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ANTAGONISTIC EFFECT OF *Bacillus pumilus* AND/OR *Trichoderma viride* AGAINST *Fusarium solani* OF COMMON BEAN

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ABSTRACT

Root rot disease, caused by *Fusarium solani f.* sp. *phaseoli,* is one of the main root diseases impacting production of common bean in Egypt. The antagonistic effects of *Bacillus pumilus* and *Trichoderma viride*, were tested against *F. solani, in vitro* and in greenhouse conditions. *In vitro* tests, *B. pumilus* and *T. viride* significantly reduced the mycelial growth of pathogenic fungi. In greenhouse experiment, *B. pumilus* and *T. viride*, as soil treatments, significantly reduced the pre and post-emergence damping off disease incidence of *F. solani* under artificial infection. The percentages of disease incidence in treated plants ranged from 26.2 to 30.0%, compared to 72.0% in control plants, in both pre and post-emergence stages, respectively. The best protection of damping off disease was obtained by *T. viride*, followed by *B. pumilus* . All treatments improved the survival plant and growth parameters. Results showed increased levels of peroxidase and polyphenoloxidase activities in treated bean plants, compared to untreated ones.

INTRODUCTION

Soil borne plant pathogens cause economic losses annually in many crops, hence, negatively effect food security. Wilt caused by *Fusarium oxysporum,* a soil borne pathogenic fungi, is considered as one of the constraints responsible for 50-100% crop losses resulting in low productivity due to early wilting (Haware and Nene, 1980, 1982). Control of *Fusarium* by chemicals is often uneconomical and has negative environmental impacts and may lead to the development of fungicidal resistance variants. Biological management is considered an environmentally acceptable alternative to existing chemical treatment methods to control soil pathogenic fungi causing wilt (Harman *et al.,* 2004; Eziashi *et al.,* 2007).

Fusarium root rot on beans is caused by the fungus *Fusarium solani f.* sp. *phaseoli.* The fungus can attack older seedlings, and is most severe on plants growing under stressfull conditions. The pathogen usually survives as thick-walled chlamydospores in soil. It is one of the most economically important root diseases of beans. Young plants are more susceptible to infection than older ones. Application of the fungicides is not economical at the long time, because they may pollute the environment, cause leave harmful residues and can lead to the development of resistant strains of the pathogen with repeated use (Vinale *et al.,* 2008). Replacement of fungicides with biocontrol agents is an alternative mean to manage the plant pathogens, produce safety food and reduce the environment pollution

(Barakat and Al-Masri, 2005). One of the most important biocontrol agents is *Trichoderma* spp.; that is the most frequently isolated soil fungi and present in plant root ecosystems (Harman *et al*., 2004). *Trichoderma* spp., also, are commercially marketed as biopesticides, biofertilizers and soil amendments. The use of *Trichoderma* fungi in agriculture can provide numerous advantages such as colonization of the root and rhizosphere of plant, control of plant pathogens by different mechanisms as parasitism, antibiosis production and inducing systemic résistance , improvement of the plant health by promotion of plant growth and stimulation of root growth (Harman *et al.,*2004).

The objective of this search was to evaluate the antagonistic potential effect of *Bacillus pumilus* and *Trichoderma viride*, as bio-control agents, against *F. solani,* the causal organism of damping off disease and nutritional status in bean plants. The antagonistic activity of *T. viride* and *B. pumilus* were tested *in vitro* and in pot. The role of bioagents in enhancing of some enzymes (peroxidase and poly phenoloxidase), related to disease control in plant was detected.The relationship between nutritional status of bean plants and application of *T. viride* and *B. pumilus* was tested.

MATERIALS AND METHODS

1- Seeds

Bean seeds (*Phaseolus vulgaris* L.) cv. Pulista was obtained from Vegetable Crops Research Department Agricultural Research Centre, Giza, Egypt.

2- Pathogens

Fusarium solani was isolated from naturally infected bean plants, showing damping-off and root rot symptoms, cultivated in Kafr El-sheikh Governorate, Egypt. The isolated fungus identified on the basis of cultural and microscopic morphological characters, according to the key given by **Barnett and Hunter (1972) and Booth (1985)**.

3- Isolation of biocontrol agents

Trichoderma viride and *Bacillus pumilus* isolated by **kamel (2010)** were kindly obtained.

