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Diversity of Endophytic Fungi and Bacteria Inhabiting the Roots of the Woodland Grass, *Festuca gigantea* (Poaceae)

Izolda Pašakinskienė [1,2,*], Violeta Stakelienė [1,2], Saulė Matijošiūtė [1,2] and Justas Martūnas [1]

[1] Botanical Garden of Vilnius University, Kairėnų 43, 10239 Vilnius, Lithuania
[2] Life Sciences Centre, Vilnius University, Saulėtekio 7, 10221 Vilnius, Lithuania
* Correspondence: izolda.pasakinskiene@gf.vu.lt; Tel.: +370-68642502

Abstract: We studied the occurrence of endophytic fungi and bacteria in the roots of *F. gigantea*, a woodland perennial grass common throughout Europe and temperate Asia. The taxonomic assignment was carried out according to the isolate's colony and cytological species morphotype characteristics and confirmed by the assessment of the standard DNA sequences, ITS, *RPB2*, *SSU*, and *TEF1-a* for fungi and 16S rDNA for bacteria. Our study has shown that *F. gigantea* roots are the habitat to a wide range of fungi and bacteria. The occurrence of fungal structures was determined in ~40% of the roots examined by Trypan Blue staining. In a surface-sterile root-cutting culture on PDA medium, we obtained isolates of six endophytic fungi species: four members of Ascomycota—*Alternaria alternata*, *Cadophora fastigiata*, *Chaetomium funicola*, and *Microdochium bolleyi*—and two of Basidiomycota—*Coprinellus* sp. and *Sistotrema brinkmannii*. In addition, we report bacteria co-occurring endophytically in the roots of this grass. The Firmicutes group was the most prevalent, consisting of four Gram-positive, endospore-forming bacteria taxa. The isolates were identified as *Bacillus pumilus*, *Bacillus* sp., *Lysinibacillus* sp., and *Priestia aryabhattai*. Moreover, two Gram-negative bacteria were detected—*Kosakonia* sp. (Proteobacteria) and *Pedobacter* sp. (Bacteroidetes). Thus, applying the isolate-culture approach, we identified a set of microorganisms in the roots of a typical grass native to the deciduous forest floor. The functional roles of these endophytes are diverse, and many of them, saprotrophs and decomposers of wood and plant debris, are linked to the decomposition of organic matter. This is the first detailed report on fungal and bacterial endophytes inhabiting the roots of *F. gigantea*. This study fills in a research gap on endophytes associated with the below-ground parts of *Festuca* spp., hitherto extensively studied for *Epichloë*/*Neotyphodium* associations in their foliar parts.

Keywords: root endophytes; Poaceae grasses; Ascomycota; Basidiomycota; Firmicutes

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1. Introduction

Endophytic fungi and bacteria are associated with almost all plants across diverse ecosystems [1–3]. Although plant endophytes are diverse, most come from the phylum Ascomycota and, to a lesser extent, from the phylum Basidiomycota. They thrive in temperate and tropical habitats up to polar sites. Endophytic fungi are common in meadows, the Alps, deserts, and forests of the middle zones [4–8]. Endophytic fungi are usually present in the plant tissues of leaves and roots without causing obvious symptoms. Many well-known endophytes of the anamorphic Ascomycota are characterized by melanized septate hyphae. Due to this feature, these fungi are assigned to a special group of dark septate endophytes, DSEs [9–11]. Endophytic fungi produce bioactive compounds that help plants resist biotic and abiotic stress, are antagonists to host pests, and are beneficial for host growth and development [11,12].

Many studies show that the roots of most grasses are home to endophytic fungi [7,13–15]. However, it should be noted that endophytes in the roots of *Festuca* spp. and closely related *Lolium* spp. have been greatly underestimated compared to a very broad literature describing

Epichloë/*Neotyphodium* (Clavicipitaceae) associations in the foliar parts and seeds of these plants [16–24].

Festuca gigantea, a member of the Schedonorus subgenus, is a woodland perennial grass common throughout Europe and temperate Asia. Unlike the closely related *F. arundinacea* and *F. pratensis*, this species is not used in agricultural pastures. It prefers shady places, damp wet habitats along ditches and riverbanks and is a common grass in forests and parks. *Festuca gigantea* is adapted to deal with light deficiency in a specific ecological niche rich in decomposing leaf litter and other plant debris shaded by the tree canopy. According to Ellenberg-type indicator value ranking, the light requirement optima for *F. gigantea* is half that for other members of the Schedonorus group, *F. arundinacea* and *F. pratensis* [25]. Like other *Festuca*, *F. gigantea* plants are associated with *Epichloë*/*Neotyphodium* fungal endophytes [19,26–28]. As mentioned above, this fungus systemically colonizes the leaves and stems of host plants but not the roots. Meanwhile, the data on root endophytes in *F. gigantea* are limited to a brief note where the presence of the fungal hyphae was recorded microscopically, but no species description was specified [29]. Thus, by investigating the occurrence of fungi and bacteria in the roots of *F. gigantea*, our study attempts to fill in the gap of root endophyte studies and is the first observation of this kind in the broad-leaved *Festuca* group.

