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Isolation and characterization of *Bacillus licheniformis* EM and its antagonistic effect against some important plant pathogens

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ABSTRACT

The antagonistic action of a newly isolated bacterium derived from an agricultural waste canal was evaluated against some economic plant pathogens. The isolate was characterized using API 20E commercial kit and molecularly identified by partial sequencing of the 16S rDNA. The phylogenetic analysis strongly related the isolate to *Bacillus licheniformis* EM. The antimicrobial activity of this isolate was evaluated *in vitro* against bacteria of crown gall *Agrobacterium tumefaciens* and soft mold *Pectobacterium carotovorum* and fungi of leaf spots and blights *Alternaria alternata*, grey mold *Botrytis cinerea*, root rot disease *Fusarium oxysporum* and damping off disease *Pythium debaryanum*. The results showed different susceptibility among the pathogens when serial concentrations of *B. licheniformis* EM late exponential phase cultures were used. Out of seven concentrations tested, the most significant antagonistic action was shown by 100, 200 and 300µl of bacterial suspension/20 ml NA medium, which showed almost complete inhibition against the tested bacteria. All the tested concentrations of *B. licheniformis* EM ranged from 20 to 500 µl/20ml PDA medium completely prevent fungi of *A. alternata* and *P. debaryanum* to grow. Therefore, the study suggests that *B. licheniformis* EM can be used as a potential biocontrol agent and recommend it for commercial formulations.

Keywords: Antagonistic activity; *B. licheniformis* EM; Antagonism, Plant pathogens.

INTRODUCTION

The use of biocontrol agents is becoming an increasingly important alternative to harmful synthetic pesticides for crop protection. Besides, chemical pesticides are not ideal for long term application due to the concerns associated with exposure risks, health and environmental hazards, residual persistence and tolerance of pests (Carson, 1962 and Radjacommare

et al., 2002). Bacteria and fungi are able to synthesize a wide range of metabolites with antimicrobial capabilities (Aneja *et al.*, 2006, Haesler *et al.*, 2008; Hernández-Rodríguez *et al.*, 2008 and Kim *et al.*, 2003). The materials based on such microorganisms have a high specificity against target plant pathogens; easy degradability; and low mass production cost.

Bacillus species offer several advantages over other Gram-negative bacteria and fungal biological control agents for protection against pathogens because of, being widely distributed in environment, having high thermal tolerance, showing rapid growth in liquid cultures and their longer shelf lives as a result of their ability to form endospores (Kim *et al.*, 2003; Cavaglieri *et al.*, 2005; Ongena and Jacques, 2008 and Perez-Garcia *et al.*, 2011). They have been reported to be effective in the biocontrol of multiple plant diseases owing to their production of several broad-spectrum antibiotics (Sharga and Lyon, 1998; Emmert and Handelsman, 1999 and Cavaglier *et al.*, 2005). Therefore, several strains of *Bacillus* species have been successfully developed as commercial biofungicides.

B. licheniformis is one of the most actively used *Bacillus* members in antagonizing plant pathogens (Lee *et al.*, 2001; Cladera-Olivera *et al.*, 2004 and Williamson *et al.*, 2007). For example it was showed a potential effectiveness against crown gall disease (Boelema, 1969), soft rot (Cladera-Olivera *et al.*, 2004), *Fusarium* wilt (Ulloa *et al.*, 2006), leaf spot (Bashon *et al.*, 1991), grey mould (Lee *et al.*, 2006 and Williamson *et al.*, 2007), and damping off in seedlings (Stankova-Opocenska and Dekker, 1970).

Many methods are available to identify and characterize bacteria. One of these available approaches is the utilization of commercial kits such as API ZYM system. This system measures the biochemical activities of the samples under test using rows of microtubules or paper stripes impregnated with various freeze-dried test substrates (Priest and Austin, 1993). These commercial kits were successfully used for differentiation between and within the species of *Bacillus* (Abdelkafi *et al.*, 2005). In contrast to phenotyping, which means study the appearance or the reaction of bacterial cell; the genotypic methods directly analyze the DNA. An important tool for genotyping is the sequencing of the PCR- amplified 16S rDNA and comparison of the data with sequences from the data bases. This has been successfully applied in determining phylogenetic relationships or identifying bacteria (Goto *et al.*, 2000 and Ki *et al.*, 2009).

