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Bio-control efficacy and chitinase production ability of *Trichoderma* spp. isolated from saline soil

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Abstract
The aim of the study was to assess the potential of Trichoderma spp. isolated from saline soil against predominant soil borne plant pathogens viz., *Fusarium oxysporum, Sclerotium rolfsii* and *Rhizoctonia bataticola.*14 Trichoderma isolates were isolated from Amravati, Akola and Buldhana districts located in saline tract of Purna valley in Vidarbha region of Maharashtra state and given code to each isolate. Efficacy of these *Trichoderma* spp. were tested by employing dual culture technique. All *Trichoderma* isolates were found significantly effective against tested pathogens. Among the isolates TrJg-11 (Jalgaon jamod) recorded maximum per cent growth inhibition of *Fusarium oxysporium* (74.79 per cent),TrBt-01 (Bhatkuli) found effective against *Sclerotium rolfsii*(73.61 per cent growth inhibition) and 66.00 per cent growth inhibition of *Rhizoctonia bataticola* was recorded by *Trichoderma* isolateTrDp-04 (Daryapur).The *Trichoderma* isolates were also assayed for estimation of chitinase enzyme and among the 14 isolates, TrNd-14 (Nandura) found to possessed highest chitinase enzyme i.e. 0.65 units/mg of protein.

Keywords: Bio-control, *Trichoderma* spp., saline soil

Introduction
Trichoderma spp. are known to exhibit mycoparasitism, antibiosis, enzyme secretion, competition and induction of systemic resistance in plants as a means to inhibit the growth and multiplication of its target fungi (Benitez *et al*., 2004) [1]. Plant disease management by *Trichoderma i*s based on complex interactions between the antagonist, the plant pathogen and the plant. Among several species of Biocontrol agents, *Trichoderma* is well documented myco-parasites and have been used successfully against certain pathogenic fungi. *T. harzianum, T. viride, T. virens, T. hamatum, T. roseum*and *T. koningii*are the species that most often used in biological control of plant pathogens.

Material and Methods
Collection of soil samples and Isolation
Soil samples were collected from saline soil of Purna valley located 14 tehsil of Amaravati, Akola and Buldhana districts of the Vidarbha region of Maharashtra State from rhizosphere soils of important crops. Serial dilution technique (Johnson and Curl, 1972) [5] was used to isolate *Trichoderma* spp. from rhizospheric soil. Prepare the stock solution by adding one gram of soil sample in nine ml distilled water. One ml of soil suspension from dilutions (10^{-3} and 10^{-4}) was aseptically poured on to sterilized Petri plates and then medium was poured at lukewarm stage. Plates were rotated gently to get uniform distribution of soil suspension in the medium. The plates were incubated at 28 ± 1 ^{0}C and observed at frequent intervals for the development of colonies.

Dual Culture
Antagonistic activity of *Trichoderma* isolates were assayed against soil borne plant pathogenic fungi by dual culture technique (Vincent, 1927) [13], 20 ml of sterilized and cooled potato dextrose agar was poured into sterilized petri plates. Isolates were evaluated by inoculating the pathogen at one side of the petri plate and the *Trichoderma* isolate was inoculated exactly on opposite side of the same plate by leaving 3 - 4 cm gap. For this, actively growing cultures were used. Control plate containing only pathogen was maintained and all the treatments were

replicated thrice. The radial mycelial growth was measured in three directions and average was recorded, per cent inhibition of growth of the test fungus was calculated (Dennis and Webster, 1971) [4].

Estimation of Chitinase enzyme

Estimation of chitinase enzyme in effective *Trichoderma* isolates was done by method suggested by Kulkarni and Ramanujam *et al.* (2010) [6]. *Trichoderma* isolated culture were grown on synthetic media (Czapek's broth) along with crab shell chitin (50 ml in 250 ml flask). After inoculating with 5 x 20^6/ml conidia, these flasks were kept on rotary shaker at 140 rpm at 25 ^{0}C for 4-5 days. Culture filtrate was collected after separating the biomass filtered with nylon cloth and dialyzed with 50 mm potassium phosphate buffer pH 6.7 (6:1) at 40 ^{0}C overnight. Sodium azide was added to keep it for further usage.

