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Fungi Detected in the Previous Year's Leaf Petioles of *Fraxinus excelsior* and Their Antagonistic Potential against *Hymenoscyphus fraxineus*

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Abstract: Studies on fungal communities in the previous year's leaf petioles of *Fraxinus excelsior* found in litter in five ash stands in southern Poland were made in 2017. Fungi were identified on the basis of isolation from 300 surface sterilized leaf petioles and by in situ inventory of fruit bodies (on 600 petioles, in spring and autumn). Identification was based on morphology of colonies and fruit bodies, and sequencing of ITS region of the rRNA gene cluster. In total, 2832 isolates from 117 taxa (Ascomycota—100; Basidiomycota—15; Mucoromycota—2 taxa) were obtained with the isolation method. The most frequent taxa (with frequency >10%) were: *Nemania serpens*, *Hymenoscyphus fraxineus*, *Alternaria* sp. 1, *Boeremia* sp., Helotiales sp. 1, *Epicoccum nigrum*, *Venturia fraxini*, *Fusarium* sp., *Fusarium lateritium*, *Nemania diffusa*, *Typhula* sp. 2 (in descending order). In total, 45 taxa were detected with the in situ inventory method. Eleven taxa were classified as dominant: *Hymenoscyphus fraxineus*, *Venturia fraxini*, *Leptosphaeria* sp. 2, *Cyathicula fraxinophila*, *Typhula* sp. 2, *Hypoderma rubi*, *Pyrenopeziza petiolaris*, *Cyathicula coronata*, *Hymenoscyphus scutula*, *Leptosphaeria sclerotioides* and *Hymenoscyphus caudatus*. Among 202 leaf petioles colonized by *H. fraxineus*, 177 petioles also showed fructification of 26 other fungi. All the isolated saprotrophs were tested in dual-culture assay for antagonism to two strains of *H. fraxineus*. Three interaction types were observed: type A, mutual direct contact, when the two fungi meet along the contact line (occurred with 43.3% of test fungi); type B, with inhibition zone between colonies (with 46.9% of test fungi); type C, when the test fungus overgrows the colony of *H. fraxineus* (with 9.8% of test fungi). The possible contribution of the fungal saprotrophs in limiting of the expansion of *H. fraxineus* in ash leaf petioles, which may result in reduction in the inoculum of ash dieback causal agent, is discussed.

Keywords: antagonism; ash dieback; biodiversity; competition; decomposition; leaf petioles

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1. Introduction

European ash (*Fraxinus excelsior* L.) started to show symptoms of serious disease in the early 1990s and, currently, dieback can be seen in most of its range in Europe [1–8]. The disease is caused by an alien ascomycete, *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya, which probably came from Eastern Asia where it occurs as an endophyte, extensive leaf colonizer and, locally, as a leaf pathogen of *Fraxinus mandshurica* Rupr. and *Fraxinus chinensis* Roxb. ssp. *rhynchophylla* (Hance) A. E. Murray [9–11]. The role of leaves, mainly petioles, as sites of infection in the *H. fraxineus* life cycle and ash dieback has been emphasized. Each year *H. fraxineus* forms apothecia, predominantly on the previous year's leaf petioles and leaflet veins, in the litter on the forest floor [5,7,8]. In Poland, apothecia of *H. fraxineus* are formed mainly in July and August. However, in some years, they can also be found in September and October [12]. In other European regions, apothecia can be found much earlier, from the middle of May [13].

Wind-disseminated ascospores of *H. fraxineus* infect leaves of *F. excelsior*, and both leaf blades and leaf petioles have been confirmed as targets for primary infections [14]. The

fungus is able to colonize all types of ash-leaf cells. In ash leaf petioles, its hyphae were observed in xylem vessels, phloem, axial parenchyma cells and also in the pith region [14].

After infection, the first symptoms in the form of local necrotic lesions appear on leaf blades, midribs and petioles [14]. The fungus can spread to woody tissues through the petiole-shoot junction prior to leaf fall [15]. The first symptoms are followed by dieback of shoots, twigs or main stems, resulting in crown dieback, bark lesions and sapwood discoloration.

During colonization of fallen leaves, *H. fraxineus* forms pseudosclerotial plates on petioles and some leaflet veins. Numerous new apothecia are formed on them in the following year, starting from early summer and completing the pathogen's one-year life cycle associated with leaves [5,8,16]. On shoots and stems, apothecia of *H. fraxineus* are formed only sporadically and have no significance in the development of epidemics [5,7,8,17,18].

Options for preventing, inhibiting or mitigating disease and damage caused by *H. fraxineus* in forest stands are very limited [8,19–21]. There are, however, some environmentally friendly alternatives to chemical control.

Considering the significance of petioles as a necessary substrate for the formation of apothecia and production of *H. fraxineus* inoculum, there is the possibility of removing (burning or composting) fallen leaves. Such methods may be possible in tree nurseries or town greenery but not, however, in forest stands [22]. In the latter, creating an environment suitable for faster decomposition of ash leaves and debris may be considered [19,20].

Another alternative considered increasingly often is microbial biocontrol, similar to that used with some success against diseases of some agriculturally important herbaceous plants and forest trees [23–25]. Several common *Fraxinus* leaf endophytes (e.g., *Boeremia exigua*, *Kretzschmaria deusta*, *Paraconiothyrium* sp., *Neofabraea alba* and *Venturia fraxini*) have been shown able to reduce germination of *H. fraxineus* ascospores [26], and some *F. excelsior* leaf and stem endophytes (e.g., *B. exigua*, *Epicoccum nigrum*, *Hypoxylon rubiginosum* and *Phoma macrostoma*) can inhibit growth of *H. fraxineus* hyphae [27–30]. Certain forest fungal communities can stimulate decomposition of litter and may be employed in the long term for indirect microbial control. The required quality of litter and its desirable microbial status can be achieved in certain conditions or after certain physical and chemical treatments [20,31–35]. For example, higher concentrations of nitrogen (N) and phosphorus (P) in litter will promote its decomposition, while a higher concentration of lignin (difficult to degrade) will often inhibit its degradation and removal [35–37]. Urea generally accelerates the decomposition of leaf debris [19] and additionally prevents the formation of *H. fraxineus* apothecia (important for dissemination of spores).

Leaves of *F. excelsior* are easily degraded compared with leaves of other broad-leaved trees in temperate forests [20,38,39] because of their lower C/N ratio and lower lignin content [40–42]. However, *F. excelsior* leaf petioles, which constitute 20% of leaf biomass, are more resistant to decomposition [20,43]. Starch concentration, considerably higher in ash petioles than in leaflets [43], ensures continuously favorable conditions for fungi. Additionally, infected petioles are the substrate for formation of pseudosclerotial plates of *H. fraxineus* which protect the fungus from biotic and abiotic stresses [16].

Fungi are the key players in litter decomposition and nutrient cycling owing to their ability to produce a wide range of extracellular enzymes which allow them to attack efficiently the recalcitrant lignocellulose matrix [44–51].

There are only few reports from qualitative and quantitative mycological analyses of *F. excelsior* petioles from litter [52,53]. More studies have been made on endophytes in petioles of living leaves, with and without symptoms, aimed at detecting possible pathogens [54–56].

The fungi of most relevance in leaf petioles from the litter are those which are antagonistic and suppressive to *H. fraxineus*, inhibiting the pathogen's growth and reducing inoculum production. Such activity depends on colonization strategies and relationships among different members of the fungal community [57,58]. This subject needs careful investigation, as in other studies on endophytes [27,28,54].

The objectives here were to evaluate: (i) fungal species diversity in leaf petioles of *F. excelsior* from the previous year, found in the litter, using the classical isolation method and an inventory made in situ, based on the occurrence of fruit bodies; and (ii) interactions between *H. fraxineus* and co-occurring members of fungal communities, in dual cultures, in vitro, and any reduction in growth rate in each fungus. An additional objective was to assess the risk of leaf petioles of *F. excelsior* being exploited as a substrate for production of inoculum by other pathogens of trees and annual plants.

2. Materials and Methods

2.1. Study Sites and Sampling

The study sites were five stands of *F. excelsior*, 27–82 years old, in Brody, Dynów, Miechów, Myślenice and Ojców, located in southern Poland (Table 1). *Fraxinus excelsior* was growing in mixed populations with other trees, mostly broad-leaved, and comprised 10–80% of the total of trees. The ash trees were showing symptoms of dieback similar to those observed over many years in other *F. excelsior* stands [59]. Leaf petioles from the previous year were collected from the litter on the forest floor between 10 May and 8 June 2017 (Table 1). A total of 6 petioles were collected beneath each of 30 trees located in various parts of each stand (180 petioles per stand). On the next day, in the laboratory, each sample was randomly divided into three subsamples, each of 60 petioles. Petioles were stored temporarily in sterile plastic boxes at 5 °C. Subsamples (I, II, III) were treated differently.

Table 1. Sampling sites and number of leaf petioles of *F. excelsior* from the previous year analyzed in the study.

Research Plots	Brody	Dynów	Miechów	Myślenice	Ojców
Coordinates	49°51′34″ N 19°40′32″ E	49°47′17″ N 22°15′20″ E	50°13′02″ N 20°02′09″ E	49°56′45″ N 19°52′48″ E	50°11′59″ N 19°48′43″ E
Altitude [m a.s.l.]	369	417	281	356	446
Fraxinus excelsior:					
age	54	60	27	82	63
Share (%)	30	10	10	50	80
Other tree species present [1]	Ag, Ap, Cb, Qr, Tc	Bp, Cb, Fs, Ps, Qr	Ap, Fs, Ld, Pt, Qr	Ap, Ag, Ld, Qr, Tc	Ap, Bp, Fs, Ps, Qr
Habitat type	Fresh upland broadleaved forest	Fresh upland broadleaved forest	Fresh upland broadleaved forest	Fresh upland broadleaved forest	Fresh upland broadleaved forest
Sampling date	10 May 2017	16 May 2017	08 June 2017	23 May 2017	30 May 2017
Number of petioles (petiole sections) used in fungal isolations	60 (720)	60 (720)	60 (720)	60 (720)	60 (720)
Number of petioles (petiole sections) yielding colonies of fungi	60 (419)	60 (582)	60 (465)	60 (487)	60 (548)

[1] Ag—*Alnus glutinosa*, Ap—*Acer pseudoplatanus*, Bp—*Betula pendula*, Cb—*Carpinus betulus*, Fs—*Fagus sylvatica*, Ld—*Larix decidua*, Ps—*Pinus sylvestris*, Pt—*Populus tremula*, Qr—*Quercus robur*, Tc—*Tilia cordata*.

2.2. Fungal Isolation and Identification

Subsample I was used for isolation of fungi and identification based on colony morphology (Table 1). In total, isolations were made from 300 petioles (3600 pieces). After removing the remains of leaflets, petioles were surface sterilized by soaking first for 1 min in 96% ethanol, then for 5 min in sodium hypochlorite solution (NaOCl, approx. 4% available chlorine) and finally for 30 s in 96% ethanol. After drying in layers of blotting paper, each petiole was divided into 12 small, equally sized pieces which were placed on the surface of malt extract agar (MEA; 20 g L^{-1} malt extract (Difco; Sparks, MD, USA), 15 g L^{-1} Difco agar) supplemented with 100 mg L^{-1} streptomycin sulphate to suppress bacterial growth, in two Petri dishes. The sequence of petiole pieces (1–12, from base to apex) was marked on the Petri dish cover, 1–6 and 7–12. The plates were incubated in darkness at 20 °C for 42 d and examined every 1–2 weeks. Emerging individual mycelia were regularly transferred to sterile Petri dishes with MEA and incubated for 3–4 weeks. Initial identification of fungi was based on morphology, which included color and structure of the colony, presence of

stromatic structures and the type of anamorphic conidiation and conidia. Isolates were grouped in morphotypes [60]. Morphology of *H. fraxineus* was compared with descriptions in previous studies [2,61,62].

2.3. In Situ Inventory of Fungi from Fruit Bodies on Petioles

This was based on the presence of fruit bodies and survival structures formed on leaf petioles, either in May–June or from the beginning of September to the end of November, in subsamples II and III, respectively [63]. Petioles from subsample II were stored at 5 °C and analyzed as quickly as possible.

Petioles from subsample III were laid out, avoiding contact between petioles from different stands, on the surface of a fenced meadow which was moderately shaded by trees and bushes (with no *Fraxinus*) and moderately humid, with extensive patches of mosses, particularly of *Atrichum undulatum* Web. et. Mohr. and *Pleurozium schreberi* (Willd. ex Brid.) Mitt., among grasses. They were examined with a magnifying lens every 10–14 days. Petioles with fructification were placed on moist filter paper in plastic boxes and transferred to the laboratory for subsequent mycological analysis. Representative, strongly colonized petioles were deposited in an herbarium. Most petioles, however, were marked with a number and returned to the meadow for further fructification. In results, assessments in May–June (subsample II) and September–November (subsample III) are referred to as "spring" and "autumn", respectively.

Identification of *H. fraxineus* was based on the presence of pseudosclerotial plates or the pathogen's anamorph (in subsample II) [16,62,64] or of apothecia formed on pseudosclerotial plates, naturally, in July–August (in subsample III). Identification of the genus *Typhula* was based on the presence of basidiocarps or ungerminated sclerotia [65,66].

Observations and photomicrographs were made with a Zeiss V12 Discovery stereo microscope and with a Zeiss Axiophot light microscope using differential interference contrast (DIC) illumination and an AxioCam MRc5 and HR3 cameras (Zeiss, Göttingen, Germany). For microscopic analysis, hand-cut sections and 1–3 slides were prepared from each fruit body being studied. Fungi were classified and identified to the lowest taxonomic rank using mycological keys and monographs.

