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Characterisation of *Trichoderma* spp. and Assessment as Biocontrol Using Dual Culture Assay Against Fungi Associated with Black Pepper (*Piper nigrum* L.) Diseases in Sarawak

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ABSTRACT

Black pepper (*Piper nigrum* L.) is one of the most widely used spices in food, beverage, cosmetics, and medicine. Black pepper production has suffered from various fungal diseases. Microbial biological control is an essential part of integrated disease management to reduce the heavy reliance on chemical fungicides. *Trichoderma* fungi comprise a large group of rhizocompetent filamentous fungi widely used in the biocontrol of plant pathogens. Three field surveys conducted on five black pepper farms in Belaga, Sarawak, identified three fungal diseases: yellowing, black berry, and foot rot. Based on the morphological and molecular characterisation, the identified fungal causal agents were *Fusarium solani* (yellowing disease), *Colletotrichum gloeosporioides* (black berry disease), and *Phytophthora palmivora* (foot rot disease). Twenty isolates of *Trichoderma* spp. were isolated from secondary forest and Biopark in Bintulu, Sarawak. *Trichoderma* isolates were characterised based on the morphological characteristics and molecular phylogenetic analysis using the rDNA internal transcribed spacer (ITS) region. *Trichoderma* isolates were separated into five distinct species, namely *T. harzianum*, *T. virens*, *T. brevicompactum*, *T. tawa*, and telomorphic *Hypocrea lixii*. Among the *Trichoderma* fungi, *T. harzianum* was the most frequently (65%) isolated species. *Trichoderma harzianum* (Isolates of TJ9, 10, and 16) showed antagonistic and inhibitory effects by 61 to 70% on *in vitro* mycelial growth against three common fungal pathogens of black pepper, *P. palmivora*, *C. gloeosporioides*, and *F. solani*. This study highlights the potential of using native *Trichoderma* fungi as biocontrol agents in the black pepper integrated disease management program.

Keywords: Anthracnose, *in vitro* antagonism, *Phytophthora* foot rot, yellowing disease

INTRODUCTION

Black pepper (*Piper nigrum* L.) is known as the king of spices for its largest share in the international spice market; it recorded an annual export value of USD 1.29 billion in 2019 (Perwara & Munggaran, 2020). Malaysia is one of the largest black pepper-producing countries. In 2019, a total of 24000 metric tons of black pepper was produced, and 98% of the total production was from Sarawak (Paulus, 2020). Besides, Sarawak black pepper was granted a geographical indication status in Malaysia for its distinctive characteristics and high-quality standards. However, fungal diseases have caused an estimated 2% annual reduction in black pepper production (Krishnamurthy *et al*., 2010; Adam *et al*., 2018). Some of the common fungal diseases of black pepper are yellowing or slow decline, black berry, foot rot, and velvet blight (Siti & Nuraini, 2020).

To mitigate the fungal infection on the black pepper, most growers heavily relied on chemical fungicides to control these diseases. Different groups of fungicides were applied to provide protection and control over diseases caused by different types of fungal pathogens. Metalaxyl and copper-based fungicides such as copper oxychloride or copper hydroxide provide reliable protection over foot rot disease caused by *Phytophthora* species. Benomyl, mancozeb, prochloraz zinc complex, carbendazim, chlorothalonil, and copper-based fungicides are used to mitigate the damage of the black berry disease caused by *Colletotrichum* species (Wong, 2010). Yellowing disease is a disease complex where the root-knot nematode (*Meloidogyne incognita*) and the burrowing

nematode (*Radopholus similis*) predispose the plant roots by providing entry points for the *Phytophthora* or *Fusarium* (Biju *et al*., 2019). Thus, the use of nematicide carbofuran to suppress the soil-borne parasitic nematode population effectively reduces yellowing disease incidence (Wong, 2010). However, the over-reliance on chemical fungicides has resulted in new challenges such as health hazards to agricultural workers, residual contamination in food, and environmental pollution (Aktar *et al*., 2009). Cultural practices and combined application of fungicide and biological control agents are put forward in response to integrated disease management. Fungal antagonists, such as *Trichoderma* species, appear to be promising biological control agents against foliar and soil-borne pathogens.

Trichoderma species (Hypocreaceae, Ascomycota) are common soil inhabitants that can be found across all climatic zones and ecosystems. Their presence in the agricultural and forest soils is typically a good indication of a healthy ecosystem (Zin & Badaluddin, 2020). They can act as endophytes colonising the above- and below ground plant tissues, saprophytes decomposing plant debris and soil organic matter, parasites to animals and plants (as opportunistic pathogens), and some strains are parasitic on soil fungi species (Mukherjee *et al*., 2014). Their versatile nutritional adaptation to different hosts and substrates can be attributed to the synthesis of various bioactive metabolites required for this purpose (Sood *et al*., 2020). Significant research effort has been directed towards exploiting the genus and its bioactive compounds in various applications.

Many *Trichoderma* species have wide applications such as biofertilisers and biofungicides, bioremediation agents for environmental pollutants, biomolecules (secondary metabolites, extracellular enzymes, and proteins) production, and biosynthesis of metallic nanoparticles (Kubicek *et al*., 2019; Zin & Badaluddin, 2020). The establishment of *Trichoderma* at the rhizosphere or the outermost layer of root cells can confer protection against various plant pathogens. The mechanisms of biocontrol exerted by *Trichoderma* on the pathogens are competition for space and nutrients, antibiosis, and mycoparasitism through the secretion of cell wall degrading enzymes or secondary metabolites (Pertot *et al*., 2015). In addition, mycoparasitic *Trichoderma* fungus can coil around and penetrate the pathogen hyphae to acquire nutrients, resulting in the collapse of fungal hyphae (Jiang *et al*., 2016). Moreover, the enzymes and metabolites released by *Trichoderma* can cause synergistic induction of induced systemic resistance (ISR) and systemic acquired resistance in host plants (Saravanakumar & Wang, 2020). These two forms of induced plant defense preconditioned the host plants against plant pathogens to achieve disease resistance.

