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Culturable Endophytic Fungi in *Fraxinus excelsior* and Their Interactions with *Hymenoscyphus fraxineus*

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Abstract: The species diversity of culturable endophytic fungi was studied in the leaves and twigs of symptomatic and asymptomatic *Fraxinus excelsior* trees. Endophytic mycobiota was dominated by Ascomycota species, with Pleosporales (44.17%) and Diaporthales (23.79%) endophytes being the most frequently observed in the tree samples. The number of endophytic isolates and species richness varied depending on the sampling date (May and October) and tissue location. Of the 54 species identified based on ITS sequences, 14 were classified as dominant. The most frequently isolated species were *Diaporthe eres*, followed by *Alternaria alternata*, *Dothiorella gregaria,* and *Fraxinicola fraxini*. The inhibitory effect of 41 species (75 isolates) of endophytes on the radial growth of a *Hymenoscyphus fraxineus* isolate was studied under in vitro conditions (dual cultures). The radial growth of *H. fraxineus* was the most inhibited by four endophytic fungi from twigs (*Fusarium lateritium*, *Didymella aliena*, *Didymella macrostoma*, and *Dothiorella gregaria*). The inhibitory effect of the four isolates was also studied under in planta conditions. The isolates artificially inoculated into the trunks of ash trees reduced the length of necroses formed by *H. fraxineus* co-inoculated in the same trunks. This effect depended on the isolate, and the inhibition was most prominent only on trunks inoculated with *F. lateritium* and *D. aliena*. Although the total length of necrotic lesions formed by the *H. fraxineus* infection was shorter in the ash trunks co-inoculated with the endophytes, the difference was not significant.

Keywords: ash dieback; European ash; endophytic mycobiota; diversity; inhibitory effect

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1. Introduction

European ash (*Fraxinus excelsior*) plantations throughout Europe have been devastated by the fungal pathogen *Hymenoscyphus fraxineus*, causing ash dieback disease since the 1990s. Investigation into the etiology of *F. excelsior* decline in Europe has revealed a rich community of other fungal species. Few species are very common, and many occur only sporadically. In addition to *H. fraxineus*, ash branches and leaves are colonized by other parasitic and saprophytic fungi [1–5], which are secondary invaders of tissue weakened or dying from ash dieback. Frequently occurring fungi include *Alternaria alternata*, *Diaporthe eres*, *Diplodia mutila*, *Epicoccum nigrum*, *Fusarium* spp., and *Phomopsis* spp. The fungus *Phyllactinia fraxini*, which causes powdery mildew disease [6], is the most common foliage pathogen of ash trees. *Plagiostoma fraxini* (anamorph *Discula fraxinea*) causes anthracnose disease in ash trees grown in relatively cool regions [7,8]. Wood-inhabiting fungal communities in ash trees include species such as *Auricularia mesenterica*, *Bjerkandera adusta*, *Inonotus hispidus*, *Perenniporia fraxinea*, and *Ganoderma* spp., causing white rot and wood degradation [9,10]. *Armillaria* species (*Armillaria cepistipes*, *A. gallica*) attack the roots of ash trees and cause root and butt rot [11].

In Slovakia, Pastirčáková et al. [12] recorded a wide spectrum of parasitic and saprophytic species of fungi commonly colonizing ash trees, but *H. fraxineus* was the most widespread species in the country [13]. *Hymenoscyphus fraxineus* causes dieback of *F. excelsior* in Europe, but in its native East Asian range, it is typically a harmless endophyte in the leaves of several *Fraxinus* species [14–16].

Endophytes can be characterized in a variety of ways [17], but in general, they are microorganisms that spend at least part of their life cycle inside plant tissues without generating visible symptoms or damage to their hosts [18]. A single plant can harbor many endophytic organisms [19]. Fungal endophytes that asymptomatically colonize plants have the capacity to promote host plant growth and can play an important role in increasing host plant tolerance to abiotic stress, plant pests, and pathogens [20–24]. Endophytic fungi are also active against pathogenic fungi. Endophytes *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., and *Penicillium* sp. from *Aristotelia chilensis* and *Embothrium coccineum* significantly inhibited the growth of the common fungal pathogen *Botrytis cinerea* [25]. An endophytic isolate of *Trichoderma koningiopsis* from *Hevea guianensis* inhibited the causal agent of *Corynespora* leaf fall disease (*Corynespora cassiicola*), both in culture and in plants [26]. Several studies have already been published on the endophytic microbiome of ash in recent years, and the protective effects of some endophytic fungi against the ash dieback pathogen have also been studied [27–34]. Although endophytes that inhibit *H. fraxineus* in vitro (e.g., *Boeremia exigua*, *Botrytis cinerea*, *Clonostachys rosea*, *Epicoccum nigrum*, *Nemania diffusa*, *N. serpens*, *Peniophora cinerea*, *Phoma macrostoma*, *Rosellinia corticium*, *Setomelanomma holmii*, and *Xylaria polymorpha*) [28,29,34] are promising candidates for a biocontrol agent for ash dieback, their efficacy should be verified by in planta tests because the in planta situation is more complex [35]. Preliminary research has revealed that *Hypoxylon rubiginosum* has an antagonistic effect on *H. fraxineus* in planta trials [31]. Little is known about the endophytic mycobiome of ash trees in Slovakia. Only a few endophytes (*Alternaria alternata*, *Dothiorella sarmentorum*, and *Fusarium oxysporum*) colonizing *F. excelsior* branches and leaves have been recorded [12,36,37]. However, there is a need to further characterize and study endophytic fungi in ash trees to understand their potential role in the biocontrol of ash dieback disease.

The aims of this study were to (i) characterize species diversity of culturable fungal endophytes in leaves and twigs from symptomatic and asymptomatic *F. excelsior* trees in spring and autumn, (ii) evaluate interactions between ash dieback pathogen *H. fraxineus* and isolated endophytes in vitro (dual cultures), and (iii) evaluate the inhibitory effect of antagonistic endophytes under in planta conditions.

2. Materials and Methods

2.1. Fungal Cultures

The strain of *H. fraxineus* (D27) used in this study was isolated from necrotic lesions on twigs of *F. excelsior* collected in central Slovakia (locality of Duchonka; 48°42′53″ N, 18°02′33″ E) in 2015 [38,39]. Malt extract agar (MEA) supplemented with 50 g/L of frozen healthy ash (*F. excelsior*) leaflets removed after autoclaving [40], was used to isolate and grow the *H. fraxineus* strain and for a dual culture bioassay. MEA supplemented with Rose Bengal 10 mg/L and antibiotics (streptomycin sulphate 50 mg/L, penicillin G 50 mg/L; both added after autoclaving) was used for fungal endophyte isolation. All of the media were autoclaved at 120 °C for 20 min, and 20 mL per plate was poured into polystyrene Petri dishes (90 × 16 mm). The fungal cultures were incubated at 25 ± 1 °C in darkness.

2.2. Study Sites and Sampling

The collection of ash samples for fungal endophyte analyses was carried out in an *F. excelsior* forest (17.03 ha; with 5% of *Robinia pseudoacacia* as an admixture species) in the locality of Jarok (southwestern Slovakia, 48°16′38″ N, 17°57′51″ E). The forest was attacked by ash dieback, which was confirmed by direct observations of disease symptoms on trees (necrotic lesions on leaflets and petioles, dieback of branches, and sporadically whole tree crowns dead) and by *H. fraxineus* apothecia presence on ash leaf petioles from the previous year on the forest floor. In total, 20 trees showing symptoms of ash dieback disease and 20 asymptomatic trees were selected for sampling on 14 May and 14 October 2019. On each sampling date, 5 leaves and 5 twigs (only those that looked healthy) were collected from 10 symptomatic and 10 asymptomatic trees. Altogether, 100 leaves and 100 twigs (two years old) were collected on each sampling occasion. The individual samples were placed in polyethylene zipper bags and stored at 5 °C overnight.

2.3. Isolation of Fungal Endophytes

Collected leaves/twigs were processed for the isolation of fungal endophytes the next day. Under aseptic conditions, 5 leaflets of individual leaf samples and a 40 mm segment of each twig were surface-disinfected (96% ethanol for 1 min, followed by 2.5% sodium hypochlorite for 5 min and 3× rinses in sterile distilled water for 1 min) and dried shortly on sterilized tissue wipes (modified after Ibrahim et al. [41]). A single 10 mm diameter leaf disc was cut off from the central part of each surface-disinfected leaflet (including a midrib) by a cork borer. Five 5 mm sections were cut from the disinfected twigs. The leaf discs and twig sections were placed on the surface of the MEA plate and incubated in the dark at 25 ± 1 °C. In total, isolations were performed from 500 fragments of 100 leaves and 500 segments of 100 twigs. The plant fragments were checked daily, and all endophytic colonies were aseptically transferred to fresh MEA plates and cultivated for 10 days. Most cultures were transferred twice to obtain pure cultures. About 10% of cultures needed 3–4 transfers. Fungal colonies on agar plates were characterized as endophytic only if mycelia grew from internal plant tissue at the edge of plant sections. The quality of the surface-disinfection method was assessed by plating three replicates of the residual third rinse water (500 µL) on MEA plates. A three-week incubation at 25 ± 1 °C resulted in no fungal colonies on these plates, which confirmed the efficacy of the disinfection procedure. Pure cultures of endophytes were used for molecular species identification, and identified cultures were included in a dual culture bioassay. The colonization rate (*CR*) of the tissue samples by endophytic fungi was calculated using the formula by Kumar and Hyde [42]:

$$\%CR = EI/SS \times 100, \quad (1)$$

where *EI* is the number of endophytic isolates obtained from the particular tissue sample (leaf or twig), and *SS* is the number of tissue sections from the tissue sample tested for endophytes on the surface of the MEA plate.