Isolation as pure cultures was attempted on each differentiated taxon, from ascospores formed in perithecia/pseudothecia, basidiospores formed in basidiocarps (for *Marasmius*, *Typhula*), conidia formed in conidiomata or sclerotia, which were spread or placed on MEA, either in a Petri dish or inside a Petri dish cover, and incubated at 20 °C [67].

Cultures representative of taxa distinguished on the basis of fruit bodies or survival structures were analyzed with the nucleotide sequences of ITS region of the rRNA gene cluster.

The term "petiole" is used for petiole and rachis. The petiole was considered as colonized by a certain taxon if it was isolated from at least 1 of 12 petiole pieces. Representative fungal isolates are stored on MEA slants at 5 °C in the Culture Collection, and exsiccates of fungi in Mycotheca of the Department of Forest Ecosystems Protection, University of Agriculture, in Kraków. The nomenclature of taxa follows Index Fungorum [68].

2.4. DNA Extraction, PCR and Sequencing

To verify the morphology-based identification and to identify non-sporulating morphotypes, the nucleotide sequences of the ITS region of the rRNA gene cluster of representative cultures were determined.

DNA was extracted from 3-week-old cultures using the Genomic Mini AX Plant Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. The ITS region of the rRNA gene cluster was amplified for sequencing. Primers used were ITS1-F [69] and ITS4 [70] or ITS5 and ITS4 [70]. Gene fragments were amplified in 25 µL reaction mixture containing 0.25 µL Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), 5 µL Phusion HF Buffer (5x), 0.5 µL dNTPs (10 mM), 0.75 µL DMSO (100%), 0.5 µL of each primer (25 µM) and 20–100 ng

μL^{-1} of template DNA. PCR amplification was performed in a Labcycler thermocycler (SensoQuest Biomedical Electronics GmbH, Göttingen, Germany) under the following conditions: an initial 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 Tm of the primers and fungal species) and 30 s at 72 °C, and a final elongation at 72 °C for 8 min. The PCR products were visualized on 2% agarose gel stained with Midori Green DNA Stain (Nippon Genetics Europe) under UV light. Amplified products were sequenced bi-directionally with the BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), at the DNA Research Centre (Poznań, Poland) using the same primers as for the PCR.

Searches using the BLASTn algorithm were performed to retrieve similar sequences from NCBI GenBank (http://www.ncbi.nlm.nih.gov accessed on 2 August 2021) [71]. Newly obtained sequences were deposited in GenBank with accession numbers presented in Supplementary Material Table S1.

Sequence matches of ≥98% were used to define species boundaries and matches ≥90% were used to define order boundaries [72]. If sequencing of the ITS region of the rRNA gene cluster was ambiguous, the taxonomy was determined by phylogenetic analysis with reference sequences from NCBI GenBank.

The ITS region of the rRNA gene cluster sequences were aligned using MAFFT v.6 [73] with the E-INS-i option, 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 [74].

Phylogenetic analyses were performed individually, for each dataset, using maximum likelihood (ML) and Bayesian inference (BI). The best-fit substitution models for dataset were established for ML and BI using the corrected Akaike Information Criterion (AICc) in jModelTest v.2.1.10 [75,76]. Maximum likelihood (ML) analysis was run in PhyML 3.0 [77] via the Montpelier online server (http://www.atgc-montpellier.fr/phyml/ (accessed on 2 August 2021)) with 1000 bootstrap replicates. The best evolutionary substitution model for the ITS region of the rRNA gene cluster was GTR+I+G. The BI analysis was carried out in MrBayes v.3.1.2 [78]. The MCMC chains were run for 10 million generations using the best-fit model. Trees were sampled every 100 generations, resulting in 100,000 trees from both runs. The default burnin, first 25% of samples, was used. The resulting phylogenetic trees (Supplementary Material Figure S1) contain all the ITS region of the rRNA gene cluster sequences generated in this study.

2.5. Dual-Culture Assays

Representative fungi of each morphotype from subsample I were screened for their ability to suppress mycelial growth of two isolates of *H. fraxineus* by in vitro dual-culture assays on malt extract agar (MEA).

The *H. fraxineus* isolates were obtained in 2017 from the previous year's leaf petioles with pseudosclerotia found in the litter, in two different *F. excelsior* stands in southern Poland. Both isolates (Hf1 and Hf2) were characterized by moderately quick and even growth (35 and 39 mm on MEA after 3 weeks at 20 °C).

Plugs (8 mm diameter.) of young, actively growing mycelium of *H. fraxineus* and the test fungus were placed simultaneously on MEA in a Petri dish, opposite each other and 25 mm from the Petri dish edge. Plates inoculated only with *H. fraxineus* served as negative controls. Fungal growth was measured after incubation for 3 weeks in darkness at 20 °C. The measurements were: (i) radial mycelial growth of *H. fraxineus* (Ri) and of the saprotrophic test fungus (Rs) taken along an axis joining the centers of the plugs of the two fungi; (ii) radial mycelial growth of *H. fraxineus* on the control plate (Rc); (iii) the average of the maximum radial mycelial growth in the upper and lower parts of the test-fungus colony (Rm). Percentage inhibition of radial growth of *H. fraxineus* and of the test fungus was calculated according to the formulae $(Rc - Ri)/Rc \times 100$ and $(Rm - Rs)/Rm \times 100$, respectively [79,80]. The width of the inhibition zone (mm) was measured along the axis

joining the plugs of the two fungi. Growth was measured with a ruler to an accuracy of 1 mm.

Previous observations showed that four types of interactions between *H. fraxineus* and a test fungus can be expected: type A, mutual direct contact, when both colonies meet along the contact line, with no inhibition zone; type B, with an inhibition zone between colonies; type C, the test fungus overgrows and covers the colony of *H. fraxineus*; type D, the *H. fraxineus* colony overgrows the colony of the test fungus [81]. For type B, the width of inhibition zone was scored as: Bs, <3 mm; Bm, 4–5 mm; Bw, 6–8 mm; Bv, >8 mm. Inhibition of mycelial growth was scored according to the amount of decreased growth as: f, no reduction; a, <25%; b, 26–50%; c, 51–75%; d, >75%.

When a test fungus overgrew and covered the *H. fraxineus* colony, its efficacy against of *H. fraxineus* was checked using re-isolations of the fungus (from 6–12 inocula taken from each combination of pathogen and test fungus).

2.6. Data Analyses

Frequency of an individual taxon was defined as percentage (%) of petioles colonized by that taxon. Diversity in microfungal communities from the five stands was analyzed with Shannon diversity index (H') and Simpson's diversity index (SDI) [82,83]. Species dominance in microfungal communities was determined with the Camargo method [84].

The association between frequency of fungi and their origin was analyzed using principal component analysis (PCA). PCA was performed for species with at least 10% frequency in subsample I (communities isolated from the previous year's leaf petioles) and 5% frequency in subsamples II and III (detected as fruit bodies on petioles).

The same dataset was used to determine whether the differences in the occurrence of fungi on particular stands were statistically significant using the Marascuilo procedure. Similarities in fungal communities from the five stands were analyzed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), clustering based on the Bray–Curtis similarity index.

Co-occurrence of taxa in two fungal communities isolated from leaf petioles (subsample I) or detected as fruit bodies (subsamples II and III) was analyzed using a co-occurrence network created with Gephi visualization software v.0.9.2 combined with the Fruchterman–Reingold layout or the Yifan Hu proportional layout graph algorithm [85], after previous calculation of Spearman's rank correlation coefficient and statistically significant difference ($p < 0.05$). All calculations were made using Statistica v.12 [86], PAST v.3.11 [87] and Marascuilo procedure in R software v.3.5.1 [88].

3. Results

3.1. Fungi Isolated from the Previous Year's Leaf Petioles

All 300 leaf petioles of *F. excelsior* from the previous year and collected from the litter yielded fungi. A total of 2832 cultures was successfully isolated from 2501 petiole pieces (69.5% of the 3600 pieces) on MEA. Cultures were obtained slightly more frequently from basal petiole pieces (72.2%) than from pieces nearer the apex (66.7%). A total of 164 morphotypes was distinguished (Supplementary Material Table S1). Since a single taxon could be represented by 1–5 visually different morphotypes, sequencing of ITS region of the rRNA gene cluster of representative isolates resulted in detection of 117 fungal taxa (Supplementary Material Tables S1 and S2). The number of taxa detected in a single stand ranged from 53 (Brody) to 70 (Dynów) (Table 2).

Table 2. Fungi isolated from the previous year's leaf petioles of *F. excelsior* in the litter in five forest stands in southern Poland in spring 2017.

Taxon	Phylum [1]	Order	Number of Colonized Ash Petioles in Study Plots [2]					
			1	2	3	4	5	Total Number (%)
Agaricomycetes sp.	B	-			4		5	9 (3.0)
Alternaria sp. 1	A	Pleosporales	11_{a} [3]	16_{a}	9_{a}	20_{a}	11_{a}	67 (22.3) [4]
Alternaria sp. 2	A	Pleosporales	1					1 (0.3)
Alternaria sp. 3	A	Pleosporales				2	7	9 (3.0)
Alternaria sp. 4	A	Pleosporales	1					1 (0.3)
Athelia sp.	B	Atheliales		1				1 (0.3)
Aureobasidium pullulans	A	Dothideales	3	2	1			6 (2.0)
Boeremia exigua	A	Pleosporales		1	1	3	2	7 (2.3)
Boeremia sp.	A	Pleosporales	4_{bc}	13_{ab}	26_{a}	17_{a}	2_{c}	62 (20.7) [4]
Chaetomium globosum	A	Sordariales		2				2 (0.7)
Chromelosporium sp.	A	Pezizales	2		1	1	1	5 (1.7)
Cladosporium sp. 1	A	Capnodiales		2	2	1		5 (1.7)
Cladosporium sp. 2	A	Capnodiales	3	1	6	1	1	12 (4.0) [4]
Clonostachys rosea	A	Hypocreales	2		2	1		5 (1.7)
Colletotrichum acutatum	A	Glomerellales	4	2	4	4		14 (4.7) [4]
Coniochaeta angustispora	A	Coniochaetales	1					1 (0.3)
Coniochaeta sp. 1	A	Coniochaetales	1		2	3	2	8 (2.7)
Coniochaeta sp. 2	A	Coniochaetales			2			2 (0.7)
Coniochaeta sp. 3	A	Coniochaetales	6	2		2	3	13 (4.3) [4]
Coniothyrium cf. *dispersellum*	A	Pleosporales				7		7 (2.3)
Coprinellus disseminatus	B	Agaricales			1	2		3 (1.0)
Coprinellus micaceus	B	Agaricales					1	1 (0.3)
Cyathicula coronata	A	Helotiales		1	1	1	2	5 (1.7)
Cyathicula fraxinophila	A	Helotiales	1	2	3	2	1	9 (3.0)
Cyathicula sp. 2	A	Helotiales	1	1			1	3 (1.0)
Cytospora pruinosa	A	Diaporthales	2		1	2		5 (1.7)
Cytospora sp.	A	Diaporthales		2	1			3 (1.0)
Dactylaria sp.	A	Rhytismatales		1				1 (0.3)
Desmazierella acicola	A	Pezizales	7	2	1	1	3	14 (4.7) [4]
Diaporthe eres	A	Diaporthales	2	1	1	16	4	24 (8.0) [4]
Discohainesia oenotherae	A	Chaetomellales		2				2 (0.7)
Epicoccum nigrum	A	Pleosporales	8_{ab}	1_{b}	9_{ab}	20_{a}	4_{b}	42 (14.0) [4]
Eupenicillium sp.	A	Eurotiales				1		1 (0.3)
Fellozyma sp.	B	Incertae sedis		1				1 (0.3)
Fusariella sp.	A	Incertae sedis	1			1	1	3 (1.0)
Fusarium lateritium	A	Hypocreales	10_{a}	4_{a}	2_{a}	12_{a}	5_{a}	33 (11.0) [4]
Fusarium sp.	A	Hypocreales	2_{b}	14_{a}	3_{ab}	11_{ab}	5_{ab}	35 (11.7) [4]
Graphilbum sp.	A	Ophiostomatales			3		1	4 (1.3)
Gyrothrix sp.	A	Incertae sedis	1	1				2 (0.7)
Helotiales sp. 1	A	Helotiales	26_{a}	0_{c}	3_{bc}	6_{bc}	14_{ab}	49 (16.3) [4]
Herpotrichia sp.	A	Pleosporales					2	2 (0.7)
Hymenoscyphus caudatus	A	Helotiales	1	5	1		1	8 (3.0)
Hymenoscyphus fraxineus	A	Helotiales	14_{b}	37_{a}	34_{a}	15_{b}	10_{b}	110 (36.7) [4]
Hymenoscyphus scutula	A	Helotiales		2	1	2	1	6 (2.0)
Hymenoscyphus sp.	A	Helotiales	2	1	1			4 (1.3)
Hypholoma fasciculare	B	Agaricales	1					1 (0.3)
Hypoderma rubi	A	Rhytismatales	2	5	1	9	6	23 (7.7) [4]
Lanzia sp.	A	Helotiales	2				1	3 (1.0)
Lasiosphaeriaceae sp.	A	Sordariales		2			3	5 (1.7)
Lemonniera sp.	A	Helotiales				2		2 (0.7)
Leptosphaeria conoidea	A	Pleosporales		1				1 (0.3)

