

From the Institute of Plant Nutrition and Soil Science at Kiel University

Rhizosphere Dynamics in Maize-Based Intercropping Systems

Dissertation

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Summary

Cereal–legume intercropping represents a promising strategy in sustainable agriculture, aiming to enhance nutrient cycling, reduce dependency on synthetic fertilizers, and support agroecosystem resilience under increasing environmental constraints. Although intercropping systems are known to improve rhizosphere interactions and microbial functionality the specific contributions of maize genotype, intercrop identity, and soil properties to rhizosphere dynamics remain insufficiently understood.

This dissertation explores how *maize genotypic diversity*, *intercropping configuration*, and *soil context* influence rhizosphere biochemical activity, microbial community dynamics, and nutrient acquisition. Using a combination of greenhouse rhizotron experiments and field trials across different environments, the research develops a trait-based framework for understanding plant–soil–microbe interactions.

In the first study, a greenhouse experiment using rhizotrons examined six genetically contrasting maize (*Zea mays* L.) genotypes intercropped with faba bean (*Vicia faba* L.). Rhizosphere processes were spatially evaluated using in situ zymography. Maize genotypes exhibiting deep axial roots and dense lateral branching induced pronounced hotspots of acid phosphatase and leucine aminopeptidase activity, particularly in the shared root zone. These biochemical responses were maize genotype-dependent, suggesting enhanced nutrient mobilization potential under legume intercropping.

The second study extended the investigation to field conditions across two agroecologically distinct sites in Switzerland, characterized by differing soil texture and organic matter content. Maize was intercropped with either field pea (*Pisum sativum* L.) or ryegrass (*Lolium perenne* L.), representing functionally distinct intercrop species. Intercropping with field pea significantly elevated rhizosphere orthophosphate availability and enriched bacterial groups associated with phosphorus solubilization. In contrast, intercropping with ryegrass favored organic phosphorus stabilization and increased the abundance of fungal biomarkers, including arbuscular mycorrhizal fungi. These microbial and biochemical responses varied with soil context. While genotype-specific influences were evident in controlled environments, the Swiss field trial emphasized the dominant roles of intercrop identity and soil characteristics in shaping rhizosphere microbial structure and phosphorus turnover.

The third study employed field-based ¹⁵N isotope tracing to quantify nitrogen uptake and the determination of arbuscular mycorrhizal fungi (AMF) colonization across three maize genotypes grown in monoculture and in intercropping with faba bean. Intercropping enhanced

both AMF colonization and nitrogen recovery from the applied ^{15}N tracer. Genotype FC1571 achieved the highest absolute nitrogen uptake, reflecting superior root exploration and total foraging capacity. Conversely, genotype A347, despite lower AMF colonization, demonstrated the strongest functional efficiency of AMF symbiosis, with a robust positive correlation between colonization levels and ^{15}N acquisition. These findings underscore that mycorrhizal responsiveness, rather than colonization level alone, is a key determinant of nutrient use efficiency.

Together, the three studies demonstrate that maize root traits, when interacting with intercrop species and site-specific soil properties, significantly shape rhizosphere biochemical profiles, microbial assembly, and nutrient acquisition dynamics. High-performing genotypes such as FC1571 and LH74 fostered synergistic microhabitats with elevated enzymatic activity and microbial diversity, particularly under legume intercropping. These outcomes are driven not only by architectural root traits, but also by genotype-dependent patterns of rhizodeposition, enzyme exudation, and symbiotic associations.

The findings provide mechanistic insight into trait-driven facilitation processes in intercropping systems and highlight the potential of genotype-informed intercropping design as a tool to enhance nutrient cycling, reduce fertilizer inputs, and improve agroecosystem resilience. This research establishes a scientific foundation for integrating root trait phenotyping, microbial profiling, and soil resource modeling into breeding programs and agroecological practices aimed at optimizing productivity in low-input, sustainable cropping systems.

Zusammenfassung

Getreide–Leguminosen-Mischkulturen gelten als vielversprechende Strategie für eine nachhaltige Landwirtschaft, da sie die Nährstoffkreisläufe optimieren, den Einsatz

synthetischer Düngemittel reduzieren und die Resilienz von Agrarökosystemen unter zunehmenden Umweltbelastungen stärken. Obwohl bekannt ist, dass Mischkulturen die Rhizosphäreninteraktionen und mikrobielle Funktionen fördern, sind die spezifischen Rollen von Genotyp, Kulturart und Bodeneigenschaften in diesen Prozessen bislang unzureichend verstanden.

Diese Dissertation untersucht, **wie die genotypische Vielfalt von Mais (*Zea mays*), die Konfiguration von Mischkulturen und die Bodeneigenschaften** die biochemische Aktivität in der Rhizosphäre, die mikrobielle Gemeinschaftsdynamik und die Nährstoffaufnahme beeinflussen. Durch die Kombination von Rhizotron-Experimenten im Gewächshaus und Feldversuchen an unterschiedlichen Standorten wird ein merkmalsbasiertes Rahmenkonzept für das Verständnis von Pflanze–Boden–Mikroben-Interaktionen in diversifizierten Anbausystemen entwickelt.

In der ersten Studie wurde ein kontrolliertes Gewächshausexperiment mit Rhizotronen durchgeführt, bei dem sechs genetisch unterschiedliche Maisgenotypen gemeinsam mit Ackerbohne (*Vicia faba* L.) angebaut wurden. Rhizosphärenprozesse wurden räumlich mithilfe von in situ Zymographie bildgebend erfasst. Genotypen mit tiefreichenden Vertikalwurzeln und dichter Seitenverzweigung zeigten ausgeprägte Hotspots der Aktivität von saurer Phosphatase und Leucin-Aminopeptidase, insbesondere im gemeinsamen Wurzelbereich. Diese biochemischen Reaktionen waren genotypabhängig, was auf ein erhöhtes Potenzial zur Nährstoffmobilisierung in Leguminosen-Mischkulturen hinweist.

Die zweite Studie übertrug die Untersuchung auf Feldbedingungen an zwei Standorten in der Schweiz, die sich in Hinblick auf Bodentextur und organischem Kohlenstoffgehalt unterschieden. Mais wurde dabei entweder mit Erbse (*Pisum sativum*) oder mit Weidelgras (*Lolium perenne*) kultiviert, zwei funktionell kontrastierenden Begleitpflanzen. Die Mischkultur mit der Erbse erhöhte signifikant die Verfügbarkeit von Orthophosphat im Wurzelraum und begünstigte bakterielle Gruppen, die mit Phosphormobilisierung assoziiert sind. Im Gegensatz dazu förderte die Mischkultur mit Weidelgras die Stabilisierung organisch gebundenen Phosphors und erhöhte die Häufigkeit pilzlicher Biomarker, einschließlich arbuskulärer Mykorrhizapilze. Diese mikrobiellen und biochemischen Reaktionen variierten abhängig vom Bodentyp.

Während maisgenotypspezifische Effekte unter kontrollierten Bedingungen klar sichtbar waren, betonte die Feldstudie in der Schweiz vor allem die dominanten Einflüsse der Mischkulturart und der Bodeneigenschaften auf die mikrobielle Struktur und den Phosphorumsatz in der Rhizosphäre.

In der dritten Studie wurde eine feldbasierte ^{15}N -Isotopenmarkierung zur Nachverfolgung der Stickstoffaufnahme durchgeführt, und die Kolonisierung durch arbuskuläre Mykorrhizapilze (AMF) bei drei Maisgenotypen im Reinanbau und in Mischkultur mit Ackerbohne bestimmt. Die Mischkultur führte zu einer verstärkten AMF-Kolonisierung und einer erhöhten Aufnahme des Stickstofftracers in die Maisbiomasse. Der Genotyp FC1571 erreichte die höchste absolute Stickstoffaufnahme, was auf eine effiziente Wurzelexploration und umfassende Erschließung des Wurzelraums hindeutet. Im Gegensatz dazu zeigte der Genotyp A347 trotz geringerer Kolonisierung die höchste funktionelle Effizienz der Mykorrhizasymbiose mit einer starken positiven Korrelation zwischen Kolonisierungsgrad und ^{15}N -Aufnahme. Diese Ergebnisse unterstreichen, dass nicht die das Ausmaß der AMF Kolonisierung, sondern die genotypspezifische Mykorrhiza-Reaktionsfähigkeit entscheidend für die Nährstoffeffizienz ist. ***Insgesamt zeigen die drei Studien***, dass Mais-Wurzeigenschaften in Wechselwirkung mit Interkulturarten und standortspezifischen Bodeneigenschaften die biochemischen Profile der Rhizosphäre, die mikrobielle Gemeinschaftsstruktur und die Nährstoffaufnahme maßgeblich beeinflussen. Leistungsstarke Genotypen wie FC1571 und LH74 förderten besonders unter Leguminoseninterkultur die Ausbildung synergistischer Mikrohabitate mit erhöhter Enzymaktivität und mikrobieller Diversität. Diese Prozesse werden nicht nur durch die Wurzelarchitektur bestimmt, sondern auch durch genotypspezifische Muster der Rhizodeposition, Enzymausscheidung und symbiotischen Interaktionen. Die Ergebnisse liefern mechanistische Einblicke in merkmalsgesteuerte Förderprozesse in Mischkulturen und verdeutlichen das Potenzial einer genotypisch fundierten Interkultur-Gestaltung zur Verbesserung der Nährstoffkreisläufe, zur Reduktion von Düngemittleinsätzen und zur Stärkung der Resilienz von Agrarökosystemen. Diese Arbeit legt damit die wissenschaftliche Grundlage für die Integration von Wurzelmerkmal-Phänotypisierung, mikrobieller Profilierung und Bodenressourcenmodellierung in Züchtungsprogramme und agrarökologische Anbaustrategien zur Produktivitätssteigerung in nachhaltigen, ressourcenschonenden Anbausystemen.

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Abbreviations

Abbreviation	Definition
%	Percent
°C	Degrees Celsius
¹⁵ N	Nitrogen-15 isotope
A347	Maize genotype A347
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
AP	Acid phosphatase
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (growth stage scale)
BS	Bean rhizosphere
BX1B73	Maize genotype BX1B73
CAT	Catalytic efficiency (enzyme kinetics)
DOC	Dissolved organic carbon
DW	Dry weight
EA-IRMS	Elemental Analyzer–Isotope Ratio Mass Spectrometry
FAME	Fatty acid methyl esters
FC1571	Maize genotype FC1571
G+	Gram-positive bacteria
G–	Gram-negative bacteria
ICP-OES	Inductively coupled plasma optical emission spectrometry
LAP	Leucine aminopeptidase
LH74	Maize genotype LH74
MB	Microbial biomass
MBS847	Maize genotype MBS847
MR	Maize rhizosphere
N	Nitrogen
NUE	Nutrient use efficiency

Abbreviation	Definition
P	Phosphorus
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acids / Phospholipid fatty acid analysis
PLFA PCA	Principal component analysis of phospholipid fatty acids
POM	Particulate organic matter
pH	Potential of hydrogen (soil acidity/alkalinity)
R ²	Coefficient of determination
REO	Rare earth oxides
RPE	Rhizosphere priming effect
RSA	Root system architecture
RZ	Rhizosphere zone
SD	Standard deviation
SEM	Standard error of the mean
SR	Shared rhizosphere (maize + bean)
V. faba	<i>Vicia faba</i> L. (faba bean)
V _{max}	Maximum enzyme velocity
WT	Wild type maize genotype
Zymo	Zymography (enzyme activity imaging)
μmol	Micromole

1 Extended summary

1.1 Intercropping as approach towards more sustainable maize production

Modern agriculture faces a critical challenge: to meet rising global demands for food, fibre, and bioenergy while also preserving soil health, minimizing environmental degradation, and adapting to climate variability (Foley et al., 2011; Tilman et al., 2011). The widespread degradation of soils, depletion of essential nutrients, and persistent reliance on mineral fertilizers have accelerated the search for ecologically viable cropping systems that can enable sustainable intensification (Cassman & Grassini, 2020).

Among these alternatives, diversified cropping systems, particularly cereal–legume intercropping, are increasingly recognized for their ability to optimize land use and biological resources. Intercropping, defined as the simultaneous cultivation of two or more crop species in the same spatial and temporal field unit, enhances complementarity in resource acquisition, improves nutrient cycling efficiency, and contributes to overall agroecosystem resilience (Bedoussac et al., 2015; Brooker et al., 2015a; Martin-Guay et al., 2018; Tang et al., 2021).

Recent advances in agronomy, plant physiology, and ecology have deepened our understanding of how crop species and genotypes interact in intercropping systems. Mechanisms such as niche complementarity, facilitation, and microtopographic variation play a central role in structuring plant interactions in intercropping systems. These processes enhance resource partitioning, contribute to disease suppression, and strengthen above–belowground integration (Brooker et al., 2015a). In cereal–legume systems, biological nitrogen fixation by legumes and improved phosphorus acquisition through modification in rhizosphere, such as enhanced microbial activity, and root exudation are critical for improving soil fertility and reducing reliance on external inputs (C. Li et al., 2020; Xue et al., 2016). These mechanisms contribute to sustainable intensification by enabling higher or more stable yields with fewer inputs.

The intercropping of maize (*Zea mays* L.) and faba bean (*Vicia faba* L.) exemplifies a biologically complementary system, particularly effective in the acquisition of nitrogen (N) and phosphorus (P). Faba bean contributes to nitrogen cycling through symbiotic nitrogen fixation and improves phosphorus mobilization via the exudation of organic acids, carboxylates, and phenolic compounds. These compounds, particularly organic acids, are especially effective in mobilizing phosphorus from sparingly available sources, especially in alkaline soils where P availability is limited by precipitation with calcium. In acidic soils, while P fixation with iron and aluminum reduces availability, organic acids and other root exudates can still enhance P mobilization through chelation and complexation mechanisms (Hinsinger

et al., 2009; Pii et al., 2015; Richardson et al., 2009). Maize, characterized by an extensive and fast-growing root system, exhibits a high demand for nutrients and a capacity for rapid uptake, making it a strong complementary species in intercropping systems (Marschner & Rengel, 2012; Oburger & Jones, 2018).

Although the agronomic and ecological benefits of such systems are increasingly documented, the genotype-specific mechanisms that underpin rhizosphere interactions and nutrient acquisition remain insufficiently explored. In particular, the effects of maize genotypic variation on root system architecture, root exudate composition, and interactions with rhizosphere microorganisms are still not fully understood. This gap is especially significant in the context of nutrient cycling, where root–microbe interactions influence the solubilization and assimilation of nitrogen and phosphorus (Demie et al., 2022; Homulle et al., 2022; Martin-Guay et al., 2018; Smith & Smith, 2011; York et al., 2015). Soil microbes, often referred to as the "unseen majority," play a central role in regulating plant productivity, diversity, and nutrient cycling in terrestrial ecosystems (Van Der Heijden et al., 2008), making them crucial players in the functioning of intercropping systems.

This dissertation addresses these knowledge gaps by integrating multiple analytical perspectives, including rhizosphere enzyme activity imaging, and kinetics, Arbuscular mycorrhizal fungi ink technique, microbial community profiling, and stable isotope tracing. These methodologies were systematically applied across controlled greenhouse experiments and complementary field studies. Specifically, six maize genotypes were compared in greenhouse conditions and in the German field trials, allowing detailed investigation of genotype-specific effects. In contrast, the Swiss field trials employed a single maize genotype to explicitly focus on how intercrop identity and soil characteristics interactively shape rhizosphere dynamics.

The overarching aim of this work is to identify genotype-specific mechanisms through which maize root traits modulate microbial dynamics in interaction with intercrop species and soil conditions. By uncovering how plant genotype influences functional rhizosphere interactions, this research provides critical insights for breeding programs and cropping system management, emphasizing traits like root architecture, exudation profiles, and microbial responsiveness. Ultimately, this supports the development of more efficient, fertile, and resilient agroecosystems through genotype-informed diversification strategies.

1.1.1 Root traits and rhizosphere enzyme activity

A greenhouse rhizotron study was conducted using 24 rhizotrons, each containing one of six maize genotypes intercropped with faba bean. Zymography was used to quantify the spatial distribution of rhizosphere enzyme activities. Pronounced genotype effects were observed for leucine aminopeptidase activity and acid phosphatase, with LAP ranging approximately from 380 to 550 pmol cm⁻² h⁻¹ and AP from 1500 to 1800 pmol cm⁻² h⁻¹ across genotypes. Maize genotypes FC1571 and LH74, which exhibited deeper and more highly branched root systems, produced pronounced enzyme activity hotspots, particularly in the shared rhizosphere with faba bean. This highlighted functionally synergistic rhizosphere zones where maize and faba bean root systems overlapped (Courty et al., 2010).

These results demonstrate that maize root architecture and genotype-specific exudation patterns are major determinants of the spatial heterogeneity of rhizosphere biochemical activity in intercropping systems. This supports the importance of genotype selection for improved nutrient cycling and sustainable crop production.

1.1.2 Microbial shifts and phosphorus dynamics

Field experiments at two sites with contrasting soil textures (loam and sand) were conducted to assess microbial and phosphorus responses to maize–legume and maize–ryegrass intercropping. Phosphorus fractionation revealed that legume-based intercrops increased labile P pools (resin- and NaHCO₃-extractable P), particularly in loamy soils with higher organic matter content. In contrast, ryegrass systems maintained larger organic P reserves (George et al., 2018; Hedley et al., 1982; Horst et al., 2001).

PLFA profiling showed that intercropping with field pea significantly increased the relative abundance of Gram-positive bacteria and Actinobacteria, microbial groups associated with phosphorus solubilization (Buyer & Sasser, 2012; Willers et al., 2015). Ryegrass intercropping enriched fungal biomarkers, especially saprotrophic fungi, consistent with slower phosphorus turnover. These biomarkers provided functional insight into microbial contributions to rhizosphere phosphorus dynamics across cropping systems.

Microbial community shifts were closely tied to rhizosphere pH dynamics and varied by site, reflecting the combined influence of soil properties and intercrop identity on rhizosphere function. Genotype effects were not evaluated at these field sites

(George et al., 2018; Marschner et al., 2001). These interactions play a key role in enhancing phosphorus use efficiency in diversified cropping systems.

1.1.3 Maize genotype-specific nitrogen acquisition and mycorrhizal colonization

In a field experiment with three maize genotypes (FC1571, B73WT, and A347), ¹⁵N-labeled ammonium sulphate was applied to intercropped plots to evaluate genotype-specific nitrogen uptake. Genotypes FC1571 and A347 demonstrated significantly higher ¹⁵N recovery in aboveground biomass (~43.2% and ~39.1%, respectively) compared to B73WT (~28.5%), despite similar shoot biomass among genotypes (Drinkwater & Snapp, 2007).

AMF colonization, quantified by ink–vinegar staining (Pelikan blue) and gridline intersection microscopy, ranged from 26% (B73WT) to 48% (FC1571). Notably, genotype A347, with moderate colonization (~35%), showed the strongest correlation between AMF presence and nitrogen recovery. This suggests a high level of mycorrhizal functional responsiveness and indicates that colonization intensity alone does not reliably predict nutrient uptake efficiency (J. Chen et al., 2023; Smith & Smith, 2011).

These findings highlight the potential of selecting maize genotypes for symbiotic efficiency, not just colonization extent, to enhance nitrogen use efficiency in legume-based intercropping systems (Berger & Gutjahr, 2021; Lehmann et al., 2012).

1.1.4 Toward phosphorus-smart cropping systems

The strategic selection of maize genotypes with favourable root traits and microbiome-mediated interactions offers a promising approach to enhance phosphorus mobilization and rhizosphere function in legume-based intercropping systems. This strategy is particularly timely given the rising cost of fertilizers, increasing climate variability, and the depletion of global phosphate reserves (Cordell et al., 2009; Vance, 2001).

“Phosphorus-smart” cropping systems emphasize plant traits such as elevated acid phosphatase activity, targeted rhizosphere exudation, and strong mycorrhizal responsiveness, mechanisms that biologically optimize phosphorus availability and reduce dependence on synthetic inputs. When adapted to specific soil conditions, genotype-informed intercropping provides a viable pathway to improve nutrient use efficiency, lower environmental impacts, and promote the long-term sustainability of agricultural systems.

Collectively, the findings of this dissertation demonstrate that maize genotype plays a critical role in shaping rhizosphere enzyme activity, nutrient acquisition, and symbiotic interactions in intercropping systems. Genotypes with fibrous root systems, elevated phosphatase activity, and high responsiveness to arbuscular mycorrhizal fungi consistently achieved superior nutrient uptake and rhizosphere biochemical performance across both greenhouse and field conditions.

These outcomes highlight the potential of trait-based genotype selection to enhance resource partitioning and microbial facilitation in cereal–legume intercropping.

Such trait-informed strategies form the basis for advancing resource-efficient, low-input agriculture by improving nutrient cycling, reducing reliance on synthetic fertilizers, and supporting microbial biodiversity. Resulting agroecosystems are more resilient to environmental stress and contribute to the restoration of long-term soil fertility (Brooker et al., 2015a).

Moreover, the integration of metagenomic sequencing, spatial enzyme imaging, and stable isotope tracing provides powerful tools to unravel multi-scale interactions among plant genotypes, microbial consortia, and nutrient fluxes. These insights establish a mechanistic foundation for breeding programs aligned with the goals of sustainable intensification and offer a roadmap for designing biologically efficient and ecologically regenerative cropping systems.

1.1.5 Outlook: Trait-based design of cropping systems

The evidence presented in this dissertation underscores the transformative potential of trait-based strategies in designing intercropping systems that enhance resource efficiency and rhizosphere function. Variations in maize root architecture, exudate chemistry, and mycorrhizal responsiveness were shown to shape nutrient acquisition and generate spatial heterogeneity in rhizosphere biochemical activity, particularly in legume-based systems.

These findings suggest that specific root and microbial traits can serve as functional indicators for selecting crop genotypes suited to ecologically intensified agriculture. For instance, combining a maize genotype with deep rooting and elevated phosphatase activity with a complementary legume species may enhance phosphorus mobilization through biochemical complementarity. Similarly, selecting maize lines with high mycorrhizal responsiveness could improve nitrogen acquisition in symbiosis-rich intercropping environments.

To fully exploit these synergies, future research should integrate high-throughput root phenotyping with genomic and transcriptomic tools to enable genome-informed selection of belowground traits. Developing a systems-level framework that links plant genetics, microbial ecology, and soil–plant feedback modeling; Figure 1 will be key to advancing precision agroecology (Brooker et al., 2015a; York et al., 2013). Such an integrative approach promises to optimize nutrient cycling, enhance microbial functionality, and support resilient, low-input cropping systems that safeguard both soil health and global food security.

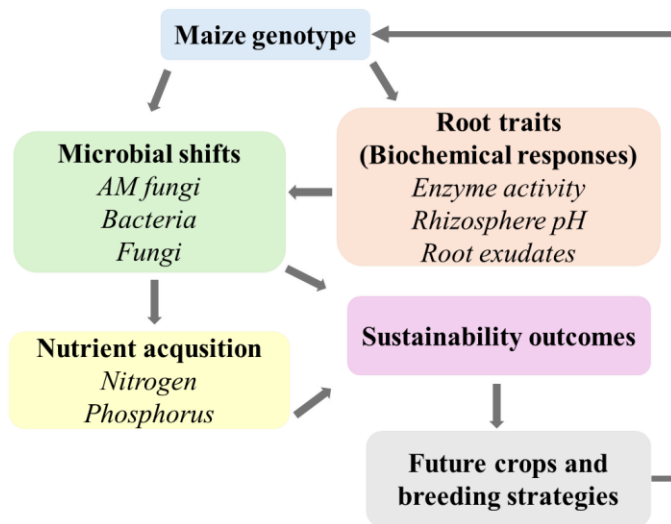


Figure 1. Genotype-driven rhizosphere processes for sustainable intercropping systems. This diagram illustrates the cascading effects of maize genotype on rhizosphere interactions through root traits and biochemical responses, leading to microbial shifts, enhanced nutrient acquisition, and sustainability outcomes. These insights ultimately inform future crop development and breeding strategies within intercropping systems.

1.1.6 Methodological integration for rhizosphere analysis

This dissertation employed a diverse and interdisciplinary methodological framework to characterize rhizosphere processes across both greenhouse and field environments. Building on recent advances, this study applied the combined use of spatially resolved zymography and planar optode imaging in transparent rhizotrons, which enabled fine-scale visualization of enzyme activity and pH gradients. These techniques advanced our understanding of genotype–microbe–soil interactions by mapping localized biochemical hotspots and acidification zones within each system.

In field experiments, ^{15}N isotope tracing quantified nitrogen uptake efficiency and supported functional assessment of arbuscular mycorrhizal fungi (AMF) colonization across intercropping treatments. Phosphorus fractionation and phospholipid fatty acid (PLFA) profiling were employed to evaluate how soil texture and intercrop identity shaped microbial community composition and nutrient partitioning. These tools demonstrated that microbial stoichiometry and functional responses are context-dependent, modulated by both genotype and environmental variables.

This integrative approach bridged plant physiology, biogeochemistry, and microbial ecology, allowing trait–function relationships, such as phosphatase activity, root architecture, and microbial responsiveness, to be analyzed across spatial and ecological gradients. Collectively, these methodologies established a foundation for linking advanced root phenotyping (Fiorani

& Schurr, 2013; Kuijken et al., 2015) with microbial diagnostics, contributing to the development of sustainable cropping systems and strategies for ecological intensification.

1.2 Objectives

The primary objective of this dissertation is to advance mechanistic understanding of how root traits in genetically diverse maize genotypes influence rhizosphere biochemical activity, nutrient mobilization, and microbial community dynamics in cereal–legume intercropping systems. Through the integration of greenhouse rhizotron experiments and multi-site field trials, this research explores genotype-dependent interactions that regulate nutrient cycling and crop–microbe relationships under diversified cropping conditions.

The specific objectives were to:

1. Evaluate how root architectural and functional traits in six maize (*Zea mays* L.) genotypes influence rhizosphere enzyme activities and microbial diversity when intercropped with faba bean (*Vicia faba* L.) under controlled greenhouse conditions (**Study 1**).
2. Assess the effects of intercropping with a legume (*Pisum sativum* L.) and a non-legume (*Lolium perenne* L.) on phosphorus availability and microbial community composition across soils differing in texture and organic matter content (**Study 2**).
3. Quantify nitrogen uptake and AMF colonization in three maize genotypes intercropped with faba bean in field conditions, using ^{15}N -labeled ammonium sulphate and ink–vinegar staining to evaluate genotype-specific nitrogen recovery and mycorrhizal responsiveness (**Study 3**).
4. Investigate how rhizosphere biochemical activity and microbial nutrient acquisition strategies are shaped by rhizosphere–microbe–enzyme feedbacks in intercropping systems, and how these processes vary by maize genotype under field conditions (**Studies 2 and 3**).
5. Synthesize genotype-specific root and microbial traits across all studies and propose a trait-based framework for optimizing nutrient cycling, crop–microbe symbioses, and agroecosystem resilience in cereal–legume intercropping systems.

Objectives 1 to 3 correspond directly to the three core experiments. Objectives 4 and 5 integrate cross-study insights to identify generalizable trait–function linkages and inform genotype-based strategies for enhancing nutrient use efficiency and long-term agroecological sustainability.

1.3 Materials and methods

1.3.1 Overview of experimental design

This chapter outlines the experimental design, soil and plant sampling protocols, and analytical approaches employed in the three core studies of this dissertation. The research included one controlled greenhouse rhizotron experiment and two field experiments. Each was designed to investigate distinct aspects of rhizosphere function, nutrient mobilization, and microbial dynamics in maize-based intercropping systems.

1.3.2 Study 1: Rhizotron experiment (Greenhouse)

1.3.2.1 Experimental setup and design

A controlled rhizotron experiment was conducted in the greenhouse facilities of Christian-Albrechts-Universität zu Kiel to examine genotype-specific rhizosphere processes in a maize–faba bean (*Vicia faba* L.) intercropping system. Six maize (*Zea mays* L.) genotypes, B73WT, BX1B73, MBS847, A347, FC1571, and LH74, were selected based on their contrasting root traits and morphological characteristics. All were intercropped with a widely cultivated European cultivar of faba bean.

Each rhizotron (60 × 30 × 8 cm) was filled with 20 kg of field-collected sandy loam (Cambic Luvisol), stratified into 10 kg of topsoil (0–30 cm) and 10 kg of subsoil (30–60 cm). Soils were sieved to 2 mm and packed to a uniform bulk density of 1.4 g cm⁻³. Soil moisture was maintained at 70% of water-holding capacity throughout the experiment.

The experimental design consisted of one maize and one faba bean plant per rhizotron. Root zones were functionally distinguished as maize rhizosphere zone, shared rhizosphere, and faba bean rhizosphere zone for the purpose of spatially resolved sampling and analysis, but roots were allowed to grow freely without physical partitions. Four biological replicates were established for each maize genotype, resulting in 24 rhizotrons in total.

Maize seeds were germinated on moist filter paper in darkness at 21 °C for 72 hours and transplanted into rhizotrons. Faba bean seeds were pre-soaked in water for 3 hours and sown once the maize plants reached the three-leaf stage. Seeds were positioned 15 cm apart to minimize early interspecific competition.

To encourage root growth along the observation window, rhizotrons were wrapped in aluminum foil to block light and inclined at a 45-degree angle. Their positions were randomized and rotated weekly to control for positional effects within the greenhouse. Environmental conditions were maintained at 20–24 °C, with a 16 h light / 8 h dark photoperiod, and light intensity ranging from 500 to 1800 μmol m⁻² s⁻¹.

1.3.2.2 Plant harvest and rhizosphere sampling

Plants were harvested at maize growth stage BBCH 30 (stem elongation). Aboveground biomass was cut at the base, and root tips were carefully extracted to minimize disturbance to the rhizosphere.

The following samples were collected from each rhizotron:

- Rhizosphere soil: Soil tightly adhering to the root (rhizosphere) was gently brushed off using sterile brushes separately from each zone (maize, shared, bean) and from both soil layers (0–30 cm and 30–60 cm depth).
- Root tips: The terminal 4 to 5 cm of root segments were sampled for analysis of arbuscular mycorrhizal fungi (AMF) colonization.

A schematic illustration of the sampling zones for zymography and rhizosphere soil collection is provided in Figure 1 (manuscript 1).

1.3.2.3 Zymography and enzyme kinetics

Spatial patterns of rhizosphere enzyme activity were visualized using in situ zymography. Polyamide membranes (0.45 μ M pore size) were pre-saturated with enzyme-specific substrates: 7-amino-4-methylcoumarin (AMC, 200 μ M) for the detection of leucine aminopeptidase (LAP), and para-nitrophenyl phosphate (pNPP, 5 mM) for acid phosphatase (AP), following the protocols described by (Spohn & Kuzyakov, 2013) and (Sinsabaugh et al., 2009).

Zymography was performed at two maize developmental stages, corresponding to the five-leaf and nine-leaf stages, in order to capture temporal variation in enzyme expression. Following incubation, membranes were imaged to detect either fluorescence (for LAP) or colour development (for AP). The resulting images were quantitatively analyzed using ImageJ software to generate spatial enzyme activity maps across different rhizosphere zones and soil depths.

In addition to spatial mapping, potential enzyme activities were quantified in freshly collected rhizosphere soil using fluorometric microplate assays. Soil suspensions were prepared in 50 mM sodium acetate buffer (pH 5.5) and incubated with a gradient of substrate concentrations. LAP activity was measured using AMC substrates with fluorescence detection at excitation/emission wavelengths of 365/450 nm. AP activity was assessed using 4-methylumbelliferyl phosphate (MUF-P) or para-nitrophenyl phosphate (pNPP), with detection via fluorescence and absorbance respectively with the same excitation/emission parameters, or pNPP measured at 405 nm absorbance.

Enzyme kinetic parameters, including maximum reaction rate (V_{max}) and the Michaelis constant (K_m), were calculated by fitting substrate–velocity data to the Michaelis–Menten model using nonlinear regression.

1.3.2.4 Arbuscular mycorrhizal fungi (AMF) colonization

Fine root segments (1–2 cm in length) were cleared in 10 percent potassium hydroxide (KOH) at 90°C for 30 minutes, rinsed thoroughly, and stained using the ink–vinegar method as described by (Vierheilig et al., 1998).

AMF colonization was assessed using the gridline intersection method under a compound microscope at 400× magnification (McGONIGLE et al., 1990a). A minimum of 100 intersects were scored per sample to quantify the presence of AMF structures, including hyphae, vesicles, and arbuscules. Colonization percentage was calculated as the proportion of intersects containing any AMF structure.

1.3.2.5 Microbial community analysis

Microbial community profiling of rhizosphere soil samples was performed by Novogene (Munich, Germany). Total genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen). Amplicon sequencing of the 16S rRNA gene (V3–V4 region) was conducted for bacterial and archaeal community characterization, and the ITS1 region was sequenced for fungal communities. Sequencing was performed on an Illumina MiSeq platform with paired-end 2×250 bp reads.

Raw sequences were subjected to quality filtering, chimera removal, and denoising using DADA2, as implemented by Novogene’s standard pipeline. Amplicon sequence variants (ASVs) were clustered and taxonomically assigned using the SILVA database (version 138) for 16S sequences and the UNITE database (version 8.2) for ITS sequences.

Basic diversity metrics (alpha and beta diversity), community composition summaries, and principal coordinate analyses (PCoA) were generated by Novogene and verified using QIIME2 (version 2022.2). Downstream statistical analysis and figure generation were conducted in R as described in Section 1.3.2.6.

1.3.2.6 Statistical analysis

All statistical analyses were performed using R software (R Core Team, 2023). Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene’s test.

Genotypic and treatment effects were evaluated using one-way analysis of variance (ANOVA), followed by Tukey’s Honest Significant Difference (HSD) post hoc test. Enzyme kinetic

parameters (Vmax and Km) were estimated through nonlinear regression using the nls function in R.

Microbial community composition was analyzed via principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA), implemented with the vegan package. Graphical representations, including boxplots and ordination plots, were generated using ggplot2.

1.3.3 Study 2: Field intercropping experiment (Switzerland)

1.3.3.1 Experimental sites and design

This study was conducted at three agricultural field sites in Switzerland, each characterized by distinct soil types and topographical features. The Siselen site (Site 1) is classified as a Haplic Luvisol. The Seedorf site (Site 2) comprises a Haplic Cambisol and a Gleyic Cambisol located in the lower topography. The Fraubrunnen site (Site 3) includes Haplic and Cutanic Luvisols, with observed local soil compaction occurring at depths between 30 and 40 cm. Soil classification followed the guidelines of the (WRB, 2022).

At each site, two intercropping treatments were implemented: (1) maize (*Zea mays* L.) intercropped with field pea (*Pisum sativum* L.), and (2) maize intercropped with ryegrass (*Lolium perenne* L.). A single hybrid maize cultivar was used across all sites to isolate intercrop and soil texture effects without confounding genotype variation.

Each treatment was arranged in an 8 × 16 m plot following a randomized complete block design (RCBD) with ten replicates per treatment. This setup resulted in a total of 60 plots across all three sites (2 treatments × 3 sites × 10 replicates). The RCBD minimized edge effects and facilitated uniform field management.

Sampling was carried out at two maize developmental stages: BBCH 32 (stem elongation) and BBCH 65 (flowering). In each plot, a central soil trench approximately 1 m deep and 1.5 m wide was excavated to access the rhizosphere zone where root interactions between maize and the intercrop species were most intense. The choice of intercropping combinations was designed to explore functional complementarity, with cereal–legume and cereal–grass pairings selected to promote resource partitioning and distinct rhizosphere processes.

1.3.3.2 Soil and rhizosphere sampling

At both BBCH 32 and BBCH 65 stages, rhizosphere soil was sampled from the Ap horizon (0–30 cm), targeting root-dense zones in the shared maize–intercrop rhizosphere. Multiple subsamples were collected from each plot and pooled to form composite rhizosphere samples.

Soil was extracted using a stainless-steel auger, gently air-dried at ambient temperature, sieved to <2 mm, and stored at 4 °C for subsequent chemical analyses.

For microbial analyses, tightly adhering rhizosphere soil was collected by gently brushing and shaking roots from the maize–intercrop interface. These samples were placed in sterile polyethylene containers and immediately stored at –20 °C for phospholipid fatty acid (PLFA) analysis. All field tools were sterilized between plots to prevent cross-contamination. Soil samples collected during this phase were later used for biochemical analyses described in Sections 1.3.3.4 and 1.3.3.5.

1.3.3.3 Soil pH measurement

Soil pH was determined in rhizosphere samples using the potentiometric method in accordance with ISO 10390:2021, which is recommended for mineral soils. Air-dried and sieved rhizosphere soil (<2 mm) was suspended in a 0.01 M calcium chloride (CaCl₂) solution at a 1:5 (weight-to-volume) ratio. The suspension was thoroughly mixed and allowed to equilibrate for 30 minutes.

