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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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VIKAS R. MHASKE, S.T. INGLE, P.R. PADGHAN AND V.D. KINWATKAR
Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola-444104, Maharashtra

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Trichoderma, soil-borne filamentous fungi, are capable of parasitizing several plant pathogenic fungi. Fourteen isolates of *Trichoderma* spp. from different locations of Amravati, Akola and Buldhana districts located in saline tract of Purna valley in Vidarbha region of Maharashtra statewere characterized for their cultural, morphological features after assigning different code to each isolate. Significant chitinase activities of all *Trichoderma* isolates has been recorded in growth medium. TrNd-14 (Nandura) was found to possess highest chitinase enzyme i.e. 0.65 units/mg of protein.

Key words: Bio-control agent, chitinase enzyme, mycoparasitism, *Trichoderma* spp.

INTRODUCTION

The use of bio-agents having bio-control and plant growth promotion (PGP) activities have been considered as naturally and environmentally acceptable alternative to minimize the use of synthetic chemicals and their hazardous effects, and to provide protection to the plants against resident pathogen populations (Lugtenberg *et al*. 2001). Fungi are the most extensively researched group of biological control agents. Over 75 years ago, the antagonistic nature of fungal species from the genus, *Trichoderma* was demonstrated. The genus *Trichoderma* is the most common saprophytic fungi in the rhizosphere and widely distributed in all types of soil and other diverse habitats (Hajieghrari *et al*. 2008).

Rapid growth rate in culture and production of numerous spores that are varying shades of green characterize this fungus (Howell, 2003; Shalini and Kotsthane, 2007). They are favoured by the presence of high levels of plant organic matter and plant roots, which they colonize readily. Some strains are highly rhizosphere competent, i.e. able to colonize and grow on roots (Harman *et al.* 2004).

*Correspondence :vikasrmhaske@gmail.com

MATERIALS AND METHODS

Collection of soil samples and isolation

Soil samples were collected from different ecological habitat of field crops of Purna valley located 14 tehsil of Amaravati, Akola and Buldhana districts of the Vidarbha region of Maharashtra State for the isolation of *Trichoderma* spp. (Table 1**).** Samples were brought to laboratory and stored at 4°C until used. Five-fold serial dilutions of each soil samples were prepared in sterilized distilled water and 0.5 ml diluted sample was poured on the surface of Trichoderma Specific Medium (TSM) (Elad *et al.*1981). Plates were incubated at 28 ± 2°C for 96 h. Morphologically different colonies appearing on the plates were purified in the Potato Dextrose Agar (PDA) (HiMedia, India). The purified isolates were preserved at 4°C and used during the course of study.

Morphological Characters of the Trichoderma Isolates

The morphological and cultural characteristics of 14 isolates of *Trichoderma* were studied in four different media viz., PDA and TSM following the

protocol of Samuels *et al.*(2016). Mycelial discs (6 mm) of young growing culture of respective isolates of *Trichoderma* was inoculated in the periphery of the Petri plates containing above said media and incubated at 28 ± 2°C for one week. Colony radius was measured at 24, 48 and 72 h. each growth rate experiment was repeated three times in triplicate and the results were averaged for each isolate. Additional characters include presence of pigments, green conidia, odor and colony appearance were also noted. Morphological observations were recorded from cultures grown on PDA plates. The following characters were measured; Phialide, conidium and presence of chlamydospores. Each character was measured from each isolates.

Estimation of Chitinase enzyme

Estimation of chitinase enzyme in effective *Trichoderma* isolates was done by method suggested by Kulkarni and Ramanujam *et al.* (2010). Isolated cultures of *Trichoderma* 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 25^0C 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 40^0C 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). 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 30^0C. 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 4^0C 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 diluted up to required concentration (50mM) and pH maintained at 6.7.

Estimation of Protein

To estimate the protein concentration Lowry's method was followed (Lowry *et. al*. 1951) *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 content was estimated from mycelia extract. 1 ml of aliquot was taken in centrifuge tube to which 1 ml of 10% Trichloro acetic acid was added to precipitate the protein. This mixture was allowed to stand and then centrifuged. Supernatant was discarded and the procedure repeated twice. This sample was used for protein estimation, using BSA as standard.

