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A baseline of *Arbutus unedo* L. microbiome for future research: *In vitro* versus *ex vitro*

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ABSTRACT

Arbutus unedo L. (strawberry tree) is a multipurpose Ericaceae tree with a circum-Mediterranean distribution and tolerant to abiotic and biotic stresses. The bioactive compounds and edible berries make this species attractive from an economical perspective, which causes a high demand for true to type plants. As micropropagation techniques are quite popular for the production of strawberry tree clonal plants, the characterization of its microbiome is essential, due to the possible impact in micropropagation success, including acclimatization. Thus, this study aims to identify the endophytic communities on strawberry tree plant tissues *in vitro*, as well as their prevalence and/or alteration upon plant acclimatization. For this purpose, the microbiome of two genotypes under micropropagation and *ex vitro* was identified using a culture-independent method. Bacterial OTUs were assigned to 7 phyla and 79 genera whereas only one Archaea genus was identified. The most abundant and diverse bacterial phylum was Actinobacteriota (48%) followed by Proteobacteria (43%), Firmicutes (6%) and Bacteroidota (3%). Noticeable differences in terms of composition and diversity were found when the microbiome of genotypes *in vitro* was compared, whereas the composition of *ex vitro* samples was similar. A significant higher diversity was found on both genotypes *ex vitro* when compared to the respective *in vitro* plants. This study presents the very approach into the identification and characterization of strawberry tree microbiome, including *in vitro* plants, using a culture-independent method. The genotype proved to be a determinant factor shaping microbiota structure. The relevance of this results for micropropagation and breeding are discussed.

1. Introduction

Arbutus unedo L. (strawberry tree) is a small Ericaceae tree with a circum-Mediterranean distribution, that thrives on marginal dry lands and rocky soils (Martins et al., 2016a). Due to its natural resilience to abiotic stress and ability to resprout after forest fires, it is recognized as one of the most important species on southern European forest biomes. Furthermore, their edible berries are a vital source of revenue for producers in rural areas and are attracting considerable interest due to several bioactive compounds with applications in the cosmetic and pharmaceutical industries (Martins et al., 2016b). For these reasons, the strawberry tree has received much attention from researchers over the last two decades and a great deal of work has been done to select superior genotypes. Due to the low level of differentiation that seems to exist between populations, caused by a high gene flow, the screening should be take into account intra- rather than inter-population diversity (Lopes et al., 2012; Takrouni et al., 2012; Takrouni and Boussaid, 2010). In order to supply producers with true-to-type plants from high quality selected genotypes, micropropagation protocols have been developed (Martins et al., 2019, 2016a). To obtain axenic cultures, micropropagation techniques require a sterilization procedure, to eliminate microorganisms from plant meristematic tissues, before *in vitro* establishment. Nevertheless, the presence of bacteria in micropropagated plants is a common scenario. Although some of these bacteria are contaminants resulting from human handling, others are endophytes that survive the sterilization procedure and persist in the cultures (Quambusch and Winkelmann, 2018). An intriguing area in this field that few researchers have addressed is the presence of latent microorganisms in *in vitro* plant cultures, that do not grow in the plant culture medium. Some studies were able to identify a considerable variety of these microorganisms in plant tissues under micropropagation from different plant species, such as *Atriplex* spp. (Lucero et al., 2011), *Pogonatherum paniceum* (Koskimäki et al., 2010) and *Prunus avium* (Quambusch et al., 2014). As micropropagation techniques are the main approach used for

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strawberry tree plant production due to their great advantages over traditional methods (Martins et al., 2019), and due to the impact of endophytes on its host, the identification and characterization of endophyte communities in the strawberry tree *in vitro* cultures, in particular those that prevail after plant acclimatization and transference to field conditions.

With this in mind, the aim of this study was the identification of latent bacteria in *A. unedo in vitro* cultures and test the effect of plant genotype on microbiota structure. Moreover, the prevalence of these endophytes upon plant transfer to *ex vitro* conditions was evaluated. Insights about possible synergies between those microorganisms and host plant are provided and the implications of these results on *A. unedo* selection and micropropagation are discussed.

2. Methods

2.1. Micropropagation

Two genotypes with different origins, genotype A (young tree) and genotype B (seedling) were selected for this study from a population growing in Coimbra region, Central Portugal (N 40°12′17.472″ W 8°23′40.929″, altitude 103 m). Plants were selected based on their physiological performance under drought (Martins et al., 2019). Both genotypes were established *in vitro* and micropropagated according to Martins et al. (2019). Briefly, young branches of genotype A were cut, dipped in 100 mg L^{-1}fungicide (Aliette, Bayer CropScience, Germany) for 10 min and rinsed with distilled water. After this treatment, branches were kept on containers, covered with a plastic bag, watered with distilled water and placed on a culture chamber (FitoClima 10,000 HP, Aralab, Portugal) for 30 days under 16-h photoperiod, an irradiance of 250 μmol m^{-2} s^{-1}, a temperature of 25 °C, and air humidity of 70%. The epicormic shoots formed were removed and surface sterilized with 70% ethanol (for 30 s) and calcium hypochlorite (5%, w/v, Sigma-Aldrich, St. Louis, MO, USA) with two drops of Tween 20for 10 min. After 3 washes with sterile distilled water, the epicormic shoots (0.5–1 cm) were placed in Anderson Rhododendron medium (Anderson, 1980; DuchefaBiochemie B.V, Haarlem, The Netherlands) with 6-benzylaminopurine (2 mg L^{-1}, Sigma-Aldrich), sucrose (3%, w/v, Duchefa) and agar (0,6%, w/v, Duchefa), on test tubes (25 × 150 mm) with plastic caps (Duran, Mainz, Germany). The pH was adjusted to 5.7 using KOH or HCl diluted solutions (0.01 M – 1 M), and the culture medium was autoclaved at 121 °C for 20 min (800–1100 g cm^{-2} gel strength after autoclaving). The culture was done in a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 μmol $m^{-2}s^{-1}$ (cool-white fluorescent lamps), and a temperature of 25 °C. For genotype B, isolated seeds were disinfected following the same methodology used for shoots and germinated on Petri dishes with filter paper discs and sterile distilled water. After 1 month of cold stratification (4 °C), the seeds were transferred to a culture chamber under 16-h photoperiod at 25 °C. After germination, the roots of the plantlets obtained were removed and the shoots placed on the medium described before. For axillary shoot proliferation, shoots were cultured on plastic containers (O118/80+OD118 with white filter, Microbox, Deinze, Belgium) with 100 mL of the previously described medium. The cultures were kept under a 16-h photoperiod at 25 °C, with culture intervals of 8 weeks for a total period of 2 years. After this period, the multiplication rate was 2.2 ± 0.4 shoots/explant in genotype A and 4.3 ± 1.4 in genotype B.

