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

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

G. Innocenti, M. Montanari, H. Righini and R. Roberti*
Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Viale G. Fanin, 46, Bologna, Italy.

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*Corresponding Author: R. Roberti
roberta.roberti@unibo.it

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Abstract

The green mould disease causes serious economic losses in *Pleurotus ostreatus* crop worldwide and also in Italy, where prochloraz is the only chemical fungicide allowed to ntrol the disease. The effectiveness of the doses 0.01, 0.05, 0.25 and 1.25 µL L^{-1} (field se) 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 showed by field dose of prochloraz, and for *T. pleuroti* also by 0.25 L^{-1}. Complete inhibition of spore germination occurred for all *Trichoderma* strains at field se, and at 0.25 µL L^{-1} for *T. pleuroti* strains. In *in vivo* assay, the effect of prochloraz doses 5, 0.25 and 1.25 µL L^{-1} on colonisation of straw substrate by *T. pleuroti*, *T. pleuroticola* d *T. guizhouense* inoculated at two spore density (1×10^2 and 1×10^5 spores mL^{-1}) immediately after *P. ostreatus* spawn was studied. *Trichoderma pleuroti* and *T. pleuroticola* were both responsible of green mould disease, whereas *T. guizhouense* was not pathogenic. *Trichoderma pleuroti* was more aggressive than *T. pleuroticola*. Prochloraz was effective inst *T. pleuroti* at the field dose, and against *T. pleuroticola* at 0.25 and 1.25 µL L^{-1} doses. Our 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 non-volatile metabolites occurred.

Introduction

Pleurotus ostreatus (Jacq.) Kumm., commonly known as oyster mushroom, is one of the most commercially important edible mushroom 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 problem in *P. ostreatus* crop. Typical symptoms of the disease are green sporulation areas on the surface of cultivation substrate that is exposed to green mould infection mostly during spawn run. Massive attacks of the disease were reported in South Korea, where 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 was also reported in North America (Sharma & Vijay, 1996). Serious cases of the green mould were detected in *P. ostreatus* farms in Italy (Woo *et al.*, 2004; Alfonzo *et al.*, 2008; Woo *et al.*, 2009; Innocenti & Montanari, 2014). Studies by Park *et al.* (2006) and Komon-Zelazowska *et al.* (2007) showed that two species of *Trichoderma*, *T. pleuroti* S. H. Yu & M.S. Park (2006; previously *T. pleurotum*) and *T. pleuroticola* S. H. Yu & M. S. Park (2006), 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* Samuels & W. Gams, the responsible of the green mould disease of *Agaricus bisporus* (Lange) Imbach (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 been found only 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 USA, Canada, New Zealand, Europe, India (Hatvani, 2008), and on 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-occurred in the cultivation substrate with no clear dominance of one or the other species (Hatvani, 2008), the latter was found to be more aggressive than *T. pleuroti* (Hatvani, 2008). The role of *T. harzianum* in the *P. ostreatus* green mould disease is 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

symptomatic areas of cultivation substrate, whereas *T. harzianum* was isolated only from disease symptomless areas.

The disease control is commonly based on the application of fungicides. Since 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 are 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 agarised medium; iii) the effect of prochloraz on green mould disease under conditions similar to those of mushroom farm; and iiii) *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 homogenised samples were 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 cheese cloth. Samples were then diluted in a Ringer's solution and 100 μL aliquots plated on Petri dishes containing *Trichoderma* Semi-selective Medium (TSM; Smith *et al.*, 1990). Dilutions and

plates were repeated twice for each sample. After three days of incubation at 25° C, *Trichoderma* colonies were transferred on Malt Extract Agar (MEA; Difco Labo-ratories, Detroit) plates at 22° C until the sporulation occurred. *Trichoderma* spores were then suspended and diluted in sterile distilled water, added with Tween 20 and monosporic colonies were obtained. DNA was extracted from the mycelium by using the NucleoSpin Plant II kit (MACHEREY-NAGEL, Düren, Germany) following the manufacturer protocol. A multiplex PCR (SimpliAmp Thermal Cycler, Applied Biosystems, CA, USA) assay with *tef1* (translation elongation factor 1-α) sequence-based primers FPforw1, FPrev1, PSrev1 was carried out under the conditions described by Kredics *et al.* (2009), to assess the belonging to the *T. pleuroticola/T. pleuroti* complex. Amplification of the fragment of *tef1* gene 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/cm 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 Polimerase Chain Reaction (PCR) using ITS1f and ITS4 as fungal primers (White *et al.*, 1990, Gardes & Bruns, 1993). Sequencing of amplified ITS was performed by Macrogen Inc. (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)

added with 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, Somycel, Langeais, France) widely cultivated in Italian farms was utilised.