Trichoderma species such as *T. harzianum*, *T. viride*, *T. atroviride*, *T. asperellum, T. longibrachiatum,* and *T. virens* have been used in the biological control of a wide range of fungal plant diseases caused by *Sclerotium*, *Phytophthora*, *Rhizoctonia*, *Pythium*, and *Fusarium* genera (Zin & Badaluddin, 2020). However, the biocontrol effectiveness of different *Trichoderma* strains is generally varied due to the differential production of hydrolytic enzymes (Alamri *et al*., 2016). Besides, the effectiveness of biocontrol is also influenced by environmental variables (soil type and agronomic practices), the complexity of the pathosystem, and the ecological origin of *Trichoderma* strains (Harman *et al*., 2004; Chaverri *et al*., 2015). *Trichoderma* strains isolated from the same geographical origin but from different substrate sources (e.g., leaf litter and soil) showed differential antagonistic responses against *Fusarium oxysporum* f.sp. *cubense* (Napitupulu *et al*., 2019).

The outbreak of fungal diseases has hampered the productivity of black pepper. In many cases, these diseases can lead to complete yield losses. Therefore, an urgent need for an economical and environmentally friendly disease control approach is needed to minimise yield loss. This has led to the screening of antagonistic *Trichoderma* fungi for biological control of the major fungal diseases in black pepper. Hence, this study commenced on i) isolation and characterisation of *Trichoderma* spp. using morphological and molecular techniques, and ii) evaluation of the biocontrol activity of *Trichoderma* spp. isolated from different ecological niches against major fungal pathogens from black pepper.

MATERIALS AND METHODS

Field Survey and Isolation of Fungi Associated with Various Diseases of Black Pepper

Field surveys were conducted on five black pepper farms located in Belaga, Sarawak. Symptomatic plants showing disease symptoms such as blackening of leaves and rot root (foot rot disease), foliar yellowing and wilt (yellowing disease), and brownish lesions on leaves and berries (black berry disease) were identified. Plant tissue samples (berry, leaf, stem, and root) were collected for the isolation of pathogenic fungi. Plant tissues were washed under running tap water and cut into segments of 5 mm^2. Plant tissues were surface sterilised using 1% (v/v) sodium hypochlorite for 1 min and rinsed twice with sterile distilled water. Plant tissues were plated onto potato dextrose agar (PDA) (Oxoid, UK) amended with 100 mg/L ampicillin and 100 mg/L rifampicin to inhibit bacterial growth, and incubated at 26 ± 2 °C for three days. The actively growing mycelia were transferred onto new PDA plates. The identification of pathogenic fungi was based on the morphological characteristics as described by Drenth and Sendall (2001) for *Phytophthora* species, Leslie and Summerell (2006) for *Fusarium* species, and Jayakumar *et al*. (2009) for *Colletotrichum* species.

Isolation of *Trichoderma* Fungi

Trichoderma species were isolated from root rhizosphere and tree barks of secondary forest, Biopark (integral deer framing and recreational system) in Bintulu, Sarawak. Information on the *Trichoderma* isolates used in this study is listed in Table 1. Samples were brought to the laboratory and kept at 4 °C until observation. A five-fold serial dilution was used to dilute the microbial communities in the soil samples. Soil samples were prepared using sterilised distilled water, and the aliquoted samples were plated onto *Trichoderma*-selective medium (TSM) (Askew & Laing, 1993) with slight modification. The collected tree barks were sterilised with 1% sodium hypochlorite for 1 min and washed twice with sterilised distilled water. Samples were sliced into pieces of approximately 5 mm in length and plated onto TSM. Plates were incubated at 26 ± 2 °C for two days. Actively growing mycelia were transferred onto new PDA (Oxoid, UK). Putative *Trichoderma* isolates were purified using the single spore isolation method to obtain pure fungal cultures.

Morphological Identification

The cultural and morphological characteristics of the pure *Trichoderma* isolates were studied on PDA incubated at 26 ± 2 °C. The first appearance of green conidia, conidiation pattern, formation of conidial pustules, and pigmentation was observed from 7-day-old cultures. Microscopic characteristics of *Trichoderma* isolates were observed under a compound microscope (Leica DM2500, USA) after being stained with lactophenol cotton blue. Identification was based on the taxonomic keys provided by Bissett (1991) and Samuels *et al*. (2012). Morphological characteristics such as conidiophores and branching patterns, phialide, and conidia shape were recorded. Colony radius of each *Trichoderma* isolate was measured at 24-, 48- and 72-hours intervals. Mycelial growth rate (mm/day) was determined from the two perpendicular diameters of each isolate. The fungal growth rate was determined according to these categories: slow (<10 mm/day), medium (10-12 mm/day), and fast (>12 mm/day) (Dubey *et al*., 2010). Experiments were performed in triplicate, and statistical analysis was performed using Analysis of Variance (ANOVA) in SAS version 9.4 program.

Molecular Characterisation

Genomic DNA was extracted from 7-day-old cultures grown in potato dextrose broth (PDB) (Oxoid, UK) at 26 ± 2 °C. Fungal mycelia (100 mg) were homogenized using BioMasher-II (Optima, Japan). DNA was extracted using the fungal/yeast genomic DNA extraction kit (BioTeke Corporation, China) according to the manufacturer's instructions. The quality of the extracted DNA was visualized by agarose gel electrophoresis and quantified by spectrophotometry. For species identification, the internal transcribed spacer (ITS) region was amplified using the primers ITS1 and ITS4. Polymerase chain reaction (PCR) was performed in a total reaction volume of 50 µl, including 1X Power Taq Master Mix (BioTeke Corporation, China), 0.4 µm of each primer, and 50 ng template DNA. PCR was performed in the MiniOpticon Real-Time PCR System (BioRad, USA). PCR program consisted of initial

denaturation of 2 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and final extension of 10 min at 72 °C. PCR products were visualized in 1% agarose gel. PCR products were purified using Gel Extraction and PCR Purification Combo Kit (BioTeke Corporation, China) according to the manufacturer's instructions. Sequencing of the purified PCR products was undertaken by Apical Scientific Sdn. Bhd., Malaysia. Consensus sequences were assembled from forward and reverse sequencing reads. All sequences obtained in this study were deposited in GenBank with the accession numbers listed in Table 1. Consensus sequences were compared to sequences in the GenBank database at National Center for Biotechnology Information (NCBI).

