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Investigation of the Biocontrol Potential of Two Ash Endophytes against *Hymenoscyphus fraxineus* Using In Vitro Plant–Fungus Dual Cultures

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Abstract: Development of effective biocontrol procedures using ash endophytes to combat an ash pathogen *Hymenoscyphus fraxineus* would be an appropriate contribution to the ongoing effort to protect European ash stands against ash decline. In this study we investigated the biocontrol potential of two ash endophytes, *Thielavia basicola* and *Minimidochium* sp., against *H. fraxineus* using in vitro plant-fungus and fungus-fungus dual cultures approach in three biocontrol models. The tests aimed to determine whether the endophytes show antagonism toward *Fraxinus excelsior* and *F. pennsylvanica*, to assess the level of antagonism of the endophytes toward *H. fraxineus* and to identify potential secondary metabolites induced by the presence of *H. fraxineus*. The results that dual culture experiments modeled according to our design may be a very useful tool to precisely study biocontrol potential of fungi, i.e., without the impact of environmental factors. Such experiments also enable the selection of most resistant ash genotypes and rapid propagation, producing large numbers of pathogen-free seedlings. It should be noted, however, that both of the endophytes tested in the dual cultures strongly inhibited the growth of *H. fraxineus*. Their growth under the influence of callus/seedlings was also inhibited. Comparison of HPLC profiles showed that the presence of *H. fraxineus* in the post-culture medium induced the production of an unknown secondary metabolite in this species. Such results suggest that some of the plant–fungus combinations examined in this study may have potential to be developed as biocontrol methods, thus increasing the survivability of ash stands under natural conditions.

Keywords: biocontrol models; dual cultures; endophytes; *Fraxinus excelsior*; HPLC; *Hymenoscyphus fraxineus*

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1. Introduction

Most tree species harbor abundant communities of microscopic fungi, including numerous species of endophytes [1]. Endophytes are microorganisms that develop asymptomatically within the host tissues, at least for a part of their life cycle, forming disjunctive microthalli that may consist of only a few cells [2,3]. In wild plants the communities of endophytes are usually very diverse, both phylogenetically and ecologically [4], resulting in a variety of trophic interactions with the host plant, ranging from parasitism to full mutualism [5,6]. For instance, the close integration of fungi within plant tissues offers numerous opportunities for increase of disease resistance in the host. Schulz et al. [6] hypothesized that this is achieved via various secondary metabolites that are secreted in response to the presence of other fungi or bacteria. This kind of endophyte-pathogen antagonism effected by secondary metabolites may be frequently observed in dual cultures where mycelial growth of particular fungi is stopped without physical contact of the colonies. Indeed, secondary metabolites can be sometimes observed directly, e.g., as

macroscopic crystalline structures on the surface of agar plates [7]. Fungal endophytes can also protect plants against pathogens through direct interaction through hyperparasitism or competition through the use of substrates. In addition, endophytes can promote plant growth, reduce their sensitivity to abiotic stress, and protect plants against herbivores [8].

Most plant endophytes belong to *Ascomycota* [5]. Some of them are able to produce abundant secondary metabolites [9]. There is substantial differentiation of species within this group, as virtually all of the species within the *Ascomycota* have their own specific profile of metabolites [10,11]. The abundance and specificity of produced metabolites may be used in further studies aiming to obtain certain compounds, and in biocontrol applications.

However, the interactions between the host plant and various endophytes and pathogens, and between various species of endophytes are intrinsically unstable and are often described as balanced antagonism. Relatively minor changes of environmental conditions or changes in the pressure exerted by competitors may cause some endophytes to become pathogens [6]. Thus, some endophytes are potential pathogens capable of causing various diseases in economically important tree species [12]. The pathogenicity of individual endophytes can be assessed using in vitro dual culture methods without the need of laborious in vivo surveys under natural conditions or controlled pathogenicity tests [13,14]. Moreover, the dual culture approach offers the opportunity to select the most resistant plant genotypes to a given fungal species. Thus, studies using plant cultures in vitro may help to assess the risk of fungal infections. Such procedures usually allow also the production of high numbers of individual plants that are characterized by increased resistance [15]. In addition to this, dual cultures allow for research on secondary metabolites [16]. This is due to the intermediary callus state that plants regenerated via somatic embryogenesis or indirect organogenesis pass through; in vitro produced seedlings are not only free of pathogens, but also may be characterized by increased levels of secondary metabolites as an effect of the resistance response [17]. All this means that in vitro callus cultures are an important experimental tool enabling the facilitation of biotechnological, phytopathological (dual cultures, biocontrol tests), pharmacological (secondary metabolites) and physiological (micropropagation) studies [18–20].

Fraxinus excelsior L. (European ash) is an economically and ecologically important tree species in Europe that is threatened by the rapid expansion of *Hymenoscyphus fraxineus* [21–24], the cause of ash dieback disease. The pathogen causes severe defoliation and the dieback of twigs and branches in the crown, frequently resulting in tree mortality at levels reaching 85% in plantations and 69% under natural forest conditions [25]. Apart from its main host, *F. excelsior*, the fungus causes similar disease symptoms in other ash species, however the intensity of disease symptoms varies significantly. *F. angustifolia*, which occurs mainly in southern Europe, is also highly susceptible [22,24].

The manna ash (*Fraxinus ornus*) is considered weakly susceptible to *H. fraxineus* [26]. *F. pennsylvanica* Marsh can be assessed as moderately susceptible, and the tests performed indicate some differentiation in pathogenicity in relation to this species of ash [22,27]. In Poland, a new species, *Hymenoscyphus pusillus*, has recently been demonstrated on the leaves of *F. pennsylvanica*, the pathogenicity of which has not yet been determined [28].

The extent of damage caused by *H. fraxineus* in Europe demonstrated the need for new procedures to protect ash stands. Thus, our study focuses on the development of biocontrol methods using ash endophytes, minimizing the negative effect of the pathogen. Indeed, recent analyses show that endophytic fungi have an evident potential to become important biocontrol agents, due to their ability to increase host vitality and resistance to pathogen infection [29]. Our studies on the optimization of endophytes' selection and application are based on the analysis of dual interactions between the pathogen, endophytes and host plant. An important aspect in these experiments was the investigation of effective interactions between pathogen/endophyte and the host using in vitro dual cultures. Our studies assessed interactions for the dual combinations of three fungi: the ash pathogen *H. fraxineus* and two ash endophytes: *Thielavia basicola* and *Minimidochium* sp., and two

types of tissues, i.e., callus cultures and callus regenerated seedlings, of two ash species: *F. excelsior* and *F. pennsylvanica*.

Thielavia basicola, occurs in close association with another hyphomycete species that traditionally has been identified as *Thielaviopsis basicola*. Apart from the mycoparasitic *T. basicola*, most of *Thielavia* occur in soil, and some of these species have been found to have economic and ecological significance [30]. Several *Thielavia* produce bioactive metabolites, such as prostaglandin synthetase inhibitors produced by *T. terricola* [31], and antifungal compounds that are active against *Candida albicans* produced by *T. subthermophila* [32]. *Minimidochium* sp., on the other hand, produces blue laccases, i.e., a group of enzymes mediating oxidation of numerous organic and inorganic compounds, such as various phenols and polyphenols, benzenethiols and metal cyanide complexes [33]. Ecologically, laccases are abundantly produced by numerous Ascomycota species which are partly responsible for white wood rot and contribute to litter decomposition through the degradation of lignin [34]. Despite the fact that *Minimidochium* sp. is known mainly as a saprotrophic fungus, it can also colonize the tissues of living plants, causing disease symptoms as described above. Thus, it acts as an endophyte. An example of such a relation is the occurrence of the well-known fungus *Desmazierella acicola*, commonly occurring on *Pinus sylvestris* [35], as well as many other species of fungi.

Development and commercialization of biocontrol techniques is not an easy process and, currently, the number of biocontrol studies targeting fungal pathogens is limited. Thus, laboratory techniques involving controlled conditions in vitro may be a useful tool for facilitating studies on potential biocontrol agents [36]. Such an approach will enable the testing of various plant-fungus and fungus-fungus interactions in controlled conditions without the added complexity resulting from the impact of the external environment under field conditions.

The aim of this study was to use the dual cultures approach to precisely investigate the mutual interactions between two endophytes, i.e., *T. basicola* and *Minimidochium* sp., ash pathogen *H. fraxineus* and two ash species *F. excelsior* and *F. pennsylvanica*. In particular, we aimed to: (i) determine the interactions between the endophytes and ash callus cultures, (ii) determine the interaction between the endophytes and in vitro regenerated ash seedlings, (iii) assess the level of antagonism of the endophytes toward the ash pathogen *H. fraxineus*, (iv) assess the usefulness of dual culture approach in selecting potential endophytic candidates to be tested as biocontrol agents and (v) identify secondary metabolites that are potentially secreted by the endophytes in reaction to *H. fraxineus*.

