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Cultural and morphological characterization of rhizospheric isolates of fungal antagonist *Trichoderma*

K. K. Sharma[1*] and U. S. Singh[2]

[1]Vivekanand Parvatiya Krishi Anusandhan Sansthan, Almora-263601 (Uttarakhand), INDIA
[2]International Rice Research Institute - 9th Floor, Aggarwal Corporate Tower, Rajendra Place, New Delhi - 110 008, INDIA
*Corresponding author. E-mail: abhay23p@yahoo.com

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Abstract: The genus *Trichoderma* contains species that are of a great economic importance due to their ability to act as biological control agents against a large variety of fungal plant pathogens. In the present investigation thirty isolates of the *Trichoderma* sp. were obtained from the rhizosphere soils of different plants at different locations at Nainital, Almora, Udham Singh Nagar, Derhadun, Haridwar and Tehri Garhwal districts of Uttarakhand (India). The isolates were characterized on the basis of their cultural and morphological characteristics. The cultural characteristics included linear growth, colony colour, pigmentation and growth pattern. Morphological characteristics studied were structure, shape and arrangement of conidiophores, phialides and conidia. Out of thirty isolates, 6 isolates namely PB10, PB13, PB23, PB26, PB27 and PB28 were identified as *T. virens* and remaining 24 isolates as *T. harzianum*.

Keywords: Characterization, Cultural, Morphological, Phialides, *Trichoderma*

INTRODUCTION

Genus *Trichoderma* have traditionally been classified as Fungi Imperfecti based upon differences in morphology under Deuteromycotina, Hyphomycetes, Phialasporace, Hyphales, Dematiaceae as they produce only asexual spores i.e. conidia (Singh *et al*., 2006). As is usually the case with other fungal genera, species of *Trichoderma* too were defined originally on the basis of morphology by workers like Rifai (1969) and Bisset (1991a, b and c). The culture sporulation pattern varied considerably within and between the two species. Although conidial shape and arrangement and hyphal branching pattern helped in distinguishing species from each other, they failed to designate *Trichoderma* species. Seaby (1996) also reported that differentiation of *Trichoderma* spp. using classical microscopic features alone was difficult since cultural morphology varied widely on different media and spore size varied significantly with incubation temperature. Moreover, variation among the isolates based on size of the phialides, and their arrangements was small. However, sporulation pattern and size of the spores within the species were highly variable. Keeping the above facts in view, the present study was undertaken to characterize and estimate cultural and morphological variability among thirty isolates of *Trichoderma* isolated from the rhizospheric soil of different crops and locations of Uttarakhand.

MATERIALS AND METHODS

Sampling and isolation: The experimental material consisted of the thirty strains of the *Trichoderma* sp. which were isolated from the rhizospheric soil samples on Trichoderma selective medium (Elad *et al*., 1981) using serial dilution technique (Krassilnikov, 1950) from different crops and locations of Nainital, Almora, Udham Singh Nagar, Derhadun, Haridwar and Tehri Garhwal districts of Uttarakhand-India (Table 1).

Cultural and morphological characterization of *Trichoderma* isolates: The cultural and morphological characteristics of the 30 *Trichoderma* isolates were determined on Potato dextrose agar (PDA) and Cornmeal dextrose agar (CMD) medium respectively. A 5 mm diameter disc using a sterile cork borer was cut from the actively growing edge of a fresh colony (before the start of conidial production) placed on Petri dish containing 20 ml medium (PDA for cultural and CMD for morphological study) approximately 1.5 cm from the edge of the Petri dish with the mycelial surface facing downwards and three replications were maintained for each isolate.

For cultural study, one set of Petri dishes were incubated at 25^0C and another set at 35^0C in darkness. The colonies were examined at 24 h intervals for pattern of conidiation, first appearance of green conidia, formation of conidial pustules, presence of any odor or yellow pigmentation and colony radius was measured from the edge of the inoculum plug after 72 h at both 25^0C and 35^0C. For morphological characterization of *Trichoderma*, the cultures were incubated at 20^0C. Microscopic preparations for morphological studies slides were prepared in 3%

KOH followed by lactophenol-cotton blue from pustules where conidia were still white, generally within a week of incubation at 20^0C. After placing the cover slip, the slide was observed under the microscope for morphological characters like conidiophores, their branching pattern, angle to main axis, phialide numbers and their arrangement, conidial shape and colour and formation of chlamydospores and their position. Species identification was based on the morphological and taxonomic keys provided by Bisset (1991a, b).

