



The microbes within: what happens inside *Acacia longifolia* nodules during development

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Abstract

Plant-microbe interactions are important for plant development, particularly mutualisms where host promiscuity allows associations with diverse microbial partners. This flexibility is crucial for adaptation, especially in invasive species. Understanding microbial community dynamics is therefore key to explain invasion success. *Acacia longifolia*, a member of the Fabaceae family, is an aggressive invader capable of establishing symbioses with several microorganisms including rhizobia within root nodules. These interactions promote growth and play a role in its invasive capacity. However, the nodulation process remains poorly understood, regarding microbial succession and the dynamics of these communities, following nodule development. We assessed microbial profiles in root nodules from 1-year-old saplings in two habitats using Next-Generation Sequencing, targeting 16 S and 25–28 S rRNA genes. Nodules were classified by size as a proxy for developmental stage. Our findings show that (i) different developmental stages have a characteristic microbial community; (ii) there is a shift in dominance (i.e., abundance) from early to fully developed stage, with nodules containing respectively more microbes from seed or from soil; (iii) microbial partners change in each habitat. The microbial succession indicates a shift in abundance over time, highlighting mostly the changes in recruitment: while several genera that dominate early-stage nodules are mostly found in seeds, fully developed nodules have a community mostly acquired from the surrounding soils and showed a much more specialized fungal community. Our study shows a dynamic assembly of root nodules communities within invasive range that might contribute to the plasticity and adaptative strategy of *A. longifolia* in these new habitats.

Keywords Nodulation · Early developed · Fully developed · Abundance · Succession

1 Introduction

Plant-microbe interactions are an interesting topic to study under the scope of climate change (Aqeel et al. 2023) and plant invasions (Coats and Rumpfo 2014). A particularly important relationship is mutualistic associations, in which both partners, the host and the symbiont, gain advantage in a positive feedback basis (Carrapiço 2010). Root nodules are an example of these interactions

that occur in the Fabaceae family, rendering these structures of utmost interest (Desbrosses and Stougaard 2011). Rhizobia, the group of nitrogen-fixing bacteria, are housed inside these specialized structures and use glucose provided by the host as an energy source to fix atmospheric nitrogen and convert it into fixed forms (e.g., ammonia), which can be used by the plant for its metabolism and synthesis of organic molecules (Dupont et al. 2012). Despite this standard process assigned to nodulation, rhizobia represent a group under constant revision and update (e.g., Kuzmanović et al. 2022), not only due to advances in identification and analytical techniques, but also because of their agricultural and ecological importance (Basile and Lepek 2021; Yermko et al. 2025). Alongside, microbial heterogeneity within root systems is well documented, with previous studies demonstrating that microorganisms are not homogeneously distributed but instead vary according to root developmental stage

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(primary vs. secondary roots) and specific root zones (e.g. root tip, lateral meristem, and border cells; Philipot et al. 2013). Consequently, exploring whether distinct microbial assemblages colonize nodules at different developmental stages and how these assemblages change over time is particularly relevant, as such dynamics may differentially influence plant performance. For instance, in *Arabidopsis thaliana*, distinct root zones along the soil depth gradient host different bacterial communities associated with contrasting metabolic functions (Loo et al. 2024).

It is also known that the interaction between rhizobia and legumes is accompanied by intricate molecular dialogues and subsequent infection processes (Dupont et al. 2012; Magne et al. 2024), making it a controlled process, especially by the host (Ferguson et al. 2019). While many crop legumes exhibit relatively high host–symbiont specificity (Wang et al. 2018), *Acacia* sensu lato tends to be more promiscuous in partner selection (Rodríguez-Echeverría et al. 2009; Keet et al. 2017; Jesus et al. 2020, 2023, 2025), associating with a broader range of rhizobial taxa rather than relying on a single specific symbiotic partner (Magne et al. 2024). Also, nodulation in legumes, in the initial step of root curling, can allow the entrance of other non-rhizobial partners and several studies have reported this promiscuity (Martínez-Hidalgo and Hirsch 2017; Hansen et al. 2020; Etesami 2022; Jesus et al. 2023). Furthermore, previous studies also showed the presence of fungal genera inside *Acacia* spp. root nodules (Scheublin and Van Der Heijden 2006; Jesus et al. 2023), which is expected having in mind the richness and diversity of the soil environment. Despite this inherent interaction between acacias and soil determining a selection of partners for symbiotic interactions in nodules, it is important to consider the contribution of vertical transmission associated with seed microbiome. Seeds also harbor a specific but diverse set of microbes that can help plants in early germination and development (Condessa et al. 2024; Jesus et al. 2025, 2026).

Acacia longifolia Andrews Willd. is a leguminous tree native to Southern Australia and is classified as highly invasive, spreading in several Mediterranean-type ecosystems, particularly in Portugal (Marchante et al. 2008, 2023; Colaço et al. 2023). Its introduction was based on its ability to prevent erosion by stabilizing sand dunes, which can be partly ascribed to its symbiotic interactions (Peperkorn et al. 2005). However, this symbiosis not only allows nitrogen fixation but gives a competitive advantage in nutrient-poor soils (Werner et al. 2010; Antunes et al. 2018a; Marchante et al. 2008; Souza-Alonso et al. 2017). This leads to a disruption of native ecosystems by altering nutrient cycles, especially

nitrogen and phosphorus, displacing native flora (Marchante et al. 2008; Ulm et al. 2017a, b), ending up with *A. longifolia* reshaping belowground microbial communities. *Acacia* sp. root nodules are of indeterminate-type starting with root hair infection; when fully developed, root nodules are elongated structures, often branched, and internally divided into four histological zones reflecting successive developmental stages: the meristematic zone (I), infection zone (II), nitrogen-fixing zone (III), and senescence zone (IV) (Jesus et al. 2023). Zone I is the active growth region and is usually free of bacteria. In zone II, infection occurs via infection threads, leading to bacterial release and differentiation into bacteroids in zone III, where nitrogen fixation takes place. Finally, zone IV, located near the root attachment, is characterized by cell degradation and loss of bacterial function (Franssen et al. 1992; Maunoury et al. 2008; Dupont et al. 2012; Giller et al. 2016). The microbiome present inside root nodules can be broader than the rhizobia group (e.g., Martínez-Hidalgo and Hirsch 2017; Jesus et al. 2020), and *A. longifolia* has been classified as a promiscuous legume interacting with both bacteria and fungi (Jesus et al. 2023). Understanding how these communities inside *A. longifolia* nodules change during nodule maturation is critical for elucidating the ecological mechanisms underpinning its invasiveness. Such shifts may reflect selective recruitment of beneficial microbes, co-evolutionary matches, or opportunistic colonization by saprophytic or pathogenic taxa (Stengel et al. 2022). Moreover, these microbial dynamics may influence not only nitrogen fixation efficiency but also broader plant–soil feedback, potentially facilitating the spread of *A. longifolia* and its long-term ecological impacts. So, if nodulation is such a dynamic process, the microbial shift throughout nodule development – from initiation to full development – is of remarkable interest, especially considering specific contexts in which mutualisms can be determinant, as for plant invasions.