2. Materials and Methods

2.1. Plant and Fungal Material

The plant material used in this study comprised either callus cultures or in vitro regenerated seedlings of two ash species: *F. excelsior* and *F. pennsylvanica*. The callus cultures were initiated using zygotic embryos (primary explants) extracted from mature ash seeds collected in October 2018 in the stand located in the Kańczuga Forest District (Southern Poland, Directorate of State Forests in Krosno, 49°59′00″ N, 22°24′42″ E, 210 MSL). Sixty-year-old ash trees were selected from the economically managed mixed species stand. Seeds were harvested from a single uninfected *F. excelsior* tree and a single uninfected *F. pennsylvanica* tree.

The procedure involved surface sterilization of ash seeds using 12% sodium hypochlorite (Sigma-Aldrich, St. Louis, MO, USA) prior to culturing being performed and following the protocol described in patent application no DP.P.433288 WIPO ST 10/C PL433288 and WO2021187995A2. All the callus cultures were systematically subcultured every 18 days.

The ash seedlings used in the study were regenerated from callus cultures via indirect organogenesis. They were obtained in vitro from secondary explants, which ensured they were free of fungal pathogens. All of the callus cultures selected for further analyses after nine months of culturing reached a diameter of 25 mm. and mass of ca. 2.15 $\pm$ 0.1 g; they were characterized by proper color, hydration, size, vitality and the content of photosynthetic pigments, indicating their good physiological condition.

The fungal material used in the biocontrol tests comprised three fungi: two endophytes: *T. basicola* (isolate C43), *Minimidochium* sp. (isolate Jp 49), and the ash pathogen *H. fraxineus* (isolate 20952). Both, *H. fraxineus* and *T. basicola*, were isolated from the plant material collected in southern Poland (50°21′69″ N, 20°02′40″ E, 298 MSL): the pathogen from dead leaf rachis of *F. excelsior* was collected in 2017 from the litter, while the endophyte from asymptomatic *F. excelsior* was collected from leaf petiole. *Minimidochium* sp., on the other hand, originated from live *F. pennsylvanica* leaves collected in the Kraków-Zakrzówek forest circle (50°02′22.62″ N, 19°54′57.60″ E, 207 MSL). The fungi were obtained from the collection of Professor Tadeusz Kowalski, Department of Forest Ecosystems Protection, University of Agriculture in Krakow. The two endophytes used in our experiments were selected based on the previous results. Among the 90 *F. excelsior* endophytes examined by Kowalski et al. [28], *T. basicila* and *Minimidochium* sp. showed the widest inhibition zone in dual cultures, with *H. fraxineus*, *T. basicila* also producing unidentified crystalline structures in vitro [28]. Surface sterilization of the plant material (ash petioles/rachises) was carried out subsequently by dipping the plant material in the following solutions: ethanol (96%) for 1 min, NaOCl (approx. 4% of available chlorine) for 5 min, and ethanol (96%) for 30 s. Next, the samples were dried by placing them between layers of blotting paper [28].

For each experimental combination, we chose cultures obtained from 100 calluses and about 25 secondary seedlings of each *Fraxinus* species. For all experiments, three controls were tested for callus tissue, secondary seedlings induced on the callus cultures, and the fungi. We used three replicates (three dishes and three Erlenmayer flasks) for each model of dual cultures.

2.2. Photosynthetic Pigments Content in Propagated Plant Material

The aim of the analyses carried out on photosynthetic pigments was to determine whether the biotic stress (fungi) caused changes in the ash assimilation apparatus compared to the control leaves (not subjected to stress).

The experiment involved estimations of chlorophyll a, chlorophyll b and the total carotenoids content in ash callus and seedlings, i.e., the parameters useful in assessing photosynthetic activity and genetic stability. The total photosynthetic pigments were extracted in temperatures of liquid nitrogen with 80% (*v*/*v*) acetone from eight-month-old non-embryogenic callus cultures and six-week-old secondary seedlings of *F. excelsior* and *F. pennsylvanica*. The extracts were centrifuged (2 min, 13,400× *g* rpm), followed by absorbance measurements of the resulting supernatant at wavelengths of 663.2; 646.8 and 470 nm [37]. The contents of particular pigments (i.e., chlorophyll a (C_a), chlorophyll b (C_b), total chlorophyll (C_{a+b}) and total carotenoids (C_{x+c})) were calculated according to the following formulas:

$$C_a = 12.25\ A_{663.2} - 2.79\ A_{646.8} \quad (1)$$

$$C_b = 21.50\ A_{646.8} - 5.10\ A_{663.2} \quad (2)$$

$$C_{a+b} = 7.15\ A_{663.2} + 18.71\ A_{646.8} \quad (3)$$

$$C_{x+c} = (1000\ A_{470} - 1.82\ C_a - 85.02\ C_b)/198 \quad (4)$$

2.3. Dual Cultures In Vitro—Model for Biocontrol Studies

The biocontrol potential of the endophytes was tested according to three bio-models. Two models included plant/fungus dual cultures using two types of plant material: callus cultures and seedlings of both ash species, coupled with all three fungi examined in this study, i.e., endophytes *T. basicola* (isolate C43) and *Minimidochium* sp. (isolate Jp49) and ash-pathogenic *H. fraxineus* (isolate 20952). In the third bio-model we tested the interactions between the endophytes and the ash pathogen *H. fraxineus*.

2.4. Interactions of F. excelsior and F. pennsylvanica Callus Tissues with H. fraxineus and the Endophytes

Dual cultures were established using callus lines representing the unique genotypes of green and European ash. Twelve cultures were identified for each species, and were characterized by the highest proliferation coefficient. Three callus cultures were used to establish control cultures without a fungal co-partner. The average callus proliferation rate was about 90%. The particular callus cultures used in this experiment were selected based on their optimal physiological parameters (diameter—1.0 cm, mass—1020 mg on average, color—green-cream, amount of photosynthetic pigments—Table 1).

Table 1. The content of photosynthetic pigments per one gram of measured sample.

Sample	Chlorophyll a (mg)	Chlorophyll b (mg)	Total Chlorophylls (mg)	Carotenoids (mg)
Callus	0.011 ± 0.004	0.010 ± 0.003	0.021 ± 0.007	0.004 ± 0.002
Seedling	0.620 ± 0.067	0.342 ± 0.160	0.962 ± 0.227	0.158 ± 0.001

The following procedure was used to establish dual cultures. A callus culture was placed in a Petri dish (diam. 90 mm) containing a modified MS medium (patented) ([38,39]—described in DP.P.433288) 0.5 cm of the dish's side. Each time a plug, with a diameter of 0.5 cm, of an actively growing fungal culture was placed 15 mm from the edge of the callus lump. All of the combinations were prepared in triplicate, including 15 dishes for the control (only plant or fungal copartner) and 18 dishes of dual cultures. Such prepared cultures enabled precise measurements of mycelium growth toward callus culture and in the opposite direction, as well as easy observations of any modifications of both copartners' phenotypes. The particularly significant development was a stop reaction that involved the complete suppression of mycelial growth toward the callus culture, potentially indicating the production of secondary metabolites by callus tissue. A development of dual culture was observed every 48 h. Callus of *F. excelsior* and *F. pennsylvanica*, as well as fungi, were incubated in a Biogenet FS 3400 phytotron at 24 °C ± 1 °C, 50% humidity, in the dark. All reagents used in this experiment were purchased from Sigma-Aldrich.

2.5. Interactions of F. excelsior and F. pennsylvanica Seedlings with H. fraxineus and the Endophytes

This experiment involved six-week-old seedlings of *F. excelsior* and *F. pennsylvanica* and all three studied fungi, *H. fraxineus*, *T. basicola* and *Minimidochium* sp. The particular seedlings used in the experiment were selected based on their optimal physiological parameters (size, number of leaves, color, and amounts of photosynthetic pigments).

Dual cultures were prepared in triplicate for combinations of both the ash species and all of the fungi mentioned above. In total, 21 cultures were established, including three control cultures (without the fungal copartner) for each of the tree fungi. The cultures were set up in a similar way as before. This time, however, we used sterile, transparent and ventilated polypropylene containers, 40 mm high, lid diam. 90 mm (Plant Culture Dish PP, SPL Life Sciences Co., Pocheon-si, Gyeonggi-do, Korea) instead of Petri dishes, a modified MS medium [39] (patent no DP.P.433288) containing 5.25 mg × dm^{-3} of indole-3-butyric acid (IBA) and white LED illumination (the remaining conditions have been tested in the previous experiment).

