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IDENTIFICATION AND SCREENING OF *TRICHODERMA* SPP. AS ANTAGONISTIC AGENTS AGAINST PHYTOPATHOGENS, *COLLETOTRICHUM* SP. AND *RHIZOCTONIA SOLANI* KÜHN

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SUMMARY

Trichoderma sp. is a promising biocontrol agent against phytopathogens that cause significant yield losses in various crops. The following study aimed to identify the *Trichoderma* spp. isolates through morphological and molecular characterizations and to select the most potential *Trichoderma* isolates antagonistic to the fungi species *Colletotrichum* sp. and *Rhizoctonia solani*. *Trichoderma* spp. colonies exhibited a flat elevation with a central regular edge, central yellow to green with white edges, and the highest degree of branching and pyramid-shaped conidiophores. The 17 isolates belonged to *Trichoderma asperellum* (13 isolates), *T. gamsii* (one isolate), *T. harzianum* (two isolates), and *T. koningiopsis* (one isolate). By dual culture assays, *T. koningiopsis* TR3 and *T. asperellum* Pan23.1 had the higher growth inhibition than others against the phytopathogen *Colletotrichum,* accounting for 75.86% and 82.76%, respectively. *T. asperellum* Kun4, *T. asperellum* Pan23.2, and *T. koningiopsis* TR3 demonstrated their supreme growth inhibition against the phytopathogen *Rhizoctonia* (85.00%, 87.50%, and 92.50%, respectively). The preliminary screening of isolates *T. asperellum (*Pan23.1, Pan23.2, and Kun4) and *T. koningiopsis* TR3 confirmed their ability as potential biocontrol agents. Therefore, further studies are essential to explore these four isolates as effective biocontrol agents for controlling the phytopathogens *Colletotrichum* sp. and *Rhizoctonia solani*.

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Keywords: *Trichoderma* spp., biocontrol agent, antagonism, *Colletotrichum* sp., *Rhizoctonia* sp., growth inhibition, crop losses, ITS

Key findings: Based on morphological and molecular characteristics, the 17 tested isolates acquired from this study attained grouping in the same cluster identified as *Trichoderma asperellum*, *T. gamsii*, *T. harzianum*, and *T. koningiopsis*. Dual culture tests demonstrated that *T. koningiopsis* TR3 and *T. asperellum* Pan23.1 had the highest growth inhibition against *Colletotrichum*. The *T. asperellum* Kun4 and Pan23.2 and *T. koningiopsis* TR3 exhibited the greatest antagonism against the phytopathogen *Rhizoctonia*.

INTRODUCTION

Plant diseases caused by pathogens, including bacteria, fungi, viruses, and nematodes, significantly hinder the potential yield of economic crops. The phytopathogens *Colletotrichum* spp. and *Rhizoctonia* spp. are widely notorious for their detrimental impact on economic crops. *Colletotrichum* spp. are responsible for anthracnose disease in papaya, potato, chili, cocoa, and tomato (Belov *et al.,* 2018; De-Silva *et al.,* 2019; El-Baky and Amara, 2021; Zakaria, 2021). Conversely, *Rhizoctonia* spp. has hosts of broad-spectrum essential food crops, such as soybean, rice, wheat, potato, chickpea, and sugar beet (Ajayi-Oyetunde and Bradley, 2018; Ram and Singh, 2018; Liu and Mundt, 2020; Kiptoo *et al.,* 2021; Li *et al.,* 2021; Misra *et al.,* 2023). Both pathogens also attack crop plants during their growth and postharvest.

Fungal species within the genus *Trichoderma* are notable for their rapid growth and crucial stress tolerance. Numerous species of *Trichoderma* exhibited a cosmopolitan distribution, thriving in diverse ecological niches (Japanis *et al.,* 2021). The adaptability of *Trichoderma* makes it a valuable tool in sustainable agriculture for managing phytopathogens and enhancing crop productivity (Yao *et al*., 2023). *Trichoderma* is a saprophytic fungus with the highest levels of diversity worldwide. Several types of this genus have the potential to produce cellulase and hemicellulose (*T. reesei*) and can antagonize and kill other fungi, especially *T. harzianum*, *T. atroviride*, *T. virens*, and *T. asperellum*, and are beneficial as biocontrol agents against phytopathogens (Halifu *et al.,* 2020; Tomah *et al.,* 2020).

