

## Original Research Article

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## In vitro Evaluation for Antagonistic Potential of Some Bio Control Isolates against Important Foliar Fungal Pathogens of Cowpea

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### ABSTRACT

Cowpea is an important leguminous vegetable crop usually grown during Pre-kharif, Kharif and Rabi season under West Bengal condition. Now days this crop is severely affected due to biotic stress. Seven foliar pathogens namely *Alternaria alternata*, *Colletotrichum capsici*, *Corynespora cassicola*, *Rhizoctonia solani*, *Fusarium ciceri*, *Sclerotium rolfsii* and *Curvularia lunata* were isolated from the diseased cowpea crop in Jaguli Instructional farm of BCKV. From the legume field crop two *Trichoderma* isolates namely *T. harzianum* and *T. viride* were isolated from rhizosphere soil of cowpea. In-vitro efficacy of this *Trichoderma* isolates were measured on Bell's scale and accordingly percent inhibition also calculated after dual plate technique. Maximum and minimum inhibition was obtained in case of *Sclerotium rolfsii* (64.4%) and *Rhizoctonia solani* (42.2%) respectively against *T. harzianum*. *T. viride* also become effective and percent inhibition range from 31.4 – 60.9%. Maximum and minimum inhibition was obtained in case of *Colletotrichum capsici* (60.9%) and *Fusarium ciceri* (31.4%) respectively. *T. harzianum* completely overgrew the pathogen in most of the cases except one or two and antagonistic potential as per Bell's scale was S<sub>1</sub> in most of the cases. In case of *T. viride* it covered 2/3<sup>rd</sup> of the petriplate and restricted the growth of the pathogen with a fine zonation. Antagonistic potential as per Bell's scale was S<sub>2</sub> in most of the cases. Finally these two isolates of *Trichoderma* may be effectively employed for managing *Sclerotium rolfsii*, *Rhizoctonia solani*, *Colletotrichum capsici* and *Fusarium ciceri* under integrated disease management (IDM) programme of cowpea.

#### Keywords

Cowpea, *T. viride*,  
*T. harzianum*,  
Foliar fungal  
pathogens.

#### Article Info

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### Introduction

Cowpea (*Vigna unguiculata* L. Walp) a dicotyledonous plant belonging to the family fabaceae, genus *Vigna* (Cronquist, 1988) is one of the important kharif legume grown in India. It is a warm season crop, grown many areas of the humid tropics and subtropical zones. Cowpea is tolerant to heat and dry conditions, but is intolerant to frost (Davis *et al.*, 2000) and it also has the useful ability to fix atmospheric nitrogen through its root

nodules. It is grown throughout India for its long, green vegetable pods, seeds, and foliage for fodder (Mandal *et al.*, 2009). In India, cowpea is grown on about 0.5 million ha with an average productivity of 600 to 750 kg grains/ha (Ahlawat and Shivakumar, 2005). The major constraints of cowpea cultivation is several cowpea diseases by various pathogens like fungi, bacteria, viruses, nematodes, and parasitic flowering plants which cause

damage to profitable cowpea production in all agro-ecological zones where the crop is cultivated. In recent survey under West Bengal condition, it has been noticed that various foliar fungal diseases are the main problem for the leguminous vegetables. As for example, Ajibade and Amusa (2001) reported that 64% of 74 cowpea lines evaluated in 1999 were found susceptible to brown blotch disease of cowpea which is caused by *Colletotrichum spp.* Moses (2006) reported that *F. solani* cause great loss in cowpea yields and they are considered as future threats to cowpea production. Kumar *et al.*, (1997) recorded serious outbreaks of disease caused by *Colletotrichum spp* during 1989-93 in various mid-hill areas of the Himachal Pradesh and so on. Almost all the recommended cultivars grown in different agro-climatic regions of the state have become susceptible to one or the other race of the pathogen. During survey for present research work, seven foliar fungal pathogens are found to be susceptible for cowpea production. These identified seven pathogens are *Alternaria alternata*, *Colletotrichum capsici*, *Corynespora cassicola*, *Rhizoctonia solani*, *Fusarium ciceri*, *Sclerotium rolfsii* and *Curvularia lunata*. So, to combat this problem management of these diseases is necessary.

Management of disease with fungicides is uneconomical and hazardous to nature (Ahmad *et al.*, 2010). In recent years, large numbers of synthetic fungicides have been banned in the western world because of their undesirable attributes such as high and acute toxicity. Besides this, fungicide causes ground water pollution, killer to some antagonistic pathogens present in soil and the pathogens may become resistance by repeated use of same fungicide. Pathogens become more virulent due to absence of the competition of soil microflora. Considering the deleterious effects of synthetic fungicides, there is an urgent need for alternative agents for the

management of pathogenic microorganisms. One of the most promising means to achieve this goal is by the use of new tools based on bio-control agents (BCAs) for pest and disease control alone or to integrate with reduced doses of chemicals in the control of plant pathogens resulting in minimal impact of the chemicals on the environment (Vinale *et al.*, 2009). Bio-control methods are successful as non-chemical and eco-friendly approach in the sustainable agricultural production.

