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ISOENZYME DIVERSITY IN MYCOPARASITIC TRICHODERMA SPP.

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ABSTRACT
In attempts to know the better understanding of biochemical variability of *Trichoderma* spp. seven isoenzyme were screened for their detectable presence and all the enzyme loci showed the polymorphic bands and number of electromorphs evidenced by each system varied between 5 in case of both catalase and malate dehydrogenase and 13 in protease. T_5 isolate of *Trichoderma* showed very less number of bands in each enzyme loci excepting glutamate dehydrogenase and glutamate synthase where 7 and 5 different alleles were present, respectively. T_1 and T_7 isolates showed highest similarity (0.680) and distantly related with T_5 (0.228) as per isozyme data. The isolates belong to T_9 and T_{10} having the highest similarity index (0.460) belongs to the *T. roseum*. The number of common and unique loci for each isoenzyme obtained in this present experiment could be used further for specific biochemical markers for taxonomic differentiation in *Trichoderma*.

INTRODUCTION

Trichoderma, a filamentous soil inhabiting mycoparasite, have been used in commercial preparation for biological control of many fungal plant diseases (Jash and Pan, 2004a, b; Pan and Jash, 2009a, b, 2010a, b). 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 enzymes such as chitinase, b-1,3 glucanase (Pan and Jash, 2009a), and proteinase and as well as secondary metabolites (Jash, 2006).

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 and spore. Morphology as a single principal criterion for the classification of fungus has been found inadequate in the identification of several species. Now a day several molecular techniques are used for studying taxonomy and as well as variability in *Trichoderma* spp. Electrophoresis of intracellular protein and enzymes, DNA hybridization and PCR based characterization of 16S intergeneric spacer region of ribosomal DNA etc. 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). 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. Enzymes encoded by different alleles or separate genetic loci possess different electrophoretic mobilities. The differences are due to variation in the amino acid content of the molecule, which in turn is dependent on the sequence of nucleotides in the DNA (Umadevi *et al.,* 2001). Isozyme analysis is a useful technique presently being used by mycologists and plant pathologists to resolve taxonomic problems, identify unknown fungal isolates, finger print patentable fungal lines, analyze the extent of genetic variability in a population, trace the geographic origin of pathogens, follow the segregation of genetic loci, and determine ploidy levels at various stages in the life cycle of a fungus (Bonde *et al.,* 1991). 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 test the variability in terms of biochemical character and as well as taxonomical implication in *Trichoderma* population collected from six agroecological zones of West Bengal by employing the isozyme 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 4^0C for further use.

Extraction of enzyme

One loop full actively growing hypha of *Trichoderma* inoculated in 50 ml basal liquid glucose yeast peptone medium (GYPM) and after 7days of incubation at 28 ± 1°C, the mycelial mat was harvested. 1 g of dried mycelial mat of each isolates was homogenized in liquid nitrogen. The mycelial paste was transferred to 50 ml Oakridge centrifuge tube. To each tube 0.5 ml of 0.6 M Tris-HCl buffer (pH 6.8) was added and centrifuged 4°C. The supernatant was collected after centrifuging the mycelia mat in 0.5 ml of 0.6 M Tris-HCl buffer (pH 6.8) at 10000 rpm for 20 min stored at -15°C.

Electrophoresis for isozyme polymorphism

The seven enzyme systems used in this study (Table 2) were resolved on 8% poly acrylamide gel electrophoresis at 4°C according to Davis (1964).

Peroxidase

The 35 ml of sample containing 25 ml frozen aliquot and 10 ml dye (5% bromo phenol blue: glycerol 1:1) was loaded in each well for electrophoresis.

The gels were pre incubated in 0.1 M phosphate buffer, pH 6.6 for 30 min, transferred to the same buffer containing 50 mg O-dianisidine (dissolved in few drop of acetic acid) for 30 min in the dark and finally transferring into the buffer containing 0.015% H_2O_2 until clear bands developed (Hislop and Stahmann, 1971).

Esterase

The gels were placed in 0.2 M Tris-HCl buffer, pH 7.0 for 30 minutes and then transferred to a staining solution containing 30 mg alpha naphthyl acetate dissolved in 2 ml of ethyl alcohol (dehydrated), 100 mg fast blue RR diazonium salt and 90 ml of 0.01 M Tris-HCl Buffer, pH 7.0 (Weber *et al*., 1967).