Measurements were performed at room temperature using a calibrated digital pH/mV meter (Thermo Orion DMP-2). The device was calibrated daily using certified pH buffer solutions (pH 4.0, 7.0, and 10.0). All pH measurements were conducted in duplicate to ensure accuracy.

1.3.3.4 Phosphorus fractionation

Phosphorus fractions were quantified following an operational extraction approach adapted from Prietzel et al. (2015), focusing on plant-relevant phosphorus pools rather than comprehensive chemical speciation. Three fractions were considered: orthophosphate phosphorus (P_Ortho), total phosphorus (P_Total), and non-citric-acid-extractable phosphorus (NCA_P).

Rhizosphere soil samples were air-dried at room temperature and sieved to <2 mm. Orthophosphate phosphorus (P_Ortho) was extracted using 1% (w/v) citric acid at a 1:10 soil-to-solution ratio and quantified colorimetrically using the molybdate–ascorbic acid method (Murphy & Riley, 1962), corresponding to the first extraction step described by Prietzel et al. (2015).

Total phosphorus (P_Total) was determined on finely ground subsamples following complete HF/HClO₄ digestion, with phosphorus concentrations measured by inductively coupled plasma–optical emission spectrometry (ICP-OES; Varian Vista Pro).

Non-citric-acid-extractable phosphorus (NCA_P) was calculated as the difference between total phosphorus and citric-acid-extractable orthophosphate ($NCA_P = P_Total - P_Ortho$). This operationally defined fraction comprises phosphorus forms not mobilized by 1% citric

acid, including mineral-bound, occluded, and organically associated phosphorus pools. Importantly, NCA_P does not represent total organic phosphorus as determined by ignition or alkaline extraction methods (Saunders & Williams, 1955), but reflects a less readily available phosphorus pool relevant to longer-term phosphorus dynamics under field conditions.

1.3.3.5 PLFA analysis (Microbial community profiling)

Microbial community composition in rhizosphere soil was assessed using phospholipid fatty acid (PLFA) profiling, following the protocol of (Bligh & Dyer, 1959), as adapted for soil matrices by (Frostegård et al., 1991), and (Gunina et al., 2014). PLFA profiling was specific to Study 2 and was not applied in the rhizotron experiment described in Section 1.3.2.

Briefly, 6 g of fresh soil was homogenized in a mixture of chloroform, methanol, and potassium citrate buffer (volume ratio: 0.8:1:2; pH 4.0). Lipids were extracted from the homogenate and fractionated on silica gel columns into neutral lipids, glycolipids, and phospholipids.

Phospholipids were then transesterified into fatty acid methyl esters (FAMES) using 12.5 M boron trifluoride in methanol, with derivatization conducted at 80°C. The resulting FAMES were identified and quantified using gas chromatography–mass spectrometry (GC-MS) with a Hewlett Packard 5890 GC equipped with a 5971A mass selective detector.

Microbial biomarker assignments followed established literature references and were further refined using loadings from principal component analysis (PCA). For specific biomarker details, see Supplementary Tables S3 to S6. PLFA concentrations were normalized to dry soil weight (per gram), and microbial groupings were derived through PCA with VARIMAX rotation to capture major community structure trends.

1.3.3.6 Statistical analysis

All statistical analyses were performed using (R Core Team, 2023). Normality of data distributions was assessed using the Shapiro–Wilk test, and homogeneity of variances was tested using Levene’s test. Depending on data characteristics, treatment effects were evaluated using either Welch’s t-test (for normally distributed data with unequal variances) or the non-parametric Wilcoxon rank-sum test.

For multivariate microbial community data derived from PLFA profiling, principal component analysis (PCA) was used to explore treatment-driven variation in microbial groups. In addition, redundancy analysis (RDA) was applied to assess the covariation between microbial community composition and phosphorus fractions, using the *vegan* package in R (Oksanen et al., 2025).

Pearson correlation coefficients were computed to examine relationships among soil pH, microbial biomarkers, and phosphorus pools. Data visualization tools such as ggplot2 and patchwork were used to generate boxplots, violin plots, and ordination biplots.

All multivariate statistical workflows and scripts were harmonized with those used in Study 1 to enable cross-experiment comparisons.

1.3.4 Study 3: Nitrogen uptake and AMF colonization in maize–faba bean field intercrops

1.3.4.1 Field site and experimental design

The field experiment was conducted during the 2024 growing season at Wurzelhof, located in Schinkel, Schleswig-Holstein, Germany (latitude 54°22'0" N, longitude 9°57'46" E), situated within a temperate climate zone. The soil at the site is classified as a Luvisol, characterized by moderate organic matter content and with a moderate pH, typical of temperate Luvisols (approx. 6.2–6.5). According to climate data from the Deutscher Wetterdienst (2024), average monthly temperatures during the growing season (April– September) ranged from 15°C to 22°C, with cumulative precipitation of approximately 450 mm.

The experimental layout followed a randomized complete block design with three replicates per treatment. Three maize genotypes (FC1571, B73WT, and A347), selected based on contrasting root phenotypes, were cultivated under two cropping systems: (i) monoculture (maize only) and (ii) maize intercropped with faba bean (*Vicia faba* L.). A total of 18 plots were established (3 genotypes × 2 cropping systems × 3 replicates), each measuring 2 m × 1.5 m.

In intercropped plots, six rows were sown lengthwise in alternating maize–bean order (1:1), with a row spacing of 15 cm and 20 cm spacing between plants within rows. Monoculture plots consisted of four maize rows with increased inter-row spacing to establish a lower-density, open-canopy architecture that mirrored the biomass density of the intercropping setup. This field-based assessment enabled the evaluation of genotype-dependent nitrogen acquisition under realistic soil–plant–microbe interactions, complementing the controlled rhizotron findings of Study 1.

1.3.4.2 ¹⁵N Isotope labeling and plant harvesting

To assess nitrogen uptake efficiency across maize genotypes and cropping systems, a ¹⁵N isotope labeling experiment was conducted at the BBCH 50 stage of maize growth (visible stem elongation with approximately ten fully unfolded leaves).

The tracer solution consisted of ¹⁵N-enriched sodium nitrate (Na¹⁵NO₃, ≥98 atom percent) and ¹⁵N-enriched ammonium sulphate ((¹⁵NH₄)₂SO₄, ≥98 atom percent), both sourced from Sigma-

Aldrich (Germany). A total of 6.49 mg of ^{15}N was uniformly distributed across the 18 experimental plots. The tracer was injected into pre-dug holes at two depths (20 cm and 70 cm) using a microinjection pipette, with 20 mL of solution applied per injection site. Applications were carried out under moist soil conditions to enhance diffusion and minimize isotopic loss. Plants were harvested seven days after labeling. Aboveground biomass (shoots) of both maize and faba bean was collected, oven-dried at 65°C for 5 to 6 days, and finely ground using a ball mill. In total, 65 biomass samples, including both labeled and control treatments, were analyzed for total nitrogen concentration and ^{15}N enrichment at the University of Göttingen using an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS; Thermo Scientific, Germany). Nitrogen uptake and ^{15}N recovery were calculated using the excess ^{15}N method described by (Hauck & Bremner, 1976). These measurements were subsequently correlated with AMF colonization data to evaluate genotype-specific differences in symbiotic nitrogen acquisition under intercropping.

1.3.4.3 Arbuscular mycorrhizal fungi (AMF) colonization

At the time of harvest, fine root samples were collected from all maize plants in each plot, including both monocropped and intercropped treatments. The staining and scoring methods followed the same protocol described in Section 1.3.2.5 (Study 1), ensuring methodological consistency across greenhouse and field conditions. Root segments (1–2 cm in length) were thoroughly washed, cleared in 10 percent potassium hydroxide (KOH) at 90°C, and stained using the ink–vinegar method as described by (Vierheilig et al., 1998).

AMF colonization was quantified under a compound microscope at 400× magnification using the gridline intersect method (McGONIGLE et al., 1990a). For each sample, a minimum of 100 intersects was examined for the presence of AMF structures, including hyphae, vesicles, and arbuscules. The total colonization percentage was calculated as the proportion of intersects containing any AMF structure relative to the total number of intersects scored.

1.3.4.4 Statistical analysis

All statistical analyses were conducted using R software (R Core Team, 2023). A two-way analysis of variance (ANOVA) was used to evaluate main and interactive effects of maize genotype and cropping system on two key outcome variables: ^{15}N -labeled nitrogen uptake and percentage AMF colonization. Experimental block was included as a random effect in the model.

Linear mixed-effects models were fitted using the lme4 package, and post hoc comparisons were conducted with Tukey's Honest Significant Difference (HSD) test using the emmeans package. Statistical significance was considered at a threshold of $p < 0.05$.

Relationships between ^{15}N uptake and AMF colonization were assessed using Pearson correlation coefficients. Data visualizations, including bar graphs and scatterplots, were produced using the ggplot2 package. Summary statistics were stratified by maize genotype and cropping system to support genotype-level interpretation of AMF responsiveness and nitrogen uptake.

1.4 Main results and discussion

1.4.1 Genotype-specific root traits and spatial–temporal rhizosphere biochemistry

The rhizotron experiments revealed strong genotype-specific effects on rhizosphere biochemical activity in maize–faba bean intercropping systems. Genotypes FC1571 and LH74 consistently exhibited highly localized hotspots of acid phosphatase (AP) and leucine aminopeptidase (LAP) activity in the shared rhizosphere compartment, as visualized using in situ zymography. These genotypes were also characterized by deeper, more extensively branched root systems with high lateral root density and wide branching angles—traits associated with increased root surface area and enhanced rhizodeposition (Yin et al., 2019; York et al., 2015).

Measured $V_{\max}$ values for AP and LAP in FC1571 reached approximately 110 and 65 $\text{nmol g}^{-1} \text{h}^{-1}$, respectively, significantly exceeding the values observed in BX1B73 and B73WT (both $<40 \text{ nmol g}^{-1} \text{h}^{-1}$; $p < 0.01$). These results align with prior studies indicating that localized enzyme secretion is a key mechanism for nutrient acquisition under phosphorus-deficient conditions (Razavi et al., 2016; Spohn & Kuzyakov, 2013).

Zymographic imaging revealed that enzymatic activity clustered at root tips and lateral branching zones, known microsites for mucilage and exudate release (Jin et al., 2013; Lambers et al., 2009). These zones are rich in rhizodeposits such as organic acids, sugars, and amino acids, which stimulate microbial metabolism and drive nutrient mineralization. Root architectural features such as branching density and total surface area strongly influenced the spatial distribution of these biochemical hotspots, shaping microbial community assembly and function (Dijkstra et al., 2013; Pétriacq et al., 2017).

Temporal dynamics of enzyme activity showed a consistent increase in AP and LAP expression from BBCH 15 to BBCH 19, with peak activity occurring in the shared rhizosphere zone. At BBCH 19, enzymatic hotspots extended up to 1.5 mm from root surfaces, particularly in intercropped FC1571 and LH74, indicating cumulative root–microbe interactions over time. Vertical soil profiling further showed enzyme activity concentrated in the 0–30 cm layer, though it remained detectable even in deeper zones for deep-rooted genotypes.

These exudation-driven patterns contributed to a structured ecological feedback loop:

1. Root architectural traits (depth, branching, surface area);
2. Rhizodeposition (organic C release);
3. Microbial enrichment (nutrient-mineralizing taxa);
4. Elevated enzyme activity and nutrient solubilization;
5. Improved nutrient uptake and plant performance.

This feedback loop was particularly amplified in legume–maize systems, especially when FC1571 or LH74 were paired with *Vicia faba*, due to complementary exudate profiles and niche partitioning. These synergistic interactions promoted microbial guild coexistence and enzymatic functionality, contributing to improved phosphorus and nitrogen acquisition and higher nutrient use efficiency (Ben-chuan et al., 2022; Berendsen et al., 2012).

In summary, rhizosphere biochemical complexity emerges from the integrated expression of genotype-specific root traits, exudation chemistry, rhizosphere acidification, and enzyme-mediated microbial processes. These results reinforce the concept that root–microbe interactions are dynamic and co-regulated, and they can be systematically optimized through trait-based genotype selection. Incorporating these traits into breeding strategies and intercropping system design offers strong potential for enhancing nutrient cycling and agroecosystem sustainability (Abalos et al., 2019; Cassman & Grassini, 2020; Moore et al., 2022).

1.4.2 Root exudates, microbial community assembly, and functional feedbacks

Microbial community profiling, via PLFA analysis (Study 2) and 16S/ITS sequencing (Study 1, greenhouse), revealed clear shifts in rhizosphere microbial structure and functional potential across different maize genotypes, intercropping partners, and soil compartments. These changes were most pronounced in shared rhizosphere zones, where interspecific root interactions intensified biochemical activity and microbial recruitment.

Legume-based intercropping, with *Vicia faba* (greenhouse and German field trials) and *Pisum sativum* (Swiss field trials), significantly increased the relative abundance of Gram-negative bacteria and Actinobacteria groups associated with high metabolic versatility, phosphorus solubilization, and degradation of complex organic substrates (Buyer & Sasser, 2012; Lin et al., 2020; Marschner et al., 2011). These microbes are known to be selectively enriched by root-derived carbon compounds, including organic acids and phenolics, which stimulate microbial metabolism and phosphatase activity (Richardson et al., 2009; Zhelnina et al., 2018).

By contrast, intercropping with ryegrass (*Lolium perenne*) favored saprotrophic fungi and arbuscular mycorrhizal fungi (AMF). This fungal-dominated nutrient cycling pathway likely reflects slower carbon turnover and more recalcitrant root exudates characteristic of ryegrass, which select for fungal decomposer communities (Georgiou et al., 2022; Hallama et al., 2019; Lavallee et al., 2020).

Microbial community profiling and literature evidence suggest that genotypes such as FC1571 and LH74 may differ in their exudation of organic acids, sugars, and amino acids, biochemical traits known to shape microbial niche heterogeneity (Dennis et al., 2010; Kuzyakov & Razavi, 2019; Pétriacq et al., 2017). This dynamic is governed by a feedback loop: root traits → rhizodeposition → microbial enrichment → enzyme activity → nutrient mobilization.

High-exudation genotypes consistently showed higher phosphatase and LAP activity, and significantly enriched microbial taxa involved in phosphorus and nitrogen mobilization (Berendsen et al., 2012; Philippot et al., 2013; Tardy et al., 2014). Indicator species analysis revealed strong enrichment of beneficial microbes in FC1571 and LH74 rhizospheres, especially under *Vicia faba* intercropping. Such microbial consortia contribute to nutrient mineralization, stress resilience, and pathogen suppression, thereby enhancing ecosystem multifunctionality. Moreover, legume–maize intercropping amplified this feedback by introducing complementary exudate profiles and niche partitioning among microbial guilds. In particular, *Vicia faba* enhanced organic nitrogen turnover and stimulated microbial cohabitation via cross-signalling mechanisms (Ben-chuan et al., 2022; Berendsen et al., 2012; Lareen et al., 2016). These dynamics reinforced the role of rhizosphere exudates as ecological filters for microbial community assembly and functionality.

Multivariate analyses (PCA, PERMANOVA) confirmed that maize genotype, intercropping partner, and rhizosphere compartment all significantly affected microbial composition ($p < 0.01$), while soil type exerted secondary modulation. Together, these results support a framework in which rhizosphere microbial function is emergent from genotype-specific root traits, carbon deposition patterns, and plant–microbe–soil feedback loops. These findings advance the concept that microbiome-driven nutrient cycling is both a consequence and co-driver of plant functional diversity in sustainable agroecosystems (Homulle et al., 2022; Van Der Heijden et al., 2008). These insights underscore the importance of selecting genotypes that modulate microbial networks through exudate-mediated mechanisms, reinforcing their role as functional levers in genotype-informed intercropping strategies.

1.4.3 Phosphorus fractionation and dynamics across sites and treatments

Phosphorus dynamics in Study 2 were evaluated using an operational fractionation framework focusing on orthophosphate phosphorus (P_Ortho), total phosphorus (P_Total), and non-citric-acid-extractable phosphorus (NCA_P). Across the Swiss field sites, which differed in soil texture and organic matter content, intercropping treatments exerted a strong influence on the distribution of these phosphorus pools, highlighting the role of biologically mediated rhizosphere processes in regulating phosphorus availability.

Legume-based intercropping systems, particularly maize–field pea, consistently enhanced P_Ortho concentrations in the rhizosphere compared with maize–ryegrass systems. This effect was most pronounced in loamy soils, where higher microbial activity and buffering capacity likely amplified biologically driven phosphorus mobilization. Increased P_Ortho availability coincided with rhizosphere acidification and elevated phosphatase activity, supporting a mechanism of pH-mediated solubilization and enzymatic phosphorus turnover rather than changes in bulk soil phosphorus stocks (George et al., 2018; Oberson et al., 2006; Tang et al., 2021).

Genotype-specific responses further modulated these patterns. Maize genotypes such as FC1571 and LH74, which exhibited high rhizosphere enzyme activity in zymographic assays, were associated with greater P_Ortho availability under legume intercropping. These findings indicate that root-driven biochemical processes, including organic acid exudation and phosphatase activity, play a central role in enhancing short-term phosphorus availability in intercropping systems (Hallama et al., 2019; Richardson et al., 2009).

In contrast, maize–ryegrass intercropping maintained higher rhizosphere pH and was characterized by comparatively lower P_Ortho concentrations but modestly elevated NCA_P. This operationally defined fraction includes phosphorus forms not mobilized by 1 % citric acid and likely reflects enhanced microbial immobilization and phosphorus retention within mineral-bound and organically associated pools. Such patterns are consistent with fungal-dominated rhizospheres and slower phosphorus turnover rates typically observed in grass-based systems (Bünemann et al., 2011; Turner, 2008; Guenet et al., 2016).

Integrating phosphorus fractions with enzyme activity measurements and planar optode-derived rhizosphere pH revealed a close coupling between biochemical conditions and phosphorus dynamics. Zones of lowered pH and elevated phosphatase activity were associated with increased P_Ortho availability, underscoring the importance of root–microbe–enzyme feedbacks in regulating phosphorus cycling at the rhizosphere scale (Oburger & Jones, 2018; Spohn & Kuzyakov, 2013).

Overall, these results support the concept of functionally distinct rhizosphere zones shaped by maize genotype and intercrop identity. Legume-based systems promote rapid, bacteria-driven phosphorus mobilization, whereas grass-based systems favor phosphorus retention and slower cycling. This functional differentiation provides a biologically grounded framework for designing low-input cropping systems that enhance phosphorus use efficiency through targeted rhizosphere processes rather than reliance on external fertilizer inputs (Lambers et al., 2015; Rose et al., 2013).

1.4.4 Nitrogen uptake efficiency and mycorrhizal symbioses

The ^{15}N tracer experiments conducted in the field revealed distinct genotype-specific differences in nitrogen (N) uptake efficiency among maize cultivars grown in intercropping systems with *Vicia faba*. Notably, genotype FC1571 exhibited the highest ^{15}N recovery in aboveground biomass, indicating superior capacity for nitrogen acquisition and assimilation. In contrast, B73WT showed significantly lower ^{15}N enrichment despite comparable shoot biomass, suggesting decoupling between biomass production and nitrogen capture in this genotype. Genotype A347 showed intermediate ^{15}N enrichment but was characterized by high rates of mycorrhizal colonization, suggesting partial compensation for limited root biomass via symbiotic N and P uptake.

These findings from our field experiments align with prior work showing that variation in root depth, branching, and exudation profiles strongly affects nitrogen foraging efficiency (Kumar et al., 2022; Lynch, 2019). In particular, deeper or more branched root systems may access N from subsurface horizons, while high exudation rates can stimulate microbial N mineralization in the rhizosphere (Liao et al., 2006; Q. Shen et al., 2018; York et al., 2013).

Microscopic assessment of arbuscular mycorrhizal fungi (AMF) colonization using the gridline intersection method revealed a positive correlation between AMF colonization and ^{15}N uptake, particularly strong in genotype A347. Although A347 had moderate biomass, its with colonization rates exceeding 65%, suggesting that mycorrhizal symbioses partially compensated for A347's limited root biomass, enhancing both N and phosphorus (P) uptake via extraradical hyphal networks (Beslemes et al., 2023; P. Chen et al., 2021; Smith & Smith, 2011). These findings align with prior work illustrating that AMF improve the uptake of both phosphorus and nitrogen forms (nitrate and ammonium), particularly under nutrient-depleted or low-input conditions (Bücking & Kafle, 2015; Thirkell et al., 2016).

Across all genotypes, intercropping consistently led to higher total plant N content compared to monoculture controls. This enhancement is underpinned by three facilitative mechanisms well-documented in legume–cereal intercropping systems:

- Biological nitrogen fixation (BNF) by *Vicia faba*, enriching rhizosphere N (Kebede, 2021; Maluk et al., 2022).
- Hydraulic redistribution and root zone complementarity improving spatial nutrient access (Bayala & Prieto, 2020; L. Zhang et al., 2024).
- Increased microbial N cycling activity (ammonification, mineralization) in intercropped rhizospheres (Brooker et al., 2015a).

These synergistic dynamics confirm that legume-based intercropping enhances NUE, especially when paired with maize genotypes expressing high root plasticity or strong AMF responsiveness. These results highlight the importance of root phenotyping and symbiotic screening in genotype selection for intercropping. Maize genotype plays a pivotal role in determining the magnitude of NUE improvements under intercropping. Importantly, root-AMF-nitrogen uptake interactions are not uniform across genotypes, highlighting the importance of trait-informed breeding and crop pairing strategies for sustainable agroecosystem design.

1.4.5 Genotype × environment × management interactions

Integrated analysis across the rhizotron (Study 1) and field experiments (Studies 2 and 3) revealed differences attributable to maize genotype, soil type, and cropping system, with consistent effects on enzyme activity, phosphorus availability, ¹⁵N uptake, and microbial community composition. In contrast, the Swiss field experiment (Study 2) used a single hybrid maize cultivar across all sites, so no genotype effects were tested in that context.

The strongest genotype-dependent effects were observed for elevated acid phosphatase (AP) and leucine aminopeptidase (LAP) activity, as well as increased AMF colonization and microbial biomass, particularly in genotypes such as FC1571 and LH74. These responses were most pronounced under legume-based intercropping conditions, especially in greenhouse and field environments with sufficient organic matter availability. Although loamy soils supported greater microbial activity in general, direct genotype comparisons were only conducted in controlled rhizotron experiments (Study 1) and in the German field site (Study 3). These findings support prior evidence that high-resource environments facilitate the expression of beneficial rhizosphere traits (Fierer et al., 2012; Hallama et al., 2019). Examples of genotype-specific responses:

- FC1571 (environmentally stable): Demonstrated consistently high enzyme activity (acid phosphatase and leucine aminopeptidase), elevated AMF colonization, and increased microbial biomass across both controlled and field conditions. These traits indicate a plastic but stable rhizosphere phenotype with robust nutrient mobilization.
- A347 (functionally responsive): Despite lower AMF colonization rates, it showed the strongest correlation between AMF presence and ¹⁵N uptake, highlighting its high functional responsiveness to symbiosis and efficient nutrient acquisition under intercropping.

These findings align with the concept of rhizosphere plasticity, the ability of root phenotypes and exudation patterns, to dynamically respond to environmental gradients such as soil pH, texture, organic content, and neighboring species (Canarini et al., 2019; Lareen et al., 2016).

Implications for breeding and agroecosystem design:

- Trait stability vs. environmental responsiveness must be assessed through multi-environment genotype screening.
- Key rhizosphere traits for selection include:
 - Root depth and total surface area
 - Carbon exudation rate and biochemical complexity
 - AMF colonization potential and responsiveness
 - Rhizosphere enzyme activity (especially phosphatases)

Breeding programs should adopt G×E×M-informed strategies, aligning genotypes with appropriate management systems (e.g., intercrop partners, amendments, or tillage) to co-optimize nutrient cycling, microbial stability, and agroecosystem resilience. These results reinforce that G×E×M interactions are central, not peripheral, to unlocking genotype potential in sustainable agriculture. Future efforts should integrate trait-informed genomics, rhizobiome characterization, and multi-site feedback trials to guide precision breeding and genotype-specific intercropping design (Jiang et al., 2024; Lynch, 2019; York et al., 2015).

1.4.6 Trait-based design and agronomic deployment of sustainable cropping systems

This dissertation demonstrates that selecting maize genotypes with advantageous root and rhizosphere traits offers a robust pathway to sustainable, low-input agriculture. Integrating these genotypes into intercropping systems can boost nutrient use efficiency, enhance soil microbial networks, and increase resilience under resource constraints.

Key rhizosphere traits driving these benefits include:

- Root system architecture: Genotypes with deeper, more branched roots and extensive lateral development enhance soil exploration, water uptake, and nutrient interception (Lynch, 2019; Paez-Garcia et al., 2015; York et al., 2015).
- Mycorrhizal responsiveness: High potential for colonization and functional symbiosis with arbuscular mycorrhizal fungi (AMF) can improve phosphorus and nitrogen acquisition via enhanced root–fungus exchange (P. Chen et al., 2021; Smith & Smith, 2011);
- Microbial recruitment capacity: The ability to enrich beneficial and functionally diverse microbial consortia contributes to improved nutrient cycling, stress tolerance, and plant growth promotion (Berendsen et al., 2012; Lareen et al., 2016; Van Der Heijden et al., 2008).

Legume-based intercropping, particularly with *Vicia faba* and *Pisum sativum*, further strengthened these traits by providing complementary root structures and exudate profiles. Notably, genotypes such as FC1571 and LH74 expressed these beneficial traits consistently in both greenhouse and field settings, though validation in more environments is needed.

To apply these findings, strategies include:

- Agroecological zoning: Integrating belowground trait profiles into cultivar zoning systems to better match genotypes with site-specific edaphic constraints (e.g., low-P or low-N soils).
- Seed system innovation: Embedding trait-based recommendations into farmer advisory platforms to facilitate site-specific maize genotype selection.
- Decision-support tools: Developing participatory or digital tools that align genotype traits with intercrop species, soil fertility status, and biotic stress profiles.

Examples of trait–function–environment alignment include:

- In phosphorus-limited soils, genotypes that secrete high levels of phosphatases can improve mobilization of less readily available phosphorus forms through enzymatic and pH-mediated processes.
- In nitrogen-deficient systems, genotypes with strong AMF responsiveness or high root exudation can enhance N uptake and support microbial-mediated N cycling.

These strategies are central to the goals of climate-smart agriculture, enabling stable productivity while minimizing ecological impact. Moreover, they promote farmer-centered innovation through actionable, science-based genotype recommendations.

Recommended implementation strategies:

- Development of trait-based selection indices that include exudate chemistry, enzyme activity (especially phosphatases), and AMF colonization responsiveness.

- Application of high-throughput rhizosphere phenotyping tools, including rhizotrons, and zymography, to accelerate trait screening and selection (Lobet et al., 2012);
- Cross-sector collaboration among breeders, agronomists, soil microbiologists, and extension services to mainstream rhizosphere traits into breeding pipelines and participatory research platforms.

In summary, trait-based genotype selection emerges as a support of agroecological design, guiding the transition toward resilient, resource-efficient, and multifunctional cropping systems.

1.4.7 Limitations and perspectives for future research

While this research provides advances understanding of rhizosphere trait expression within intercropping systems, several methodological and conceptual limitations offer clear avenues for future investigation: First, the functional capacity of microbial communities was only inferred, not directly measured; future studies should integrate metagenomics, metatranscriptomics, and metabolite profiling for greater resolution (Edwards et al., 2015; Leff et al., 2015). Second, root exudate patterns were estimated indirectly; direct chemical profiling using metabolomics and microdialysis is needed to clarify genotype-specific exudation (Oburger & Jones, 2018; Zhalnina et al., 2018). Such data are key to understanding how specific metabolites shape microbial recruitment, enzyme induction, and nutrient mobilization. Additionally, the experiments were limited to one growing season and specific environments, restricting generalization. Multi-year, multi-site trials are needed to test the stability of genotype $\times$ environment interactions across broader gradients (Mwafulirwa et al., 2021; Prus & Piepho, 2024; Zhou et al., 2024). Finally, nutrient fluxes between plants and microbes remain under-characterized; future research should employ dual-isotope tracing and sequential biomass partitioning to map real-time nutrient cycling (Dijkstra et al., 2013; Haddix et al., 2016; IAEA, 2001).

Bridging these gaps will require interdisciplinary work across plant phenotyping, metabolomics, microbial ecology, and systems modelling, crucial steps toward trait-optimized breeding and resilient, efficient cropping systems.

1.4.8 Methodological reflections on the rhizosphere toolkit

This dissertation highlights the value of integrated rhizosphere phenotyping to unravel root–soil–microbe interactions. By combining in situ zymography, ^{15}N isotope tracing, and PLFA profiling, we captured rhizosphere function across diverse environments. Each method contributed distinct insights: zymography visualized enzyme activity, ^{15}N isotope tracing

quantified nitrogen uptake, and PLFA assessed microbial structure. Nonetheless, limitations remain: optode imaging is low-throughput, zymography is difficult to scale to field studies, and PLFA/amplicon sequencing lack functional resolution. Future research should integrate metabolomics, metatranscriptomics, and machine learning to resolve exudation profiles and enable high-throughput, trait-based phenotyping for breeding and agroecological design (Leff et al., 2015; Papoutsoglou et al., 2023; Prosser, 2015; Trivedi et al., 2020).

1.4.9 Rhizosphere trait synergies: A synthesis

Across all studies, a clear network of rhizosphere traits emerged as central to nutrient cycling and plant-microbe dynamics. Genotypes like FC1571 and LH74, characterized by deep, branched roots, showed root traits associated with increased exudation, enzyme activity, and recruitment of beneficial microbes. These traits interact in feedback loops, root architecture shapes exudation, which drives microbial and enzyme activity, ultimately enhancing nutrient mobilization and plant uptake.

Despite environmental variation, these synergies consistently improved nutrient cycling and reduced fertilizer needs in intercropping systems. This supports a modular, ideotype-based approach to breeding: targeting combinations of rhizosphere traits (root depth, exudate chemistry, enzyme induction) alongside yield and resilience for designing sustainable, low-input cropping systems (Bais et al., 2006; Lareen et al., 2016; M. Liu et al., 2022).

1.5 Conclusions

This dissertation advances our mechanistic understanding of how maize genotype-specific root traits interact with intercropping strategies to shape rhizosphere biochemical activity, microbial assembly, and nutrient cycling in cereal-based agroecosystems. Our findings provide both theoretical insights and translational frameworks for designing sustainable, genotype-informed cropping systems.

- Root system architecture is a primary driver of rhizosphere function. Genotypes such as FC1571 and LH74, distinguished by deep, highly branched roots and high lateral density, generated pronounced rhizodeposition and localized hotspots of enzyme activity. This spatial structuring enabled enhanced phosphorus mobilization and nutrient acquisition, substantiating trait-based selection models for resilient cropping (Lynch, 2019; Péret et al., 2011; York et al., 2013).
- Legume-based intercropping with *Vicia faba* and *Pisum sativum* enhanced phosphorus availability and diversified microbial function. These intercropping systems particularly benefited maize genotypes with high exudation capacity, resulting in increased

orthophosphate availability and altered phosphorus partitioning and recruitment of beneficial microbial taxa (Richardson et al., 2009; Brooker et al., 2015; Li et al., 2023).

- Soil type substantially influenced genotype and intercropping effects. Loamy soils with higher organic matter content accentuated genotype-specific trait expression and microbial responses, demonstrating the importance of G×E×M (Genotype × Environment × Management) interactions for site-specific optimization (Hallama et al., 2019; Lareen et al., 2016).
- Genotype-dependent nitrogen uptake was enhanced by mycorrhizal symbiosis. ¹⁵N tracer studies highlighted FC1571 and A347 as high-performing genotypes, with mycorrhizal colonization (particularly in A347) supporting greater N recovery identifying mycorrhizal responsiveness as a breeding target for low-input systems (Chepsergon & Moleleki, 2023; Chu et al., 2013; Smith & Smith, 2011).
- Methodological integration enabled multidimensional trait profiling but has limitations. Zymography, isotope tracing, PLFA, and sequencing together mapped genotype effects, but future research should prioritize direct exudate quantification, metabolomics, and multi-season, multi-site trials to fully resolve rhizosphere complexity (Oburger & Jones, 2018; Zhalnina et al., 2018).

Implications:

Strategically deploying root-trait-informed maize genotypes in functional intercrops can reduce fertilizer dependence, enhance soil fertility, and improve system resilience. Aligning plant functional traits, rhizosphere microbial ecology, and management strategies enables the development of cropping systems that are more sustainable, adaptive, and responsive to the multifaceted challenges of modern agriculture.

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2 Manuscript

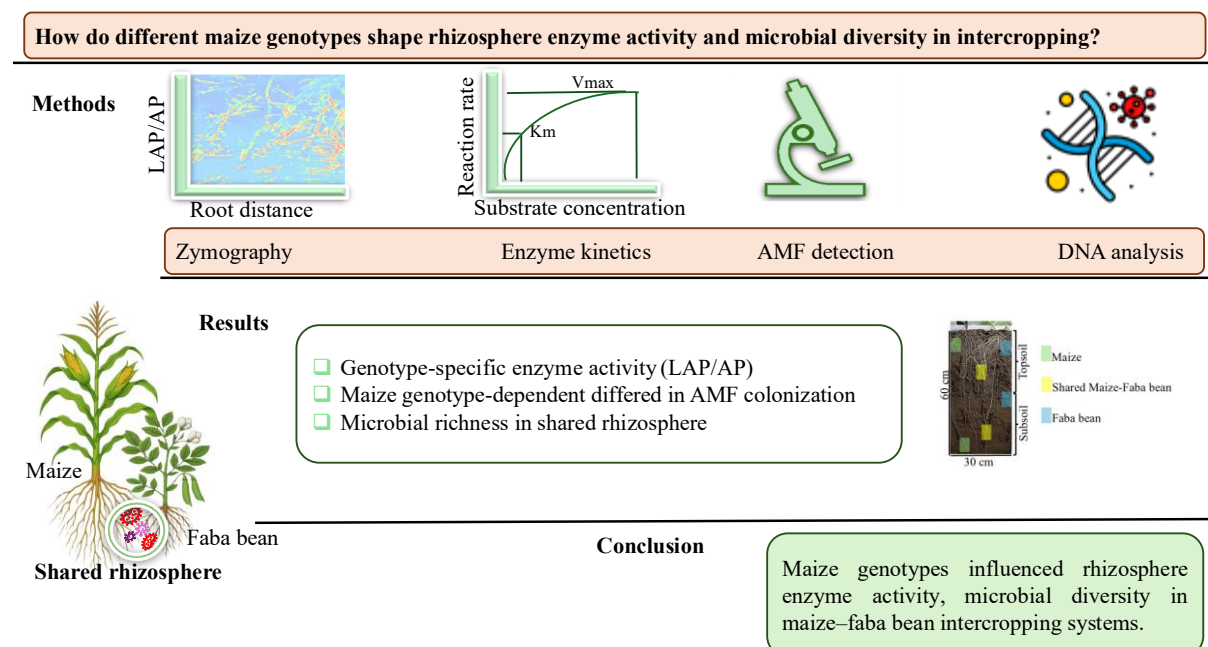
2.1 Study 1. Genotype-specific root and rhizosphere dynamics in maize-faba bean intercropping enhance soil enzyme activity and microbial diversity

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Graphical abstract



Highlights

- Maize genotype-dependent differed in AMF colonization and enzyme activity patterns

- Enzyme activity along roots' exudation gradients varied strongly among maize genotypes
- *topsoil vs subsoil*
- Intercropping reduced the relative abundance of *Fusarium solani* in most maize genotypes
- Intercropping increased fungal diversity in maize genotypes
- Maize genotypes FC1571, and LH74 promoted beneficial microbial communities

Abstract

Intercropping maize with faba bean presents a sustainable agricultural strategy enhancing resource efficiency and resilience particularly regarding space, nitrogen (N), suppressing weeds, and reducing erosion. We studied the effects of different maize genotype on the spatiotemporal distribution of rhizosphere i) enzyme activities, ii) enzyme kinetics and iii) microbial diversity in an intercropping system with faba bean in top- and the subsoil. We found maize genotype-dependent effects on AMF colonization and root traits. Enzyme activity along roots' exudation gradients varied strongly between the maize genotypes

- Clear spatial differences were observed between *topsoil vs subsoil*
- Intercropping reduced relative abundance of *Fusarium solani* for most maize genotypes
- Intercropping increased fungal diversity across maize genotypes

This study highlights the role of maize genotype selection in shaping rhizosphere enzyme activity and microbial community composition, thereby supporting more functionally diverse and potentially resilient intercropping systems.

