In-Vitro Studies of Different Culture Media and Biocontrol Agents on Growth and Sporulation of *Alternaria Alternata* (Fr.) Keissler an Incitant of Broad bean (*Vicia Faba L.*) Leaf Blight Disease

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Abstract— *Studies on different culture media and biocontrol agents on growth and sporulation of Alternaria alternata (fr.) keissler causal organism of Broad bean (Vicia faba L.) leaf blight disease was conducted in vitro. Six culture media was tested for growth and sporulation of A. Alternata. It was found in that in liquid broth media, Potato dextrose broth (607 mg, dry weight of mycelium) and in solid media, maximum linear growth recorded in Potato dextrose agar (90 mm) with maximum number of spores 6.2 x 103 spores/ml. The antagonistic studies of 5 fungal biocontrol agents in dual culture technique found that all biocontrol agents statistically produced a significant antagonistic effect on linear growth and sporulation of A. Alternata. Maximum linear growth inhibition of Alternaria alternata was found in Trichoderma harzianum (17.5mm) with 89.6% inhibition and at par with Trichoderma viride (19.5mm) with 84.4% inhibition followed by T. hamatum (22.3 mm) with 74.1% inhibition and Gliocladium virens (21.0 mm) with 70.6% and minimum significant inhibition effect in T. koningii (22.8 mm) with per cent inhibition of 67.2 over the check. Highest spore inhibition of was found in T. harzianum (89.6%) and T. viride (84.4%) followed by T. hamatum (74.1%) and G. virens (70.6%) while minimum inhibition was found in T. koningii (67.25). It was further observed that T. harzianum could overgrown the growth of pathogenic fungus within 2 days and for T. viride within 3 days where as T. hamatum and G. virens took 5 days.*

Keywords— Alternaria alternata, blight disease, broth media, solid media, efficacy, linear growth, sporulation, mycelium, biocontrol agents.

I. INTRODUCTION

Broad bean (*Vicia faba L.*) is an important leguminous crop cultivated in different parts of mild sub-tropical and temperate regions of the world. In Manipur and north eastern state of India it is mostly grown as vegetable crop, where tender green pods were used as culinary purposes and mature seeds used as dals, snacks preparation and seed purposes. This important winter vegetable crop was often attacked by various diseases and out of eight major diseases recorded, blight disease caused by *Alternaria alternata* fr keissler is most serious (Gupta, 1985). The disease first appeared as small circular, brownish colour at the margin or tip of the leaf and progressed towards the midrib or the spots coalesce together to form into an elongated, irregular necrotic dark brown lesions. The affected young plants turn pale and leaf get drooping and wilted and die later. At present there is little information on physiology of the fungus associated with the blight disease and on management practices against this disease. Therefore the present investigation was taken up to study the effect of different types of culture media on the growth and sporuration of the pathogen and also to study the antagonistic effect of various bioagents against this pathogenic fungus.(Goswami and Mittal, 2002; Mittal et al., 2005 and Goswami and Mittal, 2004)

II. MATERIALS AND METHODS

The research works was carried out in the laboratory of Department of Plant Pathology, Krishi Vigyan Kendra-Senapati district, Manipur in the year 2018-19. The details of experimental approaches and procedures adopted during the course of investigations are described as follows:

2.1 Collection of diseased specimen

Broad bean plant showing typical leaf blight symptoms were collected from different location of Senapati district of Manipur during rabi season (2018-19) and brought in the laboratory of KVK-Senapati, Hengbung, Manipur for isolation purpose and use further for study.

2.2 Isolation and purification of fungus associated with Broad bean leaf blight

The infected leaves were surface sterilized with 1% sodium hypochlorite solution for about 1 minutes and then wash by 2-3 change of sterile water and with the help of sterilized blade cut into small bits of 2-3 mm including half live and half diseased death tissue and then by using sterilized inoculating needle, 2-3 bits is inoculated aseptically in a Petri plates containing sterilized fresh potato dextrose agar (PDA). The inoculated plates were incubated at 25 ± 1^0C for 2-3 days. Purification of the isolated fungal pathogen was done by single hyphal tip culture method. The mycelium tip from the Petri plates was aseptically transferred it into PDA slants and stored at room temperature and periodically subculturing done for further investigation work.

