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Isolation and characterization of *Trichoderma erinaceum* for antagonistic activity against plant pathogenic fungi.

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Abstract

Trichoderma is reported to be one of the most widely distributed soil fungi and the biocontrol potential of *Trichoderma* has been studied against a wide range of plant pathogenic fungi. In the present study *T. erinaceum* was screened for antifungal activity against seven selected plant pathogenic fungi and for chitinase and glucanase production. In antifungal assays highest mean percent inhibition was observed against the pathogenic fungus *Rhizoctonia solani* (72.66 % ±7.6). Molecular characterization of rDNA of ITS region was made for identification and it was identified as *Trichoderma erinaceum.*

Keywords – biocontrol – chitinase – glucanase – ITS

Introduction

Trichoderma species commonly found in the soil ecosystems have gained immense importance in the last few decades due to its ability to control several plant pathogens (Chakraborty et al. 2010). Use of *Trichoderma* to control plant diseases is not harmful to the environment unlike chemical pesticides (Perveen & Bokhari 2012, Reena et al. 2013). *Trichoderma* strains have also been recognized for their ability to increase root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients (Poovendran et al. 2011). Several *Trichoderma* species are used as biocontrol agents against plant pathogenic fungi such as *Sclerotinia*, *Verticillium*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides* and *Thielaviopsis paradoxa* under both greenhouse and field conditions (Wijesinghe et al. 2010). *Trichoderma* is used in different crops like rice, wheat, pulses, vegetables, coconut, black pepper, cardamom, ginger, banana, sugarcane, sunflower, groundnut, soybean, cotton, castor and tobacco against a wide range of plant pathogens (Sriram et al. 2013). Therefore it is important to investigate the diversity of *Trichoderma* in the soil since such information can lead to the isolation of *Trichoderma* species having higher antagonistic efficiency and development of better biological control methods to manage plant pathogenic fungi. *Trichoderma*, reduce growth, survival or

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infections caused by pathogens through different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion especially extra cellular lytic enzymes such as chitinase and glucanases (Poovendran et al. 2011, Kumar et al. 2012). The present study was undertaken to determine the antifungal activity of *T. erinaceum* against seven selected plant pathogenic fungi and to determine its chitinase and glucanase production.

Materials & methods

The isolate

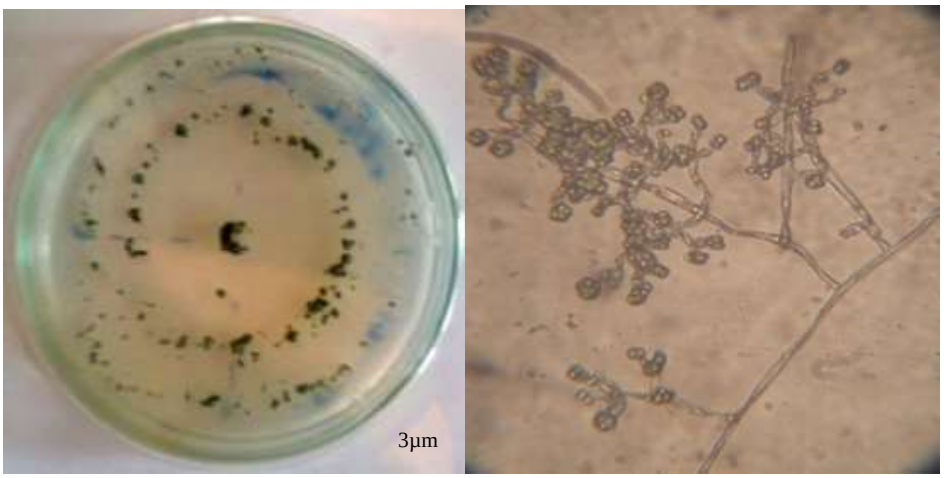
The fungal isolate used in the present study was isolated from the soil of a rubber plantation in Matugama, Sri Lanka using the soil dilution plate method with chitin selective medium. Chitin selective medium was made from 5 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$ 1, 0.3 g/L $MgSO_4.7H_2O$, 1.36g/L KH_2PO_4, 20 g/L agar with 1.5 % (w/v) colloidal chitin as the main carbon source (Susana 2006) and was characterized further.

