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Molecular Identification and Antagonistic Activity of *Trichoderma* species from Chilli Field Soil in Thiruvarur District, Tamil Nadu, India

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ABSTRACT

***Trichoderma asperellam* is a fungal species that is frequently utilized in the biological control of plant pathogenic fungi. The creation of analytical enzymes enhances its ability to control biological infections. It has been highlighted that physical traits alone are insufficient for identifying these fungal species. As a result, the study's goal was to use molecular markers to identify *Trichoderma* species and assess their antagonistic effectiveness against plant pathogens. *T. asperellam* have antagonistic and physiologically controlled activity, which inhibited the pathogenic fungus to the greatest extent possible in a dual culture approach. *T. asperellam* had the highest inhibition (62.7%), followed by *T. harzianum* (56.0%), *T. koeningii* (56.0%), *Aspergillus niger* (52.5%), *Aspergillus sulphureus* (48.5%), *Penicillium* sp. (48.41%), and *Aspergillus flavus* (47.3%).**

Keywords: *Trichoderma* sp, Biocontrol, Molecular identification, Gene sequencing

INTRODUCTION

Capsicum (*Capsicum annuum* L.) is a popular spice crop that is widely grown in the tropics and southern countries including Bangladesh, India, Pakistan, and Sri Lanka. *Capsicum* is a member of the Solanaceae family (Night shade). The Solanaceae family consists primarily of herbs and shrubs, with a few climbers thrown in for good measure. There are over 90 genera and nearly 3000 species in the family (Vidhyarttie and Tripathi, 2002; Sterm, 2000). Because of its hot flavour and nutritional value, *Capsicum* is a frequently grown crop. Heiser and Smith (1953) recognized two *Capsicum* species grown as vegetables, while varieties are all *Capsicum annuum* or *Capsicum frutescens. C. annuum* is not known in its natural condition, although it is widely cultivated as sweet pepper, bell pepper, cherry pepper, and green pepper (Messraen, 1992). Chilli peppers thrive best in temperatures ranging from 20 to 30^0C during the day. Growth and yield are frequently lowered when the temperature falls below 15°C or surpasses 32°C for long periods of time. Chillies are susceptible to a variety of illnesses caused by fungi, bacteria, viruses, nematodes, and abiotic stressors. *Pythium* species are soil-borne pathogenic fungi that cause seed rot and damping off in a variety of crops, such as chillies and tomatoes (Shah Smith and Burns, 1996).

Biological control agents have recently been employed more frequently as an alternative to chemical pesticides in the management of plant infections due to the detrimental effects of chemical pesticides on both human health and the environment. Biological control agents can be bacterial or fungal strains (Melnick *et al.*, 2008; Validov *et al.*, 2009). *Trichoderma* species are non-sexual creatures that coexist with other soil-dwelling fungi with teleomorphs of the *Hypocrea* genus (Ascomycota). The intertwining of morphological characteristics and phonetic events, especially among *Trichoderma anamorph* forms, makes establishing the morphology of *Trichoderma* challenging (Druzhinina *et al.*, 2006; Hassan *et al.*, 2014).

Molecular markers can be used to definitively establish the validity of any morphological taxonomic system and to characters fungal genotypes. Taxonomic objectives and identification of unknown isolates with morphological identification is time consuming and complex, and certain species are sexual or heterothallic, making identification with sexual structure characterization challenging.

Several PCR-based molecular approaches have been utilised to detect and distinguish bacteria in recent years. In addition, the evolutionary link of the species with *Trichoderma* has been established using DNA sequences from several genetic variations (Kullnig *et al.*, 2002; Chaverri *et al.*, 2003).

MATERIALS AND METHODS

Fungal isolates are isolated and identified.

Soil samples were taken from a chilli field in Tamil Nadu's Thiruvarur district. The dilution plate method was used to isolate the *Trichoderma* isolates, as described by Hassan and El-Awady (2011).

