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Variability in production of extracellular hydrolytic enzymes by *Trichoderma* spp. and induction of resistance in Gram (*Cicer arietinum*)

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ABSTRACT: **Ten isolates of *Trichoderma* spp. isolated from rhizosphere region of different crops were evaluated for their ability to produce extracellular hydrolytic enzymes, *viz.*, β-1, 3 glucanase, chitinase and cellulase enzymes with different concentrations of carbon sources, pH, and temperature levels and the induction of systemic resistance in gram seedlings. Highest chitinase and β-1, 3 glucanase activity were observed in TR_2 isolate whereas TH_2 isolate exhibited highest activity of cellulase. TH_1 and TH_5 were the next best isolates in their chitinase enzyme activity. Similarly, TR_1 and TH_1 isolates followed the TR_2 for β-1, 3-glucanase activity. The activity of cellulase was highest in TH_2 isolate followed by TH_5 and TR_2, which were statistically significant. The enzyme β-1,3 glucanase was produce in media with pH 4.0 to 8.0 with an optimum pH5.5. The optimum pH for chitinase and cellulase enzyme activity was recorded at 5.0. With the variable temperatures, i.e., 20, 25, 30, 35 and 40°C, the enzyme activities of all three enzymes showed highest at 30°C. The activities of enzymes β-1,3 glucanase and chitinase was recorded highest at 3.0 and 4.0% concentrations of glucan and chitin, respectively whereas 0.75% concentration of cellulose showed highest cellulase activity. The highest induced resistance by *Trichoderma* isolates in gram seedlings, was recorded by TR_1 through the increase activity of PAL and β-1,3 glucanase and TH_1 by enhance activity of peroxidase enzyme.**

KEY WORDS: β-1,3 glucanase, cellulase, chitinase, induced resistance, *Trichoderma* spp.

INTRODUCTION

Trichoderma and *Gliocladium* spp. had been established as potential biocontrol agents during past few decades have created a new milestone in non-chemical plant disease management system and organic farming. *Trichoderma* spp. are known to produce many extracellular hydrolytic enzymes viz., β-1, 3 glucanase, chitinase, cellulase, etc. by which they cause lysis or degrading outer wall of many plant pathogenic fungi. *Trichoderma* seems to be an effective biocontrol agent for protecting a number of crop plants from several soil borne plant pathogens (Mukhopadhyay *et al.*, 1992). The

mechanisms by which strains of *Trichoderma* functions are mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrients and inactivation on pathogens enzymes, etc. (Harman, 2000).

The possible mechanisms involved in antagonism of soil borne plant pathogens are antibiotic and enzyme production as well as hyphal interactions, physical contacts followed by degradation of cell wall and disorganization of cell components, coiling, hooks or appressorium like structures and penetrate the host cell wall by secreting hydrolytic enzymes such as polysaccharide lyase, protease and lipase (Chet, 1987). The ability of *T. harzianum* to produce extracellular β-1, 3 glucanase and chitinase into the medium supplemented with laminarin and chitin led several authors to postulate that the released enzymes were actively involved in microbiological control (Lewis and Papavizas, 1987; El Katanty *et al.,* 2004). Sometimes, starvation condition could induce secretion of cell wall degrading enzymes (Ramot, *et al.*, 2000), whereas in others, cell wall or cell wall components were required to trigger the enzyme (Elad *et al.*, 1982). This paper describes the variability in the secretion and regulation of some exracellular enzymes by some isolates of *Trichoderma* and induction of systemic resistance in gram seedlings.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Ten isolates of *Trichoderma* were isolated from the rhizosphere soil of different crops by dilution plate technique (Harris and Sommers, 1968) using *Trichoderma* specific medium (TSM) (Elad and Chet, 1983). The isolates were identified into different species with the help of taxonomic keys and monograph of Rifai (1969). All the identified strains of *Trichoderma* were maintained on potato dextrose agar (PDA) slant at 4°C for further use.

