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**SHORT COMMUNICATION**

# Identification of *Trichoderma asperellum* from selected fruit plantations of Sri Lanka

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**Abstract:** Three isolates of *Trichoderma asperellum* (TR1, TR2, and TP3) isolated from soil samples collected from rambutan (*Nephelium lappaceum*) and pineapple (*Ananas comosus*) plantations in Sri Lanka had similar characteristics with respect to growth at different temperature, pH and media. The three isolates are reported to be antagonistic to fungal pathogens of pineapple (i.e. *Thielaviopsis paradoxa*) and rambutan (i.e. *Colletotrichum gloeosporioides*). Analysis of genome DNA of the isolates showed that the isolated fungi were *T. asperellum*. This is the first report of the occurrence of *T. asperellum* in Sri Lanka.

**Keywords:** Biocontrol agent, fungi, *Trichoderma asperellum.*

## INTRODUCTION

Different species of *Trichoderma* have been reported as potential biocontrol agents to controll soil borne plant pathogens more effectively than chemicals[1-3]. Use of these fungi is not harmful to the environment as compared to chemical pesticides[5]. *Trichoderma* spp. are present in substantial quantity in nearly all agricultural soils and in other environments such as decaying wood. Their use has now been recognized the world over as an alternative to chemical fungicide to control plant diseases[5,6]. *Trichoderma* spp. have been used as biocontrol agents of pathogenic fungi of *Sclerotinia sclerotioum*[7], *Verticillium* spp., *Rhizoctonia solani* [8], *Fusarium oxysporum*[9,10], *Colletotrichum gloeosporoides*[11], and *Thielaviopsis paradoxa*[12] under greenhouse and field conditions.

Taxonomy of *Trichoderma* is based on morphological characteristics such as form, size, colour and ornamentation of conidia, branching patterns and short inflated phialides. Conventional methods for detecting *Trichoderma*, which employ the plate enrichment technique, do not always satisfactorily discriminate between *Trichoderma* species. Furthermore, culture techniques are labour intensive and require considerable taxonomic expertise. In contrast, molecular techniques such as PCR and DNA sequencing are very sensitive, reliable and rapid methods for species detection[13].

There is very little or no information on the occurrence of *Trichoderma* spp. in Sri Lanka. The present study was undertaken to identify the taxonomy of three *Trichoderma* isolates (TR1, TR2, and TP3) obtained from soil samples collected from pineapple and rambutan plantations in Sri Lanka. All three isolates exhibited significant antagonistic activity against common fungal pathogens of pineapple (*Ananas comosus*) and rambutan (*Nephelium lappaceum*) [12].

## METHODS AND MATERIALS

*Trichoderma* obtained from the soil of a rambutan plantation in Gampaha and a pineapple plantation in Avissawella, was used in this investigation. Fifty composite soil samples were collected into sterile polypropylene bags from 25 different locations in each orchard. The collected soil samples were brought to the laboratory within 2 h.

The isolates were TR1, TR2 (from rambutan plantation) and TP3 (from pineapple plantation). The isolates were obtained using the soil dilution plate method and maintained on potato dextrose agar during the period of study. As solid media-potato dextrose agar (PDA), Czapek-Dox agar (CZA), plate count agar (PCA) and yeast and mould agar (YMA) were used. PDA, CZA and PCA were prepared according to the Difco manual[14].

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YMA was made from 15 g agar, 20 g Dextrose, 5 g Yeast extract and 0.1 g Chloromphenicol in 1L of distilled water. Buffered CZ broth was prepared according to the procedure described previously[15].

The effect of *Trichoderma* isolates on the pineapple black rot pathogen and rambutan anthracnose pathogen was tested using *in-vitro* bioassay[12]. Four wells of diameter 4 mm were cut equidistant from each other on the outer area of plate, each having 15 mL PDA. Each well was filled separately with 75 µL of $10^7$ conidia/mL suspension of *Trichoderma* isolates (TR1, TR2 and TP3) prepared as described above. A mycelial disc (4 mm diameter) of *Th. paradoxa* or *C. gloeosporioides* obtained from the periphery of a 7 d old culture was placed at the centre of each plate prior to incubation at 28 ± 2 ºC for 9 d. The growth of *Th. paradoxa* or *C. gloeosporioides* was assessed by measuring the colony diameter. In the control, sterile distilled water was used instead of the conidial suspension. The assay was repeated three times for each pathogen with 9 replicate plates per treatment.

A mycelial disc of 4 mm diameter obtained from a 7 d old culture of the test *Trichoderma* isolate on PDA was placed at the centre of the test medium. All plates were incubated at 28 ± 2 °C and growth was assessed by measuring the colony radius along two perpendicular axes 48, 72, 120, 144, 166, and 196 h after inoculation as in a previous study[11]. The experiment was repeated twice with 4 replications.

