Biological Control of Chickpea Damping-off and Root Rot Diseases

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Abstract:

Twenty three fusarial isolates of 3 species e.g. *Fusarium oxysporum, F. solani* and *F. moniliforme* which isolated from diseased chickpea collected from different fields located at Assiut governorate were tested for their pathogenicity to chickpea. All these isolates caused pre and post emergence damping-off and root rot. *F. oxysporum* isolate (No.35) gave the highest percentage of infection. Isolates of *Trichoderma harzianum, Pseudomonas fluorescence and Bacillus subtilis* which isolated from chickpea rhizosphere inhibited growth of *F. oxysporum, F. solani and F. moniliforme*, *in vitro* and reduced pre and post emergence damping-off and root rot under greenhouse conditions. The selected antagonists varied in their inhibitory effect on radial growth of the tested pathogen and the reduction of the diseases.

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Introduction:

Chickpea (*Cicer arietinum L*) is considered the seven crops in Egypt. The cultivated area in Egypt reached in year 2011renged from 12000 to20000 Fadden. Most of this area was cultivated in upper Egypt especially Assiut governorate.

Damping-off and root rot caused by *Fusarium oxysporum, F. solani* and *F. moniliforme*, is considered sever disease in chickpea in Egypt as well as all the world(Noher *et al*., 2009; Sumanti *et al*., 2009; Khetarpal *et al*., 2009; Singh *et al*., 2010; Iqbal *et al*., 2010 and Meki *et al*., 2011) .Using fungicides is consider harmful for human and environment (Meki *et al.*, 2011) Therefore we use biological control to control diseases caused by *Fusarium oxysporum, F. solani* and *F. moniliforme*. Which consider safe and cheaper. Using *Trichoderma harzianum* which considered is one of the efficient biocontrol agents that commercially produced to prevent development of several soil pathogenic fungi(Kaushal and Sood , 2008; Alam *et al.,* 2009 and Anand and Reddy*.,* 2009) A different mechanism has been suggested as being responsible for their biocontrol activity which includes mycoparsasitism antibiosis competition for neutrant and space and serration of chitinolytic enzymes(Harman ,2000). Antifungal metabolites produced by *T. harzianum*, *Pseudomonas fluorescence* and *Bacillus subtilis* have been investigated for their antagonistic and fungal properties (Gowily *et al.,* 1995; Saikia *et al.,* 2003; Goel *et al.,* 2002 and Mujeebur *et al.,* 2004).

Materials and Methods:

Isolation and identification of pathogenic isolates:

Forty fungal isolates were collected from different chickpea fields located at Assiut governorate. Isolates were isolated from infected chickpea plants showing damping-off and root rot according the methods described by (Hajieghrari, 2009). Isolated fungi were purified using single spore technique and identified on basis of morphological and cultural characteristic according to (Domsh *et al.,* 2007). And confirmed by Assiut University, Mycological Center (AUMC)

Pathogenicity tests:

Twenty three *Fusarium* isolates were tested for their pathogenicity using Giza3 chickpea cultivar under greenhouse conditions during (2005/2006) growing season. Pathogen inocula were prepared on barley medium as described by (Singh *et al.,* 1997). Sterilized barley grains were inoculated with 5 mycelial blocks (0.5-mm in diameter) 5-days old. The inoculated flasks were kept under room temperature for 3 weeks. The flasks were shaken with hand every alternate day. The autoclaved pots (30cm in diameter) filled with autoclaved soil and inoculated with fungal inocula at the rate of 50g/kg of soil. Five pots were used as replicates. Ten seeds of chickpea Giza3 cultivar were surface sterilized by dipping in 3% sodium hypochlorite solution for 3 min. and washing with sterilized water and seeded in every pot containing infested soil. Pre and post-emergence damping- off were recorded after 15 and 35 days, re-

spectively. Root rot was determined after 60 days from planning using disease index.

