MOLECULAR SCREENING OF TRICHODERMA ISOLATES

Amna Ali, Rukhsana Bajwa,
Nasir Mehmood, Rasheda Jabeen

Institute of Mycology and Plant Pathology,
University of the Punjab, Lahore, 54590, Pakistan.

aliaamna@hotmail.com, write2amna@gmail.com

Abstract: *The molecular study is based on genomic DNA therefore DNA must be extracted in a pure and uncontaminated form. In this study, genomic DNA of twenty four isolates of Trichoderma species (T. harzianum, T. hamatum, T. koningii and T. pseudokoningii) indicated band of ~15Kb on 0.8% agarose gel and quality was determined by obtaining absorbance ratio (260/280) in the range of 1.7-1.9. Restriction fragment length polymorphism (RFLP) analyses were performed by using two restriction endonulease enzymes i.e., BamHI and HindIII. The BamHI represented results in the range of 500bp-750bp. 18S rRNA gene targeting was further carried out through optimization in ribotyping analysis.*

Keywords: *Trichoderma harzianum, T. hamatum, T. koningii, T. pseudokoningii RFLP, restriction endonuleases, 18S rRNA.*

1. Introduction

The genus *Trichoderma* is characterized "as rapidly growing colonies bearing tufted or pustulate, repeatedly branched conidiophores with lageniform phialides and hyaline or green conidia borne in slimy heads" [14]. The researchers are interested in this genus because of its novel biological properties and biotechnological applications. However, classification and phenotypic identification of *Trichoderma* species have been proved difficult, because morphological characteristics are easily changed by environmental influences [9]. Currently molecular techniques like DNA sequencing [1], Random Amplification of Polymorphic DNA (RAPD) analysis [18], Restriction Fragment Length Polymorphism (RFLP) analysis [8], internal transcribed sequences (ITS) of the ribosomal DNA (rDNA) analysis (rDNA-ITS1) and universally primed polymerase chain reaction (UP-PCR) have been used to characterize isolates of *Trichoderma* [3].

Today the concern of scientists is either in the direction of gene targeting or ribotyping, the newest fingerprinting tool for genomic DNA that contain all or part of the genes coding for 18S rRNA in eukaryotes [15]. The purpose of this study is to take advantage of advanced molecular techniques for phylogenetic analysis of indigenous isolates of *Trichoderma* to comprehend our knowledge of this genus by supplementing the phenotypic identification.

2. Materials and Methods

Trichoderma strains

The local isolates of *Trichoderma* species (fourteen isolates of *T. harzianum*, five of *T. konningii*, four of *T. pseudokonningii*, and one of *T. hamatum*.) were acquired from First Fungal Culture Bank of Pakistan (FCBP), Institute of Mycology and Plant Pathology, University of the Punjab, Lahore. All cultures were maintained on MEA (malt extract agar) medium at 4^{o}C.

Genomic DNA Extraction

The preserved cultures were inoculated on fresh ME broth medium and incubated at 20-24^{o}C for a week. Cultures were filtered and fungal mat was used for DNA extraction by CTAB method [12]. DNA pellet was washed; air dried and resuspended in 50 µL TE buffer (10mM Tris-HCL pH 8.0, 10mM EDTA). To lower RNA contamination, 5µL of RNase (20mg/mL) was added.

Spectrophotometric Quantification of DNA

The spectrophotometer was calibrated taking TE buffer as a blank. Each DNA sample was diluted by 900µL distilled water in a cuvette and mixed well [7]. The concentration was calculated on the assumption that an absorbance of 1 at 260nm corresponds to 50mg/mL double stranded DNA or 40mg/mL single stranded DNA [13]. The

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amount of DNA was quantified by using the following formula:
DNA concentration (µg/mL) = OD_{260} × Dilution factor × 50

DNA Quality analysis through Agarose Gel Electrophoresis
DNA fragments were separated by following a method derived from Hoisington *et al.* [7]. Agarose (0.8g) was added in 100mL 1.0X TAE electrophoresis buffer, warmed for 1.5 minute. The melted agarose was cooled to 60 to 50ºC before adding 5µL ethidium bromide (10mg/mL) and poured in the gel-casting tray with comb. After solidification, gel was placed in an electrophoresis tank, containing 500mL 1X TAE buffer. The samples were loaded in gel with 3µL of 6X loading dye and connected to power supply at 100V for 45min. The DNA bands were compared with the catalog of 1Kb DNA marker showing molecular sizes (Fermentas) for quality estimation as well using UV Transilluminator.