2.2. Rooting and acclimatization

For root induction, 3 cm long shoots were dipped on indole-3-butyric acid (1 g L^{-1}, Sigma-Aldrich) for 30 s and placed on containers with perlite (Siro, Mira, Portugal), under 16-h photoperiod at 25 °C and 70% humidity. The cover was gradually removed and after a one-month plants were transferred to individual containers (1700 cm^3) with a substrate composed of peat (30–0, Siro) and perlite (3:1, v/v). After one year, plants were transferred to larger containers (100 dm^3) with peat (Siro 30–0), and were kept in a greenhouse for another year under uncontrolled light, temperature and humidity conditions.

2.3. Sample preparation

Plant material was collected from two different development stages of the plants: *in vitro* shoots (A1 and B1) and two years old plants (A2 and B2). *Ex vitro* plants height was 150 ± 15 cm (A2) and 180 ± 13 cm (B2). Leaves and stems from A1 and B1 (950 ± 72 mg) were ground in liquid nitrogen with a sterile mortar and pestle, in a laminar flow cabinet, to avoid contaminations. Each sample consisted of 5 different shoots. Healthy leaves from A2 and B2 (10 ± 0.74 g) were collected from different branches and thoroughly washed in sterile water, surface-disinfected in 70% ethanol for 30 s, sodium hypochlorite (5%, w/v) for 10 min and washed three times in sterile distilled water. Finally, leaves were ground in liquid nitrogen as described before and 250 mg were used for DNA extraction.

2.4. DNA extraction and sequencing

DNA was extracted using DNeasy Powersoil Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V5-V6 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions, 0.3 μM of each PCR primer: forward primer 799F-mod3 5′–CMGGATTAGATACCCKGG-3′ (Hanshew et al., 2013) and reverse primer 1115R 5′–AGGGTTGCGCTCGTTG-3′ (Turner et al., 1999) and 50 ng of template DNA in a total volume of 25 μL. PCR conditions involved a 3 min denaturation at 95 °C, followed by 30 cycles of 98 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and paired-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

2.5. Sequencing reads and statistical analysis

Raw reads were extracted from Illumina MiSeq® System in fastq format and the obtained sequences were analyzed using the Quantitative Insights into Microbial Ecology bioinformatics pipeline, QIIME2 (version 2020.2.0; https://docs.qiime2.org/2020.2/) (Bolyen et al., 2019). The sequences were demultiplexed using the "demux" plugin according to their sequence code. The cleaning and replication of the sequences was done using the DADA2 (Divisive Amplicon Denoising Algorithm) pipeline (Callahan et al., 2016) within the QIIME2 program to remove sequencing adapters and trim bases with an average quality lower than Q25. Chimeric sequences were removed and Operational Taxonomic Units (OTU) were obtained at 3% of dissimilarity and were taxonomically classified using vsearch classifier against SILVA (release 138). OTUs were selected at a 97% similarity threshold using the open reference strategy. Rarefaction curves were performed with phyloseq package (McMurdie and Holmes, 2013) using R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria, R Core Team 2020). The normalization of the data was performed rarefying all samples to the minimum number of reads found. To analyze the endophytic microbiota structure, Venn diagram, heatmaps, correlations, Principal

Component Analysis (PCA) and diversity analysis were carried out using R software. Venn diagrams were constructed using the package VennDiagram (Chen, 2018). To evaluate the interaction and significance of the different endophyte genera on microbiota composition, correlations were calculated using the package GGally (Schloerke et al., 2018), heatmaps were constructed using the package ComplexHeatmap (Gu et al. 2016) and a PCA was performed using the package factoextra (Kassambara and Mundt, 2019). Diversity analysis - Shannon index (H'), Simpson's Index (D), Simpson's Index of Diversity (1-D') and Pielou index (J') - were obtained using the package vegan (Oksanen et al., 2019), to compare the diversity between samples from different genotypes and different ages. The similarity percentages (SIMPER)(Clarke, 1993) were performed using a Bray-Curtis dissimilarity with the PAST software (version 4.0.3). Raw Illumina sequencing data was deposited in the NCBI as SRA files within the bioproject with the accession number PRJNA703435.

3. Results

3.1. Endophytic microbiota associated with two genotypes of Arbutus unedo L

A total of 113.546 good quality 16S rRNA reads and 160 Operational Taxonomic Units (OTUs) were recovered by Illumina sequencing: A1 (22.349 reads), A2 (32.348 reads), B1 (34.773 reads) and B2 (24.076 reads). No chloroplasts reads were detected, and chimaeras, unassigned or mitochondrial reads were removed. Unassigned reads were only found in sample B1, representing 0.25% of the total good quality reads. Each sample reached the plateau phase, indicating that much of the diversity of the microbial community could be captured (Fig. S1). The level of sequencing coverage was 99% in all samples. Bacterial and Archaea communities were found: Bacterial OTUs were assigned to 7 phyla, 12 classes, 37 orders, 58 families and 79 genera where as for the Archaea communities only one unique phyla, class, order, family and genus was identified (Supplementary Table S1). The most abundant and diverse bacterial phylum was Proteobacteria (44.5%, comprising 32 genera), followed by Actinobacteriota (44.1%, comprising 26 genera), Firmicutes (6.2%, comprising 10 genera) and Bacteroidota (3.0%, comprising 8 genera) (Fig. 1). At the genus level, *Pseudomonas* was the most abundant (27.8%), followed by *Mycobacterium* (21.9%), *Cutibacterium* (16.3%), *Serratia* (7.7%), *Acinetobacter* (2.8%) and *Halococcus* (2.0), accounting for 78.5% of the total diversity (Fig. 1). Altogether, minor genera accounted for 21.0% of the diversity. The most abundant genera by phylum were: *Mycobacterium* (49.7%), *Cutibacterium* (36.9%), *Corynebacterium* (3.5%), *Lawsonella* (1.8%), *Micrococcus* (1.7%) amd *Janibacter* (1.4%) within Actinobacteriota; *Chryseobacterium* (67.2%), *Alloprevotella* (10.4%), *Sphingobacterium* (6.7%), *Capnocytophaga* (4.7%), *Empedobacter* (4.1%), *Prevotella* (2.9%), *Proteiniphilum* (2.3%) and *Hymenobacter* (1.7%) within Bacteroidota; *Staphylococcus* (29.4%), *Streptococcus* (28.6%), *Bacillus* (17.8%), *Lactobacillus* (9.9%), *Anaerococcus* (4.4%), *Carnobacterium* (4.3%) and *Gemella* (3.5%) within Firmicutes; *Pseudomonas* (62.4%), *Serratia* (17.2%), *Acinetobacter* (6.3%), *Candidatus_Portiera* (3.8%), *Wolbachia* (1.6%), *Escherichia-Shigella* (1.3%) and *Sphingomonas* (1.1%) within Proteobacteria (Fig. 1).

