70

RESEARCH ARTICLE

Molecular Characterization of *Trichoderma* spp. Isolates by Internal Transcribed Spacer (ITS) Region Sequencing Technique and its Use as a Biocontrol Agent

Ziyaul Haque[1], Mohammed S. Iqbal[1,*], Ausaf Ahmad[1], Mohd S. Khan[2] and Jyoti Prakash[1,*]

[1]*Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow, India*
[2]*Department of Biosciences, Integral University Lucknow Uttar Pradesh, Lucknow, India*

Abstract:

Objective:

In the present investigation, *Trichoderma* spp., isolated from rhizospheric soil, has been identified by Internal Transcribed Spacer (ITS) region sequencing technique and its antagonistic activity was evaluated against *A. niger*.

Methods:

The sequencing analysis was done with its ITS1 region of the rRNA gene. Using the ITS1 amplified products for all isolated fungi, a bi-directional DNA sequencing was done with high quality bases (>98% - 100%). Antagonistic activity was done using dual culture technique.

Results:

All of the ITS1 nucleotide sequences obtained in this study matched 97% - 100% with the published sequence of *Trichoderma* spp. The results confirmed the strains as *T. asperellum* and *T. viride* with gene bank accession no. (ZTa); MK937669 and (ZTv); MK503705, respectively. When phylogenetic analysis was done for the isolates, the optimal tree with the sum of branch length = 0.69585023 and 0.10077756 for *T. asperellum* and *T. viride,* respectively, was observed. There were a total of 678 and 767 for *T. asperellum* and *T. viride* positions in the final dataset, respectively. Antagonistic activity was done for the isolated strains of *Trichoderma* spp. against *A. niger,* and it was found that *T. asperellum* showed maximum antagonistic activity (79.33±7.09%).

Conclusion:

The findings prolong the genome availability for relative investigations pointing out phenotypic variances to compare with *Trichoderma* genetic diversity. The present investigation delivered the Bases of future studies for better knowledge in understanding the complicated connections of *Trichoderma* spp. to be used as an effective biocontrol agent.

Keywords: Antagonism, Biocontrol agent, Fungus, Internal transcribed spacer , *Trichoderma* spp, Rhizospheric soil.

1. INTRODUCTION

Trichoderma spp. is a kind of fungal species that assists as an impending substitute to restrain chemicals and pathogen as resistance to crop cultivars. This aggressive and competitive property of the fungus is due to their metabolic competence [1]. Specific and precise description of Fungus is necessary for accurate diagnosis of disease and their remedy of accompanying fungal contagions. *Trichoderma* species are difficult to distinguish morphologically, therefore, molecular methods, including DNA sequencing and genealogical concordance phylogenetic species recognition using several unliked genes, are needed to give accurate identification of *Trichoderma* spp [2]. Thus, the identification of fungal species by means of traditional approaches is not as definite as by the method of genotyping. The methodology of genotyping comprises of amplification of targets that are phylogenetically informative like subunit of rRNA gene [3]. The genes encoding the rRNA are an extremely conserved sequence that is crucial for the existence of all cells. The structure of proteins and rRNA involving the ribosomes are exceedingly well-

* Address correspondence to this author at the Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow, India;
Tel: +9451310257; E-mail: jpamity2020@gmail.com

maintained during the course of evolution, as they are involved in the interaction of multifarious intermolecules and intramolecules to sustain their mechanism of protein synthesis [3 - 5]. Due to the antagonistic activities of *Trichoderma* spp*.,* they are operative in contributing biocontrol of pathogens that are soil borne as they are simple inhabitants in the soil [6]. The chief characteristic of efficacious biological resistant strategies comprises of the manufacture, preparation, and the distribution of bioagents. It has utmost been characteristically suitable for systematic molecular investigation at the level of species, and also even within the species [7, 8]. The first attempt for phylogenetic exploration of the complete genus of *Trichoderma* was done by means of sequence investigation of rDNAs ITS-1 region [9]. In rDNA, an individual cluster comprises of 18S rDNA, 5.8S rDNA, 28S rDNA, External Transcribed Spacer (ETS) and Internal Transcribed Spacer 1 and 2 (ITS1 and ITS 2) [5]. For the determination of phylogenetic relationships, the spacer regions and the genes, distinctly or in grouping, are used as molecular markers [10]. However, 28S rDNA and 18S rDNA (length-conservative regions) are generally applied in the determination of phylogenetic relationships amongst groups of upper levels [10 - 12], although when considered at species level, regions of length variability like internal transcribed spacer 1 and 2 (ITS1 and ITS 2) are often used [13 - 15]. Thus, in the present study, the identification of *Trichoderma* spp. at species level was determined with consideration to the regions of length variability *i.e* Internal Transcribed Spacer (ITS).

