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Optimization of factors affecting proliferation and flourishment of *Trichoderma harzianum* in Egyptian soil

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Abstract. The present study was planned to evaluate the efficiency of potential antagonistic *Trichoderma* as biocontrol agent for damping-off of tomato and root rot of kidney bean plants, by optimization of cultural factors of *Trichoderma* in soil under laboratory conditions. Results revealed that inoculating *Trichoderma* into the soil as mycelial preparations grown on rice husks, resulted in better survival and proliferation, than when grown on corn meal at concentration of 5 % with soil moisture content of 30 % WHC and optimum temperature 28°C. The incubation period of 12 days and pH of 5.5 resulted in best dry weight and the best antagonistic potential. The optimum incubation temperature that gave the highest dry weight was 25°C while that gave the best antagonistic potential was 20°C. Using chitin and sodium nitrate as carbon and nitrogen sources induced growth and best antagonistic potential.

Key words: Antagonistic effect, *Trichoderma harzianum* , *Pythium ultimum, Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii*.

Introduction

The abundance of *Trichoderm*a in various soils is coupled with their ability to degrade organic substrates, their metabolic versatility, their resistance to microbial inhibitors, the depedence on prevailing conidions, strains involved, and active competitive saprophytic ability.

For the effective use of *Trichoderma* spp. in biological control of soilborne plant pathogens, it is necessary that the antagonist is able to survive, grow and proliferate in the soil and rhizosphere (Garrett 1956). Many reports dealt with the survival and proliferation of *Trichoderma* spp. in soil.

Increasing the antagonistic activity has been the target of some investigators. Generally, specific conditions must prevail for maximum activity. These include adjustment of environmental as well as physiological and growth conditions that should run parallel with strain improvement (Handelsman and Stabb 1996, Larkin *et al.* 1998). Also, many investigators suggested that the conditions must be optimized to obtain maximum inhibition of the pathogenic organism by the action of other antagonistic microorganisms (Cook and Baker 1983). Most of such studies have been conducted on agar, which gave presumptive results for antagonism and the results obtained in laboratory experiments provide necessary information about the antagonistic potential of microorganisms for disease control (Baker and Cook 1974).

The main purpose of this paper is to study some factors affecting the survival and proliferation of *Trichoderma harzianum* in soil.

Materials and Methods

Isolation of test fungi

Fungi used in this study to control two plant diseases were obtained as follows:

(1) *Trichoderma harzianum* (T1 isolated from rhizosphere and rhizoplane of healthy and diseased tomato plants collected at Tezmant village while T2 was isolated from rhizoplane of kidney bean plants collected from Seds village at Beni-Sueif).

(2) *Pythium ultimum* was isolated from damped-off tomatoes collected at Tezmant village at Beni-Sueif.

(3) *Macrophomina phaseolina, Rhizoctonia solani* and *Sclerotium rolfsii* were isolated from rotted roots of kidney bean plants collected from Seds village at Beni-Sueif.

Proliferation of *Trichoderma*

Effect of age and substrate used on *Trichoderma* growth in soil

Autoclaved corn meal-sand or sand-rice-husk in 500 ml Erlenmeyer flasks with distilled water (1:1:2, W/ W/ V (El-Naghy *et al*. 1998) was inoculated by *Trichoderma harzianum* and

incubated at 28°C for 7,15 and 21 days. Inocula were added to sterilized soil at 5% concentration (W/W) in 500 ml Erlenmeyer flasks containing 200 g sterilized soil (clay-sand). Moisture content was adjusted to 50% soil WHC and soil was incubated at 28°C for 60 days (El-Naghy *et al*. 1998). Dilution plate method was used for determination of *Trichoderma* count after 1, 7, 15, 30, and 60 days using modified Martin's medium (Papavizas 1982).

Effect of inoculum concentration on the mycelial growth of *Trichoderma*

This experiment was performed using *Trichoderma harzianum* inoculum of 21 days old mycelia on rice husks, which proved to be the most suitable for maintaining *Trichoderma* growth in soil. This inoculum was added to 500 ml Erlenmeyer flasks containing 200 g soil at different concentrations 0.5, 5 and 10% (W/W) and mixed very well.