2.6. Interactions between H. fraxineus and the Endophytes in Dual Cultures

The *H. fraxineus*/endophyte dual cultures involved one strain of the pathogen and one strain of the two studied endophytes, and all combinations were prepared with two replicates. The cultures were established in Petri dishes with a malt extract agar medium (MEA) (20 g × dm^{-3} malt extract, Difco—Sparks, St. Louis, MD, USA + 15 g × dm^{-3} agar, Difco), supplemented with 100 mg L^{-1} tetracycline (BioShop Canada, Burlington, ON, Canada) to suppress bacterial growth. The cultures were incubated at 19 °C ± 1 °C in the

dark for three weeks. During the incubation, the cultures were observed for macroscopic interactions between *H. fraxineus* and the endophytes, especially in terms of mycelial growth of the pathogen, and the final observations were made after three weeks.

The radius of *H. fraxineus* colonies were measured along the axis connecting the centers of the plugs of both partners. The percentage inhibition of the pathogen's radial growth was calculated according to the formula: $(Rc - Ri)/Rc \times 100$, where Ri is mycelial growth of *H. fraxineus* toward the endophytic fungus and Rc is the radius of the pathogen's colony on a control plate (Rc) [40]. The macroscopic interactions observed in dual cultures were photographically documented.

Two endophytes characterized by the strongest inhibitory effect on *H. fraxineus* growth, i.e., *T. basicola* and *Minimidochium* sp., have been tested in liquid dual cultures to estimate how their metabolite profiles are affected by *H. fraxineus* metabolites. Dual cultures were prepared using liquid cultures of *H. fraxineus*, incubated in conical flasks. All the liquid cultures had been incubated for three weeks at 120 rpm (ELMI DOS-20 shaker), in 19 °C, containing 100 mL of liquid malt extract medium (ME: g × dm^{-3} malt extract, BIO-MAXIMA Poland). After three weeks of incubation, the *H. fraxineus* cultures were centrifuged (10 min, 4500× *g* rpm) (Centrifuge 5810 R, Eppendorf, Hamburg, Germany), three falcon-type tubes (capacity 15 mL) were half filled in with the resulting supernatant. Five additional tubes were filled (7 mL each) with fresh ME medium. Two of supernatant-filled tubes and four fresh medium tubes were inoculated with an endophyte, either *T. basicola* (isolate C43) or *Minimidochium* sp. (isolate Jp49), with each combination occurring in three replicates. In addition, two fresh medium tubes were inoculated with *H. fraxineus* using the centrifuged pathogen's mycelium. The design of the experiment involving liquid cultures is summarized in Figure 1.

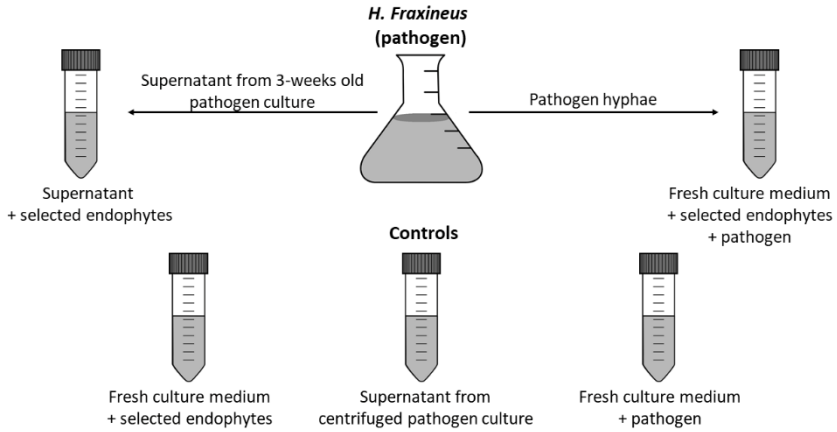

Figure 1. Scheme of the experiment with endophytic fungi *Thielavia basicola* and *Minimidochium* sp.

2.7. Preliminary Investigation of Metabolites Inhibiting Mycelial Growth of H. fraxineus

Secondary metabolites produced in response to *H. fraxineus* were examined in post-culture media of both endophytes, either co-cultured with *H. fraxineus* or cultured with addition of purified post-culture medium of *H. fraxineus*. All of the liquid cultures were further incubated for the next three weeks with 120 rpm shaking (ELMI DOS-20 shaker). After this time, liquid media from each culture were separated from mycelium by decantation, transferred to new falcon-type tubes and centrifuged (14,000× *g* rpm, 1 h, 20 °C, Centrifuge 5810 R, Eppendorf); the resulting supernatants were stored at −4 °C until further analyses.

To investigate the presence of potentially active metabolites of *H. fraxineus*, *T. basicola* and *Minimidochium* sp., high-performance liquid chromatography (HPLC) analyses were performed of the colony media purified in the previous step according to the diagram presented in Table 2. The analysis was performed using an Agilent Technologies 1260 Infinity II HPLC set, including: thermostatic autosampler, UV-Vis DAD detector and Nucleosil 100 C18.5 µm, 25 × 0.4 mm^2 column (Teknokroma, Barcelona, Spain). Prior to injections, the

samples were filtered through a 0.45 µm pore membrane filter. A volume of 100 µL of the sample was used to inject the column with 1 mL/min of effluent flow. Detection involved a DAD detector recording UV-Vis spectrum of 200–700 nm with particular measurements recorded at 234, 278, 360 and 440 nm wavelengths.

Table 2. Mobil phase gradient condition. Solvent A—acetonitrile:methanol:water (72:8:1 *v*/*v*).

Time (min)	Mobil Phase (%)			Flow Rate (mL/min)
	Water	Solvent A	Methanol	
0.00	85.00	15.00	-	1.00
10.00	-	100.00	-	1.00
15.00	-	100.00	-	1.00
16.00	-	-	100.00	1.00
21.00	-	-	100.00	1.00
22.00	85.00	15.00	-	1.00
25.00	85.00	15.00	-	1.00

2.8. Statistical Analysis

The data were analyzed statistically using software Statistica 13. One-way analysis of variance (ANOVA) and an accompanying Scheffe test were used to determine which means are significantly different between the control and biocontrol models at the significance set at *p*-values < 0.05.

Those analyses allowed us to determine if the presence of *Fraxinus excelsior* or *Fraxinus pennsylvanica* influenced the growth of each of the tested species of fungi. For this purpose, the size of the mycelium were compared after 14 and 21 days of growing, with the size of the mycelium growing according to one of the *Fraxinus* species after 14 and 21 days. The same symbols within the analyzed pair of species mean no differences, and different symbols mean statistically significant differences (Table 3). The same procedure was used to determine the effect of the two different fungi species on each other.

Table 3. The means of the radii of three fungal species in dual cultures and control cultures. Post-hoc analysis with the Scheffe test.

		The Means of the Radii (mm)			
1. Callus:	**Fungi Species:**	**Control (Fungus) Biocontrol Models [1,2,3]**			
		After 14 Days	**After 21 Days**	**After 14 Days**	**After 21 Days**
Fraxinus excelsior *Fraxinus pennsylvanica*	*Hymenoscyphus fraxineus* (isolate 20952)	33 ± 1.00^{a}	41 ± 1.73^{b}	20 ± 1.00^{c} 33 ± 1.00^{a}	24 ± 1.73^{c} 35 ± 1.73^{a}
Fraxinus excelsior *Fraxinus pennsylvanica*	*Thielavia basicola* C43	4 ± 2.00^{a}	10 ± 2.00^{b}	0^{a} 0^{a}	3^{a} 4 ± 2.00^{a}
Fraxinus excelsior *Fraxinus pennsylvanica*	*Minimidochium* sp. Jp49	40 ± 2.00^{a}	43 ± 1.00^{a}	12 ± 2.00^{b} 14 ± 1.73^{b}	22 ± 3.46^{c} 23 ± 1.00^{c}
2. Seedling:	**Fungi species:**				
Fraxinus excelsior *Fraxinus pennsylvanica*	*Hymenoscyphus fraxineus* (isolate 20952)	33 ± 1.00^{a}	41 ± 1.73^{b}	18 ± 1.00^{c} 15^{b}	24 ± 1.73^{d} 34 ± 2.65^{a}
Fraxinus excelsior *Fraxinus pennsylvanica*	*Thielavia basicola* C43	4 ± 2.00^{a}	10 ± 2.00^{b}	2 ± 1.00^{a} 0^{a}	19 ± 3.00^{c} 15 ± 1.00^{c}
Fraxinus excelsior *Fraxinus pennsylvanica*	*Minimidochium* sp. Jp49	40 ± 2.00^{a}	43 ± 1.00^{a}	10 ± 2.00^{b} 12 ± 2.00^{b}	12 ± 1.00^{b} 18^{c}
3. Fungi species:	**Fungi species:**				
Hymenoscyphus fraxineus (isolate 20952)	*Hymenoscyphus fraxineus* (isolate 20952)	34 ± 1.73^{a}	42^{b}	-	-
	Thielavia basicola C43	10 ± 2.00^{a}	16 ± 1.73^{b}	5 ± 2.00^{c}	7 ± 1.00^{ac}
	Minimidochium sp. Jp49	40 ± 2.00^{a}	43 ± 1.00^{a}	38 ± 2.65^{a}	43 ± 1.00^{a}

[1, 2, 3] Biocontrol models in vitro: callus-fungus; seedling-fungus; fungus-fungus; *p*-values < 0.05 [a, a; b, b; c, c] Same letters—no statistically significant differences. Other letters—there are statistically significant differences.

