J. Mycopathol, Res, **49(1)** : 9—19 (2011)

Enzyme activities of *Trichoderma* isolates from Andaman and Nicobar Islands

SOMESHWAR BHAGAT AND SITANSU PAN
Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia 741 252, West Bengal
Email of the corresponding author : sombhagat 73@rediffmail.com

Received : 10.12.2008 Rev. MS Accepted : 20.11.2010 Published : 25.04.2011

The effect of different substrates as carbon sources on enzyme activities of *Trichoderma* isolates in minimum synthetic medium (MSM) was studied. There was marked improvement in the chitinase activities of *Trichoderma* isolates when the medium was supplemented with either chitin or mycelial powder of *M. phaseolina* or *S. rolfsii.* similarly β-1, 3 glucanase, β-1, 4 glucanase, and cellulase activities of *Trichoderma* isolates were also significantly enhanced with addition of glucan, CMC (Carboxymethyl cellulose) and mycelial powder of *Pythium aphanidermatum* in the basal medium, respectively. The isolate TvAN-3 was most efficient with highest chitinase activity when this isolete was grown in medium supplemented with colloidal chitin (26.5 and 37.0 Pkat/ml), followed by TvAN-5, ThrAN-5, ThrAN-7, ThrWB-1 and the isolate ThmAN-10 and ThrWB-2 were the lowest chitinase producers under similar condition. Highest β-1, 3 and β-1, 4 glucanase activities were expressed with ThrAN-5 (13.6 and 14.9nkat/ml) and ThrWB-2 being the lowest enzyme activity of only 6.4 and 7.5 nkat/ml. ThmAN-4 was most efficient in secreting cellulase in th cluture medium irrespective of other carbon sources. The other best isolates of *Trichoderma* were ThrAN-5, TvAn-3, TvAN-5, ThmAN-10, ThrAN-7 and ThrWB-1 and it was found that TvAN-8 being the least effective isolates in view of their cellulase activities.

Key words: *Trichoderma* isolates, extracellular hydrolytic enzymes, SDS-PAGE

INTRODUCTION

Trichoderma spp. are among the most frequently isolated soil fungi and present in plant root systems (Harman, 2000). These fungi are opportunistic, avirulent plant symbionts (Harman *et al*, 2004), and functions as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from disease. So far, *Trichoderma* spp are among the most studied fungal BCAs and commercially marketed as biopesticides, biofertilizers and soil amendments (Harman, 2000; Harman *et al.*, 2004; Lorito *et al.*, 2004). *Trichoderma*, a filamentous soil inhabiting mycoparasite, have been used in commercial preparation for biological control of many fungal induced plant diseases (Pan *et al*, 2001; Bhagat and Pan, 2007). A wide range of prokaryotic and eukaryotic microorganisms have the potential to produce cell-wall-degrading enzymes when chitin or isolated fungal cell wall material are present in the growth medium (Shahai and Manocha, 1993; Pitson *el al*, 1993). Production of extracellular β-l, 3-glucanases, chitinase and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell wall (Carsolio *et al.*, 1994; De La Cruz *et al*, 1995; Kumar and Gupta, 1999, Roy *et al*, 2005). These observation, together with the fact that chitin, β-l, 3-glucan and protein are the main structural components of most fungal cell walls (Peberdy, 1990), are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant

pathogens (Chet and Baker, 1981). High β-1, 3-glucanase and chitinase activities are detected in dual culture when *Trichoderma harzianum* parasitized *R. solani* and *S. rolfsii* compared with low levels in absence of pathogens (Chet and Baker, 1980; Elad *et al.*, 1983; Baker and Dickman, 1993). Therefore, present investigation has been carried out to evaluate the addition of various carbon sources to induce certain extracellular hydrolytic enzymes.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Twelve isolates of *Trichoderma* were grown in a minimal synthetic medium (MSM, El-Katatny *et al.*, 2000) supplemented with different substrates i.e. yeast cell glucan, CMC, colloidal chitin, mycelial powder of *P. aphanidermatum, M. phaseolina* and *S. rolfsii* at 0.05% (w/v) as sole carbon sources. Mycelial powder of *P. aphanidermatum, M. phaseolina* and *S. rolfsii* were prepared by growing them separately in potato dextrose broth medium for 7 days at 28 ± l°C. The dried mycelial mat was made into powder form by grinding through pestler and mortar. The pH of medium was adjusted to pH 6.0 and sterilized into the Erlenmeyer conical flasks (250 ml) at 121°C for 15 min. 50 ml medium was inoculated with 1 ml spore suspension of antagonists (2 x 10^8 cfu/ml) and incubated into a BOD cum rotary shaker at 150 rpm for 12-15 hrs each day at 28 ± 1°C for 5 days. The culture filtrate was harvested by separating the mycelial mat by passing it through a Whatmann no. 42 filter paper and centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was collected into a sterilized conical flasks and tested for enzyme activity immediately

β-1, 3 glucanase (E.C. 3.2.1.58)

The reaction mixture of 0.5 ml laminarin (3.2 mg/ml distilled water), 1.0 ml of 0.05 M citrate buffer (pH 4.8) and 0.5 ml culture filtrate of test isolates of *Trichoderma*, were incubated at 40°C for 60 min. The reaction was stopped by boiling in water bath. The release of reducing sugar was estimated by dinitrosalicyclic acid method (Miller, 1959). The reaction mixture was added with 2.0 ml of dinitrosalicyclic acid reagent and incubated in boiling water bath for 15 min. The absorbance of the reaction mixture was measured at 575 nm in a spectrophotometer and compared with standard graph drawn by following same procedure but using different concentrations of glucose instead of culture filtrate. The quantity of reducing sugar was calculated from the glucose standards used in the assay and activity of β-1, 3 glucanase was expressed as nkat/ml. One nkatal is corresponds to release of 1 n mol glucose- equivalent per second under specified condition.

β-1, glucanase (endoglucanase)

A mixture of 1.0 ml of 1.0% carboxymethyl cellulose, 2.0 ml of 0.05M citrate buffer (pH 4.8) and 1.0 ml culture filtrate, was incubated at 55°C for 30 min in a water bath with periodical shaking. The reaction was stopped by boiling and 4.0 ml of dinitrosalicyclic acid reagent was added. The enzyme activity of β-1, 4 glucanase was estimated and expressed by same method followed in case of β-1, 3 glucanase activities.

