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POTENTIAL DIAZOTROPH COMMUNITIES OF *CORDIA DODECANDRA* PHYLLOSPHERE ENDOPHYTES IN AGROFORESTRY SYSTEMS

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Abstract

Background: Endophytic bacteria in the phyllosphere play a crucial role in plant growth and health. The microbiota of *Cordia dodecandra*, a Mesoamerican native species, may include diazotrophic bacteria.

Questions: This study evaluates changes in the phyllosphere microbiota of *C. dodecandra* growing in two agroforestry systems and explores its potential for nitrogen fixation.

Studied Species / Data Description: A metagenomic approach was used to analyze next-generation sequencing data from foliar DNA of *C. dodecandra*.

Study Site and Dates: Samples were collected in July 2023 from six trees located in two agroforestry systems at Rancho Juanes, Yucatán.

Methods: Tree dasometric variables and foliar nutrient contents were recorded. Diversity of endophytic bacteria was analyzed using 16S rRNA V3–V4 sequences. Potential diazotrophs were identified based on *nifH* gene sequences. Correlation analyses were performed between *nifH* abundance (estimated via qPCR), tree traits, and foliar nutrients to assess the influence of potential diazotrophic activity on tree growth.

Results: Dasometric measurements and foliar ammonium content varied notably among the six trees, while foliar nitrogen and carbon were relatively similar. A total of 420 ASVs were identified as endophytes, with nine dominant genera consistently found across all samples. *nifH* sequences were assigned to six putative diazotrophic species. A marginal positive correlation was found between *nifH* abundance and canopy cover.

Conclusions: The core endophytic microbiota of the *C. dodecandra* phyllosphere may contribute to tree growth and show diazotrophic potential. Further research is needed to understand the ecological functions of bacterial endophytes in tropical trees.

Keywords: agroforestry systems, ciricote, core microbiota, diazotroph, endophytes, *nifH* gene

Resumen

Antecedentes: Las bacterias endófitas de la filósfera son importantes en el crecimiento y salud de las plantas. La microbiota de *Cordia dodecandra*, especie mesoamericana, puede incluir diazotrofos.

Preguntas: Se evalúan cambios en la microbiota de la filósfera de *C. dodecandra* en dos sistemas agroforestales y su potencial diazotrófo**.**

Especies estudiadas / Descripción de los datos: Se realizó un análisis metagenómico con secuenciación masiva del DNA foliar de *C. dodecandra***.**

Sitio de estudio y fechas: Las muestras se recolectaron en julio de 2023 de seis árboles en Rancho Juanes, Yucatán.

Métodos: Se registraron variables dasométricas de los árboles y contenido de nutrientes foliares. La diversidad de bacterias endófitas se analizó utilizando secuencias 16S rRNA V3-V4. Los posibles diazótrofos se identificaron con secuencias del gen *nifH*. Se realizaron correlaciones entre la abundancia de *nifH* (estimada mediante qPCR), características de los árboles y nutrientes foliares para evaluar la influencia de la actividad diazotrófica potencial en el crecimiento de los árboles.

Resultados: Las mediciones dasométricas y contenido foliar de amonio variaron entre los seis árboles, mientras que el nitrógeno y carbono foliar fueron relativamente similares. Se identificaron 420 ASVs y nueve géneros dominantes en todas las muestras. Las secuencias *nifH* se asignaron a seis especies diazotróficas potenciales. La correlación entre la abundancia de *nifH* y cobertura del dosel fue marginalmente positiva.

Conclusiones: La microbiota endofítica central de la filosfera de *C. dodecandra* puede contribuir al crecimiento y tiene un potencial diazotrófico. Se necesita investigar sobre las funciones de las bacterias endófitas en árboles tropicales.

Palabras clave: sistemas agroforestales, ciricote, microbiota central, diazótrofo, endófitos, gen *nifH.*

The phyllosphere refers to the habitat encompassing the external surface and internal tissues of plant leaves, and it is the most extensive terrestrial habitat, covering nearly twice the Earth's surface (Vorholt 2012). Bacteria are the most abundant microorganisms in the phyllosphere, occupying approximately 10^6 to 10^7 cells per cm^2 of leaf tissue (Vorholt 2012). The bacteria living in the phyllosphere are epiphytic when they live on the surface and endophytic when they inhabit the internal tissues of the leaves (Vorholt 2012, Dastogeer *et al*. 2020). Both epiphytic and endophytic bacteria play an essential role in plant growth, development, and reproduction (Yadav *et al*. 2021). The endophytic microbiota of the phyllosphere consists of different taxa which are considered core microbiota when consistently present across multiple samples of the species, and other taxa that are variable and transient (Morella *et al*. 2020, Risely 2020). The contribution of core endophytic microbiota to nutrient acquisition and plant growth is assumed, as they can act as symbionts of the species. On the other hand, variable and transient (non-core) microbiota may be associated with differences in the host's habitat. For instance, in *Suaeda salsa* (L.) Pall., the microbiota diversity in young leaves increases with phosphorus content in soil, while diversity decreases in mature leaves as soil pH increases (Zhou *et al*. 2023).

Within the microbiota, we can find nitrogen-fixing bacteria known as diazotrophs or diazotrophic bacteria, which have the metabolic capacity to reduce dinitrogen (N_2) to ammonia (NH_3) through the nitrogenase enzyme (Fe-Pérez *et al.* 2015). There are three nitrogenase isoforms, the most common being Mo-nitrogenase, which is encoded by a complex of genes including *nifH,* which is responsible for mediating ATP-dependent electron transfer during catalysis (Li *et al*. 2014, Kuypers *et al*. 2018). The *nifH* gene is widely distributed in diazotrophic bacteria (Sun *et al*. 2021). Therefore, it is the most used biomarker to study phylogeny, diversity, and potential diazotrophic activity (Gaby & Buckley 2014).

