Plant Pathology (2019) **68**, 392–398 Doi: 10.1111/ppa.12953

Trichoderma species associated with green mould disease of *Pleurotus ostreatus* and their sensitivity to prochloraz

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Green mould disease causes serious economic losses in *Pleurotus ostreatus* crops worldwide, including in Italy, where prochloraz is the only chemical fungicide allowed to control the disease. The effectiveness of the doses 0.01, 0.05, 0.25 and 1.25 μL L^{-1} (field dose) of prochloraz (Sponix Flow, 450 g L^{-1}), against colony growth rate and spore germination of *Trichoderma pleuroti*, *T. pleuroticola* and *T. guizhouense* strains on wheat straw extract agar plates were evaluated. Complete inhibition of *Trichoderma pleuroti* and *T. pleuroticola* growth was shown by the field dose of prochloraz, and also by the 0.25 μL L^{-1} dose for *T. pleuroti*. Complete inhibition of spore germination occurred for all *Trichoderma* strains at field dose, and at 0.25 μL L^{-1} for *T. pleuroti* strains. In *in vivo* assays, the effect of prochloraz doses 0.05, 0.25 and 1.25 μL L^{-1} on colonization of straw substrate by *T. pleuroti*, *T. pleuroticola* and *T. guizhouense* inoculated at two spore densities (10^2 and 10^5 spores mL^{-1}) immediately after *P. ostreatus* spawn was studied. *Trichoderma pleuroti* and *T. pleuroticola* were both responsible for green mould disease, whereas *T. guizhouense* was not pathogenic. *Trichoderma pleuroti* was more aggressive than *T. pleuroticola*. Prochloraz was effective against *T. pleuroti* at the field dose, and against *T. pleuroticola* at 0.25 and 1.25 μL L^{-1}. The study on *Trichoderma* × *Pleurotus* interaction type showed that *Trichoderma* species were active against the mycelial growth of *P. ostreatus* by competition for space and nutrients, and neither hyphal interaction nor effect by volatile or nonvolatile metabolites occurred.

Keywords: green mould disease, oyster mushroom, prochloraz, *Trichoderma* spp.

Introduction

Pleurotus ostreatus, commonly known as oyster mushroom, is one of the most commercially important edible mushrooms worldwide. Italy, Hungary and Poland are the main producers in Europe (Błaszczyk *et al.*, 2013). Many biotic diseases may cause significant yield losses in *P. ostreatus* farms. The green mould disease caused by *Trichoderma* species is one of the most serious problems for the *P. ostreatus* crop. Typical symptoms of the disease are green sporulation areas on the surface of the cultivation substrate that is exposed to green mould infection, mostly during spawn run. Massive attacks of the disease have been reported in South Korea, where the first significant losses were observed (Park *et al.*, 2006), Sri Lanka (Jayalal & Adikaram, 2007), Hungary (Hatvani *et al.*, 2007), Croatia (Hatvani *et al.*, 2012), Romania (Kredics *et al.*, 2006), Spain (Gea, 2009) and Poland (Sobieralski *et al.*, 2012). The disease has also been reported in North America (Sharma & Vijay, 1996). Serious cases of green mould have been detected in *P. ostreatus* farms in Italy (Woo *et al.*, 2004, 2009; Alfonzo *et al.*, 2008; Innocenti & Montanari, 2014). Studies by Park *et al.* (2006) and Komon-Zelazowska *et al.* (2007) showed that two species of *Trichoderma*, *T. pleuroti* (previously *T. pleurotum*) and *T. pleuroticola*, are the predominant causal agents of *Pleurotus* green mould disease. They both belong to the *Harzianum* clade of *Hypocrea/Trichoderma*, which also includes *T. aggressivum*, responsible for green mould disease of *Agaricus bisporus* (Hatvani *et al.*, 2007; Komon-Zelazowska *et al.*, 2007). Morphological studies have revealed that *T. pleuroticola* shows pachybasidium-like properties, characteristic of the *Harzianum* clade, while *T. pleuroti* possesses *Gliocladium*-like conidiophore morphology (Komon-Zelazowska *et al.*, 2007). *Trichoderma pleuroti* has only been found in the area of *P. ostreatus* cultivation (Kredics *et al.*, 2009); in contrast, *T. pleuroticola* has been found in soil, plant debris or decaying wood in the USA, Canada, New Zealand, Europe and India (Hatvani, 2008), and on the basidioma surface of wild *P. ostreatus* (Kredics *et al.*, 2009). This suggests that the two species may occupy different ecological and trophic niches in nature (Hatvani, 2008). *Trichoderma pleuroti* and *T. pleuroticola* usually co-occur in the cultivation substrate, with no clear dominance of one or other species (Hatvani, 2008), although the latter was found to be more aggressive (Hatvani, 2008). The role of *T. harzianum* in the *P. ostreatus* green mould disease is

