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Biological control of damping-off and root rot of fenugreek

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Abstract

Eight *Trichoderma* and nine bacterial isolates which isolated from rhizospher and nodules of fenugreek plants. Also four isolates of rhizobacteria (PGPR) namely *Basillus subtilis* (*B.s*), *Basillus polymyxa* (*B.p*), *Basillus megaterium* (*B.m*) and *Pseudomonas fluorescens* (*P.f*) were tested in vitro for thir ability against *Fusarium solani, Rhizoctonia solani* and *Macrophomina phaseolina* which caused damping-off and root rot of fenugreek plants.The results showed that *Trichoderma* isolate number (T3) gave the highest reduction on maycelial growth of three pathogenic fungi followed by isolate number (T2) which adentified as *Trichoderma harzianum* and *Trichoderma hamatum*, respectively. *Pseudomonas fluorescens* followed by *Basillus polymyxa* , *Rhizobium* sp. isolate (Rh3), *Basillus subtilis* and *Basillus megaterium* gave highly antagonistic effect was clear against the tested fungi as will as used in greenhous experiment. A pot experiment was carried out under greenhouse conditions. Results showed that Treated seed of fenugreek caltivar (Giza 2 cv.) with *Rhizobacteria* and or treated soil with *T. harzianum* and *T. hamatum* reduced pre and post damping-off and root rot diseases of fenugreek and increased survival plant compared with the control. *Trichoderma harzianum* followed by *Rhizobium* sp. isolate (Rh3) gave the best reduction in these respects.

Keywords: biological control, fenugreek, rhizobacteria, root rot, Trichoderma.

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Introduction

Fenugreek (*Trigonella foenum graecum*) is an annual herb that belongs to the family *Leguminosae* widely grown in Egypt and Middle Eastern countries. It is commonly found growing in the Mediterranean region of the world (Bukhari et al., 2008). Fenugreek seed contains 20% protein, 50% carbohydrate, 5% fat and 25% dietary fibers lipids, cellulose starch, ash, calcium, iron and β-carotene (USDA, 2001). Also, it has been found to contain vitamin C, niacin, potassium, and diosgenin (which are a compound that has properties similar to estrogen). Other active constituents in fenugreek are alkaloids, lysine and L-tryptophan, as well as steroidal saponins. Therefore, it is used in artificial flavoring and in the production of hormones (Acharya et al., 2007a and b). Green fenugreek is a good source of iron (Fe) as well as other minerals for human beings (Chhibba et al., 2000). Fenugreek is a medicinally important plant possessing anti-diabetic, anti-cancerous, anti-microbial and hypocholesterolaemic properties (Naganand et al., 2010). Fenugreek subjected to attack by number of diseases. Among these diseases damping-off and root rot are the most important diseases of fenugreek which affects both germinating seeds, young seedlings and can reduce crop yield and caused by *Rhizoctonia solani* Kuhan, *Fusarium solani* Mart and *Macrophomina phaseolina* (Madkour & Aly) (Mohamed et al., 2013; Yadav & Anamika, 2005; Haque & Ghaffar, 1992). The alternative of synthetic chemicals is the use of certain biocontrol agents, these are inexpensive and ecofriendly and have no harmful effect on human. In general, the idea of controlling soil borne plant pathogens with chemical fungicides has been shifted to biological control that may play an important role in agriculture. Plant growth promoting rhizobacteria (PGPR) facilitate the plant growth directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Munees & Mulugeta, 2014). Researchers have reported that treated fenugreek seed or treated soil with *Trichoderma* spp., rhizobia and compost reduce damping-off and root rot of fenugreek plants (Mohamed et al., 2013; Haque & Ghaffar, 1992). This study was carried out in order to: evaluate the efficacy of rhizobacteria (PGPR) and *Trichoderma* spp. as biocontrol agents against *Rhizoctonia solani*, *Fusarium solani* and *Macrophomina phaseolina*, the causal fungi of damping-off and root rot diseases of fenugreek plants

Materials and methods

Isolation and identification of the causal pathogens: Samples of fenugreek plants showing root rot and damping-off symptoms were collected from different farms located in Assiut, Sohag and Qena governorates, Egypt. The infected roots were thoroughly washed with running tap water, cut into small fragments, superficially sterilized with sodium hypochlorite (0.5%) for 2 minutes, washed several times with sterile distilled water and dried between sterilized filter papers. The sterilized pieces were transferred into potato dextrose agar (PDA) medium supplemented with penicillin (20 Iu ml-1) and incubated at 25 ±1 ^{0}C, then examined daily for fungal growth. The fungal colonies were purified using

single spore or hyphal tip techniques suggested by Booth (1985) and Dhingra and Sinclair (1985). Then, they identified according to their morphological and microscopical characters as described by Booth (1985) and Barnett and Hunter (1972). The obtained isolates were maintained on PDA slants and kept in refrigerator at 5°C for further studies. The identification of isolates was confirmed by Mycological Research Center (AUMC), Assiut University, Egypt.

