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Plant Growth and Microbiota Structural Effects of Rhizobacteria Inoculation on Mahogany (*Swietenia macrophylla* King [Meliaceae]) under Nursery Conditions

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Abstract: *Swietenia macrophylla* is a tropical timber species of ecological and economic importance. However, its slow vegetative growth and root development in nurseries strongly limit its production. This study evaluated the effect of 10 rhizobacteria strains during the early stages of production of *S. macrophylla*. Superficially disinfected seeds were inoculated with rhizobacteria under commercial nursery conditions. Inoculation was complemented by initial fertilization without growth regulators, fungicides, or bactericides. The results indicate that the rhizobacteria strains induce different responses in plants. Significant differences in plant biomass and root architecture were found. Treatments inoculated with *Bacillus* sp., *Bacillus polyfermenticus*, and *Bacillus siamensis* strains; showed an increase of up to 41% (dry weight). Plants increased root biomass by 30% when inoculated with *S. siamensis*. All inoculated strains were identified as members of the genus *Bacillus* spp., and their presence three months after inoculation was assessed by 16S rRNA gene-based amplicon massive sequencing. We found that *Bacillus* sp. genus was only present in inoculated treatments, suggesting that inoculated bacteria could establish themselves successfully as part of the microbiota. These results support the advantages of using PGPRs in commercial tropical tree production.

Keywords: plant-microorganism interaction; root development; tropical forest trees

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1. Introduction

Swietenia macrophylla King. (Meliaceae), commonly known as Mahogany is a neotropical forest species participating in critical ecological roles as a habitat for animal species, soil conservation, and carbon capture dynamics [1]. This species also provides economic benefits for its logging, reaching a value of up to five times more than coniferous woods [2]. For these reasons, it has become a priority species grown in nurseries for restoration and commercial production purposes. However, it has been reported that early-stage *S. macrophylla* plants show slow growth and poor root system development [3,4], affecting the plant's height increase and overall quality, compromising survival during later stages in nurseries and plantations.

Several studies have identified edaphic bacteria, commonly named plant growth-promoting rhizobacteria, are capable of colonizing and significantly inducing the development of roots in plants. Described mechanisms include growth regulators production, such as auxins, cytokinins and gibberellins [5–7]; modulating ethylene levels [8]; increasing the availability of nutrients through the biological fixation of atmospheric nitrogen [9];

and mineral solubilization [10]. These properties have been primarily demonstrated in experimental and agricultural species rather than species of forestry interest.

Recent data show that *Bacillus subtillis* inoculation, in addition to fertilization in nurseries, promotes a significant increase in the biomass of *Fraxinus americana* L. [11]. This response was similar to the results of applying *Hydrogenophaga pseudoflava* to *Picea glauca* Moench. *x engelmannii*, which promoted increases of 25% in the dry matter weight of the roots and the number of shoots, as well as a 30% increase in the number of branches generated during the first year after the transplant to the field with respect to untreated plants [12]. In tropical forest species, rhizobacteria inoculation has been evaluated under uncontrolled nursery or field conditions in *Schizolobium parahyba* [13], *Shorea selanica* [14], *Tectona grandis*, *Eucalyptus* spp. [15], and *Cedrela fissilis* [16], another Meliaceae family member closely related to *S. macrophylla.* These works have demonstrated a positive impact on seedling performance [14,16,17], phytosanitary improvement [15], and wood yield [13], contributing to a growing acceptance by foresters as a component of their technological package for nursery and field establishment [18,19]. The mid-to-long-term establishment and benefits of inoculated rhizobacteria under commercial conditions are often assumed with certainty. However, bacterial prevalence depends on factors like nutrients, soil properties, plant exudates, and competition [20]. Recent work focused on evaluating rhizobacterial survival under commercial conditions demonstrated by qPCR the rapid decay within 22 to 34 days of inoculated strains, becoming undetectable by day 41 [21]. The evaluation of the microbiota architecture on inoculated plants under commercial conditions has been recently explored on annual crops [22], however forest models, where long-term plant-microbe interactions are essential, remain unexplored.

In the present work, ten non-commercial rhizobacteria strains were selected to evaluate the plant-growth-promoting effect on *S. macrophylla*, particularly exploring their impact on plant root structure under commercial nursery phase. At the end of the trial, 16S massive sequencing was used to evaluate the capabilities of inoculated rhizobacteria to get established as a long-term microbiota member in the rhizosphere and their possible effect on the establishment of rhizospheric bacterial microbiota architecture.

