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Effect of some *Bacillus* Species Combined with Chemical Resistance Inducers on Control of Pea Damping-off and Root-rot Diseases

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ABSTRACT

The isolated fungi of *Alternaria sp., Fusarium semitectum, F. solani, Rhizoctonia solani,* and fungus-like *Pythium ultimum* caused pre-and post-emergence damping–off to pea plants. In the pathogenicity test, the fungus *Fusarium solani* showed the highest percentages of pre-and post-emergence damping-off followed by *Rhizoctonia solani* and *F. semitectum.* Meanwhile, *Alternaria* sp. caused the least figures of pre-and post-damping-off followed by fungus-like *Pythium ultimum*. Data demonstrated that the tested bioagents *Bacillus megaterium, B. subtilis* and *B. thuringiensis* and the CRIs resulted in a significant reduction to the linear growth of both *F. solani* and *R. solani* compared with control treatment with reduction-concentration dependent. In addition, CRIs were less efficient in this regard than the bioagents. The obtained results revealed that soaking pea seeds in 100 mM Bion (BTH) for six hours, then pelleting them with supernatant of *B. megaterium* resulted in a significant reduction to the incidence of damping-off and root-rot severity caused by both tested fungi. Also, a significant increase to the plant height and the produced green pods yield was recorded compared to control treatment. Moreover, the most efficient treatment in reducing damping-off and root-rot severity and increasing each of plant height, number of pods/ plant and average weight of green pods (g) / plant was the combination with Bion and *B. megaterium*. Treating pea seeds with the tested *Bacillus* strains and the CRIs resulted in increasing of the total phenols in the roots pea plants compared with the plants of untreated seeds. The study recommends treatment of pea seeds in the mixture of Bion and *B. megaterium* to protect the roots and seedlings and improve the plant characteristics.

Keywords*: Bacillus spp., Fusarium. Rhizoctonia, pea, damping-off, root-rot*

1. INTRODUCTION

Pea (*Pisum sativum* L.) is considered one of the most important food legume crops in Egypt for local consumption and exportation. The economic importance of pea cultivation in the world could be explained by its high nutritional value of vitamins, protein, carbohydrates and some other nutrients. It can improve the soil fertility through nitrogen fixation.

Pea is liable to be attacked by many bacterial, fungal, viral, nematode diseases and physiological disorder. However, fungal diseases, especially damping-off and root-rot diseases are considered the major destructive diseases affecting the crop yield (Kraft and Pfleger, 2001).

Plant disease control represents a major challenge that farmers are facing in the management of cropping systems. Both *Fusarium solani* and *Rhizoctonia solani* are soilborne fungal pathogen causing pea damping off, root-rot and generating compromised quality of crops and reducing yields. Taking into consideration the demands of growing the population, food production must rise each year to feed the seasonal increase in the population. Food and beverages industry and commerce

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depend on agricultural sector as their main supplier of raw materials. The challenge is to provide more output with limited available resources. Plant disease control represents the major issue that farmers are facing in the management of cropping systems. The fungal diseases represent one of the major reasons of decrease the yield of agricultural crops all over the world (Makovitzki *et al.,* 2007). Prevention and control management of plant pathogenic fungi is achieved mainly by the use of synthetic fungicides. However, the massive and sometimes inappropriate use of the synthetic fungicides in agricultural practices resulted in severe negative effects on multiple levels. On the one hand, the plant pathogens have developed resistance to the used fungicides (Rossall, 2012). Therefore, there is a growing interest to find a new safe control formulation for plant protection, which represents a real need in the nowadays context of sustainable development in agriculture and ecology area. Plants produce multiple secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids (Butu *et al.,* 2014) being in the same time important sources of biologically active molecules possessing antibacterial, antifungal (Negi, 2012) and antioxidant properties (Butu *et al.,* 2014). Chemical treatments with traditional fungicides and integrated disease management methods used for control of *F. solani* and *R. solani* diseases seem not to be completely effective, and therefore the diseases cause and remain a persistent issue for the farmers to deal with (Huang *et al.,* 2012).

Protection of plants against plant pathogens by induction of systemic resistance is a new approach that is much safer to the environment and plant products as compared to deadly agrochemicals applied to control plant diseases (Yan *et al.,*2003).

This research aimed to study the effect of some *Bacillus* strains and chemical resistance inducer on both *F. solani* and *R. solani* in *vitro* and *in vivo*. Also, total phenolic compounds were estimated in bacterial, CRIs and pathogen on treated pea plants.

2. MATERIAL AND METHODS

2.1 Tested Pathogens

Five fungal isolates, *Alternaria sp., Fusarium semitectum, F. solani, Rhizoctonia solani,* and fungus-like *Pythium ultimum* were used in this study. The fungi were isolated from infected pea plants cv. Master B showing root-rot symptoms were collected from fields at Giza governorates, Egypt.

2.2. Bioagents (Antagonistics)

Three *Bacillus* strains, *Bacillus megaterium, B. subtilis* and *B. thuringiensis* were used as biocontrol agents to suppress the activity of the fungal isolates under investigation. The bioagents source is Rhizospheric soil samples collected from pea plants have good plant growth vigor at fields of Giza governorates, Egypt.

