Bull. Fac. Agric., Cairo Univ., 55 (2004): 121- 144.

FACTORS AFFECTING SECRETION OF THE ANTIFUNGAL CHITINASE BY *Trichoderma harzianum* AND *T. longibrachiatum*

(Received: 30.4.2003)

By
N. A. Younis and A. S. Ahmed

Plant Research Department , Nuclear Research Center, Atomic Energy Authority, Inshas, Egypt

ABSTRACT

Culture filtrate of *Trichoderma harzianum* and *T. longibrachiatum* (grown on potato dextrose broth medium) at 80% concentration suppressed completely the growth of certain root infecting fungi, *i.e., Fusarium. moniliforme, F. oxysporium, F. semitectum, F. solani, F. tabacinum, Phytophthora infestans, Rhizoctonia solani,* and *Verticillium* sp. Both fungi were able to produce chitinase enzyme when allowed to grow on both potato dextrose and Czapek's Dox broth media. Increasing incubation period led to increase enzyme productivity. Chitinase productivity was not affected by variations in inoculum concentration except with *T. harzianum*, grown on potato dextrose broth medium 15 days after incubation, where enzyme productivity increased with increasing inoculum density, $7x10^7$ spores/ml was the the best concentration for enzyme production. A wide range of temperature and pH were favorable for chitinase production by *T. harzianum* and *T. longibrachiatum* on both media used (20°C up to 30°C and pH 6, 7 & 8). The best carbon source used was cellobiose at 1&2% with *T. harzianum* after 15 days of incubation comparing with Czapek's Dox liquid medium and at 2% concentration in the case of *T. longibrachiatum*. Malt extract and ammonium phosphate induced the highest chitinase productivity compared with other tested nitrogen sources for both fungi. L- Tyrosine exhibited the highest productivity among all amino acids tested in this study after 9 days for both the tested bioagents. Exposing spore suspension of both isolates to different doses of γ-rays resulted in a slight increase by *T. harzianum*

at doses o.2, 0.4, 0.6, 0.8, 1, 2 and 4 K.Gy and by *T. longibrachiatum* at doses 0.4, 0.8, 1, 2 and 4 K.Gy.

Key words: *chitinase, environmental factors, in-vitro inhibitory effect, γ-rays,root pathogenic fungi,Trichoderma harzianum, T. longibrachiatum*

1.INTRODUCTION

Nowadays there is accumulating evidence that *Trichoderma* spp. which are easily isolated from soil and other environments and readily grown, are among the most promising biocontrol agents in a large scale applications. Biocontrol agents could be a potential alternative to the use of chemicals for management of plant diseases caused by soil borne pathogens (Ulhoa and Peberdy, 1991and 1992; Lorito *et al.*, 1993; Haran *et al.*, 1996; Benhamou and Chet, 1996 and Durman *et al.*, 1999). *Trichoderma* spp. are known to be efficient producers of polysaccharide lyses, and lipases, all of which may be involved in host cell wall degradation (Cherif and Benhamou 1990 and Harman *et al* 1993). Among the enzymes most commonly suggested to be involved in mycoparasitism are those that degrade chitin, recently, a few chitinolytic enzymes from *Trichoderma* spp. were purified and characterized. These enzymes were most frequently considered critical in biocontrol (Ulhoa and Peberdy 1991 and 1992; Haran *et al* 1996; Migheli *et al* 1998).

Ulhoa and Peberdy 1992 have purified two chitinolytic enzymes from *Trichoderma* spp. identifying them as N-acetyl-B-D-chitobiase and chitinase. Attachment of hyphae of *Trichoderma harzianum* to cells of *Rhizoctonia solani* was followed by a series of degradation events in the host, the cytochemical localization of N-acetylglucosamine residues revealed that chitin breakdown occurred gradually, suggesting a continuous production of chitinases by the antagonist (Benhamou and Chet, 1993).

Chitin is an un-branched polysaccharide composed primarily of ß-1-4 polymer of n-acetylglucosamine with occassional glucosamine residues.Chitin is hydrolyzed by the chitinase enzyme system. The chitinases have many applications *i.e.* in the control of fungal diseases of plants (Benhamou and Chet, 1996 and Haran *et al.*, 1996) in the treatment of fungal diseases of human and animals (Davies and Pope, 1977 and 1978) and in the production of single cell protein (Carroad andTom, 1978). Farkas

(1979) and Mauch *et al.*, (1988) stated that chitin is a major structural component of the cell walls of many plant pathogenic fungi.

Investigating large-scale production of microbial chitinase needs studying various parameters. Wijk *et al.*, (1998) investigated the toxicity of sodium chlorite and sodium chlorate on the growth of *T. hamatum*. The effect was lower if ammonium used as the only nitrogen source (compared with nitrate), chlorite toxicity was influenced by the source of nitrogen.

Tronsmo and Harman, (1992) found that the production of N-acetyl beta-glucosamindase, chitobiosidase and biomass by *T. harzianum* was dependent on the media composition but not on inoculum concentration. Growth of *T. harzianum* in simple mineral salt solution supplemented with a single carbon source, including chitin, gave low enzyme activity in the culture filtrate and few conidia, addition of any of several complex materials, such as (V8- juice), yeast extract or protease peptone, substantially increased enzymes and spore yield.

Janas and Targonski, (1995) studied the effect of temperature range 26-38C° on the production of cellulases, xylanases and lytic enzymes by four mutant strains of *T. reesei*. Most of the studies on the expression and regulation of these lytic enzymes were performed in liquid culture supplemented with different carbon sources, *e.g.*, chitin, purified fungal cell walls, glucose, or Glc NAc.(De la Cruz *et al.*, 1993, Garcia *et al.*, 1994, Geremia *et al.*, 1993 and Haran *et al.*, 1995). Tamada *et al.*, (1987) found that a dose of 2 KGy of γ-rays to *Trichoderma reesei* cells secreted cellulases 1.8 times as much as the untreated cells. Sidkey *et al.*,(1996) indicated that γ-rays at a dose of 6 KGy increased the yield of protease secreted by *Aspergillus parasiticus* 8.6 fold.

This research is directed towards the *in vitro* effect of the culture filtrate of *T. harzianum* and *T. longibrachiatum* on certain root pathogenic fungi and the various parameters affecting the production of chitinase secreted by both isolates.

2.MATERIALS AND METHODS

2.1.Fungal isolates and growth conditions

The isolates of *Trichoderma harzianum*, Rifai and *T. longibrachiatum*, Rifai, used in this study, were isolated from phylloplane of different plants (*Duranta* sp. and *Rosa* sp.) and also from different rotten materials (*e.g.* rotten cotton lint and old rotten books). *T. harzianum* and

T. longibrachiatum were grown on potato dextrose agar medium (PDA) at 25°C. Studying the parameters were performed on potato dextrose and Czapek's Dox liquid media.

