Potential of *Trichoderma* spp. and *Pinus sylvestris* Bark Extracts as Biocontrol Agents against Fungal Pathogens Residing in the Botryosphaeriales †

Vera Karličić 1,*, Jelena Jovičić-Petrović 1, Veljko Marojević 1, Milica Zlatković 2, Saša Orlović 2 and Vera Raščević 1

1 Faculty of Agriculture, University of Belgrade, 11 000 Belgrade, Serbia; jelenap@agrif.bg.ac.rs (J.J.-P.); veljko.maroevic@yahoo.com (V.M.); vera@agrif.bg.ac.rs (V.R.)
2 Institute of Lowland Forestry and Environment, University of Novi Sad, 21 000 Novi Sad, Serbia; milica.zlatkovic@uns.ac.rs (M.Z.); sasao@uns.ac.rs (S.O.)

† Presented at the 1st International Electronic Conference on Forests—Forests for a Better Future: Sustainability, Innovation, Interdisciplinarity, 15–30 November 2020; Available online: https://iecf2020.sciforum.net.

Abstract: Botryosphaeriales represent a diverse order of fungal pathogens of various woody plant species. In Serbia, these fungi are important pathogens of forest, ornamental, and fruit trees causing die-back, cankers, leaf blights, fruit, and root rot. The aim of this study was to evaluate the antifungal activity of *Pinus sylvestris* bark extracts and *Trichoderma* spp. against *Botryosphaeria dothidea*, *Dolichosporium sordariae*, and *Neofusicoccum parvum* (Ascomycota, Botryosphaeriales) isolated from *Picea abies*, *Thuja occidentalis*, and *Pinus laurocerasus* trees planted in urban areas in Serbia. Bark extracts were prepared in water solution at two temperatures (80 and 120 °C). The extracts were tested using two concentrations (20% and 30%). Moreover, two *Trichoderma* isolates obtained from *P. sylvestris* bark were tested against Botryosphaeriales and their antagonistic potential was evaluated in vitro using a confrontation test. Mycelial growth of *B. dothidea* and *D. sarmentorum* was significantly inhibited in the presence of bark extracts, while *N. parvum* showed no growth inhibition. *Botryosphaeria dothidea* growth was inhibited by 35 to 39% in the case of 20% extracts and by 39 to 44% in the case of 30% extracts. The growth inhibition of *D. sarmentorum* was between 48% and 56% in the case of 20% extracts and between 53% and 60% in the case of 30% extracts. The two *Trichoderma* isolates showed antifungal activity against the selected pathogens. An isolate BKG 4 showed the highest inhibition level, and it inhibited the growth of *B. dothidea*, *D. sarmentorum*, and *N. parvum* by 85%, 75%, and 62%, respectively. Preliminary results suggest that both *P. sylvestris* bark extracts and *Trichoderma* spp. could be used as biocontrol agents against *B. dothidea*, *D. sarmentorum*, and *N. parvum*, and this should be a further studied.

Keywords: Botryosphaeriales; biocontrol; pine bark extracts; *Trichoderma* spp.

1. Introduction

Sawmill industries represent a huge generator of bark waste estimated to more than three hundred million tons [1]. The disposal and storage of bark waste is a global problem since it is mostly used as an energy source through direct combustion or transported to landfills [1,2]. However, removed bark represents a raw material with a whole scale of valuable purposes. Substrate formulations, soil conditioners, plant protection, health and industrial products, and bioremediation agents are some of the potential applications of this material considered to be a waste [1,3–6]. Coniferous bark, as a component of commercial substrates, increases water and nutrient availability and exhibits antifungal, antibacterial, and insecticidal effects [3–4]. Antimicrobial and antifungal activity of extracted bark constit-
uents has been well documented [5–7], suggesting that they are environmentally sound alternatives to plant protection chemicals. In addition, different microorganisms with such roles inhabit above-ground and below-ground plant parts [8,9]. Microbial communities inhabiting plant parts mainly originate from the rhizosphere and some of them are known as plant growth-promoting microorganisms, for example, Bacillus spp., Pseudomonas spp., Azospirillum spp., Arthrobacter spp., Aspergillus spp., Penicillium spp., Piriformospora spp., Phoma spp., and Trichoderma spp. These microorganisms increase disease resistance by triggering plant defensive mechanisms [8,10]. Members of the fungal genus Trichoderma (Ascomycota, Hypocreales, Hypocreaceae) are well-known biocontrol agents with plant growth-promoting characteristics [10]. These fungi are most diverse in the above-ground plant parts such as bark, but they are also widely distributed in soils [11]. Nowadays, more than 250 *Trichoderma*-based biofungicides are commercially available worldwide [12].