Identification of the isolate

The microscopic examination of the isolate was done through Lacto-phenol Cotton Blue staining technique. Further, the molecular characterization was done using DNA sequencing and phylogenetic analysis. Genotypic identification was carried out by PCR amplification and sequencing of ITS region for the strengthening the morphological identification. The rDNA sequence of ITS region using universal primers; ITS 1(5''- TCC GTA GGT GAA CCT GCG G- 3'') and ITS 4 (5''- TCC TCC GCT TAT TGA TAT GC- 3'') primers (Chakraborty et al. 2010) were used to amplify a ~ 600 bp fragment of the ribosomal DNA (rDNA), including the 5.8S gene and the flanking intergenic transcribed spacers ITS1 and ITS2. The purified amplicon was bidirectionally sequenced using ITS1 and ITS4 primers. The resultant sequence was edited using Bio Edit version 7.2.0 and was subjected to BLAST search analysis at NCBI. The DNA sequence was submitted to GenBank under the accession number KJ381061. A comparative study of rDNA sequences of isolate with other rDNA sequence of *Trichoderma sp.* was done using BLAST algorithm at the website http://www.ncbi.nlm.nih.gov. Evolutionary analyses were conducted using PhylML (Posada 2008). Total 24 closely related *Trichoderma* sp with *T. erinaceum* were taken in to account for preparation of phylogeny tree by the method of maximum likelyhood. PhylML was used for ML analyses. Tree topologies were evaluated by performing bootstrap analysis of 100 data sets. jModeltest (Rannala & Yang 1996) was used to select the models of nucleotide substitution for ML analysis. The number of substitution schemes was set to 11, base frequencies + F, rate variation + I and + G, and the base tree for likelihood calculations was set to ML OPTIMIZED. A total of 88 models were compared. Once the likelihood scores were calculated the models were selected according to the Akaike information criterion (AIC), Bayesian information criterion (BIC) and Decision Theory performance based selection (DT). Trees for best BIC and DT models were selected for the analysis.

Antifungal activity in solid cultures

To determine the antifungal activity of *T. erinaceum*, it was tested on seven selected plant pathogenic fungi using the dual culture method (Matroudi et al. 2009, John et al. 2010). Pure cultures of plant pathogenic fungi *Fusarium oxysporum*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Corynespora cassiicola*, *Rigidoporus microporus* and *Phytophthora meadii* were obtained from the Department of Plant Sciences, University of Colombo. PDA plates were inoculated by placing a 9 mm diameter mycelial disc of the pathogenic fungus on one side obtained from a 4 day old culture on PDA. A similar disc of the *T.* erinaceum obtained from the growing edge of 4 day old culture on PDA was placed on the opposite side of the pathogenic fungus. The plates were incubated for 3 days at room temperature (28 ± 2^{o}C). The contact zones of the two colonies were observed under the light microscope for any interactions between the two fungi and radial mycelia growth of the test pathogen was determined by measuring



(A) (B)

Fig. 1 – A, Colony appearance of *T. erinaceum* grown for 5 days at 28°C on CDA; B Conidiophores and conidia of *T. erinceum*.

the radial growth. Percent inhibition (PI) was calculated as; PI (%) = (γ0-γ/ γ0) x 100 where, γ0 is the radial growth of test pathogen in the control plate and γ is the radial growth of test pathogen in test plate. All tests were carried out with three replications against seven pathogenic fungi. The data obtained were statistically analyzed using MINITAB 14. One way ANOVA and Tukey test were performed (95% simultaneous confidence intervals). For controls pathogenic fungi were inoculated separately on a one side of the petri plates containing PDA media.

