Effects of seed inoculation with *Hartmannibacter diazotrophicus* on indigenous bacterial communities in the rhizosphere and on plant yield parameters in wheat and barley over three seasons

Thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor rerum naturalium
(Dr. rer. nat.)

Submitted by

M. Sc. Santiago Andres Quiroga Quisaguano

Faculty of Agricultural Sciences, Nutritional Sciences
and Environmental Management

Institute of Applied Microbiology

Justus-Liebig University Giessen, Germany

Giessen, 2025

PUBLICATIONS

International peer reviewed scientific publications:

Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Schulz, F., Krutych, M., Zörb, C., Schnell, S., 2024. Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance. FEMS Microbiol. Ecol. 1–13. https://doi.org/10.1093/femsec/fiae023

Quiroga, S., Ratering, S., Rosado-Porto, D., Rekowski, A., Schulz, F., Zörb, C., Schnell, S., 2025. Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial. Appl. Soil Ecol. 206, 105823. https://doi.org/10.1016/j.apsoil.2024.105823

Sacharow, J., Ratering, S., **Quiroga, S**., Geißler-Plaum, R., Schneider, B., Österreicher Cunha-Dupont, A., Schnell, S., 2024. Cercozoan diversity of spring barley grown in the field is strongly plant compartment specific. Front. Microbiomes 3, 1–12. https://doi.org/10.3389/frmbi.2024.1352566

Publications under review:

Sacharow, J., Ratering, S., **Quiroga, S**., Geißler-Plaum, R., Schneider, B., Österreicher Cunha-Dupont, A., Schnell, S., 2024. Composition of Cercozoan diversity: unravelling leaf, root, and soil specificity in crop plants. Available at SSRN: http://dx.doi.org/10.2139/ssrn.5007925

Publication to be submitted:

Quiroga, S., Ratering, S., Rosado-Porto, D., Rekowski, A., Schulz, F., Zörb, C., Schnell, S., 2025. Complementary roles of eDNA and eRNA in characterizing rhizosphere bacterial communities of wheat and barley across seasons.

Quiroga, S., Ratering, S., Rosado-Porto, D., Kostner, D., Schnell, S., 2025. Absence of aboveground effects may conceal changes underground: Plant-growth promoting rhizobacteria and salt stress altered the rhizosphere microbiome of roses.

Rekowski, A., Wimmer, M., **Quiroga, S**., Rosado-Porto, D., Ratering, S., Schnell, S., Zörb, C., 2025. Seed inoculation with *Hartmannibacter diazotrophicus*: starch and protein properties in malting.

Sacharow, J., Rosado-Porto, D., **Quiroga, S**., Ratering, S., Geißler-Plaum, R., Schneider, B., Schnell, S., 2024. Which effect does the inoculation of the plant growth-promoting rhizobacterium *Hartmannibacter diazotrophicus* E19^{T} have on the microbiome of winter wheat?

Derbali, I., Derbali, W., **Quiroga, S**., Gharred, J., Manaa, A., Schnell, S., Slama, I., Koyro H., 2025. The role of plant growth promoting rhizobacteria in responses of quinoa to hyperosmotic salinity

Presentations:

Quiroga, S., Ratering, S., Rekowski, A., Krutych, M., Zörb, C., Schnell, S., 2023. Effects of seed inoculation of wheat and barley in organic farming on plant parameter and the rhizosphere microbiome. Soil as a Sustainable Resource for the Bioeconomy (BonaRes) conference. Berlin, Germany.

Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Schulz, F., Krutych, M., Zörb, C., Schnell, S., 2024. Seed inoculation of wheat and barley with *Hartmannibacter diazotrophicus* improves yield parameters without altering the rhizosphere bacterial microbiome. Soil as a Sustainable Resource for the Bioeconomy (BonaRes). Final conference. Leipzig, Germany.

Posters

Quiroga, S., Ratering, S., Rekowski, A., Zörb, C., Schnell, S., 2022. Production of wheat and barley under reduced input in organic farming. International symposium Microbe-Assisted Crop Production-Opportunities, Challenges and Needs (miCROPe). Vienna, Austria.

Sacharow, J., **Quiroga, S**., Rosado-Porto, D., Ratering, S., Dupont, A., Schnell, S., 2023. Diversity, co-occurrence, and network analysis of the rhizosphere microbiota in response to inoculation of winter wheat with *Hartmannibacter diazotrophicus* E19. 16th symposium on Bacterial Genetics and Ecology (BAGECO). Copenhagen, Denmark.

Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Zörb, C., Schnell, S., 2023. Effects of seed inoculation of wheat and barley in organic farming on the rhizosphere microbiome. Annual conference of the Association for General and Applied Microbiology (VAAM). Göttingen, Germany.

Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Schulz, F., Krutych, M., Zörb, C., Schnell, S., 2024. Seed inoculation of wheat and barley with *Hartmannibacter diazotrophicus*

improves yield parameters without altering the rhizosphere bacterial microbiome. Soil as a Sustainable Resource for the Bioeconomy (BonaRes) final conference. Leipzig, Germany.

The present work was carried out at the Institute of Applied Microbiology, Faculty of Agricultural Sciences, Nutritional Sciences, and Environmental Management, Justus-Liebig University, Giessen during the period from March 2020 to September 2024 under the guidance of Prof. Dr. Sylvia Schnell.

I Supervisor: Prof. Dr. Sylvia Schnell
Institute of Applied Microbiology
Justus-Liebig University
Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

II Supervisor: Prof. Dr. Andreas Gattinger
Department of Agronomy and Plant Breeding II
Justus-Liebig University
Karl-Glöckner-Str. 21 C, 35394 Giessen, Germany

Statement

"I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus-Liebig University Giessen for the Safeguarding of Good Scientific Practice."

Giessen, 26.02.2025 Santiago Andres Quiroga Quisaguano

Table of contents

List of abbreviations I

Summary II

Zusammenfassung III

Chapter I Introduction 1

1.1. Transition to a sustainable agriculture system 2

1.2. Plant growth promoting rhizobacteria (PGPR) 7

1.2.1. Nitrogen fixation 9

1.2.2. Phosphorus solubilization 9

1.2.3. Iron sequestration 10

1.2.4. Plant growth regulators 10

1.2.5. Understanding rhizobacteria signaling to enhance PGPR application 11

1.3. Microbial composition dynamics in response to the introduction of foreign microorganisms 15

1.4. Reported inconsistencies: Why do bioinoculants fail beyond the laboratory? 17

1.5. Monitoring Bioinoculants 19

1.6. *Hartmannibacter diazotrophicus* strain E19^{T} as PGPR 21

1.7. Aim of the study 21

1.8. References 23

Chapter II Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance 38

Chapter III Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial 52

Chapter IV Complementary roles of eDNA and eRNA in characterizing rhizosphere bacterial communities of wheat and barley across seasons 68

Chapter V Absence of aboveground effects may conceal changes underground: Plant-growth promoting rhizobacteria and salt stress altered the rhizosphere microbiome of roses 86

Chapter VI General Discussion 114

Supplements 130

Supplement 1. Seed inoculation with *Hartmannibacter diazotrophicus*: starch and protein properties in malting barley 130

Suplement 2. Which effect does the inoculation of the plant growth-promoting rhizobacterium *Hartmannibacter diazotrophicus* E19^{T} have on the microbiome of winter wheat? 132

Acknowledgements 135

List of abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AGPs	Arabinogalactan proteins
AHLs	N-acyl homoserine lactones
AIs	Autoinducers
AMF	Arbuscular mycorrhizal fungi
CFU	Colony forming units
DAS	Days after seeding
DDT	Dichloro-diphenyl-trichloroethane
DR	Detection rate
eDNA	Environmental DNA
eRNA	Environmental RNA
EPS	Extracellular polymeric substances
FISH	Fluorescence *in situ* hybridization
GH	Gladbacherhof
GMOs	Genetically modified organisms
HSI	Hyperspectral imaging
HTP	High throughput phenotyping
HVs	High-yielding crop varieties
IAA	Indol-3-acetic acid
ISR	Induced systemic resistance
KH	Kleinhohenheim
MCPs	Methyl-accepting chemotaxis proteins
MPN	Most probable number
NGS	Next generation sequencing
PGPR	Plant growth promoting rhizobacteria
PGR	Plant growth regulators
PSB	Phosphate solubilizing bacteria
qPCR	Quantitative PCR
QQ	Quorum quenching
QS	Quorum sensing
SA	Salicylic acid
SAR	Systemic acquired resistance

Summary

The use of mineral fertilizers and pesticides is not allowed in organic farming, which can reduce the yield and grain quality of cereals. In recent years, the potential of plant growth-promoting rhizobacteria (PGPR) has been recognized for a better supply of nutrients and improved resilience to abiotic and biotic plant stress. Although the mechanisms of PGPR are well studied, their effectiveness under field conditions is not consistent. There is also limited information on the effects of the use of allochthonous microorganisms on the native soil microbiome. In this study, we evaluated the effects of seed inoculation with *Hartmannibacter diazotrophicus* strain E19^T on the rhizosphere bacterial communities of wheat and barley by analyzing community changes by the strain using metabarcoding and subsequent bioinformatic evaluation. This analysis was performed at two experimental stations (Gladbacherhof and Kleinhohenheim) for organic farming in three seasons (2021-2023). For this purpose, DNA and RNA were extracted from the rhizosphere soil, the 16S rRNA gene sequences or the 16S rRNA were sequenced and analyzed bioinformatically. The effects of different row spacing and the application of organic fertilizer on winter wheat were also tested. The occurrence of *H. diazotrophicus* strain E19^T was quantified by real-time PCR at the flowering and milk-ripe/yellow-ripe stages. In order to determine the influence of the various factors (with/without seed inoculation, different row spacing, and with/without fertilization on winter wheat) on the plant, the grain and straw yield, as well as the crude protein concentration of the grains were recorded.

H. diazotrophicus strain E19^T was detected 273 and 119 days after sowing on both wheat roots (up to 3.1 x 10^5 copies g^{-1} DW) and barley roots (up to 5 x 10^5 copies g^{-1} DW) mainly at one experimental station. The abundance of *H. diazotrophicus* strain E19^T was correlated with yield parameters using linear mixed models. Significant effects were found for crude protein concentration (0.80% higher in barley and 0.30% higher in wheat compared to the average) and straw yield (453 kg ha^{-1} higher in wheat compared to the average). Although no significant effects on grain yield were found, a trend towards improvement was observed with the combination of organic fertilizer and bacterial inoculation. Interestingly, *H. diazotrophicus* strain E19^T did not change the rhizosphere community structure over three seasons. These results were based on a comparison of beta-diversity indices (Robust Aitchison Principal Component Analysis) with subsequent statistical analysis using PERMANOVA ($p > 0.05$). Similarly, alpha diversity indices, including Shannon-Wiener, observed amplicon sequence variants (ASVs) and Gini-Simpson (Wilcoxon, $p > 0.05$) showed no significant effects. Similar results were found after extraction of environmental RNA compared to DNA and its sequencing

and bioinformatic analysis. These results indicate that the indigenous bacterial rhizosphere communities were resilient to newly introduced bacteria after seed inoculation with *H. diazotrophicus* strain E19^T. The strongest changes in the bacterial community were identified between the two locations as shown by differential abundance analysis (ALDEx2). The ALDEx2 analysis identified 2860 ASVs that were different between winter wheat and spring barley at the Gladbacherhof site, while only 232 ASVs were different between these crops at Kleinhohenheim. These differences could be due to the different crop rotations at the two sites.

Zusammenfassung

Der Einsatz von Mineraldünger und Pestizide sind in der ökologischen Landwirtschaft nicht erlaubt, was den Ertrag und die Kornqualität von Getreide mindern kann. Für eine bessere Versorgung mit Nährstoffen und verbesserter Resilienz gegenüber abiotischem und biotischem Stress der Pflanzen wurde in den letzten Jahren das Potential von pflanzenwachstumfördernden Rhizobakterien (PGPR) erkannt. Obwohl die Mechanismen von PGPR gut untersucht sind, ist ihre Wirksamkeit unter Feldbedingungen nicht konsistent. Auch gibt es begrenzte Informationen, welche Auswirkungen der Einsatz von allochthoner Mikroorganismen auf das native Bodenmikrobiom hat. In dieser Studie haben wir die Auswirkungen einer Saatgutinokulation mit *Hartmannibacter diazotrophicus* Stamm E19^T auf die bakteriellen Gemeinschaften der Rhizosphäre von Weizen und Gerste evaluiert, indem wir Veränderungen der Gemeinschaft durch den Stamm mit Hilfe von Metabarcoding und anschließender bioinformatischer Auswertung analysiert haben. Diese Analyse wurde an zwei Versuchsstationen (Gladbacherhof und Kleinhohenheim) für ökologischen Landbau in drei Saisons (2021-2023) durchgeführt. Hierfür wurden DNA und RNA aus dem Rhizosphärenboden extrahiert, die 16S rRNA Gensequenzen bzw. die 16S rRNA sequenziert und bioinformatisch ausgewertet. Die Auswirkungen unterschiedlicher Reihenabstände und die Anwendung organischer Düngemittel auf Winterweizen wurden ebenfalls getestet. Das Vorkommen von *H. diazotrophicus* Stamm E19^T wurde mittels Real-Time PCR zum Zeitpunkt der Blüte und der Milchreife/Gelbreife des Getreides quantifiziert. Um den Einfluss der verschiedenen Faktoren (mit/ohne Inokulation des Saatgutes, unterschiedliche Reihenabstände und mit/ohne Düngung bei Winterweizen) auf die Pflanze zu bestimmen, wurden der Korn- und Strohertrag sowie die Rohproteinkonzentration der Körner erfasst.

H. diazotrophicus Stamm E19^T wurde 273 bzw. 119 Tage nach der Aussaat sowohl an den Weizenwurzeln (bis zu 3,1 x 10^5 Kopien g^{-1} TG) als auch an den Gerstenwurzeln (bis zu 5 x 10^5 Kopien g^{-1} TG) hauptsächlich an einer Versuchsstation nachgewiesen. Die Häufigkeit von *H. diazotrophicus* Stamm E19^T wurde durch lineare gemischte Modelle mit Ertragsparametern korreliert. Signifikante Auswirkungen wurden bei der Rohproteinkonzentration (0,80% höher bei Gerste und 0,30% höher bei Weizen im Vergleich zum Durchschnitt) und dem Strohertrag (453 kg ha^{-1} höher bei Weizen im Vergleich zum Durchschnitt) gefunden. Obwohl keine signifikanten Auswirkungen auf den Kornertrag gefunden wurden, konnte ein Trend zur Verbesserung mit der Kombination aus organischem Dünger und bakterieller Inokulation festgestellt werden. Interessanterweise veränderte *H. diazotrophicus* Stamm E19^T über drei Saisons hinweg nicht die Struktur der Rhizosphärengemeinschaft. Diese Ergebnisse basierten auf einem Vergleich der Beta-Diversität Indizes (Robust Aitchison Principal Component Analysis) mit anschließender statistischer Auswertung mittels PERMANOVA ($p > 0,05$). Ebenso zeigten die Alpha-Diversitätsindizes, einschließlich Shannon-Wiener, observed amplicon sequence variants (ASVs) und Gini-Simpson (Wilcoxon, $p > 0,05$) keine signifikanten Effekte. Ähnliche Ergebnisse wurden nach der Extraktion der Umwelt RNA im Vergleich zur DNA und deren Sequenzierung und bioinformatischer Auswertung gefunden. Diese Ergebnisse deuten darauf hin, dass die indigenen bakteriellen Rhizosphärengemeinschaften nach Samenbeimpfung mit *H. diazotrophicus* Stamm E19^T resilient gegenüber neu eingebrachten Bakterien waren. Die stärksten Veränderungen der bakteriellen Gemeinschaft wurden zwischen den beiden Standorten durch die differenzielle Abundanzanalyse (ALDEx2) gezeigt. Die ALDEx2-Analyse identifizierte 2860 ASVs, die zwischen Winterweizen und Sommergerste am Standort Gladbacherhof unterschiedlich waren, während in Kleinhohenheim nur 232 ASVs zwischen diesen Kulturen verschieden waren. Diese Unterschiede könnten durch die verschiedenen Fruchtfolgen der beiden Standorte bedingt sein.

Chapter I Introduction

1.1. Transition to a sustainable agriculture system

During the early stages of the green revolution, the use of fertilizers, pesticides, new irrigation technology systems, along with the emergence of high-yielding crop varieties (HVs), dramatically enhanced the production of essential crops for human nutrition. The development of HVs began with the identification of semi-dwarf wheats in Japan in the 1800s and semi-dwarf rice varieties several centuries ago. After breeding processes in Italy and USA, semi-dwarf wheat varieties were introduced to Mexico. This, together with research in rice carried out in the Philippines, aimed to create small varieties that prioritized grain production over straw material (Dalrymple, 1978).

Great expectations were initially raised in the field of pesticides with dichlorodiphenyltrichloroethane (DDT). Although DDT was originally synthesized in 1874, it was amply used against typhus and malaria during the Second World War. In agriculture, the recreation of DDT by Paul Müller in 1939 unveiled its effectiveness against a range of insects, including the Colorado potato beetle (*Leptinotarsa decemlineata*) and later controlling pests such as pink bollworm (cotton) or codling moth (deciduous fruits) (Metcalf, 1973). Similar success in weed control was achieved with the discovery of another chlorinated compound, the 2,4-dichlorophenoxyacetic acid (2,4-D) in the early 1940s (Peterson, 1967). The rise of synthetic fertilizers dates back to the first decade of the 19th century, with the Haber-Bosch process, which consisted of converting hydrogen and nitrogen into ammonia ($N_2 + 3H_2 \rightarrow 2NH_3$) at high temperatures and pressures. Without this outstanding breakthrough, estimates suggest that only slightly over half of the worldwide population could have been sustained (Fig. 1a) (Ritchie, 2017). Collectively, these advancements have enabled a doubling of average worldwide cereal crop production (from 1.36 t ha^{-1} in 1961 to 2.93 t ha^{-1} in 2010) while increasing cultivable land area by only 30% (for review: John and Babu, 2021; Liu et al., 2015). Nevertheless, the promising initial perspectives of chemical fertilizers and pesticides later led to unintended and unexpected side effects on the environment, as well as on animal diversity, and human health.

Pesticides and fertilizers have been overused since the green revolution, with their consumption increasing by 35-40% in more than 80 countries evaluated between 1960 and 2010 (Liu et al., 2015), thereby leading to multiple consequences. Besides the overuse of fertilizers, their application becomes inefficient as plants take up only 10-45% and 30%-50% of the applied phosphorus and nitrogen fertilizers, respectively (Singh et al., 2016). For instance, a significant increase in rice and wheat yields has been observed after nitrogen fertilization with 550-600 kg

N ha^{-1}. However, nitrogen losses doubled (Ju et al., 2009). This excessive nutrient input, as a consequence of intensive agriculture, has enriched aquatic environments through a process known as eutrophication. The overgrowth of algae and cyanobacteria increases primary production but also degradation of the produced biomass with respiration rates, leading to the depletion of dissolved oxygen and consequently, affecting the habitat and diversity of aquatic animals (Levich, 1996). Moreover, the side effects of fertilizer overuse include soil erosion due to changes in soil properties (e.g. pH-value, organic matter, salinization), nitrate NO_3^- leaching and nitrous oxide (N_2O) emission, which cause water quality depletion and contribute to air pollution (Ju et al., 2009; Pernes-Debuyser and Tessier, 2004; Rashmi et al., 2020).

In the case of pesticides, direct impacts on human health have been more evident. The initial success of DDT was based on its chemical stability, which allowed it to persist in the environment, reducing its application frequency but also accumulating in animals across the food chain and ultimately impacting human health. Carcinogenic, reproductive, and neuropsychological effects in humans, along with a decrease in animal populations (especially birds) have been associated with DDT, which was restricted after 1970 (for review: Beard, 2006). Equally critical have been the consequences for biodiversity. Beketov et al. (2013) provided evidence of species pool losses among stream invertebrates in Australia and Europe. They empirically demonstrated a significant decline in taxonomic richness, which was also supported by predictive models. The taxa abundance of stream invertebrates decreased by 25% in Australia and 42% in Europe between reference and highly contaminated sites. The authors suggested that to mitigate the effects of pesticides on biodiversity, enhanced safeguards should be applied in pesticide registration, mitigation practices, and field application methods. Nevertheless, nearly a decade later, the outlook looks bleak. A recent study evaluated the risk of pesticide pollution across 168 countries, focusing on the 92 most used active ingredients, including 19 fungicides, 21 insecticides, and 59 herbicides. The findings showed that 24.5 million km^2 of agricultural land (64% of the worldwide total) is susceptible to pesticide contamination, while 31% is facing a high threat (Fig. 1b) (Tang et al., 2021). Therefore, the development of new agricultural strategies and technologies is required to minimize the effects of fertilizers and pesticides while simultaneously optimizing crop yields without deteriorating soil quality, biodiversity, and human health.

Organic farming (OF) has its origins in the early 20^{th} century, evolved between the two World Wars and was mainly influenced by four key aspects: (i) an agricultural crisis, despite the simultaneous emergence and use of mineral fertilizers and pesticides; (ii) research orientated

from a biological perspective, integrating the role of soil microorganisms in soil fertility and the relationship between plants, roots, and soil (e.g. Lorenz Hiltner); (iii) reform movements in the German empire, known as 'Lebensreform' (or 'Life reform') and the 'Food reform' in USA, advocating for changes in lifestyle and eating habits and the adoption of organic practices; and (iv) sustainable practices carried out in East Asia (e.g., composting or recycling of municipal waste) (Vogt, 2007). However, OF gained relevance from the 1970s, and is now considered one of the main strategies for a sustainable agricultural system. By 2022, organic farmland increased to over 96 million hectares. While organic food and drink sales were estimated at 135 billion euros. Moreover, Germany is considered the largest consumer of organic products, with sales reaching 15.3 billion euros (Willer et al., 2024). Within Germany, Hessen is a leading state, with 2404 farmers who organically managed 16.4% of the agricultural area by 2023 (LLH, 2023). Compared to conventional agriculture, organic farming has shown to improve soil structure, soil carbon sequestration, increase microbial activity, and water retention (Gattinger et al., 2012; Lori et al., 2017; Shepherd et al., 2002; Williams et al., 2017). This together with crop rotation practices (e.g. cover crops) have shown not only to improve soil fertility or prevent erosion but also to increase soil microbial richness and diversity (McDaniel et al., 2014; Venter et al., 2016). Nonetheless, since the use of synthetic fertilizers and pesticides is prohibited, the main challenge of organic farming is narrowing the yield gap (19-25%) compared to conventional agriculture (De Ponti et al., 2012; Meemken and Qaim, 2018; Seufert et al., 2012). More intricate still, measuring the environmental impacts between both agricultural systems is complex due to the numerous factors involved, as well as the different management strategies and research methods employed (Clark and Tilman, 2017; Kirchmann et al., 2016; Tuomisto et al., 2012). A review conducted by Meemken and Qaim (2018) indicated that OF results less pollution than conventional agriculture when assessed per unit of land, though this advantage diminishes when evaluated per unit of output. A more recent and comprehensive meta-analysis, which included life cycling assessment (LCA) and 15 environmental parameters, compared both agricultural systems (Boschiero et al., 2023). The analysis revealed that OF had lower environmental impacts across most of the metrics analyzed, with fertilization being the main contributor to the impacts in both systems.

In addition to organic farming, innovative sustainable technologies have emerged to address concerns about feeding a constantly expanding human population, which is projected to reach 10 billion within the next 30 years (Gerten et al., 2020). Certain crops have long generation times. To address this challenge, a method known as 'speed breeding', which involves regulated temperatures and prolonged light exposures (22 hours light/ 2 hours dark), has shortened the

generation time from two-three to six per year for several crops such as barley (*Hordeum vulgare*), spring wheat (*Triticum aestivum*), or chickpea (*Cicer arietinum*) (Watson et al., 2018).

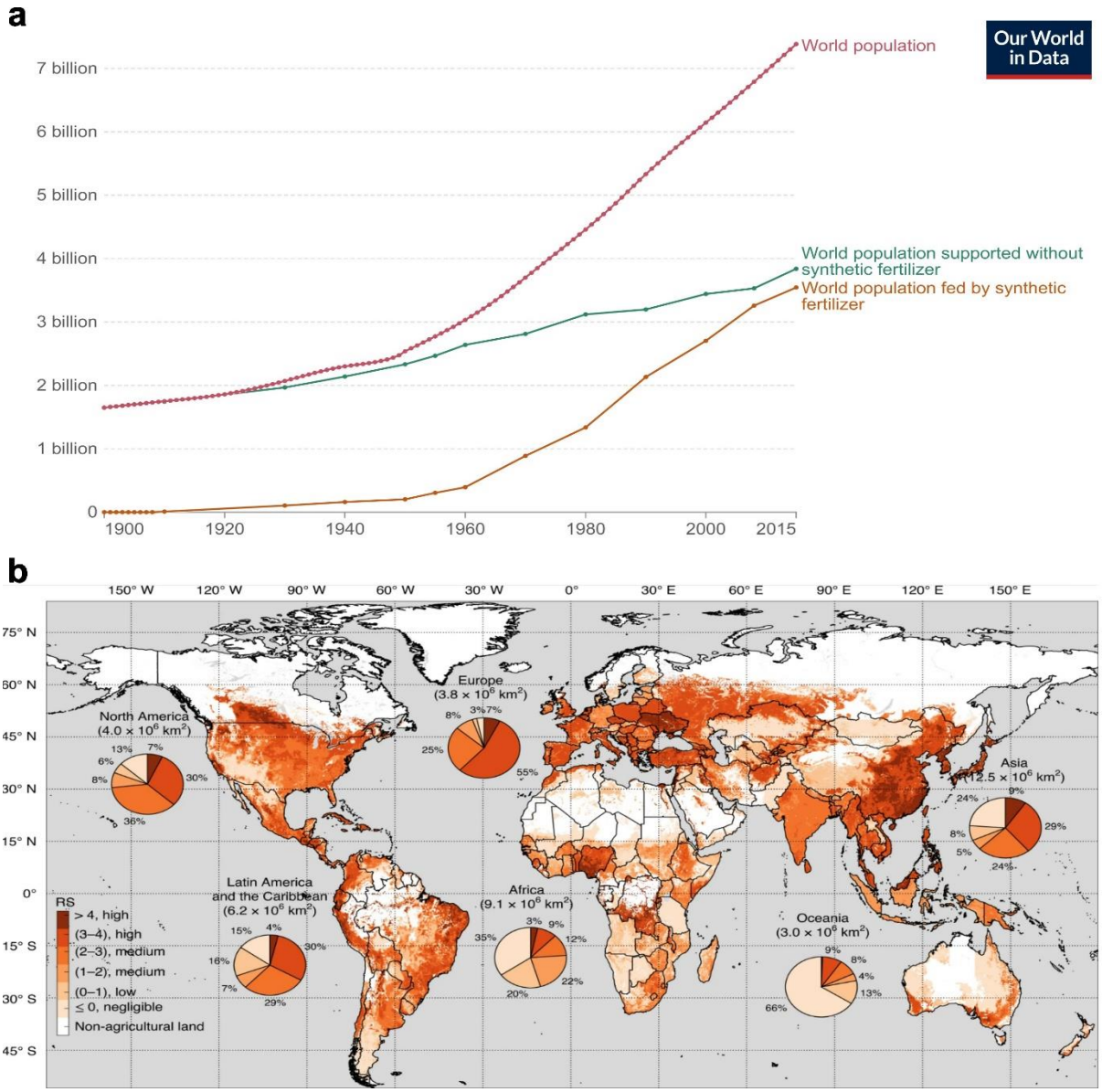


Figure 1. Two sides of the same agricultural coin. **(a)** Impact of synthetic nitrogen fertilizers on global population growth based on the Haber-Bosch process. Data obtained from Erisman et al. (2008); Smil (2002); Stewart et al. (2005), as cited by Ritchie (2017). **(b)** World pesticide risk map. In white, non-agricultural land; risk score (RS) ≤ 0 indicates negligible risk; RS 0-1, low risk; RS 1-3, medium risk; and RS > 3, high risk (Tang et al., 2021).

De novo domestication of wild or semi wild plants, achieved through the incorporation or manipulation of target genes via genome-editing, represents another promising technology for conferring plant resistance to pests or enhancing resistance to abiotic stresses. Genome editing like CRISPR-cas9 has demonstrated its efficiency in *de novo* domestication of wild tomato

(*Solanum pimpinellifolium*). In a single generation, editing six genes resulted in morphological modifications, including a threefold increase in fruit size, a tenfold increase in fruit number, and improvements in nutritional value. Lycopene accumulation in *de novo* domesticated tomato was 510 mg kg^{-1}, a 500% increase compared to the lycopene accumulation (92 mg kg^{-1}) observed in cherry tomato (*Solanum lycopersicum*) (Zsögön et al., 2018).

In addition to plant breeding methods, the incorporation of high throughput phenotyping (HTP) technologies in agriculture can contribute significantly to the development of high-yield crops and the monitoring of plant diseases. HTP platforms can examine several parameters, including plant architecture, water use efficiency, photosynthetic activity, transpiration, or pathogen detection (Danzi et al., 2019). For instance, thermal infrared cameras can distinguish between healthy and unhealthy plants by detecting variations in leaf temperature (Berdugo et al., 2014). While hyperspectral imaging (HSI) combines spectral (e.g. chemical composition) and image (e.g. color, shape, size) information to identify biotic and abiotic stress. Moshou et al. (2014) accurately identified drought-stressed plants within wavelengths of 460-900 nm, achieving over 99% accuracy after incorporating machine learning into HIS.

An important aspect of implementing the aforementioned technologies is considering consumer acceptance. This is the case with genetically modified organisms (GMOs), which have low public approval and have sparked controversial debate (Dona and Arvanitoyannis, 2009; German National Academy of Sciences Leopoldina, 2019; Lundquist, 2015; Qaim, 2020). Unlike GMOs, the use of living microorganisms such as plant growth promoting rhizobacteria (PGPR) has increasingly garnered interest and acceptance as a sustainable agricultural strategy. The capabilities of PGPR can contribute to diminishing the negative effects caused by chemical fertilizers and pesticides. Nevertheless, to transition to feasible sustainable agricultural systems, it is necessary to integrate all these technologies. On one hand, to potentiate crop yield efficiency while minimizing environmental repercussions. On the other hand, improving our understanding of the interactions between plants and their environment at different scales. In this regard, the application of high-throughput sequencing technologies has revealed various aspects related to the composition and dynamics of plant and microbial communities. The use of marker genes such as 16S rRNA, combined with shotgun metagenome analysis, has determined that microbiome differentiation in barley roots is mainly driven by host-microbe and microbe-microbe interactions occurring in the rhizosphere (Bulgarelli et al., 2015). Delving deeper, the use of metatranscriptomics provides essential information about functional gene expression. Hayden et al. (2018) reported on the functional activities of microbial communities

in the wheat rhizosphere between disease-suppressive and non-suppressive soils for *Rhizoctonia solani*. More oxidative stress (e.g. superoxide dismutase) and antibiotic (e.g. phenazine) genes were found in the non-suppressive soil, while more cold-shock and polyketide stress genes were identified in the suppressive soil. This study has begun to shed light on the functional patterns that could be used for *Rhizoctonia* suppression. Additionally, the use of metabolomics approaches has become relevant for unraveling the complexity of plant-microbe and microbe-microbe interactions. A recent study has shown how seed inoculation with *Paenibacillus alvei* and *Bacillus subtilis* can cause metabolite reprogramming in the rhizosphere and leaves of wheat. The accumulation of metabolites such as organic acids, lipids, benzenoids, and phenylpropanoids was related to plant performance, chemotaxis, and plant-microbe interactions (Mashabela et al., 2022). The integration of various high-throughput sequencing technologies will not only enhance our understanding of the dynamics and interactions between plants and microorganisms but will also improve the engineering and development of more effective products based on living microorganisms.

1.2. Plant growth promoting rhizobacteria (PGPR)

Soil harbors a vast microbial diversity, with up to 1 x 10^{10} microorganisms contained in just one gram. Over a century ago, Lorenz Hiltner introduced the term 'rhizosphere' to refer to the zone where soil is affected by plant roots (Hiltner, 1904). The rhizosphere can be defined as the narrow zone of soil surrounding plant roots where chemical, biological, and physical processes actively occur and are influenced by the root system and its exudates (Hartmann et al., 2008; Weller and Thomashow, 1994). The active interactions between roots and soil are primarily driven by root exudates, including enzymes (e.g. amylase, phosphatase), hormones (e.g. auxins, gibberellins, strigolactones), amino acids (α-alanine, histidine), organic acids (e.g. butyric acid, acetic acid), sugars (e.g. glucose, sucrose), and other substances (e.g. sterols, lipids, flavonoids) (for review: Wang et al., 2022). These organic compounds can recruit beneficial microorganisms, leading to intensive microbial activity (Fig. 2). In fact, the microbial communities in the rhizosphere are estimated to be around 1 x 10^{9} cells cm^{-3} (Weller and Thomashow, 1994). Nevertheless, in the last decade, the conception of the rhizosphere has begun to switch toward a more holistic approach. York et al. (2016) stated that the interconnectedness and dynamic processes taking place in the rhizosphere should also be considered. Therefore, the term 'holistic rhizosphere' encompasses macroorganisms (e.g. nematodes, earthworms), mucigel (soil particle agglutination), microbial community gradients (e.g. microbial hotspots), and zones of accumulation or depletion of root exudates, water,

volatiles, gases, and nutrients. Altogether, these components have led to the conception of the rhizosphere as dynamic rather than static. Thus, studying the complexity of the 'holistic rhizosphere' can enhance our understanding of plant-microbe interactions, nutrient cycling, and soil structure.

The term plant growth promoting rhizobacteria (PGPR) was first coined by Kloepper and Schroth (1978) to describe bacteria in the rhizosphere that are able to colonize plant roots and promote plant growth. Later, Kloepper (1994) pointed out that PGPR, as intrinsic characteristics, must be able to colonize plant roots, proliferate, compete with other rhizosphere microorganisms, survive, and promote plant growth. Bacteria such as *Azospirillum*, *Azotobacter*, *Burkholderia*, *Pseudomonas*, *Serratia*, *Agrobacterium*, *Rhizobium*, *Paenibacillus*, and *Bacillus* have been employed and commercialized as PGPR. Plant growth enhancement by PGPR utilizes a wide range of well-studied mechanisms such as nitrogen fixation, phosphorus solubilization, iron sequestration, phytohormone production, and the triggering of systemic disease resistance (For review: Khoso et al., 2024; Oleńska et al., 2020)

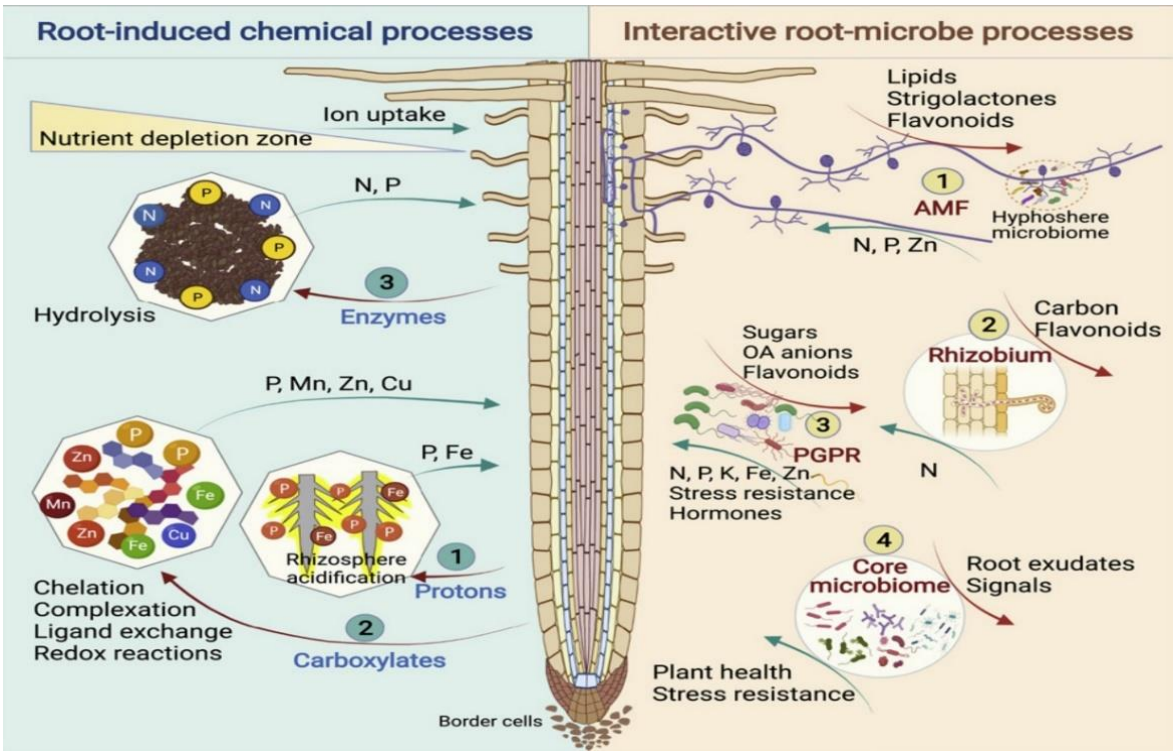


Figure 2. Schematic representation of the chemical and microbial interactions in the rhizosphere. Chemical interactions include: (**1**) roots induce changes in pH value due to proton release, (**2**) enhance nutrient availability through carboxylate secretion, and (**3**) hydrolyze organic compounds via enzyme exudation. Microbial interactions between roots and: (**1**) arbuscular mycorrhizal fungi (AMF), (**2**) nitrogen fixing bacteria, (**3**) PGPR, and (**4**) soil microbial community structure (Wang et al., 2022).

1.2.1. Nitrogen fixation

Certain bacteria are able to convert atmospheric nitrogen (N_2) into ammonia (NH_3) through the action of nitrogenase enzymes (Hardy et al., 1973). The most common nitrogen fixing bacteria used for nitrogen fixation and plant growth promotion include *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, and *Bacillus* (Bai et al., 2003; Favero et al., 2022; Figueiredo et al., 2008; Remans et al., 2008). In symbiotic nitrogen-fixing bacteria that form nodules with legume roots (e.g. *Rhizobium*), ammonia is excreted from the bacteroid to the symbiosome, where it is protonated to ammonium (NH_4^+) due to the acidic conditions (Mylona et al., 1995; Schwember et al., 2019; Whitehead et al., 1998). In free-living nitrogen-fixing bacteria (e.g. *Azotobacter*), ammonia produced inside the cell can be converted to NH_4^+, which can then be transported either into or out of the cell via ammonium transporters (Brewin et al., 1999; Day et al., 2001; Van Dommelen et al., 1998). Both ammonia and ammonium can be assimilated by plants, supporting plant nutrition and promoting plant growth. However, several authors argued that the contribution of diazotrophic bacteria to plant growth promotion in non-legumes is not primarily due to nitrogen fixation but rather to other PGPR abilities (Giller et al., 2024; Lin et al., 1983; Okon and Labandera-Gonzalez, 1994; Schultz et al., 2017; Tien et al., 1979). For instance, Hurek et al. (1994) found that plant growth promotion of rice was not linked to nitrogen fixation by *Azoarcus* sp. after using Nif^- mutants. Instead, beneficial effects were attributed to increased mineral uptake or improved water efficiency. Similarly, studies on *Azospirillum* sp. suggest that plant growth promotion is mainly due to enhanced mineral uptake or root density instead of nitrogen fixation (Lin et al., 1983; Schultz et al., 2017; Tien et al., 1979). This increase in plant biomass has also been observed in wheat and barley after the application of several PGPRs, including *Azospirillum brasilense*, in combination with boron fertilizer (Turan et al., 2012).

1.2.2. Phosphorus solubilization

Phosphorus is considered a limiting nutrient because, although it is abundant in the soil, it is mostly present in insoluble forms (e.g. $Ca_3(PO_4)_2$, $CaHPO_4$). The soluble forms of phosphate that plants take up are principally monobasic ($H_2PO_4^{1-}$) and dibasic (HPO_4^{2-}) phosphate. Moreover, phosphorus solubilization and precipitation depend considerably on pH-value. Phosphate solubilizing bacteria (PSB), such as *Rhizobium*, *Bacillus*, and *Pseudomonas*, can provide soluble phosphate forms to the plants through the secretion of organic acids, including gluconic, 2-ketogluconic, citric, oxalic, or butyric acid (Chen et al., 2006; Kim et al., 1997; Vyas and Gulati, 2009). Nevertheless, the application of PSB has shown variable results in enhancing plant growth through improved phosphorus uptake (De Zutter et al., 2022; Meyer et

al., 2019; Pande et al., 2017; Raymond et al., 2021). A recent meta-analysis of 104 studies showed that increases in P-uptake do not always have a direct relationship with plant growth parameters such as biomass or height (De Zutter et al., 2022). Similarly, a critical review by Raymond et al. (2021) suggested that other indirect mechanisms, such as increasing root growth due to the secretion of phytohormones, could promote plant growth or enhance phosphorus availability by improving root-soil contact, as analyzed in studies on wheat (Hansen et al., 2020; Liu et al., 2019; Meyer et al., 2017).

1.2.3. Iron sequestration

Similar to phosphorus, iron is predominantly found in nature in its insoluble form as ferric ion Fe(III). PGPR are able to synthesize secondary metabolites known as siderophores, which have a high affinity for iron. Siderophores bind Fe(III) ions, forming a siderophore-iron complex in the microbial membrane that facilitates the uptake of Fe(II) into the cells. Siderophores secreted by PGPR have proved to be effective in biocontrol through siderophore-mediated competition, in which PGPR restrict iron availability to pathogens (Kloepper et al., 1980; Shao et al., 2024; Shen et al., 2022). Several siderophore producers, including *Bacillus*, *Pseudomonas*, and *Streptomyces*, have shown antagonistic effects against fungal pathogens such as *Fusarium oxysporum* or *Alternaria* sp. (Chaiharn et al., 2009; Qiao et al., 2017; Wang et al., 2020). In addition, siderophores have demonstrated to form complexes with other metals, such as cobalt, zinc, manganese, and molybdenum, thereby extending their role in biological metal uptake and metal cycling (Harrington et al., 2012; for review: Kramer et al., 2020). For instance, *Pseudomonas aeruginosa* secretes two siderophores, pyochelin and pyoverdine, which can chelate other metals with lower affinity than iron, such as Al(III), Cu(II), Ni(II), Zn(II) (Andrejević et al., 2023; Braud et al., 2010). In the same way, coelibactin, a siderophore secreted by *Streptomyces coelicolor*, is capable of chelating zinc (Kallifidas et al., 2010).

1.2.4. Plant growth regulators

PGPR can synthesize, secret, and regulate organic compounds known as phytohormones or plant growth regulators (PGR), which, at low concentrations, can influence morphological, physiological, and biochemical processes. In addition to the five well-characterized PGR auxins, cytokinins, gibberellins, abscisic acid, and ethylene, several other hormones have been identified in recent decades, including salicylic acid, jasmonate, strigolactones, brassinosteroids, and nitric oxide (for review: Khan et al., 2020; Santner and Estelle, 2009).

The main naturally occurring and well-characterized auxin is indol-3-acetic acid (IAA), which is involved in several processes, including root architecture (adventitious and lateral root

development), cell division, apical dominance, and abiotic stress resistance. Cytokinins, which are associated with cell division, and gibberellins, which promote stem elongation, are also related to plant growth promotion (Aloni et al., 2006; Boiero et al., 2007). Abscisic acid (ABA) is involved in stomatal closure and also plays a role in plant growth. Finally, ethylene (C_2H_4) is a gas which is associated with root architecture, germination, and senescence. However, under stress conditions, excessive production of ethylene leads to detrimental effects on plants. Moreover, the intricate signaling network between different PGR has led to antagonistic, synergistic, and additive interactions (Fan et al., 2009; Mayak et al., 1999; Stearns et al., 2012). Root development can be modulated by cytokinins and auxins, and in combination with ethylene, these three PGR can control lateral root formation (Aloni et al., 2006). More intricately, ethylene levels can be modulated by PGPR in different ways. On one side, after plants take up IAA produced by PGPR, they increase the synthesis of 1-aminocyclopropane-1-carboxylate (ACC) through the action of the ACC synthase enzyme. As ACC is the precursor of ethylene, IAA uptake results in higher levels of ethylene. Although PGRs are linked and involved in root development, an overproduction of IAA can suppress root elongation, likely due to elevated ethylene concentrations (Mayak et al., 1999). On the other hand, PGPR can also synthesize the enzyme ACC deaminase, which, in contrast to ACC synthase, cleaves ACC into ammonia and α-ketobutyrate, thereby reducing ethylene levels. This particular capability of PGPR has been reported as an efficient way to reduce plant stress (Stearns et al., 2012). Similarly, antagonistic and synergistic relationships between ABA and salicylic and jasmonic acids have been associated with different plant-pathogen interactions (Fan et al., 2009).

1.2.5. Understanding rhizobacteria signaling to enhance PGPR application

The plant-microbe and microbe-microbe communications are mediated through the secretion of chemical signaling molecules by plants and microbes in the rhizosphere. Understanding these interactions in greater detail will contribute to improving sustainable strategies, including the use of living microorganisms such as PGPR (for review: Singh et al., 2022). As mentioned previously, one chemical signaling mechanism is mediated through the secretion of root exudates. These signaling molecules are capable of selectively choosing certain beneficial microbes through synergistic and antagonistic mechanisms among microorganisms. For instance, the secretion of arabinogalactan proteins (AGPs) from root tips is not only involved in plant development. Xie et al. (2012) showed that AGPs secreted by *Pisum sativum* can attract beneficial rhizobacteria such as *Rhizobium leguminosarum*. Meanwhile, Cannesan et al. (2012)

revealed the role of AGPs in protecting the root cap against the oomycete *Aphanomyces euteiches*.

Chemotaxis has elucidated important aspects of bacterial-root associations, including the identification of chemoreceptor proteins like methyl-accepting chemotaxis proteins (MCPs), chemotactic responses to various compounds (e.g. sugars, amino acids), and root colonization by certain rhizobacteria (Bacilio-Jiménez et al., 2003; Feng et al., 2018). Delving deeper into the specificity of several chemoreceptors, a recent systematic evaluation of 98 chemical compounds by Feng et al. (2018) identified 5 chemorepellents and 39 chemoattractants for the PGPR *Bacillus amyloliquefaciens*. After deleting eight chemoreceptor (MCP) genes, the chemotactic ability to 44 compounds was totally lost. Moreover, the authors identified a major chemoreceptor for several chemoattractants (McpA), chemoreceptors with narrow compound range (McpC), and a specific chemoreceptor responsible for arginine chemotaxis (McpR).

Microorganisms communicate through a mechanism known as quorum sensing (QS), which can occur among individuals of the same species (intraspecies communication), between different species (interspecies communication), or even across different kingdoms (interkingdom communication; Fig. 3) (Bramhachari, 2019). These cell-to-cell interactions involve the production and detection of signaling molecules known as autoinducers (AIs), which sense bacterial population density, distribution, and diffusion space within their environment. Moreover, QS has been related to the regulation of several functions, including biofilm formation, root colonization, antibiotic resistance, and virulence factors (Reimmann et al., 2002; Wei and Zhang, 2006). Biofilms are multicellular communities that adhere to surfaces and are embedded in a polymeric matrix, recently referred to as the matrixome (for review: Karygianni et al., 2020). This matrix confers structural stability, supporting the survival and proliferation of the microbial community, and it is mainly composed of extracellular polymeric substances (EPS), which include polysaccharides (e.g., exopolysaccharides Psl, Pel), proteins (e.g., TasA), nucleic acids (extracellular DNA), lipids, and lipopolysaccharides (da Cruz Nizer et al., 2024; DeFrancesco et al., 2017; Diehl et al., 2018; Spiers and Rainey, 2005). The counterpart of QS is referred to as quorum quenching (QQ), which is responsible for disrupting quorum sensing (Fig. 3; Dong et al., 2001). QQ inhibits QS through several enzymes and chemicals (e.g. lactonases, catechin, quercetin) that interfere with cell-to-cell communications and has been associated with the inhibition of biofilm formation and virulence factors, thus becoming a key biological control mechanism (Adonizio et al., 2008; Ouyang et al., 2016; Paluch et al., 2020; Vandeputte et al., 2010; Wang et al., 2004).

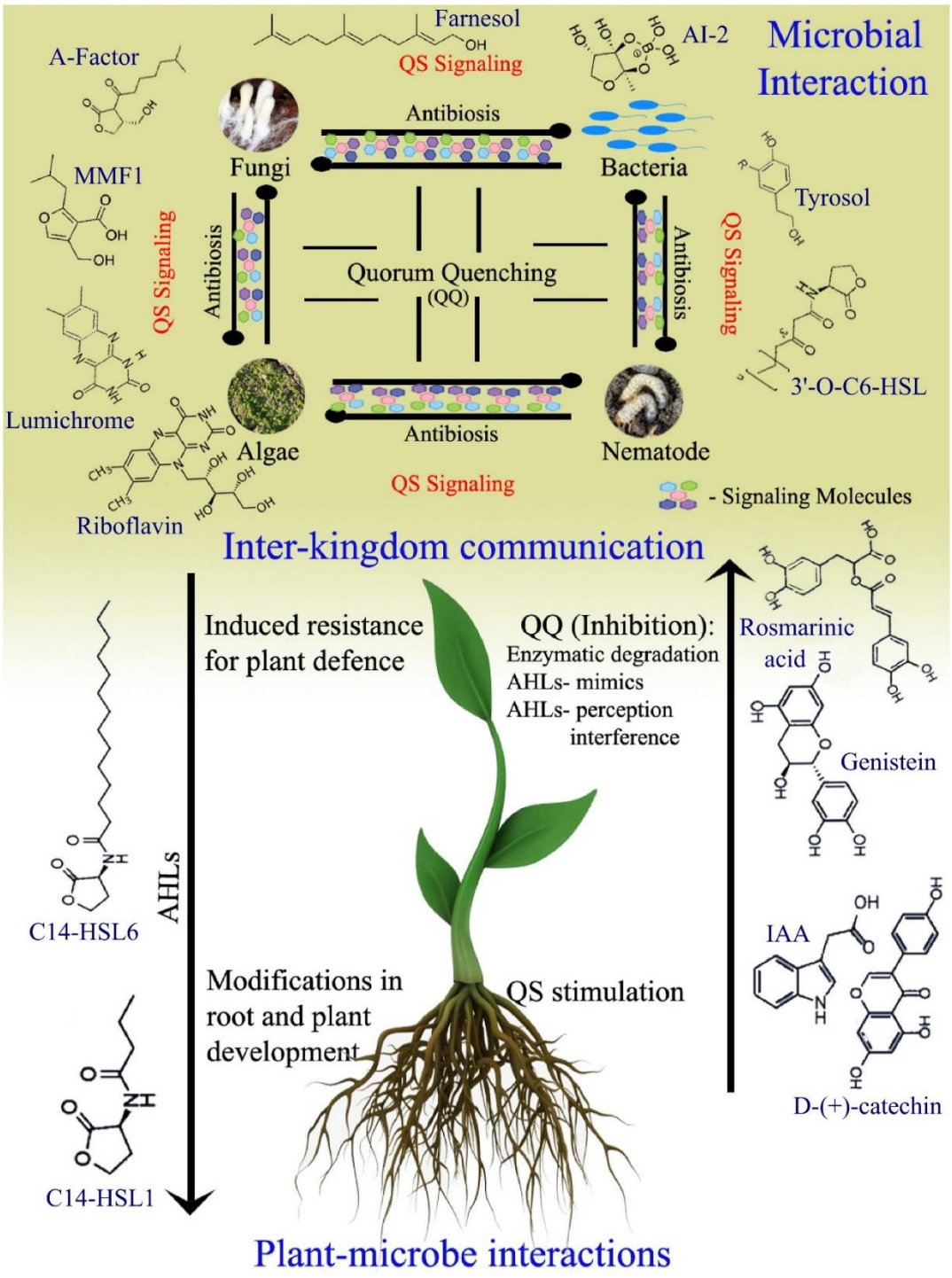


Figure 3. Signaling molecules involved during microbe-microbe and plant-microbe interactions. Interspecies, intraspecies, and interkingdom communications are mediated by the secretion of quorum sensing (QS) molecules, while quorum quenching (QQ) can disrupt these communications. Figure modified from Singh et al. (2022).

During QS, gram positive bacteria use oligopeptides as AIs, while gram negative bacteria employ N-acyl homoserine lactones (AHLs) (Hartmann et al., 2014). In contrast, QQ utilizes enzymes such as lactonases and acylases, which are responsible for hydrolyzing the lactone

ring and amide bonds of AHLs, respectively (Wang et al., 2004). AHLs can regulate gene expression, shape root architecture, and activate the systemic defense mechanisms in plants (Fig. 3). It has been stated that expressing the AHL degradation gene (aiiA), which encodes the AiiA lactonase from *Bacillus* sp., in *Pseudomonas aeruginosa* hinders its QS system, reducing the production of virulence factors and swarming motility of this opportunistic pathogen (Reimmann et al., 2002). Interestingly, AHL-mimic QS signals produced by bean (*Phaseolus vulgaris*) and rice (*Oryza sativa*) can affect biofilm formation in a plant-bacterial specific manner. While biofilm formation was improved in *Sinorhizobium fredii* by AHL-mimic QS signals from both plants, in the PGR *Pantoea ananatis*, only bean AHL-mimic QS signals inhibited biofilm formation (Pérez-Montaño et al., 2013).

Ortíz-Castro et al. (2008) identified several AHLs involved in root development in *Arabidopsis thaliana,* with N-decanoyl-HL (C10-HL) being the main AHL responsible for reshaping root morphology. Moreover, it has been reported that inoculation with AHL-producing PGPRs can trigger induced systemic resistance (ISR) (Pang et al., 2009; Schuhegger et al., 2006; Viswanath et al., 2016). For instance, Schuhegger et al. (2006) showed that AHLs elicited by *Serratia liquefaciens* MG1 and *Pseudomonas putida* IsoF triggered ISR against *Alternaria alternata* on tomato plants. A decrease of more than 70% in necrotic cell death was observed after *S. liquefaciens* inoculation. Furthermore, AHL compounds were associated with salicylic acid (SA) accumulation in roots, as well as with increased expression of genes involved in ethylene signaling, antioxidant system, and defense responses in tomato plant leaves. This study complements existing knowledge on ISR, which, along with systemic acquired resistance (SAR), have been described as plant defense mechanisms against pathogens. Their particularities are outlined in Table 1 (for review: Choudhary et al., 2007; Van Loon et al., 1998).

Unveiling the intricate networks of rhizobacterial signaling has led to a better understanding of their interactions, regulation, and suppression mechanisms with other microorganisms and host plants. The main contribution of these discoveries has been the development of novel strategies for pathogen suppression and the control of plant disease (Cannesan et al., 2012; Ortíz-Castro et al., 2008; Pérez-Montaño et al., 2013; Reimmann et al., 2002).

Beyond agriculture, knowledge of bacterial signal-response has been applied in other fields, such as the food industry and medicine. Resveratrol has been proven to disrupt biofilm formation and interfere with QS in the foodborne pathogens *Arcobacter butzleri* and *Campylobacter* spp. (Duarte et al., 2015). Similarly, curcumin has shown to inhibit the

development of uropathogens due to its anti-QS and antibiofilm activities (Packiavathy et al., 2014). Moreover, QS in *Pseudomonas aeruginosa* has been related to its CRISPR-Cas adaptive immune system, suggesting medical applications through the use of anti-QS compounds to suppress the bacterial CRISPR-Cas defense mechanism (Høyland-Kroghsbo et al., 2017). Nevertheless, plant-microbe interactions are still far from being completely understood, especially when an external microorganism is introduced into the resident soil microbial communities. The incorporation of other approaches such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics of rhizosphere communities can contribute to fill this gap.

Table 1. Typical characteristics of ISR and SAR as plant immune systems (Choudhary et al., 2007; Van Loon et al., 1998).

		Plant immune systems	
		ISR	**SAR**
Features	Pathogen spectrum	- Broad	- Broad
	Trigger mechanism	- Non-pathogenic/beneficial rhizobacteria	- Pathogen infection
	Mode of action	- Activation of defense related genes (priming)	- Expression of pathogenesis related genes (PRs)
	Signaling pathways	- Jasmonic acid (JA) - Ethylene	- Salicylic acid (SA)
	Plant defense mechanisms	- Plant defense response - Complex immune process	- Plant defense response - Complex immune process

1.3. Microbial composition dynamics in response to the introduction of foreign microorganisms

Microbial composition is constantly subjected to many disturbances, to which soil microorganisms have evolved to react and adapt. Three possible ways in which microbial communities respond to disturbances have been proposed (Fig. 4) (Allison and Martiny, 2008). Microbial composition is considered **resistant** when a disturbance does not significantly alter its original state. In fact, it has been stated that introducing an exogenous microorganism is strongly buffered by native soil microbial communities (Björklöf et al., 2003). When the microbial composition is modified by an introduced disturbance but is able to rapidly recover

to its original state, it is considered **resilient**. For instance, it has been shown that inoculation with *Bacillus subtilis* caused a temporary shift, at different degrees, in lettuce rhizosphere microbial communities, lasting three days in bacterial communities and 14 days in eukaryotic communities (Qiao et al., 2017). Lastly, microbial composition is deemed **functionally redundant** when, despite a disturbance affecting its structure, certain taxa with similar ecological functions remain, preventing an alteration of the overall ecological process (Fig. 4). In a microcosms experiment, Wohl et al. (2004) found that higher species richness is related to higher cellulose decomposition rates, indicating that functionally redundant species can positively influence ecosystem function. However, a better comprehension of functional genes and the composition of microbial communities is required to elucidate the contribution of functional redundancy to ecosystem processes.

Allison and Martiny (2008) analyzed several studies and found that microbial composition was particularly sensitive to elevated CO_2 (60%), temperature (82%), carbon amendments (83%), and N/P/K fertilization (84%). Moreover, the authors discovered that while microbial communities are susceptible to disturbances, they do not rapidly return to their original state. In fact, it was determined that after a couple years, the disturbances can still persist, and functionally redundant taxa might not be as common as originally thought, potentially having direct impacts on ecosystem processes. In this context, the impact of bioinoculants on soil microbial composition has acquired more relevance in the last decades, especially with the emergence of next generation sequencing (NGS) technologies that have enabled the analysis of different aspects of microbial communities. In agreement with these authors, a recent analysis of more than 100 studies determined that introducing exogenous microorganisms not only shifted soil microbial communities in 86% of the cases but these communities also failed to recover their original state in most of the cases (for review: Mawarda et al., 2020). Nevertheless, only 36% of these studies have been conducted under field conditions, and even fewer have been longitudinal studies (Quiroga et al., 2025). Given the marked differences between laboratory or greenhouse and field experiments, it is necessary to evaluate these impacts *in situ* conditions in order to elucidate the real effects of introducing living microorganisms into the soil. Moreover, incorporating longitudinal analysis is crucial: (i) to assess the potential long-lasting effects of bioinoculants in soil microbial populations and (ii) to gain deeper insights into microbial community dynamics over time.