A total of 37 species were identified, such as: *Bacillus thuringiensis, Kocuria rhizophila, K. palustris, Pseudomonas alcaligenes, P. mendocina, P. umsongensis, Sphingomonas azotifigens, S. mali* and *S. yabuuchiae* (Supplementary Table S2). From the identified species, 10 were found *in vitro* in genotype A and 9 in genotype B, whereas *ex vitro*, 17 and 20 species were found in genotype A and B, respectively.

3.2. Differences in the composition of the endophytic microbiota between genotypes

Comparing the microbiota structure of the two strawberry tree genotypes there were noticeable differences. A substantial difference was that the domain Archaea was only identified in genotype A - *ex vitro*. At the phylum level, considerable differences in the relative abundance were observed between genotypes *in vitro*. While Proteobacteria was the most abundant phylum on genotype A, Actinobacteriota had a higher relative abundance on genotype B (Fig. 2A). This difference in relative abundance was also observed at the genus level (Fig. 2B). *In vitro*, a considerably higher number of unique genera was identified in genotype A (16) when compared to genotype B (6). At this development stage, 15

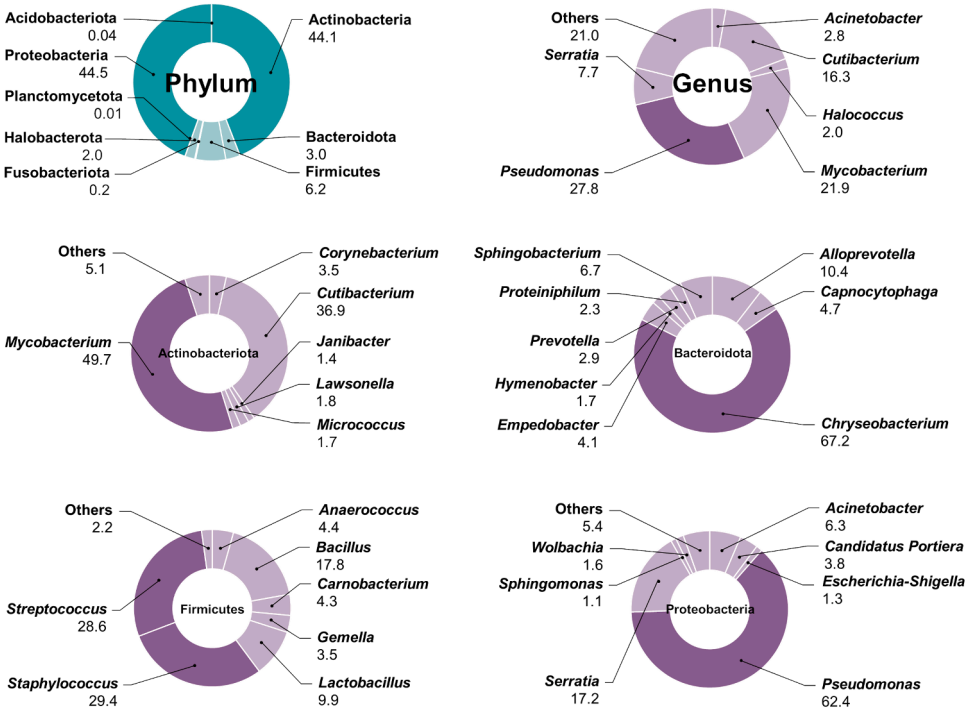

Fig. 1. Overall endophytic microbiota relative abundance (%) at the phylum and genera level.

