Ecophylogenetic patterns of rhizosphere bacterial community assembly in *Pisum* spp. (Fabaceae, Fabeae) reveal strong ecological filtering

Victor Angot[1*], Vincent Pailler[2], Abdelhamid Kebieche[3], Elodie Belmonte[2], Virginie Bourion[1], Yanis Bouchenak-Khelladi[1]

[1] Université Bourgogne Europe, Institut Agro Dijon, INRAE, UMR Agroécologie, Dijon, France

[2] INRAE, UMR GDEC, Gentyane, Clermont-Ferrand, France

[3] Institut Agro, Université d'Angers, INRAE, IRHS, SFR 4207 QUASAV, Angers, France

[*] Corresponding author: victor.angot@inrae.fr

Abstract

Plant-microorganisms interactions are among the oldest biotic relationships and play a fundamental role in shaping biological systems. These associations involve several and diverse species, each evolving on different timescales. Understanding these interactions requires approaches that integrate both the ecological dynamics and evolutionary processes, which drive the adaptation of plants and microorganisms. We investigated bacterial community assembly dynamics associated with four diverse *Pisum* spp. accessions grown in greenhouse conditions on soil. Bacterial DNA was extracted from bulk soil, rhizosphere, rhizoplane, and endosphere microhabitats across three plant growth stages, followed by full-length 16S rRNA gene sequencing. Bacterial communities varied in diversity, composition and structure across microhabitats and growth stages. Ecophylogenetic analyses indicated strong plant-induced ecological filtering, with community assembly across space and time structured by phylogenetic constraints. This illustrates the role of plant-defined ecological niches in shaping bacterial communities, where selective recruitment favors specific and closely related lineages. Our findings suggest that an ecophylogenetic framework provides valuable insights into plant–microbiota dynamics by integrating ecological and evolutionary processes, thereby offering a powerful perspective to investigate the co-adaptation of plants and their associated microbiota.

Key words: plant-microorganisms interactions, *Pisum* spp., plant development, ecophylogenetic, ecological filtering

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Introduction

Biotic interactions are responsible for major evolutionary changes, such as increased genetic diversity within populations or triggering adaptive changes leading to ecological speciation, across multiple spatial and temporal scales. These complex interactions, that can involve two or thousands of species and occur within or across trophic levels, contribute to crucial ecological patterns that structure biological systems (Schemske *et al.*, 2009; Spicer & Woods, 2022). One of the most intricate and complex biotic interaction is the association between plants and microorganisms (Lyu *et al.*, 2021), that is among the oldest relationship that may have originated around 460-475 Mya (Brundrett, 2002).Legumes (Fabaceae) is a highly diverse family of angiosperms, many of which form symbiotic associations with nitrogen-fixing bacteria and play an important role in fixing atmospheric nitrogen. Globally, grain legumes (tribe Fabeae) were estimated to fix a total of 35.5 million tons of nitrogen every year (Herridge et al., 2022). Among these, *Pisum* spp. include one of the first domesticated plant species (Zohary & Hopf, 1973). *Pisum* is divided into two species : *P. fulvum* and *P. sativum* which is subdivided in 3 sub-species : *P. sativum* subsp. *sativum*, *P. sativum* subsp. *elatius* and *P. sativum* subsp. *abyssinicum* (Smýkal et al., 2011). While the symbiotic relationship of *Pisum* spp. with *Rhizobia* is well-documented (Bourion *et al.*, 2018; Hawkins & Oresnik, 2022), the complex interactions with non-symbiotic microorganisms remain largely unknown whereas the rhizosphere microbiota play a crucial role in shaping *Rhizobia*-legumes interactions (Han *et al.*, 2020). The non-symbiotic bacteria could also improve the effectiveness of *Rhizobia*-inoculants by enhancing their ability to colonize, survive, and persist in the soil, as well as their capacity to interact with other microorganisms (O'Callaghan et al., 2022; Kong & Liu, 2022). Plant-microorganisms interactions are governed by a complex 'chemical dialogue' through the production of exudates (primary and secondary metabolites) by the plant (Duc *et al.*, 2022). Some exudates, such as organic acids (Rekha *et al.*, 2018), amino-acids (Feng *et al.*, 2018) and glucose (Zhang *et al.*, 2015), have chemo-attractive properties that facilitate bacterial colonization (Chaparro *et al.*, 2013). Other compounds, such as antimicrobial peptides, have antibiotic properties that prevent the colonization of pathogenic microorganisms (Pacheco-Cano *et al.*, 2018). In addition, root architecture and root hairs structure act as a strong environmental filter during the assembly of the rhizosphere microbial communities (Kawasaki *et al.*, 2016; Robertson-Albertyn *et al.*, 2017). Therefore, plants can strongly influence the composition and the structure of rhizosphere microbial communities, exceeding the influence of soil properties (Tkacz *et al.*, 2020). In return, the associated microbial communities play important roles such as atmospheric nitrogen fixation and nutrient availability enhancement, which can improve plant fitness (Bhattacharyya & Jha, 2012; Peix *et al.*, 2015; de la Fuente Cantó *et al.*, 2020). Rhizosphere microbiota also assists the plant in dealing with disturbances caused by biotic and/or abiotic stresses. For instance, associated rhizosphere microbiota can enhance water acquisition in water-limited and saline environments (Kasim *et al.*, 2016; Yang *et al.*, 2021; Azulay *et al.*, 2022), improve plant immunity when competing with pathogens (Li *et al.*, 2021) or facilitate the production of phytohormones (Puga-Freitas & Blouin, 2015). The assembly dynamics of microbial communities are shaped by multiple factors, requiring multifactorial analyses that account for both spatial and temporal dimensions. Different plant compartments (referred here as plant-associated microhabitats), such as the rhizosphere, rhizoplane and endosphere contain distinct microbial communities (Cregger et al., 2018; Gupta et al., 2021). Therefore, the identification of microbial associations and enrichment or impoverishment dynamics through an ecological gradient (i.e., from the rhizosphere to the endosphere) is crucial in order to characterize ecological filters associated with different plant-associated microhabitats (Zhang et al., 2017; Iven et al., 2022). While it is demonstrated that assembly of soil microbial communities is determined by biotic interactions such as mutualism (Goberna et al., 2014; Romdhane et al., 2022), facilitation or competition (Faust & Raes, 2012), the association and assembly dynamics throughout plant-associated microhabitats

