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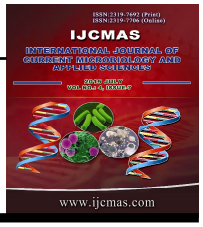

Original Research Article

Biochemical and Molecular Characterization of Native Isolates of *Trichoderma* Antagonistic against *Sclerotium* and *Pythium* Infecting Tobacco

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Keywords

Extracellular enzymes, Genetic diversity, Isoenzymes, RAPD, *Trichoderma*

A B S T R A C T

Twelve isolates of *Trichoderma* isolated from rhizosphere of tobacco antagonistic to *Sclerotium* and *Pythium* infecting tobacco were evaluated for production of HCN and extracellular enzymes, and their genetic diversity by RAPD and isoenzymes. All isolates showed HCN production ranging from 0.08 to 0.14 OD. The isolate TvJt1 produced maximum content of HCN but it was at a par with the isolate ThHt1 and ThJt1. The isolate ThJt1 showed the maximum and significantly higher chitinase activity followed by isolate TvHt2. The isolate TvHt2 showed maximum and significantly higher β-1,3-glucanase activity when compared to all other isolates. Isolate TvHt2 showed nearly 61.66% higher β-1,3-glucanase activity when compared to the isolate TvJt1. A total of 140 amplified fragments were produced by 15 primers in 12 isolates, of which 113 were polymorphic with 80.2 per cent polymorphism. The genetic similarity in RAPD was 33 to 90 % whereas it was 30 to 90% in isoenzyme markers. In RAPD, the pair-wise similarity between the isolates ThHt3 and ThJO1 was minimum (33%) whereas in isoenzyme system, the pair-wise similarity was minimum (30%) between the isolates ThJO1 and ThHt1. Clustering analysis by unweighted pair group method on arithmetic averages (UPGMA), in both the marker systems, isolates were grouped into two clusters mainly based on species specificity and region of isolation. The two marker systems showed nearly the similar results. There was no correlation between the RAPD/ isozyme pattern and the antagonistic potential of the *Trichoderma* isolates.

Introduction

Tobacco is one of the important quality conscious commercial crops grown in India. The presence of fungal diseases on tobacco and its economical consequences require the use of many fungicides. Production of healthy seedling is a prerequisite to produce good grade and quality tobacco. *Sclerotium rolfsii* causes collar rot in tobacco nursery and carry the disease to fields and the disease is wide spread in all the tobacco areas of India. *Pythium aphanidermatum* causes damping off which is a serious

disease in tobacco nurseries causing death of seedlings. There is no resistance to *S. rolfsii* and *P. aphanidermatum* in the available tobacco cultivars. The use of *Trichoderma* with all mechanisms of biological control will help to suppress these pathogens. Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated into cultural and physical controls and limited chemical usage for an effective integrated disease management (Biljana and Jugoslav, 2012).

There is not a general solution and biocontrol system must be developed for each crop. Before the development and application of a biocontrol agent, it is necessary to study the various aspects of biocontrol agents. Successful biological control systems commonly employ naturally occurring, antagonistic microorganisms that can effectively reduce activities of plant pathogens (Shishido *et al.,* 2005). Cook (1993) suggested that microorganisms isolated from the root or rhizosphere of specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species. *Trichoderma* spp. are well known biocontrol agents against a wide range of soil borne pathogens and some have a plant growth promotion capacity (Ozbay *et al.,* 2004). They are frequently associated with both biocontrol activity and promotion of plant and root growth (Chet *et al.,* 2006; Harman *et al.,* 2004). Biological control involving *Trichoderma* spp. operates by way of mycoparasitism, antibiosis and competition. *Trichoderma* spp. secret some cell wall degrading enzymes like chitinases and also secret some volatile and non volatile compounds which enter into the cell in the form of signal and triggers the secondary messengers and it alter the metabolic pathway of the pathogen. Screening of diverse population of biocontrol agents is an important requirement for developing efficient biocontrol agents. Therefore, it is imperative to index biocontrol agents prevailing in the area concerned.

Identification of species or strains by morphological characters like shape, size and formation of conidia are highly unstable and dependent on media and environment (Hermosa *et al.*, 2000). Phenotypic variation is abundant in *Trichoderma*, and expertise is required to distinguish between closely related isolates and to recognize variation within the species. With the introduction of molecular technology, it became very easy to identify the closely related organisms. Different molecular markers including randomly amplified polymorphic DNA (RAPD) have been used to characterize genetic diversity in fungal population and for different objectives and desired applications (Larissa *et al.,* 2002; Shanmugam *et al.,* 2008). The objective of the present study is to evaluate the production potential of HCN and extracellular enzymes by native *Trichoderma* isolates from tobacco rhizosphere and to assess the genetic variability by RAPD and isoenzyme markers.

