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Effect of the Combination Between Bioagents and Benzothiadiazole (BTH) on Management of Uromyces Pisi the Causal of Pea Rust

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Abstract: Antagonistic bioagents naturally occurring on pea leaves free from rust infection were isolated and evaluated for their antagonism against *Uromyces pisi*, the causal of rust. Isolates of both *Bacillus* spp., *i.e. Bacillus chitinosporus, B. megaterium, B. thuringiensis* and *B. subtilis* and *Trichoderma* spp., *i.e. Trichoderma album, T. hamatum, T. harzianum* and *T. viride* were selected, purified and identified The inhibitory effect of these isolates was assessed *in vitro* on the germination of the urediospores of the causal fungus. The inhibitory effect of *Bacillus* spp. ranged between 31.9-42.4% and *Trichoderma* spp. between 34.9-53.5%. In addition, B. thuringiensis recorded the highest inhibition to the urediospores of the causal fungus followed by *B. megaterium* then *B. subtilis* and *B. chitinosporus*. Meanwhile, *T. viride* gave the highest inhibition followed by *T. harzianum* then *T. hamatum* and *T. album*. The tested antioxidant, *i.e.* bion (BTH), chitosan and salicylic acid caused significant reduction to the germinated urediospores of *U. pisi* compared with the control. This reduction was gradually increased by increasing the concentration. In addition, BTH was the most efficient one in this regard. Under greenhouse conditions spraying of pea plants with any of *Bacillus* spp. and *Trichoderma* spp., 48 h. before inoculation with *U. pisi* on the grown plants from seeds soaked or not in 20 mM of BTH significantly reduced the severity of the disease in the range of 4.0 – 5.4, 12.0-15.8%, respectively compared with the control (48.7%). Soaking pea seeds in BTH before sowing was best method than un-soaked seeds in BTH for managing the disease. The fungicide Topas was the superior treatment followed by *B. thuringiensis* then *T. viride* in reducing rust severity and increasing the number of the produced green pods and their weight / plant compared with control. All the tested bioagents, BTH and the fungicide Topas resulted in considerable increase to sugars and phenol contents of pea leaves compared with the control., BTH was always more effective more than the tested bioagents and the fungicide Topas in this regard. Total nitrogen, the concentration of the total free amino acids and the percentages of crude protein in the seeds of Master B pea cv. were greatly increased due to spraying the tested bioagents, BTH and the fungicide Topas compared with the control. BTH was the superior treatment in increasing these components followed by the tested bioagents then the fungicide Topas. In addition, *B. thuringiensis* and *T. viride* were the best bioagents in increasing of these components.

Keywords: Pea, Antioxidants, *Bacillus* spp., *Trichoderma* spp., Biological Control, Topas, *Uromyces pisi*, Sugars, Phenol Compounds, Total Nitrogen, Total Amino Acids, Crude Protein

1. Introduction

Pea (*Pisium 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 compounds. It improves the soil fertility through nitrogen

fixation.

Pea is liable to be attack by many bacterial, fungal, viral, nematode diseases in addition to physiological disorder. However, fungal diseases, especially rust is considered one of the major destructive diseases affecting the crop yield (Hagedron, 1984 and Kraft, and Pfleger, 2001), especially in the north and middle parts of the Delta in Egypt and several countries in the world (Abada *et al.,* 1997; Gupta and Shayam, 1998 and Parilli *et al*., 2015).

The fungus *Uromyces pisi* is a heteroecious rust pathogen, completing its life cycle on two host plant species. The sexual stages are completed on *Euphorbia cyparissias* (cypress spurge), while the asexual lifecycle stages are completed on leguminous crop hosts such as *Lathyrus, Orobus, Pisum* and *Vicia* spp. *E. cyparissias* is an erect, branching, rhizomatous perennial, which typically grows to 30 cm tall. It occurs on poor and mainly dry soils, along forest edges, and roadsides. Numerous tiny flowers appear in umbel-like clusters in spring. The asexual stage commences with the release of aeciospores produced by *U. pisi* on *E. cyparissias*, which are wind dispersed and infect field pea crops. Infection by aeciospores results in the production of uredinia and subsequent urediniospores. As the host plant matures telia are produced resulting in the formation of teliospores. This leads to the formation of basidiospores, which are windborne and infect *E. cyparissi*. The sexual stage occurs on the alternate host *E. cyparissias*. The rust fungus remains latent during the winter in the roots of *E. cyparissias*, and grows with the host as it shoots in the spring. Infection of Euphorbia is restricted to the underground rhizome buds and requires an incubation period of 1-year (Parilli *et al.*, 2015). The infected host plants develop earlier in the season and are inhibited from flowering. The host plant is induced by the fungus to form pseudoflowers; yellow leaves that grow in a rosette on the top of stems and resemble true flowers in colour and shape (Pfunder and Roy, 2000). In addition, sweet smelling nectar is produced by the fungus on the surface of the yellow leaves, giving the appearance of a true flower. The nectar contains fungal gametes (spermatia) that are transferred by nectar feeding insects (including bees and ants) from one fungal mating type to another. Once fertilization has occurred, aeciospores are released which infect leguminous host plants including field peas.