Catalase

The procedure of Woodbury *et al*. (1971) was followed for staining catalase isozyme. The gel was washed in three changes of distilled water for 45 min to remove buffer and then incubated in 0.003% hydrogen peroxide solution for 10 min. After rinsing the gel in distilled water, it was incubated in a freshly mixed solution consisting equal volume of 1% potassium ferricyanide and 1% ferric chloride for about 20 min.

Protease

After electrophoresis, the gel was rinsed in water and incubated in solution containing 0.04 M Tris, pH 7.6, 2 mM $MgCl_2$ and 2 mM $CaCl_2$ overnight. Then the gel was stained with coomassie blue solution according to the method of Thimmaiah (1999)

Malate dehydrogenase

The procedure described by Shaw and Prasad (1970) was followed to visualize the band. The gel was immediately incubated in reaction mixture containing 50 mg NAD, 30 mg nitroblue tetrazolium, 2 mg phenazine methosulfate, 10 ml of 1 M sodium L-malate, 15 ml of 0.1 M Tris-HCl, pH 7.0, 70 ml of water and 5 ml of 0.1 M NaCN for 30 min and visualized the bands.

Glutamate dehydrogenase

The gel was incubated in reaction mixture containing 20 mg NADP, 30 mg nitroblue tetrazolium, 2 mg phenazine methosulfate, 25 ml of 0.5 M phosphate buffer, pH 8.0, 5 ml of 1 M sodium glutamate (pH 7.0) and 70 ml of water for 2 hr and visualized the bands (Vallejos, 1983).

Glutamate synthase

After electrophoresis, the gel was pre incubated in 0.1 M potassium phosphate buffer, pH 7.5 for 30 min. Then the gel was incubated in 0.1 M phosphate buffer, pH 7.5 containing 30 mM NADH, 15 mM a-ketoglutarate and 15 mM glutamine for 60 min at room temperature. After washing the gel three times in 0.1 M Tris-HCl buffer, pH 8.5, it was incubated in staining solution containing 50 ml of 0.1 M Tris- HCl buffer, pH 8.5, 250 mg nitroblue tetrazolium and 30 mg phenazine methosulfate (Thimmaiah, 1999).

Data analysis

A relative mobility (Rm) value was assigned to each band of enzyme 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 for each enzyme. Computer software, Numerical Taxonomy System for Multivariate Statistical Program (NTSYS-PC) version 1.60 (Exeter Publishing Co., Setauket, NY) (Rohlf, 1990) was used for analysis of isoenzymes data. The presence or absence, 1 and 0, respectively, for a particular enzyme 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 $S_{sm} = (a+b)/n$, 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 (Sneath and Sokal, 1973). A phenetic tree was developed from the matrix of similarity coefficient by the unweighted pair-group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

Seven enzymes were screened for their detectable presence in *Trichoderma* isolates and all the enzyme loci showed the polymorphic bands and number of electromorphs evidenced by each system varied between 5 in case of both catalase and malate dehydrogenase and 13 in protease (Table 3). Phenotypic banding patterns were identified based on relative position of isozymes and interpreted in terms of presumed alleles of the genetic loci coding for the isoenzymes. T_5 isolate of *Trichoderma* showed very less number of bands in each enzyme loci excepting glutamate dehydrogenase and glutamate synthase where 7 and 5 different alleles were present, respectively. Isolate with isozyme banding pattern in common were not found and all were different electrophoretic phenotypic. An electrophoretic phenotype was defined as one or more fungal isolates with distinctive combination of isoenzyme phenotype.

Seven polymorphic bands were found in esterase isozyme system (Fig 1). Rm value of band varied from 0.237 to 0.725. The top most cathodal band of Rm value 0.237 was found in all the isolates excepting the T_4 and T_5. T_9 and T_{10} isolates of *T. roseum* was similar in esterase isozyme banding pattern indicating the same electrophoretic phenotype. Again T_7 and T_8 isolates of *T. virens* were same electrophoretic phenotype

