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Trichoderma spp. from Pine Bark and Pine Bark Extracts: Potent Biocontrol Agents against Botryosphaeriaceae

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Abstract: *Pinus sylvestris* bark represents a rich source of active compounds with antifungal, antibacterial, and antioxidant properties. The current study aimed to evaluate the antifungal potential of *P. sylvestris* bark against *Botryosphaeria dothidea*, *Dothiorella sarmentorum*, and *Neofusicoccum parvum* (Botryosphaeriaceae) through its chemical (water extracts) and biological (*Trichoderma* spp. isolated from the bark) components. The water bark extracts were prepared at two temperatures (80 and 120 °C) and pH regimes (7 and 9). The presence of bark extracts (30%) caused inhibition of mycelial growth of *B. dothidea* and *D. sarmentorum* for 39 to 44% and 53 to 60%, respectively. Moreover, we studied the antagonistic effect of three *Trichoderma* isolates originating from the pine bark. *Trichoderma* spp. reduced growth of *B. dothidea* by 67%–85%, *D. sarmentorum* by 63%–75% and *N. parvum* by 55%–62%. Microscopic examination confirmed typical mycoparasitism manifestations (coiling, parallel growth, hook-like structures). The isolates produced cellulase, β-glucosidase and N-acetyl-β-glucosaminidase. The volatile blend detected the emission of several volatile compounds with antimicrobial activity, including nonanoic acid, cubenene, cis-α-bergamotene, hexanedioic acid, and verticillol. The present study confirmed in vitro potential of *P. sylvestris* bark extracts and *Trichoderma* spp. against the Botryosphaeriaceae. The study is an important step towards the use of environmentally friendly methods of Botryosphaeriaceae disease control.

Keywords: Botryosphaeriaceae; biocontrol; pine bark extracts; *Trichoderma citrinoviride*; VOCs; lytic enzymes

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1. Introduction

Pesticides are generally considered a quick, easy, and inexpensive solution against plant pathogens. However, constant reliance on chemicals has led to the emergence of more virulent strains with higher resistance to active compounds [1,2]. Understanding the seriousness of this problem has triggered an intense search for alternative solutions, among which "naturally-based" products have attracted special attention. For example, a collaboration between sawmill industries and plant health experts has led to multiple solutions for plant disease control. Sawmill industries generate enormous amounts of bark waste which are mostly burned or disposed to landfills [3,4]. Removed bark represents raw material for substrate formulations, soil conditioners, a variety of human health and industrial products, and bioremediation agents [3,5]. Moreover, it also exhibits antifungal, antibacterial, and insecticidal properties which main carriers are compounds such as terpenes, phenolics, flavonoids, tannins, and pinosylvins [4–6]. In addition, a bark represents a habitat with a complex set of niches available to various microorganisms and communities [7]. Species of the Botryosphaeriaceae (Ascomycota: Botryosphaeriales) are important pathogens of forest, ornamental, fruit trees, and agricultural plants. These fungi are distributed worldwide

causing a variety of symptoms such as crown die-back, cankers, leaf blights, and shot hole disease [8–11]. The Botryosphaeriaceae are difficult to control as they reside as endophytes and latent pathogens in wood, and the disease symptoms appear when host is under stress [12]. Moreover, these fungi colonize the xylem tissue, and most species are known as generalist pathogens, able to infect different taxonomically unrelated hosts [12–14]. This is of particular concern in urban environments which represent mixtures of native and non-native trees, conifers and broadleaves, and where environmental conditions are such that promote stress on the trees [9].

Fungi of the genus *Trichoderma* (Ascomycota: Hypocreales: Hypocreaceae) represent a large, ecologically diverse group of well-known biocontrol agents (BCA) [1]. Worldwide, more than 60% of registered biopesticides are *Trichoderma*-based [15]. The modes of biocontrol action are various and include competition, mycoparasitism, antibiosis, inactivation of the pathogen's enzymes, and induction of plant disease resistance [2,16]. Moreover, these fungi produce powerful secondary metabolites such as cell-wall degrading enzymes (cellulase, protease, chitinase), volatile organic compounds (VOCs) and non-volatile compounds. Many VOCs have been associated with *Trichoderma* spp., including sesquiterpenes, alcohols, ketones, lactones, esters, thioalcohols, thioesters, and cyclohexenes [15]. Some VOCs are directly involved in communication of *Trichoderma* spp. with their co-habitants and in antibiosis [15]. Moreover, the VOCs are usually involved in indirect antagonistic actions, by diffusing and affecting distant opponents [17].

The research of *Trichoderma* fungi as BCA has been directed towards diseases of agricultural plants and *Trichoderma* spp. proved to be effective antagonists of numerous pathogens, including *Armillaria* spp., *Botrytis* spp., *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp., *Sclerotinia* spp., and *Verticillium* spp. [18,19]. Moreover, pine bark extracts have been shown to inhibit growth of *Coniophera puteana* (Schumach.) P.Karst., *Trametes versicolor* (L.ex. Fr.) Pilát, *Botrytis cinerea* Pers., *Colletotrichum acutatum* J.H. Simmonds, *Phytophthora cactorum* (Lebert and Cohn) J. Schröt., and *Mycosphaerella fragariae* (Tul.) Lindau [6,20]. However, the research on *Trichoderma* spp. as BCA of Botryosphaeriaceae has been limited and mostly focused on grapevine trunk diseases [21]. Moreover, except for a preliminary study [22], there has been no previous research related to pine bark extracts as a biological control option for fungi classified in the Botryosphaeriaceae.

In this regard, the aims of this study were: (1) to conduct in vitro evaluation of the antifungal activity of *P. sylvestris* bark extracts and *Trichoderma* spp. against *Botryosphaeria dothidea* (Moug. ex. Fr.) Ces. et de Not., *Dothiorella sarmentorum* (Fr.) AJL Phillips, A. Alves and J. Luque, and *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and AJL Phillips (Ascomycota: Botryosphaeriaceae); (2) to conduct in vitro evaluation of antifungal effects of *Trichoderma*-VOCs on Botryosphaeriaceae mycelial growth; and (3) to detect effective VOCs and cell-wall degrading enzymes produced by *Trichoderma* spp. when confronted with the Botryosphaeriaceae.

