Research Paper

Determination of lytic enzyme activities of indigenous *Trichoderma* isolates from Pakistan

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Abstract

This study investigated lytic enzyme activities in three indigenous *Trichoderma* strains namely, *Trichoderma asperellum, Trichoderma harzianum* and *Trichoderma* sp. Native *Trichoderma* strains and a virulent strain of *Rhizoctonia solani* isolated from infected bean plants were also included in the study. Enzyme activities were determined by measuring sugar reduction by dinitrosalicylic acid (DNS) method using suitable substrates. The antagonists were cultured in minimal salt medium with the following modifications: medium A (1 g of glucose), medium B (0.5 g of glucose + 0.5 g of deactivated *R. solani* mycelia), medium C (1.0 g of deactivated respective antagonist mycelium) and medium D (1 g of deactivated *R. solani* mycelia). *T. asperellum* showed presence of higher amounts of chitinases, β-1, 3-glucanases and xylanases in extracellular protein extracts from medium D as compared to medium A. While, the higher activities of glucosidases and endoglucanses were shown in medium D extracts by *T. harzianum*. β-glucosidase activities were lower compared with other enzymes; however, activities of the extracts of medium D were significantly different. *T. asperellum* exhibited maximum inhibition (97.7%). On the other hand, *Trichoderma* sp. did not show any effect on mycelia growth of *R. solani* on crude extract.

Key words: lytic enzymes, mycoparasitic activity, *Rhizoctonia solani*, *Trichoderma*.

Introduction

Soil-borne pathogens cause diseases that result in significant loss of quality and yield of the valuable crops around the globe. Fungi, being the most aggressive soil-borne pathogens, have been intensely investigated due to inflicting damage to major crops. The main pathogenic fungal genera involved in such damage are: *Phythium, Botrytis, Rhizoctonia and Fusarium* (Djonovic *et al.*, 2007). Pesticides have been widely used to control these pathogens (Gerhardson, 2002) but their applications have been increasingly criticized due to environmental and human health concerns (Punja and Utkhede, 2003; Bues *et al.*, 2004).

In Pakistan, being an agricultural country, almost all of the economically important crops are affected by phytopathogenic fungal species such as *R. solani, Fusarium moniliforme*, and *Fusarium solani* (Ahmed *et al.*, 1997). *R. solani* lives in subterranean forms, which makes it the most resistant pathogen. Hence, chemical control is ineffective, unless highly selective fungicides are used. The search for novel bio-control agents, therefore, is a prime target of many plant pathologists (Harman *et al.*, 2004); and *Trichoderma* species, such as *T. harzianum*, *Trichoderma hamatum*, *Trichoderma reesei* or *Trichoderma virens,* are recommended by many researchers as preferred choice for controlling phytopathogens (Punja and Utkhede 2003;

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Steyaert *et al.*, 2003; Harman *et al.*, 2004; Montealegre *et al.*, 2010; Castro *et al.*, 2014). .

The genus *Trichoderma* includes cosmopolitan saprophytic fungi found in soil and have long been known to be effective against plant pathogen, *R. solani* (Weindling, 1932). *Trichoderma* acts through direct fungal penetration (Asad *et al.*, 2014) and/or by secreting antifungal compounds, such as hydrolytic enzymes, in order to inhibit the growth of phytopathogens. For instance, *T. harzianum* releases hydrolytic enzymes against *Crinipellis perniciosa*, the causative agent of cocoa (*Theobroma cacao*) disease (De Marco *et al.*, 2003). Due to the significant role of hydrolytic enzymes in the biocontrol activity of *Trichoderma* species against *R. solani,* the present study was undertaken to assess the activities of hydrolytic enzymes from three *Trichoderma* species. Furthermore, the antagonistic potential (antibiosis) of metabolites, obtained from crude extracts of selected isolates, was studied.

Materials and Methods

Microorganisms

Three *Trichoderma* strains (*Trichoderma asperellum, Trichoderma harzianum* and *Trichoderma* sp.) isolated from native agricultural soils were obtained from Fungal Culture Bank of the University of the Punjab, Lahore, Pakistan. A virulent strain of *R. solani* previously isolated from the infected bean plants was courteously supplied by Agroinnova culture bank, University of Torino, Italy (Minuto *et al.*, 2008; Asad *et al.*, 2014). All microbial cultures were grown and maintained on potato dextrose agar (Difco, Becton, Dickinson, Sparks, MD) at 4 °C. The antagonists were cultured in the minimal salt medium according to the method of Lilly and Barnett (1951) for chitinase, xylanase, β-1, 3-glucanase, endoglucanase (CMCase) and β-glucosidase activities. Minimal salt medium (Tseng *et al.*, 2008) was used with minor modifications as medium A (1 g of glucose), medium B (0.5 g of glucose + 0.5 g of deactivated *R. solani* mycelia), medium C (1.0 g of deactivated respective antagonist mycelium) and medium D (1 g of deactivated *R. solani* mycelia). Fifty milliliters of each of the mentioned media was incubated at 25 °C on a rotary shaker at 150 rpm for 0, 24, 48, 72, 96 and 120 h. Filtrates were collected by centrifugation at 3000 x *g* for 10 min at 4 °C and the filtrate was used for enzyme activity assays.

Deactivation of mycelium

Mycelia from seven-day old cultures of *Trichoderma* and *R. solani* were collected by centrifugation (3000 x *g*) for 10 min and subsequently washed twice with 50 mL of sterile and deionized water. The collected mycelia were boiled twice for 20 min to obtain the deactivated mycelia and stored at -20 °C until used.

Enzyme activity assays

Enzyme activities of *Trichoderma* strains were determined by using dinitro-salicylic acid (DNS) reagent (Miller, 1959) and sugar reduction in the respective substrates was measured.