Pathogenicity tests: Pathogenicity test of the isolated fungi were carried out according to Mohamed et al. (2013), under greenhouse conditions at the Faculty of Agriculture, Al-Azhar University Assiut, Egypt. The plastic pots (20 cm diameter) were sterilized by immersing in 5% formalin solution for 15 minutes, then left for several days to get rid of the poisonous effect of the formalin. Six isolates of *R. solani*, three isolates of *M. phaseolina* and twenty two isolates of *Fusarium* spp. were obtained from different locations. The fungi used throughout this experiment as well as the source of isolates are shown in Table (1). The inoculum which used in the foregoing studies consisted of uniform agar discs 5 mm. in diameter bearing 7-days old and grown in 500 ml. glass bottles containing the following substrate per bottle (25g. coarse sand, 75g. barley and 100ml tap water to cover the mixture in bottles). The bottles were autoclaved at 20 lp/Sq. for 30 minutes. The bottles were incubated at 25°C for two weeks to obtain sufficient growth of the fungi. The sterilized pots were filled with Sterilized clay loam soil and inoculated with the fungal inoculums at the rat 2 g /Kg. soil, then watered and lift for one week before sowing to ensure even distribution and growth of each particular fungus.

Table 1: Source of fungal isolates used in this study obtained during 2013 growing season.

Fungus	Isolate code	Source
R. solani	R1	Sohag
	R2	Assiut
	R3	Assiut
	R4	Qena
	R5	Assiut
	R6	Qena
M. phaseolina	M1	Assiut
	M2	Sohag
	M3	Sohag
F. solani	F1	Qena
	F2	Qena
	F3	Qena
	F4	Qena
	F5	Assiut
F. oxysporum	F6	Assiut
	F7	Sohag
	F8	Sohag
	F9	Sohag
F. moniliforme	F10	Assiut
	F11	Qena
	F12	Assiut
	F13	Assiut
	F14	Sohag
	F15	Sohag
F. equiseti	F16	Sohag
	F17	Qena
	F18	Qena
	F19	Assiut
F. semitectum	F20	Sohag
	F21	Assiut
	F22	Assiut

Disinfested fenugreek seeds cultivar Giza2 were sown in the infested pots at the rate of 10 seeds/pot (20 cm in diameter). Four pots were used for each isolate, (which were considered as replicates). Pots containing sterile soil mixed with barley grains free of any fungus were sown similarly with disinfested fenugreek seeds at the same rate to be used as control treatment. Pots were kept under observation and irrigated as needed. Results were recorded after 15 and 30 days of planting

for damping-off and after 45 days for root rot. The percentage of pre and post emergence damped-off as well as healthy survival plants in each treatment were determined 15 and 30 days after sowing, respectively using the formula according to El-Helaly et al., (1970) and El-Sayed-Sahar and Mousa-Abeer (2015).

$$\text{Pre-emergence (\%)} = \frac{\text{Number of Non germinated seeds x100}}{\text{Total number of sown seeds}}$$

$$\text{Post-emergence (\%)} = \frac{\text{Number of dead seedling}}{\text{Total number of sown seeds}} \times 100$$

$$\text{Survival plant (\%)} = \frac{\text{Number of survival plant}}{\text{Total number of sown seeds}} \times 100$$

The infected plants of each replicate were removed from the soil after the inoculation period, washed thoroughly to remove soil debris, then disease severity percentage (DS %) was estimated as the following:

$$\text{DS (\%)} = \Sigma\,[(1A+2B+3C+4D)/4T] \times 100$$

where, A, B, C and D are the number of plants corresponding to the numerical grade, 1, 2,3 and 4 respectively and 4T is the total number of plants (T) multiplied by the maximum discoloration grade 4, where T=A+B+C+D. To detect the different degrees of disease, plants were classified into four categories according to (Abo-Elyousr et al., 2014; Dorrance et al., 2003) with slight modifications. The root rot rating scale was as follows: 0 = no root rot; 1=1 to 25% of roots with visible lesions or root rot; 2=approximately 26 to 50% of the roots rote or damaged; 3=51 to 75% of the root rot; and 4= 76 to 100% root rot or completely damaged (Fig.1).

Figure 1: An arbitrary (0- 4) disease scale used to measure disease severity % on fenugreek cv. Giza 2, according to Dorrance et al. (2003).

Isolation of *Trichoderma* spp.: Soil samples were collected from rhizosphere of healthy fenugreek plants, growing fields in Assiut Governorate. One hundred gram from rhizosphere soil were collected into each sterile plastic bag and kept in the refrigerator at the Plant Pathology Laboratory, Faculty of Agriculture, Al-Azhar University, Assiut, Egypt for further analysis. Isolation of antagonistic *Trichoderma* spp. from rhizosphere soil was made using serial dilution technique (Belete & Ahmed 2015; Waksman, 1922). Each soil sample was thoroughly mixed and pulverized by means of mortar and pestle, and passed through a 0.5 mm soil screen sieve before 1 g was suspended in 9 ml sterile distilled water. The suspensions were made homogeneous by agitation using a vortex mixer and further serial dilutions of 10^{-2}, 10^{-3} and 10^{-4}. One milliliter of serially diluted suspension from each dilution was pipetted into potato dextrose agar (PDA) medium. The Petri plates were thoroughly shacked by gently swirling in clockwise and anti-clockwise direction to

uniformly spread the suspension. Isolates of *Trichoderma* colonies were picked for antagonism studies after incubating the plates at 25 ± 1°C for 48 h. and re-streaked on a new plate of PDA medium to obtain pure colonies. Eight *Trichoderma* isolates were identified according to Kubicek and Harman (2002) based on their conidial morphology, color and texture, and growth characteristics.