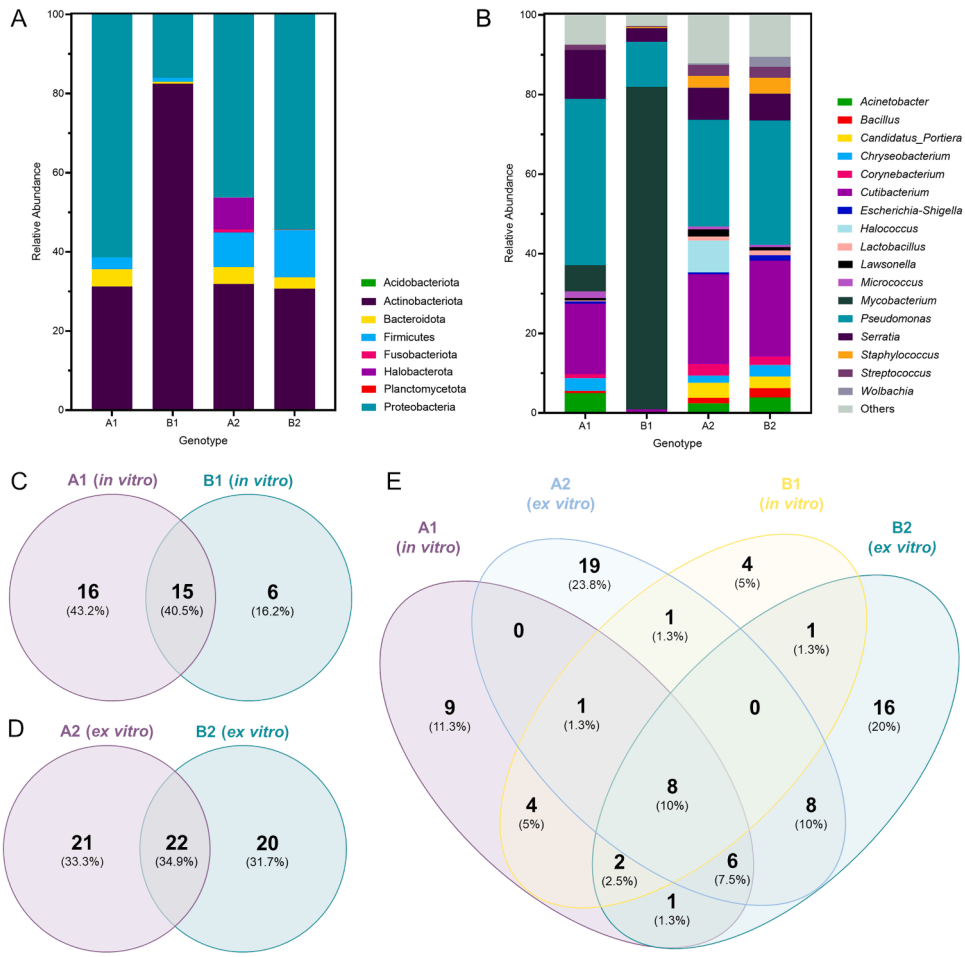

Fig. 2. Relative abundance of endophytic microbiota at the phylum (A) and genus (B) level for samples A1 (genotype A - *in vitro*), B1 (genotype B - *in vitro*), A2 (genotype A - *ex vitro*), and B2 (genotype B - *ex vitro*); Venn diagram showing the common and exclusive genera and relative abundance of the endophytic microbiota of samples A1 and B1 (C), A2 and B2 (D), and between all samples (E).

bacterial genera (40.5%) were found to be shared between genotypes (Fig. 2C, Supplementary Table S3). In the *ex vitro* samples the number of unique genera was very similar between genotypes (21 in genotype A and 20 in genotype B). Furthermore, 22 genera (34,9%) were shared between genotypes (Fig. 2D, Supplementary Table S3). Only eight bacterial genera (10%) were shared between all the samples: *Bacillus, Chryseobacterium*, an unknown genus of the family *Comamonadaceae, Cutibacterium, Pseudomonas, Serratia, Staphylococcus* and *Streptococcus* (Fig. 2E).

Concerning the distribution of OTUs among genotypes, 15 were shared on the *in vitro* samples, most belonging to the Proteobacteria phylum (7; *Pseudomonas* 5, *Burkholderia-Caballeronia-Paraburkholderia* 1 and *Serratia* 1). Twice as much as unique OTUs were identified in genotype A than in genotype B, respectively 31 unique OTUs in genotype A, most belonged to the Actinobacteria (14) followed by Proteobacteria (7); and fifteen in genotype B, most belonged to the Proteobacteria (6) followed by Firmicutes (5). The number of unique OTUs in the *ex vitro* samples was very similar between genotypes: 47 and 49 in genotypes A and B respectively. In genotype A, most OTUs belonged to the Actinobacteria phylum (21) followed by Proteobacteria (16), whereas in genotype B most belonged to Proteobacteria (18), followed by Actinobacteria (15) and Firmicutes (14). 22 OTUs were found to be shared between strawberry tree genotypes in the *ex vitro* samples (Supplementary Table S4).

3.3. Effect of plant development stage on the endophytic microbiota (in vitro vs. ex vitro)

Regarding the *in vitro* development stage of strawberry trees genotypes, Proteobacteria (61.4%) was found to be the most abundant phylum in genotype A, followed by Actinobacteria (31.3%), whereas Actinobacteria represented 82.5% of the endophytic microbiota in genotype B (Fig. 3). In contrast, samples collected from *ex vitro* strawberry trees showed a similar composition between genotypes at the phylum level, most belonging to Proteobacteria (46.2–54.4%), followed by Actinobacteria (31.8–30.7%) and Firmicutes (8.8–11.9%), in genotype A and B respectively (Fig. 3).

At the genera level, 16 unique genera (27.1%) were identified *in vitro* in genotype A compared to 28 (47.5%) *ex vitro*, whereas in genotype B 10 unique genera (19.2%) were identified *in vitro* compared to 31 (59.6%) *ex vitro* (Fig. 4A-B). The difference between *in vitro* and *ex vitro* samples was also confirmed by the log2 fold change calculation of OTUs at a phylum and genera level (Fig. 4C-D). An increase in the number of OTUs was observed between *in vitro* and *ex vitro* samples in almost all the genera, except *Acinetobacter, Chryseobacterium, Escherichia-Shigella, Micrococcus, Pseudomonas* and *Serratia* in genotype A.

3.4. Similarity analysis between microbial communities

The dissimilarity value obtained (76.1%) for single genera between the *in vitro* samples of genotypes A (A1) and genotype B (B1) demonstrated that the microbiota at this stage of development was

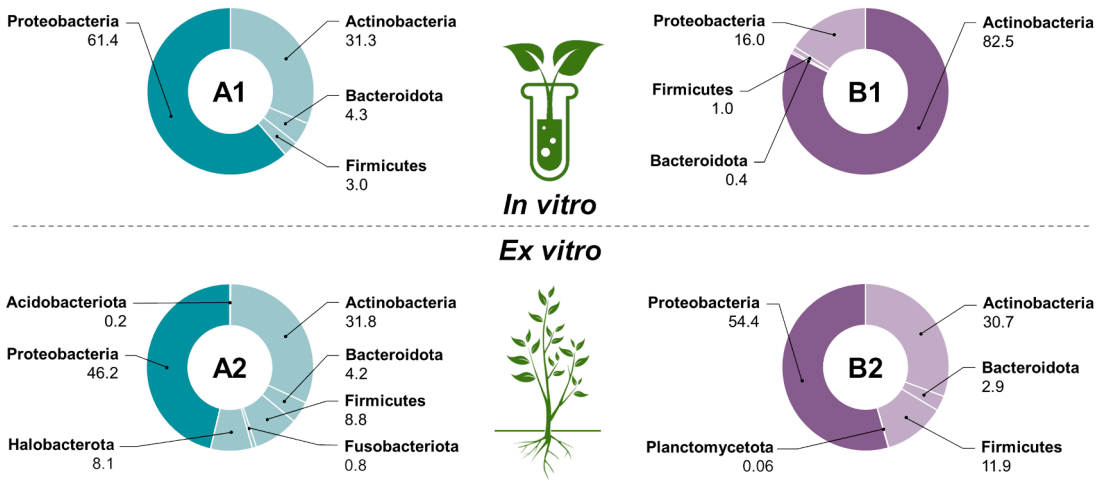

Fig. 3. Relative abundance of the endophytic microbiota at the phylum level between genotype A and B at different development stages (*in vitro* and *ex vitro*).

