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

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Abstract: The Botryosphaeriales represents 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*, *Dothiorella sarmentorum* and *Neofusicoccum parvum* (Ascomycota: Botryosphaeriales) isolated from *Picea abies*, *Thuja occidentalis* and *Prunus laurocerasus* trees planted in urban areas in Serbia. Bark extracts were prepared in water solution at two temperatures (80 and 120 ºC). 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 estimated *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 to 56% in the case of 20% extracts and 53 to 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 deserves a further study.

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]. On the other side, removed bark represents row material with a whole scale of valuable purposes. Substrate formulations, soil conditioners, plant protection, health and industrial products, bioremediation agents are some of the potential applications of this material considered as 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 constituents is well documented [5-7], suggesting them as environmentally sound alternatives to plant protecting chemicals. Also, different microorganisms with such role

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, e.g. *Bacillus* spp., *Pseudomonas* spp., *Azospirillum* spp., *Arthrobacter* spp., *Aspergillus* spp., *Penicillium* spp., *Piriformospora* spp., *Phoma* spp., *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 like 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 as city parks 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]. Besides, there is an 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: 1. to isolate *Trichoderma* spp. from *Pinus sylvestris* bark; 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 ºC and maintained at room temperature. Two different water extracts were prepared using distilled water as a solvent. Extraction was carried out in a 0.5 L Erlenmeyer flasks by immersing the bark powder (40 g dry weight) in the 400 mL of the solvent. Erlenmeyer flasks were placed in a water bath (Memmert WNB 14, Germany) for 2 hours 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 hours (Binder, Germany). The water extract yields were calculated according to the formula:

Yield of extract (%) = (W1× 100)/ W2 [22]

Where W1 is the weight of the dry extract residue after solvent removal and W2 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 identified in the 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:

$$\text{Mycelial growth inhibition (\%)} = ((DC - DT) / DC)) \times 100, \quad [7]$$

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: 0 - 25% non-toxic, 26 - 50% little 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 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. on 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. Temperature increase favors the solubility of phenolic compounds in water resulting in an increased rate of extraction and decreased extraction time [26].

Generally, water is not considered as the best solvent since it shows no selectivity in the extraction and consequently, both, phenolic and non-phenolic compounds are dissolved. On the other side, 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%) compared to benzene, ether, and ethanol as a solvent [2]. Also, 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* for 35 - 39% (Table 1) while concentration of 30% increased the level of inhibition to 39 - 44%. In the case of *D. sarmentorum* water extract prepared at 80ºC showed the highest inhibition rate (60%).

According to [24] effects of extracts on *B. dothidea* mycelium growth are considered as little 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 little 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 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 *P. sylvestris* bark water extracts.

***P. sylvestris* bark extracts**		**Water at 80ºC**	**Water at 120ºC**
B. dothidea	20%	39^{bA}	35^{aA}
	30%	44^{aA}	39^{aB}
D. sarmentorum	20%	56^{aA}	48^{aA}
	30%	60^{bA}	53^{aA}

Means in the same row followed by different lowercase letters are significantly different different by the Tukey's test ($p < 0.05$); Means in the same column and same pathogen followed by different uppercase letters are significantly different by the 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* activity was 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}

Mean values in the same row with different lowercase letters are significantly different according to the Tukey test ($p=0.05$).

The interaction established between the two *Trichoderma* isolates, *B. dothidea* and *D. sarmentorum* induced morphological changes characterized as overgrow 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 australe*, *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. in suppressing the growth of *B. dothidea*, *N. parvum* 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 microscopical 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.

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