2.3 Studies on effect of different culture media on the growth and sporulation of *Alternaria alternata* incitant of blight disease of broad bean

Six different culture media namely, Corn meal agar, Czapek's dox agar, Elliot's agar, Richard's agar, Potato dextrose agar and Host extract agar were tested on growth and sporulation of fungus both in solid and liquid broth form of the media.

Different culture media, their composition and preparation methods:

2.3.1 Corn meal agar

Ingredients composition	Quantity
(i). Corn meal	50.00 g
(ii). Dextrose	20.00 g
(iii). Agar	15.00 g
(iv). Distilled water	1000.00 ml

Preparation: 1000 ml of distilled water was heat to boiling in a pan, add 50 g of corn meal powder and dissolve 20 g dextrose and add 15 g agar, bit by bit with constant stirring by glass rod and make up the volume upto correct volume (1000ml) by adding distilled water and then dispense in conical flasks and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. For broth culture media agar is devoid.

2.3.2 Czapek's dox agar

Ingredients composition	Quantity
(i). Sodium nitrate	2.00 g
(ii). Magnesium sulphate	0.50 g
(iii). Potassium chloride	0.50 g
(iv). Ferrous sulphate	0.05 g
(v). Agar	15.00 g
(vi). Dextrose	20.00 g
(vii). Water	1000.00 ml

Preparation: Suspend the whole chemical ingredients in 1000 ml distilled water. Heat to boiling to dissolve the medium completely makes up the deficit volume by adding distilled water. Dispense the media in conical flasks, plugged with non absorbent cotton and sterilized by autoclaving at 15 lbs pressure for 15 minutes (121^0C). For broth culture media agar is devoid.

2.3.3 Elliot's agar

Ingredients composition	Quantity
(i). Sodium carbonate	1.00 g
(ii). $MgSO_4 7H_2O$	0.50 g
(iii). KH_2PO_4	1.36 g
(iv). Asperagine	1.00 g
(v). Dextrose	5.00 g
(vi). Agar	15.00 g
(vii). Distilled Water	1000.00 ml

Preparation: Suspend the whole chemical ingredients in 1000 ml distilled water. Heat to boiling to dissolve the medium completely makes up the deficit volume (upto 1000ml) by adding distilled water. Dispense the media in conical flasks, plugged with non absorbent cotton and sterilized by autoclaving at 15 lbs pressure for 15 minutes (121^0C). For broth culture media agar is devoid.

2.3.4 Richard's agar

Ingredients composition	Quantity
(i). Potassium nitrate	10.00 g
(ii). Monopotassium dehydrogen phosphate	5.00 g
(iii). Magnessium sulphate	2.50 g
(iv). Ferric chloride	0.02 g
(v). Sucrose	50.00 g
(vi). Agar	15.00 g
(vii). Distilled	1000.00 ml

Preparation: Suspend the whole chemical ingredients in 1000 ml distilled water. Heat to boiling to dissolve the medium completely makes up correct volume (1000ml) by adding distilled water. Dispense the media in conical flasks plugged with non absorbent cotton and sterilized by autoclaving at 15 lbs pressure for 15 minutes (121^0C). For broth culture media agar is devoid.

2.3.5 Potato dextrose agar

Ingredients composition	Quantity
(i). Potato	200.00 g
(ii). Dextrose	20.00 g
(iii). Agar	15.00 g
(iv). Distilled	1000.00 ml

Preparation: 200 g sliced potato were boiled in 500 ml of distilled water and half cook or till soften, collect the decoction by straining in a cheese cloth and add 20 gm of dextrose. In another beaker 500 ml of water is heated and 20 gm of agar is added bit by bit till it completely gets dissolved. Now agar solution and potato extracts dextrose solution were mixed together and the volume is brought upto correct volume (1000 ml) by adding more distilled water. Dispense the media in conical flasks and plugged with non absorbent cotton and sterilized by Autoclaving at 15 lbs pressure (121^0C) for 15 minutes. For potato dextrose broth media agar is devoid.

