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Morphological Characterization of Biocontrol Isolates of Trichoderma to Study the Correlation between Morphological Characters and Biocontrol Efficacy

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Abstract. The morphological characterization was carried out for 5 isolates of *Trichoderma harzianum* and 7 isolates of *Trichoderma viride* and tested for their biocontrol efficacy. The isolates belonging to *T.harzianum* were analogous in colony colour, culture smell, mycelial colour, conidiation, conidial shape, conidial wall and conidial colour. Correspondingly the isolates of *T.viride* showed certain similarity in colony colour, colony edge, culture smell, conidiophore branching, conidial wall, conidial colour and chlamydospores. Inter specific differences through cluster analysis based on morphological characters grouped the twelve isolates into three major clusters where all the isolates of *T.harzianum* formed a single cluster while the isolates of *T.viride* were bifurcated into two groups. The clustering was substantiated by similarity index which showed maximum similarity among *T.harzianum* isolates with only less than 20% variation among themselves. Similarly the clusters having isolates of *T.viride* also had less variation within them. The biocontrol efficacy of these twelve isolates of *Trichoderma* was experimented by dual culture test under laboratory condition and there existed some relation between the biocontrol efficacy of these isolates and morphology.

Introduction

Identification based on morphological characters consent a relatively simple method for classification of *Trichoderma* as genus, but the species perceptions are complex to construe and there is considerable confusion over the application of specific names. Bisby could identify only one species *T.viride* after examining several isolates and collection of *Trichoderma* without finding any reliable characters to distinguish them, he concluded *Trichoderma* as a monotypic genus. It was only few years ago the factual character of *Trichoderma* has been recognized [1]. Pioneers in *Trichoderma* like Rifai and Bissett observed certain cultural characters that could be used for identification and description of these species *viz,* tuft or cushion of hyphae on natural substrate composed of conidiophores, spores and some sterile hyphae, conidiophores indefinite, branched or unbranched hyphae bearing phialides laterally or terminally, phialides oust by heads rarely short or in chains, spore hyaline or brightly colored, one celled. Nonetheless, class affinities of the genus for the development of species are still very slow [14, 3, 4, and 5]. Rifai classified the *Trichoderma* into nine species aggregates [14], further it was elaborated by Bissett [2, 3, 4 and 5], covering thirty five species, their classification reflected the importance of microscopic characters for delimiting the *Trichoderma* species.

Members of the fungal genus *Trichoderma* were found to be useful as effective biological control agents for many diseases caused by soil borne pathogens. Weindling gave the first report on *Trichoderma* as a potential biocontrol agent [22], since then various workers have speculated the existence of biological control ability of *Trichoderma* for over seventy years [9]. *Trichoderma* species can act as biocontrol agents through different synergistic mechanisms. However, it is difficult to predict the degree of synergism and the behavior of a biocontrol agent in a natural

pathosystem. Considering that environmental conditions are important, the right selection of biocontrol agents, which begins with a safe characterization of biocontrol strains in the new taxonomic schemes of *Trichoderma*, is equally important since the exact identification of strains to the species level is the first step in utilizing the full potential of fungi in specific applications [11]. However the taxonomic status of this species is imprecise and the criteria used to classify and identify strains so far do not provide sufficient discrimination, especially with those isolates of interest in biocontrol programmes. Therefore the present study enumerated to characterize the cryptic species of *Trichoderma* taxa associated with biological control of certain soil borne pathogens based on morphological characters.

Material and methods

Morphological characterization

The five isolates of *T.harzianum* and seven isolates of *T.viride* examined for this study are listed in Table 1. All the isolates taken for study were already classified as *Trichoderma harzianum* and *Trichoderma viride* through biochemical analysis based on their toxicity over the plant pathogens and was reported as potential biocontrol agents [16].

Table 1. List of the *Trichoderma* isolates taken for the study

No.	Isolate name	Species
1	Th3	*T.harzianum*
2	Th10	*T.harzianum*
3	Th30	*T.harzianum*
4	Th31	*T.harzianum*
5	ThAg	*T.harzianum*
6	Tv2	*T.viride*
7	Tv4	*T.viride*
8	Tv12	*T.viride*
9	Tv15	*T.viride*
10	Tv32	*T.viride*
11	TvChen	*T.viride*
12	TvNir	*T.viride*

PDA with low sugar medium was used for the manipulation of growth rate of different isolates [13]. Few days later after the colonies become visible, mycelial mat of about 5 mm diam was taken from the actively growing edge of the colony and inoculated onto freshly prepared medium. The transfer of the mycelial mat should be before the culture start producing conidia. The mat was placed approximately 1.5cm from the edge of the petriplate and the plates were incubated under darkness at 25^0C. They were examined at 25 hourly intervals and observed for growth rate (colony radius from the edge of mat), colony edge, colour, smell and reverse colony colour. The growth tests were repeated four times at roughly weekly intervals and the average readings were taken. All micro morphological data were taken within one week from colonies grown on PDA containing the antibiotics streptomycin at 25^0C.