There are a limited number of studies of fungal and bacterial endophytes using the same experiment, whether focused on a particular plant or a particular ecosystem [3,15,30,31]. One of the best examples is Toju and co-authors' study [3] of fungal and bacterial communities in the leaves and roots across more than 100 plant species sampled in the grassland. Their observations reveal that the below-ground microbiome has different features and dynamics compared to the above-ground microbiome. Therefore, research revealing the complexity of the assemblage of endophytic fungi and bacteria in the hidden parts of plants is greatly valuable for the understanding of plant life.

A diverse group of bacteria, including strains of *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Serratia*, *Kosakonia*, and *Xanthomonas*, has been shown to promote plant growth and has been assigned as plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting bacteria (PGPB) [32,33]. In grasses, the inoculation of bermudagrass with *Bacillus* spp. strains has demonstrated beneficial effects, showing nitrogenase activity, phosphate solubilization, and siderophore production [34]. In addition, the removal of bacteria from the seeds by sterilization demonstrated significant detrimental effects on root hair development in *F. arundinacea* and *Lolium perenne* plants [35]. To date, reports on bacterial endophytes in grasses related to *F. gigantea* are limited to a set of strains isolated from surface-sterilized seeds of *F. arundinacea*, where *Bacillus*, *Pantoea*, and *Pseudomonas* bacteria were detected [36]. However, bacteria inhabiting the roots of *F. gigantea* and other related *Festuca* have not yet been documented. Thus, one of the objectives of this study was to isolate culturable bacterial strains from the roots of *F. gigantea* and determine their taxonomy.

Firstly, in the course of the microscopic examination of *F. gigantea* root apical sections, we detected fungal and bacterial morphostructures. Following this discovery, we aimed to determine the species of fungi and bacteria colonizing the roots of *F. gigantea*. A taxonomic assignment was carried out according to the typical isolate's colony and cytomorphotype characteristics of the species, and it was confirmed by the assessment of the standard DNA sequences. Based on this investigation, we describe a set of endophytic fungi and bacteria colonizing the roots of *F. gigantea*. This is the first detailed report on the endophytes inhabiting the roots of this woodland grass.

2. Materials and Methods

2.1. Root Sampling and Sterilization

For isolation of the fungi and bacteria, tiller samples of *F. gigantea* from healthy plants were collected on the edges of deciduous forest sites in the Botanical Garden of Vilnius University, Kairėnai (Lithuania, Vilnius; N 54.7362067, E 25.4034823) and Vingis Park (Lithuania, Vilnius, N 54.682574, E 25.233736) during the vegetation season in May–June.

The tillers were washed under running tap water, and the roots were removed. Tillers without roots were placed in test tubes filled with tap water. New roots, 1–2 cm long, were collected and sterilized accordingly: 50% ethanol for 90 s, 1.25% sodium hypochlorite for 90 s, and, finally, the samples were washed 3 times for 3 min with sterile water. In addition, 200 µL of final wash water was added to three Petri dishes with PDA or LB medium during the fungal and bacterial culture step, which were used as a negative control to confirm that root sterilization was adequate.

2.2. Microscopy and Estimation of the Abundance of Endophytic Fungi in F. gigantea Roots

For microscopy, newly grown roots are described in Section 2.1, (excluding the sterilization step) were collected and placed in 1.5 mL Eppendorf test tubes with a fixative of ethanol–glacial acetic acid (3:1) and kept in the refrigerator (at 2–3 °C) until use.

Prior to microscopy, all the roots were softened using enzyme treatment as follows: the sampled roots were washed twice with citrate buffer (0.1 M, pH 4.8) at 27–28 °C for 10 min. and treated with 0.5% Macerozyme R-10 at 37 °C for 25 min.

The root cross-sections were prepared without specimen staining and examined for fungal and bacterial morphostructures under a Nikon ECLICE Ci-L phase-contrast microscope. In total, ~100 roots from 25 plants were analyzed.

To determine the frequency of endophytic fungi in *F. gigantea*, tillers from the plants collected from Kairėnai and Vingis locations were used. In each location, three sampling replicates were made during the season, with two tillers from 5 plants and 2 roots from each tiller collected, making 60 root samples in total for each location. After the enzyme treatment, the roots were washed with citric citrate buffer and stained with 0.025% Trypan Blue, following the protocol described by Kiheri et al. [37] with some modifications: root staining at 90 °C for 30 min, then bleaching with a lactic acid–glycerol (1:1) mixture, repeating it three times. The first two bleaches were at 37 °C for 30 min, and the third was left at room temperature for 24 h before microscopic analysis. A lactic acid–glycerol mixture (4:1) was used to prepare the root tip sections on the microscopy slides. The presence of fungal structures was assessed using a phase contrast microscope in 10 fields of view for each root at ×400 magnification.

