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Inhibitory Effect of *Trichoderma* spp. Causing Green Mold Disease on the Edible Mushroom *Pleurotus pulmonarius* and *Pleurotus floridanus*

(Kesan Perencatan terhadap Pertumbuhan *Trichoderma* spp. yang Menyebabkan Penyakit Kulat Hijau pada *Pleurotus pulmonarius* dan *Pleurotus floridanus*)

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ABSTRACT

The popular cultivated edible oyster mushrooms in Malaysia, *Pleurotus pulmonarius* (grey oyster mushroom) and *Pleurotus floridanus* (white oyster mushroom), are susceptible to green mold disease caused by *Trichoderma* spp. The decline of the quality and yield production of the mushrooms up to 100% has been frequently observed due to infection by *Trichoderma*. The objective of this study was to investigate the competitive inhibition of *Trichoderma* spp. isolated from the farm with *Pleurotus* spp. Nine strains of *Trichoderma* were cultured *in vitro* with *Pleurotus* on potato dextrose agar and the radial growth as well as mycelial diameter growth were recorded. The direct plate assay results showed that *T. koningiopsis* and *T. harzianum* were the most pathogenic strains against *P. pulmonarius* and *P. floridanus* by overgrowing and replacing the mycelia, respectively, due to space competition and nutrient suppression. The bi-plate Petri dish approach for assessing contactless inhibition of *Trichoderma* spp. against *Pleurotus* spp. demonstrated that *T. asperellum*, *T. ghanense*, and *T. koningiopsis* were able to cross over the partition and inhibit *P. pulmonarius* mycelium. Meanwhile, inverted plate assay demonstrated that *T. ghanense* and *T. reesei* inhibited *P. pulmonarius* mycelia, respectively, indicating contactless inhibition of *Pleurotus* by *Trichoderma.* When tested with *P. floridanus*, only *T. ghanense* demonstrated inhibition in bi-plate Petri dish method and inverted plate assay. In summary, all nine *Trichoderma* spp. suppressed *Pleurotus* growth in varying degrees. Thus, besides competing with mushroom mycelia for nutrients and space, *Trichoderma* sp. could also release volatile organic compounds that act without direct contact.

Keywords: Green mold; inhibition; mushroom; pathogenic

ABSTRAK

Cendawan *Pleurotus pulmonarius* (Tiram kelabu) dan *Pleurotus floridanus* (Tiram putih) adalah cendawan yang paling digemari sebagai makanan dan ditanam di Malaysia, tetapi sering terdedah kepada penyakit kulat hijau yang disebabkan oleh *Trichoderma* spp. Jangkitan oleh *Trichoderma* sp. ini biasanya mengakibatkan kemerosotan kualiti dan hasil cendawan sehingga 100%. Objektif utama penyelidikan ini adalah untuk mengkaji kesan perencatan secara kompetitif oleh *Trichoderma* spp. yang dipencilkan daripada ladang dengan *Pleurotus* spp.. Sembilan strain *Trichoderma* dikulturkan secara *in vitro* dengan *Pleurotus* pada agar kentang dektrosa sebelum pertumbuhan radial serta jarak jejari miselia diukur. Keputusan ujian plat langsung menunjukkan bahawa *T. koningiopsis* dan *T. harzianum* masing-masing adalah strain yang paling patogen terhadap *P. pulmonarius* dan *P. floridanus* dengan pertindihan pertumbuhan miselia berlebihan disebabkan oleh persaingan ruang dan kekurangan nutrien. Bagi ujian dwi-piring petri pula, penilaian adalah berdasarkan kesan perencatan tanpa sentuhan oleh *Trichoderma* spp. terhadap *Pleurotus* spp. dengan *T. asperellum*, *T. ghanense* dan *T. koningiopsis* mampu menyeberangi penghadang dan merencat pertumbuhan miselia *P. pulmonarius*. Sementara itu, ujian piring terbalik menunjukkan bahawa *T. ghanense* dan *T. reesei* menindas miselia *P. pulmonarius*, ini membuktikan kesan perencatan boleh berlaku tanpa sentuhan oleh *Trichoderma* sp. terhadap *Pleurotus* sp.. Apabila diuji dengan *P. floridanus*, keputusan menunjukkan hanya *T. ghanense* merencat pertumbuhan miselia dalam kedua-dua ujian Petri dwi-piring dan piring terbalik. Kesimpulannya, kesemua sembilan species *Trichoderma* merencat pertumbuhan *Pleurotus* dalam kadar yang berbeza. Oleh itu, selain bersaing untuk nutrien dan ruang dengan miselia cendawan, *Trichoderma* spp. juga menghasilkan sebatian organik mudah meruap yang aktif walaupun tanpa interaksi fizikal.

Kata kunci: Cendawan; kulat hijau; patogen; perencatan

INTRODUCTION

A total of 43 edible mushroom species are commercially cultivated worldwide where *Pleurotus* species is ranked as the third largest commercially produced edible mushroom (Bakratsas et al. 2021). In Malaysia, out of the 17 types of mushrooms are cultivated, only eight species are grown commercial. Among these, *Pleurotus* is the most commonly cultivated genus (Islam et al. 2017) with *P. pulmonarius* and *P. floridanus* contribute approximately 91% to the local market (Rosmiza et al. 2016). The production of mushrooms in Malaysia has a significant increase from 5,589 tonnes in 2018 to 10,997 tonnes in 2022 (Department of Agriculture 2022).

