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SYSTEMIC RESISTANCE IN SUGAR BEET ELICIATED BY NON-PATHOGENIC, PHYLLOSPHERE-COLONIZING *Bacillus pumilus* AND *B. subtilus* AGAINST THE PATHOGEN *Cercospora beticola Sacc.*

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ABSTRACT

A total of 10 *Bacillus subtilis* (7) and *Bacillus pumilus* (3) isolates from sugar beet phyllosphere were evaluated for its biocontrol activity against sugar beet cercospora leaf spot disease (CLS) under greenhouse and field conditions compared to the fungicide Topsin M70. PR-related proteins, chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase as well as indol acetic acid were determined in treated and untreated sugar beet plants. Under greenhouse conditions the reduction in disease severity caused by *B. subtilis* and *B. pumilus* treatments ranged from 73.33 - 80% and 80 - 83% respectively. Under field conditions the reduction ranged from 56.18 to 81.27% and 62.55 to 81.27% from *B. subtilis* and *B. pumilus* respectively. The levels of the determined PR proteins significantly increased in all the treated plants either with *B. subtilis* or *B. pumilus*. In addition, the levels of IAA were higher in the treated plants compared to the untreated plants. The sucrose percentage increased in the treated plants compared to the infected untreated control. The present study suggested the role of *Bacillus subtilis* and *Bacillus pumilus* in inducing plant acquired resistance.

Keywords: *Cercospora beticola, Bacillus subtilis, Bacillus pumilus*, cercospora leaf spot, biological control, induced acquired resistance.

INTRODUCTION

Epiphytic microbs has been documented for numerous phyllosphere and rhizosphere inhabiting organisms and/or stimulating the induction of systemic resistance mechanisms within the plant (Bargabus *et al.* 2002). Understanding the behavior of a biological control agent (BCA) in the environment in which it is expected to perform may lead to improved performance of the BCA. The mechanisms operating in controlling cercospora leaf spot with bacillus isolate BacB are thought to include antibiosis, induced systemic resistance (ISR), competition, and hyperparasitism. Antibiosis and hyperparasitism have been demonstrated *in vitro* (Jacobsen *et al.* 2003), while ISR has been demonstrated in growth chamber studies (Braun-Kiewnick *et al.* 1998). Kloepper *et al.* 2004 reported strains of *B. subtilis* suppress diseases by inducing host defenses. However, it is likely that the most effective biological control strains will act via multiple mechanisms. For example, *B. subtilis* strain S499 can inhibit fungal pathogens directly through antibiosis, but it was also found to induce resistance to foliar pathogens when it was applied to the plant root (Ongena

et al. 2005 and 2007). Such induction of enhanced defensive capacity can be systemic as seed-treatment with bacteria at the time of seeding was shown to trigger protective effects on aboveground parts (Van Loon *et al.* 1998). The induced resistance constitutes an increase on the level of basal resistance to several pathogens simultaneously, which is one of the benefits under natural conditions where multiple pathogens exist (Van Loon and Glick, 2004). Bargabus *et al.* 2002 found that the biological control agent, *Bacillus mycoides* isolate Bac J (BmJ), is capable of inducing SAR in sugar beet. Grant and Loake (2000) and Van Peer *et al.* (2000) found two molecular markers associated with pathogen-induced SAR, β-1,3-glucanase and chitinase, induced during BmJ–plant interactions. In addition, BmJ has been shown to induce biphasic production of active oxygen species (AOS), they also found in incompatible plant–pathogen interactions, another instance where systemic acquired resistance is induced.

The aim of this study is to verify that induced resistance is another mechanism through which *B. pumilus* and *B. subtilus* can suppress cercospora leaf spot under both greenhouse and field conditions.

MATERIALS AND METHODS

Effect of biocontrol agents on CLS incidence under greenhouse and field conditions.

Biocontrol agents:

In this study, 10 biocontrol active isolates of *B. pumilus* (KSH-21-18A, KSH-43-15 and KSH-21-17A) and *B. subtilus* (SHR-27-5, DK-36-3B, SHR-27-17, KSH-43-11, KSH-43-G, SHR-27-11 and SHR-33-7b) originally isolated from sugar beet leaves from Kafr-Elshaikh (KSH), Sharkeia (SHR) and Dakahleia (DK) governorates during 2008. All the tested isolates were antagonistic to *C. beticola* and identified in the Sugar Crops Diseases Research Dept., SCRI, ARC, Egypt (Taghian *et al.* 2008).

