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Comparative *in Vitro* Efficacy Assessment Methods of a Bioagent *Trichoderma harzianum* THR 4 against Rice Blast Pathogen *Magnaporthe oryzae oryzae*

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Abstract

Rice is the staple food crop for a large part of the human population in the world today. Rice blast is by far the most important disease of the many diseases that attack rice caused by *Magnaporthe oryzae oryzae* (MoO). Failures of entire rice crops have resulted directly from rice blast epidemics. *Trichoderma harzianum* the well-known antagonistic fungus is widely used in agriculture as bio-fungicide. In the present study five different designs of *in vitro* dual culture techniques were evaluated on Potato Dextrose Agar (PDA) to find out the most suitable technique to interaction study between MoO and *T. harzianum* THR 4. The design where four 5.0 mm mycelia discs of *T. harzianum* THR 4 were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish in dual culture gave most satisfactory result with 92% growth inhibition of MoO in compare to control plate of MoO.

Subject Areas

Agricultural Science

Keywords

Rice Blast, *Magnaporthe oryzae oryzae* (MoO), *Trichoderma harzianum* THR 4, Biological Control

1. Introduction

Rice (*Oryza sativa* L.) is a staple food for half of the world's population [1]. It is

central to Bangladesh's economy, accounting for nearly 20 percent of gross domestic product (GDP) and providing about one-sixth of the national income of Bangladesh [2]. Rice blast caused by *Magnaporthe oryzae oryzae* (MoO) is a key concern in combating global food insecurity given the disease is responsible for approximately 30% of rice production losses globally the equivalent of feeding 60 million people [3]. These losses increase the global rice price and reduce consumer welfare and food security. As rice is the staple crop for more than half the world's population so any reduction in rice blast would have substantial beneficial effects on consumer livelihoods. *Pyricularia oryzae* (Po) isolated from infected leaf and panicle and identified based on cultural characteristics and conidia morphology and mycelia growth of *Pyricularia* isolates varied significantly with fair to excellent sporulation ability [4].

Chemicals are commonly applied for controlling rice blast disease [5] [6] [7]. However, the frequent use of fungicides on crops may cause hazards to human beings, plant health, beneficial micro-organisms, and develop fungicide resistance into the pathogens and residual toxicity in plant parts. On the other hand, some bio-control agents have proved to be most secure and have no adverse impact on environment [8] [9]. Eight botanical plant extracts have been tested *in vitro* against *Magnaporthe oryzae oryzae* and found satisfactory reduction in mycelia growth of MoO [10]. *Trichoderma* spp., the well-known antagonistic fungi are widely used in agriculture as bio-fungicides [11]. *Trichoderma* spp., inhibited the mycelia growth of rice blast fungus [12]. The use of antagonistic fungi to control the destructive plant pathogens is getting more importance since few decades [13] [14].

Bio-control agents are widely regarded as natural remedy with non-threatening affect. *Trichoderma* species act against target organisms in several ways [15]. Volatile and non-volatile compounds of *Trichoderma* spp. were analysed by GC-MS technique (Gas chromatography-mass spectrometry (GC-MS), an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample [16]. Time of the application of the *Trichoderma* is also important. The purpose of this study was to evaluate comparative efficacy assessment methods of bio-agent *Trichoderma harzianum* in controlling *Magnaporthe oryzae oryzae* (MoO) *in vitro*.

2. Materials and Methods

2.1. Materials

2.1.1. Experimental Site

The experiment was conducted in the Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

2.1.2. Experimental Period

The experiment was conducted during the period from June 2018 to December 2019.

2.1.3. Inoculum of Test Fungus *M. oryzae oryzae* (MoO)

The present study was conducted to evaluate the efficacy assessment methods of *T. harzianum* THR 4 against a virulent isolate of *Magnaporthe oryzae oryzae* MoO19 [17]. The isolate was identified based on three celled pyriform conidia (Figure 1).

2.1.4. Inoculum of antagonist *T. harzianum*THR4

The bio-control agent *T. harzianum* THR 4 was obtained from Pathology Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka [18]. *T. harzianum* was sub-cultured in *in vitro* condition for antagonism test against MoO (Figure 2 and Figure 3).