Of 11 *Trichoderma* species, 60 isolates collected from several crop rhizospheres across different geographic locations in India underwent successful authentication as potential biocontrol agents against soil-borne plant pathogenic fungi, including *Rhizoctonia solani* (Jambhulkar *et al.,* 2024). *Trichoderma's* ability to antagonize pathogens can also be favorable as an alternative to pesticides. Similarly, *Trichoderma* promotes plant growth and enhances plant resistance to pathogens, making effective biofertilizers.

Trichoderma spp. exhibit complex taxonomy and remarkable genetic diversity, making them a significant focus of contemporary research. Despite this diversity, only a few *Trichoderma* spp. have achieved successful utilization as mycofungicides. Accurate species identification is vital due to the morphological similarities among various *Trichoderma* spp. and the necessity to exclude harmful strains that could negatively impact economically important commodities, such as cultivated mushrooms. Relying solely on morphological characteristics is inadequate for reliable identification, especially within genetically diverse groups like the *T. harzianum* and *T. viridescens* complexes, which display considerable morphological variability (Cai *et al*., 2021). However, to address these identification challenges, employing molecular techniques can determine which *Trichoderma* spp. can effectively inhibit the growth of fungal species, such as *Colletotrichum* and *Rhizoctonia, in vitro*. Analysis of the rDNA sequence in the internal transcribed spacer (ITS) region is particularly useful, as it typically shows the highest variation for distinguishing fungal species (Singh *et al*., 2014).

Furthermore, Dou *et al*. (2020) introduced the multilocus identification system for *Trichoderma* (MIST) to enhance species documentation.

Thus, to fully harness the capabilities of the *Trichoderma* spp. for biocontrol applications, it is essential to accurately identify and characterize this fungus. The following study aimed to measure the most significant percentage of inhibition of *Trichoderma* spp. isolates against the phytopathogens *Colletotrichum* sp. and *Rhizoctonia solani in vitro* and to identify *Trichoderma* spp. isolates both morphologically and molecularly using the ITS sequence analysis. Moreover, the presented results could contribute to the agricultural sector in solving crop disease problems mainly caused by the fungal species of *Colletotrichum* and *Rhizoctonia*.

MATERIALS AND METHODS

Microbial isolates and culture media

Trichoderma isolates from the soil totaled 17, obtained from the collection of the Indonesian Center for Spice and Medicinal Crops Instrument Standard Testing and Indonesian Center for Soil and Fertilizer Instrument Standard Testing, Ministry of Agriculture, Bogor, West Java, Indonesia. These were recent studies on the isolation of new species of *Trichoderma* spp. from soil in the rhizosphere of rice, chili, and pepper in West Java. This rhizosphere usually provides a favorable environment with nutrient-rich and suitable pH from slightly acidic to neutral, which are suitable for *Trichoderma* spp. growth. The transfer of one dose of fungal isolates from the collection culture proceeded into the potato dextrose agar (PDA) medium containing 200 g potato extract, 20 g dextrose, and 15 g agar for one liter volume. This rejuvenation continued in a laminar airflow cabinet. The transferred isolate then grows for 3–5 days at room temperature.

Morphological observation of the *Trichoderma*

The *Trichoderma* morphology investigation ensued macroscopically and microscopically. Macroscopic observations commenced on the third day, including the colony's color, shape, texture, and shape of the colony's edge. Microscopic observations used a 10% potassium hydroxide solution for the mounting medium. The shape of the spores and conidiophores was visible under a compound light microscope (Olympus BX51). For the macroscopic observations, *Trichoderma* isolates grown on a new PDA of agar plate continued by placing mycelial plugs of 5 mm in diameter uniformly for all isolates from the rejuvenate isolates. The culture plates then sustained incubation at room temperature (27 °C ± 2 °C) for five days. A duplicate plate for each isolate prepared earlier often incurs careful observations of contaminants of distinct colonies with different growth patterns and morphology from *Trichoderma* spp. by visual assessment and microscopic examination. The diameter of each colony in mm and the color, elevation, and edge of each isolate were prominent at the end of incubation until the agar plates' full colonization. The morphological characteristics of the fungal structure received names using the terms of Gams and Bisset (1998). Only the isolates morphologically having *Trichoderma* characteristics proceeded to further confirmation by a molecular analysis for species identity.

Molecular identification

Genomic DNA extraction made from fresh culture used the recommended method (Jain *et al.*, 2020). The DNA pellet's dissolving occurred in a 1x TE buffer before storing at -20 °C. Quantitative analysis of DNA succeeded in using a 2000 Nanodrop Spectrophotometer (Thermo Scientific, USA), while qualitative analyses transpired using 1% agarose gel.