Fungi belonging to the genus *Trichoderma* and bacteria such as *Pseudomonas* are the most promising bio-control agent against a range of plant pathogens under a variety of environmental conditions (Chen *et al.*, 1997). So keeping these views in mind the objective of the present research work is biological control of cowpea foliar fungal diseases by using the antagonists *Trichoderma harzianum*, *Trichoderma viride*, *Yeast* and *Pseudomonas fluorescens*.

## **Materials and Methods**

### **Isolation of the pathogens**

Diseased leaves having numerous spots collected from the field were cut into 0.5x0.5 cm pieces containing only 1 spots and initially rinsed with sterile distilled water. Then, they were surface sterilized with 0.1% mercuric chloride (HgCl<sub>2</sub>) solution for 45 seconds followed by 5-6 times serial washing with sterilized distilled water.

Surface sterilized diseased parts were then placed at the centre of a Petri plate containing 20 ml of sterilized water agar medium supplemented with antibiotic chloramphenicol @ 50mg/litre. Plates were incubated at 28±1°C temperature for 48-72 hrs. The mycelial growth that radiated from the diseased tissue was picked up and inoculated

on to the fresh PDA slant. Inoculated PDA slants were then incubated at  $28\pm 1^{\circ}\text{C}$  temperature for 7 days. This method was used for all the seven isolated pathogens viz. *Alternaria alternata*, *Colletotrichum capsici*, *Corynespora cassicola*, *Rhizoctonia solani*, *Fusarium ciceri*, *Sclerotium rolfsii* and *Curvularia lunata*.

### Isolation of the antagonists

Soil was collected from pulse field maintained at Jaguli Instructional farm of BCKV. *Trichoderma* was isolated from the rhizosphere soil, using dilution plate method (Harris and Sommers, 1968) on TSM. The collected soil was dried under shade and ground to powder with a mortar and pestle and passed through 2mm mesh sieve.

Ten grams of powdered soil was mixed with 90 ml of sterile distilled water to prepare  $10^{-1}$  dilution. This suspension was used for serial dilutions up to  $10^{-4}$ . One ml of the suspension from  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  were plated separately on 20 ml of solidified TSM in each of the sterilized petriplates. Five plates were inoculated for each dilution from a particular sample.

The suspension was then distributed uniformly on medium surface by horizontal shaking and was incubated at  $28+ 1^{\circ}\text{C}$  for seven days. The green colonies of the antagonists usually appeared at 4<sup>th</sup> or 5<sup>th</sup> day of incubation. Each colony was studied carefully under microscope, using 0.1 % lactophenol- cotton blue stain (0.1g cotton blue mixed in 100ml of standard lactophenol solution) and compared according to the monographs of *Trichoderma* (Rifai, 1969) for identification at genus and species level. Five different strains of *Trichoderma* spp. were isolated. Out of them one identified as *T. harzianum* which was finally selected for the study.

### *In-vitro* evaluation of antagonistic potential of *Trichoderma harzianum* against all the seven isolated pathogens

Antagonistic activities of *Trichoderma* were measured through dual culture technique (Morton and Straube, 1955) against the test pathogens.

In this experiment, 6 mm diameter blocks of the pathogens and *Trichoderma* were inoculated at the same time on the opposite sides of the PDA medium in petriplates (9 cm diameter).

Both the pathogen and *Trichoderma* used were of same age. The plates containing paired cultures were incubated at  $28+ 1^{\circ}\text{C}$  for around 8 days. In each case, a control plate was also maintained. The antagonistic ability of each isolate was measured through modified Bell's scale (Bell *et al.*, 1982) developed as follows:-

$S_1$  = Antagonist completely overgrew the pathogen (100 % overgrowth)

$S_2$  = Antagonist overgrew at least 2/3 growth of the pathogen (75% overgrowth)

$S_3$  = Antagonist colonized on half of the growth of the pathogen (50% overgrowth)

$S_4$  = Antagonist and pathogen locked at the point of contact

$S_5$  = Pathogen starts overgrowing the antagonist

### Antagonistic potential of *Pseudomonas fluorescens*

Sterile PDA medium was poured in sterilized petriplates. After solidification of the medium, a loopful of bacterial culture from 24-48 hours old bacterial slants was taken and

streak was drawn at one side of petriplate. Fungal plugs were placed on opposite side of the bacterial streak. Incubation was done in BOD incubator at 30+ 2<sup>0</sup> C temp for 3- 4 days.

Observations were made by measuring the length of fungal growth, bacterial growth and zone of inhibition by scale (mm). In each case necessary replications and necessary control plates were also maintained.

### **Antagonistic potential of the yeast isolate**

Antagonistic potential of the yeast isolate was tested through dual culture technique against the test pathogens.

For this experiment, 6 mm diameter block of the pathogen from pre grown plates were inoculated on yeast extract potato dextrose agar medium at a distance of 2 cm from the periphery of the sterile plate (9 cm). On the other side of the plate a streak of yeast isolate was made at the same distance as that of the pathogen.

