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Towards the Biological Control of Devastating Forest Pathogens from the Genus Armillaria

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Abstract: Research Highlights: A large scale effort to screen, characterize, and select Trichoderma strains with the potential to antagonize Armillaria species revealed promising candidates for field applications. Background and Objectives: Armillaria species are among the economically most relevant soilborne tree pathogens causing devastating root diseases worldwide. Biocontrol agents are environment-friendly alternatives to chemicals in restraining the spread of Armillaria in forest soils. Trichoderma species may efficiently employ diverse antagonistic mechanisms against fungal plant pathogens. The aim of this paper is to isolate indigenous Trichoderma strains from healthy and Armillaria-damaged forests, characterize them, screen their biocontrol properties, and test selected strains under field conditions. Materials and Methods: Armillaria and Trichoderma isolates were collected from soil samples of a damaged Hungarian oak and healthy Austrian spruce forests and identified to the species level. In vitro antagonism experiments were performed to determine the potential of the Trichoderma isolates to control Armillaria species. Selected biocontrol candidates were screened for extracellular enzyme production and plant growth-promoting traits. A field experiment was carried out by applying two selected Trichoderma strains on two-year-old European Turkey oak seedlings planted in a forest area heavily overtaken by the rhizomorphs of numerous Armillaria colonies. Results: Although *A. cepistipes* and *A. ostoyae* were found in the Austrian spruce forests, *A. mellea* and *A. gallica* clones dominated the Hungarian oak stand. A total of 64 Trichoderma isolates belonging to 14 species were recovered. Several Trichoderma strains exhibited in vitro antagonistic abilities towards Armillaria species and produced siderophores and indole-3-acetic acid. Oak seedlings treated with *T. virens* and *T. atrobrunneum* displayed better survival under harsh soil conditions than the untreated controls. Conclusions: Selected native Trichoderma strains, associated with Armillaria rhizomorphs, which may also have plant growth promoting properties, are potential antagonists of Armillaria spp., and such abilities can be exploited in the biological control of Armillaria root rot.

Keywords: Armillaria; Trichoderma; root rot; biocontrol; antagonism; siderophore; indole-3-acetic acid

1. Introduction

Armillaria and Desarmillaria species (Physalacriaceae and Basidiomycota) are globally distributed fungal plant pathogens varying in host range and pathogenicity [1,2]. They cause white rot, a severe destructive disease (also known as Armillaria root rot) on a wide range of woody hosts growing in managed plantations, natural forests, orchards, and amenity plantings in urban areas, and their impact often leads to devastating forest damages and immense economic losses [3]. Armillaria colonies are spread in the soil by root-like rhizomorphs, which can attack host trees through root contacts, and then the penetrating hyphae colonize heartwood and invade the cambium as mycelial fans [4]. In general, Armillaria root disease results in reduced forest productivity due to direct mortality or permanent non-lethal infections affecting the health and growth of the trees [5].

It is well known that most Armillaria species exhibit specialization towards either coniferous or broadleaf hosts. Although native coniferous forests in the Northern hemisphere are predominantly inhabited by *A. ostoyae* and *A. cepistipes*, various oak and other broadleaf species are most exposed to *A. mellea*, *A. gallica*, and *Desarmillaria tabescens* [6–8].

The use of naturally occurring antagonistic fungi (e.g., certain Trichoderma species) and bacteria (e.g., Bacillus and Pseudomonas species) has uncovered great potential to successfully reduce the pathogenic activities of Armillaria. Particularly, native microorganisms isolated from soil, rhizosphere, or directly from plant roots usually have a better adaptation to that specific soil and plant environment, and thus can display more efficient control of diseases, than introduced exotic microorganisms [9]. Species of the soilborne genus Trichoderma have been widely used as biocontrol agents. Results of field trials showed that mycelia and conidia of five combined isolates of Trichoderma species significantly reduced the root colonization of *A. luteobubalina* and may have had additional inhibitory effects on fruiting body development as well [10]. Application of *T. harzianum* to the soil surrounding the wood-borne inoculum of Armillaria caused a significant reduction in the viability of the pathogen [11]. Armillaria failed to invade the stem sections colonized by *T. harzianum* and had low viability in the plant materials inoculated with Trichoderma [12]. The use of air-spading combined with *T. harzianum* inoculation also proved to be a potential joint cultural/biocontrol strategy against *A. mellea* in a forest [13].

The biocontrol abilities of Trichoderma strains are based on a wide arsenal of various antagonistic mechanisms [14]. Trichoderma species are excellent competitors for space and nutrients. Their extracellular enzyme systems, including cellulases (e.g., endocellulases, cellobiohydrolases, and β-glucosidases) and xylanases (e.g., endoxylanases and β-xylosidases), enable the efficient utilization of plant polysaccharides, while they can successfully compete for iron by the production of siderophores [14]. Antifungal secondary metabolites including pyrones, polyketides and non-ribosomal peptides play important roles in their antibiotic effects against fungal plant pathogens [14]. Direct mycoparasitism is closely associated with the production of extracellular cell wall-degrading enzymes (CWDE)—such as glucanases, chitinases, and proteases—playing an important role in the degradation of the cell wall and the penetration into the host hyphae [14,15]. Besides the above-mentioned direct mechanisms of antagonism, the ability of Trichoderma to promote plant growth via mechanisms including phosphorous mobilization, by extracellular phosphatases and the production of indole-3-acetic acid derivatives [14], and induce systemic resistance in the host plant can also be considered when screening for biocontrol agents. The antagonistic behavior of some Trichoderma strains may result from the interaction with plant roots, promotion of plant growth, and improving tolerance to abiotic stresses as well as plant resistance to diseases [13,16].

The presence of Armillaria root rot disease in the forests of the Northern hemisphere, and its economic consequences have consumed a lot of environmentally harmful and polluting fungicides. Woody plants, beyond their commercial values, provide essential components of wildlife habitats worldwide. However, Armillaria species often seem to dominate in the forests and may cause serious diseases leading to compromised seedlings. Commercial products based on Trichoderma used to protect plants have been available on the market. However, isolating and screening for antagonistic

Trichoderma strains from diverse populations distributed at different geographic regions may be more helpful for developing efficient biocontrol agents against a broad range of pathogens from the genus Armillaria. The aim of this study is to select and characterize Trichoderma strains with the potential to control Armillaria and examine their performance during application in the field.

2. Materials and Methods

2.1. Isolation of Armillaria and Trichoderma

Samples of bulk soil (soil outside the rhizosphere), upper rhizospheric soil, Armillaria rhizomorphs and their surrounding soil, as well as Armillaria fruiting bodies were collected from a heavily Armillaria-damaged oak stand (Keszthely Hills, Hungary) and healthy native spruce forests (Rosalia, Austria). The rhizomorph samples were taken as aliquots of the soil pools associated with the collected rhizomorphs. The Roth and Shaw medium [17] supplemented with 15 mg/L benomyl and 250 mg/L streptomycin was applied for Armillaria isolation from the field samples. For Trichoderma isolation, 1 g of fresh soil per sample was suspended in sterile 0.9% NaCl solution, diluted serially (the 10^{-1}, 10^{-2}, and 10^{-3} dilution) and spread on Trichoderma selective media. The composition of the media for selectively isolating Trichoderma strains was 10 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \times 7H_2O$, 20 g/L agar, amended with 0.25 mL/L 5% Rose-Bengal in water, 0.5 mL/L 0.2% dichloran in ethanol, 0.01% streptomycin, 0.01% oxytetracycline, and 0.01% chloramphenicol [18]. After 3 days of incubation at 25 ± 0.5 °C, fungal colonies including Trichoderma were detected and transferred onto potato dextrose agar (PDA).