2. Materials and Methods

2.1. Preparation of Pinus Sylvestris Bark Extracts

Pinus sylvestris bark extracts were prepared, and extraction yields calculated as described in Karličić et al. [22]. The water extracts were obtained at two temperatures (80 and 120 °C) and pH regimes (7 and 9) in three repetitions. The extraction yields represented a percentage of soluble bark powder and were 4% for extracts prepared at 80 °C and 5% for extracts prepared at 120 °C [22].

2.2. Fungal Isolates

Phytopathogenic isolates of *B. dothidea* (CMW 39314), *D. sarmentorum* (CMW 39365), and *N. parvum* (BOT 275) were isolated from *Picea abies* (L.) H. Karst., *Thuja occidentalis* L., and *Prunus laurocerasus* L., respectively. The identity and pathogenicity of the isolates was determined in previous studies [8,13].

The putative biocontrol fungal agents *Trichoderma* spp. were isolated from *P. sylvestris* bark using a serial dilution in a previous study by Karličić et al. [22] and kept in 20% glycerol at −80 °C until use. Morphological identification of the fungal isolates based on colony appearance and microscopic examination (Leica DMLS, Leica Microsystems GmbH, Wetzlar, Germany) was determined after 3-day incubation on PDA at 25 °C in darkness. Genomic DNA was extracted from five days-old cultures grown on sterile cellophane circles pierced with a sterile hypodermic needle by following the manufacturer's protocol for the ZR Soil Microbe DNA Kit (Zymo Research, Irvine, CA, USA). The ITS region of the rDNA was amplified using primers ITS1F [23] and ITS 4 [24]. Part of the *tef 1-α* gene and part of the *RPB2* gene were amplified using primers EF1 and EF2 and fRPB2-5f and fRPB2-7cr, respectively [25,26]. The 25 µL PCR reaction mixtures contained 2.5 µL of 10× Taq buffer with $(NH_4)SO_4$ (Thermo Scientific, Waltham, MA, USA), 3 µL of 25 mM $MgCl_2$ (Thermo Scientific, Waltham, MA, USA), 1 µL of 100 mM of each dNTPs (Thermo Scientific, Waltham, MA, USA), 0.5 µL of 10 µM of each primer (Invitrogen, Paisley, UK), 2 µL (40 ng) of genomic DNA, 0.3 µL (1.5 U) of Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) and 15.2 µL of autoclave-sterilized ultra-pure water The PCR was performed in an Eppendorf Mastercycler epgradient S thermal cycler (Eppendorf AG, Hamburg, Germany) under the conditions described in Kovač et al. [27]. However, the *tef 1-α* region was amplified using annealing temperature of 60 °C instead of 55 °C.

The PCR products were purified and sequenced by Macrogen Europe (Amsterdam, The Netherlands).

Nucleotide sequences were examined for sequencing errors, edited, and assembled using BioEdit v. 7.2.5, and MEGA X, whereas sequence alignments were carried out using MEGA X and MAFFT v. 7 (on-line) as described in Zlatković et al. [28]. Sequences of the three loci (*ITS*, *tef 1-α*, *RPB2*) were analysed individually and in combination following the GCPSR concept [29]. The phylogenetic analyses of the individual genes were carried out using Maximum Likelihood (ML) analyses, whereas analyses of the combined datasets (*tef 1-α* + *RPB2*), and (*ITS* + *tef 1-α* + *RPB2*) were performed using ML, Maximum parsimony (MP) and Bayesian analyses (BI). The ML and MP analyses were conducted using PhyML v. 3.0 (on-line) and PAUP v. 40b10 as described by Zlatković et al. [8,28]. The BI analyses were carried out in MrBayes v. 3.0b4 as described in Kovač et al. [27]. The DNA sequences generated in this study were deposited in the GenBank genetic sequence database (Table S1 in Supplementary Materials).

2.3. In Vitro Assessment of Antifungal Activity of Pinus Sylvestris Bark Extracts

The antifungal activity of *P. sylvestris* bark extracts towards *B. dothidea*, *D. sarmentorum* and *N. parvum* was assessed, mycelial growth inhibition was calculated and the degree of toxicity was estimated as described in Karličić et al. [22]. The experiment was repeated three times.

2.4. In Vitro Assay of Trichoderma spp. Antifungal Activity

The antifungal activity of *Trichoderma* strains towards *B. dothidea*, *D. sarmentorum* and *N. parvum* was assessed, growth inhibition calculated, and the degree of antagonistic activity estimated as described by Karličić et al. [22]. The experiment was repeated three times. At the same time, microscopic observations of the fungal interactions were performed. Small amounts of mycelia from the plates with individual fungi (negative controls) and from the interaction zones of plates with dual cultures were collected using a sterile hypodermic needle, mounted in distilled water on microscope slides and examined using an Olympus BX53F light microscope (Olympus Co., Tokyo, Japan) with Nomarski differential interference contrast (DIC) equipped with an Olympus SC50 digital camera and accompanying software.

2.5. Biochemical Characterization of Trichoderma spp. Antifungal Activity

The biochemical characterization of *Trichoderma* spp. antifungal activity included the determination of cell wall-degrading enzymes and the production of siderophores. A semiquantitative determination of cell-wall degrading enzymes (lipase, esterase-lipase, N-acetyl-β-glucosaminidase and β-glucosidase) was performed using an API ZYM kit according to the manufacturer's protocol (BioMereux, Craponne, France). The presence of cellulase was determined using carboxymethyl cellulose (CMC) agar method in three repetitions [30]. Siderophore production was detected on the Chrome azurol S (Sigma-Aldrich, St. Louis, USA) agar medium in three repetitions [31]. The chrome azurol S (CAS) agar plates were inoculated with 5-mm-diameter mycelia discs of three *Trichoderma* isolates and incubated at 28 °C for 72 h. The appearance of yellow-orange halo zones around colonies was considered as a positive result.

