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Application of Antagonistic Microorganisms to Control Powdery Mildew of Pea

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Laboratory and greenhouse studies were conducted to assess the effectiveness of five antagonistic isolates of actinomyces, bacteria and fungi for controlling powdery mildew of pea caused by *Erysiphe pisi*. All of the isolates were effective in reducing the conidial germination *in vitro* and severity of the disease in the greenhouse, when applied as foliar sprays at the seedling stage. Five bioagents, *i.e. Bacillus subtilis, Pseudomonas fluorescence, Trichoderma harzianum, Trichoderma viride* and *Streptomyces* sp. significantly reduced severity of powdery mildew on pea. On the other hand, distortion in cellular components of pea cells was observed as a result of infection. The ultrastructure investigation revealed deformation and completely damage of hyphae and haustoria of *E. pisi* in response to *B. subtilis* invasion as early as 24h after application of the antagonist.

Keywords: *Bacillus subtilis, Erysiphe pisi*, pea, powdery mildew, *Pseudomonas fluorescence, Streptomyces* sp. and *Trichoderma* spp.

Chemical control is not always completely effective in controlling plant diseases, since pathogens may develop resistance to some fungicides (Gullino and Wardlow, 1999). In addition, consumer awareness of the implications of harm to the environment and human health through the use of pesticides has intensified the search for alternative methods of disease control (Gullino *et al.*, 1999).

Biological control of fungal plant pathogens appears as an attractive and realistic approach and numerous microorganisms have been identified as biocontrol agents. The phyllosphere of above ground parts of plants is a dynamic ecosystem inhabited by specific fungi and bacteria. The interactions between microorganisms and plant hosts that lead to biocontrol can include antibiosis, competition, induction of host resistance and predation (Sobiczewski, 2002). Such properties are first exposed by the fungi *Trichoderma* spp. (Bartmanska and Dmochowska-Gladysz, 2006), bacteria from the genera *Bacillus* and *Streptomyces* (Pengnoo *et al.*, 2006).

Batta (2003) pointed out that application of formulated conidia of *T. harzianum* in invert emulsion at a concentration of $2.0X10^8$ conidia/ml significantly suppressed the disease-lesion diameter caused by *Alternaria cucumerina* on treated cucumber leaf-discs. Such application decreased the lesion diameter to 4.5 and 6.5mm or reduced it by 70.4 and 57.2% relative to control when used just before or 24h after the causal inoculation, respectively.

David (2008) stated that with increasing awareness of possible deleterious effects of fungicides on the ecosystem and growing interest in pesticide free agricultural products, biological control now appears to be a promising strategy for managing diseases in a range of crop.

This study was aimed to evaluate some bacteria and fungi collected from Beni-Suef Governorate, as microbial agents for control of powdery mildew on pea, using foliar spray technique.

Materials and Methods

Five antagonistic isolates were tested in this study, *i.e.* two species of *Trichoderma*, (*T. harzianum, and T. viride*), two bacteria species (*Bacillus subtilis and Pseudomonas fluorescence*) and *Streptomyces* sp. They were isolated from parasitized powdery mildew colonies on pea leaves and were morphologically identified according to the classical methods of Bergey *et al.* (1939), and morphological characters as described by (Gilman, 1957 & Barnett and Hunter, 1972), and were kindly confirmed by the stuff of Bacteriol. and Mycol. Dept., Fac. of Sci., Beni-Suef Univ.

Biocontrol agents were grown on potato dextrose agar (PDA) at 28±2°C under continuous fluorescent light for 3 days for bacterial agents or 10 days for fungal agents. The cultures were flooded with sterilized distilled water; colonies were gently scraped, through four layers of cheesecloth. The concentrations of the suspension were adjusted to 10^8 colony forming units (cfu)/ml for bacteria, and 10^7 spores/ml for fungi and Streptomyces (Sriram *et al.*, 2009).