2.4. Molecular Identification of Endophytes

DNA was extracted from two-week-old cultures using the EZ-10 Spin Column Fungal Genomic DNA Kit (Bio Basic Inc., Markham, ON, Canada) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal RNA gene was amplified using the primer combinations ITS1F/ITS4 [43,44] and ITS4/ITS5 [43]. PCR conditions for the primer pair ITS1F/ITS4 were set as reported by Kádasi Horáková et al. [45]. The amplification reaction conditions for primers ITS4/ITS5 included initial denaturation at 95 °C for 14 min, followed by 30 cycles of denaturation at 95 °C for 25 s, annealing at 56 °C for 50 s, elongation at 72 °C for 90 s, and final extension at 72 °C for 10 min. The PCR mix consisted of approximately 10 ng of template DNA, 10 pmol/µL of forward and reverse primers, 5x HOT FIREPol® Blend Master Mix (Solis Biodyne, Tartu, Estonia), and deionized water of molecular grade (Pro injection, B. Braun). All PCRs were performed in a total

volume of 20 µL in Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR products were visualized on 1% (*w*/*v*) TBE agarose gel stained with a SimplySafe stain (EURx, Gdansk, Poland). The target PCR fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was performed in both directions using an ABI PRISM 3130 (Applied Biosystems, Waltham, MA, USA) by SEQme Ltd. (Dobříš, Czech Republic). The retrieved sequences were processed using SnapGene® Viewer 5.0.7 (GSL Biotech LLC, San Diego, CA, USA) and compared by BLASTN ver. 2.13.0+ [46] against ITS sequences deposited in the NCBI GenBank database.

2.5. Dual Culture Bioassay

In total, 75 fungal endophytes were screened for their capacity to suppress the growth of *H. fraxineus* (strain D27) in a dual culture bioassay on MEA (supplemented with ash leaflets) in Petri dishes (90 × 16 mm). Mycelial plugs (5 mm in diameter) of the actively growing margins of the *H. fraxineus* and endophyte colonies were placed at a fixed distance of 55 mm on the MEA plates. The plugs were placed at the opposing end of the same Petri dish. Due to the slower growth rate of *H. fraxineus* compared to the endophytes, *H. fraxineus* plugs were first cultured for four days at 25 °C, and on the fifth day, plugs of endophytes were placed on the MEA plates [47]. Dual culture tests of six slow-growing endophytic isolates (TA31-4M, TA55-5M, TA93-1M, TS94-4M, LS25-1M, and LS42-3M) were started simultaneously with the *H. fraxineus* strain on the MEA plates. Ten control plates were inoculated in a similar manner, but two plugs of *H. fraxineus* were placed in Petri dishes without endophytes. The plates for all combinations of *H. fraxineus* and endophytes were incubated at 25 ± 1 °C in the dark. The interactions between the dual culture partners were checked at three-day intervals, and the radii of colonies were measured using a digital caliper on day 27 post-inoculation. Each combination of the *H. fraxineus* strain and the endophytic isolates was performed in triplicate. Inhibition of radial growth of the *H. fraxineus* strain by the endophytes in dual cultures was evaluated by the inhibition index of radial growth (*IRG*), calculated using the following formula:

$$IRG = (R1 - R2)/R1, \quad (2)$$

where *R*1 is the radius of the *H. fraxineus* colony from the control Petri dishes, and *R*2 is the radius of the *H. fraxineus* colony measured on the line between the inoculation positions of the *H. fraxineus* strain and the endophyte in the dual culture plates. The radial growth of the *H. fraxineus* colony was inhibited by the co-culturing endophyte when the *IRG* value was greater than zero. The higher the *IRG* value within the range of 0–1, the greater the inhibition effect of the endophyte.

Interactions between colonies of *H. fraxineus* and endophytes were also visually assessed, and the following interaction types [48] were recognized: physical contact of mycelia in which neither isolate was able to overgrow the other (A), *H. fraxineus* colony partially overgrown by an endophyte after initial deadlock with mycelial contact (B1), *H. fraxineus* colony overgrown by an endophyte without initial deadlock (B2), an endophyte colony overgrown by *H. fraxineus* (C), an inhibition zone present between the colonies with the width of <2 mm (D1), the inhibition zone was >2 mm (D2).

2.6. Field Bioassay

Inoculation experiments were conducted on 5-year-old *F. excelsior* trees grown in the experimental area of the Institute of Forest Ecology SAS in Nitra. The experimental trees had an average trunk diameter of 15.4 mm at the inoculation point. Inoculations were performed with one isolate of *H. fraxineus* (strain D27) and four isolates of endophytes exhibiting inhibitory activity in a dual culture bioassay (TS105-4M—*Didymella aliena*, TA63-2O—*Didymella macrostoma*, TA52-5M—*Dothiorella gregaria*, and TS94-4M—*Fusarium lateritium*). For inoculum production, sterilized discs (4 mm in diameter) of *F. excelsior* sapwood were colonized with fungal isolates on MEA plates for 3 weeks [49]. Inoculations of the trees were carried out in the tree trunk (ca. 40 cm from the base of the trunk) on 21 April

2021. Two holes (4 mm in diameter) were drilled by a cork borer in the bark tissue at a distance of 2 cm. The holes were patched with the colonized discs, one colonized with an endophyte isolate, and one colonized with *H. fraxineus*. The endophyte was inoculated upward. Combinations of *H. fraxineus* strain with each endophytic isolate were inoculated in four replicates. As a negative control, a single sterile non-colonized disc was applied to the trunk, whereas an *H. fraxineus*-colonized disc was applied to the trunk as a positive control. In the case of positive and negative controls, three replicate inoculations were performed. The discs were then covered with parafilm. Parafilm wrapping was removed after 30 days. Together, 34 trees were inoculated. The host's response to the fungal isolates was measured by the formation of a callus or necrotic tissue at 30-day intervals. The length of superficial necrosis on the bark was measured in the acropetal and basipetal directions from the inoculation point. All experimental trees were cut down 180 days after inoculation (on 18 October 2021) and transported to the laboratory for analysis. After the bark tissue was removed, the length of the cambial necrosis was measured. The tree trunk was cut at the inoculation point, and the depth of necrosis was measured.

Two samples of wood tissue, each approximately 5 × 5 mm, were taken from the margin of necrosis that formed after inoculation of *H. fraxineus* on all experimental trees. The samples were used to confirm the presence of *H. fraxineus* in the tissue. The samples were homogenized in liquid nitrogen, and the total genomic DNA was extracted according to the manufacturer's protocol using the EZ-10 Spin Column Fungal Genomic DNA Kit. DNA was suspended in 50 µL of elution buffer and stored at −20 °C. The identity of *H. fraxineus* was confirmed by species-specific primers targeting the 18S gene and the ITS-2 region of the rDNA operon [50]. The PCR components and conditions were in accordance with Pastirčáková et al. [51].

2.7. Data Analysis

Chi-square tests were performed to determine if the colonization rate by endophytic fungi was affected by the tissue type (leaf and twig), the health condition of the studied trees (symptomatic and asymptomatic), and from the sampling time (May and October). The Shannon and Simpson diversity indices [52,53] were used to determine the diversity of fungal endophytes found in ash tree samples. Species dominance was estimated for each species of fungal endophyte according to Camargo [54]. A species was considered dominant if its relative abundance was higher than $1/S$, where S denotes species richness. *IRG* data from the dual culture bioassay were arcsine transformed ($n' = arcsin\sqrt{n}$) before analysis of variance (ANOVA) was used to determine differences among fungal endophytes. If significant differences were detected, the post hoc Tukey HSD test ($p = 0.05$) was performed. All statistical analyses were performed using Minitab 17® (© 2013 Minitab Inc., State College, PA, USA).

3. Results

3.1. Endophytic Mycobiota in Ash Leaves and Twigs

Altogether, 400 tissue samples (2000 tissue sections) were cultured from the leaves and twigs of 40 ash trees. Endophytic fungi grew from all of the examined trees, and 799 tissue sections (39.95%) yielded fungal colonies. The colonization rate (*CR*) of samples by endophytes varied depending on the tissue type, the health status of the trees, and from the date of sampling (Table 1). The mean *CR* of the different tissue types ranged from 13.6 to 72.0%. The lowest rate of colonization was observed in leaves sampled in May, whereas the highest *CR* was detected in twigs collected in May. Chi-square analysis identified a significant difference in *CR* between leaves and twigs collected from both symptomatic ($X^2_{(1,N=500)} = 63.51$, $p < 0.001$) and asymptomatic trees ($X^2_{(1,N=500)} = 30.29$, $p < 0.001$). This was observed regardless of the sampling date, and twigs were colonized by endophytes at a significantly higher rate than leaves in May ($X^2_{(1,N=500)} = 392.71$, $p < 0.001$), but more endophytes were retrieved from leaves than twigs in October ($X^2_{(1,N=500)} = 40.14$, $p < 0.001$). However, the health status of the sampled trees had no significant effect on the *CR* of leaf

($X^2_{(1,N=500)}$ = 3.51, p = 0.061) and twig samples ($X^2_{(1,N=500)}$ = 0.42, p = 0.517). The date of sampling was a significant factor in retrieving endophytes from the trees. A significantly higher *CR* was detected for leaf samples collected in October from both symptomatic ($X^2_{(1,N=500)}$ = 66.59, $p < 0.001$) and asymptomatic trees ($X^2_{(1,N=500)}$ = 171.96, $p < 0.001$), but the twig samples were significantly more colonized in May ($X^2_{(1,N=500)}$ = 73.63, $p < 0.001$ for asymptomatic trees and $X^2_{(1,N=500)}$ = 62.78, $p < 0.001$ for symptomatic trees).

Table 1. Isolation of endophytic fungi on malt extract agar plates from different types of tissue samples collected from *Fraxinus excelsior* in Slovakia in 2019.