Symbiotic associations between diazotrophic bacteria and plants have traditionally been found in legume root nodules (Carvalho *et al*. 2014, Hernández-Rodríguez *et al*. 2017), where they fix nitrogen and transfer it to the host (Turner *et al*. 2013, Shin *et al*. 2016, Rosado *et al*. 2018). Diazotroph symbiotic associations are not limited to root nodules; in the last two decades, diazotrophic bacteria have been found in the phyllosphere playing an important role (Fürnkranz *et al*. 2008, Abadi *et al*. 2021). Estimates of nitrogen fixation rates in the Amazon suggest that nitrogen fixation in the phyllosphere is ten times higher than in the soil, with rates of 0.33 and 0.03 kg N ha^{-1} yr^{-1}, respectively (Moreira *et al*. 2021). Nitrogen-fixing bacterial genera such as *Bacillus*, *Pseudomonas*, *Pantoea*, *Microbacterium*, *Sphingomonas*, *Methylobacterium*, *Arthrobacter*, and *Azospirillum* are common in the phyllosphere (Shabanamol *et al*. 2018, Bao *et al*. 2019, Liang *et al*. 2019, Abadi *et al*. 2021). Studies on diazotrophic communities have been conducted on annual crops such as *Saccharum officinarum* L. (Singh *et al*. 2022) and *Zea mays* L. (Zhang *et al*. 2022); in fruit trees such as *Pyrus pyrifolia* (Burm.f.) Nakai, *Prunus armeniaca* L., *Prunus avium* (L.) L., and *Vitis vinifera* L. (Liang *et al*. 2019), and shrubs such as *Jatropha curcas* L. (Madhaiyan *et al*. 2015). Diazotrophs have also been reported in temperate timber species as epiphytic microbiota of the phyllosphere in *Quercus ilex* L. (Rico *et al*. 2014), *Pinus bungeana* Zucc. ex Endl., and *Sabina chinensis* L. (Bao *et al*. 2020). Other reports of diazotrophs of the phyllosphere of timber temperate species, found them as endophytic microbiota in *Populus trichocarpa* Torr. & A.Gray ex Hook. (Doty *et al*. 2016), *Pinus contorta* Douglas ex Loudon (Padda *et al*. 2019), and *Thuja plicata* Donn ex D.Don (Bal *et al*. 2012). To our knowledge, no studies have examined the potential diazotroph taxa inhabiting the phyllosphere of tropical American timber species.

Cordia dodecandra DC. is a tree recognized in the Yucatan Peninsula (Mexico) for its use both as timber and a fruit tree (Pennington & Sarukhán 2005). The species in managed in smallholder properties as monoculture plantations and silvopastoral systems (Campos *et al*. 2015, López-Ramírez *et al*. 2023). The endophytic microbiota in the phyllosphere of *C. dodecandra* includes *Methylobacterium*, *Actinomycetospora*, *Aureimonas*, *Allorizobium* and *Sphingomonas* as dominant genera in forest and homegarden populations across two different regions in Yucatan (May-Mutul *et al*. 2022). Hence, they may be considered part of its core microbiota. Some members of *Methylobacterium*, *Allorizobium* and *Sphingomonas* are considered to have the metabolic capacity to fix nitrogen (Madhaiyan *et al*. 2015, Stone *et al*. 2018, Camargo-Neves & Araújo 2019, Zhu *et al*. 2022). In this study, we characterized the bacterial endophytic microbiota and assessed its potential diazotrophic communities in 21-year-old *C. dodecandra* growing in smallholder agroforestry systems. Exploratory comparisons of the diversity of endophytic microbiota in monoculture plantations and silvopastoral systems were also conducted, as carbon, nitrogen, and phosphorus levels are approximately two, three, and one hundred times higher, respectively, in the soil of silvopastoral systems (López-Ramírez *et al*. 2023).

Materials and methods

Study species and sites. Cordia dodecandra, locally named as ciricote, is a deciduous tree native to southern Mexico and Central America (Miranda & Hernández-X 1963), widely used for timber (Pennington & Sarukhán 2005). Due to deforestation and the decline of wild populations, the National Forestry Commission of Mexico has identified the species as a priority for conservation and management (López-Upton *et al*. 2011). The species is an important component of Mayan homegardens (Hurtado-Torres *et al*. 2020) and agroforestry systems at the region (Campos *et al*. 2015).

This study was conducted at Rancho Juanes (Figure 1), where *C. dodecandra* plantations were established in 2002. The trees, sown simultaneously at three months of age and originating from the same nursery, were therefore 21 years old at the time of the study. The monoculture plantations are characterized by the dominance of *C. dodecandra* in the tree strata and some cacti growing under the trees, while in the silvopastoral system *Cynodon plectostachyus* (K.Schum.) Pilg. grass was sown for sheep fodder. The *C. dodecandra* trees present foliage from June to November in the monoculture plantations, while in the silvopastoral system the foliage presence is extended through all year excepting October (Campos *et al*. 2016). Pruning of *C. dodecandra* trees is performed twice a year in both monoculture plantation and silvopastoral system, while at the latter sprinkler irrigation, weed control and incorporation of sheep excreta are additional management practices (Campos *et al*. 2015).

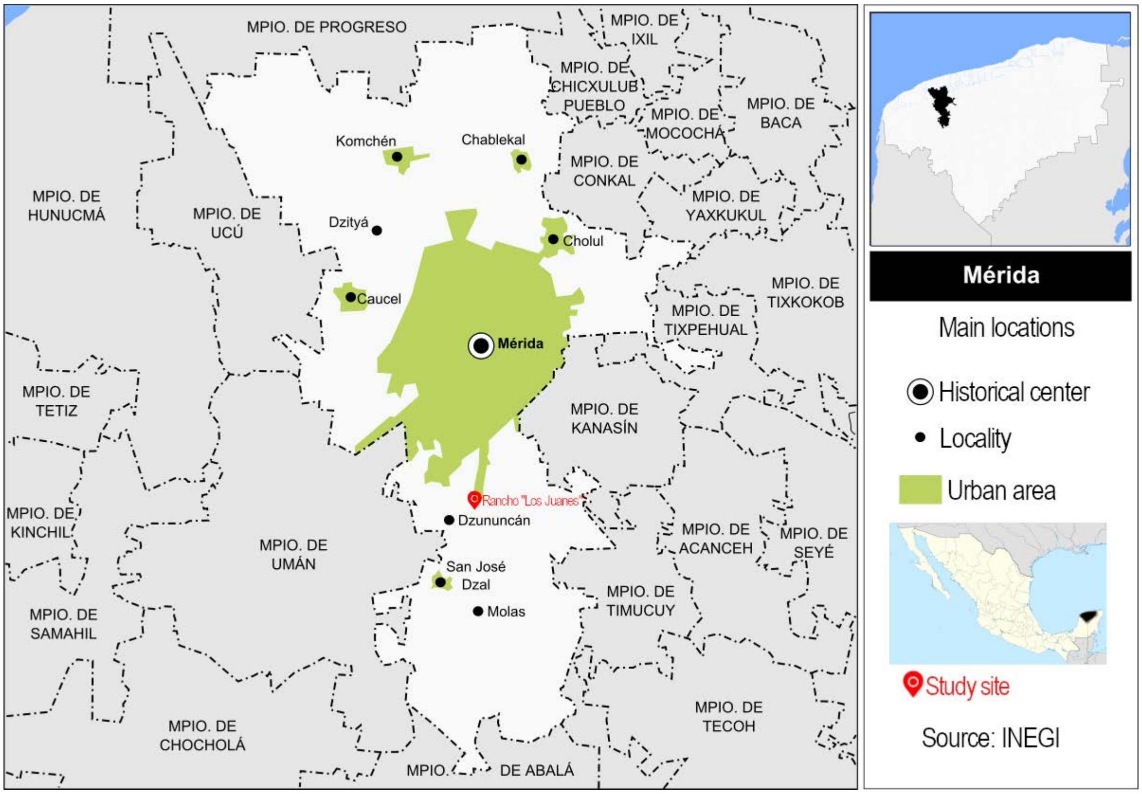

Figure 1. Location of the study site in Rancho Juanes at Xmatkuil locality in the Yucatan state of Mexico.