Measurement of Chitinase

Turbidity method

Endochitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin as per the method suggested by Kulkarni *et al*., (2010) [6]. A suspension containing 1% (w/v) or moist colloidal chitin was prepared in 50 mM potassium phosphate buffer, pH 6.7. A mixture consisting of 0.5 ml each of chitin suspension and the enzyme solution to be tested was prepared and inculcated for 24 h at 30 ^{0}C. Subsequently the mixture was diluted with 5 ml distilled water and the optical density was read at 510 nm. Enzyme activity was calculated as the percentage of reduction of a chitin suspension by 5 per cent.

Preparation of Colloidal Chitin and Phosphate Buffer

Colloidal chitin was prepared as per the method of Roberts and Selintrenikoff (1988) and stored at 4 ^{0}C for further use. Phosphate Buffer (pH 6.7) was prepared by adding Potassium Dihydrogen Phosphate (KH_2PO_4) 1 M, 136 gm in 1000 ml of distilled water and Potassium hypophosphate (K_2HPO_4) 1 M, 174 gm in 1000 ml of distilled water. Both were mixed together and dilute up to required concentration (50mM) and pH should be maintained 6.7.

Estimation of Protein

To estimate the protein concentration Lowry's method was followed (Lowry *et al*. 1951) [7] *Trichoderma* isolates culture were mass-cultivated on potato dextrose broth for 7- 10 days at 28 ± 2 °C. Towards the end of the incubation period, mycelia were harvested, washed in SDW and blot-dried. The mycelial mat was crushed in sterilized, pre-chilled pestle and mortar into a fine powder using liquid nitrogen. Protein Quantity was estimated from mycelia extract. 1 ml of aliquot was taken in centrifuge tube to which 1 ml of 10% Trichloroacetic acid was added to precipitate the protein. This mixture was allowed to stand and then centrifuged. Supernatant was discarded and the procedure is repeated twice. This sample is used for protein estimation. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 1 ml. The BSA range is 0.2 to 1 mg/ ml. The test tube with 1 ml distilled water serve as blank. Add 4.5 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well. This solution is incubated at room temperature for 10 mins. Then add 0.5 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Take the optical density (measure the absorbance) at 660 nm. Plot the absorbance against protein concentration to get a standard calibration curve. Check the absorbance of unknown sample and Estimate the amount of protein present in the given sample from the standard graph

Results and Discussion

Efficacy of *Trichoderma* isolates against soil borne plant pathogens (Per cent Growth Inhibition) at 7 DAI

Table 1: Efficacy of *Trichoderma* isolates against *Fusarium oxysporum* (Per cent Growth Inhibition) at 7 DAI

S.N.	Treatment	Code	Mean Radial Growth (mm)	Per cent Growth Inhibition (%)
1	T_1	TrBt-01	26.27	70.81
2	T_2	TrCb-02	25.65	71.50
3	T_3	TrAc-03	29.17	67.59
4	T_4	TrDp-04	26.95	70.06
5	T_5	TrMr-05	25.87	71.26
6	T_6	TrBp-06	27.00	70.00
7	T_7	TrTl-07	26.85	70.17
8	T_8	TrAt-08	31.00	65.56
9	T_9	TrAk-09	23.85	73.50
10	T_{10}	TrSg-10	27.54	69.40
11	T_{11}	TrJg-11	22.69	74.79
12	T_{12}	TrSp-12	26.58	70.46
13	T_{13}	TrMp-13	24.48	72.80
14	T_{14}	TrNd-14	28.62	68.20
	Control		90.00	0.00
	F' test		Sig.	-
	SE(m)±		0.27	-
	CD (P=0.01)		1.05	-

Data is presented in table.1 and fig 1 all *Trichoderma* isolates were found effective against *Fusarium oxysporum*. Among the *Trichoderma* isolate, T_{11} (TrJg-11) recorded maximum growth inhibition i.e.74.79 per cent. The next best isolates were T_9 (TrAk-09) and T_{13} (TrMp-13) which were at par with each other and recorded73.50 and 72.80 per cent growth inhibition. This was followed by isolates T_2 (TrCb-02), T_1 (TrBt-01) andT_{12} (TrSp-12) were statistically at par with each other and recorded 70.81, 71.50 and 70.46 per cent growth inhibition. Whereas the lowest per cent growth inhibition was observed in T_8 (TrAt-08) i.e.65.56 per cent. These findings showed similarity with the observations made by Chaudhary *et al*. (2012) [3] and Cherkupally *et al*. (2017) [2] who have tested the *Trichoderma* isolates against *Fusarium oxysporum* and recorded the significant results.