This study aims to characterize compositional and taxonomical shifts in *A. longifolia* microbial communities, to provide new insights into the dynamics of microbial communities following nodule development. Specifically, we hypothesized that (i) root nodule microbial communities vary according to nodule development; (ii) there are changes in microbial communities that reflect the contribution of both soil and seed microbiomes: while early developed nodules integrate mostly the seed microbiome, fully developed ones have a community dominated by microbes acquired from the soil; (iii) nodule is a structure that includes a microbiome influenced by the surrounding habitat, reflecting the plant's adaptation to local environmental conditions.

2 Materials and methods

2.1 Field sampling

In Spring 2023, three *Acacia longifolia* saplings (ca. one year old) were sampled in two distinct habitats, a Dune and a Forest in Mira, Centre of Portugal. Within each habitat, sampled individuals were selected, at approximately 10 m from each other, to minimize microsite overlap and to better capture within-habitat variability. Saplings were measured (height and diameter at base height) and leaves were collected; roots were carefully dug up to harvest the attached nodules. Nodules were counted, organized by size (i.e., early or small, intermediate and fully or big) before storage in separate tubes, containing silica to dehydrate until further processing. Parameters at plant, nodule and leaf levels were measured for each sapling in each habitat. Photochemical efficiency of the photosystem II was measured using Handy PEA (Hansatech Instruments), in the field before harvesting the plants (five measurements per sapling). Other leaf traits were measured and include i) water content calculated using the formula $[(\text{fresh weight} - \text{dry weight}) / \text{fresh weight} \times 100]$ (five leaves per sapling); ii) area measured using the Area meter (Licor 3100 C) (five leaves per sapling); iii) isotopic analysis performed using composite samples (five leaves from each sapling). For this analysis, samples were dried at 60 °C for one week and then converted into powder using a ball mill and weighed to further analysis in a tin capsule. Carbon and nitrogen isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) were determined by continuous flow isotope ratio mass spectrometry (CF184 IRMS) on a Sercon Hydra 20–22 (Sercon, UK) stable isotope ratio mass spectrometer

coupled to a EuroEA (EuroVector, Italy) elemental analyser. Uncertainty of the isotope ratio analysis, calculated using values from 6 to 9 replicates of secondary isotopic reference material interspersed among samples in every batch analysis, was $\leq 0.1\%$. This analysis was performed in the Stable Isotopes and Instrumental Analysis Facility (SIAF) Faculty of Sciences of the University of Lisbon.

2.2 Nodule characterization

In the laboratory, for each sapling within each habitat, nodules were carefully organized by size as a proxy for one of two developmental stages: round and smaller hereafter referred to as early developed and elongated and bigger hereafter defined as fully developed (Fig. 1). Intermediate nodules were excluded from further analysis. Composite samples of each developmental stage (five nodules per sapling) were prepared for each habitat to proceed with high throughput sequencing.

Alongside, mature seeds from the nearby adult trees and soil from the rhizospheric environment were collected in each habitat as described in Jesus et al. (2025) and microbial communities were also identified through high throughput sequencing.

2.3 Microbial profiling

Root nodules (5 nodules per sample) were organized by developmental stage for each sapling in each habitat (three replicates for each habitat, two habitats totalizing 6 samples) and were prepared for Next-generation sequencing (NGS), along with rhizospheric soils and seeds. Before

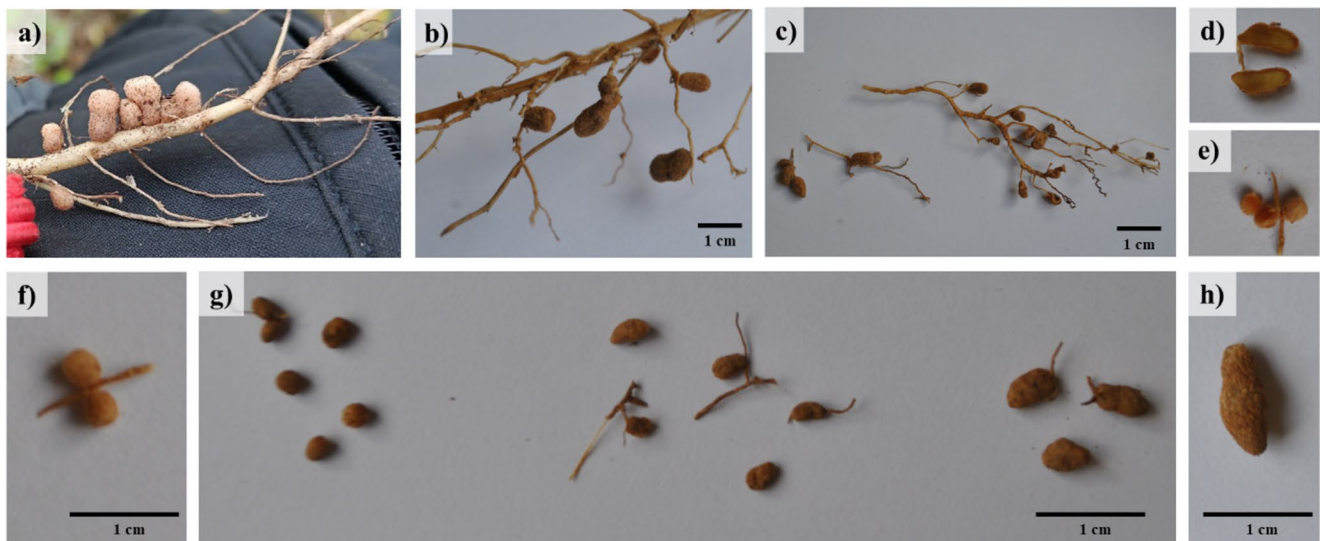


Fig. 1 Different developmental stages identified in the root system of *Acacia longifolia* saplings: nodules attached to the roots (**a–c**); functional nodule with the characterized reddish tissue (**d–e**); early devel-

oped nodule (**f**); nodules organized by size, including early developed on the left, intermediate type in the middle and fully developed on the right (**g**); fully developed nodule (**h**)