As a biocontrol agent, *Trichoderma* spp works extensively virtuous (mycoparasitism mechanisms), because of its antagonistic characteristic [16, 17]. The progression ostensibly consists of *Trichoderma* chemotropic growth, mycoparasitic identification of the host, extra-cellular enzymes secretion, hyphae permeations, and finally, host lysis [18]. Mycoparasitism encompasses outbreak of one fungal species, directly on the other [19]. This complicated progression comprises of chronological proceedings, which comprises of identification of other fungal strain by *Trichoderma* spp, followed by an outbreak of cellular machinery of the host, thereafter its permeations inside the fungal host and lastly carnage the host [20]. *Trichoderma* spp. grows to the concerning fungal host by identifying them. Such offensive remote sensing property of *Trichoderma* spp. Is, to some extent, due to the consecutive formation of pathogenesis linked proteins typically chitinase and glucanase proteases [21]. Continuous secretion of exo-chitinases by *Trichoderma* spp. damages cell-walls of the fungal host, thereby discharging oligomers, which play a crucial part in the inhibition of fungal host [17]. *Trichoderma* spp. is associated with the host pathogen, loops around it and forms appressoria, thereby discharging its content [22]. Therefore it results in the formation of peptides associated with pathogenesis that services in both the entrance of *Trichoderma* spp. hyphae as well as cell wall content digestion [23]. The degradation of the cell of host fungus due to the formation of these biologically synthesized chemicals outcomes as parasitism. Thus, the identification of such a valuable non-pathogenic fungus would help us in commercialization and ultimately developing a superior tactic to eliminate pathogens that are soil-borne.

2. MATERIALS AND METHODS

2.1. Culture Preparation

The soil sample was collected from sugarcane cultivation field (from rhizospheric plane) located at IIM Road Lucknow, Uttar Pradesh, for isolation of *Trichoderma* spp. (isolation and purification processes was done by serial dilution and sub-culturing technique). Morphologically, microscopically, and biochemically identified strain was cultured and maintained on potato dextrose broth (PDB; Hi Media) and potato dextrose agar (PDA; Hi Media) plate [24]. The isolates were assigned as ZTa and ZTv for *T. asperellum* and *T. viride,* respectively. Freshly cultured pure colony was further used for DNA isolation.

2.2. Primers

The primers obtained from Bio kart India Pvt. Ltd. India, were used for *Trichoderma* spp. *viz*. ZTa and ZTv for PCR amplification were ITS 1 (´TCCGTAGGTGAACCTTGCGG) and ITS 4 (´TCCTCCGCTTATTGATATGC) with an annealing temperature of 61°C and 53°C, respectively. These primers bind to conserved regions, with corresponding positions. The PCR product was amplified that encompasses a portion of the Internal Transcribed Spacer (ITS) region. The size of the PCR product generated was varying and according to the organism tested. The same primers were used for ZTa and ZTv for direct sequencing with the same annealing temperature of 61°C and 53°C, respectively.

2.3. DNA Isolation and Sequencing

The genomic DNA of primarily identified fungal isolates was extracted and amplified using PCR, followed by the sequencing analysis, using its ITS1 region of the rRNA gene. Primarily, for genomic DNA extractions, approximately 100 mg of mycelial powder was used; mycelial powder was obtained by grinding a small portion of the fungal culture in liquid nitrogen using a mortar and pestle. Fungal genomic DNA was isolated using a modified Phenol: Chloroform method [25]. Amplification of ITS genes was carried out with the ITS 1 (TCCGTAGGTGAACCTTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) universal primer pair, which produced ~500 bp amplicon products. PCR Master Mix (Promega ™) was used to amplify the ~500 bps region of the ITS region. A negative control (PCR mix without template DNA) was also performed in all PCR experiments. The PCR reaction conditions were set for 95°C for 2 min (1 cycle), followed by 35 cycles of denaturation at 95°C for 30s, annealing at 52°C for 30 min and extension at 72°C for 2 min, before a final extension at 72°C for 15 min (1 cycle). PCR products were subjected to purification using Omega™ PCR Purification Kit, by following the protocol of Liu *et al*. [26]. Purified PCR products were then subjected to an ethidium bromide-stained 1% agarose gel (Fisher Scientific) along with a 1 kb DNA ladder (Promega) to estimate the size of the amplified band. The purified PCR products were subjected to sequencing [27].

2.4. Gene Bank and BLAST

The ITS rRNA gene sequences were subjected to the

BLASTn search program (National Center for Biotechnology Information) to find a similarity index between sequences. Sequences are edited using MEGA (Molecular Evolutionary Genetics Analysis) software [28]. Each sequence was subjected to an individual BLAST search to be verified in Gene Bank. The newly obtained sequences were aligned with highly similar, homologous sequences from Gene Bank using the multiple sequence alignment program MUSCLE, with default parameters. The BLASTn similarity search program was used to find homologous sequences against the NCBI nucleotide database that confirmed the species level similarity with the query sequence of the isolates.

The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (500 replicates), were shown next to the branches [29]. This analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The phylogenetic tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [30, 31] and were in the units of the number of base substitutions per site. Evolutionary analyses were conducted using MEGA X [28].

2.5. Biocontrol Activity

In vitro antagonastic activity was done according to the protocol of Dennis and Webster [32]. As per the protocol, for seven days, the fungal isolates were cultured on PDA media in petri plates. The 5mm diameter disks of growing colonies of *Trichoderma* mycelium and *Aspergillus niger* were cut and transferred on other PDA media petri plates, 7 cm apart from each other. The plates were incubated for five days at 28± 1°C. A standard strain of *Trichoderma* spp. (MTCC167), obtained from Department of Biosciences, Integral University, was also taken and similar treatment was done. The percent inhibition of growth was determined by the method of Watanabe [33]. The experiment was done in triplicate (n=3).

