

Understanding wheat-associated microbiomes and root metabolites in response to nitrogen deficiency

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Submitted in total fulfilment of the requirements of
the degree of Doctor of Philosophy

June 2025

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Abstract

Nitrogen (N) is an essential element for plant growth, and its deficiency significantly constrains crop productivity in agricultural systems. While synthetic N fertilisers have been extensively applied to secure crop production, their mismanagement leads to various environmental issues, necessitating the exploration of sustainable alternatives. Soil microbial communities are indispensable regulators of the nitrogen cycle, actively modulating the conversion and retention of applied fertilisers to significantly enhance the plant's nitrogen use efficiency and minimise environmental loss. Symbiotic interactions between soil microorganisms and plants have garnered substantial interest. For instance, the symbiotic relationships of diazotrophs and arbuscular mycorrhizal fungi (AMF) with legume plants are well-documented. However, research into microbial symbiotic interactions with non-legume plants, such as wheat (a top-produced crop in Australia), presents greater challenges.

Plants engage in intensive interactions with soil microorganisms, collectively termed plant-microbe interactions. Abiotic stress profoundly alters plant metabolism, which subsequently influences these interactions. Evidence suggests that plants under abiotic stress conditions recruit and shape the rhizosphere and root endospheric microbial communities through metabolite secretion, aiding adaptation to harsh environments. This phenomenon, described as the "cry-for-help" has been reported in plants experiencing drought, flooding, heat, and salinity stresses. The soil microbes directly impact N transformation, consequently affecting plant nitrogen-use efficiency (NUE) and growth. Despite this, our understanding of plant-microbe interactions under N deficiency remains limited.

Although a recent study demonstrated a symbiotic plant-microbe interaction in maize under N deficiency, these findings may not be directly applicable to wheat due to host-specific plant-microbe interactions. Increasing evidence indicates that microbial community composition, functional profiles, and metabolic profiles vary across plant species and cultivars. Root-associated microbial community and functional profile directly affect rice NUE. However, it remains unexplored whether plant-microbe interaction responses to N deficiency differ among cultivars. Therefore, we hypothesise that (1) the plant-microbe interaction of wheat changes under N deficiency, and (2) the plant-microbe interaction response to N deficiency differs between wheat cultivars with contrasting NUE.

This thesis investigates the plant-microbe interactions of wheat cultivars, specifically those with contrasting NUE, under conditions of N deficiency.

Chapter 1 introduces plant-microbe interactions and their potential to overcome limitations associated with current biofertilisers.

Chapter 2 explores the wheat root-associated bacterial community and its functional profile, as well as the primary metabolites within wheat roots, respond to N deficiency. Through a controlled pot experiment, we analysed the bacterial communities residing in both the root endosphere and rhizosphere of two wheat cultivars, high-NUE Mace and low-NUE Gladius. Additionally, using a hydroponic experiment, we grew Mace and Gladius under varying N levels to analyse their metabolomic responses. Our findings indicate dynamic shifts in wheat-bacterial interactions under N deficiency, with notable intraspecific differences observed between wheat cultivars.

Chapter 3 extends this investigation to the wheat root-associated fungal community and its functional profile under N deficiency. Utilising data from the wheat rhizosphere samples collected in Chapter 2, we examined fungal community composition and functional profiles. While fungal composition and functional profiles differed between the wheat cultivars, consistent with the bacterial data, the fungal functional profile remained stable under N deficiency. This suggests that the interaction between wheat and fungi is more stable than bacteria in the presence of N deficiency.

Chapter 4 further explores the N-metabolism response of rhizosphere bacteria in wheat under N deficiency, drawing data from the metagenome generated in Chapter 3. This work reveals that the two wheat cultivars exhibit distinct bacterial N-metabolism responses when N is deficient.

Cumulatively, these findings provide compelling evidence that the wheat-microbe interaction shifts under N deficiency, alongside a concomitant shift in the plant's metabolite profile. Furthermore, this wheat-microbe interaction exhibits intraspecific variation between different wheat cultivars. These insights enhance our understanding of cultivar-specific wheat-microbe interactions in response to N deficiency, holding significant implications for future

agricultural management strategies and biofertiliser development. Finally, Chapter 5 synthesises the results from Chapters 2, 3 and 4, and discusses potential future research directions of biofertiliser development strategies.

Declaration

I, *Lok Hang Chan*, certify that:

- i. This thesis comprises of my original work towards the degree of Doctor of Philosophy.
- ii. Due acknowledgement has been made in text to all other material used
- iii. The thesis is no longer than the maximum word limit in length, exclusive of tables, maps, bibliographies, and appendices.

Preface and contribution of authors

The research presented in this thesis represents my original work, conducted in collaboration with various co-authors. Specific contributions from all authors are detailed in the "Author Contributions" section of each relevant chapter and are further acknowledged in the individual chapter acknowledgements where applicable. This thesis was completed under the expert supervision of Associate Professor Hang-Wei Hu (Principal Supervisor), Associate Professor Shu Kee Lam, and Professor Deli Chen from the School of Agriculture, Food and Ecosystem Sciences (SAFES) at the University of Melbourne, as well as Professor Caixian Tang from the La Trobe Institute for Sustainable Agriculture and Food (LISAF) at La Trobe University.

This research was made possible through funding from the Research Training Program Scholarship and the Australian Research Council (ARC) Hub for Smart Fertiliser. Chapters 2 and 3 have undergone peer review and been published. Specifically, the findings from Chapter 2 were published in the *Plant and Soil* journal. The results of Chapter 3 were published in the *Rhizosphere* journal. A revised version of Chapter 4 was submitted to the *Soil Security* journal and the major revision requested by the editor has been addressed. I served as the primary author for all published and submitted chapters presented within this thesis. The specific contributions made by co-authors are explicitly detailed within each chapter. Furthermore, the research undertaken for this thesis has been presented at both Australian and international conferences, as further detailed herein.

Publications arising from this thesis

- Chan LH, Lam SK, Chen DL, Tang CX, Chen QL, Roessner U, Hu HW (2025) Nitrogen deficiency drives fungal compositional shifts without functional changes in wheat rhizosphere. *Rhizosphere* 33. doi: ARTN 101030.10.1016/j.rhisph.2025.101030.
- Chan LH, Lam SK, Chen DL, Tang CX, Chen QL, Roessner U, Salazar VW, Gupta S, Dias DA, Hu HW (2024) Cultivar-specific wheat-associated bacterial communities and metabolites in response to nitrogen deficiency. *Plant and Soil*. doi: 10.1007/s11104-024-07048-0.

Conference presentations

- “Intraspecific variation of wheat-bacteria interaction under low nitrogen condition”, presented at Soil Science Australia Conference, Darwin, Australia, 2023.
- “Understanding wheat-associated microbiomes and root metabolites in response to nitrogen deficiency”, presented at Microbial Ecology-Environmental Microbiology (MEEM), Melbourne, Australia, 2023.
- “Intraspecific wheat-bacterial interaction under nitrogen deficiency”, presented at the Fifth International Symposium on the Nitrogen Nutrition of Plants, Sydney, Australia, 2023.
- “Greener food system and the help of bacteria”, presented at the three-minute thesis competition organised by the faculty of science at the University of Melbourne, Melbourne, Australia, 2023.
- “Nitrogen deficiency drives fungal compositional shifts without functional changes in wheat rhizosphere”, presented at the New Zealand Society of Soil Science and Soil Science Australia Joint Conference, Rotorua, New Zealand, 2024.

Acknowledgments

I extend my sincere gratitude to my principal supervisor, Associate Professor Hang-Wei Hu, for his exceptional guidance and unwavering patience throughout my doctoral candidature. His profound professional knowledge has been a wellspring of research ideas, and his consistent support has been instrumental to my progress. He graciously provided me with the invaluable opportunity to contribute to Theme 3 of the ARC Hub for Smart Fertiliser, which facilitated collaboration with scientists possessing diverse expertise and various industry partners. As a highly cited researcher in the field of soil molecular ecology, Associate Professor Hu's extensive experience and insightful commentary have significantly motivated my successful publications. Beyond research, his exemplary leadership and personal values have offered me profound inspiration. His unwavering support and encouragement throughout my degree have been truly invaluable.

My profound thanks are also extended to my co-supervisor, Associate Professor Shu Kee Lam, for his insightful guidance and constructive discussions. His willingness to share his extensive experience in glasshouse experiments greatly contributed to the successful execution of my own experiments. His meticulous approach to scientific writing, particularly in phrasing and grammar, has considerably enhanced my own writing skills. Beyond his supervisory role, he has been a supportive mentor and friend, providing essential care and assistance for my mental well-being throughout my doctoral studies. His critical judgment has been invaluable in deepening my understanding of experimental results from multiple perspectives.

I am also grateful to my co-supervisors, Professor Deli Chen and Professor Caixian Tang, for their expert guidance and enriching discussions concerning soil and plant science. Professor Chen is a world-renowned scientist specializing in the N cycle in soil and serves as the Chief Investigator of the ARC Smart Fertiliser Hub. Professor Tang possesses significant expertise in soil-plant interactions and was also a valuable participant in Theme 3 of the Hub. Their astute comments on experimental design and result interpretations greatly assisted my preparation of manuscripts for publication.

I would like to thank my committee members, Dr. Qinglin Chen and Professor Ute Roessner, for their invaluable discussions. Dr. Chen, an expert in antibiotic resistance genes in soil

microorganisms, provided considerable assistance with sampling procedures and offered meticulous reminders regarding experimental and data handling details. Professor Roessner, a plant biochemist specialising in plant metabolites, provided numerous insightful feedback that significantly improved my hydroponic design and metabolite sampling methods.

Finally, a special thank you to my girlfriend, Cheuk Ying (Kate) Chung, for her unwavering support and compassionate care throughout this entire journey. I am also deeply thankful to my family and friends, whose consistent support for all my decisions throughout this degree has been profoundly appreciated.

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List of Abbreviations

ABC	- ATP-binding cassette
ACC	- 1-aminocyclopropane-1-carboxylate
AHL	- N-acyl homoserine lactone
AI-2	- Autoinducer-2
ALMTs	- Aluminium-Activated Malate Transporters
AMF	- arbuscular mycorrhizal fungi
ATP	- adenosine triphosphate
BNF	- biological N fixation
DNA	- deoxyribonucleic acid
DNRA	- dissimilatory nitrate reduction to ammonium
DMPP	- 3,4-dimethylpyrazole phosphate
IAA	- indole-3-acetic acid
JA	- jasmonic acid
MAMP	- microbe-associated molecular pattern
MATE	- Multidrug and Toxic Compound Extrusion
MTI	- MAMP-triggered immunity
miRNAs	- microRNAs
miPEPs	- miRNA-encoded peptides
N	- Nitrogen
N ₂	- dinitrogen
NH ₃	- ammonia
NH ₄ ⁺	- ammonium
NO ₃ ⁻	- nitrate
NO ₂ ⁻	- nitrite
NO	- nitric oxide
N ₂ O	- nitrous oxide
NUE	- Nitrogen Use Efficiency
P	- phosphorus
PGPR	- plant growth-promoting rhizobacteria
QS	- quorum sensing
SynCom	- synthetic community
SWEETs	- Sugars Will Eventually Be Exported Transporters

sRNAs - small RNAs

siRNAs - small-interfering RNAs

VOCs - volatile organic compounds

Chapter 1 - Introduction and Literature Review

1.1 Australian wheat agriculture system and the use of N fertiliser

Global food production has substantially increased over the past half-century to meet escalating demand (Godfray et al. 2010; Tilman et al. 2011). According to the United Nations Food and Agricultural Organisation (FAO), global food security encompasses: (i) the availability of sufficient quantity and quality of food through domestic production or imports; (ii) individuals' entitlements to acquire food for a nutritious diet; (iii) the achievement of nutritional and physiological well-being through adequate diet, clean water, and sanitation; and (iv) consistent access to adequate food for all households and individuals (Nations 2022; Wheeler and von Braun 2013). Significant challenges to food security include population expansion, climate change, and environmental degradation. Global food demand is projected to increase by over 70% to sustain a human population exceeding 10 billion by 2050 (Godfray et al. 2010). A meta-analysis of literature published between 2000 and 2018 assessed global food security projections from 2010 to 2050 (van Dijk et al. 2021). This analysis indicated that a business-as-usual development scenario would lead to the largest increase in total food consumption, implying that continuous population growth under such conditions poses the most significant future food security risks (van Dijk et al. 2021).

Nitrogen (N) is a critical element in plant nutrition, essential for proper plant metabolism, growth, and development (Kumar and Brar 2021). Various forms of N fertilisers are universally applied in agricultural systems to supply N to the soil and ensure crop productivity. However, it is estimated that over 60% of applied N is lost to the environment (Wei et al. 2024), constraining crop N-use efficiency (NUE) and causing various environmental issues. Inorganic N fertilisers, synthetically derived chemical compounds such as urea, ammonium (NH_4^+), and nitrate (NO_3^-), are widely used. However, the mismanagement of synthetic fertiliser application contributes to significant environmental issues, including eutrophication, nitrous oxide (N_2O) emissions (a potent greenhouse gas), and groundwater contamination (Zhang et al. 2015). Moreover, the escalating demand for inorganic fertilisers and their rising manufacturing costs impose a substantial economic burden on farmers (Zhang et al. 2015). Consequently, researchers are actively investigating

alternative strategies to alleviate these environmental and economic pressures, promoting a transition towards sustainable agriculture. Concurrently, N-rich organic fertilisers derived from plant, animal, and mineral sources (e.g., compost, seaweed extracts, manure, and biochar) have shown promising benefits in wheat production and have attracted increased interest in recent decades (Koutroubas et al. 2016; Salim 2016; Tilston et al. 2005). Nevertheless, their efficacy is often limited by a slow nutrient release rate and lower nutrient content, which are dependent on microbial decomposition processes. While numerous studies confirm that both inorganic and organic N fertilisers significantly enhance crop production in wheat, the combined application of microorganisms with fertilisers is often reported as the most effective approach for enhancing N use efficiency (NUE) (L'Espérance et al. 2024; Zhang et al. 2019). For instance, a field study on *Nigella sativa* L. demonstrated that the co-application of chemical and biological fertilisers resulted in superior plant growth compared to applying either alone (Moradzadeh et al. 2021). This underscores the critical role of soil microorganisms in optimising N fertiliser use efficiency.

Wheat (*Triticum aestivum* L.) is the third most-produced cereal crop globally, with annual harvests exceeding 750 million tonnes (Hazard et al. 2020; Nations 2022). In Australia, wheat constitutes the primary grain production, averaging approximately 22 million tonnes annually. It is also a crucial export commodity for Australia, with an estimated 65% to 75% of production being exported and accounting for approximately 10% of global wheat exports (Centre 2022). Australia's wheat production plays a vital role in stabilising global food security by compensating for production fluctuations in other regions (Wang et al. 2018). N deficiency severely restricts plant growth and diminishes both the quality and quantity of wheat production. Wheat plants experiencing insufficient N exhibit reduced photosynthetic rates (Kumar and Brar 2021), leading to symptoms such as decreased leaf greenness, smaller leaf sizes, reduced stem height, fewer tillers and heads, and lower grain protein content. The profound impact of N deficiency on wheat grain production is well-documented in the literature; it has been reported that without sufficient N input, wheat production can decrease by more than 30% compared to conditions with adequate N availability (Hochman and Horan 2018). Hence, understanding the plant-soil microbe interaction in wheat is important for potentially improving wheat under N deficiency.

1.2 N metabolism of soil microbe and plant NUE

1.2.1 Soil microbial N-cycle and N fertiliser use efficiency

Soil microbial communities are highly diverse and perform a wide range of essential functions, including the intricate modulation of the N-cycle. This microbial N-cycling directly impacts NUE. Therefore, it is crucial to first discuss the key microbial N transformation processes. The N-cycle comprises multiple interconnected N transformation pathways, including N-fixation, nitrification, NO_3^- reduction, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), anammox, immobilisation, and mineralisation. Among these pathways, some are considered N-loss pathways (e.g., denitrification, nitrification leading to NO_3^- leaching), as they facilitate the loss of N fertilisers to the environment, thereby reducing NUE. Conversely, other pathways (e.g., N-fixation, DNRA, immobilisation) are beneficial in augmenting or retaining the soil N pool, thus holding significant potential for improving NUE.

1.2.1.1 N_2 -fixation

N-fixation is a high-energy-consuming biological process that converts atmospheric dinitrogen (N_2) into plant-available NH_4^+ , typically requiring 16 adenosine triphosphate (ATP) molecules per N_2 molecule. This enzymatic reaction is predominantly facilitated by certain bacteria and archaea through the nitrogenase enzyme complex, often encoded by *nif* genes (Bottomley and Stewart 1977; Hill et al. 1981). Organisms capable of performing N-fixation are collectively known as diazotrophs. Diazotrophs can be further classified as symbiotic or free-living, based on their beneficial relationship with a plant host.

Rhizobium is a well-studied example of symbiotic diazotrophs. Various *Rhizobium* species colonise legume plant roots, stimulating the formation of root nodules where N_2 is fixed for plant uptake. The application of *Rhizobium* has been shown to improve crop growth (Mng'ong'o et al. 2023). To harness the beneficial effects of symbiotic diazotrophs, the rotation of legume and non-legume crops in agricultural management is a common practice to enrich the soil N pool. Such management practices have significantly improved N use efficiency (NUE). Studies have demonstrated that these rotation methods can improve fertiliser NUE via the improvement of organic N-cycling (Breza et al. 2023). However,

robust evidence providing promising strategies and conclusive results for N-fixation benefiting non-legume cereal crops in agriculture remains limited.

In contrast, free-living diazotrophs, also known as planktonic diazotrophs, are soil bacteria that fix N_2 independently of direct plant association. Although free-living diazotrophs do not directly supply N to the plant host, they indirectly benefit plant growth. For example, some studies suggest that free-living diazotrophs enrich the bulk soil N pool content (Norman and Friesen 2017). Given their less stringent reliance on specific plant hosts, there is increasing research interest in their potential benefits for non-legume crops. Examples of free-living diazotrophs include bacterial species from genera such as *Azotobacter*, *Azospirillum*, *Herbaspirillum*, and *Cyanobacteria* (Ladha et al. 2022). Furthermore, studies indicate that free-living diazotrophs can positively impact the growth of other microbial communities, such as stimulating the proliferation of beneficial fungi and plant growth-promoting rhizobacteria (PGPR), which subsequently benefit plant growth (Kennedy et al. 2004).

1.2.1.2 N mineralisation

Beyond N fixation, microbial decomposition significantly enriches the pool of plant-available N through the degradation of organic materials, such as plant residues. Mineralisation, also known as ammonification, is a crucial step within this decomposition process, converting organic N into inorganic forms, thereby increasing plant-available N in the soil (Li et al. 2017).

A diverse array of bacterial and fungal species is known to possess the capability to degrade various organic compounds. This metabolic process involves numerous genes and enzymes (Raza et al. 2023). For instance, the *gdh* gene, encoding the glutamate dehydrogenase enzyme, plays a significant role in converting glutamate to α -ketoglutarate and ammonia (NH_3), consequently increasing plant-available N in the soil. Similarly, the *ure* gene family, which encodes the urease enzyme responsible for urea hydrolysis, catalyses the breakdown of urea into NH_3 , thereby substantially boosting plant-available N in the soil. While these genes and their encoded enzymes are instrumental in transforming organic N into forms usable by plants, their overall impact on N fertiliser use efficiency can be variable and complex, warranting further investigation (He et al. 2020).

1.2.1.3 Nitrification

The nitrification process, which transforms NH_4^+ into NO_3^- , significantly influences plant N uptake. Studies have demonstrated that different plant species exhibit varying uptake preferences for specific N forms, a preference that is primarily mediated by the presence and activity of root transporters, including both NH_4^+ and NO_3^- transporters (Glass et al. 2002). For example, NO_3^- is commonly reported as the preferred N source for wheat (*Triticum aestivum* L.) (Li et al. 2022b). Nitrification is a multi-step microbial transformation process. Key genes involved in this pathway include *amo*, which encodes NH_3 monooxygenase, *hao*, encoding hydroxylamine oxidoreductase, and *nxr*, encoding nitrite oxidoreductase (Levy-Booth et al. 2014).

Since NO_3^- is highly water-soluble, it is often prone to leaching, especially under irrigation. Consequently, some studies consider the nitrification pathway a significant contributor to N loss (Beeckman et al. 2018). It has been estimated that >30% of applied fertiliser N can be lost through leaching (Hussain et al. 2020). This substantially reduces the NUE of fertilisers. One strategy to mitigate this loss is to reduce nitrification, thereby minimising leaching. The enzymes involved in nitrification are primarily NH_3 monooxygenase and nitrite oxidoreductase. Chemists have targeted such enzymes to create inhibitors that slow down the nitrification process. An example of a commercially available nitrification inhibitor is DMPP (3,4-dimethylpyrazole phosphate). This product has been shown to reduce overall N loss by >50% (Friedl et al. 2020).

1.2.1.4 Denitrification

The denitrification process is a multi-step pathway that reduces NO_3^- to gaseous forms of N, which are then released into the environment. This pathway begins with the reduction of NO_3^- to NO_2^- via nitrate reductase, an enzyme encoded by the *nar* gene. Subsequently, NO_2^- is further reduced to nitric oxide (NO) through nitrite reductase, encoded by the *nir* gene. NO is an unstable compound and is then further reduced to N_2O , which is released into the atmosphere (Liu et al. 2020b). Notably, N_2O gas is a potent greenhouse gas. It was estimated that 4.5 kg N ha^{-1} of N were lost through denitrification annually in a legume-based dairy-farm pasture (Luo et al. 2000). Furthermore, a proportion of N_2O will be reduced to N_2 gas through nitrous oxide reductase, an enzyme encoded by the *nos* gene (Liu et al. 2020b).

Although N_2 gas can theoretically be returned to the soil system through N fixation, this amount is typically small in comparison to the N removed. Since denitrification ultimately removes reactive N from the plant and soil system, it is often commercially considered a significant N loss pathway. It is commonly reported that there is an increased detection of N_2O emissions under N fertilisation (Li et al. 2022a).

1.2.1.5 Dissimilatory nitrate reduction to ammonium (DNRA)

DNRA is a crucial anaerobic microbial pathway that plays a significant role in N cycling by reducing NO_3^- to NH_4^+ . This process stands in direct competition with denitrification for available NO_3^- and NO_2^- as electron acceptors in anaerobic environments (Zhao et al. 2025). The DNRA pathway is characterised by two primary enzymatic steps: First, NO_3^- is reduced to NO_2^- by various nitrate reductases, including periplasmic enzymes (e.g., encoded by the *napA* gene) and membrane-bound complexes (e.g., encoded by the *narGHJ* operon). The subsequent, critical step involves the direct, eight-electron reduction of NO_2^- to NH_4^+ . This key conversion is predominantly catalysed by multi-heme cytochrome c nitrite reductases, most notably those encoded by the *nrfA* gene (or *nrfAH* operon) (Pandey et al. 2020; Wang et al. 2024a). While other nitrite reductases, such as NADH-dependent nitrite reductases (e.g., encoded by *nirB* genes), can also reduce NO_2^- , the *nrfA*-mediated pathway is recognised as the canonical dissimilatory route for NH_4^+ production. DNRA is generally favoured under conditions of high carbon-to-nitrogen (C/N) ratios, thereby implying the recirculation of N even under low N availability. (Yang et al. 2025).

1.2.2 Beneficial soil microbe under N deficiency

One direct strategy to mitigate N limitation in agricultural systems is to increase the N content in the soil. Beyond the addition of chemical fertilisers, certain microorganisms can significantly benefit plant growth under low N conditions (Chen et al. 2019; Dellagi et al. 2020) (Table 1). For instance, diazotrophs are a group of microbes capable of performing biological N fixation (BNF), a process that converts atmospheric N_2 gas into plant-available ammonia (NH_3) via the nitrogenase enzyme complex. The activity of nitrogenases is typically controlled by *nif* genes, which are commonly targeted to assess the N-fixation capabilities of microbes (Dellagi et al. 2020). Plants experiencing N deficiency stress can actively recruit diazotrophs, thereby enhancing the N content in the surrounding soil. A diverse range of

microbial genera are recognised as diazotrophs, including *Azospirillum*, *Herbaspirillum*, *Paenibacillus*, *Rhizobium*, *Nostoc*, *Azotobacter*, *Stenotrophomonas*, and *Frankia* (Chen et al. 2019; Dellagi et al. 2020) (Table 1).

Besides direct N acquisition, microbes can also alleviate abiotic stresses by improving overall plant nutrient acquisition. Some fungi from the phylum Glomeromycota, commonly known as arbuscular mycorrhizal fungi (AMF), can stimulate alterations in plant root architecture to enhance N acquisition (Rodriguez et al. 2019). Similarly, a recent study identified *Massilia Oxalobacteraceae* as a bacterium capable of stimulating lateral root development in maize under N deficiency, thereby improving N uptake. Interestingly, *Massilia Oxalobacteraceae* achieves this without producing phytohormones or 1-aminocyclopropane-1-carboxylate (ACC) deaminase, instead mediating an alternative pathway that alters the balance between cell proliferation and differentiation during lateral root development (Yu et al. 2021) (Figure 1). However, further evidence is required to fully elucidate whether other microbial mechanisms can also stimulate root development under N deficiency stress.

Beyond directly increasing soil N content or enhancing N acquisition, microbes may also improve the N uptake efficiency of plants. A recent study investigated the root microbiota of two rice cultivars (*Oryza sativa sp. Japonica* and *Oryza sativa sp. Indica*) that exhibit contrasting NUE. This difference in NUE in rice is known to be partly attributed to the *NRT1.1B* gene, which encodes a NO_3^- transporter and sensor. Using advanced genomic techniques, this study provided evidence that the *NRT1.1B* transporter in rice coordinates the root microbiota, potentially contributing to overall plant N acquisition (Zhang et al. 2019). This finding suggests that the intricate interaction between plant N transporters and associated microbes could be a key factor influencing rice NUE, a mechanism that warrants further investigation for its potential application in improving plant NUE under N stress.

While microbes can increase soil N content and enhance N acquisition, they may also enhance other plant functions that confer a broader benefit to plant growth and stress tolerance. A functional genomic approach has uncovered an intriguing interaction between the bacterium *Stenotrophomonas maltophilia* and the intronless *AhCytb6* gene in peanut (*Arachis hypogaea*) under N deficiency stress. It was suggested that *Stenotrophomonas maltophilia* BJ01 could enhance the expression of the *AhCytb6* gene, which has been associated with positive effects on seed germination efficiency and health under both salinity

and N stress. Overexpression of the *AhCytb6* gene led to altered gene expression across various categories, including metabolism, transporters, photosynthesis, abiotic stress responses, receptor/kinase/signaling pathways, transcription factors, and antioxidant systems, suggesting a complex tolerance mechanism (Alexander et al. 2021). These results provide compelling evidence that microbes can influence plant functional genetics, leading to a cascade of functional changes that confer N stress tolerance. Future studies should delve deeper into the molecular pathways regulated by *AhCytb6* to fully elucidate these N tolerance mechanisms.

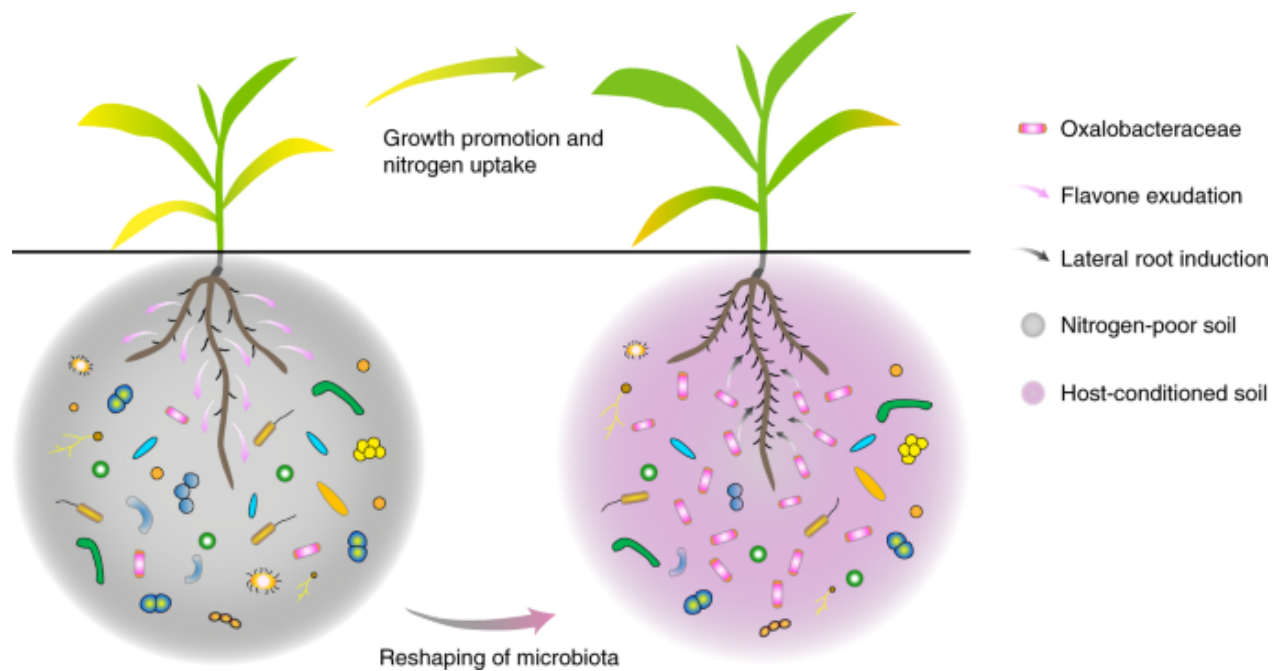


Figure 1. Proposed model for flavone-dependent, microbiota-mediated lateral root formation and plant performance (Yu et al. 2021).

Table 1. Beneficial microbes under N stress and their mechanisms.

Genera	Species	Strain	Plant	Mechanism	Reference
<i>Azopirillum</i>	<i>sp.</i>		Maize, Rice, Wheat, Finger Millet, Sorghum, Bajra, Oil seeds, angiosperms and crops	N-fixation	(Dellagi et al. 2020; Mahmud et al. 2021)
<i>Azotobacter</i>	<i>sp.</i>		Mustard, Sunflower, Grapes, Sugarcane, Banana, Watermelon, Papaya, Coconut, Plantation and Forest crops	N-fixation	(Mahmud et al. 2021)
<i>Frankia</i>	<i>sp.</i>		Actinorhizal plants	N-fixation	(Ma et al. 2015)
<i>Glomeromycota</i>	<i>sp.</i>		angiosperms and crops	N uptake stimulation	(Ma et al. 2015)
<i>Herbaspirillum</i>	<i>sp.</i>		angiosperms and crops	N-fixation	(Ma et al. 2015)
<i>Massilia</i>	<i>Oxalobacteraceae</i>		<i>Zea mays L.</i> (Maize)	specific microbe-mediated pathway alters the balance between proliferation and differentiation during lateral root development	(Yu et al. 2021)
<i>Nostoc</i>	<i>sp.</i>		Marine, aquatic and terrestrial plants	N-fixation	(Mahmud et al. 2021)
<i>Bacillus</i>	<i>sp.</i>		angiosperms and crops	N-fixation	(Dellagi et al. 2020)
<i>Pseudomonas</i>	<i>fluorescens</i>	ACC50 & ACC73	<i>Triticum aestivum L.</i> (Wheat)		(Shaharouna et al. 2008)
<i>Rhizobium</i>	<i>sp.</i>		<i>Fabaceae sp.</i> (Legume), Leguminous crops such as Cowpea, Gram, Pea, G/nut, Soybean, Berseem, Lucerna, <i>Parsaponia sp.</i> (Non-Legume)	N-fixation	(Dellagi et al. 2020; Mahmud et al. 2021)
<i>Stenotrophomonas</i>	<i>meliloti</i>		Alfalfa (lucerne)	N-fixation	(Alexander et al. 2019)
	<i>maltophilia</i>	BJ01	<i>Arachis hypogea L.</i> (Peanut)	N-fixation, Increase gene expression of AhCytb6 gene	

1.3 Limitations of current biofertiliser

1.3.1 Host-specific interaction

While symbiotic plant-microbe interactions offer significant agricultural potential, harnessing these relationships effectively in diverse cropping systems remains challenging. A primary obstacle is the phenomenon of host-specific interaction, which describes the unique ability of different plant hosts to interact with specific soil microorganisms. This specificity varies considerably across plant species and even among cultivars of the same species (Wippel et al. 2021; Yue et al. 2024).

