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Use of Secondary Metabolites of Wood-Decaying Fungi to Reduce Damping off Disease

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Abstract: Phytopathogenic fungi can cause plant diseases that are difficult to control, including mass mortality of some tree species. The *Fusarium oxysporum* complex (*sensu lato*) is one of the most dangerous groups of phytopathogenic fungi, causing the death of conifer species, including *Pinus sylvestris* seedlings in forest and ornamental nurseries. Recently, non-chemical methods of plant protection have become the basis of integrated pest management (IPM) in the European Union (EC Directive). The possibility of protection of pine seedlings against the pathogen *F. oxysporum* using active substances from wood-destroying fungi commonly found in forests was examined. Methanolic extracts of *Fomitopsis pinicola*, *Ganoderma applanatum*, and *Trametes versicolor* were found to contain substances effective in both prevention and treatment of infected seedlings. *G. applanatum* and *T. versicolor* showed particular biological activity in increasing plant resistance. Efficacy, especially of the extract of *F. pinicola*, increased with concentration. Further field trials are needed to confirm the results obtained in laboratory tests on plant protection.

Keywords: biological control; white and brown rot fungi; *Fomitopsis pinicola*; *Trametes versicolor*; *Ganoderma applanatum*; *Fusarium oxysporum*

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1. Introduction

The species complex known as *Fusarium oxysporum* includes a wide range of organisms, some of which are saprophytic and others endophytic, but there are a considerable number of pathogens, especially in nurseries of conifers. Other published papers have also reported the pathogenicity of *F. oxysporum* in forestry. *Fusarium oxysporum* has long been recognized in nurseries as the most important causal agent of root and hypocotyl rots [1]. The role of the various *Fusarium* spp. has often been questioned, but species such as *F. oxysporum*, and *F. solani* can cause death of *Pinus* seedlings [2]. *Fusarium* root disease is one of the most common diseases of conifer seedlings worldwide. In addition to root disease, *F. oxysporum* and other *Fusarium* species are often responsible for root death at earlier stages of seedling development [3].

Since the fungal complex *F. oxysporum* causes serious plant diseases worldwide, often leading to crop failure and economic decline in agricultural countries, the search for new methods of crop protection is of great importance, especially in the context of the new EU Directive on Integrated Pest Management (IPM) against pests and diseases, where all

non-chemical methods are to be used before pesticides. This approach is expected to reduce pollution and improve quality of life. The *Fusarium* spp. complexes also cause problems in Polish forestry, for example, as one of the causes of massive bud dieback in oaks [4] or as a serious threat to nursery plants. It is also predicted that the number of plant species affected by *Fusarium* spp. could increase dramatically [5–7].

Pathogenic *Fusarium* strains are among the top 10 most destructive fungal pathogens in the world [8,9]. Moreover, the range of host plants on which the pathogens can develop includes more than 120 plant species, even excluding those naturally occurring in forest ecosystems. In Poland, *Fusarium oxysporum sensu lato* was considered in forestry to cause a disease of the seedlings of many forest-forming species called damping-off. Only recently have developments in molecular biology allowed identification at the DNA level and shown that several fungal species belong to this complex [10]. These *Fusarium* spp. generally occur as saprotrophs in the soil, and most strains that can colonize plant roots are commensal endophytes that do not affect plant health [9] and are often used as biological control agents [11]. However, there are pathogenic strains among them that are commonly associated with agricultural crops [12–14]. Although most researchers believe that the impact of these organisms on forest pathology is very low, there are examples that confirm their pathogenicity to forest-dwelling species [15]. This is probably due to the transfer of virulent strains from agriculture to forest nurseries [16,17]. Natural transmission routes may include water taken from watercourses or other natural reservoirs and used to irrigate plants in nurseries. Another example is birds, whose role as vectors has been confirmed for *Phytophthora* species, which are also involved in conifer seedling mortality [18]. Currently, *F. oxysporum* is considered a species complex that can cause serious problems not only in agriculture and horticulture, but also in forestry, as it is involved in the mortality of seedlings of Scots pine (*Pinus sylvestris*) in nurseries, but also kills the buds of oak shoots [4] or other forest tree species such as beech or ash [19,20]. In nurseries, *F. oxysporum sensu lato* causes pre-emergence and post-emergence damping off seedlings with root death and stem cankers [19,21–32].

The use of chemical products such as conventional pesticides to control invasive pest species [33–35] has several drawbacks, such as environmental degradation, non-target effects and cost [34]. Therefore, the challenge is to develop new and eco-friendly alternatives for the safe control of diseases, and which pose low risk to human health and the environment. Biological control strategies can be more cost-effective, efficient, environmentally friendly, and sustainable [33]. Thus, biological control of pathogens has become an essential part of integrated pests management (IPM) in forests [34]. Scientists are conducting research to evaluate forest responses to these practices at different scales to improve outcomes and reduce the use of pesticides [34].

Preventive measures are an effective means of controlling fusariosis and consist of inducing or increasing plant resistance through the use of biopreparations and/or seed treatments [35]. Agrios's [36] research on the role of phenolic compounds emphasizes that the ability to synthesize them is related to the acquisition of resistance by the plant. Wood-decomposing fungi produce many biologically active compounds, including phenolic compounds [36–38]. Atanasova-Penichon et al. [39] reviewed the antioxidant secondary metabolites with possible involvement in resistance to *Fusarium*. Their fungicidal activity was characterized against various *Fusarium* species, and the authors proposed a ranking of phenolic acids for their toxicity to *F. graminearum* as follows: chlorogenic acid < p-hydroxybenzoic acid < caffeic acid < syringic acid < p-coumaric acid < ferulic acid. This pathogen is not common in forests, but it is important in agriculture, and the limitation of its population would be a food biosafety issue. The antifungal activity of p-hydroxybenzoic acid and cinnamic acid in *Ganoderma lucidum* against *Aspergillus fumigatus*, *A. ochraceus*, *A. niger* was also reported by Heleno et al. [40]. Culture filtrate of *Trametes versicolor* also inhibited the growth of *F. langsethiae* [41], *F. oxysporum*, and *Botrytis cinerea* [42]. Similarly, antifungal activities of *Fomitopsis pinicola* extracts against *Fusarium inflexum* and *Fusarium heterosporium* have been found [43].