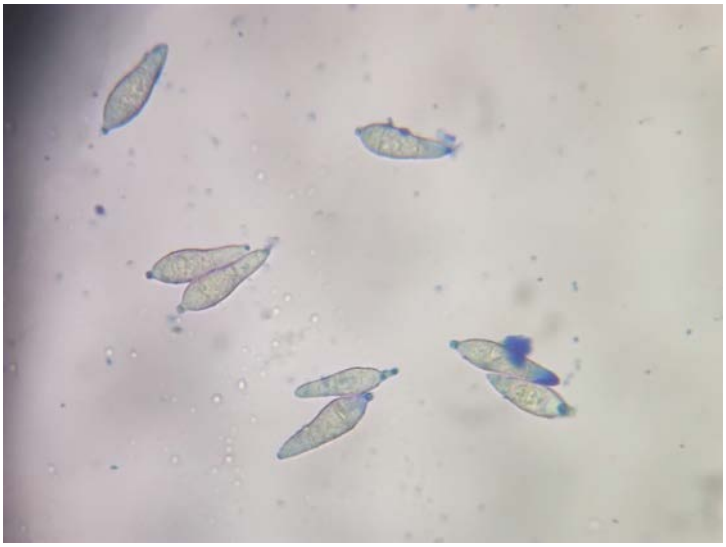

Figure 1. Three celled pyriform conidia of MoO (×40).

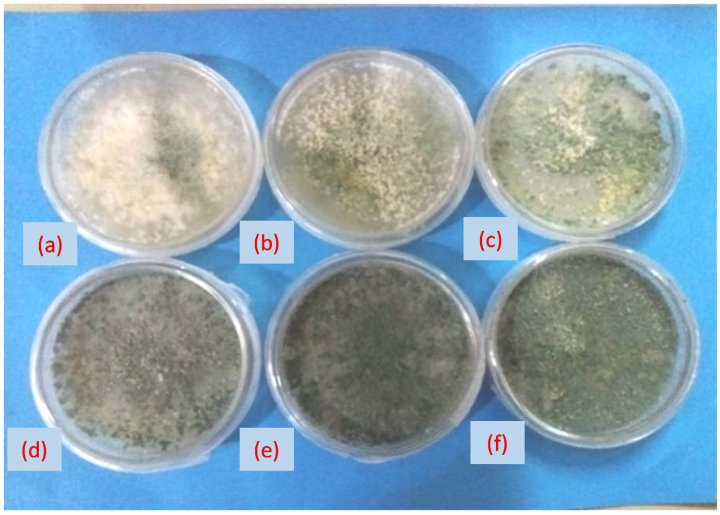

Figure 2. Pure culture of *Trichoderma harzianum* THR 4 at different days after inoculation; (a) Mycelial growth at 6DAI; (b) Mycelial growth at 7DAI; (c) Mycelial growth at 8DAI; (d) Mycelial growth at 10DAI; (e) Mycelial growth at 12DAI; (f) Mycelia growth at 14DAI.

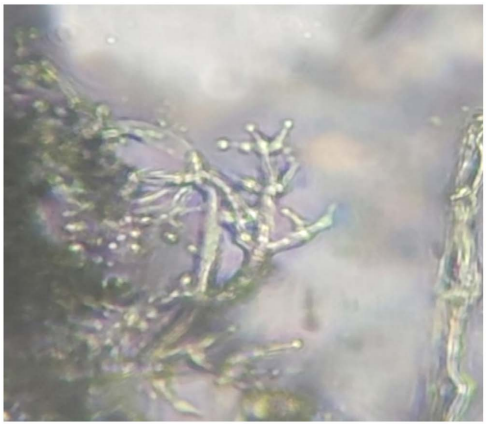

Figure 3. Mycelia, conidiophores, phialides and conidia of *T. harzianum* THR 4 (×40).