Evidence consistently demonstrates that the root-associated microbial community structures differ substantially between legume and non-legume plants, with legume-associated communities generally exhibiting a higher abundance or activity of N-fixing microbes. A study comparing the soil microbial communities of *Arabidopsis thaliana* and *Lotus japonicus* explicitly proved that soil microbial communities interact preferentially with particular plant species, thereby highlighting the profound influence of host-specific interaction (Wippel et al. 2021). This host specificity extends beyond distinct plant species to encompass variations among cultivars within the same plant species. For example, it has been reported that root-associated microbial communities vary significantly among different wheat cultivars (Chen et al. 2019), and even between wild and domesticated wheat populations (Yue et al. 2023). Similarly, research on rice cultivars has revealed substantial differences in the functional profiles of their root-associated microbial communities (Zhang et al. 2019). Collectively, such host-specific interactions significantly limit the broad-spectrum effectiveness of bioinoculants in agricultural systems.

Challenges associated with beneficial microbes in agriculture are not limited to microbe-microbe competition and suppression. Plants also interact closely with soil microbes in ways that can restrict the performance of beneficial inoculants. In agricultural soils, plant-available N exists in soluble inorganic forms (NO_3^- , NH_4^+ , NO_2^-) and various organic N forms. Proteins, as a complex form of organic N, are typically digested by proteases into smaller amino acid molecules for plant uptake (Nasholm et al. 2009). However, some plants, such as *Arabidopsis*, can directly absorb intact proteins without prior digestion (Paungfoo-Lonhienne et al. 2008). Growing evidence indicates two important paradigms: (i) plants can utilise organic N sources, such as amino acids, in addition to inorganic N (Paungfoo-

Lonhienne et al. 2008); and (ii) plants actively compete with microorganisms for N, rather than merely utilising residual N left over by soil microbes (Dunn et al. 2006; Schimel and Bennett 2004).

A study investigating N uptake in grass species (*Anthoxanthum odoratum* L. or *Festuca rubra* L.) with varying competitive abilities for N, utilised ^{15}N labelling techniques. The results revealed that plant species proficient at acquiring nutrients from the soil are not necessarily better at tolerating increased competition with microbes for these nutrients. More specifically, intense microbial competition may exert more adverse effects on the uptake of organic N by plants compared to inorganic N (Dunn et al. 2006). Therefore, the addition of beneficial microbes could potentially intensify the burden on plants to compete for nutrients, a factor that requires thorough examination before these microbes are widely deployed as biofertilisers.

In addition to nutrient competition, the efficacy of beneficial microbial applications is also limited by plant species specificity. A recent study employing a cross-inoculation method with *Lotus japonicus* and *Arabidopsis thaliana* observed a host preference phenomenon for root microbiota recruitment (Wippel et al. 2021) (Figure 2). This strongly suggests that the recruitment of specific microbes is often plant dependent. Rhizodeposition, defined as the loss of all plant-derived materials from roots, including the release of dead cells and the secretion of metabolites through root exudation, is a key driver for the selective recruitment of microbes. Root exudates comprise diverse classes of carbon compounds, including carbohydrates, amino acids, organic acids, flavonols, lignins, coumarins, auronnes, glucosinolates, anthocyanins, indole compounds, fatty acids, sterols, allomones, proteins, and enzymes (Badri and Vivanco 2009). Certain microbes are capable of chemotactically moving towards these plant-derived carbon compounds and utilising root-derived rhizodeposits as an energy source (Verbon and Liberman 2016). Thus, rhizodeposition actively shapes the plant-microbe interaction by favouring the recruitment of specific soil microbes, a topic that will be further discussed. Ultimately, the host-specific nature of microbial recruitment and composition limits the universal performance of beneficial microbes across different plant species.

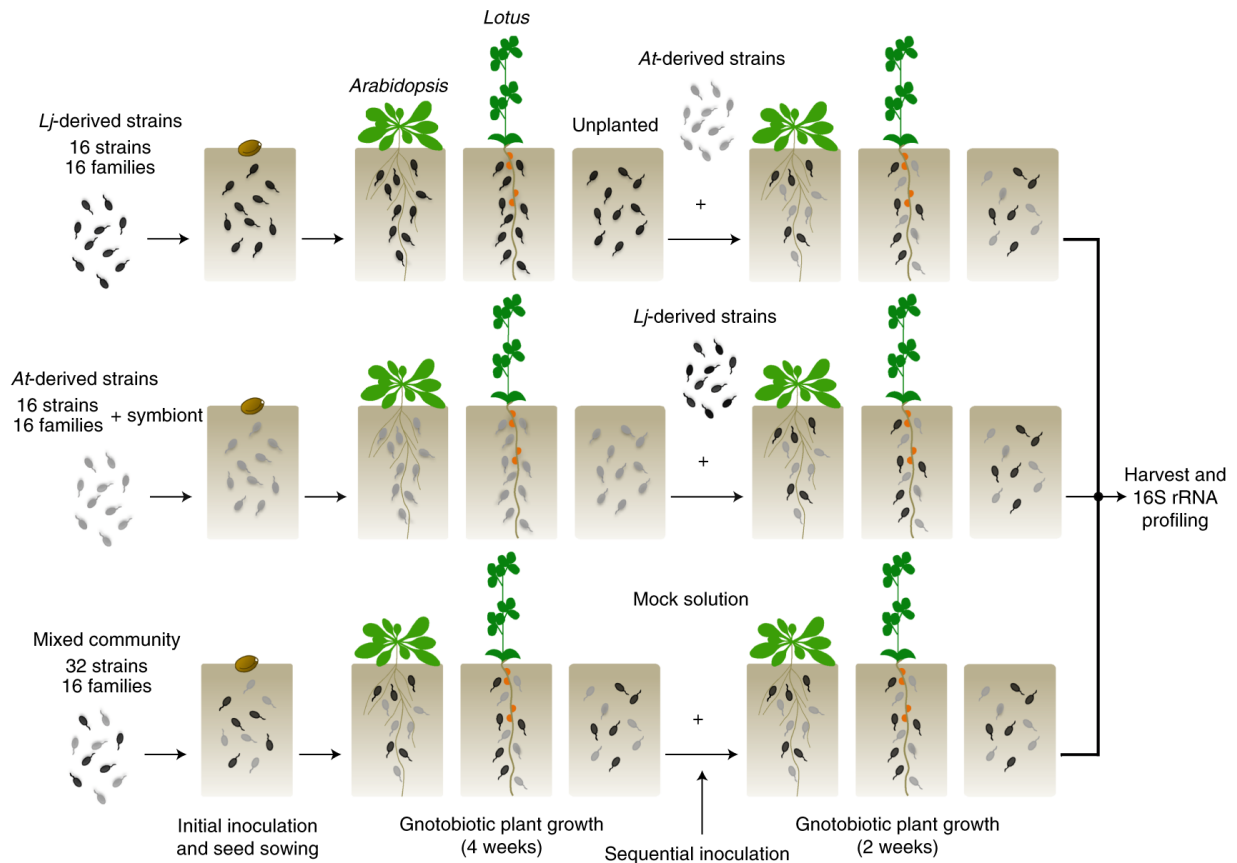


Figure 2. Cross-inoculation and co-inoculation of *Lotus japonicus* and *Arabidopsis thaliana* microbiota (Wippel et al. 2021).

1.3.2 Indigenous microbial suppression

Other than the selective interactions exerted by plant hosts, a significant barrier limiting the efficiency of biofertilisers is the microbe-microbe suppression originating from the indigenous microbial community. Soil microbiota represents a dynamic and complex network of microorganisms, stabilised by intricate microbe-microbe interactions. A key mechanism underpinning these interactions is quorum sensing (QS), which describes the process of bacterial cell-to-cell communication involving the production, detection, and response to extracellular signalling molecules or metabolites (Whiteley et al. 2018).

N-acyl homoserine lactone (AHL) is a well-studied QS molecule primarily produced by Gram-negative bacteria. AHLs have been extensively investigated and shown to act as signalling molecules not only within and between bacterial species but also between bacteria and higher organisms. Apart from QS molecules, microbes also produce microbial-produced

volatile organic compounds (mVOCs) such as terpenes, indoles, alkenes, and short-chain fatty acids (Liu and Brettell 2019). In Gram-positive bacteria, species-specific QS is predominantly facilitated through small peptides. Autoinducer-2 (AI-2), a furanosyl borate diester, has also been reported as a signal for interspecies communication (March and Bentley 2004). However, the full extent of interspecies interaction mediated by these mechanisms is still being elucidated.

Quorum sensing mechanisms have been extensively studied in both human and plant-associated microbial communities. In agricultural contexts, *Bacillus spp.* and *Pseudomonas spp.* are among the most extensively studied beneficial microorganisms in the rhizosphere (Sun et al. 2022b). Species from these genera can provide multiple beneficial effects; for example, *Pseudomonas putida* and *Bacillus subtilis* have been shown to confer both drought and salinity stress tolerance to plants. A study illustrated a cooperative microbe-microbe interaction between *Bacillus velezensis* SQR9 and *Pseudomonas stutzeri* XL272 within the cucumber rhizosphere. This research demonstrated that *B. velezensis* could stimulate the recruitment of *P. stutzeri* to form biofilms, with exopolysaccharides and *TasA* protein identified as important mediators for such cooperation (Sun et al. 2022b).

Conversely, microbial inhibition, the inverse of microbial stimulation, also occurs within soil communities. In addition to abiotic stress tolerance, microbes protect plants against biotic stresses, which negatively influence plant growth. Microbial inhibition is frequently harnessed for biocontrol strategies against plant pathogens. Beneficial non-pathogenic microbes can directly interact with pathogens by secreting chemical metabolites that suppress their growth or render them avirulent, thereby protecting the host plant (Enebe and Babalola 2020). For instance, a Gram-positive microbe *Micromonospora* obtained from the root nodules of legumes exhibited direct biocontrol by inhibiting the growth of various fungal pathogens. This microbe also induced jasmonic acid (JA) signalling defences in tomato plants exposed to the fungus *Botrytis cinerea* (Martinez-Hidalgo et al. 2015).

Furthermore, bacteria can indirectly benefit plant growth by preventing the proliferation or activity of plant pathogens through competition for space and nutrients (Badri et al. 2009), as well as by inducing the plant immune system (Yu et al. 2019). Microbes can activate microbe-associated molecular pattern (MAMP)-triggered immunity (MTI), a defence mechanism that, while limiting pathogen proliferation, can also curtail plant growth, a phenomenon known as the growth–defence trade-off (Yu et al. 2019). A recent study

inoculated *Arabidopsis thaliana* plants with the pathogen strain *Pseudomonas* 401, alongside either suppressive or non-suppressive commensals. The findings proposed that a delicate balance between suppressive and non-suppressive commensals is essential to achieve optimal plant growth and defence against pathogens (Ma et al. 2021a) (Figure 3). Consequently, the introduction of exogenous beneficial microbes into an established soil microbiota can disrupt the existing QS network, potentially leading to either positive or negative outcomes. Often, introduced microbes are suppressed or even eliminated by the indigenous soil microbiota, which significantly limits the successful application of beneficial microbes in agricultural settings.

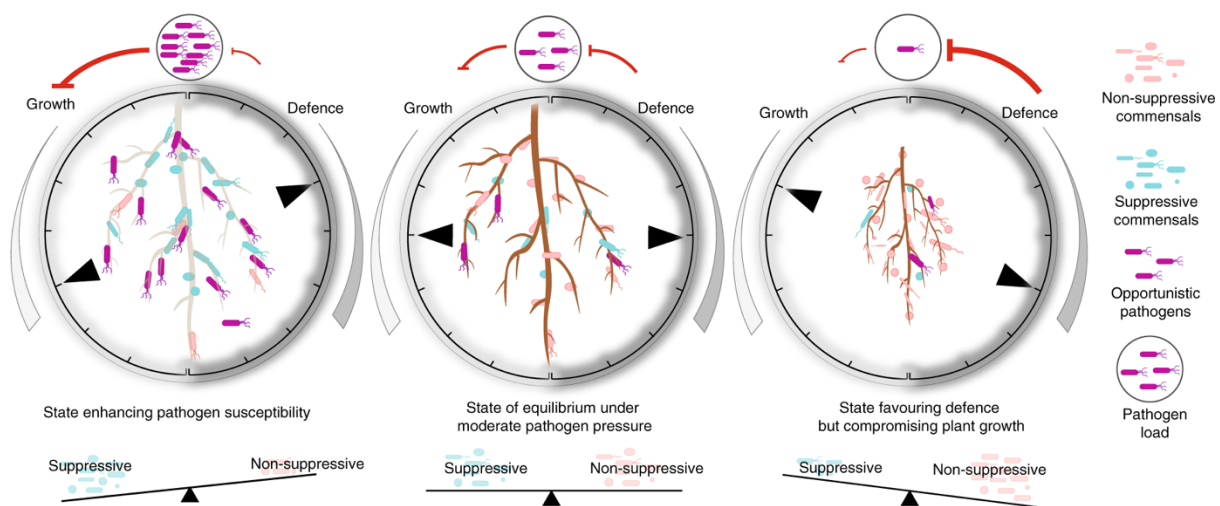


Figure 3. Proposed model that the balance between non-suppressive and suppressive strains integrates with plant innate immunity and buffers the system against pathogen challenge and defence-associated trade-off (Ma et al. 2021b; a).

1.3.3 Dynamic environment

Beneficial microbes that demonstrate efficacy under controlled laboratory conditions frequently encounter limitations or outright failure when applied in the field. A primary cause of this discrepancy lies in the uncontrolled and highly variable environmental conditions prevalent in agricultural settings, such as fluctuations in rainfall, temperature, light intensity, humidity, and nutrient availability (Liu and Brettell 2019). These dynamic factors can

directly influence the soil microbiome by activating, suppressing, or even eliminating specific microbial populations. Furthermore, they can indirectly impact the soil microbiome by altering plant physiology, which in turn influences the root exudates and microenvironments that shape microbial communities.

A study identifying beneficial microbes from the rhizosphere and roots of sorghum grown under high and low N field conditions exemplifies this challenge. While the beneficial microbial consortium was designed to approximate the natural microbiota of the rhizosphere and root endosphere, field trials demonstrated that the same groups of bacteria did not consistently perform optimally under varying field conditions (Chai et al. 2021). The multitude of interacting environmental factors influencing the soil microbiota in the field presents a significant challenge to accurately predicting the effects of introduced beneficial bacteria on plant performance.

Ultimately, despite the discovery of numerous beneficial microbes capable of assisting plants under abiotic stresses, their successful application as biofertilisers continues to face limitations. The intricate relationship between microbes, plants, and the environment forms an inseparable three-component system. Consequently, the introduction of exogenous beneficial microbes can interfere with the efficacy and stability of this established tripartite interaction. This understanding provides critical insight for biofertiliser development, emphasising that successful strategies must consider all three interactions: (i) beneficial bacteria with other microbes in the community, (ii) beneficial bacteria with the host plant, and (iii) beneficial bacteria with the prevailing environment (Figure 4).

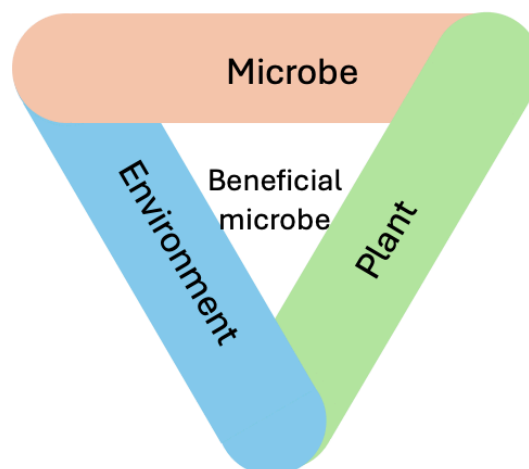


Figure 4. schematic diagram of beneficial microbe limitations.

1.4 Next-generation strategy

1.4.1 Considerations for microbial selection for biofertiliser development

The intricate and complex relationships among microbes, plants, and their environment pose significant challenges to the effective application of beneficial microorganisms in agriculture. Nevertheless, increasing evidence suggests that a microbial consortium, rather than a single strain, can provide more flexible and robust beneficial effects to plants. This has led to an expansion of research into the synthetic community (SynCom) approach, where artificially generated microbial communities are designed for specific agricultural applications, particularly as biofertilisers.

For instance, a study demonstrated that co-inoculation of PGPR and diazotrophs significantly improved root growth in wheat (*Triticum aestivum* L.) and potentially enhanced its ability to cope with environmental stresses (Dal Cortivo et al. 2017). However, despite this promise, a standardised methodology for designing effective SynComs remains elusive. The limitations associated with the application of beneficial microbes mentioned above further complicate their successful formulation as biofertilisers. Therefore, this section will discuss critical considerations for biofertiliser development from both microbial and plant-centric perspectives.

1.4.1.1 Plant-associated microbes

Beyond the intricate interactions among microorganisms, plants also engage in intensive interactions with microbes. The rhizosphere, defined as the narrow zone of soil directly influenced by plant roots, is known for its high abundance of PGPR and is consequently a frequent target for identifying beneficial microbes (Philippot et al. 2013). While beneficial microbes are prevalent in the rhizosphere, they are also found within plant tissues as endophytes. It is hypothesised that beneficial microbes capable of transitioning from the rhizosphere to colonise the plant endosphere have successfully navigated the plant immune system, thereby increasing their likelihood of conferring beneficial effects (Compant et al. 2010). Reviews indicate that microbes colonise different plant compartments, including seeds, roots, shoots, and leaves, with varying affinities (Compant et al. 2010; Finkel et al. 2017). Furthermore, even microbes from the same taxonomic family can benefit plants through non-identical mechanisms (Ferrara et al. 2012). This complexity highlights the growing interest in identifying and studying endospheric PGPR. Beneficial microbes have

been identified through divergent approaches, considering various factors such as different plant components, distinct growth stages, and diverse cultivars. Several examples and perspectives on these discoveries will be discussed below.

Root-Associated Microbiome: Direct and Dynamic Interactions

In addition to seedborne microbes, microorganisms from the rhizosphere can also enter and colonise the internal tissues of plants, conferring plant growth-promoting effects. Generally, endospheric bacteria are more likely to exhibit significant plant growth-promoting effects compared to bacteria that exclusively colonise the rhizosphere (Compant et al. 2010). Consequently, researchers have also focused on root endophytes for beneficial microbe discovery. The penetration process does not necessarily involve active microbial mechanisms; thus, many rhizosphere bacteria can potentially become endophytic at some stage of their life cycle (Compant et al. 2010; Hardoim et al. 2008). Passive penetration can occur at sites of root emergence, at cracks or wounds created by mechanical damage or deleterious microorganisms, or through root tips. However, specific adaptations have evolved for certain bacteria, such as root nodulation of bacteria, or those possessing specialised mechanisms for active penetration of the root system (Compant et al. 2010). Importantly, the successful penetration of microbes is often stimulated or facilitated by the plant itself, and plants under abiotic stress may uniquely recruit and permit the entry of specific stress-tolerant microbes. A pot experiment investigated the effects of drought on the root microbiome of rice plants (*Oryza sativa* ssp. *japonica* variety M-206), revealing distinct changes in microbial diversity within both the root endosphere and rhizosphere. Notably, a taxon of *Streptomyces* (OTU 1037355) became most abundant in the root endosphere following drought treatments. Seed inoculation with this *Streptomyces* taxon subsequently resulted in significantly longer roots (Santos-Medellin et al. 2021). This study provides a compelling example of a beneficial microbe specifically targeting the root endosphere under drought conditions.

Regarding nutrient deficiency stress, a study demonstrated that the P starvation response in *Arabidopsis thaliana* is closely related to its root microbiome. A 35-member SynCom, primarily derived from *Brassicaceae* roots, was inoculated onto *A. thaliana*. Transcriptomic analysis of *A. thaliana* revealed an enrichment of the transcriptional regulator *PHR1*, which is responsible for *PHR1* promoter binding motifs and genes involved in biological processes related to P starvation response. The results provided an example of a

root endophyte microbiome that facilitates nutrient stress tolerance, strongly supporting the continued investigation of the root microbiome for beneficial microbes (Castrillo et al. 2017). Interestingly, the inoculation of this SynCom showed a negative effect on shoot orthophosphate (the primary form of P in plants) accumulation under low orthophosphate conditions. This suggests that while plants and microbes can interact for enhanced nutrient acquisition, an environment without sufficient nutrient input will still ultimately limit plant growth. This finding aligns with a previous study that observed optimal plant growth from a combination of chemical and biological fertilisers (Moradzadeh et al. 2021).

A recent study on beneficial root microbes under N stress demonstrated that *Massilia Oxalobacteraceae* stimulated lateral root development in maize under N deficiency. Intriguingly, this effect occurred without the production of known phytohormones or ACC deaminase activity, both commonly associated with root development stimulation. This suggests a specific microbe-mediated pathway that may alter the balance between proliferation and differentiation during lateral root development (Yu et al. 2021). Due to the significant beneficial effects observed from root endospheric microbes, the investigation of beneficial microbes for SynCom development should strongly target the root endophyte. Numerous studies have elucidated the dynamic interaction between plants and their associated microbes. While microbes can penetrate roots to form a root endospheric microbiome and contribute to seeds with an adapted microbiome, the plant itself also interacts profoundly with its external environment. Review studies have summarised bacterial diversity across bulk soil, rhizosphere, and different plant compartments, consistently showing an enrichment of Proteobacteria and Firmicutes as one moves from bulk soil to plant-associated niche (Trivedi et al. 2021). The significant compositional difference between bulk soil and the rhizosphere is primarily driven by plant-microbe interactions, with the chemical compounds in root exudates hypothesised to be the main cause. Furthermore, at different plant growth stages, the root exudation process can vary, leading to distinct microbial recruitment patterns (Chaparro et al. 2014) (Figure 8). A more detailed discussion of plant-soil microbe interaction will follow.

Recent research has emphasised the importance of eco-evolutionary processes in the assembly of plant-associated microbiomes, including dispersal, selection (where biotic and abiotic effects cause fitness differences), ecological drift, and diversification (Cordovez et al. 2019). Both stochastic colonisation and historical contingency may play essential roles in

structuring the plant-associated microbiome. Rare taxa are postulated to provide a robust reservoir of ecological functions and could drive priority effects in microbiome assembly during the early stages of plant development, with variable consequences for plant performance at later stages (Trivedi et al. 2021). Under abiotic stress, plants experience different levels of stress at various growth stages and may interact uniquely with their microbiome. Thus, an approach of discovering beneficial microbes at different growth stages might uncover taxa that are growth-stage specific in conferring N stress tolerance, a crucial consideration when generating dynamic SynComs tailored to plant development.

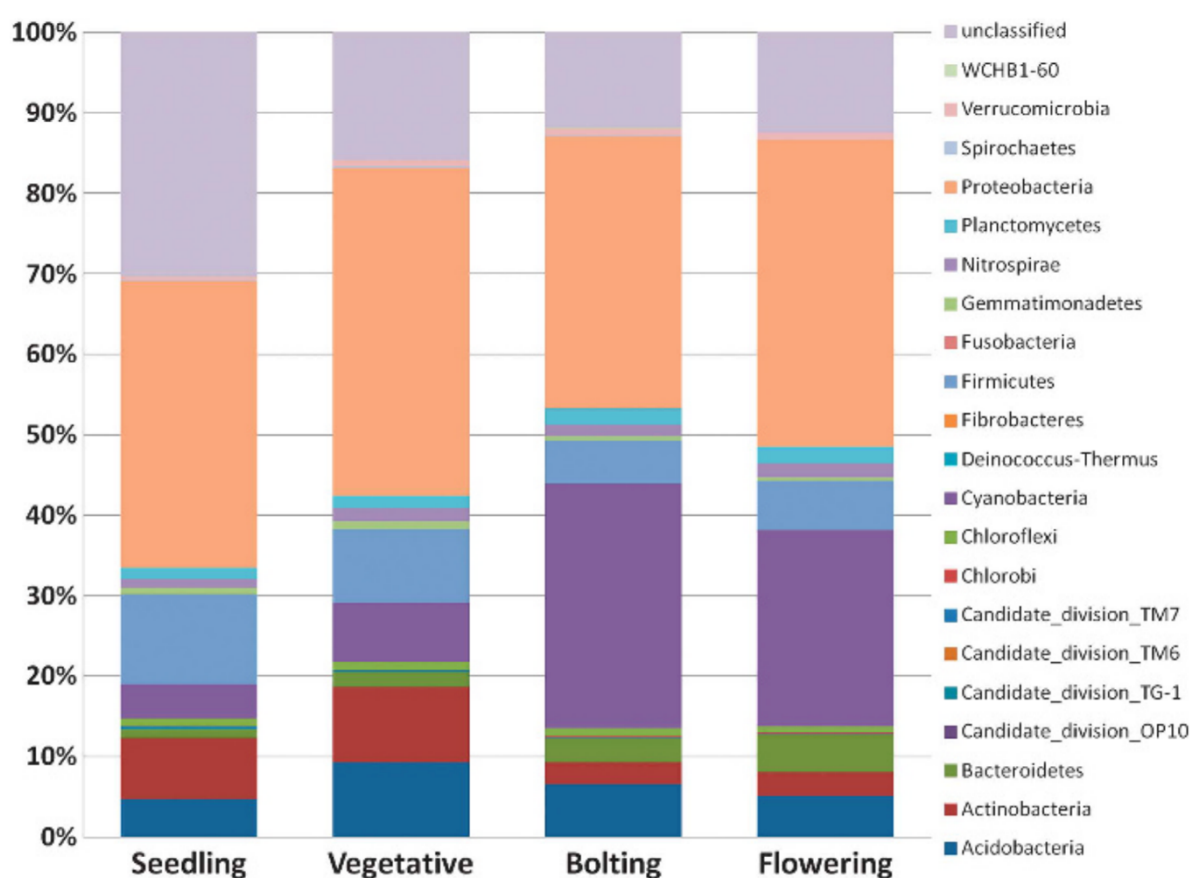


Figure 5. Relative abundance (%) of the major bacterial phyla present in the rhizosphere microbial community at each plant developmental stage (Chaparro et al. 2014).

1.4.1.2 Plant growth stages

The profound effect of plant development on the rhizosphere microbiome has been demonstrated in numerous studies. For instance, an investigation into the bacterial

community of field wheat rhizosphere at different growth stages revealed significant compositional differences between samples collected at various developmental points. This study further explored the impact of PGPR on wheat growth. A significant divergence in the bacterial community structure between control and PGPR bio-inoculation treatments indicated that seed PGPR inoculation substantially altered the bacterial community throughout the wheat's growth stages (Roesti et al. 2006). Similarly, a long-term field experiment focusing on both epiphytic and endophytic leaf and root microbes of *B. stricta* plants showed significant differences in microbial richness and composition over the host's lifetime (Wagner et al. 2016).

Given that microbial succession during early plant development is crucial for establishing the foundation for mature plant growth, many studies have specifically investigated the effects of PGPR at these nascent stages. One such study isolated five bacterial strains from the rhizosphere of field-grown wheat and barley—identified as *Bacillus megaterium* RC07, *Bacillus licheniformis* RC08, *Paenibacillus polymyxa* RC05, *Rhodobacter capsulatus* RC04, and *Pseudomonas putida* RC06—all possessing either N-fixation or P-solubilisation abilities. When these strains were inoculated with crops, the results indicated a stronger influence on root, leaf, and sugar content during the early phases of growth (Cakmakci et al. 2006). Additionally, the PGPR *Azospirillum* has exhibited a strong stimulatory effect on root development and exudation. Pre-inoculation of the strain *Azospirillum lipoferum* CRT1 significantly altered the bacterial community of maize rhizosphere grown in the field, specifically showing changes in rhizobacterial community structure during the early stages of maize growth (Baudoin et al. 2009). Another investigation into the same bacterial strain corroborated these findings, demonstrating similar changes in the early stages of maize growth and further substantiating its role in stimulating root development (El Zemrany et al. 2007).

Microbes can influence plant growth throughout both early and later developmental stages. A study examining the rhizobacterial community of field-grown *Arabidopsis thaliana* at different growth stages found that transcripts aligned to microbes involved in plant growth promotion or N fixation were more abundant during later growth stages (Chaparro et al. 2014). Collectively, these examples demonstrate that different PGPR may confer benefits to plants at various developmental time points. Consequently, when formulating SynComs, it is essential to consider incorporating PGPR that target specific growth stages.

Numerous studies have consistently shown that the rhizosphere microbiome is actively shaped during plant development via root exudation. In the same *Arabidopsis thaliana* study mentioned previously, plants grown in vitro were subjected to root exudate analysis. This revealed that both the rhizobacterial community and the root exudation profiles differed significantly across growth stages, lending strong support to the hypothesis that root exudates are a primary factor shaping the rhizobacterial community (Chaparro et al. 2014). It is also important to note that the N requirement for plant growth varies significantly at different developmental stages (Chen et al. 2014) (Figure 9), leading to different levels of N stress. This varying demand for N could subsequently affect root exudation patterns and, by extension, the composition of the rhizobacterial community.

Recent research has emphasised the importance of eco-evolutionary processes in the assembly of plant-associated microbiomes, such as dispersal, selection (where biotic and abiotic effects cause fitness differences), ecological drift, and diversification (Cordovez et al. 2019). Both stochastic colonisation and historical contingency may play essential roles in structuring the plant-associated microbiome. Rare taxa are postulated to provide a robust reservoir of ecological functions and could drive priority effects in microbiome assembly during the early stages of plant development, with variable consequences for plant performance at later stages (Trivedi et al. 2021). Under abiotic stress, plants experience different levels of stress at various growth stages and may interact uniquely with their microbiome. Thus, an approach of discovering beneficial microbes at different growth stages might uncover taxa that are growth-stage specific in conferring N stress tolerance, a crucial consideration when generating dynamic SynComs tailored to plant development.

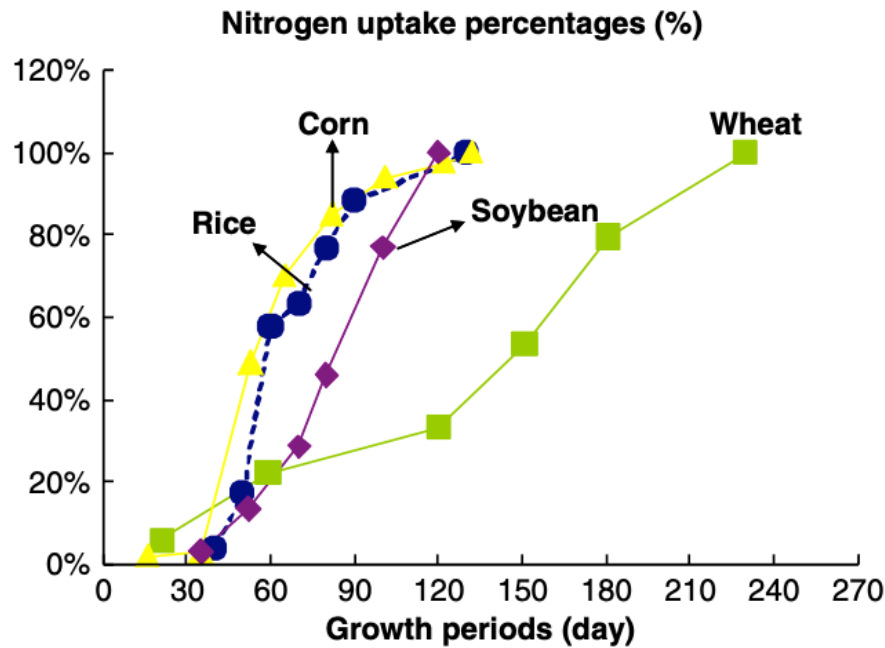


Figure 6. N uptake percentages for the different growth stages of different crops (Chen et al. 2014).

1.4.1.3 Plant genotype

Another factor that significantly influences the bacterial community associated with plants is plant genotype. Distinct genotypes can lead to diverse plant morphology, physiology, and responses to the environment, thereby differentially shaping the rhizobacterial community (Berg and Raaijmakers 2018). For instance, a study investigating *Arabidopsis* genotypes known to exhibit varying levels of *Pseudomonas fluorescens* growth reduction demonstrated that a reduction in this beneficial bacterium did not necessarily correlate with an increase in pathogen infection. The authors concluded that plant genotype is an essential determinant of whether a microbiome community confers benefit or harm to its host (Haney et al. 2015). While the rhizobacterial community has been significantly influenced by plant genotype, similar influences on both epiphytic and endophytic leaf and root microbiomes of *B. stricta* plants have also been illustrated (Wagner et al. 2016).