Materials and Methods

Soil samples were collected as given in the table 1. *Trichoderma* spp. were isolated using dilution plate and soil plate techniques on PDA. Among the various isolates, 12 isolates of *Trichoderma* were selected. *Trichoderma* colonies were identified according to the identification key based on primarily growth characters on the medium and branching of conidiophores, shape of phialides, emergence of phialides and spore characters (Gams and Bisset, 1998).

Production of volatile inhibitor compound, HCN was estimated by the method of Wei *et al.* (1991) with a slight modification. The filter paper strips soaked in picric acid solution were exposed to growing *Trichoderma* isolates in 150 ml conical flasks containing 25 ml liquid medium turned to brown. They were then soaked in 40 ml of water for 10 min for complete leaching of the brown colour from the filter papers and the OD of colour solution was read at 515 nm in spectronic 20 and the values of OD were recorded after subtracting the blank value.

Estimation of enzyme activity

For estimation of chitinase and β-1, 3-glucanse activities, the isolates were grown on minimal synthetic medium (El-Katatny *et al.* 2000) containing chitin for chitinase and laminarin for β-1,3-glucanase activity. Extraction and estimation of chitinase and β-1,3-glucanase was done as per the methods of EI-Katatny *et al.* (2000) and Elad *et al.* (1993) respectively. The activity of the enzyme expressed as pkat/ml (pmol/s). All the experiments were set up in triplicate. The data were expressed as mean ± SD (n=3). Different letters in each column represent significant differences (p<0.05).

Total genomic DNA extraction

Genomic DNA was extracted by using the CTAB method (Hexa-decyl tri-methyl ammonium bromide) modified by Doyle and Doyle (1990). For DNA isolation, fungal mycelia were harvested from the isolates grown in potato dextrose broth medium incubated for 5 days at 26^{o}C. The fungal mat was filtered through sterile filter papers and was pressed between the tissue paper to remove the excess moisture and was ground to a fine powder with liquid nitrogen. The quantity of the extracted DNA was examined under UV light following agarose gel electrophoresis (0.8% gel containing 0.5 µg/ml ethidium bromide). The DNA concentration was estimated by comparing with known concentration of lambda DNA as standard.

RAPD and Primers

A total of 20 decamer primers belonging to different series (Operon Technologies Inc, Alameda, CA, USA) were screened for RAPD analysis. Of these, 15 primers were selected for analysis of isolates based on repeatability. The PCR amplification conditions were as described by Williams *et al.* (1990) with minor modifications. Amplifications were carried out in a 25 µl reaction mixture containing 25 ng template DNA, 0.5 units of Taq DNA polymerase, 0.2 mM of each dNTP and 20 ng of each primer.

Amplification was carried out in a DNA thermal cycler (PTC-100, Peltier thermal cycler, M.J. Biotech, USA) programmed to run the following thermal profile: initial denaturation at 94^{o}C for 5 min, 40 cycles of 94^{o}C x 1 min, 37^{o}C x 1 min, 72^{o}C x 2 min, followed by final extension at 72^{o}C for 10 min. Amplification products were resolved on 1.4% agarose gel at 50 V and gels were documented in gel documentation system (Gene genius, Syngene bio-imaging system, U.K). The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

Data analysis

All the isolates were scored for presence or absence of RAPD bands. Only intense bands and those that were repeatedly amplified were scored visually. The data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected

to further analysis. The Excel file containing the binary data was imported into NT Edit of NTSYS-pc software version 2.02 (Rohlf, 1998). The 0/1 matrix was used to calculate similarity as DICE coefficient using SIMQUAL subroutine in Similarity routine. The resultant similarity matrix was employed to construct dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means Average (UPGMA) to infer genetic relationships and phylogeny.

Isoenzymes

For isozyme analysis, the mycelial mats were harvested 6 days after incubation at 26^{o}C by filtering through Whatman No.1 filter paper. They were repeatedly washed with sterile distilled water to remove excess salts adhering to the mycelial mats. Mycelial mats (500 mg) dried at room temp for 1 h and pressed between tissue paper were homogenized with 2 ml cold extraction buffer (Tris-HCl, 0.1 M, pH 7.4 containing 0.1% β-mercaptoethanol) with quartz sand in pre-chilled mortor and pestle. The tubes containing extracts were kept in ice for 30 min with occasional shaking. The samples were then centrifuged at 14,000 rpm for 20 min at 4^{o}C. The clear supernatant was collected and used for electrophoretic studies.