Table 2. *Cont.*

Taxon	Phylum [1]	Order	Number of Colonized Ash Petioles in Study Plots [2]					
			1	2	3	4	5	Total Number (%)
Leptosphaeria rubefaciens	A	Pleosporales		4	1	1		6 (2.0)
Leptosphaeria urticae	A	Pleosporales		1	1	1		3 (1.0)
Leptosphaeria sp. 1	A	Pleosporales					1	1 (0.3)
Leptosphaeria sp. 2	A	Pleosporales		1	1	1	1	4 (1.3)
Leptospora rubella	A	Incertae sedis	3		1			4 (1.3)
Leptospora sp.	A	Incertae sedis					1	1 (0.3)
Lophiostoma corticola	A	Pleosporales		1		3		4 (1.3)
Malbranchea sp.	A	Onygenales			1			1 (0.3)
Microsphaeropsis sp.	A	Pleosporales		12	4		3	19 (6.3) [4]
Mortierella sp.	M	Mortierellales					2	2 (0.7)
Mycena citrinomarginata	B	Agaricales		1			1	2 (0.7)
Mycena sp.	B	Agaricales	1				1	2 (0.7)
Mycoarthris sp. 1	A	Rhytismatales		1	5		6	12 (4.0) [4]
Mycoarthris sp. 2	A	Rhytismatales			2			2 (0.7)
Nemania diffusa	A	Xylariales	0_{b}	12_{a}	16_{a}	0_{b}	5_{ab}	33 (11.0) [4]
Nemania serpens	A	Xylariales	38_{b}	13_{c}	25_{bc}	34_{b}	54_{a}	164 (54.7) [4]
Nemania sp.	A	Xylariales		1		3		4 (1.3)
Neofabraea kienholzii	A	Helotiales		2		3		5 (1.7)
Neonectria sp. 1	A	Hypocreales					4	4 (1.3)
Neonectria sp. 2	A	Hypocreales	2	6		1		9 (3.0)
Paracucurbitaria corni	A	Pleosporales		2		2		4 (1.3)
Paraophiobolus arundinis	A	Pleosporales					1	1 (0.3)
Paraphaeosphaeria michotii	A	Pleosporales			1			1 (0.3)
Paraphaeosphaeria neglecta	A	Pleosporales		9	4			13 (4.3) [4]
Peniophora incarnata	B	Russulales	6	1	3	3	2	15 (5.0) [4]
Periconia sp. 1	A	Pleosporales	1		1		2	4 (1.3)
Periconia sp. 2	A	Pleosporales	1			1		2 (0.7)
Peziza ninguis	A	Pezizales		5		1		6 (2.0)
Peziza varia	A	Pezizales	8	7	5	3	6	29 (9.7) [4]
Pezizomycetes sp.	A	-	1					1 (0.3)
Phacidium lacerum	A	Phacidiales	1			1		2 (0.7)
Phlyctema vagabunda	A	Helotiales		2		1		3 (1.0)
Phoma sp. 1	A	Pleosporales			3			3 (1.0)
Phoma sp. 2	A	Pleosporales		2	2			4 (1.3)
Phoma sp. 3	A	Pleosporales		1	17	2	3	23 (7.7) [4]
Phoma sp. 4	A	Pleosporales		1	2	3	5	11 (3.7) [4]
Phoma sp. 5	A	Pleosporales		5		6	2	13 (4.3) [4]
Plectosphaerella cucumerina	A	Glomerellales				1	3	4 (1.3)
Plenodomus sp. 1	A	Pleosporales	1	1				2 (0.7)
Pleospora sp.	A	Pleosporales	1			3		4 (1.3)
Psathyrella piluliformis	B	Agaricales			1			1 (0.3)
Pseudocoleophoma polygonicola	A	Pleosporales	1	1			1	3 (1.0)
Pseudoophiobolus italicus	A	Pleosporales		4				4 (1.3)
Pseudoplectania nigrella	A	Pezizales	2	1				3 (1.0)
Pyrenochaeta sp.	A	Incertae sedis	1			1		2 (0.7)
Pyrenochaetopsis leptospora	A	Pleosporales		2				2 (0.7)
Pyrenopeziza petiolaris	A	Helotiales	1	10	2	6	3	22 (7.3) [4]
Pyrenopeziza sp.	A	Helotiales			1	1		2 (0.7)
Rhexocercosporidium sp.	A	Helotiales	3	4	5	4	5	21 (7.0) [4]

Table 2. *Cont.*

Taxon	Phylum [1]	Order	Number of Colonized Ash Petioles in Study Plots [2]					
			1	2	3	4	5	Total Number (%)
Rosellinia abscondita	A	Xylariales	3	2	1		1	7 (2.3)
Rosellinia corticium	A	Xylariales		1	1			2 (0.7)
Rosellinia nectrioides	A	Xylariales	1				1	2 (0.7)
Sistotrema sp.	B	Cantharellales		1				1 (0.3)
Tetracladium sp.	A	Rhytismatales		2	1			3 (1.0)
Tilletiopsis washingtonensis	B	Entylomatales					2	2 (0.7)
Trametes versicolor	B	Polyporales				2		2 (0.7)
Trichoderma viride	A	Hypocreales		1		3		4 (1.3)
Truncatella sp.	A	Amphisphaeriales		4				4 (1.3)
Typhula sp. 1	B	Agaricales		2	1	1		4 (1.3)
Typhula sp. 2	B	Agaricales	3_a	10_a	3_a	8_a	9_a	33 (11.0) [4]
Umbelopsis sp.	M	Umbelopsidales	1			1	1	3 (1.0)
Vargamyces aquaticus	A	Incertae sedis		1				1 (0.3)
Venturia fraxini	A	Venturiales	9_a	4_a	11_a	7_a	10_a	41 (13.7) [4]
Verticillium dahliae	A	Glomerellales					1	1 (0.3)
Xylaria polymorpha	A	Xylariales	6			1	1	8 (2.7)
Xylaria sp. 1	A	Xylariales	1	3				4 (1.3)
Number of petioles analyzed	-	-	60	60	60	60	60	300
Number of colonies obtained	-	-	446	696	519	576	595	2832
Species richness (S)	-	-	53	70	58	59	58	117
Sum of frequency of taxa	-	-	218	272	249	274	238	1251
Simpson's index of diversity (SID, SID = 1 − D)	-	-	0.94	0.96	0.94	0.95	0.93	0.96
Shannon index of diversity (H′)	-	-	3.31	3.67	3.36	3.49	3.40	3.86

[1] A—Ascomycota, B—Basidiomycota, M—Mucoromycota. [2] 1—Brody, 2—Dynów, 3—Miechów, 4—Myślenice, 5—Ojców. [3] Within row, values with different subscript letters are statistically different ($p < 0.05$) according to χ^2 multiple comparisons test using the Marascuilo procedure. [4] Indicates dominant species (taxa) of fungi determined according to Camargo's index [84] (1/S). A species was defined as dominant if Pi > 1/S, where Pi is the relative frequency of a species i and S represents species richness.

The fungi isolated belonged to three phyla: Mucoromycota (1.7%), Ascomycota (85.5%) and Basidiomycota (12.8%) (Supplementary Material Table S2). Mucoromycota was represented by two taxa: *Mortierella* and *Umbelopsis* (Table 2). Ascomycota was represented by 100 taxa belonging to 19 orders. Pleosporales, Helotiales and Xylariales were represented by 32, 15 and 8 species, respectively, and were dominant in the fungal community. Eight orders were each represented only by one species (Figure 1 and Supplementary Material Table S2). The highest species diversity was in *Leptosphaeria* and *Phoma* (five species), and *Alternaria*, *Coniochaeta* and *Hymenoscyphus* (four species each) (Table 2). Basidiomycota was represented by 15 taxa belonging to six orders. *Typhula* sp. 2 (11.0%), *Peniophora incarnata* (5.0%), as well as Agaricales and Russulales, were the most frequent (Figure 1 and Table 2). Yeast-like basidiomycetes were represented by two species from the genera *Fellozyma* (Pucciniomycotina) and *Tilletiopsis* (Ustilagomycotina). Ten taxa were detected only in 1–2 petioles.

Among 117 taxa identified, 27 taxa were classified as dominants (Table 2). The most frequent taxa (>10%) were: *Nemania serpens*, *Hymenoscyphus fraxineus*, *Alternaria* sp. 1, *Boeremia* sp., Helotiales sp. 1, *Epicoccum nigrum*, *Venturia fraxini*, *Fusarium* sp., *Fusarium lateritium*, *Nemania diffusa* and *Typhula* sp. 2 (Table 2).

A total of 18 of the detected taxa occurred in all five stands, while 35 taxa occurred only in one stand (Table 2). The Shannon diversity index (H′) and Simpson's diver-

sity index (SDI), indicating diversity in all five fungal communities, were H′ = 3.86 and SDI = 0.96. There most diversity was at Dynów (H′ = 3.67, SDI = 0.96) and least at Ojców (SDI = 0.93) and Brody (H′ = 3.31).

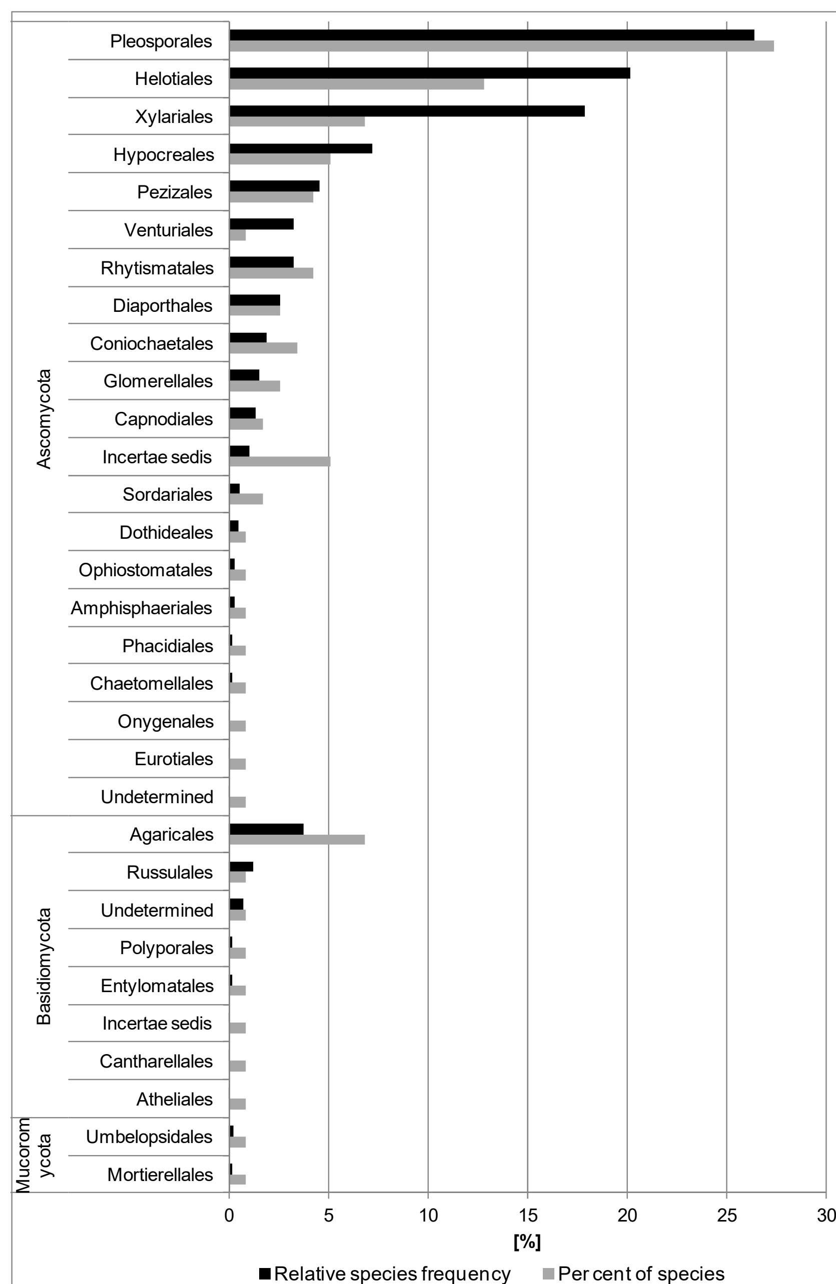

Figure 1. Frequency of fungi detected in situ on the previous year's leaf petioles of *F. excelsior*. Relative species frequency calculated as the ratio of the number of specimens in the order to all specimens n = 1251. Percent of species calculated as the ratio of the number of species in the order to all species n = 117.

Principal component analysis (PCA) separated the localities along the first axis (explaining 67.97% of the variation), mainly on the basis of prevalence of *Nemania serpens*, *H. fraxineus*, Helotiales sp. 1 and other dominant species (Figure 2). The second PCA axis explained 13.87% of the variation and separated Myślenice from other locations. This axis separated locations mainly on the basis of prevalence of *Epicoccum nigrum* and the other dominant species, e.g., *Alternaria* sp. 1, *Boeremia* sp. and *Nemania diffusa* (Figure 2). The difference in the prevalence of *E. nigrum* at Myślenice and at Ojców and Dynów was

statistically supported by the Marascuilo procedure. Similarly, *N. serpens* was significantly more frequent at Ojców than at the other locations, whereas *H. fraxineus* was significantly more frequent at Dynów and Miechów than at other locations (Table 2).