Keywords

Maize genotype, Faba bean, Enzyme activity, Microbial community, Intercropping, Arbuscular mycorrhizal fungi, DNA sequencing

Introduction

As the demand for sustainable farming grows, researchers are constantly looking for ways to improve agricultural efficiency while reducing environmental impact. One promising approach is intercropping, where two or more crops are grown together in the same field. Intercropping has a long history and remains central to modern agroecological practices due to its ability to recycle nutrients, optimize resource utilization, and increase crop yields compared to monocultures (Brooker et al., 2015; Tian et al., 2018). By combining crops with different growth patterns, intercropping also reduces the need for chemical fertilizers and pesticides, making it an excellent strategy for more sustainable and resilient agriculture (Yu et al., 2019).

Furthermore, it increases microbial diversity, and resilience to environmental stresses (Lithourgidis et al., 2011), making it a key strategy for sustainable intensification of agricultural production (Brooker et al., 2015).

Maize (*Zea mays* L.) and legumes, such as faba bean (*Vicia faba* L.), are commonly intercropped due to their complementary growth patterns and resource-sharing capabilities (Lithourgidis et al., 2011). Legumes contribute to biological nitrogen fixation, enriching the rhizosphere with nitrogen and thereby reducing the need for synthetic nitrogen fertilizers (Drinkwater et al., 1998; Brooker et al., 2024). This nitrogen enrichment (Ali et al., 2025) directly benefits maize, enhancing growth and supporting long-term soil fertility (Hua et al., 2020). Additionally, maize and faba bean possess distinct root architectures, maize roots typically extend deeper into the soil profile, while faba bean roots predominantly occupy upper soil layers (Brooker et al., 2015). Such complementarity in root structure reduces belowground competition and maximizes overall resource acquisition efficiency due to niche complementary (Homulle et al., 2022; Zhang et al., 2016).

Another major advantage of maize-faba bean intercropping is its impact on soil microbes. Legumes stimulate beneficial microbes, such as nitrogen-fixing bacteria (e.g., *Rhizobium*) and phosphorus-mobilizing microbes, which play critical roles in nutrient cycling and plant growth (Richardson & Simpson, 2011). While increased microbial diversity and functional potential are typical features of rhizosphere communities compared to bulk soil, intercropping can further enhance these effects. Studies have shown that intercropped systems promote greater enrichment of plant-growth-promoting genera such as *Pseudomonas* and *Burkholderia* (Yang et al., 2017), thereby improving nutrient uptake efficiency, nitrogen (N) dynamics and grain yield (Li et al., 2022). Contrastingly, Fusarium root rot of corn (*zea mays* L.) is yield-limiting – a study in South Dakota indicates that eight species of *Fusarium* are aggressive root rot pathogens (Okello et al., 2019) Root rot pathogens attack beans at all growth stages and cause damping-off at the seedling stage, yellowing of the leaves, stunted growth. *Fusarium* species are ubiquitous in soils and are considered as field fungi invading more than 50% of maize grains before harvest (Robledo-Robledo, 1991). In a maize-bean intercropping the species diversity was influenced by crop type with bean rhizosphere being more diverse with *Fusarium* compared to maize rhizosphere as shown by Shannon indices (Okoth & Siameto, 2011).

The importance of rhizosphere metabolites in shaping soil biodiversity and community composition should not be neglected (Singh et al., 2022), as they are actively secreted by plants to regulate microbial abundance and activity in the rhizosphere. Intercropping can stimulate changes in root and microbial exudates under nutrient-deficient conditions, promoting microbial nutrient incorporation and therefore enhancing nutrient availability in the rhizosphere, consequently facilitating greater plant nutrient uptake (Du et al., 2020). Beside the primary metabolites, plant roots exude secondary metabolites e.g. benzoxazinoids (Dick et al., 2012) which induce herbivore resistance (Maag et al., 2016) and have strong fungicidal but less pronounced antibacterial effects (Cotton et al., 2019). Bilerya et al., (2022) concluded that root hairs facilitate better plant nutrition at the shortage of available nutrients while the presence of benzoxazinoids in exudates protect plant from pathogenic microorganisms.

Therefore, root traits such as lateral root density, branching intensity, and rooting depth are increasingly recognized as key determinants of the soil microenvironment, shaping which microbial communities thrive in the rhizosphere (McGrail et al., 2020). The spatial distribution of released exudates strongly depends on root morphology because organic substances are released along the root axis (mainly at the root tips, elongation zones, and root hairs) (Kaiser et al., 2015; Peterson & Farquhar, 1996).

The extent to which these specific root traits are linked to nutrient mobilization (via extracellular enzyme activity) and rhizosphere microbial communities in top- and in subsoils in order to guarantee an intercropping success remains incompletely understood. Recently, field-based data revealed significant differences in metabolite and microbiome profiles between the rhizosphere soils of maize monoculture and intercropping. In particular, intercropping soils exhibited higher microbial diversity and metabolite chemodiversity (Jiang et al., 2024). However, it is still unclear which maize genotypes most effectively promote microbial diversity and nutrient mobilization in maize–faba bean intercropping systems. We therefore investigated six maize genotypes intercropped with faba bean under controlled conditions, focusing on differences in root traits, enzyme activities, and microbial community composition in topsoil and subsoil. Using i) soil zymography to detect the spatio-temporal distribution of enzyme activities, ii) enzyme kinetics to quantify potential activity at identified hotspots, and iii) amplicon sequencing to characterize bacterial and fungal communities, we examined genotype-specific rhizosphere processes. In addition, AMF colonization was quantified microscopically. We hypothesized that maize genotypes with deeper rooting systems and denser lateral roots would foster greater microbial abundance and diversity,

thereby enhancing the potential for nutrient mobilization in maize–faba bean intercropping. Furthermore, we expected that genotypes with higher root-associated enzyme activities and stronger AMF colonization would enrich beneficial microbial taxa involved in nitrogen and phosphorus cycling, leading to distinct rhizosphere community structures.

To our knowledge, this is one of the few studies using large rhizotrons (60 cm depth) to simultaneously monitor genotype-specific root traits and their biochemical and microbial impacts in intercropped maize–faba bean systems (Supplementary Figure S1).

Materials and methods

1.1 Maize genotype selection and key traits

This study examined one faba bean cultivar and six maize inbred lines, selected for their distinct root traits, including lateral root density, taproot depth, and root exudation patterns. The maize genotypes B73 (WT) and BX1B73 (Hamilton, 1964), were obtained from the University of Bern, while MBS847, A347, FC1571, and LH74 were provided by the University of Göttingen.

The key traits of each maize genotype are summarized below : The distinct root traits of the six maize genotypes are illustrated in Supplementary Figure S1.

- B73(WT): A wild-type maize genotype with a fibrous root system and extensive root hairs (Vetterlein et al., 2022). It efficiently accesses both topsoil and subsoil nutrients and is known for rhizosphere acidification and high acid phosphatase activity, which aid in phosphorus mobilization (Hochholdinger et al., 2018; Lippold et al., 2021).
- Bx1B73: A genetically modified derivative of the B73 genotype with reduced benzoxazinoid (BX) exudation (Hamilton, 1964), affecting microbial interactions, stress tolerance, herbivore resistance and nutrient cycling (Ahmad et al., 2011; Bilyera et al., 2021; Maag et al., 2016). BX1B73 exhibited all major root types, seminal, nodal, lateral, and crown roots (own observations).
- MBS847: An early-flowering genotype commonly used in studies on flowering time and grain yield (Durand et al., 2010; Huang et al., 2010). It develops deep taproots and a high density of root hairs (own observation).
- A347: A drought-tolerant genotype from the A321 subpopulation, known for deep taproots and moderate lateral root density (Schaefer & Bernardo, 2013).

- FC1571: A cold-tolerant European genotype with early vigour and a dense root system, making it well-suited for nutrient uptake in colder climates (Revilla et al., 2014). It develops a deep taproot system and dense root hairs (own observations).
- LH74: A forage maize genotype valued for its high stover yield and digestibility, making it a potential genetic resource for silage production (Wolf et al., 1993). It possesses several long lateral roots (own observation).

The faba bean cultivar BIRGIT used in this study is widely grown in Europe.

1.2 Soil sampling and basic soil properties

The soil used in this study was collected in September 2022 from the experimental farm at Hohenschulen of the Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts University in Schleswig-Holstein, Germany (54°19'05"N, 9°58'38"E). The soil is classified as Cambic Luvisol, with a sandy loam texture, consisting of 44% sand, 35% silt, and 21% clay. Soil pH was measured using the CaCl₂ method, revealing slightly acidic values of 6.7 for topsoil and 6.8 for subsoil. Soil was sieved to 2 mm to remove debris before rhizotron use. In order to mimic field soil conditions in the rhizotrons, samples were taken from 0–30 cm and 30–60 cm depths.

1.3 Rhizotron setup in controlled greenhouse

A rhizotron experiment was conducted in a controlled greenhouse environment from March 10, 2023, to June 28, 2023. Custom-designed rhizotrons, measuring 60 cm in height, 30 cm in width, and 8 cm in thickness, were used for plant growth. Each rhizotron was filled with 20 kg of sieved soil, with 10 kg of subsoil at the base and 10 kg of topsoil at the top. To maintain consistency, the soil was packed in layers to achieve a bulk density of 1.4 g/cm³. Soil moisture was kept at 70% of its water-holding capacity throughout the experiment.

To prevent external light exposure, and the development of cyanobacteria, the transparent observation side of each rhizotron was covered with a 50-μm aluminium-coated foil for the duration of the experiment. The foil was temporarily removed only during experimental procedures or brief root observations, and was immediately replaced afterwards to maintain darkness. The setup was positioned at a 45° angle to encourage root growth along the the transparent side and enabling accessible, non-destructive root observation and sampling when required.

Maize seeds were germinated on sterilized petri dishes lined with moistened filter paper and kept in darkness at 21°C for 72 hours. Once germinated, one seed was planted in each rhizotron. Greenhouse conditions were maintained at a temperature of 20–24°C, with an artificial light intensity ranging from 500 to 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a light-dark cycle of 16 hours light and 8 hours dark.

Faba bean seeds were soaked in tap water for three hours prior to planting and sown into the rhizotrons when maize seedlings reached the three-leaf stage, minimizing early interspecies competition. Each faba bean seed was placed 15 cm away from the maize plant to ensure adequate spacing and reduce direct competition for resources such as nutrients, water, and light.

To minimize positional bias due to uneven greenhouse conditions, rhizotrons were randomly arranged on racks and rotated weekly to ensure uniform exposure to environmental conditions. Plants were irrigated every two days with 250 mL of tap water.

Each maize genotype–faba bean combination was grown in four independent rhizotrons ($n = 4$), providing biological replicates for all plant and soil measurements except microbial community sequencing. For DNA-based microbial community analysis, equal amounts of rhizosphere soil from all four replicates per treatment and compartment were pooled prior to DNA extraction, resulting in a single composite DNA sample for sequencing. Consequently, no biological replication was available for sequencing-based community analyses.

Bacterial and fungal amplicon sequencing was performed by Novogene using 16S and universal ITS primers. As standard ITS primers do not efficiently amplify arbuscular mycorrhizal fungi (AMF; Glomeromycota), AMF were only detected in trace abundance or not at all in the sequencing data. To address this limitation, AMF colonization was quantified separately by root staining and microscopy. The rhizotron setup and genotype-specific root architectures are illustrated in Supplementary Figure S1.

1.4 Spatial imaging and rhizosphere sampling (BBCH 15 and 20)

To monitor root-zone processes over time, spatial imaging and rhizosphere sampling were conducted non-destructively at two maize growth stages: The first sampling took place at BBCH 15–16, corresponding to the 5–6 leaf stage, and the second at BBCH 19–20, which corresponds to the 9–10 leaf stage. The spatial arrangement of sampling areas within the rhizotrons (Figure 1) highlighted regions designated for enzyme activity analysis using soil

zymography membranes and rhizosphere soil sampling across two distinct soil layers (topsoil: 0–30 cm; subsoil: 30–60 cm).

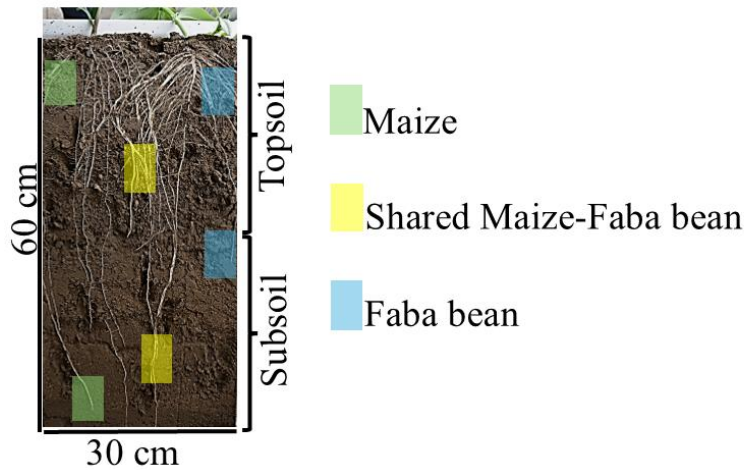


Figure 1: Schematic illustration of the root sampling zones within the rhizotron experimental setup. Colored regions indicate specific sampling areas for placing zymography membranes, and collecting rhizosphere soil at two soil depths: topsoil (0–30 cm) and subsoil (30–60 cm).

1.4.1 Soil zymography

Zymography was conducted twice, at the 5–6 leaf stage (BBCH 15–16) and again at the 9–10 leaf stage (BBCH 19–20), to monitor spatio-temporal pattern of enzyme activity. Polyamide membranes (0.45 μm , Shanghai Xingya, China) of 7×10 cm were pre-saturated with substrate solutions for enzyme detection. For acid phosphatase (AP), 4-methylumbelliferyl phosphate (MUF-P, Sigma-Aldrich) was dissolved in 0.05 M MES buffer (pH 6.5) with 2% (v/v) DMSO at a final concentration of 10 mM. For leucine aminopeptidase (LAP), L-leucine-7-amido-4-methylcoumarin (AMC, Sigma-Aldrich) was dissolved in 0.05 M Trizma buffer (pH 7.4) at a final concentration of 10 mM.

Each membrane was incubated with 3 ml of substrate solution for 1 hour at the root–soil interface in the rhizotron, shielded from light by aluminum foil and a weighted cover. After incubation, membranes were removed, briefly washed, and imaged immediately. All images were captured using a Canon EOS 6D digital camera with a Canon EF 24–105 mm 1:4L IS II USM lens, under UV illumination (excitation 365 nm, emission 450 nm). Camera settings were standardized (ISO 800, f/2.8, 1/10 s) for all images.

For quantification, 4 cm^2 pieces of polyamide membrane were soaked in a series of MUF and AMC solutions at concentrations of 0, 0.2, 0.5, 1, 2, 4, 6, 8, and 10 mM for MUF, and 0, 0.1,

0.2, 1, 2, 4, and 5 mM for AMC. These calibration membranes were imaged under identical conditions. Standard curves were used to relate grey value intensity to enzyme activity (expressed as nmol MUF or AMC released $\text{cm}^{-2} \text{h}^{-1}$). Images were analyzed with ImageJ software (Wayne Rasband, 1997), using background subtraction and thresholding following Ma et al. (2017), Tegtmeier et al. (2021), and Loeppmann et al. (2025). Signal intensities were averaged for each membrane area; mean $\pm$ 2SD was used to identify enzyme activity hotspots.

For the distance–activity profiles (Figures 2–3), enzyme activities were quantified from six selected root tips per treatment, expressed as $\text{pmol mm}^{-2} \text{h}^{-1}$ (converted from $\text{nmol cm}^{-2} \text{h}^{-1}$ by $\times 100$). This approach provides mean activity gradients with distance from the root. In contrast, the spatial distribution maps (Figures 4, S4, S5) display the full image in the original $\text{nmol cm}^{-2} \text{h}^{-1}$ scale, including all hotspots across the rhizosphere. Consequently, maxima in the maps may appear higher than the averaged profiles, as they capture localized heterogeneity beyond the selected root tips.

1.4.2 Rhizosphere soil sampling (BBCH 15–20, non- destructive)

Rhizosphere soil was gently collected from root surfaces during both imaging stages (BBCH 15–16 and BBCH 19–20) to monitor microbial and biochemical changes. These samples were stored for subsequent enzyme kinetics analysis (only BBCH 19–20 samples) and microbial (dsDNA) analysis.

1.5 Destructive sampling at BBCH 30

At BBCH 30 (stem elongation stage, typically ~ 10 – 12 leaves), plants were destructively harvested. Shoots were cut at the base, and roots were extracted carefully. During this final sampling, three types of samples were collected from each rhizotron. First, rhizosphere soil was collected as the third and final soil sample for biochemical and microbial analyses. Second, root tips were harvested specifically for the analysis of arbuscular mycorrhizal fungal (AMF) colonization. Third, root and associated soil material were sampled for subsequent dsDNA extraction and sequencing. Figure 1 shows sampling zones used throughout the experiment, including zymography locations across topsoil (0–30 cm) and subsoil (30–60 cm).

1.6 Analysis on sampled material

1.6.1 Enzyme kinetics

Enzyme kinetics were analyzed using rhizosphere soil collected during the BBCH 19–20 stage of maize growth. Rhizosphere soil suspensions were prepared by adding 1 g of fresh soil to 50 mL sterile deionized water and shaking at 120 rpm for 25 minutes. The suspensions were then

used immediately for enzyme assays. Assays followed modified protocols of German et al. (2011) and (Loeppmann et al., 2016). Each assay was carried out in black 96-well microplates with a final volume of 200 μ L per well, consisting of 50 μ L soil suspension, 50 μ L buffer, and 100 μ L substrate solution. For LAP, assays were performed in 0.05 M Trizma buffer (pH 7.4) with L-Leucine-7-amido-4-methylcoumarin (AMC; Sigma-Aldrich) as substrate. For AP, assays were performed in 0.05 M MES buffer (pH 6.5) with 2 % (v/v) DMSO using 4-methylumbelliferyl phosphate (MUF-P; Sigma-Aldrich). Stock solutions (10 mM) were prepared in sterile tubes and stored at -20°C (stable for 6 months). Working solutions (1 mM) were freshly prepared by diluting stock 1:10 in the respective buffer (stable for 6 weeks at 4°C). Eight substrate concentrations (0, 20, 40, 60, 80, 100, 200, 400 μ M) were applied. Fluorescence intensity was measured at excitation/emission wavelengths of 355/460 nm for LAP and 365/450 nm for AP using a CLARIOstar Plus microplate reader (BMG LABTECH, Germany). Plates were incubated at 25°C , and fluorescence was recorded at 0-, 30-, 60-, and 120-min. Calibration curves were prepared with AMC and MUF standards (0–400 μ M) in the respective buffers. Enzyme kinetic parameters (V_{max} and K_{m}) were estimated using nonlinear regression to the Michaelis–Menten model (Michaelis & Menten, 1913), following German et al. (2011). Rates were derived from the linear phase of the reaction.

1.6.2 Soil pH (Bulk method, pre-harvest)

Soil pH was measured prior to destructive harvest at two depths (0–30 cm and 30–60 cm) following Minasny et al. (2011), using a 1:2.5 (w/v) soil-to-0.01 M CaCl_2 slurry. These bulk pH values served as baseline indicators of soil acidity in the broader topsoil and subsoil, outside the immediate influence of the rhizosphere.

1.6.3 Staining arbuscular-mycorrhizal fungal colonization in root

The terminal 4–5 cm of root tips at BBCH 30 were cleared in 10% potassium hydroxide (KOH) at 95°C for 5 minutes, then stained with 5% Pelikan (Pelikan Holding, Hannover, Germany) blue ink-vinegar solution. Stained roots were mounted on slides for microscopic evaluation. Arbuscular mycorrhizal fungi (AMF) colonization was quantified using the gridline intersection method (McGonigle et al., 1990), analyzing 100 root intersections per sample for the presence of AM structures (hyphae, vesicles, and arbuscules), as well as non-AM fungal structures.

1.6.4 Soil DNA extraction and analysis

Genomic DNA was extracted separately from roots and rhizosphere soil samples at BBCH 30 using specialized extraction kits. Root samples were processed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), optimized for extracting high-quality DNA from plant tissues. For soil samples, microbial DNA was isolated with the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), specifically chosen due to its efficiency in recovering DNA from complex soil matrices.

DNA quantity and purity were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) with NanoDrop software version 3.7.1. DNA purity was assessed by the absorbance ratio (A_{260}/A_{280}), with values of 1.8–2.0 considered suitable for downstream analyses.

Library preparation and amplicon sequencing were performed commercially by Novogene (Munich, Germany) using the Illumina NovaSeq 6000 sequencing platform. For bacterial community profiling, the V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified and sequenced. For fungal communities, the internal transcribed spacer (ITS) regions (ITS1 and ITS2) were targeted. Sequence data were quality-filtered, denoised, and processed using the DADA2 algorithm implemented in QIIME2, resulting in amplicon sequence variants (ASVs) for downstream analysis and taxonomic classification.

1.7 Statistical analysis

All statistical analyses were performed using R version 4.3.1 (R Core Team, 2024). Prior to ANOVA, data normality was tested using the Shapiro-Wilk test, and homogeneity of variances was verified with Levene's test to ensure assumptions were met. Subsequently, one-way ANOVA was applied to evaluate differences among treatments and maize genotypes, followed by Tukey's Honest Significant Difference (HSD) post-hoc test for multiple pairwise comparisons (significance level $p < 0.05$). For enzyme kinetics data, nonlinear regression was performed according to the Michaelis-Menten equation, with kinetic parameters ($V_{\max}$ and k_m) estimated using the *nls* function in R. The Michaelis-Menten model was selected to characterize enzyme-substrate interactions accurately, as per established enzyme kinetics protocols. The Chao1 was used to estimate richness by adding a correction factor to the observed number of species (For reviews of these and other nonparametric estimators, see Colwell and Coddington Chazdon et al.) For instance, Chao1 estimates total species richness as

$$S_{\text{Chao1}} = S_{\text{obs}} + \frac{n_1^2}{2n_2}$$

where S_{obs} is the number of observed species, n_1 is the number of singletons (species captured once), and n_2 is the number of doubletons (species captured twice). this index is particularly useful for data sets skewed toward the low-abundance classes, as is likely to be the case with microbes. we use diversity measures for relative comparisons, we avoid the problem of not being able to measure bias.

All graphical representations, including boxplots, bar graphs, and enzyme kinetic curves (with fitted Michaelis-Menten curves overlaid on raw data points), were generated using the ggplot2 package in R.

2. Results

2.1 Zymography and enzyme kinetics of soil hotspots

In the maize rhizosphere at BBCH15 (Figure 2A, B), LAP activity declined with increasing distance from the root center. In the topsoil (A), genotype effects were significant at all distances (0 mm: $p < 0.0001$; 0.5 mm: $p = 0.004$; 1.0 mm: $p = 0.022$; last point: $p < 0.001$). At the root surface (0 mm), B73WT showed the highest activity ($\sim 550 \text{ pmol mm}^{-2} \text{ h}^{-1}$), significantly greater than LH74 ($\sim 350 \text{ pmol mm}^{-2} \text{ h}^{-1}$), a difference of $\sim 40\%$. FC1571, BX1B73, and A347 were intermediate, while MBS847 grouped with B73WT. At 0.5 mm, B73WT remained $\sim 15\text{--}20\%$ higher than LH74. At 1.0 mm and the last point, B73WT and FC1571 exceeded LH74, whereas BX1B73 and A347 were intermediate. In the subsoil (B), no significant genotype effects were detected (all $p > 0.05$). Activities across all genotypes declined similarly from $\sim 500 \text{ pmol mm}^{-2} \text{ h}^{-1}$ at the root surface to $\sim 250 \text{ pmol mm}^{-2} \text{ h}^{-1}$ at > 1.5 mm. In the shared maize–bean rhizosphere (Figure 2C, D), no significant differences were observed in the topsoil (C) at any distance (all $p > 0.05$). Activities started near $\sim 500 \text{ pmol mm}^{-2} \text{ h}^{-1}$ at the root and decreased to $\sim 300 \text{ pmol mm}^{-2} \text{ h}^{-1}$ at 2 mm. In the subsoil (D), genotype effects were significant at 1.0 mm ($p = 0.006$). At this distance, FC1571 and A347 showed higher activity ($\sim 420\text{--}440 \text{ pmol mm}^{-2} \text{ h}^{-1}$) than BX1B73 ($\sim 300 \text{ pmol mm}^{-2} \text{ h}^{-1}$), a difference of $\sim 30\%$. No significant differences were found at other distances. In the bean rhizosphere (Figure 2E, F), significant genotype effects occurred in both topsoil and subsoil. In the topsoil (E), effects were significant at 0.5 mm ($p = 0.004$) and 1.0 mm ($p < 0.001$). At 0.5 mm, beans grown with FC1571 and B73WT had higher activity ($\sim 500 \text{ pmol mm}^{-2} \text{ h}^{-1}$) than those with

BX1B73 ($\sim 400 \text{ pmol mm}^{-2} \text{ h}^{-1}$). At 1.0 mm, beans associated with FC1571 and B73WT remained significantly higher ($\sim 25\%$) than those with BX1B73. In the subsoil (F), significant differences were found at 1.0 mm ($p = 0.001$). Beans grown with FC1571, B73WT, and LH74 showed higher activity ($\sim 400\text{--}420 \text{ pmol mm}^{-2} \text{ h}^{-1}$) than those grown with BX1B73 ($\sim 280 \text{ pmol mm}^{-2} \text{ h}^{-1}$), a difference of $\sim 30\%$. Other distances showed no significant differences. A second zymography performed at BBCH19 revealed broadly similar LAP activity patterns across genotypes and compartments, with no additional significant effects (Supplementary Figure S2).

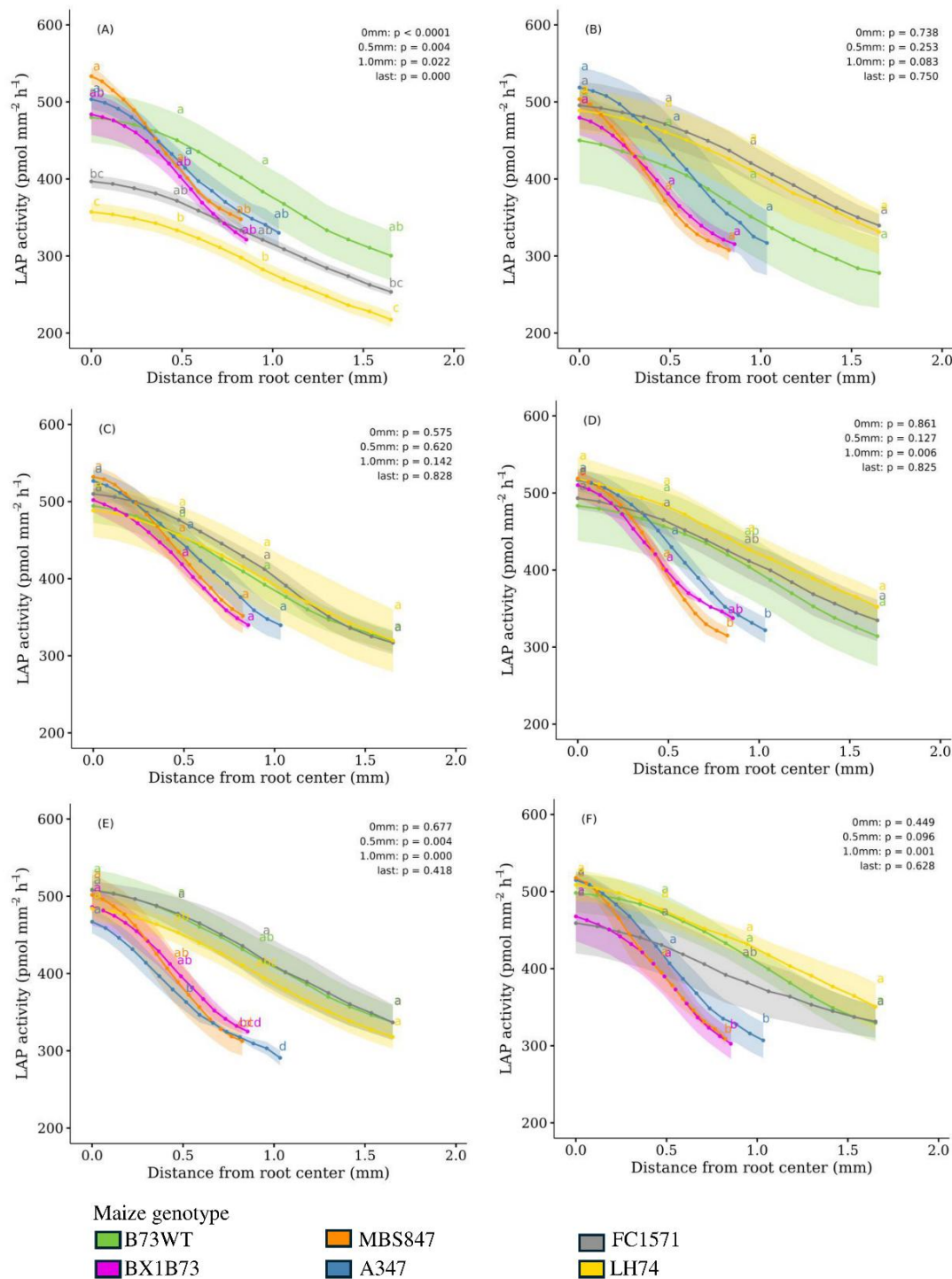


Figure 2. Leucine aminopeptidase (LAP) activity in rhizosphere samples at maize growth stage BBCH15. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean rhizosphere. Lines represent mean LAP activity with shaded areas showing standard error ($n = 4$). Different letters indicate significant differences among maize genotypes at the same distance (Tukey's HSD, $p < 0.05$). P-values from ANOVA are reported for 0, 0.5, and 1.0 mm distances, and the last measurement point.

Acid phosphatase (AP) activity (Figure 3) declined with distance from the root center. In the maize rhizosphere (A), genotype effects were significant at 0.5 mm ($p = 0.002$) and 1.0 mm ($p < 0.001$), but not at 0 mm or the last point. At 0.5 mm, FC1571, LH74, and B73WT formed the upper group (a), BX1B73 and A347 were lower (b), and MBS847 was lowest (c). At 1.0 mm, FC1571, LH74, and B73WT remained highest (a), MBS847 was lower (b), BX1B73 intermediate (bc), and A347 lowest (c). In the subsoil (B), genotype effects were again significant at 0.5 and 1.0 mm (both $p < 0.001$), but not at 0 mm or the last point. At 0.5 mm, FC1571, LH74 ($\pm$ B73WT) grouped higher (a), BX1B73 and A347 were lower (b), and MBS847 was lowest (c). At 1.0 mm, FC1571 and LH74 ($\pm$ B73WT) remained higher (a), while BX1B73, A347, and MBS847 grouped lower (b). In the shared maize–bean rhizosphere (C, D), genotype effects were significant at 0.5 and 1.0 mm in both depths (all $p < 0.001$). At 0.5 mm in the topsoil (C), LH74, FC1571, and B73WT were higher (a), A347 and MBS847 were lower (b), and BX1B73 lowest (c). At 1.0 mm, the same pattern persisted. In the subsoil (D), LH74 and FC1571 ($\pm$ B73WT) were higher (a/ab), A347 and BX1B73 were lower (b), and MBS847 lowest (c). At 1.0 mm, LH74 and FC1571 ($\pm$ B73WT) grouped higher (a), while A347, BX1B73, and MBS847 were lower (b). In the bean rhizosphere (E, F), genotype effects were again significant at 0.5 and 1.0 mm (both $p < 0.001$), but not at 0 mm or the last point. In the topsoil (E), FC1571 and B73WT were higher (a), LH74 was intermediate (ab), BX1B73 and A347 were lower (b), and MBS847 lowest (c). At 1.0 mm, FC1571, LH74, and B73WT were highest (a), BX1B73 was lower (b), MBS847 lower again (c), and A347 lowest (d). In the subsoil (F), FC1571, LH74, and B73WT grouped higher (a), while BX1B73, A347, and MBS847 were lower (b). A second zymography at BBCH19 revealed no consistent genotype effects on AP activity across compartments (Supplementary Figure S3).

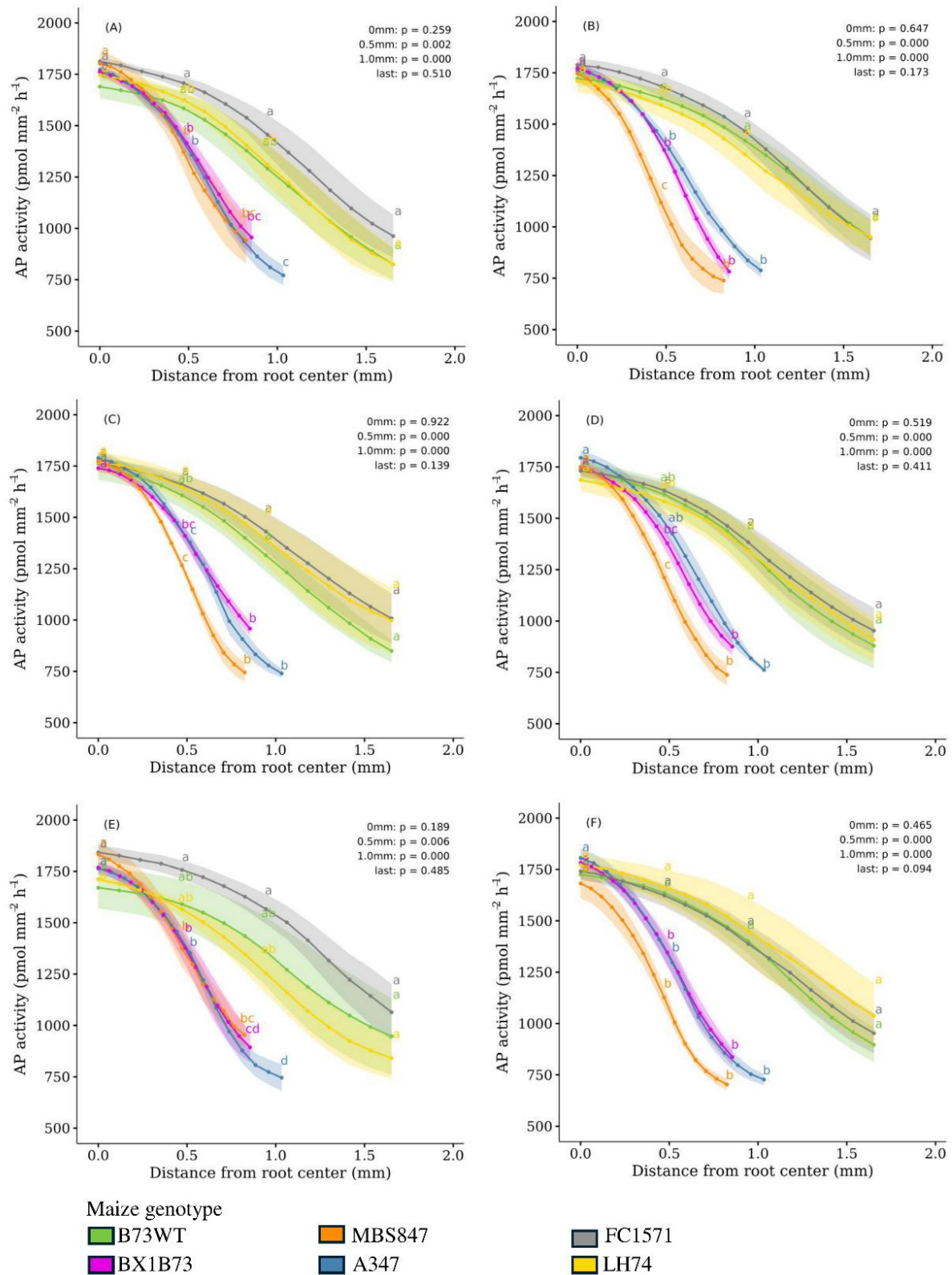


Figure 3. Acid phosphatase (AP) activity at maize growth stage BBCH15. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize-bean rhizosphere, and (E, F) bean rhizosphere. Lines represent mean AP activity with shaded areas showing standard error (n = 4). Different letters indicate significant differences among maize genotypes at the same distance (Tukey's HSD, $p < 0.05$). P-values from ANOVA are reported for 0, 0.5, and 1.0 mm distances, and the last measurement point.

