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Trichoderma as a Biocontrol Agent against *Diaporthe* and *Lasiodiplodia* Species

Hena Mahmood* **and Choudhary Sharfuddin**

Plant Pathology and Microbiology Laboratory, Department of Botany, Patna University, Patna, Bihar, India

**Corresponding author*

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A B S T R A C T

Trichoderma species have emerged as powerful biocontrol agents for controlling various diseases of crops and plants. Fungi belonging to *Diaporthe* and *Lasiodiplodia* species comprise some highly destructive plant pathogens affecting important agricultural crops. In the present study, the *Trichoderma* isolates were assessed for their antagonistic potential against *Diaporthe* and *Lasiodiplodia* species. The two methods used were dual culture assay and inverted plate assay. In the dual culture assay, the percentage inhibition of radial growth (PIRG) of *Diaporthe* species ranged from 65.16% to 73.68% with the maximum PIRG shown by isolate T4, which was identified as *Trichoderma erinaceum*; and the PIRG of *Lasiodiplodia* species ranged from 57.14% to 62.04% with the maximum PIRG shown by isolate T1, which was identified as *Trichoderma yunnanense*. In the inverted plate assay, the percentage of inhibition of *Diaporthe* species ranged from 19.79% to 60.29% and the maximum inhibition was exhibited by isolate T4. The volatile compounds produced by the *Trichoderma* isolates showed no inhibitory effect on the growth of *Lasiodiplodia* species. Thus, it can be concluded that the *Trichoderma* isolates showed more effective antagonism against *Diaporthe* than against *Lasiodiplodia*.

Introduction

Biological control or biocontrol is a technique where living organisms such as fungi and bacteria are employed to control the diseases of crops and plants caused by phytopathogenic organisms, as they are capable of killing or suppressing the growth of the phytopathogenic organisms, acting as their natural enemies. The living organisms used for the biocontrol of the plant pathogens are known as biological control agents or biocontrol agents (BCAs). Some of the microorganisms used as biocontrol agents include *Bacillus thuringiensis* (Liang *et al.,* 2025), *Pseudomonas aeruginosa* (Xie *et al.,* 2023), *Trichoderma harzianum*, *Trichoderma viride* (Yao *et al.,* 2023) and *Paecilomyces lilacinus* (Anastasiadis *et al.,* 2008).

Biocontrol agents are eco-friendly and do not harm human health or the environment. Therefore, they are being preferred over chemical pesticides, which adversely affect both human health and the environment. Chemical pesticides can cause a wide range of health

issues in humans. Human beings get exposed to pesticides through skin contact, ingestion or inhalation. Even short-term exposure to these toxic substances can cause health problems such as skin rashes, stinging eye, throat irritation, headaches, dizziness, nausea, vomiting and diarrhea. Extreme exposure or long-term exposure to these chemicals can lead to severe health conditions such as cancer, neurological disorders, kidney damage, endocrine and reproductive issues, and in some cases may even lead to death. Agricultural workers, in particular, face the highest risks due to direct, routine exposure to these harmful chemicals (Hashimi *et al.,* 2020). The reckless application of these synthetic chemicals damages the ecosystem. Chemical pesticides and their residues can persist in the environment for extended periods and cause air, water and soil pollution. These pesticides kill the beneficial organisms present in the soil and decrease the fertility of the soil. The bioaccumulation and biomagnification of these chemicals in the food chain is also a critical concern. These harmful chemicals enter the water bodies through leaching and runoff and negatively impact the aquatic life (Kashyap, Garg and Arora, 2024). The persistent use of synthetic chemicals often leads to the development of pesticide resistance in the pests requiring higher doses or stronger, more toxic chemicals to control them (Kole *et al.,* 2019).

