African Journal of Biotechnology Vol. 11(45), pp. 10350-10357, 5 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB12.599
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Significance of secondary metabolites and enzymes secreted by *Trichoderma atroviride* isolates for the biological control of Phomopsis canker disease

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Accepted 18 April, 2012

Phomopsis theae **Petch, the causative agent of Phomopsis canker diseases, is one of the major constrain in tea plants. The present study deals with development of efficient biological control agent against canker based on the secretion of several secondary metabolites and defense enzymes under *in vitro* conditions. Among the isolates evaluated, the antifungal compounds obtained from Tv1 *Trichoderma atroviride* was able to inhibit the growth of pathogen and showed 100% inhibition at concentration of 500 ppm. Synthesis of extracellular enzymes such as amylase, cellulase, polygalacturonase, protease and chitinase correlate with the antagonist activity of the isolates and maximum enzymatic activity was observed for Tv1 isolates followed by Tm3 and Tc3 isolates. The antifungal compounds responsible for the biological control activity were characterized through gas chromatography - mass spectroscopy (GC-MS) analysis.**

Key words: *Trichoderma atroviride, Phomopsis theae,* tea plants, antifungal compounds, enzymatic activity.

INTRODUCTION

Tea (*Camellia sinensis* (L.) O. Kunze) is one of the oldest, non-alcoholic and refreshment beverages widely consumed all over the world. It is cultivated in more than 50 countries across the altitudes of 2300 m above mean sea level (MSL). Tea plantations are primarily rain fed and cropping condition needs a moist climate with alternating wet and dry periods. This stable micro climatic conditions and mono cultural habitat stay behind as suitable sites for many phytopathogens to get well established. Among several diseases, Phomopsis canker caused by the fungal pathogen *Phomopsis theae* Petch is the most frequent disease which causes significant damages to tea plants. Due splash dispersal of conidia from diseased tissues, occurrence of the disease is prevalent and infected plantations are being cleared and replanted with clones (Ponmurugan and Baby, 2008).

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Application of toxic fungicides is the prime approaches for the control of this disease; however it can be overcome by the biological treatments.

Trichoderma sp. are opportunistic, avirulent plant symbionts invader, confer with fast growing nature, strong spore producer and acts as a source of various cell wall degrading enzymes and secondary metabolites (Vinale, 2008). *Trichoderma* has a wide competence to mobilize and exploit the soil nutrients (Singh et al., 2010). It is able to secrete 40 different secondary metabolites that may contribute to their mycoparasitism and antibiotic action (Sivasithamparam and Ghisalberti, 1998). These volatile and nonvolatile toxic metabolites hinder the colonization of pathogen (Reino et al., 2008; Poornima, 2011), induce resistance and promote the growth of plants to large extent (Shalini et al., 2006). *Trichoderma* based biocontrol products contributes to nearly 50% of fungal biopesticides market and frequently used as soil and growth enhancers (Whipps and Lumsden, 2001).

The canker wounds have turned out to convalesce successfully by using *Trichoderma* sp. to a greater extent

(Ponmurugan and Baby, 2007). Our earlier *in vitro* investigations has proved that indigenous *Trichoderma atroviride* isolated from Valparai gave the best inhibitory effect on *Phomopsis theae* through dual culture and antibiosis (Anita and Ponmurugan, 2011). The antagonist activity of *Trichoderma* sp. is judged by the secretions of a wide range of cell wall degrading enzymes including cellulases, pectinase and chitinases. Hence the present study was carried out with main objectives to evaluate the enzymes secreted by the *T. atroviride* isolates against *P. theae* and to determine the antifungal compounds responsible for the pathogenicity. In addition, the characteristic of secondary metabolites secreted by the pre-eminent strain was evaluated through gas chromatography - mass spectroscopy (GC-MS).

MATERIALS AND METHODS

Fungal cultures used

Trichoderma sp. used for the present study was isolated from the rhizosphere soil samples of Southern Indian tea plantation, which includes the districts of Valparai, Coonoor, Munnar, Vandiperiyar, Gudalore and Koppa. The cultures were identified as *T. atroviride* (MTCC 9641) from Microbial Type Culture Collection (MTCC), Chandigarh. *P. theae* (IMI No. 384005) was obtained from Plant Pathology Division, United Planters' Association of Southern India (UPASI) Tea Research Institute, Valparai, India for the present investigation. A standard culture of *T. atroviride* (MTCC 2461) was procured from MTCC, Chandigarh for comparisons purpose.

Extraction of secondary metabolites

The culture filtrate of the antagonist was prepared using potato dextrose broth (PDB). The mycelium mat was removed and the cultural filtrate was filtered by sterilized membrane filter. The metabolic compounds were extracted through solvent extraction process using ethyl acetate at ratio of 1:1 (v/v). The antifungal compounds diffused in the upper layer of the separating funnel were collected and concentrated using rotary evaporated at 40°C. Hence, aforementioned obtained metabolites from different isolates of *T. atroviride* were evaluated for antagonism against *P. theae*.