2. Materials and Methods

2.1. Bacterial Strains Identification

Ten rhizospheric bacterial strains (identified as 25, 29, 35, 38, 42, 46, 47, 49, 50 and 52) isolated from *Persea americana* Mill. in the "Laboratorio Planta Ambiente" from "Facultad de Agrobiología Presidente Juarez" described previously [23,24], were selected for evaluation. Molecular identification of strains was performed as follows. Genomic DNA was extracted using the modified enzymatic technique reported by [25]. The 16S rRNA gene region was amplified using the universal primers 27F (5-AGRGTTTGATYMTGGCTCAG-3') and 338R (5'-TGCWGCCWCCCGTAGGWGT-3') [26]. Polymerase chain reaction (PCR) amplification was performed in a final volume of 20 µL containing 1 µL of dimethyl sulfoxide, 10 µM each primer, 250 ng of DNA and 10 µL (2×) of GoTaq®Green Master Mix (Promega, Madison, WI, USA). Thermal cycling was performed as follows: an initial denaturation at 94 °C for 5 min followed by 30 denaturation cycles at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1.5 min, with a final extension at 74 °C for 5 min. Amplicons were sequenced using the Sanger method by Macrogen (Rockville, MD, USA). Sequences were analyzed with the search program Basic Local Alignment Search Tool (BLAST) to determine their identity, and sequences were deposited in the GenBank database (Supplementary Table S1).

2.2. Rhizobacteria Functional Validation Bioassay

The methodology proposed previously [23] was followed to produce the bacterial cultures as an inoculum source in the Mahogany bioassay. Briefly, the bacterial strains were grown in a potato-sucrose medium at 28 °C, with constant agitation at 160 rpm to reach a OD_{600} nm = 0.6, equivalent to 10^8 CFU mL^{-1}, and used immediately. Inoculation

time varied to synchronize all cultures to reach desired OD. *Arabidopsis thaliana* L. ecotype Columbia (Col-0) seeds were superficially disinfected. Subsequently, six seeds were sown in line per plate, using 10% Murashigue and Skoog (MS) medium (Caisson Labs, UT, USA). Each bacterial strain was inoculated by streaking at a distance of 5 cm from the seeds in a parallel opposing line. The trial was established under a completely randomized experimental design, with three replicates per treatment, including a control treatment. Finally, the plates were transferred to a growth chamber (ECOSHEL Mod. C800D, Mc Allen, TX, USA); placed vertically at an angle of 70 degrees, and randomly ordered. Primary root length and number of secondary roots were measured 10 and 20 days after sowing (DAS) by plate photograph and software analysis using Rhizo2 [27]. Plants were grown under 16:8 h light:dark photoperiod, light intensity of 200 µmol m^{-2} s^{-1}; 25 ºC, and 50% relative humidity.

2.3. Functional Evaluation of Rhizobacteria on S. macrophylla under Nursery Conditions

After collection from mature, healthy trees, *S. macrophylla* seeds were superficially disinfected with sequential washes of 10% (v) of commercial sodium hypochlorite solution and sterile water following procedures reported by [28]. Surface sterilization was corroborated by culturing a sample of seeds in Petri dishes containing semisolid potato-dextrose agar (PDA) medium and incubating for 48 h at 37 °C. Subsequently, seeds were submerged in corresponding liquid bacterial inoculum supplemented with carboxymethylcellulose (CMC) (DEIMAN, Mexico) in a 6 mg·mL^{-1} ratio and with constant agitation at 190 rpm for 30 min. Seeds were sown in a substrate based on expanded perlite, exfoliated vermiculite (Agrolita, Mexico), and peat moss (Premier, Quebec, Canada) at a ratio of 3:3:4 (v/v), with 3.7 g·L^{-1} Multicote™ fertilizer (Haifa Iberia S.L., Madrid, Spain). The mixture was arranged in trays with 150-mL cavities and supplemented with the corresponding bacterial inoculum at a 1:10 (v/v) ratio at the sowing time. The nursery experiment was performed in a commercial nursery with 50% shade. Fifteen days later, a second inoculation was conducted in which 10 mL of the bacterial suspension was placed [1:7 ratio (v/v)] directly on the substrate that covered the seeds, as reported previously [29]. Plant irrigation started 24 h later with crude well water (approximately 50 m^3/Ha every 48 h). For nursery trials, a completely randomized design was used, with 15 plants per replicate and 3 replicates per treatment. The duration of the experiment was three months. The whole experiment was repeated a year later (furtherly referred to as the first and second experiments). Measurements of stem height and diameter were performed each month. The plant biomass in dry weight and the number of dead plants were determined at the end of each experiment.