Trichoderma species are widely used as sustainable, eco-friendly alternative to chemical pesticides for controlling soil-borne diseases such as *Fusarium*, *Pythium*, *Rhizoctonia* due to their rapid growth and strong antagonistic action. *Trichoderma* acts through various mechanisms which include mycoparasitism where *Trichoderma* coils around and penetrates the hyphae of the harmful fungi, secreting enzymes such as chitinases to dissolve their cell walls. The other mechanism through which *Trichoderma* acts is antibiosis where it produces volatile and non-volatile secondary metabolites which have inhibitory effect on the pathogens. *Trichoderma* competes with the pathogens for nutrients and space, reducing the occurrence of diseases. *Trichoderma* also acts as a biofertilizer and plant growth promoter. It enhances root development in plants and increases their nutrient uptake. *Trichoderma* enhances plant tolerance to abiotic stresses like drought, salinity and cold. It triggers systemic resistance in plants, bolstering their natural immune systems against pests and diseases. *Trichoderma* offers a safe, low-cost, non-polluting, environmentally friendly method to improve soil health, plant vigor and crop yields. The bio fungicide formulations available commercially, comprising primarily *Trichoderma harzianum* and *Trichoderma viride*, can be used for seed treatment, root dipping or soil application. Integrating *Trichoderma* species with reduced doses of chemical agents can enhance control effectiveness. Thus, through the use of natural agents, the reliance on the synthetic chemicals for controlling plant diseases can be significantly reduced (Thambugala *et al.,* 2020; Ayaz *et al.,* 2023; Kumar *et al.,* 2023; Yao *et al.,* 2023; Nassary, 2025).

Diaporthe spp. are notorious plant pathogens responsible for diseases like stem blights, cankers, dieback and seed decay across numerous important agricultural crops (Udayanga *et al.,* 2011). *Diaporthe* species such as *Diaporthe helianthi* and *Diaporthe gulyae* cause Phomopsis stem canker of sunflower (Zambelli *et al.,* 2021). *Diaporthe eres*, *D. rostrata* and *D. sackstonii* are causal agents of walnut branch blight and dieback (Zhao *et al.,* 2025). *Diaporthe* species are also associated with grapevine trunk diseases (Billar de Almeida *et al.,* 2020). Pod and stem blight in soybeans is primarily caused by *D. sojae*. *Diaporthe* seed decay in soybeans, in which the seeds become shriveled, elongated, and chalky white, reducing seed quality and germination rates, is primarily driven by *Diaporthe longicolla* (George *et al.,* 2025). *Diaporthe cucurbitae* is known to cause fruit rot and stem blight in cucurbits like pumpkins and squash (McKeen, 1957).

Lasiodiplodia species, including *L. theobromae* are major fungal pathogens which cause severe agricultural issues like dieback, gummosis, root rot and postharvest fruit rot. *Lasiodiplodia theobromae* is a primary cause of dieback, stem-end rot, and panicle brown rot in mangoes. It causes nut fall, leaf blight, and stem-end rot in coconut. It causes crown rot in bananas and stem-end rot in figs. It also causes severe root rot in sugar beets (El-Ganainy *et al.,* 2022; da Silva França *et al.,* 2025; Rao *et al.,* 2025). It causes stem gummosis and rapid decline in rubber tree (He *et al.,* 2025). It causes destructive heart rot and rachis blight in date palm (Atallah *et al.,* 2024). Among the diseases affecting grapevine plantations, Botryosphaeria dieback, caused mainly by fungus *L. theobromae*, is a serious disease which causes major losses (Marraschi *et al.,* 2019).

In the present work, the efficacy of the *Trichoderma* isolates against the two fungal plant pathogens-*Diaporthe* and *Lasiodiplodia* species, was studied. Two methods were used for the evaluation of antagonistic activity of the *Trichoderma* isolates against the pathogens. The first

method was the direct confrontation assay (dual culture assay) and the second method was the inverted plate assay (sealed plate assay) in which the effect of volatile organic compounds (VOCs) produced by *Trichoderma* was assessed.