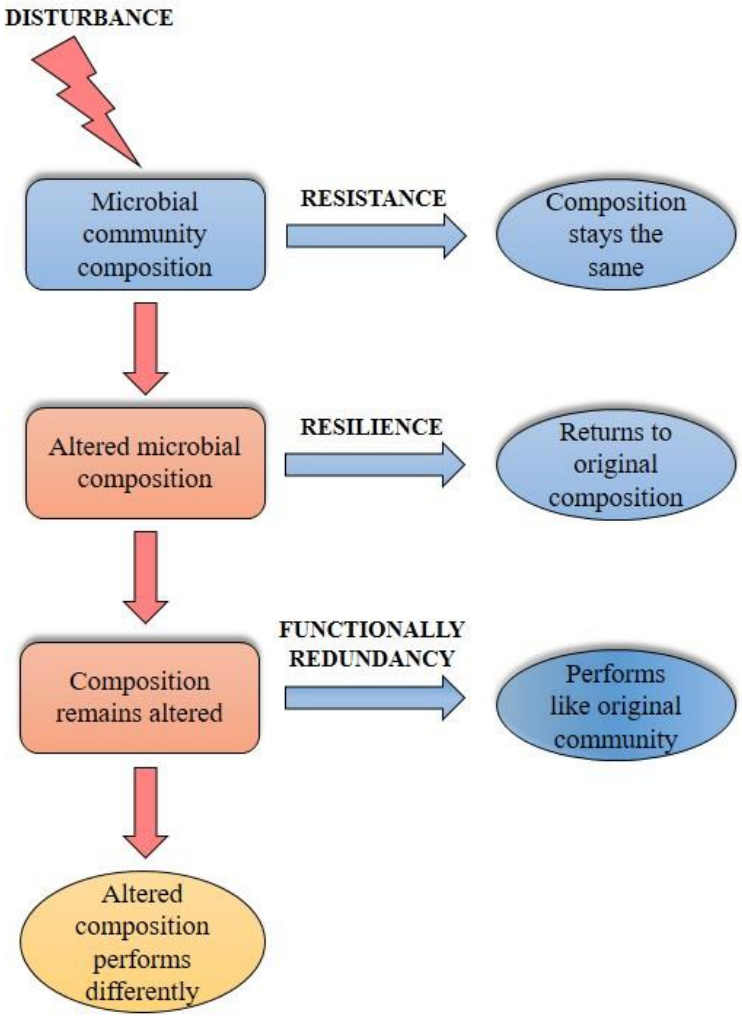


Figure 4. Schematic representation of the three possible microbial community responses to a disturbance. Figure adapted from Allison and Martiny (2008).

1.4. Reported inconsistencies: Why do bioinoculants fail beyond the laboratory?

As mentioned above, the mechanisms that PGPR employ to promote plant growth have been extensively studied over decades, primarily under gnotobiotic conditions or at a greenhouse level. However, applying PGPR under field conditions involves additional challenges and difficulties. Harsh and fluctuating environmental conditions, biotic and abiotic stresses, and competition with the native soil microbial communities have created a discrepancy about the efficiency of PGPR *in situ* conditions. This reported inefficacy is not recent. In fact, even before Kloepper and Schroth (1978) introduced the term PGPR, Brown (1974) had already analyzed inconsistencies in certain bacteria, showing that this was not the first study on this topic. Since then, several authors have documented these incongruities up to the present day (Li et al., 2023; Martínez-Viveros et al., 2010; Shaharoona et al., 2008). The reasons for the failure of bioinoculants under field conditions are multiple, complex, and cannot be excluded from each

other as they are interconnected. Starting with the soil matrix, which harbors plants and microbes, its properties, along with the environmental conditions, can influence the success of introducing a new microorganism (for review: Mendoza-Suárez et al., 2021; Rodríguez-Navarro et al., 2007). Hungria and Vargas (2000) reported that soil acidity, water stress, and elevated temperatures are the main factors affecting the capacity of rhizobia to fix nitrogen through nodulation with grain legumes in tropical soils. Interestingly, it was discussed that improving grain yield in soybean and common bean can show promising results after screening and inoculating indigenous rhizobia adapted to these abiotic stress conditions. From the plant side, root exudates or plant variety can also influence the efficacy of a bioinoculant to varying degrees (Kuzmicheva et al., 2017; Saadaoui et al., 2022; Valente et al., 2020). Saadaoui et al. (2022) observed that the response of durum wheat to PGPR inoculation varied among varieties. The Bousselam variety showed greater improvement in several parameters including germination capacity, chlorophyll content, and reductions in malondialdehyde (MDA) and proline levels, compared to Boutaleb wheat variety. More interestingly, Valente et al. (2020) tested the effect of the PGPR *Pseudomonas kilonensis* on several modern and ancient wheat varieties and found more positive interactions between PGPR and ancient wheat varieties (e.g., root colonization, gene expression) compared to the modern ones. These findings suggest that high-yield wheat varieties do not promote plant genes involved in interactions with PGPR, possibly due to the introduction of dwarf wheat varieties, as well as differences in root architecture or metabolic composition compared to ancient varieties.

The scarce information about native soil microbial communities' response to an allochthonous microorganism, as well as the dynamics (e.g. root colonization, survival) of the introduced microorganism, are important aspects for PGPR improvement. A recent publication showed that *Bacillus amyloliquefaciens* population in the rhizospheric soil fluctuates throughout the soybean plant life cycle, indicating its potential as a biocontrol agent. After root colonization, the bacterial population of *B. amyloliquefaciens* decreased in an initial stage, reached its highest concentration at flowering stage, and eventually decreased again while remaining detectable 108 days after seedling emergence (Zhang et al., 2020). Finally, the predominance of positive PGPR results over negative ones due to the academic publishing system is an important factor that can skew the real efficacy of bioinoculants (Cardinale et al., 2015; Mallon et al., 2018). In wheat and barley, the positive effects of PGPR are more prevalent (Baffoni et al., 2015; Canbolat et al., 2006; Liu et al., 2022; Turan et al., 2012), with only a few negative or inconsistent results reported (Di Salvo et al., 2018; Javaid et al., 2008; Mayer et al., 2010). Javaid et al. (2008) found non-significant effects in wheat grain yield, shoot, and root dry weight

after the inoculation of a commercial biofertilizer combined with different soil amendments, even finding adverse effects in the wheat grain yield in some of the treatments. The authors attributed differences in soil type, soil nutrient status, plant species, and cultivation history as possible factors that can affect plant responses. Similarly, in a four-year field trial conducted on an organic farm with several crops, including wheat and barley, the spraying application of two commercial bioinoculants showed significant but inconsistent effects over the years, and only when combined with an organic substrate (Mayer et al., 2010).

1.5. Monitoring Bioinoculants

Understanding how and to what extent living microorganisms colonize, compete, and survive in the environment where they have been introduced can provide important information about their success as plant growth enhancers, as well as their dynamics with the host during its life cycle. However, monitoring of bioinoculants is not a common practice despite the availability of several methods. Rilling et al. (2019) pointed out that less than 25% of studies use tracking methods. Typically, the main parameters considered to determine the effect of PGPR on plants are plant biomass or plant physiology. These authors categorized PGPR tracking methods into three groups (Fig. 5): immune-associated methods such as ELISA, immunofluorescence, and immunogold (Gyaneshwar et al., 2001; Lucas García et al., 2003; Odell and Cook, 2013; Yegorenkova et al., 2010), reporter gene-based methods such as *lacZ*, *gusA*, GFP, and *luxAB* (De Weger et al., 1997; Krzyzanowska et al., 2012; Ramos et al., 2002; Solanki and Garg, 2014), and nucleic acid base methods like quantitative qPCR or FISH (Rahman et al., 2018; Stets et al., 2015; Urrea-Valencia et al., 2021).

ELISA is the most common immunoassay method, which detects PGPR through an enzymatic reaction involving antigen-antibody reactions. For instance, *Pseudomonas fluorescens* was detected in the rhizosphere but not in the endorhizosphere of pepper seedlings using strain-specific monoclonal antibodies. This strain was found at high concentrations (CFU g^{-1} soil) up to 45 days after inoculation, linking its competence and colonization capacity with biometric plant parameters (Lucas García et al., 2003). Nevertheless, immune-associated methods can be time consuming and expensive due to antibody production or animal immunization. For instance, Yegorenkova et al. (2010) developed polyclonal antibodies in rabbits against *Paenibacillus polymyxa* exopolysaccharides to examine bacterial root colonization in wheat seedlings. The study found a positive correlation between the detection of exopolysaccharides and CFU counts, indicating that the concentration of *P. polymyxa* in the wheat roots increased over the evaluated time periods.

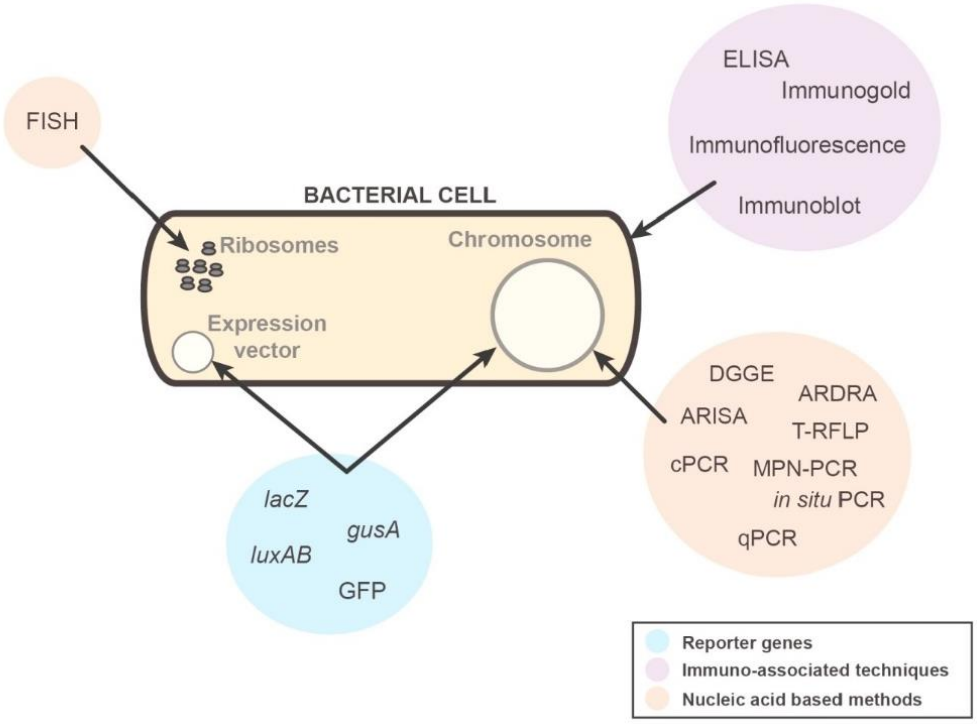


Figure 5. Plant growth promoting rhizobacteria tracking methods. FISH: fluorescence *in situ* hybridization. LacZ: β-galactosidase, gusA: β-glucoranidase, luxAB: bacterial luciferase, GFP: green fluorescent protein, ELISA: enzyme-linked immunosorbent assay, DGGE: denaturing gradient gel electrophoresis, ARISA: automatic ribosomal interspace spacer analysis, ARDRA: amplified ribosomal DNA restriction analysis, T-RFLP: terminal restriction fragment length polymorphism, MPN-PCR: most probable number PCR, cPCR: competitive PCR, qPCR: quantitative PCR (for review: Rilling et al., 2019).

Reporter gene methods can detect the expression of a target gene and visualize it through fluorescence (GFP), luminescence (luxAB), or color (lacZ, gusA) (De Weger et al., 1997; Ramos et al., 2002; Villegas and Paterno, 2008). Employing the gusA gene marker, Villegas and Paterno (2008) determined that a *Pseudomonas* strain colonized sugar cane roots through root tips and lateral root fractures, as indicated by *x*-gluc blue-stained plants. However, one limitation of this method is that it cannot be used on the field because genetically modified bacteria could represent a biosafety risk if released into the environment. On the other hand, nucleic-acid based methods address many of the disadvantages of the previously mentioned methods, as they do not require genetic transformation or antibody production (Rahman et al., 2018; Rodrigues et al., 2008; Stets et al., 2015). Fluorescence *in situ* hybridization (FISH) is a nucleic-acid based method used for PGPR detection, where fluorescently labeled oligonucleotide probes bind to specific ribosomal RNA (rRNA) sequences (16S or 23S). For example, using 16S rRNA based oligonucleotide probes, Rodrigues et al. (2008) identified 42 isolates of *Azospirillum amazonense* through FISH. Another widely used nucleic-acid based method is quantitative PCR (qPCR), which relies on the design of specific primers to ensure

high accuracy and specificity. Moreover, qPCR allows for quantitative measurements (Couillerot et al., 2010). Stets et al. (2015) detected and quantified *Azospirillum brasilense* in wheat roots up to 13 days after bacterization, with concentrations over 10^7 CFU g^{-1} fresh root under both sterile and non-sterile conditions (growth chamber). Moreover, the persistence of *A. brasilense* in maize has been demonstrated under field conditions, where the bacterial population was detected through qPCR in the soil at a concentration of 10^5 CFU g^{-1} one day after seeding (DAS), but was no longer detectable after the fifth day. In contrast, in the roots, the bacterium was found 75 DAS at a concentration of 10^4 CFU g^{-1} (Urrea-Valencia et al., 2021).

1.6. *Hartmannibacter diazotrophicus* strain E19^T as PGPR

Hartmannibacter diazotrophicus strain E19^T is a gram negative plant growth promoting rhizobacterium isolated from *Plantago winteri*, located in a natural salt meadow in Münzenberg, Germany (Suarez et al., 2014). The genus *Hartmannibacter* has been related to bacteria found in diverse environments such as the rhizosphere of *Panax gingseng* (Wei et al., 2020), high elevations (1400 m) (Martinez et al., 2021), and extreme environments such as thermal waters (Szuróczki et al., 2016). Recently, *H. diazotrophicus*, a member of the family *Pleomorphomonadaceae* (Hördt et al., 2020), has been proposed for reclassification into a new family, along with the genus *Methylobrevis* (Ludwig et al., 2021).

Research on *H. diazotrophicus* strain E19^T has shown to increase water content, as well as root and shoot dry weight, of barley plants under salt stress conditions in a greenhouse experiment (Suarez et al., 2015). Inoculation of alfalfa with the strain improved growth performance in both salt-tolerant and salt-sensitive cultivars under 10 dS m^{-1}. However, it was successful only in salt-tolerant cultivars at high salinity (20 dS m^{-1}) (Ansari et al., 2017). The use of specific FISH probes has demonstrated the presence of *H. diazotrophicus* strain E19^T mainly on the root surface (Suarez et al., 2015). The complete sequencing of its genome has identified several genes related to its plant growth promoting activities (Suarez et al., 2019).

1.7. Aim of the study

PGPR have been extensively investigated over the past 50 years, providing valuable information about the mechanisms they exert to promote plant growth (Section 1.2). Nevertheless, the long-lasting effects of PGPR on indigenous soil microbial communities, as well as their dynamics once released into the field, are not well understood (Sections 1.3 & 1.5). In the context of sustainable agriculture and organic farming, it is essential to improve yield

and grain quality while minimizing environmental impacts (Section 1.1). Grain quality is related to several parameters including protein and starch content, phytate, mineral elements, baking quality, and protein fractions such as glutenin, gliadin, and albumin/globulin. These quality parameters rely on specific requirements that vary according to the plant species. In wheat, improving nitrogen supply while increasing protein content is needed. In contrast, for brewery purposes, starch content prioritizes protein content in barley, which should be kept below 11% (Díaz et al., 2022). To cope with these challenges, the use of PGPR has gained important relevance alongside other technologies that need to be complemented (Section 1.1). However, there is a reported inconsistency when living microorganisms are applied under field conditions (Section 1.4).

Considering this background, the current research focuses more on the rhizosphere microbiome and yield parameters rather than on grain quality parameters under reduced input in organic farming. Therefore, the aims of the present study were: i) to evaluate the effects of *Hartmannibacter diazotrophicus* strain E19^{T}, row spacing, and fertilizer application on the rhizosphere bacterial communities of wheat and barley at two different organic farms over three consecutive seasons (2020-2023) through metabarcoding characterization; ii) to determine the bacterial dynamics and survival of *H. diazotrophicus* strain E19^{T} under field conditions; iii) to assess the effects of seed inoculation with *H. diazotrophicus* strain E19^{T}, row spacing, and fertilizer application on plant yield parameters.

1.8. References

Aasfar, A., Bargaz, A., Yaakoubi, K., Hilali, A., Bennis, I., Zeroual, Y., Meftah Kadmiri, I., 2021. Nitrogen fixing *Azotobacter* species as potential soil biological enhancers for crop nutrition and yield stability. Front. Microbiol. 12, 1–19. https://doi.org/10.3389/fmicb.2021.628379

Adonizio, A., Kong, K.F., Mathee, K., 2008. Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by south Florida plant extracts. Antimicrob. Agents Chemother. 52, 198–203. https://doi.org/10.1128/AAC.00612-07

Allison, S.D., Martiny, J.B.H., 2008. Resistance, resilience, and redundancy in microbial communities. Proc. Natl. Acad. Sci. U.S.A. 105, 11512–11519. https://doi.org/10.1073/pnas.0801925105

Aloni, R., Aloni, E., Langhans, M., Ullrich, C.I., 2006. Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann. Bot. 97, 883–893. https://doi.org/10.1093/aob/mcl027

Andrejević, T.P., Ašanin, D.P., Pantović, B. V., Stevanović, N.L., Marković, V.R., Djuran, M.I., Glišić, B., 2023. Metal complexes with valuable biomolecules produced by *Pseudomonas aeruginosa*: a review of the coordination properties of pyocyanin, pyochelin and pyoverdines. Dalt. Trans. 52, 4276–4289. https://doi.org/10.1039/d3dt00287j

Ansari, M., Shekari, F., Mohammadi, M.H., Biró, B., Végvári, G., 2017. Improving germination indices of alfalfa cultivars under saline stress by inoculation with beneficial bacteria. Seed Sci. Technol. 45, 475–484. https://doi.org/10.15258/sst.2017.45.2.02

Bacilio-Jiménez, M., Aguilar-Flores, S., Ventura-Zapata, E., Pérez-Campos, E., Bouquelet, S., Zenteno, E., 2003. Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. Plant Soil 249, 271–277. https://doi.org/10.1023/A:1022888900465

Baffoni, L., Gaggia, F., Dalanaj, N., Prodi, A., Nipoti, P., Pisi, A., Biavati, B., Di Gioia, D., 2015. Microbial inoculants for the biocontrol of *Fusarium* spp. in durum wheat. BMC Microbiol. 15, 8–10. https://doi.org/10.1186/s12866-015-0573-7

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

Beard, J., 2006. DDT and human health. Sci. Total Environ. 355, 78–89. https://doi.org/10.1016/j.scitotenv.2005.02.022

Beketov, M.A., Kefford, B.J., Schäfer, R.B., Liess, M., 2013. Pesticides reduce regional biodiversity of stream invertebrates. Proc. Natl. Acad. Sci. U. S. A. 110, 11039–11043. https://doi.org/10.1073/pnas.1305618110

Berdugo, C.A., Zito, R., Paulus, S., Mahlein, A.K., 2014. Fusion of sensor data for the detection and differentiation of plant diseases in cucumber. Plant Pathol. 63, 1344–1356. https://doi.org/10.1111/ppa.12219

Björklöf, K., Sen, R., Jørgensen, K.S., 2003. Maintenance and impacts of an inoculated mer/luc-tagged *Pseudomonas fluorescens* on microbial communities in birch rhizospheres

developed on humus and peat. Microb. Ecol. 45, 39–52. https://doi.org/10.1007/s00248-002-2018-8

Boiero, L., Perrig, D., Masciarelli, O., Penna, C., Cassán, F., Luna, V., 2007. Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl. Microbiol. Biotechnol. 74, 874–880. https://doi.org/10.1007/s00253-006-0731-9

Boschiero, M., De Laurentiis, V., Caldeira, C., Sala, S., 2023. Comparison of organic and conventional cropping systems: a systematic review of life cycle assessment studies. Environ. Impact Assess. Rev. 102. https://doi.org/10.1016/j.eiar.2023.107187

Bramhachari, P.V., 2019. Implication of quorum sensing system in biofilm formation and virulence. https://doi.org/10.1007/978-981-13-2429-1

Braud, A., Geoffroy, V., Hoegy, F., Mislin, G.L.A., Schalk, I.J., 2010. Presence of the siderophores pyoverdine and pyochelin in the extracellular medium reduces toxic metal accumulation in *Pseudomonas aeruginosa* and increases bacterial metal toleranceemi. Environ. Microbiol. Rep. 2, 419–425. https://doi.org/10.1111/j.1758-2229.2009.00126.x

Brewin, B., Woodley, P., Drummond, M., 1999. The basis of ammonium release in *nif*L mutants of *Azotobacter vinelandii*. J. Bacteriol. 181, 7356–7362. https://doi.org/10.1128/jb.181.23.7356-7362.1999

Brown, M.E., 1974. Seed and root bacterization. Annu. Rev. Phytopathol. 12, 181–197. https://doi.org/10.1146/annurev.py.12.090174.001145

Bulgarelli, D., Garrido-Oter, R., Münch, P.C., Weiman, A., Dröge, J., Pan, Y., McHardy, A.C., Schulze-Lefert, P., 2015. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe 17, 392–403. https://doi.org/10.1016/j.chom.2015.01.011

Canbolat, M.Y., Bilen, S., Çakmakçı, R., Şahin, F., Aydın, A., 2006. Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biol. Fertil. Soils 42, 350–357. https://doi.org/10.1007/s00374-005-0034-9

Cannesan, M.A., Durand, C., Burel, C., Gangneux, C., Lerouge, P., Ishii, T., Laval, K., Follet-Gueye, M.L., Driouich, A., Vicré-Gibouin, M., 2012. Effect of arabinogalactan proteins from the root caps of pea and *Brassica napus* on *Aphanomyces euteiches* zoospore chemotaxis and germination. Plant Physiol. 159, 1658–1670. https://doi.org/10.1104/pp.112.198507

Cardinale, M., Ratering, S., Suarez, C., Zapata Montoya, A.M., Geissler-Plaum, R., Schnell, S., 2015. Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress. Microbiol. Res. 181, 22–32. https://doi.org/10.1016/j.micres.2015.08.002

Chaiharn, M., Chunhaleuchanon, S., Lumyong, S., 2009. Screening siderophore producing bacteria as potential biological control agent for fungal rice pathogens in Thailand. World J. Microbiol. Biotechnol. 25, 1919–1928. https://doi.org/10.1007/s11274-009-0090-7

Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., Young, C.C., 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil Ecol. 34, 33–41. https://doi.org/10.1016/j.apsoil.2005.12.002

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

Clark, M., Tilman, D., 2017. Comparative analysis of environmental impacts of agricultural production systems, agricultural input efficiency, and food choice. Environ. Res. Lett. 12. https://doi.org/10.1088/1748-9326/aa6cd5

Couillerot, O., Bouffaud, M.L., Baudoin, E., Muller, D., Caballero-Mellado, J., Moënne-Loccoz, Y., 2010. Development of a real-time PCR method to quantify the PGPR strain *Azospirillum lipoferum* CRT1 on maize seedlings. Soil Biol. Biochem. 42, 2298–2305. https://doi.org/10.1016/j.soilbio.2010.09.003

da Cruz Nizer, W.S., Allison, K.N., Adams, M.E., Vargas, M.A., Ahmed, D., Beaulieu, C., Raju, D., Cassol, E., Howell, P.L., Overhage, J., 2024. The role of exopolysaccharides Psl and Pel in resistance of *Pseudomonas aeruginosa* to the oxidative stressors sodium hypochlorite and hydrogen peroxide. Microbiol. Spectr. 12, 1–18. https://doi.org/10.1128/spectrum.00922-24

Dalrymple, D.G., 1978. Development and spread of high-yielding varieties of wheat and rice in the less developed nations, Sixth. ed. Washington, D.C. https://doi.org/10.22004/ag.econ.330481

Danzi, D., Briglia, N., Petrozza, A., Summerer, S., Povero, G., Stivaletta, A., Cellini, F., Pignone, D., de Paola, D., Janni, M., 2019. Can high throughput phenotyping help food security in the mediterranean area? Front. Plant Sci. 10, 1–13. https://doi.org/10.3389/fpls.2019.00015

Day, D.A., Poole, P.S., Tyerman, S.D., Rosendahl, L., 2001. Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell. Mol. Life Sci. 58, 61–71. https://doi.org/10.1007/PL00000778

De Ponti, T., Rijk, B., Van Ittersum, M.K., 2012. The crop yield gap between organic and conventional agriculture. Agric. Syst. 108, 1–9. https://doi.org/10.1016/j.agsy.2011.12.004

De Weger, L.A., Kuiper, I., Van Der Bij, A.J., Lugtenberg, B.J.J., 1997. Use of a lux-based procedure to rapidly visualize root colonisation by *Pseudomonas fluorescens* in the wheat rhizosphere. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 72, 365–372. https://doi.org/10.1023/A:1000565413024

De Zutter, N., Ameye, M., Bekaert, B., Verwaeren, J., De Gelder, L., Audenaert, K., 2022. Uncovering new insights and misconceptions on the effectiveness of phosphate solubilizing rhizobacteria in plants: a meta-analysis. Front. Plant Sci. 13, 1–14. https://doi.org/10.3389/fpls.2022.858804

DeFrancesco, A.S., Masloboeva, N., Syed, A.K., DeLoughery, A., Bradshaw, N., Li, G.W., Gilmore, M.S., Walker, S., Losick, R., 2017. Genome-wide screen for genes involved in eDNA release during biofilm formation by *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. 114, E5969–E5978. https://doi.org/10.1073/pnas.1704544114

Di Salvo, L.P., Ferrando, L., Fernández-Scavino, A., García de Salamone, I.E., 2018. Microorganisms reveal what plants do not: wheat growth and rhizosphere microbial communities after *Azospirillum brasilense* inoculation and nitrogen fertilization under field conditions. Plant Soil 424, 405–417. https://doi.org/10.1007/s11104-017-3548-7

Díaz, A.B., Durán-Guerrero, E., Lasanta, C., Castro, R., 2022. From the raw materials to the bottled product: influence of the entire production process on the organoleptic profile of industrial beers. Foods 11, 3215. https://doi.org/10.3390/foods11203215

Diehl, A., Roske, Y., Ball, L., Chowdhury, A., Hiller, M., Molière, N., Kramer, R., Stöppler, D., Worth, C.L., Schlegel, B., Leidert, M., Cremer, N., Erdmann, N., Lopez, D., Stephanowitz, H., Krause, E., van Rossum, B.J., Schmieder, P., Heinemann, U., Turgay, K., Akbey, Ü., Oschkinat, H., 2018. Structural changes of TasA in biofilm formation of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S. A. 115, 3237–3242. https://doi.org/10.1073/pnas.1718102115

Dona, A., Arvanitoyannis, I.S., 2009. Health risks of genetically modified foods. Crit. Rev. Food Sci. Nutr. 49, 164–175. https://doi.org/10.1080/10408390701855993

Dong, Y.-H., Wang, L.-H., Xu, J.-L., Zhang, H.-B., Zhang, X.-F., Zhang, L.-H., 2001. Quenching quorum-sensing dependent bacterial infection by an N-acyl homoserine lactonase 813–817. https://doi.org/https://doi.org/10.1038/35081101

Duarte, A., Alves, A.C., Ferreira, S., Silva, F., Domingues, F.C., 2015. Resveratrol inclusion complexes: antibacterial and anti-biofilm activity against *Campylobacter* spp. and Arcobacter butzleri. Food Res. Int. 77, 244–250. https://doi.org/10.1016/j.foodres.2015.05.047

Erisman, J.W., Sutton, M.A., Galloway, J., Klimont, Z., Winiwarter, W., 2008. How a century of ammonia synthesis changed the world. Nat. Geosci. 1, 636–639. https://doi.org/10.1038/ngeo325

Fan, J., Hill, L., Crooks, C., Doerner, P., Lamb, C., 2009. Abscisic acid has a key role in modulating diverse plant-pathogen interactions. Plant Physiol. 150, 1750–1761. https://doi.org/10.1104/pp.109.137943

Favero, V.O., de Carvalho, R.H., Leite, A.B.C., dos Santos, D.M.T., de Freitas, K.M., Boddey, R.M., Xavier, G.R., Rumjanek, N.G., Urquiaga, S., 2022. *Bradyrhizobium* strains from Brazilian tropical soils promote increases in nodulation, growth and nitrogen fixation in mung bean. Appl. Soil Ecol. 175. https://doi.org/10.1016/j.apsoil.2022.104461

Feng, H., Zhang, N., Du, W., Zhang, H., Liu, Y., Fu, R., Shao, J., Zhang, G., Shen, Q., Zhang, R., 2018. Identification of chemotaxis compounds in root exudates and their sensing chemoreceptors in plant-growth-promoting rhizobacteria *Bacillus amyloliquefaciens* SQR9. Mol. Plant-Microbe Interact. 31, 995–1005. https://doi.org/10.1094/MPMI-01-18-0003-R

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

Gattinger, A., Muller, A., Haeni, M., Skinner, C., Fliessbach, A., Buchmann, N., Mäder, P., Stolze, M., Smith, P., Scialabba, N.E.H., Niggli, U., 2012. Enhanced top soil carbon stocks under organic farming. Proc. Natl. Acad. Sci. U.S.A. 109, 18226–18231. https://doi.org/10.1073/pnas.1209429109

German National Academy of Sciences Leopoldina, 2019. Towards a scientifically justified, differentiated regulation of genome edited plants in the EU.

Gerten, D., Heck, V., Jägermeyr, J., Bodirsky, B.L., Fetzer, I., Jalava, M., Kummu, M., Lucht,

W., Rockström, J., Schaphoff, S., Schellnhuber, H.J., 2020. Feeding ten billion people is possible within four terrestrial planetary boundaries. Nat. Sustain. 3, 200–208. https://doi.org/10.1038/s41893-019-0465-1

Giller, K.E., James, E.K., Ardley, J., Unkovich, M.J., 2024. Science losing its way: examples from the realm of microbial N_2-fixation in cereals and other non-legumes. Plant Soil. https://doi.org/10.1007/s11104-024-07001-1

Gyaneshwar, P., James, E.K., Mathan, N., Reddy, P.M., Reinhold-Hurek, B., Ladha, J.K., 2001. Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. 183, 2634–2645. https://doi.org/10.1128/JB.183.8.2634-2645.2001

Hansen, V., Bonnichsen, L., Nunes, I., Sexlinger, K., Lopez, S.R., van der Bom, F.J.T., Nybroe, O., Nicolaisen, M.H., Jensen, L.S., 2020. Seed inoculation with *Penicillium bilaiae* and *Bacillus simplex* affects the nutrient status of winter wheat. Biol. Fertil. Soils 56, 97–109. https://doi.org/10.1007/s00374-019-01401-7

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., Burns, R.C., 1973. The acetylene-ethylene assay for N_2 fixation. Plant Physiol 43, 1185–1207.

Harrington, J.M., Bargar, J.R., Jarzecki, A.A., Roberts, J.G., Sombers, L.A., Duckworth, O.W., 2012. Trace metal complexation by the triscatecholate siderophore protochelin: structure and stability. BioMetals 25, 393–412. https://doi.org/10.1007/s10534-011-9513-7

Hartmann, A., Rothballer, M., Hense, B.A., Schröder, P., 2014. Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. Front. Plant Sci. 5, 1–4. https://doi.org/10.3389/fpls.2014.00131

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

Hayden, H.L., Savin, K.W., Wadeson, J., Gupta, V.V.S.R., Mele, P.M., 2018. Comparative metatranscriptomics of wheat rhizosphere microbiomes in disease suppressive and non-suppressive soils for *Rhizoctonia solani* AG8. Front. Microbiol. 9, 1–19. https://doi.org/10.3389/fmicb.2018.00859

Hiltner, L.T., 1904. Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache [On recent experiences and problems in the field of soil bacteriology with special consideration of green manuring and fallowing]. Arbeit. Deut. Landw. Ges. Berlin 98, 59-78.

Hördt, A., López, M.G., Meier-Kolthoff, J.P., Schleuning, M., Weinhold, L.M., Tindall, B.J., Gronow, S., Kyrpides, N.C., Woyke, T., Göker, M., 2020. Analysis of 1,000+ type-strain genomes substantially improves taxonomic classification of *Alphaproteobacteria*. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.00468

Høyland-Kroghsbo, N.M., Paczkowski, J., Mukherjee, S., Broniewski, J., Westra, E., Bondy-Denomy, J., Bassler, B.L., 2017. Quorum sensing controls the *Pseudomonas aeruginosa* CRISPR-Cas adaptive immune system. Proc. Natl. Acad. Sci. U. S. A. 114, 131–135. https://doi.org/10.1073/pnas.1617415113

Hungria, M., Vargas, M.A.T., 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. F. Crop. Res. 65, 151–164. https://doi.org/10.1016/S0378-4290(99)00084-2

Hurek, T., Reinhold-Hurek, B., Van Montagu, M., Kellenberger, E., 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. 176, 1913–1923. https://doi.org/10.1128/jb.176.7.1913-1923.1994

Javaid, A., Bajwa, R., Anjum, T., 2008. Effect of heat-sterilization and EM (effective microorganisms) application on wheat (*Triticum aestivum* L.) grown in organic-amended sandy loam soil. Cereal Res. Commun. 36, 489–499. https://doi.org/10.1556/CRC.36.2008.3.13

John, D.A., Babu, G.R., 2021. Lessons from the aftermaths of green revolution on food system and health. Front. Sustain. Food Syst. 5, 1–6. https://doi.org/10.3389/fsufs.2021.644559

Ju, X.T., Xing, G.X., Chen, X.P., Zhang, S.L., Zhang, L.J., Liu, X.J., Cui, Z.L., Yin, B., Christie, P., Zhu, Z.L., Zhang, F.S., 2009. Reducing environmental risk by improving N management in intensive chinese agricultural systems. Proc. Natl. Acad. Sci. U.S.A. 106, 3041–3046. https://doi.org/10.1073/pnas.0813417106

Kallifidas, D., Pascoe, B., Owen, G.A., Strain-Damerell, C.M., Hong, H.J., Paget, M.S.B., 2010. The zinc-responsive regulator Zur controls expression of the coelibactin gene cluster in *Streptomyces coelicolor*. J. Bacteriol. 192, 608–611. https://doi.org/10.1128/JB.01022-09

Karygianni, L., Ren, Z., Koo, H., Thurnheer, T., 2020. Biofilm matrixome: extracellular components in structured microbial communities. Trends Microbiol. 28, 668–681. https://doi.org/10.1016/j.tim.2020.03.016

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

Khoso, M.A., Wagan, S., Alam, I., Hussain, A., Ali, Q., Saha, S., Poudel, T.R., Manghwar, H., Liu, F., 2024. Impact of plant growth-promoting rhizobacteria (PGPR) on plant nutrition and root characteristics: Current perspective. Plant Stress 11, 100341. https://doi.org/10.1016/j.stress.2023.100341

Kim, Y., Jordan, D., McDonald, G.A., 1997. Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. Biol. Fertil. Soils 26, 79–87. https://doi.org/10.1007/s003740050347

Kirchmann, H., Kätterer, T., Bergström, L., Börjesson, G., Bolinder, M.A., 2016. Flaws and criteria for design and evaluation of comparative organic and conventional cropping systems. F. Crop. Res. 186, 99–106. https://doi.org/10.1016/j.fcr.2015.11.006

Kloepper, J., Schroth, M.., 1978. Plant growth-promoting rhizobacteria on radishes. IV international conference on plant pathogenic bacteria. Fourth Int. Conf. Plant Pathog. Bact. Angers, Fr. 2, 879–882.

Kloepper, J.W., 1994. Plant growth-promoting rhizobacteria (other systems). *Azospirillum*/Plant Assoc. Y. Okon (Ed.), CRC Press. Boca Raton, FL 137–166.

Kloepper, J.W., Leongt, J., Teintzet, M., Schroth, M.N., Ruben, J.A., Bennett, A.F., 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria of activity metabolism and its possible relation to vertebrate origins. Nature 286, 885–886.

Kramer, J., Özkaya, Ö., Kümmerli, R., 2020. Bacterial siderophores in community and host

interactions. Nat. Rev. Microbiol. 18, 152–163. https://doi.org/10.1038/s41579-019-0284-4

Krzyzanowska, D., Obuchowski, M., Bikowski, M., Rychlowski, M., Jafra, S., 2012. Colonization of potato rhizosphere by GFP-tagged *Bacillus subtilis* MB73/2, *Pseudomonas* sp. P482 and *Ochrobactrum* sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. Sensors (Switzerland) 12, 17608–17619. https://doi.org/10.3390/s121217608

Kuzmicheva, Y. V., Shaposhnikov, A.I., Petrova, S.N., Makarova, N.M., Tychinskaya, I.L., Puhalsky, J. V., Parahin, N. V., Tikhonovich, I.A., Belimov, A.A., 2017. Variety specific relationships between effects of rhizobacteria on root exudation, growth and nutrient uptake of soybean. Plant Soil 419, 83–96. https://doi.org/10.1007/s11104-017-3320-z

Levich, A.P., 1996. The role of nitrogen-phosphorus ratio in selecting for dominance of phytoplankton by cyanobacteria or green algae and its application to reservoir management. J. Aquat. Ecosyst. Stress Recover. 5, 55–61. https://doi.org/10.1007/bf00691729

Li, J., Wang, J., Liu, H., Macdonald, C.A., Singh, B.K., 2023. Microbial inoculants with higher capacity to colonize soils improved wheat drought tolerance. Microb. Biotechnol. 16, 2131–2144. https://doi.org/10.1111/1751-7915.14350

Lin, W., Okon, Y., Hardy, R.W.F., 1983. Enhanced mineral uptake by *Zea mays* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Appl. Environ. Microbiol. 45, 1775–1779. https://doi.org/10.1128/aem.45.6.1775-1779.1983

Liu, H., Qiu, Z., Ye, J., Verma, J.P., Li, J., Singh, B.K., 2022. Effective colonisation by a bacterial synthetic community promotes plant growth and alters soil microbial community. J. Sustain. Agric. Environ. 1, 30–42. https://doi.org/10.1002/sae2.12008

Liu, X., Jiang, X., He, X., Zhao, W., Cao, Y., Guo, T., Li, T., Ni, H., Tang, X., 2019. Phosphate-solubilizing *Pseudomonas* sp. strain P34-L promotes wheat growth by colonizing the wheat rhizosphere and improving the wheat root system and soil phosphorus nutritional status. J. Plant Growth Regul. 38, 1314–1324. https://doi.org/10.1007/s00344-019-09935-8

Liu, Y., Pan, X., Li, J., 2015. A 1961–2010 record of fertilizer use, pesticide application and cereal yields: a review. Agron. Sustain. Dev. 35, 83–93. https://doi.org/10.1007/s13593-014-0259-9

LLH, 2023. Ökologischer Landbau [WWW Document]. URL https://llh.hessen.de/umwelt/oekologischer-landbau/

Lori, M., Symnaczik, S., Mäder, P., De Deyn, G., Gattinger, A., 2017. Organic farming enhances soil microbial abundance and activity—a meta-analysis and meta-regression. PLoS One 12, 1–25. https://doi.org/10.1371/journal.pone.0180442

Lucas García, J.A., Schloter, M., Durkaya, T., Hartmann, A., Gutiérrez Mañero, F.J., 2003. Colonization of pepper roots by a plant growth promoting *Pseudomonas fluorescens* strain. Biol. Fertil. Soils 37, 381–385. https://doi.org/10.1007/s00374-003-0608-3

Ludwig, W., Viver, T., Westram, R., Francisco Gago, J., Bustos-Caparros, E., Knittel, K., Amann, R., Rossello-Mora, R., 2021. Release LTP_12_2020, featuring a new ARB alignment and improved 16S rRNA tree for prokaryotic type strains. Syst. Appl. Microbiol. 44, 126218. https://doi.org/10.1016/j.syapm.2021.126218

Lundquist, K.A., 2015. Unapproved genetically modified corn: It's what's for dinner. Iowa Law Rev. 100, 825–851.

Mallon, C.A., Le Roux, X., Van Doorn, G.S., Dini-Andreote, F., Poly, F., Salles, J.F., 2018. The impact of failure: unsuccessful bacterial invasions steer the soil microbial community away from the invader's niche. ISME J. 12, 728–741. https://doi.org/10.1038/s41396-017-0003-y

Martínez-Viveros, O., Jorquera, M.A., Crowley, D.E., Gajardo, G., Mora, M.L., 2010. Mechanisms and practical considerations involved in plant growth promotion by Rhizobacteria. J. Soil Sci. Plant Nutr. 10, 293–319. https://doi.org/10.4067/S0718-95162010000100006

Martinez, S.J., Simão, J.B.P., Pylro, V.S., Schwan, R.F., 2021. The altitude of coffee cultivation causes shifts in the microbial community assembly and biochemical compounds in natural induced anaerobic fermentations. Front. Microbiol. 12, 1–14. https://doi.org/10.3389/fmicb.2021.671395

Mashabela, M.D., Tugizimana, F., Steenkamp, P.A., Piater, L.A., Dubery, I.A., Mhlongo, M.I., 2022. Untargeted metabolite profiling to elucidate rhizosphere and leaf metabolome changes of wheat cultivars (*Triticum aestivum* L.) treated with the plant growth-promoting rhizobacteria *Paenibacillus alvei* (T22) and *Bacillus subtilis*. Front. Microbiol. 13, 1–23. https://doi.org/10.3389/fmicb.2022.971836

Mawarda, P.C., Le Roux, X., Dirk van Elsas, J., Salles, J.F., 2020. Deliberate introduction of invisible invaders: a critical appraisal of the impact of microbial inoculants on soil microbial communities. Soil Biol. Biochem. 148, 107874. https://doi.org/10.1016/j.soilbio.2020.107874

Mayak, S., Tirosh, T., Glick, B.R., 1999. Effect of wild-type and mutant plant growth-promoting rhizobacteria on the rooting of mung bean cuttings. J. Plant Growth Regul. 18, 49–53. https://doi.org/10.1007/PL00007047

Mayer, J., Scheid, S., Widmer, F., Fließbach, A., Oberholzer, H.R., 2010. How effective are "Effective microorganisms® (EM)"? results from a field study in temperate climate. Appl. Soil Ecol. 46, 230–239. https://doi.org/10.1016/j.apsoil.2010.08.007

McDaniel, M.D., Tiemann, L.K., Grandy, A.S., 2014. Does agricultural crop diversity enhance soil microbial biomass and organic matter dynamics? a meta-analysis. Ecol. Appl. 24, 560–570. https://doi.org/10.1890/13-0616.1

Meemken, E.M., Qaim, M., 2018. Organic agriculture, food security, and the environment. Annu. Rev. Resour. Econ. 10, 39–63. https://doi.org/10.1146/annurev-resource-100517-023252

Mendoza-Suárez, M., Andersen, S.U., Poole, P.S., Sánchez-Cañizares, C., 2021. Competition, nodule occupancy, and persistence of inoculant strains: key factors in the *Rhizobium*-legume symbioses. Front. Plant Sci. 12. https://doi.org/10.3389/fpls.2021.690567

Metcalf, R.L., 1973. Century of DDT. J. Agric. Food Chem. 21, 511–519. https://doi.org/https://doi.org/10.1021/jf60188a040

Meyer, G., Bünemann, E.K., Frossard, E., Maurhofer, M., Mäder, P., Oberson, A., 2017. Gross phosphorus fluxes in a calcareous soil inoculated with *Pseudomonas protegens* CHA0 revealed by ^{33}P isotopic dilution. Soil Biol. Biochem. 104, 81–94. https://doi.org/10.1016/j.soilbio.2016.10.001

Meyer, G., Maurhofer, M., Frossard, E., Gamper, H.A., Mäder, P., Mészáros, Schönholzer-Mauclaire, L., Symanczik, S., Oberson, A., 2019. *Pseudomonas protegens* CHA0 does not increase phosphorus uptake from ^{33}P labeled synthetic hydroxyapatite by wheat grown on calcareous soil. Soil Biol. Biochem. 131, 217–228. https://doi.org/10.1016/j.soilbio.2019.01.015

Moshou, D., Pantazi, X.E., Kateris, D., Gravalos, I., 2014. Water stress detection based on optical multisensor fusion with a least squares support vector machine classifier. Biosyst. Eng. 117, 15–22. https://doi.org/10.1016/j.biosystemseng.2013.07.008

Mylona, P., Pawlowski, K., Bisseling, T., 1995. Symbiotic nitrogen fixation. Plant Cell 7, 869–885. https://doi.org/doi: 10.1105/tpc.7.7.869

Odell, I.D., Cook, D., 2013. Immunofluorescence techniques. J. Invest. Dermatol. 133, 1–4. https://doi.org/10.1038/jid.2012.455

Okon, Y., Labandera-Gonzalez, C.A., 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem. 26, 1591–1601. https://doi.org/10.1016/0038-0717(94)90311-5

Oleńska, E., Małek, W., Wójcik, M., Swiecicka, I., Thijs, S., Vangronsveld, J., 2020. Beneficial features of plant growth-promoting rhizobacteria for improving plant growth and health in challenging conditions: A methodical review. Sci. Total Environ. 743. https://doi.org/10.1016/j.scitotenv.2020.140682

Ortíz-Castro, R., Martínez-Trujillo, M., López-Bucio, J., 2008. N-acyl-L-homoserine lactones: a class of bacterial quorum-sensing signals alter post-embryonic root development in *Arabidopsis thaliana*. Plant, Cell Environ. 31, 1497–1509. https://doi.org/10.1111/j.1365-3040.2008.01863.x

Ouyang, J., Sun, F., Feng, W., Sun, Y., Qiu, X., Xiong, L., Liu, Y., Chen, Y., 2016. Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa*. J. Appl. Microbiol. 120, 966–974. https://doi.org/10.1111/jam.13073

Packiavathy, I.A.S.V., Priya, S., Pandian, S.K., Ravi, A.V., 2014. Inhibition of biofilm development of uropathogens by curcumin - an anti-quorum sensing agent from *Curcuma longa*. Food Chem. 148, 453–460. https://doi.org/10.1016/j.foodchem.2012.08.002

Paluch, E., Rewak-Soroczyńska, J., Jędrusik, I., Mazurkiewicz, E., Jermakow, K., 2020. Prevention of biofilm formation by quorum quenching. Appl. Microbiol. Biotechnol. 104, 1871–1881. https://doi.org/10.1007/s00253-020-10349-w

Pande, A., Pandey, P., Mehra, S., Singh, M., Kaushik, S., 2017. Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. J. Genet. Eng. Biotechnol. 15, 379–391. https://doi.org/10.1016/j.jgeb.2017.06.005

Pang, Y., Liu, X., Ma, Y., Chernin, L., Berg, G., Gao, K., 2009. Induction of systemic resistance, root colonisation and biocontrol activities of the rhizospheric strain of *Serratia plymuthica* are dependent on N-acyl homoserine lactones. Eur. J. Plant Pathol. 124, 261–268. https://doi.org/10.1007/s10658-008-9411-1

Pérez-Montaño, F., Jiménez-Guerrero, I., Contreras Sánchez-Matamoros, R., López-Baena, F.J., Ollero, F.J., Rodríguez-Carvajal, M.A., Bellogín, R.A., Espuny, M.R., 2013. Rice and bean AHL-mimic quorum-sensing signals specifically interfere with the capacity to form biofilms by plant-associated bacteria. Res. Microbiol. 164, 749–760.

https://doi.org/10.1016/j.resmic.2013.04.001

Pernes-Debuyser, A., Tessier, D., 2004. Soil physical properties affected by long-term fertilization. Eur. J. Soil Sci. 55, 505–512. https://doi.org/10.1111/j.1365-2389.2004.00614.x

Peterson, G., 1967. The discovery and development of 2,4-D. Agric. Hist. Soc. 41, 243–254. https://doi.org/https://www.jstor.org/stable/3740338

Qaim, M., 2020. Role of new plant breeding technologies for food security and sustainable agricultural development. Appl. Econ. Perspect. Policy 42, 129–150. https://doi.org/10.1002/aepp.13044

Qiao, J., Yu, X., Liang, X., Liu, Yongfeng, Borriss, R., Liu, Youzhou, 2017. Addition of plant-growth-promoting *Bacillus subtilis* PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. BMC Microbiol. 17, 1–12. https://doi.org/10.1186/s12866-017-1039-x

Quiroga, S., Ratering, S., Rosado-Porto, D., Rekowski, A., Schulz, F., Zörb, C., Schnell, S., 2025. Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial. Appl. Soil Ecol. 206, 105823. https://doi.org/10.1016/j.apsoil.2024.105823

Rahman, M.M., Flory, E., Koyro, H.W., Abideen, Z., Schikora, A., Suarez, C., Schnell, S., Cardinale, M., 2018. Consistent associations with beneficial bacteria in the seed endosphere of barley (*Hordeum vulgare* L.). Syst. Appl. Microbiol. 41, 386–398. https://doi.org/10.1016/j.syapm.2018.02.003

Ramos, H.J.O., Roncato-Maccari, L.D.B., Souza, E.M., Soares-Ramos, J.R.L., Hungria, M., Pedrosa, F.O., 2002. Monitoring *Azospirillum*-wheat interactions using the *gfp* and *gus*A genes constitutively expressed from a new broad-host range vector. J. Biotechnol. 97, 243–252. https://doi.org/10.1016/S0168-1656(02)00108-6

Rashmi, I., Roy, T., Kartika, K.S., Pal, R., Coumar, V., Kala, S., Shinoji, K.C., 2020. Organic and inorganic fertilizer contaminants in agriculture: impact on soil and water resources. Springer International Publishing, Cham, pp. 3–41. https://doi.org/10.1007/978-3-030-41552-5_1

Raymond, N.S., Gómez-Muñoz, B., van der Bom, F.J.T., Nybroe, O., Jensen, L.S., Müller-Stöver, D.S., Oberson, A., Richardson, A.E., 2021. Phosphate-solubilising microorganisms for improved crop productivity: a critical assessment. New Phytol. 229, 1268–1277. https://doi.org/10.1111/nph.16924

Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H., Défago, G., Hass, D., 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. Microbiology 148, 923–932. https://doi.org/10.1099/00221287-148-4-923

Remans, R., Ramaekers, L., Schelkens, S., Hernandez, G., Garcia, A., Reyes, J.L., Mendez, N., Toscano, V., Mulling, M., Galvez, L., Vanderleyden, J., 2008. Effect of *Rhizobium-Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. Plant Soil 312, 25–37. https://doi.org/10.1007/s11104-008-9606-4

Rilling, J.I., Acuña, J.J., Nannipieri, P., Cassan, F., Maruyama, F., Jorquera, M.A., 2019.

Current opinion and perspectives on the methods for tracking and monitoring plant growth–promoting bacteria. Soil Biol. Biochem. 130, 205–219. https://doi.org/10.1016/j.soilbio.2018.12.012

Ritchie, H., 2017. How many people does synthetic fertilizer feed?. Published online at OurWorldInData.org. [WWW Document]. URL https://ourworldindata.org/how-many-people-does-synthetic-fertilizer-feed

Rodrigues, E.P., Rodrigues, L.S., De Oliveira, A.L.M., Baldani, V.L.D., Teixeira, K.R.D.S., Urquiaga, S., Reis, V.M., 2008. *Azospirillum amazonense* inoculation: effects on growth, yield and N_2 fixation of rice (*Oryza sativa* L.). Plant Soil 302, 249–261. https://doi.org/10.1007/s11104-007-9476-1

Rodríguez-Navarro, D.N., Dardanelli, M.S., Ruíz-Saínz, J.E., 2007. Attachment of bacteria to the roots of higher plants. FEMS Microbiol. Lett. 272, 127–136. https://doi.org/10.1111/j.1574-6968.2007.00761.x

Saadaoui, N., Silini, A., Cherif-Silini, H., Bouket, A.C., Alenezi, F.N., Luptakova, L., Boulahouat, S., Belbahri, L., 2022. Semi-arid-habitat-adapted plant-growth-promoting rhizobacteria allows efficient wheat growth promotion. Agronomy 12. https://doi.org/10.3390/agronomy12092221

Santner, A., Estelle, M., 2009. Recent advances and emerging trends in plant hormone signalling. Nature 459, 1071–1078. https://doi.org/10.1038/nature08122

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., Van Breusegem, F., Eberl, L., Hartmann, A., Langebartels, C., 2006. Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. Plant, Cell Environ. 29, 909–918. https://doi.org/10.1111/j.1365-3040.2005.01471.x

Schultz, N., Pereira, W., de Albuquerque Silva, P., Baldani, J.I., Boddey, R.M., Alves, B.J.R., Urquiaga, S., Reis, V.M., 2017. Yield of sugarcane varieties and their sugar quality grown in different soil types and inoculated with a diazotrophic bacteria consortium. Plant Prod. Sci. 20, 366–374. https://doi.org/10.1080/1343943X.2017.1374869

Schwember, A.R., Schulze, J., del Pozo, A., Cabeza, R.A., 2019. Regulation of symbiotic nitrogen fixation in legume root nodules. Plants 8, 333. https://doi.org/10.3390/plants8090333

Seufert, V., Ramankutty, N., Foley, J.A., 2012. Comparing the yields of organic and conventional agriculture. Nature 485, 229–232. https://doi.org/10.1038/nature11069

Shaharoona, B., Naveed, M., Arshad, M., Zahir, Z.A., 2008. Fertilizer-dependent efficiency of *Pseudomonads* for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). Appl. Microbiol. Biotechnol. 79, 147–155. https://doi.org/10.1007/s00253-008-1419-0

Shao, Z., Gu, S., Zhang, X., Xue, J., Yan, T., Guo, S., Pommier, T., Jousset, A., Yang, T., Xu, Y., Shen, Q., Wei, Z., 2024. Siderophore interactions drive the ability of *Pseudomonas* spp. consortia to protect tomato against *Ralstonia solanacearum*. Hortic. Res. 11. https://doi.org/10.1093/hr/uhae186

Shen, N., Li, S., Li, S.Y., Zhang, H., Jiang, M., 2022. The siderophore-producing bacterium, *Bacillus siamensis* Gxun-6, has an antifungal activity against *Fusarium oxysporum* and promotes the growth of banana. Egypt. J. Biol. Pest Control 32.

https://doi.org/10.1186/s41938-022-00533-7

Shepherd, M.A., Harrison, R., Webb, J., 2002. Managing soil organic matter – implications for soil structure on organic farms. Soil Use Manag. 18, 284–292. https://doi.org/10.1111/j.1475-2743.2002.tb00270.x

Singh, D.P., Singh, H.B., Prabha, R., 2016. Microbial inoculants in sustainable agricultural productivity: Vol. 1: Research perspectives. https://doi.org/10.1007/978-81-322-2647-5

Singh, K., Chandra, R., Purchase, D., 2022. Unraveling the secrets of rhizobacteria signaling in rhizosphere. Rhizosphere 21, 100484. https://doi.org/10.1016/j.rhisph.2022.100484

Smil, V., 2002. Enriching the Earth: Fritz Haber, Carl Bosch, and the transformation of world food production. MIT Press. ISBN 9780262194495.

Solanki, M., Garg, F.C., 2014. The use of *lac*Z marker in enumeration of *Azotobacter chroococcum* in carrier based inoculants. Brazilian J. Microbiol. 45, 595–601. https://doi.org/10.1590/S1517-83822014000200030

Spiers, A.J., Rainey, P.B., 2005. The *Pseudomonas fluorescens* SBW25 wrinkly spreader biofilm requires attachment factor, cellulose fibre and LPS interactions to maintain strength and integrity. Microbiology 151, 2829–2839. https://doi.org/10.1099/mic.0.27984-0

Stearns, J.C., Woody, O.Z., McConkey, B.J., Glick, B.R., 2012. Effects of bacterial ACC deaminase on *Brassica napus* gene expression. Mol. Plant-Microbe Interact. 25, 668–676. https://doi.org/10.1094/MPMI-08-11-0213

Stets, M.I., Campbell Alqueres, S.M., Souza, E.M., Pedrosa, F. de O., Schmid, M., Hartmann, A., Cruz, L.M., 2015. Quantification of *Azospirillum brasilense* FP2 bacteria in wheat roots by strain-specific quantitative PCR. Appl. Environ. Microbiol. 81, 6700–6709. https://doi.org/10.1128/AEM.01351-15

Stewart, W.M., Dibb, D.W., Johnston, A.E., Smyth, T.J., 2005. The contribution of commercial fertilizer nutrients to food production. Agron. J. 97, 1–6. https://doi.org/10.2134/agronj2005.0001

Suarez, C., Cardinale, M., Ratering, S., Steffens, D., Jung, S., Montoya, A.M.Z., Geissler-Plaum, R., Schnell, S., 2015. Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress. Appl. Soil Ecol. 95, 23–30. https://doi.org/10.1016/j.apsoil.2015.04.017

Suarez, C., Ratering, S., Geissler-Plaum, R., Schnell, S., 2014. *Hartmannibacter diazotrophicus* gen. nov., Sp. nov., a phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt-meadow plant. Int. J. Syst. Evol. Microbiol. 64, 3160–3167. https://doi.org/10.1099/ijs.0.064154-0

Suarez, C., Ratering, S., Hain, T., Fritzenwanker, M., Goesmann, A., Blom, J., Chakraborty, T., Bunk, B., Spröer, C., Overmann, J., Schnell, S., 2019. Complete genome sequence of the plant growth-promoting bacterium *Hartmannibacter diazotrophicus* strain E19^{T}. Int. J. Genomics 2019, 7586430. https://doi.org/10.1155/2019/7586430

Szuróczki, S., Kéki, Z., Káli, S., Lippai, A., Márialigeti, K., Tóth, E., 2016. Microbiological investigations on the water of a thermal bath at Budapest. Acta Microbiol. Immunol. Hung. 63, 229–241. https://doi.org/10.1556/030.63.2016.2.7

Tang, F.H.M., Lenzen, M., McBratney, A., Maggi, F., 2021. Risk of pesticide pollution at the

global scale. Nat. Geosci. 14, 206–210. https://doi.org/10.1038/s41561-021-00712-5

Thilakarathna, M.S., Raizada, M.N., 2017. A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. Soil Biol. Biochem. 105, 177–196. https://doi.org/10.1016/j.soilbio.2016.11.022

Tien, T.M., Gaskins, M.H., Hubbell, D.H., 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of Pearl Millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. 37, 1016–1024. https://doi.org/10.1128/aem.37.5.1016-1024.1979

Tuomisto, H.L., Hodge, I.D., Riordan, P., Macdonald, D.W., 2012. Does organic farming reduce environmental impacts? - a meta-analysis of european research. J. Environ. Manage. 112, 309–320. https://doi.org/10.1016/j.jenvman.2012.08.018

Turan, M., Gulluce, M., Şahin, F., 2012. Effects of plant-growth-promoting rhizobacteria on yield, growth, and some physiological characteristics of wheat and barley plants. Commun. Soil Sci. Plant Anal. 43, 1658–1673. https://doi.org/10.1080/00103624.2012.681739

Urrea-Valencia, S., Etto, R.M., Takahashi, W.Y., Caires, E.F., Bini, A.R., Ayub, R.A., Stets, M.I., Cruz, L.M., Galvão, C.W., 2021. Detection of *Azospirillum brasilense* by qPCR throughout a maize field trial. Appl. Soil Ecol. 160. https://doi.org/10.1016/j.apsoil.2020.103849

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

Van Dommelen, A., Keijers, V., Vanderleyden, J., De Zamaroczy, M., 1998. (Methyl)ammonium transport in the nitrogen-fixing bacterium *Azospirillum brasilense*. J. Bacteriol. 180, 2652–2659. https://doi.org/10.1128/jb.180.10.2652-2659.1998

Van Loon, L.C., Bakker, P.A.H.M., Pieterse, C.M.J., 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36, 453–483. https://doi.org/10.1146/annurev.phyto.36.1.453

Vandeputte, O.M., Kiendrebeogo, M., Rajaonson, S., Diallo, B., Mol, A., Jaziri, M. El, Baucher, M., 2010. Identification of catechin as one of the flavonoids from combretum albiflorum bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAQ1. Appl. Environ. Microbiol. 76, 243–253. https://doi.org/10.1128/AEM.01059-09

Venter, Z.S., Jacobs, K., Hawkins, H.J., 2016. The impact of crop rotation on soil microbial diversity: a meta-analysis. Pedobiologia (Jena). 59, 215–223. https://doi.org/10.1016/j.pedobi.2016.04.001

Villegas, L., Paterno, E., 2008. Growth enhancement and root colonization of sugarcane by plant growth-promoting bacteria. Philipp. J. Crop Sci.

