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The Root Mycobiota of *Betula aetnensis* Raf., an Endemic Tree Species Colonizing the Lavas of Mt. Etna (Italy)

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Abstract: *Betula aetnensis* is an endemic tree of high conservation value, which thrives on the nutrient-poor volcanic soils of Mount Etna. Since plant–microbe interactions could play a crucial role in plant growth, resource uptake, and resistance to abiotic stresses, we aimed to characterize the root and rhizosphere microbial communities. Individuals from natural habitat (NAT) and forest nursery (NURS) were surveyed through microscopy observations and molecular tools: bacterial and fungal automated ribosomal intergenic spacer analysis (ARISA), fungal denaturing gradient gel electrophoresis (DGGE). *B. aetnensis* was found to be simultaneously colonized by arbuscular (AM), ectomycorrhizal (ECM), ericoid (ERM) fungi, and dark septate endophytes (DSE). A high diversity of the bacterial community was observed whilst the root fungal assemblage of NAT plants was richer than that of NURS. Root and rhizosphere fungal communities from NAT plants were characterized by Illumina MiSeq sequencing. Most of the identified sequences were affiliated to Helotiales, Pezizales, and Malasseziales. Ascomycota and Basidiomycota dominated roots and rhizosphere but differed in community structure and composition. ECM in the roots mainly belonged to *Tylospora* and *Leccinum*, while *Rhizopogon* was abundant in the rhizosphere. The Helotiales, including ERM (mostly *Oidiodendron*) and DSE (mostly *Phialocephala*), appeared the dominant component of the fungal community. *B. aetnensis* harbors an extraordinarily wide array of root-associated soil microorganisms, which are likely to be involved in the adaptation and resistance mechanisms to the extreme environmental conditions in volcano Etna. We argue that nursery-produced seedlings could lack the necessary microbiota for growth and development in natural conditions.

Keywords: *Betula pendula*; Mediterranean forests; primary succession; *Oidiodendron*; *Phialocephala*; mycorrhizal symbionts

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1. Introduction

Betula aetnensis Raf. (Family Betulaceae) is a tree species endemic to Sicily, where it mostly thrives on the north-eastern slopes of volcano Etna, from 1400 to 2100 m a.s.l. [1,2]. *Betula aetnensis* is thought to be originated from *Betula pendula* Roth., a European-wide distributed birch [3]. Particularly, during the last glacial period, *Betula pendula* may have reached Sicily, where the geographical isolation and the peculiar ecological conditions of Mt. Etna may have allowed the differentiation of *Betula aetnensis* [4]. Despite its taxonomic rank is still under debate, *B. aetnensis* is considered a separated taxon in the Italian flora [4]. This species has an excellent ability to colonize volcanic substrates, where it plays a prominent role as a typical pioneer species at the beginning of the primary succession, showing high ecological importance. The adaptation to such harsh habitats by *B. aetnensis* reflects its

ability to tolerate soils with limited organic matter, as well as nutrient and water availability, where it may benefit from the absence of plant competitors [5].

In this regard, *B. aetnensis* behaves similarly to other European birches, which are light-demanding species mostly linked to early successional communities, with a strong pioneer attitude, and capable of establishing even in harsh and nutrient-limited conditions [3]. For instance, *Betula pendula* is particularly successful in colonizing disturbed sites, including little developed and contaminated soils, as well as coal mining spoils [6–8]. Interestingly, *Betula pendula* stands occurring in similar volcanic soils are found in Vesuvius volcanic complex (Campania region, Italy) [9] but information about soil microorganisms is lacking.

In the last decades, the research on *B. aetnensis* has been mostly focused on understanding the reasons underlying the increasing decaying of mature trees, that seems in part related to *Armillaria mellea* (Vahl) P. Kumm., a pathogenic fungus which typically affects aged and/or stressed and/or unhealthy individuals [10]. Conversely, less attention has been paid to soil microbial communities, including mycorrhizae [11] and fungal endophytes, which are expected to play a crucial role both in early and later life stages, significantly affecting plant growth and development, resource uptake, and resistance to abiotic stresses [12–14]. The decisive importance of mutualistic interactions with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, in which higher plants may benefit from enhanced nutrient and water uptake, especially in harsh and critical environments, whereas symbiotic fungi receive the organic carbon produced by plants, is widely acknowledged for most terrestrial plants including forest species [15]. By contrast, the knowledge of other root-inhabiting species and the ecological role played by endophytic fungi is very limited [13]. Root-associated microbes such as mycorrhiza helper bacteria and nitrogen-fixing actinobacteria could benefit plant fitness in harsh environments and scarce soil nutrient conditions [16], all this without considering that symbiotic fungi community of the plant species in the edge of the distribution range [17], such as *B. aetnensis*, may be particularly affected by changes in environmental conditions (e.g., due to climate change) [18]. Furthermore, plant–soil microbe interactions may play a crucial role in shaping the structure and composition of plant communities, thus affecting biodiversity patterns and the functioning of natural ecosystems [19]. Plant–soil biota feedbacks have been increasingly recognized as important drivers of plant coexistence and competitive ability [20]. For instance, the progressive accumulation of soil-borne pathogens associated with higher densities of conspecifics has been considered a mechanism allowing the establishment of more diverse plant communities [21]. Conversely, positive feedbacks may arise from community shift, for instance due to the spread of invasive alien plants [22].

The knowledge of the root-inhabiting microbial community of *B. aetnensis* is limited as specific surveys are lacking up to now. However, this information is of key importance for elucidating the biological and ecological role of soil microorganisms, as well as for the potentially relevant consequences for the conservation of this endemic species. To fill this knowledge gap, we studied symbiotic fungi, endophytic fungi, and bacterial communities occurring in *B. aetnensis* roots. Furthermore, the comparison between individuals from the natural habitat (NAT) and nursery-grown seedlings (NURS) allowed us to assess whether the plant material and soil used for afforestation purposes bore the same soil microorganisms. Root-associated microorganisms were surveyed by means of microscope observations and molecular approaches (PCR-based DNA fingerprinting techniques), providing a rapid and sensitive detection of microbial diversity regardless of their culturability. To understand the potential contribution of plant–microbiota interactions for *Betula* survival in hostile environment as the extreme edaphic conditions in volcano Etna, we studied the root-associated and rhizosphere soil mycobiota of *B. aetnensis* individuals from the natural habitat through Illumina MiSeq. High throughput sequencing of the ribosomal internal transcribed spacer (ITS) region provides accurate semi-quantitative information about the diversity, structure, and composition of a fungal community, which is partially or not at all obtainable from traditional culture-based analyses, fingerprinting methods, or microscopy observation. At present, this technology has been widely applied to studies of

microbial diversity in roots and rhizosphere soil of plants. While an increasing number of studies have evaluated root-associated fungi, including endophytes, most studies are focused on one or two groups of microrganisms, whilst the coexistence of different mycorrhizal symbionts, fungal endophytes, and bacteria in the same root system has rarely been investigated (e.g., [23]).