2.2. Materials

2.2.1. Efficacy of Bio-Agent *T. harzianum* THR 4 in Controlling Radial Mycelia Growth of MoO *in Vitro*

Bio-control agent such as *T. harzianum* was tested under laboratory conditions against rice blast causing fungus, MoO. This experiment was done to evaluate *T. harzianum* against MoO following different methods in dual culture on PDA plates.

Four mm disc of test fungus and biocontrol agent at 2DAI (**Figure 4**) were placed at opposite sides to each other in Petri dishes in five different designs that containing sterilized PDA medium. There were 3 replications of each design containing bio-control agent and *Magnaporthe oryzae oryzae* and were incubated at 25°C. Petri dishes containing the test fungus and bio-control agents separately incubated at 30°C served as control. Colony diameter of both bio-control agent and the test fungus were recorded after each 48 hours by giving straight line in the center of both colonies with permanent marker. The interaction and mechanism of antagonism was observed when the colonies of both fungi met [19].

2.2.2. Designs of Dual Culture Technique for Observing the Efficiency of *T. harzianum* against MoO

This experiment was done following five different designs of dual culture technique to know the interactions among MoO and *Trichoderma harzianum* THR 4 and also control plates were set.

In first design, one disc of MoO was set against one disc of *T. harzianum* following dual culture technique. In second design, three discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish following dual culture technique. In third design, four discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish following dual culture technique. In fourth design, three discs of MoO were set on the periphery of the petridish surrounding one disc of *T. harzianum* on the center of the petridish following dual culture technique. In fifth design, four discs of MoO were set on the periphery of

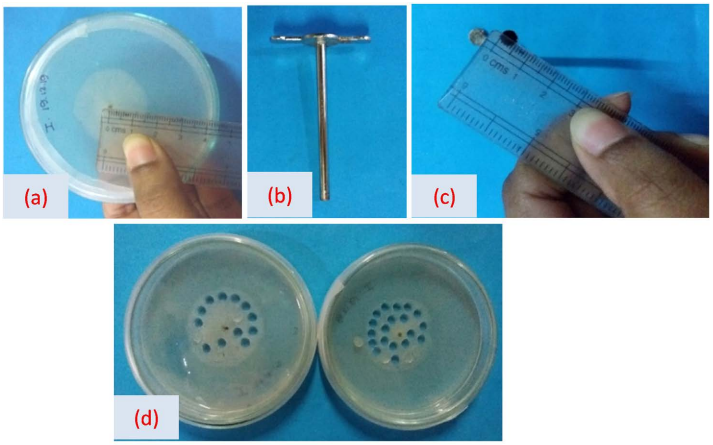

Figure 4. (a) Measuring the radial growth of THR 4 at 2DAI; (b) Block cutter; (c) Measuring the diameter of mycelial block; (d) After removing the blocks from cultured plate of *T. harziamum* THR 4.

the petridish surrounding one disc of *T. harzianum* on the center of the petridish following dual culture technique. There was also two different petridish set as control condition for both tested fungus MoO and bio-agent *Trichoderma harzianum* (**Figure 5**).

2.2.3. Experimental Design and Statistical Analysis

The experiment was done following Complete Randomized Design (CRD) with three replications and statistical analysis was done using Statistix10 software. Data were analyzed at 5% level of significance. One factor analysis of variance (ANOVA) was done and critical value for comparison was recorded as LSD value. There are different groups (a, b, etc.) in which the means are not significantly different from one another. Data are significantly different from one group to another.

3. Result

3.1. *In Vitro* Mycelia Radial Growth of MoO with Bio-Agent *Trichoderma harzianum* THR 4 in PDA

Third design where four discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish following dual culture technique successfully inhibited the fungal growth and infection of MoO (2 mm) whereas the growth of bio-agent was 16.67 mm followed by second design where three discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish inhibited the fungal growth (4.33 mm) whereas the growth of bio-agent was 18.33 mm at 6DAI (**Table 1**). In fourth and fifth design mycelia radial growth of MoO was 6.67 mm whereas the mycelia growth of bio-agent was 33.33 mm followed by first design where mycelia radial growth of MoO was 11.67 mm whereas mycelia radial growth of *T. harzianum* recorded 28.33 mm. In control condition mycelia radial growth of tested fungus and bio-agent was 25 mm and 40 mm respectively.