Enzyme assay

To determine chitinase and β-1, 3-glucanase activities of the isolate specific liquid media were used. To prepare 1 liter of chitin liquid medium, 15 g of colloidal chitin, 5 g of yeast extract, 1 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4.7H_20$, 1.36 g of KH_2P0_4 were mixed and volumed up to 1 liter with distilled water (Susana 2006). The glucan liquid medium having 5 g of β-glucan prepared using dry yeast cells according to the method described by (Zechner-Krpan et al. 2010), 1 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4.7H_20$, 0.8 g of KH_2P0_4, 0.2 g of KNO_3 were mixed and also volumed up to 1 liter with distilled water (Susana 2006). The pH of the both was adjusted to 5.5. 25 ml of the relevant liquid medium was added to 100 ml conical flasks. The liquid media in flasks were inoculated with a 9 mm diameter mycelial disc obtained from the growing edge of a 4 day old culture of the isolate. The inoculated flasks were incubated on a rotary shaker at 120 rpm at room temperature. The cultures were harvested at 24 hours intervals by filtration through whatman no 1 filter paper. Resulting filtrates were stored at 4^0C and was used to determine enzyme activity. Activities of enzymes were determined by dinitro salicylic acid (DNS) assay (EL-Katatny et al. 2000). For chitinase assay the reaction mixture contained 500 µl of 1% (w/v) colloidal chitin in sodium acetate buffer (pH 5.5) and 1 ml of culture filtrate. The mixture was incubated at 45^0C for 30 min and in a water bath at 90^0C for 10 min. Then the mixture was centrifuged at 13,000 rpm for 10 min. To the supernatant 1 ml of DNS reagent was added followed by 300 µl of potassium sodium tartrate. Thereafter the mixture was heated in a boiling water bath for 5 min and after cooled to room temperature the absorbance was recorded using a spectrophotometer at 540 nm. The enzyme blank was distilled water and the control was uninoculated liquid medium. Glucanase was also assayed as above by incubating 1 ml of 2.5% β-glucan in sodium acetate buffer (pH 5.5) with 200 µl of enzyme solution. All other steps followed were exactly as for chitinase assay. The amount of reducing sugars released was calculated from standard curves for glucose and the activities of chtinase and glucanase are expressed in pkat (pmol/s). All experiments were carried out with three replications. The data obtained were statistically analyzed using MINITAB 14. One way ANOVA and Tukey test were performed (95% simultaneous confidence intervals).