Fungi were isolated from infected roots of bean (*Phaseolus vulgaris* L.) *i.e., F. oxysporium& Phytophthora infestans* while (*F. moniliforme, F. solani, F. tabacinum,* and *R. solani.*) isolated from infected roots of lupin (*Lupinus termis* Forsk) and *F. semitectum & Verticillium* sp. isolated from infected roots of chick-pea (*Cicer arietinum* L.). Root infecting fungi were grown on (PDA) medium at 22°C for 10 days. Isolated fungi were purified and identified to the generic level by the description of Gilman (1957) at Fac. of Sci., Al Aazhar Univ.(Girls Branch).However, confirmation of identification of the fungal isolates to species level was carried out at the Botany Dept., Fac. of Sci., Aussiut Univ.

2.2. Influence of culture filtrates of *T. harzianum* and *T. longibrachiatum* on growth of the pathogenic fungi

The clear filtrate of both isolates were mixed with sterilized PDA medium at 50 and 80% concentration. Petri dishes containing PDA medium with bioagent culture filtrate were inoculated by disks of any of the tested root pathogens and incubated at 22°C for10 days.

2.3.Assay of chitinase activity

Colloidal chitin was prepared from chitin powder (Sigma Co.) according to the method described by Ried and Ogryd-Ziak, (1981).Both of *T. harzianum* and *T.longibrachiatum* were grown on both Czapek's Dox and potato dextrose ((liquid media). Measurement of chitinase activity was carried out according to the method of Monreal and Reese, (1969) one ml of the enzyme extract was added and mixed by shaking to one ml of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6). Tubes were incubated in a water bath at 37°C for 60 minutes, then cooled and centrifuged before assaying. Reducing sugars liberated were determined in 1 ml of the supernatant by Dinitrosalicylic acid (DNS) method (Miller, 1959). Optical density was determined at 540 nm.

2.4.Factors affecting chitinase secretion

The following factors were studied regarding their effect on the production and biosynthesis of chitinase by *T. harzianum* and *T. longibrachiatum:*

2.4.1. Incubation period Chitinase productivity of the two species of the genus *Trichoderma* was assayed at the end of time intervals of 3, 9 and 15 days.

2.4.2. Inoculum concentration Different inoculum densities of the two species of the genus *Trichoderma* were prepared by means of a haemocytometer. Both pH and temperature were adjusted to 7 and 30°C, respectively.

2.4.3. Incubation temperature The production medium was prepared, the optimum inoculum concentration was adjusted for both isolates to $7x10^7$ spore/ml.

2.4.4. pH The pH was adjusted at different values by using (6N NaOH) or (6N HCl). Inoculation and incubation were carried out as previously mentioned.

2.4.5. Organic and inorganic nitrogen sources Czapek's Dox liquid medium was supplied with different organic and inorganic nitrogenous sources with an equivalent amount of N_2 to that present in 0.3% (w/v) $NaNO_3$. Temperature was adjusted to 25°C.

2.4.6. Amino acids The previously mentioned medium for both fungal isolates, *T. harzianum* and *T. longibrachiatum* was supplemented with different amino acid sources, each of which contained a percentage of N_2 equivalent to that located in 0.3% (w/v) sodium nitrate. Temperature was adjusted to 25°C.

2.4.7. Carbon sources The carbon sources (chitin, grounded shrimp shells, cellobiose, cellulose powder) were added to Czapek's Dox liquid medium at 1 and 2 % concentration. Temperature was adjusted to 25°C.

2.4.8. γ-rays Czapek's Dox liquid medium was used in this respect. Spore suspension of *T. harzianum* and *T. longibrachiatum* ($7x10^7$ spore/ml) was irradiated with different doses of γ-rays (*i.e.*05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 4.0 KGy). Temperature was adjusted to 25°C.

Three replicates were used in the experiment.

3.RESULTS AND DISCUSSION

Data presented in Table(1) reveal that both the tested isoltes of *Trichoderma* are a valuable source of biocontrol of chitinous plant pathogens. The percentages of reduction in growth for the tested pathogens are shown in the table. Metabolites may affect the pathogens in various ways depending on the biological control strain, this includes an extra cellular enzyme system that decompose pathogens and prevent growth (Fravel,1988; Cherif and Benhamou 1990 and Mischke 1997). The degree of inhibition is proportional to the level of chitin in the cell wall of the target fungi (Lorito *et al.*, 1993). *Trichoderma* sp. has a substantial ability to suppress a wide range of plant pathogenic fungi by various mechanisms including the production of cell-wall degrading enzymes such as chitinase (Papavizas 1985; Chet 1987 and Goldman *et al.*,1994).

Among the enzymes most commonly suggested to be involved in mycoparasitism are those that degrade chitin. Studying some factors

Table (1): ***In vitro*** **inhibitory effect of culture filtrate of** *T. harzianum* **and** *T. longibrachiatum* **at 50 and 80% concentrations on growth of certain root infecting fungi 15 days after incubation at 22°C.**

Root pathogenic fungi	% Reduction in growth			
	Trichoderma longibrachiatum		*Trichoderma harzianum*	
	Concentration 50%	Concentration 80%	Concentration 50%	Concentration 80%
Fusarium moniliforme	67.14	100	100	100
Fusarium oxysporium	72.14	100	100	100
Fusarium semitectum	100	100	100	100
Fusarium solani	100	100	100	100
Fusarium tabacinum	67.86	100	100	100
Phytophthora infestans	100	100	100	100
Rhizoctonia solani	71.86	100	82.14	100
Verticillium sp	100	100	100	100

affecting biosynthesis of chitinase is very important. The activity of the enzyme (mg/ml) was estimated with the help of a standard curve of N-acetyl- D- Glucoseamine (2- Acetamido-2- deoxy-D-glucose).

As shown from Table (2) both *T. harzianum* and *T. longibrachiatum* were able to produce the chitinase enzyme when allowed to grow on both potato dextrose and Czapek's Dox broth media. Increasing the incubation period led to increase the enzyme productivity. Data also indicated that chitinase productivity was not affected by variations in inoculum concentration except with *Trichoderma harzianum* grown on potato dextrose broth medium after 15 days of incubation where the enzyme productivity increased with increasing inoculum concentration. $7x10^7$ spores/ml culture was the best for enzyme production. Increasing chitinase productivity by increasing the incubation period confirmed the work of Haran *et al.*, 1995& 1996; Melo *et al.*, 1997; Pleban *et al.*, 1997 and El-Mougy 2000.

Table(2):Chitinase secreted (mg/ml) by *T. harzianum* and *T.longibrachiatum* grown on potato dextrose and Czapek' s Dox broth media in relation to different inocula density at different time intervals.

