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BACILLUS SUBTILIS AS BIOAGENT USED TO CONTROL *CERCOSPORA* SUGAR BEET LEAF SPOT DISEASE

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ABSTRACT: In Egypt, *Cercospora beticola* Sacc., causes leaf-spot, the most serious diseases on sugarbeet, that dramatically reduces the yield quantity and quality. The availability of fungicides from different classes has become a crucial component in controlling the disease due to environmental hazards. Twenty one *Bacillus subtilis* isolates previously isolated from sugarbeet phyllospher at different Egyptian governorates significantly reduced Cercospora leaf spot (CLS) severity compared to the untreated control plants under greenhouse and field conditions for three successive growing seasone (2005/2007). Data showed that non significant difference was detected between the tested *B. subtilis* isolates and the fungicide treatment. Data of the third season revealed that the lowest severity was recorded with SHR-27-5 isolate (0.5%) while the highest severetity (1.0%) was detected with DK-36-3B and KSH-43-15 isolates compared to the untreated control treatment (2.67%). Data also showed that disease reduction using some selected *B. subtilis* isolates ranged from 62.55-74.91%. The present study showed the efficiency and importance of using bioagent active isolates of *B. subtilis* for controlling CLS disease on sugar beet in Egypt.

Key words: *Cercospora beticola*, sugar beet, leaf spot, *Bacillus subtilis*. and bioagent.

INTRODUCTION

Leaf-spot of sugar beet, caused by *Cercospora beticola* Sacc. is the most important foliar diseases in warm and humid areas of Egypt (El-Kholi, 1995). Sugar beet losses caused by Cercospora leaf spot

(CLS) ranging from 25 to 50% (Byford, 1996). In addition, this disease reduced yield quality and quantity. The control of leaf spot disease by extensive fungicide application added costs to producers and repeatedly has selected for fungicide-tolerant *C. beticola* strains (Weiland and Koch, 2004). The availability of fungicides from different classes has become a crucial component in the control of CLS on sugar beet. Reports have been made of *C. beticola* field isolates exhibiting resistance to fungicides in the benzimidazole class (Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974; Weiland and Halloin, 2001) and increased tolerance to organo-tin fungicides; Bugbee, 1995, as well as triazole classes (Karaoglanidis *et al.*, 2000). With the recent concern regarding pesticide residues on plants, there is a need for alternative disease-management practices that will reduce risk to the environment.

Several researchers showed the importance of using *Bacillus subtilius* as a biocontrol agent from the phyllosphere to control Cercospora leaf spot of sugar beet (Kiewnick and Jacobsen, 1998 and Collins and Jacobsen, 2003), and other diseases. *Bacillus* spp. has been used to control a number of leaf spot and post harvest diseases due to forming endospores facilitates long-term storage, easy commercialization and its resilient structures capable of surviving desiccation, heat, oxidizing agents, as well as UV and γ radiations (Chakraborty *et al.*, 1994, Setlow, 1995 and Bargabus *et al.*, 2004). Bacilli also produce a range of antibiotic compounds that are inhibitory to fungi (Utkhede and Sholberg, 1986), bacteria (Zuber *et al.*, 1993), and insects (Aranda *et al.*, 1996). Critical in choosing *Bacillus* spp. isolate as a biocontrol agent (BCA) due to its ability to form an endospore, its capacity to use chitin and β-glucan as substrates, inhibit the growth of *C. beticola in vitro*, and affect disease control better than 40% in field and glasshouse studies. Bargabus *et al.*, (2002) evaluated *Bacillus pumilus* and *Bacillus mycoides*, isolated from sugar beet phyllosphere, the bacteria are a non-pathogenic, phyllosphere inhabiting, and cause a reduction in Cercospora leaf spot (*Cercospora beticola* Sacc.) of sugar beet from 38 to 91 % in both glasshouse and field experiments. Cooksey and Moore, (1980) isolated a highly antagonistic *Bacillus megaterium* from the

phyllosphere of jute against *C. corchori*.

This work aimed to eveluated several isolates of *B. subtilis*, isolated from sugar beet phyllosphere compared with Topsin M-70 for control Cercospora leaf spot disease of sugar beet by greenhouse and field experiments.

MATERIALS AND METHODS

Source of Cercospora Isolate

A virulent isolate of *C. beticola* (isolated from Kafr El-Sheikh) was obtained from the Cercospora collection in the Department of Sugar Crops Pest and Diseases, Sugar Crops Research Institute, ARC, Giza.