According to NOM-021 (SEMARNAT 2000), the soils of the silvopastoral system and the monoculture plantation are quite similar, with high levels of nitrogen (> 0.25 %), calcium (< 10 cmol kg^{-1}), and organic matter (> 6.0 %). Nonetheless, the soil in the silvopastoral system shows higher concentrations of organic carbon, organic matter, carbonates, nitrogen, inorganic phosphorus, and calcium compared to that of the monoculture plantation. These differences are attributed to the deposition of sheep feces and the biomass left behind after grazing (López-Ramírez *et al*. 2023).

Sampling design. In July 2023, before the rainy season began, two composite samples were obtained for each tree: one from 10 young leaves and another from 10 mature leaves, both collected from the middle canopy, adding up to 12 initial samples. Leaves were sterilized with 70 % ethanol and transported on ice to the laboratory, where they were stored at -70 °C. To optimize the representation of healthy microbiota, the inclusion criteria for *C. dodecandra* trees were: 1) presence of young and mature leaves, 2) no evidence of herbivory on the leaves, and 3) no apparent fungal, viral or bacterial damage on the leaves. Only two individuals in the monoculture plantation and four in the silvopastoral system met these criteria.

Dasometric and foliar nutrient characterization of trees in the agroforestry systems. The variables height, DBH (diameter at breast height), and canopy cover, as described by Campos *et al.* (2015), were obtained as indirect measurements of tree growth (Table 1). Two weeks after initial collection nutrient analyses were performed. To estimate total nitrogen and carbon content in the foliar tissue of each tree, the Dumas method was conducted in 250 mg ground tissue with a 70-mesh-size, previously dried to constant weight at 70 °C (Simonne *et al*. 1994). The estimates were obtained by duplicates in the LECO CN 828 analyzer (LECO, St. Joseph, Michigan). The Nessler reactive method (Madero *et al*. 1998) was used to determine the foliar ammonium content with 1 g of tissue by triplicates. Due to equipment problems, nutrient content was not properly estimated on the 12 initial samples and a new composite sample of 20 leaves (young and mature) per tree were collected to analyze carbon, nitrogen (total and ammonium) as previously described.

Table 1. Dasometric measurements and foliar nutrient content of *Cordia dodecandra* trees growing in Rancho Juanes Yucatan at monoculture plantation and silvopastoral system.

Variable	Rancho Juanes	Monoculture plantation	Silvopastoral
Cover canopy (m)	22.92 ± 9.93	20.90 ± 2.8	23.92 ± 10.78
Height (m)	5.91 ± 1.31	4.92 ± 0.2	6.40 ± 1.17
DBH (cm)	43.33 ± 22.15	24.15 ± 9.05	15.02 ± 1.36
Foliar nitrogen (%)	1.96 ± 0.21	1.72 ± 0.15	2.09 ± 0.06
Foliar carbon (%)	40.75 ± 1.27	41.05 ± 1.72	40.60 ± 0.81
Foliar NH_4^+ (mg/Kg)	664.70 ± 187.47	730.26 ± 182.43	631.92 ± 172.98

Endophytic bacterial communities in agroforestry systems. After five days of collection, leaves were sterilized with 96 % ethanol and 2 % commercial sodium hypochlorite and then rinsed five times with sterile distilled water (Patel & Archana 2017). Small sections (1 cm^2) were cut from each leaf from the 12 initial samples and macerated with liquid nitrogen. DNA was isolated from 125 mg of the foliar tissue with ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, California). The concentration and purity of DNA samples were evaluated using a Multiskan™ GO microplate spectrophotometer µDrop plate with SkanIt v. 7 software (Thermo Scientific™, Whaltham, Massachusetts). We verified the DNA integrity by TBE buffer-electrophoresis in 1.2 % agarose gels that were stained with ethidium bromide and visualized with UV light. The 12 initial samples and a negative control (DNAse-RNAse free water) were then amplified for the 16S rRNA gene using primers 16S-Fw (5'-GTAGACGCTATTCCCTAAGAT-3') and 16S-Rv (5'-CTCCCAACTATACAGTACACACCTCATA- 3') (Stevanovic *et al*. 2011). A 25 µL reaction with 100 ng DNA, 12.5 µL GoTaq Green Master Mix 2X (Promega, Madison Wisconsin), and 10 µL of DNAse-RNAse free water was amplified in the C1000 Touch thermal cycler PCR system (Bio-Rad, Hercules, California). The PCR program consisted of an initial denaturation at 94 °C for 5 min followed by 34 cycles (94 °C for 30 s short denaturation, 58 °C primer annealing for 30 s, and 72 °C extension for 90 s) with a final extension at 72 °C for 5 min. Amplification was evaluated by electrophoresis as previously described. The library construction and DNA sequencing were performed in Novogene (Novogene, Shangai, China) on the Illumina NovaSeq 6000 platform using 515F (5-GTGYCAGCMGCCGCGGTAA- 3') and 907R (5'-CCGTCAATTCMTTRAGTTT- 3') bacterial universal primers for the V4 toV5 region of 16S rRNA (Pichler *et al*. 2018, Sebastião *et al*. 2015, Zhou *et al*. 2024).