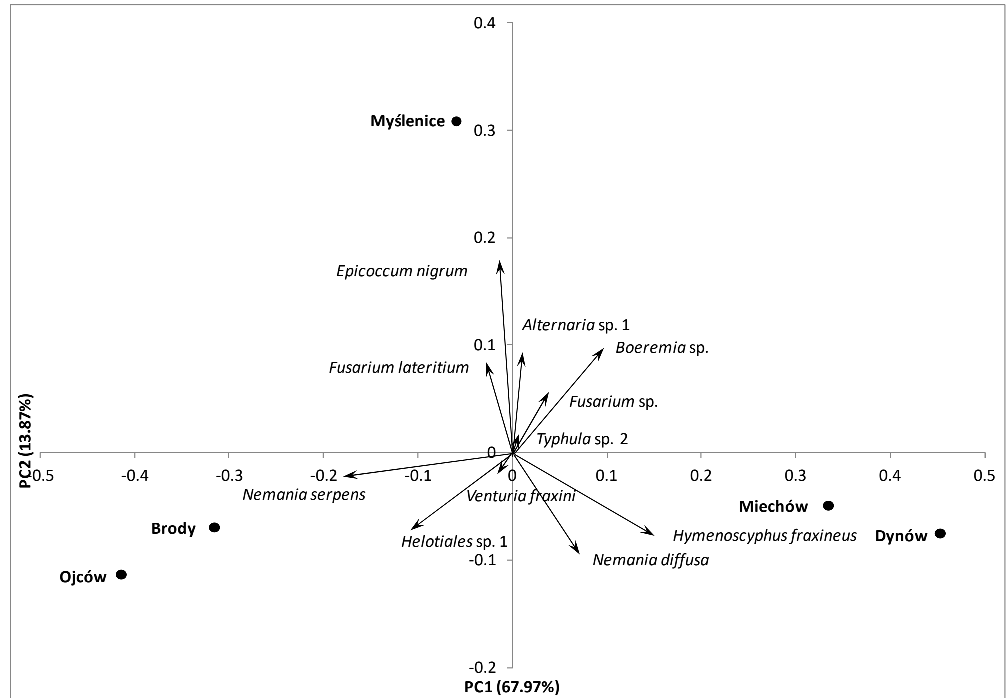

Figure 2. Principal component analysis (PCA) with frequencies of fungal variables represented by arrows and sites represented by black circles, for mycobiota isolated from the previous year's leaf petioles of *F. excelsior*.

The dendrogram based on the Bray–Curtis index showed similarity between fungal communities in stands at Dynów and Miechów and dissimilarity between them and communities at Ojców, Brody and Myślenice (Figure 3).

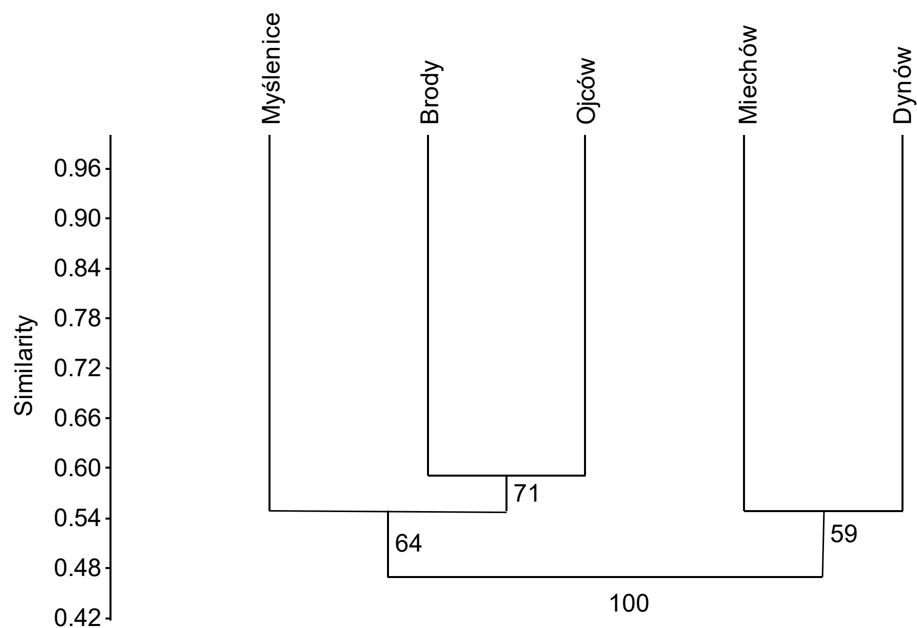

Figure 3. Similarity of fungal communities isolated from the previous year's leaf petioles of *F. excelsior* in five stands shown by dendrogram based on Bray–Curtis similarity index.

Analysis of co-occurrence of fungi in communities isolated from the previous year's leaf petioles (subsample I) made with Spearman's rank correlation coefficient showed 233 relationships that were statistically significant, including 129 positive and 104 negative correlations. There were five modules of co-occurrence. The first consisted of 16 taxa and included mostly *F. lateritium*, *N. diffusa* and *Typhula* sp. 2. The second consisted of 21 taxa and included mostly *Epicoccum nigrum*. The third consisted of 14 taxa and included mostly *Boeremia* sp. The fourth consisted of 16 taxa and included mostly *Alternaria* sp. 1. The fifth consisted of 14 taxa and included mostly Helotiales sp. 1, *H. fraxineus* and *N. serpens* (Figure 4).

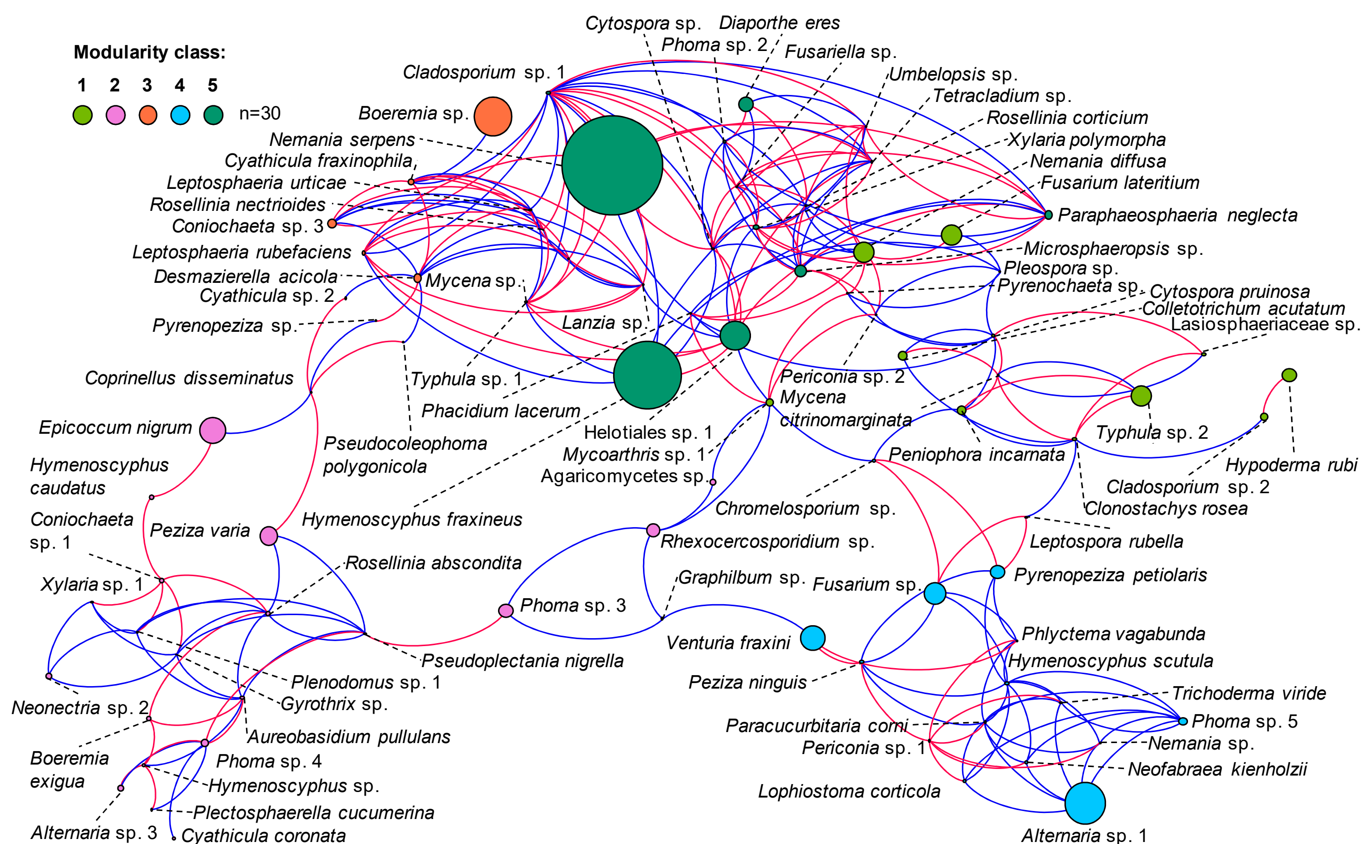

Figure 4. Network of co-occurrence of fungi isolated from the previous year's leaf petioles of *F. excelsior* in five stands, in southern Poland, determined on the basis of data grouped for the study plots. Each connection shows a strong (Spearman's $p > 0.6$) and significant ($p < 0.05$) correlation. Blue lines indicate positive and red lines negative correlation. Five modules were generated, and the same color represents fungal taxa with potential co-occurrence in the same module. The frequency of the species was indicated by the size of each node.

Taxa with the most frequent relationships with others were *Cladosporium* sp. 1, Helotiales sp. 1, *Microsphaeropsis* sp. and *Xylaria polymorpha*. Co-occurrences of *Cladosporium* sp. 1 with other taxa were equally positively or negatively correlated (with slightly higher values for the positive correlations). *Microsphaeropsis* sp. had similar numbers of positive and negative correlations. Helotiales sp. 1 and *X. polymorpha* had a positive correlation, but occurrences of Helotiales sp. 1 or *X. polymorpha* with other fungi were often negatively correlated. A negative correlation suggests that increased frequency of one taxon causes decreased frequency of another. A good example is the co-occurrence of Helotiales sp. 1 and *H. fraxineus*, the latter decreasing in the presence of the former; this is the only negative statistically significant correlation for co-occurrence of *H. fraxineus* with other fungi (Figure 4).

3.2. In Situ Inventory of Fungi Based on Fruit Bodies on Petioles

A total of 45 taxa was detected from fruit bodies present on the 600 previous year's leaf petioles (subsamples II and III) (Figure 5 and Table 3). All 45 taxa were cultured and subject to molecular analysis. Twenty-three of them had been isolated previously from petioles (subsample I). Twenty-two taxa were additional and extended the list of fungi from petioles (Tables 2 and 3). The fungi detected were Ascomycota and Basidiomycota (Figure 5). Ascomycota was represented by 41 taxa (91.1%) from nine orders. Those in the order Helotiales were most frequent (51.0%) and had most diversity (17 species) (Figure 5 and Supplementary Material Table S2).

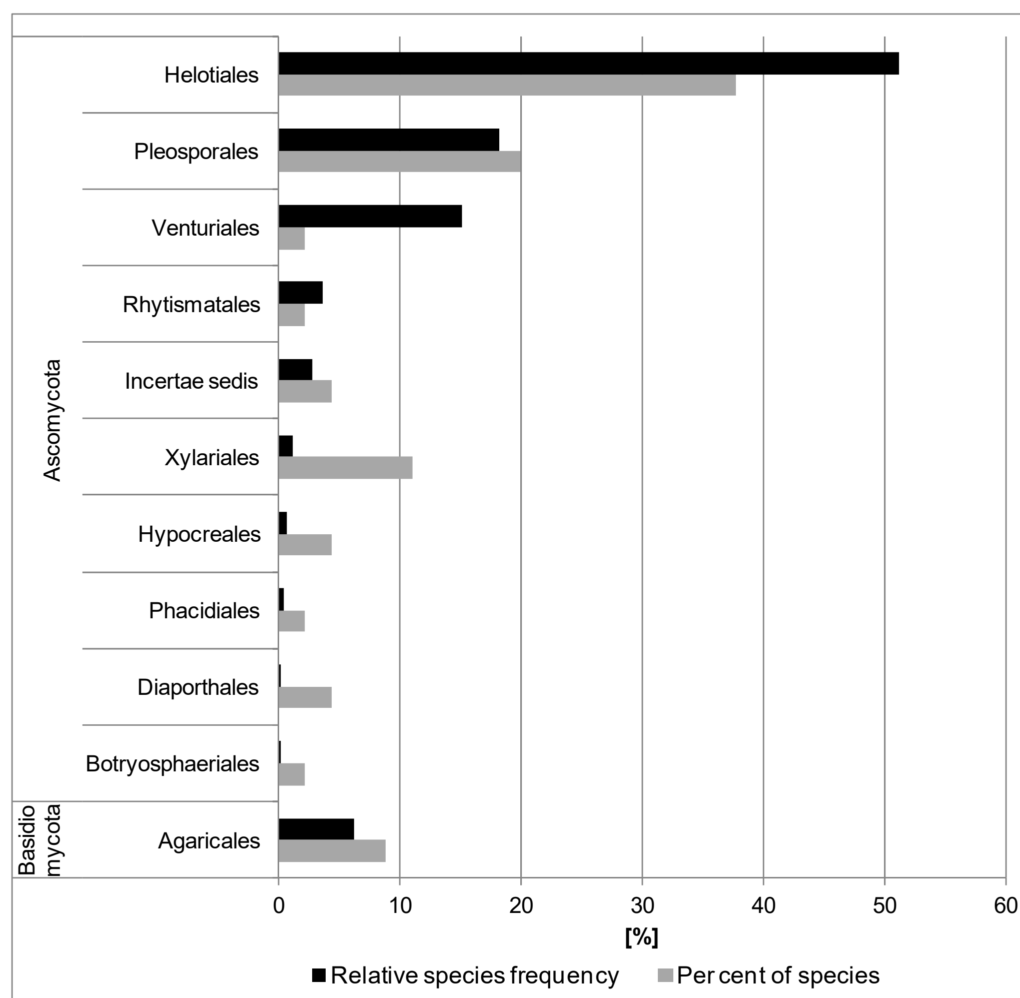

Figure 5. Frequency of fungi detected in situ from fruit bodies on the previous year's leaf petioles of *F. excelsior*. Relative species frequency calculated as the ratio of the number of specimens in the order to all specimens $n = 882$. Percent of species calculated as the ratio of the number of species in the order to all species $n = 45$.