DNA extraction, root nodules were surface disinfected to reduce contamination from microorganisms present on the nodule surface. The technology used was Oxford Nanopore © performed to infer microbial communities. Total DNA was extracted using the modified CTAB (CetylTrimethyl-Ammonium Bromide) protocol according to Yuan (2019). DNA purity was accessed by absorbance at 260 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA), and the concentration by Qubit™ 4 Fluorometer (Thermo Fischer Scientific, USA). The extracted DNA was stored at -20°C until further processing. For the rDNA gene amplification, Long Amp hot start Taq 2× master mix (New England Biolabs, MA, USA) was used at 1X along with 50 ng/μL of genomic DNA from swab samples. To amplify the full-length 16S rDNA and 25–28S rDNA bacterial and fungal gene, respectively, 0.25 μM of the primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-C GGTTACCTTGTTACGACTT-3') and RL0R_Fw (5'-AC CCGCTGAACCTTAAGC-3') and LR7_Rv (5'-TACTACC ACCAAGATCT-3') were used (Weisburg et al. 1991; Vilgalys 2016). PCRs was conducted on a Biometra UNO II, using the following conditions for 16S rDNA gene: 1 cycle of 94 °C for 1 min, 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 65 °C for 2 min, and a final extension of 65 °C for 5 min and for 25–28 S rDNA: 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 65 °C for 1 min, and a final extension of 65 °C for 5 min. Subsequently, amplification products were visualized via gel electrophoresis and purified using the Solid Phase Reversible Immobilization (SPRI) technique with magnetic beads (Stotchevoi et al. 2020). The library was prepared from 200fmol input DNA from each sample using the Sequencing Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK) in accordance with the manufacturer's protocol. Sequencing runs were performed using R10.4.1 flow cells on a PromethION sequencing platform and sequencing data were acquired in real time using MinKNOW 18.08.2 software. Sequencing data was stored in fastq files, with each file representing a batch of 4000 reads. Sequencing data was derived from 16 S to 25 S–28 S amplicons, with the exclusion of low-quality reads. Furthermore, for 16 S, sequences assigned to chloroplasts were identified during taxonomic filtering and removed prior to downstream analyses to prevent host contamination. The remaining reads underwent size selection, retaining those with lengths between 1200 bps and 1700 bps, accomplished through prinseq-lite (Schmieder and Edwards 2011). Taxonomic classification employed a Lowest Common Ancestor approach, utilizing an index based on k-mers that map to the lowest common ancestor of all genomes known to encompass a specific k-mer, using the software Kraken (Wood et al. 2019) and reads were classified according to NCBI

Taxonomy database [RefSeq+GenBank Archaea and Bacteria (May 2023) and GenBank Fungi (April 2022)].

2.4 Statistical analysis

All classified microbial operational taxonomic units (OTUs) at the genus level were used to construct a two-dimensional non-metric multidimensional scaling (NMDS) ordination to assess whether bacterial and fungal communities were segregated according to nodule developmental stage and habitat. Venn diagrams, at genus level, were constructed to show the number/percentage of common and specific genera for early and fully developed nodules, seed and soil for each habitat. Alpha diversity metrics were also calculated including Shannon-Wiener diversity (Shannon 1948), Chao richness (Chao 1984) and Pielou dominance (Pielou 1966) for all samples (nodules from both developmental stages, seed and soil) and for both habitats. To further integrate microbial community composition and infer the relative contribution of soil- and seed-associated microorganisms, heatmaps were generated using abundance data from the ten most abundant genera identified in each sample. Abundance values were z-score normalized to improve comparability among samples. Hierarchical clustering of samples (rows) and genera (columns) was performed based on Bray–Curtis dissimilarity, using the unweighted pair-group method with arithmetic mean (UPGMA) as the clustering algorithm. Data analysis was performed in R Studio (2024.12.0) (R Core Team, 2024) using the packages *pheatmap* (Kolde 2019), *stats* and *vegan* (Oksanen et al. 2022).

3 Results

Plant, nodule and leaf parameters showed differences among saplings sampled in dune and forest (Table 1). Saplings from forest exhibited greater shoot growth, while DBH values were comparable or slightly lower than those observed in dune. Nodule characterization revealed variation across developmental stages, with differing distributions between habitats. Regarding leaf traits, water content, leaf area, and yield varied among saplings, without a clear and consistent pattern between habitats. Stable isotopes ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$), as well as N and C contents, also differed between dune and forest saplings, showing variation in ecophysiological strategies.

Microbial communities were clustered considering nodule development stage ($p < 0.01$), indicating changes in bacterial and fungal compositions ($R^2 = 0.41$ and $R^2 = 0.74$, respectively) (Fig. 2). The influence of habitat in microbial communities' segregation was also checked but was not significant in both communities.

Table 1 Plant, nodule and leaf parameters assessed in the field. Plant measurement includes shoot height (cm) and Diameter at Base Height (DBH, cm); Nodule characterization includes the number of early, intermediate and fully developed nodules. Leaf traits include water content (%), area (cm²), yield, isotopic signatures for nitrogen and carbon ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, ‰) and nitrogen (N) and carbon (C) contents (%). Mean and standard deviations are presented when applicable

	Individual	Dune			Forest		
		1	2	3	1	2	3
Plant measurements	Shoot (cm)	45	70	30	75	90	90
	DBH (cm)	1.1	1.3	0.6	0.85	0.8	0.8
Nodule characterization	Early developed	46	8	24	45	21	31
	Intermediate developed	10	3	8	18	7	15
	Fully developed	30	5	16	18	7	15
Leaf traits	Water content (%)	57.03±2.5	53.73±5.6	49.8±2.2	52.86±4.6	52.23±4.7	51.24±5.2
	Area (cm ²)	14.33±4.4	11.92±3.6	9.67±3.3	10.38±5.3	9.88±1.9	8.53±1.8
	Yield	753±32.8	445±75.7	733±39.9	729±39.6	733±32.5	722±73.6
	$\delta^{15}\text{N}$ (‰)	-2.12	-2.4	-2.34	-3.42	-3.18	-2.95
	$\delta^{13}\text{C}$ (‰)	-30.25	-30.28	-27.89	-27.9	-26.84	-31.18
	N (%)	2.34	2.26	2.4	1.89	1.71	1.96
	C (%)	47.85	47.17	49.84	52.06	48.19	47.24

Furthermore, Venn diagrams show the common and specific genera present in each sample, including nodules from both developmental stages, seed and soil for both habitats (Fig. 3). We found more bacterial genera compared to fungi among samples. As well, for bacterial communities, we identified higher values of specific genera in soil (43.9 and 33.1% in dune and forest, respectively), followed by fully developed nodules (3.2 and 6.5% in dune and forest, respectively), early developed nodules (2.6 and 4.2% in dune and forest, respectively) and seed (0.4 and 1.8% in dune and forest, respectively). For fungal communities, more exclusive taxa were found in fully developed nodules (7.4 and 12.9% in dune and forest, respectively), followed by soil (7.0 and 9.8% in dune and forest, respectively), seed (2.7 and 2.2% in dune and forest, respectively) and early developed nodules (2.5 and 1.4% in dune and forest, respectively). Shared or common genera were found when comparing all samples, varying considering microbial group and habitat. Nodules, both fully and early developed, shared taxa with seeds and soil.