Every measurement of micro morphological characters were taken from material that was immersed in drop of 3% aq. KOH, which was consequently substituted by water since the microscopic preparation desiccated. For direct microscopic observations, 20 units of every character

were measured with the exclusion of chlamydospores. Light and phase contrast microscope were employed in the present study. The morphological characterization was studied by the observations made from the microscopic slides, with verification of the key provided by Rifai and Bissett [14, 2, 3, 4, and 5]. Other cultural characters like colony color, growth rate, colony edge and culture smell were also studied.

Statistical analysis

The observed phenetic characters were used as descriptors and the variation present within each descriptor were called as descriptor states. For our convenience each descriptor states was assigned with a rank which was used for our morphological analysis.

Statistical analysis was carried out using INDOSTAT package developed by Indostat Services, Hyderabad, India.

Multivariate analysis

Classification (cluster) and ordination (Principal Component Analysis) analysis were performed.

Cluster analysis

Simple matching similarity index was used to form clusters based on various quantitative and qualitative characters traits of 12 isolates.

For phylogenetic linkage study Weighted Average Linkage Clustering was done. This method takes weighted average of resemblance coefficients when revising the resemblance matrix. For example, suppose we are using a resemblance coefficient with values denoted by R_{jk}, under weighted average linkage clustering method, the value of coefficient between say, clusters 124 (i.e. containing accessions 1,2,4) and 35 (i.e. containing accessions 3,5) is

$R_{(124)(35)} = w_{13}R_{13} + w_{15}R_{15} + w_{23}R_{23} + w_{25}R_{25} + w_{34}R_{34} + w_{45}R_{35,}$ where the weights, w_{jk} are unequal.

The method gives large weights to those isolates admitted to their clusters at a more recent clustering step. This means that the earlier an isolate enters a cluster in the sequence of clustering steps, the less weight its resemblance with isolates outside its cluster is given when the resemblance between clusters is evaluated. The scored data was finally converted into binary form based on presence (1) or absence (0) for that particular character and subjected to statistical analysis using INDOSTAT package developed by Indostat services, Hyderabad, India.

Dual culture test

Invitro confrontations were studied by dual culture technique [7].This technique was used to test the antagonistic ability of *Trichoderma* isolates against the phytopathogenic fungi *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*. The host fungus and *Trichoderma* were grown on potato dextrose agar (PDA) for a week at room temperature (28±2°C). Small bocks of the target fungus were cut from the periphery and transferred to the fresh petridish containing PDA. The Petridish consisting of target fungi and *Trichoderma* was incubated at room temperature and observed periodically.

Growth parameters in all dual cultures were read after 7 days. The assay was repeated twice. As all the isolates were having biocontrol potentiality every isolates were taken for further studies. The plates containing only the target pathogenic organisms without *Trichoderma* were taken as control to evaluate the percent growth inhibition.

Results

Morphological characterization

The basic for taxonomical studies is morphological characterization which even though is a long-established technique still today it has its importance. The isolates were grown on PDA for 3-7 days as pure cultures and the various mycelial and conidial characters of different isolates were recorded as camera lucida drawings. The morphological characters were based on the fifteen characters included here viz. colony Growth rate (after 7 days in cm) at 25^0C, colony colour, reverse colony colour, colony edge, culture smell, conidiation, mycelial form, mycelial colour, conidiophore branching, phialide disposition, phialide shape, conidial shape, conidial wall etc (Table 2). The morphological observations of the twelve isolates can be recapitulated as follows. Most of the isolates belonging to *T.harzianum* were similar in colony colour, culture smell, mycelial colour, conidiation, conidial shape, conidial wall and conidial colour (Plate 1). Similarly the isolates of *T.viride* showed certain similarity in colony colour, colony edge, culture smell, conidiophore branching, conidial wall, conidial colour and chlamydospores (Plate 2). The major difference between the isolates of *T.harzianum* and *T.viride* were their conidial wall pattern, conidial shape, conidial colour, colony edge and culture smell.