The isolates of *Trichoderma* spp. were grown on a minimal synthetic medium (MSM) (El-Katatny *et al.,* 2000) supplemented with dried mycelium of *M. phaseolina* @ 5g l^{-1} as sole carbon source. The medium was adjusted to pH6.0 and sterilized in Erlenmeyer flasks at 1.4kg / cm^2 for 15minutes at a temperature of 121°C. The sterilized media (50ml) was inoculated with 1ml spore suspension @ $2x10^7$ ml^{-1} and incubated at 28 ± 1°C for 5 days with gentle shaking at 150rpm 15 hrs in a day. The culture filtrate was separated from mycellial mat by centrifugation at 4°C for 10 min at 5000rpm and the supernatant was immediately tested for enzyme activity.

Time course and carbon sources on enzyme activity

The highly effective strain of *Trichoderma* (TR_2) was grown in minimal synthetic medium (MSM) amended with various carbon sources i.e. laminarin @ 1g l^{-1} (from *Laminaria digitata*), colloidal chitin @ 2g l^{-1}, cellulose @ 5g l^{-1}, mycelial powder of *M. phaseolina* and *S. rolfsii* @ 5g l^{-1} with no sugar. The pH of the medium was adjusted to 6.0 in all cases. Six concentrations of carbon sources (0.25, 0.50, 0.75, 1.0, 1.25 and 1.50%) by amending with laminarin, colloidal chitin and cellulose were evaluated for β-1, 3 glucanase, chitinase and cellulase enzymes activity efficient strains of *Trichoderma*.

Effect of pH and temperatures on enzyme activity

Enzyme activity of *Trichoderma* isolate (TR_2), as a function of pH was determined by adjusting the pH of growth medium to 4.0–8.0 using 50mM acetate buffer (pH 4.0 to 5.5) and phosphate buffer (pH6.0 – 8.5). The fungus was grown in MSM (minimal synthetic media) for chitinase (pH6.0), β-1, 3 glucanase (pH5.5) and cellulose (pH5.5) at 20, 25, 30, 35 and 40°C temperature to determine the effect of temperature on these enzyme activities.

β-1, 3 glucanase (EC 3.2.1.58)

The reaction mixture contained 0.5ml laminarin (3.2 mg ml^{-1}), 1.0 ml 0.05M citrate buffer (pH4.8) and 0.5ml culture filtrate was incubated at 40°C for 60min and boiling stopped the reaction. The equal volume of dinitrosalicyclic acid reagent was added to the

reaction mixture and warmed in boiling water bath for 15min. The absorbance of reaction mixture was measured at 575nm in a spectrophotometer and compared with the standard graph drawn by following the same procedure but using different concentrations of glucose instead of culture filtrate. The quantity of reducing sugar was estimated and activity of β-1, 3 glucanase was expressed as nkat ml^{-1} min^{-1} and one nkat is corresponds to the release of In mol glucose per second per ml.

Chitinase (E.C.3.2.1.14)

A mixture of 0.5ml culture filtrate, 0.5ml of colloidal chitin and 1.0ml of Mcllavaines buffer (pH4.0), was incubated at 37°C for two hrs into a water bath with constant shaking. Three ml of potassium ferricyanide reagent was added and heated in boiling water bath for 15min. The amount of N-acetyl glucosamine (NAG) released was estimated and absorbance of reaction mixture was measured at 420nm comparing with the standard graph drawn by performing the same procedure but using different concentration of NAG instead of culture filtrate. The amount of reducing sugars released was calculated from the standard curve for NAG and the activity of chitinase was expressed in pkat ml^{-1}.

Cellulase (E.C. 3.2.1.4)

The reaction mixture containing 1ml of 1% cellulose, 2.0ml of 0.05M citrate buffer (pH4.8) and 1.0ml of culture filtrate was incubated for 30min at 55°C in a water bath with periodical shaking and the reaction was completed by boiling. The amount of glucose released in the reaction was estimated by dinitrosalicyclic acid reagent method and enzyme activity was expressed as released of 1ì mol glucose ml^{-1} min^{-1} for one unit.