Trichoderma morphological identification

The morphological identification of the *Trichoderma* isolates was based on the appearance and pigmentation of the various colonies, as well as microscopic characteristics such as conidiophore branching patterns, phialide organisation, and conidia shapes and sizes, as described by Kumar and Sharma (2011).

Morphological identification of *Trichoderma*

The appearance and colour of the various colonies, as well as microscopic characteristics such as conidiophore branching patterns, phialide organisation, and conidia shapes and sizes, were used to morphologically identify the *Trichoderma* isolates, as described by Kumar and Sharma (2011).

$$\text{Percentage inhibition of growth} = \frac{r - r^1}{r} x100$$

r= Growth of the fungus was measured from the centre of the colony towards the centre of the plate in the absence of antagonistic fungus.

r^1= Growth of the fungus was measured from the centre of the colony towards the antagonistic fungus.

The colony interactions between the test pathogen and the soil fungi were assessed following the model proposed by Porter (1924) and Dickinson and Broadman (1971). Five types of interaction grades as proposed by Skidmore and Dickinson (1976) have been used. They are as follows:

1. Mutual intermingling growth without any microscopic sights of interaction – Grade 1.
2. Mutual intermingling growth where the growth of the fungus is ceased, and is being over grown by the opposed fungus – Grade 2.
3. Intermingling growth where the fungus under observation is growing into the opposed fungus either above or below – Grade 3.
4. Slight inhibition of both the interacting fungi with a narrow demarcation line (1-2 mm) – Grade 4.
5. Mutual inhibition of growth at a distance of >2mm – Grade 5.

Isolation of chromosomal DNA

One gram of mycelium was ground in a grinder with 75 l of STE extraction buffer (320 mM Sucrose, 10 mM Tris-Cl, 20 mM EDTA, 75 mM NaCl, and 2.5 ml of 20% SDS), 5 mg Polyvinyl pyrolidone, and 0.1 g silica powder, and incubated at 65°C for 10 min. For 10 min, the sample was centrifuged at 13,000 rpm. After centrifugation, an equal proportion of chloroform: isoamyl alcohol was added to the supernatant. 2/3 volume isopropanol was added to the aqueous layer and centrifuged for 10 min at 13,000 rpm. The pellet was centrifuged and washed with 70% ethanol before being dried and dissolved in 50 µl TE buffer.

Analysis of DNA Purity and Quality

The DNA stock samples was quantified using UV spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µ g DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 was considered to be of good purity. Concentration of DNA was estimated using the formula. Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor Quality of DNA was again checked by agarose gel electrophoresis. The 0.8 % agarose was prepared (0.8 g agarose power/100ml X TBE), and was melted. 30 ml agarose was poured into the casting tray. The gel was allowed to solidify and the comb and tape was removed. 1 X TBE (Tris-Borate-EDTA; electrophoresis buffer) was added to the chamber until the buffer just covers the top of the gel. The samples were loaded with Bromophenol blue loading dye, taking care not to puncture the well bottoms. The power pack was turned on and run at 100V. The gel was viewed on a UV transilluminator after electrophoresis. The DNA was used further for PCR.

PCR amplification of 18S rDNA

ITS fragment was amplified by PCR from fungal genomic DNA using ITS-PCR universal primers:

Details of primers used for PCR

ITS 1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4: 5'- TCCTCCGCTTATTGATATGC-3'

PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given below.

Analysis of DNA Amplification by AGE

Standard DNA Markers

Commercially available 100 bp ladder was used as standard molecular weight DNA.

PCR-Product Electrophoresis

Loaded 5 µl of PCR product with 4 µl bromophenol blue (loading dye) in 1.5% agarose gel. Run the gel at constant voltage of 100 V and current of 45 A for a period of 1 h 20 min till the bromophenol blue has travelled 6 cm from the wells. Viewed the gels on UV transilluminator and photograph of the gel was taken.

Purification and DNA Sequencing of samples

Amplified PCR product was purified using column purification as per manufacturer's guidelines, and further used for sequencing reaction.

Sequencing of Purified ITS Gene Segment

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer (Applied Biosystems, USA).