1- Effect of bioagents suspensions on conidial germination:

Freshly prepared bioagents suspensions were sprayed individually on clean glass slides and left to air dry. Infected pea leaves showing obvious formation of powdery mildew infection were shaken on the glass slides to deposit the conidia. Glass slides were put on glass rods in Petri-dishes (two in each dish) containing sterilized distilled water (about 100% relative humidity). Conidial germination percentages were assessed at zero time of the experiment, and then the dishes were sealed with para-film and incubated at 20±1°C for 24 hours. For microscopic examination, the glass slides were stained with ethylene blue as described by Johansen (1940) and Kunoh *et al.* (1992).

2- Greenhouse experiments:

After obtaining promising results *in vitro* on their affectivity against *E. pisi*, an experiment was conducted in cages using the highly susceptible pea cultivar Master B for powdery mildew. Tested seeds of pea were sown in plastic pots (25 cm diameter), 5 seeds per pot, irrigated when it is needed and left to grow. Plants were thinned into 3 per pot 10 days after sowing and left to grow in the greenhouse until two to five leaves were developed (aged 4 weeks). Two methods of bioagents application, *i.e.* pre- and post-inoculation with *E. pisi* were evaluated against disease development as follow:

Pre-inoculation treatment:

The leaves were treated with the bioagents suspension just before inoculation with *E. pisi* conidiospores by one hour after treatment, which were shaken onto the extended leaf and allowed to settle for 20 minutes.

Post-inoculation treatment:

When the disease severity scored 46% (Falloon *et al.*, 1995), the leaves were treated with bioagents suspension four times, at 7 days intervals between each application. Inoculated plants were then returned to a dedicated cage. Plants inoculated only with *E. pisi* served as check and transferred to separate clean cage. Another check treatment was sprayed with Topsin M-70 (dimethyl [1, 2-phenylenebis (iminocarbonothioyl)] bis [carbamate]) as standard. The severity of powdery mildew disease in each treatment was recorded after each spray by 7 days.

3- Transmission electron microscopy:

This work was done in TEM Lab. FARP., Fac. of Agric., Res. Park, Cairo Univ. After 24hours from inoculation, the leaves were removed and examined using a dissecting microscope to confirm the leaf area colonized by *E. pisi*. Transmission electron micrographs interaction between pea leaf tissues and *E. pisi* treated with *B. subtilis* were carried out. Tissues were cut into small pieces about 1-2 mm., fixed in 2% glutraldhyde in phosphate buffer, pH 7.2 and subjected to a vacuum for 1-4 minutes every 15 minutes for 2 hours on ice. Prior to vacuum treatment, floating samples were poked under the buffer surface with pointed metal pokers. Rinsing took place in phosphate buffer, pH 7.2, for 45 minutes, with buffer changes at 15 and 30 minutes. Further fixation in 1% Osmium Tetraoxide in phosphate buffer, under intermittent vacuum and poking, took place for 1.5 hours. Samples were then rinsed again in the phosphate buffer (Williams and Carter 1996). Samples were dehydrated through an ethanol series in buffer: 35, 50, 70, 80, 95 and 100% for 60 minutes each. Then infiltrate with Resin as follow:

Propylene : Resin (no accelerator)	2:1	1hr
Propylene : Resin (no accelerator)	1:1	1hr
Propylene : Resin (no accelerator)	1:2	1hr
Pure Resin (no accelerator)		1hr
Pure Resin (no accelerator)		Overnight
Pure Resin + accelerator		2hr
Embed samples into moulds 60°C oven.		

Semi thin sections were prepared on glass slides through cutting at 1μ using the ultramicrotome. Sections were stained with Toludine blue for 5 min. and examined by light microscope model M-200M. Ultra-thin sections were cut using ultra-microtome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mish). Sections were stained with double stain (Uranyl acetate 2% for 10 min followed by lead citrate for 5 min) and examined by transmission electron microscope JEOL (JEM-1400) at the candidate magnification. Images were captured by CCD camera model AMT, optronics camera with 1632 x 1632 pixel format as side mount configuration. This camera uses a 1394 fire wire board for acquision (Bozzola and Russell, 1999).