Medium	Biocontrol strain	Inoculum density (spore/ml)	Chitinase productivity (mg/ml)		
			3days	9 days	15 days
Potato dextrose broth	*T. harzianum*	$7x10^7$	3.631	5.482	7.345
		$4x10^6$	3.621	5.449	6.46
		$2x10^5$	3.621	5.448	5.54
		$2x10^4$	3.458	5.43	5.41
	T. longibrachiatum	$7x10^7$	3.592	5.414	7.31
		$4x10^6$	3.639	5.517	7.276
		$2x10^5$	3.631	5.517	7.276
		$2x10^4$	3.618	5.483	7.345
Czapek' s Dox broth	*T. harzianum*	$7x10^7$	3.349	5.517	7.4
		$4x10^6$	3.16	5.478	7.1
		$2x10^5$	3.04	5.51	7.04
		$2x10^4$	3	5.493	7
	T.longibrachiatum	$7x10^7$	3.5	5.392	7.01
		$4x10^6$	3.48	5.401	7.22
		$2x10^5$	3.49	5.42	7.3
		$2x10^4$	3.02	5.4	7.1

Table (3) shows that a wide range of temperature, 20°C up to 30°C was favourable for chitinase production by *T. harzianum* and *T. longibrachiatum* on both media used. *Trichoderma* sp. is most commonly found in warm climatic regions (Danielson & Devary 1973).

Table (3): Chitinase secreted (mg/ml) by *T. harzianum* and *T. longibrachiatum* grown on potato dextrose and Czapek' s Dox broth media in relation to different incubation temperatures at different time intervals.

Medium	Biocontrol strain	Incubation temperature (°C)	Chitinase secretion (mg/ml)		
			3 days	9 days	15 days
Potato dextrose broth	*T. harzianum*	10	0.13	2.32	2.88
		20	1.14	2.67	4.23
		30	3.63	5.48	7.34
		40	0	0	0
	T.longibrachiatum	10	0.11	2.48	2.81
		20	1.14	2.64	4.02
		30	3.56	5.41	7.3
		40	0	0	0
Czapek' s Dox broth	*T. harzianum*	10	0.26	2.78	2.98
		20	1.15	2.84	4.44
		30	3.56	5.37	7.26
		40	0	0	0
	T.longibrachiatum	10	0.13	2.57	2.73
		20	1.16	2.66	4.43
		30	3.5	5.52	7.13
		40	0	0	0

A wide range of pH also was obtained (Table, 4) for enzyme production by the two fungal isolates, where pH5, 6, 7& 8 were favourable for the biosynthesis of chitinase. Similar results were obtained by El-Gamal (2000).

Table (4): Chitinase secreted (mg/ml) by *T. harzianum* and *T. longibrachiatum* grown on potato dextrose and Czapek' s Dox broth media in relation to different pH values.

Medium	Biocontrol strain	pH values	Chitinase secretion (mg/ml)		
			3 days	9 days	15 days
Potato dextrose broth	*T. harzianum*	5	0.66	1.12	2.64
		6	1.92	3.21	4.74
		7	3.52	4.87	6.76
		8	2.01	4.94	5.17
	T.longibrachiatum	5	0.61	2.49	2.98
		6	1.89	3.41	4.82
		7	3.41	4.42	6.82
		8	1.48	3.22	5.07
Czapek' s Dox broth	*T. harzianum*	5	0.73	1.88	2.49
		6	2.08	3.2	4.89
		7	3.78	4.81	7.22
		8	2.11	4.81	6.1
	T.longibrachiatum	5	0.71	2.35	2.72
		6	2.11	3.97	4.88
		7	3.66	4.81	7.12
		8	1.67	4.11	5.52

Different carbon sources were added to Czapek's Dox liquid medium as a sole source of carbon, regarding their effect on chitinase secretion by *T. harzianum* and *T. longibrachiatum* (Tables 5 & 6).The best carbon source used was cellobiose at 1&2% concentration in the case of *T. harzianum* and at 2% with *T. longibrachiatum* after 15 days of incubation comparing with Czapek's Dox liquid medium (Figs.1&2). *Trichoderma* sp. uses a wide range of compounds as carbon sources, the carbon and energy requirements of *Trichoderma* can be satisfied by monosaccharides and disaccharides as well as complex polysaccharides (Papavizas 1985).

The effect of nitrogen source on chitinase productivity was studied (Tables7&8). Malt extract and ammonium phosphate induced the highest chitinase productivity compared with other tested nitrogen sources for both *T. harzianum and T. longibrachiatum* after 15 days of incubation (Figs.3& 4). Ammonium appears to be the most readily

Table (5): Chitnase secreted (mg/ ml) by *T. harzianum* grown on Czapek's Dox broth medium in relation to different carbon sources at different time intervals.

Different carbon sources	Chitinase secretion (mg/ml)		
	3 days	9 days	15 days
Chitin 1%	3.46	4	4
Chitin 2%	3.647	5.413	5.32
Grounded shrimp shells 1%	3.644	3.48	3.37
Grounded shrimp shells 2%	3.52	3.517	3.41
Cellulose powder 1%	3.621	5.448	5.862
Cellulose powder 2%	3.594	5.483	5.463
Cellobiose 1%	3.659	5.483	7.31
Cellobiose 2%	3.644	5.483	7.31
Czapek's Dox broth medium	3.621	5.52	5.56

Table(6): Chitnase secreted (mg/ ml) by *T. logibrachiatum* grown on Czapek's Dox broth medium in relation to different carbon sources at different time intervals.

Different carbon sources	Chitinase secretion (mg/ml)		
	3 days	9 days	15 days
Chitin 1%	2.586	5.344	5.02
Chitin 2%	3.62	3.931	3.207
Grounded shrimp shells 1%	3.616	3.42	3
Grounded shrimp shells 2%	3.565	3.17	3.05
Cellulose powder 1%	3.62	4.86	4.65
Cellulose powder 2%	3.634	5.28	5.21
Cellobiose 1%	3.631	5.48	5.241
Cellobiose 2%	3.664	5.55	7.379
Czapek's Dox broth medium	3.621	5.52	5.82

utilized source of nitrogen, other sources such as urea, nitrate and even nitrite support abundant vegetative growth (Papavizas 1985). Ammonium phosphate induced the highest *β- glucosidase* and CPase (cellulase powder-ase) productivity by *A.terreus* and *P. oxalicum* more than other tested nitrogen sources (Younis *et al.*, 2001). In addition,*T.longibrachiatum* wild type strain CECT 2606 is used in transformation experiments and routinely cultured on malt extract agar at 30°C (Migheli *et al.*,1998).

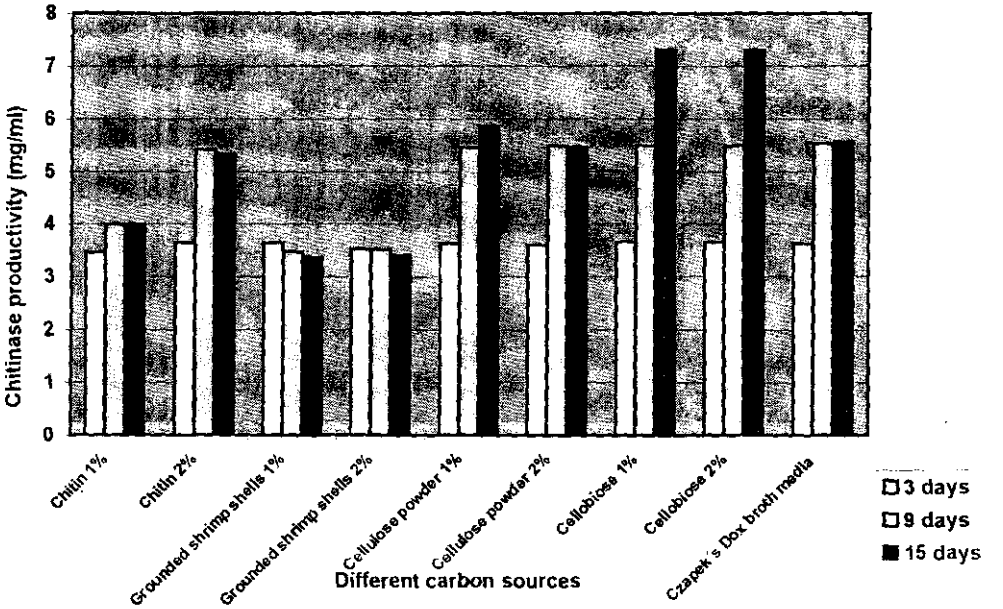


Fig.(1): Chitinase secretion (mg/ml) by *T. harzianum* grown on Czapek' s Dox broth medium in relation to different carbon sources at different time intervals.

