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Scientific Paper

THE FIRST REPORT ON MUSHROOM GREEN MOULD DISEASE IN CROATIA*

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Green mould disease, caused by *Trichoderma* species, is a severe problem for mushroom growers worldwide, including Croatia. *Trichoderma* strains were isolated from green mould-affected *Agaricus bisporus* (button or common mushroom) compost and *Pleurotus ostreatus* (oyster mushroom) substrate samples collected from Croatian mushroom farms. The causal agents of green mould disease in the oyster mushroom were *T. pleurotum* and *T. pleuroticola*, similar to other countries. At the same time, the pathogen of *A. bisporus* was exclusively the species *T. harzianum*, which is different from earlier findings and indicates that the range of mushroom pathogens is widening. The temperature profiles of the isolates and their hosts overlapped, thus no range was found that would allow optimal growth of the mushrooms without mould contamination. Ferulic acid and certain phenolic compounds, such as thymol showed remarkable fungistatic effect on the *Trichoderma* isolates, but inhibited the host mushrooms as well. However, commercial fungicides prochloraz and carbendazim were effective agents for pest management. This is the first report on green mould disease of cultivated mushrooms in Croatia.

KEY WORDS: Pleurotus ostreatus, Agaricus bisporus, Trichoderma pleurotum, T. pleuroticola, T. harzianum, *disease control*

Mushroom green mould disease

Mushrooms dominating commercial cultivation worldwide are *Agaricus bisporus* (button or common mushroom), *Lentinula edodes* (shiitake), and *Pleurotus ostreatus* (oyster mushroom) (1). Conditions under which these mushrooms are cultivated favour fast mould growth. Moulds compete for space and nutrients more effectively than the mushrooms and can produce secondary toxic metabolites, extracellular enzymes, as well as various volatile organic compounds (2, 3), that can substantially lower or even entirely block commercial production.

Agaricus bisporus

The first green mould epidemic was reported in Northern Ireland in 1985, quickly followed by outbreaks in several European countries (4-7). In the early 1990s, a similar disease appeared in mushroom crops in the United States and Canada (8-10). Aggressive biotypes had originally been identified as *Trichoderma harzianum* Th2 and Th4, but later they were re-described on the basis of morphological characteristics and of the phylogenetic analyses of the

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internal transcribed spacer (ITS) 1 region and the translation elongation factor 1-alpha (*tef1*) gene as new species *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum*, respectively (11). *Agaricus* green mould reported in Hungary seems to have been caused by *Trichoderma aggressivum* f. *europaeum* (12).

Pleurotus ostreatus

Recently, green mould disease of cultivated oyster mushroom has also been reported in several countries (12-18), and the causal agents were identified and described as new species *T. pleurotum* and *T. pleuroticola* (19, 20).

Status in Croatia

Similar to the neighbouring countries (Hungary, Serbia), the production of both *A. bisporus* and *P. ostreatus* in Croatia is affected by green mould infections. Though the disease generates serious production problems with significant economic consequences, its background has not been studied in Croatia until now.

MATERIALS AND METHODS

Isolation of fungal strains

Trichoderma strains were isolated from green mould-affected samples. Compost and substrate samples were obtained from a farm in north-western Croatia growing button mushroom and a farm in central Croatia growing oyster mushroom. Button mushroom compost samples were collected in the summer and oyster mushroom in both the summer and the winter growing seasons. Two green mould-affected and two healthy samples were collected each time. *Trichoderma* infections appeared sporadically at both farms at the time of sampling. *Trichoderma* isolates were deposited in the culture collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb under MFBF codes. Host mushrooms (button and oyster) were isolated from healthy samples collected together with the infected ones. All strains were isolated and maintained according to Hatvani et al. (12).

Species identification

DNA extraction, PCR-amplification of the internal transcribed spacer (ITS) region, DNA sequencing, and sequence analysis were performed as described by Kredics et al. (16).

In vitro *confrontation assays*

The aggressiveness of the isolates towards their corresponding host mushrooms was tested *in vitro* in dual-plate assays according to Szekeres et al. (21).