Induction of resistance by *Trichoderma* isolates

In this experiment, gram seeds (cv. Mahamaya) were surface sterilized in 2% NaOCl for 2min and thoroughly washed with sterile distilled water and sown in sterilized soil-FYM mixture (3: 1) in earthen pots. Seedlings were grown at room temperature with 11h of light and 13h of darkness for 10 days of sowing of seeds. The spore suspension of different strains of antagonist (10^7 conidia ml^{-1}) was inoculated to each pot soil after 10 days of sowing. Pot mixture without *Trichoderma* was maintained as control. Each pot was moistened by adding sterile distilled water whenever required and leaf tissue was plucked for enzyme activity.

Phenylealanine ammonia lyase (PAL, E.C.4.3.1.5)

PAL activity was determined spectrophotometrically as trans cinnamic acid formed during the enzyme reaction (Brueske, 1980). One gram of leaf tissue was extracted in 2ml of chilled 0.1M Potassium phosphate buffer (pH8.0) and filtered through a muslin cloth. The reaction mixture contained 0.1ml of enzyme extract, 0.4ml of 0.1Mborate buffer (pH8.0) and 0.5ml of 12Mml L-phenyl alanine. The reaction was stopped by addition of 1Mtrichloroacetic acid and absorbance of reaction mixture was measured at 290nm in a spectrophotometer. The enzyme activity was expressed on fresh weight basis as n mol of trans cinnamic acid released per min per gram of tissue.

β-1, 3 glucanase (E.C. 3.2.1.58)

β-1, 3 glucanase activity was colorometrically assayed by the laminarin-dinitrosalicyclic method. The enzyme extract (62.5µl) was added to 62.5µl of laminarin and incubated at 40°C for 10min. The reaction was stopped by the addition of 375 µl of dinitrosalicyclic acid reagent and heating for 5min. in a boiling water bath. The resulting coloured solution was diluted with 4.5ml of distilled water, vortexed and its absorbance was measured at 500nm. The enzyme activity was expressed as ì mol equivalent glucose released per hour per gram fresh weight of tissue.

Peroxidase (E.C. 1.11.1.7)

The peroxidase activity was determined by using ortho-dianisidine as substrate (Basan *et al.*, 1985). The reaction mixture containing 3.5ml of 0.5M phosphate buffer (pH6.5), 0.2ml of enzyme extract and 0.1ml of freshly prepared O- dianisidine solution (1mg ml^{-1} methanol) and the activity of peroxidase was measured in a spectrophotometer

at 430nm. 0.2ml of 0.2M H_2O_2 was mixed and change in the absorbance at same wavelength was recorded. The enzyme activity was measured as change in 0.01 absorbance per min per mg of protein.

RESULTS AND DISCUSSION

The results presented in Table 1 revealed that the highest chitinase and β-1, 3 glucanase activities were observed in TR_2 (42.8U and 10.2U, respectively) isolate whereas TH_2 isolate exhibited highest activity of cellulase (25.5U). Very low activity of both chitinase and â-1, 3 glucanase was noticed in case of TV_1 and TH_5 isolates, respectively. The activity of cellulase was highest in TH_2 isolate followed by TH_5 (19.8U) and TR_2 (20.5U), which were statistically significant. Based on these results, TR_2 for chitinase and β-1, 3 glucanase and TH_2 for cellulase activity were selected for all the further experiment.

The activities of chitinase, β -1,3 glucanase and cellulase enzyme were increased with incubation period and reached its peak 92.2U at 7 days, 21.5U at 3 days and 56.1U at 5 days of incubation, respectively and declined thereafter to 78.0U, 8.0U and 32.1U at eighth days of incubation (Fig. 1). The highest activity of all three enzymes was recorded when the colloidal chitin, laminarin and cellulose was added as carbon sources in the growth media.The activity of extracellular hydrolytic enzymes of *Trichoderma* isolate in response to the variable pH is presented in Fig. 2. The enzyme β-1,3 glucanase was active from pH 4.0 to 8.0 with an optimum at pH 5.5 (21U) and lowest at pH 4.0 (3.0U) whereas optimum pH for chitinase and cellulose enzyme activity was 5.0. The lowest activity of all three enzymes was recorded at pH 8.0. With the variable temperatures, i.e., 20, 25, 30, 35 and 40°C, the enzyme activities of all three enzymes showed highest at 30°C (116.5U for chitinase, 105.5U for β-1, 3 glucanase and 98.5U for