Diversity metrics were different for dune and forest habitats (Table 2). These values show that early developed nodules had higher diversity, dominance and OTU number and higher richness in dune compared to fully developed (3.69, 0.55, 782 and 1202 compared to 3.5, 0.53, 726 and 1232, respectively). In forest, the pattern was similar, with early developed nodules showing higher values of diversity and dominance and lower values of richness and OTU (3.75, 0.57 and 1138, 696 compared to 3.65, 0.56 and 1164 and 724, respectively).

Heatmap hierarchical clustering revealed distinct abundance patterns associated with both microbial sources and nodule developmental stage. For bacterial communities in dunes, both early and fully developed nodules clustered together with soil and separated from seeds (Fig. 4).

Early developed nodules were dominated by the genera “*Candidatus Portiera*” and *Synechocystis* in dune, while fully developed nodules presented *Bradyrhizobium* as the more abundant genera, followed by and *Acaryochloris* and *Pseudomonas* (Fig. 4a). In forest, early developed nodules were dominated by “*Candidatus Portiera*” and “*Candidatus Paraprochloron*”, and fully developed nodules presented a higher abundance of *Acaryochloris* (Fig. 4b). Also, seeds were more characterized by a community dominated by cyanobacteria, that were consistently present in nodules from both developmental stages, but more closely resembled to nodules in dune compared to forest. Considering soil communities, while in dune there was a higher abundance of *Planktothrix* and *Klebsiella*, in forest, *Bradyrhizobium* and *Paraburkholderia* were the dominant genera.

For fungal communities, in dune habitat, community composition showed a clear differentiation between early and fully developed nodules, as well as between nodules and their potential sources (seed and soil) (Fig. 5). In dune, early developed nodules clustered more closely with seed-associated communities and were characterized by higher relative abundances of genera such as *Phialophora*, *Fusarium* and *Acremonium* (Fig. 5a). In contrast, fully developed nodules displayed a distinct fungal profile, with increased relative abundance of genera including *Coniochaeta*, *Solicozozyma*, *Synecephalastrum*, *Sordaria* and *Serendipita*, which were also more abundant in soil communities. In forest, fungal communities exhibited a similar but more pronounced structuring pattern (Fig. 5b). Early developed nodules were more closely aligned with seed-associated communities, showing higher relative abundances of genera such as *Clonostachys*, *Phialocephala*, *Fusarium* and *Acremonium*. Fully developed nodules, however, clustered nearer to soil communities and were dominated by genera including *Metschnikowia*, *Tubaria* and *Solicozozyma*.

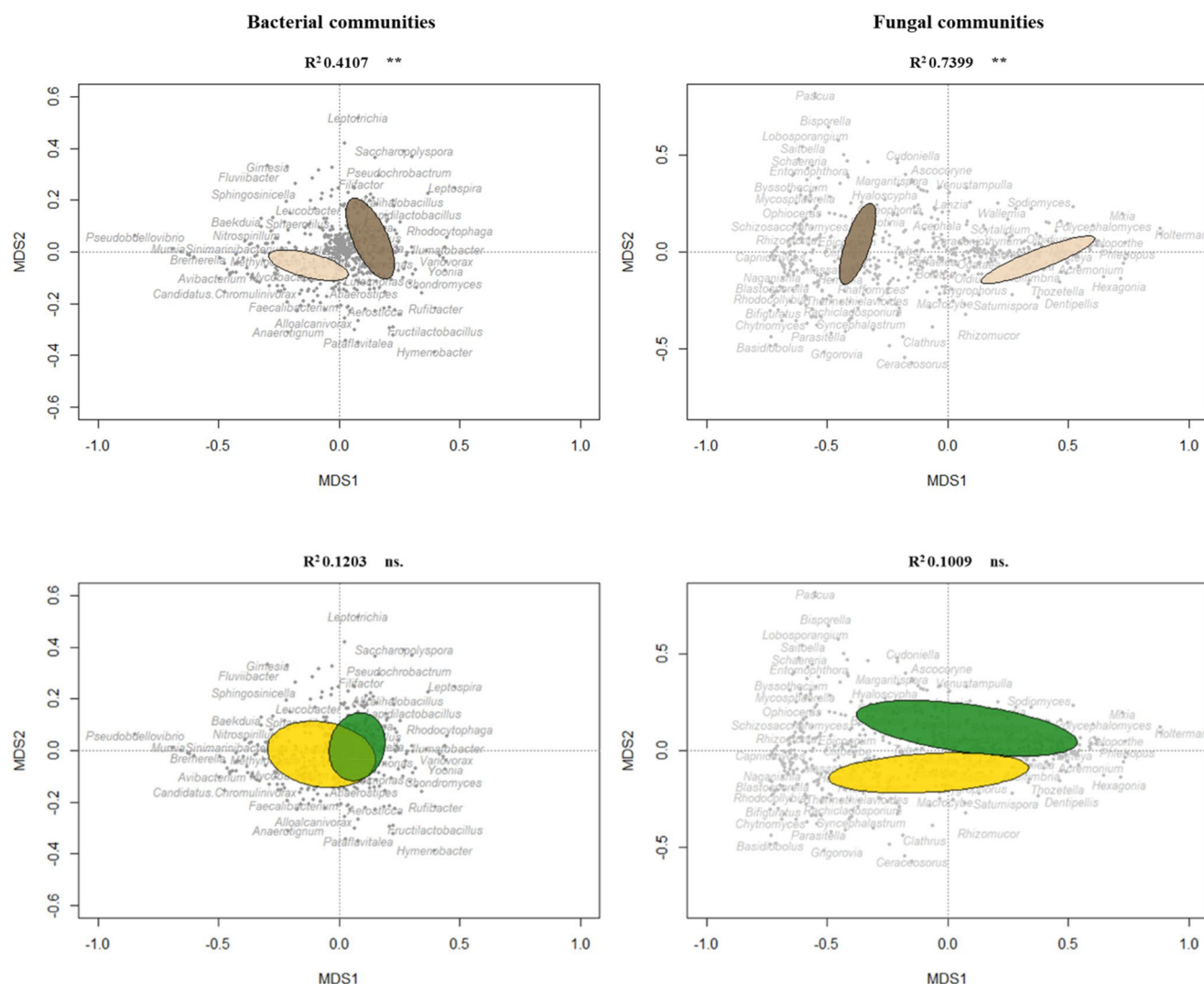


Fig. 2 Two-dimensional non-metric MultiDimensional Scaling (NMDS) based on genus level for bacterial and fungal communities (stress value = 0.07 and 0.06, respectively) for nodule developmental stage and habitat. Colors represent nodule developmental stage (light

pink for early developed and brown for fully developed) and habitat (yellow for dune and green for forest). Squared R and statistical significance are presented in each figure

