J.Agric.Chem.and Biotechn., Mansoura Univ.Vol. 8 (6): 177 – 181, 2017

Antagonistic Effect of *Bacillus* spp. against Sugar Beet Pathogens *Fusarium* Wilt

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ABSTRACT

Twenty bacterial isolates were recovered from the rhizosphere of sugar beet cultivars in Egypt. All the isolates have antagonistic effect against three phytopathogenic fungi: *Fusarium solani, Fusarium oxysporum* and *Fusarium dimerum. In vitro* all bacteria gave comparable results. Four isolates which showing highly antagonism were identified by standard tests. *In vitro* it was found that 4 strains very active to antagonism and were namely by biolog system belonging to *Bacillus* spp.; *B. subtilis, B. amyloliquefaciens, B. weihenstephanensis* and *B. pseudomycoides.In vivo*, the highest percentage of survival plants was 86, 90 and 72%by *B.amyloliquefaciens*, *B.pseudomycodies* and *B. subtilis* for the control of *F. solani, F. oxysporum* and *F. dimerum*, respectively more than the other *Bacillus* spp.All *Bacillus* strains were positive for indole acetic acid production from tryptophan, but it failed to produce HCN. Also, all *Bacillus* strains were negative for cellulase and chitinase tests.The usage of *Bacillus* spp. as seed treatment reduced the percentage of damping-off incidence of sugar beet under greenhouse conditions. This result shows that these four *Bacillus* spp. are very effective biocontrol agents and should be used for further biocontrol applications and should be classified as biocontrol agents.

Keywords : *Bacillus* spp.; Biological control ; *F .solani ; F .oxysporum ;F.dimerum ;*Antagonism.

INTRODUCTION

The most important sugar crops is Sugar beet (*Beta vulgaris* L.) in many countries of the world. In Egypt, due to the great consumption of sugar, the production of sugar beet must be increased to cover the requirements of sugar which depended sugar cane.Under Egyptian conditions sugar beet plant attacked by numerous, foliar and root diseases (Mosa and El-Kholi, 1996).

Damping-off and root-rot disease are the most important of soil diseases caused by phytopathogenic such as *Rhizoctonia solani* Kuhn, *Sclerotium rolfsii* Sacc., *Phoma* (*Pelospora*) *beta* Berl. Several species of *Fusarium* and *Phythium* were also recorded, i.e. *Fusarium solani* (Mart) Sacc, *F. oxysporum* f. sp *conglutinans* Wollenw, *F. oxysporum* Snyder Hans, *F. moniliforium* Sacc. and *F. meresmoides* Corda, *Pythium aphanidermatum*, *P. mamillatum* Meurfd, *P. ultimum* and *P. debaryanum* Hesse (El-Kholi, 2000). The economically most important diseases is damping-off disease . Damping-off caused heavy losses in different parts of the world. The disease is caused by members of *Fusarium*, *Pythium* and *Rhizoctonia* (Cook and Baker, 1983). Many rhizospheric microorganisms are known to be equipped with antagonistic potential against phytopathogenic such as *Trichoderma* spp. (Chet and Baker, 1981) and rhizobacteria (Caroline *et al*., 2013). Several *Bacillus* spp. have high antagonistic ativity *Bacillus* spp. are used as ‘‘cell factories" because of production of enzymes the best studied bacteria is *Bacillus subtilis* also, *B. amyloliquefaciens* sub sp. *plantarum* increases plant growth and protect it by producing phytohormones and antimicrobial compounds. The *B. amyloliquefaciens* sub sp. *plantarum* group, FZB42, is reported to be have a great capacity for secondary metabolites *B. amyloliquefaciens* produces lipopeptides and polyketides by some gene clusters with antimicrobial and antifungal activities (Koumoutsi *et al*., 2004 and Chen *et al*., 2006).Hydrogen cyanide (HCN) is toxic and effectively blocks the cytochrome oxidase pathway. HCN is highly toxic to all aerobic microorganisms at picomolar concentrations. The production of HCN by *Pseudomonads fluorescens* is believed to be involved in the suppression of root pathogens. *P. fluorescens* CHA0 produces siderophores, antibiotics and HCN, but *Thielaviopsis basicola* was suppressed because of HCN which caused black rot of tobacco (Voisard *et al*., 1989). Production of IAA in plants help to increase root dry weight and there by increase the plants ability to take up N, P, K compared to untreated control . It caused increase in vegetables especially pepper, cucumber and tomato (Kidoglu *et al*., 2007). Many microorganisms produce other metabolites that can involve with pathogen growth and/or activities. Many microorganisms produce and release lytic enzymes. Polymeric compounds hydrolyze by enzymes including chitin, proteins, cellulose, hemicellulose, and DNA. None the less, microbes that know a preference for colonizing and lysing plant pathogens should be classified as biocontrol agents.