Table 2. Descriptions and morphological characters of the specios used for statistical analysis

	Isolate/ characters	Th3	Th10	Th30	Th31	ThAg	Tv2	Tv4	Tv12	Tv15	Tv32	TvChen	TvNir
Over all appearance	Colony growth rate (cm)	8-9 in 3days	8-9 in 3days	8-9 in 3days	8-9 in 3days	9-10cm in 5days	9-10cm in 5days	7-8cm in 5days	6-7cm in 5days	9-10cm in 5days	9-10cm in 5days	9-10cm in 5days	9-10cm in 5days
	Colony colour	Green to dark green	Green to dark green	Green to dark green	Green to dark green	Dark green	Dark green	Dark green	Yellow to green	Olive green to Dark green	Yellow to green	Yellow to green	Dark green
	Reverse colony colour	Yellow	Colourless	Light yellow	Creamish	Deep yellow	Deep yellow	Colourless	Deep yellow	Colourless	Deep yellow	Colourless	Deep yellow
	Colony edge	Smooth	Smooth	Smooth	Smooth	Wavy	Wavy	Wavy	Wavy	Wavy	Smooth	Wavy	Wavy
	Culture smell	Malt	Malt	Malt	Malt	Coconut	Coconut	Coconut	Coconut	Coconut	Coconut	Coconut	Coconut
Mycelium	Mycelial form	Arachnoid	Floccose to Arachnoid	Floccose to Arachnoid	Floccose to Arachnoid	Floccose to Arachnoid	Arachnoid	Arachnoid	Floccose	Floccose	Floccose	Floccose	Floccose to Arachnoid
	Mycelial colour	Watery white	Watery white	Watery white	Watery white	Watery white	Watery white	White	White	Watery white	Watery white	White	Watery white
Conidiophore	Conidiation	Ring like zones	Ring like zones	Ring like zones	Ring like zones	Concentric zones	Concentric zones	Concentric zones	Ring like zones	Ring like zones	Ring like zones	Concentric zones	Concentric zones
	Conidiophore branching	Highly branched, regular 2-3µmL	Highly branched, regular 4-6µmL	Branched, regular 1-2µmL	Branched, regular 1-3µmL	Moderately branched, irregular 4-5µmL	Moderately branched, irregular 2-2.5µmL	Moderately branched, irregular 4-5µmL	Moderately branched, irregular 4-5µmL	Moderately branched, irregular 1.5-2.5µmL	Highly branched, irregular 1.5-2.5µmL	Highly branched, irregular 1.5-2.5µmL	Moderately branched, irregular 4-5µmL
Phialide	Phialide disposition	2-3 whorls	Solitary	2/3 whorls	2-3 whorls	Solitary	2/3 whorls	2/3 whorls	2/3 whorls	Solitary	Solitary	Solitary	Solitary
	Phialide shape	Globose 8-15 x 2-3 µm	Nine-Pin shape 8-15 x 2-3 µm	Nine-Pin shape 8-14 x 2.4-3 µm	Globose 8-15 x 2-3 µm	Sigmoid or hooked 8-14 x 2.4-3 µm	Sigmoid or hooked 8-14 x 2-3.5 µm	Sigmoid or hooked 8-14 x 2-3 µm	Sigmoid or hooked 8-14 x 2.4-3 µm	Nine-Pin shape 15 x 2.5-3.5 µm	Nine-Pin shape 4.5-12.5 x 2.5-3.5 µm	Nine-Pin shape 5-18 x 2-3.5 µm	Sigmoid or hooked 8-14 x 2.4-3 µm
Conidia	Conidial shape	Subglobose 3.6-4.5 µm	Subglobose 3.6-4.5 µm	Subglobose 3-4.8x3.5-4 µm	Subglobose 3.5-4.5 µm	Globose to obovoid 3.6-4.5 µm	Globose to obovoid 3-4.5 µm	Globose to obovoid 3.6-4.5 µm	Globose to subglobose 3.6-4.5 µm	Globose to subglobose 3-5 µm	Globose to subglobose 2.5-3.5 µm	Globose to subglobose 2.5-5 µm	Globose to obovoid 3.6-4.5 µm
	Conidial wall	Rough	Rough	Rough	Rough	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
	Conidial colour	Green	Green	Green	Green	Green	Green	Green	Green	Dark Green	Dark Green	Dark Green	Green
Chlamydospores		Not observed	Not observed	Present globose	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