Chitinase activity

Chitinase activity was determined according to Tseng *et al.* (2008), an artificial substrate containing 10 μL of 0.5% 4-nitrophenyl *N, N'*-diacetyl-β-D-chitobioside (Sigma-Aldrich, USA) and 250 μL of the enzyme samples were mixed in 250 μL of 100 mM acetate buffer having pH 5. After 30 min, 50 μL of 0.4 M Na_2CO_3 was added to terminate the reaction and turbidity (OD) was measured at 415 nm. One unit of the enzyme activity was defined as the amount of enzyme, required to produce 1 mmol of the product per milligram of the protein per hour.

Beta-1, 3-glucanase activity

Enzyme activity was quantified according to Masih and Paul (2002) by incubating 250 μL of the enzyme samples and 250 μL of 1% laminarin dissolved in 0.2 M acetate buffer (pH 5) at 50 °C for 40 min. The reaction was terminated by 500 μL of DNS reagent and kept for 10 min in a boiling water bath. After boiling, the solution was diluted by adding 4 mL of distilled water and after cooling, the amount of the reducing sugars was measured at 540 nm using D-glucose as benchmark. Specific activity of the enzyme was manifested as mmol of glucose released per milligram of the protein per hour.

Beta-glucosidase activity

Beta-Glucosidase activity was determined by the method of Tokao *et al.* (1985). The reaction mixtures were prepared by adding 250 μL enzyme sample and 400 μL of 17 mmol L^{-1} salicin solution dissolved in 0.2 mol L^{-1} sodium acetate buffer (pH 4.6). 250 μL distilled water was added to the solution and incubated at 50 °C for 40 min. Optical density was recorded at 540 nm using glucose as standard. Specific activity of the enzyme was expressed as mmol of glucose released per milligram of the protein per hour.

Xylanase activity

For xylanase activity, 100 μL enzyme sample was mixed with 500 μL of 1% oat spelt xylan dissolved in 0.1 mol L^{-1} phosphate buffer (pH 7), and then 400 μL of 0.1 mol L^{-1} phosphate buffer (pH 7) was added (Bailey *et al.*, 1992). The resultant mixture was incubated at 30 °C for 20 min, cooled, and turbidity was recorded at 540 nm with D-xylose as standard. One unit of enzyme activity was defined as 1 mmol of xylose released per milligram of the protein per hour.

Endoglucanase activity

Endoglucanase activity was determined as described by Ko *et al.* (2005). Briefly, 250 μL enzyme sample was added to 250 μL of 1% carboxymethyl cellulose dissolved in 0.2 mol L^{-1} sodium acetate buffer (pH 5). The enzyme containing mixture was incubated at 50 °C for 40 min and terminated by adding 1.5 mL of DNS reagent. Turbidity was recorded at 540 nm using glucose as a standard. Enzyme activity was defined as mmol of xylose released per milligram of the protein per hour.

Total protein concentration in enzyme solution was estimated using bovine serum albumin as standard (Bradford, 1976). The specific activity of the enzyme was expressed as the amount of enzyme that catalyzed formation of 1 mmol of the product per hour under the assay conditions. The experiments were performed twice with three replicates for each treatment.

Effect of crude extract on *R. solani* mycelial inhibition (Antibiosis)

Three discs of mycelial agar plugs (5 mm diameter) were inoculated in 100 mL potato dextrose broth (PDB). The samples were incubated for 7 days at 25 ± 1 °C on a rotary shaker at 100 rpm and then filtered through a Millipore filter (0.2 μm). The samples were sterilized by passing through a biological membrane filter (0.2 μm). Filtered supernatant (500 μL) from each of the *Trichoderma* species was spread over the surface of PDA plates and a 5 mm disc of *R. solani* was inoculated at the center of each plate. The plates were incubated at 25 °C until the colony spread over the surface of PDA plates in the control treatment (Dennis and Webster 1971). Radial growth of the pathogens was recorded on a daily basis. The percent inhibition of the average growth of mycelia in relation to the growth of the controls was calculated by using Equation 1 (Edington *et al.*, 1971).

$$\text{Mycelial inhibition (\%)} = \left[\frac{C_2 - C_1}{C_2}\right] \times 100 \qquad (1)$$

where C_1 = radial mycelial growth of *R. solani* in the presence of *Trichoderma*, and C_2 = radial mycelial growth of *R. solani* in control.

Experimental design and statistical analyses

The experiments were repeated twice in a complete randomized block design with four replicates for each treatment. The data were analyzed using SPSS software (version 17.0 Chicago IL, USA). Analysis of variance (ANOVA) was carried out with a significance defined at p < 0.05. Duncan's HSD multiple range test was used as a post-hoc analysis to compare means.

Results

Chitinases

Chitinase activity assays showed that all the antagonists cultured in medium D, (containing deactivated mycelium of *R. solani*) expressed the highest enzyme activity compared to other three media (Figure 1). The maximum activity (173 U mL^{-1}) was detected by *T. asperellum* in the extracts of medium D after 96 h of incubation, which was the highest among all the isolates. The medium B, containing glucose and deactivated mycelium of *R. solani*, also showed high activities (111 U mL^{-1}) as compared to medium A (glucose) and C (deactivated antagonist mycelium).

The maximum chitinase activity by *T. harzianum* was detected in medium D after 72 h of incubation (117 U mL^{-1}). The medium B showed maximum activity of 97 U mL^{-1} after 96 h of incubation. While the medium A and C expressed very low chitinases activities (Figure 1).

Trichoderma sp. also showed an increased chitinase activity (149 U mL^{-1}) until 96 h of incubation in the extracts of medium D. While in medium B, the activity increased (89 U mL^{-1}) until 72 h of incubation and then decreased. Significantly low chitinase activities were expressed in medium A and C (Figure 1).