2.2. Identification of Armillaria and Trichoderma Isolates

One-hundred mg of fresh mycelia from each fungal isolate was collected for DNA extraction following the manufacturer's instructions of the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, USA). The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal RNA gene cluster was amplified using the ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) universal primers for fungi [19]. The PCR reactions were carried out in a final volume of 25.1 µL, consisting of 2.5 µL 10× DreamTaq Buffer with 20 mM $MgCl_2$, 2.5 µL of 2 mM dNTP mix, 0.1 µL of 5 U/µL DreamTaq DNA Polymerase (Thermo Scientific), 0.5 µL of each primer (10 µM), 18 µL bidistilled water, and 1 µL template DNA. Amplifications were performed in a Doppio Thermal Cycler (VWR, Hungary). Thermal cycling parameters were as follows; initial denaturation at 94 °C for 5 min, 35 cycles of DNA denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, elongation at 72 °C for 50 s, and a final elongation at 72 °C for 7 min. For amplification of the translation elongation factor 1-alpha (*tef1α*) gene fragment, reaction mixtures were the same as described above, but with universal primers TEF-LLErev (5′-AACTTGCAGGCAATGTGG-3′) and EF1-728F: (5′-CATCGAGAAGTTCGAGAAGG-3′) [20] and the thermal cycling program, with an initial denaturation at 94 °C for 5 min, 40 cycles of DNA denaturation at 94 °C for 45 s, primer annealing at 57 °C for 30 s, elongation at 72 °C for 90 s, and a final elongation at 72 °C for 7 min. The amplicon quality was detected by 2% agarose gel electrophoresis of 4 µL samples from the reaction mixtures. Direct sequencing of the unpurified PCR products was performed by the sequencing platform of the Biological Research Centre, Szeged. The resulting sequences were analyzed by *TrichO*key 2.0 [21], *Tricho*BLAST [22] and NCBI Nucleotide BLAST. The isolated and identified Armillaria and Trichoderma strains were deposited in the Szeged Microbiology Collection (SZMC, www.szmc.hu), Szeged, Hungary, whereas the sequences were submitted to the GenBank Nucleotide database (ncbi.nlm.nih.gov) under the accession numbers listed in Table 1.

Table 1. Armillaria and Trichoderma isolates collected during the study.

Location	GPS-N	GPS-E	Collection Date	Sample	Isolate Identifier	Diagnosis	ITS (GenBank)	*tef1α* (GenBank)
Armillaria Isolates								
Rosalia, Austria	47° 41.649	16° 17.940	28.10.2016	fruiting body	SZMC 24125	*Armillaria cepistipes*	-	MN580140
	47° 41.640	16° 17.937	28.10.2016	fruiting body	SZMC 24126	*Armillaria cepistipes*	-	MN580151
	47° 41.628	16° 17.929	28.10.2016	rhizomorph	SZMC 24128	*Armillaria ostoyae*	-	MN580144
	47° 41.629	16° 17.964	28.10.2016	fruiting body	SZMC 24129	*Armillaria ostoyae*	-	MN580139
	47° 41.621	16° 17.948	28.10.2016	fruiting body	SZMC 24130	*Armillaria ostoyae*	-	MN580142
Keszthely, Hungary	46° 48.728	17° 16.992	20.07.2016	bulk soil	SZMC 24095	*Armillaria gallica*	-	MN580162
	46° 48.712	17° 16.994	20.07.2016	bulk soil	SZMC 24098	*Armillaria gallica*	-	MN580163
	46° 48.702	17° 16.987	29.10.2016	bulk soil	SZMC 24099	*Armillaria gallica*	-	MN580160
	46° 48.657	17° 16.954	29.10.2016	fruiting body	SZMC 24131	*Armillaria mellea*	MN585779	MN580137
	46° 48.671	17° 16.959	29.10.2016	fruiting body	SZMC 24132	*Armillaria mellea*	MN585780	MN580159
	46° 48.706	17° 16.949	29.10.2016	fruiting body	SZMC 24133	*Armillaria mellea*	MN585781	MN580138
	46° 48.723	17° 16.974	29.10.2016	fruiting body	SZMC 24134	*Armillaria mellea*	MN585777	MN580152
	46° 48.712	17° 16.978	29.10.2016	fruiting body	SZMC 24135	*Armillaria mellea*	-	MN580145
	46° 48.736	17° 16.992	29.10.2016	fruiting body	SZMC 24651	*Armillaria mellea*	-	MN580153
	46° 48.772	17° 16.992	29.10.2016	fruiting body	SZMC 24136	*Armillaria mellea*	MN585778	MN580155
	46° 48.760	17° 16.982	29.10.2016	fruiting body	SZMC 24137	*Armillaria mellea*	MN585776	MN580154
	46° 48.749	17° 16.936	29.10.2016	fruiting body	SZMC 24139	*Armillaria mellea*	MN585782	MN580158
	46° 48.720	17° 17.009	29.10.2016	fruiting body	SZMC 24140	*Armillaria mellea*	MN585783	MN580146
	46° 48.736	17° 16.992	03.11.2016	fruiting body	SZMC 24141	*Armillaria mellea*	-	MN580150
	46° 48.665	17° 16.993	03.11.2016	fruiting body	SZMC 24142	*Armillaria mellea*	-	MN580143
	46° 48.738	17° 16.956	03.11.2016	fruiting body	SZMC 24143	*Armillaria gallica*	-	MN580141
	46° 48.877	17° 17.143	03.11.2016	fruiting body	SZMC 24144	*Armillaria mellea*	-	MN580148
	46° 48.883	17° 17.153	03.11.2016	fruiting body	SZMC 24145	*Armillaria mellea*	-	MN580149
	46° 48.917	17° 17.062	03.11.2016	fruiting body	SZMC 24146	*Armillaria mellea*	-	MN580161
	46° 48.892	17° 16.941	03.11.2016	fruiting body	SZMC 24147	*Armillaria mellea*	-	MN580157
	46° 48.883	17° 16.937	03.11.2016	fruiting body	SZMC 24148	*Armillaria mellea*	-	MN580156
	46° 47.935	17° 16.958	03.11.2016	fruiting body	SZMC 24149	*Armillaria mellea*	-	MN580147

Table 1. *Cont.*

Location	GPS-N	GPS-E	Collection Date	Sample	Isolate Identifier	Diagnosis	ITS (GenBank)	*tef1α* (GenBank)
Trichoderma Isolates								
Rosalia, Austria	47° 41.649	16° 17.940	28.10.2016	bulk soil	SZMC 24270	*Trichoderma koningii*	MN516459	MN520036
					SZMC 24271	*Trichoderma koningii*	MN516460	MN520038
					SZMC 24272	*Trichoderma koningii*	MN516461	MN520042
					SZMC 24273	*Trichoderma koningii*	MN516462	MN520041
	47° 41.640	16° 17.937	28.10.2016	bulk soil	SZMC 24274	*Trichoderma atroviride*	MN516463	MN520048
					SZMC 24275	*Trichoderma atroviride*	MN516464	MN520049
					SZMC 24276	*Trichoderma atroviride*	MN516465	MN520050
	47° 41.618	16° 17.873	28.10.2016	bulk soil	SZMC 24277	*Trichoderma koningii*	MN516466	MN520033
					SZMC 24278	*Trichoderma koningii*	MN516467	MN520040
					SZMC 24279	*Trichoderma koningii*	MN516468	MN520039
					SZMC 24280	*Trichoderma asperellum*	MN516469	MN520031
	47° 41.629	16° 17.964	28.10.2016	bulk soil	SZMC 24285	*Trichoderma koningii*	MN516474	MN520034
					SZMC 24286	*Trichoderma koningii*	MN516475	MN520037
					SZMC 24287	*Trichoderma koningii*	MN516476	MN520035
					SZMC 24288	*Trichoderma asperellum*	MN516477	MN520032
	47° 41.621	16° 17.948	28.10.2016	bulk soil	SZMC 24289	*Trichoderma asperellum*	MN516478	MN520030
					SZMC 24290	*Trichoderma koningii*	MN516479	MN520043
	47° 40.896	16° 17.211	28.10.2016	bulk soil	SZMC 24291	*Trichoderma longipile*	MN516480	MN520056
	47° 41.628	16° 17.929	28.10.2016	rhizomorph	SZMC 24281	*Trichoderma guizhouense*	MN516470	MN520084
					SZMC 24282	*Trichoderma paraviridescens*	MN516471	MN520044
					SZMC 24283	*Trichoderma simmonsii*	MN516472	MN520079
					SZMC 24284	*Trichoderma simmonsii*	MN516473	MN520078
Keszthely, Hungary	46° 48.657	17° 16.954	29.10.2016	bulk soil	SZMC 24429	*Trichoderma simmonsii*	-	MN520069
					SZMC 24430	*Trichoderma simmonsii*	-	MN520071
					SZMC 24431	*Trichoderma simmonsii*	-	MN520077
					SZMC 26770	*Trichoderma simmonsii*	-	MN520072
					SZMC 24433	*Trichoderma simmonsii*	-	MN520081
	46° 48.671	17° 16.959	29.10.2016	bulk soil	SZMC 24434	*Trichoderma tomentosum*	-	MN520066