RESULTS AND DISCUSSION

Growth rate of Trichoderma spp. isolated from saline soil

Mycelial growth rate of the *Trichoderma* spp. isolated from saline soil.Isolates growing on PDA medium were recorded 24, 48 and 72 h after inoculation and data is presented in Table 2. The highest mycelium growth i.e.35.16, mm/day recorded by TrAc-03 isolate. The next best isolates TrMp-13, TrAk-09, TrSp-12 and TrDp-04 which were at par with each other and recorded mycelial growth rate i.e. 34.97, 34.28, 34.27 and 33.97 mm/day. The lowest radial mycelium growth rate was observed in TrTl-07 i.e.31.62 mm/day. Satyakala *et al*. (2017) and Kumar *et al.* (2018) also measured the linear mycelia growth of *Trichoderma* isolates after 24, 48 and 72 hours at different pH levels and estimated the tolerance capacity of isolates. Barakat *et al.* (2006) reported that, *Trichoderma* isolate Jn14 showed maximum growth rate i.e. 27 mm/day on PDA medium whereas lowest growth 10.67 mm/day recorded by the isolate.

Table. 1: Collection of soil samples from different locations in saline tract of Purna valley

District	Location	Crop associated	Code name
Amravati	Bhatkuli	Soybean	TrBt-01
Amravati	Chandur bazar	Pigeon pea	TrCb-02
Amravati	Achalpur	Pigeon pea	TrAc-03
Amravati	Daryapur	Soybean	TrDp-04
Akola	Murtizapur	Black gram	TrMr-05
Akola	Balapur	Pigeon pea	TrBp-06
Akola	Telhara	Soybean	TrTl-07
Akola	Akot	Pigeon pea	TrAt-08
Akola	Akola	Green gram	TrAk-09
Buldhana	Shegaon	Black gram	TrSg-10
Buldhana	Jalgaon jamod	Pigeon pea	TrJg-11
Buldhana	Sangrampur	Soybean	TrSp-12
Buldhana	Malkapur	Soybean	TrMp-13
Buldhana	Nandura	Green gram	TrNd-14

Table 2: Growth rate of *Trichoderma* spp. isolated from saline soil

Trichoderma isolates	Mean Mycelial Growth (mm) After 24.00 h	48.00 h	72.00 h	Growth rate (mm/day)
TrBt-01	13.67	38.11	80.00	33.17
TrCb-02	15.30	49.07	81.80	33.25
TrAc-03	13.89	50.02	84.22	35.16
TrDp-04	11.05	48.53	78.98	33.97
TrMr-05	12.78	46.07	80.22	33.72
TrBp-06	12.97	48.18	79.02	33.03
TrTl-07	14.25	45.25	77.50	31.62
TrAt-08	13.85	47.19	81.62	33.89
TrAk-09	15.98	49.07	84.55	34.28
TrSg-10	12.72	43.33	80.15	33.71
TrJg-11	11.50	49.83	78.22	33.36
TrSp-12	14.08	51.17	82.63	34.27
TrMp-13	14.89	52.56	84.83	34.97
TrNd-14	12.11	44.07	78.50	33.20
F- test				Sig.
SE(m)±				0.19
CD (P=0.01)				0.75

Table 3: Cultural and morphological characteristics of *Trichoderma* spp. of saline soil