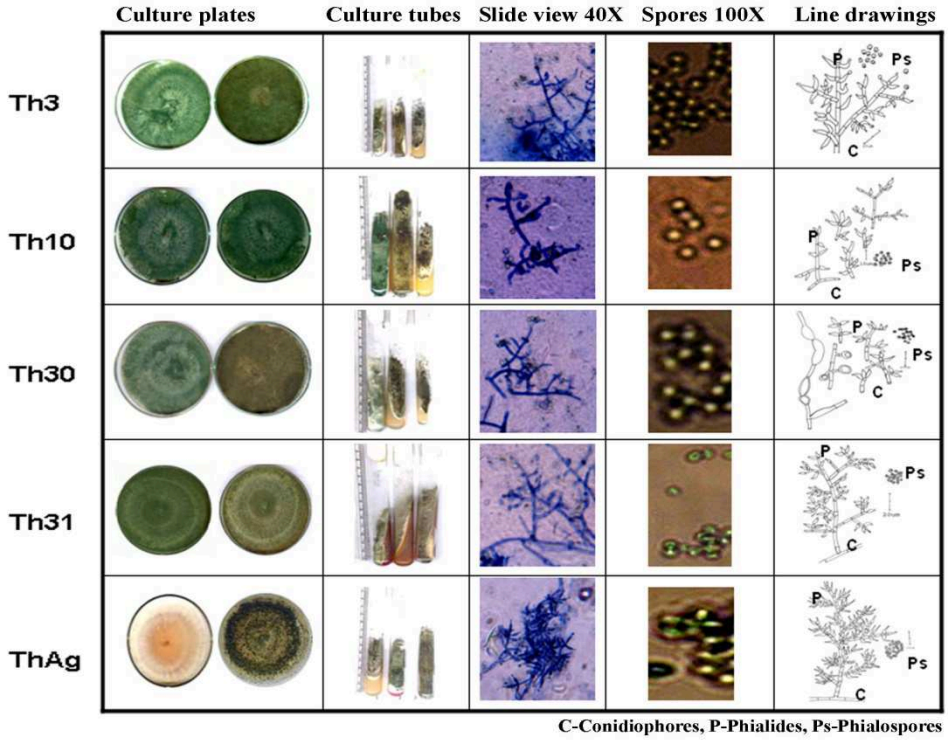

Plate 1. Morphological observation of *T.harzianum* isolates (slide view – light microscope, scale for camera lucida drawings 20µm)

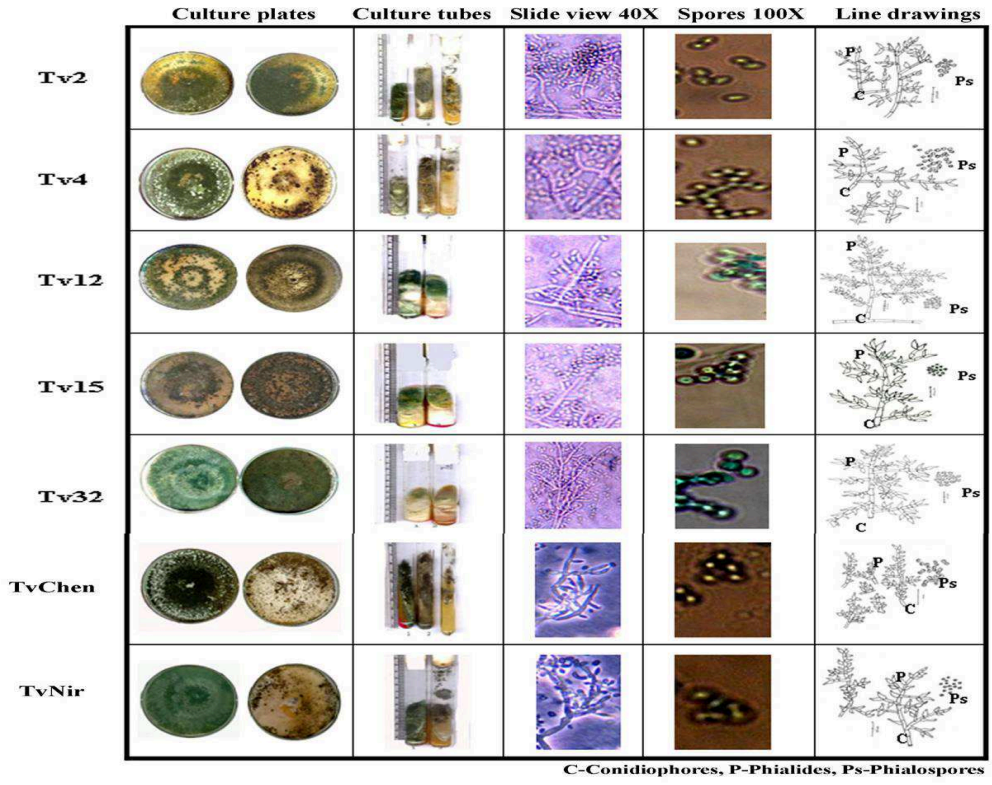

Plate 2. Morphological observation of *T.viride* isolates (slide view – light microscope, scale for camera lucida drawings 20µm)

Cluster analysis

As mentioned above, only few characters were significantly differing between the two species, a total of fifteen quantitative and qualitative characters were taken to analyze the inter specific difference. However most of characters observed were in abstract nature which may hinder the statistical analysis of the isolates. Thus variation in the characters was given specific ranking so as to facilitate the comparative study. These numerical data was again converted to binary form, which was subjected to statistical analysis. This was based on the report by Samuel *et al*. where he used the morphological characterization to analyze the variation between the isolates among *Trichoderma* species associated with green mold epidemic of commercially grown *Agaricus bisporus* [15]. Similarly Munaut *et al*. reported variation in *Colletotrichum gloeosporioides* based on morphological characterization [12].The data were used to compute simple matching similarity index and dendrogram was constructed which is shown in Fig. 1. Three distinct clusters were obtained and the morphological similarity among the isolates varied from 40-90%. The salient features of the clusters are summarized below.

