

RESEARCH ARTICLE

Assessment of Biocontrol Ability of *Trichoderma* Isolates Against White Root Disease Caused by *Rigidoporus Microporus* in Cinnamon Under Laboratory Conditions

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Abstract: White Root Disease (WRD), caused by *Rigidoporus microporus*, poses a significant threat for cinnamon cultivations in Sri Lanka. The application of synthetic fungicides to manage this disease contradicts the principles of organic cinnamon production. This study aimed to isolate *Trichoderma* species from the rhizosphere soils of cinnamon, banana, and pepper, identify them morphologically, and assess their antagonistic activity against the WRD pathogen. The research was conducted at the National Cinnamon Research and Training Centre in Palolpitiya, Thihagoda, Sri Lanka. A dual culture assay was employed to evaluate the antagonistic potential of eight isolates: four from the rhizosphere soil of cinnamon trees (MA1, MA2, MA3, and MA4) and four from the rhizosphere soil of pepper (MT) and banana (Tc, Tk, and T13). The dual culture plates were incubated for five days, during which the radial mycelial growth of the pathogen was measured, and the percentage inhibition of this growth was calculated. The results indicated that the MA1 isolate achieved the highest percentage inhibition of radial mycelial growth at 44.12%, while the MA2 isolate showed the lowest inhibition at 22.06%. Importantly, the MA1 isolate significantly inhibited the mycelial growth of *R. microporus* ($p \leq 0.05$) compared to the other isolates.

Keywords: Biological control, dual plate assay, mycelial growth, percentage inhibition, organic agriculture.

Introduction

Cinnamon (*Cinnamomum zeylanicum* Blume, Syn. *Cinnamomum verum* J. Presl.) is one of the iconic crops in the world being used for spice daily by people all over the world. Currently, cinnamon is considered as the number one spice crop grown in Sri Lanka, accounting for more than 85 % of world's true cinnamon exports (Hewavitharana *et al.*, 2022). Ceylon cinnamon obtained the first-ever Geographical Indication (GI) certification for its specific geographical location and quality-related parameters in February 2022. The long-standing reputation for Ceylon cinnamon has always been linked to its quality, colour, aroma, and flavor (Ceylon Cinnamon Cultivation in Sri Lanka, 2023).

There are numerous cinnamon species in Sri Lanka but only *C. zeylanicum* is grown commercially. Currently,

focal areas of cinnamon cultivation are located along the coastal belt from Negambo to Matara and inroads to Ratnapura and Kalutara areas (Department of Export Agriculture, 2019).

Producing high-quality cinnamon products catering to global and local demand depends on many attributes. Although cinnamon is considered a hardy plant, it is still prone to attacks from numerous insects and microbes (Rajapakse and Kumara, 2010). Various pests and diseases can cause significant losses in cinnamon cultivations on the island, reducing the yield of cinnamon bark. It can impair the quality of other associated products as well.

White root disease is considered one of the most devastating diseases to many commercially grown rubber plantations worldwide and affects cinnamon and tea cultivations. The disease caused by the necrotrophic fungus *Rigidoporus microporus* (Fr.)

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Overeem is more common during the dry seasons when plants are under stress. WRD frequently occurs when cinnamon is cultivated in lands that had earlier been used for rubber cultivation or in lands closer to rubber cultivation (Jayasinghe *et al.*, 2020).

The use of chemical fungicides to manage the white root disease in cinnamon has been practiced so far. However, the application of synthetic fungicides for larger extents of land is uneconomical and most importantly, causes severe environmental pollution. Those synthetic chemicals are known sources of health hazards for humans as well. Therefore, a more economically viable, ecologically sustainable, and socially sound approach is needed to control the white root disease in cinnamon cultivations of Sri Lanka. Biological control of diseases is an emerging trend in the world. For example, the *Chaetomium cupreum* fungal species and its metabolites have shown the ability to suppress the growth of *Rigidoporus microporus*. When formulated as both powder and oil, *C. cupreum* effectively prevented *R. microporus* from infecting rubber tree roots under field conditions (Kaewchai and Soyong, 2010).

According to the nursery trials of (Go *et al.*, 2023), rubber tree clone RRIM600, when treated with *T. asperellum* alone or in combination with *T. spirale*, showed a remarkable decrease in disease severity in rubber trees. This suggests that *T. asperellum* holds significant potential as a biocontrol agent for combating *R. microporus* infection in rubber trees. According to Prasetyo and Aeny, (2013), the effective management of WRD in smallholder rubber plantations can be achieved using a combination of botanical, biological, and chemical agents. Among these, *Trichoderma* spp. offers a promising, eco-friendly alternative to conventional methods, contributing to sustainable disease control strategies. The literature suggests that the *Trichoderma* spp., which is naturally found in soils, can be widely used as biocontrol agents against plant pathogenic fungi in agriculture.