In this research, a newly *Bacillus* isolate, *B. licheniformis* EM, was isolated and characterized using API 20E commercial kit and identified by partial sequencing of the 16S rRNA gene. The *in vitro* antagonistic effect of *B. licheniformis* EM was investigated against plant pathogenic bacteria of crown gall *Agrobacterium tumefaciens* and soft mold *Pectobacterium carotovorum* and fungi of leaf spots and blights *Alternaria alternata*, grey mold *Botrytis cinerea*, root rot *Fusarium oxysporum* and damping off *Pythium debaryanum*.

MATERIALS AND METHODS

Materials: Potato Dextrose Agar (PDA), Nutrient Broth (NB) and Nutrient Agar (NA) media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). All materials were used without further purification. API 20E kit (Biomerieux, France). DNA, dNTPs, $MgCl_2$, KCl, tris-HCl, and Taq polymerase (Pharmacia Biotech., USA). Gene Cycler™ Bio-Rad, USA.

***Bacillus* isolation and purification:** Samples have been previously collected at June 2009 from an agricultural waste canal near to Kafr El-Dawar, El-Behera Governorate, Egypt. One ml of the sample was suspended in 100 ml NB then incubated at 37°C under shaking conditions (200 rpm) for 24 h. Vegetative cells were then killed by adding chloroform (1%, v/v), vortexing and incubating at room temperature overnight. Spores were germinated by platting 0.1 ml of the spore suspension into plates containing NA medium. Colonies were picked up and further purified by streaking on NA plates.

Phenotyping: Pure cultures were examined for Gram reaction and sporulation ability, after incubation in NA plates at 37°C for 24 h. Biochemical characterization was performed using API 20E kit (Biomerieux, France). 0.1 ml of overnight *B. licheniformis* EM culture was used to inoculate each of the 22 wells, containing freeze-dried test substrates. The inoculated strips were incubated at 37°C for 7 h.

DNA extraction, amplification and purification: DNA was extracted from *B. licheniformis* EM and *B. subtilis* 168, a positive control, cultures according to Sambrook *et al.*, (1989). The 16S rDNA was amplified, approx. 1500 bp, by PCR using the forward primer, 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse one 5'-TACGGYTACCTTGTTACGACTT-3'. The PCR mixture consists of 30

pmol of each primer, 100 ng of DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 20 mM KCl, 10 mM tris-HCl pH 8.3, and 2.5 U of Taq polymerase. The 50 µl PCR mixture containing tube was placed in the DNA thermocycler, Gene Cycler™ Bio-Rad. The PCR conditions were as follows: initial denaturation of DNA at 95°C for 3 min and then 30 cycles of three-step PCR amplifications consisting of denaturation at 94°C for 1 min, primer reannealing at 55°C for 1 min and extension at 72°C for 2 min. Samples were subjected to an additional extension at 72°C for 10 min at the end of the amplification cycles (Ausubel *et al.*, 1999). The amplicons were finally purified using QIA quick PCR purification kit (Qiagen, USA).

Gel electrophoresis: Ten µl of PCR products, mixed with the loading buffer, were loaded on a 1.5% (w/v) agarose gel and electrophoresed with 1X TEA (Tris EDTA Acetate) buffer. DNA was visualized by UV transillumination after staining with ethidium bromide. The molecular size of the amplified fragments was estimated using DNA ladder of 100bp.

Data analysis and construction of the phylogenetic tree: After obtaining the sequences, homology search was performed against DDBJ (DNA Data Bank of Japan), using Blast program to find the sequences producing significant alignment with the obtained sequences. Similarity percentages among the sequences were obtained using Biology WorkBench software version 3.2. Multisequence alignment and molecular phylogeny were performed using ClustalW (a distance-based analysis program at http://www.ddbj.nig.ac.jp/) program (Saitou and Nei, 1987). The tree topology was evaluated using the neighbor-joining method based on 1000 resamplings (Saitou and Nei, 1987 and Chun and Bae, 2000).