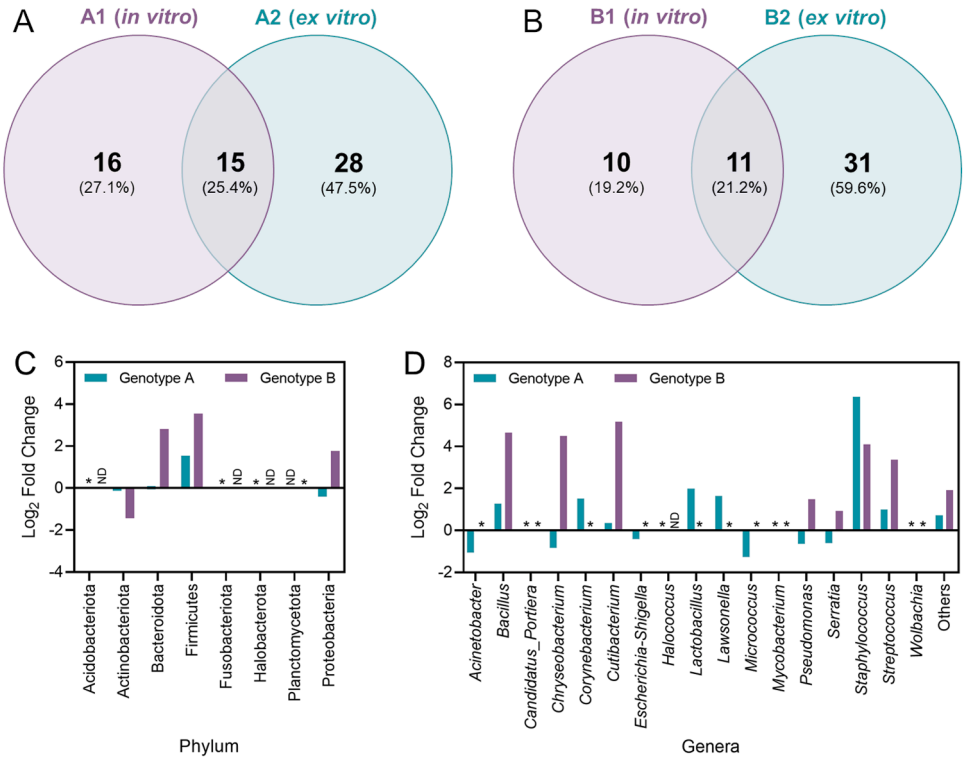

Fig. 4. Venn diagram showing the common and exclusive genera and relative abundance of the endophytic microbiota of genotype A (A) and genotype B (B)*in vitro* and *ex vitro*; Log2 fold change calculation of OTUs between *in vitro* and *ex vitro* samples at a phylum (C) and genus (D) level for genotypes A and B.

considerably different (Supplementary Table S5). *Mycobacterium, Pseudomonas* and *Cutibacterium* were the genera with the most contribution to the dissimilarity with 48.96%, 20.05% and 11.22% respectively. A lower dissimilarity value was determined between A2 and B2 (22.14%). In this case, *Halococcus, Pseudomonas* and *Wolbachia* were the genera with the most contribution to the dissimilarity with 18.24%, 10.08% and 4.89% respectively. The relative abundance of endophytic microbiota found in the two developmental stages of strawberry trees was more similar in genotype A (A1/A2) with an average dissimilarity of 37.80%, than in genotype B (B1/B2) with an average dissimilarity of 83.11%. The genera with the most contribution to the dissimilarity in genotype A were *Pseudomonas* (19.76%), *Halococcus* (10.78%) and *Mycobacterium* (8.70%), whereas in genotype B were *Mycobacterium* (48.78%), *Cutibacterium* (14.12%) and *Pseudomonas* (12.06%).

3.5. Endophytic microbiota structure

A correlation analysis carried out at the genus level revealed a positive correlation between most genera in terms of OTUs relative abundance, whereas, a negative correlation was observed between *Mycobacterium* and all other genera. Furthermore, no correlation was found between some genera (*e.g., Micrococcus* and *Serratia* with *Bacillus, Candidatus Portiera, Halococcus, Lactobacillus, Staphylococcus* and *Wolbachia*) (Fig. 5).

A PCA analysis using OTUs relative abundances at the genus level differentiated the endophytic microbiota according to the genotype (A vs B) and development stage (*in vitro* vs *ex vitro*). Each axis reflects the percentage of the variation between the samples with the X-axis representing the highest dimension of variation (PC1–62.1%) and the Y-axis

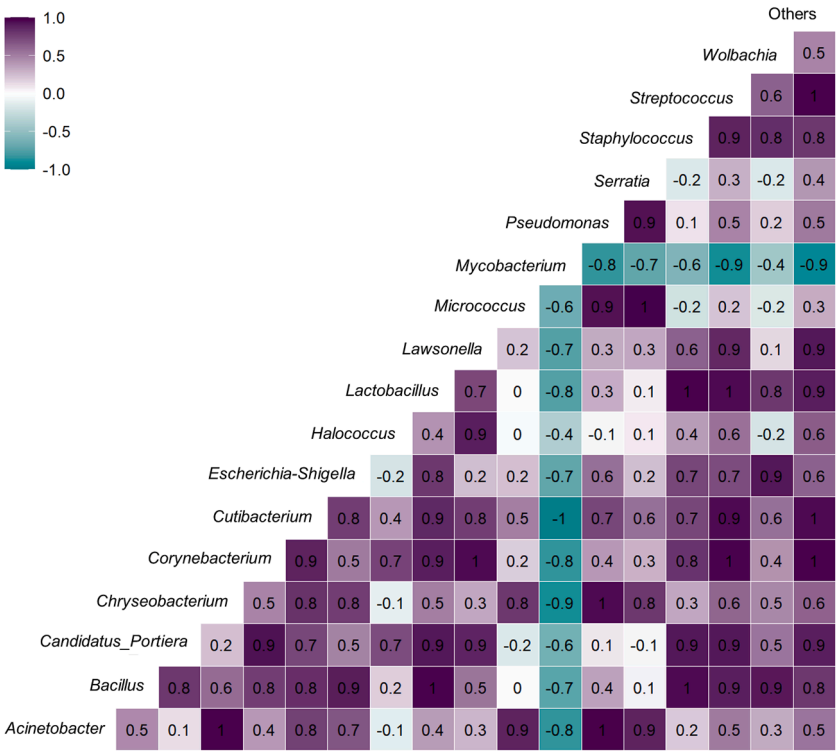

Fig. 5. Pearson correlation analysis at the genus level for all the samples. Values close to 1 and −1 indicate a high positive or negative correlation respectively, while values close to 0 indicate no correlation.