Plants exhibiting different NUE are commonly employed to study plant responses to varying N availability. High NUE plants generally possess an advantage in growing under low N environments (Hirose 2011). Consequently, genotypes with differing NUE are hypothesised to respond distinctly to N stress conditions. An investigation into the

rhizobacterial community of rice cultivars with high (V6 and V7) and low (V10 and V15) NUE revealed a higher abundance of *Rhodomicrobium* and *Holdemanella* in the high NUE cultivar, with *Rhodomicrobium* being a known diazotroph. This finding highlights changes in the rhizobacterial community associated with different NUE genotypes. Furthermore, targeted RT-qPCR analysis demonstrated that certain N-cycling genes, specifically *nirK*, *nirS*, and *nosZ* (responsible for denitrification), exhibited lower abundance in the rhizosphere of the high NUE cultivar, implying reduced N loss in the soil (Chen et al. 2022). This suggests that specific microbes in the rhizosphere may promote N-cycling processes that enhance N availability for plant growth.

Functional genes in maize with high (Lo5) and low (T250) NUE were also investigated using a meta-transcriptomic approach. Consistent with the aforementioned rice study, both the rhizobacterial community composition and gene expression profiles were influenced by genotype. The Lo5 rhizosphere was characterised by higher expression of genes involved in N mineralisation processes, such as *ureC* and *hao*, whereas the rhizosphere of the T250 line exhibited higher expression of *norB/C* and *nosZ* genes (indicative of denitrifying activity). It has been proposed that faster inorganic N depletion in the rhizosphere of the Lo5 maize line could favour higher N mineralisation, while longer retention of inorganic N in the rhizosphere soil of T250 could support higher denitrification processes (Pathan et al. 2018) (Figure 10). These significant differences in bacterial community and function underscore the profound influence of plant genotype on the microbiome in the context of N stress. Therefore, a high NUE cultivar may recruit distinct microbes that facilitate better stress tolerance, offering a valuable resource for creating N stress-tolerant SynComs.

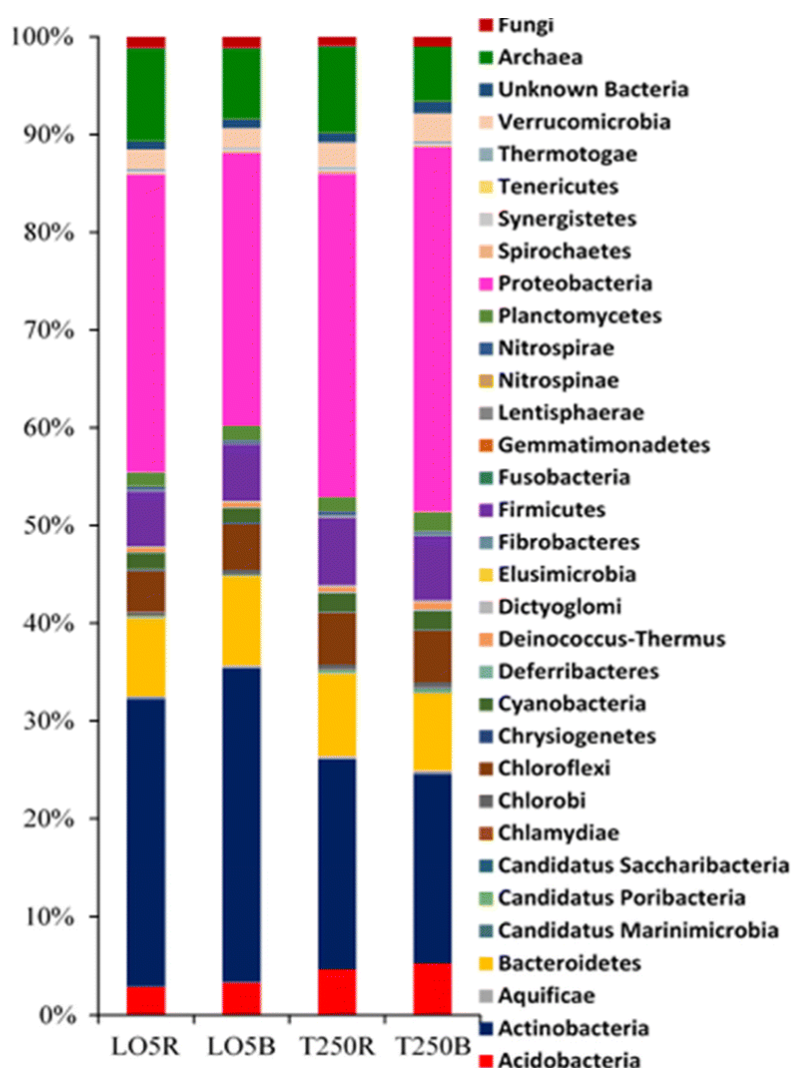


Figure 7. Relative abundance of taxonomic affiliation for maize Lo5 (high) and T250 (Low) (Pathan et al. 2018).

1.4.2 Harnessing plant metabolites signals

Beneficial microbes are critical for optimal plant growth. Numerous studies have demonstrated that plants interact intricately with microbes to achieve a variety of beneficial effects on their development. These complex interactions between plants and microbes can generally be classified into three categories: (i) plant-to-microbe, (ii) microbe-to-microbe, and (iii) microbe-to-plant (Macabuhay et al. 2022; Trivedi et al. 2021). While microbes can confer benefits to plant growth, plants themselves actively influence their associated microbial communities. Specifically, root exudation by plants plays a significant role in attracting and recruiting beneficial microbes to the rhizosphere (Korenblum et al. 2020; Middleton et al. 2021) (Figure 11).

Root exudation represents a substantial carbon cost to the plant; young seedlings, for instance, typically exude approximately 30–40% of their fixed carbon as root exudates (Badri and Vivanco 2009). These exudates primarily consist of carbon-based compounds, although they also contain released ions, inorganic acids, oxygen, and water. The organic compounds in root exudates are broadly categorised into two classes: (i) low-molecular weight compounds and (ii) high-molecular weight compounds (Badri and Vivanco 2009). Low-molecular weight compounds encompass a range of primary and secondary metabolites. Primary metabolites are directly involved in fundamental metabolic processes, while secondary metabolites are indirectly involved. Both classes of metabolites are crucial for microbe and plant growth. However, based on current knowledge, the distinction between primary and secondary metabolites is not solely limited by molecular weight and can be challenging to delineate definitively (Pichersky and Gang 2000).

Certain microbes exhibit chemotactic behaviour, moving towards plant-derived carbon compounds and utilising them as an energy source (Schulz-Bohm et al. 2018; Verbon and Liberman 2016). Plant-microbe interactions can be broadly categorised as positive or negative. Positive interactions include symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia, and PGPR. Conversely, negative interactions involve associations with parasitic plants, pathogenic microbes, and invertebrate herbivores (Badri and Vivanco 2009). Reviews suggest that plants under abiotic stress undergo changes in their morphology and physiology. These alterations can lead to distinct root exudation patterns, thereby recruiting unique bacterial consortia that confer abiotic stress tolerance (Liu et al. 2020a; Zhang et al. 2020).

Despite these observations, research into the detailed interactions between plants and microbes has been hindered by challenges in detecting and characterising root metabolites. However, advancements in metabolomics approaches have significantly propelled research in metabolite detection, thus facilitating a deeper understanding of plant-microbe interactions. Nevertheless, studies specifically targeting plant-microbe interactions under N stresses remain notably limited (Hong et al. 2022). A range of important compounds found in root exudates has been reviewed, and some of the most significant ones will be discussed in this section (Badri and Vivanco 2009; Sasse et al. 2018; Uroz et al. 2019; Vives-Peris et al. 2020).

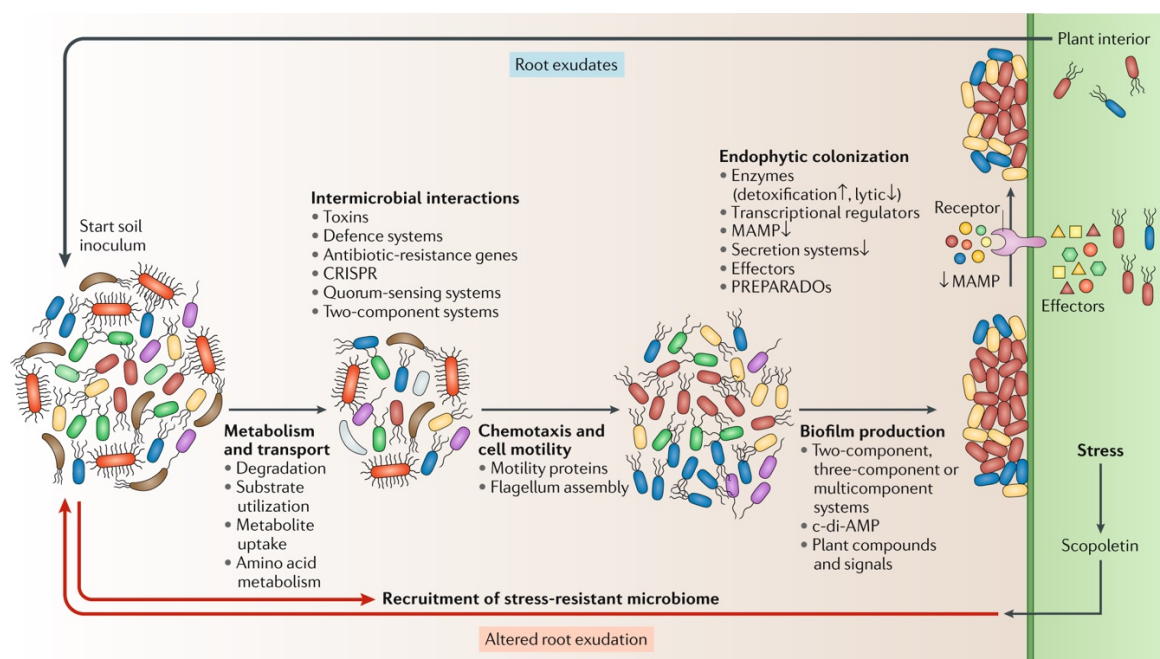


Figure 8. Plant colonisation and microbiome assembly (Trivedi et al. 2021).

1.4.2.1 Primary metabolites

Carbohydrates (sugar)

Sugars constitute a significant fraction of root exudates and serve as a primary carbon source for rhizosphere microbes (Sasse et al. 2018). The secretion of carbohydrates and specific carboxylates, such as malate and oxalate (often exuded in high quantities), is mediated by protein-based transport mechanisms across membranes, rather than simple diffusion (Vives-Peris et al. 2020). Sugars Will Eventually Be Exported Transporters (SWEETs) are a family of sugar uniporters. Intriguingly, SWEET mutant plants, which exhibit altered root sugar homeostasis and higher sugar export from roots compared to wild-type plants, have also been shown to be more susceptible to disease (Sasse et al. 2018). Despite their role in sugar transport within the plant, no transporters directly exporting sugars into the rhizosphere have been definitively characterised to date, leading to ongoing debate about whether sugar exudation is primarily a transport-driven process (Jones et al. 2009; Sasse et al. 2018). However, potential evidence for passive sugar efflux is supported by observations of higher sucrose concentrations around young, more permeable root tissue compared to older, less permeable root tissue (Sasse et al. 2018).

Sugars are a product of photosynthesis, synthesised primarily in plant leaves. N is an essential element for the synthesis of photosynthetic components. Therefore, N deficiency can lead to leaf senescence and negatively affect plant sugar synthesis, consequently altering the sugar content in root exudates (Paul and Driscoll 1997; Wingler and Roitsch 2008). This interconnectedness makes carbohydrates a critical target for studying plant-microbe interactions, particularly in the context of nutrient stress.

Amino acids

Amino acids are recognised by microbial chemoreceptors and represent a crucial fraction of root exudates, playing a key role in the early stages of root colonisation (Sasse et al. 2018). The plant's modulation of amino acid transport could serve either as a means of communication with microbes or as a direct response to their presence (Sasse et al. 2018). Studies have demonstrated that both the beneficial strain *Pseudomonas fluorescens* Pf0-1 and *Bacillus subtilis* exhibit motility towards all 20 proteinogenic amino acids. Furthermore, mechanisms in both *P. fluorescens* and *Pseudomonas putida* KT2440 reveal them to be proficient catabolisers of amino acids, particularly aspartate, glutamate, asparagine, and glutamine (Sonawane et al. 2003). Amino acids also play a significant role in the synthesis and regulation of auxin phytohormones, which are instrumental in numerous plant processes, including root development and architecture. Their presence in the rhizosphere can also be attributed to rhizosphere-dwelling bacteria, many of which synthesise indole-3-acetic acid (IAA), a common auxin (Moe 2013).

Given that N is a fundamental building block of amino acids, N deficiency profoundly affects the composition and quantity of exuded amino acids. For example, studies with maize plants cultured under low N availability have shown a reduction in amino acid exudation (Vives-Peris et al. 2020). The demonstrated participation of amino acids in plant and beneficial microbe interactions highlights them as a promising area for further investigation.

Organic acids

Beyond sugars and amino acids, organic acids constitute a significant fraction of root exudates and serve as crucial sources of microbial nutrients. The release of organic acids like

malate and citrate, often exuded in high quantities, is mediated by specific transporters such as Aluminium-Activated Malate Transporters (ALMTs) and Multidrug and Toxic Compound Extrusion (MATE) families. This exudation is frequently modulated by the presence of metal ions and microbes (Sasse et al. 2018).

Organic acids have been consistently shown to interact with various beneficial microbes. For instance, L-malic acid secreted from plant roots can specifically attract *Bacillus subtilis* FB17. Similarly, malic acid and citric acid in watermelon root exudates are known to recruit *Paenibacillus polymyxa* SQR-21 (Vives-Peris et al. 2020). A study investigating standard organic acids (oxalic acid, malic acid, citric acid, succinic acid, and fumaric acid) in cucumber and banana seedlings demonstrated that the distinct composition of root exudates directly correlated with the chemotactic response, biofilm formation, and colonisation behaviour of bacterial strains originating from their respective rhizospheres (Zhang et al. 2014).

While organic acids are not always directly linked to N metabolism, it is noteworthy that studies have shown low N fertilisation not only reduces amino acid exudation but also increases the presence of organic acids in root exudates (Carvalhais et al. 2013). Furthermore, the importance of organic acids as phytohormones, such as salicylic acid, jasmonic acid, and ethylene, in mediating biotic and abiotic stress responses has been extensively reviewed (Bari and Jones 2009). Research indicates that these phytohormones play a pivotal role in mediating microbe colonisation and inducing systemic plant immune responses (Lebeis et al. 2015). This suggests that abiotic stress can significantly influence the profile of organic acids in root exudates, and this influence is intricately linked to the N availability for the plant, thereby affecting plant-microbe interactions.

Nucleotides/purine

Plant small RNAs (sRNAs) are short nucleotide sequences categorised primarily as microRNAs (miRNAs) or small-interfering RNAs (siRNAs). Generally, miRNAs originate from single-stranded RNA precursors forming distinctive stem-loop structures, whereas siRNAs are processed from longer double-stranded RNAs. These double-stranded RNAs can be derived from inverted repeats, sense-antisense transcript pairs, or products of RNA-dependent RNA polymerases. Plant siRNAs are further classified into various types based on

their distinct biogenesis pathways, including trans-acting siRNAs or secondary phased siRNAs, natural antisense transcripts-derived siRNAs, heterochromatic siRNAs, and long siRNAs (Huang et al. 2019).

While nucleotides are known to be imported by secondary active transporters, their precise exudation mechanisms remain elusive. Extracellular ATP is well-established as a signalling molecule, and ATP-binding cassette (ABC) transporters have been proposed to mediate its cellular export (Rees et al. 2009; Sasse et al. 2018). Plant endogenous sRNAs play a crucial regulatory role in symbiotic plant-microbe interactions. For example, *miR169* regulates nodule differentiation; its association with polysomes decreases upon rhizobia infection, thereby elevating targeted HAP levels to promote nodulation (Huang et al. 2019). Additionally, the *miR171* family of miRNAs regulates the *Nodule Signalling Pathway 2* (*NSP2*) gene, the *LOST MERISTEMS 1* (*LOM1*) gene, and the *TOO MUCH LOVE* suppressor to protect and promote AMF symbiosis. This family is observed to be involved in AMF symbiosis under phosphate starvation conditions and in the early nodule organogenesis associated with diazotrophs (Huang et al. 2019). Other miRNAs also contribute to plant symbioses, often related to auxin response and organogenesis. For instance, *miR396* targets Growth-Regulating Factor genes to promote AMF infection. Furthermore, *miR393j-3p* represses the expression of *ENOD93* during rhizobia-soybean symbiosis to control meristematic activity during nodule development (Huang et al. 2019).

Beyond these examples of indirect interactions, studies have also revealed that sRNAs can directly exchange between kingdoms. The seminal discoveries that fungal sRNAs can be transferred into plant cells to promote virulence, and vice versa that plants deliver sRNAs to fungal pathogens as part of their defence program, have added a new level of complexity to our understanding of plant disease (Cai et al. 2018; Frantzeskakis et al. 2020; Weiberg et al. 2013). In both scenarios, the transmitted sRNAs can provoke gene silencing in the respective recipient organism (Frantzeskakis et al. 2020). The first documented instance of actual bi-directional cross-kingdom RNA interference through sRNA trafficking was described between *Arabidopsis thaliana* and the fungal pathogen *Botrytis cinerea* (Wang et al. 2016). Opinion studies suggest that miRNAs shape at least a portion of the rhizosphere microbiota. With more fundamental research proving the exchange of miRNAs between plants and the rhizosphere microbiota, the use of miRNA-encoded peptides (miPEPs) to stimulate the synthesis and release of specific beneficial plant microRNAs could emerge as a promising

tool for various applications in ecological engineering. The use of miPEPs offers an indirect method to modulate the rhizosphere microbiota, potentially obviating the need for exogenous inoculum additions (Middleton et al. 2021). As N is a fundamental component of nucleotides, N availability could significantly influence the quantity and profile of miRNAs in root exudates.

Fatty acid/Lipid

Various forms of lipids, including fatty acids and sterols, also significantly enrich the rhizosphere chemistry (Andersson and Hughes 2014). Fatty acid transport is essential for mycorrhizal symbiosis; mycorrhizal fungi are dependent on their hosts for the synthesis of certain fatty acids, and the current model proposes the transport of lipids by ABCG proteins within the symbiotic membrane (Sasse et al. 2018). As major components of the plasma membrane, lipids establish physical barriers on living cell surfaces, influence communication between the host and microbe, and serve as signalling molecules or providers of elicitors for recognition. Consequently, they profoundly influence the establishment or prevention of microbial colonisation (Chapman 1998; Macabuhay et al. 2022). A recent review highlights the diverse roles of lipids in planta and at different stages of plant root–microbe interactions, encompassing: (i) signalling and resource exchange; (ii) pattern recognition, signal transduction, and downstream defence mechanisms during perception and contact; and (iii) the establishment of symbiosis that can either promote plant growth and stress tolerance or lead to host death (Macabuhay et al. 2022).

During abiotic stress responses, certain lipids function as crucial secondary messengers. Phospholipase-derived phosphatidic acid, oxylipins, phosphoinositides, sphingolipids, fatty acids, lysophospholipids, N-acyl ethanolamines, and galactolipids have all been identified as operating in this capacity (Macabuhay et al. 2022). Hopanoid lipids, for example, are found in both plants and bacteria. Numerous studies across diverse bacterial species indicate that hopanoids facilitate bacterial survival under various stress conditions (e.g., high temperatures, low pH, high osmotic pressures, and during antibiotic or detergent treatments), although the specific beneficial conditions vary among bacterial strains. Furthermore, many hopanoid-producing bacteria are capable of N fixation, including *Anabaena* spp., *Azotobacter* spp., *Beijerinckia* spp., *Bradyrhizobium* spp., *Burkholderia* spp.,

Frankia spp., and *Nostoc spp.* While it has long been suggested that hopanoids facilitate N fixation metabolism, few direct tests of this hypothesis have been performed to date (Belin et al. 2018). Although lipids have been shown to influence both plant-microbe and microbe-microbe interactions, research specifically on lipids in the context of root exudates, particularly under N stress conditions, remains limited. The insights gained from studies on hopanoids, however, provide a valuable starting point for future lipidomic investigations of root exudates under N stress conditions.

1.4.2.2 Secondary metabolites

Flavonoids

In plants, flavonoids are involved in a wide array of processes, including plant–pathogen interactions, pollination, light screening, seed development, and allelopathy (Hernandez et al. 2009). Many flavonoid biosynthetic genes are induced under stress conditions, and consequently, flavonoid levels increase during exposure to both biotic and abiotic stresses, such as wounding, drought, metal toxicity, and nutrient deprivation. Flavonoids have been suggested to act as antioxidants, protecting plants from oxidative stress (Hernandez et al. 2009). These compounds are considered crucial root-released rhizosphere signal molecules that modulate the interaction of roots with microbes. Genes like flavone synthase (FNS) and chalcone synthase colorless2 (C2) are critical for flavonoid biosynthesis in maize. Furthermore, root flavonoids are essential for initiating symbiosis with rhizobia in legumes and also function as auxin transport regulators. This implies that flavonoid-mediated root–microbe interactions might modulate developmental processes within the host plant itself (Yu et al. 2021).

The involvement of flavonoids in plant-microbe interactions extends beyond pathogens to include microbes that provide beneficial effects, such as *Bradyrhizobium japonicum*, a well-known diazotroph. Flavonoids also stimulate mycorrhizal spore germination and hyphal branching, and influence quorum sensing (Philippot et al. 2013). A study on the Chinese tallow tree (*Triadica sebifera* L. Small) identified quercetin, a specific flavonoid, that significantly increased root fungal colonisation in seedlings (Tian et al. 2021). Additionally, research demonstrated that flavonoids broadly contribute to the diversity of the *Arabidopsis* root microbiome, with a particular emphasis on the attraction of

Aeromonadaceae. Specifically, *Aeromonas* sp. H1 showed increased colonisation on *Arabidopsis* pap1-D, a dominant mutant known for over-accumulating flavonoids, including anthocyanins and flavonols. Naringenin, a key intermediate molecule in flavonoid biosynthesis and a flavanone common in both legumes and non-legumes, was shown to facilitate this interaction (He et al. 2022).

While studies have shown that plant flavonoid secretion is affected by abiotic or biotic stresses, and that flavonoids can attract microbes, direct proof of abiotic stress directly shaping the microbiome via plant flavonoids is less common. However, a study investigated the interaction between maize root flavonoids and rhizosphere bacteria under N deprivation. In this research, *Colorless2-Inhibitor diffuse* (*C2-Idf*) mutants, which display highly reduced levels of apigenin-related flavonoids compared to C2 wild-type plants, were grown under N-poor conditions. When *C2-Idf* mutants were subsequently transplanted to N-poor soil where C2 wild-type plants had previously grown, reduced N deficiency symptoms were observed in the leaves of the *C2-Idf* mutants. Coupled with rhizosphere bacterial community analysis, these findings suggested that under N stress conditions, maize increases the expression of the flavone synthase-encoding gene *FNSI2*, leading to increased flavone secretion. The secreted flavones, in turn, attracted *Oxalobacteraceae*, which stimulated lateral root development to help the plant cope with N stress (Yu et al. 2021). These examples collectively demonstrate that flavonoids are an important class of metabolites to investigate in the context of plant-microbe interactions, especially under nutrient stress.

Volatile organic compounds (VOCs)

In addition to soluble secondary compounds, plants release various volatile organic compounds (VOCs) that are crucially involved in interactions with surrounding soil organisms. It is estimated that VOCs constitute about 1% of plant secondary metabolites. VOCs are small, lipophilic compounds with low molecular weight, high vapor pressure, and low boiling points (Schulz-Bohm et al. 2018). Plant VOCs are typically released in response to biotic stresses, such as herbivore and pathogen attack, and abiotic stresses caused by environmental fluctuations (Kong et al. 2021).

Reviews have provided several compelling examples of plant VOCs interacting with beneficial microbes (Fincheira and Quiroz 2018). For instance, 2R,3R-butanediol has been

shown to induce systemic drought tolerance in *Arabidopsis thaliana* seedlings via salicylic acid-ethylene and jasmonic acid signalling pathways (Cho et al. 2008). Furthermore, *Bacillus subtilis* GB03 enhances salt tolerance in *A. thaliana* seedlings through the regulation of the sodium transporter *HKT1*. This transporter is up-regulated in the shoot and down-regulated in the root, leading to a low accumulation of salt in the plant (Zhang et al. 2008). It has also been reported that VOCs released by a *Pseudomonas simiae* strain induce salinity stress tolerance in *Glycine max* L. Merrill. In this case, a reduction in Na concentration, an increase in K concentration, and accumulation of proline were observed, providing evidence of osmotic protection. The study further revealed that 4-nitroguaiacol and quinoline, specifically, induced seed germination of *G. max* under salinity stress conditions, accompanied by higher chemotaxis and altered root exudates (Fincheira and Quiroz 2018). A study utilising a glass olfactometer system and the SynCom technique demonstrated that VOCs emitted by plant roots play an important role in the long-distance attraction of bacteria from bulk soil (Schulz-Bohm et al. 2018). This long-distance attraction of microbes via VOC signalling is not limited to belowground interactions but is also linked with processes in the plant's aboveground parts (Kong et al. 2021).

While substantial evidence indicates that plant VOCs shape the rhizosphere microbiome, studies have also revealed that microbes themselves release VOCs that directly influence plant growth. For example, it was discovered that (-)-thujopsene, a low-abundant sesquiterpene in the emission profile of *Laccaria bicolor*, was sufficient to stimulate lateral root formation in both plant systems (maize and *Arabidopsis*) even in the absence of the fungus or when the fungus's sesquiterpene biosynthesis was inhibited by lovastatin (Ditengou et al. 2015). Given that robust root development is a crucial mechanism for nutrient acquisition and abiotic stress tolerance, this finding prompts further consideration of how VOCs might contribute to N stress tolerance.

1.4.3 The next-generation fertiliser approach

Beneficial microbes' roles under abiotic stress and in plant-microbe interactions have been thoroughly discussed. Moving forward, other directions that could potentially improve the development of biofertilisers warrant consideration. The application of plant-microbe interactions could extend beyond biofertiliser design to also inform the development of novel

chemical fertilisers. While the addition of N is crucial for ensuring crop productivity, reducing N loss is equally important for retaining N in the soil and maximizing its availability for plant growth. A recent perspective study proposed that a new generation of urease inhibitors, nitrification inhibitors, and controlled-release coatings is essential for achieving sustainable agriculture. Two scenarios based on such an approach have been suggested (Lam et al. 2022) (Figure 14): (i) incorporating plant signals (metabolites) into fertiliser coatings to recruit beneficial microbes that enhance N acquisition; and (ii) incorporating receptors into fertiliser coatings that release N when exposed to specific plant signals.

Lipids have been suggested as a novel field of plant metabolites to explore, given their essential role as cell wall components, such as phospholipids (Macabuhay et al. 2022). The first scenario, involving the incorporation of plant signals into fertiliser coatings, might be achievable if these lipid metabolites could be used to create a cell-wall-like coating. For the second scenario, utilising biological receptors as sensors for plant metabolites could enable dynamic interaction with the plant, especially in response to stress conditions. However, synthesizing *de novo* a receptor for this purpose could be challenging. A more efficient approach might involve isolating existing receptors from microbes that naturally respond to specific metabolites. Another challenge would be successfully implanting biological receptors into a chemical coating. Aligning with the first scenario's suggestion, isolated biological receptors might be more readily incorporated into a lipid-based coating.

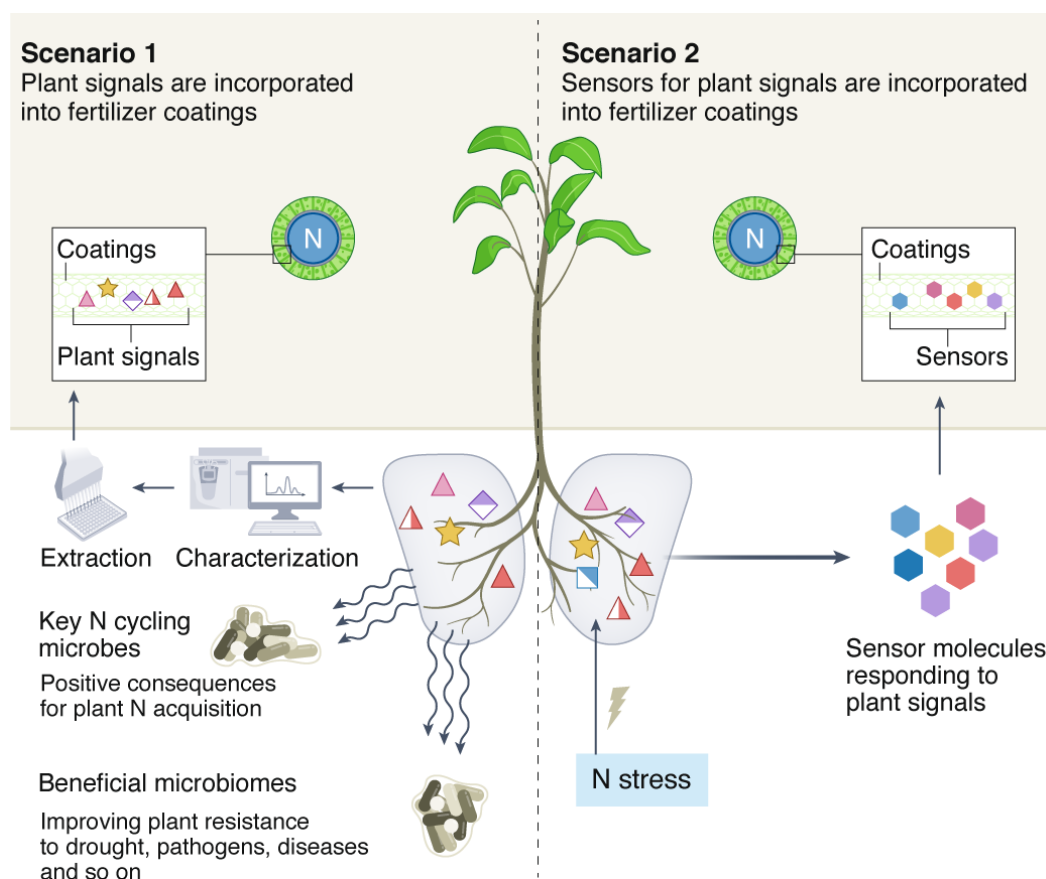


Figure 9. Proposed scenarios that harness plant signals for designing new fertiliser coatings (Lam et al. 2022).

1.5 Plant-microbe interaction under N deficiency

As an element critical for plant growth, N is widely recognised to significantly affect plant root exudation, subsequently altering interactions with the rhizosphere microbiome. N is an essential constituent of chlorophyll, nucleic acids, and proteins. Consequently, N stress impairs many fundamental plant processes, including amino acid biosynthesis, photosynthesis, and the tricarboxylic acid cycle. As a result, the root exudation profiles of N-stressed plants commonly display a marked decrease in amino acids, carboxylates, and sugars (Chai and Schachtman 2022). Studies employing N-cycling genes (*nifD*, *nifH*, *ureC*, *nasA*, *nirA*, *pmoA-amoA*, *hao*, *nirK*, *norB*, *nosZ*) targeted qPCR and *16S* rRNA amplicon sequencing have investigated maize rhizosphere dynamics and activities under N fertilisation. These studies have shown an increased abundance and activity of NH_3 -oxidizing and denitrifying bacteria. Furthermore, results have indicated increased secretion of sugars, sugar

alcohols, and phenolics in maize roots, suggesting that high N-rate fertilisation leads to changes in the rhizobiome (Zhu et al. 2016). Another study on maize rhizosphere and root exudation under N application demonstrated similar results. Community analysis revealed decreased bacterial diversity with increased N application, and the metabolic profile differed significantly, with 27 down-regulated and 40 up-regulated ions observed in high vs. low N treatments (Hao et al. 2022).