Table 1 *Minimal inhibitory concentrations (MIC, mg mL^{-1}) of natural compounds on the* Trichoderma *pathogens in comparison with control oyster and button mushrooms*

Compound	Nature	*T. pleuroticola* MFBF 10383	*T. pleuroticola* MFBF 10387	*T. pleurotum* MFBF 10386	*T. pleurotum* MFBF 10388	*T. harzianum* MFBF 10389	*T. harzianum* MFBF 10390	Oyster mushroom	Button mushroom
Tannic acid	PP	1.25	10	2.5	5	>10	>10	NT	NT
Gallic acid	PP	10	10	10	10	>10	>10	NT	NT
Curcumin	PP	>10	10	10	5	>10	>10	NT	NT
Rosmarinic acid	PA	10	5	2.5	2.5	5	10	NT	NT
Ferulic acid	PA	0.32	1.25	0.63	0.32	1.25	0.63	0.63	0.63
Caffeic acid	PA	2.5	5	2.5	2.5	10	10	NT	NT
Chlorogenic acid	PA	10	10	2.5	5	10	10	NT	NT
Chrysin	F	>10	>10	5	5	>10	>10	NT	NT
Quercetin	F	>10	10	10	10	>10	>10	NT	NT
Naringenin	F	>10	>10	10	10	>10	>10	NT	NT
Thymol	P	0.16	0.32	0.16	0.08	0.16	0.08	0.08	< 0.08
1,8-Cineole	P	2.5	2.5	2.5	2.5	2.5	2.5	NT	NT
(+)-Menthol	P	0.63	0.63	0.63	0.63	0.63	0.63	0.32	< 0.16
(-)-Menthol	P	0.63	0.63	0.63	0.63	0.63	0.63	0.32	< 0.16

PP: polyphenol, PA: phenolic acid, F: flavonoid, P: phenol, MFBF: the number of strains from the Collection of Microorganisms, Department of Microbiology, Faculty of Pharmacy and Biochemistry University of Zagreb, NT: not tested

Characterisation of the isolates

The effect of temperature, natural compounds, and commercial fungicides on the mycelial growth of two isolates from each species in comparison with their host mushrooms were tested on solid YEG medium (12). The fungi were inoculated onto the solid surface as mycelial plugs (4 mm diameter) taken from the actively growing edge of young colonies. *Trichoderma*, *P. ostreatus*, and *A. bisporus* colony diameters were measured after two days, one week, and two weeks, respectively. All experiments were repeated three times.

In order to determine the temperature growth profiles of the isolates the plates were incubated at (5, 10, 15, 20, 25, 30, 35, and 40) °C.

Stock solutions (30 mg mL^{-1}) of natural compounds quercetin, caffeic acid, chrysin, curcumin, naringenin, gallic acid, tannic acid, ferulic acid, chlorogenic acid, rosmarinic acid, (+)-menthol, (-)-menthol, thymol, and 1,8-cineole (Sigma-Aldrich) were prepared in ethanol. Two-fold serial dilution series was made in eight steps in melted and cooled (60 °C) YEG medium. Tested concentrations were (10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, and 0.08) mg mL^{-1}. The fungi were inoculated onto the solidified media as described above, and incubated at 25 °C. The minimal inhibitory concentrations (MIC) of the compounds were determined after incubation and defined as the concentrations at which no fungal growth was observed in comparison with control without compounds added.

Stock solutions (10 mg mL^{-1}) of commercial fungicides prochloraz (SPORTAK®, Bayer CropScience, Zagreb, Croatia), tebuconazol (FOLICUR® W 250, Bayer CropScience, Zagreb, Croatia), and carbendazim (BAVISTIN® FL, Chromos Agro d.d., Zagreb, Croatia) were prepared in dimethyl sulphoxide (DMSO). Serial dilution (starting from 10 µg mL^{-1} for each fungicide), inoculations, incubation, and MIC determination were performed as described above.

RESULTS

Isolation of fungal strains

Twenty *Trichoderma* strains were isolated from green mould-affected oyster mushroom substrate and twenty from button mushroom compost samples.