Viswanath, G., Sekar, J., Prabavathy, V.R., 2016. Acyl homoserine lactone-producing rhizobacteria elicit systemic resistance in plants. Microbial-mediated Induc. Syst. Resist. Plants. Springer Nature Singapore, pp. 135–146. https://doi.org/10.1007/978-981-10-0388-2_9

Vogt, G., 2007. The origins of organic farming. Org. Farming An Int. Hist. 9–29.

https://doi.org/10.1079/9780851998336.0009

Vyas, P., Gulati, A., 2009. Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. BMC Microbiol. 9. https://doi.org/10.1186/1471-2180-9-174

Wang, L., Rengel, Z., Zhang, K., Jin, K., Lyu, Y., Zhang, L., Cheng, L., Zhang, F., Shen, J., 2022. Ensuring future food security and resource sustainability: insights into the rhizosphere. iScience 25, 104168. https://doi.org/10.1016/j.isci.2022.104168

Wang, L.H., Weng, L.X., Dong, Y.H., Zhang, L.H., 2004. Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). J. Biol. Chem. 279, 13645–13651. https://doi.org/10.1074/jbc.M311194200

Wang, M., Xue, J., Ma, J., Feng, X., Ying, H., Xu, H., 2020. *Streptomyces lydicus* M01 regulates soil microbial community and alleviates foliar disease caused by *Alternaria alternata* on cucumbers. Front. Microbiol. 11, 1–13. https://doi.org/10.3389/fmicb.2020.00942

Watson, A., Ghosh, S., Williams, M.J., Cuddy, W.S., Simmonds, J., Rey, M.D., Asyraf Md Hatta, M., Hinchliffe, A., Steed, A., Reynolds, D., Adamski, N.M., Breakspear, A., Korolev, A., Rayner, T., Dixon, L.E., Riaz, A., Martin, W., Ryan, M., Edwards, D., Batley, J., Raman, H., Carter, J., Rogers, C., Domoney, C., Moore, G., Harwood, W., Nicholson, P., Dieters, M.J., Delacy, I.H., Zhou, J., Uauy, C., Boden, S.A., Park, R.F., Wulff, B.B.H., Hickey, L.T., 2018. Speed breeding is a powerful tool to accelerate crop research and breeding. Nat. Plants 4, 23–29. https://doi.org/10.1038/s41477-017-0083-8

Wei, H.L., Zhang, L.Q., 2006. Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 89, 267–280. https://doi.org/10.1007/s10482-005-9028-8

Wei, X., Wang, X., Cao, P., Gao, Z., Chen, A.J., Han, J., 2020. Microbial community changes in the rhizosphere soil of healthy and rusty *Panax ginseng* and discovery of pivotal fungal genera associated with rusty roots. Biomed Res. Int. 2020. https://doi.org/10.1155/2020/8018525

Weller, D.M., Thomashow, L.S., 1994. Current challenges in introducing beneficial microorganisms into the rhizosphere. Mol. Ecol. Rhizosph. Microorg. Wiley, pp. 1–18. https://doi.org/10.1002/9783527615810.ch1

Whitehead, L.F., Day, D.A., Tyerman, S.D., 1998. Divalent cation gating of an ammonium permeable channel in the symbiotic membrane from soybean nodules. Plant J. 16, 313–324. https://doi.org/10.1046/j.1365-313X.1998.00298.x

Willer, H., Trávníček, J., Schlatter, B., 2024. The world of organic agriculture. Statistics and emerging trends 2024 356.

Williams, D.M., Blanco-Canqui, H., Francis, C.A., Galusha, T.D., 2017. Organic farming and soil physical properties: an assessment after 40 years. Agron. J. 109, 600–609. https://doi.org/10.2134/agronj2016.06.0372

Wohl, D.L., Arora, S., Gladstone, J.R., 2004. Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. Ecology 85, 1534–1540. https://doi.org/10.1890/03-3050

Xi, J., Wang, Y., Yang, X., Tao, Y., Shao, Y., Feng, F., 2017. *Mongoliimonas terrestris* gen.

nov., sp. nov., isolated from desert soil. Int. J. Syst. Evol. Microbiol. 67, 3010–3014. https://doi.org/10.1099/ijsem.0.002067

Xie, F., Williams, A., Edwards, A., Downie, J.A., 2012. A plant arabinogalactan-like glycoprotein promotes a novel type of polar surface attachment by *Rhizobium leguminosarum*. Mol. Plant-Microbe Interact. 25, 250–258. https://doi.org/10.1094/MPMI-08-11-0211

Yegorenkova, I. V., Tregubova, K. V., Matora, L.Y., Burygin, G.L., Ignatov, V. V., 2010. Use of ELISA with antiexopolysaccharide antibodies to evaluate wheat-root colonization by the rhizobacterium *Paenibacillus polymyxa*. Curr. Microbiol. 61, 376–380. https://doi.org/10.1007/s00284-010-9622-5

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

Zhang, S., Ma, Y., Jiang, W., Meng, L., Cao, X., Hu, J., Chen, J., Li, J., 2020. Development of a strain-specific quantification method for monitoring *Bacillus amyloliquefaciens* TF28 in the rhizospheric soil of soybean. Mol. Biotechnol. 62, 521–533. https://doi.org/10.1007/s12033-020-00268-6

Zsögön, A., Čermák, T., Naves, E.R., Notini, M.M., Edel, K.H., Weinl, S., Freschi, L., Voytas, D.F., Kudla, J., Peres, L.E.P., 2018. *De novo* domestication of wild tomato using genome editing. Nat. Biotechnol. 36, 1211–1216. https://doi.org/10.1038/nbt.4272

Chapter II

Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance

Research article

Published in FEMS Microbiology Ecology

FEMS Microbiology Ecology, 2024, **100**, fiae023
DOI: 10.1093/femsec/fiae023
Advance access publication date: 16 February 2024
Research Article

Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance

Santiago Quiroga [1], David Rosado-Porto [1,2], Stefan Ratering [1], Azin Rekowski [3], Franz Schulz [4], Marina Krutych[1], Christian Zörb [3], Sylvia Schnell [1,*]

[1]Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany
[2]Faculty of Basic and Biomedical Sciences, Simón Bolívar University, 080002 Barranquilla, Colombia
[3]Institute of Crop Science, Quality of Plant Products, 340e, University of Hohenheim, 70593 Stuttgart, Germany
[4]Department of Agronomy and Plant Breeding II, Justus-Liebig University Giessen, 35394 Giessen, Germany
*Corresponding author. Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany.
E-mail: Sylvia.Schnell@umwelt.uni-giessen.de
Editor: [Anton Hartmann]

Abstract

Monitoring of bioinoculants once released into the field remains largely unexplored; thus, more information is required about their survival and interactions after root colonization. Therefore, specific primers were used to perform a long-term tracking to elucidate the effect of *Hartmannibacter diazotrophicus* on wheat and barley production at two experimental organic agriculture field stations. Three factors were evaluated: organic fertilizer application (with and without), row spacing (15 and 50 cm), and bacterial inoculation (*H. diazotrophicus* and control without bacteria). *Hartmannibacter diazotrophicus* was detected by quantitative polymerase chain reaction on the roots (up to 5×10^5 copies g^{-1} dry weight) until advanced developmental stages under field conditions during two seasons, and mostly in one farm. Correlation analysis showed a significant effect of *H. diazotrophicus* copy numbers on the yield parameters straw yield (increase of 453 kg ha^{-1} in wheat compared to the mean) and crude grain protein concentration (increase of 0.30% in wheat and 0.80% in barley compared to the mean). Our findings showed an apparently constant presence of *H. diazotrophicus* on both wheat and barley roots until 273 and 119 days after seeding, respectively, and its addition and concentration in the roots are associated with higher yields in one crop.

Keywords: coating; detection; gum arabic; PGPR; qPCR; root colonization; seed inoculation; survival

Introduction

Since Kloepper and Schroth (1978) introduced the term plant growth-promoting rhizobacteria (PGPRs), a broad spectrum of research has focused on revealing the complexity of plant–microbial interactions and how they are able to induce beneficial effects on plant immunity, growth, and productivity (Kloepper 1981, Kirchhof et al. 1997, El Zemrany et al. 2006, Burns et al. 2015, Singh et al. 2022). PGPRs enhance plant performance through different mechanisms that have been widely reported over the years, such as nitrogen fixation (Hartmann et al. 1988), phosphorus solubilization (Wani et al. 2007), phytohormone production (Goswami et al. 2014), or triggering plant defense responses (Kloepper et al. 2004). These advances in PGPR research over the last decade have been linked to a paradigm shift toward sustainable agriculture (Singh et al. 2011, Backer et al. 2018). The current global attempt to reduce the use of mineral fertilizers and chemical pesticides has become an important issue, with special attention paid to most cultivated crops because of their environmental, economic, and social impacts (Thudi et al. 2021). The excessive use of mineral fertilizers contributes to not only the release of nitrous oxide (N_2O) to the atmosphere but also soil erosion and nitrate (NO_3^-) leaching into groundwater. The latter has become a serious problem in Germany, reporting an excess of the maximum NO_3^- permissible value (50 mg l^{-1}) in several groundwater-sampling sites in recent years (Sundermann et al. 2020).

Wheat and barley are among the six most produced cereals around the world, being fundamental in human nutrition, but at the same time, their cultivation may have a negative environmental impact due to the energy consumption for the production of fertilizers and pesticides, whose excessive use can lead to eutrophication and contaminated drinking water. Thus, several strategies have been considered to enhance yield from a sustainable perspective, including molecular breeding (Würschum et al. 2017), bioformulations (Yahya et al. 2022), or disease resistance (Skoppek et al. 2022). In this context, the use of PGPR has led to the release of several biofertilizers into the market. They aim to avoid soil degradation, reduce greenhouse emissions, and, at the same time, maintain soil fertility without reducing crop

Received 1 December 2023; **revised** 9 February 2024; **accepted** 15 February 2024

production (Aloo et al. 2022). However, PGPRs have to face and overcome many challenges. The effectiveness of PGPRs tested *in vitro* under field conditions is inconsistent (Cardinale et al. 2015, Owen et al. 2015), and in contrast to the laboratory or greenhouse, where several parameters, such as temperature, humidity, water availability, or light regime, are controlled, the field conditions vary depending on the season. Microorganisms that grow in gnotobiotic conditions have to adapt and survive in the new environment, compete with other microorganisms, and face different types of biotic and abiotic stresses (Backer et al. 2018). Understanding how these factors modulate and influence PGPR colonization is fundamental to extending the knowledge about plant–microbe interactions *in situ* as well as to improve the effectiveness of bioinoculants (Burns et al. 2015). However, there is scarce information regarding the tracking and monitoring of these inoculants once they are released in the field. In fact, < 25% of the studies used tracking methods, even though their development was not recent (for review, see Rilling et al. 2019). Among the different monitoring methods, the quantitative polymerase chain reaction (qPCR) is based on the design of specific primers that confer high accuracy, specificity, and reproducibility. In contrast to other methods for tracking PGPR, such as the use of reporter genes (beta-glucuronidase gene, green fluorescent protein) (Villegas and Paterno 2008, Krzyzanowska et al. 2012) or immunoassays (enzyme-linked immunosorbent assay) (Yegorenkova et al. 2010), which are semiquantitative, culture-dependent, or involve genetically modified bacteria, qPCR enables the quantitative analysis of nucleic acids used as templates. Most qPCR studies have been performed to detect *Azospirillum* spp. in roots (Faleiro et al. 2013, Stets et al. 2015, Coniglio et al. 2022) and bulk or rhizosphere soil (Bashan et al. 1995). Nevertheless, these studies were conducted under greenhouse conditions, considering short evaluation periods (mainly 14 days) and/or sterile/artificial environments that do not reflect field conditions. qPCR has been used to monitor the populations of various types of legume-nodulating rhizobia in the field (Maluk et al. 2022, 2023). Only a few long-term studies under field conditions have been performed to monitor the dynamics of PGPR in the rhizosphere soil (Zhang et al. 2020), roots (Soares et al. 2021, Urrea-Valencia et al. 2021), or stalks (Fernandes et al. 2014) of different plants, including maize, *Brachiaria* grasses, soybean, and sugarcane.

In this study, we inoculated the plant growth-promoting rhizobacterium *Hartmannibacter diazotrophicus* strain E19^T on winter wheat (WW) (*Triticum aestivum* L., cv. Aristaro) and spring barley (SB) (*Hordeum vulgare* L., cv. Odilia) seeds in order to evaluate its plant growth-promoting effects under field conditions. In addition, we developed specific primers to perform a long-term qPCR tracking of the bacterium at different stages in both plants, quantifying and correlating these results with the different yield parameters. *Hartmannibacter diazotrophicus* is a Gram-negative, rod-shaped, strictly aerobic bacterium, isolated from the rhizosphere of *Plantago winteri* (Suarez et al. 2014). Plant growth promoting (PGP) abilities *in vitro* and *in vivo* (greenhouse) have shown that *H. diazotrophicus* is able to fix nitrogen, solubilize insoluble phosphate, reduce stress through 1-amynocyclopropane-1-carboxylate (ACC) deaminase activity, and promote plant growth under salt stress conditions (Suarez et al. 2014, 2015). Previously, successful root colonization by *H. diazotrophicus* was identified using specific Fluorescence *in-situ* hybridisation (FISH) probes in barley grown under greenhouse conditions (Suarez et al. 2015, Rahman et al. 2018), indicating its accumulation on the root surface, except at the root tips.

Materials and methods

Experimental site

Field experiments were performed at the organic experimental stations Gladbacherhof (GH) (50°23′N, 8°15′E), Giessen University, central Germany, and Kleinhohenheim (KH) (48°44′N, 9°11′E), Hohenheim University, southwest Germany, during the seasons 2020–2021 (season I) and 2021–2022 (season II). The fields are located 185 and 444 m above sea level, with an average annual precipitation of 592 and 591 mm and an annual average temperature of 10.8 and 10.5°C, respectively (2020–2022). Both sites show Haplic Luvisol (IUSS Working Group WRB 2015) as soil type and have been organically managed since 1989 (GH) and 1994 (KH). The soil properties of both fields are listed in Supplementary Table S1. Additional soil properties have been described in detail by Elfadl et al. (2010), Chen et al. (2014), Schulz et al. (2014), and Binacchi et al. (2023). At Gladbacherhof, an 8-year crop rotation with 2-year alfalfa grass (*Medicago sativa*), winter rye (*Secale cereale*), potato (*Solanum tuberosum*), WW (*T. aestivum*), field beans (*Vicia faba*), spelt (*T. aestivum* ssp. *spelta*), and SB (*H. vulgare*) has been cultivated. Potato and spelt wheat were planted in the fields before seeding WW and SB, respectively. In contrast, a 5-year crop rotation at Kleinhohenheim with clover grass (*Trifolium* spp.), intensive vegetables, summer cereal, vegetables with low N-requirement, and emmer (*T. dicoccum*) was cultivated, with clover grass always planted before WW and SB.

Bacterial culture, seed coating, and field experiments

Hartmannibacter diazotrophicus strain E19^T (LMG 27460^T), from the culture collection of the Institute of Applied Microbiology (Justus Liebig University, Germany), was cultured in a half-concentrated marine bouillon (Carl Roth GmbH, Germany) and incubated on an orbital shaker at 27°C and 150 rpm for 48 h. Then, 2000 ml of the liquid culture was centrifuged at 17 700 × *g* for 20 min and re-suspended in 400 ml of 0.03 M $MgSO_4$. The inoculation of *H. diazotrophicus* was based on and modified from the seed-coating technique described by Kloepper (1981). In our experiments, gum arabic 25% (Fisher Scientific, UK) and talc (Carl Roth GmbH, Germany) were autoclaved at 121.9°C for 25 min and pH adjusted to 7.0 ± 0.2 before coating. Subsequently, it was mixed with the bacterial re-suspension in a 1:1 ratio. Sterile $MgSO_4$ solution was used as the control treatment. The gum inoculum ($\bar{x} = 1.62 \times 10^9 \pm 4.89 \times 10^8$ bacteria ml^{-1}) was gently placed and spread over WW (*T. aestivum*, cv. Aristaro) or SB (*H. vulgare* L., cv. Odilia) seeds, mixed well, and covered with talc (2.2 times the volume of the gum inoculum). The seeds were stored at 4°C until sowing. For colony-forming unit (CFU) determination, 0.1 g of the seed-coated powder was dissolved in 9.9 ml of 0.18% sodium pyrophosphate, followed by serial dilutions in 0.9 ml of NaCl (0.9%), obtaining an average concentration for both seasons of $\bar{x} = 2.18 \times 10^8 \pm 9.56 \times 10^7$ CFU g^{-1} powder. A randomized complete block design with four replicates was performed under field conditions to evaluate the following factors: bacterial inoculation (strain E19^T, ctrl without bacteria), row spacing (15 and 50 cm), and fertilization (with fertilizer, without fertilizer, only for WW). Approximately 400 seeds m^{-2} were sowed in plots of 7.5 m^2 (5 m × 1.5 m). WW seeds were sowed between October and November 2020 (season I) and October 2021 (season II) and fertilized with organic manure in March 2021 (100.2 kg N ha^{-1}, season I) and 2022 (107.1 kg N ha^{-1}, season II) by the soil drenching method (Supplementary Table S2). SB

seeds were sowed in April 2021 (season I) and March 2022 (season II). Fertilization of SB was not performed, as it is not common in organic farming.

Parallel greenhouse experiment to evaluate seed coating and strain E19^T survival

Hartmannibacter diazotrophicus has previously proved to effectively colonize barley roots under salt stress conditions (Suarez et al. 2015). Therefore, in order to evaluate its root colonization abilities in WW, 700 g of soil from GH was milled, sieved (<4 mm), and placed in square plastic pots of 14 × 14 cm, containing 20 coated WW seeds used for seeding at GH during season I. In addition, we prepared pots with coated seeds with an initial concentration of 100 mM NaCl and then watered them three times with 50 mM NaCl, reaching a final concentration of 250 mM NaCl. All pots were maintained at 60% of their maximum water holding capacity (WHC = 239 ml). Coated seeds without bacterial inoculation (control samples) were also included in the experiments with and without salt stress, with three replicates per treatment.

Root sampling and DNA extraction

Field plants with soil at their roots were dug out from two different points in each plot (~1 m into the plot from the edge) 120 days after seeding (DAS) (only at GH, season I), then at flowering (BBCH-60), milk ripe (BBCH-75, season I), and fully ripe (BBCH-89, season II) stages. Similarly, root samples from the greenhouse experiment were collected 10 DAS (BBCH-12) and 30 DAS (BBCH-30), considering the Zadoks growth scale (Zadoks et al. 1974). Thereafter, samples were collected in plastic bags and stored at 4°C. Once in the laboratory, the soil was discarded from the roots using sterilized forceps and scissors. Samples with seed inoculation were processed at separate locations compared to those without seed inoculation. Only the upper part of the roots was placed in sterile plastic centrifuge tubes of 15 ml and stored at −80°C. For the greenhouse experiment, the roots were cut immediately after removing the soil, and the lower, middle, and upper parts were frozen separately in sterile plastic centrifuge tubes at −80°C.

Prior to DNA extraction, the roots were rinsed two to three times with sterilized deionized water to remove the soil. They were then ground with liquid nitrogen using a sterilized mortar and pestle (180°C for 5 h). Approximately 100–200 mg of fresh root weight was placed into a 2-ml screw-cap microcentrifuge tube containing 700 mg of heat-sterilized zirconium beads (0.1 mm). For DNA extraction, 1 ml of extraction buffer (0.2 M sodium phosphate buffer, 0.1 M NaCl, 0.05 M EDTA, 25 g l^{-1} SDS, pH 8) was added to the tubes. Root tissue was disrupted with a homogenizer for 45 s at 5.5 m s^{-1} (Fastprep-24™, Biomedicals, Santa Ana, CA, USA) and later centrifuged at 17 000 × *g* for 5 min at 4°C. RNA was digested by the addition of 2.5 µl RNAse (20 mg ml^{-1}), followed by incubation for 30 min at 37°C. For DNA separation from lipids and cell debris, 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tubes, mixed by inversion, and centrifuged at 17 000 × *g* for 5 min at 4°C. The upper aqueous phase from the tubes was recovered and transferred into a new 2 ml microcentrifuge tube. Subsequently, 1 ml of chloroform was pipetted into the tubes and centrifuged at 17 000 × *g* for 5 min at 4°C. The supernatant was collected in a new 2 ml microcentrifuge tube. DNA precipitation consisted of the addition of 1 ml of precipitation buffer (200 g l^{-1} polyethylene glycol 6000, 2.5 M NaCl), followed by incubation for 30 min at 4°C. The tubes were centrifuged at 17 000 × *g* for 30 min at 4°C and the supernatant was discarded by decanting. Finally, a washing step with 800 µl of ice-cold 75% ethanol was performed. The tubes were mixed by inversion and centrifuged at 17 000 × *g* for 10 min at 4°C. After ethanol removal, the DNA pellet was dried next to the Bunsen burner flame and dissolved in 30 µl of nuclease-free water.

Primer design

A specific primer pair (E19_F_932: 5′-GTCCGGCTATCCAGAGAGAT-3′; E19_R_1261: 5′-ATTAGCTGACCCTCGCAGGT-3′) targeting the 16S rRNA gene of strain E19^T was designed by aligning the 16S rRNA gene of strain E19^T and its closest relatives. The sequences were obtained from the SILVA database (Quast et al. 2012), aligned, and merged with the LTPs111 (February 2013) database (Munoz et al. 2011) using the ARB version 5.2 program (Ludwig et al. 2004). The specificity of the primer pair was checked using the online programs Probe Check (Loy et al. 2008) and SILVA TestPrime v1.0 (Klindworth et al. 2013). The specificity of the primers was also tested by cloning, using the method described by Kampmann et al. (2012a), with DNA isolated from the rhizosphere of different plants. The cloned DNA sequences from the vectors were sequenced (LGC, Berlin, Germany), and only sequences identical to the corresponding sequences of the strain E19^T were found.

Quantitative PCR standard curve

The copy numbers of the standard 16S rRNA gene segments were calculated as described by Kampmann et al. (2012b). For qPCR, 5 µl of SYBR Green JumpStart™ Ready Mix (Sigma–Aldrich, USA), plus 0.2 µl of the primers 932F (10 µM) and 1261R (10 µM), 0.1 µl BSA (20 µg $µl^{-1}$), 2 µl of PCR-water, and 2.5 µl of DNA were used for a total volume of 10 µl. The DNA for the standard curve was serially diluted 10-fold between the range in which *H. diazotrophicus* was detectable (from 6.88 × 10^5 copies $µl^{-1}$ until 6.88 × 10^0 copies $µl^{-1}$). Quantification of *H. diazotrophicus* in the samples was performed in four replicates with a Rotor Gene Q (Qiagen, Hilden, Germany) using the following program: 2 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 63°C, 45 s at 72°C, and 15 s at 84°C. Q-Rex software v1.1.04 (Qiagen, Hilden, Germany) was used for absolute quantification, normalization, and Cq calculation.

Harvesting, yields, and copy number g^{-1} DW

Harvesting of WW and SB took place in August in the years 2021 and 2022. Fresh and dry weights were determined from the seeds and straw and adjusted to 12% moisture for grain yield and straw yield calculations. In addition, the thousand-kernel weight was also included. To determine the crude protein concentration, the grain samples were dried and milled using a tube mill (MM301, Retsch, Haan, Germany). The DUMAS combustion method was used to determine the total N content in the individual flour samples using a Vario MACRO cube CHNS macro elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany). Finally, the N content was multiplied by a factor of 5.7 (Sosulski and Imafidon 1990).

The result of the qPCR in copies $µl^{-1}$ was converted to copies g^{-1} DW soil using the following formula:

$$C = \frac{Cr \times Vu \times Vt}{ms \times dm} \times 100\%,$$

where *C* is the copies per dry weight sample (copies g^{-1} DW sample), *Cr* is the copies per qPCR reaction (copies $µl^{-1}$), *Vu* is the dilution factor of the DNA in the qPCR (usually 1:10), *Vt* is the total volume of the DNA extract (30 µl), *ms* is the mass of the extracted environmental sample (g), and *dm* is the dry matter content of the extracted environmental sample (%).

Statistical analysis

Statistical evaluation of the copies g^{-1} DW was carried out using R studio software, v4.3.0 (R Core Team 2023). The qPCR data were log10 transformed. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) were performed to compare significant differences between treatments. Eventually, data visualization was performed through R packages ggstatsplot v0.11.1 (Patil 2021), palmerpenguins v0.1.1 (Horst et al. 2020), and tidyverse v2.0 (Wickham et al. 2019).

A linear mixed-model approach was applied using the R package lme4 v1.1–26 (Bates et al. 2015). In order to assess the effect of seed inoculation with *H. diazotrophicus*, each crop was analyzed separately, and the factors bacterial inoculation (categorical variable and continuous variable as $\log_{10}$ copies g^{-1} DW sample), fertilizer management (for WW), and row spacing were assessed as fixed effects. On the other side, harvest season, field location, and experimental block were used as random effects for model creation. Initially, it was determined the level of variance explained by each one of the afore-mentioned factors. Subsequently, three models were created, as described by the following formulas:

Model 1:

$$Y_i = \beta_0 + \alpha_{T(i)} + b_{0i} + e_i,$$
$$b_{0i} \sim NID(0, \sigma^2) \text{ and } e_i \sim NID(0, \sigma^2).$$

Model 2:

$$Y_{ijkl} = \beta_0 + \beta 1_j + \beta 2_k + \beta 3_l + \alpha_{T(i)} + b_{0i} + b_{1ij} + e_{ijkl},$$
$$\begin{bmatrix} b_{0i} \\ b_{1i} \end{bmatrix} \sim NID\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{01} & \sigma_1^2 \end{bmatrix}\right) \text{ and } e_{ijkl} \sim NID(0, \sigma^2).$$

Model 3:

$$Y_{ijkl} = \beta_0 + \beta 1_j + \beta 2_k + \beta 3_l + (\beta 1\beta 2)_{jk} + (\beta 1\beta 3)_{jl} + (\beta 2\beta 3)_{kl} + (\beta 1\beta 2\beta 3)_{jkl} + \alpha_{T(i)} + b_{0i} + b_{1ip} + e_{ijkl},$$
$$\begin{bmatrix} b_{0i} \\ b_{1i} \end{bmatrix} \sim NID\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{01} & \sigma_1^2 \end{bmatrix}\right) \text{ and } e_{ijkl} \sim NID(0, \sigma^2),$$

where Y_i is the yield or response of the ith plant with j bacterial inoculation, k fertilizer management, and l row spacing. β_0 is the overall fixed intercept, $\beta 1_j$ is the intercept for bacterial inoculation, $\beta 2_k$ is the intercept for fertilizer management, and $\beta 3_l$ is the intercept for row spacing. $(\beta 1\beta 2)_{jl}$ corresponds to the intercept of the interaction between bacterial inoculation and fertilizer management. $(\beta 1\beta 3)_{jl}$ is the intercept of the interaction between bacterial inoculation and row spacing. $(\beta 3\beta 2)_{kl}$ corresponds to the intercept of the interaction between fertilizer management and row spacing. $(\beta 1\beta 2\beta 3)_{jkl}$ is the intercept of the interaction of all fixed effects. α_T is the intercept of hierarchical random effects for the ith plant, b_{0i} and b_{1i} are the random intercept and random bacterial inoculation slope effects for the ith plant, and e is the random error. With the typical assumption of mutual independence of random effects and random error and normally and identically distributed effects. Model 1 represented the dependent variable without fixed effects and with only random effects. Model 2 includes fixed effects but does not assume any interaction among them. Model 3 includes fixed effects, with the assumption of interaction among them.

Following, the models were compared using the function *model.comparison* from R package flexplot v0.19.1. (Fife 2022), using the Akiake information criterion, Bayesian information criterion, and Bayes factor as the criteria for model selection.

Later, the selected model was fitted using the restricted maximum likelihood (REML) and *t*-tests and Type III ANOVAs were performed by applying the Satterthwaite's method for approximating the degrees of freedom for the *t* and F tests using the R package lmerTest v3.1–3 (Kuznetsova et al. 2017).

Results

Long-term qPCR detection of the strain E19^T under field conditions at different developmental stages

Hartmannibacter diazotrophicus was successfully detected in the roots of WW and SB during the two evaluated seasons, mostly at Gladbacherhof (Fig. 1a). As the E19^T detection was not found in all the samples collected, treatments were clustered only by bacterial inoculation, and the detection rate was calculated to estimate which percentage of the root samples under field conditions were colonized by strain E19^T. The highest copy number of *H. diazotrophicus* was observed during season II at the flowering stage for WW ($\bar{x}_{\log10\ copies} = 5.32$, $\bar{x}_{copies} = 3.1 \times 10^5$ copies g^{-1} DW) and milk ripe for SB ($\bar{x}_{\log10\ copies} = 5.37$, $\bar{x}_{copies} = 5 \times 10^5$ copies g^{-1} DW). Although some significant differences between seasons or stages were found, no clear trend was observed in either plant species (Fig. 1a). Surprisingly, no significant reduction was observed in the number of copies in WW at 120 DAS ($\bar{x} = 4.83$, Fig. 1a) compared to the advanced developmental stages ($P > 0.05$) (Fig. 1a). However, the detection rate declined with the highest value recorded at 120 DAS (75%). Moreover, the strain E19^T detected in samples at flowering was not necessarily found in the next stage, and vice versa. In contrast, the highest detection percentages in SB were observed at the milk/fully ripe stage (100% and 88% detection rates in seasons I and II, respectively). It is important to point out that in WW, strain E19^T was detected after a longer period of time (up to 273 DAS, Fig. 1a) than in SB (up to 119 DAS, Fig. 1a). Furthermore, strain E19^T was not detected in any of the control samples (roots of wheat and barley that were not seed-inoculated with E19^T), showing the specificity of the primers and the non-native presence of *H. diazotrophicus* in the soil of both organic farms. As the number of copies g^{-1} DW was zero, they were not shown in Fig. 1. Intriguingly, in the other experimental field (Kleinhohenheim), strain E19^T could not be detected during season I; it was only detected in two replicates of SB during season II ($\bar{x} = 4.87$, 25% detection rate, Fig. 1a).

qPCR detection of the strain E19^T under greenhouse conditions

In parallel to sowing WW to the field in season I and due to accessibility and better root examination, the same coated seeds were used for the detection of strain E19^T in an early growth stage of the wheat plants in a greenhouse experiment. In addition to the optimal growth conditions for wheat, salt stress was applied to evaluate whether strain E19^T could be detected under saline conditions in WW (Fig. 1b). Ten DAS, the copy number of strain E19^T under salt stress ($\bar{x} = 6.33$) was higher than that of the strain E19^T without salt stress ($\bar{x} = 5.81$). However, no significant differences were found between treatments ($P = 0.78$; Fig. 1b). Remarkably, the highest number of copies was detected in the upper part of the root under salt stress ($\bar{x}_{\log10\ copies} = 6.36$, $\bar{x}_{copies} = 2.55 \times 10^6$ copies g^{-1} DW). Furthermore, no significant decrease over time was observed in the middle and lower root sections under this stress condition (Fig. 1b). This was in contrast with the results obtained without salt after 30 days, in which the concentration significantly decreased in the middle ($\bar{x} = 4.05$) and lower parts ($\bar{x} = 4.14$) of the roots but not in the upper part ($\bar{x}_{\log10\ copies} = 5.21$, $\bar{x}_{copies} = 1.82 \times 10^5$ copies g^{-1} DW). The detection percentage was

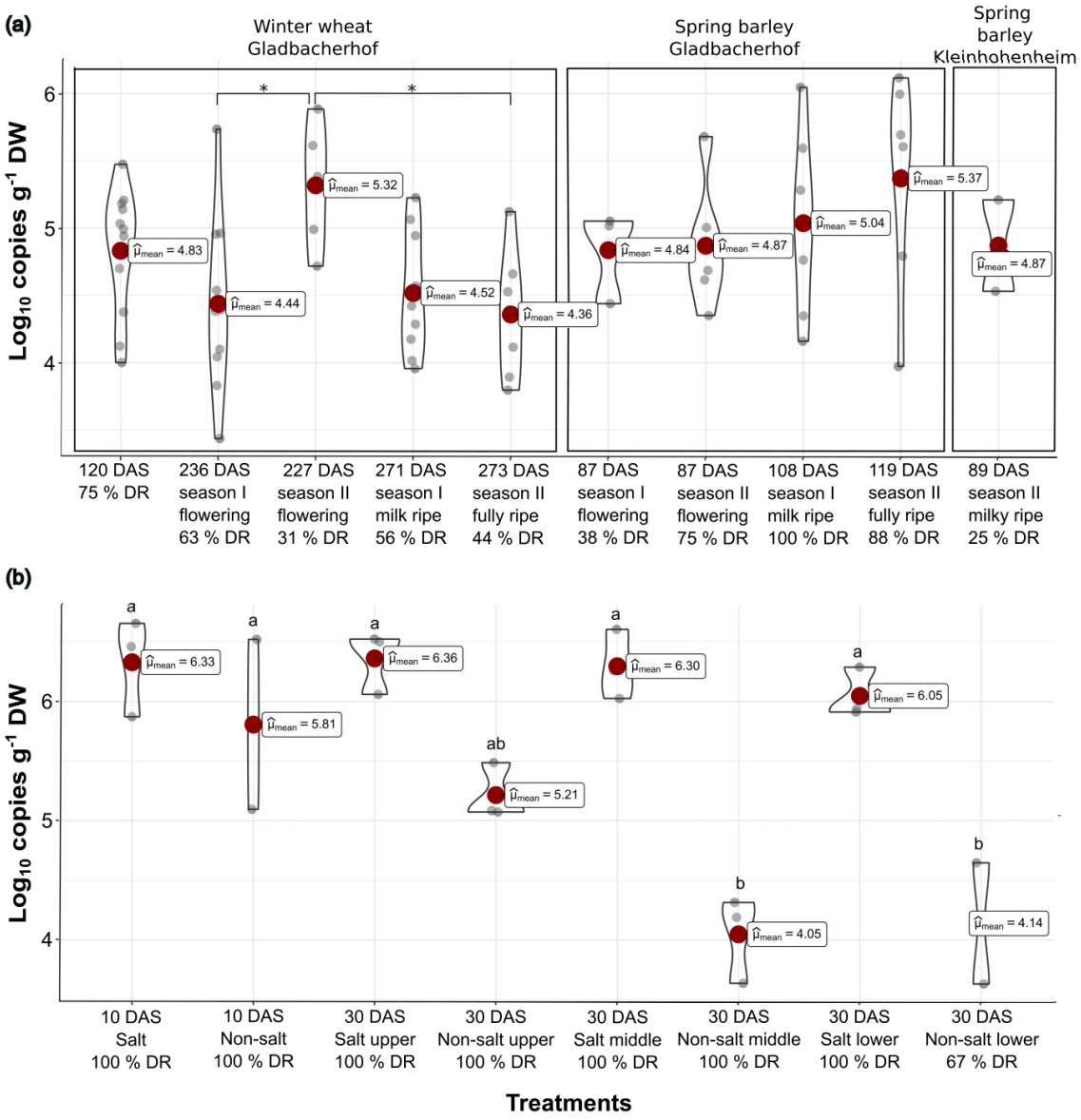


Figure 1. (a) Comparison of the number of copies per gram of DW (log10) of strain E19^T for wheat and barley during the two seasons under field conditions. Significance codes: < 0.05 '*'. Subsets for wheat (n = 5–12, Tukey's HSD, α = 0.05) and barley (n = 3–8, ANOVA, α = 0.05) were used for statistical analysis. (b) Comparison of the number of copies per gram of DW (log10) of strain E19^T for wheat under saline (salt) and non-saline conditions (non-salt). Different letters show significant differences between treatments (Tukey's HSD, α = 0.05). DR = detection rate (%); DAS = days after seeding; upper = upper section of the roots; middle = middle section of the roots; lower = lower section of the roots.

100% in all treatments, except for the lower part of the roots after 30 days without salt stress (67%, Fig. 1b).

Influence on bacterial inoculation, fertilizer, and row spacing on plant parameters

At first glance, yield parameters collected during the seasons 2020–2021 and 2021–2022 showed better performance in terms of grain yield, straw yield, and thousand-kernel weight in season II in both fields compared to the previous season, not being the case for crude protein concentration (Table 1). In fact, the linear mixed model revealed that most of the variation was determined not only by the season, but also by the location and the block (Fig. 2). The variance of these three factors varied depending on the yield parameters, with the season being the main source of variance in SB and the location the main source of variance in WW. Therefore, to control their contribution to the overall variance, season, location, and block were modeled as random effects for the linear mixed model.

Type III ANOVA of the linear mixed models of WW showed a significant effect on straw yield, with only a significant interaction

Table 1. Average and standard deviation of agronomic data for WW and SB at different locations during the seasons 2020–2021 and 2021–2022.

Season	Plant	Location	Treatment		Grain yield (kg ha^{-1})	Straw yield (kg ha^{-1})	Thousand kernel weight (g)	Crude protein concentration (%)
2020–2021	WW	Gladbacherhof	Fertilizer	With	4186 ± 331	8037 ± 1062	42.3 ± 1.3	13.6 ± 0.8
				Without	3965 ± 367	7775 ± 749	42.4 ± 1.1	11.8 ± 1.5
			Row spacing	15 cm	4136 ± 334	7693 ± 845	42.0 ± 1.1	12.6 ± 1.4
				50 cm	4014 ± 388	8120 ± 957	42.7 ± 1.1	12.8 ± 1.6
			Bacterial inoculation	Ctrl	4105 ± 361	7583 ± 807	42.4 ± 1.2	12.1 ± 1.7
				E19	4045 ± 372	8230 ± 923	42.3 ± 1.2	13.2 ± 1.0
		Kleinhohenheim	Row spacing	15 cm	3564 ± 1193	2552 ± 891	41.5 ± 2.2	9.8 ± 0.2
				50 cm	3552 ± 733	2554 ± 761	42.9 ± 1.4	9.7 ± 0.4
			Bacterial inoculation	Ctrl	3263 ± 734	2737 ± 699	42 ± 2.4	9.7 ± 0.3
				E19	3809 ± 1055	2395 ± 877	42.4 ± 1.6	9.7 ± 0.3
	SB	Gladbacherhof	Row spacing	15 cm	1414 ± 347	1050 ± 217	45.0 ± 0.5	11.8 ± 2.8
				50 cm	1240 ± 644	939 ± 297	45.9 ± 1.0	12.8 ± 1.5
			Bacterial inoculation	Ctrl	1301 ± 417	1016 ± 249	45.5 ± 0.9	10.8 ± 1.5
				E19	1353 ± 614	972 ± 281	45.5 ± 0.9	13.8 ± 1.8
		Kleinhohenheim	Row spacing	15 cm	1352 ± 125	2187 ± 382	45.3 ± 2.3	10.5 ± 0.3
				50 cm	1200 ± 146	1861 ± 391	44.9 ± 2.6	10.5 ± 0.7
			Bacterial inoculation	Ctrl	1302 ± 178	2079 ± 334	45.2 ± 1.5	10.5 ± 0.5
				E19	1250 ± 129	1969 ± 492	45 ± 3.2	10.5 ± 0.5
2021–2022	WW	Gladbacherhof	Fertilizer	With	5212 ± 825	8865 ± 1324	49.5 ± 0.9	12.5 ± 1.1
				Without	5061 ± 651	8883 ± 1154	49.3 ± 0.7	11.5 ± 1.4
			Row spacing	15 cm	5310 ± 792	9135 ± 1231	49.2 ± 0.8	11.8 ± 1.4
				50 cm	4964 ± 653	8612 ± 1194	49.6 ± 0.9	12.1 ± 1.3
			Bacterial inoculation	Ctrl	4997 ± 712	8735 ± 1273	49.2 ± 0.7	11.6 ± 1.5
				E19	5276 ± 754	9013 ± 1193	49.6 ± 0.9	12.3 ± 1.1
		Kleinhohenheim	Fertilizer	With	5045 ± 744	5323 ± 1018	42.8 ± 2.5	13.0 ± 0.4
				Without	4913 ± 941	5187 ± 1340	41.2 ± 2.8	12.4 ± 0.9
			Row spacing	15 cm	4690 ± 880	5556 ± 1374	41.5 ± 2.5	12.5 ± 0.8
				50 cm	5269 ± 703	4953 ± 871	42.5 ± 2.9	13.0 ± 0.6
			Bacterial inoculation	Ctrl	5027 ± 920	5183 ± 1285	41.9 ± 2.9	12.6 ± 0.7
				E19	4931 ± 773	5326 ± 1086	42.1 ± 2.6	12.7 ± 0.8
	SB	Gladbacherhof	Row spacing	15 cm	3832 ± 768	3597 ± 1249	48.7 ± 1.4	12.0 ± 1.3
				50 cm	3692 ± 699	3599 ± 1209	49.4 ± 1.4	11.7 ± 1.3
			Bacterial inoculation	Ctrl	3801 ± 783	3763 ± 1313	48.9 ± 1.6	10.7 ± 0.7
				E19	3723 ± 687	3433 ± 1111	49.2 ± 1.3	12.9 ± 0.5
		Kleinhohenheim	Row spacing	15 cm	5794 ± 434	3659 ± 901	46.1 ± 2.3	11.3 ± 1.2
				50 cm	5576 ± 224	3229 ± 786	48.6 ± 2.8	11.3 ± 1.4
			Bacterial inoculation	Ctrl	5581 ± 386	3430 ± 1009	47.5 ± 2.4	10.3 ± 0.9
				E19	5790 ± 301	3458 ± 718	47.2 ± 3.3	12.3 ± 0.6

Ctrl = control without bacteria, E19 = inoculation with *H. diazotrophicus*, 15 cm = row spacing of 15 cm, 50 cm = row spacing of 50 cm, and with = with fertilizer, without = without fertilizer.

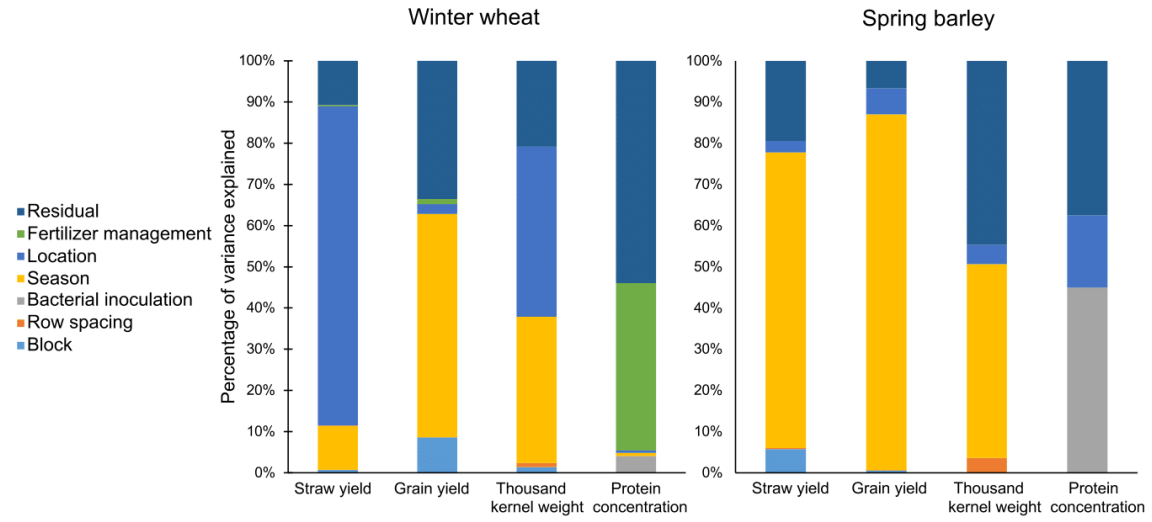


Figure 2. Percentage of variance of the different factors used for modeling the linear mixed model in WW and SB.

Table 2. Type III ANOVA with Satterthwaite's method based on linear mixed models of different yield parameters for WW and SB.

Plant	Yield parameter	Factor	Bacterial inoculation	Fertilizer	Row spacing	Bacterial inoculation: fertilizer
WW	Straw yield	F value	2.12	0.78	1.19	7.36
		Pr(>F)	0.17	0.38	0.28	**8.08×10^{-3}****
	Grain yield	F value	0.74	3.94	0.28	0.87
		Pr(>F)	0.41	0.05	0.6	0.36
	Thousand kernel weight	F value	0.74	6.13	14.44	-
		Pr(>F)	0.39	**1.51×10^{-2}***	**2.58×10^{-4}*****	-
	Crude protein concentration	F value	8.15	37.43	-	-
		Pr(>F)	**5.40×10^{-3}****	**2.08×10^{-8}*****	-	-
SB	Straw yield	F value	0.70	-	2.67	-
		Pr(>F)	0.41	-	0.11	-
	Grain yield	F value	0.14	-	4.03	-
		Pr(>F)	0.71	-	0.05	-
	Crude protein concentration	F value	39.07	-	0.37	-
		Pr(>F)	**5.92×10^{-8}*****	-	0.54	-

Empty spaces belong to factors that were not included for the linear mixed model. Numbers in bold indicate significant differences. Significance codes: "***"<0.001, "**"<0.01, "*"<0.05.

between bacterial inoculation and the addition of fertilizer (Table 2). Moreover, the t-test using Satterthwaite's method showed that bacterial inoculation with strain E19^T plus the addition of fertilizer significantly increased straw yield by 836 kg ha^{-1} compared to the mean ($\bar{x}$ = 5915 kg ha^{-1}, $P = 0.045$) (Fig. 3a). In contrast, in the absence of fertilizer, the bacterial inoculation could not perform properly, showing a significant decrease of 1080 kg ha^{-1} in straw yield compared to the mean ($P = 0.048$) (mostly caused by the absence of fertilizer, Fig. 3a). The grain yield results showed a similar trend to straw yield, but with non-significant results (Table 2, Fig. 3b). In addition, bacterial inoculation, fertilizer, and row spacing showed a significant effect on the thousand-kernel weight (fertilizer, row spacing, Table 2, Fig. 3d) and grain protein concentration (bacterial inoculation and fertilizer, Fig. 3c). The t-test using Satterthwaite's method indicated that the absence of fertilizer significantly decreased by 0.5 g and 1.25% the thousand-kernel weight and protein concentration compared to their means ($\bar{x}$ = 43.9 g, $P = 0.017$; $\bar{x}$ = 12.34%, $P = 3.41 \times 10^{-8}$, respectively). Remarkably, bacterial inoculation significantly increased the protein concentration (Fig. 3c) of WW by 0.56% compared to the mean ($\bar{x}$ = 12.34%, $P = 5.80 \times 10^{-8}$).

Non-significant effect of the factors row spacing and bacterial inoculation was found in SB for the straw yield, grain yield, and thousand-kernel weight (reduced linear model with no interaction between the fixed effects, Table 2). Nevertheless, the analysis of protein concentration revealed that bacterial inoculation significantly increased its concentration by 1.80% compared to the mean ($\bar{x}$ = 10.51%, $P = 1.19 \times 10^{-7}$, Supplementary Fig. S1b).

Similarly, mixed linear models were applied to correlate the copies g^{-1} DW of strain E19^T obtained from qPCR with the different yield parameters. The flowering stage was the best indicator compared to the ripening stages for assessing the correlation between the concentration of *H. diazotrophicus* and crop yield parameters (Supplementary Table S3). Strain E19^T copies g^{-1} DW showed the same significant effect on the straw yield of WW (Table 3), with an increment of 453 kg ha^{-1} (Fig. 4a) compared to the mean

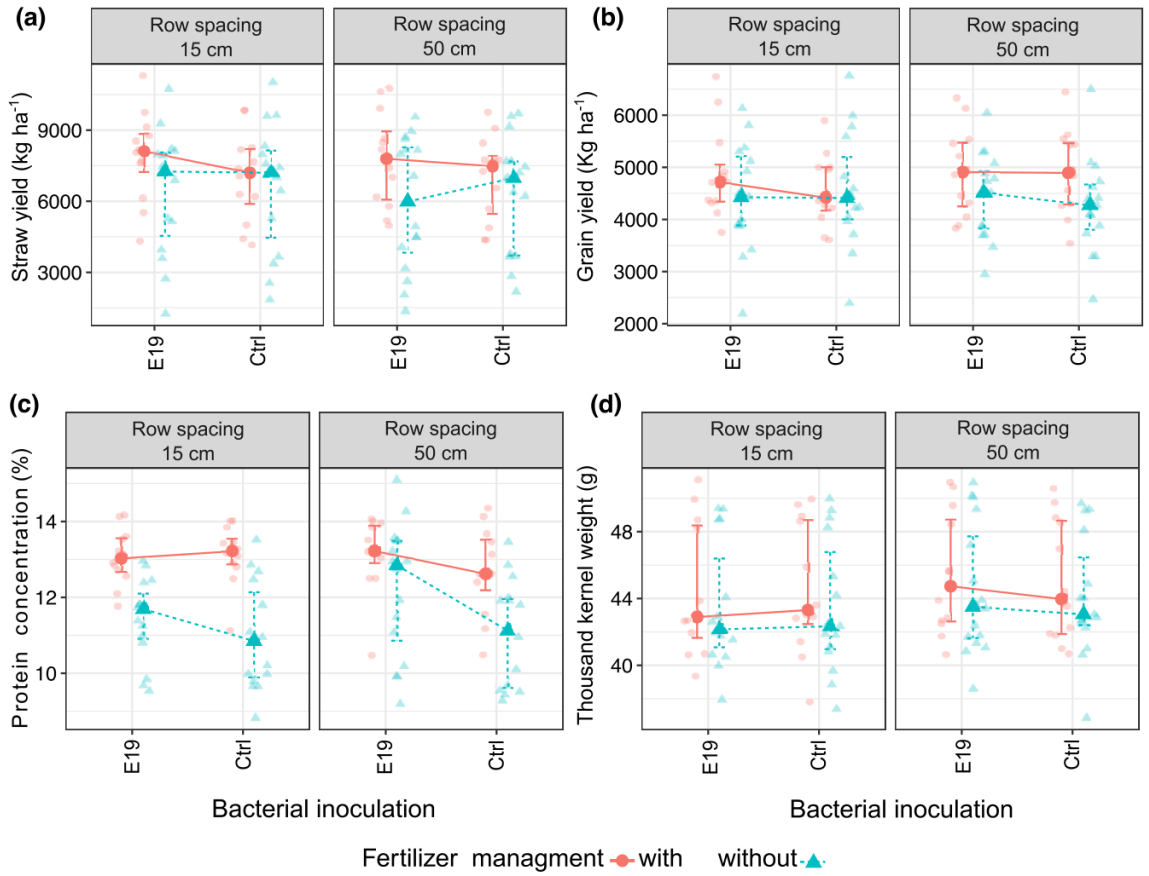


Figure 3. Linear mixed models fit by REML for WW (a) straw yield, (b) grain yield, (c) protein concentration, and (d) thousand kernel weight.

Table 3. Type III ANOVA with Satterthwaite's method based on linear mixed models of different yield parameters compared to the number of copies g^{-1} DW (log) of strain E19^T for WW and SB.

Plant	Yield parameter	Factor	Log copies E19	Fertilizer	Row spacing	Log copies E19: fertilizer
WW	Straw yield	F value	9.97	3.25	0.76	4.55
		Pr(>F)	**2.15×10^{-3}****	0.08	0.39	**3.56×10^{-2}***
	Grain yield	F value	1.73	4.76	0.21	0.23
		Pr(>F)	0.19	**3.29×10^{-2}***	0.65	0.63
	Crude protein concentration	F value	8.46	31.98	1.57	-
		Pr(>F)	**4.50×10^{-3}****	**2.00×10^{-4}*****	0.21	-
SB	Straw yield	F value	0.87	-	2.42	-
		Pr(>F)	0.35	-	0.13	-
	Grain yield	F value	4.94	-	3.82	-
		Pr(>F)	**3.09×10^{-2}***	-	0.06	-
	Crude protein concentration	F value	24.74	-	0.06	-
		Pr(>F)	**5.34×10^{-6}*****	-	0.81	-

Empty spaces belong to factors that were not included for the linear mixed model. Numbers in bold indicate significant differences. Significance codes: "***"<0.001, "**"<0.01, "*"<0.05.

($\bar{x} = 6486$ kg ha^{-1}, $P = 0.032$, *t*-test using Satterthwaite's method) and the same non-significant tendency for grain yield (Fig. 4b). Remarkably, in both WW and SB, the crude protein concentration clearly showed the effect of fertilizer application (only WW) and the number of copies g^{-1} DW of strain E19^T, indicating that higher copies g^{-1} DW of strain E19^T or fertilizer application were positively correlated with higher protein concentrations (Fig. 4c). Furthermore, higher concentrations of *H. diazotrophicus* detected in the soil were positively correlated with a significant increase of 0.30% and 0.80% in protein concentration compared to the mean

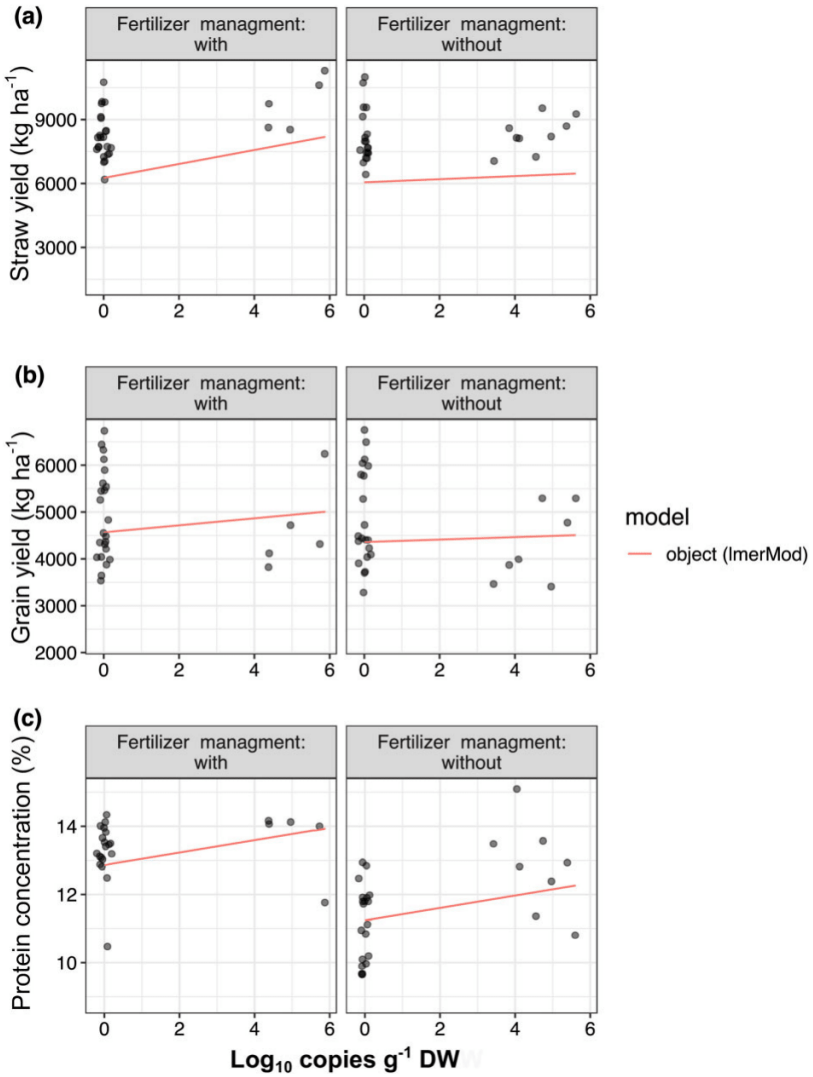


Figure 4. Linear mixed models fit by REML considering the log copies g^{-1} DW of strain E19^T for WW (a) straw yield, (b) grain yield, and (c) crude protein concentration.

of WW ($\bar{x}$ = 12.96%, P = 0.0058, Fig. 4c) and SB ($\bar{x}$ = 11.45%, $P = 2 \times 10^{-16}$, Supplementary Fig. S1a).

Surprisingly, in the case of SB, the number of copies g^{-1} DW of strain E19^T was negatively correlated with grain yield (Table 3). The t-test using Satterthwaite's method indicated that by each increment in a unit of log copies g^{-1} DW, there was a significant decreased in the grain yield of SB by 171 kg ha^{-1} compared to the mean ($\bar{x}$ = 3088 kg ha^{-1}, P = 0.025). Nevertheless, it is important to note that rather low SB yields were obtained during the season 2020–2021 (<1500 kg ha^{-1}, Table 1) due to weather conditions that caused fungal infection, affecting plant yield parameters and probably contributing to the negative effect observed by bacterial inoculation.

Discussion

In the last decade, bioinoculants have gained significant importance as a sustainable agricultural strategy. However, the lack of information on plant microbial interactions coupled with the reported inconsistency of their efficacy under agronomic conditions, as well as their persistence once released into the soil, is a current claim to be unveiled (for review, see Owen et al. 2015, Thilakarathna and Raizada 2017, Salomon et al. 2022). In this study, we developed specific primers to monitor the plant growth-promoting bacterium *H. diazotrophicus*, focusing on three important aspects: (i) bacterial root colonization after seed coating; (ii) early and long-term persistence and quantification of *H. diazotrophicus* under field experimental conditions; and (iii) the correlation between the number of copies g^{-1} DW of *H. diazotrophicus*, as well as the different factors evaluated with certain yield parameters of WW and SB.