2.4. Bacterial Structure

Bacterial diversity and relative abundance were determined at the end of the second experiment as follows: A composite sample of the roots of ten plants from each treatment was collected and milled using liquid nitrogen and mortar. Subsequently, 0.25 g of each sample was used to extract total DNA using a PowerSoil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. For 16S rRNA gene sequencing of the samples, 300 to 400 ng of total DNA was sent to an external sequencing service (RTLGenomics) using an Illumina MiSeq 2000 platform and 27F/338R set of primers. Fastq files were processed using QIIME 2 2020.6 [30] following the Moving Pictures tutorial as a pipeline. The sequences were grouped into operational taxonomic units (OTUs), and classifications were assigned using the GreenGenes 13-8 dataset release as a reference [31].

2.5. Statistical Analyses

The software Infostat 2017 (National University of Cordoba, Argentina) was used to analyze the data by means of the probabilistic Q-Q plot, as well as the homogeneity of variances by means of the Levene test, later the analysis of variance was performed (ANOVA) and Tukey's means test used to evaluate differences between treatments inoculated with PGPR bacteria.

3. Results

3.1. Taxonomic Assignation and Validation of In Vitro Growth-Promoting Effect of Rhizobacteria

Molecular identification of strains resulted in their assignation to *Bacillus* genus of bacteria (Firmicutes). Species-level was reached only (identity > 99%) for *Bacillus subtillis* (strain-25); *B. polifermenticus* (strain-38); *B. pumilis* (strain-49); and *B. siamensis* (strain-52), whereas the other six strains were identified only to genus level (*Bacillus* spp.). The plant growth-promoting functional properties of strains were firstly validated under controlled conditions in vitro on a model plant as *S. macrophylla* seedlings resulted very difficult to evaluate under these conditions. The root structure of *A. thaliana* seedlings growing in Petri dishes inoculated with each strain showed significant differences ($p \leq 0.001$ and $F \geq 7.61$). Plantlets in inoculated plates developed shorter primary roots, reducing their length to 35% compared to plants growing in control plates. Secondary root production was also affected by rhizobacteria presence, increasing up to 367% the number of these structures in relation to plants developing in non-inoculated plates 20 days after sowing (Figures 1a and 2).

3.2. Effect of Rhizobacteria Strains Inoculation on S. macrophylla

The plant-promoting effect of rhizobacteria strains was evaluated in two separate experiments. *S. macrophylla* seeds embedded with rhizobacteria were evaluated under nursery conditions. After three months of evaluation, plants showed no altered phenotype (Figure 1b). Inoculated plants reach the same germination rate compared to control non-inoculated individuals (~75%). Other parameters such as stem height and root-collar diameter showed subtle, but not statistically significant differences with respect to control plants during the experiments (Figure 1e). In contrast, dry weight evaluated three months after sowing, consistently showed significant increases ($p \leq 0.05$ and $F \geq 2.18$) in leaves and roots dry weight when compared to non-inoculated plants (Figures 1f and 3a). Notably, those plants inoculated with *B. siamensis* showed an increase of 75% in leaves and 41% in roots dry weight. Stem dry weight presented subtler differences (Figure 3a).

3.3. Bacterial Communities in the Rhizosphere of Seedling S. macrophylla in the Nursery

Bacterial microbiota from the rhizospheric environment from healthy plants was analyzed by the end of the second experiment. Microbiota analysis yields an average of 24,856 bacterial sequences per sample, with lengths of $\geq$ 280 nucleotides, yielding a total of 298,237 quality sequences taxonomically grouped into 14 phyla, 33 classes, 85 orders, 149 families, 238 genera, and 285 species. In all treatments and control samples, the most abundant phyla were Proteobacteria (37.6 to 64.3%), followed by Cyanobacteria (6.1 to 32.3%) Actinobacteria (9.8 to 17.6%), Bacteroidetes (0.5 to 2.2%), Gemmatimonadetes (0.2% to 1.3%) and Firmicutes (0.03 to 1.6%) (Figure 4a). No significant shifts were found at this taxonomic level; however, at the Class level, the Firmicutes represented by members of *Pullulanibacillus* spp. (27%), *Alicyclobacillus* spp. (0.8 to 88.2%) and *Bacillus* spp. (27.2 to 95.8%) genera were identified in all cases, except in the case of *Bacillus* spp. members that were detected in all treatments except for the control treatment (Figure 4b).