Biocontrol is a tool in plant protection and is a part of the rapidly developing biological pest management, which includes a wide range of different methods, including beneficial soil microorganisms. Biological pest control is a way to protect plants in nurseries or ecosystems from harmful pathogens and involves the use of compounds that may have a wide range of biological activities, including antifungal, cytotoxic, insecticidal, etc. [35,44]. The intensive use of synthetic pesticides in the control of plant pests and diseases is a cause for concern, mainly because of the toxicity and carcinogenicity of their chemical compounds and their persistence in the environment. There is a need to explore newer forms of crop protection that could replace traditional fungicides, which is in line with the principle of IPM currently promoted by the European Commission. With the promotion of organic farming, IPM is one of the tools for pest control with low pesticide use and must be implemented by all professional users. Annex III of the EU directive states that "Sustainable biological, physical and other non-chemical methods must be preferred to chemical methods if they provide satisfactory pest control".

Fungi are the only organisms capable of decomposing wood, and the changes in decomposing material owing to fungal activity creating niches to other organisms. This is related to two important features: the ability to biosynthesize secondary metabolites with strong biological effects and the possession of extensive enzymatic pathways that allows them to carry out complex biotransformation reactions. Most wood-destroying fungi are white rot and brown rot fungi belonging to the phylum Basidiomycota.

Bioactive substances from fungi can generally be divided into two groups: high molecular weight compounds, which include primarily polysaccharides and proteins and low molecular weight compounds, such as steroles, terpenoids, or phenols [45]. In interactions in complex environment, secondary metabolites play a crucial role. The wide spectrum of action includes antibacterial, antiviral, antifungal, immunomodulatory, signalling and cytotoxic activities [46,47]. Nevertheless, the function of many fungal secondary metabolites remains still unknown.

Fungi can be the basis to produce innovative biopreparations of natural origin. Bioactive substances from macrofungi can inhibit the growth and germination of spores of other fungi [35]. Chemical substances that increase the resistance of plants or are harmful to pathogenic fungi show the usefulness of fungal extracts in disease management [48]. Among fungal antioxidants, particularly important are phenolic acids, including caffeic acid, ferulic acid, *p*-coumaric acid, *o*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, and vanillic acid, which may support plant immune system [49,50].

Numerous studies have reported the ability of secondary metabolites to induce plant resistance [51]. Polysaccharides are essential for pathogenic mechanisms and for immune responses in fungal infections [52–55]. In turn, exopolysaccharides (EPSs) are the active fungal biomacromolecules of soil organic matter that can help protect plants from environmental stresses or unwanted interactions with other organisms [56]. Therefore, biopreparations that induce plant resistance to phytopathogens may be produced using extracts from wood-decaying fungi, these may contain polysaccharides as well as phenolic compounds or other active substances.

Three basidiomycetous species of wood decay fungi were selected for the study: *Ganoderma applanatum*, *Fomilopsis pinocola*, and *Trametes versicolor*. The purpose of this study was to find out whether these fungi, owing to their purported carcinostatic properties and enhancement of human immunity, can also protect plants against root pathogens of the *F. oxysporum*, which can cause a major disease in plant nurseries.

The use of bioactive compounds from wood-decay fungi could represent a new research direction in the field of biological protection. Preparations of natural origin could both inhibit the development of pathogenic soil fungi and limit the undesirable effects of xenobiotic pollution of the natural environment.

We considered the following two hypotheses in the present study:

(a) *Fusarium* infection and the lesions develop in spite of the application of wood-decay fungi extracts, which may contain both non-volatile compounds (NVOC) and volatile compounds (VOC).
(b) At a certain concentration, extracts from wood-decay fungi effectively restrict *Fusarium* infection or disease development in Scots pine seedlings.

2. Materials and Methods

2.1. Extracts from Wood Decaying Fungi

2.1.1. Origin of Wood-Decaying Fungi

Fruiting bodies of *F. pinicola* (host: *Picea abies*), *G. applanatum* (host: *Quercus robur*), and *T. versicolor* (host: *Quercus robur*) were collected from the trunks of infected host tree species in Białowieża Forest (Hajnówka Forest District, Poland). Species were identified morphologically following the identification key of Ryvanen at al. [57]. These are common species that do not raise taxonomic questions. Therefore, standard identification methods were used in this case. The preparations were made under a binocular magnifying glass at 10–25× magnification. Opta-Tech LAB 40 light microscope with phase contrast and Nikon Eclipse Ni with Nomarski contrast were used to observe the microscopic features. Fungi were identified based on their distinctive characteristic macroscopic features [57,58] based on dichotomous keys used in standard fungal taxonomy, analyzing macroscopic features of the fruiting bodies (1) and elements of the microscopic structure of the hymenium (2).

For archival purposes, dried fruiting bodies were stored in the Fungarium of the Scientific Research Centre in Hajnówka of the Institute of Forest Sciences (acronym BLS).