Code	Cultural and morphological characteristics: Colony Growth (mm) at 7th DAI	Colony growth type	Colony colour	Pigmentation	Phialides	Conidia shape
TrBt-01	82.90	Sub aerial and disperse	Light green	Whitish grey colour	Branched or ampuliform	Globules
TrCb-02	87.50	Flat with concentric ring	Light green to pale yellow	Light yellow colour	Frequently paired	Ellipsoidal
TrAc-03	90.00	Flat and superficial	Dark green	Ash grey colour	Ampuliform	Globules to slightly oval
TrDp-04	89.67	Flat with concentric ring	Light green to pale yellow	Amber colour	Branched	Ellipsoidal
TrMr-05	88.25	Flat with concentric ring	Greenish yellow	Light yellow colour	Frequently paired	Oblong to Ellipsoidal
TrBp-06	89.33	Sub aerial and disperse	Light green	Whitish grey colour	Branched	Globules
TrTl-07	85.67	Superficial and disperse	Milky white to light green	Creamy white colour	Frequently paired	Globules
TrAt-08	87.30	Flat with concentric ring	light Green to Yellow	Amber colour	Lageniform or bottle shaped	Ellipsoidal
TrAk-09	90.00	Flat and superficial	Dark green	Ash grey colour	Ampuliform	Globules
TrSg-10	88.50	Superficial	Light Green	Yellow colour	Ampuliform	Globules
TrJg-11	89.67	Flat with concentric ring	Greenish to pale yellow	Light Yellow colour	Frequently paired	Ellipsoidal
TrSp-12	90.00	Flat with concentric ring	Greenish to pale yellow	Amber colour	Cylindrical or slightly inflated	Ellipsoidal
TrMp-13	90.00	Sub aerial and disperse	Whitish to light green	Whitish grey colour	Lageniform or bottle shaped	Globules
TrNd-14	87.90	Flat and Disperse	Whitish to light green	Whitish grey colour	Ampuliform	Globules

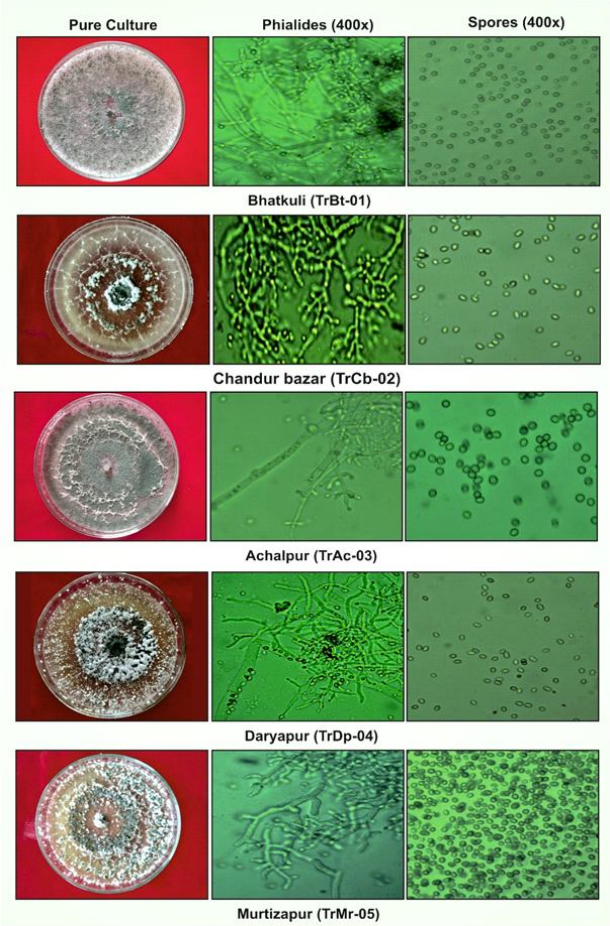

Fig. 1 : Growth of *Trichoderma* isolates (TrBt-01,TrCb-02, TrAc-03,TrDp-04,TrMr-05) in Petri plates and microscopic observation of their phialides and spores

