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Full Length Research Paper

Production and antagonistic effect of *Trichoderma* spp. on pathogenic microorganisms (*Botrytis cinerea, Fusarium oxysporium, Macrophomina phasealina* and *Rhizoctonia solani*)

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***Trichoderma* spp. are widely used as bio-fungicides in agriculture. Induction of plant defense and mycoparasitism (killing of one fungus by another) are considered to be the most important mechanisms of *Trichoderma*-mediated biological control. This study is based on the optimized production of *Trichoderma viride*. The media used for the economical production of *T. viride* conidia contain 5% jaggery and 0.5% baker's yeast. It is clear that the growth and sporulation of *Trichoderma* mycelia require different temperatures. Mycelia had significant growth at 37°C and sporulation at 24°C (low temperature). For industrial production of *T. viride* conidia, it is suggested that the culture should be incubated initially at 37°C until the mycelia are formed and then at 24°C to induce sporulation. Formulating *Trichoderma* in talc is better than doing it in oil because the spores are hydrophilic in nature. There is current understanding of the interactions of *Trichoderma* with plant pathogens such as *Botrytis cinerea*, *Fusarium oxysporium*, *Macrophomina phasealina* and *Rhizoctonia solani*, and it is concluded that *Trichoderma* has antagonistic effect against these pathogens.**

Key words: *Trichoderma*, induced resistance, biological control, mycoparasitism.

INTRODUCTION

At present, around 30% of all plant species have been destroyed by plant pathogens. Pesticides and organic compounds are widely used to control plant pathogens in many countries. However, the degradation of such compounds is very difficult and their concentration and accumulation in food chains lead to higher toxicity levels in animals (Chet, 1987; Lynch, 1990). *Trichoderma* spp. (teleomorph Hypocrea) is the most successful bio-fungicide used in today's agriculture; there are more than 60% registered biofungicides world-wide (Verma et al., 2007). *Trichoderma*-based *Trichoderma viride* species have been investigated for over 80 years. In India alone,

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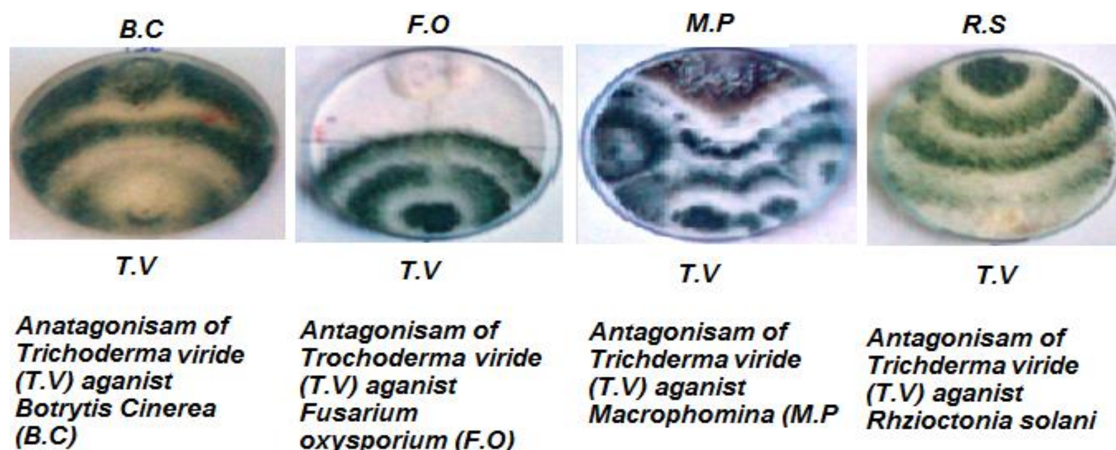

Figure 1. Antagonistic activity of *Trichoderma Viride* to different fungal Strain *Botrytis Cinerea, Fusarium Oxysporium, Macrophomina Phasealina, Rhizoctonia Solani.*