2.1.2. Preparation of Extracts from Wood Decaying Fungi

Fresh fruiting bodies of *F. pinicola*, *G. applanatum* and *T. versicolor* were chopped into approximately 1 cm^3, and 100 g of each fungus were weighed out. The procedure of extraction was as described below and it was the same for each of the fungi. The raw material was transferred to screw-capped bottle with 200 mL 99.8% methanol. The 24 h extraction process was repeated three times. Then, the extracts obtained in three processes were combined and filtered through a 5 cm diameter pleated paper filter Circles Whatman no. 1 (460 × 570 mm). The methanol was evaporated during 90 min with used a vacuum evaporator, and the dry residue was used for chemical and biological analysis; such a technique has been used before with success [59].

In addition, fungal extracts were deposited in the Fungi Extract Bank-a scientific collections of the Institute of Forest Sciences (https://fungiextractbank.com/en/, accessed on 1 October 2021), Białystok University of Technology, Poland. The Fungi Extract Bank is a collection of extracts from several hundred species of Macromycetes fungi—mostly from polypore fungi (saprotrophs and parasites).

2.1.3. Fungal Extracts Analysis by Gas Chromatography-Mass Spectrometry

Samples for GC/MS analysis were prepared as follows: 10 mg of the methanolic fungal extract (from reference and from fresh samples) was diluted with 1 mL of 99.8% (anhydrous) pyridine and 100 µL of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was added. The mixture was heated at 60 °C for 30 min. The silylated samples were separated using an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass selective detector. Injection of 1 µL of sample was performed using an Agilent 7693A autosampler. Separation was performed on a HP-5MS (30 m × 0.25 mm × 0.25 µm film thickness) fused silica column at a helium flow rate of 1 mL/min. The injector operated in a split (1:10). The injector temperature was 300 °C. The initial column temperature was 50 °C and increased to 325 °C at 3 °C/min; the final temperature was maintained for 10 min. The ion source temperature was 230 °C and the quadrupole temperature was 150 °C. Electron ionization mass spectrometry (EIMS) was performed at an ionization energy of 70 eV. Detection was performed in full-scan mode from 41 to 800 a.m.u. After integration,

the contribution of each component to the total ion current (% of TIC) was calculated. The retention indices of the analytes were determined from the retention times of alkanes.

The mass spectral data and calculated retention indices were used to identify components. Mass spectrometric identification was performed using an automated system of GC-MS data processing provided by NIST—The National Institute of Standards and Technology and data from "Identification of Biologically and Environmentally Significant Organic Compounds Mass Spectra and Retention Indices Library of Trimethylsilyl Derivatives". The retention indices of the registered compounds were compared with those in the NIST database (https://webbook.nist.gov/, accessed on 1 October 2021).

2.2. Preparation of Pathogens for Testing

An isolate IBL279f of *F. oxysporum* [12] GenBank accession number MF162321.1 (strain IBL279f) came from the collection of the Forest Research Institute in S ekocin Stary, Poland, and originated from a pine nursery that had experienced root disease issues. The isolate of *F. oxysporum* was grown in a Petri dish (90 mm) sealed with parafilm. Next, 1 cm^2 pieces of the mycelium were cut out with a sterile dissecting needle. These pieces of mycelium were transferred individually to sterile Petri dishes (90 mm) on a previously prepared PDA medium. The concentration of prepared PDA was 39 g/L. The plates containing the thus prepared refreshed *Fusarium* culture were also protected with parafilm to prevent the entry of unwanted microorganisms that could contaminate the culture. After 7 days of growing in light conditions, the hyphal plugs on the medium at room temperature, the grown colonies were stored in the refrigerator at 10 °C. Some of the grown colonies were examined for colony appearance, spore production, and other morphological characteristics, and one sample of each was stained with 2 drops of Melzer reagent (a solution of 0.75%–1.25% iodine and 2.50%–3.75% potassium iodide in a mixture of 50% water and 50% chloral hydrate) to better visualize some structures. The stained slides were fixed by briefly heating (a few seconds) over a flame from the bottom of the slide to remove air bubbles and viewed under a dissecting and light microscope (40× magnification). These cultures were the source of the 1 cm diameter hyphal plugs used in the pine seedling experiment.

2.3. Pinus Sylvestris Seedlings for Use in In Vitro Studies

2.3.1. Pine Seedlings Being Prepared for In Vitro Tests

The seeds of Scots pine came from the forest district Borne Sulinowo, Poland. The seeds were sown on sterile, moist tissue paper in Petri dishes covered with the upper lid. Depending on the need of seeds, moisture was supplied by watering with a small amount of sterilized distilled water (10 mL) every time there was a visible sign of a water loss. In addition, in order to speed up the germination process (as this did not take place on the germinator), lighting in the form of lamps (58 W) was used. The natural cycle of the day was kept: 12 h with light (day), 12 h without light (night). After 2 weeks, 3 seedlings per dish were moved. The transfer of the seedlings to the new dishes was carried out under sterile conditions. The working surface and tweezers were sterilised with 70% propanol.

2.3.2. Use of Extracts for Seedling Protection

In vitro tests were divided into two control treatments and four test treatments. Germinated 20-day-old seedlings of *P. sylvestris* were used for these tests. One drop (about 2 mL) of each fungal extract was placed onto root tips of each seedling in the following concentrations: 5%, 10%, 25%, 50% and 100%. The plants were observed after seven days in natural light and room temperature (25–26 °C), and the inhibition of pathogen development was assessed in comparison to a control.

2.3.3. Control and Test Treatments

Control treatment was inoculated with the pathogen by applying hyphal plugs (1 cm^2 square piece of myceluium with PDA) and was not treated with extracts. Inhibition studies were performed in Petri dishes by placing 1 cm^2 square pieces of *F. oxysporum* hyphal plugs

(cut out of PDA culture) on tips of plant roots (laying on sterile moist paper). In each inoculation treatment, 3 seedlings were used per extract concentration. Extracts were applied as droplets or by dipping roots into extract solution. Depending on the treatment, either one drop of the extract was applied on the *F. oxysporum* mycelium or the pine seedlings were dipped in the extract.