The quality of raw sequences from the 12 initial samples was checked using FastQC (Andrews 2010). The pipeline reported by May-Mutul *et al*. (2022), was used in QIIME2 v 2023.2 (Bolyen *et al*. 2019), for demux, denoise, and assign the taxonomic group to ASVs. The latter was performed using the 138-99 weighted database of Silva (Kaehler *et al*. 2019). For further analyses, ASVs corresponding to host chloroplasts and mitochondria were filtered. An evaluation of the abundance of sequences for the 12 initial samples showed a large variation in sample size (65 to 5,490 sequences per sample). To increase the number of sequences per sample and reduce sample size variation, the ASVs from young and mature leaves were combined for each tree, resulting in six final merged samples. The final diversity analyses were performed in R v4.3.2 (R Core Team 2023), using phyloseq package v1.50.0 (McMurdie & Holmes 2013) as outlined in the pipeline (Supplementary material S1). Due to the small number of trees grouped in the monoculture plantation (2 trees) and in the silvopastoral system (4 trees) the comparative analyses must be considered exploratory. The number of observed ASVs, Chao1, and Shannon indexes were estimated to characterize the alpha diversity. Means of the Shannon index from monoculture plantation and silvopastoral system were compared using Wilcoxon rank test on rarefied samples to even depths. To correct for differences on the observed sequences variation per sample (496 to 6,814), normalization of the six final merged samples was attained using cumulative sum scaling transformation (CSS). Principal Coordinate Analysis, ANOSIM, and PermANOVA (Zhang *et al*. 2022), were performed on the Bray-Curtis distances using both raw and normalized CSS data to test for differences on the composition and structure of endophytic communities. Relative abundance by order and genus was plotted for majoritarian taxa, defined as those representing more than 1 % of the total observed sequences. A differential abundance analysis ANCOMBC (Lin & Peddada. 2020) was performed on the relative abundance for the ASVs grouped by genus using both raw and normalized CSS data. In the result section only plots and heatmap from normalized CSS data were included.

Potential diazotroph-endophytic communities. The presence of potential diazotrophic bacteria was evaluated by endpoint PCR using the primers PolF (5'-TGCGAYCCSAARGCBGACTC 3') and PolR (5'-ATSGCCAT-CATYT-CRCCGGA) to amplify the region 115 (134) to 457 (476) of the *nifH* gene (Gaby & Buckley 2017). We used the DNA from root nodules of *Leucaena leucocephala* growing in Lerma, Campeche, as a positive control for amplification, as the presence of diazotrophs in its rhizosphere has been reported (Gibson *et al*. 1982) and the DNA from the 12 initial samples. The PCR reaction was similar to that described previously for 16S rRNA, although primers were at 10 pmol, and the program consisted of an initial denaturation at 95 °C for 3 min followed by 30 cycles (95 °C for 1 min short denaturation, 55 °C primer annealing for 1 min, and 72 °C extension for 1 min) with a final extension step at 72 °C for 7 min (Rusmana & Lestari 2015). Amplification was evaluated by electrophoresis as described before. The DNA of the 12 initial samples was sent to Novogene (Shangai, China) for library construction and sequencing of the *nifH* gene, using Pol-Fw and Pol-Rv primers, on the Illumina NovaSeq 6,000 platform. Successful amplification, library construction and sequencing in Novogene were only obtained in 3 out of the 12 initial samples. The quality of raw sequences from these 3 samples was checked using FastQC (Andrews 2010), and the pipeline in the supplementary material (Supplementary material 2) was used in QIIME2 v. 2023.2 (Bolyen *et al*. 2019), for demux and denoise with dada2. The taxonomic assignment with *nifH* genes was obtained using the Marine Microbial Ecology database of University of California, Santa Cruz (Heller *et al*. 2014). Unassigned ASVs were filtered and excluded, and only those identified at the species level are reported here as potential diazotrophs, whit its relative abundance per sample (Figure S1).

To evaluate the relative abundance of the *nifH* gene, the initial 12 samples and the positive control of DNA from root nodules of *L. leucocephala* were amplified by qPCR. The 20 µL reactions consisted of 200 nM of each primer (PolFw and PolRv), 5 µL SSoAdvanced Universal SYBR® Green Supermix (Bio-Rad, Hercules, California), 50 ng of DNA, and DNAse- & RNAse-free water. The qPCR program consisted of an initial denaturation at 98 °C for 30 s followed by 40 cycles of short denaturation at 95 °C for 15 s an annealing at 62 °C for 30 s and extension at 72 °C for 40 s (Zhang *et al*. 2022) in the CFX96™ Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California). The specificity of amplification at different temperatures was evaluated by melting curves and the relative abundance of the *nifH* gene was estimated by the fold change of amplification relative to the positive control. The threshold amplification for *nifH* gene was at cycle 14 for positive control and between cycles 23 and 25 for the 12 initial *C. dodecandra* samples. The curves started their stationary phase at cycle 32 (Table S1).

To test for an association between the relative abundance or the *nifH* gene and host tree growth, a series of Pearson correlation analysis (Holm-corrected *P*) were performed using dasometric measurements -DBH, height, and canopy cover, and the nutrient contents: carbon, nitrogen and ammonium (Table 1) against fold change values. The fold change values per tree was obtained by adding the values obtained for the young leaves and the mature leaves of the 12 initial samples for each of the six trees (Table S1).

Results

Dasometric and foliar nutrient characterization of trees in the agroforestry systems. The 21-year-old trees assessed in this study had on average a height of 5.91 m ± 1.31 m, DBH of 18.07 cm ± 7.51 cm, and a canopy cover of 22.92 m^2 ± 9.93 m^2. A large variation in the dasometric variables was observed among the six trees, with coefficients of variation of 22 % for height, and 42 and 43 % for DBH and canopy cover, respectively (Table 1). Variation among trees exceeded 50 % in DBH for trees from monoculture plantations and in canopy cover for trees from the silvopastoral system. Nutrient content in the foliar tissue was less variable among trees with an average of 1.96 ± 0.21 % and 40.75 ± 1.27 % for nitrogen and carbon, respectively, although variation in the ammonium was larger with an average of 664.70 ± 187.47 g/kg resulting in a coefficient of variation of 28.20 % for all trees, and 27.37 and 28.59 % in monoculture plantation and silvopastoral system, respectively (Table 1).