2.6. Statistical Analysis

All the experiments were performed in triplicate and calculated as mean ± SD. Two-way ANOVA was performed using MS Excel (2016).

3. RESULTS

3.1. Identification of Fungal Strains Using its Sequencing Technique

Morphologically, microscopically and biochemically identified strain, as reported by Haque *et al*. [24], were used for further identification and confirmation, using ITS sequencing molecular technique. The isolated fungal strains were identified using sequencing analysis in the present study. However, primarily, the strains were identified on the basis of morphological, microscopic, and biochemical characterization. The isolates were visually characterized on the basis of phenotypic characters like colony color (dark green and cottony whitish green colonies), growth pattern, shape and size of conidiophore, phialides and conidia were observed microscopically. The morphologically and microscopically

analyzed isolates were assigned as *T. asperellum* (ZTa) and *T. viride* (ZTv), respectively. Therefore, the present sequence analysis was performed for fungal identification using its Internal Transcribed Spacer (ITS) region. The Internal Transcribed Spacer (ITS) was referred to the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding transcribed region in the polycistronic rRNA precursor transcript. Using the ITS1 amplified products for all isolated fungi, a bi-directional DNA sequencing was completed with high quality (HQ) bases (>98% HQ–100% HQ). Analysis of the generated ITS1 nucleotide sequences confirmed species-identification of the fungal isolates. All of the ITS1 nucleotide sequences, obtained in this study, matched 97% - 100% with the published sequence of *Trichoderma* spp. with gene bank accession number of *T. asperellum* (ZTa), MK937669 and that of *T.viride* (ZTv), MK503705. No intra-specific genetic variation was noticed among the fungi. The results confirmed the *Trichoderma* spp. strains as *T. asperellum* and *T. viride.*

When phylogenetic analysis was done for the isolates of *T. asperellum* and *T.viride*, the following results were obtained using the Neighbor-Joining method [34]. The optimal tree with the sum of branch length = 0.69585023 and 0.10077756 for *T. asperellum* and *T. viride,* respectively, was observed. There was a total of 678 and 767 positions for *T. asperellum* and *T. viride,* respectively, in the final dataset. The phylogenetic analysis and the sequence obtained is given in Fig. (**1A** and **B**) for *T. asperellum and T. viride,* respectively.

3.2. Biocontrol Activity

When the antagonistic activity was tested for the isolated strains of *Trichoderma* spp. against *A. niger*, it was found that *T. asperellum* showed maximum antagonistic activity (79.33±7.09%) while minimum antagonistic activity was shown by the standard strain of *Trichoderma* spp. MTCC167 (54.33±9.50%). However, when Two-way ANOVA was applied between the three *Trichoderma* spp. strains, significant variation was observed with *p value* < 0.05 (0.024) and the effect of days on the growth were also found to be significant (*p value* < 0.05) (4.75E-06). The results are shown in Fig. (**2**).

4. DISCUSSION

The specie concept of micro-organisms (prokaryotic and eukaryotic) is well defined by Rossello´-Mora and Kampfer [35], where it was described that micro-organisms might be capable of extensive diversity range amongst the various other living organisms and can be determined by the phylogenetic analysis which shows ancestry with a common pattern. Moreover, there are various techniques for the identification of pathogenic micro-organisms for the recognition of pathogens in food crops, water, *etc* [36]. However, in a study by Castro-Escarpulli *et al*.; Bajinka and Secka; She and Bender [37 - 39], it was mentioned that molecular techniques were found to be the best for identification of micro-organisms, which confers precision and accuracy. The molecular methods like gene sequencing using 16S ribosomal RNA for fungal ITS sequence can be applied for the identification of organisms [40 - 42]. As per the previously reported findings, in the present study, we have applied the ITS sequencing technique to identify *Trichoderma* spp. isolates. The identification of fungus at

species level by the use of ITS region (as a genetic marker) of the gene was well established [43 - 45]. In the past few decades, the ITS1 region sequencing technique has been extensively applied for genotyping of fungal strains, which are pathogenic to humans causing various health ailments like cutaneous infection, meningitis, allergies, and respiratory illness, *etc* [46]. The ITS1 region sequencing technique recognized the pathogenic human yeast and 40 species out of 106 strains have been tested [47]. It was also investigated that 44 human-pathogenic mold species were identified in 201 strains using the ITS region sequencing technique [48].