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Published online 2 November 2018

Plant Pathology

still not clear. Woo *et al.* (2009) identified the majority of isolates pathogenic to *P. ostreatus* from Italian mushroom farms as *T. pleuroticola* and *T. harzianum* and less commonly as *T. pleuroti*. Hatvani *et al.* (2012) identified *T. pleuroti* and *T. pleuroticola* as causal agents of the green mould disease from samples obtained from Croatian farms. In the study of Innocenti & Montanari (2014), *T. pleuroti* and *T. pleuroticola* were isolated from areas of the cultivation substrate with symptoms, whereas *T. harzianum* was isolated only from symptomless areas.

Disease control is commonly based on the application of fungicides. Because studies on fungicide efficacy on cultivated mushrooms by agrochemical companies are rare, only few fungicides have been officially recommended. Currently only prochloraz is allowed for use in mushroom farms in Italy. The few studies carried out on the sensitivity of *T. harzianum*, *T. pleuroti* and *T. pleuroticola* to fungicides, specifically to prochloraz, showed that *T. pleuroti* and *T. pleuroticola* were more sensitive than *T. harzianum* (Hatvani *et al.*, 2012; Innocenti & Montanari, 2014).

The objectives of the present work were to study: (i) the role of *T. harzianum* in green mould disease; (ii) the sensitivity to prochloraz of *Trichoderma* species pathogenic to *P. ostreatus* on wheat straw agarized medium; (iii) the effect of prochloraz on green mould disease under conditions similar to those of mushroom farms; and (iv) the *Trichoderma* × *P. ostreatus* interaction type.

Materials and methods

Trichoderma strains: origin and species identification

Trichoderma strains were isolated from green mould-affected and symptomless samples of wheat straw substrate of *P. ostreatus* from a mushroom farm located in Emilia Romagna, Italy. Samples were homogenized with a blender. About 10 g (fresh weight) of each homogenized sample was placed in a 300 mL flask containing 90 mL extraction solution (0.1% sodium pyrophosphate), shaken for 10 min at 360 rpm and filtered through sterile cheesecloth. Samples were then diluted in a Ringer's solution and 100 μL aliquots plated on Petri dishes containing *Trichoderma* semiselective medium (TSM; Smith *et al.*, 1990). Dilutions and plates were repeated twice for each sample. After 3 days of incubation at 25 °C, *Trichoderma* colonies were transferred to malt extract agar (MEA; Difco) plates at 22 °C until sporulation occurred. *Trichoderma* spores were then suspended and diluted in sterile distilled water, Tween 20 added, and monosporic colonies were obtained. DNA was extracted from the mycelium by using the NucleoSpin Plant II kit (Macherey-Nagel) following the manufacturer's protocol. A multiplex PCR (SimpliAmp thermal cycler; Applied Biosystems) assay with *tef1* (translation elongation factor 1-α) sequence-based primers FPforw1, FPrev1 and PSrev1 was carried out under the conditions described by Kredics *et al.* (2009), to assess species belonging to the *T. pleuroticola*/*T. pleuroti* complex. Amplification of the *tef1* gene fragment was performed as follows: 1 cycle at 94 °C for 5 min; 35 cycles at 94 °C for 45 s, 63 °C for 45 s, and 72 °C for 1 min; and a final elongation at 72 °C for 10 min (Hatvani *et al.*, 2007, modified). Amplification products were separated by electrophoresis on 1.5% agarose gel in 1× TAE buffer at 100 V for about 45 min, stained with ethidium bromide and visualized under UV light.

For *Trichoderma* strains that were not identified as *T. pleuroti*/*T. pleuroticola*, the internal transcribed spacer (ITS) region of ribosomal DNA was amplified by PCR using ITS1f and ITS4 as fungal primers (White *et al.*, 1990; Gardes & Bruns, 1993). Sequencing of amplified ITS was performed by Macrogen Inc. (South Korea). Taxonomic identifications were performed comparing retrieved sequences with those available in the online databases provided by the National Centre for Biotechnology Information (NCBI) using the BLAST search program (Altschul *et al.*, 1997). For *T. harzianum* strains, a new characterization was carried out by sequencing the *tef1* fragment (Bisset *et al.*, 2015; Chaverri *et al.*, 2015) using the primers TEF1LLErev (Jaklitsch *et al.*, 2005) and EF1-728 F (Carbone & Kohn, 1999). All strains were maintained in potato dextrose broth (PDB; Difco) with added 15% glycerol at −80 °C, and deposited in the culture collection of the Department of Agricultural and Food Sciences, University of Bologna under AFS codes.

