

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.707.299>

Role of Root Colonizing *Trichoderma* Species in Management of *Alternaria* Leaf Blight of Asalio (*Lepidium sativum* L.) Caused by *Alternaria alternata*

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ABSTRACT

Lepidium sativum an important medicinal plant with immense pharmacological properties has been observed to be generally affected by many fungal pathogens in India particularly *Alternaria alternata* characterized by the appearance of brown necrotic spots on the leaf margin affecting the herb yield. Root colonizing PGPF (plant growth promoting fungi) have been reported to produce substances such as plant hormones to allow plants to utilize decomposing organic matter through mineral solubilization and to suppress plant pathogens in the rhizosphere by antagonistic mechanisms, such as the production of hydrolytic enzymes, aggressive mycoparasitism, competition for saprophytic colonization, and the induction of plant systemic resistance. The effect of six species of *Trichoderma* isolated from different crops of rhizosphere and their efficacy was assessed under *in vitro* and *in vivo* conditions. Under *in-vitro* conditions, they were screened for their qualitative traits viz., IAA production, phosphorus solubilizing activity and ammonia producing activity. Biomass determination and bioefficacy tests were performed against *Alternaria alternata*. The six *Trichoderma* species viz., *T. koningii*, *T. ressei-1* and *T. longibrachiatum* produced higher quantity of IAA. The tri-calcium phosphate solubilization activity was recorded only with *T. asperellum* and *T. harzianum*. The *T. koningii* and *T. ressei-2* medium ammonium producer while rest four *Trichoderma* species were minimum ammonium producer. Out of six species of *Trichoderma* highest suppression was recorded with *T. ressei-2* towards the *Alternaria alternata*. However, the highest inhibition was recorded by metabolite of *T. asperellum* isolates that corresponds to 38.75 percent reduction in mycelia growth when growth medium was non-amended with znso_4 . Similarly, the highest inhibition was recorded in metabolite of *T. ressei-2* isolates when growth medium was amended with znso_4 . The highest biomass production of *T. ressei-2* was recorded with znso_4 amended medium while the highest biomass of *T. ressei-1* was recorded with non znso_4 amended growth medium. The effect of inoculation of fungal bioagent along with FYM and znso_4 was found significant on relative water content (RWC), chlorophyll content, membrane stability index (MSI) and disease index under *in-vivo* conditions. The minimum disease incidence of *Alternaria* leaf blight was recorded with the soil application of either *T. ressei-2*+FYM + znso_4 or *T. ressei-1*+FYM + znso_4 .

Keywords

Root colonizing *Trichoderma*, *Alternaria* leaf blight, Asalio (*Lepidium sativum* L.), *Alternaria alternata*

Article Info

Accepted:
17 June 2018
Available Online:
10 July 2018

Introduction

Lepidium sativum also known as common cress, garden cress, garden pepper cress, pepper grass or pepperwort (english) and chandrasur, chansur (hindi); is an annual herb, belonging to Brassicaceae family. *Lepidium* seed is an important source of iron, folic acid, calcium and vitamins A, C and E. The seed also contains arachidic, linolic fatty acids and rich in protein (2.6 g/100 g), whereas the leaves are an excellent source of vitamin A, C and folate (Doke and Guha, 2014). However, chandrasur an important medicinal plant with significant pharmacological properties has been observed to be generally affected by many fungal pathogens in India. Among them *A. alternata* causes severe leaf spot in the northern Indian plains. *Alternaria* leaf spot disease symptoms in *L. sativum* are characterized by the appearance of brown necrotic spots on the leaf margin. The necrosis spreads towards the midrib and as a result the leaf curls up and dries, affecting herbal yield. Root colonizing fungi in the genus *Trichoderma* frequently increases root growth development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Harman *et al.*, 2004a). Many studies have shown an increase in growth and P-uptake by plants through the inoculation of PSMs (one of the component of PGPF) in pot experiments (Vassilev *et al.*, 2006) and as well as in field conditions (Valverde *et al.*, 2006). Therefore, to overcome the menace of this pathogen, the Plant Growth Promoting Fungi (PGPF) with special reference to *Trichoderma* species have been used to manage the disease with following objectives;

Isolation and characterization of Plant Growth Promoting Fungi (PGPF) with special reference to *Trichoderma* species

Establishment of bio-control potential by against *Alternaria alternata* by *Trichoderma* species under In-vitro and In-vivo Conditions

Materials and Methods

Collection of diseased specimens and purification of the pathogen

Diseased *Asalio* plants exhibiting typical symptoms of *Alternaria alternata* infection were collected from the experimental field of AICRP on Medicinal Aromatic Plants and Betelvine of Jawaharlal Nehru Krishi Vishwa Vidyalaya (22°49'- 22° 80'N; 78°21'- 80°58'E), Jabalpur in the Central India during 2016-17. The affected disease portions of plant (like leaf, branches etc.) were cut with the help of sharp razor and rinsed with sterilised water to remove traces of dirt. These were surface sterilised by dipping in 1:1000 mercuric chloride solution for one minute and washed twice with sterile water. These pieces were transferred aseptically to sterilised Petri-dishes containing solidified PDA in a laminar air flow. The Petri-dishes were incubated at 25±2°C. The growth of fungus was observed after 72 hours and isolations were made from developing colonies for further study. The pathogen was further purified through single spore method and sub-cultured on PDA slants and kept at 4 °C for further use.

Treatment details of mycoflora used under in-vitro and in-vivo studies

<i>Trichoderma</i> Species
<i>T. koningii</i>
<i>T. ressei-1</i>
<i>T. harzianum</i>
<i>T. asperellum</i>
<i>T. longibrachiatum</i>
<i>T. ressei-2</i>
Control

The field experiment was conducted with six treatments and three replications in randomized block design with plot size of two square meters during 2016-17. The beneficial fungi (@ 2ml/m²) were combined with ZnSO₄ (@ 200ppm) were applied in soil.

IAA producing activity

The presence of IAA-like substances was detected by following the method of Sarwar and Kremer (1995) in L-tryptophan agar. The fungi were grown on L-tryptophan agar medium in triplicate and incubated at $28 \pm 2^{\circ}\text{C}$ for seven days in the dark. After seven days of incubation, the fungus grown on L-tryptophan agar medium was added with freshly prepared Salkowsky reagent (Sarwar and Kremer, 1995) in triplicate, for each bioagent grown on Petri dish and incubated in the dark for 30 min for development of pink colour. The amount of IAA production was expressed by + and – sign. The - indicates no IAA production; + faint pink colour and small amount of IAA production; ++ pink colour and medium amount of IAA production; +++ dark pink colour and high amount of IAA production.