Disease index:

Root system discoloration index was determined for external discoloration according to the index described by (Achenbach and Jennifer 1996). Readings were converted to discoloration index using

$$\text{Disease severity \%} = \frac{0A+1B+2C+3D+4E}{4T} X100$$

Where A, B, C, D and E are the number of the plants corresponding to the numerical grade 0, 1, 2, 3, and 4, respectively and (4T) is the total number of plants (T) multiplied by the maximum disease grade 4, Where, T=A+B+C+D+E

The grade of external discoloration:

The numerical grade of external root system suggested by Achenbach and Jennifer (1996) with modification were used

0= No infection

1=1- 25% root tissue exhibiting discoloration

2= 26-50% root tissue exhibiting discoloration

3= 51-75% root tissue exhibiting discoloration

4=More than 75% root tissue exhibiting discoloration or plant died.

Source of antagonists:

Isolation of *Trichoderma harzianum*

T. harzianum isolates were isolated from chickpea rhizosphere using method described by (Mujeebur *et al*., 2004). Antagonistic isolates were purified using single spore isolation and identified on basis of morphological and culture characteristic according to(Domsh *et al.,* 2007). The identification was confirmed by Assiut University, Mycological Center (A.U.M.C).

Isolation of *Bacillus subtilis*.

B. subtilis isolates were isolated from chickpea rhizosphere using method described by (Hervas *et al.*, 1998). and identified according to their morphological and biochemical activities described by (Bergey's 1978).

Source of *Pseudomonas fluorescence* isolates.

P.fluorescence (isolate No. 1) was obtained from bacterial collection of Institute of Plant Protection University of Georg-August, Gottingen, Germany. (Isolates No .2 and 3) obtained from Agriculture Research Center, Ministry of Agriculture Giza Egypt.

The antagonistic effect of *Trichoderma* harzianum against *Fusarium* isolates.

The antagonistic capability of three fungal isolates isolated from chickpea rhizosphere was tested against the tested pathogenic fungi *in vitro*. The highly pathogenic isolates of *F.oxysporum* (Isolates No.12, 16 and 35), *F. solani* (Isolates No.4, 8 and 14) and *F. moniliforme*. (Isolates No.22, 24 and 32) were selected for this study. Petri dishes (9cm in diameter) each containing PDA medium (pH7) were inoculated with 5-mm equal disks of *T. harzianum* obtained from 4 days old cultures grown on PDA medium at 25±1°C.

A disc (5- mm in diameter) of *F. oxysporum, F. solani* and *F. moniliforme*, was inoculated at equal distance of the opposite side of Petri dish. Plates inoculated with pathogenic fungi only were used as control and five replicates were used for each test. The inoculated plates were incubated at 25±1°C.

Observation on antagonism and or mycoparasitism of the tested fungi recorded when the growth of the

pathogenic fungi completely covered the plate surface in control.

Culture filtrate (nonvolatile) and early volatile metabolites testes:

Culture filtrates of three *T. harzianum* isolates showed over growth upon pathogen mycelium were tested by growing fungi in conical flasks (250 ml) each contained 100 ml of Czapek's solution liquid medium at 25±1°C.After 14 days incubation period, mycelia mats were discarded by filtration through filter papers and culture filtrates were sterilized by passing through Sitz filter. Sterilized culture filtrates were added to autoclaved Potato Dextrose Agar (PDA) medium to get 10% concentration (v/v) before dispensing medium in Petri dishes, when the temperature of the medium was about 50. Medium of PDA without addition of culture filtrates of antagonists was used as control. Disk 5-mm. in diameter, from 5-days old cultures of each pathogenic fungus was transferred to the center of dishes and incubated at 25±1°C.Three replicates were used for each treatment. After 6 days incubation period, linear growth of tested pathogens was recorded and percentage of inhibition was calculates as

$$\% \text{ of growth reduction} = \frac{\text{Growth in control- growth in treatment}}{\text{Growth in control}} \times 100$$

Antagonistic effect of *P. fluorescence* and *B. subtilis* isolates against the causal pathogens of *Fusarium in vitro*.