The commercial *P. ostreatus* 'Spoppo' (Sylvan) widely cultivated in Italian farms was used.

Effect of prochloraz on *Trichoderma* colony growth and spore germination

The experiment was conducted on wheat straw extract agar (WSEA; Rajendran *et al.*, 1991) amended with the fungicide prochloraz (Sponix Flow, 450 g L^{-1}; Adama Makhteshim Ltd) at 0.01, 0.05, 0.25, 1.25 μL L^{-1} doses, poured into 9 cm plates (Da Silva & Neves, 2005). The concentration range, dilution factor 1:5, was chosen to include a maximum dose of 1.25 μL L^{-1}, which is the field dose usually applied in Italian mushroom farms. The fungicide was added to the medium after autoclaving at 121 °C, when the medium had cooled to approximately 40 °C. One 0.5 cm diameter MEA plug from an actively growing colony of each fungus was inoculated in the centre of each plate. Untreated WSEA plates inoculated with each fungus acted as controls. Three plates (replicates) were used for each dose, and for the control. Plates were incubated at 25 °C in the dark in a completely randomized experimental design. Colony diameters were measured along two perpendicular axes 2, 4 and 6 days after inoculation. Daily radial growth was then calculated (Reeslev & Kjoller, 1995). The effect of 0.01, 0.05, 0.25, 1.25 μL L^{-1} prochloraz doses was also tested on *P. ostreatus* 'Spoppo' (PoSp) colony growth in WSEA plates.

In spore germination studies, conidia were removed from a 7-day-old colony of each *Trichoderma* isolate growing on MEA, by adding sterile water with Tween 80 to obtain a final concentration of 10^3 spores mL^{-1}. An aliquot of 0.1 mL was spread on the surface of each 9 cm diameter WSEA plate unamended (control) or amended with the fungicide as reported above. The percentage of spore germination was estimated 12 h after inoculation by using an Eclipse TE2000-E microscope (Nikon) at ×600 magnification. Spores were considered germinated when the germ tube length was equal to or greater than the spore width. Four replicates for each prochloraz dose and control were performed.

In vivo assay

In this assay, PoSp, and *Trichoderma* strains Tg 501, Tpa 432 and Tpi 492 were used. *Pleurotus ostreatus* commercial spawn consisted of sterile millet seeds colonized by the mycelium of the

fungus. Each *Trichoderma* strain was cultured on MEA plates at 25 °C for 4 days in the dark, then under natural light, to obtain abundant sporulation. The growing substrate was prepared using a technique similar to that used in mushroom farms. Chopped wheat straw (2–5 cm) was steam pasteurized at 90 °C for 1 h (Sobieralski *et al.*, 2012) and inoculated with the PoSp spawn (2% v/w). For each sample, 100 g of substrate + PoSp was then distributed in a perforated transparent plastic food container (15 × 10 × 8 cm), and prochloraz was applied by adding 20 mL water solution of the fungicide corresponding to 0.05, 0.25 and 1.25 μL L^{-1} doses. Finally, 3 mL of water spore suspension of each *Trichoderma* strain was added. Two densities of *Trichoderma* (10^2 and 10^5 spores L^{-1}) were used separately to simulate low and high level attacks by the pathogen. Each container was then wrapped in a plastic bag closed by a cotton plug. Controls consisted of containers with PoSp + *Trichoderma*, with 20 mL of water. The prochloraz treatment time used was comparable to that on the farms, where the fungicide is applied to the substrate at spawn. Three replicates were performed for each treatment for a total of 72 containers that were located in a growth chamber following a complete randomized design at 23–25 °C and 80% relative humidity. Four weeks later, during the spawn run phase, the colonization of substrate by *Trichoderma* was visually assessed for each container using a five-point scale, where: 0, no colonization; 1, sporadic growth, few small green areas; 2, light growth, <20% of substrate colonized by green mould; 3, medium growth, 20–50% of colonized substrate; 4, heavy growth, >50% colonization by green mould. The colonization rate (index) was then calculated as the mean of the values of the replicates. At the end of the experiment, *T. pleuroticola* and *T. pleuroti* were reisolated from the substrate and identified as previously described.

