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Trichoderma Species Problematic to the Commercial Production of *Pleurotus* in Italy: Characterization, Identification, and Methods of Control

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Abstract: Nine isolates of *Trichoderma* were obtained from the diverse phases of compost preparation and the production of different commercial varieties of oyster mushrooms *Pleurotus* spp. with the apparent presence of green mould disease. The isolates were morphologically and genetically characterized. Molecular fingerprinting indicated that the isolates belonged to the species *T. pleuroticola* and *T. harzianum*. In order to identify control measures, changes in temperature, pH, and application of fungicides were tested on the present isolates, using known *Trichoderma* biocontrol strains as a reference. Fungicide effects on the growth of *Pleurotus* isolates were also assessed. The optimal growth temperatures were 25 °C for *Trichoderma* isolates and 28 °C for *Pleurotus* isolates, and *Trichoderma* always grew faster than *Pleurotus*. In particular, a reduction of about 30% was recorded for some of the *Trichoderma* isolates when comparing the colony growth at 25 and 28 °C. *Trichoderma* isolates developed well within a wide pH range, with the best growth occurring between pH 5 and 7, whereas *Pleurotus* preferred more alkaline conditions (pH 8 to 9). Prochloraz and metrafenone were found to inhibit *Trichoderma* growth with different dose responses that did not affect the growth of *Pleurotus* spp. In particular, metrafenone was the most effective active ingredient that inhibited the majority of the *Trichoderma* isolates (1–25% growth reduction).

Keywords: *Pleurotus* spp.; *Trichoderma pleuroticola*; *Trichoderma harzianum*; mushroom; molecular characterization; fungicides

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1. Introduction

Filamentous fungi belonging to the genus *Trichoderma* can live as ubiquitous saprophytes in both natural and agricultural ecosystems. They are active secondary opportunists commonly found in the soil, grow rapidly on many diverse substrates, and are prolific producers of spores that are readily transported by wind or dust. In addition, their distribution is associated with the movement of humans, animals, insects, and mites, and they are frequently disseminated by agricultural machinery and products [1–4]. Some species of this genus have been extensively studied as biological control agents and utilized as the active ingredient in many commercial biopesticides and biostimulants [5–7].

In 1985, an epidemic of green mould disease caused by *Trichoderma* spp. surfaced in the production of *Agaricus bisporus* in Northern Ireland, subsequently followed by severe

infestations in mushroom farms in other locations of the United Kingdom, Spain, Germany, the Netherlands, the United States, and Canada. It has been estimated that the worldwide economic losses incurred by the champignon mushroom industry in the 10 years following the initial outbreak exceeded USD 30 million, with production losses oscillating between 30 and 100% [8–16]. In Italy, a problem with *Trichoderma* infestations appeared in the production of another edible commercial mushroom, *Pleurotus ostreatus* (oyster mushroom). The disease initially appeared around 2002 in the northern regions and expanded in the following years to the southern (and other) Mediterranean regions. The disease caused dramatic yield losses and a drastic decrease in production within the oyster mushroom industry [17].

The compost used in industrial mushroom production is host to both innocuous as well as aggressive isolates of *Trichoderma*, which cannot be morphologically distinguished from one another [13,18–20]. The richness of the compost/substrate in holocellulose compounds, i.e., hemicellulose, cellulose, and lignin, which are high energy sources for fungal populations, encourages *Trichoderma* proliferation [21,22]. Among the isolates obtained from the infested *A. bisporus* compost, four different biological forms of *Trichoderma* were found. Two were not pathogenic to *A. bisporus*, namely *T. harzianum* (formerly classified as *T. harzianum* biotype Th1), which taxonomically includes the ex-neotype strain of *T. harzianum* [20] as well as many biological control strains [13,19,23,24], and *T. atroviride* (formerly classified as *T. harzianum* biotype Th3). The pathogenic form found in Europe (formerly classified as *T. harzianum* biotype Th2) [25,26] was identified as *T. aggressivum* forma *europaeum* [13]. Further, the *Trichoderma* species typically found in North America, pathogenic to champignon mushrooms, differed from the European species and was taxonomically designated as *T. aggressivum* f. *aggressivum* (formerly designated as *T. harzianum* biotype Th4 [13,27,28]).

Interestingly, it has been determined that some *Trichoderma* species associated with *Pleurotus* production are taxonomically different from those found aggressive to *A. bisporus*, and these species were identified as *T. pleuroticola* and *T. pleuroti* [23,29,30]. The distribution of *T. pleuroticola* and *T. pleuroti* species in *Pleurotus* compost appears variable; for example, the majority of the Hungarian isolates belonged to *T. pleuroti*, whereas most of the Italian isolates belonged to *T. pleuroticola* [30].

The problem with *Trichoderma* spp. infestions in *Pleurotus* production is relatively new in comparison to the situation with *Agaricus* [13,17,20,31].

As characteristic of all substrates infected with *Trichoderma*, the typical symptoms or signs of the disease can be recognised by the formation of green mould (due to the conidia) on the cultivated mushrooms and compost, which, in cases of acute outbreaks, results in a total loss of production due to contamination [32,33].

T. pleuroticola has been found on the natural substratum of the oyster mushroom, both on the commercially cultivated varieties as well as the wild *P. ostreatus*, whereas *T. pleuroti* has been found only on the cultivated *P. ostreatus* and its substrate [34,35].

Woo et al. [17] reported that *Trichoderma* spp. was only found at the initial phase of substrate preparation and disappeared after pasteurization at 60 °C for 10 h. However, *Trichoderma* spp. was detected again at the spawn run stage when temperatures reached ca. 30 °C. The optimal mycelial growth of the green mould occurred at 80% relative humidity. A substrate of around pH 8–9 may slow the growth of *Trichoderma* spp., resulting in a decrease in infection spread [2].

In the present study, we characterized the *Trichoderma* spp. associated with *Pleurotus ostreatus* and *P. eryngii* displaying signs of green mould disease in order to (1) monitor fungal presence during the processing phases; (2) isolate and identify the associated *Trichoderma*; (3) test potential control methods, in particular by manipulation of physical factors affecting *Trichoderma* spp. growth and development; and (4) test two approved fungicides to determine the effective doses for controlling *Trichoderma*, without producing undesirable consequences for *Pleurotus* spp.

2. Materials and Methods

2.1. Fungal Isolation from Oyster Mushroom Production Chain

Trichoderma spp. were isolated from green mould-infected samples collected during different phases of compost preparation used for growing *Pleurotus ostreatus* and *P. eryngii* at the company Saipan S.r.l. (Cava de Tirreni, Salerno, Italy) and at nearby mushroom houses during the first fruiting stage of oyster mushrooms in 2007. During this period, Saipan produced about 1 million substrate bales (approx. 17 kg each), pre-inoculated with different isolates of *Pleurotus*, which were transferred after about 20 days of incubation to mushroom farms throughout the country.