2.6. The Effect of Trichoderma VOCs on Mycelial Growth of Botryosphaeriaceae

The effect of VOCs produced by *Trichoderma* strains on the mycelial growth of Botryosphaeriaceae was tested using the method of confronted cultures without contacts of the two mycelia [32]. The two Petri dishes containing 20 mL of potato dextrose agar (PDA, Himedia, India) were individually inoculated with 5-mm-diameter mycelia discs of a pathogen (Botryosphaeriaceae) and an antagonist (*Trichoderma* spp.). Inoculated plates were sealed with Parafilm®, arranged to face each other and incubated at 25 °C in a microbiological incubator (Binder, Tuttlingen, Germany) in the dark until fungi in the control plates (plates with individual fungi, negative control) reached edges of plates. The experiment was repeated three times. The effects of volatile metabolites were estimated as percentage of mycelial growth inhibition (MGI) calculated using the following equation:

$$\text{MGI (\%)} = ((\text{DC} - \text{DT})/\text{DC})) \times 100, \quad (1)$$

where MGI is mycelial growth inhibition, 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 [33].

The antagonistic levels were estimated as described in Ruiz-Gómez et al. [34] and classified as low (MGI 50%); medium (50% < MGI ≤ 60%); high (60% < MGI ≤ 75%); and very high (MGI > 75%).

2.7. Collection of VOCs and GC−MS Analysis

To determine VOCs emitted by *Trichoderma* strains penicillin bottles containing 5ml PDA were inoculated with *Trichoderma* spp. The VOCs were collected six days after inoculation using headspace solid-phase micro-extraction followed by gas chromatography (Agilent Technologies 7890 B GC System, AIM, Littleton, CO, USA) coupled with mass spectrometry (Agilent Technologies 5977A MSD, AIM, Littleton, CO, USA). Briefly, the 0.2 g of the sample (PDA + *Trichoderma* strain) was placed in a headspace vial, followed by the addition of 0.5 mL of sterilized distilled water. Bottles containing only sterile PDA served as negative controls. Each vial was sealed using a cap with PTFE/silicone septa and incubated at 70 °C. The solid phase microextraction fiber (Polydimethylsiloxane (PDMS) 100 µm, Agilent Technologies, AIM, Littleton, CO, USA) was inserted into the head space of the vial containing the sample solution. The extraction was carried out at 70 °C with 90 min of fiber-exposed time. After sampling, the SPME fiber was withdrawn into the needle, removed from the tube, and inserted into the hot injector port (270 °C) of the GC system where the extracted analyte was desorbed and transferred to the analytical column (HP-5, Agilent Technologies, AIM, Littleton, CO, USA). A relatively long desorption time in the injector (10 min) was selected to avoid carryover between runs to ensure full desorption of analyte from the fiber. Ultra-high purity 5.0 grade helium (Messer Tehnogas AD, Belgrade, Serbia) was used as a carrier gas at a flow rate at 1.2 mL/min along with the spitless injection. The oven temperature was programmed for an initial 50 °C for 2 min and was

then increased in two steps: 50–80 °C at a rate of 20 °C/min and held for 6 min at this temperature; 80–280 °C at a rate of 15 °C/min and 240–280 °C and held for 6 min at this temperature. During the analysis, data acquisition was carried out in full scan mode (*m/z* 27–350) operated in the electron ionization mode at 70 eV with a source temperature of 230 °C. Volatile compounds were identified by comparison with the National Institute of Standards and Technology (NIST) database. The VOCs that showed mass spectra with match factor ≥80% were considered as identified substances.

2.8. Statistical Analyses

The data were subjected to ANOVA followed by Tukey's HSD post-hoc comparison tests to determine if there were statistically significant differences between the means ($p = 0.05$). All statistical analyses were performed using Statistica 12.0 (StatSoft, Tulsa, OK, USA).

3. Results

3.1. In Vitro Assessment of Antifungal Activity of Pinus Sylvestris Bark Extracts

The antifungal properties of pine bark extracts were tested at two concentrations, i.e., 20% and 30% (Table 1). The concentration of 20% inhibited growth of *B. dothidea* for 34%–39% while the concentration of 30% increased the level of inhibition to 39%–44%. The pine bark extracts inhibited *D. sarmentorum* mycelia for 48%–66%. The lowest inhibition (48%) was obtained using neutral water extracts prepared at 120 °C, while alkaline water extract prepared at 120 °C showed the highest inhibition rate (66%). Pine bark extracts showed no signs of inhibition of radial growth of *N. parvum* (Table 1). However, the aerial mycelium of this fungus was sparse compared to the control plate.

Table 1. Mycelial growth inhibition (%) by pine bark water extracts.

Pine Bark Water Extracts		Water pH7/80 °C	Water pH9/80 °C	Water pH7/120 °C	Water pH9/120 °C
B. dothidea CMW 39314	20%	39 ^{bA}	36 ^{abA}	35 ^{aA}	34 ^{aA}
	30%	44 ^{aB}	43 ^{aB}	39 ^{aB}	40 ^{aB}
D. sarmentorum CMW 39365	20%	56 ^{aA}	56 ^{aA}	48 ^{aA}	60 ^{aA}
	30%	60 ^{abA}	62 ^{abB}	53 ^{aA}	66 ^{bA}
N. parvum BOT 275	20%	NI	NI	NI	NI
	30%	NI	NI	NI	NI

NI—no inhibition; mean values in the same row with different lowercase letters are significantly different according to the Tukey test ($p = 0.05$); mean values in the same column and same pathogen with different uppercase letters are significantly different according to the Tukey test ($p = 0.05$).

3.2. Identification of Trichoderma spp.

Morphology of the three isolates from the *P. sylvestris* bark was characterized by scarce mycelium producing typical diffusing yellow pigment which was particularly pronounced in isolate *T. citrinoviride* DEMf TR4. Conidiophores were sparsely branched, representing a long strongly developed central axis from which lageniform and mostly solitary phialides arise, bearing smooth-walled subglobose to ellipsoidal conidia. These morphological features corresponded to common features of the *Longibrachiatum* section of the genus *Trichoderma* [35]. Considering the difficulties differentiating *Trichoderma* species belonging to this section, the additional molecular analyses were performed. *Trichoderma* spp. were identified using phylogenetic analyses of the *ITS rDNA*, *TEF 1-α*, and *RPB2* genes. The concatenated datasets with the two loci (*tef 1-α* + *RPB2*) and three loci (*ITS* + *tef 1-α* + *RPB2*) had 1532 characters of which 577 characters were parsimony informative and 2164 characters of which 623 characters were parsimony informative, respectively. The PHT test showed that the loci can be combined ($p = 0.01$ for both concatenated datasets). The MP analyses of the concatenated dataset of the two loci produced a tree with CI = 0.6, RI = 0.7, TL = 1850, whereas the analyses of the three loci resulted in two equally most

parsimonious trees with CI = 0.6, RI = 0.7, TL = 1948. The ML, BI and MP analyses of each concatenated dataset yielded trees with the similar topology (Figures 1, S1 and S2).