Interaction between *Trichoderma* and *P. ostreatus*

To verify the mechanism of interaction between *Trichoderma* spp. and PoSp, dual culture and volatile and nonvolatile metabolite techniques were used (Dennis & Webster, 1971a,b; Innocenti *et al.*, 2015). In the dual culture assay, 5 mm diameter MEA plugs from actively growing colonies of PoSp were inoculated on WSEA plates, and 5 mm diameter plugs of *Trichoderma* were inoculated 40 mm apart on the same plates 48 h later. All combinations of *Trichoderma* × PoSp were performed in triplicate. All plates were maintained at 25 °C in the dark. For measuring the competitive capacity of *Trichoderma* versus PoSp, the colonization of substrate by *Trichoderma* was evaluated 24, 48 and 72 h after inoculation using the method of Camporota (1985) with the formula $(D_1 \times 100)/D_2$, where D_1 is the distance covered by *Trichoderma* along the line which connects the two inoculated plugs, and D_2 is the distance between the two plugs (40 mm).

The hyphal interactions were studied on WSEA plates covered by a sterile cellophane layer (Safta) and inoculated as specified above with both fungi. After 24 h of incubation, the cellophane was removed and a 1 cm^2 portion from the interaction area was stained with 0.5% trypan blue (1:1:1; lactic acid:glycerol:water), then inspected by using an Eclipse TE2000-E microscope at ×400 magnification. The types of hyphal interaction were classified as coiling, penetration, vacuolation and coagulation of cytoplasm (Dennis & Webster, 1971c).

The ability of *Trichoderma* to produce nonvolatile metabolite(s) against PoSp colony growth was determined by the method of Dennis & Webster (1971a). A 5 mm diameter plug of each *Trichoderma* strain was inoculated in the centre of a sterile cellophane disc laying on a 9 cm WSEA plate. After 24 h, the cellophane with the colony was removed and a 5 mm diameter plug of PoSp was inoculated in the centre of the plate. The diameter of the PoSp colonies was determined after 48, 72 and 96 h, and compared with that of PoSp WSEA *Trichoderma*-free plates. Four replicates for each treatment were considered.

The ability of *Trichoderma* to produce volatile metabolite(s) active against PoSp mycelial growth was determined by the apparatus of Camporota (1985), consisting of two bottom halves of WSEA plates on top of each other, separated by an inox lid with a central hole closed by a sliding flap. One bottom half was previously inoculated with a 5 mm plug of each *Trichoderma* strain separately, and the other with a 5 mm plug of PoSp. After 72 h of incubation in the dark at 24–25 °C, the hole was opened to allow volatile metabolites from *Trichoderma* to act against PoSp mycelium growth. Then, after 48, 72 and 96 h, the diameter of PoSp colonies was measured as specified above.

All *in vitro* and *in vivo* experiments were repeated once with similar results. The data of one experiment are reported.

Statistical analysis

Data of colony growth rate at different prochloraz doses were analysed by two-way (two factors: fungal strain and fungicide dose) ANOVA, and compared by Student–Newman–Keuls (SNK) test. Data of *Trichoderma* colonization rate were analysed by three-way (three factors: fungal species, fungal density and prochloraz dose) ANOVA, and compared by SNK test. Statistical procedures were carried out with STATGRAPHIC PLUS v. 2.1 software (Statistical Graphics Corp.).

Results

Isolation and identification of *Trichoderma* strains

Fourteen *Trichoderma* strains were selected on the basis of morphological features. The multiplex PCR assay with *tef1* sequence-based primers FPforw1, FPrev1 and PSrev1 showed eight samples with two bands of 447 bp and 218 bp, indicating the presence of *T. pleuroti*, and four samples with a single band of 447 bp, indicating the presence of *T. pleuroticola*. Two samples, AFS 501 and AFS 446, were not amplified (Fig. S1). The ITS sequence of AFS 501 showed 100% identity with *T. harzianum* (MG832456.1), and that of AFS 446 showed 99% identity with *T. harzianum* (MH333257.1) deposited in NCBI. Subsequent identification by *tef1* sequence-based primers TEF1LLErev and EF1-728 F showed 100% identity with *T. guizhouense* ex-type HGUP 0038/CBS 131803 (JN215484) for both strains. The *tef1* sequences of *T. guizhouense* AFS 501 and AFS 446 were deposited in the NCBI GenBank database with accession numbers MH922983 and MH922984, respectively.