The high level of taxonomic diversity was demonstrated by the diversity of fruit bodies on the dead petioles (Figure 6a–t). Colonization of petioles by certain fungi was associated with discoloration of the surface of the colonized petiole pieces, e.g., *Hymenoscyphus scutula*, Helotiales sp. 1, *Hypoderma rubi* and *L. sclerotioides* caused, respectively, grey-brown, rusty-brown, light-cream or dark-grey discoloration (Figure 6d,e,g,j).

Table 3. Fungi detected in situ by inventory of fruit bodies on the previous year's leaf petioles of *F. excelsior* in five forest stands, in southern Poland in 2017.

Taxon	Order [1]	Phylum [2]	Taxa No.	Number of Petioles Colonized in Study Plots [3]					Total Number (%)	Hf [6]	Accession	BLASTn	Identities %
				1	2	3	4	5					
Amniculicola sp.	P	A	FeF402				2		2 (0.3)		MZ492963	MK353143	92.42
Anthostoma turgidum	X	A	FeF425		2				2 (0.3)		MZ493125	KC774617	99.84
Apiognomonia hystrix	D	A	FeF401					1	1 (0.2)		MZ493111	KX776442	100
Boeremia exigua	P	A	FeF403	2		6			8 (1.3)	1	MZ493112	MN540289	100
Calycina sp.	H	A	FeF404				1	1	2 (0.3)		MZ493113	JF908571	100
Cyathicula coronata	H	A	FeF405		1	16	7	3	27 (4.5) [5]	16	MZ492976	MH858141	100
Cyathicula fraxinophila	H	A	FeF406	21_{a} [4]	11_{a}	18_{a}	12_{a}	20_{a}	82 (13.7) [5]	31	MZ492980	MK584998	99.09
Cyathicula sp. 1	H	A	FeF407	3					3 (0.5)		MZ492983	MK584998	98.7
Cyathicula sp. 2	H	A	FeF408	2			1	2	5 (0.8)		MZ492978	MK584998	98.7
Cyathicula sp. 3	H	A	FeF409	6	1		6	2	15 (2.5)	5	MZ493114	MK584998	98.35
Cyathicula sp. 4	H	A	FeF445			1		2	3 (0.5)		MZ492982	MK584998	99.09
Diaporthe sp. 1	D	A	FeF446		1				1 (0.2)	1	MZ493057	EU552122	99.67
Diplodia fraxini	B	A	FeF410	1			1		2 (0.3)	2	MZ493115	MT587347	100
Helotiales sp. 1	H	A	FeF413	2			1	4	7 (1.2)		MZ492996	GU174285	98.97
Helotiales sp. 2	H	A	FeF414					1	1 (0.2)		MZ492997	MH578458	98.41
Helotiales sp. 3	H	A	FeF415	2	2		1	2	7 (1.2)	1	MZ493124	KY430513	99.44
Hymenoscyphus caudatus	H	A	FeF416	3	4	5	4	5	21 (3.5) [5]	7	MZ492985	KM114539	99.38
Hymenoscyphus fraxineus	H	A	FeF417	30_{bc}	59_{ab}	66_{a}	21_{c}	26_{c}	202 (33.7) [5]		MZ492992	LLCC00000000	100
Hymenoscyphus scutula	H	A	FeF418	8	2	4	6	7	27 (4.5)	12	MZ492989	MK674606	99.83
Hymenoscyphus sp.	H	A	FeF419			1	1	2	4 (0.7)		MZ492987	MK674606	97.03
Hypoderma rubi	R	A	FeF420	8	4	3	13	4	32 (5.3) [5]	13	MZ493025	GU138741	99.64
Lasionectria sp.	Hy	A	FeF421	1					1 (0.2)		MZ493040	KY607542	96.47
Leptosphaeria sclerotioides	P	A	FeF422		7		14	2	23 (3.8) [5]	5	MZ492959	FJ179155	100
Leptosphaeria sp. 2	P	A	FeF423	29_{ab}	17_{ab}	17_{ab}	38_{a}	11_{b}	112 (18.7) [5]	39	MZ492957	KC965764	95.33
Leptospora rubella	Is	A	FeF424	2	2	1		1	6 (1.0)	5	MZ493116	MH857603	99.83
Lophiostoma corticola	P	A	FeF426		2		1	1	4 (0.7)	2	MZ493117	KU712227	100
Marasmius epiphyllus	A	B	FeF427					1	1 (0.2)		MZ493079	FN293008	99.66
Neonectria sp. 2	Hy	A	FeF411	2			1	2	5 (0.8)	1	MZ493037	JF735313	99.82
Paraophiobolus arundinis	P	A	FeF428		2		2	5	9 (1.5)	6	MZ492953	MG520945	100
Phacidium lacerum	Ph	A	FeF429	2	1		1		4 (0.7)	3	MZ493005	KU942438	100
Plenodomus sp. 2	P	A	FeF430					1	1 (0.2)		MZ492947	KU973715	100
Pleosporales sp.	P	A	FeF431				1		1 (0.2)		MZ492961	KC965786	96.51

Table 3. *Cont.*

Taxon	Order [1]	Phylum [2]	Taxa No.	Number of Petioles Colonized in Study Plots [3]					Total Number (%)	Hf [6]	Accession	BLASTn	Identities %
				1	2	3	4	5					
Pseudocoleophoma polygonicola	P	A	FeF432	1					1 (0.2)		MZ492973	NR_154274	99.62
Pyrenochaeta sp.	Is	A	FeF433	11	2		4	2	19 (3.2)	2	MZ492948	MT236853	98.98
Pyrenopeziza petiolaris	H	A	FeF434	7	10		3	11	31 (5.2) [5]	12	MZ493015	MH857804	99.29
Trichopeziza sp. 1	H	A	FeF435	8	1	1		1	11 (1.8)	1	MZ493009	AB481285	97.93
Trichopeziza sp. 2	H	A	FeF436	1	1		1		3 (0.5)		MZ493008	JN033398	99.23
Typhula erythropus	A	B	FeF439			1	1	1	3 (0.5)	2	MZ493118	MT232359	100
Typhula sp. 1	A	B	FeF437	1	3	1	6	4	15 (2.5)	3	MZ493119	KR673716	99.86
Typhula sp. 2	A	B	FeF438		10	2	15	10	37 (6.7) [5]	16	MZ493081	NR_132792	88.65
Venturia fraxini	V	A	FeF440	26 $_{a}$	24 $_{a}$	27 $_{a}$	27 $_{a}$	29 $_{a}$	133 (22.2) [5]	53	MZ493120	EU035457	99.83
Xylaria sp. 1	X	A	FeF441		2				2 (0.3)		MZ493122	MF774330	99.83
Xylaria sp. 2	X	A	FeF442			1	1		2 (0.3)	1	MZ493121	MF774330	99.48
Xylaria sp. 3	X	A	FeF443				1	2	3 (0.3)	1	MZ493059	MH578518	97.86
Xylaria sp. 4	X	A	FeF444	1					1 (0.2)		MZ493123	MH578518	97.1
Number of petioles analyzed	-	-	-	120	120	120	120	120	600	-	-	-	-
Species richness (S)	-	-	-	25	24	17	30	31	45	-	-	-	-
Sum of frequency of taxa	-	-	-	180	171	171	194	166	882	241	-	-	-
Simpson's index of diversity (SID, SID=1-D)	-	-	-	0.9	0.84	0.79	0.91	0.91	0.89	-	-	-	-
Shannon index of diversity (H′)	-	-	-	2.61	2.36	1.99	2.72	2.82	2.73	-	-	-	-

[1] Order: P—Pleosporales, X—Xylariales, D—Diaporthales, H—Helotiales, B—Botryosphaeriales, R—Rhytismatales, Hy—Hypocreales, Is—Incertae sedis, A—Agaricales, Ph—Phacidiales, V—Venturiales. [2] A—Ascomycota, B—Basidiomycota. [3] Study plots: 1—Brody, 2—Dynów, 3—Miechów, 4—Myślenice, 5—Ojców. [4] Within row, values with different subscript letters are statistically different ($p < 0.05$) according to χ^2 multiple comparisons test using the Marascuilo procedure. [5] Indicates dominant species (taxa) of fungi determined according to Camargo's index (1/S) [84]. A species was defined as dominant if Pi > 1/S, where Pi is the relative abundance of a species i and S represents species richness. [6] Number of petioles with *H. fraxineus* and co-occurring fungus.

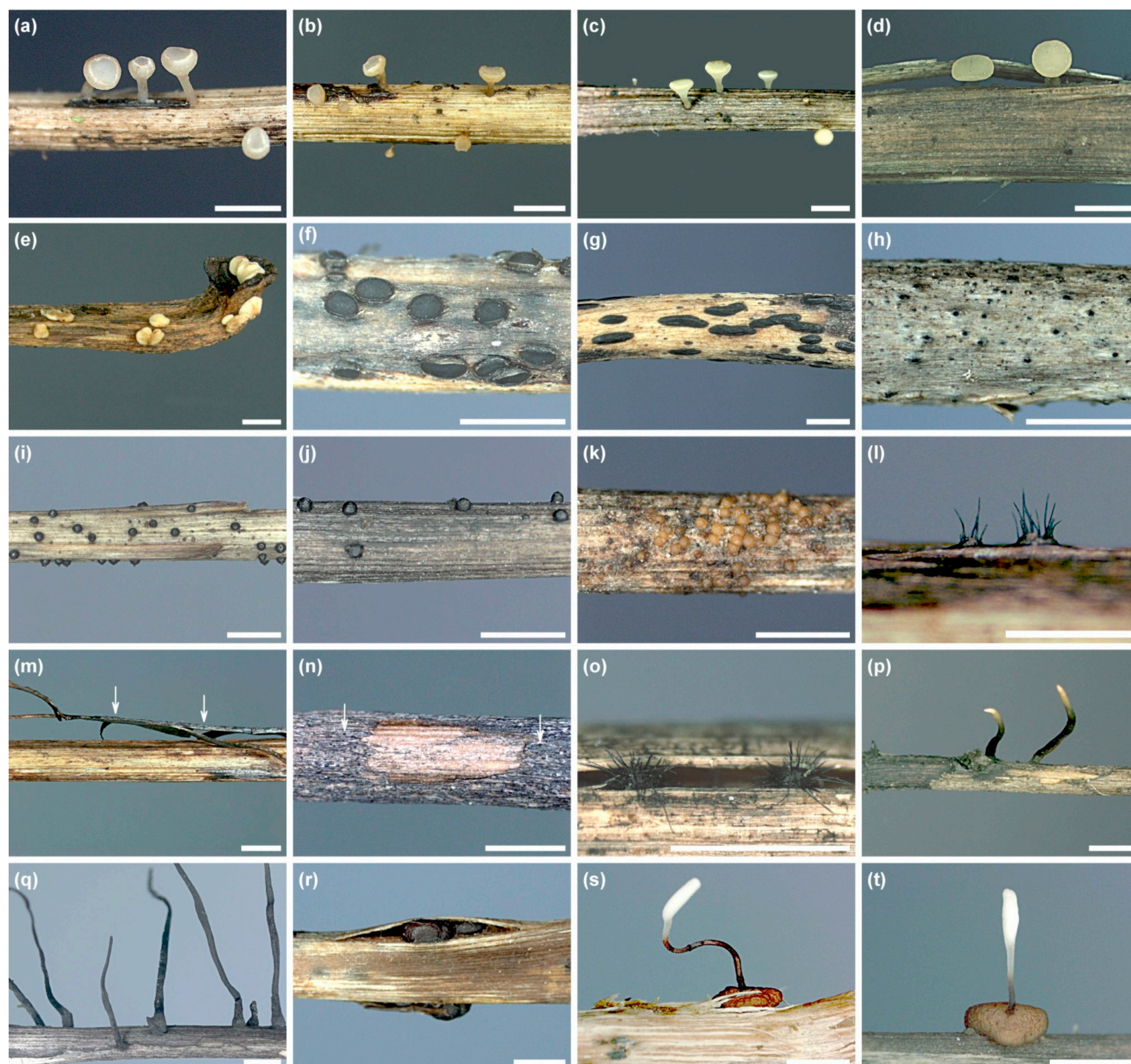

Figure 6. Fungal fruit bodies on the previous year's leaf petioles of *F. excelsior* in the litter, in 2017: (**a**–**f**) apothecia: a—*Cyathicula fraxinophila*, (**b**) *Cyathicula* sp. 2, (**c**) *Hymenoscyphus caudatus*, (**d**) *Hymenoscyphus scutula*, (**e**) Helotiales sp. 1, (**f**) *Pyrenopeziza petiolaris*, (**g**) *Hypoderma rubi*, hysterothecia and pycnidia, (**h**) *Paraophiobolus arundinis* pseudothecia, (**i**) *Leptosphaeria* sp. 2, pseudothecia, (**j**) *Leptosphaeria sclerotioides*, pycnidia, (**k**) *Neonectria* sp. 2, perithecia, (**l**–**n**) *Venturia fraxini*, with setae lining the ostiolum of the pseudothecium (**l**), pseudothecia on the exfoliated periderm (**m**), pseudothecia on petioles with damage made by small fauna (**n**), (**o**) setaceous pycnidia of *Pyrenochaeta* sp., (**p**) anamorph of *Xylaria* sp. 1, (**q**) anamorph of *Xylaria* sp. 3, (**r**,**s**)—*Typhula* sp. 2, sclerotia (**r**) and carpogenic sclerotium (**s**), (**t**) *Typhula erythropus*, carpogenic sclerotium.

Basidiomycota were represented by four taxa, all from the order Agaricales (Figure 5, Table 3 and Supplementary Material Table S2).

The formation of fruit bodies and sclerotia of some fungi, including *H. scutula*, *Pyrenochaeta* sp. or *Typhula* sp. 2, may contribute to loosening and desquamation of the peripheral tissue of petioles (Figure 6d,o,r,s). Additionally, the petiole's surface tissues may be eaten by small animals living in the litter, exposing the inner tissues to colonization but also causing loss of taxa that fruit on the eaten tissue edges (Figure 6n).