ITS Sequence Analysis

Each nucleic acid sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequence at the 3 'and 5' ends (considering peak and quality values for each base) using the sequence analysis tools. The edited sequences (ITS gene) were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) program in the NCBI Gene Bank (www.ncbi.nlm.nih.gov) DNA database for identifying the fungal strains.

Phylogenetic analysis

The D_2 region of 18S rRNA gene sequence was used to carry out BLAST with the NR database of NCBI gene bank database (URL http://www, ncbi.n/m.nih.g). Based on maximum identity scores first ten sequences were selected and Global pair wise sequence similarity between the sequence were performed using Needleman and Wunsch algorithm available with the emboss sequence analysis suite. Multiple sequence analysis was performed using alignment program CLUSTAL W. The phylogenetic tree was constructed using MEGA 4 software.

The evolutionary history was inferred using the Neighbor joining method of Saitou and Nei (1987). The bootstrap consensus tree inferred from 500 replicated was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicated tree in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches; the evolutionary distances were computed using the Kimura 2- parameter method (Kimura, 1980) and are in the units of the number of base substation gaps substations per site codon position included were 1^{st} + 2^{nd} + 3^{rd} + noncoding. All position gaps and missing data were eliminated from the dataset (complete deletion option). There was a total of 663 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.,* 2007). Tree visualization was done with the tree view program.

18S rRNA Secondary structure prediction of fungal isolate

The Secondary structure of *Trichoderma asperellum* were predicted using the Bioinformatics tools available in online www.genebee.msu.su/service/ma 2-reduced.html.

Restriction site analysis of 18S rRNA of fungal isolate

The restriction sites in 18S rRNA of *Trichoderma asperellum* were analyzed using NEB cutter program version 2.0 in online www.neb. com/NEBcutter 2/index.phb.

RESULTS AND DISCUSSION

Control of pathogen using antagonistic fungi by dual culture technique

Growth of *Pythium debaryanum* towards the centre of the plate in the absence of antagonistic fungi (control) was 45mm measurement was taken in to after 72 h.

The type of interactions of the pathogen with soil fungi were as follows.

Aspergillus fumigatus	- Grade 1
A. sydowi	- Grade 2
A. niger, A. sulphureus, and *T. harzianum*	- Grade 3
Penicillium sp. *T.asperellum,* and *T. koeningii*	- Grade 4
A. flavus	- Grade 5

The maximum percentage inhibition of *T. asperellum* (62.7%), followed by *T. harzianum* (56.0%), *T. koeningii* (52.5%), *A. niger* (52.5%), *A. sulphureus* (48.5%), *Penicillium* sp. (48.4%), *A. flavus* (47.3%), *A. fumigatus* (41.6%), and *A. sydowi* (41.1%) was inhibited against *P. debaryanum* respectively (**Figure 1**).

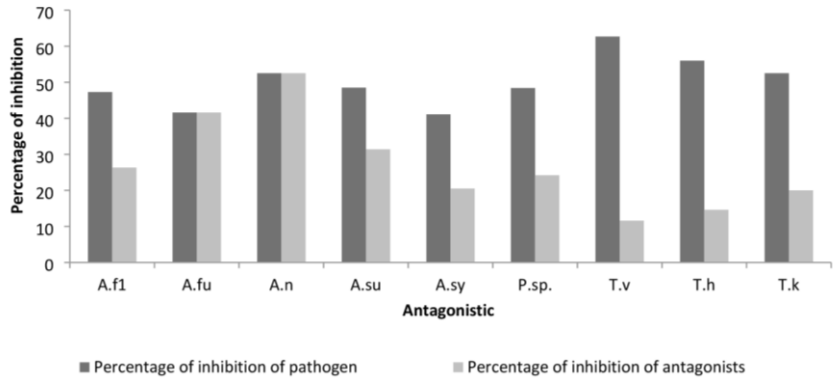

Figure 1: Colony interaction between *Pythium debaryanum* and Soil fungi in dual culture experiment.