Species of the Botryosphaeriales (Ascomycota) are distributed worldwide and occur on various tree species. These fungi are known as endophytes, latent pathogens, pathogens, and saprophytes and they usually cause disease when their hosts are under stress [9]. In Serbia, Botryosphaeriales are important pathogens of woody and vascular plants causing die-back, cankers, leaf blights, shot hole disease, fruit, and root rot [13–18]. The pathogenicity of Botryosphaeriales is of a particular importance in urban environments such as city parks which are mostly comprised of plants belonging to diverse taxonomic groups and these fungi can infect multiple hosts [19]. Botryosphaeriales species are difficult to control because these pathogens reside in vascular woody tissues and cause cankers [20]. Additionally, there is increasing concern about the environmental effects and safety of conventional fungicide-based control measures against diseases caused by these fungi [21].

The aims of the present study were the following: (1) to isolate *Trichoderma* spp. from *Pinus sylvestris* bark and (2) to evaluate the antifungal activity of *P. sylvestris* bark extracts and *Trichoderma* spp. against *Botryosphaeria dothidea*, *Neofusicoccum parvum*, and *Dothiorella sarmentorum* in vitro.

2. Material and Methods

2.1. Isolation of *Trichoderma* spp.

*Trichoderma* spp. were isolated from *P. sylvestris* bark obtained from the Eko Farm Kovačević (Gornji Milanovac, RS), using a serial dilution method. One milliliter of $10^{-3}$ dilution was spread evenly on potato dextrose agar plates (PDA, HiMedia, India) that were kept at 25 ± 1 °C for 7 days. The colonies with morphological and cultural characteristics of *Trichoderma* spp. (yellow-green colonies with a characteristic conidiophore branching pattern) were selected, purified, and kept in 20% glycerol at −80 °C, until use.

2.2. Extract Preparation

*Pinus sylvestris* bark was ground with a mill, sifted through 0.5 mm sieve, dried at 50 °C and maintained at room temperature. Two different water extracts were prepared using distilled water as a solvent. Extraction was carried out in 0.5 L Erlenmeyer flasks by immersing the bark powder (40 g dry weight) in 400 mL of the solvent. The Erlenmeyer flasks were placed in a water bath (Memmert WNB 14, Germany) for 2 h at 80 °C and at 120 °C in an autoclave (Panasonic MLS-37812) for 20 min. The extraction mixtures were filtered with a Whatman® Grade 52 filter paper (Merck, Germany) and the residues were dried in a convection oven at 105 °C for 24 h (Binder, Germany). The water extract yields were calculated according to the following formula [22]:

$$\text{Yield of extract (\%) = } \left(\frac{W_1 \times 100}{W_2}\right)$$

where $W_1$ is the weight of the dry extract residue after solvent removal and $W_2$ is the initial weight of bark powder (40 g). Prepared WEs were kept in a refrigerator (4 °C) until used.
2.3. Assay of Water Bark Extracts’ Antifungal Activity In Vitro

The antifungal activity assay was performed against the growth of *B. dothidea* (CMW39314), *D. sarmentorum* (CMW39365), and *N. parvum* (BOT275). The *B. dothidea*, *D. sarmentorum*, and *N. parvum* were isolated from *Picea abies*, *Thuja occidentalis*, and *Prunus laurocerasus*, respectively, and have been identified in previous studies [14,19,23]. These isolates are maintained at −80 °C in the culture collection of the Institute of Lowland Forestry and Environment (ILFE), University of Novi Sad. Before use, the isolates were subcultured onto freshly prepared PDA and allowed to grow for one week at 25 °C, in the dark.