3. Results

The control calluses were successfully initiated from 85% of *F. excelsior* and 89% of *F. pennsylvanica* seeds. The rates comprise the proportions of embryos that initiated viable and potentially morphogenetic callus cultures (i.e., those that were not spontaneously killed by bacteria or fungi) to the overall number of embryos cultured. The control callus was yellow-green, compact and hydrated at ca. 50%, and was morphogenetically directed toward granular growth (Figure 2).

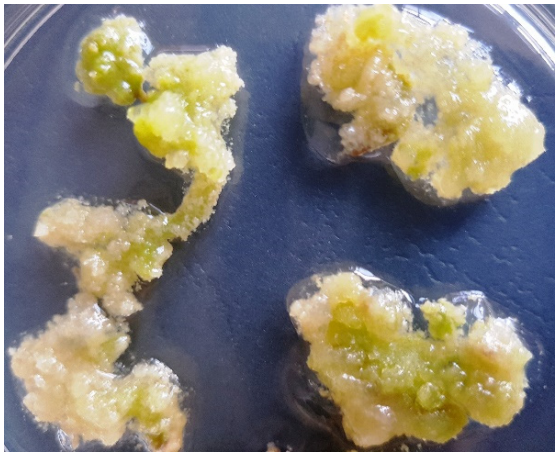

Figure 2. Callus cultures of *Fraxinus excelsior* used as the control for dual culture experiments in vitro.

All of the callus cultures were further propagated in vitro and used to produce seedlings via indirect organogenesis. In total, regeneration yielded 73 individual seedlings; 42 for *F. excelsior* and 31 for *F. pennsylvanica*. All of the seedlings were physiologically well developed, properly rooted and contained adequate levels of chlorophyll pigments (Table 1). They had 14 to 16 viable and photosynthetically active leaves (Figure 3).

Figure 3. Seven-week-old seedlings of *Fraxinus excelsior* regenerated in vitro (control).

3.1. Photosynthetic Pigments Content in Propagated Plant Material

No statistically significant differences in the photosynthetic pigment content have been observed between *F. excelsior* and *F. pennsylvanica* seedlings produced in vitro. Both types of seedlings contained high concentrations of chlorophyll a, and they were approximately 50% and 75% higher than those of chlorophyll b and carotenoids, respectively (Table 1).

Similarly, the concentrations of photosynthetic pigments did not differ significantly between the callus cultures of both ash species. However, they proved to contain much lower amounts of the pigments than seedlings (Table 1).

3.2. Biocontrol Tests

Results of the seedling vs. fungus cultures clearly demonstrated that *T. basicola* C43 was the only fungus whose growth was greatly inhibited in the dual culture with the ash seedling (biocontrol model no. 2). This was confirmed by the performed statistical analysis (Table 3).

The average radius of *T. basicola* colonies growing in the presence of callus tissue (bio-model 1) was not statistically affected by callus cultures, either of *F. excelsior* or *F. pennsylvanica*. The results of the Scheffe test (Table 3) show that the mycelial growth of H. fraxineus colonies toward *T. basicola* in dual cultures (bio-model 3) was statistically significantly reduced, compared to the control. Moreover, *T. basicola* growth rate in the control (all three bio-models) measured after 14 and 21 days was statistically different (the diameter of the mycelium was about 6 mm larger) from the other tested fungi. The fungus did not kill the callus, either of *F. excelsior* or *F. pennsylvanica*, after 60 days of culturing (without passaging). On the contrary, *T. basicola* mycelium formed a characteristic white coating preventing the hyphae from reaching the callus tissues. In all likelihood, the mycelial growth was stopped by the callus tissue producing secondary metabolites. After such a long culturing time, the calluses themselves usually remained—especially in the case of *F. pennsylvanica*—fully viable, at least partially green and optimally hydrated. The endophyte also caused yellow discoloration of the MS medium (Figure 4). *Minimidochium* sp., on the other hand, started to grow faster in dual cultures, as early as in the fourth day of culturing. After 21 days it reached the callus tissue of both, *F. excelsior* and *F. pennsylvanica*, but did not overgrow them causing only slight browning at the points of contact (Table 3, Figure 5). Dual cultures involving the callus of both ash species and *H. fraxineus* resulted, inevitably, in the dieback of the callus co-partner, which occurred no later than after seven days. The callus cells became necrotic and not capable of morphogenesis (Figure 6). Thus, callus cultures, as well as their regenerated seedlings (see below), were systemically infected by *H. fraxineus* without any signs of defense response; that is, the callus tissue did not produce any substances secondary to the medium (e.g., in the form of metabolites) and callus and seedlings did not inhibit, even for one day, the pathogen mycelium.

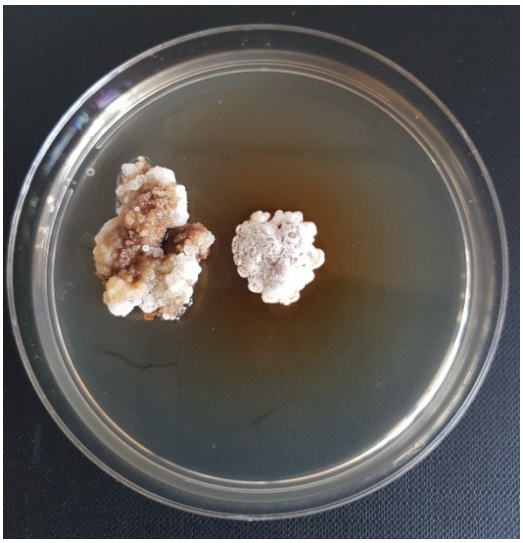

Figure 4. Interaction of *Fraxinus excelsior* callus cultures with endophyte *Thielavia basicola*.

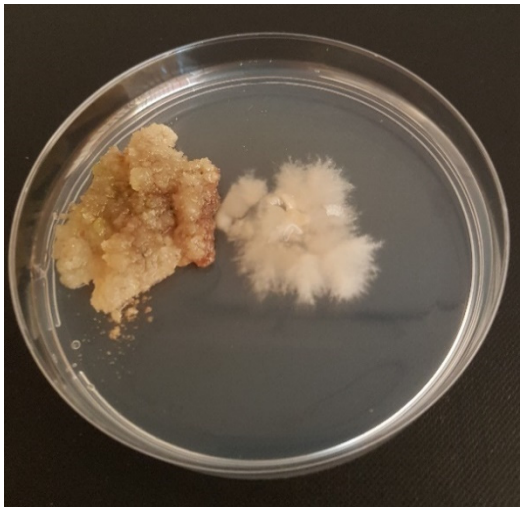

Figure 5. Interaction of *Fraxinus excelsior* callus cultures with endophyte *Minimidochium* sp.

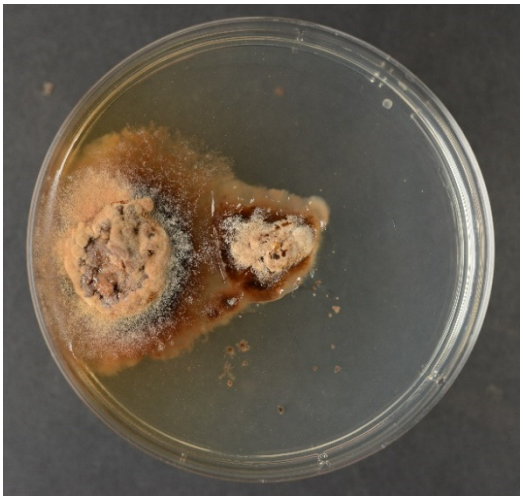

Figure 6. Interaction of *Fraxinus excelsior* callus culture with pathogen *Hymenoscyphus fraxineus*.

The growth rates of *T. basicola* toward *F. excelsior* and *F. pennsylvanica* callus are shown in Figure 7. The data shown indicate that the growth of the endophyte stopped in the 18th day of culture for approximately 22 days. It then resumed and continued for the next eight days (form 42th to 50th day of culturing), after which time the stop reaction appeared again and was not overcame as the experiment ended.

In general, the seedlings showed very similar defense reactions to endophytes *T. basicola* and *Minimidochium* as previously observed in callus cultures. After 21 days of dual cultures, they were completely viable and contained high chlorophyll concentrations, comparable to the control plants (Table 1, Figure 8A). Similar to callus cultures, no defense response was observed in seedlings in reaction to the presence of *H. fraxineus*, resulting in their complete dieback.

