academicJournals

Vol. 16(20), pp. 1168-1175, 17 May, 2017
DOI: 10.5897/AJB2017.15905
Article Number: 670A81264285
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Exploration of Sri Lankan soil fungi for biocontrol properties

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Received 20 January, 2017; Accepted 29 March, 2017

Sri Lankan soil is a rich source of fungi with new strains that are not well explored to date. In the present study, a total of 83 soil fungi were isolated from different locations of Sri Lanka using chitin and β-1,3-glucan selective media. Of the 83 isolates, 22 isolates showed high biocontrol activities against seven selected plant pathogenic fungi in dual culture plate assay. These 22 isolates were further analyzed for chitinase, glucanase production and for antifungal activities. *Trichoderma* sp. 1 showed significantly high mean chitinase (0.60±0.012 nkat/ml) and glucanase (0.42±0.031 nkat/ml) activities after 24 h and 96 h of incubation respectively compared to other isolates. Furthermore, it revealed the highest mean percentage (76.66±7.6) of inhibition against *Phytophthora meadii* in the well diffusion method using chitinase filtrates. *Penicillium* sp. 1 showed the highest mean percentage (64.75±1.6) of inhibition against *Rigidoporus microporus* in well diffusion method with glucanase filtrates. Three *Trichoderma* isolates that unveiled high enzyme activities as well as high antifungal activities were identified as *Trichoderma erinaceum* (*Trichoderma* sp. 1), *Trichoderma virens* (*Trichoderma* sp. 5) and *Trichoderma asperellum* (*Trichoderma* sp. 8) using molecular characterization.

Key words: Chitin, glucan, antifungal, *Trichoderma*, *Phytophthora, Rigidoporus.*

INTRODUCTION

Soil fungi such as *Trichoderma, Rhizopus, Aspergillus* and *Penicillium*, that have strong biocontrol activity which produced chitinases and glucanases which have been studied extensively. In Sri Lanka, current estimates suggest that there could be as many as 25,000 of fungi species, of which only a little more than 2,000 are presently known (Karunarathna et al., 2012). Having special environmental characteristics and being rich in soil fungi populations, the mycology of Sri Lankan soil has not been well explored, constituting an excellent source for the search of new strains. The soil samples used in this study were collected from a range of locations in Sri Lanka (9 districts) including natural forests, a compost heap, chitin bated soil, market garden and from rubber plantations.

Chitin and β-1,3-glucan selective media were used to

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isolate soil fungi, that produced chitinases and glucanases. The importance of β-1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) as key enzymes responsible for pathogenic fungal cell and sclerotial wall lysis and degradation has been reported (EL-Katatny et al., 2000). Therefore, use of chitinase and glucanase producing micro organisms as biological control agents against many fungal pathogens has been reported.

Most of the previous investigations indicated that, *Trichoderma* species are the best biocontrol agents among other soil fungi (Bell et al., 1982; Elad and Kapat, 1999; Ramezani, 2009). However there is still considerable interest in searching for new biocontrol fungi isolates especially in Sri Lankan soil which has not been well explored although it is known to be rich in biodiversity. By considering all these facts, the present study was undertaken to explore Sri Lankan soil, to isolate fungi that have strong biocontrol activities with high chitinase and β-1,3-glucanase production.

MATERIALS AND METHODS

Collection sites

For isolation of glucanolytic and chitinolytic fungi, soil samples were obtained from five different types of locations in Sri Lanka covering 9 districts including rubber plantations (Mathugama, Kuruwita and Kegalle), natural forests (Dambulla, Sigiriya, Polonnaruwa and Mathara), compost heap, market garden (Kandy) and from a baited method.

Isolation of chitinolytic and glucanolytic fungi

The soil samples were collected into sterile polypropylene bags, brought to laboratory within 12 h and used to isolate the fungi using the soil dilution plate method, with either chitin or glucan selective media. Chitin selective medium contained; yeast extract (5 g/L), $(NH_4)_2SO_4$ (1 g/L), $MgSO_4.7H_2O$ 0.3 (g/L), KH_2PO_4 (1.36 g/L) and Agar (20 g/L) with 1.5% colloidal chitin as the main carbon source (Severgnini, 2006).