Endophytic bacterial communities in agroforestry systems. We obtained 2,474,697 paired reads of 250 pb in length that were down to 15,632 sequences after filtering host's chloroplasts and mitochondria. The number of sequences ranged from 496 to 6,814 per final merged samples (six trees). The rarefaction of the sequences to an even depth of 496 per sample, were plotted in curves that began to flatten at depths of 250 for Shannon index, but did not for the observed ASVs, therefore we did not perform comparisons for observed ASVs and Chao index (Figure 2A). The endophytic microbiota of *C. dodecandra* phyllosphere included 420 ASVs. The comparison of Shannon diversity index values between monoculture plantation and silvopastoral system was no significant ($P = 1$) and ranged from 2.84 to 3.72 (Figure 2B). However taxonomic assignment was higher than 90 % at the family level and less than 65 % for genus and species taxa. The majoritarian orders (with a relative abundance higher than 1 % and present in all samples) were i) Rhizobiales and Sphingomonadales of the class Alphaproteobacteria; and ii) Kineosporiales, Frankiales, Micrococcales, and Pseudonocardiales of the class Actinobacteria (Figure 3A). The majoritarian endophytic bacteria included 18 classified at the genus level, six grouped at the family level, and two grouped at class level (Figure 3B). Only *Methylobacterium-Methylorubrum*, *Aureimonas*, *Sphingomonas*, *Microbacterium*, *Quadrisphaera*, *Pseudokyneococcus*, *Curtobacterium*, and *Actinomycetospora* were present in all samples (Figure 3B). The endophytic communities of the phyllosphere from silvopastoral system were grouped closer according to the principal coordinate analysis (Figure 4). However, no significant differences were observed in the Bray-Curtis distances between endophytic communities of monoculture plantation and silvopastoral according to ANOSIM-permANOVA ($P_{adj} = 0.4$). No differences in the abundance at the genus level were observed either between agroforestry systems, and the heatmap plotting the results from ANCOMBC suggests that each tree has different genera of endophytic bacteria enriched in their phyllosphere (Figure 5).

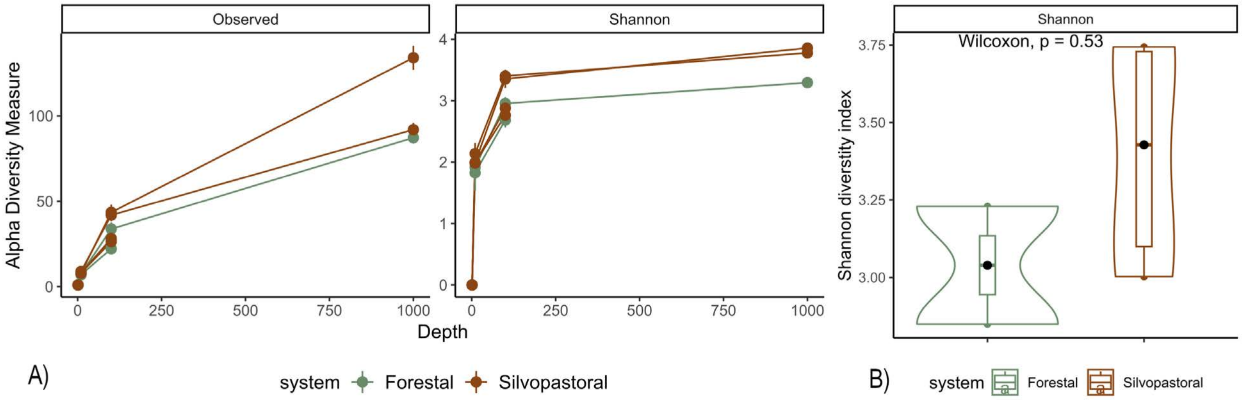

Figure 2. Rarefaction curves of alpha diversity indices for the endophytic microbiota in the phyllosphere of *Cordia dodecandra*, based on observed ASVs and the Shannon diversity index. A Wilcoxon test was used to compare Shannon diversity between trees growing in monoculture plantations (green) and silvopastoral systems (brown) in Yucatán

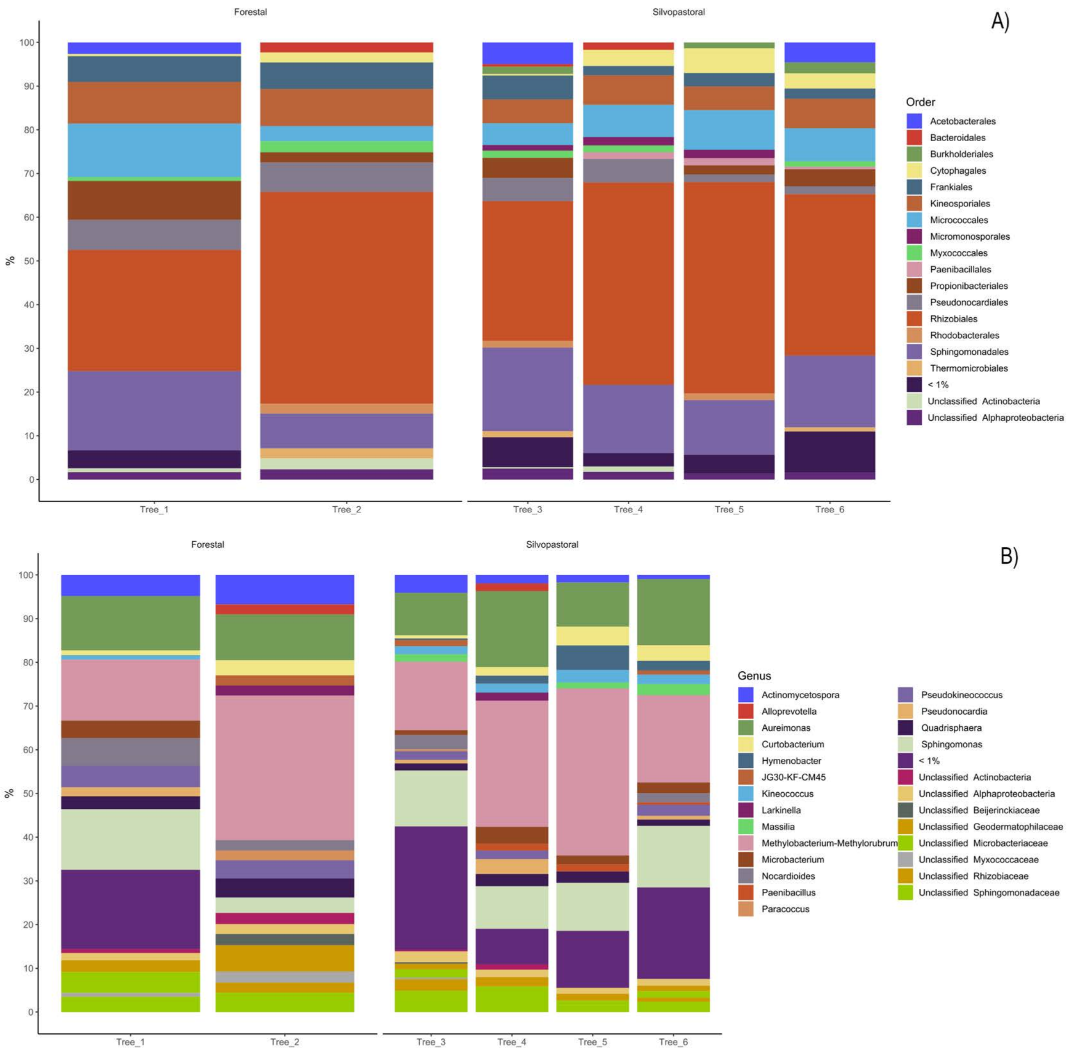

Figure 3. Relative abundance of the majoritarian (relative abundance < 1 %) taxa classified at the A) order and B) genus level of the 420 ASVs of endophytic microbiota in the phyllosphere of *Cordia dodecandra* trees growing in monoculture plantation and silvopastoral systems in Yucatan.