Cellulase (E.C. 3.2.1.4)

1.0 ml cellulose (1.0%), 2.0 ml of 0.05 M citrate buffer (pH 4.8) and 1.0 ml of culture filtrate (enzyme source) was mixed thoroughly and incubated at 55°C for 30 min into a water bath with constant shaking. The reaction mixture was stopped by boiling. The units of glucose released from reaction mixture was estimated by dinitrosalicyclic acid method and enzyme activity was expressed as 1μmol glucose/ml/min for one Unit

Chitinase (E.C. 3.2.1.14)

The reaction mixture contained 0.5 ml suspension of colloidal chitin (0.5%), 1.0 ml McIlvaine's buffer (pH 4.0) and 0.5 ml culture filtrate (enzyme source), was mixed thoroughly and incubated at 37°C for 20 min in a water bath with periodical shaking. The reaction was stopped by boiling the mixture for 3 min in boiling water bath. 3.0 ml potassium ferricyanide reagent was added and warmed in boiling water bath for 15 min. The amount of N-acetyl glucosamine (NAG) released was calculated from the absorbance of reaction mixture at 420 nin and comparing with standard graph drawn by following same procedure except using different concentrations of commercial NAG suspension instead of culture filtrate. The activity of chitinase was expressed as pkat (p mol/s) per milliliter.

Electrophoretic patterns of proteins and enzyme activities

SDS-polyacrylamide gel electrophoresis was used to determine the variation in production of extra cellular proteins and hydrolytic enzymes by some isolates of *Trichoderma*. The protein as well as enzyme activities of antagonists were studied by SDS-PAGE method with 12.5% gel based on the method of Laemmli (1970).

Culture condition and extraction of protein

Twelve isolates of Trichoderma were grown in basal glucose yeast peptone (GYPM) medium. Four mycelial discs (6 mm dia) of actively growing culture of *Trichoderma* was inoculated into the Erlenmeyer conical flask (250 ml) containing 100 ml GYPM medium and incubated at 28 ± 1°C for 5 days. The mycelial mat was harvested by passing it through Whatmann No.42 filter paper with the help of Buchner funnel followed by 2-3 washing with distilled water to remove the trace of media, if any. Mycelial mat was dried by soaking it with sterilized blotting paper and air dried at 10-15°C. Five hundred (mg) of dried mycelial mat of each isolate was crushed into a centrifuge tube (2.5 ml) with the help of a stick (double pointed) after adding 500 μ1 of urea buffer (pH 6.8). The mixture was boiled for 2 min, stirred and again boiled for 2 min and finally centrifuged at 15000 rpm for 10 min at 4°C. The supernatant was collected without disturbing the upper lipid layer or the pellet into an eppendrof tube (1.0 ml) and immediately used or stored at 20°C. Before separation of enzymes, the concentration of enzyme in the extracted aliquot was measured by Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as standard. The variation in protein pattern of test isolates by SDS-PAGE was done using protein marker (42 KDa) as control.

Enzyme activity of Trichoderma isolates by SDS-PAGE

The culture filtrate (enzyme source) was prepared as described earlier with supplementation of different carbon sources. The β-1, 3 glucanase (from *B subtilis*, Fluca-Biochemica E.C. 3.2.1.6), chitinase (*L. marscense*, Sigma, E.C.) and cellulase enzymes (from *A. niger*, Fluca- Biochemica, E.C. 3.2.1.4) were as marker and the pattern of band in each isolate was compared with respective marker. The distance traveled by protein (enzyme) band concerned in test isolates of *Trichoderma*, was compared with pure form of three enzymes viz., β-1,3 glucanase, cellulose and chitinase.

RESULTS AND DISCUSSION

Enzyme activities of Trichoderma isolates

The results presented in Figs. 1 - 4, revealed that all isolates of *Trichoderma* significantly produced different cell wall degrading enzymes (β-1, 3 glucanase, β-1, 4 glucanase, cellulase and chitinase) when the basal medium (Minimal synthetic media) was supplemented with different carbon sources. The pattern of enzyme activities by *Trichoderma* isolates suggested that chitin adaptation of the antagonist up to 5th successive generations did not enhanced their enzyme activities when only basal medium was used. However, there was marked improvement in the enzyme activities of *Trichoderma* when the medium was supplemented with either chitin or mycelial powder of *M. phaseolina* or *S. rolfsii* in case of chitinase activity. While addition of glucan, CMC (Carboxymethyl cellulose) and mycelial powder of *Pythium aphanidermatum* significantly stimulated their β-1, 3 glucanase, β-1, 4 glucanase, and cellulase activities, respectively. The result also suggested that β-1, 3 glucanase, β-1, 4 glucanase, and cellulase were both adaptive and constitutive in nature while chitinase was purely inducible since they need some substrates with chitin oligomers, may be in the form of fungal cells or pure chitin. All the *Trichoderma* isolates did not showed chitinase activities when they were grown in medium containing only minimal synthetic medium regardless of their chitin or non-chitin adaptation, as there was devoid of chitin in that medium.

Highest β-1, 3 glucanase activity (Fig. 1) was recorded with the antagonist isolates ThrAN-5 (13.6 and 14.9 nkat/ml) followed by TvAN-3 (14.0 and 14.7 nkat/ml), TvAN-5 (13.8 and 14.9 nkat/ml), ThrAN-7 (11.7 and 12.8 nkat/ml), ThrAN-13 (10.4 and 11.7 nkat/ml), TvAN-10 (10.9 and 11.5 nkat/ml), ThmAN-4 (10.5 and 11.5 nkat/ml. Similar results were also recorded with β-1, 4 glucanase activities of *Trichoderma* isolates (Fig. 2), where ThrAN-5, TvAN-3, TvAN-5 and ThrWB-1 consistently shown their potential with high β-1, 4 glucanase activities. Highest β-1, 4 glucanase activity of *Trichoderma* isolates was found when they grown in the basal medium duly supplemented with Caboxymethyl cellulose sodium salt, followed by mycelial powder of *P. aphanidermatum* and lowest enzyme activities by *Trichoderma* isolates was recorded in medium containing colloidal chitin.