2. Materials and Methods

2.1. Collection Sites and Plant Traits

Betula aetnensis individuals were collected in the natural habitat (NAT) and in a forest nursery (NURS) (Table 1) with their rhizosphere soil (zone immediately surrounding the roots) (Figure 1). The natural habitat (NAT, "Mareneve" locality) is located at an altitude of 1574 m a.s.l., in the north-eastern slopes of Etna volcano (Figure 2), within the upper supramediterranean humid-hyperhumid bioclimatic belt [2]. In the nearest weather station (Piano Provenzana, Etna Nord, altitude of 1825 m a.s.l.), the mean annual temperature is 8.4 °C and mean annual precipitation is 930 mm. The study site is undergoing primary succession, with lavas in the process of plant colonization, characterized by pedogenic substrates, affected by strong abiotic stresses and limited nutrient and water availability [5]. The natural vegetation of the area is dominated by scattered individuals of *Pinus nigra* subsp. *calabrica* (Loud.) A. E. Murray, sometimes forming small thickets, and, more rarely, by mature plants of *B. aetnensis* and *Populus tremula* L. Since the natural regeneration of *B. aetnensis* in the study area is very limited, and young plants are crucial for the conservation of the species, only two individuals fully established were collected (Table 1). One-year old seedlings were collected from the regional forestry nursery "Flascio" (NURS), which is located at an altitude of 865 m a.s.l. Since soil and plant material for the nursery were collected in natural sites, they could be considered representative of field conditions. Seedlings in the nursery were subject to regular watering but not to fertilization treatments. Because of the rarity of this endemic species and the importance of nursery-grown individuals for reforestation activities, we could not use more than three seedlings.

Table 1. Main characteristics of the surveyed individuals and quantitative assessment of ectomycorrhizal and arbuscular mycorrhizal colonization of *Betula aetnensis* roots. NAT: Natural habitat; NURS: Forest nursery; F%: frequency of root infection; M%: colonization intensity of the root cortex; m%: colonization intensity of the mycorrhizal root cortex; A%: abundance of arbuscules in the root cortex; a%: abundance of arbuscules in the mycorrhizal root cortex. The above reported parameters were calculated according to Trouvelot et al. [24].

Sample Code	Height (cm)	Basal Diameter (cm)	Age (Years)	Ectomycorrhizal Colonization			Arbuscular Mycorrhizal Colonization				
				Observed Root Tips (N)	Observed Root Length (cm)	Colonization (%)	F%	M%	m%	A%	a%
NAT1	39	0.40	8	2.730	217.0	68.4	94.4	54.0	55.9	15.0	27.8
NAT2	50	0.55	12	1.077	115.0	81.0	95.7	46.3	48.5	7.35	15.8
NURS1	37	0.47	1	3.534	275.3	93.1	97.2	51.8	53.7	11.2	21.6
NURS2	37	0.63	1	4.081	404.3	95.3	95.8	55.6	57.4	12.2	21.9
NURS3	36	0.63	1	5.496	650.0	94.3	98.7	57.0	58.8	11.4	20.0

Plant height and basal diameter (both in cm) of all individuals were measured soon after the collection. After taking the cross-sectional discs, the age of NAT individuals was determined by counting annual rings. Tree ring boundaries were marked under a binocular and the width of the increment zones was measured to the nearest 0.01 mm along at two disk radii using a moving table and software TSAP-Win Time Series Analysis (Frank Rinn, Heidelberg, Germany) [25].

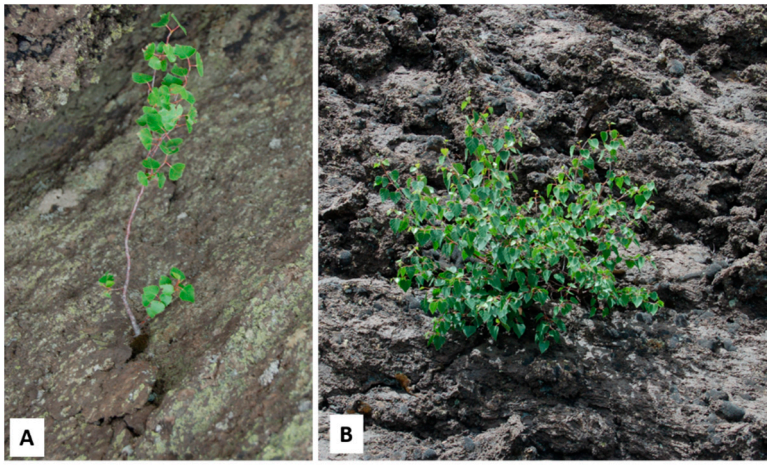

Figure 1. *Betula aetnensis* growing in its typical habitat; (**A**) young individual thriving on a lava slope, (**B**) an older plant growing on bare volcanic soils. Note that the area is totally devoid of other plants and only mosses and lichens are present.

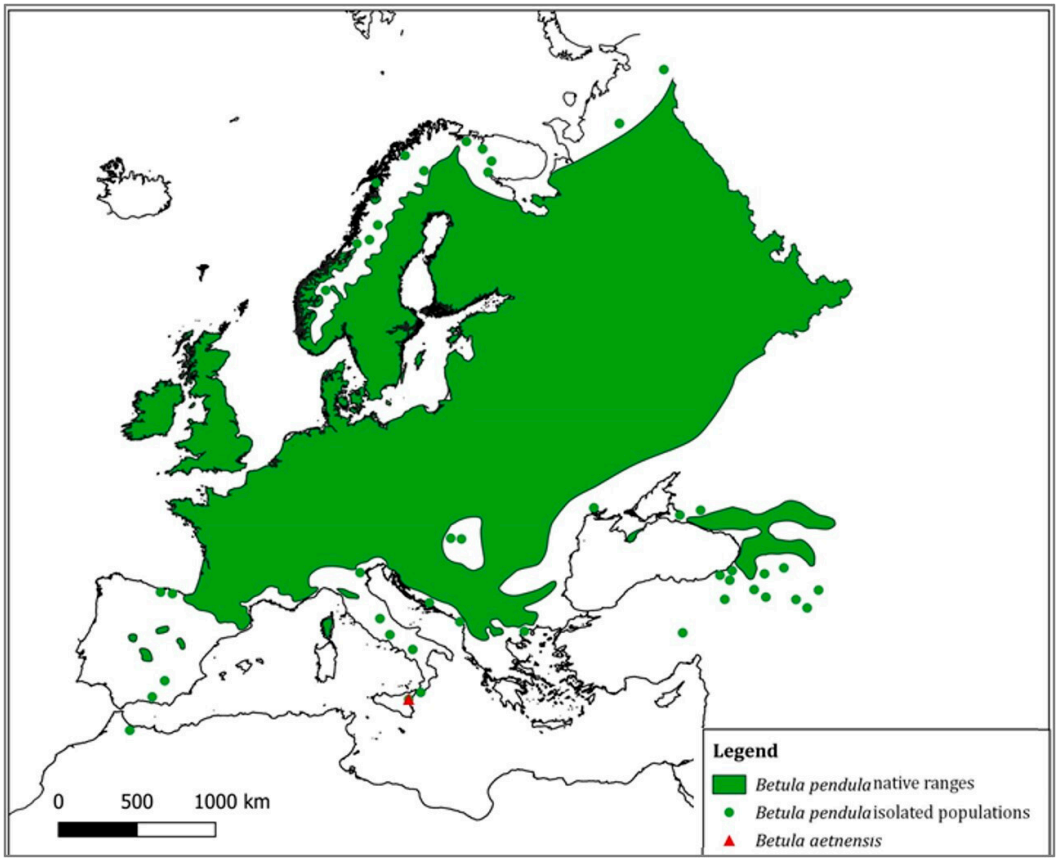

Figure 2. The narrow range of *Betula aetnensis* in Sicily in the context of the wide European distribution of *Betula pendula*, the most taxonomically close birch species (from Caudullo et al. [26], modified, licensed under a Creative Commons Attribution 4.0 International license).

2.2. Soil Analysis

Soil was sampled near the two plants in the natural habitat and also directly collected from the pot of each nursery-grown seedling. Soil analysis was carried out to evaluate soil organic matter (SOM), carbon content (C) soil nitrogen (N), C/N ratio, and pH, which were assessed using the methods described in Schuelke [27]. Available soil phosphorus

(P_2O_5) was determined through extraction with sodium bicarbonate ($NaHCO_3$) according to the Olsen method [28], while K content (as K_2O) was determined using the barium chloride-triethanolamine method of Mehlich [29].