Among 45 taxa identified, 11 were classified as dominants (Table 3). This group included *Hymenoscyphus fraxineus*, *Venturia fraxini*, *Leptosphaeria* sp. 2, *Cyathicula fraxinophila*, *Typhula* sp. 2, *Hypoderma rubi* and *Pyrenopeziza petiolaris* (in descending order, with frequency 33.7%–5.2%).

A total of 8 of the detected taxa occurred in all five stands, while 13 occurred only in one stand (Table 3). The numbers of taxa detected in a single stand ranged from 17 (Miechów) to 31 (Ojców) (Table 3).

The Shannon diversity index (H′) and Simpson's diversity index (SDI), indicating the diversity over all five stands, were H′ = 2.73 and SDI = 0.89. There was most diversity at Ojców (H′ = 2.82, SDI = 0.91) and least at Miechów (H′ = 1.99, SDI = 0.79) (Table 3).

PCA separated the localities along the first axis (explaining 73.98% of the variation), mainly on the basis of the prevalence of *H. fraxineus*. The second PCA axis explained 16.69% of the variation and separated Ojców from the other locations. This axis separated locations mainly on the basis of the prevalence of *Leptosphaeria* sp. 2 as well as the other dominant species, *H. fraxineus*, *C. fraxinophila* and *V. fraxini* (Supplementary Material Figure S2). The difference in prevalence of *H. fraxineus* at Miechów from that at Brody, Ojców and Dynów was statistically supported by the Marascuilo procedure. Similarly, *Leptosphaeria* sp. 2 was statistically more frequent at Myślenice than at Ojców (Table 3).

The dendrogram based on the Bray–Curtis index showed similarity between fungal communities in stands at Dynów and Miechów and dissimilarity between them and communities in stands at Ojców, Brody and Myślenice (Supplementary Material Figure S3). These results support those of analysis based on isolation of fungi from petioles (subsample I).

The fungi detected varied depending on their time of fructification on *F. excelsior* petioles (Figure 7). Six taxa, including *P. petiolaris*, fruited only in spring (Figure 7). Thirteen taxa fruited in both spring and autumn, with variable frequency observed in some of them, particularly *V. fraxini* (Figure 7). Only *H. rubi* fruited in spring and autumn with similar frequency, although in spring the fungus produced only immature hysterothecia (as well as the anamorph) (Figure 6e). Most fungi, including *C. coronata*, *C. fraxinophila*, *H. caudatus*, *H. scutula* and *Pyrenochaeta* sp., fruited in autumn (Figure 7). The autumn-fruiting group also includes basidiomycetous *Typhula* spp. It should be emphasized, however, that, in spring, *Typhula* formed only sclerotia with no sporophores (Figure 7).

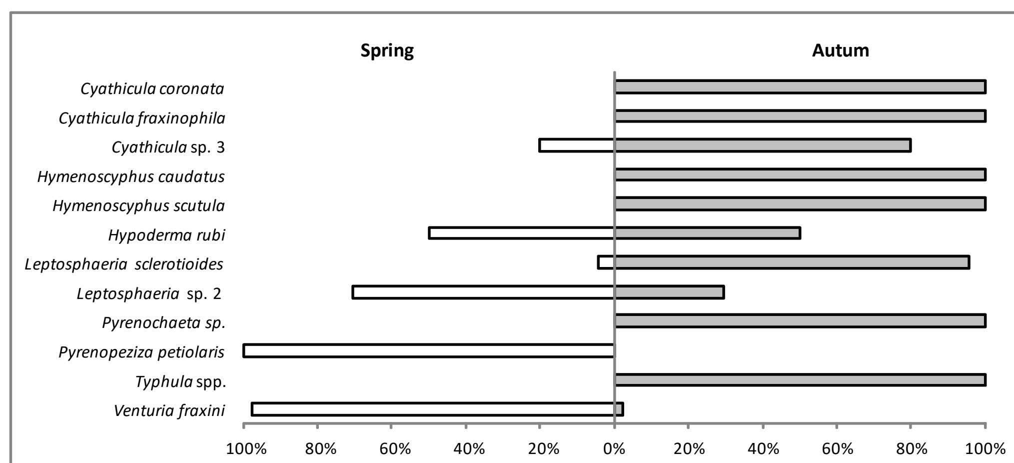

Figure 7. Fruit bodies of fungi (with frequency >2%) from the previous year's leaf petioles of *F. excelsior* according to season. In spring, *Typhula* spp. produced only sclerotia with no sporophores.

In spring, identification of *H. fraxineus* was based on the presence of pseudosclerotial plates with phialides and endoconidia (Figure 8a–c). Apothecia appeared on pseudosclerotial plates at the beginning of July (Figure 8d). Careful morphological analysis excluded the presence of the related species, *H. albidus*.

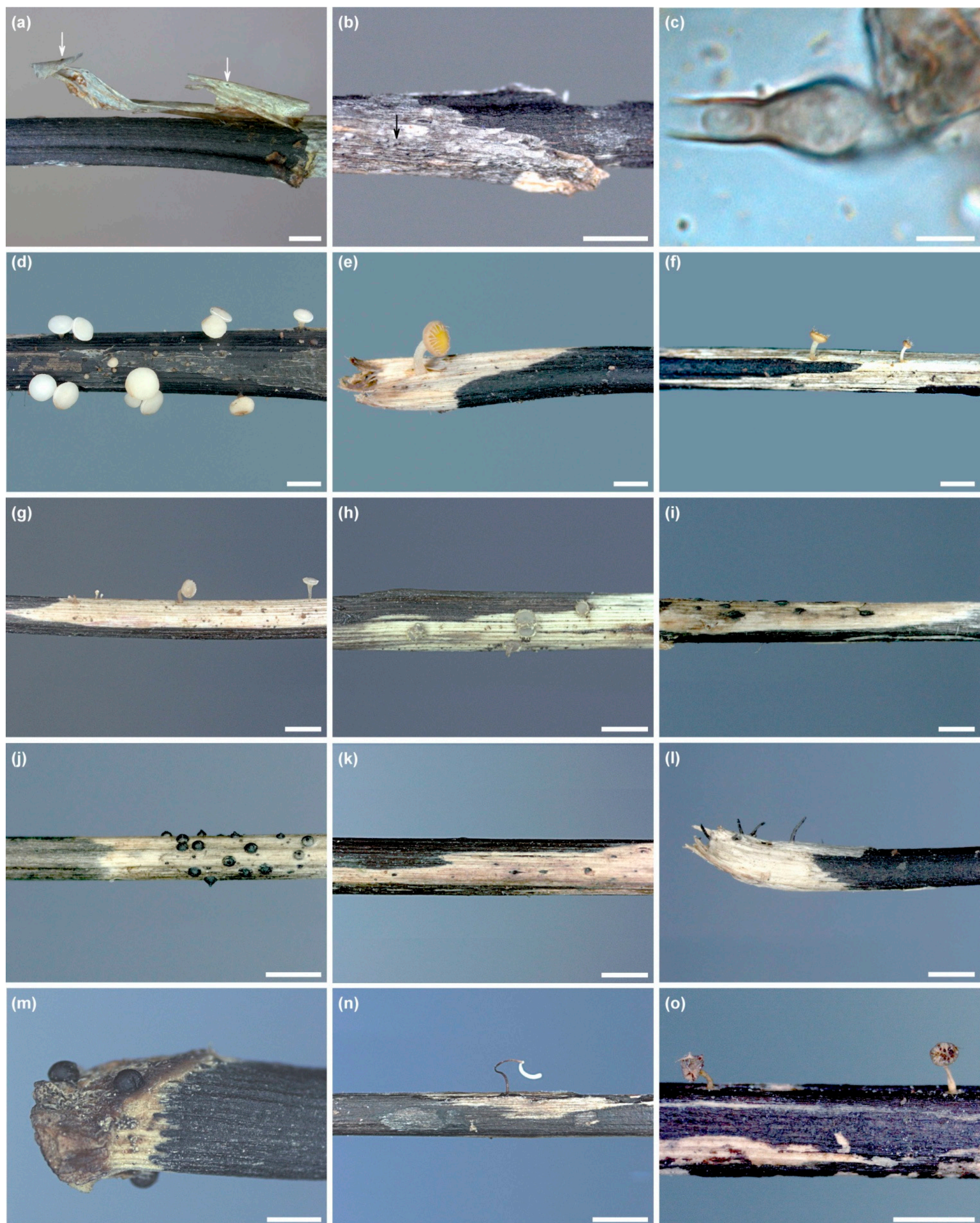

Figure 8. *Hymenoscyphus fraxineus* and other fungi on the previous year's leaf petioles of *F. excelsior* in the litter: (**a**,**b**) *H. fraxineus* pseudosclerotial plate revealed by the exfoliating epidermis, with pseudothecia of *Venturia fraxini* (arrows), (**c**) phialide and endoconidium of *Chalara fraxinea* on pseudosclerotial plate, (**d**) apothecia of *H. fraxineus* (July 2017), (**e**–**n**) pseudosclerotial plate of *H. fraxineus* and: (**e**,**f**) *Cyathicula coronata*, (**g**) *Cyathicula fraxinophila*, (**h**) *Trichopeziza* sp. 1, (**i**) *Pyrenopeziza petiolaris*, (**j**) *Leptosphaeria* sp. 2, (**k**) *Lophiostoma corticola*, (**l**) *Xylaria* sp. 2, (**m**,**n**) *Typhula* sp. 2, sclerotium at the base of petiole (**m**), carpogenic sclerotium (**n**), (**o**) apothecia of *C. coronata* on pseudosclerotial plate of *H. fraxineus*.

Fruit bodies of 26 other fungal species were present on 177 out of 202 leaf petioles colonized by *H. fraxineus* (Table 3). One, two and three additional taxa co-occurred on 124, 42 and 11 petioles, respectively. Usually, the petiole pieces colonized by them were visually different, being distinctly brighter than those with *H. fraxineus* pseudosclerotia (Figure 8a–o). On one petiole, *Cyathicula coronata* produced its apothecium on the *H. fraxineus* pseudosclerotium (Figure 8o).

Observation showed that *H. fraxineus* may co-exist spatially in ash petioles with *V. fraxini* in a way that is different from its co-existence with some other fungi. Pseudosclerotia of *H. fraxineus* were formed subepidermally and appeared in late spring, after desquamation of the epidermis (Figure 8a,b), which led to the removal of pseudothecia of *V. fraxini* formed in the epidermis earlier. Desquamation of the epidermis could be caused by *H. fraxineus*, but also by other physical and biological factors (Figure 6m,n).

Co-occurrence of fungi was positive or negative (data not shown) and statistically significant if strongly positively correlated. Increased frequency of *H. fraxineus* was associated with increased frequency of *Leptosphaeria sclerotioides*, *Leptospora rubella* and *Paraophiobolus arundinis* (Supplementary Material Figure S4 and Table 3).

3.3. Dual-Culture Assays

Three types of interaction (A, B, C) were observed on MEA in dual-culture assays with either of two isolates of *H. fraxineus*, originating from two stands of *F. excelsior*, and 117 fungal species representing 164 morphotypes, isolated from the previous year's leaf petioles (Figure 9a–t and Table 4, Supplementary Material Table S1). The fourth type (D) listed in Methods did not occur (Table 4, Supplementary Material Table S1). The types occurred with different frequencies. Types A and B were significantly more frequent than type C (Table 4). The difference in frequency between type A and type B was statistically non-significant. The frequency of any type did not depend on the *H. fraxineus* isolate used (Table 4). One species of test fungus was represented by 1–5 morphotypes (Supplementary Material Table S1). Differences in interaction type between different morphotypes of one species were sporadic and often temporary, e.g., *D. acicola* FeF42 had an inhibition zone only for the first 3 weeks, then covered the zone and finally had an effect similar to that observed with *D. acicola* FeF119 (Supplementary Material Table S1).

Table 4. Interaction types in dual cultures of *H. fraxineus* and test fungus from the previous year's leaf petioles of *F. excelsior*.

Interaction Types	***Hymenoscyphus fraxineus* (Strain) Number (%)**		
	Hf 1 [2]	**Hf 2** [2]	**Total** [3]
A (mutual direct contact of mycelia)	73 (44.5) $_{a}$	69 (42.1) $_{a}$	142 (43.3) $_{a}$
B (inhibition zone) [1]	75 (45.7) $_{a}$	79 (48.2) $_{a}$	154 (46.9) $_{a}$
C (overgrowth of *H. fraxineus* colony by a saprotroph)	16 (9.8) $_{a}$	16 (9.8) $_{a}$	32 (9.8) $_{b}$
D (overgrowth of saprotroph's colony by *H. fraxineus*)	0	0	0 $_{c}$
Number of pathogen/co-partner combinations in dual cultures	164	164	328
[1] inhibition zone width			
Bs (up to 3 mm)	29 $_{a}$	31 $_{a}$	60 (18.3) $_{a}$
Bm (4–5 mm)	25 $_{a}$	26 $_{a}$	51 (15.5) $_{ab}$
Bw (6–8 mm)	10 $_{a}$	12 $_{a}$	22 (6.7) $_{b}$
Bv (>8 mm)	11 $_{a}$	10 $_{a}$	21 (6.4) $_{b}$

[2] Within rows in two columns (Hf 1 and Hf 2), values with the same letter are not significantly different ($p > 0.05$) according to χ^2 test.
[3] Within column, values with different subscript letters are statistically different ($p < 0.05$) according to χ^2 multiple comparisons test using the Marascuilo procedure separately for "interaction types" and "inhibition zone width" datasets.