Managing plant diseases with fungicides sometimes gives good results. However, improper use of fungicides leads mostly to environmental pollution, disasters throughout the world and the phenomena of resistance to the causal pathogens (Brewer and Larkin, 2005). Therefore, to overcome these difficulties, it is urgent to apply alternative safe efficient methods against such disease or at least rationalization their application.

Biological control is considered an important approach of agricultural biotechnology in recent years for controlling many fungal plant pathogens. Both *Bacillus* and *Trichoderma* spp. are the most promising and effective bioagents against various plant pathogenic fungi (Deshmukh *et al.,* 2010; Barakat *et al.,* 2014 and Ragab *et al.*, 2015). Trichoderma as antagonist is much more complex, that is nutrient competition, hyperparasitism, antibiosis, space and cell wall degrading enzymes (Abd-El-Khair *et al.*, 2010 and Junid *et al.,* 2013).

It was also found that there is a large variety of volatile secondary metabolites produced by *Trichoderma* spp. such as ethylene, carbon dioxide, hydrogen cyanide, aldehydes and ketones which play an important role in controlling many plant pathogens (Heydari and Pessarakli, 2010; Nagendra and Kumar, 2011; Zaher *et al.,* 2013; Abada and Ahmed, 2014; Barakat *et al.,* 2014; Bhattacharjee and Dey, 2014 and Ragab *et al.,* 2015).

Biological control using antagonistic bacteria has been reported as an attractive alternative due to their ability to antagonize the pathogen by different modes of action, and to effectively colonize distinct plant habitats (Raaijmakers *et al.,* 2002). Most attention has been focused on the use of gram-positive *Bacillus* species, however, possess several advantages that make them good candidates for use as biological control agents (BCA). First, their antagonistic effect is caused by their ability to produce different types of antimicrobial compounds, such as antibiotics (e.g., bacilysin, iturin, mycosubtilin) (Shoda, 2000). Second, they are able to induce growth and defense responses in the host plant. Furthermore, *Bacillus* spp. are able to produce spores resistant to UV light and desiccation, which allows them to resist adverse environmental conditions, and permits easy formulation for commercial purposes (Raaijmakers *et al.,* 2002 and Bhattacharjee and Dey, 2014).

The aim of this work is to evaluated the efficiency of some bacterial and fungal bioagents as well as antioxidants on the germination of the urediospores of *U. pisi in vitro*. Also, management of pea rust with *B. megaterium, B. thuringiensis, T. harzianum* and *T. viride* in combination with BTH under greenhouse conditions. Furthermore, to assess the effect of these treatments on the sugars and phenol compounds content as well as total nitrogen, the concentration of total free amino acids and crude protein.

2. Materials and Methods

2.1. Plant Materials

Pea seeds cv. Master B were obtained from Legume Crops Res. Dept., Agric. Res. Cent., Giza, Egypt.

2.2. The Fungal Pathogen

Pea leaves bearing the uredial sori of an isolate of *Uromyces pisi* was frequently collected from Dakahlia governorate, which was used throughout this study.

2.3. Isolation, Purification and Identification of the Antagonists

Microorganisms naturally occurred on pea leaves surface were isolated from the phylloplane of healthy plants,

collected from Dakahlia governorate using dilution plate technique. Serial dilution plate technique was used to isolate native antagonistic *Trichoderma* spp. on PDA medium and *Bacillus* spp. on nutrient agar medium (Oedjijono and Dragar, 1993).