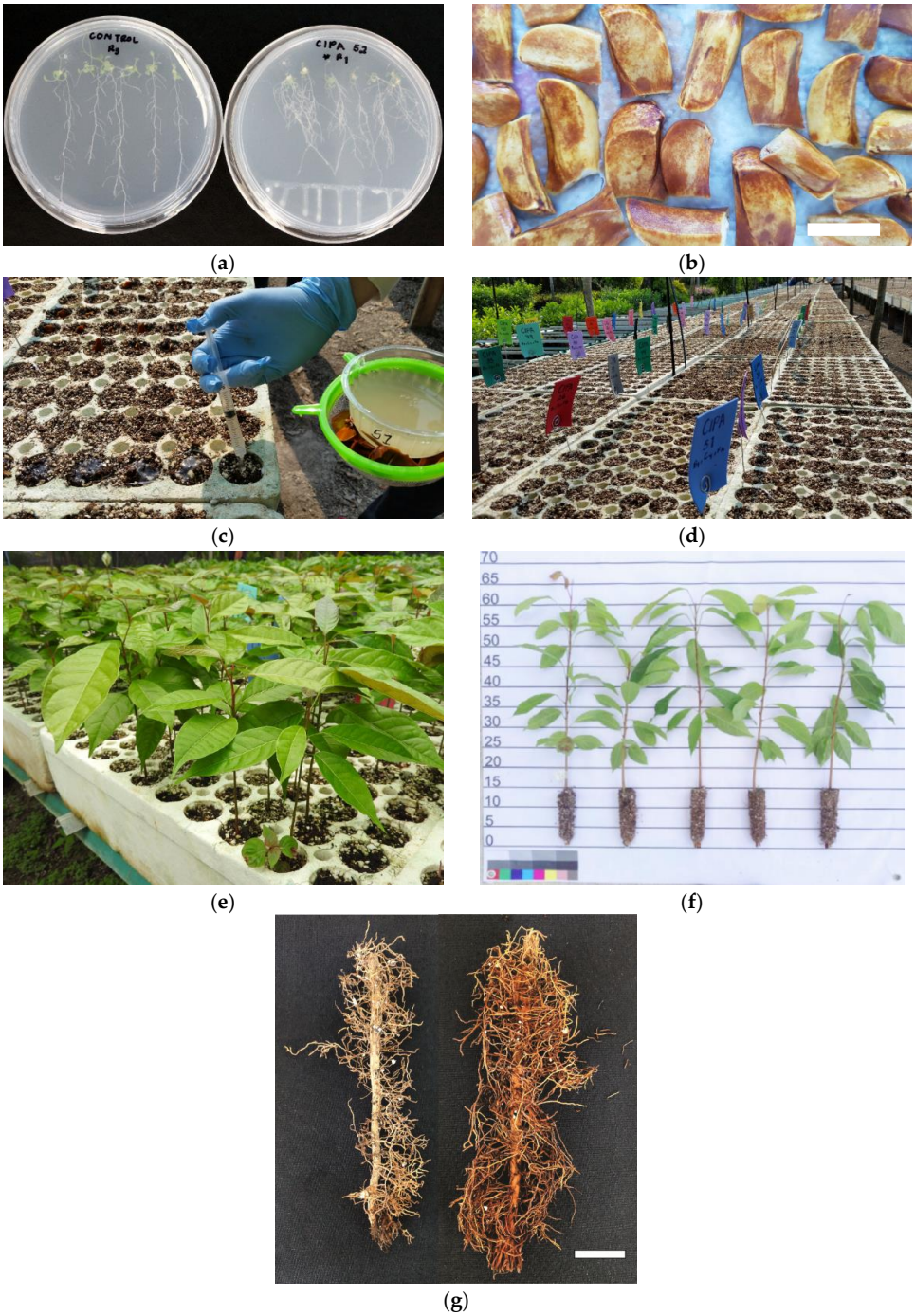

Figure 1. General aspects of key procedures and results. Photographic evidence of (**a**) *A, thaliana* in vitro assay showing the different phenotypes found in seedlings without rhizobacteria inoculates (**left**), where straight roots with scarce secondary roots are formed in comparison to seedlings growing in the presence of one of the strains used in this work (**right**) *B. siamensis* (strain-52). (**b**) Surface-disinfected *S. macrophylla* seeds (see Section 2). The white bar in the bottom-left of the picture corresponds to 1 cm. (**c**) In-nursery re-inoculation of rhizobacteria strains. Notice that at this point, seeds are already covered by the substrate. (**d**) Nursery once established the bioassay within a commercial production process. (**e**) *S. macrophylla* plants two months after germination growing in the nursery. (**f**) An aspect of plants three months after germination. Scale