remain largely unknown (Moroenyane *et al.*, 2021). Insights from human microbiomes indicate that microbial recruitment can follow an underdispersion assembly pattern (Darcy *et al.*, 2020) (i.e. taxa are more likely to be recruited if a phylogenetically close relative was previously established). Whether similar processes govern the mode and tempo of rhizosphere microbial recruitment by plants remains largely speculative. The use of ecophylogenetic frameworks is therefore needed to reveal association patterns with host plants (Wang & Sugiyama, 2020). The soil microbial community assembly across plant-associated microhabitats may be attributed to phylogenetic niche conservatism (Losos, 2008; Pyron et al., 2015), when closely related species inhabit similar ecological niches. However, instances of competitive exclusion have been shown, such as in floral nectar of *Mimulus aurantiacus,* where closely related yeast species inhibited each other's recruitment (Peay *et al.*, 2012). This reflects the tendency of related species to occupy similar ecological niches which would lead to competitive exclusion. Resource availability can influence the intensity of competition and is a key factor in determining the ecological dynamics of communities (Pekkonen et al., 2013). Similarly, the intensity of plant rhizodeposition and its ability to provide ecological niches for bacteria could affect the pattern of phylogenetic association. Additionally, plants are capable of recruiting different bacterial communities within their various associated microhabitats depending on environmental conditions (Bai *et al.*, 2015; de la Fuente Cantó *et al.*, 2020; Marian *et al.*, 2021; Santoyo, 2022). The specialization of microbiota on the plant's physiological needs and on its microhabitats suggest that plant-microbiota interactions prioritize specific functions over particular microbial taxa (Castellano-Hinojosa & Strauss, 2021). Since closely-related species often have similar ecological functions (Burns & Strauss, 2011; Martiny *et al.*, 2015; Anacker & Strauss, 2016), using phylogenetic approaches to study bacterial communities present in different plant-associated microhabitats and throughout growth stages could provide valuable insights for understanding microbial recruitments patterns and whether they are consistent with phylogenetic relatedness (as proposed by Aguirre de Cárcer (2019)).

In this study, we conducted a greenhouse experiment with four *Pisum spp.* accessions to study the composition and structure of plant-associated belowground bacterial communities at different growth stages using both ecological and phylogenetic approaches. Our hypothesis was that the bacterial communities associated with the plant are specific to the distinct microhabitats it provides and change with growth stages. We suggested that ecological filtering imposed by the plant on bacterial communities change along spatial and temporal gradients and may be related to bacterial phylogenetic constraints as they reflect functional attributes.

Material and Methods

Experimental design and data collection

Four *Pisum* accessions from the Pea Genetic Resource Centre at INRAE Dijon were selected: (i) one *Pisum fulvum* (IPIP10023) and (ii) one *Pisum sativum* subsp*. elatius* (IPI200733) as two wild species accessions, one *Pisum sativum* subsp*. abyssinicum* 'ATAR' (IPI200596) and one *Pisum sativum* subsp*. sativum* 'CAMEOR' (IPI200293) as two accessions representative of domestication or selection events. The seeds were stored in a cold room prior to sowing. Seeds from *Pisum fulvum* and *Pisum sativum* subsp*. elatius* were scarified using a scalpel to enable germination. Soil was collected at Auxonne, Côte d'Or, France (47.185N, 5.403E) from an organic farm and sieved to 4mm. The physical and chemical characterization of soil was assessed at the laboratory "SADEF Agronomy & Environment", and the result of the analysis can be found in supplementary data (see Appendix 1). The experiment was conducted as a completely randomized design and was carried out in a climate-controlled glasshouse located at INRAE Dijon from February to May 2023. The greenhouse was equipped with a temperature control system that maintained a temperature of 20°C during the day and 15°C at night, and a 16-hours

132 photoperiod. Seeds were sown into sterilized 2L pots filled with sifted soil. Two seeds were sown in
133 each pot to ensure the successful germination of at least one. Pots were top-watered with osmotic
134 water with an automatic dripper system until germination. Afterwards, pots were watered daily with
135 100mL of a nutritive solution (MgSO4+7H2O 1mL/L, CaCl2 0,63ml/L, K2SO4 1,39mL/L, NaCl 0,1mL/L,
136 Oligo 1mL/L, and iron versenate 1mL/L; pH=6,5). Plants were harvested at three successive growth
137 stages: the first stage (S1) was defined as 5 to 6 leaf nodes, the second stage (S2) was the beginning of
138 flowering (appearance of the first flower); and the third stage (S3) was the beginning of seed filling
139 (with the first pod containing internal seeds at least 8mm in length). The aim was to have nine plants
140 for each growth stage and for each accession (nine replicates x three growth stages x four accessions)
141 resulting in a total of 108 plants, with 27 plants per accession. However, not all seeds germinated, but
142 only 25/27 for *Pisum sativum* subsp*. abyssinicum,* 21/27 *for P. sativum* subsp*. sativum, 15/27 for P.*
143 *fulvum and 15/27 for P. sativum* subsp*. elatius*, which led to a total of 76 plants. Eight pots were left
144 unsown to provide bulk soil samples without any association with *Pisum*, serving as controls. After
145 retrieving the plant, the roots were transferred to a sterile centrifuge tube with 35mL of 1X PBS and
146 kept at 4°C. Roots were initially sonicated for one minute and 30 seconds to collect rhizosphere
147 microorganisms (R), then transferred to a new tube containing 35mL of 1X PBS and subjected to a
148 second round of sonication for 10 minutes to isolate rhizoplane microorganisms (Rp). The R and Rp
149 tubes were then centrifuged at 5000xg for 10 minutes, and 250mg of the resulting pellet was sampled
150 for DNA extraction. Additionally, 400mg of the central root pivot (the first 3cm below seed insertion)
151 were sampled and grinded to retrieve the endosphere (E) microbial communities. At the start of the
152 experiment, and at each stage, bulk soil samples were collected as controls and analyzed using the
153 same procedure as for R and Rp. Some plant nodules were also sampled, however, due to the low
154 number of plants that formed nodules, the results concerning this microhabitat could not be included
155 in this study. In total, we collected 260 samples that included all plant-associated microhabitats at 3
156 growth stages and 32 bulk soils.

