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Members of the *Trichoderma harzianum* Species Complex with Mushroom Pathogenic Potential

Henrietta Allaga [1,2], Anuar Zhumakayev [1,2], Rita Büchner [1,2], Sándor Kocsubé [1], Attila Szűcs [1], Csaba Vágvölgyi [1], László Kredics [1] and Lóránt Hatvani [1,*]

[1] Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép Fasor 52, 6726 Szeged, Hungary; henrietta.allaga@gmail.com (H.A.); anuar_zhumakaev@mail.ru (A.Z.); cristinagodd@gmail.com (R.B.); shigsanyi@gmail.com (S.K.); a.szunyi@gmail.com (A.S.); mucor1959@gmail.com (C.V.); kredics@bio.u-szeged.hu (L.K.)

[2] Doctoral School of Biology, Faculty of Science and Informatics, University of Szeged, Közép Fasor 52, 6726 Szeged, Hungary

* Correspondence: lorant.hatvani@gmail.com; Tel.: +36-62-544-849

Abstract: Previously, severe green mould infections could be attributed mainly to *Trichoderma aggressivum* Samuels & W. Gams, as well as *T. pleuroti* S.H. Yu & M.S. Park and *T. pleuroticola* S.H. Yu & M.S. Park in the case of *Agaricus bisporus* (J.E. Lange) Imbach (button mushroom) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (oyster mushroom), respectively. The purpose of our study was the examination of green mould agents deriving from the growing facilities of button mushroom, oyster mushroom and shiitake (*Lentinula edodes* (Berk.) Pegler) located in various countries of Europe, and initially classified into the *Trichoderma harzianum* Rifai species complex (THSC). Species identification was carried out using the multilocus sequence typing analysis of the internal transcribed spacer regions, as well as translation elongation factor 1-alpha, calmodulin and RNA polymerase B subunit II gene sequences. In vitro confrontation assays were applied to test the aggressiveness of the isolates towards mushrooms, while the effect of commercial fungicides on the growth of the strains was examined by the macrodilution method. Six *Trichoderma* species, namely *T. afroharzianum* P. Chaverri, F.B. Rocha, Degenkolb & Druzhin., *T. atrobrunneum* F.B. Rocha, P. Chaverri & Jaklitsch, *T. guizhouense* Q.R. Li, McKenzie & Yong Wang, *T. harzianum sensu stricto*, *T. pollinicola* F. Liu & L. Cai and *T. simmonsii* P. Chaverri, F.B. Rocha, Samuels, Degenkolb & Jaklitsch were detected in the different samples, with *T. harzianum, T. pollinicola* and *T. simmonsii* being the most aggressive. Prochloraz was found to have strong in vitro inhibitory effect on mycelial growth on most strains, however, *T. simmonsii* isolates showed remarkable tolerance to it. Our data suggest that *T. harzianum* and *T. simmonsii* may also be considered as potential causal agents of mushroom green mould.

Keywords: cultivated mushrooms; *Agaricus bisporus*; *Lentinula edodes*; *Pleurotus ostreatus*; green mould; *Trichoderma harzianum* species complex; fungicide susceptibility

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1. Introduction

Complying with the rising customer demand, world mushroom production has increased more than 30-fold during the past four decades [1]. The five dominant genera, accounting for 85% of the global supply include the species *Agaricus bisporus* (button mushroom/champignon), *Pleurotus ostreatus* (oyster mushroom) and *Lentinula edodes* (shiitake). Numerous factors, such as water activity, the ratio of carbon and nitrogen content, temperature, humidity, pH, luminosity, air composition, as well as the quality of growing substrate/compost and casing material play key roles in mushroom cultivation, having substantial influence on the formation and development of fruiting bodies [2,3]. In addition, mushroom yield can be severely affected by different pests including insects, mites, nematodes, viruses and bacteria, but the most serious crop losses are attributed worldwide to fungal diseases such as cobweb, dry bubble, wet bubble and particularly green mould, caused *Cladobotryum* spp. [4–6],

Lecanicillium fungicola (Preuss) Zare & W. Gams [7,8], *Hypomyces perniciosus* Magnus (formerly *Mycogone perniciosa*) [9–12], and *Trichoderma* spp. [13–15], respectively.

Despite the large number of *Trichoderma* species (*T. citrinoviride* Bissett, *T. crassum* Bissett, *T. hamatum* (Bonord.) Bainier, *T. koningii* Oudem., *T. longibrachiatum* Rifai, *T. spirale* Bissett) found in mushroom compost [16], green mould outbreaks in the cultivation of *A. bisporus* had been attributed initially to *T. harzianum* [17–19]. The most aggressive biotypes, Th2 and Th4, were subsequently introduced as the new species *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum*, causing green mould infections in European countries and North America, respectively [20]. In contrast, being the only *Trichoderma* species detected in green mould affected samples of compost and casing material, *T. harzianum* was identified as the real causal agent of a severe outbreak in Croatia [21].

Green mould outbreaks have also been reported in the cultivation of *P. ostreatus* worldwide. The major causal agents are known to belong to the species *T. pleuroti* and/or *T. pleuroticola* [22] in numerous countries, including Korea [22], Italy, Hungary, Romania, Canada, Iran, the Netherlands, Germany, New Zealand [23–25], Croatia [21], Poland [26], as well as Serbia and North Macedonia [15]. However, Woo et al. [27] also identified *T. harzianum* (later re-classified as *T. pleuroticola* [25]) as a common pathogen of oyster mushroom in Italy, while in Hungary and Poland *T. harzianum* and *T. atroviride* Bissett were found in high proportions in addition to *T. pleuroti* and *T. pleuroticola* [24,26].