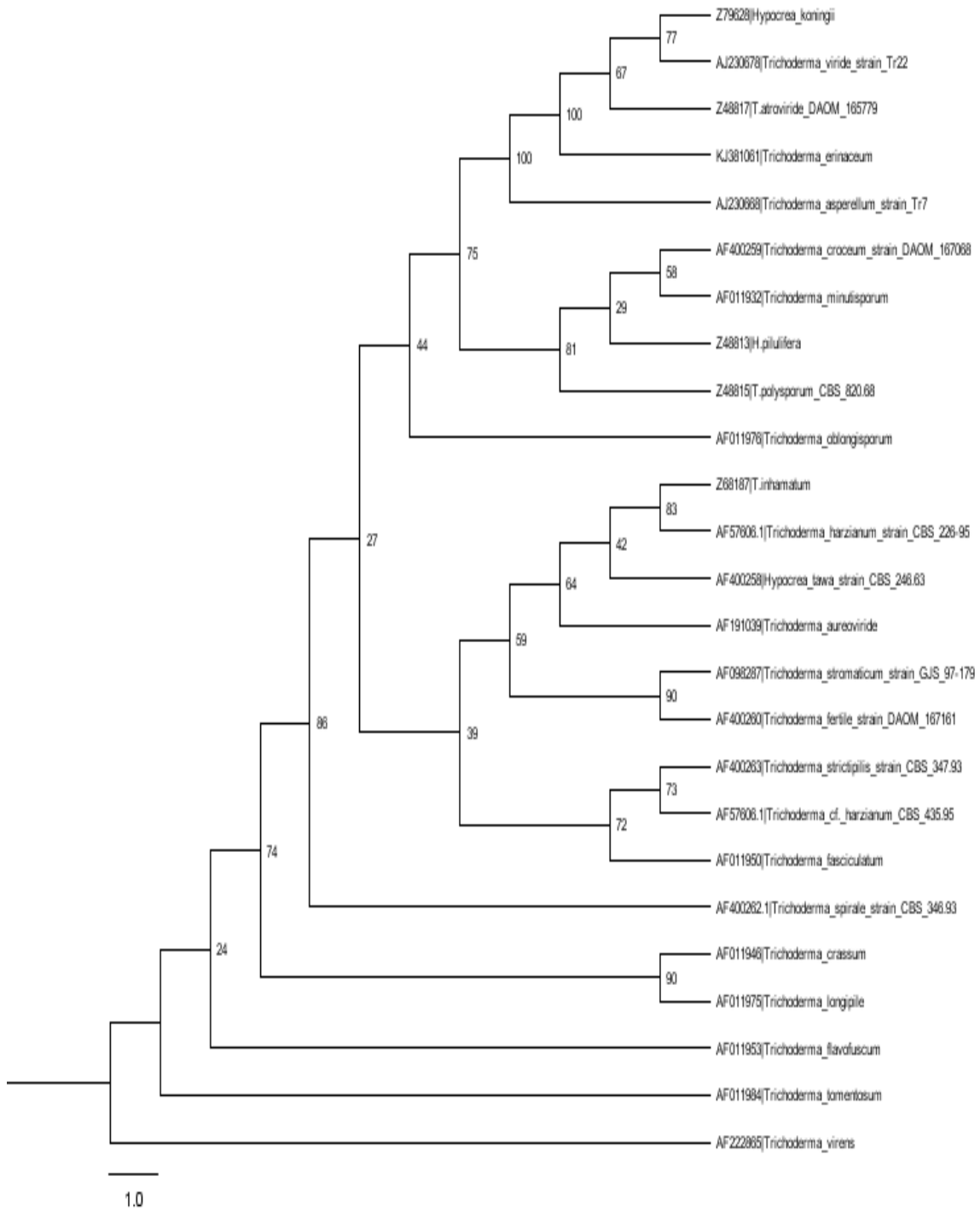


Fig. 2 – The phylogenetic tree obtained by sequence analysis of ITS1 and ITS2 sequence of the isolated *T. erinaceum* and the sequences of other 24 *Trichoderma* species obtained from NCBI, GenBank.

Determination of antifungal activity in liquid cultures of the *T. erinaceum*

To test the effect of *T. erinaceum* culture filtrates on growth of the pathogenic fungi the well diffusion method was used (Fenice et al. 2006, Bosah et al. 2010). Plates having 25 ml of PDA were prepared and 4 wells were made in the PDA media equal distant to each other and 2 cm from the center. Then an 8 mm diameter agar plug obtained from the edge of 4 day old culture of the pathogenic fungus on PDA was placed at the centre of the plate. Thereafter 3 of the 4 wells on each plate were filled with 50 µl of fungal filtrates and the 4th well was filled with 50 µl of the boiled fungal filtrate which was the control. The plates were inoculated at room temperature for 4 days

and radial mycelia growth of the test pathogen was determined by measuring the radius. Percent inhibition (PI) was calculated as; PI (%) = (γ^0-γ/ γ^0) x 100 where, γ^0 is the radius of test pathogen in the control (liquid medium) and γ is the radius of test pathogen in the enzyme solution. All tests were carried out with three replications against seven pathogenic fungi. The data obtained were statistically analyzed using MINITAB 14. One way ANOVA and Tukey test were performed (95% simultaneous confidence intervals).

Results

Isolation and morphological characterization

Primarily, the isolate was identified by studying the colony morphology on Czapeckdox agar (CDA) medium and microscopic analysis of reproductive structure. The isolate grew rapidly on CDA forming a cottony green colony with concentric rings. The conidiophores were erect and arose from short side branches. The size of conidia was 2.5 µm ± 0.1 and was globose, pale green with smooth walls. Conidia occurred in clusters (Fig. 1). Based on the above microscopic observations the isolate was tentatively identified and assigned to the genera *Trichoderma* (Coomaraswamy & Fonseka 1981).

Amplification of ITS region of *T. erinceum*

The ITS region was successfully amplified and sequenced. The homology search against the GenBank data base revealed a 100% similarity to the ITS region of *T. erinaceum*. The isolate was thus designated as *T. erinaceum.* The phylogenetic tree obtained by sequence analysis of ITS region of *T. erinaceum* and the sequences of 24 other *Trichoderma* species obtained from NCBI, GenBank is given in below.

Bootstrap analysis with 100 bootstrap replications demonstrated five major branches. (Fig. 2) *T. erinaceum* belongs to *viride* clade. This cluster is supported by more than 86% of bootstrap values.