Preparation of the phyllospheric microorganisms inoculum.

Bacterial isolates were grown in 250 ml nutrient broth at 28 ±2°C for 48 hr on a rotary shaker. The bacterial suspensions then diluted by sterilized distilled water up to 1000 ml with adding 0.1 ml Tween-80 as described by Vereijssen *et al.* (2003) and Esh, (2005) and adjusted to $1x10^6$ cfu/ml to be ready to spray on the experimental plants.

Preparation of *C. beticola* inoculum.

Colonies of *C. beticola* 30-days old culture were flooded with 10 ml sterile distilled water and rubbed with a glass rod. 500 ul of this suspension used to inoculate sugar beet leaf broth (SBLB) then incubated at 28 ±2°C under a 16-hr photoperiod (fluorescent lights) for 30 days. After incubation, cultures were blinded separately in a partial sterilized (by ethanol 70%) electrical blinder for 5 min. The fungal suspension diluted by distilled water to reach 3×10^4 cfu/ml to spray on the experimental plants. (Vereijssen *et al.* 2003 and Esh, 2005).

Inoculation of sugar beet plants:

Greenhouse trials.

Sugar beet plants variety Kawimera 8 weeks old (grown in 30 cm diameter pots) were treated by the tested phyllospheric bacteria two folds before inoculation with *C. beticola* in 7 days intervals. One week after the last treatment the conidial suspension 3 x 10^4 cfu/ml of *C. beticola* was prepared and atomized on sugar beet leaves from all directions until run off. After inoculation, 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 done to increase the greenhouse humidity to above 90%. After 5 days, the plastic sheets removed, and plants kept on the bench to allow disease development (Esh, 2005). Three replicates (3 plants each) were used for each bacterial treatment with a positive control (untreated infected) and negative control (untreated uninfected).

Open field trials.

This experiment was conducted in Sakha Experimental Station, Kafr El-Shaikh governorate. The field divided to (3x12.5 meter) plots and each plot consists of six rows. The experimental design used was complete randomize design with three replicates (plots) for each treatment. The results only recorded for the inner four rows of each plot. The same procedures and sugar beet variety used in the greenhouse experiment were used in the field trails, with one exeption that the plants were left for the natural environmental conditions after inoculation with *C. beticola* without covering the plants with plastic bags.

The results of cercospora severity (DS_{AGR}, Agronomica diagram) was calculated according to Battilani key (0-5 scale) of severity (Battilani *et al.* 1990).

Roots of treated sugar beet plants were collected at the end of experiment (harvest time, 200-day) and sucrose polarmetrically determined (Pol. %) on a lead acetate extract of fresh macerated roots according to (Le-Docte, 1927).

Effect of application with the bioagent on some compounds related to induce resistance in sugar beet plants.

Preparation of enzyme source.

From the green house experiment, treated and untreated sugar beet leaves samples with the bioagent were grounded with liquid nitrogen (L-N_2) as fine powder with a mortar. One gram of the grounded tissues was mixed with one ml of extraction buffer phosphate buffer pH 6.0 according to (Bollage *et al.* 1996). Samples were vortexed and centrifuged at 13000 r.p.m. for 15 min. under 4°C to remove cell debris. The clear supernatant (crude enzyme source) was collected and kept at -80°C for further study (Soltis and Soltis 1990).

Determination of indol acetic acid (IAA):

A colorimetric technique was performed using the Van Urk Salkowski reagent (1 ml of 0.5 M $FeCl_3$ and 50 ml of 35% $HClO_4$ in water), 1 ml of the extract mixed with 2 ml of the reagent and incubated for 25 min. at room

temperature. The optical density was measured using the wavelength 530 nm. A standard curve of pure IAA (Sigma-Aldrish) was used (Bric *et al.* 1991).