In a preliminary dual plate assay, all *T. pleuroti* strains showed similar antagonistic activity towards *P. ostreatus* colony growth. Two strains, *T. pleuroti* AFS 492 (Tpi 492) and AFS 497 (Tpi 497), were used in *in vitro* experiments. *Trichoderma pleuroticola* strains also showed similar antagonistic activity against *P. ostreatus*, and two

strains, AFS 432 (Tpa 432) and AFS 488 (Tpa 488), were selected. Both *T. guizhouense* AFS 446 (Tg 446) and AFS 501 (Tg 501) were also used.

Effect of prochloraz on *Trichoderma* colony growth and spore germination

The colony growth rate of *Trichoderma* strains at different prochloraz doses are reported in Table 1. Two-way ANOVA indicated that for all *Trichoderma*, the dose factor was significant ($P < 0.05$), whereas the strain factor and the interaction between the two factors were not significant. For Tg 446 and Tg 501, the highest doses of prochloraz, 0.25 and 1.25 μL L^{-1}, significantly reduced the mean colony growth rate by 22.3% and 86.7%, respectively compared with the untreated control. For Tpa 432 and Tpa 488, all doses significantly reduced the mean colony growth rate. The 1.25 μL L^{-1} dose completely inhibited colony growth and the 0.25 μL L^{-1} dose reduced mean colony growth by 89.5%, while the two lowest doses (0.05 and 0.01 μL L^{-1}) decreased the mean colony growth rate by 45.4% and 19.0%, respectively, for Tpa 432 and Tpa 488. For Tpi 492 and Tpi 497, all doses significantly reduced the mean colony growth rate. The 1.25 and 0.25 μL L^{-1} doses completely inhibited colony growth, while the 0.05 and 0.01 μL L^{-1} doses decreased mean colony growth rate by 69.3% and 15.1%, respectively, for Tpi 492 and Tpi 497.

None of the prochloraz doses showed any effect on PoSp colony growth. The values of colony growth were 10.2 ± 0.0 mm per day for the control, and 10.1 ± 0.0, 10.1 ± 0.1, 10.0 ± 0.1 and 9.8 ± 0.5 mm per day for prochloraz doses of 0.01, 0.05, 0.25 and 1.25 μL L^{-1}, respectively.

The effect of prochloraz on spore germination of *Trichoderma* strains is reported in Table 2. Complete inhibition of all *Trichoderma* strains occurred at 1.25 μL L^{-1}, and of both Tpi strains at 0.25 μL L^{-1}. The spore germination of both Tpa strains gradually decreased from 0.05 to 0.25 μL L^{-1}, and of both Tpi strains from 0.01 to 0.05 μL L^{-1}.

In vivo assay

Because the two strains of each *Trichoderma* showed similar results in *in vitro* experiments, only one strain of each species, namely Tpa 432, Tpi 492 and Tg 501, was used for the *in vivo* assay. No symptoms of green mould disease were observed in any containers inoculated with Tg 501. The growth of PoSp mycelium was optimal in the untreated control inoculated with the highest *Trichoderma* concentration. Colonies of Tg 501 developed from samples of infected control substrate plated on TSM.

Table 1 Colony growth rate (mm day^{-1}) of *Trichoderma guizhouense* 446 and 501, *T. pleuroticola* 432 and 488, and *T. pleuroti* 492 and 497 at different prochloraz doses.

	Prochloraz dose (μL L^{-1})				
Trichoderma	0	0.01	0.05	0.25	1.25
T. guizhouense					
446	32.5 ± 2.1	32.8 ± 1.9	32.8 ± 2.9	27.3 ± 1.3	3.9 ± 1.4
501	34.8 ± 1.4	34.8 ± 2.6	35.8 ± 1.6	24.8 ± 1.2	5.2 ± 1.4
Mean	33.6 ± 2.0 c	33.8 ± 2.3 c	34.3 ± 2.7 c	26.1 ± 1.8 b	4.5 ± 1.4 a
T. pleuroticola					
432	37.4 ± 1.9	31.3 ± 1.3	21.4 ± 2.8	4.3 ± 1.6	0.0 ± 0.0
488	38.8 ± 1.9	30.3 ± 1.5	20.3 ± 1.9	3.7 ± 1.1	0.0 ± 0.0
Mean	38.1 ± 1.9 e	30.8 ± 1.4 d	20.8 ± 5.2 c	4.0 ± 8.7 b	0.0 ± 0.0 a
T. pleuroti					
492	26.1 ± 1.3	22.4 ± 1.4	8.1 ± 2.1	0.0 ± 0.0	0.0 ± 0.0
497	26.8 ± 1.9	22.3 ± 1.0	8.1 ± 1.8	0.0 ± 0.0	0.0 ± 0.0
Mean	26.4 ± 2.8 d	22.4 ± 1.1 c	8.1 ± 1.8 b	0.0 ± 0.0 a	0.0 ± 0.0 a

For each *Trichoderma* species, the dose factor was significant, whereas the strain and the dose × strain interaction factors were not significant according to two-way ANOVA ($P < 0.05$). The data are the means ± SD. Data in the row followed by the same letter did not differ significantly according to Student-Newman-Keuls test ($P < 0.05$).