The most relevant *Trichoderma* pathogens of shiitake were reported to involve *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii* Rifai, *T. polysporum* (Link) Rifai, *T.* cf. *stramineum* P. Chaverri & Samuels, *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx and *Trichoderma* sp. [28], as well as *T. mienum* Chang S. Kim, Nakagiri & N. Maek. and *T. pseudolacteum* Chang S. Kim & N. Maek. in Japan [29,30], while in Korea *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. polysporum*, *T. longibrachiatum*, *T. viride* Pers., *T. pseudogelatinosum* (M. Komatsu & Yoshim. Doi) Chang S. Kim and *T. pseudostramineum* (Yoshim. Doi) Chang S. Kim [31], as well as *T. deliquescens* (Sopp) Jaklitsch were detected [32,33]. In China, *T. harzianum* and *T. atroviride* proved to be the predominant infectious agents of shiitake preceding *T. viride*, *T. pleuroticola*, *T. longibrachiatum* and *T. oblongisporum* Bissett [34], while Cao et al. [35] identified *T. oblongisporum* as the cause of the disease.

One of the most widespread members of the genus *Trichoderma*, *T. harzianum* was considered initially to be an aggregate species, potentially covering a number of morphologically cryptic species with different biological characteristics [36], and currently it is recognized as the "*Trichoderma harzianum* species complex" (THSC) [37], containing a progressively growing number of described species [38–41]. Members of the THSC occupy a broad range of habitats worldwide, including soil, living fungi and plants as well as decaying plant material [37,38], and certain species are known as mycoparasites [42,43], which explains their frequent appearance in the growing facilities of cultivated mushrooms [15]. Among the members of the THSC, the association of *T. atrobrunneum*, *T. simmonsii* and *T. guizhouense* with cultivated mushrooms has been reported so far in addition to *T. harzianum* [44–46]. Despite the comprehensive taxonomical studies, there are still knowledge gaps to be filled in terms of the biology of "*T. harzianum*" [47]. Therefore, in addition to the precise species identification of THSC strains isolated from green mould-affected samples of different cultivated mushrooms and their substrates, the purpose of our research was to examine the antagonistic potential of the isolates towards their hosts, as well as the potential applicability of commercial fungicides against them as means of disease control.

2. Materials and Methods

In the present study, *Trichoderma* strains recovered from button mushroom (*A. bisporus*), oyster mushroom (*P. ostreatus*) and shiitake (*L. edodes*) producing facilities located in different European countries were investigated. According to the results of a preliminary, ITS sequence-based analysis, the isolates were classified into the *Trichoderma harzianum* species complex. The fungi were grown on potato dextrose agar (PDA) medium at 25 °C. The examined strains are listed in Table 1.

Table 1. *Trichoderma* strains examined in the study.

Species	Strain Number	Source	GenBank Accession Numbers				Reference
			ITS	*tef1*	*cal1*	*rpb2*	
T. afroharzianum	SZMC 12432	*Pleurotus ostreatus*, Spain	MZ754498	MZ773449	MZ773454	MZ773428	Present study
T. afroharzianum	SZMC 25728	*P. ostreatus*, North Macedonia	MZ754499	MZ773444	MZ773455	MZ773423	Present study
T. afroharzianum	SZMC 26672, KG10	*P. ostreatus*, Serbia	MT876582	MZ773435	MZ773456	MZ773414	[15]
T. atrobrunneum	SZMC 25744	*Lentinula edodes*, Serbia	MZ754500	MZ773440	MZ773457	MZ773419	Present study
T. atrobrunneum	SZMC 26673	*P. ostreatus*, North Macedonia	MZ754501	MZ773434	MZ773458	MZ773413	Present study
T. guizhouense	SZMC 22514	*P. ostreatus*, Croatia	MZ754502	MZ773448	MZ773459	MZ773427	Present study
T. guizhouense	SZMC 25749, T58	*L. edodes*, Serbia	MT876594	MZ773439	MZ773460	MZ773418	[15]
T. guizhouense	SZMC 25750, T59	*L. edodes*, Serbia	MT876595	MZ773438	MZ773461	MZ773417	[15]
T. guizhouense	SZMC 26669	*P. ostreatus*, Serbia	MZ754503	MZ773437	MZ773462	MZ773416	Present study
T. harzianum	SZMC 1764, C08	*Agaricus bisporus*, Hungary	MZ754504	MZ773453	MZ773463	MZ773432	[15]
T. harzianum	SZMC 1814, C22	*A. bisporus*, Hungary	MZ754505	MZ773452	MZ773464	MZ773431	[24]
T. harzianum	SZMC 1844	*A. bisporus*, Croatia	MZ754506	MZ773451	MZ773465	MZ773430	Present study
T. harzianum	SZMC 1848	*A. bisporus*, Croatia	MZ754507	MZ773450	MZ773466	MZ773429	Present study
T. harzianum	SZMC 25729	*P. ostreatus*, North Macedonia	MZ754508	MZ773443	MZ773467	MZ773422	Present study
T. harzianum	SZMC 25730	*P. ostreatus*, North Macedonia	MZ754509	MZ773442	MZ773468	MZ773421	Present study
T. pollinicola	SZMC 24399	*L. edodes*, Hungary	MZ754510	MZ773446	MZ773469	MZ773425	Present study
T. pollinicola	SZMC 24445	*L. edodes*, Hungary	MZ754511	MZ773445	MZ773470	MZ773424	Present study
T. simmonsii	SZMC 24248	*L. edodes*, Hungary	MZ754512	MZ773447	MZ773471	MZ773426	Present study
T. simmonsii	SZMC 25740	*L. edodes*, Serbia	MZ754513	MZ773441	MZ773472	MZ773420	Present study
T. simmonsii	SZMC 26671	*P. ostreatus*, Serbia	MZ754514	MZ773436	MZ773473	MZ773415	Present study
T. simmonsii	SZMC 26674	*P. ostreatus*, North Macedonia	MZ754515	MZ773433	MZ773474	MZ773412	Present study
T. aggressivum f. *aggressivum*	SZMC 23834	*A. bisporus*, Hungary	nr	nr	nr	nr	[13]
T. pleuroticola	SZMC 23033, TUCIM 2104	*P. ostreatus*, Hungary	nr	nr	nr	nr	[25]
T. reesei	SZMC 22614, TUCIM 917	nr	nr	nr	nr	nr	[48]