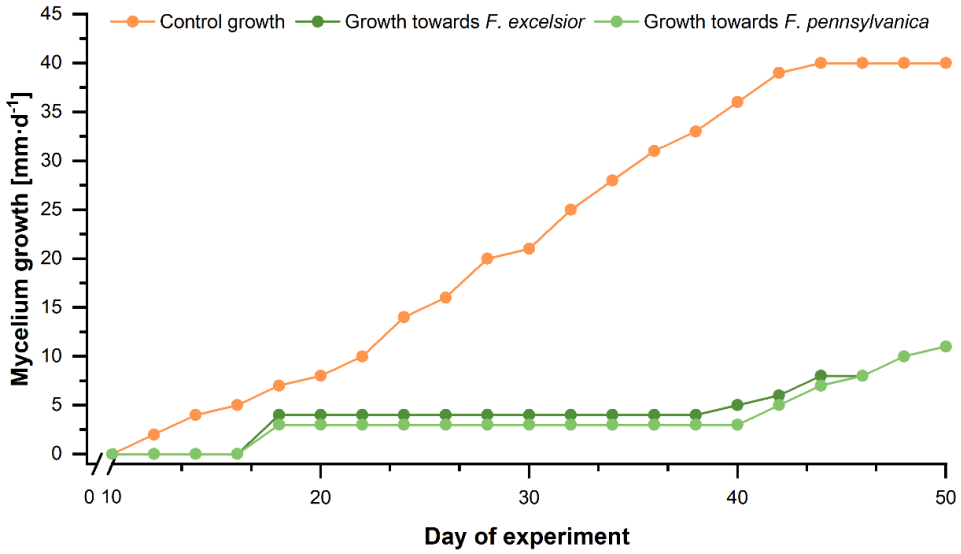

Figure 7. Growth rate of the endophytic fungus *Thielavia basicola* towards callus tissues of *Fraxinus excelsior* and *Fraxinus pennsylvanica*.

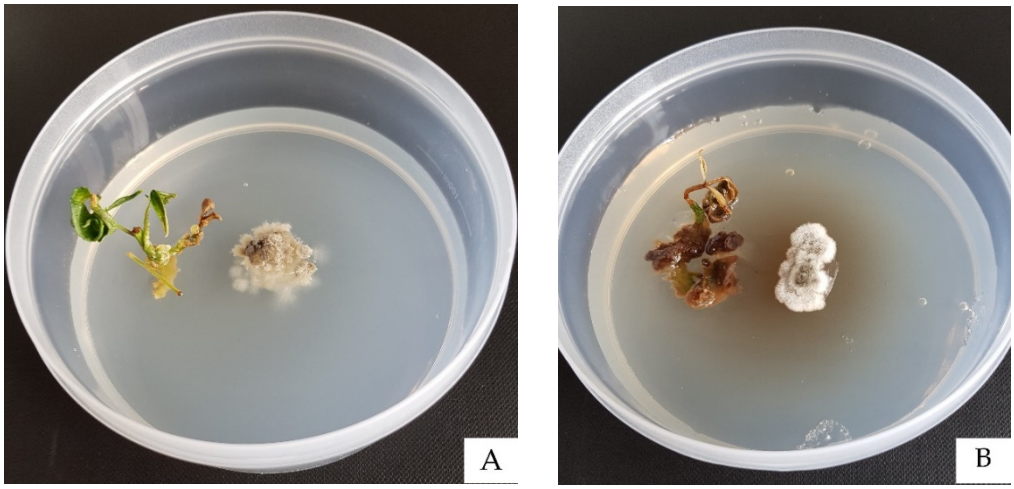

Figure 8. Interaction of *Fraxinus excelsior* seedlings with endophyte *Thielavia basicola* (fungus in the center of the dish); (**A**) after 21 days of dual cultures, (**B**) after 40 days of dual cultures.

In the last biocontrol experiment we tested the interactions between the endophytic fungi and the pathogen themselves. Both the endophytes tested in this study had a strong inhibitory effect on the growth of *H. fraxineus* which was measured as a reduction in the colony radius. After three weeks of culturing, the colony diameter in the control reached 0.7 cm for *T. basicola* (C43), and 4.3 cm for *Minimidochium* sp. (Jp 49). For both the endophytes, the colony radius toward *H. fraxineus* (strain No 20952) was significantly reduced, by 47.9% and 77.2% for *T. basicola* and *Minimidochium* sp., respectively (Table 3). All of the reactions described above were recorded in one-month-old dual cultures. It should be noted, however, that both endophytes tested in dual cultures strongly inhibited the growth of *H. fraxineus*. Their growth under the influence of callus/seedlings was also inhibited. Apart from very strong inhibition of the pathogen's growth, *T. basicola* also produced dark pigment which was released to the medium in both the individual and dual cultures involving this species. This was visible as brown discoloration (dark on the revers) of the medium in areas directly surrounding its colonies. Whereas visually, stronger inhibition of *H. fraxineus* growth was observed for *Minimidochium* sp., which was the only endophyte isolated from green ash.

Macroscopic observations of interactions between *H. fraxineus* and the endophytes showed that both *T. basicola* and *Minimidochium* sp. have a high inhibitory effect on the pathogen's growth. On average, they reduced the radius of *H. fraxineus* colonies by 46.9% ± 1% or 80.0% ± 1% respectively. Another effect observed in *T. basicola* vs. *H. fraxineus* dual cultures involved the production of unidentified secondary metabolite secreted to the medium that was visible in the form of dark beige crystal-like structures (Figure 9). No additional readily visible effects (e.g., medium discoloration or production of secondary metabolites) were observed in *Minimidochium* sp. vs. *H. fraxineus* dual cultures (Figure 10).

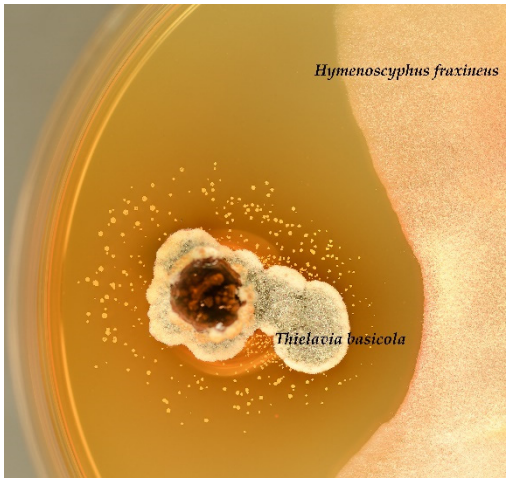

Figure 9. Interaction between: *Hymenoscyphus fraxineus* (one pathogen on the right) and *Thielavia basicola* (one endophyte on the left).

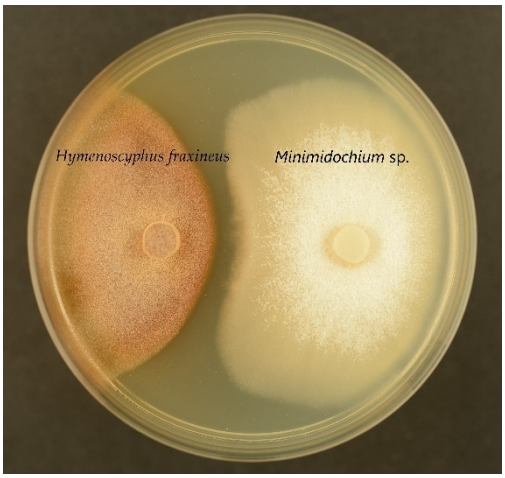

Figure 10. Interaction between: *Hymenoscyphus fraxineus* (one pathogen on the left) and *Minimidochium* sp. (one endophyte on the right).

3.3. Preliminary Investigation of Metabolites Produced in Response to H. fraxineus

The sought-after potentially active substances produced by endophytic fungi possibly inhibited the growth and development of *H. fraxineus*. Analyses of changes in the profile of metabolites secreted by endophytic fungi showed that, indeed, *T. basicola* HPLC produces an additional compound (Figure 11—indicated with an arrow, retention time of 7 min), but only in the presence of the purified, three-week-old post-culture medium of *H. fraxineus* (Figure 11). The substance was not produced in liquid *T. basicola* vs. *H. fraxineus* dual cultures, nor in individual cultures of *T. basicola* on liquid medium (Figure 8B). No such

development was observed for *Minimidochium* sp. liquid cultures, either individual, dual or with the addition of pathogen's post-culture medium (Figure 12).