157 Bacterial DNA extraction, amplification and sequencing

158 DNA was extracted from all samples using DNeasy® PowerSoil® Pro Kits (QIAGEN®, Hilden, Germany)
159 following the manufacturer's protocol and stored at -20°C. Concentrations and purity of the
160 extractions were assessed using a Nanodrop2000™ spectrophotometer (Thermo Fischer Scientific™,
161 Waltham, USA). DNA extraction yielded insufficient concentration and purity in 11 samples, which
162 were therefore excluded from amplification. The amplification of full-length 16S rRNA gene (V1-V9
163 regions) was performed according to the PacBio® procedure and general best practices
164 recommendations. Single-molecule Real-time long reads sequencing was performed at Gentyane
165 Sequencing Platform (Clermont-Ferrand, France) with a PacBio Sequel II Sequencer (Pacific
166 Biosciences®, Menlo Park, CA, USA). Amplicons were normalized at 5ng/µL, pooled and purified with
167 1.3X SMRTbell cleanup beads. The SMRTbell library was prepared using a SMRTbell prep kit 3.0,
168 following the "Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0" protocol. A Femto
169 Pulse (Agilent Technologies®, Santa Clara, CA, USA) assay was used to assess the fragments size
170 distribution. Pools were carried into the enzymatic reactions to remove the single-strand overhangs
171 and to repair any damage that may be present on the DNA backbone. An A-tailing reaction followed
172 by the overhang adapter ligation was conducted to generate the SMRTbell template. After nuclease
173 treatment, pools were cleanup with 1.3X SMRTbell cleanup beads. The SMRTbell libraries were quality
174 inspected and quantified on a Femto Pulse (Agilent Technologies®) and a Qubit fluorimeter with Qubit
175 dsDNA HS reagent Assay kit (Life Technologies®). A ready-to-sequence SMRTbell Polymerase Complex
176 was created using a Binding Kit 3.1 (PacBio®). The PacBio Sequel II instrument was programmed to load
177 a 150pM library and sequenced in CCS mode on a PacBio SMRTcell 8M, with the Sequencing Plate 2.0

178 (Pacific Biosciences®, Menlo Park, CA, USA) with 0.5 hour of pre-extension time and acquiring one
179 movie of 10 hours.

180 Four SMRTcells were used and the CCS reads were generated from raw data (i.e., subreads) with "ccs"
181 and each sample was demultiplexed from each pool using "lima". These were performed using "SMRT-
182 Link v13.0" software (Pacific Biosciences®, Menlo Park, CA, USA). Two hundred and forty-nine samples
183 were analyzed in a fastq file format. The DADA2 package (Callahan *et al.*, 2016) in R was used to
184 perform taxonomic affiliations of the samples (See supplementary material). Primers were removed
185 and strands orientation was conducted. Then, reads were filtered according to their sizes (between
186 1,000 and 1,600bp), followed by a dereplication and denoising steps to obtain the amplicon sequence
187 variants (ASV). Taxonomic affiliations were done using the "silva_nr99_v138" database (Yilmaz *et al.*,
188 2014), and ASVs present in less than 2 samples were removed. The total number of ASVs retrieved was
189 19,747 (ccs reads data available in Genbank; SAMN45805597-SAMN45805845). Chloroplast,
190 mitochondrial or eukaryote 16S rRNA sequences were removed, which resulted in 18,335 ASVs for 249
191 samples (See supplementary material).

192 Ecological analyzes

193 The statistical analyses were performed using RStudio software (version 4.3.0, see supplementary
194 material). To ensure data quality and that sampling of the different microhabitats was properly
195 conducted, outlier samples from the initial set of 249 were identified using two approaches. Firstly,
196 richness of the samples was calculated, and 62 outliers with aberrantly low or high richness were
197 removed (likely due to technical issues that do not reflect true biological variation). Secondly, a Bray-
198 Curtis dissimilarity matrix was constructed, and Nonmetric Multidimensional Scaling (NMDS) analysis
199 was performed for each microbial community compartment using the "vegan" package (Oksanen *et*
200 *al.*, 2022). To identify outliers, 95% confidence ellipses were drawn based on microbial communities'
201 structure, resulting in the removal of 14 more outliers (see Appendix 2) and a final set of 173 samples.
202 Finally, we removed ASVs whose sequencing depth was less than 0.01% of the total. This resulted in a
203 total of 1,604 ASVs across 173 samples. In order to check that the removal of samples with aberrant
204 species richness did not bias the results, we performed community ecology analyses using only
205 community structure outliers and compared the results (N=229). Analyses of alpha and beta diversity,
206 as well as community composition, indicated that the overall trends and interpretations were
207 consistent between the two datasets (see Appendix 3).The completeness of community coverage was
208 confirmed using rarefaction curves (Schöler *et al.*, 2017). Shannon index (H) and species richness (S)
209 were calculated using the *diversity* function in the "vegan" package. Analyses of the alpha diversity
210 indexes were conducted using multiple Kruskal-Wallis test from the package "agricolae" (de
211 Mendiburu, 2023). Structures of microbial communities were analyzed using Bray-Curtis dissimilarity
212 index and visualized through distance-based redundancy analysis (db-RDA with 10,000 permutations)
213 using the "vegan" package (Oksanen *et al.*, 2022). This multivariate technique has proven to be useful
214 when measuring multispecies responses to structured multifactorial designs (Legendre & Anderson,
215 1999).

216 Bacterial phylogenetic inference

217 In order to infer a bacterial phylogeny including ASVs we retrieved from our samples, we aligned 1,604
218 16S rRNA sequences using MUSCLE 3.6 (Edgar, 2004) and used Gblocks 0.91 (Castresana, 2000) to
219 eliminate poorly aligned positions. The resulting matrix included 1604 taxa and 1,195 characters
220 (16S rRNA sequence matrix is available with the online version of the manuscript). Phylogenetic
221 inferences were performed using BEAST 2.6.4 (Bouckaert *et al.*, 2019). The substitution model used
222 was GTR +I+G (Yang, 1994); three runs of 100 million chains were performed, sampling every 5,000
223 generations. We checked for convergence and viewed statistics for each run using TRACER 1.7.2

(Rambaut *et al.*, 2018). After combining the three runs and removing 40% of burn-in samples, we summarized the results using a maximum clade credibility (MCC) tree.

We used the *rao.diversity* function implemented in the "SYNCA" package (Debastiani & Pillar, 2012) to compute the Rao's quadratic entropy (FD_Q, Rao, 1982) in order to estimate the effects of plant-associated microhabitats and growth stages on phylogenetic diversity. Temporal phylogenetic dispersion model of bacterial communities was estimated by calculating the empirical dispersion parameter (*D*) for each plant-associated microhabitats, following the method developed by Darcy *et al.* (2020). The dispersion parameter identifies the extent to which a newly recruited taxon over time is associated with the phylogenetic distance of a taxon previously retained (Darcy et al., 2020). This metric allows to determine whether newly recruited bacteria over time are phylogenetically distant from previous ones (i.e. are newly recruited bacteria more closely related to previous recruits than expected by chance?). All above analyses were performed using the MCC tree.