Isolation of rhizobium from root nodules of fenugreek plants: Root nodules were collected from young and healthy seedling of fenugreek plants (*Trigonella foenum-graecum*) from field at different locations in Assiut governorate, Egypt. Fenugreek plants were uprooted carefully so as to get intact are obtained. These plants were brought in laboratory without any delay. Healthy and pink nodules were detached from the root, washed in tap water to remove the adhering soil particles from nodule surface. Nodules were dipped in 0.1% mercuric chloride ($HgCl_2$) solution for 30 seconds and later washed successively three times with sterilized distilled water to remove the traces of toxic HgCl2. Surface sterilized nodules were transferred in test tube containing 5 ml sterilized distilled water. These nodules were crushed with the help of sterilized glass rod to obtain a milky suspension of bacteriods, and then streaked on yeast extract mannitol agar (YEMA) containing Congo red 0.0025% (w/v). The plates were incubated at 28±1^{o}C for 24-48 h. *Rhizobium* colonies were remained white, translucent, elevated and mucilaginous, after 24-72 h, where a contaminations turned red as described by Vincent (1970), Vishal and Abhishek (2014) and Jain et al. (2012). The colonies were picked up and transferred to YEMA slant for further characterization.

Evaluation of antagonistic activity of *Trichoderma* spp. against the pathogenic fungi: Eight different species of *Trichoderma* were screened against the pathogenic fungi *in vitro*. The antagonistic effects of each *Trichoderma* spp. against *F. solani, M. phaseolina* and *R. solani* were tested using dual culture technique (Coskuntuna & Ozer, 2008; Abdel-Kader et al., 2002). The tested isolates of *Trichoderma* spp. were grown on PDA medium at 25°C, for 6 days and used as inocula. Discs from each isolate of *Trichoderma* spp. (5 mm in diameter) were inoculated on PDA medium in one side of Petri plate and the opposite side was inoculated by pathogenic fungi. Four replicates were used for each treatment. Inoculated plates with pathogenic fungi only were used as the control. After five days incubation period at 25°C, the linear growth of the tested pathogen was recorded when the growth of the pathogen covered the plate surface in the control treatment. The percentages of mycelial growth inhibition were calculated according to following formula:

$$\text{Mycelial growth inhibition (\%)} = [A-B/A] \times 100$$

Where: A = the length of the hyphal growth in the control, B = the length of hyphal growth of the tested fungus.

The antagonistic *Trichoderma* isolates which gave a higher percentage of mycelia growth inhibition were identified *Trichoderma harzianum* and *T.*

hamatum by Assiut University Mycological Research Center, Egypt. Therefore, these antagonistic fungi were used in greenhouse experiment.

Evaluation of antagonistic activity of rhizobacteria against pathogenic fungi: Four isolates of rhizobacteria namely *B. subtilis, B. megaterium, B. polymyxa* and *P. fluorescens* were obtained from MERCIN, Faculty of Agriculture, Ain Shams University, Egypt. Nine isolates of *Rhizobium* were isolated form root nodules of fenugreek plants. They were tested against the pathogenic fungi *F. solani, R. solani,* and *M. phaseolina* under *in vitro* conditions. The tested isolates of bacteria were grown on Nutrient Sucrose Agar medium (NSA) (Peptone 5 g, beef extract 3 g, sucrose 5 g, agar 20 g, and distilled water 1liter) and incubated at 28°C for one day, and used as inocula (Sallam-Nashwa et al., 2013). Petri plates (9 cm in diameter) containing potato dextrose agar (PDA) medium were inoculated in the middle by discs (5 mm in diameter) of pathogenic fungi, then inoculated with the tested bacterium on two opposite side of the tested pathogen. Four replicates were used for each treatment. Inoculated plates with pathogenic fungi only, were served as the control. After five days incubation period at 25°C, the linear growth of the tested pathogens was recorded when the growth of the pathogens covered the plate surface in the control treatment. The percentages of mycelial growth inhibition were calculated according to the following formula:

$$\text{Mycelial growth inhibition (\%)} = [A-B/A] \times 100$$

Where: A = the length of the hyphal growth in the control, B = the length of hyphal growth in the tested isolate.

The highly antagonistic isolates of rhizobacteria; *P. fluorescens, B. polymyxa, B. subtilis, B. megaterium* and *Rhizobium* sp. isolate number (Rh3) were selected and used in greenhouse experiment.