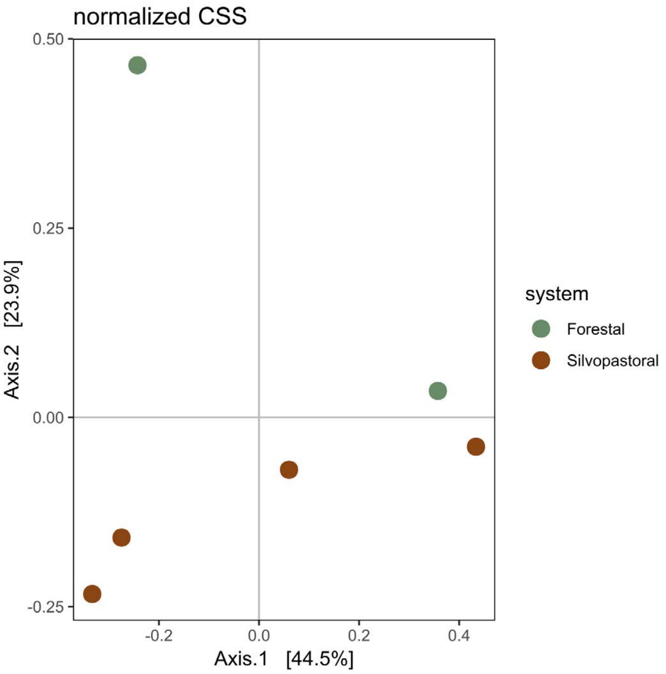

Figure 4. Principal coordinate analysis (PCoA) of the endophytic microbiota in the phyllosphere of *Cordia dodecandra* trees growing in monoculture plantation (green) and silvopastoral systems (brown) in Yucatan.

Potential diazotroph-endophytic communities. A total of 495,747 paired 360 bp reads of the *nifH* gene were obtained for the three samples that were successfully amplified and sequenced. After exclusion of the unclassified ASVs, 26 remained, and sample size of the three samples ranged from 1,898 to 10,307 sequences. The ASVs were assigned to six potential diazotrophs species (Table 2). The most abundant order among potential diazotrophs was Nostocales with abundances ranging from 60 to 90 %. At the species level, *Hassalia byssoidea* and *Nostoc commune* were the most abundant potential diazotrophs with relative abundances of 60 and 10 %, respectively (Figure S1).

All 12 initial samples from young and mature leaves for each one of the trees were successfully amplified using qPCR. Values of the fold change were similar between monoculture plantation and silvopastoral system ($t = 0.15$, $P = 0.88$). After adding *nifH* gene fold change values for each tree, the correlations with values obtained to characterize each tree was determined, and only canopy cover had a marginally significant and positive correlation ($r = 0.76$, $P = 0.06$) with the *nifH* relative abundance. No significant correlations were observed with DBH and height ($r = 0.26$, $P = 1$ and $r = 0.12$, $P = 1$, respectively) or with carbon, total nitrogen and ammonium ($r = -0.64$, $P = 0.17$, $r = 0.10$, $P = 0.89$ and $r = 0.31$, $P = 0.52$, respectively) (Figure 6).

Discussion

Dasometric and foliar nutrient characterization of trees in the agroforestry systems. In this study, *C. dodecandra* displayed high variability in dasometric measurements and foliar ammonium content, but relatively low variability in foliar carbon and nitrogen content. Although the trees examined originated from the same nursery and were established simultaneously, the recorded variability may have genetic and environmental biases. Campos *et al.* (2015) report that the trees from Rancho Juanes agroforestry systems came from four different nurseries. They also found that differences in management, mainly irrigation, weeding and pruning are associated with variations in the DBH, height and canopy cover. They observed significant differences in these variables between the monoculture plantations and the silvopastoral system; however, our study failed to find this effect, largely because of the small sample size of trees included. The foliar nitrogen and carbon measured in our study are intermediate compared to those reported for

Table 2. Classification for the diazotroph-endophytic microbiota in the phyllosphere of *Cordia dodecandra* in Yucatan using *nifH* database from The Marine Microbial Ecology database and ID and authors of the Genbank accession.

Species	Taxonomic clasification	Genbank ID	Authors
Alcaligenes faecalis	D_Bacteria. p_Pseudomonadota. c_Betaproteobacteria. o_ Burkholderiales. f_Alcaligeniaceae	CCF77362.1	Shahid 2013
Ensifer americanum	D_Bacteria. p_ Proteobacteria. c_Alphaproteobacteria. o_ Rhizobiales. f_Rhizobiaceae	AIX97566.1	Cordero *et al.* 2016
Methanosaeta concilii	D_Archaea p_ Stenosarchaea. c_ Methanomicrobia. o_ Methanotrichales. f_ Methanotrichaceae	AEB67348.1	Barber *et al.* 2011
Chroococcidiopsis thermalis	D_Archaea p_Cyanobacteriota. c_ Cyanophyceae. o_ Chroococcidiopsidales. f_ Chroococcidiopsidaceae	AAQ99146.1	Boison *et al.* 2004
Hassallia byssoidea	D_Archaea p_Cyanobacteriota. c_ Cyanophyceae. o_ Nostocales.f_ Tolypothrichaceae	KIF38087.1	Singh *et al.* 2015
Nostoc commune	D_Archaea p_Cyanobacteriota. c_ Cyanophyceae. o_ Nostocales. f_ Nostocaceae	ABF81842.1	Yeager *et al.* 2007

trees in homegardens and forest populations (Estrada-Medina *et al*. 2023), suggesting that the species has phenotypic plasticity to reallocate these nutrients to its foliage from the soil or other sources. The ammonium (NH_4+) found on the *C. dodecandra* leaves was variable with three out of the six trees having almost two times more than the others (Table 1). This variability may reflect differences in the potential absorption of atmospheric N_2, nitrogen absorption and fixation or protein metabolism (Shin *et al*. 2016). Further studies to determine the nitrogen source of ammonium within the foliage are needed, particularly as potential diazotroph endophytes were found in this study as we will discuss in the next sections.