Results

Bacterial community composition and structure

Bacterial communities were characterized from a total of 173 samples. To ensure a good coverage of the bacterial communities, we generated rarefaction curves using the full set of 18,335 ASVs, illustrating the increase in observed ASVs as a function of sequencing depth (see Appendix 4). All samples reached an asymptote in their rarefaction curves, indicating that the bacterial communities were fully sampled in all microhabitats and that rarefaction was unnecessary. Community structure differed significantly among the four Pisum accessions, although only a small fraction of the variance was explained (see Appendix 5). Thus, the subsequent results of this part are based on data compiled for the four accessions. Bacterial community diversity varied across the different plant-associated microhabitats (Figure 1A, B). Specific richness was highest in the bulk soil (bulk, median S = 558), followed by the rhizosphere (R, S = 406) and rhizoplane (Rp, S = 411), with the lowest richness observed in the endosphere (E, S = 70; Figure 1A, $p < 0.001$). The Shannon diversity index significantly decreased across the microhabitats, from 5.77 in bulk to 5.45 in R, 5.25 in Rp, and 3.66 in E (Figure 1B, $p < 0.001$). Although specific richness and Shannon diversity were not significantly influenced by growth stages (Figure 1C, D), it is noteworthy that the initial community exhibited the highest diversity, with S = 664 and H = 6.04 in the initial bulk soil (i.e., soil sampled before sowing) (Figure 1C, D).

Bacterial communities' structure differs across plant-associated microhabitats, growth stages and host *Pisum* genotypes ($R^2 = 0.51$, $p < 0.001$, Figure 2A). Changes in structure were best explained by microhabitats (27.63%, $p < 0.001$, Figure 2A). There was no overlap of 95% CI ellipses between microhabitats that were not in close physical proximity (Figure 2A). The highest overlap in 95% CI was observed between R and Rp, while the overlaps with the other microhabitats were small. Growth stages had a lower but significant effect on bacterial communities' structure (4.27%, $p < 0.001$) and this effect was dependent on microhabitats (3.93%, $p < 0.001$, Figure 2A). Time had no significant effect on the bacterial communities in the bulk soil ($p = 0.24$; see Appendix 6A). In contrast, growth stages had a significant and increasingly pronounced effect on the bacterial communities in the plant-associated microhabitats, explaining respectively 9.09%, 11.70%, and 13.48% of the variance in R, Rp and E ($p < 0.001$; see Appendix 6B, C, D). The host genotype (species effect) had a significant but marginal influence on the associated bacterial communities (4.07%, $p < 0.001$). Interestingly, this effect varied across plant-associated microhabitats and growth stages (2.91%, $p < 0.05$ and 3.79%, $p < 0.001$).

The taxonomic composition of bacterial communities at the genus level varied across plant-associated microhabitats (Figure 2B). In the bulk, two major genera were present: *Bacillus*, which constituted 21.8% of the community, and Vicinamibacterales (order level, unidentified genus), accounting for

4.6%, while the rest of the community included many other genera with low abundances (others = 72%; see Appendix 7). While *Bacillus* was dominant in the bulk, its relative abundance decreased across the plant-associated microhabitats to 15.9% in R, 7.8% in Rp and 0.8% in E. Similarly, Vicinamibacterales decreased to 3.4% in R, 1.4% in Rp and was absent in E. In contrast, several genera which were nearly absent in the bulk increased in abundance within the plant-associated microhabitats. Notably, *Rhizobium* rose from 0.5% in bulk to 2.8% in R, 5.6% in Rp, and 19% in E. *Rhizobacter* increased from 0.1% in bulk to 1.2% in R, 4.7% in Rp, and 7.1% in E. *Noviherbaspirillum* increased from 0.2% in bulk to 2% in R, 3.5% in Rp and 4.5% in E. *Rubrivivax* increased from approximately 0% in bulk to 1.2% in R, 2.6% in Rp, and 5.1% in E. *Methylobacillus* increased from approximately 0% in bulk to 1.5% in R, 2.1% in Rp, and 3% in E. *Hydrogenophaga* increased from 0.1% in bulk to 1.2% in R, 3.5% in Rp, and 3.6% in E. *Flavobacterium* increased from 0.46% in bulk to 4.7% in R, 1.4% in Rp, and 3.4% in E. Two genera exhibited inconsistent increases across the plant-associated microhabitats: *Paeniglutamicibacter* showed an important increase from around 0% in bulk to 1.5% in R and 8.2% in Rp, but was absent in E while *Pseudomonas* accounted for 0.4% in bulk, 1.6% in R, 5.8% in Rp and 3.2% in E. Lastly, *Flavobacterium* was more abundant in the plant-associated compartments but did not vary significantly across them, accounting for approximately 0% in bulk, 3.1% in R, 2.9% in Rp and 3.1% in E (see Appendix 7A).

The taxonomic composition of the bulk communities remained stable over time, while significant changes were observed across plant-associated microhabitats (Figure 2B; see Appendix 7B). The relative abundance of four major genera significantly increased across growth stages, from the vegetative (S1) to the seed filling stage (S3). *Bacillus* increased by +10.2% in R, +2.8% in Rp and +1.4% in E ($p < 0.05$). Paeniglutamicibacter increased by +4% in R and +13.2% in Rp ($p < 0.001$). Hydrogenophaga and Vicinamibacterales increased only in Rp, by +2.9% and +0.47%, respectively ($p < 0.05$, Figure 2b). In contrast, relative abundance of five major genera significantly decreased through growth stages: *Rhizobacter* decreased by -1.8% in R, -8.3% in Rp and -14.1% in E ($p < 0.001$); Noviherbaspirillum by -2.55% in Rp and -4.66% in E ($p < 0.001$); Flavobacterium by -6.4% in R, -2.7% in Rp and -2.3% in E ($p < 0.001$); *Rubrivivax* by -2% in R, 2.9% in Rp and -7.2% in E ($p < 0.001$); *Methylobacillus* by -1.2% in R ($p < 0.05$) and by -1.32% in Rp ($p < 0.001$, Figure 2b). The relative abundance of *Pseudomonas* and *Rhizobium* did not vary significantly across growth stages in any of the microhabitats.