Phosphorus solubilizing activity

Phosphate solubilizing fungi were isolated by the dilution plate methods modified by Johnson *et al.*, (1959) on PVK medium (Pikovskaya, 1948) with tri-calcium phosphate as insoluble inorganic phosphate source. Rose Bengal as bacteriostatic agent was added (10 ml/l) at concentration 1/15000 (Smith and Daws, 1944). Total fungal counts were calculated in triplicate after 7 days of isolation by multiplying average number of colonies in each plate with inverted dilution factors. The isolates were identified on the basis of colony morphology, spore characteristics and microscopic examination according to Moubasher (Moubasher, 1993).

Pikovskaya's medium with rose Bengal addition was prepared. Sterilized PVK media was poured into sterilized plates, after solidification of the media, fungal strains were placed on the center of plates under aseptic conditions. They were incubated at $28 \pm 2^{\circ}\text{C}$ for 5 days with continuous observation for

colony diameter. The P solubilizing fungi were detected by the formation of clear halo around their colonies. The performance of each fungus was marked by assigning them + and – sign. The - indicates no phosphorus solubilization, + small amount of phosphorus was dissolved, ++ medium amount of phosphorus was dissolved and +++ high amount of phosphorus was dissolved.

Ammonia producing activity

For the detection of ammonia production, all species were grown in Petri-dishes containing peptone water agar (peptone: 10.0 g; NaCl: 5.0 g; distilled water: 1000 ml; 7.0 pH). The Petri-dishes were inoculated with seven days old culture of bioagent's and incubated at $30 \pm 1^{\circ}\text{C}$ for 5 days.

The accumulation of ammonia was detected by adding Nessler's reagent (0.5 ml per plate). A faint yellow colour indicated a small amount of ammonia, and deep yellow to brownish colour indicated medium to maximum production of ammonia.

Determination of biomass production

The testing of biomass production by the beneficial fungi was done by growing them on potato dextrose broth and amended with and without ZnSO_4 (@200ppm) prepared in 100 ml Erlenmeyer flask and final pH was adjusted to 6.5 to 7.0. They were later inoculated aseptically with 5mm actively grown culture disc of the fungus. Three replications were maintained. The entire set up was incubated for 7 days at 25°C to attain maximum growth and sporulation. Mycelial mat was obtained by filtering on pre-weighed filter paper (Whatman filter paper no.1) (as fresh weight) and dried in hot air oven at 60°C until a constant weight (dry weight) was obtained (Hall and Bell 1961).

Evaluation of antagonistic potential of beneficial fungi through dual culture technique

The antagonistic potentials of bioagents such as *Trichoderma* species were evaluated against test pathogen (*Alternaria alternata*) through dual culture technique (Denis and Webster, 1971). Per cent inhibition of growth of the pathogens was calculated by using the following formula.

Inhibition =

$$\frac{\text{Radial growth in control (C)} - \text{Radial growth in the treatment (T)}}{\text{Radial growth in control(C)}}$$

Per cent inhibition= Inhibition x100

Assessment of culture filtrates of beneficial mycoflora added with or without ZnSO₄ by poison food technique

Effect of culture filtrate of *Trichoderma* species combined with and without ZnSO₄ was assessed against mycelial growth of *Alternaria alternata* by Dennis and Webster (1971) method. Filtrate of antagonist(s) culture in PDA broth grown for 10 days with or without addition of ZnSO₄ (@ 200ppm).

Fungi-toxicity of beneficial fungi was expressed as inhibition of radial growth of test pathogen by following formula:

$$\text{Percentage of inhibition} = \frac{R1-R2}{R1} \times 100$$

R1 –Radial growth of the pathogen in control plate,

R2 - Radial growth of the pathogen in test plate

Relative water content (RWC)

Measurements of RWC (Barrs and Weatherly, 1962) were performed on leaves collected from Asalio plants. Leaves were always collected from the mid section of either branches or seedlings, in order to minimize age effects. Individual leaves were first removed from the stem with tweezers. A sharp razor blade was used to cut the leaf base and leaves were then immediately weighed (fresh mass, FM). The FM obtained from each sample was minimum 1 gram. In order to obtain the turgid mass (TM), leaves were floated in distilled water inside a closed Petri dish. At the end of the imbibition period, leaf samples were placed in a pre-heated oven at 80 °C for 48 h, in order to obtain the dry mass (DM). Values of FM, TM, and DM were used to calculate RWC, using the following equation:

$$\text{RWC (\%)} = \frac{[(\text{FM} - \text{DM}) / (\text{TM} - \text{DM})] \times 100.}$$

A leaf sample was made up of ten to fifteen leaves, collected from the same branch or seedling. For data analysis, each leaf sample was treated as an experimental unit. The experimental units were organized following a Random block design.

Chlorophyll content index

Chlorophyll Content Index was estimated through the portable chlorophyll meter (Peng *et al.*, 1992). Fully expanded leaf sample from three places of each plant of different treatments has been selected for estimation of chlorophyll content index.

The mean of triplicate readings taken using SPAD-502 (SPAD-502, Minolta, Japan) around the mid-point near the midrib of each sample were recorded for different treatment of Asalio leaf and averaged.

Membrane stability index (MSI)

The membrane stability index (MSI) was determined according to the method of Deshmukh *et al.*, (1991). Leaf discs (0.2 g) of control and treated plants were thoroughly washed in running tap water and double distilled water and were placed in 20 ml of doubled distilled water at 40 °C for 30 minutes, after that electrical conductivity (EC) was recorded by conductivity bridge (C1).

Subsequently, the same samples were placed in boiling water bath (100°C) for 10 minutes and the electrical conductivity was recorded (C2). The membrane stability index was calculated by using the formula:

$$\text{MSI} = [1 - C1 / C2] \times 100$$

Results and Discussion

Qualitative characterization of beneficial attributes of plant growth promoting rhizosphere fungi

The three *Trichoderma* species viz., *T. koningii*, *T. ressei-1* and *T. longibrachiatum* produced higher quantity of IAA as shown by development of dark pink colour of growth medium while rest three (*T. asperellum*, *T. harzianum* and *T. ressei-2*) were minimum IAA producer. The tri-calcium phosphate solubilization activity was recorded only with *T. asperellum* and *T. harzianum*. The *T. koningii* and *T. ressei-2* medium ammonium producer while rest four *Trichoderma* species were minimum ammonium producer.(table.1)

Screening of different *Trichoderma* species against *Alternaria alternata* under in-vitro conditions

The effects of six species of *Trichoderma* were assessed against mycelia growth of *A. alternata* and all the species were found highly suppressive towards the test pathogen. The suppression of mycelia growth of test pathogen by different species of *Trichoderma*

varied between 21.98 mm to 27.61mm. The highest (21.98 mm) suppression was recorded with T₆(*T. ressei-2*) which was statistically at par with the suppression recorded with T₂ (22.24 mm). The highest (40.28 %) inhibition percent was recorded with T₆(*T. ressei-2*), while least (24.99 percent) with T₁(*T. koningii*).(table.2)