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

Sl. No.	Code	Taxa	*Crop associated*	Location
1.	T_1	*T. viride*	Potato field	Falakata, Jalpaiguri
2.	T_2	*T. harzianum*	Chilli field	Kakdwip, South 24 Pgs
3.	T_3	*T. viride*	Mustard field	Bishnupur, Bankura
4.	T_4	*T. harzianum*	Brinjal field	Kalyani, Nadia
5.	T_5	*T. harzianum*	Sunflower field	Namkhana, South 24 Pgs.
6.	T_6	*T. virens*	Gladiolus field	Kalimpong, Darjeeling
7.	T_7	*T. virens*	Jute field	Arambagh, Hooghly
8.	T_8	*T. virens*	Cabbage field	Ranaghat, Nadia
9.	T_9	*T. roseum*	Potato field	Alipurduar, Jalpaiguri
10.	T_{10}	*T. roseum*	Rice field	Sehara Bazar, Bardhaman

Table 2: Enzymes with detectable activity, abbreviations, commission number and buffer systems used in this study

Enzymes	Abbreviation	E.C. number	Buffer system
Esterase	EST	3.1.1.1	LB
Peroxidase	POD	1.11.1.7	TG
Catalase	cat	1.11.1.6	TG
Protease	pro	-	tc
Malate dehydrogenase	mdh	1.1.1.37	tm
Glutamate dehydrogenase	GDh	1.4.1.3	tg
Glutamate synthase	gogat	2.6.1.53	tg

Table 3: Alleles detected at seven enzyme loci for ten isolates of *Trichoderma* spp.

Isolate	Enzyme* EST	POD	CAT	RRO	MDH	GDH	GOGAT
T_1	6	5	4	13	4	7	6
T_2	4	6	4	8	3	8	4
T_3	3	5	5	9	4	4	3
T_4	4	4	3	5	3	6	5
T_5	2	2	2	4	3	7	5
T_6	3	3	4	6	4	5	5
T_7	5	3	3	10	4	7	5
T_8	5	4	4	7	5	8	5
T_9	2	5	4	5	4	8	7
T_{10}	2	6	3	5	2	3	6

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

due to similar in isozyme banding pattern. T_5 isolate of *T. harzianum* only produced dimorphic band of Rm value of 0.550 and 0.650. Esterase isoenzyme system may be useful in distinguishing the isolates between the *T. roseum* and *T. virens*.

Peroxidase isozyme showed the 10 polymorphic bands in the 10 isolates of *Trichoderma* spp. (Fig. 2). Top most cathodal band (Rm value 0.237) was present in all the isolates except in T_5. Two isolates of *T. virens* (T_6 and T_7) showed similar banding pattern. T_5 isolate showed only two separate bands and was totally different from others. Similarly, T_1 and T_3 isolates under the *T. viride* group showed almost equal banding pattern and difference was that only one additional dark band in T_1 isolate.

When the catalase activity was analyzed, each of the isolates had several isozymes that were variable in both mobility and number (Fig. 3). Two bands with Rm value of 0.037 and 0.062 were present in all the isolates except in T_5 isolate. T_1 isolate of *T. viride,* T_2 of *T. harzianum*, T_6 and T_8 of *T. virens* and T_9 isolate of *T. roseum* showed equal banding pattern in catalase isozyme. The only one band with mobility value of 0.225 made the difference between T_2 and T_4 of *T. harzianum,* T_7 and T_8 of *T. virens* and T_9 and T_{10} of *T. roseum*.

The banding pattern of protease isozyme was more complex than others (Fig. 4). The total 21 polymorphic bands were observed in protease isozyme of which T_1 isolate showed 13 polymorphic bands. Rm value of band varied from 0.025 to 0.962 indicating wide variation in enzyme polymorphism. There was no distinct relationship between the different species of *Trichoderma*. Only a single band of Rm value 0.262 was present in all the 8 isolates of *Trichoderma* having absent in T_1 and T_7. T_1 isolate of *T. viride* possessed another two bands (Rm 0.775 and 0.887), which were absent in rest of the isolates and similarly T_7 with one band of Rm value of 0.650.

Some variation was noted in malate dehydrogenase isoenzyme pattern among the isolates within a group (Fig. 5) and a total of 8 polymorphic bands were found in the 10 isolates. T_8 isolates of *T. virens* produced highest polymorphic bands where as only dimorphic band in T_{10} isolate. A band of Rm value 0.375 was common in all the *Trichoderma* isolates except in T_5. T_6 and T_7 isolates of *T. virens* showed similar banding pattern in

Table 4: Relative mobility (Rm) of alleles of enzyme loci for ten isolates of *Trichoderma* spp.

