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Isolation and characterization of *Trichoderma asperellum* for antagonistic activity against different soil-borne plant pathogens

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Abstract
Rhizospheric soil samples were collected from Akola, Pune, Solapur, Amravati, Sangli and Nagpur and abbreviated as Tv1, Tv2, Tv3, Tv4, Tv5 and Tv6, respectively for the isolation of *Trichoderma asperellum* on *Trichoderma* Selective Medium (TSM) by serial dilution and pour plate technique. Morphological characteristic *viz*., radial growth and colony characters of these isolates were studied on PDA. The maximum radial growth (mm) was recorded in Tv1 and Tv4 i.e. 90.00 mm collected from Akola and Amravati districts from cotton and soybean crop, respectively whereas, minimum was 87.50 mm observed inTv3 of Solapur district from Jowar crop. The antagonistic efficacy of *Trichoderma asperellum* against the soil born plant pathogens *viz*., *Fusarium solani*, *Sclerotium rolfsii* and *Rhizoctonia bataicola* were tested. The maximum per cent inhibition (82.44%) and minimum radial growth (15.80 mm) of *Fusarium solani* was observed in isolate Tv5. In case of *Sclerotium rolfsii* the minimum radial growth (17mm) with maximum per cent inhibition (81.11%) was recorded in isolate Tv3 collected from Solapur district. Antagonism between *Trichoderma asperellum* and test pathogen *Rhizoctonia bataticola* showed maximum growth inhibition (80.33%) in Tv3 isolate.

Keywords: *Trichoderma asperellum*, isolation, soil-borne pathogen, location, antagonism

Introduction
The *Trichoderma* spp. serves as a potential alternative to chemical control measure and growing pathogen resistance crop cultivars. *Trichoderma* is easily identified in culture media, which produces large number of characteristics small, green or white conidia, from phialides present on the profusely or meagerly branched conidiophores. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters of the species. Molecular analysis of several strains revealed that classification based on morphological data has been, erroneous to great extent resulting in re-classification of several isolates and species (Samuels, 1996) [14].

The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, ability to survive under unfavourable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms. These properties have made *Trichoderma* a ubiquitous genus present in any habitat and at high population densities (Chet *et al*., 1997) [5].

Mechanisms involved in the biocontrol activity of *Trichoderma* spp. against plant pathogens are important in designing effective and safe biocontrol strategies. Different proposed mechanisms include: mycoparasitism (attack and killing of pathogen) and competitive inhibition for space and nutrients. *Trichoderma* are also known to produce different antibiotic substances e.g. Gliotoxin, Gliovirin, Viridin, and Trichoviridin. *Trichoderma* have also been known to inhibit the growth of pathogenic fungi by modifying the rhizosphere. Moreover, infestation of *Trichoderma* in the rhizosphere helps plant to promote nutrient / fertilizer uptake, seed germination and photosynthetic rates (Asad *et al*. 2014) [2].

There are many biological control agents which have been reported as an efficient alternative to reduce the use of fungicides. However, in order to continue management of new strain / races of soil borne fungal pathogens being evolved in the nature, there is necessity to evolve

new effective biocontrol agents. Amongst many effective bio control agents, *Trichoderma* is one of them whose species have been reported to be inhibitory to many soil borne pathogens (Harman *et al*., 2004) [7].

Material and Methods

Selection of sites for sampling and soil sample collection

Soil samples were collected from different districts of Maharashtra. The approachable locations of different districts were selected and visited for soil sampling (Table No.1). Generally healthy plants were selected from standing crop of that location and rhizospheric soil was collected. For rhizospheric soil, plant was gently and carefully uprooted, soil tightly adhering the root was collected, such five samples were collected randomly from the crop field, mixed and 1/4th part was used as composite rhizospheric soil sample of the region.

Table 1: Soil samples collected from different districts of Maharashtra

Sr. No.	Location	Crop associated
1.	Akola	Cotton
2.	Pune	Maize
3.	Solapur	Jowar
4.	Amravati	Soybean
5.	Sangli	Brinjal
6.	Nagpur	Pigeon pea

Isolation of *Trichoderma asperellum* from rhizospheric soil by serial dilution method

The *Trichoderma asperellum* was isolated from the soil collected from the different locations by serial dilution technique. The *Trichoderma* selective medium was used for the isolation of *Trichoderma asperellum*. 1 ml of soil suspension from dilutions (10^{-3} and 10^{-4}) was aseptically added to sterile petriplates containing twenty ml of *Trichoderma selective* medium and incubated 37ºC for 3 days. After incubation, well separated individual colonies with yellow green and whitish green pigments were marked. The individual colonies were picked up with sterile loop and transferred to Potato Dextrose agar media plates and the pure cultures so obtained were stored in a refrigerator at 40 ºC for further use. (Arumugam K. *et al*. 2013) [1].