Zymography maps for maize genotype MBS847 intercropped with faba bean confirmed that AP activity was closely associated with root surfaces across all rhizosphere compartments (Figure 4). In the maize rhizosphere of MBS847, activity was concentrated along primary and lateral root axes, forming continuous hotspots in the topsoil (A) and more localized zones in the subsoil (D). In the shared maize–bean rhizosphere adjacent to MBS847 (B, E), AP activity was similarly root-associated but extended further into the surrounding soil matrix in the topsoil. Bean rhizospheres from the same rhizotron (C, F) displayed a more patchy distribution of AP activity, with only small, localized hotspots in the subsoil. These spatial maps illustrate the heterogeneous distribution of AP activity and complement the averaged distance–activity profiles (Figure 3), which summarize gradients across all six maize genotypes.

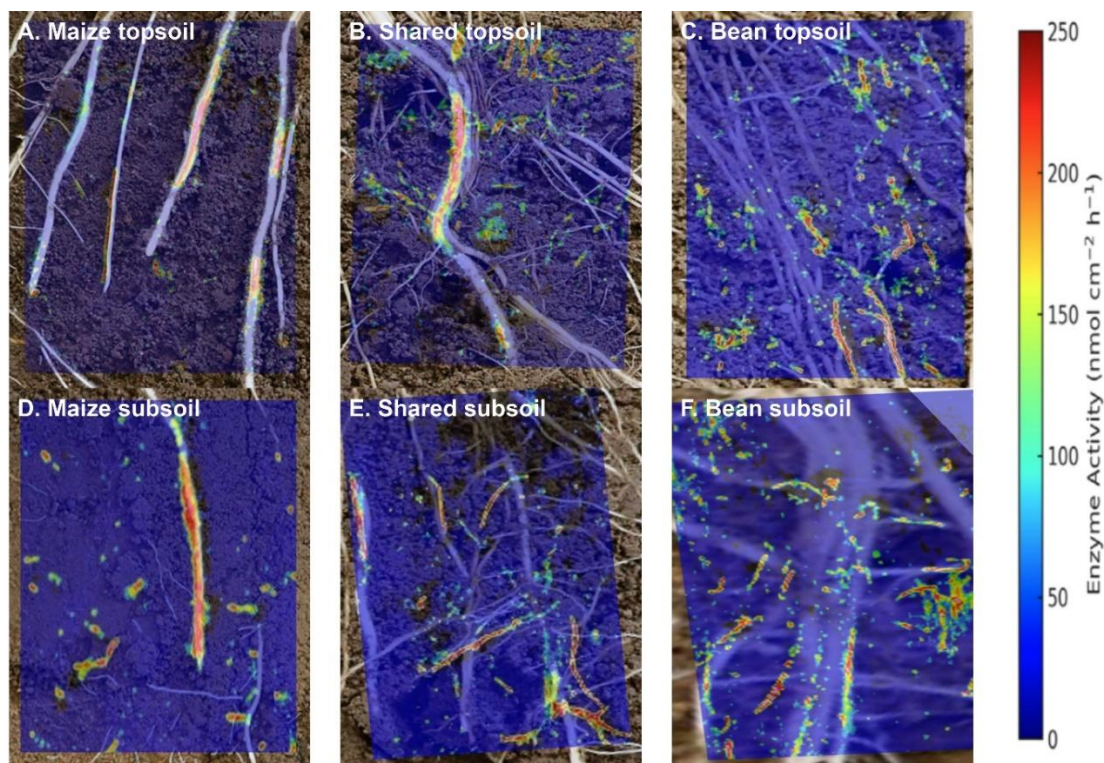


Figure 4. Spatial distribution of acid phosphatase (AP) activity at maize growth stage BBCH15 for maize genotype MBS847 intercropped with faba bean. Panels (A, D) show the MBS847 maize rhizosphere, (B, E) the shared maize–bean rhizosphere adjacent to MBS847, and (C, F) the bean rhizosphere from the same rhizotron, with topsoil (0–30 cm) in A–C and subsoil (30–60 cm) in D–F. Hotspots of enzyme activity are visualized by zymography, with color intensity indicating activity ($\text{nmol cm}^{-2} \text{ h}^{-1}$). The images shown are representative of four biological replicates and illustrate the spatial heterogeneity of AP activity along root axes and in the surrounding soil.

One-way ANOVA revealed genotype effects on LAP V_{max} at BBCH19 (Figure 5). In the maize rhizosphere, genotype had a marginal effect in the topsoil ($F(5,18) = 2.54$, $p = 0.066$) but a significant effect in the subsoil ($p = 0.018$), where LH74 showed higher activity than B73WT. In the shared maize–bean rhizosphere, genotype significantly affected LAP V_{max} in

both topsoil ($p < 0.001$) and subsoil ($p = 0.001$). In the topsoil, MBS847 and LH74 had higher activity than B73WT and BX1B73, with A347 and FC1571 intermediate. In the subsoil, BX1B73 exhibited the highest activity, while other genotypes grouped lower. In the bean rhizosphere, genotype effects were significant in the topsoil ($p < 0.001$), where FC1571 and A347 showed higher activity than B73WT, BX1B73, and LH74, and MBS847 was lowest. In contrast, no significant genotype effects were detected in the bean subsoil ($F(5,18) = 0.79$, $p = 0.568$). Pairwise comparisons are shown in Supplementary Figure S4.

One-way ANOVA revealed clear genotype effects on AP Vmax at BBCH19 (Figure 6). In the maize rhizosphere, genotype significantly affected activity in both topsoil ($F(5,18) = 4.28$, $p = 0.0097$) and subsoil ($F(5,18) = 7.45$, $p = 0.0006$). In the shared maize–bean rhizosphere, genotype effects were highly significant in topsoil ($F(5,18) = 7.62$, $p = 0.0005$) and subsoil ($F(5,18) = 14.29$, $p < 0.0001$). In the bean rhizosphere, genotype effects were not significant in topsoil ($F(5,18) = 1.93$, $p = 0.140$), but were highly significant in subsoil ($F(5,18) = 13.28$, $p < 0.0001$). Pairwise comparisons among genotypes are presented in Supplementary Figure S5.

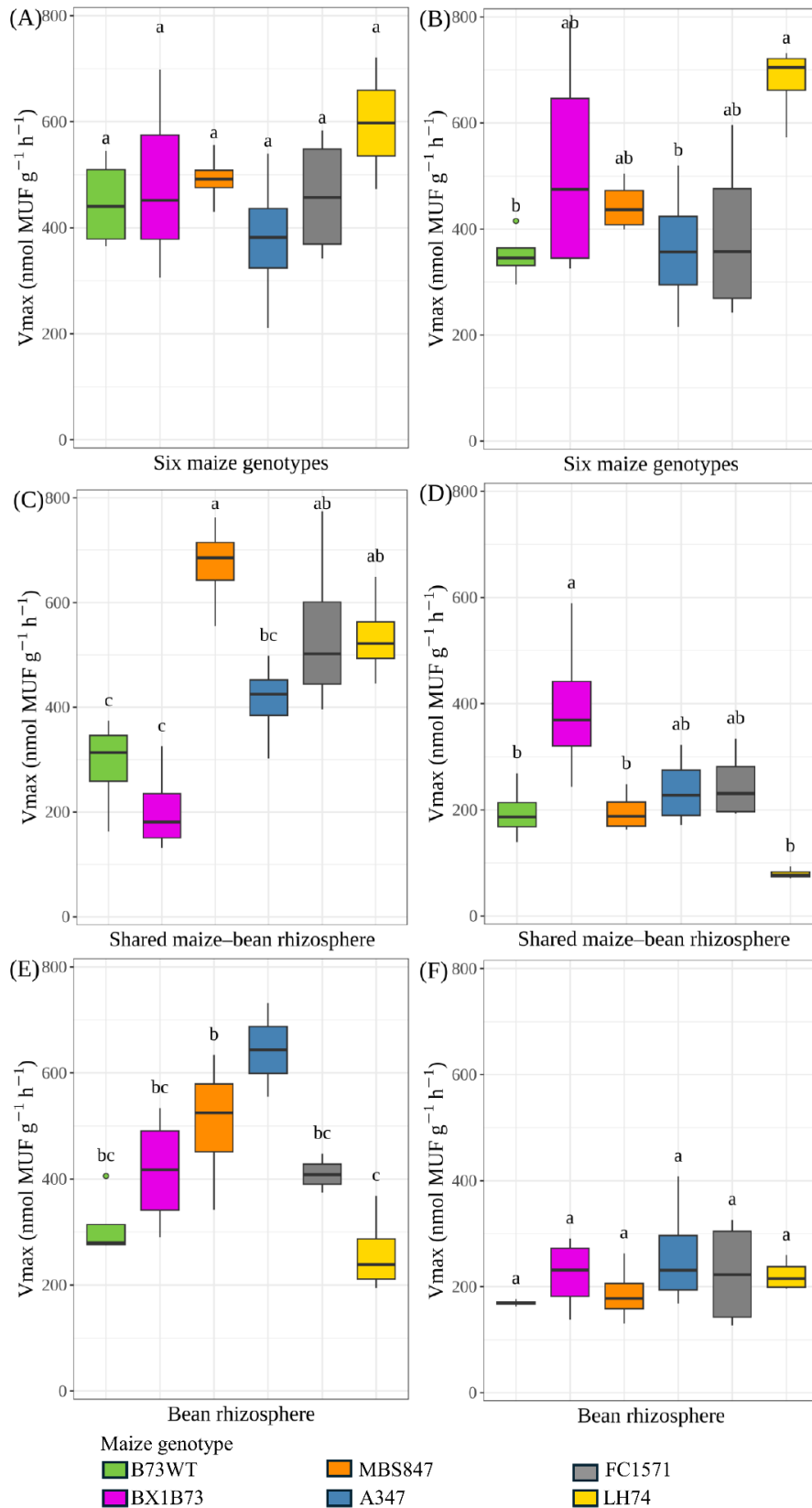


Figure 5. Maximum potential activity (V_{max}) of leucine aminopeptidase (LAP) in rhizosphere samples at maize growth stage BBCH19. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean rhizosphere. Different letters above boxplots indicate significant differences among maize genotypes according to Tukey's HSD test ($p < 0.05$). Boxplots represent four biological replicates ($n = 4$).

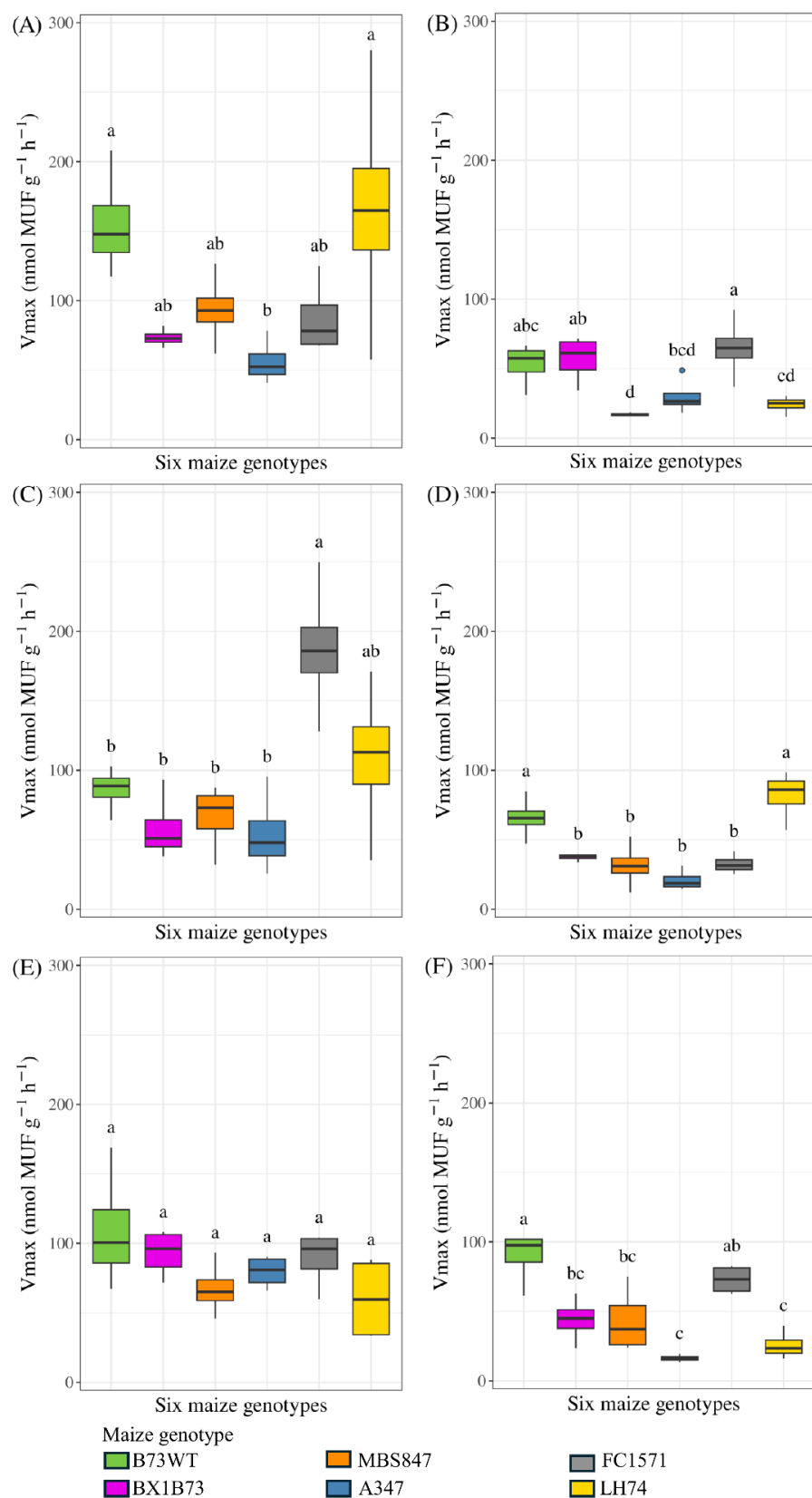


Figure 6. Maximum potential activity (V_{max}) of acid phosphatase (AP) in rhizosphere samples at maize growth stage BBCH19. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean rhizosphere. Different letters above boxplots indicate significant differences among maize genotypes according to Tukey's HSD test ($p < 0.05$). Boxplots represent four biological replicates ($n = 4$).

2.2 Arbuscular mycorrhizal fungal (AMF) colonization in maize roots and rhizosphere microbial community composition

Arbuscular mycorrhizal fungi (AMF) colonization rates at BBCH 30 varied significantly among the six maize genotypes (Figure 7). Mean colonization percentages ranged from 46% (B73WT) and 46.75% (BX1B73) up to 84.75% in FC1571. Intermediate colonization was observed in A347 (67.5%) and LH74 (73.25%). MBS847 exhibited lowest colonization (45.75%). Statistical analysis (Tukey's HSD) grouped genotypes into three categories: FC1571 with the highest colonization, LH74 and A347 as intermediate, and BX1B73, MBS847, and B73WT as low ($p < 0.001$). Genotype FC1571 displayed the narrowest spread among high colonizers, while BX1B73 and B73WT showed consistently low values with limited variation. Regression analyses of colonization components (arbuscular, hyphal, vesicular, and overall colonization frequency) against shoot biomass (Supplementary Figure S6) showed weak associations. Only arbuscular colonization showed a significant relationship with shoot biomass ($R^2 = 0.19$, $p = 0.034$), while all other colonization were non-significant.

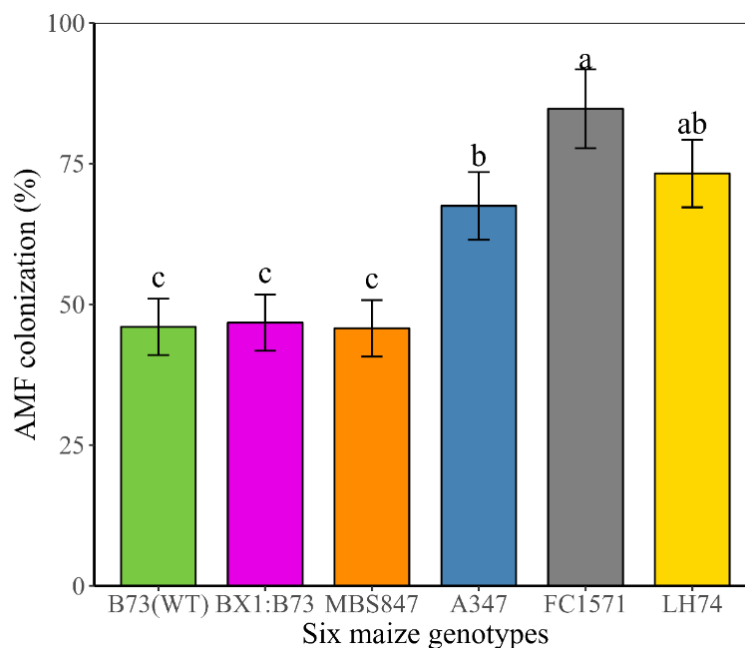


Figure 7. Arbuscular mycorrhizal fungi (AMF) root colonization (%) across six maize genotypes at BBCH 30 on the root tips, visualized using the ink-staining technique. Bars represent mean values $\pm$ standard deviation ($n = 4$). Lower-case letters indicate significant differences among genotypes based on Tukey's HSD test ($p < 0.001$).

Fungal community composition

The relative abundance of fungal taxa differed among maize genotypes and rhizosphere compartments (Figure 8). Across all samples, five dominant species were consistently detected: *Fusarium solani*, *Trichoderma gamsii*, *Fusarium venenatum*, *Talaromyces purpureogenus*, and *Penicillium polonicum*.

In the maize rhizosphere, *Fusarium solani* was the most abundant species in all genotypes, with highest levels in A347 and LH74. *Talaromyces purpureogenus* occurred at moderate abundance across most genotypes, whereas *Trichoderma gamsii* was comparatively lower, particularly in MBS847. *Fusarium venenatum* and *Penicillium polonicum* showed the lowest relative abundances. In the shared maize–bean rhizosphere, *Fusarium solani* remained dominant, with elevated values in LH74 and moderate abundance in MBS847 and FC1571. *Talaromyces purpureogenus* and *Trichoderma gamsii* were detected at low to moderate levels, while *Fusarium venenatum* and *Penicillium polonicum* consistently contributed the least.

In the bean rhizosphere, *Fusarium solani* again predominated, especially in LH74, while the remaining taxa occurred at lower relative abundances. Hierarchical clustering revealed grouping patterns in which *Fusarium venenatum* and *Penicillium polonicum* were consistently separated from the other fungal taxa.

Fungal alpha diversity

Alpha diversity metrics (Supplementary Figure S7) indicated moderate variation among maize genotypes and rhizosphere compartments. Chao1 richness was highest in B73WT and LH74 in the maize rhizosphere, while FC1571 showed lower richness. In the shared maize–bean rhizosphere, richness was reduced for most genotypes but remained elevated in LH74. Bean rhizospheres displayed intermediate richness values across genotypes. Shannon diversity indices ranged between ~3.5 and 5.0, with limited variation across compartments, reflecting relatively even fungal communities.

Bacterial community composition

Bacterial communities were also dominated by a small set of genera across all compartments (Figure 9). Five genera were consistently detected: *Pseudomonas*, *Rhizobium*, *Bacillus*, *Clostridium*, and Actinobacteria.

In the maize rhizosphere, *Pseudomonas* was most abundant in all genotypes, with enrichment in FC1571 and LH74. *Rhizobium* occurred at moderate levels, particularly in A347 and LH74. *Bacillus* and *Clostridium* were present at low to moderate levels, while Actinobacteria showed the lowest abundances across genotypes.

In the shared maize–bean rhizosphere, *Pseudomonas* remained dominant, especially in FC1571 and LH74. *Rhizobium* exhibited more variable abundance compared to the maize rhizosphere, whereas *Bacillus* and *Clostridium* remained low to moderate. Actinobacteria again represented the smallest proportion.

In the bean rhizosphere, *Pseudomonas* was again the most abundant, with highest values in FC1571 and LH74. *Rhizobium* occurred at higher levels compared to maize or shared rhizospheres, while *Bacillus*, *Clostridium*, and Actinobacteria remained minor contributors.

Bacterial alpha diversity

Alpha diversity indices of bacterial communities (Supplementary Figure S8) showed consistently higher richness and evenness compared to fungi. Chao1 richness exceeded 3000 OTUs in all compartments, with BX1B73 and LH74 generally highest. In the shared and bean rhizospheres, richness was slightly reduced compared to the maize rhizosphere but remained above 3000 for all genotypes. Shannon diversity indices ranged from ~9.5 to 10.5 and did not differ strongly among compartments or genotypes, indicating high evenness across bacterial taxa.

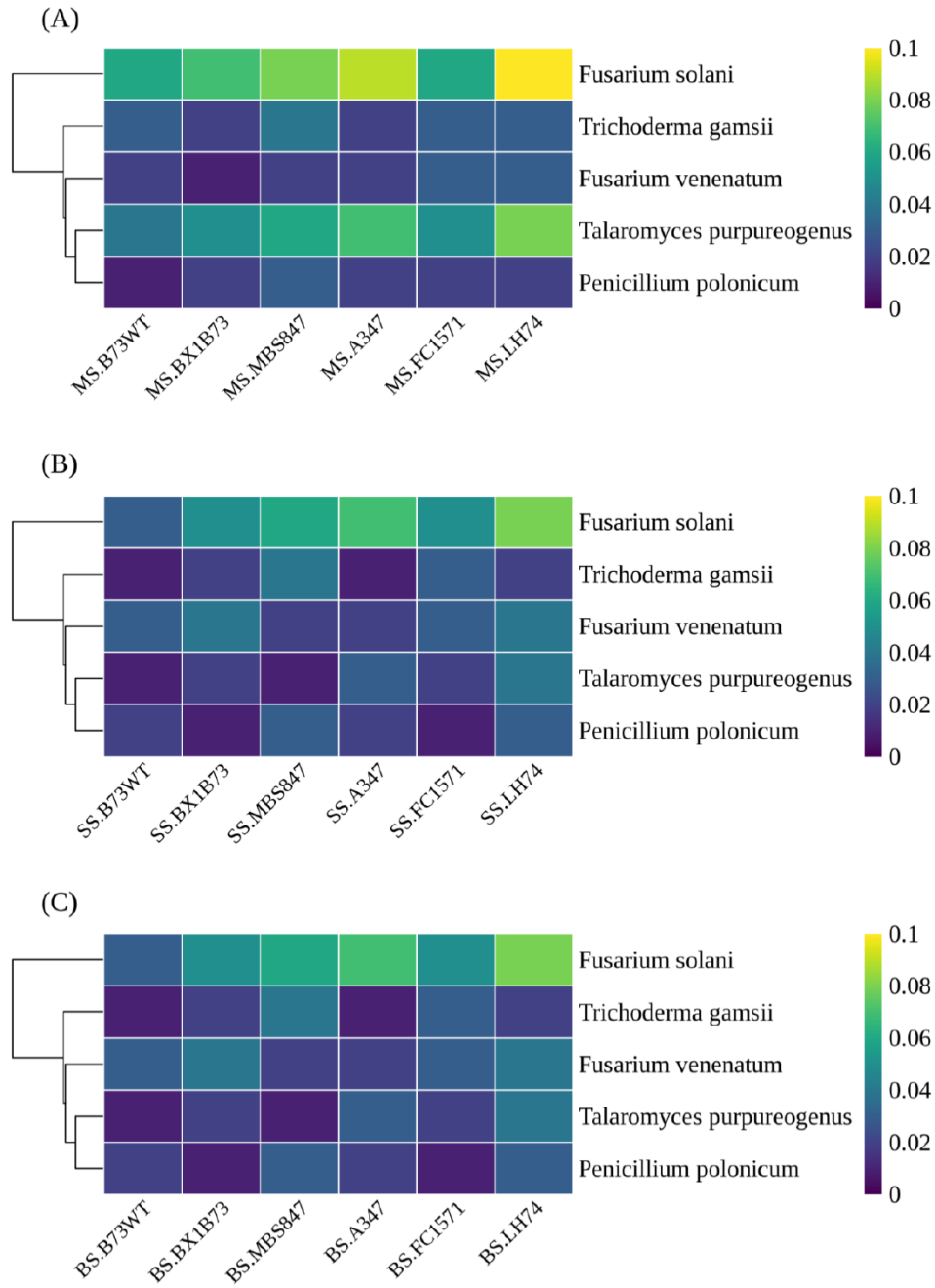


Figure 8. Heatmaps showing the relative abundance of fungal species in the (A) maize rhizosphere (MS), (B) shared maize–bean rhizosphere (SS), and (C) bean rhizosphere (BS) across six maize genotypes at the BBCH 30 growth stage. Abundance values are based on normalized sequence counts.

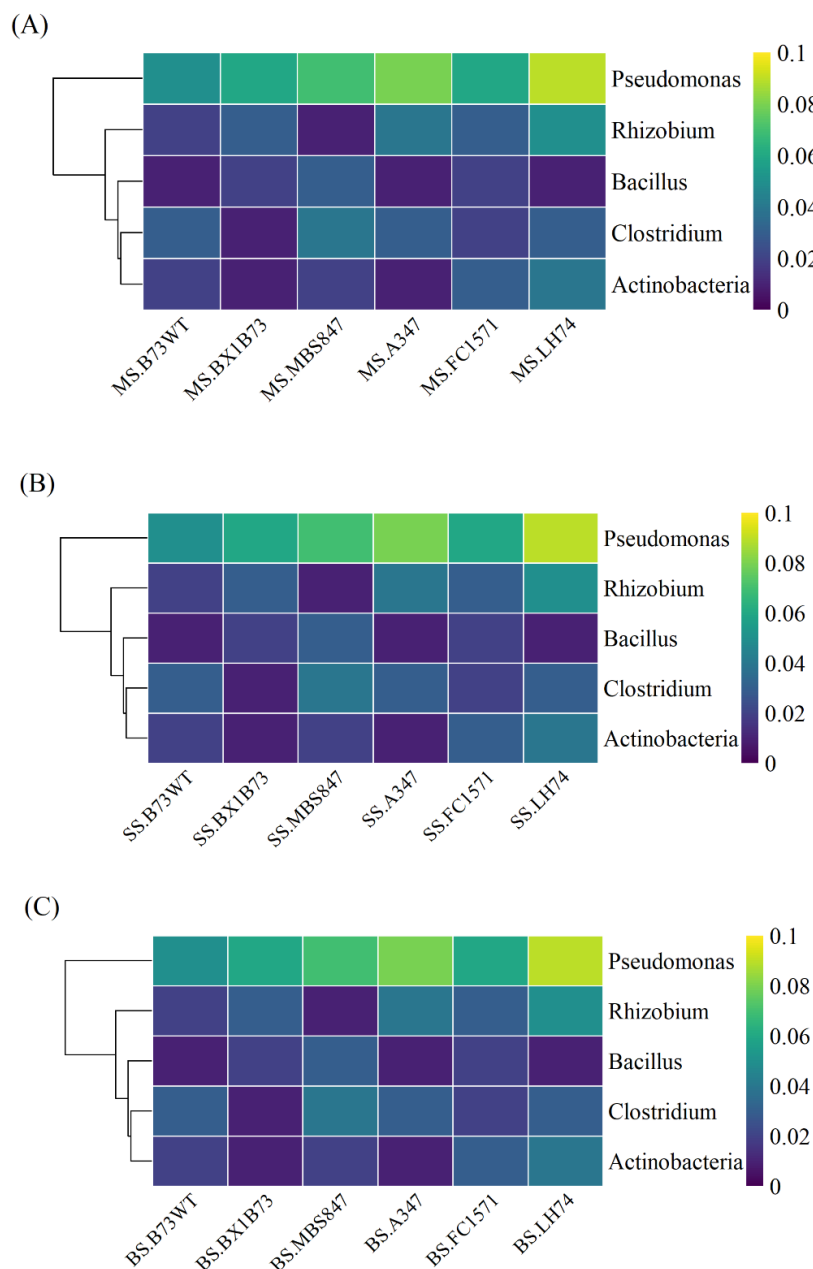


Figure 9. Heatmaps showing the relative abundance of bacterial genera in the (A) maize rhizosphere (MS), (B) shared maize-bean rhizosphere (SS), and (C) bean rhizosphere (BS) across six maize genotypes at the BBCH 30 growth stage. Abundance values are based on normalized sequence counts.

3. Discussion

3.1 Genotype-dependent rhizosphere enzyme activity

Our study demonstrates that maize genotypes strongly determine rhizosphere enzyme activity fields. Zymography at BBCH15 revealed clear genotype-specific gradients of leucine aminopeptidase (LAP) and acid phosphatase (AP) (Figures 2–3). In the maize rhizosphere, LAP was highest in B73WT and MBS847, with activities at the root surface up to ~40% greater than in LH74 (Figure 2A). AP activity showed even stronger separation, with FC1571 and

LH74 consistently ranking highest across maize, shared, and bean rhizospheres, while BX1B73, A347, and MBS847 remained low (Figure 3A–F). These patterns highlight how root exudation chemistry and architecture generate distinct enzyme activity fields that extend beyond the immediate root surface into the soil matrix, consistent with recent work on genotype-specific rhizosphere microgradients (Lanzavecchia et al., 2024).

3.2 Depth sensitivity and intercropping effects

Depth strongly modulated genotype expression. LAP genotype differences were pronounced in the topsoil but disappeared in the subsoil, suggesting that proteolysis is more dependent on resource availability and root density (Figure 2A vs 2B). By contrast, AP maintained robust genotype separation at 0.5–1.0 mm across depths and compartments (Figure 3), reflecting the stability of phosphomonoester hydrolysis even under subsoil constraints. Notably, genotype effects propagated across plant borders: in both shared and bean rhizospheres, enzymatic profiles reflected the identity of the neighboring maize genotype. For example, bean roots paired with FC1571 and B73WT showed ~25–30% higher LAP or AP activities than those paired with BX1B73 (Figures 2E–F, 3E–F). Such cross-partner imprinting demonstrates that maize genotypes not only structure their own rhizospheres but also influence their intercrop partner's microenvironment.

3.3 Functional links to AMF colonization

The enzymatic patterns correspond closely with arbuscular mycorrhizal fungal (AMF) colonization (Figure 7). FC1571 and LH74 exhibited the highest colonization (~85%), coinciding with strong AP activity, whereas BX1B73 and B73WT showed markedly lower colonization (~45–50%) and reduced phosphatase expression. These results suggest a functional axis linking root exudation, AMF recruitment, and phosphorus mobilization, consistent with evidence that AMF enhance P uptake by extending enzymatic activity beyond the depletion zone (S. Jiang et al., 2021; Smith & Smith, 2011). Conversely, BX1B73 demonstrated high LAP potential but weak AMF colonization, reflecting a trade-off between nitrogen- and phosphorus-related strategies. Such contrasts underline the importance of considering genotype-specific trait combinations in breeding for nutrient-efficient intercrops.

3.4 Microbial community assembly and diversity

Microbial sequencing confirmed that genotype and rhizosphere type shape community composition (Figures 8–9). Fungal communities were dominated by *Fusarium solani* across all compartments, with higher relative abundance in A347 and LH74, whereas *Talaromyces* and

Trichoderma occurred at moderate levels. Bacterial communities were consistently dominated by *Pseudomonas* and *Rhizobium*, with genotypes FC1571 and LH74 supporting the highest abundances of these taxa. Such associations mirror the high AP and AMF colonization observed in the same genotypes, suggesting that root traits filter beneficial microbial taxa. Diversity analyses (Figures S7–S8) further indicated that bacterial richness and evenness were high and relatively stable across compartments, while fungal diversity varied more strongly with genotype. These findings are in line with evidence that root exudates structure fungal communities more tightly than bacterial ones (Guo et al., 2017; Zhu et al., 2021).

3.5 Toward trait-informed intercropping design

Together, our results highlight clear genotype-specific strategies for nutrient mobilization in maize–faba bean intercropping. FC1571 and LH74 combined high AP activity, strong AMF colonization, and enrichment of beneficial microbes, positioning them as promising genotypes for phosphorus-limited systems. By contrast, BX1B73 excelled in LAP activity, suggesting strong proteolytic potential for nitrogen cycling but limited P acquisition capacity. Importantly, these genotype effects extended across intercrop partners, demonstrating that selecting a maize genotype with favorable traits can enhance nutrient cycling in both maize and bean rhizospheres. The use of large rhizotrons allowed us to capture these dynamics at both topsoil and subsoil depths, revealing that genotype-specific root traits translate into stable, depth-resolved impacts on rhizosphere functioning. Such trait-informed selection strategies could be instrumental for improving nutrient use efficiency and resilience in intercropping systems under future climate and resource constraints.

4 Conclusion

This study provides comprehensive insights into how maize–faba bean intercropping influences rhizosphere enzyme activity, AMF colonization, microbial diversity, and soil biochemical functioning. By integrating zymography, enzyme kinetics, AMF staining, and DNA-based microbial community analysis in a large rhizotron system with topsoil and subsoil compartments, we were able to resolve genotype-specific belowground interactions at a depth and spatial resolution rarely achieved in intercropping studies.

Our results demonstrated that leucine aminopeptidase (LAP) activity was consistently higher in maize rhizospheres, with genotypes FC1571 and BX1B73 showing particularly strong proteolytic activity, indicative of enhanced nitrogen mobilization potential. In contrast, acid phosphatase (AP) activity was most pronounced in bean rhizospheres and in maize genotypes

FC1571 and LH74, highlighting the central role of these genotypes and the legume partner in phosphorus mobilization. Intercropped zones exhibited enhanced enzymatic efficiency, as indicated by higher V_{max} values, pointing to synergistic effects of complementary root exudation and microbial co-stimulation.

AMF colonization further revealed clear genotype-dependent patterns. FC1571 and LH74 showed the highest colonization rates (approximately 85%), while BX1B73 and B73WT were lowest (around 46%). These patterns suggest that root exudate chemistry, including potential trade-offs linked to benzoxazinoid production, underlies compatibility with AMF. Genotypes with high AMF colonization also supported broader AP activity fields and greater abundances of phosphorus-mobilizing microbial taxa, underscoring a functional synergy between symbiosis and enzymatic nutrient acquisition.

Microbial community analysis showed that the maize rhizosphere was enriched in *Pseudomonas*, *Bacillus*, and *Lysobacter*, taxa associated with phosphorus solubilization and disease suppression, whereas the bean rhizosphere favored *Rhizobium*, *Neorhizobium*, and *Streptomyces*, key contributors to nitrogen fixation and organic matter decomposition. Shared compartments displayed the highest bacterial and fungal diversity, suggesting that intercropping promotes synergistic microbial interactions not present in monocultures. These findings emphasize that maize genotype selection in intercrops not only affects the maize rhizosphere itself but also propagates to the partner legume, restructuring microbial communities and their functional potential.

Overall, our study highlights the potential of genotype-informed maize–faba bean intercropping as a sustainable agroecological strategy to enhance nutrient turnover, microbial diversity, and soil fertility, particularly in subsoil layers where nutrient availability is limited. Genotypes such as FC1571 and LH74 appear especially promising for phosphorus mobilization, while BX1B73 provides strong proteolytic capacity for nitrogen cycling. These results underline the importance of multi-trait optimization, integrating enzyme activity, microbial associations, and symbiotic compatibility, when designing intercropping systems.

5 Future research directions

Future work should integrate metagenomic and metabolomic approaches to identify microbial genes and metabolic pathways responsible for enzyme regulation and nutrient cycling in intercropping systems. Long-term field trials will be required to test the stability and resilience of genotype-specific enzyme and microbiome patterns under variable environmental and

management conditions. In addition, targeted AMF inoculation and optimization of maize–bean planting ratios may further enhance microbial facilitation and crop productivity. Translating rhizotron-level insights into field-scale breeding and management strategies will be crucial for achieving sustainable intensification of intercropping systems.

6 Final remarks

By combining spatially resolved zymography, enzyme kinetics, AMF colonization, and microbial sequencing in a large rhizotron system, this study demonstrates that maize genotype identity is a central determinant of rhizosphere functioning in intercrops. Genotype effects were expressed not only at the root surface but extended into the subsoil and across crop partners, influencing enzymatic activity and microbial assembly at multiple scales. These insights provide a mechanistic understanding of how plant traits propagate through rhizosphere processes, offering a foundation for breeding strategies that explicitly integrate microbial ecology into genotype selection. In this way, intercropping systems can be designed to optimize nutrient cycling, soil fertility, and agroecosystem resilience under the dual pressures of climate change and resource limitation.

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8 Ethical statements

This research was conducted in compliance with institutional, national, and international guidelines for environmental and agricultural research. No human participants or animal subjects were involved. All plant cultivation, soil sampling, and microbial analyses were performed with the necessary permissions from relevant authorities, in accordance with best practices to minimize environmental impact. No genetically modified organisms (GMOs) were used beyond standard agronomic practices. The authors declare no conflicts of interest related to this work.

