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Trichoderma harzianum Th azad/6796: From Isolation to Bioformulation

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Trichoderma **belongs to a group of filamentous fungi which is an effective biocontrol agent too against various soil borne pathogens such as *Fusarium, Pythium, Alternaria, Sclerotinia*, etc. to name a few. The strains in this species exhibit a wide range of functionalities that prove to be fruitful to the ecology. *Trichoderma* has been validated as a potential bioagent due to its antagonistic activity against destructive pathogens. There are several antagonistic mechanisms exercised by *Trichoderma* species such as nutrient competition, antibiotic production and mycoparasitism, which justify its biocontrol activity, but, mycoparasitism has been taken into prior consideration in this study. *Trichoderma* has attained importance as a substitute for chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters. The study aims at identifying a fungal strain of Trichoderma from the soil of an Indian farm located at Meja in the state of Uttar Pradesh. A set of universal primers (Internal Transcribed Spacer regions) was used for the amplification of an 18S rRNA gene fragment and eventually leading to the identification of the strain as *Trichoderma harzianum Th azad/6796.* Phylogenetic analysis of the newly identified strain was carried out through bioinformatics tools to validate the results and assign this strain as the type strain of a species of the genus *Trichoderma*. Thus, an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation. The strain has also been used in a talc-based bioformulation and its shelf life has been studied that could help in commercializing the specific strain of Trichoderma for the agricultural industry.**

Keywords: *Trichoderma;* Phylogenetic analysis; Internal Transcribed Spacer; DNA sequencing.

Trichoderma, commonly available in soil and root ecosystem, has gained immense importance since last few decades due to its biological control ability against several plant pathogens. Some strains of *Trichoderma* like *Trichoderma harzianum*, *T. atroviride*, *T. viride*, *T. virens and T. koningii* are efficient biocontrol agents which have the ability to inhibit pathogen growth in the soil, thereby, improving the overall health of the plant. Antagonistic microorganisms such as *Trichoderma* reduce growth and survival of the pathogen by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. Such microorganisms are now available commercially and used in crop management and practices. Though *Trichoderma* species were earlier discovered as potent biocontrol agents but their commercial applications have been started in the recent years. Biocontrol agents are widely regarded by the general public as "natural" and therefore non-threatening products, although risk

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assesments must clearly be carried out on their effects on non-target organisms. Moreover, knowledge concerning the behaviour of such antagonists is essential for their effective use (Sonika *et al*, 2014b). The morphological and microscopic figures of *Trichoderma harzianum Th azad*/ 6796 are given below:

Accurate and definitive fungal identification is essential for correct disease diagnosis and treatment of associated fungal infections. Thus, a morphological study was carried out to identify the strain of Trichoderma under optimum growth conditions and in a suitable media. Morphology of Trichoderma species includes various identification characteristics such as colony growth rate (cm), colony color, colony edge, colony smell, mycelial form and mycelial color. The morphology of conidia and phialids, conidia color, shape and size were also studied.

As, fungal species characterization using classical methods, is not very specific so genotypic approach is used in this study. The genotypic approach used here includes the amplification of a small-subunit (18S) rRNA gene[1]. The reason for selecting this particular gene fragment is that rRNA and its encoding genes hold significant conservation in the fungal evolution. As reported in the studies of Sacchi *et al.*, Hillis *et al.* and Woese, the conservation of rRNA is due to the presence of inter and intramolecular interactions between the proteins that make up the complete ribosome or protein synthesizing machinery[2-4].

Several scientists[5-9] first attempted to understand the phylogeny of the complete genus of Trichoderma using the sequence analysis of ITS regions of rDNA. Internal Transcribed Spacer (ITS) is considered to be the universal primer for the identification of fungal strains, thus, the present study includes the use of ITS sequences as primers to identify the *Trichoderma harzianum* strain and also to test the combination of ITS and rRNA based analysis to identify novel strains.

This study summarizes the methods of isolation and identification of an unknown fungal strain present in the soil of a farm in the Meja town of Allahabad district using 18S rRNA gene sequence as reported in bacterial rRNA gene[10] to characterize the strain *Th azad* as a member of the *Trichoderma* species. The soil sample has great potential for biodiversity and biological conservation due to which it has gained popularity among the people. The internal transcribed spacer (ITS) region of the rDNA is perhaps the most widely sequenced DNA region in fungi and has typically been most useful for molecular systematic study at species level, and even within species[10].

