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Structure and Abundance of *Fusarium* Communities Inhabiting the Litter of Beech Forests in Central Europe

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Abstract: Members of the genus *Fusarium* and related genera are important components of many ecosystems worldwide and are responsible for many plant diseases. However, the structure of beech litter-inhabiting *Fusarium* communities and their potential role in reducing the natural regeneration of European beech are not well understood. To address this issue, we examined *Fusarium* communities in the litter of uneven-aged, old-growth beech-dominated forests in the Carpathians (Poland) and in the Alps (Austria), and in a managed beech stand (Poland). The fungi inhabiting beech litter were investigated using beechnuts and pine seedlings as bait. The pathogenicity of the most common species was investigated by inoculating beech germinants. *Fusarium* spp. were identified based on morphology and DNA sequence comparisons of *RPB2* and *TEF1-α* genes, combined with phylogenetic analyses. Twelve fungal species were identified from 402 isolates, including nine known and three currently undescribed species. The isolates resided in three species complexes within the genus *Fusarium*. These were the *F. oxysporum* (one taxon), *F. sambucinum* (three taxa), and *F. tricinctum* (six taxa) species complexes. In addition, one isolate was assigned to the genus *Neocosmospora*, and one isolate could be placed within the genus *Fusicolla*. The most frequently isolated fungi from beechnuts and beech germinants were *F. avenaceum* (Fr.) Sacc., *F. sporotrichioides* Sherb. and *Fusarium* sp. B. The structure and abundance of species within *Fusarium* communities varied by beech forest type. The species richness of *Fusarium* spp. was greatest in old-growth beech-dominated stands, while abundances of *Fusarium* spp. were higher in managed beech-dominated stands. Pathogenicity tests showed that all four *Fusarium* species isolated from beechnuts and beech germinants could cause germinants to rot beech, suggesting that these fungi may play a negative role in the natural beech regeneration.

Keywords: European beech; *Fusarium*; *Fagus sylvatica*; litter; natural regeneration; soil-borne fungi

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1. Introduction

European beech (*Fagus sylvatica* L.) is the main broadleaved tree species in Europe's montane regions, where beech is commonly a component of mixed-species forests that includes silver fir (*Abies alba* Mill.), and Norway spruce (*Picea abies* (L.) H. Karst.) [1,2]. Beech seeds are very rich in nutrients and are consumed by many animals, including numerous bird species, mammals (primarily rodents), and several insects and molluscs. Beechnuts buried in soil for a long time may be colonized by fungi that could cause serious diseases of beechnuts and cause the rot of beech germinants [3]. Fungi are ubiquitous in soil and may affect seed survival directly by decomposition or pathogenesis [4]. Previous studies on beechnuts collected in forests showed they can be colonized by saprotrophic fungi such as *Alternaria* spp., *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Mucor hiemalis* Wehmer, *Penicillium* spp., *Rhizopus nigricans* Ehrenb., *Trichoderma koningii* Oudem., or serious pathogens such as *Rhizoctonia solani* Kühn or *Fusarium* spp. [5–19]. These fungi can lead to seed death or alter seedling survival following germination. The results reported by

Korpel' [10] and Bílek et al. [11] indicated a possible 80–90% loss of germination capacity, especially under wet and mild winter conditions. Large differences in beech seedling densities among regeneration patches have been reported [12], in part due to favorable climatic conditions for soil-borne fungi that become active in beech litter and cause high levels of seed mortality during the early stages of germination. In forest beech ecosystems, the population structure of soil-borne pathogens is very poorly understood. Past studies showed that among the pathogens that can cause beechnut rot, Cylindrocarpon-like fungi, such as *Neonectria* and *Ilyonectria* spp. are dominant [13]. However, the diversity of *Fusarium* spp. that potentially could cause beechnuts and germinants to rot under natural regeneration conditions is largely unknown.

Members of the genus *Fusarium* occupy various ecological niches as pathogens, as endophytes or saprotrophs in different climatic zones, particularly in tropical and subtropical regions [14]. Although *Fusarium* species are recognized as a threat mainly in agricultural settings, they can also induce serious losses in forestry, e.g., pitch cankers on numerous pine species caused by *Fusarium circinatum* Nirenberg & O'Donnell, especially in South Africa [15]. *Fusarium* spp. are also well known as agents of important diseases that limit the production of seedlings in forest nurseries worldwide [16]. In forest nurseries, *Fusarium* species cause pre- and post-emergence damping-off, root decay, seedling wilt, stem cankers, and seed decay (e.g., [16–26]). Germinating seeds and young seedlings in nurseries can be challenged by a variety of *Fusarium* species such as *F. oxysporum* Schltdl. sensu lato, *F. commune* K. Skovg., O'Donnell & Nirenberg, *F. proliferatum* (Matsush.) Nirenberg, *F. circinatum*, *F. acuminatum* Ellis & Everh., *F. avenaceum*, *F. equiseti* (Corda) Sacc., *F. chlamydosporum* Wollenw. & Reinking, *F. tricinctum* (Corda) Sacc., *F. moniliforme* J. Sheld., and *Necosmospora solani* (Mart.) L. Lombard & Crous (formerly known as *Fusarium solani* (Mart.) Sacc.). Among these species, members of *F. oxysporum* sensu lato species complex are the most dominant threat [17,21,22,26].

Fusarium spp. are generally soil-borne pathogens causing root and/or collar rot symptoms on young seedlings or older trees. However, information about similar disease symptoms on seedlings or trees in natural and semi-natural forests is very limited in Europe. *Fusarium* spp. were detected in roots of European ash (*Fraxinus excelsior* L.) in the former Czechoslovakia [27], and in roots of pedunculate oak (*Quercus robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl.) in Austria [28], and Poland [29]. There is no information on the occurrence of *Fusarium* spp. in association with the natural regeneration of forest trees, although members of this plant-pathogenic fungal group have been isolated from forest soil and litter. For example, *Fusarium* sp. and *F. proliferatum* were found in the soil of Scots pine (*Pinus sylvestris* L.) forests in Scotland [30] and Poland [31], while *F. avenaceum* and *F. lateritium* Nees were found on sessile oak and sycamore (*Acer pseudoplatanus* L.) litters in the UK [32]. The majority of ecological studies on *Fusarium* spp. have been performed with respect to agricultural systems, therefore members of the genus *Fusarium* are more frequently encountered in open land soil samples [33] compared to samples collected in regenerating and mature forests.

Outside Europe, *Fusarium* spp. were found in roots of naturally regenerating Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings in Canada [34], and in deciduous seedlings with damping-off symptoms in Japan [35]. Pathogenic *Fusarium* spp. have been reported to be associated with roots and rhizosphere soil of forest trees in the west of Iran [36], while *F. avenaceum* and *F. longipes* Wollenw. & Reinking have been recovered as damping-off pathogens in natural regenerating *Eucalyptus obliqua* L'Hértier and *E. radiata* Sieber ex DC. in Australia [37]. Recently, *F. solani*, *F. oxysporum*, *F. verticillioides* (Sacc.) Nirenberg, *F. equiseti*, *F. fujikuroi* Nirenberg, *F. pseudocircinatum* O'Donnell & Nirenberg and *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas were isolated from seeds of *Aspidosperma polyneuron* Müll. Arg. and their ability to cause darkening of the leaf limb have been confirmed in pathogenicity trials [38]. Members of the genus *Fusarium* are also commonly found in soil and forest litter in other parts of the world. For example, the existence of diverse *Fusarium* species populations in forest soils have been reported in

Canada [39], Australia [40], Sri Lanka [41], India [42], Malaysia [43], Argentina [44], and Indonesia [45].