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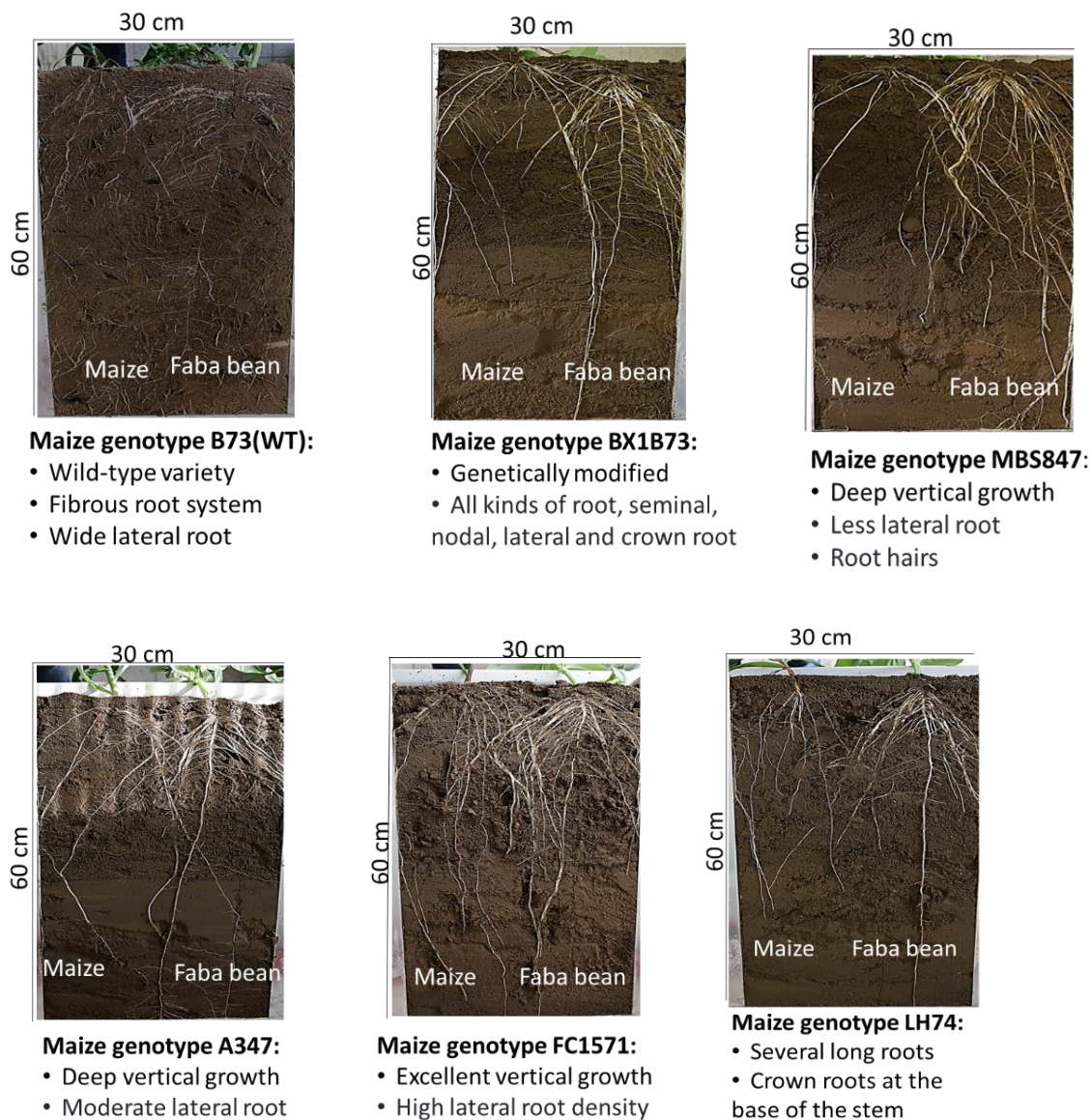
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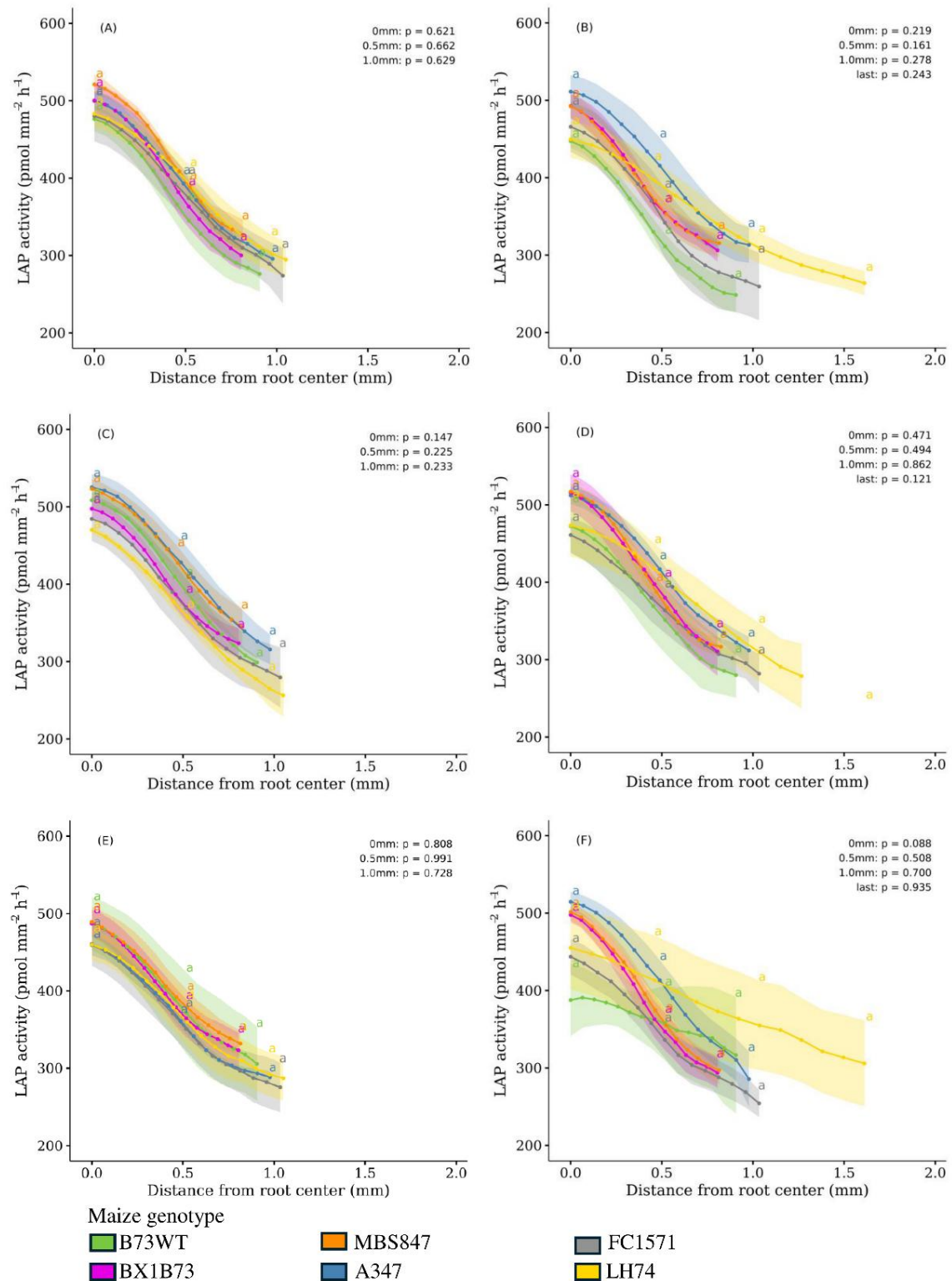
Supplementary

Characterization of distinct root traits specific to individual maize genotypes utilized in this study (this order of the genotype we will see in all experiment)

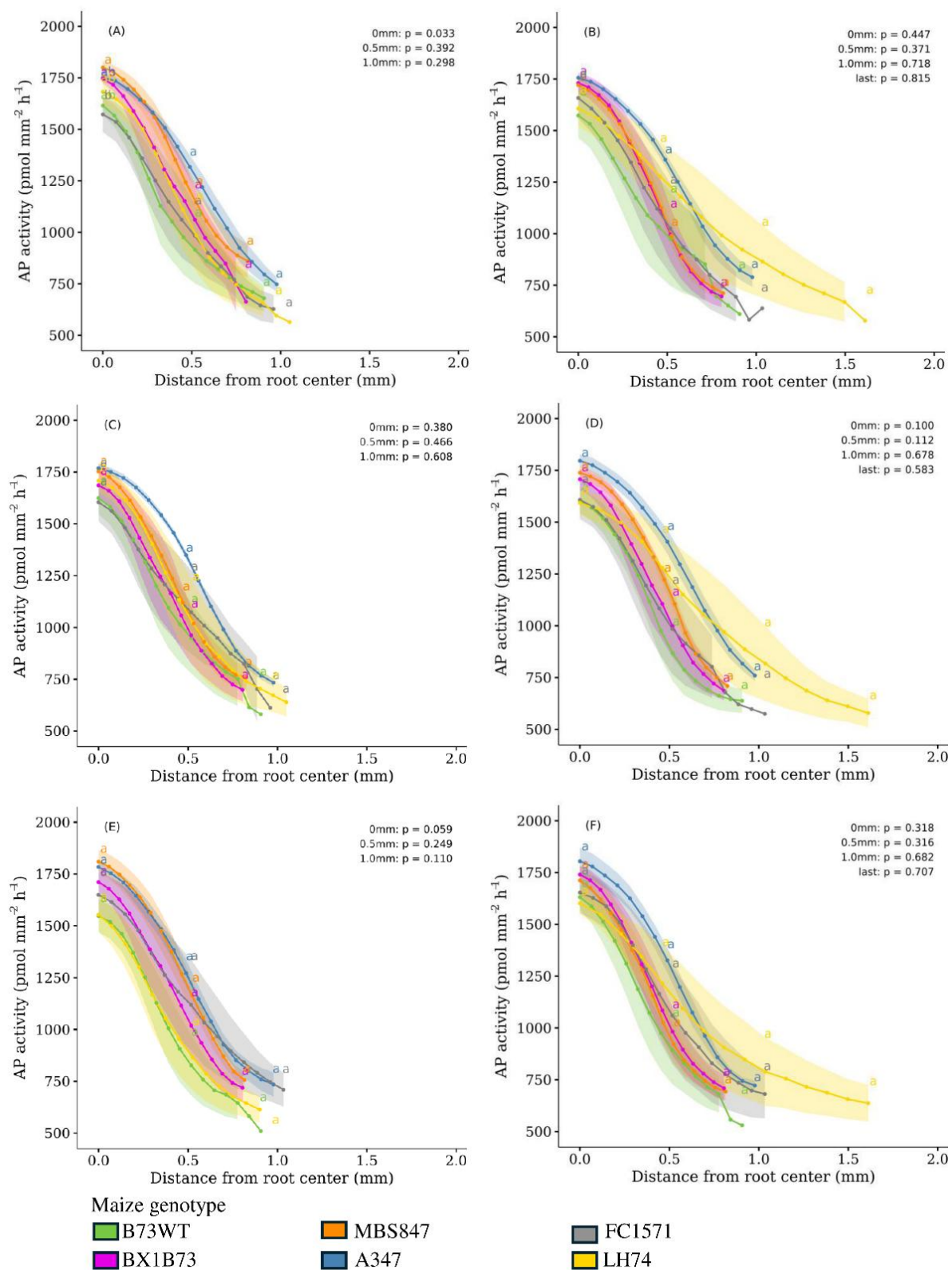


Supplementary Figure S1. Rhizotron setup for intercropping six maize genotypes with faba bean. Each rhizotron ($60 \times 30 \times 8$ cm) was filled with topsoil (0–30 cm) and subsoil (30–60 cm). Maize (left side) and faba bean (right side) were planted 15 cm apart, separated by root barriers with membrane windows for zymography and sampling. Pictures illustrate root traits of individual maize genotypes: B73WT (fibrous root system, wide lateral spread), BX1B73 (diverse root types including seminal, nodal, and crown roots), MBS847 (deep growth, sparse lateral roots, pronounced root hairs), A347 (deep growth with moderate lateral branching), FC1571 (dense lateral root network with strong vertical growth), and LH74 (several long roots with crown roots at stem base). Images represent characteristic root system architecture at the BBCH 30 growth stage ($n = 4$).

Zymography was conducted at two developmental stages: at BBCH 15 (first zymography) and BBCH 19-20 (second zymography). Figures S2 and S3 show enzyme activity profiles from the second zymography at BBCH 19-20.



Supplementary Figure S2. Leucine aminopeptidase (LAP) activity at BBCH19. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean rhizosphere. Lines represent mean LAP activity profiles with shaded areas showing standard error ($n = 4$). Reported p -values correspond to ANOVA results at 0, 0.5, and 1.0 mm distances, and the last measurement point.



Supplementary Figure S3. Acid phosphatase (AP) activity at BBCH19. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean rhizosphere. Lines represent mean AP activity with shaded areas showing standard error (n = 4). Reported p -values correspond to ANOVA results at 0, 0.5, and 1.0 mm distances, and the last measurement point. No significant genotype effects were detected.

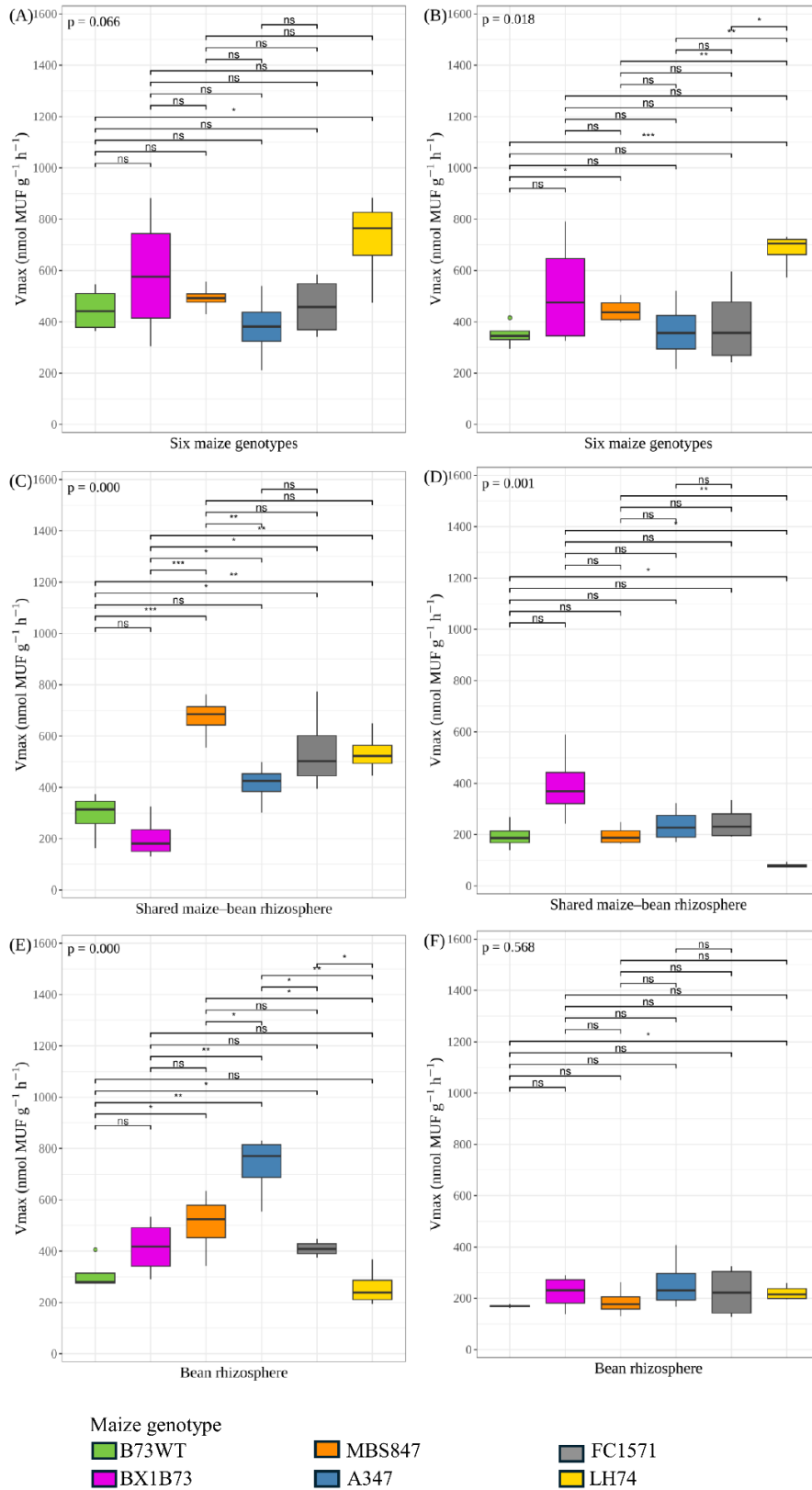


Figure S4. Pairwise comparisons of leucine aminopeptidase (LAP) V_{max} in rhizosphere samples at maize growth stage BBCH19. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean rhizosphere. Significance levels are indicated as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and ns = not significant. Boxplots represent the six maize genotypes with four biological replicates each ($n = 4$).

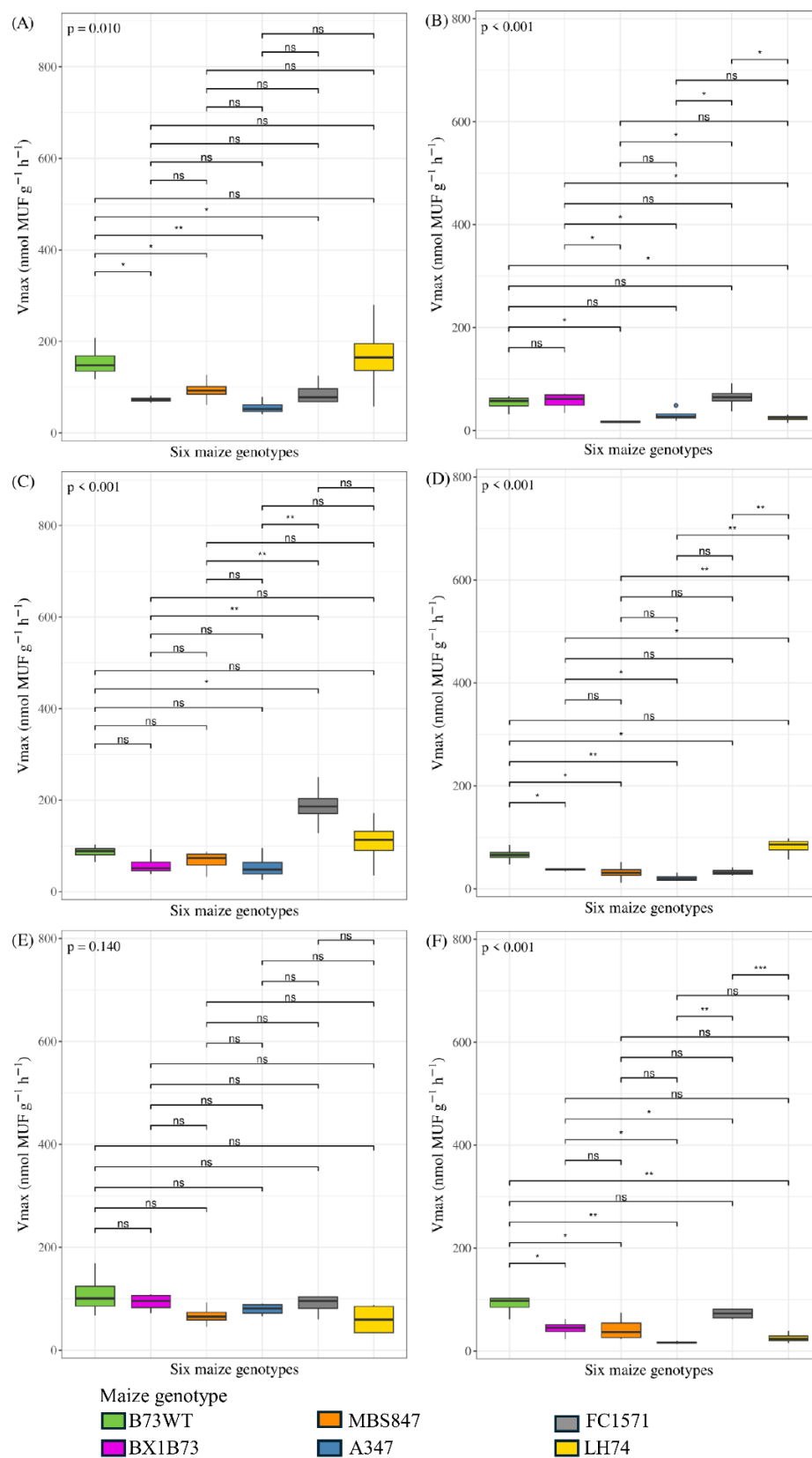
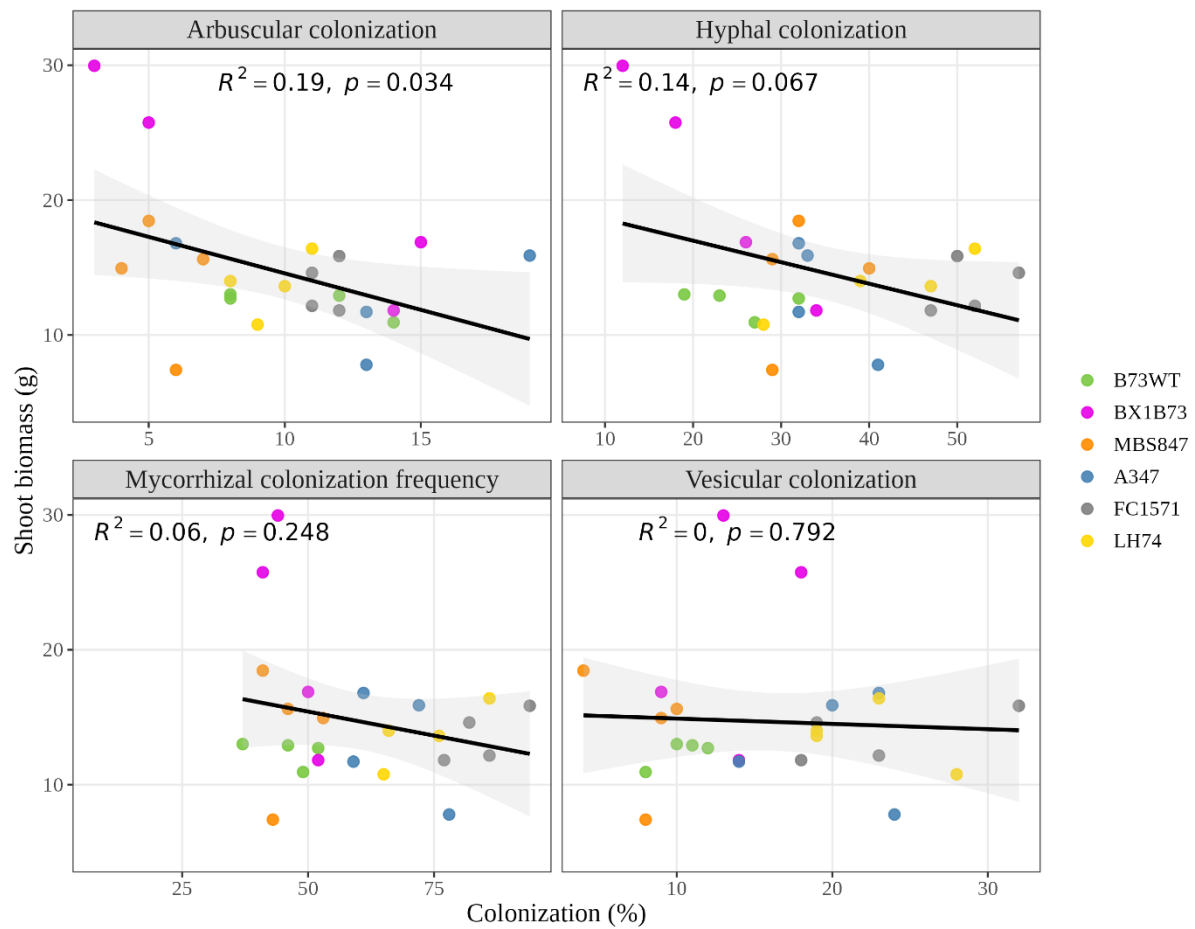


Figure S5. Pairwise comparisons of acid phosphatase (AP) V_{max} in rhizosphere samples at maize growth stage BBCH19. Panels (A, C, E) show topsoil (0–30 cm), and panels (B, D, F) show subsoil (30–60 cm). (A, B) Maize rhizosphere, (C, D) shared maize–bean rhizosphere, and (E, F) bean

rhizosphere. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = not significant. Different colored boxplots represent the six maize genotypes ($n = 4$).



Supplementary Figure S6. Linear regressions between maize shoot biomass and different components of arbuscular mycorrhizal fungal (AMF) colonization at BBCH 30. Panels show relationships with arbuscular colonization, hyphal colonization, mycorrhizal colonization frequency, and vesicular colonization. Colored points represent six maize genotypes (B73WT, BX1B73, MBS847, A347, FC1571, LH74). Regression statistics (R^2 and p -values) are indicated within each panel.

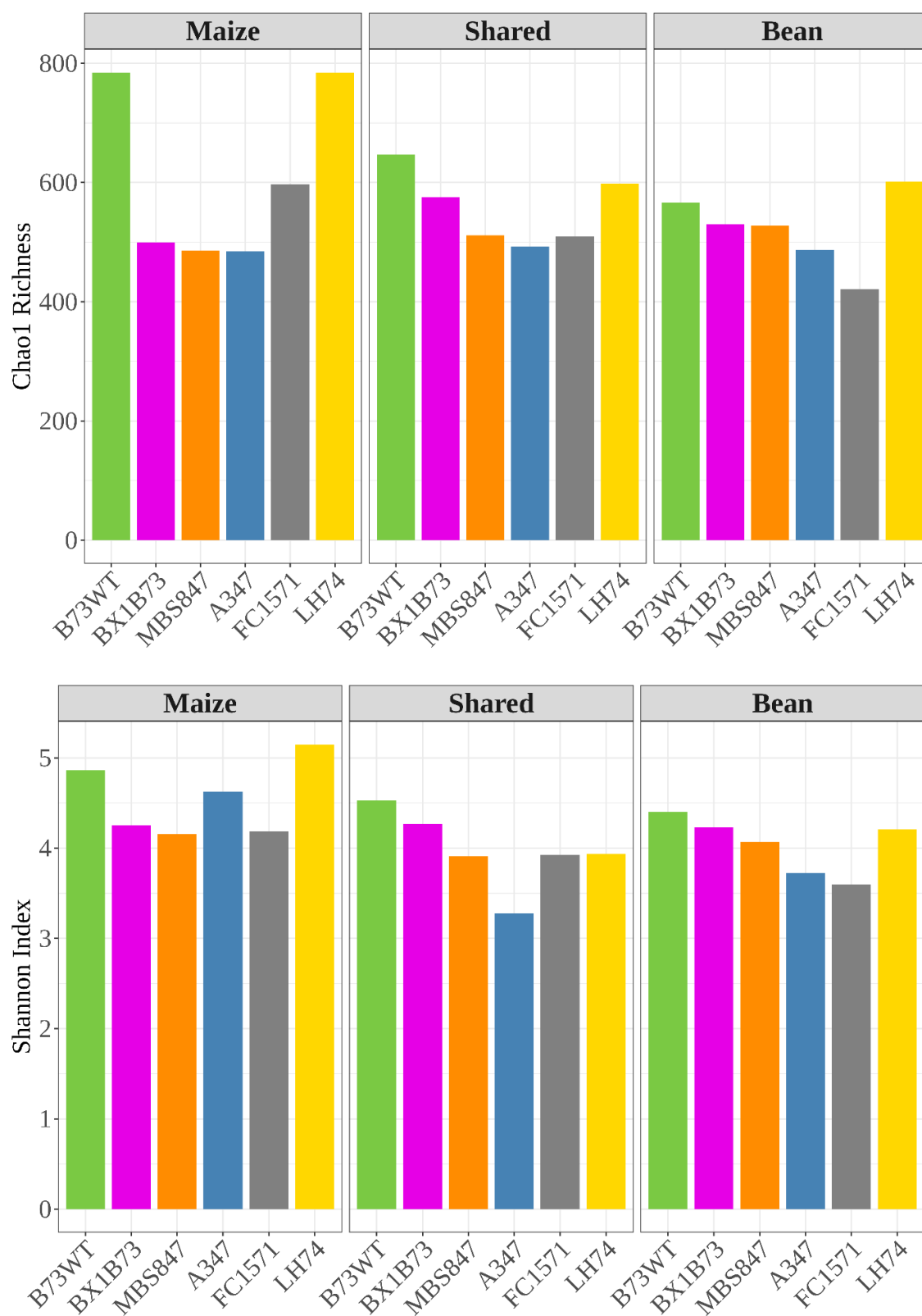


Figure S7. Alpha diversity of fungal communities across maize genotypes and rhizosphere compartments. (Top panels) Chao1 richness and (bottom panels) Shannon diversity index are shown for each maize genotype in the maize rhizosphere, shared maize–bean rhizosphere, and bean rhizosphere at BBCH 30. Bars represent mean values per genotype and compartment.

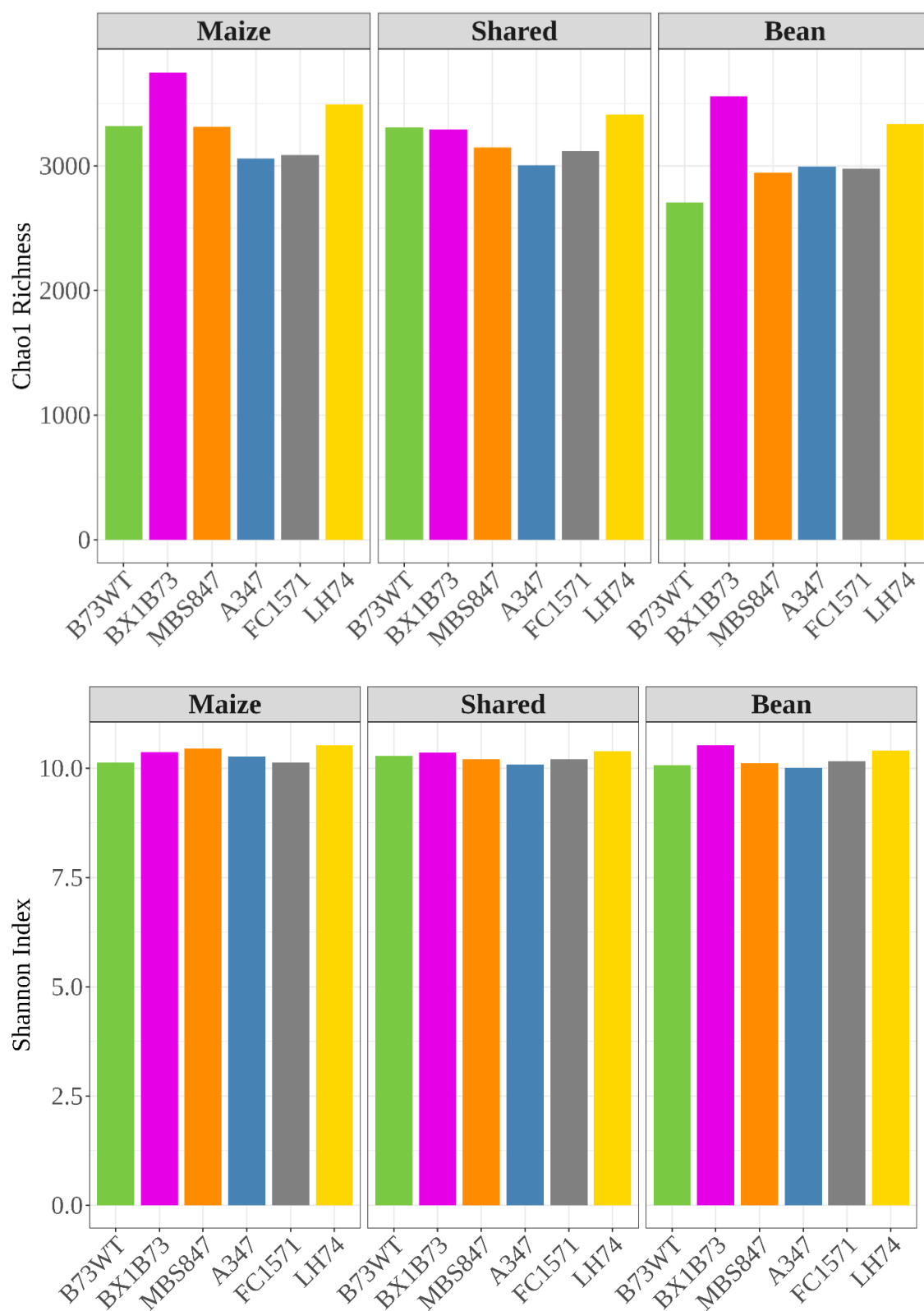
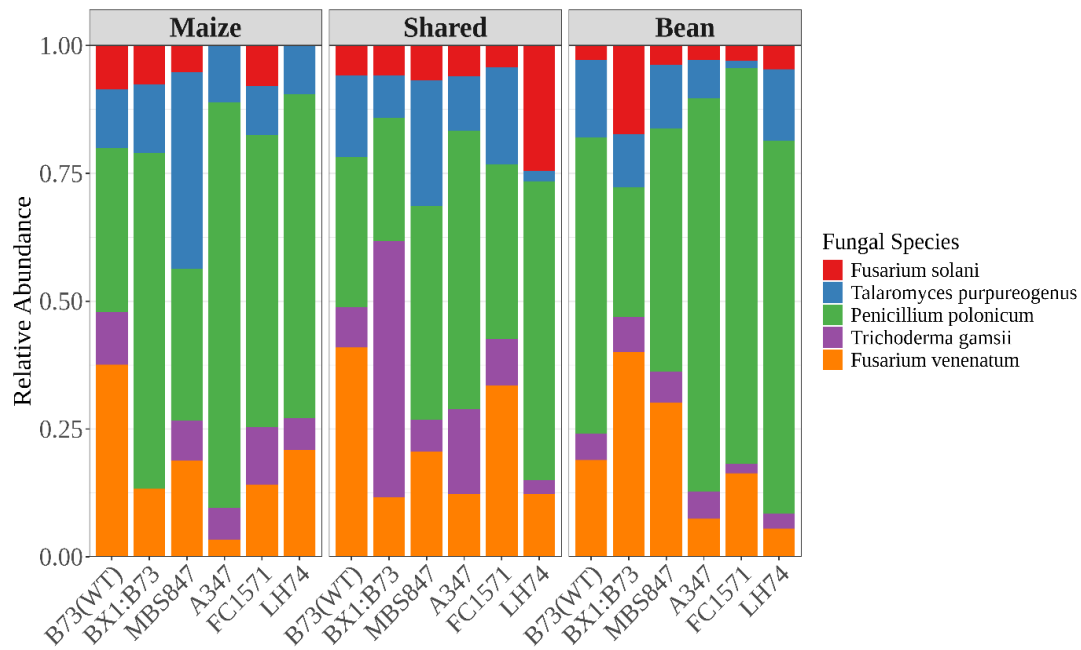
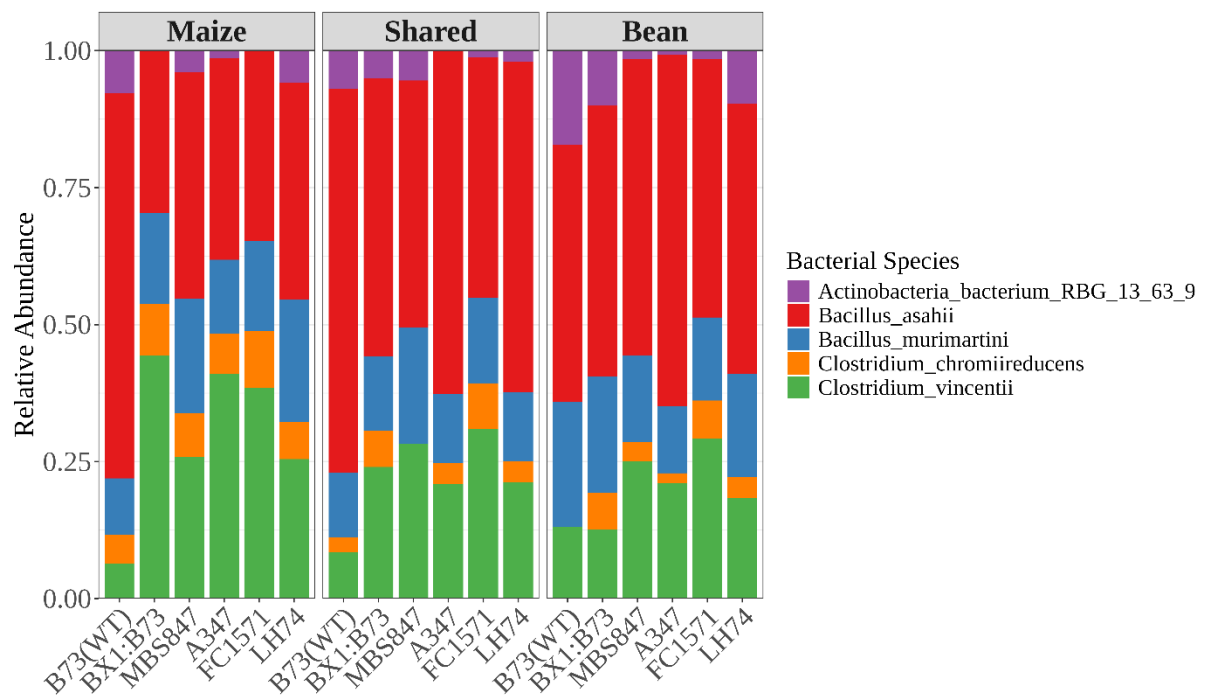


Figure S8. Alpha diversity of bacterial communities across maize genotypes and rhizosphere compartments. (Top panels) Chao1 richness and (bottom panels) Shannon diversity index are shown for each maize genotype in the maize rhizosphere, shared maize–bean rhizosphere, and bean rhizosphere at BBCH 30. Bars represent mean values per genotype and compartment.