Bacterial suspension was prepared from 24h old cultures of *P. fluorescence* or *B. subtilis* and stroked at the center of Petri dish containing (PDA) medium, then two disks (5mm in diameter) from 4 days old culture of the causal pathogens were inoculated at equal distance of the opposite side of Petri dish and three replicates were used. When the pathogenic fungi covered the plate surface of control without bacterial treatment, the distance between the edge of bacterial colonies and the fungus was measured.

Biological control of chickpea damping-off and root rot by *T. harzianum* under greenhouse conditions.

The effect of *T. harzianum*, on control of chickpea damping-off and root rot disease was carried out under greenhouse conditions during growing seasons (2007/2008) and (2008/2009). Clay loam soil with 1% organic maters was used. Completely randomize design was accomplished in greenhouse. Inocula of pathogens and *T. harzianum* were prepared as above mentioned described in pathogenicity tests.

Isolates of *F. oxysporum* (No.35), *F. solani* (No.4), *F. moniliforme* (No.22) and isolates of *T. harzianum* (1, 2 and 3) were used in this study.

Pots (30-cm in diameter) were filled with sterilized clay loam soil. *T. harzianum* inoculum was added at the same time of soil infestation with pathogens inoculum at rate of 50g/kg. Pots seeded with 10 seeds of Giza 3 chickpea cultivar. Pots were irrigated and fertilized as recommended, five pots were used as replicates and five replicates without treatment were used as control. Pre and post emergence damping-off were recorded after 15 and 35 days, respectively. Disease index of root rot determined 60 days after planting.

Biological control of chickpea damping-off and root rot by *P. fluorescence* or *B. subtilis* under greenhouse conditions.

The effect of three isolates of each *P. fluorescence* (No. 1,2 and 3)

and *B. subtilis* (No. 1,2and 3) on control chickpea damping-off and root rot were carried out under greenhouse conditions at Assiut University during growing seasons (2007/2008) and (2008/2009). Pots 30cm in diameter containing autoclaved soil were infested with the pathogenic isolates of *F. oxysporum* (No.35), *F. solani* (No.4) and *F. moniliforme* (No.22) 7days before planting at rate of 50g/kg .Inocula of pathogen were prepared as above mentioned described in pathogenicity tests . Bacterial suspension of *P. fluorescence* and *B. subtilis* was prepared by growing them in 250 ml conical flasks, each containing 100 ml of Martins medium at 27±2°C. After 7 days of incubation period, the obtained cultures were centrifuged for 5 minutes at 3000 r.p.m. After centrifugation, bacterial cells were re-suspended in sterile distilled water to give concentration of $1x10^5$ cell/ml.of the antagonistic isolates of *P. fluorescence* and concentration of $1x10^7$ cell/ml.of *B. subtilis*. The bacterial suspention was added to the soil 5 days before infestation with the pathogen. Ten sterilized chickpea seeds were planted in each pot. Five replicates were used for each individual treatment. Plants watered when necessary. Pre and post emergence damping-off were recorded after 15 and 35 days respectively and root rot determined after 60 days from planting. Pots containing non- infested soil mixed with 50g/kg. of sterilized barely medium were used as control.

Statistical analysis:

All data were subjected to statistical analysis and means were compared using LSD test Gomez and Gomez, (1984).

Results:

Data in Table (1) indicated that all tested isolates were able to infect chickpea plants of Giza 3 cultivar and causing root rot and damping-off diseases. The tested fungal isolates were varied in their virulence. In general, *F. oxysporum* isolate (No.35) caused the highest percentage of pre and post-emergence damping-off (60%, 40%) respectively, followed by *F. oxysporum* isolates (No.16) (64%, 30%, and 92.5%). The lowest percentage of pre and post-emergence damping-off and root rot caused by *F. oxysporum* isolate (No. 40) (30%, 2%, and 31.25%).