The isolates grown on 100 mL CZ-broth was dispersed in 250 mL *Erlenmeyer* flask, buffered with citrate phosphate buffer at pH values 3.5, 4.0, 4.5 and 5.0. Each flask was inoculated with a 4 mm mycelial disc from 7 d old culture of *Trichoderma* isolates (TR1,TR2, TP3) and incubated at 28 ± 2 °C. To assess the growth, cultures were harvested at 48 h intervals by filtering through sterile Whatman No.1 filter paper and the residual mycelium was dried to a constant weight at 80 °C in a hot air oven for 6-8 h. The weight was determined as described in previous studies[6,15]. The experiment was repeated twice with 4 replications.

PDA plates were inoculated as described above and incubated at temperatures of 4, 12, 16, 20, 23, 25, 28 and 30 °C. Growth was assessed by measuring the colony radius along two axes at right angles[15].

Genomic DNA was extracted using the method of Plaza *et al.*[16] with minor modifications. The mycelium of *Trichoderma* isolates (TR1, TR2, and TP3) was directly collected from 7 d old culture plates and 200-500 mg of mycelium material was added to 1.5 mL microcentrifuge tubes. Sterile white quartz sand was added to the sample at a ratio of 3 g of sand per 1 g of tissue. Sufficient extraction buffer [100 mM Tris-HCl (pH 8.0), 20 mM $Na_2EDTA$, 0.5 M NaCl and 1% sodium dodecylsulfate] was added to the eppendorf tube. A mixture of buffer saturated with phenol, chloroform and isoamyl alcohol was added at a ratio of 0.5 mL per 1g of tissue. The mixture was ground vigorously for 30 s with a pestle to form a thick paste. Then 2 mL of the extraction buffer and 1 mL of buffered phenol/isoamyl alcohol was added per 0.5 g of starting tissue and the solution was mixed thoroughly. The mixture was transferred to an eppendorf tube and capped and centrifuged (MicroSPin 24S Sorvall) at 16,000 rpm for 5 min at room temperature. The supernatant was taken out and mixed with 0.6 mL of isopropanol, incubated for 10 min at room temperature and in ice for 5 min and centrifuged for 20 min at 16,000 rpm (MicroSPin 24S Sorvall) to obtain a pellet. The pellet was rinsed with 95% ethanol and centrifuged for 3 min (MicroSPin 24S Sorvall) at 10,000 rpm. The pellet was air-dried and was resuspended in 340 µL of TE [10 mM Tris-HCl, 1 mM $Na_2EDTA$ (pH 8.0)] containing ribonuclease A at 20 µg/mL. The samples were incubated at 37 °C for 30 min and extracted with 0.3 mL of phenol, chloroform and isoamyl alcohol followed by centrifugation at 5000 rpm for 5 min. The resulting aqueous phase was mixed with half volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol and mixed well. The sample was incubated at -20 °C for 30 min and centrifuged for 20 min at 10,000 rpm. The resulting DNA pellet was rinsed with 95% ethyl alchohol, air-dried and resuspended with 100 µL of TE. Extracted DNA was stored in 100 µL Tris EDTA buffer at -20 °C.

Primers ITS1 and ITS4 were used to amplify fragments of the ribosomal DNA (rDNA), including the 5.8S gene and the flanking intergenic transcribed spacers ITS1 and ITS2. DNA amplification was performed in a total volume of 25 µL by mixing 5 µL of the template DNA with 10 µM concentrations of each forward and reverse primer, 10 mM concentrations of each dNTPs, and 2 units of *Taq* DNA polymerase (Promega) and 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl). These reactions were subjected to an initial denaturation of 5 min at 94 °C, followed by 35 cycles of 30 at 94 °C, 1 min at 52 °C and 2 min at 72 °C, with a final extension of 7 min at 72 °C in a thermal cycler (RoboCycler GRADIENT 40).

PCR Primers[17]:
ITS 1 (Forward): 5''- TCC GTA GGT GAA CCT GCG G- 3''
ITS 4 (Reverse): 5''- TCC TCC GCT TAT TGA TAT GC- 3''
The amplified PCR products were visualized on a 2% agarose gel (w/v) in 0.5 X TBE containing ethidium bromide by UV transillumination. The band size observed

was approximately 570-600 bp[18], which was estimated by using an appropriate DNA molecular marker.

Data analysis of growth parameters was done by calculating the standard errors of the mean values of the results. The sequence obtained was blasted against the sequences that was reported in the Gene Bank so far and compared. For further confirmation the sequence was aligned with the Gene Bank deposited sequences using the CLUSTAL-W software package.