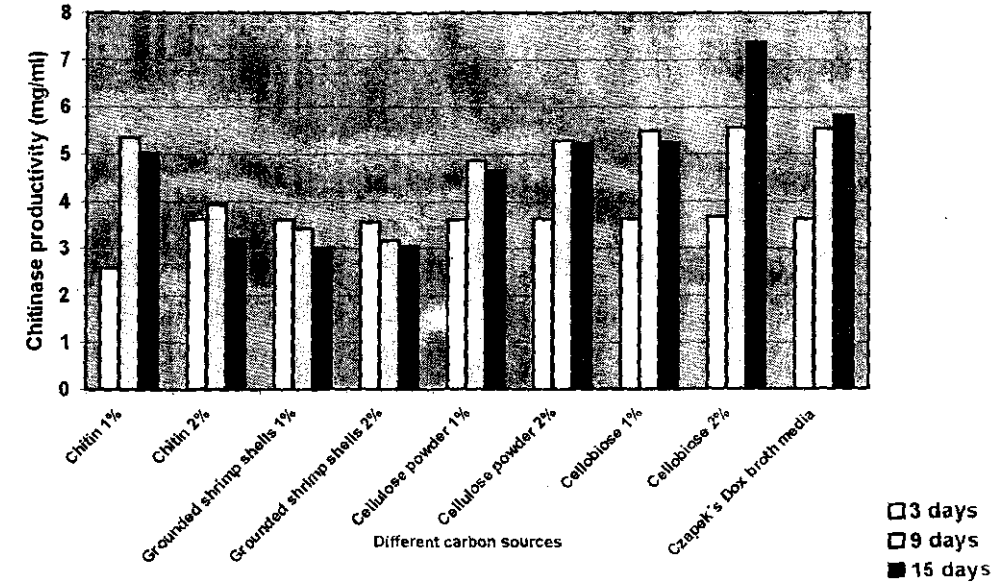


Fig.(2):Chitinase secretion (mg/ml) by T. longibrachiatum grown on Czapek' s Dox broth medium in relation to different carbon sources at different time intervals.

Table(7): Chitnase secreted (mg/ ml) by *T.harzianum* grown on Czapek's Dox broth medium in relation to different organic and inorganic nitrogen sources at different time intervals.

Different organic and inorganic nitrogen sources	Chitinase secretion (mg/ml)		
	3 days	9 days	15 days
Malt extract	1.505	5.424	7.31
Yeast extract	3.162	5.172	5.2
Pepton	3.121	5.414	5.3
Sodium nitrate	3.349	5.517	5.531
Ammonium chloride	3.152	3	3.2
Ammonium sulphate	1.143	1.5	1.6
Ammonium phosphate	3.72	4.689	7.31
Ammonium nitrate	2.42	2.759	2.76
Ammonium oxalate	1.34	1.95	1.97
Urea	3.32	5.206	5.21

Table(8): Chitnase secreted (mg/ ml) by *T. logibrachiatum* grown on Czapek's Dox broth medium in relation to different organic and inorganic nitrogen sources at different time intervals.

Different organic and inorganic nitrogen sources	Chitinase secretion (mg/ml)		
	3 days	9 days	15 days
Malt extract	3.693	5.448	7.328
Yeast extract	3.621	4.552	4
Pepton	3.766	3.42	3
Sodium nitrate	3.693	5.512	5.584
Ammonium chloride	3.639	3.71	3.7
Ammonium sulphate	3.592	4.103	4
Ammonium phosphate	3.621	5.483	7.345
Ammonium nitrate	3.589	3.61	3.6
Ammonium oxalate	3.685	3.59	3.6
Urea	3.631	3.8	3.5

Concerning amino acids (Tables 9&10) the results for chitinase productivity by *T. harzianum* and *T. longibrachiatum* indicated that L-Tyrosine exhibited the highest productivity among all amino acids tested in this study after 9 days of incubation as shown from (Figs 5 & 6).

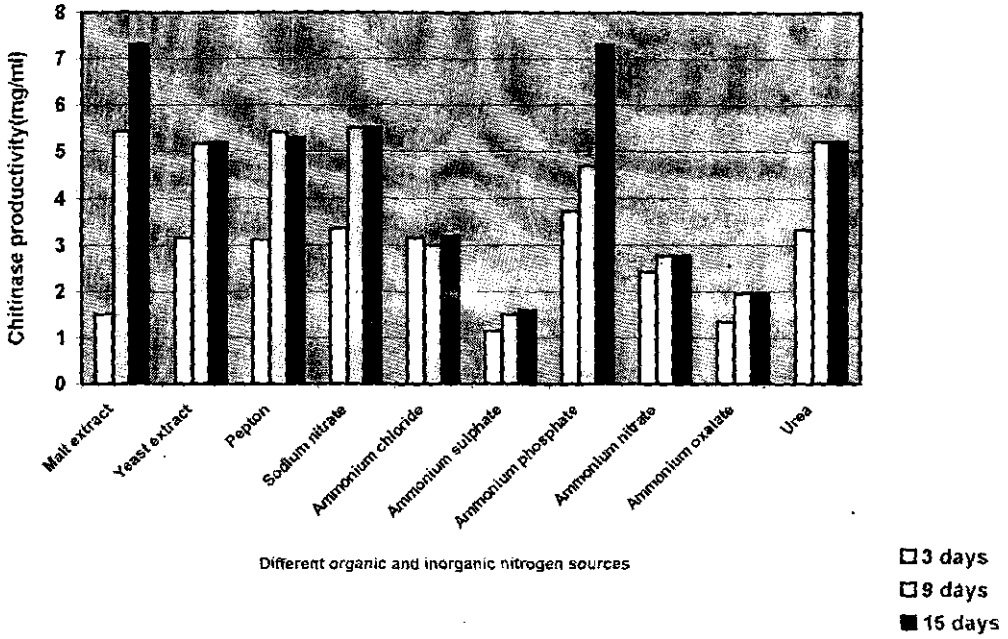


Fig.(3): Chitinase secretion (mg/ml) by *T. harzianum* grown on Czapek's Dox broth medium in relation to different organic and inorganic nitogen sources at different time intervals.