β- 1, 3-glucanase

The assays for β-1,3-glucanase showed that all the antagonists were able to express much higher enzyme activity in extracts of medium D than the extracts of other media. After 48 h of incubation, *Trichoderma* sp. showed maximum activity of 149 U mL^{-1}, while *T. asperellum* and *T. harzianum* exhibited activity of 146 and 137 U mL^{-1}, respectively. *T. harzianum* maintained maximum activity till 120 h, while the activities decreased in the extracts of *T. asperellum* and *Trichoderma* sp. after reaching the maximum level (Figure 2). Very high activities were observed in the extracts of medium B. The activities dropped to minimum after 72 and 96 h of incubation, in the case of *T. asperellum* and *T. harzianum* respectively, while for *Trichoderma* sp., the activity retained at 102 U mL^{-1} until 120 h of incubation. The results showed that the glucanase activities of all the strains in extracts of medium A and C were non-significant (Figure 2).

β- glucosidase

Assays indicated that β-glucosidase activities were comparatively lower than that of the chitinase and glucanase. The enzyme activity was significantly higher in the extracts of medium D. The maximum activities were 92 U mL^{-1} by *T. asperellum,* and 91 U mL^{-1} by *T. harzianum* and *Trichoderma* sp. (Figure 3). These activities were expressed within first 24 h of the incubation, which decreased immediately thereafter. *T. asperellum* showed almost similar activities in all the three media, where maxi-
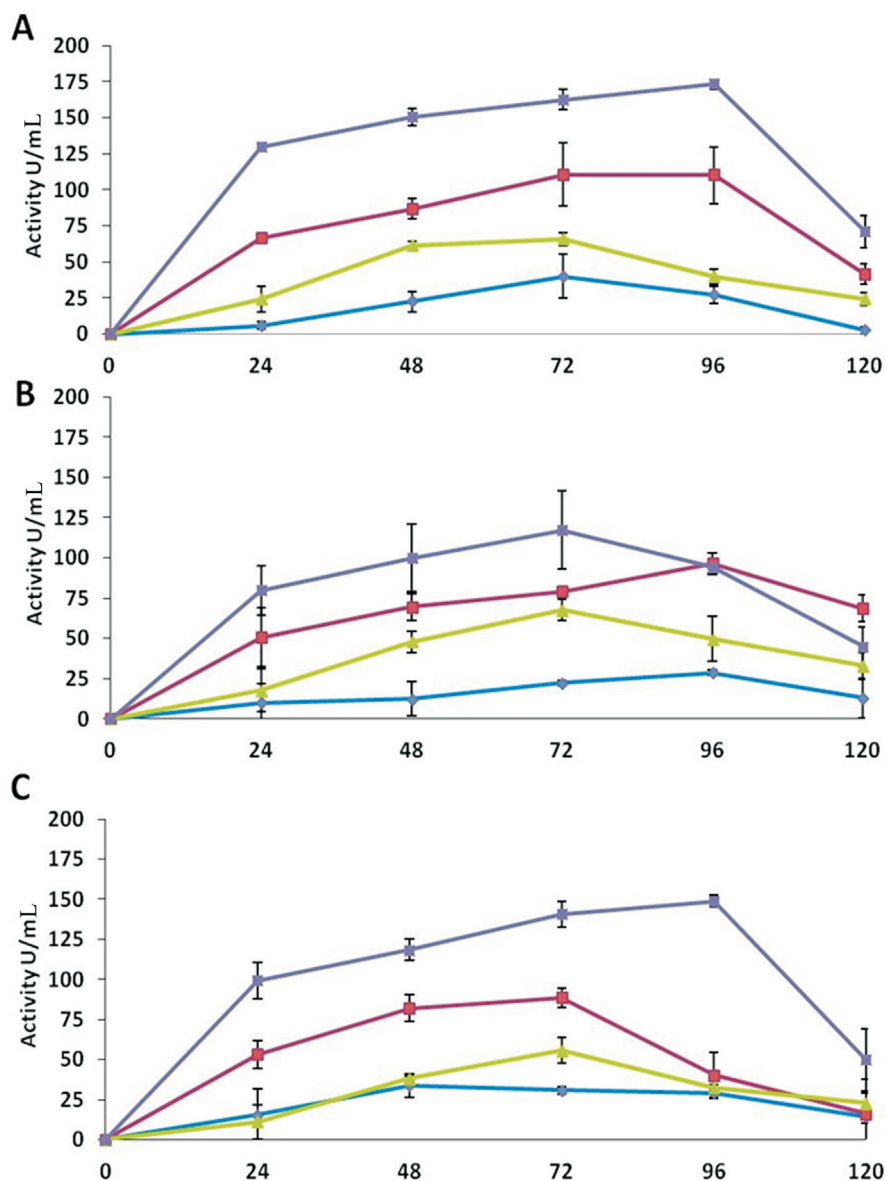
Figure 1 - Chitinase activities of the extracellular protein extracts of *Trichoderma* in different media over an incubation period of 120 h. A: *T. asperellum*, B: *T. harzianum*, C: *Trichoderma* sp. The activities of the enzyme are denoted in different media as: medium A (-◆-), medium B (-■-), medium C (-▲-), and medium D (-x -). Values are means of four replicates, and error bars represent SE.

mum activities were 51 U mL^{-1} in medium A after 72 h of incubation and 50 and 55 U mL^{-1} after 24 h of incubation in medium B and C, respectively. *T. harzianum* and *Trichoderma* sp. exhibited very low enzyme activities in the extracts of all the three media. These activities were also lower than the activities expressed by *T. asperellum* in the respective media (Figure 3).