The strain of *Trichoderma* under study was further mixed in talc powder to prepare a bioformulation that could be used on farms against pathogenic fungi such as *Fusarium*. The bioformulation thus prepared would prove helpful to the farmers as it would be inexpensive and easily accessible to them.

MATERIALS AND METHODS

Isolation and identification of *Trichoderma*

The soil samples for the identification of Trichoderma were collected from the experimental fields located at Meja town of Allahabad district of India. Isolate of *Trichoderma* species was isolated and identified in potato dextrose agar (PDA) with low sugar medium[11]. The identification of *Trichoderma* isolates were confirmed both by morphological and molecular characters (ITS) and deposited in Indian Type Culture Collection (ITCC), IARI, Pusa, New Delhi.

DNA isolation of *Trichoderma*

Pure culture of the target fungus was grown overnight in liquid Potato Dextrose Broth medium for the isolation of genomic DNA using a method described by Hiney[12]. The total genomic DNA was extracted from isolate of *Trichoderma harzianum Th azad*/6796 based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method of Crowhurst *et al*[13] with minor modification.

Molecular characterization

The Internal Transcribed Spacer (ITS) regions of the rDNA repeat from the 32 -end of the 18S and the 52 -end of the 18S gene were amplified using the two primers, ITS-1 and ITS-4, respectively, which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene[14]. The PCR amplification reactions were performed in a 50 ml mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM $MgCl_2$, 200 mM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2µmM of each primer, 40ng/ml of template and 2.5U of Taq polymerase

(Shahid *et al*, 2013b). . The cycle parameters included an initial denaturation for 5 minutes at 94°C; followed by 40 cycles of denaturation for 1 minute at 94°C; primer annealing at 55°C for 2 minutes; primer extension for 3 minutes at 72°C, and, a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1mg/ml) and the complete electrophoresis gel setup was carried out for 3 hours at 60 volts in TAE buffer. A marker of 1 Kb ladder (MBI, Fermentas) was used in the gel. The gel was observed in a trans-illuminator over ultraviolet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit for purification.

Purification of PCR product

The PCR product was purified by QIAGEN gel extraction kit using the protocol as described here. The DNA fragment was excised from the agarose gel with a clean sharp scalpel. The gel slice was then weighed in an eppendorf and 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~ 100 ml). The mixture was then incubated at 50°C for 10 min. The gel was dissolved in a vortex mixer until the mixture color is uniformly yellow. Further, 1 volume of isopropanol was added to the sample and mixed. A QIAquick spin column is then placed in a 2 ml collection tube provided. The sample is applied to the QIA quick column followed by centrifugation for 1 minute so that DNA binds to the column. The supernatant is then discarded and the QIAquick column is placed back in the collection tube. A volume of 0.75 ml of PE was added to QIAquick column and centrifuged for 1 minute to wash. The supernatant is again discarded and the QIAquick column centrifuged for an additional 1 minute at 10000x g. The QIAquick column is now placed into a clean 1.5 ml eppendorf. We then added 50ml of Eluent Buffer (EB) (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 minute to elute the DNA.

DNA sequencing of the 18S rDNA fragment

The 18S rDNA amplified PCR product (100 ng concentration) was used for sequencing with the single 18S rDNA 20F ITS-1 forward primer: 5'-TCCTCCGCTTATTGATATGC-3' and 22R ITS-4 reverse primer: 5'-GGAAGTAAAAGTCG TAACAAGG-3' synthesized by DNA Sequencer at Merck laboratory (Bangalore, India).

Sequence analysis

Sequence analysis of the sequenced gene was initiated with the use of a similarity searching algorithm such as BLAST (Basic Local Alignment Search Tool). The gene of interest, 18S rRNA of the test strain, was searched for similar gene sequences using nucleotide BLAST program against a non-redundant nucleotide (nr/nt) database[15]. The database sequences that were found to be ~90% similar to the test sequence were selected as the best matching homologs and were then subjected to a Multiple Sequence Alignment in the ClustalW program[16].

Based on the multiple sequence alignment of the selected sequence set, an evolutionary distance matrix and a phylogenetic tree were then computed using the Neighbor-Joining method. MEGA (Molecular Evolutionary Genetics analysis) version 4.0 was used for phylogenetic and molecular evolutionary analyses[17,18].