2.3. Isolation of Fungi

To make Potato Dextrose Agar (PDA) medium, 200 g of peeled, sliced potatoes (Lithuanian var. Rasa) were boiled in 1 L of distilled water for 30 min. The potato mass was filtered through cheesecloth, saving effluent. The potato infusion was poured into flasks 200 mL at a time. A total of 4 g of dextrose and 4 g of agar were added to each flask. Mixtures were autoclaved at 121 °C for 20 min. The medium was enriched with ampicillin sodium salt (final concentration—100 µg/mL) and streptomycin sulfate (final concentration—100 µg/mL) to selectively inhibit bacterial growth.

The experiment was repeated in 8 replicates, with 4 replicates from Kairėnai and 4 from Vingis locations. In total, 200 root fragments were evaluated for the cultivation of endophytes. Five cuttings of sterile roots were placed in each Petri dish with a PDA medium. Before isolation, the roots were squashed with a sterile needle to facilitate the proliferation of endophytic fungi. The root cuttings in Petri dishes with PDA were incubated in the dark at 27 °C. After 7–14 days, we observed the growth of fungal colonies in proximity to the root segments. The obtained fungal isolates are deposited in the collection of the Laboratory of the Botanical Garden of Vilnius University.

2.4. Isolation of Bacteria

A total of 25 g of LB powder (Fisher BioReagents, Waltham, MA, USA) were dissolved in 1 L of purified water, heated and agitated until completely dissolved, and sterilized by autoclaving at 121 °C for 15 min. Sterile root cuttings were prepared as described in Section 2.1. Root cuttings in Petri dishes with LB medium were incubated at 37 °C in the dark. After 1–2 days, we observed bacterial colonies in proximity to the root segments. The

experiment was repeated twice; in total, 50 root fragments were evaluated. The obtained bacterial isolates are deposited in the collection of the Laboratory of the Botanical Garden of Vilnius University.

2.5. DNA Extraction from Fungi and Bacteria Culture Colonies

The fungi and bacteria's genomic DNA was isolated using the Quick-DNA™ HMW MagBead Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's guidelines.

For the extraction of the genomic DNA of the fungi, 10-day-old fungal colonies were used, sampling 100 mg of mycelial biomass. For bacteria, 1–2-day-old colonies were used. Each bacterial colony was grown in a liquid LB medium, and the next day, genomic DNA was isolated.

2.6. Standard DNA Amplification and Sequencing

For standard DNA amplification, the primer pairs used in the PCR reactions are listed in Tables 1 and 2 for fungi and bacteria. The total volume of the PCR mix for amplification was 50 µL. PCR was conducted under the temperature profile of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 49–61 °C [calculated according to the primer's Ta = Tm − (0–4 °C)] for 30 s and 72 °C for 1 min, and the final extension at 72 °C for 5 min.

Table 1. The list of primers in PCR reactions for amplification of fungi DNA sequences.

Locus	Primers	Primer Sequences (5′–3′)	Tm °C	Reference
ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	54	[38]
TEFa	EF1-728F EF-2	CATCGAGAAGTTCGAGAAGG GGARGTACCAGTSATCATGTT	54	[39]
SSU	NS1 NS4	GTAGTCATATGCTTGTCTC CTTCCGTCAATTCCTTTAAG	49	[38]
RPB2	RPB2-5F2 fRPB2-7cR	GGGGWGAYCAGAAGAAGGC CCCATRGCTTGYTTRCCCAT	58	[40]

Table 2. The list of primers in PCR reactions for amplification of standard bacteria 16S rDNA sequences.

Locus	Primers	Primer Sequences (5′–3′)	Tm °C	Reference
16S rDNA	27f CM 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	52	[41]
16S rDNA	704F 765R	GTAGCGGTGAAATGCGTAGA CTGTTTGCTCCCCACGCTTTC	56	[41]
16S rDNA	S-D-Bact-0341-b-S-17 S-D-Bact-0785-a-A-21	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC	56	[42]

The PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The sequencing was performed by BaseClear B.V., Leiden, Netherlands. The fungal and bacterial colonies representing sequences were analyzed against NCBI reference data using the BLAST tool https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 17 July 2024).

2.7. Morphological Characterization of Endophytic Fungi

The fungal isolates were characterized by their morphological characteristics, including structure, color, and colony edge. A mixture of glycerol–lactic acid (1:4) was used for analyzing and photographing the fungal mycelium specimens. For viewing, a microscopic Nikon ECLICE Ci-L phase-contrast microscope was used.

2.8. Photography

The images of the colonies were taken by a Sony Alpha a6300 camera (Sony Corporation, Tokyo, Japan) with a Sigma 56 mm f/1.4 lens. Root section cuttings and mycelium samples were analyzed under the phase-contrast microscope, and the NIS-Elements D software (version 6.02.01) program was used for microscopic photography and analysis.

3. Results

3.1. Cytological Morphotypes of Endophytes and Their Abundance in the Roots of F. gigantea

Firstly, in the course of a microscopic examination of *F. gigantea* root apical sections, we detected patterns of fungal and bacterial morphostructures.