Loci	Rm value Esterase	Peroxidase	Calalase	Protease	Malate dehydro-genase	Glutamate dehydro- genase	Glutamate synthase
1	0.237	0.237	0.037	0.025	0.075	0.052	0.084
2	0.475	0.028	0.062	0.062	0.100	0.173	0.231
3	0.500	0.350	0.225	0.087	0.312	0.191	0.368
4	0.550	0.512	0.262	0.112	0.375	0.278	0.431
5	0.587	0.550	0.300	0.237	0.562	0.330	0.484
6	0.650	0.562	0.725	0.262	0.675	0.426	0.557
7	0.725	0.600	0.775	0.287	0.762	0.486	0.642
8		0.612		0.325	0.875	0.539	0.778
9		0.637		0.375		0.713	0.915
10		0.712		0.400		0.739	
11				0.512		0.878	
12				0.550		0.921	
13				0.575			
14				0.600			
15				0.650			
16				0.712			
17				0.725			
18				0.775			
18				0.837			
20				0.887			
21				0.962			

Table 5: Genetic similarity between electrophoretic phenotypes of *Trichoderma* isolates through isoenzyme analysis

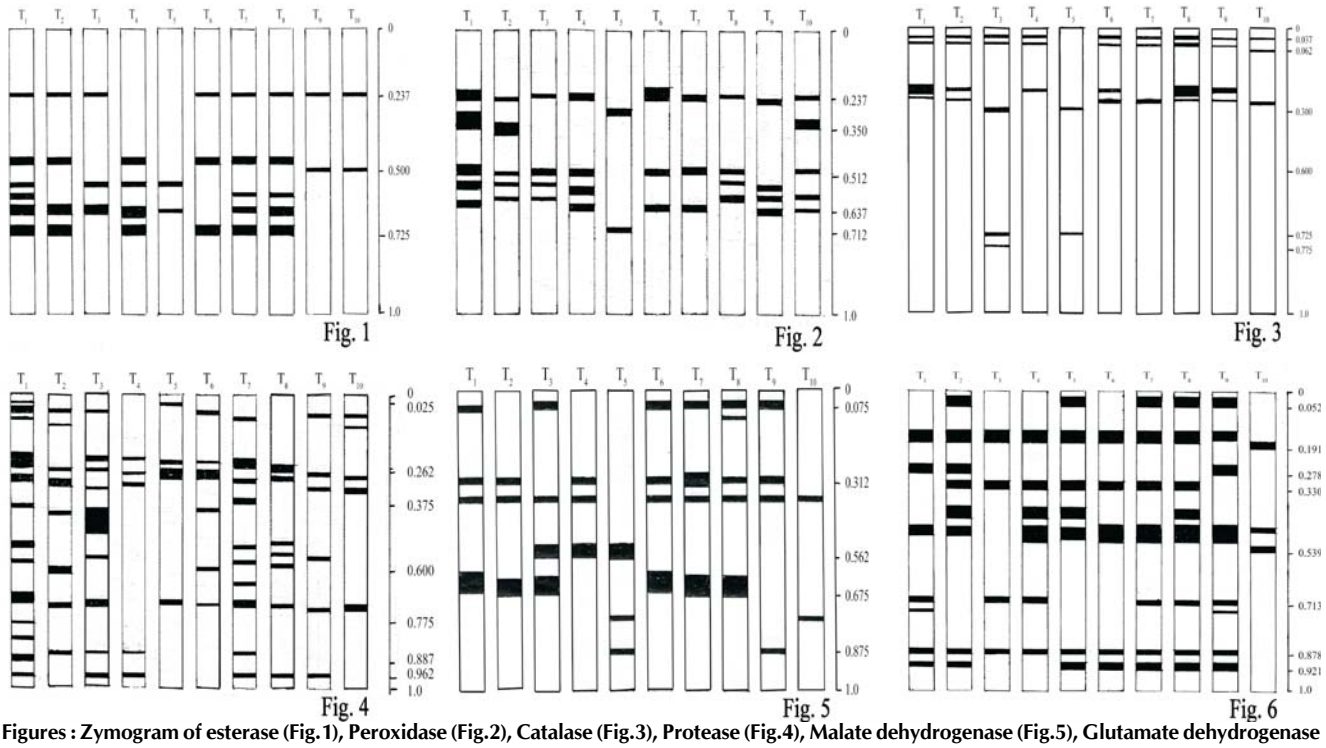
Isolate	Electrophoretic phenotype T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
T_1	1.000									
T_2	0.509	1.000								
T_3	0.436	0.440	1.000							
T_4	0.509	0.458	0.466	1.000						
T_5	0.228	0.260	0.311	0.357	1.000					
T_6	0.500	0.545	0.422	0.512	0.279	1.000				
T_7	0.680	0.480	0.403	0.543	0.254	0.604	1.000			
T_8	0.627	0.520	0.469	0.521	0.312	0.619	0.673	1.000		
T_9	0.454	0.460	0.352	0.395	0.250	0.477	0.396	0.460	1.000	
T_{10}	0.263	0.326	0.270	0.255	0.155	0.325	0.222	0.250	0.409	1.000