The DNA amplification ran using ITS1 (5'-TCTGTAGGTGAACCTGAGG) and ITS4 (5'-

TCCTCCGCTTATTGATATGC) primers. The PCR cocktails comprised 12.5 µL DNA Taq Polymerase, 1 µL of 10 pmol ITS1 primer (forward), 1 µL of 10 pmol ITS4 primer (reverse), 8.5 µL sterile ddH_2O, and 2 µL of 100 ng/µL DNA template. The PCR reaction continued in a T1 Thermocycler (Biometra, Germany) with the following program: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 35 s, and elongation at 72 °C for 30 s. The reaction ended with a final elongation at 72 °C for 7 min. The PCR products' separation utilized 1% agarose gel electrophoresis in 0.5x TAE buffer before staining with ethidium bromide (EtBr) and visualizing with the help of UV light using ChemiDoc.

Sanger sequencing proceeded with the PT. Genetika Science. The *Trichoderma* sequences' alignment among themselves used Clustal the Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) to identify the nucleotide variations among them. Then, matching of sequencing results with the database collection in Gene Bank occurred, and their analysis employed the BLASTN (Basic Local Alignment Search Tool-Nucleotide) program on the NCBI (National Center for Biotechnology Information) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The non-redundant (nr) gained selection in the BLAST process with the nucleotide query. The phylogenetic tree among the sequences obtained from this study and collected accessions from the NCBI underwent construction using MEGA 5.1.

Antagonistic test

The antagonistic ability of *Trichoderma* against the fungal species *Colletotrichum* and *Rhizoctonia in vitro* materialized using the dual-test method at room temperature (27 °C ± 2 °C) and relative humidity in the range of 80%–90% for optimal fungal activity (Svetlana *et al*., 2010). Pieces of each fungal colony (± 5 mm ∅) from the edge of six-day-old culture on PDA medium continued transfer onto a PDA medium in a petri plate. The distance between two pieces of tested fungal colony was about 4 cm away (Bunbury-Blanchette and Walker, 2019). The radial growth measurement of each fungal colony emerged daily, with the percentage of growth inhibition (PGI) calculated using the following formula:

$$PGI\ (\%) = KR\text{-}R1/KR \times 100\%$$

Where, PGI = the percent of growth inhibition (%), KR = the distance from the point of inoculation to the colony margin on the control dishes (mm), and R1 = the distance of fungal growth from the point of inoculation to the colony margin (mm).

Three isolates of the species *Trichoderma* with the highest PGI values sustained testing for further antagonist treatment with inoculation time and three replicates. These three isolates were Pan23.1 and Pan23.2 (*T. asperellum*) and TR3 (*T. koningiopsis*). The first treatment involved initially culturing the pathogen, followed by culturing *Trichoderma* after 24 hours. The second treatment involved culturing *Trichoderma* for 24 hours in advance, using a PDA medium in a petri plate in this dual culture test. The petri plates' incubation began under room conditions, avoiding exposure to direct light. The colony growth measurement of each fungus happened daily until the colony reached the edge of the plate. All data on antagonistic incur analysis for ANOVA using Rv.4.2.0 (https://www.r-project.org/), and comparison for significant differences between treatments ensued using HSD at a p-value < 0.05.

RESULTS

Morphological characteristics

The *Trichoderma* isolates have flat elevations with regular edges. The colors produced by all the 17 isolates varied in the center, starting from white (isolates TR3 and TR4), yellowish (isolates I4, OA9, D4, and PB_2), and yellow (isolate O5) to green (isolates Pan23.1, Kun4, Gam4, Bo17, Pan23.2, GO9, PO3, and N34), while the edges were white (Table 1).

Table 1. Morphological characteristic of 17 *Trichoderma* sp. isolates.

No.	Isolates	Color		Elevation	Edge
		Center	Edge		
1	O5	Yellow	White	Flat	Regular
2	Pan23.1	Green	White	Flat	Regular
3.	Kun4	Green	White	Flat	Regular
4	I4	Yellowish	White	Flat	Regular
5	Gam4	Green	White	Flat	Regular
6	OA9	Yellowish	White	Flat	Regular
7	D4	Yellowish	White	Flat	Regular
8	Bo17	Green	White	Flat	Regular
9	Pan23.2	Green	White	Flat	Regular
10	PC5	Yellow	White	Flat	Regular
11	TR4	White	White	Flat	Regular
12	RR9	Yellow	White	Flat	Regular
13	PB_2	Yellowish	White	Flat	Regular
14	GO9	Green	White	Flat	Regular
15	PO3	Green	White	Flat	Regular
16	N34	Green	White	Flat	Regular
17	TR3	White	White	Flat	Regular