2.3.6 Host extract agar

Ingredients composition	Quantity
(i). Broad bean leaves	100.00 g
(ii). Dextrose	20.00 g
(iii). Agar	15.00 g
(iv). Distilled	1000.00 ml

Preparation: 100 gm fresh broad bean leaves were boiled in 500 ml of distilled water till soften. Collect the decoction by straining in a cheese cloth and to it add 20 g dextrose. In another beaker 500 ml of water is heated and 15 gm of agar is added bit by bit till it completely gets dissolved. Now agar solution and broad bean leaf extracts dextrose solution were mixed together and the volume is brought upto correct volume (1000 ml) by adding more distilled water, dispensed the media in conical flasks and plugged with non absorbent cotton and sterilized by Autoclaving at 15 lbs pressure (121^0C) for 15 minutes. For host leaf extract broth media agar is devoid.

1) Liquid Broth media test

50 ml each of the broth media to be tested was dispensed in 100 ml capacity conical flasks and autoclaved at 15 lb pressure (121^0C) for 15 minutes. Each treatment was replicated into four times. Mycelial discs of 3 days old test fungus mycelium was cut by using sterilize 5 mm cork borer and with the help of sterilized inoculating needle a mycelial disc was aseptically inoculate into those conical flasks containing the test media. The inoculated conical flasks was plugged again and incubated at 25 ± 1^0C for 10 days. The mycelial mats was harvested through pre-weighed Qualigens No. 651 A (11.0 cm diameter) filter paper and dried at 60^0C in a hot air oven for 72 hours and then cooled in desiccator for 24 hours and re-weighed in an electronic balance. The mycelial dry weight for each treatment was recorded and actual growth was calculated.

2) Solid media test:

50 ml each of different solid medium to be tested was autoclaved and then dispensed 20 ml of it in the sterilized Petri plates of 9 cm diameter. Each treatment was replicated into four. Mycelial discs were cut from 3 days old actively growing colony of fungal culture by using sterilized 5 mm diameter cork borer. One mycelial disc was inoculated at the centre of each test plates containing sterilized media and then incubated at 25±1^0C. The radial growth was recorded in 24 hours intervals till the fungus covered the whole plate. The differences in the growth rate were calculated for all the treatments by using the formula Vincent (1927).

$$I = \frac{(C-T)}{C} \times 100 \quad (1)$$

Where, I=percent inhibition

C=growth in control

T=growth in treatment

For estimation of fungal spores the method described by Devi (1991) was followed. For this, 1 sq.cm mycelial block was cut from each plate and the mycelial mats were removed from the medium. It was then transferred into a test tube containing 5ml distilled water and shaken it for 5 minutes. The spore density/ml of this homogemous spores solution was estimated by using a Haemocytometer.

2.4 Effect of biocontrol agents on the growth and sporulation of *Alternaria alternate*

The efficacy of 5 biocontrol agents namely *Trichoderma viride, Trichoderma harzianum, Trichoderma hamatum, Trichoderma koningii* and *Gliocladium virens* was tested on growth and sporulation of *Alternaria alternata* by dual culture technique. The biocontrol agents were collected from ITCC, IARI, New Delhi vide Dept. of Plant Pathology, College of Agriculture, CAU, Imphal.

Biocontrol agents and pathogen was multiplied in sterilized potato dextrose agar (PDA) plates separately. Mycelial discs were cut with a sterilized cork borer of 5 mm diameter from actively growing 3 days old fungal colony. Each mycelial discs of both pathogen and bio-agents was placed in opposite pole 3cm apart in fresh sterilized potato dextrose agar (PDA) plates. The control plate was inoculated only with pathogenic fungal mycelial disc. Each treatment was replicated into four times. The seeded plates were then incubated at 25 ± 1^0C. The observation was taken at 24 hours interval till the pathogen and biocontrol agent comes in contact with each other. The per cent inhibition on the fungal radial growth was calculated.

