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

Kamel, S. M .H. 1 and Nagwa M. M. El-Khateeb2

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 chlamydomspores 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 rhizosphere of plant, control of plant pathogens by different mechanisms as parasitism, antibiosis production and inducing systemic resistance, 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 (0.5 cm) 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 x 100
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 hemocytometer slide (Adjusted at 10⁷ 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 (10⁶ 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 10⁶ spore or 10⁸ bacterial cell/gm. A week 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)

\[
\text{Disease Incidence} \% = \frac{\text{(Total number of infected plants)}}{\text{(Total number of plants)}} \times 100
\]

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.

1. Peroxidase assay

Peroxidase activity was measured by incubating 0.1 ml of enzyme extract with 4 ml of guaiacol for 15 minutes at 25°C and absorbance at 470 nm was determined. The guaiacol solution consisted of 3 ml of 0.05 M potassium phosphate, pH 7, 0.5 ml of 2 % guaiacol and 0.5 ml of 0.3 % H2O2 (Abeles et al., 1971). Peroxidase activity was expressed as the increase in absorbance at 470 nm/gram fresh weight/15 minutes.

2. 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 catechol 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Antagonistic effect</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mycelial Diameter (cm)of</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.00 a</td>
<td>0.0</td>
</tr>
<tr>
<td><em>T. viride</em></td>
<td>3.10 b</td>
<td>65.6</td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>3.90 b</td>
<td>56.7</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Damping off (%)</th>
<th>Survival plants (%)</th>
<th>(Disease incidence) D.I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-emergence</td>
<td>Post-emergence</td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>4.0 d</td>
<td>8.0 c</td>
<td>88.0b a</td>
</tr>
<tr>
<td>Control (infested with <em>F. solani</em>)</td>
<td>50.0 a</td>
<td>26.0 a</td>
<td>24.0 e</td>
</tr>
<tr>
<td><em>F. solani</em> + <em>B. pumilus</em></td>
<td>14.0 c</td>
<td>18.0 b</td>
<td>68.0 c</td>
</tr>
<tr>
<td><em>F. solani</em> + <em>T. viride</em></td>
<td>20.0 b</td>
<td>18.0 b</td>
<td>62.0 d</td>
</tr>
<tr>
<td><em>F. solani</em> + <em>B.pumilus</em> + <em>T. viride</em></td>
<td>16.0 bc</td>
<td>16.0 b</td>
<td>68.0 c</td>
</tr>
<tr>
<td><em>F. solani</em> + Vitavax T</td>
<td>12.0 c</td>
<td>14.0 b</td>
<td>74.0 b</td>
</tr>
<tr>
<td>LSD at 0.05%</td>
<td>5.498</td>
<td>4.237</td>
<td>5.189</td>
</tr>
</tbody>
</table>

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.

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

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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Peroxidase Activity</th>
<th>Polyphenoloxidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (infested with F. solani)</td>
<td>0.395 d</td>
<td>-</td>
</tr>
<tr>
<td>F. solani + B. pumilus</td>
<td>0.781 b</td>
<td>97</td>
</tr>
<tr>
<td>F. solani + T. viride</td>
<td>0.811 a</td>
<td>105</td>
</tr>
<tr>
<td>F. solani + B. pumilus + T. viride</td>
<td>0.792 b</td>
<td>100</td>
</tr>
<tr>
<td>F. solani + Vitavax T</td>
<td>0.699 c</td>
<td>77</td>
</tr>
</tbody>
</table>

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 Kutl (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).

REFERENCES


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النشاط التضادي لكل من بكتيريا بسيلس بيوملس وفطر تريكورديما فيردى ضد
الإصابة بفطر فيوزاريوم سولاني على نباتات الفاصوليا

سامح محمد حسن كامل ونجوى محمد محمد الخطب

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يعتبر مرض عفن الجذور المسبب عن فطر الفيوزاريوم سولاني فاصولاه أحد
أهم أمراض الجذور تأثيرا على انتاجية نباتات الفاصوليا في مصر. تم اختبار التأثير
التضادي لكل من بكتيريا بسيلس بيوملس وفطر التريكورديما فيردى ضد المسبب المرضي
فيوزاريوم سولاني، معامليا. وكذلك تحت ظروف الصويا، أوضحنت النتائج المتصل عليها
تستمأوف العمل إن استخدام بكتيريا بسيلس وفطر تريكورديما أدى إلى خفض معنوي
في النمو البسيمولي لتلفة الممرض. وتحت ظروف العدوى الصناعية في الصويا أثناء
استخدام بكتيريا بسيلس وفطر التريكورديما (كمفاعلة تربة) إلى إخفاء معنوي في موت
الذكور قبل وبعد الأنبات. وكذلك نسبة الإصابة بفطر فيوزاريوم سولاني. حيث تراوحت
نسبة الإصابة في النباتات المعالمة من 26-30% بالمقارنة بـ 72% في الكنترول. يذكر
وقد كان أفضل حماية من مرض موت البيوتاتaquainted عند المعاملة بفطر التريكورديما
فريدى ثم بليها بكتيريا بسيلس بيوملس. أثبتت النتائج أن كل المعاملات أدت إلى تحسن
عداد النباتات المقاومة في مقابلة الفقار. ظهرت النتائج متفقة من نشاط
النتباثات البروكسيزد والبول فينول أو كسيديز في النباتات المعالمة، بالمقارنة مع النباتات
غير المعالمة (الكنترول).

قام بتحكيم البحث

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