Determination of chitinase:

A mixture of crude enzyme source (1 ml) and suspension of colloidal chitin (1 ml, 0.1% in 50 mM sodium acetate buffer, pH 5.0) was incubated at 38°C in a water bath with constant shaking. After 2 hr., the release of *N*-acetylglucosamine in the reaction mixture was estimated according to Reissig *et al.* (1955). The enzyme activity was determined using *N*-acetylglucosamine (Sigma) as the standard. Absorbance was measured at 660 nm using a spectrophotometer Milton Roy Spectronic 1201. One unit of chitinase is defined as the amount of enzyme producing 1 μmol *N*-acetylglucosamine/min in 1 ml of reaction mixture under standard assay conditions. Specific activity was expressed as units per milligram of protein.

Determination of β 1, 3 glucanase

The reaction mixture was the substrate laminarin (Sigma-Aldrich) (2.5% w/v) in 10 mM ammonium acetate, pH 6.0, and 1 mM Dithiothreitol (DTT). The reaction incubated at room temperature for 24hr. samples were assayed for the release of reducing sugars according to the Somogyi-Nelson method Nelson (1944) modified by Naguib (1964 and 1965). Absorbance was measured at 660 nm using a spectrophotometer Milton Roy Spectronic 1201. Standard curve of glucose was used as reference (Lim *et al.* 1991). β 1,3 glucanase activity was determined as 1 nmol of glucose released per minute per ml.

Determination of polyphenoloxidase.

Leaf samples were extracted according to the method described by Malik and Singh (1990) .The enzyme extract was prepared by grounding 5 g leaves in 0.1 M sodium phosphate buffer pH 7.0 (2 ml / g fresh weight), then centrifuged at 6000 rpm for 30 min. under 4 ^{o}C. The clear extract was collected, completed to 15 ml volume using phosphate buffer and used as crude enzyme source.

The reaction mixture contained 0.2 ml of crude enzyme source, 1 ml of phosphate buffer pH 7.0, 1 ml of 10^{-3} M catechol and complete with distilled water up to 6 ml .The reaction was incubated for 30 min. at 30 ^{o}C. One unite of polyphenol oxidase was expressed as the change in absorbance at 420 nm / 30 min. / 1 g fresh weight (Matta and Dimond, 1963).

Determination of peroxidase:

The reaction mixture extraction as described by Malik and Singh **(**1990**)** was contained 0.5 ml phosphate buffer pH 7.0, 0.2 ml enzyme source, 0.3 ml of 0.05 M pyrogallol, 0.1 ml of 1%(v/v) H_2O_2 and distilled water was added up to obtained 3 ml .The reaction mixture was incubated at 30 ^{o}C for 5 min. then the reaction stopped by adding 0.5 ml of 5 %(v/v) H_2O_2 (Kar and Mishra, 1976). One unit of peroxidase activity was expressed as the changes in absorbance at 425 nm / min. / 1 g fresh weight.

Statistical analysis

Data of the present work were statistically analyzed by analysis of variance according to Snedecore and Cochran (1982) using the ANOVA on Computer package MSTATC (Anonymous 1986).

RESULTS AND DISCUSSION

Effect of biocontrol agents on CLS incidence under greenhouse and field conditions

Data in Table (1) show that, all the tested bacterial isolates significantly decreased the CLS severity under greenhouse conditions compared to the control treatment.

Table (1): Efficiency evaluation of the selected efficient biocontrol activity phyllospheric bacterial on decreasing the severity of sugar beet Cercospora leaf spot disease on sugar beet variety Kawimera when sprayed twice (two weeks intervals) before artificial infection with *C. beticola* under greenhouse conditions.

	Bioagent	Disease severity (D.S.)	D.S. reduction (%)
B. subtilis	SHR-27-5	1	73.33
	DK-36-3B	0.75	80
	SHR-27-17	1	73.33
	KSH-43-11	0.87	76.67
	KSH-43-G	1	73.33
	SHR-27-11	0.88	76.53
	SHR-33-7b	0.75	80
B.pumilus	KSH-21-17A	0.75	80
	KSH-21-18A	0.75	80
	KSH-43-15	0.62	83.33
Topsin M70		0.75	80
Infected control		3.75	
Uninfected control		0	
LSD at 0.05		0.52	

The bacterial isolates KSH-43-15, DK-36-3B, KSH-21-17A, KSH-21-18A and SHR-33-7b recorded the lowest significant disease severity (0.62 for the first isolate and 0.75 for the others) compared to the untreated control treatment which recorded (3.75) and caused a percentage of disease severity inhibition ranged from 80.0 to 83.33%. The other tested isolates also significantly decreased the disease severity.