RESULTS

Cultural characterization of *Trichoderma* isolates: The cultural characteristics and growth rates of the 30 *Trichoderma* isolates were determined on Potato dextrose agar medium (Table 2**)**. All the isolates were fast growing reaching a radius of 42.5 to 56.5 mm after 72 h at 25^0C and 20 to 37.8 mm after 72 h at 35^0C. At 35^0C, the isolates produced abnormal compact colonies with irregular margin. Conidiation in the *T. harzianum* isolates was predominantly effuse covering the entire surface of the plates. However, some isolates initially produced flat pustules in concentric rings. The pustules appeared powdery due to dense conidiation. *T. virens* isolates showed predominantly effuse conidiation without formation of any pustule. Some isolates produced yellow pigment on PDA. Conidial colour change was observed from white to varying shades of green. In most isolates conidia were formed by 48 h and turned green within 72 h.

Morphological characterization of *Trichoderma* isolates: In the morphological studies of *Trichoderma*, primarily two types of arrangement of conidiophores and phialides was observed among the 30 isolates (Plates 1, 2, Table 3). Twenty four isolates except PB 10, 13, 23, 26, 27 and 28 were characterized by highly branched divergent and dendritic conidiophores. Longest branches form near the base of the hypha and nearest the main axis. Branches toward the tip and secondary branches tended to be held at 90° with respect to the axis from which they arise. Cells supported the phialides equivalent in width to, or at most only slightly wider than, the base of phialides arising from them. Divergent phialides were typically arranged in whorls of 3-5 and held at 90° with respect to the hyphae from which they arose, or solitary. Those in whorls were typically flask-shaped, enlarged in the

Table1. Soil samples collected from different locations of Uttarakhand.

L. No.	Sample code	Crop	Location	Soil pH	Isolate code
1	R1KG	Rice	Kathgodam-Haldwani	6.9	PB1
2	R3H	Rice	Halduchaur-Haldwani	6.8	PB2
3	R2LCb	Rice	Lamachaur-Haldwani	7.0	PB3
4	R1Da	Rice	Kherna-Almora	6.9	PB4
5	R1Db	Rice	Kherna-Almora	6.8	PB5
6	R2Da	Rice	Kherna-Almora	7.0	PB6
7	R2Db	Rice	Kherna-Almora	7.0	PB7
8	SPC1	Rice	SPC-Pantnagar	6.8	PB8
9	SPC2	Rice	SPC-Pantnagar	6.8	PB9
10	1a	Rice	Rudrapur-U.S. Nagar	7.2	PB10
11	1ab	Rice	Rudrapur-U.S. Nagar	7.2	PB11
12	1bc	Rice	Rudrapur-U.S. Nagar	7.2	PB12
13	3	Rice	Rudrapur-U.S. Nagar	7.1	PB13
14	5	Rice	Rudrapur-U.S. Nagar	7.1	PB14
15	AM	Apple	Mukteshwar-Almora	6.7	PB15
16	BM	Broccoli	Mukteshwar-Almora	6.8	PB16
17	PM1	Pea	Mukteshwar-Almora	6.8	PB17
18	PM2	Pea	Mukteshwar-Almora	6.8	PB18
19	SM	Strawberry	Mukteshwar-Almora	6.8	PB19
20	WM	Walnut	Mukteshwar-Almora	6.8	PB20
21	RP1	Rice	Premnagar-Dehradun	6.6	PB21
22	TR1	Mustard	Premnagar-Dehradun	6.7	PB22
23	A	Maize	Dhalwala-Rishikesh	6.8	PB23
24	B	Maize	Bhaniawala-Dehradun	7.0	PB24
25	B1	Rice	Bhaniawala-Dehradun	7.1	PB25
26	C1	Rice	Mazra-Ranipokhri	6.9	PB26
27	D	Maize	Geetanagar Rishikesh	6.8	PB27
28	D1	Rice	Raipur-Dehradun	6.8	PB28
29	F1	Rice	Raiwala-Hardwar	7.1	PB29
30	G1	Rice	Nagani, Tehri Garhwal	7.0	PB30

Table 2. Cultural characteristics of different *Trichoderma* isolates.

