Identification of \textit{Trichoderma} BPS-1, a Biological Control Agent against Pea Wilt Pathogen \textit{Fusarium Oxysporum} F.sp. \textit{Pisi}.

V.K. Mishra$^1$, A.K. Passari$^2$ and B.P. Singh$^3$

$^1$Department of Biotechnology, Mizoram University, Tanhril, Aizawl, INDIA.
$^{2,3}$Department of Biotechnology, Mizoram University, Tanhril, Aizawl, INDIA.

Abstract

Screening for biocontrol agent was carried out against the most devastating diseases of pea from different agro-climatic zones of Mizoram. In total, 74 Trichoderma isolates were isolated from different regions of Mizoram and maintained. Dual plate assay was performed against Fusarium oxysporum f. sp. pisi (MTCC-2480) and found out that the Trichoderma BPS-1 was showing the maximum antagonistic activity against the pathogen. Morphological characters and colony growth are similar as other species of Trichoderma, including the newly recognized species \textit{T. asperellum}. Internal transcribed spacer (ITS) region (Approx- 514bp) along with 5.8S gene (ITS1-5.8S-ITS2) of the fungus was compared with known species to determine the phylogenetic relationship of the isolate. The Sequence was completely identical with Trichoderma pseudokiningii. On the basis of results we concluded that Trichoderma BPS-1 was Trichoderma pseudokiningii. This strain can be used as an effective biocontrol agent against pea wilt pathogen Fusarium oxysporum f. sp. pisi and other fungal diseases of important agricultural crops.

Keywords: Trichoderma Spp.; ITS-RFLP; Fusarium oxysporum f.sp. pisi; Biocontrol.

1. Introduction

Phytopathogens play major role in causing diseases to many agriculturally important crops; resulting in loss of plant yield. Fungicides and other pesticides accumulate
hazardous toxic compounds which posses threat to human life and the surrounding environment. Pathogens are also found to develop resistance against several pesticides. In order to tackle the problems caused by chemical pesticides efficient alternatives are being developed (Anand S. and Reddy J., 2009). Biological control was introduced that uses microorganism, which interferes with pathogens and pests of various crops to overcome the problems caused by chemical means of plant protection (Anand S. and Reddy J., 2009). Fungal growth can be controlled by means of organisms which attack or compete with fungi, such as nematodes, other fungi and bacteria. Biological control can limit the instances of plant diseases caused by fungus and bacteria and have specific advantages over synthetic fungicides, including fewer non-target and environmental effects, efficacy against fungicide-resistant pathogens and reduced probability of resistance development (Matroudi et al., 2009).

Among fungi, the most widely used biofungicides are Trichoderma sp. (Mukherjee et al., 2013). India alone is having more than 250 commercial formulations which are being used against many crops for sustainable agriculture (Mukherjee et al., 2013, Singh et al., 2012). Major mechanisms are mycoparasitism, antibiosis, competition through rhizosphere competence and production of cell wall degrading enzymes. Trichoderma sp. is widely abundant in all climates over different geographically abundant regions. Even though most Trichoderma sp. found on wild mushrooms and trees; soil or rhizospheric soil has been viewed as its main habitat (Mukherjee et al., 2013, Druzhinina et al., 2011). They are known as significant decomposers of woody substrates and characterized by assimilation of wide range of substrates, rapid growth rates and their antimicrobial properties.

Wilt caused by Fusarium oxysporum f. sp. pisi is one of the most devastating disease of pea. Pea wilt and root rot alone are responsible for 93% of total yield in India (Sharma et al., 2006). Trichoderma atroviride, Trichoderma harzianum and Trichoderma longibrachiatum were found to have antagonistic effect on Sclerotinia sclerotiorum, the causal agent of canola stem rot (Matroudi et al., 2009). Species of Trichoderma are more resistant to the products of glucose oxidase activity than many plant pathogens (Brunner et al., 2005, Kim et al., 1993). The availability of a versatile expression system for T. atroviride based on application of biocontrol-related promoters (Brunner et al., 2005, Mach et al., 1999, Zeilinger et al, 1999) suggests that it might also be possible to improve this biocontrol agent by developing its disease control effect.