2.3. Chemical resistance inducers (CRIs)

Three chemical resistance inducers, Bion (benzothiadiazole), Humic acid ($C_{187}H_{186}O_{89}N_9S_1$) and Salicylic acid (monohydroxy benzoic acid) were tested in this study. All tested chemical inducers were obtained from Elgomhuria Company for Trading, Drugs, Chemicals and Medical Supplies, Cairo, Egypt.

2.4. Pea seeds

Pea seeds (cv. Master B) were obtained from the Ministry of Agriculture, Giza governorates, Egypt.

2.5. Isolation, purification and identification of the causal fungi

Samples were collected from infected pea roots, washed in running tap water for several times, then cut into small pieces and surface sterilized with 1% sodium hypochlorite for 2 minutes under aseptic condition. The plant pieces were rinsed in sterilized water for several times and dried between sterilized Whatman 1 filter papers then plated on potato-dextrose-agar (PDA) medium and incubated for 7 days at 25±1° C.

The developed fungal colonies were purified using hyphal tip technique and identified depending on their morphological features based on the description of Gilman (1957) and Booth (1971).

2.6. Pathogenicity test

The inoculum of the tested fungi was grown in bottles contained autoclaved barley sand medium for two weeks. Formalin sterilized soil was infested with the inoculums of the isolated fungi at the rate of 2% inoculums level and distributed in plastic pots (25cm in diam.). Uninfected formalin sterilized soil was used as control treatment. All pots were sown with pea seeds (cv. Master B). Five seeds were sown in

each pot and three replicates were used for each treatment. Pre and post emergence damping–off were recorded 15 and 30 days after sowing, respectively. Re-isolation of the pathogens was carried out from the damped off seedlings.

2.7. Isolation, purification and identification of the antagonists

Rhizospheric soil samples collected from pea plants have good plant growth vigor was used to isolate the antagonists. Serial dilution plate technique was used to isolate native *Bacillus spp.* on nutrient agar medium (Oedjijono and Dragar, 1993).

The isolated *Bacillus* species were selected, purified and identified depending on the description of Parry *et al.*, (1983) and Holt and Krieg (1984).

2.8. Effect of culture filtrate of the tested bioagents on the linear growth of *F. solani* and *R. solani*

The effect of the culture filtrate of the three *Bacillus* isolates on the growth of both pathogenic fungi was studied. One hundred ml of nutrient, broth medium was put in each 250 ml flask and sterilized by steamer for three successive days. The medium was inoculated with a loop of any of the bacterial bioagent(s) taken from two-day-old culture. Inoculated flasks were incubated on a rotary shaker at 200 rpm for 2 days at 28 ± 2°C. The growth was filtered through double layer of filter paper (Watman 1). The calculated amounts of the bioagents culture filtrate were mixed with PDA medium after sterilization to obtain a final concentration of 20, 40, 60 and 80%. The medium was then steamed for three successive days and poured into the Petri-dishes (20 ml/plate). After solidification, the Petri-plates were inoculated with 5 mm. discs of any of the tested two pathogens cut from the five days old culture. PDA plates inoculated with the test pathogens, but not amended with culture filtrate were maintained as control. Plates were then incubated at 28±2 °C. Five replicates were maintained for each treatment. Periodic observations on the linear growth of the mycelium were recorded. The inhibition percentage of mycelia growth of the tested pathogens was calculated according to the formula: $I = (C- T)/ C X100$

Whereas;

I= Percent of inhibition in growth of tested pathogen.

C= Linear growth of the pathogen (mm) in control.

T= Linear growth of pathogen (mm) in treatment.

2.9. Effect of Chemical resistance inducers (CRIs) on the linear growth of *F. solani* and *R. solani*

The chemical resistance inducers (CRIs) Bion (benzothiadiazole), Humic acid ($C_{187}H_{186}O_{89}N_9S_1$) and Salicylic acid (monohydroxy benzoic acid) were prepared at 20, 40, 60, 80 and 100 mM based on their molecular weight. The calculated amounts of the CRIs were mixed with PDA medium before sterilization to obtain a final concentration. The medium was then steamed for three successive days and poured into the Petri-dishes (20 ml/plate). After solidification the Petri-plates were inoculated with 5 mm. discs of any of the tested two pathogens cut from the five days old culture. PDA plates inoculated with the test pathogens, but not amended with CRIs were maintained as control. Plates were then incubated in an incubator at 28±2°C. Five replicates were maintained for each treatment. Periodic observations on the linear growth of the mycelium were recorded. Inhibition percentage of mycelial growth of the tested pathogens was calculated as mentioned before.

2.10. Effect of bacterial bioagent and CRI Bion on damping-off. root-rot severity and some crop parameters

One hundred ml of nutrient broth medium were put in each 250 ml flask and sterilized by steamer for three successive days. The medium was inoculated with a loop of any of the bacterial bioagent, *B. megatewrium* (BM) taken from two days old culture. Inoculated flasks were incubated on a rotary shaker at 200 rpm for 2 days at 28 ± 2°C.

The suspension of the inoculated flasks by any of the tested bioagents was centrifuged in sterile 50-ml plastic tubes at 6000 rpm for 10 min to make pellet. The pellet (30×10^6 cfu / ml) was mixed with sterilized talc powder at the rate of 1:1 (v/w), then dried at room temperature.