Polyacrylamide gel electrophoresis (PAGE) was performed by using 4% stacking gel and 8% resolving gel in Tris-glycine buffer for isoenzymes (Laemmli, 1991) and SDS-PAGE for total soluble proteins (Sambrook *et al.,* 1989). Staining for the detection of isozymes of esterase, malate dehydrogenase, superoxide dismutase and acid phosphatase was done by the method of Shaw and Prasad (1970). Isoenzymes of peroxidase, catalase and polyphenol oxidase were detected by the methods given by Sadasivam and Manikam (1992). The zymograms were prepared to indicate relative mobility of isozyme bands. Separate runs were made to record the banding patterns of different isozymes. For soluble proteins, SDS-PAGE gel was stained in Coomassie blue solution. The isozyme profiles were scored across lanes for the presence (1) or absence (0) of bands at specific loci and analyzed as in case of RAPD.

Results and Discussion

In vitro production of HCN and extracellular hydrolytic enzymes by *Trichoderma* isolates

All isolates showed HCN production ranging from 0.08 to 0.14 OD. The isolate TvJt1 showed maximum HCN production (Table 2) followed by ThHt1 and minimum by isolates ThJnt and ThRt1. The isolate TvJt1 produced maximum content of HCN but it was at a par with the isolate ThHt1 and ThJt1.

The isolate ThJt1 showed the maximum and significantly higher chitinase activity compared to other isolates (Table 2). The isolate TvHt2 was the second highest producer of chitinase and significantly higher than other isolates. The isolates ThDt1 and ThDt2 collected from Dinhata were at a par in chitinase activity. The isolate TvHt2 showed maximum and significantly higher β-1,3-glucanase activity when compared to all other isolates (Table 2). The isolate ThJt1 showed second highest activity of β-1,3-glucanase. All other isolates showed β-1,3-glucanase activity but they were all at a par. Isolate TvHt2 showed nearly 61.66% higher β-1,3-glucanase activity when compared to the isolate TvJt1.

Antagonistic abilities of *Trichoderma* spp. are a combination of several mechanisms, including direct mycoparasitism, which

involves the production of cell-wall degrading enzymes (CWDE) and production of secondary metabolites which inhibit the growth of pathogens (Lorito *et al.*, 2010; Qualhato *et al.*, 2013). In the present study, all the isolates collected from the rhizosphere of tobacco from different regions and a commercial isolate produced HCN, chitinase and β-1, 3-glcanase. The isolates ThJt1 and TvHt2 produced maximum chitinase and β-1, 3-glcanase respectively and also showed maximum inhibition of the pathogens *Sclrotium* and *Pythium* (data not shown).

RAPD

A total of 20 random decamer primers were used, out of which 15 primers were chosen for analysis based on clear reproducible RAPD patterns. A total of 140 amplified fragments were produced by 15 primers in 12 isolates, of which 113 were polymorphic with 80.2 per cent polymorphism (Table 3). The number of amplified fragments in RAPD ranged from 3 to 13 per primer with an average of 9.3 across all the isolates. A total of 27 amplified fragments were common to all the isolates indicating the evolutionary conservation in genome of *Trichoderma* spp. The primers OPAB18 and OPD12 produced 7 and 6 amplified fragments respectively which were monomorphic. The size of the amplified fragments varied from 150 to 2200 bp. The primers OPB20, OPD8, OPAB16 and OPAA11 produced 100% polymorphic fragments. Some of the RAPD profiles showed specific pattern for some isolates. Representative agarose gel was given in figure 1.

The genetic similarity among the 12 isolates of *Trichoderma* varied from 33 to 90%. The isolates ThHt3 and ThJO1 showed the minimum genetic similarity (33%) whereas the isolates ThRt1 and ThRt2 showed maximum genetic similarity. The isolate TvHt2 and TvJt1 (*T. viride*) showed genetic similarity ranging from 41 to 58 per cent with isolates of *T. harzianum.*