Well diffusion assay

The potato dextrose agar medium amended with 50, 100, 250 and 500 ppm concentrations of metabolites obtained from different isolates of *T. atroviride* was transferred into 90 mm Petri-plates. The plates were inoculated with 5 mm mycelium plug of *P. theae* in the centre. The control was maintained without metabolites. The linear growth measurement was recorded and percentage of inhibition was calculated.

Evaluation of enzymatic activity of *T. atroviride* isolates

The secretion of the different kinds of enzymes such as amylase, cellulase, polygalacturonase, protease and chitinase were carried out for all the isolates and was assessed at 3rd, 4th, 7th and 9th day. The mycelium plug of 9 mm was prepared and inoculated in Czapek Dox media by replacing carbon source such as starch, carboxymethyl cellulose, pectin and colloidal chitin for amylase, cellulase, polygalacturonase and chitinase production, respectively. For the estimation of proteases activity, sodium nitrate was replaced in the medium by casein as the enzyme source. The culture broth was centrifuged and the supernatant was used for enzyme assay and reaction mixture with heat-killed enzyme served as the reagent blank for all the estimations. Activities of amylase and cellulase were carried out by measuring the reducing sugars as glucose by dinitrosalicylic acid method (Miller, 1959). The absorbance was read at 540 nm using an ultra violet-visible spectrum (UV-VIS) spectrophotometer (Hitachi, Japan).

One unit of enzyme activity is defined as the amount of enzyme which released 1 µg of reducing sugar per minute under experimental conditions. Polygalacturonase was determined by the method outlined by Neukon (1960). The reaction mixture contained 4 ml of 1% polygalacturonic acid, 1 ml of 0.1 M acetate buffer (pH 6.0) and 1 ml of 0.01 M calcium chloride ($CaCl_2$) (pH 6.0) and 4 ml of crude enzyme extract. The absorbance was determined at 515 nm. One unit activity was defined as the amount of enzyme liberating 1 µg galactronic acid mg protein^{-1} h^{-1} under assay conditions. Protease activity was determined by the method of Mahadevan and Sridhar (1989). Equal volume of cultural filtrate and 1% casein solution prepared using 0.1 M phosphate buffer (pH 7.0) was mixed well and incubated at 30°C in a water bath. The reaction was stopped by adding 1 ml of ninhydrin to the reaction mixture. The purple color of the solution was read at 560 nm.

One unit activity of enzyme is defined as the amount of amino acid changing in OD h^{-1} under assay conditions. Chitinase activity was determined by the method of Reissig et al. (1955). The reaction mixture consists of 1 ml of crude enzyme with 1 ml of 0.1% colloidal chitin prepared in 0.5 M sodium acetate buffer (pH 5.2). To 0.5 ml of reaction mixture, 0.1 ml of potassium tetraborate was added followed by boiling in a water bath for 3 min. To this, 3 ml of diluted p-dimethylaminobenzaldehyde reagent was added and incubated at 37°C for 15 min and read at 585 nm. One unit activity was defined as the amount of chitinase enzyme, which produces 1 µmol of N-acetylglucosamine in 1 min.

Characterization of secondary metabolites through GC-MS analysis

GC-MS was preformed to analyse the metabolic compounds synthesized by the pre-eminent isolate. It was performed on GC Clarus 500 Perkin Elmer equipment with Turbo mass gold detector. The column used was Elite-5MS with 30 m × 0.25 mm capillary column with a particle size of 0.25 µm. The injection temperature was set at 250°C. The helium gas flow rate through the column was 1 ml/min, ions were generated at an electron impact (EI) of 70 kV, the ion source temperature was set at 200°C, and the mass range was m/z 50 to 1,000. The column temperature was maintained at 110°C for 2 min and then raised to 200°C at a rate of 10°C/min.

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and the significant means were segregated by critical difference (CD) at various levels and standard error (SE) was also calculated (Gomez and Gomez, 1984). The means were separated according to Duncan's multiple square tests at $P \leq 0.05$.

RESULTS AND DISCUSSION

Antagonist activity of *T. atroviride* isolate

Percentage of inhibition in terms of linear growth

Table 1. Effect of metabolites synthesized by *T. atroviride* isolates on the growth of *P. theae*.