Pleurotus spp., commonly known as oyster mushrooms, are globally cultivated and commercialized as food source and nutraceuticals as well as biotherapeutic molecules (Adebayo et al. 2013). *Pleurotus* mushrooms are rich in carbohydrates, minerals, crude fiber, crude protein, and lipids (Effiong et al. 2023). The protein content in *Pleurotus* mushrooms is more nutritionally beneficial than superior to the plant-based proteins (Wang & Zhao 2023). Besides, the crude fat content of *Pleurotus* mushrooms is relatively low, ranging from 1.0% to 6.7% (Wang et al. 2014). *Pleurotus* mushrooms contain bioactive compounds such as tocopherol, adenine, niacin, riboflavin, and thiamine (Effiong et al. 2023). These compounds are closely associated to the mushroom's medicinal potential, which include antioxidant, antitumor, and antimicrobial activities (Adebayo & Oloke 2017).

From the late 1990s to early 2000s, multiple reports were brought up on the loss of button mushroom (*Agaricus bisporus*) yields due to green mold disease, which originated in Northern Ireland in 1985 and quickly spread across Europe (Colavolpe, Mejía & Albertó 2014). This disease has caused a significant 37% decrease in the gross production index of mushrooms between 1998 and 2006 in the United Kingdom and Northern Ireland (Food and Agriculture Organization of the United Nations 2022). The economic impact of green mold disease is not only affecting the button mushroom but also oyster mushroom cultivation. The first outbreak of green mold disease on *P. ostreatus* was reported in North America (Sharma & Vijay 1996). In the United States of America, the disease caused the declined in the production of oyster mushroom by 23% between 2017 and 2019 (Manjit, Kamal & Sharma 2021), led to 3-6% price increase due to reduced supply and increased of demand (Grand View Research 2022). Severe outbreaks of green mold disease in *P. ostreatus* cultivation farms have also been also reported in Korea, Italy and Hungary (Hatvani et al. 2007).

Green mold disease can lead to complete crop losses, affecting economic impact for oyster mushroom growers (Park, Bae & Yu 2006), including those in Malaysia (Ajis, Tan & Chai 2024). Hence, extensive research has been studied to identify the cause of this widespread disease and to develop effective management strategies. These studies showed that *Trichoderma* species are closely associated with green mold disease, with thick and green fungal sporulation observed on the oyster mushrooms substrates and the presence of greenish mycelium in compost (Park, Bae & Yu 2006). Although *Trichoderma* is recognized as a beneficial biocontrol agent in agriculture, it is a destructive pathogen in mushroom cultivation. Hence, this study focuses on the pathogenic behavior of *Trichoderma* as a contaminant in the mushroom cultivation.

Trichoderma pleuroti, T. pleuroticola in Korea and Poland (Błaszczyk et al. 2013); *T. harzianum* in Sri Lanka (Jayalal & Adikaram 2007) and Europe (Allaga et al. 2021); *T. simmonsii*, *T. guizhouenese,* and *T. afroharzianum* in Europe (Allaga et al. 2021) have been identified and responsible for green mold disease in *Pleurotus* cultivation. *Trichoderma longibrachiatum* caused the significant damage, up to 78.6%, in *Pleurotus eryngii* production (Choi et al. 2003). According to Lee et al. (2020), *P. eryngii* failed to produce fruiting bodies when co-cultivated with *T. pleuroti*. Besides, *Trichoderma pleuroti* (Ponnusamy et al. 2021), *T. harzianum* and *T. ghanese* (Ajis, Tan & Chai 2024) were also identified as prevalent fungal contaminant in the *P. pulmonarius* on sawdust substrates in Malaysia.

The rapidly growing *Trichoderma* spp. spread spores easily through water, insects such as sciarid flies, contaminated tools, and air, substrate, and grower apparel (Šašić Zorić et al. 2023). According to Kredics et al. (2010), *Trichoderma* can grow on the surface or colonise the mushroom substrate without showing any symptoms of green mold disease for 10 to 35 days after the spawning process. The dense, whitish mycelium initially produced by *Trichoderma* closely resembles mushroom mycelium, making it difficult to distinguish between the two. The mycelial mat on the casing layer eventually becomes green or dark green due to extensive sporulation of *Trichoderma* (Luković et al. 2020). As a result, the mushroom mycelium which colonise the mushroom substrate have to compete with *Trichoderma* for nutrients and space. Most of the *Trichoderma* species have been reported to be mycoparasites where they are able to secrete enzymes like chitinases, ß–glucanase, and cellulases, which function to hydrolyse cell walls of *Pleurotus* mycelium (Jayalal & Adikaram 2007).

Much research on *Trichoderma* disease has focuses on *Agaricus bisporus, Lentinula edodes*, and *Pleurotus ostreatus* (Dang et al. 2023). However, it remains unclear which *Trichoderma* species exhibit the most significant inhibitory effect on *P. pulmonarius* and *P. floridanus*. The uncontrolled inhibitory effect of *Trichoderma* spp. could jeopardize the oyster mushrooms cultivation by indirectly causing a decline in yield production and mushroom quality, which would subsequently affect the market and lead to significant losses in one of the most highly sought food sources (Sobieralski et al. 2012). Hence, this study aimed to investigate the competitive inhibition and contamination risk of *Trichoderma* spp. on *Pleurotus*

pulmonarius and *P. floridanus in vitro.* We also evaluated the morphological changes and growth patterns of both fungi under co-cultivation conditions to better understand the contamination pattern.

MATERIALS AND METHODS

PREPARATION OF MEDIA AND FUNGAL STRAINS

Two *Pleurotus* species (*P. floridanus* and *P. pulmonarius*) were obtained from Mushroom Research Centre, Universiti Malaya. Nine different *Trichoderma* spp. (*T. pleuroti* (CH1F3), *T. pleuroti* (D30(11)), *T. asperellum* (A11), *T. afroharzianum* (WA1), *T. harzianum* (D10-110), *T. ghanense* (D10-14), *T. koningiopsis* (A23), *T. reesei* (A232B), *T. parareesei* (A233B)) were isolated from various sources during the mushroom cultivation process (Iqbal et al. 2017). For the following assays, a 10-day-old *Pleurotus* culture was inoculated for three days before inoculating a 3-day-old *Trichoderma* culture on potato dextrose agar (PDA) (Difco) in 90 mm Petri dishes and was incubated at 28 ± 2 °C in the dark.

