J. Mycopathol. Res. ***55(4)*** *: 391-399, 2018;* (ISSN 0971-3719)

Morphological variability and protein profiling of mycoparasitic *Trichoderma* spp.

SUBHENDU JASH*, GOLAM MOINUDDIN AND ARINDAM SARKAR
Regional Research Station (Red and Laterite Zone), Bidhan Chandra Krishi Viswavidyalaya, Jhargram 721507, West Bengal

Received : 22.08.2017 Accepted : 15.09.2017 Published : 29.01.2018

In attempts to know the better understanding of biochemical variability of *Trichoderma* spp. along with morphological characters for taxonomical significance protein profiling of mycelial protein of different isolates was studied. Morphological parameters i.e. shape, size of phialide, phialospore, chlamydospore and colony morphology were observed and identification was done based on the morphometry. All the isolates showed polymorphic bands through electrophoresis of mycelia proteins. Most of the protein bands are concentrated with their mobility value of 0.200 to 0.575. T9 and T10 isolates which were identified as *T. roseum* showed almost equal banding pattern with genetic similarity coefficient of 0.888. T6 and T8 isolates of *T. virens* have highest similarity of 0.833. Intra specific similarity was distinct in *T. virens* and *T. roseum* species as per electrophoresis analysis in this experiment.

Key words: Morphometry, protein polymorphism, *Trichoderma*, variability

INTRODUCTION

Trichoderma, a filamentous soil inhabiting mycoparasite, have been used in biological control of many soil borne fungal plant diseases (Bose *et al.* 2005; Pan *et al.* 2009; Pan and Jash, 2009a, b, 2010a,b). The production of volatile and non volatile antibiotics (Jash *et al.* 2005; Roy *et al.* 2005; Khalko *et al.* 2005), release of extracellular hydrolytic enzymes (Pan and Jash, 2009a), mycoparasitism (Jash, 2006), and induction of defense response (Pan and Jash, 2010a) are the key mechanism by which *Trichderma* suppress the plant pathogens in soils. However, the numerous mechanisms have been developed during last forty years or so to explain the biocontrol activity of *Trichoderma*, the process is still more complex. The interaction between *Trichoderma* and pathogenic fungi involves chemotropism, lectin mediated recognition and formation of trapping and penetration structures (Jash, 2006). This process is further supported by the secretion of extracellular hydrolytic enzymes such as chitinase, cellulose, b-1,3 glucanase, proteinase (Pan and Jash, 2009a), and as well as secondary metabolites. Tolerance to some common fungicides and herbicides (Khalko *et al.* 2006; Pan and Jash, 2009c) and ability to colonize the rhizosphere rapidly (Jash and Pan, 2007) helps the *Trichoderma* to survive in soil for long time.

Trichoderma possess great genetic variability. Some strains have a wide spectrum of activity and few strains may control only specific pathogens, while still others may have little or no biocontrol efficacy (Jash and Pan, 2004a, b). Polynucleate cells in *Trichoderma* thalli produce more stable homokaryotic conidia and it seems reasonable that wild strains may be heterokaryons. If so, isolation of homokaryons from mass by single spore isolation may give rise to different and more stable strains (Stasz *et al.* 1988). Most of the *Trichoderma* species are not encountered in association with sexual stages and are considered to be strictly mitotic, clonal fungi. The apparent lack of sexuality is a barrier to understand interrelationship within and among *Trichoderma* spp. (Samuels, 1996).

Characteristics of primary importance in fungal taxonomy include measurements of spores and spore bearing structures, as well as characters of the vegetative mycelium and the colour of mycelium

*Corresponding author : drsubhendujash@gmail.com

and spore. Morphological characters used in species recognition in *Trichoderma* have been outlined by many taxonomists (Rifai, 1969; Bissett, 1984). Morphology as a single principal criterion for the classification of fungus has been found inadequate in the identification of several species. Any biochemical characters which might help to define these species or to separate this group from the rest of genus, could be of value, but it is necessary first to ascertain whether the character under consideration is constant within a given species, and any variation show sufficient correlation with morphological variation to make comparison with other species of value (Peberdy and Turner, 1968). Now a day several molecular techniques are used for studying variability and as well as taxonomical significance in *Trichoderma* spp. namely electrophoresis of intracellular protein and enzymes, DNA hybridization and PCR based characterization of 16S intergeneric spacer region of ribosomal DNA etc. The Poly acrylamide gel electrophoresis is more sensitive and gives better resolution of the protein. The neutral independent markers are ideal for analysis of pathogen population structure because usually they are not exposed to the strong selection pressure of the host. It is simple, efficient and inexpensive technique for evaluating the taxonomy, genetics, virulence and epidemiology of plant pathogens (Micales and Bonde, 1995). So, the present study was designed with an objective to study the biochemical variability along with morphological characters for taxonomical significance in *Trichoderma* population collected from six agroecological zones of West Bengal by employing the intracellular protein profiling.