It should be noted that both the yeast and the antagonist were of the same age when inoculated. A similar plate but without the yeast isolate was also set up as a control for the experiment. The plates containing the paired cultures were then incubated at 28+ 1<sup>0</sup> C for about 6 days.

Inhibition percentage of pathogens were calculated based on the following the formula as suggested by Sundar *et al.*, (1995):

$$\% \text{ Inhibition} = \frac{X-Y}{X} \times 100$$

Where, X is the mycelial growth of the pathogen in the absence of antagonist and Y is the mycelial growth of the pathogen in the presence of the antagonist.

## **Results and Discussion**

### ***In-vitro* antagonistic potential of *Trichoderma harzianum***

The *in-vitro* antagonistic potential of *Trichoderma harzianum* and *T. viride* were evaluated against seven fungal pathogens viz *Alternaria alternata*, *Curvularia lunata*, *Corynespora cassicola*, *Fusarium ciceri*, *Sclerotium rolfsii*, *Colletotrichum capsici* and *Rhizoctonia solani* by dual plate method. The antagonistic potential was rated on Bell's scale. Percentage inhibition was also calculated and the results have been presented in the following table-1.

From the table-1, it is revealed that *Trichoderma harzianum* possess a significant antagonistic property against these seven pathogens. Percentage inhibition ranged from 42.2- 64.4%, indicating that it is effective in controlling these pathogens. Maximum and minimum inhibition was obtained in case of *Sclerotium rolfsii* (64.4%) and *Rhizoctonia solani* (42.2%) respectively. Except one or two, in most of the cases the growth of the pathogen was totally restricted and *T. harzianum* completely overgrew the pathogen. Antagonistic potential as per Bell's scale was S<sub>1</sub> in most of the cases (Plate-1). *Trichoderma viride* is also effective in controlling the pathogens but its aggressiveness is less than *T. harzianum*. Percentage inhibition ranged from 31.4- 60.9 %. Maximum and minimum inhibition was obtained in case of *C. capsici* (60.9%) and *F. ciceri* (31.4%) respectively. In most of the cases, *T. viride* covered 2/3<sup>rd</sup> of the petriplates and restricted the growth of pathogen with a fine zonation. Antagonistic potential as per Bell's scale was S<sub>2</sub> in most of the cases (Plate-2).

The isolated fungal pathogens were tested for their growth and behaviour in presence of

antagonistic bacteria *Pseudomonas fluorescens* and antagonistic fungi yeasts in dual culture plates. Percentage inhibition was also calculated and the results have been presented in the following table-2.

From the results of this table-2, it is clear that *Pseudomonas fluorescens* is not highly effective against these pathogens. Efficiency is moderate to low. Percentage inhibition over control varied from 11.1- 36.3 %.

Maximum inhibition was obtained in case of *Colletotrichum capsici* (36.3%), while least inhibition was obtained in case of *Rhizoctonia solani* (11.1%) revealing the fact that they are resistant to this particular strain of bacterial antagonist. Whereas in case of Yeast, it is revealed that among these pathogens, some were significantly inhibited by the yeast isolate.

Inhibition percentage varied from 13.3 - 57.0%, maximum and minimum being obtained in case of *Sclerotium rolfisii* (57%) and *Fusarium ciceri* (13.3%) respectively. Inhibition zone was recorded in case of *Corynespora cassicola* only (0.6 cm). In rest of the cases, where inhibition was recorded, growth of the pathogen was restricted immediately after touching the streak.

Four biocontrol agents were evaluated for their efficacy in controlling the growth and progress of the seven isolated plant pathogens of cowpea under *in vitro* condition. Percentage inhibition was calculated to determine the efficiency of the biocontrol agents.

Antagonism of *T. harzianum* against *S. rolfisii* was reported by Mukherjee and Tripathi (2000). Manibhusan Rao *et al.*, (1989) reported antagonism against *R. solani* & *S. rolfisii* possibly by the mechanism of antibiosis and mycoparasitism. Parab *et al.*,

(2009) reported antagonistic activity of *Trichoderma harzianum* against *Fusarium spp.*, causing damping off and root rot disease under *in vitro* conditions.

Rahman *et al.*, (2013) reported antagonistic activities of different *Trichoderma* strains under *in vitro* condition against *Colletotrichum capsici*, a causal agent of anthracnose fruit rot of chilli.

Dual culture test showed that *Trichoderma* strains effectively inhibited mycelia growth of the pathogen. *T. harzianum* IMI-392433 showed the highest inhibition (81.96 %) and mycelial overgrowth (78.98%).

Pandey (2010) also studied comparative antagonistic properties of *T. harzianum* and *T. viride* against *Alternaria alternata* under *in vitro* condition. The experiment was allowed to run for 10 days. Results indicated that *T. harzianum* reduced the growth of *A. alternata* by 67.07% and was found to be more effective in controlling the growth of test pathogen. *T. viride*, causing a reduction of 66.67% was also found to be a suitable biocontrol agent.