Phylogenetic Analysis

ITS sequence alignment was carried out using ClustalW and visually edited using MEGA X (Stecher *et al*., 2020). The interleaved Nexus file was formatted for the MrBayes v3.2.7 program (Ronquist & Huelsenbeck, 2003). The statistical selection of the best nucleotide substitution model was carried out using jModelTest (Posada, 2008). The GTR+G model was selected based on the Akaike information criteria (AIC) analysis (Akaike, 1974). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed at one million generations, and trees were sampled at every 100 generations. A Bayesian consensus tree was generated after burn-in 25% for one million generations.

Confrontation Assay

Nineteen *Trichoderma* isolates were screened from their growth inhibition and antagonistic activity on the three black pepper pathogens: *Phytophthora palmivora*, *Colletotrichum gloeosporioides*, and *Fusarium solani* using the dual culture method. Seven-day-old fungal cultures with optimum mycelial growth were used for the assay. The assay was performed in triplicate on PDA. The mycelium discs (5 mm in diameter) of *Trichoderma* isolates and tested isolates were inoculated at 10 mm from the edge, opposite site from each other in a Petri dish (90 mm in diameter). The plates were incubated at 26 ± 2 °C for 10 days, and culture diameters were measured at every 24-hour interval to monitor the mycelial growth of each fungus. The percentage inhibition of radial growth of pathogens (PIRGP) was determined by using the calculation as described by Ezziyyani *et al*. (2004): [(R1 - R2)/R1]x100, where R1 is the radius of pathogen without *Trichoderma* inoculation (control) and R2 is the radius of the pathogens with *Trichoderma* inoculation. The assay was repeated three times. Mycoparasitism of *Trichoderma* isolates against fungal pathogens was evaluated at 10 days after inoculation using the following antagonistic degree: 0 - no invasion on pathogen colony, 1 - 25% invasion on pathogen colony, 2 - 50% invasion on pathogen colony, 3 - 100% invasion on pathogen colony, 4 - 100% invasion and sporulation on pathogen colony (Ezziyyani *et al*., 2004; Gonzalez *et al*., 2020).

RESULTS

Black Pepper Pathogenic Fungi Isolation

Black pepper plants with symptoms resembling foot rot, black berry, and yellowing diseases were surveyed on five black pepper farms located in Belaga, Sarawak. Foot rot disease symptoms include sudden wilting, defoliation, and root rot. It should not be confused with the yellowing disease, where diseased plants display yellowing, defoliation, and root rot symptoms. The formation of black lesions on pepper fruit is a typical symptom of black berry disease (Figure 1). Black berry and yellowing diseases were identified in all five black pepper farms. However, foot rot disease has a low disease prevalence in Belaga where the disease was found only on one black pepper farm. Fungal pathogens were isolated from different plant parts such as roots, stems, and fruits, exhibiting various disease symptoms. Pathogenicity test is recommended to verify further the role of these fungi on the collected diseased black pepper. However, these isolated fungal isolates have been previously reported as common pathogens causing several diseases of black pepper in Sarawak and various countries outside Malaysia (Biju *et al*., 2019; Siti & Nuraini, 2020). A total of 23 fungal isolates were collected: two *Phytophthora* isolates, nine *Fusarium* isolates, and twelve *Colletotrichum* isolates. Based on the morphological and molecular characterisation, the pathogenic fungi isolated from the diseased black pepper plants were *F. solani* (yellowing disease), *C. gloeosporioides* (black berry disease), and *P. palmivora* (foot rot disease). The

growth of *P. palmivora* was slow on PDA with scanty fluffy whitish mycelium. It produced ovoid to papillate sporangia and globose chlamydospores. *Colletotrichum gloeosporioides* appeared fast-growing with grey aerial mycelium and orange concentric ring on PDA. The conidia were cylindrical in shape. *Fusarium solani* were morphologically indistinguishable with moderately fast-growing whitish
mycelium and pale orange pigments. *Fusarium solani* produced three types of mitotic spores on PDA: sickle-cell shaped macroconidia, ovoid microconidia, and globose chlamydospores (Figure 2). ITS sequence analysis has confirmed the identity of all isolated pathogenic fungi. One isolate of each pathogenic species was selected for confrontation assay. ITS sequence of *F. solani* isolate used in confrontation assay is available with NCBI GenBank accession number MT328730.

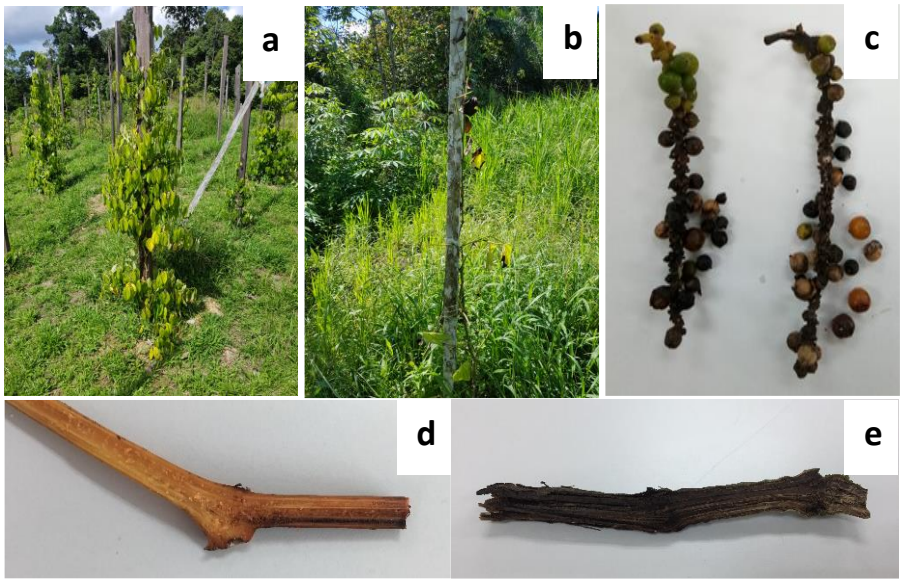

Figure 1. Symptoms of (a) yellowing, (b) foot rot, and (c) black berry diseases. (d) Discoloration of vascular tissue was found in black pepper stem infected with the yellowing disease. (e) Basal stem rot was associated with foot rot disease. Photos were taken from black pepper farms located in Belaga