Temporal and spatial recruitment in a phylogenetic context

Using our phylogenetic tree of 1,604 bacterial ASVs, we examined the phylogenetic diversity of bacterial communities across the different microhabitats and calculated the phylogenetic dispersion parameter (*D*) to assess the phylogenetic patterns of bacterial recruitment throughout growth stages in each microhabitat. Phylogenetic diversity was the highest in the bulk with $FD_Q = 0.68$ ($p < 0.001$, Figure 3A) and progressively decreased across the plant-associated compartments with $FD_Q = 0.65$ in R, $FD_Q = 0.60$ in Rp and $FD_Q = 0.44$ in E ($p < 0.001$, Figure 3A). The phylogenetic diversity significantly varied across growth stages only within the endosphere, rising from $FD_Q = 0.39$ at S1 to $FD_Q = 0.45$ at S2 and $FD_Q = 0.49$ at S3 ($p < 0.01$, data not shown). The phylogenetic dispersion parameter differs across plant-associated microhabitats (Figure 3B). Bacterial communities in R exhibited overdispersion pattern with $D > 0$ ($D = 0.34$, $p < 0.05$, Figure 3B). In Rp, *D* was lower than in R ($p < 0.1$) and not different to $D = 0$, indicating a neutral phylogenetic dispersion pattern ($D = 0.03$, $p > 0.05$, Figure 3B). Communities in E showed underdispersion, with $D = -0.40$, significantly lower than in Rp and different from the neutral model ($p < 0.05$, Figure 3B), indicating that closely related ASVs have more probabilities of being recruited throughout growth stages.

Discussion

Microbial community assembly

Bacterial communities were different across the different microhabitats. These offer distinct ecological niches, which is reflected by the significant changes in abundance, composition, and structure of bacterial communities. The highest diversity was observed in the bulk soil and gradually decreased from the rhizosphere to the endosphere. Plant-associated microhabitats act as ecological filters along the spatial gradient, where communities from the bulk soil are shaped in the rhizosphere, which are further influenced by the rhizoplane, ultimately resulting in only a small part of these communities being present in the endosphere. Bacterial communities were very specific to the plant-associated microhabitats with very small overlaps of 95% CI. Among the most important changesacross microhabitats, ASVs affiliated to *Pseudomonas* increased from bulk soil to the rhizosphere. This genus is frequently found in the endosphere and nodules of legumes (Mayhood & Mirza, 2021; Hossain *et al.*, 2023) and may influence the outcome of the root-nodule symbiosis (Crosbie *et al.*, 2022). The highest significant shift in relative abundance between the rhizosphere and endosphere was observed for an ASV from *Rubrivivax*, a genus of diazotrophic bacteria capable of nitrogen fixation (Zhou *et al.*, 2021). However, not all *Rubrivivax* ASVs exhibited an increase in the endosphere, highlighting the need to examine plant-bacteria associations at a high infrageneric resolution. ASVs from *Flavobacterium* and *Noviherbaspirillum* were also enriched in plant-associated microhabitats. Members of *Noviherbaspirillum* have been suggested to promote grain biomass in legumes (Barros *et al.*, 2022) and *Flavobacterium* plays a role in phosphate solubilization (Walitang *et al.*, 2017). Conversely, the greatest decreases in abundance across microhabitats were observed for the Vicinamibacterales, with some of which have been identified as potential pathogens for *Vaccinium corymbosum* (highbush blueberry) (Jia *et al.*, 2024), altough their interactions with legumes remain unexplored.

Host growth stages influenced the structure and composition of bacterial communities. One of the highest variations in taxonomic composition through growth stage is the increase in *Paeniglutamicibacter* abundance at later stages in the rhizoplane. While the role of *Paeniglutamicibacter* in plant interactions has not been studied yet, its involvement in hydrocarbon degradation in soils has been demonstrated (Brzeszcz *et al.*, 2024). Since hydrocarbons share structural similarities with many secondary plant metabolites (Singer, 2006; Rohrbacher & St-Arnaud, 2016), it is plausible that this metabolic capacity enables *Paeniglutamicibacter* to respond to root exudates (Pang *et al.*, 2021). Time did not significantly affect the structure of bacterial communities in the control bulk soils, highlighting the importance of the host influence on the temporal dynamic of bacterial communities. Even if further work is necessary to understand the mechanistic processes driving plant-bacterial recruitment throughout plant development, numerous studies have illustrated the influence of plant growth stages on the non-symbiotic microbial communities (Qiao *et al.*, 2017; Dai *et al.*, 2019; Larrouy *et al.*, 2023). These variations seem to be tied to shifts in the plant's requirements throughout its development, as well as to changes in the quality and quantity of rhizodeposits produced by the host (Hütsch et al., 2002; Houlden et al., 2008; Chaparro et al., 2013). Indeed, rhizodeposition is dependent on plant carbon allocation (Shamoot et al., 1968) which varies through growth stages (Morin et al., 2022). Carbon allocation in the roots is important during the vegetative stage (Voisin et al., 2003b). At the flowering stage, the concentration of carbon increases to supply shoot apical meristem and induces floral transition (Cho et al., 2018). During the reproductive stage, carbon is remobilized from the leaves, stem and roots to fill the seeds (Weber et al., 1997). Because the microorganisms associated with *Pisum spp.* can enhance its nitrogen acquisition, the dynamics of the rhizosphere microbiota are linked to the plant's nitrogen needs, which vary at different growth stages (Du et al., 2023). During the vegetative phase, nitrogen requirements are significant to ensure plant growth and to build reserves in the above-ground parts (Voisin et al., 2007). During seed filling stages,

361 nitrogen acquisition is reduced and the plant uses its nitrogen stocks to fill the seeds (Voisin et al.,
362 2003a). Both carbon and nitrogen fluxes variations impact the rhizosphere microbiota; however, the
363 influence of developmental stages on the recruitment of microbial communities remains uncertain
364 (Biget et al., 2024), especially given the potential confounding effect of the time since sowing (Dibner
365 et al., 2021).

366 Our findings reveal that plants host diverse bacterial communities that vary across the microhabitats
367 they provide and throughout their growth stages. These shifts in community composition and structure
368 reflect the plant's capacity to act as an ecological filter, creating distinct niches that select for particular
369 bacterial taxa. At the same time, microorganisms also contribute to shaping their environments,
370 feeding back into the construction and modification of plant-associated microhabitats. Given the
371 complexity of integrating spatial and temporal factors in host–microbiota systems, moving toward an
372 eco-evolutionary framework appears particularly valuable. Such an approach would help disentangle
373 the mechanisms driving community recruitment, persistence, and turnover across plant development.