MATERIALS AND METHODS

Isolation of the Trichoderma spp.

Ten isolates of *Trichoderma* were isolated from the rhizosphere soil of different ecological habitat of West Bengal by dilution plate technique using modified *Trichoderma* specific medium (TSM). The isolates were identified (Table 1) based on monograph of Bissett (1984). All the identified strains of *Trichoderma* were maintained on potato dextrose agar (PDA) slant at 4°C for further use.

Phenotype analysis

Phenotype characterization of all the isolates was performed in order to unequivocally verify the taxa. The phenotypic evaluation consisted of growth trials, colony appearance and measurements of the anamorphic part. Colony characters and the characteristic of conidiophores and conidia were taken from culture grown on oat meal agar (OMA) and PDA. To obtain growth rates and colony characters, cultures were first inoculated onto freshly prepared PDA in petri plates. When the colonies were actively growing at 28°C, a 5mm diameter plug of culture was taken from actively growing edge and inoculated onto a freshly made 9 cm diameter petri plates containing 20ml OMA. Morphological character i.e. conidiophore shape, length and width, width of phialide at the base, phialide width at the widest, phialide length and shape, phialospore shape, length and width, chlamydospore shape, length and width etc. were obtained from cultures grown on OMA at 28°C, alternating 12 hr dark and 12 hr cool white fluorescent light. All conidiophores and conidia were first hydrated in 3% KOH and the measurements immediately made directly from KOH according to the protocol of Lieckfeldt *et al.* (2001). Measurements of the colony radius were taken every 24 hr at the same time of the day for several days until the colony covered the plate. Basic statistics of micromorphology were made based on 50 measurements except where indicated.

Extraction of proteins and proteins profiling

Ten isolates of *Trichoderma* were grown in basal liquid glucose yeast peptone medium (GYPM). Three mycelial discs each of 5 mm diameters cut from the actively growing culture on PDA were inoculated into 50 ml of GYPM in each 250 ml Erlenmeyer flask. After 7days of incubation at 28±1°C, the mycelial mat was harvested by filtration through Whatman No. 1 filter paper on a Buchner funnel followed by three washing with distilled water. Mycelial mat was dried by soaking it with sterilized blotting paper. 1g of dried mycelial mat of each isolates was homogenized in chilled mortar and pestle in liquid nitrogen followed by 1ml of 0.6 M Tris-HCl buffer pH 6.8. The mycelial paste was transferred to centrifuge tube. To each tube 0.5 ml of 0.6 M Tris-HCl buffer (pH 6.8) was added and centrifuged at 4°C in 10000 rpm for 20 min. The supernatant was collected from the tube without disturbing the lipid upper layer or the pellet, dispensed into 1ml Eppendrof tube and stored at -15°C. The protein concentration of the extracted aliquot was measured by Lowry *et al.* (1951)

method, using bovine serum albumin (BSA) as a standard.

Poly acrylamide gel electrophoresis (PAGE) was used to separate soluble protein extracted from mycelium by sodium dodecyl sulfate discontinuous buffer system using 10% gel based on the method of laemmli (1970). Protein concentration in each sample was adjusted to 150 mg protein / 50 ml of sample by mixing sample buffer. The samples were mixed thoroughly in Eppendrof tube and heated in boiling water bath for 2-3 min to ensure complete interaction between proteins and SDS. After cooling 50 ml sample was loaded in the individual well of the stacking gel by a micropipette. The power supply unit was switched on and regulated to 15 mA for few min until the sample traveled through the stacking gel. Then the run was continued at 2 mA for about 2 hr until the bromophenol blue reached the desire distance. The gel was stained with coomassie blue staining solution for 1 hr and then destained in the same solution without stain and Rm value of each protein band was calculated.