Lytic enzymes are glucanases, proteases (Dunne *et al*., 1997), cellulases, and chitinases. Bacteria could parasitize fungi caused diseases by the production of these enzymes.

Biocontrol abilities of the producing bacteria have been associated with production of extracellular cell wall degrading enzymes (El-Tarabily, 2006).

This work aims at evaluating the antagonistic effect of some selected bacterial isolates against sugar beet pathogens *Fusarium* wilt and their effects on damping-off and survival sugar beet plants infected with the pathogenic fungi.

MATERIALS AND METHODS

Source of soil samples:

Soil samples were collected from the rhizosphere of healthy sugar beet seedling at different types of soil. Intact root system was dug out. Soil samples were put in plastic bags and stored at 4^{o} C.

Isolation and purification of Bacteria:

Ten grams of soil samples was suspended in 90 ml of sterile tap water and serial dilution were made. 1 ml from each dilution was transferred to Petri-dishes. Thereafter, nutrient agar medium was added and mixed thoroughly. Three replicates were prepared from each dilution. Colony units were obtained after two days of incubation at 30^{0}C and purified on nutrient agar medium (NA).The bacterial isolates were maintained on NA at 4^{0}C for further use (Johnson and Curt 1972).

Fungi isolates:

Three pathogenicfungal isolates namely: *Fusarium solani, F. oxysporum* and *F. dimarium* were obtained from Plant Pathol. Res. Institute, Agric. Res. Center (A.R.C), Giza, Egypt.

In vitro* experiments:*

Antagonistic effect of the bacterial isolates against the causal pathogenic fungi

The antagonism between the isolated bioagent and the causal pathogenic fungi was tested *in vitro* using PDA medium. Petri dishes containing 10ml of PDA medium were inoculated with equal disks (7mm diam.) of *Fusarium* spp. obtained from 5 days old culture which placed at the periphery of the plate. The antagonistic bacterium was streaked at the center of each plate by a loop loaded with 48 hr. old bacterial culture grown at 27-30°C on nutrient broth. Three replicates were used for each particular treatment. Antagonistic effect was determined by measuring the diameter of inhibition zone between the antagonistic bacteria against the tested pathogenic fungi. Petri dishes inoculated with *Fusarium* spp. only were served as control check (Abo-Elnaga, 2006).

Identification of bacterial isolates:

Four isolates of bacteria were selected randomly in different shape and color because all bacteria gave comparable results. These bacterial isolates were identified using standard tests according to Bergy's Manual of Systematic Bacteriology (2005).

Bacteria identification by Biolog system:

The best antagonistic bacteria were identified using Biolog system in the Cairo MIRCEN, Faculty of Agriculture, Ain Shams Univ.(Biolog, 2013).

Greenhouse experiment

5mm mycelial disk from 5 days old cultures of *Fusarium* spp. were prepared by growing in sterilized glass bottles (500ml) containing barley medium (150g sorghum seeds, 50g clean sand, 4g glucose and 200 ml water) and autoclaved in two consecutive days and incubated at 27±1^{0}C for 15 days. Pots (35 cm. in diameter) were sterilized by immersing in 5% formalin solution for 10 mins. and left to dry in open air.

At the same day of sowing, surface sterilized seeds were soaked for one hour before sowing in 3days old cultures of *Bacillus* spp. (10^8-10^9cfu/ml) growing in a liquid nutrient broth medium. Ten seeds were sown in each pot and four replicates were made for each treatment. Surface sterilized un-soaked seeds were served as a control check.

Percentages of pre- and post-emergence damping-off were calculated after 15 and 45 days of sowing. These experiments were carried out under greenhouse conditions for growing season 2014/2015.

Percentages of pre and post-emergence damping-off and survival plants were calculated from planting to 45 days as follows:

- % of pre-emergence damping-off = (No. of non-emergedseeds/No. of sown seeds) x 100
- % of post-emergence damping-off = (No. of killed seedlings/ total No. of emerged seedlings) x 100
- % of survival plants = (No. of un-infected plants/total No. of plants) x 100

Detection of antagonistic compounds

Detection of Hydrogen Cyanide (HCN) Production:

Production of HCN was detected according to the method of Lorck (1948). Freshly grown cells were spread on a tryptone soy agar (30 g) and glycine were added 4.5 g/l and a sterilized filter paper were saturated with 1% solution of picric acid and 2% sodium carbonate was placed in the upper lid of the Petri dish, the plates incubated at 30°C for 4 days, a change in colour of the filter paper from yellow to reddish brown is the presence of cyanogenic activity.