Table 1. *Cont.*

Location	GPS-N	GPS-E	Collection Date	Sample	Isolate Identifier	Diagnosis	ITS (GenBank)	*tef1α* (GenBank)
	46° 48.706	17° 16.949	29.10.2016	bulk soil	SZMC 24435	*Trichoderma simmonsii*	-	MN520075
					SZMC 26771	*Trichoderma simmonsii*	-	MN520080
					SZMC 24436	*Trichoderma simmonsii*	-	MN520068
					SZMC 24403	*Trichoderma simmonsii*	-	MN520076
					SZMC 24404	*Trichoderma simmonsii*	-	MN520082
	46° 48.723	17° 16.974	29.10.2016	bulk soil	SZMC 26772	*Trichoderma atrobrunneum*	-	MN520090
					SZMC 24405	*Trichoderma atrobrunneum*	-	MN520091
	46° 48.712	17° 16.978	29.10.2016	bulk soil	SZMC 26773	*Trichoderma simmonsii*	-	MN520083
					SZMC 26774	*Trichoderma virens*	-	MN520058
					SZMC 24408	*Trichoderma simmonsii*	-	MN520067
	46° 48.736	17° 16.992	29.10.2016	bulk soil	SZMC 24409	*Trichoderma hamatum*	-	MN520028
					SZMC 24410	*Trichoderma hamatum*	-	MN520029
	46° 48.772	17° 16.992	29.10.2016	rhizosphere	SZMC 26778	*Trichoderma atroviride*	MN516444	MN520052
					SZMC 26779	*Trichoderma atroviride*	MN516445	MN520051
					SZMC 26780	*Trichoderma atroviride*	MN516446	MN520053
	46° 48.769	17° 16.961	29.10.2016	bulk soil	SZMC 24411	*Trichoderma paratroviride*	-	MN520045
					SZMC 26775	*Trichoderma citrinoviride*	-	MN520054
					SZMC 26776	*Trichoderma citrinoviride*	-	MN520055
					SZMC 26777	*Trichoderma simmonsii*	-	MN520070
					SZMC 24412	*Trichoderma simmonsii*	-	MN520074
	46° 48.749	17° 16.936	29.10.2016	rhizosphere	SZMC 24413	*Trichoderma atroviride*		MN520047
					SZMC 24414	*Trichoderma atroviride*		MN520046
	46° 48.738	17° 16.956	20.07.2016	rhizomorph	SZMC 24292	*Trichoderma virens*	MN516447	MN520059
					SZMC 24293	*Trichoderma virens*	MN516448	MN520061
					SZMC 24294	*Trichoderma virens*	MN516449	MN520062
					SZMC 24295	*Trichoderma virens*	MN516450	MN520060
					SZMC 24296	*Trichoderma virens*	MN516451	MN520064

Table 1. *Cont.*

Location	GPS-N	GPS-E	Collection Date	Sample	Isolate Identifier	Diagnosis	ITS (GenBank)	*tef1α* (GenBank)
	46° 48.758	17° 16.959	20.07.2016	rhizomorph	SZMC 24297	*Trichoderma atrobrunneum*	MN516452	MN520087
					SZMC 24298	*Trichoderma simmonsii*	MN516453	MN520073
					SZMC 24299	*Trichoderma atrobrunneum*	MN516454	MN520086
					SZMC 24300	*Trichoderma crassum*	MN516455	MN520057
					SZMC 24301	*Trichoderma atrobrunneum*	MN516456	MN520088
					SZMC 24302	*Trichoderma atrobrunneum*	MN516457	MN520089
					SZMC 24303	*Trichoderma virens*	MN516458	MN520063
	46° 48.722	17° 16.993	20.07.2016	rhizomorph	SZMC 24205	*Trichoderma virens*	-	MN520065
					SZMC 24206	*Trichoderma atrobrunneum*	MN516443	MN520085

2.3. Antagonistic Activity Assessment In Vitro by Dual Culture Assay

Trichoderma isolates were screened for their antagonistic abilities against Armillaria isolates in vitro using dual-culture confrontation test. During the experiments, 25 Armillaria isolates were confronted with 62 Trichoderma isolates on PDA plates. Armillaria strains were inoculated with agar plugs (5 mm in diameter, cut from the edge of 14-day-old colonies) 1.5 cm from the center of PDA plates. After 14 days, the Trichoderma isolates were inoculated in a similar way, 1.5 cm from the center of PDA plates in the opposite direction, resulting in a distance of 3 cm between the two inoculation positions. After a further 5 days of incubation, image analysis of plate photographs was performed by ImageJ. Biocontrol Index (BCI) values were calculated with Microsoft Excel 2010 according to the formula BCI = (area of Trichoderma colony/total area occupied by the colonies of both Trichoderma and the plant pathogenic fungus) × 100 [23]. All confrontation tests were repeated three times under the same experimental conditions. Values were recorded as the means with standard deviations for triplicate experiments.

2.4. Extracellular Enzyme Activity Measurements

Conidiospores (2×10^5/plate) of Trichoderma strains were transferred into Petri plates (9 cm in diameter), each containing 3 g spelt bran and 10 mL distilled water. After 9 days of incubation at room temperature, the enzyme extraction was carried out in 25 mL distilled water at 5 °C for 3 h, followed by filtering through gauze, to remove fungal hyphae and spelt bran, and centrifugation of the crude extract in a Heraeus Multifuge 3SR (Thermo Fisher Scientific, Hungary) at 4300 *g* for 10 min. One mg/mL stock solutions were prepared from chromogenic substrates in distilled water. β-Glucosidase, cellobiohydrolase, β-xylosidase, and phosphatase enzyme activities were measured with p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-cellobioside, p-nitrophenyl phosphate, p-nitrophenyl-β-D-xylopyranoside, and disodium salt hexahydrate (all from Sigma-Aldrich, Hungary), respectively. One-hundred microliters of substrate solution, 25 µL 10-fold diluted culture supernatant, and 75 µL distilled water were mixed in the wells of a microtiter plate. After 1 h of incubation at room temperature, 50 µL 10% Na_2CO_3 was added to stop the reaction. The optical densities were measured with a Spectrostar Nano microplate reader (BMG Biotech) at 405 nm. Background values of the crude extract and the value resulting from the self-degradation of the substrate were subtracted from the optical density of the enzymatic reactions. The U/mL values were calculated according to the formula $((A/\varepsilon \times l) \times 10^6)/60$, where "A" is the absorbance of the solution at 405 nm, "ε" is the molar extinction coefficient (for p-nitrophenol: 1.75×10^4 M^{-1} cm^{-1}) and "l" is the pathlength of the light in the solution. All measurements were carried out in 3 biological replicates.

2.5. Quantitative Analysis of Indole-3-Acetic Acid Production

The indole-3-acetic acid (IAA) production of Trichoderma isolates was analyzed by colorimetric analysis using Salkowsky's reagent [24] with some modification. The isolates were inoculated into 20 mL tryptone soy broth (TSB) (15 g/L tryptone, 5 g/L peptone from soy, 5 g/L NaCl, 1 mg/mL tryptophan) and incubated for 7 days at 25 °C with shaking at 150 rpm. After the incubation period, 2 mL of each culture was centrifuged in a Heraeus Fresco 17 Microcentrifuge (Thermo Fisher Scientific, Hungary) at 8000 rpm for 15 min. The supernatant was preserved and 100 µL was mixed with 200 µL of Salkowski's reagent (300 mL H_2SO_4 (98%), 15 mL $FeCl_3$ (0.5 M), and 500 mL distilled water) and incubated at room temperature in the dark for 1 h. The optical density (OD) was measured at 530 nm with a Spectrostar Nano microplate reader (BMG Labtech, Germany) after 30 min. The IAA concentration was determined using a calibration curve of standard IAA solutions. All measurements were carried out in 3 biological replicates.

2.6. Siderophore Production

Siderophore production of Trichoderma isolates was determined by using a modified chrome azurol S (CAS) agar test [25]. One half was CAS blue agar and the other half was an iron-free medium in 9 cm diameter Petri plates. The CAS agar was prepared according to Schwyn and Neilands [26]. The iron-free medium was MEA agar medium (10 g/L glucose, 12.5 g/L yeast extract, 5 g/L malt extract, and 20 g/L agar). A fungal mycelial disc (4 mm) of active culture was transferred to the plates with iron-free medium. Orange and purple halos around the colonies on the blue medium were indicative of siderophore production. All measurements were carried out in 3 biological replicates.