Evaluation of bioefficacy of culture filtrates of *Trichoderma* species against *Alternaria alternata* under in-vitro conditions

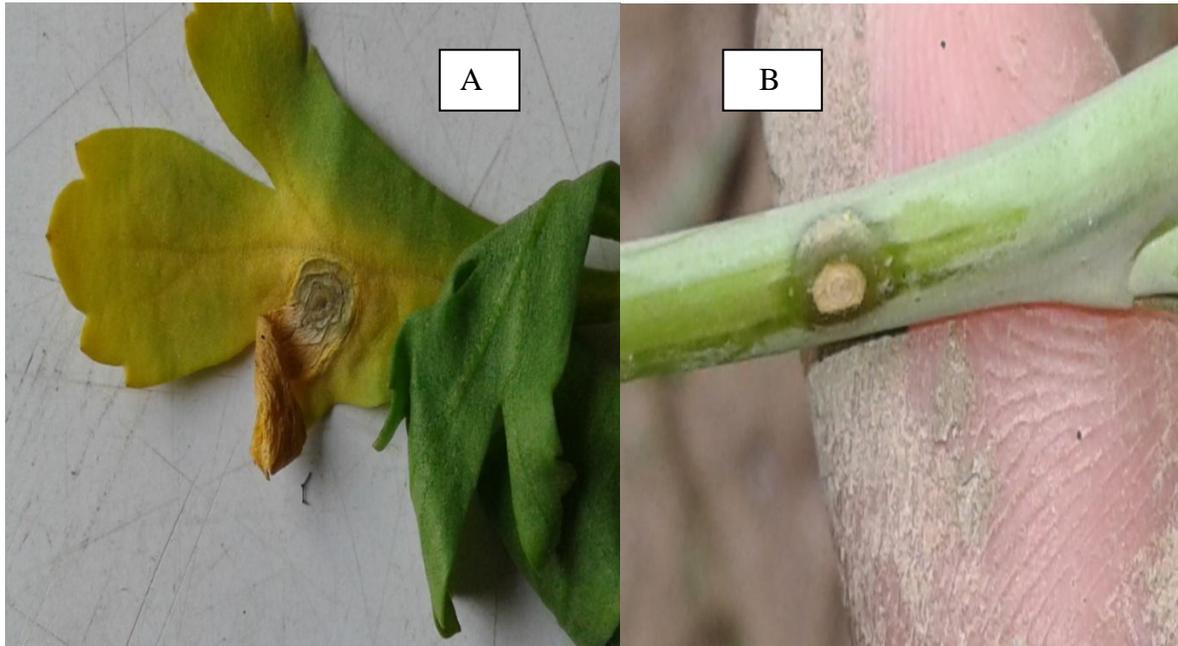
The inhibition of mycelia growth of *Alternaria alternata* under poison food technique by culture filtrate of different *Trichoderma* species varied within themselves and also with time. The highest (23.75mm) inhibition was recorded with T₄ that correspond to 38.75 percent mycelia growth reduction followed by T₆ (25.58mm) and T₂(25.68mm) that were identical to each other in growth inhibition.

The growth of the pathogen had increased with time but increase was comparatively slower from 72hours (27.22mm) to 96hours (28.66mm) hours but significantly increased at 120hours.(table.3)

Evaluation of bioefficacy of culture filtrates of *Trichoderma* species amended with ZnSO₄ against *Alternaria alternata*

The culture filtrate of different *Trichoderma* species amended with ZnSO₄ had significantly inhibited the mycelial growth of *A. alternata* when tested under poison food technique (Table4.7) at different time intervals. The maximum (23.75mm) inhibition was recorded with T₆ followed by T₄ (24.61mm) while least inhibition was recorded with T₃ (28.54). Almost similar inhibition was recorded with T₁ (27.96mm), T₅ (27.80mm) and T₂ (27.41). The mycelia growth of test pathogen increased from 48 hours (25.96mm) to 120 hours (30.85mm) (Table 4).

Plate.1 Plant parts affected by *Alternaria alternata*



(A) Necrotic and concentric leaf spots at margin of leaf (B) Concentric spots on stem

Plate.2 Isolation and purification of *Alternaria alternata*



Plate.3 Different species of *Trichoderma* isolated from different crop rhizosphere