Xylanase

All antagonists showed a higher xylanase activity in medium D compared to other media. *T. asperellum* exhibited maximum xylanase activity of 162 U mL^{-1} in the extracts of medium D after 72 h of incubation, which

decreased to 81 U mL^{-1} after 120 h of the incubation (Figure 4). The extracts from medium B exhibited activity of 140 U mL^{-1} at 72 h of the incubation and then decreased thereafter to 45 U mL^{-1}.

In the extracts of medium A and C, some activity was shown by *T. asperellum* after 72 h, which then diminished at the end of the incubation period. The xylanase activities by *T. harzianum* were also comparatively high in extracts of medium D. The maximum activity was 152 U mL^{-1} after 72 h of incubation. A significant activity was expressed by the extracts of medium B, while the extracts of medium A and C showed very low activities (Figure 4).
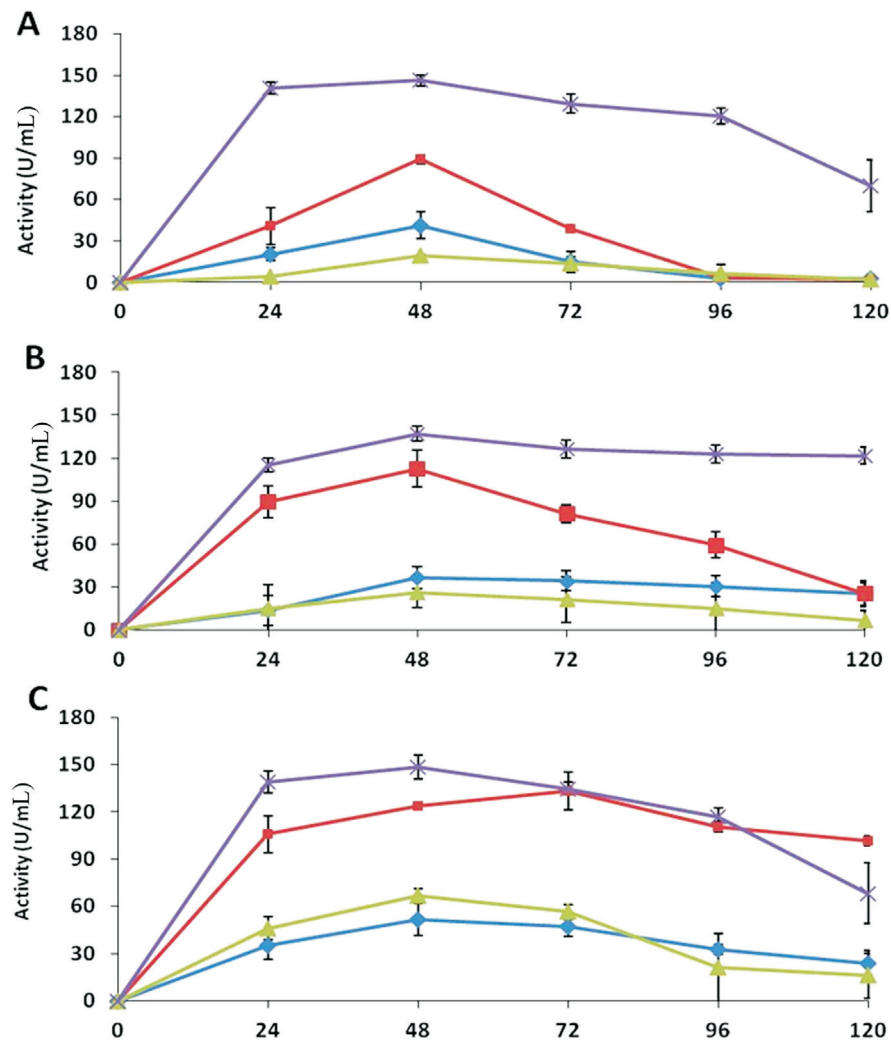
Figure 2 - β-1, 3-glucanase activities of the extracellular extracts of *Trichoderma* in different media over an incubation period of 120 h. A: *T. asperellum*, B: *T. harzianum*, C: *Trichoderma* sp. The activities of the enzyme are denoted in different media as: medium A (-◆-), medium B (-■-), medium C (-▲-), and medium D (-x -). Values are means of four replicates, and error bars represent SE.

Trichoderma sp. showed highest maximum xylanase activity of 175 U mL^{-1} in the extracts of medium D after 72 h of the incubation. The medium B also showed significantly higher activities upon induction by deactivated pathogen mycelium, while in the extracts of other media, the enzyme activities were comparable to other isolates (Figure 4).

Endoglucanase

As shown in Figure 5, *T. asperellum* showed much higher endoglucanase activity than other enzymes in the extracts of all media. There was no effect of the presence of pathogen mycelium in medium D compared with the extracts of medium A, containing only glucose, the same level of enzyme activity was exhibited.

The maximum activities in medium A and B were 147 and 139 U mL^{-1}, respectively; while in media C and D both showed about 153 U mL^{-1} activity after 48 h of incuba-

tion. At the end of the time course, the activities significantly decreased to 77, 80, 63 and 20 U mL^{-1} in medium A, B, C, and D, respectively (Figure 5).