Test plant pathogenic microorganisms: Bacteria of crown gall *Agrobacterium tumefaciens* (Family: Rhizobiaceae; Class: Alpha Proteobacteria) and soft rot *Pectobacterium carotovorum* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria) and fungi of leaf spots and blights *Alternaria alternata* (Family: Dematiaceae; Class: Deuteromycetes), grey mold *Botrytis cinerea* (Family: Moniliaceae; Class: Deuteromycetes), root rot *Fusarium oxysporum* (Family: Tuberculariaceae; Class: Deuteromycetes) and damping off *Pythium debaryanum* (Family: Pythiaceae; Class: Oomycetes) were provided by the Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt.

***In vitro* antagonism assay:** The *in vitro* antagonistic effect was detected by mixing 5-500 µl of *B. licheniformis* EM suspension/cell-free supernatant, obtained by shaking at 37°C till late exponential phase ($O.D_{550}$ = 0.9), with around 20 ml of warm NA (for bacteria) or PDA (for fungi) and inoculating the pathogen to the solidified plates. For *P. carotovorum* and *A. tumefaciens*, 0.1 ml of the bacterial cultures ($O.D_{550}$ = 1.2 and 1, respectively) was separately spread on the surface of *B. licheniformis* EM plates that containing different volumes (10 - 300 µl/20ml NA medium) and incubated at 30°C for 24 hrs. The control culture was set inoculating the bacteria in NA not inoculated with *B. licheniformis* EM. For the plant pathogenic fungi, 10 to 500 µl of *B. licheniformis* EM culture ($O.D_{550}$ = 0.9) was spread on 9-cm-diameter Petri dishes containing 20 ml PDA medium and immediately after this, 5-mm plugs from the leading edge of a 5-days old fungal culture were placed in the centre of each Petri dish. The control culture was set inoculating the fungus in PDA not inoculated with bacteria. The plates were incubated for 7 days at 28°C. The fungal inhibition was scored by measuring radial growth of the fungus in cm in every plate.

RESULTS

Isolation and identification of isolated bacteria: *B. licheniformis* EM, as a Gram positive, spore forming and rod-shaped bacterium was isolated from a highly polluted agricultural waste canal. This bacterium has been biochemically characterized using API 20E kit and the results are shown in Table (1). It has the ability to produce some enzymes such as β-galactosidase, arginine dihydrolase, lysine decarboxylase, omithine decarboxylase, urease, tryptophan deaminase, gelatinase, oxidase and catalase. In addition, it can utilize glucose and citrate. For molecular identification of the new isolate, the 16S rDNA (approx 1500 bp) was amplified using RP and LP pair of primers as shown in Figure (1). The same primers were used to partially sequence the 16S rDNA (approx. 485 bp). The sequences were deposited in the GenBank and the accession number was HM246696. Phylogenetic analysis of the target sequence and other homologous sequences from the data base revealed that this bacterium is strongly belonging to *B. licheniformis* Biology WorkBench 3.2 program showed that there is a 99% similarity between the target sequence, *B. licheniformis* EM, and other *B. licheniformis* strains (Figure 2).

Table (1). Characterization of *B. licheniformis* EM using API 20E

Characteristic	Result
β-Galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Tryptophan deaminase	+
H_2S production	-
Urease production	+
Indole production	-
Acetoin production	+
Gelatinase production	+
Oxidase production	+
Catalase production	+
Citrate utilization	+
D-Glucose utilization	+
D-Mannitol utilization	-
Inositol utilization	-
D-Sorbitol utilization	-
D-Rhamnose utilization	-
D-Sucrose utilization	-
D-Melibiose utilization	-
Amygdalin utilization	-
L-Arabinose utilization	-