Table 2: Efficacy of *Trichoderma* isolates against *Sclerotium rolfsii* (Per cent Growth Inhibition) at 7 DAI

S.N.	Treatment	Code	Mean Radial Growth (mm)	Per cent Growth Inhibition (%)
1	T_1	TrBt-01	23.75	73.61
2	T_2	TrCb-02	32.09	64.35
3	T_3	TrAc-03	25.25	71.95
4	T_4	TrDp-04	30.29	66.34
5	T_5	TrMr-05	29.40	67.33
6	T_6	TrBp-06	27.85	69.06
7	T_7	TrTl-07	30.58	66.02
8	T_8	TrAt-08	28.70	68.11
9	T_9	TrAk-09	31.11	65.43
10	T_{10}	TrSg-10	30.55	66.06
11	T_{11}	TrJg-11	29.06	67.71
12	T_{12}	TrSp-12	28.25	68.61
13	T_{13}	TrMp-13	31.19	65.34
14	T_{14}	TrNd-14	26.22	70.87
	Control		90.00	00.00
	F' test		Sig.	-
	SE(m)±		0.39	-
	CD (P=0.01)		1.50	-

Data is presented in table.2 and fig.2 all *Trichoderma* isolates were found effective against *Sclerotium rolfsii*. Among the *Trichoderma* isolate,T_1 (TrBt-01) recorded maximum growth inhibition i.e.73.61per cent. The next best isolate were T_3 (TrAc-03) and T_{14} (TrNd-14) which were at par with each other and recorded 71.95 and 70.87 per cent growth inhibition respectively. This was followed by isolates T_6 (TrBp-06), T_8 (TrAt-08), T_{11} (TrJg-11) andT_{12} (TrSp-12) were statistically at par with each other and recorded 69.06, 68.11, 67.71 and 68.61 per cent growth inhibition respectively. The lowest per cent growth inhibition was observed in T_2 (TrCb-02) i.e.64.35 per cent. Similar findings were recorded by Meher *et al.* (2018) [8] and Srinivasulu *et al.* (2005) [12] who reported maximum mycelial inhibition of *S. rolfsii* with *Trichoderma* spp. *in vitro*.

Table 3: Efficacy of *Trichoderma* isolates against *Rhizoctonia bataticola* (Per cent Growth Inhibition) at 7 DAI

S.N.	Treatment	Code	Mean Radial Growth(mm)	Per cent Growth Inhibition (%)
1	T_1	TrBt-01	32.53	63.85
2	T_2	TrCb-02	34.17	62.04
3	T_3	TrAc-03	37.08	58.80
4	T_4	TrDp-04	30.60	66.00
5	T_5	TrMr-05	36.40	59.56
6	T_6	TrBp-06	31.30	65.22
7	T_7	TrTl-07	32.83	63.52
8	T_8	TrAt-08	34.32	61.87
9	T_9	TrAk-09	38.07	57.70
10	T_{10}	TrSg-10	41.02	54.42
11	T_{11}	TrJg-11	33.07	63.26
12	T_{12}	TrSp-12	35.46	60.60
13	T_{13}	TrMp-13	31.00	65.56
14	T_{14}	TrNd-14	33.67	62.59
	Control		90:00	00.00
	F' test		Sig.	-
	SE(m)±		0.42	-
	CD (P=0.01)		1.63	-