Unweighted pair group method on arithmetic averages (UPGMA) method of clustering, the isolates separated in to two clusters. The isolates TvHt2 and TvJt1 were formed cluster 2 with 68 per cent of genetic similarity. The 10 isolates belonging to *T. harzianum* formed cluster 2 (Fig. 2). In the cluster1, the 10 isolates grouped into three sub-clusters. Sub-cluster 1a and sub-cluster 1b were formed by 3 and 5 isolates respectively whereas the sub-cluster 1c was formed by two isolates. The sub-cluster 1a included the isolates ThC, ThDt1 and ThDt2. The isolate ThC was a commercial isolate and showed 75% genetic similarity with isolates ThDt1 and ThDt2 which were present in the same group. The isolates ThDt1 and ThDt2 were grouped together with 84% of genetic similarity, which were isolated from Dinhata, West Bengal. The sub-cluster 1b included 5 isolates i.e., two isolates from Flue-cured Virginia tobacco rhizosphere, Rajahmundry, two isolates from tobacco rizhosphere, and one from oil palm rhizosphere, Jeelugumilli, West Godavari Dt, Andhra Pradesh (Fig. 2). The isolate ThRt1 and ThRt2 were grouped together with maximum genetic similarity (90%). The isolate ThJt1 from FCV tobacco rhizospere, Jeelugumilli linked with this group with 75% and 81% genetic similarity with ThRt1 and ThRt2 respectively. The isolate ThJnt, from *natu* tobacco rhizosphere, Jeelugumilli linked with isolate ThJt1 of the same region with 66% genetic similarity. The isolate ThJO1, from oil palm rhizosphere from the same region was also included along with isolate ThJt1 and ThJnt with 61 and 72 per cent genetic similarity. The sub-cluster 1c formed by two isolates (ThHt1 and ThHt3), which were isolated from FCV tobacco rhizosphere, Hunsur,

Karnataka. The commercial isolate ThC showed 50% and 41% with TvHt2 and TvJt1 respectively whereas it showed genetic similarity ranging from 53 to 80% with other isolates of *T. harzianum*. The isolate from oil palm rhozosphere showed genetic similarity ranging from 33 to 72% with the isolates of *T. harzianum*. The genetic similarity among 5 isolates of FCV tobacco rhizosphere varied from 50 to 80%.

Thus the clustering pattern was mainly based on the region and species specificity. The isolate ThJt1, which produced maximum chitinase was grouped along with other isolates (ThJnt, ThRt1, ThRt2 and ThJO1) with genetic similarity ranging from 50 to 80 per cent indicated that the trait of producing higher levels of chitinase was not playing any role in cluster formation. The isolate ThJO1 from oil palm rhizosphere was also included in the cluster formed by isolates from Jeelugumilli but it showed more genetic dissimilarity with other isolates of the same group. Some of the RAPD primers gave specific pattern to some isolates which can be used as markers in tracing the isolates if used as biocontrol agents. Misra and Gupta (2009) reported that the RAPD technique was found to be advantageous over other molecular techniques for the genetic characterization of *Trichoderma* spp. due to the possibility of detecting DNA polymorphisms among very closely related strains. Siameto *et al.* (2010) characterized seven isolates using RAPD and reported the genetic similarity from 0.231 to 0.857 among isolates and also suggested that the technique of RAPD was efficient in demonstrating intraspecific genetic variability. Chakraborty *et al.* (2010) studied genetic variability using RAPD among nineteen isolates of *T. viride* and *T. harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural fields of North Bengal region and reported genetic similarity from 0.67 to 0.95 with the formation of two distinct clusters of *T. viride* and *T. harzianum* with five sub-groups of each cluster. Sagar *et al.* (2011) used RAPD markers and reported the existence of high level of genetic diversity among the isolates.

Isoenzymes

Isoenzymes of peroxidase, esterase, acid phosphatase, catalase, superoxide dismutase, polyphenol oxidase, malate dehydrogenase and soluble protein profiles were used to study the genetic variability among the isolates of *Trichoderma*.

Esterase: Isoenzymes of esterase resolved on 8% polyacrylamide gel showed a maximum of 12 major loci (Fig. 3). The maximum numbers of bands (9) were present in isolate ThHt1 whereas minimum numbers of isozyme bands were in the isolate ThC. The isozyme band with Rf value 0.45 was present in all the isolates whereas the band with Rf value 0.35 was present in all the isolates except ThC and ThHt3. The isozyme band with Rf value 0.52 was specific to the isolate ThHt3. The isozyme band with Rf value 0.12 was present only in isolates ThC, ThDt1 and ThHt3. Many isolates have 6 to 8 isozyme bands.

Catalase: Isoenzymes of catalase showed a maximum of 7 loci. The isozyme band with Rf value 0.7 was present in all isolates. The maximum number of loci (3) was present in the isolates ThRt1, ThJnt, TvHt2 and ThHt3. The isozyme bands with Rf value 0.3 and 0.4 were present only in the isolate ThHt3 whereas the isozyme bands with Rf value 0.42 was present in isolates ThHt1 and TvHt2.