The aim of this study was to use Fusarium oxysporum f.sp. pisi as a model microorganism to test the biocontrol activity of species of Trichoderma with antifungal activity by using dual culture methods and their identification. Identification will be done by amplifying ITS region. These genes are relatively conserved among fungi which give a molecular basis of establishing phylogenetic relationships (Pandey et al., 2003, White et al., 1990). We concluded that Trichoderma pseudokiningii is a potential candidate to develop biocontrol agent against pea wilt pathogen.
2. Materials and Methods
2.1 Isolation and identification of Trichoderma isolates
Total of 74 isolates of Trichoderma spp. were isolated by using fungal specific media from soils collected from different rhizospheric soils from Aizawl, Mizoram. Plant pathogens Fusarium oxysporum (CABI-293942), Fusarium oxysporum f.sp. pisi (MTCC-2480) and Fusarium graminarum (MTCC-1893) were used in this study were obtained from Microbial Type Culture Collection (MTCC) and (CABI-UK). Soil samples were serially diluted and plated on Potato Dextrose Agar (PDA) media containing 30µg each of Chloramphenicol and Streptomycin sulphate to inhibit any bacterial growth. Pure cultures of Trichoderma spp. were obtained by subculturing on PDA medium. The inoculated cultures were incubated for 4-5 days at 28 °C in order to achieve full growth. Initial identification of isolates was done morphologically by observing the color and growth pattern of mycelium in PDA plates. Fungus having white or green color mycelium were expected as Trichoderma isolates and were analyzed microscopically and confirmed by looking on appearance of their hyphal and conidial characteristics.

2.2 Antagonistic effect of Trichoderma spp. by Dual culture technique
Dual culture method of all the isolates was performed as described by Kamala et al, 2011. It implies evaluation of antagonistic effect of Trichoderma spp. against plant pathogens on Potato Dextrose Agar by measuring their respective radial growth after a certain incubation period. Mycelial plug of 5 mm diameter of Trichoderma spp. were placed on PDA plates of about 1 cm each from the corner of the plate. Plant pathogen was placed 4 cm away from the test fungi. Control plates of plant pathogens were also maintained on PDA by placing 5 mm of mycelial plug, without test fungus. The plates were allowed to incubate at 28 °C for 5-6 days. Antagonistic effect of the test fungi was estimated by measuring their radial growth in comparison to the control plates by the following formula (Fokkema 1976) - Antagonistic effect - (A – B/A) x 100 where, A is the diameter of mycelial growth of pathogenic fungus in control and B is the diameter of mycelial growth of pathogenic fungus with Trichoderma isolate. Antagonistic activity by Trichoderma isolates against tested three phytopathogens were analysed.

2.3 DNA extraction
Genomic DNA of the potential isolates was extracted by method described by Lee and Taylor with minor modifications (Yao et al., 1991). Fungi were grown in PDA plates for 3-4 days for isolation of genomic DNA. The crushed mycelia was incubated in lysis buffer (50 mM tris–Hcl, 50mm EDTA, 10% SDS) at 65°C for 90 mins in water bath followed by centrifugation at 12,000 rpm for 15 mins. Supernatant was extracted by adding water saturated phenol followed by centrifugation at 12,000 rpm for 15 mins. The supernatant was treated by adding equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) followed by further centrifugation. This step was repeated twice. 5M sodium acetate and isopropanol was added to the supernatant in order to
precipitate the DNA. DNA was pelleted by centrifugation at 12,000 rpm for 15 mins. Pellets were washed with 70% ethanol followed by centrifugation at 10,000 rpm for 10 mins. Pellets were air dried and re suspended in TE buffer (pH 8.0). RNA contamination was removed by adding 50µg RNase and incubating at 37°C for 60 mins. Quality and quantity of DNA was determined both by spectrophotometrically using Eppendorf Biophotometer Plus.