S/N	*T. atroviride* isolate	Linear growth (mm) of *P. theae* at different concentration of metabolites from *T. atroviride* isolate			
		50 ppm	150 ppm	250 ppm	500 ppm
1	Tv1	41.67 ± 2.89 (53.70[a])	30.67 ± 1.15 (65.93[a])	16.67 ± 1.53 (81.48[a])	0.00 (100[a])
2	Tc3	57.00 ± 1.73 (36.67[c])	46.67 ± 1.53 (48.15[c])	38.33 ± 0.58 (57.41[c])	19.33 ± 1.15 (78.52[c])
3	Tm3	52.67 ± 0.58 (41.48[b])	41.67 ± 1.53 (53.70[b])	34.00 ± 1.00 (62.22[b])	16.33 ± 1.53 (81.85[b])
4	Tvan4	62.00 ± 2.00 (31.11[d])	53.00 ± 1.00 (41.11[d])	43.67 ± 1.15 (51.48[d])	31.33 ± 1.15 (65.19[d])
5	Tg2	63.33 ± 1.53 (29.63 [d])	56.67 ± 1.53 (37.04[e])	48.67 ± 1.15 (45.93[d])	42.00 ± 1.00 (53.33[e])
6	Tk2	62.00 ± 1.00 (31.11 [d])	49.33 ± 1.15 (45.19[f])	43.67 ± 1.15 (51.48[e])	33.67 ± 1.53 (62.59[f])
7	T MTCC*	70.33 ±1.53 (21.85[e])	63.33 ± 1.53 (29.63[g])	51.00 ± 1.73 (43.33[f])	43.33 ± 1.53 (51.85[f])
	SE ±	7.6116	8.6977	9.4824	12.7454
	CD at P=0.05	27.0559	30.9166	33.7056	45.3041

Values in parantheses indicate the percentage of inhibition of *P. theae* by *T. atroviride* isolates.*Standard culture used for comparison. Means within a column followed by the same superscript letters(s) are not significant according to Duncan's multiple square test (P≥0.05).

exhibited by *P. theae* in the presence of metabolites secreted by *T. atroviride* isolates through well diffusion assay is illustrated in Table 1. Tv1 *T. atroviride* isolates showed highest percentage of inhibition ranging from 53.70, 65.93, 81.48 and 100 with the different ranges of concentration of 50, 150, 250 and 500 ppm, respectively. The vast significant at ($P \leq 0.05$) was observed among the isolates. There was rapid decrease observed in the linear growth of the pathogen with increased concentration of the metabolites. Also Tm3 and Tc3 isolates obtained from Munnar and Coonoor soil samples showed 81.85 and 78.52% of inhibition at the concentration of 500 ppm. However, no significant difference was observed at lower concentration of 50 ppm among Tvan4, Tg2 and Tk2 isolates. The standard culture was reported to produce minimum percentage of inhibition of 51.85 at 500 ppm concentration. Biocontrol activity of *Trichoderma* sp. involves direct interaction with pathogen by synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics (Benı´tez et al., 2004). From earlier research conducted by Eziashi et al. (2006) has reported that *Trichoderma viride* and *Trichoderma polysporum* has inhibited the mycelium growth of C*yanophora paradoxa* with higher percentage of inhibition of 81 and 79.This highly correlates with the present study that Tv1 *T. atroviride* can be able to arrest the growth of *P. theae* inhibit completely at 500 ppm concentration.

Enzyme assay

Trichoderma sp. through the mechanisms of myco-parasitism can be able to cleave the hyphae cell wall of the pathogen by secreting enzymes such as glucanases and chitinase (Eduardo et al., 2011). *T. atroviride* has been reported to produce array of enzymes with antimicrobial effect against the test pathogen. These hydrolytic enzyme secreted at a constitutive level detects the presence of another fungus by sensing the molecules released from the host by enzymatic degradation (Harman et al., 2004; Woo and Lorito, 2007). In the present study, six strains of *T. atroviride* isolated from soil samples from southern Indian tea plantation along with a standard culture showed a diverse extracellular enzymatic activity. The results depicted in Figure 1 evidently prove that, all the isolates produced substantial amount of amylase enzyme. The maximum activity was recorded for Tv1 isolate which was followed by Tc3 and Tm3 isolates. Vast significant difference was observed between indigenous strains and standard culture. The activity of amylase was reported to be gradually increased and attains its peak within 7th day of incubation and declined after 9th day for all the isolates.

Trichoderma sp. is known to produce cellulases enzymes (Nevalainen and Penttila, 1995). In accordance, all the seven isolates were originated to produce considerable quantity of this enzyme. The results pointed out in Figure 2 depicts that *T. atroviride* Tv1 isolate attain its peak production of 48U mg^{-1}on 7th day and Tc2 and Tm3 among others were also good producers of cellulases. Moreover Tg2 and Tk2 produced equal amount of 35U mg^{-1} followed by 32U mg^{-1} produced by standard culture. However no significant difference was observed between Tvan4, Tg2, Tk2 and T MTCC isolates for the production of cellulases. As clearly shown in Figure 3, quantity of polygalacturonase synthesized by isolates showed no significant difference on 3rd day, and continued to be improved on 5th day and vital significant difference was observed among the isolates on 7th day. The quantity of enzymes was found to be diminishing for all the isolates on 9th day, except Tm3, which showed minor improvement.