Restriction Fragment Length Polymorphism (RFLP) Analysis
RFLP analysis was carried out as described by Ranganath *et al.*, [11]. DNA were digested using 1U restriction endonuclease enzymes *Bam*HI (G↓GATCC) and *Hind*III (A↓AGCTT) with 2.0 µL restriction buffer (1X) at 37ºC overnight. The restriction activities of enzymes were checked on 1% agarose gel stained with ethidium bromide at 100 volts for 45 minutes and examined under UV transilluminator and photographed.

18S Ribosomal RNA (rRNA) gene targeting
Ribotyping was carried out by the method described by Borneman and Hartin [2] with some modifications. rRNA gene amplification reaction mixture was contained in PCR Buffer (1.0X), $MgCl_2$ (1.5-3.0mM), dNTPs (0.2mM), forward and reverse primer (100 pMole/µL), Template DNA (0.5-1µg), Taq Polymerase (1U) constituting final volume of 50 µL. List of fungal specific rRNA primers and amplification profile are in Table: 1.

Temperature Cycling Condition of 18S rRNA gene Amplification
18S rRNA amplification was conceded out in Master cycler gradient PCR (thermalcycler) with initial denaturation at 94°C, followed by 40 cycles of denaturation again at 94°C, primer annealing at 65°C, and primer extension at 72°C. The thermal cycles were terminated by a final extension at 72°C with final hold at 4.0°C and preheat lid temperature at 105°C.

Analysis of Amplified 18S rRNA gene fragment
The amplified product was checked on 1% agarose gel with 5µL of 6X gel loading dye at 100 volts for 45 minutes and examined under UV light and photographed on the Gel Documentation System.

3. Results

Quality of extracted DNA
The DNA bands of 24 isolates of *Trichoderma* species were compared with marker DNA bands and indicated the presence of genomic DNA intact band of ~15Kb. It was further utilized in downstream analysis in RFLP and rRNA gene targeting ribotyping (Plate-1).

Spectrophotometric Quantification of Extracted DNA
Ratio of OD_{260}/OD_{280} is determined to assess the purity of the sample. The ratio of absorbance 260/280nm (1.8-1.9 for pure DNA preparations) provided an estimate of the purity of the DNA (Table-2).

RFLP Analysis
Plate-2a represents RFLP analysis, along with the negative control of twenty four different isolates of *Trichoderma* species subjected to restriction using *Bam*HI enzyme. In *Trichoderma hamatum* isolated from *Syzygium cumini,* stem bark and soil only one band was observed at 750bp. However in *Trichoderma koningii* (*Mangifera indica*, wood) and *Trichoderma pseudokoningii* (Tannery effluent) two bands were observed, one at ~600bp and other at 750bp. *Trichoderma harzianum* (*Mangifera indica*, rhizospheric soil) showed only one band at 500bp.

Analysis of 18S rRNA gene targeting/Ribotyping
The rRNA gene amplified band was observed at 600bp in the case of *Trichoderma hamatum* isolate (*Syzygium cumini* stem bark, FCBP accession number 769) while in remaining isolates bands were in slightly smeared form. Furthermore, rRNA gene amplification conditions were optimized by altering different Tm and $MgCl_2$ concentrations (Plate-2b).

4. Discussion

In recent years, efforts involving molecular methods have been made for the identification and taxonomic investigation of different *Trichoderma* species, including members of the section *Longibrachiatum* [5]. The genomic DNA can serve as long term storage of information. Therefore advance molecular techniques can be used to study the variability in the genome of organism. RFLP are the initial steps for screening the genome of any organism. The optimized conditions of rRNA gene amplification were also conceded out for proceeding towards ribotyping. The good quality fungal genomic DNA extraction is the primary step towards molecular exploration of the genome. He *et al.* [6] have described the improved protocols for molecular analysis in the pathogenic fungus *Aspergillus flavus*. Correspondingly in this recent study, twenty four isolates of *Trichoderma* were subjected to the optimized DNA extraction protocol and in all cases 0.1µg/µL – 0.3 µg/µL genomic DNA was obtained. Furthermore quality estimation of extracted genomic DNA was done by taking the absorbance at 260nm and 280nm as proposed by Hoisington *et al.* [7]. As in this study ratio ranging 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids, ratio lower than 1.8 indicated the presence of proteins and/or other UV absorbers. While ratio more than 2.0 indicated that the samples may be tainted with chloroform or phenol. Proper quantification of DNA is quiet essential before proceeding towards downstream molecular investigations; similar conclusions were also anticipated by De Mey *et al.* [4].