about 250 products are available for field applications (Singh et al., 2009). Recently, they have been used as biological control agents and their isolates have become commercially available (Mukherjee et al., 2012). This development is largely as a result of a change in public attitude towards the use of chemical pesticides and fumigates such as methyl bromide (Elad et al., 1980). In this respect, *T. viride* spp. have been studied as biological control agents against soil-borne plant pathogenic fungi (Küçük, 2000; Chet and Inbar, 1994). Results from different studies showed that several strains of *T. viride* had a significant reducing effect on plant diseases caused by pathogens such as *Rhizoctonia Solani, Sclerotium rolfsii, Phythium aphanidermatium, Fusarium oxysporum, F. culmorum and Gaeumannomyces graminis* var. *tritici* under greenhouse and field conditions (Basim et al., 1999; Sivan and Chet, 1993; Chet and Baker, 1981; Dolatabadi et al., 2011; Ephrem et al., 2011). Isolates of *T. viride* can produce lytic enzymes (Küçük, 2000; Haran et al., 1996) and antifungal antibiotics (Dennis and Webster, 1971; Brewer et al., 1987; Almassi et al., 1991). They can also be competitors of fungal pathogens (Whipps, 1987) and promote plant growth (Inbar et al., 1994). They have ability to grow in a wide range of temperatures, are capable of antagonizing plant pathogens, using lignocellulosic materials for growth. Antibiosis and hyperparasitism make *T. viride* isolates possible bio-control agents (Haran et al., 1996; Rifai, 1969; De La Cruz et al., 1992). For these reasons, *T. viride* has become a successful bio-control agent in agriculture field. Many companies recently started large scale production of *T. viride* conidia for agricultural use like biopesticide. As a beneficial biological agent, *Trichoderma* is a filamentous fungus which has gained attention because of its multi-prolonged action against various plant pathogens (Harman et al., 2004; Shabir-U-Rehman, et al., 2013). One of the most important things considered in the design of a mass production procedure is the compatibility of the product with both formulation and application techniques. For example, the use of oil formulations for application at ultra low volume rates requires the production of lipophilic conidia which suspend easily in oils (Khurana et al., 1993). Submerged conidia are hydrophilic and are not easily formulated in oils. Blastospores are similarly produced in submerged liquid fermentation, but are also hydrophilic and have been found to lose viability relatively quickly during storage. Due to these problems, there is the necessity to develop cost-effective methods to produce conidia of high quality in large-scale. In this study, we attempt to test the bio-efficacy of *T. viride* against fungal pathogens, optimize the culture medium for the production of conidia, and formulate it in a non-lipophilic solid material (talc) instead of using oil. Certain strains of *Trichoderma* species were reported to induce transcriptomic changes in plants and some are known to protect plants from diseases and abiotic stresses (Bailey et al., 2006).

MATERIALS AND METHODS

Fungal strains

The microbial strains used in this study are *Botrytis cinerea, F. oxysporium, Macrophomina phasealina, Rhizoctonia solani* and the antagonistic fungi *T. viride* is obtained from depositary laboratory (Figure 1).

Media optimization for the mass production *of Trichoderma viride*

For the mass production of *T. viride*, different types of media were used (which are cost effective). The media mainly contained Jaggery, yeast extract, glucose and baker's yeast (Figures 2 and 3). 5% baker's yeast and peptone are taken; 3, 4, 5, 10 and 15% of jaggery and 0.5, 1, 1.5, 2, and 2.5% glucose are used to get the optimal composition for the mass production. Acidic condition is

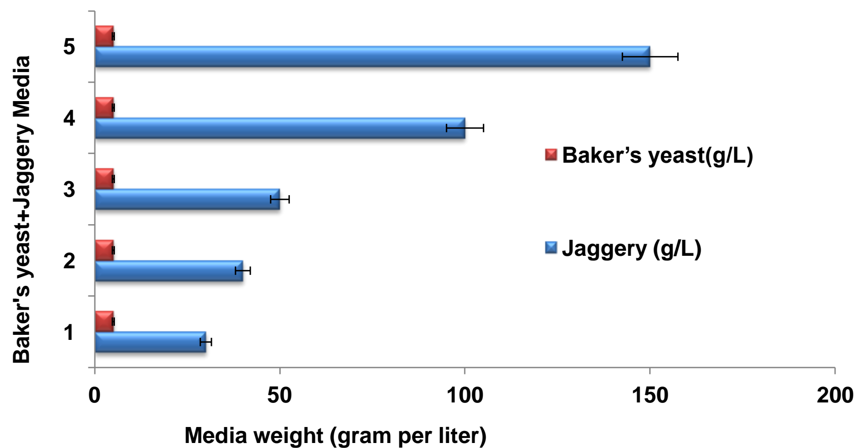

Figure 2. Media composition of baker's yeast and Jaggery.