For estimation of spores, 1 sq. cm. mycelial blocks were cut from each plate at the point of contact between pathogen and biocontrol agent. The mycelial mat was removed from the medium and then transferred it to different test tubes containing 5 ml sterile distilled water and shaken for 5 minutes, the spore density per ml from each treatment was estimated by using haemocytometer.

III. RESULT AND DISCUSSION

TABLE 1
EFFICIENCY OF DIFFERENT TYPES OF BROTH CULTURE MEDIA ON MYCELIUM GROWTH OF *ALTERNARIA ALTERNATA*

S. No.	Types of culture media	Growth (mg)*
1.	Corn meal	195
2.	Czapek's dox	230
3.	Elliot's broth	255
4.	Richard's medium	125
5	Potato dextrose	607
6.	Host extract	465
	C.D. $_{5\%}$	4.2

**** Mean of four replication***

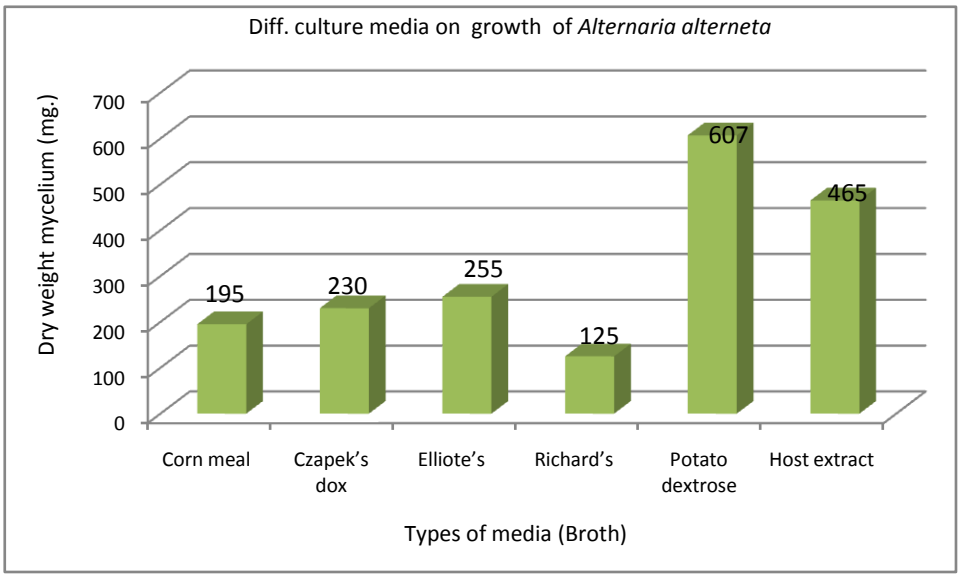

FIGURE 1: Performance of different culture media on growth of *Alternaria alternata*

The data presented in the above Table 1, fig.1, is the results of different types of broth culture media test on the growth of fungi. Among the media statistically significant and maximum growth of *Alternaria alternata* (dry wt. mycelium) was found in Potato dextrose (607mg) followed by Host extract (467mg) and Elliote's media (255mg) while minimum growth was found in corn meal (195) and Richard's medium (125). Our present investigation was in corroborate with that of Lipps and Herr (1980) who reported that maximum growth of *Alternaria helianthi* was observed on potato dextrose broth and V-8 juice, semi synthethic medium. Similarly, Singh (1994) also reported that *Alternaria alternata*, an incitant of stalk rot of sunflower could grow well on Potato dextrose broth among various media under trial.