Pea seeds were surface sterilized with 1.0% solution of sodium hypochlorite for 1 min and then thoroughly washed with tap water. Formalin sterilized sandy clay soil were divided into fifteen treatments (Table, 4) as follows:

1- Pea seeds dressed with the inoculums of the tested bioagent (BM) (5g/kg. Seeds) were sown in five pots contained sterilized soil.
2- Pea seeds soaked for six hours in the CRI Bion (100 mM) were sown in five pots contained sterilized soil.
3- Pea seeds dressed with the inoculums of the tested bioagent (5g/kg. Seeds) were sown in pots contained infested soil with 2% inoculum level of *F. solani*.
4- Pea seeds dressed with the inoculums of the tested bioagent (5g/kg. Seeds) were sown in pots infested soil with 2% inoculum level of *R. solani*.
5- Pea seeds soaked for six hours in the Bion (100 mM) were sown in pots contained infested soil with 2% inoculum level of *F. solani*.
6- Pea seeds soaked for six hours in the Bion (100 mM) were sown in pots contained infested soil with 2% inoculum level of *R. solani*.
7- Pea seeds soaked for six hours in the Bion (100 mM) and dressed with the inoculums of the tested bioagent (5g/kg. Seeds) were sown in pots contained infested soil with 2% inoculum level of *F. solani*.
8- Pea seeds soaked for six hours in the Bion (100 mM) and dressed with the inoculums of the tested bioagent (5g/kg. Seeds) were sown in pots contained infested soil with 2% inoculum level of *R. solani*.
9- Pea seeds dressed with the inoculums of the tested bioagent (5g/kg. Seeds) were sown in pots contained infested soil with 2%inoculum level of both *F. solani* and *R. solani*.
10- Pea seeds soaked for six hours in the Bion (100 mM) were sown in five pots contained infested soil with both *F. solani* and *R. solani*.
11- Pea seeds soaked for six hours in the Bion (100 mM) and dressed with the inoculums of the tested bioagent, (5g/kg. seeds) were sown in pots contained soil infested soil with 2% inoculum level both *F. solani* and *R. solani*.
12- Untreated pea seeds with any of the tested bioagent and Bion were sown in pots contained infested soil with 2% inoculum level of *F. solani*.
13- Untreated pea seeds with any of the tested bioagent and Bion were sown in pots contained infested soil with 2% inoculum level of *R. solani*.
14- Untreated pea seeds with any of the tested bioagent and Bion were sow in pots contained infested soil with 2% inoculum level of both *F. solani* and *R. solani*.
15- Untreated pea seeds with any of the tested bioagent and Bion were sown in pots contained sterilized soil.

Five seeds were sown in each pot and seven pots were used for each treatment. The incidence of pre-and post-emergence damping-off as well as root-rot severity were calculated 15, 30 and 90 days after sowing, respectively. Also, the plant height (cm), number and weight (g) of pods / plant were estimated and recorded at the end of the experiment (90 days).

2.11. Assessment of disease severity

Incidence of pre-and post-emergence damping-off were calculated 15 and 30 days after sowing. Also, the severity of root-rot was assessed three months after sowing on the roots of the grown plants, by bull-off the plants, after irrigation, from randomly two pots, using the devised scale (0-5) and disease severity was assessed using the following formula described by Salt (1982):

Disease severity % = £ (nxv) / 5N X 100

Whereas:

n = Number of infected roots in each category.

v = Numerical values of each category.

N = Total number of the infected roots.

2.12. Estimation of total phenolic compounds in bacteria, Bion and pathogen treated pea plants

One gram of pea roots sample was extracted with 10 ml of 80% methanol at 70 °C for 15 min. Reaction mixture was containing 1 ml of methanolic extracts, 5 ml of distilled sterilized water, and 250 µl of Folin–ciocalteau reagent (1 N). This solution was kept at 25 °C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Gallic acid was used as the standard. The amount of phenolic compounds was expressed as mg gallic acid per g plant material (Zieslin and Ben-Zaken,1993).

2.13. Statistical analysis

Data were statistically analyzed using the standard procedures for complete randomize block and split designs as mentioned by Snedecor and Cochran (1967). The averages

were compared at 5% level using least significant differences (L.S.D) according to Fisher (1948).

3. RESULTS

3.1. Isolation and identification of the fungal isolates and antagonistic *Bacillus* strains under investigation

Isolation trials from the rotten roots of pea roots yielded fungal isolates of *Alternaria sp., Fusarium semitectum, F. solani, Rhizoctonia solani,* and the fungus-like, *Pythium ultimum.* The fungi were used to conduct pathogenicity test.

The isolated anatgonistic *Bacillus* strains were purified and identified as *Bacillus megaterium , B. subtilis* and *B. thuringiensis*.

3.2. Pathogenicity tests

Data presented in Table (1) show that the isolated fungi resulted in causing pre-and post-emergence damping–off to pea. *Fusarium solani* caused the highest percentages of pre-and post-emergence damping-off, being 26.7 and 20.0 % followed by *Rhizoctonia solani,* being 20.0and 23.3 % then *F. semitectum,* being 16.7 and 20.0 %, respectively. Meanwhile, *Alternaria* sp. caused the lowest figures of pre-and post-emergence damping-off, being 10.0 and 3.3 % followed by *P. ultimum,* being 16.7 and 13.3 %, respectively.