DIRECT PLATE ASSAY

Pleurotus and *Trichoderma* spp. plugs were placed at 15 mm from the edge of the Petri dish on PDA media, respectively (Figure 1(A)). For the control, a plug of *Pleurotus* was placed on one side of the plate without any *Trichoderma* culture, and vice versa for the *Trichoderma* plate. Each treatment was performed in triplicates and incubated at 28 ± 2 °C. The radius for the mycelium growth of *Pleurotus* and *Trichoderma* spp. were observed and measured daily for five days using a standard ruler.

GROWTH INHIBITION

The growth rate of each culture was measured, and the percentage of inhibition was calculated for direct plate assay (Naser et al. 2022):

$$Percentage\ of\ inhibition = \frac{Gc - Gt}{Gc} \times 100$$

where G_c refers to mycelia growth of *Pleurotus* on the control plate; and G_t is mycelia growth of *Pleurotus* on the tested plate.

INHIBITION RATING SCALE FOR DIRECT PLATE ASSAY

The inhibitory effect of *Trichoderma* against *Pleurotus* was determined using the inhibition rating scale (Badalyan, Innocenti & Garibyan 2002) with modifications. The score was assigned as described in Table 1. The inhibitory index (II) was calculated for each fungal species using the following formula: $II = A(n \times 1) + B(n \times 2) + B_{A1}(n \times 2.5)$, where n is the frequency of each type or subtype of reaction.

The frequency (f) of type and subtype of competition between *Pleurotus* spp. and *Trichoderma* spp. in direct plate assay was calculated herewith:

$$f = \frac{\text{Total score of each type or subtype of reaction}}{\text{Total inhibitory index}} \times 100\%$$

BI-PLATE PETRI DISH METHOD

Bi-plate Petri dish method was used following the method described by Toral et al. (2021) with modification to monitor any indirect inhibition of *Trichoderma* spp. against the growth of *Pleurotus*. *Pleurotus* spp. and *Trichoderma* sp. were inoculated onto a standard two-compartment Petri dish (Nest Biotechnology) containing PDA media (Figure 1(B)). A plate with *Pleurotus* culture alone was served as control and vice versa for each *Trichoderma* spp. The experiment was set up in triplicate and incubated at 28 ± 2 °C. The interaction among the mycelium was recorded over five days by measuring the growth radius of both *Pleurotus* and *Trichoderma* species mycelium.

INVERTED PLATE ASSAY

The inverted plate assay was used to study the effects of indirect volatile metabolites released by *Trichoderma* spp. on *Pleurotus* as described by Ruangwong et al. (2021). A mycelial plug of *Pleurotus* was inoculated at the center of PDA for three days prior to the inoculation of three-day-old *Trichoderma* sp. Then, the plate covers were removed, and the *Trichoderma* plate was placed on top of the *Pleurotus* plate; sealed both of the plates together with parafilm (Figure 1(C)). For the control treatment, the bottom plate contained only *Pleurotus* culture and while top plate contained only *Trichoderma* culture. Each treatment was conducted in triplicates and incubated at 28 ± 2 °C.

TABLE 1. Inhibition index of *Pleurotus* by *Trichoderma*

Subtypes	Inhibition category	Score
A	deadlock with mycelial contact	1
B	overgrowth without initial deadlock	2
B_{A1}	partial replacement after initial deadlock with contact	2.5

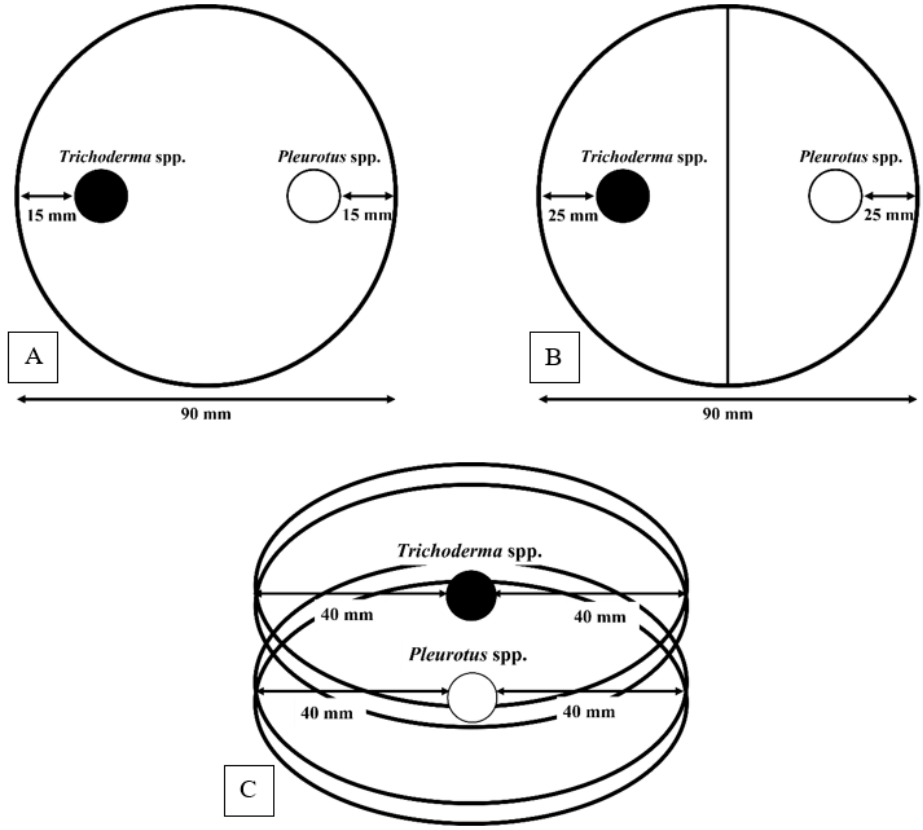


FIGURE 1. Overview of the experimental setup of (A) direct plate assay, (B) bi-plate Petri dish method, and (C) inverted plate assay between two mycelia plugs

MORPHOLOGICAL ANALYSES

The treated and control samples were observed macroscopically for any sign of growth inhibition, discoloration or infection. The mycelium was also observed under a light microscope (Olympus BH-2 BHT) with 400x magnification.