Trichoderma selective medium (TSM) (Elad *et al*., 1981: Mukherjee, 1991) [6, 11] was used for isolation of *Trichoderma asperellum*. The ingredients are as follows:

Table 2: Ingredients of *Trichoderma* Selective Medium (TSM)

Chemicals	Quantity
$MgSO_4.7H_2O$	0.2 g
K_2HPO_4	0.9 g
KCl	0.15 g
NH_4NO_3	1.0 g
Glucose	3.0 g
Chloramphenicol	0.20 g
Apron 35SD	0.015 g
Captan	0.2 g
Rose Bengal	0.15 g
Agar-agar	20.0 g
Distilled water	To make volume 1Litr.

Table 3: Location and code of *Trichoderma asperellum* isolates

Sr. No.	Location	Crop associated	Code name
1.	Akola	Cotton	Tv1
2.	Pune	Maize	Tv2
3.	Solapur	Jowar	Tv3
4.	Amravati	Soybean	Tv4
5.	Sangli	Brinjal	Tv5
6.	Nagpur	Pigeon pea	Tv6

Purification of *Trichoderma asperellum* cultures

Trichoderma asperellum isolates were purified by single spore culture. The spores of the isolates were inoculated into a Petri dish seeded with PDA medium. Sub- culturing was done from the growing front of the single new colony. Small amount of spores were taken on the tip of a sterilized inoculating needle and streaked on PDA poured Petri dishes. This process was repeated by taking inoculum from edge of colonies growing in the freshly streaked Petri plate, and again streaking it in PDA plates. Colony arising from single spore was picked up and inoculated on a fresh plate. This culture was used for further studies.

Study of morphological characteristics and growth rate of *Trichoderma asperellum* isolates

The morphological characteristics and growth rates of the 6 isolates of *Trichoderma asperellum* were determined on Potato dextrose agar (PDA) medium. A 5 mm diameter plug was cut from the actively growing edge of a fresh colony (before the start of conidial production) of the isolates, using a sterile cork borer. The disc was placed in a 90 mm Petri dish, containing 20 ml of PDA medium, approximately 1.5 cm from the edge of the Petri dish with the mycelial surface facing downwards. Three replications were maintained for each isolate. The Petri dishes were incubated in darkness at 28±10 ^{0}C.The colonies were examined at 24 h intervals and colony radius was measured from the edge of the inoculum plug after 7 days. The following observations on growth rate and cultural characters of the isolates were recorded:

1. Colony diameter on PDA after 7 days
2. Colony growth type
3. Colony colour
4. Pigmentation in the colony

Collection of pure cultures of soil inhibiting plant pathogens

Pure cultures of soil born plant pathogen *viz*., *Fusarium solani*, *Sclerotium rolfsii* and *Rhizoctonia bataticola*, were collected from Department of Plant Pathology, Dr. P.D.K.V, Akola, which previously known pathogenic nature.

Dual culture Technique

Antagonistic activity of *Trichoderma* isolates were assayed against *Rhizoctonia bataticola*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. Sp *solani* by using dual culture inoculation technique described by Vincent, (1927) [19], Mandal *et al*., (1999) [9] in Petri plates. Five mm disc from the periphery of actively growing pathogen on PDA was placed in centre of 90 mm diameter Petri plates containing PDA. Three discs of each actively growing isolates of *Trichoderma asperellum* were placed at equidistance on all four sides 30

mm apart from centre disc of pathogenic fungus. The plates were incubated at ambient condition under alternate dark and light cycle up to 7 days. Simultaneously the pathogenic fungus disc (5mm) was incubated on PDA Petri plates alone and incubated under similar condition for same period. Plates were observed every day for nothing the behaviour at the point of intermating of two cultures under stereoscopic microscope. On seventh days after incubation, the growth of pathogenic test fungus was measured and per cent growth inhibition was calculated using the following formula.

$$\text{Per cent Growth inhibition} = \frac{C - T}{C} \text{ X } 100$$

Where

C = Mycelial growth (mm) in control plate.