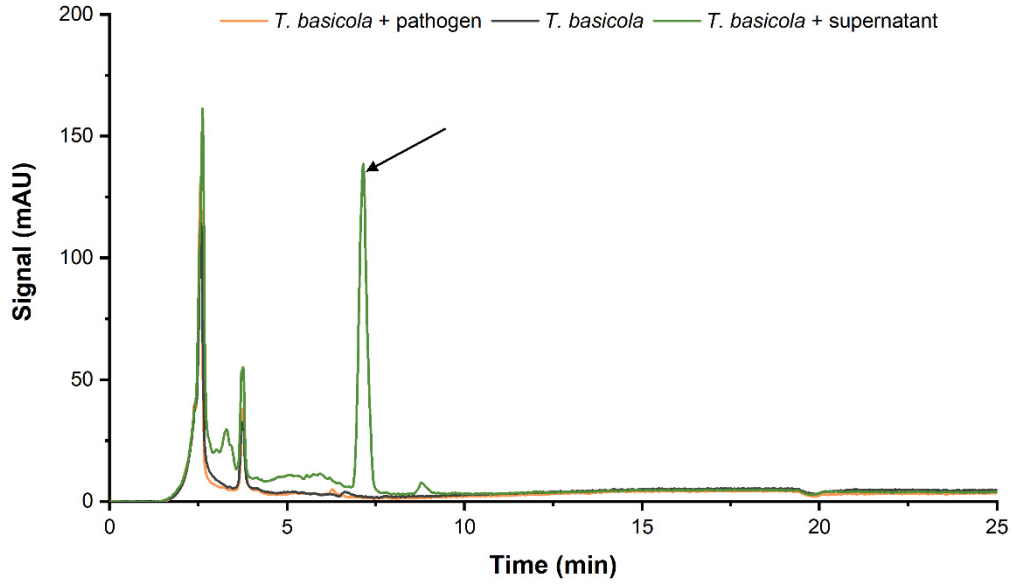

Figure 11. HPLC chromatograms of: *Thielavia basicola* + pathogen—supernatant from dual culture of endophyte with pathogen; *T. basicola*—supernatant from endophyte single culture; *T. basicola* + supernatant—supernatant from culture of endophyte growing in purified medium from 3-week old pathogen culture. Marked peak—additional substance. Signal recorded at 360 nm.

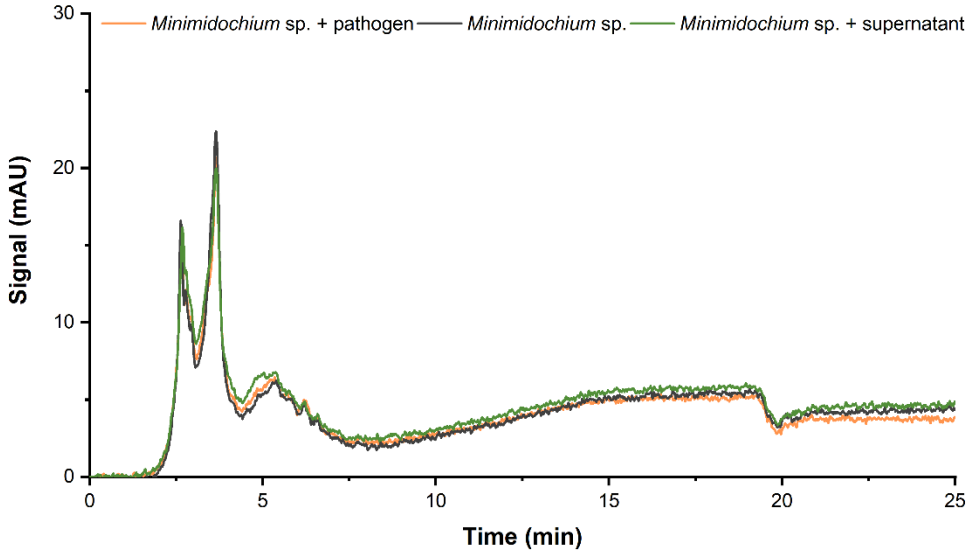

Figure 12. HPLC chromatograms of: *Minimidochium sp* + pathogen—supernatant from dual culture of endophyte with pathogen; *Minimidochium* sp.—supernatant from endophyte single culture; *Minimidochium sp* + supernatant—supernatant from culture of endophyte growing in purified medium from 3-weeks old pathogen culture. Signal recorded at 360 nm.

4. Discussion

Culturing of fungi in the presence of their plant hosts in vitro is not only a very useful model in studies of pathogenesis at the level of initiation, proliferation and growth of plant cells and tissues, but also enables us to select and to quickly propagate host genotypes that are most resistant to a given fungal agent; either a pathogen or an endophyte. However, this kind of experiment in vitro requires sterile plant and fungal material. One of the

sources of such plant material may be seeds that can be used to produce callus tissue and seedlings that are completely free of any microorganism. The process requires a variety of biotechnological procedures in vitro and is not easy. The literature lacks any information on the micropropagation procedures developed for some forest tree species including *F. excelsior*. Indeed, our results confirmed that one of the problems preventing the development of such micropropagation techniques for European ash is a difficult access to pathogen-free plant material. The ubiquitous occurrence of *H. fraxineus* and its related disease causes severe damage and frequent mortality of entire trees, especially in young ash stands. Unfortunately, the ash dieback disease seems to be a lasting problem, as it has managed to spread throughout most of Europe, including the British Isles [41]. The same applies to ash seeds, approximately 70% of which are infected by fungal pathogens [42]. It turned out that endophytic fungi are also a large problem. They also cause diseases on *Fraxinus*. Therefore, in this work, biocontrol studies were undertaken. For this purpose, three biological models were introduced in dual cultures in vitro. At the same time, in Germany, together with the Spaniards, research was conducted on the development of a biocontrol agent using endophytic fungi to control ash dieback, a disease that endangers the very existence of the *Fraxinus excelsior*. According to the researchers, such a protective endophyte should produce metabolites toxic to the invasive fungus *H. fraxineus*, the causal agent of ash dieback. From a total of 340 fungal endophytes from *F. excelsior*, the interactions with 13 exemplary isolates are presented here. In co-cultures between *H. fraxineus* and endophyte, there was almost always reciprocal inhibition between the pathogen and endophyte. Endophytes that inhibited *H. fraxineus* more than they were inhibited by the pathogen were inoculated into axenically cultured *F. excelsior* to select those that grew asymptomatically in the host. Most of the endophytes caused disease symptoms in axenically cultured *F. excelsior* seedlings, highlighting the importance of not only relying on co-culture experiments, but also using in planta experiments [14]. Thus, the application of the plant/fungus dual culture approach in pathogenicity studies of European ash has not been possible until recently due to a lack of effective micropropagation procedures, including disinfection protocols for ash seed material and optimization of culturing media. For instance, our study is the first time that our newly patented medium composition has been used (described in DP.P.433288, WO2021187995A2). Another difficulty in pathogenicity/biocontrol studies using dual cultures results from the fact that they can be complex and labor-intensive. Usually this means, that only a limited number of fungal species can be properly investigated. Thus, the species need to be selected carefully to provide clear answers as to why the defense response of the plant co-partner is activated in vitro and how it develops with time. For this reason, it seems reasonable to include in dual culture tests a variety of experimental designs (bio-models), as was done in this study, as well as to test fungi of various trophic classes, i.e., pathogens, endophytes, and sometimes even saprotrophs [43].

Synthesis of secondary metabolites [44], as well as defense proteins [40], in plant tissues or fungal thalli is activated as a part of the defense reaction, the response that may be triggered by various (most of) endophytes. Some pathogens, on the other hand, such as *H. fraxineus*, induce a localized response of plant or fungal tissues/thallus as early as a few days after penetration. This reaction usually takes the form of a hypersensitive response (HR) where the host causes necrosis of the small tissue/thallus fragment directly surrounding the infection site via programmed cell death (PCD). This functions to restrict the pathogen's growth to the affected fragment, preventing it from spreading throughout the rest of the host. An example of such a development can be seen in Figure 6 where the *F. excelsior* callus turns brown, necrotic and dies due to attack by *H. fraxineus*. Numerous metabolic and physiological changes follow a hypersensitive response, including changing the transcription and phosphorylation patterns of multiple proteins, increased Ca^{2+} outflow from the cells and the stiffening of cell walls by increased deposition of callose, lignin and suberin [45]. Another defense reaction triggered by the invasion of pathogens is oxidative burst, i.e., a sudden, but temporary, release of reactive oxygen species (ROS), whose concentrations reach toxic levels. It should be mentioned that infected ash tissues

(callus or regenerated seedlings) probably produce phytoalexins, i.e., natural antimicrobial compounds, that accumulate rapidly in areas of pathogen infection. Induction of various numerous pathogenesis-related proteins (PR) is another antimicrobial reaction triggered by a hypersensitive reaction [46]. These proteins include various hydrolases degrading cell walls, especially those of fungal pathogens (chitinases, glucanases) [47].