STATISTICAL ANALYSES

Statistically significant differences among tested samples were analysed using one-way analysis of variance (ANOVA). Statistically significant was set when p-value less than 0.05 using Tukey's HSD test (IBM SPSS Statistics 26).

RESULTS AND DISCUSSION

DIRECT INHIBITION ON MUSHROOM MYCELIAL GROWTH BY *Trichoderma* spp.

Macroscopically, all tested plates showed that *Pleurotus* spp. exhibited a slow mycelium growth rate with visible signs of suppression or infection upon direct contact with *Trichoderma* in its mycoparasite stage, observed within 48 to 72 h after post-inoculation. Specifically, *P. pulmonarius* and *P. floridanus* were inhibited by *T. parareesei* (A233B) and *T. asperellum* (A11), characterised by thickened mycelium mat (Figure 2(c), 2(d), 2(i), 2(j)). Conversely, light yellowish-brown discoloration of mycelia was observed at the infected regions of *Pleurotus* spp. when challenged with *T. koningiopsis* (A23) (Figure 2(a), 2(b)), *T. pleuroti* (CHIF3, D30(11)) (Figure 2(e)-2(h)), *T. afroharzianum* (WA1) (Figure 2(k), 2(l)), *T. harzianum* (D10-110) (Figure 2(m), 2(n)), *T. ghanense* (D10-14) (Figure 2(o), 2(p)), and *T. reesei* (A232B) (Figure 2(q), 2(r)). The thickening of the cell walls induced by *Trichoderma* could potentially generate mycelial interference, thereby, reducing the progression and penetration of pathogens (Sood et al. 2020). Besides, the browning observed in the infected mycelium of *P. floridanus* may be attributed to the secretion of metabolites, such as ethanolic acids, phenols and terpenoid compounds (Illuri et al. 2022) which could mitigate the infiltration and damage caused by *Trichoderma* (Sood et al. 2020).

All the mushroom mycelia were observed to have thin, short and sparsely branched hyphae under the microscope. This is probably because *Trichoderma* inhibits the mycelia by secreting cell wall-degrading enzymes and

antibiotics such as isocyano metabolites, anthraquinones, β-glucanases, and chitinase as well as breaking down or reducing the hyphal cells of the mushrooms (Wang et al. 2016). *P. pulmonarius* and *P. floridanus* mycelia were inhibited by *T. koningiopsis* where the hyphae were found to be coiled, curled, and disrupted (Figure 2(a), 2(b)). The presence of *T. koningiopsis* conidia caused the coiling of *Pleurotus* mycelia through mycoparasitism and exhibited its inhibitory effect on various mushroom species.

The cultivation *of Pleurotus eryngii* was severely affected by *T. koningiopsis* as this destructive pathogen outcompeted its mycelia, colonised the entire mushroom substrate rapidly, and completely or partially inhibited primordia formation (Kim et al. 2013). Similarly, Chen et al. (2021) documented a 60-70% reduction in the growth of *Dictyophora rubrovolvata* (bamboo mushroom) during the early stages of cultivation due to *T. koningiopsis*, causing either the direct death of mycelia or the inhibition of fruiting bodies development. Besides, the hyphal tips of the *P. pulmonarius* hyphae were affected by *T. parareesei* (A233B), which caused them to swell into small spherical shapes (Figure 2(c)).

P. floridanus mycelia were colonised and overgrown by rapidly growing *T. koningiopsis* (A23), *T. parareesei* (A233B), *T. pleuroti* (D30(11)), *T. harzianum* (D10-110), and *T. reesei* (A232B), and forming a high density of *Trichoderma* conidia and hyphae present within the *P. floridanus* mycelia (Figure 2(b), 2(d), 2(h), 2(n), 2(r)). The growth of *P. ostreatus* mycelium was significantly inhibited by 90% due to colonisation of *T. harzianum*, which formed green-coloured mycelial and conidia masses, accompanied by the production of diffusible metabolites (Jayalal & Adikaram 2007). Similarly, Mumpuni, Sharma and Brown (1998) reported that the mycelia growth of *Agaricus bisporus* was suppressed by up to 90% due to toxic diffusible metabolites produced by *T. harzianum* which inhibited the formation of fruiting bodies and caused green mold disease.