Date of Sampling	Health Status of Trees *	Tissue Location on Trees	Mean Colonization Rate of Tissue Samples (%) **	Total Number of Isolates	Mean Number of Isolates per Tissue Sample **
May 2019	Asymptomatic	Leaf	13.6 ± 2.0	34	0.7 ± 0.1
		Twig	68.4 ± 4.8	197	4.0 ± 0.3
	Symptomatic	Leaf	19.2 ± 2.9	48	1.0 ± 0.2
		Twig	72.0 ± 4.7	198	4.0 ± 0.3
October 2019	Asymptomatic	Leaf	68.0 ± 4.1	179	3.6 ± 0.3
		Twig	41.2 ± 3.3	103	2.0 ± 0.2
	Symptomatic	Leaf	54.4 ± 3.2	136	2.7 ± 0.2
		Twig	44.8 ± 2.9	112	2.2 ± 0.2

* Trees displaying symptoms of ash dieback diseases were categorized "symptomatic", and trees without symptoms of the diseases were considered "asymptomatic" in this study; ** mean with standard error (±SE).

Altogether, 1007 isolates of endophytic fungi were obtained from the analyzed ash trees. The number of isolates obtained was not uniformly distributed across the collected samples and was dependent on the sampling date, tree health, and tissue type (Table 1). The highest number of isolates was retrieved from the twigs collected in May (197 and 198 isolates from asymptomatic and symptomatic trees, respectively) and the asymptomatic leaves sampled in October (179 isolates). The lowest number of cultures was isolated from the leaves in May. The mean number of isolates per tissue sample (twig or leaf) varied from 0.7 to 4.0 (Table 1) with a significant difference ($F_{(7,72)}$ = 15.57, $p < 0.001$) among sample types, sampling dates, and the health status of trees. For example, leaf samples collected in May yielded significantly fewer isolates than twig samples on the same collection date or leaf samples in October (Figure 1). The sampling date was an important factor in obtaining endophytes from a particular tissue type. In October, a significantly higher mean number of isolates was recovered from leaves than in May, whereas more isolates were obtained from twigs in May than in October.

Based on macromorphological characteristics, 206 endophytic isolates were selected for molecular identification, 199 isolates were identified at the species level, 5 isolates were determined at the genus level, and 2 isolates could only be placed in the order rank (Table 2). Endophytic mycobiota isolated from the tissue samples comprised 54 species from 42 genera and 13 orders. Sequences of four isolates provided a BLAST match with the GenBank database of less than 98% and might therefore be different taxa than those listed in Table 2. Interestingly, *H. fraxineus* was not isolated from the tissue samples of symptomatic trees using the isolation method employed in this study. The numbers of species varied depending on the sampling dates (May and October) and tissue locations (leaf and twig) (Figure 2). As many as 43 endophytes were detected in either from leaves or twigs, and 16 endophytes were identified in both tissue types. On both sampling dates, 20 endophytes were detected. Twelve endophytes were detected in both tissue types and on both sampling dates.

Table 2. Endophytic fungi identified from leaves and twigs of ash trees displaying (symptomatic) or not displaying (asymptomatic) symptoms of ash dieback disease collected in Slovakia in May and October 2019.

Order	Taxon	Number of Fungal Isolates					GenBank Acc. No. ITS	BLASTn	Identities %
		AL (May/Oct)	SL (May/Oct)	AT (May/Oct)	ST (May/Oct)	Total Number (May/Oct)			
Agaricales	*Hygrophorus* sp. *	0/1				0/1		LT716040	78.49
Amphisphaeriales	*Lepteutypa fuckelii*			2/0	1/0	3/0	OM950730	MZ045855	100
Botryosphaeriales	*Diplodia fraxini* **		2/0	1/0	2/0	5/0	OM950731	MT587349	99.82
	Dothiorella gregaria **		0/1	6/1	2/2	8/4	OM950732	MN685280	99.44
	Microdiplodia sp.			1/0		1/0	OM950733	FJ228194	99.11
Cladosporiales	*Cladosporium allicinum*	0/1				0/1	OM950734	MT573471	100
	Cladosporium cladosporioides	1/0			1/0	2/0	OM950735	MT635286	100
	Cladosporium tenuissimum		0/1		0/1	0/2	OM950736	LT603045	100
Diaporthales	*Cytospora pruinosa*				1/0	1/0	OM950737	MW447045	99.83
	Diaporthe eres **	0/1		8/0	17/3	25/4	OM950738	OM442980	100
	Diaporthe nobilis			1/0	2/0	3/0	OM950739	KJ609011	99.65
	Diaporthe oncostoma **	1/2	1/2	0/1	0/1	2/6	OM950740	LN714541	99.82
	Diaporthe rudis **		0/1	2/0	3/0	5/1	OM950741	MW032267	99.66
	Diaporthe vacuae				2/0	2/0	OM950742	MZ127189	99.66
Dothideales	*Aureobasidium pullulans*	1/1			1/0	2/1	OM950743	MW560221	100
Eurotiales	*Aspergillus pseudoglaucus*				1/0	1/0	OM950744	KX696361	100
Helotiales	*Neofabraea vagabunda*		0/1			0/1	OM950745	KT923785	99.63
Hypocreales	*Fusarium avenaceum*				1/1	1/1	OM950746	MW016661	100
	Fusarium lateritium **			4/1	2/1	6/2	OM950747	JQ693397	100
Mycosphaerellales	*Ramularia endophylla*	0/1				0/1	OM950748	MH859364	100

Table 2. *Cont.*

Order	Taxon	Number of Fungal Isolates					GenBank Acc. No. ITS	BLASTn	Identities %
		AL (May/Oct)	SL (May/Oct)	AT (May/Oct)	ST (May/Oct)	Total Number (May/Oct)			
Pleosporales	*Alternaria alternata* **	0/3	1/8	0/7	5/2	6/20	OM950749	MK183818	100
	Alternaria infectoria **	0/2	0/2	0/1		0/5	OM950750	MK461063	100
	Alternaria longipes		0/1			0/1	OM950751	MH712187	99.82
	Aposphaeria corallinolutea	0/1				0/1	OM950752	MT177916	99.80
	Ascochyta medicaginicola			1/2		1/2	OM950753	KF293990	100
	Ascochyta pisi			0/1		0/1	OM950754	MH854853	99.61
	Comoclathris incompta **			1/1	1/1	2/2	OM950755	KU973715	99.83
	Coniothyrium ferrarisianum *				1/0	1/0		MH860854	97.09
	Didymella aliena **		1/1		2/3	3/4	OM950756	KC311486	99.81
	Didymella glomerata			0/1	2/0	2/1	OM950757	MN075513	100
	Didymella macrostoma			0/1		0/1	OM950758	MH858090	99.81
	Epicoccum nigrum **		0/1	0/2	2/1	2/4	OM950759	KX664321	100
	Epicoccum thailandicum *				1/0	1/0		MG975626	80.79
	Foliophoma camporesii				1/1	1/1	OM950760	MN244200	99.65
	Lophiostoma corticola			0/1	1/0	1/1	OM950761	KT004559	99.62
	Muriphaeosphaeria viburni				1/0	1/0	OM950762	MW446984	99.48
	Neodidymelliopsis camporesii		0/1		1/1	1/2	OM950763	MN244199	99.81
	Neosetophoma aseptata			2/0		2/0	OM950764	NR164449	99.12
	Nothophoma spiraeae				2/0	2/0	OM950765	OM287410	99.43
	Paracucurbitaria corni			1/0		1/0	OM950766	MT547826	98.70
	Phaeosphaeria sp.			0/1	0/1	0/2	OM950767	LC171698	99.81
	Phoma herbarum			0/1		0/1	OM950768	KP739881	99.62
	Phoma sp.				0/1	0/1	OM950769	MG098303	99.31
	Pleosporales sp.		2/0			2/0	OM950770	MT777338	100

Table 2. *Cont.*

Order	Taxon	Number of Fungal Isolates					GenBank Acc. No. ITS	BLASTn	Identities %
		AL (May/Oct)	SL (May/Oct)	AT (May/Oct)	ST (May/Oct)	Total Number (May/Oct)			
	Praetumpfia obducens				1/0	1/0	OM950771	NR147688	98.30
	Pseudocamarosporium brabeji			0/1		0/1	OM950772	KR909143	99.32
	Pyrenophora triseptata			0/1		0/1	OM950773	MT548680	99.84
	Sporormiella minima			2/0		2/0	OM950774	MG098329	99.43
	Stemphylium vesicarium **	1/2	0/2	0/1	1/0	2/5	OM950775	MZ452063	100
Sordariales	*Dichotomopilus erectus*	0/1				0/1	OM950776	MN956887	99.64
	Chaetomium globosum *	0/1				0/1		MH858130	74.45
	Sordaria fimicola		0/1	1/0	1/0	2/1	OM950777	MN341410	99.82
	Sordaria lappae	0/1				0/1	OM950778	MH858210	100
Venturiales	*Fraxinicola fraxini* **	2/1	6/0			8/1	OM950779	MW447009	99.63
Xylariales	*Anthostoma amoenum*	0/1				0/1	OM950780	KC774569	98.67
	Hypoxylon fragiforme			0/1		0/1	OM950781	EF155508	100
	Nemania diffusa	0/1				0/1	OM950782	MZ078701	99.65
	Nemania serpens				1/0	1/0	OM950783	MF161316	99.48
	Rosellinia corticium **	1/1	0/1		1/0	2/2	OM950784	KY593990	99.81
	Total number of isolates	7/22	13/24	33/26	61/20	114/92			
	Species richness (S)	6/17	6/14	14/17	30/14	38/41			
	Simpson's index of diversity (D)	0.816/0.934	0.722/0.852	0.872/0.895	0.899/0.910	0.925/0.929			
	Shannon's index of diversity (H)	1.748/2.471	1.525/2.179	2.331/2.502	2.931/2.528	3.152/3.232			

Abbreviations: AL—leaf from asymptomatic trees, SL—leaf from symptomatic trees, AT—twig from asymptomatic treeKPs, ST—twig from symptomatic trees. * Indicates a BLAST match below 98% and likely a different species than listed; the sequences were not submitted to GenBank. ** Indicates the dominant species of endophytes, determined according to Camargo [54].