Colloidal chitin was prepared as described by Ahmad et al. (2010). The glucan selective medium contained; $(NH_4)_2SO_4$ (1 g/L), $MgSO_4.7H_2O$ (0.3 g/L), KH_2PO_4 (0.8 g/L), KNO_3 (0.2 g/L) and agar (20 g/L) with 0.5% β-glucan prepared using dry yeast cells according to the method described by Zechner-Krpan et al. (2010) which is used as the main carbon source.

The pH of both media was adjusted to 5.5. To prevent bacterial growth culture media, 50 mg/L were amended with ampicillin. The pure cultures of fungi isolates obtained were maintained on potato dextrose agar during the period of study.

Screening of soil fungi isolates for biocontrol activities against pathogenic fungi using dual culture plate assay

Soil isolates (83) were 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.

Potato Dextrose Agar (PDA) plates were inoculated, by placing a 9 mm diameter mycelial disc of the pathogenic fungus on one side, obtained from a 4 days old culture on PDA. A similar disc of soil isolate, obtained from the growing edge of 4 days 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°C). The contact zones of the two colonies were observed under the light microscope for any interactions between the two fungi. For controls, only the pathogenic fungi were inoculated separately on a one side of the Petri plates, containing PDA media. In this dual culture plate assay, soil isolates were identified as effective biocontrol agents based on their ability to overgrow, form an inhibition zone and then growth inhibition of pathogen.

Morphological identification of soil isolates

Fungal mycelia and their reproduction structures were examined microscopically and identified upto genus level, using the mycological key (Coomaraswamy and Fonseka, 1981). Spore size, shape and nature of conidiophores were considered to identify fungi species.

Measurement of enzyme activities

The chitin and glucan liquid media were prepared as previously described without adding agar to solid media 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 isolates.

The inoculated flasks were incubated on a rotary shaker of 120 rpm at room temperature. The cultures were harvested at 24, 42, 72 and 96 h intervals, by filtration through whatman no 1 filter paper. Resulting filtrates were stored at 4°C and was used to determine enzyme activity.

The activities of enzymes were determined by dinitrosalicylic acid (DNS) assay (EL-Katatny et al., 2000). The chitinase assay was conducted using 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°C for 30 min followed by incubation at 90°C for 10 min. Then, the mixture was centrifuged at 13,000 rpm for 10 min in a water bath.

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.

The glucanase activity was also assayed similarly 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 were expressed in pkat (pmol/s). All experiments were carried out in triplicate. The data obtained were statistically analyzed using MINITAB 14. One way ANOVA and Tukey test were performed using 95% simultaneous confidence intervals.

Well diffusion method to detect antifungal activity of isolates against pathogenic fungi

Petri plates containing 25 ml of PDA were prepared and 4 wells were made with an equal distant to each other and 2 cm from the center. Then an 8 mm diameter agar plug obtained from the edge of 4 days old culture of the pathogenic fungus on PDA was placed

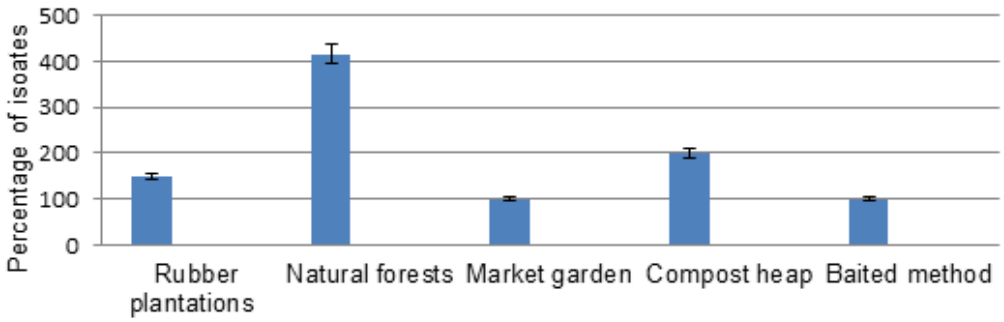

Figure 1. Percentage values of soil isolates of different sites.

at the center 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 four days and radial mycelia growth of the test pathogen was determined by measuring the radius. Percent inhibition (PI) was calculated as; PI (%) = (γ°-γ/ γ°) x 100 where, γ° 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, for both chitinase and glucanase filtrates of all 22 isolates against 7 pathogenic fungi. The data obtained were statistically analyzed using MINITAB 14.