Relative abundance stacked bar plot Fungi



Relative abundance stacked bar plot Bacteria

Figure S9. Stacked bar plots showing the relative abundance of dominant fungal (top panel) and bacterial (bottom panel) species in the maize rhizosphere, shared maize–bean rhizosphere, and bean rhizosphere at BBCH 30. Each bar represents the mean relative abundance for individual maize genotypes within each compartment. Major fungal and bacterial taxa are indicated in the legends.

2.2 Study 2. Crop-specific microbial restructuring and phosphorus dynamics under maize intercropping systems across contrasting soils

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Abstract

Optimizing phosphorus (P) use in agriculture remains a critical challenge, especially for systems that minimize external inputs. This study examines how intercropping maize (*Zea mays* L.) with either field pea (*Pisum sativum* L.) or ryegrass (*Lolium perenne* L.) affects phosphorus (P) availability through rhizosphere acidification and microbial community shifts across three distinct agricultural soil types.

We quantified total (P_Total), orthophosphate phosphorus (P_Ortho), and non-citric-acid extractable phosphorus (NCA_P) alongside soil pH and microbial community composition derived from phospholipid fatty acid (PLFA) profiling. Field pea intercropping consistently lowered rhizosphere pH (mean 4.77) and significantly increased P_Ortho concentrations, while ryegrass maintained a higher pH in the rhizosphere (mean 6.26) and was associated with greater NCA_P concentrations. Across all sites, a strong negative correlation between rhizosphere pH and P_ortho ($r = -0.54$, $p = 0.002$) indicating legume-driven P mobilization via rhizosphere acidification was observed.

PLFA analysis revealed that field pea rhizosphere was strongly bacterial-dominated, whereas ryegrass rhizospheres, although also dominated by bacteria, exhibited a much higher relative abundance of fungi compared to field pea. This highlights that intercrop identity distinctly structures microbial pathways involved in phosphorus cycling. These findings underscore the importance of intercrop selection in modulating both biogeochemical and microbial processes, offering a targeted strategy to improve phosphorus efficiency in intercropping systems.

Keywords

Intercropping, phosphorus cycling, rhizosphere pH, maize, microbial communities, phospholipid fatty acid (PLFA)

Highlights

- Field pea intercropping lowered pH, increasing orthophosphate P.
- Ryegrass intercropping maintained neutral pH, conserving organic P.
- Strong negative correlation between pH and orthophosphate P.
- Bacterial dominance in field pea, fungal enrichment in ryegrass.
- Crop selection strongly shapes soil P dynamics and microbes.

1. Introduction

Feeding a growing global population while preserving soil resources remains one of the most pressing challenges in agriculture today. Among the strategies improving resource use efficiency, intercropping, the practice of growing two or more crops together, has gained renewed attention for its ecological and agronomic benefits (Brooker et al. 2015). Intercropping systems can enhance yield stability, reduce pest and disease pressure, improve nutrient cycling, and support long-term soil health (Zhang et al. 2020; Zhu et al. 2023).

Of particular interest are cereal-legume and cereal-grass systems, such as maize (*Zea mays* L.) grown with field pea (*Pisum sativum* L.) or ryegrass (*Lolium perenne* L.). In particular, maize–field pea intercropping integrates functionally distinct plant types with complementary traits, such as differences in rooting depth, nutrient acquisition strategies, and canopy structure (Cong et al. 2015; Zhang et al. 2020). In contrast, maize–ryegrass intercropping combines two grass species (*Poaceae*) with more similar functional traits (Brooker et al. 2015). Legumes are known to acidify the rhizosphere by exuding organic acids, a process that enhances phosphorus (P) solubilization and supports biological nitrogen (N) fixation (Hinsinger 2001; Dakora and Phillips 2002; Marschner et al. 2011). In contrast, grasses tend to maintain near-neutral pH levels and promote topsoil nutrient retention and physical soil structure development through dense root networks (Hinsinger et al. 2009; Li et al. 2023). These contrasting plant strategies can reduce interspecies competition and promote more efficient use of soil phosphorus (Willey 1979; Brooker et al. 2015), particularly in cereal–legume mixtures. Phosphorus commonly limits crop productivity, especially in low-input or organic systems (MacDonald et al. 2011; Withers et al. 2014). Although total phosphorus levels in soil can be high, much of it is chemically bound to iron, aluminum, or calcium, making it unavailable for plant uptake (Hinsinger 2001; McDowell and Haygarth 2025; Lucas et al. 2025). Improving phosphorus use efficiency (PUE), defined as the crop yield or biomass produced per unit of phosphorus

taken up, is therefore a critical goal for sustainable nutrient management. This is especially urgent in light of shrinking global phosphate reserves and the environmental consequences of inefficient phosphorus use (Veneklaas et al. 2012).

Microbial communities play a central role in soil P cycling through enzymatic mineralization, solubilization, and direct competition for phosphorus in the rhizosphere (Richardson and Simpson 2011; Heuck et al. 2018). These microbial processes are highly sensitive to pH and root exudates, which differ significantly between plant species (Marschner et al. 2011; George et al. 2018).

Soil phosphorus exists in functionally distinct pools: orthophosphate (P_Ortho) is the most bioavailable form, while less available forms; including organic phosphorus (P_Organic), occluded, and mineral bound P, require microbial or chemical processes before plants can access them. The distribution of these pools is shaped by interactions among crop species, soil chemistry, and microbial activity (Turner et al. 2002; Veneklaas et al. 2012; George et al. 2018), and can be defined in this study as orthophosphate phosphate (P_Ortho) and non-citric-acid extractable phosphorus (NCA_P).

To study such interactions, phospholipid fatty acid (PLFA) analysis is a widely used method for assessing microbial community structure and functional changes in response to shifts in nutrient status and rhizosphere conditions (Buyer and Sasser 2012; Frostegård et al. 2011). However, field studies that integrate PLFA profiling with phosphorus fractionation and soil pH under intercropping, especially across diverse soil types remain scarce (Li et al. 2007; Lizcano-Toledo et al. 2021; Yang et al. 2022).

In this study, we investigate how intercropping maize with field pea or ryegrass alters rhizosphere pH, phosphorus availability, and microbial community composition across three contrasting field sites. Specifically, we tested the following hypotheses:

Rhizosphere pH varies significantly between intercropping systems and field sites;

The distribution of phosphorus fractions reflects crop induced changes in rhizosphere pH;

Microbial community composition, as inferred from PLFA biomarkers, differs by intercrop species and corresponds with phosphorus dynamics.

By linking chemical and biological indicators of phosphorus availability, this study aims to provide practical insights into designing intercropping systems that enhance phosphorus use efficiency under real field conditions.

2. Material and methods

2.1 Experimental sites and intercropping design

This study was conducted at three agricultural sites in Switzerland, each characterized by distinct soil types and topographic features. The Siselen site (Site 1) is classified as a Haplic Luvisol, Seedorf (Site 2) includes Haplic Cambisol and Gleyic Cambisol in its lower topography, and Fraubrunnen (Site 3) comprises a mix of Haplic and Cutanic Luvisols, with localized compaction observed at 30–40 cm soil depth (IUSS Working Group WRB 2022).

At each site, two intercropping systems were established: (1) maize (*Zea mays* L.) intercropped with field pea (*Pisum sativum* L.), and (2) maize intercropped with ryegrass (*Lolium perenne* L.). Treatments were replicated ten times in a randomized complete block design (RCBD), resulting in 60 plots across the three locations (2 treatments $\times$ 3 sites $\times$ 10 replicates). Each plot measured 8 $\times$ 16 m, ensuring sufficient area for field operations and minimizing edge effects. Sampling was carried out at two crop development stages: BBCH 32 (stem elongation) and BBCH 65 (flowering). Plots were sampled at BBCH 32 and at BBCH 65. Within each plot, a soil trench approximately 1 m deep and 1.5 m wide was excavated at the center to access the shared rhizosphere zone, the area where maize and intercrop roots overlapped most extensively.

These specific intercropping combinations were selected based on prior evidence showing that cereal-legume and cereal-grass systems leverage functional complementarity. Such combinations promote deeper resource partitioning, nitrogen fixation (only cereal-legume mixture), and increased microbial stimulation in the rhizosphere (Li et al. 2020; Zhang et al. 2020; Zhu et al. 2023).

2.2 Soil sampling

Soil samples were collected at BBCH 32 and BBCH 65 from the shared rhizosphere, targeting zones of maximal root interaction between maize and its intercrop species. For phosphorus fractionation (orthophosphate and non-citric-acid-extractable P) and pH analyses, composite rhizosphere samples were taken from the Ap horizon (0–30 cm), following standard field protocols (Schachtman et al. 1998; Prietzel et al. 2015). Soil was carefully extracted from root-dense areas, reflecting the active chemical environment experienced by both crops.

Samples were collected using a stainless-steel auger, and multiple subsamples from each plot were combined to account for spatial heterogeneity. Soil intended for chemical analyses was gently air-dried at room temperature, passed through a 2 mm sieve to remove coarse material, and stored in sealed polyethylene bags at 4 °C.

For microbial community assessment, rhizosphere soil was collected by gently shaking the root system to remove loosely attached soil, followed by brushing to detach soil tightly adhering to the root surface. Special attention was given to root channels where both maize and intercrop roots co-occurred. Samples were transferred into sterile polyethylene containers and frozen at -20°C immediately after collection, following established PLFA protocols (Frostegård et al. 1991), and adapted for soil systems by Gunina et al. (Gunina et al. 2014). All tools were sterilized between plots to prevent cross-contamination.

2.3 Soil pH measurement

Soil pH was measured using samples from the shared rhizosphere zone between maize and the intercrop species. This approach reflects the immediate chemical environment experienced by both plants and provides a more accurate assessment of biologically relevant pH conditions. Measurements followed ISO 10390:2021 standards for mineral soils, using the potentiometric method (International Organization for Standardization 2021). Dried and sieved rhizosphere samples ($<2\text{ mm}$) were suspended in a 0.01M calcium chloride (CaCl_2) solution at a 1:5 (w/v) soil-to-solution ratio. The mixture was stirred thoroughly and allowed to equilibrate for 30 minutes (Carter and Gregorich 2008).

pH was measured using a calibrated digital pH/mV meter (Thermo Orion DMP-2) at room temperature. Calibration was performed daily using certified buffer solutions (pH 4.0, 7.0, and 10.0), and electrode performance was verified with repeated readings of the pH 7.0 standard. All rhizosphere samples were analyzed in duplicate to ensure accuracy. This method provides a reliable estimate of pH under field-relevant root-zone conditions (Thomas, G. W. 1996).

2.4 Phosphorous fraction

Phosphorus fractions were analyzed following the method of Prietzel et al. (2015) with modifications. We quantified two directly measured and one derived fraction: orthophosphate phosphorus (P_Ortho), total phosphorus (P_Total), and non-citric-acid-extractable phosphorus (NCA-P).

Rhizosphere soil samples were air-dried at room temperature and sieved through a 2 mm mesh (Carter and Gregorich 2008). P_Ortho was extracted using a 1% (w/v) citric acid extracts, prepared at a 1:10 soil-to-solution ratio using the molybdate- ascorbic acid method (Murphy and Riley 1962), this correspond to the first extraction step described in Prietzel et al. (2015). P_Total was quantified on finely ground soil subsamples after complete HF/HClO_4 digestion, with phosphorous concentrations determined by inductively coupled plasma-optical emission

spectrometry (ICP-OES; Varian Vista Pro)(Soltanpour et al. 2018). The non-citric-acid-extractable phosphorus (NCA_P) was calculated as the difference between P_Total and P_Ortho ($\text{NCA_P} = \text{P_Total} - \text{P_Ortho}$). This operationally defined pool includes all phosphorus forms not extracted by 1% citric acid, including mineral-bound, occluded, and organic P. While this fraction may contribute to long-term P availability through mineralization or desorption processes, it does not reflect total organic P as determined by ignition or alkaline extraction methods (Saunders and Williams 1955).

2.5 PLFA analysis

Microbial community composition was evaluated using phospholipid fatty acid (PLFA) profiling. Lipids extraction followed the one-phase lipid protocol of Bligh and Dyer (1959), refined for soil application by Frostegård et al. (1991), and modified by Gunina et al. (2014). To account for losses during lipid extraction and clean-up, 25 µg of dinonadecanoylglycerol-phosphatidylcholine, internal standard one (IS1; 1 µg µL⁻¹, Larodan Lipids, Malmö, Sweden), was added to approximately 6 g (±0.2 g) of soil prior to extraction. Samples were extracted twice with a 2:1:0.8 mixture of methanol, chloroform, and 0.15 M citric acid/KOH buffer adjusted to pH = 4 following Bligh and Dyer (1959) as adapted by Frostegård et al. (1991). Phospholipids were separated from neutral lipids and glycolipids by solid-phase extraction using activated silica gel columns (Silica gel Merck, particle size 0.063–0.200 mm), and were then derivatized to fatty acid methyl esters (FAMES). Alkaline saponification of the purified phospholipids was performed with 0.5 mL of 0.5 M NaOH dissolved in MeOH for 10 min at 100 °C, followed by methylation with 0.75 mL of BF₃ dissolved in methanol (~1.25M) at 80 °C for 15 min. FAMES were extracted by liquid-liquid extraction with hexane (three times). Before the measurements of the FAMES, 15 µL internal standard two (IS2; 1 µg µL⁻¹) tridecanoic acid methyl ester and 185 µL of toluene were added to the samples. Samples were transferred to 1.5 mL GC vials and analyzed using a GC IsoLink II gas chromatograph (ThermoFisher Scientific) coupled to an ISQ Single Quad Massspectrometer as described in Gunina et al. (2014). PLFA biomarkers were identified using retention time and mass spectra comparisons with an external FAME standard mix. PLFA concentrations were normalized to nanomoles per gram of dry soil (nmol g⁻¹).

PLFA groupings were derived using principal component analysis (PCA) and factor analysis with VARIMAX rotation. Each PLFA was assigned to the microbial group corresponding to its highest factor loading (see Supplementary; Tables S2–S6). Grouping criteria were adapted from Apostel et al. (2018) and include PLFAs like *10Me16:0* for Actinobacteria and *cy19:0*

for Gram-negative bacteria. PCA biplots and heatmaps were used to visualize microbial community structure across treatments and sites (Supplementary Figures S1–S5).

2.6 Statistical analysis

All statistical analyses were performed using R software (R Core Team 2023). Data normality was assessed using the Shapiro–Wilk test. Depending on distribution, comparisons between crop treatments were performed using either Welch’s *t*-test (for normally distributed data) or the Wilcoxon rank-sum test (for non-parametric data).

Pearson correlation coefficients were calculated to explore relationships among microbial groups, rhizosphere pH, and phosphorus fractions (P_Ortho, P_Total, and NCA-P). Multivariate differences in microbial community composition were assessed using principal component analysis (PCA) based on standardized PLFA profiles, implemented via the vegan package (Oksanen et al. 2001). All visualizations were produced using ggplot2 (Wickham 2016), including boxplots, bar graphs, PCA biplots, and confidence ellipses. Plot layouts were arranged using patchwork (Pedersen 2024), and axis scaling was adjusted using scales (Wickham et al. 2023b). Data wrangling and preparation were conducted using dplyr (Wickham et al. 2023a). See statistical methods in supplementary information for test assumptions and software details.

3. Results

3.1 Crop-specific rhizosphere pH patterns

Significant differences in rhizosphere pH were observed between field pea and ryegrass intercropping treatments across all three experimental sites. Rhizosphere soils, specifically sampled from zones of maximal root interaction, and at Site 1 ($n = 10$ per crop), field pea had a mean pH ($\pm$ SD) of 4.96 ± 0.32 , while ryegrass had 6.23 ± 0.28 . At Site 2, field pea pH was 4.69 ± 0.31 and ryegrass 6.18 ± 0.21 . At Site 3, field pea pH was 4.65 ± 0.29 and ryegrass 6.38 ± 0.27 . For each site, the difference between crops was highly significant (Welch’s *t*-test, all $p < 0.001$; see Supplementary Table S1). Figure 1 shows boxplots of pH values per treatment and site, with each point representing a single plot ($n = 10$).

Density plots (Figure 2) show the distribution of rhizosphere pH values for field pea and ryegrass at each experimental site. At Site 1, field pea pH values ranged from 4.21 to 4.95 (mean 4.71), while ryegrass pH ranged from 5.81 to 6.71 (mean 6.35). At Site 2, field pea pH

ranged from 4.29 to 5.29 (mean 4.71), and ryegrass from 5.66 to 6.71 (mean 6.20). At Site 3, field pea pH ranged from 4.19 to 5.67 (mean 4.89), and ryegrass from 5.67 to 6.60 (mean 6.29). Across all sites, the density curves for field pea were consistently shifted toward lower pH values, while those for ryegrass were centered at higher, near-neutral values. Each tick mark in the plot represents an individual measurement ($n = 10$ per crop and site).

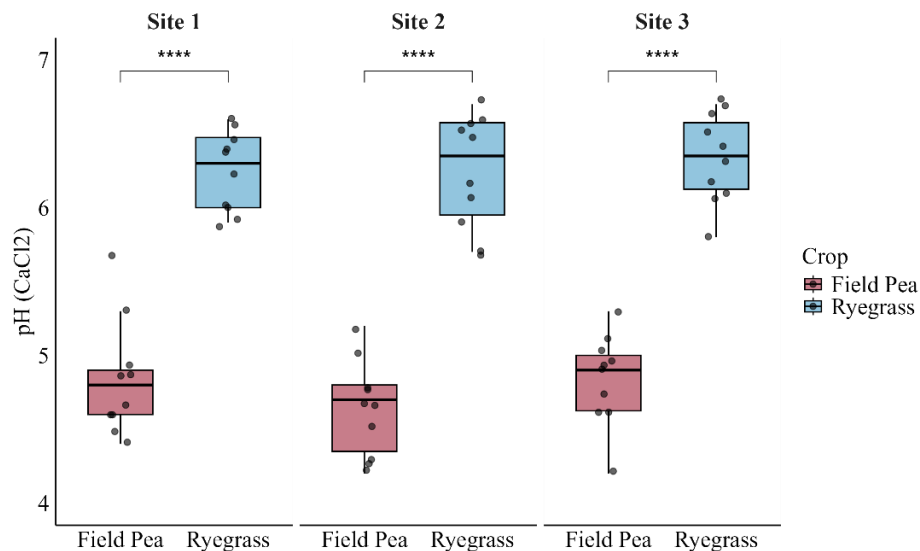


Figure 1. Rhizosphere pH (CaCl₂) under field pea and ryegrass intercropping at three experimental sites. Boxplots show means from $n = 10$ plots per treatment per site. Error bars represent standard deviation. Asterisks indicate significance levels based on Welch's t -tests (**** $p < 0.0001$).

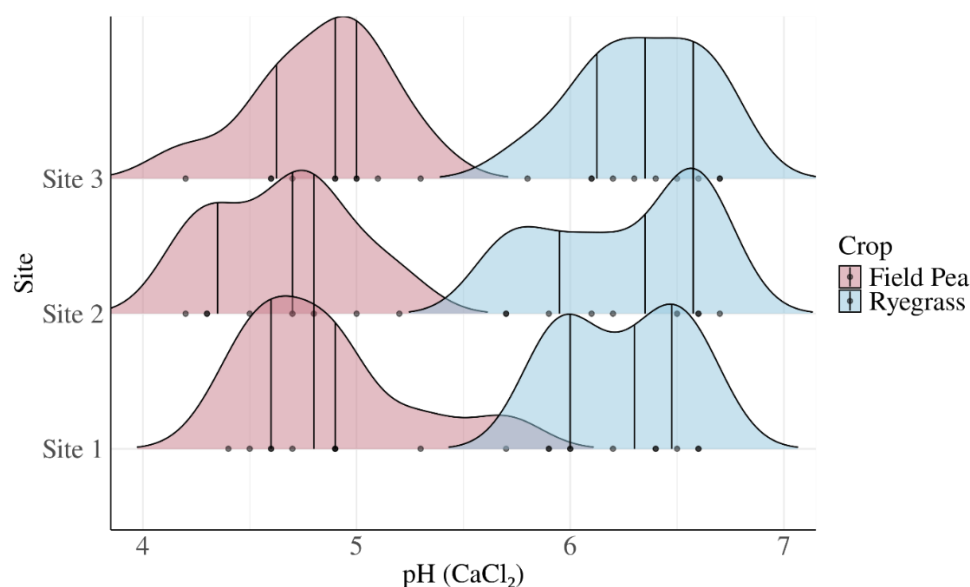


Figure 2. Split ridge plot (joy plot) showing the distribution of soil pH (CaCl₂) under Field Pea and Ryegrass at three field sites. Density curves represent pH distributions from $n = 10$ plots per treatment per site.

3.2 Crop-specific orthophosphate phosphorus and organic phosphorus concentrations

Orthophosphate phosphorus (P_Ortho) concentrations were significantly higher in the rhizosphere soils of field pea compared to ryegrass across all sites. On average, field pea plots had mean P_Ortho values of 0.070 ± 0.022 mg/g, whereas ryegrass plots showed lower values of 0.031 ± 0.014 mg/g ($p < 0.001$; Fig. 3). Site-wise means and standard deviations are presented in Supplementary Table S2. The difference in P_Ortho between crops was statistically significant at each site (Tukey's HSD test, $p < 0.05$; Fig. 3). Non-citric-acid-extractable phosphorus (NCA_P) concentrations were modestly higher under ryegrass (0.064 ± 0.015 mg/g) relative to field pea (0.061 ± 0.018 mg/g), with a statistically significant difference observed only at Site 2 ($p < 0.05$; Supplementary Table S2).

The ratio of P_Ortho to P_Total was consistently greater in field pea than in ryegrass across all sites (see Fig. 3). Violin plots show the full distributions for each treatment and site ($n = 10$ per crop per site). Pearson correlation analysis revealed a significant negative association between rhizosphere pH and P_Ortho concentrations ($r = -0.536$, $p = 0.0023$; Fig. 4B). Linear regression further confirmed that pH was a strong predictor of P_Ortho, explaining 38% of the total variance (adjusted $R^2 = 0.38$, $p = 0.0014$). However, when regression analyses were stratified by crop, the relationship was only statistically significant in field pea ($R^2 = 0.14$, $p = 0.042$), and not in ryegrass ($R^2 = 0.07$, $p = 0.163$), as shown in Supplementary Figure S1.

Total phosphorus (P_Total; Fig. 4A) and organic phosphorus (P_Organic; Fig. 4C) both showed weak, statistically non-significant correlations with pH ($|r| < 0.25$, $p > 0.1$).

The ratio of P_Ortho to P_Total decreased significantly with increasing pH (global model: $R^2 = 0.20$, $p = 0.013$; Fig. 4D), with crop-specific analysis showing significance in field pea only ($R^2 = 0.20$, $p = 0.013$).

Bar plots (Fig. 5) display the mean concentrations of P_Total, P_Ortho, and NCA_P for field pea and ryegrass at each site, with error bars representing one standard deviation ($n = 10$ per crop per site).

All full site-level data, including means, standard deviations, and detailed statistical outputs, are provided in Supplementary Tables S2 and S6.

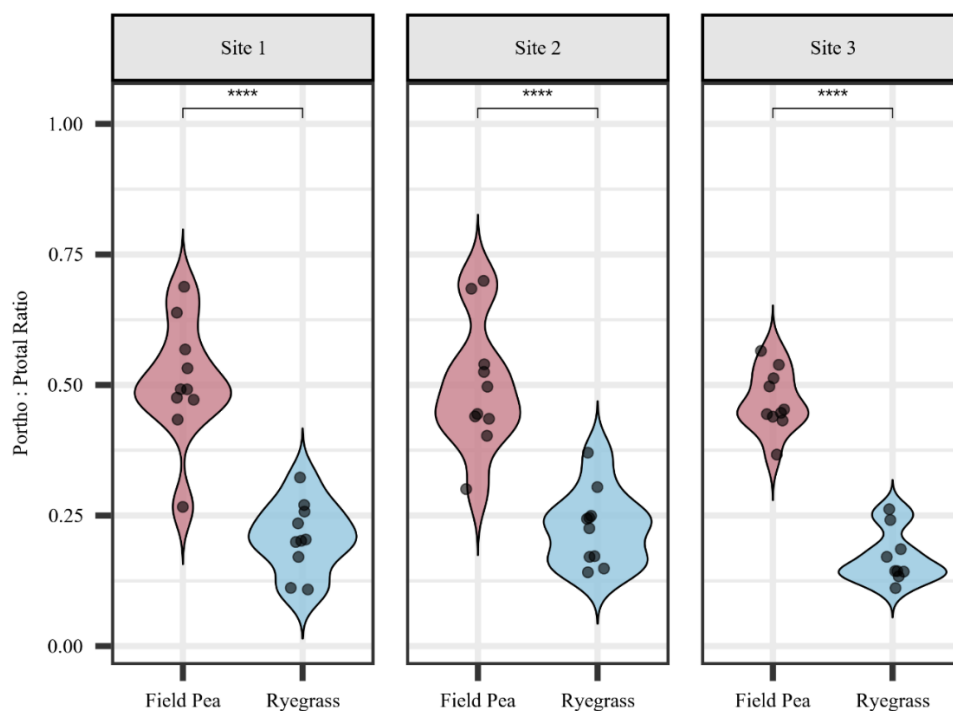


Figure 3. Ratio of orthophosphate phosphorus (P_Ortho) to total phosphorus (P_Total) in rhizosphere soil from field pea and ryegrass intercropping systems across three experimental sites. Violin plots illustrate treatment distributions. Letters denote statistically significant differences based on Tukey's HSD test ($p < 0.05$). $n = 10$ plots per treatment per site.

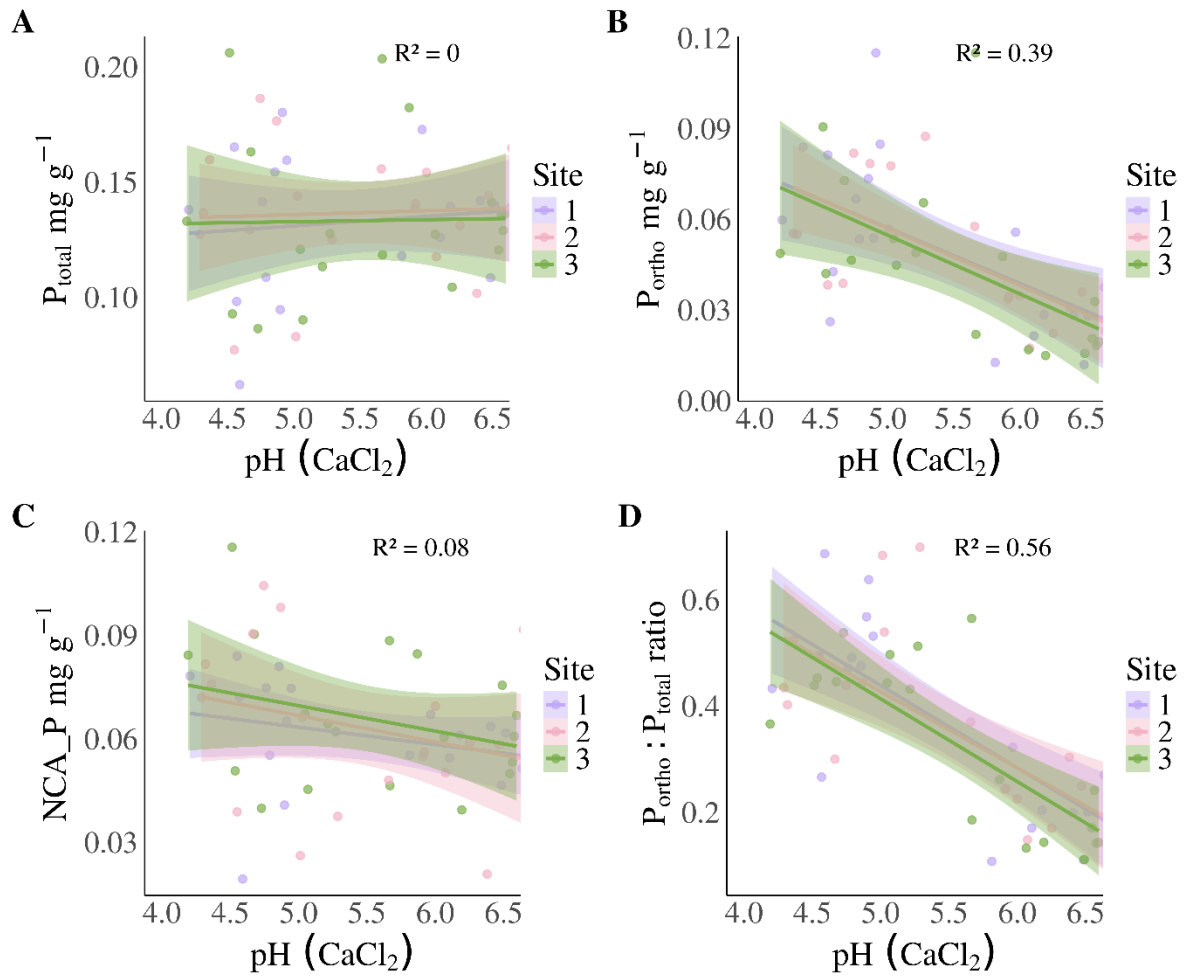


Figure 4. Linear regression plots showing the relationship between rhizosphere pH (CaCl₂) and phosphorus fractions across all three experimental sites. (A) Total phosphorus (P_{Total}); (B) Orthophosphate phosphorus (P_{Ortho}); (C) Non-citric-acid-extractable phosphorus (NCA_P); (D) Ratio of P_{Ortho} to P_{Total} . Each point represents an individual plot-level observation ($n = 10$ per treatment per site). Data points are color-coded by site (Site 1: purple, Site 2: pink, Site 3: green). Solid lines represent fitted linear regression models; shaded areas denote 95% confidence intervals.

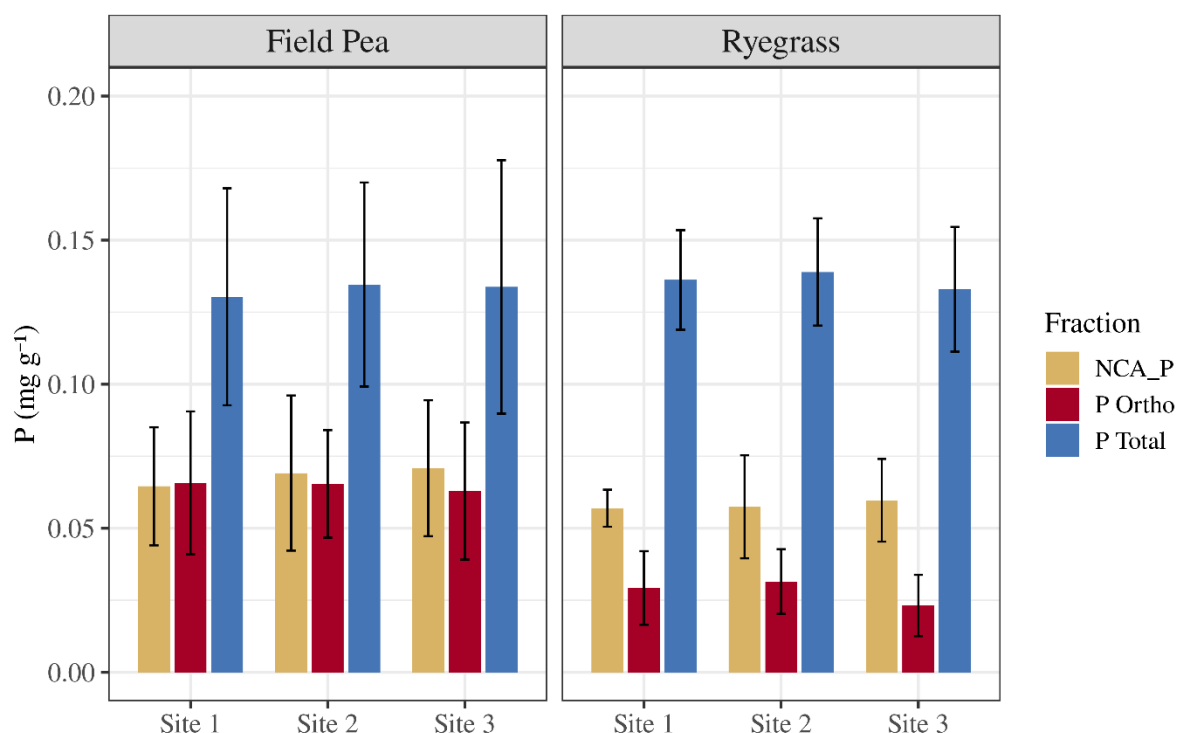


Figure 5. Total phosphorus (P_Total), orthophosphate phosphorus (P_Ortho), and Non-citric-acid-extractable phosphorus (NCA_P) concentrations in rhizosphere soil under field pea and ryegrass intercropping across three experimental sites. Bars represent mean $\pm$ SD ($n = 10$ per treatment per site). Color scheme corresponds to P fractions: blue (P_Total), red (P_Ortho), and gold (NCA_P).

3.3 Microbial community differences

Phospholipid fatty acid (PLFA) analysis showed rhizosphere soils under field pea consistently had higher relative abundances of bacterial biomarkers, particularly Gram-positive taxa and Actinobacteria (e.g., i15:0, a15:0, 10Me16:0, 10Me18:0; Supplementary Fig. S2; Tables S3–S5). In contrast, ryegrass plots consistently exhibited elevated levels of fungal PLFAs, including arbuscular mycorrhizal fungi (AMF) indicators such as 18:2 ω 6,9 and 16:1 ω 5c. These trends were observed at all three experimental sites (see Supplementary Fig. S2).

Bacteria-to-fungi PLFA ratios (Fig. 6) were significantly higher in field pea rhizosphere compared to ryegrass at each site (Site 1: mean $\pm$ SD: 7.8 ± 1.2 vs. 3.8 ± 1.1 , $p < 0.05$; Site 2: 7.2 ± 0.9 vs. 2.9 ± 1.0 , $p < 0.01$; Site 3: 8.6 ± 1.5 vs. 3.1 ± 1.3 , $p < 0.05$; Welch's t-test; $n = 10$ per treatment per site).

Protozoa-to-fungi PLFA ratios (Fig. 7) were also higher in field pea compared to ryegrass at Site 1 (mean $\pm$ SD: 2.1 ± 0.8 vs. 1.1 ± 0.7 , $p < 0.05$), but not significantly different at Sites 2 or 3 ($p > 0.05$ for both comparisons).

Principal component analysis (PCA) of PLFA profiles (Supplementary Figs. S3–S5) showed distinct clustering by crop type at all three sites. PC1 explained 38% of the total variance and

was associated primarily with bacterial markers, while PC2 explained 22% of the variance and was associated with fungal biomarkers.

All underlying data, including group means, standard deviations, and full biomarker profiles per site and crop, are provided in Supplementary Tables S3–S5.