Re-isolation from the damped-off seedlings proved the pathogenicity of the tested fungi.

3.3. Effect of culture filtrate of three *Bacillus* strains on the linear growth of both pathogenic fungi (*F. solani* and *R. solani*)

Data shown in Table (2) showed that all the test three *Bacillus* strains caused significant reduction to the linear growth of *F. solani* and *R. solani* compared with the control treatment. This reduction was gradually increased by increasing the concentration of the tested bioagents. In addition, the linear growth of the two tested pathogens was completely inhibited by adding of *B. megaterium* at the concentration of 80 %. Furthermore, *B. megaterium* was the most efficient bioagent in this regard followed by *B. thuringiensis* then *B. subtilis,* being 27.8, 37.6 and 40.6 mm, respectively. Therefore, *B. megaterium* was chosen to test its capability as biological control agent against the tested two pathogens under greenhouse conditions.

No significant difference was found regarding the effect of the tested bioagents on *F. solani* and *R. solani*, being 49.0 and 48.8 mm., respectively.

3.4. Effect of three CRIs on the linear growth of *F. solani* and *R. solani*

Table (3) showed that all tested CRIs resulted in a significant reduction to the linear growth of the two pathogenic fungi compared with the control treatment. This reduction was gradually increased by increasing the concentration of the tested CRIs, being 76.4, 64.0, 48.9, 37.2 and 22.5 mm. at 20, 40, 60, 80 and 100 mM., respectively. All tested antioxidants caused a

Table (1): Pathogenicity of the isolated fungi on pea plants (cv. Master B), pot experiment.

Treatments		% Damping off	
		Pre–emergence*	Post–emergence**
1	*Alternaria* sp.	10.0	3.3
2	*F.semitectum*	16.7	20.0
3	*P.ultimum*	16.7	13.3
4	*F. solani*	26.7	20.0
5	*R. solani*	20.0	23.3
6	Control	0.0	0.0

* Assessed 15 days after sowing, ** Assessed 30 days after sowing.

Table (2): Effect of culture filtrate of three *Bacillus* strains on the linear growth of *F. solani* and *R. solani*, five days after incubation at 28 ±2°C.

Bacillus strains	The pathogenic fungi	Average linear growth (mm) at concentration (%) 20	40	60	80	Mean	General mean
B. megaterium	*F. solani*	66.4	30.2	10.0	0.0	26.7	
	R. solani	68.6	33.0	13.8	0.0	28.9	27.8
B.subtilis	*F. solani*	79.8	49.0	29.0	9.2	39.3	
	R. solani	51.0	28.8	10.0	41.8	40.6	40.0
B.Thuringiensis	*F. solani*	74.8	41.4	28.0	9.0	38.3	
	R. solani	73.4	39.8	26.0	8.2	36.	37.6
	F. solani	90.0	90.0	90.0	90.0	90.0	
Control	*R. solani*	90.0	90.0	90.0	90.0	90.0	90
Mean	*F. solani*	77.8	52.7	38.5	27.1	49.0	-----
	R. solani	77.3	53.5	37.2	27.1	48.8	------
General mean		77.6	53.1	37.9	27.1	-----	------

L.S.D. at 5% for:
Bacillus strains (B)= 2.6, Fungi (F)= n.s, Concentration(C)=3.6 and B x C = n.s,
BxF= n.s, Bx C= 1.8, Fx c= 3.4.

Table (3): Effect of three CRIs on the linear growth of *F. solani* and *R. solani*, five days after incubation at 28 ±2°C.

Inducer resistance chemicals	The pathogenic fungi	Average linear growth (mm) at concentration of (mM) 20	40	60	80	100	Mean	General mean
Bion	*F. solani*	70.6	55.0	34.0	18.0	0.0	35.5	
	R. solani	68.4	53.2	33.6	16.8	0.0	34.4	35.0
Humic acid	*F. solani*	74.8	57.8	37.0	22.2	0.0	38.4	
	R. solani	73.2	58.0	36.6	21.4	0.0	37.8	38.1
Salicylic acid	*F. solani*	72.6	54.0	35.0	20.0	0.0	36.3	
	R. solani	71.4	53.8	34.6	18.4	0.0	35.7	36.0
Control	*F. solani*	90.0	90.0	90.0	90.0	90.0	90.0	
	R. solani	90.0	90.0	90.0	90.0	90.0	90.0	90.0
Mean	*F. solani*	77.0	64.1	49.0	37.6	22.5	50.0	----
	R. solani	75.8	63.8	48.7	36.7	22.5	49.5	----
General mean		76.4	64.0	48.9	37.2	22.5	----	----

L.S.D. at 5% for: CRIs (I)= 2.1, Fungi (F)= n.s, Concentration(C)=3.9, B x C = n.s, BxF= n.s, Bx C= 1.8, Ix Fx c= 3.5.

complete inhibition of the two tested fungi at 100 mM.. Bion was the most efficient inhibitor in this regard followed by Salicylic acid then Humic acid, being 35.0, 36.0 and 38.1 mm, respectively. Therefore, Bion was chosen to test its capability to the control of the tested two pathogens under greenhouse conditions.