Source of Phyllospheric Microorganisms

Twenty one bacterial isolates of *Bacillus subtilis*, with an *in vitro* antagonistic activity against *Cercospora beticola*, formally isolated from sugar beet phyllosphere drived from five governorates, Behera (BH), Dakahleia (DK), Giza (GZ), Sharkeia (SHR) and Kafr El-Shaikh (KSH) were obtained from Sugar Crops Pathology Lab, Sugar Crops Research Institute, Agriculture Research Center, Giza-Egypt.

Preparation of *Bacillus subtilis* Bioagent

Bacillus subtilis isolates were grown on nutrient broth medium (pepton 10g, yeast extract 5g, Nacl 5g) for 3 days on rotary shaker at 28°C then the cell density adjusted to $3x10^6$ cfu/ml by distilled water according to the method described by Douglas *et al.* (2003).

Preparation of *C. beticola* Inoculum

Colonies of *C. beticola*, in Petri dishes, were flooded with 10 ml sterile distilled water and rubbed with a glass rod. One mililiter of this suspension used to inoculate sugar beet leaf broth (SBLB) medium then incubated at 28°C under a 16-hr photoperiod (fluorescent light, 2000 Lux) for 30 days. After incubation, cultures were blinded separately in a partial sterilized (using 70% ethanol) electrical blinder for 5 minutes then diluted with sterilized distilled water to reach 3×10^4 cfu/ml (Vereijssen *et al.*, 2003 and Esh, 2005).

Inoculation of Sugar Beet Plants

Sugar beet variety (Raspoly), recorded as a susceptible variety, (El-Kholi, 1995 and Esh, 2005) was used in both greenhouse and field experiments for three agricultural seasons (2004/2005,

2005/2006, and 2006/2007). Greenhouse experiment was conducted in Sugar Crops Research Institute, at Giza, Egypt. Sugar beet plants were grown in 30 cm diameter pots filled with 3kg (sand: peat moss: clay soil) (1:1:1). The experiment designed in a complete randomized block design with five replicates. The field experiment was conducted in an experimental field located at Sakha Research Station, Kafr El-Sheikh Governorate. Plot area was $21m^2$ with five rows for each. The experimental design used was complete randomized design with three replicates (plots) for each treatment.

Sugar beet plants (16 weeks old) were sprayed by the tested *B. subtilis* isolates, 2 times before inoculation with *C. beticola* in 7 days intervals. One week after the last bacterial treatment, the conidial suspension of *C. beticola* 3×10^4 cfu/ml was atomized on sugar beet leaves from all directions until run-off. (Vereijssen *et al.*, 2003 and Esh, 2005).

In the greenhouse, after inoculation with *C. beticola*, plants were irrigated and covered with transparent plastic bags to serve as a moist chamber and the greenhouse fog system was kept running for 5-days. Both procedures were used to increase the greenhouse humidity to above 90%. After 5 days, the plastic sheets were removed, and plants were kept on a bench to allow for disease development. On the other hand, field inoculation was done using the same method used in the greenhouse, but the plants were left for the natural environmental conditions without covering the plants with plastic bags after the inoculation with *C. beticola* (Esh, 2005). In both greenhouse and field experiments an additional 2 treatments were conducted instead of the bacterial isolate spray. The first treatment carried out with water to serve as a negative control and the second treatment with the recommended CLS fungicide (Topsin M-70) that was used to serve as a positive control. The results of Cercospora severity was calculated according to Battilani key of severity as shown in Table 1 (Battilani *et al.*, 1990). In the field experiment the results only recorded for the inner three rows of each plot.

Statistical Analysis

Data were statistically analyzed by analysis of variance according to Snedecor and Cochron, (1982) using SPSS system version 8, (1997).