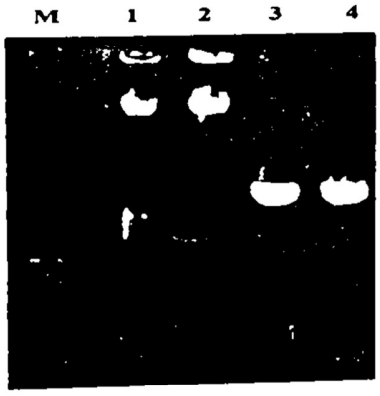

Figure (1). Gel electrophoresis of genomic DNA of *B. licheniformis* EM and *B. subtilis* 168 as a control (lanes 1 and 2, respectively), and approx. 1500 bp of the 16S rRNA gene from *B. licheniformis* EM and *B. subtilis* 168 (lanes 3 and 4, respectively). M, is 100 bp genetic marker.

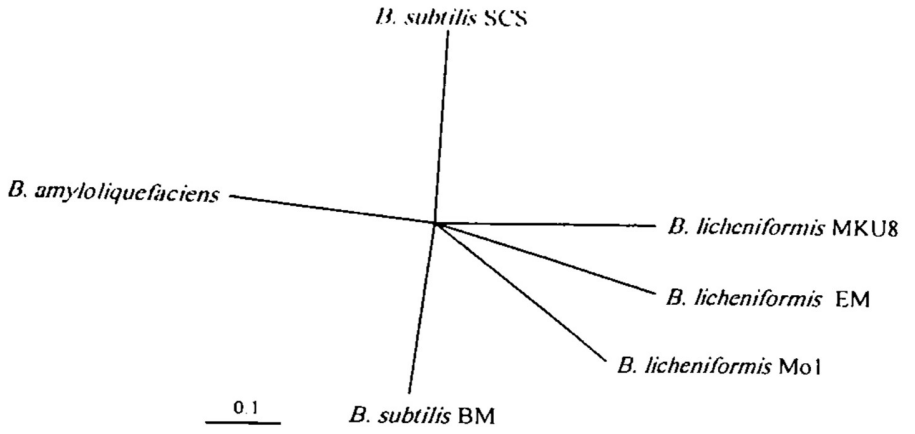

Figure (2). Phylogenetic tree based on partial sequences (approx 485 pb) of the 16S rRNA gene. The tree constructed by neighbor-joining method using ClustalW software. The scale indicates substitutions per site.

***In vitro* antagonistic activity between plant pathogenic bacteria and *B. licheniformis* EM**: *B. licheniformis* EM was used in this study as an antagonistic bacterium against plant pathogenic bacteria of crown gall bacterium *P. carotovorum* ($O.D_{550}$ = 1.2) and soft mold *A. tumefaciens* ($O.D_{550}$ = 1). As shown in Table (2), *B. licheniformis* suspension was more efficient than the cell-free supernatant. Using different volumes of *B. licheniformis* culture, grown till late log phase ($O.D_{550}$ = 0.9) at 35°C and 200 rpm, showed different sensitivity levels among the two pathogens. As shown in Figure (3), *P. carotovorum* exhibited more sensitive response than *A. tumefaciens* and its growth was weakened at all the tested treatments of *B. licheniformis*. Out of seven concentrations tested, the most significant antagonistic action was shown by 100, 200 and 300µl of bacterial suspension/20 ml NA medium, which showed almost a complete inhibition against both of *A. tumefaciens* and *P. carotovorum*.

***In vitro* antagonistic activity between plant pathogenic fungi *and B. licheniformis* EM**: The *in vitro* antifungal activity of *B. licheniformis* EM against four plant pathogenic fungi of *A. alternata*, *B. cinerea*, *F. oxysporum* and *P. debaryanum* is presented in Table 3 and Figure 4. The growth inhibition of the tested fungi by *B. licheniformis* was determined after a week of incubation. The interesting finding was that only 10 µl of the

antagonistic suspension was enough to prevent the growth of *A. alternata* and *P. debaryanum* (Figure 4A and B, respectively). In addition, a dramatic decrease in mycelia growth of *B. cinerea* and *F. oxysporum* was detected upon using 25 µl of *B. licheniformis* culture (Table 3) however, 500 µl of the bacterial suspension was required to almost inhibit the growth of both fungi (Figure 4C and D, respectively). It can be noticed that the inhibition percentages of the mycelial growth of *F. oxysporum* were higher (72.22 - 82.22%) than that obtained with *B. cinerea* (45.56 - 79.63%).