The samples were collected at different stages of mushroom production. Ten grams of compost material were suspended in 100 mL of distilled water and agitated for one hour at room temperature, and aliquots from a logarithmic dilution series were spread onto potato dextrose agar (PDA; Himedia laboratory, Mumbai, India). Standard microbiological protocols were used for determining the number of colony-forming units (CFUs) over a 5 day period, and for isolating, purifying, and storing the *Trichoderma* isolates. Three biological control agents of *Trichoderma*: *T. atroviride* strain P1 (ATCC74058), *T. atroviride* strain A6, and *T. afroharzianum* strain T22 (ATCC20847) were included in the experiments for comparison to the putative pathogenic isolates.

Different commercial oyster mushroom spawns (or seeds) were obtained from the compost producer comprising eleven strains of *P. ostreatus* (coded as HK45, M6, P6, P15, P24, P77, P107, P145, PC3, PF4, and PLC), and one isolate each of *P. eryngii* (P47, "cardoncello" mushroom), and *Pholiota aegerita* (AFO, "pioppino" mushroom). Regardless of the genus or species, all the oyster mushroom isolates will be referred to hereafter as "*Pleurotus*". The grains of "seed" inoculum were placed on PDA plates to obtain mycelium cultures that were conserved as PDA slants under mineral oil.

2.2. Effect of Temperature on Fungal Growth

Young starter fungal cultures of *Trichoderma* and *Pleurotus* were maintained on PDA at 25 °C. A 5 mm disk of each culture was transferred to the centre of a new Petri plate (90 mm diameter) containing PDA and incubated in the dark at temperatures of 25, 28, and 35 °C to determine the effect of temperature on mycelium development [13,36]. The colony diameter was measured at 24 h intervals. In addition, the time of the appearance of green spores, the characteristics of the colony growth, and the pigmentation of the mycelium were recorded. The experiment consisted of three replicates for each fungal isolate and was repeated on three separate occasions.

2.3. Effect of pH on Fungal Growth

A 5 mm disk of young PDA cultures of *Trichoderma* and *Pleurotus* was transferred to 24-well plates containing 1 mL of Potato Dextrose Broth (PDB, Himedia laboratory, Mumbai, India) adjusted to pH values of 4, 5, 6, 7, 8, or 9 with HCl or KOH (control = PDA at pH 5.5) and incubated at 25 °C in the dark. The colony growth was evaluated at 24 hour intervals by using an established evaluation scale based on five values: 0 = no apparent fungal growth (dead or severely inhibited, dimension of the initial inoculum unchanged), 1 = minimal fungal growth (1 to 25% of the growth of the control, growth barely exceeds the initial inoculum), 2 = medium fungal growth (26 to 50% of the control), 3 = good fungal growth (51 to 75% of the control), and 4 = very good fungal growth (no apparent inhibition, 76 to 100% of the control).

2.4. Genetic Characterization

Genomic DNA was extracted from all *Trichoderma* isolates following the modified protocol of Manganiello et al. [37]. The isolates were grown in PDB on a rotary shaker at 150 rpm for 96 h at 25 °C. Fresh mycelium was collected after vacuum filtration through No. 4 Whatman filter paper (Whatman Biosystems Ltd., Maidstone, UK), frozen in liquid nitrogen, ground to a fine powder, and immediately processed.

Total genomic DNA was extracted from 100 mg of ground mycelium using the Macherey-Nagel™ NucleoSpin™ Soil (MN) (Düren, Germany) according to the manufacturer's protocol. The internal transcribed spacer and translation elongation factor 1α (TEF1) amplifications were performed using Phusion polymerase in a MiniAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Primers ITS-1 (5′-CTTGGTCATTTAGAGG AAGTAA-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify a fragment (~0.6 kb) of rDNA, including ITS-1 and ITS-2 and the 5.8S rDNA gene (White et al., 1990), whereas the 5′ portions of translation elongation factor 1α (~0.8 kb) were amplified with primers TEF1-F (5′-ATGGGTAAGGARGACAAGAC-3′) and TEF1-R (5′-GGARGTACCAGTSATCATGTT-3′) (O'Donnell et al., 1998) by using the following parameters: denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s; annealing at 55 °C for 30 s; extension at 72 °C for 40 s; final extension at 72 °C for 5 min.

Amplicons were separated by gel electrophoresis on 1% *w*/*v* agarose supplemented with SYBR Safe DNA Gel Stain (Invitrogen, Paisley, UK). Amplicon sizes were determined by comparison to a 1 Kb bp DNA ladder marker (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA). PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The concentrations of DNA were determined using a Qubit™ fluorometer with a dsDNA BR Assay Kit™ (Thermo Fisher Scientific, Wilmington, DE, USA). Samples were sent for sequencing to Eurofins Genomics, and then DNA sequences were blasted against the NCBI GenBank and Trichoblast databases; all sequences obtained in this study were deposited to NCBI GenBank.

Phylogenetic relationships among the nine *Trichoderma* isolates were investigated based on ITS and TEF1 sequences. The blasted DNA sequences were aligned using the Clustal W algorithm [38] with MEGAX software [39]. Multiple and pairwise alignment parameters were applied using a gap penalty of 15 and a gap length penalty of 6.66. Final alignment adjustments were made manually to remove artificial gaps. The analysis was conducted separately for the two partial gene sequences. The aligned sequences were then concatemerized and used for phylogenetic reconstruction. The evolutionary history was inferred by using the maximum likelihood method and the Tamura–Nei model [40]. The bootstrap consensus tree inferred from 1000 replicates was considered to represent the evolutionary history of the taxa analyzed. The initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura–Nei model and then selecting the topology with a superior log likelihood value. Evolutionary analyses were conducted using MEGA X.

2.5. Dual Confrontation Assays

The antagonistic activity of *Trichoderma* isolates against *Pleurotus* spp., phytopathogens (*Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani,* and *Sclerotinia sclerotiorum*), and *Trichoderma-Trichoderma* combinations were evaluated using a dual culture assay in vitro. Two mycelial plugs (5 mm diameter), obtained from each 7-day-old fungal culture, were placed at 0.25 mm from the border, on opposite sides of a Petri plate (9 cm diameter) containing PDA, then incubated at 25 °C. Plate cultures with the single isolate of each *Trichoderma* spp., were performed to themselves and to the different pathogens, or *Pleurotus* spp. Evaluation of the radial growth was recorded 7 days post-inoculation.

The growth inhibition percentage was calculated using the following formula:

$$Growth\ inhibition(\%) = 100 - \frac{R - DC}{R}$$

where R = isolate radial growth in the control and DC = radial growth of the same isolate in the dual culture.

2.6. Effect of Fungicides on Fungal Growth

Prochloraz (Mirage® 45 EC, 450 g L^{-1}; Adama Makhteshim Ltd., Beer Sheva, Israel) and metrafenone (Vivando® SC, 500 g L^{-1}; BASF SE, Ludwigshafen am Rhein, Germany) effectively control many ascomycetes and deuteromycetes [41], such as *Trichoderma*, with minimal effects on basidiomycetes, such as *Agaricus* or *Pleurotus*.

A 5 mm disk of a young PDA culture of *Trichoderma* or *Pleurotus* was transferred to a 24-well plate containing 1 mL of PDB with different concentrations of fungicide (control containing untreated PDB) and then incubated at 25 °C in the dark. In particular, prochloraz was applied at 1, 0.7, 0.5, and 0.2 mL L^{-1}, whereas metrafenone was applied at 10, 7, 5, and 2.5 µL L^{-1}.