No significant difference was found regarding the effect of the tested CRIs on *F. solani* and *R. solani* being 50.0 and 49.5 mm, respectively.

3.5. Effect of the bioagent, *B. megaterium* and the CRI, Bion on the incidence of pea damping-off and severity of root-rot under greenhouse conditions

Data presented in Table (4) show the effect of the bioagent *B. megaterium* and the CRI Bion on the incidence of pea damping-off and severity of root-rot under the greenhouse conditions. Results indicated that both Bion and *B. megaterium*, either each used alone or in combination, resulted in significant reduction to damping–off and root-rot severity caused by the two tested pathogenic fungi compared with pea plants grown in soil infested with any of the two tested pathogenic fungi. Moreover, the most efficient treatment in reducing damping- off and root-rot severity was the combination with Bion and *B. megaterium* in case of soil infested with any of *F. solani* and *R. solani* alone, being, 4% damping-off (for both fungi) and 2.5 and 3.0 % root-rot severity, respectively, without significant difference. No damping-off and root-rot severity were seen in case of pea plant grown from the treated seeds with any of Bion and *B. megaterium* and grown in uninfected soil with any of the two pathogenic fungi as well as control treatment (untreated seeds grown in uninfected soil). In addition, single treatment with any of Bion and *B. megaterium* was of low efficiency in reducing damping-off and root-rot severity than the combined treatment with Bion and *B. megaterium*, especially in case of soil infested with any of the tested fungi than that infested with both fungi.

Soil infested with both *F. solani* and *R. solani* recorded the highest percentages of pre and post emergence damping-off as well as and root-rot severity, being 40 and 24, 31.7%, compared with soil infested with each of them alone, being 32 and 20 and 27.8 % for *F. solani* and 36, 16 and 27.0 % for *R. solani*, respectively. The mortality of pre-emergence damping-off was significantly higher than post-emergence damping-off, being 14.7 and 10.4 %, respectively.

Table (4): Effect of the bioagent, *B. megaterium* (BM) and the CRI, Bion, each alone or in combination, on the incidence of pea damping-off and severity of root-rot caused by *F. solani* and *R. solani*, greenhouse experiment.

Treatments	% Damping-off		Mean	% Root-rot severity
	Pre-emergence	Post- emergence		
B. megaterium (BM)	0.0	0.0	0.0	0.0
Bion (B)	0.0	0.0	0.0	0.0
BM+ *F. solani*	12	12	12	7.1
BM+ *R. solani*	16	8	12	6.9
B+ *F. solani*	16	8	12	7.5
B+ *R. solani*	16	8	12	5.2
BM+B+ *F. solani*	4	4	4	2.5
BM+B+ *R. solani*	4	4	4	3.0
BM+*F. solani*+ *R. solani*	16	12	14	4.8
B+ *F. solani*+ *R. solani*	16	12	14	6.0
BM+B+ *F. solani* +*R. solani*	12	8	10	4.1
F. solani	32	20	27	27.8
R. solani	36	16	27	27.0
F. solani+ *R. solani*	40	24	32	31.7
Control (Uninfected soil)	0.0	0.0	0.0	0.0
Mean	14.7	10.4	-----	LSD at 5%= 3.7

LSD at 5 % for: Treatments (T)= 3.0, Disease severity (D) =2.1 and Tx D = 4.0.

3.6. Effect of the bioagent *B. megaterium* and the CRI Bion on the plant height and pod yield under greenhouse conditions

Results shown in Table (5) show that both Bion and *B. megaterium*, either each used alone or in combination, resulted in significant increase to the plant height, number of pods / plant and average weight of the green pods (g) / plant of pea plants grown in soil infested with any of the two tested pathogenic fungi or both compared with the plant grown in soil infested with any of the two tested pathogenic fungi or both and untreated by Bion and *B. megaterium*. Moreover, the most efficient treatment in increasing plant height, number of pods/ plant and the average of produced green pod yield (g) / plant was the combination with Bion and *B. megaterium* in case of soil infested with any of *F. solani* and *R. solani* alone, being 68.6 cm, 15.2 pod and 80.5 g for *F. solani* and 68.0 cm, 15.0 pod and 69.0 g. for *R. solani*, respectively. The highest figures of plant height, number of pod yield / plant and average weight of green pods (g) / plant was obtained from plants grown from seeds treated with Bion and grown in uninfected soil with any fungus, being 76.3 cm, 21.6 pod and 116.0 g. followed by that treated with *B. megaterium* and grown in uninfected soil with any fungus, being 74.8, 20.2 pod and 111.6 g, respectively. Meanwhile, untreated seeds with any of Bion and *B. maegaterium* and soil infested with any of the two tested fungi, either alone or in combination, recorded the lowest figures of plant height and pod yield.