So, these findings obtained from the present experiment of dual culture assay stands conformation with the previous results obtained by Mukherjee and Tripathi (2000), Manibhusan Rao *et al.*, (1989), Parab *et al.*, (2009), Rahman *et al.*, (2013) etc.

*Pseudomonas fluorescens* was moderately effective against some of the pathogens such as *C. capsici* (36.3%), *C. cassicola* (31.3%) etc; while its efficacy was poor against *F. ciceri* (28.9%), *S. rolfisii* (28.9%), *R. Solani* (11.1%) etc. Inhibition zone was observed in case of *A. alternata* (0.4cm), and *C. capsici* (0.2 cm). Probably it was the inhibitory effect due to production of secondary metabolites by the bacteria.

**Table.1** Antagonistic potential of *Trichoderma harzianum* determined by dual culture method

Sl. No.	Pathogen	Point of contact (DAI)	<i>Trichoderma harzianum</i>				<i>Trichoderma viride</i>			
			Distance covered (cm) at final day of observation by		Antagonistic potential on modified Bell's scale (at final day of observation)	Percentage inhibition (%)	Distance covered (cm) at final day of observation by		Antagonistic potential on modified Bell's scale (at final day of observation)	Percentage inhibition (%)
			Pathogen	Antagonist			Pathogen	Antagonist		
1.	<i>Alternaria alternata</i>	2 days	1.2	5.5	S <sub>1</sub>	52.0 (5.05)	1.2	5.5	S <sub>1</sub>	52.0 (5.05)
2.	<i>Curvularia lunata</i>	2 days	1.5	5.5	S <sub>1</sub>	48.3 (4.95)	1.5	5.5	S <sub>1</sub>	48.3 (4.95)
3.	<i>Corynespora cassicola</i>	2 days	1.2	5.6	S <sub>1</sub>	44.0 (4.84)	1.2	5.6	S <sub>1</sub>	44.0 (4.84)
4.	<i>Fusarium ciceri</i>	2 days	1.5	5.5	S <sub>1</sub>	55.4 (5.13)	1.5	5.5	S <sub>1</sub>	55.4 (5.13)
5.	<i>Sclerotium rolfsii</i>	2 days	1.6	5.5	S <sub>1</sub>	64.4 (5.36)	1.6	5.5	S <sub>1</sub>	64.4 (5.36)
6.	<i>Rhizoctonia solani</i>	1 day	2.6	5.4	S <sub>1</sub>	42.2 (4.80)	2.6	5.4	S <sub>1</sub>	42.2 (4.80)
7.	<i>Colletotrichum capsici</i>	1 day	0.9	5.5	S <sub>1</sub>	59.5 (5.24)	0.9	5.5	S <sub>1</sub>	59.5 (5.24)
<b>SEm ±</b>						0.07				0.07
<b>CD (p=0.05)</b>						0.21				0.21

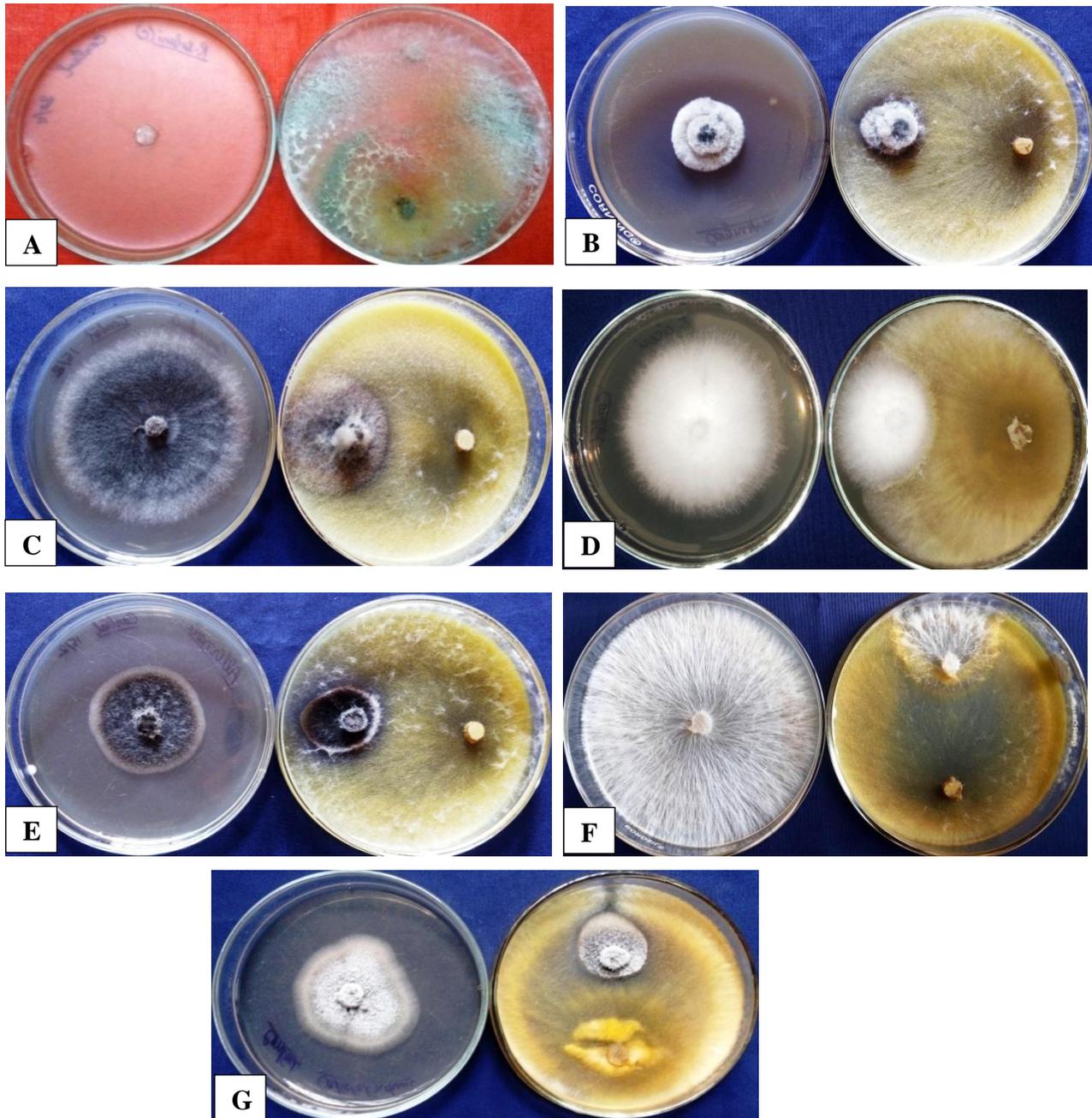
\*Figures in parenthesis are angular transformed value

