

Spatial heterogeneity in the rhizosphere microbiome and root nitrogen assimilation gene expression in soil-based and soilless cucumber and tomato organic greenhouse systems

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Abstract

Aims

The rhizosphere microbiome plays a central role in plant nutrition, yet it is often treated as a spatially homogeneous zone of plant influence. In organic greenhouse systems, where nutrients are supplied through surface-applied organic fertilizers, resource gradients may generate spatially heterogeneous rhizospheres. This study investigated how rhizosphere selection and sampling depth shape microbial communities, root nitrogen (N) assimilation gene expression, and their relationships with crop N offtake.

Methods

A split-plot experiment was conducted over two crop cycles (cucumber and tomato) in soil-based and soilless greenhouse systems under four organic fertilization regimes. Rhizosphere and bulk samples were collected at 0–5 and 10–15 cm, and microbial community composition, functional profiles, and root expression of N-related genes were analyzed.

Results

Rhizosphere selection interacted with sampling depth to shape prokaryotic and fungal community composition in both systems. Prokaryotic communities in the 0–5 cm rhizosphere were enriched in putative N-cycling functions relative to bulk soil and the 10–15 cm rhizosphere, particularly in the soilless system. Saprotrophic fungal communities were more evenly distributed across depths. Glutamine synthetase type 1 expression was consistently higher in roots from the 0–5 cm layer and was positively associated with numerous microbial taxa. Sampling depth also influenced the relationships among rhizosphere community composition, root N-assimilation gene expression, and crop N offtake.

Conclusions

These findings demonstrate that the rhizosphere is spatially heterogeneous and that accounting for this heterogeneity can improve understanding and management of root-soil-microbiome interactions in agroecosystems.

Introduction

The rhizosphere is a highly dynamic zone of interaction between plants and their associated microbial communities (Berendsen et al. 2012; Bulgarelli et al. 2013). It hosts a diverse microbiome, many members of which contribute to plant nutrition alongside a wide range of other ecological functions. Plant–microbe interactions begin early in plant development and intensify following the onset of photosynthesis, when plants allocate an estimated 10–40% of newly fixed carbon (C) belowground as

root exudates (Pausch and Kuzyakov 2018; Sasse et al. 2018; Zhalnina et al. 2018). These compounds serve as a major energy source for rhizosphere microorganisms and is a primary mechanism through which plants shape microbial community assembly. Consequently, compared with bulk soil, rhizosphere communities are often characterized by lower taxonomic diversity but greater microbial abundance and activity due to enhanced resource availability (Berendsen et al. 2012; Ling et al. 2022).

Traditionally, the rhizosphere has often been treated as a relatively homogeneous zone of plant influence (Wang et al. 2020). However, growing evidence suggests that rhizosphere processes are highly heterogeneous in space. Variations in root architecture, root age, exudation patterns, oxygen availability, moisture, and nutrient concentrations can generate distinct microhabitats within the rooting zone (Kuzyakov and Razavi 2019; Wang et al. 2020). Recent studies have begun to link spatial variation in root exudation to the spatial organization of microbial communities (Schultes et al. 2025). Consequently, this concept of rhizosphere heterogeneity has emerged as an important framework for understanding plant–microbe interactions (Wang et al. 2020; Schneider et al. 2026). Root systems play a central role in generating this heterogeneity. Root architecture is highly plastic and responds strongly to local environmental conditions, particularly nutrient availability (Forde 2014).

Organic greenhouse systems are highly intensive production environments in which high-yielding crops such as cucumber and tomato are cultivated for extended periods in either soil-based or soilless growing systems. Soil-based systems rely on the native mineral soil, providing crops with access to a relatively large soil volume (Dion et al. 2020). In contrast, soilless systems are characterized by cultivation in containers filled with peat-based or coir-based growing media (Burnett et al. 2016; Gonnella and Renna 2021); in Canada, crops with production cycles exceeding six months must be provided with a minimum of 60 L of growing medium per square meter of photosynthetic area (CAN/CGSB-32.310-2026, 2026). Owing to the high nutrient demand of these crops and the long duration of production cycles, organic fertilizers are commonly applied repeatedly as surface topdressing throughout the growing season (Voogt 2014; Dorais 2019). Such fertilization practices can result in nutrient inputs remaining concentrated near the growing medium surface, creating vertical gradients in nutrient availability. Nutrient gradients, particularly in nitrate availability, are known to influence root architecture, growth, and physiological responses (Mounier et al. 2014). Because roots actively forage for nutrients and adjust their metabolism to local resource conditions, spatial variation in nutrient availability may generate depth-dependent patterns in root functioning and rhizosphere microbial community assembly (Schneider et al. 2026). Despite growing recognition that rhizosphere processes are spatially heterogeneous, relatively little attention has been paid to how agricultural management practices contribute to the formation of spatially structured rhizospheres (Wang et al., 2020).

Understanding these processes is particularly important for nitrogen (N) cycling, especially in organic growing systems. Plant N acquisition in organic systems depends on tightly coupled interactions between roots and microorganisms because organic fertilizers and organic matter must first undergo microbial decomposition before nutrients become available for uptake. N transformations occurring in the rhizosphere are often more closely synchronized with plant demand than those occurring in bulk soil

(Schmidt et al. 2019). Furthermore, roots can influence N cycling in their immediate vicinity through rhizosphere-mediated processes such as rhizosphere priming and the regulation of nitrification and denitrification (Subbarao et al. 2013; Bardon et al. 2014; Moreau et al. 2019; Ling et al. 2022). Despite the central role of these interactions, it remains unclear to what extent nutrient gradients generated by surface-applied fertilization structure rhizosphere microbial communities and their functional potential along the root profile in organic soil-based and soilless greenhouse systems.

Crop N metabolism is itself a dynamic process involving multiple genes that respond to environmental conditions and local nutrient availability (Migocka et al. 2013). Consequently, depth-dependent nutrient availability may also generate spatial variation in root N assimilation. However, the extent to which fertilization practices shape vertical patterns of root N assimilation remains poorly understood. Likewise, whether such patterns are associated with corresponding changes in rhizosphere microbial community structure and function remains largely unexplored. Linking plant functional responses to microbiome assembly represents an emerging area of research. For example, expression of the nitrate transporter NRT1.1B has been associated with enhanced N-use efficiency and enrichment of specific N-cycling taxa in the rhizosphere of rice indica varieties (Zhang et al. 2019).

Here, we investigated how surface application of organic fertilizers influences the vertical organization of rhizosphere microbial communities and root N assimilation gene expression in organic greenhouse systems. We conducted a greenhouse experiment over two crop cycles (cucumber, *Cucumis sativus* var. *sativus* followed by tomato, *Solanum lycopersicum* var. *lycopersicum*) to compare two contrasting organic greenhouse growing systems (a soil-based and a soilless system using peat-based growing media) under four fertilization regimes. Specifically, we examined how rhizosphere selection, the process by which plant roots selectively recruit microorganisms from the surrounding bulk soil to shape a distinct rhizosphere microbiome, and sampling depth influence the taxonomic and functional composition of prokaryotic and fungal communities, how root expression of N assimilation genes varies across depths, and whether this plant and microbial responses are associated with total crop N offtake.

We hypothesized that, in addition to the combined effect of the crop type and the fertilization regime: (1) rhizosphere selection would interact with sampling depth to shape the structure and functional potential of the rhizosphere microbiome, leading to spatially heterogeneous rhizospheres; (2) expression of N-related genes in secondary roots would vary as a function of sampling depth and would be associated with patterns in the structure of the rhizosphere microbiome; (3) the 0–5 cm soil zone, in close proximity to the surface-applied organic fertilizer layer, would exhibit rhizosphere microbial and root transcriptional patterns most strongly associated total crop N offtake. We expected these effects to be present in both growing systems.

Materials and methods

Site description and experimental design

The experiment was carried out in a 400 m² certified organic greenhouse located at the Institut National d'Agriculture Biologique (INAB) in Victoriaville, Québec, Canada (46° 03' N, 71° 58' W). Two crop cycles were studied: a cucumber crop grown from May 12th to August 11, 2023, and a tomato crop grown from December 7, 2023, to May 9, 2024. In the soil-based system, a cycle of unfertilized mixed crops (lettuce, kale, zucchini) was grown from September 15th to November 15, 2023, and was not included in this study. Growing media in the soilless system was renewed between both crop cycles. Both crop cycles were established following the same split-plot experimental design, with growing system as the main factor and fertilization regime as the subplot factor. All growing system x fertilization regime combinations were replicated four times.