The variable type of hyphae morphology observed demonstrates that the fungi living inside the roots represent different fungi species (Figure 1A–E). In some tissue specimens, we found both hyaline, cystidia-like hyphae (~130 µm long) and thin, melanized, dichotomously branched hyphae present in the same root (Figure 1B,C). The majority of the fungal structures were extracellular (Figure 1A–C,E,H,I), although some appeared intracellular (Figures 1D and S1). Some hyphae were clustered together in abundant groups, which indicates rapid proliferation of endophytic fungi (Figure 1H,I). The *Alternaria*-type conidia recorded show that *Alternaria* sp. has an active developmental cycle inside the root tissues of *F. gigantea* (Figure 1F).

Figure 1. Cytological view of fungal and bacterial endophyte morphotypes in the root meristem of *F. gigantea*: (**A**–**E**) images of endophytic fungi hyphae; (**F**) *Alternaria*-type conidia; (**G**) agglomerates of fungal spores; (**H**,**I**) clusters of hyphae; (**J**) large bunches of Bacillus-type endophytic bacteria; (**K**) chains of filamentous bacteria. Scale bar = 10 µm.

In addition, we viewed images of endophytic bacteria (Figure 1J,K). Large bunches of scattered rod-type bacteria indicated active *Bacillus* sp. spread (Figure 1J). Moreover, the occurrence of fungi and bacteria together was quite common in some root specimens.

The abundance of endophytic fungi was analyzed in the root tip cross-sections stained with Trypan blue. At the Kairėnai and Vingis locations, the fresh-grown roots were sampled in three replicates from the tillers collected during the vegetation season in May–June. The microscopical analysis of 60 roots for each location in total (no significant differences between replicates were observed) showed the occurrence of fungal structures in about ~40% of the roots examined (Table 3).

Table 3. Endophytic fungi structure abundance in *F. gigantea* root tip tissues.

Location	Roots Fragments		Roots with Endophytic Fungi, %	Microscopical Fields of View		
	* No. Analysed	No. with Fungi Structures Detected		No. Analysed	No. with Fungi Structures Detected	Endophytic Fungi Abundance, %
Kairėnai	60	28	46.7	600	155	25.8
Vingis	60	25	41.7	600	141	23.5

* No.—number.

3.2. Fungal Endophyte Isolation and Taxonomic Assignment

Endophytic fungi were isolated from the surface-sterile fresh root cuttings of *F. gigantea* placed on a PDA medium. Twenty-five root segments were planted in eight replicates, four from Kairėnai and four from Vingis locations. Seven fungal isolates were obtained from a total of 200 root segments planted. Four fungal species identified belong to Ascomycota, and two are from Basidiomycota (Figure 2, Table 4). No colony growth was detected in control Petri dishes with the final root-wash water after sterilization.

Table 4. Taxonomic assignment of the endophytic fungi isolated from the roots of *F. gigantea* according to the standard DNA data.

Fungus	Isolate Code	DNA Locus	DNA Identities, bp	Congruence, %	BLAST ID
Alternaria alternata	BSG001	ITS	440/440	100.0	PP218262.1
		RPB2	587/587	100.0	MN922279.1
	BSG002	*SSU*	647/647	100.0	OR453387.1
		TEF	373/373	100.0	MK386655.1
Cadophora fastigiata	BSG003	ITS	531/531	100.0	MN833359.1
			508/508	100.0	MF077223.1
Chaetomium funicola	BSG039	ITS	248/249	99.6	FN394680
			570/570	100.0	PP165499.1
		RPB2	527/540	97.6	XM_062782192.1
		SSU	417/417	100.0	AF048794.1
Microdochium bolleyi	BSG008	ITS	460/460	100.0	MT276137.1
		RPB2	543/543	100.0	MN817764.1
Coprinellus sp.	BSG004	ITS	390/397	98.2	JN689938
			464/471	98.5	FN386275
Sistotrema brinkmannii	BSG005	ITS	514/514	100.0	DQ093653.1
			650/650	100.0	JQ912675.1
		SSU	567/567	100.0	KM222227.1

The taxonomic assignment was based on the colony morphology and the cytomorphological characteristics of the species (Figure 2) and confirmed by the alignment of the PCR-produced ITS, *RPB2*, *SSU*, and *TEF1-a* sequences with the reference fungal DNA data using the BLAST tool [43] (Table 4).

In the isolate culture using PDA medium, the fungal community obtained from the roots of *F. gigantea* was dominated by Ascomycota species represented by *Alternaria alternata*, *Cadophora fastigiata*, *Chaetomium funicola*, and *Microdochium bolleyi*.

Alternaria alternata (Fr.) Keissler (1912) isolate's colony view and cytomorphology images are shown in Figure 2(A1–A4). The colonies on the PDA medium are fast-growing, black to olivaceous-black or greyish, floccose; conidiophores single or in small groups; conidia multicell, ovoid or ellipsoidal, often with a short conical beak, present singly or in acropetal chains, having both transverse and longitudinal septations, pale brown or golden-brown to brown, ~20–50 × 6–10 µm; hyphae subhyaline, septate. Typical *A. alternata*

conidia were observed microscopically in both isolate mycelium (Figure 2(A3)) and root section specimens (Figure 1F).