4- Evaluation of antagonistic bioagents against *F. solani*

The antagonistic effect of the tested two biocontrol agents against *F. solani* was examined. *T. viride* and *F. solani* were cultured on PDA medium for 7 days at 28-30°C. Then, a disc (0.5 cm diameter) of the antagonistic fungal colony was cut and placed opposite to the colony of the pathogen. On the other hand, a streak of the bacterial strain was placed on PDA plates at 28°C for 24 h., then a mycelial disc (0.5cm) of the test fungi was placed onto PDA plates at 0.5 cm distant from the bacterial colony. Four replicates were prepared in each experiment. Inoculated plates were incubated at 28°C until the fungal growth of the control plates reached the edge of the plate. The increase and decrease in mycelial growth of the pathogenic fungus were calculated according to Fokemma (1973) as follow**:**

Antagonistic effect =A-B/A x100

Where, A is the diameter of mycelial growth of pathogenic fungus in control, B is the diameter of mycelial growth of pathogenic fungus with *T. viride* or *B. pumilus*.

5- Preparation of antagonistic inocula

The propgules suspension of *T. viride* was prepared in sterile distilled water from 7-days-old- culture on PDA (Rojo *et al.,* 2007) . The fungal inoculum was harvested by flooding the culture with sterile distilled water and then rubbing the culture surface with a sterile glass rod . the fungal propgules concentration in suspension was determined by counting using a heamocytometer slide (Adjusted at 10^7 spores / ml) as described by Sivan *et al*., (1984). Meanwhile, cultures of *B. pumilus* isolates grown on nutrient broth medium were obtained after 4 days of incubation period using shaking incubator. Cell suspension was adjusted after counted by plate count technique (10^8 CFU/ ml) as described by Mosa *et al*., (1997).

6- pot experiment

The pot experiment was carried out at the Dept. of vegetable diseases, Plant Pathology Institute, Agric. Research Center, Giza, Egypt, during October 2011. Antifungal activity of *B. pumilus* and *T. viride* against *F. solani* pathogen was evaluated in pots under artificially infestation conditions. The experiment was designed under greenhouse conditions, using pots (40 cm in diameter) containing 4 kg of sterilized loamy clay soil. Soil was infested with pathogenic fungus grown on sorghum-sand medium at a rate of 5 g/ kg soil in different pots 7 days before application of biocontrol agents. Soil was inoculated with either spore suspensions of *T. viride* or cell suspensions of *B. pumilus* isolates to have soil containing concentration of 10^7 spore or 10^8 bacterial cell/ gm. Aweek later, ten bean seeds (cv. Pulista) were sown in each pot. Five pots as replicates were used for each treatment in completely randomized experimental design. The experiment included the following treatments: 1) non-infested soil (control), 2) soil treated with *F. solani* only, 3) *F. solani* + *B. pumilus*, 4) *F. solani* + *T. viride*, 5) *F. solani* + combination of two bioagents and 6) *F. solani* + Vitavax T (2g/kg seeds). Pots were kept under greenhouse conditions till the end of the experiment. Disease incidence of pre and post- emergence of damping off disease incidence and survival (%) of bean plants were recorded after 15, 30 and 45 days, respectively, as described by Phillips and Hayman (1970) .

The disease incidence was recorded by using the following formula as Ajmal *et al*.,(2001)

Disease Incidence (%) = (Total number of infected plants / Total number of plants) X 100

7- Effect of the tested bioagents on

a- Disease assessment

Effect of the tested *T. viride* and *B. pumilus* in reducing the damping off disease incidence at pre and post-emergence stages, as well as the percentages of survival healthy plants were recorded after 15 , 30 and 45 days after sowing.

b- Plant growth and yield parameters

Random samples of ten bean plants were collected at 60 days after sowing for each bioagent treatment as well as the control plants. The plant growth parameters, as fresh and dry weight of plant and length of root and shoot per plant were estimated.

c- Some plant enzymes activity

Effect of *T. viride* and *B. pumilus* application on the activity of peroxidase and polyphenoloxidase enzymes related to plant defense against pathogens infection were determined in leaves of bean plants at the end of experiment.

Extraction of enzymes

Plant tissue (g) was homogenized with 0.2 Tris Hcl buffer (pH 7.8) containing 4mM
Mercaptoethanol at the rate of 1/3 w/v. The homogenate was centrifuged at 3000 rpm for 15 minutes. The supernatant was used to determine peroxidase and polyphenoloxidase activities according to Tuzun *et al*., 1989.

i. Peroxidase assay

Peroxidase activity was measured by incubating 0.1 ml of enzyme extract with 4 ml of guaiacol for 15 minutes at $25^{O}C$ and absorbance at 470 nm was determined.The guaiacol solution consisted of 3 ml of 0.05 M potassium phosphate. pH7, 0.5 ml of 2 % guaiacol and 0.5 ml of 0.3 % H_2O_2 (Abeles *et al*., 1971). Peroxidase activity was expressed as the increase in absorbance at 470 nm/gram fresh weight/15 minutes.

ii. Polyphenoloxidase assay

Polyphenoloxidase activity was determined using the colorimetric method, described by Matta and Dimond (1963). The reaction mixture contained 1.0 ml of crude enzyme extract, 1.0 ml 0.2 M sodium phosphate at pH 7.0 and 1.0 ml of 10 M catecholl brought to final volume of 6.0 ml with distilled water (Morsy, 2005). The activity of polyphenoloxidase was expressed as the optical density at 475 nm.