3.7. Estimation of total phenolic compounds in bacterial, Bion and pathogen treated and untreated pea plants

Induction of defense-related biochemicals like total phenolic compounds was studied in bacterial, CRIs and pathogens-treated pea plants (Table, 6). It was noticed that *Bacillus* strains and CRIs induced considerable higher production of phenolic compounds compared with control treatment and both fungi. However, low change was observed total phenolic contents of untreated control (0.32, 0.33 and 0.36 mg gallic acid / g plant fresh roots after 15, 30 and 45 days, respectively). Meanwhile, the tested bioagents and CRIs caused considerable increase by lengthening the time of assessment of total content of phenolic compounds. Considerable increase was observed in the total phenolic compounds of plants treated with the tested CRIs than those treated with the bacterial bioagents.

Table (5): Effect of the bioagent, *B. megatewrium* (BM) and the CRI, Bion on plant height and the produced pod yield (g) / plant under greenhouse conditions.

Treatments	Plant height (cm)	Average no. of pods / plant	Average weight of pods (g) / plant
B. megaterium (BM)	74.8	20.2	111.6
Bion (B)	76.3	21.6	116.0
BM+ F. solani	62.9	12.0	71.1
BM+ R. solani	63.1	11.0	65.9
B+ *F. solani*	64.0	10.4	58.4
B+ *R. solani*	64.7	10.8	60.2
BM+B+ *F. solani*	68.0	15.2	80.5
BM+B+ R. solani	68.6	15.0	79.0
BM+ *F. solani*+ *R. solani*	65.5	11.0	65.2
B+ F. solani+ *R. solani*	66.8	11.5	67.0
BM+B+ *F. solani* +*R. solani*	70.0	11.0	66.4
F. solani	43.9	6.9	41.0
R. solani	41.0	7.0	41.8
F. solani+ *R. solani*	39.0	6.0	25.2
Control (Uninfected soil)	75.0	18.0	105.5
L.S.D. at 5 %	3.7	2.8	4.3

Table (6): Effect of *Bacillus* species, CRIs and pathogens-treated and untreated pea plants under different combinations on the total content of phenolic compounds, 15, 30 and 45 days after inoculation with bioagents and pathogen.

Treatments	Gallic acid in mg / g plant roots after (days)			Mean
	15	30	45	
B. megaterium	0.40	0.57	0.63	0.53
B. subtilis	0.40	0.56	0.61	0.52
B. thuringiensis	0.40	0.57	0.62	0.53
Bion	0.40	0.66	0.71	0.59
Humic acid	0.40	0.65	0.68	0.58
Salicylic acid	0.40	0.68	0.70	0.59
F. solani	0.40	0.53	0.63	0.52
R. solani	0.40	0.51	0.62	0.51
Control (Uninfected soil)	0.32	0.33	0.36	0.37
Mean	0.40	0.60	0.63	------

The increase in phenolic compound by the tested bioagents nearby and/or equal to those resulted from the pathogenic fungi. Both BTH and salicylic resulted in the highest total content of phenolic compounds, being 0.59mg gallic acid / g plant fresh roots followed by Humic acid, being 0.58. mg gallic acid / g plant fresh roots.

4. DISCUSSION

Plant disease management represent a major challenge that farmers are facing in the control of cropping systems. It is well known that *Fusarium solani* and *Rhizoctonia solani* are soil-borne fungal pathogen causing damping-off and root-rot diseases to many crops and generating compromised quality of crops and reducing yields.

A distinct broad-spectrum resistance response in the plant either of below- and above-ground parts could be induced by colonization of plant roots with selected strains of nonpathogenic bacteria, such as various species of the genus *Bacillus* (Kloepper *et al.,*2004. This type of resistance to diseases is named as induced systemic resistance (ISR) (van Loon, *et al.,* 1998; van Loon, 2007 and De Vleesschauwer *et al.,*2009). Both of *F. solani* and *R. solani* are the most dominant and virulent soil-borne plant pathogens and are widely distributed in various soil types worldwide. Recently, non-pathogenic bacteria have attracted the attention of many researchers due to their effectiveness as beneficial biological agents and disease resistance in a variety of crops. Application of some *Bacillus* strains to the soil and/ or seed coating has been found effective for suppressing soil-borne diseases and has successfully induced systemic resistance in the treated plants (Kloepper *et al.,* 2004 and Szczech and Shoda, 2007). Various species of *Bacillus* have been shown to exhibit (inducer systemic resistance) ISR activity. Elicitation of ISR by *Bacillus* strains has been demonstrated in greenhouse and field trials on many crops (Kloepper *et al.,* 2004).

Isolation trials from the rotten roots of pea roots collected from Giza governorate yielded five fungal isolates. The isolated fungi were purified and identified as *Alternaria sp*., *Fusarium semitectum, F. solani*, *Pythium ultimum* and *Rhizoctonia solani*.

The isolated fungi caused pre and post emergence damping–off to pea. The fungus *F. solani* caused the highest percentages of pre-and post-emergence damping-off followed by *R. solani* then *F. semitectum.* Meanwhile, *Alternaria* sp. caused the lowest figures of pre and post emergence damping-off followed by *P. ultimum.*

The isolated fungi were previously isolated from the roots of pea plants and proved their pathogenicity (Abada *et al.,*1992, Masoodi *et al.,*2000, Kraft and Pfleger,2001, Zue, 2003,

Hamid, 2012, Sharma-Poudyal *et al.*,2015 and Muhanna *et al.,* 2018)

The tested bioagents and the CRIs resulted in significant reduction to the linear growth of both *F. solani* and *R. solani* compared with control treatment. This reduction was gradually increased by increasing the incorporated concentration to PDA medium. In addition,,CRIs were less efficient in this regard than the bioagents.