in the left is expressed in cm. (**g**) Representative dried roots of *S. macrophylla* plants corresponding to non-inoculated plants (**left**) and plants inoculated with *B. siamensis* (strain-52) (**right**). The white bar in the bottom-left of the picture corresponds to one cm.

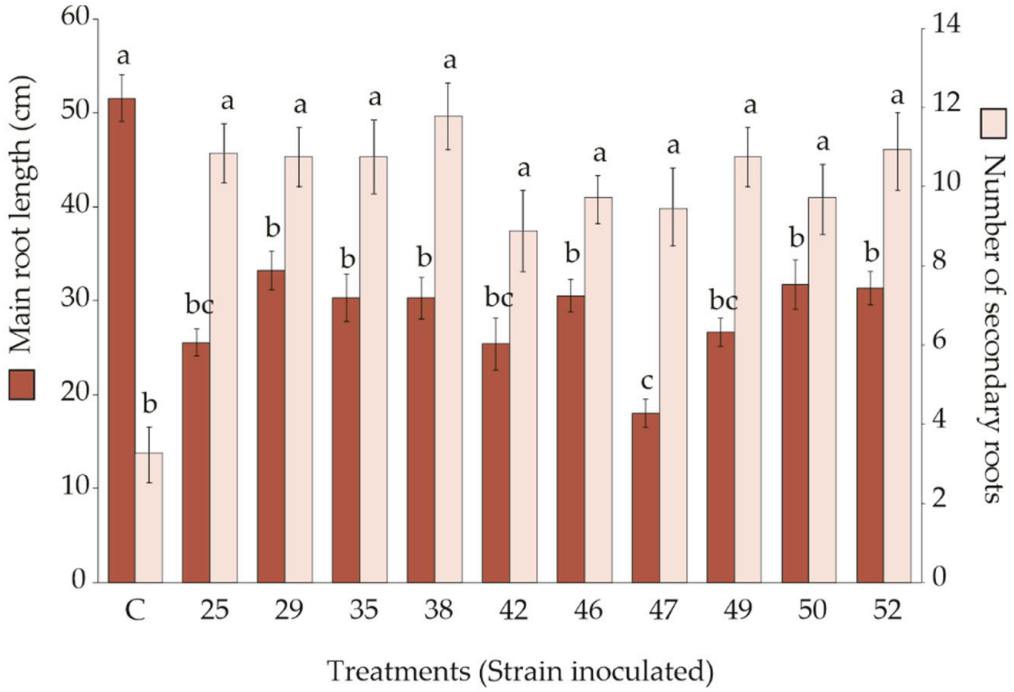

Figure 2. Effect of rhizobacteria strains on the root system of *A. thaliana*. Main root length (dark bars) and the number of secondary roots (light bars) were evaluated in vitro 20 days after germination in the presence of different bacteria strains. The number in each treatment corresponds to the number of each strain. Error bars represent the standard error. Letters over error bars indicate significant differences between the means (n = 30) of the treatments using Tukey's post hoc test ($p \leq 0.05$). Letter C refers to identical treatment of seeds and plants except for absence of bacterial inoculation.