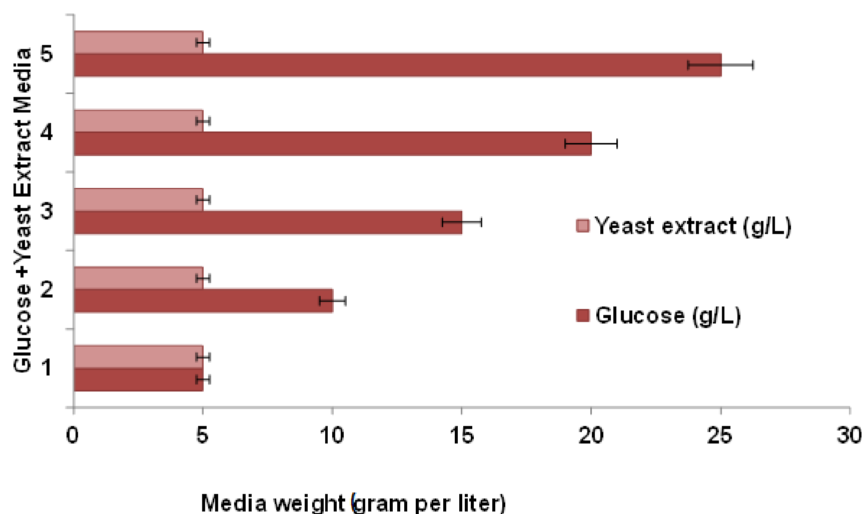

Figure 3. Media composition of yeast extract and glucose.

maintained by adjusting the pH to 5 with 0.1 N HCl. The media are distributed into 20 flasks as each flask contains 50 ml of medium. All the flasks were labeled properly and autoclaved at 121°C and 15 lbs pressure for 30 min. Then the bottles were cooled to room temperature.

Preparation of inoculums

The media used to inoculate *T. viride* for spore production were yeast extract (2%), peptone (0.5%), glucose (3%), and pH was maintained to 5 with 0.1 N HCl. The media were autoclaved at 121°C and 15 lbs pressure for 20 min. *Trichderma viride* spores were taken from PDA slant. The spores were added as the concentration of spores reached 5×10^4 per ml; 0.01% of sodium lauryl sulphate was added to the spore suspension. The flask was kept on the shaker for two hours to achieve uniform distribution of spores. Sodium lauryl sulphate helps to separate the spore clumps. 1 ml of spore suspension was added to each mass production medium flask in a sterile condition and mixed properly. Two flasks from each type of medium were not inoculated with spores and used as a control. Among 20 flasks of each type, 9 inoculated flasks and 1 control flask were incubated at 24°C. Nine (9) inoculated flasks and 1 control flask were incubated at 37°C ; the flasks were incubated for seven days (Figure 4).

Measurement of wet weight

The inoculated media were centrifuged at 4000 rpm for 20 min and the pellet was separated. The supernatant was stained with lactophenol cotton blue and observed under 40x magnification of microscope. If spores were found in supernatant, it was centrifuged again at 5000 rpm for 20 min. Then the weight of the pellet was measured. 1 g of pellet was taken and mixed with 1 kg of sterile talc and 0.5 g of carboxy methyl cellulose (CMC); and sieved carefully. Then 1 g of sample was taken and serially diluted with sterile distilled water. Few dilutions were taken to count the colony forming units (CFU) on potato dextrose agar plates. The medium