Molecular identification of *Trichoderma* isolates

Genotypic identification was carried out by PCR amplification and sequencing of ITS region. 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'') (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 amplified fragments (~600 bp) were separated by agarose gel (1%) electrophoresis and the excised fragments were purified using PCR Clean-Up System-Wizard®SV Gel (Promega), according to the manufacturer's instructions. The purified amplicons were bidirectionally sequenced using ITS1 and ITS4 primers. The resultant sequences were edited using Bio Edit version 7.2.0 and were subjected to BLAST search analysis at NCBI. DNA sequences were submitted to GenBank under accession numbers; KJ381061, KP641614 and KY270875.

RESULTS

In this study, 83 chitinolytic and glucanolytic fungi were isolated from soil samples, collected from different locations in Sri Lanka, using chitin and glucan based selective media. Out of 83, 48 were isolated from glucan selective medium and 35 were isolated from chitin selective medium.

According to the results, the highest percentage of isolates was obtained from the natural forests (Figure 1). Out of 83 isolates, 22 showed biocontrol activity against seven pathogenic fungi in dual culture plate assay. They produced inhibition zones with pathogenic fungi colony, mycelia degradation and in some cases coiling structures were observed when examined under the microscope (Figure 2).

Enzyme assay

Chitinase activity of the isolates ranged from (0.60 - 0.00) and (0.54 - 0.00) nkat/ml, respectively (Table 1). In most of the species the optimum chitinase activity after 24 h of growth and optimum glucanase activity after 96 h of growth. *Trichoderma* sp. 1 and *Penicillium* sp. 1 (0.60±0.012 nkat/ml and 0.55±0.042 nkat/ml), showed significantly high mean chitinase activities after 24 h of incubation. *Trichoderma* sp. 1 and *Trichoderma* sp. 8 (0.42±0.031 nkat/ml and 0.54±0.009 nkat/ml) showed significantly high mean glucanase activities after 96 h of incubation.

Well diffusion method to detect antifungal activity of isolates against pathogenic fungi

In chitinase filtrate assay, the highest mean percentage was observed in *Trichoderma* sp. 1(76.66 ± 7.6) against *P. meadii*. *Trichoderma* sp. 1 and *Penicillium* sp 4 showed significantly high mean percent inhibition values than others isolates, against the seven tested pathogenic fungi ($P<0.05$) (Table 2). In glucanase filtrate assay, the highest mean percentage was observed in *Penicillium* sp 1(64.75±1.6) against *R. microporus*, *Trichoderma* sp 1 and *Penicillium* sp 1. *Aspergillus* sp 1 and *Trichoderma* sp 8 showed significantly high mean percent inhibition values than the rest of the isolates against seven tested pathogenic fungi ($P<0.05$) (Table 3) (Table 4).

Amplification of ITS region of *Trichoderma* isolates

The ITS regions of three *Trichoderma* isolates (Table 4) were successfully amplified and sequenced. A comparative analysis of rDNA sequences of the three Trichoderma isolates against rDNA sequences of