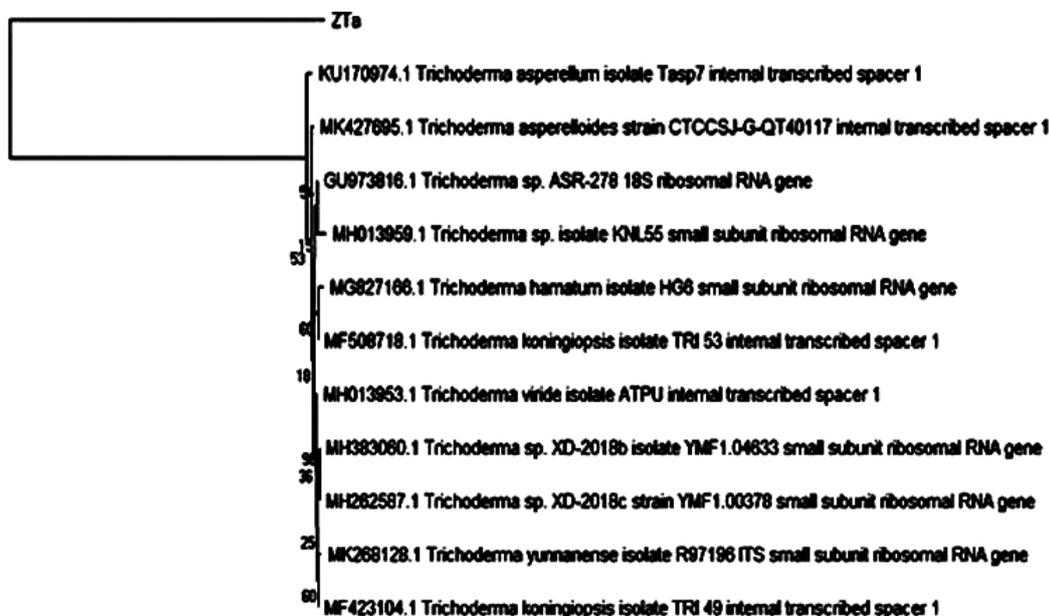It was further reported that ITS1 region sequencing technique was used to identify and distinguish five species of *Rhizopus,* which can cause meningitis in human beings [49]. Infection due to *Rhizopus* causing intestinal mucormycosis was investigated efficaciously using the ITS region sequencing technique [50]. Since the ITS region sequencing technique is promptly being envisioned as a barcodes, suggestions have been put forward to construct a distinct set of public reference data for ITS sequences of various fungal species [51]. Efforts have been made to form a guiding principle for the genuineness and consistency of newly produced sequences of fungal ITS [52]. The query sequence of isolate *T. asperellum* (MK937669) showed 97.16% identity with *T. asperellum* (MH013956), and isolate *T. viride* (MK503705) showed 99.82% with *T. viride* of (JF304319). The findings clearly indicate that the isolated strains were confirmed as *Trichoderma* spp.

(A)

Sequence *T. asperellum*

ACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCGCGCTCCCGGTGCGAGTT
GTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGT
CCCGATCCCCAACGCCGATCCCCCGGAGGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAAT
ACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCT
GCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTG
TAAGAAATACGTCCGCGAGGGGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCT
GCGACGCACCCGGGGGCGTGGACCCCGGCGAAGGCAACAGTTTGGTAACGTCCCCATTGGGGTTGGGGAATTGG
AAAACTCCGGAAATGGATCCCCTCCGAAGGTTCCCCCAAGGGG

Phylogenetic analysis

(B)

Sequence *T. viride*

GCTCCGAGCTCACTCCCAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCG
CAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTG>TAGTCCCCTCGCGGACGTATTTCTTAC
AGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTA
TTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGAC
CCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACT
CGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAA
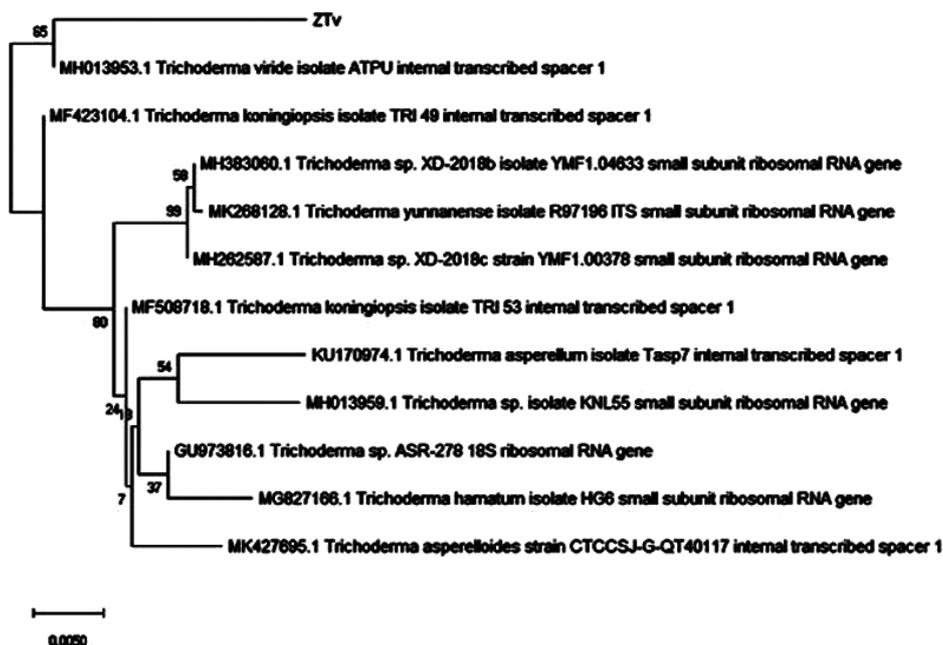TACCCGCTGAACTTAAGCATATCTTAAAAGGGGGGAGAA

Phylogenetic analysis

Fig. (1A and B). Sequence and phylogenetic tree of **(A)***T. asperellum;* ZTa and **(B)***T. viride;* ZTv.