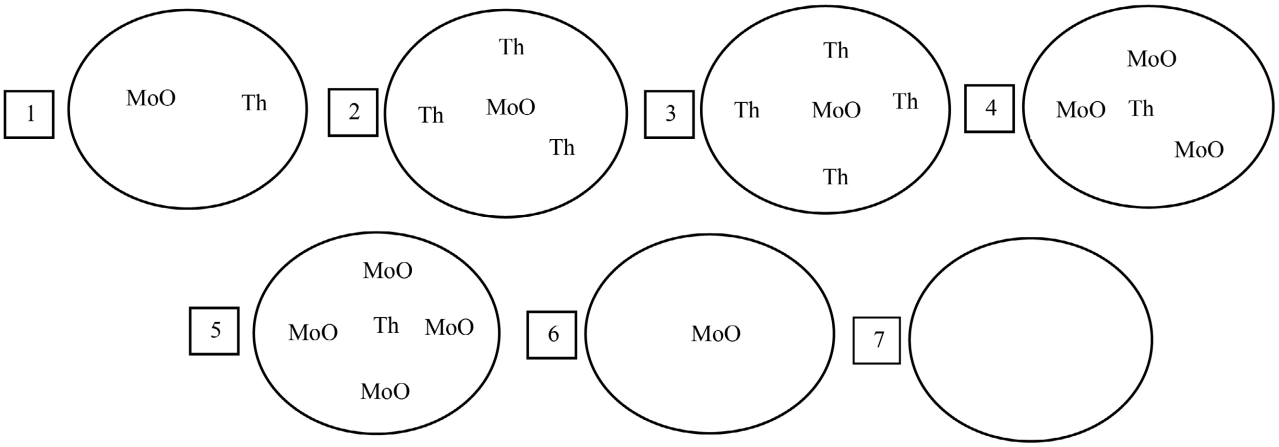

Figure 5. Five treatment and two control designs to interaction study between *T. harzianum* THR 4 and MoO. MoO denotes *Magnaporthe oryzae oryzae* and Th denotes *T. harzianum* THR 4.

Table 1. Mycelia radial growth inhibition of MoO and *T. harzianum* THR 4 by each other in different *in vitro* treatment design in PDA.

Treatment Design	Mycelia radial growth of MoO (mm)	% Inhibition of growth	Mycelia radial growth of Th (mm)	% Inhibition of growth
1. MoO and Th disks were set in the opposite periphery.	11.67 b	53.32	28.33 c	29.18
2. MoO disk in the center and three Th disks on the periphery of the dish in equal distance	4.33 d	82.68	18.33 d	54.18
3. MoO disk in the center and four Th disks on the periphery of the dish in equal distance	2.00 e	92.00	16.67 d	58.33
4. Th disk in the center and three MoO on the periphery of the petridish in equal distance	6.67 c	73.32	33.33 b	16.68
5. Th disk in the center and four discs of MoO on the periphery of the petridish in equal distance	6.67 c	73.32	33.33 b	16.68
6. Control condition for MoO	25.00 a	0.00	-	-
7. Control condition for Th	-	-	40.00 a	0.00
LSD (0.05)	1.83		2.26	

MoO = *Magnaporthe oryzae oryzae* and Th = *Trichoderma harzianum* THR 4.

In *in vitro* condition third design where four discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish following dual culture technique gave most satisfactory result with 92% growth inhibition of MoO in compare to control treatment (**Figure 6**).