2.3. Ectomycorrhizal Colonization

The whole root system of each individual was deeply cleaned and laid on a horizontal plane. Then, along a vertical section, the whole root system was split into two identical parts, half of which was observed for searching ectomycorrhizal structures. Ectomycorrhizal colonization was assessed by means of a stereoscope at 4–40× magnification. The most typical features of ectomycorrhizal symbiosis, such as root tip shape, branching, and color were considered [30]. The total root length was measured, and the ectomycorrhizal colonization was quantified according to Brundrett et al. [31].

2.4. Arbuscular Mycorrhizal and Endophytic Colonization

The half of the root system not used for ectomycorrhizal assessment was analyzed for observation of arbuscular mycorrhizal and endophytic structures. Root fragments not more than 0.5–1 mm thick were thoroughly cleaned and split in small pieces 2–3 cm long. The colonization by arbuscular mycorrhizal fungi (AMF) and by endophytic fungi was assessed following the standard procedure by Phillips and Hayman (1970), which involves three main phases: root clearing with KOH (10%), acidification with HCl (2%), and staining with Trypan blue (0.05%) in glycerol. Stained roots were then immersed in lactic acid and stored in Petri dishes at 4 °C. Observations were made under a light microscope (Leica Microsystems, Heerbrugg, Switzerland, Leica DFC 420C©). The most peculiar fungal structures of AMF (i.e., non-septate hyphae, arbuscules, vesicles, and coils) and of fungal endophytes (i.e., septate hyphae and microsclerotia) were searched at 20 and 40× magnification. The presence of arbuscules, vesicles, coils, and septate hyphae was expressed as a percentage of the number of observed root fragments. Then, the quantitative assessment of AM colonization was made following the procedure by Trouvelot et al. [24] and using the spreadsheet provided by Mercy (2017). Such method allows to determine the following parameters: frequency of root infection (F%), colonization intensity of the root cortex (M%), colonization intensity of the mycorrhizal root cortex (m%), abundance of arbuscules in the root cortex (A%), and abundance of arbuscules in the mycorrhizal root cortex (a%). For the presence of AMF, we took into account the occurrence of intracellular hyphae and, above all, of arbuscules inside the cortical cells, as they are the most distinctive feature of this peculiar plant–fungus symbiosis [32].

2.5. DNA Extraction

Total DNA of *B. aetnensis* roots and rhizosphere soil (the zone immediately surrounding the roots) was extracted from randomly selected thin roots, considering each individual from natural habitat (NAT1, NAT2) and pooled samples from nursery (NURS1+2+3). Rhizosphere soil was collected after digging all around the selected plants, pulling up the root system and shaking it vigorously; then, the soil still attached to roots was collected as rhizosphere soil [33]. Before DNA extraction, a scalpel blade was used to cut the plant roots in sections of about 2 cm, the plant roots were surface sterilized by immersion in 75% ethanol for 5 min, and immersed in sodium hypochlorite solution (0.9%, w/v) for 10 min. The roots were then washed with sterile water for 5 min to remove surface sterilization agents and blotted dry with sterile filter paper. Analyses were carried out using the FastDNA Spin kit for soil (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's instructions. For DNA extraction, 500 mg of root samples or rhizosphere soil were used. Root samples were cut into 2 mm fragments and were frozen in liquid nitrogen in 1.5 mL tubes, crushed thoroughly using a micropestle. Root samples fragments or rhizosphere soil were placed into 2.0 mL tubes containing Lysing Matrix E. Homogenization in the FastPrep®® instrument was performed for 40 s at a speed setting of 6.0. The proteins were precipitated with PPS buffer, the DNA solution was transferred to a SPIN Filter Tube

and washed with SEWS-M Solution. The purified DNA was eluted in 50 µL of DNase-RNase-free water. Yield was increased by incubation of SPIN™ Filter DNA binding for 5 min at 55 °C in a heat block. The DNA samples were analyzed by electrophoresis in 1% agarose gel with 1% ethidium bromide and stored at −20 °C until further analysis. DNA quality and concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

2.6. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The structure of microbial communities was investigated by the bacterial and fungal automated ribosomal intergenic spacer analysis (B-ARISA and F-ARISA, respectively), using the bacterial primers ITSF/ITSReub [34], and the fungal primers 2234C/3126T [35] (Table S1). The forward primer (2234C) was modified including a degenerated position [36], improving the sensitivity of analyses and aiming to limit biases. The PCR fragments were labeled as described in Schuelke [37], using a sequence-specific forward primer with M13 tail at its 5′ end (TGT AAA ACG ACG GCC AGT), a sequence-specific reverse primer not modified, and the universal fluorescent-labeled M13 primer (FAM-TGT AAA ACG ACG GCC AGT-3′). PCR mixtures (30 µL) contained buffer Phire Hot Start 1X (Thermo Scientific, Waltham, MA, USA), dNTP 0.2 mM, sequence-specific forward primer with M13 tail 0.8 µM, sequence-specific reverse primer 0.3 µM, the universal fluorescent-labeled M13primer, BSA (New England Biolabs, Massachusetts, USA) 0.1%, Phire Hot Start II DNA Polymerase (Thermo Scientific, Waltham, MA, USA) 0.4 µL, and 20 ng of DNA. The amplification was as follows: 98 °C for 30 s, followed by 35 cycles consisting of 98 °C for 15 s, 50 °C for 15 s, 72 °C for 30 s; 72 °C for 1 min. PCR products (5 µL) were loaded on a 2% agarose gel and separated by electrophoresis. Gels were stained with 1% ethidium bromide. The samples were separated on a capillary electrophoresis Bioanalyzer ABIPrism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed as described in Novara et al. [38].

2.7. PCR-DGGE of Fungal Endophytes

Nested PCR was used for the amplification of the ITS1 region of fungal rDNA to increase the resolution yield of denaturing gradient gel electrophoresis (DGGE) [39]. A fragment comprising both ITS1 and ITS2 was amplified in the first round of amplification using the universal primers ITS1F [40] and ITS4 [41] (Table S1). PCR amplification was performed in a total volume of 30 µL, containing 20 ng of template DNA, 0.5 mM of each primer, 0.3 µM of dNTPs, buffer Phire Hot Start 1× (Thermo Scientific, Waltham, MA, USA), Phire Hot Start II DNA Polymerase (Thermo Scientific, Waltham, MA, USA) 0.4 µL. The amplified reaction was performed using an initial denaturation at 84 °C for 30 s, followed by 35 cycles of 98 °C for 15 s, 50 °C for 15 s, and 72 °C for 10 s, with a final extension phase of 1 min at 72 °C. The amplification product from the first PCR round was diluted 10-fold; 1 µL of the dilution was used as the template for the second round of PCR. The ITS1 region was specifically amplified using the ITS1f [40] and ITS2 primers [41]. A GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) [42] was added to the 5′ end of the ITS1f primer for denaturing gradient gel electrophoresis (DGGE) analysis. PCR and cycling conditions were as described above, except that the annealing temperature that was set to 55 °C. All amplification products were electrophoresed in 1.5% (*w*/*v*) agarose gels, stained with ethidium bromide, and visualized under UV light. Molecular analysis of arbuscular mycorrhizal fungi (AMF) was performed using NS1 and NS41 [43] primers as described in Yergeau et al. [44] and AM1 [45] and the NS31-GC primers [46] according to Santos et al. [47]. Denaturing gradient gel electrophoresis (DGGE) analyses were performed on a 20–50% denaturant gradient (100% is defined as 40% (*v*/*v*) deionized formamide and 7 M urea) using a INGENY phor-U2 system instrument (Ingeny, Leiden, NL [48]. The gels were 1.5 mm thick (20 × 20 cm) and contained 9% (*w*/*v*) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) plus 1× Tris-Acetate-EDTA buffer [38]. Approximately 500 ng of each nested PCR product was loaded onto the gels and electrophoresis was performed in 1× TAE at 60 °C for 17 h. Gels were

stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions, and the gel image was captured with a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA). Some dominant DGGE bands were excised with a sterile pipette tip and resuspended over-night in 20 µL DNA/RNA free water (GIBCO, Milano, Italy) at 4 °C. Then, 2 µL of the eluted DNA was used as the template and amplified under the same conditions described above.