SZMC: Szeged Microbiology Collection (Department of Microbiology, FSI, University of Szeged, Hungary). TUCIM: TU Collection of Industrially Important Microorganisms, Vienna University of Technology [49]. nd: no data. nr: not relevant.

Trichoderma isolates were identified at the species level by the sequence analysis of the internal transcribed spacer (ITS) 1 and 2 regions of the ribosomal DNA, as well as fragments of the translation elongation factor 1-alpha (*tef1*), calmodulin (*cal1*) and RNA polymerase B subunit II (*rpb2*) genes. The isolates were grown on PDA plates covered by cellophane membrane for 1 day at 25 °C, then genomic DNA was extracted from the harvested fresh mycelia using an E.Z.N.A.® Fungal DNA Mini Kit (Omega BIO-TEK, Norcross, GA, USA), following the instructions of the manufacturer. Amplification and sequencing were performed as described by Hatvani et al. [50]. Multiple sequence alignments were made by MAFFT v7.312 [51], using the E-INS-i option. The dataset was partitioned to exons and introns in the case of *cal1* and *tef1* sequences, while the ITS dataset was partitioned by ribosomal and internal transcribed spacer region. On the concatenated dataset, the best fitting model for each partition (Table 2) was selected by using ModelTest-NG v0.1.4 [52], based on the corrected Akaike information criterion (AICc). Maximum likelihood analysis was carried out by RAxML-NG v0.9.0 [53], with 1000 tbootstrap replicates. The corresponding sequences of the type strains of closely related *Trichoderma* species were used as reference based on recent taxonomic studies in connection with the THSC [39,54].

Table 2. Best fit evolutionary models selected by ModelTest-NG.

Genomic Region	Selected Model
cal1 intron	TPM1uf + G4
cal1 exon	TIM1 + G4
ribosomal region	TPM1uf + G4
internal transcribed spacer	TPM2uf + G4
rpb2	GTR + G4
tef1 intron	TIM2 + G4
tef1 exon	TIM1 + G4

A set of isolates sufficiently representing each detected species, host mushrooms and geographical locations was selected for the subsequent analyses based on the results of species identification.

The aggressiveness of the *Trichoderma* strains towards their host mushrooms was tested using in vitro confrontation assays. The protocol of Szekeres et al. [55], adapted to mushroom pathogenic *Trichoderma* strains [56] was applied with minor modifications. Agar discs (5 mm in diameter) cut from the growing front of the mushroom (*A. bisporus* SZMC 23395, *P. ostreatus* SZMC 23392, *L. edodes* SZMC 24442) colonies were inoculated onto PDA medium at a distance of 3 cm from the edge of the Petri plates (9 cm in diameter). *Trichoderma* isolates were inoculated on the plates at 3 cm from the center of the mushroom colony in the same way after 7, 3 and 3 days of incubation at 25 °C in the case of *A. bisporus*, *P. ostreatus* and *L. edodes*, respectively. The non-mycoparasitic *T. reesei* E.G. Simmons SZMC 22614 strain was used as negative control, while isolates *T. aggressivum* f. *aggressivum* SZMC 23834 and *T. pleuroticola* SZMC 23033 were used in the case of *A. bisporus*, as well as *P. ostreatus* and *L. edodes*, respectively, as positive controls. The experiments were carried out in 2 replicates. The plates were photographed after 7 days of co-cultivation at 25 °C, and the pictures were processed by the IrfanView v3.95 software. Aggressivity index (AI, %) values were calculated from the areas occupied by the fungal colonies, using a previously developed image-analysis-based biocontrol index (BCI) assessment method [55,56], according to the following formula (1).

$$AI = \text{area covered by the fungal pathogen/total covered area (pathogen and mushroom)} \times 100 \quad (1)$$

The susceptibility of the isolates to the two commercial fungicides authorized for mushroom cultivation in Europe, Harvinta (BASF S.E., Ludwigshafen, Germany; active

ingredient: metrafenone 500 g/L) and Sporgon 50 WP (BASF Agro B.V., Zürich Branch, Switzerland; active ingredient: prochloraz 461 g/kg) [15] was examined on PDA medium amended with the fungicides. The effect of Harvinta and Sporgon 50 WP on mycelial growth was tested at 16, 8, 4, 2 and 1 mg/L concentrations, compared to the controls containing no fungicide. The *Trichoderma* strains were inoculated to the center of the plates as described above in 3 replicates. After 7 days of incubation at 25 °C, colony radius values were measured, and the rate of growth inhibition (GI, %) was calculated using the formula below (2), where r_f is the radius of colonies that appeared in the presence of the fungicides at different concentrations, while r_c is the colony radius values measured on the control plates.