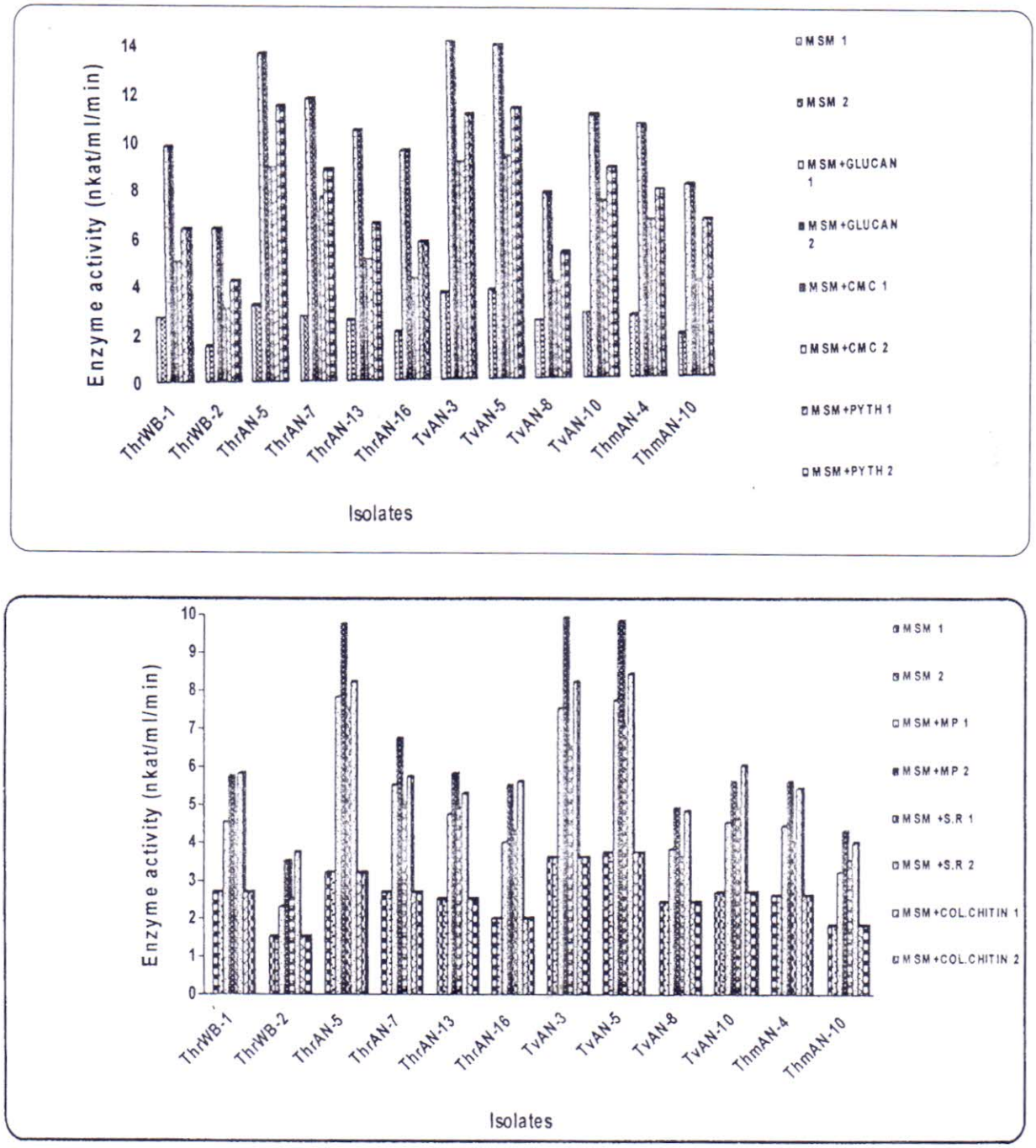


Fig 1: β-1, 3 glucanase activities by *Trichoderma* spp.

In case of cellulase activity (Fig. 3), chitin adaptation of *Trichoderma* isolates up to 5th consecutive generations did not improved the enzyme activity of antagonist tested only in MSM (minimal synthetic medium). However, there was slight improvement in enzyme activities of antagonists when they were grown in medium containing either mycelial powder of *M. phaseolina* or *S. rolfsii*, or chitin, but there was a significant increase in cellulase activity of antagonists in medium supplemented with mycelial powder of *P. aphanidermatum* or CMC (carboxymethyl cellulose) or glucan. Highest cellulase activity of *Trichoderma* isolates was recorded in the medium supplemented with mycelial powder of *P. aphanidermatum* followed by CMC (Carboxymethyl cellulose) and glucan. Among the *Trichoderma* isolates, ThmAN-4 was most efficient in secreting cellulase in the culture medium irrespective of other carbon sources. The other best isolates of *Trichoderma*