Our results clearly show that such a defense response of both callus tissue and callus regenerated seedlings of both ash species is insufficient to prevent infection by *H. fraxineus*. The pathogen was able to cause complete necrosis of entire callus cultures (Figure 6) and seedlings just after few days of dual cultures. The opposite situation was observed in cultures involving endophytes *T. basicola* and *Minimidochium* sp., where a state of equilibrium was developed allowing for coexistence of the plant and endophytic copartners. This observation indicates that the two endophytes examined in this study represent good models for studying molecular mechanisms underlying the non-pathogenic coexistence of ash and fungal endophytes, as well as for the development of a biocontrol method to control *H. fraxineus* with, otherwise harmless, endophytes. *T. basicola* produced a metabolite. On day 40 of the double in vitro culture (biocontrol model 2: *F. excelsior* seedling—*T. basicola* endophytes) it was visible in the medium (Figure 8). Perhaps, on the 21st day of cultivation, the plant started a defense reaction and the synthesis of a metabolite by the fungus. This was confirmed by the performed statistical analysis (Table 3), which showed that *T. basicola* was the only fungus whose growth was significantly inhibited in double cultivation with an ash seedling. Secondary metabolites are also produced by the fungi of the genus *Hymenoscyphus*. [48,49]. Research by scientists from Germany and Spain allowed for a better understanding of the role of secondary metabolites involved in the disease process. They investigated cultures of *H. fraxineus* and its native European relative *Hymenoscyphus albidus* for their production of bioactive compounds over the past years and already reported novel natural products from both species. A virulent culture of *Hymenoscyphus fraxineus*, the causal agent of ash dieback, was investigated for its production of secondary metabolites in a 70 L batch fermentation. Chemical analysis of the mycelial extract by means of flash chromatography and preparative HPLC led to the isolation of a new ergostane-type steroid and a new related lanostane triterpenoid, both revealing the same glycosylation pattern [48]. Our research concerns the role of secondary metabolites, but produced by endophytes as a result of, among others, their defense reaction against pathogens, which is an important element in the fight for the survival of the species. It is believed that the number of secondary metabolites produced by plants, fungi and bacteria [50] reaches dozens of thousands. This group of compounds recently received a lot of attention, mostly due to their biological activity, not only in the field of plant protection, but also in medical applications, including potential anticancer drugs [51,52].

This paper describes the experiments aiming to develop experimental designs for biocontrol testing and to identify microorganisms whose metabolic profiles should be further investigated in order to identify mechanisms of biocontrol interactions, potentially increasing ash survivability. These studies are continued at the Department of Forest Ecosystem Protection. They refer to endophytic fungi as biotic stress factors, but are also extended to abiotic stress factors (heavy metals and salinity of the substrate) [53]. The setup is also useful in testing for production of biologically active metabolites, including compounds with potential medicinal applications. Our studies on the metabolites produced by *F. excelsior* and *F. pennsylvanica* callus cultures and by endophytes will continue, including their identification and investigation of their role in inducing acquired resistance of plants to fungal infections; much-needed work especially for tree species, whose very survival is threatened by emergent fungal disease.

5. Conclusions

The newly development micropropagation method enabled very efficient screening of fungi for their pathogenicity toward European ash using dual culture approach. Such methods vastly facilitate selection process, what most probably will result in selection

of ash genotypes resistant to fungal infections in near future. However, for the selection process to produce reliable and precise results multiple parameters need to be taken into account. This is why the tests should include various experimental designs (biomodels) and fungi belonging to various trophic groups (pathogens, endophytes, or even saprotrophs). Our studies showed that a European ash endophyte *Thielavia basicola* in the presence of ash tissues produces an unidentified secondary metabolite, whose presence can be detected using HPLC. Presumably, secondary metabolites secreted to the growth medium in dual cultures induce defense response of the plant tissue preventing their colonization by the fungus. For this reason, results produced in vitro using dual culture approach in biocontrol models such as ours are not only important for plant protection applications, but may also prove significant from the point of view of medicine and pharmacology.

6. Patents

Patent No. DP.P.433288. https://ewyszukiwarka.pue.uprp.gov.pl/search/pwpdetails/P.433288?lng=pl (accessed on 18 March 2020). Method of obtaining common ash seedlings (*Fraxinus excelsior* L.) and nutrients suitable for use in this method. Worldwide Patent No. WO2021187995A2. https://worldwide.espacenet.com/patent/search/family/075954228/publication/WO2021187995A2?q=PCT%2FPL2021%2F050017 (accessed on 18 March 2021). The Method of obtaining saplings of the common ash (*Fraxinus excelsior* L.) and the media suitable for use in this method.

Author Contributions: Individual contributions: conceptualization, K.N.-C.; methodology, K.N.-C., D.L. and N.M.-R.; software, K.N.-C., D.L. and N.M.-R.; writing—original draft preparation, K.N.-C.; visualization, K.N.-C., D.L. and N.M.-R.; funding acquisition K.N.-C. All authors have read and agreed to the published version of the manuscript.

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References

1. Wemheuer, B.; Wemheuer, F. *Assessing Bacterial and Fungal Diversity in the Plant Endosphere*; Metagenomics Humana Press: New York, NY, USA, 2017; pp. 75–84.
2. Stone, H.S. *High-Performance Computer Architecture*; Addison-Wesley Longman Publishing Co., Inc.: Boston, MA, USA, 1987.
3. Saikkonen, K.; Faeth, S.H.; Helander, M.; Sullivan, T.J. Fungal endophytes: A continuum of interactions with host plants. *Annu. Rev. Ecol. Syst.* **1998**, *29*, 319–343. [CrossRef]
4. Murphy, B.R.; Doohan, F.M.; Hodkinson, T.R. Fungal endophytes of barley roots. *J. Agric. Sci.* **2014**, *152*, 602–615. [CrossRef]
5. Sieber, T.N. Endophytic fungi in forest trees: Are they mutualists? *Fungal Biol. Rev.* **2007**, *21*, 75–89. [CrossRef]
6. Schulz, B.; Haas, S.; Junker, C.; Andrée, N.; Schobert, M. Fungal endophytes are involved in multiple balanced antagonisms. *Curr. Sci.* **2015**, *109*, 39–45.
7. Weber, G.; Hübner, T.; Gieren, A.; Sonnenbichler, J.; Kowalski, T.; Holdenrieder, O. X-Ray Structure Analysis and Spectroscopic Data of the Antibiotic 8-(Dichloroacetyl)-5-hydroxy-2, 7-dimethyl-1, 4-naphthoquinone from the *Fungus mollisia* sp. *Z. Nat. C* **1987**, *42*, 1–3. [CrossRef]
8. Terhonen, E.; Blumenstein, K.; Kovalchuk, A.; Asiegbu, F.O. Forest tree microbiomes and associated fungal endophytes: Functional roles and impact on forest health. *Forests* **2019**, *10*, 42. [CrossRef]
9. Hanson, J.R. *Chemistry of Fungi*; Royal Society of Chemistry: London, UK, 2008.
10. Barklund, P.; Kowalski, T.J.C.J. Endophytic fungi in branches of Norway spruce with particular reference to *Tryblidiopsis pinastri*. *Can. J. Bot.* **1996**, *74*, 673–678.