4 Discussion

Root nodules are fundamental structures for leguminous plant development due to their pivotal role in biological nitrogen fixation (Dupont et al. 2012; Magne et al. 2024). In legumes such as *Acacia longifolia*, a species widely recognized for its invasive capacity, nodulation appears to be a key mechanism underlying ecological adaptation and, consequently, invasive success (Rodríguez-Echeverría 2007; 2009; 2010; Souza-Alonso et al. 2017; Jesus et al. 2025). Bacterial communities inhabiting legumes' root nodules have been extensively characterized since the work of Franssen et al. (1992), which revealed a predominance of classical rhizobia, including genera such as *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Burkholderia*

and *Paraburkholderia*. More recent studies have expanded this view, highlighting the presence of diverse non-rhizobial microsymbionts (Martínez-Hidalgo and Hirsch 2017; Jesus et al. 2020, 2025), as well as a complex fungal community associated with nodules (Jesus et al. 2023). Despite these advances, the temporal dynamics and successional patterns of microbial communities throughout nodule development remained poorly understood. The present study proposes that nodule-associated microbial communities exhibit successional patterns following developmental stage, despite some microbes being acquired within the same temporal window (i.e., root infection step). Shifts in microbial abundance were generally aligned with differential contributions from seed- and soil-derived microorganisms; in particular, our findings suggest that rhizobial partners are not vertically

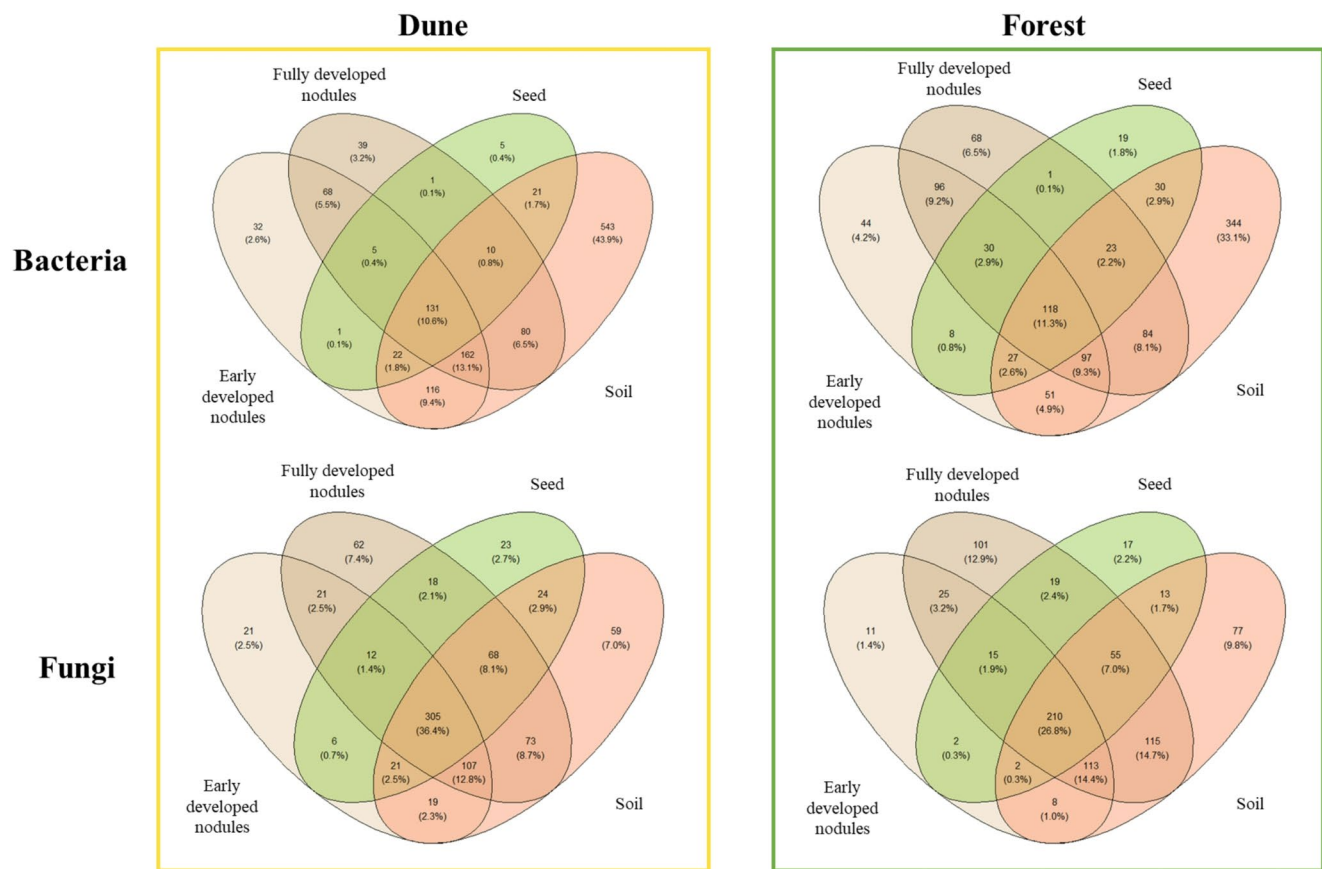


Fig. 3 Venn diagrams at genus level presenting the shared and the exclusive bacterial and fungal taxa for each nodule developmental stage (early and fully developed nodules), seed and soil, in each habitat (dune and forest)

Table 2 Diversity metrics including Diversity (Shannon-Wiener), Richness (Chao1) and Dominance (Pielou) and Operational Taxonomic Units (OTUs), for each habitat (dune and forest) and for each sample (Early and fully developed nodules, seed and soil). Standard deviations are presented when applicable