2.8. Illumina MiSeq Sequencing and Data Analysis

The root and rhizosphere mycobiota of *B. aetnensis* was identified by high throughput sequencing of the ribosomal ITS region. The PCR was conducted with the ITS3 and ITS4 primers [41] (Table S1) using a 50 µL total volume with the following components: 25 µL PCR Master Mix (Roche, Indianapolis, IN, USA), 1.5 µL 10mM primers, and 2 µL DNA template. PCR consisted of 95 °C (5 min), followed by 36 cycles of 95 °C (30 s), 55 °C (30 s), and 72 °C (60 s) for each cycle extension, and 72 °C (10 min) for final extension. Amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform according to the standard protocols. Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.17). Operational units (OTUs) were clustered with 97% similarity cutoff, chimeric sequences were identified and removed using UPARSE-OTU algorithm. A taxonomic identity for each representative phylotype was performed using the fungal UNITE ITS database (https://unite.ut.ee/, accessed on 10 March 2021) [49]. To assign putative ecology to detected sequences, a literature review was conducted considering the highest match(es) obtained for each sequence.

3. Results

3.1. Plant Traits and Substrate Characteristics

The comparison of plant traits (Table 1) showed a large difference in the growth rate between NAT and NURS plants. Indeed, 8–12 years old NAT plants (mean height 44 cm, mean basal diameter 0.48 cm) were almost similarly developed in respect to one-year old nursery-grown plants (mean height 36 cm, basal diameter 0.58 cm), although being much older, confirming the strong influence of substrate and harsh environmental conditions for *B. aetnenis* growth. Soils from the natural habitat were not identical although sampled in the same area and they were much poorer than soils from the forest nursery (NURS) (Table S2). Soil organic carbon content was about 14 times higher in the NURS than in the NAT soil (2.45 vs. 0.17%), whilst phosphorus and nitrogen availability were more than 10 times higher and about 6 times higher in the NURS, respectively. In any case, soil data confirmed the low edaphic fertility of both substrates. Potassium in the NAT was lower than the detection limit of the method used. The C/N ratio was notably higher in the nursery (≈8) than in the natural habitat (≈3), while the pH was very similar, being 7.5–7.6 in all soil samples. Such large differences are due to the fact that the soils for the forest nursery came from lava flows older than those in the natural habitat, and were therefore more developed and fertile, as well as deeper. Conversely, the substrates in the natural habitat are only little evolved, being at the beginning of the primary succession.

3.2. Ectomycorrhizal Structures

At least 1000 root tips per individual were observed, ranging from 1021 to 5496 per individual, and approaching a total of almost 18,000 root tips (Table 1). Overall, about 330 cm of root length in NAT individuals and 1430 cm of root length in NURS plants were observed. *Betula aetnensis* roots exhibited a high level of colonization in all samples, with ectomycorrhizal tips (Figure 3) exceeding, on average, 74% of total observed tips in the natural soil, and 75% in the nursery soil. The ectomycorrhizal colonization was, on average, 74.7% in the natural soil and 77.8% in the nursery soil. Clear morphological and chromatic differences in the ectomycorrhizal root tips suggested the occurrence of different fungal species. Although there were notable qualitative differences in color, shape, and type of branching of the ectomycorrhizal roots, the most frequently observed root tips were

brown-black in color, dichotomous branching, and straight (Figure 3). A small number of roots had an ivory-bronze color and regular dichotomous branching. The microscopic observations showed that *Betula aetnensis* mycorrhizal root tips resembled those formed by typical ectomycorrhizal genera, also described in *Betula pendula* [50].

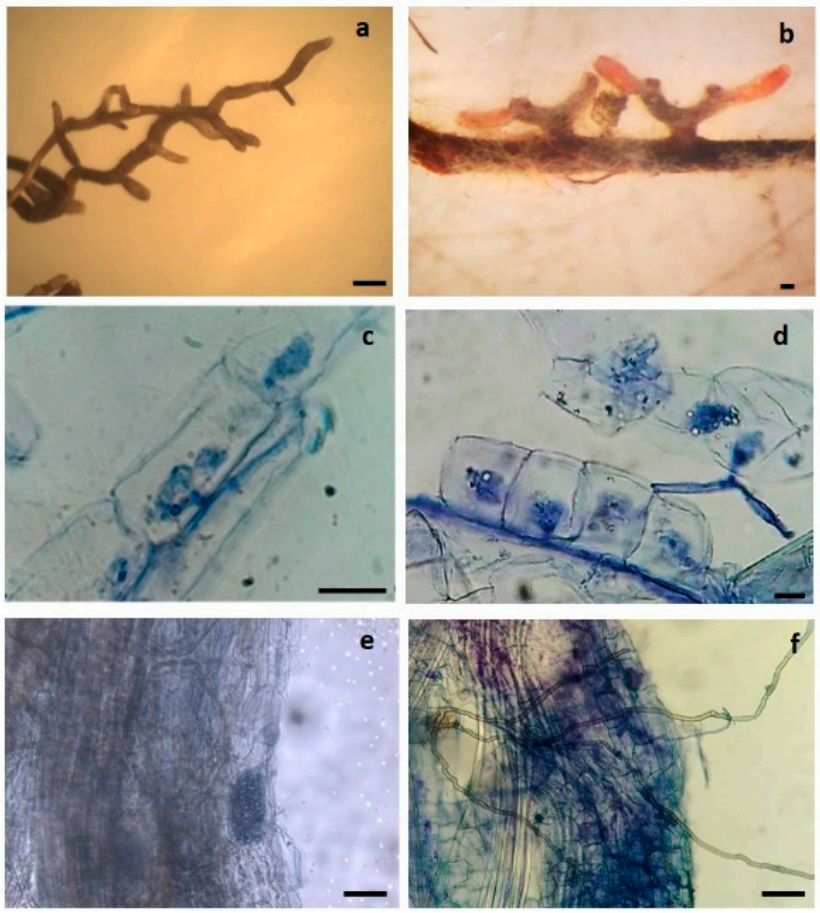

Figure 3. Microscope views of *Betula aetnensis* roots. (**a**,**b**) ectomycorrhizal root tips; (**c**,**d**) arbuscular mycorrhizal structures, with arbuscules in evidence; (**e**) structures of endophytic fungi, particular of an intracellular microsclerotium, (**f**) septate hyphae of endophytes. Bars = 200 µm (**a**,**b**); 20 µm (**c**–**f**).

3.3. Arbuscular Mycorrhizal and Endophytic Structures

Overall, 100 1-cm long root fragments per specimen were observed. The typical AM structures observed in *Betula aetnensis* roots (Figure 3) were arbuscules, that occurred in about half of the root samples (on average, in 50.5% of NAT roots and 46.8% of NURS roots, data not shown), and above all vesicles, which occurred, on average, in 80.0% of NAT roots and in 89.3% of NURS roots. Coils were also well represented, ranging from 77 to 85% in all root samples. According to Trouvelot et al. [24] estimations (Table 1), the frequency of root infection (F%) was high in all the individuals, ranging from 95% in natural habitat and 97% in the nursery but the abundance of arbuscules was similarly low both in NURS and NAT roots. Typical endophytic structures were also observed, including microsclerotia and septate hyphae (Figure 3). Septate hyphae occurred, on average, on 43% of NAT roots and on 52% of NURS roots (data not shown).