*A.f-Aspergillus flavus; A.fu-A. fumigatus; A.n-A. niger; A. su-A. sulphureus; A.sy-A. sydowi; P.*sp.*-Penicillium* species; *T.h-Trichoderma harzianum; T.k-T. koeningii; T.v-Trichoderma asperellum*

Molecular characterization of fungi

Molecular characterization of PCR amplification of 18S rRNA

PCR amplification of the 18S rRNA gene was used to assess the molecular features of *T. asperellum*. In an agarose gel, the genomic DNA and amplified products were separated.

18S rRNA sequencing of fungal isolates

Using a specific 18S rRNA sequence primer (TCCGTAGGTGAACCTGCGG-3' forward, TCCTC CGCTTATTGATATGC-3' reverse), the 18S rRNA genes of *T. asperellum* isolated from chilli field soil were partially sequenced. To obtain an accession number, the sequence of 18S rRNA was deposited in Gene bank (NCB1 EMBL and DDBJ). HM438946.1 is the accession number for *T. asperellum.*

Phylogenetic tree analysis

T. asperellum 18S rRNA gene sequences were compared to sequences from the Gene Bank using BLAST, and 100 percent similarity was determined. The results revealed that the most closely related isolates of *T. viride* HM438946.1 and *Trichoderma asperellum* GU723446.1 were *T. viride* HM438946.1 and *Trichoderma asperellum* GU723446.1 (100 percent similarity). The *T. asperellum* phylogenetic relatedness was investigated using the Neighbor joining method (**Figure 2)**.

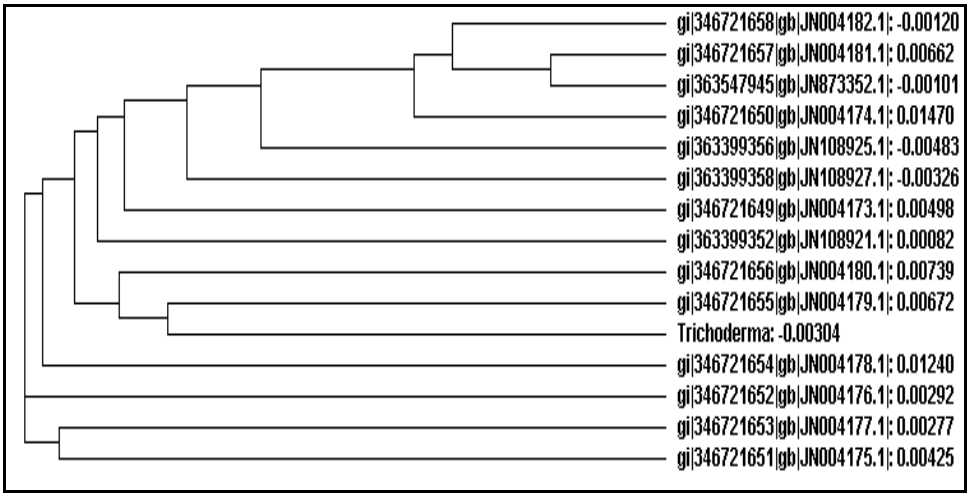

Figure 2: *Trichoderma asperellum* ribosomal RNA gene, partial sequence Phylogenetic tree.

18SrRNA Secondary structure prediction of fungal isolates

Bioinformatic tools were used to predict the secondary structures of *T. asperellum*. The secondary structure of *T. asperellum* (GU 723446.1) 18S rRNA revealed 23 stems, 16 bulg loops, and 11 hairpin loops. The free energy structure of *T. asperellum* 18S rRNA secondary structure was -90.8 kKal/mol (**Figure 3**).