All the fungal cultures of *Trichoderma* spp. were isolated and purified by hyphal tip method and then identified on the basis of cultural and microscopic morphological characters (Rifia, 1969 and Bissctt, 1991).

Also, the isolated *Bacillus* spp. were purified and identified using the description of Parry *et al.* (1983) and Holt and Krieg (1984).

2.4. Effect of the Tested Bioagents and Antioxidants on Urediospores Germination

The antagonistic effect of the isolated bioagents on the germination of the urediospores of *U. pisi* was assessed in vitro. Flasks (250 ml.) containing nutrient medium were inoculated with loops of the culture of any of the tested bacteria and incubated at 28±2 for 48 h. to grow. The bacterial suspension was adjusted to contain $1x10^2$, $1x10^4$ and $1x10^6$ cfu /ml. Also, *Trichoderma* spp. were grown on gliotoxin fermentation medium (GFM) as described by Brain and Hemming (1945) for 7 days. 20 ml. of sterile water were added to each Petri-dish and growth (spores and mycelium) was gently crushed by sterilized camel brush and collected in sterile 500 ml conical flask. The collected growth was filter through 3 layer of cheesecloth and the filtrate was adjusted to contain $1x10^2$, 10^4 and 10^6 conidia using a haemocytometer.

The effect of different antioxidants on the germinated urediospores of *U. pisi* was carried out *in vitro*. The concentrations of 2, 5**,** 10 and 20 mM of the antioxidants**,** *i.e.* bion benzothiadiazole (BTH), chitosan (cellulose with the hydroxyl at position C_2 substituted with an acetamido group) and salicylic acid (monohydroxybenzoic acid) were prepared depending on their molecular weight.

Freshly urediospores of the pathogen were added to each concentration of the tested bacterial and fungal bioagents as well as antioxidants. One m1. of uredial suspension was placed on each sterilized slide, borne on two glass rods in a sterilized Petri-dish (two slides in each Petri-dish) containing a piece of wetted cotton by sterilized distilled water to provide high relative humidity. The same was made for a spore suspension put in distilled sterilized water only as control treatment. Preparations were incubated in darkness at 25±1°C for 24 hour. Four Petri dishes for each treatment were used as replicates. The percentages of uredial germination were counted in a total of 100 urediospore. The germinated uredia were counted and mean of percentages of germination was calculated and recorded for each treatment.

2.5. Greenhouse Experiment

Antifungal activity of the four species of both *Bacillus* (*B. chitinosporus, B. megaterium, B. subtilis* and *B. thuringicensis*) and *Trichoderma* genera (*T. album, T. hamatum, T. harzianum,* and *T. viride*) as well as BTH were evaluated for their efficiency in controlling pea rust caused by *U. pisi* in pots under artificial inoculation conditions in comparison with the fungicide Topas (tubaconazole).

Pea seeds (cv. Master B) were divided into two groups. The first on was soaked in 20 mM BTH for six hours before sowing and the second one was soaked in water only for the same time.

Seven pea seeds were sown in each plastic pot (30 cm in diameter) containing formalin sterilized silt soil. The emerged seedlings were thinned into five plants in each pot, 10 days after sowing. Ten replicates of 40 days old plants (mid of April, 2016) for each treatment were sprayed with any of the tested bioagents, i.e. Bacillus spp.($1x10^6$ cfu / ml water) and *Trichoderma* spp. ($1x10^6$ spore / ml water) three sprays; the first was two days before inoculation with the urediospores suspension of the causal fungus, the second 10 days after the first spray and the third 10 days after the second spray. The fungicide Topas was also sprayed as check three times also. Control plants were sprayed with urediospores suspension of *U. pisi* only and sprayed with water only. Few drops (0.5 ml/ l preparation) from Tween 20 were added to the sprayed bioagents and the fungicide as adherent material. All pots were covered with polyethylene bags for 48 h as a moist chamber at 18-25°C in the greenhouse. The plants received all the recommended agriculture practices.

Disease severity was recorded using the devised scale (0-9) proposed by Mayee and Datar (1986). Also, the average number of pods and weight of green pods / plant were assessed.

2.6. Disease Assessment

The artificially infected plants were carefully examined to estimate the severity of the infection by rust depending on the devised scale (0-9) by Mayee and Datar (1986) using the following formula:

$$\% \text{ Disease severity} = \frac{£(n \times v)}{9N} x100$$

Where:

n = Number of infected leaves in each category.

v = Numerical values of each category.