374 Phylogenetic constraints on bacterial community assembly

375 Our bacterial phylogeny based on full-length high fidelity 16S rRNA gene sequencing of 1,604 ASVs is
376 congruent with a recently published bacterial phylogeny based on genomic data (Zhu *et al.*, 2019). The
377 Proteobacteria form a monophyletic group, with Gammaproteobacteria being sister to
378 Alphaproteobacteria. The Desulfobacteriota are correctly placed in our phylogeny, being a sister group
379 to Proteobacteria (see Appendix 8). The Bacteroidota clade is separated into three main orders:
380 Chitinophagales, Flavobacteriales, and Cytophagales. The Acidobacteriota form a monophyletic group
381 but its placement is not congruent with the phylogeny of Zhu et al. (2019), which retrieved it as a sister
382 group to the Desulfobacteria. However, the number of Acidobacteriota sampled by Zhu *et al.* (2019) is
383 small (56 out of 10,575 taxa) compared to our sampling (128 out of 1,604). Firmicutes, Actinobacteria,
384 Chloroflexi, and Cyanobacteria are monophyletic and form a set of related sister groups. Finally, the
385 Planctomycetota are not present in Zhu et al. (2019) phylogeny, but according to Rensink *et al.* (2020),
386 they are a sister to Thermotogae. Thermotogae are inferred to be sister to Cyanobacteria in Zhu et al.
387 (2019) phylogeny, supporting the placement of Planctomycetota in our phylogeny (see Appendix 8).
388 These results suggest that, although recombination and horizontal gene transfer processes affecting
389 the 16S rRNA gene can weaken the concordance of 16S rRNA phylogenies (Hassler *et al.*, 2022), using
390 full-length sequencing enables the construction of phylogenies that are congruent with phylogenomic
391 inferences. In addition, 16S rRNA gene phylogenies were shown to reflect the ecological and functional
392 traits of soil bacterial communities (Borenstein *et al.*, 2008). The phylogenetic diversity (PD) varied
393 across microhabitats, with the highest PD being observed in the bulk soil followed by a gradual
394 decrease across the rhizosphere, rhizoplane, and endosphere. The ecological filtering associated with
395 increasing plant interaction intensity does not randomly reduce bacterial diversity but rather selects
396 for specific, phylogenetically related lineages, likely by intensifying niche constraints. Goldford *et al.*
397 (2018), using ex situ cultivation of microbial communities from different habitats, showed that
398 communities assembled at family-level, suggesting that this pattern may reflect functional selection
399 linked to competitive advantages. Our results support the prediction that phylogenetically conserved
400 set of traits allow specific bacteria lineages to pass through ecological constraints imposed by the host
401 plants (Aguirre de Cárcer, 2019), suggesting that selective recruitment is correlated with bacterial
402 functions required for plant development, as previously reported by Mougel *et al.* (2006). In addition,
403 the temporal phylogenetic pattern of community assembly varied across the plant-associated
404 microhabitats. The model we used considers the likelihood that newly recruited taxa are similar to
405 closely related taxa previously recruited (Darcy et al., 2020). Overdispersion observed in the
406 rhizosphere suggests that interspecific competition among closely related bacteria plays a crucial role
407 in shaping the community (Nemergut *et al.*, 2013). Closely related taxa, which share similar niches, are

408 competitively excluded, leading to phylogenetic overdispersion of bacterial community. The
409 rhizosphere, as an open and nutrient-rich microhabitat, could provide a diverse array of ecological
410 niches. These niches can support the coexistence of distantly related bacteria, probably with distinct
411 ecological functions. Conversely, the underdispersion pattern observed in the endosphere indicates
412 the opposite process: the bacterial community is not mainly structured by competition among closely
413 related bacteria but rather by strong environmental filtering (Mayfield & Levine, 2010). The
414 endosphere, as a highly selective and closed microhabitat, imposes significant environmental
415 constraints that can be abiotic (physical barriers of root cells) or biotic (plant-derived chemical
416 compounds; Lumibao *et al.*, 2020). It shows that closely related bacteria are able to coexist in a
417 community (Webb *et al.*, 2002), with specific functional traits, often conserved among phylogenetically
418 related taxa. Significant phylogenetic community structure, in our case underdispersion in the
419 endosphere, is suggested to reflect that traits are under ecological selection (Webb *et al.*, 2002;
420 Gerhold *et al.*, 2015). If the traits being selected for are horizontally transferred between bacteria
421 lineages with no connection with their relatedness, we would not observe any phylogenetic signature
422 (Darcy et al., 2020). Selection of bacterial communities or specific strains within these communities by
423 a plant host has been shown in several studies (Bertin *et al.*, 2003; Bulgarelli *et al.*, 2013; Sasse *et al.*,
424 2018; Bourion *et al.*, 2018; Xiong *et al.*, 2021). Over evolutionary timescales, this selective recruitment
425 could have driven the evolutionary history of plant-associated bacterial communities. The interaction
426 between plants and microorganisms, which impacts the fitness of both, could be a keystone in the
427 evolutionary trajectory of land plants and a mark of coevolution (Fitzpatrick et al., 2018). The outcomes
428 of these interactions are context-dependent and change over evolutionary time in response to local
429 selection pressures among the involved species (Koskella & Bergelson, 2020).

430 Altogether, our results illustrate the strong influence of plants on the soil bacterial communities with
431 which they associate, shaping both their ecological and evolutionary dynamics across spatial and
432 temporal scales. Bacterial communities were highly specific to underground plant-associated
433 microhabitats, and their assembly was structured by phylogenetic constraints, with host filtering taxa
434 according to their evolutionary relatedness, consistent with the recruitment of phylogenetically
435 conserved traits. However, investigating the heritability of bacterial communities is crucial for
436 understanding whether these associations are shaped by (i) horizontal transfer from the environment
437 to plant-associated microhabitats, (ii) plant-specific traits, or (iii) vertical transmission across plant
438 generations. Also, the effects of bacterial functions on plants' physiology (for instance, increases in
439 auxins and/or siderophors in plant rhizosphere) and bacterial functional changes over time were not
440 measured in this study. Bacterial functions may constitute an important parameter for explaining the
441 mechanisms involved in plant-bacteria interaction patterns. The extent to which bacteria influence
442 physico-chemical properties of plants microhabitats was not examined in this study but is needed to
443 further disentangle the complex and intricate mechanisms involved in plant-bacteria interactions.