Polyphenol oxidase: Isoenzymes of polyphenol oxidase showed a maximum of 6

loci. The maximum number of bands (5) were present in isolate ThHt1. The isozyme band with Rf value 0.28 was present in all the isolates except ThC, ThJO1 and ThHt3. The isozyme band with Rf value 0.02 was present only in ThHt1 and ThHt3 whereas the isozyme band with Rf value 0.25 was present in isolate ThC.

Acid phosphatase: Isoenzymes of acid phosphatase showed maximum of 6 loci. The isolate ThHt3 showed maximum number of isozymes whereas the isolate ThRt2 showed a single isozyme. The isozyme bands with Rf value 0.25 and 0.29 were present in many isolates.

Superoxide dismutase: Isoenzymes of SOD resolved into 7 loci. The maximum numbers of loci (3) were present in the isolates ThDt1, ThDt2 and ThHt1. The isozyme band with Rf value 0.15 was present only in the isolates ThDt1 and ThDt2 whereas the isozyme band with Rf value 0.35 was present in the isolates ThJt1 and ThHt3.

Malate Dehydrogenase: Isoenzymes of MDH showed a maximum of 5 loci. The isozyme bands with Rf value 0.45 was present in all the isolates except ThRt2, ThJt1, TvHt2 and ThHt3. The isozyme bands with Rf value 0.58 was present in ThJnt, ThHt1 and TvHt2. The isolate TvHt2 showed maximum number of (4) isozyme bands whereas the isolates ThC, ThJO1, ThRt1 and ThHt3 showed only one isozyme.

SDS-PAGE of soluble protein profiles showed a number of bands with very low variation among the isolates with exceptions. Isolates ThJO1 and ThJnt showed specific protein bands which were absent in other isolates. SDS-PAGE pattern was not taken into consideration for genetic diversity estimation.

Based on isozyme analysis, the genetic similarity among the 12 isolates varied from 30 to 90%. The maximum genetic similarity was observed between the isolates ThRt1 and ThRt2 whereas the least genetic similarity was observed between isolates ThJO1 and ThHt1. The isolate TvHt2 and TvJt1 showed genetic similarity ranging from 41 to 57 and 35 to 61% with isolates of *T. harzianum*. The isolates TvJt1 and TvHt2 belong to *T. viride* showed 61% of genetic similarity between them.

Unweighted pair group method on arithmetic averages (UPGMA) method of clustering, the isolates divided into two clusters (Fig. 4). Ten isolates of *T. harzianum* were formed cluster1 whereas the two isolates of *T. viride* were grouped in cluster 2. The 10 isolates in the cluster 1 were divided into two sub-clusters. Sub-cluster 1a consists of two groups. The first group was formed by isolates ThRt1 and ThRt2 and the commercial isolate ThC linked to this group. The isolates ThRt1 and ThRt2 were isolated from FCV tobacco rhizosphere, Rajahmundry, were grouped together with maximum genetic diversity (90%). The commercial isolate ThC linked to this group with 80 and 88 per cent genetic similarity with ThRt1 and ThRt2 respectively. The second group in sub-cluster 1a was formed by isolates ThJO1 (oil palm rhizosphere) and isolate ThJnt (natu tobacco rhizosphere) with 80% genetic similarity and both were from the same area (Jeelugumilli, West Godavari Dt. Andhra Pradesh) whereas the isolate ThJt1 from FCV tobacco rhizosphere from the same area was also linked to this group with 60 and 76% genetic similarity with isolate ThJO1 and ThJnt respectively.

The sub-cluster 1b consists of four isolates which formed into two groups. The isolates ThDt1 and ThDt2 from *Motihari* tobacco,

Dinhata, West Bengal were grouped together with 84% of genetic similarity. The isolates ThHt1 and ThHt3 were grouped together with 71% of genetic similarity. The cluster 2 was formed by two isolates TvJt1 and TvHt2 from FCV tobacco rhizosphere, Jeelugumilli and Hunsur, Karnataka respectively with a genetic similarity of 68%.

The commercial isolate ThC showed genetic similarity ranging from 42 to 88% with isolates *T. harzianum* and, 35% and 42% with isolates TvHt2 and TvJt1 respectively. The isolate ThJO1 from oil palm rhizosphere showed genetic similarity ranging from 30 to 80% with isolates of tobacco rhizosphere and 52 and 61% with TvHt2 and TvJt1 respectively. The genetic diversity varied from 37 to 71 per cent among the five isolates from FCV tobacco rhizosphere belonging to *T. harzianum* (ThRt1, ThRt2, ThHt1, ThHt3 and ThJt1). The isolates ThHt1 and ThHt3 from FCV tobacco rhizosphere and same region showed maximum genetic similarity whereas they showed 37% genetic similarity with isolate ThJt1 of FCV tobacco rhizosphere from Jeelugumilli region.