Two contrasting growing systems were studied. The soil-based system relied on native mineral soil, a dystic brunisol (Table 1; Rompré et al., 1984), which had been managed organically for six years under diversified vegetable production and fertilized primarily with feather meal during the three years preceding the study (see Table S1 for initial chemical parameters). One experimental unit of the soil-based system consisted of 8 m² plots (2 m x 4 m), with each plant grown in a volume of 80 L of soil, although root growth was not constrained between plants. The soilless system consisted of container cultivation using a peat-based growing medium (Table 1; Berger, QC, Canada). One experimental unit (4.4 m²) corresponded to four 60 L containers (0.55 m x 0.40 m x 0.32 m), with each plant grown in a volume of 27.5 L, although root growth was not constrained between the two plants grown in the same container. Crop management was standardized across systems, with cucumber (*Cucumis sativus* CV. 'Katrina', Johnny's seeds) and tomato (*Solanum lycopersicum* CV. 'Rebelski', Johnny's seeds) grown at 3 plants m⁻². Both crops were started as seedlings in a peat-based growing medium (Agro mix OS, Fafard, Qc, Canada) and transplanted after four (cucumber) or ten (tomato) weeks.

Four fertilization regimes were tested: (1) standard fertilization (StdFert), supplying 80% of N with feather meal and 20% with pelleted poultry manure; (2) alfalfa meal (AM) fertilization (100%AM), supplying N with only alfalfa meal; (3) 50%AM, combining alfalfa meal and StdFert in equal N proportions; and (4) control, receiving only the initial base fertilization. The StdFert formulation was chosen to meet crop N requirements while limiting excess inputs of P (Koller et al. 2016). Organic fertilizer rates were adjusted for nutrient composition, dry matter content, and estimated mineralization (Table S1; Dion et al., 2020) and surface-applied without incorporation on a weekly (cucumber) or biweekly (tomato) basis to reflect commercial organic greenhouse practices. N application rates fell within established agronomic recommendations, and all fully fertilized regimes received a total of 66.5 g of available N (N_{av}) m⁻² for both crop cycles combined (Table S3). To ensure plant establishment in the soilless system, all treatments received an initial feather meal application corresponding to 25% (cucumber) or 12.5% (tomato) of total available N. Supplemental Mg, K, P, and micronutrients were applied weekly. Additional details can be found in Giard-Laliberté et al. (2026).

Table 1
Main characteristics of the soil-based and soilless growing systems

Parameter	Soil-based	Soilless
Substrate	Mineral soil (dystric brunisol: 72% sand, 13% silt, 15% clay)	Peat-based growing medium composed of peat (55%), wood fiber (23%), perlite (12%), and compost (10%) (v/v)
Cultivation history	6 years under organic cultivation	None
Crop density	3 plants m ⁻² (for both crop cycles)	3 plants m ⁻² (for both crop cycles)
Depth of substrate available for roots	~ 50 cm	25 cm
Depth of the A horizon	15 cm	-
Exploitable substrate volume per plant	~ 80 L (0.8 m width × 0.2 m length × 0.5 m depth)	27.5 L (0.4 m width × 0.275 m length × 0.25 m depth)
Physical root barrier between plants	None	None (two plants per container)
Renewal of soil/growing media between crop cycles	No	Yes

Greenhouse climate and irrigation were automatically regulated using a Priva environmental control system (Priva, De Lier, The Netherlands). During the cucumber cycle, day and night air temperatures were maintained at 26°C and 22°C, respectively, without supplemental lighting. For tomato production, temperatures were set at 25°C during the day and 22°C at night, with additional high-pressure sodium lighting providing approximately 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for a 16 h photoperiod, corresponding to an additional daily light integral of about 20 $\text{mol m}^{-2} \text{day}^{-1}$. Irrigation was triggered by cumulative light exposure and differed between systems: soilless containers were watered using micro-sprinklers (720 $\text{mL m}^{-2} \text{min}^{-1}$), whereas soil beds were irrigated via drip lines delivering 120 $\text{mL m}^{-2} \text{min}^{-1}$. Mean daily irrigation volumes were 3.88 and 4.82 $\text{L m}^{-2} \text{day}^{-1}$ for the soil-based and soilless systems, respectively, during cucumber production, and 3.49 and 3.38 $\text{L m}^{-2} \text{day}^{-1}$ during the tomato cycle. Average growing media/soil (0–15 cm) temperatures and water-filled pore space (WFPS) were 23.3°C/20.9°C and 63.4%/45.3% (cucumber/tomato), respectively in the soilless system, compared with 24.5°C/20.3°C and 42.3%/35.1%, respectively, in the soil-based system.

Data collection and analysis

Soil sampling

Root-exclusion cylinders were used as in the soilless systems, roots are constrained to a very limited volume, leaving little or no uncolonized growing media after a crop cycle. Cores were constructed from acrylonitrile butadiene styrene (ABS) water pipe (15 mm internal diameter, 30 mm in length; Johnson et al., 2001; Marian et al., 2019). Three rectangular openings were cut into each cylinder, representing approximately 50% of the belowground external surface area. Nylon mesh (35 μ m pore size) was affixed to the openings and the base of the cylinder. Cylinders were installed in each plot at the beginning of each crop cycle and filled with soil or growing medium prior to transplanting. Sampling was conducted at the end of each crop cycle while plants were at the harvest stage. Bulk soil (i.e., soil within the root-exclusion cylinder) and rhizosphere soil or rhizosphere growing medium were collected at two depths (0–5 cm and 10–15 cm) to contrast the zone immediately influenced by surface-applied organic fertilizers with a deeper layer within the active root zone, corresponding to the A horizon in mineral soils and to root-colonized growing media in the soilless system. For rhizosphere sampling, a 10 $\times$ 10 $\times$ 15 cm (W $\times$ L $\times$ H) block of soil or growing medium was extracted from each experimental unit and processed immediately. Residual surface fertilizer was carefully removed prior to subsampling. Rhizosphere soil was defined as the soil tightly adhering to roots and was collected from three secondary-root subsamples per depth (Kawasaki et al. 2016). Roots were gently shaken to remove loosely bound soil, and the resulting root–soil mixture (~ 25 mL) was transferred to 50 mL centrifuge tubes, with the volume adjusted to 45 mL using sterile 0.9% NaCl solution. Samples were vortexed twice for 30 s, with roots removed between steps, then centrifuged for 2 min and the supernatant discarded (Barillot et al. 2013). For bulk soil, three subsamples per depth were collected within cores and composited. Approximately 25 mL of the homogenized sample was transferred to a 50 mL tube and processed following the same procedure as for rhizosphere samples, except for the root removal step. The resulting soil or growing-medium pellet was stored at – 20°C. All tools and gloves were cleaned and sterilized between samples to prevent cross-contamination. Due to a defect in some of the exclusion cylinders (the side seams were not properly sealed), roots were found in some bulk soil samples. These samples were therefore excluded from subsequent analyses, resulting in the removal of four experimental units from the cucumber trial and one experimental unit from the tomato trial.

Root sampling

Root samples for RT-qPCR were collected concurrently with soil sampling from the same rhizosphere sampling block. At each depth, composite samples consisted of three subsamples of secondary roots. Roots were gently rinsed with sterile water to remove adhering debris, placed in pre-labeled aluminum envelopes, flash-frozen in liquid nitrogen, and stored at – 80°C until analysis.

Total crop N offtake

Crop N offtake is defined here as the cumulative amount of N removed from the soil or growing medium and recovered in harvested and removed aboveground plant tissues. To quantify total crop N offtake, leaf trimmings, fruits, and final aboveground biomass were collected from two plants per experimental

unit, weighed fresh, and dried at 50°C for 48 h. Fruits were collected at each harvest event (daily for cucumber and biweekly for tomato), leaf trimmings were collected weekly, and the final aboveground biomass was collected at the end of the trial. Total crop N offtake was calculated as the sum of total N and NO₃⁻-N contents across all plant compartments, determined by using an Agilent MP-AES (Santa Clara, CA, USA; Giard-Laliberté et al., 2026).