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

The experiment was conducted on wheat straw extract agar (WSEA, Rajendran *et al.*, 1991) ended with the fungicide prochloraz (Sponix Flow, 450 g L^{-1}, Adama Makhteshim LTD, er-Sheva, Israel) 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 considering as ximum concentration a 1.25 µL L^{-1} dose, which is usually applied in Italian mushroom farms (field dose). The fungicide was added to the medium after autoclaving at a temperature of approximately 40 °C. One 0.5 cm diam. plug from a malt extract agar (MEA, Difco) actively growing colony of each fungus was inoculated in the centrum 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 randomised experimental design. Colony diameters were measured along two perpendicular axes two, four and six 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 towards *P. ostreatus* Spoppo (PoSp) colony growth in WSEA plates.

In spore-germination studies, conidia were removed from 7-d-old colony of each *Trichoderma* isolate growing on MEA, by adding sterile water with Tween 80 to obtain the al concentration of 10^3 spores mL^{-1}. An aliquot of 0.1 mL was spread on the surface of

each 9 cm diam. 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 a Nikon ECLIPSE TE2000-E microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands) 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, Tg 501, Tpa 432 and Tpi 492 were used. *Pleurotus ostreatus* commercial spawn consisted in sterile millet seeds colonised by the mycelium of the fungus. Each *Trichoderma* strain was cultured on MEA plates at 25 °C for four days in the dark, then under natural light, to obtain an abundant sporulation. The growing substrate was prepared by using the following technique similar to that used in mushroom farm. Chopped wheat straw (2-5 cm) was steam pasteurised at 90 °C for 1 h (Sobieralski *et al.*, 2012) and inoculated with the PoSp spawn (2% v/w). An amount of 100 g of substrate + PoSp was then distributed in each perforated transparent plastic food container (15×10×8 cm), and prochloraz was applied by ding 20 mL water solutions of the fungicide corresponding to 0.05, 0.025 and 1.25 µL L^{-1} doses. Finally, 3 mL of water spore suspension of each *Trichoderma* strain was added. Two nsity of *Trichoderma* (1×10^2 and 1×10^5 spores L^{-1}) were used separately to simulate a low and a massive attack by the pathogen. Each container was then wrapped in a plastic bag closed by a cotton plug. Controls consisted in containers with PoSp + *Trichoderma*, added with 20 mL of water. The time of prochloraz treatment we used is comparable with that of the farm, where the fungicide is applied to 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 relative humidity at 80%. Four weeks later, during the spawn run phase, the colonisation of substrate by *Trichoderma* was visually assessed for each container using a five-point scale where: 0, no colonisation; 1, sporadic growth, few small green areas; 2, light growth, less than 20% of substrate colonized by green mould; 3, medium growth, 20-50% of colonized substrate; 4, heavy growth, more than 50% colonisation by green mould. The colonisation rate (index) was then calculated as the mean of the value of the replicates. At the end of the experiment, Tpa and Tpi were re-isolated from substrate and identified as previously described.

Interaction between *Trichoderma* and *Pleurotus ostreatus*

To verify the mechanism of interaction between *Trichoderma* spp. and PoSp, dual culture and volatile and non-volatile metabolite techniques were utilised (Dennis & Webster, 1971a, 1971b; Innocenti *et al.*, 2015). In dual culture assay, 5-mm diam. plugs from actively MEA growing colonies of PoSp were inoculated on WSEA plates, and 5-mm diam. plugs of *Trichoderma* were inoculated 40 mm apart in the same plates 48 h later. All combinations *Trichoderma*- PoSp were performed in triplicate. All plates were maintained at 25 °C in the dark. For measuring the competitive capacity of *Trichoderm*a *vs* PoSp, the colonisation of substrate by *Trichoderma* was evaluated 24, 48 and 72 h from inoculation using the method of Camporota (1985) with the formula (D1 × 100)/D2, where D1 is the distance covered by *Trichoderma* along the line which connects the two inoculated plugs, and D2 is the distance between the two plugs (40 mm).