Table 1. Activity of extracellular enzymes produced by different isolates of *Trichoderma*

Isolates	Chitinase (p kat ml^{-1})	β-1, 3 glucanase (n kat ml^{-1})	Cellulase (glucose ml^{-1})
TH_1	35.7	7.9	13.0
TH_2	18.2	4.1	25.5
TH_3	24.0	6.5	6.9
TH_4	16.9	4.6	12.3
TH_5	30.2	3.9	19.8
TV_1	15.9	6.0	18.0
TV_2	21.7	4.5	14.2
TV_3	28.0	6.4	9.5
TR_1	30.5	8.8	17.0
TR_2	42.8	10.2	20.5
S Ed	1.14	0.29	0.14
LSD (P = 0.01)	2.66	0.99	1.08

cellulose enzyme) (Fig. 3). The enzyme activities were found to increase from 20 to 30°C and thereafter declined sharply. The enzyme activities of chitinase, β-1,3 glucanase and cellulase with different concentrations of chitin and laminarin, is presented in Fig.4, which revealed that enzyme activities were increased with increase in concentration of respective substrate and reached its peak at 3.0% concentration for β-1,3 glucanase (80.1U) and 4.0% for chitinase (85.2U) whereas cellulase (69.5U) enzyme reached its peak at 0.6% concentration of cellulose (Fig. 5).

The results of systemic induced resistance in gram seedlings following application of *Trichoderma* isolates to the root system is presented in Fig. 6, which indicates that there was an increase in activity of L-phenylalanine ammonia

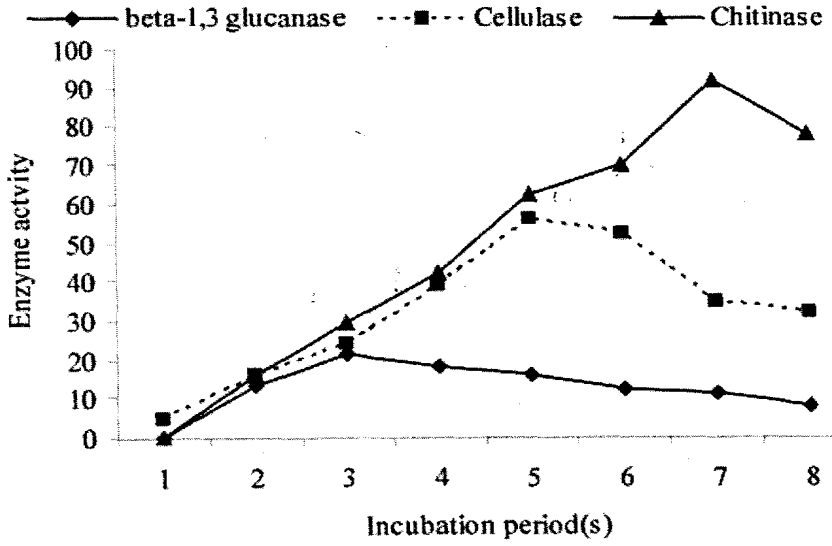

Fig. 1. Effect of incubation period (days) on enzyme activity of TR2 and TH2 isolates