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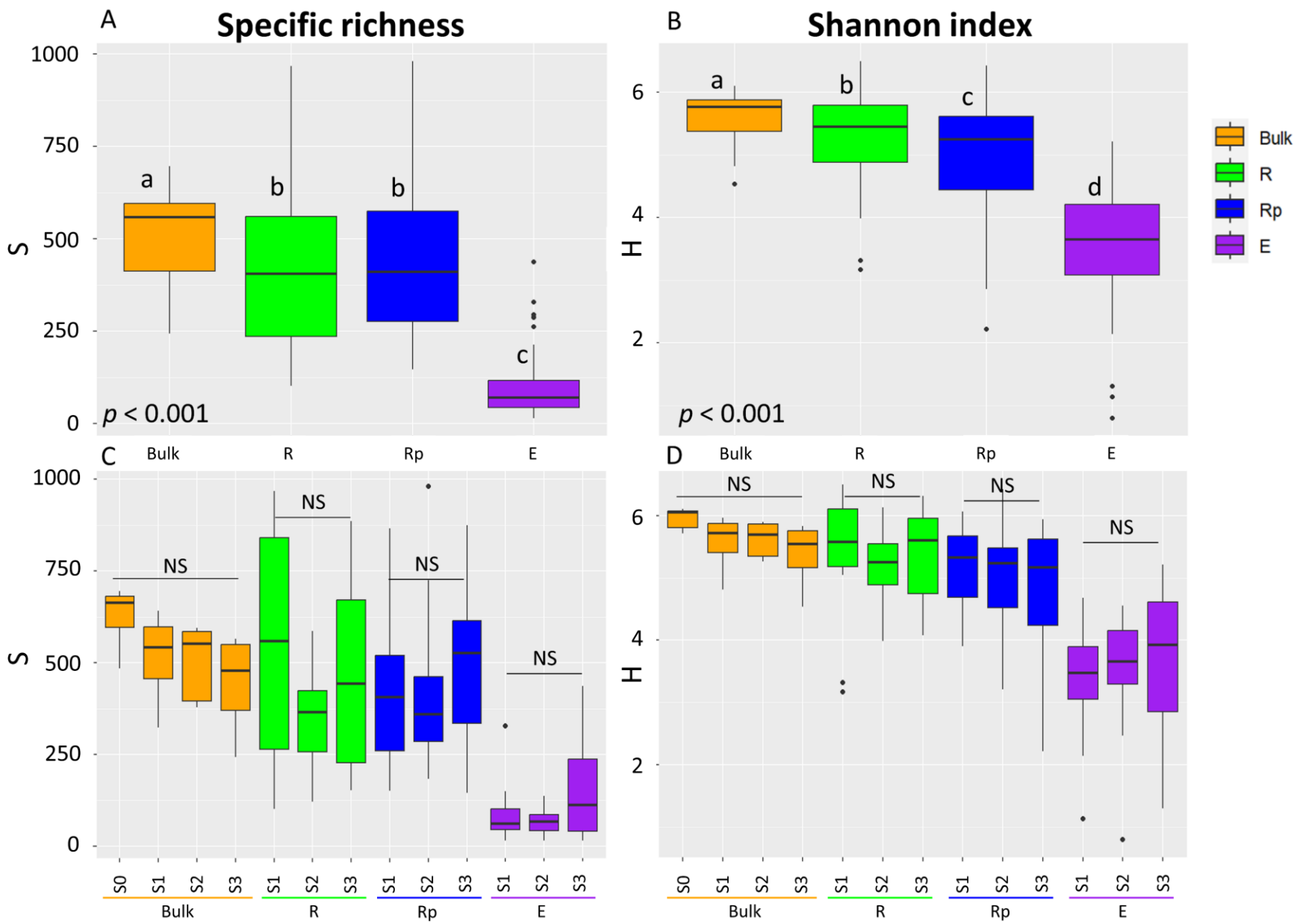

Figure 1: Specific richness and Shannon diversity index of bacterial communities across bulk soil and the different plant-associated microhabitats (a, b) and coupled with growth stages (c, d). Multiple Kruskal-Wallis comparisons (N_{Bulk} = 20, N_R = 45, N_{Rp} = 52, N_E = 56). NS indicates non-significant differences.