**Table.2** Antagonistic potential of *Pseudomonas fluorescens* & Yeast determined by dual culture method

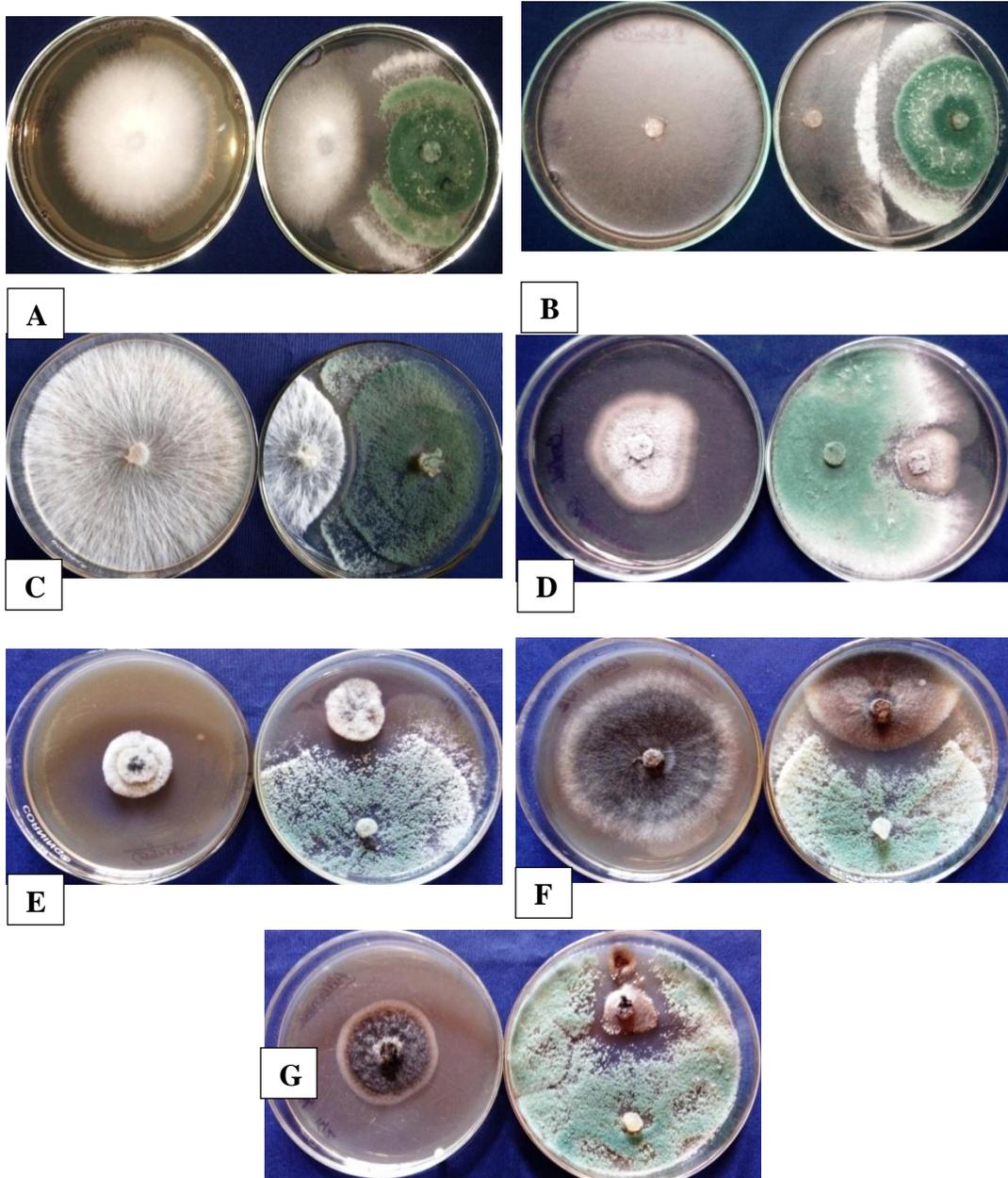
Sl. No.	Pathogen	Inhibition zone (cm)	Distance covered (cm) by pathogen in		Percentage inhibition	Inhibition zone (cm)	Distance covered (cm) by pathogen in		Percentage inhibition
			Dual culture	Control			Dual culture	Control	
1	<i>Alternaria alternata</i>	0.4 cm	2.7	3.5	23 (4.26)	–	2.4	2.9	16.2 (4.01)
2	<i>Curvularia lunata</i>	–	3.5	4.3	18.6 (4.1)	–	2.2	4	45.0 (4.87)
3	<i>Corynespora cassicola</i>	–	2	3	31.3 (4.51)	0.6	1.8	3	40.0 (4.74)
4	<i>Fusarium ciceri</i>	–	3.2	4.5	28.9 (4.44)	–	2.6	3	13.3 (3.88)
5	<i>Sclerotium rolfsii</i>	–	3.2	4.5	28.9 (4.44)	–	1.8	4	57.0 (5.17)
6	<i>Rhizoctonia solani</i>	–	4	4.5	11.1 (3.77)	–	3.3	4.5	26.7 (4.37)
7	<i>Colletotrichum capsici</i>	0.2 cm	2.2	3.5	36.3 (4.64)	–	2.7	4.2	35.7 (4.63)
<b>SEm ±</b>					0.07				0.08
<b>CD (p=0.05)</b>					0.21				0.25