T. harzianum showed higher activities in extracts of medium D. The maximum enzyme activity (194 U mL^{-1}) was expressed within first 24 h of the incubation and then decreased to 143 U mL^{-1} after 120 h. In other media, the activities increased after 48 h of incubation to 144, 131 and 145 U mL^{-1} in medium A, B, and C, respectively; these activities were retained as 114, 104 and 75 U mL^{-1} for the three media, respectively, after 120 h (Figure 5). *Trichoderma* sp. also showed high endoglucanase activity (214 U mL^{-1}) in medium D extracts after 72 h of incubation. In medium A, B and C maximum endoglucanase activities were 151, 145 and 128 U mL^{-1} after 48 h of incubation, which decreased to 116, 99 and 92 U mL^{-1}, respectively, by the end of the time course (Figure 5).
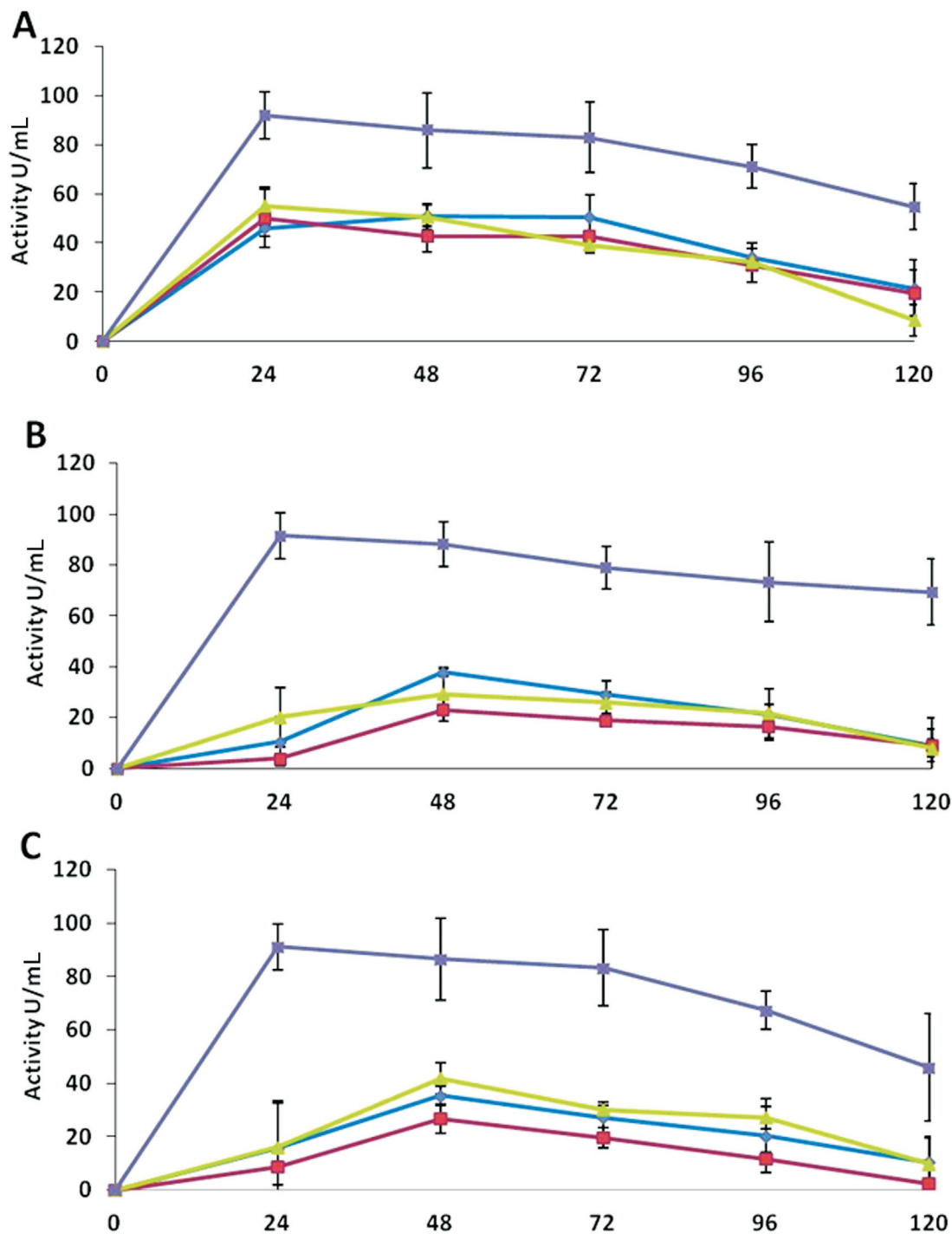
Figure 3 - β- glucosidase activities of the extracellular protein extracts of *Trichoderma* species in different media over an incubation period of 120 h. A: *T. asperellum*, B: *T. harzianum*, C: *Trichoderma* sp. The activities of the enzyme are denoted in different media as: medium A (-◆-), medium B (-■-), medium C (-▲-), and medium D (-x -). Values are means of four replicates, and error bars represent SE.

Effect of crude extracts of different species of *Trichoderma* on the growth inhibition of *R. solani*

The effect of the metabolites present in the crude extracts of the media was statistically significant for all the strains (p < 0.05). *T. asperellum* was most effective to inhibit the mycelial growth of *R. solani* by 97.7%, while *T. harzianum* was effective with 72.7% of inhibition, and *Trichoderma* sp. was failed to show any effect on the mycelial growth (Table 1).

Table 1 - Effect of crude extracts of different *Trichoderma* species on growth inhibition of *R. solani*.

Isolates	Radial growth of *R. solani* (cm)			% mycelial inhibition
	24 h	48 h	72 h	
T. asperellum	0.0 ± 0.0*	0.0 ± 0.0	0.1 ± 0.0	97.7a**
T. harzianum	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	72.7b
Trichoderma sp.	1.3 ± 0.2	2.5 ± 0.2	3.4 ± 0.0	22.7c
Control (*R. solani*)	1.5 ± 0.3	3.4 ± 0.2	4.4 ± 0.1	-