The conidiophores and phialides of the *Trichoderma* isolates were variable and showed complex structures critical for asexual reproduction. All isolates revealed the highest level of branching in conidiophores with pyramid-shaped branches and spores that cluster like green grapes. Phialides seem to be oriented parallel to the main axis of the conidiophores at various angles. However, isolates demonstrated their highly branched conidiophores with clusters of phialides, while other isolates featured simpler, less branched conidiophores and with less clustered phialides. Although some isolates also exhibited compact conidiophore structures with dense clusters of phialides.

Molecular characteristics and genetic relationship

The presented study used specific primers to amplify the ITS regions of *Trichoderma* isolates. Primers typically used were ITS1 (forward primer) and ITS4 (reverse primer), which flank the ITS region. All the *Trichoderma* isolates used gave amplicons ranging from 600 to 700 bp. The amplification of *Trichoderma* genomic DNA using primers ITS1 and ITS4 revealed clear and visible bands.

The ITS region with produced amplicons received sequencing, and comparing with reference databases allowed for precise species identification. As many as 13 of 17 isolates emerged as *T. asperellum*, one isolate was of *T. gamsii*, one isolate was of *T. koningiopsis*, and the remaining two isolates were of *T. harzianum*. The sequences showed 99%–100% similarity with the NCBI accession collection. The *Trichoderma* isolates Pan23.1, Pan23.2, PC5, and Kun4 showed 100% similarity with an E value of 0.0 to *T. asperellum* strain TR11. In this study, the similarity of *Trichoderma* sequences with database collections appears in Table 2.

The alignment among the 17 *Trichoderma* sequences displayed the nucleotide variations in ITS regions. Several single nucleotide polymorphisms (SNPs) existed in the consensus region of the ITS among the different isolates, suggesting the highest variation even though they belong to the same species. Some insertions and deletions developed a large gap, approximately 17 nucleotides (TAATCTGAGCCTTCTCG), separating PB_2 and N34 isolates from the others (Figure 1a). According to homology analysis (Table 2), isolates PB_2 and N34 were distinct as *T. harzianum* species. This result

Table 2. Sequence similarity among 17 *Trichoderma* sp. isolates.

Isolates	Description	Value E	Similarity (%)	Accessions
O5	*Trichoderma asperellum strain* TR11	0.0	99	KC859432.1
Pan23.1	*Trichoderma asperellum strain* TR11	0.0	100	KC859432.1
Kun 4	*Trichoderma asperellum strain* TR11	0.0	100	KC859432.1
I4	*Trichoderma asperellum strain* UTP-16	0.0	99	FJ640576.1
Gam4	*Trichoderma asperellum strain* UTP-16	0.0	99	FJ640576.1
OA9	*Trichoderma asperellum isolate* T8	0.0	99	JX422014.1
D4	*Trichoderma asperellum strain* TR11	0.0	99	KC859432.1
Bo17	*Trichoderma asperellum strain* TR11	0.0	99	KC859432.1
Pan23.2	*Trichoderma asperellum strain* TR11	0.0	100	KC859432.1
PC5	*Trichoderma asperellum strain* TR11	0.0	100	KC859432.1
TR4	*Trichoderma gamsii strain* UNISS 11-62	0.0	99	EF488141.1
RR9	*Trichoderma asperellum strain* TR11	0.0	99	KC859432.1
PB_2	*Trichoderma harzianum isolate* A1S4-2	0.0	99	KJ767087.1
G09	*Trichoderma asperellum isolate* M29	0.0	99	JX422010.1
P03	*Trichoderma asperellum strain* TR11	0.0	99	KC859432.1
N34	*Trichoderma harzianum strain* LIPIMC0572	0.0	99	KC847182.1
TR3	*Trichoderma koningiopsis strain* F-2-46	0.0	99	KF751670.1

further enunciated an interesting variation that could correlate to unique characteristics distinguishing this species from other *Trichoderma* species and requires addressing in the following study.