Therefore, the objective of this study was to isolate naturally existing *Trichoderma* spp. from cinnamon growing lands, morphologically identify them, and *in vitro* evaluate the antagonistic effects of different *Trichoderma* isolates against the causal agent of white root disease to identify the most effective isolate against *Rigidoporus microporus* among them.

Material and Methods

Soil sample collection

Soil samples were collected from different locations (minimum 10 m apart) in the cinnamon growing areas of the National Cinnamon Research and Training Centre, Department of Cinnamon Development, Palolpitiya, Thihagoda, from the rhizosphere within the top 20 cm of the soil, using simple random sampling. The samples were air-dried on a laboratory bench at room temperature for several days to reduce the bacterial population and facilitate fungal isolation. After drying, the soil samples were stored at room temperature.

Isolation of *Trichoderma* spp.

The serial dilution technique was employed to isolate *Trichoderma* from the soils, following the method described by Go *et al.*, (2015). For each dilution series, 1 g of soil was dissolved in 10 ml of sterile distilled water in a test tube. Then, 1 ml of the solution was further diluted dissolved in 9 ml of distilled water to create a dilution of 10^{-1} . The series was made up to 10^{-4} dilution. Using a micropipette, 1000 μ L of each dilution was transferred to the center of sterile PDA petri plates. The petri dishes were placed inverted to prevent condensation on the lids and were incubated at 28°C for two weeks. Fungal colonies resembling *Trichoderma* spp. were purified through repeated sub-culturing.

Hyphal tip transfer technique

The emerging *Trichoderma* colonies were purified using hyphal tip transfer technique (Rangaswami and Mahadevan, 1998). In this method the pathogenic fungal culture was inoculated at the center of a petri dish containing plain agar. Then the fungus was allowed to grow in the form of thin hyphal strands. These strands were observed and located under low power of the microscope, with the dish inverted. The hyphal tips away from the place of inoculation were marked with a glass marking pen. Then the marked hyphal tip was carefully cut along with a bit of agar bearing it. The cut agar piece was placed on a nutrient agar slant, allowing the mycelial colony to grow.

Identification of *Trichoderms* isolates

The colony morphology of each *Trichoderma* isolate was recorded based on the macroscopic characteristics (Siddiquee, 2017). Microscopic slides of the isolates

were prepared by touching a sterile inoculation loop on the conidia formed on the mycelia of the isolates and mounting on a drop of Lactophenol cotton blue stain positioned on the slide. A cover slip was used to fix the slide. Observations were taken using a compound light microscope (Carl Zeiss Microscopy GmbH, Germany). Microscopic characteristics such as the arrangement of conidiophores, phialides, and conidia were observed using the same microscope according to the sticky tape method described by Flegel, (1980).

Culture acquisition of the pathogen

A pure isolate of *R. microporus* isolated from a white root disease infected cinnamon fields was obtained from the culture collection of the National Cinnamon Research and Training Centre, Palolpitiya, Thihagoda.

In-vitro growth study of *Trichoderma* isolates

The growth of the eight *Trichoderma* isolates was observed for five days. The isolates were grown on Potato Dextrose Agar (PDA) plates and incubated at 28 ± 2 °C. Daily measurements of colony radii of *Trichoderma* isolates were taken, allowing for the determination of growth rates. These data were utilized to assess each isolate's overall vigor, allowing us to compare their potential as biocontrol agents in the following antagonistic activity studies.

Antagonistic activity of *Trichoderma* on pathogens

The antagonistic potential of eight *Trichoderma* isolates against *R. microporus* was verified in a dual culture assay, following the method described by (Fokkema, 1978), using 9 cm diameter Petri plates containing 20 ml of sterile PDA medium. Mycelial plugs (7 mm in diameter) of the 7-day-old fungal antagonist and the pathogen were cut using a sterile cork borer and placed on the same Petri dish, each positioned 1 cm away from the edge of the Petri dish on opposite sides. The control samples were prepared similarly, except that only pathogenic isolates were placed on the Petri dishes. Inoculated samples were then incubated at 28 ± 2 °C. All dual cultures were replicated four times for each treatment and arranged in a completely randomized design.

The radial growth of the pathogen in both the control and dual culture plates was measured using a caliper after five days. The Percentage Inhibition of Radial Growth (PIRG) was computed using the data taken

five days after incubation, referring to a method described by Korsten *et al.*, (1995) as follows.

$$PIRG = (R_c - R_d) \times \frac{100}{R_c}$$

Radial growth of the pathogen in control plates (R_c) and radial growth of the pathogen in the dual culture plates (R_d) were recorded five days after incubation.