representing the second-highest dimension of variation (PC2–24.3%) (Fig. 6A). The analysis allowed clear differentiation among the development stage (*in vitro* vs *ex vitro*), especially in genotype B. The relative abundances of genera that contributed to the microbiotas divergence by development stage were mainly *Mycobacterium* for sample B1, and *Acinetobacter, Chrysobacterium, Micrococcus, Pseudomonas* and *Serratia* for sample A1. The differences observed between genotypes in the *in vitro* samples did not translate into *ex vitro*, which have a similar composition and relative abundance at the genus level. A high positive correlation was also obtained between samples A1, A2 and B2, whereas no correlation was found between those samples and B1 (Fig. 6B). These results were also confirmed in the heatmap, which shows the contribution of the bacteria genera mentioned before to the microbiota structure. The hierarchical clustering analysis identified the same relation between samples priorly revealed by the PCA analysis (Fig. 6C).

3.6. Alpha diversity analysis

Diversity was estimated for each sample through the measurement of the Shannon index (H'), Simpson's Index, Simpson's Index of Diversity (1- D, sometimes called Dominance Index) and Pielou index (J'). All diversity indices support that the endophytic microbiota of strawberry trees varies according to genotype and/or developmental stage (Fig. 7). The Shanon (H_{A1}= 2.04 and H_{B1}=0.79) and Simpson's (D_{A1}= 0.77 and D_{B1}=0.33) indexes from *in vitro* shoots supported that genotype was a structuring factor of the endophytic microbiota. On the other hand, this factor had no impact on the endophytic microbiota from the *ex vitro* samples (H_{A2}= 2.57 and H_{B2}=2.45; D_{A2}= 0.86 and D_{B2}=0.83). Simpson's Index of Diversity showed greater diversity on *ex vitro* samples when compared to *in vitro* in both genotypes (1-D_{A2} = 0.14 and 1-D_{B2} =0.17). This index also supports the low diversity found in the genotype B *in vitro* (1-D_{B1}= 0.67) as a consequence of the high relative abundance found for the *Mycobacterium* genus. Pielou's evenness index (J'), which measures the distribution of genera by sample showed a very heterogeneous genus distribution on *in vitro* samples from genotype B (J'$_{B1}$= 0.26), once again supported by the high relative abundance of the genus *Mycobacterium* found, whereas *ex vitro*, the homogeneity found between genotypes is also supported by this index (J'$_{A2}$= 0.68 and J'$_{B2}$=0.66).

4. Discussion

For a long time, *in vitro* cultures were believed to be axenic, thus free of any microorganism contamination. However, this is far from the truth, and several archaea, bacteria and fungi species have been identified inside plant tissues growing *in vitro* using culturable methods. The fact that most of these microorganisms are latent and do not cause any symptoms or grow in the plant growth medium is undoubtedly the reason why they remain undetected. Nonetheless, culture-independent methods can bypass this limitation and usually reveal an unexpectedly high diversity. Strawberry tree *in vitro* cultures are not an exception and have been found to harbor a considerable diversity of bacteria. Most of the dominant genera identified in this study have already been found on other micropropagated species. *Acinetobacter*was identified in *Ipomoea batatas* (Izarra et al., 2020) and *Staphylea pinnata* (Szewczyk-Taranek et al., 2020), while *Cutibacterium*was found on *Triticum* spp (Kuźniar et al., 2020). *Mycobacterium*was found in *Aglaonema* sp. (Fang and Hsu, 2012), *Pogonatherumpaniceum* (Koskimäki et al., 2010)and *Prunus avium* (Quambusch et al., 2014), while *Pseudomonas*was detected in *Ipomoea batatas* (Izarra et al., 2020), *Aglaonema* sp. (Fang and Hsu, 2012) and *Triticum* spp. (Kuźniar et al., 2020).*Staphylococcus*, another bacterial genus identified on strawberry tree is also commonly identified on *in vitro* growing plant tissues, such as *Atriplex* spp. (Lucero et al., 2011) and *Ipomoea batatas* (Izarra et al., 2020), as well as *Bacillus* that have been identified in plant material of poplar, larch and spruce, that had been micropropagated for at least 5 years (Ulrich et al., 2008).Other bacteria genera found on strawberry tree, such as *Chryseobacterium, Pseudomonas* and *Serratia* are also commonly found in other plants (Afzal et al., 2019).

Several authors hypothesized that the presence of endophytes might influence micropropagation success at different stages of the process (Laukkanen et al., 2000; Pirttilä et al., 2008). The endophyte