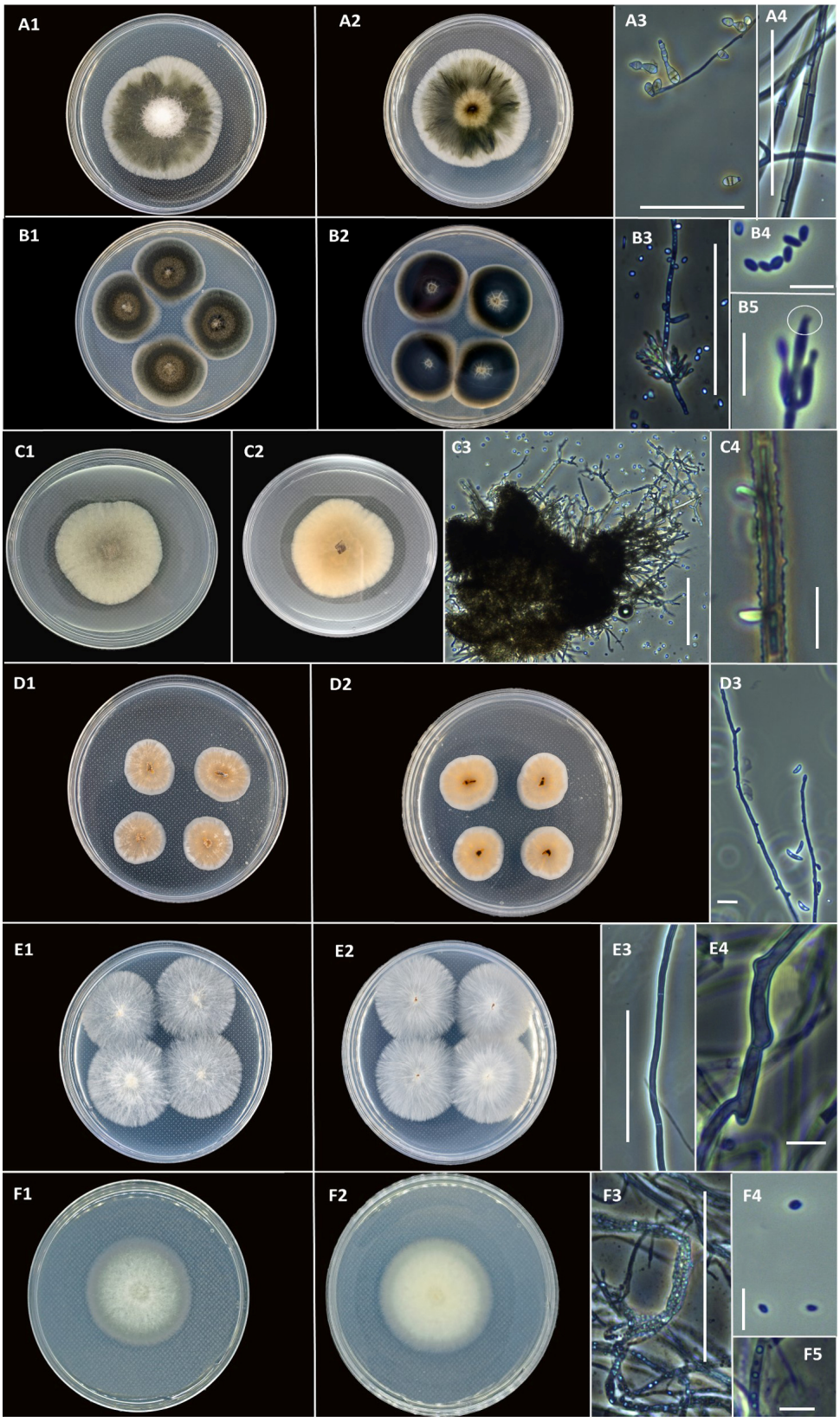

Figure 2. The 7–10-day isolate colonies on PDA medium from the roots of *F. gigantea* and cytomorphological images obtained from mycelium: (**A1**,**A2**) *Alternaria alternata* (isolate BSG001) top

and reverse view, (**A3**) multicelled, obclavate conidia with short conical beaks, and (**A4**) segmented hyphae; (**B1**,**B2**) *Cadophora fastigiata* (BSG003) top and reverse views, (**B3**,**B5**) conidiophores (funnel-shaped collarette marked) and segmented hyphae, and (**B4**) conidia; (**C1**,**C2**) *Chaetomium funicola* (BSG039) front and reverse views, (**C3**) ascomata, and (**C4**) ascomata hair and ascospores sticking to it; (**D1**,**D2**) *Microdochium bolleyi* (BSG008) top and reverse, (**D3**) conidia on cylindrical conidiogenous cells; (**E1**,**E2**) *Coprinellus* sp. (BSG004) top and reverse view, and (**E3**,**E4**) segmented hyphae; (**F1**,**F2**) *Sistotrema brinkmannii* (BSG005) top and reverse view, (**F3**,**F5**) hyphae, and (**F4**) spores. Scale bar: (**A3**,**A4**,**B3**,**C3**,**E3**,**F3**) = 100 µm; (**B4**,**B5**,**C4**,**D3**,**E4**,**F4**,**F5**) = 10 µm.