4- Disease assessment:

Artificially inoculated plants were carefully examined to estimate the severity of the infection by powdery mildew depending on the devised and modified scale (0 - 10) for disease score that applies to each position on the key according to Falloon *et al.* (1995). These conversions were made arithmetically using the following equations:

Y = 5X (for disease scores <4);
Y = 13.3X-33 (for disease scores >4); where Y = % of surface area diseased, and
X = mean disease score.

Reduction of disease severity was calculated relative to the check according to the following formula:

$$\text{Reduction (\%)} = \frac{\text{Severity of check - Severity of treatment}}{\text{Severity of check}}$$

5- Statistical analysis:

Data were statistically analyzed for computering L.S.D. according to the procedure described by Snedecor and Cochran (1989).

Results

Effect of bioagents suspensions on conidial germination in vitro:

According to the obtained results, the inhibitory effect of the tested bioagents suspensions, *i.e.* two species of *Trichoderma* (*T. viride* and *T. harzianum* 1×10^7), two species of bacteria (*P. fluorescence* and *B. subtilis* 2×10^8) and *Streptomyces* sp. 1×10^7 was assessed against spore germination of *E. pisi*. Data summarized in Table (1) and illustrated by Fig. (1) indicate that all suspensions of bioagents tested significantly decreased the percentages of spore germination of *E. pisi* compared to check treatment.

Table 1. Effect of bioagents suspensions (Bacteria (1×10^8), *Streptomyces* sp. (1×10^7) and *Trichoderma* spp. (1×10^7) on spore germination of *E. pisi*, 48h after incubation at 20±1°C.

Antagonistic agent	Spore germination (%)	Reduction of spore germination (%)*
B. subtilis	4	86.21
P. florescence	2	93.11
Streptomyces sp.	2	93.11
T. harzianum	5	82.76
T. viride	4	86.21
Control	29	-----

* Reduction of spore germination in comparison to control.

