Trichoderma asperellum, Identified as a Novel Fungal Biocontrol Agent for the Control of Plant Pathogen

Thilagavathi Rasu, Nakkeeran Sevugapperumal, Raguchander Thiruvengadam and Samiyappan Ramasamy

Abstract—Twenty isolates of biocontrol Trichoderma species were isolated from rhizosphere soils of different host plants in various locations of Tamil Nadu, India. Of which, TTH1 exhibited better inhibition (64.4%) of mycelial growth of the pathogen, S. rolfsii followed by TV 1 (60%) and TED 1 (60%) under in vitro conditions. These three isolates were further tested for their chitinase activity on agarose gel amended with chitin. TTH 1 produced more integrated dense value (IDV) of 105015 on agarose gel followed by TV 1 (96385) and TED 1 (77487) isolates. It indicated that TTH 1 produced more chitinase to degrade the cell wall of pathogen while tested in dual plate technique. The ITS1 to ITS2 region of TTH 1 isolate was amplified and compared with nucleotide sequences from the GenBank database after sequencing. UPGMA tree was constructed using CULSTAL X 1.81 for their similarity. Finally it was confirmed as Trichoderma asperellum.

Keywords—Anatagonistic potential, chitinase, plant pathogen, Trichoderma asperellum Trichoderma isolates

I. INTRODUCTION

TRICODERMA is a genus of fungi present in all soils. Many species in this genus have been developed as biocontrol agents against several plant pathogenic fungi. The genus has attracted considerable scientific attention and gained immense importance since last few decades due to its biological control ability [6]. Superior biocontrol potential may then be found in strains having a high capacity to produce cell wall degrading enzymes [7]. Production of chitinase enzyme by Trichoderma spp. [5]-[12] is one among broad spectrum mode of action against plant pathogens, which act on cell walls of plant pathogens [11]. Inhibition of plant pathogenic fungai by different species of Trichoderma was studied under in vitro conditions by many researchers [1]-[3]-[10]-[15]. The present study was proposed with an objective to collect Trichoderma isolates from different host plants in different locations of Tamil Nadu, India and to identify potential biocontrol Trichoderma sp. based on in vitro evaluation and chitinase production for control of plant pathogenic fungi, S. rolfsii.

II. MATERIALS AND METHODS

A. Isolation of Fungal Biocontrol Agents

Fungal biocontrol agents belonging to the genus Trichoderma sp. were isolated from rhizosphere soil of different crops including sugarbeet, banana, sunflower, groundnut, blackgram, cotton, brinjal, tomato, jasmine, jatropha, tapioca etc. grown in different locations of Tamil Nadu. From each crop, the soil particles tightly adhered with root surface were removed separately and suspended in 10ml of sterile distilled water. After serial dilution, one ml suspension from 10⁶ dilution was transferred to sterile Petri dishes containing Trichoderma specific medium (TSM). Observations were taken after five days for the presence of Trichoderma sp. colonies.

B. In vitro Screening

Different isolates of Trichoderma sp. were tested against a devasting soil borne plant pathogen, S. rolfsii under in vitro conditions using dual culture technique [2]. Nine mm disc of Trichoderma sp. from three days old culture was placed five mm away from the periphery of the PDA plate and opposite to the culture disc of S. rolfsii. Control plate was maintained with S. rolfsii alone and incubated at room temperature (27±2°C). Four replications were maintained. Observations were taken after the S. rolfsii reached full growth in the control plate. The radial mycelial growth of the S. rolfsii and per cent reduction over control was calculated by using the formula, Per cent inhibition over control

\[ \text{Percentage Inhibition} = \frac{(C-T)}{C} \times 100 \]  

(1)

Where, C- mycelial growth of S. rolfsii in control; T- mycelial growth of S. rolfsii in dual plate.

C. Chitinase Production

The Trichoderma sp. isolates (TV 1, TTH 1 and TED 1), performed better against S. rolfsii under in vitro conditions.
were further tested for their chitinase (cell wall degrading enzyme) production. Mycelial discs from actively growing cultures of Trichoderma sp. isolates were inoculated into PD broth and incubated for 48 h at 22°C in a rotary shaker at 200 rpm.

The mycelium was recovered by filtration through Whatman filter paper, resuspended in CZ medium supplemented with the crabshell chitin (0.5% w/v) and further incubated for 48 h. The cultures were filtered through Whatman No.1 filter paper. The culture filtrates were used for chitinase assay [8]. A one per cent (w/v) agarose solution was prepared in sodium phosphate (0.01 M; pH 5.5) and heated to boiling point. One milliliter of a 1% glycol chitin solution was added to 100 ml of the agarose solution. The resulting suspension was stirred to ensure homogenous distribution of the substrate, and 30 ml aliquots were poured into polypropylene Petri dishes (9 cm dia). The agarose was allowed to cool and solidified for 20 to 25 min. Small wells (3 mm dia) were carved in the agarose gels at a distance of 1.5 cm from each other to form a grid. The agarose plugs were removed by needle. Five microliter samples of the enzyme solution were loaded into each well. The plate was incubated for 2 h at 37°C by floating Petri dishes in a water bath. After incubation, 50 ml of 0.5 M solution of Tris-HCl (pH 8.9) with 0.01% calcoflour brightener 28 was added to the plate and incubated for 10 min to stop the reaction and stain the plate. The gel was rinsed twice and flooded with distilled water followed by overnight color development in the dark. Then observed under UV light for the presence of lytic zones around the wells. The area of activity was calculated using software present in the gel documentation system [13].