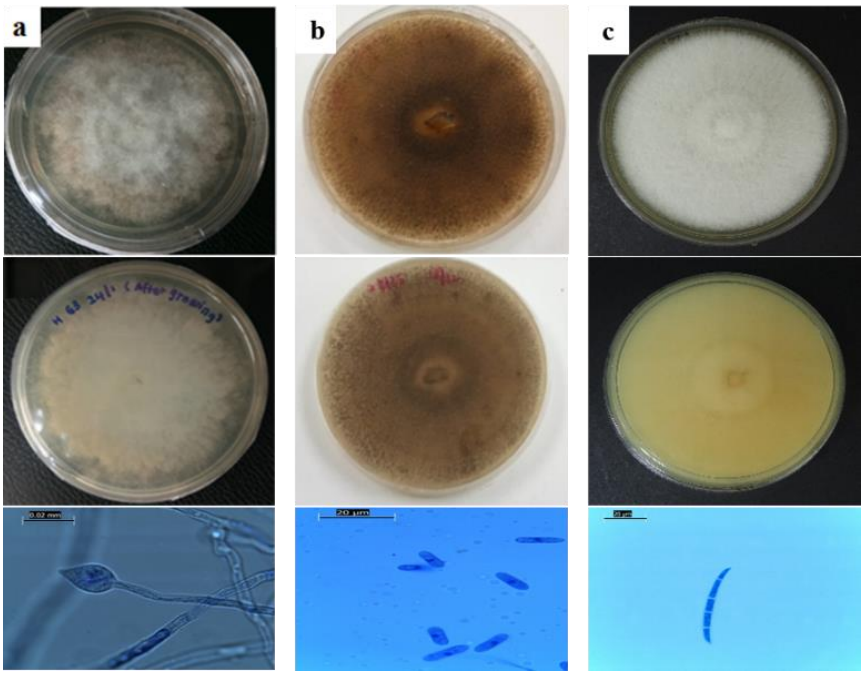

Figure 2. Colony morphology (upper and underside) of a) *Phytophthora palmivora*, b) *Colletotrichum gloeosporioides*, c) *Fusarium solani* and their mitotic spores with scale bar

Table 1. *Trichoderma* spp. isolated in this study. The origin, identification, and GenBank accession numbers of all isolates are provided

Isolate No.	Location and Source	Species	ITS Accession Number [a]	Maximum Identity (%)	NCBI Best Match Sequence
TJ1	Biopark, root rhizosphere	*Trichoderma virens*	MZ489555	99.82%	MT529862
TJ2	Biopark, root rhizosphere	*Trichoderma virens*	MZ489556	100.00	MT529862
TJ3	Biopark, root rhizosphere	*Trichoderma virens*	MZ489557	99.82	MT529406
TJ4	Biopark, root rhizosphere	*Trichoderma harzianum*	MZ489558	99.82	MN889412
TJ5	Biopark, root rhizosphere	*Trichoderma harzianum*	MZ489559	99.82	MT229305
TJ6	Borneo Base Forest, root rhizosphere	*Trichoderma harzianum*	MZ489560	100.00	MG519511
TJ7	Borneo Base Forest, root rhizosphere	*Trichoderma harzianum*	MZ489561	100.00	MG489889
TJ8	Borneo Base Forest, root rhizosphere	*Trichoderma harzianum*	MZ489562	99.82	MG519511
TJ9	Nyabau Forest Reserve, root rhizosphere	*Trichoderma harzianum*	MZ489563	99.82	MK116422
TJ10	Nyabau Forest Reserve, root rhizosphere	*Trichoderma harzianum*	MZ489564	100.00	MK116422
TJ11	Nyabau Forest Reserve, root rhizosphere	*Trichoderma harzianum*	MZ489565	99.82	MK552405
TJ12	Biopark, root rhizosphere	*Trichoderma harzianum*	MZ489566	99.82	MT229305
TJ13	Biopark, root rhizosphere	*Trichoderma harzianum*	MZ489567	99.82	MN889412
TJ14	Biopark, root rhizosphere	*Trichoderma harzianum*	MZ489568	100.00	MN889412
TJ15	Bintulu recreational park, tree bark	*Trichoderma tawa*	MZ489569	100.00	MH625704
TJ16	Bintulu recreational park, tree bark	*Trichoderma harzianum*	MZ489570	100.00	MK943766
TJ17	Bintulu recreational park, root rhizosphere	*Hypocrea lixii*	MZ489571	100.00	HQ229942
TJ18	Bintulu recreational park, root rhizosphere	*Trichoderma brevicompactum*	MZ489572	100.00	MK120912
TJ19	Bintulu recreational park, root rhizosphere	*Trichoderma brevicompactum*	MZ489573	100.00	MK253291
TJ20	Bintulu recreational park, root rhizosphere	*Trichoderma harzianum*	MZ489574	99.82	MT229305

[a] GenBank accession number nuclear rDNA ITS1-ITS4 (ITS) sequences

Morphological Characterisation of *Trichoderma*

Fungal isolates were collected from the rhizosphere and bark of different plants, and 20 were identified as *Trichoderma* isolates. The morphological characteristics and growth rate of the twenty *Trichoderma* isolates were observed on PDA. All the isolates were fast-growing, reaching a daily growth rate of 22.2 - 28.8 mm/day at 26 ± 2 °C (Table 2). Initially, *Trichoderma* isolates produced white arachnoid to floccose aerial mycelium, fully covering the Petri dish after three days of incubation. In most isolates, conidia were formed within 48-72 hours of incubation and developed into yellowish green to dark green colonies scattered in minute tufts of mycelium form (Figure 3). Conidia on aerial mycelium formed concentric rings with alternating light and dark-coloured zones. Pustules were formed from conidial condensation, where the conidia were loosely arranged in it. Pustules were generally green and white. Some isolates produced white pustules on the green conidial mat. Conidial pustules distributed around the center and near the fringe were noted after four days of incubation. The microscopic characteristics observed in this study were the branching pattern of conidiophores and the shape of conidia. Conidiophores were highly branched with asymmetrical phialides arranged in whorls, cylindrical base, and giving a flask-shaped appearance (Figure 4). Globose-ellipsoidal, single-celled conidia were clustered at the terminal whorl of the phialides. Terminally and intercalary chlamydospores were observed in some isolates.