Statistical Analysis

The experimental data in four replicates were statistically analyzed using Minitab Inc., 2013. *Minitab* 17 (computer software), following One-Way Analysis of Variance (ANOVA). The treatment means were separated by the Tukey test and were determined by using the significant level of 0.05 .

Results and Discussion

Isolation and identification of *Trichoderma* spp.

Morphological characteristics persist as a potential method in identifying *Trichoderma* up to the genus level (Samuels *et al.*, 2002). Four different *Trichoderma* isolates were identified from the collected soil samples. The identification was carried out according to the morphological and microscopic characteristics described by Siddiquee, (2017).

All four isolates exhibited dense white mycelia within the first 3 to 4 days after culturing on PDA, a characteristic of *Trichoderma* spp. (Šašić Zorić *et al.*, 2023). All the isolates produced green colour conidia followed by sporulation, which is a characteristic feature in the *Trichoderma* genus (Luković *et al.*, 2020). This was an important criterion in the morphological identification of the isolates as *Trichoderma* strains. Some *Trichoderma* strains generate diffusible pigments that vary from greenish yellow to reddish shades; however, colorless strains may also be produced (Reghmit, 2023). Isolate MA1 showed a bright yellow colour pigmentation 2-3 days after inoculation. There are several *Trichoderma* spp. that produce yellow colour pigment on PDA. *Trichoderma reesei* produces a typical yellow colour pigment, a secondary metabolite during its growth (Derntl *et al.*, 2016).

According to Lebeau *et al.*, (2020), *Trichoderma harzianum* is also known to be a yellow-brown pigment producer.

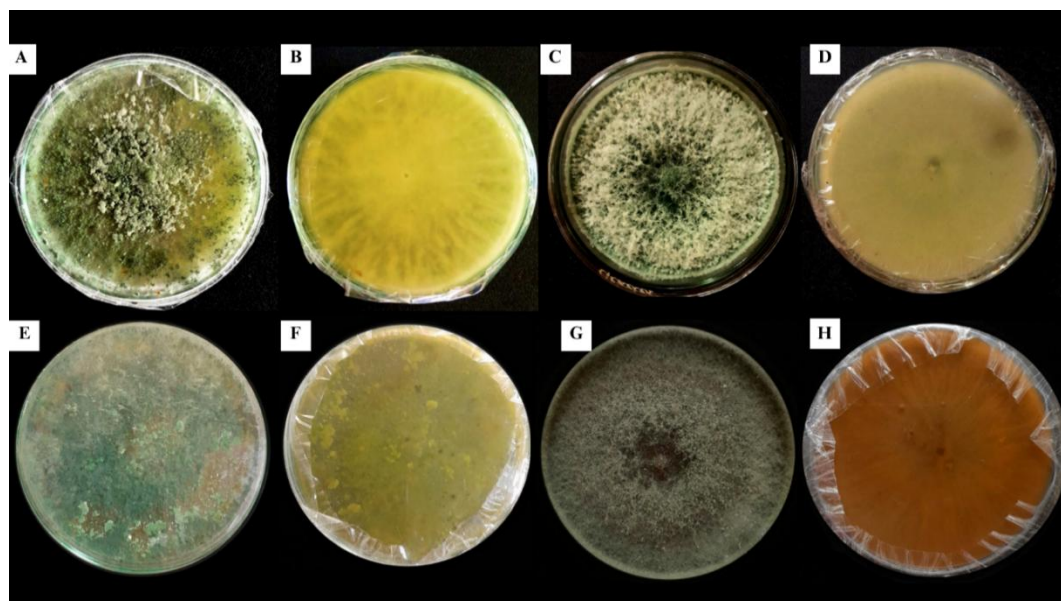


Figure 01 shows the colony morphology of the four *Trichoderma* isolates MA1, MA2, MA3, and MA4 obtained from cinnamon rhizosphere soils

MA2 and MA3 isolates did not show a noticeable pigment production. However, isolate MA4 showed a dark brown colour pigmentation in the colony (Figure 01).

Microscopic characteristics inherent to *Trichoderma* genus were utilized to confirm the isolates' classification within the genus. Generally, *Trichoderma* spp. produce translucent phialides formed on the fertile branches of conidiophores (Lu *et al.*, 2004). As stated by Jayasuriya and Thennakoon, (2007), the production of phialides is an important criterion in selecting an antagonist as a biocontrol agent. Therefore, the isolates MA1, MA2, MA3 and MA4 were microscopically identified as *Trichoderma* spp. due to the presence of branched conidiophores with phialides (Figure 02).

In addition to the isolates obtained in this study, four *Trichoderma* isolates were used. One isolate (MT) was obtained from the rhizosphere soil of pepper, sourced from the Plant Pathology Division, Central Research Station, Matale. The other three isolates (Tc, Tk, and T13) were obtained from the rhizosphere soil of banana plants and were provided by the Department of Botany, University of Ruhuna.