In the entire samples intact genomic DNA band was observed at ~15Kb indicating the fact that extracted DNA has very less degradation and can be used further for molecular analysis. Werner and Irzykowska [16] have made similar observations while working on fungus *Fusarium oxysporum*. RFLP analysis was applied to all isolates of *Trichoderma* species by using *Bam*HI and *Hind*III restriction endonuclease enzymes. In case of *Bam*HI, five isolates showed restriction profiles with DNA band sizes in the range of 500bp-750bp. In case of *Hind*III limited number of restriction DNA fragments were observed in the genomic DNA of these *Trichoderma* isolates. Pipe and Shaw [10] have reported contradictory results in which the genomic DNA from *Phytophthora infestans* digested by *Hind*III resulted in the best separation of end-fragments. The nucleotide sequences of small ribosomal subunit genes (16S and 18S rRNA genes) have been studied by Woese [17]. The genes encoding the small ribosomal subunit (SSU, 16S rRNA gene in bacteria and 18S gene in eukaryotes) were selected because these genes contain both conserved and variable regions. On the contrary, only rRNA gene amplification condition was optimized by checking different melting temperature (Tm) conditions in our study. The 65°C (Tm) was found to be appropriate but requires some further optimization because sufficient amount of the target gene is required for further molecular analysis.

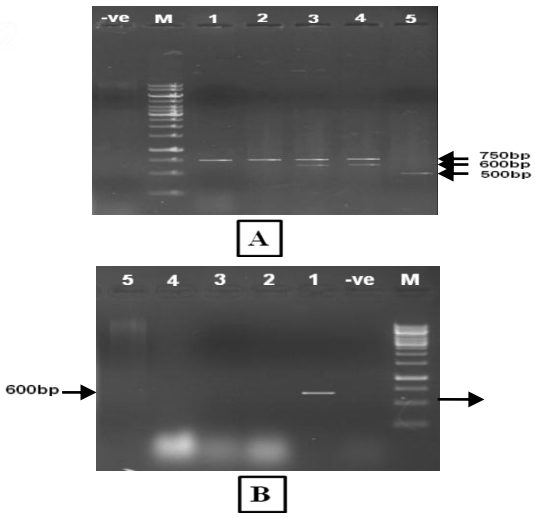

Plate 1: Genomic DNA extraction from different isolates of *Trichoderma* species by CTAB method. **M:** DNA marker (1Kb ladder), **1:** *T. hamatum* (*Syzygium cumini*, stem bark), **2:** *T. hamatum* (soil mycoflora), **3:** *T. koningii* (citrus fruit), **4:** *T. koningii* (*M. indica* stem), **5:** *T. koningii* (Oat seeds), **6:** *T. koningii* (wood), **7:** *T. koningii* (*M. indica*, wood), **8:** *T. pseudokoningii* (Green Chilli), **9:** *T. pseudokoningii* (Citrus fruit), **10:** *T. pseudokoningii* (Tannery effluent), **11:** *T. pseudokoningii* (Isolate sent by Dr. S.M. Khan), **12:** *T. harzianum* (*Mangifera indica*, rhizospheric soil), **13:** *T. harzianum* (Leaf litter), **14:** *T. harzianum* (Decaying Wood), **15:** *T. harzianum* (Air mycoflora), **16:** *T. harzianum* (Basidiocarp of *Hydnum* sp.), **17:** *T. harzianum* (Mushroom contamination), **18:** *T. harzianum* (Polluted water), **19:** *T. harzianum* (Soil mycoflora), **20:** *T. harzianum* (Air mycoflora), **21:** *T. harzianum* (contamination of *M. phaseolina*), **22:** *T. harzianum*

(*Helvela elastica* fruiting body), **23:** *T. harzianum* (Decaying wood) and **24:** *T. harzianum* (Rhizispheric soil).