$$GI = 100 - (r_f/r_c \times 100) \quad (2)$$

The statistical analysis of the obtained data was performed using the software R (version 4.0.3) [57] and RStudio Desktop (version 1.3.1093) [58]. The effect of the fungicide in different concentrations on fungal growth was analyzed by one-way ANOVA. Assumptions for parametric tests as normal distribution of the residuals and homoscedasticity (similar variances in all groups) were checked before applying statistical test. The variants not corresponding to the mentioned assumptions for parametric tests were analyzed using non-parametric alternative for ANOVA Kruskal–Wallis test. Each significant ANOVA result ($p < 0.05$) was subjected further to post-hoc pair-wise comparisons (Tukey's HSD (honestly significant difference) test). Unless specified, the data are presented as means of 3 replicates ± standard deviation (SD).

3. Results

3.1. Species Identification of Green Mould Agents

A total of 21 *Trichoderma* strains isolated from specimens showing green mould symptoms at the growing facilities of *A. bisporus*, *P. ostreatus* and *L. edodes* from different European countries was examined in the study. On the basis of their ITS sequences, they were classified to the *Trichoderma harzianum* species complex (THSC), however, the subsequent multilocus sequence typing (MLST) analysis of the ITS, *tef1*, *cal1* and *rpb2* sequences of the isolates revealed various species (Figure 1). *T. harzianum sensu stricto* was the only species found to be associated with *A. bisporus* both in Hungary and Croatia, and it was also detected at a North Macedonian *P. ostreatus* growing farm. The samples of both oyster mushroom and shiitake appeared to be inhabited by *T. atrobrunneum*, *T. guizhouense* and *T. simmonsii* in various countries. *T. afroharzianum* was found to prefer the conditions of *P. ostreatus* cultivation regardless of geographical location, while *T. pollinicola* was detected exclusively at a Hungarian farm producing *L. edodes* (Table 1).