***In-vitro* growth study of the *Trichoderma* isolates**

The morphological features of *Trichoderma* isolates, including colony color, reverse color, form, elevation, and growth rates, show considerable variation among species. These traits are essential for accurately identifying and classifying isolates, which can impact their effectiveness as biological control agents (BCAs) in sustainable agriculture (Kubicek *et al.*, 2019)

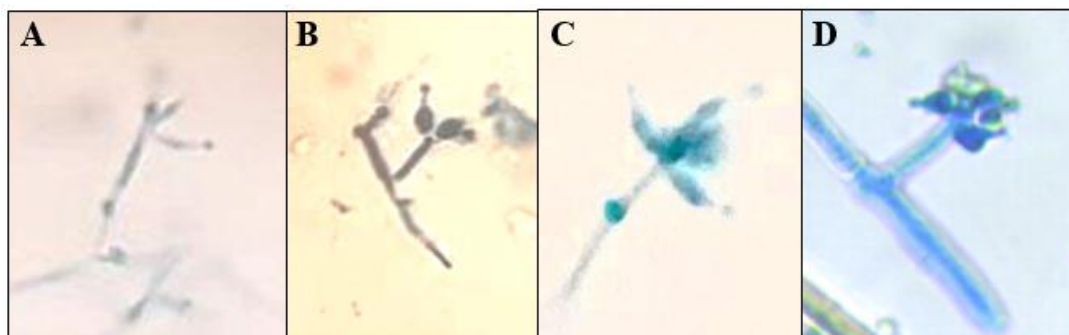


Figure 2. Presence of phialides in isolates obtained from cinnamon rhizosphere soils under 400x

Table 1: Colony colour, colony form, colony margin and colony elevation of eight *Trichoderma* isolates grown on PDA medium

Isolate	Colony Colour	Colony Reverse Colour	Colony Form	Colony Margin	Colony elevation
MA1	Hazel green	Husk yellow	Round	Initially ciliate, later undulate	Flat
MA2	Rifle green	Pale brown	Concentric	Initially ciliate, later undulate	Flat
Tc	Cactus green	Pale brown	Concentric	Initially ciliate, later undulate	Flat
Tk	Cactus green	Pale brown	Concentric	Initially ciliate, later undulate	Flat
T13	Camo green	Pale brown	Concentric	Initially ciliate, later undulate	Flat
MA3	Hazel green with white pustules	Pale brown	Concentric	Initially ciliate, later undulate	Flat
MA4	Rifle green	Pastel brown	Concentric	Initially ciliate, later undulate	Flat
MT	Hazel green	Pale brown	Round with raised margins	Ciliated	Flat

Table 1 outlines the characteristics of eight different *Trichoderma* isolates 10 days after incubation on PDA media, focusing on colony margin, form, reverse colour, colony colour, and growth rate. The colony colours varied from hazel green to different shades of green, such as cactus and rifle green. Producing green colour conidia is a characteristic of the species in *Trichoderma* genus (Błaszczuk *et al.*, 2014). These colour differences in colony colours might indicate genetic diversity among the isolates (Bhatnagar *et al.*, 1999).

The reverse colours of the colonies mainly ranged from shades of yellow to brown, identified in isolate MA1 and pale brown observed in the others. This feature could be connected to the metabolic byproducts of the fungal growth or the pigments produced during that growth (Contreras-Cornejo *et al.*, 2016). Additionally, the coloration can offer valuable information about the physiological condition of the colonies.

Colony forms mainly produced concentric conidial rings. Except for the isolates MA1 and MT. A notable characteristic of *Trichoderma* is its ability to produce concentric rings of conidia when exposed to alternating light and dark conditions, as well as a single ring of conidia in response to a sudden burst of light (Steyaert *et al.*, 2010). The colony margins of the isolates exhibited some diversity, with most initially displaying a ciliate form followed by an undulated form. However, all the colonies were flat with reference to the height they grew.

Growth rates of the eight *Trichoderma* isolates

Table 2 shows how the growth rates of *Trichoderma* isolates varied in this study, with values ranging from 1.5 cm/day (isolate Tk) to 2.1 cm/day (isolate MA4). Isolate MA4 demonstrated the highest growth rate. Isolates MA1, T13, and MT showed moderate growth rates between 1.6 to 1.7 cm/day, suggesting a balanced growth pattern that could be useful for steady, consistent establishment in various environments. In contrast, the slower growth rate of Tk (1.5 cm/day) might imply a more gradual colonization, which could still offer benefits in applications focused on long-term soil health. This range of growth rates highlights the potential diversity within *Trichoderma* strains and their possible specialized uses depending on the application requirements.

Table 2: Growth rates of the eight *Trichoderma* isolates 3 days after incubation, grown on Potato Dextrose Agar (PDA) plates incubated at 28 ± 2°C.