It is worthy to mention that there were no significant differences between the all tested isolates nor with the protectant fungicide treatment which recorded a disease severity (0.75).

Data in Table (2) show that, no significant difference detected between the tested bacterial isolates and the fungicidal treatment. The lowest severity recorded with the isolate SHR-27-5 and KSH-21-17A (0.5) while the highest disease severity was 1.17 recorded by the bacterial isolate SHR-27-11 compared to the untreated control treatment (2.67). No significant difference detected between the tested bacterial isolates and the fungicidal treatment (Table 2). The lowest severity recorded with the isolate SHR-27-5 and KSH-21-17A (0.5) while the highest disease severity (1.17) recorded by

the bacterial isolate SHR-27-11 compared to the untreated control treatment (2.67). The obtained data also showed that, the reduction in disease severity by using the selected bacterial isolates ranged from 56.18-81.27%.

Table 2: Efficiency evaluation of the selected efficient biocontrol activity phyllospheric bacterial on decreasing the severity of sugar beet Cercospora leaf spot disease on sugar beet variety Kawimera when sprayed twice (two weeks intervals) before artificial infection with *C. beticola* under field conditions

	Isolates	Disease severity (D.S.)	D.S. reduction (%)
B. subtilis	SHR-27-5	0.5	81.27
	DK-36-3B	1	62.55
	SHR-27-17	0.83	68.91
	KSH-43-11	0.83	68.91
	KSH-43-G	0.83	68.91
	SHR-27-11	1.17	56.18
	SHR-33-7b	0.67	74.91
B. pumilus	KSH-21-17A	0.5	81.27
	KSH-21-18A	0.67	74.91
	KSH-43-15	1	62.55
Topsin M70		0.67	74.91
Infected control		2.67	-
Uninfected control		0	-
LSD at 0.05		0.5	81.27

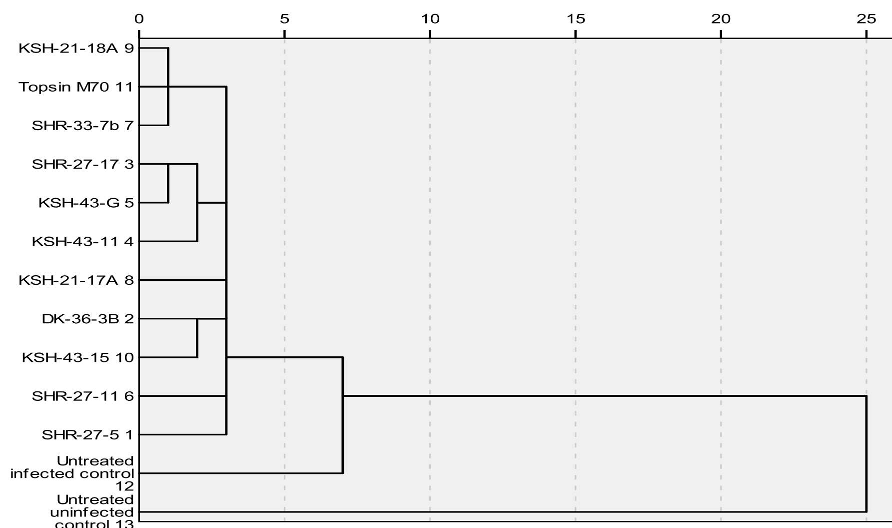

Fig.1: Dendrogram showing the combined data clustering of *C. beticola* disease severity recorded in greenhouse and field after two treatments with the selected *B. subtilis* and *B. pumilus* isolates before inoculation with the *C. beticola*.

The greenhouse and field evaluations of the selected putative biocontrol agents showed the efficiency of using biocontrol active bacterial isolates for controlling CLS disease .The data showed no significant differences between the tested biocontrol agents and the recommended fungicide Topsin® M70 under greenhouse and field conditions.

The Dendrogram (Fig.1) showing the combined data clustering of *C. beticola* disease severity recorded in greenhouse and field, after two folds treatment with the selected *B. subtilis* and *B. pumilus* isolates before inoculation with the pathogen, divided the treatments to two main groups the first group contained the untreated uninfected control while the second group divided to two subgroups the first one contained the untreated infected control while all the bacterial treatments (10) were under the second subgroup and divided to 6 groups. It is worthy to mention that the fungicide treatment and the bacterial treatments with KSH-21-18A (*B. pumilus*) and SHR-33-7B (*B. subtilis*) were under the same sub-sub group with no difference.