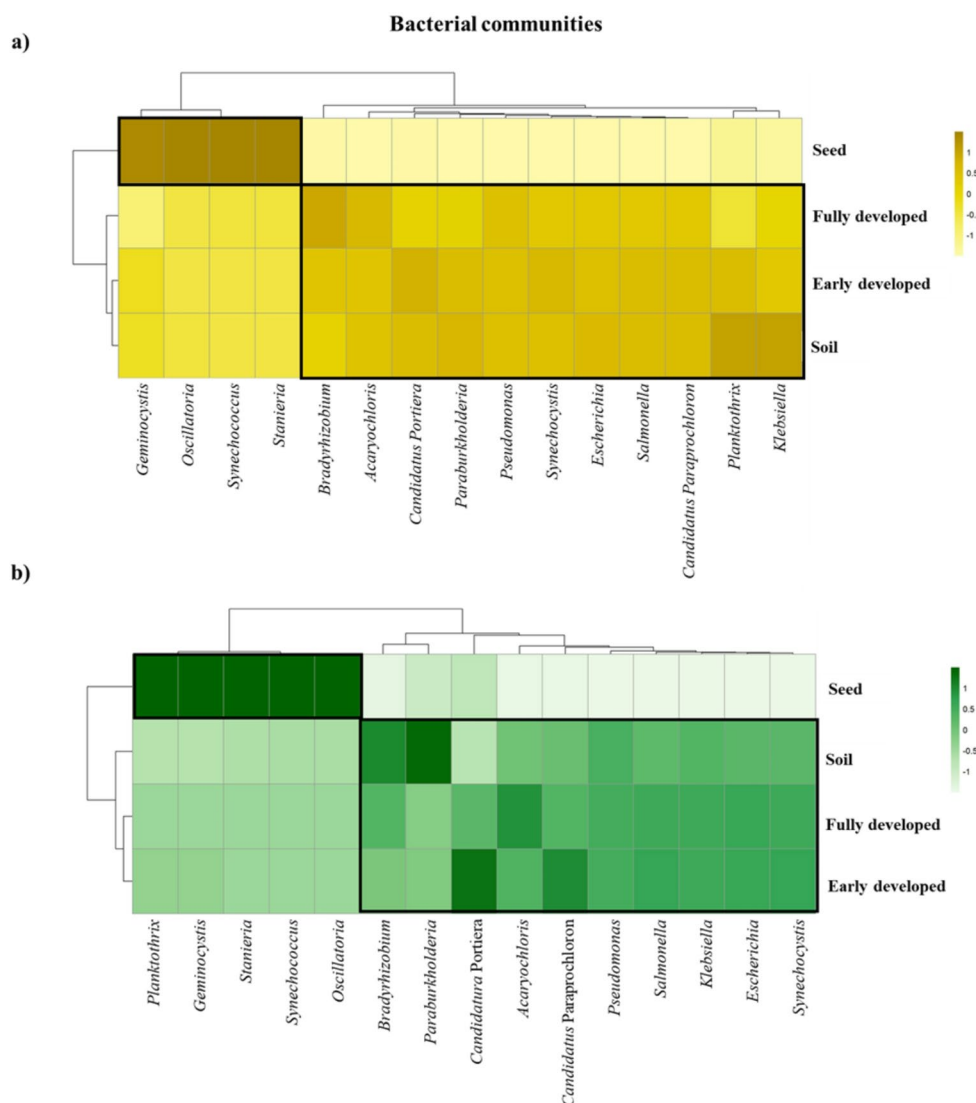
Habitat	Sample type	Diversity	Dominance	Richness	OTU
Dune	Early developed	3.69±0.06	0.55±0.01	1202±19	782±33
	Fully developed	3.50±0.25	0.53±0.04	1232±76	726±26
	Seed	0.998	0.18	538	265
	Soil	3.561	0.45	3496	2602
Forest	Early developed	3.75±0.06	0.57±0.01	1138±74	696±22
	Fully developed	3.65±0.07	0.56±0.01	1164±12	724±23
	Seed	1.13	0.19	875	370
	Soil	3.62	0.49	2330	1682

transmitted but are instead recruited from the surrounding soil. Consistent with previous findings from our group demonstrating a seed contribution to nodule microbiome (Jesus et al. 2025), here we found that early developed nodules were characterized by a higher relative abundance of seed-associated fungi and soil-derived bacteria. Fully developed nodules showed an increased abundance of soil microbes, which seems to be the case of rhizobia that are not vertically transmitted. This highlights a dynamic process of microbial community assembly occurring during nodule maturation.

Alongside, we identified a succession that reflects in dominating genera, but within habitat specificity.

Microbial communities inside *A. longifolia* nodules have a conserved set of bacterial taxa, varying in abundance, that is shared across developmental stages and habitats, showing however some inherent variability. The presence of this rich and highly diverse microbial community within *A. longifolia* nodules may be host-related, particularly considering the early stages of nodulation (Ferguson et al. 2019). Leguminous plants release flavonoids that specifically attract