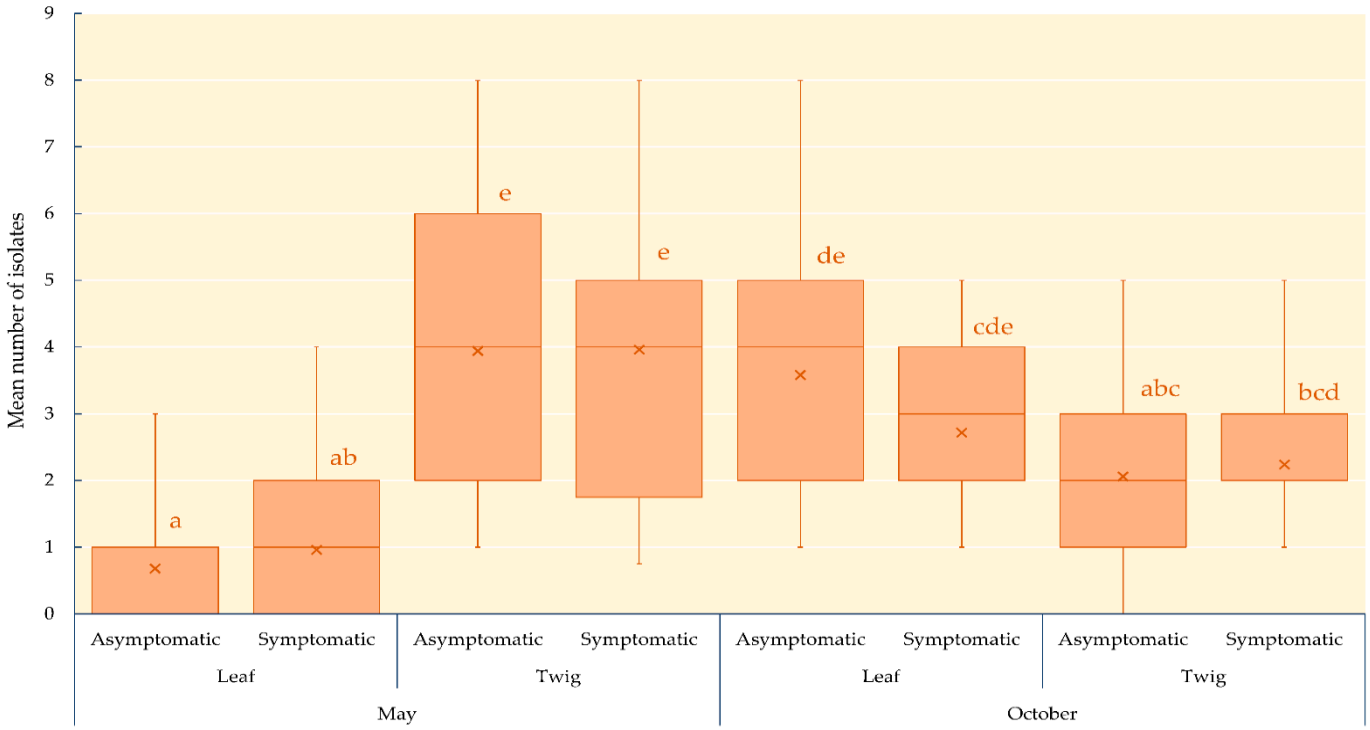

Figure 1. Box and whisker plot showing the mean number of isolates of endophytic fungi per tissue sample (leaves or twigs) of ash trees displaying (symptomatic) or not displaying (asymptomatic) symptoms of ash dieback disease collected in May and October 2019; mean values indicated by the same letter are not significantly different (Tukey HSD test; $p = 0.05$).

The mycobiota of the endophytes from the ash tissue were significantly dominated by Ascomycota (205 isolates, 99.51%) and included 13 orders. One isolate was identified as *Hygrophorus* sp. (Agaricales) from Basidiomycota, however, with a low percent identity (78.49%) after BLAST in the GenBank database. Therefore, the identity of this isolate must be considered doubtful. Pleosporales endophytes were the most frequently identified (91 isolates, 44.17%) from the tissue samples, and this order was the most species-rich (29 species, 49.15%), followed by Diaporthales, with 23.79% of relative isolate frequency (49 isolates) and six species (10.17%) (Figure 3c). The remaining fungal orders were represented by $\leq$5 species and $\leq$18 isolates. Relative frequencies of isolates by taxonomic order and percentage proportions of species numbers in particular orders were similar on both sampling dates (Figure 3a,b). Although the number of fungal orders discovered in May and October was comparable (11 orders in May vs. 12 orders in October), the structure of the identified orders varied. On both sampling dates, Pleosporales was the most species-rich order (19 species in May and 20 species in October). Diaporthales was the second most abundant order. Although only six Diaporthales species were detected in May, the relative frequency of isolates reached 33.33% (38 isolates), which was higher than the relative frequency of the Pleosporales isolates (29.82%, 34 isolates). Diaporthales were less abundant in October (11 isolates, 11.58%) than in May. While Amphisphaeriales and Eurotiales (each represented by a single species) were only detected in May, Helotiales, Mycosphaerellales, and Agaricales (each represented by a single species) were detected only in October. The species richness in the genera was variable. The most species-rich genus, *Diaporthe*, was represented by five species; *Cladosporium*, *Alternaria*, and *Didymella* were each represented by three species (Table 2). Most genera (29) were represented by a single species. Of the 54 identified species, 14 were classified as dominant. *Diaporthe eres* was the most frequently isolated species (25 isolates in May, and 4 isolates in October), followed by *Alternaria alternata* (6 isolates in May, and 20 isolates in October), *Dothiorella*

gregaria (8 isolates in May, and 4 isolates in October), and *Fraxinicola fraxini* (8 isolates in May, and 1 in October).

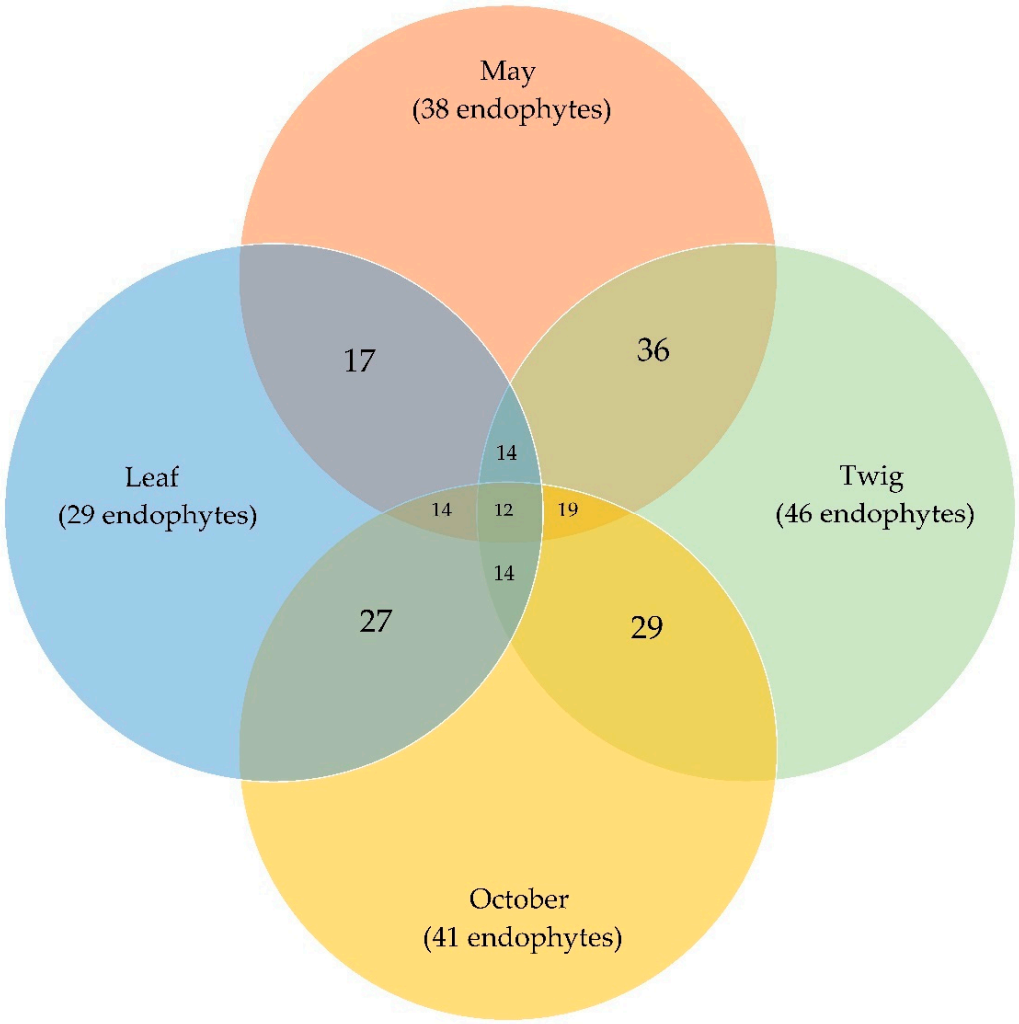

Figure 2. Venn diagram showing the number of identified endophytes shared among different sampling dates (May and October) and tissue locations (leaf and twig) on *Fraxinus excelsior*.

The species diversity indices calculated for all samples in May reached 0.925 and 3.152 for Simpson's (D) and Shannon's (H) indices, respectively, and they were similar to the indices for samples collected in October (D = 0.929; H = 3.232) (Table 2). The highest diversity indices were recorded for endophyte populations in asymptomatic leaves in May (D = 0.934; H = 2.471), and the lowest values were recorded for symptomatic leaves in May (D = 0.722; H = 1.525).