The colony growth was evaluated daily for the first 7 days using the established measurement scale based on five values: 0 = no apparent growth, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% good fungal growth and colony development compared to the untreated control (PDB).

The same experiment was performed by using a spore suspension of each *Trichoderma* isolate (1×10^5 sp mL^{-1}) to determine the effect on the germination of conidia. The assay was terminated 30 days after spore inoculation. Each experiment included three replicates per treatment and was repeated twice.

2.7. Statistical Analyses

One-way ANOVA was performed using GraphPad Prism Software to test the effects of temperature and fungicide applications on *Trichoderma* strains, as well as on data from the dual culture assay *Trichoderma*-phytopathogens. The analysis of variance was corrected for multiple comparisons by LSD post hoc test considering a p-value ≤ 0.05 for the temperature assay and Tukey test (p-value ≤ 0.05) for all the other assays.

3. Results

3.1. Determination of Microbial Concentration

The number of colony-forming units (CFUs) obtained from the different *P. ostreatus* and *P. eryngii* production phases was variable, and the highest microbial counts were detected during the fermentation and fruitification phases (Table 1). Bacteria were detected in all stages of preparation, even after pasteurization, and were always present in concentrations greater than the fungi, ranging from 2.4×10^8 CFUs per gram of compost (CFU g^{-1}) after pasteurization to 9.5×10^{11} CFU g^{-1} during fermentation. In comparison, *Trichoderma* spp. were found initially in the compost preparation, and at later stages during the incubation and fruiting stages of the *Pleurotus* in concentrations ranging from 7.1×10^2 to 5.8×10^8 CFU g^{-1}, respectively. No fungi were obtained after pasteurization or during the phase immediately after seeding. *Trichoderma* spp. was always identified in the general fungi counts associated with the different processing stages. Further, both bacterial and fungal microbes were detected in the source of recycled water that was used throughout the compost preparation for wetting the substrate material.

Table 1. Microbial concentrations found in compost sampled at different phases of *P. ostreatus* and *P. eryngii* production, the number of colony-forming units (CFU g^{-1}) of bacteria and fungi, and specifically the presence of *Trichoderma* spp.

	Production Phase	Bacteria (CFU g^{-1})	Fungi (CFU g^{-1})	Presence of *Trichoderma*
1	Uninoculated—crude substrate material	5.1×10^9	5.2×10^6	Yes
2	Fermentation tunnel	9.5×10^{11}	2.9×10^8	Yes
3	After treatment in a pasteurization tunnel	2.4×10^8	0	No
4	Seeding of grain spawn, bale packaging	2.5×10^8	0	No
5	Inoculated bale, incubation at 25 °C	6.9×10^8	7.1×10^2	Yes
6	Fruiting stage, incubation at 25 °C in a mushroom grow house	7.1×10^8	5.8×10^8	Yes
7	Recycled water used in production	7.6×10^7	9.2×10^4	Yes

3.2. Fungal Growth and Morphological Characterization

A morphological analysis was conducted on the *Trichoderma* spp. isolated from infected compost (NZ-1 and NZ-2), 20 days after spawn seeding (GP-6-1, GP-6-2, GP-BI-4, GP-BI-6, GP-BI-8), in the phase of *P. ostreatus* fruitification (NZ-3), and in recycled water (GP-2-2).

The nine *Trichoderma* isolates were grown at different temperatures (25, 28, and 35 °C), and optimal mycelial growth and sporulation were observed at 25 °C two days after incubation (Figure 1A). No significant differences were noted in colony growth among the isolates of different *Trichoderma* species at 25 °C with the exception of NZ-2, which was the only isolate that grew better at 28 °C than at 25 °C (Figure 1A,B). In particular, a reduction of 35, 34, 31, and 14% was recorded for NZ-1, T22, A6, and GP-6-2, respectively, comparing the colony diameters at 25 and 28 °C (Figure 1A).