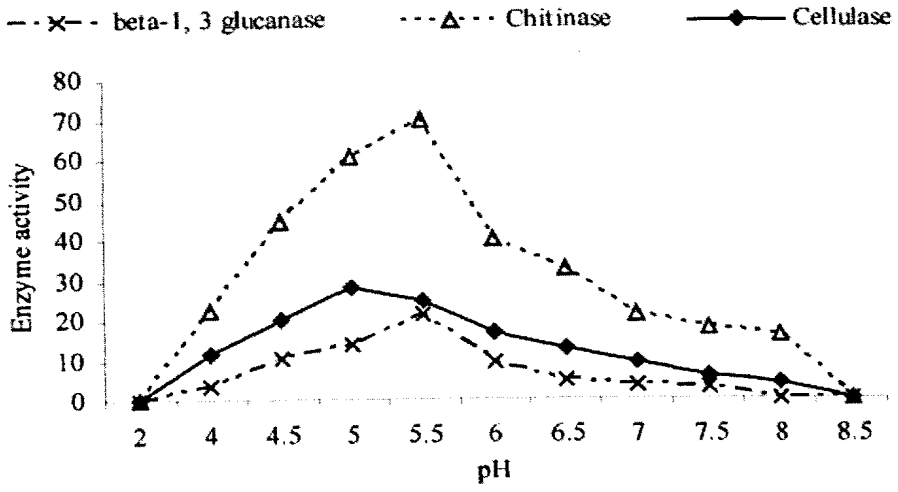

Fig. 2. Effect of pH on the enzyme activity of TR2 isolate

lyase (PAL) activity. The degree of this enzyme activity was varied with different isolates of *Trichoderma* spp. TR_1 isolate of *T. roseum* induced highest PAL activity (4.8U) followed by TH_1 isolate of *T. harzianum* (4.5U) and TV_3 (3.9U) with an increased enzyme activity of 71.4, 60.7 and 39.3, respectively, over non-inoculated plant. Activity of β-1,3 glucanase activity was also increased in host plant after root inoculation with isolates of *Trichoderma*. Maximum activity was recorded with TR_1 isolate (275U) followed by TR_2 (265U) as compared to 150U in non-treated control, whereas very low (6.6%) increased activity was observed in host plant in case of TV_2 isolate (Fig. 7). Similarly, the increased level of peroxidase activity was observed in the gram seedlings inoculated with different isolates of *Trichoderma* spp. (Fig. 8). Highest activity was noticed in the seedlings inoculated with TH_1 isolate (190U) followed by TR_1 (180U).

It is well known fact wide range of prokaryotic and eukaryotic microorganisms have the potential to produce cell wall degrading enzymes by using the materials that are present in the growth medium (Jijakli and Lepoivre, 1998; Guiliano *et al.*, 2001). Production of hydrolytic enzymes such as β-1,3 glucanase, chitinase, cellulase and proteinase increased significantly when *Trichoderma* spp. were grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (Kumar and Gupta, 1999; Roy *et al.*, 2005). Kumar and Gupta (1999) reported that cell walls of *M. phaseolina* and *S. rolfsii* are known to have glucan and chitin that should have resulted in the induction of glucanase and chitinase in mycelial mat amended media. High β-1,3 glucanase and chitinase activities were detected in dual culture when *T. harzianum* parasitized *R. solani* and *S. rolfsii* compared with low levels of substrates or in absence of pathogen (Elad *et al.*, 1983).

A low level of β-1,3 glucanase and cellulase and no chitinase was observed when the antagonistic strain was deprived of a carbon source and its growth was low in present investigation. No production or low level of chitinase activity in deprived carbon sources confirms that chitinase enzyme is produced inducibly not constitutively. Expression of these cell wall degrading enzymes frequently has been reported to be induced by fungal cell wall components and repressed by carbon catabolite repressor such as glucose (Tronsmo and Harman, 1993; Donzelli *et al.*, 2001; Guiliano *et al.*, 2001).

Chitinase, β-1,3 glucanase and cellulase enzymes production were favoured by acidic pH (5.0, 5.5 and 5.0, respectively). Acidic pH was also reported to be an important growth parameter in production of chitinase and β-1,3 glucanase in *T. harzianum* (Elad *et al.*, 1982). Ulhoa and Peberdy (1991) found that the production of chitinase was markedly affected by pH with an optimum pH of 6.0. The pH and temperature optima of these three enzymes in present investigation is duly supported by the findings of Jijakli and Lepoivre (1998) and Harman *et al.* (1993).