3.4. Bacterial and Fungal Diversity

The automated ribosomal intergenic spacer analysis (ARISA) was performed to analyze the structure of root microbial communities of the two *Betula aetnensis* individuals collected in the natural habitat (NAT1 and NAT2), and the three young individuals from the nursery and the NURS root sample (NURS1+2+3). We detected 25 distinct prokaryotic

OTUs and 10 fungal OTUs with range sizes from 51 to 424 bp (data not shown). Bacterial richness was higher than fungal richness in all the root systems, and a higher microbial diversity was observed in the NAT samples with respect to the NURS sample. NAT2, in particular, showed the highest root microbiota diversity (Figure 4).

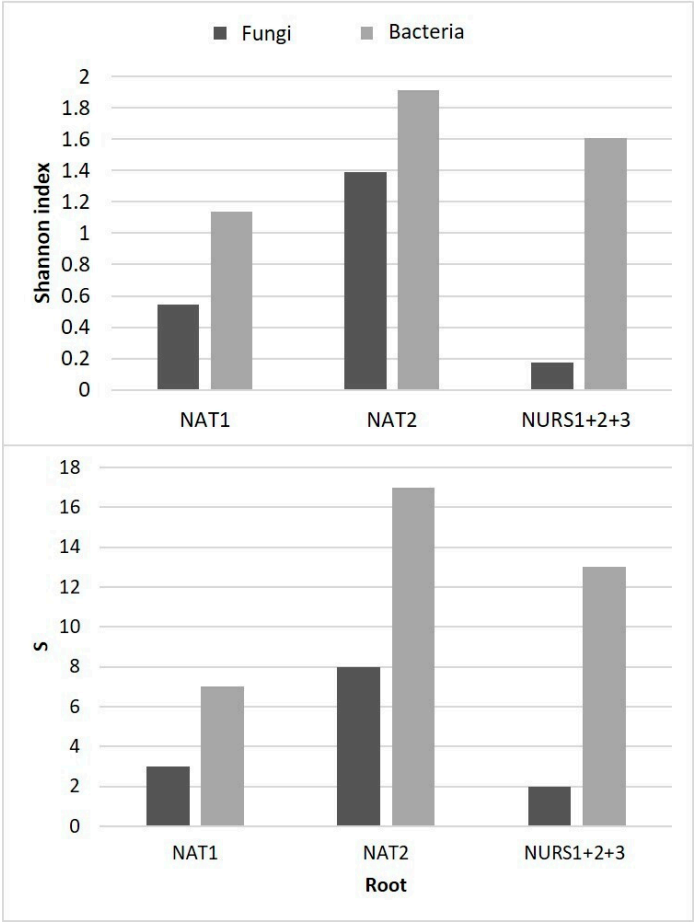

Figure 4. Diversity (Shannon index) and richness (S) of the bacterial and fungal communities estimated from automated ribosomal intergenic spacer analysis (ARISA) in *Betula aetnensis* roots from the natural habitat (NAT1, NAT2) and nursery-grown seedlings (NURS1+2+3).

3.5. Diversity of Fungal Root Endophytes

Diversity and composition of the *Betula aetnensis* root mycobiota was analyzed by ITS PCR–DGGE. About 6–8 discernible bands were observed for each sample using the primers targeting fungal ribosomal intergenic spacer ITS1-ITS2 with variable intensities revealing similar and restricted putative OTUs in all the samples. Cluster analysis based on the DGGE profiles indicated that the fungal communities from the natural habitat were grouped together and were separated from those of the forest nursery (data not shown). Sequences of 12 random bands DGGE gel of *B. aetnensis* from NAT and NURS roots were affiliated to Ascomycota (92%) and Basidiomycota (8%) division (Table 2). The unique sequence affiliated to Basidiomycota division, detected only in the natural habitat, belonged to the genus *Malassezia*. Within the dominant division Ascomycota, Helotiales was the most abundant order (82%), followed by Pezizales (genus *Tricharina*) (18%); both were detected in NAT and NURS roots. The most abundant sequences affiliated to the Helotiales belonged to the genus *Phialocephala*, known as a DSE, while the remaining sequences were related to uncultured Helotiales. Surprisingly, no PCR product was obtained using primers targeting AMF sequences.

Table 2. Phylogenetic affiliation of *Betula aetnensis* root endophytes detected by DGGE analysis and band sequencing. NAT: Natural habitat; NURS: Forest nursery. DSE: dark-septate endophytes; ERM: ericoid mycorrhizae; SAP: saprotrophs. Putative ecology was assigned by referring to the definition reported in the literature for the genus.

Sample	Band n°	Sequence Length (bp)	Division	Class	Order	Putative Ecology	Closest Sequence Match	Similarity (%)	Accession Number
NAT1	1	142	Ascomycota	Leotiomycetes	Helotiales	DSE, ERM, SAP	Uncultured *Phialocephala* clone otu19_mt2015	98%	AB354287.1
	3	259					Uncultured *Phialocephala*	99%	HF947843.1
	4	242					*Phialocephala helvetica* isolate RSF_Q104	95%	EU103612.1
	6	169		Pezizomycetes	Pezizales	DSE	Uncultured *Tricharina* isolate DGGE gel band ZA4	94%	KM200057.1
	5	240	Basidiomycota	Exobasidiomycetes	Malasseziales	DSE	Uncultured *Malassezia* clone TS1-9013 18S	95%	KC525787.1
NAT2	9	177	Ascomycota	Leotiomycetes	Helotiales	DSE, ERM, SAP	*Phialocephala fortinii* isolate FFP810 18S	97%	JQ711957.1
	10	239					Uncultured *Phialocephala* clone WD_S2_8_55a_1 1	95%	JX630399.1
	11	243					Uncultured *Helotiales* clone AhedenL36	98%	FJ475791.1
	13	148					*Phialocephala fortinii* isolate m14	98%	MH931279.1
NURS1+2+3	15	177	Ascomycota	Leotiomycetes	Helotiales	DSE, ERM, SAP	*Phialocephala fortinii* isolate m14	98%	MH931279.1
	16	258					*Phialocephala fortinii* isolate m14	97%	MH931279.1
	20	257		Pezizomycetes	Pezizales	DSE	Uncultured *Tricharina* isolate DGGE gel band ZA4	96%	KM200057.1

3.6. Identification of B. aetnensis Root Mycobiota

The root and rhizosphere mycobiota of *B. aetnensis* grown in the natural habitat were analyzed to a finer level by Mi-seq Illumina sequencing of the fungal ITS region. After chimera removal, a total of 80,372 and 102,820 reads were obtained from NAT1 and NAT2 roots (NAT1R and NAT2R), that were grouped into 43 and 121 OTUs, respectively. From rhizosphere soil samples NAT1RS and NAT2RS, 82,505 and 71,990 reads were obtained and were grouped in 89 and 101 OTUs, respectively. Diversity indices calculated at genus level (Table 3) were higher in NAT2 than in NAT1 both for roots and rhizosphere soil confirming the trends obtained from ARISA (Figure 4). *B. aetnensis* roots and rhizosphere were both dominated by Ascomycota and Basidiomycota. Ascomycota were more abundant than Basidiomycota both in the roots (67 vs. 29–32%) and in the rhizosphere soil (48–90 vs. 8–48%) (Figure 5). Other less abundant sequences (<5%) were assigned to the phyla Chytridiomycota, Rozellomycota, and Mucoromycota. Ascomycota were mainly represented by the order Helotiales in all samples, with abundances higher than 50% (Figure 3), followed by Eurotiales and Pleosporales, which were more abundant in the rhizosphere soil than in roots. Saccharomycetales, Capnodiales, and Dothideales were exclusively present in roots, while Venturiales, Sordariales, Hypocreales, and Chaetothyriales were exclusively detected in the rhizosphere soil. Basidiomycota were represented by Malasseziales, Agaricales, and Boletales in the roots and mainly by Boletales in the rhizosphere soil. At genus level, the root mycobiota was clearly distinct from the rhizosphere mycobiota (Figure 6), and differences between the two NAT plants were also found. In the roots, only *Phialocephala* (Dark Septate Endophytes, DSE) reached the same high abundance in both NAT1 and NAT2. *Malassezia* (DSE) and *Oidiodendron* (ERM, Ericaceous mycorrhizal fungi) genera were more abundant in NAT1 whilst *Gyoerffyella* (ERM) was more abundant in NAT2. The most

remarkable difference was in the contribution of ECM fungi, with six genera detected only in NAT2, and including *Tylospora*, *Leccinum* and *Cladosporium* (Figure 6). The sequences of *Oidiodendron* were identified at the species level and were affiliated to *O. maius*, while *Meliniomyces* sequences were affiliated to *M. bicolor*. In contrast, the rhizosphere-associated fungal community of *B. aetnensis* was characterized by high abundance of *Rhizopogon*, *Helicodendron*, *Cladophialophora*, and *Penicillium*.