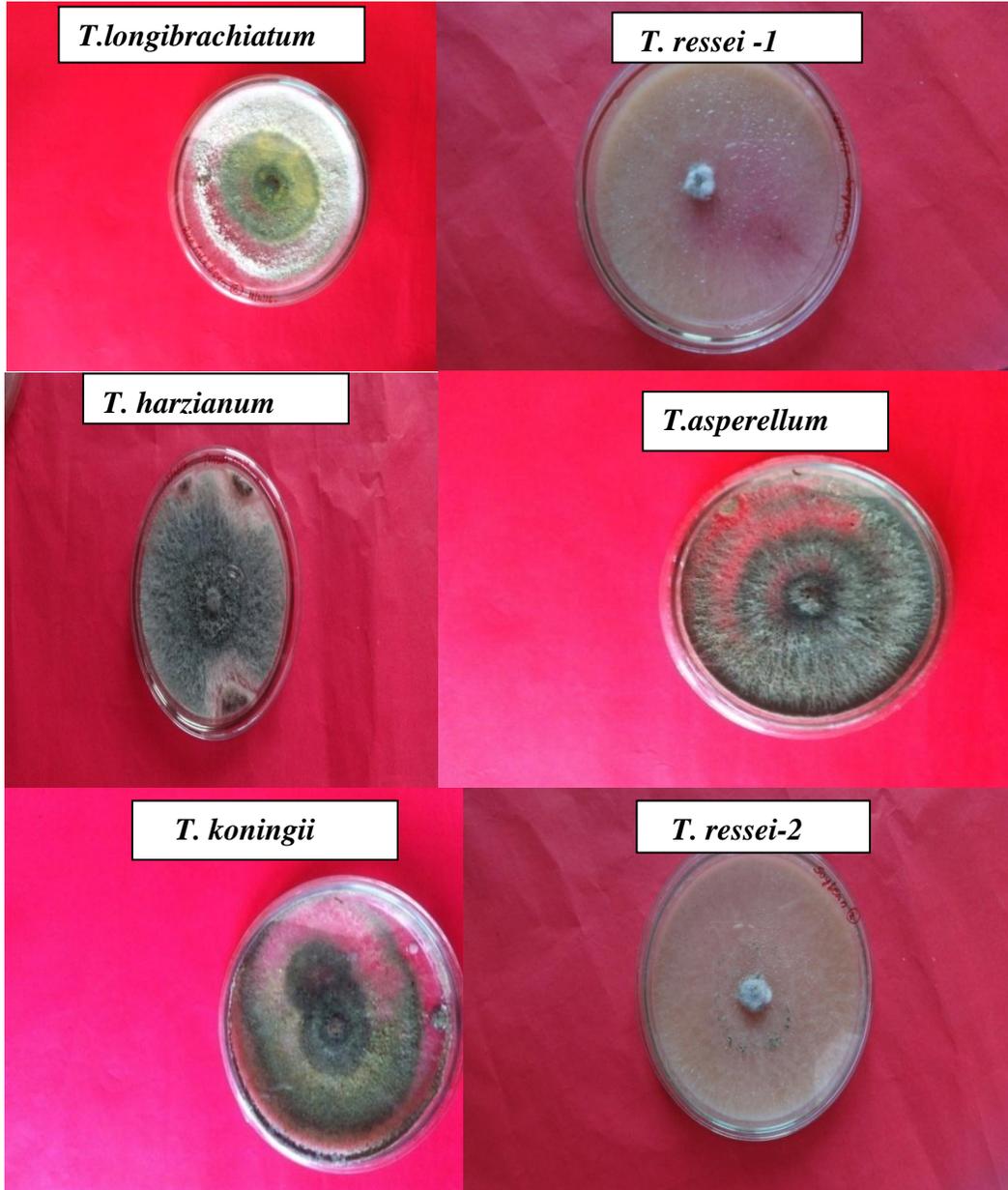
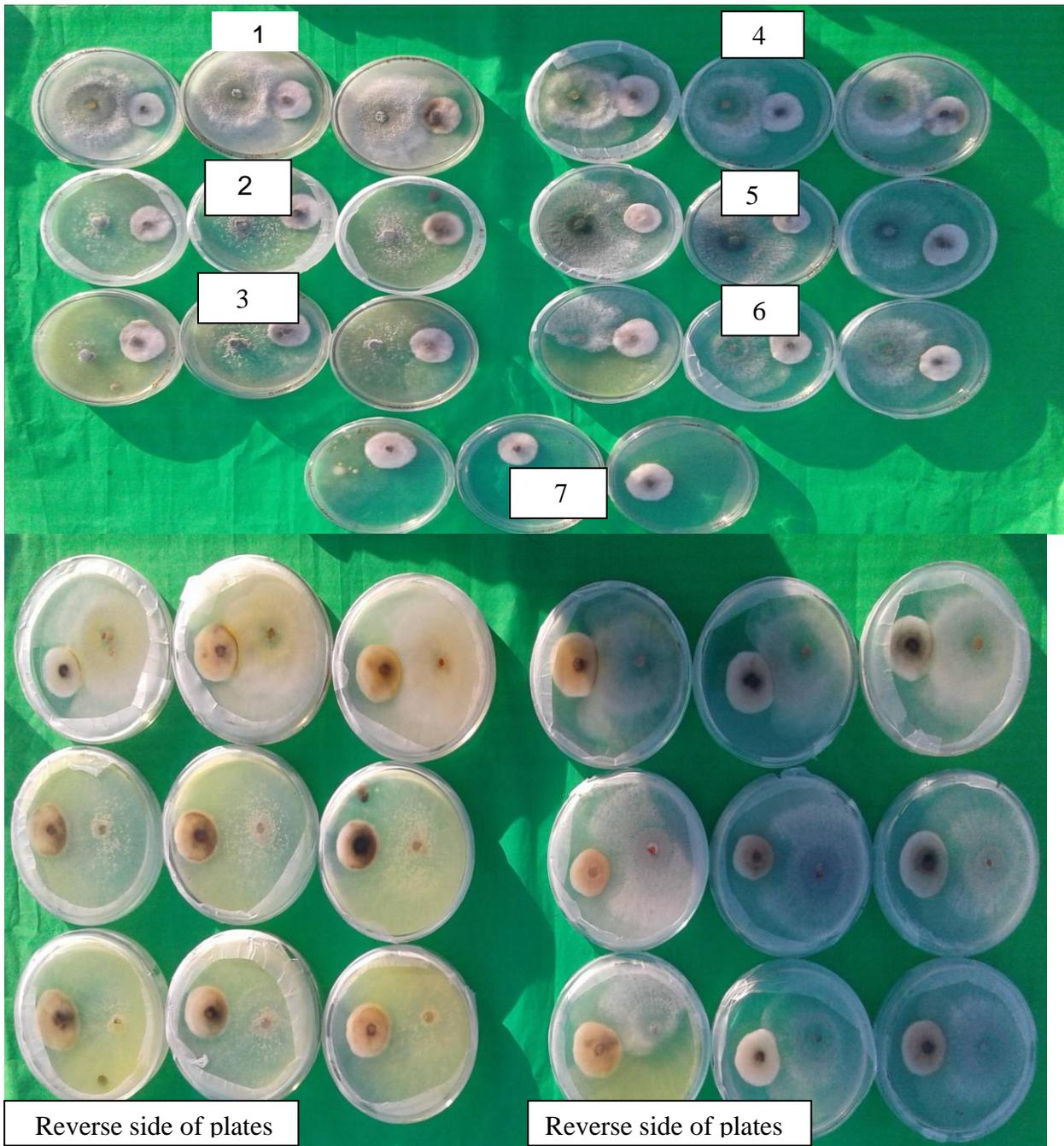


Plate.4 Suppression of mycelial growth of *Alternaria alternata* by *Trichoderma* species



(1) *T.longibrachiatum*, (2) *T. ressei-1*, (3) *T.ressei-2*, (4) *T. koningii*, (5) *T.asperellum*, (6) *T. harzianum* (7)Control

Plate.5 Evaluation of bioefficacy of culture filtrate of *Trichoderma* species against *Alternaria alternata* under *in-vitro* conditions