Fungal proteases play a significant role in cell wall lyses by catalyzing the cleavage of peptide bonds in proteins (Mata et al., 2001). Tv1 isolate showed enormous secretion of protease enzyme and maximum activity was observed on 7th day of production (Figure 4). This obviously proves the antagonist activity of Tv1

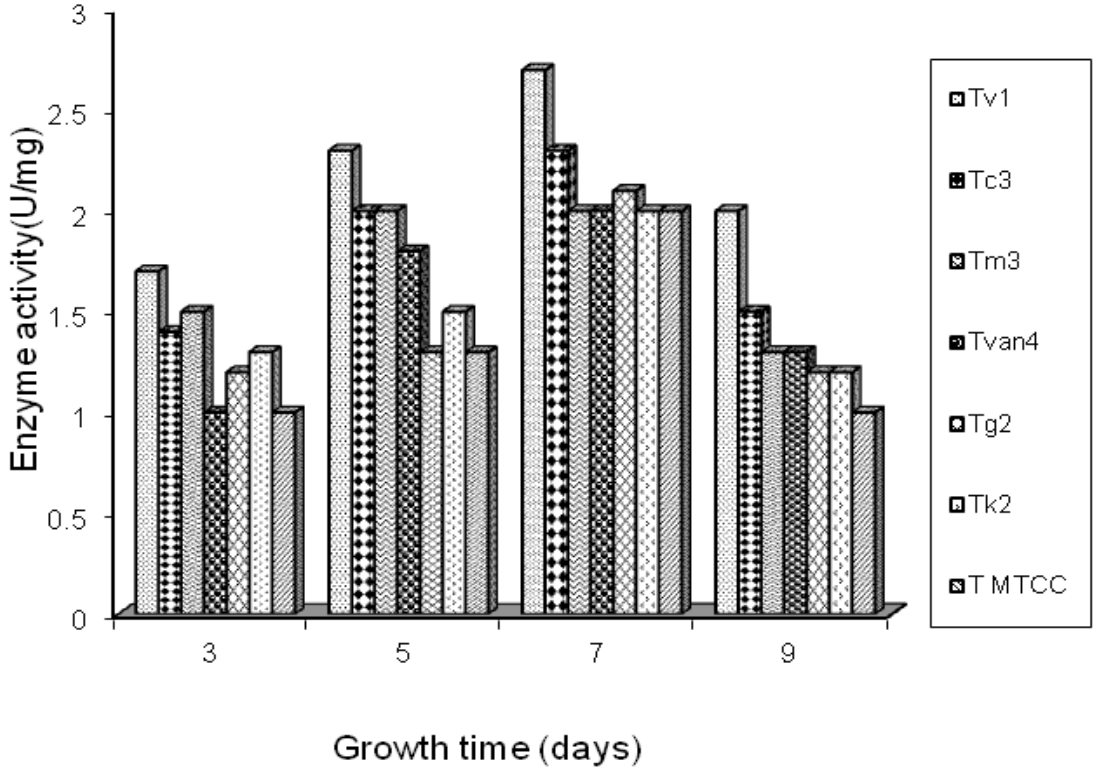

Figure 1. Comparative analysis of Amylase synthesized by *T. atroviride* isolates.