## RESULTS AND DISCUSSION

All three isolates of *Trichoderma* grew rapidly on PDA forming a smooth-surfaced, watery white mycelia mat which soon became whitish green and then dull green with the production of conidia. The isolates possessed repeatedly branched conidiophores. The size of conidia ranged from 2.8-3.5 µm and they were green in colour, smooth walled with sub-globe shape.

In the well diffusion assay, clear inhibition zones were seen initially in all three isolates against both *Th. paradoxa* and *C. gloeosporioides* respectively. Eventually, the *Trichoderma* isolates came into contact with *Th. paradoxa* and *C. gloeosporioides* on the third day after inoculation and thereafter *Trichoderma* started to overgrow the pathogen. The plates were covered exclusively by *Trichoderma* on the seventh day after the inoculation and it was not possible to re-isolate the pathogen from the assay plates. There were no differences in the three *Trichoderma* isolates on the ability to inhibit the growth of *Th. paradoxa* and *C. gloeosporioides* respectively (Figure 1 & 2 ).

The growth rate of the three isolates (TR1, TR2 and TP3) did not differ significantly. All isolates showed a significantly higher growth rate on PDA (Figure 3). The growth was optimum at 28 °C for all three isolates (TR1, TR2 and TP3). There was a rapid decrease in growth below 20 °C and above 30 °C in all three isolates in all media.

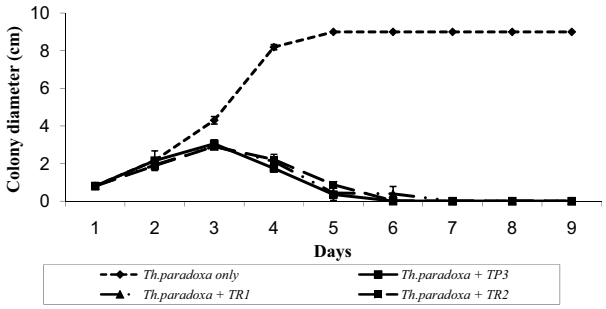



**Figure 1:** The antagonistic effect of *Trichoderma* isolates spore suspension on the radial mycelial growth of *Th. Paradoxa.* Values are the means of nine replicates. Vertical bars indicate standard errors of the mean.

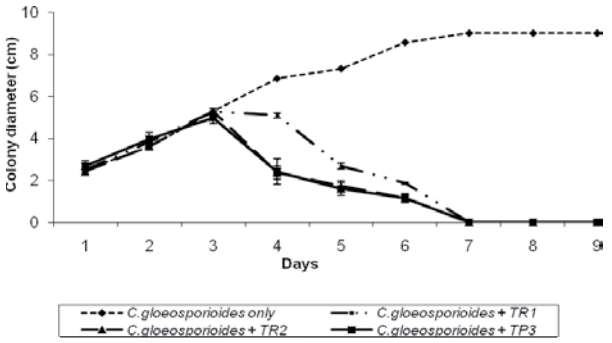



**Figure 2:** The antagonistic effect of *Trichoderma* isolates spore suspension on the radial mycelial growth of *C. gloeosporioides*. Values are the means of nine replicates. Vertical bars indicate standard errors of the mean.

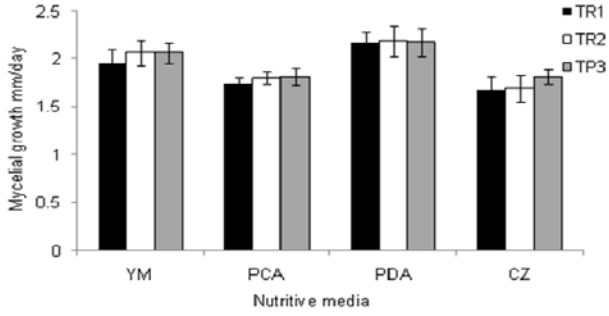



**Figure 3:** Growth of *Trichoderma* isolates (TR1, TR2 and TP3) in different media. Values are the means of four replicates. Vertical bars indicate standard errors of the mean.

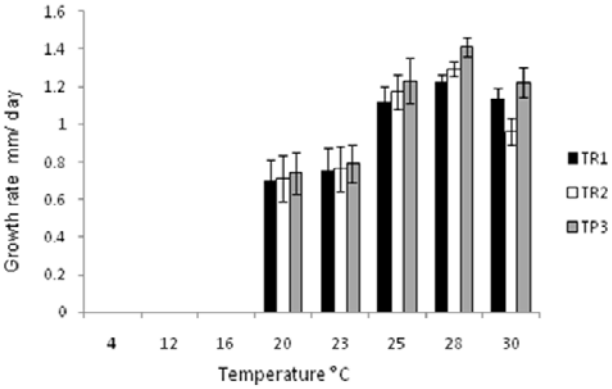



**Figure 4:** Growth rate of *Trichoderma* (TR1, TR2 and TP3) isolates at different tempretures. Values are the means of four replicates. Vertical bars indicate standard errors of the mean.