Table (2). Growth of *P. carotovorum* and *A. tumefaciens* in plates containing different concentrations of *B. licheniformis* EM suspension or cell-free supernatant, after incubation of 24 hrs at 30°C.

B. licheniformis EM (µl/20 ml NA)	*B. licheniformis* Suspension		*B. licheniformis* Supernatant	
	P. carotovorum	*A. tumefaciens*	*P. carotovorum*	*A. tumefaciens*
0	+++	+++	+++	+++
10	++	+++	+++	+++
20	+	+++	++	+++
40	+	+++	++	+++
50	-	++	+	+++
100	-	+	-	+++
200	-	-	-	+
300	-	-	-	-

+++, good growth; ++, medium growth; +, weak growth; and -, no growth.

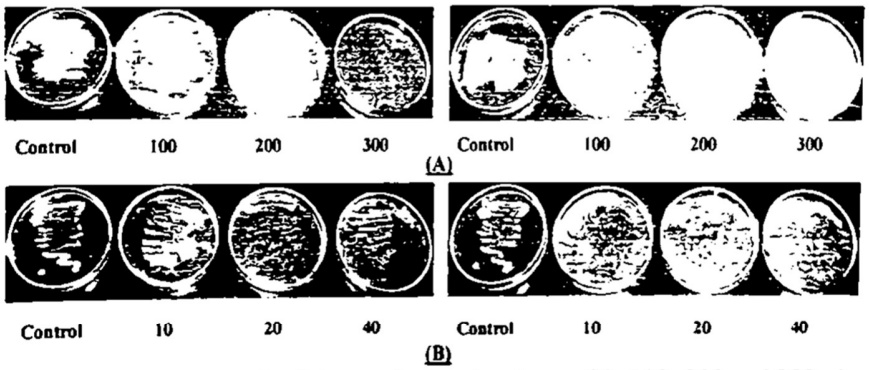

Figure (3). A: Growth of *A. tumefaciens* in plates of 0, 100, 200 and 300 µl supernatant (left) and suspension (right), respectively of *B. licheniformis* EM late exponential cultures/20 ml NA medium. B: Growth of *P. carotovorum* in plates of 0, 10, 20 and 40 µl supernatant (left) and suspension (right), respectively of *B. licheniformis* EM late exponential cultures/20 ml NA medium.

Table (3). ***In vitro*** **antagonistic activity of** ***B. licheniformis*** **EM as a biocontrol agent against fungi of** ***A. alternata, B. cinerea, F. oxysporum*** **and** ***P. debaryanum.***

B. licheniformis EM (µl/20 ml PDA)	Inhibition of mycelial growth (%) ± SE			
	A. alternata	*B. cinerea*	*F. oxysporum*	*P. debaryanum*
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
10	100±0.0	45.56±1.11	72.22±0.64	100±0.0
25	100±0.0	67.04±0.37	73.33±0.64	100±0.0
50	100±0.0	70.37±0.98	74.44±0.64	100±0.0
100	100±0.0	72.22±0.64	77.78±0.64	100±0.0
300	100±0.0	76.67±1.70	79.26±1.34	100±0.0
500	100±0.0	79.63±0.98	82.22±0.64	100±0.0