Isolate	Growth rate ± SE (cm/day)
MA1	1.7 ± 0.13
MA2	1.9 ± 0.20
Tc	1.9 ± 0.22
Tk	1.5 ± 0.21
T13	1.6 ± 0.18
MA3	1.9 ± 0.22
MA4	2.1 ± 0.16
MT	1.6 ± 0.28

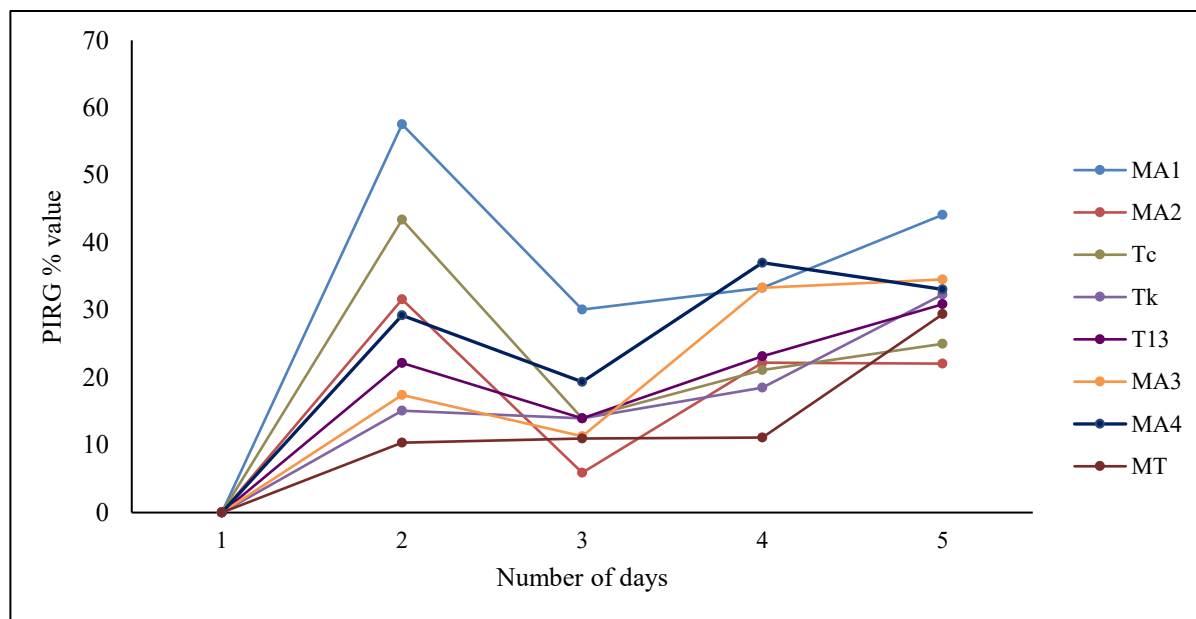


Figure 3. Percentage inhibition of radial growth (PIRG) values for the dual-culture assays of the eight *Trichoderma* isolates against *R. microporus* for 5 consecutive days

Biological control abilities of *Trichoderma* species

The biocontrol ability of the *Trichoderma* isolates was tested by analyzing the Percentage Inhibition of Radial Growth (PIRG) values. A total of eight *Trichoderma* isolates were screened against the *R. microporus*. Figure 3 shows the PIRG values for the dual-culture assays. The results revealed distinct inhibition patterns over the five-day incubation period. On the first day, no inhibition was observed across all isolates. However, by the second day, some isolates showed significant antagonistic activity: MA1 recorded the highest inhibition at $57.55\% \pm 2.8$, followed by Tc at $43.4\% \pm 4.1$, indicating strong early suppression of *R. microporus*. Other isolates had moderate to lower inhibition levels, such as MA2 at $31.6 \pm 3.5\%$ and Tk at 15.09% . On the third day, PIRG values generally decreased, which may reflect competitive interactions or adaptation between the organisms.

By the fourth day, inhibition increased again for most isolates, with MA4 and MA1 demonstrating the highest effectiveness at $37.04\% \pm 2.2$ and $33.33\% \pm 2.9$, respectively. These findings emphasize the variability in inhibition dynamics among different *Trichoderma* isolates and the temporal nature of their antagonistic interactions. However, the biological control ability is measured on the 5th day of a dual culture assay.

Table 3: Effects of eight *Trichoderma* isolates on Percentage Inhibition of Radial mycelial Growth (PIRG) of *R. microporus* in dual culture after five days of culture in Petri dishes on PDA

Isolate	PIRG %
MA1	44.12 ± 3.39^A
MA2	22.06 ± 5.63^B
MA3	34.56 ± 6.06^{AB}
MA4	33.09 ± 4.41^{AB}
MT	29.41 ± 10.74^{AB}
T13	30.88 ± 5.63^{AB}
Tc	25.00 ± 8.82^B
Tk	32.32 ± 7.59^{AB}

Values re the means of four replicates $\pm$ Standard deviation. Within each column, mean with different letters are significantly different according to the Tukey's multiple comparison test ($p < 0.05$).