Figure 9. Various types of interactions observed in dual cultures (MEA, 3 weeks, 20 °C), *Hymenoscyphus fraxineus* (Hf1 or Hf2) from the left, test fungus on the right: (**a**–**d**) type A interaction, (**a**) *Hymenoscyphus fraxineus* (FeF164), (**b**) *Alternaria* sp. 1 (FeF152), (**c**) *Microsphaeropsis* sp. (FeF92b), (**d**) *Pyrenochaeta* sp. (FeF13), (**e**–**l**) type B interaction, (**e**) *Phacidium lacerum* (FeF246), (**f**) *Plectosphaerella cucumerina* (FeF161b), (**g**) *Hypoderma rubi* (FeF203), (**h**) *Pleospora* sp. (FeF80), (**i**) *Paraphaeosphaeria neglecta* FeF109), (**j**) *Lanzia* sp. (FeF26), (**k**) *Malbranchea* sp. (FeF243), (**l**) *Pseudocoleophoma polygonicola* (FeF201), (**m**–**t**) type C interaction, (**m**) *Nemania diffusa* (FeF104), (**n**) *Nemania serpens* (FeF245), (**o**) *Rosellinia corticium* (FeF167), (**p**) *Rosellinia nectrioides* (FeF187) (with inhibition zone only partly covered, see text), (**q**) *Xylaria* sp. 1 (FeF97a), (**r**) *Xylaria polymorpha* (FeF30), (**s**) *Desmazierella acicola* FeF119, (**t**) *Hypholoma fasciculare* (FeF241).

In type A interactions, the size of the *H. fraxineus* colony and the shape of the contact line between two colonies often depended on the rate of growth of the test fungus (Figure 9b–d).

Type B interactions, the formation of the inhibition zone, were the most common. The most frequent width of the inhibition zone was Bs, and the least frequent was Bv. The Bs width (Figure 9e,f) occurred significantly more frequently than Bw or Bv (Table 4).

Very wide inhibition zones (>8 mm) were observed in 6.4% of cases, in pairings of *H. fraxineus* with *Coniochaeta angustispora*, *Cytospora pruinosa*, *Fusarium* sp., *Lanzia* sp. FeF26, *Malbranchea* sp. (inhibition zone 16 mm), *Peziza ninguis*, *Plenodomus* sp. 1, *Pseudocoleophoma polygonicola* (inhibition zone 27 mm) or *Pseudoophiobolus italicus* (Figure 9j–l and Supplementary Material Table S1). Visible morphological and physiological changes in colonies were often observed: *Hypoderma rubi* produced intensive, brownish-red pigment at the border with *H. fraxineus* (Figure 9g); *Lanzia* sp. FeF26 caused the formation of blackish areas in *H. fraxineus* colonies, possibly from melanization of the pathogen's hyphae (Figure 9j); *Plenodomus* sp. 1 or *Pleospora* sp. FeF80 caused the production of a black, melanin-like substance which stained the agar (Figure 9h).

Type C interactions, in which the test fungus overgrows the colony of *H. fraxineus*, occurred in assays with morphotypes of 12 taxa: *Clonostachys rosea*, *Desmazierella acicola* FeF119 (thin cover), *Hypholoma fasciculare*, *Lasiosphaeriaceae* sp. (thin cover), *Nemania diffusa*, *N. serpens*, *Peniophora incarnata*, *Rosellinia corticium*, *R. nectrioides*, *Trichoderma viride*, *Xylaria polymorpha* and *Xylaria* sp. 1 (Figure 9m–t and Supplementary Material Table S1). This type of interaction was often complex and preceded by: (i) earlier, considerable inhibition of the *H. fraxineus* colony; (ii) formation of an inhibition zone caused by accumulation of non-volatile metabolites produced by *H. fraxineus* (Figure 9p); and (iii) overgrowth of the inhibition zone (Figure 9p). *Hypholoma fasciculare* and *T. viride* entirely covered the inhibition zone and the *H. fraxineus* colony after 3 weeks (Figure 9t), while *Lasiosphaeriaceae* sp. and *D. acicola* FeF119 covered them only slightly. *Rosellinia abscondita* started to cover the narrow inhibition zone (width Bs < 3 mm) and the *H. fraxineus* colony after 4 weeks, and so could not be assessed.

Only the test fungi were re-isolated from colonies of *H. fraxineus* overgrown by the test fungi.

The reduction in growth rate of *H. fraxineus* mycelium, in the region growing towards the test fungus, was determined in all dual-culture combinations by comparison with the control. Amounts of reduction differed. Most often it ranged between 51 and 75% and only sporadically was >75% (Table 5). The origin of *H. fraxineus* did not affect the amount of inhibition (Tables 4 and 5). Two statistically different groups of taxa were obtained according to the amount of growth reduction they caused in *H. fraxineus*. The first group included taxa causing 26–50 or 51%–75% reduction; the second group included other taxa (Table 5). A reduction in the growth of the saprotrophic partners towards *H. fraxineus* was observed in 90.0% of dual cultures. Most often (61.3%), the reduction rate was between 26 and 50% (Table 5).

Table 5. Reduction in *H. fraxineus* and the test fungi colonies in dual cultures.

Reduction Rate	*Hymenoscyphus fraxineus*			Fungal Co-Partners		
	Hf 1 [1]	**Hf 2** [1]	**Total Number (%)** [2]	**Hf 1** [1]	**Hf 2** [1]	**Total Number (%)** [2]
a (<25%)	5 $_a$	5 $_a$	10 (3.0) $_b$	37 $_a$	46 $_a$	83 (25.3) $_b$
b (26–50%)	68 $_a$	57 $_a$	125 (38.1) $_a$	104 $_a$	97 $_a$	201 (61.3) $_a$
c (51–75%)	85 $_a$	93 $_a$	178 (54.3) $_a$	5 $_a$	6 $_a$	11 (3.4) $_c$
d (>75%)	6 $_a$	9 $_a$	15 (4.6) $_b$	0	0	0 $_c$
f (0%)	0	0	0 (0.0) $_b$	18 $_a$	15 $_a$	33 (10.0) $_{bc}$
Total	164	164	328 (100.0)	164	164	328 (100.0)

[1] Within rows in two columns (Hf 1 and Hf 2), values with the same letter are not significantly different ($p > 0.05$) according to χ^2 test.
[2] Within columns, values with different subscript letters are statistically different ($p < 0.05$) according to χ^2 multiple comparisons test using the Marascuilo procedure.

4. Discussion

4.1. Fungal Diversity

Analyses of fungal communities from dead leaf petioles of *F. excelsior* from five locations show that they have high diversity of both common and rare species and are comparable with fungal communities from other tree species. Taxonomically, the fungi detected belonged mostly to the Ascomycota, less often to the Basidiomycota and occasionally to the Mucoromycota. The results are consistent with earlier findings on fungal communities from litter [47,48,50,51,89,90], although the relative occurrences of Ascomycota and Basidiomycota depend on the stage of decomposition of the substrate. Ascomycota are usually relatively more abundant in the early stages of decomposition while Basidiomycota increase over time, becoming most frequent at the advanced decomposition stage [48,51,89–91]. Ascomycetous fungi can even decrease the rate of decomposition by exclusion of secondary saprotrophic Basidiomycota, so affecting the microbiological status of woody tissues [34].

The classical method of isolation of fungi on agar medium led to detection of more taxa than the in situ inventory of fungi based on the presence of fruit bodies. The latter method supplemented the former. There were, however, a few taxa, such as *Cyathicula fraxinophila* and *Leptosphaeria* sp. 2, which were detected more often in situ. This could have resulted from the possible domination of the successful fungus or from the method of disinfection used. The fungus detected might be faster growing in vitro, especially from the surface epidermal and subepidermal layers, and resistant to the disinfectants used [92].

A total of 117 taxa was detected by isolation, and 45 taxa by in situ inventory based on the presence of fruit bodies. Among the 45 taxa producing fruit bodies on petioles, 23 were also isolated on agar medium. Therefore, a total of 139 taxa was detected on the dead leaf petioles of *F. excelsior* in litter.

The difference between the two methods in number of taxa detected may be expected. Among the reasons for fewer taxa being detected in situ are that: (i) some fungi do not produce fruit bodies in their life cycle, (ii) some fungi produce filamenous anamorphs rather than teleomorphs, and (iii) yeast forms may dominate in situ [93]. Some taxa detected produce fruit bodies on ash leaves less often than on other substrates, such as tree logs, branches and stumps (e.g., *Coniochaeta*, *Nemania*, *Peniophora*, *Trametes*, *Xylaria*) or on tree stems and twigs (e.g., *Cytospora pruinosa*, *Diplodia fraxini*) [94–98].

The ascomyceous taxa detected belong to 20 orders, indicating high diversity among the Ascomycota. Most diversity was observed in the Pleosporales, which were comparably abundant and diverse as endophytes in living ash leaves [29,81,99]. The findings support those of Hyde et al. [100], Promputtha et al. [101] and De Silva et al. [102], which showed that some fungal species associated with living leaves were also found in leaf litter. These are the host-specific fungal endophytes that most often switch to become saprotrophs [103]. Certain taxa found in the dead *F. excelsior* petioles, namely *Boeremia exigua*, *Colletotrichum acutatum*, *Cytospora pruinosa*, *Diaporthe eres*, *Fusarium lateritium*, *Nemania diffusa*, *N. serpens*, *Venturia. fraxini* and *X. polymorpha*, have been found previously in living, symptomless leaflets and petioles of *F. excelsior* [54,81,99].

Ascomycetous fungi detected in this study differed in their trophic character, host range, tissue specificity, lifestyle and significance in forests. Most frequent among the potential pathogens was *Venturia fraxini* which, in favorable conditions, can cause withering and premature leaf fall in *F. excelsior* stands [104,105], although in some regions it is a dominant species in symptomless leaflets or petioles of *F. excelsior* [81,99,106]. It is one of the rare groups of fungi that easily produce fruit bodies on ash petioles, with mature ascospores appearing very early, in May-June, before infectious ascospores of *H. fraxineus*. This predestines the fungus as a very early endophyte colonizing living leaves of *F. excelsior*. Both *H. fraxineus* and *V. fraxini* can survive in ash petioles in the litter and the early colonization of leaves by *V. fraxini* may contribute to temporary protection against *H. fraxineus*. This is not supported, however, by the assays in vitro and observations in situ. *Venturia fraxini* was not among the fungi most antagonistic to *H. fraxineus*: it colonizes the surface tissues of petioles which does not prevent *H. fraxineus* from colonizing other,

empty spatial niches and formation of pseudosclerotia [16,106]. Other frequent potential pathogens were *Boeremia exigua*, *Cytospora pruinosa* and *Diplodia fraxini*. The first two were found previously on *F. excelsior* with symptoms of decline [55,97,107]. In dual cultures, both fungi were strongly antagonistic towards *H. fraxineus* which may suggest their effectiveness against *H. fraxineus* in nature. *Diplodia fraxini* (with pycnidia on the sampled petioles) was found on ash leaves for the first time. It has previously been associated with cankers and dieback of *F. excelsior* [98].

Most of the taxa detected in leaf petioles of *F. excelsior* are, however, known exclusively or generally to have a saprotrophic lifestyle on leaf residues of forest trees, dead wood or the remains of herbaceous plants. The group of the most frequent *saprotrophs* included representatives of the genera *Cyathicula*, *Hypoderma*, *Hymenoscyphus* and *Pyrenopeziza*, and the family *Xylariaceae*. *Cyathicula species* (Helotiales) are known from their common occurrence in ash leaf petioles [108,109]. These fungi were shown here not to have been eliminated by *H. fraxineus* from the petiole tissues. *Cyathicula fraxinophila* occurred most frequently. This fungus was often observed previously on ash petioles in the Czech Republic [53] but not in Ukraine [52]. It is host specific to *F. excelsior* and the epidemics of ash dieback in Europe may be associated with a decrease in its population. Another member of the genus, *C. coronata*, occurs on a wide range of herbaceous plants [108]. *Hypoderma rubi* has been reported on leaves, petioles and twigs of a wide range of woody plants, mostly dicotyledons; its main, most colonized host is *Rubus* [110]. It has been found on *F. excelsior* and other species of ash [111,112]. Its high frequency in dead leaf petioles of ash is shown and emphasized here. Unexpectedly frequent was also *Pyrenopeziza petiolaris* (Helotiales), which has been found previously on numerous broad-leaved trees (*Acer*, *Aesculus*, *Ailanthus*, *Betula*, *Fagus*, *Populus*, *Sorbus* and *Tilia*), particularly on *Acer pseudoplatanus* [109,113]. It is possible that *A. pseudoplatanus* occurred frequently in the sampled stands and so may have contributed to the frequent occurrence of *P. petiolaris* on *F. excelsior*. Apothecia of *P. petiolaris* mature in late spring, and the involvement of ascospores in early development of an endophytic phase in living leaves, followed by a saprotrophic life cycle and survival in dead leaf petioles until the following year, cannot be excluded.

Two members of the genus *Hymenoscyphus* found in this study, *H. caudatus* and *H scutula*, can colonize various species of plants. *Hymenoscyphus caudatus* occurs mostly in leaf debris of broad-leaved trees, including *Aesculus*, *Corylus*, *Populus* and *Salix*. *Hymenoscyphus scutula* occurs mostly on herbaceous stems of dicotyledons. Both fungi have been found previously on *F. excelsior* but with much lower frequency [52,109,114,115]. We did not detect *H. albidus*. Studies in other countries, such as the Czech Republic and Norway, suggest it was absent or occurred at low frequency [53,116].

Representatives of the *Xylariales* on *F. excelsior* petioles only sporadically produced stromata with conidia and were detected only on the basis of isolation, although numerous species from this order form fruit bodies on wood of many species of broad-leaved trees [95,96]. They are common endophytes in young leaflets and petioles of *F. excelsior* and other species of broad-leaved trees [81,95,99,117]. This endophytic phase can help them in further colonization of leaves after their fall.