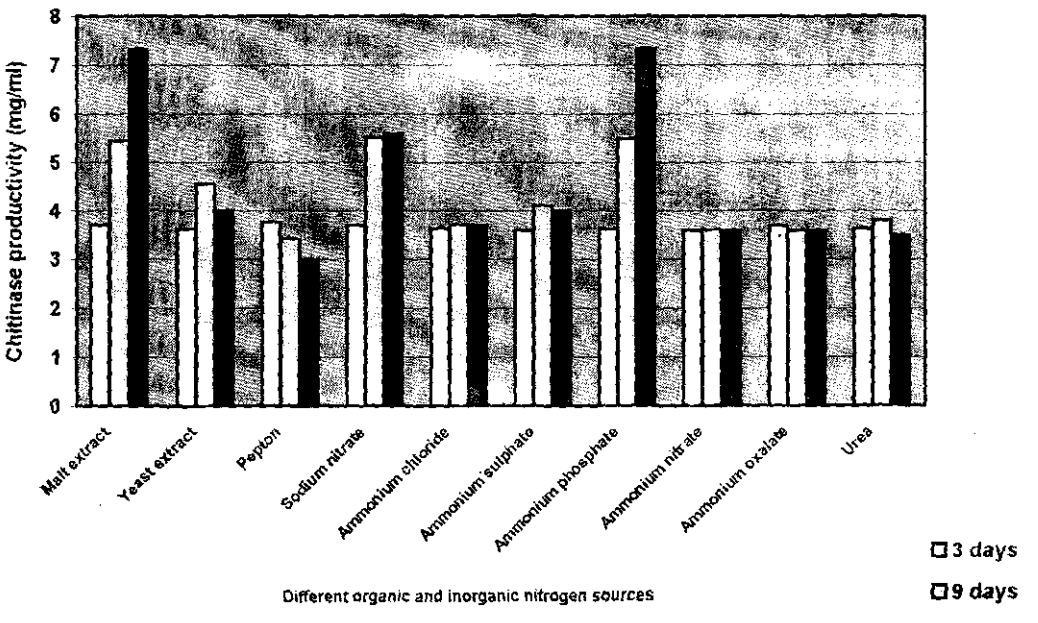


Fig.(4): Chitnase secretion (mg/ml) by *T. longibrachiatum* grown on Czapek' s Dox broth medium in relation to different organic and inorganic nitrogen sources at different time intervals.

Table(9): Chitnase secreted (mg/ ml) by *T.harzianum* grown on Czapek's Dox broth medium in relation to different amino acids at different time intervals.

Different amino acids	Chitinase secretion (mg/ml)		
	3 days	9 days	15 days
DL- valine	0.675	0.603	0.586
LB-Phenylalanine	3.512	3.1	3.1
Glycine	3.481	3.5	3.5
L-Tyrosine	3.12	3.879	3.88
L-Lysine	1.345	1.3	1.3
L-Arginine	0.654	0.65	0.65
DL-Isoleucine	2.366	2.4	2.4
L-Serine	0.649	0.6	0.6
Leucine	2.464	2.5	2.5
Czapek´s Dox broth medium	3.349	5.517	5.52

Table(10): Chitnase secreted (mg/ ml) by *T.longibrachiatum* grown on Czapek's Dox broth medium in relation to different amino acids at different time intervals.

Different amino acids	Chitinase secretion(mg/ml)		
	3 days	9 days	15 days
DL- valine	2.278	2.26	2.6
LB -Phenylalanine	3.574	3.4	3.4
Glycine	2.436	2.3	2.3
L-Tyrosine	3.633	5.12	5.12
L-Lysine	3.512	3.4	3.4
L-Arginine	3.62	3.2	3.2
DL-Isoleucine	3.63	3.6	3.6
L-Serine	1.84	1.7	1.7
Leucine	3.514	3.4	3.4
Czapek´s Dox broth medium	3.504	5.01	5.01

Exposing spore suspensions of *T. harzianum* and *T. longibrachiatum* to different doses of γ-rays in relation to chitinase induction is illustrated in (Tables 11 &12). A slight increase was recorded by *T. harzianum* at doses o.2, 0.4, 0.6, 0.8, 1, 2 and 4 KGy and by *T. longibrachiatum* at doses 0.4,

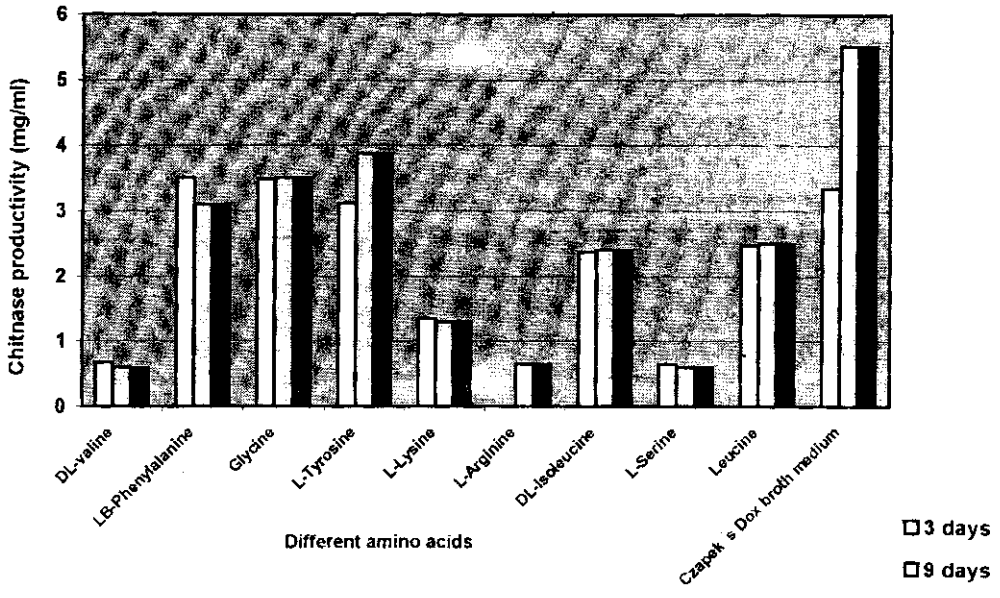
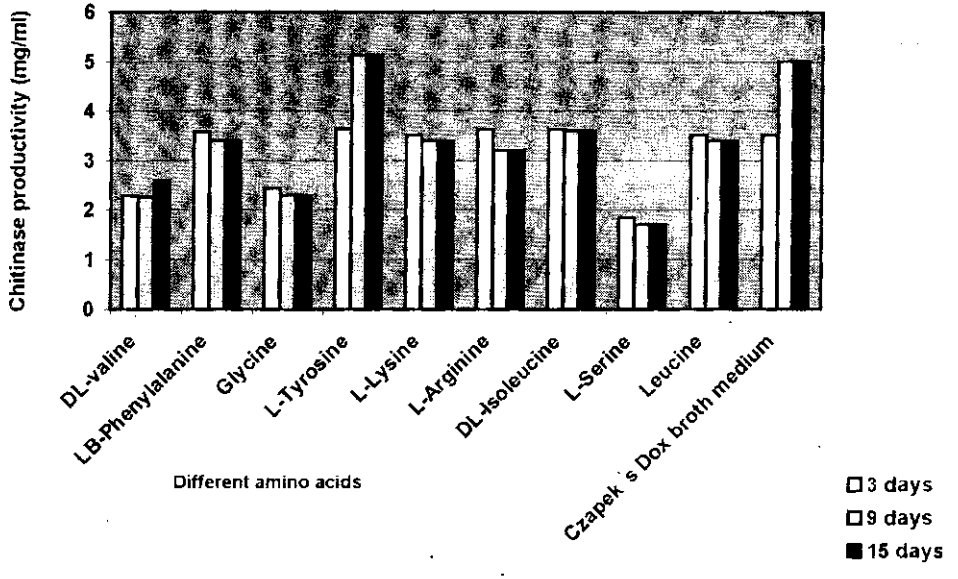