According to Table 03, the PIRG mean value of isolate MA1 (44.12 ± 3.39^A) is significantly higher than that of isolate Tc (25.00 ± 8.82^B) and isolate MA2 (22.06 ± 5.63^B), as indicated by the differing letter notations. Isolates MA3, MA4, MT, T13, and Tk, marked with "AB," do not show significant differences when compared with either the highest or lowest values,

suggesting that they fall within an intermediate range of PIRG effectiveness.

The results show that the MA1 isolate is the most effective isolate among the isolates used against *R. microporus*, showing 44.12 % inhibition of the radial growth of the pathogen. MA2 isolate is the least effective in inhibiting the pathogen's radial growth, exhibiting only 22.06 % inhibition. An PIRG exceeding 40 % is considered high, indicating that the specific *Trichoderma* isolate serves as a more potent biological control agent (Kannangara *et al.*, 2017). Isolates MA1, MA3, and MA4 were isolated from the rhizosphere soils of the cinnamon trees at NCR&TC, are the three most effective bio control agents in inhibiting the pathogen's radial growth under *in-vitro* conditions compared to the other isolates used in the study. This inhibition is more than 33%. According to

previous studies, the PIRG values can vary depending on factors such as the location of isolation, time of isolation, weather conditions prevailed at the time of isolation, host plant etc. (Li *et al.*, 2016).

Figure 04 shows the observations of the dual culture assays of the four *Trichoderma* isolates obtained from cinnamon rhizosphere soils against *R. microporus* on five consecutive days starting from the day of culturing.

The study used four other *Trichoderma* isolates obtained from the rhizosphere soil of pepper (MT) and bananas (Tc, Tk, and T13), to evaluate the antagonistic effects of different *Trichoderma* isolates against the causal agent of white root disease, to identify the most effective isolate against *R. microporus* among them (Figure 05).