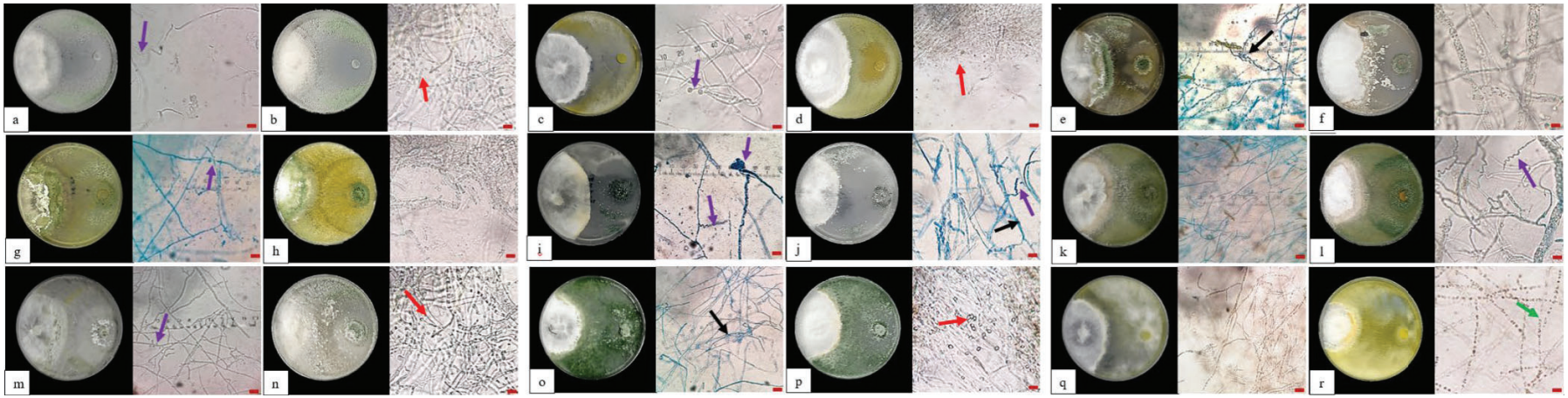
The inhibitory index values, and the frequency of each type and subtype of reaction were shown in Tables 2 and 3. These index values measured the competitive inhibition between *Pleurotus* spp. and *Trichoderma* spp. The difference of total inhibitory index of 49.5 and 46.6 for *P. pulmonarius* and *P. floridanus,* respectively, suggested that *P. pulmonarius* might be more susceptible to inhibition by *Trichoderma* compared with *P. floridanus*. Based on the inhibitory index values, *Trichoderma* species were divided into two groups: weakly inhibitors (II<10), which included *T. asperellum* (A11), *T. reesei* (A232B) and *T. parareesei* (A233B); and moderate inhibitors (10-15), which included *T. pleuroti* (CHIF3, D30(11)), *T. afroharzianum* (WA1), *T. harzianum* (D10-110), *T. ghanense* (D10-14), and *T. koningiopsis* (A23) (Table 2).

The frequency of replacement (84.4%) was higher than that of deadlock (15.6%) (Table 3). The frequent replacement of *Pleurotus* spp. by *Trichoderma* clearly indicates that most *Trichoderma* species exhibited strong inhibitory effects against *Pleurotus*. Both *P. pulmonarius* and *P. floridanus* reached a deadlock with mycelial contact when confronted with *T. asperellum* (A11) and *T. reesei* (A232B). Besides, *P. floridanus* reached deadlock against *T. parareesei* (A233B). In all three replicates, *P. pulmonarius* was overgrown by *T. pleuroti* (D30(11)), *T. afroharzianum* (WA1), *T. harzianum* (D10-110), *T. ghanense* (D10-14), *T. koningiopsis* (A23), and *T. parareesei* (A233B) without any initial deadlock. Meanwhile, *P. floridanus* was overgrown by *T. pleuroti* (CHIF3, D30(11)), *T. afroharzianum* (WA1), *T. ghanense* (D10-14), and *T. koningiopsis* (A23,) indicating that the growth of *P. floridanus* was suppressed and competitively inhibited by the presence of *Trichoderma* species.

For *P. pulmonarius,* all triplicates were partially replaced after an initial deadlock when in contact with *T. pleuroti* (CHIF3) while *P. floridanus* was partially replaced after an initial deadlock when in contact with *T. harzianum* (D10-110). The growth of both *P. pulmonarius* and *P. floridanus* were significantly inhibited by all *Trichoderma* spp. after 72 h of post-inoculation (Figure 3), demonstrates the strong competitive ability of *Trichoderma* against *Pleurotus*, leading to deadlock, overgrowth and eventual replacement of *Pleurotus* mycelia. Among all the *Trichoderma* species, *Trichoderma koningiopsis* (A23) showed the highest inhibitory effect against *P. pulmonarius* mycelia with 99% inhibition whereas *T. harzianum* (D10-110) showed the highest inhibitory effect against *P. floridanus* with 90% inhibition (Figure 3). In contrast, *T. afroharzianum* (WA1) showed the least inhibition against *P. pulmonarius* mycelia at 49%, and *T. parareesei* (A233B) recorded the lowest inhibition rate at 64 % (Figure 3).

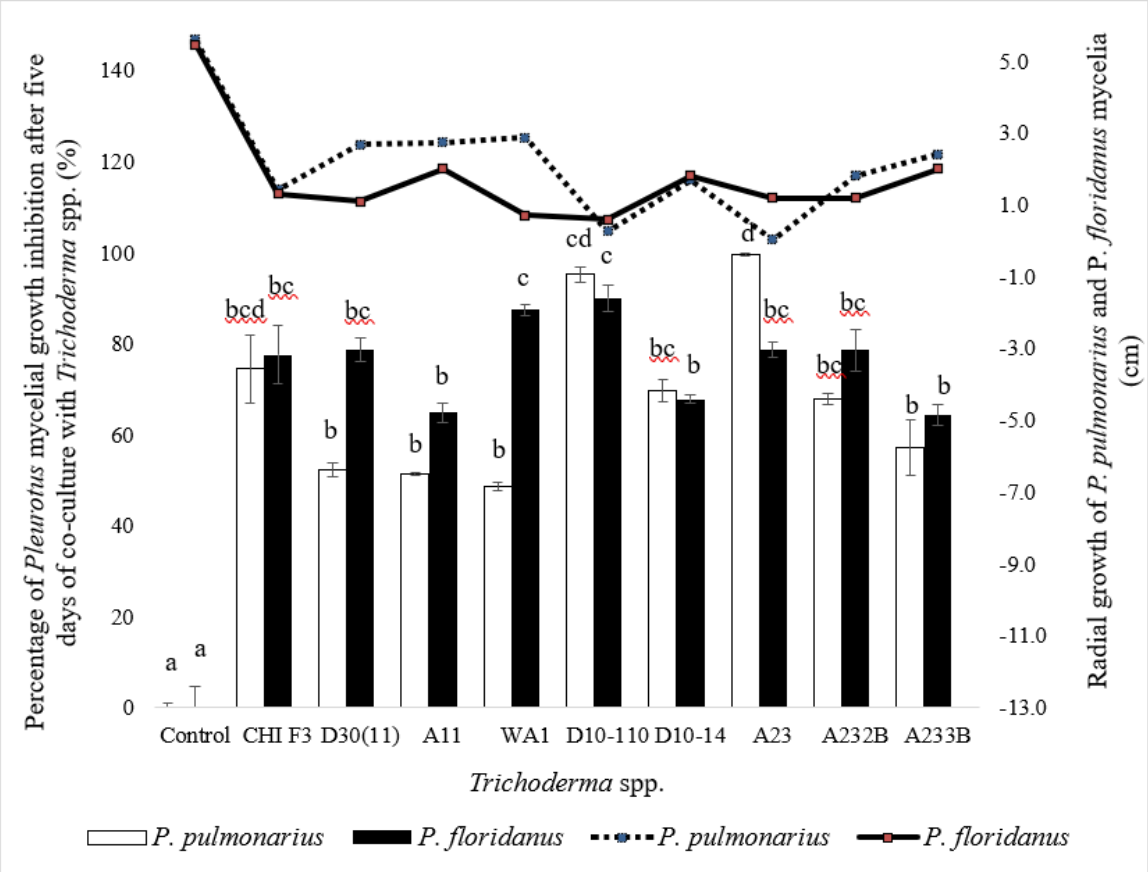
INDIRECT INHIBITION ON MUSHROOM MYCELIA GROWTH BY *Trichoderma* spp.