Effect of certain bioagents on incidence of fenugreek root rot and damping-off diseases caused by the tested pathogens under greenhouse conditions: Inocula of *T. harzianum* and *T. hamatum* were prepared on sterilized barley medium in 500 ml glass bottles.Each bottles contained (75 g barley grains, 25 g sand and 75 ml tap water). Each bottle was inoculated with discs (0.5 cm in diameter) of 4 days-old cultures of a desired antagonist. Bottles were incubated at 25±1 ^{0}C for 15 days. The content of bottles was thoroughly mixed in plastic container and used as a source of inoculum. Inoculum of each antagonist was added to infested pots at the rate of 3% w/w at the time of planting (Ahmed-Hoda et al., 2000). The antagonistic bacteria, *i.e. P. flourescens, B. megaterium,B. polymyxa, B. subtilis* and *Rhizobium* sp. were grown in nutrient broth medium (NB) (Abd-Alla et al., 2007). All the tested bacteria were incubated on a rotary shaker at 200 rpm for 48 h at 28 ± 2°C. The bacterial cells were harvested by centrifugation at 6,000 rpm for 10 min, washed twice in 0.05 M. phosphate buffer pH 7.0, and re-suspended in sterilized distilled water. The concentration of bacterial cells in the suspensions was adjusted to 3X10^{6} cells per milliliter (cfu/ml) (Abdel-Kader et al., 2012) according to its turbidity using

spectrophotometer at 400nm. Fenugreek seeds were sterilized in 0.5% sodium hypochlorite for 2 min then thoroughly washed in sterilized distilled water and left to air dry under aseptic conditions. Fenugreek seeds were thoroughly immersed in bacterial suspension for 5 minutes. Sucrose was added to the mixture to enable the bacteria stick to the seed surface and also to offer initial nutrients for the bacteria, then left to dry for 2 hrs. in a laminar flow before planting. The effects of biocontrol agents *T. harzianum,T. hamatum, B. megaterium, B. polymyxa, B. subtilis, Rhizobium sp*.(Rh3) and *P. flourescens* were evaluated individually against fenugreek damping-off and root rot diseases incited by *R. solani, F. solani* and *M. phaseolina* under greenhouse conditions. This experiment was carried out during 2015 and 2016 growing seasons, in the greenhouse of Agricultural Botany Department, Faculty of Agriculture, Al-Azhar University, Assiut, Egypt. Plastic pots were filled with sterilized soil and mixing with fungal inocula as described before at rate 2-3 % of clay soil (w\w), one week before planting. The antagonistic fungi were added to the infested soil at rate 3% of soil (w\w) in pots, at the time of planting. Each pot was sown with 10 treated seeds of fenugreek Giza 2 cv., four pots were used for each treatment as replicates. The infested pots individually with pathogenic fungi only were sown with disinfested fenugreek seeds and served as control. The treatments can be summarized as follows:

1- Soil infested with *M. phaseolina+T.hamatum* T3
2- Soil infested with *M. phaseolina+T.hamatum* T2
3- Soil infested with *F. solani* + *T.harzianum* T3
4- Soil infested with *F. solani* + *T.hamatum* T2
5- Soil infested with *R. solani* + *T.hamatum* T2
6- Soil infested with *R. solani* + *T.harzianum* T3
7- Seed treated with *B. subtilis*
8- Seed treated with *B.megaterium*
9- Seed treated with *B. polymyxa*
10- Seed treated with *P. flourescens*
11- Seed treated with *Rhizobium*
12- Control (untreated)

Results were recorded after 15 and 30 days of planting for the percentage of pre and post emergence damped-off as well as healthy survival plants in each treatment, respectively. At the end of the experiment plants were uprooted washed, rated for Disease severity (DS %) was estimated as described before.

Statistical analysis: Data were subjected to statistical analysis using analysis of variance and means were compared using the LSD test according to Gomez and Gomez (1984).

Results and Discussion

Isolation and identification of fenugreek root rot and damping-off causal fungi: Thirty one fungal isolates were isolated from infected roots of fenugreek plants collected from different localities in Assiut, Sohag, and Qena governorates, Egypt. Fungal isolates were identified by using the morphological features of mycelia and spores as described by Barnet and Hunter (1977) and Booth (1985) and confirmed by Mycological Research Center (AUMC), Assiut University, Egypt. Table (1) shows that the isolated fungi were identified as four isolates both of *Fusarium oxysporum* Schlecht and *F. equesti* Corda, five isolates of *F. solani* Mart, six isolates both of *F. moniliforme*

Saccardo and *R. solani,* three isolates both of *F.semitectum* Berk & Ravenel and *M. phaseolina* Madkour & Aly.