Cluster 1 This cluster consists of all the isolates of *T.harzianum* characterized by colony colour, culture smell, mycelial colour, conidiation, conidial shape, conidial wall and conidial colour.

Cluster 2 Four isolates of *T.viride* formed this cluster having characters like similar colony edge, culture smell, conidiophore branching, phialide shape, conidial wall and chlamydospores.

Cluster 3 Tv32, TvChen and TvNir belonging to *T.viride* formed this cluster which consisted of similar growth rate, colony colour, mycelial form, phialide disposition, phialide shape, conidial shape, conidial wall, conidial colour and chlamydospores.

The cluster analysis grouped the twelve isolates into three major clusters. The inter and intra cluster distances are shown in Table 3. The inter cluster distance between clusters 1 and 3 was the highest (0.605) followed by cluster 1 and 2 (0.458). Intracluster distance was highest for cluster 2 (0.332) followed by cluster 1 (0.20) and cluster 3 (0.175).From the intra cluster distances it can be inferred that the cluster 2 was more diverse than cluster 1 and 3 which was supported by shaded matrix index. The inter cluster distances showed that cluster 1 was highly distant from cluster 3 followed by cluster 2. This finding confirmed the result of cluster analysis where *T.harzianum* formed an entirely distinct group from the isolates of *T.viride*. The result of PCA was also in consistent with this finding.

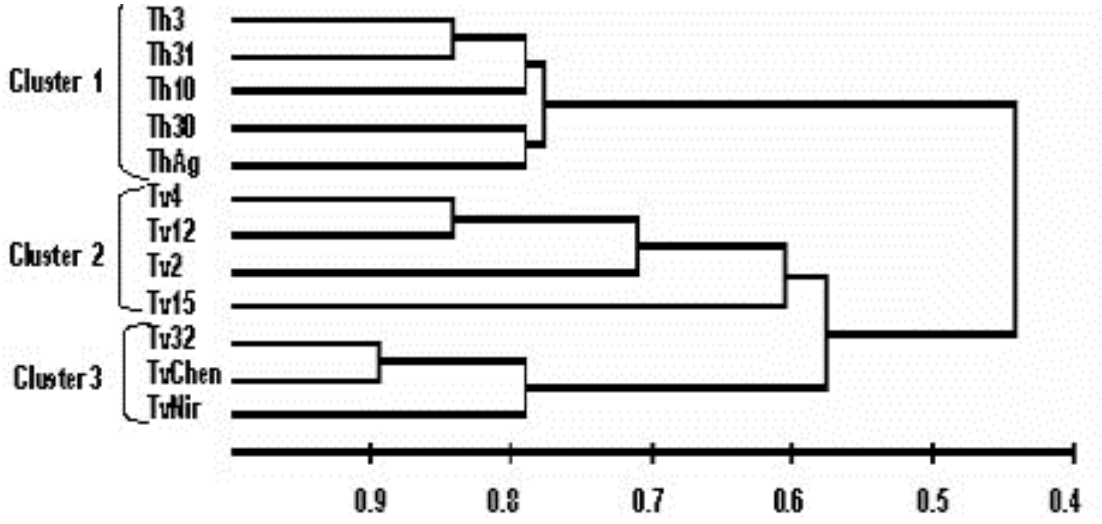

Fig 1. Inter-relationship between the *Trichoderma* isolates based on the morphological characters. The value 0.4 – 0.9 indicates the simple matrix similarity index.

Table 3. Inter and Intra cluster distances for the isolates of *Trichoderma* isolates.

Clusters	Cluster 1	Cluster 2	Cluster 3
Cluster 1	0.202	0.458	0.605
Cluster 2		0.332	0.453
Cluster 3			0.175

Similarity matrix

A band graph analysis was carried out for the 12 isolates. The graphic depiction showed similar band alignment for the isolates with similar characters (Fig. 2).