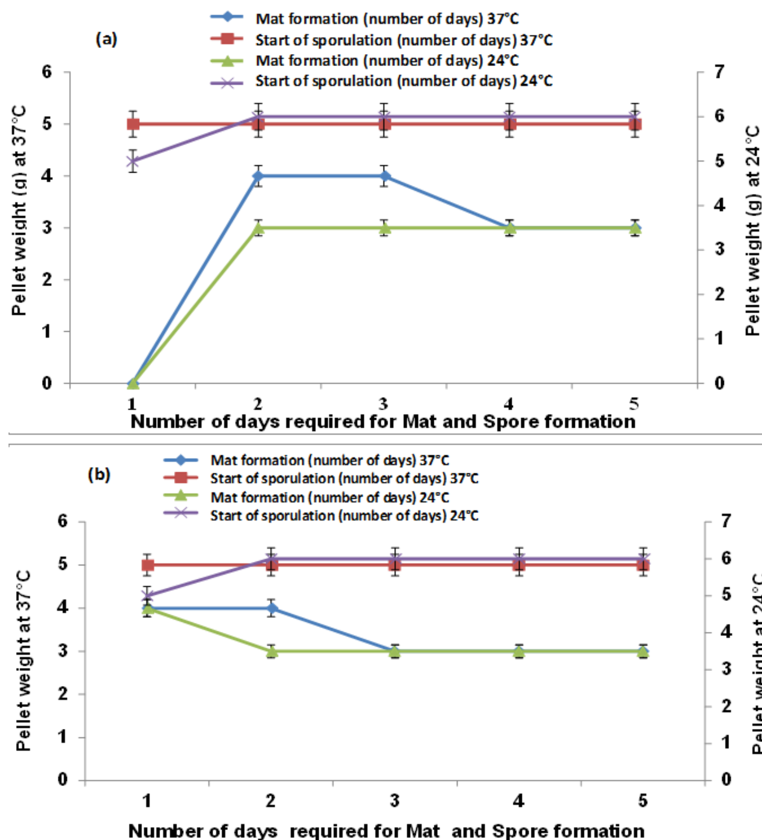

Figure 4. Effect of medium concentration (weight) on mat and spore formation at different temperature: (a) low concentration (b) high concentration.

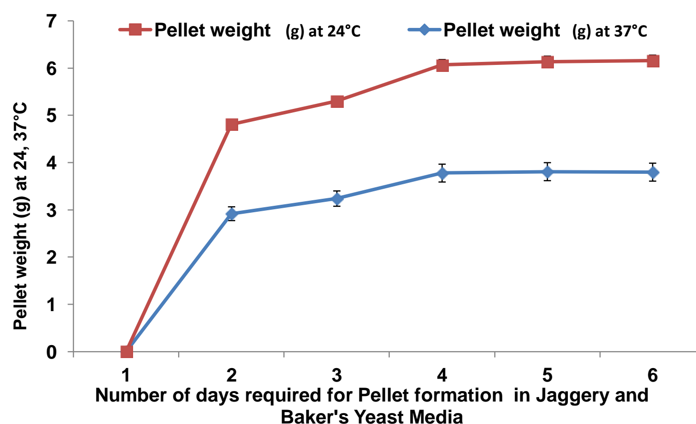

Figure 5. Pellet formation at different temperature of Jaggery and baker's yeast.

with the maximum spore count was selected for submerged fermentation in 25 L fermenter (Figures 5 and 6).

Submerged fermentation in 25 L fermenter

The optimized medium was used for submerged fermentation in a 25 L fermenter. 25 L of medium was prepared based on the composition and poured into the fermenter. Impellers were set properly and fermenter was closed and autoclaved at 121°C and 15 lbs pressure for 20 min. After autoclaving, the fermenter was cooled for two days.

T. viride spore suspension was prepared in spore suspension medium and spore concentration was adjusted to 2.5×10^{6}/ml. The spores were suspended evenly by adding sodium lauryl sulphate and keeping on shaker for 2 h. 100 ml of spore suspension was

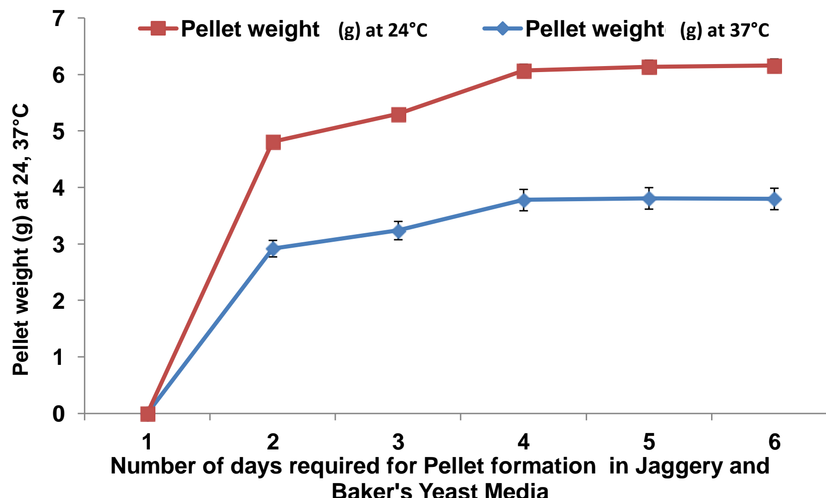

Figure 6. Pellet formation at different temperature of glucose and yeast extract Media.

inoculated into fermenter using vacuum pump in a laminar air flow. Temperature and aeration were continuously monitored for seven days. After every 24 h, samples were collected to observe the spores. After seven days of incubation, 10 ml of sample was collected and centrifuged at 4000 rpm for 20 min, of which the pellet was separated. The supernatant was stained with lactophenol cotton blue and observed under 40x magnification of microscope. If spores were found in supernatant, it was centrifuged again at 5000 rpm for 20 min. Then the weight of the pellet was measured. The pellet was formulated in talc, and colony forming unit (CFU) was counted on potato dextrose agar plates.