A relative mobility (Rm) value was assigned to each band of protein activity detected and it was calculated by using the formula, Rm= distance moved by the protein band/ distance moved by the dye, and zymograms were prepared. Computer software, Numerical Taxonomy System for Multivariate Statistical Program (NTSYS-PC) was used for analysis of protein profiling data. The presence or absence, 1 and 0, respectively, for a particular protein band was recorded. A matrix of simple matching coefficient for each pair of isolates was constructed using a similarity programme (SIMQUAL) within NTSYS-PC by the formula Ssm = (a+b)/n, where a = the number of bands common in the pair of isolates, b = the number of band absent in the pair of isolates but present in at least one isolate, and n = the total number of bands. A phenetic tree was developed from the matrix of similarity coefficient by the unweighted pair-group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

Phenotype analysis

Morphological parameters i.e., shape, size, ornamentation and arrangement of anamorphic characters of ten isolates were studied in different nutrient media presented in Table 2. Phialide of T. viride isolates (T1 and T3) varied from straight to irregularly bent, 6.7 - 8.6 µm in length, 3.5 to 3.8 µm in width. Phialospores were globose, distinctly roughened, and size varied from 3.4- 4.4 x 3.1-4.2 µm. Conidiophores long upto 85.2 ìm, pyramidally branched, short branches near the tip and longer one with repeated branching in the lower part, arising from the surface of agar or from whitish hyphae. Chlamydospores were globose to ellipsoidal, up to 10.4 µm long.

Table 1: Isolates of *Trichoderma* investigated and their sources

Code	Taxa	Crop associated	Locations in West Bengal
T_1	*T. viride*	Potato field	Falakata, Jalpaiguri
T_2	*T. harzianum*	Chilli field	Kakdwip, South 24 Pgs
T_3	*T. viride*	Mustard field	Bishnupur, Bankura
T_4	*T. harzianum*	Brinjal field	Kalyani, Nadia
T_5	*T. harzianum*	Sunflower field	Namkhana, South 24 Pgs.
T_6	*T. virens*	Gladiolus field	Kalimpong, Darjeeling
T_7	*T. virens*	Jute field	Arambagh, Hooghly
T_8	*T. virens*	Cabbage field	Ranaghat, Nadia
T_9	*T. roseum*	Potato field	Alipurduar, Jalpaiguri
T_{10}	*T. roseum*	Rice field	Sehara Bazar, Bardhaman

T2, T4 and T5 isolates of T. harzianum produced ampulliform to subglobose phialide with constriction at the base and swollen in the middle. Among the three isolates, T2 produced large sized phialide (7.4 - 9.2 x 2.9 - 3.9 µm) while largest phialospore was found in T3 (3.4 - 4.3 x 3.1 - 3.9 µm). Phialospore was smooth walled. Conidiophores straight or flexuous, highly branched, primary branches arise nearly at right angles, usually in whorls of 2-3, longer than the *T. viride* isolates.

T6, T7 and T8 isolates were included in the genus *T. virens*. Phialide lageniform to ampulliform, constricted at base, swollen in the middle, attenuated to the apex, forming a broad penicillus toward the tip, 7.8 - 9.4 µm long, 2.2 - 2.7 µm wide at the base. Phialospore was broadly ellipsoidal, both end rounded and smooth walled with maximum size of 4.5 - 4.8 x 3.9 - 4.3 µm in T8 isolate. Among three, T7 produced longer conidiophore (107.2 - 128.9 x 4.3 - 4.9 µm) while in T8 it was very shorter (22.2 - 57.7 x 4.4 - 4.7 µm).

Lastly, T9 from terai and T10 from old alluvial zone were identified as *T. roseum*. Colony characteristic and reproductive morphology of these two isolates were somewhat different from others.

Table 2: Anamorphic morphological characters of the isolates and taxa of *Trichoderma*