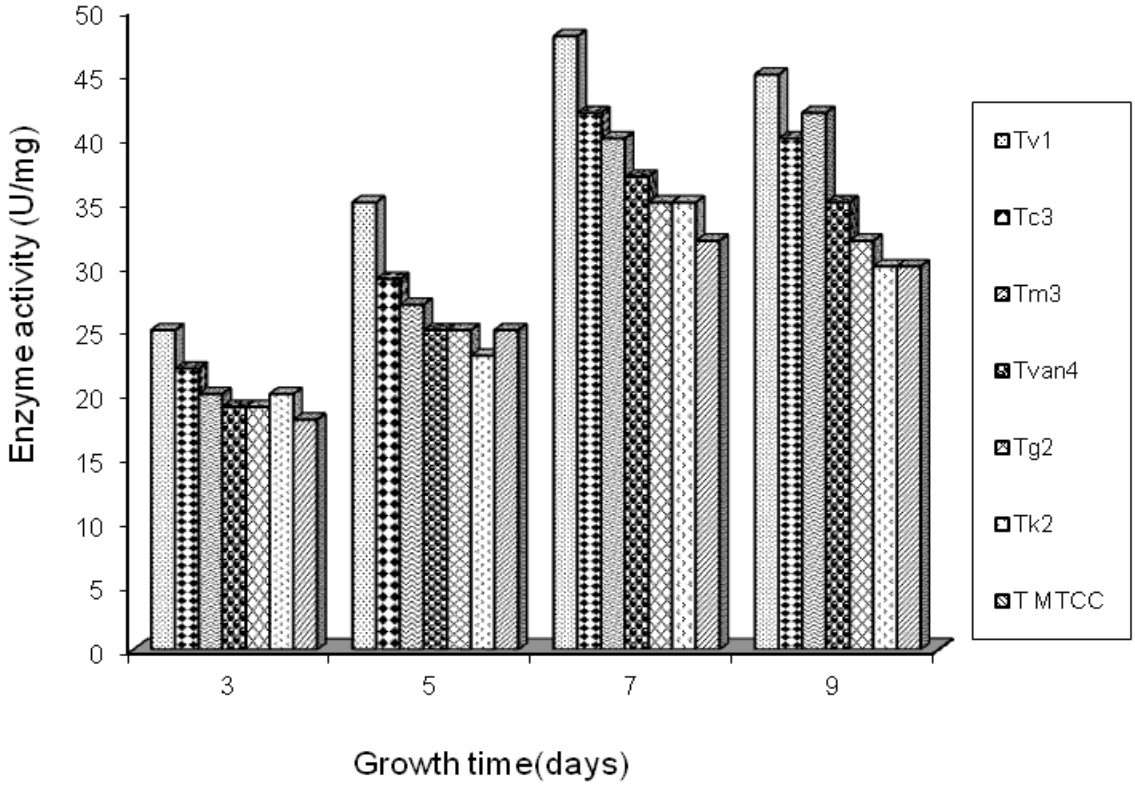

Figure 2. Comparative analysis of cellulase synthesized by *T. atroviride* isolates.

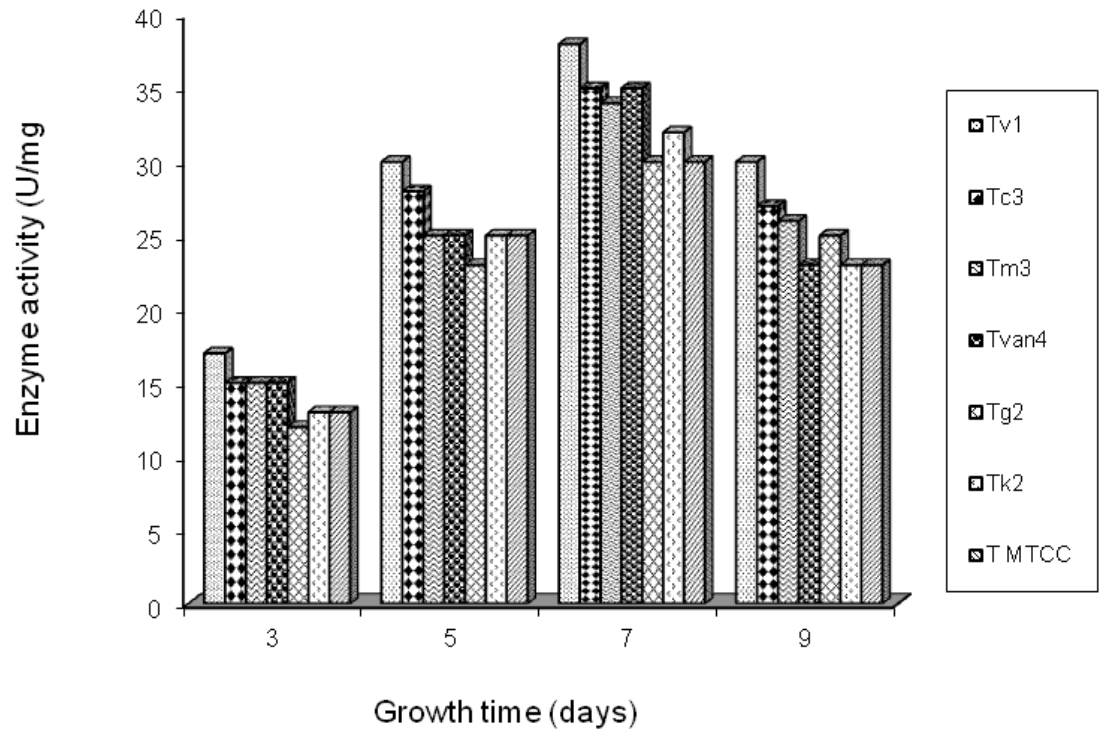

Figure 3. Comparative analysis of polygalacturonase synthesized by *T. atroviride* isolates.