The outgroup accessions comprised *T. viride*, *T. arundinaceum*, and *T. reesei* sequences. The phylogenetic tree revealed those three outgroup accessions appeared in different clusters (Figure 1b). Meanwhile, the 17 isolates of *Trichoderma* acquired from this study resulted in the same cluster, which greatly agreed with the alignment results to the NCBI collection. In the phylogenetic tree, the isolates N34 and PB_2 showed separation from *T. harzianum* isolates AIS4-2 and LIPIMC0572 sequences. Both N34 and PB_2 existed in a cluster between *T. asperellum* and *T. koningiopsis*, while *T. harzianum* isolates AIS4-2 and LIPIMC0572 emerged in another cluster with an outgroup sequence, in contrast to homology results.

Antagonistic test

The dual culture assay of *Trichoderma* spp. isolates inhibited the radial growth of the fungal species *Colletotrichum* sp. and *Rhizoctonia solani*. The average percentage of their inhibition against *Colletotrichum* was less compared with the species *Rhizoctonia*. The highest inhibition came from the isolate Pan23.1 (82.76%). The greatest magnitude of the inhibition value of *Trichoderma* against *Rhizoctonia* was 92.50% by the isolate TR3. Isolates with an inhibition value >80% were Kun4, Pan23.2, RR9, N34, and TR3 (Table 3). The antagonist test for isolates Pan23.2 and TR3 against the species *Rhizoctonia* is visible in Figure 2. The selected isolates of TR3, Pan23.1, and Pan23.2, grown 24 hours before the two pathogens of *Colletotrichum* sp. and *Rhizoctonia* sp., indicated their significant growth inhibition, mostly higher than 95% (Table 4).

DISCUSSION

Macroscopic morphology observations proceeded on 17 isolates of *Trichoderma* at the age of three days in the form of edges and elevations, as well as, the color of the isolate. All the isolates have white edges with flat elevations and regular edges. Susila *et al*. (2023) findings also gave similar results regarding macroscopic observations of *Trichoderma*, including growth rate, spore color, and spore distribution patterns. Several isolates can produce the yellow pigment that diffuses into the agar medium, namely, PB_2, Pan23.1, N34, and Kun4, accompanied by a flat surface with a soft and wool-like structure.