We determined successful root colonization of *H. diazotrophicus* through qPCR at the greenhouse level, simulating field conditions (GH soil and coated seeds) for WW seeds. The bacterium was detected 10 DAS in 100% of the samples analyzed at a high concentration (Fig. 1b), indicating on one side the seed coating effective-

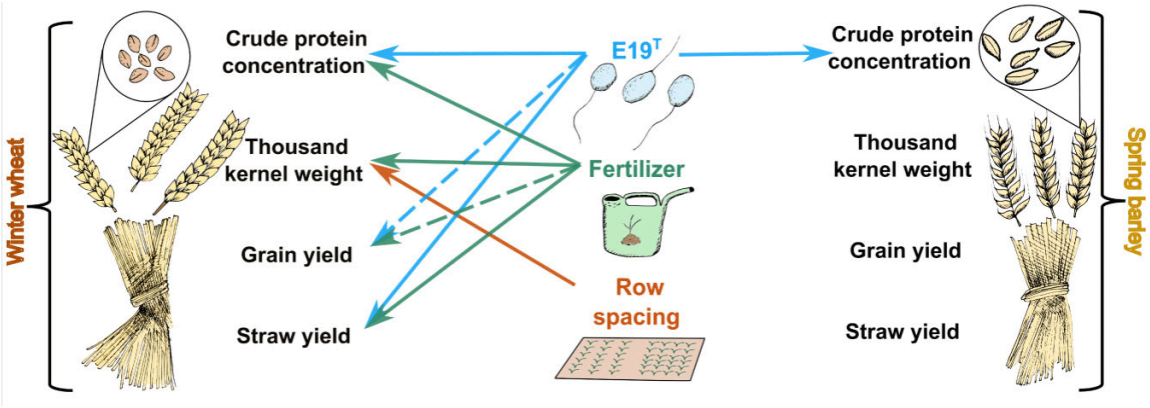


Figure 5. Illustrative representation of the different correlations obtained from linear mixed model analysis of WW and SB. Solid lines represent a significant effect of the factor evaluated. Dashed lines show non-significant interactions but a positive trend with the factors.

ness technique and on the other side the root colonization ability of *H. diazotrophicus*, not only for barley but also for wheat. In the E19^T genome, several genes have been related to rhizosphere/root colonization, such as those involved in quorum sensing (*aiiA*, *lux*, *rhtB*), chemotaxis (*che*, *tsr*), motility (*flg*, *fli*), secretory protein systems (*tat*, *sec*), and antibiotic resistance (*acr*, *emr*), which were described in the detailed gene list of Suarez et al. (2019) in their Supplementary Tables S12 and S15. Earlier, other groups, e.g. Compant et al. (2010) and Schikora et al. (2016), pointed out that the above-mentioned bacterial traits of PGPR are important for root colonization. Furthermore, in a recent metatranscriptomic study by Vannier et al. (2023), several genes have been associated with root colonization success of PGPR. All these genes are also present in the genome of E19^T, like the PstABCS operon, which encodes the phosphate starvation system (Pst) and promotes phosphate assimilation of E19^T. Similarly, the iron import gene *exBD* and the general stress response gene *typA*, which are important for root colonization (Vannier et al. 2023), were also present in the E19^T genome.

In agreement with the results for barley seeds obtained by Suarez et al. (2015), salt stress proved to play a fundamental role in the performance and persistence of strain E19^T on the roots of WW ($\bar{x}_{\log10\ copies} = 6.33$, Fig. 1b). To this point of evaluation, our results were in agreement with similar results obtained at a greenhouse level by qPCR in wheat roots (Stets et al. 2015) or maize roots (Couillerot et al. 2010, Faleiro et al. 2013) observed up to 18 days after inoculation with *Azospirillum* spp. Nevertheless, as time progressed, we observed at 30 DAS a significant decrease in the number of copies g^{-1} DW in the middle ($\bar{x}_{\log10\ copies} = 4.05$) and lower parts ($\bar{x}_{\log10\ copies} = 4.14$), but not in the upper part ($\bar{x}_{\log10\ copies} = 5.21$, Fig. 1b) of the WW root samples without salt stress, indicating that although the bacterium could be detected in all root sections, its survival began to decline (Fig. 1b). Xiang et al. (2010) propose three possible scenarios for introduced bacteria: a long-term persistence, a sharp decline some days/weeks after inoculation, or a gradual decrease of bacterial population. Based on our results, we observed that *H. diazotrophicus* was able to colonize the roots at an early stage (greenhouse results), gradually decrease its cell number, and stabilize over time (detected in advanced developmental stages), but not in 100% of the plants (Fig. 1a), showing lower detection percentages in WW (31%–63%, probably due to time lapse and hard climate conditions) and higher percentages in SB according to the results of field experiments (up to 100%). When an exogenous microorganism is introduced into a new habitat, it has to find or compete with native microorganisms for a niche that could be already occupied (niche overlapping), but it could also lead to dynamic character displacement, resulting in either competitive exclusion or coexistence with indigenous microorganisms (Hemmerle et al. 2022). Moreover, according to the "kill the winner" hypothesis (Thingstad 2000), resident viruses are able to maintain equilibrium and coexistence between bacterial communities, attacking the highly abundant microbial populations, especially when foreign bacteria are introduced (Russ et al. 2023). Bioinoculants usually contain highly concentrated inocula and mechanisms to ensure root colonization (Rocha et al. 2019), as previously described for E19^T.

Monitoring of bioinoculants under field conditions has increased in the last few years, revealing the dynamics of introduced microorganisms (Zhang et al. 2020, Urrea-Valencia et al. 2021). Soares et al. (2021) found differences between *Brachiaria* cultivars after inoculation with *Azospirillum baldaniorum*. In our study, the location was a determinant of *H. diazotrophicus* detection. We cannot clearly explain its unsuccessful detection at KH during season I and only in very few samples in season II (Fig. 1a). Therefore, seed transportation, interference during DNA extraction, or qPCR must be reconsidered. Nevertheless, we speculate that, although the soils of both organic farms share the same type (Haplic Luvisol) and similar soil properties (Supplementary Table S1), the native soil microbial communities could be different and play a pivotal role in the survival of *H. diazotrophicus* (Thingstad 2000, Hemmerle et al. 2022).

An illustrative summary of the correlation results is presented in Fig. 5. The addition of fertilizer to WW significantly improved the performance of *H. diazotrophicus* in terms of straw yield (836 kg ha^{-1} compared to the mean). This correlation was also observed with the copy number g^{-1} DW (453 kg ha^{-1}). Previous crop fertilization (e.g. potato at GH) combined with the low fertilizer level applied (<110 kg N ha^{-1}) could have contributed to the non-significant differences obtained in grain yield. The combination of PGPR and fertilizer could be related to better crop yield performance (Urrea-Valencia et al. 2021). Shaharoona et al. (2008) obtained similar results for wheat with the inoculation of *Pseudomonas fluorescens* and low levels of fertilizer, arguing that ACC deaminase producers (like *H. diazotrophicus*) reduced the inhibitory effect of the ethylene secreted in the roots at low nutrient levels, showing better growth and yield.

Remarkably, *H. diazotrophicus* significantly increased the grain raw protein concentration in both crops (0.56% and 1.80% for wheat and barley, respectively, compared to the mean; Fig. 3c and Table 2), correlating higher number of copies g^{-1} DW with higher protein concentrations (0.30% and 0.80% for wheat and barley; Fig. 4c and Table 3). This increase could be indirectly related to the nitrogen fixation activity reported for *H. diazotrophicus* (Suarez et al. 2014) and the 14 *nif* genes and fixation-related genes identified in its genome (Suarez et al. 2019). Nevertheless, our approach will be corroborated by further experiments, such as the use of ^{15}N isotope techniques that have been used to determine the nitrogen fixation activity of other diazotrophs (Urquiago et al 2012). In general, higher wheat grain protein concentrations, especially in the conditions of organic agriculture, are required to improve baking quality; however, relevant grain protein subunits should also be considered (Rekowski et al. 2020). In contrast, for a beer brewery, the barley protein concentration needs to be <11% in order to favor carbohydrate levels and reduce off-flavors (Díaz et al. 2022). In this context, inoculation with *H. diazotrophicus* is not recommended for the production of barley for brewery purposes, but for animal feed. Nevertheless, further research is required to confirm this finding. The lack of consistent positive effects of strain E19^T on the other yield parameters of SB was hidden by the susceptibility of the cultivar Odilia to fungal infections (Federal Plant Variety Office 2021) and subsequently low yields. Strain E19^T possesses genes that encode for phenazine biosynthesis and γ-aminobutyric acid (biocontrol agents) (Suarez et al. 2019). However, a threshold in the density of bioinoculants is required to show an effective response against phytopathogens (Raaijmakers et al. 1995). Similarly, the success of PGPR in controlling fungal infections is usually limited to low fungal density (Siddiqui and Shaukat 2002), which requires good fungal monitoring under field conditions. Future experiments shall explore the biocontrol potential, particularly because the genome of strain E19^T contains a quorum quenching system with a gene for *N*-acetyl-homoserine lactone degradation. This system has the potential to influence both pathogen infection and defense mechanisms.

Conclusion

In summary, the development of specific primers to track *H. diazotrophicus* strain E19^T has undoubtedly demonstrated its root colonization ability at early developmental stages, not only in barley as shown in previous research, but also in wheat, indicating its adaptability to colonize different plant species. We successfully detected the strain E19^T through qPCR from the greenhouse level (30 DAS) until advanced developmental stages (milk/fully ripe) under field conditions. Although high concentrations and detection rates of strain E19^T, with or without salt stress, were observed until 30 DAS, once beyond the greenhouse the detection rate was reduced. It gradually decreased but could be detected until advanced developmental stages, which suggests possible bacterial stabilization in the roots over time. These results also suggest that successful root colonization in the early stages does not directly imply high survival rates in later growth stages. Furthermore, the effect of exogenous bacterial inoculation on the native microbial soil communities needs to be evaluated in addition to interactions among each other, which help to elucidate the survival of strain E19^T. Finally, WW straw yield and grain protein concentration were significantly enhanced by inoculation with *H. diazotrophicus* and addition of organic fertilizer. Furthermore, *H. diazotrophicus* favored an increase in the SB crude protein concentration.

Acknowledgements

We warmly acknowledged to Rita Geissler-Plaum and Bellinda Schneider for their technical support. We are also grateful to Simon Schäfer for his support during field experiments at Kleinhohenheim. We acknowledge Elnaz Salehi-Mobarakeh for her help with qPCRs and Arizbeth Sevilla for her help with the illustration. This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

Santiago Quiroga (Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft), David Rosado-Porto (Data curation, Visualization, Writing – original draft), Stefan Ratering (Conceptualization, Investigation, Project administration, Writing – review & editing), Azin Rekowski (Data curation, Formal analysis, Investigation, Writing – original draft), Franz Schulz (Investigation), Marina Krutych (Investigation), Christian Zörb (Conceptualization, Formal analysis, Supervision, Writing – review & editing), and Sylvia Schnell (Conceptualization, Funding acquisition, Supervision, Writing – review & editing).

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

Conflict of interest: The authors declare no competing interests.

Funding

This work was fully funded by the Federal Ministry of Education and Research (BMBF) of Germany under the BONARES program (Rhizo4bio) BreadAndBeer 031B0906A and 031B0906B.

Data availability

Yield parameters and qPCR data are available at the BONARES repository under the name "Production of wheat and barley under reduced input in organic farming."

References

Aloo BN, Tripathi V, Makumba BA *et al.* Plant growth-promoting rhizobacterial biofertilizers for crop production: the past, present, and future. *Front Plant Sci* 2022;**13**:1–15.

Backer R, Rokem JS, Ilangumaran G *et al.* Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front Plant Sci* 2018;**871**:1–17.

Bashan Y, Puente ME, Rodriguez-Mendoza MN *et al.* Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. *Appl Environ Microbial* 1995;**61**:1938–45.

Bates D, Mächler M, Bolker BM *et al.* Fitting linear mixed-effects models using lme4. *J Stat Soft* 2015;**67**:1–41.

Binacchi F, Niether W, Brock C *et al.* Demystifying the agronomic and environmental N performance of grain legumes across contrasting soil textures of central Germany. *Agric Ecosyst Environ* 2023;**356**:108645.

Burns JH, Anacker BL, Strauss SY *et al.* Soil microbial community variation correlates most strongly with plant species identity, followed by soil chemistry, spatial location and plant genus. *AoB Plants* 2015;**7**:plv030.

Cardinale M, Ratering S, Suarez C *et al.* Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress. *Microbiol Res* 2015;**181**:22–32.

Chen H, Fan M, Kuzyakov Y *et al.* Comparison of net ecosystem CO_2 exchange in cropland and grassland with an automated closed chamber system. *Nutr Cycl Agroecosyst* 2014;**98**:113–24.

Compant S, Clément C, Sessitsch A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* 2010;**42**:669–78.

Coniglio A, Larama G, Molina R *et al.* Modulation of maize rhizosphere microbiota composition by inoculation with *Azospirillum argentinense* Az39 (formerly *A. brasilense* Az39). *J Soil Sci Plant Nutr* 2022;**22**:3553–67.

Couillerot O, Bouffaud ML, Baudoin E *et al.* Development of a real-time PCR method to quantify the PGPR strain *Azospirillum lipoferum* CRT1 on maize seedlings. *Soil Biol Biochem* 2010;**42**:2298–305.

Díaz AB, Durán-Guerrero E, Lasanta C *et al.* From the raw materials to the bottled product: influence of the entire production process on the organoleptic profile of industrial beers. *Foods* 2022;**11**:3215. https://doi.org/10.3390/foods11203215.

El Zemrany H, Cortet J, Peter Lutz M *et al.* Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. *Soil Biol Biochem* 2006;**38**:1712–26.

Elfadl E, Reinbrecht C, Claupein W. Evaluation of phenotypic variation in a worldwide germplasm collection of safflower (*Carthamus tinctorius* L.) grown under organic farming conditions in Germany. *Genet Resour Crop Evol* 2010;**57**:155–70.

Faleiro AC, Pereira TP, Espindula E *et al.* Real time PCR detection targeting *nif*A gene of plant growth promoting bacteria *Azospirillum brasilense* strain FP2 in maize roots. *Symbiosis* 2013;**61**:125–33.

Fernandes P, Simões-Arajo J, Varial de Melo L *et al.* Development of a real-time PCR assay for the detection and quantification of *Gluconacetobacter diazotrophicus* in sugarcane grown under field conditions. *African J Microbiol Res* 2014;**8**:2937–46.

Fife D. Flexplot: graphically-based data analysis.*Psychol Methods* 2022;**27**:477–96.

Goswami D, Pithwa S, Dhandhukia P *et al.* Delineating *Kocuria turfanensis* 2M4 as a credible PGPR: a novel IAA-producing bacteria isolated from saline desert. *J Plant Interact* 2014;**9**:566–76.

Hartmann A, Fu HA, Burris RH. Influence of amino acids on nitrogen fixation ability and growth of *azospirillum* spp. *Appl Environ Microb* 1988;**54**:87–93.

Hemmerle L, Maier BA, Bortfeld-Miller M *et al.* Dynamic character displacement among a pair of bacterial phyllosphere commensals in situ. *Nat Commun* 2022;**13**:2836. https://doi.org/10.1038/s41467-022-30469-3.

Horst A, Hill A, Gorman K. Palmerpenguins: palmer archipelago (antarctica) penguin data. R package version 0.1.0. 2020. https://doi.org/10.5281/zenodo.3960218 (23 February 2024, date last accessed).

IUSS Working Group WRB. World reference base for soil resources 2014, update 2015 international soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No 106 FAO, Rome. 2015. https://www.fao.org/3/i3794en/I3794en.pdf (13 February 2024, date last accessed).

Kampmann K, Ratering S, Baumann R *et al.* *Hydrogenotrophic methanogens* dominate in biogas reactors fed with defined substrates. *Syst Appl Microbiol* 2012;**35**:404–13.

Kampmann K, Ratering S, Kramer I *et al.* Unexpected stability of *Bacteroidetes* and *Firmicutes* communities in laboratory biogas reactors fed with different defined substrates. *Appl Environ Microb* 2012;**78**:2106–19.

Kirchhof G, Schloter M, Abmus B *et al.* Molecular microbial ecology approaches applied to diazotrophs associated with nonlegumes. *Soil Biol Biochem* 1997;**29**:853–62.

Klindworth A, Pruesse E, Schweer T *et al.* Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 2013;**41**:e1.

Kloepper J, Schroth M. Plant growth-promoting rhizobacteria on radishes. IV international conference on plant pathogenic bacteria article. *Proceedings of the 4th international conference on plant pathogenic bacteria, Angers, France*. 1978;**2**:879–82.

Kloepper JW, Ryu CM, Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 2004;**94**:1259–66.

Kloepper JW. Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. *Phytopathology* 1981;**71**:590.

Krzyzanowska D, Obuchowski M, Bikowski M *et al.* Colonization of potato rhizosphere by GFP-tagged *Bacillus subtilis* MB73/2, *Pseudomonas* sp. P482 and *ochrobactrum* sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. *Sensors* 2012;**12**:17608–19.

Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: tests in linear mixed effects models. *J Stat Soft* 2017;**82**:1–26.

Loy A, Arnold R, Tischler P *et al.* ProbeCheck—a central resource for evaluating oligonucleotide probe coverage and specificity. *Environ Microbiol* 2008;**10**:2894–8.

Ludwig W, Strunk O, Westram R *et al.* ARB: a software environment for sequence data. *Nucleic Acids Res* 2004;**32**:1363–71.

Maluk M, Ferrando-Molina F, Lopez del Egido L *et al.* Fields with no recent legume cultivation have sufficient nitrogen-fixing rhizobia for crops of faba bean (*Vicia faba* L.). *Plant Soil* 2022;**472**:345–68.

Maluk M, Giles M, Wardell GE *et al.* Biological nitrogen fixation by soybean (*Glycine max* [L.] Merr.), a novel, high protein crop in Scotland, requires inoculation with non-native bradyrhizobia. *Front Agron* 2023;**5**:1–16.

Munoz R, Yarza P, Ludwig W *et al.* Release LTPs104 of the all-species living tree. *Syst Appl Microbiol* 2011;**34**:169–70.

Office FPV. Descriptive list of varieties: cereals, maize, oil and forage plants, legumes, beet, cover crops. 2021;100–62. https://www.bundessortenamt.de/bsa/en/variety-testing/descriptive-variety-lists/downloading-descriptive-variety-lists (23 February 2024, date last accessed).

Owen D, Williams AP, Griffith GW *et al.* Use of commercial bioinoculants to increase agricultural production through improved phosphrous acquisition. *Appl Soil Ecol* 2015;**86**:41–54.

Patil I. Visualizations with statistical details: the "ggstatsplot" approach. *JOSS* 2021;**6**:3167.

Quast C, Pruesse E, Yilmaz P *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2012;**41**:D590–6.

R Core Team. *R: a Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing,2023. http://www.R-project.org.

Raaijmakers JM, Leeman M, Van Oorschot MM *et al.* Dose-response relationships in biological control of fusarium wilt of radish by *Pseudomonas* spp. *Phytopathology* 1995;**85**:1075–81.

Rahman MM, Flory E, Koyro HW *et al.* Consistent associations with beneficial bacteria in the seed endosphere of barley (*Hordeum vulgare* L.). *Syst Appl Microbiol* 2018;**41**:386–98.

Rekowski A, Wimmer MA, Hitzmann B *et al.* Application of urease inhibitor improves protein composition and bread-baking quality of urea fertilized winter wheat. *J Plant Nutr Soil Sci* 2020;**183**:260–70.

Rilling JI, Acuña JJ, Nannipieri P *et al.* Current opinion and perspectives on the methods for tracking and monitoring plant growth—promoting bacteria. *Soil Biol Biochem* 2019;**130**:205–19.

Rocha I, Ma Y, Souza-Alonso P *et al.* Seed coating: a tool for delivering beneficial microbes to agricultural crops. *Front Plant Sci* 2019;**10**: 1357. https://doi.org/10.3389/fpls.2019.01357.

Russ D, Fitzpatrick CR, Teixeira PJPL *et al.* Deep discovery informs difficult deployment in plant microbiome science. *Cell* 2023;**186**:4496–513.

Salomon MJ, Demarmels R, Watts-Williams SJ *et al.* Global evaluation of commercial arbuscular mycorrhizal inoculants under greenhouse and field conditions. *Appl Soil Ecol* 2022;**169**: 104225. https://doi.org/10.1016/j.apsoil.2021.104225.

Schikora A, Schenk ST, Hartmann A. Beneficial effects of bacteria-plant communication based on quorum sensing molecules of the N-acyl homoserine lactone group. *Plant Mol Biol* 2016;**90**:605–12.

Schulz F, Brock C, Schmidt H *et al.* Development of soil organic matter stocks under different farm types and tillage systems in the organic arable farming experiment Gladbacherhof. *Arch Agron Soil Sci* 2014;**60**:313–26.

Shaharoona B, Naveed M, Arshad M *et al.* Fertilizer-dependent efficiency of *Pseudomonas* for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). *Appl Microbiol Biotechnol* 2008;**79**:147–55.

Siddiqui IA, Shaukat SS. Resistance against the damping-off fungus *rhizoctonia solani* systemically induced by the plant-growth-promoting rhizobacteria *Pseudomonas aeruginosa* (IE-6S+) and *P. fluorescens* (CHA0). *J Phytopathol* 2002;**150**:500–6.

Singh JS, Pandey VC, Singh DP. Efficient soil microorganisms: a new dimension for sustainable agriculture and environmental development. *Agric Ecosyst Environ* 2011;**140**:339–53.

Singh K, Chandra R, Purchase D. Unraveling the secrets of rhizobacteria signaling in rhizosphere. *Rhizosphere* 2022;**21**:100484.

Skoppek CI, Punt W, Heinrichs M *et al.* The barley HvSTP13GR mutant triggers resistance against biotrophic fungi. *Mol Plant Pathol* 2022;**23**:278–90.

Soares IC, Pacheco RS, da Silva CGN *et al.* Real-time PCR method to quantify Sp245 strain of *Azospirillum baldaniorum* on *Brachiaria* grasses under field conditions. *Plant Soil* 2021;**468**:525–38.

Sosulski FW, Imafidon GI. Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods. *J Agric Food Chem* 1990;**38**:1351–6.

Stets MI, Campbell Alqueres SM, Souza EM *et al.* Quantification of *Azospirillum brasilense* FP2 bacteria in wheat roots by strain-specific quantitative PCR. *Appl Environ Microb* 2015;**81**:6700–9.

Suarez C, Cardinale M, Ratering S *et al.* Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress. *Appl Soil Ecol* 2015;**95**:23–30.

Suarez C, Ratering S, Geissler-Plaum R *et al.* *Hartmannibacter diazotrophicus* gen. nov., sp. nov., a phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt-meadow plant. *Int J Syst Evol Microbiol* 2014;**64**:3160–7.

Suarez C, Ratering S, Hain T *et al.* Complete genome sequence of the plant growth-promoting bacterium *Hartmannibacter diazotrophicus* strain E19^T. *Int J Genomics* 2019;**2019**:1–12. https://doi.org/10.1155/2019/7586430.

Sundermann G, Wägner N, Cullmann A *et al.* Nitrate pollution of groundwater long exceeding trigger value: fertilization practices require more transparency and oversight. DIW Weekly Report, ISSN 2568-7697, Deutsches Institut für Wirtschaftsforschung (DIW), Berlin, Vol. 10, Iss. 8/9. 2020, pp. 61–72. https://www.diw.de/de/diw_01.c.741058.de/publikationen/weekly_reports/2020_08_1/nitrate_pollution_of_groundwater_long_excceding_trigger_valu_tilization_practices_require_more_transparenca_and_oversight.html (23 February 2024, date last accessed).

Thilakarathna MS, Raizada MN. A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. *Soil Biol Biochem* 2017;**105**:177–96.

Thingstad TF. Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnol Oceanogr* 2000;**45**:1320–8.

Thudi M, Palakurthi R, Schnable JC *et al.* Genomic resources in plant breeding for sustainable agriculture. *J Plant Physiol* 2021;**257**:153351.

Urquiaga S, Xavier RP, de Morais RF *et al.* Evidence from field nitrogen balance and ^{15}N natural abundance data for the contribution of biological N_2 fixation to Brazilian sugarcane varieties. *Plant Soil* 2012;**356**:5–21.

Urrea-Valencia S, Etto RM, Takahashi WY *et al.* Detection of *Azospirillum brasilense* by qPCR throughout a maize field trial. *Appl Soil Ecol* 2021;**160**:103849. https://doi.org/10.1016/j.apsoil.2020.103849.

Vannier N, Mesny F, Getzke F *et al.* Genome-resolved metatranscriptomics reveals conserved root colonization determinants in a synthetic microbiota. *Nat Commun* 2023;**14**:8274. https://doi.org/10.1038/s41467-023-43688-z.

Villegas L, Paterno E. Growth enhancement and root colonization of sugarcane by plant growth-promoting bacteria. *Philipp J Crop Sci* 2008;**33**:3–24.

Wani PA, Khan MS, Zaidi A. Co-inoculation of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth, yield and nutrient uptake in Chickpea. *Acta Agron Hungarica* 2007;**55**:315–23.

Wickham H, Averick M, Bryan J *et al.* Welcome to the tidyverse. *JOSS* 2019;**4**:1686.

Würschum T, Langer SM, Longin CFH *et al.* A modern green revolution gene for reduced height in wheat. *Plant J* 2017;**92**:892–903.

Xiang SR, Cook M, Saucier S *et al.* Development of amplified fragment length polymorphism-derived functional strain-specific markers to assess the persistence of 10 bacterial strains in soil microcosms. *Appl Environ Microb* 2010;**76**:7126–35.

Yahya M, Rasul M, Sarwar Y *et al.* Designing synergistic biostimulants formulation containing autochthonous phosphate-solubilizing bacteria for sustainable wheat production. *Front Microbiol* 2022;**13**:1–16.

Yegorenkova IV, Tregubova KV, Matora LY *et al.* Use of ELISA with antiexopolysaccharide antibodies to evaluate wheat-root colonization by the rhizobacterium *paenibacillus polymyxa*. *Curr Microbiol* 2010;**61**:376–80.

Zadoks JC, Chang TT, Konzak CF. A decimal code for the growth stages of cereals. *Weed Res* 1974;**14**:415–21.

Zhang S, Ma Y, Jiang W *et al.* Development of a strain-specific quantification method for monitoring *Bacillus amyloliquefaciens* TF28 in the rhizospheric soil of soybean. *Mol Biotechnol* 2020;**62**:521–33.

Received 1 December 2023; **revised** 9 February 2024; **accepted** 15 February 2024

Chapter III

Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial

Research article

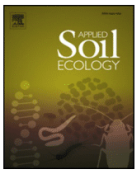
Published in Applied Soil Ecology

Applied Soil Ecology 206 (2025) 105823

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



Research paper

Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial

Santiago Quiroga [a], Stefan Ratering [a], David Rosado-Porto [a,b], Azin Rekowski [c], Franz Schulz [d], Christian Zörb [c], Sylvia Schnell [a,*]

[a] Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany
[b] Faculty of Basic and Biomedical Sciences, Simón Bolívar University, 080002 Barranquilla, Colombia
[c] Institute of Crop Science, Quality of Plant Products, 340e, University of Hohenheim, 70593 Stuttgart, Germany
[d] Department of Agronomy and Plant Breeding II, Justus-Liebig University Giessen, 35394 Giessen, Germany

ARTICLE INFO

Keywords:
PGPR
Bio-inoculant
Indigenous microbiome
Bacterial community composition
Differential abundance
Longitudinal analysis

ABSTRACT

The effects of plant growth promoting bacteria used for inoculation on native microorganisms remain unexplored under field conditions and, to a lesser extent, in longitudinal studies using different crops. This study, spanning three seasons across two organic fields, examined through 16S rRNA gene sequencing how the seed inoculation of *Hartmannibacter diazotrophicus* influenced the rhizosphere bacterial communities of wheat and barley. In addition to bacterial inoculation, the effects of row spacing and organic fertilizer application were also assessed. Together with previous results, we determined that *H. diazotrophicus* could improve crop yield parameters without altering the bacterial community composition. Alpha and beta diversity indices showed non-significant effects for most of the three factors evaluated. The 19 most prevalent taxa at the genus level were identified in both crop species, which mainly encompassed the phyla *Pseudomonadota*, *Acidobacteriota*, and *Actinomycetota*. Differential abundance analysis showed that the location significantly influenced the recruitment of different bacterial communities by the same crop species. While in one organic farm, 2860 ASVs were affected by crop species, 232 ASVs were impacted at the other location. Further analyses, including longitudinal analysis, linear mixed model effects, and diversity indices, showed a significant effect of location, crop species, and season on the dynamics of bacterial communities. Our results are unusual compared with most of the studies reported and indicate the resilience of rhizosphere bacterial populations after the incorporation of an allochthonous microorganism such as *H. diazotrophicus*.

1. Introduction

The use of living microorganisms has emerged as a strategy to address the unwanted effects of chemical pesticides and mineral fertilizers. By 2023, a market research report valued the global fertilizer market at over 200 billion USD (Global Market Insights, 2024). For biofertilizers, another report projects a market value of 2.70 billion USD by the current year (Market Data Forecast, 2024). As a sustainable agricultural strategy, biofertilizers are expected to grow, with projections indicating a market value of 4.78 billion USD with a compound annual growth rate (CAGR) of 12.09 % by 2029 (Market Data Forecast, 2024). Along with this continuous increase, several concerns have arisen regarding the environmental impacts of using living microorganisms to enhance plant performance. The current debate aims to elucidate the effects of bio-inoculants, broadly referred to beneficial microorganisms, once they are released into the soil. From an analysis of 108 studies, Mawarda et al. (2020) determined that soil microbial communities were altered in 86 % of cases when bio-inoculants were introduced. Only a few studies have shown that shifts in microbial community structure can negatively influence plant growth promotion (Ciccillo et al., 2002;

* Corresponding author at: Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany
E-mail addresses: Santiago.Quiroga@umwelt.uni-giessen.de (S. Quiroga), Stefan.Ratering@umwelt.uni-giessen.de (S. Ratering), David.Rosado-Porto@agrar.uni-giessen.de (D. Rosado-Porto), Azin.ghabelrahmat@uni-hohenheim.de (A. Rekowski), Franz.Schulz@agrar.uni-giessen.de (F. Schulz), Christian.zoerb@uni-hohenheim.de (C. Zörb), Sylvia.Schnell@umwelt.uni-giessen.de (S. Schnell).

https://doi.org/10.1016/j.apsoil.2024.105823
Received 25 April 2024; Received in revised form 21 October 2024; Accepted 9 December 2024
Available online 15 December 2024

Kozdrój et al., 2004). This bias towards reporting primarily positive results may distort the perceived efficacy of bio-inoculants (Cardinale et al., 2015).

Plant growth promoting rhizobacteria (PGPR), a category within bio-inoculants, enhance plant growth through mechanisms including 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, nitrogen fixation, iron sequestration, or induction of systemic resistance (for review: El-Saadony et al., 2022; Nagrale et al., 2023). Further studies have determined that bio-inoculants can alter soil physicochemical properties such as soil aggregation, soil organic matter decomposition, and nutrient cycling (Dar et al., 2021; Sun et al., 2020; Zhou et al., 2020). Nevertheless, the positive or negative environmental impacts of introducing bio-inoculants are complex and go beyond determining alterations in soil microbial populations. In natural environments, microbial communities shift in different plant compartments during the plant life cycle (Wagner et al., 2016, 2014).

The introduction of PGPRs has been correlated with changes in community composition and abundance of bacteria involved in plant growth promotion (e.g. *Chryseobacterium* and *Bradyrhizobium*) (Zhong et al., 2019). Interestingly, exogenous bacterial inoculation has been related to an increase in the abundance of biocontrol agents (e.g. *Cryptococcus*) during soil pathogen suppression (Fu et al., 2017). However, it is also crucial to consider factors prior to inoculation. Keswani et al. (2019) highlighted the re-categorization of several PGPR as biosafety level 2 microorganisms (moderate individual risk and low community risk). To establish an appropriate environmental and human safety index (EHSI) and adequate regulatory frameworks, whole genome sequencing is highly recommended. In this regard, differentiating a microorganism at species and strain levels can contribute to an accurate assessment of its biosafety level based on phylogenomic information (Vílchez et al., 2016; Keswani et al., 2019).

Field experiments often present complexities such as harsh environmental conditions, competition with native soil microbial communities, and different agronomical practices. Interestingly, these challenges have begun to provide valuable insights into the efficacy of bio-inoculants. For instance, bio-inoculants have shown to be more effective in dry regions compared to other climatic zones (Schütz et al., 2018) and re-inoculating microorganisms from local fields may offer plant growth promotion advantages due to their intrinsic environmental adaptability (Jiang et al., 2023). Furthermore, bacterial community assembly has been shown to be influenced by different crop rotation practices (Zhou et al., 2023). Nevertheless, the effects of PGPR in the field are limited. Of the studies analyzed by Mawarda et al. (2020), only 36 % were conducted under field experimental conditions. Additionally, 25 % used surface-sterilized seeds prior to inoculation under greenhouse conditions (Appendix A, Supplementary Table S1). This may give the bio-inoculant a partial advantage that does not accurately reflect in situ conditions and may manifest as part of the reported inconsistencies observed when bio-inoculants are applied in the field (Mayer et al., 2010; Jansson et al., 2023; de-Bashan and Nannipieri, 2024). Therefore, understanding the dynamics of bio-inoculants with indigenous soil microbial communities and their surrounding environment is essential for improving their efficacy. Current efforts to standardize the application of bio-inoculants in field studies may enhance the validity and reproducibility of these experiments (Neuhoff et al., 2024).

Hartmannibacter diazotrophicus strain E19^T is a PGPR, originally isolated from a natural salt meadow. In vitro and greenhouse experiments with barley have demonstrated its capability to solubilize phosphate, fix nitrogen, and enhance plant growth through ACC deaminase activity (Suarez et al., 2014, 2015). Later on, these PGPR abilities were corroborated by the identification of several relevant genes in its genome (Suarez et al., 2019). Recently, *H. diazotrophicus* strain E19^T was monitored on the roots of spring barley and winter wheat under field conditions. The bacterium was detected on the roots of both crops up to 273 and 119 days after seeding, mainly in one of the two organic farms evaluated. Additionally, its abundance was correlated with several yield parameters (Quiroga et al., 2024). Nevertheless, its impact on rhizosphere bacterial communities has not been addressed. Current molecular and omics approaches can help to disentangle the interactions between native microbial communities and allochthonous microorganisms, as well as the effects of abiotic and biotic factors (for review: Rai et al., 2023). Therefore, in this study, metabarcode sequencing was performed on soil bacterial communities at two organic farms across three seasons to examine how these populations were affected by strain E19^T inoculation, row spacing, and fertilizer application.

2. Materials and methods

2.1. Experimental site, seed coating, and field experiments

Two organic experimental stations were considered for the experiments: Kleinhohenheim (KH) (48° 44′ N, 9° 11′ E) located in southwest Germany and Gladbacherhof (GH) (50° 23′ N, 8° 15′ E) located in central Germany. The organic fields belong to the Hohenheim and Giessen universities, respectively. The experiments were conducted during three consecutive seasons: season I (2020−2021), season II (2021−2022), and season III (2022−2023). The soil type at each location was classified as Haplic Luvisol (IUSS Working Group WRB, 2015). The farms have been organically managed since 1994 (KH) and 1989 (GH). Detailed information regarding the experimental sites, soil properties, and crop rotation is provided in Appendix A (Supplementary Tables S2 and S4,) and Appendix B (Supplementary Table S6). Seed-coating of winter wheat (WW) and spring barley (SB) seeds was adapted from Kloepper (1981). Briefly, a resuspension in 0.03 M $MgSO_4$ of *Hartmannibacter diazotrophicus* strain E19^T (LMG 27460^T), cultured in a half-concentrated marine bouillon (Carl Roth GmbH, Germany), was mixed in a ratio 1:1 with gum Arabic 25 % and pH-value adjusted to 7.0 ± 0.2 ($\bar{x} = 1.73 \times 10^9 \pm 7.86 \times 10^8$ bacteria ml^{-1}). The mixture was spread over WW (*Triticum aestivum*, cv. Aristaro), and SB (*Hordeum vulgare* L., cv. Odilia/RGT-planet) seeds, properly mixed, and coated with talcum powder (Carl Roth GmbH, Germany). Colony forming unit (CFU) from the seed coated powder for all the seasons was estimated in $\bar{x} = 1.78 \times 10^8 \pm 1.05 \times 10^8$ CFU g^{-1} powder. To assess bacterial-specific effects, control treatments included only 0.03 M $MgSO_4$ without bacteria, following the previously described steps.

All field experiments were carried out similarly in a randomized complete block design (RCBD). Four replicates for the combination of the following factors were considered: fertilizer management (with fertilizer, without fertilizer, only for WW), row spacing (15 cm, 50 cm), and bacterial inoculation (strain E19^T, ctrl without bacteria). About 400 seeds m^{-2} were sown in plots of 7.5 m^2. Winter wheat was sown between October and November, whereas spring barley was sown between March and May (Appendix A, Supplementary Table S2). Only WW was fertilized with organic liquid manure by the soil drenching method with an average of 97.6 ± 11 kg N ha^{-1} (<110 kg N ha^{-1}, Appendix A, Supplementary Table S3). Fertilization of SB was not carried out because brewery standards require that protein concentration must be <11 % (Díaz et al., 2022).

2.2. Rhizosphere soil sampling, DNA extraction, and soil analysis

Roots of field plants and corresponding soil were collected from two distinct points in each plot (~1 m from the edge of the plot) at two plant growth stages: flowering (BBCH-60) and ripe (BBCH-75/BBCH-89), according to the Zadoks growth scale (Zadoks et al., 1974). During transportation, the samples were stored at 4 °C. The soil directly in contact with the roots was sieved into a glass bowl next to a Bunsen burner flame, using a metal sieve of 1.5 mm pore size and an ethanol-sterilized spoon. To avoid cross-contamination, newly sterilized glass dishes and sieves (180 °C for 5 h) were used for each sample. The tables were cleaned, disinfected, and covered with sterile covers for each

sample. Additionally, bacterial treatments and controls were processed in separate areas within the same room while maintaining aseptic conditions. The obtained rhizosphere soil was stored in sterile plastic centrifuge tubes at −80 °C for DNA extraction and in plastic bags at −20 °C for chemical analyses. DNA extraction and chemical analyses were performed within three months and five months after storage, respectively. To ensure cell disruption, approximately 700 mg of heat-sterilized zirconia beads (0.1 mm) were placed inside two ml screw-cap microcentrifuge tubes, prior to the addition of 400–500 mg of rhizosphere soil. DNA extraction procedure was described in detail by Quiroga et al. (2024). For this study, we modified the protocol by adjusting the amount of rhizosphere soil used, as well as the volumes of reagents: 0.7 ml of extraction buffer (0.2 M sodium phosphate buffer, 0.1 M NaCl, 0.05 M EDTA, 25 g l^{-1} SDS, pH 8), 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1), and 0.5 ml of chloroform. Dried DNA pellets were dissolved in 50 µl of nuclease-free water.

Ammonium (NH_4^+) and soil microbial biomass (SMB) were determined from rhizosphere soil samples from seasons I and II, as described by Kandeler and Gerber (1988) and Turner et al. (2001), respectively. For NH_4^+, two grams of soil were used, and colorimetrically determined after extraction with 1 M KCl at 660 nm. For SMB, 12.5 g of soil was employed for each fumigated and unfumigated samples, and the UV absorbance was measured at 280 nm with 0.5 M K_2SO_4. Additional chemical parameters that showed no significant differences, including the carbon/nitrogen ratio, nitrate, and soil microbial respiration, were measured as described by Rosado-Porto et al. (2023).

2.3. Metabarcoding characterization

Soil-extracted DNA was diluted (1:50) and the V4-V5 regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) using Kapa High Fidelity (KAPA/Hifi) polymerase (Roche diagnostics GmbH, Mannheim, Germany). Universal 16S rRNA gene primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTCMTTTRAGTTT) (Integrated DNA Technologies, Belgium) were used for amplification (Claesson et al., 2009; Engelbrektson et al., 2010). Detailed information on PCR conditions, barcode and adapter addition, as well as elution, purification, quantification, and sequencing of the PCR products via Ion Torrent PGM is provided in the Supplementary section of Kaplan et al. (2019). To avoid contamination, all sensitive steps were carried out under a class II biosafety cabinet, and the corresponding negative controls were included to detect possible contamination.

2.4. Bioinformatics and statistical analysis

Sequencing data were analyzed using QIIME2–2022.11 version (Bolyen et al., 2019) and R studio software, v4.3.2 (R Core Team, 2023). In QIIME2, sequences were demultiplexed using the cutadapt QIIME2 plugin (Martin, 2011). Quality control, denoising, dereplicate of single-end sequences, and chimera filtering were performed using the QIIME2 plugin of the software package DADA2 (Callahan et al., 2016). Sequences for all seasons were trimmed at 15 bp and truncated at 325 bp. Amplicon Sequence Variants (ASVs) produced with DADA2 were taxonomically affiliated with a trained fitted classifier (Pedregosa et al., 2011; Bokulich et al., 2018b) based on the SILVA database (release 138) (Quast et al., 2012). After taxonomic affiliation unassigned, mitochondrial and chloroplast sequences were filtered out.

The R package microViz v0.12.1 (Barnett et al., 2021) was used for data curation. Subsequently, the 50 most prevalent taxa at genus level were considered to create a heatmap with Kendall correlation and compositional data based on centered log-ratio (clr) transformation. In addition, the categorical variables grouped as location, bacterial inoculation, fertilizer management, crop species, row spacing, and plant growth stage were transformed into a binary format. A value of 1 was assigned to GH, strain E19^T, with fertilizer, WW, 15 cm row spacing, and flowering stage, respectively. Similarly, prevalent taxa at the genus level were filtered and plotted based on relative abundance considering ASVs with a detection threshold of 0.20 % and a prevalence of 75 % through R package Microbiome v1.24.0 (Lahti and Shetty, 2019). Differential abundance of compositional data was conducted using the R package ALDEx2 v1.34.0 (Fernandes, 2014) at different grouping levels, including bacterial inoculation, row spacing, fertilizer, plant growth stage, crop species, location, and season. Previously, ASV counts lower than 99 were filtered out through the R package Phyloseq v1.46.0 (McMurdie and Holmes, 2013). To ensure the identification of taxa that were substantially affected, effect size values (≥ 1 or ≤ -1) and adjusted p-values ($p < 0.05$) obtained from the Benjamini-Hochberg correction of Welch's t-test (we.eBH) were utilized, as recommended by the authors.

Alpha diversity analyses were carried out using the R package Vegan v2.6.4 (Oksanen et al., 2019). The samples were rarefied and the diversity indices Observed ASVs, Shannon-Wiener, and Gini-Simpson were estimated and plotted. Pairwise comparisons of each alpha diversity index were obtained through Wilcoxon rank-sum test with Holm adjustment. The QIIME2 Gemelli plugin with Robust Aitchison PCR (RPCA) was used for beta diversity analysis. Subsequently, the significant differences were identified through permutational multivariate analysis of variance (PERMANOVA, 999 permutations) with Benjamini-Hochberg correction (Martino et al., 2021). Similarly, to visualize which taxa were related to changes over time, the q2-longitudinal plugin based on machine learning regressors was used ("longitudinal feature-volatility" action) (Bokulich et al., 2018a). Thereafter, the significant effects across time were assessed using linear mixed-effects (LME) models based on Shannon entropy included within the q2-longitudinal plugin ("linear-mixed-effects" action). For the LME, location, crop species, and season were treated as fixed effects. Finally, to evaluate the effect of bacterial inoculation and fertilizer management on NH_4^+ and SMB concentrations, linear mixed models (LMM) were applied using the R packages lme4 v1.1.35.1 (Bates et al., 2015) and lmerTest v3.1.3 (Kuznetsova et al., 2017). Bacterial inoculation and fertilizer management (only for WW) were considered as fixed effects. Whereas season, location, and experimental block were set as hierarchical random effects, and three models were developed using the following formulas:

Model 1:

$$Y_i = \beta_0 + \alpha_{T(i)} + b_{0i} + e_i$$

$$b_{0i} \sim NID\left(0, \sigma^2\right) \text{ and } e_i \sim NID\left(0, \sigma^2\right)$$

Model 2:

$$Y_{ijk} = \beta_0 + \beta 1_j + \beta 2_k + \alpha_{T(i)} + b_{0i} + b_{1ij} + e_{ijk}$$

$$\begin{bmatrix} b_{0i} \\ b_{1i} \end{bmatrix} \sim NID\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{01} & \sigma_1^2 \end{bmatrix}\right) \text{ and } e_{ijk} \sim NID\left(0, \sigma^2\right)$$

Model 3:

$$Y_{ijk} = \beta_0 + \beta 1_j + \beta 2_k + (\beta 1 \beta 2)_{jk} + \alpha_{T(i)} + b_{0i} + b_{1ip} + e_{ijk}$$

$$\begin{bmatrix} b_{0i} \\ b_{1i} \end{bmatrix} \sim NID\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{01} & \sigma_1^2 \end{bmatrix}\right) \text{ and } e_{ijk} \sim NID\left(0, \sigma^2\right)$$

Where $\boldsymbol{Y_i}$ = chemical parameter concentration or response of the $\boldsymbol{i}$-th crop species, $\boldsymbol{j}$ = bacterial inoculation, $\boldsymbol{k}$ = fertilizer management, $\boldsymbol{\beta_0}$ = overall fixed intercept, $\boldsymbol{\beta 1_j}$ = intercept for bacterial inoculation, $\boldsymbol{\beta 2_k}$ = intercept for fertilizer management, $\boldsymbol{(\beta 1 \beta 2)_{jk}}$ = intercept of the interaction between both fixed effects, and $\boldsymbol{\alpha_T}$ = intercept of hierarchical random effects for the $\boldsymbol{i}$-th crop species. Finally, $\boldsymbol{b_{0i}}$ = random intercept, $\boldsymbol{b_{1i}}$ = random bacterial inoculation slope effects for the $\boldsymbol{i}$-th crop species, $\boldsymbol{e}$ = random error, and **NID** = normally and identically distributed effects. Model 1 illustrates the dependent variable (chemical parameter concentration) with only random effects and without fixed effects. Model 2 shows fixed effects but does not infer any interaction between

them. Model 3 considers fixed effects and assumes the interactions between them. Finally, the models assumed mutual independence among the variability originated from differences between groups (random effects) and the unexplained variability within groups (random error).

3. Results

3.1. Nineteen prevalent bacterial taxa at genus level identified in winter wheat and spring barley rhizosphere over a three-year field trial

A total of 13,052,032 sequences were obtained across all seasons, with a minimum of 5037 sequences and a maximum of 65,316 sequences per sample obtained after demultiplexing and quality control using QIIME2. After performing taxonomic cleaning in R, the taxa bar plots were grouped according to crop species, location, and season. ASVs with a relative abundance lower than 0.20 % were excluded. This filtering enabled the identification of the 19 most prevalent taxa at the genus level, along with their respective phyla, for each crop at GH and KH, and over three consecutive seasons (Fig. 1). The results revealed that the rhizosphere of WW and SB shared 19 taxa at the genus level, encompassing five phyla (*Pseudomonadota*, *Acidobacteriota*, *Actinomycetota*, *Bacillota*, and *Candidatus* Methylomirabilota). Of the most prevalent taxa, 47 % were within the phylum *Pseudomonadota*, whereas 89 % belonged to the phyla *Pseudomonadota*, *Acidobacteriota*, and *Actinomycetota*. Differences in the percentage of relative abundance were observed across crop species, location, and seasons in genera considered as PGPR (e.g. *Bradyrhizobium*, *Lysobacter*, and *Microvirga*) or involved in nutrient cycling (e.g. *Arenimonas*, *Luteimonas*).

3.2. Alpha diversity indices significantly varied between locations

After conducting an exploratory analysis, these differences were evaluated using different species diversity measurements. No significant effects of bacterial inoculation, fertilizer, or row spacing were observed on any of the alpha diversity indices evaluated over the three seasons ($p > 0.05$). Instead, the treatments were categorized according to location (GH, KH), crop species (WW, SB), and plant growth stage (flowering/ripe) for each individual season. In addition, the species richness for those indices was estimated (rarefaction, 95 % confidence intervals, Fig. 2). A significant influence of location on all alpha diversity indices was determined across all three seasons for both crops. This was observed in comparisons between GH (WW/SB at the flowering stage) and KH (WW/SB at the flowering/ripe stage). In the case of WW, the Shannon-Wiener and Gini-Simpson indices at KH were significantly higher than those at GH for all alpha diversity indices. In contrast, this trend was not completely followed by SB, where the indices were significantly higher at KH than at GH during seasons II and III, but not in season I (Fig. 2A). Interestingly, a significant variation between the flowering and ripe stages was observed only for WW across all seasons in the three alpha diversity indices (comparison only at GH), with the exception of Simpson index in season II (Fig. 2B). In the case of SB, this trend could not be observed beyond season I (Fig. 2A), when no significant differences were detected for any of the indices during seasons II (Fig. 2B) and III (Fig. 2C). Conversely, crop species showed significant differences mainly in all alpha diversity indices at the same location and stage across seasons. However, exceptions occurred in season I (Simpson index for KH, Fig. 2A) and season II (Observed ASVs for KH, and Simpson index for GH, Fig. 2B), when WW and SB were not significantly different.

3.3. Strain E19^T did not alter the rhizosphere bacterial microbiome, whereas bacterial communities were significantly different between locations

Rhizosphere bacterial communities in WW and SB were assessed by RPCA through the Gemelli plugin. The findings showed that bacterial inoculation, fertilizer management, and row spacing did not significantly alter the bacterial community structure. This was consistent across all seasons, as confirmed by the PERMANOVA analysis, showing no significant differences ($p > 0.05$, 999 permutations). The only exception was observed in the last season, when a significant effect of fertilizer on WW was found at GH during the ripe stage (Table 1). Additionally, significant differences were detected in season III, when fertilizer and bacterial inoculation were combined, attributing the main effect to the fertilizer (Table 1). This trend was not observed in the other two seasons or locations ($p > 0.05$). Similar to the alpha diversity, we

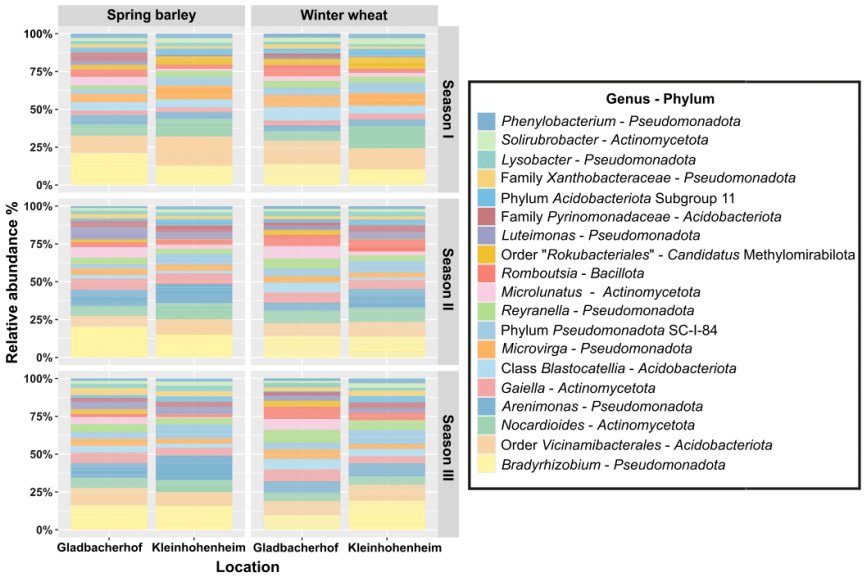


Fig. 1. Rhizosphere bacterial composition based on the relative abundance (%) of winter wheat and spring barley at different locations over three seasons. Taxa were collapsed at the genus level after removing ASVs with a relative abundance lower than 0.20 %.

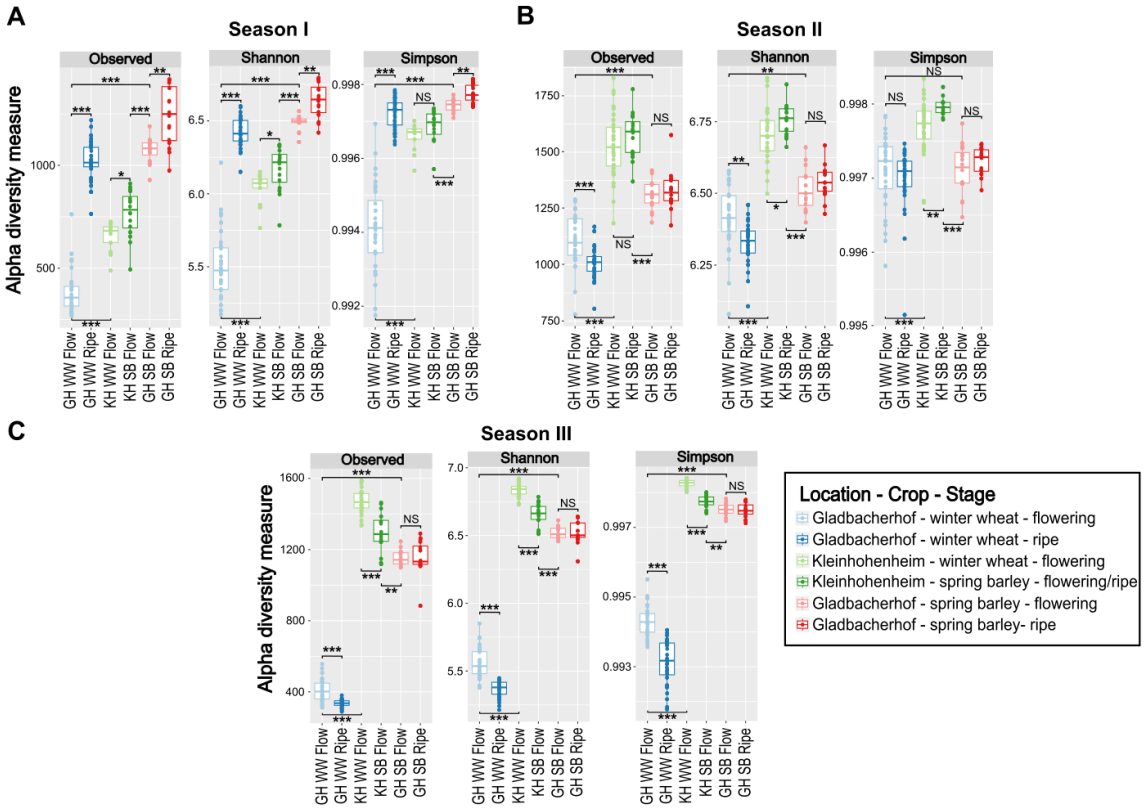


Fig. 2. Wheat and barley rhizosphere bacterial alpha diversity indices across seasons. The observed ASVs, Shannon-Wiener, and Gini-Simpson indices were grouped according to location, crop species, and plant growth stage during **(A)** season I (2020–2021), **(B)** season II (2021–2022), and **(C)** season III (2022–2023). Normalization was performed using rarefaction. NS, not significant. Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05, '*'.

determined how the rhizosphere bacterial communities were affected by location, crop species, and plant growth stage in each season (Fig. 3). The RPCA showed clear distinctions between location and crop species across seasons. These findings were corroborated by the PERMANOVA analysis (Table 1), indicating that the rhizosphere bacterial communities at GH differed significantly from those at KH. Additionally, the results revealed that, within the same location, crop species were able to recruit different bacterial communities. However, these differences varied depending on the location. At GH, distinct clusters were consistently found between WW and SB during the three seasons. Conversely, at KH although these clusters were closer together in the RPCA, PERMANOVA analysis showed significant differences only during seasons I and III (Fig. 3B-C).

When the plant growth stage was evaluated, the same consistent trend observed in the alpha diversity was found. WW bacterial communities (only at GH) significantly changed from the flowering to the ripe stage in all seasons (Table 1). In contrast, this pattern was not observed in SB, in which the bacterial communities differed between stages only during season I (Fig. 3A).

3.4. Differential abundance showed the effect of location on the recruitment of different bacterial communities by the same crop species

Following the analysis of ecological diversity indices in terms of bacterial diversity, we delved deeper to determine the taxa that were influenced by fertilizer, bacterial inoculation, and row spacing. Additionally, the variation of these taxa across location, plant growth stage, crop species, and season was also examined. This issue was addressed in two ways: first, through a correlation heatmap using Kendall correlation, and second, by analyzing the differential abundance employing ALDEx2 and considering effect size values ≥1 or ≤ −1.

Most correlations determined in the heatmap were given by location, crop species, season, and, to a lesser extent, by fertilizer and plant growth stage (Fig. 4A). The correlation heatmap mainly showed negative correlations for location and crop species, indicating that the 50 most prevalent taxa at the genus level were negatively correlated with GH and WW (Fig. 4A). Interestingly, several taxa were positively correlated as time progressed from season I to III. In contrast to bacterial inoculation and row spacing, in which almost no correlations were found, fertilizer application showed negative correlations. Based on these findings, we transitioned our approach to conduct a more specific analysis using ALDEx2 differential abundance. To corroborate whether the three factors evaluated had an effect on taxa abundance, each location, crop species, and season were analyzed separately at the flowering stage for comparison purposes. This approach was based on the observation that most of the variation in taxa abundance (Fig. 4A) was explained by these variables. ALDEx2 differential abundance showed no significant differences for the we.eBH ($p > 0.05$) with effect size values≥ -1 and ≤ 1 for any of the factors evaluated. These findings indicate that fertilizer management, bacterial inoculation, and row spacing had no significant effect on taxa abundance. Thereafter, the effect of crop species on differential abundance was examined at both locations for each season. The results showed that depending on the location, crop species were significantly influenced by different taxa

Table 1
PERMANOVA pairwise comparisons of beta diversity. Treatments were grouped according to location, crop species, and plant growth stage as well as by fertilizer and bacterial inoculation plus fertilizer.

Comparison	Evaluated factor	Season I	Season II	Season III
GH WW Flowering vs GH WW Ripe	stage	0.001***	0.0012**	0.0011**
GH WW Flowering vs KH WW Flowering	location	0.001***	0.0012**	0.0011**
GH SB Flowering vs GH SB Ripe	stage	0.001***	0.361	0.903
GH SB Flowering vs KH SB Flowering	location	0.001***	0.0012**	0.0011**
GH WW Flowering vs GH SB Flowering	crop species	0.001***	0.0012**	0.0011**
KH WW Flowering vs KH SB Flowering	crop species	0.001***	0.953	0.0011**
GH WW Ripe with fertilizer vs GH WW Ripe without fertilizer	fertilizer	0.758	0.250	0.0023**
GH WW Ripe Ctrl with fertilizer vs GH WW Ripe E19 without fertilizer	fertilizer + bacterial inoculation	0.763	0.699	0.028*
GH WW Ripe E19 with fertilizer vs GH WW Ripe Ctrl without fertilizer	fertilizer + bacterial inoculation	0.906	0.344	0.0073**
GH WW Ripe E19 with fertilizer vs GH WW Ripe E19 without fertilizer	fertilizer + bacterial inoculation	0.917	0.459	0.0015**

Gladbacherhof (GH), Kleinhohenheim (KH), winter wheat (WW), spring barley (SB). The adjusted *p*-value is shown, considering 999 permutations. Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05, '*'.

($p_{we.eBH} < 0.05$). In fact, it was originally intended to plot all taxa at the genus level with effect size values ≥ 1 and ≤ -1. Nonetheless, due to the large number of taxa significantly affected between SB and WW, higher effect size thresholds were set for plotting at GH (≥ 2.3 to 2.6 and ≤ -2.3 to −2.6) and at KH (≥ 1.2 to 1.6 and ≤ -1.2 to −1.6) (Fig. 4B). At GH, 2860 ASVs were significantly affected by crop species in at least one season, including 15 of the 19 most prevalent taxa. While at KH, 12 times fewer ASVs were found (232 ASVs), including ten of the 19 most prevalent taxa (Appendix B, Table S5). Despite the difference between locations, the taxa in Fig. 4B clearly illustrate that identifying a consistent trend in differential abundance for a specific taxon was difficult. For instance, while the prevalent genus *Microlunatus* was negatively affected by WW at GH across all seasons, the genera *Luteimonas*, *Arenimonas*, and *Bradyrhizobium* were only negatively influenced by WW in one of the three seasons. In contrast, at KH, *Nocardioides* was negatively influenced by WW compared to SB in seasons I and III, but positively affected during season II. These findings show, first, the pivotal role of location in the recruitment of different taxa by the same crop species, and second, the positive or negative influence of season on the same taxa.