Quantification of transcription of plant N assimilation genes

Root samples were ground under liquid N to a fine powder. Total RNA was extracted from 250 mg of frozen root material using the EZ-10 Spin Column Plant RNA Mini-Preps Kit (Bio Basic, Markham, ON, Canada), following the manufacturer's protocol. Residual genomic DNA was removed using RNase-free DNase, and RNA integrity was verified by agarose gel electrophoresis. RNA concentration was quantified using a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA synthesis was performed using 500 ng of total RNA per sample with the PrimeScript RT reagent kit (Perfect Real Time; Takara, San Jose, CA, USA), according to the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) was carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR Green qPCR Master Mix (New England Biolabs, Ipswich, MA, USA), in reaction volumes of 35 µL. For each target gene, all samples were analyzed within the same 96-well plate to avoid inter-run variation. All RT-qPCR followed the same thermal cycling conditions: 95°C/30s, then 44 cycles of 95°C/10s, 60°C/30s, 68°C/30s and a melt curve from 65°C to 95°C (0.5°C increments). Primer sequences for target genes and candidate reference genes are listed in Table 2. The genes of interest were involved in the crop assimilation of N. Glutamine synthetase (GS genes) are involved in the assimilation of ammonium into amino acids (Bernard and Habash 2009) and are often correlated with increased N assimilation (Sestili et al. 2018). Nitrate transporters (NRT genes) are involved in the nitrate uptake from the soil solution into the root cells (Migocka et al. 2013). Reference genes are defined as transcripts exhibiting stable expression across experimental conditions, tissues, and treatments, and are commonly used to normalize gene expression data in the absence of absolute quantification standards (Warzybok and Migocka 2013). Reference gene stability was assessed using the geNorm algorithm implemented in the *NormqPCR* package. Candidate housekeeping genes were evaluated across all experimental conditions and ranked according to expression stability. The two most stable reference genes were selected for each crop (CsACT and CsEF for cucumber; LeACT 2 and LeSAND for tomato) and used to calculate a sample-specific normalization factor based on their mean C_q values. C_t values were corrected according to primer efficiency assessed with PCR miner (http://118.190.66.83/miner/data_submit.htm). Relative expression of target genes was then determined using the ΔC_q method and expressed as 2^{-ΔC_q}.

Table 2
List of genes and associated primers used in RT-qPCR reactions.

Gene	Crop	primer sequence (5'-3')	Fonction	Reference
CsNRT2.1	Cucumber	F: CTGTAGCTTCCCCGATCGTC R: TCGTCAGGTTGAGGTTGTCTG	Nitrite and nitrate reductase	Gou et al., 2020
CsGS1	Cucumber	F: TTCTTTCTTTTGATCCAAAACCA R: ATGTCGCCCTGTGAGACGACGCT	Glutamine synthetase	Ma et al., 2019
CsACT	Cucumber	F: CCGTTCTGTCCCTCTACGCTAGTG R: GGAAGTGTCTTTGCAGTCTCGAG	Housekeeping gene	Warzybok & Migocka, 2013
CsCACS	Cucumber	F: TGGGAAGATTCTTATGAAGTGC R: CTCGTCAAATTTACACATTGGT	Housekeeping gene	Migocka et al., 2013
CsEF	Cucumber	F: ACTTTATCAAGAACATGATTAC R: TTCCTTCACAATTTTCATCG	Housekeeping gene	Migocka et al., 2013
LeGS1	Tomato	F: ACACGGAGAAGGAAGGAAAGGGAT R: TCCGCGATCATAGAGGTCACAACA	Glutamine synthetase	Sestili et al., 2018
LeNRT2.3	Tomato	F: AATGCATGGTGTACTGGTAGAGAG R: CTAATAATAGGGACTAAAGGGGCTG	Nitrite and nitrate reductase	Sestili et al., 2018
α -tubulin	Tomato	F: TGAGGTCTTCTCACGCATTGACCA R: AATCCTTCTCGAGGGCAGCAAGAT	Housekeeping gene	Sestili et al., 2018
LeACT 2	Tomato	F: CATTGTGCTCAGTGGTGGTTC R: CTGCTGGAAGGTGCTAAGTG	Housekeeping gene	Gonzalez et al., 2016
LeSAND	Tomato	F: TTGCTTGGAGGAACAGACG R: GCAAACAGAACCCCTGAATC	Housekeeping gene	Gonzalez et al., 2016

Soil DNA extraction and library construction and microbial analyses

Soil DNA extraction and library construction and microbial analyses

Total DNA was extracted from 0.5 g (fresh weight) of the pellet of rhizosphere soil or growing media using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. DNA yield and purity were quantified using a Tecan microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Amplicon libraries were sequenced in paired-end mode (2 × 250 bp) on an AVITI platform (Element Biosciences, San Diego, CA, USA) at Génome Québec (McGill University, Montréal, QC, Canada), following the library preparation protocol described by Jeanne and colleagues (2022). For prokaryotic communities, the V4 region of the 16S rRNA gene was amplified using primers 515 F and 806R, while fungal communities were targeted by amplifying the ITS1 region with primers BITS-ITS1 and B58S3 (Bokulich and Mills 2013). Sequence data were processed using QIIME 2 (Bolyen et al. 2019), with denoising, read merging, and chimera removal performed via the q2-dada2 pipeline (Callahan et al. 2016). After quality control, a total of 12.3 million 16S reads were retained, yielding 28,108 amplicon sequence variants (ASVs), and a total of 13.6 million ITS reads produced 8,182 ASVs. Taxonomic assignments were carried out using a naïve Bayes classifier (classify-sklearn) trained on the SILVA v138 database for 16S sequences and the UNITE v9 database for ITS sequences. Predicted microbial functional traits were inferred using the FAPROTAX and FungalTraits databases for bacterial and fungal communities, respectively (2020; Sansupa et al. 2021). For prokaryotes, only functions related to the C and N cycles were kept for analysis. For fungi, only feeding strategies were analyzed.

Data analysis

All statistical analyses were performed in R v4.3.3 (R Core Team 2024). Analyses on alpha and beta diversity and putative functions were conducted separately for each growing system using the combined dataset from both crop cycles and all fertilization treatments except the unfertilized control. Alpha and beta diversity metrics were calculated from rarefied ASV tables (16,235 reads per sample for 16S rRNA and 12,237 reads per sample for ITS).

The Shannon diversity was analyzed using linear mixed-effect models fitted with the 'nlme' package. Fixed effects included fertilization regime, crop cycle, rhizosphere compartment, sampling depth, and all pairwise interactions, while blocks, fertilization regime within the block, and crop cycle within fertilization regime were included as random effects. Model assumptions (normality and homoscedasticity) were verified, and variance structures were incorporated when necessary. Significance of fixed effects was assessed using ANOVA followed by Tukey post hoc tests. Similar models were applied to root gene expression data, although analyses were conducted separately for each crop type.

Effects of fertilization regime, crop cycle, rhizosphere compartment, sampling depth, and all pairwise interactions on microbial community composition were tested using permutational multivariate analysis of variance (PERMANOVA; *adonis2*, 'vegan', 999 permutations) based on Bray–Curtis dissimilarities, with plot included as a random effect. Pairwise PERMANOVA was used to assess differences among group centroids. Community patterns were visualized using canonical analysis of principal coordinates (CAP; *capscale*, 'vegan'), while accounting for crop cycle and fertilization regime effects.

Differences in functional potential among rhizosphere compartment × sampling depth combinations were tested using Kruskal–Wallis tests (*kruskal.test*, ‘stats’), followed by Benjamini–Hochberg-corrected Dunn post hoc comparisons (‘agricolae’). Associations between microbial community composition and root N-related gene expression were assessed using Spearman rank correlations (*cor.test*, ‘stats’) based on Bray–Curtis PCoA axis scores calculated separately for each crop × growing system combination. Taxa associated with gene expression were identified using genus-level count data transformed by centered log-ratio (CLR) transformation (*aldex.clr*, ‘ALDEx2’) followed by correlation analysis (*aldex.corr*).

Finally, partial least squares structural equation modeling (PLS-SEM) was used to evaluate the direct and indirect effects of microbial community structure and root N-related gene expression on total plant N offtake at each sampling depth. Analyses were performed using ‘semnir’, with standardized variables and single-item composites. Model parameters were estimated using *estimate_pls*, and path significance was assessed by bootstrapping (*bootstrap_model*, 1000 resamples).

Results

Effect of rhizosphere selection and sampling depth on the structure of prokaryotic microbial communities

Across both crop cycles and considering only full-fertilization regimes (control excluded), sampling depth significantly influenced the alpha diversity of soil-based prokaryotic communities. In the soil-based system, both 0–5 cm bulk and rhizosphere samples exhibited lower Shannon diversity compared to their 10–15 cm counterparts ($p < 0.0001$; Fig. 1a). In the soilless system, a significant interaction between rhizosphere selection and sampling depth was observed, with the highest alpha diversity found in bulk and rhizosphere samples at 10–15 cm, followed by the 0–5 cm rhizosphere ($p < 0.0001$; Fig. 1b). Prokaryotic beta diversity (Bray–Curtis) was significantly affected by sampling depth, rhizosphere selection, and their interaction in both systems (PERMANOVA; Fig. 1c, d). Sampling depth explained the largest share of compositional variation (soil-based: $R^2 = 0.20$, $p = 0.001$; soilless: $R^2 = 0.19$, $p = 0.001$) while rhizosphere selection (soil-based: $R^2 = 0.03$, $p = 0.002$; soilless: $R^2 = 0.05$, $p = 0.001$) and the interaction term ($R^2 = 0.02$, $p = 0.002$ in both systems) accounted for a smaller but significant proportion of the variance. Analysis of multivariate dispersion (betadisper) further revealed that community variability differed among factors. In the soil-based system, multivariate dispersion was significantly affected by both rhizosphere selection ($p = 0.04$) and sampling depth ($p = 0.001$), with the greatest variability observed in bulk samples and in the 0–5 cm layer (data not shown). In contrast, in the soilless system, dispersion was influenced by sampling depth only ($p = 0.001$), with higher variability at 0–5 cm, while no significant effect of rhizosphere selection was detected ($p = 0.42$; data not shown).