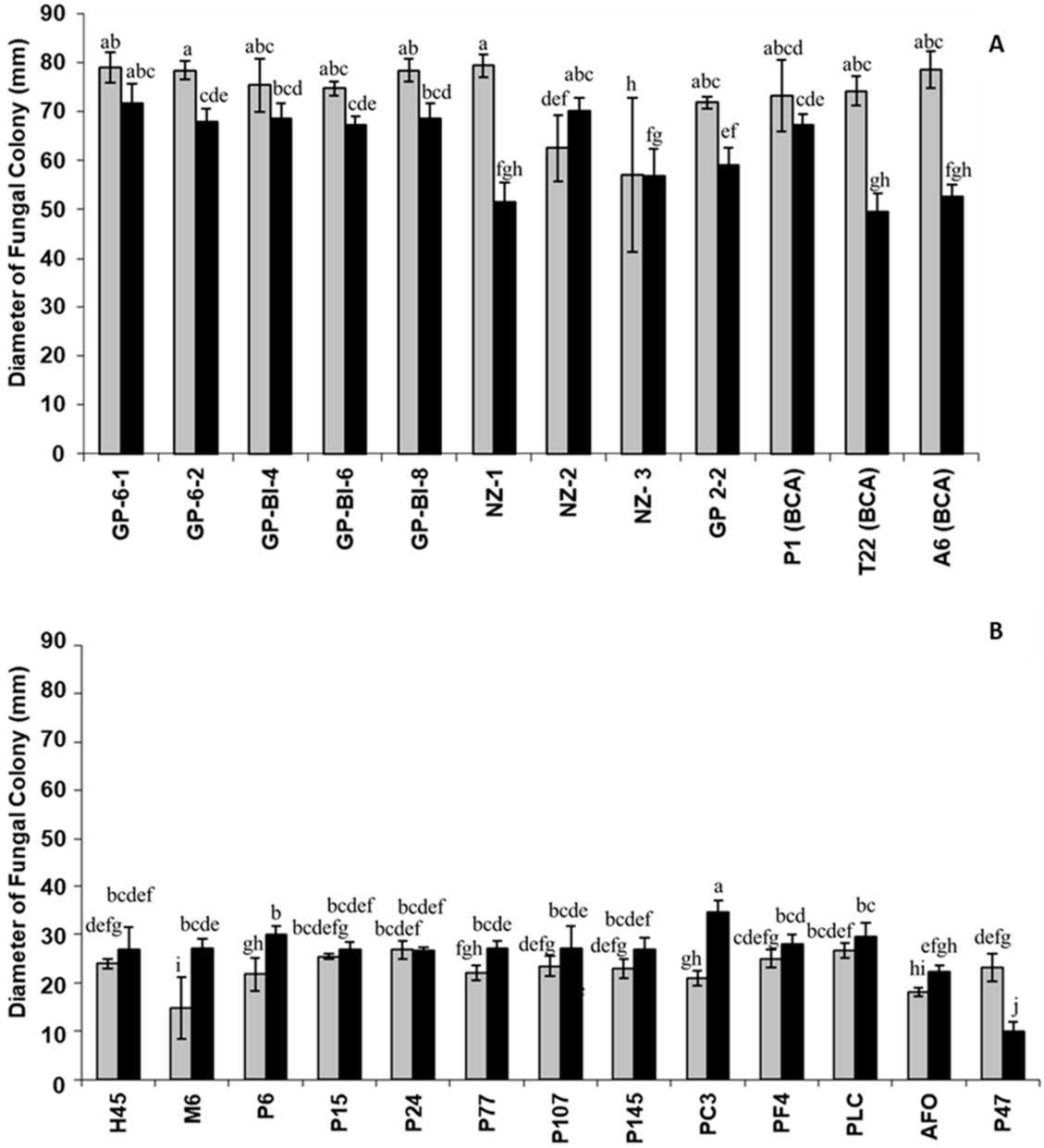

Figure 1. Mycelial growth, determined by measuring the colony diameter (mm) of fungal isolates at 25 °C (grey bars) and 28 °C (black bars) of (**A**) *Trichoderma* at two days and (**B**) *Pleurotus* at three days after inoculation on PDA. Bars with different letters are significantly different (p-value ≤ 0.05) according to one-way ANOVA followed by the LSD post hoc test.

In general, the isolates of *Pleurotus ostreatus* and *Pholiota aergerita* (AFO) preferred a temperature of 28 °C for growth after three days, whereas *Pleurotus eryngii* (P47) grew better at 25 °C (Figure 1B). All *Trichoderma* isolates grew faster than all the tested species of *Pleurotus* at both growing temperatures assessed.

All *Trichoderma* isolates were able to grow over a wide range of pH values, but the optimal growth for most isolates occurred on substrates with a pH value of around 6 to 7 (Figure 2A). In contrast, the *Pleurotus* species grew better on alkaline substrates with the optimum for mycelium development at pH 8 to 9 (Figure 2B).

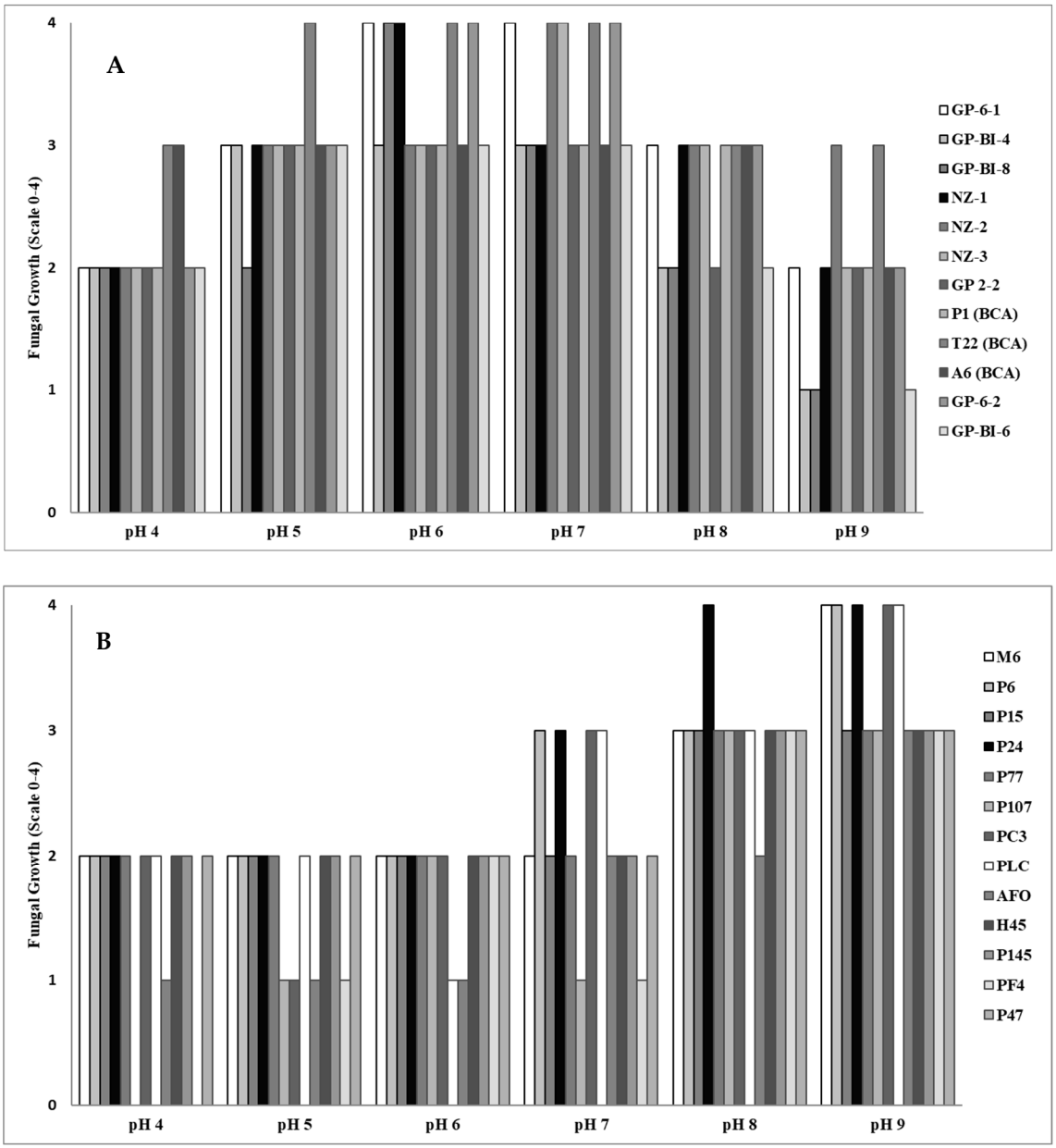

Figure 2. Evaluation of fungal mycelium growth in Potato Dextrose Broth adjusted to different pH values as determined by measurement on a five-value scale, where 0 = no apparent fungal growth, 1 = growth 1–25% of the control (water), 2 = growth 26–50% of the control, 3 = growth 51–75% of the control, and 4 = growth 76–100% of the control of different isolates of (**A**) *Trichoderma* at two days and (**B**) *Pleurotus* at three days after inoculation.

3.3. Genetic and Morphological Characterization of Trichoderma Isolates

Partial sequences of translation elongation factor 1α (TEF1 ~ 600 bp), ribosomal polymerase β subunit (RPB ~ 800 bp), and internal transcribed spacers (ITS ~ 600 bp) genes were submitted to NCBI GenBank under accession numbers as reported in Table 2. Sequences were analyzed separately and compared to the NCBI non-redundant nucleotide database. TEF1 and ITS concatemeric sequences were found to be informative for the identification of new isolates. The multilocus phylogenetic analysis revealed that GP-6-1, GP-6-2, GP-BI-4, GP-BI-6, GP-BI-8, NZ-1, NZ-2, and NZ-3 isolates belonged to *Trichoderma pleuroticola* while strain GP-2-2 was identified as *T. harzianum*. Phylogenetic analysis was conducted with *T. atroviride* P1 and A6, *T. afroharzianum* T22, *T. harzianum* T1, and *T. pleuroticola* T1295 as references, and *T. reesei* AIT TRMS44c2 as the outgroup (Figure 3).