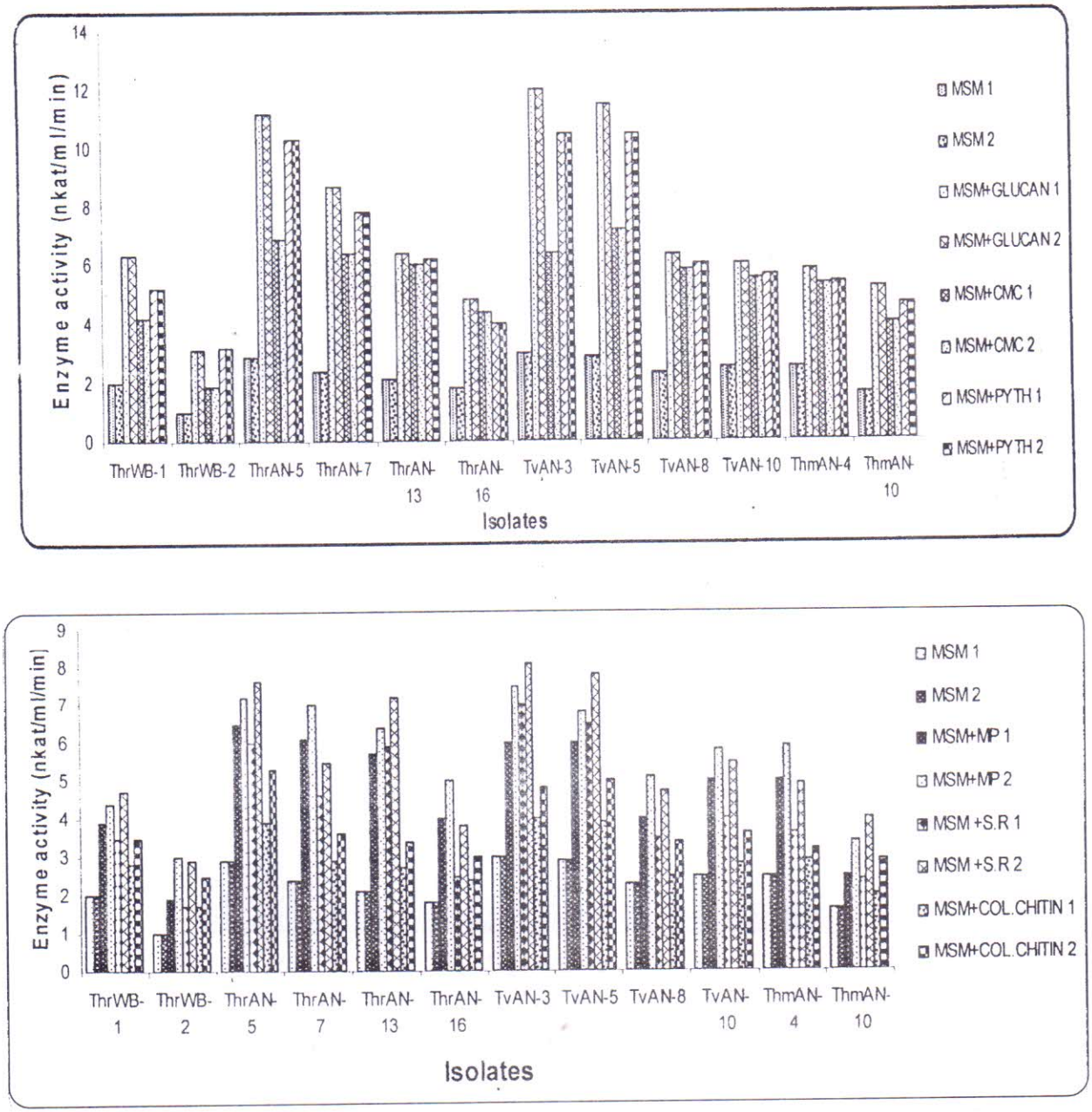


Fig 2: β–1,4 glucanase activities by *Trichoderma* spp.

were ThrAN-5, TvAN-3, TvAN-5, ThmAN-10, ThrAN-7, ThrWB-1 and TvAN-8 being the least effective isolates in view of their cellulase activities.

The chitinase activities of all *Trichoderma* isolates were much more when they were grown in the medium supplemented with mycelial powder of *M. phaseolina*, *S. rolfsii* and chitin as a sole source of carbon. Among the isolates of *Trichoderma,* the isolate TvAN-3 was most efficient with highest chitinase activity (Fig. 4) when this isolate was grown in medium supplemented with colloidal chitin (26.5 and 37.0 Pkat/ml), mycelial powder of *M. phaseolina* (19.0 and 29.5 Pkat/ml) and *S. rolfsii* (19.0 and 28.8 Pkat/ml), followed by TvAN-5, ThrAN-5, ThrAN-7, ThrWB-1 and the isolate ThmAN-10 and ThrWB-2 were the lowest chitinase producers under similar condition.

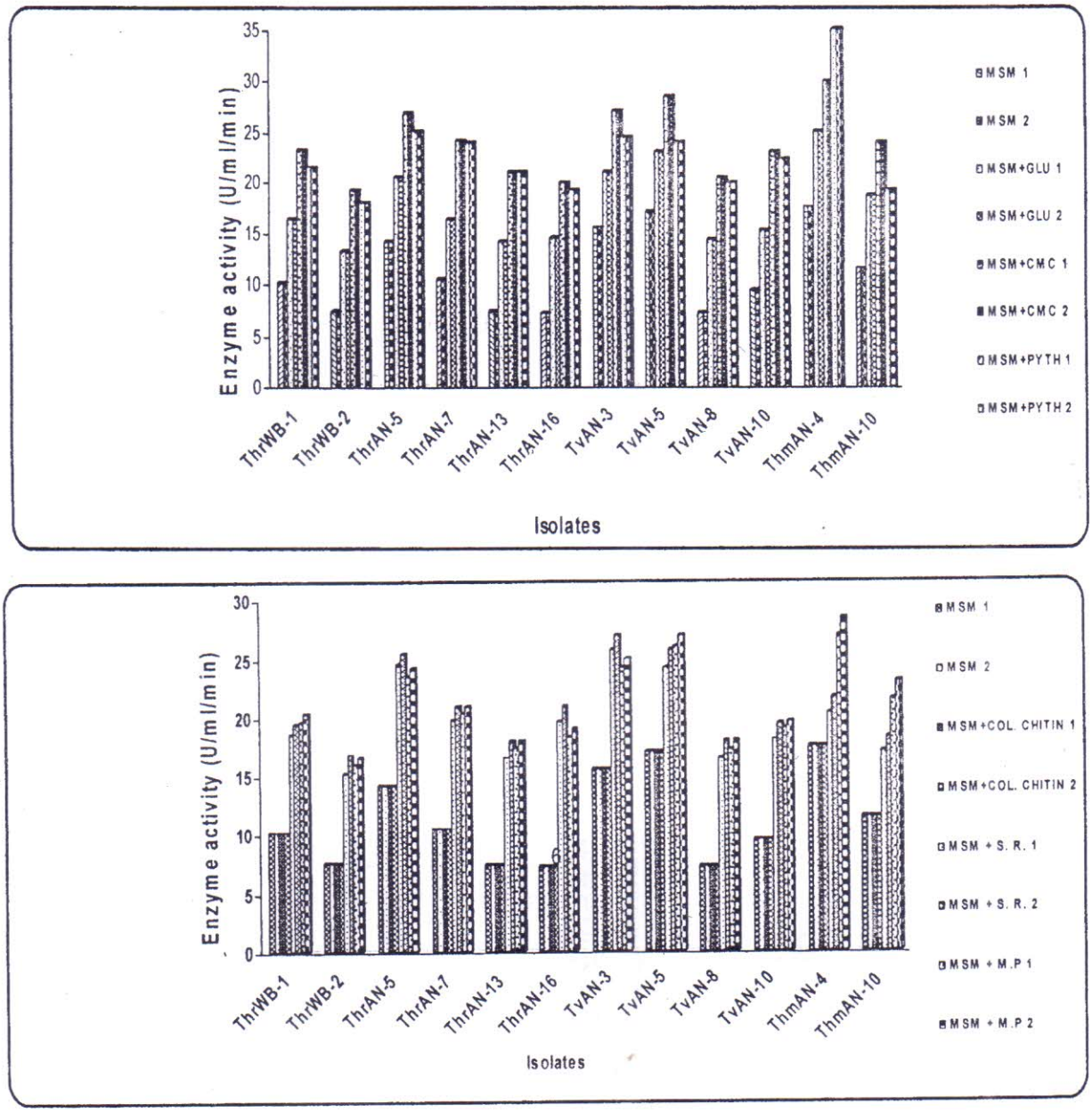


Fig 3: Cellulase activities by *Trichoderma* spp.