The number of germinated beechnuts may be reduced by fungal pathogens inhabiting the litter layer. A recent study showed that habitants of beech litter, namely *Neonectria* and *Ilyonectria* species are pathogenic and may adversely affect the natural regeneration of *F. sylvatica* [13]. The relatively small number of *Fusarium* spp. reported from forestry systems might leave the impression that forests do not harbor a large diversity of these fungi, although it may also indicate that forest plants have been poorly explored in this regard. We are inclined towards the second option and hypothesize that there are many *Fusarium* spp. including undiscovered species associated with juvenile stages of forest trees. As part of a fungal diversity survey of beech litter in Central Europe conducted in 2011 [13], several members of the genus *Fusarium* occurring in larger numbers were recorded. Therefore, the objectives of this study were: (i) to characterize the diversity of *Fusarium* spp. and related genera in beech litter, (ii) to characterize pathogenicity of several isolates of the most frequently recovered *Fusarium* spp. associated with beech litter. Accordingly, this study will provide knowledge for understanding the role of *Fusarium* species on natural beechnuts germination and hence the survival of beech natural regeneration.

2. Materials and Methods

2.1. Study Area

The research conducted from 2010–2012 was carried out in three study sites: natural uneven-aged old-growth beech forests in the Carpathians (Babia Góra National Park, Poland, 49°33′ N, 18°34′ E) and the Alps (Rothwald, Dürrenstein, Austria, 47°47′ N, 15°04′ E) and one even-aged (60–80 years old) managed beech forest in the Krakowsko-Częstochowska Highland (Zabierzowski Forest, Zabierzów, Poland, 50°09′ N, 19°78′ E). Both old-growth stands were composed of European beech, silver fir, and Norway spruce, with beech the most abundant tree species. In the Western Carpathians (Babia Góra), the elevation of the study area was between 940 and 1010 m, and the bedrock was Carpathian flysch, a mixture of sandstone and shales. The mean annual temperature was approximately 4 °C, and the annual precipitation ranged between 1300 and 1400 mm. In the Alps (Rothwald), the elevation of the study area was between 900 and 1400 m, and the bedrock was primarily dolomite and banked limestone. The mean annual temperature was between 4 and 5 °C, and the mean annual precipitation was 2200 mm. In the Zabierzowski Forest, the elevation was between 290 and 310 m; the bedrock was loess covering deeper layers of limestone. The climate was mild with a mean annual temperature of approximately 8 °C and a mean annual precipitation of 700 mm. The stand was composed of 70% European beech; the other tree species were European ash, sycamore (*Acer pseudoplatanus* L.), and pedunculate oak. Detailed information regarding these locations can be found in Jankowiak et al. [13].

2.2. Fungal Isolation

Fusarium spp. were collected via trapping using beechnuts (experiment 1, *in situ*) and Scots pine (*Pinus sylvestris* L.) seedlings (experiment 2, in the laboratory). *In situ* experiments were conducted using a network of permanent sample plots established in Babia Góra National Park in 1995 and in Rothwald in 2001 for monitoring seed production and dispersal by forest trees [46]. In the Zabierzowski Forest, the experiment was conducted along a line transect as described by Jankowiak et al. [13]. In the laboratory experiment, the litter used was taken from some permanent sample plots established in Babia Góra. Pine seedlings were used to isolate a broad-spectrum of pathogenic fungi, including those related to conifers. The methodological details for both experiments have been described previously by Jankowiak et al. [13].

Experiment 1: In the stands, fifty or 25 beechnuts were placed inside perforated plastic boxes (0.08 m^3 or 0.02 m^3), mixed with leaf litter collected near to the site of the box in the sample plot, and finally the box was filled with litter. Then, the boxes were covered

with a thin layer of litter and left in the forest. The boxes were placed in the autumn (October–November) in 38 sample plots in Babia Góra, 30 sample plots in Rothwald, and 10 sample plots in the Zabierzowski Forest. After wintering, the boxes were recovered in the spring (late April to late May) and taken to the laboratory. All beechnuts and beech germinants were collected and analysed. The numbers of healthy and diseased specimens were counted. The beechnuts and beech germinants affected by necrosis, and other discoloration were used for fungal isolation (Figure 1A–D).

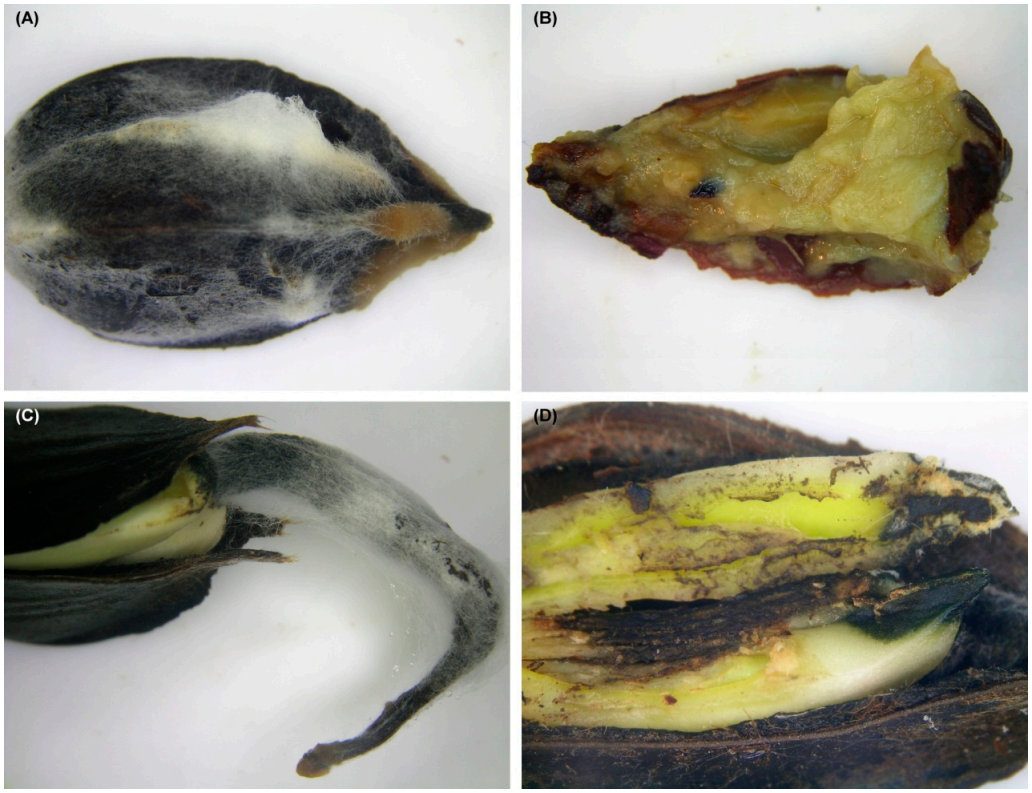

Figure 1. *Fusarium*-infected beechnuts and beech germinants collected from the litter in old growth beech-dominated forest (Babia Góra Forest) (**A**) beechnuts.; (**B**) late stage of cotyledon rot in beechnuts; (**C**) rot of beech germinant; (**D**) early stage of cotyledon necrosis.