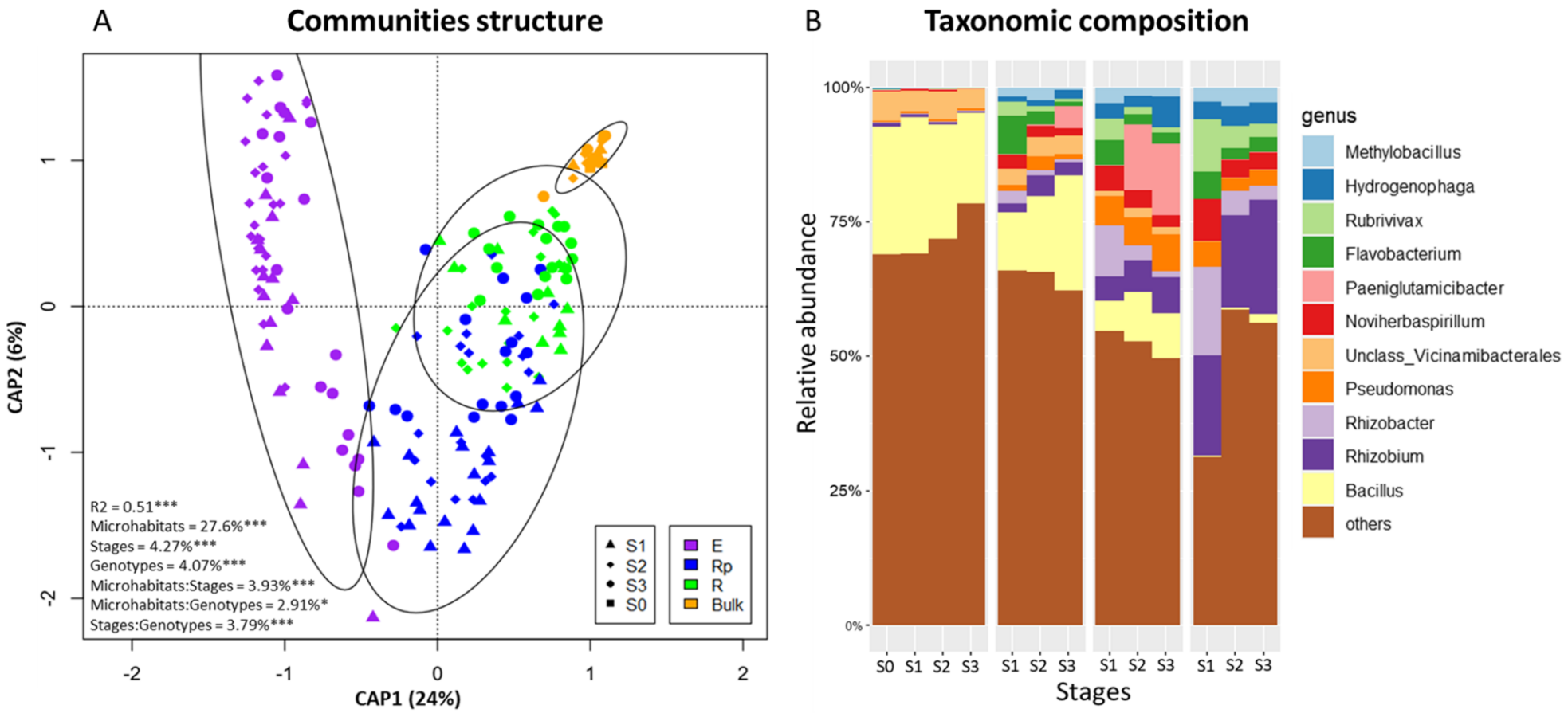

Figure 2: Bacterial communities' structure and composition across bulk soil and the different plant-associated microhabitats and growth stages. **Panel A:** Bray-Curtis distance-based redundancy analysis (similar to Constrained Analysis of Principal Coordinates). The model was tested using PERMANOVA with 10,000 permutations. The R-squared value indicates the percentage of variance explained by the model, including the three variables (microhabitat, growth stages and host genotype) and their interactions. Note that for figure clarity, host genotypes are not displayed. Significance code: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **Panel B:** Average taxonomic composition of bacterial communities based on relative abundance at the genus level across bulk soil, plant-associated microhabitats and growth stages. Only the eleven most abundant genera are shown, with the remaining genera grouped together and labeled as "others". N = 173. Note that data from all host genotypes were compiled.

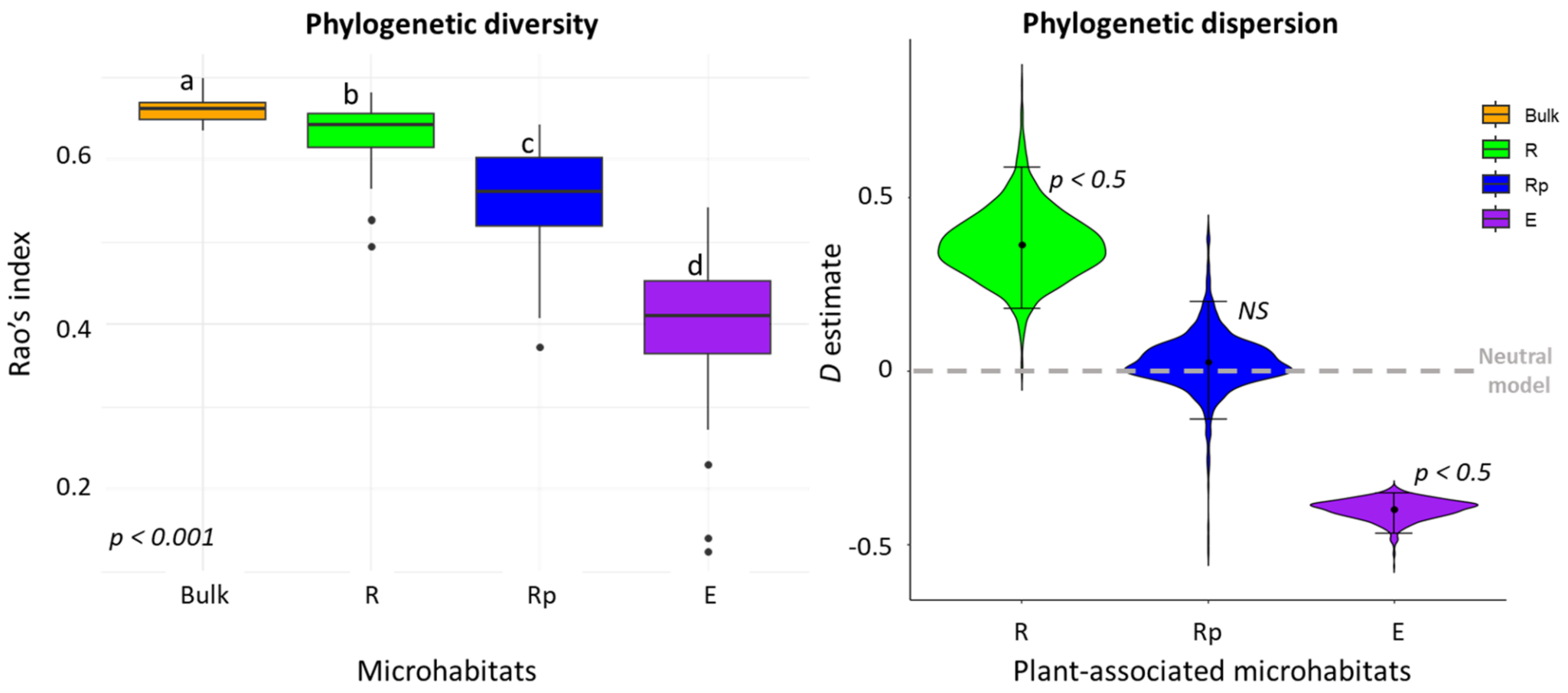

Figure 3: Bacterial communities' structure and composition across bulk soil and the different plant-associated microhabitats and growth stages. **Panel A:** Bray-Curtis distance-based redundancy analysis (similar to Constrained Analysis of Principal Coordinates). The model was tested using PERMANOVA with 10,000 permutations. The R-squared value indicates the percentage of variance explained by the model, including the three variables (microhabitat, growth stages and host genotype) and their interactions. Note that for figure clarity, host genotypes are not displayed. Significance code: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **Panel B:** Average taxonomic composition of bacterial communities based on relative abundance at the genus level across bulk soil, plant-associated microhabitats and growth stages. Only the eleven most abundant genera are shown, with the remaining genera grouped together and labeled as "others". N = 173. Note that data from all host genotypes were compiled.