3.2. Inhibitory Effect of Endophytes against H. fraxineus on Artificial Medium

As many as 75 isolates of 41 species of endophytic fungi were tested for their inhibitory effect on the radial growth of *H. fraxineus* in dual cultures. Various types of mycelial interactions were observed between the *H. fraxineus* isolate and the endophytic fungi after 27 days of co-incubation on MEA (Table 3, Figure 4). The most frequent type of interaction was the formation of an inhibition zone between the co-partners in the dual cultures. This interaction type was observed for 49 endophyte isolates (31 species). The width of the inhibition zone depended on the endophyte isolates. It was greater than 2 mm (D1 interaction type) for 25 isolates and less than 2 mm (D2 interaction type) for 24 isolates. The largest mean zone of growth inhibition was formed around the colonies of *Phoma herbarum* (9.33 mm) and *Phaeosphaeria* sp. (8.67 mm), both isolated from asymptomatic

twigs collected in October. The *H. fraxineus* colony was overgrown by 20 isolates (13 species) of endophytic fungi, 14 isolates (9 species) partially overgrew the *H. fraxineus* colony after an initial deadlock with mycelial contact (B1 interaction type), and 6 isolates (4 species) overgrew *H. fraxineus* without an initial deadlock (B2 interaction type). The capacity of *H. fraxineus* to grow over endophyte colonies was also observed (C interaction type). Such a situation was observed for one isolate of *Sporormiella minima* (Figure 4w) when the *H. fraxineus* isolate partly overgrew the edge of the endophyte colony. The interaction of five isolates of endophytic fungi from leaves resulted in physical contact with the colony of *H. fraxineus* (A interaction type). In this case, neither the endophyte nor *H. fraxineus* could overgrow the co-partner after mutual contact of mycelia. There was no observed variation in the interaction type between replicates of the same isolate; however, isolates of the same species could form different types of interactions with *H. fraxineus*, e.g., *Didymella aliena* isolates: LS25-3O type B1, TS105-4M type D1, and TS43-1M and LS102-1M type D2. In the dual culture experiment, the inhibitory effect of endophytes on the radial growth of *H. fraxineus* was observed for 57 isolates (35 species). The radius of *H. fraxineus* colonies adjacent to their endophytic co-partners was reduced compared to that in the control. The mean inhibition index (*IRG*) varied significantly ($F_{(56,114)} = 6.37$, $p < 0.001$) among the endophytes (Table 3). The *IRG* values for most endophytes (52 isolates) did not exceed 0.25. The highest value of *IRG* was observed for *Fusarium lateritium* isolate TS94-4M, reaching 0.575 ± 0.017. The radial growth of *H. fraxineus* was the most reduced by four endophytic isolates from twigs (TS94-4M *Fusarium lateritium*, TS105-4M *Didymella aliena*, TA63-2O *Didymella macrostoma*, and TA52-5M *Dothiorella gregaria*) (Table 3). These isolates were selected for the in planta bioassay. Eighteen endophytic isolates (17 species) did not show an inhibitory effect on the growth of *H. fraxineus*. In this case, the radius of the *H. fraxineus* colonies was greater in the dual cultures than in the control.

Table 3. Inhibitory effect of endophytic fungi on the radial growth of *Hymenoscyphus fraxineus* in a dual culture experiment.

Isolate Name	Endophytic Fungus	Interaction Type *	Mean Inhibition Index ± SE **
TS94-4M	*Fusarium lateritium*	D2 (4.00)	0.575 ± 0.017 a
TS105-4M	*Didymella aliena*	D1	0.326 ± 0.019 b
TA63-2O	*Didymella macrostoma*	D2 (3.33)	0.318 ± 0.020 bc
TA52-5M	*Dothiorella gregaria*	D2 (3.33)	0.280 ± 0.041 bcd
LS92-2O	*Alternaria alternata*	D1	0.267 ± 0.026 bcde
TA93-2O	*Dothiorella gregaria*	D2 (3.00)	0.212 ± 0.010 bcdef
TA35-3O	*Ascochyta medicaginicola*	D1	0.202 ± 0.050 bcdef
LS25-3O	*Didymella aliena*	B1	0.197 ± 0.007 bcdef
TA31-4M	*Sporormiella minima*	C	0.196 ± 0.018 bcdef
TA31-3O	*Pyrenophora triseptata*	D1	0.195 ± 0.001 bcdef
LA45-4O	*Rosellinia corticium*	B2	0.191 ± 0.043 bcdef
LA74-1O	*Sordaria lappae*	D1	0.189 ± 0.029 bcdef
TS85-2M	*Diaporthe rudis*	D2 (2.00)	0.188 ± 0.038 bcdef
LS62-2O	*Alternaria alternata*	D1	0.176 ± 0.020 bcdef
TS102-1O	*Diaporthe eres*	B1	0.175 ± 0.007 bcdef
TA63-1O	*Ascochyta medicaginicola*	D1	0.174 ± 0.020 bcdef
TA34-1M	*Sordaria fimicola*	B1	0.153 ± 0.015 bcdef
TA23-1O	*Phoma herbarum*	D2 (9.33)	0.150 ± 0.019 bcdef
TA102-2O	*Didymella glomerata*	D1	0.139 ± 0.007 bcdef
TA63-1M	*Ascochyta medicaginicola*	D1	0.134 ± 0.024 bcdef
TA93-1M	*Microdiplodia* sp.	D2 (6.00)	0.128 ± 0.051 bcdef
TS31-1M	*Epicoccum nigrum*	B1	0.126 ± 0.007 bcdef
TS91-2M	*Rosellinia corticium*	D2 (5.00)	0.119 ± 0.014 bcdef
TA52-2O	*Hypoxylon fragiforme*	B1	0.117 ± 0.072 bcdef
LS21-1O	*Stemphylium vesicarium*	A	0.114 ± 0.028 bcdef

Table 3. *Cont.*

Isolate Name	Endophytic Fungus	Interaction Type *	Mean Inhibition Index ± SE **
LA44-5O	*Diaporthe eres*	B1	0.111 ± 0.031 bcdef
LA25-3O	*Stemphylium vesicarium*	A	0.106 ± 0.044 bcdef
LS31-2O	*Diaporthe rudis*	D1	0.104 ± 0.022 bcdef
TS43-1M	*Didymella aliena*	D2 (4.00)	0.101 ± 0.035 bcdef
LS102-1M	*Didymella aliena*	D2 (3.33)	0.100 ± 0.020 bcdef
LA94-1M	*Rosellinia corticium*	B2	0.100 ± 0.023 bcdef
LS61-2O	*Neodidymelliopsis camporesii*	D1	0.100 ± 0.028 bcdef
TS41-1M	*Diaporthe eres*	D2 (3.33)	0.098 ± 0.027 cdef
LA13-3O	*Alternaria alternata*	B1	0.086 ± 0.055 def
LS73-3O	*Sordaria fimicola*	B1	0.083 ± 0.032 def
LA52-1O	*Fraxinicola fraxini*	D1	0.077 ± 0.140 def
TS64-1O	*Cladosporium tenuissimum*	D1	0.077 ± 0.050 def
LA72-1O	*Anthostoma amoenum*	A	0.074 ± 0.017 def
LS22-1M	*Diplodia fraxini*	D1	0.072 ± 0.028 def
TA61-1M	*Dothiorella gregaria*	B1	0.071 ± 0.024 def
TS35-2O	*Diaporthe oncostoma*	D1	0.071 ± 0.021 def
LS103-3O	*Alternaria infectoria*	D1	0.070 ± 0.012 def
LA25-4O	*Nemania diffusa*	B2	0.068 ± 0.021 def
TS64-4O	*Epicoccum nigrum*	D2 (6.67)	0.057 ± 0.093 def
TA82-1O	*Stemphylium vesicarium*	D1	0.052 ± 0.011 ef
TA94-1O	*Phaeosphaeria* sp.	D2 (8.67)	0.052 ± 0.054 ef
TA91-3O	*Ascochyta pisi*	D2 (3.00)	0.052 ± 0.026 ef
TA55-5M	*Lepteutypa fuckelii*	B2	0.042 ± 0.022 ef
TA75-3M	*Diaporthe nobilis*	B1	0.041 ± 0.017 ef
LS44-1O	*Cladosporium tenuissimum*	D1	0.035 ± 0.006 f
TS52-2M	*Diaporthe eres*	B1	0.031 ± 0.044 f
TS43-1O	*Comoclathris incompta*	D2 (2.33)	0.030 ± 0.030 f
TS35-3O	*Foliophoma camporesii*	D1	0.018 ± 0.054 f
TS12-3M	*Diaporthe eres*	B1	0.017 ± 0.030 f
TA35-3M	*Neosetophoma aseptata*	B1	0.012 ± 0.022 f
TA55-2M	*Comoclathris incompta*	B2	0.010 ± 0.072 f
LA65-2O	*Aposphaeria corallinolutea*	D1	0.009 ± 0.034 f
TS62-1O	*Neodidymelliopsis camporesii*	D2 (4.33)	−0.005 ± 0.045
LS15-1O	*Diaporthe oncostoma*	D1	−0.006 ± 0.015
TS12-1O	*Phoma* sp.	D2 (8.00)	−0.015 ± 0.029
TS101-2O	*Fusarium lateritium*	D2 (3.33)	−0.017 ± 0.045
LA21-5O	*Ramularia endophylla*	D2 (6.00)	−0.019 ± 0.028
TS31-5M	*Diaporthe rudis*	D1	−0.022 ± 0.016
LS72-2O	*Rosellinia corticium*	B2	−0.027 ± 0.024
TA63-2M	*Dothiorella gregaria*	D2 (3.33)	−0.027 ± 0.024
LA51-2O	*Aureobasidium pullulans*	D2 (3.00)	−0.038 ± 0.008
TS24-1M	*Diaporthe eres*	B1	−0.039 ± 0.079
LS11-1O	*Neofabraea vagabunda*	D2 (8.00)	−0.042 ± 0.040
TA92-1O	*Lophiostoma corticola*	A	−0.053 ± 0.038
LS25-1M	*Fraxinicola fraxini*	D2 (3.33)	−0.102 ± 0.029
LS42-3M	*Fraxinicola fraxini*	D1	−0.106 ± 0.007
TA71-6M	*Diaporthe rudis*	D1	−0.109 ± 0.030
LA93-1O	*Cladosporium allicinum*	A	−0.111 ± 0.045
TS44-2M	*Diaporthe eres*	D1	−0.140 ± 0.009
LS85-2M	*Diaporthe oncostoma*	D2 (5.33)	−0.201 ± 0.010

* Observed interaction types: A—physical contact of mycelia; B1—*H. fraxineus* colony partially overgrown by an endophyte after initial deadlock with mycelial contact; B2—*H. fraxineus* colony overgrown by an endophyte without initial deadlock; C—an endophyte colony overgrown by *H. fraxineus*; D1—an inhibition zone present with a width of <2 mm; D2—an inhibition zone of >2 mm; the average width of the inhibition zone (mm) in parenthesis; ** mean inhibition indices followed by the same letter are not significantly different ($p = 0.05$).