T = Mycelial growth (mm) in treatment plate.

Table 4: Antagonistic efficacy of *Trichoderma asperellum* isolates against *Fusarium solani* in dual culture technique

Treatment No.	Code name	Description
T_1	Tv1	*Trichoderma asperellum* (Tv1) + *Fusarium solani*
T_2	Tv2	*Trichoderma asperellum* (Tv2) + *Fusarium solani*
T_3	Tv3	*Trichoderma asperellum* (Tv3) + *Fusarium solani*
T_4	Tv4	*Trichoderma asperellum* (Tv4) + *Fusarium solani*
T_5	Tv5	*Trichoderma asperellum* (Tv5) + *Fusarium solani*
T_6	Tv6	*Trichoderma asperellum* (Tv6) + *Fusarium solani*
T_7	Control	*Fusarium solani* culture

Table 5: Antagonistic efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii* in dual culture technique

Treatment No.	Code name	Description
T_1	Tv1	*Trichoderma asperellum* (Tv1) + *Sclerotium rolfsii*
T_2	Tv2	*Trichoderma asperellum* (Tv2) + *Sclerotium rolfsii*
T_3	Tv3	*Trichoderma asperellum* (Tv3) + *Sclerotium rolfsii*
T_4	Tv4	*Trichoderma asperellum* (Tv4) + *Sclerotium rolfsii*
T_5	Tv5	*Trichoderma asperellum* (Tv5) + *Sclerotium rolfsii*
T_6	Tv6	*Trichoderma asperellum* (Tv6) + *Sclerotium rolfsii*
T_7	Control	*Sclerotium rolfsii* culture

Table 6: Antagonistic efficacy of *Trichoderma asperellum* isolates against *Rhizoctonia bataticola* in dual culture technique

Treatment No.	Code name	Description
T_1	Tv1	*Trichoderma asperellum* (Tv1) + *Rhizoctonia bataticola*
T_2	Tv2	*Trichoderma asperellum* (Tv2) + *Rhizoctonia bataticola*
T_3	Tv3	*Trichoderma asperellum* (Tv3) + *Rhizoctonia bataticola*
T_4	Tv4	*Trichoderma asperellum* (Tv4) + *Rhizoctonia bataticola*
T_5	Tv5	*Trichoderma asperellum* (Tv5) + *Rhizoctonia bataticola*
T_6	Tv6	*Trichoderma asperellum* (Tv6) + *Rhizoctonia bataticola*
T_7	Control	*Rhizoctonia bataticola* culture

Results and Discussion

Morphological Characters of *Trichoderma asperellum*

Morphological characters of *Trichoderma asperellum* with respect to radial growth and colony characters were studied on PDA.

The radial growth (mm) of all isolates was measured at 7th days after inoculation. The maximum radial growth (mm) was recorded in Tv1 and Tv4 i.e. 90.00 mm collected from Akola and Amravati Districts on cotton and soybean crop respectively whereas minimum was (87.50 mm) observed inTv3 of Solapur District from Jowar crop.

Among six isolates of *Trichoderma asperellum* Tv1, Tv5 and Tv6 are milky white to dark green in colour with white yellow and amber colour pigmentation, respectively having subaerial and disperse mycelial growth. The isolate Tv2 produce greenish yellow colony colour with yellow pigmentation having flat and disperse mycelial growth. The isolate Tv3 had subaerial mycelial growth, milky white to grayish green colony colour with yellow pigmentation. Inisolate Tv4, flat and subaerial mycelial growth was observed with light grey to greenish yellow colony colour and white pigmentation on PDA.

The present results are in agreement with Soesanto *et al.* (2011) [16] and Khang *et al*. (2013) [8], who were isolated *Trichoderma* spp. and studied the colony colour as velvetinous with white and dark green flocouse surface along with scattered green patches and yellow to green pigmentation on PDA medium.