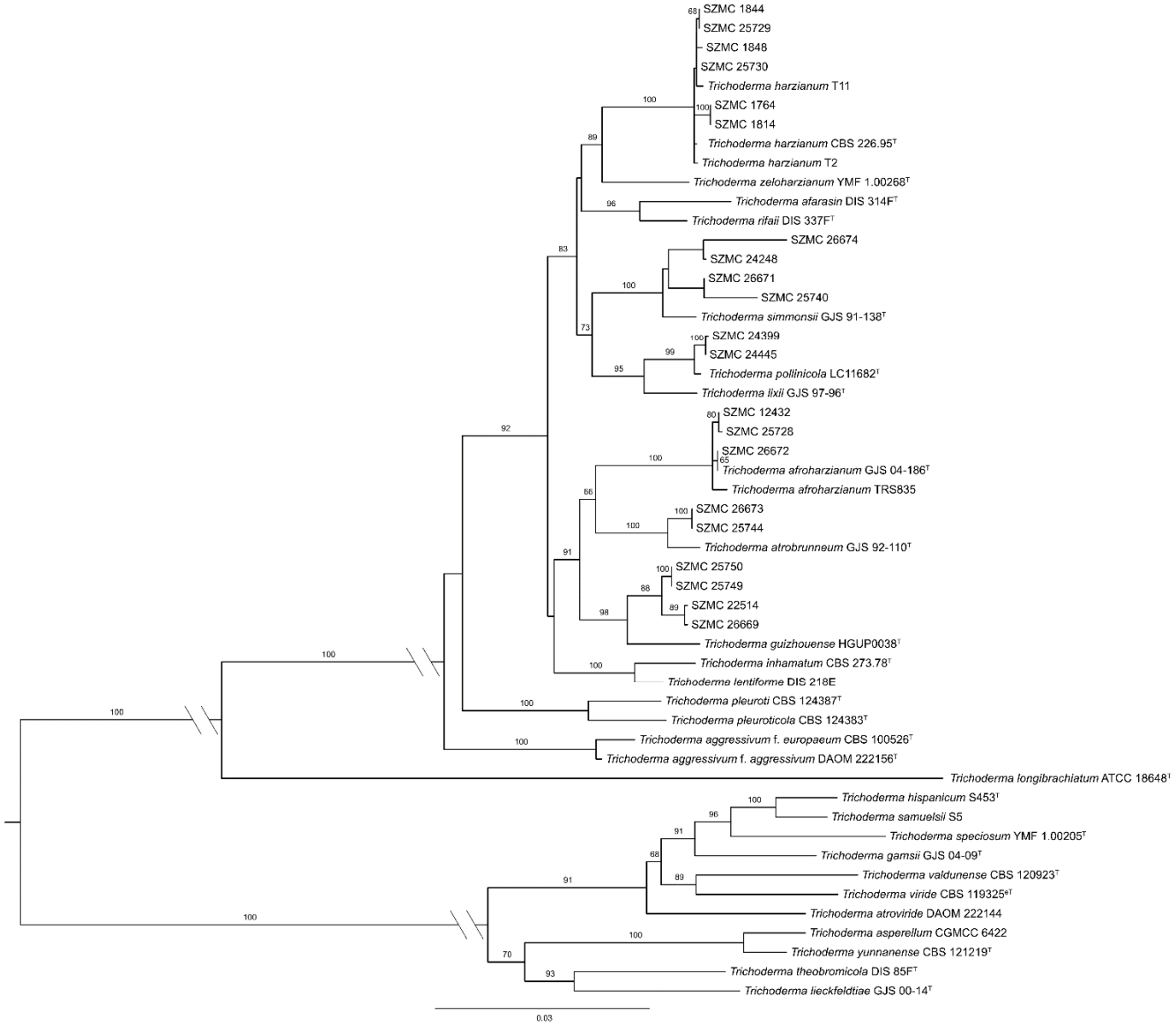

Figure 1. Maximum likelihood phylogenetic analysis of *Trichoderma* strains isolated from mushroom growing facilities based on the concatenated translation elongation factor 1-alpha (*tef1*), internal transcribed spacer (ITS), second largest subunit of RNA polymerase II (*rpb2*), and calmodulin (*cal1*) sequences. T = ex-type, eT = ex-epitype. Numbers above branches are bootstrap values. Only values higher than 70% are shown.

3.2. Antagonistic Potential of Trichoderma Isolates against Cultivated Mushrooms In Vitro

The antagonistic potential of the *Trichoderma* isolates towards their corresponding host mushrooms was tested using dual plate assays, and the results are shown in Table 3. Aggressiveness was considered as high, moderate and low if AI values were >85, 60–85 and <60, respectively. Button mushroom was devastated by the examined *T. harzianum* strains (AI: 98.67 and 100.00), similarly to *T. aggressivum* f. *aggressivum* (AI: 100.00), but interestingly, the mushroom showed the same level of susceptibility to the non-mycoparasitic *T. reesei* as well. Likewise, both *T. simmonsii* and *T. pollinicola* could completely overgrow the colony of shiitake (AI: 100.00), while it appeared to be less susceptible to *T. guizhouense* (AI: 57.58) (Figure 2). Surprisingly, higher AI values were observed in the case of *T. reesei* than the pathogenic *T. pleuroticola* (AI: 80.80 and 73.15, respectively). Oyster mushroom also proved to be highly susceptible to the examined *T. simmonsii* isolates (AI: 87.65 and 88.70), but remarkable growth inhibition was caused by *T. guizhouense* and *T. harzianum* as well. In contrast, moderate resistance to *T. afroharzianum* was observed.

Table 3. Aggressivity index (AI) values of *Trichoderma* strains confronted with cultivated mushrooms (means of 2 replicates ± standard deviations).

***Trichoderma* Strains**	**AI (%)**		
	Button Mushroom	**Oyster Mushroom**	**Shiitake**
T. afroharzianum SZMC 12432	-	80.90 ± 1.22	-
T. afroharzianum SZMC 25728	-	83.84 ± 0.98	-
T. afroharzianum SZMC 26672	-	80.97 ± 2.81	-
T. atrobrunneum SZMC 25744	-	-	81.99 ± 1.87
T. atrobrunneum SZMC 26673	-	81.90 ± 0.00	-
T. guizhouense SZMC 22514	-	87.33 ± 2.45	-
T. guizhouense SZMC 25749	-	-	57.58 ± 1.92
T. guizhouense SZMC 26669	-	85.67 ± 1.37	-
T. harzianum SZMC 1764	98.67 ± 1.15		-
T. harzianum SZMC 1844	100.00 ± 0.00	-	-
T. harzianum SZMC 25730	-	86.44 ± 0.78	-
T. pollinicola SZMC 24399	-	-	100.00 ± 0.00
T. simmonsii SZMC 24248	-	-	100.00 ± 0.00
T. simmonsii SZMC 25740	-		100.00 ± 0.00
T. simmonsii SZMC 26671	-	88.70 ± 1.67	
T. aggressivum f. *aggressivum* SZMC 23834	100.00 ± 0.00	-	-
T. pleuroticola SZMC 23033	-	87.83 ± 1.41	73.15 ± 1.61
T. reesei SZMC 22614	100.00 ± 0.00	82.66 ± 0.00	80.80 ± 2.11

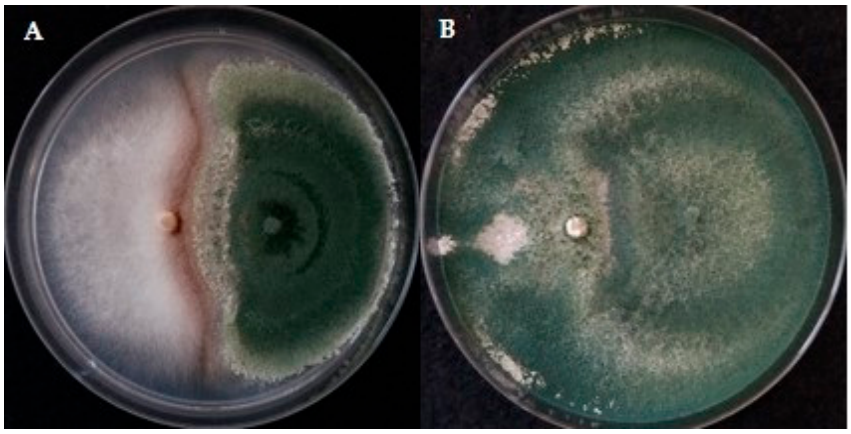

Figure 2. In vitro confrontation of *Lentinula edodes* (left) and *Trichoderma* isolates (right). (**A**) *T. guizhouense* SZMC 25749, (**B**) *T. simmonsii* SZMC 24248.