*The results are the mean value (± SE) of two independent experiments, each with four replicates.
**Values followed by the different letters are statistically different by Duncan's multiple range test (p < 0.05).
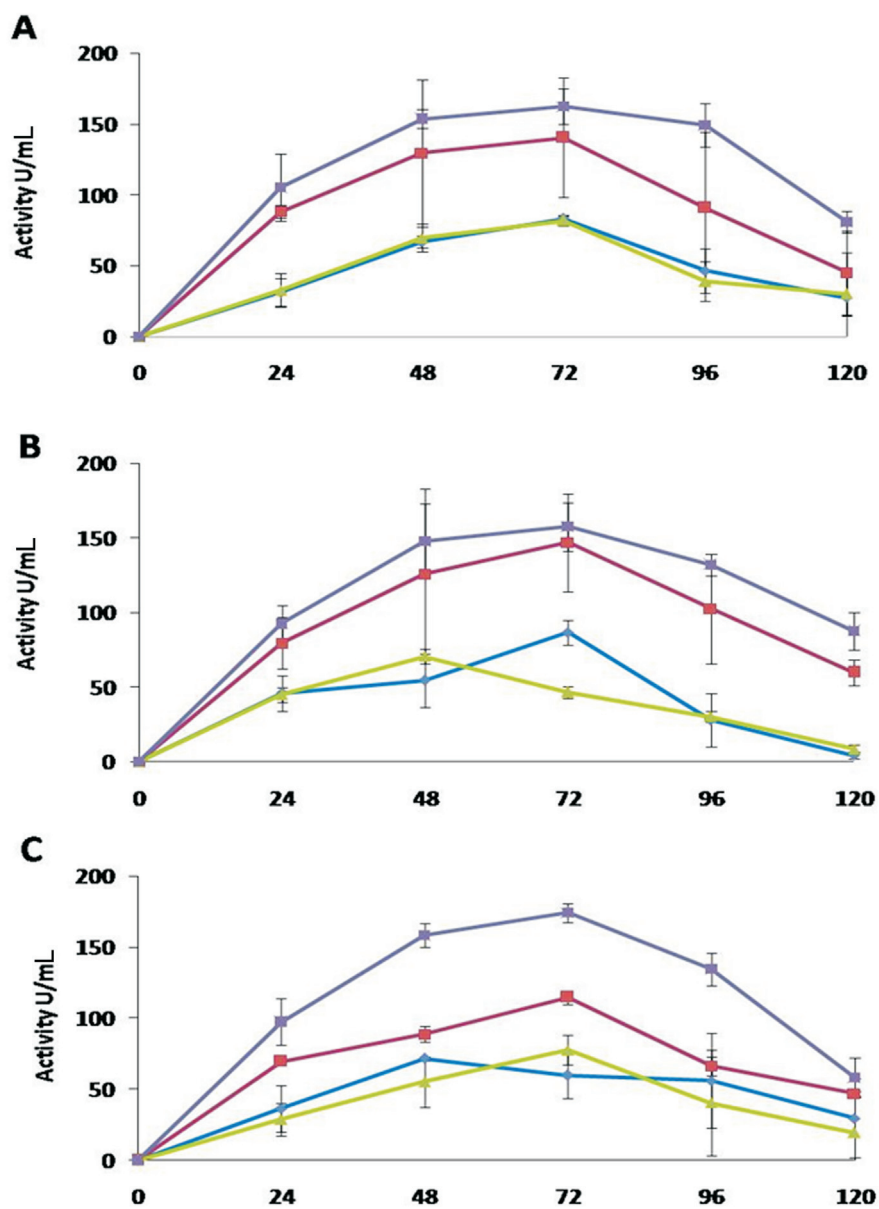

Figure 4 - Xylanase activities of the extracellular protein extracts of *Trichoderma* in different media over an incubation period of 120 h. A: *T. asperellum*, B: *T. harzianum*, C: *Trichoderma* sp. The activities of the enzyme are denoted in different media as: medium A (-◆-), medium B (-■-), medium C (-▲-), and medium D (-x -). Values are means of four replicates, and error bars represent SE.
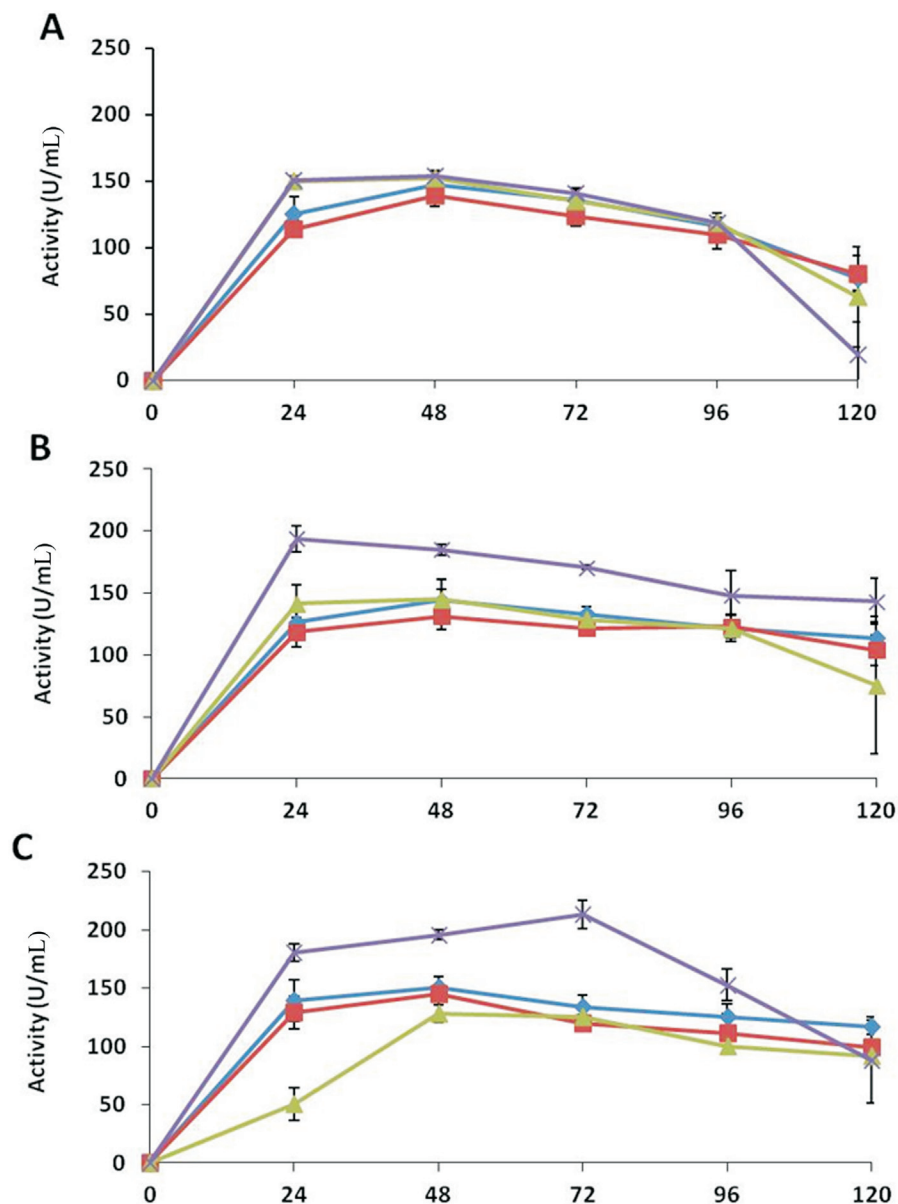
Figure 5 - Endoglucanase activities of the extracellular protein extracts of *Trichoderma* in different media over an incubation period of 120 h. A: *T. asperellum*, B: *T. harzianum*, C: *Trichoderma* sp. The activities of the enzyme are denoted in different media as: medium A (-◆-), medium B (-■-), medium C (-▲-), and medium D (-x -). Values are means of four replicates, and error bars represent SE.