Enzyme activities of Trichoderma spp. through SDS-PAGE

The enzyme activities ((β-1, 3 glucanase, cellulase and chitinase) by *Trchoderma* isolates in the liquid growth medium (MSM) duly supplemented with 0.5% of different substrates (CMC, mycelial powder of *P. aphanidermatum, S. rolfsii,* and *M. phaseolina* and colloidal chitin) as sole carbon sources, were visualized by SDS-PAGE gel electrophoresis method. The results presented in Fig. 1, indicated that all isolates of *Trichoderma* have shown their enzymes activities which was confirmed by comparing the bands produced by marker as pure enzyme sources. It was also suggested that the (β-1,3 glucanase and cellulase enzymes produced bands at both upper and lower end with low as well as high molecular weight proteins, but comparatively high molecular weight proteins were observed for chitinase enzymes in the assay condition. The molecular weight of pure (β-1,3 glucanase varied from 8- 85 kDa, whereas it varied from 24-75 kDa and 12-41 kDa for cellulase and chitinase enzymes, respectively.

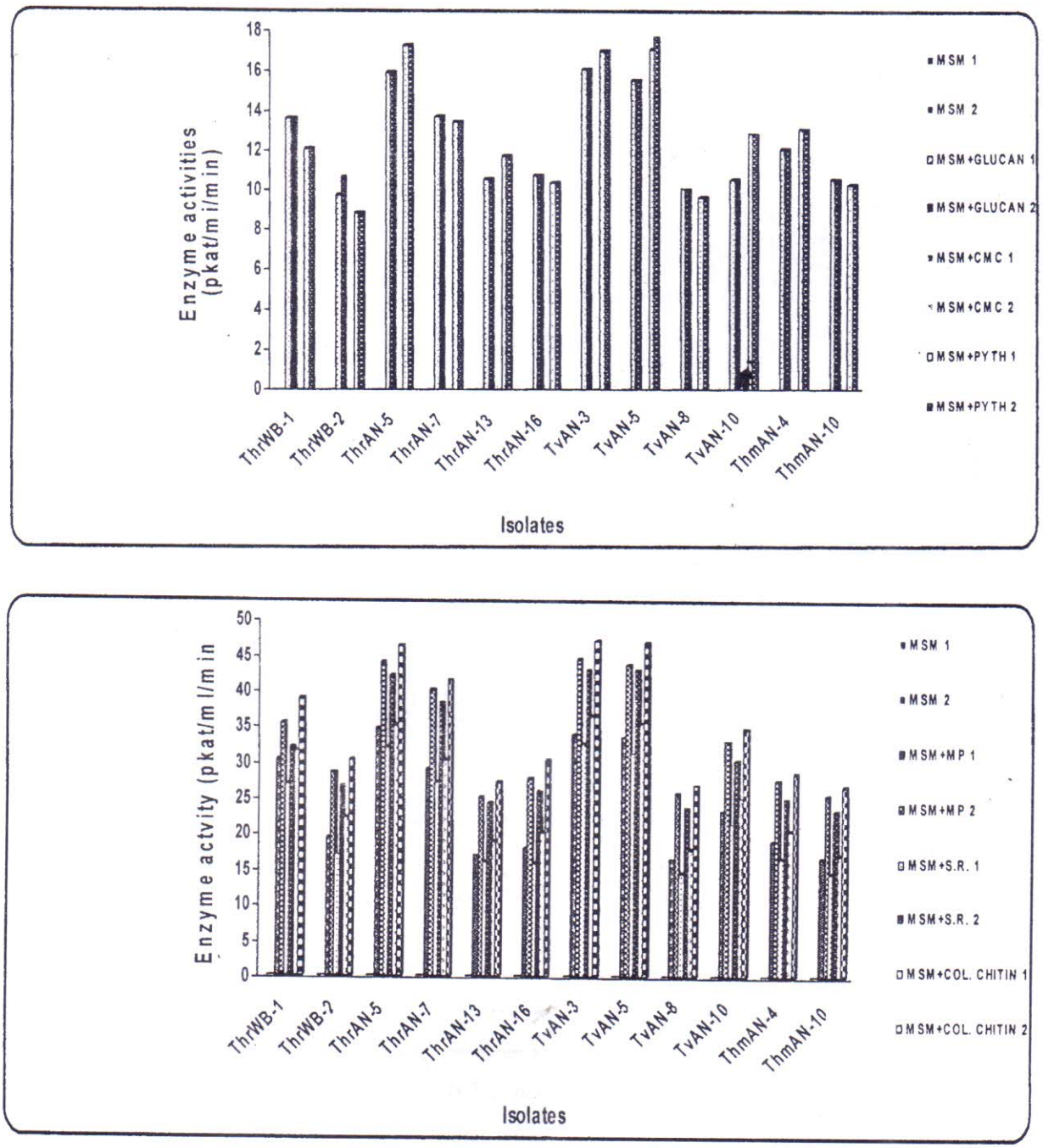
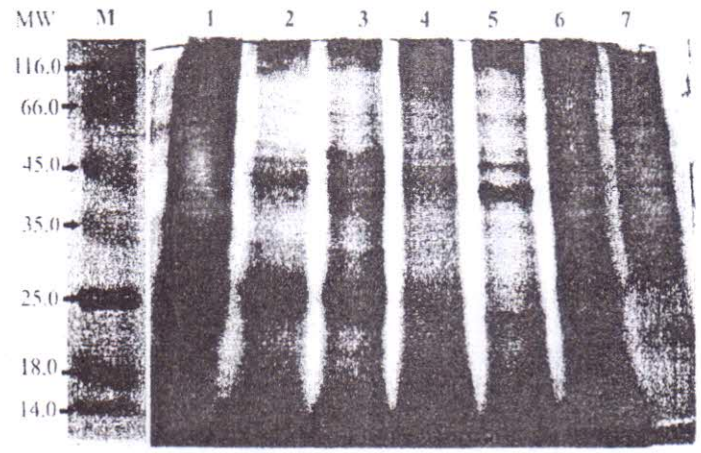


Fig 4: Chitinase activities by *Trichoderma* spp.