Experiment 2: Litter collected from 16 sample plots in Babia Góra was mixed with a sterile peat–vermiculite substrate (a 2:1 ratio of litter to substrate) and then placed in plastic pots (volume 330 mL). Thirty surface-sterilised *P. sylvestris* seeds had been sown in each of the five replicate pots (30 seeds per pot). The experiment was conducted in a phytotron chamber for 10 weeks. During that period, any *P. sylvestris* seedlings with damping-off symptoms were collected for fungal isolation.

Samples taken from both experiments, i.e., symptomatic tissues of beechnuts, beech germinants, and *P. sylvestris* roots were surface sterilized for 10 s in 96% ethyl alcohol followed by 3 min in 4% v/v sodium hypochlorite (NaOCl) (Chempur, Piekary Śląskie, Poland). Next, the tissues were rinsed three times for 3 min in sterile distilled water, dried on sterile blotting paper, and finally cut into smaller fragments (5 mm × 5 mm) and placed onto 2% malt extract agar (MEA) medium (Biocorp, Warszawa, Poland) containing 2 μg mL^{-1} tetracycline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). In summary, fungi were isolated from 842 beechnuts and 781 beech germinants (experiment 1) and the roots of 134 damped-off *P. sylvestris* seedlings (experiment 2).

The isolation plates were incubated in the dark at 22 °C for one week. Isolates were then transferred to fresh 2% MEA by hyphal tip transfer for purification. Isolates with fusarioid spores were retained for further study and stored at 4 °C.

2.3. Morphological Identification

Isolates identified as *Fusarium* spp. were purified by single-spore culturing. Single-spore derived isolates were cultivated on potato dextrose agar (39 g PDA l^{-1}, Biomaxima, Poland) and synthetic low nutrient agar (SNA) [47] and were grouped into morphotypes based on their macro- and microscopic features [48]. Depending on the number of isolates belonging to the same morphotype, 1–15 isolates per morphotype were chosen for molecular identification. The isolates are maintained in the culture collection of the Department of Forest Ecosystems Protection, the University of Agriculture in Krakow, Poland (Table 1).

Table 1. Cultures used in this study and GenBank accession numbers for sequences.

Taxon	Isolate [1]	Isolation Source	Site	GenBank Accession no.	
				RPB2	*TEF1-α*
Fusarium acuminatum	36HS	*Fagus sylvatica, germinants*	Poland, Babia Góra	MZ078936	MZ078975
Fusarium avenaceum	P49HS	*Fagus sylvatica*, germinants	Austria, Rothwald	MZ078937	MZ078976
	P40HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078938	MZ078977
	43HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078939	MZ078978
	P56HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078940	MZ078979
	3HS	*Pinus sylvestris*, seedlings	Poland, Babia Góra	MZ078941	MZ078980
	P53HS	*Fagus sylvatica*, germinants	Austria, Rothwald	MZ078942	MZ078981
	35HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078943	MZ078982
	17HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078944	MZ078983
	1HS	*Pinus sylvestris*, seedlings	Poland, Babia Góra	MZ078945	MZ078984
	34HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078946	MZ078985
	37HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078947	MZ078986
	20HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078948	MZ078987
	53HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078949	MZ078988
	46HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078950	MZ078989
	P41HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078951	MZ078990
Fusarium graminearum	5HS	*Pinus sylvestris*, seedlings	Poland, Babia Góra	MZ078952	MZ078991
Fusarium oxysporum	23HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078953	MZ078992
Fusarium sambucinum	**14HS**	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078954	MZ078993
	47HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078955	MZ078994
Fusarium sporotrichioides	4HS	*Pinus sylvestris*, seedlings	Poland, Babia Góra	MZ078956	MZ078995
	42HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078957	MZ078996
	48HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078958	MZ078997
Fusarium tricinctum	P55HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078959	MZ078998
Fusarium A	8HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078924	MZ078963
	7HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078925	MZ078964
Fusarium B	54HS	*Fagus sylvatica*, germinants	Poland, Zabierzowski Forest	MZ078926	MZ078965
	10HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078927	MZ078966
	31HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078928	MZ078967
	11HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078929	MZ078968
	18HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078930	MZ078969
	29HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078931	MZ078970
	30HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078932	MZ078971
	61HS	*Fagus sylvatica*, germinants	Austria, Rothwald	MZ078933	MZ078972
	62HS	*Fagus sylvatica*, germinants	Austria, Rothwald	MZ078934	MZ078973
Fusarium C	27HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078935	MZ078974
Neocosmospora solani	25HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078960	MZ078999
	26HS	*Fagus sylvatica*, beechnuts	Poland, Babia Góra	MZ078961	MZ079000
	50HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	MZ078962	MZ079001
Fusicolla sp.	16HS	*Fagus sylvatica*, germinants	Poland, Babia Góra	-	MZ079002

[1] In bold isolates used in pathogenicity tests.

2.4. DNA Extraction, Amplification and Phylogenetic Analysis

DNA was extracted using the Genomic Mini AX Plant Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. DNA was amplified in a 25 µL reaction mixture containing 0.25 µL of Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), 5 µL of Phusion HF buffer (5X, 0.5 µL of dNTPs (10 mM), 0.75 µL of DMSO (100%) and 0.5 µL of each primer (25 µM). Amplification of the gene regions was performed under the following conditions: a denaturation step at 98 °C for 30 s followed by 35 cycles of 5 s at 98 °C, 10 s at 52–64 °C (depending on the optimal melting temperature of the primers and fungal species) and 30 s at 72 °C and a final chain elongation step at 72 °C for 8 min. The amplification reactions were performed using a LabCycler thermocycler (SensoQuest Biomedical Electronics GmbH, Göttingen, Germany). For sequencing and phylogenetic analyses, two loci were amplified: RNA polymerase second largest subunit (*RPB2*), and the translation elongation factor 1- alpha (*TEF1-α*). The primers used for PCR and sequencing of the various gene regions were as follows: RPB2-5F2

and fRPB2-7cR or fRPB2-7cF and fRPB2-11aR [49,50] for *RPB2*; EF1/EF2 [51] for *TEF1-α*. The isolates were identified to the species level by conducting Basic Local Alignment Search Tool (BLAST) searches with *Fusarium*-ID [52] and GenBank sequence data. BLAST searches [53] using the BLASTn algorithm were performed to retrieve similar sequences from GenBank (http://www.ncbi.nlm.nih.gov, accessed on 1 April 2021). The reference sequences came from several taxonomic reports [54–58], including ex-type cultures of *Fusarium* spp. Datasets were curated with the Molecular Evolutionary Genetic Analysis (MEGA) v6.06 program [59]. The first analysis based on *TEF1-α* sequences was conducted to assess the preliminary identification of the isolates and their phylogenetic affinity among the different species complexes (SC) of *Fusarium* and related genera. In this study, *TEF1-α* combined with *RPB2* were used for phylogenetic analysis of four *Fusarium* SC and for the genus *Necosmospora*.