Fig.(5):Chitinase secretion (mg/ml) by *T. harzianum* grown on Czapek' s Dox broth medium in relation to different amino acids at different time intervals.



Fig(6): Chitinase secretion (mg/ml) by *T. longibrachiatum* grown on Czapek' s Dox broth medium in relation to different amino acids at different time intervals.

0.8, 1, 2 and 4 KGy (Figs.7 & 8).Gamma-irradiation as a tool in the production of mutated fungal isolates with enhanced enzyme productivity was recorded (Tamada *et al.*, 1987; Gbdemah and Awofo 1990; Kumakura 1993; Sidky *et al.*, 1996; Younis 1999 and Younis *et al.*, 2001).On the other hand, γ-rays at a dose 0.1 KGy did not increase the secretion of chitinase enzyme in the case of *T. harzianum,* and also at doses 0.2 & 0.6 KGy with *T. longibrachiatum.* These doses may induce cell lysis (Tamada *et al.*,1987). The increased enzyme production in the culture of irradiated cells resulted from the recovery of radiation damage after irradiation (Kumakura 1993). The obvious alteration in the chitinase secretion by the same producing strains in response to the treatment with different doses of γ-rays is in complete accordance with that reported by Sinha and Chakrabarty,(1977) who found that a mutant of *Aspergillus wentii Wehmer* obtained after γ-rays treatment altered amylase activity and exerted a marked variation in amylase activity, *i.e.*,an increase, decrease or even complete absence in such activity was noticed.

Table(11): Chitnase secretion (mg/ ml) by *T.harzianum* grown on Czapek's Dox broth medium in relation to different doses of gamma-rays at different time intervals.

γ-rays dose (kGy)	Chitinase secretion(mg/ml)		
	3days	9 days	15 days
0	3.62	5.517	5.53
0.05	3.62	5.448	6.413
0.1	1.078	5.448	5.432
0.2	3.649	5.517	7.379
0.4	4.034	5.517	7.345
0.6	1.601	5.414	7.379
0.8	2.121	5.414	7.379
1	3.592	5.414	7.31
2	3.636	5.483	7.31
4	3.659	5.483	7.31

From the results reported above it is noticed that *T.harzianum* was more aggressive than *T. longibrachiatum* against *F.moniliform, F.tabacinum, F.oxysporium* and *R.solani* at concentration 50% (Table, 1), despite their chitinase productivity was almost equal. Chitinase may not be

Table(12): Chitnase secretion (mg/ ml) by *T. longibrachiatum* grown on Czapek's Dox broth medium in relation to different doses of gamma-rays at different time intervals.

γ-rays dose (kGy)	Chitinase secretion(mg/ml)		
	3 days	9 days	15 days
0	3.62	5.517	5.828
0.05	3.62	5.517	6.241
0.1	3.61	5.483	6.966
0.2	3.633	5.482	5.473
0.4	2.474	5.552	7.345
0.6	3.587	5.517	5.49
0.8	3.631	5.483	7.241
1	3.621	5.448	7.275
2	3.621	5.414	7.03
4	3.683	5.414	7.276

the only enzyme responsible for the degradation of fungi cell walls, it is likely that the coordinated action of several hydrolyses (*i.e.*,ß-1,3-glucanses, lipases and proteases) are required for a complete degradation (Benhamou and Chet 1993).

In conclusion, the obtained data emphasized the possibility of applying *T. harzianum* and *T. longibrachiatum* as an effective biocontrol agent of several important plant-pathogenic fungi responsible for serious damage on many crops and minimizing the use of fungicides. Both isolates are capable of secreting chitinase enzyme, the development for detection of fungal chitinase may be now applied for an additional study as expression of chitinase during mycoparasitism and purification of the released enzyme. The discovery of new biological control agents and the demonstration of their value in reducing disease hazard have opened new promising avenues for practical applications in agriculture and lowering environmental pollution.

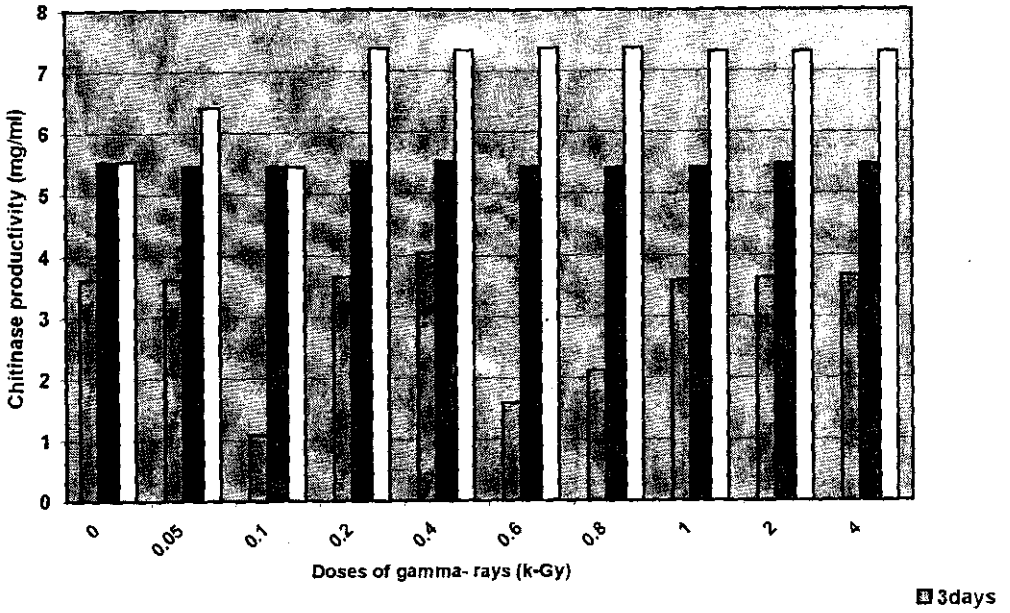


Fig.(7): Chitinase secretion (mg/ml) by *T. harzianum* grown on Czapek's Dox broth medium in relation to different doses of gamma rays at different time intervals. .