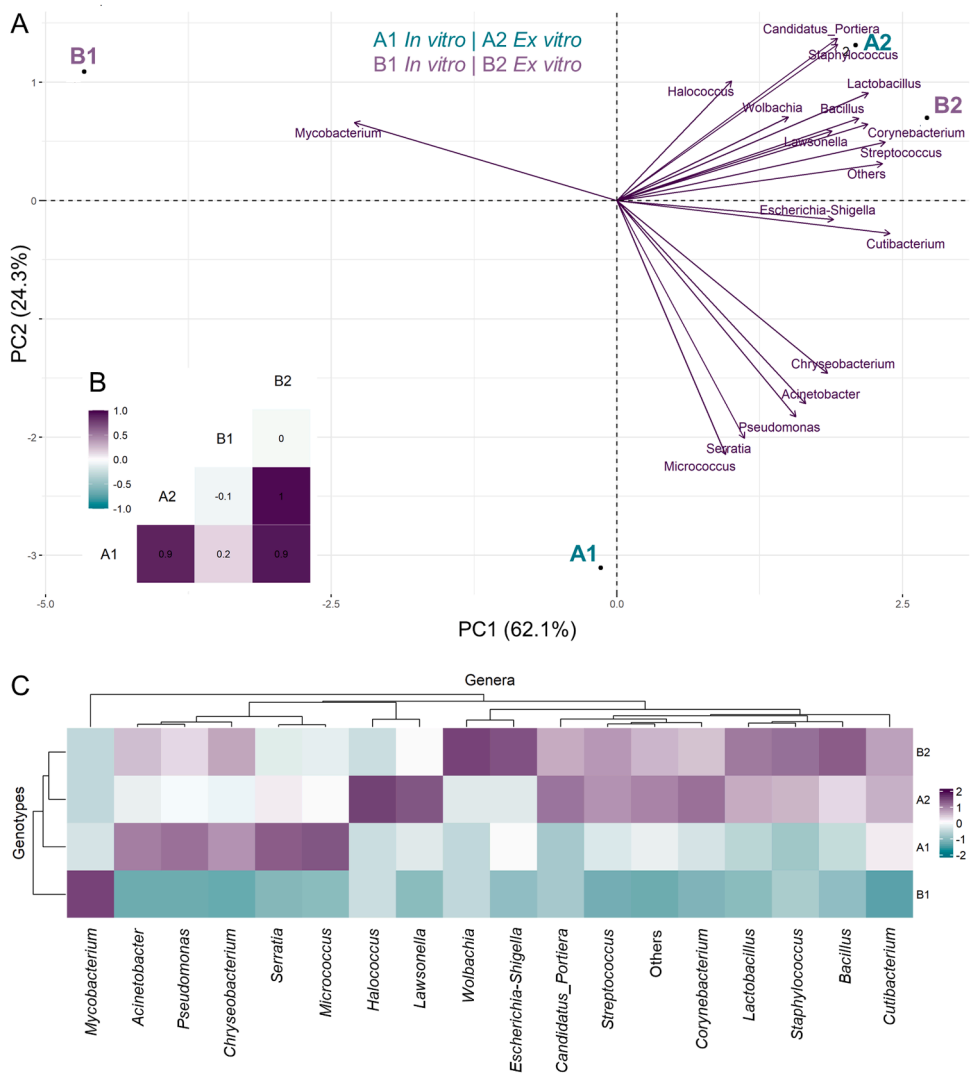

Fig. 6. PCA analysis based on the OTUs relative abundance for genotypes A and Bat different developmental stages (*in vitro* and *ex vitro*) (A); Correlation analysis (B) and Heatmap with a hierarchical clustering analysis using OTUs relative abundances for all samples.

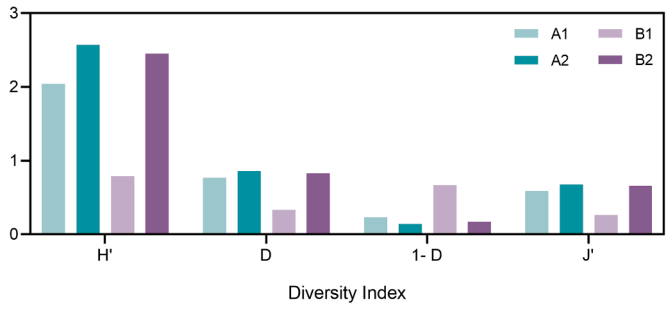

Fig. 7. Alpha diversity indexes for each sample through the measurement of the Shannon index (H'), Simpson (D), Simpson's Index of Diversity (1- D) and Pielou index (J').

composition, together with the plant genotype and tissue culture conditions are even pointed as the key elements in the process, influencing the regeneration capacity of the plant material (Pirttilä et al., 2008). The negative effects on plant micropropagation caused by different endophytes have been reported, such as those caused by *Methylobacterium* and *Mycobacterium* on *Pinus sylvestris* (Laukkanen et al., 2000; Pirttilä et al., 2008). *Mycobacterium* was once reported as a widespread and recurrent contaminant in plant tissue culture, due to its resistance to disinfection procedures (Taber et al., 1991),and recent studies indicated the presence of *Mycobacterium* on the rhizosphere of different plant species (Bouam et al., 2018). This bacterial genus was found on both strawberry tree genotypes sampled but only under *in vitro* conditions, which seems to indicate that this genus did not cope well with the *ex vitro* environment. Furthermore, as the relative abundance of *Mycobacterium* was the highest on genotype B, it would be expected that the micropropagation could be affected. However, this was not the case, and genotype B presented high micropropagation rates (data not shown).

Although these possible undesired effects, most plant endophytes seem to produce a positive effect on the host. Several mechanisms can be involved in endophyte-plant interaction, promoting plant growth and fitness, such as hormone production (IAA - indole-acetic acid and gibberellic acid), siderophores, cell-wall degrading enzymes and other metabolites, etc. Additionally, endophytes might also stimulate plant immunity, leading to metabolic changes in the host plant. All in all, these mechanisms often confer protection against abiotic and biotic stresses which translates into high crop yields (Radhakrishnan et al., 2017). Among the genera found on strawberry tree tissues, *Acinetobacter, Bacillus, Burkholderia, Paraburkholderia, Pseudomonas* and *Serratia* are some that might promote the mentioned positive effects. For example, *Acinetobacter* sp. strains isolated from the rhizosphere of

Pennisetum glaucum were able to solubilize phosphates and produce IAA and siderophores. Additionally, *in vitro* assays showed they caused inhibition of *Fusarium oxysporum* and also enhanced the shoot height, root length, and root dry weights of inoculated *Pennisetum glaucum* plantlets (Rokhbakhsh-Zamin et al., 2011). Similarly, a *Serratia* sp. strain isolated from *Achyranthes aspera* was able to solubilize phosphate, produce ammonia, IAA and siderophores, greatly promoting plant growth (Devi et al., 2016). In this study, *Burkholderia* and *Paraburkholderia* were only found *in vitro*, with a very low relative abundance. *Acinetobacter* was found in all samples except B1, whereas *Bacillus, Pseudomonas* and *Serratia* were identified in all samples. In fact, *Pseudomonas* is the genera with the highest relative abundance in samples A1, A2 and B2.