2.7. Field Study in the Keszthely Hills

A field study was set up on the 13 April 2017 in the Keszthely Hills, in a forest clearing surrounded by a 2-meter-high fence, located in the central part of a heavily Armillaria-damaged Turkey oak (*Quercus cerris*) stand. A total of 235 two-year-old, bare rooted seedlings of *Q. cerris* from the nursery of the Bakonyerdő Ltd. forestry company, with a stem length of 10–52 cm, a main root length of minimum 25 cm, and a stem base diameter of minimum 6 mm, were planted. Before planting, 10 L plastic buckets were used to soak the roots of 115 seedlings for at least 2 h in tap water (control group), whereas the roots of the other 120 seedlings were soaked in tap water containing conidia of *T. virens* SZMC 24205 and *T. atrobrunneum* SZMC 24206, both at a concentration of 10^6 conidia per mL (treated group) for at least 2 h. The seedlings were planted in groups of 40 into parcels of 6.4 × 6.3 m resulting in a density of 1 seedling per m^2. The allocation of the parcels was random in a block design of 3 × 4 parcels, with 3 parcels treated, 3 parcels untreated (control), and 6 parcels left empty, to cover a larger area for balancing the eventual differences in soil quality and distribution of potential Armillaria inoculum in the soil. Seedlings were planted into 20 cm deep holes made with 10 cm wide drain spades. Seedling stem height (in mm) and stem base diameter (in mm with one decimal precision) were recorded individually for each tree with measuring tape and slide calipers, respectively. From the recorded values a biomass index (BMI) was calculated for each seedling according to the following formula: $(BD/2)^2 \times \pi \times L$, where BD is the stem base diameter and L is the stem length. The area received no further treatment. Half a year later, on the 17 October 2017, the seedlings were evaluated for survival, their L and BD values recorded again, and the BMI values calculated. A seedling was recorded as "dead" if it was degraded or showed a dry brown appearance without any leaves, and it was not possible to excoriate the surface around the stem base with the orifice of a 1 mL plastic pipette tip. Stem height extensions (dL), stem base diameter extensions (dBD), and BMI changes (dBMI) were calculated. Seedlings with green leaves and an increase in biomass production were taken as "growing" live plants. All other seedlings without a significant biomass extension but with stems still green under the bark and slightly damp to the touch were considered "surviving" ones. In the end, after the second round of the measurements, size values lower than the ones measured directly after planting were considered as the result of measurement error and were removed from the total pools. The percentage of dead and surviving seedlings was calculated for each parcel, and their total numbers were compared between the control and treated groups by testing "independence" with the aid of the χ^2 test with Yates's correction.

3. Results

3.1. Diversity of the Genera Armillaria and Trichoderma in Healthy and Armillaria-Damaged Forests

Armillaria and Trichoderma strains were isolated from different locations of healthy and Armillaria-infested forests. The sampling sites were two different regions, one in Northwest Hungary (Keszthely-hills) and one in Northeast Austria (Rosalia Mountains) (Table 1). Four Armillaria species could be identified by the sequence analysis of a fragment of the *tef1α* gene: the conifer-specific species *A. cepistipes* (2 isolates) and *A. ostoyae* (3 isolates) were abundant in the neighboring Rosalia spruce forest stands, whereas the presence of *A. mellea* (18 isolates) and *A. gallica* (4 isolates) was revealed in the Keszthely oak stand (Table 1).

A total of 64 strains showing typical morphology of Trichoderma were also isolated from soil, rhizosphere or Armillaria rhizomorph-associated samples collected in the two examined forest areas (Table 1). Forty-two and 22 isolates were collected from the oak stand near Keszthely (Hungary) and the spruce forest at Rosalia (Austria), respectively. As the ITS sequences did not enable an exact, species-level identification in the case of many Trichoderma isolates, the species identification was set up based on the sequence of a tef1α gene fragment. The isolates proved to represent 14 Trichoderma species: *T. simmonsii* (17), *T. koningii* (11), *T. virens* (8), *T. atroviride* (8), *T. atrobrunneum* (7), *T. asperellum* (3), *T. hamatum* (2), *T. citrinoviride* (2), *T. tomentosum* (1), *T. paratroviride* (1), *T. crassum* (1), *T. guizhouense* (1), *T. paraviridescens* (1), and *T. longipile* (1) (Table 1). The diversity of Trichoderma species showed a difference between the two forests. Only two species—*T. atroviride* and *T. simmonsii*—were isolated from both locations. The species *T. virens*, *T. atrobrunneum*, *T. citrinoviride*, *T. hamatum*, *T. tomentosum*, *T. paratroviride*, and *T. crassum* were only isolated from the oak stand near Keszthely (Hungary), whereas *T. koningii*, *T. asperellum*, *T. guizhouense*, *T. paraviridesens*, and *T. longipile* were only found in the spruce forest at Rosalia (Austria) (Table 1). Eleven samples revealed isolates from a single species. A frequent species pair detected in Rosalia samples was *T. koningii–T. asperellum*, whereas in the Keszthely samples, the co-occurrence of *T. simmonsii–T. virens* and *T. virens–T. atrobrunneum*. Communities consisting of more than 2 species in the same sample were *T. guizhouense–T. paraviridescens–T. simmonsii* (Rosalia), *T. paratroviride–T. citrinovirie–T. simmonsii* (Keszthely), and *T. atrobrunneum–T. simmonsii–T. crissum–T. virens* (Keszthely).

3.2. In Vitro Antagonism of the Isolated Trichoderma Strains Towards Armillaria Species

All 8 isolates of *T. virens*, and some isolates of *T. simmonsii*, *T. atrobrunneum*, *T. guizhouense*, T. *atroviride*, *T. citrinoviride*, *T. paratroviride*, *T. hamatum*, and *T. tomentosum*, proved to be highly effective against the 25 examined Armillaria isolates. Figure 1 shows a representative set of plate photographs taken during the in vitro antagonism experiments and reflecting all species combinations of Trichoderma and Armillaria. In many cases, antagonistic Trichoderma isolates were able to overgrow Armillaria colonies and intensely produce conidia on their surface, thereby potentially restricting Armillaria growth. Isolates of *T. virens*, such as SZMC 24205, SZMC 24294, SZMC 24303, SZMC 24293, and SZMC 26774, proved to be the best in vitro antagonists with BCI values above 80 for more than 17 out of 25 Armillaria strains (Table 2). Isolates of *T. simmonsii* showed high in vitro antagonistic abilities with BCI values above 80 for more than 15 out of the 25 tested Armillaria isolates, whereas the *T. koningii*, *T. asperellum*, *T. paraviridescens*, and *T. longipile* isolates had the lowest BCI values against almost all of the tested Armillaria isolates. The distribution of antagonistic Trichoderma species with higher BCI values showed a geographical pattern. Except for the two species—*T. simmonsii* and *T. atroviride*, isolated from both the oak stand in Keszthely—and the spruce forest in Rosalia, having relatively high antagonistic activities, species that were only isolated from the Keszthely Hills, including *T. virens*, *T. atrobrunneum*, *T. hamatum*, *T. citrinoviride*, *T. paratroviride*, and *T. tomentosum*, exhibited good in vitro antagonistic abilities against most of the tested Armillaria isolates. All isolates of *T. koningii*, *T. asperellum*, *T. paraviridesens*, and *T. longipile*, which seem to dominate in the soil of the Rosalia forest, showed lower BCI values against most Armillaria isolates.