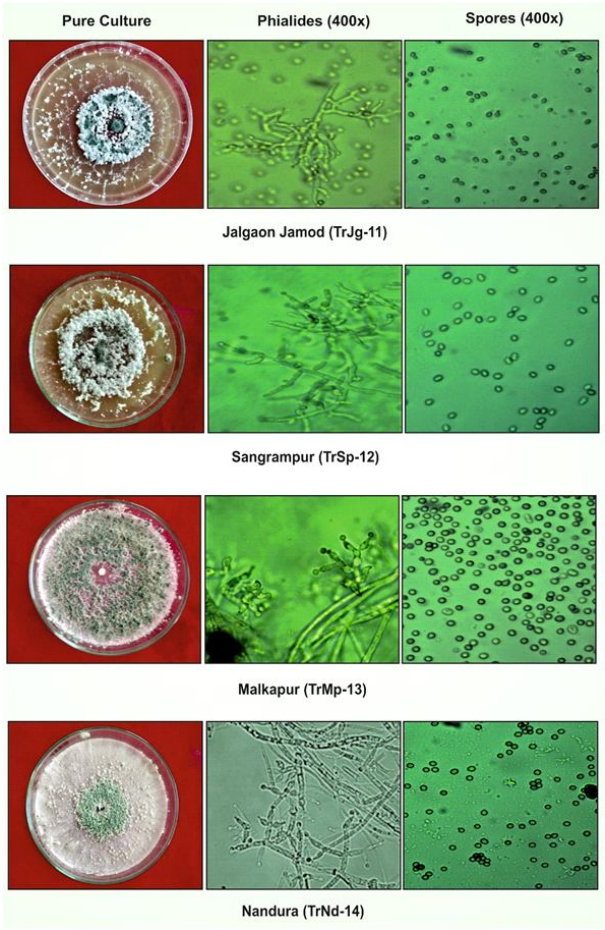

Fig. 3: Growth of *Trichoderma* isolates (TrJg-11, TrSp-12, TrMp-13, TrNd-14) in Petri plates and microscopic observation of their phialides and spores

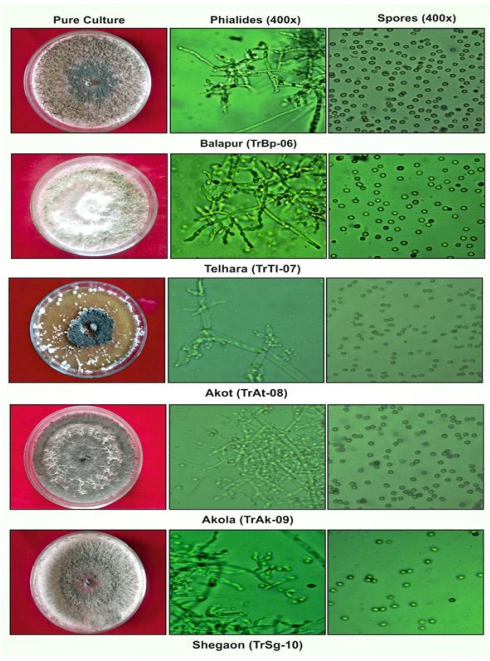

Fig. 2: Growth of *Trichoderma* isolates (TrBp-06,TrTl-07, TrAt-08, TrAk-09, TrSg-10) in Petri plates and microscopic observation of their phialides and spores

Cultural and morphological characteristics of Trichoderma isolates

The *Trichoderma* isolates maintained on the PDA medium showed variation. The morphological study was based on different parameters like colony growth, colony color, and pigmentation. Colony characters of *Trichoderma* isolates were studied using 7 days old cultures that were incubated at 28°.C ± 35°C. At 28°C, all *Trichoderma* isolates grew well and formed conidia within 4 days. The radial growth (mm) of all isolates was measured at 7^{th} days after inoculation. Also the spore size, spore shape, spore count and phialides were recorded (Table 3, Figs 1-3).

Chitinase Activity

The data presented in Table 4 revealed that *Trichoderma* isolate T_{14} (TrNd-14) had maximum chitinase activity, i.e. 0.65 chitinase enzyme units/ mg of protein, which was at par with T_6 (TrBp-06),

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

Treatme	Code	Chitinase enzyme units/ mg of protein			Mean Chitinase enzyme units/ mg of protein
		RI	RII	RIII	
T_1	TrBt-01	0.50(1.00)*	0.50(1.00)*	0.49(0.99)*	0.50(1.00)*
T_2	TrCb-02	0.50(1.00)	0.49(0.99)	0.49(0.99)	0.49(1.00)
T_3	TrAc-03	0.61(1.05)	0.62(1.06)	0.61(1.05)	0.61(1.06)
T_4	TrDp-04	0.58(1.04)	0.58(1.04)	0.58(1.04)	0.58(1.04)
T_5	TrMr-05	0.56(1.03)	0.56(1.03)	0.57(1.03)	0.56(1.03)
T_6	TrBp-06	0.65(1.07)	0.64(1.07)	0.64(1.07)	0.64(1.07)
T_7	TrTl-07	0.60(1.05)	0.58(1.04)	0.62(1.06)	0.60(1.05)
T_8	TrAt-08	0.46(0.98)	0.46(0.98)	0.45(0.97)	0.46(0.98)
T_9	TrAk-09	0.61(1.05)	0.60(1.05)	0.61(1.05)	0.61(1.05)
T_{10}	TrSg-10	0.37(0.93)	0.37(0.93)	0.38(0.94)	0.37(0.93)
T_{11}	TrJg-11	0.54(1.02)	0.54(1.02)	0.54(1.02)	0.54(1.02)
T_{12}	TrSp-12	0.48(0.99)	0.49(0.99)	0.48(0.99)	0.48(0.99)
T_{13}	TrMp-13	0.60(1.05)	0.59(1.04)	0.60(1.05)	0.60(1.05)
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