Discussion

Apart from other mechanisms, antagonistic ability of *Trichoderma* also includes production of lytic enzymes that hydrolyze the cell wall of the host fungus (Woo *et al.*, 2006). In most fungi, chitin and glucans are the most abundant microfibrilar components in the cell wall, while proteins and glucans act as cementing matrix (Peberdy, 1990). In the current study, deactivated *R. solani* mycelium was used rather than cell walls of the pathogen to simulate production of natural metabolites by *Trichoderma* which resulted in significant activities of chitinase, xylanase and β-1, 3-glucanase. The higher activities of extracellular enzymes in medium D and B as compared to medium A (containing only glucose) illustrate that these enzymes were induced by the presence of deactivated mycelium of *R. Solani*, this observation concords with the study of Tseng *et al.* (2008). This behavior can be correlated to the reduction of *R. solani* mycelium after seven days of incubation, when co-inoculated *in vitro*. Chitin is the major component of fungal cell walls (Free, 2013). Hence, the chitinases were considered to be involved in the degradation of cell wall of *R. solani* by *T. virens* (Baek *et al.*, 1999). Simi-

lar observations were recorded by Viterbo *et al.* (2002), where Chitinase catalyzed cleavage of β-1,4 linkages between N-acetyl-β-D-glucosamine units. Our results demonstrated that *T. asperellum* produced higher amount of chitinases compared with other species, and *T. harzianum* produced lowest one. The enzyme activities were observed until 96 h and 72 h of incubation by *T. asperellum* and *T. harzianum*, respectively, which decreased significantly after 120 h (Figure 1). De Marco *et al.* (2003) also reported increase in chitinase activity by *Trichoderma* isolates within 72 h of incubation. The chitinolytic system of *Trichoderma* comprises many enzymes, including 1,4-β-acetylglucosaminidase, endochitinases, and exochitinases (Brunner *et al.*, 2003). The chitinases are thought to be the major enzyme reported participating in mycoparasitic interaction. In current study, increased production of the enzymes upon induction by pathogen's deactivated mycelium indicated their possible role in antagonistic activity in dual culture assays. Harighi *et al.* (2007) purified chitinase-42 (Chi42) from *Trichoderma atroviride,* which showed a strong potential to lyse *R. solani* cell wall and inhibit mycelial growth. *T. virens* mutants over-expressing Chi42 showed an enhanced biocontrol activity against *R. solani* in cotton seedlings compared to the wild types (Howell, 2003). Haran *et al.* (1996) detected the presence of GlcNAcases (1,4-β-acetylglucosaminidase), Chit73, and Chit102 in *T. harzianum* TM and *T. asperellum*. However, Kullnig *et al.* (2000) reported that endochitinases were regulated by *chit33*, *chit36* and *chit42* genes during stress. Dana *et al.* (2001) suggested that *chit33* is not expressed during overgrowth of *R. solani*, but expressed only during the contact phase; whereas, *chit36Y* expression does not require direct contact with the pathogen. Two proteins, 1,3-β-glucosidase and a 42 kDa endochitinase, were identified by Grinyer *et al.* (2004) in the culture supernatant of *T. Atroviride*, grown in the medium containing cell walls of *R. solani* as the sole carbon source.

The major component of *R. solani* cell wall has been identified as β-glucan in addition to chitin (Lahsen *et al.*, 2001); therefore, glucanases can play important role in antagonistic activity. β-1,3-glucanases cleave β-1,3 linkages between two molecules of glucose (Viterbo *et al.*, 2002). In the present study, medium D, containing deactivated mycelium of the pathogen, showed highest activity of β-1,3-glucanase for all the three isolates; and, in addition, *T. harzianum* retained the activity to a maximum level throughout the incubation period (Figure 2). In contrast to this, De Marco *et al.* (2003) reported increase in glucanase activity by *Trichoderma* isolates within 72 h of incubation. De La Curz *et al.* (1995) and Vázquez-Garcidueñas *et al.* (1998) also showed an increased activity of glucanases by *Trichoderma* sp. within 48 h of induction, in the presence of different substrates having hydrolytic ability in combination with other enzymes. However, Lorito *et al.* (1994) pu-

rified an endo-β-1,3-glucosidase involved in the inhibition of spore germination of *B. cinerea,* establishing role of glucanase in mycoparasitism. El-Katatny *et al.* (2001) also purified a glucanase having a potential to inhibit the growth of *S. rolfsii* from *T. harzianum* isolate T-24. Therefore, the activities of glucanases by all *Trichoderma* species can be speculated to be involved in the antagonistic potential in dual culture assays.