3.3. Influence of Commercial Fungicides on Mycelial Growth

The influence of the commercial fungicides prochloraz and metrafenone on the mycelial growth of the green mould agents was tested using the macrodilution method, the results are presented as the rate of growth inhibition (GI, %) (Tables 4 and 5). Prochloraz could effectively inhibit the growth of the majority of the examined *Trichoderma* isolates at low concentration (2–16 mg/L, with no significant differences ($p > 0.05$)), however, certain *T. simmonsii* strains showed tolerance to the fungicide, being able to grow remarkably even in the presence of the substance at the highest tested concentration (16 mg/L) as compared to the rest of the isolates ($p < 0.05$) (Table 4). Although metrafenone was able to substantially inhibit

the growth of certain *Trichoderma* strains (*T. atrobrunneum* SZMC 26673, *T. simmonsii* SZMC 26674 and SZMC 25740, *T. guizhouense* SZMC 25749) at 2–16 mg/L compared to other isolates ($p < 0.05$), the vast majority of the examined strains were just moderately affected by this fungicide, as it was not able to prevent their growth without significant differences between the tested concentration values, including the highest dose (16 mg/L, $p > 0.05$) (Table 5).

Table 4. Inhibitory effect of prochloraz on the mycelial growth of *Trichoderma* strains isolated from mushroom production (GI, %).

***Trichoderma* Strains**	**Prochloraz (mg/L)**						
	16	**8**	**4**	**2**	**1**	**0.5**	**0.25**
T. afroharzianum SZMC 12432	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	97.31 ± 2.33	94.63 ± 1.16	91.27 ± 2.33	75.17 ± 5.07
T. afroharzianum SZMC 25728	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	96.55 ± 3.16	95.86 ± 0.00	78.62 ± 1.20
T. afroharzianum SZMC 26672	100.00 ± 0.00	96.72 ± 5.68	93.44 ± 5.68	96.72 ± 5.68	88.52 ± 7.51	96.72 ± 5.68	62.30 ± 12.38
T. atrobrunneum SZMC 25744	100.00 ± 0.00	89.91 ± 4.37	92.44 ± 4.37	84.03 ± 2.91	65.55 ± 5.25	71.43 ± 2.91	15.12 ± 5.83
T. atrobrunneum SZMC 26673	100.00 ± 0.00	93.14 ± 1.70	87.25 ± 3.40	88.24 ± 5.10	77.45 ± 6.12	78.43 ± 1.70	19.61 ± 7.40
T. guizhouense SZMC 22514	95.24 ± 3.72	96.43 ± 4.72	93.75 ± 3.79	94.64 ± 3.09	80.36 ± 3.09	80.35 ± 6.44	33.93 ± 0.00
T. guizhouense SZMC 25749	95.56 ± 7.70	88.89 ± 3.85	86.67 ± 0.00	91.11 ± 7.70	80.00 ± 11.55	84.45 ± 16.78	57.78 ± 10.18
T. guizhouense SZMC 26669	95.58 ± 0.96	91.16 ± 0.95	90.61 ± 0.95	85.08 ± 1.66	75.14 ± 8.77	79.56 ± 4.17	37.57 ± 4.17
T. harzianum SZMC 1764	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	96.53 ± 6.02	100.00 ± 0.00	84.03 ± 1.21
T. harzianum SZMC 1844	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	95.58 ± 1.54	97.35 ± 2.66	78.76 ± 0.00
T. harzianum SZMC 25730	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.28 ± 1.25	92.09 ± 4.49	95.68 ± 5.71	81.29 ± 4.49
T. pollinicola SZMC 24399	100.00 ± 0.00	97.87 ± 3.68	96.45 ± 3.25	88.65 ± 1.23	83.69 ± 2.46	82.27 ± 1.23	40.43 ± 5.63
T. simmonsii SZMC 24248	89.63 ± 3.39	88.89 ± 0.00	88.15 ± 1.28	82.22 ± 4.45	75.56 ± 2.23	71.11 ± 3.85	28.15 ± 5.59
T. simmonsii SZMC 25740	89.70 ± 2.10	83.63 ± 6.30	71.82 ± 1.29	64.24 ± 4.20	41.82 ± 3.64	50.30 ± 11.69	12.73 ± 3.15
T. simmonsii SZMC 26671	82.68 ± 3.49	82.12 ± 1.93	77.09 ± 3.49	59.22 ± 10.10	41.90 ± 4.22	51.40 ± 6.71	18.99 ± 4.84
T. simmonsii SZMC 26674	95.42 ± 0.00	95.42 ± 2.29	91.60 ± 2.64	87.02 ± 1.32	79.39 ± 2.29	82.44 ± 4.77	35.12 ± 17.49

Table 5. Inhibitory effect of metrafenone on the mycelial growth of *Trichoderma* strains isolated from mushroom production (GI, %).