Character	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
Phialide:										
Shape	Straight to irregularly bent	Ampulliform to subglobose, constricted at base	Straight to irregularly bent	Ampulliform to sub globose constricted at base, swollen in the middle	Ampulliform, usually attenuate to a narrow short cylindrical	Lagenifo rm to ampullifo rm, constricted at base, swollen in the middle, attenuated to the apex	Lagenifo rm to ampullifo rm, constricted at base, swollen in the middle, attenuated to the apex	Lagenifo rm to ampullifo rm, constricted at base, swollen in the middle, attenuated to the apex	Straight, formed in closely appressed whorls of 4-7, conidial head discrete	Straight formed in closely appressed whorls of 4-6, conidial head discrete
Length (µm)	7.9 – 8.6	7.4 – 9.2	6.7 – 8.4	6.8-7.5	7.1-9.1	8.7-9.4	7.8-9.1	8.2-8.9	11.2-13.0	10.8-12.7
Width at the base (µm)	2.2 – 2.5	2.3 – 2.4	2.2 – 2.4	2.3-2.5	2.2-2.5	2.4-2.7	2.2-2.5	2.3-2.5	2.1-2.3	2.2-2.4
Width at the widest (µm)	3.6 – 3.8	2.9 – 3.9	3.5 – 3.7	3.2-3.7	3.1-3.9	4.1-4.4	3.8-4.2	3.9-4.2	2.9-3.2	2.9-3.4
Phialospore:										
Shape	Globose, distinctly roughened	Subglobose to ovoid, smooth walled	Globose, distinctly roughened	Subglobose to ovoid, smooth walled	Subglobose to ovoid, smooth walled	Ellipsoidal to ovoid, both ends rounded, smooth walled	Subglobose to ovoid, both end rounded and smooth walled	Short ellipsoidal, smooth walled,	Elongate, asymmetrical apex, base slightly protruding, smooth walled	Elongated, asymmetrical apex, base slightly protruding, smooth walled
Size (µm)	3.5-4.4x 3.1-4.2	3.0-3.2 x 2.2-2.4	3.4-4.3 x 3.1-3.9	2.9-3.2 x 2.1 – 2.4	2.8-3.3 x 2.2-2.5	4.4-4.9 x 3.6-3.8	4.3-4.7 x 3.9-4.1	4.5-4.8 x 3.9-4.3	4.8-5.1 x 3.1-3.6	4.9-5.6 x 3.2-3.6
Conidiophore:										
shape	Pyramidally branched, short branches near the tip and long repeated one in lower part	Straight or flexuous highly branched, primary branches arise nearly at right angles, usually in whorls of 2 or 3	Pyramidally branched, short branches near the tip and long repeated branching in the lower part	Straight or flexuous, highly branched primary branches arising nearly at right angles	Straight or flexuous, highly branched, primary branches arise at rightly angles in whorls of 2- 3	Irregularly branched, apex bearing a terminal whorl of appressed branches	Branching irregularly, apex bearing a terminal whorl of appressed branches arising at right angles	Irregularly branched, apex frequently bearing a terminal whorl of appressed branches	Primary conidiophores with divergent, verticilliate type, secondary one densely penicillate type, smaller than earlier	Penicillioid type of long conidiophores with highly branches at upper portion
Size (µm)	32.5 – 70.4 x 4.1 – 4.3	42.4-92.4 x 4.0-4.4	43.9-85.2 x 3.9-4.4	22.1-103.4 x 4.2-4.7	38.6-87.1 x 4.0-4.3	37.2-69.8 x 4.3-5.4	107.2-128.9 x 4.3-4.9	22.2-57.7 x 4.4-4.7	92.2-129.6 x 4.3-4.8	87.8-132.1 x 4.4-4.8

(*Contd. part table 2*)

Chlamydospore:										
Shape	Globose to ellipsoidal	Subglobose to ellipsoidal or pyriform	Globose to ellipsoidal	Subglobose to ellipsoidal	Subglobose to ellipsoidal or pyriform	Subglobose to ellipsoidal	Subglobose to ellipsoidal	Subglobose to ellipsoidal	Subglobose to ellipsoidal	Subglobose to ellipsoidal
Size (µm)	8.4-10.2 x 7.9-8.6	8.2-11.4 x 6.1-8.4	8.5-10.4 x 7.8-8.6	8.2-10.9 x 6.2-8.4	8.4-10.6 x 6.7-8.5	8.4-9.4 x 7.6-8.6	9.2-11.4 x 7.9-10.0	8.4-9.1 x 6.2 – 7.8	6.7-9.1 x 5.8-7.2	7.2-9.2 x 6.4-7.8
Growth rate on:										
PDA	3-4 days	3 days	3-4 days	3 days	3 days	3 days	3 days	3 days	6 days	6 days
OMA	4-5 days	3-4 days	5 days	4 days	3-4 days	3 days	4 days	3 days	7 days	7 days
Pigmentation:										
PDA	Yellowish	Absent	Dull yellowish	Slightly yellowish	Absent	Yellowish	Yellowish	Dull yellow	Absent	Absent
OMA	Yellowish	Absent	Dull yellowish	Absent	Absent	Amber	Dull yellowish	Dull yellow	Absent	Absent
Colony character:										
PDA	Greenish fluffy growth with slow sporulation	Aerial mycelium floccose, white to grayish, sporulation is uniform and green	Light green sporulation throughout the plate,	Grayish green, fluffy colony with profuse mycelium, sporulation in concentric ring	Deep green, granular submerged mycelial growth	Aerial mycelium floccose, white to grayish, rapid dark green sporulation covered the entire surface	Deep green granular, submerged mycelial growth	Dark green raised nodular colony with white mycelium at the margin	Colonies granular, white in colour, turned to slightly green after long sporulation	Colonies granular, white in colour, with scanty mycelial growth
OMA	Greenish growth on centre and whitish at margin with very slow sporulation	Green raised colony with profuse mycelial growth	Sporulation very slow, more in margin than centre	Dark green appressed colony with sporulation throughout the plates	Dark green, submerged with little raised white mycelial growth at the margin	Dark green raised colony with white mycelials growth at the margin	Dark green, submerged with little raised white mycelial growth	Dark green raised colony with white mycelial growth at the margin	Whitish granular colony, looks light green after sporulation	Whitish with slightly green in colour