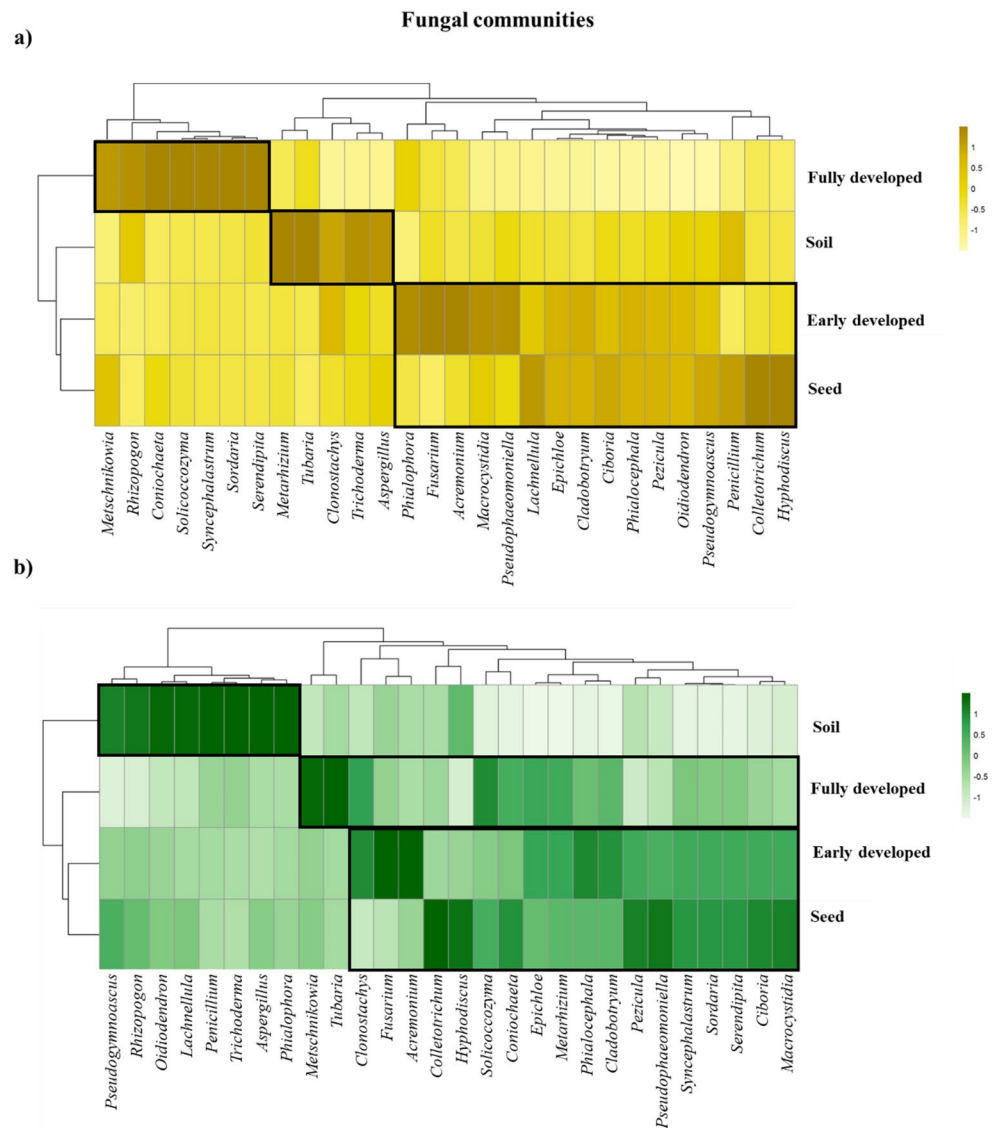
Fig. 4 Heatmap based on bacterial communities at genus level (top 10) considering each sample (soil, seed and fully and early developed nodules) for each habitat, dune (a) and forest (b). Samples (rows) and genera (columns) were clustered hierarchically based on relative abundance' profiles. These values were standardized in z-scores (varying between -1 and 1, against each mean value)



bacteria to their roots (Dupont et al. 2012). Alongside rhizobia, other microorganisms may also be recruited as “hitchhikers” during this process (Hassan and Mathesius 2012). In the case of invasive plants, the outcome of these interactions may differ from those observed in the native range already proposed by other authors (e.g., Callaway et al. 2008; Delavaux et al. 2017). In this study, potential hitchhikers include cyanobacteria, which have previously been reported within root nodules (e.g., Jesus et al. 2023; 2025) and are known to establish symbiotic associations with a wide range of plants (Álvarez et al. 2023). This ability to symbiotically interact with plants and their capacity for nitrogen fixation (Singh 2014; Mishra et al. 2021; Nawaz et al. 2024) may facilitate their entry and persistence within *A. longifolia* nodules. This may help explain the high relative abundance of cyanobacteria in early developed nodules, potentially acquired via seeds, including the genera *Synechococcus*, *Oscillatoria*, *Planktothrix*, and *Geminocystis*. These taxa were firstly

identified in aquatic systems however recent research has been showing that they can colonize terrestrial ecosystems being present in soils and rhizospheric environments (Kaštrovský 2024). Notably, these genera were consistently detected throughout nodule maturation, suggesting stable persistence once established. Their early presence may be further facilitated by the transition of an initially oxygen-rich nodule environment, which later becomes microaerophilic, providing the conditions required for effective nitrogen fixation (Dupont et al. 2012). However, it has not been demonstrated that cyanobacteria are active nitrogen fixers inside these nodules, so its full role remains to be clarified. Given that *A. longifolia* is a highly efficient invasive species with effective water and nutrient-use strategies (Marchante et al. 2008), these patterns may reflect an integration of these cyanobacteria within the seed that are passed vertically to the novel plant generation enhancing the ecological performance in the invaded range.

Fig. 5 Heatmap based on fungal communities at genus level (top 10) considering each sample (soil, seed and fully and early developed nodules) for each habitat, dune (**a**) and forest (**b**). Samples (rows) and genera (columns) were clustered hierarchically based on relative abundance profiles. These values were standardized in z-scores (varying between -1 and 1, against each mean value)



As expected, *Bradyrhizobium* dominated fully developed nodules in dunes, where nitrogen levels are a limitation to plant growth, and rendering symbiotic nitrogen fixation essential for plant performance (e.g., Ehinger et al. 2014; Birnbaum et al. 2016). Along with *Pseudomonas*, these genera constitute important plant-growth promoting bacteria (PGPB) and have been studied for their plant benefits (Dorjey et al. 2017; Martínez-Hidalgo and Hirsch 2017). On the other hand, in the forest habitat, fully developed nodules were dominated by *Acaryochloris*, differing from previous studies (e.g., Rodríguez-Echeverría et al. 2007; 2012; Jesus et al. 2020; 2023; 2025) and suggesting an influence of surrounding soil conditions. Interestingly, *Acaryochloris* is another cyanobacterial genus; its capacity to fit in diverse ecological niches may promote successful soil colonization and survival within nodules (Swingley et al. 2008). Although phylogenetically distinct, Cyanobacteriota

and Pseudomonadota both contain nitrogen-fixing taxa and therefore contribute to the functional group of diazotrophs (Coba de la Peña et al. 2018), which may further facilitate establishment and functional integration, potentially contributing to nitrogen fixation (Latysheva et al. 2012; Baroch 2022). Another abundant genus was “*Candidatus Portiera*” that belongs to phylum Pseudomonadota. It is known that *nod* and *nif* genes are often located in symbiotic islands or even plasmids being easily transmitted horizontally across genera or species (Moulin et al. 2001; Andrews and Andrews 2018; Liu et al. 2023); so, this genus can easily be integrated into nodule community, possibly explaining why it was more abundant in early developed nodules from both habitats. Both *Synechocystis* and “*Candidatus Paraprochloron*” are members of the phylum Cyanobacteriota and were present with higher abundance in early developed nodules from dune and forest, respectively. *Synechocystis* has been shown

to support heterotrophic microorganisms' growth, including genera classified as plant growth-promoting bacteria (PGPB) through close microbial interactions (Abdulaziz et al. 2016; Tanweer et al. 2022). Although these interactions have been shown only in culture-based systems, they can provide a basis for the potential role of Cyanobacteria in plant-microbe processes within root nodules. "*Candidatus Paraprochloron*" is a cyanobacterial symbiont, that has been mainly described in symbiosis with marine invertebrates (Chen et al. 2021). Therefore, its detection and high relative abundance in nodules is intriguing and highlights the need for future studies to determine whether these cyanobacteria may also play a role in plant-associated symbioses.