The obtained results revealed that soaking pea seeds in 100 mM Bion for six hours then dressed with the bioagent, *B. megaterium* resulted in significant reduction to the incidence of damping-off and root-rot severity caused by both fungi with significant increase to the plant height (cm) and the produced green pods yield (g)/ plant compared with the control treatment.

Non-pathogenic rhizobacteria may activate inducible defense mechanisms in the plant in a similar manner to pathogenic microorganisms. Such mechanisms can include reinforcement of plant cell walls, production of anti-microbial phytoalexins, synthesis of pathogenesis related proteins (PRs) (Hammond-Kosack and Jones, 1996), as well as an enhanced capacity to express these defense responses upon challenge inoculation with a pathogen, a mechanism known as priming' (Conrath *et al.,* 2006). Activation of defense reactions suggests that even a beneficial rhizobacterium may be perceived by the plant as a potential threat, and that such perception involves the production of resistance-eliciting compounds that act mechanically similar to compounds produced by plant pathogenic fungi and bacteria.

Gram-positive *Bacillus* species, however, possess several advantages that make them good candidates for use as biological control agents. First, their antagonistic effect is caused by their ability to produce different types of antimicrobial compounds, such as antibiotics (*e.g.,* bacilysin, iturin, mycosubtilin) and siderophores (Shoda, 2000). Second, they are able to induce growth and defense responses in the host plant (Raupach and Kloepper, 1998). Furthermore, genus *Bacillus* is able to produce resistant spores to UV light and desiccation, which allows them to resist adverse environmental conditions, and permits easy formulation for commercial purposes (Raaijmakers *et al.,* 2002).

The tested CRIS are systemic acquired resistance elicitors, which reduces many fungal diseases by their exogenous applications as a systemic acquired resistance elicitor (Barilli *et al.,* 2015 and Abada *et. al*., 2018 and Attia *et al*.,2022). This protection is known to be related with the induction of the phenol pathway, but the particular metabolites involved have not been determined yet. This suggests fungal growth impairment by both direct toxic effect as well as plant cell wall reinforcement (Barilli *et al.,* 2015).

The stimulation of seed germination and the recovery from damping-off of that were caused by both the pathogenic fungi were apparent as a promotion of growth relative to appropriate control plants. However, in reality they were the result of disease suppression. Many bacteria in soil have similar properties (Compant *et al*., 2005), but in a number of cases rhizobacteria can enhance plant growth in the absence of potentially pathogenic microorganisms, as has been shown in *e.g.* gnotobiotic systems (van Loon and Bakker, 2003).

Treating pea seeds with the tested *Bacillus* strains and the antioxidant Bion resulted in increasing total phenolic compounds in the roots compared with untreated seeds and that treated with the fungicide Mon cut.

Many authors report increases in stress-related enzyme activities such as phenylalanine ammonia-lyase, peroxidase, polyphenoloxidase, ß-1,3-glucanase and chitinase, as well as induction of specific PRs in leaves of plants of which the roots were colonized by resistance-inducing PGPR (van Loon and Bakker, 2006).

Farkas and Kiraly (2008) declared that the participation of an endogenous supply of phenolic compound in the plant disease resistance is dependent upon active phenol oxidase system. Also, Lattanzio *et al.,* (2006) mentioned that pre-formed antibiotic compounds such as phenolic and polyphenolic compounds are ubiquitous in plants and play an important role in non-host resistance to filamentous fungi. The term "phytoanticipin" has been proposed to distinguish these preformed antifungal compounds from phytoalexins, which are synthesized from remote precursors in response to pathogen attack. Some antibiotic phenolics are stored in plant cells as inactive bound forms but are readily converted into biologically active antibiotics by plant hydrolyzing enzymes (glycosidases) in response to pathogen attack. These compounds can also be considered as preformed antibiotics since the plant enzymes

that activate them are already present but are separated from their substrates by compartmentalization, enabling rapid activation without a requirement for the transcription of new gene products (Osbourn, 1996). In such cases, free phenolics are likely to be much more toxic to the invading organism than the bound forms. In addition, even if preformed antifungal phenolics are present in healthy plants at levels that are anticipated to be antimicrobial, their levels may increase further in response to challenge by pathogens. Pit well known that phenolic content is the compounds whose quantity is raised when a plant comes under attack by a pathogen (Waterman and Mole, 1995). Systemic induction of phenolic compounds under influence of bacterial strains was first reported by van Peer *et al*., (1991). However, this alone is not reliable for indication of disease resistance in plant tissues (Waterman and Mole, 1995). Akram *et al.,* (2013) reported that a significant increase in total phenolic contents was observed in bacterial-treated plants. They added that pathogen alone was able to induce phenolic formation in plants but with slightly increased levels.