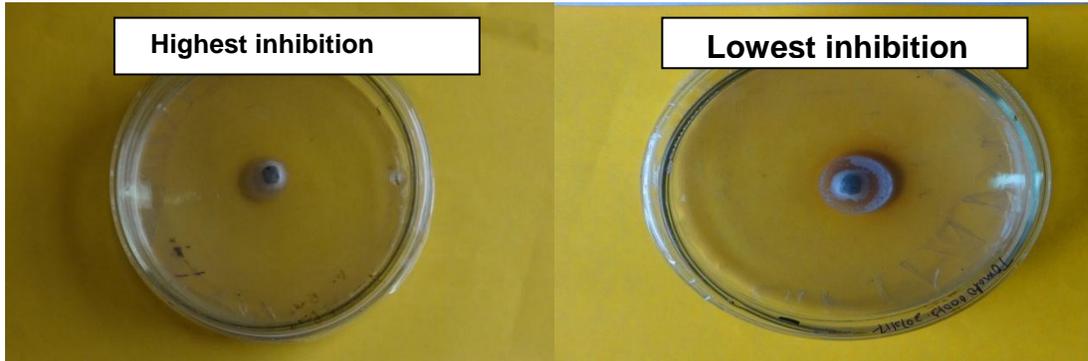


Plate.6 Evaluation of bioefficacy of culture filtrate of *Trichoderma* species amended with $ZnSO_4$ against *Alternaria alternata*

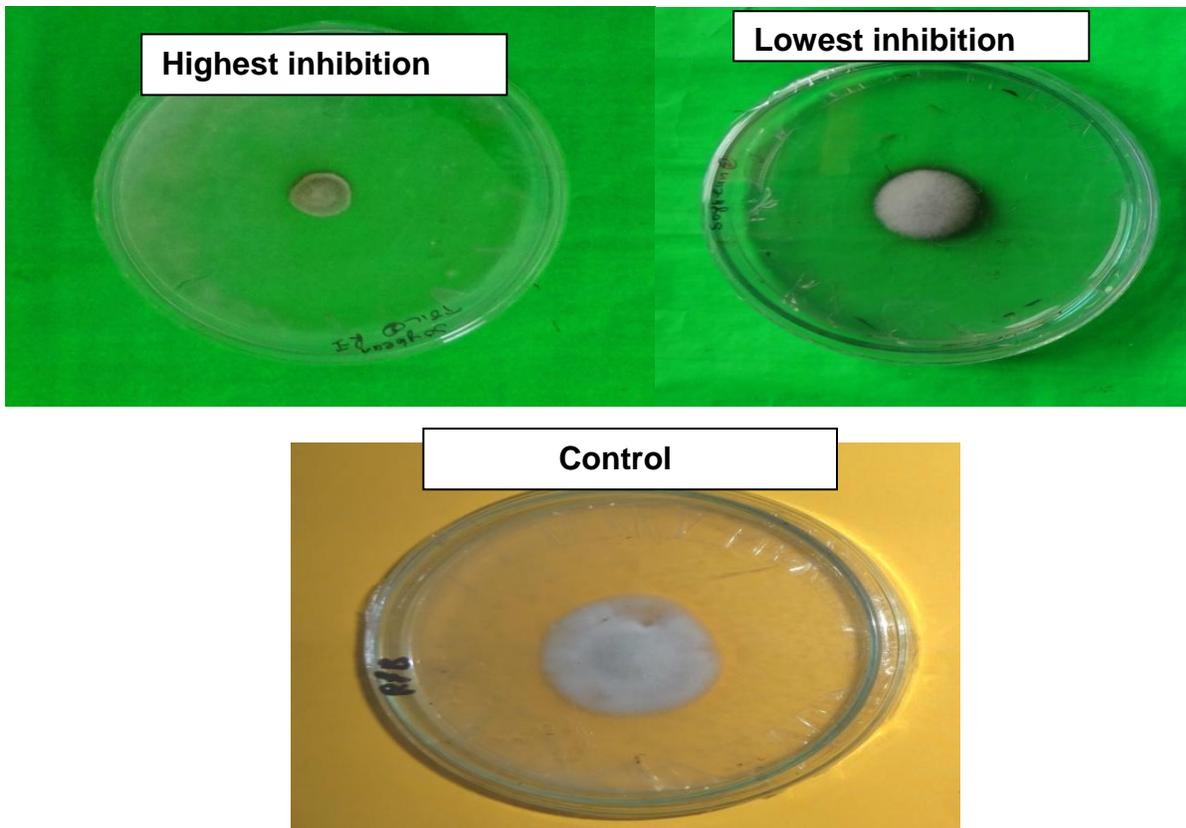


Plate.7 Multiplication of *Trichoderma* species in potato dextrose broth



Plate.8 Multiplication of *Trichoderma* species in potato dextrose broth amended with ZnSO₄



Table.1 Qualitative characterization of beneficial attributes of plant growth promoting rhizosphere fungi

PGPF isolates different Rhizosphere soils	IAA Producing activity	Phosphorus solubilizing activity	Ammonia Producing activity
<i>T. koningii</i>	+++ (dark pink) maximum	+ (minimum)	++ (deep yellow) medium
<i>T.ressei-1</i>	+++ (dark pink) maximum	- absent	+ (faint yellow) minimum
<i>T.asperellum</i>	++ (faint pink) medium	+ (minimum)	+ (faint yellow) minimum
<i>T. harzianum</i>	++ (faint pink) medium	+ (minimum)	+ (faint yellow) minimum
<i>T.longibrachiatatum</i>	+++ (dark pink) maximum	- absent	+ (faint yellow) minimum
<i>T.ressei-2</i>	++ (faint pink)medium	- absent	+ + (deep yellow) medium

Table.2 Screening of different *Trichoderma* species against *Alternaria alternata* under *in-vitro* conditions

<i>Trichoderma</i> species	Growth in (mm)				
	48hours	72hours	96hours	mean	Percentage inhibition
T ₁ (<i>T.koningii</i>)	27.73 (21.66)	31.52 (27.33)	23.57 (16.00)	27.61	24.99
T ₂ (<i>T.ressei-1</i>)	27.50 (21.33)	25.90 (19.33)	13.34 (5.33)	22.24	39.58
T ₃ (<i>T.harzianum</i>)	27.62 (21.50)	30.43 (25.66)	22.51 (14.66)	26.85	27.05
T ₄ (<i>T.asperellum</i>)	26.80 (20.33)	20.60 (12.66)	23.82 (16.33)	23.74	35.50
T ₅ (<i>T.longibrachiatatum</i>)	27.03 (20.66)	24.81 (17.66)	21.69 (13.66)	24.51	33.41
T ₆ (<i>T.ressei-2</i>)	27.15 (20.83)	25.45 (18.66)	13.34 (5.33)	21.98	40.28
Control	31.08 (26.66)	36.45 (35.33)	42.89 (46.33)	36.81	-
Mean	27.84	27.88	23.02		
CV	7.14				
Fungus CD(P≤0.05)	1.78				
Hours CD(P≤0.05)	1.17				
Fungus x Hours	3.09				

The values in the parenthesis are original value

Table.3 Evaluation of bioefficacy of culture filtrates of *Trichoderma* species against *Alternaria alternata* under *in-vitro* conditions.