Solid state fermentation

For mass production of *T. viride spores* in solid state fermentation, substrates such as sorghum seeds, rice and wheat flour were used.

Solid state fermentation with sorghum seeds

Sorghum seeds were washed for 20 min under running tap water. Then they were washed with distilled water for two times and allowed to dry. The seed coats were ruptured carefully in mixer. Then the seeds were soaked in distilled water for 20 min; excess water was removed. 150 g of wet seeds was weighed and autoclaved at 121°C and 15 lbs pressure for 30 min.

Solid state fermentation with rice and wheat flour

For solid state fermentation with rice and wheat flour, same protocol was followed. 100 g of flour was weighed, 30 ml of water was added and autoclaved at 121°C and 15 lbs pressure for 30 min. After autoclaving, seeds (150 g) or flour (100 g) were spread in each tub. Each tub was inoculated with 5 ml of spore suspension containing 10^6 spores/ml. Then the tubs were covered with plastic sheets and incubated at 27°C for a week. After a week, the seeds were sieved on a mesh to obtain the spores. The weight of the spores formed per 100 g of the seed was measured. 1 g of spore was taken and formulated in talc; and CFU count was calculated on potato dextrose agar plates.

Determination of antagonistic efficacy of *Trichoderma viride*

Antagonistic efficacy of *T. viride* was tested on potato dextrose agar plates. Antagonistic efficacy was observed by plating both the organisms on same Petri plate. Presence of inhibition zone indicates that *T. viride* is antagonistic to given pathogen. The four fungal pathogens such as *Botrytis cinerea, F. oxysporium, M. phasealina, R. solani* and *T. viride* were separately inoculated and labeled as control; on the other plates a line was drawn in the middle. On one side, *T. viride* was inoculated; on the other side, *B. cinerea* was inoculated. Likewise, *F. oxysporium, M. phasealina, and R. solani* were inoculated opposite to *T. viride.* Plates were incubated at 27°C for a week; then the plates were observed for zone of inhibition.

Statistical analysis

Data were analyzed by Descriptive Statistics in MS-EXCELL-2007 Software.

RESULTS AND DISCUSSION

T. viride was cultured on different media for seven days at different temperatures. Mycelial mat formation and sporulation started at different intervals in different media (Figure 4). As the results suggest, at low concentrations of jaggery and glucose, mycelial mat formation was not satisfactory. As their concentration increased, mat formed completely and within less time. At low concentrations of jaggery and glucose, due to low nutrition, sporulation was induced at early stage and hence it was observed on the 5th day. As the medium became nutrient rich, induction of sporulation was delayed. Therefore, it indicates that sporulation can be induced by nutrient starvation. Leo et al. (2010) show that at a concentration of 4 g Kg^{-1} under field conditions, an abiotic stress tolerant *T. viride* was effective against root rot disease and enhanced yield of

Table 1. The intervals at which mycelial mat formation, sporulation occurred.

Medium composition (g/L)		Mat formation (No. of days) 37°C	Start of sporulation (No. of days) 37°C	Mat formation (No. of days) 24°C	Start of sporulation (No. of days) 24°C
Jaggery	**Baker's yeast**				
30	5	4±0.02	5±0.03	4±0.02	5±0.03
40	5	4±0.02	5±0.03	3±0.01	6±0.04
50	5	3±0.01	5±0.03	3±0.01	6±0.04
100	5	3±0.01	5±0.03	3±0.01	6±0.04
150	5	3±0.01	5±0.03	3±0.01	6±0.04
Glucose	**Yeast extract**				
5	5	-	5±0.03	-	5±0.03
10	5	4±0.02	5±0.03	3±0.01	6±0.04
15	5	4±0.02	5±0.03	3±0.01	6±0.04
20	5	3±0.01	5±0.03	3±0.01	6±0.04
25	5	3±0.01	5±0.03	3±0.01	6±0.04

Table 2. Spore formation at different medium composition, at 24 and 37°C temperature.