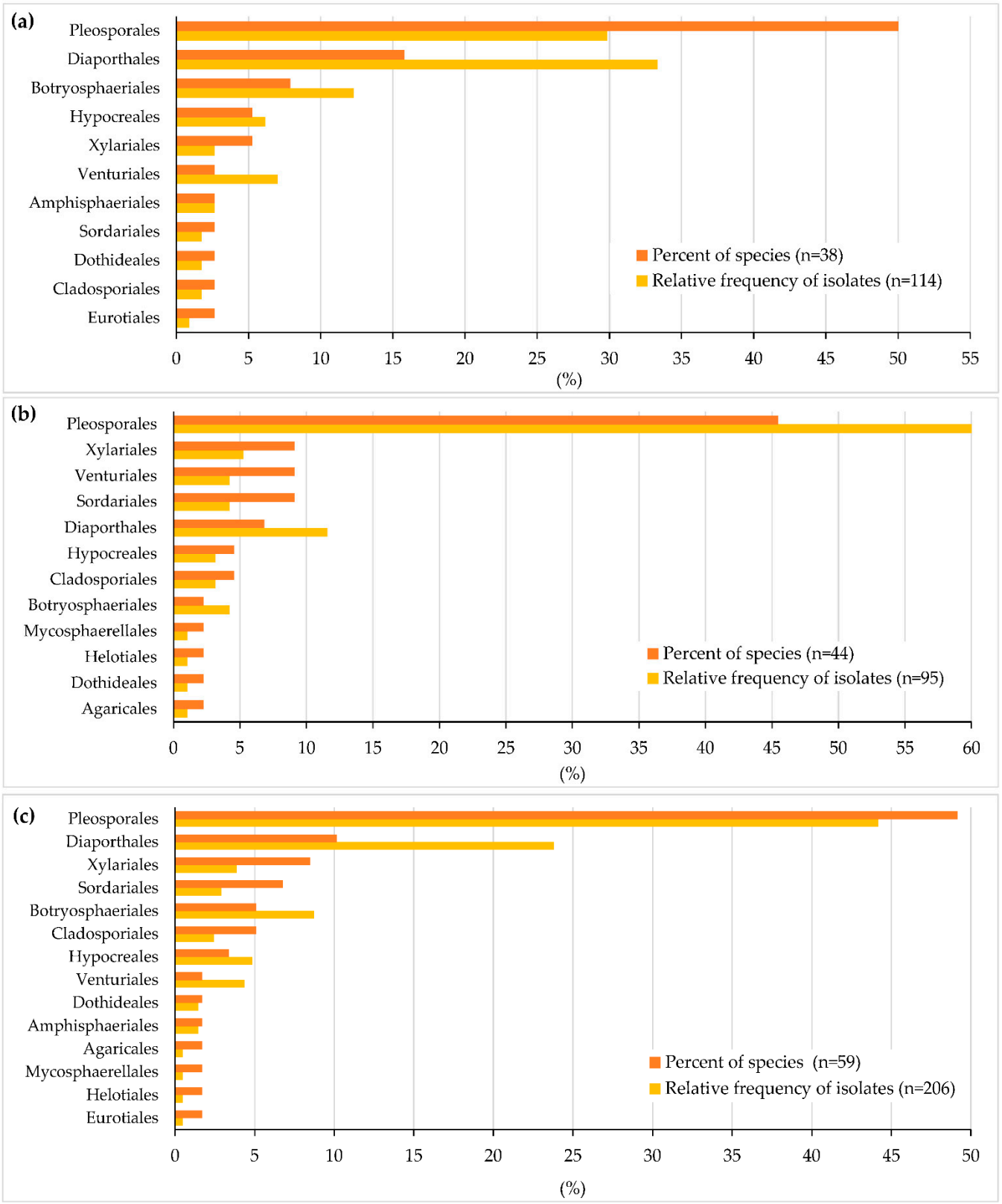

Figure 3. Relative frequency of isolates by taxonomic order and percentage proportions of species number in a particular order to the overall number of isolated species; (**a**) analysis of isolates from May 2019; (**b**) analysis of isolates from October 2019; (**c**) total number of isolates in 2019.

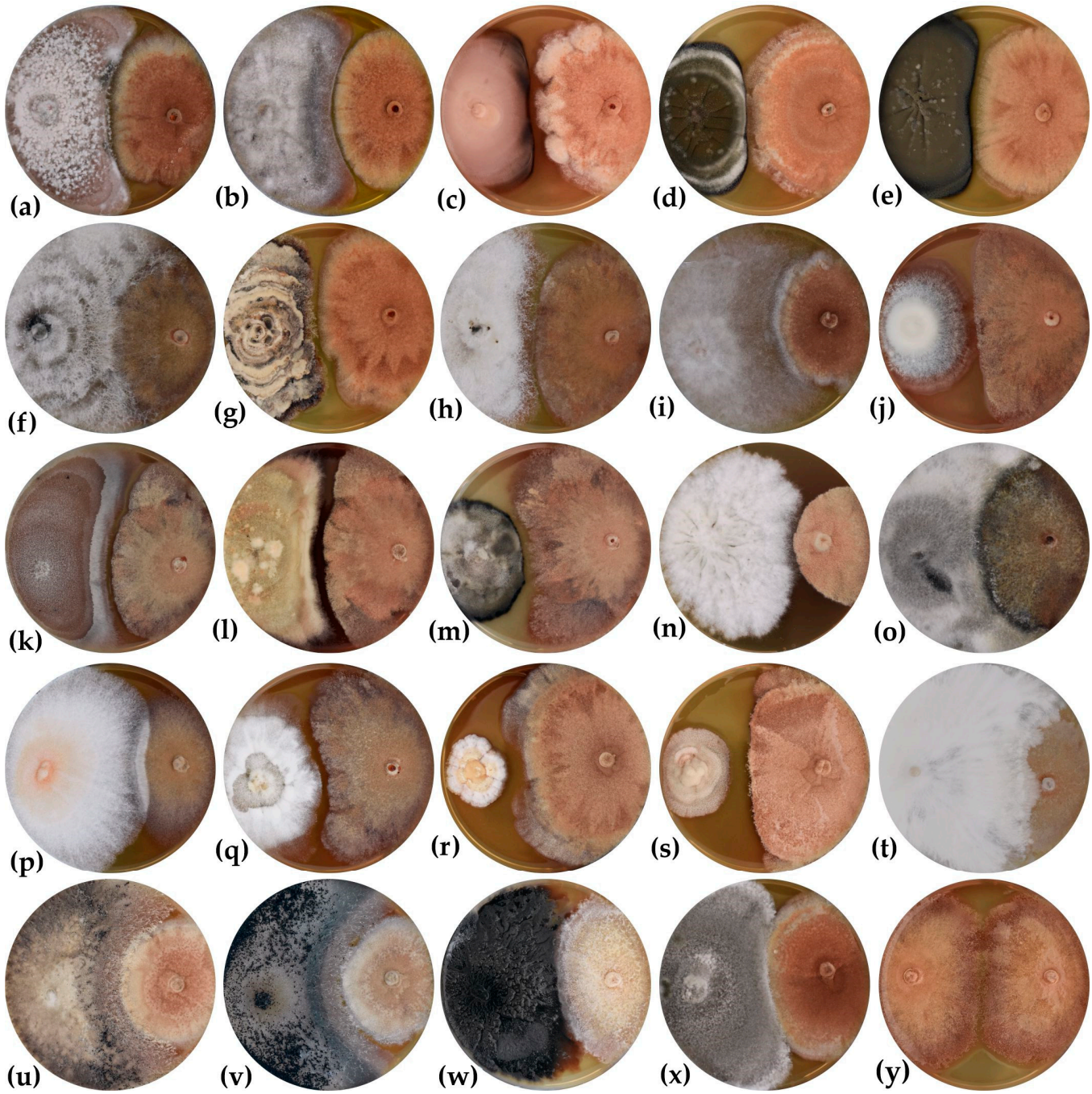

Figure 4. Mycelial interactions after 27 days of co-incubation between the isolate of *Hymenoscyphus fraxineus* (right) and selected isolates of endophytic fungi (left): (**a**) *Alternaria infectoria*, (**b**) *Ascochyta medicaginicola*, (**c**) *Aureobasidium pullulans*, (**d**) *Cladosporium allicinum*, (**e**) *Cladosporium tenuissimum*, (**f**) *Diaporthe eres*, (**g**) *Diaporthe oncostoma*, (**h**) *Diaporthe rudis*, (**i**) *Didymella aliena*, (**j**) *Didymella macrostoma*, (**k**) *Dothiorella gregaria*, (**l**) *Epicoccum nigrum*, (**m**) *Fraxinicola fraxini*, (**n**) *Fusarium lateritium*, (**o**) *Hypoxylon fragiforme*, (**p**) *Lepteutypa fuckelii*, (**q**) *Microdiplodia* sp., (**r**) *Neofabraea vagabunda*, (**s**) *Phaeosphaeria* sp., (**t**) *Rosellinia corticium*, (**u**) *Sordaria fimicola*, (**v**) *Sordaria lappae*, (**w**) *Sporormiella minima*, (**x**) *Stemphylium vesicarium*, (**y**) control—two plugs of *H. fraxineus* taken from the same colony.