The moisture content was adjusted at 50% soil WHC and inoculated soil samples were incubated at 28°C for 60 days and examined for *Trichoderma* growth development by using the dilution plate method after 1, 7, 15, 30, and 60 days using modified Martin's medium.

Effect of soil moisture content on the mycelial growth of *Trichoderma*

Flasks (each containing 200 g of clay-sand soil) were inoculated with 5% inoculum of 21 days old *Trichoderma harzianum* mycelia grown on rice husk meal. The moisture content was adjusted at 30, 50, or 75% soil WHC by adding sterilized distilled water. All flasks were incubated at 28°C. Fungal growth determination was made after 1, 7, 15, 30, and 60 days using the dilution plate method and modified Martin's medium.

Effect of temperature on the mycelial growth of *Trichoderma*

Each conical flask (500 ml capacity) received 200 g of loamy sand soil inoculated with 5% inoculum of *Trichoderma harzianum* (21 days old) grown on rice husk sand. Moisture content was kept constant throughout the experimental period (60 days) at 20, 28, 35 and 45°C. Fungal growth determination was made after 1, 7, 15, 30, and 60 days of incubation using the dilution plate method and modified Martin's medium.

Effect of environmental conditions and nutritional requirements on the growth of *Trichoderma harzianum* and its potential as a bioagent

T. harzianum T2 was selected to carry out this study as it was more virulent and showed the best antagonism against all test pathogens under laboratory conditions.

Effect of incubation period

Trichoderma harzianum (T2) was grown on liquid Czapek-Dox's basal medium (Davet and Rouxel 2000) for periods of 3, 6, 9, and 12 days. PH values were adjusted at 5.5 before sterilization.

The medium was dispensed in 250 ml capacity Erlenmeyer conical flasks where each flask received fifty ml. Flasks were sterilized and inoculated with 5 mm discs of growing *Trichoderma* and incubated at 28 °C for the proposed periods of experiment. After the elapse of each period of incubation, 3 flasks were taken to estimate both of growth (as dry weight) and efficiency of the filtrate in inhibiting the growth of *Pythium ultimum*, *Macrophomina phaseolina, Rhizoctonia solani*, and *Sclerotium rolfsii*. This efficiency was estimated by sterilization of the culture filtrates using 0.45 or 0.2 µ-pore membrane filter and then added at 50% (v/v) to double strength potato dextrose agar medium (PDA) (Davet and Rouxel 2000). The filtrate supplemented with PDA was dispensed at 10 ml aliquots in 90 mm diameter Petri-dishes. Plates containing no culture filtrates were used as controls. Pathogenic fungi were inoculated in the center of plates and linear growth was measured at right angles, for three replicate plates per treatment, after 3 days of incubation in constant light at 28 °C (Calistru *et al.* 1997). The percentage of inhibition (I) was calculated according to Topps and Wain equation (1957).

Effect of pH values

Trichoderma harzianum was grown in Czapek's Dox broth medium previously adjusted at different pH values (5, 5.5, 6, 6.5, and 7) in three replicates of Erlenmeyer flasks (using 0.1 N HCL and 0.1 N Na OH) and incubated at 28 °C. After 12 days of incubation (which gave best growth and antagonistic potential), the growth of *T. harzianum* was measured as mentioned above. The pH of the filtrate was neutralized before testing its inhibition for the growth of the pathogenic fungi as mentioned above.

Effect of temperature

Czapek's Dox broth medium that adjusted at pH 5.5 (which gave best growth and antagonistic potential) was dispensed in 250 ml Erlenmeyer flasks. Media were inoculated with *Trichoderma harzianum* and incubated at different degrees of

temperature (20, 25, 35 and 45°C) for 12 days. At the end of incubation period, the fungal growth was determined in three replicate flasks as usual.

Effect of carbon source

Sucrose recommended in Czapek's Dox basal broth was replaced by one of the following carbon sources: glucose, starch, cellulose, and chitin at 2% concentration. Sugars under test were separately sterilized by adding drops of diethyl ether, left to dry and then incorporated separately with the autoclaved basal broth. pH values were adjusted to 5.5 before autoclaving. Media were then inoculated with the organism and incubated for 12 days at 20°C. Thereafter, fungal growth and potency were determined.