In present investigation, it was found that chitinase β-1,3 glucanase and cellulase enzyme activity was increased with increase in concentration of respective substrate. This finding is in accordance with Ulhoa and Peberdy (1991) where he suggested that chitinase activity was substrate's concentration dependent above 0.5% (w/v) chitin there was no further synthesis of the growth medium by *T. harzianum* was increased upto 1% concentration, whereas β-1,3 glucanase enzyme production increase upto 1% concentration of laminarin but decreased at higher concentrations. This may be due to the fact that at higher concentration of sugar activity of this enzyme was inhibited.

Trichoderma spp., a potential biocontrol agents has led to the proposal that besides their recognized antifungal properties, such organisms could also act as elicitors of plant defense reactions, thereby promoting the expression of plant defense related genes. The results of present investigation, demonstrates that different defense related enzymes reduced resistance in host plant following the treatment of *Trichoderma* isolates. Application of *Trichoderma* to the root system resulted in a significant increase of PAL, mRNA in leaves both

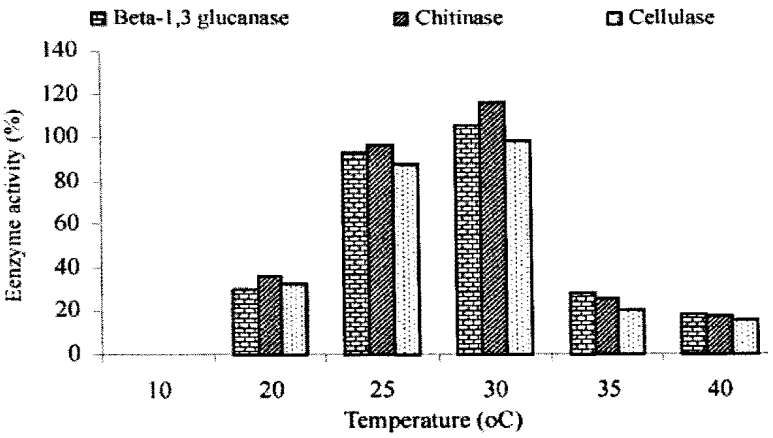

Fig. 3. Effect of temperature on the enzyme activity of TR2 isolate

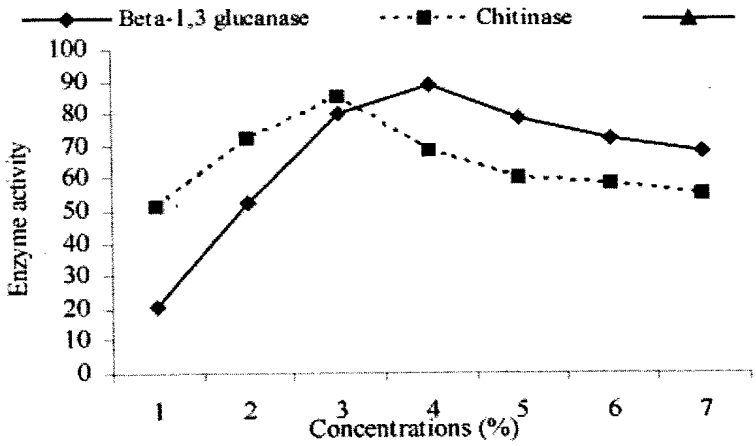

Fig. 4. Effect of variable concentrations of chitin, beta 1,3 glucan and cellulose on enzyme activity of TR2 isolate

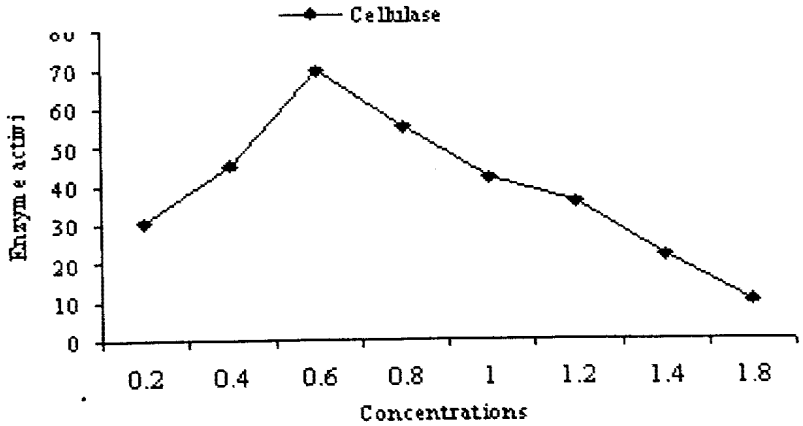

Fig. 5. Effect of variable concentrations of cellulose on cellulase activity of TH2