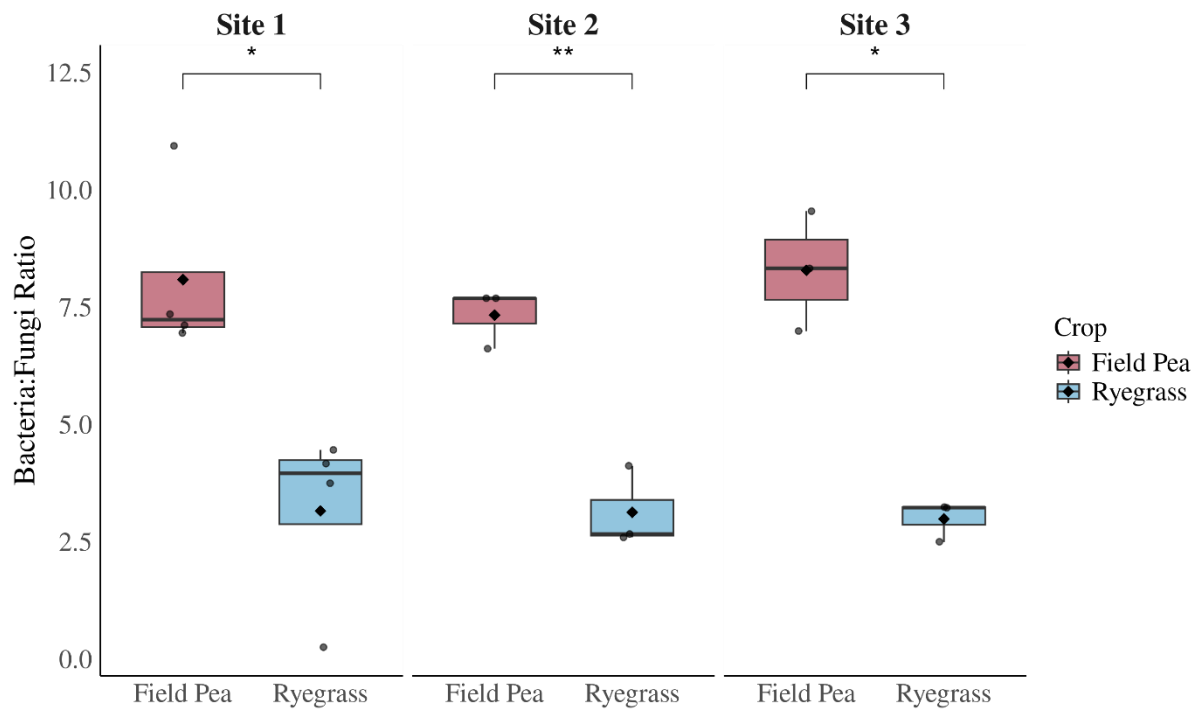


Figure 6. Bacterial-to-fungal PLFA ratio in rhizosphere soils under field pea and ryegrass intercropping across three experimental sites. Boxplots show medians and interquartile ranges; diamonds represent group means. Asterisks indicate significance levels ($p < 0.05 = *$, $p < 0.01 = **$; Welch's t -test). $n = 10$ plots per treatment per site.

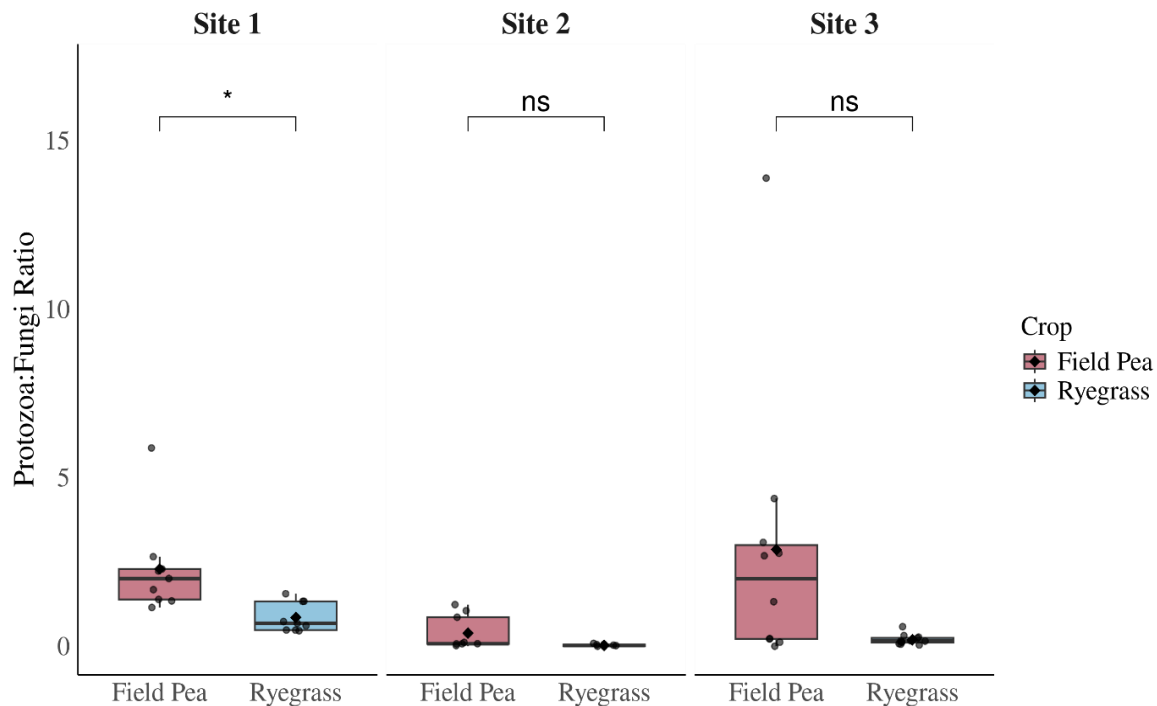


Figure 7. Protozoa-to-fungi PLFA ratio in rhizosphere soils under field pea and ryegrass intercropping across three experimental sites. Boxplots show medians and interquartile ranges; diamonds represent group means. Asterisks indicate significance levels ($p < 0.05 = *$); “ns” denotes no significant difference (Welch’s t -test). $n = 10$ plots per treatment per site.

4. Discussion

4.1 Phosphorus acquisition strategies in maize–field pea intercropping systems

Our results demonstrate that field pea significantly alters rhizosphere conditions in maize-based intercropping, with important consequences for phosphorus (P) availability. Across all three experimental sites, field pea intercropping consistently lowered rhizosphere pH (Supplementary Table S1). This acidification was closely associated with increased orthophosphate phosphorus (P_Ortho) concentrations, as shown by a significant negative correlation ($r = -0.54$, $p = 0.0023$; Fig. 4B). Regression analysis further showed that pH was a significant predictor of P_Ortho in field pea plots ($R^2 = 0.14$, $p = 0.042$), but not in ryegrass. This pattern aligns with previous research demonstrating that legumes, including field pea, release organic acids (e.g., citrate, malate) (Etesami et al. 2021) that lower rhizosphere pH and mobilize mineral-bound phosphorus (Hunter et al. 2014; Ma et al. 2021; Chen et al. 2023c), thus increasing P bioavailability (Hinsinger et al. 2011; Maltais-Landry et al. 2014) for both the legume and companion maize (Dakora and Phillips 2002; Hinsinger et al. 2009; Richardson et al. 2009; Tang et al. 2014). For example, Richardson et al. (2009) and Pantigoso et al. (2023) reported that root exudates from legumes facilitate P solubilization in mixed cropping

systems, while Ferguson et al. (2013), Mitran et al. (2018), and Chen et al. (2023a) observed similar pH–P_Ortho relationships in field settings. The ratio of P_Ortho to total phosphorus (P_Total) also declined with increasing pH ($R^2 = 0.20$, $p = 0.013$; Fig. 4D), further supporting the acidification effect in increasing the proportion of bioavailable P under field pea. These findings are in line with those of Hinsinger (2001), and Latati et al. (2014), who demonstrated that legume-driven acidification enhances P solubility and turnover.

Microbial community analysis via PLFA indicated that field pea rhizospheres were enriched in Gram-positive bacteria and Actinobacteria (e.g., i15:0, a15:0, 10Me16:0; see Table S3). These bacterial groups are known for their resilience in acidic conditions and their role in organic P mineralization through phosphatase activity (Marschner 2003; Fierer et al. 2007; Zhao et al. 2020). The bacteria-to-fungi PLFA ratio was significantly higher under field pea at all sites (Fig. 6), indicating a bacterial-dominated system. These results support recent findings by (Jiang et al. 2024), who found that bacterial dominance under legumes accelerates nutrient cycling and P turnover.

Taken together, our results show that field pea, through acidification and stimulation of bacterial mineralizers, enhances P availability in the maize–field pea system. This supports a functional complementarity where legumes facilitate P acquisition via both direct (acidification) and indirect (microbial shifts) mechanisms.

4.2 Phosphorus acquisition strategies in maize–ryegrass intercropping systems

In contrast, ryegrass intercropping maintained higher rhizosphere pH, with comparatively lower P_Ortho concentrations but modestly elevated non-citric-acid-extractable phosphorus (NCA_P), especially at Site 2 ($p < 0.05$). The P_Ortho to P_Total ratio was consistently lower in ryegrass plots (Figs. 3, 5). Correlations between pH and both P_Total and NCA_P were weak and not significant ($|r| < 0.25$, $p > 0.1$; Figs. 4A, 4C). Microbial PLFA profiles indicated fungal-dominated communities under ryegrass, including increased AMF-associated biomarkers (16:1 ω 5c; Table S3) and higher fungal PLFAs overall (Supplementary Fig. S2). These findings are in line with (Turner 2005) and (Ngosong et al. 2012), who reported that grasses tend to form strong associations with AMF and promote fungal-driven nutrient cycling. For instance, (Finney et al. 2021) and (Bünemann et al. 2012) observed greater NCA_P pools under grass-dominated systems, suggesting microbial P immobilization is favored in such conditions. Importantly, P immobilization is not considered a plant acquisition strategy;

instead, it reflects slower microbial turnover and the stabilization of phosphorus in organic matter by fungi (Dippold and Kuzyakov 2013; Blagodatskaya et al. 2014).

The higher abundance of AMF markers supports the role of mycorrhizal associations in facilitating maize access to organically bound P, albeit more slowly than the acidification-driven processes observed with field pea.

Protozoa-to-fungi PLFA ratios showed significant crop effects only at Site 1 (Fig. 7), indicating that predator–decomposer interactions may vary with site-specific conditions and crop composition (Hobbie and Villóeger 2015; Li et al. 2021).

Principal component analysis (PCA) further confirmed distinct clustering of microbial communities by crop type (Supplementary Fig. S6), underscoring the influence of plant species on rhizosphere microbiota and, consequently, P cycling.

In summary, maize–ryegrass systems tend towards a more fungal-driven, mycorrhizal network that favour organic P accumulation and gradual nutrient release (e.g., ryegrass cover crops; increased phosphatase & organic P) (Hallama et al. 2019; Soltangheisi et al. 2020; Hansen et al. 2022; Chen et al. 2023b) whereas maize–field pea systems exhibit rapid, bacteria-driven P cycling, as shown by microbial community and enzyme activity shifts (Lu et al. 2023)

4.3 Implications for maize nutrition

Collectively, our results indicate that the identity of the intercrop (field pea vs. ryegrass) has direct and indirect impacts on P availability for maize in intercropping systems. Field pea promotes faster P solubilization and availability via acidification and bacterial activity, while ryegrass fosters a more conservative, fungal-dominated pathway. The choice of intercrop can thus be used strategically to modulate phosphorus cycling and optimize maize nutrition according to management goals.

5 Conclusion

This study demonstrates that intercrop identity in maize-based intercropping systems plays a critical role in shaping rhizosphere phosphorus (P) dynamics, primarily through its influence on soil pH and microbial community composition. Field pea consistently induced rhizosphere acidification across all sites, a change that correlated with increased mobilization of orthophosphate phosphorus (P_Ortho). This shift was likely mediated by enhanced bacterial activity, particularly Actinobacteria, which are known to promote organic P mineralization under acidic conditions.

In contrast, ryegrass maintained a more neutral rhizosphere pH, favoring the retention of non-citric-acid-extractable phosphorus (NCA_P) and enriching fungal communities, most likely arbuscular mycorrhizal fungi (AMF). This operational fraction includes phosphorus forms not extractable by 1% citric acid, including both organic and mineral-bound species. This highlights a potential shift toward microbial P retention and slower nutrient cycling in grass-based systems.

Overall, our findings emphasize that plant species selection in intercropping systems can drive distinct belowground processes with direct consequences for soil phosphorus availability and cycling. Future work incorporating functional assays and high-resolution microbial community profiling would further clarify the mechanisms underpinning these crop-specific effects.

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7 Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary

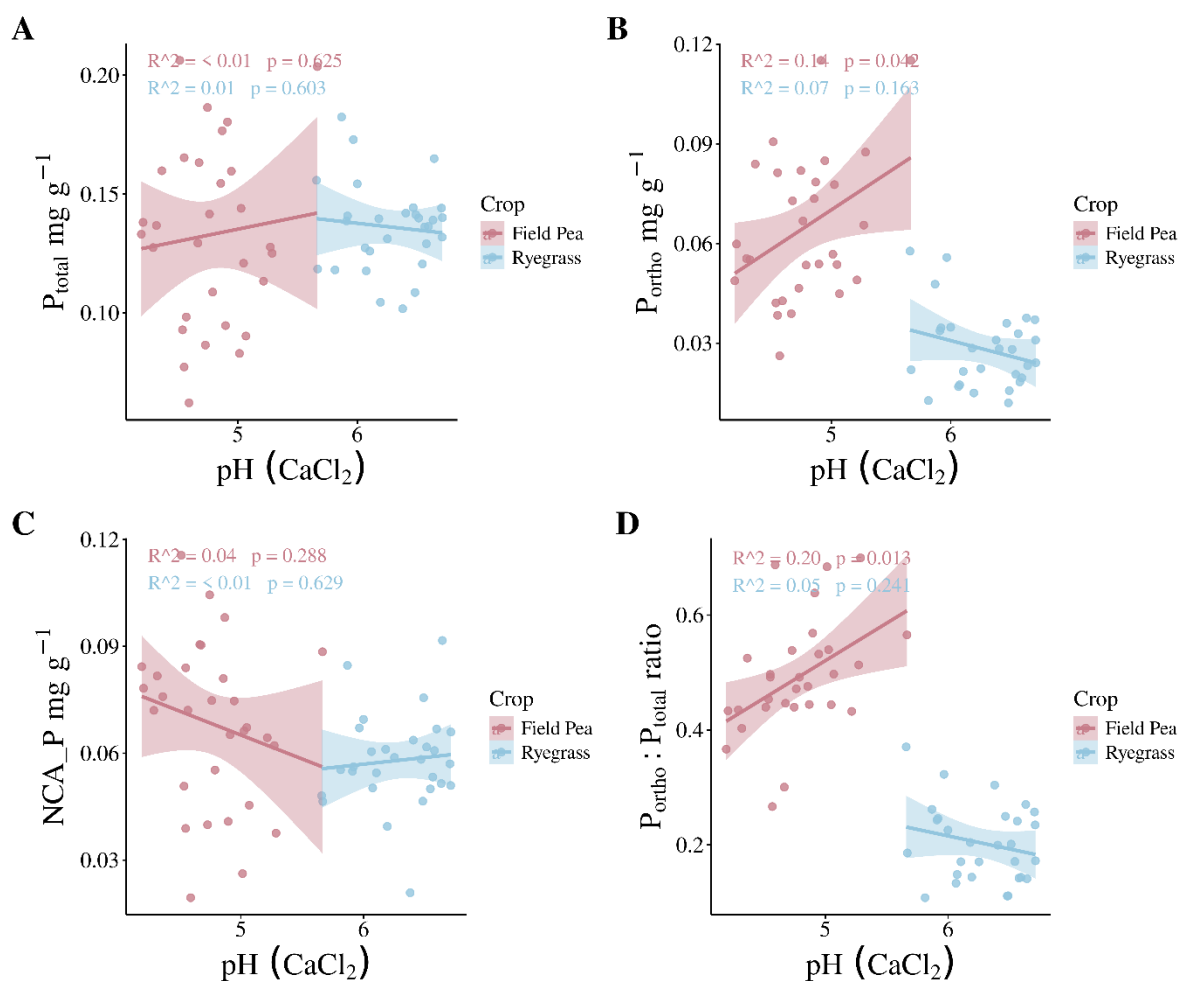
Site	Comparison	Mean pH (Field Pea)	Mean pH (Ryegrass)	Test	p-value
Site 1	Field Pea vs Ryegrass	4.96 ± 0.32	6.23 ± 0.28	Welch's t-test	< 0.001
Site 2	Field Pea vs Ryegrass	4.69 ± 0.31	6.18 ± 0.21	Welch's t-test	< 0.001
Site 3	Field Pea vs Ryegrass	4.65 ± 0.29	6.38 ± 0.27	Welch's t-test	< 0.001

Supplementary Table S1. Site-specific rhizosphere pH values under field pea and ryegrass intercropping. Table reports mean ± standard deviation (SD) for each crop at three field sites. Statistical comparisons were conducted using Welch's *t*-tests (*n* = 10 plots per treatment per site). All differences were highly significant (*p* < 0.001).

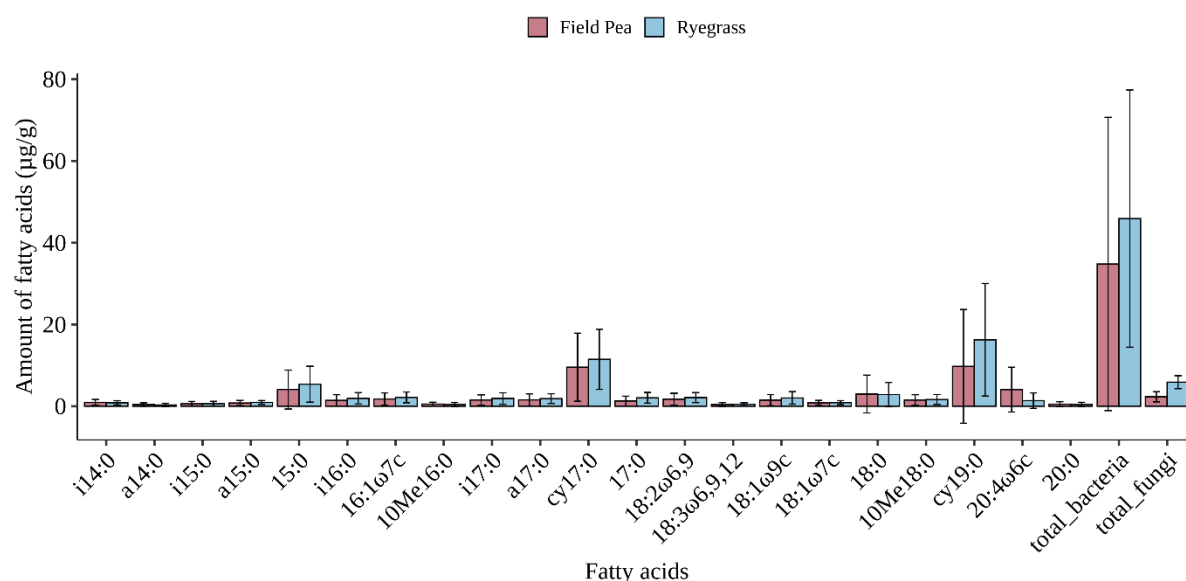
Comparison	Test Type	Statistic	p-value	Significant
P_Ortho: Field Pea vs Ryegrass	Welch's t-test	<i>t</i> = 7.82	< 0.0001	Yes
P_Total: Field Pea vs Ryegrass	Welch's t-test	<i>t</i> = 1.35	0.181	No
P_Organic: Field Pea vs Ryegrass	Welch's t-test	<i>t</i> = −1.67	0.101	No
pH ~ P_Ortho	Pearson Correlation	<i>r</i> = −0.536	0.0023	Yes
pH ~ P_Organic	Pearson Correlation	<i>r</i> = +0.204	0.2811	No
pH ~ P_Total	Pearson Correlation	<i>r</i> = +0.153	0.4187	No

Comparison	Test Type	Statistic	p-value	Significant
Regression: pH in P_Ortho model	Linear Regression	$t = -3.44$	0.0014	Yes
Regression: Crop (Ryegrass) in model	Linear Regression	$t = -4.52$	< 0.0001	Yes
Regression: Site 2 in model	Linear Regression	$t = 0.21$	0.834	No
Regression: Site 3 in model	Linear Regression	$t = -0.91$	0.366	No
Crop-specific: pH ~ P_Ortho (Pea)	Linear Regression	$R^2 = 0.14$	0.042	Yes
Crop-specific: pH ~ P_Ortho (Ryegrass)	Linear Regression	$R^2 = 0.07$	0.163	No
Crop-specific: pH ~ P_Ortho/P_Total (Pea)	Linear Regression	$R^2 = 0.20$	0.013	Yes
Crop-specific: pH ~ P_Ortho/P_Total (Ryegrass)	Linear Regression	$R^2 = 0.05$	0.241	No

Supplementary Table S2. Statistical comparisons and correlation summaries for phosphorus fractions and rhizosphere pH under field pea and ryegrass intercropping. Table includes t -tests, Pearson correlations, and regression models from both global and crop-specific analyses. Significance defined as $p < 0.05$.



Supplementary Figure S1. Crop-specific linear regression plots showing relationships between rhizosphere pH (CaCl₂) and phosphorus fractions under field pea (red) and ryegrass (blue) intercropping. Each point represents a plot-level observation ($n = 10$ per treatment per site). Panels show: (A) Total phosphorus (P_Total), (B) Orthophosphate phosphorus (P_Ortho), (C) Non-citric-acid-extractable phosphorus (NCA_P), and (D) P_Ortho: P_Total ratio. R^2 and p -values are reported per crop. Shaded areas represent 95% confidence intervals. Significant relationships were observed only for field pea in panels (B) and (D), suggesting a legume-specific acidification effect on phosphorus availability.



Supplementary Figure S2. Relative abundance (mean $\pm$ SD) of individual phospholipid fatty acid (PLFA) biomarkers extracted from rhizosphere soil in maize intercropped with field pea (red) and ryegrass (blue). Biomarkers are categorized by microbial groups, including Actinobacteria (e.g., 10Me16:0), Gram-positive and Gram-negative bacteria (e.g., i15:0, cy19:0), fungi (e.g., 18:2ω6,9), and general microbial indicators. Values represent means of 10 replicate plots per treatment and site. Groupings used for microbial community structure analysis are based on factor analysis detailed in Supplementary Table S2.

Table S3: Phospholipid fatty acid standards used in GC–MS analysis

FA Type	Name (IUPAC)	Common Name	Abbreviation	Retention Time (s)*	Microbial Indicator Group
Saturated	Tetradecanoic acid	Myristic acid	14:0	150	General bacteria
	Pentadecanoic acid	—	15:0	355	General bacteria
	Hexadecanoic acid	Palmitic acid	16:0	630	General bacteria
	Heptadecanoic acid	Margaric acid	17:0	1010	General bacteria
	Octadecanoic acid	Stearic acid	18:0	1520	General bacteria
	Eicosanoic acid	Arachidic acid	20:0	2930	—

FA Type	Name (IUPAC)	Common Name	Abbreviation	Retention Time (s)*	Microbial Indicator Group
Branched-chain	11-Methyltridecanoic acid	Anteisomyristic	a14:0	100	Gram-positive bacteria
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	90	Gram-positive bacteria
	12-Methyltetradecanoic acid	12-Methylmyristic	a15:0	290	Gram-positive bacteria
	13-Methyltetradecanoic acid	13-Methylmyristic	i15:0	270	Gram-positive bacteria
	13-Methylpentadecanoic acid	Anteisopalmitic	a16:0	545	Gram-positive bacteria
	Methylpentadecanoic acid	14-Isopalmitic	i16:0	525	Gram-positive bacteria
	14-Methylhexadecanoic acid	14-Methylpalmitic	a17:0	890	Gram-positive bacteria
	15-Methylhexadecanoic acid	15-Methylpalmitic	i17:0	860	Gram-positive bacteria
Cyclopropane	cis-9,10-Methylenehexadecanoic acid	Methylpalmitic	cy17:0	940	Gram-negative bacteria
	cis-9,10-Methyleneoctadecanoic acid	Dihydrosterculic acid	cy19:0	2025	Gram-negative bacteria
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic	10Me16:0	780	Actinobacteria
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	1745	Actinobacteria
Mono-unsaturated	9-Tetradecenoic acid	Myristoleic acid	14:1 ω 5c	130	–
	cis-11-Hexadecenoic acid	–	16:1 ω 5c	595	AM fungi
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1 ω 7c	570	Gram-negative bacteria
	cis-Octadecenoic acid	Cis-vaccenic acid	18:1 ω 7c	1400	Gram-negative bacteria
	cis-9-Octadecenoic acid	Oleic acid	18:1 ω 9c	1375	General bacteria
	11-Eicosenoic acid	Eicosenoic acid	20:1 ω 9c	2700	–
Poly-unsaturated	cis,cis-9,12-Octadecadienoic acid	Linoleic acid	18:2 ω 6,9	1320	Fungi
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4 ω 6	2320	Protozoa
	cis,cis,cis-6,9,12-Octadecatrienoic acid	γ -Linolenic acid	18:3 ω 6,9,12	1355	–

Supplementary table S3. List of phospholipid fatty acids (PLFAs) included in the external standard used for GC–MS analysis. Table includes IUPAC names, common names, standard abbreviations,

retention times (in seconds) based on a 45 m × 0.5 mm GC column (±15 s tolerance), and their microbial group assignments where applicable. These markers were used to classify microbial communities in rhizosphere soil samples under maize–intercrop systems.

Supplementary table S4

Top PLFA biomarker loadings from PCA (5 components, VARIMAX rotated). Values shown are unrounded for clarity. Bold values indicate the highest loading per biomarker across components.

PLFA Biomarker	PC1	PC2	PC3	PC4	PC5
i17:0	0.263	−0.062	0.046	−0.023	−0.040
i16:0	0.260	−0.068	0.085	−0.007	−0.051
16:1ω5c	0.256	−0.067	0.033	−0.020	−0.163
cy17:0	0.255	−0.085	−0.045	−0.071	−0.015
a15:0	0.255	−0.079	0.048	−0.040	−0.016
10Me18:0	0.254	−0.011	0.030	0.086	−0.143
i15:0	0.242	−0.160	−0.002	0.014	0.026
16:0	0.238	−0.146	−0.048	0.086	0.192
10Me16:0	0.230	−0.045	0.073	−0.248	−0.126
18:1ω9c	0.229	−0.165	−0.151	−0.024	−0.063
14:0	0.229	0.158	0.082	−0.062	0.151
18:3ω6,9,12	0.221	−0.105	−0.290	0.071	−0.183
cy19:0	0.221	−0.184	−0.040	−0.117	0.097
a16:0	0.204	0.174	−0.229	0.069	−0.221
14:1ω5c	0.194	0.169	−0.339	−0.044	0.085
18:0	0.192	−0.018	−0.074	0.292	0.440
17:0	0.174	0.261	0.003	−0.063	−0.029
16:1ω7c	0.156	−0.038	0.556	0.045	0.086
20:0	0.141	0.286	−0.125	0.051	0.034

PLFA Biomarker	PC1	PC2	PC3	PC4	PC5
i14:0	0.127	0.273	0.118	−0.008	−0.067

Supplementary table S5

Microbial groupings based on dominant PCA loadings per biomarker. Grouping uses ecological function and PCA dominance (≥ 0.22 loading on PC1 or strongest elsewhere).

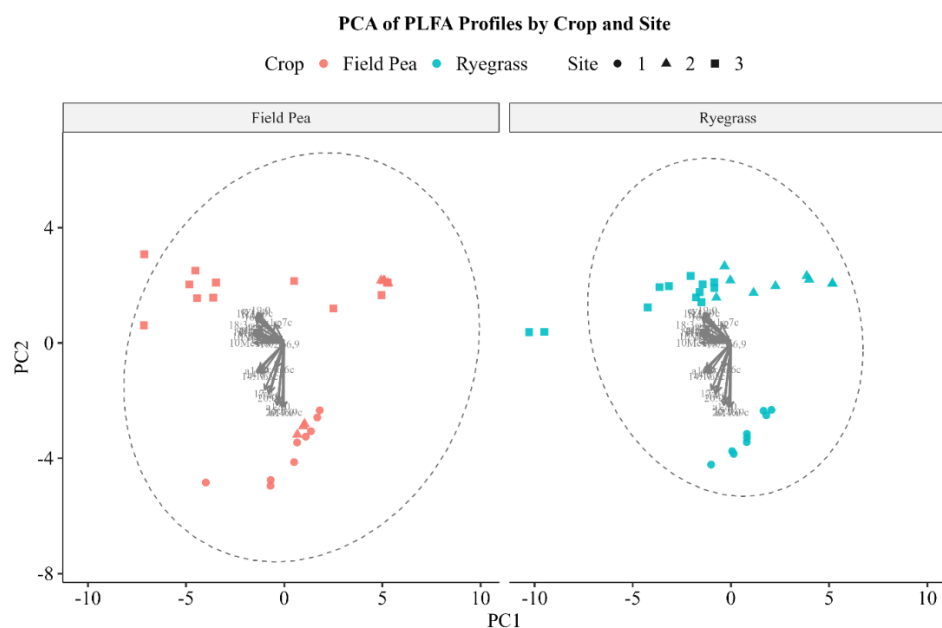
PLFA Biomarker	Assigned Group	Justification
i15:0, i16:0, i17:0, a15:0, a16:0, a17:0	Gram-positive bacteria	Branched-chain saturated markers
cy17:0, cy19:0, 16:1 ω 7c, 18:1 ω 7c	Gram-negative bacteria	Cyclopropane/unsat markers typical
10Me16:0, 10Me18:0	Actinobacteria	Methylated fatty acids
18:2 ω 6,9, 18:3 ω 6,9,12	Fungi	Polyunsaturated markers
16:1 ω 5c	AM fungi	Mycorrhizal-specific marker
14:0, 16:0, 17:0, 18:0	General bacterial biomass	Saturated core membrane FAs

Supplementary table S6

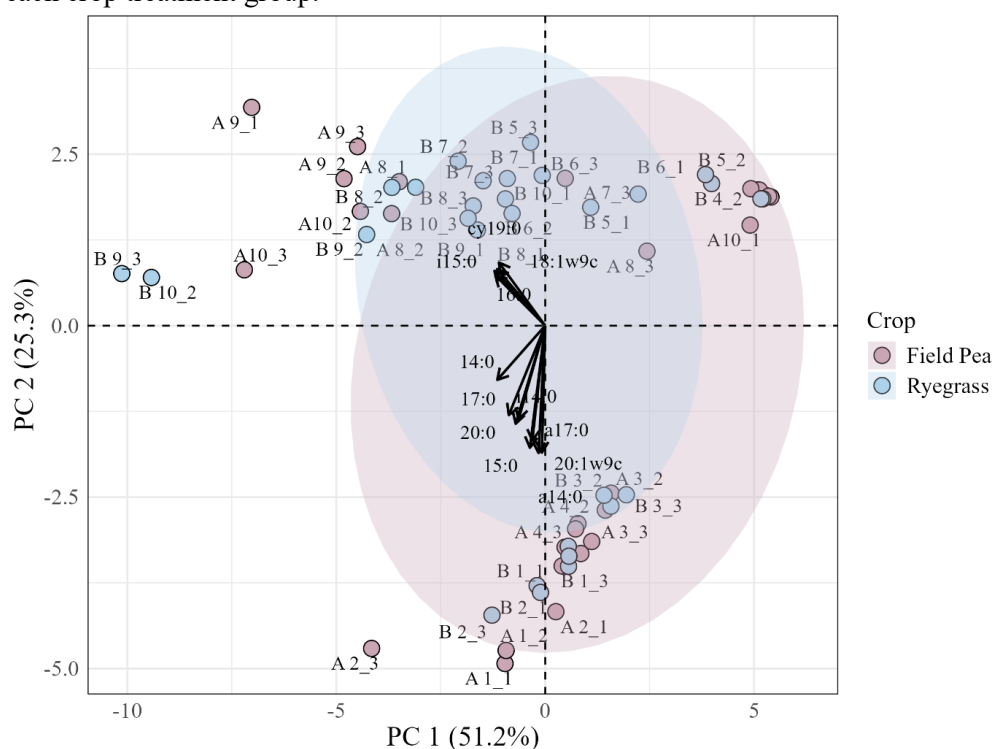
Microbial groups associated with top PCA components based on biomarker loadings. Each factor represents dominant ecological signals.

PCA Axis (Component)	Dominant Microbial Group	Biomarkers
PC1	General & Gram-positive bacteria	i15:0, a15:0, i16:0, 16:0, i17:0
PC2	Saturated markers / Gram-neg.	i14:0, 14:0, 17:0, 20:0
PC3	Gram-negative bacteria	16:1 ω 7c, 18:1 ω 7c, 14:1 ω 5c
PC4	Actinobacteria / Fungi mix	10Me16:0, 18:2 ω 6,9, 10Me18:0
PC5	Fungi	18:3 ω 6,9,12, 18:0

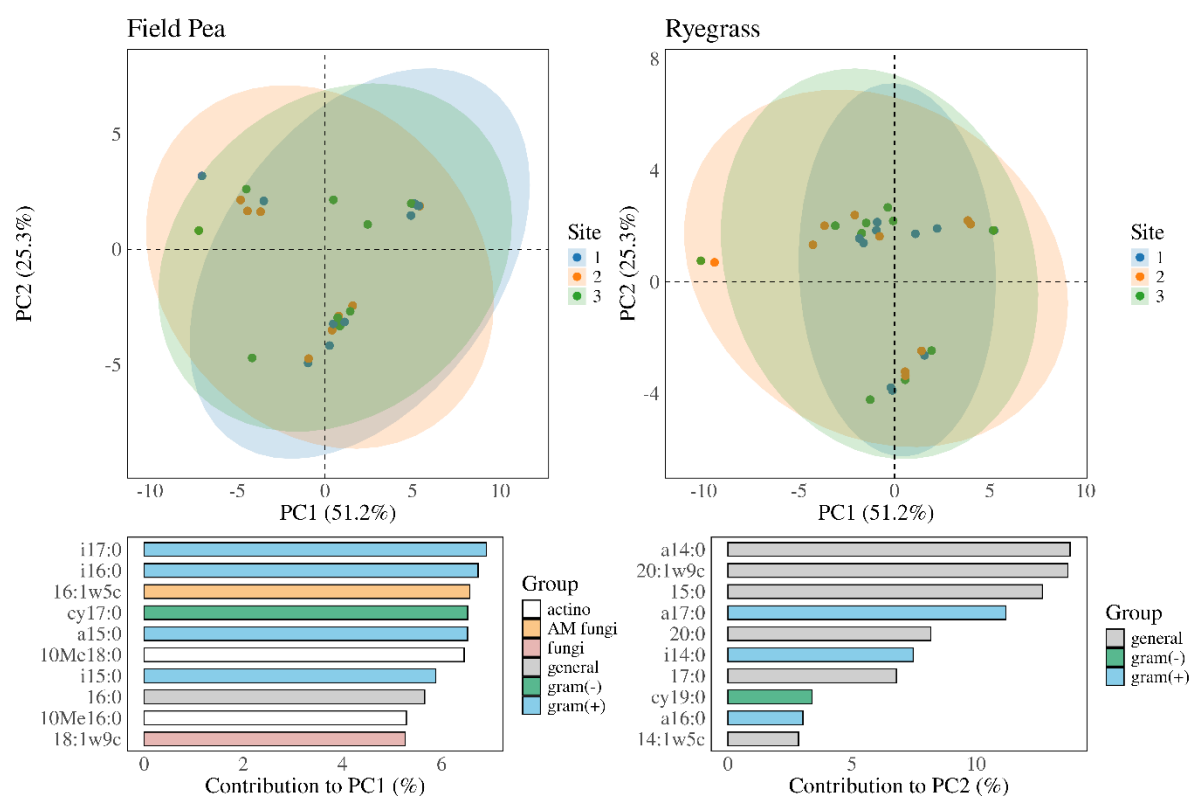
Supplementary tables S4–S6. Results of principal component analysis (PCA) of 27 PLFA biomarkers derived from maize rhizosphere samples. Table S2 shows the top 20 loading values from a 5-component VARIMAX-rotated PCA. Table S3 assigns each PLFA to microbial functional groups based on diagnostic literature and loading patterns. Table S4 summarizes dominant microbial groups associated with each PCA component axis.