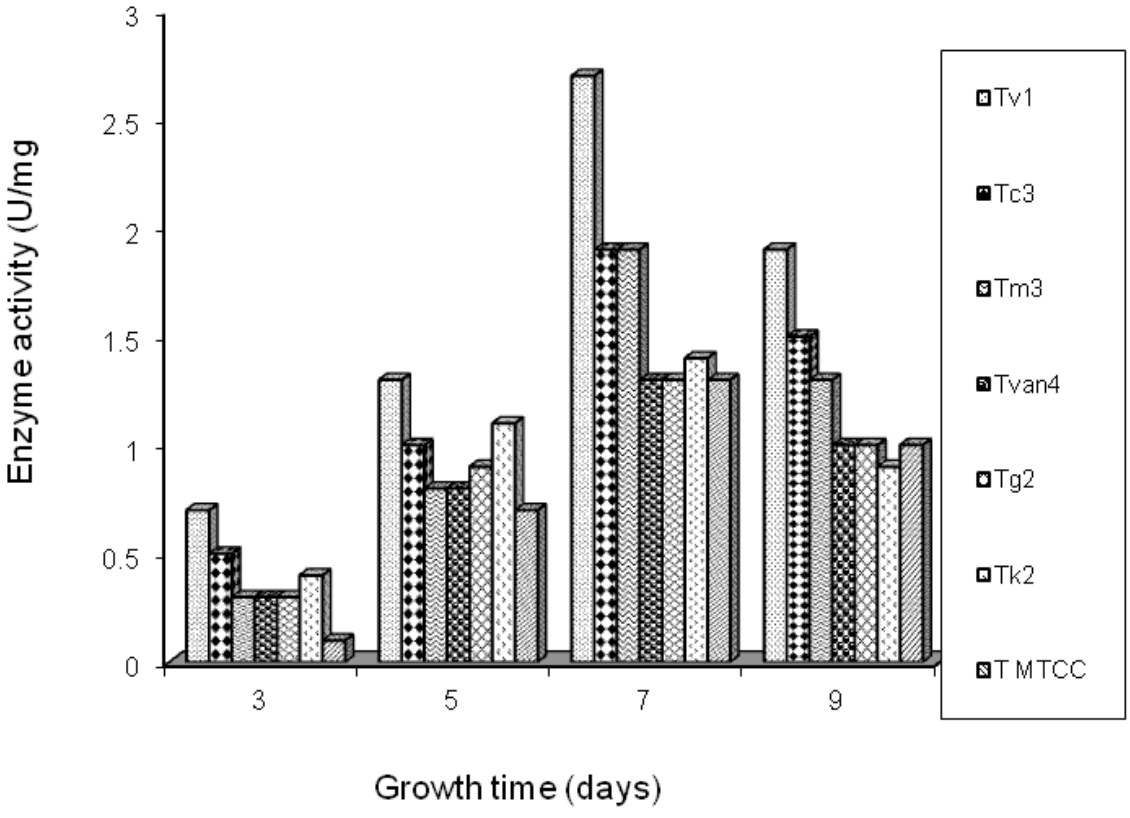

Figure 4. Comparative analysis of protease synthesized by *T. atroviride* isolates.

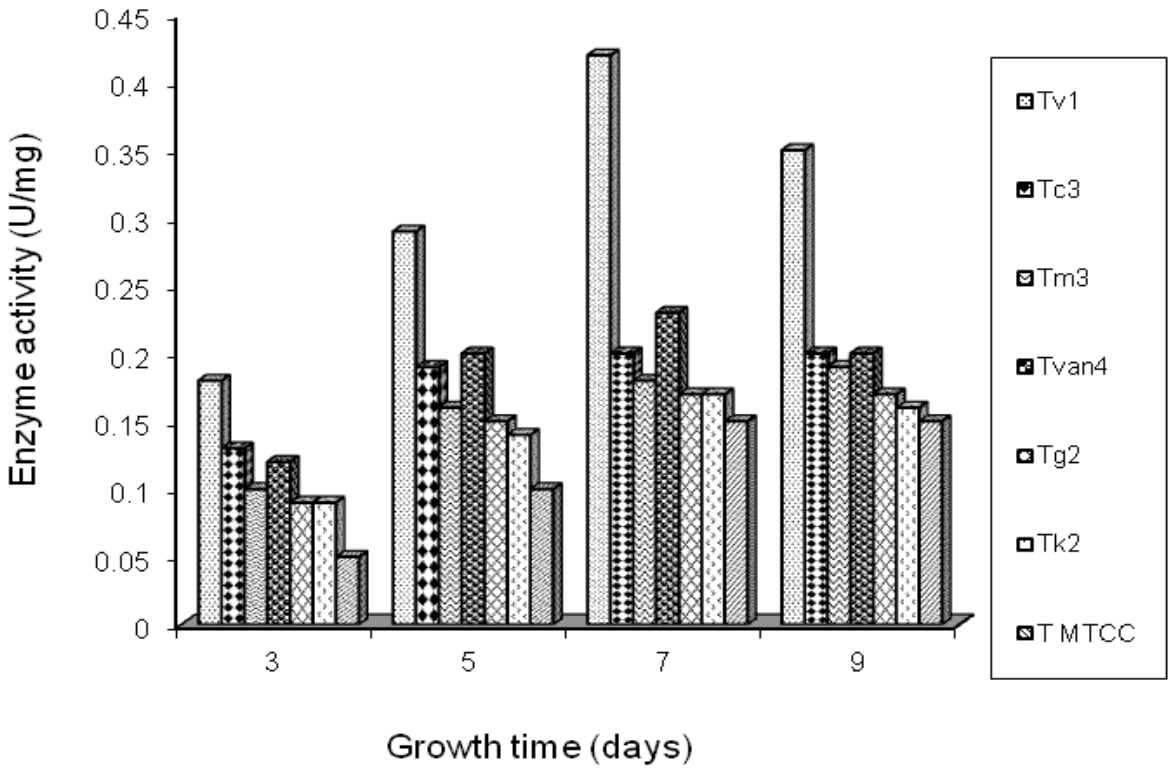

Figure 5. Comparative analysis of chitinase synthesized by *T. atroviride* isolates.