8- Statistical analysis:

Data obtained were subjected to computer statistical package (ASSTATE) originated by Silva, *et al.* (2009).

RESULTS

1. Evaluation of fungal and bacterial isolates for antagonistic activities against *F. solani in vitro*

Data in Table (1) show that the bioagent isolates succeeded in reducing the mycelial growth of *F. solani*. *T. viride* was more effective than *B. pumilus* for reducing the mycelial growth of *F. solani,* being 3.1 and 3.9 cm, respectively. Moreover, *T. viride* inhibited the over growth of *F. solani*, comparing with *B. pumilus*. Both of *T. viride* and *B. pumilus* strains reduced growth by 65.6 and 56.7 %, respectively, comparing with the control (Table 1). This behaviour represents an important approach for controlling a root rot disease of bean plants. The potentialities of the used isolates could be

attributed to their effect to secrete hydrolytic enzymes or antifungal metabolites.

Table (1): Effect of *T. viride* and *B. pumilus* treatments on the linear growth of *F. solani in vitro*.

Treatments	Antagonistic effect	
	mycelial Diameter (cm)of	Reduction (%)
Control	9.00 a	0.0
T. viride	3.10 b	65.6
B. pumilus	3.90 b	56.7

Means in each column followed by the same letter are not significantly different according to LSD test (p= 0.05).

2. Efficiency of the two antagonistic biocontrol agents under greenhouse conditions:

Data presented in Table (2) reveal that soil infested with *F.solani* significantly increased damping-off of bean seedlings and severely reduced survival rate (24 %) compered to untreated control (88 %) or Vitavax T. treatment (74 %). Inoculation with *T. viride* or *B.pumilus,* significantly increased survival plant compared with the *F. solani* infested soil, ranging between 68 and 62%, respectively (Table 2). However, higher percentage of survival plants of bean seedlings were attained in response to treatment with dual bioagents (68%) than the individual one. Also, data indicate that among the bioagents no significant different were found. *B. pumilus* was found to be highly effective in reducing the incidence percentage of *Fusarium* root rot of bean. The least disease incidence was recorded in treatment with *B. pumilus* (26.2%) followed by mix bio agents (28.0%) and *T. viride* (30.0%) compared with untreated control(72%),while fungicide Vitavax T was (23.0 %).

Table (2): Influence of two antagonistic isolates on bean plants grown in pots containing *F. solani* infested soil under greenhouse condition.

Treatments	Damping off (%)		Survival plants (%)	(Disease incidence) D. I (%)
	Pre-emergence	Post-emergence		
Control (untreated)	4.0 d	8.0 c	88.0b a	14.6 d
Control(infested with *F. solani*)	50.0 a	26.0 a	24.0 e	72.0 a
F.solani+B. pumilus	14.0 c	18.0 b	68.0 c	26.2 bc
F. solani +T. viride	20.0 b	18.0 b	62.0 d	30.0 b
F.solani +B.pumilus +T. viride	16.0 bc	16.0 b	68.0 c	28.0 bc
***F.solani* +Vitavax T**	12.0 c	14.0 b	74.0 b	23.0 c
LSD at 0.05%	5.498	4.237	5.189	5.389

Means in each column followed by the same letter are not significantly different according to LSD test (p= 0.05).

Data presented in Table (3) indicate the effect of bioagents on some plant growth parameters viz., plant height, fresh and dry weights, length of roots and shoots of common bean. Results reveal that treatment with *B.*

pumilus, T. viride and mix of the two bioagents increased plant height, compared to the control plants. *B. pumilus* gave the highest records (51.2 cm), followed by mix of bioagents (49.8 cm) and *T. viride* (49.3 cm), while the control plants gave (37.4 cm). No significant differences were recorded among bioagent treatments, while significant ones were recorded between bioagent treatments and the control plants.