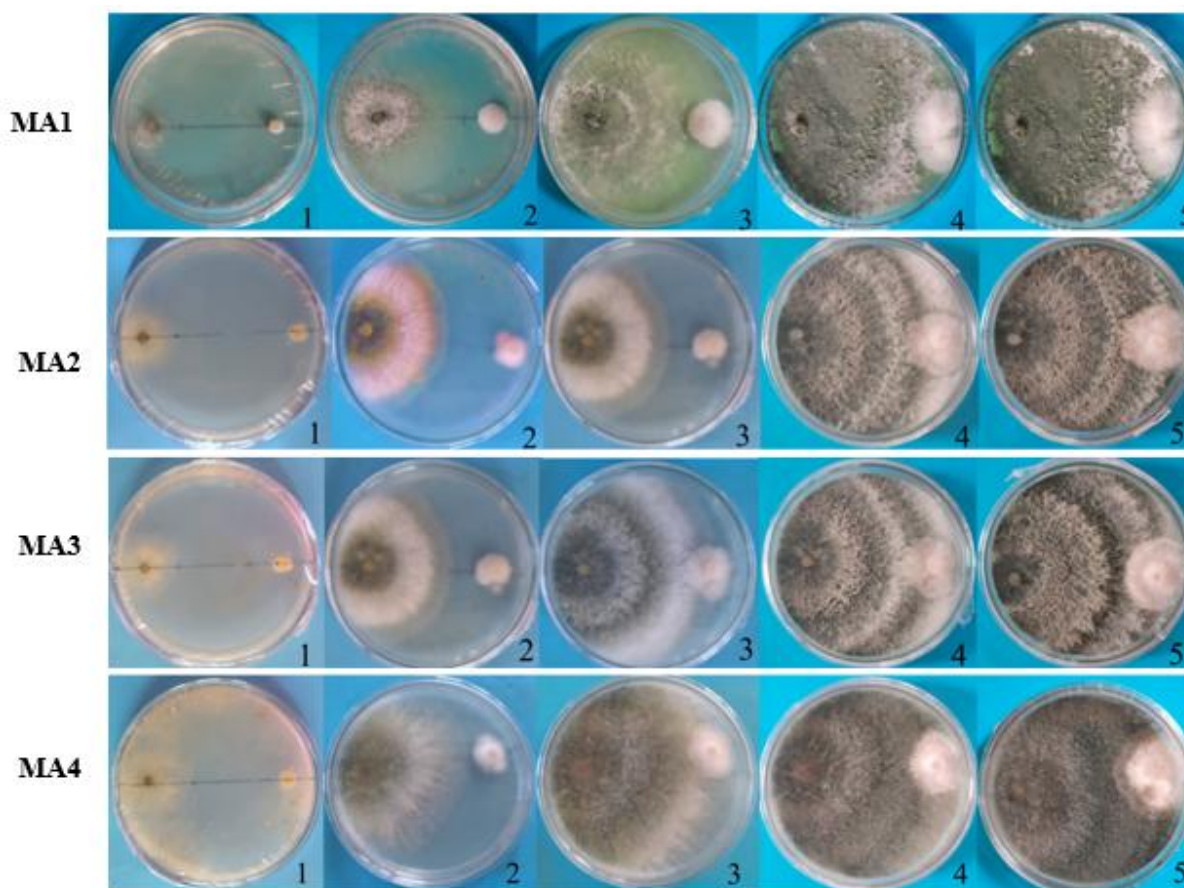


Figure 4. Dual culture assays of *Trichoderma* isolates obtained from cinnamon rhizosphere soils (left) vs. *R. microporus* (right) for five consecutive days after culturing. MA1, MA2, MA3 and MA4 are *Trichoderma* isolates and 1-5 indicate dates after culturing.

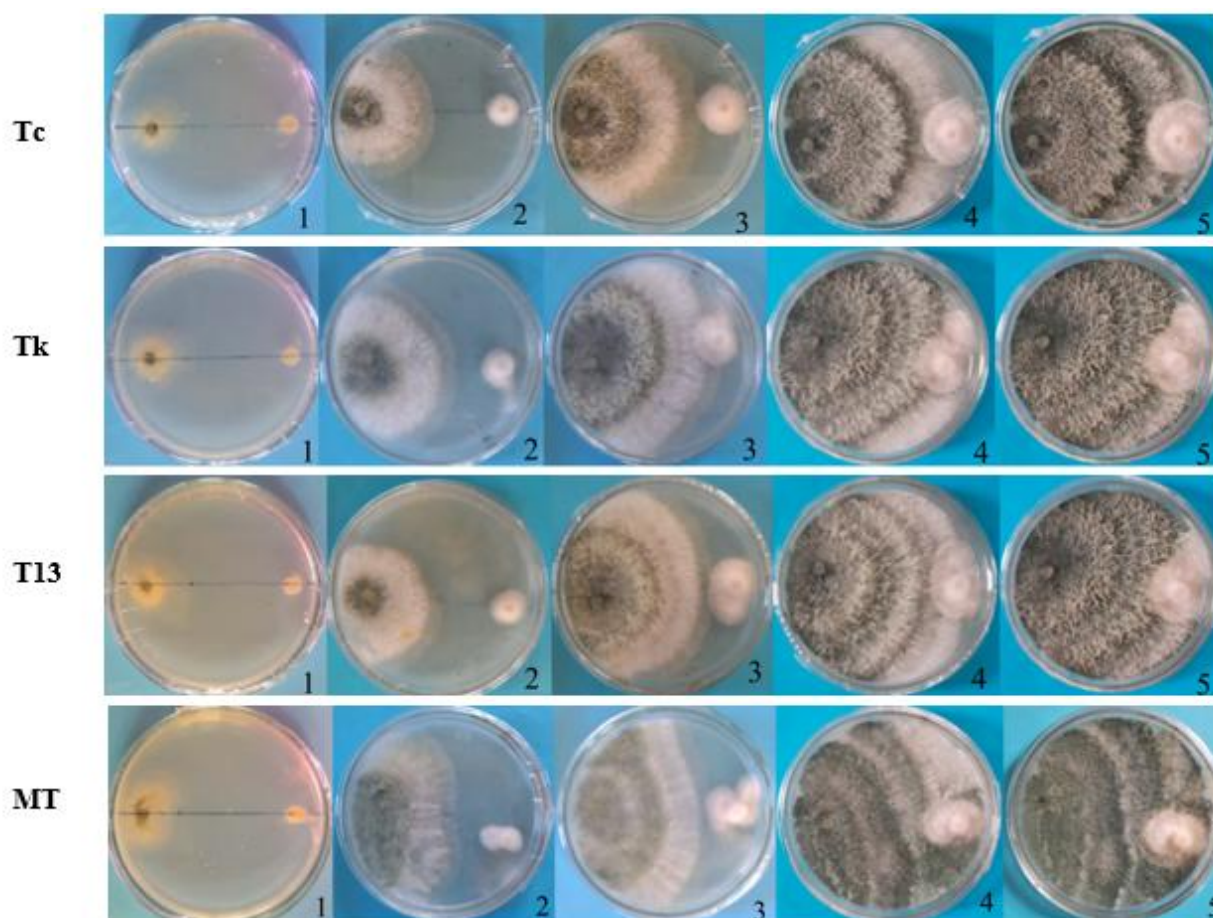


Figure 5. Dual culture assays of *Trichoderma* isolates obtained from pepper and banana rhizosphere soils (left) vs. *R. microporus* (right) for five consecutive days after culturing. Tc, Tk, T13 and MT are *Trichoderma* isolates and 1-5 indicate dates after culturing.)