3.3. In Planta Evaluation of the Inhibitory Effect of Endophytes

Four endophytic isolates from ash twigs of symptomatic (TS94-4M *Fusarium lateritium* and TS105-4M *Didymella aliena*) and asymptomatic (TA63-2O *Didymella macrostoma* and TA52-5M *Dothiorella gregaria*) trees that inhibited the *H. fraxineus* strain on MEA plates were selected for the in planta bioassay. The endophytic isolates used to artificially inoculate the trees formed no necroses, and the wounds made on the bark during inoculation were covered by protective callus tissue and were almost totally regenerated 180 days after inoculation (Figure 5b). The same situation was observed in the negative control when the trees were treated with sterile, non-colonized discs. Following *H. fraxineus* inoculation, typical ash dieback lesions appeared surrounding the inoculation points (Figure 5a). The necrotic lesions developed on the surface of the bark and in the wood tissue. The length of superficial necroses developed on the bark by the artificially inoculated *H. fraxineus* isolate (Figure 6a) varied depending on whether (the positive control) endophytic fungal isolates were co-inoculated on the experimental trees. Necroses were shorter on trees co-inoculated with endophytes TS94-4M and TS105-4M ($\overline{x}$ = 24.25 ± 4.77 and 17.50 ± 4.29, respectively) than on trees inoculated only with *H. fraxineus* ($\overline{x}$ = 34.00 ± 12.50 mm). This indicates a suppressive effect of the endophytes on *H. fraxineus* growth and corresponds with the results of the in vitro bioassay. However, the difference in necrosis length was not significant ($F_{(2,8)}$ = 1.305, $p > 0.05$). No suppressive effect on *H. fraxineus* was observed in trees inoculated with the endophytic isolates TA63-2O and TA52-5M. The lesions were of the same length ($\overline{x}$ = 34.00 ± 12.29 mm) or longer ($\overline{x}$ = 37.25 ± 8.41 mm) than lesions in the positive control.

The lengths of superficial necroses (on the bark) were shorter than the lengths of cambial necroses (Figure 6a,b), but the difference was not significant ($p > 0.05$). The average length of cambial necroses in the positive control reached 39.83 ± 14.61 mm, and the necroses on the experimental trees co-inoculated with the endophytes TS94-4M and TS105-4M were shorter (25.75 ± 4.46 mm and 17.75 ± 4.27 mm, respectively) than the control necrosis.

The necroses did not develop evenly above or below the inoculation points. On average, the length of necrosis in the acropetal direction (to the endophyte inoculation point) was shorter by 79.26 ± 18.37% (superficial necroses) and 75.25 ± 14.69% (cambial necroses) than the length in the basipetal direction (Figure 5a). However, the difference was not significant ($F_{(1,30)}$ = 3.734, $p > 0.05$ for superficial necroses, and $F_{(1,30)}$ = 3.304, $p > 0.05$ for cambial necroses). The growth of necroses depended on co-inoculated endophytes. Necroses on experimental trees co-inoculated with isolates TA52-5M and TS94-4M grew evenly in both directions, while necroses on trees co-inoculated with TA63-2O and TS105-4M grew predominantly in the basipetal direction. Necrotic wood depth at the inoculation point reached the pith of the tree trunk (Figure 5a), and the mean depth varied from 5.25 ± 0.25 mm to 7.50 ± 2.25 mm, depending on the co-inoculated endophytes.

The presence of *H. fraxineus* was confirmed in all necrotic lesions by species-specific PCR, which produced an amplicon of 456 bp.

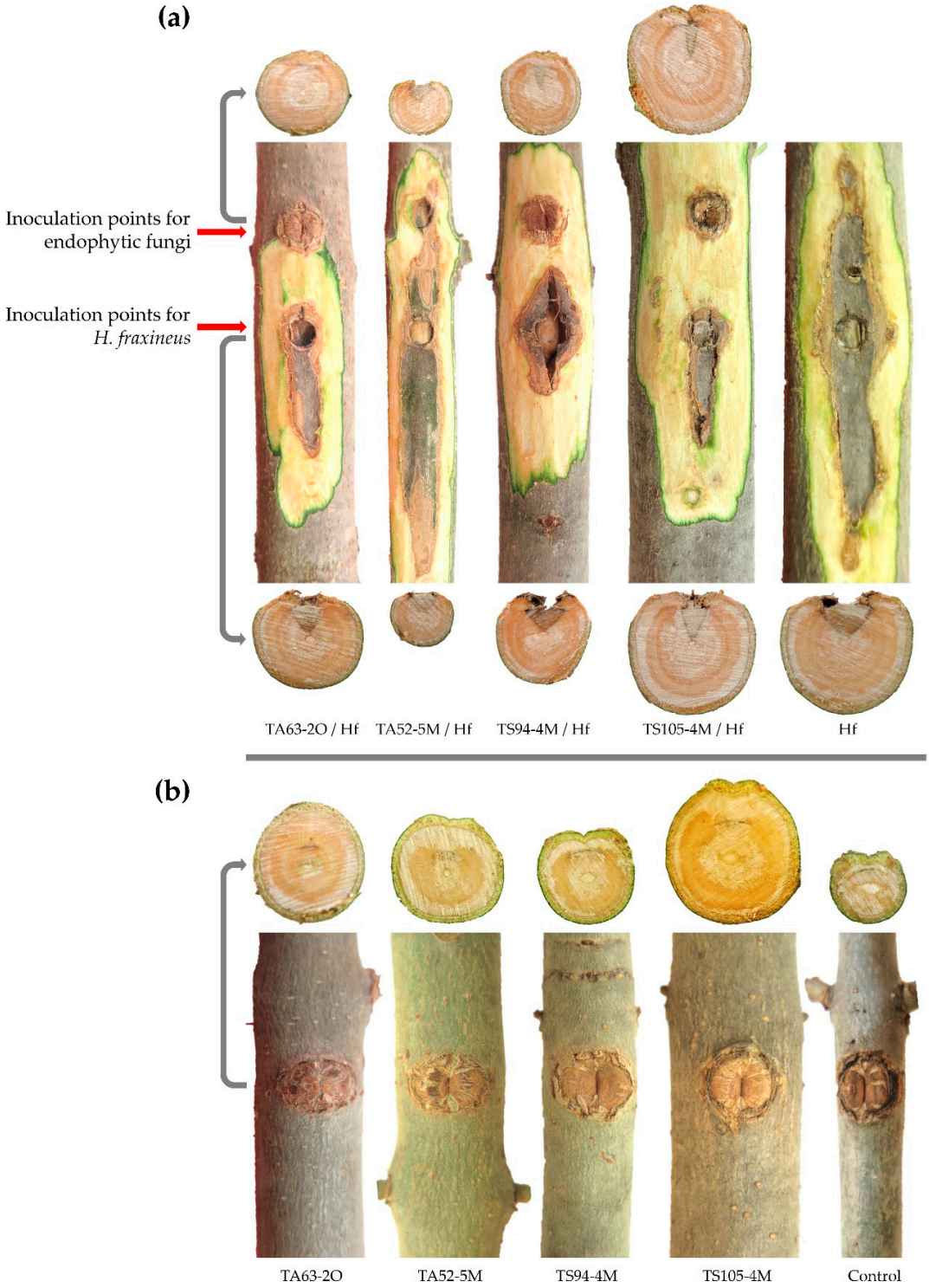

Figure 5. Necrotic lesions on the stems of five-year-old *Fraxinus excelsior* trees developed 180 days after their artificial inoculation with *Hymenoscyphus fraxineus* (Hf) and endophytic fungi *Didymella macrostoma* (TA63-2O), *Dothiorella gregaria* (TA52-5M), *Fusarium lateritium* (TS94-4M), and *Didymella aliena* (TS105-4M): (**a**) necroses developed after inoculation of the bark with *H. fraxineus* alone (the positive control) or in combination with endophytic fungal isolates including cross-sections through the ash stems at the inoculation points; (**b**) trees recovered from wounds performed on the bark of ash trees during inoculation with endophytic fungal isolates, including non-inoculated tree (the negative control).

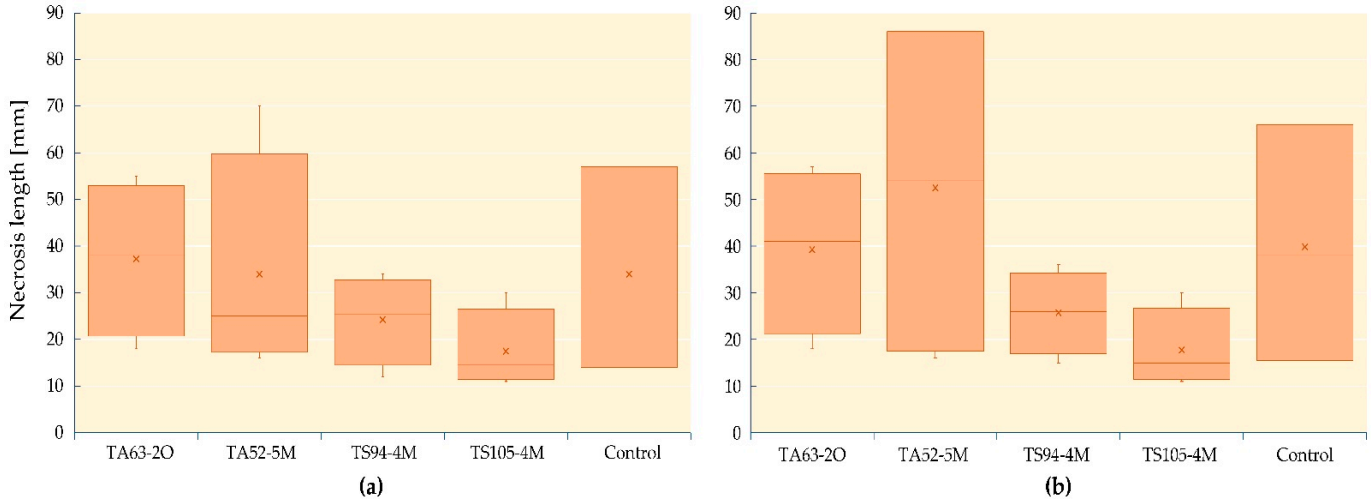

Figure 6. Box and whisker plots showing the length of superficial (**a**) and cambial (**b**) necroses developed by artificially inoculated *Hymenoscyphus fraxineus* on the bark of ash trees colonized or not colonized (control) by selected endophytic fungal isolates (TA63-2O—*Didymella macrostoma*, TA52-5M—*Dothiorella gregaria*, TS94-4M—*Fusarium lateritium*, TS105-4M—*Didymella aliena*).