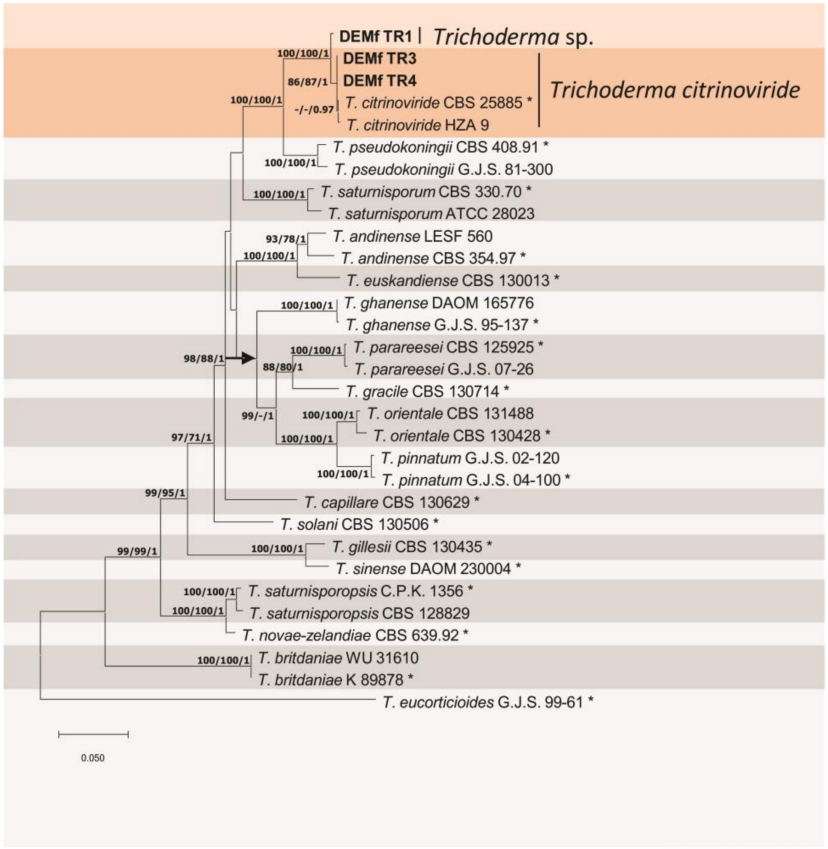

Figure 1. Phylogenetic tree generated from maximum likelihood analysis (ML) based on concatenated alignments of ITS, tef 1-α and RPB2 sequence data showing the position of *Trichoderma citrinoviride* in relation to its closely related species belonging to the *Longibranchiatum* clade. ML and maximum parsimony (MP) bootstrap support values greater than 70% and Bayesian posterior probability values (PP) greater than 0.95 are indicated at the tree nodes (ML/MP/PP). The type strains are marked with an asterisk and isolates sequenced in this study are shown in bold. *T. eucorticioides* G.J.S. 99-61 (clade Hypocreanum) is included as an outgroup. Scale bar indicates expected number of substitutions per site.

In the phylogenetic analyses of both single and combined loci isolates sequenced in this study clustered within a clade strongly supported in the analyses of the *RPB2* gene and fully supported in the analyses of the *tef 1-α* and combined analyses of the two and three loci (Figures 1 and S2). Isolates DEMf TR3 and DEMf TR4 from this study clustered within a sub-clade corresponding to *T. citrinoviride* (Figures 1 and S2). These isolates had only two single nucleotide polymorphisms (SNPs) that differentiated them from the type strain of *T. citrinoviride* CBS 25885 (Table S2). The sub-clade corresponding to *T. citrinoviride* was well-supported in all three analyses (86/87% ML, MP bootstrap support; posterior probability: 1).

Isolate DEMf TR1 clustered within a sub-clade corresponding to *T. citrinoviride* only in the individual analyses of the *ITS* and *tef 1-α* (Figure S1). In analyses of the *RPB2*, and combined analyses of *tef 1-α/RPB2* and *ITS/tef 1-α/RPB2* genes, the isolate clustered within a sister clade to *T. citrinoviride* (Figures 1 and S2). There were 14 bp differences that differentiated this isolate from the type strain of its phylogenetically closest relative *T. citrinoviride* (Table S2). Based on phylogenetic analyses, isolates from this study were identified as *T. citrinoviride* and *Trichoderma* sp.

3.3. In Vitro Assay of *Trichoderma* spp. Antifungal Activity

The *Trichoderma* isolates caused inhibition of Botryosphaeriaceae in confrontation test (Table 2). The highest percentage of *B. dothidea* growth inhibition (85%) was achieved by *T. citrinoviride* DEMf TR4, followed by *Trichoderma* sp. DEMf TR1. This level of antagonistic activity is characterized as very strong. Moreover, *T. citrinoviride* DEMf TR4 and *Trichoderma* sp. DEMf TR1 showed very high antagonistic activity towards D. sarmentorum. *T. citrinoviride* DEMf TR4 was the only *Trichoderma* isolate to show high antagonistic activity against *N. parvum*.

Table 2. Mycelial growth inhibition (%) of *B. dothidea*, *D. sarmentorum* and *N. parvum* by *Trichoderma* spp. isolated from *P. sylvestris* bark.

Pathogen	*Trichoderma* sp. DEMf TR 1	*T. citrinoviride* DEMf TR3	*T. citrinoviride* DEMf TR4
B. dothidea CMW 39314	76 ^{ab}	67 ^{a}	85 ^{b}
D. sarmentorum CMW 39365	75 ^{b}	63 ^{a}	75 ^{b}
N. parvum BOT 275	59 ^{b}	55 ^{a}	62 ^{c}

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

The confrontation test revealed different competition strategies. Interaction of *Trichoderma* strains with *B. dothidea* and *D. sarmentorum* resulted in overgrowth with replacement [36]. Interaction of *Trichoderma* strains with *N. parvum* (Figure 2) was labeled as a deadlock at distance [36] manifested through the presence of an inhibition zone. Moreover, the co-inoculation of these fungi resulted in enhanced production of dark pigmentation in *N. parvum*.