For phylogenetic analyses, sequence alignments were performed using the online version of MAFFT v7 [60]. The *RPB2* and *TEF1-α* datasets were aligned using the E-INS-i strategy with a 200PAM/κ=2 scoring matrix, a gap opening penalty of 1.53, and an offset value of 0.00. The alignments were checked manually with BioEdit v.2.7.5 [61]. The resulting alignments and trees were deposited into TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S28194, accessed on 4 May 2021).

Phylogenetic trees were inferred for each of the datasets using three different methods: Maximum likelihood (ML), Maximum Parsimony (MP), and Bayesian inference (BI). For ML and BI analyses, the best-fit substitution models for each aligned dataset were established using the corrected Akaike Information Criterion (AICc) in jModelTest 2.1.10 [62,63]. ML analyses were carried out with PhyML 3.0 [64], utilizing the Montpelier online server (http://www.atgc-montpellier.fr/phyml/, accessed on 15 April 2021). The ML analysis included bootstrap analysis (1000 bootstrap pseudoreplicates) to assess node support values and the overall reliability of the tree topology.

MP analyses were performed using PAUP* 4.0b10 (Swofford D.L., Sunderland, MA, USA) [65]. Gaps were treated as the fifth state. Bootstrap analysis (1000 bootstrap replicates) was conducted to determine the levels of confidence for the nodes within the inferred tree topologies. Tree bisection and reconnection (TBR) was selected as the branch swapping option. The tree length (TL), Consistency Index (CI), Retention Index (RI), Homoplasy Index (HI), and Rescaled Consistency Index (RC) were recorded for each analysed dataset after the trees were generated.

BI analyses using Markov Chain Monte Carlo (MCMC) methods were carried out with MrBayes v3.1.2 [66]. The four MCMC chains were run for 10 million generations applying the best-fit model for each data set. Trees were sampled every 100 generations, resulting in 100,000 trees. The Tracer v1.4.1 program [67] was utilized to determine the burn-in value for each dataset. The remaining trees were utilized to generate a 50% majority-rule consensus tree, which allowed for calculating posterior probability values for the nodes.

All sequences generated in this study were deposited in NCBI GenBank (Table 1) and are presented in the phylogenetic trees. All analyses were first run independently for each gene partition. The resulting trees were visually compared for topological incongruences. Gene partitions showing no topological incongruence were combined for the final analyses presented here. The different partitions and conditions used for each analysis are shown in Table 2.

2.5. Pathogenicity Tests

Fusarium avenaceum, *F. sambucinum* Fuckel, *F. sporotrichioides*, and *Fusarium* sp. B associated with symptomatic beechnuts and/or beech germinants were used for inoculation (two isolates per species) (Table 1). Five healthy germinants (about 10–15 mm long) were placed on a 9-cm plastic Petri plate covered with sterile moistened blotting paper. The germinants were inoculated with 5-mm plugs taken from the margin of a 10–14-day-old fungal culture growing on a PDA medium. Five germinants were inoculated with sterile PDA as negative controls. The plates were wrapped with Parafilm® (Amcor, Zürich,

Switzerland) and stored at 15 °C in the dark. After 14 days, the number of germinants with visible necroses were recorded. *Fusarium* was re-isolated from symptomatic germinants on a PDA medium. The experiment was repeated twice.

Table 2. Characteristics of the gene partitions used in this study.

Dataset [1]	Locus [2]	Substitution Model [3]	Number of Sites				Parsimony Statistics			
			Total	Cons. [4]	Var. [5]	Phy.i. [6]	CI [7]	RI [8]	RC [9]	HI [10]
Neocosmospora spp.	Combined *RPB2+TEF1-α*	GTR + I + G	1396	909	181	306	0.504	0.638	0.322	0.496
Fusarium tricinctum SC	Combined *RPB2+TEF1-α*	GTR + I + G	1407	1010	33	364	0.785	0.917	0.72	0.215
Fusarium sambucinum SC	Combined *RPB2+TEF1-α*	GTR + G	1647	1109	15	523	0.804	0.929	0.747	0.196
Fusarium oxysporum SC	Combined *RPB2+TEF1-α*	HKY + G	1497	1305	90	102	0.835	0.861	0.719	0.165

[1] SC species complex; [2] RPB2 RNA polymerase second largest subunit; TEF translation elongation factor 1-α; [3] GTR generalized time-reversible model; G gamma distribution; I proportion of invariable sites; HKY Hasegawa-Kishino-Yano; [4] Conserved; [5] Variable; [6] Phylogenetically informative; [7] CI consistency index; [8] RI retention index; [9] RC rescaled CI; [10] HI homoplasy Index.

3. Results

3.1. Fungal Isolation

In total, 402 fungal isolates resembling *Fusarium* spp. have been collected. Of these, 375 isolates were isolated from beechnuts and beech germinants (Experiment 1), whereas 27 isolates were obtained from pine roots (Experiment 2).

3.2. Fungal Identification and Phylogenetic Analysis

Comparison of the *TEF1-α* sequences with sequences in GenBank and FUSARIUM-ID database confirmed their phylogenetic affinities among the different species complexes (SC) of *Fusarium* and related genera. The isolates were distributed into three *Fusarium* SC, namely the *F. oxysporum* SC (FOSC, one isolate), *F. sambucinum* SC (FSAMSC, six isolates), and the *F. tricinctum* SC (FTSC, 29 isolates). Three isolates (25HS, 26HS, and 50HS) belonged to the genus *Neocosmospora*, and one isolate (16HS) represented the genus *Fusicolla*.

Within the *F. oxysporum* SC, one isolate was identified as *F. oxysporum* sensu stricto (Figure 2). The *F. sambucinum* SC was represented by three species. In this species complex, *RPB2* and *TEF1-α* sequences of one isolate were identical to that of *F. graminearum* Schwabe, three isolates represented *F. sporotrichioides*, while two isolates were *F. sambucinum* (Figure 3). The *RPB2* and *TEF1-α* sequences for 29 isolates in the *F. tricinctum* SC showed that these isolates represented three known and three unknown taxa. The known species were represented by *F. acuminatum* (one isolate), *F. avenaceum* (15 isolates), and *F. tricinctum* (one isolate) (Figure 4). Two isolates named as *Fusarium* sp. A, and nine isolates named as *Fusarium* sp. B formed two distinct lineages most closely related to *F. avenaceum* (Figure 4). Within the FTSC, *Fusarium* sp. A formed a new lineage, while isolates of *Fusarium* sp. B belonged to the FTSC 5 (Figure 4). One of the isolates referred to herein as *Fusarium* sp. C, formed a separate clade close to *F. acuminatum* and *F. tricinctum* (Figure 4). The genus *Neocosmospora* was represented by one species: *Neocosmospora solani* (Figure 5).