Data is presented in table.3 and fig 3, all *Trichoderma* isolates were found effective against *Rhizoctonia bataticola.* Among the *Trichoderma* isolates, T_4 (TrDp-04) recorded maximum growth inhibition i.e. 66.00 per cent, which was at par with T_6 (TrBp-06) and T_{13} (TrMp-13) which were recorded 65.22 and 65.56 per cent growth inhibition respectively. The next best isolates were T_1 (TrBt-01), T_7 (TrTl-07) and T_{11} (TrJg-11) which was at par with each other and recorded 63.85,63.52 and 63.26 per cent growth inhibition respectively. Whereas the lowest per cent growth inhibition was observed in T_{10} (TrSg-10) i.e.54.42 per cent. Nagamani *et al.* (2017) [9] also evaluated twenty *Trichoderma* isolates for their efficacy against soil borne plant pathogen *R. bataticola.* In case of *R. bataticola, T. asperellum* inhibited the mycelial growth of test pathogen by 82.5 per cent followed by *T. asperellum* (ATPU 1and KNPG 3) with 80.6 per cent inhibition over control and least recorded in *T. viride* (KJ 12) with 64.7 per cent.

Table 4: Chitinase enzyme units/ mg of protein in *Trichoderma* isolates of saline soil

S.N.	Treatment	Code	Chitinase enzyme units/ mg of protein			Mean Chitinase enzyme units/ mg of protein
			RI	RII	RIII	
1	T_1	TrBt-01	0.50(1.00)*	0.50(1.00)*	0.49(0.99)*	0.50(1.00)*
2	T_2	TrCb-02	0.50(1.00)	0.49(0.99)	0.49(0.99)	0.49(1.00)
3	T_3	TrAc-03	0.61(1.05)	0.62(1.06)	0.61(1.05)	0.61(1.06)
4	T_4	TrDp-04	0.58(1.04)	0.58(1.04)	0.58(1.04)	0.58(1.04)
5	T_5	TrMr-05	0.56(1.03)	0.56(1.03)	0.57(1.03)	0.56(1.03)
6	T_6	TrBp-06	0.65(1.07)	0.64(1.07)	0.64(1.07)	0.64(1.07)
7	T_7	TrTl-07	0.60(1.05)	0.58(1.04)	0.62(1.06)	0.60(1.05)
8	T_8	TrAt-08	0.46(0.98)	0.46(0.98)	0.45(0.97)	0.46(0.98)
9	T_9	TrAk-09	0.61(1.05)	0.60(1.05)	0.61(1.05)	0.61(1.05)
10	T_{10}	TrSg-10	0.37(0.93)	0.37(0.93)	0.38(0.94)	0.37(0.93)
11	T_{11}	TrJg-11	0.54(1.02)	0.54(1.02)	0.54(1.02)	0.54(1.02)
12	T_{12}	TrSp-12	0.48(0.99)	0.49(0.99)	0.48(0.99)	0.48(0.99)
13	T_{13}	TrMp-13	0.60(1.05)	0.59(1.04)	0.60(1.05)	0.60(1.05)
14	T_{14}	TrNd-14	0.65(1.07)	0.65(1.07)	0.66(1.08)	0.65(1.07)
		F' test				Sig.
		SE(m)±				0.021
		CD (P=0.01)				0.063

The data presented in table 4. revealed that *Trichoderma* isolate T_{14} (TrNd-14) content maximum i.e. 0.65 chitinase enzyme units/ mg of protein, which was at par with T_6 (TrBp-06), T_3 (TrAc-03), T_9 (TrAk-09), T_7 (TrTl-07) and T_{13} (TrMp-13) i.e. 0.64, 0.61, 0.61, 0.60 and 0.60 unit /mg of protein respectively. The next best isolates were T_4 (TrDp-04), T_5 (TrMr-05) and T_{11} (TrJg-11) which contained chitinase enzyme units/ mg of protein 0.58, 0.56 and 0.54 respectively. Whereas the lowest chitinase enzymei.e.0.37 enzyme units/mg of protein was estimated in T_{10} (TrSg-10). Kulkarni S. and Ramanujam *et al*. (2010) [6] also studied the ability of *Trichoderma* isolates to produce chitinase enzyme through polyacralamide gel electrophoresis (SDS-PAGE) method related to their antagonistic ability will help to identify the markers and it can be inserted in to the plant itself through genetic engineering to evolve resistant varieties or these markers may be inserted into *Trichoderma species* itself to promote its antagonistic ability.