malate dehydrogenase system while T_8 isolate of *T. virens* showed one additional band.

In case of glutamate dehydrogenase isozyme system (Fig. 6) more number of common alleles was noticed. Band with relative mobility of 0.173 and 0.878 were absent in T_{10} isolate. Another band of Rm value 0.486 was present in all the isolates excepting T_3. Among the 3 polymorphic bands in T_{10}, two were not common in any other isolates. Similar type of trends in common band were also noticed when zymogram of glutamate synthase isoenzyme was drawn (Fig.7).

Numerical analysis of isoenzyme data with UPGMA resulted in splitting of the all isolates into different groups (Table 5, Fig. 8). T_6 and T_8 isolates of *T. virens* showed highest similarity coefficient of 0.67 and T_5 isolate alone was in totally diverse line. The first group composed of three clusters containing the six isolates of which three identified as *T. virens*, two as *T. harzianum* and one as *T. viride*. T_3 isolate of *T. viride* occupied a rather separate position in the phenetic tree. T_9 and T_{10} isolates of *T. roseum* were in another separate cluster. Intraspecific similarity was distinct in the *T. virens* and *T. roseum* species through UPGMA analysis.

The seven enzyme systems were selected because of their high resolution and reproducibility on poly acrylamide gel electrophoresis. In the 1960s and 1970s, analytical techniques were developed for utilizing isozyme data for numerical taxonomy (Nei, 1972). Because the majority of animal and plant populations for which these techniques were developed were diploid, sexually reproducing species, several of the assumptions that must be met to apply these techniques were not applicable to haploid asexual organisms such as fungi. Although multiple bands for a given enzyme may result from post translational modifications or multiple subunits, as well as different alleles or loci, several crosses that would classify these relationships are not possible with this fungus. Thus the bands can only be treated as putative loci and alleles. The absence of bands in certain isolates was considered as the expression of presumed null alleles or other alternative mechanisms may be involved. Micales *et al.*, (1987) explained the absence of bands may be due (i) low levels of detection of certain enzymes, (ii) determination of enzymes during sample



Figures : Zymogram of esterase (Fig.1), Peroxidase (Fig.2), Catalase (Fig.3), Protease (Fig.4), Malate dehydrogenase (Fig.5), Glutamate dehydrogenase (Fig.6)

preparations (iii) lack of expression of inducible enzymes in culture medium and (iv) inability of isolates to produce certain enzymes, because of different selection pressure that they face in culture medium.

The amount of variation that was found among *Trichoderma* isolates was rather high as compared to the results of other studies. Petrunak and Christ (1992) examined the 44 isolates of *Alternaria solani* and 96 isolates of *A. alternata* from various host and geographic locations for isozyme variability and found that the average genetic diversity for a given enzyme ranged from 0.000 to 0.763; the average genetic diversity was 0.500. In bean rust study Linde *et al.* (1990) found that most distantly related isolates in the study had approximately 36% similarity, but most of the isolates had similarity coefficient of 84% or greater. Laroche *et al.* (1992) differentiated the different anastomosis groups of *R. solani* into genetically distant groups on the basis of principal component analysis and cluster analysis using isozyme electrophoresis. Chen *et al.* (1992) used isozyme analysis of 13 enzymes to compare 204 isolates of 10 *Pythium* spp. from diverse geographical locations and found that morphologically distinct species could be differentiated by banding pattern. Analysis of isozyme banding pattern of á- and â-estarease has also been utilized for differentiating Alternaria spp. in ornamental plants (Ambesh *et al.*, 2014, Marak *et al.*, 2014). Roy Barman and Dutta (2016) reported intracellular protein and isoenzyme variability in *Xanthomonas campestris* pv. *campestris*.