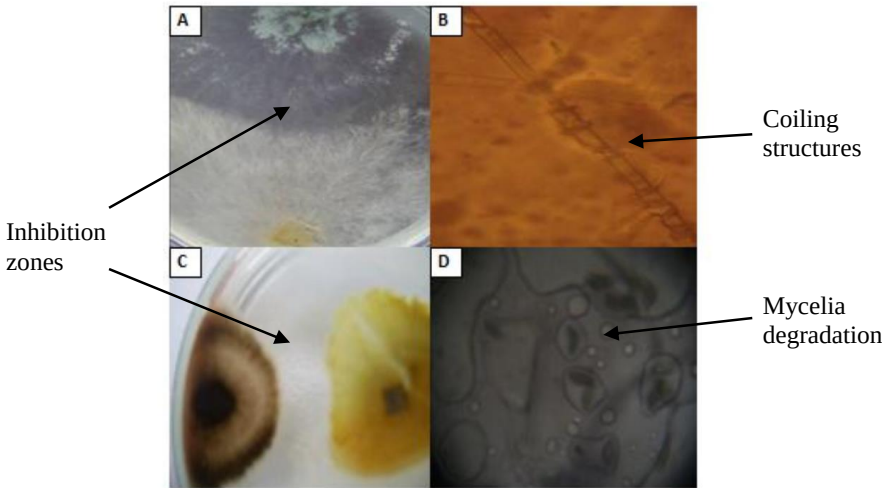

Figure 2. A. *Trichoderma* sp (1) against *Rhizoctonia solani* showing inhibition zone. **B.** Microscopic view of coiling structures between *Trichoderma* sp. (1) against *Rhizoctonia solani*. **C.** *Penicillium* sp. (8) against *Curvularia* sp showing inhibition zone. **D.** Microscopic view of mycelia degradation of *Curvularia* sp.

Table 1. Chitinase and glucanase activities of soil fungal isolates.

Soil isolate	Chitinase activity (nkat/ml)				Glucanase activity (nkat/ml)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Aspergillus sp. 1	0.25±0.01	0.06±0.01	0.06±0.00	0.03±0.00	0.07±0.00	0.12±0.01	0.21±0.01	0.15±0.01
Penicillium sp. 1	0.55±0.04	0.24±0.00	0.12±0.03	0.11±0.00	0.11±0.01	0.14±0.00	0.16±0.01	0.15±0.00
Aspergillus sp. 2	0.48±0.01	0.09±0.02	0.03±0.01	0.01±0.05	0.04±0.03	0.02±0.01	0.03±0.03	0.12±0.00
Trichoderma sp. 1	0.60±0.01	0.23±0.00	0.15±0.02	0.06±0.05	0.01±0.00	0.08±0.10	0.24±0.01	0.42±0.03
Trichoderma sp. 2	0.19±0.04	0.06±0.00	0.03±0.00	0.03±0.00	0.01±0.00	0.17±0.05	0.14±0.00	0.24±0.00
Trichoderma sp. 3	0.04±0.01	0.02±0.00	0.02±0.04	0.02±0.00	0.00±0.00	0.01±0.00	0.00±0.00	0.03±0.01
Trichoderma sp. 4	0.29±0.09	0.14±0.01	0.13±0.01	0.12±0.00	0.00±0.00	0.00±0.00	0.06±0.05	0.19±0.00
Trichoderma sp. 5	0.19±0.00	0.11±0.01	0.09±0.00	0.07±0.00	0.04±0.03	0.19±0.01	0.23±0.01	0.24±0.00
Penicillium sp. 2	0.33±0.00	0.17±0.01	0.13±0.00	0.10±0.00	0.08±0.00	0.06±0.00	0.06±0.00	0.08±0.00
Penicillium sp 3	0.25±0.01	0.17±0.01	0.09±0.01	0.07±0.01	0.04±0.01	0.04±0.00	0.05±0.01	0.10±0.00
Trichoderma sp. 6	0.11±0.00	0.18±0.02	0.172±0.0	0.14±0.00	0.04±0.00	0.08±0.00	0.08±0.00	0.04±0.00
Penicillium sp. 4	0.30±0.01	0.16±0.04	0.12±0.01	0.10±0.00	0.03±0.01	0.00±0.00	0.03±0.00	0.01±0.00
Penicillium sp. 5	0.28±0.00	0.09±0.01	0.02±0.01	0.02±0.00	0.01±0.00	0.04±0.01	0.07±0.00	0.09±0.01
Aspergillus sp. 3	0.11±0.00	0.08±0.00	0.02±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.03±0.01	0.01±0.00
Penicillium sp. 6	0.13±0.00	0.09±0.00	0.04±0.00	0.05±0.00	0.05±0.00	0.06±0.00	0.04±0.00	0.05±0.00
Penicillium sp. 7	0.09±0.00	0.05±0.00	0.01±0.00	0.07±0.00	0.03±0.00	0.01±0.00	0.01±0.00	0.10±0.00
Penicillium sp. 8	0.07±0.03	0.04±0.01	0.04±0.00	0.00±0.00	0.03±0.01	0.02±0.00	0.00±0.00	0.04±0.00
Aspergillus sp. 4	0.05±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.00
Trichoderma sp. 7	0.19±0.07	0.20±0.00	0.11±0.02	0.09±0.04	0.12±0.00	0.11±0.01	0.17±0.00	0.24±0.01
Trichoderma sp. 8	0.04±0.00	0.06±0.02	0.24±0.00	0.35±0.00	0.53±0.00	0.52±0.02	0.47±0.00	0.54±0.00
Fusarium sp. 1	0.09±0.03	0.02±0.00	0.01±0.00	0.02±0.00	0.07±0.01	0.05±0.00	0.02±0.00	0.04±0.00
Penicillium sp. 9	0.20±0.04	0.04±0.00	0.04±0.01	0.03±0.00	0.12±0.00	0.10±0.00	0.05±0.00	0.09±0.00