Subtle morphological variation was observed among the isolates. These morphological differences were used for species identification. Morphological features of each isolate were compared to the taxonomic keys for *Trichoderma*. However, morphological characteristics may be insufficient to differentiate between closely related species. Hence, molecular characterisation was included in this study for closely-related species identification. Based on this polyphasic approach, five named species: *T. virens* (3 isolates), *T. harzianum* (13 isolates), *T. brevicompactum* (2 isolates), *T. tawa* (1 isolate), and teleomorphic *Hypocrea lixii* (1 isolate) were identified. The general morphological features of each species were compared. All *T. virens* isolates formed dark bluish green to dull green conidia throughout the colony, pale yellow pigmentation, formed green pustules of conidia, appressed phialides, and globose single-celled conidia. Variation in the intensity of colony colour was found within *T. harzianum*, which produced colony colour ranging from whitish green to yellowish green and dull green. *Trichoderma harzianum* isolates can be distinguished by their amber pigmentation which was not produced by other species. This species tended to form a yellowish green colony with dense and uniformly distributed globose conidia, the presence of chlamydospores, and

phialides were held at a right angle to the hyphae they arise. On PDA, colonies of *T. brevicompactum*, *T. tawa* and *H. lixii* appeared white in colour. *Trichoderma brevicompactum* and *H. lixii* formed green conidia and white pustules only around the point of inoculum and near the plate margin, whereas *T. tawa* produced hyaline conidia forming a white arachnoid to floccose colony. *Trichoderma brevicompactum* was characterised by appressed phialides, and ellipsoidal single-celled conidia. Conidiophores of *T. tawa* formed unpaired branching at acute angles with the main hyphae, phialides of non-gathering type, and ellipsoidal conidia. *Hypocrea lixii* formed highly branched conidiophores, paired branching, non-gathering type phialides, globose single-celled conidia, and chlamydospores.

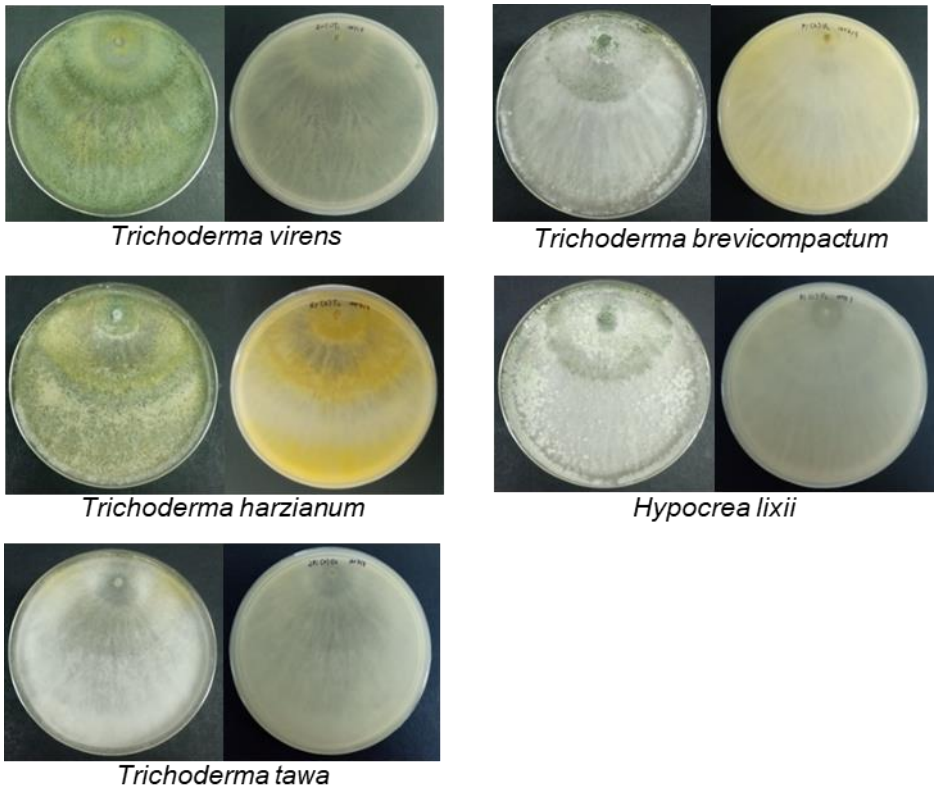

Figure 3. Cultural and morphological characteristics of *Trichoderma* spp. Colony appearance (upper and underside) of *Trichoderma* spp. grown for 7 days on PDA at 26 ± 2 °C

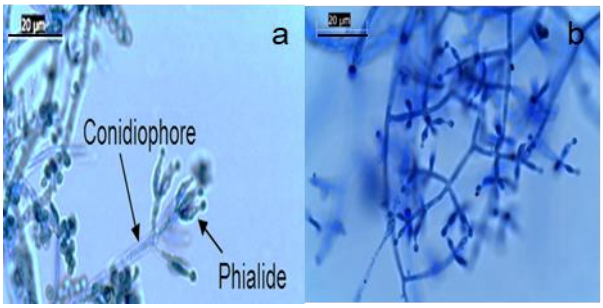

Figure 4. Conidiophore and phialide observed under 1000x magnification with scale bar of 20 µm. a) *Trichoderma virens* formed appressed phialides, and globose single-celled conidia, b) *Trichoderma harzianum* phialides were held at a right angle with the main hyphae