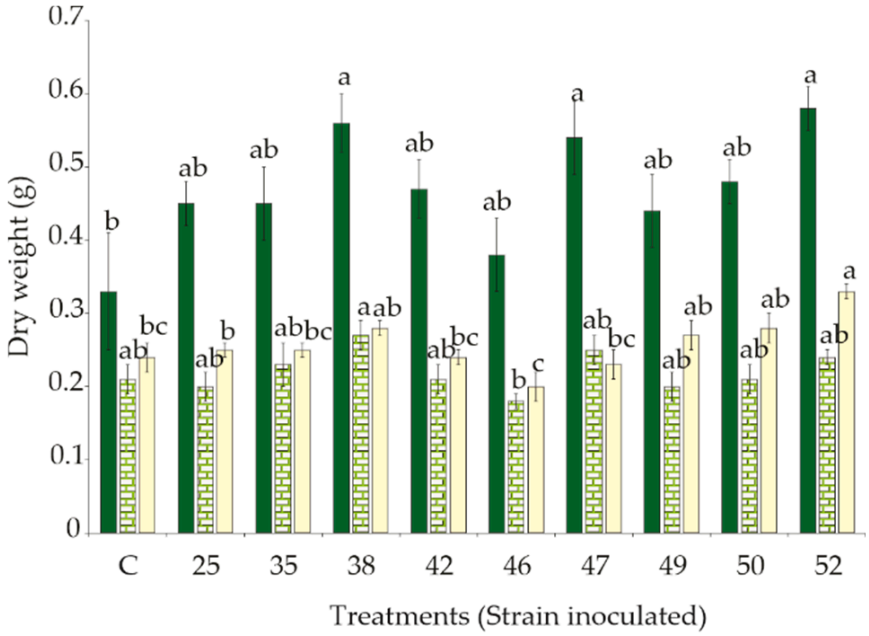

Figure 3. Effect of rhizobacteria strains on biomass and survival over nursery *S. macrophylla*. Total dry weight of leaves (dark bars), stems (patterned-filled bars), and roots (light bars) was determined three months after germination and inoculation with different rhizobacteria strains. C letter refers to control treatment (non-inoculated). Letters over error bars indicate significant differences between the means (n = 30) of the treatments using Tukey's post hoc test ($p \leq 0.05$).