Table 2 Effect of different prochloraz doses on spore germination (%) of *Trichoderma guizhouense*, *T. pleuroticola* and *T. pleuroti* strains, 12 h after inoculation.

	T. guizhouense		*T. pleuroticola*		*T. pleuroti*	
Dose (μL L^{-1})	446	501	432	488	492	497
0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
0.01	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	80.0 ± 5.0	70.3 ± 2.1
0.05	100 ± 0.0	100 ± 0.0	59.7 ± 4.5	67.3 ± 8.7	35.0 ± 7.0	36.7 ± 2.9
0.25	60.3 ± 6.5	72.6 ± 7.8	25.3 ± 2.1	22.7 ± 3.5	0.0 ± 0.0	0.0 ± 0.0
1.25	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

The data are the means ± SD.

Table 3 Multifactor ANOVA for substrate colonization by *Trichoderma pleuroticola* 432 and *T. pleuroti* 492 during spawn run phase of *Pleurotus ostreatus* 'Spoppo' cultivation cycle.

Source	d.f.	Sum of squares	Mean square	*F*
Trichoderma species (TS)	1	36.75	36.75	190.56***
Trichoderma concentration (TC)	1	3.0	3.0	15.56***
Prochloraz dose (D)	3	79.1667	26.3889	136.83***
TS × TC	1	0.75	0.75	3.89
TS × D	3	18.4167	6.13889	31.83***
TC × D	3	1.16667	0.388889	2.02
Error	35	6.75	0.192857	
Corr. total	47	146		

***$P \leq 0.001$.

Molecular identification confirmed that the fungus was present, but it was not responsible for the green mould disease. Therefore, Tg 501 data were not considered for statistical analysis. Multifactor ANOVA (Table 3) based on Tpa 432 and Tpi 492 data indicated that *Trichoderma* species (TS), *Trichoderma* concentration (TC) and prochloraz dose (D) factors, and the interaction TS × D were significant ($P \leq 0.001$). Figure 1 shows the effect of any single factor on colonization growth rate by *Trichoderma*. Tpi 492 colonized the substrate more efficiently than Tpa 432 (Fig. 1a), and 10^5 spores mL^{-1} concentration determined the highest colonization (Fig. 1b). Independently of *Trichoderma* species and spore concentration, 1.25 and 0.25 μL L^{-1} fungicide doses significantly reduced substrate colonization (Fig. 1c). Data of TS × D interaction are reported in Table 4. In the untreated substrate, *Trichoderma* species showed different colonization scores: 2.2 for Tpa 432 and 4.0 for Tpi 492. Upon prochloraz treatment, the growth of Tpi 492 was significantly inhibited only at 1.25 μL L^{-1}, whereas that of Tpa 432 was inhibited both at 0.25 and 1.25 μL L^{-1}.

Trichoderma × *Pleurotus* interaction

The substrate colonization values of Tg 501, Tpa 432 and Tpi 492 in dual culture with PoSp are reported in Table 5. Tpi 492 was the most, and the Tg 501 the least competitive strain. After 4 days, Tpa 432 and Tpi 492 colonies were able to completely overgrow the PoSp colony with intense conidiation, whereas Tg 501 did not overgrow the PoSp colony after contact. No *Trichoderma* strain caused any inhibitory effect by volatile or nonvolatile metabolites on PoSp growth (data not shown). Regarding hyphal interactions, most of the *Trichoderma* hyphae grew parallel to PoSp hyphae, and numerous hyphal tips of PoSp were dichotomous. Coiling and hyphal penetration were not observed.