Effect of nitrogen source

Sodium nitrate in the basal medium broth was replaced by one of the following nitrogen sources: sodium nitrite, ammonium sulphate, peptone and asparagine. After inoculation and growth at 20°C for 12 days, the fungal growth and potency were determined as usual.

Statistical analysis

One-way ANOVA (Snedecor and Cochran 1962) followed by the least significant difference test (LSD) were used to verify the significance of the difference between the mean values of the different treatments or parameters at 0.05 level of probability.

Results and Disccusion

In this study, evaluation of *Trichoderma harzianum* growth and its antagonistic potential under varying environmental conditions (incubation period, pH and incubation temperature) and different carbon and nitrogen sources was carried out under laboratory conditions.

Proliferation of *Trichoderma*:

Sterilized soils inoculated with mycelial preparations of *T. harzianum* grown for different periods (7, 14 and 21 days) on sand-corn meal and sand-rice husks at a concentration of 5% showed that mycelial preparations of 21 days gave more population densities than the other ages and the 7-day-old mycelial preparation gave the least population density. In no case, there was a decrease in the colony count of *T. harzianum* during the incubation period than the initial count at the beginning of experiment indicating better survival and proliferation of *Trichoderma* in soil (Table 1). As the intensities of the above mentioned parasitic interactions in the soil are lower than observed in the dual-culture experiments, probably because of the lower concentration of nutrients in the soil supporting *Trichoderma* growth (Inbar *et al.* 1996). *Trichoderma* inoculum added to the soil in the form of hyphae grown on a natural medium such as rice husks or corn meal did not appear to be subjected to fungistasis as reported by Lockwood (1977) and the hyphae are less sensitive to fungistasis than the conidia.

The comparison of rice-husks with corn meal as substrates supporting growth of *T. harzianum* revealed the significant superiority of rice-husks over corn meal in supporting fungal growth in soil during the whole incubation period (Table 1). The older inocula of *Trichoderma* gave higher population densities than younger ones, probably because of higher inoculum potential.

Results presented in Figure (1) indicated that the final count of *Trichoderma* in case of the 5% inoculum was more than that of the 10 % concentration. The results of the present investigation revealed that at 5 % concentration of the mycelial hyphae introduced into the soil was more persistent than other concentrations for the whole incubation periods, indicating its stability for further experimentation as suggested previously by El-Naghy *et al.* (1998).

To determine the optimum moisture content which encourages mycelial proliferation of *T. harzianum* in soil, 21-day-old mycelial preparations of the fungus grown on sand rice-husk were added to the soil at 5% concentration. Soil moisture was adjusted to different levels (Fig. 2)

Obtained results on the effect of moisture content and temperature on proliferation of the antagonist in soil support the mesophilic nature of the fungus (optimum growth was 28°C (Fig. 3) and better growth at good aeration conditions (30 % m. c.) in sterile soil as suggested previously by Shaban (1986) and El-Naghy *et al*. (1993, 1998) and Haq *et al.* (2009).

The highest significant mycelial weight of *T. harzianum* T2 was obtained after 12 days of incubation. Also, the inhibitory effect of *T. harzianum* T2 against the growth of *P. ultimum*, *M. phaseolina*, *R. solani* and *S. rolfsii* was observed after 12 days Table (2). Similarly,

Table (1): Proliferation of *Trichoderma* (colony count/mg) introduced into sterile soil as mycelial inoculum with different ages grown on rice husks and corn meal. Values indicated are means followed by standard deviations. LSD at $P = 0.05$.