isolate in controlling the growth of *P. theae*. The afore-mentioned results highly correlate with recent research conducted by Haggag et al. (2006) that proteases secreted by *Trichoderma harzianum* were effective in reducing brown spot disease severity and pathogen sporulation on faba bean leaves inoculated with *Botrytis fabae*. Cell wall of the phytopathogen includes chitin and other complex macro molecules. In order to metabolize these compounds, the antagonistic fungi need to synthesize chitinase to break chitin and other allied compounds. Hence quantification of this enzyme relates to the extent of mycoparasitism shown against the pathogen.

In this present study, all the isolates showed least amount chitinolytic activity and no significant difference was observed, except Tv1 isolate which showed maximum of 0.42U mg-1 on 7^{th} of production (Figure 5). All the enzyme productions were found to be proportional with mycelium dry weight of the subsequent isolates. The present study evidently proves that *T. atroviride* Tv1 isolate showed various extracellular enzymatic activities such as cellulolytic, amylolytic, chitinolytic, pectinolytic and proteolytic in substantial quantity than compared to other isolates. These findings are found to be parallel with the work done by Gachomo and Kotchoni (2008) which reveals that, the more diverse enzymatic activities, the more effective are the antagonist in suppressing the growth of pathogen.

Analysis of secondary metabolites through GC-MS

Characteristics of secondary metabolites synthesized by Tv1 *T. atroviride* through GC-MS are specified in Table 2. The culture filtrate of the antagonist showed the presence of several compounds such as N-phenylethylenediamine (Figure 7a), phenol, 2-(2-benzoxazolyl) (Figure 7b), phthalic acid (Figure 7c), diallylamine (Figure 7d), 1,2,4,5-tetrazine, 3,6-bis(1-methylethyl) (Figure 7e), propanal, 2-methyl-oxime (Figure 7f), N-(2-propynyl)-2,2-dimethylaziridine (Figure 7g). The chromatogram analysis of GC-MS (Figure 6) illustrated that phthalic acid, diallylamine and 1,2,4,5-tetrazine3,6-bis (1-methylethyl) obtained at the retention time of 20.91, 21.83 and 23.26, respectively showed higher elevated peaks. These secondary metabolites perhaps serve the antagonist for survival function by competing against the pathogen.

The results of the present study support the consi-deration of the work done by Dubey et al. (2011) where the presence of wide range of secondary metabolite obtained from the cultural filtrate of *T. harzianum*, *T. viride* and *T. virens* which were responsible for biological control of *Rhizoctonia solani*. To conclude the study, Tv1 *T. atroviride* was able to produce various defense enzymes and secondary metabolite containing antibiotics to substantial amount under *in vitro* conditions and found to be highly significant compared to other isolates. Additional researches are to be carried out with this

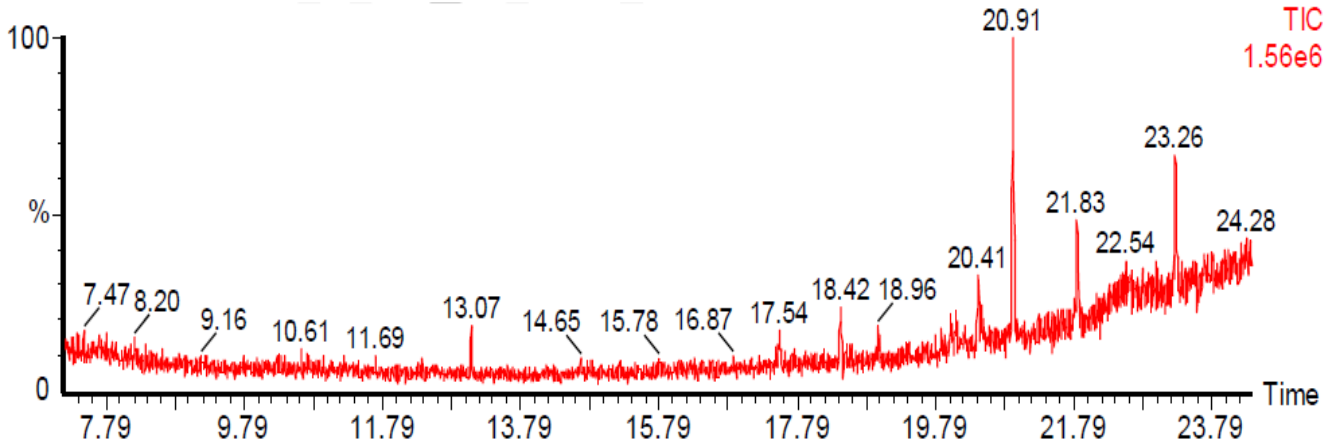

Figure 6. GC-MS chromatogram of Tv1 *T. atroviride* isolate obtained from Valparai region.