Thus clustering pattern mainly based on region of isolation and species specificity. The isolate ThJt1, which produced maximum chitinase was grouped with isolates ThJnt and ThJO1 with genetic similarity 76 and 60% respectively, indicating the trait of production of higher levels of chitinase was not played any role in the cluster formation. The isolate ThJO1 from oil palm rhizosphere was also linked with isolates ThJt1 and ThJnt from tobacco rhizosphere indicating that the region of the isolates played important role in cluster formation. Differences in isoenzyme and protein patterns in various fungal species have been reported (Rani and Kumar 2007). Lavanya *et al.* (2008) reported positive activity for 8 of 9 isozymes tested in native PAGE for all isolates of *T. harzianum* and the genetic similarity between 0.22 and 0.61 among the isolates.

Table.1 *Trichoderma* isolates and their origin of isolation used in the present study

S. No	Isolate	Source of isolate
1	ThRt1	*T. harzianum*, Rajahmundry, FCV tobacco
2	ThRt2	*T. harzianum*, Rajahmundry, FCV tobacco, nursery
3	ThJT1	*T. harzianum*, Jeelugumilli, FCV tobacco
4	Th Jnt	*T. harzianum*, Jeelugumilli, *Natu* tobacco
5	Tv Jt1	*T. viride*, Jeelugumilli, FCV tobacco
6	Th D t1	*T. harzianum*, Dinhata, WB, *Mothihari* tobacco
7	Th D t2	*T. harzianum*, Dinhata, WB, *Jati* tobacco
8	ThHt1	*T. harzianum*, Hunsur, Karnataka, FCV tobacco
9	TvHt2	*T. viride*, Hunsur, Karnataka, FCV tobacco
10	ThHt3	*T. harzianum*, Hunsur, Karnataka, FCV tobacco
11	ThJO1	*T. harzianum*, Jeelugumilli, oil palm
12	Th C	*T. harzianum*, Commercial isolate

Table.2 Production of HCN and activity of extracellular enzymes by *Trichoderma* isolates

Isolates	HCN (OD)	Chitinase (p kat/ml)	β-1,3-glucanase (n kat/ml)
ThRt1	0.09±0.005 ef	40.26±1.96 f	4.26±0.19 c
ThRt2	0.12±0.017 bc	42.82±1.49 ef	5.22±0.10 c
ThJt1	0.126±0.003 ab	62.12±3.15 a	6.75±0.14 b
ThJnt	0.08±0.006 f	40.12±1.61 f	4.06±0.13 c
TvJt1	0.14 ±0.004 a	52.12±2.42 bc	3.86±0.17 c
ThDt1	0.106±0.004 cde	48.24±1.61 cd	4.26±0.07 c
ThDt2	0.098±0.003 de	46.14±1.22 de	4.34 ±0.27c
ThHt1	0.128±0.004 ab	41.34±1.50 f	4.86±0.25 c
TvHt2	0.096±0.007 de	56.24±0.59 b	9.94±0.19 a
ThHt3	0.11±0.006 d	48.26±1.48 cd	5.08±0.11 c
ThJO1	0.102±0.002 de	46.12±1.41 de	4.75±0.21 c
Th C	0.094±0.005 ef	38.26±1.14 f	4.48±0.30 c

Table.3 Data on RAPD markers among the 12 *Trichoderma* isolates

Primer	Total no. of bands	No. of polymorhic bands	Polymorphism (%)
OPC2	3	2	66.6
OPC6	5	3	60
OPC12	9	8	88.8
OPA18	7	0	0
OPB4	12	11	91
OPB14	12	11	91
OPB20	13	13	100
OPL8	7	7	100
OPL12	6	0	0
OPP16	5	3	60
OPM4	11	10	90.9
OPM11	12	12	100
OPM15	13	11	84.6
OPAB13	13	12	76.9
OPAB16	12	12	100

Figure.1 RAPD profile amplified by primers OPM11, OPM15 and OPB 20: 1. ThC, 2. ThDt2, 3. ThDt1, 4. ThHt1, 5. ThHt3, 6. TvHt2, 7. ThJO1, 8. ThJt1, 9. ThJnt, 10. TvJt1, 11. ThRt1, and 12. ThRt2. M= DNA Marker