Table 3. Diversity indices of fungal communities at genus level estimated by Illumina sequencing in the roots (NAT1R and NAT2R) and rhizosphere soil (NAT1RS and NAT2RS) of *Betula aetnensis* individuals from the natural habitat.

Index	Sample			
	NAT1R	NAT2R	NAT1RS	NAT2RS
Taxa_S	7	15	9	12
Simpson_1-D	0.686	0.8706	0.6042	0.8407
Shannon_H	1.295	2.342	1.269	2.043
Evenness_eˆH/S	0.5217	0.6933	0.3953	0.6428

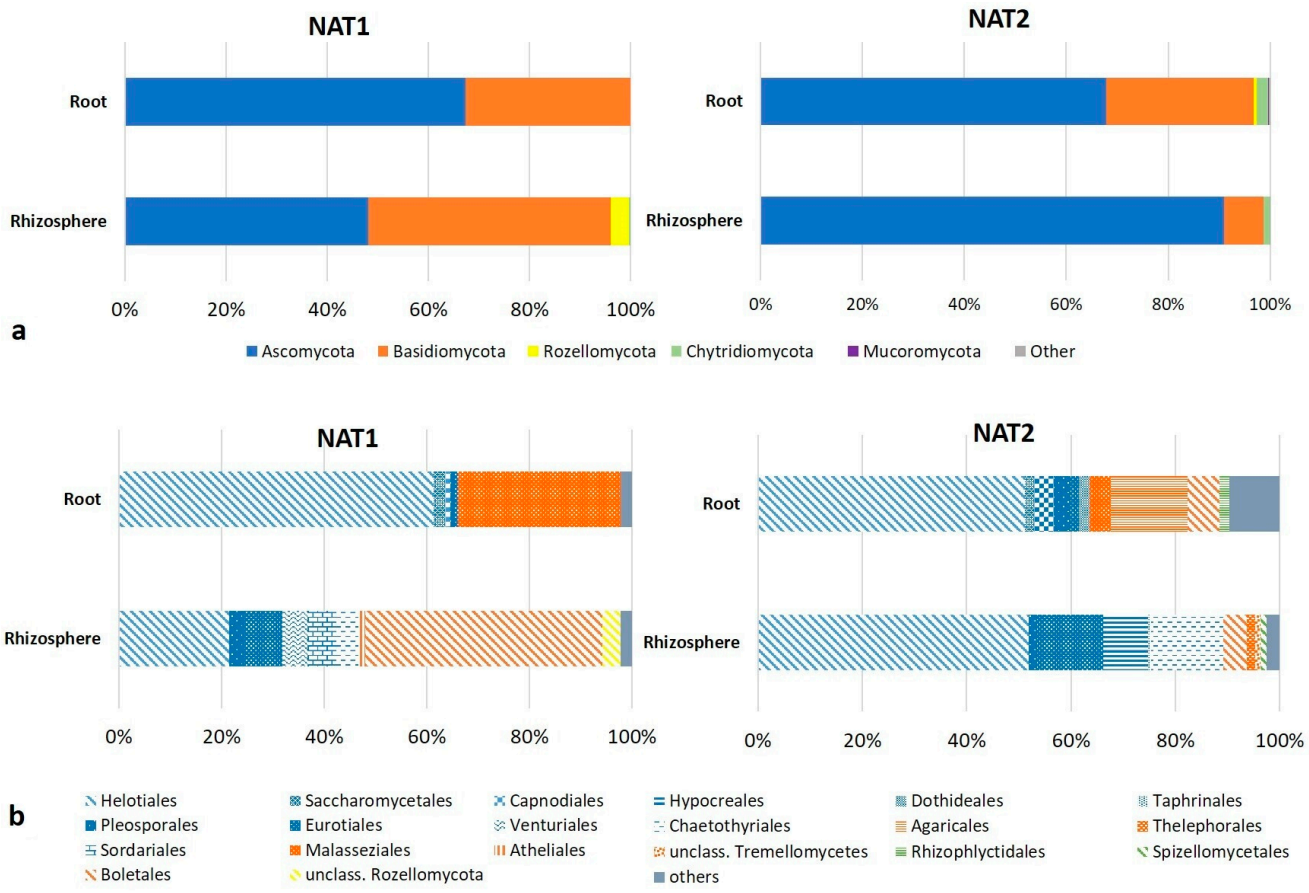

Figure 5. Relative abundance of fungal (**a**) phyla and (**b**) orders detected by Illumina sequencing in the roots and rhizosphere soil of *Betula aetnensis* individuals (NAT1 and NAT2) from the natural habitat. Only the taxa >0.5% are shown.