The four test treatments differed in the method of application of the extract and in the timing of application before or after infection (prevention versus cure). In two treatments (I and II), one drop of the extract were applied using a sterile disposable pipette. In two other treatments (III and IV), the roots of seedlings were dipped into the extract. These treatments are described in Table 1.

Table 1. Treatments of extracts application to the seedlings' roots.

Treatment	Description
I	One drop of the extract was applied to the root tip 24 h after the seedling was inoculated with a 1 cm^2 sized mycelial plug of *F. oxysporum*.
II	One drop of the extract was applied to the root tip 24 h before each seedling was inoculated with a 1 cm^2 sized mycelial plug of *F. oxysporum*.
III	Seedling roots were dipped in the extract for 1 min and inoculated with a 1 cm^2 sized mycelial plug of *F. oxysporum* immediately after removal from the extract.
IV	Seedling roots were dipped in the extract for 1 min and inoculated with a 1 cm^2 mycelial plug of *F. oxysporum* 24 h after that.

2.3.4. Assessment of Disease Symptoms on Pine Seedlings

After 7 days, the length of necrosis on roots was measured, together with an evaluation of seedling health (root length, discoloration). The differences in the measurements were examined in terms of statistical significance. Additionally, the chemical composition of the extracts was examined by a mass spectrometer to determine which substances could be responsible for the obtained effects.

2.4. Statistical Analysis

Data processing and statistical analysis of data were performed using SAS 9.4 (SAS Institute, Cary, NC, USA) software using the SAS Enterprise Guide user interface and SAS/Stat procedures [60]. A t-test was conducted to verify statistical significance of differences between two populations, at $p < 0.05$, with PROC TTEST. Analysis of variance (ANOVA) was used for pariwise multiple comparisons with Tukey's HSD (honestly significant difference) test, at $p < 0.05$, with PROC GLM. PROC REG was used to estimate linear regression coefficients and corresponding *p*-values.

3. Results

3.1. Chemical Composition of Extracts from Wood Decaying Fungi

Chemical composition of methanol extracts from *F. pinicola*, *G. applanatum* and *T. versicolor* were analyzed by GC-MS and the results are listed in tables in Supplementary Materials. The main components of fungi extracts were carbohydrates as well as fatty acids and fatty acid esters. The carbohydrate content in *F. pinicola*, *G. applanatum* and *T. versicolor* extracts was 54.8, 35.8 and 72.7%, respectively. The content of fatty acid and fatty acid esters was 8.5, 37.4 and 10.4%, respectively.

Hydroxy acids were found in all analyzed extracts from fungi fruiting bodies. The *F. pinicola* extract contained lactic acid, and the *G. applanatum* extract furthermore had glycolic acid as well as methyl esters of malic acid and 2-hydroxyoctadecanoic acid. The *T. versicolor* extract was rich in hydroxy acids such as malic acid (0.89%), citric acid (0.68%) and lactic acid (0.19%). Dicarboxylic acids (succinic acid, fumaric acid, azelaic acid) and aromatic acids (4-hydroxybenzoic acid, vanillic acid, benzoic acid) were only identified in methanol

extract from *T. versicolor*. This extract was also distinguished by free amino acid content (5%). The highest content of sterols (18.17%) was in the *G. applanatum* extract.

3.2. Effects of Extract Concentrations on the Length of Pine Root Lesion

The main objective of the study was to verify whether the application of the extracts reduced compared to the control the length of the root lesion (necrosis) of the 20-day-old pine seedlings and whether this depended on the extract concentration.

The lowest applied concentration of 5% resulted in a significant reduction in necrosis length (Figure 1). As the concentration of the extracts increased, the preventive or curative effect was generally stronger, as reflected by a significant decrease in necrosis length. The calculated linear regression model to test the statistical significance of this trend was confirmed. The relationship between necrosis length and extract concentration is not linear, but has a more exponential decay form. Therefore, a suitable logarithmic transformation of the concentration value was performed to test the significance of the coefficient in the linear regression models.

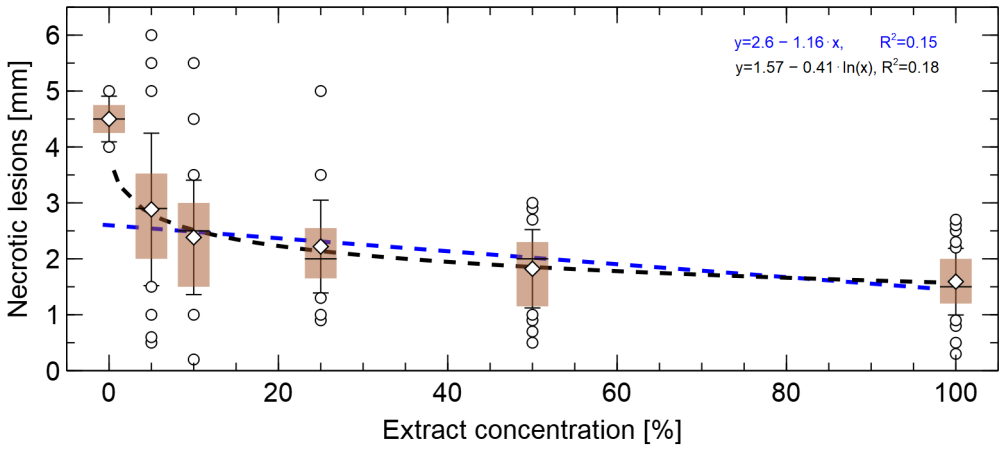

Figure 1. The relation between necrosis length and extract concentration for all measured roots samples. The concentration of 0% represents the control. The box spans from the 1st to 3rd quantile. Horizontal line in the box represents sample median, diamond sign represents mean value, the whiskers show standard deviation from the mean, and the circles outside whiskers represent outlier observations. The blue and black dashed lines represent regression fit lines, for which the corresponding parameters and R^2 values are typed in the figure. The *p*-values for all regression coefficients are below 0.001.