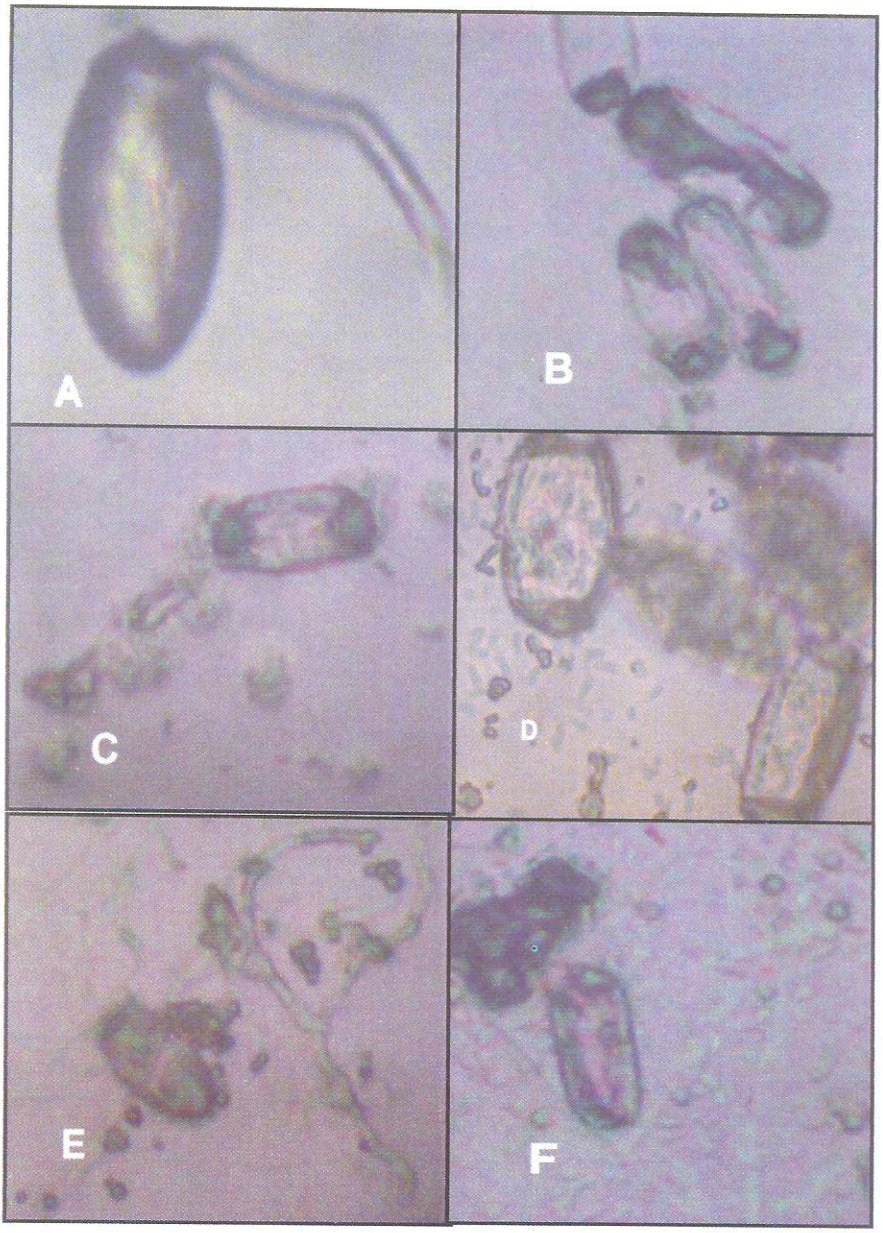

Fig. 1. B, C, D, E and F show the effect of *B. subtilis*, *P. fluorescence*, *Streptomyces* sp, *T. viride*, and *T. harzianum* suspensions, respectively, on spore germination of *E. pisi* compared to the non-treated (A). (400x)

Among the antagonist suspensions tested, suspension of *Streptomyces* sp. and *P. fluorescence* decreased percent of the maximum conidiospore germination of *E. pisi* (2%) followed by *B. subtilis* and *T. viride* (4%) compared to the check treatment (29%). Whereas, spore suspension of *T. harzianum* was the least effective as it resulted in the least inhibition (5%).

Effect of bioagents suspensions on disease severity under greenhouse:

In pre-pathogen inoculation, data presented in Table (2) show that all the used bioagents significantly decreased disease severity on pea plants compared to check treatment.

Table 2. Effect of some bio-agents applied before inoculation by *E. pisi* on disease severity of powdery mildew under greenhouse conditions

Antagonistic agent	Disease severity (%)					
	1st week	2nd week	3rd week	4th week	Mean	Reduction of * disease severity (%)
B. subtilis	0.0	0.0	0.0	5.0	1.3	95.58
P. florescence	0.0	0.0	0.0	0.0	0.0	100.00
Streptomyces sp.	0.0	0.0	0.0	0.0	0.0	100.00
T. harzianum	0.0	0.0	5.0	5.0	2.5	91.15
T. viride	0.0	0.0	0.0	5.0	1.3	95.58
Topsin M-70	0.0	0.0	5.0	5.0	2.5	91.15
Control	5.0	15.0	33.0	60.0	28.3	-
Mean	0.7	2.1	6.1	11.4	-	-
L.S.D. at 5% for: Bioagents (B)= 2.75. Disease severity (S)=4.17. B X S= 5.82.						

*Reduction of disease severity in comparison to control.

Results indicated that the best reduction of disease severity was obtained when *Streptomyces* sp. and *P. fluorescence* were sprayed on pea plants than other treatments, being 100% followed by *B. subtilis* and *T. viride*, being 95.58%, both *T. harzianum* and Topsin M-70 caused the same effect on reducing disease severity (91.15%), however the differences were not significant among the bioagents, on the average.

In post-pathogen inoculation, a similar trend was observed (Table 3). All treatments tested significantly decreased percentages of disease severity than check treatment. *P. fluorescence* was significantly the superior treatment (98.29%), followed by *B. subtilis* and *Streptomyces* sp. (96.58%), compared to *T. viride* and *T. harzianum* (94.86, 91.44%), respectively. In general, all used treatments gave better results than those obtained when the fungicide Topsin M-70 was used (88.01%).