Phialospore was elongated, slightly asymmetrical, apex obliquely rounded, base slightly protruding and smooth walled and larger (4.9 - 5.6 x 3.2 - 3.6 µm in T10).

All the isolates grew faster, excepting T9 and T-10, reaching 90 mm diameter within 3 to 5 days on OMA and PDA media at an optimum temperature of 28°C. Growth rate of *T. roseum* strains (T9 and T10) was very slow requiring 6 days on PDA and 7 days on OMA for covering full agar plate. Colony characteristics were distinguishable among the isolates both in PDA and OMA media. Greenish fluffy growth with dense profuse mycelial mat was found in both *harzianum* and *viride* isolates where as *virens* isolates produced floccose aerial mycelium comprised of short hyphae that formed a uniform lawn over the colony. T9 and T10 isolates produced a distinctive sweet coconut odour in the culture.

In the present investigation soil samples were col-

Table 3: Alleles detected and their relative mobility (Rm) value for ten isolates of *Trichoderma* spp.

Taxa	No of allele*	Relative mobility (Rm) value									
		Loci 1	Loci 2	Loci 3	Loci 4	Loci 5	Loci 6	Loci 7	Loci 8	Loci 9	Loci 10
T_1	6	0.141	0.175	0.238	0.302	0.429	0.492	-	-	-	-
T_2	10	0.254	0.286	0.333	0.380	0.412	0.444	0.460	0.492	0.508	0.571
T_3	10	0.079	0.190	0.238	0.302	0.396	0.413	0.444	0.476	0.492	0.571
T_4	9	0.190	0.254	0.397	0.413	0.444	0.476	0.524	0.571	0.635	-
T_5	6	0.238	0.302	0.381	0.429	0.444	0.476	-	-	-	-
T_6	10	0.190	0.238	0.317	0.349	0.381	0.413	0.429	0.460	0.571	
T_7	4	0.317	0.381	0.444	0.524	-	-	-	-	-	-
T_8	10	0.190	0.254	0.302	0.349	0.381	0.413	0.476	0.524	0.603	-
T_9	9	0.095	0.206	0.270	0.317	0.413	0.476	0.524	0.603	0.000	-
T_{10}	8	0.095	0.190	0.317	0.413	0.476	0.540	0.619	-	-	-

*Alleles were numbered sequentially from the anodal end of the gels

Table 4: Genetic similarity between electrophoretic phenotypes of *Trichoderma* isolates through protein profiling

Isolate	Electrophoretic phenotype									
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
T_1	1.000									
T_2	0.333	1.000								
T_3	0.333	0.538	1.000							
T_4	0.250	0.583	0.583	1.000						
T_5	0.333	0.454	0.600	0.363	1.000					
T_6	0.333	0.818	0.666	0.727	0.454	1.000				
T_7	0.250	0.400	0.400	0.300	0.428	0.400	1.000			
T_8	0.285	0.692	0.692	0.615	0.500	0.833	0.333	1.000		
T_9	0.363	0.583	0.583	0.500	0.250	0.583	0.300	0.500	1.000	
T_{10}	0.272	0.500	0.636	0.545	0.272	0.636	0.333	0.538	0.888	1.000