Table 7: Morphological characteristics of *Trichoderma asperellum* collected from different Districts of Maharashtra

Sr. No.	Isolates	Radial growth (mm) at 7 DAI	Colony characters		
			Colony growth type	Colony colour	Pigmentation
1.	Tv1	90.00	Sub aerial and disperse	Milky white to dark green	White colour
2.	Tv2	89.67	flat and disperse	Greenish yellow	Yellow colour
3.	Tv3	87.50	Sub aerial and disperse	Milky white to grayish green	Yellow colour
4.	Tv4	90.00	Flat and Superficial	Light grey to greenish yellow	White colour
5.	Tv5	87.90	Disperse and superficial	Milky white to dark green	Yellow colour
6	Tv6	89.33	Sub aerial and disperse	Milky white to dark green	Amber colour

Antagonistic efficacy of *Trichoderma asperellum* isolates against *Fusarium solani, Scleritium rolfsii and Rhizoctonia bataticola* (per cent growth inhibition) at 7 DAI

The maximum per cent inhibition (82.44%) and minimum radial growth (15.80 mm) of *Fusarium solani* was observed in isolate Tv5 which was at par with isolate Tv3 *i.e* 80.52% inhibition with 17.53 mm radial growth, whereas minimum per cent inhibition (73.78%) was recorded in Tv6 isolate.

The results are in conformity with the findings of Belete *et al.* (2015) [4] and Sonawane *et al.* (2015) [17] who found that the isolate *Trichodrma asperellum* showed the maximum (96.00%) per cent inhibition of *fusarium.* In this purview *Trichoderma asperellum* has been investigated as an important antagonistic soil fungus having the ability to reduce the soil borne disease incidence.

In case of *Sclerotium rolfsii*, statistically significant differences were obtained among the different *Trichoderma asperellum* isolates against test pathogen over control. The minimum radial growth (17mm) with maximum per cent inhibition (81.11%) was recorded in isolate Tv3 collected from Solapur district which was at par with Tv4 (80.41%) and Tv5 (80.11%). However, the isolate Tv6 (78.11%) showed minimum per cent inhibition over control.

The present findings are in conformity with Bagwan N. B. (2011) [3], who tested *Trichodrma* spp. Isolates against *Sclerotium rolfsii* in which, isolate T043 showed maximum (98.70%) growth inhibition. Rao and Kulkarni (2003) [13] also reported the maximum (58.50%) growth inhibition of *Sclerotium rolfsii* by *Trichoderma asperellum.* Shrinivasulu (2005) [18] also reported that *Trichoderma asperellum* was very effective in reducing the radial growth of *Sclerotium rolfsii.*

Table 8: Antagonistic efficacy of *Trichoderma asperellum* isolates against *Fusarium solani, Scleritium rolfsii and Rhizoctonia bataticola* (per cent growth inhibition) at 7 DAI

Sr. No.	Isolates	Radial growth (mm) at 7th DAI			Per cent inhibition over control		
		F. solani	*S. rolfsii*	*R. bataticola*	*F. solani*	*S. Rolfsii*	*R. bataticola*
1.	Tv1	20.32	19.30	26.57	77.43 (59.94)*	78.55 (62.43)*	70.47 (57.08)*
2.	Tv2	19.17	18.47	22.00	78.70 (62.51)	79.47 (63.07)	75.55 (60.36)
3.	Tv3	17.53	17.00	17.70	80.52 (63.82)	81.11 (64.23)	80.33 (63.67)
4.	Tv4	18.20	17.63	24.93	79.78 (63.27)	80.41 (63.72)	72.3 (58.24)
5.	Tv5	15.80	17.90	18.53	82.44 (65.22)	80.11 (61.68)	79.41 (63.00)
6.	Tv6	23.60	19.70	24.87	73.78 (59.19)	78.11 (60.41)	72.36 (58.28)
7.	control	90	90.00	90.00	00.00	00.00	00.00
	F test	Sig	Sig	Sig.	Sig	Sig	Sig.
	S.E (M)±	0.50	0.58	0.50	0.74	1.03	0.36
	C.D. at (p= 0.01)	2.13	2.42	2.12	3.10	4.34	1.53

The per cent growth inhibition of *Rhizoctinia bataticola* by *Trichoderma asperellum* isolates was shown in Table 8. Antagonism between *Trichoderma asperellum* and test pathogen *Rhizoctonia bataticola* indicated that, the test pathogen stops growing upon contact with the antagonist *Trichoderma asperellum.* The maximum growth inhibition (80.33%) of *Rhizoctonia bataticola* was exerted by Tv3 isolate with minimum radial growth (17.70 mm) which was at par with Tv5 isolate (79.41%).

This study is further supported by Shalini and Kotasthane (2006) [15], Mayo *et al*. (2015) [10] Naeimi *et al*. (2010) [12] who studied the *in vitro* antifungal ability of the different *Trichoderma* isolates was based on the ability to produce metabolites that may inhibit the growth of *R. solani.*

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