Determination of Indole Acetic Acid Production (IAA):

Eight grams of nutrient broth (Merck) were suspended in 500 ml of distilled water; 1 ml of bacterial fresh cultures were inoculated into 10 ml nutrient broth to which tryptophan had been added (1 mg and 3 mg tryptophan) in each test tube.Cultures were incubated at 30°C for 48 h. Each test tube was removed 4 ml culture from it .The culture centrifuged at 10.000 rpm for 15 min. An aliquot of 1 ml of supernatant was transferred into a fresh tube to which 50 µl of 10 mM orthophosphoric acid and a 2 ml of Salkowski reagent consist of (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated for 25 minutes at room temperature. The presence of a pink colour indicated the presence of indole acetic acid (Patten and Glick, 2002). The absorbance of the pink solution from each isolate was measured and recorded at 530 nm using spectrophotometer (Thermo Spectronic, Merck, SA).

Cellulase production:

Aerobic cellulose decomposition was determined using Dubos medium (Allen, 1959) .(Positive tubes were characterized by yellowish-brown discoloration and gradual degradation of the filter paper strips present in tubes.

Chitinase production:

Colloidal chitin was prepared according modified method by Faramarzi *et al* .(2009) .Ten grams of chitin were added into 100 ml concentrated HCl (37%) .Chitin were kept in for 2 h at room temperature or until chitin dissolved. The suspension was precipited by slowly added to 500 ml of ice-cold absolute ethanol and neutralized pH with 10 N NaOH .The mixture was centrifuged at 8000 rpm for 10 mins and the precipitate was ready to use as a medium substrate .colloidal chitin (4.5) g/l) and bromocresol purple (0.15g/l) were directly made to prepare chitinase medium .Change to yellow-color and retained enough bromocresol purple even after pH was adjusted to 4.7 and sterilization at 121°C for 15 min .(Gomez *et al*2004 ; Fen *et al* 2006; Wirth and Wolf 1990).

Statistical analysis:

The obtained data were subjected analysis of variance (ANOVA) (Steel and Terrie 1960) . Least significant differences (L.S.D.) and Duncan's multiple range test (DART) were applied for comparing means under study (Duncan, 1955).

RESULTS AND DISCUSSION

The antagonistic effect:

Twenty bacterial isolates were tested *in vitro* against the isolates of damping-off fungi. Data in Table (1) showed that all the bacterial isolates have antagonistic effect against

the pathogenic fungal isolates. Seven bacterial isolates (No.1, 2, 3, 5,7,13 and 19) showed high level of antagonism against the tested fungal isolates while the others showed low level of antagonism.These results are in agreement with Adebayo and Ekpo (2005),they found that *Bacillus subtilis* inhibited fungal growth. Also, Ashour and Afify 2016 reported that *Bacillus* spp. are known to reduce fungi damping-off. Similarly, in the current study, several species of the genus *Bacillus* are effective in controlling a variety of fungal plant diseases (Williams and Asher 1996; Ashour and Afify 1999 and Commare *et al.,* 2002). *F. oxysporum* was suppressed by *Bacillus* spp. *in vitro*. *Bacillus* spp. isolated from chickpea are very effective in controlling *F. oxysporum* isolates including *F. o. ciciris, F. o. phaseoli* and *F. o.melonies* (Land *et al*., 1997).

Table 1. Screening of different bacterial isolates to antagonism against *Fusarium* spp.

Bacterial isolates No.	Inhibition zone (mm) Fungal species		
	F. solani	*F. oxysporum*	*F. dimarium*
1	1.8abcde	1.4efg	2.0b
2	1.6cdef	1.2fg	0.5e
3	1.7bcdef	2.1abc	1.0d
4	2.1ab	2.1abc	.1o ef
5	1.8abcde	2.4a	1.5c
6	1.5 def	2.1abc	1.0d
7	1.3 fg	1.8cde	1.3 cd
8	1.4efg	2.3ab	1.5c
9	1.3fg	1.0g	1.2cd
10	1.0g	1.5def	1.5c
11	1.6cdef	1.2fg	0.3ef
12	2.2a	1.5def	2.0b
13	2.0abc	0.5h	2.0b
14	2.1ab	2.4ab	2.0b
15	1.53 def	2.3ab	2.2ab
16	1.5def	1.4efg	2.5a
17	1.4efg	2.2abc	2.0b
18	1.0g	2abc	2.1ab
19	1.86abcd	1.8cde	1.0d
20	2.0abc	1.9bcd	1.5c
control	0.0h	0.0i	0.0f
LSD 0.05	464 .0	0.429	0.42