N = Total number of the infected leaves.

(Table, 1) shows rating scale used for scoring pea rust (severity according to Mayee and Datar, 1986).

Table 1. *Scale ranging, content six degrees from 0-9.*

Category	Disease severity description
0	No symptoms on leaf.
1	Rust pustules small, scattered covering 1% or less of leaf area.
3	Rust pustules more in number covering 1-10% of leaf area.
5	Typical rust pustules covering 11-25% of leaf area.
7	Typical rust pustules covering 26-50% of leaf area. Leaf shedding.
9	Typical rust pustules covering 51% or more of leaf area and defoliation severe.

2.7. Biochemical Changes Associated with the Infection by Pea Rust and the Treatment with the Tested Bioagents and BTH

2.7.1. Sugars and Phenol Compounds in the Treated Pea Leaves

Fresh plant sample (10 g) from each treatment was cut into small pieces and immediately macerated into 95% boiling ethanol for 10 min. The macerated were transferred into Soxhlet unites containing 75% ethanol as an extraction solvent. The extract process resumed for 12 hrs. Ethanol extracts were filtrated and evaporated until the complete removal of ethanol. The dried residue was dissolved in 5ml isopropanol 50% and kept in freezer till analysis. The extracts were used, later for analysis of sugars and phenols.

Reducing, non-reducing and total were spectrophotometeric determined at 540 nm using the picric acid technique as described by (Thomas and Dutcher, 1924) as follows.

A volume of 0.5ml of each extract was placed in test tubes; containing 5ml of distilled water and 4ml picric solution were added. The mixture was boiled for 10 min. After cooling, 1ml sodium carbonate solution 20% was added and the mixture was boiled again for 15 min. After it was cooled, the tubes were completed to 10 ml with distilled water. Thereafter, the density of developed color was determined at 540 nm using spectrophotometer (spectronic 106) in presence of blank and using glucose as a standard.

The content of non-reducing sugars was calculated as the difference between the total sugars and reducing sugars.

Determination of total phenol compounds was carried out as described by (Simons and Ross, 1971). Concentrate hydrochloric acid (0.25 ml) was added to 0.2 ml of the sample extract in test tube and mixed. The mixture was then boiled for about 10min. After cooling, 1ml Folin reagent and 5ml sodium carbonate solution (20%) were added and diluted to 10 ml using distilled water. After 30 min the density of the developed blue color was determined at 520 nm using chatichole as standard. Phenol compounds were calculated as milligrams equivalent of catechol /g fresh weight (Mayer *et al*., 65).

Free phenols determination was carried out using the same described method with some exception, since, 1ml Folin reagent and 3ml sodium carbonate solution (20%) were added to 0.2 ml of the sample extract, diluted with distilled water to 10 ml. After 30 min, the density of the developed blue color was determined at same wavelength.

2.7.2. Determination Total Nitrogen, Total Amino Acids and Crude Protein Constitutes of Pea Dry Seeds

Pea seeds (20 g) were taken, dried in an electric oven at 70° till constant weight and ground. Samples were extracted according to Goldschmidt *et al.*, (1968). For determination of total nitrogen calorimetrically by using orange G dye method according to Hafez and Mikkelsen (1981). Crude protein of seeds was calculated by multiplying total N% × 6.25.

Total free amino acids were determined according to Moore and Stein (1954). Free amino acids were calculated as milligrams equivalent of argenin /g fresh weight.

2.8. Statistical Analysis

Data were statistically analyzed using the standard procedures for 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. Inhibitory Effect of the Tested Bioagents and Antioxidants on the Germinated Urediospores of U. pisi

The inhibitory effect of the antagonistic bioagents against the germinated urediospores of *U. pisi in vitro* are shown in Tables (2 and 3). All the tested bioagents decreased the germinated urediospores of *U. pisi* compared with the control. This decrease was gradually decreased by increasing the concentration of the cfu and spore suspension.