These results are in agreement with those reported by Brewer and Larkin, (2005) who found that, some of the biological control treatments reduced disease as well as the chemical control, suggesting synergistic effects. In addition, these results might be due to the use of the fungicide seven days before the inoculation with the pathogen. There are several fungicides currently labeled for use in controlling CLS. They fall into two categories, protectant fungicides that rely on direct contact with the pathogen, and systemics that are absorbed by the plant and distributed systemically. The protectant fungicides include an organo-metal, triphenyltin hydroxide (TPTH) (SuperTin®, AgriTin®), and the ethylene bis-dithiocarbamates (Maneb®, Mancozeb®, Manex® and Penncozeb®). The protectant fungicides offer consistent disease control but kill only germinating spores and have no ability to affect the fungus once infection has occurred. These fungicides are active against several biochemical sites, reducing the likelihood of resistance development in the pathogen. However, isolates of *C. beticola* tolerant to TPTH have been detected in the Sidney (Australia) growing area Jacobsen *et al.* (2003) as well as in Minnesota-USA (Bugbee, 1995). Both protectant and systemic fungicides will persist for approximately two weeks. However, systemic fungicides have “kick-back” activity that allows the curing of infections 24-72 hours after an infection has taken place. This allows for greater flexibility in the application timing of systemics. The systemic fungicides include the benzimidazole, thiophanate methyl (Topsin-M®), the triazole, tetraconazole (Eminent®), and the strobilurins, azoxystrobin (Quadris®) and trifloxystrobin (Gem® and Headline®). These systemic fungicides have very specific modes of action that allow for the development of resistance. Resistance development limits the number of applications recommended in each growing season and requires that steps be taken to reduce the development of fungicide resistance such as rotating or alternating with fungicides with different modes of action (Köller and Wilcox, 1999 and Karaoglanidis *et al.* 2000 and 2001).

Effect of application with the bioagent on some compounds related to induce resistance in sugar beet plants:

Determination of indol acetic acid IAA in treated sugar beet leaves:

Data presented in Table (3) show that, sugar beet plants response to the treatment with the biocontrole agents differed in the levels of IAA. The data showed a high significant difference between the IAA levels in the treated plants compared with the untreated healthy control and the untreated artificially infected control. The data also showed that, no correlation between the biocontrol agent treatment and biocontrol activity. It was noticed that, the levels of IAA in the untreated healthy control plants was lower almost two folds than the levels determined in the untreated infected plants. On the other hand, the data obtained showed that, the treatment with the biocontrol agent increased the levels of IAA in plant leaves more than the levels detected in both of the untreated healthy and infected control. The increase of IAA levels resulted by the treatment of sugar beet plants by some of the selected biocontrol bacterial were two to three folds higher than the levels recorded in the untreated healthy and infected control. In contrary of that, some other isolates decreased the level of IAA in the plants to the level of the untreated healthy control. It is worthy to mention that, the leave samples used in this experiment were collected from selected treatments which showed the highest biocontrol activity, in other words, from those treatments recorded the lowest disease severity in the greenhouse experiment.

Data in Table (3) also show that, the highest IAA levels were recorded when sugar beet plants treated with isolates SHR-27-17 (11.5647 mg/ml), KSH-21-17A (10.5133 mg/ml), SHR-33-7b (6.6584 mg/ml) and KSH-21-18A (3.6621 mg/ml) compared to the untreated healthy and infected control which recorded (1.080 and 2.425 mg/ml, respectively). The treatment with other tested bacterial isolates recorded levels of IAA which almost equal or slightly greater than the levels recorded in the untreated healthy and infected control.

Changes in the content of IAA in inoculated plants observed at the end of the experiment may attributed to the presence of bacteria in phyllosphere environment directly or, more likely, to the well-known ability of hormones to influence the rate of synthesis and decay of each other (Evans, 1984). The latter suggestion seems more likely, since IAA and amino buteric acid (ABA) accumulated later than cytokinins in treated plants. This suggests that, IAA and ABA content might have been modified by accumulation of cytokinins in inoculated plants. The presence of cytokinin producing microorganisms can therefore be expected to influence not only cytokinin content in treated plants but also that, of other hormones (Arkhipova *et al.* 2005).