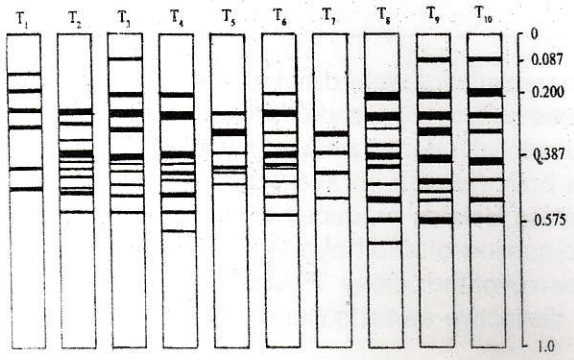

Fig. 1 : Zymogram of mycelial proteins through SDS-PAGE

lected from the rhizosphere of different crop plant under different agroecological niches which was fully rich in *Trichoderma* population. In West Bengal *Trichoderma viride*, *T. harzianum*, *T. virens*, *T.roseum* were abundant in the six agroclimatic zones of which population of *T. harzianum* was more abundant (Bose, 2006). There may be a geographic bias in the respective distribution of *Trichoderma* spp. Jash *et al.* (2003) studied the micrometric and morphometric characters of *T. virens* isolates collected from different zones of West Bengal and results showed no differences

from those made in the present investigation. Morphometric studies showed that length/breadth ratio of both phialide and phialospore is important tools for differentiation of isolates in *T. harzianum* (Bose *et al.* 2005; Jash *et al.* 2006).

Samuels (1996) observed that a relatively weak medium like CMA with 2% dextrose permits better conidial aggregation when compared to media such as PDA or OMA, where conidial production is generally much more profuse. These findings also

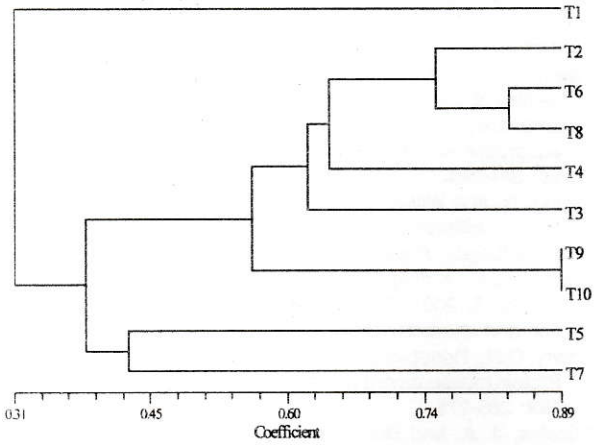

Fig. 2 : Dendrogram of *Trichoderma* isolates through SDS-PAGE

support the results of present investigations. Isolates of *T. roseum* produced a sweet coconut odour in culture and it may be due to production of volatile antibiotic 6-pentyl-á-pyrone. Rey *et al.* (2001) reported this type of odour in the culture of *T. atroviride* and *T. viride*.

Protein polymorphism

In the present investigation protein patterns of a given isolate did not vary in successive gels in that all bands were equally well resolved on each occasion. On the basis of these invariant bands, the ten isolates of *Trichoderma* were separated into different electrophoretic phenotypes (Table 3 ;Fig.1). Some bands were common in most of the isolates regardless of the species concerned or ecological habitat from which the fungus was collected. That is the genus *Trichoderma* was characterized by a particular band or set of bands. Most of the protein band concentrated with their mobility value of 0.200 to 0.575. Band with lower Rm values appeared sharper and stained more intensely than those of greater Rm. T9 and T10 isolates of *T.roseum* showed almost equal banding pattern having genetic similarity coefficient of 0.888. Genetically distant isolates were observed between T1 and T4, T1 and T7 and T5 and T9 isolates with similarity coefficient of only 0.250. However, there was some identical protein component in the isolates of a particular species but it was more difficult to establish a suitable marker protein for as species character.

Numerical analysis of protein profiling data with UPGMA resulted in splitting of the all isolates into different groups (Table 4 ; Fig. 2). T6 and T8 isolates of *T. virens* showed highest similarity coefficient of 0.833 whereas T7 isolate alone was in totally diverse line. T1 isolates of *T. viride* was distantly related to all the other isolates having highest similarity of only 0.333. However, T5 isolate of *T. harzianum* and T7 isolates of *T. viride* was in the nearest in the tree but both having similarity coefficient of only 0.428. T9 and T10 isolates of *T. roseum* were in another separate cluster having highest similarity. Intraspecific similarity was distinct in the *T. virens* and *T. roseum* species through UPGMA analysis.