Discussion

Pleurotus ostreatus is one of the most cultivated mushrooms worldwide, and its production is increasing;

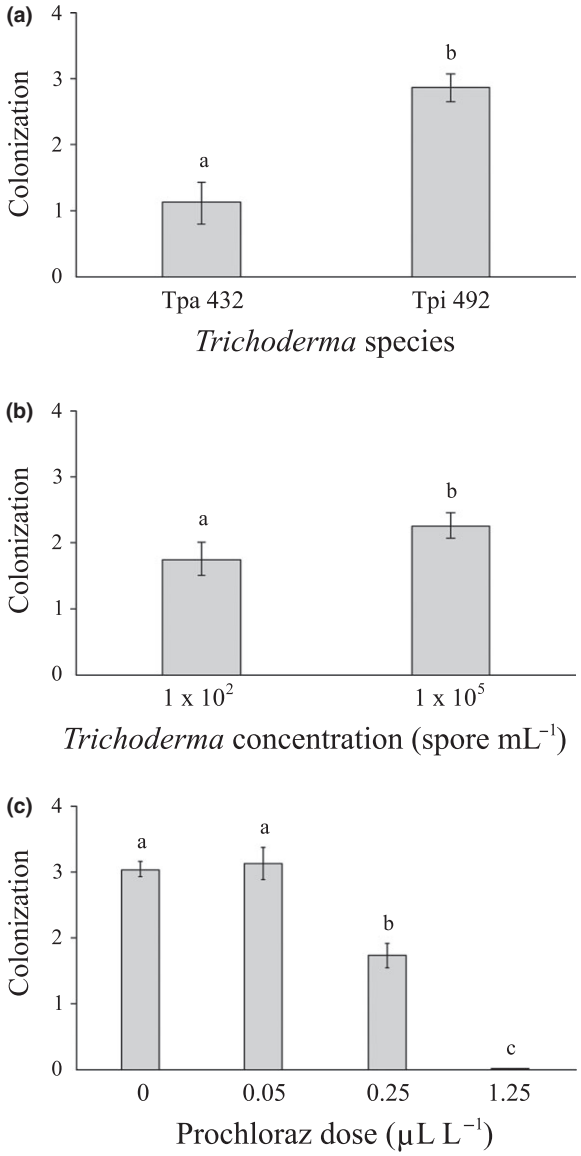

Figure 1 *In vivo* assay: main effects of each factor ($P \leq 0.0001$), *Trichoderma* species (a), *Trichoderma* concentration (b), and prochloraz dose (c) on substrate colonization rate (0–4) by *T. pleuroticola* (Tpa 432) and *T. pleuroti* (Tpi 492) during spawn run. Each error bar represents SD. Different letters indicate significant differences according to Student–Newman–Keuls test ($P < 0.05$).

Table 4 *In vivo* assay: effect of different doses of prochloraz on substrate colonization (0–4) by *Trichoderma pleuroticola* (Tpa 432) and *T. pleuroti* (Tpi 492) 3 weeks after prochloraz treatment.

	Prochloraz dose (μL L^{-1})			
Species	0	0.05	0.25	1.25
Tpa 432	2.2 ± 1.0 aB	2.3 ± 0.8 aB	0.0 ± 0.0 aA	0.0 ± 0.0 aA
Tpi 492	4.0 ± 0.0 bB	4.0 ± 0.0 bB	3.5 ± 0.8 bB	0.0 ± 0.0 aA

Mean values ± SD followed by the same uppercase letter in a row and by the same lowercase letter in a column are not significantly different according to Student-Newman-Keuls test ($P < 0.05$).

Table 5 Colonization rate (0–100) of wheat straw extract agar substrate by *Trichoderma guizhouense* (Tg 501), *T. pleuroticola* (Tpa 432) and *T. pleuroti* (Tpi 492) in dual culture with *Pleurotus ostreatus* 'Spoppo' measured 24, 48 and 72 h after *Trichoderma* inoculation.

Time (h)	Tg 501	Tpa 432	Tpi 492
24	16.7 ± 2.9 a	24.2 ± 1.4 b	40.8 ± 3.8 c
48	34.2 ± 2.9 a	55.8 ± 1.4 b	66.7 ± 2.9 c
72	Contact[a]	Overgrowth[b]	Overgrowth[b]

Mean values ± SD followed by the same letter in a row are not significantly different according to Student-Newman-Keuls test ($P < 0.05$).
[a]Mutual inhibition at mycelial contact.
[b]Overgrowth of *Trichoderma* on *P. ostreatus* mycelium.