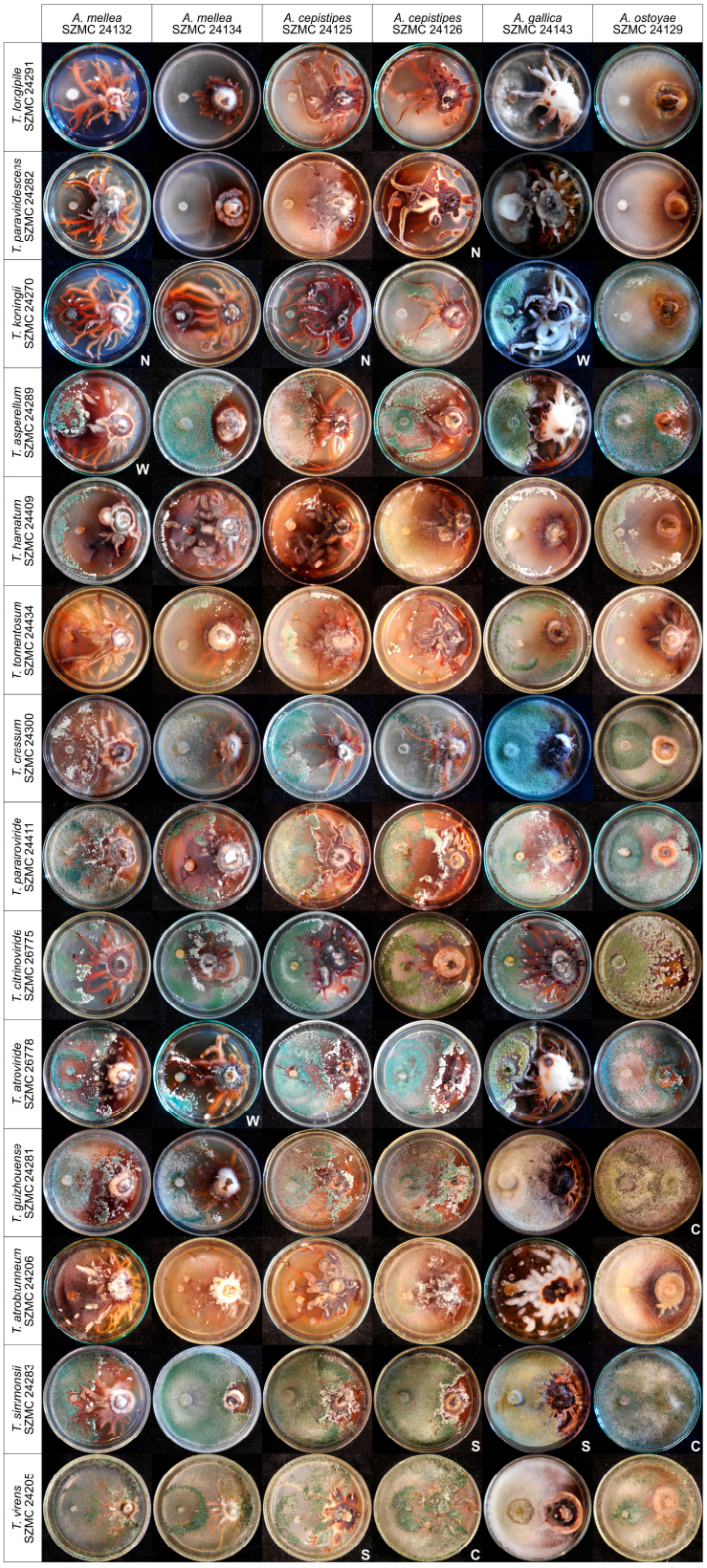

Figure 1. In vitro antagonism of Trichoderma strains from different species against *Armillaria mellea*, *A. cepistipes*, *A. gallica*, and *A. ostoyae*. Example plates are marked with N: no inhibition; W: weak inhibition; S: strong inhibition; C: complete overgrowth of Armillaria by Trichoderma.

Table 2. In vitro antagonistic abilities of Trichoderma isolates towards Armillaria isolates.

Species	Isolate	Number of Tested Armillaria Isolates	BCI Values ≥ 80	Percentage of BCI ≥ 80 (%)
T. tomentosum	SZMC 24434	25	15	60.00
T. paratroviride	SZMC 24411	25	19	76.00
T. crassum	SZMC 24300	25	13	52.00
T. hamatum	SZMC 24409	25	9	36.00
T. hamatum	SZMC 24410	25	16	64.00
T. citrinoviride	SZMC 26775	25	12	48.00
T. citrinoviride	SZMC 26776	25	13	52.00
T. virens	SZMC 26774	25	24	96.00
T. virens	SZMC 24205	25	17	68.00
T. virens	SZMC 24292	25	22	88.00
T. virens	SZMC 24293	25	22	88.00
T. virens	SZMC 24294	25	23	92.00
T. virens	SZMC 24295	25	22	88.00
T. virens	SZMC 24296	25	22	88.00
T. virens	SZMC 24303	25	24	96.00
T. atrobrunneum	SZMC 26772	25	19	76.00
T. atrobrunneum	SZMC 24405	25	15	60.00
T. atrobrunneum	SZMC 24206	25	6	24.00
T. atrobrunneum	SZMC 24297	25	10	40.00
T. atrobrunneum	SZMC 24299	25	6	24.00
T. atrobrunneum	SZMC 24301	25	11	44.00
T. atrobrunneum	SZMC 24302	25	10	40.00
T. simmonsii	SZMC 24430	25	15	60.00
T. simmonsii	SZMC 24431	25	17	68.00
T. simmonsii	SZMC 26770	25	21	84.00
T. simmonsii	SZMC 24433	25	16	64.00
T. simmonsii	SZMC 24435	25	19	76.00
T. simmonsii	SZMC 24436	25	18	72.00
T. simmonsii	SZMC 24403	25	17	68.00
T. simmonsii	SZMC 24404	25	18	72.00
T. simmonsii	SZMC 26773	25	19	76.00
T. simmonsii	SZMC 24408	25	10	40.00
T. simmonsii	SZMC 26777	25	17	68.00
T. simmonsii	SZMC 24412	25	16	64.00
T. simmonsii	SZMC 24298	25	13	52.00
T. simmonsii	SZMC 24283	25	18	72.00
T. simmonsii	SZMC 24284	25	16	64.00
T. atroviride	SZMC 26778	25	12	48.00
T. atroviride	SZMC 26779	25	8	32.00
T. atroviride	SZMC 26780	25	10	40.00
T. atroviride	SZMC 24413	25	16	64.00
T. atroviride	SZMC 24414	25	14	56.00
T. atroviride	SZMC 24274	25	14	56.00
T. atroviride	SZMC 24275	25	12	48.00
T. atroviride	SZMC 24276	25	10	40.00
T. koningii	SZMC 24270	25	9	36.00
T. koningii	SZMC 24271	25	9	36.00
T. koningii	SZMC 24272	25	9	36.00
T. koningii	SZMC 24273	25	7	28.00
T. koningii	SZMC 24277	25	9	36.00
T. koningii	SZMC 24278	25	7	28.00
T. koningii	SZMC 24279	25	10	40.00
T. koningii	SZMC 24285	25	7	28.00
T. koningii	SZMC 24286	25	8	32.00
T. koningii	SZMC 24287	25	7	28.00
T. koningii	SZMC 24290	25	10	40.00
T. asperellum	SZMC 24280	25	9	36.00
T. asperellum	SZMC 24288	25	7	28.00
T. asperellum	SZMC 24289	25	8	32.00
T. guizhouense	SZMC 24281	25	16	64.00
T. paraviridescens	SZMC 24282	25	6	24.00
T. longipile	SZMC 24291	25	6	24.00

3.3. Extracellular Enzyme Production of the Trichoderma Isolates

The extracellular enzyme measurements revealed that isolates of the same Trichoderma species have similar enzyme activity values (Figure 2). Altogether, most of the isolates could be characterized by high β-glucosidase (Figure 2a) and phosphatase (Figure 2d), but lower cellobiohydrolase (Figure 2b) and β-xylosidase (Figure 2c) activities. The 11 *T. koningii* isolates, along with *T. asperellum* SZMC

24288, SZMC 24280, and *T. paraviridescens* SZMC 24282, showed good β-glucosidase and β-xylosidase activities. Among them, *T. paraviridescens* SZMC 24282 had good phosphatase, whereas *T. koningii* SZMC 24286 and SZMC 24270 good cellobiohydrolase activities as well. The examined *T. virens*, *T. atrobrunneum*, *T. simmonsii*, and *T. atroviride* isolates showed lower activity levels for all enzymes tested, except for *T. atroviride* SZMC 26780, which had a very high β-xylosidase activity.

Figure 2. *Cont*.