The morphological and physiological characteristics of *Trichoderma* spp. were evaluated by growing cultures on PDA for 96 h at 35 °C. *T. atroviride* isolates did not grow, and no spores or pigmentation was observed. In contrast, *T. afroharzianum* T22 grew rapidly, more extensively (50 to 86 mm), and sporulated well; *T. harzianum* GP-2-2 grew slower, forming clear concentric rings of white, cottony aerial mycelium alternating with zones of green spores (Figure 4).

On the contrary, *T. pleuroticola* growth was particular and distinct from the other species. The colony diameters ranged from 30 to 53 mm, with irregular growth, exhibiting a dense circular white-cream mycelium at the centre, bordered externally with a dark yellow to yellow-brown zone. Moreover, multiple sectors of finely diffuse mycelium extending outwards and culminating with variable, lobed, wavy margins were observed. Sporulation was non-existent or very sparse in the central region of the colony (Figure 4).

Table 2. Isolates of *Trichoderma* used in this study and their origins. NAP—isolates conserved in the collection at the University of Naples, Portici (NA), Italy. This study *: isolates obtained in the present study and described in Komoń-Zelazowska et al. [30].

Species (Biotype)	Isolate Code	Source	Geographic Origin	Fungal Source, Compost, Phase of Production	GenBank Number ITS	GenBank Number EF 1α	GenBank Number RPB-II
T. atroviride	P1	NAP	Norway	Biological control strain	OQ360634	OR146259	OR146262
T. atroviride	A6	NAP	Italy	Biological control strain	OR045890	OR146261	OR146263
T. afroharzianum	T22	NAP	USA	Biological control strain (ATCC20847)	OQ360633	OR146260	OR146264
T. harzianum	GP-2-2	This study	Italy	*Pleurotus*: recycled water	OQ002336	OQ026348	OQ026374
T. pleuroticola	GP-6-1	This study	Italy	*Pleurotus*: 20 days after seeding	OQ002319	OQ026371	OQ026387
T. pleuroticola	GP-6-2	This study	Italy	*Pleurotus*: 20 days after seeding	OQ002330	OQ026370	OQ026383
T. pleuroticola	GP-BI-4	This study *	Italy	*Pleurotus*: 20 days after seeding	OQ002335	OQ026366	OQ029696
T. pleuroticola	GP-BI-6	This study *	Italy	*Pleurotus*: 20 days after seeding	OQ002331	OQ026364	OQ026385
T. pleuroticola	GP-BI-8	This study	Italy	*Pleurotus*: 20 days after seeding	OQ002321	OQ026362	OQ029698
T. pleuroticola	NZ-1	This study *	Italy	*Pleurotus*: infected compost	OQ002327	OQ026360	OQ029699
T. pleuroticola	NZ-2	This study *	Italy	*Pleurotus*: infected compost	OQ002328	OQ026359	OQ029700
T. pleuroticola	NZ-3	This study *	Italy	*Pleurotus*: in fruitification	OQ002326	OQ026358	OQ029701

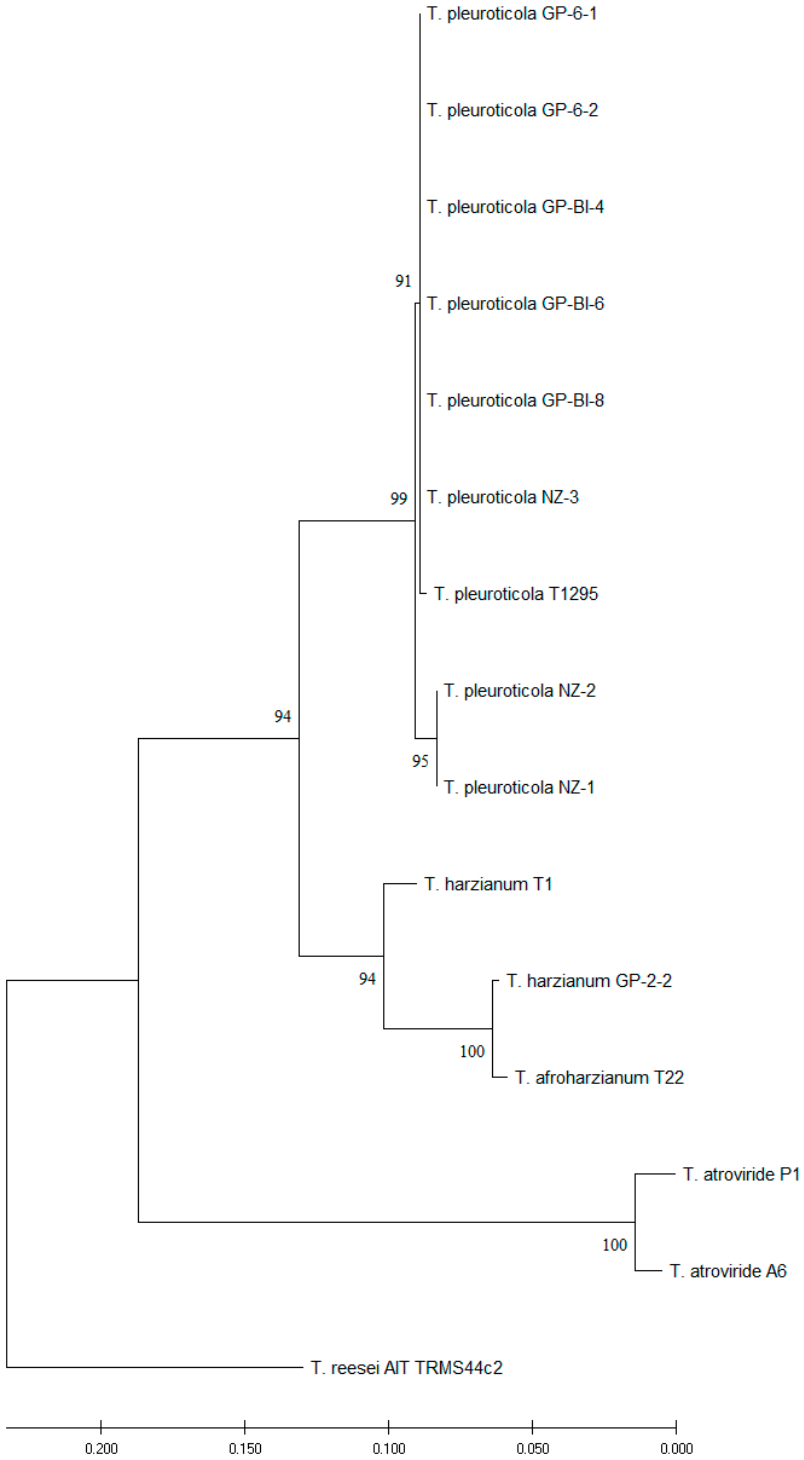

Figure 3. Evolutionary analysis of ITS and TEF sequences performed using the maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method and the Tamura–Nei model [40]. The bootstrap consensus tree inferred from 1000 replicates [42] was used to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [42]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura–Nei model and then selecting the topology with a superior log likelihood value. This analysis involved 15 nucleotide sequences. Evolutionary analyses were conducted using MEGA X [39].