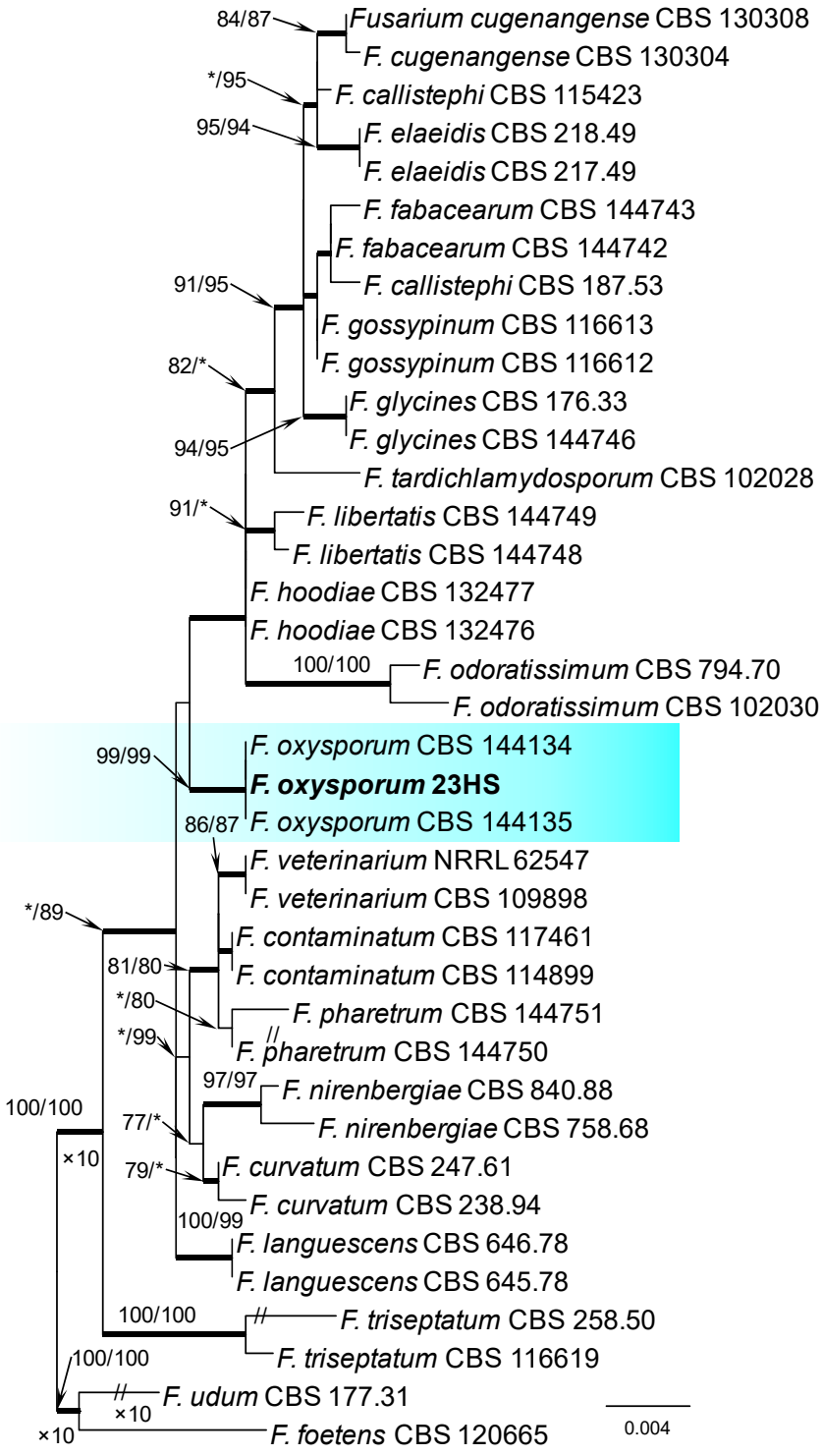

Figure 2. Phylogram obtained from Maximum Likelihood (ML) analysis of the combined dataset of *RPB2+TEF1-α* for selected species of the *Fusarium oxysporum* SC. Sequence obtained during this study is presented in bold font. The Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. * Bootstrap values < 75%. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Fusarium foetens* and *Fusarium udum* represent the outgroup in analyses of the combined dataset of *RPB2+TEF1-α*.