Table 2. Summary of morphological characteristics and growth rate of *Trichoderma* isolates

Isolate No.	Species	Mycelial Form	Colony Colour	Pigmentation	Concentric Ring	Pustule	Growth Rate (mm/day)
TJ1	*T. virens*	Scattered in minute tufts	Dark bluish green	Pale yellowish	Yes	Yes	25.4
TJ2	*T. virens*	Scattered in minute tufts	Dark bluish green	Pale yellowish	Yes	Yes	25.6
TJ3	*T. virens*	Scattered in minute tufts	Dull green	Pale yellowish	Yes	Yes	25.4
TJ4	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	26.4
TJ5	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	25.9
TJ6	*T. harzianum*	Arachnoid to floccose	Yellowish green	White	Yes	Yes	28.0
TJ7	*T. harzianum*	Arachnoid to floccose	Whitish green	White	Yes	No	28.5
TJ8	*T. harzianum*	Scattered in minute tufts	Dull green	White	Yes	No	28.3
TJ9	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	28.5
TJ10	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	27.8
TJ11	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	28.7
TJ12	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	28.8
TJ13	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	28.6
TJ14	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	28.6
TJ15	*T. tawa*	Arachnoid to floccose	White	White	Yes	Yes	26.8
TJ16	*T. harzianum*	Scattered in minute tufts	Dull green	Amber	Yes	Yes	26.3
TJ17	*H. lixii*	Scattered in minute tufts	Whitish green	White	Yes	Yes	26.4
TJ18	*T. brevicompactum*	Scattered in minute tufts	White	Pale yellowish	Yes	Yes	24.0
TJ19	*T. brevicompactum*	Scattered in minute tufts	White	Pale yellowish	Yes	Yes	22.2
TJ20	*T. harzianum*	Scattered in minute tufts	Yellowish green	Amber	Yes	No	26.2

Molecular Identification and Phylogenetic Analysis

Molecular identification of *Trichoderma* isolates was carried out using the ITS rDNA region. PCR amplification has resulted in a single band of approximately 600 bp. Compared to the sequences available on NCBI GenBank, all *Trichoderma* isolates were identified to species level with homology of 99.82 to 100% (Table 1). Multiple sequence alignment has revealed a substantial difference between the ITS rDNA sequences of different *Trichoderma* species. A phylogenetic tree was constructed based on ITS rDNA region to elucidate the genetic relatedness between the species. Other *Trichoderma* species with ITS rDNA sequences were retrieved from the NCBI GenBank database, and *T. brunneoviride* was used as an outgroup to determine the position of the root (Table 3). Using the Bayesian phylogenetic program, the phylogenetic tree constructed based on ITS rDNA sequences showed five main clades (Figure 5). Sequence variability was detected among the *T. harzianum, T. virens,* and *T. brevicompactum* isolates, leading to the formation of sub-clades. The differences may not support cryptic species recognition but were sufficient for phylogenetic reconstruction. *Trichoderma harzianum* isolate TJ16 was excluded from the analysis because it was positioned in a separated clade from the other *T. harzianum* isolates. Interestingly, isolate TJ16 has a distinct dull green colonial colour compared to other *T. harzianum* isolates.

Confrontation Assay

A curve with concavity orientated towards the pathogen was observed at the interaction zone between the two fungal colonies on the dual culture plates. The degree of curvature was determined by the differential growth rate of the interacting colonies. A curve was formed when one colony had a faster growth rate than the other, whereas a straight line was formed when both colonies shared the same growth rate (Hassan *et al*., 2014). Growth inhibition of pathogenic fungi was evident after four days of inoculation. *Trichoderma* isolates have a varied range of PIRGP against *P. palmivora* (47.2-62.9%), *C. gloeosporioides* (60.2-70.7%), and *F.*

solani (59.7-70.2%) (Table 4). Strong mycoparasitism was indicated by the invasion and sporulation of *Trichoderma* isolates on the pathogen colony. *Trichoderma* isolates showed varying degrees of antagonistic activity against the pathogens. Most of the *Trichoderma* isolates have grade two to three antagonistic activity against three tested fungal pathogens. Three isolates of *T. harzianum* (isolate TJ4, TJ10, and TJ16) showed the highest average of 62% PIRGP against *P. palmivora* with grade three antagonistic activity. Among the isolates, *T. tawa* (isolate TJ15) showed the highest inhibitory activity against *C. gloeosporioides* with 70.7% PIRGP and grade three antagonistic activity, followed by *T. harzianum* (isolate T5) with 70.3% PIRGP and grade three antagonism. *Trichoderma virens* isolates showed the weakest antagonistic activity against *C. gloeosporioides* with an average of 61% PIRGP. A clear inhibition zone was clearly seen between the colonies at ten days after inoculation. The highest growth inhibition of an average of 70% against *F. solani* was demonstrated by two *Trichoderma* species, *T. harzianum* (isolate TJ7 and TJ8) and *T. tawa* (isolate TJ15). However, statistical analysis revealed no significant difference in PIRGP among these isolates. Strong antagonistic activity (grade four) was observed in *T. tawa* (isolate TJ15). The lowest antagonistic activity was observed in *T. brevicompactum* (isolate TJ18), where a near straight curve line was formed at the interacting zone.