2.4 Amplification of ITS rDNA gene

Internal transcribed spacer rDNA gene (ITS1-5.8S-ITS2) from the isolates was amplified using the polymerase chain reaction (PCR) with universal primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-GCT-TAT-GTA-TAT-GC 3') developed by White et al., (1990) by the PCR conditions described by Sim et al., 2010 with some modifications. The PCR amplification reactions were performed with a total 25 µl of reaction that comprised of 100 ng of genomic DNA, 2.5 µl of 10x PCR buffer with 25 mM MgCl2, 2 µl of 10 mM dNTPs, 1 unit of Taq polymerase and 10 pmol of each primer. PCR conditions were 5 min at 94°C for initial denaturation, 35 cycles each of 1 min at 94°C for denaturation, 1 min at 54°C for annealing, 1 min 20 secs. at 72°C for extension, and a final extension at 72°C for 5 min in Applied Biosystems GneAmp PCR System 9700 thermal cycler. The amplified PCR products was run by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml), and the PCR product were visualized under a Bio Rad Gel Doc XR+ gel documentation system.

2.5 Sequencing, Molecular identification and Phylogenetic Placement of Trichoderma BPS-1

Trichoderma BPS-1 was found to be the most potent inhibitor of growth of the phytopathogens among all screened isolates. The rDNA region of Trichoderma BPS-1 was sequenced in the Department of Biochemistry, south campus, University of Delhi, New Delhi, India. The sequences were aligned using Clustal W packaged in the MEGA 5 (version 5.05) software (Tamura et al., 2011). A nucleotide BLAST search was performed with the sequence of Trichoderma BPS-1 with the public database. It showed maximum identity of 99% and maximum query coverage with Trichoderma pseudokoningii. A phylogenetic tree was generated by Neighbor joining (NJ) tree with Trichoderma sequences retrieved from NCBI along with sequence of Trichoderma BPS-1 with bootstrap support (5000 replicates) for each particular clade.

3. Conclusions

In total 74 Trichoderma isolates were obtained from soil samples of Dampa Tiger Reserve Forest, Murlen National Park and Phawngpui National Park. All the cultures were grown and maintained on PDA media. Identification of Trichoderma sp. based on morphological features, colony colour and conidial structures under microscope was done. Internal transcribed region of rDNA was amplified using primers ITS1 (5' TCC-
Identification of Trichoderma BPS-1, a Biological Control Agent against Pea GTA-GGT-GAA-CCT-GCG-G 3’) and ITS-4(5’ TCC-TCC-GCT-TAT-TGA-TAT-GC 3’). Amplified product was obtained in the range of 500-600 bp. All the isolates were screened for their antagonistic activity by dual plate method against some important phytopathogens for identification as potential biocontrol agents. Trichoderma BPS-1 showed maximum antagonistic activity against *Fusarium oxysporum* f. sp. *pisi* (MTCC-2480). Based on its ability to act as potential biocontrol agent against pea wilt pathogen *Fusarium oxysporum* f. sp. *pisi*; Internal transcribed spacer (ITS) region of *Trichoderma* BPS-1 was sequenced. Nucleotide BLAST search results of the sequence identifies *Trichoderma* BPS-1 as *Trichoderma pseudokoningii*.

![Fig. 1](image1.png)

**Fig. 1**: Antifungal activity shown by *Trichoderma* BPS-1 (A: Control & B, C, D, E-Test; E-Antagonistic activity shown by *Trichoderma* BPS-1 against *Fusarium oxysporum* and *Fusarium oxysporum* f.sp. *pisi*

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<th>Sl. No.</th>
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A Phylogenetic tree was generated by NJ method by comparing Internal transcribed spacer (ITS) region (approx. 514 bp) along with 5.8S gene (ITS1-5.8S-ITS2) with known species of *Trichoderma* retrieved from NCBI nucleotide database to determine the phylogenetic relationship of the isolate. *Fusarium oxysporum* was taken as outgroup to reveal the situation at the root.

**Fig. 2:** Inhibition effect of *Trichoderma* sp. on some phytopathogens.

**Fig. 3:** Phylogenetic placement of *Trichoderma* BPS-1.
It was found that Trichoderma BPS-1 clustered with Trichoderma pseudokoningii which was in accordance with the BLAST results. This led us to identify the isolate Trichoderma BPS-1 as Trichoderma pseudokoningii having potent antagonistic activity against pea wilt pathogen Fusarium oxysporum f. sp. pisi which can further be used as an effective biocontrol agent against pea wilt. This strain can therefore be used for assessment of field biocontrol against Fusarium oxysporum f. sp. pisi.

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References