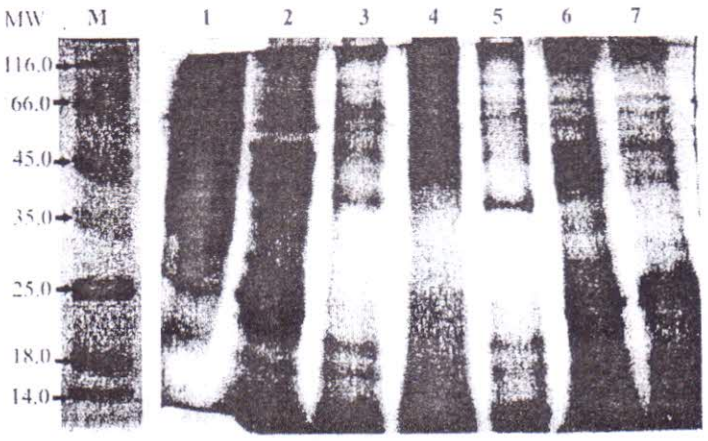
Electrophoretic patterns of mycelial proteins by Trichoderma isolates

The results on SDS-PAGE of extracellular protein by some isolates of *Trichoderma* revealed that all isolates differed with each other by producing different bands. The banding patterns in the gel electrophoresis (Fig. 2) revealed that there was clear variation in number as well as position of bands (mobility) in the assay conditions. Some bands were common in all test isolates with or without same intensity. Few bands appeared in few isolates and hence denoted the variation in production of proteins by *Trichoderma* isolates. Highest numbers of protein bands were produced by ThrAN-5 isolate and lowest number was recorded with ThmAN-10. The darker and thick bands indicated that the protein of specific molecular weight was prominently expressed in assay condition and was in higher quantity whereas light



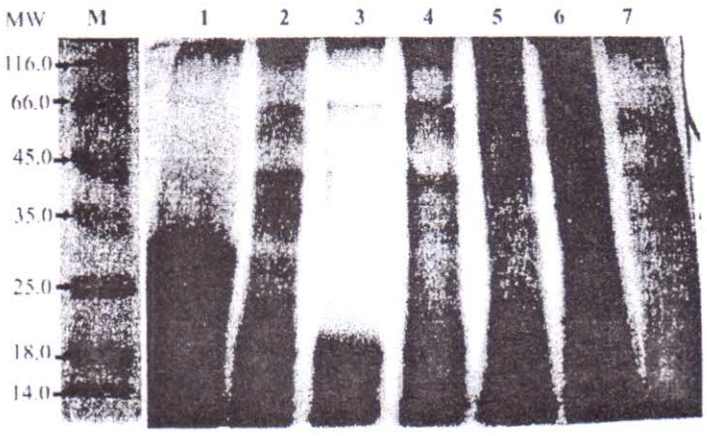
M - Protein Marker
Lane 1- Glucanase
Lane 2- TvAN-5
Lane 3- ThrAN-13
Lane 4- TvAN-10
Lane 5- TvAN-3
Lane 6- ThrAN-5
Lane 7- Cellulase

A. β-1,3 glucanase activities



M: Protein Marker
Lane 1- Cellulase
Lane 2- ThrAN-13
Lane 3- ThrAN-7
Lane 4- TvAN-3
Lane 5- ThrAN-5
Lane 6- TvAN-10
Lane 7- TvAN-5

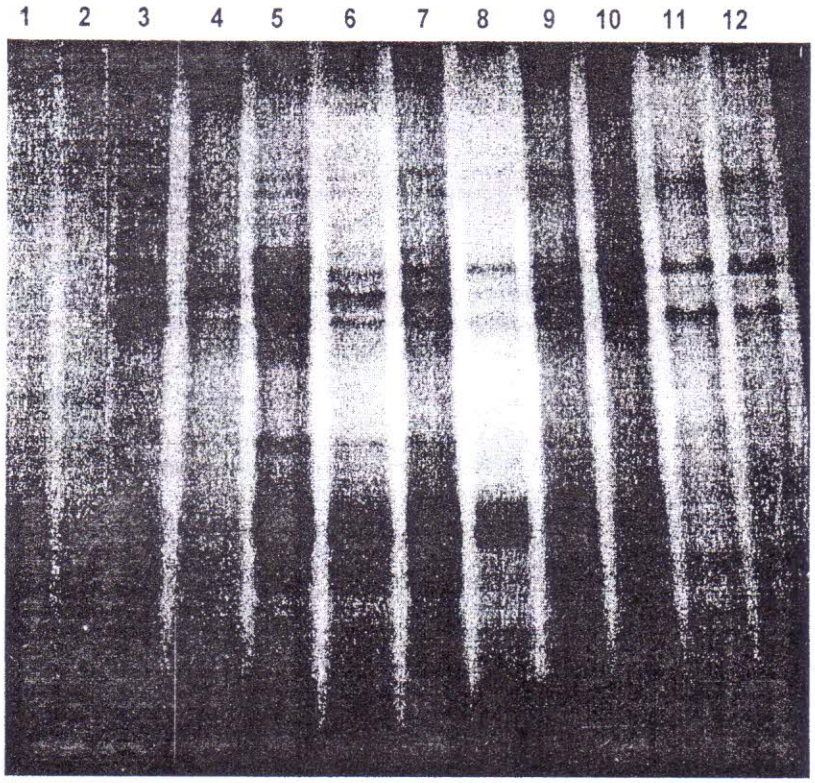
B. Cellulase activities



M: Protein Marker
Lane 1- Chitinase
Lane 2- TvAN-5
Lane 3- ThrAN-5
Lane 4- ThrAN-7
Lane 5- TvAN-10
Lane 6- TvAN-3
Lane 7- ThrAN-13

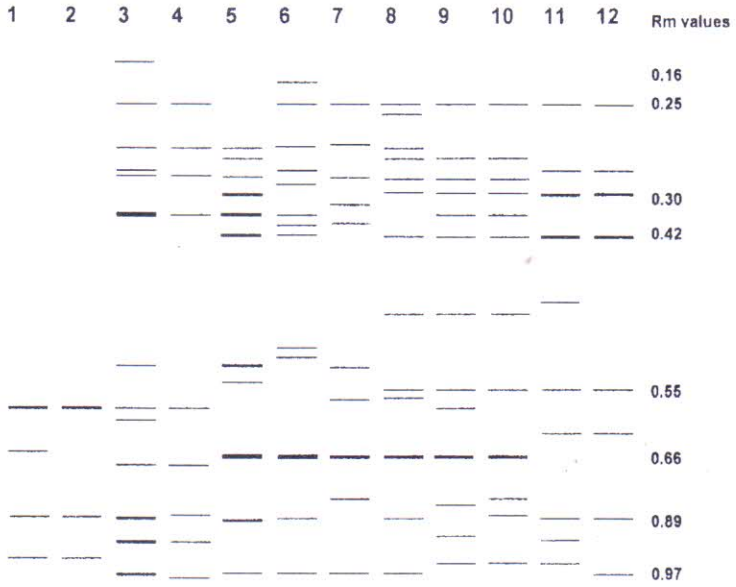
C. Chitinase activities

Fig 5; Enzyme activities of *Trichoderma* isolates



(A)