According to a study conducted by (Go *et al.*, 2023), using isolates from *Trichoderma* species *T. koningiopsis*, *T. spirale*, *T. asperellum* and *T. reesei* against *R. microporus* isolated from rubber fields in Malaysia, the PIRG values had varied as 10.20 %, 70.00 %, 78.80 % and 5.90 %, respectively. The PIRG values of the present study fall between 22.06 % and 44.12 %, where the lowest PIRG value exceeds the PIRG values of *T. koningiopsis* and *T. reesei* in Go's study. However, even the isolates from the same species may exhibit different PIRG values depending on factors such as location, weather conditions, host plant, etc. at the time of isolation (Li *et al.*, 2016).

The most effective *Trichoderma* isolate against *R. microporus* of this study was the MA1 isolate, exhibiting 44.12 % radial growth inhibition of the pathogen. There were several special characteristics that were observed in this isolate when compared to the other isolates. The ring formation was not prominent in MA1 isolate. A clear inhibition zone was

visible in the dual culture assay after 4 days of incubation (Figure 6).

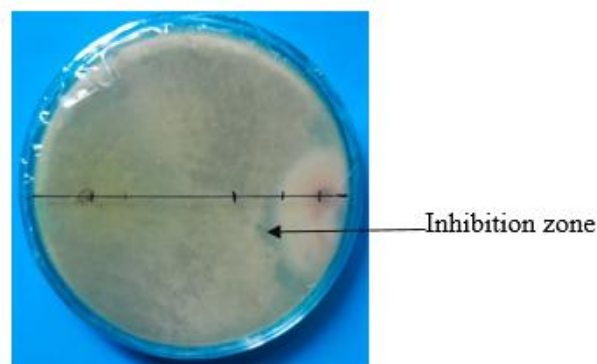


Figure 6. The inhibition zone of the MA1 isolate after 4 days in the dual culture assay.

Yellow colour pigmentation was observed after two to three days of inoculation of MA1 isolate (Figure 7) on PDA.

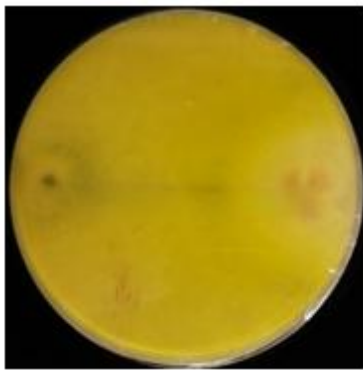


Figure 7. Yellow colour pigment produced by MA1 isolate in the dual culture assay

Trichoderma species are well known for producing secondary metabolites, many of which have important bioactivities that can act as antimicrobials, antioxidants, pigments, and hormones. Sorbicillinoids are generally known as a yellow pigment synthesized by several Ascomycetes fungi, including some *Trichoderma* species. Most of these pigments have the above-mentioned antimicrobial properties (Hou *et al.*, 2022). The yellow colour pigment produced by the MA1 isolate can be a sorbicillinoid with an antimicrobial property as MA1 isolate showed the highest percentage inhibition of radial growth of the causal agent of the white root disease.

In comparison to similar studies on WRD in rubber, the PIRG values found in the current study against the white root pathogen in cinnamon were notably lower. Consequently, additional research is needed to identify or develop more potent *Trichoderma* strains specifically designed for WRD management in cinnamon, which could lead to more effective biocontrol strategies for this crop, as well as to ensure the robustness and generalizability of the findings from the current study.

Conclusions and Recommendations

In conclusion, the MA1 isolate exhibited the highest Percentage Inhibition of Radial Growth (PIRG), while the other isolates showed no significant differences in their ability to inhibit *R. microporus*. The PIRG values for these isolates ranged from 22.06 % to 34.56 %. The in vitro inhibition of *R. microporus* can be ranked as follows: MA1 > MA3 > MA4 > Tk > T13 > MT > Tc > MA2. This ranking highlights the differing effectiveness of the isolates, with MA1 demonstrating the strongest inhibitory effect. Among the 8 *Trichoderma* isolates, 3 isolates, including isolate MA1, MA2, and MA4, obtained from cinnamon

rhizosphere soils, showed higher antagonistic potential against *R. microporus* than isolates obtained from pepper and banana rhizosphere soils. Further studies should focus on conducting extensive studies by isolating more virulent *Trichoderma* isolates and screening against *R. microporus* under laboratory as well as under field conditions.

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