Cadophora fastigiata Lagerb. & Melin (1928) [=*Phialophora fastigiate* (Lagerb. & Melin) Conant (1937)] isolate's colony view and cytomorphology images are shown in Figure 2(B1–B5). The colonies on the PDA are medium greyish-brown, suede-like, reverse dark brown to black, producing ray-like strands towards the center; conidiophores pale brown, straight, unbranched, producing terminal phialides; phialides appear clustered or singly; conidiogenous cells phialidic, pale brown, smooth, with funnel-shaped collarette; conidia ovoid, smooth, light brown, nonseptate, ~5 × 2.5 µm; hyphae subhyaline to pale brown, septate.

Chaetomium funicola Cooke (1872) (=*Dichotomopilus funicola* (Cooke) X. Wei Wang & Samson 2016) isolate's colony view and cytomorphology images are shown in Figure 2(C1–C4). The ten-day isolate colonies on the PDA medium are white with a grey tint, reverse pale orange, floccose, hyphae segmented; ascomata dark brown, dichotomously hairy, globose to ovoid, brown, 150–220 µm; terminal hairs dichotomously branched 2–5 times, forming a dense head; ascospores brown, ovate, slightly apiculate at both ends, ~5 × 3 µm.

Microdochium bolleyi (R. Sprague) de Hoog & Herm-Nijh. (1977) isolate's colony view and cytomorphology images are shown in Figure 2(D1–D3). The colonies on the PDA are peachy orange, suede-like or floccose, in reverse black-spotted at the center, white at edges; hyphae hyaline, septate; conidiogenous cells ampullate or cylindrical; conidia crescent-shaped, hyaline, one-celled, thin-walled, smooth, ~6 × 2 µm.

Two isolate cultures of Basidiomycota, *Coprinellus* sp. and *S. brinkmannii*, were obtained on a PDA medium from the surface-sterile root fragments of *F. gigantea*.

Coprinellus sp. (Pers.) J. E. Lange (1938) isolate's colony view and cytomorphology images are shown in Figure 2(E1–E4). The isolate colonies on the PDA medium are white to yellowish, growing fluffy, with aerial mycelial tufts filling Petri; hyphae subhyaline, septate, and some clamp projections visible.

Sistotrema brinkmannii (Bres.) J. Erikss. (1948) isolate's colony view and cytomorphology images are shown in Figure 2(F1–F5). The isolate colonies on the PDA medium are pale yellowish-white, floccose to fluffy, with concentric rings; "chain chlamydospores" are visible microscopically, which is consistent with Potvin and co-authors [44]; basidiospores are dark, ellipsoid, ~4 × 2 µm.

3.3. Bacterial Endophyte Isolation and Taxonomic Assignment

In the culture, from fifty root segments of *F. gigantea* incubated on an LB medium, we obtained isolated cultures of six bacterial taxa. The group of Firmicutes bacteria was the most prevalent and was represented by *Bacillus pumilus*, *Bacillus* sp., *Lysinibacillus* sp., and *Priestia aryabhattai* (Table 5). All these four taxa belong to Gram-positive, endospore-forming bacteria. In addition, from the root tissues of *F. gigantea*, we obtained isolates of two Gram-negative, non-spore-forming bacteria—*Kosakonia* sp. (=*Enterobacter* sp.) (Proteobacteria) and *Pedobacter* sp. (Bacteroidetes). No colony growth was detected in control Petri dishes with the final root-wash water after sterilization. The taxonomic assignment of the bacteria was confirmed by the BLAST results of 16S rDNA sequences (Table 5). The obtained 16S DNA sequences of our bacterial isolates were deposited in the GenBank (Table S1).

Table 5. Taxonomic assignment of endophytic bacteria isolated from the roots of *F. gigantea* according to standard 16S rDNA sequences.

Bacteria	Bacteria Isolate Code	Colony Characteristics			Congruence, %	BLAST ID
		Colony Shape, Surface, Edge Shape	Color	DNA Identities, bp		
Bacillus pumilus	BSB021	Almost round, opaque, shiny, uneven edge	Yellow	1086/1087	99.91	MK521063.1
Bacillus sp.	BSB013	Round, opaque, rough, uneven edge	White	986/1012	97.43	CP026662.1
Lysinibacillus sp.	BSB054	Round, flat, opaque, smooth edge	White	1084/1087	99.72	MH385002.1
Priestia aryabhattai	BSB045	Round, straight edges, shiny oil, fluff-shaped colony	Cream	1084/1088	99.63	MH321608.1
Kosakonia sp.	BSB028	Round, the surface is smooth, shiny, uneven edge	White	1068/1088	98.16	MG835978.1
Pedobacter sp.	BSB034	Round, convex, opaque, shiny, smooth edge	Pink	1039/1092	95.15	CP079218.1

4. Discussion

Phylogenetically diverse fungi, bacteria, and archaea are widely found coexisting in plant aerials and below-ground parts. They make communities that affect plant life by playing pivotal roles in nutritional chains and ensuring well-being by providing resistance to stress, diseases, and pests [1,2,12,45–47]. In this study, we describe the set of endophytic fungi and bacteria found in the roots of *F. gigantea*. This is the first detailed report on fungal and bacterial associations in the roots of this woodland grass, which is native to Europe and much of Asia.