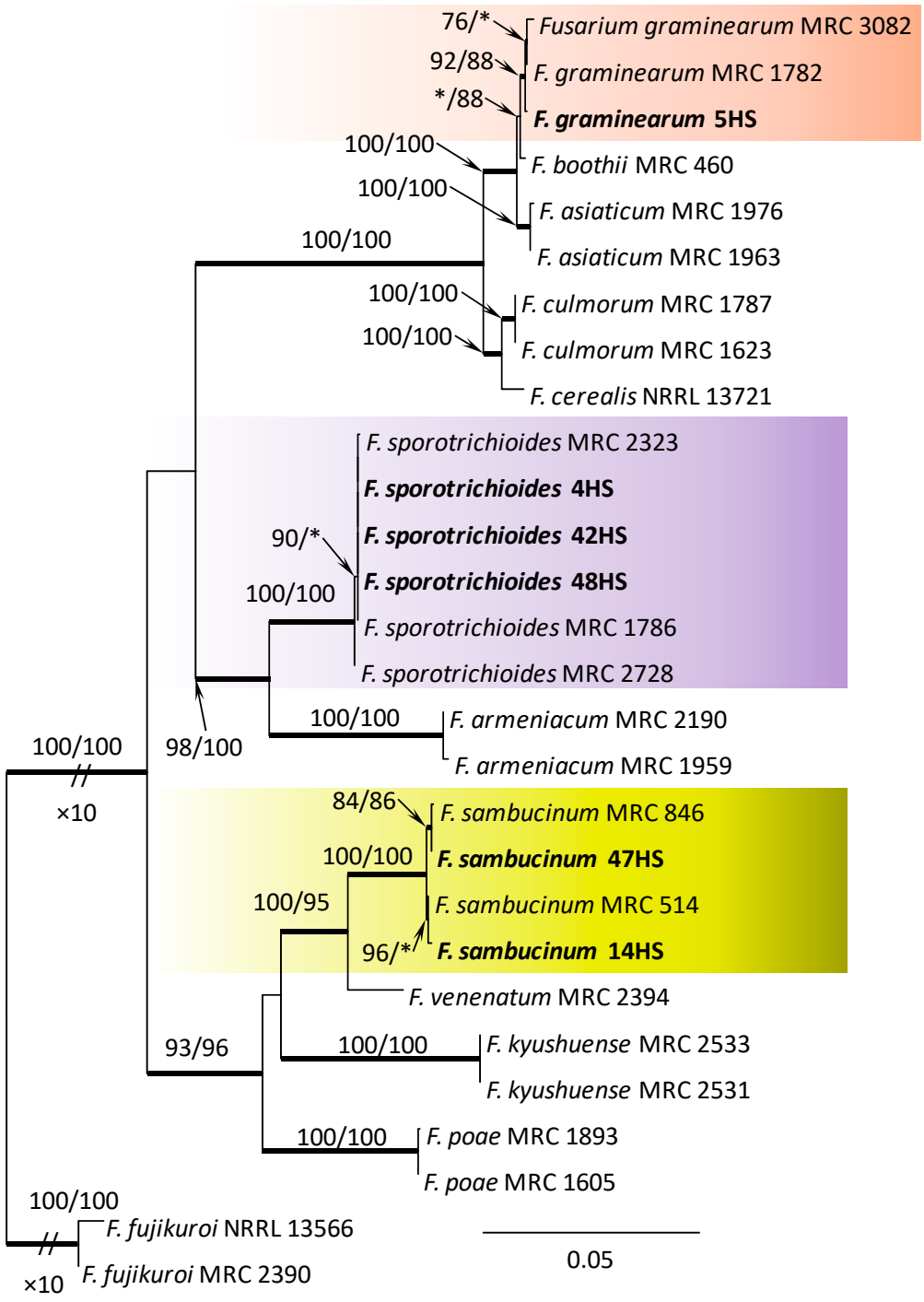

Figure 3. Phylogram obtained from Maximum Likelihood (ML) analysis of the combined dataset of *RPB2+TEF1-α* for selected species of the *Fusarium sambucinum* SC. Sequences obtained during this study are presented in bold font. The Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. * Bootstrap values < 75%. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Fusarium fujikuroi* represents the outgroup in the analysis of the combined dataset of *RPB2+TEF1-α*.

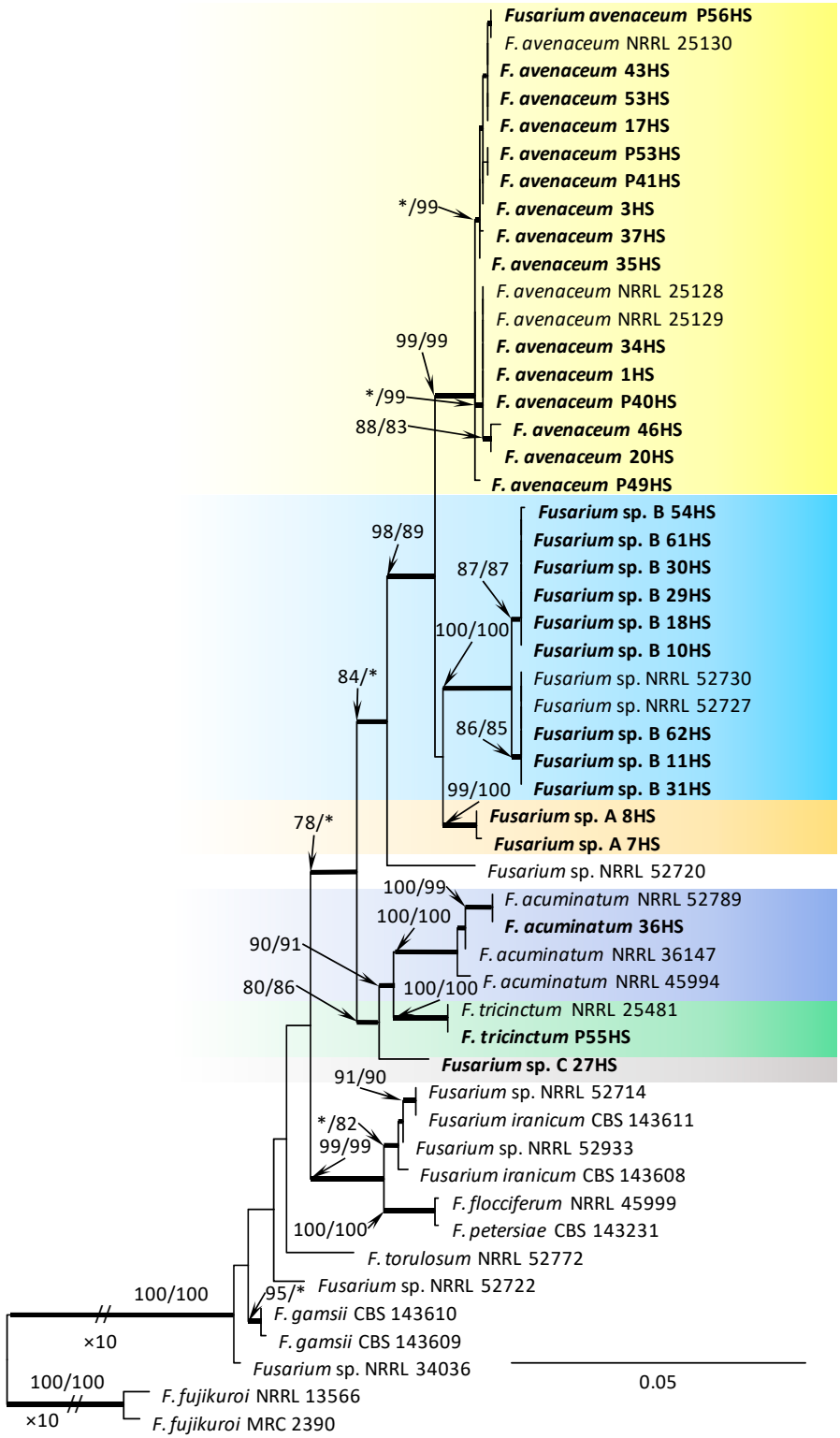

Figure 4. Phylogram obtained from Maximum Likelihood (ML) analysis of the combined dataset of *RPB2+TEF1-α* for selected species of the *Fusarium tricinctum* SC. Sequences obtained during this study are presented in bold font. The Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. * Bootstrap values < 75%. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Fuarium fujikuroi* represents the outgroup in the analysis of the combined dataset of *RPB2+TEF1-α*.