Materials and Methods

Isolation and identification of *Trichoderma* species

Trichoderma species were isolated from the soil samples collected from the agricultural fields, gardens and parks located in various districts of Bihar. *Trichoderma* species are free-living fungi which frequently inhabit the rhizosphere, the area of soil surrounding plant roots. Therefore, rhizosphere soil was collected from a depth of 10-15 cm for the isolation of *Trichoderma*. The soil samples were brought to the Plant Pathology and Microbiology Laboratory, Department of Botany, Patna University, Patna. Then they were air-dried at room temperature. Serial dilution technique was used to prepare different dilutions of soil sample for inoculation. For this, 1 g of soil was thoroughly mixed in 9 mL of sterile distilled water by vortexing the mixture to make a 10^{-1} dilution. The next dilution of 10^{-2} was obtained by transferring 1 mL of the 10^{-1} dilution to 9 mL of sterile distilled water. Further dilutions were prepared in the same manner by taking 1 mL from the previous dilution and mixing it in 9 mL of sterile distilled water to get the next dilution.

Dilutions from 10^{-2} to 10^{-6} were used as inoculum. Inoculation was done using spread plate technique. A small amount of diluted sample (0.1 mL) was pipetted out and transferred to Potato Dextrose Agar (PDA) media supplemented with chloramphenicol to suppress bacterial growth. A spreader was used to spread the sample evenly over the surface of the PDA media. The plates were incubated at 27±2 °C (Aneja, 2007). *Trichoderma* species were identified based on morphological and microscopic characteristics with the help of the manual for identification of fungi by Barnett and Hunter.

The species of *Trichoderma* isolates were confirmed by sequencing of the Internal Transcribed Spacer (ITS) gene (600 bp). The complete procedure was carried out at National Collection of Industrial Microorganisms, Council of Scientific and Industrial Research-National Chemical Laboratory, Pune, India.

Isolation and identification of phytopathogenic fungi

Diseased plant materials were collected. The infected part showing the symptoms of the disease along with some adjacent healthy tissue was cut out from the diseased plant material with the help of a flame-sterilized knife. Then it was further sectioned into small pieces of size 3-5 mm. These specimen pieces were transferred to Petri dish containing 2% sodium hypochlorite solution with the help of flame-sterilized forceps and left for one minute for surface sterilization. After one minute, these pieces were removed from sodium hypochlorite solution and rinsed three times with sterile distilled water. Then they were placed on sterile filter paper and left to dry. These specimen pieces were then transferred to PDA media supplemented with chloramphenicol. The PDA plates were incubated at 27±2 °C. After few days, growth of fungi was observed on the plate.

The pure culture of the fungi was obtained by subculturing it. The isolated fungi were identified based on the appearance of their colony such as colony color, texture, growth pattern and their microscopic characteristics. The microscopic features were studied by preparing slides using lactophenol cotton blue (Thilagam, Kalaivani and Hemalatha, 2018; Kavitha and Ranganayakulu, 2025).

The identification of *Diaporthe* species was confirmed by sequencing of the ITS region done at National Collection of Industrial Microorganisms, Council of Scientific and Industrial Research-National Chemical Laboratory, Pune, India. For this purpose, first of all, the genomic DNA was extracted. Then, the amplification of ITS gene (600 bp) was carried out via Polymerase Chain Reaction (PCR). The primers used were ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.,* 1990). The PCR product was purified and sequenced. The resulting sequence was analyzed by Basic Local Alignment Search Tool (BLAST) with closest culture sequence retrieved from the National Center for Biotechnology Information (NCBI) database (Altschul *et al.,* 1990).

The identification of *Lasiodiplodia* species was confirmed with the support of Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India.

Assessment of antagonistic activity of *Trichoderma* isolates through dual culture assay

The dual culture assay was carried out following the standard protocol while making certain modifications (Morton and Stroube, 1955). For this experiment, both the antagonist (*Trichoderma* spp.) and the pathogen were inoculated in the same Petri dish at opposite ends. The inoculation was done at a distance of 1.5 cm away from the periphery of 100 mm Petri plate. The mycelial discs of 6 mm diameter taken from the margin of 5-day-old cultures of *Trichoderma* isolates and the pathogens (*Diaporthe* and *Lasiodiplodia*) grown on PDA media at 27±2° C were used for inoculation. The experiment was carried out in triplicate. The plates were incubated at 27±2 °C. The pathogen grown alone in a Petri dish without the antagonist was treated as control.