Fig 1: Antagonistic activity of *Trichoderma* isolates against *F. oxysporum* at 7 DAI

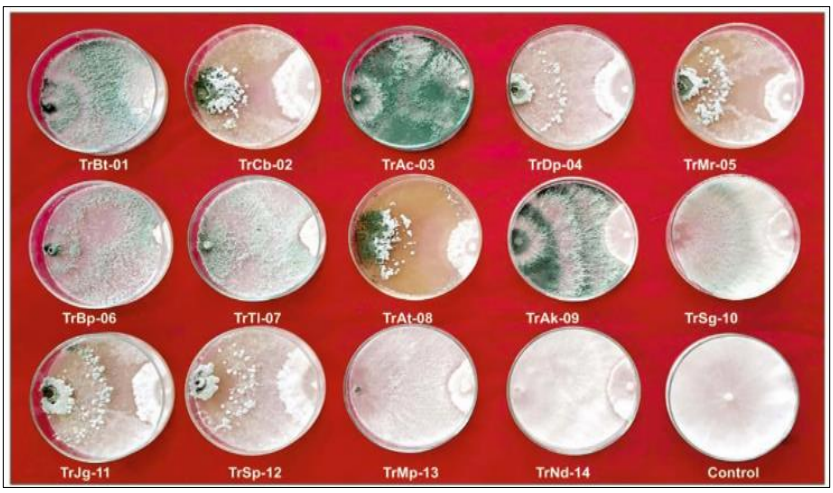

Fig 2: Antagonistic activity of *Trichoderma* isolates against *S. rolfsii* at 7 DAI

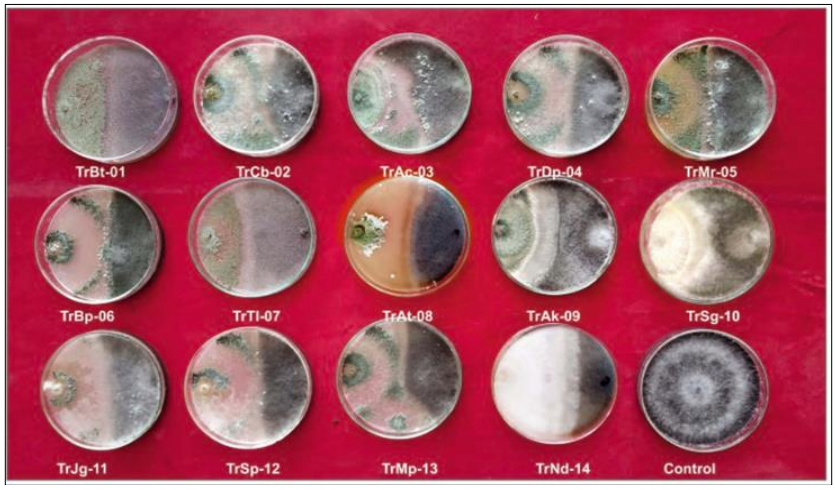

Fig 3: Antagonistic activity of *Trichoderma* isolates against *Rhizoctonia bataticola* at 7 DAI

Conclusion

- There is existence of *Trichoderma* in saline tract (Less than 8.5) of Purna valley located in 14 tehsils of Amravati, Akola and Buldhana districts in Vidarbha region of Maharashtra state.
- Isolates were differed in their antagonistic potential when tested against soil borne plant pathogen *viz*., *Fusarium oxysporum. Sclerotium rolfsii* and *Rhizoctonia bataticola*
- Maximum growth inhibition of *Fusarium oxysporium* (74.79 per cent), *Sclerotium rolfsii* (73.61 per cent) *and Rhizoctonia bataticola* (66.00 per cent) were recorded by *Trichoderma* isolates TrJg-11, TrBt-01 and TrDp-04 respectively.
- The *Trichoderma* isolates TaNd-14, TrBp-06, TrAc-03, TrAk-09, TrTl-07, and TrMp13 exhibited highest chitinase enzyme unit /mg of protein.

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