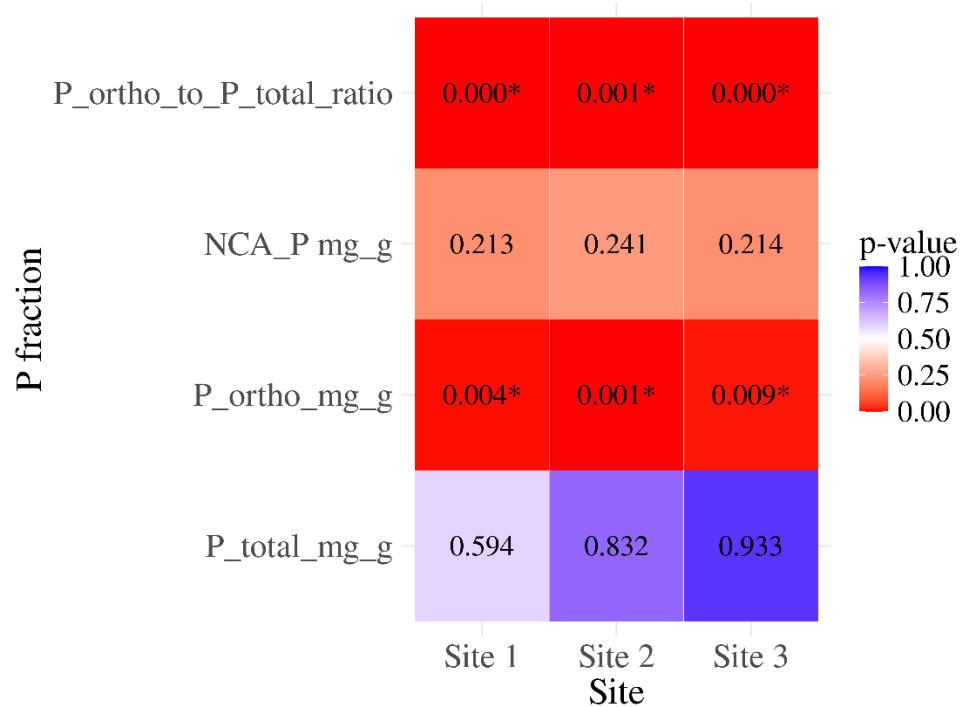
Supplementary Figure S3. Principal Component Analysis (PCA) of phospholipid fatty acid (PLFA) profiles in rhizosphere soil under maize intercropped with field pea (red) and ryegrass (blue), across three sites (symbols). PCA was performed on 27 standardized PLFA biomarkers. The first two components (PC1, PC2) explain the major variance in microbial community structure. Microbial markers are shown as vectors indicating loadings. Dotted ellipses denote 95% confidence intervals for each crop treatment group.

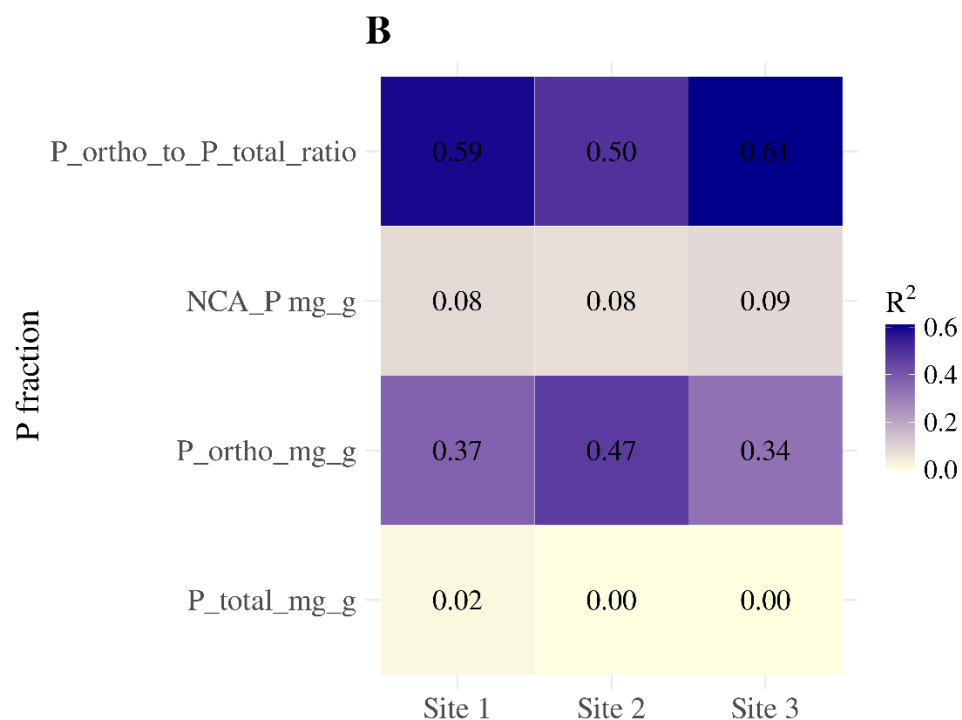


Supplementary Figure S4. Principal Component Analysis (PCA) of phospholipid fatty acid (PLFA) profiles from rhizosphere soil in maize–intercropping systems with field pea (purple) and ryegrass (blue). Individual samples are plotted based on their PC1 (51.2%) and PC2 (25.3%) scores. Ellipses represent 95% confidence intervals around each crop group. Vectors represent the loadings of individual PLFA biomarkers (e.g., *18:1 ω 9c*, *cy19:0*, *10Me16:0*) contributing to microbial community separation along the two principal components.



Supplementary Figure S5. Principal component analysis (PCA) of rhizosphere microbial communities under maize–intercropped Field Pea and Ryegrass systems. Top panels display PCA score plots of individual samples for each crop, with 95% confidence ellipses. Bottom panels show the relative contributions of individual phospholipid fatty acid (PLFA) biomarkers to PC1 (left) and PC2 (right), grouped by microbial functional categories: actinobacteria (*actino*), arbuscular mycorrhizal fungi (*AM fungi*), fungi, general bacteria, Gram-negative (*gram(-)*), and Gram-positive bacteria (*gram(+)*).





Supplementary Figure S6. (A) p-values from site-wise Pearson correlations between rhizosphere pH (CaCl₂) and phosphorus fractions (P_Ortho, NCA_P, P_Total, and P_Ortho : P_Total ratio) across three experimental sites. Significant correlations ($p < 0.05$) are marked with asterisks. (B) Corresponding R^2 values from linear regressions between pH and P fractions. Strongest explanatory power was observed for the P_Ortho : P_Total ratio, with R^2 values up to 0.62.

2.3 Study 3. Genotype matters: Shaping nitrogen uptake and fungal symbiosis in maize–faba bean intercrops

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Abstract

Improving nutrient efficiency while reducing fertilizer dependence is a key goal for sustainable agriculture. Intercropping cereals with legumes enhances nutrient cycling and soil health, but the role of cereal genotype in shaping root-microbe interactions remains underexplored. In this field study, we examined how three different maize genotypes (FC1571, B73WT, and A347) influence nitrogen (N) uptake and colonization by arbuscular mycorrhizal fungi (AMF) when grown alone or intercropped with faba bean (*Vicia faba* L.). A ¹⁵N tracer was used to track nitrogen recovery, while root samples were assessed for AMF colonization.

Intercropping generally enhanced ¹⁵N recovery and AMF colonization, though the magnitude of response varied significantly by genotype. A347, despite having lower AMF colonization levels, exhibited the strongest correlation between AMF presence and ¹⁵N uptake ($R^2 = 0.64$), suggesting highly functional symbiosis. In contrast, FC1571 had the highest AMF colonization overall but showed a weaker relationship with N recovery ($R^2 = 0.15$), indicating less efficient coupling.

These findings highlight the critical role of maize genotype in modulating microbial interactions and nutrient responsiveness. Selection for genotypes with not only high colonization but also functional mycorrhizal efficiency may unlock the full potential of intercropping systems in sustainable, low-input agriculture.

Keyword: Intercropping, Maize genotype, Faba bean, Arbuscular mycorrhizal fungi (AMF), Nitrogen uptake, ¹⁵N tracer

Highlight

- Intercropping with faba bean significantly increased AMF colonization in maize roots.
- Maize genotype FC1571 showed highest ¹⁵N uptake but weak AMF–N coupling ($R^2 = 0.15$).
- A347 exhibited strong AMF–N correlation ($R^2 = 0.64$) despite low colonization rates.

- Genotype $\times$ cropping system interaction revealed trait-dependent intercrop performance.
- Functional responsiveness to AMF is more predictive of nitrogen recovery than colonization level.

1 Introduction

Improving nutrient efficiency while reducing fertilizer dependency remains a critical challenge in sustainable agriculture. Intercropping cereal crops with legumes is a promising approach to enhance nutrient dynamics, improve soil structure, and promote long-term system productivity. Among such systems, maize (*Zea mays* L.) intercropped with faba bean (*Vicia faba* L.) is particularly valuable due to their complementary functions. Legumes contribute fixed atmospheric nitrogen through symbiosis with rhizobia, while maize benefits from this added nitrogen pool via spatial and temporal niche differentiation. This synergy can reduce external nitrogen inputs and increase nutrient use efficiency (Brooker et al., 2015a; L. Li et al., 2014). Beyond nutrient contributions, intercropping supports beneficial soil microbial associations, particularly with arbuscular mycorrhizal fungi (AMF). These fungi establish mutualistic relationships with most terrestrial plants, facilitating the uptake of key nutrients, especially phosphorus and nitrogen, and enhancing tolerance to abiotic stress in nutrient-limited environments (Smith & Smith, 2011). Within intercropping systems, AMF networks may connect host species, forming a shared nutrient conduit that improves system-level productivity (Hodge & Fitter, 2010). Additionally, AMF contribute to soil aggregation, carbon cycling, and resilience to environmental fluctuations, reinforcing their significance in sustainable cropping systems (Rillig & Mummey, 2006).

While species-level interactions in intercropping have been extensively studied, the role of intraspecific genotypic variation is less understood. In maize, genotypic differences in root morphology, exudation profiles, and mycorrhizal responsiveness significantly influence nutrient foraging capacity and belowground microbial interactions (Sawers et al., 2008; X.-X. Wang et al., 2020). Genotypes with more finely branched or fibrous root systems are thought to be more responsive to AMF and more efficient in nutrient foraging. However, whether these differences translate into functional benefits particularly under field condition remains an open question.

Most current understanding of these interactions comes from controlled-environment studies, which do not fully replicate the heterogeneity and complexity of real field conditions. Factors such as soil texture, microbial diversity, and weather variability can significantly influence nutrient dynamics and microbial colonization (Van Der Heijden et al., 2008). There remains a

clear gap in field-based research evaluating how maize genotypes differ in their ability to engage with microbial partners and acquire nitrogen when grown in intercropped systems. This study addresses this gap by evaluating three maize genotypes FC1571, B73WT, and A347 grown alone and in intercropping with faba bean. We used ^{15}N isotope tracing to quantify nitrogen uptake and measured AMF colonization via root staining and microscopy. Importantly, we examined the relationship between AMF colonization and ^{15}N recovery to determine whether colonization intensity translates into functional nitrogen benefits. We hypothesize that maize genotypes differ not only in their degree of AMF colonization, but also in the effectiveness of these interactions in enhancing nitrogen uptake. Identifying such genotype-specific traits may inform breeding programs focused on optimizing root-microbe cooperation for sustainable, low-input agriculture.

2 Materials and methods

2.1 Field site and experimental design

The field experiment was conducted during the 2024 growing season at Wurzelhof, Schinkel, Schleswig-Holstein, Germany (54°22'0" N, 9°57'46" E), situated in a temperate climate zone. The soil at the site is classified as a Hortic Anthrosol, with high organic matter content in the topsoil. Average monthly temperatures during the growing season ranged from 15°C to 22 °C, with a total precipitation of approximately 450 mm during the April–September growing season (Deutscher Wetterdienst (DWD), 2024). The experimental layout followed a randomized complete block design with three replicates per treatment. Treatments consisted of three maize (*Zea mays* L.) genotypes representing distinct root phenotypes: FC1571; fibrous, highly branched root system, B73WT; standard wild-type genotype with a fibrous root system and extensive root hairs, adapted to diverse soil environments, A347; sparse, deep-reaching root architecture. Each genotype was cultivated under two cropping systems: Monoculture (maize only), and intercropped with faba bean (*Vicia faba* L.), a commonly used European legume cultivar. A total of 18 plots were established (3 maize genotypes $\times$ 2 cropping systems $\times$ 3 replicates), each measuring 2 m $\times$ 1.5 m. Six rows were planted lengthwise in alternating maize–bean order (1:1), with 15 cm row spacing and 20 cm between plants within rows. Monocropped plots included four maize rows with wider inter-row spacing to reflect the absence of faba bean, creating a lower-density, open-canopy structure.

2.2 ^{15}N isotope labeling and plant harvesting

To investigate nitrogen uptake efficiency across maize genotypes in intercrop systems, a ^{15}N labeling experiment was conducted during the 2024 field season. At the time of tracer application, maize plants were at BBCH stage 50, flowering.

A tracer solution was prepared using ^{15}N -enriched sodium nitrate $\text{Na}^{15}\text{NO}_3 \geq 98 \text{ atom } \% ^{15}\text{N}$; (Sigma-Aldrich, Germany) ^{15}N -enriched ammonium sulphate ($(^{15}\text{N} \text{ H}_4)_2\text{SO}_4, \geq 98 \text{ atom } \% ^{15}\text{N}$) (Sigma-Aldrich, Germany). The tracer was applied to pre-drilled holes at two soil depths (20 cm: topsoil and 70 cm: subsoil), with 20 mL of solution injected per hole using a syringe attached to a tube with perforations at the targeted depth. Data from all genotypes were grouped by cropping system and analyzed for both topsoil (20 cm) and subsoil (70 cm) injection depths. Plants were harvested seven days after labeling. We collected aboveground biomass (shoots) and roots of maize and faba bean. Aboveground biomass samples were oven-dried at 65°C for 5–6 days and then ground into a fine powder using a ball mill. Root tips (4–5 cm) designated for AMF analysis were carefully washed and stored at -20°C to preserve structural integrity and prevent microbial activity until staining. The remaining root material used for nitrogen analysis was processed following the same drying and grinding protocol as the shoot biomass. Subsamples ($\sim 2 \text{ mg}$) were weighed using a precision microbalance ($\pm 0.001 \text{ mg}$) and analyzed for total nitrogen content and ^{15}N enrichment using an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, Thermo Scientific, Germany). ^{15}N recovery and nitrogen uptake were calculated following the excess ^{15}N method described by (Hauck & Bremner, 1976).

2.3 Assessment of AMF colonization

Maize-root segments (1–2 cm in length) were washed thoroughly and cleared in 10% potassium hydroxide (KOH) at 90°C , then stained using the ink–vinegar method (Vierheilig et al., 1998). Arbuscular mycorrhizal fungi (AMF) colonization was quantified using the gridline intersect method under a compound microscope at $400\times$ magnification. For each sample, a minimum of 100 intersects were scored for the presence of AMF structures, including hyphae, vesicles, and arbuscules. Total colonization (%) was calculated as the proportion of intersects containing any AMF structure relative to the total number of observed intersects.

2.4 Statistical analysis

All statistical analyses were performed in (R Core Team, 2023). A two-way analysis of variance (ANOVA) was used to assess the effects of maize genotype and cropping system on ^{15}N uptake and AMF colonization, with field block included as a random factor. The lme4 package was used to fit linear mixed-effects models, and post hoc comparisons were conducted using Tukey's Honest Significant Difference (HSD) test via the emmeans package. Statistical

significance was defined at $p < 0.05$. Relationships between ^{15}N uptake and AMF colonization were evaluated using Pearson correlation coefficients with the stats package. Data visualization was conducted using ggplot2 for plotting and dplyr for data manipulation. All results were summarized by genotype and cropping system, and visualized as grouped bar charts by cropping system within each genotype for both soil depths.

3 Results

3.1 Genotypic variation in ^{15}N recovery across cropping systems and depths

Application of ^{15}N -labeled nitrogen enabled detailed quantification of nitrogen uptake across maize genotypes under monocropped and intercropped conditions. Results revealed strong genotype-dependent patterns in tracer recovery, with intercropping enhancing uptake generally enhancing uptake, particularly from the topsoil (20 cm) (Figure 1).

Among the genotypes, A347 showed the highest ^{15}N recovery under intercropped conditions from the topsoil, with values exceeding 11% of applied tracer, despite exhibiting relatively low AMF colonization. In contrast, recovery from the subsoil (70 cm) remained low ($<2\%$) across all cropping systems (Figure 2).

B73WT displayed moderate uptake, with slightly higher values under intercropping than monoculture. Topsoil uptake was higher ($\sim 5\%$) compared to the subsoil, where recovery dropped below 2%. Interestingly, faba bean grown with B73WT also absorbed tracer nitrogen, and beans paired with FC1571 showed comparable ^{15}N enrichment levels.

FC1571 exhibited consistently strong ^{15}N uptake, especially in the intercropped topsoil treatments, reaching up to $\sim 10\%$. Subsoil uptake was also measurable for FC1571, though lower in magnitude.

Across all genotypes and cropping systems, ^{15}N recovery was consistently higher from the topsoil (20 cm) than from the subsoil (70 cm), confirming the dominant role of the upper rooting zone in nitrogen uptake (Figures 1 and 2). This trend was particularly evident in A347 under intercropping, whereas FC1571 exhibited a broader vertical uptake profile, recovering ^{15}N from both depths. These depth-specific patterns suggest that genotype-specific root traits and microbial interactions jointly influence the vertical distribution of nitrogen acquisition.

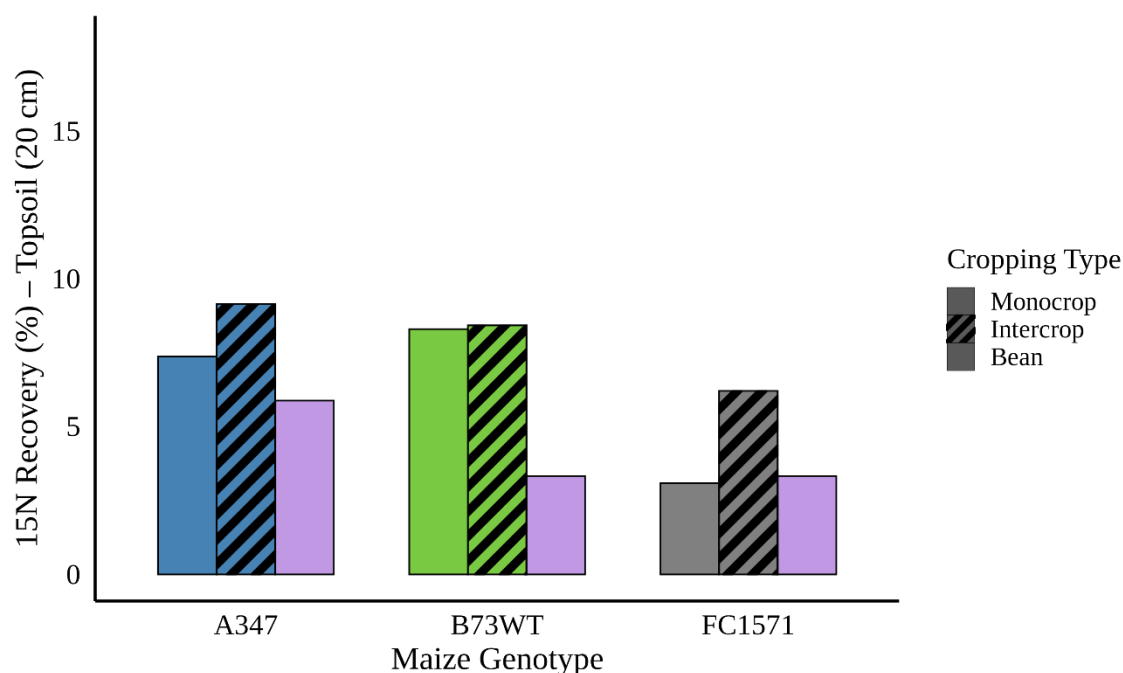


Figure 1. ^{15}N recovery in shoots of maize genotypes (A347, B73WT, FC1571) and intercropped faba bean from the topsoil (20 cm). Within each genotype group, bars represent monocropped maize, intercropped maize (striped), and intercropped bean (solid lilac). Bars show means; error bars represent standard error (SE).

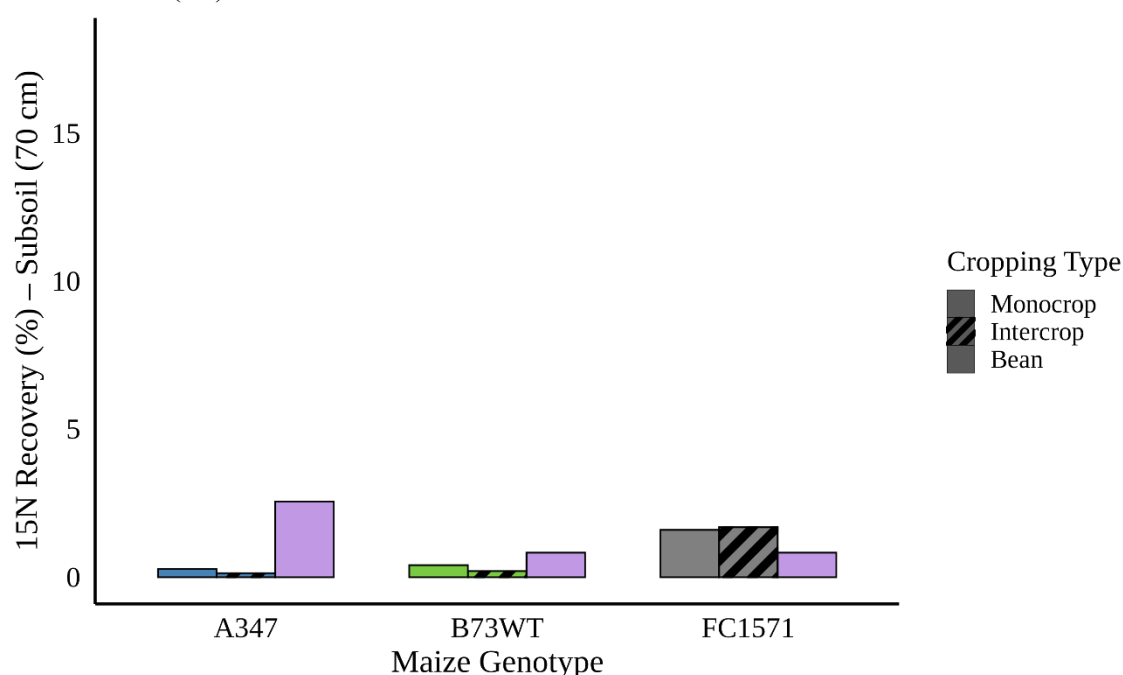


Figure 2. ^{15}N recovery in shoots of maize genotypes (A347, B73WT, FC1571) and intercropped faba bean from the subsoil (70 cm). As in Figure 1, cropping systems are shown side-by-side for each genotype, with black stripes denoting intercropped maize. Bars show means; error bars represent standard error (SE).

3.2 Mycorrhizal colonization patterns and functional response

Arbuscular mycorrhizal fungal (AMF) colonization varied across genotypes and cropping systems (Figure3). Intercropping consistently enhanced colonization levels in maize roots

relative to monoculture. A two-way ANOVA revealed a main effect of cropping design on AMF colonization ($p = 0.0228$), with no significant genotype $\times$ cropping system interaction suggesting a positive effect of faba bean on mycorrhizal association. Among the genotypes, FC1571 had the highest mean colonization (59.0% intercropped vs. 48.7% monocropped), followed by B73WT (53.3% vs. 45.7%) and A347 (47.3% vs. 37.3%). While the general trend of increased AMF colonization under intercropping was evident, the magnitude of the response varied, being most pronounced in FC1571 and less distinct in A347 and B73WT.

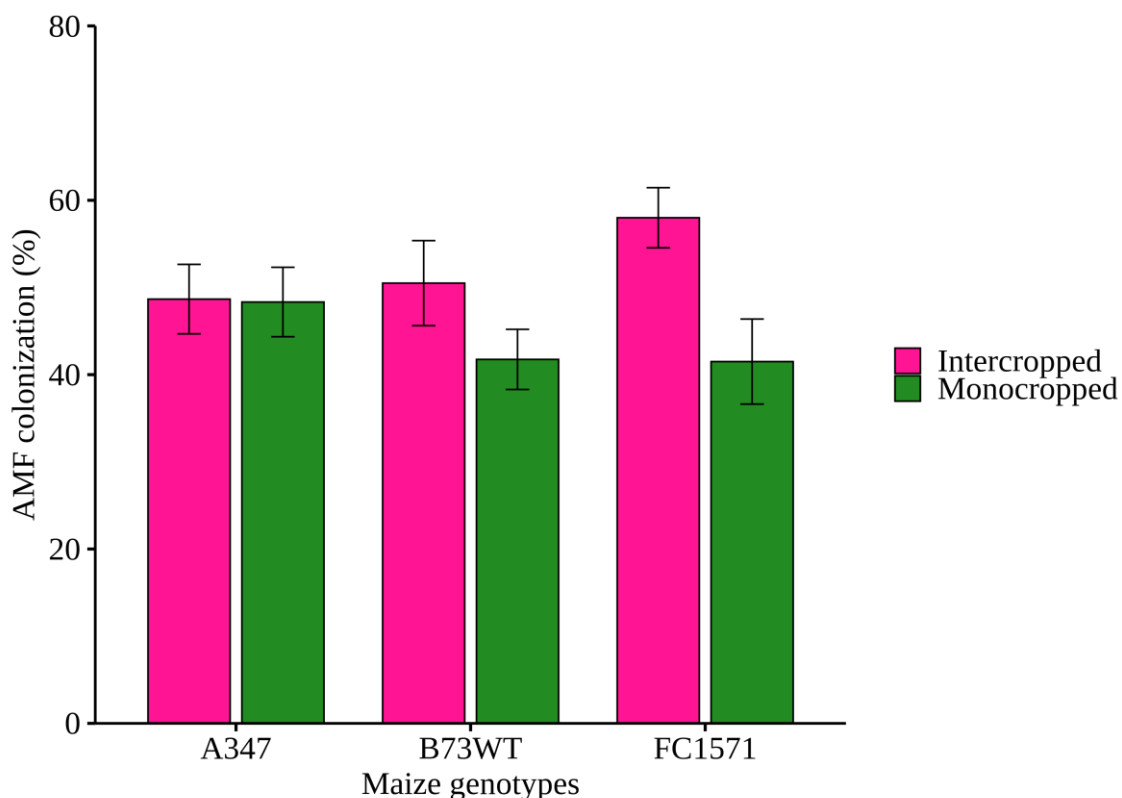


Figure 3. Total AMF colonization (%) in maize roots across three genotypes (B73WT, FC1571, A347) under monocropped and intercropped systems. Bars represent mean $\pm$ SE ($n = 3$). (ANOVA $p = 0.0228$).

3.3 Genotype-specific AMF responsiveness

To evaluate the functional role of AMF colonization in nitrogen acquisition, genotype-specific linear regression was performed between total AMF colonization (%) and ^{15}N recovery (Figure 4). Each point represents an individual maize plant ($n = 6$ per genotype; 3 replicates per cropping system). While all genotypes were colonized by AMF, their responsiveness in terms of ^{15}N uptake varied substantially.

Genotype A347 exhibited the strongest positive correlation ($R^2 = 0.64$), suggesting an efficient mycorrhizal contribution to nitrogen uptake despite having the lowest overall colonization percentage. This indicates that a functionally optimized symbiosis can lead to high nutrient

acquisition even at modest colonization levels. In contrast, FC1571 showed moderate colonization but a weaker correlation ($R^2 = 0.15$), while B73WT showed little functional linkage ($R^2 = 0.04$), despite moderate colonization levels. These results suggest that the genotype-specific nature of AMF functionality, likely driven by differences in root traits or exudation patterns that influence fungal-mediated nutrient transfer.

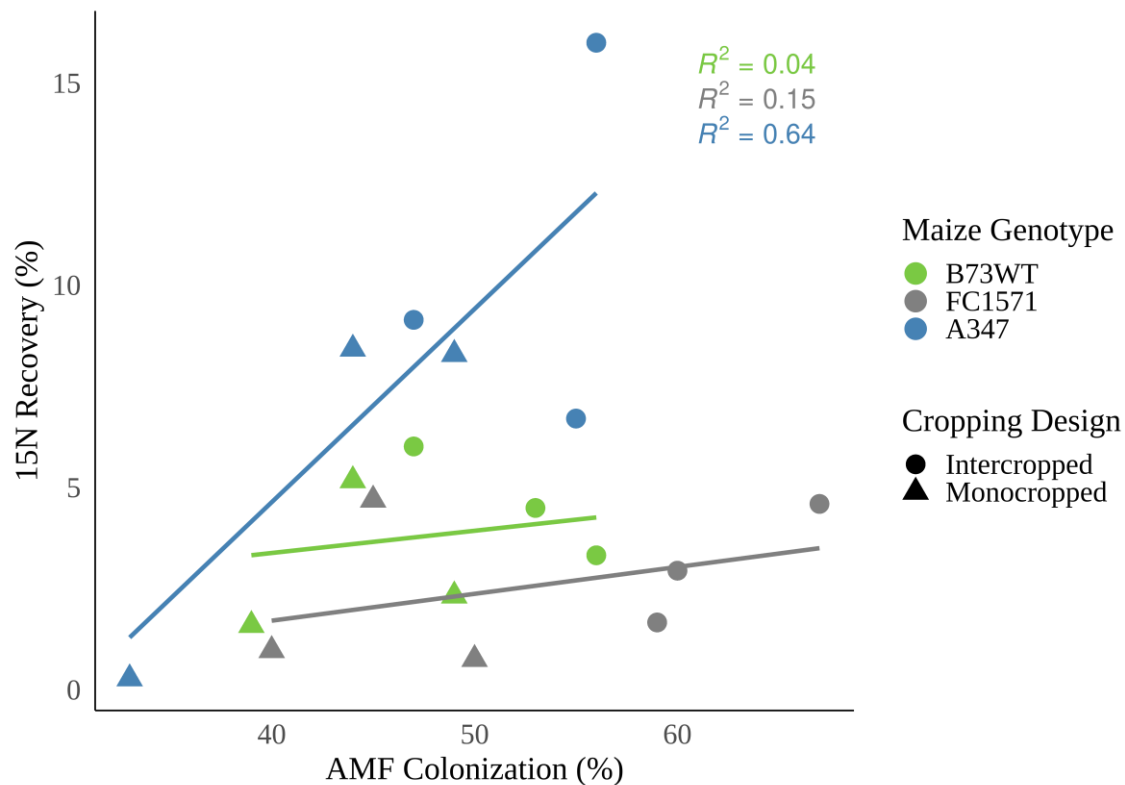


Figure 4 . Relationship between arbuscular mycorrhizal fungi (AMF) colonization and ^{15}N recovery in three maize genotypes under intercropping and monocropping. Each point represents an individual plant ($n = 6$ per genotype). Linear regression lines and R^2 values are shown for each genotype. A347 showed the strongest functional correlation ($R^2 = 0.64$), followed by FC1571 ($R^2 = 0.15$) and B73WT ($R^2 = 0.04$).

4 Discussion

4.1 Genotypic variation governs nutrient uptake efficiency

This study provides compelling evidence that maize genotype plays a pivotal role in shaping N acquisition dynamics under intercropping. Among the tested genotypes, FC1571 consistently outperformed both B73WT and A347 in ^{15}N uptake, regardless of cropping system. This suggests that intrinsic root traits, such as greater surface area, root branching, or exudation patterns, may enhance access to soil nitrogen pools, including those enriched by legume activity. These findings align with those of (Jemo et al., 2017), who highlighted the role of root morphology in enhancing nutrient capture under legume–cereal systems.

4.2 Depth-dependent ^{15}N dynamics reveal rooting zone preference

Consistently greater ^{15}N recovery from the 20 cm depth compared to 70 cm across genotypes reveals that nutrient uptake in early maize growth stages is predominantly confined to upper soil layers. However, FC1571 also showed relatively stronger uptake at 70 cm, particularly in intercropped treatments. This may indicate deeper rooting or improved vertical nutrient access, possibly facilitated by root complementarity or microbial mobilization in intercropping, as shown by (Reddy et al., 2020).

4.3 AMF colonization and N assimilation

Intercropping with faba bean significantly increased AMF colonization in maize roots across all genotypes likely due to rhizodeposition and root proximity effects (Gosling et al., 2006; Willis et al., 2013). The observed correlation between AMF colonization and ^{15}N uptake ($r = 0.72$, $p < 0.01$) supports a symbiotic role for AMF in enhancing nitrogen acquisition (Mäder et al., 2000).

While traditionally associated with phosphorus uptake (Smith & Smith, 2011), AMF have increasingly been linked to N acquisition, particularly under low-input or intercropped conditions, via improved root architecture, or enhanced access to microsites (Jansa et al., 2019; Mäder et al., 2011). This suggests that AMF act as a nutrient bridge in cereal–legume intercrops, supporting both hosts' nutritional demands (Hodge & Fitter, 2010).

4.4 Genotype-driven AMF responsiveness

The relationship between AMF colonization and nitrogen uptake varied markedly among genotypes. While FC1571 exhibited the highest colonization (up to 59%), the functional correlation with ^{15}N uptake was weak ($R^2 = 0.15$). Although FC1571 also recovered ^{15}N from both the topsoil and subsoil, this did not translate into greater functional efficiency, as reflected by its low AMF correlation. This indicates that its nitrogen acquisition may not be primarily AMF-mediated, but rather driven by other root-related mechanisms such as increased surface area or exudation activity.

In contrast, A347 displayed the strongest functional correlation ($R^2 = 0.64$), despite having the lowest colonization rates. This implies a more efficient or specialized AMF interaction when colonization occurred, likely facilitated by compatible AMF strains or local root plasticity. Such genotype-driven AMF responsiveness is well supported in prior studies (Sawers et al., 2008; X.-X. Wang et al., 2020).

B73WT, though intermediate in colonization, showed a negligible relationship ($R^2 = 0.04$), indicating a passive or inefficient mycorrhizal interaction. These findings challenge the assumption that colonization intensity equates to functional benefit and instead emphasize

qualitative responsiveness over quantitative colonization (Sawers et al., 2008; Smith & Smith, 2011).

4.5 Implications for sustainable intercropping systems

From an agroecological perspective, these findings reinforce the idea that genotype choice is critical in optimizing intercrop performance (Brooker et al., 2015a; X.-X. Wang et al., 2020). FC1571 may be a suitable candidate in systems prioritizing total nitrogen recovery, whereas A347 appears ideal where microbial efficiency and resource use per unit colonization matter most. Breeding programs should therefore prioritize genotypes that not only root aggressively but also engage symbiotically in functionally meaningful ways (Sawers et al., 2008).

By linking AMF function to ^{15}N tracer recovery under field conditions, this study adds to the growing evidence that microbial mutualisms should be explicit breeding targets, especially in low-input systems. These results align with prior work (Brooker et al., 2015a; Hodge & Fitter, 2010; Smith & Smith, 2011), and extend the current understanding by offering empirical genotype-based comparisons under real field conditions.

5 Conclusion

This field study demonstrates that maize genotype significantly modulates both nitrogen uptake and AMF colonization in cereal–legume intercropping. Intercropping with faba bean consistently enhanced both processes, but the magnitude and efficiency of benefit were strongly genotype-dependent.

While FC1571 achieved the highest absolute nitrogen uptake and colonization, A347 exhibited the most efficient functional relationship between AMF colonization and nitrogen recovery. These findings underscore that functional responsiveness to AMF, not colonization alone, should be a key selection criterion in genotype development for sustainable agriculture.

Future intercropping strategies and breeding programs should integrate root architecture traits, microbial compatibility, and functional symbiosis metrics to develop resource-efficient, resilient, and ecologically aligned cropping systems.

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Supplementary Information – Third Manuscript

Table S-1. Root colonization data per plot (field experiment)

Plot	Genotype	Treatment	Hyphae (%)	Vesicles (%)	Arbuscular (%)	Total colonization (%)
1	FC1571	Intercropped	41	15	4	60
2	FC1571	Monocropped	32	5	3	40
3	FC1571	Monocropped	33	12	4	49
4	FC1571	Intercropped	35	11	2	50
5	FC1571	Intercropped	38	14	4	56
6	FC1571	Monocropped	31	5	2	47
7	B73WT	Intercropped	39	22	6	67
8	B73WT	Monocropped	22	8	3	33
9	B73WT	Intercropped	25	22	3	50
10	B73WT	Monocropped	27	17	3	47
11	B73WT	Intercropped	22	28	5	55
12	B73WT	Monocropped	29	19	1	49
13	A347	Intercropped	15	24	1	40
14	A347	Monocropped	17	18	4	39
15	A347	Intercropped	27	17	0	44
16	A347	Monocropped	21	21	2	44
17	A347	Intercropped	33	22	4	59
18	A347	Monocropped	27	17	0	44

Table S-2. Mean total AMF colonization (%) by genotype and treatment

Genotype	Monocropped (%)	Intercropped (%)
A347	42.33	47.67
B73WT	43.00	57.33
FC1571	45.33	55.33