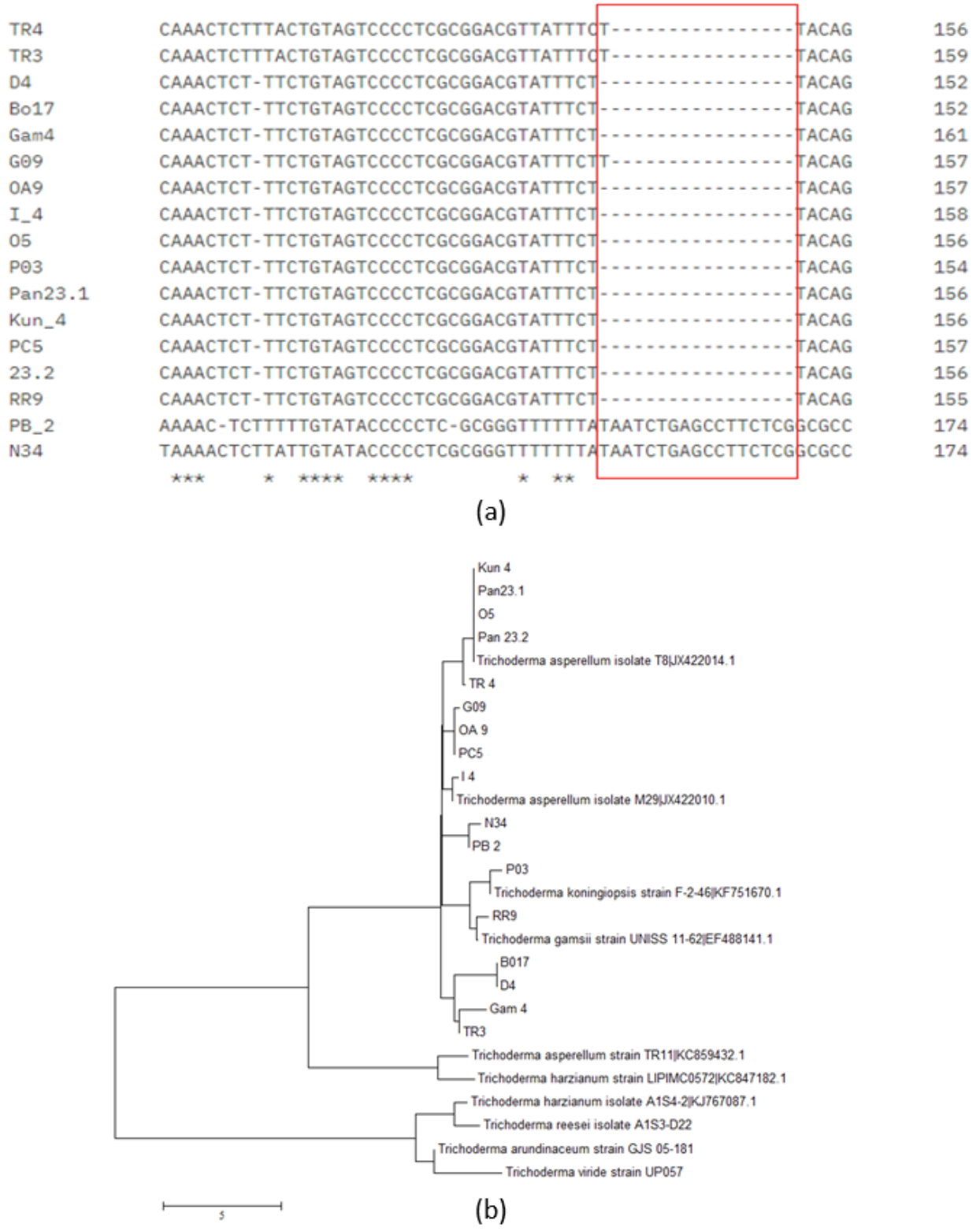

Figure 1. ITS sequences of *Trichoderma* sp. isolates. (a) Nucleotide variations in ITS sequence, (b) phylogenetic tree of *Trichoderma* sp. isolates from ITS sequencing results compared to NCBI database and outgroup sequences.

Table 3. Antagonistic test of 17 isolates of *Trichoderma* sp. against pathogenic fungi.

No.	Isolate	Inhibition (%)	
		Colletotrichum sp.	*Rhizoctonia* sp.
1	O5	55.17ef	65.00f
2	Pan23.1	82.76a	72.50de
3	Kun4	58.62de	85.00b
4	I4	51.72f	65.00f
5	Gam4	55.17ef	72.50de
6	OA9	62.07cd	60.00g
7	D4	58.62de	70.00e
8	Bo17	51.72f	75.00d
9	Pan23.2	65.52c	87.50b
10	PC5	65.52c	70.00e
11	TR4	65.52c	75.00d
12	RR9	65.52c	80.00c
13	PB_2	51.72f	60.00g
14	GO9	58.62de	62.50fg
15	PO3	62.07cd	72.50fe
16	N34	62.07cd	80.00c
17	TR3	75.86b	92.50a

Means followed by the same letters are not significantly different according to the HSD test with a p-value < 0.05.

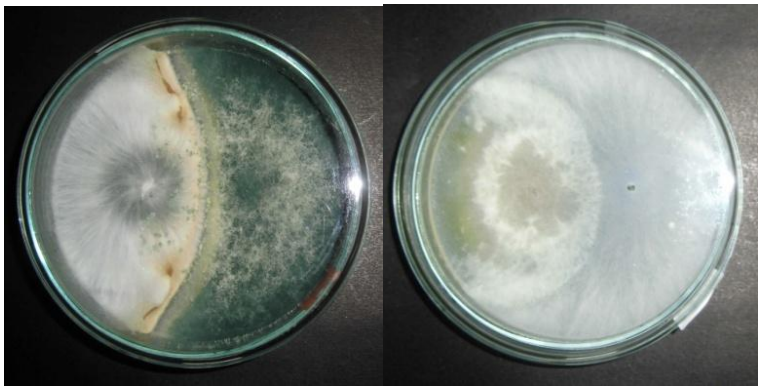

Figure 2. Growth inhibition of *Trichoderma asperellum* Pan23.2 (left) and *T. koningiopsis* TR3 (right).

Table 4. Growth inhibition of *Trichoderma koningiopsis* and *T. asperellum* against the fungal species *Colletotrichum* and *Rhizoctonia*.

Treatments	Antagonist	Growth Inhibition (%)
Colletotrichum sp. was inoculated 24 hours prior *Trichoderma* spp.	*T. koningiopsis* TR3	34.48a
	T. asperellum Pan23.1	45.98a
	T. asperellum Pan23.2	42.53a
Trichoderma was inoculated 24 hours prior *Colletotrichum* sp.	*T. koningiopsis* TR3	95.40a
	T. asperellum Pan23.1	96.55a
	T. asperellum Pan23.2	93.10a
Rhizoctonia sp. was inoculated 24 hours prior *Trichoderma* spp.	*T. koningiopsis* TR3	63.33a
	T. asperellum Pan23.2	65.00a
	T. asperellum Kun4	63.33a
Trichoderma was inoculated 24 hours prior *Rhizoctonia* sp.	*T. koningiopsis* TR3	97.50a
	T. asperellum Pan23.2	94.17a
	T. asperellum Kun4	96.67a

Means followed by the same letters are not significantly different according to the HSD test with a p-value < 0.05.