Incubation period (days)	Food base	Age (days)			LSD
		7	14	21	
1	Corn meal	55±1	190±10	120±2	11.8
	Rice husks	35±2	150±5.0	195±5	8.40
	LSD	3.50	17.90	8.60	
7	Corn meal	40±5	160±5	140±15	19.12
	Rice husks	80±3	155±4	200±5	8.15
	LSD	9.30	10.30	25.30	
15	Corn meal	35±4	80±10	125±3	12.89
	Rice husks	95±5	210±5	220±2	8.40
	LSD	10.20	17.90	5.77	
30	Corn meal	35±5	70±3	55±5	8.86
	Rice husks	55±5	85±2	150±2	6.60
	LSD	11.30	5.77	8.60	
60	Corn meal	30±3	40±5	55±1	8.6
	Rice husks	20±5	25±1	130±2	6.3
	LSD	9.34	8.17	3.50	

Table (2): Dry weights of *Trichoderma harzianum* (T2) and its potential as a bioagent expressed as % of the growth reduction of the pathogenic fungi at different incubation periods. Values indicated are means followed by standard deviations. LSD at $P = 0.05$.

Incubation periods (days)	*Trichoderma* dry weight	% of growth reduction			
		P. ultimum	*M. phaseolina*	*R. solani*	*S. rolfsii*
1	0.00±0.000	00.0±0.0	00.0±0.0	00.0±0.0	00.0±0.0
3	0.22 ±0.010	00.0±0.0	00.0±0.0	00.0±0.0	00.0±0.0
6	0.26±0.010	00.0±0.0	20.8 ±0.6	18.5 ±0.5	31.3±0.3
9	0.27 ±0.001	12.9 ±0.5	25.5 ±0.1	18.5 ±0.03	42.9 ±0.1
12	0.41 ±0.002	27.3 ±0.3	29.3 ±0.1	20.8±0.3	0.50 ±0.0
LSD	0.012	0.47	0.5	0.48	0.26

Table (3): Dry weights of *Trichoderma harzianum* (T2) (after 12 days incubation period) and its potential as a bioagent expressed as % of the growth reduction of the pathogenic fungi at different pH values. Values indicated are means followed by standard deviations. LSD at $P = 0.05$

pH	*Trichoderma* dry weight	% of growth reduction			
		P. ultimum	*M. phaseolina*	*R. solani*	*S. rolfsii*
5.0	0.31±0.001	00.0±0.00	3.7 ±0.299	7.3 ±0.4	8.90 ±0.10
5.5	0.33±0.002	29.3 ±0.20	29.6 ±0.6	23.6 ±1.0	11.10 ±0.10
6.0	0.32±0.00	3.40 ±0.19	29.6 ±0.6	21.8 ±0.5	4.40 ±0.30
6.5	0.30 ±0.01	3.40 ±0.40	1.9 ±0.01	00.0±0.0	0.00±0.00
7.0	0.20 ±0.01	0.00±0.00	00.0±0.0	00.0±0.0	0.0±0.00
LSD	0.012	0.40	0.73	0.97	0.27

Calistru *et al*. (1997) found that the culture filtrates of *T. harzianum* and *T. viride* grown for 12 days were inhibitory to *Fusarium moniliforme* and *Aspergillus flavus*. On the other hand, Mousa (2004) reported that *T. harzianum* exhibited the maximum antagonistic activity after six days of incubation.

The optimum pH value for maximum growth and antagonistic activity of *T. harzianum* T2 was 5.5 (Table 3). Several investigators found that the acidic side was preferable for different *Trichoderma* spp. to give maximum antagonistic potential against various phytopathogenic fungi (Chet and Baker 1981, Khalaf 1993, Kredics *et al*. 2004).

The optimum incubation temperature for growth of *T. harzianum* T2 was 25°C, while the optimum incubation temperature for maximum antagonistic potential was 20°C Table (4). Similarly, Goldfarb *et al*. (1989) reported that the maximum antagonistic activity of *T. harzianum* was 20 °C. Moreover, the results are almost in agreement with those of Elmougy (2000) who found that the optimum temperature for the growth of *T. harzianum*, *T. viride* was 20-30°C. Santamarina and Rosello (2006) observed that *T. harzianum* inhibited the development of *R. solani* and *Verticillium dehaliae* upon contact at 15 and 25°C. They also found that the growth decreased as temperature dropped from 25 to 15°C.