RESULTS AND DISCUSSION

The present experiments were carried out to evaluate the biocontrol activities of 21 isolates

Table 1. Battilani key for disease assessment of *Cercospora* leaf spot disease on sugar beet. Cited from Vereijssen *et al.* (2003)

Infection degree	Symptoms description	Infection degree	Symptoms description
0	Healthy foliage	3	Fully and almost fully grown leaves show several Coalesced necrotic areas of 1-2 cm diameter, that don't lead to large necrotic areas.
0.5	A single isolated spot on some leaves	3.5	Some 2-4 outer leaves show relatively large necrotic areas (20 – 30% of the leaf area).
1	50% of the outer leaves (fully grown or old) show one to a few spots (20). Coalescence of maximum 2 spots can appear.	4	For the first time some leaves (2-8) show 80 to 100% severity.
1.5	Outer leaves (50% of foliage) show 20 to 200 spots per leaf. Coalescence of maximum 2 spots can appear.	4.5	The entire foliage is strongly affected.
2	Nearly all outer leaves are affected by several spots, still isolated. Coalescence of maximum 2 spots can appear.	5	The original foliage is completely destroyed.
2.5	Some (2-4) outer leaves show Coalescence of spots to necrotic areas. First spot appear on inner leaves.	6	For every week scale, 5 continuous 0.5 is added. This phase shows flushes of growth, which can be affected in turn.

Bacillus subtilis isolated from sugar beet phyllosphere. In term of using biological control agents for foliar application, Raj *et al.* (2005) reported that foliar spray was found to be a more efficient delivery method than seed or slurry treatment. Data shown in Table 2 show that the tested isolates gave a different biocontrol activity against Cercospora leaf spot in both of greenhouse and filed experiments. All tested isolates significantly reduced CLS severity compared to the untreated control plants. Generally, it was noticed that the disease severity in the greenhouse was always higher than in the field, this might be due to the favorable conditions for *C. beticola* spores to germinate and penetrate the infection sites were much favouralile under greenhouse conditions (High Humidity, optimal temperature for infection) rather than under field conditions. Also the biocontrol activities of the tested *B. subtilis* isolates differed in both greenhouse and field experiments. These results in agreement with those reported by Knudsen and Hudler, (1987) and Peng and Sutton (1991) who reported that the field applications of biocontrol agents to reduce preharvest diseases have not often met with much success due to the environment conditions. Also Schisler *et al.* (2002) preformed a comparison study on the biological control activities of *B. subtilis* and other bioagents under the field and greenhouse conditions. They found that the relative performance indices for used antagonists calculated from greenhouse and field results demonstrated that the bioassay location influenced the relative performance of antagonists.

In growing season 2004-2005, the greenhouse experiment, revealed that isolates KSH-43-15, DK-36-3B, SHR-33-7b, KSH-43-11, KSH-21-16, KSH-43-2, SHR-27-17, SHR-27-5, KSH-39-2 and KSH-43-17 decreased the disease severity to (0.70%, 0.80%, 1.00%, 1.10%, 1.67%, 2.00%, 2.00%, 2.00%, 2.33% and 2.33%, respectively) compared to the untreated control treatment which recorded 4.66% with no significant difference with the fungicide (Topsin M-70) treatment which recorded 0.8. These results are in the harmony with those reported by Tronsmo and Ystaas 1980, Korsten, *et al.*, 1997, Brewer and Larkin 2005 and Maketonl *et al.*, 2008 who found that some of the biological control treatments were equivalent to commercial fungicides,

Table 2. Effect of spraying sugar beet plants with the tested *Bacillus subtilis* isolates twice before infection on CLS incidence and the percentage of disease inhibition compared to the untreated control, under greenhouse and field conditions (season 2004-2005)

Bacillus subtilis isolates	First season 2004-2005			
	Greenhouse		Field	
	Disease severity	% of disease inhibition	Disease severity	% of disease inhibition
BH-45-2A	3.33	28.54	0.82	75.88
DK-17-15	2.67	42.70	1.15	66.18
DK-36-3B	0.80	82.83	1.04	69.41
GZ-3-11	3.00	35.62	1.16	65.88
KSH-21-16	1.67	64.16	1.22	64.12
KSH-21-21A	2.67	42.70	1.67	50.88
KSH-21-7	2.67	42.70	1.73	49.12
KSH-39-1A	3.33	28.54	0.93	72.65
KSH-39-2	2.33	50.00	1.24	63.53
KSH-42-23	3.33	28.54	1.52	55.29
KSH-43-11	1.10	76.39	1.63	52.06
KSH-43-d	3.00	35.62	0.72	78.82
KSH-43-15	0.70	84.98	1.34	60.59
KSH-43-17	2.33	50.00	0.92	72.94
KSH-43-2	2.00	57.08	1.85	45.59
KSH-43-B	3.33	28.54	1.11	67.35
KSH-44-9	3.33	28.54	1.80	47.06
SHR-27-17	2.00	57.08	1.32	61.18
SHR-27-5	2.00	57.08	1.20	64.71
SHR-33-7b	1.00	78.54	1.61	52.65
SHR-38-8	3.33	28.54	0.83	75.59
Topsin M70	0.80	82.83	1.00	70.59
Untreated Control	4.66		3.40	
L.S.D at 0.05 =	**1.7**		**0.35**	