The essay was conducted using PDA plates containing 20% and 30% of bark extract. Agar plugs (5 × 5 mm) of the 7-day-old cultures of *B. dothidea*, *D. sarmentorum*, and *N. parvum* were placed in the centre of 85 mm Petri dish with *P. sylvestris* bark extracts. The control plates contained only PDA. Each treatment was replicated three times. Inoculated Petri dishes were incubated at 25 °C, in the dark. Radial growth of the mycelium was measured in two directions until the control fungus had reached the edge of the plate. The mycelial growth inhibition percentage was calculated according to the formula [7]:

\[
\text{Mycelial growth inhibition (\%) = } \left( \frac{(DC - DT)}{DC} \right) \times 100,
\]

where DC is the average diameter of a fungal colony of the control group, and DT is the average diameter of a fungal colony of the treatment group.

The degree of extracts toxicity was estimated as follows: 0–25% non-toxic, 26–50% slightly toxic, 51–75% moderately toxic, and >75% as toxic [24].

2.4. Assay of Trichoderma spp. Antifungal Activity In Vitro

The ability of *Trichoderma* spp. to antagonize *B. dothidea*, *D. sarmentorum*, and *N. parvum* was assessed using a confrontation assay on PDA plates. The plates were double inoculated with agar plugs (5 × 5 mm) of a 7-day-old culture of *B. dothidea*, *D. sarmentorum*, *N. parvum*, and *Trichoderma* spp. at a 3 cm distance. Each treatment was replicated three times. Inoculated Petri dishes were incubated at 25 ± 2 °C, in the dark, until the control fungus had reached the edge of the plate. The mycelial growth inhibition percentage was calculated using the same formula as above. The degree of antagonistic activity was estimated as: very high (% > 75), high (% = 61–75), moderate (% = 51–60), and low (% < 51) [25].

2.5. Statistical Analyses

The statistical analyses were performed using the analyses of variance (ANOVA) and software Statistica (StatSoft, Tulsa, OK, USA). Mean values of data were compared using the Tukey test at a significance level of \( p = 0.05 \).

3. Results and Discussion

After extraction, the water-soluble fraction from the non-soluble particulate material was separated by filtering. Weight losses detected after bark drying revealed different levels of extraction depending on the temperature regime. Extraction yields of *P. sylvestris* bark at 80 °C were 4.10%, while yields were 5.14% at 120 °C. A temperature increase favors the solubility of phenolic compounds in water resulting in an increased rate of extraction and decreased extraction time [26].

Generally, on the one hand, water is not considered to be the best solvent since it shows no selectivity in the extraction and, consequently, both phenolic and non-phenolic compounds are dissolved. On the other hand, industrial usage gives preference to water over other solvents [2]. The highest extraction yields of *P. sylvestris* bark were obtained with hot water (4.80–13.70%) as compared with benzene, ether, and ethanol as a solvent [2]. In addition, several solvents (water, ethanol, and acetone) were used for *P. radiata* bark extraction and the highest yields were achieved with water (11.99%) at 120 °C [27].
The antifungal properties of the extracts were tested using two concentrations (20 and 30%). Extract concentration of 20% inhibited mycelial growth of *B. dothidea* by 35–39% (Table 1), while 30% concentration increased the level of inhibition to 39–44%. The case of *D. sarmentorum* water extract prepared at 80 °C showed the highest inhibition rate (60%).

According to [24], the effects of extracts on *B. dothidea* mycelium growth are considered to be slightly toxic, while the same treatments showed a moderately toxic effect on *D. sarmentorum* growth. Similarly, hydrophilic extracts of *P. sylvestris* applied in 1% concentration caused slight toxicity towards *Schizophyllum commune* (48.00%), *Gloeophyllum trabeum* (49.14%), and *Fibroporia vaillantii* (47.03%), while 5% concentration was classified as moderately toxic in all three cases with an inhibition percentage over 55% [28]. *Pinus sylvestris* bark extracts showed no inhibition of radial growth of *N. parvum*, but the sparse aerial mycelium was noted. The water extracts increased asexual sporulation of *Trichoderma* spp., but did not cause growth inhibition.