All isolates of *Trichoderma* sporulated by the end of the experiment and produced conidia that were globose to subglobose (length-to-width ratio 1.1 to 1.2), smooth, and green in colour (light yellow-green to dark green-blue). All *Trichoderma* were able to grow over the *Pleurotus* fungal colonies, exhibiting "normal" mycelium and spore development, similarly to the biocontrol reference isolates. A fungal antagonistic effect was clearly observed when confrontations were conducted between the *Trichoderma* isolates (in particular NZ-2), including the biological control strains (i.e., P1), and various fungal plant pathogens, such as *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, or *Fusarium oxysporum* (Table 3). As shown in Figure 5, in some *Trichoderma-Trichoderma* combinations, particularly those involving the biocontrol isolates, a clear inhibition zone and/or competition for space and nutrients were observed in confrontations: GP-6-1 vs. P1, A6 vs. NZ-3, NZ-1 vs. T22, and P1 *vs.* T22, indicating that an antagonistic response can be produced among members of the same fungal genus (interspecies).

Table 3. Dual culture assay of best-performing *Trichoderma* isolates against different phytopathogens (*Botrytis cinerea*, *Fusarium oxysporum, Rhizoctonia solani*, and *Sclerotinia sclerotiorum*) evaluating the percentage of radial growth inhibition. Statistical data analysis was performed using one-way ordinary ANOVA followed by Tukey's multiple comparisons test considering a p-value ≤ 0.05; ns: not significant; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001; ****: p-value ≤ 0.0001; CI: confidence interval for a difference between means.

Fungal confrontation	Summary	Adjusted p Value	% Inhibition
T22 vs. *B. cinerea*	ns	0.3195	
GP-6-2 vs. *B. cinerea*	**	0.0022	−12%
P1 vs. *B. cinerea*	***	0.0002	−15%
NZ-2 vs. *B. cinerea*	***	0.0001	−16%
T22 vs. *F. oxysporum*	ns	0.659	
GP-6-2 vs. *F. oxysporum*	**	0.005	−7%
P1 vs. *F. oxysporum*	**	0.0024	−8%
NZ-2 vs. *F. oxysporum*	***	0.0004	−9%
T22 vs. *R. solani*	ns	0.6214	
GP-6-2 vs. *R. solani*	ns	0.2546	
P1 vs. *R. solani*	*	0.0202	−12%
NZ-2 vs. *R. solani*	****	<0.0001	−25%
T22 vs. *S. sclerotiorum*	***	0.0004	−13%
GP-6-2 vs. *S. sclerotiorum*	****	<0.0001	−19%
P1 vs. *S. sclerotiorum*	****	<0.0001	−26%
NZ-2 vs. *S. sclerotiorum*	****	<0.0001	−30%

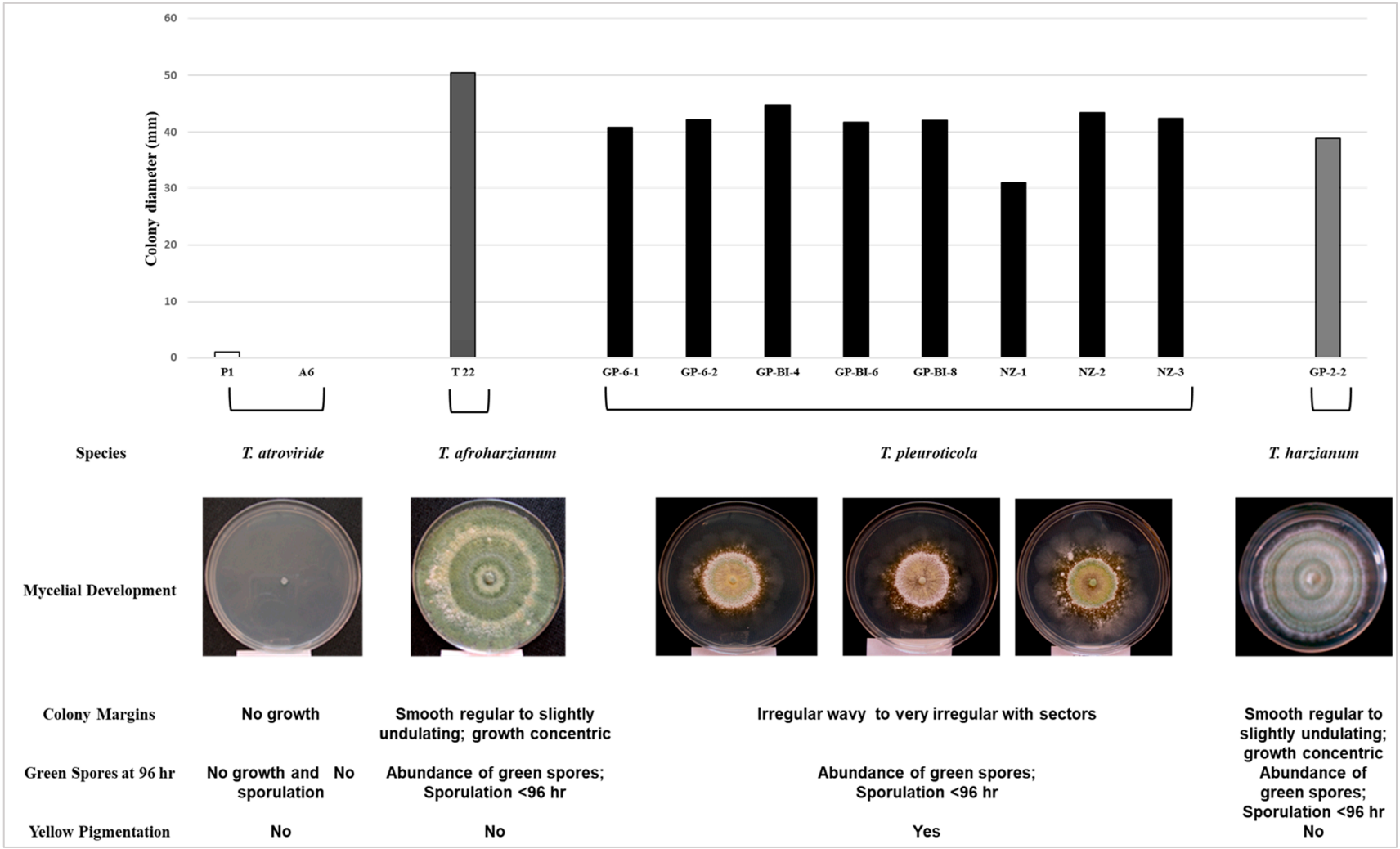

Figure 4. Morphological and physiological characteristics of *Trichoderma* isolates grown for 96 h at 35 °C on PDA described as colony diameter (mm): fungal growth; species: *Trichoderma* species identifications determined by NCBI blast analysis; mycelial development: phenology of fungal colony; colony margin: form of colony; green spores and yellow pigmentation: sporulation and presence of green conidia and/or yellow pigmentation.