Table 3. Effect of some bio-agents applied after inoculation *by E. pisi* on disease severity of powdery mildew under greenhouse conditions

Antagonistic agent	Disease severity (%)					
	1st Spray	2nd Spray	3rd Spray	4th Spray	Mean	Disease severity * reduction (%)
B. subtilis	5.0	5.0	0.0	0.0	2.5	96.58
P. fluorescence	5.0	0.0	0.0	0.0	1.3	98.29
Streptomyces sp	5.0	5.0	0.0	0.0	2.5	96.58
T. harzianum	15.0	5.0	5.0	0.0	6.3	91.44
T. viride	10.0	5.0	0.0	0.0	3.8	94.86
Topsin M-70	20.0	10.0	5.0	0.0	8.8	88.01
Control	46.0	60.0	86.0	100.0	73.0	-
Mean	15.4	12.9	13.7	14.3	-	-
L.S.D. at 5% for: Bioagents (B)= 3.65 Disease severity (S)= 2.02 B x S= 7.30						

*Reduction of disease severity in comparison to control.

Transmission electron microscope (TEM):

Transmission electron microscope observations performed on inoculated-untreated pea leaf tissues demonstrated that *E. pisi* hyphae were well developed and adhering to the surface of the host's body (Fig. 2A), epidermal cells generally appeared vacuolated, grand of chloroplasts irregular in shape and cytoplasm was condensed without distortion (Fig. 2B). In transverse section, the haustorial lobe appeared in the epidermal cell (Fig. 2C), walls were deposited disruption of plasma membrane and degrading chloroplasts membrane were also observed (Fig. 2D). By 24h after inoculated with *B. subtilis*, *E. pisi* hyphae suffered from severe damage and it were still recognizable even if several ultrastructure modifications were evident (Fig. 3 A, B, C), in fact, a thinning of the hypha wall at the site of attempted penetration can be seen (Fig. 3A), the hypha of the fungus showed severe vacuoles (Fig. 3B), cytoplasm was condensed by time (Fig. 3C).

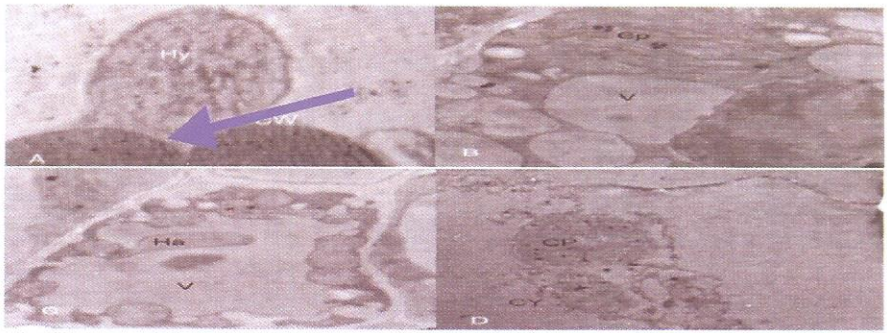

Fig. 2. Thin-section transmission electron microscope of the interaction between pea leaf tissues and *E. pisi*. A- Hypha of the fungus (Hy) adhering host cell wall (CW), arrow 8000X. B- Irregular chloroplast (CP) ad numerous vacuoles (V) in the epidermal cell, 12000X. C- Ultrastructural aspect of the haustorial body (Ha) formed in the infected epidermal host cell, 5000X. D- Distortion of the cytoplasm (Cy) and degrading chloroplast (CP), 5000 X.