3.3. Influence of Fungal Extracts on Root Lesions Development Caused by F. oxysporum

A comparison of different extracts was made on the reduction of necrosis length (Figure 2). The extract from *F. pinicola* had a significantly weaker effect than the other two extracts. No difference was observed between extracts from *G. applanatum* and *T. versicolor*.

3.4. Preventive or Curative Effects of Extracts on Disease on P. sylvestris Germinants

The curative or preventive effect of the extract for treatment and control was tested by comparing necrosis length (Figure 3a). The shortest necroses were observed in the samples to which the curative treatment I was applied with the extract. The Tukey's test shows a significant difference between the control and the other treatments. No difference was found at the *p*-value level of 0.05 in pairwise comparisons between pairs of treatments I versus III and II versus IV.

Since *F. pinicola* extract had the weakest effect in reducing necrosis, further analysis was focused on the effects of extracts from *G. applanatum* and *T. versicolor* (Figure 3b). A significant difference between treatments was confirmed by Tukey's test at *p*-value level of 0.05.

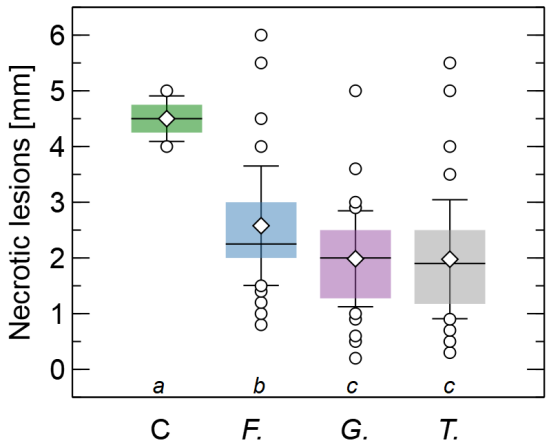

Figure 2. Necrosis length for various extracts: C–control, *F.–Fomitopsis pinicola*, *G.–Ganoderma applanatum*, and *T.–Trametes versicolor*. Treatments, for which statistically significant difference at $p < 0.05$ was found using Tukey's multiple comparisons test, are marked by different characters in the bottom part of the chart.

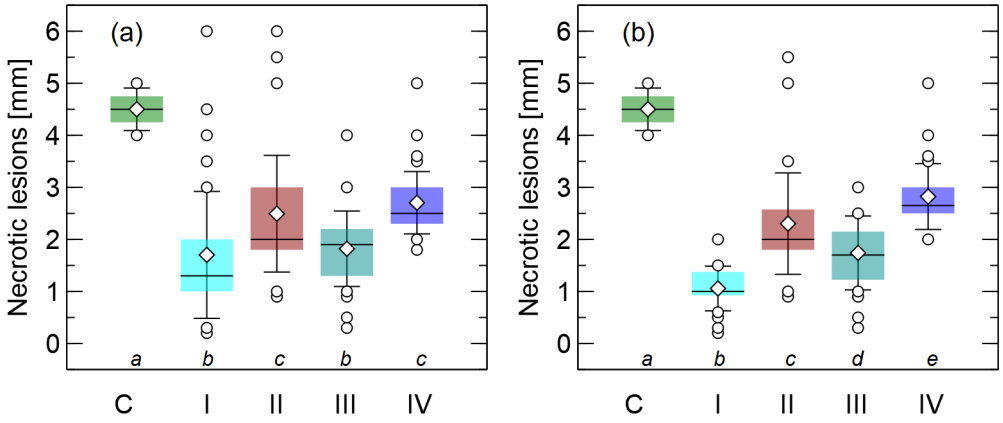

Figure 3. Necrosis lengths for the four treatments of extracts application (I—IV) and control (C). In panel (**a**), all measured samples are presented, in (**b**), samples for which the extract of *Fomitopsis pinicola* was applied have been excluded. Treatments, for which a statistically significant difference at $p < 0.05$ was found using Tukey's multiple comparisons test, are marked by different characters in the bottom part of the chart.

The next step of the analysis concerned the combination of pairs of factors that distinguished the different sample types. The aim of this test was to determine whether the trend toward decreasing necrosis length with increasing extract concentration persisted when the data were analyzed for subgroups defined by the treatment of extract application (preventive or curative) and the type of extract (fungal species) (Figure 4). The observed decreases in lesion length along with extract concentration were significant for all cases, except for treatment I. The *p*-value for the slope coefficient of 0.08 for treatment I indicate that such relation is weak and the effect of the extracts on the fungi may be similar regardless of the applied extract concentration.

Necrosis length by type of extract used (fungal species) and treatment of extract application (preventive, curative) are shown in Figure 5, necrosis length was most reduced in treatment I when *G. applanatum* or *T. versicolor* extracts were applied. The Tukey's multiple comparison test performed for these data confirms statistical significance at *p*-value level of 0.05 between treatment I, *Ganoderma* and *Trametes* extracts and other variants except the cases: treatment II *Ganoderma*, treatment III *Fomitopsis* and *Tramentes*.