The hyphal interactions were studied on WSEA plates covered by a sterile cellophane layer (Safta, Piacenza, Italy) and inoculated as specified above with both fungi. After 24 h of ubation, the cellophane was removed and a portion of 1 cm^2 from interaction area was

stained with 0.5% Trypan blue (1:1:1; lactic acid:glycerol:water), then inspected by using a Nikon ECLIPSE TE2000-E microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands) 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 non-volatile metabolite/s against Po Sp colony growth was determined by the method of Dennis & Webster (1971a). A 5 mm diam. plug of each *Trichoderma* strain was inoculated in the centrum of a sterile cellophane disc laying on 9 cm WSEA plate. After 24 h, the cellophane with the colony was removed and a 5 mm diam. plug of PoSp was inoculated in the centrum of the plate. 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) constituted by two bottoms of WSEA plates overlapped and separated by an inox lid with a central hole closed by a sliding flap. One bottom 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 by *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 Neuman Keuls (SNK) test. Data of *Trichoderma* colonisation rate were analysed by three-way (three factors:

fungal species, fungal density and prochloraz dose) ANOVA, and compared by SNK test. Statistical procedure was carried out with the software Statgraphic Plus version 2.1 (Statistical Graphics Corp., USA 1996).

Results

Isolation and identification of *Trichoderma* strains

Fourteen *Trichoderma* strains were selected on the base of morphological features. The multiplex PCR assay with *tef1* sequence-based primers FPforw1, FPrev1, PSrev1 showed 8 samples with two bands of 447 bp and 218 bp respectively, indicating positivity to *T. pleuroti*, and 4 samples with a single band of 447 bp, indicating positivity to *T. pleuroticola*. Two samples, AFS 501 and 446, were not amplified (Fig. 1). The ITS sequence of AFS 501 showed 100% identity with *T. harzianum* (MG 832456.1), and that of AFS 446 99% showed identity with T. *harzianum* (MH 333257.1) deposited in NCBI. Subsequent identification by *tef1* sequence-based primers TEF1LLErev and EF1-728 F showed 100% identity with *T. guizhouense* Q.R. Li, McKenzie & Yong Wang bis, ex-type HGUP 0038/CBS 131803 (JN215484) for both strains. The *tef1* sequences of *T. guizhouense* AFS 501 and 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, then two strains namely *T. pleuroti* AFS 492 (Tpi 492) and 497 (Tpi 497) were used in *in vitro* experiments. *Trichoderma pleuroticola* strains also showed similar antagonistic activity against *P. ostreatus*, then two strains namely AFS 432 (Tpa 432) and 488 (Tpa 488) were selected. Both T. *guizhouense* AFS 446 (Tg 446) and 501 (Tg 501) were 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 nificant. For Tg 446 and Tg 501 the highest doses of prochloraz (0.25 and 1.25 µL L^{-1}) nificantly reduced the mean colony growth rate by 22.3% (0.25 µL L^{-1}) and 86.7% (1.25 L^{-1}), respectively compared with untreated control. For Tpa 432 and 488, all doses nificantly reduced the mean colony growth rate. The 1.25 µL L^{-1} dose completely inhibited d 0.25 µL L^{-1} dose reduced by 89.5% colony growth, while the two lowest doses (0.05 and 1 µL L^{-1}) decreased colony growth rate by 45.4 and 19.0%, for Tpa 432 and 488, respectively. For Tpi 492 and 497, all doses significantly reduced the mean colony growth e. The 1.25 µL $L^{-1}$and 0.25 µL L^{-1} doses completely inhibited colony growth, while 0.05 d 0.01 µL L^{-1} doses decreased colony growth rate by 69.3 and 15.1% for Tpi 492 and 497, respectively.