The18S rRNA gene sequence of the test strain was again compared with a different set of sequence databases such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA) using Ribosomal RNA BLAST program[19,20]. 18S rRNA gene sequence of test strain is also compared against those sequences in Ribosomal Database Project[21] by using the RDP Classifier check Program. The annotated information for the sequence in the database to which 18S rRNA aligns is used for the fungal identification (Shahid *et al* 2014 ab).

Bioformulation and its Validation

Talc powder was evaluated as a carrier material to produce bioformulation of *Trichoderma* species. The carrier was dried under sun, powdered (sieve pore: 1mm) and sterilized at 1.05 kg/cm^2 pressure for 30 minutes. The substrate was mixed with a 7 days old culture of *Trichoderma harzianum* previously grown on PDA in 2:1 (solid culture) w/v and 5g/kg CMC was added as an adjuvant. Observations on colony forming units of Trichoderma species was recorded initially and at monthly intervals for up to 6 months for shelf life study.

RESULTS

Rapid identification of bioagents is very

necessary and important in the pathological laboratory to take decision for installment of commercialized bioformulation.

The identified *Trichoderma harzianum Th azad* isolate was confirmed both by morphological and molecular characters (ITS) and deposited in Indian Type Culture Collection (ITCC), IARI, Pusa, New Delhi. The particular strain was then allotted with an ITCC Accession No. 6796.

The morphological descriptors used in the characterization of *Trichoderma harzianum* have been described in this section as they hold significance in rapid visual identification of the strain under study.

a) Colony Growth Rate: 8-9 in 3 days
b) Colony Color: Dark green
c) Colony Edge: Wavy
d) Mycelial Form: Floccose to Arachnoid
e) Mycelial Color: Watery white
f) Conidiation: Ring like zones
g) Conidiophore branching: Highly branched, regular
h) Conidial Wall: Smooth
i) Conidial Color: Dark green

The genomic DNA was extracted from isolated fungal strain *Trichoderma harzianum Th azad*-6796/09 and universal ITS-1 forward primer and ITS-4 reverse primer were used for the amplification and sequencing of the 18S rRNA gene