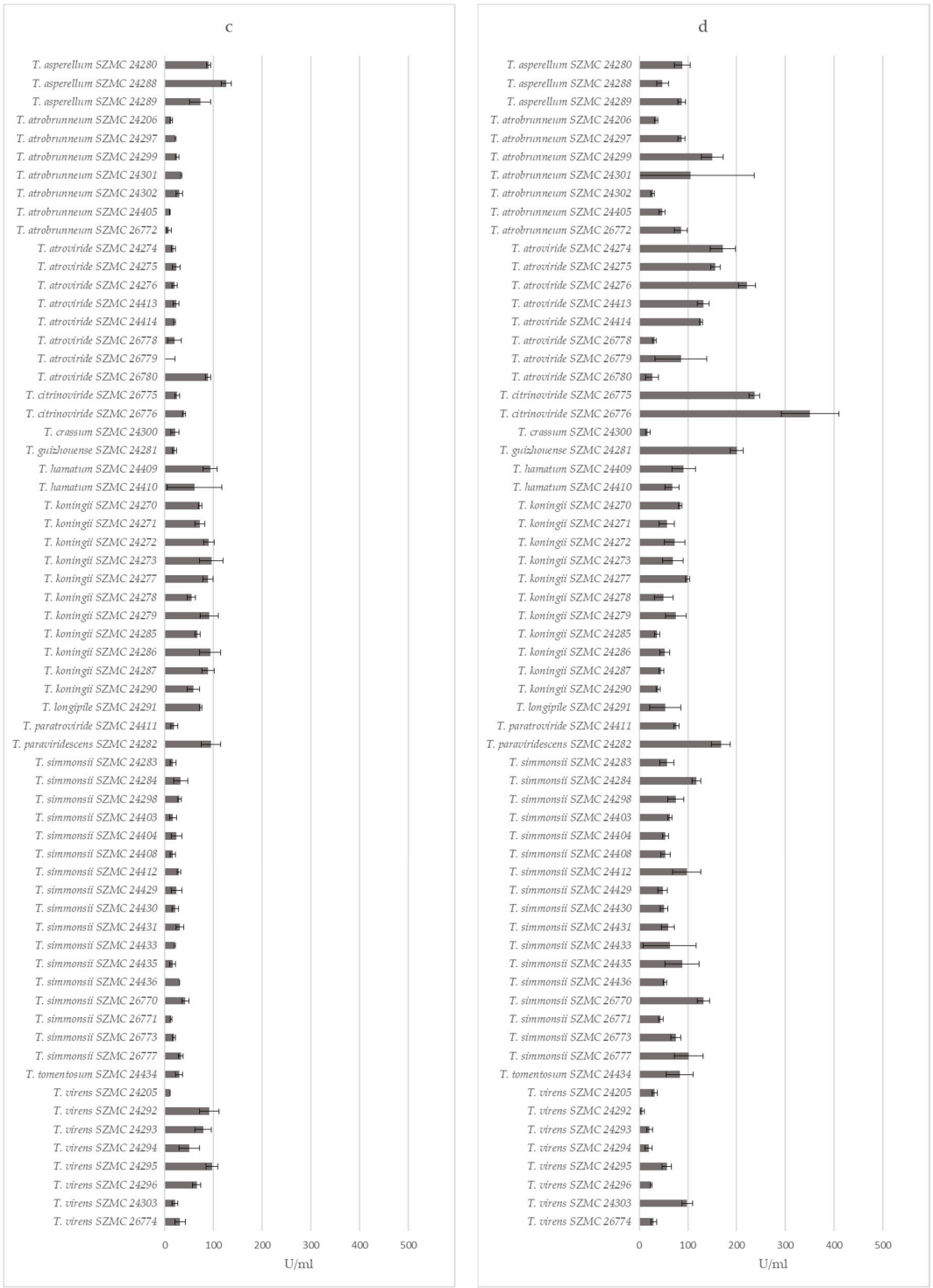

Figure 2. Extracellular enzyme activities of Trichoderma isolates derived from forest soil samples: (**a**) β-glucosidase, (**b**) cellobiohydrolase, (**c**) β-xylosidase, and (**d**) phosphatase.

3.4. Potential Plant Growth-Promoting Traits of the Isolated Trichoderma Strains

All of the examined isolates from *T. atrobrunneum*, *T. simmonsii*, *T. hamatum*, and *T. citrinoviride*, along with the single isolates of *T. tomentosum*, *T. longipile*, *T. paratroviride*, and *T. guizhouense*, proved to be IAA producers, whereas the *T. atroviride* isolates, as well as the examined single isolates of *T. paraviridescens* and *T. crissum*, were unable to produce this metabolite (Figure 3). Both producers and non-producers were found among the examined isolates of *T. virens*, *T. koningii*, and *T. asperellum.* The

highest amounts of IAA were detected in the case of the isolates of *T. tomentosum*, *T. citrinoviride*, *T. hamatum*, as well as certain isolates of *T. atrobrunneum*, *T. simmonsii*, and *T. koningii*.

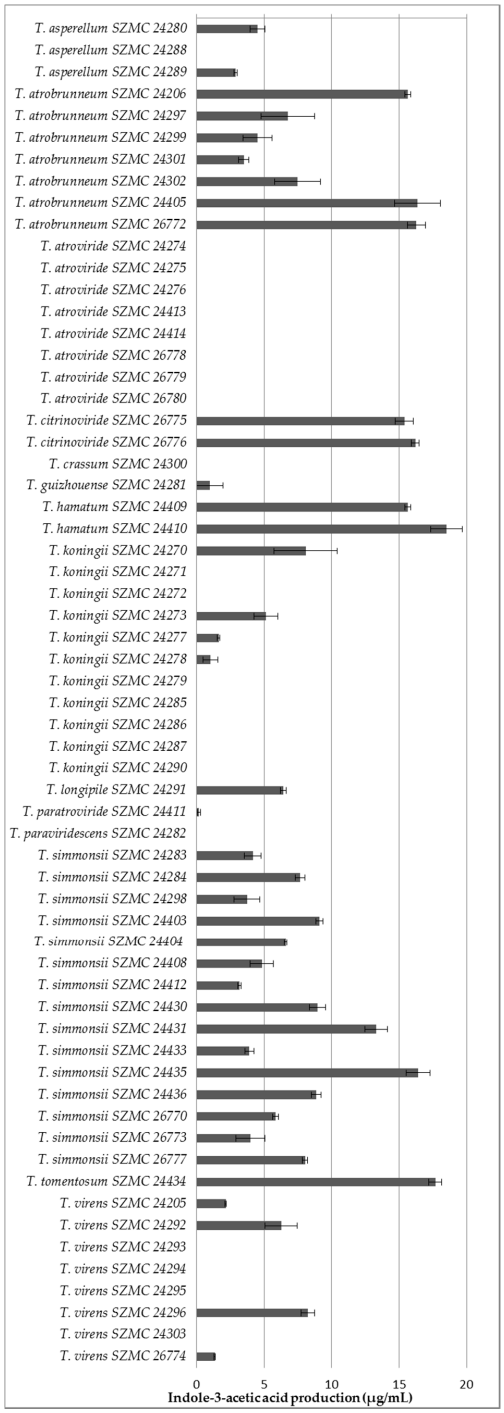

Figure 3. Indole-3-acetic acid production of Trichoderma isolates derived from forest soil samples.

All the *Trichoderma* isolates tested were able to produce siderophores, which was indicated by the change of the color of blue medium to orange or purple (Figure 4). The different colors of the medium suggested that the produced siderophores were structurally different. There are two major groups of siderophores, known as catechol-type and hydroxamate-type [25]. In the case of catechol-type siderophores the medium turns to purple, which was detected in the case of the *T. atroviride*, *T. paraviridescens*, and *T. koningii* isolates, whereas the hydroxamate-type siderophores result

in an orange color, as it was the case for all other examined species (Figure 4). The isolates of the species *T. asperellum* seemed to produce both types of siderophores (see plate 8 on Figure 4).