### Table 1. Mycelial growth inhibition (%) by *Pinus sylvestris* bark water extracts.

| Pathogen     | *P. sylvestris* Bark Extracts | Water at 80 °C | Water at 120 °C |
|--------------|--------------------------------|----------------|-----------------|
| *B. dothidea* | 20%                           | 39 bA          | 35 AB           |
|              | 30%                           | 44 aA          | 39 aB           |
| *D. sarmentorum* | 20%                           | 56 aA          | 48 AB           |
|              | 30%                           | 60 bA          | 53 aA           |

Different lowercase letters (a, b) in the same row indicate a significance difference among means (Tukey’s test, *p* < 0.05); Different uppercase letters (A, B) in the same column and same pathogen indicate a significance difference among means (Tukey’s test, *p* < 0.05).

Fungal isolations from *P. sylvestris* bark yielded two isolates of *Trichoderma* spp. (BKG 3 and 4) which were subjected to a confrontation test with *B. dothidea*, *D. sarmentorum*, and *N. parvum* (Table 2). The test showed that *Trichoderma* spp. was able to inhibit the growth of the pathogens five days after the confrontation. *Trichoderma* sp. BKG 4 showed very high antagonism against *B. dothidea* and *D. sarmentorum*. In the test with *N. parvum*, the activity qualified as high. *Trichoderma* sp. BKG 3 showed high antagonism against *B. dothidea* and *D. sarmentorum*, while the effects on *N. parvum* mycelial growth were declared as moderate.

### Table 2. Mycelial growth inhibition (%) by two *Trichoderma* spp. isolated from *P. sylvestris* bark.

| Pathogen     | *Trichoderma* sp. BKG 3     | *Trichoderma* sp. BKG 4 |
|--------------|-----------------------------|-------------------------|
| *B. dothidea* | 67 a                        | 85 b                    |
| *D. sarmentorum* | 63 a                        | 75 b                    |
| *N. parvum*   | 55 a                        | 62 c                    |

Different lowercase letters in the same row indicate a significance difference among means according to the Tukey’s test (p < 0.05).

The interaction established between the two *Trichoderma* isolates, *B. dothidea* and *D. sarmentorum*, induced morphological changes characterized as overgrown with replacement which indicates mycoparasitism as the mode of the action [29,30]. The confrontation test of *N. parvum* with *Trichoderma* isolates resulted in a deadlock at a distance with enhanced production of dark pigmentation in *N. parvum*. This is expected as pigments indicate the meeting of the two genetically different mycelia while an inhibition zone indicates the diffusion of antifungal metabolites [30,31].

Biocontrol potential of *Trichoderma* strains has been widely studied, documented, and commercialized [12]. *Trichoderma atroviride* can be used as biological protection of grapevine pruning wounds against species of the Botryosphaeriales [32]. The authors showed that *T. atroviride* reduced incidence of *Neofusicoccum australis*, *N. parvum*, *Diplodia seriata*, and *Lasiodiplodia theobromae* by 78%, 80%, 85% and 92%, respectively.
Preliminary results of the study suggested, for the first time, in vitro potential of *P. sylvestris* bark extracts and *Trichoderma* spp. for suppressing the growth of *B. dothidea*, *N. parva*um, and *D. sarmentorum* isolated from ornamental trees with the die-back symptoms, in Serbia. It is also the first study to indicate the in vitro biocontrol potential of *P. sylvestris* bark extracts against diseases caused by Botryosphaeriales. However, more research is needed to further examine different antagonistic mechanisms using biochemical and microscopic examinations and to identify *Trichoderma* spp. Moreover, future work should also focus on the extraction of active compounds and on optimization of the extraction procedure.

**Author Contributions:** Conceptualization, V.R. and M.Z.; methodology, J.J.-P., V.K. and V.M.; writing—original draft preparation, V.K. and M.Z.; writing—review and editing, V.R., J.J.-P. and S.O.; visualization, V.K.; funding acquisition, V.R. and S.O.

**Funding:** This research was funded by the Serbian Ministry of Education, Science and Technologi cal Development, contract number 451-03-68/2020-14/200116 and project number 451-03-68/2020-14/200197.

**Conflicts of Interest:** The authors declare no conflict of interest.

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