The growth of *Pleurotus* mycelium was inhibited by the rapid growth of *T. asperellum* (A11), *T. ghanense* (D10-14), *T. koningiopsis* (A23), *T. afroharzianum* (WA1), and *T. reesei* (A232B), which crossed over partition (Figure 4(a)-4(h)). *Pleurotus pulmonarius* and *P. floridanus* mycelia at the regions infected by *Trichoderma asperellum* and *T. ghanense* showed abnormal hyphae growth, forming with dense mycelium mat (Figure 4(c)-4(f)). Moreoever, *P. pulmonarius* mycelia at infected area developed a yellowish-brown line when challenged with *T. koningiopsis* (Figure 4(a)). Qiu et al. (2017) demonstrated that the growth of *Pleurotus* mycelium may be inhibited by the rapid growth of *T. asperellum,* which outcompetes the mycelia growth and produces abundance of conidia. Furthermore, *Trichoderma* conidia germinate into filamentous hyphae upon attachment to mushroom mycelia (Qiu et al. 2017). Optimum temperature enhances the conidia spreading, which results in agglutination reaction between *Pleurotus* mycelia and *T. asperellum* conidia, thus causing extensive green mold infection (Figure 4(c),4(d)). *Trichoderma ghanense*, a member of the *Longibraciatum* clade, is a



(Magnification = 400×; Bar scale = 10 µm)

FIGURE 2. Direct plate assay of inhibition effect of *Trichoderma* spp. against *Pleurotus* spp. *Pleurotus* was inoculated on the left side, while *Trichoderma* was inoculated on the right side of the Petri dish. The left panel showed the macroscopic morphology of the inhibitory effect, while right panel showed the microscopic morphology (a,b) Interaction of *T. koningiopsis* against *P. pulmonarius* (a) and *P. floridanus* (b); (c,d) *T. parareesei* against *P. pulmonarius* (c) and *P. floridanus* (d); (e,f) *T. pleuroti,* CH1 F3 against *P. pulmonarius* (e) and *P. floridanus* (f); (g,h) *T. pleuroti,* D30(11) against *P. pulmonarius* (g) and *P. floridanus* (h); (i,j) *T. asperellum* against *P. pulmonarius* (i) and *P. floridanus* (j); (k,l) *T. afroharzianum* against *P. pulmonarius* (k) and *P. floridanus* (l); (m,n) *T. harzianum* against *P. pulmonarius* (m) and *P. floridanus* (n); (o,p) *T. ghanense* against *P. pulmonarius* (o) and *P. floridanus* (p); (q,r) *T. reesei* against *P. pulmonarius* (q) and *P. floridanus* (r). Red arrows indicate attachment of *Trichoderma* conidia on *Pleurotus* hyphae, suppressing its growth; black arrows indicate coiling of hyphae; green arrow indicates lysis of hyphae and purple arrows indicate abnormal *Pleurotus* hyphae growth



Different letters indicate significant differences by Tukey's HSD test ($p < 0.05$)

FIGURE 3. Percentage inhibition (%) of *Pleurotus* mycelial growth and radial growth measurements of *P. pulmonarius* and *P. floridanus* mycelia (cm) after five days of co-culture with *Trichoderma* spp.