Antifungal activity in solid cultures

In dual cultures the *T. erinaceum* inhibited the growth of all seven test pathogens. Clear inhibition zones were seen in contact zones of *T. erinaceum* with the pathogens *F. oxysporum, R. solani* and *C. gloeosporioides.* The highest mean percent inhibition (40.35% ± 0.031) was observed against *R. solani* and the lowest mean percent inhibition (26.18% ± 0.025) was observed against *R. microporus*. Microscopic observation of the contact zone of *T. erinaceum* with *R. solani* showed degradation of mycelia of *R. solani* and presence of coiling structures (Fig. 3). The *T. erinaceum* did not produce inhibition zones against *C. lunata, C. cassiicola, R. microporus* and *P. meadii*, but grew over the pathogen's colony and inhibited further growth of pathogen. All pathogens were grown fully on control plates after prolonged incubation.

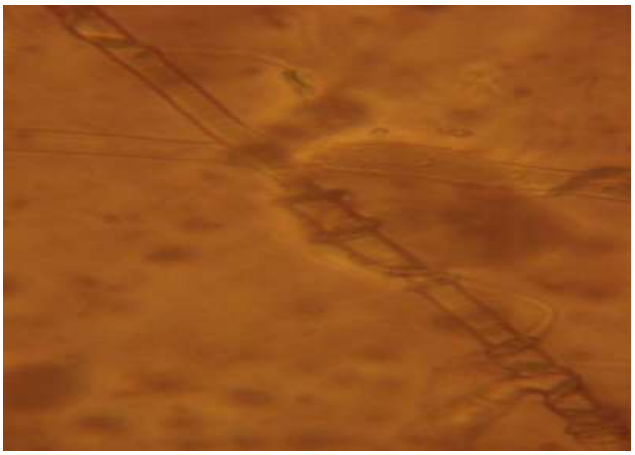


Fig. 3 – Coiling structures of *T. erinaceum* around mycelia of *R. solani*.

Enzyme assay

The isolate showed optimum chitinase activity (0.604 nkat/ml ± 0.012) after 24 hours of incubation and optimum glucanase activity (0.416 nkat/ml ± 0.031) after 96 hours of incubation (Fig. 4).

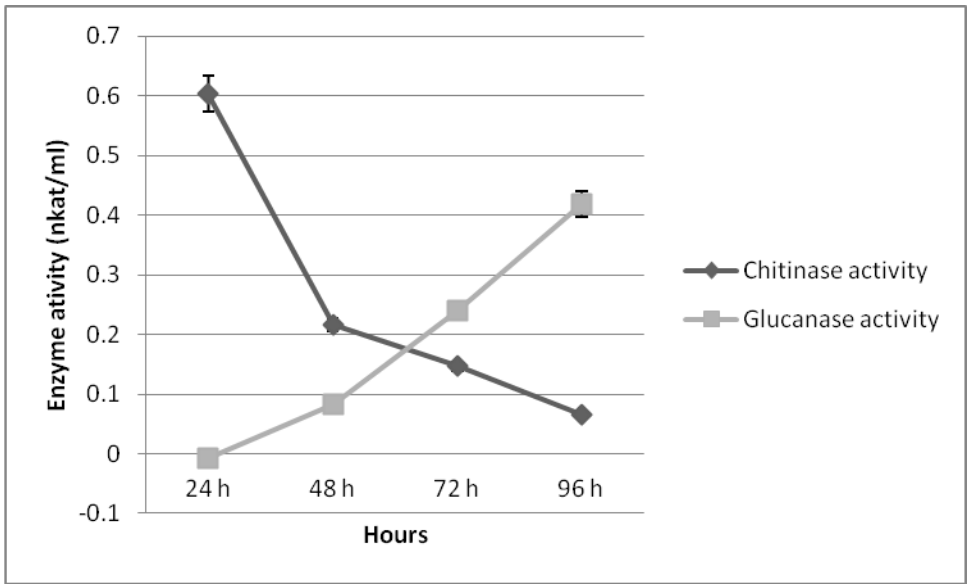


Fig. 4 – Time effect on chitinase and glucanase production by *T. erinaceum*. Values are the means of three replicates. Vertical bars indicate standard errors of the mean.

Determination of antifungal activity in liquid cultures of *T. erinaceum*

In antifungal tests using liquid culture filtrates, inhibition zones were produced against all pathogens. In chitinase filtrates the highest mean percent inhibition (66.38 % ±7.6) was obtained against *P. meadii* and the lowest mean percent inhibition (16.41% ±6.41) was obtained against *C. gloeosporioides*. In glucanase filtrates the highest mean percent inhibition (72.66 % ±3.81) was obtained against *R. solani* and the lowest mean percent inhibition (20.9% ±5.65) was obtained against *F. oxysporum* (Fig. 5).