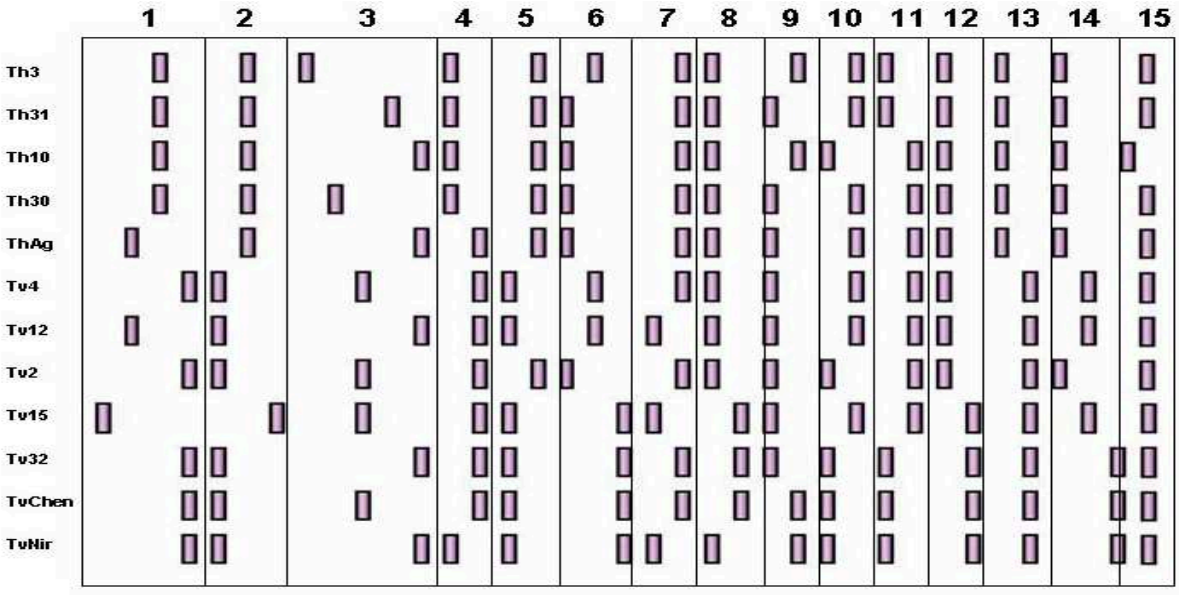

Fig 2. Graphical depiction of the descriptors for the 12 Trichoderma isolates. No's 1-15 are the fifteen quantitative and qualitative characters taken for statistical analysis

The shaded similarity matrix was used to make pair wise comparison for all isolates to confirm their similarity. The dark colour region of the matrix showed the maximum similarity of 90-100% between the isolates while the light colored region showed the minimum similarity between the isolates (Fig. 3). The maximum similarity was between the isolates Tv12 and Tv4, while minimum similarity was between isolates TvChen and Th30.

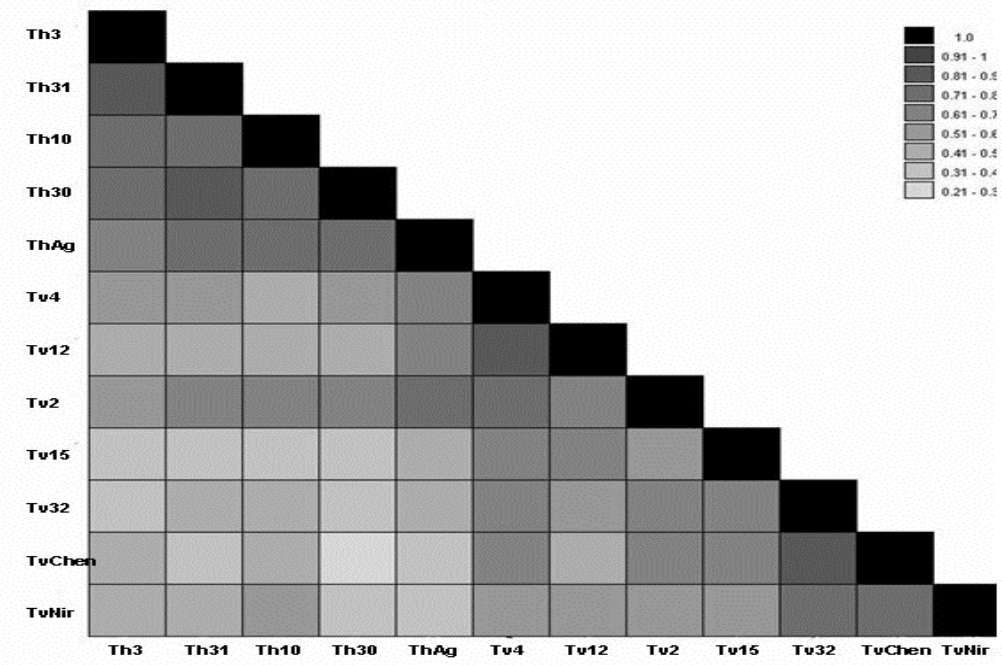

Fig 3. Graphical representation of the pair wise similarity index between the *Trichoderma* isolates

Principle component analysis (PCA)

PCA performed on studied traits showed that first two most informative components accounted for about 58% variation and the plot showed *T.harzianum* and *T.viride* as two distinct components confirming the cluster analysis (Fig. 4).