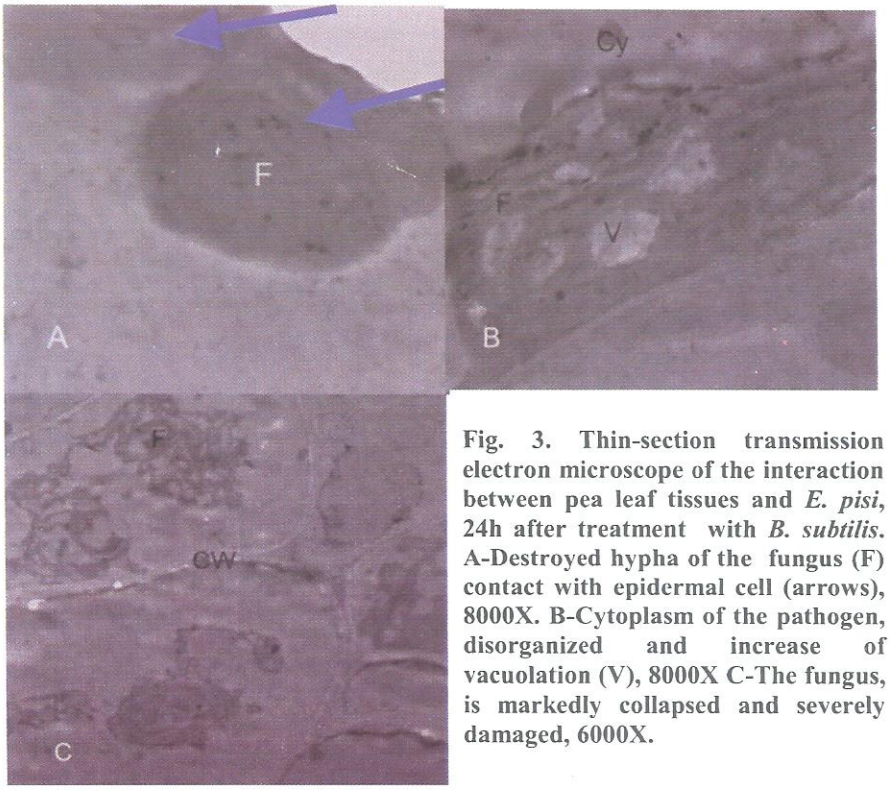

Fig. 3. Thin-section transmission electron microscope of the interaction between pea leaf tissues and *E. pisi*, 24h after treatment with *B. subtilis*. A-Destroyed hypha of the fungus (F) contact with epidermal cell (arrows), 8000X. B-Cytoplasm of the pathogen, disorganized and increase of vacuolation (V), 8000X C-The fungus, is markedly collapsed and severely damaged, 6000X.

Discussion

From the previously presented data, it is clear that biocontrol agents can protect pea plants against powdery mildew. In this study all bioagents suspensions significantly inhibited conidial germination. The probable mode of action of *Trichoderma* species such as *T. harzianum* and *T. viride* against *Alternaria cucumerina* might be through production of viridin and gliotoxin. (Batta, 2003). One idea that has been advanced is that enzymes such as chitinases and/or glucanases produced by the biocontrol agent are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitin, and β-glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity (Metcalf and Wilson, 2001 and Elad and Kapat, 1999).

Some investigators explained the mode of action of *P. fluorescence* and *Streptomyces* sp. Fluorescent pseudomonads are capable of producing a wide spectrum of biocontrol factors such as: siderophores, antibiotics, cyanide and extracellular enzymes against numerous plant pathogenic fungi or bacteria, (Fakhouri *et al.*, 2001). In addition, there was multi-fold increase in peroxidase,

polyphenoloxidase, phenylalanine ammonia lyase, ß-1, 3 glucanase, chitinases and phenolics in plants treated with *P. fluorescence* + azoxystrobin. (Anand *et al.*, 2009). While the effect of *B. subtilis* in controlling powdery mildew could be interpreting on the light of its antibiotics production (Bacteriocin and subtilisin), which act as inhibitors to pathogenic fungi, (Demoz and Korsten, 2006). On the other hand, *Streptomyces* sp. beside producing many antibiotics, it gives an increment of peroxidase and polyphenoloxidase enzymes activity, which indicates a positive relationship between increases in peroxidase and polyphenoloxidase enzymes activity and reduction in disease incidence and severity.