Trichoderma sp. available in GenBank data base werecarried out separately for each using BLAST algorithum at the website http://www.ncbi.nlm.nih.gov. The homology search against the GenBank data base revealed a 100% similarity to the ITS region of *T. erinaceum* (*Trichoderma* sp 1):KJ38061, *T. virens* (*Trichoderma* sp 5):KP641614 and *T. asperellum* (*Trichoderma* sp 8): KY270875.

Table 2. Mean percent inhibition of chitinase filtrates of isolates on seven pathogenic fungi.

S/N	Isolates	Pathogenic fungi						
		Colletotrichum	*Corynespora*	*Curvularia*	*Fusarium*	*Phytophthora*	*Rigidoporus*	*Rhizoctonia*
1	*Aspergillus sp.1*	40.7±8.50	1.23±2.14	42.66±4.6	33.33±7.6	64.75±1.6	2.97±2.94	0.00±0.00
2	*Penicillium sp .1*	06.1±5.30	27.7±25.4	8.33±14.4	8.33±14.4	0.00±0.00	0.87±1.51	0.00±0.00
3	*Aspergillus sp. 2*	10.0±17.3	3.70±3.20	11.6±10.4	27.26±7.8	2.22±3.85	1.96±3.39	0.00±0.00
4	*Trichoderma sp.1*	16.0±6.90	41.6±7.21	48.33±2.8	31.6±16.1	76.66±7.6	38.66±2.3	3.70±6.41
5	*Trichoderma sp.2*	47.1±5.88	18.7±16.5	40.73±6.4	36.66±5.7	14.3±24.7	6.16±2.13	0.00±0.00
6	*Trichoderma sp.3*	16.6±14.4	0.00±0.00	0.00±0.00	0.00±0.00	9.00±0.00	0.00±0.00	0.00±0.00
7	*Trichoderma sp. 4*	2.22±3.85	0.00±0.00	0.00±0.00	0.00±0.00	38.33±7.6	0.00±0.00	0.00±0.00
8	*Trichoderma sp. 5*	3.70±6.41	47.9±7.21	9.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	4.00±4.00
9	*Penicillium sp. 2*	50.0±05.0	11.7±2.95	0.00±0.00	3.17±2.74	38.33±7.6	20.0±4.00	9.06±9.09
10	*Penicillium sp. 3*	3.50±3.04	0.00±0.00	6.66±8.82	5.55±4.80	0.00±0.00	16.0±4.00	46.66±2.9
11	*Trichoerma sp. 6*	04.0±04.0	21.1±7.69	28.2±22.8	18.17±4.5	5.92±4.62	0.00±0.00	26.6±15.3
12	*Penicillium sp. 4*	45.0±8.66	49.3±2.30	10.0±10.0	0.00±0.00	66.66±0.0	58.33±7.2	41.33±2.3
13	*Penicillium sp. 5*	0.00±0.00	0.00±0.00	20.0±10.0	0.00±0.00	2.74±2.71	0.00±0.00	0.00±0.00
14	*Aspergillus sp .3*	1.66±2.89	1.62±1.40	38.4±3.84	3.17±2.74	0.72±1.25	11.11±9.6	22.8±39.6
15	*Penicillium sp. 6*	13.33±7.6	45.0±6.61	32.2±1.92	20.0±8.66	7.84±4.49	21.73±2.2	0.95±1.65
16	*Penicillium sp. 7*	14.3±12.5	4.44±5.09	37.01±3.7	0.00±0.00	3.00±3.00	1.96±1.69	0.00±0.00
17	*Penicillium sp .8*	1.85±3.20	12.0±08.0	43.75±3.1	7.51±2.57	24.44±8.4	31.11±1.9	0.00±0.00
18	*Aspergillus sp. 4*	16.0±04.0	12.8±2.56	18.16±4.5	9.72±8.67	0.98±1.69	4.76±1.64	0.00±0.00
19	*Trichoderma sp. 7*	2.38±4.12	51.6±17.5	41.1±12.6	1.90±1.65	28.33±5.8	2.22±3.85	0.00±0.00
20	*Penicillium sp. 9*	2.56±4.44	13.33±1.4	3.88±4.19	7.62±5.95	0.74±1.28	6.66±6.66	0.00±0.00
21	*Trichoderma sp. 8*	56.0±04.0	11.33±2.3	18.66±2.3	67.94±2.2	2.38±2.38	22.22±5.1	0.00±0.00
22	*Fusarium sp. 1*	0.00±0.00	0.00±0.00	8.89±3.84	9.05±4.54	2.43±2.44	36.66±3.3	0.00±0.00