\*Figures in parenthesis are angular transformed values

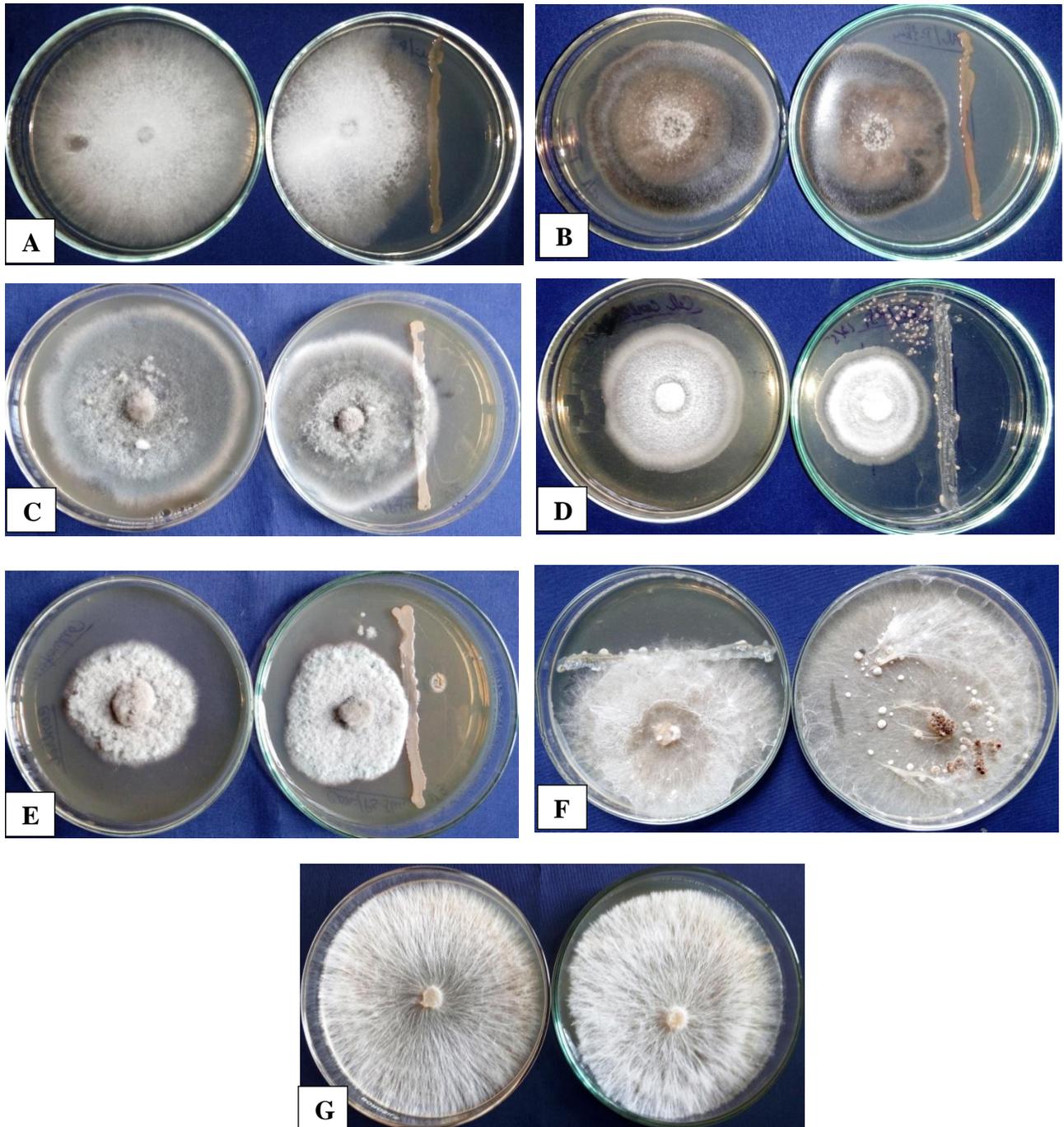
**Plate.1** Antagonistic response of *Trichoderma harzianum* against *R. solani* (A), *C. cassicola* (B), *C. lunata* (C), *F. ciceri* (D), *A. alternata* (E), *S. rolfii* (F) and *C. capsici* (G)