Fig. 1. *Trichoderma harzianum Th azad*/6796 *strain in PDA medium*

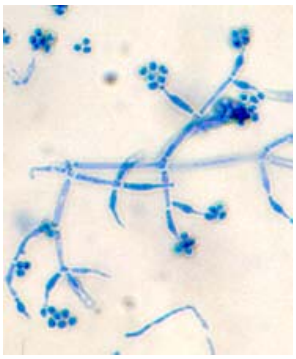

Fig. 2. *Trichoderma harzianum Th azad*/6796 *strain under microscope*

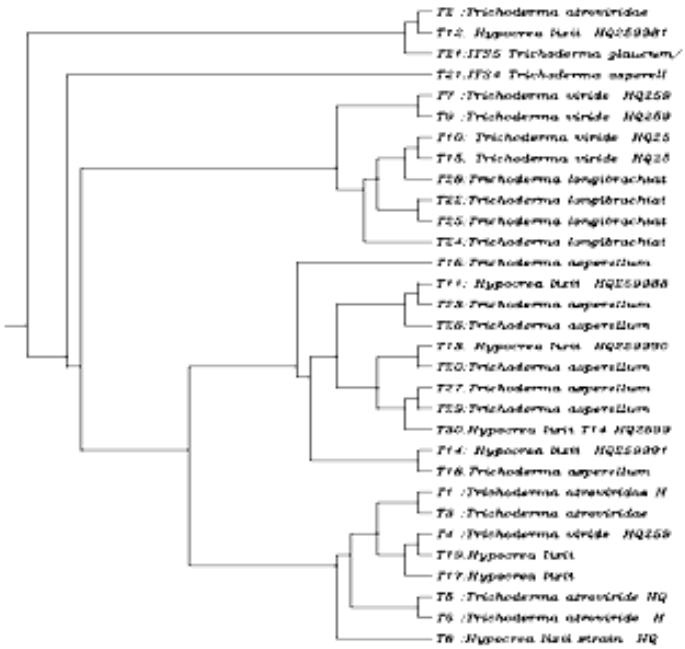

Fig. 3. Phylogenetic and molecular variability analysis of *Trichoderma* species

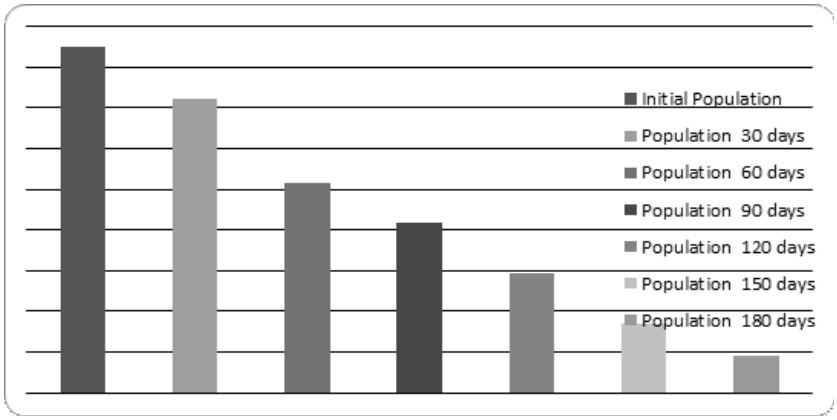

Fig. 4. Effect of talc carrier on the population of *Trichoderma harzianum Th azad*

fragment. The 18S rRNA gene of 546 base pairs thus sequenced was used for the identification of isolated fungal strain from the soil sample.

Furthermore, the phylogenetic tree (based on 18S rRNA gene sequence) showing the evolutionary relationship between the test strain *Th azad* and selected homologs of the genus *Trichoderma* is given in Figure 1. It is evident from the phylogenetic analysis of 18S rRNA gene that the isolate *Th azad* represents an individual genomic species in the genus *Trichoderma*. Comparison of test strain against known sequences of SSU rRNA and LSU rRNA databases showed that the gene sequence of isolate *Th azad* has 90% sequence similarity (Score=546 bits, Expect=0.0) with 18S rRNA gene sequence of *Trichoderma* (Genbank Accession No.: KC800922). Similarity rank program classifier available at the Ribosomal Database Project[20] classified the isolate *Th azad* as a novel genomic species of the genus *Trichoderma* with a confidence threshold of 90% (Figure 3). The 18S rRNA gene sequence of isolate *Th azad* was deposited in GenBank and allotted with the accession number i.e., KC800922.

The shelf life of *Trichoderma harzianum Th azad* in talc as a carrier material, determined at an interval of 30 days, indicated that the decline in the number of propagules started from 30th day onwards. Talc based bioformulation was found to be the best material as it can retain the maximum number of viable propagules (which was 29.7x10^6 cfu/g at 180 days of storage). The growth rate of Trichoderma harzianum Th azad in talc carrier is shown in Figure 4.

DISCUSSION

The sequence analysis and estimating the phylogeny thereupon has been done taking the rRNA sequences into account in all of the major organismal domains.

In eukaryotes, genes encoding small subunit (SSU) and large-subunit (LSU) rRNA have been found to support a close subgeneric relationship.

The genetic variability within 69 biocontrol isolates of *Trichoderma* collected from different geographic locations and culture collections and their phylogenetic analysis were done with the help of the sequence data obtained from the ITS-1 region of ribosomal DNA and a fragment of the translation elongation factor 1 (tef1) and reported that more than 50% of the potential biocontrol strains were grouped within *Trichoderma* section Pachybasium[21,22].

The identification of different *Trichoderma* isolates by molecular methods using ITS sequences has further characterized it into three main clades. Thus, a reliable phylogenetic tree was constructed with this data containing *Trichoderma harzianum* isolates[23].

The shelf life study of Trichoderma has earlier been carried out on other strains such as *Trichoderma longibrachaitum* and *Trichoderma atroviride*[24]. The shelf life of *T. longibrachiatum* in three different carrier materials (talc, lignite and charcoal) was determined at 30 days interval. The effect of temperature, pH and culture media for

growth and sporulation of Trichoderma species was also done in a way to find out the ways to increase the shelf life of the bioformulation.

CONCLUSION

Trichoderma strains have been recognised as biological agent for the control of plant diseases such as wilting caused by *Fusarium* especially in leguminous and solanaceous crops. *Trichoderma* species not only prevents the soil borne pathogens to cause diseases but also play an important role in increasing root growth and development, crop productivity, resistance to abiotic stress and uptake and use of nutrients.

Biocontrol agents belonging to the genus *Trichoderma* prove to be potent agents against soil borne pathogens. Thus, a directive approach for their isolation, identification, molecular characterization and their phylogenetic analysis would help the plant pathologists to benefit the agricultural industry. This would also lead to the commercialization of Trichoderma and its formulations at low costs.

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