3.2. Interaction between MoO and *Trichoderma harzianum* THR 4 in Dual Culture Designs

Data were recorded to understand the interactions between tested fungus MoO and bio-agent *Trichoderma harzianum* THR 4. The third design where four discs

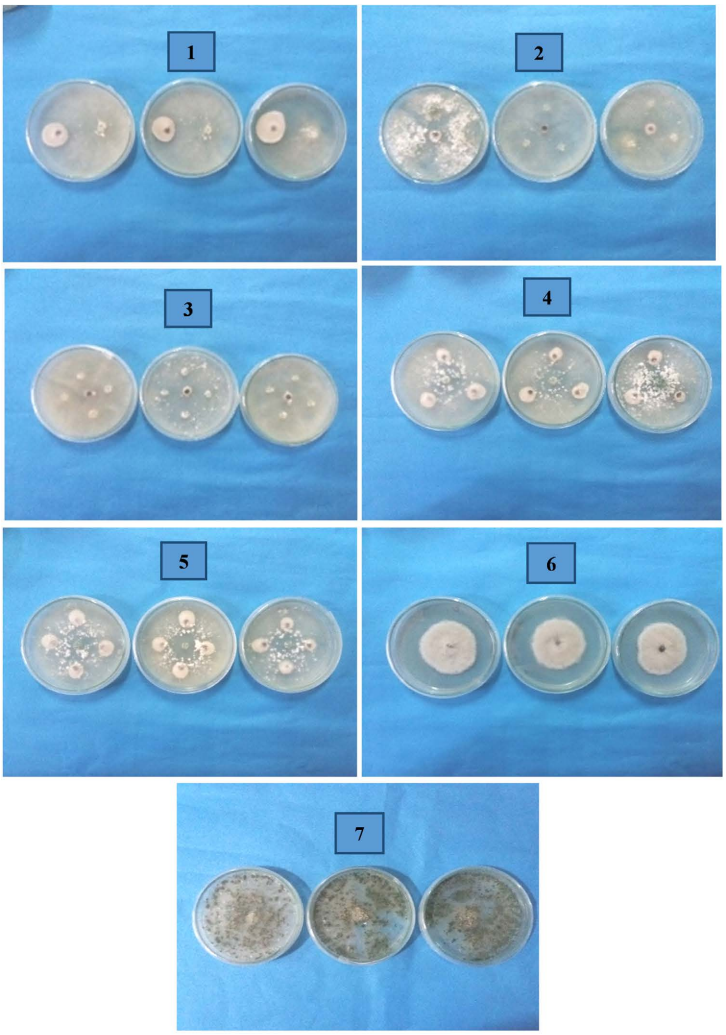

Figure 6. Growth of *Magnaporthe oryzae oryzae* (MoO) and *Trichoderma harzianum* (Th) in both dual culture technique and in control condition in PDA at 6 DAI; 1 = One disc of MoO was set against one disc of Th, 2 = Three discs of Th were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish, 3 = Four discs of Th were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish, 4 = Three discs of MoO were set on the periphery of the petridish surrounding one disc of Thon the center of the petridish, 5 = Four discs of MoO were set on the periphery of the petridish surrounding one disc of Thon the center of the petridish and 6 = Control plates of MoO and 7 = Control plates of Th.

of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish following dual culture technique successfully inhibited the fungal growth and infection of MoO towards bio-agent was 2mm whereas the growth of bio-agent towards the tested fungus was 15 mm followed by second design where three discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish inhibited the fungal growth (4 mm) towards bio-agent whereas the growth of bio-agent towards tested pathogen was 18 mm at 6DAI (**Table 2** and **Figure 7**). In fourth design mycelia radial growth of MoO towards *T. harzianum* was

Table 2. Interaction of *Magnaporthe oryzae oryzae* (MoO) with *Trichoderma harzianum* THR 4 (Th) in dual culture designs in PDA at 6 DAI.