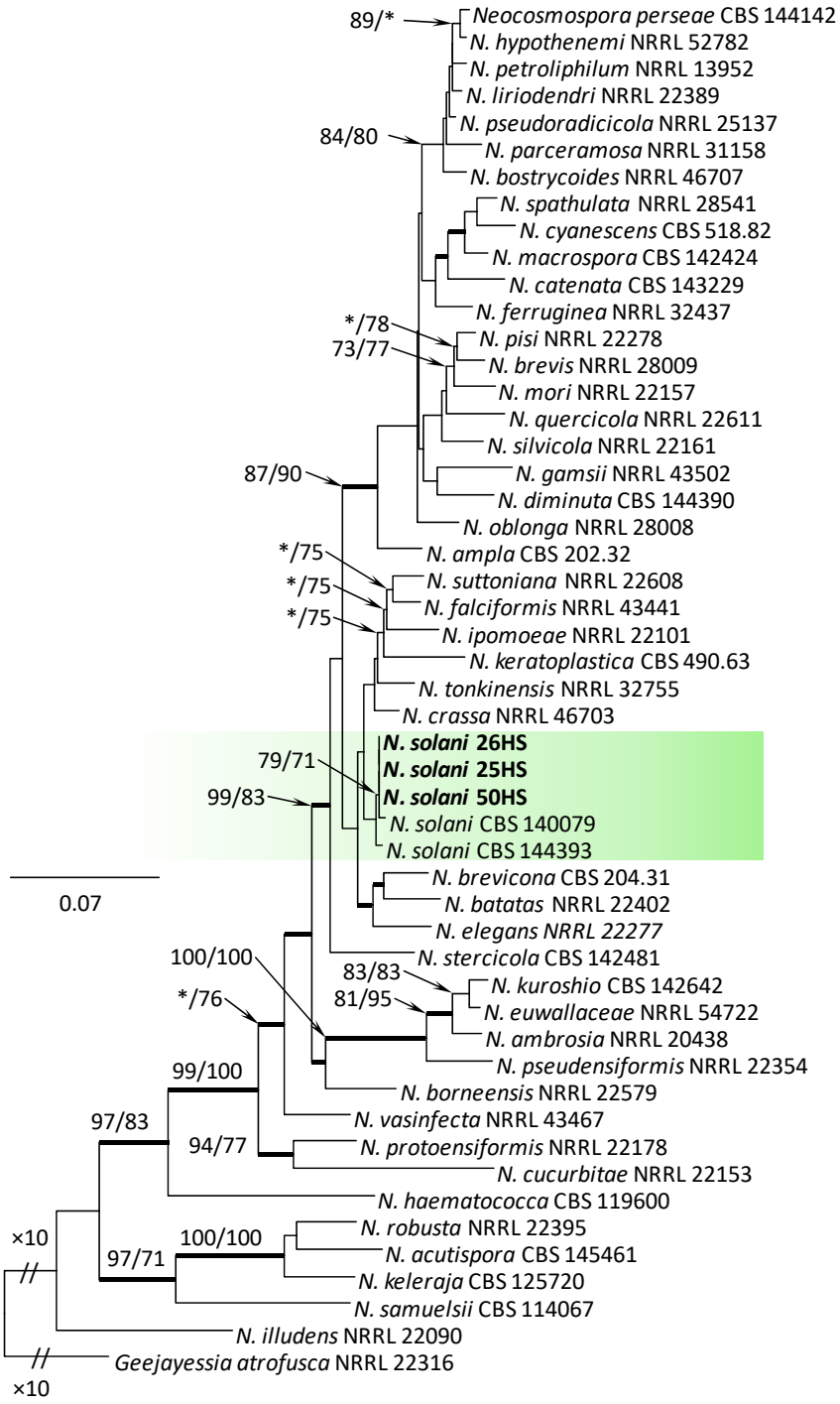

Figure 5. Phylogram obtained from Maximum Likelihood (ML) analysis of the combined dataset of *RPB2+TEF1-α* for selected species of *Neocosmospora* spp. Sequences obtained during this study are presented in bold font. The Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. * Bootstrap values < 75%. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Geejayessia atrofusca* represents the outgroup in the analysis of the combined dataset of *RPB2+TEF1-α*.

The *TEF1-α* sequence of isolate 16HS was 99% identical to *Fusicolla aquaeductuum* (Radlk. & Rabenh.) Gräfenhan, Seifert & Schroers (isolate CBS 835.85) [68].

3.3. Frequency of Isolation of *Fusarium* spp. and Related Genera from Beech Litter

Experiment 1: The members of the *F. tricinctum* SC were the most commonly isolated, and *F. avenaceum* was the dominant member among the *Fusarium* spp. isolated, found in 9.3% to 65.2% of beechnuts and beech germinants. This fungus had the highest isolation frequency (65.2%) from the Zabierzowski Forest. The lowest isolation frequency of *F. avenaceum* (9.3%) was obtained in Babia Góra (Table 3). *Fusarium* sp. B, the next most frequently isolated *Fusarium* species from beechnuts and beech germinants were isolated from 2.1 to 5.8% of specimens (Table 3). Additionally, *F. sporotrichioides* was isolated at varying frequencies having the highest isolation frequency (10.1%) in the Zabierzowski Forest, and the lowest isolation frequency (from 0% to 0.8%) in Babia Góra and Rothwald (Table 3). Other *Fusarium* species, *Fusicolla* sp., and *Neocosmospora solani* were sporadically isolated (from 0.1% to 2.9%) (Table 3). The genera *Fusarium*, *Fusicolla*, and *Neocosmospora* were most commonly isolated in the managed forest, Zabierzowski Forest (79.7%). In contrast, the frequencies of these fungi in the natural forests (Babia Góra and Rothwald) were considerably lower (19.3% and 16.8%, respectively) (Table 3).

Table 3. Isolation frequency (%) of *Fusarium* and related genera from the beech litter collected at the three study sites.

Taxon	Locations			
	Babia Góra		Rothwald	Zabierzowski Forest
	Experiment 1 *In Situ* (Beechnuts or Beech Germinants)	Experiment 2 in Laboratory (Pine Seedlings)	Experiment 1 *In Situ* (Beechnuts or Beech Germinants)	Experiment 1 *In Situ* (Beechnuts or Beech Germinants)
Fusarium acuminatum	1.6			
Fusarium avenaceum	9.3	16.7	13.2	65.2
Fusarium graminearum		1.4		
Fusarium oxysporum	1.6			
Fusarium sambucinum	0.5		0.2	2.9
Fusarium sporotrichioides		1.4	0.8	10.1
Fusarium tricinctum	0.3			
Fusarium sp. A	0.7			
Fusarium sp. B	4.3		2.1	5.8
Fusarium sp. C	0.1			
Fusicolla sp.	0.1			
Neocosmospora solani	0.2		0.8	
Fusarioid species (*Fusarium*, *Fusicolla* and *Neocosmospora*), total	18.9	19.6	16.8	79.7
Cylindrocarpon-like species (*Ilyonectria* and *Neonectria*), total [1]	31.9	48.3	43.2	39.1

[1] Data from Jankowiak et al. [13].

Experiment 2: Only three *Fusarium* species were found in the litter from Babia Góra. Like experiment 1, the most abundant species was *F. avenaceum*, found in 16.7% of dying *P. sylvestris* seedlings (Table 3). *Fusarium graminearum* and *F. sporotrichioides* were sporadically isolated (1.4%). Isolates of *F. graminearum* have been detected only in this experiment (Table 3).

3.4. Pathogenicity

Inoculation of beech germinants with isolates of *F. avenaceum*, *F. sambucinum*, *F. sporotrichioides*, and *Fusarium* sp. B resulted in extensive necrosis on all germinants; no necrosis occurred in control germinants (Figure 6A–F). All the fungal species were successfully re-isolated from the inoculated germinants.

Figure 6. Necrosis on beech germinants inoculated with: (**A**) *Fusarium avenaceum* (53HS); (**B**) *F. avenaceum* (17HS); (**C**) *F. sambucinum* (14HS); (**D**) *F. sporotrichioides* (48HS); (**E**) *Fusarium* sp. B (10HS); (**F**) control.

4. Discussion

This study resulted in the recovery of 402 isolates of *Fusarium* and related genera from natural and semi-natural beech-dominated woodlands in Central Europe, where these fungi are largely unstudied. The fungi included species of *Fusarium*, *Neocosmospora solani,* and *Fusicolla* sp. The phylogenetic analysis showed that these isolates could be assigned to 12 distinct taxa, of which four represented undescribed species; and *F. avenaceum*, *F. sporotrichioides,* and *Fusarium* sp. B were the most commonly detected. Isolates of *F. avenaceum, F. sambucinum, F. sporotrichioides*, and *Fusarium* sp. B were also determined to be virulent on beech germinants.