After 5 days of incubation, the radial growth of fungal pathogen in the dual culture assay plate as well as the control plate was measured. The percentage inhibition of radial growth (PIRG) was calculated by using the following formula (Vincent, 1947; Begum *et al.,* 2008):

$$PIRG\ (\%) = [(R_1-R_2) / R_1] \times 100$$

Where R_1 was the mean of the radial growth of the pathogen on the control plate and R_2 was the mean of the radial growth of the pathogen on the treated plate.

Assessment of antagonistic activity of *Trichoderma* isolates through inverted plate assay

The inhibitory effect of volatile organic compounds (VOCs) released by *Trichoderma* on the phytopathogenic fungi was evaluated by sealed plate method, also known as inverted plate method. Certain modifications were made to the standard protocol (Dennis and Webster, 1971; Nagamani *et al.,* 2017). For this assay, the experimental set-up consisted of two bottoms of the PDA plates facing each other, sealed together using parafilm, one containing the antagonist (*Trichoderma*) inoculated at the center and the other containing the pathogen inoculated at the center, incubated in such a position that the PDA plate containing the antagonist was below and the PDA plate containing the pathogen was above. Both the antagonist and the pathogen were inoculated on the same day using 3-day-old culture as inoculum. The mycelial discs of 6 mm taken from the margin of the colonies of the antagonist and the pathogen grown on PDA media at 27±2° C were used for inoculation. After inoculation and sealing the plates together, they were incubated at 27±2° C for 3-5 days. The control was an identical, sealed culture plate containing the pathogen but devoid of the antagonistic organism. The experiment was carried out in triplicate. The percentage inhibition of radial growth (PIRG) was calculated after 4 days of incubation by using the same formula used for dual culture assay.

Results and Discussion

Identification of *Trichoderma* and the pathogens

Trichoderma was identified based on the observation of the colony characteristics and microscopic features with the help of authentic manuals available for the identification of fungi (Figures 1 and 2). The *Trichoderma* isolates started their growth on PDA media as white, cottony mycelium that later turned various shades of green on maturation. They showed rapid growth. The *Trichoderma* colonies often developed distinct concentric rings. The isolates T1 and T4 were white or colorless on the reverse side. The isolates T2 and T3 produced yellow pigments on the reverse side (Singh and Sharma, 2020). The *Trichoderma* isolates showed microscopic features such as highly branched conidiophores; presence of phialides; single-celled conidia which were hyaline, ovoid and borne in small terminal clusters (Barnett and Hunter, 1972; Gams and Bissett, 2002). *Trichoderma* isolate T1 was identified as *Trichoderma yunnanense*, isolates T2 and T3 were identified as *Trichoderma afroharzianum* and isolate T4 was identified as *Trichoderma erinaceum* on the basis of the sequence analysis.

Diaporthe was identified on the basis of macroscopic and microscopic features and sequencing of ITS rRNA gene (Figure 3). The *Diaporthe* colonies on PDA media were white and fast-growing. The colonies were initially white and then they turned greyish with age, with a darker, brownish reverse side. The development of black, pycnidial structures (conidiomata) was observed on the surface of the agar. *Diaporthe* produces hyaline and aseptate conidia (Barnett and Hunter, 1972; Udayanga *et al.,* 2011; Gomes *et al.,* 2013). BLASTn searches of the ITS sequence data revealed that the isolate showed 98.39% identity and 96% query coverage with a *Diaporthe sackstonii* sequence available in the NCBI database.

Lasiodiplodia species showed rapid growth on PDA media (Figure 4). The fungi grew quickly, filling the 100 mm Petri dish within 3-4 days when incubated at a temperature of 27±2° C. The colony appeared off-white initially but later changed to grey, dark grey and eventually black color. The surface was fluffy with dense aerial mycelium. The reverse side of the plate appeared black. After several days of incubation, the colony produced black, embedded to erumpent pycnidia. When slide was prepared by teasing the pycnidia, it released both mature and immature spores which were visible under microscope. Immature conidia were unicellular (aseptate) while mature conidia were dark brown, thick-walled and one-septate (Barnett and Hunter, 1972; Atallah *et al.,* 2024; He *et al.,* 2025). The identification of the isolate was confirmed as *Lasiodiplodia theobromae* with the help of Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India.