<i>Trichoderma</i> species	Growth in (mm)					
	48hours	72hours	96hours	120hours	mean	Percentage inhibition
T ₁ (<i>T.koningii</i>)	26.68 (20.16)	26.80 (20.33)	26.80 (20.33)	27.50 (21.33)	26.94	30.53
T ₂ (<i>T.ressei-1</i>)	24.84 (17.66)	25.47 (18.50)	25.72 (18.83)	26.68 (20.16)	25.68	33.78
T ₃ (<i>T.harzianum</i>)	25.96 (19.16)	27.03 (20.66)	27.03 (20.66)	28.52 (22.83)	27.14	30.01
T ₄ (<i>T.asperellum</i>)	22.51 (14.66)	23.18 (15.50)	24.34 (17.00)	24.97 (17.83)	23.75	38.75
T ₅ (<i>T.longibrachiatatum</i>)	27.03 (20.66)	27.03 (20.66)	27.74 (21.66)	28.19 (22.33)	27.50	29.08
T ₆ (<i>T.ressei-2</i>)	22.78 (15.00)	25.96 (19.16)	27.13 (20.83)	26.44 (19.83)	25.58	34.03
Control	30.00 (25.00)	35.03 (33.00)	41.84 (44.50)	48.25 (55.67)	38.78	-
Mean	25.69	27.22	28.66	30.08		
CV	2.23					
FungusCD(P≤0.05)	0.51					
Hours(P≤0.05)	0.38					
Fungus x Hours	1.02					

The values in the parenthesis are original value transformed into arch sin

Table.4 Evaluation of bioefficacy of culture filtrates of *Trichoderma* species amended with ZnSO₄ against *Alternaria alternata*

Trichoderma species	Growth in (mm)					Percentage inhibition
	48hours	72hours	96hours	120hours	mean	
T ₁ (<i>T.koningii</i>)	(26.07) 19.33	(27.03) 20.66	(27.96) 22.00	(28.42) 22.66	27.96	29.39
T ₂ (<i>T.reesei-1</i>)	(26.03) 19.33	(27.24) 21.00	(27.94) 22.00	(28.41) 22.66	27.41	30.78
T ₃ (<i>T.harzianum</i>)	(27.71) 21.66	(28.18) 22.33	(28.75) 23.16	(29.54) 24.33	28.54	27.92
T ₄ (<i>T.asperellum</i>)	(23.04) 15.33	(24.59) 17.33	(25.09) 18.00	(25.71) 18.83	24.61	37.85
T ₅ (<i>T.longibrachiatum</i>)	(25.96) 19.16	(25.50) 21.33	(28.65) 23.00	(29.10) 23.66	27.80	29.79
T ₆ (<i>T.reesei-2</i>)	(22.50) 14.66	(23.31) 15.66	(23.83) 16.33	(25.35) 18.33	23.75	40.02
Control	(30.42) 25.66	(36.45) 32.33	(42.12) 45.00	(49.41) 57.66	39.60	
Mean	25.96	27.76	29.19	30.85		
CV	3.64					
FungusCD(P≤0.05)	0.84					
Hours(P≤0.05)	0.64					
Fungus x Hours	1.69					

Table.5 Effect of micro-nutrient on biomass production of beneficial fungi

Fungal bioagents	With ZnSO ₄				Without ZnSO ₄			
	Fresh weight	Dry weight	Biomass (%)	pH	Fresh weight	Dry weight	Biomass (%)	pH
<i>T. koningii</i>	8.54(2.20)	2.01(0.12)	94.45	5.1	8.79(2.33)	2.78(0.23)	90.20	4.1
<i>T.longibrachiatum</i>	8.68(2.28)	2.06(0.13)	94.10	5.0	8.99(2.44)	3.20(0.31)	87.00	5.8
<i>T.asperellum</i>	8.24(2.05)	2.19(0.13)	93.23	3.8	8.75(2.31)	2.29(0.16)	88.93	3.8
<i>T. reesei-1</i>	8.27(2.07)	2.47(0.18)	91.12	5.8	12.98(5.04)	1.98(0.12)	97.60	5.1
<i>T. reesei-2</i>	13.11(5.15)	2.11(0.13)	97.44	5.2	12.00(4.32)	2.16(0.15)	96.46	4.1
<i>T.harzianum</i>	7.89(1.84)	1.89(0.11)	93.67	3.7	8.06(1.96)	2.19(0.15)	92.03	3.9
CV	0.65	4.51			0.19	2.22		
CD(0.05)	0.10	0.17			0.03	0.09		

Table.6 Effect of biological treatments on physiological and disease incidence of Asalio crop

Fungal bioagents	Relative water content (%)	Chlorophyll content(%)	Membrane stability index (%)	Percent disease index(%)
<i>T. harzianum</i>	71.82	48.42	84.20	48.63
<i>T. koningii</i>	66.90	45.14	86.15	46..66
<i>T. reesei-1</i>	68.49	44.82	79.63	45.00
<i>T.asperellum</i>	57.03	38.90	90.36	53.63
<i>T.longibrachiatum</i>	53.32	38.39	88.51	52.00
<i>T. reesei-2</i>	60.76	43.53	68.82	34.32
Control	48.31	25.39	94.49	54.33
CV	0.46	0.03	0.01	0.45
Fungus CD(P≤0.05)	0.50	0.02	0.02	0.38

Figure.1 *In vitro* management of *Alternaria alternata* by *Trichoderma* species; (a) Dual culture; (b) Poison food study; (c) Poison food amended with ZnSO₄