N deficiency profoundly restrains plant growth, leading to reduced crop productivity and altered metabolic profiles and rhizosphere communities. Plants can adapt their nutrient acquisition strategies according to N availability, which, in turn, affects the soil microbial community structure, functions, and activities (Hao et al. 2022; Ma et al. 2021a). While the close relationship between soil microorganisms and the plant, facilitated by metabolites, was described as a plant-microbe interaction, the changes in this interaction when adapting to stressed conditions were described as the 'cry-for-help' phenomenon (Chai and Schachtman 2022). Changes in metabolic profiles under N stress have been observed in various plant species. For instance, lower concentrations of sugars were detected in bean plants, and reduced concentrations of amino acids were secreted by the roots of maize, pine, potato, and bean under N-limited conditions (Carvalhais et al. 2013; Zhu et al. 2016).

Secondary metabolites are known to be induced by N stress and function to modulate microbial activity involved in the N cycle, thereby enhancing plant N acquisition (Coskun et al. 2017a). Increased exudation of flavonoids, for example, is commonly observed in both legumes and non-legumes under N stress, facilitating root interactions with N-fixing bacteria (Chai and Schachtman 2022). When exposed to N-limiting conditions, leguminous plants release larger amounts of flavonoids to attract diazotrophs. Phenolic compounds also appear to be important for plants to attract beneficial microbes, as demonstrated by the production of benzoxazinoids (tryptophan-derived secondary metabolites) by maize roots to attract microbes that prime plant defence (Liu et al. 2020a). Specifically, increased levels of the flavones apigenin and luteolin in the maize rhizosphere under N deficiency have been demonstrated to recruit beneficial *Oxalobacteraceae*, which stimulate lateral root development (Yu et al. 2021). In general, under N stress, a reduction in the exudation of primary organic compounds such as organic acids, sugars, and amino acids, coupled with an increase in flavonoids, is typically observed. These changes provide valuable indicators of plant N stress.

1.6 Research questions

Although extensive research has investigated plant-microbe interactions, studies specifically focusing on these interactions under N deficiency stress remain limited. Given the evidence demonstrating host specificity of the microbiome, understanding these plant-microbe interactions becomes even more critical. This research will target wheat (*Triticum aestivum* L.), one of the most widely consumed crops globally and a primary export crop for Australia.

We hypothesise the following:

1. Wheat recruits beneficial soil microorganisms via the secretion of specific metabolites under N deficiency.
2. Wheat cultivars with contrasting NUE interact with soil microorganisms differently under N deficiency.

Based on these hypotheses, we will address the following key research questions:

1. How do soil microorganisms interact with wheat under N deficiency?
2. How does the metabolite profile in wheat change under N deficiency?
3. How do soil microorganisms interact with different wheat cultivars under N deficiency?
4. How does the metabolite profile differ in wheat cultivars under N deficiency?

We have addressed the first and third questions in Chapters 2, 3, and 4, focusing on the wheat root-associated microbial community response under N deficiency. The second and fourth questions have been addressed in Chapter 2 as well, focusing on the wheat root metabolite response to N deficiency.

Chapter 2 - Wheat root-associated bacterial community and metabolite profile under N deficiency

Connecting statements

Chapter 2 initiated our investigation by focusing on how the wheat root-associated bacterial community and wheat root metabolite profile respond to N deficiency. A key objective was also to explore the differential N response of these bacterial communities and metabolite profiles between wheat cultivars exhibiting contrasting reported NUE. To achieve this, we conducted both a controlled pot experiment and a hydroponic pot experiment. These experiments utilised two selected wheat cultivars (Mace-high NUE & Gladius-lowNUE) grown under three distinct N application rates. We employed a comprehensive suite of molecular biology techniques, including quantitative polymerase chain reaction (qPCR), amplicon sequencing, and metagenomic analysis, to study changes in the root-associated bacterial abundance, community composition, and functional profile under N deficiency. Simultaneously, we used Gas Chromatography-Mass Spectrometry (GC-MS) to analyse the non-targeted primary metabolite profile of wheat roots. This work has been published in the journal *Plant and Soil* and titled “Cultivar-specific wheat-associated bacterial communities and metabolite in response to nitrogen deficiency”.

Cultivar-specific wheat-associated bacterial communities and metabolites in response to nitrogen deficiency

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Keywords:

Nitrogen-use efficiency, Wheat, Plant-microbe interaction, Metagenomics, Metabolomics

Abstract**Background and Aims**

Nitrogen (N) deficiency in soil constrains plant growth, which beneficial soil bacterial communities may potentially alleviate. However, there is limited knowledge of the plant-bacteria interactions of wheat cultivars with different N-use efficiency (NUE) under N deficiency.

Methods

We investigated the responses of soil and root endosphere bacterial communities as well as root metabolites of two wheat cultivars (cv. Mace and Gladius) with reported high and low NUE, respectively, using a glasshouse experiment and a hydroponic experiment with three N levels.

Results

The rhizosphere bacterial community of Mace shifted under N deficiency but not in its root endosphere. Conversely, the rhizosphere bacterial community of Gladius remained unchanged under N deficiency but shifted in its root endosphere. The metagenomic analysis illustrated increased detection of genes related to bacterial growth and motility in the rhizosphere of Mace, but not of Gladius, under N deficiency. A four-fold increase in octadecanoic acid in the root of Mace, but not Gladius, under N deficiency, suggesting the potential role of octadecanoic acid in shaping the rhizobacterial community in Mace with higher reported NUE.

Conclusion

Our study highlights the divergent responses of wheat-associated microorganisms and root metabolites to N deficiency in the two cultivars. We found that wheat cultivars with higher NUE increased octadecanoic acid secretion, potentially shaping the rhizobacterial communities and enhancing their growth under N-limited conditions.

Abbreviations:

N – Nitrogen

NUE – Nitrogen Use Efficiency

ASVs – Amplicon Sequence Variants

COGs - Clusters of Orthologous Genes

PCoA – Principal Coordinate analysis

PCA – Principal Components analysis

ANOVA – Analysis Of Variance

ANOSIM – Analysis Of Similarity

PERMANOVA - Permutational Multivariate Analysis of Variance

CPM - count per million

PGPR- Plant Growth Promoting Rhizobacteria

Introduction

Nitrogen (N) is essential for plant growth and development (Andrews et al. 2004), and its deficiency in soil has reduced the yield of some major crops, including wheat (Guo et al. 2022; van Grinsven et al. 2023; Xu et al. 2022). Synthetic N fertilisers promote crop growth, but their mismanagement adversely affects soil microbial community structure (Ding et al. 2020; Enebe and Babalola 2020; Yang et al. 2022) and environmental quality (Canfield et al. 2010; Pahalvi et al. 2021). Alternative strategies that reduce dependency on chemical fertilisers without compromising crop yield should be explored. Microorganisms establish mutualistic associations with plants by obtaining essential carbon sources from plant metabolites (Bai et al. 2022). In return, they enhance plant nutrient acquisition, thereby improving plant adaption to diverse environmental conditions (Ahlawat et al. 2022; Nerva et al. 2022; Pang et al. 2020). Although bio-fertilisers leverage the beneficial effects of soil microorganisms to improve plant growth (Bhardwaj et al. 2014) and soil health (Sun et al. 2020), the efficacy of bioinoculants remains inconsistent (Fagorzi et al. 2023). Harnessing plant metabolites to modulate soil microbial interactions in the development of next-generation fertilisers potentially enhances bio-fertiliser efficiency (Lam et al. 2022). However, our understanding of the interaction between plant metabolites and soil microbiomes under N deficiency remains limited (Hong et al. 2022). Addressing this knowledge gap is crucial for advancing sustainable agricultural practices and optimising fertiliser strategies under N-deficient conditions.

The "cry-for-help" theory posits that plants under stress alter their physiological processes and immune responses, actively recruiting microbes to adapt to challenging conditions (Castrillo et al. 2017; Liu et al. 2020; Song and Haney 2021). Substantial evidence suggests the notion that plants recruit soil microorganisms to withstand extreme environmental conditions such as drought, heat and salinity (Fadiji et al. 2023; Hong et al. 2022; Kumar and Verma 2018). N deficiency poses stress on plant growth and influences the metabolic interaction with microbes (Chai and Schachtman 2022). In non-leguminous plants, the modulation of the N cycle by soil bacteria has been shown to benefit plant growth under low-N conditions (Chai and Schachtman 2022; Coskun et al. 2017). For example, maize grown in N-limited soil enhanced the contribution of soil bacteria to the N cycle (Jiang et al. 2023). This finding has also been recently validated in *Viola epidendroides* and *Baptisia macrantha*, suggesting that plants experiencing N deficiency maintain close associations with diverse N-cycling microorganisms (Camargo et al. 2023). In addition to regulating the N cycle, a recent study provided evidence that maize recruits *Massilia* sp. to enhance N-use efficiency (NUE) under N deficiency. This enhancement is accomplished through the secretion of flavones, which are identified as chemotactic attractants for these microorganisms. While the study did not detect the production of plant growth hormone, it confirmed that *Massilia* sp. stimulated lateral root growth. The study also proposed that *Massilia* sp. altered the balance between the proliferation and differentiation of root cells (Yu et al. 2021). This highlights the critical role of plant metabolites in recruiting soil bacteria to shape a beneficial microbiome, particularly in adapting to N deficiency (Haney et al. 2015).

Advances in breeding and domestication have improved plant root growth, development (Zhang et al. 2019) and morphology (Cormier et al. 2016). These advancements enhance plant N acquisition (Chen et al. 2021) and reshape the interactions between plants and microbes (Gholizadeh et al. 2022; Quiza et al. 2023; Yue et al. 2023). Consequently, this results in improved NUE and increased resilience in low-N conditions (Chen et al. 2020). Notably, studies indicate that maize breeding lines with varying NUE distinctly influence the

rhizosphere microbiome, particularly concerning N turnover (Pathan et al. 2015). Microbes can enhance plant NUE through various mechanisms, including increased microbial diversity (Zhang et al. 2019), enhanced N-cycling processes (Zhang et al. 2019), and alterations in root architecture (Yu et al. 2021). While rhizobacteria play a crucial role in plant NUE, their sensitivity to diverse soil fertility conditions must also be acknowledged (Chai et al. 2021). A growing body of evidence reveals variations in the metabolome among different plant species (McLaughlin et al. 2023) and cultivars/varieties (Kogel et al. 2010; Zheng et al. 2021). However, our understanding of genotypic variation in plant-microbe interactions in response to N deficiency remains limited. Further investigations into these interactions will elucidate the intricate dynamics between plants and microbes under N deficiency. Ultimately, this will facilitate the development of strategies to enhance NUE in crops.

In this study, we employed amplicon sequencing and metagenomic technology to compare the root-associated bacterial communities of two wheat cultivars (cv. Mace and Gladius) with high and low reported NUE, respectively (Alhabbar et al. 2018), under different N availability conditions. The GC-MS metabolomics approach was further conducted to determine the primary metabolic profile in the roots of N-deficient wheat grown in a hydroponic system. We hypothesised that (1) wheat cultivars with contrasting NUE establish distinct soil bacterial communities when subjected to N deficiency and (2) these cultivars alter root metabolite composition in response to N deficiency.

Materials and methods

Soil collection and glasshouse experiment

Soil samples used in this study were collected in March 2022 from a wheat experimental farm on the Dookie Campus at The University of Melbourne, Australia (36.382° S, 145.711° E). The experimental field has a history of wheat-pasture rotation. We collected approximately 400 kg of soil (0-10 cm depth) using a shovel after removing the root debris for the pot experiment. The soil is classified as a Dermosol (Isbell 2024) with the following physicochemical properties (measured by the Nutrient Advantage lab, Victoria, Australia): total C, 0.021 g kg⁻¹ soil, total N, 0.002 g kg⁻¹ soil, organic C, 0.018 g kg⁻¹ soil, nitrate-N, 56 mg kg⁻¹ soil, ammonium-N, 2.9 mg kg⁻¹ soil, and soil pH of 6.3. The collected soil samples were sieved to 1 cm, air-dried, and homogenised before the glasshouse experiment. Pots with an inner diameter of 16.5 cm were each filled with 4.6 kg of soil. Two wheat cultivars, high-NUE Mace and low-NUE Gladius, provided by the Australian Grain Technology (AGT) company, were selected for the pot experiment. Five uniform-sized seeds were sown at a depth of 3 cm and seedlings were thinned to three per pot at the two-leaf stage. To establish varying N levels, ammonium sulphate was applied at three rates: (1) N0, without N addition, (2) N1, 45 kg N ha⁻¹, and (3) N2, 90 kg N ha⁻¹, with four replicates for each N treatment. The soil moisture content was maintained at around 80% field capacity throughout the experiment. Plant growth conditions were maintained at 24°C /10°C for 12 h/12 h day/night, 65% humidity and ~225 µmol m⁻² s⁻¹ during the daytime throughout the study.

The wheat rhizosphere and bulk soils, as well as plant roots, were destructively sampled at the stem-elongation and the mid-anthesis stages. The wheat root system was placed in a sampling bag and gently shaken to obtain rhizosphere soil samples. Soils that remained in the pots were randomly collected as the bulk soil. Wheat roots (5-20 cm) were separated from the plant and were gently rinsed with reverse osmosis (RO) water to remove large soil particles. Root samples were subsequently shaken with 30 mL of phosphate-buffer solution

(PBS) in a falcon tube at 250 rpm to remove any adhered soil and briefly dried with filter paper. The soil and root samples were then stored at -20 °C.

Plant and soil physicochemical characterisation

The fresh above-ground plant materials were oven-dried at 60°C for seven days for biomass measurement. The dried plant material was ground to a 0.2-mm fine powder using the ultra-centrifugal mill (ZM200 Ultra Centrifugal Mill, RETSCH). A set of 48 bulk soil samples (two growth stages, two cultivars, 3 N levels and four replicates) were oven-dried at 40°C for seven days, and then ball milled using a tissue grinder (TissueLyser II, QIAGEN). The resulting, finely ground plant and soil samples were subjected to the CN analyser (LECO TruMac Series) for total C and N measurements (Figure S1). Another set of 48 bulk soil samples was oven-dried at 105°C for two days to estimate soil moisture content. For soil pH and electrical conductivity (EC) measurements, an alternative set of fresh soil was suspended in Milli-Q water at a 1:5 ratio and shaken at 250 rpm for 60 min. Soil pH was measured with a professional benchtop pH meter (HANNA), and EC was measured with the conductivity meter (Multiparameter Laboratory Benchtop Conductivity meter, SmartCHEM-LAB).

DNA extraction and amplicon sequencing

Frozen root samples were ground into a fine powder with liquid N. Total DNA was extracted from 250 mg of the frozen rhizosphere soil and root samples using the DNeasy PowerSoil Pro Kits (QIAGEN), and quality was assessed using a Nanodrop (NanoDrop One, Thermo Scientific). A total of 48 rhizosphere and 48 root endosphere DNA samples were subjected to polymerase chain reaction (PCR) amplification. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified with the 341F/806R primer pairs (Klindworth et al. 2013). The amplified region was sequenced on the Illumina MiSeq System platform at the Australian Genome Research Facility, Melbourne, Australia.

Amplicon sequencing was processed using QIIME 2 (v.2022.11) (Bolyen et al. 2019). PCR primer sequences were removed from the raw sequences using cutadapt (v.4.2) (Martin 2011) and were filtered with an average quality score cutoff >20 using DADA2 (v1.26.0) (Callahan et al. 2016). Filtered reads were further subject to DADA2 for denoising (Callahan et al. 2016). Non-chimeric amplicon sequence variants (ASVs) were obtained by merging read pairs that overlapped at least 12 bp and removing PCR chimeras. Taxonomic assignment of ASVs was performed with QIIME 2 (v.2022.11) (Bolyen et al. 2019) using the SILVA database (v.138.1) (Quast et al. 2013) for the 16S rRNA gene of the rhizosphere (Supplementary data 1) and root endosphere samples (Supplementary data 2). A minor proportion of archaeal ASVs were detected, and their alpha and beta diversity had no significant differences across samples (Figures S2 & S3). Hence, ASVs belonging to mitochondria, chloroplast, archaea and *Triticum aestivum* were removed before the downstream analysis. The count matrix was rarefied using microeco (v.1.1.0) (Liu et al. 2021) to ensure even read depth for sample comparisons.

Metagenomic analysis

To further investigate the functional changes of rhizosphere bacterial communities in response to N deficiency, shotgun metagenomic sequencing was performed on the extracted DNA of the rhizosphere samples

of Mace and Gladius under the two contrasting treatments, N0 and N2. A total of 16 metagenomic libraries of the rhizosphere soil DNA were generated with the NovaSeq 6000 sequencing platform using the Nextera Flex primer (Bruinsma et al. 2018), yielding 2×150 bp reads at the Australian Genome Research Facility, Australia. The resultant metagenome sequences (24 Gb per sample) were subjected to quality checks using fastqc (v.0.11.9) (Andrews 2010) and adapter trimming using trimmomatic (v.0.39) (Bolger et al. 2014). Samples were decontaminated using the *Triticum aestivum* genome (IWGSC) retrieved from the EnsemblPlants (Yates et al. 2022) using bowtie2 (v.2.4.2) (Langmead and Salzberg 2012). Clean reads were processed using the Metaphor (v.1.7.9) pipeline (Salazar et al. 2022). Two co-assemblies were generated by pooling the reads from samples of the same cultivar and assembled with MEGAHIT (v.1.2.9) (Li et al. 2015). Genes were predicted over the assembled contigs using Prodigal (v.2.6.3) (Hyatt et al. 2010) and functionally annotated with DIAMOND (v.2.1.0) (Buchfink et al. 2015) against the Clusters of Orthologous Genes (COGs) (v.1.0) database (Tatusov et al. 2000).

Metaphor outputs the relative abundance of contigs falling in different COGs functions (Supplementary data 3) and categories (Supplementary data 4). These files were used to compare the functional similarity between treatment samples. We integrated the contig count and COGs annotation output from Metaphor to explore the N metabolism functions in bacterial genera. Contig depth was estimated by the reads per kilobases (RPK) method (Supplementary data 5), and the contig annotation file contained COGs taxonomy and function information generated from DIAMOND (Supplementary data 6). We first filtered out contigs related to N-metabolism and then searched for the presence of the bacterial genera in Mace or Gladius under N deficiency in their corresponding samples.

Hydroponic experiment

The seeds of two wheat cultivars were sterilised with 80% ethanol for 1 min, followed by 2.5% sodium hypochlorite (NaOCl) for 15 min and rinsed with Milli-Q water five times. Sterilised seeds were germinated on petri dishes with Milli-Q water inside a fume cupboard, covered in dark, at room temperature, until the two-leaf stage was reached (Lu et al. 2021). Two uniform seedlings of each cultivar were transferred into a 4.3-L hydroponic system. The hydroponic pots were filled with a modified Hoagland solution with the following composition: 1 mM KH_2PO_4 ; 1 mM MgSO_4 ; 0.05 mM H_3BO_3 ; 0.01 mM Fe-EDTA; 0.009 mM MnSO_4 ; 0.0007 mM ZnSO_4 ; 0.0003 mM CuSO_4 ; 0.0001 mM NaCl and 0.0001 mM H_2MoO_4 . Three N levels, (1) N0, 2 mM, (2) N1, 4 mM and (3) N2, 8 mM were achieved by adding various amounts of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, KNO_3 and $(\text{NH}_4)_2\text{SO}_4$, with a NH_4^+ to NO_3^- molar ratio of 1:4 across all N treatments (Thomas and Paparozzi 2013). Additional K_2SO_4 and CaCl_2 were introduced to balance the levels of K^+ and Ca^{2+} in the solution. The hydroponic solution was renewed with a half-strength Hoagland solution once every two weeks before the stem elongation stage and with a full-strength solution weekly thereafter. The solution pH was adjusted to maintain at the range of 5.5-6.5 using 0.1 M KOH, while the water level was maintained using RO water. An aeration system was implemented in the hydroponic pots at the onset of tillering, and the arrangement of hydroponic pots was randomised on a weekly basis.

Root exudates were collected, but metabolite was under the detection limit. Hence, this study focused on the metabolite inside the wheat root. During the mid-anthesis stage, wheat root samples were collected and rinsed with 0.2 mM CaCl_2 , followed by immersion in 800 mL of 0.2 mM CaCl_2 for three hours with bubbling to

remove adhered salt and microbes from the root surface (Liu et al. 2019). The root was separated from the plant and immediately stored in a freeze room at 20°C. Frozen root samples were freeze-dried for two weeks, cut into ~3 cm pieces, and homogenised (Saiman et al. 2012). Freeze-dried root samples were ground to fine powder with liquid N and stored at -20°C for metabolite extraction.

GC-MS analysis for polar metabolites

Organic compounds in root were extracted from 60 ± 2 mg of the stored lyophilised and ground materials using 500 μ L of 100% MeOH containing 4% (v/v) of [$^{13}\text{C}_6$] sorbitol/[$^{13}\text{C}_5^{15}\text{N}$] valine (0.5 mg mL $^{-1}$). The supernatant was stored in a 2-mL reaction tube as the first extraction product. A second extraction was performed on the remaining pellet with 500 μ L of Milli-Q water, vortexed and centrifuged. The two extracted supernatants were combined and 100 μ L of the combined supernatant was dried using a speed vacuum dryer (John Morris Scientific Pty Ltd). The derivatisation of the dried extracts was performed with 20 μ L of methoxyamine hydrochloride in pyridine and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), as described by (Dias et al. 2015). Specifically, a solution of 30 mg mL $^{-1}$ methoxyamine hydrochloride in pyridine was added to all samples, followed by a derivatisation process at 37°C for 120 min with agitation at 500 rpm (61.0 g). Subsequently, 20 μ L of BSTFA was added to the samples and were further incubated on a thermomixer (500 rpm/61.0 g) at 37 °C for an additional 30 min. Samples were settled for 60 min before the injection.

The samples were analysed with a GC-MS system comprised of an autosampler (Gerstel 2.5.2), a gas chromatograph (7890A Agilent), and a quadrupole mass spectrometer (5975C Agilent) (Agilent, Santa Clara, United States). An injection volume of 1 μ L was used for each derivatised sample. The mass spectrometer was tuned using tris-(perfluorobutyl)-amine (CF43) following the manufacturer's recommendations. Chromatography was carried out on a 30 m column (J&W VF-5MS Agilent) with a film thickness of 0.25 μ m and an internal diameter of 0.25 mm. A 10 m Integra guard column was additionally equipped. The inlet temperature was set at 250 °C; the transfer line of MS was at 280 °C; the ion source was adjusted to 230 °C; and the quadrupole was maintained at 150 °C. Helium was served as the carrier gas at a flow rate of 1 mL min $^{-1}$.

Metabolite data pre-processing

Data were pre-processed in silico for mass spectral deconvolution, with peak picking and identification using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (National Institute of Standards and Technology, Gaithersburg, MD, USA). The target component library was created with the PBQC file and peak identification was confirmed with the Agilent MassHunter Qualitative Analysis B.05.00 (Agilent Technologies, Inc., 2011). The peak area data was exported with the Agilent MassHunter Quantitative Analysis software version B.08.00/Build 7.0.457.0 (Agilent Technologies, Inc., 2008). The peak area data was normalised against the internal standard [$^{13}\text{C}_6$] sorbitol and the weight of root samples to generate response values, which were log-transformed for the downstream analysis (Gupta et al. 2022) (Supplementary data 7).

Statistical analyses

All statistical analyses were performed using R (v.4.2.1) and all figures were generated with ggplot2 (v.3.4.4). Wheat growth parameters were statistically compared using the analysis of variance (ANOVA). The number of samples used for statistical analysis is provided in Table S1. Shannon index was used to estimate the

bacterial diversity and the differences between treatments were compared using the nonparametric Kruskal-Wallis test. Principal coordinates analysis (PCoA) based on the Bray-Curtis distance matrix was performed to assess the dissimilarity of the bacterial community compositions between treatments. Analysis of similarity (ANOSIM) with the ‘vegan’ package (Oksanen et al. 2022) was performed to statistically compare the similarity of bacterial community between treatment samples. Bacterial biomarkers were determined between N0 and N2 samples at the mid-anthesis stage. The linear discriminant analysis effect size (LEfSe) was quantified at the ASV level using the count per million (CPM) transformation with microeco (v.1.1.0). ASVs were considered significantly enriched with the criteria of $p < 0.05$ and a linear discriminant analysis (LDA) effect score > 2 . For the metagenomic data, the relative abundance of COG functional code from Metaphor was subjected to the PCoA and ANOSIM analyses (Supplementary data 3). The relative abundance of COG categories was further compared between the N0 and N2 treatments using the Kruskal-Wallis test (Supplementary data 4). For the GC-MS metabolomics analysis, unsupervised learning principal component analysis (PCA) was performed to assess the similarity of metabolite profiles between treatments. The metabolite composition between treatment samples was statistically compared with the Permutational Multivariate Analysis of Variance (PERMANOVA) using the ‘vegan’ package (Oksanen et al. 2022). The fold-change of non-redundant known metabolites was analysed on the response values through multiple Student’s t-tests with false discovery rate (FDR) adjustment (adj. $p < 0.05$). A significance threshold of $p < 0.05$ was applied to all statistical results.

Results

Changes in the diversity and structure of bacterial communities under N deficiency

The 16S rRNA gene sequencing yielded a total of 9,566 and 4,558 bacterial ASV after filtering, in the rhizosphere and root endosphere samples, respectively. The Kruskal-Wallis test revealed no significant changes in the alpha diversity of bacterial communities across N treatments and cultivars at both the stem elongation and the mid-anthesis stages (Figure S4). The PCoA ordination plots revealed that the bacterial community structure of Mace rhizosphere soil significantly changed across N treatments at the mid-anthesis stage (ANOSIM $p < 0.05$) (Figure 1a), while N treatments had a less significant impact on the bacterial community structure of Gladius rhizosphere, with $R^2 = 0.19$ (ANOSIM $p < 0.05$) (Figure 1b). For the root endosphere, N treatments did not significantly affect the bacterial community structure of Mace (ANOSIM $p > 0.05$) (Figure 1c), but significantly shifted the bacterial community in Gladius, with N0 samples clustering away from N2 samples (ANOSIM $p < 0.05$) (Figure 1d).

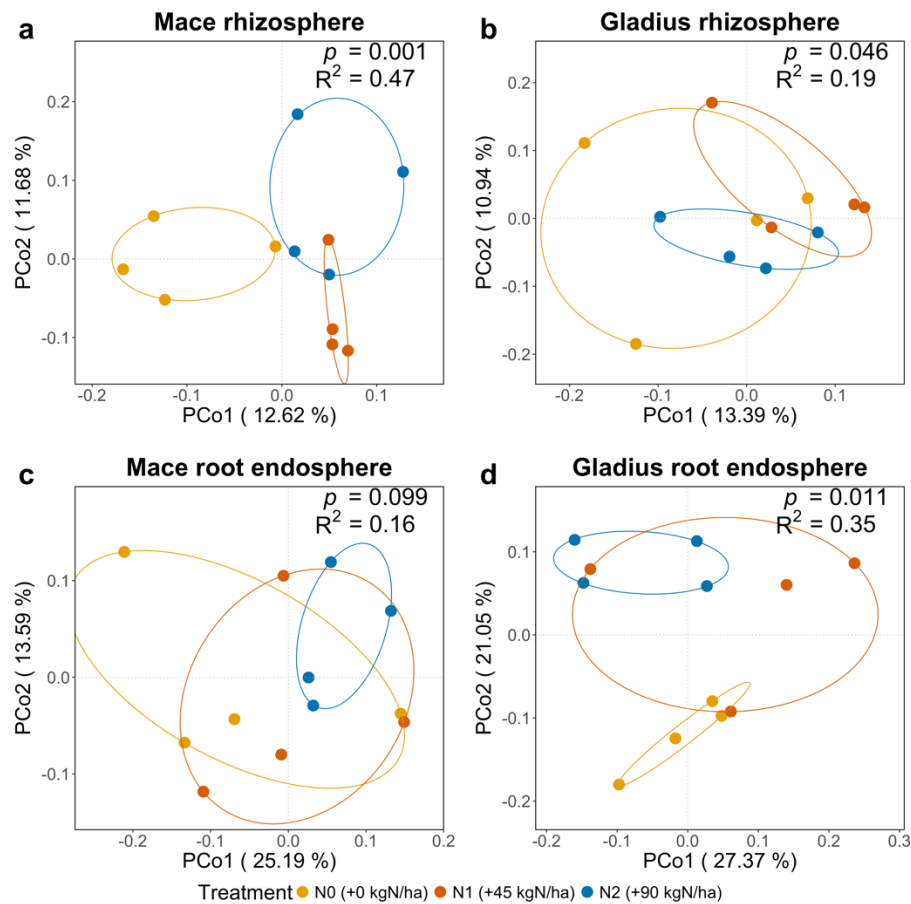


Figure 1. Principal coordinates analysis (PCoA) of the bacterial community composition in the rhizosphere soils of (a) Mace and (b) Gladius and in the root endosphere of (c) Mace and (d) Gladius at the mid-anthesis stage, under N treatments, as revealed by the amplicon sequencing analysis. Samples are colour-coded by N treatments: yellow for N0 (+0 kg N ha⁻¹), orange for N1 (+45 kg N ha⁻¹) and blue for N2 (+90 kg N ha⁻¹). The ANOSIM statistics are indicated on the top-right of each PCoA plot.

Functional changes in rhizosphere soil bacterial communities under N deficiency

We further explored the functional changes in the rhizosphere soil bacterial communities through metagenomic analyses of the N0 and N2 samples of both cultivars at the mid-anthesis stage. Across all samples, a total of 4311 functions belonging to 25 categories were identified from the COGs database. The PCoA ordinations revealed that N treatments significantly shifted the functional composition of the Mace rhizobacteria (ANOSIM $p < 0.05$) (Figure 2a), while that of Gladius rhizobacteria remained largely unchanged (ANOSIM $p > 0.05$) (Figure 2b). The COG functional categories were further analysed with the Kruskal-Wallis statistical test, which revealed enrichment of signal transduction mechanisms (T) in the rhizobacteria of both cultivars under N deficiency ($p < 0.05$) (Figure 2c). Further comparisons between the cultivars revealed that the Mace rhizobacteria had enhanced mobility and development, evident by functional enrichment in the cell wall membrane, envelope and biogenesis (M), post-translational modification, protein turnover and chaperones (O), intracellular trafficking, secretion and vesicular transport (U), cell motility (N), extracellular structure (W) and cytoskeleton (Z) ($p < 0.05$) (Figure 2c).

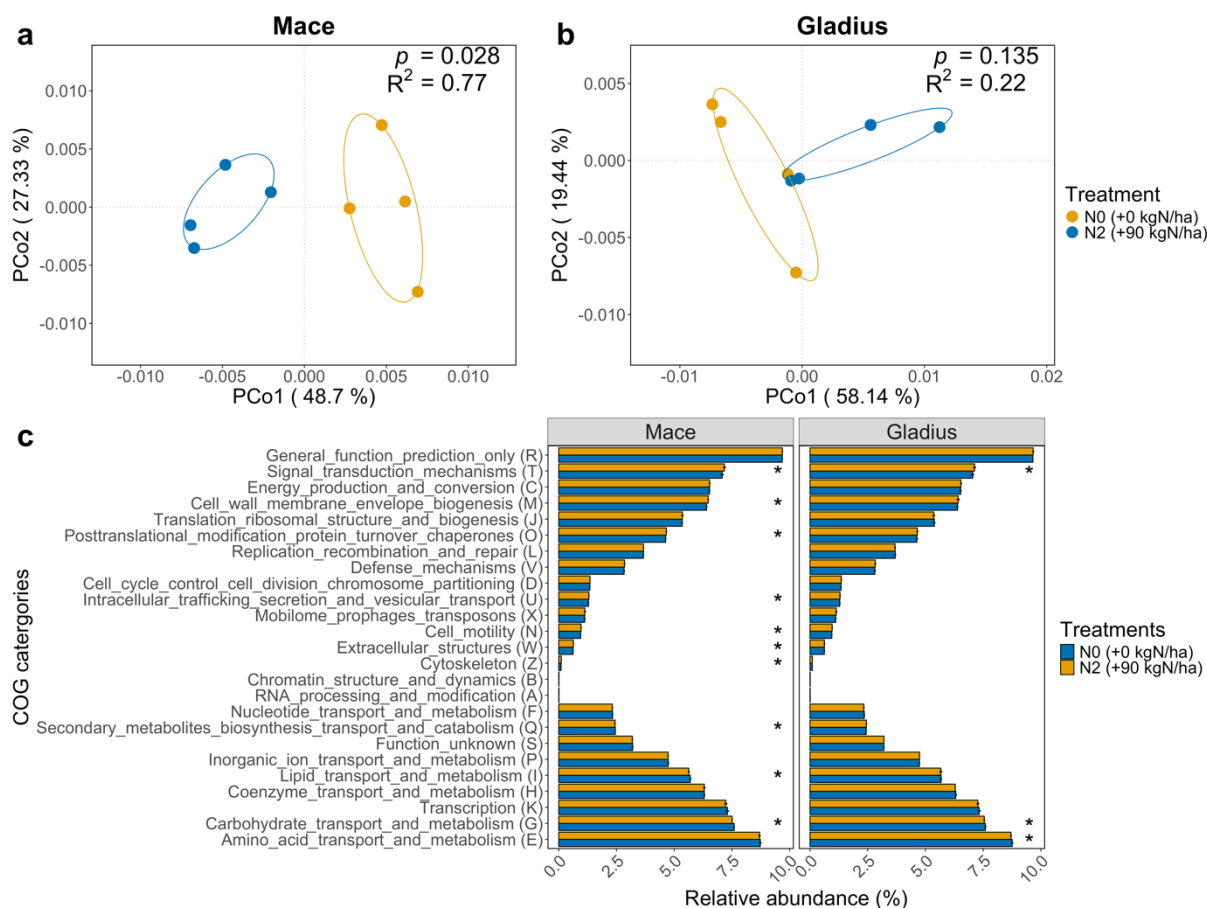


Figure 2. Principal coordinates analysis (PCoA) of the rhizobacterial COG functions detected in (a) Mace and (b) Gladius between the N0 and N2 treatments at the mid-anthesis stage, as revealed by the metagenomic analysis. The samples were coloured brown and green, for N0 and N2 treated samples, respectively. The ANOSIM statistics are indicated on the top-right of each PCoA plot. (c) The relative abundance of the grouped functional COG categories in N0- (yellow) and N2- (blue) treated Mace and Gladius with significant difference indicated on the bar (* = $p < 0.05$). The letter code description is indicated in the bracket.