Table (2) indicates that *B. thuringicensis* was the most efficient in this regard followed by *B. megaterium* then *B. subtilis* and *B. chitinosporus,* being 31.9, 35.5, 36.7 and 42.4% germination and 74.7, 62.2, 59.4 and 53.1% efficacy, respectively

Table 2. *Effect of different antagonistic Bacillus strains on the germination of U. pisi urediospores, 24 h after incubation at 25±2°C.*

Bioagents	% Uredial germination at 1x10* (cfu)			Mean	%, Efficacy
	2	4	6		
B. chitinosporus	78.4	42.2	16.6	42.4	53.1
B. megaterium	67.4	33.0	0.0	33.5	62.2
B. subtilis	73.8	36.4	0.0	36.7	59.4
B. thuringiensis	65.0	30.6	0.0	31.9	74.7
Control	90.4	90.4	90.4	90.4	------
Mean	76.9	49.9	26.8	-------	--------

* The initial percentage of urediospores germination was 1.4%.
L. S. D. at 5% for:
Bioagents (B) = 2.3.Conc. (C) = 3.2, B x C = 4.

Data presented in Table (3) reveal that the fungus *T. viride* gave the highest effect on reducing the germinated urediospores followed by *T. harzianum* then *T. hamatum* and *T, album*., being 34.9, 42.5, 51.9 and 53.5% germination, and 61.4, 53.0, 42.9 and 40.9% efficacy respectively. Control treatment recorded 90.4% germination. Control treatment recorded 90.4% germination.

Results shown in Table (4) show that the tested antioxidants, *i.e.* bion , chitosan and salicylic acid resulted in significant reduction to the germinated urediospores of the causal fungus, being 31.9 ,43.4 and 41.2% with efficacy of 65.5 ,53.5 and 55.6 and %, respectively. Control treatment recorded 91.2% germination. This reduction was gradually increased by increasing the concentration.

Therefore, both *B. thuringicensis* and *B. megaterium* strains and the two strains of. *T. viride* and *T. harzianum* in addition to BTH were tested for their efficiency on managing pea rust under greenhouse conditions.

Table 3. *Effect of different antagonistic Trichoderma strains on the germinated urediospores of U. pisi, 24 h after incubation at 25±2°C.*

Bioagents	% Uredial germination at 1x10*(spore)			Mean	% Efficacy
	2	4	6		
T. album	81.4	54.8	24.2	53.5	40.9
T. hamatum	80.6	53.0	22.0	51.9	42.9
T. harzianum	76.8	40.4	10.2	42.5	53.0
T. viride	70.0	34.8	0.0	34.9	61.4
Control	90.4	90.4	90.4	90.4	------
Mean	80.0	54.7	27.3	------	------

* The initial percentage of urediospores germination was 1.4%.
L. S. D. at 5% for:
Bioagents (B) = 2.0, Conc. (C) = 3.1, B x C = 4.2

Table 4. *Effect of different antioxidants on the germinated urediospores of U. pisi, 24 h after incubation at 25±2°C.*

Antioxidants	% Uredial germination at (mM)				Mean	%. Efficacy
	2	5	10	20		
Bion	76.8	40.4	10.2	0.0	31.9	65.5
Chitosan	81.4	54.8	24.2	13.0	43.4	53.5
Salicylic acid	80.0	53.0	22.0	9.8	41.0	55.6
Control	92.4	92.4	92.4	92.4	92.4	-----
Mean	82.7	60.4	37.2	28.8	------	----

* The initial percentage of urediospores germination was 1.8%.
L. S. D. at 5% for:
Antioxidants (A) = 2.9, Conc. (C) = 3.4, A x C = 3.8

3.2. Greenhouse Experiment

3.2.1. Effect of Some Antagonists and BTH on the Severity of Pea Rust and the Produced Green Pods Under Green- House Conditions

Spraying of pea plants either grown from soaked seeds or not in BTH with any of the tested antagonists of *Bacillus* spp., *i.e. B. megaterium* and *B. thuringiensis* and *Trichoderma* spp., i.*e. T. harazianum* and *T. viride* as well as those sprayed with the fungicide Topas two days before inoculation with *U. pisi* significantly reduced rust severity under greenhouse conditions (Table, 5) compared with the control.