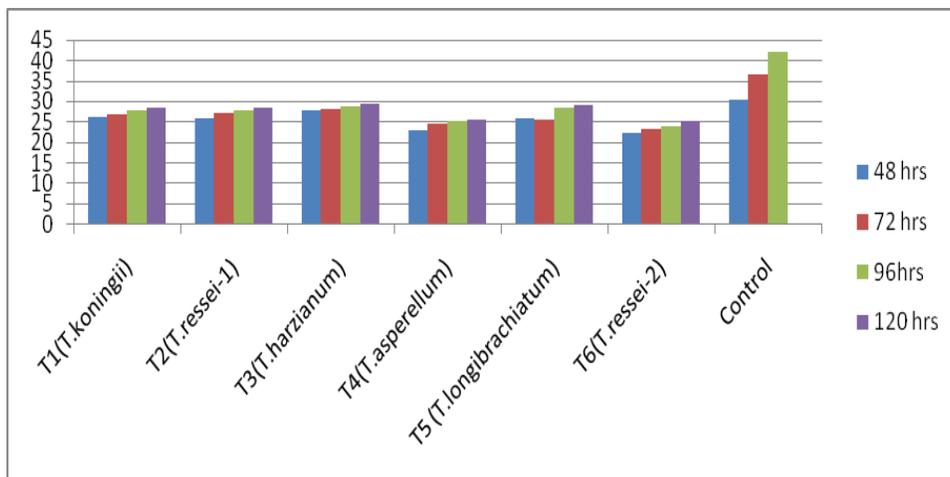
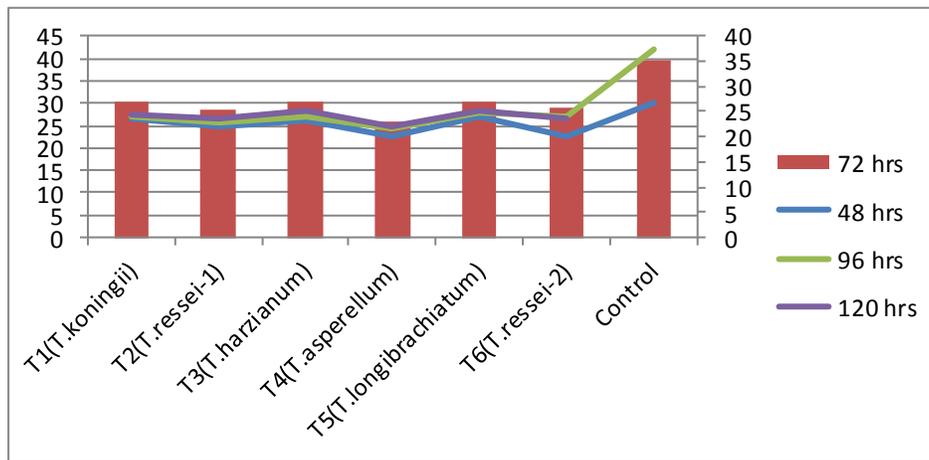
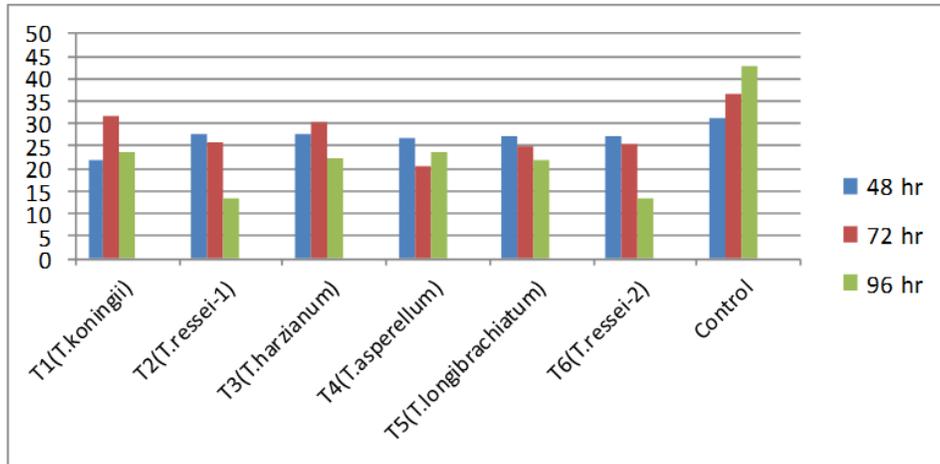
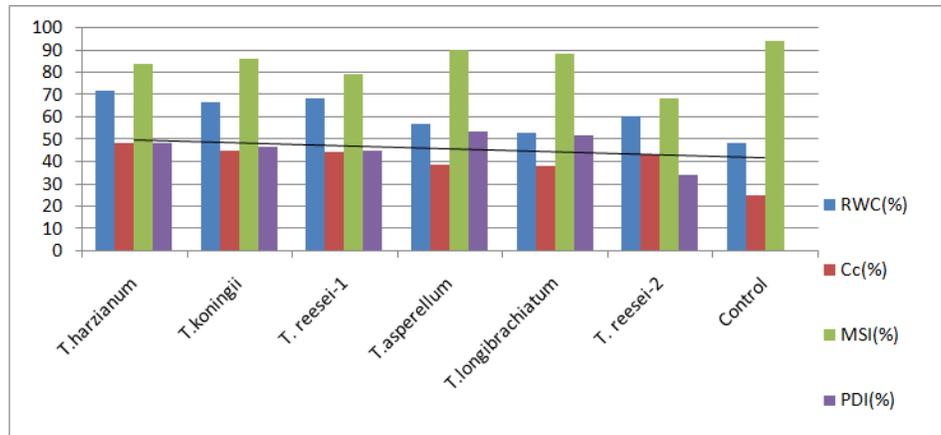


Figure.2 Effect of *Trichoderma* species on ZnSO₄ on biological parameter and bio-control of *Alternaria* leaf blight



Effect of micro-nutrient on biomass production of beneficial fungi

To evaluate the effect of micro-nutrient on biomass production, the growth medium of beneficial fungus was amended with and without ZnSO₄. The fresh weight of bio-agents varied from 7.89 to 13.11 gm while dry weight from 1.89 gm to 2.47 gm and pH from 3.7 to 5.8 in ZnSO₄ amended growth medium. The highest (97.44%) biomass production was recorded with *T. reesei-2* while least (91.12%) with *T. reesei-1*. However, almost identical but higher biomass production of *T. koningii* (94.45%) and *T. longibrachiatum* (94.10%) was recorded in ZnSO₄ amended medium. The pH of *Trichoderma* species grown on ZnSO₄ amended medium ranged from 3.7 to 5.8.

The highest *T. reesei-2* (12.98gm) fresh weight and dry weight (3.20gm) of *T. longibrachiatum* was recorded in growth medium (potato dextrose broth) not amended with ZnSO₄. However, the highest (97.60%) biomass of *T. reesei-1* was recorded in the same growth medium. The *T. reesei-2* (96.46%) was the next best to *T. reesei-1* in biomass production followed by *T. harzianum* (92.03%). The highest pH of *Trichoderma*

species growth media potato dextrose broth was recorded (3.9 to 5.8) (Table 5).

Effect of biological treatments on physiological and disease incidence of Asalio crop

The effect of inoculation of fungal bioagent along with FYM and ZnSO₄ was found significant on relative water content (RWC), chlorophyll content, membrane stability index (MSI) and disease index under *in-vivo* conditions. Although the highest (71.82%) RWC was recorded in *T. harzianum*. Lowest (53.32) RWC was recorded in *T. longibrachiatum*. Similarly, the highest chlorophyll content (48.42) was recorded in *T. harzianum* which was statistically at-par with *T. longibrachiatum* (45.14) and *T. reesei-2*. The lower and similar chlorophyll content was recorded in *T. longibrachiatum* (38.39) and *T. asperellum* (38.90). The minimum (68.82) MSI was recorded in *T. reesei-2*. The highest MSI was recorded in *T. asperellum* (90.36) that followed by *T. longibrachiatum* (88.51), *T. koningii* (86.00) but all similarly affected on MSI of Asalio crop. The minimum disease incidence was recorded *T. reesei-2* (34.32).