All doses of prochloraz did not show any effect on PoSp colony growth. The values of colony wth varied from 10.2 ± 0.0 (control), 10.1 ± 0.0 (0.01 µL L^{-1}), 10.1 ± 0.1 (0.05 µL L^{-1}), 0 ± 0.1 (0.25 µL L^{-1}) to 9.8 ± 0.5 mm/day (1.25 µL L^{-1}).

The effect of prochloraz on spore germination of *Trichoderma* strains is reported in Table 2. mplete inhibition of all *Trichoderma* strains occurred at 1.25 µL L^{-1} and 0.25 µL L^{-1} doses for Tpi strains. The spore germination of both Tpa strains gradually decreased from 0.05 to 5 µL L^{-1}, and of both Tpi strains from 0.01 to 0.05 µL L^{-1}.

***In vivo* assay**

Since in *in vitro* experiments the two strains of each *Trichoderma* showed similar results, only one strain, namely Tpa 432, Tpi 492 and Tg 501 were used. No symptoms of green mould disease were observed in all 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. Molecular identification confirmed that the fungus was present, but it was not responsible of 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 2 shows the effect of any single factor on colonisation growth rate by *Trichoderma*. As indicated in Fig. 2, Tpi 492 ain colonized the substrate more efficiently than Tpa 432 (Fig. 2A), and 1×10^5 spore mL^{-1} concentration determined the highest colonisation (Fig. 2B). Independently of *Trichoderma* cies and spore concentration, 1.25 and 0.25 µL L^{-1} fungicide doses significantly reduced substrate colonisation (Fig. 2C). Data of TS × D interaction were reported in Table 4. In untreated substrate, *Trichoderma* species showed different colonization rate: 2.2 for Tpa 432 and 4.0 for Tpi 492. Upon prochloraz treatment, the growth of Tpi 492 was significantly ibited only at 1.25 µL L^{-1}, whereas that of Tpa 432 was inhibited both at 0.25 and 1.25 µL .

***Trichoderma –Pleurotus* interaction**

The substrate colonisation 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 less competitive strain. After four days, Tpa 432 and Tpi 492 colonies were able to overgrowth completely PoSp colony with an intense conidiation, whereas Tg 501 did not overgrow PoSp after colony contact. No *Trichoderma* strain caused any inhibitory effect by volatile or not volatile metabolites on Po Sp growth (data not shown). Regarding hyphal interactions, most of *Trichoderma* hyphae grew parallel to PoSp hyphae, and numerous hyphal tips of PoSp were dichotomous. Coiling and hyphal penetration were not observed.

Conclusions

Pleurotus ostreatus is one of the most cultivated mushroom worldwide, its production is increasing, however it is susceptible to a variety of disease. One of the most serious disease is the green mould caused by some *Trichoderma* species i.e. *T. harzianum, T. pleuroticola and T. pleuroti* (Hatvani *et al.*, 2007; Woo *et al.*, 2009; Hatvani *et al.*, 2012). The role of *T. harzianum* is not still clear. Our *T. harzianum* isolate, re- identified as *T. guizhouense* (Bisset *et al*., 2015; Chiaverri *et al*., 2015), was not responsible of the disease, also 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 it is in contrast with Woo *et al*. (2009) that 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. We found that *T. pleuroti* both *in vitro* and *in vivo* experiments was more aggressive than *T. pleuroticola* against *P. ostreatus*.

Prochloraz is the only fungicide registered for the application in Italian mushroom farms. Few studies 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). Our 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, consistently to 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 cultivation cycle, no green mould disease symptoms by both *Trichoderma* pathogenic species were observed.

However, despite of the evident prochloraz efficacy and its diffuse use in mushroom farms, the disease is increasing. Based on our data, we could hypothesize that prochloraz, is able to control the primary, but not the secondary infections by air borne spores of *Trichoderma*, that occur when the fungicide was no longer to be effective, because of the decline of its concentration (Potočnik *et al.*, 2015).