Enrichment pattern of intraspecific rhizobacteria under N deficiency

We conducted LEfSe analysis of the 16S rRNA gene sequences, revealing distinct responses of rhizobacteria to N deficiency between Mace and Gladius. Specifically, 21 ASVs were significantly enriched in Mace but not in Gladius ($p < 0.05$, LDA score > 2). In comparison, 20 ASVs were significantly enriched in Gladius but not in Mace ($p < 0.05$, LDA score > 2) (Figure 3a & 3b). A comparison of ASVs at the genus level revealed ten genera observed exclusively in N-deficient Mace and 7 in N-deficient Gladius (Figure 3c). The functional profile of bacterial genera *Haliangium*, *Clostridium*, *Ilumatobacter* and *Lysinibacillus* observed in N-deficient Mace, as well as *Massilia*, *Blastococcus*, *Gemmatimonas*, *Conexibacter* and *Bacillus* observed in N-deficient Gladius, were also detected in the metagenomic data. The metagenomics analysis further revealed that rhizobacterial genera enriched in N-deficient Mace exhibited a distinct N metabolism compared to those enriched in N-deficient Gladius, particularly in categories related to N regulation, transport, nitrification, and denitrification (Figure 3d).

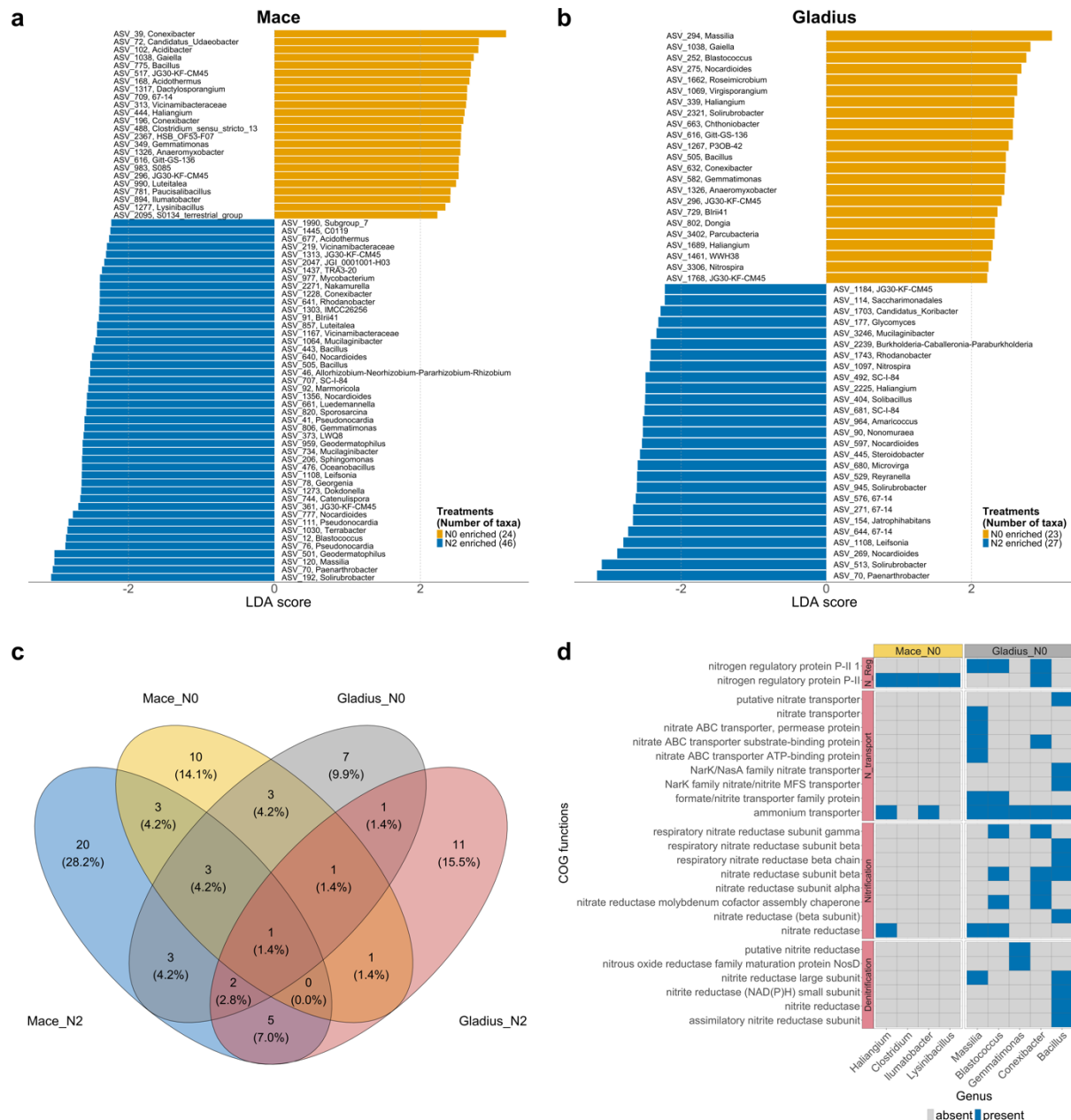


Figure 3. LefSe analysis of (a) Mace and (b) Gladius between the N0 and N2 treatments at the mid-anthesis stage, showing the bacterial ASVs with significant enrichment ($P < 0.05$, LDA score > 2). (c) Overlap of bacterial genera enriched in the N0 and N2 treatments between Mace and Gladius, based on the amplicon sequencing. (d) The presence of N metabolism functions in the bacterial genera enriched in Mace and Gladius in the N0 treatment, as revealed by the metagenomic analysis.

Intraspecific metabolite production in wheat under N deficiency

The GC-MS metabolomics analysis identified 78 compounds across all samples, including 40 non-redundant known organic compounds. Among the identified metabolites, 20 were amino acids, ten sugars, three organic acids, and two fatty acids. The PCA analysis revealed a significant N treatment effect on the metabolite profiles for both cultivars ($p < 0.05$) (Figure 4a & 4b). This result was further validated by a heatmap presenting the log-transformed response value, with a clear distinction between N treatments for both cultivars (Figure 4d). The PCA plot revealed a marked disparity in the metabolite composition between Mace and Gladius under the

N0 treatment ($p < 0.05$), but not under the N2 treatment ($p > 0.05$) (Figure 4c). The fold-change of metabolites between the N0 and N2 treatments in both cultivars revealed a predominant effect of N supply on the accumulation of organic compounds, with glycine, tyrosine and glucose exhibiting a significantly greater response to the N2 treatment for both cultivars (adj. $p < 0.05$, fold-change > 2) (Figure 4e). Furthermore, the diverse sensitivity of wheat metabolites to between N0 and N2 treatments was evident, with octadecanoic acid, gluconic acid, and maltose significantly changed in Mace; and alanine, leucine, thymine, cysteine, glycolic acid, trehalose, sorbose, glucopyranose, sucrose and glycerol-3-phosphate significantly changed in Gladius (Figure 4e). In particular, the levels of octadecanoic acid in N-deficient Mace were 4-fold higher in N0 than in N2 (Figure 4e).

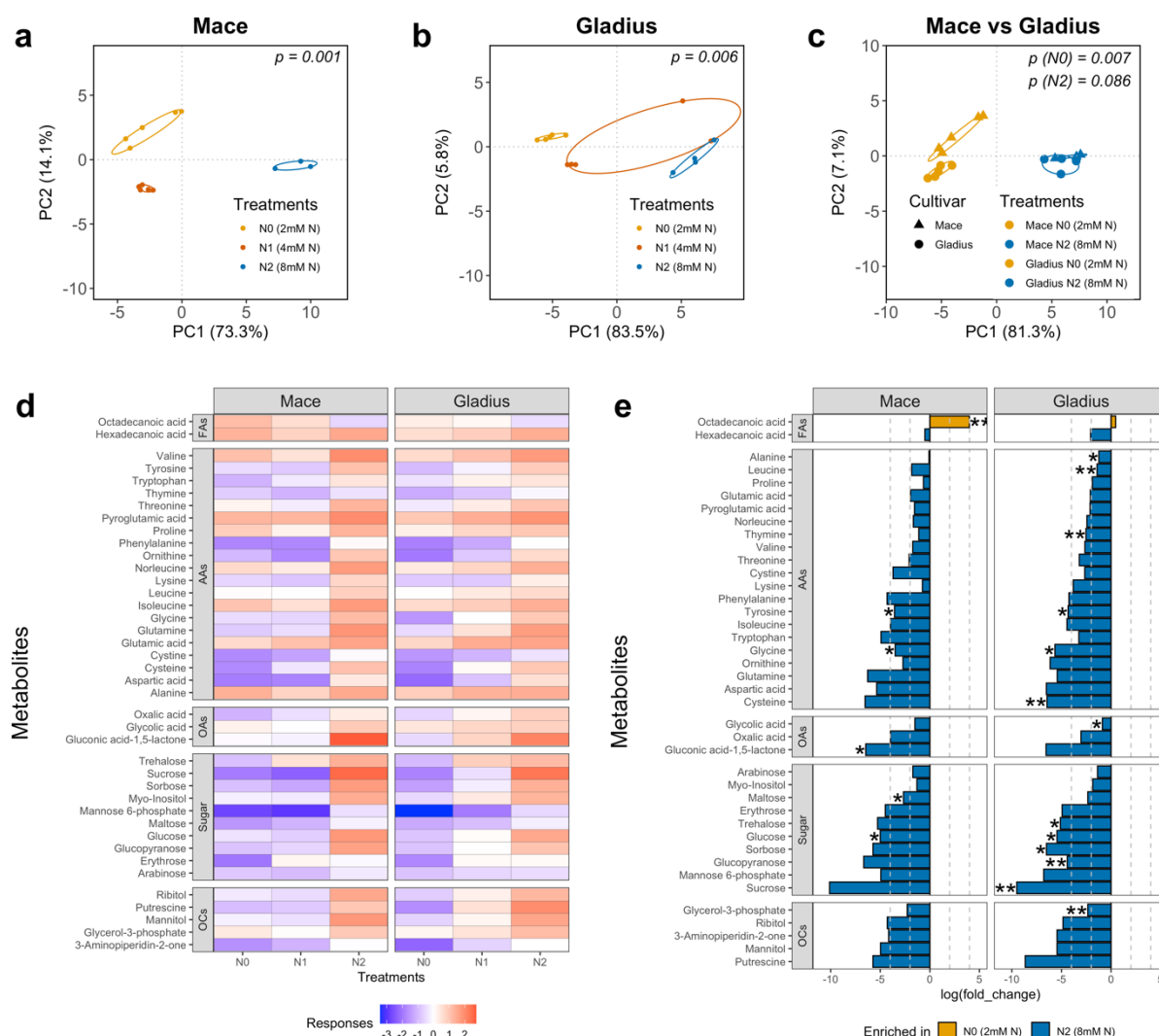


Figure 4. Principal component analysis (PCA) comparing polar metabolites detected in roots across N treatments of (a) Mace and (b) Gladius at the mid-anthesis stage. Samples are colour-coded by N treatments: yellow for N0 (2mM N), orange for N1 (4mM N) and blue for N2 (8mM N). (c) PCA comparing polar metabolites detected in roots of Mace and Gladius under N0 and N2 treatments. Samples of Mace are represented by triangles, while Gladius are represented in dots. The PERMANOVA statistics are indicated on the top-right of each PCoA plot. (d) Heatmap of the log-transformed response values of metabolites in Mace and Gladius across N treatments. (e) Log scaled fold-changes of metabolites of Gladius and Mace between the N0

and N2 treatments with significant changes indicated on the bar (* adj. $p < 0.05$, ** adj. $p < 0.001$) (FAs = fatty acids, AAs = amino acids, OAs = organic acids, and OCs = organic compounds).

Discussion

Understanding the intricacies of plant-microbe interactions in N-poor environments is crucial for better N management for sustainable agriculture (Lam et al. 2022). While previous research has demonstrated that plant metabolites facilitate beneficial plant-microbe interaction (Yu et al. 2021), our understanding of these interactions in wheat cultivars differing in reported NUE under N deficiency remains limited. We found that the bacterial community in the rhizosphere differed significantly across N treatments in Mace, but not in Gladius (Figure 1a & b). In contrast, the bacterial community in the root endosphere varied significantly by N treatments in Gladius, but not in Mace (Figure 1c & d). This finding suggests that Mace and Gladius, with their different reported NUE, interact differently with bacterial communities under N deficiency. Further examination of bacterial enrichment in the rhizosphere under N deficiency revealed a distinct set of bacterial genera enriched in Mace compared to Gladius (Figure 3). Although studies comparing the genotypic influences on plant-associated bacterial communities under N deficiency are limited, different wheat genotypes distinctly shape the root-associated bacterial community, enabling adaptation to abiotic stress (Azarbad et al. 2018; Corneo et al. 2016; Yin et al. 2019). These results underscore the primary role of the genetic variation among cultivars in bacterial recruitment. We have identified *Haliangium*, *Clostridium*, *Ilumatobacter* and *Lysinibacillus* as bacterial genera distinctly enriched in Mace, while *Massilia*, *Blastococcus*, *Gemmatimonas*, *Conexibacter* and *Bacillus* were notably enriched in Gladius (Figure 3). Species from the genera *Haliangium*, *Massilia*, *Gemmatimonas* and *Bacillus* have previously been recognised as wheat-associated plant growth promotion rhizobacteria (PGPR) (Kavamura et al. 2021). This indicated that Mace and Gladius interact with different PGPRs under N deficiency. Overall, our sequencing data demonstrated that wheat cultivars with distinct NUE exhibit differential interaction with PGPR in response to N deficiency.

The functional composition of the rhizobacterial community in Mace and Gladius under N deficiency revealed distinct functional responses to N deficiency (Figure 2a). This finding aligns with a recent study reporting variation in the relative abundance of the rhizosphere bacterial functional genes involved in multiple nutrient cycles among rice cultivars with differing plant cadmium accumulation (Cheng et al. 2023). Such results support the notion that crop cultivars have a predominant effect on the rhizobacteria functions. The comparison of functional categories showed a notable increase in the detection of genes associated with signal transduction mechanisms in both Mace and Gladius, under N deficiency (Figure 2c). Additionally, a significant increase in the detection of genes related to rhizobacterial growth and motility was observed specifically in Mace, under N deficiency, a phenomenon not observed in Gladius (Figure 2c). Genes associated with signal transduction mechanisms and extracellular structures encode important functions that enhance bacterial flagellar motility, as recently reported (Ramoneda et al. 2024). Bacterial chemotaxis, defined as the movement of bacteria toward chemical compounds, plays a critical role in facilitating symbiotic relationships with host plants (Raina et al. 2019). A recent study established a connection between rhizobacterial chemotaxis and improved N acquisition in maize cultivated with rhizobacteria possessing chemotaxis genes (Sun et al. 2023). Collectively, these findings imply that the higher NUE cultivar, Mace, may exhibit enhanced rhizobacterial chemotaxis in response to N deficiency, in contrast to Gladius. Moreover, the N metabolism of the bacterial community

differed between two rice cultivars, indicating that bacterial N metabolism directly influences rice NUE (Zhang et al. 2019). A distinct pattern of N metabolism was observed among bacterial genera, with functions related to N transport, nitrification and denitrification being absent in Mace but present in Gladius under N deficiency (Figure 3d). Given that nitrification and denitrification contribute to N losses that can reduce plant NUE in agricultural systems (Saud et al. 2022), it appears that Mace interacts with rhizosphere bacteria to adapt to N deficiency through strategies beyond N metabolism. Indeed, *Lysinibacillus* sp. is recognised for various plant-beneficial traits, including N fixation, phosphorus solubilisation, auxin production (Hernández-Santana et al. 2022; Pantoja-Guerra et al. 2023b; Passera et al. 2021) and virus biocontrol (Passera et al. 2021). A recent study demonstrated that *Lysinibacillus* sp. can stimulate root growth and development in Arabidopsis, highlighting its potential to enhance nutrient acquisition (Pantoja-Guerra et al. 2023a). This suggests that the enhanced bacterial chemotaxis ability of *Lysinibacillus* sp., may play a critical role in driving the difference in NUE between cultivars under N deficiency.

Primary metabolites secreted by plant roots act extensively as energy sources (Zhalnina et al. 2018) and chemotactic attractants for bacteria (Raina et al. 2019), playing a crucial role in shaping the rhizosphere microbiota. In our hydroponic experiment, the primary metabolites in the wheat root exudate were found to be below the detection limit for GC-MS measurement. Primary metabolites within the root, such as amino acids, organic acids and sugars are secreted in root exudates through diffusion driven by concentration gradients (Canarini et al. 2019). Therefore, we analysed the primary metabolic profile within the wheat roots, which reflects the secretion to some extent. Our metabolomic data revealed a significant shift in both Mace and Gladius under varying N availability (Figure 4a & 4b), indicating that both Mace and Gladius are sensitive to the N treatments. Furthermore, the metabolite composition highlighted differences in organic compound synthesis between the wheat cultivars, particularly under N-deficient conditions (Figure 4c & 4d). We conducted an enrichment analysis between the N0- and N2-treated samples, revealing a significant reduction in the synthesis of glucose, tyrosine and glycine under N deficiency in both cultivars (Figure 4e). This reduction has also been demonstrated in multiple crops (Sung et al. 2015; Tawaraya et al. 2018; Zhen et al. 2016). Moreover, the enrichment analysis of metabolites showed a notable increase in octadecanoic acid, known as stearic acid (C18:0), in Mace under N deficiency, whereas such accumulation was not observed in Gladius (Figure 4e). These findings demonstrate genotype-specific metabolic processes in Mace and Gladius in response to N deficiency. Interestingly, a previous study reported no effect of N deficiency on stearic acid production in wheat (Nasiroleslami et al. 2021), which contrasts with our results for Mace but aligns with those for Gladius. We hypothesise that stearic acid plays an important role in the plant-microbe interaction of Mace under N deficiency. Indeed, stearic acid accumulation in the root nodules of *Lotus japonicas* cv. GIFU indicates its potential role in symbiotic N fixation (Desbrosses et al. 2005). Additionally, stearic acid has been identified as a chemotactic attractant for the PGPR *Pseudomonas* isolate RP2 (Ankati et al. 2019). Together, the GC-MS metabolomic data suggest that stearic acid may be secreted in Mace but not in Gladius under N deficiency, potentially shaping the intraspecific wheat-bacterial community.

Our data revealed a differential rhizobacterial community and root metabolite profile between wheat cultivars of contrasting NUE in response to N deficiency. Notably, the accumulation of stearic acid in the roots of the higher NUE cultivar, Mace, suggests a potential increase in root exudates, which may influence the soil bacterial community under N deficiency. Furthermore, our metagenomic analysis indicates that Mace may

stimulate rhizobacterial motility and development while enhancing the enrichment of PGPR under N deficiency. These responses present distinct differences compared to those observed in *Gladius*. Enhancing our understanding of the chemotactic ability of PGPR towards stearic acid in wheat-grown soils could open promising avenues for developing next-generation bio-fertilisers, thereby contributing to sustainable agriculture.

Acknowledgements

This research was supported by the Australia Research Council's Industrial Transformation Research Program funding scheme (IH200100023). The wheat seeds for this research were provided by the Australian Grain Technology (AGT). The computational resources were supported by The University of Melbourne's Research Computing Services and the Petascale Campus Initiative. The authors wish to thank Pongsathorn Sukdanont for glasshouse assistance.

Competing interests

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

Author contributions

LHC, HWH, SKL, DC, and CT designed the glasshouse experiments, performed by LHC and supervised by HWH, SKL, DC, and CT. CT & UR supported the development of the metabolite collection method. QC supported DNA extraction. SG and DAD performed primary metabolite extraction and the GC-MS metabolomic analysis. LHC performed amplicon sequencing, metagenomic and metabolomic data analysis. HWH and QC supported amplicon sequence data analysis. VWS supported metagenomic analysis. SG supported metabolomic data analysis. LHC prepared the manuscript draft. HWH, SKL and LHC edited and revised the manuscript. All authors read and approved the final version of the manuscript.

Data availability

Raw sequencing files can be publicly accessed from the NCBI database with the accession number PRJNA1126416. The QIIME2 output of the 16s amplicon sequencing is provided in supplementary data 1 and 2. The Metaphor outputs of the metagenome are provided in supplementary data 3 and 4. Supplementary data 5 and 6 are available on reasonable request. The processed metabolite data is provided in supplementary data 7. Other datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Chapter 3 - Wheat root-associated fungal community under N deficiency

Connecting statements

Acknowledging that the fungal community in soil plays a vital role in supporting plant growth, particularly under low N conditions, Chapter 3 extended our investigation beyond bacteria. Building upon the findings in Chapter 2, which demonstrated shifts in the wheat root-associated bacterial community in response to N deficiency, Chapter 3 specifically focused on characterising the wheat root-associated fungal community's response to N deficiency across the two selected wheat cultivars. Utilising samples from the pot experiment detailed in Chapter 2, we performed amplicon sequencing and metagenomic analysis to comprehensively study changes in fungal abundance, community composition, and functional profiles under N deficiency. A distinct bioinformatics pipeline was employed to specifically explore the fungal functional profile. Our results revealed that while the wheat root-associated fungal community differed significantly between the two cultivars (Mace-high NUE & Gladius-lowNUE), it remained remarkably stable in its overall response to N deficiency. This work has been published in the journal *Rhizosphere*, titled “Nitrogen Deficiency drives fungal compositional shifts without functional changes in wheat rhizosphere”.

Nitrogen deficiency drives fungal compositional shifts without functional changes in wheat rhizosphere

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Abstract

Nitrogen (N) deficiency reduces crop yield, but this effect may be mitigated by symbiotic interactions between crops and fungi. However, the response of wheat-fungal interactions to N deficiency remains unclear. We hypothesised that N deficiency would induce shifts in the fungal community composition and functional profile within the wheat rhizosphere. A glasshouse experiment was conducted to examine the effects of N deficiency on the rhizosphere fungal communities of wheat (*Triticum aestivum* L.) cultivars Gladius (low N-use efficiency) and Mace (high N-use efficiency). Plants were grown until the mid-anthesis stage in a Dermosol soil treated with either 0 (Low-N) or 90 kg N ha⁻¹ (High-N). The rhizosphere fungal communities were characterised using quantitative PCR, ITS rRNA metabarcoding, and metagenomics. The abundance and diversity of the rhizosphere fungal community were not significantly influenced by N deficiency in either Mace or Gladius cultivars ($P > 0.05$). However, the fungal community composition showed significant variation with N treatments in Gladius ($P < 0.05$), whereas no such effect was observed in Mace ($P > 0.05$). Differential abundance analysis and fungal trait predictions indicated a reduction in fungal symbionts in both cultivars under N deficiency. Metagenomic analysis demonstrated that fungal functional profiles, particularly those involved in amino acid metabolism, remained unaffected by N deficiency ($P > 0.05$). This study reveals intraspecific variation in rhizosphere fungal responses to N deficiency between Mace and Gladius. The metabarcoding and metagenomic data suggest functional redundancy within the fungal community, which may enhance wheat resilience under N-deficient conditions. These findings highlight the potential of using fungal community stability in developing biofertiliser products for sustainable agriculture.

Keywords

Nitrogen-use efficiency, Wheat, Plant-microbe interaction, Fungal community, Metagenomics

1. Introduction

Nitrogen (N) deficiency constrains plant growth and development, subsequently reducing crop yields (Khan et al. 2021). While synthetic fertilizers play a crucial role in enhancing agricultural productivity, their improper management has contributed to various environmental challenges (Ding et al. 2020). The biofertilisers harnessing beneficial plant-microbe interactions may offer a sustainable alternative by reducing reliance on synthetic fertilisers (Bhardwaj et al. 2014). The symbiotic associations with ectomycorrhiza and endomycorrhiza, distinguished by their colonisation mechanisms (Bonfante and Genre 2010), have been demonstrated to improve plant N acquisition (Odoh et al. 2020). However, the overuse of N fertiliser reduces mutualistic fungal colonisation while enhancing pathogenic fungal colonisation (Lekberg et al. 2021). In contrast, plants under N deficiency initiate a “cry-for-help” process and facilitate symbiotic fungal interaction to enhance plant N acquisition (Zhang et al. 2023) and alleviate N deficiency stress (Hestrin et al. 2019). The arbuscular mycorrhizal (AM) fungi, a predominant endomycorrhiza in crops, form symbiont within the root cells of different crop species in adaptation to N-poor conditions (Bonfante and Genre 2010) (Yan et al. 2019) (Semchenko et al. 2022). However, the performance of AM fungi on wheat is inconsistent, due to the host-specificity and variable field conditions (Elliott et al. 2021; Seeliger et al. 2024; Torppa et al. 2023).

Fungi significantly contribute to soil N metabolism with different molecular mechanisms compared to bacteria (Singh 2021). Soil fungi decompose hydrocarbon from dead plant materials and subsequently improve plant N acquisition through the expansion of hyphal network, stimulation of lateral root growth, and regulation of N transport activity (Gutjahr and Paszkowski 2013; Yu et al. 2016; Zhang et al. 2023; Zhao et al. 2023). The fungal hyphal network facilitates plant growth by hosting nitrogen transporters that uptake and deliver soil N to plants (Bonfante and Genre 2010; Zhang et al. 2023). For example, root colonisation by *Serendipita bescii* enhanced lateral root development and expression of N metabolism genes, suggesting its beneficial role in wheat N acquisition under N deficiency (Ray et al. 2021). Besides the direct influence on N metabolism, the increased relative abundance of *Mortierella* sp. under N-poor conditions indicates its beneficial potential on wheat growth (Kruczynska et al. 2023; Li et al. 2020a). The colonisation of *Mortierella hyaline* modulates amino acid homeostasis in Arabidopsis root under N starvation, demonstrating its supporting role in plant growth (Svietlova et al. 2023). Although studies have shown that fungi may benefit plant

growth through the modulation of N metabolism, other fungal functional response to N deficiency remains limited.

Crop domestication and breeding have improved plant morphology and physiology (Cormier et al. 2016), leading to improvements in wheat N use efficiency (NUE) for better adaptation to N-poor environments (Chen et al. 2020; Nerva et al. 2022; Sun et al. 2016). Increasing evidence highlights variation in the root-associated fungal community across different wheat genotypes and their beneficial effect on plant NUE (Latz et al. 2021; Sawers et al. 2018; Yue et al. 2023). Elliott et al. (2021) demonstrated that root colonisation by AM fungi varies across wheat cultivars, suggesting that the genetic variation in wheat germplasm determines the N-acquisition benefit from AM fungi. While variations in fungal communities across wheat cultivars have been observed, most studies have mainly focused on AM fungal activities (Mao et al. 2023; Rani et al. 2023). In rice, a study on *Japonica* and *Indica* cultivars with contrasting NUE demonstrated that the *NRT1.1B* transporter contributes to the recruitment of distinct root endospheric microbiota that mineralise organic N, suggesting a cultivar-specific contribution of plant-microbe interaction to rice NUE (Zhang et al. 2019). However, our knowledge of the intraspecific wheat-fungal interaction in response to N deficiency remains limited.

The present study investigated the rhizosphere fungal community of two wheat cultivars, Mace and Gladius, differing in NUE (Alhabbar et al. 2018), under N deficiency. It was hypothesised that nitrogen deficiency would induce shifts in the fungal community composition and functional profile within the wheat rhizosphere. The rhizosphere fungal community composition and functional profiles were studied through metabarcoding of the fungal ITS region and metagenomic analysis, respectively. The findings demonstrated that the fungal functional profile of the wheat rhizosphere remained stable under N deficiency, providing valuable insights for the development of microbial community-based biofertilizers and their potential role in promoting sustainable agricultural practices.

2. Materials and Methods

2.1 Glasshouse experiments and sample collection

Soil (0-20 cm) classified as Dermosol (Isbell 2024), was collected in March 2022 from an experimental farm with a history of wheat cultivation at the Dookie Campus (36.38° S, 145.71° E), The University of Melbourne, Australia. The soil exhibited the following physicochemical properties: total C, 0.021 g kg⁻¹ soil; total N, 0.002 g kg⁻¹ soil; organic C, 0.018 g kg⁻¹ soil; ammonium-N, 2.9 mg kg⁻¹ soil; nitrate-N, 56 mg kg⁻¹ soil; and soil pH 6.3.

Prior to the glasshouse experiment, soil samples were sieved to 1 cm, air-dried, and homogenised. Each pot was filled with 4.6 kg of soil and had a diameter of 24.5 cm. Five wheat seeds were sown at a depth of 3 cm. Thinning was carried out at the two-leaf stage, leaving three seedlings per pot for further growth.

Two wheat cultivars, high-NUE Mace and low-NUE Gladius were grown under two different N levels: (1) Low-N, without N addition; and (2) High-N, with an application rate of 90 kg N ha⁻¹, with four replicates for each treatment. Soil moisture was maintained at 80% field capacity throughout the experiment. Plants were grown in a controlled condition, maintained at 24°C /10°C for 12 h/12 h day/night, 65% humidity and light intensity of ~225 µmol m⁻² s⁻¹ during the daytime. Wheat rhizosphere soil and root was destructively sampled at the mid-anthesis stage and was stored at -20 °C for molecular analysis.

2.2 DNA extraction and amplicon sequencing

Total DNA was extracted from 0.25 g of frozen rhizosphere soil or root samples using the DNeasy PowerSoil Pro Kits (QIAGEN), and DNA quality was assessed using a Nanodrop spectrophotometer (NanoDrop One, Thermo Scientific). The fungal ITS1–ITS2 hypervariable region was amplified with the ITS1F/ITS2 primer pairs (Gardes and Bruns 1993) and sequenced using the Illumina MiSeq platform at the Australian Genome Research Facility, Australia. Primer sequences were removed from the raw sequences using cutadapt (v.4.2) (Martin 2011) and reads with an average quality score < 20 were quality-filtered using DADA2 (v1.26.0) (Callahan et al. 2016). High-quality reads were subjected to demultiplexing and denoising using DADA2 (Callahan et al. 2016). Non-chimeric amplicon sequence variants (ASVs) were obtained by merging read pairs that overlapped at least 12 bp and removing PCR chimeras. Taxonomic assignment of ASVs was performed with QIIME 2 (Bolyen et al. 2019) against the UNITE (v.9.0) database (Abarenkov et al. 2010). ASVs with < 50 reads were filtered and the count matrix was subjected to R (v4.3.3) for statistical analysis.