Species identification

DNA was extracted from the isolates, and the ITS regions were amplified by PCR. The amplicons were subjected to automatic sequencing (external service), and the DNA sequences analysed by *Trich*OKEY v. 2.0 software (22, www.isth.info). All the 20 isolates collected from the button mushroom compost were identified as *T. harzianum*, while strains isolated from the oyster mushroom substrate included *T. pleurotum* and *T. pleuroticola* (9 and 11 isolates, respectively). Alignments (20) revealed that the ITS sequences of the isolates belonging to the same species did not share the same pattern. The differences suggested that they belonged to different strains within the species, but this did not affect the results of identification.

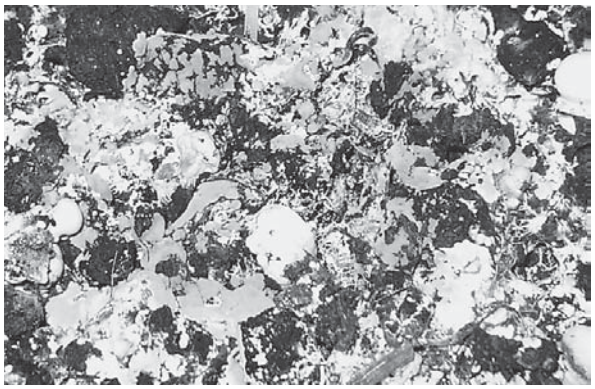


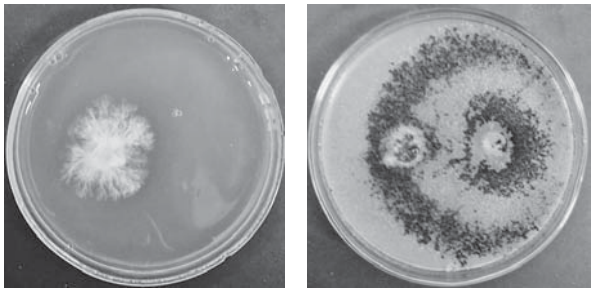
Figure 1 *Symptoms of green mould disease in the button mushroom compost*

Dual-plate assays

At the sampling sites we observed heavy colonisation of the button mushroom compost (Figure 1) and the oyster mushroom substrate by *Trichoderma*. The *in vitro* confrontation assays performed between the isolates and the colonies of button and oyster mushroom also revealed high aggressiveness of the *Trichoderma* strains towards their hosts. No significant difference was found between the antagonistic activity of the isolates obtained from the same samples; all of them overgrew the mushroom colonies completely after four days of incubation and produced conidia on their surface. Figure 2 shows button mushroom inoculated as a single colony (A) and in confrontation with *Trichoderma harzianum* MFBF 10389 (B).

Characterisation of the isolates

As all *Trichoderma* isolates showed similarly high antagonistic activity towards their hosts, two strains of each species with different ITS types (see section



A B

Figure 2 *Button mushroom inoculated as a single colony (A) and in confrontation with* Trichoderma harzianum *MFBF 10389 (B)*

"Species identification" in results) were selected for further characterisation in comparison with the button and oyster mushroom, namely *T. harzianum* MFBF 10389 and 10390, *T. pleuroticola* MFBF 10383 and 10387, and *T. pleurotum* MFBF 10386 and 10388.

The temperature growth profiles of the *Trichoderma* isolates and their hosts were found to be highly overlapping, with optima between 25 °C and 30 °C. Figure 3 shows the temperature growth profiles of the button mushroom (A) and oyster mushroom pathogenic *Trichoderma* isolates (B) in comparison with their host mushrooms.

The effect of the 10 natural compounds on the mycelial growth of the *Trichoderma* isolates and the mushrooms was also tested. Thymol, ferulic acid, (+)-menthol, and (-)-menthol showed remarkable inhibitory effect on the *Trichoderma* isolates at low concentrations (between 0.08 mg mL^{-1} and 1.25 mg mL^{-1}, Table 1). However, at these concentrations they entirely blocked the growth of the mushroom strains as well.