Pathogenicity tests: Thirty one fungal isolates were tested for their pathogenicity on fenugreek plants (Giza 2 cv.) under greenhouse conditions during 2014 growing season. Data in Table (2) illustrate that all tested fungal isolates were able to infect fenugreek plants caused root rot and damping-off diseases. All the tested isolates significantly caused root rot disease compared with control. *F. solani* (F5) gave the highest percentage of disease severity followed by *R. solani* (R2) then, *M. phaseolina* (M1) and *R. solani* (R6). Isolates of *F. equesti* (F17), *M. phaseolina* (M2 and M3), *F. moniliforme* (F12 and F14), *R.solani* (R1, R3, R4 and R5) and *F. oxysporum* (F6) showed moderate effect of disease severity followed by isolates of *F. oxysporum* (F8), and *F. solani* (F2). The other tested isolates significantly showed the lower effect of disease severity. As the regard isolates of *F. equiseti* (F16) and *F. solani* (F4) gave the lowest disease severity followed by *F. oxysporum* (F9) and *F.moniliforme* (F10). Data also exhibited that, all the tested isolates significantly caused damping-off disease except *R. solani* (R3) and *F. equiseti* (F16). *R. solani* (R2) gave the highest percentage of damping-off disease followed by *F. solani* (F5) then *R. solani* (R6), *F. semitectum* (F20) and *R. solani* (R1). Isolates of *M. phaseolina* (M1) and *F. semitectum* (F21) showed moderate effect of damping-off disease. The low effect of disease incidence was observed in other tested isolates. However, the lowest damping-off disease was found with isolate of *F. oxysporum* (F9) followed by *F. oxysporum* (F8) and *F.moniliforme* (F13 and F15) .Such results are in agreement with those obtained by Haque and Ghaffar (1992) ,Yadav and Anamika (2005), Khokhar et al. (2012) and Mohamed et al. (2013). They were reported that *R. solani, F. solani, M. phaseolina* and *Fusarium* spp. caused damping-off and root rot diseases of fenugreek under greenhouse and field conditions.

Preliminary tests for antagonistic capability of fungi and bacteria against growth of pathogenic fungi *in vitro*: Data in Table (3) and Figure (2) show that the antagonistic fungal isolates (*Trichoderma* spp.) were able to inhibit mycelial growth of the tested pathogenic fungi *i.e. M. phaseolina* (M1), *R. solani* (R2) and *F. solani* (F5) compared with the control. *Trichoderma* (T3) gave the greatest reduction of mycelial growth of the pathogens followed by isolate (T2), then isolate (T1). But, the other tested antagonistic showed moderate inhibition against the tested pathogenic fungi. The least reduction of mycelial growth of the tested pathogenic fungi was found in case of *Trichoderma* (No. T4) followed by T8. The highly antagonistic fungal isolates T3 and T2 were selected and identified as *Trichoderma harzianum* Rifai and *T. hamatum* Bon respectively by Assiut University Mycological Research Center and were used in greenhouse experiments. These results are in agreement with those recorded by Mishar (2013), Abo-Elyousr et al. (2014) and Belete et al. (2015). In this respect, antagonistic potential of different *Trichoderma* species arrange of mechanisms have to be considered. One:

production of antibiotic, volatile and non-volatile chemicals. These substances influence the permeability of cell membranes and result in an efflux of the cytoplasm (Howell, 1998). Two: mycoparasitism and excretion of lytic enzymes. The antifungal enzyme system of *Trichoderma* spp. plays an important role for detection and destroying the pathogenic cell wall (Schirmbock et al., 1994). Three: competitiveness is based on rapid growth and the production of various asexual generated conidia and chlamydospores (Chet et al., 1998; Chet, 1990). The direct influence of *Trichoderma* spp. against pathogens through colining their hyphae around the hyphae of the pathogens to prevent their continued growth (Adekunle et al., 2006).

Table 2: Percentage of fenugreek damping–off and root rot disease severity (%) of 31 fungal isolates under greenhouse conditions during 2014 growing season.

The tested fungi	Isolate No.	Damping –off			Root rot (%)
		Pre-emergency damping-off after 15 days (%)	Post-emergency damping-off after 30 days (%)	Survival (%)	
F. solani	F1	10	0	90	33.6
F. solani	F2	13.3	3.3	83.4	36.0
F. solani	F3	16.6	3.3	80.1	27.3
F. solani	F4	6.6	6.6	86.8	16
F. solani	F5	60	6.6	33.4	89.1
F. oxysporum	F6	10	0	90	63
F. oxysporum	F7	16.6	0	83.4	29.3
F. oxysporum	F8	6.6	0	93.4	37.8
F. oxysporum	F9	3.3	0	96.7	23.1
F. moniliforme	F10	10	3.3	86.7	26.5
F. moniliforme	F11	13.3	0	86.7	30.6
F. moniliforme	F12	10	0	90	45.0
F. moniliforme	F13	6.6	0	93.4	33.9
F. moniliforme	F14	16.6	0	83.4	45.6
F. moniliforme	F15	6.6	0	93.4	30.1
F. equiseti	F16	0	0	100	13.6
F. equiseti	F17	16.6	0	83.4	41.1
F. equiseti	F18	13.3	0	86.7	27.2
F. equiseti	F19	16.6	3.3	80.1	34.1
F. semitectum	F20	40	3.3	56.7	35.2
F. semitectum	F21	26.6	0	83.4	34.5
F. semitectum	F22	6.6	10	83.4	33.8
R. solani	R1	40	0	60	52.8
R. solani	R2	76.6	0	23.4	79.1
R. solani	R3	0	0	100	50.2
R. solani	R4	13.3	10	76.7	42.6
R. solani	R5	16.6	0	83.4	49.3
R. solani	R6	43.3	10	46.7	42.7
M. phaseolina	M1	30	6.6	63.4	78.6
M. phaseolina	M2	6.6	3.3	90.1	51.6
M. phaseolina	M3	13.3	6.6	80.1	66.4
Control		0	0	100	0
L.S.D. at 5%		4.56	1.32		1.20