2.3 Quantitative PCR (qPCR)

The abundance of fungi in the wheat rhizosphere was estimated using the ITS1F/ITS2R primers (Gardes and Bruns 1993) to target the ITS rRNA gene. The 10 µl qPCR reactions contained 5 µl of SYBR MasterMix (Thermo Fisher), 0.4 µl of each 10 µM forward and reverse primer, 2.2 µl of sterile DNA-free water, and 2 µl of DNA template. The reaction was carried out using the CFX96 Touch Real-Time PCR Detection System (BIO-RAD). The number of ITS gene copies was normalised by the amount of soil from which DNA was extracted.

2.4 Metagenomic analysis

The functional profile of the rhizosphere fungi in the Low-N and High-N treatments was investigated with shotgun metagenomic sequencing. A total of 16 metagenomic libraries from the rhizosphere soil DNA were generated on the NovaSeq 6000 sequencing platform using the Nextera Flex primer (Bruinsma et al. 2018), resulting in 2×150 bp reads. Metagenome sequences (24 Gb per sample) were subjected to fastqc for quality checks (v.0.11.9) (Andrews 2010) and trimmomatic for adapter trimming (v.0.39) (Bolger et al. 2014). Decontamination of the host genome is performed using bowtie2 (v.2.4.2) (Langmead and Salzberg 2012) against the *Triticum aestivum* genome (IWGSC) retrieved from the EnsemblPlants (Yates et al. 2022). The Metaphor pipeline was used to process the resulting clean reads (v.1.7.9) (Salazar et al. 2022). By pooling the reads of samples replicated from the same cultivar, two co-assemblies were generated using MEGAHIT (v.1.2.9) (Li et al. 2015). Prodigal (v.2.6.3) (Hyatt et al. 2010) was used for gene prediction on the assembled contigs, and the predicted contigs were further annotated using eggNOG-mapper (v.2.1.12) (Cantalapiedra et al. 2021) against the eggNOG database (v5.0.2) (Huerta-Cepas et al. 2019). High-quality fungi-related contigs were selected based on the taxonomy annotation of eggNOG Orthologous Groups (COG) with an e-value $< 1e^{-10}$. Contigs detected in $> 75\%$ of the samples were normalised using the count per million (CPM) method before statistical analysis.

2.5 Statistical analyses and fungal guild prediction

All statistical analyses were conducted using R (v.4.3.3), and all figures were generated using the ggplot2 (v.3.4.4) package. The difference in ITS gene copy numbers between N treatments was statistically compared using the Tukey's Honestly Significant Difference (HSD) test. The Shannon index was used to estimate fungal alpha-diversity, and the differences between treatments were statistically compared using the nonparametric Kruskal-Wallis test. Principal coordinates analysis (PCoA) based on the Bray-Curtis distance matrix was performed to assess the dissimilarity of the fungal community compositions between N treatments and cultivars. The dissimilarity of the fungal community compositions across N treatments and cultivars were compared with the two-way PERMANOVA using the 'vegan' package (Oksanen et al. 2022). The similarity of the fungal community compositions across N treatments was compared with the analysis of similarity (ANOSIM) using the 'vegan' package (Oksanen et al. 2022). The linear discriminant analysis (LDA) on the effect size (LEfSe) of ASVs was conducted to determine differential abundant fungal ASVs between N treatments with the

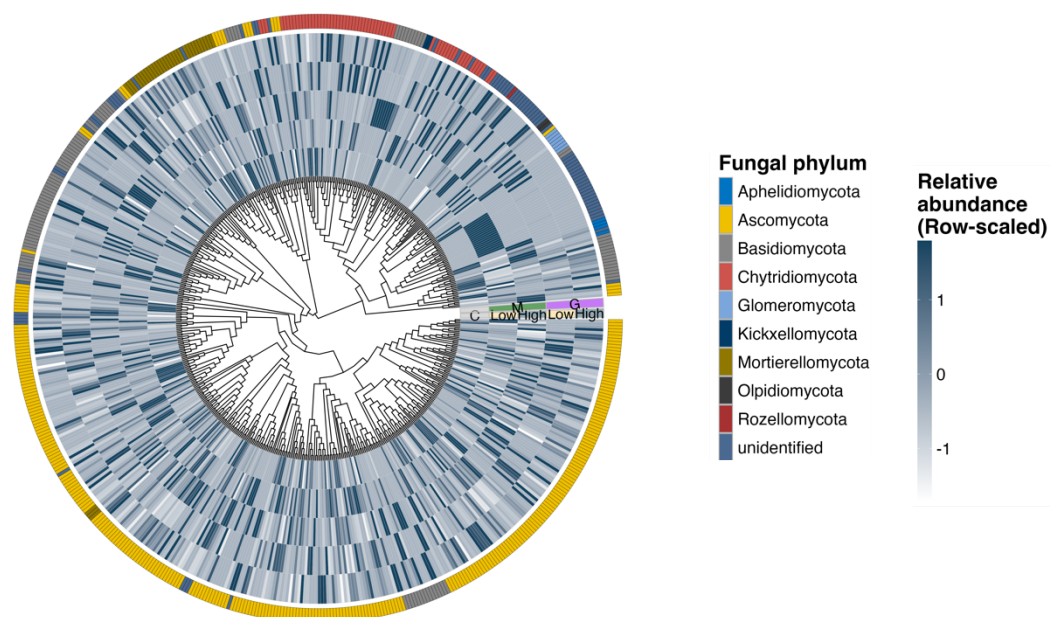
microeco (v.1.1.0) package (Liu et al. 2021). Differential abundant ASVs were selected with a threshold of P -value <0.05 and LDA >2 . “Highly probable” and “Probable” guild predictions of the differential abundant ASVs in the rhizosphere were performed with the microeco (v.1.1.0) package (Liu et al. 2021) against the FUNGuild database (Nguyen et al. 2016). PCoA and two-way PERMANOVA analyses were conducted to assess the differences in the fungal gene compositions between treatments and cultivars. LEfSe was performed to compare the differential abundant fungal genes between Mace and Gladius. For all statistical tests, a threshold of P -value <0.05 was considered significantly different unless specified.

3. Results

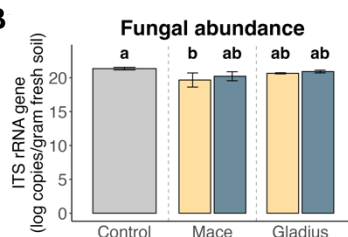
3.1 Abundance, diversity and community composition of fungi in the rhizosphere

The ITS region sequencing identified a total of 589 fungal ASVs across all rhizosphere samples. The rhizosphere fungal community was dominated by the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Mortierellomycota (Figure 1A). Multiple Tukey’s HSD tests indicated that fungal abundance in the rhizosphere did not significantly differ across cultivars or N treatments ($P > 0.05$) (Figure 1B). Similarly, fungal diversity was not significantly affected by N treatments in either cultivar ($P > 0.05$) (Figure 1C). PCoA ordinations revealed a significant shift in fungal community composition within the rhizosphere of both wheat cultivars compared to the fungal community in unplanted soil ($P < 0.05$) (Figure 1D & 1E). Furthermore, PCoA ordination combined with two-way PERMANOVA analysis showed a significant difference in fungal community composition between the cultivars Mace and Gladius ($P < 0.05$), while no significant difference was observed between the N treatments ($P > 0.05$) (Supplementary Figure 1). Additional PCoA analysis indicated a marginally significant shift in the rhizosphere fungal community composition of Gladius under N treatment ($P < 0.1$), whereas no significant shift was detected for Mace ($P > 0.05$) (Supplementary Figure 2).

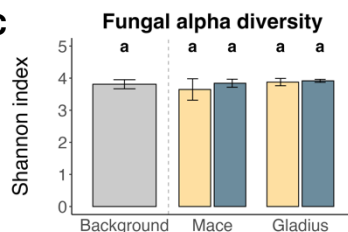
A



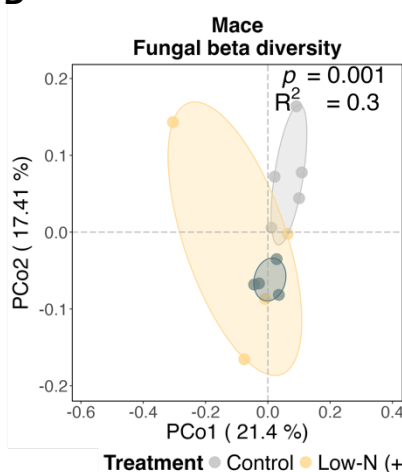
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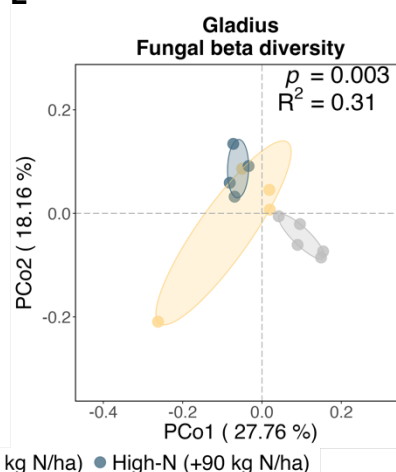


Figure 1. Fungal communities of the wheat rhizosphere at the mid-anthesis stage under N treatments. (A) Phylogenetic distribution of fungal ASVs detected in the untreated soil control (grey) and the rhizosphere of Mace (green) and Gladius (purple) under Low-N (yellow) and High-N (blue) treatments. The scaled relative abundance of ASVs is shown in the heatmap and the assigned phylum is indicated by different colours. (B) Fungal abundance measured by the qPCR analysis of ITS rRNA gene. The results of HSD tests are indicated on top of the bars. (C) Fungal alpha diversity of ITS metabarcoding data estimated by the Shannon index. Kruskal-Wallis's test is indicated on top of the bars. (D-E) Principal coordinates analysis (PCoA) of fungal beta diversity across the untreated soil control, Low-N and High-N treatments of (D) Mace and (E) Gladius, based on the relative abundances of fungal ASVs. ANOSIM statistics are indicated in the top right corner of the PCoA plots.

3.2 Fungal trait prediction of ASVs in response to N deficiency

Differential abundance analysis revealed distinct fungal responses to N treatment between Mace and Gladius. Specifically, under N deficiency, 7 ASVs were enriched and 11 were reduced in Mace ($P < 0.05$), whereas 9 ASVs were enriched and 9 were reduced in Gladius ($P < 0.05$) (Figure 2A & 2B). When comparing the two cultivars, *ASV_403* was consistently enriched under N deficiency, while *ASV_69*, *ASV_55*, and *ASV_67* were consistently reduced in both cultivars (Figure 2). Fungal trait predictions indicated that *ASV_69*, *ASV_55*, and *ASV_67*, which were reduced under N deficiency, are pathogenic, whereas *ASV_403*, which was enriched, exhibited an unidentified trophic mode (Figure 2A & 2B). Additionally, the fungal trait prediction revealed a reduction in symbiotic ASVs under N deficiency. In Mace, *ASV_5* and *ASV_13* were reduced, while in Gladius, *ASV_236* showed a similar decline (Figure 2A & 2B). An exploration of root-associated fungal taxa revealed that *ASV_5*, *ASV_13*, *ASV_69*, *ASV_38*, *ASV_43*, *ASV_83*, and *ASV_20* potentially colonised the root endosphere of Mace. In contrast, *ASV_69* and *ASV_III* were identified as potential colonisers of the root endosphere in Gladius (Supplementary Figure 3).

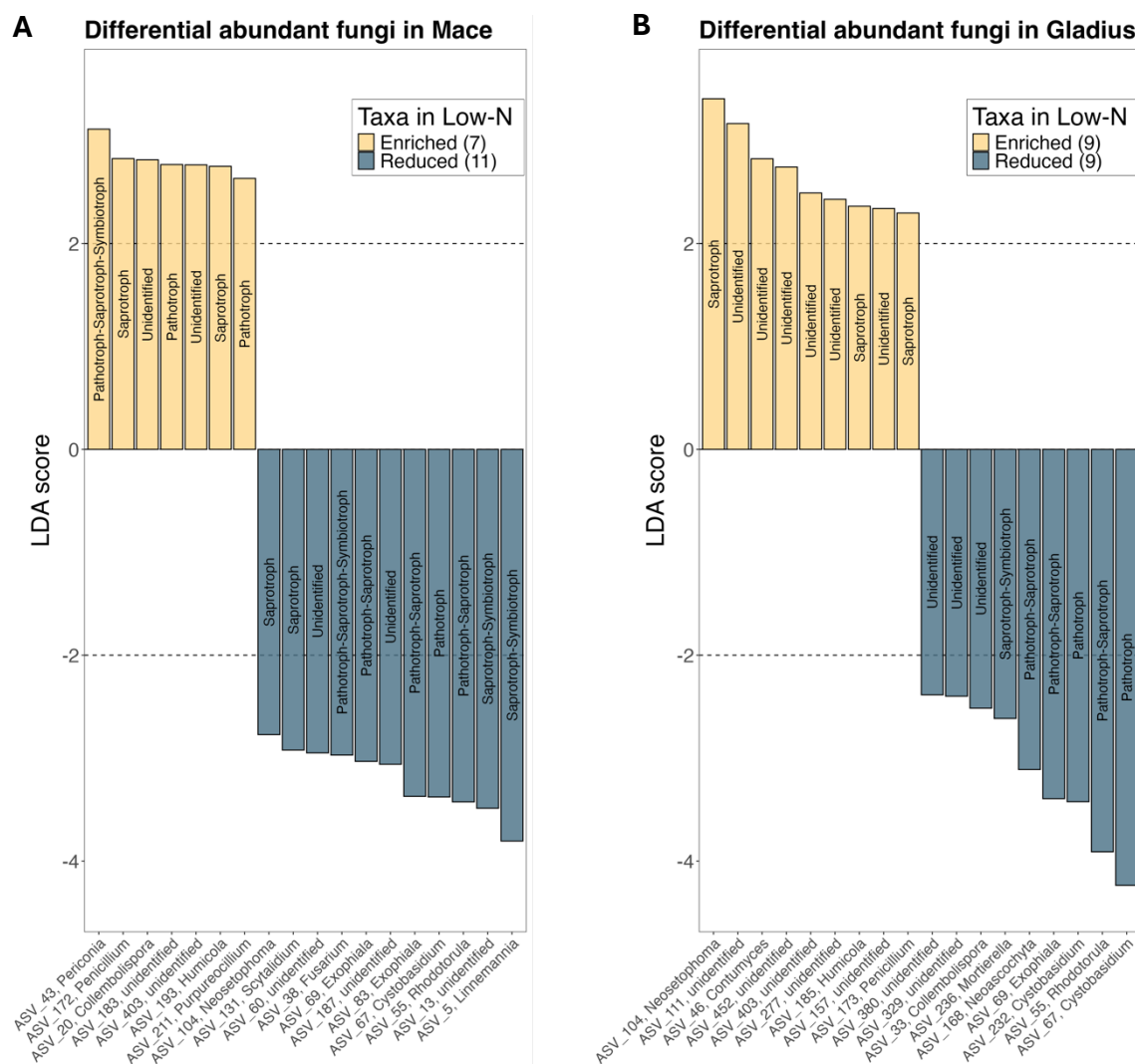


Figure 2. Fungal trait prediction of differential abundant rhizosphere fungi detected in the wheat rhizosphere. Differential abundant fungal ASVs between the Low-N and High-N treatments of (A) Mace and (B) Gladius. Bars in yellow indicated fungal ASVs enriched in the Low-N treatment compared to the High-N treatment whereas bars in blue indicated ASVs reduced in the Low-N treatment compared to the High-N treatment. The dotted line indicates a threshold of LDA score >2. The tropical mode of fungi predicted by FUNguide is indicated in the bar.

3.3 Rhizosphere fungal functional compositions of Mace and Gladius

The fungal functional community in the rhizosphere under Low-N and High-N treatments was analysed through metagenomics at the mid-anthesis stage. A total of 810 contigs were identified as fungi-related using eggNOG, corresponding to 121 functional genes (Supplementary Figure 4). Most of these genes were associated with amino acid transport and metabolism (E) (22.70%), carbohydrate transport and metabolism (G) (12.67%), coenzyme

transport and metabolism (H) (12.61%), energy production and conversion (C) (9.99%), and signal transduction mechanisms (J) (9.74%) (Figure 3A). PCoA ordination revealed no significant differences in the fungal functional community between N treatments ($P > 0.05$), although significant differences were observed between the wheat cultivars ($P < 0.05$) (Figure 3B). Differential abundance analysis indicated that the *cob* gene was significantly more abundant in Mace compared to Gladius, whereas 15 other genes were significantly less abundant in Mace than in Gladius ($P < 0.05$) (Figure 3C).

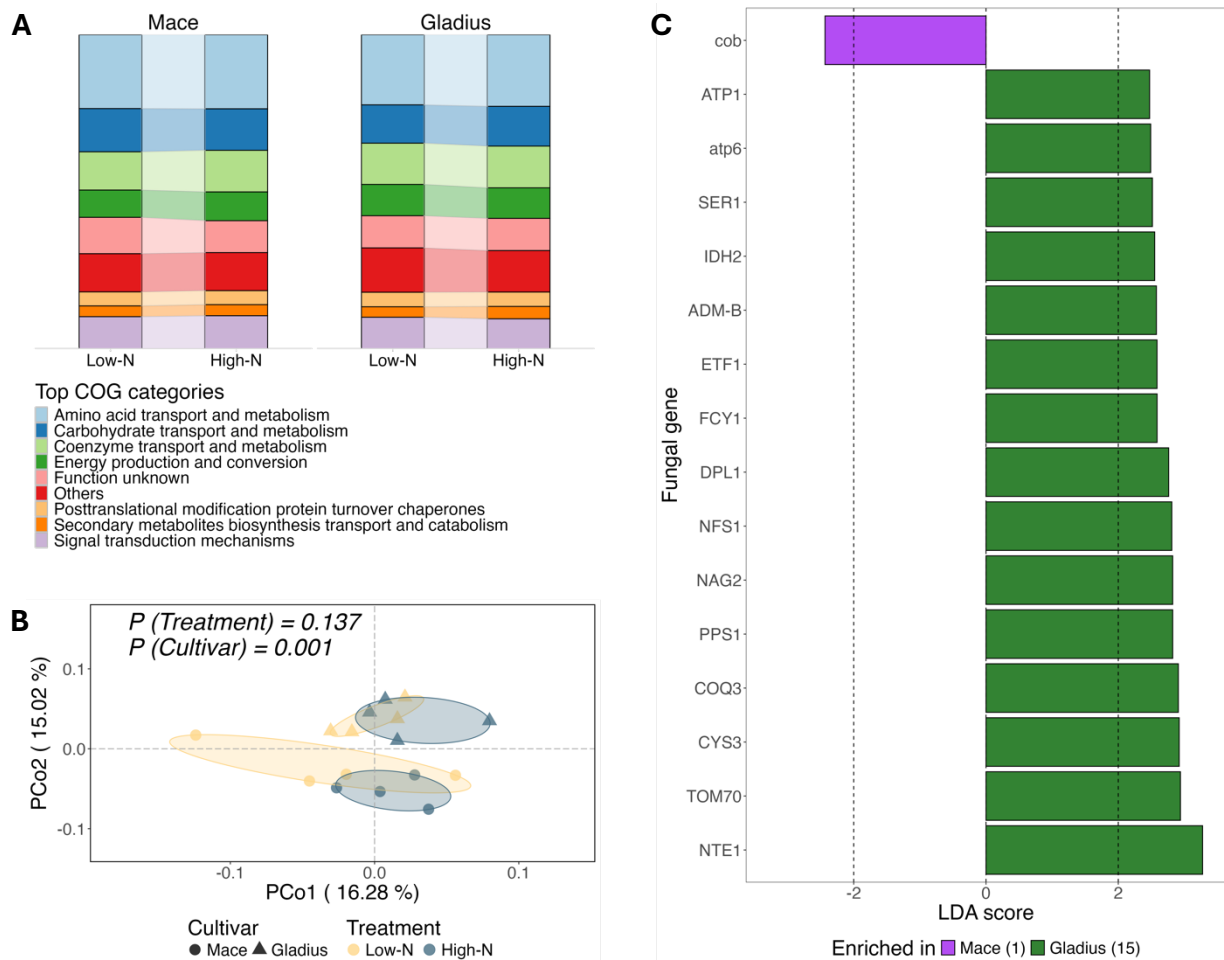


Figure 3. Fungal functional profiles of the wheat rhizosphere across N treatments. (A) Composition of the fungal-related genes in Mace and Gladius under Low-N and High-N conditions, grouped by the COG categories. (B) Principal coordinates analysis (PCoA) of fungal-related genes between Low-N and High-N treatments in Mace and Gladius. Low-N and High-N samples are represented by yellow and blue, respectively, while Mace and Gladius samples are represented by dots and triangles, respectively. Two-way PERMANOVA statistics

are shown in the top left corner of the plot. (C) Differential abundant analysis on fungal genes between the sample of Mace (purple) and Gladius (Green).

4. Discussion

4.1 Intraspecific wheat rhizosphere fungal response to N deficiency

Fungi associated with plants can potentially help their hosts adapt to harsh environments (Chai and Schachtman 2022). Understanding wheat-fungal interactions in N-poor environments is crucial for the development of microbiome-based sustainable agricultural N management strategies (Lam et al. 2022). The metabarcoding analysis revealed an intraspecific fungal community response under nitrogen deficiency (Figure 1). Although the response of the rhizosphere fungal community to N deficiency remains limited between wheat cultivars, evidence suggests that the fungal composition of wheat cultivars can differ, potentially enhancing their resistance to abiotic stress (Gqozo et al. 2020; Yue et al. 2024). The significant difference between the fungal composition of the unplanted soil and the wheat rhizosphere confirmed that wheat shapes the soil fungal community (Figure 1D & 1E). The significant difference in fungal community and functional composition between Mace and Gladius, as demonstrated by the metabarcoding and metagenomic analysis respectively, suggested that our selected cultivars, with distinct reported NUE, have different abilities to shape the soil fungal community (Figure 3B, 3C & Supplementary Figure S1). Previous literature has shown that different wheat cultivars shape the fungal community differently through host-specific wheat-fungal interactions (Wippel et al. 2021).

The differential fungal community composition response to N deficiency, demonstrated by the metabarcoding data, suggests an intraspecific wheat-fungal response to N deficiency (Supplementary Figure S2). While the results for Gladius align with previous long-term studies that reported altered interactions between rhizosphere fungi and wheat plants under N deficiency (Babalola et al. 2022; Guo et al. 2019; Wang et al. 2019), the unchanged fungal community composition in Mace has suggests a higher resilience to N deficiency. Although direct evidence linking a stable fungal community to wheat performance under N deficiency is lacking, it has been shown that the stability of the fungal community and network could benefit plants in tolerating abiotic stresses (Hernandez et al. 2021). AM fungi from the Glomeromycota phylum are known to benefit plant growth by improving N acquisition (Hodge and Storer 2015). A small proportion of fungal ASVs from the Glomeromycota phylum were detected across our rhizosphere samples (Figure 1a). However, it has been reported that a tiny fraction of Glomeromycota fungi is found in wheat plants, with variations across soil types and plant

hosts (Seeliger et al. 2024; Torppa et al. 2023). The enrichment analysis showed that none of the ASVs responding to N deficiency belonged to the Glomeromycota phylum, suggesting that N treatments have a minimal effect on the interaction between wheat and AM fungi (Figure 2). The enrichment analysis with the fungal trait prediction highlighted an intraspecific reduction in symbiotic fungi under N deficiency (Figure 2). Symbiont from the *Lennemannia* genus and an unidentified genus were reduced in Mace (Figure 2A), while an ASV from the *Mortierella* genus was reduced in Gladius (Figure 2B) under N deficiency. In addition, all predicated symbionts are from the *Mortierellaceae* family. This suggests that wheat cultivars may interact with different symbiotic fungal ASVs from the same family, though these interactions generally decrease with decreasing N availability. Our metabarcoding data of the root endosphere fungal community estimate that the predicted symbionts have colonised the roots of Mace (Supplementary Figure S3). *Mortierellaceae* fungi have been shown to benefit wheat growth by interacting with rhizobacterial metabolism and producing high-energy lipids, which subsequently improve N uptake efficiency in wheat (Li et al. 2020a; Yue et al. 2024). The beneficial effect of *Mortierellaceae* fungi across wheat species was demonstrated in a field experiment involving wild wheat, durum wheat and bread wheat, which showed a positive correlation between *Mortierellaceae* fungi and total plant N (Abdullaeva et al. 2024). Although the positive impact of *Mortierellaceae* fungi is well-documented, its growth is suppressed under N deficiency (Kruczynska et al. 2023). Notably, the enrichment analysis identified fungal *ASV_403* as enriched in both Mace and Gladius under nitrogen deficiency, suggesting its potential to benefit wheat growth under nitrogen deficiency. However, it could not be classified to any phylum, nor could its trophic mode be predicted. The metabarcoding data partially addressed our hypothesis, showing an intraspecific response of the rhizosphere fungal community to nitrogen deficiency, particularly with the reduction of symbiotic interactions.

4.2 Functional redundancy of wheat rhizosphere fungal community across N treatments

Since our metabarcoding data has demonstrated an intraspecific fungal community composition shift in response to N deficiency, we then investigated the fungal functional profile using metagenomic. The metagenomic data again indicated an intraspecific functional profile of the rhizosphere fungal community with resilience to N deficiency (Figure 3B & 3C). This contradicts our hypothesis that N-deficient wheat alters the fungal community and its functional profile. This finding differs from a previous study on perennial grass, which showed an increase in carbon acquisition genes but a reduction in N acquisition genes in the fungal

community under N fertilisation (Treseder et al. 2018). While the functional profile of the rhizosphere microbiome is highly dependent on the host genotype of wheat (Yue et al. 2024), fungal genes related to N metabolisms were not detected in either cultivar (Supplementary Figure 4). This could be related to the low occurrence of AM fungi (Saary et al. 2020; Singh 2021; Zhao et al. 2020). Despite a significant shift in the fungal composition of *Gladius* under nitrogen deficiency (Supplementary Figure 2), there were no corresponding changes in its functional profile (Figure 3B), suggesting functional redundancy across fungal species (Mueller et al. 2014; Zeng et al. 2023). It has been demonstrated that the shift in fungal growth patterns does not influence fungal carbon mineralisation, indicating functional redundancy within the fungal community (Banerjee et al. 2016). A field study of the wheat and barley fungal community under N fertilisation demonstrated that host selective rare fungal taxa carry high functional redundancy and play an important role in host resilience to N deficiency (Xiong et al. 2021).

The fungal trait prediction highlighted the responses of saprotrophic fungi to N treatments (Figure 2). Saprotrophic fungi are involved in the organic matter decomposition process and could enhance carbon and N availability to benefit plant growth (Etesami and Adl 2020; Geiser et al. 2015; Zhang et al. 2019). They dominate the rhizosphere fungal community (Schmidt et al. 2019) and can colonise the root endophyte of durum wheat with hyphal extension for better N acquisition (Abdellatif et al. 2009). The gene composition analysis highlighted amino acid metabolism as a major contributor to the fungal functional profile (Figure 3A). This function has been shown to play a crucial role in enhancing plant N acquisition (Wang et al. 2020). In contrast to previous studies that reported shifts in fungal taxonomy and function under N fertilisation (Chen et al. 2019; Li et al. 2020b), our findings indicate that although the fungal composition in *Gladius* has shifted, the functional profile was maintained. This functional stability suggests that the rhizosphere fungi may assist wheat in tolerating N deficiency.

5. Conclusion

In summary, this study revealed that the wheat rhizosphere fungal community response to N deficiency is intraspecific. Additionally, the fungal functional profile demonstrated resilience to N deficiency, with a high proportion of functions associated with amino acid metabolism. A deeper understanding of the mechanisms underpinning fungal stability could enhance the resilience of other microorganisms in N-deficient environments. This approach

has the potential to inform the development of microbial community-based biofertilisers, thereby contributing to sustainable agricultural practices.

CRedit authorship contribution statement

Lok Hang Chan: Writing – original draft, conceptualization, data curation, formal analysis, methodology, investigation. **Shu Kee Lam:** Writing – review and editing, conceptualization, Funding acquisition, methodology, Supervision. **Deli Chen:** Funding acquisition, Project administration, methodology, Supervision. **Caixian Tang:** Writing – review and editing, Funding acquisition, methodology, Supervision. **Qinglin Chen:** Writing – review and editing, data curation, formal analysis. **Ute Roessner:** Funding acquisition, methodology. **Hang-Wei Hu:** Writing – review and editing, conceptualization, Funding acquisition, Project administration, Resources, methodology, Supervision.

Declaration of competing interest

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

Acknowledgements

This research was supported by the Australia Research Council's Industrial Transformation Research Program funding scheme (IH200100023). The wheat seeds for this research were provided by the Australian Grain Technology (AGT). The computational resources were supported by The University of Melbourne's Research Computing Services and the Petascale Campus Initiative.

Data availability

Raw ITS amplicon and metagenomic sequencing files can be publicly accessed from the NCBI database with the accession numbers PRJNA1195643 and PRJNA1126416, respectively. Other datasets generated during the current study are available from the corresponding author upon reasonable request.

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Chapter 4 - Rhizosphere bacteria DNRA response in wheat cultivars under N deficiency

Connecting statements

Following the observations in Chapters 2 and 3, which indicated that the wheat rhizosphere bacterial community exhibits a greater responsiveness to N deficiency compared to the fungal community, we hypothesised a shift in bacterial modulation of the soil N cycle under these conditions. We postulated that this N-cycle response would differ between the two selected wheat cultivars (Mace-high NUE & Gladius-lowNUE), potentially accounting for their contrasting reported NUE. To test these hypotheses, we modified a bioinformatics pipeline specifically targeting bacterial N-cycling genes within the metagenomic data collected in Chapter 2. Our analysis revealed that the N-cycle remained stable in the low-NUE wheat cultivar, whereas the high-NUE wheat cultivar exhibited an increased dissimilatory nitrate reduction to ammonium (DNRA) potential. This finding suggests that wheat may enhance its NUE through an increased capacity for DNRA under N-deficient conditions. Note that the N treatments were renamed as from N0 and N2 to -N and +N, respectively. This work has been submitted to the *Soil Security* journal, and completed the major revision requested by the editor, titled “Differential modulation of rhizosphere dissimilatory nitrate reduction to ammonium (DNRA) by wheat cultivars under nitrogen deficiency”.

Differential modulation of rhizosphere dissimilatory nitrate reduction to ammonium (DNRA) by wheat cultivars under nitrogen deficiency

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Abstract

Nitrogen (N) deficiency constrains crop growth, which could potentially be mitigated by the plant-microbe interaction. However, the interaction between wheat and bacterial N metabolism in response to N deficiency remains poorly understood. A glasshouse experiment was conducted to investigate the rhizosphere bacterial N metabolism gene profile in the wheat rhizosphere with (+N) and without (-N) N application. Two wheat cultivars, Mace and Gladius, with contrasting reported N use efficiencies (NUE), were grown in an agriculture soil previously cropped with wheat. Metagenomic analysis, was used to examine the N metabolism gene profile of rhizobacteria under different N treatments. The result highlighted a significant difference in the rhizosphere bacterial N metabolism gene composition between Mace and Gladius. Differential abundance analysis revealed a significantly higher gene abundance involved in dissimilatory nitrate reduction to ammonium (DNRA) (*nrfA*, *nrfD*, and *nrfH*), in Mace under N deficiency. In contrast, no significant changes were found in Gladius. Correlation analysis highlighted a significant negative correlation between the abundance of *nrfA* and *nrfH* genes and plant total N content. We found that under N deficiency, Mace demonstrated a higher capacity for DNRA, consistent with its reported NUE. Our findings provide important information that the rhizosphere bacterial DNRA pathway response to N deficiency is different between wheat cultivars.

Keywords

Nitrogen-use efficiency, Wheat, Plant-microbe interaction, Metagenomics, Nitrogen metabolism, DNRA

1. Introduction

Synthetic nitrogen (N) fertilisers have been intensively applied in modern agriculture system to ensure crop productivity. However, N use efficiency (NUE) remains low, with less than half of the applied N being assimilated by crops and the remaining either temporarily immobilised in the soil or lost to the environment (Wang et al. 2024b). Soil microorganisms can establish mutualistic associations with plants, enhancing N acquisition and adaptation to nutrient-poor environments (Chai and Schachtman 2022). This suggests a potential for improving fertiliser NUE, as supported by the “cry-for-help” theory (Chai and Schachtman 2022). For example, maize grown in N-deficient soil can recruit *Massilia* sp. to stimulate lateral root growth and enhance N uptake (Yu et al. 2021). Consequently, plant growth-promoting rhizobacteria (PGPR) that benefit crop growth under N deficiency are being

targeted for modern biofertiliser development (Sun et al. 2020). However, the host-specific nature of plant-microbe interactions poses challenges in selecting ideal microorganisms for targeted crop growth (Wippel et al. 2021). Advancements in crop domestication and breeding influenced plant-microbe interaction that benefit plants in adapting low N conditions (Nerva et al. 2022). A study on rice cultivars with contrasting NUE have shown that the N metabolism of root endospheric microorganisms contributes to rice NUE (Zhang et al. 2019).