Lane 1- ThmAN-4
Lane 2- ThmAN-10
Lane 3- ThrAN-13
Lane 4- ThrAN-7
Lane 5- TvAN-3
Lane 6- TvAN-10
Lane 7- ThrAN-5
Lane 8- ThrAN-16
Lane 9- TvAN-5
Lane 10- ThrWB-1
Lane 11- ThrWB-2
Lane 12- TvAN-8



(B)

Lane 1- ThmAN-4
Lane 2- ThmAN-10
Lane 3- ThrAN-13
Lane 4- ThrAN-7
Lane 5- TvAN-3
Lane 6- TvAN-10
Lane 7- ThrAN-5
Lane 8- ThrAN-16
Lane 9- TvAN-5
Lane 10- ThrWB-1
Lane 11- ThrWB-2
Lane 12- TvAN-8

Fig 6; Electrophoretic pattern (A) and Zymogram (B) of mycelial protein of *Trichoderma* isolates

band indicated that the same protein with low quantity.

The antifungal arsenal of *Trichoderma* spp. includes a great variety of lytic enzymes (Lorito *et al*, 1994; Lorito, 1998), most of which play a role in biocontrol (Kubicek *et al*, 2001; Bhagat and Pan, 2008; Vinale *et al*, 2008). Many cell wall degrading enzymes from different *Trichoderma* strains have been purified and characterized (Lorito, 1998). Interestingly, when tested alone or in combinations, the purified proteins showed antifungal activity towards a broad spectrum of fungal pathogens (i.e. species of *Rhizoctonia, Fusarium, Alternaria, Ustilago, Venturia* and *Colletotrichum*, as well as against Oomycetes, *Pythium* and *Phytophthora* which lack chitin in their cell walls (Tronsmo, 1991; Lorito *et al*, 1994). The selective production of antifungal compounds produced by *Trichoderma* may be performed by modifying the growth conditions i.e., type and composition of culture medium, temperature of incubation and pH, etc. (Lorito and Scala, 1999; Woo and Lorito, 2007). The presence of different carbon sources such as mono-or-polysaccharides, colloidal chitin or fungal tissues, has been shown to induce the secretion of $CWDE_S$ (Mach *el al*, 1999; El -Katatny *et al*, 2000, 2001). Present findings are consistent with the earlier findings (Mach *el al*, 1999; El-Katatny *et al*, 2000) where they have reported that the addition of some carbon sources in growth medium significantly improved the secretion of certain cell wall degrading enzymes. Present findings have also suggested that the isolates of *Trichoderma* do not secrete the chitinase only in the basal (Minimal Synthetic Medium) medium but do so only after addition of either cell walls of chitin bearing fungi i.e. *M. phaseolina* and *S. rolfsii* and pure colloidal chitin while the other three enzymes secreted some enzymes even in minimal synthetic medium and it is increased when grown in medium containing either glucan or CMC or mycelial powder, of *P. aphanidermatum* and even also cell walls of *M. phaseolina* and *S. rolfsii*. So, this result has indicated that chitinase enzyme of *Trichoderma* is purely inducible, but β-1, 3 glucanase, β-1,4 glucanase and cellulase are both inducible or somewhat constitutive. Interestingly, the *Trichoderma* isolates release appreciable amount of β-1,3 glucanase, β-1,4 glucanase and cellulase even in medium supplemented with mycelial powder of *M. phaseolina, S. rolfsii*, since both fungi contain chitin and glucan in their cell walls (Tronsmo 1991; Vinale *et al* 2007). The antagonist isolates also secrete these enzymes in the presence of pure chitin in the medium as these enzymes are released constitutively. Expression of extracellular enzymes frequently has been reported to be induced by components of fungal cell wall and repressed by carbon catabolite repressors, such as glucose (Peterbaur *et al*, 1996; Donzelli *et al*, 2001). In some cases, starvation conditions alone could trigger $CWDE_S$ production (Ramot *et al*, 2000) while in other cell walls or cell wall components are needed (Elad *et al*, 1982). These reports support the present findings of increased enzyme activity of β-1,3 glucanase, β-1,4 glucanase and cellulase activity, as well as chitinase activities in carbon starved condition and also supplementation of mycelial powder of *M. phaseolina, S. rolfsii, P.aphanidermatum* and yeast cell glucan.

Morphological characteristics of *Trichoderma* isolates are single major criterion to study the variability among the different species, but these have been found inadequate in differentiation and identification of several species of *Trichoderma* spp. Electrophorectic separation of soluble proteins and enzymes is a useful technique presently used by mycologist and plant pathologists to resolve taxonomic problems, identify unknown fungal isolates and analyze the extent of genetic variability in a population, trace the geographic origin of pathogens, follow the segregation of genetic loci and determine ploidy levels at various stages in life cycle off fungus (Bonde *et al.* 1991). Four major factors are thought to contribute to genetic diversity in fungi including *Trichoderma* spp.: population size, mutation, migration and selection (Barrett, 1987; Nei, 1988). This may be due to the capability of *Trichoderma* isolates to produce large number of spores in a short period of time. This combined with natural mutation rates, could lead to a relatively high level of diversity.

REFERENCES

Baker, R.R. and Dickman, M. B. 1993. Biocontrol with fungi. In: *Soil Microbial Ecology Application in Agricultural and Environmental Management* (Ed., Metting, F.B.), Marcel Dekker, Inc, New York, pp. 275-306

Bhagat, S. and Pan, S. 2007. Mass multiplication of *Trichoderma harzianum* on agricultural byproducts and their evaluation against seedling blight of mungbean and collar rot of groundnut. *Indian Journal of Agricultural Sciences* **77**: 583-588.