Table 3. *Trichoderma* ITS sequences retrieved from the NCBI GenBank database

No.	Accession No.	Isolate No.	*Trichoderma* Species	Source	Origin
1	KP056781	F1d5c1	*T. virens*	Soil	Malaysia
2	KT363921	159c	*T. virens*	*Elaeis guineensis*	Malaysia
3	MZ677295	NNC109	*T. virens*	Soil	South Africa
4	MK459340	NECC31240	*T. harzianum*	-	China
5	MT229305	FL8	*T. harzianum*	Mining camp	China
6	MZ664267	2-3-9-1	*T. harzianum*	*Eupatorium chinense*	China
7	KX092002	TB003	*T. brevicompactum*	-	Malaysia
8	EU330943	CBS 112443	*T. brevicompactum*	Soil	United States
9	MK120900	BTbr6	*T. brevicompactum*	Mungbean rhizosphere	India
10	MK346243	IAUYFAAKHR8	*T. tawa*	Sewage	Iran
11	MH625704	TF9	*T. tawa*	Soil	Nigeria
12	NR134376	CBS 121130	*T. brunneoviride*	-	United States

Table 4. Inhibitory effects of *Trichoderma* isolates against pathogenic fungi isolated from black pepper

Isolate No.	Species	Percentage inhibition of radial growth of pathogens (PIRGP) (%)		
		Phytophthora palmivora	*Colletotrichum gloeosporioides*	*Fusarium solani*
TJ1	*T. virens*	52.2 ± 1.7 ^{b}	61.0 ± 0.1 ^{ab}	59.7 ± 0.3 ^{ab}
TJ2	*T. virens*	51.3 ± 0.9 ^{b}	61.2 ± 0.3 ^{ab}	60.0 ± 0.7 ^{ab}
TJ3	*T. virens*	47.2 ± 1.7 ^{c}	60.2 ± 0.7 ^{ab}	60.0 ± 0.7 ^{ab}
TJ4	*T. harzianum*	62.9 ± 1.4 ^{ab}	63.5 ± 0.3 ^{ab}	63.8 ± 0.3 ^{ab}
TJ5	*T. harzianum*	58.8 ± 0.9 ^{b}	66.1 ± 0.3 ^{a}	67.5 ± 0.6 ^{a}
TJ6	*T. harzianum*	57.1 ± 1.7 ^{b}	66.7 ± 0.1 ^{a}	67.5 ± 0.6 ^{a}
TJ7	*T. harzianum*	53.8 ± 1.7 ^{b}	67.1 ± 0.3 ^{a}	69.9 ± 0.3 ^{a}
TJ8	*T. harzianum*	55.5 ± 0.1 ^{b}	65.1 ± 0.3 ^{a}	70.2 ± 0.7 ^{a}
TJ9	*T. harzianum*	61.2 ± 2.2 ^{ab}	64.8 ± 0.6 ^{a}	65.8 ± 0.3 ^{a}
TJ10	*T. harzianum*	62.1 ± 1.7 ^{ab}	66.1 ± 0.7 ^{a}	66.8 ± 0.3 ^{a}
TJ11	*T. harzianum*	53.8 ± 0.9 ^{b}	64.1 ± 0.7 ^{ab}	65.8 ± 0.3 ^{a}
TJ12	*T. harzianum*	53.8 ± 1.7 ^{b}	64.1 ± 0.7 ^{ab}	66.8 ± 0.3 ^{a}
TJ13	*T. harzianum*	53.0 ± 1.4 ^{b}	64.5 ± 0.3 ^{a}	66.1 ± 0.3 ^{a}
TJ14	*T. harzianum*	58.8 ± 1.7 ^{b}	64.5 ± 0.3 ^{a}	66.1 ± 0.7 ^{a}
TJ15	*T. tawa*	56.3 ± 0.9 ^{b}	70.7 ± 0.6 ^{a}	69.9 ± 0.9 ^{a}
TJ16	*T. harzianum*	62.1 ± 1.7 ^{ab}	70.3 ± 0.3 ^{a}	67.8 ± 0.3 ^{a}
TJ17	*H. lixii*	-	-	-
TJ18	*T. brevicompactum*	47.2 ± 1.7 ^{c}	62.2 ± 0.7 ^{ab}	59.7 ± 0.3 ^{ab}
TJ19	*T. brevicompactum*	52.2 ± 1.7 ^{b}	62.8 ± 0.1 ^{ab}	60.7 ± 0.7 ^{ab}
TJ20	*T. harzianum*	53.8 ± 1.7 ^{b}	67.4 ± 0.7 ^{a}	68.8 ± 0.3 ^{a}

* Values are mean of three replications. Values with different lower-case letters are significantly different; P≤0.01%.

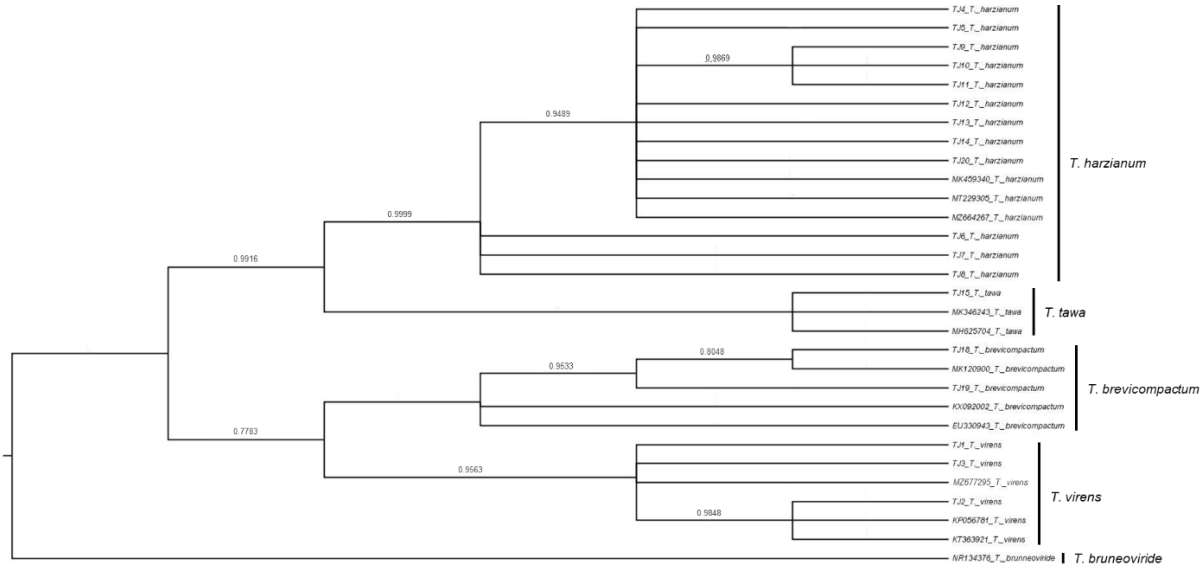

Figure 5. Phylogenetic analysis of *Trichoderma* isolates based on ITS sequences. Bayesian consensus tree was generated after burn-in 25% for one million generations. Posterior probabilities are shown at each node