Sample code	Isolate code	Colony growth (mm) after 72 hrs at 25^0C	Colony Growth (mm) After 72 hrs at 35^0C	Culture Colour	Conidiation E/F (Effuse/ flat pustule)	Pigmentation	Sporulation initiate After (hrs)
R1KG	PB1	50.5	32.4	Dark green	E	Brownish yellow	48
R3H	PB2	51.3	34.0	Light Green	E+F	-	36
R2LC	PB3	52.5	35.6	Dark green	E	Brownish yellow	48
R1Da	PB4	53.5	32.7	Whitish green	E	-	72
R1Db	PB5	56.0	31.4	Whitish green	*E+F*	-	48
R2Da	PB6	55.5	31.0	Whitish green	*E+F*	-	48
R2Db	PB7	52.5	33.0	Light Green	E	-	48
SPC1	PB8	56.0	36.0	Dark green	E+F	-	48
SPC2	PB9	52.5	35.5	Dark green	E+F	-	48
1a	PB10	50.6	27	Light Green	E	Yellow	48-72
1ab	PB11	50.4	32.9	Yellowish green	E	Yellow	48-72
1bc	PB12	42.5	20	Dark green	E	-	48-72
3	PB13	50.5	36.0	Whitish green	E	-	72
5	PB14	52.5	34.2	Whitish green	E	-	48-72
AM	PB15	56.5	37.8	Dark green	E	-	48
BM	PB16	53.0	35.1	Whitish green	F	-	72
PM1	PB17	54.1	32.3	Dark green	E+F	Yellow	48
PM2	PB18	55.0	34.2	Dark green	E	-	48
SM	PB19	55.5	35.6	Dark green	E	-	48
WM	PB20	46.5	28.4	Whitish green	F	-	72
RP1	PB21	52.5	33.7	Dark green	E+F	-	48
TR1	PB22	51.5	32.6	Yellowish green	E	Yellow	48-72
A	PB23	50.5	31.6	Light Green	E	Yellow	72
B	PB24	52.5	34.7	Light Green	E	-	48
B1	PB25	53.5	34.0	Dark green	E	-	48
C1	PB26	51.5	33.5	Whitish green	E	Yellow	48
D	PB27	50.3	34.5	Yellowish green	E	Yellow	48
D1	PB28	53.5	35.0	Dark green	E	-	72
F1	PB29	52.5	33.0	Light Green	F	Yellowish brown	48
G1	PB30	50.3	30.7	Whitish green	F	-	48-72

middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base. Terminal phialides in a whorl or solitary, were typically cylindrical or at least not conspicuously swollen in the middle and longer than the subterminal phialides (Plate 1). These above characteristics resembled with *Trichoderma harzianum*. The remaining 6 isolates (PB 10, 13, 23, 26, 27 and 28) exhibited hyaline conidiophores arising in clusters from aerial mycelium, branching toward the tip, each branch terminating in a penicillus of 3-6 closely appressed and divergently branched phialides towards the apex, with a sterile stipe (Plate 2). The characteristics of these isolates typically resembled with *T. virens*.