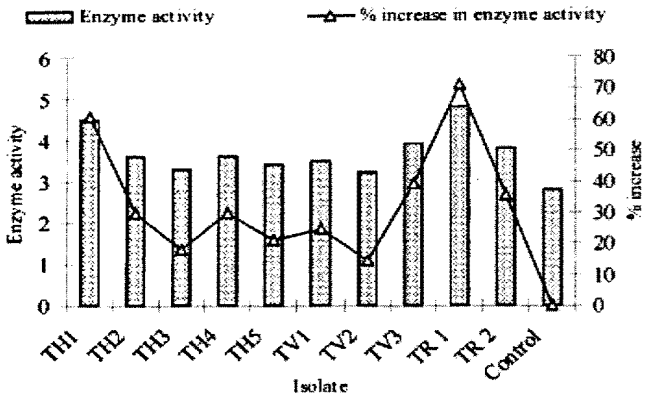

Fig. 6. Induced PAL activity in gram seedling following root drenching with *Trichoderma* ssp.

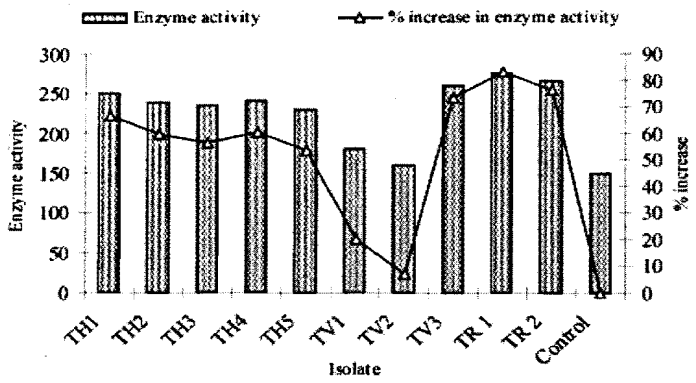

Fig. 7. Induced Beta-1-3 glucanase activity in gram seedling following root drenching with *Trichoderma* ssp.

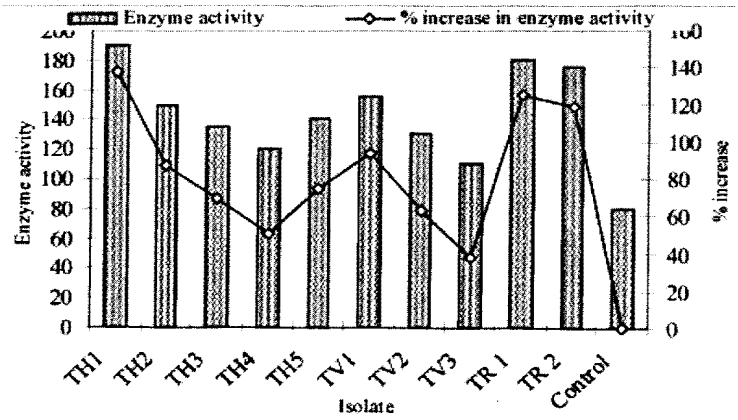

Fig. 8. Induced peroxidase activity in gram seedling following root drenching with *Trichoderma* ssp.

locally and systematically. In present investigation, enzyme activation was measured at 48 hr post elicitation with different *Trichoderma* isolates. A similar time course for PAL activity was observed by Martinez *et al.*(2001) using active cellulase from *T. longibrachiatum*. β-1,3 glucanase and peroxidase, being pathogenesis related proteins have important role in defense mechanism in plant against many plant pathogens. However, the amount of phenol was not measured in young leaf tissue because several studies have established the role of phenol in the defense response of plant. Changes in PAL activity following root treatment indicate the induction of phenolic compound.

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