4. Discussion

The results of this study revealed high species variability of culturable fungal endophytes in leaves and twigs from asymptomatic and symptomatic ash trees. Species from Pleosporales and Diaporthales were the most common endophytes, which corresponds to the findings of earlier research on endophyte diversity in ash trees in Europe [4,29,55]. Only limited information is available on fungal endophytes in ash trees in Slovakia [12,36,37], and this work considerably expanded the existing data. Most endophytic fungi identified in this study are not host-specific, e.g., *Alternaria alternata*, *Cladosporium cladosporioides*, and *Epicoccum nigrum*, and only two are strictly host-specific fungi, namely, *Diplodia fraxini* and *Fraxinicola fraxini*. The generalists were anticipated to predominate in the analyzed samples because the method of tissue examination for endophytes favored this group of fungi. *Nemania serpens*, *Diaporthe eres*, *Venturia fraxini* (a synonym for *Fraxinicola fraxini*), and *Diaporthe* sp. were the most frequent endophytes found in the leaf petioles of *F. excelsior* in Poland, as shown in a recent study [34]. Regardless of tissue type, the species structure of the most frequent endophytes identified from the ash trees in this study was different. Although *D. eres* was also the most frequently isolated species, *F. fraxini* and *N. serpens* were recovered from only nine leaf samples or a single twig sample, respectively. This discrepancy is possible because endophyte communities are not equal throughout the leaf tissue. For example, Schlegel et al. [56] discovered considerable variations in endophyte communities between leaf petioles and leaf laminae of ash and maple trees. It was evident that *D. eres* dominated in twig samples in May, whereas the second most frequent endophyte, *A. alternata*, was more prevalent in leaf and twig samples collected in October. The colonization frequency and species richness of endophytes increased with the age of leaf tissue but slightly decreased with the age of twig tissue. These differences may reflect the short life span of the leaves, and the nutrient composition and physiochemical variations between these two environments. We presume that leaf endophytes are transmitted horizontally, and that leaf colonization exists via infections directly from the environment, particularly in the case of generalist species that predominate in the analyzed samples. Different species of fungi require different lengths of time to produce spores, colonize tissue, and establish themselves in the hosts. Therefore, the colonization rate and species richness were lower in the samples collected in the spring (May) than in the fall season (October). It is generally accepted that the colonization rate of plant tissues by endophytic fungi increases with tissue age. Numerous studies have observed this finding [57–60]. In contrast to the leaf samples, the rate of colonization in twigs did not increase during the

season but decreased. The decrease was more prominent in twigs from symptomatic trees, and we presume that ash dieback disease has a negative effect on endophyte colonization. The species diversity indices calculated for both sampling dates were similar to those observed by Bilański and Kowalski [34] for endophytes in the leaf petioles of *F. excelsior*. The diversity increased slightly with the age of the leaves, but not with the age of the twigs.

Factors influencing the abundance and diversity of endophyte communities in woody plants have recently been discussed in detail by Sieber [61]. Because the identification of endophytes in this study was limited to culturable fungi, the true diversity and abundance of the endophytic community in the ash trees remained undiscovered. Many nonculturable and obligate biotrophic species could not be detected by the method used to examine endophytes in ash tree samples. Another important selection factor was the use of only one type of agar medium for endophyte isolation. However, we expect that incorporating ash leaf extract into the cultivating medium might facilitate the isolation of species that have closer interactions with the host trees. Slower-growing endophytic fungi could be outcompeted or inhibited in the medium by faster-growing species, and to eliminate this effect, Rose Bengal was used to retard the growth of fast-growing species [62], while allowing slower-growing fungi to emerge from ash tissues. Culture-dependent techniques tend to favor dominant endophytic fungi [63], and rarer species with irregular occurrences in ash trees might be missed with only two sampling occasions. Due to the limitations of the methodology used in this study, it is possible that a range of potential candidate endophytes with biocontrol capabilities was overlooked.

Fungal endophytes may interact with host plants in manifold ways, and a potential protective effect against plant pathogens is one of them [63,64]. The use of endophytic microorganisms to control plant pathogens is receiving increasing attention as a sustainable alternative to synthetic pesticides. In the last decade, the mutual relationships between endophytes and plant hosts have been studied to understand the effects of endophytes on plant pathogens and their potential use for biological control. The endophyte-based biocontrol strategy is not a novel idea and has been studied in many agricultural and horticultural crops [65–67]. Treatment of trees with endophytes with the purpose of inhibiting the development of plant-pathogenic fungi is also under consideration [21,63,68–70]. Recently, several studies have been published that have determined the antagonistic potential of fungal endophytes against *H. fraxineus* [27,29,34,71]. This study confirmed the antagonistic activity of several local endophytic isolates against *H. fraxineus* in a dual culture bioassay. The strongest antagonistic effect was observed for *F. lateritium*, *D. aliena*, *D. macrostoma*, and *D. gregaria*. The antagonistic effect was coupled with the production of a wide inhibition zone in the cases of *F. lateritium*, *D. macrostoma*, and *D. gregaria*, which indicated a release of metabolites into the culture medium with an inhibitory effect against *H. fraxineus* growth. In a similar dual culture experiment, *F. lateritium* was listed among five endophytes with the strongest inhibition effect against *H. fraxineus* [34]. This fungus is also known as a natural antagonist of the plant pathogen *Eutypa armeniacae* (synonym of *Eutypa lata*), causing sapwood necrosis in fruit trees, grapevines, and ornamental plants [72]. However, *F. lateritium* is known as a globally distributed plant pathogen and has been reported in approximately 180 hosts, mainly woody plants, where it causes wilt, tip, or branch dieback, and cankers [73,74]. *Dothiorella gregaria* is also linked to branch and trunk canker in several plants [75]. However, the *Didymella* genus mainly includes saprobes commonly found in living or dead parts of plants [76]. In a recent study, another endophyte from the ash tree *Hypoxylon rubiginosum* appeared to be a promising biocontrol endophyte with strong fungitoxic properties and an antagonistic effect on *H. fraxineus* in planta [31]; however, it is also a mild pathogen, sometimes causing cankers on plants, including ash trees [77]. In other in vitro bioassays, different endophytes demonstrated inhibitory effects against isolates of *H. fraxineus*. For example, *Sclerostagonospora* sp., *Setomelanomma holmii*, *Epicoccum nigrum*, *Boeremia exigua*, and *Fusarium* sp. [29] or *Plenodomus biglobosus* and *Paracucurbitaria corni* [71] inhibited the growth of *H. fraxineus* in dual cultures. The inhibitory effect on the germination of *H. fraxineus* ascospores has also been documented for several leaf en-

dophytes isolated from *F. excelsior*, e.g., *Fraxinicola fraxini*, *Paraconiothyrium* sp., *Boeremia exigua*, *Kretzschmaria deusta*, *Pezicula* sp., *Neofabraea alba* (synonym of *Neofabraea vagabunda*), and *Ampelomyces quisqualis* [27].

Although several studies have confirmed the antagonistic effect of fungal endophytes against *H. fraxineus* under in vitro conditions, their effect under in planta conditions has not yet been verified. Laboratory studies may not be good predictors of biocontrol agents' protective capacity, and, unfortunately, most research on tree pathogen–endophyte interactions has been conducted in the laboratory. It is uncertain how the interactions would change in the face of changing environmental conditions and existing competition with other species in the tree ecosystem; therefore, in planta bioassays are necessary. Moreover, the modes of action of most endophytes as biocontrol agents are still unknown, and bioassays on host plants may explain them. For example, the antagonistic effect of fungal endophytes on trunk necrosis development has already been documented in plants. Endophytic *Trichoderma aureoviride* used to inoculate the trunk was able to significantly reduce the necrosis size compared to the control on 30-year-old beech trees artificially inoculated with *Phytophthora plurivora* [69]. We evaluated the trunk inoculations of the four endophytic isolates, showing a strong inhibitory effect in the laboratory against inoculated *H. fraxineus*. Although the total length of necrotic lesions formed by the *H. fraxineus* infection was shorter in the ash trunks co-inoculated with the endophytes than in the trunks without the endophytes, the difference was not significant. The presence of *H. fraxineus* was confirmed in the necrotic lesions on all trunks (inoculated and non-inoculated with endophytes) six months after inoculation, which demonstrated that the endophytes could not eliminate the pathogen. The effect of endophytes on the development of necrosis was most prominent in the direction of the inoculation points of *Didymella macrostoma* and *Didymella aliena*. The weakest effect on *H. fraxineus* was *Dothiorella gregaria*. Although trunk inoculations did not produce optimistic results in this research, trunk inoculation with endophytes against phytopathogens has potential. It is likely that some fungal species can stimulate the ash's immune system against *H. fraxineus* infection, and this supports the importance of further research in the fight against this pernicious pathogen.

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