*Figures in parentheses are square root transformed values

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 activity i.e.0.37 enzyme units/ mg of protein was estimated in T_{10} (TrSg-10). Kulkarni and Ramanujam *et al*. (2010) also studied the ability of *Trichoderma* isolates to produce chitinase enzyme through polyacralamide gel electrophoresis (SDS-PAGE) method and suggested that this will help to identify the markers which 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.

CONCLUSION

There is existence of *Trichoderma* in saline tract (pH Less than 8.5) of Purna valley located in 14 tehsils of Amravati, Akola and Buldhana districts in Vidarbha region of Maharashtra state.Isolates differed in their growth rate with the highest mycelium growth i.e.35.16, mm/day recorded by TrAc-03. The *Trichoderma* isolatesTaNd-14, TrBp-06, TrAc-03, TrAk-09, TrTl---07, and TrMp13 exhibited highest chitinase enzyme unit /mg of protein.

REFERENCES

Barakat, R.,Al- Masri, M.I.,Al- Mahareeq, F. 2006. Biological Control of Sclerotium rolfsii by Using Indigenous *Trichoderma* spp. Isolates from Palestine. *Hebron Univ Res J.* 2.

Elad, Y., Chet, I., &Henis, Y. (1981). A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytotoparasitica* **9**: 59-67.

Hajieghrari, B., Mousa Torabi-Giglou, M., Mohammad Reza Mohammadi, M.R.,Davari, M.

2008 Biologicalpotantial of some Iranian Trichoderma isolates in the control of soil borne plant pathogenic fungi. *Afr. J.Biotechnol.***7**: 967-972

Howell, C.R. 2003. Mechanisms Employed by Trichoderma Species in the BiologicalControl of Plant Diseases:The History and Evolution of Current Concepts. *Plant Dis*.**87**: 4-10.

Harman,G. E .2004.*Trichoderma* species—opportunistic, avirulent plant symbionts. *Nature Rev.Microbiol.***2**: 43-56

Kulkarni S., B, R.J Ramanujam, M. Rabindra. Nagesh and N. R. Roa, 2010. Isolation, identification and documentation of efficient chitinase enzyme production ability strains of *Trichoderma*. *J .Pl. Dis. Sci.* **5**:198-202.

Kumar, M., Sharma, P. 2016. Morphological Characterization of Biocontrol Isolates of *Trichoderma* to Study the Correlation between Morphological Characters and Biocontrol efficacy. *Inter. Lett. Natur. Sci.***55**: 57-67.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

Lugtenberg, B.J.J., Dekkers, L., Bloemberg, G.V.2001. Molecular determinants of rhizosphere colonization by bacteria. *Annu. Rev. Phytopathol.***39**:461-90.

Roberts, W. K., Selitrennik, C. P. 1988. Plant and Bacterial Chitinases Differ in Antifungal Activity. *J. Gen. Microbiol.* **134**: 169 –176.

Samuels G.J., Dodd, S.L., Lu, B-S., Petrini, O., Schroers, H-S., Druzhinina, I.S. 2006. The *Trichoderma koningii* aggregate species. *Studies Mycol.***56**: 67-133.

Satyakala, K., Alladi, A., Thakur, K. D. 2017. Effect of Physiological Parameters on Growth of *Aspergillus niger* and *Trichoderma harzianum*, *Int. J. Pure App. Biosci.***5** :1808-1812.

Vishwanath, K., Gopal, K., Gopi, V. 2008. Isolation of potential *Trichoderma* spp. associated with Dry Rot infected Acid Lime (*Citrus aurantifolia* Swingle). *J.Plant Dis.Sci.* **3**:165-168.