Means within a column with the same letter are not significantly different (P>0.05)

Identification of bacterial isolates:

According to the results of the morphological, biochemical characteristics of bacterial isolates and also, biolog system the tested bacteria are belong to members of the genus Bacillus. They were identified as *B. subtilis* (isolate No.1),*B.amyloliquefaciens* (isolate No.2), *B.weihenstephanensis* (isolate No.3*)* and *B. pseudomycodies* (isolateNo.5)Table2.

***In vivo* experiments:**

Data in Table (3) indicated that all treatments with *Bacillus* spp. decreased damping-off and increased healthy plants infected with *F. solani* compared with the control treatment. The highest percentage of survival plants was 86%by *B. amyloliquefaciens* followed by *B.weihenstephanensis, B. subtilis* and *B.pseudomycodies* (82%, 70% and 66%) respectively. Similar results were found by Caroline *et al*. (2013) they found that *B. amyloliquefaciens* inhibited the growth of *F. solani* 95.2% followed by*B. cereus, B. pumilus* and *B. subtilis*. They added that four *Bacillus* spp.are very effective biocontrol agents and should be harnessed for further biocontrol applications.

Table 2. Some morphological and biochemical characteristics of the most effective biocontrol bacterial isolates

Tests	Bacterial isolates No.			
	1	2	3	5
Gram stain	+	+	+	+
Spore forming	+	+	+	+
Motility	+	+	-	-
Capsule formation	+	+	-	-
Measurement (micron)	(1x4)	(4x1.2)	(4x1)	(4x1)
Indole production	-	-	-	-
Voges proskauer	+	+	+	+
Methyl Red	+	+	+	+
Citrate utilization	+	+	+	+
Catalase production	+	+	+	+
Starch hydrolysis	+	+	+	-
Casein hydrolysis	+	+	+	+
Gelatin liquefaction	+	+	+	-
Cellulase production	-	-	-	-
Glucose assimilation	+	+	+	+
Manitol assimilation	+	-	-	+
Sucrose assimilation	+	+	+	+
Fructose assimilation	+	+	+	+

Table 3. Effect of *Bacillus* spp. against *F.solani* in controlling sugar beet seedling damping-off and survival plants under greenhouse condition:

Bacterial isolates	Damping – off (%)		Survival plants (%)
	Pre-emergence	Post-emergence	
B. subtilis	17.5 bc	30 b	70 b
B.amyloliquefaciens	10 c	15 c	86 a
B.weihenstephanensis	10 c	17.5 c	82 a
B.pseudomycodies	25 ab	35 b	66 b
Un treated control	32.5a	55 a	46 c
LSD 0.05	10.11	10.83	10.83

Means within a column with the same letter are not significantly different (P<0.05)

In the case of *F.oxysporum,* data in Table (4) indicated that the highest percentages of survival plants infected with *F.oxysporum* and treated with *B. pseudomycodies* and *B. subtilis* were 90 and 80% ,respectively. On the other hand, the survival percentages were74% and 68% in infected plants treated with *B. amyloliquefaciens* and *B.weihenstephanensis* ,respectively. Sakalauskas *et al*. (2014) reported that *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *Serratia odorifera* and *Pseudomonas* spp. were found to express antagonistic *in vitro* activity against *Gaeumannomyces graminis* var. *graminis* DSM1463.

Table 4. Effect of *Bacillus* spp. against *F.oxysporum* in controlling sugar beet seedling damping – off and survival plants under greenhouse condition:

Bacterial isolates	Damping – off (%)		Survival plants (%)
	Pre-emergence	Post-emergence	
B. subtilis	15 b	20 cd	80 ab
B.amyloliquefaciens	17.5 b	25 bc	74 bc
B.weihenstephanensis	15 b	32.5 b	68 c
B.pseudomycodies	5 c	10 d	90 a
Un treated control	35 a	60 a	44 d
LSD 0.05	9.921	10.83	10.83

Means within a column with the same letter are not significantly different (P<0.05)

In the case of *F. dimerum,* data in Table (5) indicated that the highest percentage of survival plants infected with *F. dimerum* was 72% by *B. subtilis* followed by *B. pseudomycoides, B. amyloliquefaciens* and *B.weihenstephanensis* (70, 68 and 66%), respectively. Similar results were found by Abd-Alla *et aI*. (2003) they observed that *Bacillus subtilis* has been recommended as an essential biological control agent against many fungal pathogens.