Among the commercial fungicides tested, tebuconazol was more inhibitory to mushrooms than to the *Trichoderma* isolates. Prochloraz and carbendazim showed promising features for controlling green mould disease in cultivated button and oyster mushrooms, as they inhibited the growth of the examined *Trichoderma* strains even at low concentrations without affecting their hosts (Table 2). Based on the higher MIC, *T. harzianum* isolates were more tolerant to all fungicides tested than the *Pleurotus*-pathogenic species (*T. pleurotum* and *T. pleuroticola*).

DISCUSSION

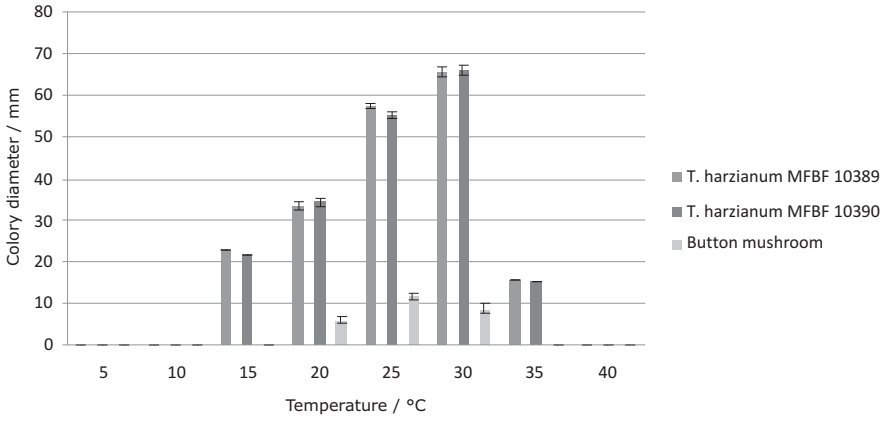
In this study, the causal agents and potential means of disease control of mushroom green mould were examined based on Croatian samples. Oyster mushroom green mould disease was caused by *T. pleurotum* and *T. pleuroticola*, which is in accordance with findings from other countries (16-20). At the same time, in the cultivation of button mushroom the sole isolated pathogen was *T. harzianum*. These results add a new name to the list of the potential mushroom-pathogenic *Trichoderma* species, as earlier studies from other countries identified only *T. aggressivum* as the button mushroom pathogen (11, 12). This finding suggests a continuous evolving of green mould disease in cultivated mushrooms and underlines the importance of monitoring these infections.

In order to find proper means of disease control, we investigated the effects of temperature, natural compounds, and commercial fungicides on pathogen and host mycelial growth. Similar to an earlier report by Woo et al. (15), the temperature profiles of the pathogens and their hosts highly overlapped (Figure 3), showing no room for effective disease control. Green mould isolates tolerated most of the natural compounds even at concentrations above 10 mg mL^{-1}. However, thymol, ferulic acid, (+)-menthol, and (-)-menthol inhibited their growth at concentrations

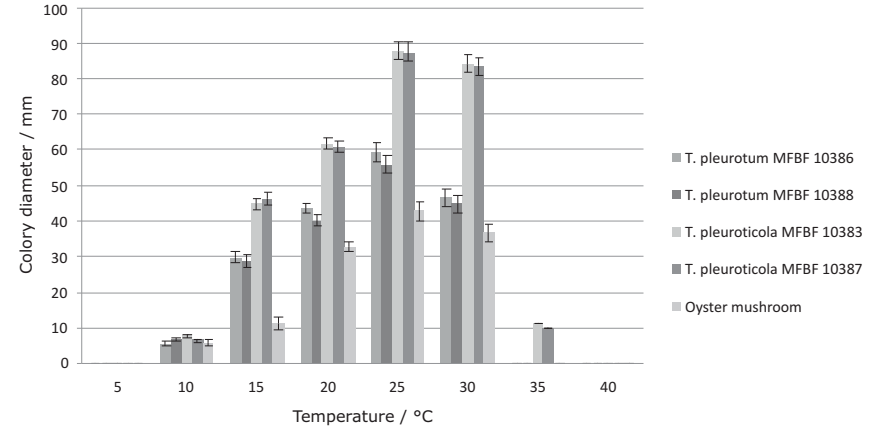
Table 2 *Minimal inhibitory concentrations (MIC, μg mL^{-1}) of commercial fungicides on the* Trichoderma *pathogens in comparison with control oyster and button mushrooms*