***Trichoderma* Strains**	**Metrafenone (mg/L)**						
	16	**8**	**4**	**2**	**1**	**0.5**	**0.25**
T. afroharzianum SZMC 12432	24.32 ± 2.34	31.09 ± 5.73	27.03 ± 8.11	18.92 ± 7.02	20.27 ± 2.34	22.98 ± 5.73	22.97 ± 7.02
T. afroharzianum SZMC 25728	27.63 ± 2.28	25.00 ± 5.59	22.37 ± 6.03	32.90 ± 6.84	30.26 ± 2.27	30.26 ± 6.03	32.89 ± 3.95
T. afroharzianum SZMC 26672	32.79 ± 17.27	38.53 ± 10.43	37.71 ± 12.38	37.71 ± 19.88	47.54 ± 17.27	45.90 ± 9.84	52.46 ± 11.36
T. atrobrunneum SZMC 25744	26.67 ± 7.64	25.00 ± 8.66	13.33 ± 2.89	20.00 ± 5.00	26.67 ± 10.41	13.33 ± 10.41	20.00 ± 5.00
T. atrobrunneum SZMC 26673	73.68 ± 0.00	45.61 ± 8.04	57.90 ± 22.32	59.65 ± 13.24	63.16 ± 13.92	43.86 ± 3.04	61.40 ± 8.04
T. guizhouense SZMC 22514	33.33 ± 3.71	34.57 ± 2.14	25.93 ± 0.00	30.87 ± 5.66	23.46 ± 13.01	28.40 ± 4.27	27.16 ± 9.32
T. guizhouense SZMC 25749	59.26 ± 12.83	62.96 ± 23.13	61.11 ± 23.57	59.26 ± 12.83	51.85 ± 12.83	44.44 ± 0.00	70.37 ± 6.41
T. guizhouense SZMC 26669	17.78 ± 1.92	15.56 ± 1.93	18.89 ± 3.85	15.56 ± 1.93	20.00 ± 5.77	24.45 ± 3.85	12.22 ± 5.09
T. harzianum SZMC 1764	21.82 ± 11.36	21.82 ± 15.74	15.46 ± 19.28	10.91 ± 31.01	5.45 ± 3.15	12.73 ± 0.00	5.45 ± 3.15
T. harzianum SZMC 1844	11.43 ± 4.95	27.14 ± 4.29	18.58 ± 6.06	11.43 ± 2.48	24.28 ± 4.95	21.43 ± 8.92	22.86 ± 4.29
T. harzianum SZMC 25730	27.85 ± 3.80	31.65 ± 7.60	24.05 ± 0.00	24.05 ± 0.00	29.12 ± 4.39	26.58 ± 5.80	22.79 ± 5.80
T. pollinicola SZMC 24399	26.15 ± 4.62	44.62 ± 13.06	23.08 ± 2.66	26.15 ± 0.00	27.69 ± 5.33	29.23 ± 2.67	26.15 ± 4.62
T. simmonsii SZMC 24248	31.51 ± 6.28	35.62 ± 4.75	35.62 ± 4.75	38.36 ± 4.11	39.73 ± 6.28	36.99 ± 12.55	36.99 ± 2.37
T. simmonsii SZMC 25740	52.78 ± 6.37	44.44 ± 2.40	50.00 ± 5.90	40.28 ± 10.48	38.89 ± 6.36	55.56 ± 2.40	36.11 ± 18.79
T. simmonsii SZMC 26671	10.12 ± 8.77	20.26 ± 6.58	7.59 ± 4.39	15.19 ± 2.19	6.33 ± 5.80	11.39 ± 2.19	10.13 ± 5.80
T. simmonsii SZMC 26674	50.00 ± 10.20	40.54 ± 6.19	40.54 ± 11.70	27.03 ± 7.03	25.68 ± 6.19	44.60 ± 13.03	33.79 ± 11.70

4. Discussion

Green mould, attributed primarily to *T. aggressivum*, *T. pleuroti* and *T. pleuroticola*, leads to substantial losses in the worldwide mushroom industry [14]. Our study has revealed that a broad range of further species belonging to the *Trichoderma harzianum* species complex are also able to cause severe green mould symptoms in the growing facilities of different edible mushrooms. The exclusive presence of *T. harzianum sensu stricto* was shown in infested samples of button mushroom in Croatia, and it was also found in *A. bisporus* cultivation in Hungary, confirming the previous findings of Hatvani et al. [21,24]. Moreover, we detected the species in an oyster mushroom farm in North Macedonia as well. Originally *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum* [20] were recognized as biotypes Th2 and Th4 of *T. harzianum*, being responsible for *Agaricus* green mould in Europe and North America, respectively [17–19]. Green mould disease caused by *T. harzianum*, affecting different cultivated edible mushrooms (such as *Pleurotus* spp., *A. bisporus*, *L. edodes*, *Flammulina velutipes* (Curtis) Singer, *Ganoderma lucidum* (Curtis) P. Karst., *Volvariella volvacea* (Bull.) Singer and *Calocybe indica* Purkay. & A. Chandra) has been reported in numerous studies even since then [26,28,34,59–65]. However, the *Trichoderma* isolates examined in the aforementioned studies were identified based on morphological characteristics, ITS or *tef1* sequences. To ensure the precise differentiation of species within the THSC, the analysis of multiple molecular markers, such as *cal1*, *rpb2* or *chi18-5* (chitinase 18-5) sequences can be recommended [38,39,47,54]. Using a multilocus approach, Oskiera et al. [44] identified several members of the THSC, namely *T. harzianum sensu stricto*, *T.* cf. *harzianum*, *T. atrobrunneum* and *T. simmonsii*, in samples collected at Polish mushroom farms, while *T. harzianum* was found on *A. bisporus* in Turkey [66] and also in shiitake production in Korea [67]. In addition to *T. harzianum*, in green mould affected oyster mushroom samples we could recover strains of *T. afroharzianum*, *T. atrobrunneum*, *T. guizhouense* and *T. simmonsii*, while in shiitake production *T. atrobrunneum*, *T. guizhouense*, *T. simmonsii* and *T. pollinicola* were found. Our analysis resulted in the identification of the THSC isolates T58, T59 and KG10 [15] as *T. guizhouense* and *T. afroharzianum*, respectively. The occurrence of *T. atrobrunneum*, *T. simmonsii* and *T. guizhouense* in mushroom cultivation has already been documented [44–46,68] however, to the best of our knowledge, *T. afroharzianum* and *T. pollinicola* have not been reported to cause mushroom green mould disease so far.