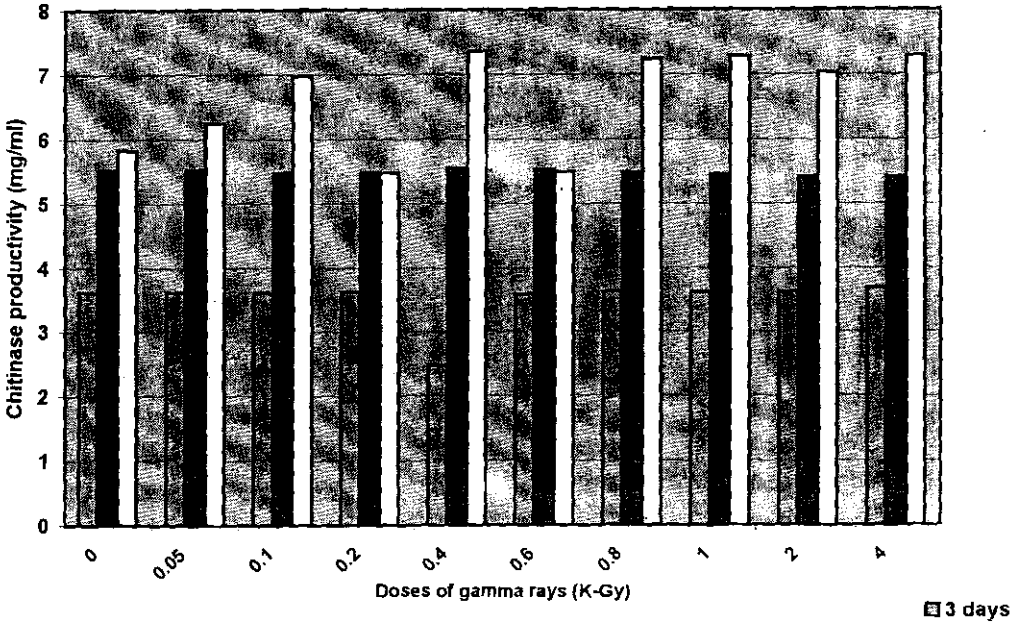


Fig.(8): Chitinase secretion (mg/ml) by *T. longibrachiatum* grown Czapek' s Dox broth medium in relation to different doses of gamma rays at different time intervals.

4.REFERENCES

Benhamou N. and Chet I. (1993). Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani:* Ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology, 83:1062 – 1071.

Benhamou N. and Chet I. (1996). Parasitism of *Sclerotium rolfsii* by *Trichoderma harzianum*: Ultrastructural and cytochemical aspects of the interaction. Phytopathology, 86: 405-416.

Carroad P. A. and Tom R.A. (1978). Bioconversion of shellfish chitin wastes: Process conception and selection of microorganisms.J. Food.Sci.,43: 1158- 1161.

Cherif M. and Benhamou N. (1990).Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporium* f.sp.*radicis-lycopersici.* Phytopathology, 80:1406-1414.

Chet I.(1987). Trichoderma: Application, mode of action, and potential as a biocontrol agent of soil borne pathogenic fungi. in:Novative Approaches to Plant Disease Control. I. Chet, ed. John Wiley& Sons, New York. Pages 137-160.

Danielson R.M. and Davey C.B.(1973). Non- nutritional factors affecting the growth of *Trichoderma* in culture. Soil Biol. Biochem., 5:495-504.

Davies D.A. L. and Pope A. M. S. (1977). Lytic enzymes as antifungal agents; a new approach to antimycotic therapy. Bull. Br. Mycol. Soc.,11: 144.

Davies D.A. L. and Pope A. M. S. (1978). Mycolase, a new kind of systemic antimycotic. Nature, 273, 235-236.

De la Cruz J., Rey M., Lora J. M., Hidalgo-Gallego A., Dominguez F., Pintor- Toro, J.A., Llobell A. and Benitez. T. (1993). Carbon source control on β- glucanses, chitobiase and chitinase from *Trichoderma harzianum.* Arch. Microbiol.,159: 316-322.

Durman S., Menedez A. and Godeas A.(1999). Evaluation of *Trichoderma* spp. as antagonist of *Rhizoctonia solani in-vitro* and as biocontrol of greenhouse tomato plants. Revista Argentina de Microbiologia, 31 (1):13-18.

El-Gamal, Nadia G.S.(2000). Biological and chemical control for root diseases of tomato plant. Ph.D. Thesis, Fac. of Agric. Cairo. Univ.

El-Mougy Nehal S.A. (2000). Effect of certain fungicides, biocides and bioagents in controlling root-rot disease of pea. Ph.D. Thesis, Fac. of Agric. Cairo Univ.

Farkas V. (1979). Biosynthesis of cell walls in fungi. Microbiol. Rev., 43: 117-144.

Fravel D.R. (1988). Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol., 26: 75-91.

Garcia L., Lora J.M. De la Cruz J., Benitez T., Llobell A. and Pintortaro J.A. (1994). Cloning and characterization of a chitinase (CHIT42) c DNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr. Genet., 27 : 83-89.

Gbdemah C.M. and Awofo V.(1990). The effect of γ-radiation on the cellulolytic, pectinolytic and amylolytic enzyme activity of some 'garri' fermenting microorganism. International Atomic Energy Agency, Vienna (Austria).Final Report of Co-ordinated Research Programme on Nuclear Techniques in the Improvement of Traditional Fermentation Practice in Developing Countries with Particular Emphasis on Cassava 1986 -1989.149: 77-83.

Geremia R., Goldman G., Jacobs D., Ardiles W., Vila S., Van Montagu M. and Herrera-Estrella A. (1993). Molecular characterization of the proteinase- encoding gene, prbl, releated to mycoparasitism by *Trichoderma harzianum*.Mol. Microbiol., 8: 603-613.

Gilman J.C. (1957). Soil fungi. Oxford and IBH publishing Co. alcutta, Bombay, New Delhi.

Goldman H.G., Hayes C. and Harman G.E.(1994). Molecular and cellular biology of biocontrol by *Trichoderma* spp. Trends Biotechnol., 12: 478-482.

Haran S., Schickler H., Oppenheim A. and Chet I. (1995). New components of the chitinolytic system of *Trichoderma harzianum*. Mycol. Res., 99: 441-446.

Haran S., Schickler H., Oppenheim A. and Chet I. (1996). Differential expression of *Trichoderma harzianum* chitinase during mycoparasitism. Phytopathology, 86: 980-985.

Harman G.E., Hayes C.K., Lorito M., Broadway R.M., DiPietero A., Peterbauer C.and Tronsmo A.(1993). Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. Phytopathology, 83: 313-318.

Janas P. and Targonski Z.(1995). Effect of temperature on the production of cellulases, xylanases and lytic enzymes by selected *Trichoderma reesi* mutants. Acta Mycologica, 30 (2): 255-264.

Kumakura M.(1993). Dose dependency of radiation enzyme production in *Trichoderma reesi.* Radiation and Environmental-Biophysics, 32(1), 41-46.

Lorito M., Harman G.E., Hayes C.K., Broadway R.M., Tronsmo A., Woo S.L.and DiPietro A.(1993). Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiodase. Phytopathology, 83: 302-307.