It is well known that *Trichoderma* are important biocontrol agents against several plant pathogens, and that they utilize 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). We found that the mycelium of *Trichoderma* was more competitive than oyster mushroom mycelium for space and nutrients, whereas we did not observe neither hyphal interaction nor effect by volatile or non-volatile metabolites. We suppose that also enzyme systems such as proteases, lipase, chitinase and glucanase could 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 our experimental conditions, T. *guizhouense* was not responsible of the 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 cultivation cycle. Unfortunately, only one fungicide application is possible, because the substrate is bagged immediately after spawn. Therefore, it is important to prevent contaminations by *Trichoderma* spores by improving farm hygiene conditions.

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Legends to Tables

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

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

Table 3. Multifactor ANOVA for substrate colonisation by *Trichoderma pleuroticola* 432 and *T. pleuroti* 492 during spawn run phase of *Pleurotus ostreatus* Spoppo cultivation cycle.

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

Table 5. Colonisation 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 *P. ostreatus* Spoppo measured 24, 48 and 72 h after *Trichoderma* inoculation.

Legend to Figure

Figure 1. 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 100bp DNA Ladder (Promega Corporation, USA); 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, re pectively; lanes 7 and 9, *T. pleuroti* AFS 492 and AFS 497, respectively; lanes 13 and 14, AFS 446 and AFS 501.

Figure 2. In *in vivo* assay: main effects of each factor ($P \leq 0.0001$), *Trichoderma* species (A), *Trichoderma* concentration (B), and prochloraz dose (C) on substrate colonisation 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 SNK test ($P < 0.05$).

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

Trichoderma	Prochloraz dose (μL L^{-1})				
	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 line followed by the same letter did not differ significantly according to SNK test ($P < 0.05$).

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

se (μL L^{-1})	*T. guizhouense*		*T. pleuroticola*		*T. pleuroti*	
	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 colonisation by *Trichoderma pleuroticola* 432 and *T. pleuroti* 492 during spawn run phase of *Pleurotus ostreatus* Spoppo cultivation cycle.

Source	**Df**	**Sum of squares**	**Mean square**	***F* values**
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 x TC	1	0.75	0.75	3.89
TS x D	3	18.4167	6.13889	31.83 ***
TC x D	3	1.16667	0.388889	2.02
Error	35	6.75	0.192857	
Corr. total	47	146		

*** = $P \leq 0.001$.

Table 4. In *in vivo* assay: effect of different doses of prochloraz on substrate colonisation (0-4) by *Trichoderma pleuroticola* (Tpa 432) and *T. pleuroti* (Tpi 492) three 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 upper case letter in a line and by the same lower case letter in a column are not significantly different according to SNK test ($P < 0.01$).

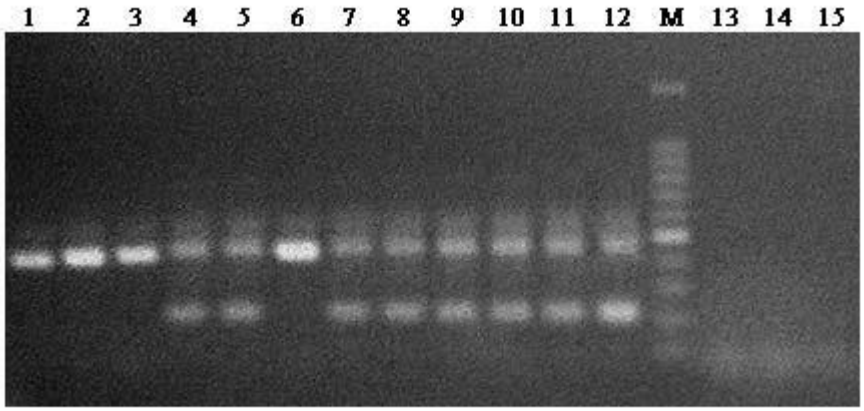
Table 5. Colonisation rate (0-100) of wheat straw extract agar substrate by *Trichoderma guizhouense* (Tg 501), *T. pleuroticola* (Tpa 432) and *T. pleuroti* (Tpi 492) in dual culture with *P. ostreatus* Spoppo measured 24, 48 and 72 h after *Trichoderma* inoculation.