Table 5. *Effect of spraying of some antagonistic biagents on pea plants grown from soaked seeds in BTH or not on the severity of pea rust (cv. Master B), under greenhouse conditions.*

Treatments	% Disease severity of plants		Mean
	Soaked in BTH	Soaked in water	
B. megaterium	4.8	14.6	9.7
B. thuringiensis	4.0	12.0	8.0
T. harzianum	5.4	16.5	11.0
T. viride	5.0	15.8	10.4
Topas	1.3	4.7	3.0
Control	21.0	40.8	30.9
Mean	6.9	17.4	------

L. S. D. at 5% for:
Treatments(T) = 2.8, Soaking (S) = 3.4, T x S = 3.7

In general, treatments of soaked pea seeds in the BTH were more efficient in managing the disease than those soaked in water only, 6.9 and 17.4%, respectively. The severity of the disease after the treatment with the tested bioagents was 7.9, 8.0, 11.0 and 10.4%, on the average respectively. Plants sprayed with the fungicide Topas recorded 3.0% disease severity on the average. Control plants recorded 30.9% disease severity on the average.

Therefore, *B. thuringicensis*, T. viride and BTH were tested for their efficiency in managing pea rust under the field conditions.

Table (6) shows pea plants either grown from soaked seeds or not in BTH with any of the tested antagonists of *Bacillus* spp., *i.e. B. megaterium* and *B. thuringiensis* and *Trichoderma* spp*., i.e. T. harazianum* and *T. viride* as well as those sprayed with the fungicide Topas two days before inoculation with *U. pisi* significantly increased the number of the produced green pods and their weight compared with the control treatment. Pea seeds soaked in the BTH were more efficient in producing high number and weight of green pods than those soaked in water only, being 15.2 and11.7 pod and 82.0 and 67.5 g./ plant, respectively. In addition, the fungicide Topas resulted in yielding the highest pod yield followed by *B. thuringiensis*, being 17.4 pod and 82.3 g./ plant and 14.0 pod and 79.2 g./ plant, on the average., respectively. Control plants produced poor yield, being 8.0 and 47.1 g./ plant, on the average.

Table 6. *Effect of spraying the tested antagonistic bioagents on pea plants grown from soaked seeds in BTH or not on the produced green pod yield (cv. Master B) under greenhouse conditions.*

Treatments	Average number of green pod yield / plant of plants		Mean	Average weight of green pod yield (g) / plants of plants		Mean
	Soaked in BTH	Soaked in water		Soaked in BTH	Soaked in water	
B. megaterium	15.8	11.6	13.7	85.7	69.5	77.6
B. thuringiensis	16.0	12.0	14.0	87.4	71.0	79.2
T. harzianum	15.4	11.5	13.5	87.1	70.0	78.6
T. viride	16.0	12.8	13.4	87.8	70.4	79.1
Topas	19.0	15.7	17.4	91.2	73.3	82.3
Control	9.0	7.0	8.0	53.4	40.8	47.1
Mean	15.2	11.7	----	82.0	67.5	----

L. S. D. at 5% for:
Treatments(T) = 3.1 2.9
Soaking (S) = 3.8 3.3
T x S = 3.3 4.5

3.4.1. Determination of Total Nitrogen, Total Amino Acids and Crude Protein Constitutes of Pea Dry Seeds

Data shown in Table (7) reveal that the percentages of total nitrogen, the concentration of total amino acids and the percentage of crude protein in the seeds of cv. Master B pea were greatly increased compared with the control. BTH was the superior treatment in increasing these components, being 5.98%, 0.232 mg/ g. dry weight and 36.46%, followed by *B. thuringiensis*, being 5.78%, 0.218 mg/ g. dry weight and 35.27% respectively compared. In addition, both *B. thuringiensis* and *T. viride* were the best bioagents in increasing of these components than both *T. harzianum* and *T. viride*. The percentages of total nitrogen total amino acids and the percentage of the crude protein in the seeds of the control treatment recorded 4.70%, 1.45 mg/ g dry weight and 30.41%, respectively.

3.4.2. Sugars (Reducing, Non-reducing and Total Sugars) Phenol Compounds (Free and Total Phenols)

The estimated values of reducing, non-reducing and total sugars as well as free and total phenols in pea leaves due to the infection by rust and the treatment with the tested bioagents and BTH are shown in Table (8). All the tested bioagents, BTH and the fungicide Topas resulted in considerable increase to these components compared with the control. However, BTH was always more effective more than the tested bioagents and the fungicide Topas.