T. Asperellum (MK937669)   *T. viride* (MK503705)   Standard (MTCC167)

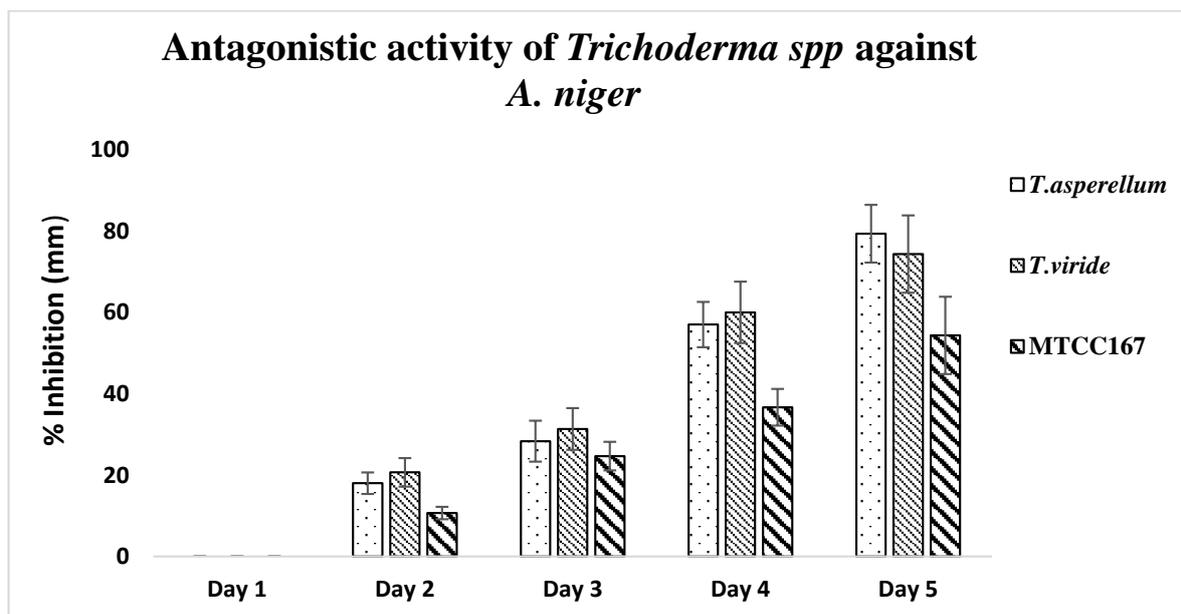## Antagonistic activity of *Trichoderma spp* against *A. niger*

Fig. (2). Antagonistic activity of *Trichoderma* spp. against *A. niger* (n=3).

The antagonistic activity of *Trichoderma* spp. showed ZTa (MK937669), the better strain against *A. niger*. The results are in good agreement with the report of Gajera and Vakharia [53] where twelve isolates of *Trichoderma* spp. showed antagonistic activity against *A. niger*. In another study by Kurt *et al*. [54], *Trichoderma* spp. strain SJ3-4, which expresses the *A. niger* glucose oxidase-encoding gene, *goxA*, under a homologous chitinase (*Nag1*) promoter, increased the competence as a biocontrol agent. The mechanism of *Trichoderma* spp. as a biocontrol agent is well established. The synthesis of siderophores by *Trichoderma* spp. makes it an effective biocontrol fungus. It was elucidated that due to the synthesis of iron-chelating siderophores by *Trichoderma* spp*.,* to survive against micronutrient insufficiency, makes itas an effective biocontrol agent. It has been further reported that the development of iron-chelating siderophores by *Trichoderma* spp. is due to the presence of other pathogenic fungi [55, 56]. Struggle for space by the pathogen from *Trichoderma* spp. as a biocontrol agent causes hindered root colonization, resulting in feeble establishment and poor disease cause. Thus, the observations of the present study showed that the isolated *Trichoderma* spp. strains are of virtuous biocontrol agents and method (ITS sequencing) [57] used for its genetic identification confirmed its occurrence.

CONCLUSION

Since fungi are very diverse at the specie level, there are several molecular techniques that have been developed for the identification of fungal species. DNA Barcoding technique is applied to a small and standardized DNA region with unique pattern and is considered as one of the most accurate and rapid method to categorize unidentified fungal species. In DNA barcoding, Internal Transcribed Spacer (ITS) region is predominantly used as it is the most sequenced region for fungal identification at species and within the species level. The ITS region is extremely polymorphic and non-coding with adequate taxonomic parts which enables to isolate sequences at species level. Molecular based techniques have endorsed more in-depth experimentation on *Trichoderm,* howeverthe outcomes are limited due to the lack of information of genome sequence. With this consideration, the identification of genome ZTa (MK937669) and ZTv (MK503705) is a contribution to understand the mode of action as a biocontrol agent by the use of genetic markers, which are activity-specific. The findings prolong the genome availability for relative investigations pointing to compare phenotypic variances with *Trichoderma* genetic diversity. It can be concluded that this investigation delivers the base for future studies for better knowledge of complicated connections of *Trichoderma* with multiple objectives for the improvement of effective *Trichoderma* strains as biocontrol agent.