Data in Table (4) and Figure (3) also exhibited that all the tested antagonistic rhizobacteria (PGPR)I inhibited growth of the tested pathogenic fungi. The highest reduction of the tested pathogenic linear mycelial growth was

displayed by *P. fluorescens* followed by *B. polymyxa*, *Rhizobium* sp. (Rh3) and *B. subtilis* respectively, then *B. megaterium*. The other tested antagonistic rhizobacteria showed moderate reduction of linear mycelia growth of the tested pathogenic fungi. The lowest inhibition growth of the pathogenic fungi observed with *Rhizobium* sp. isolate (Rh8). The strongest antagonistic rhizobacteria *P. fluorescens*, *B. polymyxa*, *Rhizobium* sp. isolate (Rh3), *B. subtilis* and *B. megaterium* which were selected and used in greenhouse experiments. These results are in line with those recorded by Rakib et al. (2012), Manoj et al. (2014) and Haggag-Karima et al. (2015). Results in Table (4) and Figure (3) also show that all the tested antagonistic rhizobacteria (PGPR) inhibited growth of the tested pathogenic fungi, The highest reduction of the tested pathogenic linear mycelial growth was displayed by *P. fluorescens* followed by *B. polymyxa*, *Rhizobium* sp. isolate (Rh3) and *B. subtilis* bioagents, respectively, then *B. megaterium*, except in case of *M. phaseolina* non antagonistic observed. The other tested antagonistic rhizobacteria showed moderate reduction of linear mycelia growth of the tested pathogenic fungi. The lowest inhibition growth of the pathogenic fungi observed with *Rhizobium* sp. (Rh8). Bacterial bioagents showed antifungal potential against the tested fungi, which might be attributing to mechanism of diffusible antagonistic substances and volatile metabolites depending on the bacterium and the pathogen combination. The diffusible substances include antibiotics (pyrrolnitrin) and siderophores (enterobactin and aerobactin) and volatilic metabolites include hydrogen cyanide and acetoin (Neupane et al., 2013; Rakh et al., 2011).

Table 3: Effect of some antagonistic fungi on mycelial growth of the tested fungi *in vitro*.

Isolates	Mycelial growth inhibition (%)			Mean
	R. solani R2	*M. phaseolina* M1	*F. solani* F 5	
Trichoderma sp. (T1)	26.9	22.17	26.35	25.1
Trichoderma sp. (T2)	39.95	34.67	59.8	44.8
Trichoderma sp. (T3)	57.72	48.35	73.02	59.6
Trichoderma sp. (T4)	15.5	18.57	14.97	16.3
Trichoderma sp. (T5)	28.82	23.05	13.85	21.9
Trichoderma sp. (T6)	29.4	20.22	19.12	22.9
Trichoderma sp. (T7)	9.12	27.45	25.5	20.6
Trichoderma sp. (T8)	22.45	14.12	23.85	20.1
Control	0	0	0	-
L.S.D. at 5%	5.40	6.73	4.35	

Effect of seed or soil treatment with bio-control agents on incidence of roo trot and damping-off diseases of fenugreek caused by three tested fungi under greenhouse conditions: Effect of soil treatment with highly antagonistic fungi (*T. harzianum* and *T. hamatum* and seed treatment with highly antagonistic rhizobacteria (PGPR) *B. subtilis*, *P. fluorescens*, *B. polymyxa* and

B.megaterium and *Rhizobium* sp. isolate (Rh3) on incidence of root rot and damping-off diseases of fenugreek plants (Giza 2 cv.) caused by the tested fungi was carried out under greenhouse conditions during 2015 and 2016 growing seasons. Data presented in Tables (5 and 6) show that treated seeds with each of antagonistic rhizobacteria and treated soil with antagonistic fungi significantly reduced the percentage of disease severity of root rot disease as well as pre and post the emergence damping-off disease of fenugreek caused with the tested fungi and increased survival plant compared with the control.