Antagonistic effect of *Trichoderma* isolates through dual culture assay

The *Trichoderma* isolates showed inhibition of the *Diaporthe* species in dual culture assay with percentage inhibition of radial growth ranging from 65.16% to 73.68% (Table 1). The maximum inhibition of *Diaporthe* was shown by isolate T4, identified as *Trichoderma erinaceum* exhibiting percentage inhibition of mycelial growth of 73.68%. The minimum inhibition (65.16%) was shown by isolate T1, identified as *Trichoderma yunnanense.* Isolate T2, identified as *Trichoderma afroharzianum*, showed an inhibition percentage of 65.23% which was almost similar to the inhibition exhibited by the isolate T1. Isolate T3 displayed an inhibition percentage of 66.32% which was slightly higher than the inhibition exhibited by the isolates T1 and T2. After incubation for 7 to 9 days, all the isolates of *Trichoderma* were able to overgrow *Diaporthe* and kill it.

Lasiodiplodia species are rapidly growing fungi and they show very fast growth. The percentage inhibition of radial growth of *Lasiodiplodia* by the various isolates of *Trichoderma* after 5 days of incubation ranged from 57.14% to 62.04% (Table 1). Isolate T1 showed maximum inhibition percentage of 62.04% while the minimum inhibition was shown by isolate T4 exhibiting an inhibition percentage of 57.14%. The isolate T2 displayed an inhibition percentage of 61.06% and isolate T3 displayed an inhibition percentage of 59.67%. All the isolates were able to overgrow and kill the pathogen when they were incubated for 8 to 11 days.

Previous researchers have also demonstrated the efficacy of *Trichoderma* species against these plant pathogens. Alshammari *et al.,* (2024) showed that *Trichoderma harzianum* strains had antagonistic effects against wood decay pathogens which included *Physalospora rhodina*, *Diaporthe citri*, and *Nattrassia mangiferae* through *in vitro* and *in vivo* studies. Begum *et al.,* (2008) screened six isolates of *Trichoderma* belonging to four species (*T. harzianum*, *T. longibrachiatum*, *T. koningii* and *T. virens*) for their antagonistic activity against *Diaporthe phaseolorum* var. *sojae* through dual culture test and found that all the isolates were able to inhibit the radial growth of *D. phaseolorum* var. *sojae* with *Trichoderma harzianum* (UPM40) showing highest antagonistic potential against the pathogen. Li *et al.,* (2022) reported inhibition of radial growth of *Lasiodiplodia theobromae* by *T. hamatum*, exhibiting a percent inhibition of 56.3% in dual confrontation assay. Studies suggest that competition for space and nutrients between *Trichoderma* and pathogens is a key mechanism of biocontrol. Mycoparasitism is another common mechanism used by *Trichoderma* to inhibit the growth of pathogen when there is direct contact between *Trichoderma* and the pathogen (Abdul-Halim *et al.,* 2023).

Antagonistic effect of *Trichoderma* isolates through inverted plate assay

The effectiveness of volatile organic compounds produced by *Trichoderma* isolates in inhibiting the pathogens was assessed by sealed plate method. As the antagonist and the pathogen were grown in two different Petri dishes, they did not share the media and had no physical interaction. The inhibition of the pathogen was only due to volatile compounds which were released by *Trichoderma* into the space inside the two sealed plates. *Trichoderma* isolates showed inhibition of *Diaporthe* spp. to different degrees (Table 2). The percentage inhibition of mycelial growth ranged from 19.79% to 60.29% for *Diaporthe* spp. in the inverted plate assay. The highest percentage inhibition of mycelial growth of *Diaporthe* was shown by isolate T4 which was 60.29%. The lowest percentage inhibition of mycelial growth was exhibited by isolate T3 which was 19.79%. The isolate T2 showed slightly higher inhibition (22.68%) than isolate T3. The isolate T1 showed percentage inhibition of mycelial growth of 48.09%.