Table (1) Pathogenic capability of twenty three *Fusarium* isolates on Giza (3) chickpea (cv) under greenhouse conditions during growing season (2005/2006)

No. of isolets	*Fusarium* isolates	Source of fungal	Emergence damping-off%		Disease index %
			pre	post	
1	*F. oxysporum*	El-Ghanayim	56	12	65
2	*F. oxysporum*	El-Kussiah	52	12	62.5
5	*F. oxysporum*	El-Kussiah	28	10	35
11	*F. oxysporum*	Sahil- Selim	32	8	40
12	*F. oxysporum*	Sahil- Selim	56	18	72.5
16	*F. oxysporum*	Al-badary	64	30	92.5
20	*F. oxysporum*	Sahil- Selim	24	22	43.8
21	*F. oxysporum*	Abnoub	58	2	60
25	*F. oxysporum*	Assiut	58	8	63.8
26	*F. oxysporum*	Assiut	50	12	61.3
31	*F. oxysporum*	Sahil- Selim	52	8	60
34	*F. oxysporum*	Abnoub	38	4	41.5
35	*F. oxysporum*	Abo-Teg	60	40	**
36	*F. oxysporum*	Assiut	60	20	80
38	*F. oxysporum*	Assiut	50	4	52.5
39	*F. oxysporum*	Al-Fath	32	12	42.5
40	*F. oxysporum*	Al-Fath	30	2	31.3
4	*F. solani*	El-Kussiah	70	20	90
8	*F.solani*	El-Kussiah	28	6	32.5
14	*F. solani*	Abo-Teg	56	14	70
22	*F. moniliforme*	Sahil- Selim	70	10	80
24	*F. moniliforme*	Abo-Teg	52	6	55
32	*F. moniliforme*	Abo-Teg	66	20	83.8
	Control		0	0	0
L.S.D. 0.05%			**5.58**	**4.57**	**8.574**

** : All plants dead

Effect of culture filtrates of *T .harzianum* on reduction of linear growth of pathogenic *Fusarium* isolates

Data presented in Table (2) indicated that all culture filtrates of tested *T. harzianum* which gave over growth upon *Fusarium* mycelium decreased significantly linear growth of tested *Fusarium* isolates. The greatest inhibition of growth caused when *T. harzianum* isolate (No. 2) tested with *F. solani* isolate (No.14) and (No.4) while the lowest inhibition caused by *T. harzianum* isolate (No.3) against *F. solani* (No.4), *F. moniliforme* (No.22) and (No.32).

Table (2) Effect of culture filtrates of *T.harzianum* on linear growth of pathogenic *Fusarium* isolates

Pathogenic isolates	No. of isolate	Bioagints	Linear growth (mm)	% Inhibition zone (mm)
F. oxysporum	12	*T. harzianum* 1	64	35
		T. harzianum 2	47	42
		T. harzianum 3	58	41
		control	89	0.0
F. oxysporum	16	*T. harzianum* 1	56	33
		T. harzianum 2	63	26
		T. harzianum 3	69	21
		control	89	0.0
F. oxysporum	35	*T. harzianum* 1	64	25
		T. harzianum 2	56	33
		T. harzianum 3	69	21
		control	89	0.0
F. solani	4	*T. harzianum* 1	70	19
		T. harzianum 2	33	56
		T. harzianum 3	75	14
		control	89	0.0
F. solani	8	*T. harzianum* 1	36	53
		T. harzianum 2	50	39
		T. harzianum 3	64	25
		control	89	0.0
F. solani	14	*T. harzianum* 1	61	28
		T. harzianum 2	28	61
		T. harzianum 3	36	53
		control	89	0.0
F.moniliforme	22	*T. harzianum* 1	61	28
		T. harzianum 2	44	45
		T. harzianum 3	67	22
		control	89	0.0
F.moniliforme	24	*T. harzianum* 1	56	33
		T. harzianum 2	50	49
		T. harzianum 3	61	28
		control	89	0.0
F.moniliforme	32	*T. harzianum* 1	47	42
		T. harzianum 2	39	50
		T. harzianum 3	67	22
		control	89	0.0
L.S.D at	5%		6.08	4.52

Antagonistic effect between P. *fluorescence* isolates and the tested *Fusarium* isolates *in vitro*.