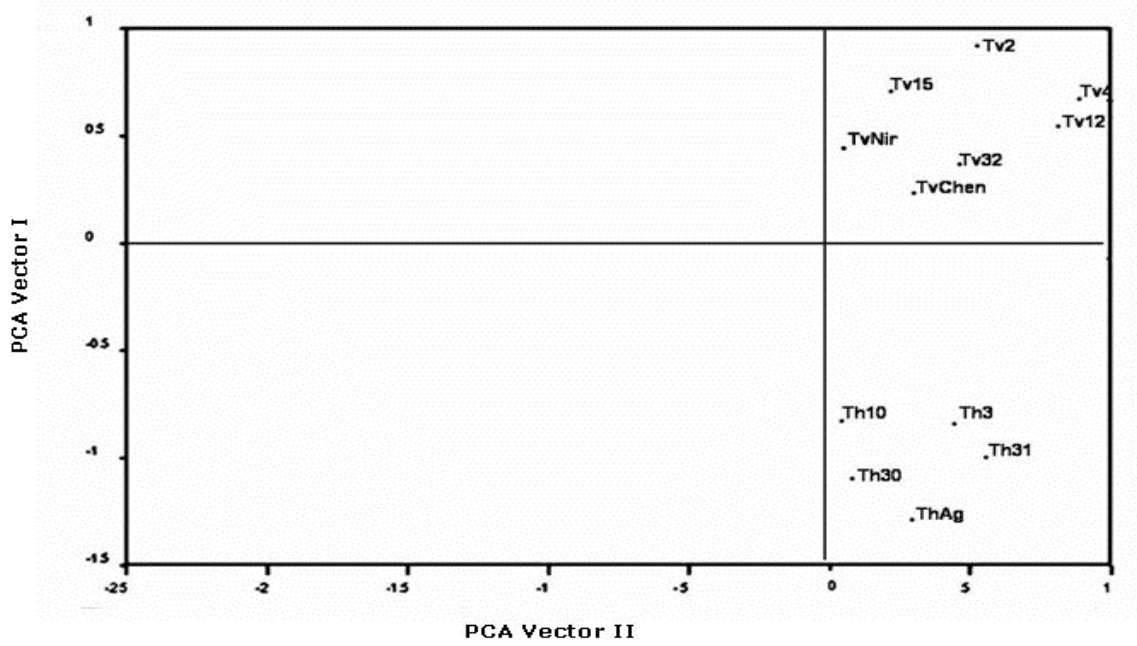

Fig 4. Principal Component Analysis of the *Trichoderma* isolates based on morphology. The vectors I and II are the first two most informative components accounted for about 58% variation

Biocontrol efficacy

The Bioefficacy of *Trichoderma* has been reviewed by several authors, and reported the biocontrol potential over different plant pathogens [21, 10, and 8]. As we know that *Trichoderma* is one of the organisms that grow well under laboratory conditions; it was easy to perform the confrontation assay through dual culture with the soil borne pathogens *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*. The isolates taken for the present study has been already established as biocontrol agents by various antagonistic and biochemical studies [16]. The Bioefficacy of these isolates were again confirmed by dual culture assay.

The confrontation assay to study the antagonistic effect of *Trichoderma* isolates on *Pythium aphanidermatum* and *Sclerotinia sclerotiorum* was done. The study revealed that *T.harzianum* isolates were more aggressive in checking the growth of the pathogenic fungi than *T.viride*. Among the *T.harzianum* isolates, Th3 had more profuse growth of 23cm^2 which overlapped *P.aphanidermatum* inhibiting its growth by 86% which was followed by Th10 and Th31. The isolate ThAg had the least effect of 74% among the *T.harzianum* isolates. TvChen had highest antagonistic property among the *T.viride* isolates against *P.aphanidermatum* which was followed by Tv4 (75%) and TvNir (74%). Similar results were obtained against *S.sclerotiorum* also, where Th3 exhibited 90% inhibition followed by Th10 and Th30. The isolate TvNir had maximum impact of 85% against the target fungi, followed by Tv2, TvChen and Tv4 (Table 4).The biocontrol potentiality of the *Trichoderma* isolates obtained in the confrontation assay was in consistent with the earlier reports of Sharma *et al*.,Sharma and Sain, Sharma and Dureja [20, 18, 19, 17]. Now an attempt was made to corroborate phenetic characters with the antagonistic ability.