Table.1 Percentage inhibition of radial growth of the two pathogens in dual culture assay

Trichoderma isolates	Percentage Inhibition of Radial Growth (PIRG) of the pathogens in Dual Culture Assay (%)	
	Diaporthe spp.	*Lasiodiplodia* spp.
T1	65.16	62.04
T2	65.23	61.06
T3	66.32	59.67
T4	73.68	57.14

Table.2 Percentage inhibition of mycelial growth of the two pathogens in inverted plate assay

Trichoderma isolates	Percentage Inhibition of Radial Growth (PIRG) of the pathogens in Inverted Plate Assay (%)	
	Diaporthe spp.	*Lasiodiplodia* spp.
T1	48.09	0
T2	22.68	0
T3	19.79	0
T4	60.29	0

Figure.1 Culture of different isolates of *Trichoderma* on PDA media

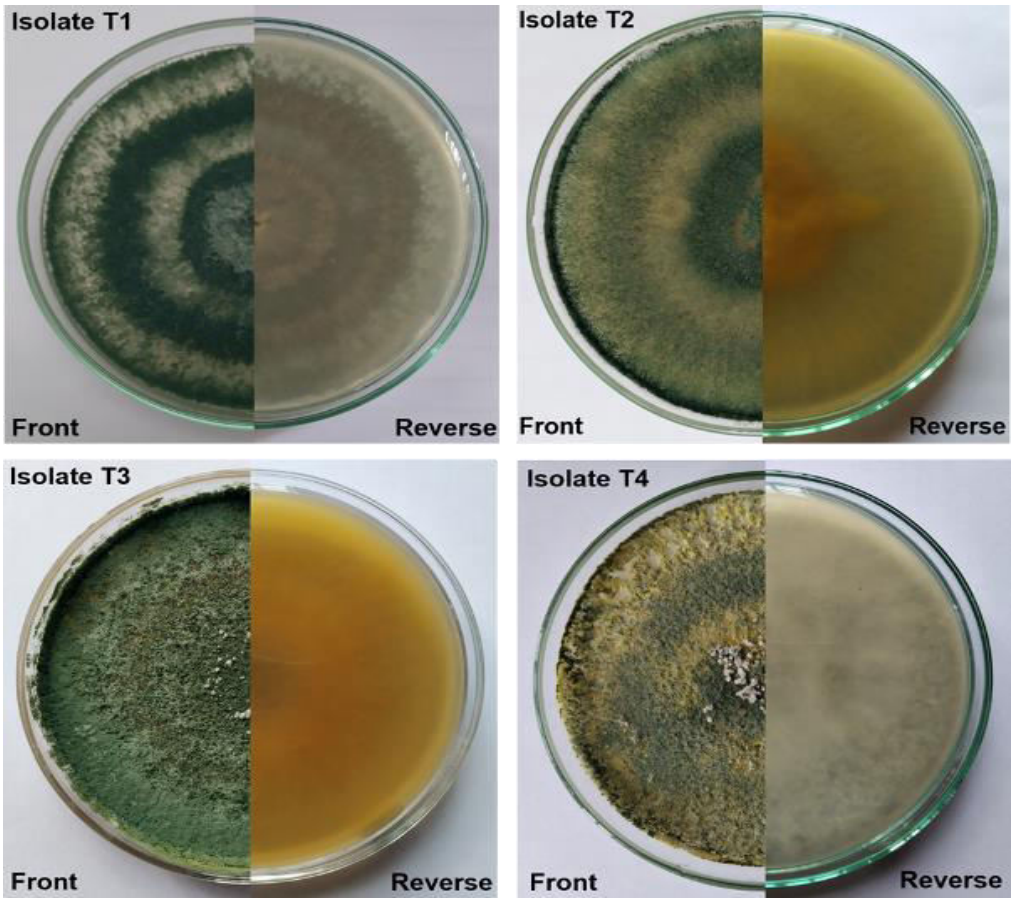


Figure.2 Microscopic view (400X magnification) of different isolates of *Trichoderma*: (a) Isolate T1; (b) Isolate T2; (c) Isolate T3; (d) Isolate T4