Treatment Design	Mycelia radial growth of MoO towards Th at 6 DAI (mm)	% Inhibition of MoO growth in 6 DAI	Mycelia radial growth of Th towards MoO at 6 DAI (mm)	% Inhibition of Th growth in 6 DAI
1. MoO and Th disks were set in the opposite periphery	8.33 b	66.68	28.33 c	29.17
2. MoO disk in the center and three Th disks on the periphery of the dish in equal distance	4.00 cd	84.00	18.00 d	55.00
3. MoO disk in the center and four Th disks on the periphery of the dish in equal distance	2.00 d	92.00	15.00 e	62.50
4. Th disk in the center and three MoO on the periphery of the petridish in equal distance	4.67 c	81.32	35.33 b	11.67
5. Th disk in the center and four discs of MoO on the periphery of the petridish in equal distance	5.00 c	80.00	35.00 b	12.50
6. Control condition for MoO	25.00 a	0.00	-	-
7. Control condition for Th	-	-	40.00 a	0.00
LSD (0.05)	2.26		2.26	

MoO = *Magnaporthe oryzae oryzae* and Th = *Trichoderma harzianum* THR 4.

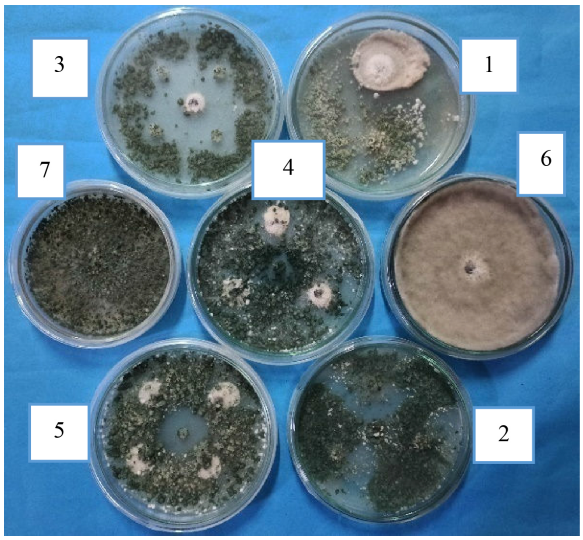

Figure 7. Growth of *Magnaporthe oryzae oryzae* (MoO) and bio-agent *Trichoderma harzianum* THR 4 (Th) in dual culture and control condition on PDA; 1 = One disc of MoO was set against one disc of *T. harzianum,* 2 = Three discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish, 3 = Four discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish, 4 = Three discs of MoO were set on the periphery of the petridish surrounding one disc of *T. harzianum* on the center of the petridish, 5 = Four discs of *M. oryzaeoryzae* were set on the periphery of the petridish surrounding one disc of *T. harzianum*on the center of the petridish and 6 = Control plates of *M. oryzae oryzae* and 7 = Control plates of *T. harzianum* THR 4.

4.67 mm whereas the mycelia growth of bio-agent towards MoO was 35.33 mm followed by fifth design where mycelila radial growth of MoO was towards *T. harzianum* was 5 mm whereas mycelia radial growth of *T. harzianum* towards MoO was recorded 35 mm and in first design where mycelia radial growth of MoO was towards *T. harzianum* was 8.33 mm whereas mycelia radial growth of *T. harzianum* towards MoO was recorded 28.33 mm. In control condition mycelia radial growth of tested fungus and bio-agent was 25 mm and 40 mm respectively.

In vitro condition third design where four discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish following dual culture technique gave most satisfactory result.

4. Discussions

Trichoderma species have been investigated as biological control agents over 70 years [20]. In our study all five designs of *in vitro* studies shown reduced growth of MoO compared to control plates. Among the five designs of our experiment the third design where four discs of *T. harzianum* was set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish in dual culture technique gave most satisfactory result in both cases of mean mycelium growth inhibition and interactions with MoO. In a previous study the highest percentage inhibition of radial growth (PIRG) values was observed with *T. harzianum* IMI-392432 using two dual culture methods, 63.80% in Method I and 80.82% in Method II [21]. In the first method (Method-I), an agar disc (6 mm) was taken from 4-day-old PDA culture plates of each *Trichoderma* isolate and placed at the periphery of the PDA plates (9 mm). Another agar disc of the same size of *C. paradoxa* was also placed at the periphery but on the opposing end of the same Petri dish. In the second method (Method-II), an agar disc (6-mm) of the antagonist, *Trichoderma* (T), was placed 2 cm away from the periphery of the Petri dish, and a same sized agar disc of the test fungus, *C. paradoxa* (C), was similarly placed 2 cm away from the edge of the Petri plate but on the end opposite of *Trichoderma* sample. As a control, *C. paradoxa* was placed in a similar manner on a fresh PDA plate. They also used poison agar, and direct methods to assess the ability of *Trichoderma virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433, and *T. harzianum* IMI-392434 to control *C. paradoxa*, which causes the pineapple disease of sugarcane. In another study it had been observed that *Trichoderma* sp. isolated from soil and tested *in vitro* against soil borne pathogens viz. *Sclerotium rolfsii*, *Rhizoctonia solani*, *Sclerotinia sclerotium* and *Fusarium solani* using dual culture technique and 100% growth inhibition was found in case of *Sclerotium rolfsii [22]*. *T. viride* (MO) also reduced the colony area of *Macrophomina phaseoli* by 19.2 and 34.9% using the dual culture and cellophane methods, respectively reported in other study [23].