The data obtained showed that all the used biocontrol agents suspensions significantly reduced disease severity of pea powdery mildew accompanied with improving total green colour as well as disappearance of white patches typical of powdery mildew infection caused by *E. pisi*. Most of the epidermal cells in these areas were healthy, although a few hand non functional cytoplasm. Moreover the obtained results show that percentage of infected leaves was reduced by 98.11% due to the application of *P. fluorescence* treatment followed by 96.23% in *Streptomyces* sp. and *B. subtilis* treatments. The least effective treatments were *T. viride* and *T. harzianum* that scored 94.34 and 90.57%, respectively.

Transmission electron microscope observations showed that the antagonistic bacteria occurred preferentially in the plant epidermal cell functions, where availability of nutrients seems to be ensured, supporting the permanence of bacterial microcolonies as vegetative cells rather than dormant endospores (Collins and Jacobsen, 2003 and Demoz and Korsten, 2006). This ability to colonize and to form microcolonies provides protection to the bacteria but also has ecological implications involving the exclusion of other microorganisms from the occupied niche or the increased production of antimicrobials (Stein, 2005). Several *B. subtilis* strains are receiving great attention because of their versatility in conferring protection on plants. The antagonistic mechanisms encompass antibiosis, competition or induction of systemic plant responses (Emmert and Handelsman, 1999; Shoda, 2000 and Ongena *et al.*, 2005). Considering the epidemiology of powdery mildews, it has been assumed that the production of antifungal able to repress spore germination should successfully control the disease (Bélanger *et al.*, 1998 and Avis and Bélanger, 2001). Early studies pointed out that antibiosis could be involved in the disease protection provided by *B. subtilis* strains (Romero *et al.*, 2004). It has been demonstrated that antifungal compounds from the iturin and fengycin families of lipopeptides are produced by these strains which play a major role in the antagonism towards *Podosphaera fusca* determined to a large extent by the suppression of conidial germination (Romero *et al.*, 2007).

The presence of bacterial cells in close relation to visibly collapsed *E. pisi* hyphae suggests the local secretion of antifungal substances at sufficient concentrations to induce structural damage, resulting in the concomitant inhibition of vegetative growth. Moreover, similar study has shown the occurrence of iturin and fengycin upon leaves treated with *B. subtilis*, providing clear evidence for production *in situ* and secretion of these antifungal compounds to the surroundings (Romero *et al.*, 2007). Taken together, the correlation between bacterial density and

disease reduction, the production *in situ* of antifungal, and the microscopic alterations which, *B. subtilis* strains induced in *E. pisi* strongly highlight the relevant role of antibiosis as the main mechanism used by these strains to protect pea plants against powdery mildew.

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استخدام الكائنات المضادة لمكافحة مرض البياض الدقيقى فى البسلة

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أجريت بعض الدراسات فى المعمل وفى الصوبة على حد سواء لتقييم كفاءة خمس عزلات من الاكتينوميسيس والبكتيريا و الفطريات التى تم عزلها من على سطح أوراق البسلة لمقاومة مرض البياض الدقيقى فى البسلة و الذى يسببه الفطر *Erysiphe pisi*.

أثبتت الدراسات أن جميع هذه العزلات قد أظهرت كفاءة عالية فى تقليل نسبة إنبات الجراثيم الكونيدية لهذا الفطر فى المعمل و ايضا شدة الإصابة المرضية فى الصوبة وذلك عندما تتم معاملتها رشا على الأوراق فى مرحلة البادرة، وهى *Pseudomonas fluorescence, Bacillus subtilis, Trichoderma viride, Trichoderma harzianum, Streptomyces* sp., وكان لهذه الكائنات دورا هاما فى خفض شدة الإصابة بالمرض بصورة معنوية واثبتت كفاءة عالية فى مقاومة البياض الدقيقى فى البسلة. وهذا ما اظهرته نتائج الدراسات التشريحية بالميكروسكوب الألكترونى من تاثير الفطر الممرض على خلايا العائل بتدميرها وتحلل محتوياتها ، كما يظهر الفحص تشوه وتدمير تام لهيفات و ممصات الفطر *Erysiphe pisi* بعد ٢٤ ساعه من معامله اوراق البسله ببكتيريا *Bacillus subtilis*.