All treatments significantly increased fresh and dry weight of plants, though they were insignificantly different between each other. However, *T.viride* achieved highly significant increase in length of root (15.7 cm), followed by *B. pumilus* and mix of bioagents (12.5, 13.3 cm), respectively, compared with control (9.3 cm). Also, fungicide Vitavax T gave the higher effect in length of root (15.9 cm). *B. pumilus*, *B. pumilus* +*T. viride* and *T. viride* recorded the best results in shoot length (14.7, 13.5 and 12.5 cm), respectively, compared with infested control (9.4 cm).

Table (3):Effect of soil inoculations with *T.* viride and/or *B. pumilus* on some plant growth parameter of bean plants infested with *F. solani* under greenhouse conditions.

Treatments	Plant Height (cm)	Fresh weight /plant(g)	Dry weight / plant(g)	Length of Root (cm)	Length of shoot (cm)
Control (infested with *F. solani*)	37.4 a	5.42 b	0.59 b	9.3 c	9.4 d
F. solani*+*B. pumilus	51.2 b	7.96 a	0.80 a	12.5 b	14.7 ab
F. solani*+*T. viride	49.3 b	7.56 a	0.78 a	15.7 a	12.5 c
F.solani*+*B.pumilus* + *T. viride	49.8 b	7.70 a	0.79 a	13.3 b	13.5 bc
***F. solani* +Vitavax T**	52.7 b	8.04 a	0.81 a	15.9 a	14.3 a

Means in each column followed by the same letters are not significantly different according to LSD test (p= 0.05).

Data in Table (4) showed that both bioagents and mix of bioagents treatments stimulated activity of peroxidase and polyphenoloxidase enzymes, comparing with the control (untreated) Table (4).

Table (4): Determination of peroxidase and polyphenoloxidase activity in bean plants treated with biocontrol in pots under greenhouse conditions.

Treatments	Enzymatic activities			
	Peroxidase		Polyphenoloxidase	
	Activity	Increase (%)	Activity	Increase (%)
Control (infested with *F. solani*)	0.395 d	-	0.189 c	-
F. solani*+ *B. pumilus	0.781 b	97	0.423 a	124
F. solani* +*T. viride	0.811 a	105	0.441 a	133
F. solani* +*B. pumilus* + *T. viride	0.792 b	100	0.432 a	128
***F. solani* +Vitavax T**	0.699 c	77	0.338 b	79

Means in each column followed by the same letter are not significantly different according to LSD test (p= 0.05).

Data show that the optical density of peroxidase activity was in the range of 0.781 to 0.811 in bean plants under bioagents application, compared to 0.395 in untreated bean plants. The peroxidase enzymatic activity was in the range 97 to 105 % in bioagents treatments application. *T. viride* significantly increased the activity of peroxidase about (105 %), followed by mix of bioagents (100 %) and *B. pumilus* (97 %). Results showed that the optical density of polyphenoloxidase were in the range of 0.423 to 0.441 in treated bean plants, compared to control plants(0.189) . Bioagents application enhanced the activity of polyphenoloxidase enzyme in bean plants from 124 to 133 %. *T. viride, B. pumilus* and mix of bioagents significantly increased the enzyme activity (133, 124 and 128 %), respectively.

DISCUSSION

Data are in harmony with those obtained by Montealegre *et al.*(2005), who,reported that *Trichoderma* spp. secreted chitinase and B 1,3 glucanase in supernatants. Moreover *Trichoderma* spp. is a well-known producer of cell wall-degrading enzymes and the antibiotics ,thus, could act synergistically with other mechanisms (Vinale *et al*., 2006). Also, Mausam *et al.,*(2007) reported that *Trichoderma* spp. are known as biological mycoparasites, and which are used commercially as biocontrol agents a range of plant pathogenic fungi such as *Fusarium*, *Pythium*, and *Rhizoctonia* strains, as well as it is a product of ecological interest.

Sarhan, *et al.,* (2001) and Montealegre *et al., (*2005) pointed that the cell free culture filtrate of *B. subtilis* inhibited the mycelial growth, radial growth, spore germination and germ-tubes length of *F. oxysporum*. Moreover, Alippi and Monaco (1994) reported that *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin which have an inhibitory effect on fungal pathogens. Pusey and Wilson (1984) reported that *B. subtilis* exerted a heat stable antibiotic interfering with spore germination, or early germ tube development of stone fruit brown root pathogen. *Bacillus sp*. also, grows very fast and occupies the court of infection and preventing pathogen spores to reach susceptible tissues in competition for spaces (Wolk and Sorkar, 1994).