however, it is susceptible to a variety of diseases. One of the most serious diseases is the green mould caused by some *Trichoderma* species, i.e. *T. harzianum*, *T. pleuroticola* and *T. pleuroti* (Hatvani *et al.*, 2007, 2012; Woo *et al.*, 2009). The role of *T. harzianum* is not still clear. The *T. harzianum* isolate here, reidentified as *T. guizhouense* (Bisset *et al.*, 2015; Chaverri *et al.*, 2015), was not responsible for the disease, even when it was inoculated in the growing substrate at a high concentration. This finding is in accordance with Komon-Zelazowska *et al.* (2007), Hatvani (2008) and Hatvani *et al.* (2012), who stated that *T. pleuroticola* and *T. pleuroti* were the causal agents for the oyster mushroom green mould disease, and is in contrast to Woo *et al.* (2009) who considered *T. harzianum* problematic to *P. ostreatus*. Hatvani (2008) and Hatvani *et al.* (2012) reported that *T. harzianum* was not detected from green mould-affected samples of the growing substrate collected from oyster mushroom farms. The present study found that *T. pleuroti*, in both *in vitro* and *in vivo* experiments, was more aggressive than *T. pleuroticola* against *P. ostreatus*.

Prochloraz is the only fungicide registered for application in Italian mushroom farms. Few studies have investigated the sensitivity to fungicides of *Trichoderma* species pathogenic to *P. ostreatus* (Hatvani, 2008; Woo *et al.*, 2009; Hatvani *et al.*, 2012). In these studies prochloraz controlled spore germination and mycelium growth of *Trichoderma* isolates without negative effects to *P. ostreatus* (Hatvani, 2008; Woo *et al.*, 2009). The present study confirmed, as expected, that prochloraz was not toxic to *P. ostreatus*, and that the fungicide was very effective against both *Trichoderma* pathogenic species. *Trichoderma pleuroti* and *T. pleuroticola* strains on WSEA plates showed a similar sensitivity to prochloraz at field dose, consistent with the results of Hatvani *et al.* (2012) on yeast extract-glucose medium. These *in vitro* results were confirmed in the *in vivo* assay. Indeed, when the fungicide was applied at field dose in a small-scale experiment reproducing the spawn run phase of the cultivation cycle, no green mould disease symptoms were observed by either *Trichoderma* pathogenic species.

However, despite the evident efficacy of prochloraz and its widespread use in mushroom farms, the disease is increasing. Based on the present data, it could be hypothesized that prochloraz is able to control the primary infections by airborne spores of *Trichoderma*, but not the secondary ones, which occur when the fungicide concentration has declined and is no longer effective (Potočnik *et al.*, 2015).

It is well known that *Trichoderma* are important biocontrol agents against several plant pathogens, and that they use several mechanisms such as mycoparasitism, antibiosis, competition for carbon, nitrogen and other growth factors, together with competition for space or specific infection sites (Harman, 2006). Here, it was found that the mycelium of *Trichoderma* was more competitive than oyster mushroom mycelium for space and nutrients, whereas neither hyphal interaction nor any effect by volatile or nonvolatile metabolites was observed. It is supposed that enzyme systems such as proteases, lipase, chitinase and glucanase could also be involved in the mycoparasitic potential of *T. pleuroti* and *T. pleuroticola* towards *P. ostreatus*, as showed by Hatvani (2008).

Finally, this study has provided evidence that, under the given experimental conditions, *T. guizhouense* was not responsible for green mould disease, and that prochloraz is effective against both *T. pleuroticola* and *T. pleuroti*. However, the treatment at spawn is not sufficient to ensure protection during the whole oyster mushroom cultivation cycle. Unfortunately, only one fungicide application is possible, because the substrate is bagged immediately after spawn. Therefore, it is important to prevent contamination by *Trichoderma* spores by improving farm hygiene.

Acknowledgements

The authors thank Dr Federico Magnani, Fungar, Coriano, RN, Italy, for his suggestions for the *in vivo* assay and Dr Antonio Prodi, Distal, University of Bologna, Italy, for assistance in *T. guizhouense* identification.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. DNA fragments amplified from 14 isolates of *Trichoderma* spp. in a multiplex PCR using primers specific for *Trichoderma pleuroti* and *T. pleuroticola*. Lane M, Bench Top 100 bp DNA ladder (Promega); lanes 1, 2, 3 and 6, *T. pleuroticola*; lanes 4, 5, 7, 8, 9, 10, 11 and 12, *T. pleuroti*; lanes 13 and 14, no DNA amplification; lane 15, negative control (no template DNA). Lanes 2 and 6, *T. pleuroticola* AFS 432 and AFS 488, respectively; lanes 7 and 9, *T. pleuroti* AFS 492 and AFS 497, respectively; lanes 13 and 14, AFS 446 and AFS 501, respectively.