Some species found unexpectedly on *F. excelsior* leaf petioles include *Phacidium lacerum*, *Leptosphaeria conoidea*, *Leptosphaeria urticae* and *Pseudoophiobolus italicus*. The first is known in Europe from its occurrence on needles of *Pinus sylvestris*. It invades the older generations of needles in the crown and persists in the fallen needles [118]. So far, *L. conoidea* has been found on dead stems of various herbaceous plants, especially *Asteraceae* (Compositae) or *Apiaceae* (Umbelliferae) [119], *L. urticae* on dead stems of *Urtica dioica* [120] and *P. italicus* on dead stems of *Onobrychis viciifolia* [121].

The frequent occurrence on petioles of species in the genera *Cyathicula*, *Hymenoscyphus* (other than *H. fraxineus*), *Hypoderma* or *Typhula* may have resulted from their time of fructification, during the leaf-fall season. This would give them priority of access among the other endophytes and pathogens that had colonized leaves in the tree crown before autumn leaf fall.

A similar interpretation may explain the differences in frequency of *Peziza varia* (9.7%) and *P. ninguis* (2.0%). The first one fruits in the leaf-fall season and the second one much later, at the spring thaw [122]. Ascospores of *P. varia* colonize the, as yet, uninhabited (in autumn) petioles of the fallen leaves, while *P. ninguis* has to compete with fungi that had colonized petioles earlier (during 3–4 months, in late autumn and winter).

Basidomycota detected in leaf petioles of *F. excelsior* from the previous year perform various ecological functions. It seems that some *Typhula* species are saprotrophs that contribute to decomposition of plant debris. There are, however, phytopathogenic *Typhula* species, *T. ishikariensis* and *T. incarnata*, which cause important diseases of cereals and grasses [65,66,123,124]. According to Yang et al. [66], sporophores of most *Typhula* species are usually found in autumn. Our results support this; only sclerotia were found in spring. *Peniophora incarnata* and *Trametes versicolor* are known decomposers of wood. Their degradation of all cell-wall constituents causes intensive white rot in logs, branches and stumps. Species of *Sistotrema cause* brown rot when carbohydrates are extensively removed and lignin is degraded only to a limited extent. *Coprinus* species, *Hypholoma fasciculare* and *Psathyrella piluliformis* form basidiocarps on stumps, often continuously, even from spring to autumn [94,125–127]. According to Dix and Webster [128], litter-inhabiting Basidiomycota in the genera *Marasmius*, *Mycena*, *Clitocybe* or *Collybia* are associated with the final stages of litter decay. Their preferences for pre-decayed substrate could limit their frequency in the previous year's leaf petioles. *Fellozyma* sp. and *Tilletiopsis washingtonensis* are representatives of the basidiomycetous yeasts. Neither taxon had preference for any dominant tree type [129,130]. Despite the significant proportion of yeasts in fungal communities in forest soil and litter, they were found sporadically at the decomposition stage of the ash petioles with the methods being used.

Examples of the relationships between fungi in ash leaf petioles demonstrate their complexity and that of the habitat, as is also indicated by different values of Shannon's and Simpson's diversity indices.

4.2. Competition between H. fraxineus and Saprotrophs

In addition to observations on simultaneous occurrence of *H. fraxineus* and other fungi in dead leaf petioles of *F. excelsior* in situ, further information on the competitive abilities of saprotrophs against *H. fraxineus* was obtained from dual-culture assays in vitro. The results show that some of the saprotrophs found can be considered as very effective antagonists of *H. fraxineus*.

It is well-known that *H. fraxineus* can produce numerous metabolites, including those with mycotoxic of phytotoxic properties [131–135]. The inhibition of mycelial growth of colonizers of ash stems and branches by *H. fraxineus* has already been observed in vitro at the beginning of studies on ash dieback [136]. The present results showed much variation among interactions between *H. fraxineus* and individual saprotrophs. Generally, *H. fraxineus* was able to reduce mycelial growth of most saprotrophs. However, all tested saprotrophs also reduced the growth of *H. fraxineus*. It may be assumed that the extent of inhibition depended mostly on the growth rate of the saprotroph and the type of mutual relationship. The present results on *H. fraxineus*–saprotroph interactions are broadly similar to those of others [27–29,137].

The dual-culture assay is expected to demonstrate the different strategies used by various groups to gain occupation of their niche and obtain the nutrition required for survival [57,128,138].

One of these groups includes the fast growing fungi, *Alternaria* spp., *Chromelosporium* sp., *Colletotrichum acutatum*, *Coniochaeta* sp. 2, *Epicoccum nigrum*, *Phacidium lacerum*, *Phoma* spp. and *Trametes versicolor*, which strongly inhibited growth of *H. fraxineus* in vitro, in agreement with results of Haňáčková [28] or Becker [139]. They may dominate during early substrate colonization. In nature, the overall effect may depend on the frequency of particular taxa. The frequent detection of the fast growing fungi in laboratory studies is often due to their affiliation to "pioneer communities of fungi" which may include

numerous "weed" species [128]. Their fast growth rate compensates for low metabolic (e.g., antibiotic) activity, in contrast to "later dominants", which may be highly antagonistic because of production of metabolic inhibitors and mycoparasitism [57,128,140].

The formation of an inhibition zone, regardless of its main producer (*H. fraxineus* and/or saprotroph), was the type of interaction seen most often. The most potentially valuable saprotrophs were those that inhibited growth of *H. fraxineus* from the greatest distance and produced wide inhibition zones. Such results were obtained with *Malbranchea* sp. (Onygenales) and *Pseudocoleophoma polygonicola* (Pleosporales). The former colonizes decomposed plants or cellulose-containing substrate, is thermophilic and is an important source of lignocellulolytic enzymes [141,142]. *Pseudocoleophoma polygonicola* is a recently described species with unknown ecology, previously found only in Japan on dead polygonaceous plants [143]. The related species, *Coleophoma empetri*, produces an antifungal echinocandin-like compound [144], suggesting promise for *P. polygonicola* which may have similar properties.

Other saprotrophs that produced inhibition zones are also known producers of antifungal metabolites or degrading enzymes. This group includes, among others, *Aureobasidium pullulans*, *Coniochaeta* spp., *Epicoccum nigrum* and *Fusarium lateritium*. *Aureobasidium pullulans* produces a group of antifungal metabolites described as aureobasidins, which are derivatives of cyclic deosipeptides, and secretes cell-wall degrading enzymes, including chitinase and β-1,3 glucanase, which contribute to lysis of pathogen hyphae [145,146]. *Coniochaeta* species produce antibacterial and antifungal coniosetin and coniochaetone-a and -b [147]. The frequently found *E. nigrum* produces flavipin, epicorazine and epipyrone A [145,148]. The antifungal activities of some of them has led to development of biological control products based on *E. nigrum* mycelium, spores and metabolites [148]. Moreover, this species secretes cell-wall degrading enzymes which contribute to lysis of pathogen hyphae. *Fusarium lateritium* produces five antifungal sesquiterpenes, including microsphaeropsisins D and E [149].

Melanins, important natural pigments, may also have an important role in establishing relationships among microorganisms. It is belived that they contribute to survival of fungi in diverse hostile environments [150]. In the present study, the production of dark-colored pigmentation was observed in tests with *H. fraxineus* and some members of the Pleosporales. Pukalski et al. [151] recently showed that *Plenodomus biglobosus* (Pleosporales) isolated from living leaf petioles of *F. excelsior* can produce pheomelanin, which is rarely produced by fungi.

Only a few fungi overgrew and covered the *H. fraxineus* colony in dual cultures, an effect that may indicate their potential for antagonism in nature. The strongest effects occurred with ascomycetous *Clonostachys rosea*, *Trichoderma viride* (Hypocreales), *Nemania*, *Rosellinia* and *Xylaria* (Xylariales), and basidiomycetous *Hypholoma fasciculare* (Agaricales) and *Peniophora incarnata* (Russulales). *Clonostachys rosea* and *T. viride* are well-known antagonists of many species of fungi [152–155] and their suppressive effects were expected.

Re-isolations of fungi from the covered colonies seemed to indicate successful replacement of *H. fraxineus* by the saprotrophs. Replacement of *H. fraxineus* by some endophyes from ash shoots in vitro has also been observed [28]. Boddy [156] pointed out that similar interactions in dead wood, in situ, may also lead to replacement of the weaker competitor in woody tissues. Successful and effective replacement of *H. fraxineus* in leaf petioles, however, when the pathogen is often surrounded by outer and inner pseudosclerotial plates, may be difficult [16]. Successful replacement could be demonstrated by the formation of the saprotroph's fruit bodies on pseudosclerotia of *H. fraxineus* which is rare. The protective value of pseudosclerotial plates can be compared with that of similar structures formed by saprotrophic *Xylaria hypoxylon*, which were so firm that they were able to prevent colonization of beech timber blocks by other saprotrophic fungi [157].

Interpretation of dual-culture assays generally supports results on colonization of leaf petioles. On 87.6% of petioles colonized by *H. fraxineus,* there were also other fungi which colonized other parts of the petiole, possibly making those parts unavailable to *H. fraxineus.*

In situ, growth of *H. fraxineus* was apparently limited by the same fungi that produced inhibition zones or made physical contact by overgrowth and replacement of *H. fraxineus*. These results show that the spectrum of fungi able to limit growth of *H. fraxineus* in situ is wide and includes more than 50% of species fruiting on leaf petioles. Significantly, most of these fungi are not host specific for *F. excelsior*. They occur on many species of trees, bushes and even herbaceous plants. Among this group, detected in the dead leaf petioles, are phytopathogenic *Discohainesia oenotherae*, *Leptosphaeria sclerotioides* and *Verticillium dahliae*. *Discohainesia oenotherae* is generally a pathogen or saprotroph on many woody species and cultivated plants, including strawberries, and occurs worldwide [158,159]. *Leptosphaeria sclerotioides* generally does not occur on trees. It is a pathogen on herbaceous legumes, particularly sweet clover (*Melilotus alba*) and lucerne (*Medicago sativa*). It may also be pathogenic on grasses and cereals exposed to low temperature [119,160]. *Verticillium dahliae* is a dangerous, vascular pathogen. It survives easily and efficiently in the form of microsclerotia in soil and plant debris. Its occurrence additionally in ash stands suggests a risk for economically important agricultural or horticultural production and the health of forests [161].

Ash dieback has caused a reduction in *F. excelsior* stands in many regions of Europe. This is being followed by a decreased frequency of fungal colonizers of ash [162]. Current studies suggest, however, that they may be replaced by other fungi which are colonizers of other species of trees and shrubs and even of herbaceous plants.

Further studies on antagonism of mycobiota against *H. fraxineus* in situ are needed. Assessment of the chance of fungi being able to compete with *H. fraxineus* must take into account the important fact that *H. fraxineus* has the advantage of having early, pre-competitive infection and colonization of living leaves while still in the crown.

5. Conclusions

An important stage in the life cycle of *H. fraxineus* on *F. excelsior* is its successful survival until the following year in dead leaves, particularly in leaf petioles which, in summer, will be the substrate for fruit-body development. This stage can be affected by co-colonizers. Co-colonizers of 139 fungal taxa, including Ascomycota (120 species), Basidiomycota (17 species) and Mucoromycota (2 species), were identified in dead leaf petioles of *F. excelsior* from the previous year. Many of these fungi showed antagonism to *H. fraxineus*. Few of these taxa were host specific to *F. excelsior*. Fungal taxa that occur in a wide range of trees, shrubs and herbaceous plants were more frequent, which is important considering that *F. excelsior* has a decreased share in European stands owing to ash dieback. The structure of fungal communities in leaf petioles of *F. excelsior* emphasizes the significance of plant species diversity in forests and the necessity for accumulation of wood debris, which serves as substrate for fruit-body and spore production by potential antagonists of *H. fraxineus*, including species of *Coniochaeta*, *Nemania*, *Peniophora*, *Rosellinia*, *Trametes* and *Xylaria*.

Supplementary Materials: The following are available online at: https://www.mdpi.com/article/10.3390/f12101412/s1, Table S1: List of morphotypes from the previous year's leaf petioles of *Fraxinus excelsior* in five forest stands, in southern Poland in 2017. Sequences deposited in GenBank. Reference sequences from GenBank. Results of interactions of test fungi with two isolates of *Hymenoscyphus fraxineus* (Hf 1/Hf 2) in vitro; Table S2: Fungi isolated from the previous year's leaf petioles of *Fraxinus excelsior* (subsample I) and fungi detected in situ from fruit bodies on the previous year's leaf petioles of *Fraxinus excelsior* (subsample II and III); Figure S1: Phylogram obtained from maximum likelihood (ML) analyses of the ITS1/2 data for the isolated fungal taxa. Sequences obtained during this study are presented in bold type. The Bootstrap values ≥75% for ML and Bayesian inference (BI) analyses are presented at branch as follows: ML/BI. * Bootstrap values <75%. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Fellozyma inositophila* and *Fellozyma* sp. represent the outgroup; Figure S2: Principal component analysis (PCA) with frequencies of fungal variables represented by arrows and sites represented by black circles, for mycobiota detected in situ from fruit bodies on the previous year's leaf petioles of *F. excelsior*;

Figure S3: Similarity of fungal communities detected in situ from fruit bodies on the previous year's leaf petioles of *F. excelsior* in five stands shown by dendrogram based on Bray–Curtis similarity index; Figure S4: Network of co-occurrence of fungi detected in situ from fruit bodies on the previous year's leaf petioles of *Fraxinus excelsior* in five stands, in southern Poland, determined on the basis of data grouped for the study plots. Each connection shows a strong (Spearman's $p > 0.6$) and significant ($p < 0.05$) correlation. Blue lines indicate positive correlation. The same color represents fungal taxa with potential co-occurrence in the same module. The frequency of the species was indicated by the size of each node.

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