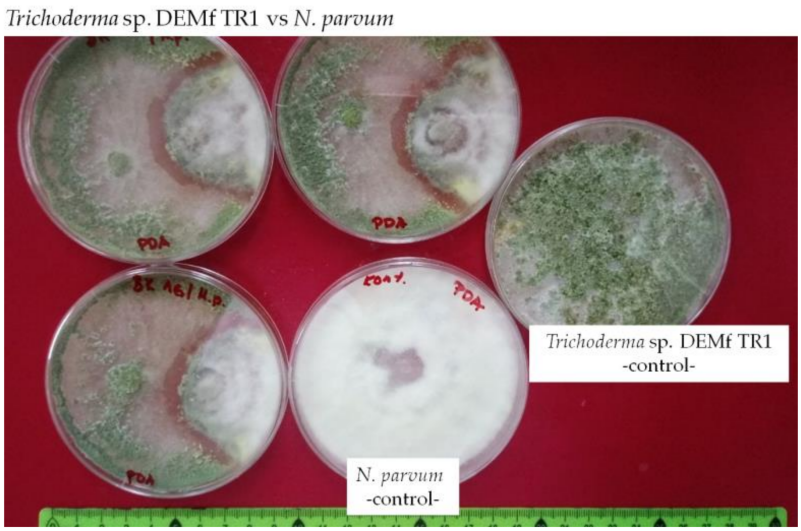

Figure 2. Plate confrontation assay of *Trichoderma* sp. DEMf TR 1 and *Neofusicoccum parvum*.

Microscopic observations of the *Trichoderma*-pathogen meeting zone (Figure 3) revealed different manifestations of BCA action and suggested mycoparasitism as a mode of action. Briefly, the hyphae of *Trichoderma* sp. DEMf TR1 grew alongside and coiled compactly around the *B. dothidea* hypha. The hook-like structures of *Trichoderma* spp. were formed around *B. dothidea* and *D. sarmentorum* hyphae. Moreover, several other morphological alternations were observed in *B. dothidea*, *D. sarmentorum* and *N. parvum* hyphae, such as vesicle-like structures, vacuolation and cytoplasmatic coagulation as a response to *Trichoderma* spp. presence. In addition, the micrograph of the meeting zone showed that both *Trichoderma* spp. and Botryosphaeriaceae isolates, reacted to mutual recognition by

intensive production of chalmydospores whereas chlamydospores were rarely formed in the control Petri dishes.

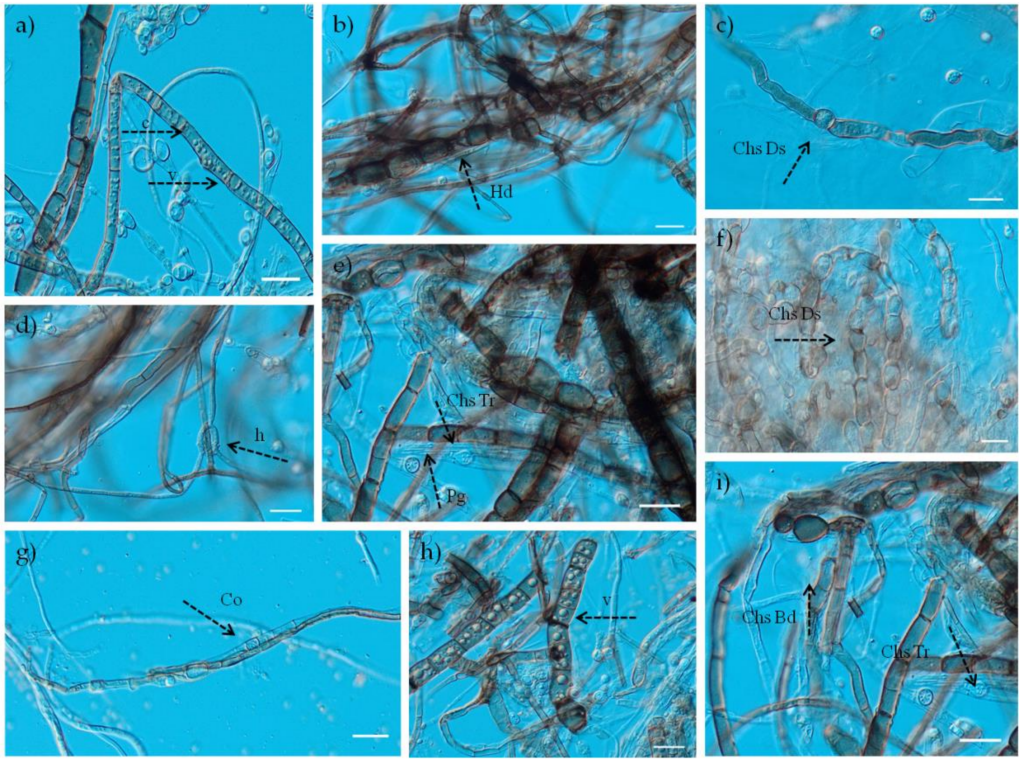

Figure 3. Observations of mycelial interactions between *Trichoderma* spp. and *B. dothidea* and *D. sarmentorum*; (**a**): c—cytoplasmatic coagulation, v—vacuolation; (**b**): Hd—hyphal desintegration; (**c**): Chs Ds—chlamydospores of *D. sarmentorum*; (**d**): h—hook-like structures; (**e**): ChsTr—chlamydospores of *T. citrinoviride* DEMf TR4, Pg—parallel growth; (**f**): Chs Ds—chlamydospores of *D. sarmentorum*; (**g**): Co—coiling; (**h**): v—vacuolation; (**i**): Chs Bd—chlamydospores of *B. dothidea*, ChsTr-chlamydospores of *T. citrinoviride* DEMf TR4; scale bar 20 µm.

3.4. Biochemical Characterization of Trichoderma spp. Antifungal Activity

Semiquantitative analyses of *Trichoderma* spp. enzymatic profiles showed the ability of these fungi to produce lipase and esterase-lipase at a moderate level as well as high amounts of N-acetyl-β-glucosaminidase. *T. citrinoviride* DEMf TR3 produced high amounts of β-glucosidase; *Trichoderma* sp. DEMf TR1 was marked as a moderate producer, while *T. citrinoviride* DEMf TR4 produced low amounts of the enzyme (Table 3). The three *Trichoderma* isolates were capable of producing cellulase. The CAS assay confirmed the ability of *Trichoderma* spp. to produce siderophores.