3.5. Longitudinal analysis revealed the effects of location, crop species, and season on bacterial community dynamics

To shed more light on how these taxa fluctuated over time, longitudinal feature volatility analysis was performed using the q2-longitudinal plugin. The 35 most potentially relevant taxa were identified (Table 2). These results were in accordance with the 13 prevalent taxa observed in Fig. 1, and were also detected in the correlation heatmap and ALDEx2 analysis (Fig. 4). The feature volatility plot showed how the relative frequency varied according to different factors over time for each taxon. From all of them (Table 2), four prevalent taxa were selected: *Luteimonas* (top one, Table 2), *Reyranella* (top two, Table 2), *Arenimonas*, and *Lysobacter* (observed in both fields after differential abundance analysis, Fig. 4B). Thereafter, their fluctuations were monitored according to crop species and location (Fig. 5).

In general, the relative frequency changed depending on the location and crop species, differentiating GH and KH, as well as WW and SB, mainly in all taxa. This differentiation was not observed when the other previously analyzed factors were considered (e.g. bacterial inoculation). The fluctuations in *Arenimonas*, *Lysobacter*, and *Luteimonas* (Fig. 5A-C) correctly fit the results obtained after differential abundance analyses, at least with high effect size values (Fig. 4B, Appendix B, Table S5). For instance, the relative frequency of *Arenimonas* increased during season II (sample point 2.0, Fig. 5B) for SB at GH, whereas the differential abundance showed a negative correlation with WW during this season (Fig. 4B). At KH, the relative frequency was higher in season III (sample point 3.0) for SB than for WW, which was in accordance with the ALDEx2 negative correlation between WW and SB. Similarly, the positive WW correlation obtained for the differential abundance of *Lysobacter* at GH during season II and *Reyranella* for all seasons (Appendix B, Table S5) was related to higher relative frequencies in WW at those sample points (Fig. 5C-D). Nevertheless, several taxa, including some of the taxa analyzed, did not fully match with the differential abundance results (Appendix B, Table S5).

The inconsistent trend observed after differential abundance analyses was better understood with the longitudinal feature volatility plot visualization. A taxon that initially did not show any difference at one sampling point, could increase/decrease at the next point, or converged at another (e.g. *Arenimonas*). These findings indicate that plant-microbe interactions are affected over time by different variables. To corroborate this, LME models based on Shannon entropy within the q2-longitudinal plugin were used to determine the effects of location, crop species, and season (fixed effects) on microbial communities. None of the fixed variables showed any significant effects (Table 3). Nevertheless, Shannon entropy was significantly associated with the interaction of sample point (season) and location ($p = 0.001$), sample point and crop species ($p < 0.001$), and sample point, location, and crop species ($p = 0.001$, Table 3). The findings revealed that the Shannon entropy of the bacterial rhizosphere microbiome significantly increased by a factor of 0.24 at KH as the season progressed. In contrast, the bacterial microbiome decreased by a factor of 0.18 in WW across all seasons. Finally, LME also showed that the bacterial rhizosphere microbiome increased over time by a factor of 0.29 in KH and WW. These results corroborated and complemented the alpha and beta diversity results, unveiling how the rhizosphere microbiome is influenced across seasons, which could not be clearly determined by previous analyses.

3.6. Strain E19^T plus fertilizer showed a positive trend increasing ammonium and microbial biomass concentrations

The last approach attempted to correlate the differential abundance with several soil chemical parameters, including ammonium, microbial biomass, carbon/nitrogen ratio, nitrate, and respiration activity, using different carbon sources (glucose, galactose, *N*-acetylglucosamine, and arginine). Nevertheless, ALDEx2 analysis did not show any relevant correlation for any of the parameters previously mentioned (data

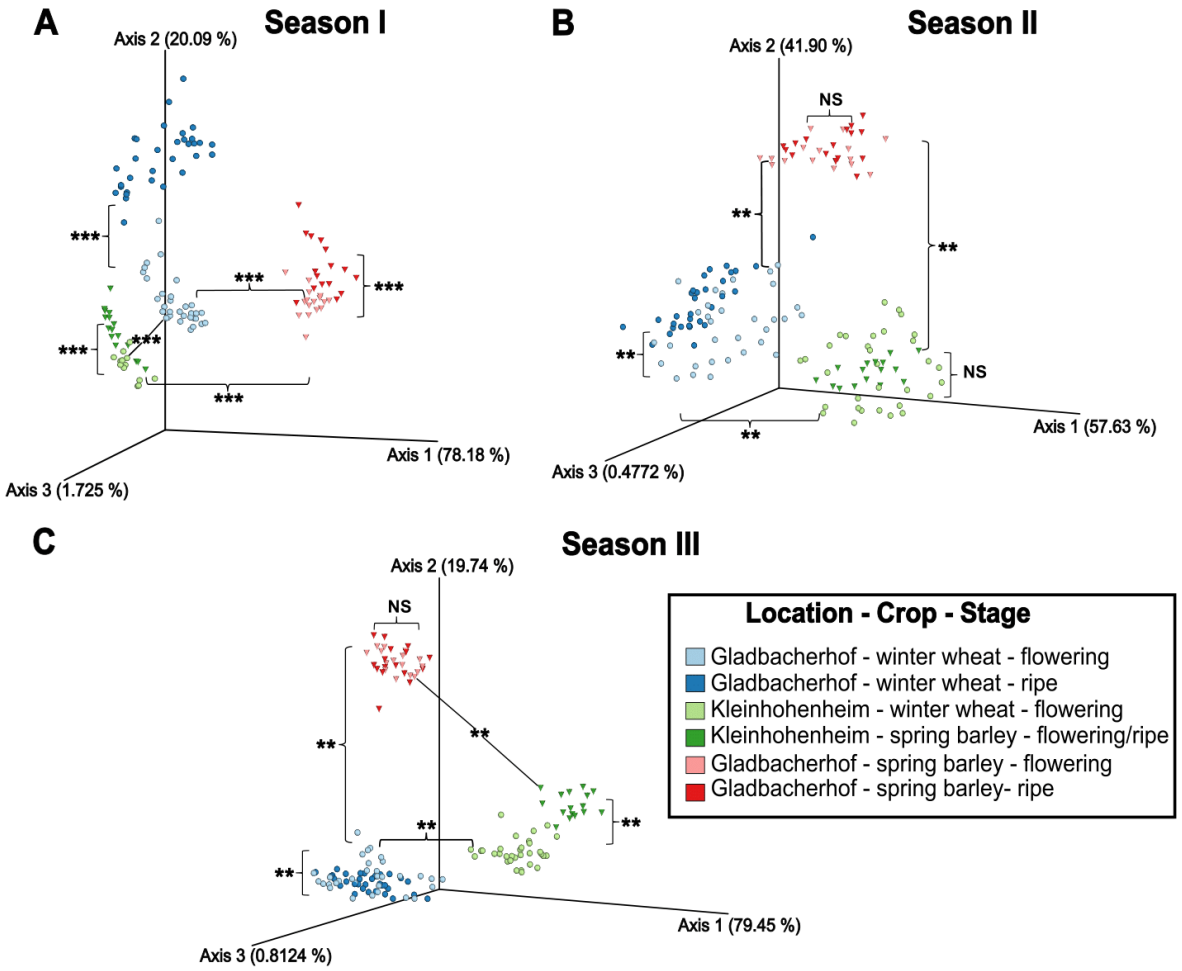


Fig. 3. Beta diversity analysis using robust Aitchison RPCA for winter wheat and spring barley at different locations and plant growth stages. **(A)** Season I (2020–2021), **(B)** season II (2021–2022), and **(C)** season III (2022–2023). NS, not significant. Significance codes: ≤ 0.001 '***', ≤ 0.01 '**'.

available at the BONARES repository). Instead, we decided to evaluate through LMMs whether the combination of fertilizer management and bacterial inoculation (fixed effects) had a significant effect on the soil chemical parameters. For this purpose, location, season, and block were modelled as hierarchical random effects. Significant effects on ammonium and microbial biomass were found in WW (Fig. 6), but not in SB ($p > 0.05$). A *t*-test using Satterthwaite's method showed significant differences only in the variable fertilizer management for microbial biomass (Table 4). However, type III analysis of variance revealed significant effects of fertilizer management on both parameters, ammonium ($p = 0.0021$) and microbial biomass ($p = 0.0004$). The results indicated a significant decrease when fertilizer was not applied by 39.4 µg SMB C g^{-1} DW soil compared to the mean ($\overline{x} = 415.5$ µg SMB C g^{-1} DW soil, Table 4). Even though a significant effect of bacterial inoculation or its combination with fertilizer could not be found (Table 4), a positive trend was observed for E19^T plus the addition of fertilizer to increase ammonium and microbial biomass concentrations (Fig. 6).

4. Discussion

4.1. Resilience of rhizosphere bacterial communities after non-native inoculation of H. diazotrophicus under field conditions

Few studies have focused on the effects of bio-inoculants under field conditions, and to a lesser extent, over an extended period (Fu et al., 2017; Gui et al., 2017; Xu et al., 2018). This study evaluated whether *H. diazotrophicus* strain E19^T could alter the rhizosphere bacterial community structure and how the interactions with these communities differed depending on crop species and location. Remarkably, strain E19^T did not alter the rhizosphere bacterial communities across all seasons (alpha and beta diversity indices). Resident microbial populations can exhibit resistance, resilience, or functional redundancy against disturbances (Allison and Martiny, 2008). It has been stated that an exogenous microorganism can be strongly buffered by native soil microbial communities (Björklöf et al., 2003). However, through mechanisms such as niche competition or antagonism, an external microorganism can be incorporated into native soil microbial communities (Russ et al., 2023). The findings in this study showed the resilience of the resident soil bacterial populations to restructuring themselves after probable disruptions in early plant growth stages due to the high

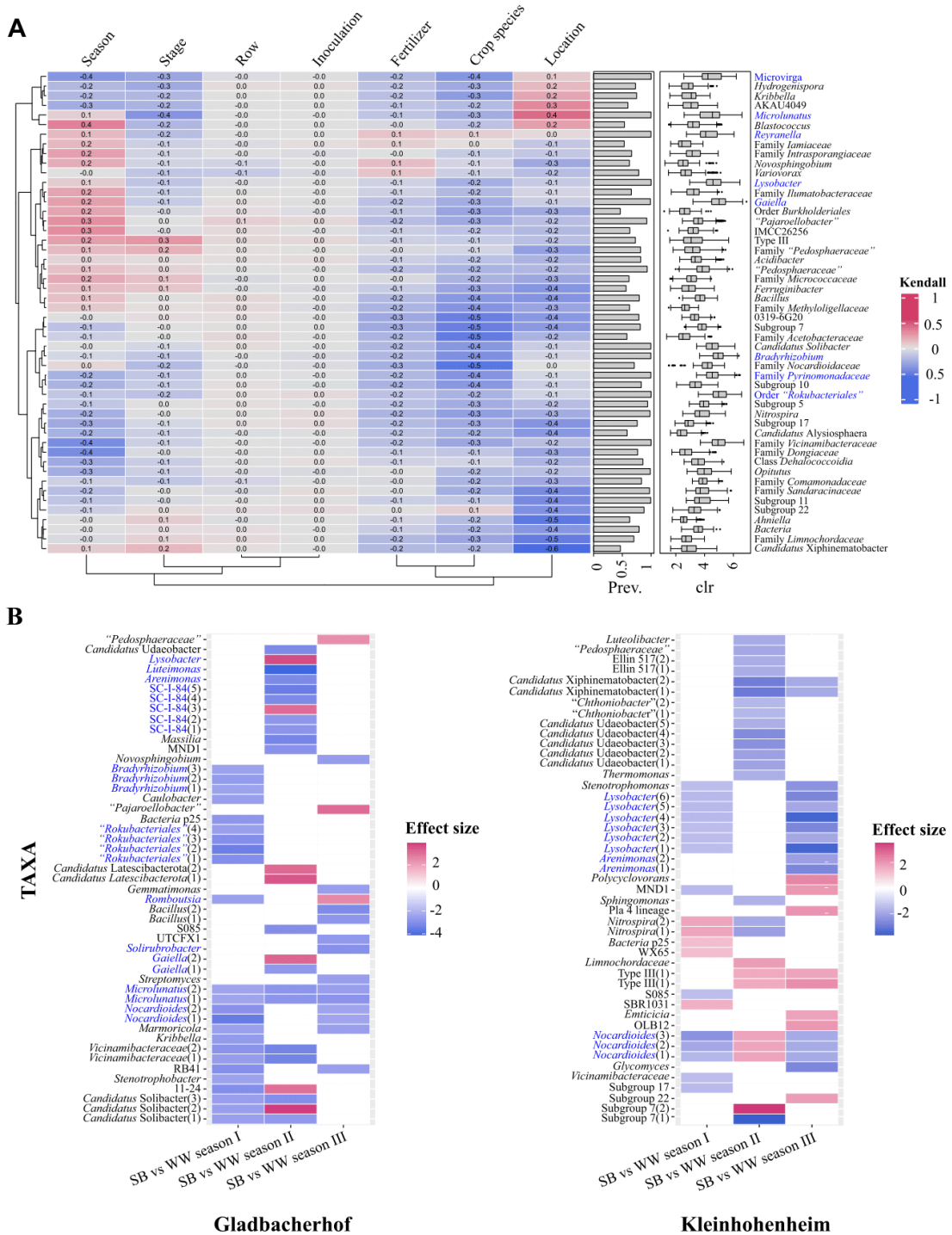


Fig. 4. Heatmaps showing the effects of different variables on taxa at the genus level across all seasons. **(A)** Correlation heatmap with Kendall correlation for the 50 most prevalent taxa **(B)** Heatmap between winter wheat (WW) and spring barley (SB) at two locations based on ALDEx2 differential abundance after removing ASVs with relative abundances <99 ASV counts. Due to the excessive number of taxa that were significantly affected, the effect size threshold varied depending on the location and season. At Gladbacherhof, effect sizes for seasons I and III: ≥ 2.3 and ≤ -2.3, for season II: ≥ 2.6 and ≤ -2.6. At Kleinhohenheim, effect size for season I: ≥ 1.2 and ≤ -1.2, for season II: ≥ 1.4 and ≤ -1.4, and for season III: ≥ 1.6 and ≤ -1.6.

Table 2
Top 35 most potentially relevant taxa at the genus level obtained from longitudinal feature volatility analysis. The taxa were organized according to their importance. Prevalent taxa are highlighted in bold.

Taxa	Importance	Cumulative average change	Global variance	Global mean
Luteimonas	0.192	0.012	5.5×10^{-5}	0.010
Reyranella	0.161	0.014	6.4×10^{-5}	0.019
Vicinamibacteraceae	0.128	−0.037	2.3×10^{-4}	0.038
Tepidisphaerales WD2101	0.098	0.008	6.0×10^{-5}	0.010
Uncultured *Gemmatimonadaceae*	0.071	−0.025	1.7×10^{-4}	0.046
Gaiella	0.061	0.036	3.0×10^{-4}	0.040
Opitutus	0.052	−0.012	3.0×10^{-5}	0.014
Nitrospira	0.044	−0.006	1.7×10^{-5}	0.011
Candidatus Latescibacterota	0.041	−0.017	1.6×10^{-4}	0.033
Entomoplasmatales type III	0.024	0.000	4.7×10^{-5}	0.008
Vicinamibacterales	0.020	−0.002	3.4×10^{-5}	0.018
Microvirga	0.018	−0.004	7.2×10^{-5}	0.021
Pyrinomonadaceae RB41	0.010	−0.018	1.0×10^{-4}	0.025
Anaerolineaceae UTCFX1	0.008	0.001	3.6×10^{-5}	0.013
Uncultured *Gaiellales*	0.007	0.020	2.2×10^{-4}	0.032
Candidatus Solibacter	0.007	−0.002	5.7×10^{-5}	0.024
Holophagae Subgroup 7	0.006	−0.012	6.4×10^{-5}	0.013
Burkholderiales SC-I-84	0.006	0.032	2.1×10^{-4}	0.057
"*Pedosphaeraceae*"	0.005	−0.001	4.0×10^{-5}	0.013
Candidatus Entotheonellaceae	0.005	0.010	1.6×10^{-4}	0.020
Microlunatus	0.003	0.030	2.0×10^{-4}	0.025
Bacillus	0.003	0.002	5.3×10^{-5}	0.012
Solirubrobacter	0.003	0.007	1.3×10^{-5}	0.010
"*Pajaroellobacter*"	0.003	0.007	1.6×10^{-5}	0.011
Xanthomonadaceae	0.003	0.002	3.5×10^{-5}	0.010
Myxococcota bacteria p25	0.002	−0.011	5.5×10^{-5}	0.019
Uncultured *Sandaracinaceae*	0.002	−0.005	2.3×10^{-5}	0.012
Bradyrhizobium	0.002	0.000	1.4×10^{-4}	0.036
Lysobacter	0.002	0.017	9.1×10^{-5}	0.027
Arenimonas	0.002	0.039	3.6×10^{-4}	0.036
Blastocatellia 11–24	0.002	−0.002	1.1×10^{-4}	0.022
Nocardioidaceae	0.001	0.004	7.7×10^{-5}	0.012
***Acidobacteriota* Subgroup 11**	0.001	−0.004	1.3×10^{-5}	0.012
Acidobacteriota Subgroup 5	0.001	−0.006	2.3×10^{-5}	0.014
Romboutsia	0.001	0.008	3.3×10^{-4}	0.020

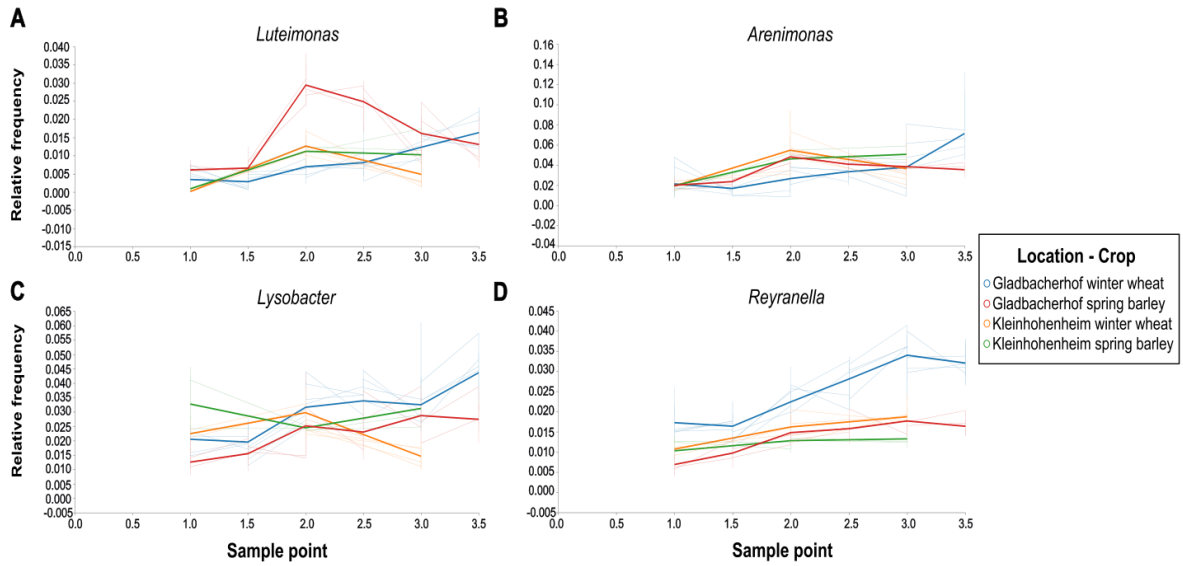


Fig. 5. Longitudinal feature volatility plot for the rhizosphere bacterial microbiome of WW and SB at the two locations. Relative frequencies of the genera **(A)** *Luteimonas*, **(B)** *Arenimonas*, **(C)** *Lysobacter*, and **(D)** *Reyranella* were plotted from the 35 most potentially relevant taxa identified in Table 2. Sample point 1, season I flowering; sample point 1.5, season I ripe; sample point 2, season II flowering (ripe only for KH-SB); sample point 2.5, season II ripe; sample point 3, season III flowering; sample point 3.5, season III ripe.

Table 3
Linear mixed-effects model for the rhizosphere microbiome based on Shannon entropy across three consecutive seasons (2020−2023). Location, crop species, and sample point were considered as fixed effects.

Variables	Estimate	Z-score	$P > \|z\|$
Intercept	8.77	26.03	<0.001***
Location [Kleinhohenheim]	−0.49	−1.02	0.31
Crop species [winter wheat]	−0.18	−0.38	0.701
Location[Kleinhohenheim]:crop species [winter wheat]	−0.01	−0.02	0.985
Sample point	−0.03	−0.77	0.442
Sample point:Location [Kleinhohenheim]	0.24	3.41	0.001***
Sample point:crop species [winter wheat]	−0.18	−3.88	<0.001***
Sample point:Location [Kleinhohenheim]:Crop species [Winter wheat]	0.29	3.21	0.001***

Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05 '*'.

bacterial concentration (Coniglio et al., 2022). A few studies conducted under field conditions have shown that resilient microbial populations can completely or partially recover their original state after several days or weeks (Kröber et al., 2014; Liu et al., 2018; Yin et al., 2013). The observed plasticity in microbial communities is unusual. In 80 % of the extended studies outlined by Mawarda et al. (2020), these communities failed to reestablish their original state. Nevertheless, it is important to emphasize that these studies represent a minor portion of the studies analyzed, as most of them were performed in the short term under greenhouse conditions (Appendix A, Supplementary Table S1). Further research is required to elucidate whether these alterations are transient or permanent.

4.2. Different rhizosphere bacterial communities could affect the persistence of H. diazotrophicus strain E19^T

Previously, strain E19^T showed its adaptability to colonize WW and SB roots, as well as its persistence over time in both crops, but was only detected through qPCR at GH (Quiroga et al., 2024). Our findings demonstrated that the rhizosphere bacterial communities varied significantly between the two locations in both diversity indices, indicating that soil bacterial communities play a fundamental role in the survival of strain E19^T. In addition, the higher values in alpha diversity indices (seasons II and III) and the different bacterial communities assembled at KH compared to GH suggest stronger competition of strain E19^T for a niche. It has been reported that high species richness is inversely correlated with successful microbial invasion due to resource competition (Van Elsas et al., 2012). This may result in niche overlap with native communities at KH, impacting its persistence over time. However, species richness alone is insufficient to explain microbial invasion success, as specific network configurations have also been shown to play a role (Wei et al., 2015). At GH, a possible lower niche overlap could lead to dynamic character displacement, which eventually led to competition but also coexist with the resident rhizosphere bacterial communities (Hemmerle et al., 2022; Russ et al., 2023).

Equally important, environmental factors like pH-value and precipitation are important drivers of soil bacterial communities (Xue et al., 2018; Zhou et al., 2018). In this study, both locations had similar soil characteristics, including soil type, pH-value, total N, and C/N ratio (Appendix A, Supplementary Table S4). Lower precipitation at GH and higher levels at KH during season II (Appendix B, Supplementary Table S6) could be related to higher alpha diversity indices observed at KH in this season, but not in the other two. Li et al. (2022) found that the alpha diversity indices were not significantly affected by precipitation in alpine grasslands. Nonetheless, precipitation significantly altered bacterial community composition and may contribute to the substantial changes observed between stages of WW at GH, where precipitation varied considerably between sampling points (June and July). In contrast, this might partially explain the stage shifts in SB only during season I, but not in the other seasons. Although environmental factors are key drivers of microbial communities, our results align more closely with several studies indicating the role of plant species in recruiting different microbial communities (Abedini et al., 2021; Burns et al., 2015; Kuzmicheva et al., 2017). These populations can vary throughout plant development, primarily due to differences in root exudation over time (Chaparro et al., 2013; Zhalnina et al., 2018). Plant developmental stage has been reported as an important driver of microbial communities (Chaparro et al., 2014; Xiong et al., 2021). Marked changes have been identified at initial stages (e.g. seedling) compared to advanced stages (e.g. reproductive stage), where microbial communities tend to stabilize

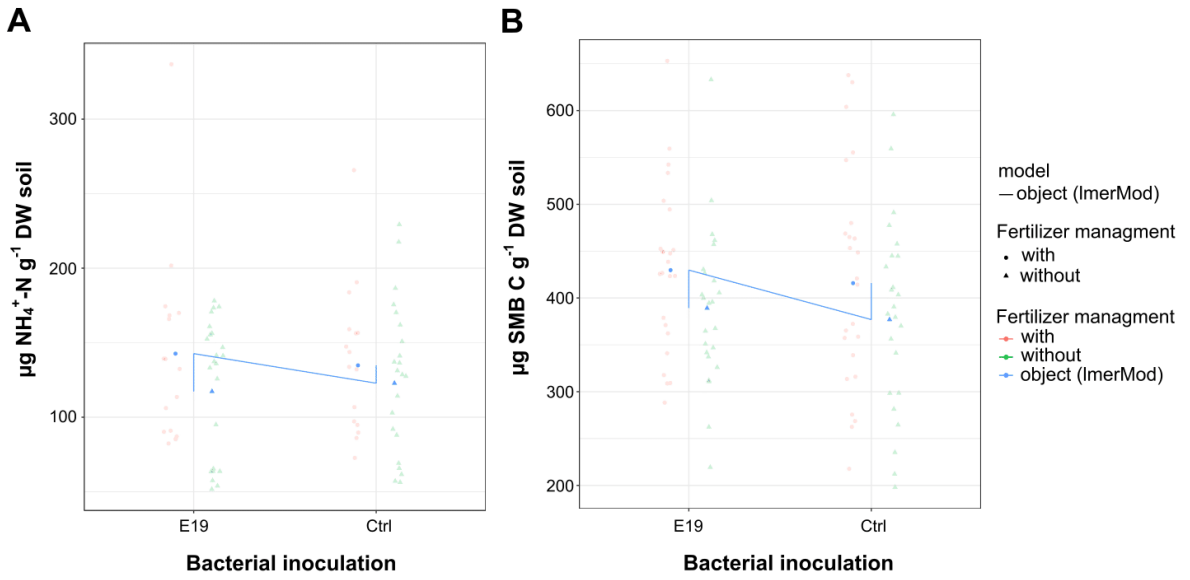


Fig. 6. Linear mixed models fit by REML for WW related to **(A)** ammonium and **(B)** microbial biomass concentrations.

Table 4
Linear mixed models fitted by REML for WW related to ammonium and microbial biomass concentrations. Fertilizer management and bacterial inoculation were considered as fixed effects.

Soil parameter	Variables	Estimate	*t* value	$P > \|t\|$
Ammonium	Intercept	134.8	6.42	0.005**
	Bacterial inoculation [E19]	7.9	0.92	0.360
	Fertilizer managment [without]	−12.0	−1.46	0.149
	Bacterial inoculation [E19]: Fertilizer managment [without]	−13.4	−1.19	0.239
Microbial biomass	Intercept	415.5	16.97	1.06×10^{-13}***
	Bacterial inoculation [E19]	14.0	0.81	0.418
	Fertilizer managment [without]	−39.4	−2.54	0.013*
	Bacterial inoculation [E19]: Fertilizer managment [without]	−0.9	−0.042	0.967

Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05 '*'.

(Zhang et al., 2018). Nevertheless, a recent study has shown that time influences microbial communities to a greater extent than plant developmental stage per se (Dibner et al., 2021).

The evaluation of different fertilizers has been shown to influence the bacterial communities of WW, increasing the Shannon index, as well as the relative abundance of the phylum *Pseudomonadota* (Liang et al., 2020). The non-significant effects of organic fertilizer application in two of the three seasons could be related to the low concentration used during the experiments (<110 kg N ha^{-1}) compared with that used in the aforementioned study (200 kg N ha^{-1}). To enhance robustness of the results, simplified experimental designs with four to six replicates are recommended for field experiments involving PGPR (Neuhoff et al., 2024). Our study considered four replicates for the combination of the three factors, resulting in a complex design.

Differential abundance analysis, including chemical parameters, corroborated the non-significant effects observed in the diversity indices, showing that no relevant taxa were affected by the factors evaluated. Based on this, questions arise about the mechanisms employed by strain $E19^T$ to improve yield parameters without altering chemical parameters, or shifting bacterial community structure, or being undetected in advanced developmental stages at one organic farm (Quiroga et al., 2024). In recent years, new mechanisms have begun to shed light on how PGPR can promote plant growth (for review: Kong and Liu, 2022). Kong et al. (2019) found that PGPR inoculation increased co-occurrence interactions among bacterial communities. Although the bacterial community composition changed minimally a few days after inoculation, the more complex co-occurrence associations were linked to plant growth promoting effects. Additionally, Chen et al. (2022) associated changes in DNA methylation in roots during the early phase with lasting PGPR effects observed in later phases, even after the removal of the inoculant from the microbiome. Although genes related to nitrogen fixation have been identified in the genome of *H. diazotrophicus* strain $E19^T$ (Suarez et al., 2019), ACC-deaminase activity appears to be one of the mechanisms by which strain $E19^T$ promotes plant growth (Suarez et al., 2015). Additionally, these new insights into microbiome improvement may be related to strain $E19^T$ plant growth promotion and the positive but not significant trend observed in ammonium and microbial biomass concentrations obtained from LMM analyses.

Several authors have emphasized the importance of investigating plant-microbial dynamics over time (Horton et al., 2014; Wagner et al., 2016; de-Bashan and Nannipieri, 2024). Of the four prevalent genera included in the longitudinal analysis, *Reyranella* and *Arenimonas* have been identified as denitrifiers (Duan et al., 2023; Xing et al., 2018), while *Luteimonas* and *Lysobacter* have been associated with disease suppression (Liu et al., 2020). *Arenimonas* has shown to be more abundant in early stages and decreased as the plant grew. Similarly, *Lysobacter* has been negatively associated with root growth response but has also shown opposite results depending on the plant species (Li et al., 2014; Zhalnina et al., 2018). As aforementioned, our alpha and beta diversity results are consistent with the role of plant species in modulating microbial communities between stages. Nevertheless, the differential abundance, linear mixed models, and longitudinal analyses conducted in this study over three consecutive seasons at two locations revealed that bacterial community dynamics can be not only plant-driven but also multifactorial (Becker et al., 2022; Wei et al., 2022). Zhang et al. (2020) determined that geographic distance had a greater influence on modulating microbial communities than seasonal variations. Nonetheless, rapidly changing environmental conditions were associated with the seasonal variability of microbial community structure.

4.3. Different crop rotation management strategies could result in different microbial communities

The results, based on alpha and beta diversity indices at different locations, were consistent with the extended concept that soil is an important force modulating microbial populations (Bulgarelli et al., 2012; Oberholster et al., 2018; Schreiter et al., 2014). Intriguingly, although both organic fields contained Haplic Luvisol soil types and similar soil properties, crop rotation was different and could be related to the differences observed in the diversity indices. In fact, agricultural management practices have shown to influence microbial populations (Schmidt et al., 2019; Zhou et al., 2023). Interestingly, at GH, the previous crops for WW and SB were potato and spelt, respectively. While at KH, clover grass was commonly used as an intercrop before sowing both cereals (Appendix A, Supplementary Table S2). Several of the most prevalent taxa observed in our study have been reported as part of the rhizosphere microbiome of these crops. The phyla *Pseudomonadota* and *Actinomycetota* have shown to dominate the rhizosphere and root microbiome of the clover grass species *Trifolium pratense* (Wahdan et al., 2021). Similarly, genera such as *Bradyrhizobium*, *Arenimonas*, *Lysobacter*, *Microvirga*, *Nocardioides*, and *Gaiella* have been associated with the co-occurrence network of several potato varieties (Martins et al., 2024). While no reports exist on the rhizosphere bacterial microbiome of spelt, the endophytic microbiome in different plant compartments has been documented (Kuźniar et al., 2020). These different crop management strategies could be related not only to the differences already described between both locations, but also to the marked difference between the bacterial community composition of WW and SB at GH ($p < 0.01$, Table 1). In contrast to KH, where WW and SB were not significantly different during season II ($p = 0.953$, Fig. 3).

4.4. Recruitment of different bacterial communities by the same crop species is affected by location

Our findings are in agreement with previous reports indicating that microbial communities are recruiting and shaping in a plant-dependent manner, even varying at different plant growth stages (Oberholster et al., 2018; Smalla et al., 2001; Wagner et al., 2016). Nevertheless, bacterial communities between flowering and ripe stages were more stable for SB than for WW (Table 1). These results suggest that fluctuations across stages could also be plant-dependent. Further research is required to confirm this hypothesis. Additionally, differential abundance analysis corroborated how bacterial communities were significantly influenced by crop species, but also varied depending on location and season (ALDEx2 analysis, Appendix B, Supplementary Table S5). Despite these fluctuations, the 19 most prevalent taxa at the genus level were identified in both crops across seasons. The phyla *Pseudomonadota*,

Acidobacteriota, and *Actinomycetota*, which encompassed 89 % of the most prevalent taxa, were consistent with the rhizosphere core microbiome phyla previously identified in wheat and barley (Escudero-Martinez et al., 2022; Simonin et al., 2020). Nevertheless, there is still controversy regarding the use of the term 'core microbiome' (Risely, 2020). It has become challenging to identify, for instance, a common genus in all studies due to the different methods and tools employed (Kavamura et al., 2021).

4.5. Limitations of 16S rRNA gene sequencing

H. diazotrophicus was only detected in one sample belonging to WW with fertilizer plus E19^T during season III at flowering stage. The limitations of 16S rRNA gene sequencing include short-length reading, intragenomic gene redundancy, sequencing errors, and other factors that can lead to difficulties during sequence assembly (Poretsky et al., 2014). Considering these constraints, *H. diazotrophicus* was detected through qPCR on the roots of WW and SB under field conditions up to a concentration of 5×10^5 copies g^{-1} DW (Quiroga et al., 2024). Nevertheless, metagenomic analyses are still limited to microbial composition. Therefore, for future research, we suggest a metatranscriptomic approach to better understand the associations of functional genes and metabolic pathways in these communities. Even though there are certain challenges to be addressed regarding metatranscriptomics, such as sample collection and preservation, mRNA enrichment, or restricted transcriptome databases (Bikel et al., 2015; Bashiardes et al., 2016; Rosado-Porto et al., 2023).

5. Conclusion

Seed inoculation of *H. diazotrophicus* strain E19^T in a three-year field trial showed no significant alteration in the diversity indices of the rhizosphere bacterial microbiome of wheat and barley. Differential abundance and linear mixed models corroborated the non-effect of bacterial inoculation and row spacing. Although fertilizer management significantly affected the bacterial microbiome during one season (beta diversity), a significant trend over time was not identified, probably because of the low nitrogen application rate. Furthermore, although both organic fields share the same Haplic Luvisol soil type, the rhizosphere bacterial communities were different, possibly due to different crop management practices. These findings showed not only how the same crop species recruited, assembled, and influenced different bacterial communities depending on the location but also how this influence was affected to different degrees over time. Finally, these results highlight the importance of longitudinal studies in understanding microbial dynamics and the long-lasting effects of introducing bioinoculants into the soil.

CRediT authorship contribution statement

Santiago Quiroga: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Stefan Ratering:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **David Rosado-Porto:** Writing – review & editing, Methodology, Data curation. **Azin Rekowski:** Writing – review & editing, Investigation. **Franz Schulz:** Writing – review & editing, Investigation. **Christian Zörb:** Writing – review & editing, Funding acquisition, Conceptualization. **Sylvia Schnell:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Funding

This work was fully funded by the Federal Ministry of Education and Research (BMBF) of Germany under the BONARES program (Rhizo4bio).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are warmly acknowledged to Rita Geissler-Plaum and Bellinda Schneider for their technical support. We are also grateful to Simon Schäfer for his support during field experiments at Kleinhohenheim.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105823.

Data availability

All DNA sequences are available on the NCBI/GenBank database repository under the bioproject accession numbers PRJNA1030754 (season I), PRJNA1030767 (season II), and PRJNA1095458 (season III). Moreover, all the chemical parameters analyzed during seasons I and II can be found at the BONARES repository under the name "Production of wheat and barley under reduced input in organic farming". The graphical abstract was created using Inkscape v.1.3.2.

References

Abedini, D., Jaupitre, S., Bouwmeester, H., Dong, L., 2021. Metabolic interactions in beneficial microbe recruitment by plants. Curr. Opin. Biotechnol. 70, 241–247. https://doi.org/10.1016/j.copbio.2021.06.015.

Allison, S.D., Martiny, J.B.H., 2008. Resistance, resilience, and redundancy in microbial communities. Proc. Natl. Acad. Sci. USA 105, 11512–11519. https://doi.org/10.1073/pnas.0801925105.

Barnett, D., Arts, I., Penders, J., 2021. microViz: an R package for microbiome data visualization and statistics. J. Open Source Softw. 6, 3201. https://doi.org/10.21105/joss.03201.

Bashiardes, S., Zilberman-Schapira, G., Elinav, E., 2016. Use of metatranscriptomics in microbiome research. Bioinform. Biol. Insights. 10, BBI-S34610. https://doi.org/10.4137/BBI.S34610.

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–41. https://doi.org/10.18637/jss.v067.i01.

Becker, M.F., Hellmann, M., Knief, C., 2022. Spatio - temporal variation in the root - associated microbiota of orchard - grown apple trees. Environ. Microbiol. 17, 31. https://doi.org/10.1186/s40793-022-00427-z.

Bikel, S., Valdez-Lara, A., Cornejo-Granados, F., Rico, K., Canizales-Quinteros, S., Soberón, X., Del Pozo-Yauner, L., Ochoa-Leyva, A., 2015. Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: towards a systems-level understanding of human microbiome. Comput. Struct. Biotechnol. J. 13, 390–401. https://doi.org/10.1016/j.csbj.2015.06.001.

Björklöf, K., Sen, R., Jørgensen, K.S., 2003. Maintenance and impacts of an inoculated mer/luc-tagged *Pseudomonas fluorescens* on microbial communities in birch rhizospheres developed on humus and peat. Microb. Ecol. 45, 39–52. https://doi.org/10.1007/s00248-002-2018-8.

Bokulich, N.A., Dillon, M.R., Zhang, Y., Rideout, J.R., Bolyen, E., Li, H., Albert, P.S., Caporaso, J.G., 2018a. q2-longitudinal: longitudinal and paired-sample analyses of microbiome data. mSystems 3, 10–1128. https://doi.org/10.1128/msystems.00219-18.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., Gregory Caporaso, J., 2018b. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 1–17. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Bulgarelli, D., Rott, M., Schlaeppi, K., Loren, Ver, van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F.O., Amann, R., Eickhorst, T., Schulze-Lefert, P., 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488, 91–95. https://doi.org/10.1038/nature11336.

Burns, J.H., Anacker, B.L., Strauss, S.Y., Burke, D.J., 2015. Soil microbial community variation correlates most strongly with plant species identity, followed by soil

chemistry, spatial location and plant genus. AoB Plants 7, plv030. https://doi.org/10.1093/aobpla/plv030.
Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.
Cardinale, M., Ratering, S., Suarez, C., Zapata Montoya, A.M., Geissler-Plaum, R., Schnell, S., 2015. Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress. Microbiol. Res. 181, 22–32. https://doi.org/10.1016/j.micres.2015.08.002.
Chaparro, J.M., Badri, D.V., Bakker, M.G., Sugiyama, A., Manter, D.K., Vivanco, J.M., 2013. Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PLoS One 8, e55731. https://doi.org/10.1371/journal.pone.0055731.
Chaparro, J.M., Badri, D.V., Vivanco, J.M., 2014. Rhizosphere microbiome assemblage is affected by plant development. ISME J. 8, 790–803. https://doi.org/10.1038/ismej.2013.196.
Chen, C., Wang, M., Zhu, J., Tang, Y., Zhang, H., Zhao, Q., Jing, M., Chen, Y., Xu, X., Jiang, J., Shen, Z., 2022. Long-term effect of epigenetic modification in plant–microbe interactions: modification of DNA methylation induced by plant growth-promoting bacteria mediates promotion process. Microbiome 10, 36. https://doi.org/10.1186/s40168-022-01236-9.
Ciccillo, F., Fiore, A., Bevivino, A., Dalmastri, C., Tabacchioni, S., Chiarini, L., 2002. Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizospheric bacterial diversity. Environ. Microbiol. 4, 238–245. https://doi.org/10.1046/j.1462-2920.2002.00291.x.
Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., de Vos, W. M., Ross, R.P., O'Toole, P.W., 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4, e6669. https://doi.org/10.1371/journal.pone.0006669.
Coniglio, A., Larama, G., Molina, R., Mora, V., Torres, D., Marin, A., Avila, A.I., Lede NoirCarlan, C., Erijman, L., Figuerola, E.L., de Souza, E.M., Cassán, F., 2022. Modulation of maize rhizosphere microbiota composition by inoculation with *Azospirillum argentinense* Az39 (Formerly *a. brasilense* Az39). J. Soil Sci. Plant Nutr. 39, 3553–3567. https://doi.org/10.1007/s42729-022-00909-6.
Dar, A., Zahir, Z.A., Iqbal, M., Mehmood, A., Javed, A., Hussain, A., Bushra, Ahmad, M., 2021. Efficacy of rhizobacterial exopolysaccharides in improving plant growth, physiology, and soil properties. Environ. Monit. Assess. 193, 1–15. https://doi.org/10.1007/s10661-021-09286-6.
de-Bashan, L., Nannipieri, P., 2024. Recommendations for plant growth-promoting bacteria inoculation studies. Biol. Fertil. Soils 60, 259–261. https://doi.org/10.1007/s00374-024-01798-w.
Díaz, A.B., Durán-Guerrero, E., Lasanta, C., Castro, R., 2022. From the raw materials to the bottled product: influence of the entire production process on the organoleptic profile of industrial beers. Foods 11, 3215. https://doi.org/10.3390/foods11203215.
Dibner, R.R., Weaver, A.M., Brock, M.T., Custer, F., Morrison, H.G., Maignien, L., Weinig, C., 2021. Time outweighs the effect of host developmental stage on microbial community composition. FEMS Microbiol. Ecol. 97, fiab102. https://doi.org/10.1093/femsec/fiab102.
Duan, M., Wang, L., Song, X., Zhang, X., Wang, Z., Lei, J., 2023. Assessment of the rhizosphere fungi and bacteria recruited by sugarcane during smut invasion. Braz. J. Microbiol. 54, 385–395. https://doi.org/10.1007/s42770-022-00871-6.
El-Saadony, M.T., Saad, A.M., Soliman, S.M., Salem, H.M., Ahmed, A.I., Mahmood, M., El-Tahan, A.M., Ebrahim, A.A.M., Abd El-Mageed, T.A., Negm, S.H., Selim, S., Babalghith, A.O., Elrys, A.S., El-Tarabily, K.A., AbuQamar, S.F., 2022. Plant growth-promoting microorganisms as biocontrol agents of plant diseases: mechanisms, challenges and future perspectives. Front. Plant Sci. 13, 923880. https://doi.org/10.3389/fpls.2022.923880.
Engelbrektson, A., Kunin, V., Wrighton, K.C., Zvenigorodsky, N., Chen, F., Ochman, H., Hugenholtz, P., 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J. 4, 642–647. https://doi.org/10.1038/ismej.2009.153.
Escudero-Martinez, C., Coulter, M., Alegria Terrazas, R., Foito, A., Kapadia, R., Pietrangelo, L., Maver, M., Sharma, R., Aprile, A., Morris, J., Hedley, P.E., Maurer, A., Pillen, K., Naclerio, G., Mimmo, T., Barton, G.J., Waugh, R., Abbott, J., Bulgarelli, D., 2022. Identifying plant genes shaping microbiota composition in the barley rhizosphere. Nat. Commun. 13, 3443. https://doi.org/10.1038/s41467-022-31022-y.
Fernandes .A., D., Reid, J., Macklaim .J., M., McMurrough, T.A., Edgell, D.R., Gloor . G., B., 2014. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome 2, 1–13. https://doi.org/10.1186/2049-2618-2-15.
Fu, L., Penton, C.R., Ruan, Y., Shen, Z., Xue, C., Li, R., Shen, Q., 2017. Inducing the rhizosphere microbiome by biofertilizer application to suppress banana fusarium wilt disease. Soil Biol. Biochem. 104, 39–48. https://doi.org/10.1016/j.soilbio.2016.10.008.
Global Market Insights, 2024. Fertilizer market - by form (dry, liquid) by product (organic, inorganic), by application (agriculture, horticulture, gardening), forecast 2024–2032. Available online at: https://www.gminsights.com/industry-analysis/fertilizer-market. Accessed on: February 2024.
Gui, H., Purahong, W., Hyde, K.D., Xu, J., Mortimer, P.E., 2017. The arbuscular mycorrhizal fungus *Funneliformis mosseae* alters bacterial communities in subtropical forest soils during litter decomposition. Front. Microbiol. 8, 1120. https://doi.org/10.3389/fmicb.2017.01120.
Hemmerle, L., Maier, B.A., Bortfeld-Miller, M., Ryback, B., Gäbelein, C.G., Ackermann, M., Vorholt, J.A., 2022. Dynamic character displacement among a pair of bacterial phyllosphere commensals in situ. Nat. Commun. 13, 2836. https://doi.org/10.1038/s41467-022-30469-3.
Horton, M.W., Bodenhausen, N., Beilsmith, K., Meng, D., Muegge, B.D., Subramanian, S., Vetter, M.M., Vilhjálmsson, B.J., Nordborg, M., Gordon, J.I., Bergelson, J., 2014. Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. Nat. Commun. 5, 5320. https://doi.org/10.1038/ncomms6320.
IUSS Working Group WRB, 2015. World reference base for soil resources 2014, update 2015 international soil classification system for naming soils and creating legends for soil maps. World soil resources reports N°. 106. FAO, Rome. Springer water. doi: https://doi.org/10.1007/978-3-319-24409-9_25.
Jansson, J.K., McClure, R., Egbert, R.G., 2023. Soil microbiome engineering for sustainability in a changing environment. Nat. Biotechnol. 41, 1716–1728. https://doi.org/10.1038/s41587-023-01932-3.
Jiang, M., Yergeau, E., Delgado-baquerizo, M., Yuan, M.M., Ding, J., Zhou, J., Crowther, T.W., Liang, Y., 2023. Home-based microbial solution to boost crop growth in low-fertility soil. New Phytol. 239, 752–765. https://doi.org/10.1111/nph.18943.
Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils 6, 68–72. https://doi.org/10.1007/BF00257924.
Kaplan, H., Ratering, S., Felix-Henningsen, P., Schnell, S., 2019. Stability of in situ immobilization of trace metals with different amendments revealed by microbial ^{13}C-labelled wheat root decomposition and efflux-mediated metal resistance of soil bacteria. Sci. Total Environ. 659, 1082–1089. https://doi.org/10.1016/j.scitotenv.2018.12.441.
Kavamura, V.N., Mendes, R., Bargaz, A., Mauchline, T.H., 2021. Defining the wheat microbiome: towards microbiome-facilitated crop production. Comput. Struct. Biotechnol. J. 19, 1200–1213. https://doi.org/10.1016/j.csbj.2021.01.045.
Keswani, C., Prakash, O., Bharti, N., Vílchez, J.I., Sansinenea, E., Lally, R.D., Borriss, R., Singh, S.P., Gupta, V.K., Fraceto, L.F., de Lima, R., Singh, H.B., 2019. Re-addressing the biosafety issues of plant growth promoting rhizobacteria. Sci. Total Environ. 690, 841–852. https://doi.org/10.1016/j.scitotenv.2019.07.046.
Kloepper, J.W., 1981. Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. Phytopathology 71, 590–592. https://doi.org/10.1094/phyto-71-590.
Kong, Z., Liu, H., 2022. Modification of rhizosphere microbial communities: a possible mechanism of plant growth promoting rhizobacteria enhancing plant growth and fitness. Front. Plant Sci. 13, 920813. https://doi.org/10.3389/fpls.2022.920813.
Kong, Z., Wu, Z., Glick, B.R., He, S., Huang, C., Wu, L., 2019. Co-occurrence patterns of microbial communities affected by inoculants of plant growth-promoting bacteria during phytoremediation of heavy metal-contaminated soils. Ecotoxicol. Environ. Saf. 183, 109504. https://doi.org/10.1016/j.ecoenv.2019.109504.
Kozdrój, J., Trevors, J.T., Van Elsas, J.D., 2004. Influence of introduced potential biocontrol agents on maize seedling growth and bacterial community structure in the rhizosphere. Soil Biol. Biochem. 36, 1775–1784. https://doi.org/10.1016/j.soilbio.2004.04.034.
Kröber, M., Wibberg, D., Grosch, R., Eikmeyer, F., Verwaaijen, B., Chowdhury .S.P., P., Hartmann, A., Pühler, A., Schlüter, A., 2014. Effect of the strain *Bacillus amyloliquefaciens* FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front. Microbiol. 5, 252. https://doi.org/10.3389/fmicb.2014.00252.
Kuzmicheva, Y.V., Shaposhnikov, A.I., Petrova, S.N., Makarova, N.M., Tychinskaya, I.L., Puhalsky, J.V., Parahin, N.V., Tikhonovich, I.A., Belimov, A.A., 2017. Variety specific relationships between effects of rhizobacteria on root exudation, growth and nutrient uptake of soybean. Plant Soil 419, 83–96. https://doi.org/10.1007/s11104-017-3320-z.
Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest package: tests in linear mixed effects models. J. Stat. Softw. 82, 1–26. https://doi.org/10.18637/jss.v082.i13.
Kuźniar, A., Włodarczyk, K., Grządziel, J., Goraj, W., Gałązka, A., Wolińska, A., 2020. Culture-independent analysis of an endophytic core microbiome in two species of wheat: *Triticum aestivum* L. (cv. 'Hondia') and the first report of microbiota in *Triticum spelta* L. (cv. 'Rokosz'). Syst. Appl. Microbiol. 43, 126025. doi:https://doi.org/10.1016/j.syapm.2019.126025.
Lahti, L., Shetty, S., 2019. Microbiome R Package. Available online at: http://microbiome.github.io. Accessed on: November 2020.
Li, X., Rui, J., Mao, Y., Yannarell, A., Mackie, R., 2014. Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. Soil Biol. Biochem. 68, 392–401. https://doi.org/10.1016/j.soilbio.2013.10.017.
Li, X., Yan, Y., Lu, X., Fu, L., Liu, Y., 2022. Responses of soil bacterial communities to precipitation change in the semi-arid alpine grassland of northern Tibet. Front. Plant Sci. 13, 1036369. https://doi.org/10.3389/fpls.2022.1036369.
Liang, R., Hou, R., Li, J., Lyu, Y., Hang, S., Gong, H., Ouyang, Z., 2020. Effects of different fertilizers on rhizosphere bacterial communities of winter wheat in the North China plain. Agronomy 10, 93. https://doi.org/10.3390/agronomy10010093.
Liu, C., Yang, Z., He, P., Munir, S., Wu, Y., Ho, H., He, Y., 2018. Deciphering the bacterial and fungal communities in clubroot-affected cabbage rhizosphere treated with *Bacillus subtilis* XF-1. Agric. Ecosyst. Environ. 256, 12–22. https://doi.org/10.1016/j.agee.2018.01.001.
Liu, L., Huang, X., Zhang, J., Cai, Z., Jiang, K., 2020. Deciphering the relative importance of soil and plant traits on the development of rhizosphere microbial communities. Soil Biol. Biochem. 148, 107909. https://doi.org/10.1016/j.soilbio.2020.107909.

Market Data Forecast, 2024. Global biofertilizers market size, share, trends, & growth forecast report. Available online at: https://www.marketdataforecast.com/market-reports/global-biofertilizers-market. Accessed on: September 2024.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. Journal. 17, 10. https://doi.org/10.14806/ej.17.1.200.
Martino, C., Shenhav, L., Marotz, C.A., Armstrong, G., McDonald, D., Vázquez-Baeza, Y., Morton, J.T., Jiang, L., Dominguez-Bello, M.G., Swafford, A.D., Halperin, E., Knight, R., 2021. Context-aware dimensionality reduction deconvolutes gut microbial community dynamics. Nat. Biotechnol. 39, 165–168. https://doi.org/10.1038/s41587-020-0660-7.
Martins, B.R., Radl, V., Treder, K., Michałowska, D., Pritsch, K., Schloter, M., 2024. The rhizosphere microbiome of 51 potato cultivars with diverse plant growth characteristics. FEMS Microbiol. Ecol. 100. https://doi.org/10.1093/femsec/fiae088.
Mawarda, P.C., Le Roux, X., Dirk van Elsas, J., Salles, J.F., 2020. Deliberate introduction of invisible invaders: a critical appraisal of the impact of microbial inoculants on soil microbial communities. Soil Biol. Biochem. 148, 107874. https://doi.org/10.1016/j.soilbio.2020.107874.
Mayer, J., Scheid, S., Widmer, F., Fließbach, A., Oberholzer, H.R., 2010. How effective are "effective microorganisms® (EM)"? Results from a field study in temperate climate. Appl. Soil Ecol. 46, 230–239. https://doi.org/10.1016/j.apsoil.2010.08.007.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.
Nagrale, D.T., Chaurasia, A., Kumar, S., Gawande, S.P., Hiremani, N.S., Shankar, R., Gokte-Narkhedkar, N., Renu, Prasad, Y.G., 2023. PGPR: the treasure of multifarious beneficial microorganisms for nutrient mobilization, pest biocontrol and plant growth promotion in field crops. World J. Microbiol. Biotechnol. 39, 100. https://doi.org/10.1007/s11274-023-03536-0.
Neuhoff, D., Neumann, G., Weinmann, M., 2024. Testing plant growth promoting microorganisms in the field - a proposal for standards. Front. Plant Sci. 14, 1324665. https://doi.org/10.3389/fpls.2023.1324665.
Oberholster, T., Vikram, S., Cowan, D., Valverde, A., 2018. Key microbial taxa in the rhizosphere of sorghum and sunflower grown in crop rotation. Sci. Total Environ. 624, 530–539. https://doi.org/10.1016/j.scitotenv.2017.12.170.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., Hara, R.B.O., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., 2019. Vegan: Community Ecology Package. R package version 2.5-6.. R Foundation for Statistical Computing, Vienna.
Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Müller, A., Nothman, J., Louppe, G., Prettenhofer, P., Weiss, R., Dubourg, V., Vanderplas, J., Passos, A., Cournapeau, D., Brucher, M., Perrot, M., Duchesnay, E., 2011. Scikit-learn: machine learning in Python. J. Mach. Learn. Res. 12, 2825–2830.
Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., Konstantinidis, K.T., 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS One 9, e93827. https://doi.org/10.1371/journal.pone.0093827.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.
Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Schulz, F., Krutych, M., Zörb, C., Schnell, S., 2024. Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance. FEMS Microbiol. Ecol. 100, fiae023. https://doi.org/10.1093/femsec/fiae023.
R Core Team, 2023. R: A Language and Environment for Statistical Computing. R foundation for statistical computing, Vienna, Austria. http://www.R-project.org.
Rai, S., Omar, A.F., Rehan, M., Al, A., Alka, T., Noshin, S., Sayyed, I.R.Z., 2023. Crop microbiome : their role and advances in molecular and omic techniques for the sustenance of agriculture. Planta 257, 27. https://doi.org/10.1007/s00425-022-04052-5.
Risely, A., 2020. Applying the core microbiome to understand host–microbe systems. J. Anim. Ecol. 89, 1549–1558. https://doi.org/10.1111/1365-2656.13229.
Rosado-Porto, D., Ratering, S., Wohlfahrt, Y., Schneider, B., Glatt, A., Schnell, S., 2023. Elevated atmospheric CO_2 concentrations caused a shift of the metabolically active microbiome in vineyard soil. BMC Microbiol. 23, 46. https://doi.org/10.1186/s12866-023-02781-5.
Russ, D., Fitzpatrick, C.R., Teixeira, P.J.P.L., Dangl, J.L., 2023. Deep discovery informs difficult deployment in plant microbiome science. Cell 186, 4496–4513. https://doi.org/10.1016/j.cell.2023.08.035.
Schmidt, J.E., Kent, A.D., Brisson, V.L., Gaudin, A.C.M., 2019. Agricultural management and plant selection interactively affect rhizosphere microbial community structure and nitrogen cycling. Microbiome 7, 1–18. https://doi.org/10.1186/s40168-019-0756-9.
Schreiter, S., Ding, G.C., Heuer, H., Neumann, G., Sandmann, M., Grosch, R., Kropf, S., Smalla, K., 2014. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Front. Microbiol. 5, 144. https://doi.org/10.3389/fmicb.2014.00144.
Schütz, L., Gattinger, A., Meier, M., Müller, A., Boller, T., Mäder, P., Mathimaran, N., 2018. Improving crop yield and nutrient use efficiency via biofertilization-a global meta-analysis. Front. Plant Sci. 8, 2204. https://doi.org/10.3389/fpls.2017.02204.
Simonin, M., Dasilva, C., Terzi, V., Ngonkeu, E.L.M., Diouf, D., Kane, A., Béna, G., Moulin, L., 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. https://doi.org/10.1093/femsec/fiaa067.
Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. 67, 4742–4751. https://doi.org/10.1128/AEM.67.10.4742-4751.2001.
Suarez, C., Cardinale, M., Ratering, S., Steffens, D., Jung, S., Montoya, A.M.Z., Geissler-Plaum, R., Schnell, S., 2015. Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress. Appl. Soil Ecol. 95, 23–30. https://doi.org/10.1016/j.apsoil.2015.04.017.
Suarez, C., Ratering, S., Geissler-Plaum, R., Schnell, S., 2014. *Hartmannibacter diazotrophicus* gen. Nov., sp. nov., a phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt-meadow plant. Int. J. Syst. Evol. Microbiol. 64, 3160–3167. https://doi.org/10.1099/ijs.0.064154-0.
Suarez, C., Ratering, S., Hain, T., Fritzenwanker, M., Goesmann, A., Blom, J., Chakraborty, T., Bunk, B., Spröer, C., Overmann, J., Schnell, S., 2019. Complete genome sequence of the plant growth-promoting bacterium *Hartmannibacter diazotrophicus* strain E19^T. Int. J. Genomics. 2019, 7586430. https://doi.org/10.1155/2019/7586430.
Sun, B., Bai, Z., Bao, L., Xue, L., Zhang, S., Wei, Y., Zhang, Z., Zhuang, G., Zhuang, X., 2020. *Bacillus subtilis* biofertilizer mitigating agricultural ammonia emission and shifting soil nitrogen cycling microbiomes. Environ. Int. 144, 105989. https://doi.org/10.1016/j.envint.2020.105989.
Turner, B.L., Bristow, A.W., Haygarth, P.M., 2001. Rapid estimation of microbial biomass in grassland soils by ultra-violet absorbance. Soil Biol. Biochem. 33, 913–919. https://doi.org/10.1016/S0038-0717(00)00238-8.
Van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Elhottova, D., Krištůfek, V., Salles, J.F., 2012. Microbial diversity determines the invasion of soil by a bacterial pathogen. Proc. Natl. Acad. Sci. USA 109, 1159–1164. https://doi.org/10.1073/pnas.1109326109.
Vílchez, J.I., Navas, A., González-López, J., Arcos, S.C., Manzanera, M., 2016. Biosafety test for plant growth-promoting bacteria: proposed environmental and human safety index (EHSI) protocol. Front. Microbiol. 6, 1514. https://doi.org/10.3389/fmicb.2015.01514.
Wagner, M.R., Lundberg, D.S., Coleman-Derr, D., Tringe, S.G., Dangl, J.L., Mitchell-Olds, T., 2014. Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild *Arabidopsis* relative. Ecol. Lett. 17, 717–726. https://doi.org/10.1111/ele.12276.
Wagner, M.R., Lundberg, D.S., Del Rio, T.G., Tringe, S.G., Dangl, J.L., Mitchell-Olds, T., 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat. Commun. 7, 12151. https://doi.org/10.1038/ncomms12151.
Wahdan, S.F.M., Tanunchai, B., Wu, Y.T., Sansupa, C., Schädler, M., Dawoud, T.M., Buscot, F., Purahong, W., 2021. Deciphering *Trifolium pratense* L. holobiont reveals a microbiome resilient to future climate changes. MicrobiologyOpen 10, e1217. https://doi.org/10.1002/mbo3.1217.
Wei, Y., Quan, F., Lan, G., Wu, Z., Yang, C., 2022. Space rather than seasonal changes explained more of the spatiotemporal variation of tropical soil microbial communities. Microbiol. Spectr. 10, e01846–22. doi:https://doi.org/10.1128/spectrum.01846-22.
Wei, Z., Yang, T., Friman, V.P., Xu, Y., Shen, Q., Jousset, A., 2015. Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health. Nat. Commun. 6, 8413. https://doi.org/10.1038/ncomms9413.
Xing, W., Li, J., Li, D., Hu, J., Deng, S., Cui, Y., Yao, H., 2018. Stable-isotope probing reveals the activity and function of autotrophic and heterotrophic denitrifiers in nitrate removal from organic-limited wastewater. Environ. Sci. Technol. 52, 7867–7875. https://doi.org/10.1021/acs.est.8b01993.
Xiong, C., Singh, B.K., He, J.Z., Han, Y.L., Li, P.P., Wan, L.H., Meng, G.Z., Liu, S., Wang, J., Wu, C., Ge, A., Zhang, L., 2021. Plant developmental stage drives the differentiation in ecological role of the maize microbiome. Microbiome 9, 1–15. https://doi.org/10.1186/s40168-021-01118-6.
Xu, J., Feng, Y., Wang, Y., Lin, X., 2018. Effect of rhizobacterium *Rhodopseudomonas palustris* inoculation on *Stevia rebaudiana* plant growth and soil microbial community. Pedosphere 28, 793–803. https://doi.org/10.1016/S1002-0160(18)60043-8.
Xue, P., Carrillo, Y., Pino, V., Minasny, B., Mcbratney, A.B., 2018. Soil properties drive microbial community structure in a large scale transect in south eastern Australia. Sci. Rep. 8, 11725. https://doi.org/10.1038/s41598-018-30005-8.
Yin, D., Wang, N., Xia, F., Li, Q., Wang, W., 2013. Impact of biocontrol agents *Pseudomonas fluorescens* 2P24 and CPF10 on the bacterial community in the cucumber rhizosphere. Eur. J. Soil Biol. 59, 36–42. https://doi.org/10.1016/j.ejsobi.2013.09.001.
Zadoks, J.C., Chang, T.T., Konzak, C.F., 1974. A decimal code for the growth stages of cereals. Weed Res. 14, 415–421. https://doi.org/10.1111/j.1365-3180.1974.tb01084.x.
Zhalnina, K., Louie, K.B., Hao, Z., Mansoori, N., Da Rocha, U.N., Shi, S., Cho, H., Karaoz, U., Loqué, D., Bowen, B.P., Firestone, M.K., Northen, T.R., Brodie, E.L., 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. Nat. Microbiol. 3, 470–480. https://doi.org/10.1038/s41564-018-0129-3.
Zhang, J., Zhang, N., Liu, Y., Zhang, X., Hu, B., Qin, Y., Xu, H., Wang, H., Guo, X., Qian, J., Wang, W., Zhang, P., Jin, T., Chu, C., Bai, Y., 2018. Root microbiota shift in rice correlates with resident time in the field and developmental stage. Sci. China Life Sci. 61, 613–621. https://doi.org/10.1007/s11427-018-9284-4.