suggesting synergistic effects. This reduction of severity is ranged from 50% (KSH-39-2 and KSH-43-17) to 84.98% (KSH-43-15). The other tested isolates gave a lower reduction in CLS severity ranged from 28.54% (SHR-38-8) to 42.70 % (DK-17-15).

On the other hand, the results of field experiment showed a different order in biocontrol activity. Isolate KSH-43-d significantly decreased CLS severity compared to the fungicide treatment. While the other isolates BH-45-2A, SHR-38-8, KSH-43-17 and KSH-39-1A decreased the disease severity to (0.72, 0.83, 0.92 and 0.93, respectively) with no significant difference with the fungicide treatment which recorded 1.0% while the untreated control recorded the highest severity 3.4.

The highest reduction in disease severity recorded by the isolate KSH-43-d (78.82%) while the lowest reduction in disease severity recorded by the isolate KSH-43-2 (45.59%).

The highest ten *B. subtilis* isolates reduced CLS severity in the first season of greenhouse experiment were chosen in the second season based on that the environmental conditions and the stress of the disease were much higher and controlled than in the field. Romero *et al.* (2007) reported that fungal and bacterial biocontrol agents against cucurbit powdery mildew performed better under greenhouse conditions of high relative humidity (90–95% RH).

Data in Table 3 show that all the tested isolates significantlly decreased CLS severity compared to the control treatment. Isolate KSH-43-15 recorded the lowest other treatments and disease severity (0.63%) compared with the untreated control .Both of KSH-39-2 and KSH-43-17 isolates recorded 1.25% disease severity. The highest reduction in CLS disease compared to the control was 77.27% (KSH-43-15) and the lowest was 54.55% (KSH-39-2 and KSH-43-17). It is worthy to mention that, all the tested isolates didn't show any significant difference with the fungicide treatment (0.75%). As in season one it was noticed also that the overall severity of the CLS disease in the greenhouse was always higher than in the field experiment. On the other hand, data from field experiment showed that all the tested isolates significantly decreased the disease compared with the control treatment while no significant difference detected between the isolates or between

the fungicide treatment and the bacterial isolates. The results are in agreement with those reported by Bargabus *et al.* (2002) who evaluated *Bacillus pumilus* and *Bacillus mycoides*, isolated from sugar beet phyllosphere and caused a reduction in Cercospora leaf spot of sugar beet from 38 to 91 % in both greenhouse and field experiments.

This experiment was conduced in the third season (2006/2007) to evaluated the most efective 6 tested isolates in second season under feild conditions. Data in Table 4 show that no significant differences were detected among the tested isolates and the fungicide treatment. The lowest severity was recorded with the isolate SHR-27-5 (0.5%) while the highest severity 1.0% was recorded by DK-36-3B and KSH-43-15 isolates compared to the untreated control treatment (2.67%). Data also showed that the disease reduction by using the selected *B. subtilis* isolates ranged from 62.55 to 74.91%. The obtained results are in harmony with those reported by (Kiewnick and Jacobsen, (1998), Bargabus et al., (2002) and Collins and Jacobsen, (2003) who mentioned that different Bacillus species isolated from the phyllosphere caused potential to control Cercospora leaf spot of sugar beet. Several researchers reported different mechanisms explaining the biocontrol action of *Bacillus subtilius*. Utkhede and Sholberg, (1986) reported that Bacilli produce a range of antibiotic compounds that are inhibitory to fungi. On the other hand, its capacity to use chitin and β-glucan as substrates, inhibit the growth of *C. beticola in vitro*, and affect disease control more than 40% in field and glasshouse studies. Bargabus *et al.* (2002).

The present research work showed the efficiency of using biocontrol active isolates of B. subtilis for controlling CLS disease. The data showed non significant differences between the tested biocontrol agents and the recommended fungicide Topsin M-70 under greenhouse and field conditions.