D. Identification of Trichoderma asperellum

The best performed TTH 1 isolate was observed under microscope for its morphological characters. Genomic DNA of TTH 1 isolate was isolated by following CTAB DNA extraction procedure [16]. Universal PCR primers (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4, 5'-TCC TCT TAT TGA TTA TAT GC-3') were used for amplification of the ITS region between the small and large ribosomal subunits. The amplified PCR products were separated by electrophoresis on 1.5% agarose gel and stained with the ethidium bromide and documented in AlphaImager documentation and analysis system (Alpha Innotech Corporation, San Leandro, California).

The amplified PCR product of approximately 560 bp was loaded in the 1% agarose gel. After electrophoresis, the gel with PCR fragment (560 bp) was excised under the UV trans illuminator (Medox Biotech India Pvt. Ltd., India) with the help of sterile surgical blade. Then they were purified with the QIAQuick gel extraction kit (Qiagen, Inc., Chatsworth, California). Finally, purified PCR product was checked on 1.5% agarose gel and then subjected to sequencing. DNA sequencing was performed at Bioserve biotech Pvt. Ltd., Hyderabad. The sequences for entire PCR products, approximately 560 bp in length was determined by using direct double pass sequencing using gene specific primers. Sequence was determined by blasting the sequence with the available Genbank resources using NCBI-BLAST search.

III. RESULTS AND DISCUSSION

Twenty isolates of Trichoderma collected from rhizosphere soil of different host plants were evaluated for their antagonistic potential against the plant pathogen S. rolfsii. The result showed that isolate TTH 1 exhibited better inhibition of mycelial growth of the pathogen (64.4%) followed by TV 1 (60%), TED 1 (60%). Other isolates were not effective in inhibiting mycelial growth of the pathogen (Table 1). Most Trichoderma strains produce volatile and nonvolatile toxic metabolites that impede colonization by antagonized microorganisms; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoalactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described by Vey [14]. Metabolites of Trichoderma sp. inhibited the growth of Phytophthora erythrosepti [3] and Thielaviopsis paradoxa [10] on PDA. Inhibition of pathogenic growth by the strains of Trichoderma sp. through production of antifungal antibiotics and or hydrolytic enzymes have been reported [9]-[15]. In the present study, chitinase (hydrolytic or cell wall degrading enzyme) production was measured for the effective Trichoderma isolates. Three isolates selected from in vitro evaluation were tested on agarose gel amended with chitin for their chitinase activity. The result showed that TTH 1 isolate produced more integrated dense value (IDV) of 105015 on the agarose gel followed by TV 1 (96385) and TED 1 (77487) isolates. It indicated that TTH 1 isolate produced more chitinase enzyme to degrade the cell wall of pathogen (Table 1). Chitinases produced by Trichoderma sp. inhibited the growth of S. rolfsii and thereby easily penetrate the mycelium as discussed by Gurnede [5] and Szekeres [12]. Buragohain [1] investigated the inhibiting effect of culture filtrate of Trichoderma sp. on S. rolfsii. In the present study, microscopic observation of TTH 1 isolate showed characteristic conidia, conidiophores and chlamydospores of the genus Trichoderma (Fig. 1). The genomic DNA of TTH 1 isolate was subjected to PCR using universal primers to
amplify the ITS1 and ITS2 regions between the small and large nuclear rDNA, including the 5.8S rDNA. These primers amplified a DNA fragment of about 560bp (Fig. 2) and it was sequenced using double pass reaction and compared with nucleotide sequences from the GenBank database. Finally it was confirmed as *Trichoderma asperellum* and submitted to National Centre for Biotechnology Information (NCBI), GenBank, New York, USA with the accession number of GQ913985. UPGMA tree was constructed using CULSTAL X 1.81 for the already existing strains of *T. asperellum* from GenBank database along with TTH1 for their similarity (Fig 3). The present study clearly indicates the potential of *T. asperellum* in inhibiting the mycelial growth of pathogen and production of cell wall degrading enzyme. Hence, *T. asperellum* would be a superior biocontrol agent for the control of plant pathogens. The work has to be intensified to study other mechanisms involved in the inhibition of pathogen growth under *in vitro* conditions.

Table I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Per cent Inhibition of <em>S. rolfsii</em> over control</th>
<th>Mean IDV on agarose gel for chitinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV1</td>
<td>60.0% (50.8)</td>
<td>96385&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TTH1</td>
<td>64.4% (53.4)</td>
<td>105015&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TEO1</td>
<td>60.0% (50.8)</td>
<td>77487&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSBO1</td>
<td>58.1% (49.7)</td>
<td>-</td>
</tr>
<tr>
<td>TSBO2</td>
<td>54.4% (47.5)</td>
<td>-</td>
</tr>
<tr>
<td>TSBO3</td>
<td>45.9% (42.6)</td>
<td>-</td>
</tr>
<tr>
<td>TSFCE1</td>
<td>39.2% (38.8)</td>
<td>-</td>
</tr>
<tr>
<td>TED 2</td>
<td>28.1% (32.0)</td>
<td>-</td>
</tr>
<tr>
<td>TED 3</td>
<td>34.1% (35.8)</td>
<td>-</td>
</tr>
<tr>
<td>TDH 1</td>
<td>58.1% (49.7)</td>
<td>-</td>
</tr>
<tr>
<td>TCOO1</td>
<td>58.1% (49.7)</td>
<td>-</td>
</tr>
<tr>
<td>TTNJ1</td>
<td>54.4% (47.5)</td>
<td>-</td>
</tr>
<tr>
<td>Other isolates</td>
<td>No inhibition</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are mean of four replications. Means followed by a common letter are not significantly different at 5% level by DMRT. Data in parentheses are arcsine transformed values. IDV – Integrated Dense Value.

**REFERENCES**