In the soil-based system, only a limited number of shifts in putative functional potential reflected the interaction between rhizosphere selection and sampling depth, whereas most patterns were associated with sampling depth alone (Fig. 1e). Denitrification and aromatic compound degradation potential were highest in the 0–5 cm rhizosphere, hydrocarbon degradation in the 0–5 cm bulk soil, and nitrification

potential in the 10–15 cm bulk soil. More broadly, several putative functions showed clear depth-related patterns, with cellulolysis, chitinolysis, fermentation, methylotrophy, nitrate reduction, and ureolysis potential enriched in the 0–5 cm layer (both rhizosphere and bulk), while methanotrophy was highest at 10–15 cm. In the soilless system, a larger number of C- and N-related putative functions were influenced by the interaction between rhizosphere selection and sampling depth (Fig. 1f). Denitrification, N fixation, ureolysis, and xylanolysis potential were highest in the 0–5 cm rhizosphere, whereas cellulolysis, chitinolysis, fermentation, and hydrocarbon degradation potential were highest in the 0–5 cm bulk growing medium, and putative nitrification peaked in the 10–15 cm bulk. Sampling depth alone explained variation in a smaller subset of putative functions, including aromatic hydrocarbon degradation, which was highest at 0–5 cm, and methanotrophy, which was highest at 10–15 cm.

Effect of rhizosphere selection and sampling depth on the structure of fungal microbial communities

In both systems, fungal alpha diversity (Shannon index) was strongly influenced by sampling depth ($p < 0.0001$; Fig. 2a, b) and by the interaction between rhizosphere selection and sampling depth ($p \leq 0.002$). In the soil-based system, diversity was highest in the 10–15 cm bulk and lowest in the 0–5 cm bulk soil. In the soilless system, diversity was also highest at 10–15 cm, while 0–5 cm bulk and rhizosphere both had the lowest value. Fungal beta diversity (Bray–Curtis) was significantly affected by sampling depth (soil-based: $R^2 = 0.10$, $p = 0.001$; soilless: $R^2 = 0.22$, $p = 0.001$), rhizosphere selection ($R^2 = 0.02–0.05$, $p \leq 0.002$), and their interaction ($R^2 = 0.02–0.03$, $p \leq 0.003$) in both systems (PERMANOVA; Fig. 2c, d). Analysis of multivariate dispersion (betadisper) indicated that fungal community variability differed among factors. In the soil-based system, dispersion was significantly influenced by rhizosphere selection, with bulk communities showing the greatest variability ($p = 0.03$; data not shown). In the soilless system, both rhizosphere selection and sampling depth affected dispersion ($p \leq 0.02$), with the highest variability observed in bulk samples and at 10–15 cm depth.

Fungal saprotrophic lifestyles varied with sampling depth, rhizosphere selection, and their interaction. In the soil-based system, the relative abundance of litter, and soil saprotrophs were highest in the 10–15 cm bulk soil, with litter saprotrophs showing similar abundance in the 10–15 cm rhizosphere (Fig. 2e). In the soilless system, rhizosphere selection structured fungal trophic guilds, with pathotrophs being more abundant in the bulk growing medium and root-associated biotrophs enriched in the rhizosphere (Fig. 2f). Depth-related patterns were also observed, with dung saprotrophs more abundant at 0–5 cm, and soil saprotrophs enriched at 10–15 cm. A limited number of interaction effects were detected, with plant and wood saprotrophs showing the highest relative abundance in the 0–5 cm rhizosphere.

Associations between rhizosphere microbial communities and root N assimilation gene transcripts

Relative expression of CsGS1 (cucumber) and LeGS1 (tomato) varied with sampling depth, with higher transcript levels in 0–5 cm roots compared to 10–15 cm in both soil-based and soilless systems ($p < 0.05$; Fig. 3a, f, k, p). Unconstrained ordinations (Bray–Curtis PCoA) were performed separately for each

crop × growing system combination to show sampling depth effects for both prokaryotic and fungal communities (Fig. S1). GS1 expression was significantly associated with rhizosphere prokaryotic community structure, as indicated by correlations with PCoA axes across all crop and growing systems ($\rho \geq |0.52|$; $p < 0.002$; Fig. 3b, g, l, q).

In soil-based cucumbers, 48 prokaryotic and 4 fungal genera were positively correlated with CsGS1 expression, including 13 prokaryotic taxa remaining significant after Benjamini–Hochberg (BH) correction (Table S4). In soilless cucumbers, 28 prokaryotic and 2 fungal genera were positively correlated, none remaining significant after BH correction. In soil-based tomatoes, 48 prokaryotic (29 after BH correction) and 4 fungal genera were positively correlated, whereas in soilless tomatoes, 41 prokaryotic (8 after BH correction) and two fungal taxa were identified. Genera positively correlated with GS1 expressions were predominantly affiliated with the Proteobacteriota and Actinobacteriota phyla (Fig. 3c, h, m, r).

Notably, *Paracoccus* and *Rhodococcus* were detected across all four crop × growing system combinations (although not always among the top-ranked taxa; Table S7). *Pseudomonas* was among the ten most strongly correlated genera in three of the four combinations (all except the soil-based tomato). Within the Actinobacteriota, *Aeromicrobium*, *Agromyces*, *Cellulosimicrobium*, *Longivirga*, and *Microbacterium* were detected in three crop × growing system combinations. Similarly, within the Proteobacteriota, *Bosea*, *Devosia*, and *Pseudoxanthomonas* were identified in three combinations, along with *Algoriphagus* (Bacteroidota) and the Chloroflexi lineage JG30-KF-CM45.

GS1 expression was also significantly associated with rhizosphere fungal community structure, as indicated by correlations with PCoA axes across all crop and growing systems ($\rho \geq |0.48|$; $p < 0.02$; Fig. 3b, g, l, q). A few genera were positively correlated to GS1 expression, none after BH-corrections (Fig. 3e, j, o, t). Most genera were Ascomycota with an unclassified Chaetomiaceae found in three of the four combinations. The genus *Mucor* from Mucoromycota phylum was correlated to GS1 in both soil-based and soilless tomatoes.

Relative expression of CsNRT2.1 (cucumber) was significantly affected by sampling depth in the soil-based system ($p = 0.03$), but not in the soilless system ($p = 0.09$; Fig. 4a, f). Expression of LeNRT2.3 (tomato) was influenced by the interaction between fertilization regime and sampling depth only in the soilless system ($p = 0.0001$; Fig. 4j, n). Only the prokaryotic community structure of the soil-based system was correlated to expression patterns of CsNRT2.1 ($p = 0.02$; Fig. 4b, d, g, i). In total, 33 prokaryotic genera (none remaining significant after BH correction) and one fungal genus were positively correlated with CsNRT2.1 in the soil-based system, compared with seven prokaryotic genera (none remaining significant after BH correction) and no fungal genera in the soilless system (Table S4). Prokaryotic taxa were mainly affiliated with Proteobacteriota, Actinobacteriota, and Bacteroidota (Fig. 4c, h), while the fungal taxa belonged to Basidiomycota (Fig. 4e, j). No correlation was found between microbial community structures and LeNRT2.3 expression (Fig. 2k, m o, q). Two and three prokaryotic genera were positively associated with LeNRT2.3 in the soil-based and soilless system, respectively.

Most belonged to the phylum Proteobacteriota, with one genus affiliated with Actinobacteriota in crop cycle and growing system combinations except soilless tomatoes. Only one fungal genus, which remained unclassified, associated with *LeNRT2.3* in the soilless system.