Endophytic bacterial communities in agroforestry systems. The endophytic communities of the phyllosphere of *C. dodecandra* are relatively rich with up to 420 ASVs and Shannon diversity indexes around 3. The endophytic communities did not differ between the agroforestry systems. The small sample size (six trees) and the large variation in the number of 16 S rRNA reads obtained per sample diminished the power of the test implemented in our study. The composition of endophytic communities varied among trees, with different genera enriched in each tree sample (Figure 5). Variation in the endophytic communities of the phyllosphere may be associated with the position of leaves within the canopy and the height relative to the base of the tree (Laforest-Lapointe *et al*. 2016, Herrmann *et al.* 2021). Additionally, specific endophyte species may help individual trees cope with distinct microenvironmental stresses. Soil physicochemical characteristics, such as phosphorus availability, redox potential, and sand content, also varied considerably in both the monoculture plantation and the silvopastoral system (López-Ramírez *et al*. 2023), with potential impacts on tree growth and development. Determining how canopy and soil conditions associated with each tree affect the diversity of endophytic microbiota in the phyllosphere of *C. dodecandra* requires further experimental investigation.

Figure 5. Heatmap showing the relative abundance of endophyte bacteria of the *C. dodecandra* phyllosphere at the genus level based on differential abundance analysis. No significant differences among the relative abundance of the genera were found between monoculture plantations and silvopastoral systems, according to ANCOM-BC results (see text for details).

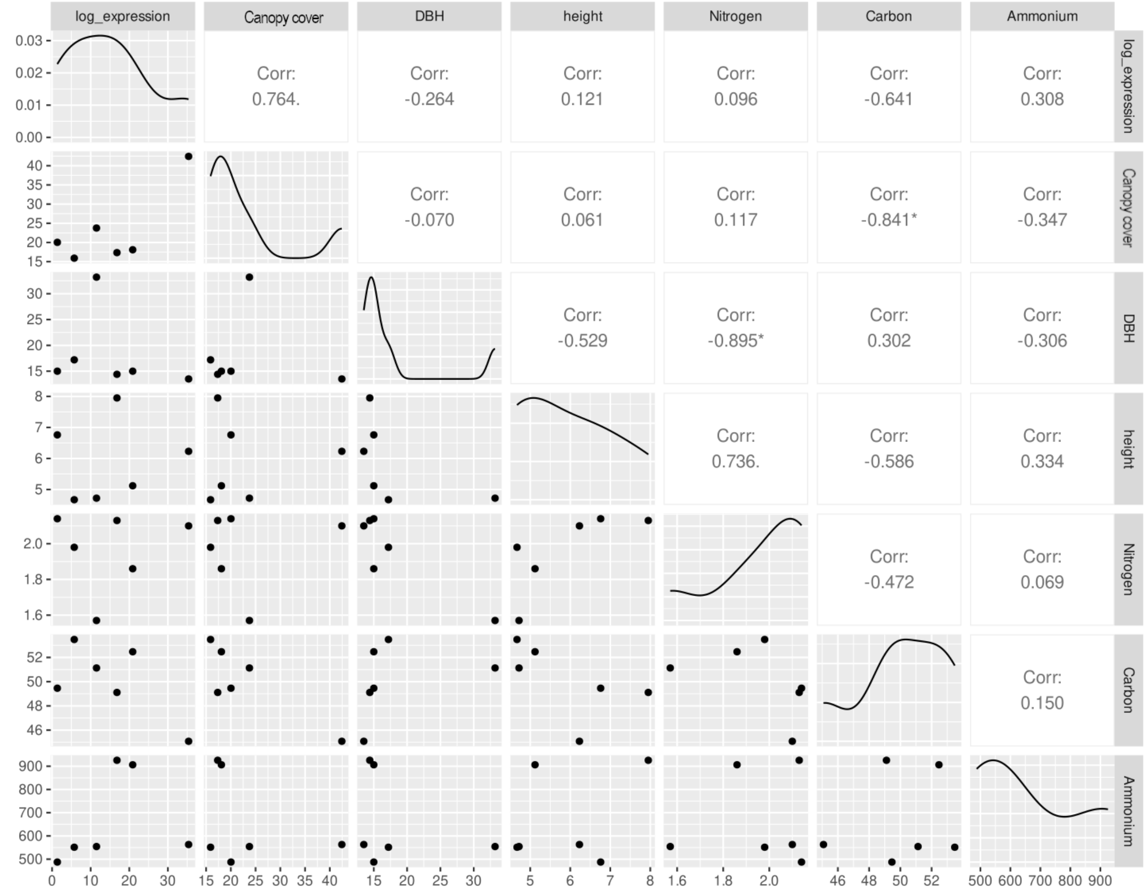

Figure 6. Correlation analysis between the relative expression of *nifH* genes in the diazotrophic endophytic microbiota and the dasometric and foliar nutrient contents estimates of *Cordia dodecandra*.

Some of the most abundant bacterial orders, such as Rhizobiales, Sphingomonadales, and Pseudonocardiales, have previously been described in the rhizosphere of *C. dodecandra* (López-Ramírez *et al*. 2023), as well as among phyllosphere endophytes in different populations (May-Mutul *et al*. 2022). Among the eight genera present in all samples with relatively large abundance (< 1 %), six were also recorded as the majoritarian genera in homegarden and forest populations from two different regions in Yucatan (May-Mutul *et al*. 2022). We propose that *Methylobacterium-Methylorubrum*, *Aureimonas*, *Sphingomonas*, *Quadrisphaera, Curtobacterium*, and *Actinomycetospora* may constitute the core endophytic microbiome of *C. dodecandra* phyllosphere. In addition to those genera, *Microbacterium*, *Quadrisphaera*, and *Pseudokineococcus* may play important roles in the agroforestry systems of Rancho Juanes. Several of these genera have been identified as endophytes in the phyllosphere of other plant species: *Quadrisphaera* in rice (*Oryza sativa* L.) (Muangham *et al.* 2019) and peanut (*Arachis hypogaea* L.) (Li *et al*. 2023); *Aureimonas* in *Jatropha curcas* (Madhaiyan *et al*. 2013) and *Colobanthus quitensis* (Kunth) Bartl. (Perazzolli *et al.* 2022), and *Pseudokineococcus* in *Morus* spp. (Chen *et al*. 2022). *Methylobacterium* and *Sphingomonas* have been reported in the phyllosphere of *Saccharum* spp. (Hamonts *et al*. 2018), *Glycine max* (L.) Merr., *Trifolium repens* L., and *Arabidopsis thaliana* (L.) Heynh. (Thapa & Prasanna 2018), while *Curtobacterium* has been found in *Zea mays* and *Glycine max* (Dunleavy 1989). The widespread presence of these genera across various angiosperms suggests that a core group of endophytic microbiota may be adapted to the phyllosphere, potentially establishing long-term symbiotic relationships with their hosts. We propose that *Actinomycetospora*, *Methylobacterium*, *Sphingomonas*, and *Curtobacterium* may contribute to plant growth in both monoculture plantations and silvopastoral systems. For example, *Actinomycetospora* has been shown to increase the dry weight of *Lemna aequinoctialis* Welw. by 1.3 to 1.4 times (Saimee *et al*. 2024). Additionally, *Methylobacterium*, *Sphingomonas*, and *Curtobacterium* have been reported to promote plant growth and suppress pathogens (Chase *et al*. 2016, Garrido *et al*. 2016). Therefore, it is plausible that these dominant genera play a key role in the growth and health of *C. dodecandra*, and their functions merit further investigation.