ETHICS APPROVAL AND CONSENT TO PARTI-CIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available within the article.

REFERENCES

[1]   Rifai MA. A revision of the genus *Trichoderma.* Mycological Papers 1969; 116: 1-56.

[2]   Druzhinina IS, Kopchinskiy AG, Kubicek CP. The first one hundred of *Trichoderma* species is characterized by molecular data. Mycoscience 2006; 47: 55-64.
[http://dx.doi.org/10.1007/S10267-006-0279-7]

[3]   Sacchi CT, Whitney AM, Reeves MW, Mayer LW, Popovic T. Sequence diversity of *Neisseria meningitidis* 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. J Clin Microbiol 2002; 40(12): 4520-7.
[http://dx.doi.org/10.1128/JCM.40.12.4520-4527.2002]   [PMID: 12454145]

[4]   Woese CR. Bacterial evolution. Microbiol Rev 1987; 51(2): 221-71.
[http://dx.doi.org/10.1128/MMBR.51.2.221-271.1987]   [PMID: 2439888]

[5]   Hillis DM, Moritz C, Porter CA, Baker RJ. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science 1991; 251(4991): 308-10.
[http://dx.doi.org/10.1126/science.1987647]   [PMID: 1987647]

[6]   Bastakoti S, Belbase S, Manandhar S, Arjyal C. Trichoderma species as biocontrol agent against soil borne fungal pathogens. Nepal J Biotechnol 2017; 5(1): 39-45.
[http://dx.doi.org/10.3126/njb.v5i1.18492]

[7]   Kubicek CP, Mach RL, Peterbauer CK, Lorito M. *Trichoderma.* From genes to biocontrol. J Plant Pathol 2000; 83: 11-23.

[8]   Lee CF, Hseu TH. Genetic relatedness of *Trichoderma* sect. Pachybasium species based on molecular approaches. Can J Microbiol 2002; 48(9): 831-40.
[http://dx.doi.org/10.1139/w02-081]   [PMID: 12455615]

[9]   Kindermann J, El-Ayouti Y, Samuels GJ, Kubicek CP. Phylogeny of the genus *trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA cluster. Fungal Genet Biol 1998; 24(3): 298-309.
[http://dx.doi.org/10.1006/fgbi.1998.1049]   [PMID: 9756711]

[10]   Wheeler WC, Schuh RT, Bang R. Cladistic relationships among higher groups of Heteroptera: Congruence between morphological and molecular data sets. Insect Syst Evol 1993; 24: 121-37.
[http://dx.doi.org/10.1163/187631293X00235]

[11]   Xie Q, Bu W, Zheng L. The Bayesian phylogenetic analysis of the 18S rRNA sequences from the main lineages of Trichophora (Insecta: Heteroptera: Pentatomomorpha). Mol Phylogenet Evol 2005; 34(2): 448-51.
[http://dx.doi.org/10.1016/j.ympev.2004.10.015]   [PMID: 15619455]

[12]   Schuh RT, Weirauch C, Wheeler WC. Phylogenetic relationships within the Cimicomorpha (Hemiptera: Heteroptera): A total-evidence analysis. Syst Entomol 2009; 34: 15-48.
[http://dx.doi.org/10.1111/j.1365-3113.2008.00436.x]

[13]   Marcilla A, Bargues MD, Ramsey JM, *et al.* The ITS-2 of the nuclear rDNA as a molecular marker for populations, species, and phylogenetic relationships in Triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. Mol Phylogenet Evol 2001; 18(1): 136-42.
[http://dx.doi.org/10.1006/mpev.2000.0864]   [PMID: 11161750]

[14]   Damgaard J, Andersen NM, Meier R. Combining molecular and morphological analyses of water strider phylogeny (Hemiptera-Heteroptera, Gerromorpha): Effects of alignment and taxon sampling. Syst Entomol 2005; 30: 289-309.

[http://dx.doi.org/10.1111/j.1365-3113.2004.00275.x]

[15]   Calleros L, Panzera F, Bargues MD, *et al.* Systematics of Mepraia (Hemiptera-Reduviidae): Cytogenetic and molecular variation. Infect Genet Evol 2010; 10(2): 221-8.
[http://dx.doi.org/10.1016/j.meegid.2009.12.002]   [PMID: 20018255]

[16]   Guo Y, Ghirardo A, Weber B, Schnitzler J-P, Benz JP, Rosenkranz M. *Trichoderma* species differ in their volatile profiles and in antagonism toward ectomycorrhiza *Laccaria bicolor.* Front Microbiol 2019; 10: 891.
[http://dx.doi.org/10.3389/fmicb.2019.00891]   [PMID: 31105677]

[17]   Gajera H, Domadiya R, Patel S, Kapopara M, Golakiya B. Molecular mechanism of *Trichoderma* as bio-control agents against phytopathogen system – A review. Curr Res Microbiol Biotechnol 2013; 1: 133-42.