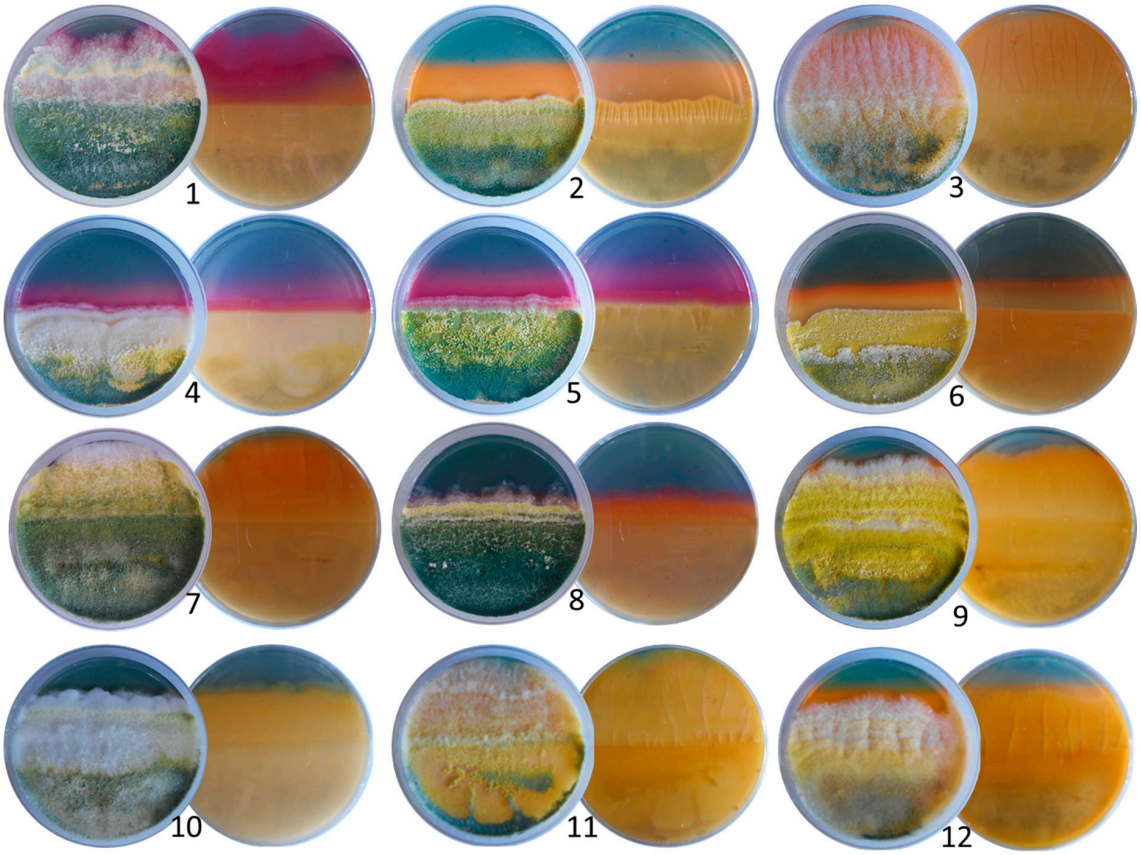

Figure 4. Production of siderophores on modified CAS agar medium by forest-derived Trichoderma isolates belonging to different species. (**1**) *T. atroviride* SZMC 24275, (**2**) *T. virens* SZMC 24205, (**3**) *T. hamatum* SZMC 24410, (**4**) *T. paraviridescens* SZMC 24282, (**5**) *T. koningii* SZMC 24287, (**6**) *T. citrinoviride* SZMC 26776, (**7**) *T. simmonsii* SZMC 24431, (**8**) *T. asperellum* SZMC 24280, (**9**) *T. atrobrunneum* SZMC 24206, (**10**) *T. guizhouense* SZMC 24281, (**11**) *T. tomentosum* SZMC 24434, and (**12**) *T. crassum* SZMC 24300.

3.5. Field Experiment in a Heavily Armillaria-Damaged Forest in the Keszthely Hills

Two Trichoderma isolates—*T. virens* SZMC 24205 and *T. atrobrunneum* SZMC 24206—were selected for a field experiment. Both strains were isolated from a Keszthely soil sample associated with decaying Armillaria rhizomorphs, which have not revealed any Armillaria growth upon isolation attempts; furthermore, both exerted very good in vitro antagonistic abilities towards the tested Armillaria isolates and were able to produce hydroxamate-type siderophores. The isolates were applied to Turkey oak seedlings as a root treatment before planting in the form of a conidial suspension (10^6 conidia per mL for both). The total survival rates calculated after 6 months for 120 treated and 115 control trees were 84.3% and 54.7%, respectively (Table 3), indicating that the applied treatment had a beneficial effect on the survival of oak seedlings planted into the soil of an Armillaria-infested forest area.

Table 3. Survival of Trichoderma-treated and control trees 6 months after planting into heavily Armillaria-infested soil.

Parcel	Total No.	[1] Dead No.	[2] Growing No.	[3] Survivor No.	[4] FM No.	[5] Corrected Total No.	Dead %	Growing %	Growing + Survivor %
1 (treated)	40	5	24	3	8	32	15.6	75.0	84.4
2 (treated)	40	5	27	5	3	37	13.5	73.0	86.5
3 (treated)	40	7	28	4	1	39	17.9	71.8	82.1
Total	**120**	**17**	**79**	**12**	**12**	**108**	**15.7**	**73.1**	**84.3**
4 (untreated)	40	23	5	2	10	30	76.7	16.7	23.3
5 (untreated)	39	13	19	2	5	34	38.2	55.9	61.8
6 (untreated)	36	7	24	0	5	31	22.6	77.4	77.4
Total	**115**	**43**	**48**	**4**	**20**	**95**	**45.3**	**50.5**	**54.7**

[1] already degraded/disappeared and dry seedlings; [2] positive biomass production; [3] still alive but no biomass production; [4] failed measurement (size values after 6 month lower than the ones measured directly after planting); [5] Total—FM.

4. Discussion

Armillaria fruiting bodies, rhizomorphs, and soil samples were collected at previously established study sites from both spruce and oak stands; all of them with abundant rhizomorph and mushroom production. The conifer sampling sites selected in Rosalia represented a native environment for Norway spruce with single clones of *A. ostoyae* and *A. cepistipes* colonies appearing only around relatively freshly cut trunks. All identified genets appeared non-damaging and tolerable by the surrounding live trees. In contrast, the Turkey oak stand from Keszthely, Hungary was a heavily infested area with multiple *A. mellea* and *A. gallica* clones merged to form a continuous coverage of the whole stand. All remaining standing trees were showing symptoms of Armillaria infections. The same bulk, rhizospheric and rhizomorph-associated soil samples were also subjected to Trichoderma isolation. The reported diversity of Trichoderma had expanded to ~75 species in temperate Europe [27,28]. All the Trichoderma species collected in this study from Keszthely and Rosalia (*T. tomentosum*, *T. paratroviride*, *T. crassum*, *T. hamatum*, *T. citrinoviride*, *T. atrobrunneum*, *T. virens*, *T. simmonsii*, *T. atroviride*, *T. koningii*, *T. asperellum*, *T. guizhouense*, *T. paraviridescens*, and *T. longipile*) had already been reported from Southern Europe [29]. Among them, the species *T. citrinoviride*, *T. atroviride*, *T. koningii*, *T. paraviridescens*, and *T. longipile* were also identified from Central Europe [30]. The *T. harzianum* species complex (also known as *T. harzianum sensu lato*) from the Harzianum clade of the genus Trichoderma was supposed to comprise at least 14 species [31], including the more recently described, biocontrol-relevant species of *T. atrobrunneum*, *T. guizhouense*, and *T. simmonsii* that were also found at both locations of our current investigation.

The application of biocontrol agents as alternatives to chemical fungicides reduces the impacts and risks on human health as well as on the environment [32]. Trichoderma species as effective biocontrol agents against diverse genera of pathogenic fungi can be used for plant disease management, especially in the case of soilborne diseases. Strains of the *T. koningii*, *T. asperellum*, *T. atroviride*, *T. hamatum*, *T. virens*, and *T. harzianum* species complex and other Trichoderma taxa have been officially registered and commercialized as crop protection products and microbial fungicides throughout the world including the European countries [33].

Antagonistic activity assessment in vitro by dual culture assay has demonstrated in this study that, besides *T. virens*, *T. atroviride*, and *T. hamatum* and the members of the *T. harzianum* species complex (*T. simmonsii*, *T. atrobrunneum*, and *T. guizhouense*), strains of *T. citrinoviride*, *T. paratroviride*, and *T. tomentosum* also proved to be effective in vitro antagonists of Armillaria species with the potential to be used as biocontrol agents against Armillaria root rot. On the other hand, certain species and strains of Trichoderma showed weak antagonistic abilities against Armillaria strains reflected by low BCI values. For example, all the tested isolates of *T. koningii* and *T. asperellum* had lower BCI values than the isolates of *T. virens*, *T. simmonsii*, *T. atrobrunneum*, *T. atroviride*, and *T. hamatum*. Previously, *T. koningii* and *T. asperellum* showed excellent antagonistic activities during the application against other plant pathogens. For instance, *T. koningii* showed the highest growth inhibition of *Rhizoctonia solani*

causing root rot in cotton, followed by *T. viride*, *T. harzianum*, and *T. virens* [34]. Similarly, *T. asperellum* showed effective antagonistic activity against the white-rot fungus *Phellinus noxius*, the causal agent of an epidemic brown root rot disease of various coniferous and broad-leaved tree species [35].