Zhang, K., Delgado-Baquerizo, M., Zhu, Y., Chu, H., 2020. Space is more important than season when shaping soil microbial communities at a large spatial scale. Msystems 5, 10–1128. https://doi.org/10.1128/msystems.00783-19.
Zhong, Y., Yang, Y., Liu, P., Xu, R., Rensing, C., Fu, X., Liao, H., 2019. Genotype and *Rhizobium* inoculation modulate the assembly of soybean rhizobacterial communities. Plant Cell Environ. 42, 2028–2044. https://doi.org/10.1111/pce.13519.
Zhou, J., Zang, H., Loeppmann, S., Gube, M., Kuzyakov, Y., Pausch, J., 2020. Arbuscular mycorrhiza enhances rhizodeposition and reduces the rhizosphere priming effect on the decomposition of soil organic matter. Soil Biol. Biochem. 140, 107641. https://doi.org/10.1016/j.soilbio.2019.107641.
Zhou, Y., Yang, Z., Liu, J., Li, Xudong, Wang, X., Dai, C., Zhang, T., Carrión, V.J., Wei, Z., Cao, F., Delgado-Baquerizo, M., Li, Xiaogang, 2023. Crop rotation and native microbiome inoculation restore soil capacity to suppress a root disease. Nat. Commun. 14, 8126. https://doi.org/10.1038/s41467-023-43926-4.
Zhou, Z., Wang, C., Luo, Y., 2018. Response of soil microbial communities to altered precipitation: a global synthesis. Glob. Ecol. Biogeogr. 27, 1121–1136. https://doi.org/10.1111/geb.12761.

Chapter IV

Complementary roles of eDNA and eRNA in characterizing rhizosphere bacterial communities of wheat and barley across seasons

Research article

To be submitted to Applied Soil Ecology

Complementary roles of eDNA and eRNA in characterizing rhizosphere bacterial communities of wheat and barley across seasons

Santiago Quiroga[1], Stefan Ratering[1], David Rosado-Porto[1,2], Azin Rekowski[3], Franz Schulz[4], Christian Zörb[3], and Sylvia Schnell[1]

Santiago Quiroga
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
Santiago.Quiroga@umwelt.uni-giessen.de
ORCID-ID 0009-0001-1392-8241

Stefan Ratering
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
Stefan.Ratering@umwelt.uni-giessen.de
ORCID-ID 0000-0001-7572-6306

David Rosado-Porto
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
[2]*Faculty of Basic and Biomedical Sciences, Simón Bolívar University, 080002 Barranquilla, Colombia*
David.Rosado-Porto@agrar.uni-giessen.de
ORCID-ID 0000-0001-6199-0114

Azin Rekowski
[3]*Institute of Crop Science, Quality of Plant Products, 340e, University of Hohenheim, 70593, Stuttgart, Germany*
Azin.ghabelrahmat@uni-hohenheim.de
ORCID-ID 0000-0003-0179-663X

Franz Schulz
[4]*Department of Agronomy and Plant Breeding II, Justus-Liebig University Giessen, 35394 Giessen, Germany*
Franz.Schulz@agrar.uni-giessen.de
ORCID-ID 0000-0002-7151-6202

Christian Zörb
[3]*Institute of Crop Science, Quality of Plant Products, 340e, University of Hohenheim, 70593, Stuttgart, Germany*
Christian.zoerb@uni-hohenheim.de
ORCID-ID 0000-0003-0000-5138

Sylvia Schnell
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
Sylvia.Schnell@umwelt.uni-giessen.de
ORCID-ID 0000-0003-3903-6089

Corresponding author*:* Sylvia Schnell

Sylvia.Schnell@umwelt.uni-giessen.de

Abstract

During metabarcoding characterization, few studies have compared environmental DNA (eDNA) and environmental RNA (eRNA). Given that eRNA can reveal active biological communities, it has emerged as a powerful tool for studying responses to environmental changes. This study compares eDNA and eRNA from rhizosphere samples of wheat and barley across two consecutive seasons in two organic fields. Additionally, we aimed to elucidate the extent to which an eRNA-approach can reveal shifts in bacterial communities after seed inoculation with *Hartmannibacter diazotrophicus* strain E19^{T}, as well as other factors in both crops and locations. While metabarcoding characterization identified a high proportion of ASVs common to both environmental nucleic acids (eNA) (up to 90%), eRNA revealed a notable percentage (27%) of ASVs that differed from eDNA. Moreover, alpha diversity indices varied between eNA depending on the season and did not consistently show higher metrics for eDNA. Rhizosphere bacterial communities identified through eRNA differed significantly from those identified through eDNA but exhibited similar differences previously reported for eDNA. Remarkably, differential abundance analysis revealed certain taxa that were either positively or negatively affected between eDNA- and eRNA-approach. More interestingly, most of the identified taxa (e.g. family *Candidatus* Entotheonellaceae) followed the same trend regardless of plant species, location, and season. Our results indicate that an eRNA-approach is a complementary tool in metabarcoding characterization, revealing active taxa that may be masked by eDNA and are likely more sensitive to be detected by eRNA.

Keywords

Metabarcoding, eNA, eRNA, eDNA, PGPR, rhizosphere, bacterial communities

Introduction

Environmental DNA (eDNA) and environmental RNA (eRNA), both referred as environmental nucleic acids (eNA), have been extensively used to characterize community composition and diversity across a wide range of habitats (Adamo et al., 2020; Zhang et al., 2024). However, high-throughput sequencing technologies primarily focus more on environmental eDNA than eRNA. This imbalance is explained as eDNA possesses more stability, less degradation to environmental factors (e.g. temperature, UV, pH-value), and lower analysis cost compared to eRNA. Nevertheless, eDNA cannot differentiate between deceased populations ("legacy" DNA) and living organisms (Macher et al., 2024; Taberlet et al., 2012). In contrast, since eRNA is less stable, it degrades quickly, and thus reflects a snapshot of living organisms, metabolically active, at a specific time (for review: Veilleux et al., 2021). This differentiation has become particularly relevant and more studied in marine environments, which face several challenges including wide biological spectrum (from bacteria to arthropods), complex dynamics, and specific requirements for marine biosecurity (Zaiko et al., 2018; Zhang et al., 2024). Conversely, the use of eRNA, as well as comparisons between eDNA and eRNA in soil environments is limited and remains unexplored. The findings coming from these studies have mainly revealed significant changes in the bacterial and fungal community composition between eRNA and eDNA of different crop species including wheat, maize, and oilseed rape (Gkarmiri et al., 2017; Kuramae et al., 2013; Li et al., 2021). However, species richness has shown variable results ranging from lower to higher or nuanced differences between eRNA and eDNA (Kuramae et al., 2013; Lasa et al., 2019; Li et al., 2021). In recent years, it has been shown a direct influence of cropping systems in the active rhizosphere and endosphere bacterial community (Bay et al., 2021). More interestingly, eRNA has contributed to disentangle the effect of elevated CO_2 on soil microbial populations, which differences where previously attributed to moisture gradient or soil conditions (Rosado-Porto et al., 2023). Similarly, more positive network correlations between bacterial communities have been found in the eRNA compared to eDNA of a 27 year fertilization trial, which included wheat and maize rotation (Li et al., 2021). Therefore, analyzing eRNA and eDNA can offer a more comprehensive view of the microbial community dynamics. Previously, we employed only eDNA to analyze the effect of introducing the plant growth promoting rhizobacterium (PGPR) *Hartmannibacter diazotrophicus* strain E19^T on the rhizosphere bacterial communities of wheat and barley. The results showed no alteration in the bacterial community structure of wheat and barley due to PGPR inoculation, but marked differences in the recruitment of bacterial communities in both crops at different locations (Quiroga et al., 2025). In the current study, we aimed to evaluate

the extent to which the rhizosphere bacterial communities of wheat and barley differ between eRNA and eDNA from field samples collected for two consecutive seasons at the two organic farms. Moreover, we also analyzed whether eDNA masked a possible shift in the bacterial community structure due to the seed inoculation with strain E19^T.

Materials and Methods

Field experiments

A detail explanation about the experimental site characteristics, field experiments, sample collection, DNA extraction, metabarcoding characterization, and statistical analysis are provided in our previous studies (Quiroga et al., 2025, 2024). Briefly, field experiments were performed at two organic fields located in Germany: Gladbacherhof (GH) (50° 23' N, 8° 15' E) and Kleinhohenheim (KH) (48° 44' N, 9° 11' E). Seasons considered for this study were season I (2020-2021) and season II (2021-2022). Inoculation of *Hartmannibacter diazotrophicus* strain E19^T (LMG 27460^T) on *Triticum aestivum*, cv. Aristaro (winter wheat, WW) and *Hordeum vulgare* L., cv. Odilia (spring barley, SB) seeds was modified based on the seed coating protocol described by Kloepper (1981), using gum arabic 25%, pH-value adjusted to 7.0 $\pm$ 0.2, and talc powder, reaching a final concentration of $\bar{x}$ = 2.18 x 10^8 $\pm$ 9.56 x 10^7 CFU g^{-1} powder (Quiroga et al., 2024). Three factors were evaluated during field experiments: bacterial inoculation (strain E19^T, ctrl without bacteria), fertilizer management (with fertilizer, without fertilizer), and row spacing (15 cm, 50 cm). Fertilization was performed only on WW. Rhizosphere soil samples collected at the flowering stage (BBCH-60) were considered for this study, with the exception of SB at KH during season II, which was collected at milk ripe stage (BBCH-75).

eDNA and eRNA extraction, metabarcoding characterization

Extraction of both eNA was performed from the same rhizosphere soil sample (400-500 mg of rhizosphere soil). In total, 1.4 ml of extraction buffer was used (0.2 M sodium phosphate buffer, 0.1 M NaCl, 0.05 M EDTA, 25 g l^{-1} SDS, pH 8), separating half of the supernatant in two different microcentrifuge tubes (one for eDNA, one for eRNA) after cell disruption and centrifugation, and following the steps described by Quiroga et al. (2025) and Quiroga et al. (2024). In the case of eRNA, the addition of RNase and the incubation for 30 min at 37°C were not performed. Both eNA were dissolved in 50 µl nuclease-free water. DNA digestion was carried out using the RNase-Free DNase set (QIAGEN GmbH, Germany) following the manufacturer's instructions. A PCR was performed using the universal 16S rRNA gene primers 27F (AGAGTTTGATCMTGGATCMTGGCTCAG) and 1492R

(GGTTACCTTGTTACGACTT) (Lane, 1991; Weisburg et al., 1991) followed by electrophoresis to confirm the absence of remaining DNA. Subsequently, RNA cleanup and reverse transcription were performed using the RNA clean & concentrator^{TM}-5 Kit (Zymo Research, Irvine, CA, USA) and the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Inc., Cedar Creek, TX, USA), respectively, according to the manufacturer's instructions. For metabarcoding characterization, the universal 16S rRNA gene primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTCMTTTRAGTTT) (Claesson et al., 2009; Engelbrektson et al., 2010) (Integrated DNA Technologies, Belgium) were used for PCR amplification through the Kapa High Fidelity (KAPA/Hifi) polymerase (Roche diagnostics GmbH, Mannheim, Germany). More detailed information about subsequent steps is provided by Kaplan et al. (2019).

Bioinformatics and statistical analysis

Sequencing analyses were performed in QIIME2 version 2022.11 (Bolyen et al., 2019) and R studio software, v4.3.2 (R Core Team, 2023). Sequences of both eNA were trimmed and truncated at 15 bp and 325 bp, respectively. The subsequent steps were identically performed as mentioned by Quiroga et al. (2025). Data curation was carried out through the R package microViz v0.12.1 (Barnett et al., 2021). To identify and visualize the most prevalent taxa at the genus level, a detection threshold of 0.25% and a prevalence of 75% were applied separately to the amplicon sequence variants (ASVs) of each eNA based on relative abundance. This analysis was performed with the R package Microbiome v1.24.0 (Lahti, L., Shetty, 2019).

Alpha and beta diversity analyses were performed through the R package Vegan v2.6.4 (Oksanen et al., 2019) and the QIIME2 Gemelli plugin with Robust Aitchison PCA (RPCA), respectively (Martino et al., 2021). Pairwise comparisons for alpha diversity indices (Observed ASVs, Shannon-Wiener, and Gini-Simpson) were conducted using the Wilcoxon rank-sum test with Holm adjustment. For beta diversity, permutational multivariate analysis of variance (PERMANOVA, 999 permutations) with Benjamini-Hochberg correction was employed to identify significant differences between treatments.

Filtering of ASV counts lower than 99 was carried out through the R package Phyloseq v1.46.0 (McMurdie and Holmes, 2013). Thereafter, differential abundance of compositional data between eDNA and eRNA at different grouping levels was performed using the R package ALDEx2 v1.34.0 (Fernandes et al., 2014). Treatments were organized by location (GH, KH), eNA (eDNA, eRNA), and plant (WW, SB), and plotted for both seasons.

Results

Analysis in QIIME2 revealed a total of 16,632,222 sequences for both eNA, with a minimum of 3,167 sequences and a maximum of 165,222 sequences per sample. To compare the most prevalent taxa at genus level, each eNA was separately analyzed using the same thresholds, and the results were plotted as shown in Fig. 1. Ten and 11 prevalent taxa were identified for eDNA and eRNA, respectively. Differences in relative abundance (%) were clearly observed between eDNA and eRNA, and to a lesser extent between locations and seasons. Both eNA shared three phyla, being the phylum *Pseudomonadota* the most abundant, with 50% and 45% of all prevalent taxa for eDNA and eRNA, respectively. This phylum together with the phyla *Acidobacteriota*, and *Bacillota* encompassed 90% (eDNA) and 73% (eRNA) of all prevalent taxa. Nevertheless, while only one phylum was different for eDNA (*Actinobacteriota*), eRNA comprised three different phyla (*Candidatus* Entotheonellaeota, *Myxococcota*, and *Candidatus* Latescibacterota) which represented 27% of all the taxa. Similarly, four prevalent genera including *Bradyrhizobium*, *Microvirga*, *Romboutsia*, and *Reyranella*, and the prevalent order *Vicinamibacterales* were shared by both eNA.

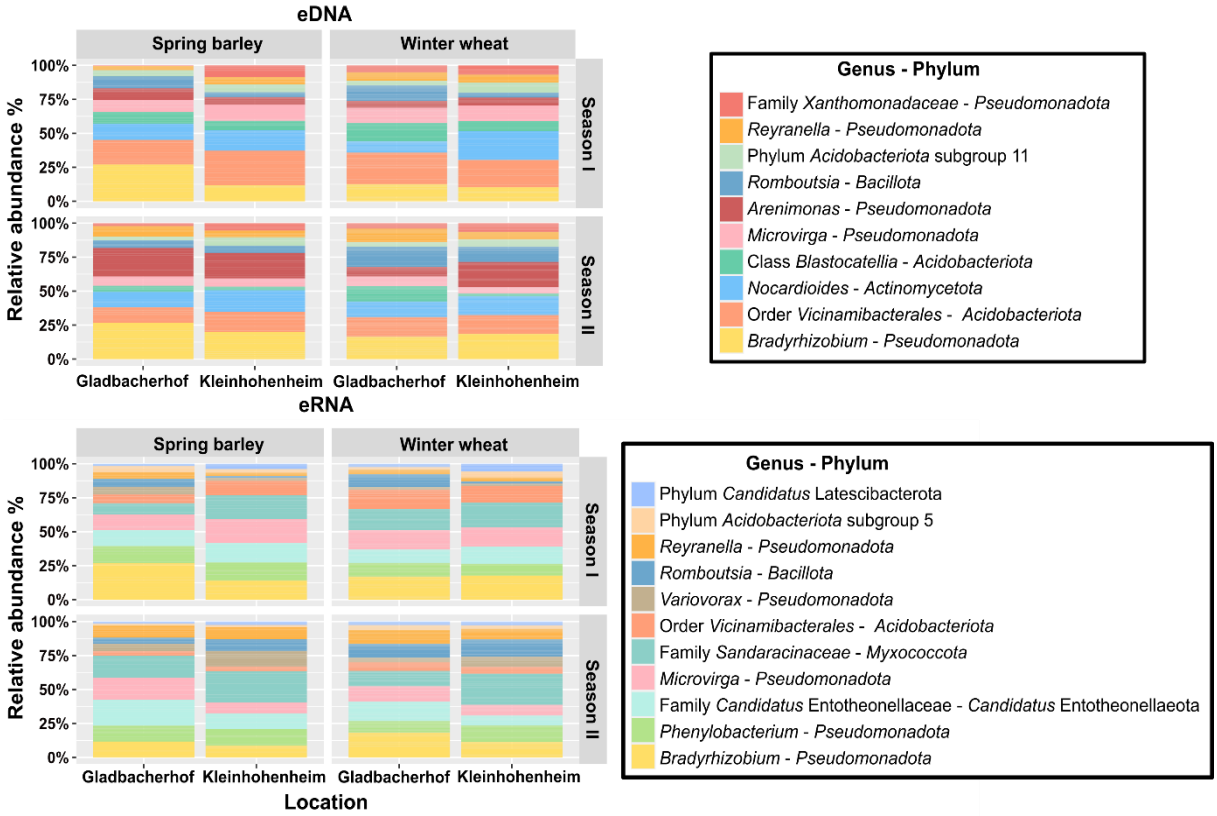


Fig. 1. Rhizosphere bacterial composition between eDNA and eRNA. Most prevalent taxa at a genus level were obtained after filtering out ASVs with a relative abundance lower than 0.25%.

Treatments were grouped by eNA and season, and alpha diversity indices were analyzed after confirming that the factors bacterial inoculation, fertilizer, and row spacing did not show significant differences over the two seasons for each eNA ($p > 0.05$). When treatments were grouped by location and crop species, similar results were obtained for eRNA as previously reported for eDNA (Quiroga et al., 2025). Instead, a comparison between each eNA over the seasons was carried out. Significant differences were found between eRNA and eDNA for all alpha diversity indices over the two seasons, with the exception of Shannon-Wiener index between eRNA and eDNA during season I ($p = 0.24$) (Fig. 2). Interestingly, season influenced each alpha diversity index to varying degrees. While consistent significant differences were found between eDNA and eRNA in the Gini-Simpson index over the two seasons, this trend was not observed with the Shannon index, in which eDNA was significantly different from eRNA during season II but not at season I. In contrast, eRNA showed significantly higher values than eDNA for Observed ASVs during season I and opposite results during season II (Fig. 2).

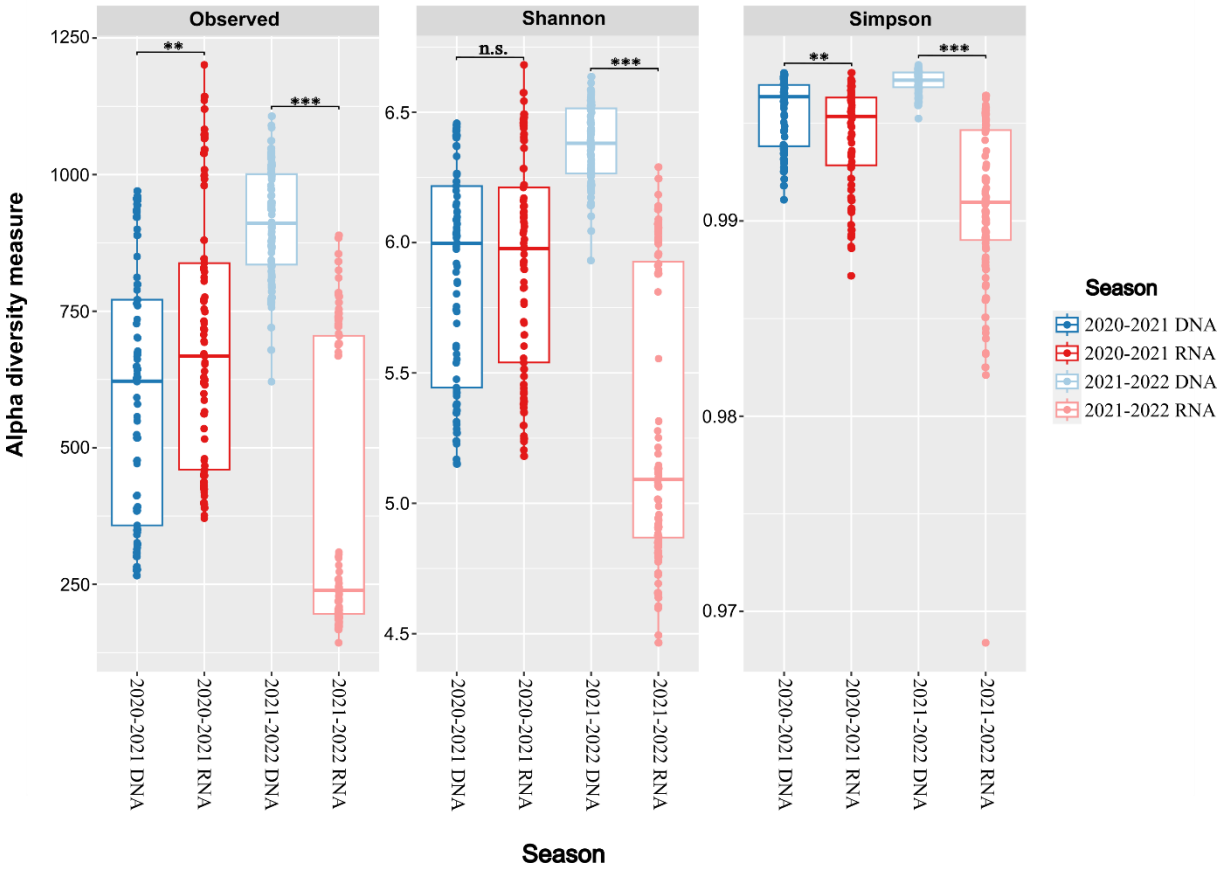


Fig. 2. Alpha diversity indices observed ASVs, Shannon-Wiener, and Gini-Simpson for eDNA and eRNA over two seasons. Normalization was performed using rarefaction. NS, not significant. Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05, '*'.

Similar to alpha diversity, PERMANOVA analysis for beta diversity of bacterial communities revealed no significant effects from the three evaluated factors for each eNA ($p > 0.05$, 999 permutations). An exception was observed during the season II, where eRNA showed significant differences in spring barley at KH between $E19^T$ and Ctrl (PERMANOVA, $p = 0.019$, 999 permutations) whereas this difference was not observed with eDNA (PERMANOVA, $p = 0.91$, 999 permutations). This trend was not observed during season I, where no significant effects were found for either eNA ($p > 0.05$, 999 permutations). Therefore, RPCA was plotted and analyzed for each season based on location, plant, and eNA. In general, a clear separation between eDNA and eRNA was identified in both seasons, indicating that rhizosphere bacterial communities differed significantly between eDNA and eRNA (PERMANOVA, $p = 0.001$, 999 permutations) (Fig. 3). During season I, eDNA differed from eRNA between SB and WW at KH. While eRNA showed no significant effect (PERMANOVA, $p = 0.18$, 999 permutations), eDNA revealed significant differences between the two plants at KH (PERMANOVA, $p = 0.0011$, 999 permutations). Nevertheless, this trend was reversed in the subsequent season, where eRNA showed significant differences between SB and WW at KH (PERMANOVA, $p = 0.0011$, 999 permutations), while eDNA did not (PERMANOVA, $p = 0.056$, 999 permutations). Aside from these differences, the remaining treatments varied significantly by plant, location, and eNA.

Differential abundance between eDNA and eRNA through ALDEx2 was determined according to plant species and location as shown in Fig. 4. Considering effect size values ≥ 1 and ≤ -1, SB showed more ASVs significantly affected than WW in both locations. While 839 ASVs at GH and 431 ASVs at KH were identified for SB, 347 ASVs at GH and 286 ASVs at KH were identified for WW. Given the varied number of affected ASVs, effect size thresholds were adjusted to visualize the most representative taxa at genus level (Fig. 4). Interestingly, special trends could be identified across both seasons. The first clear observation was that the family *Candidatus* Entotheonellaceae was positively affected by eRNA, regardless of plant or location, with a particularly noticeable effect at GH (Fig. 4). Interestingly, most of the shared taxa, such as *Microvirga*, *Phenylobacterium*, *Gaiella*, and *Nocardioides*, were either positively or negatively affected by eRNA in at least one season, with the same effect consistently observed across plants and locations. Although this trend cannot be generalized to all taxa (e.g. *Nitrospira*) and seasons, we observed that only 0.29% of the ASVs at GH and 1.75% of the ASVs at KH for WW showed a significant reverse effect between seasons. In the case of SB, these differences reached 13.5% at GH and 12.3% at KH. These findings suggest that the

abundance of certain taxa, including beneficial bacteria or prevalent taxa, can be either masked or revealed depending on the eNA used.

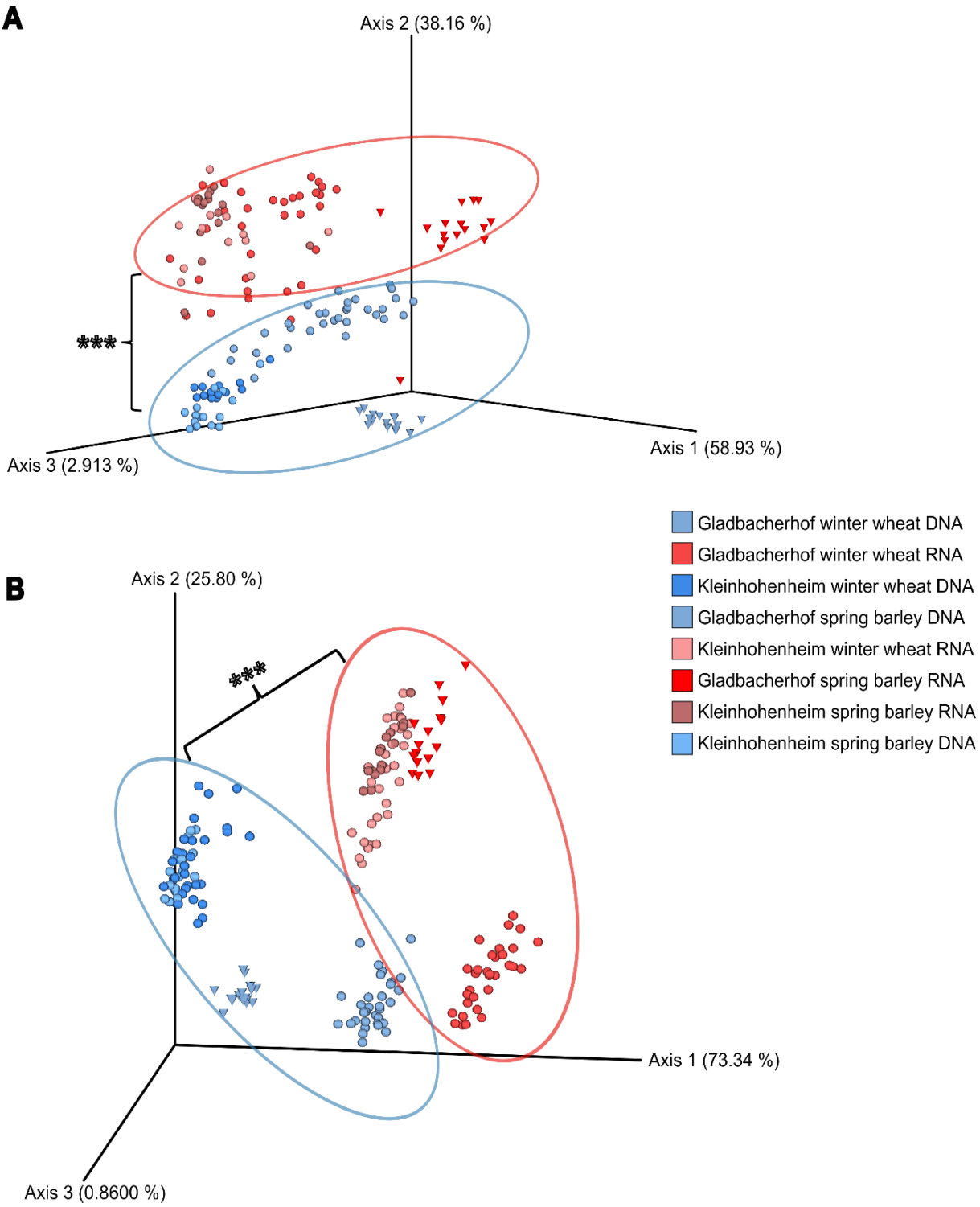


Fig. 3. Beta diversity ordination using robust Aitchison RPCA between eDNA and eRNA over two consecutive seasons for winter wheat and spring barley. **(A)** Season I (2020-2021), **(B)** season II (2021-2022). Triangles: spring barley at GH, circles: rest of the treatments. NS, not significant. Significance codes: ≤ 0.001 '***', ≤ 0.01 '**'.

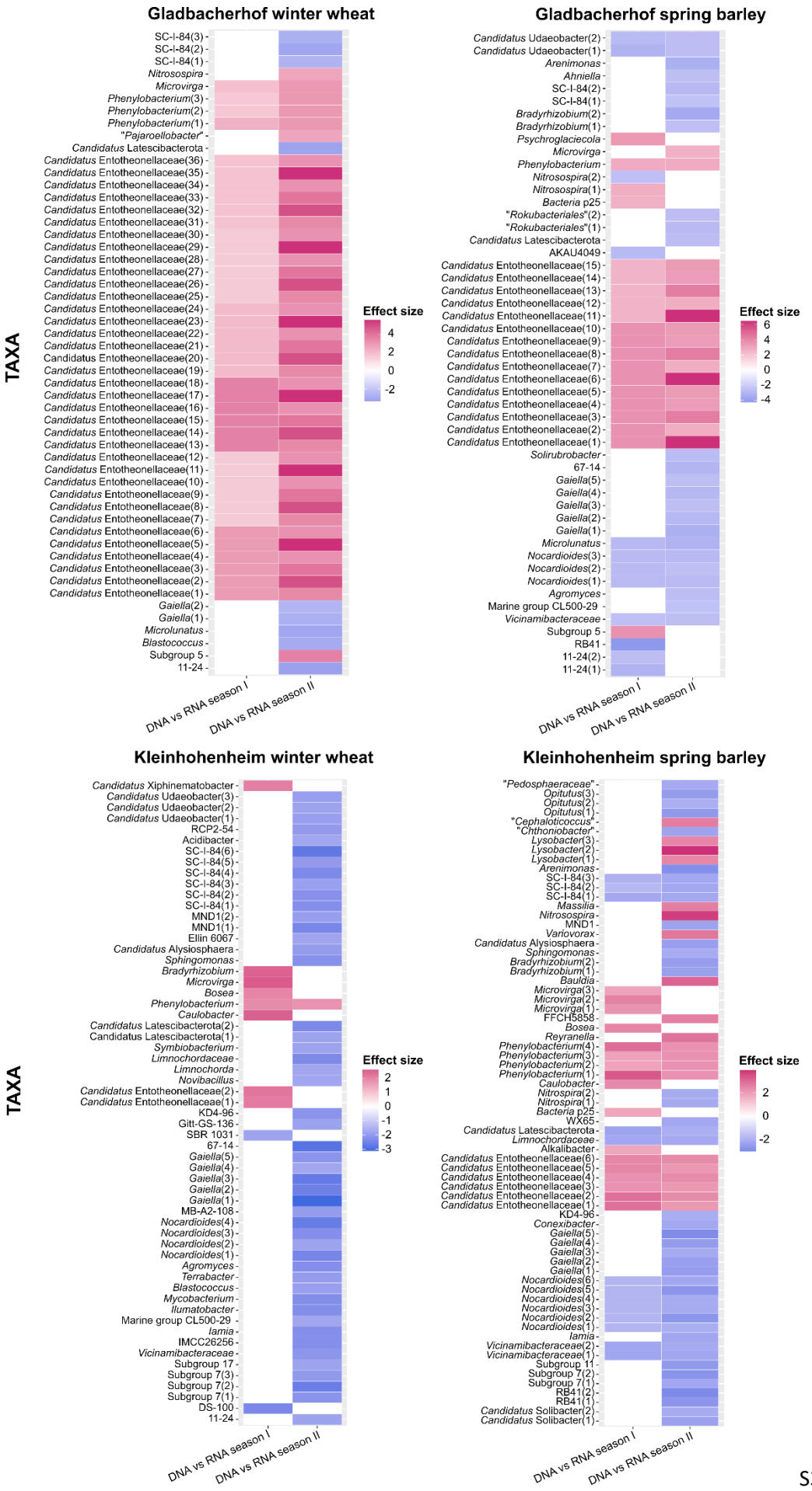

Gladbacherhof winter wheat
Gladbacherhof spring barley
Kleinhohenheim winter wheat
Kleinhohenheim spring barley
TAXA
Effect size
DNA vs RNA season I
DNA vs RNA season II

Fig. 4. Heatmaps using ALDEx2 differential abundance between eDNA and eRNA. Each heatmap compares seasons I and II for treatments grouped according to eNA, plant species, and location. Effect sizes: for WW at GH during season I: ≥ 1.5 and ≤ -1.5, for WW and SB at GH during seasons I (only SB) and II and ≥ 2.5 and ≤ -2.5, for WW and SB at KH during seasons I and II (only WW): ≥ 1.75 and ≤ -1.75, and for SB at KH during season II: ≥ 2 and ≤ -2.

Discussion

The results of the present study are in accordance with several reports indicating a clear differentiation in the microbial community composition between eDNA and eRNA (Angel et al., 2013; Baldrian et al., 2012; Giroux et al., 2022). Importantly, we conducted a deeper analysis, identifying ASVs significantly impacted not only between both eNA, but also how they varied across seasons, plant species, and location. A consistent positive trend in detecting the family *Candidatus* Entotheonellaceae by eRNA was observed, independently of location, plant, or season. Members of this family are known for their role in producing secondary metabolites and have been primarily investigated in marine environments due to their symbiotic relationships with sponges like *Theonella swinhoei* (Schmidt et al., 2000; Wilson et al., 2014). These investigations have unveiled a promising medical potential, including the identification of bioactive compounds such as the polyketide psymberin, which exhibits anticancer properties (Peters et al., 2023). Additionally, the niche occupancy of these organisms has also been extended to freshwater and soil habitats, including the rhizosphere or root endosphere (Garcia Mendez et al., 2024; Vendruscolo et al., 2022). Remarkably, our results aligned with the findings performed by Bai et al. (2023) in which the phyla *Entotheonellaeota* and *Myxococcota* were also identified as prevalent taxa in the active bacterial community of slow sand filters used for drinking water production. Our results agree with these and other authors' assertions regarding that eRNA can unveil low abundance but active taxa, which could probably exert a significant role in the microbial communities but masked by the inflated abundance of eDNA (Giroux et al., 2022; Lasa et al., 2019). Interestingly, the consistent responses (positively or negatively) of certain ASVs across locations, plants, and seasons suggests that eRNA could be more sensitive for characterizing certain taxa compared to eDNA. This can become particularly meaningful when a specific taxon needs to be identified or evaluated, for instance, as a biomarker (Bai et al., 2023).

In general, microbial communities are susceptible to different environmental changes such as temperature, pH-value, precipitation, or to the incorporation of non-native species (Allison and Martiny, 2008; Mawarda et al., 2020). It has been claimed that these factors could be more pronounced in eRNA than eDNA (Bay et al., 2021; Littlefair et al., 2022). Our analyses partially

corroborated this assertion as similar results were obtained with eRNA and eDNA, which could clearly identify significant effects in the bacterial community composition between locations and plant species across two consecutive seasons (Quiroga et al., 2025). We originally hypothesized that eRNA could reveal, more broadly than eDNA, an alteration in the bacterial community structure due to the inoculation with *H. diazotrophicus*. Nevertheless, although finding significant differences between E19^T compared to the Ctrl using eRNA only for SB at KH during season II, this trend was not followed in the other plant species, location, or season at KH. This lack of consistency could be attributed to other factors such as low statistical power due to statistical replicates or a more complex experimental design than a real alteration caused by the inoculation of *H. diazotrophicus* (Neuhoff et al., 2023). However, these limitations were compensated when different locations over consecutive seasons were included for the evaluation of the impacts of *H. diazotrophicus*. Longitudinal evaluation requires special emphasis not only to provide more robustness to the analysis but also to get a better comprehension of microbial community dynamics (Becker et al., 2022; Wagner et al., 2016). In this regard, in contrast to beta diversity, we observed varying results for the alpha diversity indices, especially between seasons. Our results showed that alpha diversity metrics for eDNA are not always higher than those for eRNA. This may be influenced by seasonal changes and could provide insights into the variability observed in the metrics reported by other studies (Kuramae et al., 2013; Lasa et al., 2019; Li et al., 2021).

Conclusion

The evaluation of the rhizosphere bacterial communities of wheat and barley revealed primarily similarities but also notable differences in the most prevalent taxa between eDNA and eRNA. Differential abundance between both eNA determined which taxa were significantly affected. Most of the taxa such as the family *Candidatus* Entotheonellaceae or the genus *Gaiella* that were positively or negatively affected between eDNA and eRNA mainly followed the same trend regardless of location, plant species, or season. Our results suggest that eRNA not only can reveal hidden taxa, but also that certain taxa are more likely to be detected using eRNA during metabarcoding characterization. In addition, the comparison between eNA complemented and corroborated our previous findings, indicating no significant effects on the total (eDNA) and active (eRNA) rhizosphere bacterial communities due to the inoculation with *H. diazotrophicus* strain E19^T. Moreover, although both eNA significantly differed in the bacterial community composition, similar changes were obtained when location, plant species, and season were evaluated.

Acknowledgments We are grateful to Bellinda Schneider, Rita Geissler-Plaum for their technical support. We also acknowledge Simon Schäfer for his support during field experiments at Kleinhohenheim.

Author contribution

Santiago Quiroga: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Stefan Ratering:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Supervision, Conceptualization. **David Rosado-Porto:** Writing –review & editing, Data curation, Software, Validation. **Azin Rekowski:** Writing – review & editing, Conceptualization. **Franz Schulz:** Writing – review & editing, Methodology, Conceptualization. **Christian Zörb:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Sylvia Schnell:** Writing – review & editing, Methodology, Funding acquisition, Supervision, Conceptualization

Funding This work was fully funded by the Federal Ministry of Education and Research (BMBF) of Germany under the BONARES program (Rhizo4bio).

Data availability All DNA/RNA sequences are available on the NCBI/GenBank database repository under the accessions numbers PRJNA1030754 (season I), PRJNA1030767 (season II) for eDNA and PRJNA1218684 (season I), and PRJNA1218727 (season II) for eRNA

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

Financial interests The authors have no relevant financial or non-financial interests to disclose

References

Adamo, M., Voyron, S., Chialva, M., Marmeisse, R., Girlanda, M., 2020. Metabarcoding on both environmental DNA and RNA highlights differences between fungal communities sampled in different habitats. PLoS One 15, 1–21. https://doi.org/10.1371/journal.pone.0244682

Allison, S.D., Martiny, J.B.H., 2008. Resistance, resilience, and redundancy in microbial communities. Proc. Natl. Acad. Sci. U. S. A. 105, 11512–11519. https://doi.org/10.1073/pnas.0801925105

Angel, R., Pasternak, Z., Soares, M.I.M., Conrad, R., Gillor, O., 2013. Active and total prokaryotic communities in dryland soils. FEMS Microbiol. Ecol. 86, 130–138. https://doi.org/10.1111/1574-6941.12155

Bai, X., Dinkla, I.J.T., Muyzer, G., 2023. Shedding light on the total and active core microbiomes in slow sand filters for drinking water production. Water Res. 243, 120404. https://doi.org/10.1016/j.watres.2023.120404

Baldrian, P., Kolaiřík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., Žifčáková, L., Šnajdr, J., Rídl, J., Vlček, Č., Voříšková, J., 2012. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME J. 6, 248–258. https://doi.org/10.1038/ismej.2011.95

Barnett, D., Arts, I., Penders, J., 2021. microViz: an R package for microbiome data visualization and statistics. J. Open Source Softw. 6, 3201. https://doi.org/10.21105/joss.03201

Bay, G., Lee, C., Chen, C., Mahal, N.K., Castellano, M.J., Hofmockel, K.S., Halverson, L.J., 2021. Agricultural management affects the active rhizosphere bacterial community composition and nitrification. mSystems 6. https://doi.org/10.1128/msystems.00651-21

Becker, M.F., Hellmann, M., Knief, C., 2022. Spatio - temporal variation in the root - associated microbiota of orchard - grown apple trees. Environ. Microbiome 1–21. https://doi.org/10.1186/s40793-022-00427-z

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., O'Toole, P.W., 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4, e6669. https://doi.org/10.1371/journal.pone.0006669

Engelbrektson, A., Kunin, V., Wrighton, K.C., Zvenigorodsky, N., Chen, F., Ochman, H., Hugenholtz, P., 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J. 4, 642–647. https://doi.org/10.1038/ismej.2009.153

Fernandes, D., A., Reid, J., Macklaim, M., J., McMurrough, T.A, Edgell, D.R., Gloor, B., G., 2014. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-

seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome 2, 1–13. https://doi.org/10.1186/2049-2618-2-15

Garcia Mendez, S., Fordeyn, S., De Tender, C., Goormachtig, S., Willems, A., 2024. Do seasons impact the root microbiome of wild *Poa annua* populations? Rhizosphere 31. https://doi.org/10.1016/j.rhisph.2024.100929

Giroux, M.S., Reichman, J.R., Langknecht, T., Burgess, R.M., Ho, K.T., 2022. Environmental RNA as a tool for marine community biodiversity assessments. Sci. Rep. 12, 1–13. https://doi.org/10.1038/s41598-022-22198-w

Gkarmiri, K., Mahmood, S., Ekblad, A., Alström, S., Högberg, N., Finlay, R., 2017. Identifying the active microbiome associated with roots and rhizosphere soil of oilseed rape. Appl. Environ. Microbiol. 83. https://doi.org/10.1128/AEM.01938-17

Kaplan, H., Ratering, S., Felix-Henningsen, P., Schnell, S., 2019. Stability of *in situ* immobilization of trace metals with different amendments revealed by microbial ^{13}C-labelled wheat root decomposition and efflux-mediated metal resistance of soil bacteria. Sci. Total Environ. 659, 1082–1089. https://doi.org/10.1016/j.scitotenv.2018.12.441

Kloepper, J.W., 1981. Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. Phytopathology 71, 590–592. https://doi.org/10.1094/phyto-71-590

Kuramae, E.E., Verbruggen, E., Hillekens, R., de Hollander, M., Röling, W.F.M., van der Heijden, M.G.A., Kowalchuk, G.A., 2013. Tracking fungal community responses to maize plants by DNA- and RNA-based pyrosequencing. PLoS One 8. https://doi.org/10.1371/journal.pone.0069973

Lahti, L., Shetty, S., 2019. Microbiome R Package. Available online at: http://microbiome.github.io. [WWW Document].

Lane, D.J., 1991. 16S/23S rRNA sequencing. Nucleic acid Tech. Bact. Syst. 115–175.

Lasa, A. V., Fernández-González, A.J., Villadas, P.J., Toro, N., Fernández-López, M., 2019. Metabarcoding reveals that rhizospheric microbiota of *Quercus pyrenaica* is composed by a relatively small number of bacterial taxa highly abundant. Sci. Rep. 9, 1–13. https://doi.org/10.1038/s41598-018-38123-z

Li, J., Wen, Y., Yang, X., 2021. Understanding the responses of soil bacterial communities to long-term fertilization regimes using DNA and RNA sequencing. Agronomy 11. https://doi.org/10.3390/agronomy11122425

Littlefair, J.E., Rennie, M.D., Cristescu, M.E., 2022. Environmental nucleic acids: a field-based comparison for monitoring freshwater habitats using eDNA and eRNA. Mol. Ecol. Resour. 22, 2928–2940. https://doi.org/10.1111/1755-0998.13671

Macher, T.H., Arle, J., Beermann, A.J., Frank, L., Hupało, K., Koschorreck, J., Schütz, R., Leese, F., 2024. Is it worth the extra mile? comparing environmental DNA and RNA metabarcoding for vertebrate and invertebrate biodiversity surveys in a lowland stream. PeerJ 12. https://doi.org/10.7717/peerj.18016

Martino, C., Shenhav, L., Marotz, C.A., Armstrong, G., McDonald, D., Vázquez-Baeza, Y., Morton, J.T., Jiang, L., Dominguez-Bello, M.G., Swafford, A.D., Halperin, E., Knight, R., 2021. Context-aware dimensionality reduction deconvolutes gut microbial community dynamics. Nat. Biotechnol. 39, 165–168. https://doi.org/10.1038/s41587-020-0660-7

Mawarda, P.C., Le Roux, X., Dirk van Elsas, J., Salles, J.F., 2020. Deliberate introduction of

invisible invaders: a critical appraisal of the impact of microbial inoculants on soil microbial communities. Soil Biol. Biochem. 148, 107874. https://doi.org/10.1016/j.soilbio.2020.107874

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Neuhoff, D., Neumann, G., Weinmann, M., 2023. Testing plant growth promoting microorganisms in the field - a proposal for standards. Front. Plant Sci. 14, 1324665. https://doi.org/10.3389/fpls.2023.1324665

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., Hara, R.B.O., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., 2019. Vegan: community ecology package. R package version 2.5-6. Vienna: R Foundation for Statistical Computing.

Peters, E.E., Cahn, J.K.B., Lotti, A., Gavriilidou, A., Steffens, U.A.E., Loureiro, C., Schorn, M.A., Cárdenas, P., Vickneswaran, N., Crews, P., Sipkema, D., Piel, J., 2023. Distribution and diversity of 'Tectomicrobia', a deep-branching uncultivated bacterial lineage harboring rich producers of bioactive metabolites. ISME Commun. 3, 1–12. https://doi.org/10.1038/s43705-023-00259-z

Quiroga, S., Ratering, S., Rosado-Porto, D., Rekowski, A., Schulz, F., Zörb, C., Schnell, S., 2025. Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial. Appl. Soil Ecol. 206, 105823. https://doi.org/10.1016/j.apsoil.2024.105823

Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Schulz, F., Krutych, M., Zörb, C., Schnell, S., 2024. Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance. FEMS Microbiol. Ecol. 100, fiae023. https://doi.org/10.1093/femsec/fiae023

R Core Team, 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

Rosado-Porto, D., Ratering, S., Wohlfahrt, Y., Schneider, B., Glatt, A., Schnell, S., 2023. Elevated atmospheric CO_2 concentrations caused a shift of the metabolically active microbiome in vineyard soil. BMC Microbiol. 23, 46. https://doi.org/10.1186/s12866-023-02781-5

Schmidt, E.W., Obraztsova, A.Y., Davidson, S.K., Faulkner, D.J., Haygood, M.G., 2000. Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel δ-proteobacterium, "*Candidatus* Entotheonella palauensis." Mar. Biol. 136, 969–977. https://doi.org/10.1007/s002270000273

Taberlet, P., Coissac, E., Hajibabaei, M., Rieseberg, L.H., 2012. Environmental DNA. Curr. Biol. 32, R1250–R1252. https://doi.org/10.1016/j.cub.2022.09.052

Veilleux, H.D., Misutka, M.D., Glover, C.N., 2021. Environmental DNA and environmental RNA: Current and prospective applications for biological monitoring. Sci. Total Environ. 782, 146891. https://doi.org/10.1016/j.scitotenv.2021.146891

Vendruscolo, E.C.G., Mesa, D., de Souza, E.M., 2022. Corn rhizosphere microbial community in different long term soil management systems. Appl. Soil Ecol. 172, 104339.

https://doi.org/10.1016/j.apsoil.2021.104339

Wagner, M.R., Lundberg, D.S., Del Rio, T.G., Tringe, S.G., Dangl, J.L., Mitchell-Olds, T., 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat. Commun. 7, 12151. https://doi.org/10.1038/ncomms12151

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Wilson, M.C., Mori, T., Rückert, C., Uria, A.R., Helf, M.J., Takada, K., Gernert, C., Steffens, U.A.E., Heycke, N., Schmitt, S., Rinke, C., Helfrich, E.J.N., Brachmann, A.O., Gurgui, C., Wakimoto, T., Kracht, M., Crüsemann, M., Hentschel, U., Abe, I., Matsunaga, S., Kalinowski, J., Takeyama, H., Piel, J., 2014. An environmental bacterial taxon with a large and distinct metabolic repertoire. Nature 506, 58–62. https://doi.org/10.1038/nature12959

Zaiko, A., Pochon, X., Garcia-Vazquez, E., Olenin, S., Wood, S.A., 2018. Advantages and limitations of environmental DNA/RNA tools for marine biosecurity: management and surveillance of non-indigenous species. Front. Mar. Sci. 5. https://doi.org/10.3389/fmars.2018.00322

Zhang, Y., Qiu, Y., Liu, K., Zhong, W., Yang, J., Altermatt, F., Zhang, X., 2024. Evaluating eDNA and eRNA metabarcoding for aquatic biodiversity assessment: From bacteria to vertebrates. Environ. Sci. Ecotechnology 21, 100441. https://doi.org/10.1016/j.ese.2024.100441

Chapter V

Absence of aboveground effects may conceal changes underground: Plant-growth promoting rhizobacteria and salt stress altered the rhizosphere microbiome of roses

Research article

To be submitted to International Microbiology

Absence of aboveground effects may conceal changes underground: Plant-growth promoting rhizobacteria and salt stress altered the rhizosphere microbiome of roses

Santiago Quiroga[1], Stefan Ratering[1], David Rosado-Porto[1,2], David Kostner[3], and Sylvia Schnell[1]

Santiago Quiroga
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
Santiago.Quiroga@umwelt.uni-giessen.de
ORCID-ID 0009-0001-1392-8241

Stefan Ratering
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
Stefan.Ratering@umwelt.uni-giessen.de
ORCID-ID 0000-0001-7572-6306

David Rosado-Porto
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
[2]*Faculty of Basic and Biomedical Sciences, Simón Bolívar University, 080002 Barranquilla, Colombia*
David.Rosado-Porto@agrar.uni-giessen.de
ORCID-ID 0000-0001-6199-0114

David Kostner
[3]*Industrial Biotechnology and Bioeconomy, University of Applied Sciences Munich, 80335 Munich, Germany*
david.kostner@hm.edu
ORCID-ID 0000-0001-7633-1795

Sylvia Schnell
[1]*Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany*
Sylvia.Schnell@umwelt.uni-giessen.de
ORCID-ID 0000-0003-3903-6089

Corresponding author*:* Sylvia Schnell

Sylvia.Schnell@umwelt.uni-giessen.de

Abstract

The benefits of plant growth-promoting rhizobacteria (PGPR) are currently considered an alternative for sustainable agriculture including ornamental plants. Roses (*Rosa* sp.) occupy a considerable part of the ornamental plant market worldwide and require resources, such as water, fertilizer, and crop protection. Herein, we evaluated the PGPR abilities of three bacterial strains (*Bacillus subtilis, Bacillus amyloliquefaciens,* and *Paenibacillus polymyxa*) on the development of *Rosa* sp. 'Flower Carpet' with and without salt stress and the changes in the bacterial microbiome of the rhizosphere caused by the addition of PGPR and salt by metabarcoding of the 16S rRNA gene. While no effect of PGPR addition on salt stress was found in the plant parameters, the bacterial microbiome analysis revealed differences both between PGPR addition and no addition, and salt and no salt. Both with and without salt stress, and with and without PGPR, the alpha diversity indices were different. The diversity was reduced due to salt and bacteria addition. Beta diversity analysis showed significant differences before and after salt stress ($p = 0.001$) and with and without PGPR addition ($p = 0.002$). Differential abundance analysis with ALDEx2 showed salt stress affected 14 amplicon sequence variants (ASVs). After addition of *B. amyloliquefaciens* and *P. polymyxa*, there was a different abundance in more ASVs (27 and 32 ASVs) than after addition of *B. subtilis* (2-3 ASVs) due to bacterial inoculation.

Keywords

Rosa sp., 16S rRNA genes, salt stress, rhizosphere microbiome, PGPR inoculation

Introduction

Since ancient times, roses (*Rosa* sp.) have sparked human interest because of their use in gardening, industry (perfumery, vitamin C), and medicine (natural oils) (Gudin, 2000). Rose trade around the world encompasses nearly 30% of the ornamental plant market (Smulders et al., 2011). A large part of the roses are grown in greenhouses and irrigated artificially. In recent years, due to the lack of good irrigation water, poorer-quality water or recycled/reused water is used, leading to an accumulation of salts in the greenhouse soil (Cabrera et al., 2009; Nirit et al., 2006). The environmental and economic impacts of salinity are hastened by climate change. As temperature rises, surface evaporation and plant transpiration also increase, leading to salinization (Zaman et al., 2018). In addition, roses in humid cold areas are often exposed to higher salt concentrations due to road salting, as they are popularly planted as ornamental plants in front gardens, traffic islands, etc. Moreover, the use of de-icing materials instead of salt is known to induce toxic effects on plants near streets (Cekstere et al. 2008).

While in recent years the rose breeders have focused on establishing robust cultivars that do not require the use of pesticides or high–maintenance, the number of publications dealing with salt tolerance is limited (for review: Niu and Sun 2019). Roses are in general salt-sensitive, capable to tolerate up to 3.5 dS m^{-1} electrical conductivity (EC) (Cabrera and Perdomo, 2003), with resistant cultivars such as *Rosa* × *hybrida* L. “New Dawn” or “RADrazz” whose growth is not substantially affected at higher concentrations (8.0 dS m^{-1} EC). However, under salt stress, roses undergo biochemical changes such as accumulation of Na^+ and Cl^- ions in the leaves, decrease of stomatal conductance, or alteration of photosystem II efficiency, which eventually lead to tissue damage, growth damage, and therefore, loss of aesthetic value (Cai et al., 2014).

Besides breeding more salt resistant cultivars, another option for making roses more salt-resistant is to use plant growth-promoting rhizobacteria (PGPR). In general, PGPR are capable of inducing plant growth, reduce abiotic and biotic stress, and even trigger plant defense mechanisms (e.g., induced systemic resistance) that act as antagonists of several pathogens (Van Loon et al., 1998). Among the wide variety of PGPR, the genus *Bacillus* is one of the most extensively reported, and *Bacillus subtilis* is considered the main growth promoter (Islam et al. 2016). Several *B. subtilis* strains synthesize phytohormones (e.g., abscisic acid or indole acetic acid) that contribute to plant growth enhancement, immune defense responses, or salt stress tolerance (Mohamed and Gomaa, 2012). Another *Bacillus* species, *B. amyloliquefaciens*, not only stimulates plant growth or confers tolerance to abiotic stress (Liu et al., 2017), but also shows extraordinary potential to produce antimicrobial metabolites (Chen et al., 2007).