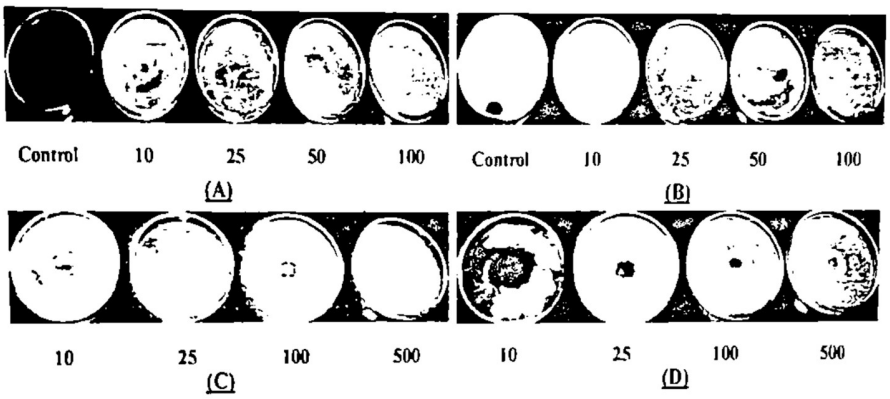

Figure (4). Growth of *A. alternata* (A), *P. debaryanum* (B), *B. cinerea* (C) and *F. oxysporum* (D) in plates containing different concentrations of *B. licheniformis* EM late exponential cultures/20 ml PDA medium.

DISCUSSION

A pure culture of Gram- positive and spore-forming rods of *B. licheniformis* EM has been characterized, identified and evaluated for its potential to antagonize a number of economically important plant pathogens. API system tests have been shown to be rapid, accurate and reproducible characterization tools. This semi-quantitative method has been successfully used in previous studies for separation between members belonging to genus *Bacillus* (Hernandez *et al.*, 1998 and Waldeck *et al.*, 2006). The use of molecular genetic characteristics to classify an organism

and place it in a map showing the relationship between this organism and other related ones is called molecular phylogeny and a tree/map showing such a relation is called phylogenetic tree (Li and Graur, 1991). Partial sequences (aprox. 485 bp) of the 16S rDNA were obtained for the new isolate and their phylogeny revealed that the 5' end region is a very efficient index for rapid identification. A similarity of about 99% was found between the novel isolate, *B. licheniformis* EM, and some other *B. licheniformis* strains derived from the GenBank. Goto *et al.*, (2000) have proved that the 5' end of the 16S rRNA gene is a hyper variant region and can be efficiently used for grouping of *Bacillus* species.

Bacillus species have often been reported to be among the most beneficial bacteria as antagonists of fungi (Melent'ev *et al.*, 2006 and Hao *et al.*, 2011), bacteria (Hagelin *et al.*, 2004 and Hammami *et al.*, 2008) and insects (Aranda *et al.*, 1996 and Quesada-Moraga *et al.*, 2004). Therefore, they are commonly used in the control of several plant pathogens due to their ability to produce a multitude of broad spectrum antibiotic compounds.

In the present study, late exponential phase *B. licheniformis* EM culture and cell-free supernatant were separately used to antagonize the growth of *P. carotovorum*, a causative agent of soft rot disease (Cladera-Olivera *et al.*, 2006), and *A. tumefaciens*, a causative agent of crow gall (Boelema, 1969). *B. licheniformis* produces a wide range of antimicrobial substances (Yakimov *et al.*, 1996 and Munimbazi and Bullerman, 1998). For instance, *B. licheniformis* strain P40 has been found to produce a bacteriocin-like substance (BLS) that inhibits the growth of *P. carotovorum* and other pathogens (Cladera-Olivera *et al.*, 2006 and Teixeira *et al.*, 2009). *B. licheniformis* EM was used efficiently, in the current study, to inhibit the growth of both *P. carotovorum* and *A. tumefaciens*. Interestingly, the antagonistic effect was more efficient when cultures rather than cell-free supernatants were used. This result suggests that *B. licheniformis* may acts by other mechanisms such as competition to nutrients and space to antagonize the pathogens in addition to its ability to produce antimicrobial compounds such as BLS (Hammami *et al.*, 2008).