Associations between rhizosphere microbial communities, root N assimilation gene transcripts and crop N offtake

Partial least squares (PLS) models were used to evaluate how crop total N offtake could be predicted from root and rhizosphere parameters (Fig. 5; see Table S6 for crop N offtake results). PLS models were run separately for each sampling depth (0–5 and 10–15 cm) and for the prokaryotic and fungal community. In the prokaryotic rhizosphere, the model based on the 0–5 cm rhizosphere explained a greater proportion of the variability in crop N export than the 10–15 cm model ($R^2 = 0.79$ vs. 0.70). In both depth-specific models, prokaryotic community structure, represented by the PCoA1 axis, exerted a significant effect on crop N export ($p < 0.01$). In the 0–5 cm rhizosphere, PCoA2 axis and prokaryotic richness (Shannon index) also had a direct effect ($p < 0.01$; Fig. 5a). In addition, GS1 gene expression was significantly associated with the PCoA axis ($p < 0.05$), while NRT gene expression was associated with the PCoA2 axis ($p < 0.05$). In contrast, in the 10–15 cm rhizosphere, crop N export was influenced by NRT gene expression directly ($p < 0.05$; Fig. 5b). In the fungal rhizosphere, the model based on the 10–15 cm rhizosphere explained a greater proportion of the variability in crop N export than the 0–5 cm model ($R^2 = 0.75$ vs. 0.61). Associations were very similar in both models, with the axis 1 of the PCoA exerting a direct effect, and GS1 an indirect effect in both models. The only difference was the direct effect of the NRT gene expression (Fig. 5d).

Discussion

In this study, conducted across two contrasting growing systems, four organic fertilization regimes, and two crop cycles, we investigated how rhizosphere selection and sampling depth independently and interactively shaped microbial community structure and functional potential under surface-applied organic fertilization. Our results support the hypothesis of rhizosphere heterogeneity, whereby rhizosphere selection interacted with sampling depth to shape microbial communities, although the magnitude of this interaction differed between growing systems. In general, rhizosphere microbial communities from the two sampling depths (0–5 cm and 10–15 cm) were more compositionally like one another than bulk communities across depths (Fig. 1a–d; Fig. 2a–d), suggesting a homogenizing effect of plant-mediated selection. However, this homogenization remained constrained by sampling depth. Indeed, rhizosphere communities were more similar to their corresponding bulk communities at the same sampling depth (0–5 cm bulk vs. rhizosphere; 10–15 cm bulk vs. rhizosphere) than to rhizosphere communities from the other depth.

Shared and system-specific patterns of rhizosphere heterogeneity

The direction and magnitude of rhizosphere selection varied depending on both the parameter measured and the growing system. Prokaryotic alpha diversity was affected by the interaction between rhizosphere selection and sampling depth only in the soilless system, where diversity in the 0–5 cm rhizosphere was lower than in the 10–15 cm rhizosphere and bulk soil, but higher than in the 0–5 cm bulk growing media. While rhizosphere microbial communities are often described as taxonomically filtered subsets of bulk soil communities, resulting in reduced alpha diversity in many agricultural systems (Ling et al. 2022), this pattern is not universal (Schmidt et al. 2019), 2019). Root-driven filtering is commonly attributed to the release of diverse C-based compounds through rhizodeposition, which can selectively stimulate specific microbial groups. However, in systems receiving surface-applied organic fertilization, additional C inputs may modify rhizosphere filtering by alleviating C limitations for microbial growth while simultaneously selecting for specific microbial groups adapted to exploit these inputs. The consistently lower richness observed in the 0–5 cm layer across both growing systems, for both prokaryotes and fungi, supports the presence of strong environmental filtering near the surface, likely driven by highly concentrated inputs. In this context, the higher (soilless cucumber and soil-based tomato) or equal (soil-based cucumber and soilless tomato) diversity observed in the 0–5 cm rhizosphere relative to 0–5 cm bulk soil suggests that root-derived C inputs may partially offset these constraints by increasing niche availability. This pattern is mainly documented in forest ecosystems, where rhizosphere and detritosphere C inputs overlap, supporting elevated microbial diversity compared to bulk (Cui et al. 2019; Ling et al. 2022). However, further research is needed to determine the extent to which externally derived C interacts with plant-driven rhizosphere signals in highly intensive agroecosystems such as organic greenhouse systems. At 10–15 cm depth, fungal richness was lower in the rhizosphere than in bulk soil across both systems, indicating a classical rhizosphere filtering effect under conditions away from surface organic inputs. In the soil-based system, there was no effect of rhizosphere selection on prokaryotic alpha diversity and community composition as also illustrated by the absence of interacting effect between rhizosphere selection and sampling depth. Species composition is generally known to be more sensitive to environmental changes than univariate measure of diversity, which mask changes in relationship between microbial groups (Hartmann et al. 2015).

Functional potential related to C and N cycling was also investigated. Only a selection of N-related putative functions showed responses related to rhizosphere selection and sampling depth consistent across growing systems, namely denitrification and nitrification potential. Although potential does not necessarily reflect *in situ* activity, this pattern is consistent with previous observations showing that plants can stimulate denitrification through enhanced rhizodeposition, likely by increasing the availability of labile C substrates (Moreau et al. 2019). A general enrichment of denitrification potential in the rhizosphere has also been reported across a wide range of agroecosystems (Ling et al. 2022). In contrast, the same study reported the depletion of nitrification potential in the rhizosphere, which is in line with our results. This pattern is commonly attributed to oxygen dynamics, as nitrification is an obligatory aerobic process, whereas the rhizosphere -being a microbial hotspot fueled by root-derived C- tends to exhibit elevated oxygen consumption relative to bulk soil (Ling et al. 2022). This could also be caused by biological nitrification inhibition, a localized process in which roots exposed to NH_4^+ release

compounds that inhibit key enzymes of the nitrification pathway, including ammonia monooxygenase and hydroxylamine oxidoreductase (Moreau et al. 2019). In line with this, nitrification potential was higher in bulk soil than in the rhizosphere at 10–15 cm depth. However, the lowest nitrification potential was observed in the 0–5 cm layer for both rhizosphere and bulk soil, suggesting that the surface layer, in direct contact with organic fertilization, may also act as a microbial hotspot where oxygen becomes limiting.

Other rhizosphere selection x sampling depth interactions in functional potential were mostly system specific. In particular, the strong impact of rhizosphere selection on N-related functional potential in the 0–5 cm rhizosphere of the soilless system suggests that plant–microbe interactions were intensified under these conditions. Soil mineral N content (0–15 cm) was much lower in the soilless growing medium compared to the mineral soil (Table S5), which likely increased plant reliance on microbial processes for nutrient acquisition. Under nutrient-limited conditions, plants are known to invest more in rhizosphere-mediated strategies, including the recruitment and stimulation of microbial communities involved in nutrient cycling, as well as the modulation of microbial functional groups (Congreves et al. 2021).

In the soilless system, the 0–5 cm bulk growing medium was characterized by a higher relative abundance of C-related functional potential than the 0–5 cm rhizosphere, particularly for functions associated with the degradation of complex organic compounds. At this depth, both rhizosphere and bulk microbial communities had access to substantial inputs of complex C from organic fertilizers. The greater functional potential for complex C processing observed in the bulk suggests that this community may have been more strongly selected for the decomposition of recalcitrant substrates, whereas rhizosphere communities may have relied more on labile, root-derived C inputs. This pattern was not observed in the soil-based system, which had a long history of intensive organic management and repeated organic inputs. Although the peat-based growing medium is characterized by very high total organic C (34.8% vs. 5.8% in mineral soil) and an elevated C:N ratio (30:1), much of this C is structurally complex and poorly bioavailable (Taparia et al. 2021; Pot et al. 2022). This may create conditions of relative C limitations, selecting for microbial communities capable of degrading recalcitrant substrates (Grunert et al. 2016; Müller et al. 2025). Consistent with this interpretation, a lower copiotroph-to-oligotroph ratio was observed in the soilless system compared to the soil-based system across the 0–15 cm profile (Giard-Laliberté et al., 2026).

Furthermore, the homogenizing effect of the rhizosphere was less evident when considering functional profiles, as few putative functions showed similar relative abundances between the 0–5 cm and 10–15 cm rhizosphere. This contrasts with the findings of Yan and colleagues (2017), who reported a strong homogenization of microbial functional traits in the rhizosphere, compared to bulk soil. However, their study did not account for spatial variability within the rhizosphere itself, which may explain the differences observed here. Finally, the stronger rhizosphere selection effect observed in the soilless system may reflect the relatively recent establishment of microbial communities in the growing medium. Unlike mineral soils with a long history of plant–microbe interactions, the growing medium represents a

more nascent system in which microbial communities are still assembling under comparatively lower levels of bioavailable nutrients (Pot et al., 2022). This is especially relevant in the present study, where the growing medium was replaced between the two crop cycles. This may increase the sensitivity of these communities to plant-driven selection and contribute to the stronger interaction effects observed. In contrast, in the soil-based system, the legacy effect of previous crops (Hannula et al. 2021; Nannipieri et al. 2023) as well as the greater nutrient availability could explain the weaker rhizosphere effect observed.