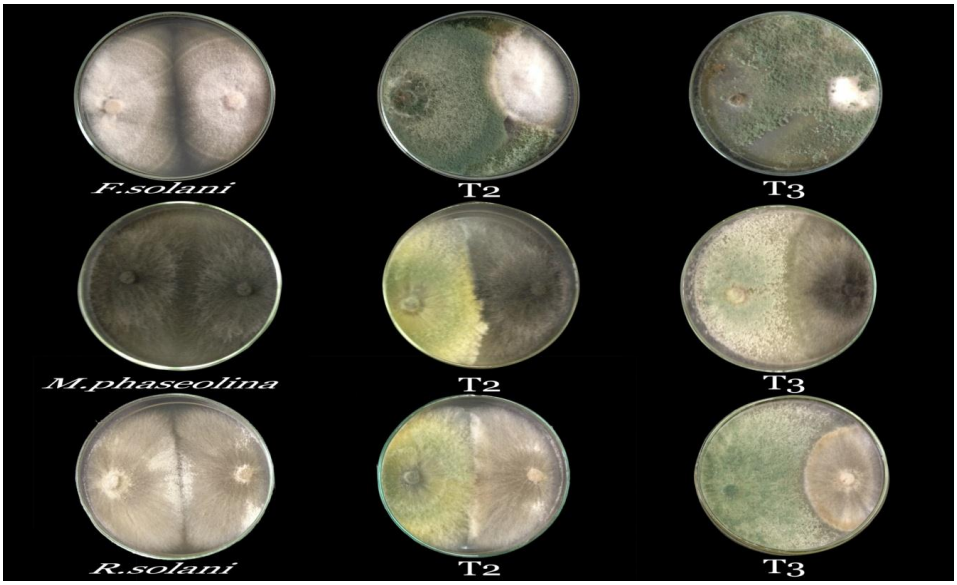

Figure 2: Effect of *T. harzianum* (T3) and *T. hamatum* (T2) on mycelial growth of the tested pathogenic fungi *in vitro*. Where: T3= *T. harzianum*, T2= *T. hamatum*.

Table 4: Effect of some antagonistic rhizobacteria on mycelial growth of the tested fungi *in vitro*.

The tested rhizobacteria	Mycelial growth inhibition (%)			Mean
	R. solani R2	*M. phaseolina* M1	*F. solani* F5	
P. fluorescens	72.45	59.67	80.87	70.9
B. subtilis	24.95	35.37	48.57	36.2
B. polymyxa	51.9	41.62	56.2	49.9
B. megaterium	45.1	0	62.45	35.8
Rhizobium spp. Rh1	32.21	26.38	38.58	32.3
Rhizobium spp. Rh2	26.66	0	31.66	19.4
Rhizobium spp. Rh3	38.28	36.06	45.94	40
Rhizobium spp. Rh4	22.21	31.38	36.06	29.8
Rhizobium spp. Rh5	28.88	19.44	29.44	25.9
Rhizobium spp. Rh6	24.99	23.88	34.69	27.8
Rhizobium spp. Rh7	35.80	29.16	22.22	29
Rhizobium spp. Rh8	16.94	0	14.72	10.5
Rhizobium spp. Rh9	18.33	16.38	26.66	20.4
Control	0	0	0	-
L.S.D. at 5%	6.75	6.45	6.46	-

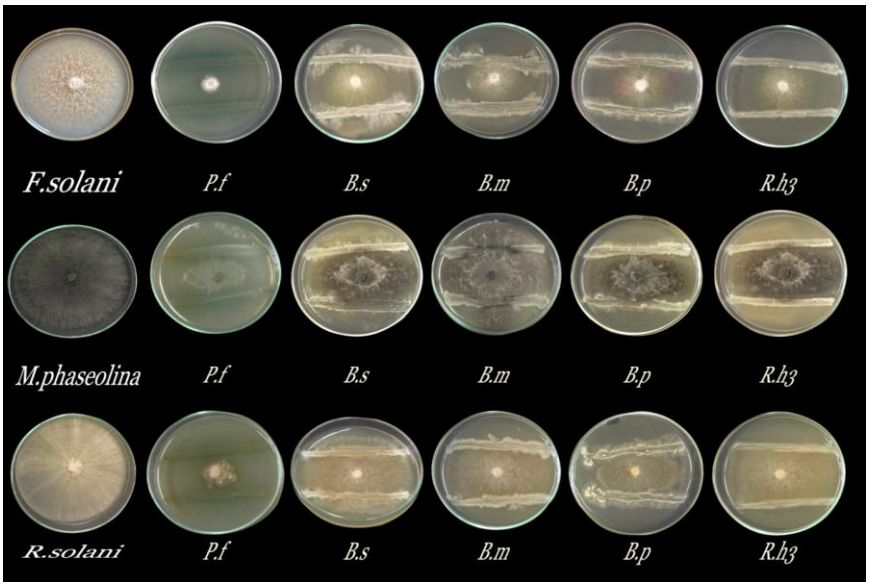

Figure 3: Effect of some antagonistic rhizobacteria on mycelial growth of the tested fungi *in vitro*. Where: *P.f = P. fluorescens, B.s = B. subtilis, B.m= B. megaterium*, *B.p = B. polymyxa* and Rh3 = *Rhizobium* sp.

Table 5: Effect of different bio-agents on incidence of root rot disease of fenugreek under greenhouse conditions during 2015 and 2016 growing seasons**.**