In vitro confrontation assays have revealed high aggressiveness of *T. harzianum* towards *A. bisporus*, similarly to *T. aggressivum* f. *aggressivum*. The results are in agreement with previous findings [21,25,64,69], which also documented the colonization of *A. bisporus* by *T. harzianum* in vitro, while in the study of Aydoğdu et al. [66] it was found to be less harmful to button mushroom than *T. aggressivum* f. *aggressivum* both in vitro and in vivo. The mushroom showed high sensitivity to the non-mycoparasitic *T. reesei* as well, confirming the results obtained by Komoń-Zelazowska et al. [25]. The shiitake culture was entirely colonized by *T. simmonsii* and *T. pollinicola*, whereas *T. guizhouense* demonstrated considerably lower aggressiveness. In contrast, the artificial inoculation of shiitake fruiting bodies with *T. guizhouense* strains T58 and T59 led to the development of severe green mould symptoms [15]. Oyster mushroom was strongly antagonized by *T. simmonsii*, as well as by *T. harzianum* and *T. guizhouense* strains. The effect was similar to that of *T. pleuroticola*, a known *Trichoderma* pathogen of *P. ostreatus* [21,25,45]. Contrary to our observations, *T. guizhouense* did not show high pathogenicity to oyster mushroom in the study of Innocenti et al. [45]. In our experiments, *T. afroharzianum* appeared to be only moderately aggressive towards *P. ostreatus*, supporting the findings of Luković et al. [15].

In our study, the commercial fungicide metrafenone (Harvinta) showed inhibitory effect on the growth of the examined *Trichoderma* strains just to a certain degree, while according to Luković et al. [15] it could efficiently suppress green mould isolates belonging to the THSC, including *T. guizhouense* T58 and *T. afroharzianum* KG10, which may be due to the differences in the applied incubation temperature (25 and 20 °C, respectively). In contrast, we found prochloraz (Sporgon 50 WP) to be highly successful in inhibiting the growth of the majority of the examined green mould agents, in accordance with previous

observations [15,21,45,64,70–72]. Nevertheless, *T. simmonsii* isolates were able to grow even if the fungicide was applied in the highest concentration (16 mg/L).

Even if a fungicide proves to be efficient in controlling green mould disease, there is always a risk for the development of resistance to it in the causal agents. Therefore, there is an increasing demand for the development of alternative means of pathogen control, to be applied within the frames of integrated disease/pest management (IDM/IPM) strategies [73]. Basically, a good quality substrate may prevent the development of green mold infection [3]. In champignon cultivation, the pasteurization of compost and the use of wood as a building material of growing spaces resulted in minimal green mould infestation, high yields and the occurrence of a larger number of flushes [74,75]. Catlin et al. [75] suggested a six-hour treatment at 60 °C for pasteurization after the harvest. However, this method is not always effective: green mould has already been isolated from freshly pasteurized compost [76], as pathogens can survive for some time at 60 °C, but it is also likely that this temperature cannot be reached in all parts of the substrate. In addition, sterilization of fungal substrates may promote the growth of *Trichoderma* due to a decrease in the natural microbiota in the substrate, which in turn increases the potential for *Trichoderma* colonization due to the lower frequency of competitive microbiota [77,78]. Controlling the pH of the casing material is also a possible method to treat green mould [79]. Attention should also be paid to the quality of construction sites used for mushroom growing. Contamination of green mould is more likely to persist on rougher surfaces such as concrete or wood than on smoother, glazed surfaces [80]. In *L. edodes* cultivation, the shiitake mycelium infected in the cultured bags became rotten, withered, yellow, and eventually perished, the surface of the breeding bags being covered with dark green fungal colonies. Above 20 °C, an incidence of almost 100% was found in some mushroom farms [35]. Wang et al. [34] reported that metabolites of different *Trichoderma* species inhibited the growth of *L. edodes* mycelium, and its hyphae were distorted and swollen in vitro. A number of *Bacillus* and *Streptomyces* isolates proved to be efficient in suppressing the growth of mushroom pathogenic *Trichoderma* strains [70–72,81,82]. Moreover, a broad range of plant extracts, essential oils and compost teas were also found to sufficiently inhibit different fungal pathogens of mushrooms, including *Trichoderma* species [73], suggesting the possibility of involving these agents in IPM approaches to successfully combat mushroom green mould disease.

5. Conclusions

The results of the present study indicate a widening spectrum of potential mushroom pathogenic *Trichoderma* species, as in addition to the known causal agents (*T. aggressivum*, *T. pleuroti*, *T. pleuroticola*), different members of the *T. harzianum* species complex, particularly *T. harzianum* and *T. simmonsii*, are also able to cause green mould disease in mushroom cultivation. According to our data, the infections can be efficiently controlled using different chemical fungicides, especially prochloraz, however, the development of alternative, biological means of pest management is highly recommended and encouraged.

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Data Availability Statement: DNA sequences obtained during the present study are openly available in NCBI GenBank [83] under the provided accession numbers (Table 1).

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