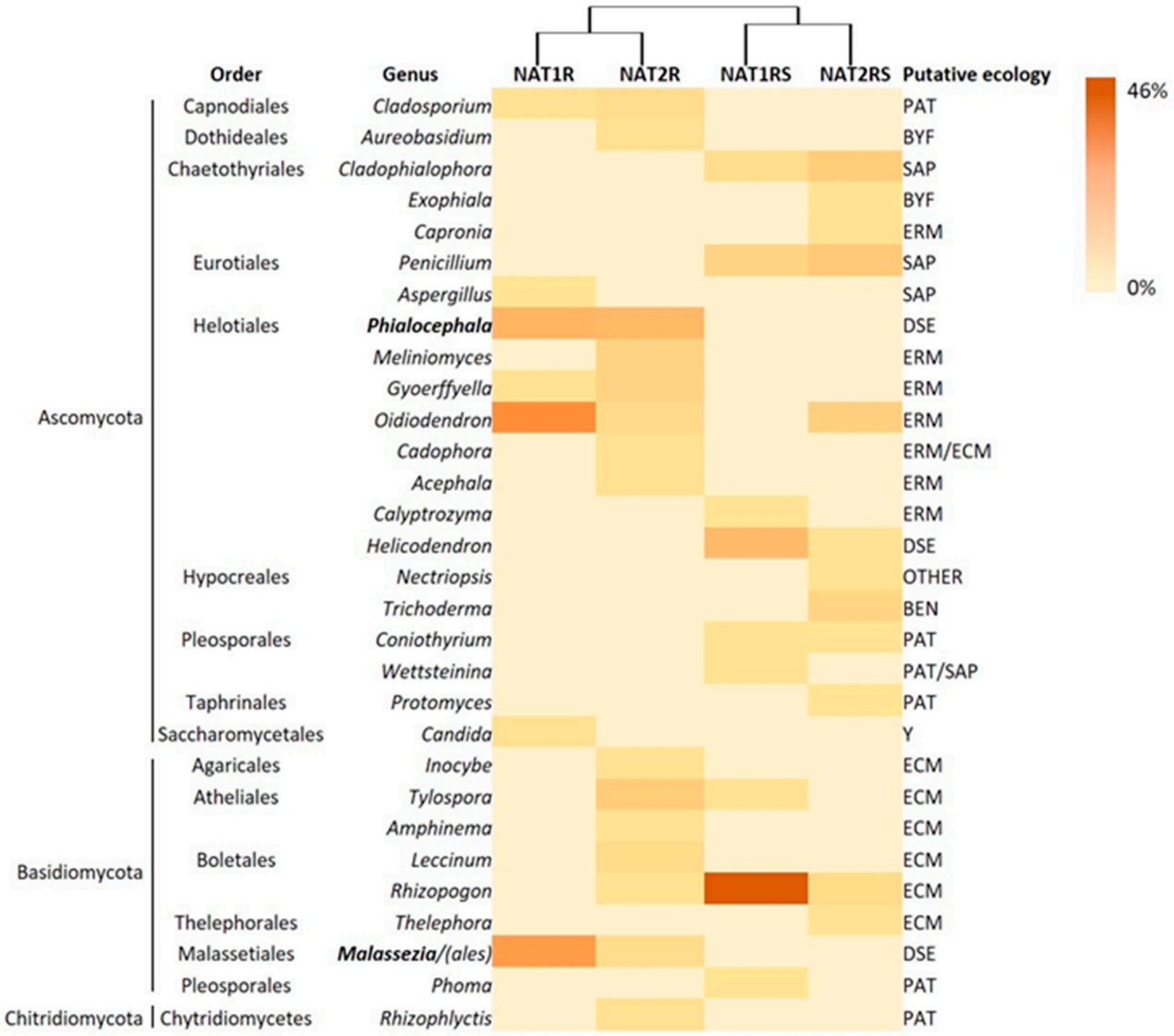

Figure 6. Heatmap showing the most abundant fungal genera detected in roots and rhizosphere soil of *Betula aetnensis* individuals (NAT1 and NAT2) from the natural habitat. The colors correspond to the relative abundance of each genus in the sample (indicated by the color legend). The genera also detected by DGGE analysis are in bold. The four assemblages were clustered according to Bray-Curtis. NAT1R, NAT2R: root samples; NAT1RS, NAT2RS: rhizosphere soil samples; PAT: pathogens; BYF: black yeast fungi; SAP: saprotrophs; ERM: ericoid mycorrhizal fungi; DSE: dark-septate endophytes; ECM: ectomycorrhizal fungi; BEN: beneficial fungi; Y: yeasts. Putative ecology was assigned by referring to the definition reported in the literature for the genus.

4. Discussion

Microscope observations, fingerprinting methods, and high-throughput Illumina sequencing analyses provided for the first time a detailed overview of the root microbiota of *Betula aetnensis*, an endemic tree species of high conservation value, native to Sicily and thriving on volcanic lavas of Mt. Etna [2]. We assessed the root-inhabiting bacterial and fungal community of plants grown in the natural habitat and the surrounding rhizosphere soil, as well as of nursery-grown seedlings. Due to the particularly threatened condition of the species, only two adult plants growing in the natural habitat were collected and studied. We recognize this is a limitation of the research, but at the same time, we deem it to provide interesting and novel insights into the belowground microbiota of *B. aetnensis*, which could serve to implement successful conservation strategies of this species. Root-associated microbial communities were highly diverse, showing the colonization by a rich

community of endophytic bacteria and a diversified array of soil fungi, encompassing mycorrhizal fungi (AM, ECM, and ERM) endophytes with an interesting or poorly known ecological role, such as dark septate endophytes (DSE) and others producing secondary metabolites with potential beneficial effects for plants. Fingerprinting analysis ARISA showed a higher diversity and richness of the prokaryotic and eukaryotic community in the roots of plants from the NAT habitat compared to the NURS. These rich microbial communities detected in natural habitat may be crucial for plant survival in the nutrient-limited volcanic substrates of Mt. Etna (C = 0.17%, N = 0.05%, P = 0.0004%). Indeed, soil organic carbon in NAT soils was in line with that of volcanic soils at Mt. Etna at an early developmental stage [51]. Conversely, SOC of NURS soils was within the range found in more developed soils of Mt. Etna, as well as in volcanic islands in the Mediterranean basin [27,52,53]. Nitrogen content in our study sites (0.03–0.31%) fell within the range found in literature (0.01–0.74%) [27,52,53], confirming the low nutrient content of primary succession sites. P and K content were much lower than those found in deeper and more fertile volcanic soils used for agricultural crops [54].

To identify the fungal communities inhabiting *B. aetnensis* roots, having a key role in survival in the nutrient-limited natural habitat, we performed microscopic observations and molecular analyses. Members of Betulaceae are predominantly ectomycorrhizal [11,15,32] and our observations followed this general trend. The typical ECM symbiotic structures were observed (e.g., mycorrhizal root tips), with high abundance, both in the NAT and in the NURS habitat. Interestingly, we showed for the first time that *B. aetnensis* also harbors AM fungi, as arbuscules, non-septate hyphae, and vesicles were clearly identified by microscopy. The co-occurrence of ECM and AM fungi in the same root system has been rarely observed in tree species [55]. These fungal symbionts are suspected to act at different stages of plant life [56,57], with AM being more frequently observed in the early stages, while being progressively replaced by ECM, as found in *Eucalyptus dumosa* A. Cunn. ex J. Oxley [58]. However, we did not find evidence of a similar behavior in *B. aetnensis*. Indeed, ECM and AMF structures were detected both in 1 year-old NURS seedlings and in 8–12 years old NAT plants. Hence, we could hypothesize that under very stressful environmental conditions, such as the nutrient-limited lavas of our study site, AMF could persist over time, while maintaining a low occurrence of arbuscules, the functional structures for nutrient exchange. Moreover, the marked difference in growth rate between natural and nursery-grown plants may help explain this pattern. In the nursery, seedlings growing under better edaphic conditions showed a growth rate about ten-fold faster than in the natural habitat. Conversely, the very slow growth rate in the volcanic soils of Mt. Etna probably determines a higher need for complex groups of root microbes, including both ECM and AMF. We could hypothesize a synergic effect of the co-occurence of ECM and AM fungi in *B. aetnensis*, with AM fungi involved in making available soil phosphorus, and ECM fungi in improving the uptake of mineral nutrients and water, together increasing plant survival and growth in nutrient-poor environments [58,59].

Denaturing gradient gel electrophoresis of the ITS-PCR amplicons was performed to elucidate fungal communities diversity associated with *B. aetnensis* from the NAT and NURS habitat. Random DGGE bands were selected, excised, and sequenced to identified phylotypes. All sequences displaying reasonable similarity (94% or higher) to fungal sequences in GenBank were considered, while sequences with less than 94% similarity were discarded as possible band artifacts which could be due to multiple sequences associated with a single band position [60]. The sequence analysis mainly revealed the presence of Ascomycetes (*Phialocephala* and *Tricharina*) and Basidiomycetes (*Malassezia*) [61]. Among them, *Phialocephala fortinii* is known as a root endophyte capable of establishing ECM associations [62].

Surprisingly, we failed in the detection of typical ECM and AM fungi by PCR using ECM primers and two different couples of Glomalean specific primers that were successfully used in other plant species [45,63]. The absence of sequences affiliated to typical ECM and AM fungi could be due to biases that ITS primers may introduce during PCR

amplification, as primer mismatches might favor some taxonomic groups in respect to others [64]. Moreover, the lack of detection of AMF could be due to the low levels of AMF hyphae in *Betula* roots, as hypothesized for *Betula pendula* [23].