The application of *T.ressei-2*+FYM+ZnSO₄ and *T.ressei-1*+FYM+ZnSO₄ was found effective in reducing the *Alternaria* leaf blight disease as it reduced disease incidence 34.32 (Table 6).

Different PGPF were isolated from the rhizosphere of different crops, the five isolates of six species of *Trichoderma* have been screened against *Alternaria alternata* under *in-vitro* and *in-vivo* conditions. All the tested bioagents showed significant mycelia growth suppressing ability under laboratory conditions and disease suppression under field conditions apart from positively affecting physiological parameters of crop. It has been reported that biocontrol agents having both antagonistic and plant growth promoting activity, could be more effective in controlling plant diseases (Borrero *et al.*, 2006) and suppression of deleterious microorganisms in the rhizosphere (Sabuquillo *et al.*, 2006).

The IAA producing activity of *T.koningii*, *T.ressei-1* and *T.longibrachiatum* was maximum among the *Trichoderma* species.. *Trichoderma* species was found to produce higher amount of ammonium. Moreover, medium ammonium production was record *T.koningii*. This could be due to the differential ability of bioagents to produce different enzymes and hormones. However, Oliveira *et al.*, (2012) confirmed that the tested isolates from *Trichoderma* genus had the ability to solublize calcium phosphate. Rudresh and co-workers. (2005) reported that the different *Trichoderma* isolates solubilized insoluble tri-calcium phosphate (TCP) to various extents. *Trichoderma viride* (TV 97), *Trichoderma virens* (PDBCTVs 12) and *Trichoderma virens* (PDBCTV) solubilized 70% of that solubilized by *Bacillus megaterium*. However, most of the *Trichoderma* genus isolates produce maximum indole acetic acid (IAA), with or

without the L-tryptophan precursor has been reported by Oliveira *et al.*, (2012).

The *T.ressei-2* and *T.ressei-1* were highly and equally suppressive towards *A. alternata*, out of six species of *Trichoderma*. The *T.asperellum* was more suppressive than *T.logibrachiatum* but equal to *T.ressei-1*. The suppression of mycelia growth of test pathogen by different species of *Trichoderma* varied between 21.98 mm to 27.61mm. There was no significant increase in mycelia growth of the pathogen was recorded from 48 hours to 72 hours with exception to *T.koningii* and *T.harzianum* where growth of test pathogen increased but significant decrease was recorded at 96hours. The presence of an inhibition zone in dual culture suggests the secretion of diffusible non-volatile inhibitory substance/ mycoparasitism by the *Trichoderma* species. Previous studies have demonstrated that before mycelia of fungi interact, *Trichoderma* sp. produces low quantities of extracellular exochitinases (Brunner *et al.*, 2003). *Trichoderma* strains inhibit the infections caused by plant pathogens using different biocontrol mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion (Poovendran *et al.*, 2011).

The result obtained in poison food technique was different from that of dual culture technique. In poison food technique, the culture filtrate of *T.asperellum* was found highly inhibitory towards the test pathogen. The growth of the pathogen had increased with time but increase was comparatively slower from 72hours to 96hours but significantly increased at 120 hours in non ZnSO₄ amended culture filtrate of *Trichoderma* species. The mycelia growth of *A. alternata* remained almost identical in culture filtrate of *T.koningii* and *T.longibrachiatum* from 48hours to 96 hours with minor increase at 120 hours. The growth

of test pathogen in culture filtrates of *T.asperellum* was slower throughout the studied time to rest. Culture filtrate of *Trichoderma* species amended with ZnSO₄ exhibited comparatively lower mycelial growth inhibition in comparison to non amended culture filtrate of *Trichoderma* species. Although the growth of the pathogen had increased with time but increase was comparatively slower after 96 hours to 120 hours. The growth of test pathogen in culture filtrates of *T.ressei-2* remained slower throughout the studied time compared to rest *Trichoderma* species. Hajieghrari *et al.*, (2008) evaluated six isolated of *Trichoderma* sp. against phytopathogenic fungi in dual culture techniques and through production of volatile and non-volatile inhibitors. The different effects of *T.harzianum* culture filtrates collected at different incubation times between tested pathogenic fungi due to *T.harzianum* ability to produce various defense enzymes and secondary metabolite containing antibiotics with varied nature, quantity and quality at different incubation times (Anita *et al*, 2012). *Trichoderma* spp. induces gene expression of proteins such as chitinase, glucanase, and peroxidase against antagonistic microbes (Hanson *et al.*, 2004).

The fresh weight of bio-agents varied from 7.89 to 13.11gm while dry weight from 1.89 gm to 2.47gm and pH from 3.7 to 5.8 in ZnSO₄ amended growth medium. The highest biomass production of *T. reesei-2* was recorded with ZnSO₄ while least identical with *T.reesei-1*. The highest fresh weight *T.reesei-1* was recorded in growth medium not amended with ZnSO₄. While highest biomass of *T. ressei-1* was recorded in the same medium. The *T. ressei-2* was next the best to *T. ressei-1* in biomass production followed by *T.harzianum*. The pH of *Trichoderma* species grown on ZnSO₄ amended medium ranged from 3.7 to 5.8. The lower pH of medium was recorded on which were grown in *Trichoderma* species in non

zinc-sulphate amended medium. The change in biomass production by different bioagents could be due to the differential requirement for the micronutrient by the bioagent's. Similarly, change in pH may be due to the different metabolites produced by different pathogen and their reaction with ZnSO₄. Mycelial growth can be stimulated by some heavy metals (Al, Fe, Mo, Pb) and inhibited by others (Cd, Co, Ni, Se) was reported by Galus (1997). Copper salts, zinc salts, calcium hydroxide, potassium hydroxide and selected nitrogen sulfur and molybdenum compounds have been found to be highly toxic to pathogenic fungi. Metal ions applied at certain concentrations under laboratory conditions may lead to the death of the tested microorganisms, where in a natural environment they can stimulate microbial growth. The effects exerted by metal ions are determined by their chemical form availability and environmental factors (Durska, 2006).

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How to cite this article:

Surya Prakash Redd, M., Vibha and Sunil Kumar Pandey 2018. Role of Root Colonizing *Trichoderma* Species in Management of *Alternaria* Leaf Blight of Asafotila (*Lepidium sativum* L.) Caused by *Alternaria alternata*. *Int.J.Curr.Microbiol.App.Sci.* 7(07): 2544-2561.
doi: <https://doi.org/10.20546/ijcmas.2018.707.299>