*Values are mean of three replicates ± SD. (P<0.05).

Table 3. Mean percent inhibition of glucanase filtrates of isolates on seven pathogenic fungi.

S/N	Isolates	*Colletotrichum*	*Corynespora*	*Curvularia*	*Fusarium*	*Phytophthora*	*Rigidoporus*	*Rhizoctonia*
1	*Aspergillus sp.1*	46.7±2.88	49.33±2.3	50.0±0.00	36.5±7.27	63.33±1.4	60.0±3.33	0.00±0.00
2	*Penicillium sp.1*	47.4±5.26	63.8±4.36	59.04±3.3	33.33±5.7	50.0±12.5	64.75±1.6	0.00±0.00
3	*Aspergillus sp. 2*	2.38±4.12	0.00±0.00	6.67±6.67	1.66±2.88	11.6±11.5	0.00±0.00	18.2±18.2
4	*Trichoderma sp.1*	22.6±4.61	42.59±3.2	44.41±7.3	20.9±5.65	42.6±2.30	36.6±14.4	46.6±3.81
5	*Trichoderma sp.2*	42.4±5.24	0.00±0.00	1.11±1.92	42.85±7.1	53.33±6.6	25.0±8.33	0.00±0.00
6	*Trichoderma sp.3*	0.00±0.00	0.00±0.00	28.89±3.8	26.6±6.67	28.89±3.8	0.00±0.00	0.00±0.00
7	*Trichoderma sp.4*	0.00±0.00	0.00±0.00	10.0±0.00	30.3±13.8	12.9±11.5	0.00±0.00	20.0±18.0
8	*Trichoderma sp.5*	4.00±4.00	0.00±0.00	0.00±0.00	18.2±15.7	0.00±0.00	0.00±0.00	0.00±0.00
9	*Penicillium sp.2*	9.06±9.09	5.71±2.85	52.09±4.7	26.66±2.8	54.3±5.65	8.55±5.35	0.83±1.44
10	*Penicillium sp.3*	46.66±2.9	25.71±2.9	28.06±3.0	43.05±8.6	1.07±1.85	24.0±21.0	22.5±2.50
11	*Trichoerma sp.6*	26.6±15.2	42.66±2.3	32.0±13.8	29.8±8.06	14.66±6.1	3.00±3.00	0.95±1.65
12	*Penicillium sp.4*	34.9±7.27	47.0±1.47	58.1±10.0	19.67±5.2	0.92±1.59	3.00±3.00	0.95±1.65
13	*Penicillium sp.5*	0.00±0.00	41.6±14.4	44.0±4.00	44.0±0.00	0.00±0.00	66.6±1.65	0.00±0.00
14	*Aspergillus sp.3*	25.0±21.7	5.88±2.94	36.4±22.8	7.23±6.62	0.85±1.47	0.00±0.00	0.00±0.00
15	*Penicillium sp.6*	10.53±2.6	11.1±3.70	0.00±0.00	12.66±5.5	1.66±2.88	13.33±3.3	4.33±7.50
16	*Penicillium sp.7*	25.9±6.41	0.00±0.00	12.5±0.00	0.00±0.00	0.00±0.00	4.00±4.00	6.66±5.77
17	*Penicillium sp.8*	21.67±5.7	49.59±5.1	45.71±4.9	3.17±2.74	4.51±4.50	11.76±0.0	0.00±0.00
18	*Aspergillus sp.4*	0.00±0.00	6.66±1.65	60.0±12.0	48.33±2.9	4.00±4.00	41.6±14.4	2.32±2.32
19	*Trichoderma sp.7*	3.08±4.07	11.09±7.2	35.7±14.3	22.79±8.0	24.4±10.2	4.44±7.69	0.95±1.65