Fungal communities have a more distinct pattern when comparing early and fully developed nodules. We found *Coniochaeta* as an important yeast previously reported for *A. longifolia* (Jesus et al. 2023) and being abundant in fully developed nodules. *Coniochaeta* has been reported as an Ascomycota that triggers symbiotic associations (Challacombe et al. 2019), suggesting its acquisition from seeds and presence since an early stage of nodule formation. Early developed nodules were associated with a more consistent set of fungi between habitats, including mostly Ascomycota and saprotrophs, while fully developed nodules were characterized by a higher relative association with Basidiomycota and more specialized plant-associated fungi, suggesting increasing community specialization during nodule maturation. Genera *Fusarium* and *Acremonium* are generally associated with roots and have a role in mobilizing and solubilizing organic phosphorus, respectively (Villanueva et al. 2021; Ma et al. 2025), and were dominant in early developed nodules from both habitats. Also, this early development stage was dominated by *Phialophora* and *Clonostachys*, in dune and forest respectively. Both genera belong to Ascomycota and have been described as mutualistic fungi with an important role in plant protection against pathogens (Schulz 2006; Piombo et al. 2023; Qin et al. 2024; Islam 2025). Notably, fungi associated with phosphorus metabolism, and plant protection were more abundant in the early stages of nodule development. This is particularly relevant considering phosphorus a key nutrient for effective nodulation (Israel 1987; Li et al. 2021) and that legumes can be exposed to microbial "hitchhikers" with opportunistic or pathogenic potential (Grundy et al. 2023). In the fully developed stage, fungi that inhabit nodules from both habitats included a higher abundance of the genera *Metschnikowia*, *Solicoccozyma* and *Tubaria*. Both *Metschnikowia* (Ascomycota) and *Solicoccozyma* (Basidiomycota) are yeasts typically found in the rhizosphere and associated with plant-microbe interactions. Along with *Tubaria*, a Basidiomycota, these fungi are known for their role in nutrient cycling and plant immunity (Matheny et al.

2007; Haniffadli et al. 2024; Alborno et al. 2025). Two other important fungi are *Sordaria* (Ascomycota) and *Syncephalastrum* (Mucoromycota) that were more abundant in dunes and have been described as important decomposers of organic matter, allowing a faster nutrient turnover in such a nutrient-poor ecosystem (Hudson 1968; Voigt et al. 2021). Together with *Serendipita*, a relevant root endophytic fungus (Saleem 2025), *Sordaria* is described as important in establishing advantageous partnerships with plants promoting their growth and stress resilience (Voigt et al. 2021; Saleem 2025). Altogether, this "myco-community" illustrates a tendency for specialization following nodule maturation, highly related with habitat characteristics. Communities are more diverse and potentially more multifunctional in dune compared to forest.

Our study shows that *A. longifolia* can have both early and fully developed nodules co-occurring in its roots. So, the nodules microbial diversity present in both development stages contribute to the plasticity associated with this invasive species. We also found differences in ecophysiological traits between dune and forest saplings, suggesting shifts in biomass allocation and resource acquisition and use, that may be linked to both environmental conditions and belowground microbial interactions. In particular, the more depleted $\delta^{15}\text{N}$ values observed in forest saplings may indicate a greater reliance on biological nitrogen fixation or differences in nitrogen sources and cycling. On the other hand, higher leaf nitrogen content in dune saplings could reflect enhanced soil nitrogen availability, higher uptake efficiency or a slower growth rate. Low variability in $\delta^{13}\text{C}$ values across individuals suggests differences in water-use efficiency, potentially reflecting either contrasting water availability or microenvironmental conditions. These isotopic parameters align with previous studies that also showed that *A. longifolia* is an efficient resource-user, allowing greater plasticity as an invasive plant (Hellmann et al. 2011; Rascher et al., 2011; 2012; Ulm et al. 2017a, b; Antunes et al. 2018b). Furthermore, the differences observed in nodule developmental stages (number and sizes) may further contribute to these patterns, as nodules at different stages are known to vary in nitrogen fixation activity and metabolic demand (Sprent 2009; Van Kessel and Hartley 2000). These ecophysiological traits measured in dune and forest saplings suggest a potential link with nodule number and the distinct microbial communities identified. It is plausible that nodules at different developmental stages support distinct and functionally complementary microbial communities, which may enhance plant performance under contrasting environmental conditions.

Despite the exploratory design of this study (only three saplings in each habitat), nodule development stage is important along with a possible habitat-related filter

evidenced when comparing dune and forest conditions, highlighting the relevance of upscaling this assessment. To fully address the questions raised, next approaches should consider (i) plant life cycle, by including not only age (saplings vs. adult trees), but also vegetative and flowering seasons, (ii) the inclusion of non-differentiated roots to infer the microbial vertical transmission, mainly the taxa extensively found from Cyanobacteriota, and (iii) the native perspective to infer if this shift also occurs within native range. Altogether, these findings set the stage to what happens inside *A. longifolia* nodules during development through a successional process that modulates microbial community composition.

5 Conclusions

Root nodules of *Acacia longifolia* host a dynamic microbial composition influenced by nodule development stage. Despite a conserved bacteria set and more variable fungal communities, successional shifts seem to occur as nodules develop, reflecting differential contributions from seed- and soil-derived microorganisms. Early developed nodules are characterized by microbial taxa associated with nutrient mobilization and plant protection, whereas fully developed nodules show increased microbial community specialization. The co-occurrence of nodules at different development stages within the same plant may contribute to the ecological success of *A. longifolia* as an invasive species. Overall, these findings emphasize the importance of considering nodule developmental stage and habitat filtering when investigating legume–microbiome interactions by providing new insights into the role of microbial succession in supporting plant adaptation under the scope of biological invasions.

Authors' contributions JGJ and AA led the data collection and curation. JGJ led the formal analysis, visualization and writing – original draft. CM and HT were responsible for supervision, validation and writing – review & editing. AA was involved in validation and writing – review & editing. HT and CM was involved in funding acquisition. All authors were responsible for conceptualization and gave final approval for publication.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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