	T. pleuroticola MFBF 10383	*T. pleuroticola* MFBF 10387	*T. pleurotum* MFBF 10386	*T. pleurotum* MFBF 10388	*T. harzianum* MFBF 10389	*T. harzianum* MFBF 10390	*Oyster mushroom*	*Button mushroom*
Carbendazim	0.63	0.63	0.63	0.63	2.5	1.25	> 10	> 10
Tebuconazol	5	5	5	5	>10	>10	5	0.08
Prochloraz	1.25	1.25	1.25	1.25	5	5	> 10	10

MFBF: the number of strains from the Collection of Microorganisms, Department of Microbiology, Faculty of Pharmacy and Biochemistry University of Zagreb



A



B

Figure 3 *Temperature growth profiles of button mushroom (A) and oyster mushroom pathogenic* Trichoderma *isolates (B) and their hosts (colony diameters in mm)*

as low as 0.08 mg mL^{-1} to 1.25 mg mL^{-1} (Table 1). These results show that phenols have a remarkable inhibitory effect on the growth of green mould isolates. Notable differences were observed between species and isolates in their susceptibility to ferulic acid and thymol. Šegvić Klarić et al. (23) showed the inhibitory effect of thymol on the growth of moulds, including *Trichoderma* spp., even at very low concentrations (MIC 1.6 µg mL^{-1} to 6.72 µg mL^{-1}). In contrast, our *Trichoderma* isolates tolerated this compound at much higher concentrations (0.08 mg mL^{-1} to 0.32 mg mL^{-1}). However, as thymol, ferulic acid, and menthol blocked the growth of the host mushroom as well, their use in pest management is not possible.

Of the commercial fungicides tested, prochloraz and carbendazim proved to be efficient in inhibiting the green mould isolates at very low concentrations (0.63 µg mL^{-1} to 5 µg mL^{-1}) and did not influence the growth of their host mushrooms (Table 2), which is similar to findings reported by Woo et al. (15) in oyster mushroom. Therefore they might be considered as potential chemical control agents to prevent or stop the spreading of mushroom green mould disease.

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Sažetak

PRVI IZVJEŠTAJ O BOLESTI ZELENE PLIJESNI U HRVATSKOJ

Bolest zelene plijesni uzrokovane vrstama roda *Trichoderma* velik je problem pri uzgoju gljiva u cijelom svijetu, uključujući i Hrvatsku. Vrste *Trichoderma* izolirane su iz komposta onečišćenog zelenom plijesni pri uzgoju šampinjona (*Agaricus bisporus*), kao i iz uzoraka supstrata uzgoja bukovača (*Pleurotus ostreatus*), s farma gljiva u Hrvatskoj. Pri infekciji bukovača izolirani su i identificirani uzročnici vrsta *Trichoderma pleurotum* i *T. pleuroticola*, što odgovara nalazima u drugim zemljama, dok je iz uzgoja šampinjona izolirana samo *vrsta T. harzianum*. Navedeni su podaci različiti od prijašnjih nalaza i upućuju na to da se širi broj infektivnih uzročnika pri uzgoju gljiva. Temperaturni profil izolata i njihovih domaćina preklapao se, a komercijalni fungicidi prokloraz i karbendazim nađeni su kao potencijalno dobri kandidati za učinkovito suzbijanje ovih infekcija. Ferulična kiselina i neke fenolne tvari kao što je timol pokazuju značajan fungistatski učinak na izolate vrsta roda *Trichoderma*, ali su također inhibitorni i za domaćine - gljive. Ovo je prvo izvješće o bolesti izazvanoj zelenom plijesni pri uzgoju gljiva šampinjona i bukovača u Hrvatskoj.

KLJUČNE RIJEČI: Agaricus bisporus, *kontrola zaraze*, Pleurotus ostreatus, T. harzianum, T. pleuroticola, Trichoderma pleurotum

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