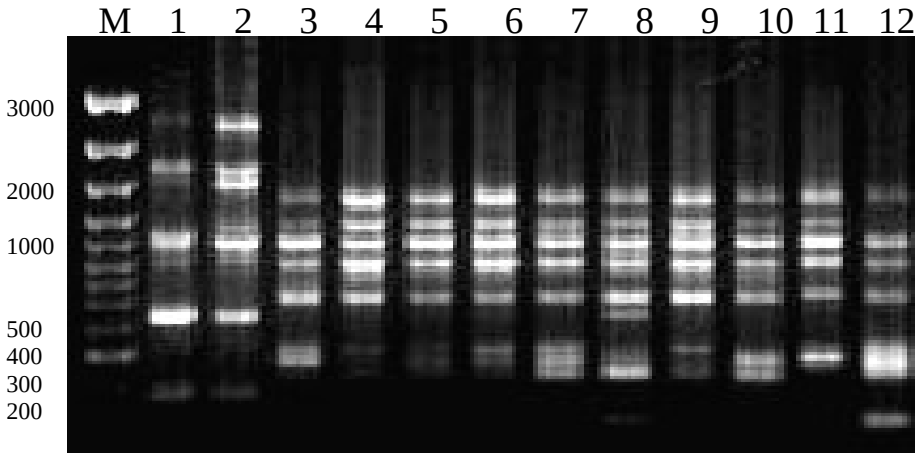


Figure.2 Dendrogram showing the relationship among the *Trichoderma* isolates based on RAPD pattern

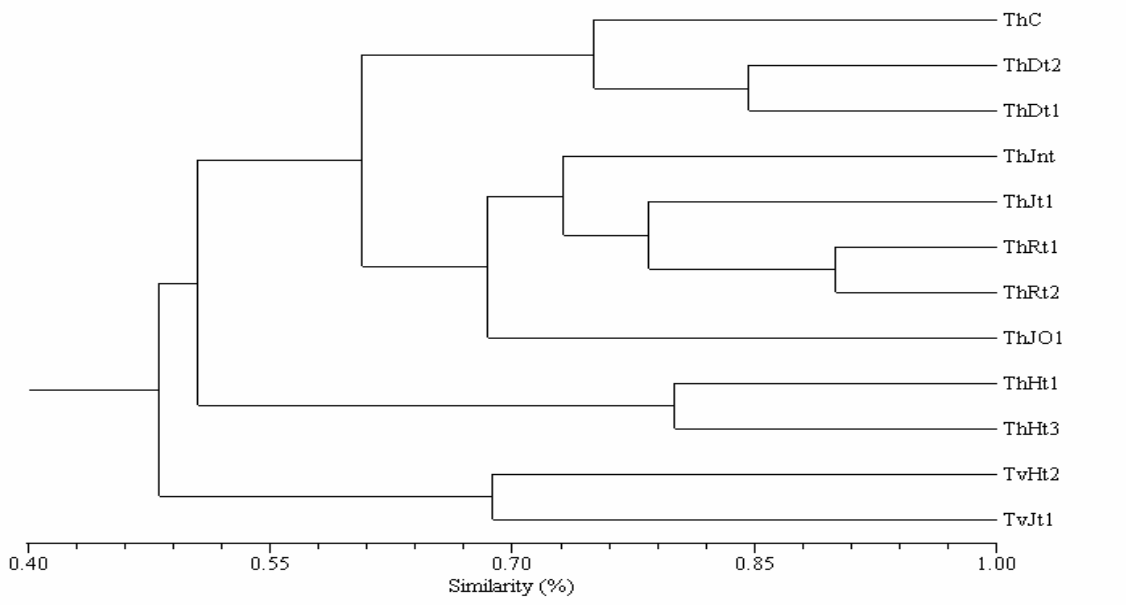


Figure.3 Isoenzyme profiles of esterase. 1. ThC, 2. ThJO1, 3. ThRt1, 4. ThRt2, 5. ThJt1, 6. ThJnt, 7. TvJt1, 8. ThDt1, 9. ThDt2, 10. ThHt1, 11. TvHt2 and 12.ThHt3