Data presented in Table (3) indicated that all *P. fluorescence* isolates inhibited the growth of the tested *Fusarium* isolates in *vitro* compared with the control. In general *P. fluorescence isolate* (No.1and 3) more effective in reducing the growth of all tested pathogens in *vitro*.

Table (3) Antagonistic effect between P. *fluorescence* isolates and the tested *Fusarium* isolates *in vitro* monitored as inhibition zone (mm)

***Pathogenic* isolates**	**No. of isolates**	**Bioagenets**	**Inhibition zone (mm)**
F. oxysporum	12	*P. fluorescence* 1	21
		P. fluorescence 2	4.9
		P. fluorescence 3	24
F. oxysporum	16	*P. fluorescence* 1	24.2
		P. fluorescence 2	19.2
		P. fluorescence 3	22.5
F. oxysporum	35	*P. fluorescence* 1	17.7
		P. fluorescence 2	17.3
		P. fluorescence 3	17.3
F. solani	4	*P. fluorescence* 1	16.8
		P. fluorescence 2	6. 7
		P. fluorescence 3	21.3
F. solani	8	*P. fluorescence* 1	9.3
		P. fluorescence 2	10.5
		P. fluorescence 3	15.3
F. solani	14	*P. fluorescence* 1	22.8
		P. fluorescence 2	6
		P. fluorescence 3	10. 7
F. moniliforme	22	*P. fluorescence* 1	11.2
		P. fluorescence 2	9.5
		P. fluorescence 3	15. 7
F. moniliforme	24	*P. fluorescence* 1	19.8
		P. fluorescence 2	4.3
		P. fluorescence 3	15.2
F. moniliforme	32	*P. fluorescence* 1	16
		P. fluorescence 2	8. 7
		P. fluorescence 3	19.2

L.S.D at 5% 2.9

Antagonistic Effect of *B. subtilis* isolates against the tested *Fusarium* isolates *in vitro*.

Data presented in Table (4) indicated that all *B. subtilis* isolates inhibited growth of all tested isolates of *Fusarium* in *vitro*. The inhibition zone between *B. subtilis* and the tested *Fusarium* was varied with *Fusarium* and *B. subtilis* isolates. Isolate of *B. subtilis* (No.2) with *F. oxysporum* isolates (No.16) and *F. solani* isolates (No.14) gave the highest inhibition zone (25 and 25.3 mm)

Table (4) Antagonistic Effect of *B. subtilis* isolates against the tested *Fusarium* isolates *in vitro* monitored as inhibition zone (mm)

***Pathogenic* isolates**	**No. of isolate**	**Biogenets**	**Inhibition zone (mm)**
F. oxysporum	12	*B. subtilis* 1	15.5
		B. subtilis 2	10
		B. subtilis 3	11.5
F. oxysporum	16	*B. subtilis* 1	14.7
		B. subtilis 2	25
		B. subtilis 3	19.7
F. oxysporum	35	*B. subtilis* 1	12.3
		B. subtilis 2	21.8
		B. subtilis 3	18.7
F. solani	4	*B. subtilis* 1	13.7
		B. subtilis 2	10
		B. subtilis 3	20
F. solani	8	*B. subtilis* 1	9.2
		B. subtilis 2	9.3
		B. subtilis 3	8.5
F. solani	14	*B. subtilis* 1	17.2
		B. subtilis 2	25.3
		B. subtilis 3	21.2
F. moniliforme	22	*B. subtilis* 1	10.5
		B. subtilis 2	10.2
		B. subtilis 3	2.8
F. moniliforme	24	*B. subtilis* 1	13
		B. subtilis 2	9.7
		B. subtilis 3	10
F. moniliforme	32	*B. subtilis* 1	6.8
		B. subtilis 2	6.5
		B. subtilis 3	8.7
L.S.D at 5%			2.91

Discussion:

Damping-off and root-rot caused by *F. oxysporum, F. solani and F. moniliforme* were important diseases of chickpea plants. Twenty three *Fusarium* isolates were tested on chickpea Giza 3 (cv). All these isolates caused pre and post emergence damping-off and root- rot. *F. oxysporum* gave the highest percentage of infection. According to the available literature these pathogens were isolated from chickpea (Singh *et al.*, 1997; Soregaon and Ravikumar 2010 and Meki *et al.,* 2011) Three isolates of *T. harzianum* showed over growth upon tested pathogenic *Fusarium* isolates and their culture filtrates inhibited the growth of the tested *Fusarium* isolates *in vitro* .These results are in accordance with those obtained by (El-Nashar *et al.,* 2001; Atef- Najwa 2008; Hajieghrari *et al.,* 2008; Waheed and Khilare 2010 and Gajera *et al.,* 2012). They reported *T. harzianum* treatment reduce the mycelia growth of the pathogenic fungi due to the rapid growth of *T. harzianum* which colonized medium surface and substrate or reduced hyphal coils over the interaction zone or produced chitins and β-(1-3)- gluconase .Soil treatment with *T. harzianum* reduced chickpea damping-off and root- rot diseases under greenhouse conditions such results in accordance with those reported by (Nawar-Lubna 2007; Kucuk *et al.,* 2007; Atef-Najwa 2008;Haggag-Wafaa *et al.,* 2011 and EL-Bramawy and EL-Sarag (2012). *In vitro*, isolates of *P. fluorescence* and *B. subtilis* inhibited linear growth of tested *Fusarium* isolates. The antagonists varied in their inhibitory effect on linear growth of the tested pathogen .Under greenhouse condition addition of *P. fluorescence* and *B. subtilis* to soil reduced incidence of pre and post emergence damping-off and root- rot of chickpea .The reduction of the diseases varied according to the pathogen and antagonistic isolates which used. These results agree with (Gowily *et al.,* 1995; Inam *et al.,* 2009; Priyanka, 2010; and Rajput *et al.,* 2010). *Pseudomonas fluorescence* produce siderphore (iron chelating compounds) which strongly reacted with free Fe and inhibit the fungal mycelia growth and spore germination produce antibiotic substances produces cyanic acid (Abo-Alnaga- Heidi,2002). Antifungal metabolites produced by bacteria like *Pseudomonas fluorescence* and *Bacillus subtilis* have been investigated for their antagonistic and fungal properties by (Gowily *et al.,* 1995; Goel *et al.,* 2002; Saikia *et al.,* 2003; and Mujeebur *et al.,* 2004).

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المكافحه البيولوجيه لمرض سقوط البادرات وعفن الجذور فى الحمص

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الملخص:

تم عزل ثلاثة وعشرون عزله من فطر فيوزاريوم تشمل ثلاثة أنواع (*Fusarium oxysporum, F. solani* and *F. moniliforme*) من جذور الحمص صنف جيزه 3 المصابه فى أماكن مختلفة بمحافظه أسيوط قد سببت أمراض سقوط البادرات المفاجئ وعفن الجذور فى الحمص وكانت عزله الفطر *F. oxysporum* رقم (35) أكثرهم أحداثا للأصابه. تحت ظروف المعمل والصوبه ووجد ان فطر *Trichoderma harzianum* أو راشحه لهم القدره على تثبيط النمو الطولي لجميع العزلات الفطريه الممرضه المختبره وكذلك بكتريا *Pseudomonas fluorescence and Bacillus subtilis* أحدثت تثبيط لنمو جميع العزلات الفطريه المختبره ولوحظ في معظم الحالات أن معامله التربة باستخدام *T. harzianum, P. fluorescence and B. subtilis* أحدثت خفض فى الاصابه بسقوط البادرات قبل وبعد الظهور وحدوث عفن الجذور لجميع الفطريات ألممرضه ألمختبره بالمقارنة بالكنترول تحت ظروف الصوبه خلال تجربتين عامى 2007و2008 واختلفت هذه العزلات فى قدرتها على المكافحه باختلاف العزلات الممرضة.