Future research should concentrate into the bioagent metabolites produced and used mode of action for them when they are applied in combination, since individual application of these bioagents could not control the plant disease. Although, greenhouse conditions differ from field plantations, this research work showed feasibility in employing biological control agents for plant diseases, further eliminating the need for chemical pesticides.

Table 3. Effect of spraying sugar beet plants with the ten selected *Bacillus subtilis* isolates twice before infection on CLS incidence and the percentage of disease inhibition compared with the untreated control, under greenhouse and field conditions in season 2005-2006

B. subtilis isolates	Second season 2005-2006			
	Greenhouse		Field	
	Disease severity	% of disease inhibition	Disease severity	% of disease inhibition
DK-36-3B	0.75	72.73	0.16	94.18
KSH-21-16	1.00	63.64	0.50	81.82
KSH-39-2	1.25	54.55	0.75	72.73
KSH-43-11	0.88	68.18	0.16	94.18
KSH-43-15	0.63	77.27	0.42	84.73
KSH-43-17	1.25	54.55	0.58	78.91
KSH-43-2	1.00	63.64	0.58	78.91
SHR-27-17	1.00	63.64	0.41	85.09
SHR-27-5	1.00	63.64	0.83	69.82
SHR-33-7b	0.75	72.73	0.50	81.82
Topsin M-70	0.75	72.73	0.16	94.18
Untreated Control	3.75		2.75	
L.S.D at 0.05	0.52		0.76	

Table 4. Final evaluation of biocontrol activities of the six selected *Bacillus subtilis* isolates on Cercospora leaf spot incidence and the percentage of disease inhibition compared with the untreated control, under field conditions in 2006-2007 growing season

B. subtilis isolates	Disease severity	% of disease inhibition
DK-36-3B	1.00	62.55
KSH-43-11	0.83	68.91
KSH-43-15	1.00	62.55
SHR-27-17	0.83	68.91
SHR-27-5	0.50	81.27
SHR-33-7b	0.67	74.91
Topsin M-70	0.67	74.91
Untreated Control	2.67	
LSD at 0.05	0.58	

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استخدام *باسيلوس ساتليس* (*Bacillus subtilis*) كعامل حيوي لمقاومة مرض التبقع السركسبورى فى بنجر السكر

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يعتبر الفطر سركوسبورا بتيكولا أحد أهم و أخطر المسببات المرضية التي تصيب نباتات بنجر السكر محدثا مرض تبقع الأوراق والذي يؤثر بالسلب في إنتاجية المحصول كمـا و نوعا. و يعد انتشار استخدام المبيدات الفطرية بأنواعها المختلفة في مقاومة المرض يشكل حرجا بما له من أخطار مباشرة علي البيئة . تم استخدام ٢١ عزله *باسـيلوس سـاتليس* ***Bacillus subtilis*** المعزولة من فيلو سفير بنجر السكر وذلك من أمـاكن مختلفـة فـي المحافظات المنتجة للبنجر.وقد تم تقيم قدرة هذه العزلات التضادية على فطر سركوسـبورا بتيكولا على مستوى المعمل والحقل خلال ثلاث مواسم زراعية متتالية . وقد أظهرت نتائج اختبارات قدره العزلات على تثبيط حدوث مرض تبقع الأوراق السركسبورى تأثير معنـوي فى خفض شده الإصابة مقارنه بالغير معامل بالبكتريا وذلك على مستوى تجارب الصـوبه والحقل. اظهر التقييم النهائي للعزلات خلال الثلاثة مواسم زراعية انه لم تظهر أية فـروق معنوية بين العزلات والمبيد الفطري المستخدم (توبسين-٧٠). و كذلك دلت النتائج علي أن أقل شده أصابه سجلت من العزله SHR-27-5 حيث أعطت(٠,٥%) شده أصـابه بينمـا أعلى شده أصابه كانت من كلا من DK-36-3B و KSH-43-15 أعطت (١%) وذلك مقارنه بتجربة المقارنة الغير معامله بالعزلة و التي سجلت شـدة أصـابه مقـدارها (% ٢,٦٧). أظهرت النتائج أن استخدام عزلات البكتيريا *باسيلوس ساتليس* أدي إلي خفـض شده المرض بدرجه تراوحت بين.74.91-62.55%. أثبتت نتـائج هـذا البحـث كفـاءة البكتيريا *باسيلوس ساتليس* فى مقاومه مرض تبقع الأوراق السركسبورى علـى نبـات بنجر السكر فى مصر.