In the soil-based system, saprotrophic fungal lifestyles were most abundant in the 10–15 cm bulk soil. This contrasts with the general expectation that saprotrophic fungi are enriched in environments receiving organic C inputs (Clocchiatti et al. 2020) and in the rhizosphere (Pausch et al. 2016; Clocchiatti et al. 2021), although their distribution in the rhizosphere appears to be context-dependent (Hennecke et al. 2023). One possible explanation is the role of N availability: high levels of mineral N have been shown to suppress fungal decomposer communities (Paungfoo-Lonhienne et al. 2015; Zhou et al. 2016). This interpretation is supported by the contrasting pattern observed in the soilless system, which was characterized by markedly lower soil mineral N content (Table S5) and where some saprotrophic lifestyles were more abundant in the 0–5 cm layer.

Root N assimilation gene expression is partly depth dependent

In line with our hypothesis, root gene expression related to N assimilation was influenced by sampling depth, with higher expression in the 0–5 cm layer, close to the organic fertilizer layer. This is consistent with the well-established ability of roots to respond to spatial heterogeneity in nutrient availability by adjusting their architecture, morphology, and activity (Sasse et al. 2018; Singh et al. 2023). In our study, glutamine synthetase 1 genes (CsGS1 and LeGS1) exhibited clearer and more consistent depth-related patterns than high affinity nitrate transporter genes (CsNR2.1 and LeNRT2.3). NRT2 transporters are known to be rapidly induced under low external nitrate concentrations but downregulated when nitrate or reduced N metabolites accumulate (Krapp et al. 1998; Migocka et al. 2013). As a result, their expression is highly dynamic and sensitive to short-term fluctuations in N availability, which may explain the weaker depth-related patterns observed here. GS1 plays a central role in N metabolism and is involved in the assimilation of ammonium into organic compounds (Bernard and Habash 2009; Sestili et al. 2018). Because GS1 expression reflects N use within the plant rather than N acquisition, it may be less sensitive to transient environmental variability, leading to the more consistent depth-related patterns observed across both crops and growing systems.

Positive associations between rhizosphere microbial taxa and root N assimilation gene expression

While roots are known to adapt to heterogeneous nutrient gradients in soil, how this adaptive response shapes the rhizosphere microbiome and its spatial organization remains poorly understood (Wang et al. 2020). At the whole-plant level, changes in plant nutritional status are known to restructure rhizosphere

microbial communities (Yang and Crowley 2000; Jacoby et al. 2017; Henneron et al. 2020). In this study, we examined associations between root expression of genes involved in N metabolism and microbial community structure in the rhizosphere. Although root genes involved in N uptake and assimilation do not directly represent the mechanisms through which plants recruit microbial partners, their expression may reflect the plant's economic interest in shaping a specific microbiome (Liu et al. 2023). Expression of low-affinity nitrate transporters has been linked to the enrichment of specific N-related taxon in the rhizosphere of rice indica varieties (Zhang et al. 2019). In our study, spatially localized variation in root N assimilation was associated with distinct microbial compositional patterns, suggesting that plant-driven selection can operate at fine spatial scales.

The prokaryotic genera associated with root N-assimilation patterns (GS1) encompassed taxa consistent with multiple, complementary roles in rhizosphere functioning, including responses to plant-derived C inputs, decomposition of organic matter, and N transformations. Several taxa, such as *Pseudomonas* and *Bacillus*, are well-established rhizosphere colonizers that respond rapidly to labile C compounds released through root exudation (Lugtenberg et al. 2001; Bhadrecha et al. 2023; Feng et al. 2026). Consistently, Wu and colleagues (Wu et al. 2025) showed that vigorous poplar individuals harbored *Pseudomonas* populations that actively responded to specific root exudates and contributed to enhanced plant growth and N acquisition. In addition, several genera identified here are consistent with N transformation pathways. For example, *Paracoccus*, *Hydrogenophaga*, and *Achromobacter* include species capable of denitrification or nitrate reduction, suggesting that localized zones of root activity may also correspond to sites with enhanced N turnover (Gaimster et al. 2018; Duffner et al. 2022; Fan et al. 2023). Complementing these processes, taxa such as *Cellulosimicrobium* and *Rhodococcus* are associated with the degradation of structurally complex organic compounds, thereby contributing to organic matter turnover and the potential release of organically bound N (Gabani et al. 2012; Krivoruchko et al. 2023). Taken together, these results support the interpretation that spatial variation in root N assimilation is associated with microbial communities integrating C and N cycling processes.

The fungal genera positively associated with variation in root N assimilation were predominantly consistent with saprotrophic lifestyles involved in the decomposition of organic matter, suggesting a strong link between localized C turnover and N availability in the rhizosphere of both organically managed growing systems. The addition of organic inputs is known to stimulate saprotrophic fungi in the rhizosphere, where they can utilize a broad spectrum of C substrates ranging from plant-derived polymers to root exudates, and contribute to the initial breakdown of complex organic matter into simpler compounds that subsequently fuel prokaryotic activity (Clocchiatti et al. 2021). Taxa such as *Agaricus*, members of the Chaetomiaceae, *Humicola*, and *Cladorrhinum* include species capable of degrading a range of organic materials through the production of extracellular enzymes, including lignocellulose and cellulose (Longoni et al. 2012; Floudas et al. 2015; Kabel et al. 2017; Bajaj and Mahajan 2019; Martins et al. 2024). These processes are central to soil functioning, as fungal-mediated decomposition releases nutrients bound in organic compounds, thereby contributing indirectly to N mineralization and bioavailability (Crowther et al. 2012). Other taxa detected in this study, such as *Mucor* and *Apiotrichum*, are broadly consistent with fungi exploiting labile or intermediate carbon substrates.

Members of Mucorales are typically fast-growing saprotrophs associated with nutrient-rich organic matter, while soil yeasts such as *Apiotrichum* are increasingly recognized as active components of soil microbial communities involved in the transformation of soluble carbon compounds (Yurkov 2018; Walther et al. 2019). Together, the fungal taxa positively associated with N assimilation are consistent with organic matter decomposition processes and encompass a range of carbon-use strategies, from labile substrates to more complex material. Importantly, given the limitations of taxonomic inference, these results should be interpreted with caution. Future studies should explore how these patterns are reflected in *situ* microbial activity as well as confirm these exploratory results through mechanistic approaches dwelling into linking direct root activity related to microbial recruitment.

Depth-dependent associations among rhizosphere microbial communities, root N assimilation gene expression, and crop N offtake.

Finally, we used PLS models to explore associations among rhizosphere microbial community structure, root expression of N metabolism genes, and crop N offtake across sampling depths. In line with our hypothesis, the 0–5 cm prokaryotic model showed the stronger association with crop N offtake. Because crop N offtake integrates both plant N concentration and biomass production, it likely reflects not only N acquisition but also broader aspects of plant performance. This may explain why microbial community structure (PCoA1) showed stronger associations with crop N offtake than root N assimilation gene expression. In contrast, transcription of root N assimilation genes primarily reflects plant responses to local N availability and internal N status, which do not necessarily translate into enhanced biomass production or crop yield (Bernard and Habash 2009; Xu et al. 2012). Interestingly, for the fungal rhizosphere, the 10–15 cm model showed stronger associations with crop N offtake. While prokaryotic communities in the 0–5 cm layer showed clearer enrichment in putative C–N functional potential, this pattern was less pronounced for fungal feeding strategies, particularly in the soil-based system, where several saprotrophic groups were relatively more abundant at 10–15 cm depth. This pattern is consistent with the weaker imprint of rhizosphere selection on fungal communities in the 0–5 cm layer (Fig. 2c) and suggests that fungal associations with nutrient cycling and plant performance may be distributed more evenly across the soil profile, or relatively more pronounced at deeper layers compared to prokaryotes. Overall, these exploratory models suggest that depth-related heterogeneity in rhizosphere community structure may be associated with crop N offtake differently for prokaryotic and fungal communities, highlighting distinct spatial organization patterns within the rhizosphere microbiome.

Methodological considerations and limitations

Our exploratory study presents several limitations that should be considered when interpreting the results. First, depth-related variations in key environmental parameters (e.g., temperature, moisture, and nutrient availability) were not explicitly quantified, although they are likely to contribute to the observed structuring of rhizosphere microbial communities. Second, the sampling methodology introduces inherent constraints: the selection of specific root types, the operational definition of the rhizosphere, and the discrete sampling depths don't capture the fine-scale spatial heterogeneity of the root–soil

interface. Third, root architectural traits are known to play a decisive role in shaping rhizosphere microbiomes. Microbial community composition is closely aligned with traits such as root diameter, branching intensity, root hair density, and overall root system architecture, which together govern resource acquisition patterns and microhabitat formation (Scheider et al., 2026). These factors were not explicitly characterized in this study and may therefore confound depth-related interpretations. Finally, functional inference based on taxonomic composition provides only an indirect assessment of ecosystem processes. This approach relies on taxon–function relationships derived from cultured organisms and assumes functional homogeneity within taxa, while overlooking intra-taxon variability, horizontal gene transfer, and the large proportion of uncultured lineages (Barnett et al. 2021). Consequently, predicted functions should be interpreted as approximations and considered hypothesis-generating rather than direct evidence of in situ activity (Prosser 2015; Louca et al. 2018).