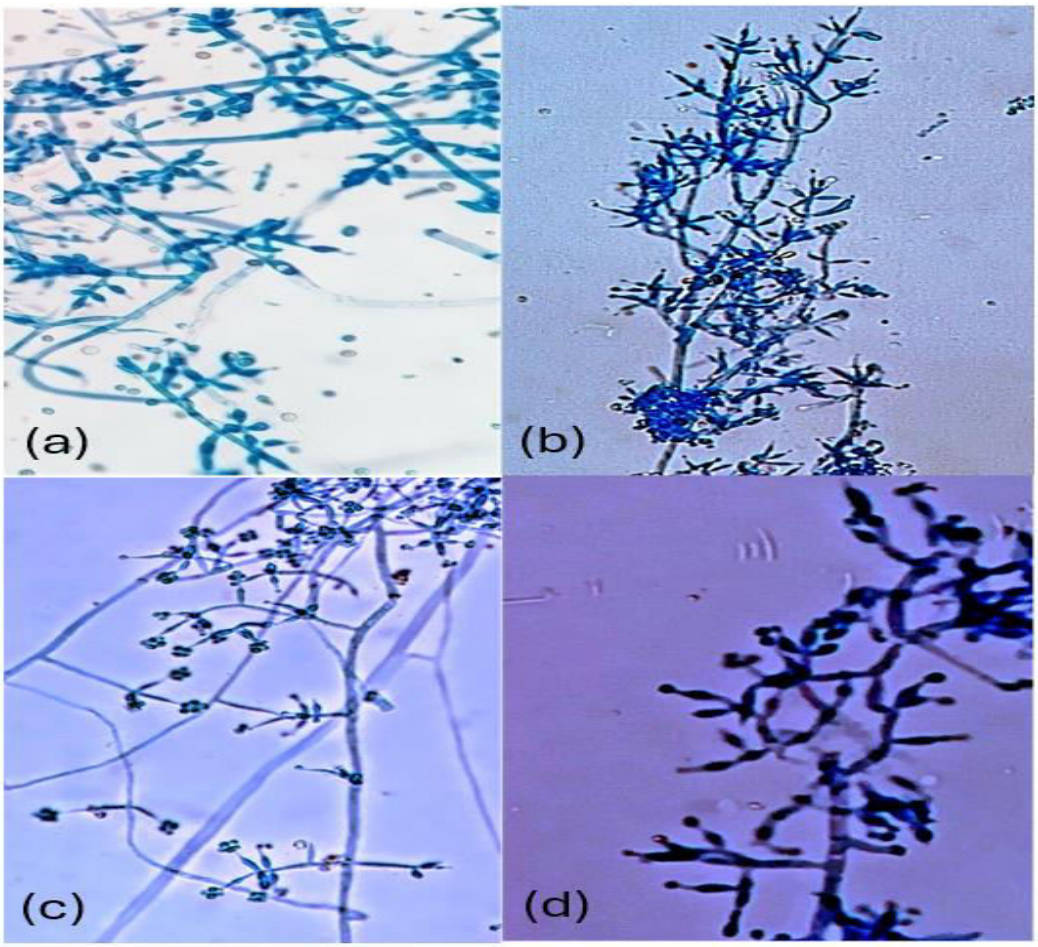
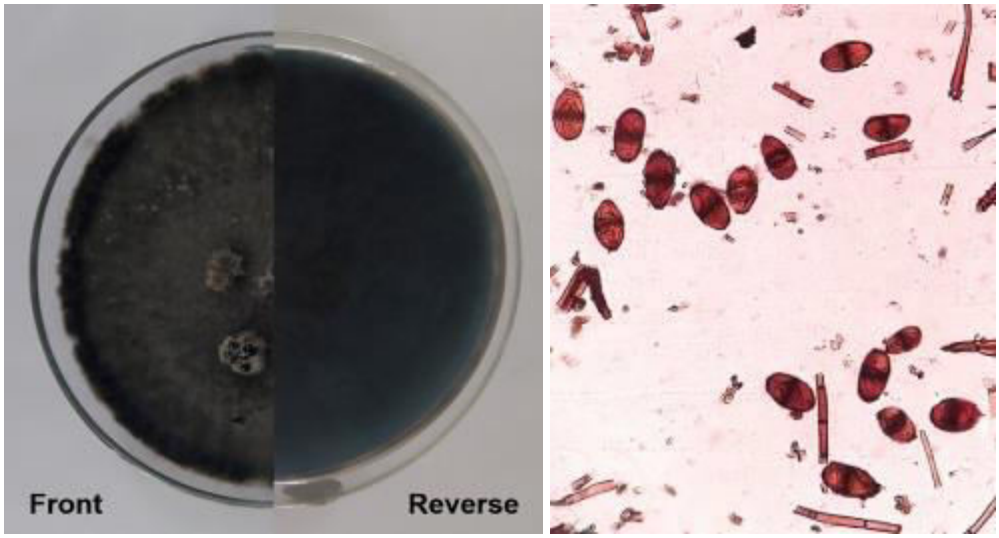