Due to the incongruences between DGGE sequencing and microscopic observations, which showed the presence of ECM and AM structures, high throughput sequencing approach and taxonomic identification (UNITE database) were performed, which provided a much more accurate picture of root and rhizosphere soil mycobiota of *B. aetnensis* in its natural habitat. A large difference in the community structure and composition of root-inhabiting fungi between the two NAT plants was observed. The root mycobiota was much richer in NAT2 than in NAT1 (8 and 12 years old, respectively), with only four genera in common, but differing in abundance levels. Hence, we may hypothesize that a progressive shift in community assemblage occurred, with the colonization by ECM fungi mostly focused in later stages of plant growth, while AMF and DSE are soon needed in the early life stages [57]. However, due to the very limited samples number in our research, further surveys, possibly non-destructive, are needed to elucidate this aspect, once we shed light on the overall diversity of the belowground microbial communities of *B. aetnensis*.

The most abundant ECM basidiomycete retrieved in *B. aetnensis* roots was *Tylospora*, followed by *Leccinum, Anphinema, Inocybe*, and *Rhizopogon*. All these genera were detected only in the root system of NAT2, except *Rhizopogon*, which was also abundant in the rhizosphere soil. Much information is available about the mycorrhizal status, and the promoting effect of fungal symbionts, for the taxonomically closest birch to *B. aetnensis*, i.e., *Betula pendula* [23], due to its widespread occurrence throughout temperate and boreal Europe [3,65,66]. ECM were found to enhance seedling growth rates, due to the improved uptake of mineral nutrients and water, especially in low fertility substrates [58]. Therefore, we may suggest a similar favorable effect could exist for *B. aetnensis*.

Beyond ECM, Illumina sequencing revealed the abundant presence of endomycorrhizal fungi classified as ericoid mycorrhizal fungi (ERM), affiliated to Helotiales order. The dual role as endophytic and mycorrhizal fungi is one of the most intriguing aspects of ERM [67]. The most abundant species was *Oidiodendron maius* in the phylum Ascomycota, which is known both as a root symbiont of Ericaceae (e.g., on *Vaccinium myrtillus* L. and *Calluna vulgaris* (L.) Hull), and as an endophyte of non-ericaceous woody plants such as *Betula*, *Picea* and *Abies* in boreal forests [68]. Interestingly, *Oidiodendron maius* enhanced the plant growth of *Betula pendula* [69] and was found to inhibit in vitro *Heterobasidium annosum* [70], a well-known root pathogen of *B. aetnensis* [10]. Other Helotiales ERM fungi detected in *B. aetnensis* were *Meliniomyces*, *Gyoerffyella*, and *Acephala*. *Meliniomyces* was found to significantly improve the growth rate of *Betula pendula* plants, by transferring carbon and nitrogen [71]. The ericoid fungus *Gyoerffyella* spp. is also known as a DSE present in *Picea abies* (L.) H. Karst. roots, where it improves nutrient mobilization from soil organic matter to the plant [72].

Among non-ERM Helotiales, the genus *Phialocephala* was the most relevant in *B. aetnensis* roots, being first detected by DGGE band sequencing and then confirmed as highly abundant through Illumina sequencing. *Phialocephala* spp. are known as DSE and are suggested to behave similarly to mycorrhizae, utilizing plant photosynthates and providing mutual advantages under certain conditions, while being neutral or negative under others [62]; yet only *Acephala applanata* Grünig and T.N. Sieber established typical ectomycorrhizal structures [73]. Stressful conditions and ecosystems with high ecological constraints could trigger stronger and more positive interactions between DSE and host plants [74,75]. Since the natural habitat of *B. aetnensis* is characterized by strong abiotic stresses, being severely water- and nutrient-limited, and endophytic fungi were abundant in its roots, the ecological role of DSE for this species could be high, for instance, acting to potentially make available mineral nutrients for plants [76].

Some peculiar associations between DSE and ECM in soils seem to exist as specific pairs DSE-ECM were found in clustered distribution [77]. In this respect, the members of the Helotiales are considered of major interest, including both OTUs which are exclusive

either of AMF or ECM symbionts, as well as OTUs with a generalist behavior, indifferently associated to both symbioses [78]. Such evidence proves that the co-occurrence of root-inhabiting fungi may give rise to different and unpredictable responses in host plants with respect to single colonization patterns. Hence, ECM, AMF, and DSE could act in synergy for improving the nutritional status of *B. aetnensis* in its stressful habitat.

Interesting insights were retrieved from the comparison between root-inhabiting mycobiota of *B. aetnensis* individuals growing in NAT habitat and the surrounding rhizosphere soil. The rhizosphere fungal community was distantly related to the root mycobiota, showing a lower diversity of ECM, ERM, and DSE, and a higher incidence of taxa with pathogenic activity. The rhizosphere was mainly dominated by extremophilic fungi (i.e., black fungi known for their ability to cope with stressful environmental conditions [79], P-solubilizing saprobes, biotrophic fungal pathogens, saprotrophs, and fungi producing secondary metabolites with beneficial effects for plants such as *Trichoderma, Penicillum*, *Aspergillus*, and *Cladosporium* [80].

B. aetnensis individuals from the nursery showed the same colonization levels by ECM and AM fungi as NAT plants but molecular fingerprinting analyses showed a much lower fungal diversity in the NURS roots, suggesting that the adaptation of NURS plants to natural conditions could be hampered by a poor mycobiota. Conversely, the prokaryotic diversity was similar between NAT and NURS plants. From a practical point of view, our results suggest that the use in forest nurseries of an apparently less evolved substrate may, in fact, have potentially higher beneficial effects for planted individuals, harboring richer and more complete soil microbial communities.

5. Conclusions

The knowledge of the co-occurrence of different root-inhabiting fungi (symbionts and endophytes) in host plants is limited up to now. In this study, we reported for the first time, through microscopic observations and molecular analyses, that root-associated microbial communities of *Betula aetnensis* encompass both typical symbiotic fungi and other fungi with an undefined or little known ecological role, including DSE and an oligospecific community of endophytic prokaryotes, occurring both in the natural habitat and the forest nursery regardless of the differences in soil organic carbon and nutrient content. This suggests that, in the harsh and nutrient-limited volcanic substrates of Mt. Etna, *B. aetnensis* plants are able to find enough propagules to establish such complex multipartite interactions, which are expected to be crucial for plant establishment, growth, and survival. It is extremely difficult to forecast the overall effects of different co-occurring fungal groups, with recognized (ECM, AMF, and ERM) and still under-investigated functions (DSE), while interacting and competing with each other in the same root system. Hence, future research is needed to understand the relative role of different root-inhabiting fungi for the conservation of this endemic tree species, including the assessment of its mycorrhizal dependence. In addition, the bacterial diversity of *B. aetnensis* deserves to be further explored. Soil microbial communities are involved in the strategies to acquire water and nutrients in limited conditions, as well as in overcoming abiotic and biotic stress, conditions which are frequently faced by *B. aetnensis* in its natural habitat. Such wealth of knowledge could be used to start targeted nursery programs aimed to produce plantlets bearing the necessary beneficial soil microorganisms and excluding species with suspected or demonstrated pathogenicity, thus improving the conservation status of this endemic and localized tree taxon. This work contributes to the knowledge of root-inhabiting microbiota of *B. aetnensis*, showing that complex belowground webs are involved encompassing not only mycorrhizal fungi but also diverse non-mycorrhizal fungal endophytes, as well as soil bacteria. This research also contributes to the study of the ecology of primary succession. Therefore, a full understanding of the functioning of symbiosis in trees would never be complete without taking into account the entire association networks involving the whole microbial community.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12121624/s1, Table S1: Primers used in this work for amplification of bacterial or fungal ribosomal DNA; Table S2: Soil characteristics of the study areas. NAT = Natural; NURS = Nursery.

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Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article and/or its Supplementary Materials.

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