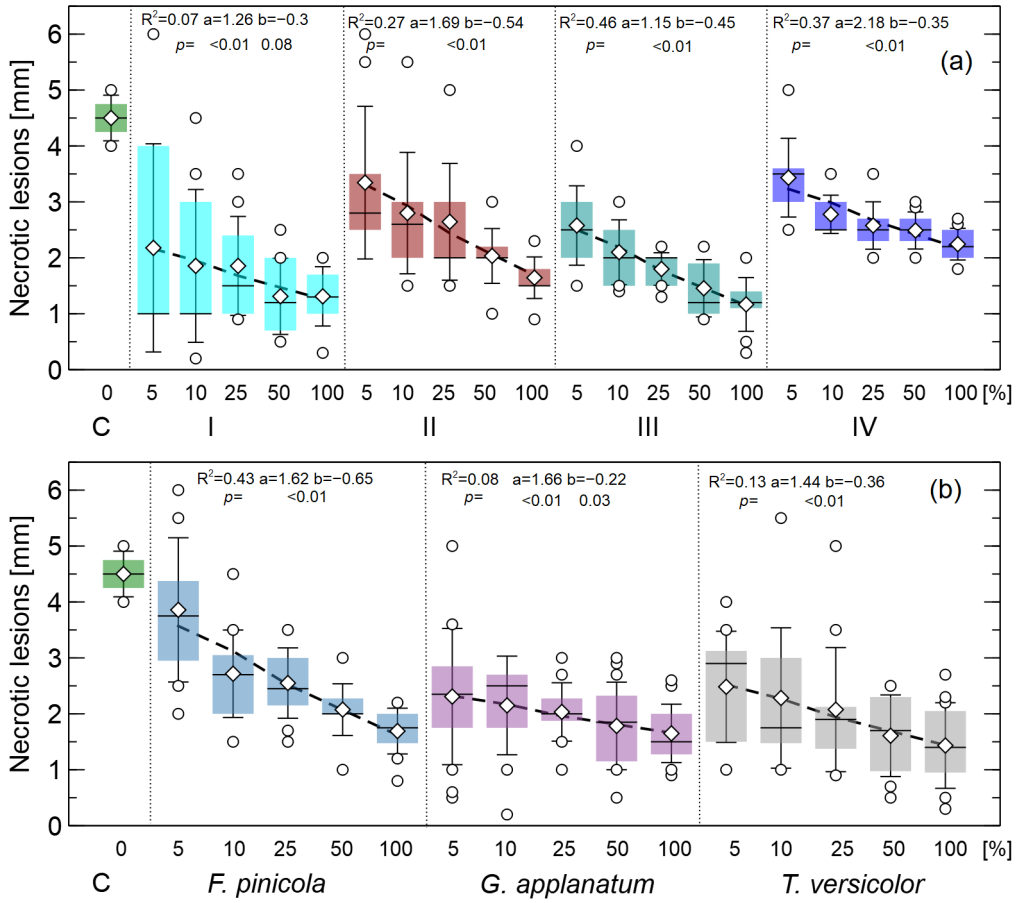

Figure 4. The relation between necrosis length and extract concentration in the control (C) and extract treatments. Comparison of (**a**) treatments with various extract application, and (**b**) various types of extracts. The dashed lines represent fitted regression lines $y = a + b \cdot \ln(x)$. The R^2 of the models, regression coefficients and corresponding p-values are typed in subfigures.

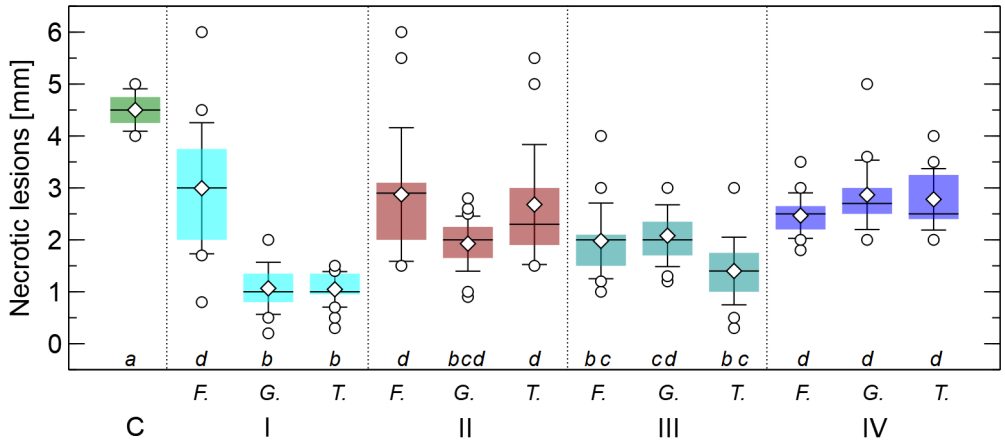

Figure 5. Necrosis length for various extracts: C–control, *F.–Fomitopsis pinicola*, *G.–Ganoderma applanatum*, *T.–Trametes versicolor* and various extracts treatments (I—IV). Treatments, for which statistically significant difference at $p < 0.05$ was found using Tukey's multiple comparisons test, are marked by different characters in the bottom part of the chart.

Since treatment I had the best effect for the extracts *G. applanatum* and *T. versicolor*, various concentrations of extracts from these two fungi were tested for treatments I–IV. Such analysis is presented in Figure 6. For treatment I, there was no difference between length and extract concentration. Such behavior is visible in this figure, also the calculated

regression slope coefficient has p-value = 0.36, which indicates that there may be no dependence of the concentration of extract on the necrotic lesions. This signifies that for treatment I, the curative effect was achieved already at the lowest studied concentration. For treatments II–IV, the slope coefficients in the regression model indicates that the the effect of extracts increases with applied concentration.