The source of carbon and nitrogen was found to affect the growth and antagonistic ability of *T. harzianum* T2. Chitin was the most favorable carbon source for the growth of *T. harzianum* T2 and its antagonistic effect on the tested phytopathogenic fungi (Table 5). Asparagine, peptone and sodium nitrate were the best nitrogen sources for the growth of *T. harzianum* T2 but sodium nitrate was the best for the maximum antagonistic ability followed by asparagine and peptone (Table 6).

In this concern, Papavizas (1985) reported that species of *Trichoderma* use a wide range of compounds as sole carbon and nitrogen sources. The carbon and energy requirements of *Trichoderma* can be satisfied by monosaccharides and disaccharides as well as polysaccharides. Kücük and Kivanc (2004) stated that *T. harzianum* isolates grown on chitin as a sole carbon source produced chitinolytic enzymes. These enzymes are needed for maximum efficiency for the biological control of the chitin containing plant pathogenic fungi. On the other hand, Elmougy (2000) recorded the highest antagonistic ability of *Trichoderma* spp. on medium containing glucose. Also, she reported that sodium nitrate was the best nitrogen source for increasing the antagonistic ability followed by asparagine and peptone. Shoulkamy *et al*. (2006) examined the induction of β-1,3-glucanase and chitinase in liquid culture medium amended with fungal dried cell walls as sole carbon source by *Trichoderma* and *Gliocladium* species and reported that the culture filtrate showed antifungal effect against *F. oxysporum.*

Results of this paper, initiate a possible biocontrol measure of some pathogenic fungi. Emphasis should be given to clarify whether the ability of biocontrol agents is stable or transient and the consistency of their performance must be improved. Accomplishing this will require more research in many diverse areas, because biological control is the culmination of complex interaction among the host, pathogen(s), antagonist(s) and environment.

It should be mentioned that most of the potential biocontrol agents appeared effective under controlled conditions, but further improvement of their efficacy is often required to provide economically acceptable and reliable control under field conditions.

Table (4): Dry weights (in mgm) of *Trichoderma harzianum* (T2) (after12 days incubation period at pH 5.5) and its potential as a bioagent expressed as % of the growth reduction of the pathogenic fungi at different incubation temperatures. Values indicated are means followed by standard deviations. LSD at $P = 0.05$.

Incubation temperature (^oC)	*Trichoderma* dry weight	% of growth reduction			
		P. ultimum	*M. phaseolina*	*R. solani*	*S. rolfsii*
20	0.30±0.010	30.3±0.30	70.2±0.20	73.4±3.4	74.1±0.08
25	0.36±0.001	18.2±0.01	63.9±0.99	43.8±0.4	62.5±2.0
35	0.27±0.01	28.8±0.80	54.4±0.40	37.5±0.5	28.1±1.1
45	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.0	0.00±0.00
LSD	0.013	0.80	1.03	3.25	2.15

Table (5): Dry weights (in mgm) of *Trichoderma harzianum* (T2) (after 12 days incubation period at pH 5.5 and incubation temperature 20 ºC) and its potential as a bioagent expressed as % of the growth reduction of the pathogenic fungi using different carbon sources. Values indicated are means followed by standard deviations. LSD at $P = 0.05$

Carbon source	*Trichoderma* dry weight	% of growth reduction			
		P. ultimum	*M. phaseolina*	*R. solani*	*S. rolfsii*
Glucose	0.33±0.002	10.3±0.30	31.6±1.59	26.1±0.10	20.3±1.0
Sucrose	0.32±0.010	6.40±0.50	25.3±0.011	20.9±0.01	18.6±0.30
Starch	0.31±0.001	33.3±3.30	22.8±1.50	37.7±2.70	6.80±1.60
Cellulose	0.37±0.020	41.0±1.00	22.8±0.80	17.4±0.30	8.40±0.39
Chitin	0.39±0.010	50.5±2.00	40.5±0.50	40.9±0.10	30.5 ±0.05
LSD	0.020	3.27	1.90	2.36	1.58

Table (6): Dry weights (in mgm) of *Trichoderma harzianum* (T2) (after12 days incubation period at pH 5.5 and incubation temperature 20 ºC) and its potential as a bioagent expressed as % of the growth reduction of the pathogenic fungi using chitin as a carbon source with different nitrogen sources. Values indicated are means followed by standard deviations. LSD at $P = 0.05$