Mauch F., Hadwiger L.A. and Boller T. (1988). Antifungal hydrolyses in pea tissue: Purification and characterization of two chitinases and two β-1,3 glucanses differentially regulated during development and in response to fungal infection. Plant Physiol., 87 : 325-333.

Melo I. S., Faull J.L. and Graemecook K.A. (1997). Relationship between *in vitro* cellulase production of uv. induced mutants of *Trichoderma harzianum* and their bean rhizosphere competence.Mycol. Res., 101: (11) 1389-1392.

Migheli Q., Candelas L.G., Dealessi L., Camponogara A. and Vidal D.R.(1998). Trasformants of *Trichoderma longibrachiatum* overexpressing the β-1,4- Endoglucanse Gene egl1 show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology, 88 : 673-677.

Miller G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal.Chem., 31, 426-428.

Mischke S. (1997). A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. Mycopathologia, 137: (1) 45-52.

Monreal J. and Reese E.T.(1969).The chitinase of *Serratia marcescens*. Canadian. J. of Microbiology, 15: 689-696.

Papavizas G.C.(1985). Trichoderma and Gliocladium : Biology, ecology and the potential for biocontrol. Annu. Rev. Phytopathol., 23: 93-121.

Pleban S., Chernin L. and Chet I. (1997). Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Letters in Appl. Microbiol., 25: (4) 284-288.

Ried J.D. and Ogryd-Ziak D.M. (1981). Chitinase over-producing mutant to *Serratia marcescens*. Appl. and Environ. Microbiol., 41 :664-669.

Sidkey, Nagwa M., Younis, Nahed A. and Soliman, Kawther M.(1996). Biosynthesis of certain extracellular enzymes by *Aspergillus parasiticus*,NRLL 2999 in relation to γ- radiation treatment. Al-Azhar Bull. Sci., 7(2) :1379-1390.

Sinha S. and Chakrabarty S.L. (1977). Relative effectiveness of mutagens on amylase production in *Aspergillus wentii*. Indian-J. of Experimental Biology,15 (11) :1068 - 1069.

Tamada M., Kasai N. and Kaetsu I. (1987). Effect of γ-ray irradiation on cellulase secretion of *T. reesei*. J. Ferment. Technol., 65 (6): 703-705.

Tronsmo A. and Harman G.E. (1992). Co production of chitinolytic enzymes and biomass for biological control by *Trichoderma harzianum* on media containing chitin. Biological control, 2 (4): 272-277.

Ulhoa C.J. and Peberdy J.F.(1991). Purification and characterization of an extracellular chitobiosidase from *Trichoderma harzianum*. Curr. Microbiol., 23: 285-289.

Ulhoa C.J. and Peberdy J.F.(1992). Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. Enzyme Microb. Technol., 14 :236-240.

Wijk D.J.V., Kroon S.G.M. and Garttener-Arends I.C.M. (1998). Toxicity of chlorate and chlorite to selected species of algae, bacteria and fungi. Ecotoxicology and Environmental safety, (B) Environmental Research, 40 (3): 206-211. (C.f. Rev. Pl. Pathol., 77: (12) 9669).

Younis, Nahed A.(1999). A comparative study on protease, α- amylase and growth of certain fungal strains of *Aspergillus* sp. after exposure to γ-rays. Arab Journal of Nuclear Sciences and Applications, 32(2): 257-264.

Younis, Nahed A. , Sidky, Nagwa M., Ammar M.S. and Ouda, Sahar M. (2001). Recycling of Water Hyacinth for production of cellulases by *Aspergillus terreus*, I_5 and *Penicillum oxalicum*, I_8 under solid state fermentation. Al- Azhar Bull. Sci., 12 (2): 353-373.

العوامل المؤثرة على افراز المضاد الفطرى الكيتينيز المفرز بواسطة تريكودرما هارزيانم وتريكودرما لونجيبراكياتم

ناهد على يونس- اشرف صبرى احمد

قسم البحوث النباتية- مركز البحوث النووية (انشاص) - هيئة الطاقة الذرية

ملخص

وجد أن استخدام الراشح الفطرى لكل من الفطرين تريكودرما هارزيانم و تريكودرما لونجيبراكياتم عند التركيز ٨٠٪ أحدث تثبيطا كاملا لنمو بعض من الكائنات الممرضة لجذور النباتات مثل :

Fusarium. moniliforme, F. oxysporium, F. semitectum, F. solani, F. tabacinum, Phytophthora infestans, Rhizoctonia solani, and Verticillium sp.

وقد ثبت أن لكلا النوعين مقدرة على إنتاج إنزيم الكيتينيزعند تنميتهما على بيئتى تشابكس دوكس ودكستروز البطاطس السائلة. كما تناول البحث دراسة تأثير الظروف البيئية على إ نتاج الإنزيم- حيث وجد أن زيادة فترة التحضين أدت الى زيادة إنتاج الإنزيم. هذا ولم يكن لاختلاف تركيز اللقاح تأثيرعلى إنتاج الإنزيم الاعند نمو الفطر تريكودرما هارزيانم على بيئة دكستروز البطاطس السائلة بعد ١٥ يوما من التحضين حيث كان هناك زيادة للإنزيم بزيادة كثافة اللقاح وكان التركيز ٧ x ١٠^{٧} جرثومة / ملى هوالأفضل لإنتاج الإنزيم . وجد أن المدى من الحرارة والحموضة المناسب لإنتاج الكيتينيز لكلا من النوعين هما (٢٠-٣٠°م & ٦- ٨) على التوالى. وبدراسة تأثير المصادر الكربونية المختلفة على إنتاج الإنزيم وجد أن أنسب مصدر كربون هو السيليوبيوز بتركيز ١و٢ ٪ للتريكودرما هارزيانم وبتركيز ٢٪ للتريكودرما لونجيبراكياتم بعد ١٥ يوما من التحضين مقارنة ببيئة تشابكس دوكس السائلة. وعند مقارنة المصادر النتروجينية المختلفة على إنتاجية الإنزيم كان مستخلص المولت وفوسفات الأمونيوم هما الأفضل لكل من النوعين المستخد مين. وقد أدى استخدام الحامض الأمينى (تيروسين) إلى أعلى إنتاجية للإنزيم عند مقارنتة بباقى الأحماض الأمينية الأخرى المستخدمة فى التجربة وذلك بعد ٩ أيام من التحضين لكل من الفطرين. وعند تعريض المعلق الجرثومى للفطرين لجرعات مختلفة من أشعة جاما وجدت زيادة طفيفة فى إنتاج الإنزيم المفرز بواسطة الفطر تريكودرما لونجيبراكياتم عند الجرعات ٤و& ٨و & ١& ٢& ٤ ك.جراى وباستخدام الجرعات٢و&٤ و & ٦و & ٨و & ١& ٢& ٤ ك.جراى لفطرالتريكودرما هارزيانم .

المجلة العلمية لكلية الزراعة - جامعة القاهرة - المجلد (٥٥) العدد الاول (يناير ٢٠٠٤) :١٢١-١٤٤.