Conclusion

Our exploratory study illustrated that, under surface-applied organic fertilization, the rhizosphere should not be considered a spatially homogeneous zone of plant influence, but rather a vertically structured environment. By integrating root gene expression, microbial community structure, and agronomic variables, we provide novel insights suggesting that spatial heterogeneity is an important organizing principle of the rhizosphere, operating at a scale that is often overlooked in agroecosystem studies.

We also observed that many of the effects of rhizosphere selection and sampling depth were system-specific, highlighting the influence of the growing environment on the rhizosphere assembly and functioning. While the soil-based system consisted of a long-established mineral soil with initial high fertility, the soilless system relied on a peat-based growing medium, meaning a younger environment with low initial fertility, high carbon content and a nascent initial microbial community. Together, our findings suggest that the patterns and effect of rhizosphere heterogeneity vary among growing systems and should not be assumed to be universal.

Neglecting this spatial heterogeneity may therefore lead to an incomplete understanding of root-soil interactions and limit the effective application of rhizosphere knowledge in agricultural systems. Future research should combine isotopic tracing and functional approaches to directly link microbial activity, root N assimilation, and spatial heterogeneity across agroecosystems.

Declarations

Competing interests

The authors have no financial or proprietary interests in any material discussed in this article.

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Data availability statement

The raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1468402. The BioProject is available at: <http://www.ncbi.nlm.nih.gov/bioproject/1468402>. Additional processed data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

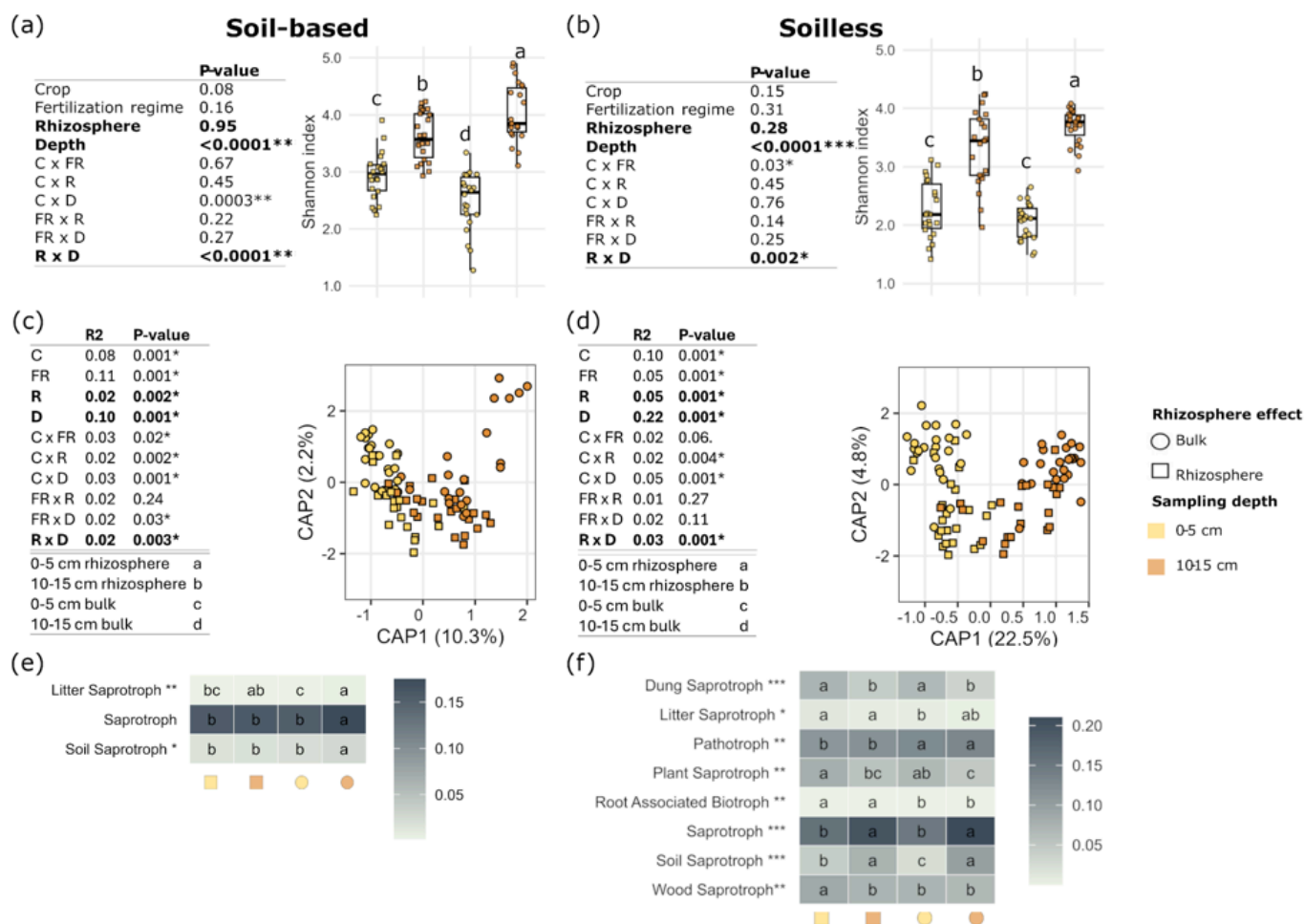


Figure 1

Effect of rhizosphere selection and sampling depth on prokaryotic community structure in the soil-based and soilless system. Shannon index (a-b), PERMANOVA results and canonical analysis of principal coordinates (CAP) (c-d) and heatmap of N- and C-related functions (e-f) in the soil-based and soilless system respectively. Control fertilization regime was excluded from these analyses. Differences in alpha diversity were tested using two-way ANOVA followed by Tukey post hoc tests. Functional profiles were inferred using FAPROTAX (via the microeco package) and analyzed using Kruskal–Wallis tests with Benjamini–Hochberg-corrected Dunn post hoc comparisons; only functions with $p < 0.05$ are shown. Functions: A.C. degradation = aromatic compound degradation; A.H. degradation = aromatic hydrocarbon degradation, H. degradation = hydrocarbon degradation. Significance levels: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$.