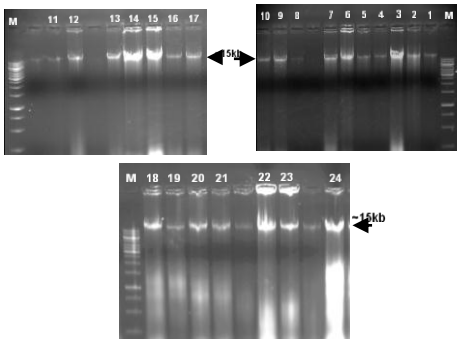

Plate 2: (A) RFLP analysis of different isolates of *Trichoderma* species by using restriction endonuclease enzyme (*Bam*HI). **–ve:** negative control, **M:** DNA marker (1Kb ladder), **1:** *Trichoderma hamatum* (*Syzygium cumini*, stem bark), **2:** *T. hamatum* (soil mycoflora), **3:** *Trichoderma koningii* (*Mangifera indica*, wood), **4:** *Trichoderma pseudokoningii* (Tannery effluent) and **5:** *Trichoderma harzianum* (*Mangifera indica*, rhizospheric soil). **(B)** rRNA gene amplification Ribotyping of five isolates of *Trichoderma* species by using fungal specific rRNA primers. **1:** *T. hamatum* (*Syzygium cumini*, stem bark), **2:** *T. hamatum* (soil mycoflora), **3:** *T. koningii* (*Mangifera indica*, wood), **4:** *Trichoderma pseudokoningii* (Tannery effluent) and **5:** *Trichoderma harzianum* (*Mangifera indica*, rhizospheric soil).

Table 1: List of Fungal Specific rRNA Primers used in rRNA Gene Targeting

Order	Name	Sequence (5' 3')	Tm	nmol
So- 36	nu-SSU-0817 (Forward)	TTA GCA TGG AAT AAT RRA ATA GGA	74.4	44.1
So- 37	nu- SSU- 1196 (Reverse)	TCT GGA CCT GGT GAG TTT CC	59.4	54.0
So- 38	nu-SSU- 1536 (Reverse)	ATT GCA ATG CYC TAT CCC CA	56.6	54.0

Table 2: DNA Concentration Values (µg/Ml) of Different Isolates of *Trichoderma* Species (FCBP Accessions)

Acc #	Name of species	$O.D_{260}$	$O.D_{280}$	Actual $O.D_{260}$ in µg/mL	Quality = 260/280
769	*T. hamatum*	0.0384	0.0523	193.92	1.8
907	*T. hamatum*	0.0326	0.0423	164.63	1.8
191	*T. koningii*	0.0366	0.0022	184.83	1.7
585	*T. koningii*	0.0361	0.0212	131.31	1.9
692	*T. koningii*	0.0310	0.0639	156.55	1.8
747	*T. koningii*	0.0373	0.0563	188.36	1.9
765	*T. koningii*	0.0304	0.0344	153.52	1.8
946	*T. koningii*	0.0345	0.0369	174.22	1.7
212	*T. pseudokoningii*	0.0322	0.0396	163.61	1.8
213	*T. pseudokoningii*	0.0438	0.0469	221.19	1.7
489	*T. pseudokoningii*	0.0427	0.0459	215.63	1.9
54	*T. pseudokoningii*	0.0463	0.0485	233.81	1.7
84	*T. harzianum*	0.0134	0.0401	65.65	1.6
125	*T. harzianum*	0.0225	0.0283	111.1	1.7
139	*T. harzianum*	0.0361	0.0378	182.30	1.7
140	*T. harzianum*	0.0236	0.0551	116.15	1.6
193	*T. harzianum*	0.0345	0.0364	174.22	1.7
210	*T. harzianum*	0.0105	0.0124	230.25	1.8
249	*T. harzianum*	0.0364	0.0865	183.82	1.7
325	*T. harzianum*	0.0249	0.0363	125.74	1.8

Acc #	Name of species	$O.D_{260}$	$O.D_{280}$	Actual $O.D_{260}$ in µg/mL	Quality = 260/280
496	*T. harzianum*	0.0125	0.0512	160.63	1.8
732	*T. harzianum*	0.0485	0.0396	244.92	1.8
755	*T. harzianum*	0.0302	0.0381	182.81	1.8
779	*T. harzianum*	0.0336	0.0375	169.68	1.9
860	*T. harzianum*	0.0362	0.0388	182.81	1.9

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