Potential diazotroph-endophytic communities. We identified six species that have the diazotroph potential inhabiting as endophytes of *C. dodecandra* phyllosphere in only three out of twelve samples of the agroforestry systems. The species did not belong to the majoritarian genera of the endophytic microbiota, and were not successfully amplified and sequenced from all trees. This suggests that the potential diazotrophic endophtyes may be rare species; however, the qPCR successful amplification allows us to establish that all individual trees have endophytic microbiota with a potential diazotroph function. The six potential diazotrophs identified in *C. dodecandra* phyllosphere by their sequence of the *nifH* gene, have been reported as microbiota of the phyllosphere, except for *Alcaligenes faecalis*. *Alcaligenes faecalis*, originally isolated from *Oriza sativa* with strain A15 was considered a diazotroph (You & Zhou 1989). *Alcaligenes faecalis* has been found as a diazotroph in rhizosphere soils on *Sesbania aculeata* Poir. and *Atriplex lentiformis* (Torr.) S.Watson (Babar *et al*. 2021) with the ability to consume nitrite and nitrate (Chen *et al*. 2021) and on the leaves of *Ixora chinensis* Lam., *Ervatamia divaricata* (L.) Burkill, *Hibiscus rosa-sinensis* L., and *Amaranthus cruentus* L. (Undugoda *et al*. 2016). The second potential diazotroph identified was *Ensifer americanum*, which has been found as a diazotroph in roots of *Phaseolus filiformis* Benth. (Rocha *et al*. 2020), *Anthyllis henoniana* Coss. Ex Batt. (Yan *et al*. 2016), *Glycine* sp., *Psoralea corylifolia* L., *Sesbania cannabina* (Retz.) Pers., *Medicago lupulina* L. (Wang *et al*. 2013), and *Acacia* spp. (Lloret *et al*. 2007, Sakrouhi *et al*. 2016). The third potential diazotroph identified was *Methanosaeta concilii* (syn. *Methanothrix concilii*), a free-living bacterium (Auman *et al*. 2001) that is frequently found in soils (Bae *et al*. 2018). The fourth potential diazotroph identified was *Chroococcidiopsis thermalis* a facultative diazotroph (Boison *et al*. 2004, Lan *et al*. 2021) widely distributed in various xeric freshwater and marine habitats, forming cyanolichens for nitrogen fixation (Villanueva *et al*. 2018). The fifth potential diazotroph was *Hassallia byssoidea*, a diazotrophic heterocyst with antibiotic properties (Rossi *et al*. 2012), sometimes associated with streptophytic algae (Singh *et al*. 2015, Mukhin *et al*. 2018) such as *Chaetosphaeridium globosum* (Knack *et al*. 2015). The sixth potential diazotroph was *Nostoc commune*, a heterocystous cyanobacteria (Peng & Bruns 2019) that has been found in soil biocrusts (Lan *et al*. 2021) and in basidiocarps (Mukhin *et al*. 2018); also considered drought resistant (Hata *et al*. 2022). In addition to N_2 fixation, *Nostoc commune* can establish surface consortia that immobilize and retain inorganic nitrogen when it is in excess (Mukhin *et al*. 2018).

The presence of the potential diazotroph microbiota in *C. dodecandra* phyllosphere may play other functional roles. It has been proposed that diazotroph communities may also influence leaf growth by promoting the synthesis of phytohormones (Dobbelaere *et al*. 2003) such as auxins, cytokinins, gibberellins, and N-acyl homoserine lactones, as experimentally observed in *O. sativa* (Defez *et al*. 2017). Tree and herb growth is enhanced when endophytic diazotrophs are present in *Salix sitchensis* Sanson ex Bong. and *Populus trichocarpa* (Knoth *et al*. 2013); in *Saccharum officinarum* (Antunes *et al*. 2022); in *Trifolium repens* and *Lolium multiflorum* Lam. (Hahn *et al*. 2014). In *C. dodecandra* a marginally significant positive correlation was found between canopy cover and *nifH* gene abundance, suggesting that endophytic diazotrophs in the phyllosphere may promote canopy growth in *C. dodecandra*, although it was not correlated with nitrogen and ammonium content in the foliage tissue. Previous studies suggest that there is a linear relationship between N_2 fixation rates and relative *nifH* gene abundance (Reed *et al*. 2010); future studies on nitrogen fixation or *nifH* gene expression in *C. dodecandra* are needed to know whether nitrogenase is active in the phyllosphere.

The trees of *C. dodecandra* growing in agroforestry systems harbor a potential diazotrophic community that was variable among individual trees. The relatively large abundance and prevalence among samples of seven genera: *Methylobacterium*-*Methylorubrum*, *Aureimonas*, *Actinomycetospora*, *Sphingomonas*, *Microbacterium*, *Quadrisphaera*, and *Pseudokineococcus* place them as the potential core endophyte community of the phyllosphere of *C.*

dodecandra. Six potential diazotroph species form part of the endophytic community of the phyllosphere of *C. dodecandra*, *Alcaligenes faecalis*, *Ensifer americanum*, *Methanosaeta concilii*, *Chroococcidiopsis thermalis*, *Hassallia byssoidea*, and *Nostoc commune*. Future experimental work is needed to deepen our understanding of the specific functions of these dominant taxa and to investigate nitrogen fixation by diazotrophic endophytic microbiota in the phyllosphere of this and other tropical timber species.

Supplementary material

Supplemental material for this article can be accessed here: https://doi.org/10.17129/botsci.3684

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