11. Frisvad, J.C.; Thrane, U.; Filtenborg, O. *Role and Use of Secondary Metabolites in Fungal Taxonomy*; Chemical Fungal Taxonomy CRC Press: Boca Raton, FL, USA, 2020; pp. 289–319.
12. Slippers, B.; Wingfield, M.J. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. *Fungal Biol. Rev.* **2007**, *21*, 90–106. [CrossRef]
13. Haňáčková, Z.; Havrdová, L.; Černý, K.A.R.E.L.; Zahradnik, D.; Koukol, O.N.D.Ř.E.J. Fungal endophytes in ash shoots–diversity and inhibition of *Hymenoscyphus fraxineus*. *Balt. For.* **2017**, *23*, 89–106.
14. Halecker, S.; Wennrich, J.P.; Rodrigo, S.; Andrée, N.; Rabsch, L.; Baschien, C.; Steinertd, M.; Stadlerab, M.; Surup, F.; Schulz, B. Fungal endophytes for biocontrol of ash dieback: The antagonistic potential of *Hypoxylon rubiginosum*. *Fungal Ecol.* **2020**, *45*, 100918. [CrossRef]
15. Nawrot-Chorabik, K. Interactions between embryogenic callus of *Abies alba* and *Heterobasidion* spp. *in dual cultures*. *Biol. Plant.* **2014**, *58*, 363–369. [CrossRef]
16. Sumarah, M.W.; Adams, G.W.; Berghout, J.; Slack, G.J.; Wilson, A.M.; Miller, J.D. Spread and persistence of a rugulosin-producing endophyte in *Picea glauca* seedlings. *Mycol. Res.* **2008**, *112*, 731–736. [CrossRef]
17. Pichersky, E.; Gang, D.R. Genetics and biochemistry of secondary metabolites in plants: An evolutionary perspective. *Trends Plant Sci.* **2000**, *5*, 439–445. [CrossRef]
18. Cusidó, R.M.; Palazón, J.; Navia-Osorio, A.; Mallol, A.; Bonfill, M.; Morales, C.; Piñol, M.T. Production of Taxol® and baccatin III by a selected *Taxus baccata* callus line and its derived cell suspension culture. *Plant Sci.* **1999**, *146*, 101–107. [CrossRef]
19. Das, A.; Chakraborty, B.; Sood, A.K. Raman spectroscopy of graphene on different substrates and influence of defects. *Bull. Mater. Sci.* **2008**, *31*, 579–584. [CrossRef]
20. Nawrot-Chorabik, K.; Pietrzykowski, M. Ecophysiological aspects of in vitro biotechnological studies using somatic embryogenesis of callus tissue toward protecting forest ecosystems. *J. For. Res.* **2019**, *30*, 1159–1166. [CrossRef]
21. Kowalski, T. *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *For. Pathol.* **2006**, *36*, 264–270. [CrossRef]
22. Gross, A.; Sieber, T.N. Virulence of *Hymenoscyphus albidus* and native and introduced *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *Fraxinus pennsylvanica*. *Plant Pathol.* **2016**, *65*, 655–663. [CrossRef]
23. Gross, A.; Holdenrieder, O.; Pautasso, M.; Queloz, V.; Sieber, T.N. *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. *Mol. Plant Pathol.* **2014**, *15*, 5–21. [CrossRef]
24. Enderle, R.; Stenlid, J.; Vasaitis, R. An overview of ash (*Fraxinus* spp.) and the ash dieback disease in Europe. *CAB Rev.* **2019**, *14*, 1–12. [CrossRef]
25. Coker, T.; Rozsypálek, J.; Edwards, A.; Harwood, T.; Butfoy, L.; Buggs, R. Estimating mortality rates of European ash (*Fraxinus excelsior*) under the ash dieback (*Hymenoscyphus fraxineus*) epidemic. *Plants People Planet* **2019**, *1*, 48–58. [CrossRef]
26. Kirisits, T.; Schwanda, K. First definite report of natural infection of *Fraxinus ornus* by *Hymenoscyphus fraxineus*. *For. Pathol.* **2015**, *45*, 430–432. [CrossRef]
27. Kowalski, T.; Bilański, P.; Holdenrieder, O. Virulence of *Hymenoscyphus albidus* and *H. fraxineus* on *Fraxinus excelsior* and *F. pennsylvanica*. *PLoS ONE* **2015**, *10*, e0141592.
28. Kowalski, T.; Bilański, P. *Hymenoscyphus pusillus*, a new species on leaves of *Fraxinus pennsylvanica* in Poland. *For. Pathol.* **2019**, *49*, e12481.
29. Latz, M.A.; Jensen, B.; Collinge, D.B.; Jørgensen, H.J. Endophytic fungi as biocontrol agents: Elucidating mechanisms in disease suppression. *Plant Ecol. Divers.* **2018**, *11*, 555–567. [CrossRef]
30. Wang, X.W.; Bai, F.Y.; Bensch, K.; Meijer, M.; Sun, B.D.; Han, Y.F.; Crous, P.W.; Samson, P.A.; Yang, F.Y.; Houbraken, J. Phylogenetic re-evaluation of Thielavia with the introduction of a new family Podosporaceae. *Stud. Mycol.* **2019**, *93*, 155–252. [CrossRef] [PubMed]
31. Kitahara, N.; Endo, A.; Furuya, K.; Takahashi, S. Thielavin A and B, new inhibitors of prostaglandin biosynthesis produced by *Thielavia terricola*. *J. Antibiot.* **1981**, *34*, 1562–1568. [CrossRef]
32. Qadri, M.; Rajput, R.; Abdin, M.Z.; Vishwakarma, R.A.; Riyaz-Ul-Hassan, S. Diversity, molecular phylogeny, and bioactive potential of fungal endophytes associated with the Himalayan blue pine (*Pinus wallichiana*). *Microb. Ecol.* **2014**, *67*, 877–887. [CrossRef]
33. Sandhi, J.; Singh, J.P.; Kaur, T.; Ghuman, S.S.; Singh, A.P. Involvement of progesterone receptors in ascorbic acid–mediated protection against ischemia-reperfusion–induced acute kidney injury. *J. Surg. Res.* **2014**, *187*, 278–288. [CrossRef]
34. Viswanath, B.; Rajesh, B.; Janardhan, A.; Kumar, A.P.; Narasimha, G. Fungal laccases and their applications in bioremediation. *Enzym. Res.* **2014**, *2014*, 163242. [CrossRef]
35. Kowalski, T. Zur Pilzflora toter Kiefernnadeln (eng. Contribution to the mycoflora of dead pine needles). *Zeitschrift für Mykologie* **1988**, *54*, 159–174.
36. Spadaro, D.; Gullino, M.L. Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop. Prot.* **2005**, *24*, 601–613. [CrossRef]
37. Lichtenthaler, H.K. Chlorophylls and carotenoids. Pigments of photosynthetic biomembranes. *Plant Cell Membr.* **1987**, *148*, 350–382.
38. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–494. [CrossRef]

39. Nawrot-Chorabik, K.; Latowski, D. Patents No. DP.P.433288 (2020), WO2021187995A2 (2021). Method of Obtaining Common Ash Seedlings (*Fraxinus excelsior* L.) and Nutrients Suitable for Use in this Method; Sposób Pozyskiwania Sadzonek Jesionu Wyniosłego (*Fraxinus excelsior* L.) Oraz Pożywki Nadające się do Stosowania w Tym Sposobie. Available online: https://ewyszukiwarka.pue.uprp.gov.pl/search/pwp-details/P.433288?lng=pl (accessed on 18 March 2021).
40. Lahlali, R.; Hijri, M. Screening, identicationand evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. *FEMS Microbiol. Lett.* **2010**, *311*, 152–159. [CrossRef] [PubMed]
41. McKinney, L.V.; Nielsen, L.R.; Collinge, D.B.; Thomsen, I.M.; Hansen, J.K.; Kjær, E.D. The ash dieback crisis: Genetic variation in resistance can prove a long-term solution. *Plant Pathol.* **2014**, *63*, 485–499. [CrossRef]
42. Mason, G.N.; Van Arsdel, E.P. Fungi associated with *Pinus taeda* seed development. *Plant Dis. Rep.* **1978**, *62*, 864–867.
43. Nawrot-Chorabik, K.; Grad, B.; Kowalski, T. Interactions between callus cultures of *Pinus sylvestris* and pine fungi with different trophic properties. *For. Pathol.* **2016**, *46*, 179–186. [CrossRef]
44. Verma, N.; Shukla, S. Impact of various factors responsible for fluctuation in plant secondary metabolites. *J. Appl. Res. Med. Aromat. Plants* **2015**, *2*, 105–113. [CrossRef]
45. Blume, Y.B.; Krasylenko, Y.A.; Yemets, A.I. The role of the plant cytoskeleton in phytohormone signaling under abiotic and biotic stresses. *Mech. Plant Horm. Signal. Stress* **2017**, 127–185.
46. Van Loon, L.C.; Van Strien, E.A. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* **1999**, *55*, 85–97. [CrossRef]
47. Sels, J.; Mathys, J.; De Coninck, B.M.; Cammue, B.P.; De Bolle, M.F. Plant pathogenesis-related (PR) proteins: A focus on PR peptides. *Plant Physiol. Biochem.* **2008**, *46*, 941–950. [CrossRef]
48. Surup, F.; Halecker, S.; Nimtz, M.; Rodrigo, S.; Schulz, B.; Steinert, M.; Stadler, M. Hyfraxins A and B, cytotoxic ergostane-type steroid and lanostane triterpenoid glycosides from the invasive ash dieback ascomycete Hymenoscyphus fraxineus. *Steroids* **2018**, *135*, 92–97. [CrossRef]
49. Halecker, S.; Surup, F.; Kuhnert, E.; Mohr, K.I.; Brock, N.L.; Dickschat, J.S.; Junker, C.; Schulz, B.; Stadler, M. Hymenosetin, a 3-decalinoyltetramic acid antibiotic from cultures of the ash dieback pathogen, *Hymenoscyphus pseudoalbidus*. *Phytochemistry* **2014**, *100*, 86–91. [CrossRef] [PubMed]
50. Russel, J.; Røder, H.L.; Madsen, J.S.; Burmølle, M.; Sørensen, S.J. Antagonism correlates with metabolic similarity in diverse bacteria. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 10684–10688. [CrossRef] [PubMed]
51. Singh, B.; Bhat, T.K.; Singh, B. Potential therapeutic applications of some antinutritional plant secondary metabolites. *J. Agric. Food Chem.* **2003**, *51*, 5579–5597. [CrossRef]
52. Xu, F.; Wang, S.; Li, Y.; Zheng, M.; Xi, X.; Cao, H.; Cui, X.; Guo, H.; Han, C. Yield enhancement strategies of rare pharmaceutical metabolites from endophytes. *Biotechnol. Lett.* **2018**, *40*, 797–807. [CrossRef] [PubMed]
53. Nawrot-Chorabik, K.; Sułkowska, M.; Osmenda, M.; Mohytych, V.; Surówka, E.; Latowski, D. The impact of biotic and abiotic stress factors on development of European ash tissue cultures. *Forests* **2021**, in review.