Almost all the isolates displayed conidiophores branching, which was relatively high and shaped like a pyramid. It was under the primary morphological results, which showed very tall branching conidiophores of *Trichoderma* with a pyramid-like pattern. The spores observed were light green to dark green, round in shape, and spread around the conidiophores. Athul and Jisha (2013) also stated *Trichoderma* conidia were elliptical, unicellular, and produced from conidiophores branching with a pyramid arrangement.

The amplification processes using the polymerase chain reaction (PCR) technique in the internal transcribed spacer (ITS) area were functional in this study. The sequence of PCR-ITS has already been effective for identifying and DNA barcoding various species of *Trichoderma* (Kumar *et al.*, 2019; Cai and Druzhinina, 2021). DNA barcoding is a valuable technique for detecting and classifying a species by sequencing a short DNA sequence from a conserved and well-identified gene (Purty and Chatterjee, 2016). Additionally, ITS areas have pretty high levels of variation, even within the same species. These characteristics make ITS sequences suitable for identifying fungal isolates at the species level. The ITS region includes ITS1 and ITS2, separated by the *5.8S* gene, located between the 18S and 28S.

In the presented study, the DNA amplification process used ITS1 and ITS4 primers, which worked in three regions, namely, ITS1, ITS2, and the *5.8S* gene, with the highest level of conservation suitable for inter- and intra-species identification (Singh *et al.,* 2014). The amplification with DNA bands had varying thicknesses, but their size was 600–700 bp for all the *Trichoderma* isolates. Previous reports stated the utilization of ITS1 and ITS4 primers in several studies that successfully showed their power to identify several fungal species, such as *Colletotrichum* (Aiello *et al.,* 2015), *Candida* (Ali *et al.,* 2015), *Cortinarius* (Itoo *et al.,* 2015), and *Aspergillus flavus* (Al-Shuhaib *et al.,* 2018).

Homology analysis of 17 isolates of *Trichoderma* continued by comparing the DNA sequencing results to the nucleotide query on the NCBI website using the BLASTN program. The homology analysis with the database showed *T. asperellum* acquired from this study subdivided into four types—strain TR11, strain UTP-16, isolate T8, and isolate M29. The *T. harzianum* cut into two types: isolate A1S4-2 and strain LIPIMC0572. The homology results obtained provided good data quality, reflected by an E-value of 0.0 for 17 isolates of *Trichoderma*. The low E-value indicated the high level of homology between the two sequences and vice versa. The E-value was an estimated value for the two sequences based on statistical calculations.

A percent total of 76.47% of the 17 isolates identified were of *T. asperellum*, 11.76% were of *T. harzianum*, and the remaining 5.8% were of *T. gamsii* and *T. koningiopsis*. Consistent with previous research, the *T. asperellum* was the most common isolate found in tropical areas (Perera *et al*., 2023). Quero *et al*. (2024) also reported the common species found in tropical areas were the *T. asperellum* and *T. harzianum*. The homology results with the database showed the classification of *T. asperellum* obtained in this study has four types, i.e., strain TR11, strain UTP-16, isolate T8, and isolate M29. However, *T. harzianum's* classification was into two types, viz., isolate A1S4-2 and strain LIPIMC0572.

Homology analysis expressed the similarity between those two isolates with *T. harzianum* isolate AIS4-2 and LIPIMC0572; however, the phylogenetic tree revealed a separation between them. These results may have happened due to several reasons, such as the complexity of *T. harzianum* species, since their ability to perform sexual reproduction (as *Hypocrea lixii*) could possibly lead to genetic variations. Other reasons are the ability of *T. harzianum* species to live in a cosmopolitan way with various substrates, as well as the limited number of primers used in this study (Chaverri *et al.,* 2015). Therefore, using more barcoding primers should be necessary in the future study.