Figure.3 Culture and microscopic view (400X magnification) of *Diaporthe*

Figure.4 Culture of *Lasiodiplodia* and microscopic view (400x magnification) of its conidia



Lasiodiplodia is a fast-growing pathogen. It covered the entire 100 mm Petri dish in 3 to 4 days. The volatile compounds released by *Trichoderma* isolates had no effect on the growth of *Lasiodiplodia* species (Table 2). After 4 days of incubation, no inhibition was observed by any of the *Trichoderma* isolates. *Lasiodiplodia* covered the entire Petri dish in 3 days in the control plate and in case of isolates T1, T2 and T3 while it took 4 days to fill the entire Petri dish in case of isolate T4.

Several researchers have demonstrated in the past that the volatile compounds released by *Trichoderma* species have an inhibitory effect on various phytopathogens such as *Fusarium oxysporum, Alternaria panax*, *Botrytis cinerae* (Joo and Hussein, 2022), *Pyrenophora teres* (Moya *et al.,* 2018), *Lasiodiplodia theobromae* (Rabuske *et al.,* 2023) and many others. Rabuske *et al.,* (2023) observed that volatile metabolites produced by *Trichoderma asperellum* isolates inhibited the mycelial growth of *Lasiodiplodia theobromae* and *Pseudofusicoccum kimberleyense*. Bhadra *et al.,* (2014) screened four *Trichoderma* isolates (*T. harzianum*, *T. koningii*, and two strains of *T. viride*) for their inhibitory effect on *L. theobromae* and found that the volatile metabolites produced by only one of them (*T. viride* green strain) inhibited the pathogen while the volatile metabolites of other isolates had no inhibitory effect on the growth of *L. theobromae* which is similar to the result obtained in the present study.

In conclusion, *Trichoderma* uses various mechanisms for inhibiting the growth of the pathogen. Besides direct hyphal interaction, secondary metabolites including both volatile and non-volatile compounds produced by *Trichoderma* play an important role in suppressing the pathogen. In the present research work, it was found that all the isolates of *Trichoderma* had inhibitory effect on the two test pathogens in the dual culture assay. The isolates showed higher percentage of inhibition for *Diaporthe* as compared to *Lasiodiplodia*. The volatile compounds released by *Trichoderma* isolates inhibited the mycelial growth of *Diaporthe* but they had no inhibitory effect on the growth of *Lasiodiplodia*. Thus, it can be concluded that the *Trichoderma* isolates were effective in inhibiting the two pathogens to different extent. Although *Trichoderma* has been established as an effective biocontrol agent, further research can help in identifying strains with higher efficiency against specific diseases and better field results. More research into the compounds produced by *Trichoderma*, and the mechanisms through which they control the spread of pathogens, can eventually help develop better strategies for plant disease management.

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Author Contributions

Hena Mahmood: Conceptualization, Methodology, Investigation, Original Draft Preparation, Funding Acquisition. Choudhary Sharfuddin: Review and Editing, Supervision.

Data Availability

The data generated or analysed in this study are included within the article.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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