The fungal cell wall is a highly dynamic structure which is continuously subjected to changes during cell expansions and divisions. The cell wall degrading enzymes are glycosyl hydrolases that degrade chitin and glucan polymer which comprises important structural elements in fungal cell walls (Peberdy, 1990). *B. licheniformis* has been also known to produce antifungal

agents against phytopathogenic fungi (Neyra and Sadasivan, 1996). A recent study of Kamil *et al.*, (2007) showed that *B. licheniformis* was a potential biocontrol agent against *F. culmorum, Pythium* sp., *A. alternata* and others due to its chitinolytic activity. Moreover, they showed that *B. licheniformis* has significantly reduced the damping off disease in a green-house experiment. In the current study, growth inhibition of four economically important plant pathogenic fungi, *A. alternata, B. cinerea, F. oxysporum,* and *P. debaryanum* was detected up on using different concentrations of *B. licheniformis* late log phase cultures. This antagonistic effect may be due to the chitinolytic activity of *B. licheniformis* EM. Anitha and Rabeeth (2010) have been shown that *Fusarium* species cell wall is resistant, to some extent, to chitinase. Moreover, Sivan and Chet, (1986 and 1989) have argued that *Fusarium* species cell walls contain more proteins than do walls of other fungi. This may explain the higher concentration of *B. licheniformis* EM culture (500µl/20 ml PDA) was required for inhibition of *F. oxysporum* growth rather than that was required (10µl/20 ml PDA) for *A. alternata* and *P. debaryanum* inhibition. However, 500µl of bacterial culture/20 ml medium was also required for a complete antagonism against *B. cinerea.*

In conclusion, our preliminary studies evaluate the effectiveness of the recently isolated and molecularly identified *B. licheniformis* EM bacterium to antagonize some important plant bacterial and fungal pathogens. Although a number of pesticides are available for plant pathogens control, this novel bacterial isolate is a potential biocontrol agent that is strongly recommended for commercial formulations of efficient antimicrobial effects and could be used in agriculture for disease control in plants.

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عزل وتوصيف بكتيريا باسيلاس ليتشينيفورميس وتأثيراتها المضادة ضد بعض مسببات الأمراض النباتية الهامة

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تم تقييم الفعل الإبادى للبكتيريا المعزولة حديثا المستمدة من قناة صرف المخلفات الزراعية لمكافحة مسببات امراض النبات الاقتصادية. العزلة تم التعرف عليها وتحديدها من قبل التسلسل الجزئي لل rDNA . تحليل تطور السلالات ذات الصلة يثبت أن العزلة هى بكتيريا باسيلاس ليتشينيفورميس (ا م) (*Bacillus licheniformis* EM). تم تقييم النشاط الإبادى البكتيري لهذه السلالة ضد البكتيريا المسببة لمرض التدرن التاجى *Agrobacterium tumefaciens* والبكتيريا المسببة للعفن الطرى لثمار الخضروات والفاكهه *Pectobacterium carotovorum* و ضد كلا من فطر *Alternaria alternata* المسبب لتبقع الأوراق, فطر *Botrytis cinerea* المسبب للعفن الرمادى, فطر *Fusarium oxysporum* المسبب لعفن الجذور وسقوط البادرات وفطر *Pythium debaryanum* المسبب لمرض الذبول الطرى فى البادرات. وأظهرت النتائج حساسية مختلفة بين تلك المسببات المرضية عندما استخدمت سلسلة تركيزات من معلق البكتيريا المختبرة. من اصل سبعة تركيزات تم اختبارها، تبين أن أهم فعل تثبيطى لهذه السلالة كان من قبل 100، 200 و 300 ميكرونيتر من معلق البكتيريا/ 20 مل من البيئة, والتي أظهرت تثبيط كامل تقريبا ضد البكتيريا المسببة لمرض التدرن التاجى *A. tumefaciens* والبكتيريا المسببة للعفن الطرى لثمار الخضروات والفاكهه *P. carotovorum*. كل التركيزات المختبرة من سلالة البكتيريا والتى تراوحت من 20 إلى 500 ميكرونيتر/ 20 مل من البيئة منعت نمو كلا من فطر *A. alternata* وفطر *P. debaryanum*. ولذلك، تقترح الدراسة انه يمكن إستخدام هذه السلالة من البكتيريا كعامل مكافحة بيولوجية ويوصي بها أن تحضر بتجهيزات تجارية.