Table 5. Effect of *Bacillus* spp. against *F.dimerum* in controlling sugar beet seedling damping–off and survival plants under greenhouse conditions:

Bacterial isolates	Damping – off(%)		Survival plants (%)
	pre - emergence	Post-emergence	
B. subtilis	12.5b	25b	72a
B. amyloliquefaciens	7.5b	30b	68a
B. weihenstephanensis	15b	32.5b	66a
B.pseudomycodies	17.5b	27.5b	70a
Control	35a	52.5a	48c
LSD 0.05	14.69	14.16	16.16

Means within a column with the same letter are not significantly different (P<0.05)

Production of antagonistic compounds:

Data in Table (6) showed the chemicals and enzymes produced by *Bacillus* spp.which might be involved in the antagonistic effect. Results indicated that all *Bacillus* isolates were negative for HCN. Similar results were reported by Singh *et al*. (2008). Data on IAA production revealed that all the *Bacillus* spp. produced IAA from tryptophan, *B. weihenstephanensis* and *B. subtilis* have the highest absorbance from the test tube having 3mg tryptophan while *B.amyloliquefaciens* and *B.pseudomycodies* had the least absorbance from the 3 mg test tube. This is similar to production of auxin which is the commonest form of IAA by *B. amyloliquefaciens* KPS46 which also supported growth of soybean (Buensanteai *et al*., 2008).All *Bacillus* isolates were negative for cellulase and chitinase production Broad –spectrum antifungal activity that may be production of multiple degradative enzymes and antimicrobial factors (Jeffrey, *et al*., 2007)

Table 6. Production of antagonistic properties from *Bacillus* spp.

Bacterial isolates	HCN	IAA µg/ml		Cellulase production	Chitinase production
		1mg tryptophan	3mg tryptophan		
B. subtilis	-	1.60	1.92	-	-
B. amyloliquefaciens	-	0.29	1.05	-	-
B.weihenstephanensis	-	2.51	3.28	-	-
B.pseudomycodies	-	0.24	0.92	-	-

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تأثير التضاد لأنواع جنس الباسيلس فى مقاومة الذبول الفيوزاريومى فى بنجر السكر

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تم فى هذه الدراسة عزل 20 عزلة بكتيرية من المجال الجذرى لأصناف بنجر السكر في مصر،وتم اختبارالتضاد الحيوى فى المعمل ضد ثلاثة فطريات مسببة للذبول الفيوزاريومى لنبات بنجر السكروهى فيوزاريوم سولاني فيوزاريوم أوكسيسبوريوم، و فيوزاريوم ديمريم ..اشارت النتائج في المختبر ان جميع العزلات البكتيرية أعطت نتائج متشابهة .تم تعريف اكفأ العزلات بالاختبارات القياسية وقد وجد أن أكفأ اربع سلالات كانت تنتمي الى جنس الباسلس وهي:الباسلس ساتلس والباسلس اميلوليكويفاشنس والباسلس وينستيفانسيس والباسلس سيدوميكودس وقد اظهرت السلالات نتائج متباينة فى الصوبة فكانت كما يلى: نسبة النباتات الحية بعد المعاملة بجنس الباسلس اميلوليكويفاشنس (86%) فى حالة المقاومة لفطر الفيوزاريم سولانى اما جنس الباسلس سيدوميكودس والباسلس ساتلس فكانت اكفأ الاجناس فى مقاومة كل من الفيوزاريم اوكسبوريم والفيوزاريم دايمريم فكانت النسبة72و 90% على التوالى. أظهرت سلالات الباسلس النتائج الاتية فى ميكانيكية المقاومة فقد فشلت كل اجناس الباسلس فى انتاج سيانيد الهيدروجين بينما أعطت نتيجة ايجابية فى انتاج اندول حمض الخليك من التربتوفان .بالنسبة لانتاج انزيم الكيتينيز والسليوليز لم تنتجة العزلات الاربعة،وعنداستخدام جنس الباسلس لمعاملة بذور بنجر السكرأدى الى تخفيض النسبة المئوية للذبول الفيوزاريومى تحت ظروف الصوبة الزراعية.اثبتت الزراعة ان الميكروبات التابعة لجنس الباسلس فعالية فى المقاومة الحيوية وينبغي استخدامها من أجل المزيد من تطبيقات المقاومة الحيوية.