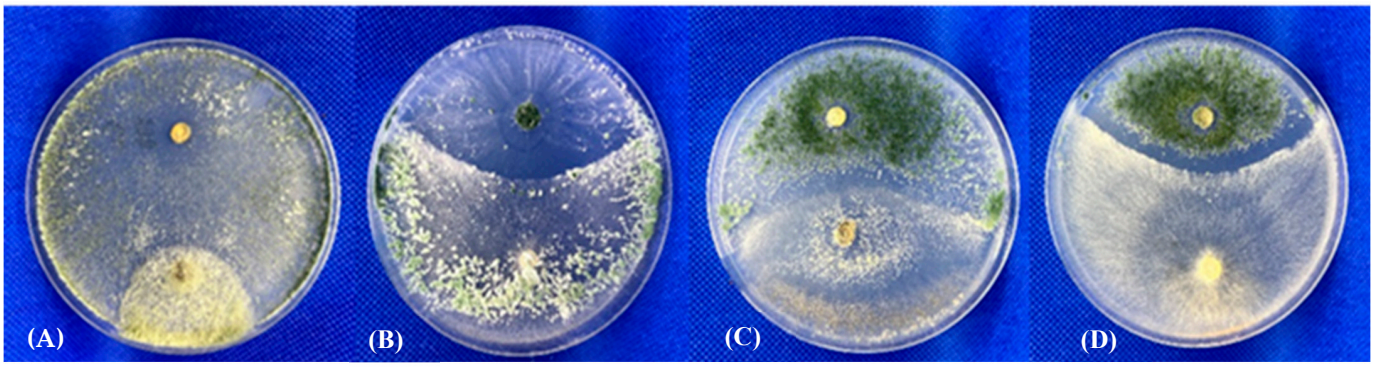

Figure 5. Dual culture assay of *Trichoderma-Trichoderma* interaction. (**A**) *T. pleuroticola* GP-6-1 (bottom) vs. *T. atroviride* P1 (top); (**B**) *T. atroviride* A6 (bottom) vs. *T. pleuroticola* NZ-3 (top); (**C**) *T. pleuroticola* NZ-1 (bottom) vs. *T. afroharzianum* T22 (top); (**D**) *T. atroviride* P1 (bottom) vs. *T. afroharzianum* T22 (top).

3.4. Treatment with Chemical Fungicides

Chemical fungicides containing prochloraz (Mirage® 45 EC, 450 g L^{-1}; Adama Makhteshim Ltd., Beer Sheva, Israel) and metrafenone (Vivando® SC, 500 g L^{-1}; BASF SE, Ludwigshafen am Rhein, Germany), applied at different doses, were effective in inhibiting both the mycelium development and spore germination of the inoculated *Trichoderma* species after three days (Figure 6A,B).

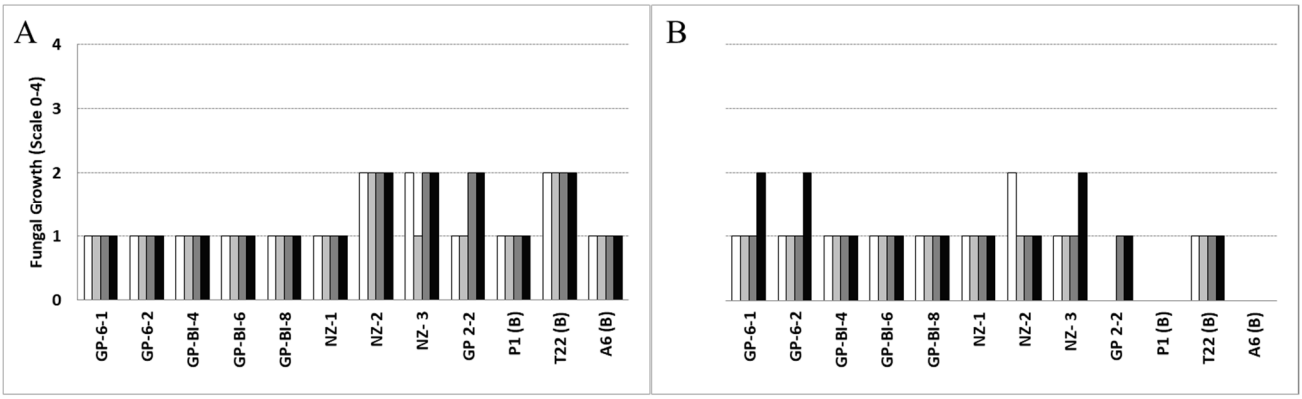

Figure 6. Effect of different fungicides, applied at various concentrations (a.i. □ = highest dose, ▫ = second dose, ▪ = third dose, ■ = lowest dose tested) of the active ingredient in 1 L of water, on the mycelial growth of *Trichoderma* isolates three days after treatment with (**A**) prochloraz (1, 0.7, 0.5, 0.2 mL) and (**B**) metrafenone (10, 7, 5, 2.5 µL). Fungal growth was measured on a five-value scale, where 0 = no apparent growth of the inoculum, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% fungal colony development with respect to the control (PDB).

T. atroviride (P1 and A6) and some *T. pleuroticola* isolates were more susceptible to prochloraz, remaining at 25% growth compared to the control, whereas NZ-2, NZ-3, GP-2-2, and T22 reached 50% growth, suggesting a lower efficacy of the active ingredient at the tested doses (Figure 6A). Generally, the control effects on the fungal mycelium growth observed 3 days after fungicide treatments were maintained for up to 30 days following the initial application.

In addition, when the same dose of prochloraz was applied to a *Trichoderma* spore suspension (10^5 sp mL^{-1}), an inhibitory effect on spore germination and spore tube elongation was observed at a similar level to that recorded for the mycelium growth inhibition (1–50%), even up to 30 days after treatment (data not shown).

The applications of metrafenone were effective in controlling the growth of the *Trichoderma* isolates (25%) at higher dose concentrations. An exception was NZ-2, which

demonstrated 50% growth at the highest doses of fungicide; however, since there was minimal growth at lower doses, this was probably an experimental artifact. *T. atroviride* A6 and P1 did not grow at any of the assayed fungicide doses of metrafenone, and *T. harzianum* GP-2-2 was inhibited at 10 and 7 µL concentrations (Figure 6B). On the other hand, treatments with the same active ingredient directly applied to conidium suspensions completely inhibited spore germination (0%) of all *Trichoderma* isolates, an effect that was maintained after 30 days.

None or minimal negative effects were observed on mycelium growth of commercial *Pleurotus* varieties treated with prochloraz or metrafenone applied at the same doses. In particular, *P. ostreatus* PC3 resulted in the only isolate significantly inhibited by the highest doses of both fungicides (Table 4).

Table 4. Effect of chemical fungicides (prochloraz and metrafenone) applied at different doses on *P. ostreatus* PC3 isolate. Statistical data analysis was performed using one-way ordinary ANOVA followed by Tukey's multiple comparisons test considering a p-value ≤ 0.05; ns: not significant; *: p-value ≤ 0.05; **: p-value ≤ 0.01.