The Trichoderma isolates collected during this study were characterized for their abilities to produce polysaccharide-degrading enzymes of the cellulolytic (β-glucosidase and cellobiohydrolase) and xylanolytic (β-xylosidase) enzyme systems that are important for efficient competition in habitats rich of plant-derived polysaccharides, as well as acidic phosphatase playing a role in phosphorus mobilization. Interestingly, the isolates of species with the best in vitro antagonistic abilities against Armillaria (*T. virens*, *T. atrobrunneum*, *T. simmonsii*, and *T. atroviride*) were among the worst producers of these extracellular enzymes and vice versa, suggesting that the main antagonistic mechanism of these Trichoderma species against Armillaria may be mycoparasitism of hyphae and rhizomorphs rather than competition for polysaccharides or increasing phosphorous availability to the tree roots. Certain Trichoderma species (e.g., *T. reesei*) can be characterized with a predominantly saprophytic behavior, while others (e.g., *T. virens* and *T. atroviride* and members of the Harzianum clade) are described as successful mycoparasitic species [14]. Extracellular hydrolytic enzymes are known as key players of both the saprophytic and the mycoparasitic behavior: the former is relying on the production of plant polysaccharide-degrading enzyme systems like cellulases or xylanases, whereas the latter is based on CWDEs targeting the cell wall of the fungal host (glucanases, chitinases, and proteases).

The competition for iron may also contribute to the anti-Armillaria activity of the examined Trichoderma iolates, as the production of siderophores proved to be a general feature among them. Previous studies reported that certain strains of *T. asperellum*, *T. atrobrunneum*, *T. atroviride*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. polysporum*, *T. reesei*, *T. virens*, *T. paratroviride*, *T. pyramidale*, *T. rufobrunneum*, *T. thermophilum*, *T. viridulum*, *T. guizhouense*, and *T. simmonsii* were mainly used as biocontrol agents due to their siderophore producing abilities [36–40]. Wang and Zhuang [40] firstly reported the siderophore-producing ability of *T. guizhouense* and *T. simmonsii*. To the best of our knowledge, the production of siderophores by *T. citrinoviride*, *T. koningii*, *T. crassum*, *T. longipile*, and *T. paraviridescens* strains is firstly demonstrated in the present study.

From the forest-derived Trichoderma isolates of our study, 40 were able to produce IAA with *T. hamatum* SZMC 24410, *T. citrinoviride* SZMC 26776, and *T. atrobrunneum* SZMC 24206 producing the highest quantities (18.49, 16.198, and 15.64 µg/mL, respectively). Data in the literature about the IAA-producing ability of Trichoderma strains is limited. Chagas et al. [41] investigated the IAA production of *T. harzianum*, *T. pinnatum*, *T. longibrachiatum*, and *T. asperelloides*, as well as two strains of *T. virens*, and recorded production values of 2.9–3.2 µg/mL. In the present study, the detected values were in a wider concentration range (1.349–8.248 µg/mL). A previous study used a similar method to show that strains of *T. atrobrunneum*, *T. guizhouense*, *T. paratroviride*, and *T. simmonsii* produce IAA at concentrations of 6.6, 10.3–21.8, 4.1–8.5, and 6.0–7.2 µg/mL [40]. In our study, the examined *T. guizhouense* and *T. paratroviride* isolates produced lower amounts of IAA. We also present the first data about the IAA production of *T. koningii*, *T. longipile*, *T. tomentosum*, *T. hamatum*, and *T. citrinoviride*.

A comparison of the data about the in vitro antagonism and the production of indole-3-acetic acid, siderophores as well as extracellular β-glucosidase, cellobiohydrolase, β-xylosidase, and phosphatase enzymes among the Trichoderma isolates mostly revealed very similar values for isolates deriving from the same sample and belonging to the same species, suggesting that the respective isolates are clonal and represent the same strain, which is, in many cases, also supported by identical sequences of the *tef1α* fragment used for species-level identification (Table 1). Examples for probable clonality are the isolate groups *T. koningii* SZMC 24277/24278/24279, *T. atroviride* SZMC 24274/24275/24276 and SZMC 24413/24414, *T. simmonsii* SZMC 24435/26771/24436/24403/24404 and SZMC 26777/24412, or *T. atrobrunneum* SZMC 26772/24405. On the other hand, in certain cases, the differences in the physiological parameters or *tef1α* sequences clearly revealed the presence of multiple strains from the same species in the same sample, e.g., *T. koningii* SZMC 2470/2471 vs. *T. koningii* SZMC 2472/2473, *T. simmonsii* SZMC 26770 vs. SZMC 24429/24430/24431/24433, *T. hamatum* SZMC 24409 vs. SZMC 24410,

or *T. simmonsii* SZMC 26773 vs. SZMC 24408—the difference of the latter two isolates is also supported by a series of single nucleotide polymorphisms in the analyzed *tef1α* gene fragment (Table 1).

Only limited information is available in the literature about field studies evaluating the applicability of Trichoderma strains against Armillaria root rot of trees. Otieno et al. [12] screened Trichoderma isolates for antagonism to Armillaria in tea stem sections buried in the soil and selected a *T. harzianum* strain, the wheat bran culture of which significantly reduced the viability of Armillaria in woody blocks of inoculum. The selected strain also exhibited high efficiency in the biocontrol of the destructive tree and bush pathogens from the genus Armillaria [13]. Schnabel et al. [42] applied biannual drenches of *T. asperellum* and *T. gamsii*, formulated as Remedier WP, onto peach trees planted in spots where a tree had declined from Armillaria root rot during the previous season, but did not find any statistical significance in survival between the treated and control trees. However, the surviving Remedier WP-treated trees were found to have significantly larger tree trunks compared to control trees three and four years after planting at one of the two replant sites involved in the study. In another study, spraying a combination of *T. harzianum* and *T. koningii* at concentrations of 2×10^7 CFU/mL and 3×10^7 CFU/mL, respectively, into holes made in an avocado orchard previously infested with *A. mellea* did not increase the survival rates of grafted peach (*Prunus persica*) saplings [43]. The lack of Trichoderma effect on the survival of peach trees in the above studies may partly be due to the Trichoderma species applied: our study demonstrated that isolates of *T. asperellum* and *T. koningii* are not among the good in vitro antagonists of Armillaria species. Furthermore, the success of a tested control strategy may also rely on the thorough selection of the Trichoderma strains, which should also consider the origin of the isolates. The two strains involved in the field test of our study were derived from a soil sample associated with Armillaria rhizomorphs which have not revealed any Armillaria growth, suggesting that the isolation of Trichoderma strains from naturally decaying Armillaria rhizomorphs and the soil surrounding them may increase the chances to find promising candidates for the successful biocontrol of Armillaria root rot. A similar strain isolation strategy from Armillaria rhizomorphs and soil samples around Armillaria-infected roots of cherry and almond trees revealed isolates of *T. virens* and *T. harzianum sensu lato* efficiently inhibiting both colony growth and rhizomorph formation of *A. mellea* [44].

5. Conclusions

One of the possible reasons behind the so-far limited success of Armillaria biocontrol by the application of Trichoderma strains in forests may lie in the selection strategy of the biocontrol strains. The results of our study suggest that certain Trichoderma species are known as successful biocontrol agents and components of commercially formulated products, e.g., *T. asperellum* or *T. koningii*, may not be the agents of choice for this purpose, as many isolates from these species were shown to lack good in vitro antagonistic abilities against Armillaria isolates. On the other hand, this study demonstrates that decaying rhizomorphs are potential sources of antagonistic Trichoderma strains with the potential to control Armillaria species and increase the survival rate of seedlings planted in the Armillaria-infested forest areas.

Another limitation of Trichoderma application in forest stands is arising from the difficulties of delivery, as the regular treatment of large forest areas with a biocontrol product is not economically feasible. An obvious time point of intervention is the planting time of the seedlings, as their roots can be easily treated with microbial products by soaking. A further promising strategy could be the conditioning of the seedlings with microbial products before planting, which could be performed in nurseries under more controlled circumstances than the ones allowed by field conditions.

A further crucial point is the exact, species-level molecular identification of the candidate Trichoderma strains for Armillaria biocontrol, which should be performed by the sequence analysis of a fragment of the tef1α gene fragment, as, according to our recent knowledge about the taxonomy of the genus Trichoderma, ITS sequence analysis is not allowing an exact diagnosis in many cases.

The interactions of introduced Trichoderma strains with other beneficial microorganisms such as mycorrhizal fungi need further investigations, as they may represent both advantages and

disadvantages to the host plant [45]. Trichoderma may act negatively on mycorrhizal fungi via competition for the colonization sites and nutrients [46], or via direct mycoparasitic attack, which, however, may also increase the uptake of phosphorous by the mycorrhizal fungus as the result of stress reaction [47]. Using beneficial fungi in forestry therefore requires the adjustment of Trichoderma–mycorrhizal fungus combinations to the host tree, as well as the optimization of the inoculation methods and the applied sylvicultural practices.

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