Nitrogen source	*Trichoderma* dry weight	% of growth reduction			
		P. ultimum	*M. phaseolina*	*R. solani*	*S. rolfsii*
Sodium nitrate	0.31±0.01	49.8±0.5	42.5±1.0	44.4±0.4	25.9±0.6
Sodium nitrite	0.26±0.02	25.3±0.3	20.4±2.0	23.7 ±2.2	5.6±0.4
Peptone	0.32±0.00	32.0±1.0	33.4 ±2.4	40.1 ±0.1	23.3±0.3
Amm. sulphate	0.24±0.03	20.0±5.0	10.4 ±1.0	18.6 ±0.4	6.6±0.6
Asparagine	0.34±0.02	41.0±1.0	40.5 ±0.50	44.4 ±0.8	25.4±0.4
LSD	0.035	4.25	2.81	1.96	0.87

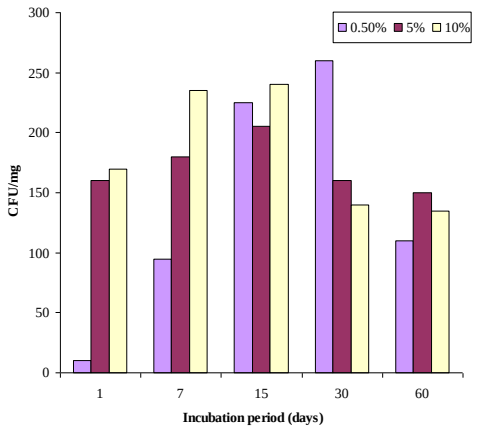

Figure (1): Proliferation of *Trichoderma* introduced into sterile soil, 21-day-old mycelial preparation grown on rice husks at different concentrations (0.5, 5 and 10%) at 50% WHC. CFU = Colony forming unit.

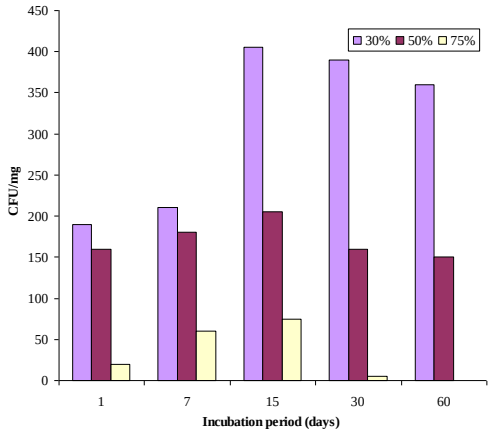

Figure (2): Proliferation of *Trichoderma* introduced into sterile soil, 21-day-old mycelial preparation grown on rice husks at concentration 5% with different WHC (30, 50 and 75%). CFU = Colony forming unit.

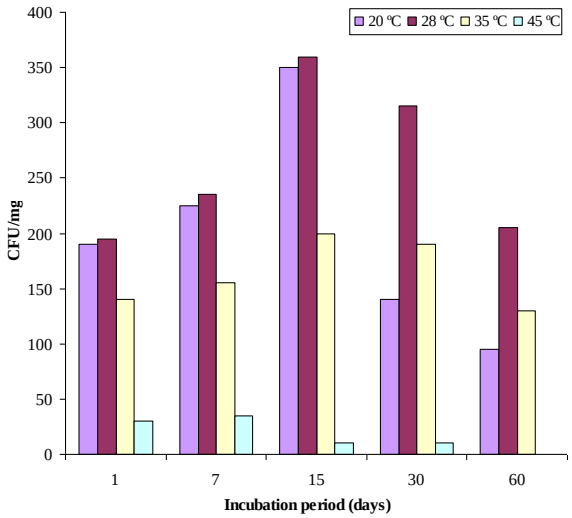

Figure (3): Proliferation of *Trichoderma* introduced into sterile soil 21-day-old mycelial preparation grown on rice husks at different concentrations 5% at different incubation temperatures (20, 28, 35 and 45 ºC) at 30% WHC. CFU = Colony forming unit.

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