The biogeochemical N metabolism is greatly influenced by soil microorganisms in the rhizosphere, which subsequently impacts plant N uptake (Coskun et al. 2017a). While the symbiotic interaction between N-fixing *Rhizobium* and leguminous plants is well-established (Hungria et al. 1998; Webster et al. 1998), non-leguminous crops lack nodule structures and derive limited benefit from N-fixing bacteria. Furthermore, N fixation in non-legumes is often suppressed by intense microbe-microbe competition for energy (Etesami and Adl 2020). Consequently, research has increasingly focused on other N transformation processes to enhance crop NUE. For example, nitrification inhibitors, both biological and chemical, are gaining interest for their ability to suppress nitrification, thereby reducing N losses through nitrate leaching and nitrous oxide emission, and ultimately improving plant NUE (Coskun et al. 2017b; Sun et al. 2016). A study on maize demonstrated that inoculation with a *Bacillus subtilis*-containing biofertiliser reduced urea use by 50% through modulation of N-cycling processes (Sun et al. 2020). This highlighted the importance of bacterial N metabolism in plant NUE. Besides the N-fixation process, microbial contribution to the reduction of nitrate to ammonium can mitigate N loss and extend plant N uptake, potentially enhancing fertiliser NUE (Cheng et al. 2023).

The two main pathways included the assimilatory nitrate reduction to ammonium (ANRA) and the dissimilatory nitrate reduction to ammonium (DNRA). While the ammonium from ANRA is immobilised by microorganisms, the ammonium from DNRA is released to the environment as a byproduct of anaerobic respiration (Stein and Klotz 2016). The preference of nitrate reduction pathways is influenced by the C:N in soil (Cheng et al. 2023). Under N fertilisation, where C:N is lowered, the activity of ANRA and DNRA pathways are reduced. This is evidenced by a study in wheat, demonstrating lower ANRA related gene detection under high N fertilisation rate (Shen et al. 2025). On the other hand, plants under N deficiency stress increase the secretion of carbon compounds in their root exudates, thereby shaping the rhizosphere microbiome (Chai and Schachtman 2022). This may subsequently increase the C:N ratio in the rhizosphere and could be favourable to DNRA bacteria (Wang et al. 2022; Zhou et al. 2022). It was reported that the root exudate of

Arabidopsis thaliana, containing amino acids, sugars, and other organic compounds, increased the ammonium (NH_4^+) production of *Shewanella loihica* via the DNRA pathway (Biswal and Chang 2022). A field study on wheat reported a negative correlation between the abundance of the DNRA bacterial community and shoot dry weight without N fertilisation (Lewin et al. 2024), suggesting the important role DNRA in wheat growth under low N conditions. Under high N fertilisation, the competitive pathway of denitrification is generally favoured over DNRA, as demonstrated by a ^{15}N tracing study (Wang et al. 2022). It was also demonstrated that wheat straw and grain yield were significantly and positively correlated with nitrate reductase activity (Eilrich and Hageman 1973), highlighting that nitrate reduction has a profound impact on wheat yield. However, our understanding of the bacterial nitrate to ammonium transformation process of wheat, in response to N deficiency, remains limited.

We hypothesised that the higher NUE wheat cultivar has a higher abundance of bacterial DNRA genes, and wheat cultivars with contrasting NUE have different bacteria N metabolism responses to N deficiency. In this study, we employed metagenomic analysis to investigate the N metabolism of rhizobacteria associated with two wheat cultivars, Mace (with a high reported NUE) and Gladius (with a low reported NUE), under varying N availability. This provides valuable insights for understanding the rhizosphere bacterial DNRA pathway response to N deficiency between wheat cultivars with contrasting reported NUE.

2. Materials and methods

2.1. Soil collection and glasshouse experiment

Soil samples (0-20 cm) were collected in March 2022 from a field with a history of wheat cultivation at the Dookie Campus of The University of Melbourne, Australia (36.382° S, 145.711° E). The soil is classified as a Dermosol (Isbell, 2024) and exhibited the following physicochemical properties: total carbon (TC), 0.021 g kg⁻¹; total N (TN), 0.002 g kg⁻¹; organic carbon (C), 0.018 g kg⁻¹; nitrate-N, 56 mg kg⁻¹; ammonium-N, 2.9 mg kg⁻¹; and pH 6.3. Soil samples were sieved to 1 cm, air-dried, and homogenised before use in a glasshouse experiment.

Two wheat cultivars, Mace and Gladius, were selected for the pot experiment. Pots with an inner diameter of 16.5 cm were filled with 4.6 kg of soil. Uniform-sized seeds were sown at a depth of 3 cm, and seedlings were thinned to three per pot at the two-leaf stage. To establish varying N levels, ammonium sulfate was applied at two rates: (1) -N, no N addition, and (2) +N, 90 kg N ha⁻¹, with four replicates per treatment. The +N was considered a

common agricultural practice for Australian wheat farms in the present study. Soil moisture content was maintained at approximately 80% field capacity throughout the experiment. Plants were grown under controlled conditions: 24°C/10°C Day/night temperature cycle (12 h/12 h), 65% humidity, and a photosynthetic photon flux density (PPFD) of $\sim 225 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the day. At the mid-anthesis stage, the wheat rhizosphere soils were sampled by gently shaking the root system within a sampling bag and stored at -20°C for subsequent analyses.

2.2. Plant and soil physicochemical characterisation

The number of wheat heads was counted at the mid-anthesis stage. Aboveground plant materials were oven-dried at 60°C for 7 days to determine dry biomass, and ground to a 0.2 mm fine powder using an ultra-centrifugal mill (ZM200 Ultra Centrifugal Mill, RETSCH). Rhizosphere soil samples were oven-dried at 40°C for 7 days and subsequently ball-milled using a tissue grinder (TissueLyser II, QIAGEN). The resulting finely ground plant and soil samples were analysed for C and N using a CN analyser (LECO TruMac Series). For soil pH and electrical conductivity (EC) measurements, fresh soil samples were suspended in Milli-Q water at a 1:5 ratio and shaken at 250 rpm for 60 mins. Soil pH was measured using a professional benchtop pH meter (HANNA), and EC was measured using a conductivity meter (Multiparameter Laboratory Benchtop Conductivity meter, SmartCHEM-LAB).

2.3. DNA extraction and qPCR

Total DNA was extracted from soil samples using the DNeasy PowerSoil Pro Kit (QIAGEN), and DNA quality was assessed using a NanoDrop spectrophotometer (NanoDrop One, Thermo Scientific). The abundance of bacteria in the wheat rhizosphere was estimated by quantifying the 16S rRNA gene using the 506F/816R primer set (Caporaso et al. 2011). Each 10 μL quantitative PCR (qPCR) reaction contained 5 μL of SYBR Green Master Mix (Thermo Fisher), 0.4 μL of each 10 μM forward and reverse primer, 2.2 μL of sterile DNA-free water, and 2 μL of DNA template. The reactions were performed using a CFX96 Touch Real-Time PCR Detection System (BIO-RAD). The number of 16S rRNA gene copies was normalised to the amount of soil from which the DNA was extracted.

2.4. Metagenomic analysis

Shotgun metagenomic sequencing was performed on the Illumina MiSeq System platform at the Australian Genome Research Facility, Melbourne, Australia, to investigate differences in N metabolism within the rhizosphere microbiome under N-deficient (-N) and N-fertilised (+N) treatments. A total of 16 metagenomic libraries were generated from rhizosphere soil DNA using the NovaSeq 6000 sequencing platform and Nextera Flex primers (Bruinsma et al. 2018), yielding 2×150 bp paired-end reads. The resulting metagenome sequences (24 Gb per sample) were quality checked using FastQC (v0.11.9) (Andrews 2010) and adapters were trimmed using Trimmomatic (v0.39) (Bolger et al. 2014). Samples were decontaminated by mapping reads against the *Triticum aestivum* genome retrieved from EnsemblPlants (Yates et al. 2022) using Bowtie2 (v2.4.2) (Langmead and Salzberg 2012). Clean reads were processed using the Metaphor (v1.7.9) pipeline (Salazar et al. 2022). Two co-assemblies were generated by pooling reads from samples of the same cultivar and assembled using MEGAHIT (v1.2.9) (Li et al. 2015). Genes were predicted from the assembled contigs using Prodigal (v2.6.3) (Hyatt et al. 2010), and contigs were annotated using eggNOG-mapper (v2.1.9) to identify genes involved in N metabolism. Contigs counts were transformed to count per million (CPM) for downstream analysis and raw contigs count were used for differential abundant analysis.

2.5. Statistical analysis

All statistical tests were performed in R (v4.3.3), with a significance threshold of $P < 0.05$. Analysis of variance (ANOVA) was used to compare the 16S rRNA gene copy numbers and other soil and plant parameters. Principal coordinate analysis (PCoA) was used to compare N-cycling gene composition between treatments and cultivars. Permutational analysis of variance (PERMANOVA) was performed using the “vegan” package (v2.6-6.1) to assess dissimilarity between treatment replicates (Oksanen et al. 2022). Linear discriminant analysis of effect size (LEfSe) analysis was performed to identify differentially abundant genes (linear discriminant analysis (LDA) score > 2 , $P < 0.05$). The LDA score reflects the discrimination power of input taxa and ranks the importance of each biomarker. The LEfSe analysis compared taxa at the order level between the -N and +N treatments. A positive LDA score indicates a significantly higher gene abundance in the -N treatment, while a negative LDA score indicates a significantly higher gene abundance in the +N treatment. As differential abundant genes was observed in Mace, downstream analyses were focused on Mace. PCoA and PERMANOVA were used to compare the bacteria order composition

between treatments. LEfSe analysis was performed to identify differentially abundant orders. Spearman's rank correlation was used to assess the correlation of bacterial orders and N-cycling gene counts with soil and plant parameters using the “Hmisc” package (v5.1-3). Correlation was considered statistically significant at $P < 0.05$, with a correlation coefficient > 0.7 .

3. Results

3.1. Measurement of soil and plant parameters under N deficiency

N addition did not significantly alter rhizosphere 16S rRNA gene abundance, soil TC, soil TN, or plant TC content across Mace and Gladius ($P > 0.05$) (Figure 1A-C & 1F). N addition significantly increased soil pH but significantly reduced soil EC in both Mace and Gladius ($P < 0.05$) (Figure 1D & 1E). The aboveground biomass, plant TN content and head count of Mace and Gladius were significantly reduced in -N compared to +N ($P < 0.05$), suggesting that both wheat cultivars were N-deficient under -N (Figure 1G-I). Specifically, the aboveground biomass of Mace and Gladius was significantly reduced by 26.7% and 23.0%, respectively, compared the -N treatment to the +N treatment ($P < 0.05$) (Figure 1H). The TN content in Mace and Gladius was also significantly reduced by 44.0% and 36.3%, respectively, compared the -N treatment to the +N treatment ($P < 0.05$) (Figure 1G). Notably, plant TN content was significantly lower in Mace compared to Gladius under both -N and +N treatments ($P < 0.05$) (Figure 1G).

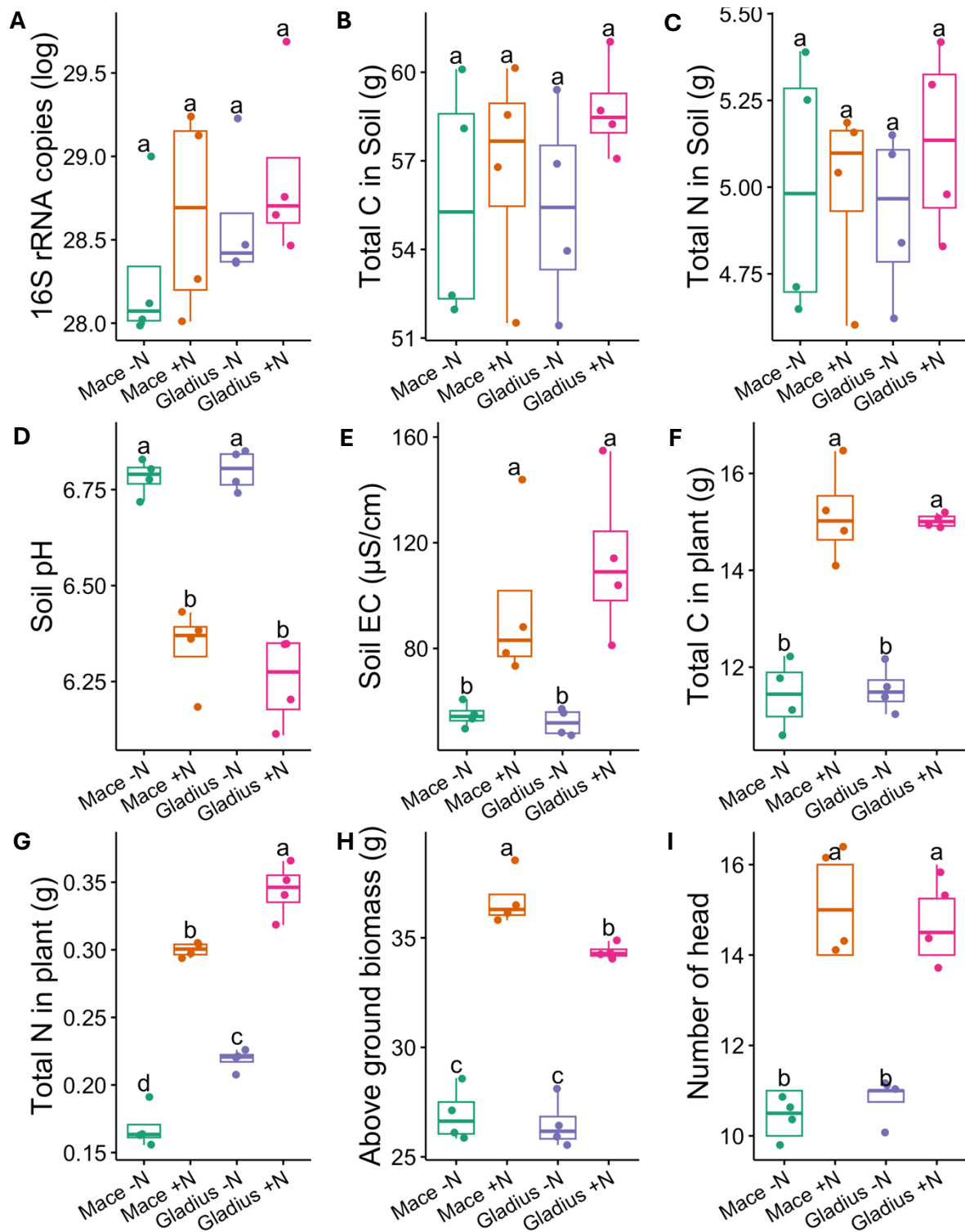


Figure 1. The effect of N deficiency on soil and plant growth in Mace and Gladius. (A) Bacterial abundance in rhizosphere soils estimated by qPCR analysis of 16S rRNA gene copy number. (B-C) Soil TC and TN content in grams. (D-E) Soil pH and electrical conductivity (EC). (F-G) Plant C and N content of the above ground plant materials in grams. (H-I) Above ground biomass and the number of heads. ANOVA results are shown above each bar, with different letters indicating significant differences between treatments ($P < 0.05$).

3.2. Bacterial N metabolism genes under N deficiency

The eggNOG-mapper identified 10,964 bacterial genes, including 56 genes directly and indirectly involved in N transformation (Figure 2A). In both Mace and Gladius, the number of bacterial gene involved in denitrification and DNRA had an observable higher abundance than other detected N metabolism (Figure 2B). PCoA revealed no significant differences in bacterial N transformation between N treatments ($P > 0.05$) but significant differences between samples from Mace and Gladius ($P < 0.05$) (Figure 2C). The heatmaps indicated that a higher abundance of bacterial N metabolism genes in Mace than Gladius in general (Figure 2A & 2B). The LEfSe analysis revealed that while no differentially abundant genes were detected in Gladius, and the abundance of bacterial *nrfD*, *nrfA*, *nrfH*, *nasE* and *nasC* genes in Mace significantly changed under N deficiency (Figure 2D). In particular, the abundance of the *nrfD*, *nrfA* and *nrfH* genes involved in bacterial DNRA significantly increased ($P < 0.05$), where the *nasE* and *nasC* genes involved in bacterial ANRA significantly decreased ($P < 0.05$), under N deficiency (Figure 2D).

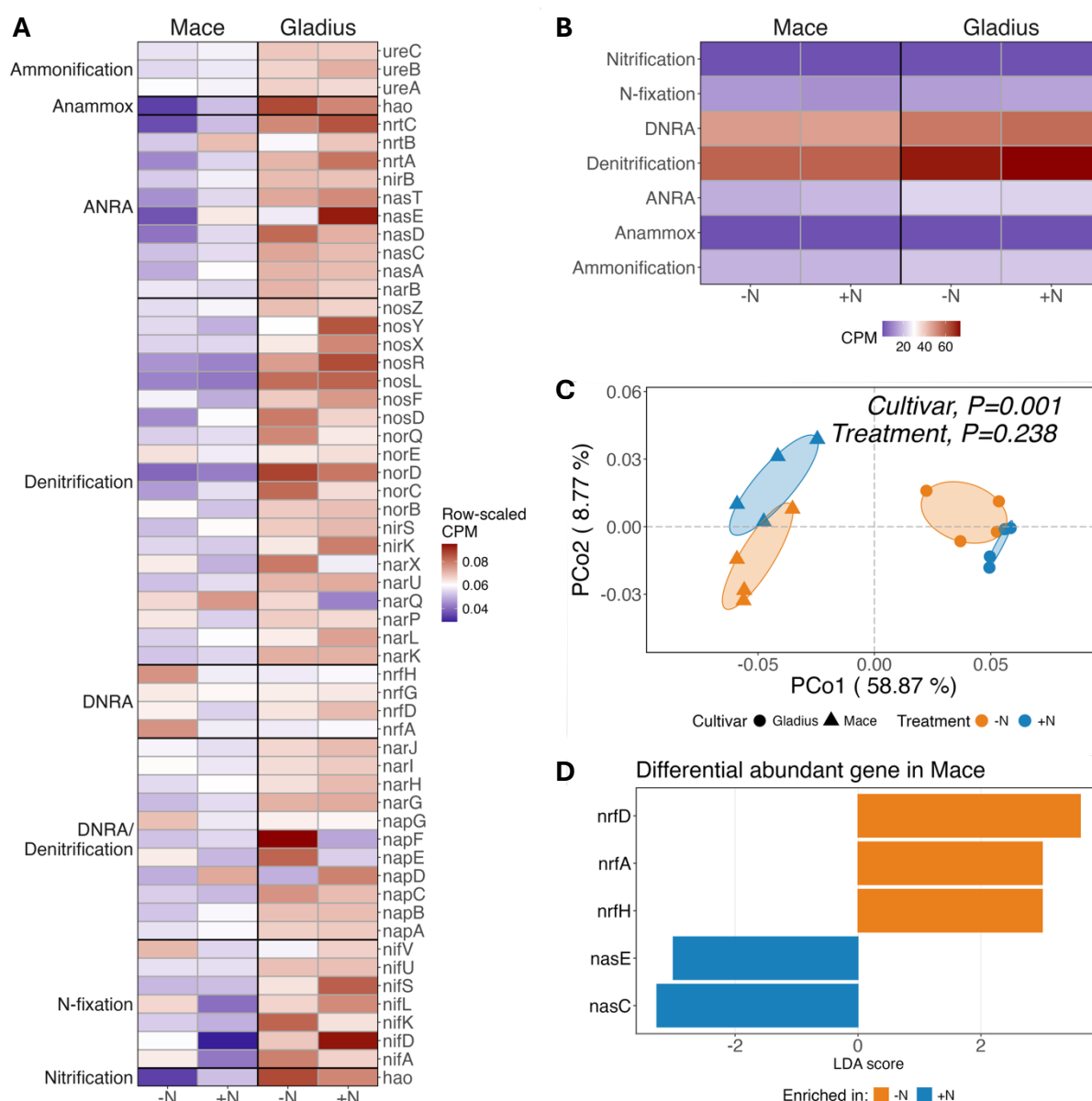


Figure 2. Bacterial N metabolism genes abundance in Mace and Gladius under N deficiency. (A) Heatmap presenting the row-scaled genes count (CPM) involved in bacterial N metabolism for Mace and Gladius. (B) Heatmap presenting the total genes count (CPM) involved in each bacterial N transformation process in Mace and Gladius. (C) Principal coordinate analysis (PCoA) plots showing N metabolism gene composition in Mace and Gladius across N treatments. PERMANOVA statistics are indicated in the top-right corner of the PCoA plots. (D) Bar plot showing the differential abundant genes in Mace under N deficiency.

For the correlation plot, the size of the dot represents the correlation coefficient, and statistical significance is indicated by the star ($P < 0.05$).

4. Discussion

Plants under N deficiency actively recruit beneficial bacteria to improve N acquisition, through the secretion of root exudates (Chai and Schachtman 2022). Changes in root exudation can alter plant-microbe interactions, thereby affecting bacterial nitrate reduction process (Putz et al. 2018). Our aboveground biomass data confirmed that both Mace and Gladius plants without addition (-N treatment) were N-deficient relative to the +N treatment at the mid-anthesis stage (simulating common agricultural practice) (Figure 1H). Although the plant TN content in Mace was consistently lower than in Gladius across both +N and -N treatments (Figure 1G), this difference is likely attributable to the N relocation to the root system under N deficiency stress (Huang et al. 2024; Wang et al. 2024c). The higher NUE plant cultivars often possess a larger root system (Huang et al. 2024). Crucially, the significantly higher biomass of Mace compared to Gladius in the +N treatment supports that Mace possesses a higher NUE than Gladius under typical N fertilisation (Figure 1H). This finding is consistent with previous reports on these cultivars (Alhabbar et al. 2018).

Our metagenomic data demonstrated differences in the N metabolism gene composition in the rhizosphere of Mace and Gladius (Figure 2A, 2B & 2C). This suggested that wheat cultivars interact and modulate rhizosphere bacteria N metabolism differently, aligning with previous study (Chen et al. 2019). The differential abundance analysis highlighted that five bacteria N metabolism genes had a significant alteration in Mace under N deficiency (Figure 2D), a finding absent in Gladius. Maize cultivars showed differential expression of the *amoA*, *nirS*, and *nifH* genes in their rhizosphere. This implies that the cultivars possess different N metabolism potentials (Favela et al. 2021). Moreover, a study showed a higher expression of N metabolism functional genes in the higher NUE rice cultivar Indica, than that of the lower NUE rice cultivar Japonica, suggesting the contribution of microbes in rice NUE (Zhang et al. 2019). These together support our hypothesis that wheat cultivars with contrasting reported NUE have a different modulation in the bacterial soil N metabolism.

Despite PCoA indicating no significant difference in N metabolism gene composition across treatments, differential abundance analysis highlighted significantly higher abundance of the bacterial *nrfD*, *nrfA*, and *nrfH* genes in the Mace rhizosphere under N deficiency (Figure 2A & 2D). This finding points to an increased modulation of the DNRA process. On

the other hand, the reduced abundance of the bacterial *nasC* and *nasE* genes reflected a reduced modulation in the ANRA process under N deficiency (Figure 2A & 2D). While the DNRA and ANRA processes both reduce nitrate to ammonium and significantly contribute to the N metabolism, the ANRA process involved N immobilisation for microorganisms growth, and the DNRA releases ammonium to the environment (Stein and Klotz 2016). This indeed suggested Mace increased rhizosphere bacterial N recycling via an enhanced DNRA process, simultaneously reducing immobilisation from the ANRA process under N deficiency. We focused on Mace, the cultivar with higher reported NUE, and further revealed that the abundance of bacterial *nrfA* and *nrfH* genes exhibited a significantly negative correlation with plant TN content (Figure 3D). This supports that Mace simulates soil DNRA under N-limited conditions, aligned with a previous discovery in rice paddies (Pandey et al. 2019). A field study on wheat reported a negative correlation between DNRA bacteria and shoot dry weight (Lewin et al. 2024), suggested an important role of DNRA under low N conditions.

A study using the ^{15}N tracer method reported a positive correlation between fertiliser N recovery efficiency and bacterial DNRA functional genes in the maize rhizosphere. It was suggested that bacterial-driven DNRA influences soil N retention, as well as other N-cycling processes, such as nitrification and denitrification, across different soil types (Xiao et al. 2024). Furthermore, previous studies have demonstrated that the C concentration have a direct impact favouring DNRA over denitrification (Carlson et al. 2020; Wang et al. 2025). A long-term field trial with ^{15}N trace element and qPCR gene quantification highlighted that a crop field with ley rotation enhanced DNRA activity through the enriched soil organic matter (Putz et al. 2018). Although our measurement in soil C indicates no significant changes under N deficiency (Figure 1B), root exudation under stress conditions may increase C availability for bacterial growth, subsequently enhancing DNRA (Cheng et al. 2022). This is supported by another study showing that wheat rhizodeposition can enrich DNRA through elevated rhizosphere C content (Lewin et al. 2024). Overall, our data indicate that Mace, with higher reported NUE, stimulated the rhizosphere bacterial DNRA process under N deficiency, aligned with our hypothesis.

Further investigation into bacterial taxa that potentially benefit Mace growth under N deficiency was conducted at the order level. We observed a significant shift in the bacterial community composition of Mace (Figure 3A), with the orders Rhodocyclales, Nitrosomonadales, Hydrogenophilales, and Bdellovibrionales showing significantly higher abundances under N deficiency (Figure 3B). This suggests their potential in providing benefits for Mace growth under N deficiency. The correlation analysis highlighted that the

abundance of Rhodocyclales was negatively correlated with the Mace TN content (Figure 3C). Despite the absence of detectable DNRA genes at the order level in our metagenomic data, such genes were identified within the Betaproteobacteria class, to which Rhodocyclales are affiliated. Studies across different systems have demonstrated that bacteria from the Rhodocyclales order are involved in the DNRA process (Coyotzi et al. 2017; Rasigraf et al. 2017). Several genera in the Rhodocyclales order, including *Azoarcus*, *Thauera* and *Azonexus*, have been reported as some key microorganisms involved in DNRA and the high C:N ratio drives the enrichment of DNRA bacteria (Wang et al. 2022; Zhou et al. 2022). Improved ammonia recovery was confirmed in a study that utilised a static magnetic field to stimulate DNRA in *Thauera* sp., thereby highlighting DNRA's potential to enhance NUE (Xie et al. 2024). It was also demonstrated that a mixed culture of Rhodocyclales species reduced N₂O emission (Conthe et al. 2018), suggesting their role in improving soil N retention and reducing N gas emission. Besides the direct contribution in N metabolism, Rhodocyclales bacteria have also been reported as PGPR to stimulate root growth to improve N acquisition and enhance stress tolerance (Numan et al. 2018). A study inoculated plant growth promoting *Bacillus* sp. in *Pyrus betulifolia* under N-limited conditions and reported a significant increase in the Rhodocyclales bacteria with unclassified genera, suggesting a potential role for Rhodocyclales bacteria in benefiting *Pyrus betulifolia* growth under N deficiency (Huang et al. 2022). Together, these suggested that Mace potentially interacted with bacteria from the Rhodocyclales order in improving NUE under N deficiency.

5. Conclusion

Our metagenomic data suggested that Mace and Gladius, with contrasting reported NUE, exhibit different interactions with bacterial N metabolism. Furthermore, we found that the gene abundances involved in soil DNRA pathway were significantly increased in the Mace rhizosphere under N deficiency, indicating that its modulation could be important for improving N fertiliser use efficiency. It is also worth noting that the Rhodocyclales order interacted with Mace, potentially acting as PGPR that may benefit wheat growth under N deficiency. These findings provide important information that the rhizosphere bacterial DNRA in response to N deficiency differed between wheat cultivars and the importance of wheat cultivar selection for sustainable agriculture.

CRedit authorship contribution statement

Lok Hang Chan: Writing – original draft, conceptualisation, data curation, formal analysis, methodology, investigation. **Shu Kee Lam:** Writing – review and editing, conceptualisation, Funding acquisition, methodology, Supervision. **Deli Chen:** Funding acquisition, Project administration, methodology, Supervision. **Caixian Tang:** Writing – review and editing, Funding acquisition, methodology, Supervision. **Qinglin Chen:** Writing – review and editing, data curation, formal analysis. **Hang-Wei Hu:** Writing – review and editing, conceptualisation, Funding acquisition, Project administration, Resources, methodology, Supervision.

Declaration of competing interest

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

Acknowledgements

This research was supported by the Australia Research Council (IH200100023, FT230100158). The wheat seeds for this research were provided by the Australian Grain Technology (AGT). The computational resources were supported by The University of Melbourne's Research Computing Services and the Petascale Campus Initiative.

Data availability

Raw sequencing files can be publicly accessed from the NCBI database with the accession number PRJNA1126416. Other datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Chapter 5 - General discussion and conclusions

Understanding the intricate interactions between wheat and microorganisms under N deficiency is paramount for advancing future biofertiliser development and fostering sustainable agricultural practices. In this chapter, we synthesise and discuss the key findings from Chapters 2, 3, and 4. We'll delve into the wheat root-associated microbial response and the wheat metabolite response to better comprehend the complex wheat-microbe interactions that occur under N-deficient conditions. Building upon our research findings, we also propose compelling directions for future research.

5.1 Wheat root-associated microbial response to N deficiency at the mid-anthesis stage

To elucidate how bacteria and fungi interact with wheat in response to N deficiency, we conducted a glasshouse experiment incorporating various N application levels and employed next-generation sequencing to analyse the associated microbial communities. Plant total N content and dry biomass suggested a significant N effect at the mid-anthesis stage. A consistent pattern was observed with both *16S* rRNA gene and *ITS* gene amplicon sequencing: the root-associated bacterial and fungal communities exhibited shifts under N deficiency at the mid-anthesis stage, but not at the stem elongation stage. This observation suggests a dynamic wheat-microbe interaction that responds to N deficiency specifically at the mid-anthesis stage. Numerous studies have reported that root-associated microbial communities change across different plant growth stages, indicating the dynamic nature of plant-microbe interactions (Cavaglieri et al. 2009; Iannucci et al. 2021; Sun et al. 2022a). Furthermore, research on wheat has shown that N treatments exert a more significant influence on the soil microbial community during later growth stages (Chen et al. 2019).

We investigated the root-associated bacterial and fungal communities, in both the root endosphere and rhizosphere, in response to N deficiency. Our amplicon sequencing revealed that the bacterial and fungal communities within the root endosphere remained relatively stable, whereas the rhizosphere bacterial and fungal communities significantly shifted under varying N treatments, particularly between the N0 (no N) and N2 (optimal N) treatments.

This finding strongly suggests a dynamic plant-microbial interaction occurring primarily within the wheat rhizosphere in response to N deficiency. This phenomenon is likely attributable to the "cry-for-help" theory, which posits that plants actively recruit rhizosphere beneficial microorganisms to cope with nutrient-deficient conditions (Liu et al. 2020a). While some studies suggest that bacterial and fungal communities in the root endosphere, having bypassed the plant immune system, may offer selective beneficial effects for plant growth, our results indicate that the microbial community in the wheat rhizosphere is more responsive to N treatments and potentially plays a greater role in assisting wheat to adapt to N-deficient conditions. It has been well-demonstrated that plants grown in non-sterile soil exhibit better growth than those in sterile soil, highlighting the beneficial role of soil microorganisms in plant development (Ochieno 2022). Consequently, our research specifically focused on the microbial interaction within the wheat rhizosphere at the mid-anthesis stage, where the most significant responses to N deficiency were observed.

5.2 Wheat-microbe interaction shifted under N deficiency

To further explore the functional responses of the rhizosphere microbial community, metagenomic analysis was performed. The results revealed significant changes in COG categories related to cell wall/exoskeleton and motility under N deficiency. Specifically, an increase in gene functions associated with bacterial mobility was observed, suggesting a potential enhancement of bacterial movement under N-deficient conditions. Chemotaxis, the directed movement of bacteria towards favourable chemical compounds, is a critical process in facilitating plant-microbe interactions (Raina et al. 2019). Prior research has similarly indicated increased flagellar activities and cell motility in bacteria interacting with wheat (O'Neal et al. 2020), but not under N deficiency.