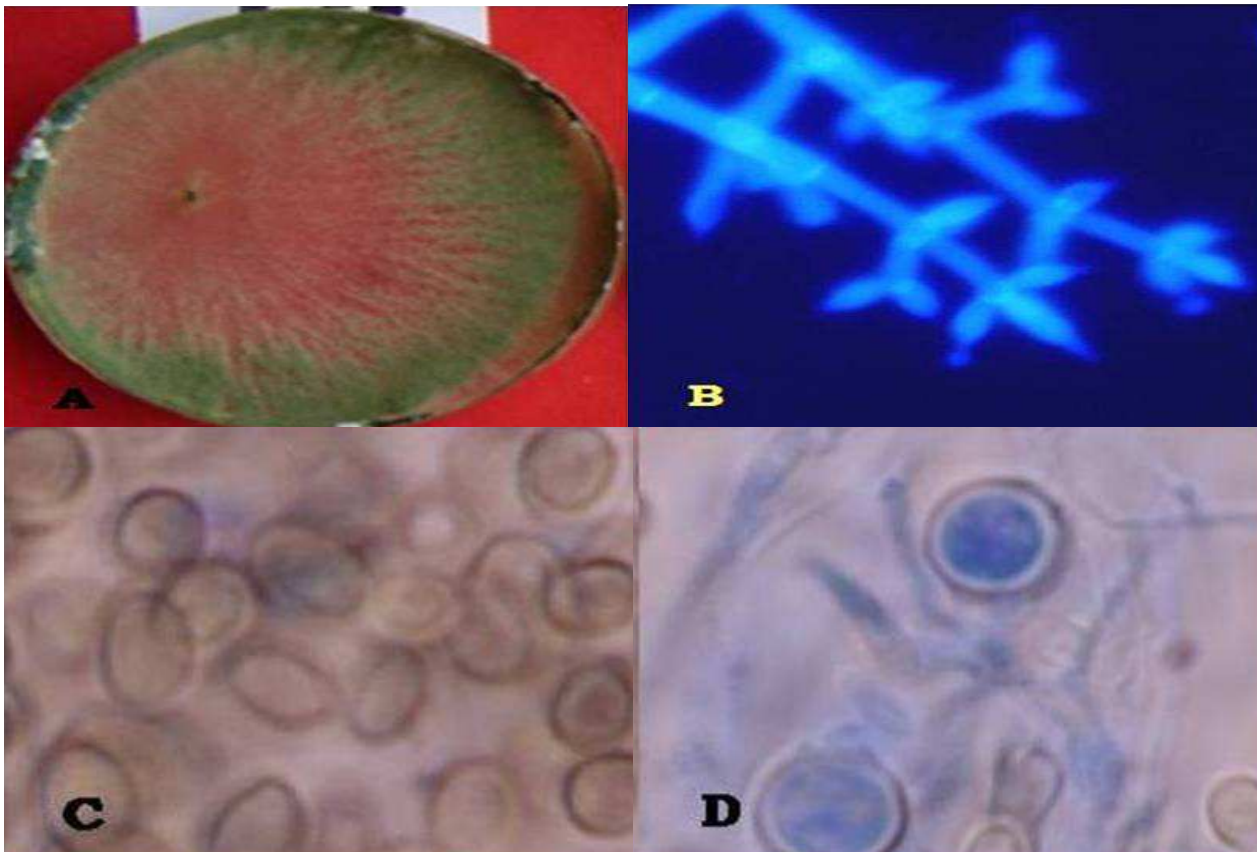

Plate 1. *Isolate PB2. Trichoderma harzianum; A. Culture B. Conidiophore and Phialides C. Conidia D. Chlamydospores*