TABLE 2
EFFECT OF SOLID CULTURE MEDIA ON GROWTH AND SPORULATION OF *ALTERNARIA ALTERNATA*

S. No.	Media	Growth (mm)*	No. of spores/ml*
1.	Corn meal agar	77	2.6×10^3
2.	Czapek's dox agar	78	2.8×10^3
3.	Elliot's agar	81	2.9×10^3
4.	Richard's agar	62	1.9×10^3
5.	Potato dextrose agar	90	6.2×10^3
6.	Host extract agar	83	3.5×10^3
	C.D. $_{5\%}$	0.6	-

**** Mean of four replication***

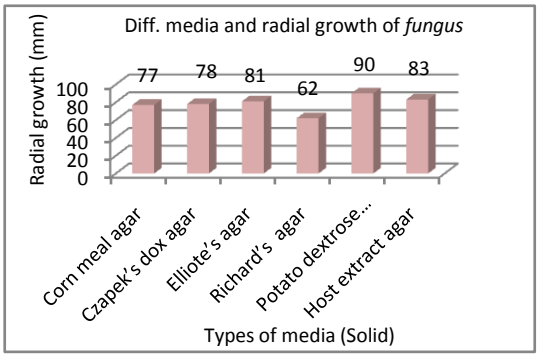

FIGURE 2: Different culture media and its effect on radial growth of *Alternaria alternata*

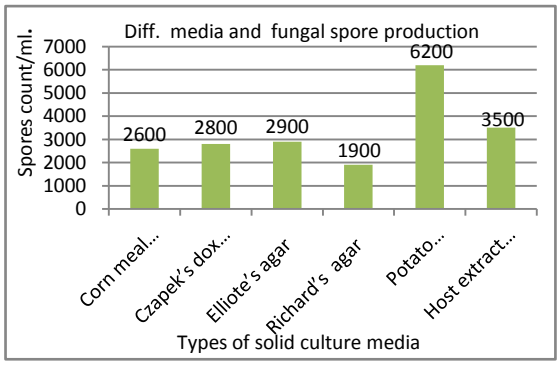

FIGURE 3: Different culture media and its effect on sporulation of *Alternaria alternata*

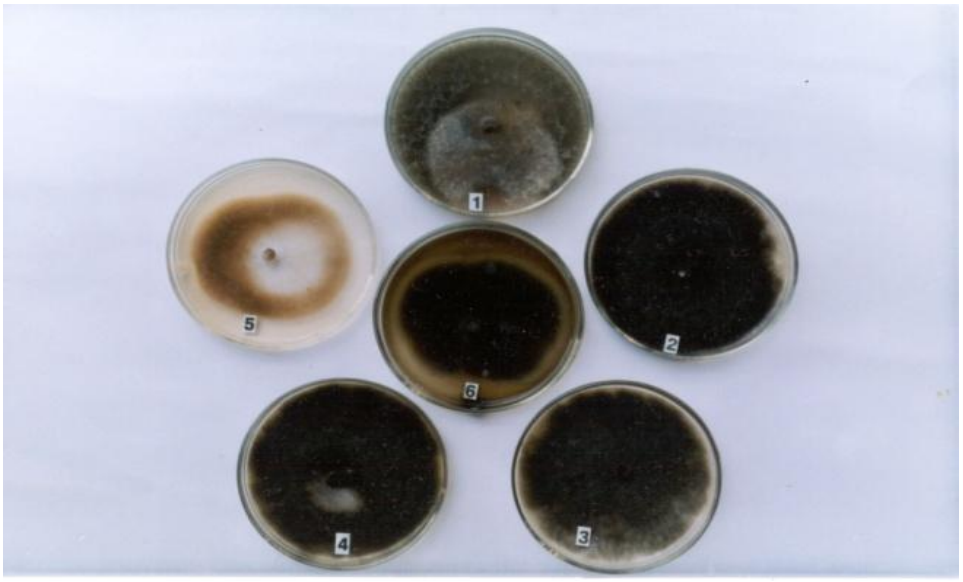

PHOTO 1: Different culture media on linear growth of *A. alternate*

(1).Elliot's agar (2).Host extracts agar (3). Potato dextrose agar (4).Czapek's dox agar (5).Richard's agar, (6).Corn meal agar