This study not only illustrated the widespread nature of *Fusarium* distribution in the litter of beech forests in Central Europe, but also established, for the first time that *Fusarium* spp. can be major germinants pathogens in the natural regeneration of beech in this area. The pathogenicity tests in this study showed that *Fusarium* spp. caused severe symptoms on the beech germinants. These findings highlight the importance of beech

litter as a disease reservoir for germinating beechnuts. This agrees with findings from a study in Poland [13], wherein the same samples from beech forests, isolation of pathogenic *Ilyonectria* spp. and *Neonectria* spp. ranged from 32 to 48%. Our work confirmed that beechnuts lying on the litter surface or buried within the litter are exposed to a wide range of potentially damaging agents. Among them, fungal pathogens belonging to *Fusarium* spp., *Ilyonectria* spp., and *Neonectria* spp. seems to be very important for beechnuts viability and therefore have a negative role in the natural regeneration of beech. We suggest that this is an important mechanism contributing to the lack of seedlings after mast years when the beechnuts that survived until spring on the forest floor were numerous but almost no seedlings are present.

In the present study, among the 10 recorded *Fusarium* species, there were five species namely *F. avenaceum*, *F. oxysporum*, *F. sambucinum*, *F. sporotrichioides* [69], and *N. solani* [8] that had been previously detected on beechnuts after harvest and after drying in Poland. These pathogens have also been reported as causal agents of damping-off in tree seedlings in forest nurseries [16,18,25]. It cannot be excluded that *Fusarium* spp. inhabiting beech litter may be disseminated via seed and spread within forest nurseries. A similar phenomenon in seed-borne *Fusarium* pathogens of *Pinus ponderosa* Dougl. ex C. Lawson were observed by Salerno et al. [70], who showed that many different *Fusarium* species are carried on seeds of *P. ponderosa* in Argentina that may serve as inoculum sources for damping-off and root rot diseases in forest nurseries.

Fusarium avenaceum was the most frequently encountered species in our study, found in association with beechnuts and beech germinants at all study sites. It indicates that *F. avenaceum* is a major agent responsible for the decline of beechnuts in Central Europe. This fungus can cause damage to many agricultural crops worldwide, including forest nurseries [19,71,72]. *Fusarium avenaceum* has also been recorded in the natural regenerating of *Eucalyptus* seedlings with causing damping-off symptoms in Australia [37]. This species has also been isolated from the roots of forest trees in Iran [36], forest litter in Sri Lanka [41], oak and sycamore litter in the UK [32], and from symptomatic shoots of *Q. robur* in Poland [73,74].

Fusarium sporotrichioides and *Fusarium* sp. B were also isolated relatively frequently from infected beechnuts and beech germinants in this survey. The pathogenicity tests in this study showed that isolates of both *Fusarium* species were able to induce necrosis on beech germinants, which may suggest that these species have the potential to reduce the natural regeneration of beech. *Fusarium sporotrichioides* is widespread across tropical and temperate regions [75] and is commonly associated with seedling diseases in forest nurseries [16], wilt symptoms and needle dieback on mature trees [76] or cankers [77]. This fungus was also found in beechnuts [8] and insect-damaged acorns of *Q. robur* [78]. In turn, an undescribed species named as *Fusarium* sp. B is the member of *Fusarium tricinctum* species complex (FTSC 5), and the phylogenetic analysis showed that this taxon is closely related to *F. avenaceum*. The *RPB2* and *TEF1-α* sequences of *Fusarium* sp. B obtained in this study were identical to isolates of *Fusarium* sp. reported from Turkey (NRRL 52730, NRRL 52227) [55,58]. Interestingly, isolates matching *Fusarium* sp. B have been recovered from the symptomatic stems of young seedlings in naturally regenerated oaks in Poland [79], indicated that this *Fusarium* species is commonly distributed in hardwood forests in Poland. The *Fusarium tricinctum* species complex (FTSC) was also represented by two other unknown taxa: *Fusarium* sp. A and *Fusarium* sp. C. All unknown *Fusarium* species detected in this study (*Fusarium* sp. A–C) probably represent new taxa and will be described elsewhere.

Fusarium oxysporum, the most common *Fusarium* damping-off pathogen in bareroot forest nurseries [3,16], was detected only sporadically in diseased tissues of beechnuts and beech germinants, although it is known from other types of forests. It has been recovered in forest litter and soil in many regions of the world (e.g., [39,40,43,44,80–82]) as well as in the roots of forest trees in Iran [36]. This study provides the first information about the presence of *F. oxysporum* in the litter in beech forests.

There were large quantitative and qualitative differences in the *Fusarium* spp. composition between the two examined types of forests, i.e., natural old-growth beech forests with a small admixture of *A. alba* and *P. abies* (Babia Góra, Rothwald), and managed beech forest with a small admixture of different hardwood species (Zabierzowski Forest). The highest species richness value was found in a natural old-growth beech forest in Babia Góra (10 species) while the lowest species richness value occurred in a managed beech forest in Zabierzowski Forest (4 species). It is possible that old beech forest enriched with conifers could promote some *Fusarium* species. On the contrary, the least complex *Fusarium* community was found in managed and relatively tree species poor Zabierzowski Forest. Recent studies have shown that, the most important factor that shape soil microbiome community assemblages are the tree hosts promoted by complex interactions, including alteration of the microclimate (temperature and moisture), production of litter, production of root exudates, or direct interactions with root-symbiotic and root-associated microorganisms [83,84].

The abundance of various *Fusarium* spp. also differed among the forest types. The abundance of *Fusarium* spp., especially *F. avenaceum*, *F. sambucinum*, and *F. sporotrichioides* in managed beech-dominated stand was considerably higher than in an old growth beech-dominated forest. The high *Fusarium* spp. abundance in the managed beech-dominated stand may be due to their host preference for hardwood trees. A comparative analysis of the occurrence of *Fusarium* spp. and *Cylindrocarpon*-like fungi (*Ilyonectria* spp. and *Neonectria* spp.) that have been collected from the same sites by Jankowiak et al. [13] showed that *Cylindrocarpon*-like fungi had no host preferences although the occurrence of *Ilyonectria* species appeared to be more closely related to the presence of conifers in temperate forests.

5. Conclusions

We showed that *Fusarium* spp. are diverse and important components of the litter mycobiota in beech forests and that they may play a negative role in the natural regeneration of beech. The results suggest that the structure and abundance of *Fusarium* communities inhabiting the litter may vary depending on the beech forest composition. However, further studies on the presence of *Fusarium* spp. on different forest trees are needed. Although, an increasing number of studies on soil fungal communities in forest ecosystems have recently been conducted in many countries, the results of our study highlight the fact that only a small proportion of litter fungi are currently known. This survey of *Fusarium* spp. in beech litter revealed three new *Fusarium* species belonging to the *Fusarium tricinctum* species complex (FTSC) for which the taxonomic status needs to be clarified in further studies. This supports the view that the diversity of *Fusarium* species and related genera in forest litter in Central Europe is high.

Author Contributions: H.S. conceived the idea and performed the fungal isolation and pathogenicity experiment, R.J. and P.B. analyzed the molecular data, H.S., R.J. and P.B. wrote the manuscript, and G.H. reviewed and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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