Table 3. Biochemical characteristics of *Trichoderma* spp. used in this study.

Strains	Cell-Wall Degrading Enzymes					Sid
	Lipase	Est-Lip	Na-β	β-Glu	Cell	
Trichoderma sp. DEMf TR 1	2	2	3	2	+	+
T. citrinoviride DEMf TR3	2	2	3	3	+	+
T. citrinoviride DEMf TR4	2	2	3	1	+	+

(est-lip)—esterase-lipase, (Na-β)—N-acetyl-β-glucosaminidase, (β-glu)—β-glucosidase, (cell)—cellulase, (sid)—siderophores; 1—low production, 2—moderate production, and 3—high production according to the API ZYM reading color scale; + positive reaction.

3.5. Effect of Trichoderma VOCs on Mycelial Growth of Botryosphaeriaceae

The VOCs emitted by *Trichoderma* sp. DEMf TR 1, *T. citrinoviride* DEMf TR3 and *T. citrinoviride* DEMf TR4 inhibited mycelial growth of *D. sarmentorum* for 35%, 40%, and 41%, respectively. Moreover, the VOCs induced loss of mycelia pigmentation in *B. dothidea* (Figure S3). The *N. parvum* growth was not inhibited. The GC-MS analysis of *Trichoderma* isolates used in this study confirmed equal profiles of the volatile compounds emitted by *Trichoderma* sp. DEMf TR1 and *T. citrinoviride* DEMf TR4. *T. citinoviride* DEMf TR3 produced six compounds and showed different spectra of VOCs. The exception was trichoacorenol which was produced by all three *Trichoderma* isolates (Figure 4).

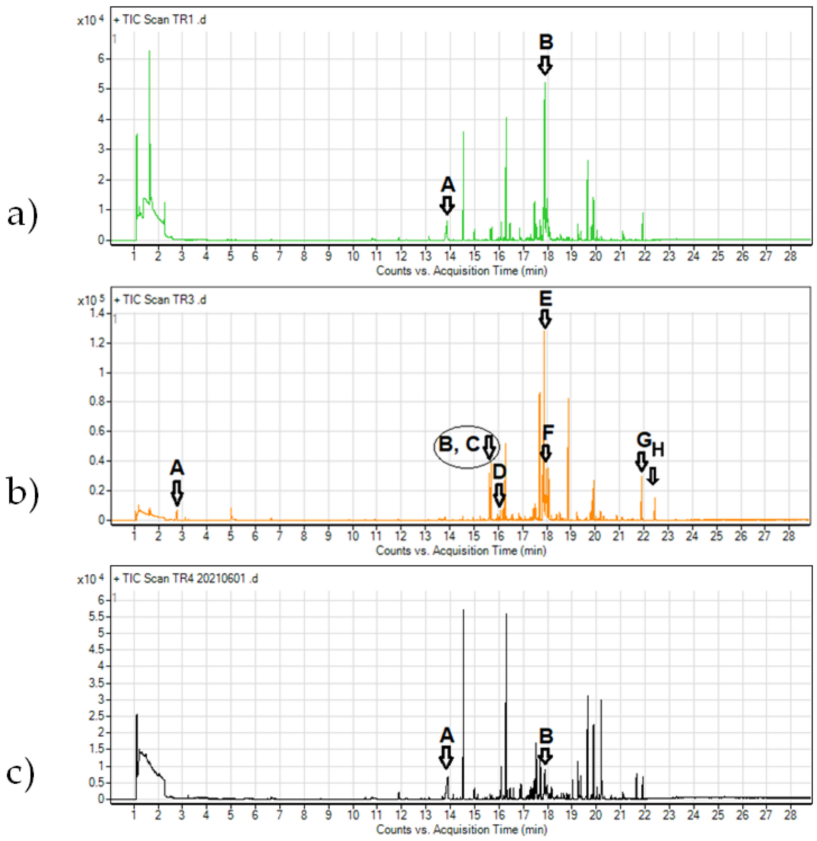

Figure 4. HS-GC-MS profiles of VOCs emitted by *Trichoderma* isolates: (**a**) *Trichoderma* sp. DEMf TR1: A: Nonanoic acid, B: Trichoacorenol; (**b**) *T. citrinoviride* DEMf TR3: A: Acetic acid, B: Cubenene, C: Cubenene, D: cis-α-bergamotene, E: Trichoacorenol, F: Trichoacorenol, G: Verticillol, H: Hexanedioic acid, bis(2-ethylhexyl) ester; (**c**) *T. citrinoviride* DEMf TR4: A: Nonanoic acid, B: Trichoacorenol.

Two compounds were detected in *Trichoderma* sp. DEMf TR1 and *T. citrinoviride* DEMf TR4 VOCs spectrum, i.e., trichoacorenol and nonanoic acid. An acetic acid, cubenene, cis-α-bergamotene, trichoacorenol, verticillol, hexanedioic acid, and bis(2-ethylhexyl) ester were detected in *T. citrinoviride* DEMf TR3 spectrum (Table 4). The amount of trichoacorenol in *Trichoderma* sp. DEMf TR1 and *T. citrinoviride* DEMf TR3 was similar and higher compared to the amount of this compund detected in *T. citrinoviride* DEMf TR4. Nonanoic acid emitted by *Trichoderma* sp. DEMf TR1 and *T. citrinoviride* DEMf TR4 were absent in *T. citrinoviride* DEMf TR3 VOCs profile, but another fatty acid, namely hexanedioic acid, was detected in VOCs spectra of this isolate. Cubenene (syn. naphthalene) was the second most abundant compund in *T. citrinoviride* DEMf TR3 VOCs blend with 8.25 relative abundance.

Table 4. VOCs emitted by *Trichoderma* sp. DEMf TR1, *T. citrinoviride* DEMf TR3 and *T. citrinoviride* DEMf TR4 identified by HS-GC-MS.