In the present study, all tested *Trichoderma* species showed some activity in medium A containing only glucose until 48 h of incubation and then decreased (Figure 2). However these findings are contrary to Ramot *et al.* (2000), who concluded that glucose inhibited the secretion of β-1,3-glucanases. Similarly, Tseng *et al.* (2008) hypothesized that *Trichoderma* secrets β-1,3-glucanases at a very low level to detect long-chain β-1,3-glucans. The enzymes are secreted for a short period in a glucose-rich medium and degrade if no polysaccharides with β-1,3-linkages are present. However, in a β-1,3-glucan-rich medium, oligosaccharides with β-1,3-linkages are generated that induce β-1,3-glucanases to cleave long chains of β-1,3-glucan producing glucose, which disintegrates cell wall of the pathogen. Xylanase catalyzes hydrolysis of xylan to xylose, and thus xylan is used as a nutrient source but its role in bio-control is not yet established. *Trichoderma reesei* produces two specific, xylan-inducible xylanases encoded by *xyn1* and *xyn2* to degrade the β-1,4-D-xylan backbone of hemicelluloses (Zeilinger *et al.*, 1996; Mach *et al.*, 1996). The role of the enzymes in bio-control has not been studied possibly due to the fact that *R. solani* cell wall lacks xylan. All the tested strains of *Trichoderma* showed a high activity of xylanase, while grown in a medium containing deactivated pathogen mycelium (Figure 4). Therefore, β-1,3-glucanase activities by *Trichoderma* species were assumed to exhibit xylanase activity, which was in agreement with Tseng *et al.* (2008). It might have contributed to the combined antagonistic activities of *Trichoderma* strains against *R. solani* in dual culture assays.

Trichoderma species are known to produce cellulases and β-glucosidase which hydrolyze β-1,4-glucans (De Marco *et al.*, 2003). The *cre1* gene in the filamentous fungi *Trichoderma reesei* and *T. harzianum* regulates cellulase expression (Ilmén *et al.*, 1996). The mycoparasitic interaction relieves binding of the Cre1 carboncatabolite repressor protein to promoter sequences of *ech42* (endochitinase-encoding) gene in *T. harzianum* (Strauss *et al.*, 1995; Lorito *et al.*, 1996; Portnoy *et al.*, 2011; Silva-Rocha *et al.*, 2014). β-1,4-glucans contribute to the cell wall of the *R. solani* (Lahsen *et al.*, 2001). In the present study, *T. harzianum* was found to produce β-glucosidase and cellulase enzymes on induction by deactivated mycelium of the pathogen within 24 h of growth, which contributed the antagonistic behavior in dual culture assay. However, *T. asperellum* did not show any significant effect on induction by deactivated

mycelium on the activity of cellulytic enzymes, suggesting that the major enzymes involved in the mycoparasitism by *T. asperellum* do not include cellulases. De Marco *et al.* (2003) reported increased activities of cellulytic enzymes within 24 h of incubation in the presence of specific substrates; *Trichoderma* may be involved in the hydrolysis of cell wall of pathogen, which differs from Tseng *et al.* (2008), who reported an insignificant cellulose activity by *T. harzianum*.

Mycoparasitism is associated with the production of cell wall-degrading enzymes such as chitinase and glucosidase and their induction as a response to infection by pathogen (Zhang *et al.*, 2010); and these two classes of the hydrolytic enzymes show synergistic activity against several pathogenic fungi (Qin *et al.*, 2003). The cell wall-degrading enzymes of *Trichoderma* are of special importance to induce the defense mechanisms of plants (Jayalakshmi *et al.*, 2009). Abd-El-Khair *et al.* (2010) also reported the induction of enzymes such as chitinase and peroxidase by *Trichoderma*, which play an important role in the defense mechanisms of plants against pathogens.

The production of antibiotics as well as secondary metabolites was determined by applying crude cell free extract of *Trichoderma* indigenous isolates on pathogen growth to establish antagonistic potential. The inhibition of mycelia growth of *R. solani* by crude extracts produced in liquid medium was significantly different among *Trichoderma* species. *T. asperellum* showed maximum inhibition of 97%, while *Trichoderma* sp. did not show any effect on mycelia growth of the pathogen (Table 1), which indicated that the secondary metabolites/antibiotics production can be related to the combined antagonistic activity by *T. asperellum* against *R. solani*. These results are in accordance with Castillo *et al.* (2011), who observed 100% mycelial growth inhibition of the pathogen by *T. asperellum*. Similar findings were reported by Etebarian (2006), showing the antifungal effect of metabolites secreted by *T. harzianum* and *T. virens* isolates causing 100% inhibition of mycelia growth of *Macrophomina phaseolina*. However, Vinale *et al.* (2006) isolated and characterized secondary metabolites from culture filtrates of two commercial *T. harzianum* isolates (T22 and T39) and reported their production in relation to mycoparasitic interaction with *R. solani*.

Conclusions

T. asperellum produced higher amounts of chitinases, β-1,3-glucanases and xylanases in a medium containing deactivated *R. solani* mycelium than the medium containing only glucose. *T. asperellum* showed maximum inhibition (97.7%) of mycelial growth of *R. solani*, while *Trichoderma* sp. did not show any effect. This study established antagonistic potential of *Trichoderma* using crude protein extract.

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