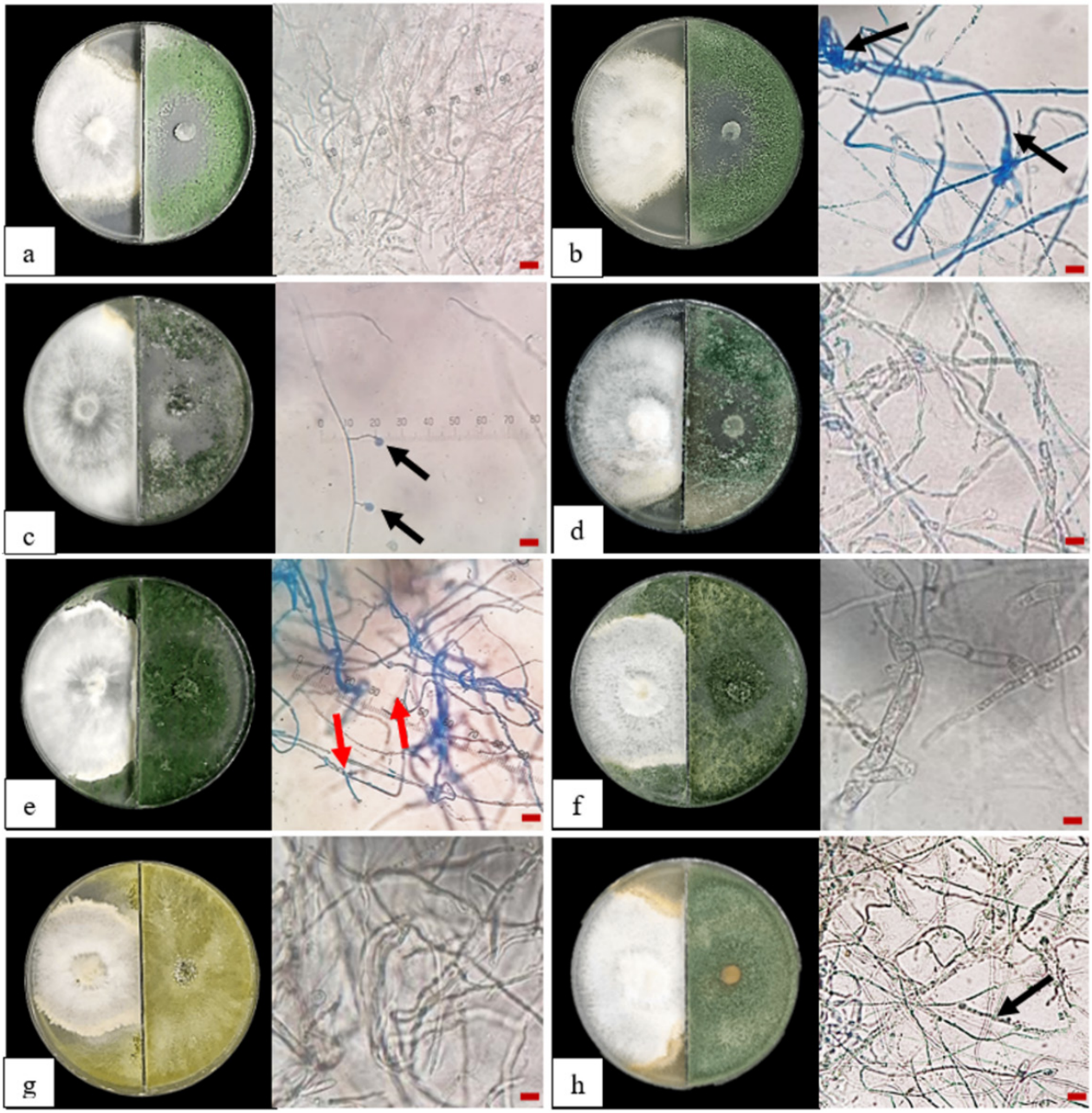
TABLE 2. Inhibitory index values and competition between *Pleurotus* spp. and *Trichoderma* spp. in direct plate assay on PDA medium

Mushroom samples	Pathogenic fungi									Total inhibitory index
	CH1F3	D30(11)	A11	WA1	D10-110	D10-14	A23	A232B	A233B	
P. pulmonarius	$3B_{A1}$	3B	3A	3B	3B	3B	3B	3A	3B	49.5
P. floridanus	3B	3B	3A	3B	$3B_{A1}$	3B	3B	3A	3A	46.5

A, deadlock with mycelial contact (score 1); B, overgrowth without initial deadlock (score 2); B_{A1}, partial replacement after initial deadlock with contact (score 2.5)

TABLE 3. Frequency of type and subtype of competition between *Pleurotus* spp. and *Trichoderma* spp. in direct plate assay on PDA medium

Deadlock		Replacement of mushrooms by pathogenic fungi		Replacement of pathogenic fungi by mushrooms	
Subtype	%	Subtype	%	Subtype	%
A	15.6	B	68.8	B	0
		B_{A1}	15.6	B_{A1}	0
Total	15.6	Total	84.4	Total	0



Black arrows indicate abnormal hyphae growth and red arrows indicate coiling of hyphae (Magnification = 400×; bar scale = 10 µm)

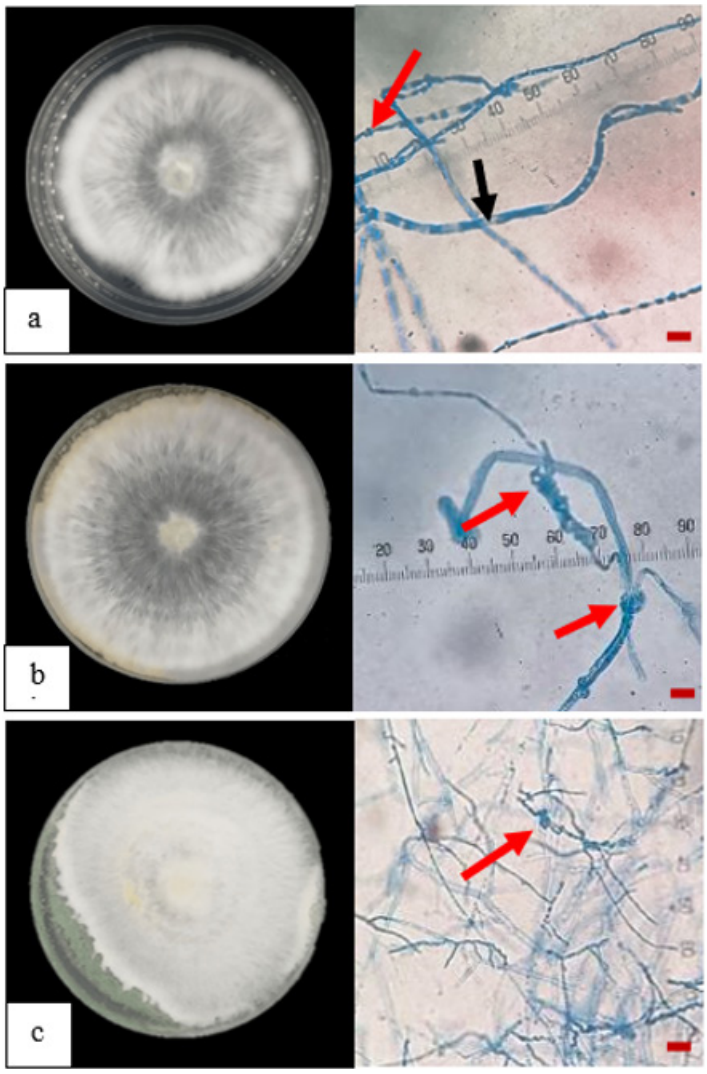
FIGURE 4. Bi-plate Petri dish method of inhibition effect of *Trichoderma* spp. against *Pleurotus*. spp. *Pleurotus* was inoculated on the left compartment, while *Trichoderma* was inoculated on the right compartment of the Petri dish. The left panel showed the macroscopic morphology of the inhibitory effect, while right panel showed the microscopic morphology. (a, b) Interaction of *T. koningiopsis* against *P. pulmonarius* (a) and *P. floridanus* (b); (c, d) *T. asperellum* against *P. pulmonarius* (c) and *P. floridanus* (d); (e, f) *T. ghanense* against *P. pulmonarius* (e) and *P. floridanus* (f); *T. reesei* against *P. floridanus* (g) and *T. afroharzianum* against *P. floridanus* (h)