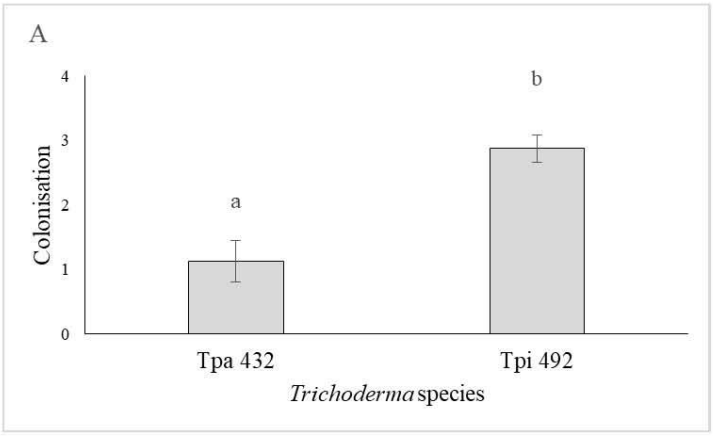
Time (h)	Tg 501	Tpa 432	Tpi 492
24	16.7 ± 2.9 a	24.2 ± 1.4 b	40.8 ± 3.8 c
48	34.2 ± 2.9 a	55.8 ± 1.4 b	66.7 ± 2.9 c
72	contact[a]	overgrowth[b]	overgrowth[b]

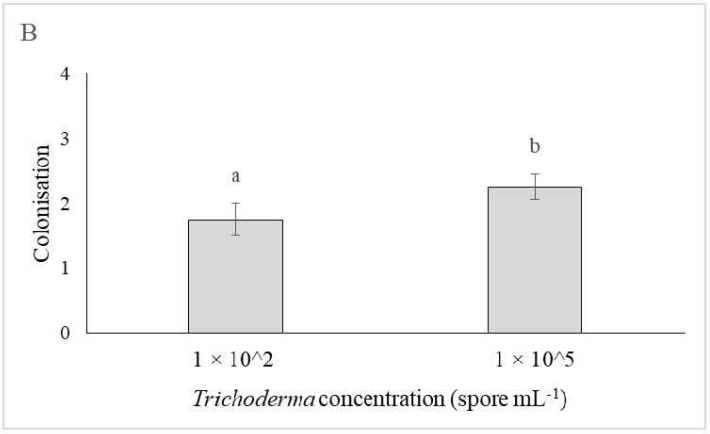
Mean values ± SD followed by the same letter in a line are not significantly different according to SNK test ($P < 0.05$).

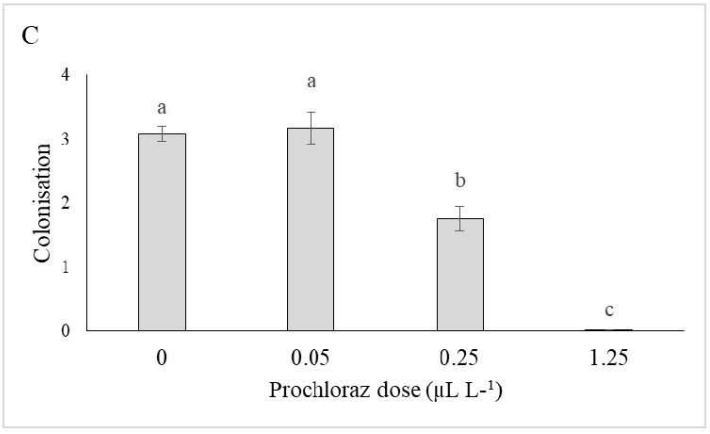
[a] Mutual inhibition at mycelial contact.

[b] Overgrowth of *Trichoderma* on *P. ostreatus* mycelium.




A
Colonisation
4
3
2
1
0
a
b
Tpa 432
Tpi 492
Trichoderma species


B
Colonisation
4
3
2
1
0
a
b
1 × 10^2
1 × 10^5
Trichoderma concentration (spore mL-1)


C
Colonisation
4
3
2
1
0
a
a
b
c
0
0.05
0.25
1.25
Prochloraz dose (µL L-1)