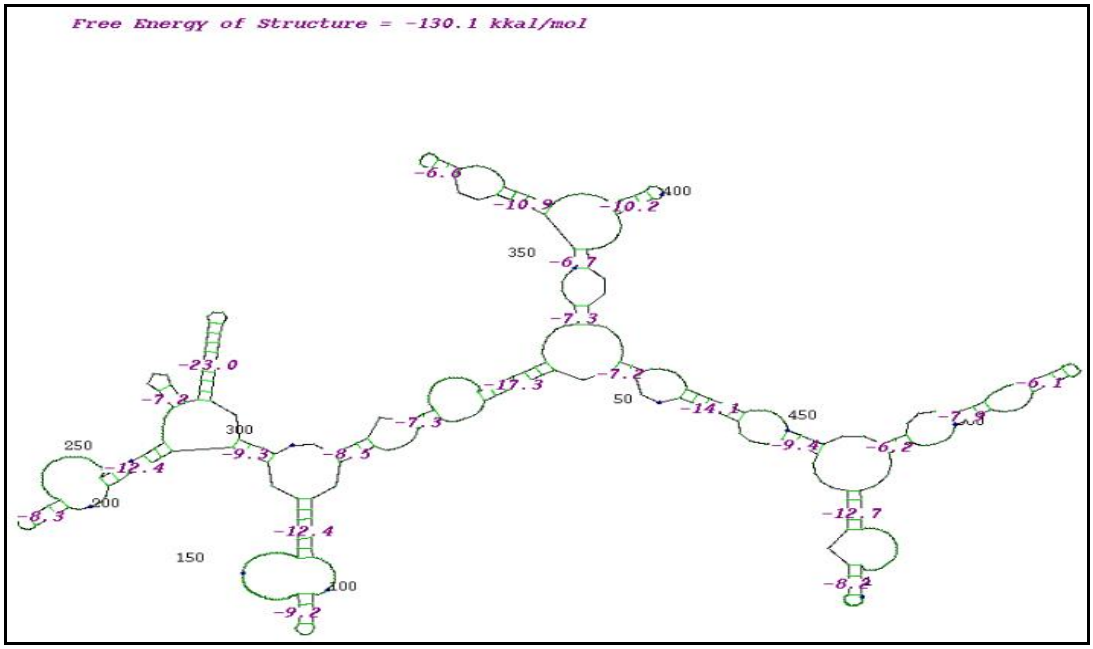

Figure 3: Secondary structure prediction of *Trichoderma asperellum.*

Restriction site analysis of 18SrRNA of fungal isolate

The 18S rRNA restriction site map of *T. asperellum* GU-723446 was investigated. There are 51 ambiguous restriction sites reported in the rRNA. In the fungal isolates, there were a lot of restriction enzyme sites. The GC-56 and AT-44 percent in *T. asperellum* were calculated using the NEB cutter software (**Figure 4**).