Radial Growth of PC3 on Chemical Fungicide Enriched Media	Summary	Adjusted p Value
PC3 on prochloraz 1 mL	**	0.0019
PC3 on prochloraz 0.7 mL	*	0.0279
PC3 on prochloraz 0.5 mL	ns	0.1342
PC3 on prochloraz 0.2 mL	ns	0.4498
PC3 on metrafenone 10 µL	**	0.0019
PC3 on metrafenone 7 µL	ns	0.1342
PC3 on metrafenone 5 µL	ns	0.2603
PC3 on metrafenone 2.5 µL	ns	0.2603

4. Discussion

The *Trichoderma* species found to be associated with the production of *Pleurotus* mushrooms in Italy were identified as *T. harzianum* and *T. pleuroticola*. These species could be readily cultured on a common fungal medium such as PDA and grew after incubation at 25°–28° C for 96 h. When assessed at 35 °C, the reference isolates *T. atroviride* P1 and A6 grew minimally or did not grow, T22 appeared normal, whereas irregular mycelial growth was noted for eight isolates of *T. pleuroticola* and the single *T. harzianum* (GP2-2) obtained from *Pleurotus*. Further, the *T. pleuroticola* species could be readily distinguished from the others because they grew slower and produced a yellow/yellow-brown pigmentation in the substrate.

In this study, the production process of oyster mushrooms involved cultivation in a pasteurized straw-based compost, inoculation with seed spawn, and packaging into bales covered with perforated plastic. The bales were subsequently piled together and placed under controlled conditions of pre-incubation at 25 °C to permit growth and diffusion of the *Pleurotus* mycelium in the compost, thus reducing the time to fruiting body formation after distribution to mushroom producers. Microbial analysis conducted during all phases of compost preparation indicated that *Trichoderma* spp. was eliminated by pasteurization at 60 °C (Table 1). *T. pleuroticola* was isolated from phases after pasteurization (from infected compost), 20 days after spawn seeding, and at the stage of fruiting body formation. This suggested that the heat-treated compost needed to be separated and isolated from the other zones of production to avoid the cross-contamination of fungal spores (air movement, dust, etc.) from the infected material after pasteurization. In addition, the spawn should be seeded as soon as possible to freshly pasteurized compost in a closed or air-filtered clean environment, and then baled immediately afterwards to maintain its integrity. Furthermore, *T. harzianum* was isolated from the recycled water used in the process. Importantly, any material found infested with *Trichoderma* spp. during the production phases should be removed and eliminated to prevent distribution and infection.

At 25 °C, no differences were observed between the colony growth of *T. pleuroticola*, *T. harzianum*, *T. afroharzianum*, and *T. atroviride*. The initial occurrence of any *Trichoderma* in the mushroom compost at spawning and subsequently during incubation at 25 °C, provides a colonization advantage for the pathogen over the cultivated *Pleurotus*, which grows preferentially at 28 °C and develops much slower. This is in contrast to the findings of Rakhmonov et al., [43], who detected an optimum temperature for *Pleurotus* growth in the substrate at 26 °C. Only growth at 35 °C differentiated the morphological characteristics of the *Trichoderma* species in plate cultures (Figure 4). Although *T. pleuroticola* growth was somewhat inhibited at higher temperatures, the development of other species such as *T. harzianum* was not; therefore, temperature cannot be considered a good physical method to control *Trichoderma* infestations.

All the tested *Trichoderma* species grew similarly and preferentially at a neutral pH (pH 6 to 7). In general, the crude straw-based substrate has a pH of about 7.5 and is mixed with alkaline compounds such as volcanic tuff rock (locally found around Naples) and/or calcium carbonate powders to increase the alkalinity to pH 8.5 or higher. However, the subsequent processing and growth of the oyster mushrooms rapidly decrease the pH of the compost such that a developing *Pleurotus* bale is reduced to pH 6.7 after one week and to pH 6.2 after two weeks of incubation. Considering that alkaline pH inhibits *Trichoderma* spp. development, it is important to establish a substrate that maintains alkalinity, particularly in the beginning phases of production, to maintain the conditions preferential for *Pleurotus* growth and development.

A literature search was conducted to identify fungicides other than prochloraz and metrafenone suggested for controlling *Trichoderma* spp. in *Agaricus*. These included benzimidazole, thiabendazole or thiophanate-methyl, and azole fungicides, which are inhibitors of ergosterol synthesis such as propiconazole [44–46]. In addition, a mix of thiabendazole, thiofanate-methyl, and dicloran was reported to be effective for the control of *Trichoderma* isolates that attack champignons, whereas carbendazim was found to be very successful [44,46,47]. Prochloraz (application dose 1 L ha^{-1}) and metrafenone (10 L ha^{-1}) are chemical fungicides used to control various fungal pathogens, with minimal effects on cultivated edible mushrooms. Both products were effective in controlling the development of established *Trichoderma* mycelium in vitro after three days; an inhibitory effect that was maintained or increased 30 days after treatment. Further, the same doses applied to the resistant fungal structures such as spores were able to inhibit both germination and hyphal growth. The application of metrafenone was more effective in controlling the growth of *Trichoderma* isolates at all tested concentrations. In particular, in agreement with Luković et al. and Allaga et al. [18,48], the commercial fungicide metrafenone showed an inhibitory effect on the growth of the examined *Trichoderma* strains, efficiently suppressing green mould isolates belonging to *T. harzianum* and *T. pleuroticola* species.

No detrimental effects of these compounds were observed on the majority of *Pleurotus* isolates at all tested concentrations, with the exception of PC3 isolate, which was significantly inhibited at the highest doses of both fungicides.

5. Conclusions

Our findings revealed that *T. pleuroticola* and *T. harzianum* associated with the production of *Pleurotus* emerged as opportunistic invaders that readily infected and colonized the compost substrate, thus resulting in competitors for oyster mushroom cultivation. All isolated *Trichoderma* species were genetically identified using molecular sequencing approaches and assigned to *Trichoderma pleuroticola* and *T. harzianum* species. The results highlighted that it is possible to manipulate physical parameters, including temperature and pH, that control *Trichoderma* spp. infections, but can favour *Pleurotus* growth. In addition, chemical treatments with metrafenone were effective in containing *Trichoderma* growth in vitro, suggesting that its application directly to the *Pleurotus* spawn or to the compost at the beginning can provide efficient control of potential *Trichoderma* infections, thereby permitting the oyster mushrooms to establish on the substrate. In general, good

sanitation practices in *Pleurotus* cultivation are required to avoid *Trichoderma* infection, dissemination, and contamination, and to reduce risks of outside infestations by the immediate removal and destruction of any infected material, and the isolation of the compost-producing/seeding zone from the growing/incubation and transport zones. If possible, the production should be conducted in an enclosed zone, with the filtration of circulating air in the ventilation system and the filtration of recycled water used in the process. Other methods to reduce potential *Trichoderma* attacks include the preparation of compost with an alkaline pH 8-9, an initial fungicide treatment of the *Pleurotus* spawn with prochloraz and/or metrafenone at seeding, followed by incubation at higher temperatures of 28 °C in order to aid the development and establishment of oyster mushroom cultures.

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