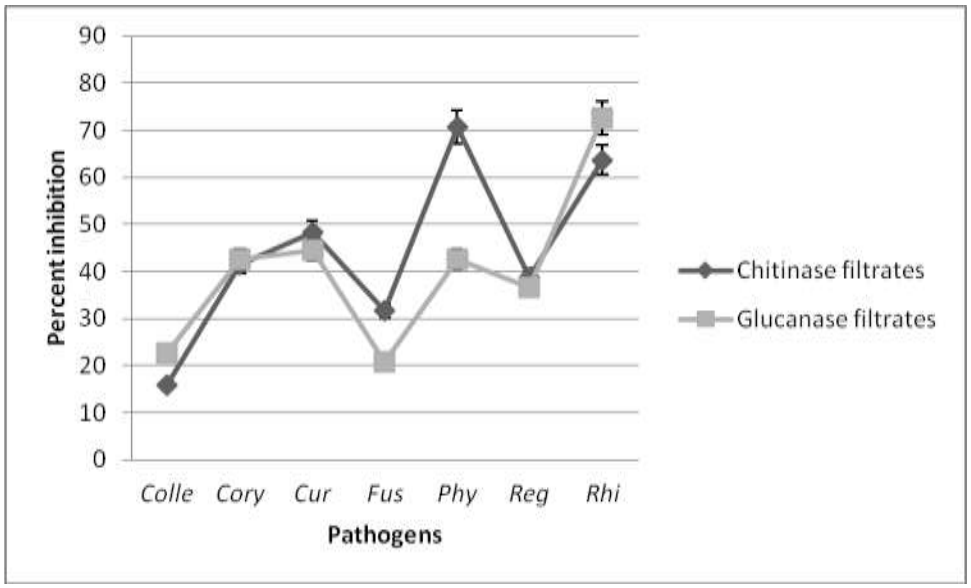


Fig. 5 – Mean percent inhibitions of pathogens for chitinase and glucanase filtrates. Values are the means of three replicates. Vertical bars indicate standard errors of the mean.

Discussion

The results of this study clearly suggest that the isolate *T. erinaceum* has the ability to inhibit the growth of all tested pathogenic fungi. *T. harzianum* and *T. viride* were reported by several workers as the best antagonists for growth inhibition against several plant pathogens by 60%-80% (Rahman et al. 2009, Siameto et al. 2010, Abdollahi et al. 2012, Kumar et al. 2012). However in present study the radial growth of tested fungi, were reduced by *T. erinaceum* with greatest reduction occurring in *R. solani* by 72.66%. Therefore the *T. erinaceum* can be used successfully to control *R. solani* than other tested pathogenic fungi. It is likely that the effect is due to the production of chitinase and β-1,3-glucanase by the *T. erinaceum*. Chitinase and β-1,3-glucanase of *Trichoderma* species act by degrading chitin and β-1,3-glucan responsible for the rigidity of pathogenic fungi cell walls, thereby destroying cell wall integrity and limiting the growth of the fungus (Siameto et al. 2011). Many studies have investigated *Trichoderma* species especially *T. harzianum, T. virens* and *T. viride* which are known to have strong antifungal activities against a range of plant pathogens (Lunge & Patil 2012). However there is still considerable interest in finding new *Trichoderma* isolates having stronger antifungal activity for development of more efficient biocontrol agents. The present isolate which produce chitinase and β-1,3-glucanase and forms coils around target mycelia appears to have the potential to be developed as a biocontrol agent. In molecular characterization our isolate *T. erinaceum* is belong to the viride clade (e.g. *T. atroviride, H. koningii, T. viride*). This finding is in accordance with (Irina et al. 2006, Kumar et al. 2012) where they had grouped the *T. erinaceum* in *viride* clade by phylogenetic analysis using only ITS region of them. To the best of our knowledge this is the first report of isolation of *T. erinaceum* from Sri Lanka. However, studies are essential to examine field conditions of the isolate before its use in biocontrol in agriculture.

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