DISCUSSION

The multifunctional traits of *Trichoderma* species to act as biocontrol agents for plant disease, natural decomposers for nutrient cycling, plant growth promoter, bioremediation agents for excess synthetic fertiliser, and pesticides have accelerated the search for novel *Trichoderma* isolates (Zin & Badaluddin, 2020). However, isolates must be carefully identified, and their biological properties must be assessed before the targeted application. For biological control, isolates should be selected based on their compatibility with the targeted field, host plants, and pathogens to reduce disturbance to the native microbial biodiversity. Understanding the differences among different species of the genus would also facilitate this process. In this study, native Sarawak *Trichoderma* isolates were identified, and phylogenetic analysis was performed before being tested for their antagonistic activities against three major fungal pathogens of black pepper in Sarawak.

No specific host-associated pattern was observed in this study since most *Trichoderma* species were isolated from a wide range of plant species. Host specialisation was not reported in *Trichoderma* compared to other fungal groups such as endophytic ascomycetes and mycorrhizal fungi. A polyphasic approach was carried out using a combination of morphological and molecular methods to identify the isolates. This is a more accurate and reliable method for *Trichoderma* species identification. The current *Trichoderma* taxonomy was based on the descriptions and keys proposed by Bissett (1991) with reference to the species complexes described by Rifai (1969), where members of the *Trichoderma* genus were organised into five sections. Species identification based on morphological characteristics is proved difficult for the *Trichoderma* genus. This resulted from a high degree of morphological similarities at the species level. Morphological identification has resulted in up to 50% of misidentification where external environment influences such as culture medium, pH, light, and temperature can lead to extensive phenotypic and physiological variation (Siddiquee *et al*., 2007; Hassan *et al*., 2014). Nevertheless, morphological characteristics are helpful for the preliminary identification of the *Trichoderma* genus. Three different groups, *T. virens*, *T. harzianum*, and *Trichoderma* spp. were identified based on distinctive morphological characteristics. Although *T. virens* and *T. harzianum* represent the *Pachybasium* section, the grouping was possible based on colony colour, pigmentation, and the branching pattern of conidiophores and phialides. Phialides of *T. harzianum* had whorls of 2 to 6 and were arranged at a right angle with conidiophore, where the phialides of *T. virens* were bent and appressed. The morphological description made in this study followed the findings of the previous studies (Bissett, 1991; Samuels *et al*., 2012).

The introduction of DNA barcoding system has led to the exponential identification of new *Trichoderma* species, with up to 375 species recognised as of July 2020 (Cai & Druzhinina, 2021). The complete ITS rDNA, specifically the ITS1-ITS2 region, was used as the primary DNA barcode for *Trichoderma* identification. ITS region allowed an easy amplification and modification to ribotyping, and access to large publicly available reference databases makes it the first locus used for rapid fungal identification. However, overlapping species identification was observed during species identification using NCBI database. In this study, different *Trichoderma* species were found to share the same ITS phylotype. Thus, recent studies have urged to include secondary DNA barcodes such as translational elongation factor EF-1 alpha (TEF1), calmodulin (CAL), and RNA polymerase II (RPB2) for accurate identification of sister species, e.g., *T. koningii* and *T. ovalisporum* (Kotasthane *et al*., 2015). This inability to distinguish between sister species was resulted from the high occurrence of the homoplasious site due to convergent evolution (Chaverri *et al*., 2015). In addition, the formation of phylogenetically different lineages among the *Trichoderma* species may have practical relevance to the identification of biocontrol agents. It may reflect differences in growth requirements and the production of secondary metabolites directly related to biocontrol potential. Among the *Trichoderma* species identified in this study, *T. harzianum* isolates were most effective in controlling fungal pathogens *in vitro*. *T. harzianum* and *T. virens* have been used for the biocontrol of damping off, wilt, blight, and rot diseases caused by various soil-borne pathogens (Zin & Badaluddin, 2020). In this study, several *T. harzianum* isolates showed significant *in vitro* mycelial growth inhibition and antagonistic activity against black pepper fungal pathogens. Nevertheless, these activities not only varied according to *Trichoderma* species but also the target pathogens. Thus, the selection of the most effective *Trichoderma* strains should be made in accordance with the target diseases. The antagonism of *Trichoderma* isolates can involve indirect and direct mechanisms depending on the target pathogen, host plant, and environmental factors. Environmental nutrient supplies play an essential role in triggering the interaction and interspecific competition between *Trichoderma* and plant pathogens. Under limited nutrient conditions, *T. harzianum* was found to produce harzianic acid showing fungicidal, siderophoric, and plant growth-promoting activities that altered the soil environment (Vinale *et al*., 2013). Next, *Trichoderma* can trigger ISR response that primes the production of phytohormones and cell wall degrading enzymes such as chitinase, glucanase, glucosaminidase, acid phosphatase, acid protease, and alginate lyase before the interaction with pathogens (Qualhato *et al*., 2013; Kumar *et al*., 2019). Therefore, a thorough understanding of molecular mechanisms of interactions between the potential biocontrol candidates, target pathogen, and host plant is important for effective biocontrol. A glasshouse trial is now underway to evaluate the biocontrol potential of candidate *Trichoderma* isolates against fungal pathogens on black pepper.

CONCLUSION

A polyphasic approach involving morphological and molecular characterisation is important for accurate species identification. Further molecular characterisation would be necessary using additional DNA barcode loci to reverify the species identity of the isolated *Trichoderma* isolates. *Trichoderma* isolates showed *in vitro* mycelial growth inhibition and antagonistic activity against three fungal pathogens of black pepper. This result suggested that *Trichoderma* isolates are potential biocontrol agents for sustainable black pepper production. Candidate isolates will be further tested in glasshouse and field experiments to evaluate their protection potential against black pepper fungal pathogens.

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