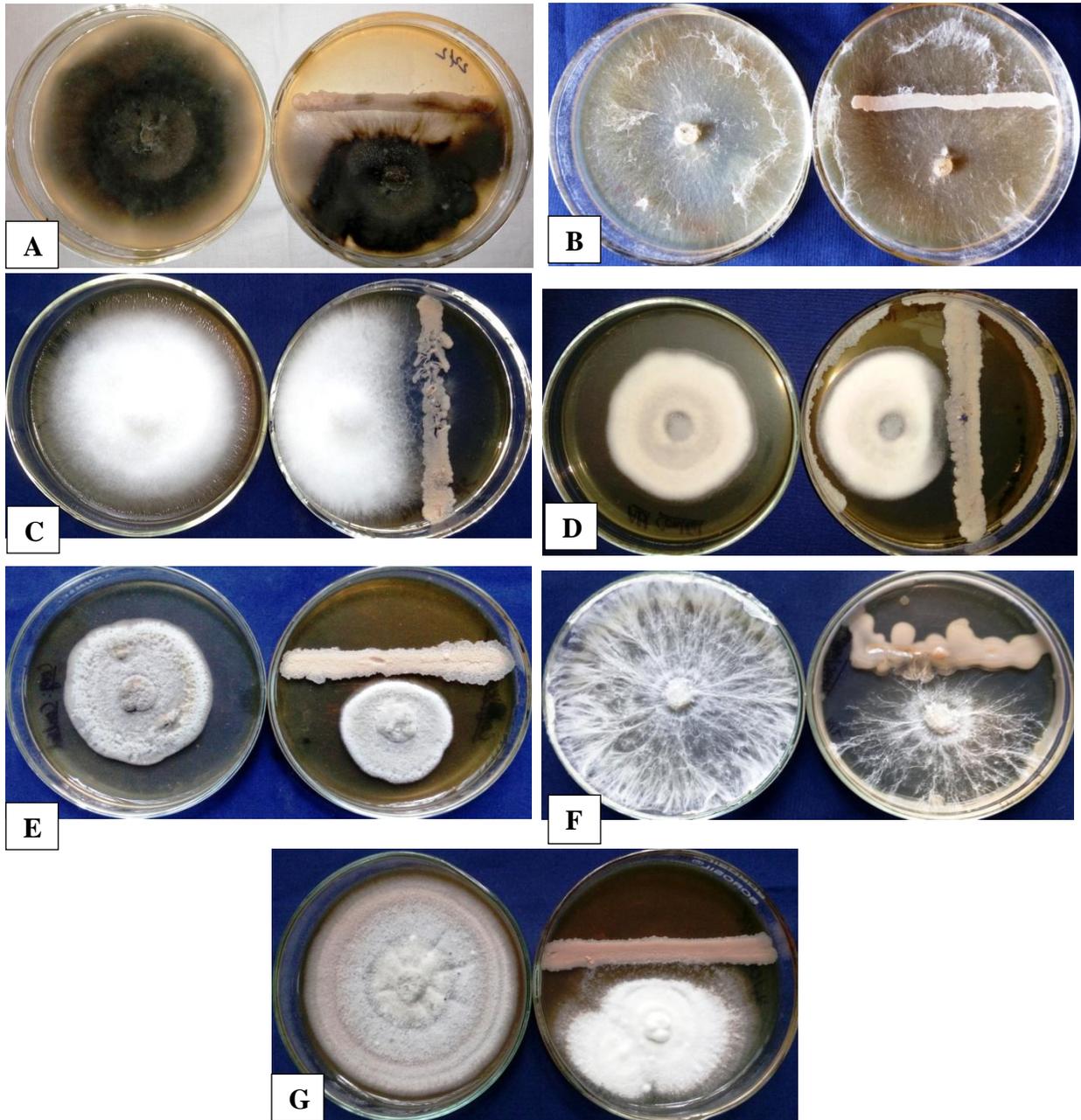
**Plate.2** Antagonistic response of *Trichoderma viride* against *F. ciceri* (A), *R. solani* (B), *S. rolfsii* (C), *C. capsici* (D), *C. lunata* (E), *C. cassicola*(F) and *A. alternata*(G)