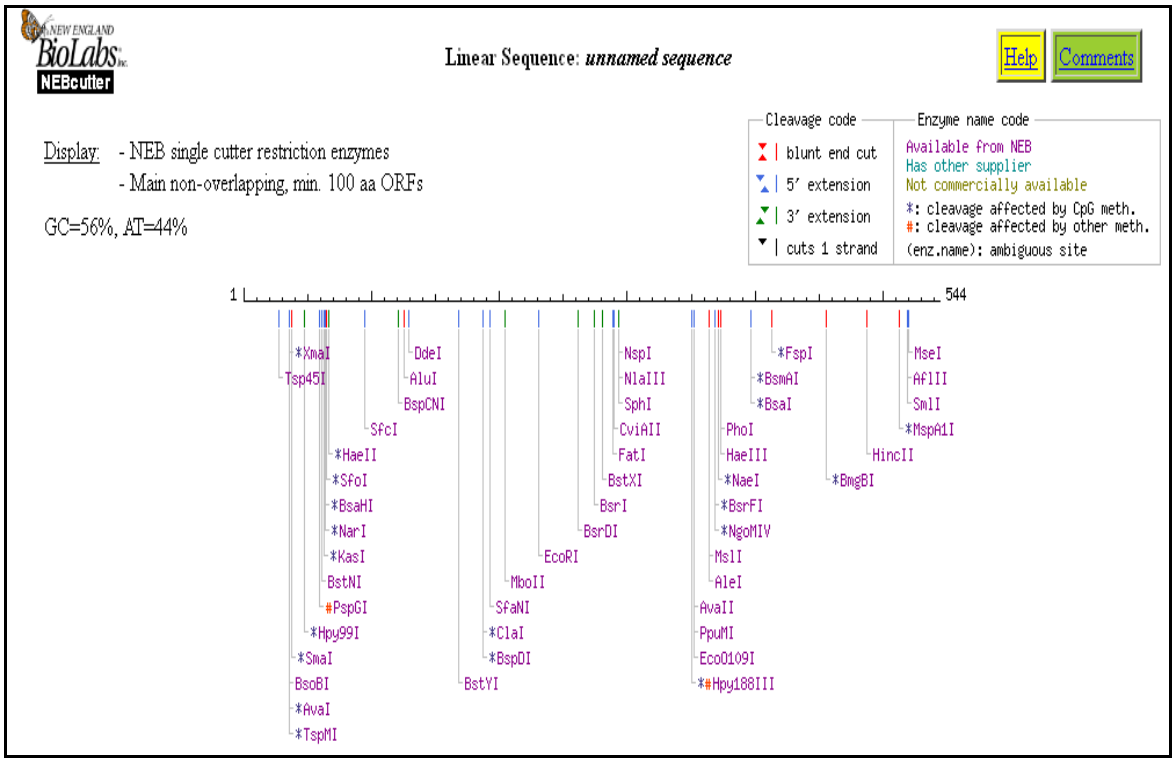

Figure 4: Restriction Site Analysis.

Trichoderma viride, *T. harzianum*, *T. hamatum*, and *Trichoderma* sp. were found to have different levels of antagonistic activity against *P. aphanidermatum*, according to Muthukumar *et al.* (2008). *Trichoderma* sp. demonstrated the most inhibition, with a reduction of 43.33 percent over control, followed by *Trichoderma viride* with a reduction of 37.78 percent over control. *T. viride* has been linked to the production of antibiotics such as viridin, gliotoxin, and trichodermin (Allen and Haenseler, 1935; Dennis and Webster, 1971; Papavizas and Lumsden, 1989). These authors also proposed various mechanisms for effective *T. viride* antagonism, including lysis, competition, and mycoparasitism. Dumitars and Fratilescu-Sesan (1979) observed an antagonistic reactivity of *T. viride* against many *Pythium* species.

Trichoderma virens and *T. harzianum* have greater antifungal activity against *Pythium* sp. Than *Trichoderma koeningii*, according to Jun and Kim (2004), and Dharmaputra *et al.* (1994) evaluated two isolates of *T. harzianum* and one strain of *T. viride* against three isolates of *Ganoderma* from oil palms. The pathogen's mycelial proliferation was reduced by all of the *Trichoderma* isolates.

In this study, *in-vitro* dual culture experiments were used to investigate the antagonistic activity of *Aspergillus*, *Penicillium* and *Trichoderma* against *Pythium debaryanum*, *Trichoderma* species from all over the world were shown to be able to inhibit the disease. However, the percentage of inhibition in different species varied. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. sydowi*, *A. sulphureus*, *Penicillium* sp., and three biocontrol agents, *Trichoderma asperellum*, *T. harzianum*, and *T.* koeningii, were evaluated against *Pythium debaryanum*, a plant pathogen.