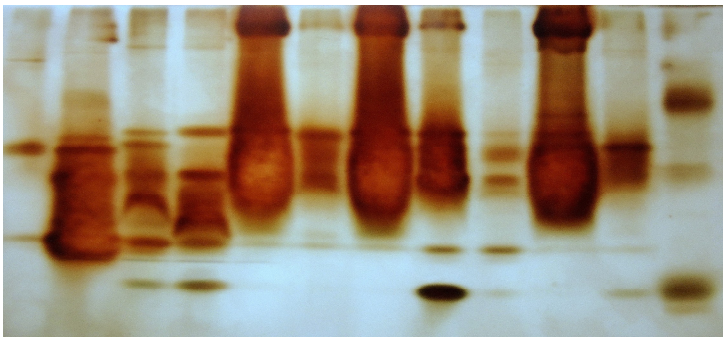
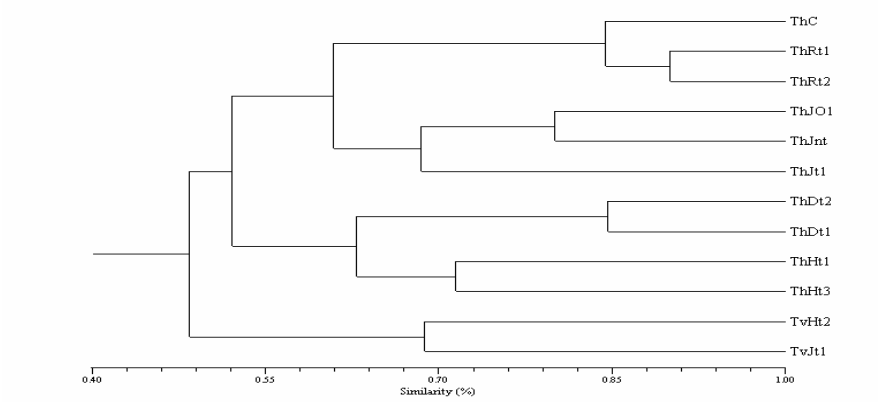


Figure.4 Dendrogram showing the relationship among the *Trichoderma* isolates based on isoenzymes pattern



Virulence and molecular markers are two parameters which are most often used to study fungal diversity. Fungal population structure inferred from physiological data may not reflect the true genetic diversity and evolutionary history of the isolates examined. Molecular marker analysis is a useful technique presently being used to resolve taxonomic problems, identify unknown fungal isolate, analyze the extent of genetic variability in the population etc. (Bonde *et al.*, 1991). In the present study, the genetic diversity among the *Trichoderma* isolates was studied by two marker systems *i.e.*, RAPD and isoenzymes. The genetic similarity in RAPD was 33 to 90% whereas it was 30 to 90% in isoenzyme markers. In RAPD, the pair-wise similarity between the isolates ThHt3 and ThJO1 was minimum (33%) whereas in isoenzyme system, the pair-wise similarity was minimum (30%) between the isolates ThJO1 and ThHt1. The two marker systems showed nearly the similar results. In both the marker systems the isolates were grouped into two clusters mainly based on species specificity and region of isolation. The isolate ThJt1, which produced maximum chitinase was grouped with isolates ThJnt and ThJO1 with genetic similarity 76 and 60% respectively in isoenzyme markers whereas it was grouped along with other isolates (ThJnt, ThRt1, ThRt2 and ThJO1) with genetic similarity ranging from 50 to 80% in RAPD,

indicating the trait of production of higher levels of chitinase was not played any role in the cluster formation. The isolate ThJO1 from oil palm rhizosphere was also linked with ThJt1 and ThJnt from tobacco rhizosphere indicating that the region of the isolates played important role in cluster formation as these isolates were collected from Jeelugumilli, West Godavari Dt., Andhra Pradesh. Existence of genetic variability among the strains of same region as well as strains from different regions was well documented (Muthumeenakshi *et al.*, 1998). There were no correlations between the RAPD pattern and the antagonistic potential of the *Trichoderma* isolates. Chen and Zhang (1994) reported the clustering of *Alternaria* isolates from Cruciferous hosts into 3 groups and banding pattern was not related to host or geographical distribution. DNA banding pattern obtained in the present study will be useful for the differentiation of various *Trichoderma* isolates from different hosts/regions. In our study, the characterization of *Trichoderma* isolates by RAPD has proved useful in separating all the isolates from each other. It has also provided us with primer markers that can be used to separate and distinguish isolates ThJt1 and TvHt2, which were shown to be the most effective strains for biological control of the collar rot and damping off of tobacco diseases and gave a distinct banding pattern with most of the primers. This possibility of distinguishing different isolates by a rather simple technique of genomic fingerprinting based on PCR-RAPD could be of great importance for the use in patent protection of fungal strains of biotechnological use, where additional more easily detectable markers are not available.

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