We identified six species of endophytic fungi, four members of Ascomycota—*A. alternata*, *C. fastigiata*, *Ch. funicola*, and *M. bolleyi*—and two of Basidiomycota—*Coprinellus* sp. and *S. brinkmannii*. In addition, we report six bacteria taxa; four isolates were identified from Firmicutes—*B. pumilus*, *Bacillus* sp., *Lysinibacillus* sp., and *P. aryabhattai*—along with *Kosakonia* sp. (Proteobacteria) and *Pedobacter* sp. (Bacteroidetes), colonizing the root tissues of *F. gigantea*.

In the roots of Poaceae and other plant taxa, Ascomycota endophytes are the most common, accounting for about 90% of the community, with Basidiomycota making a minor component [3,48–50]. In contrast, we found this proportion rather different. Two Basidiomycota species were detected out of 6 in total, which makes 1/3 of this association. This could be explained by the fact that *F. gigantea* is native to forest sites where the occurrence of Basidiomycota is highly expected.

The fungal endophytes obtained in this study are taxonomically diverse. Both Ascomycota and Basidiomycota endophytes represent unrelated taxa at the order level, and their functional role and distribution are diverse. In meta-data studies of grassland endophyte communities, the members of Heliotales and Phleosporales are among the most prevalent [3,10,51]. This is consistent with the occurrence of *C. fastigiata* and *A. alternata* in the roots of *F. gigantea* grass found in our culture-dependent study. *Alternaria alternata* (Phleosporales) is a highly common saprophyte found in soil or decaying plant debris and a pathogen that causes leaf spots, rots, and blights on different plant parts [52–55]. In addition, many studies show that *Alternaria* spp. can exist asymptomatically within a wide host range, and they have been shown to be communities' dominant components in many grasses [56–60]. In the taxonomic group linked to *F. gigantea*, *A. alternata* endophyte was recorded among the most abundant taxa in the roots of *Festuca rubra* subsp. *purinosa* from

harsh ecological niches in marine cliffs [51], whereas in *Lolium multiflorum* it was found to cause leaf spot disease [61].

Species of the genus *Cadophora* are widespread geographically. They inhabit different plant roots [4,5,10,62–64] and may even appear as grapevine trunk pathogens [65,66]. Although *Cadophora* endophytes are found colonizing very different plants, Sánchez Márquez and co-authors [56], in summarizing their research with the findings of other authors, do not list *Cadophora* species among the dominant taxa in grass mycobiota associations; they appear more linked to wood/tree habitats. Many samples of *C. fastigiata* (=*Phialophora fastigiata*) (Heliotales), which is a type *Cadophora* species, come from dying wood; they are often found growing on the wood pulp and roots of *Pinus sylvestris*, *Picea excelsa*, and *Fagus sylvatica* (references listed in Schol-Schwarz [67]). This indicates that *C. fastigiata* is a common inhabitant in boreal forests, which can explain its occurrence in *F. gigantea* collected under the forest canopy in our study.

Cheatomium funicola (=*Dichotomopilus funicola*) (Sordariales) fungus is common in terrestrial habitats; it has been found as a decomposer on plant debris and in soil [68]. *Chaetomium funicola* has been found widely occurring in very specific ecosystems, such as coastal dunes in Spain and Caatinga semiarid tropical sites in Brazil [69–71]. *Cheatomium* spp. are known as endophytes of many plants; they are often found in the roots of Poaceae [48,69,72]. However, their occurrence in *Festuca* spp. has not yet been reported. Notably, products obtained from different *Chaetomium* spp. are widely used as biofungicides and biostimulants [73,74]; however, *Ch. funicola* is not specified in these agricultural applications.

Microdochium bolleyi (Amphisphaeriales) is a DSE fungus that typically resides endophytically in plant roots, especially in herbaceous plants, including many Poaceae [5,69,72,75,76]. David and colleagues [77] reported strains of *M. bolleyi* as the most abundant in the roots of coastal herbaceous plant species along the Pacific Northwest coast of the United States. Although the effects of *M. bolleyi* on herbaceous hosts have not yet been fully qualified, *M. bolleyi* is generally considered a commensal or weak pathogen. *M. bolleyi* is often found on the roots of cereal crops and grasses, where it appears as a weak pathogen under certain conditions [78,79]. Within Poaceae grasses, Hong and co-authors [80] found *M. bolleyi* associated with the basal rot of creeping bentgrass, *Agrostis stolonifera*.