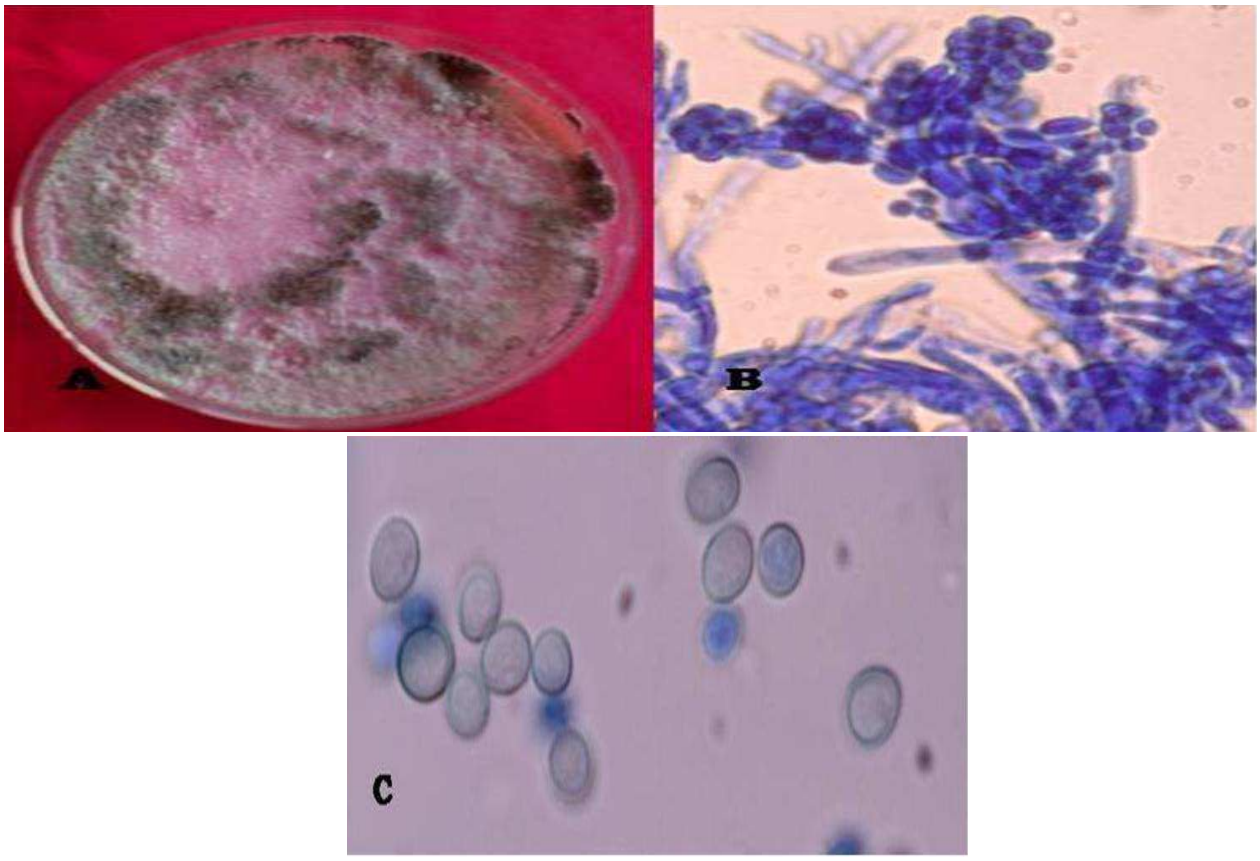

Plate 2. *Isolate PB 23. Trichoderma virens A. culture, B. Conidiophore and phialides C. Conidia*

Table 3. Morphological characteristics of *Trichoderma* isolates.

S. No.	Isolate code	Divergently Branched conidiophore at 90^0	Clutered conidiophore terminating with penicullus	Divergently branched phiallids with fertile apex	Closely appressed phiallids with sterile stipe	Chlamydospore	*T. harzianum* / *T. virens*
1	PB 1	√	-	√	-	√	*T. harzianum*
2	PB 2	√	-	√	-	√	*T. harzianum*
3	PB 3	√	-	√	-	√	*T. harzianum*
4	PB 4	√	-	√	-	√	*T. harzianum*
5	PB 5	√	-	√	-	-	*T. harzianum*
6	PB 6	√	-	√	-	√	*T. harzianum*
7	PB 7	√	-	√	-	√	*T. harzianum*
8	PB 8	√	-	√	-	√	*T. harzianum*
9	PB 9	√	-	√	-	√	*T. harzianum*
10	PB 11	√	-	√	-	√	*T. harzianum*
11	PB 12	√	-	√	-	√	*T. harzianum*
12	PB 14	√	-	√	-	√	*T. harzianum*
13	PB 15	√	-	√	-	√	*T. harzianum*
14	PB 16	√	-	√	-	-	*T. harzianum*
15	PB 17	√	-	√	-	√	*T. harzianum*
16	PB 18	√	-	√	-	√	*T. harzianum*
17	PB 19	√	-	√	-	√	*T. harzianum*
18	PB 20	√	-	√	-	-	*T. harzianum*
19	PB 21	√	-	√	-	√	*T. harzianum*
20	PB 22	√	-	√	-	-	*T. harzianum*
21	PB 24	√	-	√	-	√	*T. harzianum*
22	PB 25	√	-	√	-	√	*T. harzianum*
23	PB 29	√	-	√	-	√	*T. harzianum*
24	PB 30	√	-	√	-	-	*T. harzianum*
25	PB 10	-	√	-	√	√	*T. virens*
26	PB 13	-	√	-	√	√	*T. virens*
27	PB 23	-	√	-	√	-	*T. virens*
28	PB 26	-	√	-	√	√	*T. virens*
29	PB 27	-	√	-	√	√	*T. virens*
30	PB 28	-	√	-	√	-	*T. virens*