Table 8. *Effect of the tested bioagents, BTH and the fungicide Topas on pea leaves content of sugars and phenol compounds.*

Treatments	Sugars			Phenols	
	Reducing	Non-Reducing	Total	Free	Total
B. megaterium	0.424	0.173	0.597	23.37	26.67
B. thuringiensis	0.430	0.177	0.607	23.43	26.78
T. harzianum	0.414	0.166	0.580	23.25	26.62
T. viride	0.426	0.171	0.597	23.39	27.35
BTH	0.435	0.183	0.618	23.86	27.35
Topas	0.398	0.161	0.559	22.43	23.21
Control	0.293	0.141	0.434	17.88	18.39

Table 7. *Effect of the tested bioagents, BTH and the fungicide Topas on total nitrogen, total amino acids and crude protein of pea dry seeds the tested bioagents and the fungicide Topas.*

Treatments	%, Total nitrogen	Total free amino acids (mg/g. dry weight)	%, Crude protein
B. megaterium	5.71	0.215	35.24
B. thuringiensis	5.78	0.218	35.47
T. harzianum	5.55	0.210	35.34
T. viride	5.62	0.212	35.28
BTH	5.98	0.232	36.46
Topas	5.40	0.205	35.05
Control	4.70	0.145	30.41

4. Discussion

Many microorganisms play an important role in the management of some plant diseases. The obtained data revealed that there was a promising antagonistic species of bacteria and fungi prevalent on pea leaves, which could be exploited for the control of pea rust. The genera of *Bacillus* and *Trichoderma* comprise a great number of bacterial and fungal strains that act as bioagents (Shoda, 2000; Junid *et al.,* 2013 and Bhattacharjee and Dey, 2014). All the tested antagonistic bacteria and fungi as well as the antioxidants decreased the germinated urediospores of *U. pisi*. The antagonistic isolates of *Trichoderma* spp. overcome and inhibited the infection by *U. pisi*.

The tested antioxidants*, i.e.* bion (BTH), chitosan and salicylic acid resulted in significant reduction to the germinated urediospores of *U. pisi* compared with the control. This reduction was gradually increased by increasing the concentration. In addition, BTH was the most efficient one in this regard.

Results indicated that, spraying of plants two days before

inoculation with the tested pathogen with any of the tested *Bacillus* spp. and *Trichoderma* spp. on pea plants grown from pea seeds soaked in BTH or not significantly reduced rust severity compared with the control. Both *B. thuringiensis* and *T. viride* were the highest antagonistic isolates followed by *B. subtilis* and *T. harzianum.* This may be due to an effect on germ-tube elongation and to a lesser extension of germination rate (Zimand *et al*., 1996 and Junid *et al.,* 2013).

Trichoderma spp. are known to control pathogens either indirectly by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and enhancing plant defensive mechanisms and antibiosis, or directly by inhibition of growth and sporulation of the pathogen mechanisms such as mycoparasitism and enzyme production (Zimand *et al.,* 1996; Bouhassan *et al.,* 2004 and Junid *et al.,* 2013).

Biological control has emerged as an alternative and most promising means of the management of plant pathogens. The earlier studies revealed that antimicrobial metabolites produced by *B. subtilis* and *Trichoderma* spp. are effective against a wide range of phytopathogenic fungi (Svetlana *et al*., 2010; Junid *et al.,* 2013; Zaher *et al.,* 2013; Barakat *et al.,* 2014 and Abo-Shosha, 2016).

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The earlier studies also revealed that antimicrobial metabolites produced by *Trichoderma* spp. are effective against a wide range of phytopathogenic fungi (Svetlana *et al*., 2010; Junid *et al.*, 2013; Zaher *et al.,* 2013 and Ragab *et al.,* 2015).

The obtained results showed that *B. thuringiensis* resulted in the maximum inhibition to the germinated urediospores of the causal fungus followed by *T. harzianum* compared to the control. The inhibitory activity of the tested Trichoderma bioagents on the development of germ tube of the pathogen could be explained by the ability of *Trichoderma* spp. to produce volatile substances that are able to limit and even stop the development of the pathogen. Also it is found that there is large variety of volatile secondary metabolites produced by Trichoderma strains such as ethylene, carbon dioxide, hydrogen cyanide, aldehydes and ketones, which play an important role in controlling the plant pathogens (Vey *et al.,* 2001; Nagendra and Kumar, 2011; Junid *et al.,* 2013 and Bhattacharjee and Dey, 2014).