The data presented in the above Table 2. Fig. 2 & 3 were the results of different culture media and its effect on radial growth of fungus and production of spores. The results found among the tested media that maximum linear growth was found in Potato dextrose agar (90 mm) followed by Host extract agar (83 mm), and Elliot's agar (81 mm) whereas mimimum radial growth was recorded in Richard's agar (62 mm) and corn meal agar (77 mm). Our finding was in agreement with Allen et al. (1993) who reported best colony growth of *Alternaria helianthi* on Potato dextrose agar (PDA), whereas Pandey and Vishwakarma (1998) reported that maximum radial growth of *Alternaria alternata* was recorded in Capsicum dextrose agar and Radish dextrose agar, they also found that synthetic media like Czapek's dox agar and Richard's agar supported poor linear growth of fungus. This might be due to higher nutrient content of the vegetable base media to support growth of the fungus than those of synthetic media.

It was further observed that all media induce sporulation however, among the tested media maximum sporulation was found in Potato dextrose agar (6.2 x 10^3) and Host extract agar (3.5 x 10^3) followed by Elliote's agar (2.9 x 10^3) and Czapek's dox agar (2.8 x 103) and minimum sporulation was found in Richard's agar (1.9 x 10^3) and Corn meal agar (2.6 x 10^3). Our present investigation agreed with that of Shane et al (1981) who asserted that conidia of Alternaria helianthi per colony was maximum on Potato dextrose agar (PDA). Singh (1994) also reported that maximum sporulation of Alternaria alternata was found in Potato dextrose broth. Potato dextrose agar supported best mycelia growth and sporulation of Alternaria alternata (fr.) keissler a causal organism of chickpea blight disease, Singh Virendra et al (2001).

TABLE 3
EFFECT OF BIOCONTROL AGENTS ON GROWTH AND SPORULATION OF *ALTERNARIA ALTERNATA*

S. No.	Treatment	Time taken for meeting of bioagent and pathogenic fungi (days)	Linear growth of *A.alternata* (mm)*	Spores/ml*	% inhibition over (check)	
					Linear growth	Spores
1.	*Trichoderma koningii*	5	22.8	1.9×10^3	29.4	67.2
2.	*Trichoderma hamatum*	4	22.3	1.5×10^3	31.0	74.1
3.	*Trichoderma harzianum*	2	17.5	0.6×10^3	45.7	89.6
4.	*Trichoderma viride*	3	19.5	0.9×10^3	39.5	84.4
5.	*Gliocladium virens*	4	21.0	1.7×10^3	34.9	70.6
6	A. *Alteriaria alternata* (check)	-	32.3	5.8×10^3	0	0
	B. C.D.$_{5\%}$	-	2.6	-	-	-

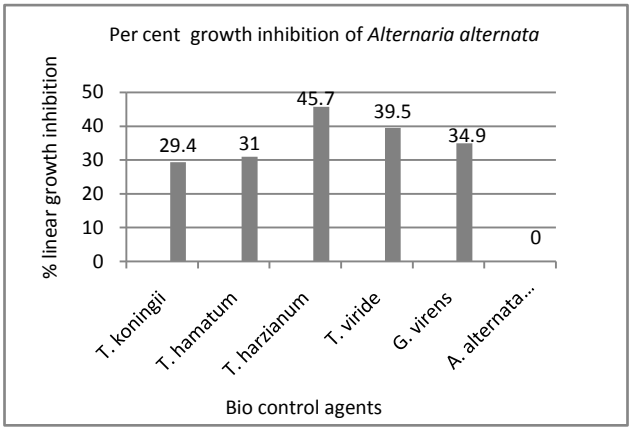

FIGURE 5: Per cent linear growth inhibition of A. alternata by different bioagents