[18]   Zeilinger S, Galhaup C, Payer K, *et al.* Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genet Biol 1999; 26(2): 131-40.
[http://dx.doi.org/10.1006/fgbi.1998.1111]   [PMID: 10328983]

[19]   Schuster A, Schmoll M. Biology and biotechnology of *Trichoderma.* Appl Microbiol Biotechnol 2010; 87(3): 787-99.
[http://dx.doi.org/10.1007/s00253-010-2632-1]   [PMID: 20461510]

[20]   Mendoza JL, Pérez MI, Prieto JM, Velásquez JD, Olivares JG, Langarica HR. Antibiosis of *Trichoderma* spp strains native to northeastern Mexico against the pathogenic fungus Macrophomina phaseolina. Braz J Microbiol 2015; 46(4): 1093-101.
[http://dx.doi.org/10.1590/S1517-838246420120177]   [PMID: 26691467]

[21]   Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. *Trichoderma* species-opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2004; 2(1): 43-56.
[http://dx.doi.org/10.1038/nrmicro797]   [PMID: 15035008]

[22]   Mukherjee M, Mukherjee PK, Horwitz BA, Zachow C, Berg G, Zeilinger S. *Trichoderma*-plant-pathogen interactions: Advances in genetics of biological control. Indian J Microbiol 2012; 52(4): 522-9.
[http://dx.doi.org/10.1007/s12088-012-0308-5]   [PMID: 24293705]

[23]   Howell CR. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Dis 2003; 87(1): 4-10.
[http://dx.doi.org/10.1094/PDIS.2003.87.1.4]   [PMID: 30812698]

[24]   Haque Z, Iqbal MS, Ahmad A, Khan MS, Singh SP, Prakash J. Explorations of tolerant *Trichoderma* spp. as plant growth promoter and biocontrol against *Colletotrichum falcatum.* J Pure Appl Microbiol 2020; 14(1): 6081.
[http://dx.doi.org/10.22207/JPAM.14.1.34]

[25]   Josep S, David RW. Cold Spring Harbor Laboratory Molecular cloning: A laboratory manual / Joseph Sambrook, David W. 3rd ed. N.Y: Russell Cold Spring Harbor Laboratory Cold Spring Harbor 2001.

[26]   Liu Y, Zheng Z, Gong H, *et al.* DNA preservation in silk. Biomater Sci 2017; 27;5(7): 1279-92.
[http://dx.doi.org/10.1039/C6BM00741D]

[27]   Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 1975; 94(3): 441-8.
[http://dx.doi.org/10.1016/0022-2836(75)90213-2]   [PMID: 1100841]

[28]   Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 2018; 35(6): 1547-9.
[http://dx.doi.org/10.1093/molbev/msy096]   [PMID: 29722887]

[29]   Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985; 39(4): 783-91.
[http://dx.doi.org/10.1111/j.1558-5646.1985.tb00420.x]   [PMID: 28561359]

[30]   Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 2004; 101(30): 11030-5.
[http://dx.doi.org/10.1073/pnas.0404206101]   [PMID: 15258291]

[31]   Varin C, Nancy R, David F. An overview of composite likelihood methods. Stat Sin 2011; 21(1): 5-42. www.jstor.org/stable/24309261

[32]   Dennis CJ, Webster J. Antagonism properties of species groups of *Trichoderma,* III Hyphal interaction. Trans Br Mycol Soc 1971; 57: 363-9.
[http://dx.doi.org/10.1016/S0007-1536(71)80050-5]

[33]   Watanabe N. Antagonism by various kind of *Trichoderma* fungi to soil born plant pathogen Bulletin of Faculty of Agriculture. Maiji University, Japan 1984; 66: 45-50.

[34]   Saitou N, Nei M. The neighbor-joining method: A new method for

reconstructing phylogenetic trees. Mol Biol Evol 1987; 4(4): 406-25.
[PMID: 3447015]

[35]   Rossello´-Mora R, Kampfer P. Defining microbial diversity-the species concept for prokaryotic and eukaryotic micro-organisms. Microbial diversity and bioprospecting. Washington, DC: ASM Press 2004.
[http://dx.doi.org/10.1128/9781555817770.ch3]

[36]   Rajapaksha P, Elbourne A, Gangadoo S, Brown R, Cozzolino D, Chapman J. A review of methods for the detection of pathogenic microorganisms. Analyst 2018; 144(2): 396-411.
[http://dx.doi.org/10.1039/c8an01488d] [PMID: 30468217]

[37]   Castro-Escarpulli G, Alonso-Aguilar NM, Sánchez GR, *et al.* Identification and typing methods for the study of bacterial infections: A brief review and mycobacterial as case of study. Arch Clin Microbiol 2015; 7: 1.