Although the assemblage identified in this study consists mainly of Ascomycota grass-host generalist species, we also obtained isolates of two Agaricomycetes from Basidiomycota—*Coprinellus* sp. and *S. brinkmannii*, co-occurring in the root tissues of woodland grass *F. gigantea*. In our study, obtaining the isolate of *Coprinellus* sp. (Agaricales) from the roots of *F. gigantea* was rather surprising, as these cosmopolitan saprotrophs are not known to colonize the roots of plants as endophytes. There are data about a *C. disseminatus* isolate culture obtained from an achlorophyllous orchid, *Epipogium roseum* [81,82]. Recent studies demonstrated that this fungus forms mycorrhizal structures and facilitates the germination and seedling formation of orchids in tropical forests [83]. In addition, *C. disseminatus* culture isolates were reported from *Holcus lanatus* (Poaceae) leaves [72], and some were obtained from the living stems of trees, including tropical species [84,85].

Sistotrema brinkmannii (Cantharellales) is a wood-rotting fungus that is widespread on the bark of trees and also found in soil. It was isolated from various substrates, including decayed *Pinus sylvestris* roots [86] and *Pinus contorta* wood [87], and was also found in healthy *Ipomea* leaves [88]. Sometimes, this fungus was found to be involved in mycorrhizal associations in the roots of some trees; however, its exact function remains to be elucidated since typical endo- or ectomycorrhizae were not formed [44]. Notably, *S. brinkmannii* shows highly antagonistic activity against *Heterobasidion* spp., the pathogen causing Heterobasidion root disease of conifers [89]. In summary, *S. brinkmannii* is not a typical grass endophyte, so its discovery as a root associate in *F. gigantea* shows that its habitat diversity could be border rather than wood association. However, considering the wide distribution of the *S. brinkmannii* fungus in boreal forests [90], finding this endophyte in woodland grass seems quite possible. To the best of our knowledge, *S. brinkmannii* occurrence within the roots of grasses has not yet been reported.

Although there is still some possibility that the fungus spores or external hyphae may have survived after surface sterilization, and further inoculation studies are needed to fully verify that *Coprinellus* sp. and *S. brinkmannii* have originated from the endosphere, the distinct cystidia-like structures in the root tip cross-sections (Figure 1B) that can be assigned to Basidiomycota but not to Ascomycota are indicative of Basidiomycota in the roots of *F. gigantea*. Besides endophytic fungi, plants are hosts to diverse endophytic bacteria that are beneficial to plant growth and development [32–34]. In the roots of grasses, diverse bacterial communities were reported across many species [1,3,15,91,92]. In our study, most, four out of six, of the bacteria detected come from Bacillaceae; these are *B. pumilus*, *Bacillus* sp., *Lysinibacillus* sp., and *P. aryabhattai*. Network analysis of microbial communities involved in the decomposition of organic matter shows that bacteria of the genus *Bacillus* and fungi representing *Chaetomium* spp. and *Alternaria* spp. are among the most common keystone taxa [31]. This is related to the endophytic representatives that we found in the roots of *F. gigantea*, a grass native to the forest floor, where organic matter normally decomposes. Notably, our analysis detected *Pedobacter* sp., a member of Sphingobacteriales that, according to Banerjee and co-authors [2], is also among the keystone taxa in woodland and grassland ecosystems. Therefore, even though the list of endophytes detected in this study is relatively short compared to the meta-analysis data, we demonstrate the existence of the pivotal microbial community players in the roots of a single woodland grass, *F. gigantea*.

This study shows that *F. gigantea* roots are the habitat for a wide range of root endophytes, fungi, and bacteria, which fills in a gap in the studies of *Festuca* spp. and other closely related grasses for root endophytes, hitherto widely investigated for their associations with *Epichloë*/*Neotyphodium* in the foliar parts. The functional roles of the identified endophytes are diverse, and many of them, saprotrophs and decomposers of wood and plant debris, are linked to the decomposition of organic matter. It should be noted that for isolation, we used a single medium, PDA for fungi and LB for bacteria; therefore, future studies using other media and taxonomic identification from a molecular meta-analysis will likely detect a much broader microbial community. On the other hand, a culture-dependent assessment allows for microorganism identification at the species level, whereas a bulk sequence meta-analysis is usually limited to the order/genera level. The obtained isolates preserved in our collection can serve as the basis for further physiological and ecological studies disclosing the interactions between endophytes and *F. gigantea* plants. Among the broad-leaved *Festuca*, *F. gigantea* belongs to a certain ecological niche; this grass prefers a shady and moderately moist environment, typical of the deciduous forest floor. Further studies, including the related *Festuca* species from the contrasting ecological sites (open field grasslands), may demonstrate differences in root endophyte assemblages and elucidate different functional roles of endophyte coexistence.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16080453/s1, Figure S1: Endophytic fungi structures in Festuca gigantea root tip cross-sections. Table S1: The list of obtained 16S DNA sequences of bacterial isolates deposited in the GenBank.

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