However, gram-positive *Bacillus* species possess several advantages that make them good candidates for use as biological control agents (BCA). 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 and Bhattacharjee and Dey, 2014). Second, they are able to induce growth and defense responses in the host plant (Raupach and Kloepper, 1998). Furthermore, Bacillus is able to produce spores resistant to UV light and desiccation, which allows them to resist adverse environmental conditions, and permits easy formulation for commercial purposes (Raaijmakers *et al.*, 2002 and Bhattacharjee and Dey, 2014).

Barilli *et al.* (2015) mentioned that BTH is a systemic acquired resistance elicitor, which reduces rust penetration in pea through phytoalexins pathway. It has been previously shown that pea rust infection can be reduced by exogenous applications of systemic acquired resistance elicitors such as BTH. This protection is known to be related with the induction of the phenol pathway but the particular metabolites involved have not been determined yet. They added that following BTH treatment, it was observed an increase in scopoletin, pisatin and medicarpin contents in all, excreted, soluble and cell wall-bound fraction. This suggests fungal growth impairment by both direct toxic effect as well as plant cell wall reinforcement. Also, the response mediated by BTH was genotype-dependent, since coumarin accumulation was observed only in the resistant genotype. In addition, exogenous application to the leaves of scopoletin, medicarpin and pisatin lead to a reduction of the different fungal growth stages, confirming a role for these phytoalexins in BTH-induced resistance against *U. pisi* hampering pre-and post-penetration fungal stages.

Farkas and Kiraly (1967) and Morkunas and Gemerek (2007) reported that peroxidase enzyme oxidizes the phenolics to more fungal toxic compounds such as quinines, which inhibit both spore germination and fungal growth. Also, peroxidase was found to be participate in the synthesis of lignin. Moreover, Melo *et al*. (2006) declared that the participation of an endogenous supply of phenol compound in the plant disease resistance is dependent upon active phenol oxidase system.

The percentage total nitrogen (N), the concentration of total amino acids and crude protein in the seeds of cv. Master B pea were greatly increased compared with the control. BTH was the superior treatment in increasing these components, being 5.96%, 0.219 mg/ g. dry weight and 36.26%, followed by the fungicide Topas, being 5.90%, 0.211 mg/ g. dry weight and 36.15% respectively compared with tested bacterial and fungal bioagents. In addition, *B. thuringiensis* and *T. viride* were the best bioagents in increasing of these components

Abd-El-Khair *et al*., (2011) and Ragab *et al.*, (2015) reported that reduced sugars increased in bean plants treated with the bioagents due to the increase in the biological activity. The increase in biological activity reduced sugars to be used in energy production of the causal pathogens. Abo-Shosha (2016) found the highest amount of reduced sugars and amount of protein were obtained when a mixture of *B. subtilis* and *T. harzianum* was used before planting time of bean seeds in soil infested with soil borne fungi.

The tested bioagents, BTH and the fungicide Topas resulted in considerable increase in total nitrogen, total amino

acid and crude protein in pea seeds compared with the control. In this regard the BTH was the most efficient in this regard followed by the fungicide Topas then the other treatments. The positive influence of the tested plant bioagents and BTH on the plant growth and yield could be due to the hormone-like activities present in the tested treatments that are involved indirectly in respiration, photosynthesis, oxidative phosphorylation, protein synthesis, anti-oxidant reactions, and various enzyme. Although BTH is known to increase plant growth, resulting in yield responses similar to those induced by plant hormones, it has not yet been shown conclusively whether salicylic acid contain hormone-like components (Muscolo *et al*., 1993).

It has been found that pre-formed antibiotic compounds such as phenol 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 (Lattanzio *et al.*, 2006). They added that some antibiotic phenolics are stored in plant cells as inactive bound forms but are readily converted into biologically active antibiotics by plant hydrolysing 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). 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.

5. Conclusion

This study showed that there were promising antagonistic species of bacteria and fungi prevalent on pea leaves, which can be exploited for the management of pea rust. Both the genera of *Bacillus* and *Trichoderma* comprise great numbers of bioagent strains that act as biological control agents for the control of plant diseases and for their ability to increase plant growth, the antagonistic properties of which are based on the activation of multiple mechanisms. The antagonistic nature may be due to antibiosis, nutrient competition and cell wall degrading enzymes. The present study clearly showed the effect of the tested genera against *U. pisi.* Based on the present investigation a new strategy will be developed for managing pea rust *in vivo*.

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