The phylogenetic tree disclosed the relationship among the 17 isolates *of Trichoderma*. Three outgroup sequences, taken randomly, compared the relationship among the species outside the identified species. The

phylogenetic tree showed all the *Trichoderma* sequences analyzed cut into two main clusters. Grouping of all 17 *Trichoderma* isolates occurred in the same cluster, indicating their close genetic relationship. However, the three outgroup sequences and *T. harzianum* isolate A1S4-2 exhibited separation from the *Trichoderma* isolates, revealing their high genetic distance. The alignment results among 17 isolates of *Trichoderma* indicated a greater nucleotide variation, especially insertion and deletion, approximately 17 nucleotides, arising among PB_2 and N34 isolates with the others.

Antagonistic ability analysis used a multiple-test method by measuring the percentage of inhibition based on the comparison of the treated and controlled pathogenic radii. The inhibitory value measurement helped determine the inhibitory effects of the antagonistic fungus *Trichoderma* against the growth of pathogen colonies. The growth of the fungal species *Colletotrichum* and *Rhizoctonia* began to appear inhibited on the second day after inoculation. The colony size of *Colletotrichum* was smaller than the colony area of *Trichoderma*. This could be due to the growth rate of *Trichoderma* being relatively quicker (>8–9 cm after three days), indicating most common characteristics of this species.

The inoculation time treatment took place on the three *Trichoderma* isolates with the highest inhibition value for each pathogen. Further antagonist testing of *Colletotrichum* against isolates Pan23.2, Tr3, and Pan23.1 showed significantly different mean inhibition between treatments I and II. The same thing happened when further testing of the antagonists of *Rhizoctonia* continued against the isolates Kun4, Tr3, and Pan23.2. The presence of *Trichoderma* allows producing enzymes that can attack pathogenic fungi and protecting plants from disease and increase the production. The *Trichoderma* can also produce several extracellular enzymes, such as chitinase, β-1,3 glucanase, protease, β-1,4 glucanase, and lipase (Ghasemi *et al.,* 2020). It also strengthens the *Trichoderma* role as a biocontrol agent because chitin and β-1,3 glucan are the main components of pathogen cell walls. The protease and lipase produced can also lyse the proteins and lipids in pathogen cell walls.

A variation appeared in the color of the pathogens *Rhizoctonia* and *Colletotrichum* in part directly adjacent to *Trichoderma* (Figure 2). This seemed to be due to the response of the respective fungi to the production of secondary metabolites by the *Trichoderma* when encountering pathogenic fungi. Light brown is an indication of the development of hard and resistant mycelium of *Rhizoctonia*, while that kind of characteristic is lacking in the group of *Colletotrichum*. Trichoderma also produces other secondary metabolites, such as antifungal antibiotics (Khan *et al.,* 2020).

Another inhibition that *Trichoderma* can carry out is competition for nutrients and mycoparasitism. The mode of action of *Trichoderma* sp. involves the colony inhibition growth of those two fungi, which could be the same, since conducting the assay was under laboratory conditions, and it was just at the early step of interactions. The secondary metabolite productions also had influences from species and strain, the environment, as well as genes, but the gene expressions are often cryptic under standard laboratory conditions (Kumari and Srividhya, 2020).

The experiments to find out the antagonistic effect of *Trichoderma* sp. either to *Colletotrichum* or *Rhizoctonia* have been progressive with many scientists (Es-Soufi *et al.*, 2020; Swati *et al*., 2024; Ibrahim, 2017). Those studies found *Trichoderma* is a biocontrol agent that can help prevent plant diseases and promote plant growth and is suitable for integration in the integrated pest management (IPM) (Tyśkiewicz *et al*., 2022; Kumar *et al*., 2023; Yao *et al*., 2023).

CONCLUSIONS

The study concluded indigenous *Trichoderma* spp. isolates from West Java demonstrated significant potential for biocontrol of the phytopathogens. Seventeen *Trichoderma* isolates showed the highest level of similarity to *T. asperellum* (13 isolates), *T. harzianum* (two isolates), *T. gamsii* (one isolate), and *T. koningiopsis* (one isolate). Most of the

Trichoderma isolates exhibited a close genetic relationship, except for *T. harzianum* A1S4-2. Out of four superior isolates against phytopathogens, TR3 and Pan23.1 have the maximum antagonistic ability against the *Colletotrichum* sp. and *Rhizoctonia solani*, respectively. *T. koningiopsis* TR3 exhibited a broad-spectrum antagonistic ability against both *Colletotrichum* sp. and *Rhizoctonia solani*. Therefore, further study is essential in plant testing to evaluate the biocontrol efficacy under field conditions to validate their efficacy in diverse environmental conditions.

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