Antifungal phenolics are present in healthy plants at levels that are anticipated to be antimicrobial, their levels may increase further in response to challenge by exogenous factors, pathogens, bioagents, insects, elicitor ect. (Waterman and Mole, 1995 and Akram *et al.*, 2013). Melo *et al*. (2006) and Farkas and Kiraly (2008) mentioned that the participation of an endogenous supply of phenol compound in the plant disease resistance is dependent upon active phenol oxidase system.

One of the properties of antimicrobials is their production of phenolic compounds that could affect microorganisms. This is due to the ability of hydroxyl groups of these compounds to bind the active sites of key enzymes and modify the metabolism of microorganisms (Gyawali and Ibrahim, 2014). Antimicrobial activity depends on the position of the hydroxyl substitution in the aromatic ring, as well as on the length of the saturated side-chain (Cueva *et al.*,2010). For example, it has been demonstrated that caffeic acid possesses higher antimicrobial activity than *p*-coumaric acid because the first one has more hydroxyl groups substituted in the phenolic ring (Stojković *et al.*,2013).

Conclusions

In this research, the tested bioagents, *Bacillus* species and CRIs resulted in considerable decrease to the linear growth of both *F. solani* and *R. solani in vitro* in addition to pea damping-off and root-rot diseases *in vivo*. The non-pathogenic rhizobacteria, *B. megaterium* and the CRI, Bion activate inducible defense mechanisms against damping-off and root-rot diseases and this resulted in an increase in the produced green pods yield and phenolic compounds in pea roots.

Author contribution

The author did the conceptualization, methodology, software, validation, formal analysis investigation, resources, data curtain, writing the original draft preparation, writing, review, editing, supervision and funding acquisition. The author has read and agreed to the published version of the manuscript.

Competing interests

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the research reported in this manuscript.

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تأثير خلط أنواع من بكتريا الباسيلس مع المواد الكيميائية المحفزة للمقاومة على مكافحة أمراض سقوط البادرات وعفن الجذور في البازلاء

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ملخص

تسببت الفطريات المعزولة فيوزاريوم سولاني وفيوزاريوم سيمتيكتم وريزوكتونيا سولاني والفطر ألترناريا وشبيه الفطر بيثيوم أولتيمم، في حدوث الإصابة بمرض سقوط بادرات البازلاء قبل وبعد ظهورها فوق سطح التربة (موت البادرات المفاجئ). وعند اختبار المرضية، لوحظ أن الفطر فيوزاريوم سولاني سبب أعلى نسبة إصابة بالموت المفاجئ قبل وبعد ظهورالبادرات، يليه الفطر ريزوكتونيا سولاني، والفطر فيوزاريوم سيمتيكتم بينما تسبب الفطر ألترناريا في أقل نسبة إصابة بالمرض يليه البيثيوم أولتيممو. اختبر التأثير المثبط للمعاملة بأنواع من البكتريا المضادة مثل بكتيريا باسيلس ميجاتيريوم، وباسيللس ساتيليس وباسيلس ثيوريجينسيس بالإضافة إلى المعاملة بالمواد الكيميائية المحفزة للمقاومة" (CRIs) وحمض الهيوميك وحمض الساليسيليك، وذلك بغرض تحديد تأثيرها التثبيطي ضد فطريات الفيوزاريوم والريزوكتونيا المسببين لأمراض سقوط بادرات البازلاء وعفن الجذور في المعمل والصوبة.

وقد أظهرت النتائج أن العوامل الحيوية المختبرة والمواد الكيميائية المحفزة للمقاومة أدت إلى انخفاض كبير في النمو الخطي لكل من فطريات الفيوزاريوم والريزوكتونيا بالمقارنة مع معاملة الكونترول. وزاد هذا الانخفاض تدريجياً بزيادة التركيز في بيئة البطاطس أجار دكستروز (PDA) بالإضافة إلى ذلك، كان مركب بيون أقل كفاءة في هذا الصدد من العوامل الحيوية البكتيرية المضادة.

وقد أظهرت النتائج أن نقع بذور البازلاء في 100 ملي مول من مادة "بي تي إتش (BTH) " لمدة ست ساعات ثم عدواها بمعلق من البكتيريا باسيللس ميجاتيريوم أدى إلى تقليل ملحوظ في شدة المرضية لسقوط البادرات وعفن الجذور في النباتات المعداة بالفطريات مع زيادة معنوية في طول النبات وإنتاجية القرون الخضراء مقارنة بمعاملة المقارنة (الكونترول). علاوة على ذلك، فإن المعاملة بخليط بيون مع البكتيريا كانت الأكثر كفاءة حيث أدت إلى تقليل شدة الإصابة بسقوط البادرات وتعفن الجذور وكذلك زيادة طول كل نبات، وعدد القرون / نبات ومتوسط وزن القرون الخضراء (جم) / نبات. كما أدت معالجة بذور البازلاء بسلالات الباسيللس المختبرة وبالمواد الكيميائية المحفزة للمقاومة " آي آر سي" بيون إلى زيادة الفينولات الكلية في جذور نباتات البازلاء مقارنة بنباتات البذور غير المعاملة . وتوصي الدراسة بمعاملة بذور تقاوي البسلة بخليط من بيون مع البكتيريا باسيلس ميجاتيريوم لحماية جذور وبادرات البسلة وتحسين صفات النبات.