.envexpbot.2013.09.014

Osono, T., and Mori, A. J. M. (2003). Colonization of Japanese beech leaves by phyllosphere fungi. *Mycoscience* 44, 437–441. doi: 10.1007/s10267-003-0135-y

Oukala, N., Aissat, K., and Pastor, V. (2021). Bacterial endophytes: The hidden actor in plant immune responses against biotic stress. *Plants* 10:1012.

Porras-Alfaro, A., and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annual review of phytopathology* 49, 291–315.

Rabha, A. J., Naglot, A., Sharma, G. D., Gogoi, H. K., and Veer, V. (2014). In vitro evaluation of antagonism of endophytic Colletotrichum gloeosporioides against potent fungal pathogens of *Camellia sinensis*. *Indian J. Microbiol.* 54, 302–309. doi: 10.1007/s12088-014-0458-8

Rothenberg, D. O., and Zhang, L. (2019). (2019) mechanisms underlying the anti-depressive effects of regular tea consumption[J]. *Nutrients* 11:1361. doi: 10.3390/nu11061361

Santoyo, G., Moreno-Hagelsieb, G., Orozco-Mosqueda, M. C., et al. (2016). Plant growth-promoting bacterial endophytes. *Microbiol. Res.* 183, 92–99. doi: 10.1016/j.micres.2015.11.008

Seghers, D., Wittebolle, L., Top, E. M., Verstraete, W., and Siciliano, S. D. (2004). Impact of agricultural practices on the *Zea mays* L. endophytic community. *Appl Environ Microb* 70, 1475–1482. doi: 10.1128/AEM.70.3.1475-1482.2004

Shan, W., Zhou, Y., Liu, H., and Xiaomin, Y. (2018). Endophytic Actinomycetes from tea plants (*Camellia sinensis*): isolation, abundance, antimicrobial, and plant-growth-promoting activities[J]. *Biomed. Res. Int.* 2018:1470305. doi: 10.1155/2018/1470305

Shyam, K., Pierre, J., and Sharon, D. J. M. (2010). Bacterial Endophyte colonization and distribution within plants. *Microorganisms.* 5:77. doi: 10.3390/microorganisms5040077

Singh, M., Kumar, A., Singh, R., and Pandey, K. D. (2017). Endophytic bacteria: a new source of bioactive compounds. 3. *Biotech* 7, 1–14.

Spaepen, S., Vanderleyden, J., and Okon, Y. (2009). Plant growth-promoting actions of rhizobacteria. *Adv. Botan. Res.* 51, 283–320.

Su, J. Q., Wang, G. H., and Yang, M. H. (2010). Mixed culture of endophytic fungi isolated from Camellia sinensis enhancing the antagonistic on plant pathogenic fungi. *Mycosystema* 29, 753–759.

Sun, J., Chang, M., Li, H., Zhang, Z., and Wan, X. (2019). Endophytic bacteria as contributors to theanine production in camellia sinensis. *J. Agric. Food Chem.* 67, 10685–10693. doi: 10.1021/acs.jafc.9b03946

Suryanarayanan, T. S., and Thennarasan, S. (2004). Temporal variation in endophyte assemblages of *plumeria rubra* leaves. *Fungal Divers.* 21, 367–377. doi: 10.1002/yea.1100

Wani, Z. A., Ashraf, N., and Mohiuddin, T. (2015). Plant-endophyte symbiosis, an ecological perspective. *Applied Microbiology and Biotechnology* 99, 2955–2965.

Wei, W., Yu, Z., Chen, F., Yan, X., Lai, Y., Wei, C., et al. (2018). Isolation, diversity, and antimicrobial and immunomodulatory activities of endophytic actinobacteria from tea cultivars zijuan and yunkang-10 (camellia sinensis var. assamica). *Front. Microbiol.* 9:1304. doi: 10.3389/fmicb.2018.01304

Xie, L. H. (2007). *Preliminary study on the isolation and interaction of Endophytic Fungi from tea trees [M]*. China: Fujian Normal University.

Xie, L. H., Xu, Y. P., Wang, G. H., et al. (2006). Effect of tea variety, leaf age and chemical component on Endophytic Fungi. *Journal of Fungal Research.* 3, 43–49.

Yan, X., Zhi, W., Yu, M., Wang, L., Xu, W., Xu, Q., Su, P., Yu, Z., Wei, C.. (2018). Isolation, diversity, and growth-promoting activities of Endophytic Bacteria From tea cultivars of Zijuan and Yunkang-10. *Front. Microbiol.* 9:1848. doi: 10.3389/fmicb.2018.01848

Zeng, S. R., Xu, C. D., Wang, H. K., et al. (2000). A preliminary study on the mechanism of Endophytic Fungi in medicinal plants and their hosts. *Chin. Tradit. Herb. Drug* 4, 68–70.

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