Table 2. Antifungal compounds identified through GC-MS obtained from the culture filtrate of Tv1 *T. atroviride* isolate.

S/N	Compounds identified	Retention time	Molecular weight of the compound
1	N-Phenylethylenediamine	13.07	136
2	Phenol, 2-(2-benzoxazolyl)-	18.42	211
3	Phthalic acid	20.91	222
4	Diallylamine	21.83	97
5	1,2,4,5-Tetrazine, 3,6-bis(1-methylethyl)-	23.26	66
6	Propanal, 2-methyl-oxime	24.67	87
7	N-(2-Propynyl)-2,2-dimethylaziridine	26.06	109

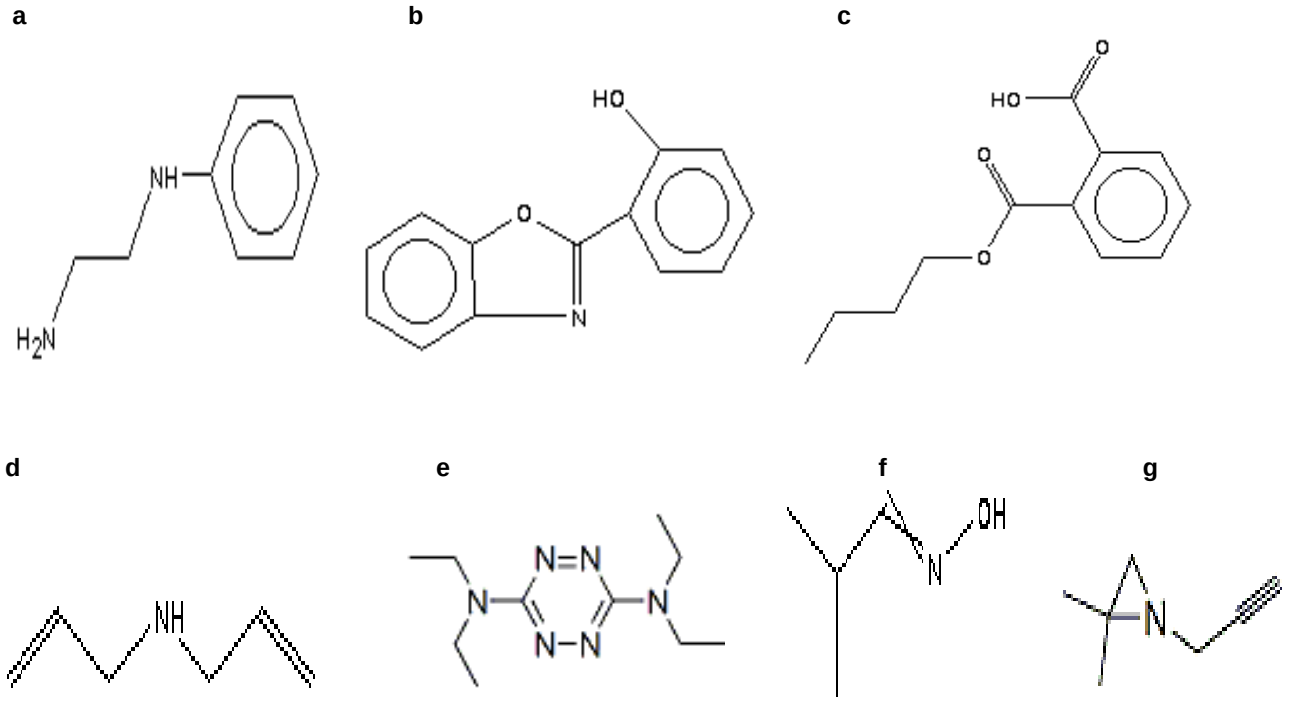

Figure 7. (a) N-Phenylethylenediamine; (b) phenol, 2-(2-benzoxazolyl)-; (c) phthalic acid; (d) diallylamine; (e) 1,2,4,5-tetrazine, 3,6-bis (1-methylethyl)-; (f) propanal, 2-methyl-oxime; (g) N-(2-propynyl)-2,2-dimethylaziridine.

isolate for optimization these bioactive compounds at various physico-chemical conditions, so that prospective of this novel isolates may be utilized for various biofungicide formulations.

ACKNOWLEGEMENTS

We would like to express our special thanks to Lion Dr. K. S. Rangasamy, Chairman. K. S. R. institutions and Dr. K. Thyagarajah, Principal, K. S. Rangasamy College of Technology, Tiruchengode, Tamilnadu, India, for providing facilities and encouragement.

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