In the present investigation, the cluster analysis of isoenzyme data confirms the Rifai (1969) taxonomy in most of the cases. *T. roseum* isolates (T_9 and T_{10}) were in same cluster which was easily separated from *T. virens* cluster. Among the three *virens* isolates, T_6 and T_8 were almost similar and present in same cluster while T_7 isolates possess another adjacent cluster having similarity coefficient of 0.603 with T_8 isolates. The results were not too much satisfactory in *T. harzianum* isolates because T_5 isolate was totally genetically different from another isolates and placed in separate 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 isoenzyme obtained in this present experiment could be used further for specific biochemical markers for taxonomic differentiation in *Trichoderma*.

Stasz *et al.* (1988b) 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 on isozyme polymorphism they 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. Despite the individual strain Stasz *et al.* (1989a) gave widely divergent allozyme patterns, the core groups coincided well with Rifai aggregate species *T. pseudokoningii, T. koningii, T. hamatum* and *T. viride*. Strains used under the name of *T. harzianum* aggregates species in their experiment fell into one or two cluster. These results indicated that, in part there was a genetic basis for the morphological aggregates species and also confirm Rifai's own contention that each aggregate is genetically heterogeneous. Leuchtmann *et al.* (1996) used an isozyme

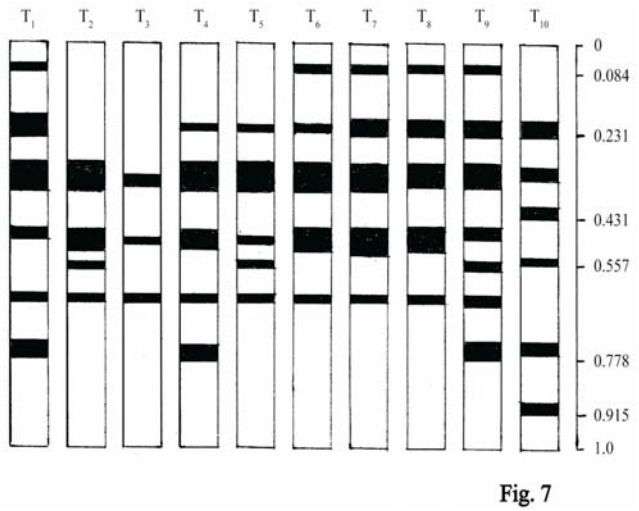


Fig. 7

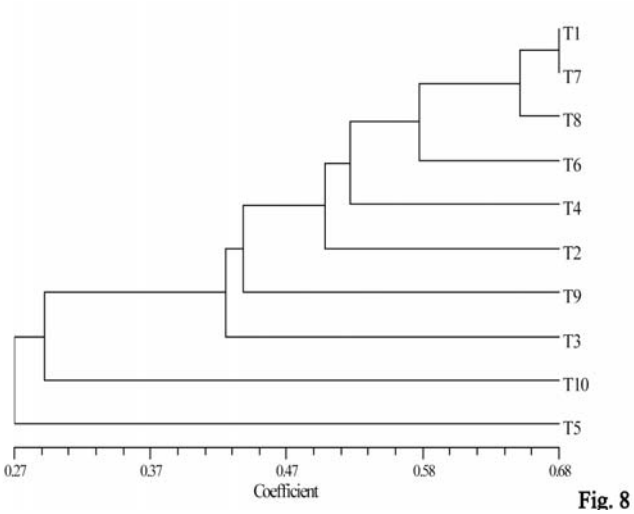


Fig. 8

Figures: Zymogram of Glutamate synthase (Fig.7), Dendogram tree of *Trichoderma* isolates based on isozyme profiling (Fig.8)

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

Beside 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. are 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 isozyme analysis. However, a greater number of isolates from a broader geographic range and additional loci would have to be analyzed to support such a conclusion.