Table 4. Bioefficacy of *Trichoderma* isolates on *Pythium aphanidermatum* and *Sclerotinia sclerotiorum.*

Isolate no:	***Trichoderma* sp.**	***Pythium aphanidermatum***		***Sclerotinia sclerotiorum***	
		Percent inhibition of mycelial growth (%) of pathogen	**Mean mycelial growth (cm^2) of *Trichoderma***	**Percent inhibition of mycelial growth (%) of pathogen**	**Mean mycelial growth (cm^2) of *Trichoderma***
Th3	*Trichoderma harzianum*	86.4	23.04	90.2	25.00
Th10	*Trichoderma harzianum*	83.0	21.16	88.1	24.01
Th30	*Trichoderma harzianum*	78.3	18.49	85.0	23.04
Th31	*Trichoderma harzianum*	80.0	20.25	74.5	16.81
ThAg	*Trichoderma harzianum*	74.5	16.81	72.7	14.40
Tv2	*Trichoderma viride*	68.0	14.44	83.3	23.24
Tv4	*Trichoderma viride*	75.0	16.81	80.5	18.49
Tv12	*Trichoderma viride*	70.0	15.21	74.7	16.81
Tv15	*Trichoderma viride*	56.5	12.96	60.9	12.96
Tv32	*Trichoderma viride*	65.8	14.44	50.5	12.25
TvChen	*Trichoderma viride*	77.0	17.64	82.0	20.25

Discussion

Thus the morphological observations of the twelve isolates can be recapitulated as follows. Most of the isolates belonging to *T.harzianum* were similar in colony colour, culture smell, mycelial colour, conidiation, conidial shape, conidial wall and conidial colour. Similarly the isolates of *T.viride* showed certain similarity in colony colour, colony edge, culture smell, conidiophore branching, conidial wall, conidial colour and chlamydospores. The major difference between the isolates of *T.harzianum* and *T.viride* were their conidial wall pattern, conidial shape, conidial colour, colony edge and culture smell.

As mentioned above, only few characters were significantly differing between the two species, a total of fifteen quantitative and qualitative characters were taken to analyze the inter specific difference. However most of characters observed were in abstract nature which may hinder the statistical analysis of the isolates. Thus variation in the characters was given specific ranking so as to facilitate the comparative study. These numerical data was again converted to binary form, which was subjected to statistical analysis. This was based on the report by Samuel *et al*. where he used the morphological characterization to analyze the variation between the isolates among *Trichoderma* species associated with green mold epidemic of commercially grown *Agaricus bisporus* [15]. Similarly Munaut *et al*. reported variation in *Colletotrichum gloeosporioides* based on morphological characterization [12].

The cluster analysis illustrated all the isolates of *T.harzianum* formed a single cluster while the isolates of *T.viride* were bifurcated into two groups. The main contributing characters which distinguished the *T.viride* isolates were phialide shape, conidial shape and conidial colour. The clustering was substantiated by similarity index which showed maximum similarity among *T.harzianum* isolates with only less than 20% variation among themselves. Similarly the clusters having isolates of *T.viride* also had less variation within them. However, when the cluster 1 constituting all *T.harzianum* isolates compared with cluster 2 consisting of Tv4, Tv12, Tv2 and Tv15, they shared only 50% similarity and when the cluster 1 was compared with cluster 3 having Tv32, TvChen and TvNir they had only 20% in common.

From the intra cluster distances it can be inferred that the cluster 2 was more diverse than cluster 1 and 3 which was supported by shaded matrix index. The inter cluster distances showed that cluster 1 was highly distant from cluster 3 followed by cluster 2. This finding confirmed the result of cluster analysis where *T.harzianum* formed an entirely distinct group from the isolates of *T.viride*. The result of PCA was also in consistent with this finding.

Relation between morphology and biocontrol efficacy

The morphological markers grouped all *T.harzianum* isolates together and did not establish much variation within these isolates. Nevertheless these isolates varied in their pathogenic ability against the two target fungi studied. The isolate Th3 which was found to be best biocontrol agent through confrontation study, was unable to differentiate itself from other isolates with morphological markers. Thus no relation could be established between biocontrol efficacy and phenetic characters. On the other hand the isolates TvChen and TvNir which had higher antagonistic effect against the target fungi formed a separate group during the cluster analysis. Therefore it can be inferred that these isolates differed from other isolates at phenetic level which can be related to their pathogenic ability. Even though the present study established a vague relationship between bioefficacy and phenetic diversity it can be confirmed only when the biochemical factors like chitinase, antibiotics etc are studied in detail.

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Volume 55
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Morphological Characterization of Biocontrol Isolates of *Trichoderma* to Study the Correlation between Morphological Characters and Biocontrol Efficacy
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