Isolate	Retention Time(min)	Peak	Volatile Compound	Relative Abundance
Trichoderma sp. DEMf TR 1	13.8756	A	Nonanoic acid	4.84
	17.8761	B	Trichoacorenol	17.68
T. citrinoviride DEMf TR3	2.7580	A	Acetic acid	1.19
	15.6375	B	Cubenene	3.75
	15.7034	C	Cubenene	4.54
	16.2008	D	cis-α-bergamotene	0.93
	17.8873	E	Trichoacorenol	18.88
	17.9652	F	Trichoacorenol	1.54
	21.9053	G	Verticillol	3.43
	22.4483	H	Hexanedioic acid	1.64
T. citrinoviride DEMf TR4	13.9	A	Nonanoic acid	5.38
	17.8773	B	Trichoacorenol	3.78

4. Discussion

The present study showed biocontrol potential of *P. sylvestris* bark estimated through chemical (water extracts) and biological (*T. citrinoviride* and *Trichoderma* sp.) components against three Botryospaheriaceae species, i.e., *B. dothidea*, *D. sarmentorum* and *N. parvum*. The *Trichoderma* isolates showed the ability to use multiple biocontrol mechanisms and to produce various antifungal substances from cell-wall degrading enzymes, siderophores to VOCs. The VOCs spectra analyses revealed presence of many volatile compounds among which nonanoic acid, cubenene, cis-α-bergamotene, hexanedioic acid, and verticillol were previously unknown as *T. citrinoviride* metabolites.

In this study, *P. sylvestris* bark extracts showed antifungal effects against *B. dothidea*, *D. sarmentorum* and *N. parvum*. The effects on *B. dothidea* mycelium growth were characterized as little toxic following the classification of Mori et al. [37]. Moreover, the bark extracts showed moderately toxic effect on *D. sarmentorum* while extracts were non-toxic towards *N. parvum*. These results are in accordance with the research of Alfredsen et al. [38] who reported slightly toxic effects of Scots pine extracts toward *Heterobasidion annosum* (Fr.) Bref., *Nectria ditissima* Tul. and C.Tul., *Ceratocystis polonica* Siem. (C. Moreau) and moderate level of its toxicity towards *Phacidium coniferarum* (Hahn) DiCosmo, Nag Raj and W.B. Kendr. Similarly, Vek et al. [39] used 5% pine bark extracts and reported moderate level of its toxicity towards several fungi, including *Schizophyllum commune* Fr., *Gloeophyllum trabeum* (Pers.) Murrill, and *Fibroporia vaillantii* (DC.) Parmasto. Several studies indicate the high toxicity of the extract to phytopathogens. In a study by Minova et al. [6] pine and spruce bark extracts (2%) showed high effectiveness in growth inhibition of several strawberry pathogens, including *B. cinerea*, *C. acutatum*, and *P. cactorum*.

Lomeli-Ramírez et al. [40] reported toxic effects of extracts from *P. strobus, P. douglasiana, P. caribea and P. leiophylla* toward *T. versicolor*. Similarly, Maritime pine extracts exhibited a significant antifungal potency against *C. puteana* and *T. versicolor* which mycelia growth was reduced by 89% and 87%, respectively [20]. In the study, even though water extracts were prepared on different temperature, duration and pH regimes, in most cases, the method of preparation did not influence the antifungal effects on *B. dothidea*, *D. sarmentorum* and *N. parvum.* However, chemical composition of the extracts was not determined and future work should also include further optimization of the extraction conditions. Nevertheless, this is the first study to show that *P. sylvestris* bark extracts could serve as biocontrol agents against Botryosphaeriaceae. Further studies should include *in planta* screening of the bicontrol potential of the extracts. The representatives of *Trichoderma* gender are well-known as antifungal agents with a long history of usage as BCA against a wide range of pathogens [21]. Several products based on *Trichoderma* are already used as protectants against grapevine trunk diseases caused by botryosphaeriaceous fungi [21].

The assay of *Trichoderma* spp. antagonistic capabilities toward three representatives of Botryospheriaceae showed that all three isolates obtained from the pine bark are effective in reducing mycelial growth. The antagonistic activity of *Trichoderma* spp. was marked as high to very high in the case of *B. dothidea* and *D. sarmentorum* and the isolates inhibited the mycelial growth of the pathogens by up to 85% and 75%, respectively. The *Trichoderma* isolates expressed moderate to high antagonistic activity against *N. parvum* by inhibiting mycelial growth by up to 62%. These results are consistent with several in vitro experiments conducted with botryosphaeriaceous fungi [21,41]. Úrbez-Torres [21] reported high antagonistic activity of *T. atroviride* who inhibited the growth of *Diplodia seriata* De Not. by 70%, and *T. koningiopsis* who inhibited the growth of *N. parvum* by 74%. Mondello et al. [41] reviewed *T. atroviride* and *T. harzianum* as highly efficient against *D. seriata*, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl, *Neofusicoccum australe* Slippers, Crous and M.J. Wingf., *N. parvum* and *T. longibrachiatum* as highly efficient against *D. seriata*. Marraschi et al. [42] reported *T. asperelloides*, *T. asperellum*, and *T. koningiopsis* as antagonists of *L. theobromae*. Moreover, *T. citrinoviride* expressed high antagonistic activity toward *Rhizoctonia solani* J.G. Kühn, *B. cinerea*, *Alternaria panax* Whetzel, *Cylindrocarpon destructans* Gerlach and L. Nilsson, *P. cactorum* and *Pythium* spp. by inhibiting mycelial growth up to 90% [43]. Similarly, Kuzmanovska et al. [44] confirmed strong antagonistic properties of *T. asperellum* and *T. harzianum* against *B. cinerea* whose growth was inhibited 77%–97% and 71%–94%, respectively.

In vitro confrontations of three *Trichoderma* isolates and representatives of Botryosphaeriaceae revealed two antagonistic strategies, i.e., overgrowth and production of antifungal compounds. This is in accordance with the work of Kotze et al. [45] who reported formation of inhibition zones between *T. atroviride* and *D. seriata*, *L. theobromae*, and *N. australe*., and *T. atroviride* overgrowth of *N. parvum*. According to Pellan et al. [46] overgrowth of the pathogen suggests stabile interaction of BCA with pathogen metabolism which indicates mycoparasitism as the main mechanism. Moreover, microscopical observations (coiling, parallel growth, hook-like structures) suggested mycoparasitic nature of *Trichoderma* isolates. Similarly, Kotze et al. [45] reported coiling and hyphal adhesion during interactions of *T. atroviride* with *N. parvum*. Moreover, in a study by Park et al. [43] *T. citrinoviride* showed ability to stick closely and coil around hyphae of *B. cinerea* and *R. solani*. Similar microscopic manifestations of direct mycoparasitism were observed during *T. asperellum* and *T. harzianum* confrontation with *B. cinerea* [44]. Microscopic observations conducted in this study revealed changes in the cytoplasm (coagulation, vacuolation) which are of great importance since they lead to hyphae disintegration and death [47]. According to Krause et al. [36] those changes indicate that antagonistic action is a result of biocontrol mechanisms such as production of antibiotic compounds or competition for nutrients.