well-known producer of cellulolytic enzyme (An et al. 2022). We hypothesize that the excessive enzyme may degrade the cell wall of the *Pleurotus* mycelia thus restrain their growth development.

Microscopic examination showed that conidia of *T. asperellum* and *T. ghanense* were present in infected *Pleurotus* regions, where mushroom mycelia appeared became coiled with minimal branching (Figure 4(c)-4(f)). Besides, *Pleurotus* mycelia challenged with *T. koningiopsis* were observed to be short, scattered, and reduced branching (Figure 4(a), 4(b)).

INDIRECT CONTACT INHIBITION OF *Trichoderma* spp. ON *Pleurotus* spp.

The growth of *P. pulmonarius* mycelium was inhibited by *T. ghanense* and *T. reesei,* possibly due to the accumulation of volatile organic compounds (VOCs) and caused abnormalities in mycelial structures (Figure 5(a), 5(b)). However, only *T. ghanense* showed visible signs of interaction with *P. floridanus*, characterised by green sporulation and the formation of a yellowish-brown ring within four days (Figure 5(c)). The hyphae of *P. pulmonarius* became thin, short, and less branched (Figure 5(b)), while the infectious side of *P. floridanus* was dominantly occupied by *T. ghanense* conidia and hyphae (Figure 5(c)).



(Magnification = 400×; Bar scale = 10 µm)

FIGURE 5. Inverted plate assay of inhibition effect of *Trichoderma* spp. against *Pleurotus* spp. The left panel showed the macroscopic morphology of the inhibitory effect, while right panel showed the microscopic morphology. Interaction of *T. reesei* against *P. pulmonarius* (a); (b,c) *T. ghanense* against *P. pulmonarius* (b), and *P. floridanus* (c). Black arrows indicate lysis of hyphae and red arrows indicate coiling of hyphae

While numerous studies have highlighted the biocontrol applications of *Trichoderma* VOCs in plant agriculture (Gualtieri et al. 2022), their potential role as pathogens causing green mold disease in *Pleurotus* cultivation remains underexplored. For instance, Krupke, Castle and Rinker (2004) reported that the growth of *Agaricus bisporus* was inhibited by 3,4-dihydro-8-hydroxy-3-methylisocoumarin, a compound produced by *T. aggressivum* f. *aggressivum.* This inhibition allowed *Trichoderma* to proliferate, consuming nutrients released by extracellular enzymes from the complex compost constituents of *Agaricus*. In another study, *T. asperelloide* emitted 2-phenylethanol (2-PE), a rose-like fragrant compound, which inhibited the growth of various fungi, including *Corynespora cassiicola*, *Fusarium incarnatum*, *Neopestalotiopsis clavispora,* and *Sclerotium rolfsii* at 48%, 64%, 68%, and 78%, respectively (Ruangwong et al. 2021). Besides, 2-PE was also suspected to attract *Megaselia pluralis* or other mushroom fly species (Kamm, Buttery & Robinson 1987) which may spread *Trichoderma* spores upon landing on mushroom cultivation substrate, thereby increasing the chances of green mold disease development.

The growth of *P. pulmonarius* and *P. floridanus* were further inhibited by the strong competition posed by *Trichoderma ghanense*, possibly due to the secretion of VOCs (Figure 5(b), 5(c)). Despite this, *T. ghanense* has rarely been reported as pathogen affecting *Pleurotus* species, hence, leaving the potential correlations between the *Trichoderma* species and *Pleurotus* largely unexplored (Samuels et al. 2012).

CONCLUSIONS

All nine *Trichoderma* species exhibited significant inhibitory effects on the growth of both *Pleurotus* species. The mycelium of *P. pulmonarius* was severely inhibited, overgrown, and eventually replaced by *Trichoderma koningiopsis*, which may be due to direct competition for nutrients and space as well as production of enzymes that induced coiling of hyphae and lyse the mushroom hyphal cells. Similarly, *P. floridanus* mycelium was inhibited and discoloured by *T. ghanense* across all assays, attributed to its capabilities for mycoparasitism, and the secretion of volatile and non-volatile metabolites. Early detection of *Trichoderma* and strict sanitation measures are essential for controlling and preventing the spread of green mold disease across mushroom cultivation farms. Further research is required to better understand the role of mycoparasitism and the contamination mechanisms of *Trichoderma* that inhibits mushroom growth.

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