Table 3. Contd.

19	*Trichoderma sp.7*	3.08±4.07	11.09±7.2	35.7±14.3	22.79±8.0	24.4±10.2	4.44±7.69	0.95±1.65
20	*Penicillium sp. 9*	3.07±4.06	3.96±1.37	2.66±2.30	31.1±16.8	27.0±6.17	2.22±3.85	0.00±0.00
21	*Trichoderma sp. 8*	45.0±5.00	42.6±4.61	43.33±8.8	43.93±2.6	60.0±2.50	54.44±3.8	0.00±0.00
22	*Fusarium sp.1*	8.89±3.84	0.00±0.00	40.0±6.67	25.0±0.00	9.26±5.78	6.25±0.00	0.00±0.00

*Values are mean of three replicates ± SD. (P<0.05).

Table 4. Summary results of fungal isolates showing significantly high enzyme activities and antifungal potential..

Test	Significantly high active isolates	Selected isolates for molecular characterization
Chitinase enzyme assay	*Trichoderma* sp. 1 *Penicillium* sp. 1	*Trichoderma* sp. 1
Glucanase enzyme assay	*Trichoderma* sp. 1 *Trichoderma* sp. 5 *Trichoderma* sp. 8	*Trichoderma* sp. 1 *Trichoderma* sp. 5 *Trichoderma* sp. 8
Chitinase antifungal test	*Trichoderma sp.* 1 *Penicillium sp. 4*	*Trichoderma sp.* 1
Glucanase antifungal test	*Aspergillus sp.* 1 *Penicillium sp.* 1 *Trichoderma sp.* 1 *Trichoderma sp.* 8	*Trichoderma sp.* 1 *Trichoderma sp.* 8

DISCUSSION

In this study, soil samples collected from five different locations in nine districts of Sri Lanka were explored for fungi of interest and a baited strategy was tested to enrich the soil flora for fungi of interest.

Initially, 83 pure cultures were obtained from different locations. Out of 83, 35 were isolated from chitin selective media and 48 were isolated from glucan selective media. Highest percentages (416.66%) of isolates, with prominent biodiversity were obtained from natural forests soil samples. This may be due to high content of nutrients and adequate moisture in forest soil. Among them, only seven isolates evidence biocontrol activity in dual culture plate assay. These include two *Aspergillus* species, two *Trichoderma* species, two *Penicillium* species and one *Fusarium* species. Only *Trichoderma* species showed the coiling structures around mycelia of *R. solani* while other isolates showed only the inhibition zones between the two colonies.