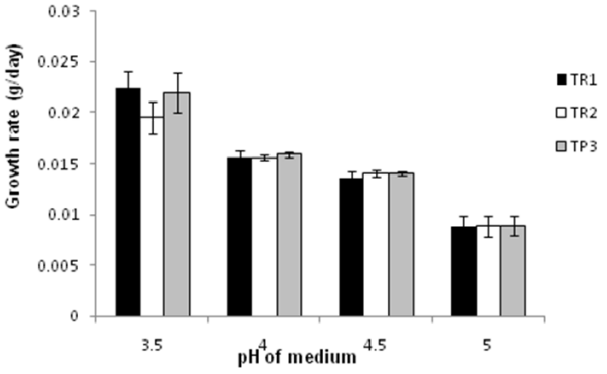


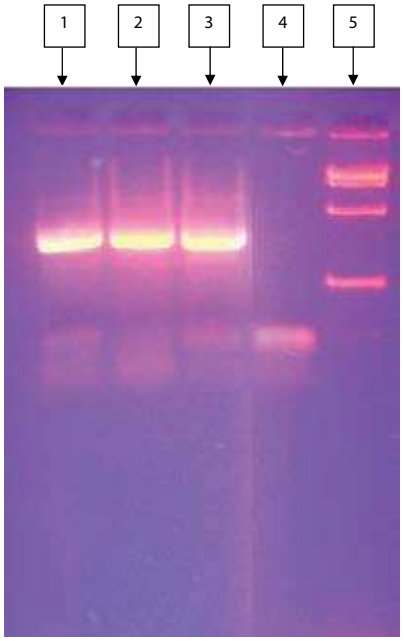

**Figure 5:** Growth rate of *Trichoderma* isolates (TR1, TR2 and TP3) in Czapek-Dox liquid medium buffered at different pH values. Values are the means of four replicates. Vertical bars indicate standard errors of the mean.

There were no significant differences in growth rates of all three isolates at different temperatures (Figure 4). All isolates showed maximum growth at pH 3.5 and the growth of isolate TR2 was significantly different from the other two isolates (TR1 and TP3) (Figure5).

Genomic DNA of all three isolates of *Trichoderma* were analyzed by PCR amplification of rDNA gene including 5.8S gene and the flanking intergenic transcribed spacers ITS1 and ITS2. All three isolates produced PCR products of similar size for the r RNA region (Figure 6).

Each sequence after editing was submitted to the Gene Bank and homology searches done against all the published *Trichoderma* sequences using BlastN and fastA programmes [National Centre for Biotechnology Information (NCBI), USA]. Basic Local Alignment search Tool (BLAST) search results of each sequence giving the closest match to the test sample was used to determine the species of *Trichoderma* isolates. For further reconfirmation and phylogenetic analysis of the strains, sequences were aligned using CLUSTAL-W (Multiple Sequence Alignment) software tool. According to the NCBI BLAST search against the Gene Bank sequence database, isolates were designated as *T. asperellum.*

The reliance on morphology or cultural characteristics for species determination and identification of fungi is difficult as the characteristics of isolates can change widely under varying environmental conditions[19]. In fungal genomes, ribosomal DNA (rDNA) genes include the 18S, 5.8S and 28S segments that code for ribosomal RNAs (rRNA). These are highly conserved genes that are separated by two less conserved regions, the internal transcribed spacers 1 and 2 (ITS1 and ITS2). ITS sequences generally vary among different species, and are used widely as informative regions for PCR assays. These ITS regions have several advantages for sequencing and phylogenetic analysis of fungal species. The rate of change is appropriate for studies at the species and genus levels, the alignment of the sequences is relatively simple and results can be interpreted phylogenetically. These regions are large enough to provide potential characteristics for phylogenetic reconstruction. Further, these ITSs are flanked by regions that are highly conserved within genera and species. PCR amplification and sequencing are much easier than for other parts of genomic DNA. According to the best of our knowledge this is the first report of isolation of *T. asperellum* from Sri Lanka.




1 – TR1, 2 - TR2, 3 – TP3, 4 – Negative control,
5 – Molecular marker
(100 bp ladder. Starting at 500bp.)

**Figure 6:** Agarose gel analysis of the PCR products of the rDNA region of *Trichoderma* isolates by ITS-1 and ITS-4 primers. The band size observed is approximately 570-600 bp.

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