Trichoderma viride was evaluated under laboratory conditions against some

common phyto-pathogens belonging to different groups of fungi, effectively inhibited the growth of the tested pathogens in dual cultures by hyperparasitism and by secretion of volatile and non-volatile metabolites. In the dual culture experiment, maximum inhibition was recorded for *Fusarium oxysporum* followed by *Rhizoctonia solani* and least for *Alternaria zinnia [24]*. Other than mycelia interaction and hyperparasitism by the *Trichoderma* species, scientists have also considered the action use of antibiotic metabolites as a contributing mechanism in the biocontrol of plant pathogens [25].

Volatile and non-volatile compounds of *Trichoderma* spp. were analyzed by GC-MS technique and the properties of distinguished compounds showed antifungal, antimicrobial and antibiotic activities [26]. Volatile compounds of *T. harzianum* and *T. viride* showed highest percent abundance for glacial acetic acid (45.32%) and propyl-benzene (41.75%), respectively. In case of non-volatile compounds, *T. harzianum* and *T. viride* showed D-Glucose, 6-O-α-D-galactopyranosyl (38.45%) and 17-Octadecynoic acid (36.23%), respectively. The results of present study confirmed that *T. harzianum* can be used as a promising biological control agent.

Our results are in accordance to those reported by [27] who agreed to the statement that antagonistic such as *T. harzianum* gave 70%~88% mycelia and conidial inhibition of *M. oryzae*. Similarly, an interaction study different species of *Trichoderma* against 24 airborne plant pathogens including *M. oryzae* were studied where *T. hamatum, T. harzianum, T. koningii, T. pseudokoningii* and *T. viride* were found to have strong antagonistic potential. He also found that selective isolates of *T. harzianum* and *T. viride* showed severe antagonism against *M. oryzae*, while *T. polysporum* was weaker antagonist [28]. Most of the antagonistic fungi were effectively inhibited the spore production of the plant pathogens where the growth inhibition (GI) of the spore production were greatly increased in some of the antagonistic fungi used [29]. This was happened due to the activity of enzymes produced by the antagonistic fungi such as *Trichoderma* sp. and *Penicillium* sp. The isolate T39 of *Trichoderma harzianum* which can be regarded as a model to demonstrate biocontrol under commercial conditions and the mechanisms involved [30].

5. Conclusion

Five different designs of dual culture technique were used to know the interactions between *Magnaporthe oryzae oryzae* (MoO) and *T. harzianum* THR 4. In *in vitro* condition third design where four discs of *T. harzianum* were set on the periphery of the petridish surrounding one disc of MoO on the center of the petridish in dual culture technique gave most satisfactory result with 92% growth inhibition of MoO. All the designs of dual culture were effective *in vitro* test against the test fungus *Magnaporthe oryzae oryzae.*

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Competing Interests

Authors have declared that no competing interests exist.

Authors' Contributions

This work was carried out in collaboration among all authors. Author ZN conducted the research work. Author FMA designed and supervised the study and edited the manuscript. Author LL and MLA managed the literature searches. All authors read and approved the final manuscript.

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