According to Ha (2010), laboratory and field testing revealed that *Trichoderma* species were able to inhibit the proliferation of fungal plant diseases while also enhancing plant growth and development. Experiments on *Phytophthora* sp., *Rhizoctonia solani*, *Fusarium* sp., *Sclerotium rolfsii*, and *Pythium* sp. have shown that some *Trichoderma* strains can diminish severe illnesses caused by fungal pathogens such as *Phytophthora* sp., *Rhizoctonia solani*, *Fusarium* sp., *Sclerotium rolfsii*, and in addition, Siameto *et al.* (2010) investigated the antagonistic effect of five soil-borne phytopathogenic fungus against *T. harzianum* on pathogen mycelia growth in dual cultures compared to controls. Yasser *et al.* (2020) have isolated 12 *Trichoderma* isolates based on molecular markers and evaluated their antagonistic effectiveness against numerous plant diseases. The 12 *Trichoderma/Hypocrea* isolates were collected from the rhizosphere of healthy tomato plants in the Saudi Arabian city of Abha. Both morphological and molecular approaches, such as sequencing the 5.8S-ITS region, were used to identify these isolates. Furthermore, these isolates were divided into two primary groups based on their taxonomy and phylogeny. The 12 isolates were then tested against *Rhizoctonia solani*, *Pythium ultimum*, and *Alternaria solani* in confrontational assays. The majority of the *Trichoderma* isolates had considerable antagonistic activity, which could be seen by SEM. The potential for *T. harzianum* to be used in IPM programmes is highlighted in this study.

Using the sequencing analysis of the ITS1 region of the rDNA, Kindermann *et al.* (1998) performed a first phylogenetic analysis of the genus *Trichoderma.* Phylogenesis based on single gene sequences, however, is now widely criticized, particularly when it comes to the use of ITS1 and/or ITS2, as several fungi and plants have been found to contain paralogous copies (Lieckfeldt and Seifert, 2000). Taylor *et al.* (1999) presented five or more gene tree phylogenetic species concepts. These interrelationships between species, when paired with phenotypic features, can lead to a taxonomy that is accurate and reflects evolutionary relationships Druzhinina *et al.* (2004) were able to identify 70 of the 77 *Trichoderma* species studied out of a total of 77. Seventy-eight isolates of *Trichoderma* were positively identified as *T. harzianum* by Kubicek *et al.* (2003); other species included *T.virens* (16 strains), *T. spirale* (8 strains), *T. koeningii* (3 strains), *T. aureoviride* (3 strains), *T. asperellum* (4 strains), *Hypocrea jecorina* (2 strains), *T. viride* (2 strains), T. (1 strain). When compared to other *Trichoderma* isolates, Ospina-Giraldo *et al.* (1999) found that phylogenetic analyses were closely related to *T. harzianum* isolates.

Based on the sequence analysis of the ITS-1 and ITS-2 sections of the rRNA gene, the goal of this work was to establish a species of *T. asperellum* gene sequences in chilli field isolates. Evidently, Chakraborty *et al.* (2011) extracted genomic DNA of *Macrophomina phaseolina* isolated from mandarin rhizosphere and used genus specific ITS-1 and ITS 4 primers for PCR amplification of 18S rRNA. The amplified product (550 bp) was sequenced and matched using BLAST against ex-type *M. phaseolina* strain sequences from the NCB1 Gene Bank, and phylogenetic analysis was performed using MEGA 4 software.

T. atroviride, *T. virens*, *T. velutinum*, *T. harzianum*, *T. asperellum*, *T. koningiopsis*, *T.aureoviride*, *H.lixii*, and *T. koeningii* were among the 18 *Trichoderma* strains identified from soil samples taken from the South Korean wetland habitat. *In-vitro* antagonistic assays were used to screen these strains against the diseases *Macrophomina phaseolina* (MP), *Fusarium graminearum* (FG), and *Botrytis cineara* (BC). *T.*

aureoviride (SKCGWO13) was shown to have more antagonistic activity against the pathogens than the other isolates. The SKCGW013 strain was also employed for metabolite extraction, purification, and analysis using column chromatography (CC) and gas chromatography mass spectrometry (GCMS). The RT-qPCR was used to investigate the expression of secondary metabolites regulating genes of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS). The results revealed the existence of eight dominating chemicals in the ethyl acetate fraction of the strain SKCGW013, which were subsequently screened against phytopathogens using a molecular modelling method. Furthermore, an RT-qPCR analysis revealed considerable expression of metabolite-related genes. A molecular docking analysis revealed that the chemicals from strain SKCGW13 suppressed the pathogens in a synergistic manner. 2H-Pyran, 3-bromo-2-butoxytetrahydro-, cis were shown to have high docking inhibitory energy against the targeted proteins FgSwi6 and Bcpmr1 from FG and BC, respectively. Overall, the metabolite profile of *T. aureoviride* SKCGW013 demonstrated that it was a great source for discovering novel metabolites as biocontrol agents, as evidenced by its antifungal activity (Saravanakumar and wang, 2020)