The amount of variation that was found among *Trichoderma* isolates was rather high as compared to the results of other studies. In the present investigation, the cluster analysis of protein profiling data confirms the Rifai (1969) taxonomy in most of the cases. *T. roseum* isolates (T9 and T10) were in same cluster which was easily separated from *T. virens* cluster. For the taxonomical point of view only 10 isolates were not sufficient to find a distinct relationship or to separate the species by using these banding patterns. However, a number of common and unique loci for each polymorphic data obtained in this present experiment could be used further for specific biochemical markers for taxonomic differentiation in *Trichoderma*.

Stasz *et al.*(1988) showed that there was a great deal of isozyme variability within *Trichoderma* and *Gliocladium* spp. and identified a series of isozymes that give unequivocal differences among strains. One hundred nine alleles were observed at 16 loci among 71 strains tested. In nearly all cases, only a single activity band was detected. Later in another experiment Stasz *et al.* (1989) concluded that morphological species are not characterized by specific alleles at single loci or specific patterns of allele at multiple loci that they called core group of morphological species. Leuchtmann *et al.* (1996) used an isozyme analysis to test the

morphologically based taxonomic hypotheses proposed by Bissett (1984) respectively for one group of *Trichoderma* spp. and results revealed that existence of biochemically defined group within Rifai (1969) *T. longibrachiatum* aggregate.

Aside from the taxonomic implication of the present investigation, the results provide more information relative to genetic diversity and evolutionary biology of *Trichoderma* spp. Four major factors are thought to contribute to genetic diversity in fungi: population size, mutation, migration and selection (Nei, 1988). One explanation for the level of variation detected within the same species may be natural mutation. *Trichoderma* spp. is capable of producing very large number of spores in a short period of time. This combined with natural mutation rates, could lead to a relatively high level of diversity. Leung and Williams (1986) have discussed isozyme variability in fungi with high reproductive capacity; they stated that mutation in these fungi could produce significant isozyme variation , if isozymes are indeed neutral with respect to fitness. Exposure of geographically isolated population to non-preferred new environment would results in new selection pressure and added genetic diversity in *Trichoderma*. In the present investigation there was no obvious differentiation of the isolates according to geographic origin by intracellular protein profiling. However, a greater number of isolates from a broader geographic range and additional loci would have to be analyzed to support such a conclusion.

REFERENCES

Bissett, J. 1984. A revision of the genus *Trichoderma* I. Section *Longibrachiatum* sect. nov. *Canadian J. Bot.* **62**: 924-931.

Bose, S. 2006. *Studies on Bioefficacy of Some Low Cost Bioformulations of Trichoderma harzianum Rifai*. Ph.D. Thesis, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, 126 p.

Bose, S., Jash, S., Roy, M., Khalko, S. and Pan, S. 2005. Evaluation of different isolates of *Trichoderma harzianum* against soil borne plant pathogens. *J. Interacad.* **9**: 329-334.

Jash, S and Pan, S. 2004a. Stable mutants of *Trichoderma virens* for biological control against *Rhizoctonia solani* that causes seedling blight in green gram. *J. Mycol. Pl. Pathol.* **34**: 476-480.

Jash, S and Pan, S. 2004b. Evaluation of mutant isolates of *Trichoderma harzianum* against *Rhizoctonia solani* causing seedling blight of green gram. *The Indian J. Agril. Sci.* **74**: 190-193.

Jash, S and Pan, S. 2007. Variability in antagonistic activity and root colonizing behavior of *Trichoderma* isolates. *J. Trop. Agril.* **40**: 114-118.

Jash, S. 2006. *Recent approaches of biological control of plant disease with Trichoderma*. In: Trends in Organic Farming in India, Purohit, S. S. and Gehlot, D.(eds.), Agrobios (India), Jodhpur, India pp. 298-315.

Jash, S., Khalko, S., Bose, S., Roy, M. and Pan, S. 2005. Evaluation of tolerance in *Fusarium oxysporum* f.s.p. *lycopersici* and resident biocontrol agents to fungicides for integration of biological and chemical methods of disease management. *J. Mycopathol. Res.* **43**: 239-243.

Jash, S., Khalko, S., Bose, S., Roy, M. and Pan, S. 2006. Morphological and physiological characterization of some mutant isolates of *Gliocladium virens*, a potential mycoparasite of sclerotial plant pathogens. *Indian J. Agril. Res.* **40**: 114-118.

Jash, S., Mazumdar, N. and Pan, S. 2003. Comparative morphometry and asexual spore productivity among some species and isolates of *Gliocladium*. *J. Interacad.* **7**: 265-268.