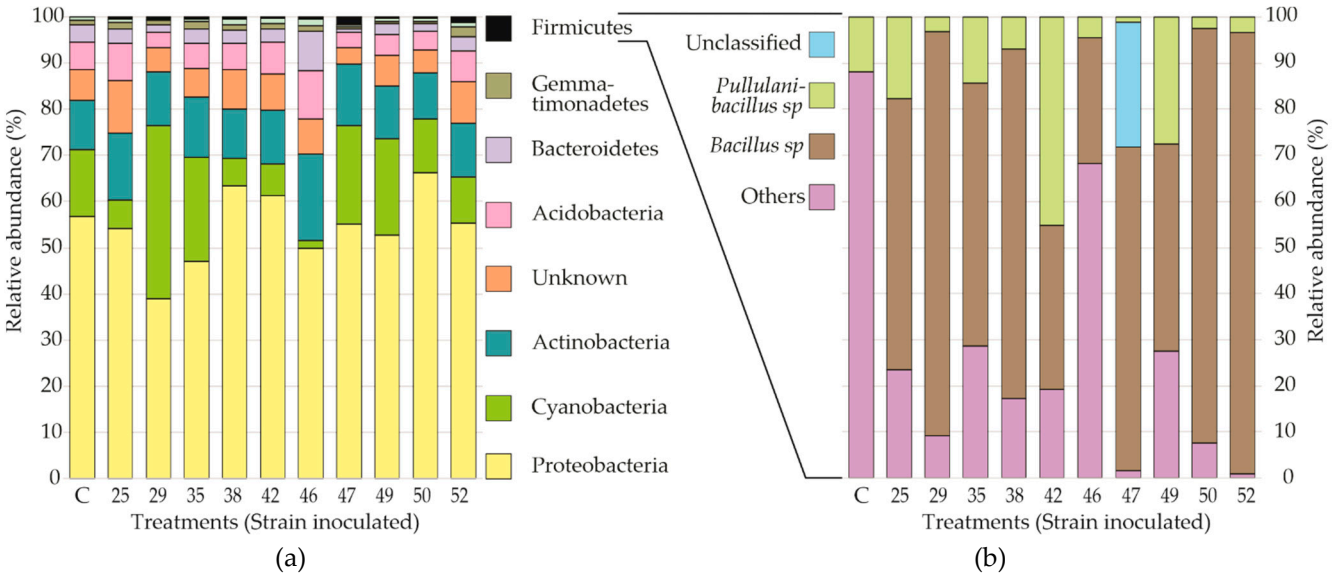

Figure 4. Composition of the bacterial community of the rhizosphere of *S. macrophylla*. 16S amplicon assessment of the microbiota present in the rhizosphere of plants three months after germination and inoculation with different rhizobacteria strains. Relative abundance (> 1%) and diversity of the primary bacterial strains in the rhizosphere at (**a**) Phylum or (**b**) Genus level graph showing only phylotypes belonging to the Firmicutes Phylum. The number in each treatment corresponds to the number of each strain. C letter refers to control treatment (non-inoculated).

4. Discussion

The use of rhizobacteria to improve the performance of forest trees during the commercial nursery phase is widely accepted [18]. This work evaluated the effect on *S. macrophylla* of ten rhizobacteria strains under nursery conditions. The results of this study demonstrate the root structure altering effect of evaluated rhizobacteria on *S macrophylla* plants. The phenotypic impact reported here suggests that these strains have a physiological effect on the root performance of seedlings under nursery conditions. Results derived from in vitro validation using *A. thaliana*, also demonstrated clear effects of rhizobacteria inoculation on root architecture. Similar morphological changes in response to rhizobacteria inoculation have been reported in a wide number of tropical tree species as *Cedrela fissilis* [16], *Acacia auriculiformis* [19], *Cecropia pachystachya, Heliocarpus popayanensis Trema micrantha, Cabralea canjerana, Cariniana estrellensis, Trichilia elegans* [32], *Eucalyptus nitens* [33], *Jatropha curcas* [34] and others. Reported changes in root architecture are associated with the modulation of cell division and the differentiation of the apical meristem and lateral root primordia sites [35]. This phenomenon is primarily due to auxin-dependent mechanisms [36]. Shifts in the concentration of plant growth regulators can be promoted by the activity of several rhizobacteria species through the production of molecules such as indole-3-acetic acid [29,37], N-acyl-homoserine lactones [38], cyclodipeptides [39], and volatile compounds [6,40]. In this regard, although many studies have performed in vitro analyses of rhizobacteria using model plants, few studies have evaluated the effect of these strains on forest species under commercial conditions.

The inoculation of rhizobacteria in forest species has demonstrated several benefits, like increases in height, stem diameter, and total biomass in *Pinus pinea*, and *Quercus ilex* seedlings where the inoculation of *Pseudomonas latus* and *Chryseobacterium balustinum* increased the biomass of shoot dry weight by 25% and 35.5% respectively four months after inoculation [41]. In *Eucalyptus nitens*, treatments inoculated with strains belonging to the genus *Bacillus* spp. promoted increases close to 140% in the aerial biomass and 130% in the root biomass [29], whereas in *Fraxinus americana*, *B. subtilis* added with fertilizer increased root biomass by 19.6 %, 22.9 % in shoot biomass, and 19.4% in leaf biomass [11].

The results here reported relative to the increasing effect of root and leaves dry weight on *S. macrophylla* plants inoculated with rhizobacteria strains are consistent with previous reports, clearly showing that these microorganisms changed root system structure. Even root confinement to tray cavities may impact overall root architecture; the observed effect of inoculated rhizobacteria on root dry weight could indicate an increase of secondary root number (Figures 1g and 3a). The effect of evaluated rhizobacteria at different levels suggests the presence of various molecular mechanisms. The mode of action of the most promising strains, such as *B. polifermenticus* (strain-38) or *B. siamensis* (strain-52), should be explored in the future. The positive effect on *S. macrophylla* plants by the *Bacillus* spp. strains evaluated in this work was observed even when the strains were initially isolated from *Persea Americana* (Lauraceae); a phylogenetically distant species; this observation supports the idea that plant-growth promotion by rhizobacteria is a peculiarity of each strain, that can be expressed in taxonomically-different plant hosts. This kind of non-specific plant-microbe association suggests the existence of adaptative plasticity, previously demonstrated by the functional capabilities of commercial strains isolated from annual crops on tropical tree species [42].