T. asperellum genomic DNA was extracted from chilli field soil and used for PCR amplification of 18S rRNA using genus-specific ITS1 and ITS4 primers. The amplified product (544 bp) was sequenced and matched against *T. asperellum* extype strain sequences from NCB1 Gene Bank using BLAST, and phylogenetic analysis was performed using MEGA 4 software.

The phylogenetic connections of many of the genus *Trichoderma* members are still unknown, therefore Bissett's (1991) notions of "species aggregation" and "section" have helped explain the location of contradictory species such as *T. harzianum, T. viride*, and *T. atroviride* within the genus. Environmental influences on morphological and physiological traits, on the other hand, have made correct identification extremely challenging (Lieckfeldt *et al.,* 1998). The sequencing of the ITS1 region of the majority of recognised *Trichoderma* species has been studied in this study and prior studies (Rehner and Samuels, 1994). The information supplied in these investigations is more relevant in determining the taxonomy of *Trichoderma*. *T. viride*'s 18S rRNA sequence was used to obtain an accession number (HM 438046.1), and *T. asperellum* 18S rRNA sequence was used to obtain an accession number (HM 438046.1). Using the NEB cutting software, 56 and 44 percent of this species were determined. The 18S rRNA of *T. asperellum* was deposited in Gene Bank http://www.ncbi.nl.1h.gov/ genebank in the current study. Bioformulation of these strains would be more appropriate with the availability of this genomic data (Kannan *et al.,* 2022). The Neighbor – joining approach was also used to create a phylogenetic tree. Based on nucleotide homology and phylogenetic analysis, the present study suggests that *T. viride* culture is closely related to *Trichoderma asperellum* (at a 100% level). *T. asperellum* 18S rRNA (GU 723446.1) had 23 stems, 16 bulging loops, and 11 hairpin loops in their secondary structure. In the fungal isolates, there were a lot of restriction enzyme sites. *T. asperellum*, on the other hand, has 51 unclear cleavage sites and restriction enzyme nature. The content of GC and AT in Thangaraj and Meenupriya (2011) previously reported phylogenetic relationships in four *Aspergillus* species and hypothesized that the ITS1 secondary structure may be used to identify fungi at the genetic level. In this study, a phylogenetic tree was also employed to identify *T. viride* fungal species. The development of agriculture requires the use of microorganisms in the management of phytopathogens as a way to compensate for the use of chemical pesticides, in order to produce healthy crops (Migul *et al.,* 2021).

SUMMARY

Trichoderma asperellum is a fungal species that is extensively utilized in the biological control of plant pathogenic fungus. The creation of analytical enzymes enhances its ability to control biological infections. It has been highlighted that physical characteristics alone are insufficient for identifying these fungal species. *Trichoderma* sp. exhibits antagonistic and biologically control activity, according to various research, with *T. asperellum* inhibiting the pathogenic fungus for the longest time in dual culture technique. *T. asperellum* demonstrated the most inhibition (62.7%), followed by *T. harzianum* (56.0%) and *T. koeningii* (56.0%). *Aspergillus niger* (52.5%), *A. sulphureus* (48.5%), *Penicillium* sp. (48.41), *Aspergillus flavus* (47.3%), *A. fumigatus* (41.6%), and *A. sydowi* (41.55), respectively DNA from a Genomic Sample.

CONCLUSION

Both morphological and molecular approaches are important methods to identify *Trichoderma* isolates. Furthermore, the *Trichoderma* isolate displayed a strong antagonistic activity against plant pathogen. The antagonistic activity suggested that *Trichoderma* sp. can be used as a biological control agent in agriculture. These isolates may further be evaluated in field experiment and the production of commercial fertilizer.

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