Bhagat, S. and Pan, S. 2008. Variability in production of extra cellular hydrolytic enzymes by *Trichoderma* spp. and induction of disease resistance in gram (*Ciccer arietinum*).

Journal of Biological Control (Article in press).

Carsolio, C.A; Gutierrez, A; Jimenez, B; van Montagu, M. and Herrera-Estrella, A. 1994. Primary structure and expression pattern of the 33 kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. *Proceedings of the National Academy* of Science, USA **91**: 10903-10907.

Chet, I. and Baker, R. 1981. Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani*. *Phytopathology* **71**: 286-290.

De La Cruz, L; Pintor-Too, JA.; Benitez, T. and Llobell, A. 1995. Purification and characterization of an endo b-1, 6 glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *Journal of Bacteriology* **177** : 1864-1871.

Donzelli, B.G.G.; Lorito, M.; Scala, F. and Harman, G.E. 2001. Cloning sequence and structure of a gene encoding an antifungal glucan 1, 3-β-glucosidase from *Trichoderma atroviride (T. harzianum)*. *Gene* **277:** 199-208.

Elad, Y; Chet, I. and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Canadian Journal of Microbiology* **28** : 719-725.

El-Katatny, M.H.; Somitsch, W.; Robra, K.H; El-Katatny, M.S. and Gubitz, G.M. 2000. Production of chitinase and β-1,3 glucanase by *Trichoderma harzianum* for control of the phytopathogenic fungus *Sclerotium rolfsii*. *Food Technology and Biotechnology* **38:** 173-180.

Harman, G.E.; 2000. Myth and dogmas of biocontrol; changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* **84**: 377-393.

Harman, G.E.; Howell, C.R.; Viterbo, A; Chet, I. and Lorito, M. 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nature Review Microbiology* **2**: 43-56

Kulling, C.; Mach, R.L.; Lorito, M. and Kubicek, C.P. 2000. Enzyme diffusion from *atroviride* (*T. harzianum* PI) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma ech42* gene expression before mycoparasitic contact. *Applied Environmental and Microbiology* **66**: 2232-2234.

Kumar, A. and Gupta, J.P. 1999. Variation in enzyme activity of tebuconazole tolerant biotypes of *Trichoderma viride*. *Indian Phytopathology* **54**: 263-266

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lorito, M.; D'Ambrosio, M.D.; Woo, S.L; Kubicek, C.P.; Harman, G.E; Hayes, C.K. and Scala, F. 1996. Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. *Molecular Plant Microbe Interactions* **9**:206-213.

Lorito, M.; Harman, G.E; Hayes, C.K; Broadways, R.M; Tronsmo, A.; Woo, S.L. and Di Pitro, A. 1999. Chitinilytic enzymes produced by *Trchoderma harzianum* : antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* **83**: 302-307.

Lorito, M.; Hayes, C.K.; Di Pietro, A; Woo, S.L. and Harman, G.E. 1994. Purification, characterization and synergistic activity of a glucan 1, 3-β- glucosidase and an N- acetyl-β-D-glucosaminidase from *Trichoderma harzianum*. *Phytopathology* **84**: 398-405.

Lorito, M.; Woo, S.L. and Scala, F. 2004. Le biotechnologie utili alla difesa sostenible delle piante: i funghi. *Agroindustria* **3**: 181-195.

Miller, G.L. 1959. Use of dinitrosalicyclic acid reagent for the determination of reducing sugars. *Analytical Chemistry* **31**: 426.

Pan, S.; Q; Ye; X.S. and Kuc, J. 1991. Association of β-1, 3 glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. *Physiological and Molecular Plant Pathology* **39**: 25-39.

Peberdy, J.F. 1990. Fungal cell wall : A Review. In : *Biochemistry of Cell Wall and Membranes in Fungi*. (Eds., Kuhn, P.J., Trinci, A.P.J., Jung, M.J., Goosey, M.W. and Copping, L.G.), Springer Verlag, Berlin, pp. 5-30.

Peterbauer, C.K.; Lorito, M.; Hayes, C.K.; Harman, G.E. and Kubicek, C.P. 1996. Molecular cloning and expression on the *nagl* gene (N-acetyl-b-Dglucosaminidase encoding gene) from *Trichoderma harzianum* PI. *Current Genetics* **30**: 325-331.

Pitson, S.M; Seviour, R.J. and McDougall, B.M. 1993. *Enzyme in Microbial Technology* **15**:178-182.

Ramot, O; Kupiek-Cohen, R. and Chet, I. 2000. Regulation of b-1, 3 glucanase by carbon starvation in the mycoparasite *Trichoderma harzianum*. *Mycological Research* **104**: 415-420.

Shahai, A.S. and Manocha, M.S. 1993. Chitinases of fungi and plants: Their involvement in morphogenesis and host-parasite interactions. *FEMS Microbiology Review* **11**: 317-338.

Tronsmo, A. and Harman, G.E. 1992. Coproduction of chitinolytic enzymes and biomass for biological control by *Trichoderma harzianum* on media containing chitin. *Biological Control* **2**: 272-277.

Tronsmo, A. and Harman, G.E. 1994. Isolation and sequence of an endochitinase encoding gene from a cDNA library of *Trichoderma harzianum*. *Gene* **138**: 143-148.

Vinale, F.; Marra, R.; Scala, F; Ghisalberti, E.L.; Lorito, M. and Sivasithamparam, K., 2007. Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. *Letters in Applied Microbiology* **43**: 143-148.

Vinale, F; Sivasithamparam, K; Ghisalberti, E.L; Marra, R; Woo, S.L. and Lorito, M. 2008. *Trichoderma*- plant - pathogen interactions. *Soil Biology and Biochemistry* **40**: 1-10.

Woo, S.L. and Lorito, M. 2007. Exploiting the intreations between fungal antagonists, pathogens and the plant for biocontrol. In : Vurro, M., Gressel, J. (Eds). *Novel Biotechnologies for Biocontrol Agent Enhancement and Management*. IOS, Springer press, Amsterdam, the Netherlands, pp. 107-130.

Woo, S.L; Donzelli, B.; Scala, F.; Mach, R.; Harman, G.E; Kubicek, C.P.; Del Sorbo, G. and Lorito, M. 1999. Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum*. *Molecular Plant Microbe Interactions* **12**: 419-429.

Zeilinger, S. and Omann, M. 2007. *Trichoderma* Biocontrol: Signal Transduction Pathaways Involved in Host sensing and Mycoparasitism. *Gene Regulation and Systems Biology* **1**: 227-234.