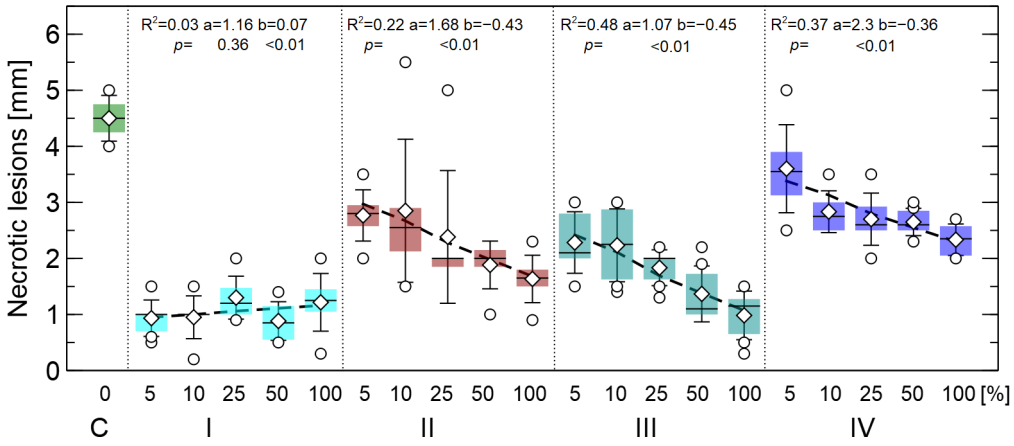

Figure 6. The relation between necrosis length and concentration of the applied extracts in treatments I–IV. Only samples, to which *Ganoderma applanatum* and *Trametes versicolor* were applied are shown here. C refers here to Control treatment, for which no extracts were applied. The dashed lines represent fitted regression lines $y = a + b \cdot \ln(x)$. The R^2 of the models, regression coefficients and corresponding p-values are typed in subfigures.

4. Discussion

4.1. Choice of Fungal Extracts

Trametes versicolor is a widespread fungal species of the order Basidiomycota, belonging to the family Polyporaceae, which facilitates the collection and preparation of extracts for plant protection. Moreover, its use in traditional medicine of the Far East has a long tradition, proving its biological activity. Extensive chemical studies have demonstrated the presence of polysaccharides, protein-polysaccharide complexes, phenols, terpenes and other substances in the fruiting bodies. At this stage of our research, we do not know which compound is responsible for inhibiting the *Fusarium* mycelium. The best known compounds isolated from *Trametes versicolor* are polysaccharide K-crestin (PSK) and polysaccharidopeptide (PSP). PSK is used in Japan as an adjuvant in cancer therapy and is the first drug of fungal origin [61]. PSP is used in China for its immunomodulatory properties. Extracts from the fruit bodies, as well as individual isolated compounds, exhibit potent biological effects, including antioxidant, antibacterial, antiviral, etc. [62].

Ganoderma applatantum, on the other hand, was selected because it is a widespread species in Poland and we anticipated that its chemical activity might produce an effective extract for preventive pre-treatment or curative post-treatment of plants inoculated with *Fusarium* species in nurseries. A closely related species *G. lucidum* is known for its biological activity and use in biomedicine, but unfortunately it is rare in Europe [63], including Poland and neighboring countries. The difference between the extracts of the three tested mushrooms was due to their biology. Only *F. pinicola* causes brown rot of the wood, while the other two fungi cause white rot. In the case of *F. pinicola*, the fungus synthesizes the enzyme cellulase, which decomposes cellulose, and the remaining undecomposed lignin turns brown, and eventually dries, cracks and turns to dust when rubbed. In white rot, the lignin is decomposed mainly by ligninase and the wood containing cellulose becomes white, soft and fibrous. In our experiment, the two extracts of the above fungi that cause white rot (lignin is decomposed faster than cellulose) were the most effective (stronger than the extract of the fungus that causes brown rot).

Fomitopsis pinicola is a common brown-rot fungal species found in coniferous forests in temperate regions throughout Europe and Asia. *F. pinicola* has been intensively studied because it has anti-tumor, anti-fungal, antioxidant, immunomodulation, and neuroprotective activities [64]. However, in our experiment, its extracts showed the weakest biological activity, so it will not be included in future in vivo studies. However, it is the most common species in the local forests, developing on dead spruce trees as a result of infestation by *Ips typographus*.

4.2. Chemical Composition of Fungal Extracts

As for the chemical composition of fungal extracts, polysaccharides are mainly responsible for their biological activity, but in the case of methanolic extracts, there are usually not too many of them [65]. This is because they are bound to the cell wall of the fungus and their amount is much higher when hot water extracts are used [66]. Therefore, in the future, we can consider preparing extracts with hot water to check if the biological activity is higher than observed now. In the methanolic extracts, mainly low molecular weight compounds are found. Quite a number of compounds have antioxidant, antibacterial, antiviral and immunomodulatory potential [47]. Some of these compounds are also found in fruits or herbs, while others are found only in fungi and in small amounts. A compound may also be specific (like a fingerprint) to a fungal species. Wood decay fungi produce ligninolytic and cellulolytic enzymes, e.g., laccases, peroxidases, and manganases, but these are not usually obtained by extraction. It is noteworthy that extract-based drugs usually contain whole fractions rather than single compounds. Hundreds of bioactive compounds can act synergistically and multidirectionally. Thus, to understand the effect of an extract, the chemical properties must be carefully evaluated. Therefore, it is important to keep in mind that the GC-MS analysis of extracts may not identify about 20% of the compounds, especially macromolecular compounds such as polysaccharides, for which other methods must be used. For this reason, we focused on demonstrating the efficacy of the preventive and curative effects of the studied fungal extracts against a dangerous pathogen in forestry, agriculture and horticulture—the fungus *F. oxysporum*. In the future, we will try to find biologically active compounds in the fungal extracts, but this was not the aim of our research at this stage.