Furthermore, strains of *Acinetobacter, Burkholderia, Serratia* and *Bacillus thuringiensis* (identified on sample A2), have been reported to have the ability to solubilize zinc, thus improving its content in plants and yield of several crops (Hakim et al., 2021). Additionally, the application of *B. thuringiensis*, an auxin producing bacteria, promoted growth of wheat plants under drought stress (Hakim et al., 2021). *Sphingomonas azotifigens* and *Pseudomonas mendocina* (both identified in sample B2) also proved their benefic effects on plants, through nitrogen fixation and nutrient absorption (Kohler et al., 2006; Videira et al., 2009). *Kocuria palustris*, identified on *in vitro* samples, showed great resistance to arsenic (Zacaria Vital et al., 2019), whereas *Kocuria rhizophila*, identified on sample A2, greatly improved the absorption of nickel (Anum et al., 2019). *Streptomyces mirabilis* (identified in sample A1), also showed great resistant to heavy metals (Schütze et al., 2014), showing the potential of the aforementioned bacteria on heavy metal mobilization and consequent benefits on plants growing in contaminated soils. Finally, *Pseudomonas alcaligenes* (identified in B2), showed an inhibitory activity of more than 80% against *F. oxysporum* f.sp. *lycopersici* suppressing the disease when a bacterial suspension was applied on tomato (Widnyana et al., 2013). Likewise, *Sphingobacterium multivorum*, a chitosan-degrading bacteria (identified in A1), was also able to inhibit mycelial elongation of the same pathogen (Matsuda et al., 2001).

The difference observed in the microbiota composition between strawberry tree genotypes under *in vitro* conditions was expected. In fact, among the factors that govern plant microbiome structure, the genotype of the host plant and its age and developmental stage are pointed to play a crucial role (Compant et al., 2019; Turner et al., 2013; Whipps et al., 2008). Differences in the bacteria endophytic populations associated with different genotypes were also observed in shoots of *P. avium* growing *in vitro* (Quambusch et al., 2014). The fact that strawberry tree genotype A was established *in vitro* from an adult plant, whereas genotype B was obtained from a seed, might also be the reason for the difference observed in the microbiota structure between these genotypes under *in vitro* conditions. Because seed microbiome is believed to be recruited mainly from the mother plant (Nelson, 2017), the aforementioned factors might also be decisive influencers on plant microbiome. Nevertheless, bacteria associated with seed are usually within the Actinobacteria, Bacteroidota, Firmicutes and Proteobacteria phyla (Nelson, 2017), which is in accordance with our findings.

Changes in the structure of the microbiota can also be expected throughout the micropropagation process through successive colonization by bacterial communities as was observed in *Ananas comosus* (Abreu-Tarazi et al., 2010).

The transition between *in vitro* to *ex vitro* conditions translated into an increase in diversity. This was an expected result as endophytic populations are usually lower in plant tissues growing *in vitro* due to the small amount of tissue used to initiate the micropropagation as well as the specific conditions of these cultures (Quambusch et al., 2014). Other possible explanation is related to horizontal transmission of these microorganisms. As the substrate used after plant acclimatization was sterilized, no microorganisms were transmitted into the plant by the soil. However, the surrounding environment is also an important source of organisms that might enter the plant through different paths integrating its microbiome (Wassermann et al., 2019). Moreover, a dramatic change was observed in genotype B microbiota composition when *in vitro* and *ex vitro* samples are compared. Furthermore, the composition of both genotypes *ex vitro* is quite similar, possible due to horizontal transmission, which in this particular case seems to suggest a minor role of the genotype on shaping endophytic microbiota. Finally, the diversity of archaea was considerably low on adult plants and were not detected on *in vitro* shoots. Although archaea species are commonly associated with plants, *e.g.* in *Zea mays* roots (Chelius and Triplett, 2001) and associated with *Eruca sativa* (Taffner et al., 2019), as far as we concern, there is hardly any report of their presence on plant tissues under micropropagation, possible because archaea might not be resilient enough to survive the sterilization procedures before *in vitro* establishment and/or struggle to thrive under the specific conditions of the *in vitro* cultures.

Due to the influence of endophytic bacteria in micropropagation, which is the major vegetative propagation technique used on *A. unedo*, microbiome studies are essential to improve the process. Furthermore, endophytes might mitigate the negative consequences of stress on plants and ameliorate their general fitness. Additionally, due to their biological control ability, some species might be ecological friendly alternatives to synthetic pesticides (Radhakrishnan et al., 2017). For example, bacteria from the genera *Bacillus* (Fira et al., 2018; Radhakrishnan et al., 2017), *Burkholderia* (Sandani et al., 2019), *Paraburkholderia* (Baccari et al., 2019) and *Pseudomonas* (Sandani et al., 2019) have been found on strawberry tree and according to several reports might have potential as biological control agents. Furthermore, several species identified on strawberry tree, such as *Bacillus thuringiensis, Kocuria palustris, K. rhizophila, Pseudomonas alcaligenes, P. mendocina, Sphingomonas azotifigens, S. multivorum* and *Streptomyces mirabilis*, might provide great benefits to their host plants as mentioned before (Anum et al., 2019; Hakim et al., 2021; Kohler et al., 2006; Matsuda et al., 2001; Schütze et al., 2014; Videira et al., 2009; Widnyana et al., 2013; Zacaria Vital et al., 2019). For this reason, the inoculation of microplants with bacterial formulations might be a great benefit not only during the multiplication phase but especially during *ex vitro* acclimatization which is a key step of the process but causes great stress to plants (Orlikowska et al., 2017). Our work provided the first insights on *A. undeo* microbiome composition, and both genotype and type of explant used for micropropagation seems to be a fundamental driver of the microbiome structure. However, more research is still required to fully characterize and understand the contribution of endophytic organisms on different aspects of strawberry tree micropropagation, including regeneration rates, rooting and acclimatization success, which might have great repercussions on plant selection, either for micropropagation or breeding purposes.

Credit author statement

On behalf of all authors (João Martins, Aitana Ares, Joana Costa and myself) I declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Moreover, I inform that this work is original and has not been published elsewhere, nor is it currently under consideration for publication in another journal or as book chapter. We do not need any permission from the copyright owners and credit any sources because the article is original and no data appearing on it have been published elsewhere.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2021.110657.

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