Similarly, *Paenibacillus polymyxa* (previously known as *Bacillus polymyxa*) can synthesize either phytohormones that improve plant growth, or peptide antibiotics such as fusaricidins and polymyxins against fungi and bacteria (Luo et al., 2018). These three bacterial strains are also capable of producing and releasing volatile organic compounds (e.g. acetoin), which have been identified as an alternative way to promote plant growth or induce systemic resistance (Lee et al., 2012; Ryu et al., 2003). Although the beneficial effects of PGPR have been evaluated in several plants (mainly crop plants), only few studies have been focused on roses (Karthikeyan et al. 2007; Tariq et al. 2016; Araujo et al. 2020). Likewise, metagenomic analyses of the rhizosphere soil of roses are rare and shed light on its rhizosphere microbiome (Yuan et al., 2022) and how it can be affected by diseases (Yim et al., 2020). Even more unusual, the impact of exogenous bacterial inoculation (bioproducts) in the soil microbial communities is poorly understood, even in crop plants, and it has been only a concern in the last recent years (Yasuda et al. 2022; Daraz et al. 2021; Shi et al. 2022). Understanding plant-microbe interactions is required to elucidate the mechanisms that could contribute to flower development, stress tolerance, or disease resistance in *Rosa* sp.

Therefore, in this study, we evaluated plant growth promoting abilities of *B. subtilis*, *B. amyloliquefaciens*, and *P. polymyxa* on the development of *Rosa* sp. 'Flower Carpet' (Noack, 1990), a common ground cover rose (also known as 'Heidetraum' or 'Naotraum') used as decoration and ornament in many outdoor areas due to its easy handling and disease resistance against the main rose pathogens like rust, black spot, and powdery mildew (Noack 1990, 1998). In addition, we used metabarcoding to characterize soil bacterial communities and evaluate the influence of exogenous PGPR inoculation on these communities before and after salt stress.

Materials and methods

Plant material and bacterial strains

An overview of the experimental design is presented in Table 1. *B. amyloliquefaciens* (BA) and *P. polymyxa* (PP) were grown in a high-density sporulation medium (DMS medium), whereas *B. subtilis* (BS) was grown in DMS medium and in a low density (non-sporulation) or Bio Medium A (BioA). Culture media and bacterial strains were provided by Fritzmeier Umwelttechnik GmbH & Co.KG (Aying, Germany) and tested for plant growth promotion using 'Flower Carpet' rose variety. To obtain bacterial-free cultural supernatant, bacterial cultures were centrifuged for 15 min at 3345 x g and 25 °C (Heraeus Megafuge 1.02, Thermo Fisher Scientific, Asheville, USA), and then filtered using sterile syringe filters with a pore size of 0.2 μm (Sarstedt AG & Co. KG, Nümbrecht, Germany). The bacteria-free culture filtrate

contained bacterial soluble substances and remaining substrates. In addition, unsterile tap water (Ctrl water), sterile DMS, and BioA culture media without inoculated bacteria (Ctrl DMS and Ctrl BioA, respectively) were used as controls.

For each treatment, four or five bare-root roses (Rosenhof Schultheis e.K., Bad Nauheim, Germany) were planted in Mitscherlich pots of 8.8 L capacity (28 cm high, 20 cm diameter), and filled with approximately 8 L of rose soil (4.6 kg DW) (Rosenerde EUFLOR GmbH, Schermbeck, Germany). All treatments were randomized and watered by the soil drenching method once per week for 14 weeks with 10 ml of different bacterial cultures at the manufacturer's concentration expressed as colony-forming unit (CFU) ml^{-1} (BioA BS = 3.4 x 10^6 CFU ml^{-1}, DMS BS = 3.9 x 10^8 CFU ml^{-1}, DMS PP = 5.4 x 10^7 CFU ml^{-1}, DMS BA = 5 x 10^8 CFU ml^{-1}) or the culture media without bacteria (controls) dissolved in 90 ml of unsterile tap water. Plant growth tests were performed at the plant experimental station of Justus Liebig University located in Giessen, Germany, between April and October 2019 (23 weeks). The light and temperature conditions were determined by the external environment. As a marker of health, the total number of flowers in the filtered and unfiltered treatments was determined at the 12^{th} week of treatment, when most of the roses bloomed. Completely opened rose buds were considered to be blooming.

Rhizosphere soil sampling and induction of salt stress

Rhizosphere soil samples from only the unfiltered treatments (32 pots, Table 1) were collected, sifted, and stored at -80 °C before (week 15^{th}) and after salt stress (week 23^{rd}) for further microbiome analysis. For salt stress induction, unfiltered treatments were watered four times with 800 ml of a solution of NaCl 600 mM (1% NaCl) until reach a final concentration of 4% NaCl. The application of salt started in the 16^{th} week of treatment and continued for approximately two weeks. After salt application (18^{th} week), the roses were rewatered with tap water for five weeks, and finally, the fresh and dry weight were determined. Fallen leaves were not considered for the dry weight.

Table 1 Experimental design for rose growth promotion and salt stress

Abbreviation of treatment	Culture medium	Bacterial strain	Filtered / unfiltered	N° replications	Salt stress at 16th week
Ctrl water	Water	-	-	5	Applied
Ctrl Bio A	Bio A	-	-	4	Applied
BioA BS	Bio A	*B. subtilis*	Unfiltered	5	Applied
BioA BS filt	Bio A	*B. subtilis*	Filtered	5	-
Ctrl DMS	DMS	-	-	4	Applied
DMS BS	DMS	*B. subtilis*	Unfiltered	5	Applied
DMS BS filt	DMS	*B. subtilis*	Filtered	5	-
DMS PP	DMS	*P. polymyxa*	Unfiltered	5	Applied
DMS PP filt	DMS	*P. polymyxa*	Filtered	4	-
DMS BA	DMS	*B. amyloliquefaciens*	Unfiltered	4	Applied
DMS BA filt	DMS	*B. amyloliquefaciens*	Filtered	4	-
Bulk soil	-	-	-	2	-

Metabarcoding characterization

DNA extraction was performed using the NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany), following the manufacturer's instructions. Soil-extracted DNA was diluted (1:10) and amplified by polymerase chain reaction (PCR) using Kapa High Fidelity (KAPA/Hifi) polymerase (Roche diagnostics GmbH, Mannheim, Germany) and the universal 16S rRNA gene primers 520F (AYTGGGYDTAAAGNG) (Claesson et al., 2009) and 926R (CCGTCAATTCMTTTRAGTTT) (Engelbrektson et al., 2010) (Integrated DNA Technologies, BVBA, Leuven, Belgium). PCRs and sequencing of the PCR products were

performed using an Ion Torrent PGM (Thermo Fisher Scientific, Asheville, USA) according to Kaplan et al. (2019).

Bioinformatics and statistical analysis

Statistical evaluation of the total number of flowers and plant dry weight was performed using R studio software, v4.3.0 (R Core Team, 2023). Analysis of variance (ANOVA) or the Kruskal-Wallis test were performed to compare significant differences between treatments. Sequencing data were analyzed using QIIME2 version 2020.6 (Bolyen et al., 2019) and R studio software, v4.3.0. In QIIME2, sequences were demultiplexed with cutadapt QIIME2 plugin (Martin, 2011). Quality control, denoising, dereplicate of single-end sequences, and chimera filtering were performed using the QIIME2 plugin of the DADA2 software package (Callahan et al., 2016). All sequences were trimmed at 15 bp and truncated at 360 bp. The amplicon sequence variants (ASVs) produced with DADA2 were taxonomically affiliated with a trained fitted classifier (Bokulich et al., 2018; Pedregosa et al., 2011) based on the SILVA database (release 138) (Quast et al., 2012). After taxonomic affiliation, mitochondrial and chloroplast sequences were filtered out.

Prevalent taxa at the genus level were filtered and plotted based on relative abundance considering ASVs with a detection threshold of 0.35% and a prevalence of 99% through R package Microbiome v1.10.0 (Lahti and Shetty, 2019). A Venn diagram at the genus level was constructed using the full dataset of Ctrl water before, Ctrl water after, and Bulk soil with the package MicEco v0.9.11 (Russel, 2021). Differential abundance of compositional data was conducted using the R package ALDEx2 v1.20.0 (Fernandes et al., 2014). Previously, ASVs with relative abundances lower than 0.25% were filtered out (min. samples = 2, min. reads = 5554, min. abundance = 0) through R package DAtest (Russel et al., 2018). A heat map was plotted at different grouping levels considering effect size values ≥ 1 or ≤ -1 and significant *p*-values ($p < 0.05$) with Benjamini-Hochberg correction of Welch's *t*-test (we.eBH) (Welch, 1947). Alpha and beta diversity analyses were carried out using the R packages Phyloseq v1.32.0 (McMurdie and Holmes, 2013) and Vegan v2.5-6 (Oksanen et al., 2019). For alpha diversity, samples were rarefied, and the diversity indices (Observed ASVs, Shannon-Wiener, and Gini-Simpson) were estimated and plotted. Pairwise comparisons of each alpha diversity index were obtained through the Wilcoxon rank-sum test (Wilcoxon, 1945) with Holm adjustment. For beta diversity analyses, dissimilarity distance matrices based on Aitchison distance of compositional data were created and visualized through Principal Component Analysis (PCA) (Jollife and Cadima, 2016). Significant differences were determined by

permutational multivariate analysis of variance (PERMANOVA) with Hochberg's adjustment and 999 permutations using the Adonis method (Anderson, 2001). Due to the low number of replications (n = 2), “Bulk soil” was not included for multiple comparisons.

All DNA sequences are available in the NCBI/GenBank database repository under the bioproject accession number PRJNA941209.

Results

Evaluation of plant growth promotion and induction of salt stress

The exploratory data analysis of the total number of flowers at the 12th week of treatment is depicted in Fig. 1, when most treatments reached the highest number of flowers bloomed. However, some replications (especially in “DMS BA”) did not bloom at the time of data collection (Fig. S1), but in the following weeks. The bloom heterogeneity, with both low and high numbers of flowers in the same treatment led to high standard deviations that were detected in most of the treatments even after filtering out the outliers (Komsta, 2011) (Fig. 1). The controls “Ctrl BioA” ($\bar{x}$ = 59.25), “Ctrl DMS” ($\bar{x}$ = 69.50), and “BioA BS” ($\bar{x}$ = 56.60) showed the highest number of flowers compared with “Ctrl water” ($\bar{x}$ = 46.33) and the other bacterial strains (filtered and unfiltered). Similarly, the mean values of “DMS BS” ($\bar{x}$ = 48.33), “DMS BS filt” ($\bar{x}$ = 50.20), “DMS PP” ($\bar{x}$ = 55.25), and “DMS BA filt” ($\bar{x}$ = 55.75) were higher than “Ctrl water” but lower than “Ctrl DMS”. Statistical analysis revealed no significant differences among the treatments, neither for the number of flowers (p = 0.66, ANOVA, α=0.05), or dry weight (p = 0.162, Kruskal-Wallis, α=0.05, Fig. S2). As the ANOVA test did not reveal any differences between the filtered and unfiltered treatments, only unfiltered treatments were considered for salt stress induction.

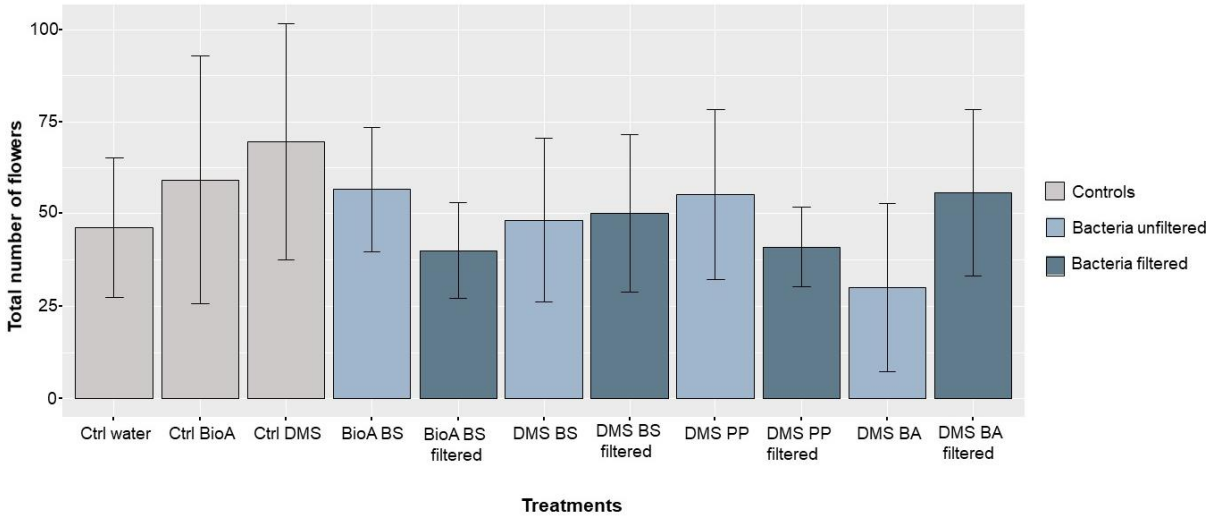


Fig. 1 Bar plot of the total number of flowers at the 12th week of treatment. Non-significant differences were found between the treatments (n = 3-5 ± SD, ANOVA, α = 0.05).

Metagenomic analysis of bacterial microbiota in *Rosa* sp. Rhizosphere

Sequencing analysis performed in QIIME2 revealed 2,221,571 sequences (min: 15,540; max: 59,985 sequences per sample) after demultiplexing and quality control. Taxa bar plots were grouped into treatments before and after salt stress, as well as controls (Ctrl water, Ctrl DMS, and Ctrl Bio A) and bacteria (Bio A BS, DMS BS, DMS PP, and DMS BA), and plotted with the bulk soil (Fig. 2a). At different grouping levels, the 20 most prevalent ASVs (after removing ASVs with relative abundances lower than 0.35%) at the genus level were identified in the bulk soil and in the rhizosphere soil of either the controls or bacteria, before and after salt stress. The 20 most prevalent ASVs belonged to nine genera, as well as to unidentified ASVs in five families, five classes, and one order (Fig. 2a). In addition, the Venn diagram at the genus level between "Bulk soil" and rhizosphere soil of roses, which were only treated with water "Ctrl water" before and after salt stress showed 49% common genera, representing the highest percentage shared between the rhizosphere and the bulk soil (Fig. 2b).

Alpha diversity

Boxplots of species richness using rarefaction with 95% confidence intervals were grouped before and after salt stress to evaluate the effects of salinity (Fig. 3). The indices Observed ASVs, and Shannon-Wiener showed significantly higher richness before salt stress compared with those after salt stress ($p_{\text{Observed ASV}} = 0.0036$, $p_{\text{Shannon-Wiener}} = 0.014$). In contrast, no significant differences were found in the Gini-Simpson index ($p = 0.25$) (Fig. 3a). On the other hand, the highest richness observed in the Ctrls before significantly decreased compared to bacteria before ($p_{Observed\ ASV} = 0.00154$, $p_{\text{Shannon-Wiener}} = 0.00107$, $p_{Gini\text{-}Simpson} = 0.00016$), indicating that richness is affected by bacterial inoculation. Moreover, the significant decrease in bacteria after ($p_{\text{Observed ASV}} = 0.00660$, $p_{\text{Shannon-Wiener}} = 0.00474$, $p_{\text{Gini-Simpson}} = 0.00067$) was influenced more by salt stress than bacteria inoculation (no significant differences between Ctrls after and bacteria after, $p > 0.05$) (Fig. 3b).

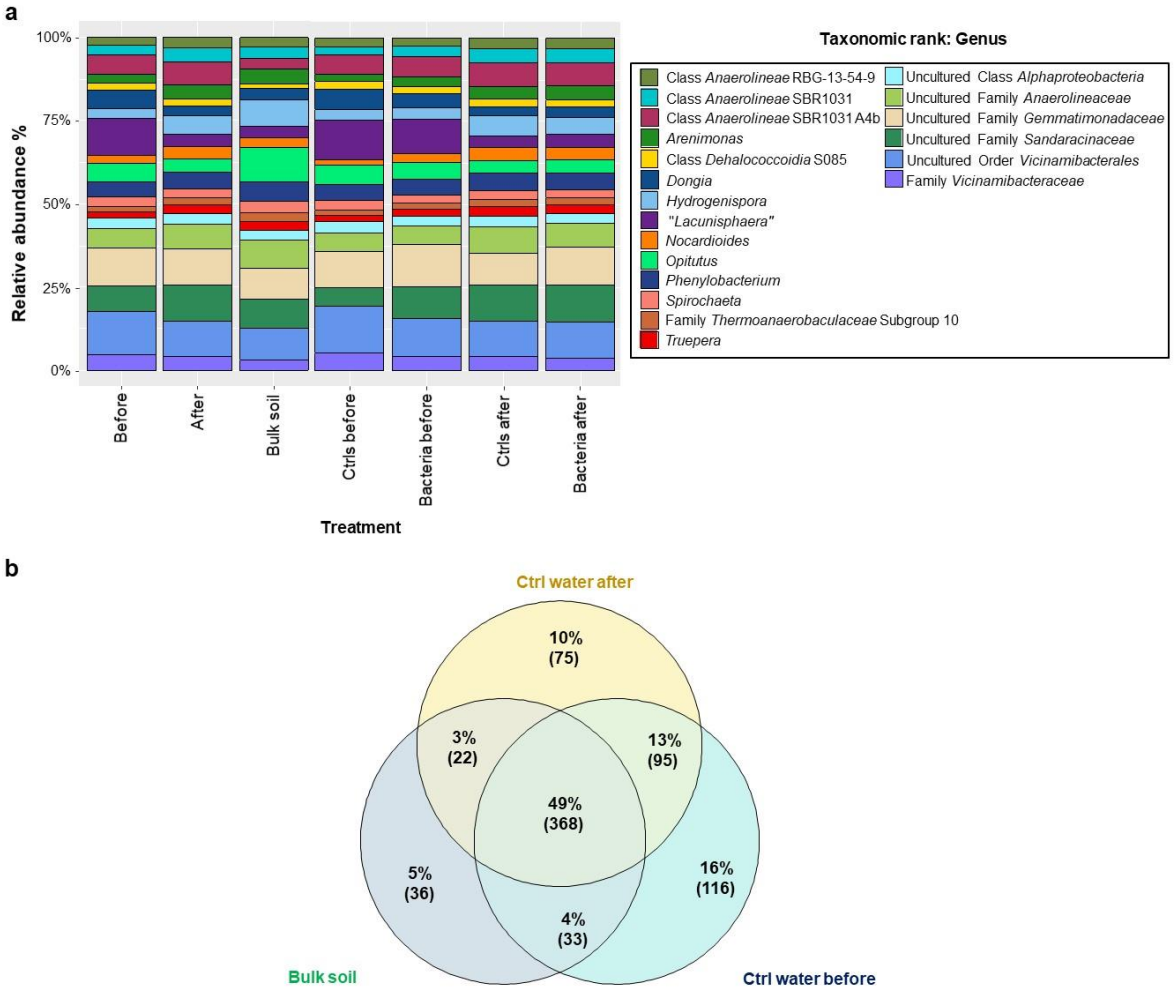


Fig. 2 (a) Bacterial composition of rhizosphere soil of *Rosa* sp. 'Flower Carpet' is shown as taxa bar plots of prevalent ASVs at the genus level (collapsed) **(b)** Venn diagram at the genus level between "Bulk soil" and the rhizosphere soil of roses treated with water "Ctrl water" (Ctrl = Control treatment) before and after salt stress (all treatments without culture media or PGPR inoculation).

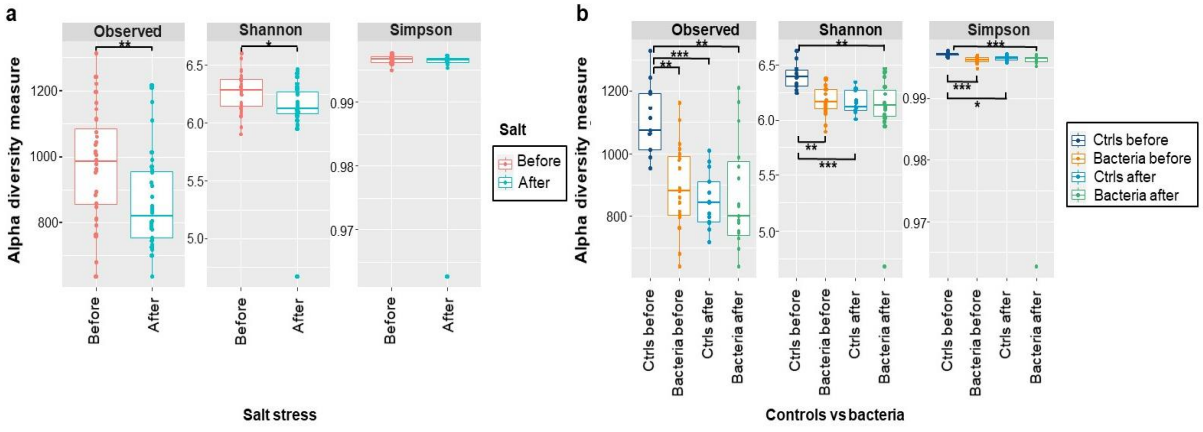


Fig. 3 Box plots of the bacterial alpha diversity indices Observed ASVs, Shannon-Wiener, and Gini-Simpson grouped **(a)** before and after salt stress, and **(b)** between controls and bacterial inoculation before and after salt stress. Normalization was performed using rarefaction. Significance codes: <0.001 '***', <0.01 '**', <0.05, '*'.

Beta diversity

Beta diversity was visualized in R software using principal component analysis (PCA) after "clr" transformation (Fig. 4). A significant shift in the rhizosphere bacterial communities due to salt stress (PERMANOVA with Hochberg's adjustment, p = 0.001) was observed when samples were clustered before and after salt stress, distinguishing two clear groups in the PCA space (Fig. 4a).

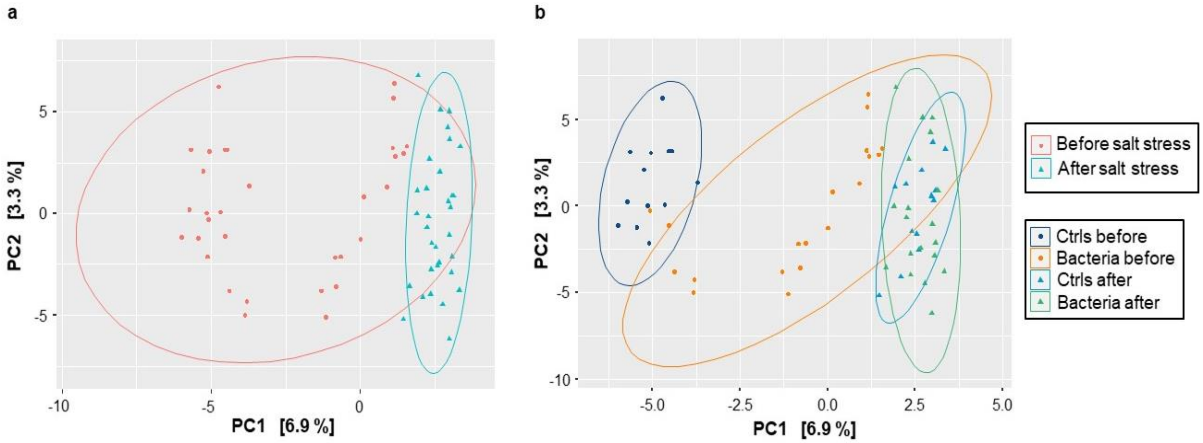


Fig. 4 PCA based on "clr" transformation and Aitchison distance. Treatments grouped **(a)** before and after salt stress and **(b)** control treatments and PGPR (bacteria) before and after salt stress.

Similarly, rhizosphere bacterial communities were significantly altered due to bacterial inoculation, when treatments were grouped into controls and bacteria before and after salt stress (Table 2). Distinct clusters were identified in the PCA space, especially for the controls and bacteria before (Fig. 4b). Remarkably, the rhizosphere bacterial community composition was significantly affected by the inoculation of each individual bacterial strain compared to "Ctrl water before" (Table 3). In addition, salt stress did not significantly change the bacterial community in the treatments inoculated with *B. amyloliquefaciens* "DMS BA" (p = 0.054) and *P. polymyxa* "DMS PP" (p = 0.054) after its application.

Differential abundance (ALDEx2) before and after salt stress

As we detected a shift in the alpha and beta diversity indices caused by either bacterial inoculation or salt stress, we considered ALDEx2 effect size (values ≥ 1 and ≤ -1) to identify a significant differential abundance of taxa at the genus level in order to observe the influence of these two main factors. In total, 42 ASVs (20 could be identified at genus level, 9 at family level, 5 at class level, 3 at order level, and 5 at phylum level) were significantly affected by the arrangement of different treatments (Fig. 5). Salt stress negatively affected six of 14 taxa (effect

Table 2 PERMANOVA pairwise comparisons of beta diversity from rhizosphere bacterial communities based on "clr" transformation between treatments grouped as Controls and Bacteria. *p*-values are shown considering Hochberg's adjustment and 999 permutations.

Treatment	Ctrls before	Bacteria before	Ctrls after
Bacteria before	0.002**	-	-
Ctrls after	0.002**	0.002**	-
Bacteria after	0.002**	0.002**	0.030*

Significance codes: <0.01 '**', <0.05, '*'

Table 3 PERMANOVA pairwise comparisons of beta diversity from rhizosphere bacterial communities based on clr transformation between bacterial strains compared with Ctrl water before and after salt stress. *p*-values are shown considering Hochberg's adjustment and 999 permutations.

Treatment	Ctrl water before	BioA BS before	Ctrl water after	DMS BS before	DMS PP after	DMS BA before
BioA BS before	0.034*	-	-	-	-	-
Ctrl water after	0.034*	0.034*	-	-	-	-
BioA BS after	0.034*	0.034*	0.035*	-	-	-
DMS BS before	0.022*	-	0.022*	-	-	-
DMS BS after	0.022*	-	0.483	0.022*	-	-
DMS PP before	0.026*	-	0.026*	-	0.054	-
DMS PP after	0.026*	-	0.026*	-	-	-
DMS BA before	0.02*	-	0.02*	-	-	0.054
DMS BA after	0.02*	-	0.02*	-	-	-

Significance codes: <0.05, '*'

size ≤ -1, red), including three of the nine most prevalent genera (*Dongia*, *"Lacunisphaera"*, and *Opitutus*) reported above (Fig. 2a). While, salt application positively affected eight taxa (effect size ≥ 1, blue) including the prevalent genus *Hydrogenispora* and the prevalent class *Anaerolineae* SBR1031.

When we compared the effect of bacterial inoculation (all the strains) with the controls before stress, eight taxa showed a significant effect, but only the prevalent genera *Dongia* and *Spirochaeta* were negatively affected by bacterial inoculation (effect size ≤ -1, red Fig. 5). However, when each bacterial strain was individually compared to the controls, taxa were affected at different degrees depending on the strain. The inoculation of DMS PP (*P. polymyxa*) and DMS BA (*B. amyloliquefaciens*) significantly influenced 27 and 32 taxa, respectively. In both cases, five prevalent taxa (*Dongia*, *Spirochaeta*, uncultured *Alphaproteobacteria*, uncultured *Vicinamibacterales*, and *Vicinamibacteraceae*) were negatively influenced (effect size ≤ -1, red), whereas only an ASV of the prevalent uncultured family *Sandaracinaceae* showed a significant positive effect (Fig. 2a). Moreover, the inoculation with DMS BA had a significant effect on five additional prevalent taxa (*Anaerolineae* SBR1031 A4b, *Arenimonas*, *Dehalococcoidia* S085, *Lacunisphaera*, and *Opitutus*). In contrast, DMS PP had only a positive influence on one additional prevalent genus (*Nocardioides*). Additionally, the inoculation with BioA BS (*B. subtilis*) only positively affected the genus *Methylomicrobium*, and negatively affected the genus *Pseudolabrys*. While DMS BS (*B. subtilis*) negatively affected an ASV of the family *Nitrosomonadaceae* MND1, and ASVs of the phyla *Candidatus* Parcubacteria and ″WS2″. Finally, we reported only the taxa with an effect size ≥ 1 and ≤ -1, which showed significant *p*-values ($p > 0.05$) obtained from Welch's *t*-test with Benjamin-Hochberg correction (we.eBH) (Table S1). An additional evaluation of genus-genus co-occurrences before and after salt stress using the R packages SPIEC-EASI v1.1.1 (Kurtz et al., 2015) and the Semi-Parametric Rank-based approach for INference in Graphical model (SPRING) v1.0.4 (Yoon et al., 2019) can be found in Fig. S3.

Discussion

Although the beneficial effects of PGPRs have been extensively reported over the last decades (Glick 1995; Islam et al. 2016), a complete understanding of the complex plant-microbe interactions involved in plant growth promotion is still lacking (Cardinale et al., 2015). In this work, no statistically positive relationship was found between the three bacterial strains and their influence on the number of rose flowers as an indicator of plant growth promotion (Fig. 1, S1) or salt stress tolerance (Fig. S2). Inoculation of PGPR at the seedling stage could be a

fundamental factor that notably changes the rhizosphere microbiota and improves crop yield (Zhang et al., 2019). However, rose plants and many others are not only propagated by seeds but also by cuttings, grafting, or stenting in order to obtain more uniform and high-quality plants (Van De Pol and Breukelaar, 1982). Moreover, characteristic features of 'Flower Carpet' rose variety like its extraordinary resistance against several pathogens (Noack 1990, 1998), and the optimal growth conditions (Fig. S1) could have interfered with the observation and correlation of additional beneficial effects of PGPRs. In fact, many PGPR experiments are carried out under certain stress conditions (e.g. biotic and abiotic stress, nutrient limitation) to determine how PGPRs enhance plant growth or reduce stress (Luo et al., 2019). Therefore, the effects of PGPR under salt stress conditions were also tested (Fig. S2). However, the final salt concentration used in our experiment (4% NaCl, 4 times 600 mM NaCl) caused severe detrimental effects on the 'Flower Carpet' variety (e.g. defoliation of most of the leaves), and was a critical factor in determining whether these strains could mitigate the effect caused by salt (Fig. S2). Lower NaCl concentrations (200 mM) are often used to induce salt stress (Singh and Jha, 2017), but similar salt concentrations have also been tested. Suarez et al. (2015) reached a concentration of 4.4% NaCl, with a significant increase in the root and shoot dry weight of barley plants inoculated with *Hartmannibacter diazotrophicus* E19^{T}. Similarly, Babar et al. (2021) found an increase in plant height and leaf area of wheat plants treated with two halo-tolerant bacteria (SBN01 and SBNO2) and 600 mM NaCl.

Metabarcoding characterization of the rhizosphere bacterial microbiome of 'Flower Carpet' rose variety revealed a high diversity in all the treatments, including bulk soil (Fig. 2). The Venn diagram showed that the highest percentage of common genera (49%) was shared between bulk and rhizosphere soil (Fig. 2b). These results are in agreement with previous studies, which indicated that the rhizosphere microbial community is appreciably influenced by bulk soil (De Ridder-Duine et al., 2005; Zhao et al., 2019), as well as by plant roots (Smalla et al., 2001) or stress conditions such as pH-value or salinity (Lozupone and Knight, 2007).

Acidobacteriota (*Thermoanaerobaculaceae*, *Vicinamibacteraceae*, *Vicinamibacterales*), *Actinomycetota* (*Nocardioides*), *Chloroflexota* (*Anaerolineaceae*, *Anaerolineae*, *Dehalococcoidia)*, *Bacillota* (*Hydrogenispora*), *Gemmatimonadota* (*Gemmatimonadaceae*), *Myxococcota* (*Sandaracinaceae*), *Pseudomonadota* (*Arenimonas*, *Dongia*, *Alphaproteobacteria*, *Phenylobacterium*), *Spirochaetota* (*Spirochaeta*), *Deinococcota* (*Truepera*), and *Verrucomicrobiota* (*Opitutus*, *"Lacunisphaera"*), were the ten phyla that

encompassed the 20 most prevalent taxa at the genus level identified in the rhizosphere of 'Flower Carpet' rose variety.

Fig. 5 Differential abundance between treatments at different grouping levels after filtering out ASVs with relative abundances lower than 0.25%. Heat map was built using the effect size obtained after ALDEx2 analysis, considering values ≥ 1 or ≤ -1. Positive effect size values (blue) show taxa positively affected by either salinity or bacterial inoculation, while negative effect size values (red) show taxa negatively affected by these two factors. Prevalent taxa are highlighted in red.

In accordance with our findings, the phyla *Acidobacteriota*, *Actinomycetota*, *Pseudomonadota*, and *Verrucomicrobiota* have also been reported as part of the main bacterial communities in the rhizosphere soil of *Rosa rugosa* (Yuan et al., 2022) and *Rosa corymbifera* (Yim et al., 2020). However, the phyla *Chloroflexota*, and *Bacillota* were found only in *R. corymbifera*, while *Gemmatimonadota* was found only in *R. rugosa*, suggesting variations in the bacterial community structure among different rose species.

A significantly higher number of observed ASVs before salt stress as well as the Shannon-Wiener index, but not in the *Gini-Simpson* index, indicates a general trend of decreasing bacterial richness after salt application (Fig. 3a). However, salt stress may also increase the community richness, leading to the emergence of opportunistic pathogens due to shifts in the bacterial communities (Yaish et al. 2016). On the other hand, a significant reduction in bacterial treatments in all alpha diversity indices was observed only before salt stress (Fig. 3b). This contrasts to the results obtained by Zhang et al. (2019), who found that inoculation of pepper (*Capsicum annuum*) with *B. velezensis* significantly increased the rhizosphere bacterial richness (Chao1 and Shannon-Wiener indices) at a mature stage.

Beta diversity analysis showed significant differences between the treatments grouped before and after salt stress (Fig. 4), suggesting that salinity altered the rhizosphere bacterial community composition after its application. Similar findings were reported by Mukhtar et al. (2018), who analyzed rhizospheric soil from various saline environments and concluded that the significant differences found in alpha and beta diversity analyses are correlated with the increase in soil salinity. The most remarkable result from the PERMANOVA analysis was the statistically significant differences observed between bacterial inoculation, either individually (Table 2) or grouped (Table 3), when compared to "Ctrl water" or "Ctrls" before and after salt stress. These results indicate that inoculation with *B. subtilis*, *P. polymyxa* and *B. amyloliquefaciens* may be correlated with a shift in the community composition of the rhizosphere soil of 'Flower Carpet' rose variety. The effects of PGPR inoculation on rhizosphere community composition remain underexplored. Recent studies using 16S rRNA gene sequence have shown how PGPRs can influence and alter soil bacterial communities (Ju et al., 2019; Zhang et al., 2019). The impact of salinity on microbial communities is well documented, which is not the case for PGPR inoculation and its impact on soil microbial communities, and even less in non-crop plants such as *Rosa* sp.

ALDEx2 analysis between the control treatments and bacterial strains before salt stress showed that only the prevalent genera *Dongia* and *Spirochaeta* were significantly affected.

Nevertheless, in a closer comparison, ASV abundances were affected at different levels by each individual bacterial strain. DMS BA and DMS PP significantly affected 16 and seven prevalent ASVs, respectively (Fig. 5), showing a considerable impact of bacterial inoculation on bacterial communities. In contrast, no prevalent ASVs were affected by inoculation with BioA BS and DMS BS (Fig. 6). Although it has been reported that exogenous inoculation of *Bacillus* sp. is able to modulate the bacterial community structure (Daraz et al., 2021; Shi et al., 2022), Xie et al. (2022) observed more significant changes in soil bacterial diversity after *B. subtilis* application than with the inoculation of *B. amyloliquefaciens* in ginger plants.

Conclusion

The rhizosphere bacterial microbiome of *Rosa* sp. consists of ASVs identified in the rhizosphere of many plants. However, very typical for the rose rhizosphere were *Acidobacteriota, Actinomycetota, Chloroflexota, Bacillota, Gemmatimonadota, Myxococcota, Pseudomonadota, Spirochaetota, Deinococcota* and *Verrucomicrobiota,* which encompassed the most prevalent genera of the phyla identified in this study. Furthermore, high salinity was not only able to cause significant modifications in the abundance but also to alter the co-occurrences established between genera of several prevalent taxa, showing the effect of salt on the microbial communities. Although a clear effect of PGPRs on plant growth promotion and salt stress alleviation could not be observed, alpha and beta diversity indices indicated that species richness and bacterial community composition present in the rhizosphere of 'Flower Carpet' are influenced by salt. Finally, exogenous PGPR inoculation was able to alter the bacterial community composition of this rose variety.

Acknowledgments We are grateful to Birgit Lewandowski and Fritzmeier Umwelttechnik for all the help and cooperation. We also warmly acknowledge to Bellinda Schneider, Rita Geissler-Plaum for their technical support, and Julian Rojas for his help during the application of salt stress. We also acknowledge Dietrich Steffens for his support at the experimental station of the university.

Author contribution SQ and SS designed the experimental setup. SQ carried out experiments, data analysis, and writing. DR and SR contributed with data analysis, review, and editing. DK, SR and SS revised the manuscript and approved it for publication. All the authors read and approved the manuscript.

Funding This work was partially funded by Fritzmeier Umwelttechnik GmbH & Co. KG.

Data availability All DNA sequences are available on the NCBI/GenBank database repository under the accession number PRJNA941209.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

Financial interests The authors have no relevant financial or non-financial interests to disclose.

References

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46. http://dx.doi.org/10.1046/j.1442-9993.2001.01070.x

Araujo NAF, Brandão RM, Barguil BM, Cardoso M das G, Pasqual M, Rezende R, Pereira MMA, Buttrós VHT, Dória J (2020) Plant growth-promoting bacteria improve growth and modify essential oil in rose (*Rosa hybrida* L.) cv. Black Prince. Front Sustain Food Syst 4:1–9. https://doi.org/10.3389/fsufs.2020.606827

Babar M, Saif-ur-Rehman, Rasul S, Aslam K, Abbas R, Athar H ur R, Manzoor I, Kashif Hanif M, Naqqash T (2021) Mining of halo-tolerant plant growth promoting rhizobacteria and their impact on wheat (*Triticum aestivum* L.) under saline conditions. J King Saud Univ - Sci 33:101372. https://doi.org/10.1016/j.jksus.2021.101372

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:1–17. https://doi.org/10.1186/s40168-018-0470-z

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857. https://doi.org/10.1038/s41587-019-0209-9

Cabrera RI, Perdomo P (2003) Reassessing the salinity tolerance of greenhouse roses under soilless production conditions. HortScience 38:533–536. https://doi.org/10.21273/hortsci.38.4.533

Cabrera RI, Solís-Pérez AR, Sloan JJ (2009) Greenhouse rose yield and ion accumulation responses to salt stress as modulated by rootstock selection. HortScience 44:2000–2008. https://doi.org/10.21273/hortsci.44.7.2000

Cai X, Niu G, Starman T, Hall C (2014) Response of six garden roses (*Rosa×hybrida* L.) to salt stress. Sci Hortic (Amsterdam) 168:27–32. https://doi.org/10.1016/j.scienta.2013.12.032

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869

Cardinale M, Ratering S, Suarez C, Zapata Montoya AM, Geissler-Plaum R, Schnell S (2015) Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress. Microbiol Res 181:22–32. https://doi.org/10.1016/j.micres.2015.08.002

Cekstere G, Nikodemus O, Osvalde A (2008) Toxic impact of the de-icing material to street greenery in Riga, Latvia. Urban For Urban Green 7:207–217. https://doi.org/10.1016/j.ufug.2008.02.004

Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O, Junge H, Voigt B, Jungblut PR, Vater J, Süssmuth R, Liesegang H, Strittmatter A, Gottschalk G, Borriss R (2007) Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol 25:1007–1014. https://doi.org/10.1038/nbt1325

Claesson MJ, O'Sullivan O, Wang Q, Nikkilä J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4. https://doi.org/10.1371/journal.pone.0006669

Daraz U, Li Y, Sun Q, Zhang M, Ahmad I (2021) Inoculation of *Bacillus* spp. modulate the soil bacterial communities and available nutrients in the rhizosphere of vetiver plant irrigated with acid mine drainage. Chemosphere 263:128345. https://doi.org/10.1016/j.chemosphere.2020.128345

De Ridder-Duine AS, Kowalchuk GA, Klein Gunnewiek PJA, Smant W, Van Veen JA, De Boer W (2005) Rhizosphere bacterial community composition in natural stands of *Carex arenaria* (sand sedge) is determined by bulk soil community composition. Soil Biol Biochem 37:349–357. https://doi.org/10.1016/j.soilbio.2004.08.005

Engelbrektson A, Kunin V, Wrighton KC, Zvenigorodsky N, Chen F, Ochman H, Hugenholtz P (2010) Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J 4:642–647. https://doi.org/10.1038/ismej.2009.153

Fernandes, D. A, Reid, J., Macklaim, M. J, McMurrough, T.A, Edgell, D.R., Gloor, B. G (2014) Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome 2:1–13. https://doi.org/10.1186/2049-2618-2-15

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117. https://doi.org/10.1139/m95-015

Gudin S (2000) Rose: genetics and breeding. Plant breeding reviews. John Wiley & Sons Inc. 159-189

Islam MT, Rahman M, Pandey P, Jha CK, Aeron A (2016) Bacilli and Agrobiotechnology. Springer

Jollife IT, Cadima J (2016) Principal component analysis: a review and recent developments. Philos Trans R Soc A Math Phys Eng Sci 374. https://doi.org/10.1098/rsta.2015.0202

Ju W, Liu L, Fang L, Cui Y, Duan C, Wu H (2019) Impact of co-inoculation with plant-growth-promoting rhizobacteria and *Rhizobium* on the biochemical responses of alfalfa-soil system in copper contaminated soil. Ecotoxicol Environ Saf 167:218–226. https://doi.org/10.1016/j.ecoenv.2018.10.016

Kaplan H, Ratering S, Felix-Henningsen P, Schnell S (2019) Stability of in situ immobilization of trace metals with different amendments revealed by microbial ^{13}C-labelled wheat root decomposition and efflux-mediated metal resistance of soil bacteria. Sci Total Environ 659:1082–1089. https://doi.org/10.1016/j.scitotenv.2018.12.441

Karthikeyan M, Bhaskaran R, Mathiyazhagan S, Velazhahan R (2007) Influence of phylloplane colonizing biocontrol agents on the black spot of rose caused by *Diplocarpon rosae*. J Plant Interact 2:225–231. https://doi.org/10.1080/17429140701701071

Komsta L (2011) outliers: tests for outliers. R package version 0.14. https://cran.r-project.org/package=outliers

Kurtz ZD, Müller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA (2015) Sparse and compositionally robust inference of microbial ecological networks. PLoS Comput Biol

11:1–25. https://doi.org/10.1371/journal.pcbi.1004226
Lahti, L., Shetty S (2019) Microbiome R package. Available online at: http://microbiome.github.io (accessed November 10, 2020).
Lee B, Farag MA, Park HB, Kloepper JW, Lee SH, Ryu CM (2012) Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa*. PLoS One 7:1–11. https://doi.org/10.1371/journal.pone.0048744
Liu S, Hao H, Lu X, Zhao X, Wang Y, Zhang Y, Xie Z, Wang R (2017) Transcriptome profiling of genes involved in induced systemic salt tolerance conferred by *Bacillus amyloliquefaciens* FZB42 in *Arabidopsis thaliana*. Sci Rep 7:1–13. https://doi.org/10.1038/s41598-017-11308-8
Lozupone CA, Knight R (2007) Global patterns in bacterial diversity. Proc Natl Acad Sci U S A 104:11436–11440. https://doi.org/10.1073/pnas.0611525104
Luo Y, Cheng Y, Yi J, Zhang Z, Luo Q, Zhang D, Li Y (2018) Complete genome sequence of industrial biocontrol strain *Paenibacillus polymyxa* HY96-2 and further analysis of its biocontrol mechanism. Front Microbiol 9:1–14. https://doi.org/10.3389/fmicb.2018.01520
Luo Y, Wang F, Huang Y, Zhou M, Gao J, Yan T, Sheng H, An L (2019) *Sphingomonas* sp. Cra20 increases plant growth rate and alters rhizosphere microbial community structure of *Arabidopsis thaliana* under drought stress. Front Microbiol 10. https://doi.org/10.3389/fmicb.2019.01221
Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17:10. https://doi.org/10.14806/ej.17.1.200
McMurdie PJ, Holmes S (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome Census Data. PLoS One 8. https://doi.org/10.1371/journal.pone.0061217
Mohamed HI, Gomaa EZ (2012) Effect of plant growth promoting *Bacillus subtilis* and *Pseudomonas fluorescens* on growth and pigment composition of radish plants (*Raphanus sativus*) under NaCl stress. Photosynthetica 50:263–272. https://doi.org/10.1007/s11099-012-0032-8
Mukhtar S, Mirza BS, Mehnaz S, Mirza MS, Mclean J, Malik KA (2018) Impact of soil salinity on the microbial structure of halophyte rhizosphere microbiome. World J Microbiol Biotechnol 34:1–17. https://doi.org/10.1007/s11274-018-2509-5
Nirit B, Asher BT, Haya F, Pini S, Ilona R, Amram C, Marina I (2006) Application of treated wastewater for cultivation of roses (*Rosa hybrida*) in soil-less culture. Sci Hortic (Amsterdam) 108:185–193. https://doi.org/10.1016/j.scienta.2006.02.001
Niu G, Sun Y (2019) Salt tolerance in roses – a review. Acta Hortic 1232:143–150. https://doi.org/doi:10.17660/ActaHortic.2019.1232.22
Noack W (1990) U.S. Patent Application No. 07/346,393
Noack W (1998) U.S. Patent Application No. 08/635,177
Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, Minchin PR, Hara RBO, Simpson GL, Solymos P, Stevens MHH, Szoecs E (2019) Vegan: community ecology package. R package version 2.5-6. Vienna: R Foundation for Statistical Computing.
Pedregosa F, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Dubourg V, Pedregosa F, Gramfort A, Michel V, Thirion B, Pedregosa F, Weiss R (2011) Scikit-learn : machine learning in python. J Mach Learn Res. 12:2825-2830
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596. https://doi.org/10.1093/nar/gks1219
R Core Team (2023) R: a language and environment for statistical computing. R Foundation

for Statistical Computing, Vienna, Austria. URL http://www.R-project.org
Russel J (2021) Russel88/MicEco: v0.9.15 (v0.9.15). Zenodo.
Russel J, Thorsen J, Brejnrod A, Bisgaard H, Sorensen S, Burmolle M (2018) DAtest: a framework for choosing differential abundance or expression method. bioRxiv. https://doi.org/10.1101/241802
Ryu C-M, Farag MA, Hu C-H, Reddy MS, Wei H-X, Pare PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci 100:4927–4932. https://doi.org/10.1073/pnas.0730845100
Shi JW, Lu LX, Shi HM, Ye JR (2022) Effects of plant growth-promoting rhizobacteria on the growth and soil microbial community of *Carya illinoinensis*. Curr Microbiol 79:1–12. https://doi.org/10.1007/s00284-022-03027-9
Singh RP, Jha PN (2017) The PGPR *Stenotrophomonas maltophilia* SBP-9 augments resistance against biotic and abiotic stress in wheat plants. Front Microbiol 8. https://doi.org/10.3389/fmicb.2017.01945
Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microbiol 67:4742–4751. https://doi.org/10.1128/AEM.67.10.4742-4751.2001
Smulders MJM, Arens P, Koning-Boucoiran CFS, Gitonga VW, Krens FA, Atanassov A, Atanassov I, Rusanov KE, Bendahmane M, Dubois A, Raymond O, Caissard JC, Baudino S, Crespel L, Gudin S, Ricci SC, Kovatcheva N, Van Huylenbroeck J, Leus L, Wissemann V, Zimmermann H, Hensen I, Werlemark G, Nybom H (2011) *Rosa* in: wild crop relatives: genomic and breeding resources. Springer, Berlin, pp 243–275
Suarez C, Cardinale M, Ratering S, Steffens D, Jung S, Montoya AMZ, Geissler-Plaum R, Schnell S (2015) Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress. Appl Soil Ecol 95:23–30. https://doi.org/10.1016/j.apsoil.2015.04.017
Tariq U, Riaz A, Jaskani MJ, Zahir ZA (2016) Screening of PGPR isolates for plant growth promotion of *Rosa damascena*. Int J Agric Biol 18:997–1003. https://doi.org/10.17957/IJAB/15.0200
Van De Pol PA, Breukelaar A (1982) Stenting of roses; a method for quick propagation by simultaneously cutting and grafting. Sci Hortic (Amsterdam) 17:187–196. https://doi.org/10.1016/0304-4238(82)90012-7
Van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483. https://doi.org/10.1146/annurev.phyto.36.1.453
Welch ABL (1947) The generalization of "Student's" problem when several different population variances are involved. Biometrika 34:28-35
Wilcoxon F (1945) Individual comparisons by ranking methods author (s): Frank Wilcoxon published by : International Biometric Society. Biometrics Bull 1:80–83
Xie K, Sun M, Shi A, Di Q, Chen R, Jin D, Li Y, Yu X, Chen S, He C (2022) The Application of tomato plant residue compost and plant growth-promoting rhizobacteria improves soil quality and enhances the ginger field soil bacterial community. Agronomy 12. https://doi.org/10.3390/agronomy12081741
Yaish MW, Al-Lawati A, Jana GA, Patankar HV, Glick BR (2016) Impact of soil salinity on the structure of the bacterial endophytic community identified from the roots of caliph medic (*Medicago truncatula*). PLoS One 11:1–17. https://doi.org/10.1371/journal.pone.0159007
Yasuda M, Dastogeer KMG, Sarkodee-Addo E, Tokiwa C, Isawa T, Shinozaki S, Okazaki S (2022) Impact of *Azospirillum* sp. B510 on the rhizosphere microbiome of rice under field conditions. Agronomy 12:1–14. https://doi.org/10.3390/agronomy12061367

Yim B, Baumann A, Grunewaldt-Stöcker G, Liu B, Beerhues L, Zühlke S, Sapp M, Nesme J, Sørensen SJ, Smalla K, Winkelmann T (2020) Rhizosphere microbial communities associated to rose replant disease: links to plant growth and root metabolites. Hortic Res 7. https://doi.org/10.1038/s41438-020-00365-2

Yoon G, Gaynanova I, Müller CL (2019) Microbial networks in SPRING - Semi-parametric rank-based correlation and partial correlation estimation for quantitative microbiome data. Front Genet 10. https://doi.org/10.3389/fgene.2019.00516

Yuan YH, Liu LX, Wang L, Dong GZ, Liu YG (2022) Effects of different seasons on bacterial community structure in rose rhizosphere soil. Appl Microbiol Biotechnol. https://doi.org/10.1007/s00253-022-12290-6

Zaman M, Shahid SA., Heng L (2018) Guideline for salinity assessment, mitigation and adaptation using nuclear and related techniques. Springer Nature

Zhang Y, Gao X, Shen Z, Zhu C, Jiao Z, Li R, Shen Q (2019) Pre-colonization of PGPR triggers rhizosphere microbiota succession associated with crop yield enhancement. Plant Soil 439:553–567. https://doi.org/10.1007/s11104-019-04055-4

Zhao M, Yuan J, Shen Z, Dong M, Liu H, Wen T, Li R, Shen Q (2019) Predominance of soil vs root effect in rhizosphere microbiota reassembly. FEMS Microbiol Ecol 95:1–11. https://doi.org/10.1093/femsec/fiz139

Supplementary Material

Absence of aboveground effects may conceal changes underground: Plant-growth promoting rhizobacteria and salt stress altered the rhizosphere microbiome of roses

Santiago Quiroga, Stefan Ratering, David Rosado-Porto, David Kostner, and Sylvia Schnell

Table S1 Effect size and p-values for the Welch's t-test with Benjamini-Hochberg correction (we.eBH) obtained from ALDEX2 analysis at different grouping levels after filtering out ASVs less than 0.25%. Only effect size values ≥ 1 or ≤ -1 and significant *p*-values < 0.05 were considered.

Taxa	Effect	we.eBH
	Salt before vs salt after	
Anaerolineae SBR1031	1.1	1.17×10^{-7}
Bryobacteraceae	-1.0	1.03×10^{-6}
Dokdonella	2.0	1.12×10^{-14}
Dongia	-1.4	4.72×10^{-10}
Fimbriimonadaceae	-1.2	1.19×10^{-8}
Hydrogenispora	1.1	1.36×10^{-7}
"Lacunisphaera"	-1.4	3.03×10^{-10}
Limnobacter	1.1	1.02×10^{-7}
Luteimonas	1.2	1.71×10^{-8}
Opitutus	-1.0	6.38×10^{-7}
Pseudolabrys	1.2	8.85×10^{-8}
Pseudoxanthomonas	1.5	1.12×10^{-10}
Reyranella	1.2	1.08×10^{-8}
Treponema	-1.7	6.91×10^{-9}
	Ctrls before vs DMS PP	
Anaerolineae SBR1031	1.2	4.30×10^{-2}
Bradyrhizobium	-1.9	2.60×10^{-3}
Bryobacteraceae	-2.7	2.96×10^{-3}
Burkholderiales TRA3-20	1.8	2.92×10^{-3}
Candidatus Kaiserbacteria	2.4	1.25×10^{-4}
Candidatus Saccharimonadales	4.1	9.54×10^{-7}
Candidatus Sumerlaea	-1.7	2.63×10^{-2}
Dokdonella	1.7	3.42×10^{-3}
Dongia	-2.5	2.34×10^{-3}
Limnobacter	1.3	1.27×10^{-2}
Nitrosomonadaceae Ellin6067	2.8	8.41×10^{-5}
Nitrosomonadaceae MND1	-2.3	2.05×10^{-2}
Nocardioides	1.4	1.43×10^{-3}
Pseudolabrys	1.7	2.78×10^{-2}
Pseudoxanthomonas	1.7	2.40×10^{-2}
Reyranella	2.2	1.85×10^{-4}

Spirochaeta	-1.5	4.33 x 10^{-2}
Thermomonas	2.0	4.82 x 10^{-3}
Thiobacillus	2.2	1.22 x 10^{-3}
Treponema	-2.8	3.34 x 10^{-4}
Uncultured *Alphaproteobacteria*	-1.8	2.61 x 10^{-2}
Uncultured *Reyranellaceae*	-1.9	7.89 x 10^{-3}
Uncultured *Rhodothermaceae*	4.1	4.25 x 10^{-7}
Uncultured *Sandaracinaceae*	2.2	8.94 x 10^{-4}
Uncultured *Vicinamibacterales*	-2.3	1.77 x 10^{-3}
"Vermiphilaceae"	1.8	1.42 x 10^{-2}
Vicinamibacteraceae	-2.7	8.36 x 10^{-4}
	Ctrls before vs DMS BA	
Anaerolineae SBR1031 A4b	-1.2	2.16 x 10^{-2}
Arenimonas	2.2	8.10 x 10^{-4}
Bradyrhizobium	-3.6	1.97 x 10^{-4}
Bryobacteraceae	-3.2	9.15 x 10^{-3}
Candidatus Parcubacteria	-1.3	1.61 x 10^{-2}
Candidatus Saccharimonadales	2.1	2.98 x 10^{-2}
Dehalococcoidia S085	-1.1	4.02 x 10^{-2}
Desulfuromonadia PB19	-1.6	1.68 x 10^{-2}
Dongia	-3.6	1.15 x 10^{-3}
Fimbriimonadaceae	-1.8	1.88 x 10^{-2}
Gemmatimonadota S0134	1.7	2.49 x 10^{-3}
"Lacunisphaera"	-2.6	9.19 x 10^{-5}
Limnobacter	2.0	5.15 x 10^{-4}
Luteitalea	-1.9	2.41 x 10^{-3}
Methylomicrobium	1.8	3.55 x 10^{-4}
Nitrosomonadaceae Ellin6067	2.3	4.36 x 10^{-3}
Nitrosomonadaceae MND1	-1.6	4.44 x 10^{-2}
Opitutus	-1.9	5.60 x 10^{-3}
Pseudoxanthomonas	1.7	1.76 x 10^{-2}
Reyranella	1.7	8.13 x 10^{-4}
Spirochaeta	-1.6	1.77 x 10^{-2}
Thermomonas	2.0	9.57 x 10^{-4}
Thiobacillus	2.6	8.60 x 10^{-5}
Treponema	-3.5	4.08 x 10^{-4}
Uncultured *Alphaproteobacteria*	-2.3	6.97 x 10^{-3}
Uncultured *"Desulfobacterota"*	-5.5	9.52 x 10^{-4}
Uncultured *Reyranellaceae*	-2.4	1.94 x 10^{-3}
Uncultured *Rhodothermaceae*	2.4	5.51 x 10^{-3}
Uncultured *Sandaracinaceae*	2.0	3.02 x 10^{-3}
Uncultured *Vicinamibacterales*	-4.3	3.74 x 10^{-5}
"Vermiphilaceae"	1.4	2.68 x 10^{-2}
Vicinamibacteraceae	-6.0	3.17 x 10^{-5}
	Ctrls before vs BIOA BS	
Methylomicrobium	2.1	2.31 x 10^{-2}
Pseudolabrys	-2.0	2.24 x 10^{-2}
	Ctrls before vs DMS BS	
Candidatus Parcubacteria	-1.5	4.28 x 10^{-2}

Nitrosomonadaceae MND1	-2.0	1.51×10^{-2}
″WS2″	-1.4	4.43×10^{-2}
	Ctrls before vs bacteria before	
Bradyrhizobium	-1.4	1.03×10^{-4}
Bryobacteraceae	-1.2	6.66×10^{-4}
Dongia	-1.1	8.13×10^{-4}
Nitrosomonadaceae Ellin6067	1.2	1.21×10^{-3}
Nitrosomonadaceae MND1	-1.1	5.32×10^{-4}
Spirochaeta	-1.0	2.68×10^{-3}
Uncultured *″Desulfobacterota″*	-1.2	1.05×10^{-2}
Uncultured *Rhodothermaceae*	1.6	2.92×10^{-5}

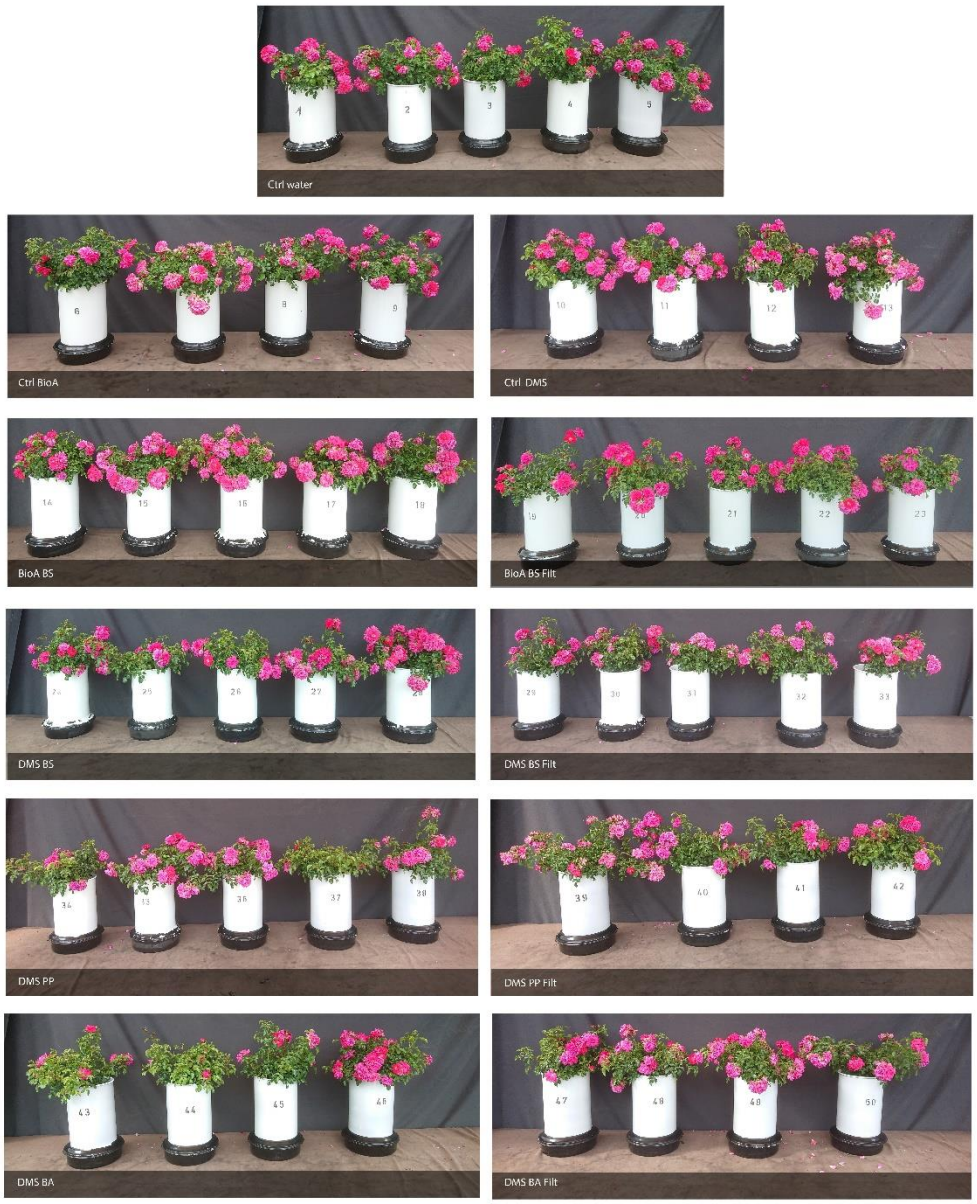


Fig. S1 Front view of rose replicates at the 12th week of treatment. Control treatments without PGPR are shown on the top. PGPR strains grown in BioA or DMS media are shown on the left, whereas their corresponding filtered treatments are shown on the right.