DISCUSSION

Cultural and morphological characterization: Cultural characteristics comprising growth rate, colony colour and colony appearance were regarded as taxonomically useful characteristics for *Trichoderma* (Samuels *et al.*, 2002a). Studies revealed that all thirty rhizospheric isolates did not much differ in cultural characteristics with most isolates exhibiting rapid growth, effuse conidiation and/or loosely arranged conidia in pustules. The same findings like pale or yellowish colour of reverse of colonies, rapid growth at 25°C to 30°C of majority of *Trichoderma* isolates and typically not growing at 35°C were recorded by Samuels *et al.* (2002a). Gams and Bissett (2002), Lin and Heitman (2005) and Samuels *et al.* (2002a) also confirmed the presence of terminal and/or intercalary chlamydospores in cultures.

Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains as a potential method to identify *Trichoderma* species (Anees *et al.*, 2010; Gams and Bissett 2002; Samuels *et al.*, 2002a). On the basis of description and keys given by Gams and Bissett (1998), *Trichoderma* isolates could be classified here into two groups as *Trichoderma harzianum* (80 %), *Trichoderma virens* (20 %). However, in the present study, the emphasis was given on conidiophores spread type i.e. gathering or non-gathering type and phialides type fertile or non-fertile (Gams and Bissett, 1998 and 2002; Rahman *et al.*, 2011). Morphological study revealed two types

of arrangement of conidiophores and phialides within 30 isolates. Conidiophores of the first group of 24 isolates were spread to the top and smooth or rounded, wide near the base. Phialides were arising mostly in crowded but had an angle with conidiophore, and had whorls of 2~6 on the terminal branches. Conidia subglobose to ellipsoidal, apex broadly rounded, base more narrowly rounded (Samuels *et al.*, 2002). These characteristics of the species *T. harzianum* sect. *Pachybasium* were same as described by Bisset (1991a, b)**.** More than ever, second group of 6 isolates (PB 10, 13, 23, 26, 27 and 28) was characterized by predominant conidiation, many divided branches, gathering all finger to top, and fertile to the apex that was penicillate type. Conidiophores of *T. virens* were smoothly bent, gather and not spread to top. Conidia broadly rounded to obovoid, both ends broadly rounded or with the base narrower. Phialides were hung like banana in the conidiophore, base and apex were more narrow than middle. All these descriptions are in agreement with the findings of earlier investigators (Bisset, 1991a and b, Samuals *et al.*, 1996, Gams and Bissett, 1998 and 2002, Samuels *et al.*, 2002, Samuels *et al.*, 2002a, Choi *et al.*, 2003, and Shah *et al.*, 2012 who recorded the similar observations.

Conclusion

Our study conclude that out of thirty isolates, 6 isolates namely PB10, PB13, PB23, PB26, PB27 and PB28 were identified as *T. virens* and remaining 24 isolates as *T. harzianum* on the basis of morphological characteristics. The cultural characteristics were not highly variable. Isolates except PB 12 and PB 20 showed fast growth reaching a radius of 50.3 mm to 56.5 mm after 72 hours at 25^0C and conidiation was predominantly effuse.

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