4.3. Preventive Measures

The current study with fungal extracts also showed a protective effect against *F. oxysporum*. GC-MS analysis showed that the most abundant compounds in the species studied were carbohydrates (35.72–72.65) (see supplemental materials). This is consistent with several publications indicating the antifungal activity of such compounds [65,67–69]. The second common group detected in this study was fatty acids, which accounted for 8.54%, 37.40% and 10.35% of the extracts from *F. pinicola*, *G. applanatum* and *T. versicolor*, respectively. Potent antifungal effects of monoacylglycerols, short-chain and long-chain fatty acids were reported [70–73].

Induced resistance, which relies on the host's natural defenses, is an effective measure to control plant diseases and meets the strategic needs of pesticide use and agricultural product safety worldwide. *Ganoderma lucidum* polysaccharide (GLP), the main active molecule of *G. lucidum*, is widely used in functional foods and clinical medicine [74,75]. However, there are few reports on the use of GLP for plant disease prevention and control. Cör et al. [75] reported the direct antibacterial effect of GLP against certain plant pathogens and foodborne fungi and bacteria. GLP can also induce systemic resistance to *Botrytis cinerea* in tomato plants and promote tomato seed germination and seedling growth [76]. GLP spray and irrigation root treatments can promote cotton growth. After soaking in GLP, both seedling height and Fusarium wilt resistance of cotton increased to some extent. The increased expression of genes related to the jasmonic acid pathway suggests that this pathway may be important for plant resistance induced by GLP plant resistance [77].

4.3.1. Protective Effect of Fungal Extracts

Methanol extracts of *G. applanatum* and *T. versicolor* showed the best results in inhibiting *F. oxysporum*. Probably, the active compounds contained in them have a direct toxic effect on the hyphae limiting the development of the pathogen, but most importantly they induce plant resistance. According to Oviasogie et al. [78], the antifungal activity of *Ganoderma* species is due to bioactive substances such as β-triterperinoids, polysaccharides, proteins, amino acids, nucleotides, alkaloids, steroids, lactones, fatty acids and enzymes [78,79]. On the other hand, Boh et al. [80] demonstrated the bioactive effects of lucidic acid and ganodermic acid. They also isolated ganodermic acid, ganolucidic acid, applanoxic acid, a group of triterpenoid aldehydes (ganoderals), and a group of triterpenoid alcohols (ganoderols) from *G. applanatum* fruiting bodies [80]). Nagaraj et al. [81] showed that this fungus produces phytochemicals such as saponins, phenols, steroids, glycosides, terpenoids, and flavonoids. The studies of Jonathan and Awtona [82] confirmed the properties of fungi of the genus *Ganoderma* in limiting the development of *F. oxysporum* [82]. Other studies on *T. versicolor* confirm the possibility of using extracts from this fungal species to inhibit pathogenic soil fungi, including *Fusarium* fungi [83]. Similarly, Waithaka et al. [84] point out the great potential of using *T. versicolor* extracts to limit the development of *F. oxysporum*.

Test results are also affected by many variables, for example, results can vary depending on the solvent used for the extract (methanol, ethanol, hexane, water extract). For this reason, scientists argue about how the extracts are used and what their concentrations are [83,85,86]. Even the substrate on which a particular fungal species grows has an influence on the content of certain secondary metabolites as well as their amount [87]. This in turn is associated with potentially different properties of the biologically active compounds.

4.3.2. Potential Applications

The curative treatment (Treatment I) seemed to be more efficacious than the preventive treatment (Treatment II) and the two dipping treatments.

Perhaps the protection provided by a 24 h post-inoculation treatment (Treatment I) might have better coincided with the stimulation of defense responses in the plant, and also had some direct inhibitory effects on hyphal growth, which had reached that part of the roots. In the curative treatment scenario, we speculate that after *F. oxysporum* inoculum is applied, there is some initial stimulation of root cell defense, which is enhanced systemically when the treatment is applied 24 h later. In the absence of the pathogen, the substances contained in the extracts may induce resistance, but since wood-destroying fungi are not strong pathogens acting against live host plant cells and likely do not produce phytotoxins, this elicits a weaker response from the plant. However, when the plant cells are in direct contact with a pathogen, the defense response should be stronger. This combined with direct activity against the fungal hyphae may lead to decreased disease.

The potential of using wood-decomposing fungi to produce active ingredients that increase plant resistance is enormous [88]. To achieve optimal results, many variables must be considered, and the research must be conducted in many iterations, which means it will require many years of work. However, biologically active compounds can be used not only to increase the resistance of the plants themselves, but also to treat seeds (bioimprinting) from which the disease-resistant seedlings will grow [89–92]. This is even more valuable as soil-borne fungi gradually mutate and form new varieties that are more resistant to existing methods of protection, particularly the chemicals used [7,93–95]. Due to the ongoing negative phenomenon of environmental chemization, there is a need to search for new, environmentally friendly methods of crop protection.

5. Conclusions

Methanolic extracts of wood-destroying material showed a positive effect in protecting pines as active preparations against soil-borne pathogen *F. oxysporum*. The fungi

G. applanatum and *T. versicolor* are potential biological control agents whose secondary metabolites can be used against the pathogen *F. oxysporum*.

In summary:

- All three fungal extracts studied showed a curative or preventive effect, resulting in a reduction in the length of root necrosis caused by infection with *F. oxysporum*.
- The strongest effects were obtained with extracts of *G. applanatum* and *T. versicolor*.
- The strongest effect was obtained in treatment I, in which one drop of the extract was applied to the root tip 24 h after infection (curative).
- The full preventive effect of the application of these two extracts in treatment I was achieved at a 5% concentration of the extract—further increasing the concentration did not change the results.

Further studies are needed to understand the mechanism of action of the extracts. Field trials (in vivo) are needed to confirm the results of plant protection efficacy. In addition, isolation and testing of the active compounds responsible for the effect of the extracts could be one of the future topics of research.

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