Compost samples are rich in organic material that provides nutrients to the soil. Second highest percentage (200%) of isolates, was obtained from compost samples. However none of the isolates had any biocontrol activity. All twelve isolates (148%) obtained from soil samples of rubber plantations (seven *Penicillium* species, three *Trichoderma* species and two *Aspergillus* species), revealed high biocontrol activity during initial screening test. Those include seven *Penicillium* species, three *Trichoderma* species and two *Aspergillus* species.

Baiting method was proved to be a productive method for enriching the surrounding soil flora, for chitinolytic and gluconolytic species (Severgnini, 2006). Only two *Trichoderma* species were isolated from having biocontrol activity. Agricultural soils are often subjected to fungicides and fertilizers and these contain very low fungal population. Hence, the market garden yielded the lowest number of isolates with one *Trichoderma* species having biocontrol activity.

The results of this dual culture test clearly suggested that out of 83 isolates, 22 showed biocontrol activity against pathogenic fungi. There are nine *Penicillium* species, eight species of *Trichoderma*, 4 species of *Aspergillus* and 1 species of *Fusarium*. Hence, these 22 isolates were selected to carry out further analysis. According to the enzyme assay results, *T. erinaceum* (*Trichoderma* sp 1) showed significantly high activities for both enzyme assays. Production of chitinase using a medium containing colloidal chitin reached a maximum after 24 h of growth for all isolates and decreased thereafter.

In contrast, synthesis of β-1,3-glucanase increased rapidly after 96 h peaking at the fourth day for most of isolates. These results are accordance with previous

studies (EL-Katatny et al., 2000; Matraudi et al., 2009; Sharaf et al., 2012).

The results of both assays and the two well diffusion tests indicated that, *T. erinaceum* (*Trichoderma* sp. 1), *T. virens* (*Trichoderma* sp. 5) and *T. asperellum* (*Trichoderma* sp. 8) can be used as an efficient biocontrol agents against all tested fungal pathogens, especially against *R. solani* and *P. meadii* which showed high mean percent inhibition values on them. In this study, some *Aspergillus* and *Penicillium* species acted as biocontrol agents while *Trichoderma* species acted best as reported by several workers earlier.

Chitinolytic and glucanolytic fungi showed great variations towards cell wall degradation, ranging from weak to strong antifungal activity depending on inherited characters. Most studies revealed that *T. harzianum* is among the most potential species of *Trichoderma* that are commonly used for phytopathogen control (Jayalakshmi et al., 2009; Muhammad and Amusa, 2003; Lorito et al., 1998; Shabir et al., 2012).

In this study, *T. erinaceum* (*Trichoderma* sp 1), *T. virens* (*Trichoderma* sp 5) and *T. asperellum* (*Trichoderma* sp. 5) were found to be highly active isolates among 83 soil isolates. It is revealed from the present study that the ability of the above selected *Trichoderma* species to control pathogenic fungi especially *R. solani* and *P. meadii* may be due to chitinase and glucanase production. These observations were in close resemblance with previous studies such as that of Lorito et al. (1998), Ramezani (2009) and Sharma et al. (2014). *Trichoderma* spp. employs several mechanisms to combat pathogens by competition for space and nutrients, secretion of cell wall degrading enzymes (chitinases and gucanases), induction of resistance etc. (Rifai, 1969).

In both well diffusion tests, boiled enzymes were used as controls. The results suggest that proteins in the suspension were likely to affect the growth of pathogens. In this study, purified enzymes were not used. If purified, enzymes were used for better inhibitions which may be obtained. Lorito et al. (1994) reported that, incubated plant pathogens such as *R. solani*, *Ustilago avenae*, *Uncinula necator* with *Trichoderma harzianum* purified endochitinase, found complete inhibition (100%) of hyphal elongation.

However understanding the antagonistic mechanisms used by *Trichoderma* species on a wide range of pathogens is important in optimizing their use as biocontrol agents. It is also important to collect information about the effect of temperature of fungal filtrate enzyme activity, elements in supernatant and incubation time on the inhibition of pathogenic fungi growth.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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