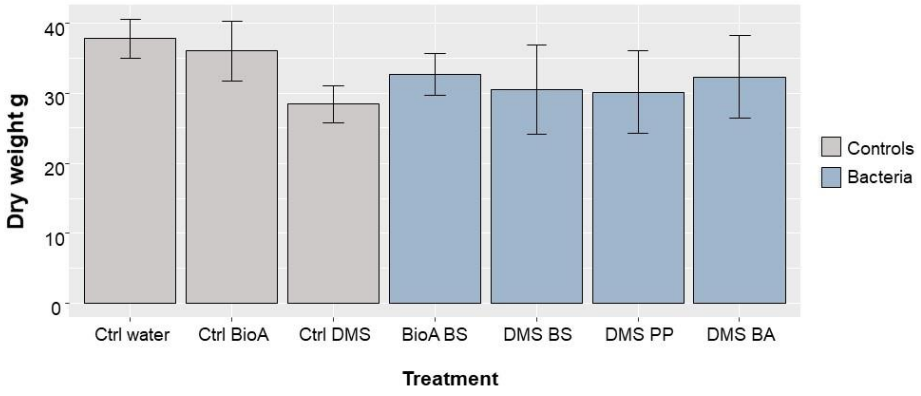


Fig. S2 Dry weight of leaves and stems after salt stress. Non-significant differences were found between treatments (n= 4-5 ± SD, ANOVA, α = 0.05).

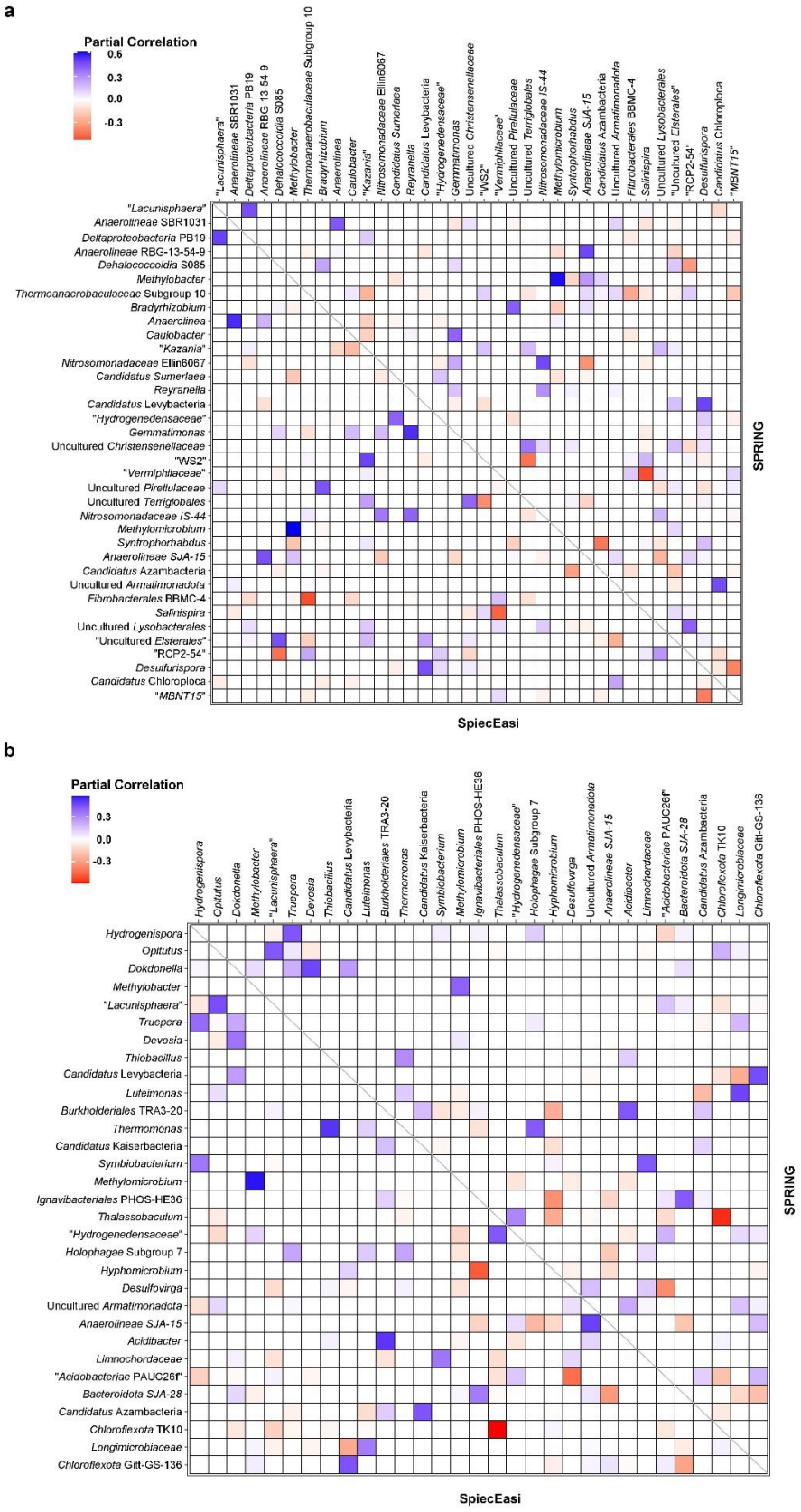


Fig. S3 Genus-genus co-occurrences using SpiecEasi (lower triangular section) and SPRING (upper triangular section), **(a)** Co-occurrences before salt stress and, **(b)** Co-occurrences after salt stress considering genera with at least one strong co-occurrence $\geq |0.4.|$

Chapter VI

General Discussion

With increased awareness about reducing chemical fertilizers and pesticides, the use of bioinoculants has gained relevance over the past few decades. Current efforts are not only shedding light on the monitoring of these inoculants in natural environments but also unveiling the impacts of their incorporation into the soil (Fu et al., 2017; Urrea-Valencia et al., 2021; Xu et al., 2018; Zhang et al., 2020). Addressing these gaps is critical both for developing more efficient inoculants and for understanding their interactions with native soil microbial communities and their long-lasting environmental repercussions. However, several factors influence the success of a bioinoculant, including the selection of potential microbial candidates, the initial microbial concentration, biotic and abiotic stresses, the ability to compete and survive in an external environment, and the plant/location specificity required for the desire inoculant (Björklöf et al., 2003; Valente et al., 2020; Van Elsas et al., 2012). Cardinale et al. (2015) questioned the effectiveness of current pure culture screening methods for identifying the best PGPR candidates. Interestingly, isolates with several plant growth promoting traits, such as *Pseudomonas brassicacearum* and *Microbacterium natoriense*, did not enhance plant growth in a pot experiment, with the exception of *H. diazotrophicus*, which was selected between the three most potential candidates. Instead, an isolate with fewer PGPR activities, like *Curtobacterium flaccumfaciens*, significantly increased barley growth under salt stress. The authors suggested re-evaluating these methods in order to avoid missing potential PGPR candidates.

Although greenhouse experiments under controlled conditions are fundamental for scaling up to agronomic applications, several established protocols need to be re-formulated and adapted for this scale (For review: O'Callaghan et al., 2022; Santos et al., 2019). Suarez et al. (2015) used disinfected barley seeds (less than 1000 seeds, ~ 0.050 kg) and incubated them for one hour in a suspension of *H. diazotrophicus*. Nevertheless, inoculating several kilograms of seeds using this methodology would be time consuming and resource intensive. Therefore, the seed coating technique suggested by Kloepper (1981) was modified by reducing the gum Arabic concentration from 50% to 25%, and adjusting the amount of talc from five to 2.2 times the volume of the gum-inoculum. Additionally, pH-value was adjusted to neutral to minimize stress on *H. diazotrophicus*, considering that autoclaved gum Arabic at 25% has a pH-value of 4.5, while the pH-value of the *H. diazotrophicus* culture medium was 7.73. Some publications include pH-value adjustment as part of their formulations (Ramamoorthy et al., 2002; Zhang et al., 2022). In contrast, in case of plants such as roses (Chapter V), which aim uniform high-quality plants, seed coating cannot be considered as they are usually propagated by cuttings, stenting, or grafting (Van De Pol and Breukelaar, 1982). Instead, soil drenching method was

chosen. Significant effects between inoculation methods such as seed coating, seed soaking, and soil drenching have been reported (Mangmang et al., 2015; Moussa et al., 2013) and could probably influence plant growth promoting abilities of the three different bacterial strains used for roses (Chapter V).

Although qPCR confirmed successful root colonization of *H. diazotrophicus* under both greenhouse and field conditions (Quiroga et al., 2024), further research is needed to scale the seed coating technique for industrial use. To guarantee proper root colonization, multiple applications as in the case of roses (during 14 weeks at concentrations between 10^6 and 10^8 CFU ml^{-1}, Chapter V) or a high initial inoculum concentration is typically suggested (10^8- 10^9 CFU ml^{-1}, $E19^T$ = 1.78 x 10^8 CFU g^{-1} powder), which eventually will increase production and application costs (Gholami et al., 2009; Martínez-Viveros et al., 2010; Moussa et al., 2013).

However, ensuring a sufficient threshold bacterial concentration might be sufficient to obtain similar results (Bankhead et al., 2004; Renoud et al., 2022; Stoll et al., 2021). Bankhead et al. (2004) demonstrated that a concentration of 10^4 CFU g^{-1} soil of different *Pseudomonas fluorescens* strains was sufficient to colonize wheat roots, indicating the high rhizosphere competence of the strains. Renoud et al. (2022) showed that low concentrations of *Azospirillum lipoferum* (10^{4-5} cells $seed^{-1}$) were still able to promote maize growth at different levels compared to the standard inoculation (10^{5-6} cells $seed^{-1}$). Interestingly, Stoll et al. (2021) reported positive results using an adjusted concentration of *Bacillus velezensis* (reduced from ~10^9 CFU ml^{-1} to ~10^7 CFU ml^{-1}), highlighting the effectiveness of inoculation during early crop stages. Initially, it was hypothesized that *H. diazotrophicus* might not require a high concentration to ensure root colonization and persistence. Al Ibrahim (2023) demonstrated that *H. diazotrophicus* was able to survive at a high concentration (over 10^8 CFU ml^{-1}) for 61 days at 5°C when it was mixed with xanthan gum at concentrations ranging from 0.125% to 0.5% under sterile conditions. In contrast, once in contact with undisinfected barley seeds, the bacterial population drastically declined from 1.87 x 10^7 to 3.33 x 10^2 CFU ml^{-1} within 16 days, despite using 0.5% xanthan gum and storing at 4°C (Zill, 2024). To extend the shelf-life of the coated seeds, the inclusion of adjuvants in the formulation, such as trehalose or molasses could be beneficial (Marina et al., 2018; Mhada et al., 2021). Indeed, the unsuccessful detection of strain $E19^T$ at Kleinhohenheim during the first season and only in a few samples in the second season drove us to speculate that a possible explanation may be the reduction in viable cells during transportation. Despite ensuring improved transportation of coated spring barley seed in season III, the percentage of detection at KH remained low (12.5%), compared to GH at the

flowering stage. However, at 30 DAS, root samples at Kleinhohenheim showed a high concentration and detection percentage ($\bar{x}_{copies}$ = 3.51 x 10^5 copies g^{-1} DW, $\bar{x}_{Log10\ copies}$ = 5.55, 75% DR). Our results align with those mentioned above, suggesting that bacterial concentration may not be a determining factor for root colonization and persistence. Instead, the competition and adaptation of *H. diazotrophicus* with the indigenous microbial communities in both the soil and on the seeds appear to be critical for its survival. Interestingly, the non-detection of *H. diazotrophicus* at KH did not affect the positive significant effects observed on grain protein concentration, straw yield (Quiroga et al., 2025), and protein fractions such as hordeins and albumin/globulin-protein Z (Supplement 1). The advantages provided by qPCR for monitoring strain $E19^T$, could not be applied for tracking the three bacterial strains in roses. This would be particularly interesting as compared with $E19^T$, all the strains altered the rhizosphere microbiome of roses but could not be identified after metabarcoding characterization. As result, the same 16S rRNA gene sequencing limitations discussed by Quiroga et al. (2025) were found for roses (Chapter V).

Frey-Klett et al. (1997) found that a high initial bacterial concentration and extended survival period of *Pseudomonas fluorescens* were not necessary to observe a helper effect during the mycorrhizal establishment between the fungus *Laccaria bicolor* and the tree *Pseudotsuga menziesii*. They found out that after nine weeks, the bacterial population in the rhizosphere had decreased below the detection threshold. In addition, the mycorrhizal index was enhanced five weeks after inoculation, when the bacterial population was only 30 CFU g^{-1} DM, suggesting that beneficial effects could take place in early stages of fungal growth. Consistent with the last statement, a recent study showed that early developmental stages of wheat, such as seedling and tillering, can determine wheat grain quality. Moreover, several parameters, including the abundance of key N-cycle genes (*amo*A, *nos*Z, *nir*K), alpha diversity indices, beta diversity dissimilarity values, and the consumption of substrates for carbon utilization (putrescine, L-Arginine, alpha-cyclodextrin) were chosen as the best predictors of grain quality (Asad et al., 2023). Forecasting these parameters at early stages could be a valuable tool for developing prevention and improvement strategies. Furthermore, these results challenge the former belief that the grain filling stage is the crucial period for determining grain quality (for review: Barneix, 2007; Zörb et al., 2018).

Previous studies have demonstrated that *H. diazotrophicus* enhances plant growth under salt stress conditions in barley (Cardinale et al., 2015; Suarez et al., 2015). More recently, high concentrations of the bacterium were detected in winter wheat under the same stress conditions

in a greenhouse experiment (Quiroga et al., 2024). Although *H. diazotrophicus* was detected at advanced developmental stages under field conditions, it was not present in 100% of the samples. However, it is likely that the bacterium could exhibit higher concentrations, detection percentages, and improved plant yield performance under salinity conditions, given its original natural environment (Hungria and Vargas, 2000; Suarez et al., 2014, 2015). A reduction in bacterial population and a shift in soil microbial communities due to the high initial inoculum is usually expected (Coniglio et al., 2022; El Zemrany et al., 2006; Liu et al., 2018). Besides competition with indigenous microbial communities or the "kill the winner" hypothesis, bacterial predation by protozoa could also be involved in the decline of *H. diazotrophicus* populations (Frey-Klett et al., 1997; Heynen et al., 1988; Thingstad, 2000). Heynen et al. (1988) reported that the addition of bentonite can modify soil structure, creating micro-niches that are inaccessible to predators like protozoa, thereby conferring bacterial protection against them.

Native soil microbial communities can exhibit resistance to allochthonous microorganisms, creating an initial barrier alongside the abiotic stress conditions of the new environment (Björklöf et al., 2003). High species richness has been shown to be inversely correlated with successful microbial invasion, principally due to high resource competition (Van Elsas et al., 2012; Vivant et al., 2013). Consistent with this statement, the higher alpha diversity indices observed at Kleinhohenheim during seasons II and III, compared to Gladbacherhof, as well as the differences in microbial community structure between the two organic farms, could be related to the unsuccessful detection of *H. diazotrophicus* at advanced developmental stages. This may be due to stronger competition for resources at one location (Quiroga et al., 2025). In addition to high species richness, specific network configurations have also shown to influence microorganism invasion (Poisot et al., 2013; Thébault and Fontaine, 2010). Wei et al. (2015) found that communities in tomato rhizosphere with high connectance, low nestedness, and high niche overlap were more effective in preventing *Ralstonia solanacearum* invasion. The authors stated that a high interaction density within bacterial communities (high connectance) creates a stabilized configuration where generalist and specialist species coexist without intense competition for the same resources (low nestedness). Additionally, similar resource competition among native communities and the invader (niche overlapping) enhanced protection against pathogen invasion. In other words, high resistance to invasion appears to be influenced more by the native community's capabilities to consume different nutrient sources and its network configuration, rather than by species richness per se. This results in less competition among indigenous species but greater competence in resisting invaders (Eisenhauer et al., 2013; for review: Saleem et al., 2019).

H. diazotrophicus did not significantly alter the bacterial community structure over three consecutive seasons at any location and plant species evaluated (Quiroga et al., 2025). Moreover, these results were also corroborated in the active rhizosphere bacterial microbiome, using eRNA, of wheat and barley rhizosphere soil during seasons I and II (Chapter IV). Remarkably, *H. diazotrophicus* was detected in one sample of the active rhizosphere microbiome. To be specific, in sample 49 belonging to spring barley inoculated with $E19^T$ during season I. This complements the detection in sample 422 corresponding to winter wheat with fertilizer inoculated with $E19^T$, during season III reported by Quiroga et al. (2025). This result is particularly relevant as detection of $E19^T$ by qPCR or 16S rRNA sequencing with its limitations (Poretsky et al., 2014) was performed using eDNA. This raised questions about the status of the detected *H. diazotrophicus*, as eDNA cannot differentiate between living and deceased organisms (Pochon et al., 2017; Yates et al., 2021). Therefore, the detection of $E19^T$ using eRNA suggests that this bacterium remains active on the roots at least until flowering stage, when it was detected (Chapter IV).

While an ideal bioinoculant, especially for organic farming, should improve plant performance without significantly impacting the microbial community structure, it is important to mention that alterations in microbial populations are not always detrimental (Eltlbany et al., 2019; Magalhães et al., 2024; Zhou et al., 2023). While much more research is needed to understand the relationship between bioinoculants and their surrounding environment, including potential risks (Keswani et al., 2019; Vílchez et al., 2016), any changes should be interpreted within the specific context and dynamics of the environment (Abdullaeva et al., 2024; Rosado-Porto et al., 2022; Zhou et al., 2023). For instance, alterations in the rhizosphere microbiome caused by elevated atmospheric CO_2 can impact the nitrogen cycle by increasing nitrogen-fixing and denitrifying taxa, which may eventually lead to higher N_2O emissions (Rosado-Porto et al., 2022). Moreover, shifts in microbial populations have also been identified in the rhizosphere microbiome of wheat as a consequence of domestication, associating them with a reduced natural capability to mitigate fungal pathogens (Abdullaeva et al., 2024). In contrast, Magalhães et al. (2024) discovered positive interactions among the rhizosphere and plant pollinators. After inoculating different tomato accessions with *B. amyloliquefaciens*, the authors observed improvements not only in floral volatile organic compound emission but also in pollen levels, which impacted the foraging behavior of *Melipona quadrifasciata*. In addition, the introduction of bioinoculants could contribute to stabilizing microbial populations, as well as restoring or enhancing microbial community structure in order to suppress pathogen infections (Bharti et al., 2015; Song et al., 2022; Zhou et al., 2023).

Bharti et al. (2015) indicated that the mitigation by PGPR of a substantial shift due to stress such as salinity can improve the resilience of microbial communities. These authors found that after inoculating three PGPR strains with and without salt stress, the microbial communities did not differ to the same extent as the control especially after salt stress conditions. Interestingly, we found the same trend with bacterial communities of *rosa* sp. after the application of *Bacillus subtilis* (DMS BS), *B. amyloliquefaciens* (DMS BA), and *Paenibacillus polymyxa* (DMS BP) under salt stress. Although a shift in the rhizosphere bacterial microbiome before salt stress was observed, the bacterial communities did not significantly vary compared to the control after salt stress (DMS BS) or with their respective treatment under salinity conditions (DMS BA and DMS BP). More interestingly, differential abundance analysis showed that each strain altered the bacterial communities to different degrees (Chapter V).

In the last decade, longitudinal studies have shown that microbial communities are not static; rather, their structure and functionality can undergo significant shifts throughout the plant life cycle, even without the introduction of an exogenous microorganism (Horton et al., 2014; Wagner et al., 2016). Research on *Arabidopsis thaliana* and maize has indicated that microbial communities experience compositional changes that are clearly distinguished at early developmental stages (e.g. seedling) (Chaparro et al., 2014, 2013; Li et al., 2014). In *A. thaliana*, alpha diversity indices did not show significant changes across all the stages evaluated, whereas beta diversity, as measured by the Bray-Curtis index, showed significant differences at the seedling stage compared to vegetative, bolting, and flowering stages (Chaparro et al., 2014). In contrast, the findings of the present study identified significant changes in both alpha and beta diversity indices between the flowering and ripe stages in winter wheat across all seasons (Quiroga et al., 2025). However, this trend was not observed in spring barley, suggesting that the dynamics of bacterial communities may be plant-dependent. Zhalnina et al. (2018) demonstrated that root exudates influenced microbial communities in a species-specific manner. The authors found that the highest concentrations of homoserine and sucrose in *Avena barbata* occurred at the seedling stage. During the vegetative stage, carboxylic acids with aromatic rings and amino acids increased, while plant hormones and quaternary ammonium salts were more abundant during senescence. These changes in root exudate abundance led to shifts in bacterial phyla, with *Pseudomonadota* increasing and *Actinomycetota*, decreasing their relative abundances as *A. barbata* grew.

Changes in microbial composition can also vary across different plant compartments (Agoussar et al., 2021; Edwards et al., 2015; Quiza et al., 2023). Edwards et al. (2015) found that microbial communities in rice differed in the rhizosphere, rhizoplane, and endosphere. However, under field conditions, plant compartment was relegated to the second most significant driving force of microbial composition, with location exerting a greater influence on shaping the microbial community structure. Indeed, cultivation practices (organic vs. conventional) explained a considerable part of the variation in microbiome assembly. Similar results were found in the bacterial communities of the wheat rhizosphere at two locations, where, in addition to differences in soil types, crop rotation also varied among locations (Donn et al., 2015). Our results are consistent with these studies, despite sharing the same soil type, differences in crop rotation practices showed clear distinctions between Gladbacherhof and Kleinhohenheim in terms of differential abundance, longitudinal analysis, and alpha and beta diversity indices over three consecutive seasons.

Although a row spacing of 15 cm did not show significant effects on grain and straw yield, a general trend toward improvement in both yield parameters was observed, especially in spring barley (Quiroga et al., 2024). Similar results suggest that narrow row spacing of 15 cm leads to better yield parameters (Safdar et al., 2022). McCollough and Melander (2022) assessed the influence of row spacing (15 cm and 20 cm) and crop density (plants m^{-2}) in spring barley, determining better yield performance with 15 cm, and better weed control, though without yield enhancement, as crop density increased. Soil moisture, light interception for plant maintenance, evapotranspiration, and nutritional seed composition could also be influenced by row spacing (Flajšman et al., 2019; Timlin et al., 2001).

Sustainable agriculture aims to maintain plant productivity while reducing input energy loss and minimizing detrimental effects on biodiversity and soil quality (Kennedy and Smith, 1995). To evaluate the influence of strain $E19^T$ on soil quality, several soil parameters including microbial biomass, ammonium, nitrate, sulfate, and C:N ratio were determined before sowing and after harvesting for both crops during seasons I and II at Gladbacherhof (Jungbecker, 2023). Although some parameters significantly increased after harvesting in the inoculated treatments with *H. diazotrophicus* alone or with the addition of fertilizer, no significant trend could be determined for any parameters, with the exception of sulfate, in any crop over the seasons. Jungbecker (2023) determined an increase in the soil ammonium and nitrate between strain $E19^T$ and Ctrl (SB, season II). This could be related to the PGPR ability to fix nitrogen originally reported in *H. diazotrophicus* (Suarez et al., 2014) and later corroborated with the identification

of *nif* genes in its genome (Suarez et al., 2019). Similarly, this capability could be attributed to the significant increase observed in the total protein concentration of barley grains, along with its protein fractions such as albumin/globulin-protein Z and hordeins (Supplement 1). Nonetheless, nitrogen fixation could be either indirectly related as discussed by Quiroga et al. (2024), or more likely, it could not represent a substantial contribution to plant growth as recently reported (Giller et al., 2024). At this point, a pivotal question arises about how *H. diazotrophicus* could improve some soil, yield, and grain quality parameters, as nitrogen fixation cannot significantly contribute to this aim. More intriguingly, without altering the rhizosphere bacterial microbiome, or even could not be detected at the threshold level in one of the organic fields (Kleinhohenheim). Several authors suggest that PGPR can enhance plant growth through mechanisms such as increasing root density, root biomass, and root architecture, improving mineral uptake, or reducing stress through ACC deaminase (Hansen et al., 2020; Schultz et al., 2017; Stearns et al., 2012), rather than through nitrogen fixation or phosphorus solubilization (Giller et al., 2024; Raymond et al., 2021). Likely, the main mechanism employed by strain E19^T is ACC deaminase, as well as increasing root biomass, as reported by Suarez et al. (2015). On the other hand, we align with novel studies suggesting that bioinoculants can provoke changes in other plant compartments during early plant developmental stages or improve the co-occurrence interactions between microbial communities (Chen et al., 2022; Kong et al., 2019; Kong and Liu, 2022). In this regard, we have found evidence of higher co-occurrences in the root microbiome of winter wheat plants inoculated with *H. diazotrophicus* E19^T but not yet published (Supplement 2).

In summary, the results of this study demonstrated that *H. diazotrophicus* strain E19^T was detected on the roots of wheat and barley until advanced developmental stages, in at least one organic farm, and remained likely active up to the flowering stage. Moreover, significant correlations were found between the bacterium abundance and the improvement of plant yield parameters such as straw yield and grain protein concentration, including the protein fractions hordeins and albumin/globulin-protein Z. Nevertheless, evaluating this bacterium under field conditions determined that its survival does not occur in 100% of the plants, providing deeper insights into its dynamics. These findings were complemented by alpha and beta diversity indices, which indicated a non-significant shift in rhizosphere bacterial communities due to seed inoculation but significant changes between the two locations across three consecutive seasons. These differences may be related to the non-detection of *H. diazotrophicus* strain E19^T in one of the farms, where microbial competition for niches and resources might be higher, thereby impacting the bacterium's survival. Additionally, we observed that location played a

significant role in assembling bacterial communities within the same plant species. While longitudinal analyses revealed fluctuations in these communities, indicating that their complex dynamics can be influenced by several factors such as location, plant species, and season. Finally, similar trends were found when eRNA and eDNA were compared.

References

Abdullaeva, Y., Ratering, S., Rosado-Porto, D., Ambika Manirajan, B., Glatt, A., Schnell, S., Cardinale, M., 2024. Domestication caused taxonomical and functional shifts in the wheat rhizosphere microbiota, and weakened the natural bacterial biocontrol against fungal pathogens. Microbiol. Res. 281, 127601. https://doi.org/10.1016/j.micres.2024.127601

Agoussar, A., Azarbad, H., Tremblay, J., Yergeau, E., 2021. The resistance of the wheat microbial community to water stress is more influenced by plant compartment than reduced water availability 1–11. https://doi.org/10.1093/femsec/fiab149

Al Ibrahim, H., 2023. Methoden zur Samenbeimpfung mit pflanzenwachstumsfördern-den Bakterien – Optimierung der Beimpfung mit Xanthan Gum und *Hartmannibacter diazotrophicus* (E19^{T}) [Methods for seed inoculation with plant growth-promoting bacteria – optimization of inoculation with xanthan gum and *Hartmannibacter diazotrophicus* (E19^{T})]. Master thesis, Justus Liebig Universität Gießen.

Asad, N.I., Wang, X.B., Dozois, J., Azarbad, H., Constant, P., Yergeau, E., 2023. Early season soil microbiome best predicts wheat grain quality. FEMS Microbiol. Ecol. 99, 1–13. https://doi.org/10.1093/femsec/fiac144

Bankhead, S.B., Landa, B.B., Lutton, E., Weller, D.M., Gardener, B.B.M.S., 2004. Minimal changes in rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains. FEMS Microbiol. Ecol. 49, 307–318. https://doi.org/10.1016/j.femsec.2004.04.005

Barneix, A.J., 2007. Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. J. Plant Physiol. 164, 581–590. https://doi.org/10.1016/j.jplph.2006.03.009

Bharti, N., Barnawal, D., Maji, D., Kalra, A., 2015. Halotolerant PGPRs prevent major shifts in indigenous microbial community structure under salinity stress. Microb. Ecol. 70, 196–208. https://doi.org/10.1007/s00248-014-0557-4

Björklöf, K., Sen, R., Jørgensen, K.S., 2003. Maintenance and impacts of an inoculated *mer*/*luc*-tagged *Pseudomonas fluorescens* on microbial communities in birch rhizospheres developed on humus and peat. Microb. Ecol. 45, 39–52. https://doi.org/10.1007/s00248-002-2018-8

Cardinale, M., Ratering, S., Suarez, C., Zapata Montoya, A.M., Geissler-Plaum, R., Schnell, S., 2015. Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress. Microbiol. Res. 181, 22–32. https://doi.org/10.1016/j.micres.2015.08.002

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

Chaparro, J.M., Badri, D. V, Bakker, M.G., Sugiyama, A., Manter, D.K., Vivanco, J.M., 2013. Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PLoS One 8, e55731. https://doi.org/10.1371/journal.pone.0055731

Chen, C., Wang, M., Zhu, J., Tang, Y., Zhang, H., Zhao, Q., Jing, M., Chen, Y., Xu, X., Jiang, J., Shen, Z., 2022. Long-term effect of epigenetic modification in plant–microbe interactions: modification of DNA methylation induced by plant growth-promoting bacteria mediates promotion process. Microbiome 10, 36. https://doi.org/10.1186/s40168-022-01236-9

Coniglio, A., Larama, G., Molina, R., Mora, V., Torres, D., Marin, A., Avila, A.I., Lede NoirCarlan, C., Erijman, L., Figuerola, E.L., de Souza, E.M., Cassán, F., 2022. Modulation of maize rhizosphere microbiota composition by inoculation with *Azospirillum argentinense* Az39 (Formerly *A. brasilense* Az39). J. Soil Sci. Plant Nutr. 39, 3553–3567. https://doi.org/10.1007/s42729-022-00909-6

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

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N.K., Bhatnagar, S., Eisen, J.A., Sundaresan, V., Jeffery, L.D., 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. U. S. A. 112, E911–E920. https://doi.org/10.1073/pnas.1414592112

Eisenhauer, N., Schulz, W., Scheu, S., Jousset, A., 2013. Niche dimensionality links biodiversity and invasibility of microbial communities. Funct. Ecol. 27, 282–288. https://doi.org/10.1111/j.1365-2435.2012.02060.x

El Zemrany, H., Cortet, J., Peter Lutz, M., Chabert, A., Baudoin, E., Haurat, J., Maughan, N., Félix, D., Défago, G., Bally, R., Moënne-Loccoz, Y., 2006. Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. Soil Biol. Biochem. 38, 1712–1726. https://doi.org/10.1016/j.soilbio.2005.11.025

Eltlbany, N., Baklawa, M., Ding, G.-C., Nassal, D., Weber, N., Kandeler, E., Neumann, G., Ludewig, U., van Overbeek, L., Smalla, K., 2019. Enhanced tomato plant growth in soil under reduced P supply through microbial inoculants and microbiome shifts. FEMS Microbiol. Ecol. 95, 1–11. https://doi.org/10.1093/femsec/fiz124

Flajšman, M., Šantavec, I., Kolmanič, A., Kocjan Ačko, D., 2019. Bacterial seed inoculation and row spacing affect the nutritional composition and agronomic performance of soybean. Int. J. Plant Prod. 13, 183–192. https://doi.org/10.1007/s42106-019-00046-8

Frey-Klett, P., Pierrat, J.C., Garbaye, J., 1997. Location and survival of mycorrhiza helper *Pseudomonas fluorescens* during establishment of ectomycorrhizal symbiosis between *Laccaria bicolor* and Douglas fir. Appl. Environ. Microbiol. 63, 139–144. https://doi.org/10.1128/aem.63.1.139-144.1997

Fu, L., Penton, C.R., Ruan, Y., Shen, Z., Xue, C., Li, R., Shen, Q., 2017. Inducing the rhizosphere microbiome by biofertilizer application to suppress banana Fusarium wilt disease. Soil Biol. Biochem. 104, 39–48. https://doi.org/10.1016/j.soilbio.2016.10.008

Gholami, A., Shahsavani, S., Nezarat, S., 2009. The effect of plant growth promoting rhizobacteria on seed germination and seedling growth of maize. Int. J. Agric. Biosyst. Eng. 3, 9–14.

Giller, K.E., James, E.K., Ardley, J., Unkovich, M.J., 2024. Science losing its way: examples from the realm of microbial N_2-fixation in cereals and other non-legumes. Plant Soil. https://doi.org/10.1007/s11104-024-07001-1

Hansen, V., Bonnichsen, L., Nunes, I., Sexlinger, K., Lopez, S.R., van der Bom, F.J.T., Nybroe, O., Nicolaisen, M.H., Jensen, L.S., 2020. Seed inoculation with *Penicillium bilaiae* and *Bacillus simplex* affects the nutrient status of winter wheat. Biol. Fertil. Soils 56, 97–109. https://doi.org/10.1007/s00374-019-01401-7

Heynen, C.E., van Elsas, J.D., Kuikman, P.J., van Veen, J.A., 1988. Dynamics of *Rhizobium leguminosarum biovar trifolii* introduced into soil; the effect of bentonite clay on predation by protozoa. 20, 483–488. https://doi.org/10.1016/0038-0717(88)90062-4

Horton, M.W., Bodenhausen, N., Beilsmith, K., Meng, D., Muegge, B.D., Subramanian, S., Vetter, M.M., Vilhjálmsson, B.J., Nordborg, M., Gordon, J.I., Bergelson, J., 2014. Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. Nat. Commun. 5, 5320. https://doi.org/10.1038/ncomms6320

Hungria, M., Vargas, M.A.T., 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. F. Crop. Res. 65, 151–164. https://doi.org/10.1016/S0378-4290(99)00084-2

Jungbecker, L., 2023. Analyse von Bodenparametern in verschiedenen ökologischen Anbausystemen zur Beurteilung der Nachhaltigkeit [Analysis of soil parameters in different organic farming systems for assessing sustainability]. Bachelor thesis, Justus Liebig Universität Gießen.

Kennedy, A.C., Smith, K.L., 1995. Soil microbial diversity and the sustainability of agricultural soils. Plant Soil 170, 75–86. https://doi.org/10.1007/BF02183056

Keswani, C., Prakash, O., Bharti, N., Vílchez, J.I., Sansinenea, E., Lally, R.D., Borriss, R., Singh, S.P., Gupta, V.K., Fraceto, L.F., de Lima, R., Singh, H.B., 2019. Re-addressing the biosafety issues of plant growth promoting rhizobacteria. Sci. Total Environ. 690, 841–852. https://doi.org/10.1016/j.scitotenv.2019.07.046

Kloepper, J.W., 1981. Development of a Powder Formulation of Rhizobacteria for inoculation of potato seed pieces. Phytopathology 71, 590–592. https://doi.org/10.1094/phyto-71-590

Kong, Z., Liu, H., 2022. Modification of rhizosphere microbial communities: a possible mechanism of plant growth promoting rhizobacteria enhancing plant growth and fitness. Front. Plant Sci. 13, 920813. https://doi.org/10.3389/fpls.2022.920813

Kong, Z., Wu, Z., Glick, B.R., He, S., Huang, C., Wu, L., 2019. Co-occurrence patterns of microbial communities affected by inoculants of plant growth-promoting bacteria during phytoremediation of heavy metal-contaminated soils. Ecotoxicol. Environ. Saf. 183, 109504. https://doi.org/10.1016/j.ecoenv.2019.109504

Li, X., Rui, J., Mao, Y., Yannarell, A., Mackie, R., 2014. Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. Soil Biol. Biochem. 68, 392–401. https://doi.org/10.1016/j.soilbio.2013.10.017

Liu, C., Yang, Z., He, P., Munir, S., Wu, Y., Ho, H., He, Y., 2018. Deciphering the bacterial

and fungal communities in clubroot-affected cabbage rhizosphere treated with *Bacillus subtilis* XF-1. Agric. Ecosyst. Environ. 256, 12–22. https://doi.org/10.1016/j.agee.2018.01.001

Magalhães, D.M., Lourenção, A.L., Bento, J.M.S., 2024. Beneath the blooms: unearthing the effect of rhizospheric bacteria on floral signals and pollinator preferences. Plant Cell Environ. 47, 782–798. https://doi.org/10.1111/pce.14771

Mangmang, J.S., Deaker, R., Rogers, G., 2015. Early seedling growth response of lettuce, tomato and cucumber to *Azospirillum brasilense* inoculated by soaking and drenching. Hortic. Sci. 42, 37–46. https://doi.org/10.17221/159/2014-HORTSCI

Marina, T.A.-E., Maythsulene, I.S.O., Valacia, L.S.-L., Marta, C.C.F., Amadou, H.B., Edemilson, C.C., Marcio, V. de C.B.C., 2018. Shelf life enhancement of plant growth promoting rhizobacteria using a simple formulation screening method. African J. Microbiol. Res. 12, 115–126. https://doi.org/10.5897/ajmr2017.8787

Martínez-Viveros, O., Jorquera, M.A., Crowley, D.E., Gajardo, G., Mora, M.L., 2010. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J. Soil Sci. Plant Nutr. 10, 293–319. https://doi.org/10.4067/S0718-95162010000100006

McCollough, M.R., Melander, B., 2022. Improving upon the interrow hoed cereal system: the effects of crop density and row spacing on intrarow weeds and crop parameters in spring barley. Weed Sci. 70, 341–352. https://doi.org/10.1017/wsc.2022.14

Mhada, M., Zvinavashe, A.T., Hazzoumi, Z., Zeroual, Y., Marelli, B., Kouisni, L., 2021. Bioformulation of silk-based coating to preserve and deliver *Rhizobium tropici* to *Phaseolus vulgaris* under saline environments. Front. Plant Sci. 12, 1–11. https://doi.org/10.3389/fpls.2021.700273

Moussa, T. A.A., Almaghrabi, O.A., Abdel-Moneim, T.S., 2013. Biological control of the wheat root rot caused by *Fusarium graminearum* using some PGPR strains in Saudi Arabia. Ann. Appl. Biol. 163, 72–81. https://doi.org/10.1111/aab.12034

O'Callaghan, M., Ballard, R.A., Wright, D., 2022. Soil microbial inoculants for sustainable agriculture: limitations and opportunities. Soil Use Manag. 38, 1340–1369. https://doi.org/10.1111/sum.12811

Pochon, X., Zaiko, A., Fletcher, L.M., Laroche, O., Wood, S.A., 2017. Wanted dead or alive? using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. PLoS One 12, 1–19. https://doi.org/10.1371/journal.pone.0187636

Poisot, T., Mouquet, N., Gravel, D., 2013. Trophic complementarity drives the biodiversity-ecosystem functioning relationship in food webs. Ecol. Lett. 16, 853–861. https://doi.org/10.1111/ele.12118

Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., Konstantinidis, K.T., 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS One 9, e93827. https://doi.org/10.1371/journal.pone.0093827

Quiroga, S., Ratering, S., Rosado-Porto, D., Rekowski, A., Schulz, F., Zörb, C., Schnell, S., 2025. Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial. Appl. Soil Ecol. 206,

105823. https://doi.org/10.1016/j.apsoil.2024.105823

Quiroga, S., Rosado-Porto, D., Ratering, S., Rekowski, A., Schulz, F., Krutych, M., Zörb, C., Schnell, S., 2024. Long-term detection of *Hartmannibacter diazotrophicus* on winter wheat and spring barley roots under field conditions revealed positive correlations on yield parameters with the bacterium abundance. FEMS Microbiol. Ecol. 100, fiae023. https://doi.org/10.1093/femsec/fiae023

Quiza, L., Tremblay, J., Pagé, A.P., Greer, C.W., Pozniak, C.J., Li, R., Haug, B., Hemmingsen, S.M., St-Arnaud, M., Yergeau, E., 2023. The effect of wheat genotype on the microbiome is more evident in roots and varies through time. ISME Commun. 3, 1–10. https://doi.org/10.1038/s43705-023-00238-4

Ramamoorthy, V., Raguchander, T., Samiyappan, R., 2002. Enhancing resistance of tomato and hot pepper to Pythium diseases by seed treatment with fluorescent pseudomonads. Eur. J. Plant Pathol. 108, 429–441. https://doi.org/10.1023/A:1016062702102

Raymond, N.S., Gómez-Muñoz, B., van der Bom, F.J.T., Nybroe, O., Jensen, L.S., Müller-Stöver, D.S., Oberson, A., Richardson, A.E., 2021. Phosphate-solubilising microorganisms for improved crop productivity: a critical assessment. New Phytol. 229, 1268–1277. https://doi.org/10.1111/nph.16924

Renoud, S., Abrouk, D., Prigent-Combaret, C., Wisniewski-Dyé, F., Legendre, L., Moënne-Loccoz, Y., Muller, D., 2022. Effect of inoculation level on the impact of the PGPR *Azospirillum lipoferum* CRT1 on selected microbial functional groups in the rhizosphere of field maize. Microorganisms 10. https://doi.org/10.3390/microorganisms10020325

Rosado-Porto, D., Ratering, S., Cardinale, M., Maisinger, C., Moser, G., Deppe, M., Müller, C., Schnell, S., 2022. Elevated atmospheric CO_2 modifies mostly the metabolic active rhizosphere soil microbiome in the Giessen FACE Experiment. Microb. Ecol. 83, 619–634. https://doi.org/10.1007/s00248-021-01791-y

Safdar, M.E., Qamar, R., Maqbool, R., Ali, H.H., Nadeem, M., Nadeeem, M.A., Abbas, T., Hayyat, M.S., Ashraf, M.A., 2022. Management strategies for optimizing barley yield through row spacing under variable weed competition duration. Pakistan J. Agric. Sci. 59, 467–476. https://doi.org/10.21162/PAKJAS/22.9887

Saleem, M., Hu, J., Jousset, A., 2019. More Than the Sum of Its Parts: Microbiome biodiversity as a driver of plant growth and soil health. Annu. Rev. Ecol. Evol. Syst. 50, 145–168. https://doi.org/10.1146/annurev-ecolsys-110617-062605

Santos, M.S., Nogueira, M.A., Hungria, M., 2019. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express 9. https://doi.org/10.1186/s13568-019-0932-0

Schultz, N., Pereira, W., de Albuquerque Silva, P., Baldani, J.I., Boddey, R.M., Alves, B.J.R., Urquiaga, S., Reis, V.M., 2017. Yield of sugarcane varieties and their sugar quality grown in different soil types and inoculated with a diazotrophic bacteria consortium. Plant Prod. Sci. 20, 366–374. https://doi.org/10.1080/1343943X.2017.1374869

Song, Q., Deng, X., Song, R., Song, X., 2022. Plant growth-promoting rhizobacteria promote growth of seedlings, regulate soil microbial community, and alleviate damping-off disease caused by *Rhizoctonia solani* on *Pinus sylvestris* var. mongolica. Plant Dis. 106, 2730–2740. https://doi.org/10.1094/PDIS-11-21-2562-RE

Stearns, J.C., Woody, O.Z., McConkey, B.J., Glick, B.R., 2012. Effects of bacterial ACC

deaminase on *Brassica napus* gene expression. Mol. Plant-Microbe Interact. 25, 668–676. https://doi.org/10.1094/MPMI-08-11-0213

Stoll, A., Salvatierra-Martínez, R., González, M., Cisternas, J., Rodriguez, Á., Vega-Gálvez, A., Bravo, J., 2021. Importance of crop phenological stages for the efficient use of PGPR inoculants. Sci. Rep. 11, 1–9. https://doi.org/10.1038/s41598-021-98914-9

Suarez, C., Cardinale, M., Ratering, S., Steffens, D., Jung, S., Montoya, A.M.Z., Geissler-Plaum, R., Schnell, S., 2015. Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress. Appl. Soil Ecol. 95, 23–30. https://doi.org/10.1016/j.apsoil.2015.04.017

Suarez, C., Ratering, S., Geissler-Plaum, R., Schnell, S., 2014. *Hartmannibacter diazotrophicus* gen. nov., sp. nov., a phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt-meadow plant. Int. J. Syst. Evol. Microbiol. 64, 3160–3167. https://doi.org/10.1099/ijs.0.064154-0

Suarez, C., Ratering, S., Hain, T., Fritzenwanker, M., Goesmann, A., Blom, J., Chakraborty, T., Bunk, B., Spröer, C., Overmann, J., Schnell, S., 2019. Complete genome sequence of the plant growth-promoting bacterium *Hartmannibacter diazotrophicus* strain E19^{T}. Int. J. Genomics 2019, 7586430. https://doi.org/10.1155/2019/758643

Thébault, E., Fontaine, C., 2010. Stability of ecological communities and the architecture of mutualistic and trophic networks. Science 329, 853–856. https://doi.org/10.1126/science.1188321

Thingstad, T.F., 2000. Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. Limnol. Oceanogr. 45, 1320–1328. https://doi.org/10.4319/lo.2000.45.6.1320

Timlin, D., Pachepsky, Y., Reddy, V.R., 2001. Soil water dynamics in row and interrow positions in soybean (*Glycine max* L.). Plant Soil 237, 25–35. https://doi.org/10.1023/A:1013385026500

Urrea-Valencia, S., Etto, R.M., Takahashi, W.Y., Caires, E.F., Bini, A.R., Ayub, R.A., Stets, M.I., Cruz, L.M., Galvão, C.W., 2021. Detection of *Azospirillum brasilense* by qPCR throughout a maize field trial. Appl. Soil Ecol. 160. https://doi.org/10.1016/j.apsoil.2020.103849

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

Van De Pol, P.A., Breukelaar, A., 1982. Stenting of roses; a method for quick propagation by simultaneously cutting and grafting. Sci. Hortic. (Amsterdam). 17, 187–196. https://doi.org/10.1016/0304-4238(82)90012-7

Van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Elhottova, D., Krištůfek, V., Salles, J.F., 2012. Microbial diversity determines the invasion of soil by a bacterial pathogen. Proc. Natl. Acad. Sci. U. S. A. 109, 1159–1164. https://doi.org/10.1073/pnas.1109326109

Vílchez, J.I., Navas, A., González-López, J., Arcos, S.C., Manzanera, M., 2016. Biosafety test for plant growth-promoting bacteria: proposed environmental and human safety index (EHSI) protocol. Front. Microbiol. 6, 1514. https://doi.org/10.3389/fmicb.2015.01514

Vivant, A.L., Garmyn, D., Maron, P.A., Nowak, V., Piveteau, P., 2013. Microbial diversity and

structure are drivers of the biological barrier effect against *Listeria monocytogenes* in soil. PLoS One 8, 1–11. https://doi.org/10.1371/journal.pone.0076991

Wagner, M.R., Lundberg, D.S., Del Rio, T.G., Tringe, S.G., Dangl, J.L., Mitchell-Olds, T., 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat. Commun. 7, 12151. https://doi.org/10.1038/ncomms12151

Wei, Z., Yang, T., Friman, V.P., Xu, Y., Shen, Q., Jousset, A., 2015. Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health. Nat. Commun. 6, 8413. https://doi.org/10.1038/ncomms9413

Xu, J., Feng, Y., Wang, Y., Lin, X., 2018. Effect of rhizobacterium *Rhodopseudomonas palustris* inoculation on *Stevia rebaudiana* plant growth and soil microbial community. Pedosphere 28, 793–803. https://doi.org/10.1016/S1002-0160(18)60043-8

Yates, M.C., Derry, A.M., Cristescu, M.E., 2021. Environmental RNA: a Revolution in ecological resolution? Trends Ecol. Evol. 36, 601–609. https://doi.org/10.1016/j.tree.2021.03.001

Zhalnina, K., Louie, K.B., Hao, Z., Mansoori, N., Da Rocha, U.N., Shi, S., Cho, H., Karaoz, U., Loqué, D., Bowen, B.P., Firestone, M.K., Northen, T.R., Brodie, E.L., 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. Nat. Microbiol. 3, 470–480. https://doi.org/10.1038/s41564-018-0129-3

Zhang, S., Ma, Y., Jiang, W., Meng, L., Cao, X., Hu, J., Chen, J., Li, J., 2020. Development of a strain-specific quantification method for monitoring *Bacillus amyloliquefaciens* TF28 in the rhizospheric soil of soybean. Mol. Biotechnol. 62, 521–533. https://doi.org/10.1007/s12033-020-00268-6

Zhang, Y., Li, Y., Liang, S., Zheng, W., Chen, X., Liu, J., Wang, A., 2022. Study on the preparation and effect of tomato seedling disease biocontrol compound seed-coating Agent. Life 12, 1–16. https://doi.org/10.3390/life12060849

Zhou, Y., Yang, Z., Liu, J., Li, Xudong, Wang, X., Dai, C., Zhang, T., Carrión, V.J., Wei, Z., Cao, F., Delgado-Baquerizo, M., Li, Xiaogang, 2023. Crop rotation and native microbiome inoculation restore soil capacity to suppress a root disease. Nat. Commun. 14, 8126. https://doi.org/10.1038/s41467-023-43926-4

Zill, A.-S., 2024. Überlebensfähigkeit von *Hartmannibacter diazotrophicus* E19^{T} nach Beimpfen von Gerstensamen unter der Verwendung unterschiedlicher Agenzien zur Ummantelung [Survivability of *Hartmannibacter diazotrophicus* E19^{T} after inoculation of barley seeds using different coating agents]. Bachelor thesis, Justus Liebig Universität Gießen.

Zörb, C., Ludewig, U., Hawkesford, M.J., 2018. Perspective on wheat yield and quality with reduced nitrogen supply. Trends Plant Sci. 23, 1029–1037. https://doi.org/10.1016/j.tplants.2018.08.012

Supplements

Supplement 1. Seed inoculation with *Hartmannibacter diazotrophicus*: starch and protein properties in malting barley

Research article

To be submitted to Cereal Chemistry

Azin Rekowski [1], Monika. A. Wimmer [1], **Santiago Quiroga** [2], David Rosado-Porto [2,3], Stefan Ratering [2], Sylvia Schnell [2], Christian Zörb [1*]

[1] Institute of Crop Science, Quality of Plant Products, 340e, University of Hohenheim, 70593 Stuttgart, Germany

[2] Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, 35392 Giessen, Germany

[3] Faculty of Basic and Biomedical Sciences, Simón Bolívar University, 080002 Barranquilla, Colombia

[*] Corresponding author: Institute of Crop Science, Quality of Plant Products, 340e, University of Hohenheim, 70593 Stuttgart, Germany. E-mail: azin.ghabelrahmat@uni-hohenheim.de

Abstract

Background: The organic cultivation of spring barley is becoming increasingly popular, but the prohibition of using herbicides as well as the reduction of mineral fertilizers bear difficulties to achieve optimal yield and quality. While the use of Plant Growth Promoting Rhizobacteria (PGPR) has been suggested to enhance soil fertility and crop productivity, its impact on the starch and protein quality of malting barley remains unclear.

Aims: This study seeks to assess whether the application of PGPR (strain E19^T) and wide row spacing influence grain storage protein subunits and starch concentrations in spring barley, and whether the effect of E19^T varies across different locations.

Methods: The study was conducted over two consecutive years at three locations in Germany. Protein subunits were analyzed using SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), while amylose and total starch content were determined fotometrically.

Results: Bacterial inoculation with strain E19^T significantly increased total protein and amylose concentrations, as well as specific protein fractions such as hordeins and albumin/globulin-protein Z, particularly under organic farming conditions. The effects of E19^T varied by location, reflecting the influence of environmental factors on its efficacy. Wider row spacing slightly increased hordein and glutelin levels, likely due to improved light absorption of the plant canopy and improved nutrient availability.

Conclusions: Microbial inoculation with strain E19^T and wider row spacing can contribute to improve the quality traits of spring barley by enhancing nutrient availability and protein synthesis. However, the effectiveness of these practices varies by location, highlighting the need for a site-specific approach to optimize barley production. Integrating microbial inoculants with strategic agronomic practices supports sustainable agriculture while improving crop performance.

Key words: Spring barley, Seed inoculation, Protein concentration and composition, Starch, Malting quality

Suplement 2. Which effect does the inoculation of the plant growth-promoting rhizobacterium *Hartmannibacter diazotrophicus* E19^T have on the microbiome of winter wheat?

Research article

To be submitted to Microbiological Research

Julia Sacharow[1*], David Rosado-Porto[1,2], **Santiago Quiroga[1]**, Stefan Ratering[1], Rita Geißler-Plaum[1], Bellinda Schneider[1] and Sylvia Schnell[1]

[1] Institute of Applied Microbiology, IFZ, Justus-Liebig-University Giessen, Giessen, Germany Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

[2] Simón Bolívar University, Faculty of Basic and Biomedical Sciences, Barranquilla, Colombia Cl. 58 #55-132, Nte. Centro Historico, Barranquilla, Atlántico, Colombia

[*] Correspondence:
Julia Sacharow
Heinrich-Buff-Ring 26
35392 Giessen (Germany)
Julia.Sacharow@en.uni-giessen.de

Keywords

Triticum aestivum, *Hartmannibacter diazotrophicus* E19^T, PGPR, bacterial composition, fungal composition, Cercozoan composition.

ORCID ID

J. S.: 0000-0002-8296-0624; Julia.Sacharow@en.uni-giessen.de

D. R. P.: 0000-0001-6199-0114; David.Rosado-Porto@agrar.uni-giessen.de

S. Q.: 0009-0001-1392-8241; Santiago.Quiroga@umwelt.uni-giessen.de

S. R.: 0000-0001-7572-6306; Stefan.Ratering@umwelt.uni-giessen.de

R. G. P.: -; Rita.Geissler-Plaum@umwelt.uni-giessen.de

B. S.: -; Bellinda.Schneider@umwelt.uni-giessen.de

S. S.: 0000-0003-3903-6089; Sylvia.Schnell@umwelt.uni-giessen.de

Author's contribution

J. S.: Conceptualization, Data curation, formal analysis, funding acquisition, investigation, methodology, writing – original draft.

D. R. P.: Data curation, formal analysis, investigation, methodology, visualization, writing – original draft.

S. Q.: Methodology, writing – review & editing.

S. R.: Methodology, supervision, writing – review & editing.

R. G. P.: Methodology, writing – review & editing.

B. S.: Methodology, writing – review & editing.

S. S.: Funding acquisition, methodology, project administration, resources, supervision, writing – review & editing.

Funding

This research received financial support through a fully funded doctoral fellowship awarded to Julia Sacharow by the German Federal Environment Foundation (Deutsche Bundesstiftung Umwelt) and by the Federal Ministry of Education and Research (BMBF) of Germany under the BONARES program (Rhizo4bio). The funding sources were not involved in the conceptualization of the project or the writing of the manuscript.

Data availability

The sequence data have been submitted to the GenBank database under accession number PRJNA1052028. All other data available upon reasonable request.

Competing interests statement

The authors have no competing interests to declare.

Declaration of interest

Declaration of interest: none.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT 3.5 (OpenAI, 2024) in order to improve the readability, clarity, style, and grammar of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Abstract

The utilization of plant growth-promoting rhizobacteria (PGPR) for seed, soil, and plant inoculation is gaining momentum due to the environmental pressures resulting from the extensive use of chemical treatments in plant production. In this study, we investigated the impact of *Hartmannibacter diazotrophicus* E19^{T} seed inoculation on the winter wheat microbiome composed of bacteria, fungi, and Cercozoan protists. Our findings reveal that the inoculation of *H. diazotrophicus* E19^{T} has no significant effect on the microbiome of bacteria, fungi, and Cercozoan protists in the bulk soil, rhizosphere soil and roots of winter wheat. Instead, the composition of the winter wheat microbiome appears to be more influenced by the analysed plant material and sampling time. Minor effects, manifested through positive and negative correlations with various amplicon sequence variants, were observed. These findings confirm that the microbiome composition of winter wheat undergoes only minimal changes upon inoculation with *H. diazotrophicus* E19^{T}.

Acknowledgements

This work would not have been possible without the invaluable help and support I received in different aspects of my academic and personal journey. I would like to take this opportunity to express my sincere gratitude.

To Prof. Dr. Sylvia Schnell, Institute of Applied Microbiology, IFZ, Justus-Liebig University Giessen, for her exceptional guidance, support, and above all, her kindness and humanity. Her encouragement motivated me to push beyond my academic limits and reinforced my faith in the goodness of people.

To Dr. Stefan Ratering, for his constant support, thoughtful suggestions, and exceptional intelligence, which not only contributed to the success of my PhD but also serve as sources of inspiration.

To Prof. Dr. Andreas Gattinger, Department of Agronomy and Plant Breeding II, Justus-Liebig University Giessen, for his willingness to dedicate his time and provide support as second supervisor.

To Dr. David Rosado-Porto, for all his support as a friend and scientist, always willing to share his hard-earned knowledge and for his substantial contributions to my PhD.

To the Federal Ministry of Education and Research (BMBF) of Germany, under the Rhizo4bio program, for its financial support of the BreadAndBeer project.

To Prof. Dr. Christian Zörb and Dr. Azin Rekowski, for their invaluable coordination and support throughout the project.

To Dr. Franz Schulz and Simon Schäfer, for their exceptional support during the field experiments at Gladbacherhof and Kleinhohenheim.

To Rita Geissler-Plaum and Bellinda Schneider, for their outstanding technical assistance, always willing to help during the sample collection, processing, and chemical and molecular analyses. Similarly, to the Applied Microbiology group: Julia, Haoran, Alessandra, Christian, Renate, Martina, and Gundula, who accompanied me over these years.

To my family, especially my parents Aníbal y Elcira, whose love and hard work paved the way that has allowed me to reach this point in my life. Without you and the love of my sisters Sonia, Talia, and Grace, nothing would have been possible.

To my lovely Marline Younan, for being my support and shelter, and most importantly, for being an example of resilience and strength, not only for me but also for many others.

Finally, and just as importantly, to my second family, my new and old friends, with whom I have been lucky to cross paths, and without whom life would have no meaning.