The obtained results indicated that, *B. pumilus* effectively reduced mycelial growth of *F. solani* and inhibited the linear growth . These results are in agreement with the findings of Berg *et al.,*(1994); Saddlers,(1996); Sankar and Jeyarajan, (1996) and Wang *et al.,*(1999). They stated that *B. pumilus* was able to suppress wilt disease caused by *F. solani*.

Results showed that the use of *Trichoderma* spp. as biocontrol agents induced the accumulation of some enzymes such as peroxidase and polyphenoloxidase which play an important role in plant defense mechanisms against pathogens infection. Results cleared that the enzymatic activity in treated bean plants increased more than in untreated control. Nawar and Kuti (2003) reported that there were positive relationships between peroxidase and resistance development in plants.

Caruso *et al*., (2001) also, experimentally, supported the idea that peroxidase play a defense role against invading pathogens. Hassan *et al*., (2007) obtained the lowest percentages of chocolate spot disease severity and the highest levels of peroxidase activities in faba bean plants. Treatments with *Trichoderma* spp. gave a highly protected of bean seedlings against damping-off disease. It may be related to the ability of *Trichoderma* spp. to stimulate the enzymes in bean plants associated with increasing the protection against disease.

Trichoderma is listed both in Europe and USA as an active principal ingredient, permitted for use in organic farming for plant disease control. *Trichoderma* spp utilize various mechanisms including nutrient competition, antibiosis, antagonism, inhibition of pathogen or plant enzymes; processes of biodegradation, carbon and nitrogen cycling; complex interactions with plants in the root zone of the rhizosphere, which involve various processes such as colonization, plant growth stimulation, biocontrol of diverse plant pathogens, decomposition of organic matter, symbiosis, and nutrient exchange (Howell, 2003 and Harman, 2006). Harman *et al.*, (2004) indicated that these fungi can induce systemic resistance in plants, thus, increasing the plant defense response to diverse pathogen attack.

B. pumilus excreted metabolites had been defined as surface active materials, which directly inhibited the pathogen, and indirectly enhanced the host plants stand against the pathogen through *Pseudoperonospora cubensis* increasing of the plant enzymes responsible for resistance.(El-Grami *et al.,* 2012)

Mixtures of two or more antagonists may increase the efficacy or decrease the variability associated with biocontrol treatments (Guetsky *et al.*, 2001; Haggag and Nofal 2006). Antagonist mixtures have been thoroughly investigated in soil systems, especially against pathogenic taxa such as *Fusarium* spp. and *Pythium* spp., some studies reported that enhanced disease control of root infecting pathogens can be attained with multi-strain mixtures (Lemanceau *et al.,* 1992), other data suggests that not all mixtures are better at disease suppression than the most effective antagonist used alone (Thrane *et al.*, 2000 and Roberts *et al*., 2005).

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النشاط التضادى لكل من بكتيريا باسيلس بيوملس وفطر تريكوديرما فيردى ضد الاصابة بفطر فيوزاريوم سولانى على نباتات الفاصوليا

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يعتبر مرض عفن الجذور المتسبب عن فطر الفيوزاريوم سولانى فاصولاى أحد أهم أمراض الجذور تاثيرا على انتاجية نباتات الفاصوليا فى مصر. تم اختبار التاثير التضادى لكل من بكتيريا باسيلس بيوملس وفطر التريكوديرما فيردى ضد المسبب المرضى فيوزاريوم سولانى ،معمليا، وكذلك تحت ظروف الصوبة.أوضحت النتائج المتحصل عليها تحت ظروف المعمل ان أستخدام بكتيريا باسيلس وفطر تريكوديرما ادى الى خفض معنوى فى النمو الميسيليومى للفطر الممرض. و تحت ظروف العدوى الصناعية فى الصوبة أدى استخدام بكتيريا باسيلس وفطر التريكوديرما (كمعاملة تربة) الى إنخفاض معنوى فى موت البادرات قبل وبعد الانبات، وكذلك نسبة الاصابة بفطر فيوزاريوم سولانى. حيث تراوحت نسبة الاصابة فى النباتات المعاملة من ٢٦-٣٠% بالمقارنة ب ٧٢% فى الكنترول.وكذلك وجد ان افضل حماية من مرض موت البادرات تحققت عند المعاملة بفطر التريكوديرما فيردى ثم يليها بكتيريا باسيلس بيوملس. أثبتت النتائج أن كل المعاملات ادت الى تحسن فى اعداد النباتات المقاومة وفى مقاييس النمو. اظهرت النتائج مستويات مرتفعة من نشاط انزيمات البيروكسديز والبولى فينول أوكسيديز فى النباتات المعاملة، بالمقارنة مع التباتات غير المعاملة(الكنترول).

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