Beyond bacteria, we utilised the eggNOG database to extract and investigate fungal-related genes, aiming to understand the fungal functional profile in response to N deficiency. In contrast to the bacterial community, our metagenomic data indicated that the fungal gene functional profile remained stable under N deficiency. This suggests that the wheat-fungal interaction is relatively stable under N-deficient conditions, especially when compared to the dynamic wheat-bacterial interaction. Studies have consistently demonstrated that soil fungal communities play a vital role in stabilizing the overall soil microbial community and often

exhibit greater stability than bacterial communities under abiotic stress conditions (de Vries et al. 2018; Li et al. 2024). The dynamic mobility observed in bacteria suggests their greater potential for utilisation in future biofertiliser development (Zhalnina et al. 2018).

Plant primary metabolites are crucial for supporting the growth and development of soil microorganisms, thereby facilitating plant-microbe interactions (Zhalnina et al. 2018). These primary metabolites, particularly from roots, are frequently secreted into the soil environment via root exudates (Chai and Schachtman 2022; Hu et al. 2018; Zhalnina et al. 2018). Our investigation into the root-internal metabolite profile identified various amino acids, organic acids, and sugars, all known to serve as energy sources for soil microorganisms (McLaughlin et al. 2023). The metabolite profile exhibited significant separation across N treatments, indicating an alteration in metabolite exudation under N deficiency in both Mace and Gladius cultivars. We observed a reduction in amino acids, sugars, and organic acids under N deficiency, a finding consistent with most previous reports (Qiu et al. 2024). Conversely, fatty acids generally increased under N-deficient conditions, suggesting their significant role in facilitating plant-microbe interactions (Liu et al. 2020c). It has been proposed that plant-secreted fatty acids are essential for microbial cell wall synthesis and play an enormous role in supporting the growth and stability of soil microorganisms (Macabuhay et al. 2022). Overall, our combined glasshouse and hydroponic experiments have clearly demonstrated a significant shift in the rhizosphere bacterial community and the wheat metabolite profile under N deficiency. The increased detection of fatty acids under low N conditions highlights their potential in shaping the wheat-bacterial interaction during N deficiency.

5.3 Intraspecific plant-microbe interaction under N deficiency

Symbiotic plant-microbe interactions are known to benefit wheat growth, particularly under N-poor conditions. However, the inherent host-specific nature of these interactions presents a significant barrier to the widespread effectiveness of bio-based fertilisers (Rai et al. 2023; Wagner et al. 2016; Wippel et al. 2021). Therefore, understanding cultivar-specific wheat-microbial interactions is crucial for enhancing the efficacy of these sustainable agricultural inputs. We investigated the rhizosphere bacterial and fungal communities of Mace and Gladius, two wheat cultivars with documented contrasting NUE from a previous

field study (Alhabbar et al. 2018). Our results revealed significant differences in both bacterial and fungal community composition between the two wheat cultivars. This aligns with prior research demonstrating distinct rhizosphere bacterial and fungal communities among different wheat cultivars (Abdullaeva et al. 2024; Chen et al. 2019). A recent study further explored root-associated microbial communities in two rice cultivars, Japonica and Indica, characterised by high and low NUE, respectively, and found them to be significantly different. The microbial community in Japonica rice exhibited higher gene expression related to N metabolism, suggesting that differences in plant-microbe interaction contribute to superior plant NUE (Zhang et al. 2019). Despite these insights, direct evidence linking specific plant-microbe interactions to wheat NUE has been lacking. Consequently, a key objective of our study was to explore the plant-microbe interaction response to N deficiency in Mace and Gladius.

Our amplicon sequencing data demonstrated that the rhizosphere bacterial and fungal communities of Mace shifted significantly under N deficiency, a pattern not observed in Gladius. This finding strongly suggests that Mace and Gladius, due to their contrasting NUE, exhibit differential microbial interactions in response to N deficiency. To further elucidate the relationship between soil microbes and wheat growth, we investigated the N metabolism of soil microorganisms associated with both cultivars. Functional prediction and metagenomic analysis were employed to examine microbial N metabolism, revealing that the N-cycle is heavily modulated by the bacterial community. The metagenomic data further showed that the N metabolism gene profile was significantly different between Mace and Gladius. This supports the conclusion that Mace and Gladius, with their contrasting NUE, exert distinct modulations on bacterial N metabolism. Previous studies have reported that N cycling gene profiles vary among different plant cultivars and may contribute to differences in plant NUE (Kaur et al. 2022; Wu et al. 2016; Zhang et al. 2019; Zhao et al. 2020). Beyond the N-cycle, our metagenomic analysis indicated increased bacterial chemotaxis in Mace under N deficiency, but not in Gladius, accompanied by an increased detection of genes related to cell motility and the cytoskeleton. A review study has suggested that the chemotactic ability of bacteria significantly influences the success of symbiotic interactions, including the recruitment of diazotrophs and PGPR (Raina et al. 2019). This observation suggests that Mace, with its superior reported NUE, may actively recruit chemotactic bacteria to support its growth under N deficiency.

In our exploration of plant primary metabolites, we found that the metabolite profiles of Mace and Gladius were distinctly separated under low N availability yet remained similar under high N availability. This indicates that their metabolite profiles exhibit differential responses to N deficiency. While previous studies have shown interspecific differences in the metabolite profiles of wheat cultivars (Mwendwa et al. 2021; Zheng et al. 2021), our results revealed that the metabolite profile differed only under N deficiency. This suggests that intraspecific microbial interactions may become more selective under N deficiency. Interestingly, despite the similarity in metabolite profiles between Mace and Gladius under high N availability, the microbial community and functional profile were significantly different under N fertilisation. This highlights that N availability exerts a greater effect on the rhizosphere microbial community than on plant metabolites. Collectively, our data strongly suggest the existence of an intraspecific bacterial interaction in response to N deficiency.

5.4 Potential wheat associated PGPR genera under N deficiency

Soil microorganisms significantly support plant growth in adapting to challenging environmental conditions (Kumar and Verma 2018). Our results imply increased mobility within the rhizosphere bacterial community of Mace under N deficiency, suggesting an enhanced bacterial chemotaxis potential for adaptation to low-N conditions. Bacterial chemotaxis is a crucial modulator of the successful establishment of PGPR symbiotic interactions with plants (Raina et al. 2019). Differential abundance analysis identified an increased detection of specific bacterial taxa belonging to the genera *Haliangium*, *Clostridium*, *Ilumatobacter*, and *Lysinibacillus* in Mace under N deficiency. While bacteria from these genera have been reported as PGPR (Hernández-Santana et al. 2022; Pantoja-Guerra et al. 2023; Passera et al. 2021), their specific beneficial effects on wheat growth under low-N conditions remain underexplored. Our metagenomic data indicated that bacteria within these genera made minimal direct contributions to the N-cycle. Instead, previous reports suggest these bacteria primarily benefit plant growth through improved N acquisition rather than direct modulation of the N cycle (Cocking 2003).

We further investigated the microbial N-cycle in the wheat rhizosphere using both functional prediction and metagenomic analyses. Functional prediction highlighted a significant reduction in taxa related to N-fixation in Mace under N deficiency, while no such changes were observed across N treatments in Gladius. However, metagenomic analysis did

not detect any genes directly related to N-fixation, suggesting that the predicted taxa might contribute to Mace's growth through alternative functions. Notably, the taxa predicted as N-fixing were predominantly *Rhizobium*, which are well-known symbiotic microorganisms with legume plants but not typically associated with N-fixation in wheat (Antoun et al. 1998; Hungria et al. 1998). Other studies have reported that *Rhizobium* can indeed act as PGPR, stimulating general plant growth and development and enhancing nutrient acquisition (Antoun et al. 1998; Lian et al. 2002).

Furthermore, our metagenomic data revealed a similar pattern for the DNRA gene, which increased in Mace under N deficiency but remained stable in *Gladius* across N treatments. The DNRA pathway, which converts NO_3^- back to NH_4^+ , is often considered a N retention process that improves plant NUE (Pandey et al. 2020). Some studies have shown that this process is enhanced under N-limited conditions, suggesting a potential mechanism for improving plant NUE in low-N environments (Ma et al. 2025). We further explored bacterial taxa involved in nitrate reduction and found that the genus *Optiutus* exhibited increased detection under N deficiency, suggesting its potential role in N retention under these conditions. Research has investigated *Optiutus* taxa and reported their capacity to enhance DNRA and improve soil N availability for plant use (Yoon et al. 2019).

5.5 Potential metabolite signal under N deficiency

While numerous studies have demonstrated that beneficial bacteria significantly influence plant growth, their effectiveness is greatly challenged by dynamic environmental conditions (Rai et al. 2023). Consequently, a deeper understanding of plant-microbe interactions holds considerable potential for the development of more promising biofertilisers. Our comparison of non-targeted metabolite profiles identified a significant increase in stearic acid in Mace under N deficiency, a response not observed in *Gladius*. This finding suggests that Mace, with its superior reported NUE, potentially interacts with soil microorganisms under N-deficient conditions through the secretion of stearic acid. Collectively, the metagenomic and metabolomic data imply that stearic acid may potentially modulate the bacterial community, including PGPR and N-cycling microorganisms. Several studies have reported increased accumulation of stearic acid in plant roots and its secretion in root exudates under N-limited conditions (Li et al. 2018; Liu et al. 2020c). Although limited

research has specifically investigated its potential in facilitating beneficial wheat-microbe interactions, a soil microcosm study demonstrated that stearic acid application reduced N₂O emissions. This suggests that stearic acid may have a beneficial effect in reducing N loss through the suppression of denitrification (Umar et al. 2023). However, its specific mechanism of action on the microbial N-cycle remains to be fully elucidated.

Stearic acid, a saturated fatty acid commonly found in wheat, plays diverse roles in microbial energy metabolism and signal transduction (Fracchia et al. 2024). It has been demonstrated that stearic acid can enhance the *che* genes in a *Pseudomonas* strain, indicating an enhanced chemotactic response of *Pseudomonas* towards stearic acid (Ankati et al. 2019). The *che* genes encode proteins involved in signal transduction pathways, facilitating microbial chemotaxis (Silverman and Simon 1977). Another study revealed that stearic acid enhanced the swarming motility and growth of a *Bacillus* species, a known PGPR, upon inoculation (Xiong et al. 2020). These findings collectively demonstrate the potential of stearic acid as a chemotactic attractant for PGPRs.

5.6 Conclusion

In conclusion, our experiments have provided evidence regarding the intraspecific variations in wheat metabolite profiles and root-associated microbial communities in response to N deficiency. We demonstrated that wheat exhibits a dynamic interaction with its rhizosphere bacterial community and a relatively stable interaction with its fungal community under N deficiency. These findings suggest that bacteria play a more prominent role than fungi in benefiting wheat growth and adaptation to N-deficient conditions.

Furthermore, our research highlighted significant intraspecific wheat-microbe interactions, revealing distinct differences in both the root-associated microbial community composition and root metabolite profiles between wheat cultivars with contrasting reported NUE. Specifically, we observed an enrichment of DNRA genes in the rhizosphere and a notable accumulation of stearic acid in the roots of cultivars exhibiting higher reported NUE under N deficiency. This strongly suggests a crucial role for stearic acid in modulating the wheat N-cycle and facilitating adaptation to N-deficient environments.

Collectively, our findings offer valuable insights that can inform the development of next generation biofertilisers aimed at enhancing wheat NUE under N deficiency. Such advancements hold significant potential to optimise agricultural cropping system management practices, promoting more sustainable and productive outcomes.

5.7 Future perspectives

Biofertiliser greatly benefit plant growth and have a great potentially in supporting plant growth under abiotic stresses. However, the efficacy of biofertilisers has been significantly constrained by numerous external factors, underscoring the urgent need for novel strategies to enhance their performance. This section proposes several perspectives for future research.

(1) Our findings indicate an increased potential for DNRA in the wheat rhizosphere and an elevated accumulation of stearic acid within wheat roots under N deficiency. Although direct evidence linking stearic acid to increased DNRA activity in the wheat rhizosphere is currently lacking, existing literature suggests that a high C:N ratio can boost DNRA activity in soil (Yang et al. 2025). Additionally, plants experiencing N deficiency often release carbon-rich compounds, such as fatty acids and organic acids, into the environment (Chai and Schachtman 2022). Therefore, exploring the potential of plant metabolites, particularly carbon-only compounds, in stimulating soil DNRA could represent a promising avenue for improving plant NUE.

(2) Beyond direct N metabolism, we have demonstrated increased bacterial mobility under N deficiency, providing evidence of the chemotactic ability of bacteria interacting with wheat under N-deficient conditions. Bacterial chemotaxis plays a critical role in facilitating the successful establishment of symbiotic interactions (Raina et al., 2019). While many studies have investigated bacterial chemotaxis using in vitro microfluidic devices (Salek et al., 2019), these setups may not accurately reflect chemotactic abilities within the complex soil environment due to the intricate nature of soil pore structures. A recent study developed an innovative experimental setup that integrates soil within a microfluidic system to investigate bacterial chemotaxis in a more realistic soil environment (Monteiro et al., 2025).

We propose that future research should explore the chemotactic capabilities of beneficial bacteria directly within soil environments.

(3) Future studies should investigate the potential of incorporating plant metabolites into biofertiliser development. Metabolite-based biofertilisers are gaining interest due to their enhanced stability compared to living microbial inoculants (Richa, 2024). It has been suggested that formulating metabolite applications to target multiple soil bacteria, thereby fostering a bacterial consortium, could be a valuable future research direction (Richa, 2024). However, a limited understanding of specific plant-microbe interactions currently constrains the rational design of such metabolite formulations. Given that several primary metabolites are commonly found both inside and exuded from the roots across diverse plant species (McLaughlin et al., 2023), we suggest that future research in metabolite formulation could prioritise these "core metabolites", potentially yielding benefits across a broader range of plant species.

(4) The strategy of co-inoculation involving both metabolites and PGPR warrants further investigation. Current research often focuses solely on the effect of microbial inoculation on plant metabolites or the application of plant metabolites on soil microbial profiles (Jiang et al. 2023; Xiao et al. 2021). However, using either microbial inoculants or metabolite inoculants alone has frequently proven to be less effective. The suppression of microbial inoculants by indigenous soil microorganisms is a key factor limiting the efficiency of traditional biofertilisers (Rai et al. 2023). While metabolite-based biofertilisers can be selective toward certain microorganisms, they are generally not unique to a single species (Calvo et al. 2014). Therefore, we propose that future studies should focus on the co-inoculation of microbial and metabolite biofertilisers, as this combined approach may support PGPR survivability and maximise their efficiency in benefiting plant growth.

(5) Both microbial inoculants and metabolites typically possess limited inherent nutrient content, such as N. Their efficacy is also significantly restricted by competition from indigenous microbes and by environmental conditions like temperature (Calvo et al. 2014; Rai et al. 2023). Biofertiliser carriers are traditionally employed to encapsulate and protect microorganisms from external environmental stresses (Kaur and Kaur 2023). Future research could explore the potential of co-inoculating both microorganisms and metabolites within biofertiliser carriers. This approach could simultaneously enrich nutrient availability and

provide enhanced protection for PGPRs from adverse external influences, further optimising biofertiliser performance.

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Appendices

Appendix 1. Supplementary material in chapter 2

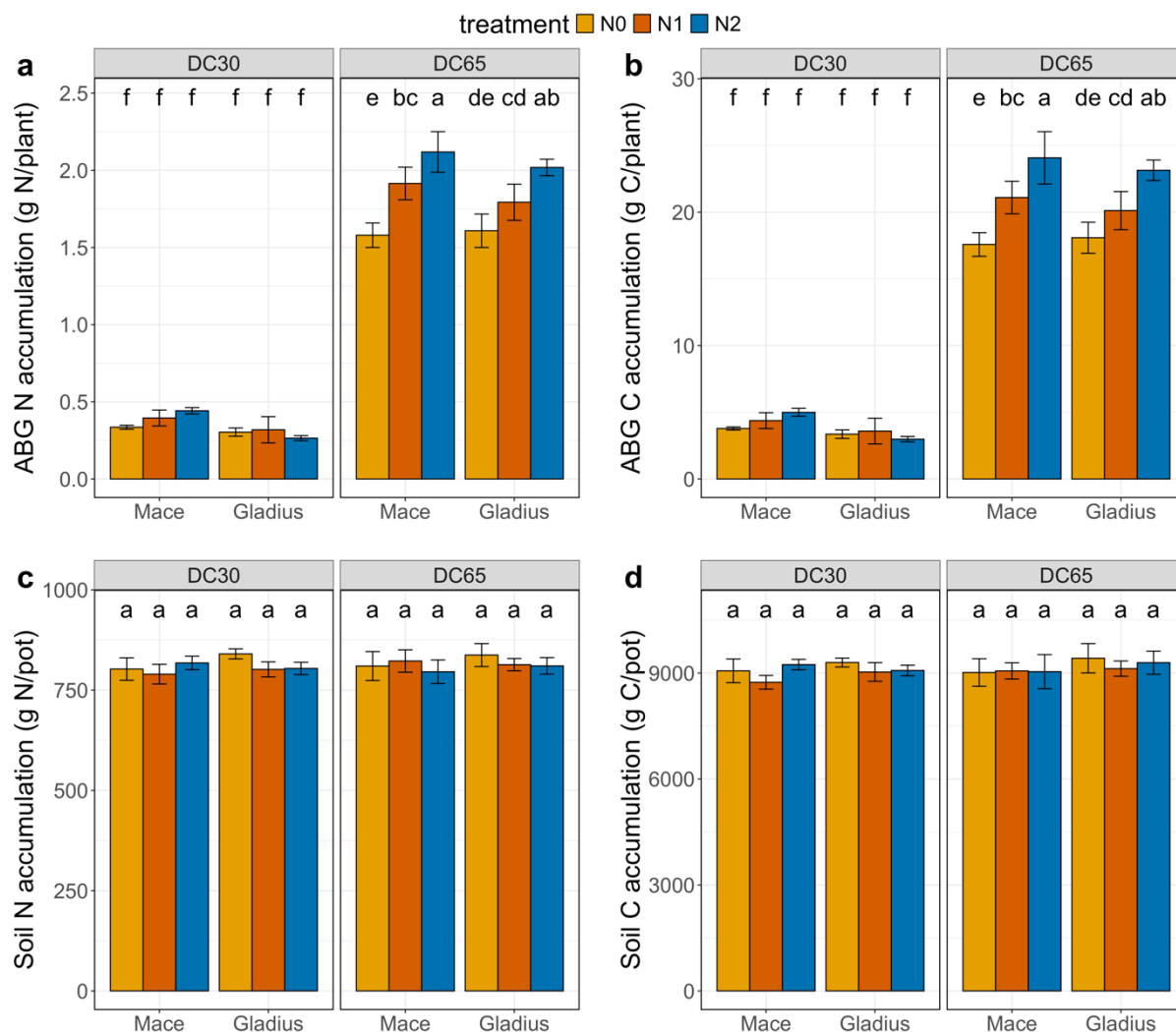


Figure S1. Gladius and Mace growth performance under N treatments at the stem elongation (DC30) and mid-anthesis (DC65) stages. The accumulation of (a) N and (b) C in the wheat-dried above-ground biomass (ABG) and the accumulation of (c) N and (d) C in the soil remained in the pot. N and C content between N treatments was statistically compared by ANOVA indicated on top of the bars.

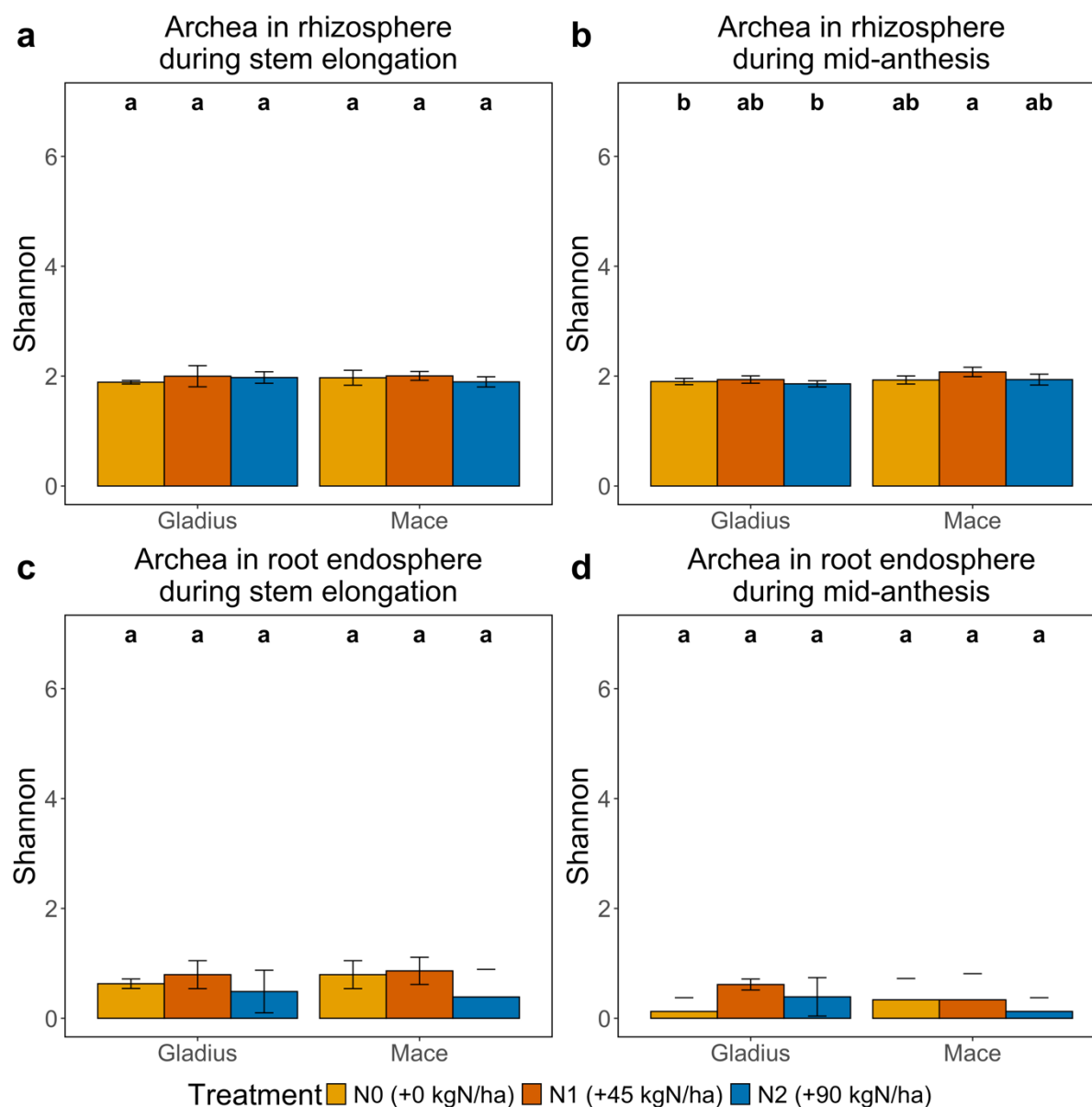


Figure S2. Wheat root-associated archaea estimated by Shannon index in the rhizosphere at (a) stem elongation and (b) mid-anthesis stages, and in the root endosphere at (c) stem elongation and (d) mid-anthesis stages. Kruskal-Wallis statistical test represented by the letters above the bar.

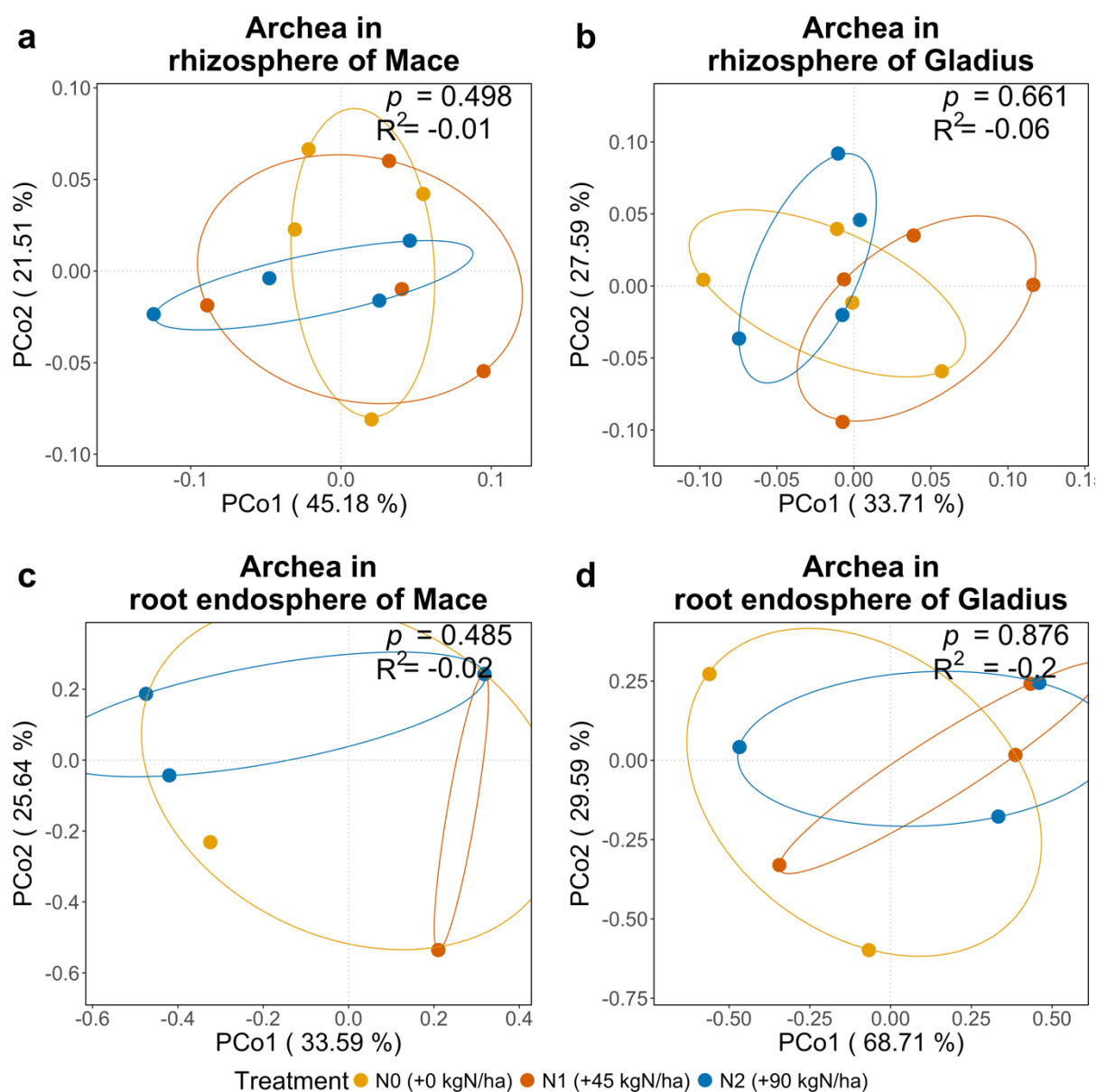


Figure S3. Principle coordinates analysis (PCoA) of wheat root-associated archaea ASVs, in the rhizosphere of (a) Mace and (b) Gladius, and in the root endosphere of (c) Mace and (d) Gladius. Sample similarity is measured by the non-parametric analysis of similarities (ANOSIM). The ANOSIM statistics are indicated on the top-right of each PCoA plot.

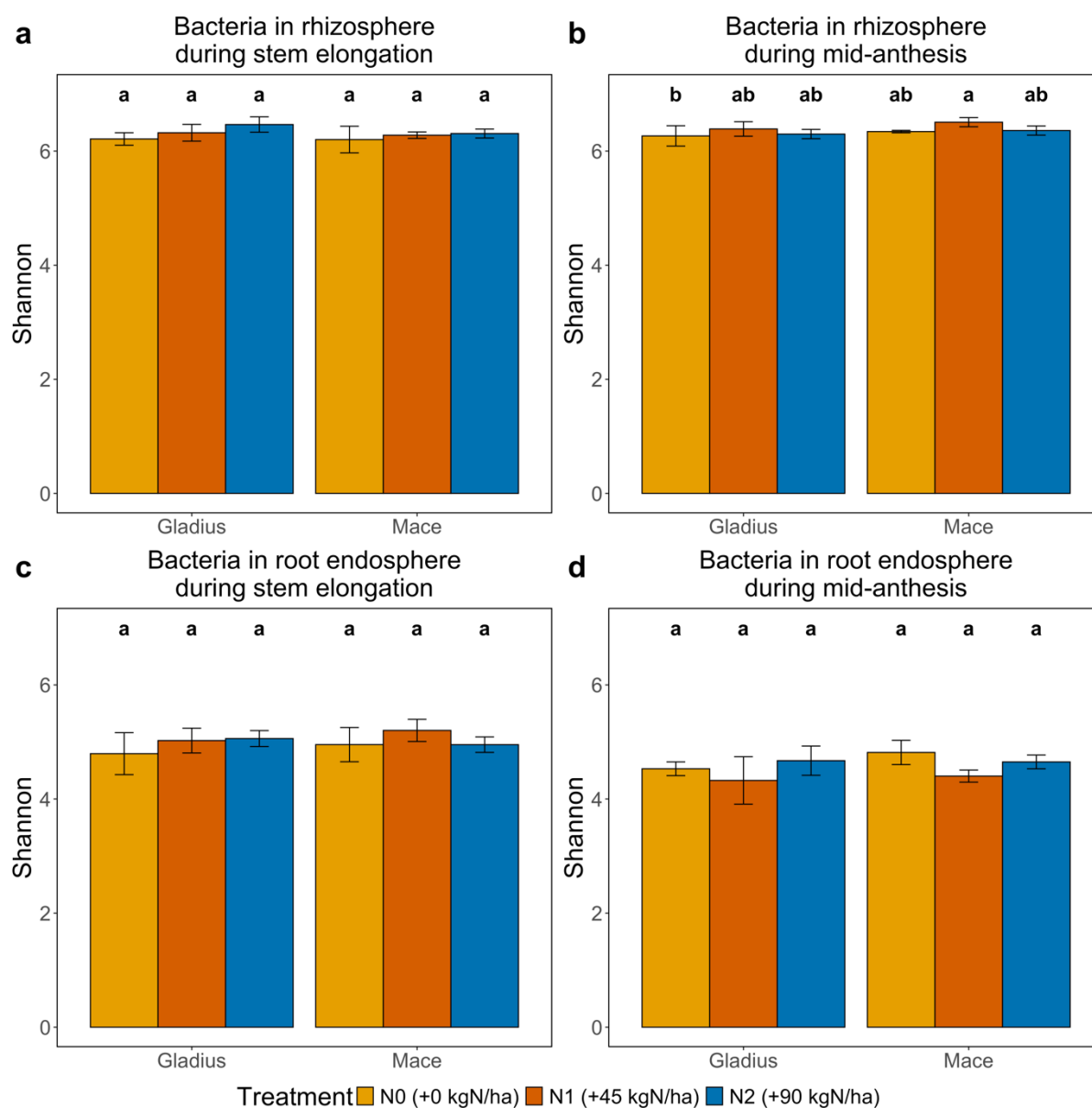


Figure S4. Wheat root-associated bacteria estimated by Shannon index in the rhizosphere at (a) stem elongation and (b) mid-anthesis stages, and in the root endosphere at (c) stem elongation and (d) mid-anthesis stages. The Kruskal-Wallis statistical test is represented by the letters above the bar.

Table S1. Samples description for different performed analyses.

Analysis	<i>16S</i> amplicon sequencing		Metagenomic	Metabolomic (GC-MS)
Compartment	Rhizosphere	Root	Rhizosphere	Root
No. samples	48 samples	48samples	16samples	27samples
Description	3x N treatments, 2x cultivars, 2x stages, 4 replicates	3x N treatments, 2x cultivars, 2x stages, 4 replicates	3x N treatments, 2x cultivars, 1x stages, 4 replicates	3x N treatments, 2x cultivars, 1x stages, 5 replicates Note: 3 plants were not sampled as they were damaged during the experiment. This included two sample of N2-treated Mace and one sample of N2-treated Gladius.