Khalko, S., Jash, S., Bose, S., Roy, M. and Pan, S. 2005. Evaluation of tolerance in *M. phaseolina, T. harzianum, T. viride* and *G. virens* to fungicides. *J. Mycopathol. Res.* **43**: 121-123.

Khalko, S., Subhalaksmi, T., Jash, S., Bose, S. and Pan, S. 2006. Herbicidal tolerance of *Trichoderma* sp., a potential biocontrol agent of soil borne plant pathogens. *The Indian J. Agril. Sci.* **76**: 443-446.

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Leuchtmann, A., Petrini, O. and Samuels, G. J. 1996. Isozyme subgroups in *Trichoderma* section *Longibrachiatum*. *Mycologia* **88**: 384-394.

Leung, H. and Williams, P. H. 1986. Enzyme polymorphism and genetic differentiation among geographic isolates of the rice blast fungus. *Phytopathol.* **76**: 778-783.

Lieckfeldt, E., Kullnig, C.M., Kubicek, C.P., Samuels, G. J. and Borner, T. 2001. *Trichoderma aureoviride*: Phylogenetic position and characterization. *Mycol. Res.* **103**: 313-322.

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

Micales, J. A. and Bonde, M. R. 1995. *Isozyme: methods and application*. In: Moleculars methods in plant pathology, R. P. Singh and U. S. Singh (eds). CRC Press, Boca Raton, pp115-130.

Nei, M. 1988. Relative roles of mutation and selection in the maiatenance of genetic variability. *Philosophy Transac. Royal Soc. London*, B **319**: 615-629.

Pan, S. and Jash, S. 2009a. Production and regulation of cell wall degrading hydrolytic enzyme in mycoparasitic *Trichoderma* spp. *J. Mycol. Pl. Pathol.* **39**: 208-215.

Pan, S. and Jash, S. 2009b. Variability in sclerotial antagonism of mycoparasitic *Trichoderma* spp. against *Rhizoctonia solani* and *Sclerotium rolfsii*. *J. Mycol. Pl. Pathol.* **39**: 409-415.

Pan, S. and Jash, S. 2009c. Tolerance of *Trichoderma* isolates to new herbicides. *J. Mycol. Pl. Pathol.* **39**: 452-457

Pan, S. and Jash, S. 2010a. Variability in induction of defense response in Bengal gram against *Trichoderma* species. *Indian Phytopath.* **63**: 35-41.

Pan, S. and Jash, S. 2010b. Variability in biocontrol potential and microbial interaction of *Trichoderma* spp. with soil inhabiting antagonistic bacteria *Pseudomonas fluorescens*. *Indian Phytopath.* **63**: 158-164.

Pan, S., Bose, S. and Jash, S. 2009. Management of root rot of cabbage (*Rhizoctonia solani* and ground nut collar rot (*Sclerotium rolfsii*) with formulation of *Trichoderma harzianum*. *J. Mycol. Pl. Pathol.* **39**: 203-207.

Peberdy, J. F. and Turner, M. 1968. The esterase of *Mortierella ramanniana* in relation to taxonomy. *J. Gen. Microbiol.* **51**: 303-312.

Rey, M., Delgado-Jarana, J. and Benitez, T. 2001. Improved antifungal activity of a mutant of *Trichoderma harzianum* CECT 2413 which produces more extracellular proteins. *Applied Microbiol. Biotech.* **55**: 604-608.

Rifai, M. A. 1969. A revision of the genus *Trichoderma*. *Mycol. Papers* **116**: 1-56.

Roy, M., Bose, S., Jash, S., Khalko, S. and Pan, S. 2005. Biological control of *Macrophomina phaseolina* by some stable mutants of *Trichoderma harzianum*. *J. Mycopathol. Res.* **43**: 245-249.

Samuels, G. J. 1996. *Trichoderma*: a review of biology and systematics of the genus. *Mycol. Res.* **100**: 923-935.

Stasz, T. E. Weeden, N. F. and Harman G. E. 1988. Methods of isozyme electrophoresis for *Trichoderma* and *Gliocladium* species. *Mycologia* **80**: 870-874.

Stasz, T. E., Nixon, K., Harman, G. E. and Weeden, N. F. and Kuter, G. A. 1989. Evaluation of phenetic species and phylogenetic relationship in the genus *Trichoderma* by cladistic analysis of isozyme polymorphism. *Mycologia* **81**: 391-403.