The ability of inoculated bacteria to establish in the rhizosphere and promote physiological responses in the long-term depends on their fitness within the native microbiota structure [43]; for this reason, the elucidation composition of the bacterial community after the inoculation of rhizobacteria becomes relevant, and new methods are being developed to monitor population dynamics [21]. The 16S-based metagenomic analysis performed to *S. macrophylla* rhizosphere showed a very low relative abundance of sequences associated with the genus *Bacillus* spp. ranging below 1% of total reads in all inoculated treatments in contrast to non-inoculated plants, where sequences belonging to this taxon were absent. Assuming that *Bacillus* sp. sequences belong to inoculated strains, this may suggest that regardless of the high amount of inoculated CFU, in the long-term, only a low proportion remains, probably displaced by environmental-borne microbial populations more capable of occupying ecological niches in the rhizosphere. However, even the low relative abundance of *Bacillus* sp. found on inoculated treatments appears to be enough to induce the observed phenotypes on inoculated plants, which supports the idea that rhizobacteria establish a tight relation with the hosting plants in which the quality, and not the microbial population size is relevant for plant fitness. Moreover, inoculated rhizobacteria may induce subtle microbiota shifts or indirect guild shifts of more abundant representatives causing observed phenotypes. As has been previously demonstrated, several rhizobacteria species are capable to produce secondary metabolites capable to modulate both microbiota structure and plant-microbe interactions [44].

The high diversity of the microbiota found is interesting, since the substrate used is from a non-edaphic origin and is of completely foreign to tropical region, hence any substantial contribution of bacteria that could establish an interaction with *S. macrophylla* plants was unexpected to be substrate-borne. The significant diversity of the communities observed may be probably originated from the environment through water irrigation, the air, as well as the possible contribution from seed endophytic bacteria [45]. However, it should be noted that the relative abundance of certain specific groups found in the microbiota of pristine habitats, where *S. macrophylla* is distributed, differs significantly from that present in the microbiota analyzed in the commercial substrate at the end of the nursery experiment. For example, the relative abundance of the phylum Firmicutes exhibited a relative abundance of between 3 and 7.6% in tropical forest samples [46], but only 0.03 to 1.6% in samples from the present study, whereas the phylum Proteobacteria exhibited an abundance of close to 30% in samples from other studies in the same area [46–48]. These results demonstrate that the genus *Bacillus* spp., -to which many rhizobacteria belong-used in this study was not detected in the control treatment. This result suggests that the inoculated bacteria may have been established in the communities of these treatments and were directly or indirectly responsible for the phenotypic effect observed for the plants in the different treatments. In addition, an analysis of the data shows that the communities present

in treatments show differences in diversity and abundance at distinct taxonomic levels, both between treatments and with respect to the control, suggesting that the effect of bacterial strains is intrinsic and that the impact on the phenotype of plants, may be due to synergistic contributions that can cause subtle changes in the abundance of the inoculated bacteria. Similar observations were reported recently for *Zea mays* rhizospheric communities, where massive 16S sequencing reveals that rhizobacterial inoculation has a subtle impact in the native microbiota [22] A deeper examination of the microbiota (including fungi, protozoans, microalgae, lichens, and viruses) and time courses monitoring in nursery developing plants [21] could reveal complex networks of microscopic consortia contributing to the successful early development of *S. macrophylla* under commercial conditions in nursery and plantations.

5. Conclusions

Evaluated rhizobacteria strains were able to promote growth of *S. macrophylla* plants under forest nursery conditions. The activity of these strains was demonstrated by the increase of aerial and root biomass. Serendipitous evidence suggests that some of the evaluated strains may also provide a health-promoting effect. Finally, the analysis of microbiota structure of *S. macrophylla* rhizosphere evaluation demonstrated that three months after sowing, a complex bacterial community had been established, including members of *Bacillus* sp. genus present only in those treatments where rhizobacteria strains were inoculated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13101742/s1, Supplementary Table S1: Taxonomic assignation.

Author Contributions: Conceptualization, A.T.C.-B. and Y.J.P.-R.; investigation, F.I.T.-E., P.A.B.-L., A.A.B.-L. and J.E.M.-H.; data curation and statistical analysis N.Y.L.-S.; writing—original draft preparation, F.I.T.-E. and Y.J.P.-R.; funding acquisition, Y.J.P.-R., A.T.C.-B., F.I.T.-E. and N.Y.L.-S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The samples analyzed may be available upon request after a share transfer agreement. The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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