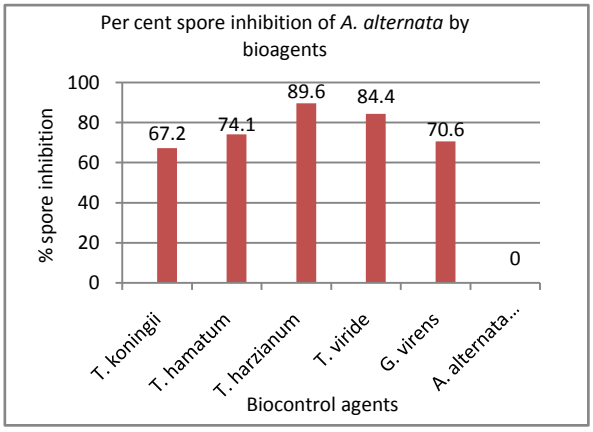

FIGURE 6: Per cent spore inhibition of A.alternata by antagonistic effect of different bioagents

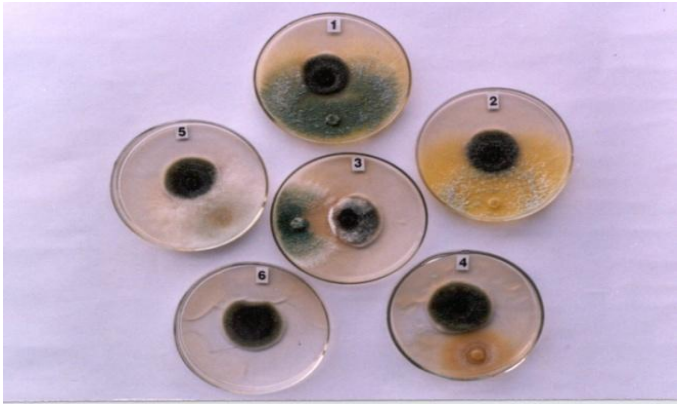

PHOTO 2: Antagonistic effect of biocontrol agent on linear growth of pathogenic fungus (*Alternaria alternata*) in dual culture test (1). *T. koningii* (2). *T. hamatum* (3). *T. harzianum* (4). *T. viride* (5). *G. virence* (6). *A. alternata* (check)

IV. CONCLUSION

The data presented in the above Table 3, Fig. 4 & 5 is the antagonistic effect of biocontrol agents on the growth and sporulation of *Alternaria alternata* during dual culture test. It was found all biocontrol agents statistically produced a significant antagonistic effect on linear growth and sporulation of *A. alternata*. However, among the bioagents, maximum

linear growth inhibition of the pathogenic fungi (*A. alternata*) was found in *Trichoderma harzianum* (17.5mm) with 89.6% inhibition over the check which was found at par with *Trichoderma viride* (19.5mm) with 84.4% inhibition followed by *Trichoderma hamatum* (22.3 mm) with 74.1% inhibition and *Gliocladium virens* (21.0 mm) with 70.6% and minimum significant inhibition effect was recorded in *Trichoderma koningii* (22.8 mm) with per cent inhibition of 67.2 over the check. It was also observed that highest per cent inhibition of sporulation of *A. alternata* was found in *T. harzianum* (89.6) and T. viride (84.4) followed by T. hamatum (74.1) and G. virens (70.6) while minimum per cent inhibition was found in T. koningii (67.2). It was observed that *T. harzianum* could over grown the pathogenic fungus within 2 days and T. viride within 3 days whereas *T. hamatum* and *G. virens* took 5 days. Our finding was in agreement with Basim and Katircioglu (1990) reported that in dual culture technique, bioagent Bacillus subtilis isolates could produce antagonistic activity against 7 (seven) fungal pathogens including *Alternaria alternata*. Similarly, Rajeshwari et al. (1998) reported that in dual culture test maximum inhibition of Macrophomina phaseolina was found in *Trichoderma harzianum, Trichoderma viride* and *Gliocladium virens* but least in *Trichoderma koningii*. Sudhakar et al. (1998) also reported that in dual culture technique *Trichoderma viride* was found superior in inhibiting the growth of *Rhizoctonia solani*, causal agent of rice sheath blight to *Trichoderma harzianum*. They also found lysis of the pathogen with the biocontrol agents.

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