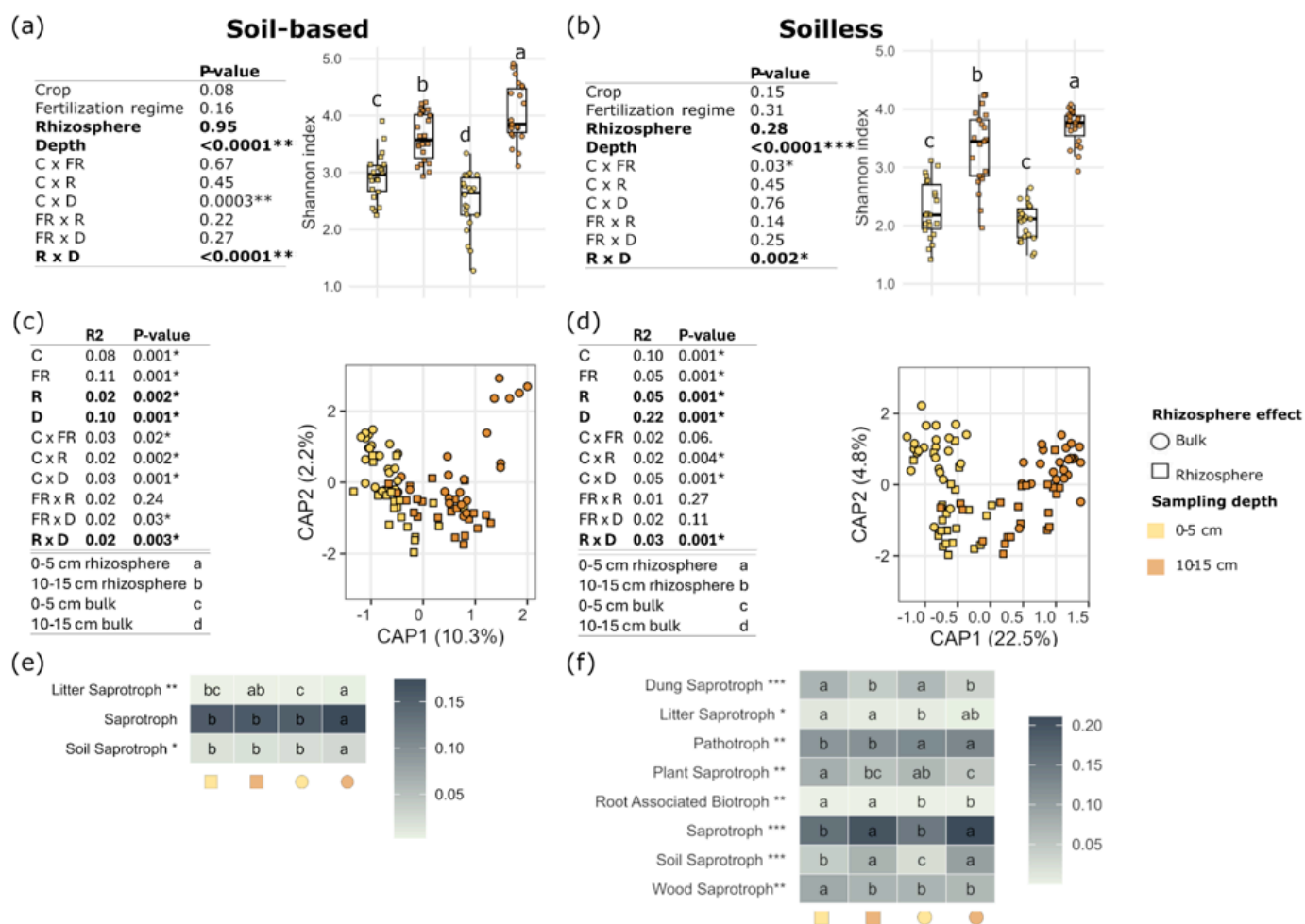


Figure 2

Effect of rhizosphere selection and sampling depth on fungal community structure in the soil-based and soilless system. Shannon index (a-b), PERMANOVA results and canonical analysis of principal coordinates (CAP) (c-d) and heatmap of saprotrophic lifestyles (e-f) in the soil-based and soilless system respectively. Control fertilization regime was excluded from these analyses. Differences in alpha diversity were tested using two-way ANOVA followed by Tukey post hoc tests. Saprotrophic lifestyles were inferred using FungalTraits (via the microeco package) and analyzed using Kruskal–Wallis tests with Benjamini–Hochberg-corrected Dunn post hoc comparisons; only functions with $p < 0.05$ are shown. Significance levels: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$.

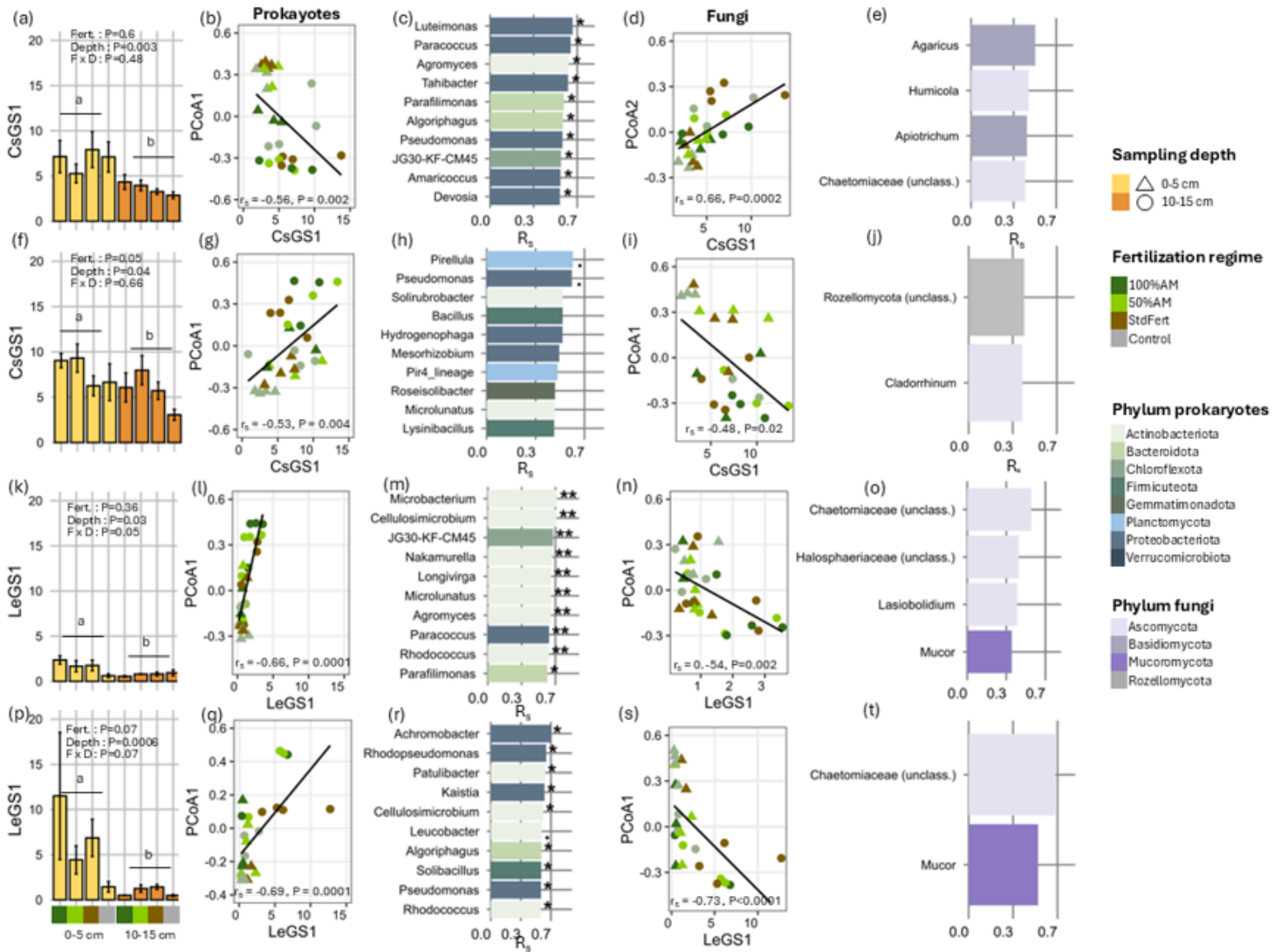


Figure 3

Root expression of glutamine synthetase and correlations with rhizosphere microbial community structure of the cucumber and tomato crop in the soil-based and soilless systems. Panels show relative expression of GS1 genes (CsGS1 in cucumber and LeGS1 in tomato) (a, f, k, p), the relationship between GS1 and one prokaryotic PCoA axis (b, g, l, q), prokaryotic genera positively correlated with GS1 (c, h, m, r), the relationship between GS1 and one fungal PCoA axis (d, i, n, s), and fungal genera positively correlated with GS1 (e, j, o, t), in soil-based (a-e) and soilless (f-i) cucumber and soil-based (j-n) and soilless (o-s) tomato. Relative expression of CsGS1 was normalized using reference genes (cucumber : CsACT and CsEF, tomato: LeSAND and LeACT2) and calculated using the $2^{-\Delta Cq}$ method. The effects of fertilization regime (Fert.), sampling depth (Depth), and their interaction (F × D) were assessed by ANOVA (n = 24 for each crop × growing system combination). Genera whose CLR-transformed relative abundance was correlated with relative expression of N-related genes were identified using ALDEx2 (aldex.corr). Only genera with p < 0.05 are shown. When more than 10 genera met this criterion, only the 10 genera with the highest rho values are presented (see Table S7 for the full results). Genera with significant Benjamini–Hochberg-corrected P-values are indicated as follows: ***p < 0.0001, **p < 0.01, *p

combination). Genera whose CLR-transformed relative abundance was correlated with relative expression of N-related genes were identified using ALDEx2 (aldex.corr). Only genera with $p < 0.05$ are shown. When more than 10 genera met this criterion, only the 10 genera with the highest rho values are presented (see Table S7 for the full results). Genera with significant Benjamini–Hochberg-corrected P-values are indicated as follows: *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, . $p \leq 0.10$. r_s = Spearman’s rho. Fertilization regimes: 100%AM = 100% of N supplied by alfalfa meal; 50%AM = 50% of N supplied by alfalfa meal; StdFert = standard organic fertilization regime based on feather meal and pelleted poultry manure.



Figure 5

Direct and indirect effects of root N assimilation gene expression and rhizosphere microbial community structure on crop N offtake across both crops and growing systems, evaluated using PLS

models. PLS-models for the prokaryotic 0–5 cm (a) and 10–15 cm (b) and fungal 0–5 cm (c) and 10–15 cm (d) rhizospheres. Models were run separately for the 0–5 cm and 10–15 cm rhizosphere layers. Root genes included CsGS1, LeGS1, CsNRT1.5A, and LeNRT2.3, while microbial structure was represented by the Shannon diversity and PCoA1–PCoA2 ordination axes. Arrow colors: Green = significant positive effect; red = significant negative effect ($p < 0.05$); gray = non-significant ($p \geq 0.05$). Significance levels: *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, . $p \leq 0.10$.

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