[38]   Bajinka O, Secka O. Integration of molecular methods into microbiological diagnostics. Appl Microbiol Open Access 2017; 3: 130.
[http://dx.doi.org/10.4172/2471-9315.1000130]

[39]   She RC, Bender JM. Advances in rapid molecular blood culture diagnostics: healthcare impact, laboratory implications, and multiplex technologies. J Appl Lab Med 2019; 3(4): 617-30.
[http://dx.doi.org/10.1373/jalm.2018.027409] [PMID: 31639730]

[40]   Buszewski B, Rogowska A, Pomastowski P, Złoch M, Railean-Plugaru V. Identification of microorganisms by modern analytical techniques. J AOAC Int 2017; 100(6): 1607-23.
[http://dx.doi.org/10.5740/jaoacint.17-0207] [PMID: 28703095]

[41]   Edgar RC. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics 2018; 34(14): 2371-5.
[http://dx.doi.org/10.1093/bioinformatics/bty113] [PMID: 29506021]

[42]   Edgar R. Taxonomy annotation and guide tree errors in 16S rRNA databases. PeerJ 2018; 6e5030
[http://dx.doi.org/10.7717/peerj.5030] [PMID: 29910992]

[43]   Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson KH. Intraspecific ITS variability in the kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol Bioinform Online 2008; 4: 193-201.
[http://dx.doi.org/10.4137/EBO.S653] [PMID: 19204817]

[44]   Pitkäranta M, Meklin T, Hyvärinen A, *et al.* Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Appl Environ Microbiol 2008; 74(1): 233-44.
[http://dx.doi.org/10.1128/AEM.00692-07] [PMID: 17981947]

[45]   Zhao Z, Li L, Wan Z, Chen W, Liu H, Li R. Simultaneous detection and identification of Aspergillus and mucorales species in tissues collected from patients with fungal rhinosinusitis. J Clin Microbiol 2011; 49(4): 1501-7.
[http://dx.doi.org/10.1128/JCM.02262-10] [PMID: 21325541]

[46]   Sulaiman IM, Jacobs E, Simpson S, Kerdahi K. Molecular identification of isolated fungi from unopened containers of greek yogurt by DNA sequencing of internal transcribed spacer region.

Pathogens 2014; 3(3): 499-509.
[http://dx.doi.org/10.3390/pathogens3030499] [PMID: 25438008]

[47]   Chen YC, Eisner JD, Kattar MM, *et al.* Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. J Clin Microbiol 2001; 39(11): 4042-51.
[http://dx.doi.org/10.1128/JCM.39.11.4042-4051.2001]   [PMID: 11682528]

[48]   Rakeman JL, Bui U, Lafe K, *et al.* Multilocus DNA sequence comparisons rapidly identify pathogenic molds. J Clin Microbiol 2005; 43(7): 3324-33.
[http://dx.doi.org/10.1128/JCM.43.7.3324-3333.2005]   [PMID: 16000456]

[49]   Nagao K, Ota T, Tanikawa A, *et al.* Genetic identification and detection of human pathogenic Rhizopus species, a major mucormycosis agent, by multiplex PCR based on internal transcribed spacer region of rRNA gene. J Dermatol Sci 2005; 39(1): 23-31.
[http://dx.doi.org/10.1016/j.jdermsci.2005.01.010] [PMID: 15978416]

[50]   Woo PC, Leung SY, To KK, *et al.* Internal transcribed spacer region sequence heterogeneity in *Rhizopus microsporus*: Implications for molecular diagnosis in clinical microbiology laboratories. J Clin Microbiol 2010; 48(1): 208-14.
[http://dx.doi.org/10.1128/JCM.01750-09] [PMID: 19906897]

[51]   Kõljalg U, Nilsson RH, Abarenkov K, *et al.* Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 2013; 22(21): 5271-7.
[http://dx.doi.org/10.1111/mec.12481] [PMID: 24112409]

[52]   Nilsson RH, Tedersoo L, Abarenkov K, *et al.* Five simple guidelines for establishing basic authenticity and reliability of newly generated fungal ITS sequences. MycoKeys 2012; 4: 37-63.
[http://dx.doi.org/10.3897/mycokeys.4.3606]

[53]   Gajera HP, Vakharia DN. Production of lytic enzymes by *Trichoderma* isolates during in vitro antagonism with *Aspergillus niger*, the causal agent of collar rot of peanut. Braz J Microbiol 2012; 43(1): 43-52.
[http://dx.doi.org/10.1590/S1517-83822012000100005]   [PMID: 24031802]

[54]   Brunner K, Zeilinger S, Ciliento R, *et al.* Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. Appl Environ Microbiol 2005; 71(7): 3959-65.
[http://dx.doi.org/10.1128/AEM.71.7.3959-3965.2005]   [PMID: 16000810]

[55]   Benítez T, Rincón AM, Limón MC, Codón AC. Mecanismos de biocontrol de cepas de *Trichoderma*. Int Microbiol 2004; 7: 249-60.
[PMID: 15666245]

[56]   Nelson EB. Exudate molecules initiating fungal responses to seed seeds and roots. Plant Soil 1990; 129: 61-73.
[http://dx.doi.org/10.1007/BF00011692]

[57]   Edgar RC. Accuracy of taxonomy prediction for 16S rRNA and fungal ITS sequences. PeerJ 2018; 6e4652
[http://dx.doi.org/10.7717/peerj.4652] [PMID: 29682424]