In this study, enzymatic profiles of *Trichoderma* spp. confirmed the ability of these fungi to produce cell-wall degrading enzymes such as esterase-lipase, N-acetyl-β-glucosaminidase, β-glucosidase, and cellulase. The common constituents of fungal cell-walls are chitin and glucan susceptible to the presence of the extracellular enzymes such as lipases, chitinase, N-acetyl-β-glucosaminidase, β-glucosidase and protease [48,49]. *Trichoderma* isolates expressed high production of N-acetyl-β-glucosaminidase which is already recognized by Lorito et al. [50] as a metabolite of *T. harzianum* and an effective inhibitor of *B. cinerea* spore germination. Similarly, Park et al. [43] reported a low level of β-glucosidase activity of *T. citrinoviride* which is consistent with enzymatic activity of *Trichoderma* isolates in this study. The three isolates of *Trichoderma* spp. produced cellulase and this coincides with previous studies which described *T. citrinoviride* as a producer of strong cellulases [43]. Moreover, Nidhina et al. [48] showed that this enzyme inhibited mycelia growth of *Phytophthora* spp. Furthermore, *Trichoderma* isolates from this study expressed another significant biocontrol mechanism, i.e., production of siderophores. This is in accordance with the study of Chen et al. [51] who reported siderophore production by *T. citrinoviride* and *T. atroviride*.

The results of in vitro confrontations from this study conducted to estimate VOCs revealed inhibitory effect of volatile metabolites on growth of *D. sarmentorum.* Similarly, in the study of Chen et al. [52] *T. koningiopsis* VOCs showed inhibitory effects on Epicoccum nigrum Link growth. *B. dothidea* and *N. parvum* growth was not inhibited and this is in accordance with the study of Stracquadanio et al. [17] who reported the absence of inhibition of *Neofusicoccum batangarum* Begoude, Jol. Roux and Slippers and *N. parvum* by *T. asperellum* and *T. atroviride* VOCs.

The volatile compounds detected in this study ae already recognized as part of *Trichoderma* spp. VOCs spectra [1,15]. However this study represents the first report of *T. citrinoviride* as producer of nonanoic acid, cubenene, cis-α-bergamotene, hexanedioic acid, and verticillol. *Trichoderma* sp. DEMf TR1 and *T. citrinoviride* DEMf TR4 produced nonanoic acid. Similarly, in a study by Aneja et al. [53] nonanoic acid was detected as part of *T. harzianum* VOCs spectra and showed inhibitory effects on fungal growth and spore germination of *Crinipellis perniciosa* Stahel and *Moniliophthora roreri* Cif. H.C. Evans. However, in this study we focused only on the effect of VOCs on the mycelial growth of Botryosphaeriaceae, and the effect of nonanoic acid on spore germination of these fungi will remain as a task for a future study. Moreover, verticillol was detected in *T. citrinoviride* DEMf TR3 VOCs spectra. Similarly, in a study of Zhang et al. [19] verticillol was one of the main components of *T. harzianum* volatile metabolites that caused growth inhibition of *Fusarium oxysporum* Schlecht. Emend. Snyder and Hansen. *T. citrinoviride* DEMf TR3 produced cubenene and hexanedioic acid which are an antioxidant and antibacterial compounds [54,55]. Considering that trichoacorenol was produced by all three *Trichoderma* isolates, and that similar levels of *D. sarmentorum* inhibition were observed when co-inoculated with each of the three *Trichoderma* spp., we hypothesize that trichoacorenol could represent the antifungal component of VOCs. Trichoacorenol together with cis-α-bergamotene belongs to the group of volatile sesquiterpenes with biological potential in suppression of microbial growth [15].

5. Conclusions

The present study showed biocontrol potential of *P. sylvestris* bark through its chemical (water extracts) and biological (*Trichoderma* spp.) components against three Botryosphaeriaceae species, i.e., *B. dothidea*, *D. sarmentorum* and *N. parvum*.

Trichoderma spp. were capable to activate multiple antifungal mechanisms, from mycoparasitism, production of cell-wall degrading enzymes, competition for nutrients (siderophores) to antifungal volatile metabolites. The study showed the ability of *T. citrinoviride* to produce nonanoic acid, cubenene, cis-α-bergamotene, hexanedioic acid and verticillol, which represent volatile compounds with antimicrobial activity. Moreover, it represents the first report of inhibitory effect of *Trichoderma* spp. on mycelial growth of *D. sarmentorum*.

Biological control is an important component of an integrated pathogen management, and this in vitro study is the first and promising step towards the introduction of *P. sylvestris* bark extracts and *Trichoderma* spp. in biological control programs for landscape pathogens such as Botryosphaeriaceae. Future work should examine the in planta activity of these potential biocontrol agents.

Supplementary Materials: The following are available https://www.mdpi.com/article/10.3390/f12121731/s1, Figure S1: Phylogenetic trees generated from Maximum likelihood (ML) analyses based on a single gene alignment of RPB2, tef 1-α and ITS sequence data, Figure S2: Phylogenetic tree generated from a maximum likelihood analysis (ML) based on a concatenated alignment of tef 1-α and RPB2 sequence data showing the position of *Trichoderma citrinoviride* in relation to its closely related species belonging to the *Longibranchiatum* clade, Figure S3: Effects of VOCs of *Trichoderma citrinoviride* DEMf TR4 on mycelial growth of *D. sarmentorum* (a) and *B. dothidea* (b), Table S1: Sequences used in the phylogenetic analyses, Table S2. Nucleotide differences between *Trichoderma* sp. and its closest phylogenetic relative *T. citrinoviride*.

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