Bioagents	Pathogens	Root rot severity %			
		Season 2015		Season 2016	
		Root rot	Mean	Root rot	Mean
	F. solani	30.81		21.25	
P. fluorescens	*R. solani*	27.69	26.1	28.12	22.7
	M. phaseolina	20		18.75	
	F. solani	28.63		25.95	
B. subtilis	*R. solani*	30.12	28.3	30.83	26.9
	M. phaseolina	26.3		24.16	
	F. solani	35.41		29.30	
B. polymayxa	*R. solani*	32.5	30.5	33.12	27.8
	M. phaseolina	23.75		21.10	
	F. solani	37.25		31.25	
B. megaterum	*R. solani*	35	32.2	34.37	29.3
	M. phaseolina	24.57		22.5	
	F. solani	12.5		13.12	
T. harzianum	*R. solani*	20.81	17.2	20.27	17.6
	M. phaseolina	18.43		19.65	
	F. solani	19.37		17.5	
T. hamatum	*R. solani*	26.10	23.4	24.86	21.5
	M. phaseolina	24.86		22.43	
	F. solani	21.37		18.75	
*Rhizobium spp.*Rh3	*R. solani*	24.65	22.6	23.12	20.8
	M. phaseolina	21.87		20.76	
	F. solani	81.25		75.62	
Control	*R. solani*	76.04	76.6	73.95	73.4
	M. phaseolina	72.65		70.08	
L.S.D. at 5% for					
Bioagents (A)		0.73		0.71	
Pathogen (B)		0.39		0.38	
Interaction (A×B)		1.13		1.08	

Trichoderma harzianum followed by *Rhizobium* sp. isolate (Rh3), and then *Trichoderma hamatum* gave the best reduction of root rot disease of fenugreek during two successive seasons. In treated seeds with *B. megaterium* followed by *B. polymyxa* gave the lowest percentage of disease severity of root rot disease during 2015 and 2016 growing seasons. The other treatments showed moderate effect of root rot disease. Concerning with damping–off disease *T.harzianum* followed by *Rhizobium* sp. (Rh3) Then *P. fluorescens* gave the best reduction of damping-off disease percentage. While treated seed with *P .Polymyxa* followed by *B.subtilis* gave the lowest percentage of damping-off disease. Treated seed with antagonistic rhizobateria and treated soil with antagonistic fungi in infested soil with *R. solani* was recorded the highest root rot disease severity followed by *F. Solani* then *M. phaseolina*. These results are in the same trend with that obtained by Haque and Ghaffar (1992), Shaban and El-Bramawy (2011), El–Mohamdy and Abd Alla (2013) and Farfour- Safinaz and Mahmoud (2014). The results might be attributed to biocontrol agents comprise of multiple beneficial characters such as rhizosphere competence, antagonistic potential, and ability to produce antibiotics, lytic enzymes and toxins.

Table 6: Effect of different bio-agents on incidence of damping-off disease of fenugreek under greenhouse conditions during 2015 and 2016 growing seasons.

Bioagents	Pathogens	Damping-off %							
		Season 2015			Mean	Season 2016			Mean
		Pre.	Post	Survival		Pre	Post	Survival	
	F. solani	5	0	95		2.5	2.5	95	
P. fluorescens	*R. solani*	15	0	85	91.6	12.5	2.5	85	92.5
	M. phaseolina	5	0	95		2.5	0	97.5	
	F. solani	12.5	0	87.5		10	0	90	
B. subtilis	*R. solani*	12.5	0	87.5	85.8	10	2.5	87.5	90
	M. phaseolina	7.5	0	92.5		7.5	0	92.5	
	F. solani	15	2.5	82.5		12.5	2.5	85	
B. polymayxa	*R. solani*	17.5	2.5	80	84.1	15	0	85	87.5
	M. phaseolina	7.5	2.5	90		5	2.5	92.5	
	F. solani	7.5	2.5	90		7.5	0	92.5	
B. megaterum	*R. solani*	10	2.5	87.5	90.8	7.5	2.5	90	93.3
	M. phaseolina	5	0	95		2.5	0	97.5	
	F. solani	5	0	95		0	2.5	97.5	
T. harzianum	*R. solani*	12.5	0	87.5	94.1	10	0	90	95.8
	M. phaseolina	0	0	100		0	0	100	
	F. solani	12.5	2.5	85		10	0	90	
T. hamatum	*R. solani*	10	2.5	87.5	88.3	12.5	0	87.5	90.8
	M. phaseolina	5	2.5	92.5		5	0	95	
	F. solani	7.5	5	87.5		7.5	0	92.5	
*Rhizobium spp.*Rh3	*R. solani*	7.5	5	87.5	91.6	10	0	90	93.3
	M. phaseolina	0	0	100		2.5	0	97.5	
	F. solani	57.5	0	42.5		52.5	2.5	45	
Control	*R. solani*	70	5	25	43.3	72.5	0	27.5	45.8
	M. phaseolina	37.5	0	62.5		30	5	65	
L.S.D. at 5% for									
Bioagents (A)		0.41	0.30	-		0.36	0.38	-	
Pathogen (B)		0.24	0.25	-		0.21	0.23	-	
Interaction (A×B)		0.68	0.73	-		0.59	0.67	-	

These biological control activities are exerted either directly through antagonism of soil-borne pathogens or indirectly by eliciting a plant-mediated resistance response. The mechanisms of biocontrol involve antibiosis, parasitism, competition for nutrients and space, cell wall degradation by lytic enzymes and induced disease resistance (Singh, 2014). Generally, the use of *Trichoderma* spp. and rhizobacteria (PGPR) have greatly reduced the pre and post emergence damping-off and root rot incidence, as well as enhanced the growth of fenugreek plants. The use of bioagents is highly beneficial as environmentally friendly application and can be used as an alternative for fungicides to enhance the plant growth and reduce disease incidence, resulting in higher yield.

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