Ambesh, B., Ngomle, S., Marak, T. and Das, S. 2014. Morphological and biochemical variation of some *Alternaria* species infected in different floricultural plants**.** *The Ecoscan: Special issue.* **VI:** 361-368

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

Bonde, M. R., Peterson, G. L., Emmett, R. W. and Menge, J. A. 1991. Isozyme comparisons of *Septoria* isolates associated with citrus in Australia and the United States. *Phytopathol.* **81:** 517-521.

Chen, W., Schneider, R. W. and Hoy, J. W. 1992. Taxonomic and phylogenetic analysis of ten *Pythium* species using isozyme polymorphism. *Phytopathol.* **82:** 1234-1244.

Davis, B. J. 1964. Disc electrophoresis II. Method and application of human serum proteins. *Annals New York Acad. Sci.* **121:** 404-427.

Hislop, E. C. and Stahmann, M. A. 1971. Peroxidase and ethylene production by barley leaves infected with *Erysiphe graminis* f.sp. *hordei*. *Physiol. Pl. Pathol.* **1:** 297-312.

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 Plant 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. 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.

Laroche, J. P., Jabaji- Hare, S. H. and Charest, P. M. 1992. Differentiation of two anastomosis groups of *Rhizoctonia solani* by isozyme analysis. *Phytopathol.* **82:** 1387-1393.

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.

Linde, D. C., Groth, J. V. and Roelfs, A. P. 1990. Comparison of isozyme and virulence diversity pattern in the bean rust fungus *Uromyces appendiculatus*. *Phytopathol.* **80:** 141-147.

Marak, T. R., Ambesh, B. S. and Das, S. 2014. Cultural, morphological and biochemical variation of *Alternaria solani* causing disease on solanaceous crops. *The Bioscan*. **9:** 1295-1300.

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*, pp.115-130.

Micales, J. A., Stipes, R. J. and Bonde, M. R. 1987. On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. *Mycologia*. **79:** 707-720.

Nei, M. 1972. Genetic distance between populations. *American Nature* **106:** 283-292.

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 Plant Pathol.* **39:** 208-215.

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. and Jash, S. 2010b. Variability in sclerotial antagonism of mycoparasitic *Trichoderma* spp. against *Rhizoctonia solani* and *Sclerotium rolfsii*. *J. Mycol Plant Pathol.* **39:** 409-415.

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

Petrunak, D. M. and Christ, B. J. 1992. Isozyme variability in *Alternaria solani* and *A. alternata*. *Phytopathol.* **82:** 1343-1347.

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

Rohlf, F. J. 1990. *NTSYS-PC. Numerical Taxonomy and Multivariate Analysis System.*Version 1.60. *Exeter Publishing, Setauket, NY.*

Roy Barman, A. and Dutta, S. 2016. Intracellular protein and isoenzyme variability of *Xanthomonas campestris* pv. *campestris*. *The Ecoscan*. **10:** 55-62.

Shaw, C. R. and Prasad, R. 1970. Starch gel electrophoresis of eyzymes, a compilation of recipes. *Biochem Genet.* **4:** 297-320.

Sneath, P. H. A. and Sokal, R. R. 1973. *Numerical Taxonomy: The Principles and Practices of Numerical Classification*. W.H. Freeman, San Francisco, California. p. 573.

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.

Thimmaiah, S. R. 1999. *Standard Methods of Biochemical analysis,* Kalyani Publishers Ludhiana, Haryana, India. p. 545.

Umadevi, G., Pal, M. and Mitter, N. 2001. Isozyme variability among isolates of *Ascochyta rabiei*. *Indian Phytopath.* **54:** 16-22.

Vallejos, E. 1983. Enzyme activity staining. In: *Isozymes in Plant Genetics and Breeding* Part A. Tanskley, S. D. and Orton, T. J.(eds)., *Elsevier Science Publishers, B.V., Amsterdam*. pp. 469-511.

Weber, O. J., Clare, B. and Stahmann, M. A. 1967. Enzymic changes associated with induced and natural resistance of sweet potato to *Ceratocystis fimbriata*. *Phytopathol.* **57:** 421-424.

Woodbury, W., Spencer, A. K. and Stahmann, M. A. 1971. An improved procedure using ferricyamide for detecting catalase isozymes. *Annals Biochem.* **44:** 301-305.