**Plate.3** Antagonistic response of *Pseudomonas fluorescens* against *F. ciceri* (A), *A. alternata* (B), *C. lunata* (C), *C. capsici* (D), *C. cassicola* (E), *R. solani* (F) and *S. rolfii* (G)



**Plate.4** Antagonistic response of Yeast against *C. lunata* (A), *R. solani* (B), *F. ciceri* (C), *A. alternata* (D), *C. cassicola* (E), *S. rolfsii* (F) and *C. capsici* (G)



Mosa *et al.*, (1997) reported antagonism of *P. fluorescens* against *R. solani*. Heber *et al.*, (1991) isolated a number of antagonistic bacteria including *P. fluorescens*, *P. putida* etc. from roots and corms of sunflower (*Helianthus annuus* L.) on nutrient agar and

King's B medium. The isolates inhibited the *in vitro* growth of *Alternaria helianthi*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Macrophomina phaseolina* causing leaf spot, wilt and root rot diseases respectively.

Linu and Jisha (2013) screened out effective isolates of *Pseudomonas* sp. against *Colletotrichum capsici*. Two isolates of *Pseudomonas* sp were tested against *Colletotrichum capsici* on PDA by dual culture technique. Isolate P<sub>1</sub> showed 78% of reduction whereas isolate P<sub>6</sub> showed 89% of the radial growth of the test pathogen *Colletotrichum capsici*.

Results obtained from the present experiment reveals that *Pseudomonas fluorescens* is poorly effective in controlling *Fusarium ciceri* and *Sclerotium rolfsii*. Although the result is different, findings of some previous workers justify it. A study was conducted to isolate and select the highest potential activities of Pseudomonads group of bacteria from 7 provinces in northeastern region of Thailand against *Sclerotium rolfsii*. Thirteen of 329 isolates were screened as antagonistic bacteria to inhibit *S. rolfsii* by dual culture assay. But high percentages of inhibition were found only in three isolates of UD1EBa-2, KK1EBa-3 and KK11EBa-3 with 51.25%, 56.25% and 60.00%, respectively (Natedara *et al.*, 2014). Rest 326 isolates of Pseudomonads did not respond to *S. rolfsii*.

Seventy-four strains of fluorescent *Pseudomonas* were tested for their ability to reduce the incidence of fusarium wilt of flax when applied either alone or in association with one preselected non-pathogenic strain of *Fusarium oxysporum* (Fo47). Four classes were established, based on the effect of bacteria on disease severity, on their own or in association with Fo47. Most of the strains did not modify the percentage of wilted plants. However 10.8% of them, although having no effect on their own, significantly improved the control attributable to Fo47 (Lemanceau and Alabouvette, 1991).

So, the results obtained from the dual culture experiment with *Pseudomonas* stands partly

conformation with the previous finding of Natedara *et al.*, (2014), Lemanceau & Alabouvette (1991) etc.

The yeast isolate was moderately effective against some of the pathogens, such as *S. rolfsii* (57%), *C. lunata* (45%), *C. cassicola* (40%), *C. capsici* (35.7%) etc. In case of *C. cassicola* remarkably the pathogenic growth was restricted and inhibition zone was also detected. At the same time for rest of the pathogens except 2-3, the mycelial growth was stopped immediately after touching the streak. At the vicinity of the streak, the mycelial density was gradually thinner. The colony colour of *Colletotrichum capsici* was significantly changed during dual culture with yeast in respect to the control.

El-Tarabily (2004) reported that the application of three rhizosphere yeasts, namely *Candida valida*, *R. glutinis* and *Trichosporon asahii* obtained from sugar beet rhizosphere, individually or in combination, significantly reduced post emergence damping-off of seedlings and crown and root rots of mature sugar beet caused by *R. solani* AG-2-2 under glasshouse.

Antagonistic yeasts to *Colletotrichum capsici* were isolated from rhizosphere soil, fruits and leaves of chili plants. The majority of yeast isolates (60 isolates; 31.09%) were isolated from rhizosphere soil. In dual culture tests, five of the isolates screened (HS6, SS11, SLD5, SS10 and PLN13) were found to inhibit *C. capsici* growth with biocontrol efficacies as 43.12%, 42.50%, 41.87, 41.25 and 40.62%, respectively (Punika *et al.*, 2013).

Except *S. rolfsii*, *C. lunata*, *C. cassicola* and *C. capsici* for rest of the pathogens the yeast isolate did not respond effectively. Being a foliar antagonist, yeast gave significant results against *Sclerotium rolfsii*.

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