Medium composition (g/L)		Pellet weight(g) 37°C	Pellet weight g) 24°C
Jaggery	**Baker's yeast**		
30	5	2.92±0.001	1.89±0.00
40	5	3.24±0.03	2.06±0.001
50	5	3.78±0.03	2.29±0.01
100	5	3.81±0.03	2.33±0.01
150	5	3.80±0.03	2.36±0.01
Glucose	**Yeast extract**		
5	5	1.69±0.00	1.14±0.00
10	5	2.31±0.01	1.38±0.00
15	5	2.68±0.01	1.44±0.00
20	5	2.81±0.02	1.66±0.00
25	5	3.04±0.03	1.84±0.00

Vigna mungo when applied as seed dresser. With respect to incubation temperature, sporulation and mycelial vegetative growth were favored by different temperatures. When the cultures were grown at 37°C (Tables 1 and 2), mycelia grew rapidly; but initiation of sporulation was delayed. At low temperatures, mycelia growth was not satisfactory and complete mat formation was delayed; but at this temperature, mycelia were converted into spore within less duration. Therefore, it is suggested that mycelial vegetative growth is favored by room temperature (37°C), and sporulation is favored by low temperature. The pellets were collected upon centrifugation, and the weight of the pellet was measured to compare the growth on different media.

These values indicate that the most efficient growth of *T. viride was* found when jaggery concentration exceeded 50 g/L. After this, there is no significant increase in the pellet weight. It is better to use jaggery than glucose (Figure 5 and 6). Pellet weight was measured at high room temperature instead of low temperature (Figure 5). This might be due to the less vegetative growth at low temperature. As the growth of *T. viride* is found far better in jaggery and baker's yeast medium, at 37°C, these samples were formulated in sterile talc to find out the number of colony forming units in one gram of pellet obtained. The Colony Forming Units count decreased gradually with increased jaggery concentration. This might be due to incomplete sporulation as the jaggery concentration increased. As the jaggery concentration increased, medium became rich in nutrient and sporulation was delayed. So such cultures require more time to be completely converted into spores. In these cultures, vegetative cells are still present at the time of harvest and centrifugation. On the basis of these results, the medium containing 50 g of Jaggery and 5 g of baker's yeast is selected as the best medium for submerged fermentation

Table 3. Colony forming units count at different Jaggery concentration and 0.5% Baker's yeast.

Medium composition (g/L)		No. of CFU/g of pellet
Jaggery	Baker's yeast	
30	5	3.12×10^9
40	5	2.98×10^9
50	5	2.88×10^9
100	5	2.82×10^9
150	5	2.8×10^9

(Table 3). When these samples are formulated, it was concluded that 3×10^{12} spores were present in 10 ml of medium.

In vitro antagonistic tests

The isolate *T. veride* (Tr 8) showed 70, 68.2, 70, 73.3, 69.3 and 70.1% growth inhibition against *R. solani, S. rolfsii, M. phaseolina, A. alternata, F. solani and C. capsici*, respectively (Mishra et al., 2011). The antagonistic activity of *T.viride* against *B. cinerea, F. oxysporium* (Farkhondeh et al., 2013; Shi et al., 2012), *M. phasealina and R. solani* (Rahman et al., 2014) inhibited growth, after the 4th day of dual plate culture. *Trichoderma* started sporulation on day 9th and 10th in *B. cinerea, M. Phasealina* and *Rhizoctonia Solani* respectively. But in *F. Oxysporium* dual plate *Trichoderma* sporulation was inhibited by *F. Oxysporium*. *T. viride* was more effective against *Botrytis Cinerea* compared to other strains (Figure 1). But *F. Oxysporium* was resistant to *Trichoderma* antagonistic activity, hence *Trichoderma* was unable to overgrow and sporulate the strain in dual plate.

Conclusion

Being biotechnologically important, mycoparasitic *Trichoderma* spp. are broadly researched for both field applications as well as basic biology .This is a simple and cost effective medium for dual plate culture. *T. viride* showed antagonistic activity against all the pathogens such as *B. cinerea, F. oxysporium, M. phasealina* and *R. solani;* therefore, *T. viride* can be used as a biopesticide against all the phytopathogens.

Conflict of interests

The authors did not declare any conflict of interest.

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