Determination of peroxidase (PO) in treated sugar beet leaves:

Peroxidase levels in the untreated control of sugar beet plants dramatically decreased compared to the healthy control and those sugar beet plants treated with the selected biocontrol active bacterial isolates (Table 3). Again, like in IAA levels determination, it is noticed that, the levels of peroxidase resulted by the treatment of sugar beet plants increased by some

of the selected biocontrol agents were two to three folds higher than the levels recorded in the untreated healthy and infected control.

The data indicated that, treating sugar beet plants with the bacterial isolates SHR-27-11, KSH-21-18A and SHR-27-17 significantly increased the levels of peroxidase to (2.33, 2.28 and 2.24 unite/min. /g fresh weight respectively) compared to the healthy untreated control (1.75 unite/min. /g fresh weight) and the untreated infected control (0.75 unite/min. /g fresh weight). The treatments by the other tested bacterial isolates were significantly higher in peroxidase levels compared to the untreated infected control.

Determination of polyphenoloxidase (PPO) in treated sugar beet leaves:

The levels of PPO in treated sugar beet leaves (Table 3) significantly increased compared to the healthy untreated control in the same time they were significantly lower than the untreated infected control. The levels of PPO ranged from 0.17 to 0.6 unite/30 min./g fresh weight compared with the healthy untreated control (0.14 unite/30 min./g fresh weight) and the infected untreated control (unite/30 min. /g fresh weight).

Table (3): Determination of indol acetic acid (IAA) , polyphenoloxidase (PPO) as well as peroxidase (PO), chitinolytic and β1,3-glucanase activity in treated sugar beet plants (variety Kawimera) with the candidate phyllospheric bacterial two weeks before the Infection with *C. beticola*.

Isolates		IAA	PO	PPO	Chitinase	β-1,3 glucanase
		(mg/ ml)	unite/min./g fresh weight.	unite/30 min./g fresh weight	Enzyme activity as µM of glucose released / ml /hr.	
B. subtilis	**SHR-27-5**	1.98	1.35	0.6	300.5	68.6
	DK-36-3B	1.3142	1.33	0.57	251.6	34.9
	SHR-27-17	11.565	2.24	0.21	176.5	97
	KSH-43-11	1.6471	1.72	0.32	324.4	95.5
	KSH-43-G	1.1389	1.08	0.25	134.8	45.2
	SHR-27-11	1.1565	2.33	0.31	231.4	48.5
	SHR-33-7b	6.6584	2.25	0.29	229	108
B. pumilus	**KSH-21-17A**	10.513	1.49	0.46	176.5	97
	KSH-21-18A	3.6621	2.28	0.39	258.8	131.5
	KSH-43-15	2.1202	1.29	0.44	164.6	125.2
Infected control		2.425	0.74	0.71	52.1	38
Uninfected control		1.08	1.75	0.14	13.9	10.3
LSD at 0.05		**0.289**	**0.22**	**0.015**	**1.61**	**1.53**

Determination of chitinase and β 1, 3 glucanase in treated sugar beet leaves:

The selected bacterial isolates dramatically increased chitinase activity in the treated sugar beet plants compared to the untreated healthy and infected control (Table 3). Among the tested bacterial isolates the isolate KSH-43-11 recorded the highest chitinase activity (229 µM of glucose

released / ml /hr.) while the bacterial isolates KSH-43-G recorded the lowest chitinase activity 134.8 µM of glucose released / ml /hr. It is worthy to mention that, the chitinase activity in the untreated healthy control recorded (13.9 µM of glucose released /ml /hr.) while the enzyme activity in the untreated infected control recorded (52.1 µM of glucose released /ml /hr.).

The combined data clustering analysis of the determined PR proteins in the treated sugar beet leaves classified the treatments to two main groups the first one contained the untreated uninfected control and the second one divided to 2 subgroups the first one contained the infected untreated control and the second one divided the treatments to two sub-sub groups the first one contained the isolates (KSH-43-11, SHR-27-5), (SHR-27-11 and DK-36-3b). The second one sub sub sub divided to 3 groups (KSH-21-18A and SHR-33-7b) and (KSH-43G) and the third one divided to tow groups (KSH-43-15) and (KSH-21-17A and SHR-27-17).

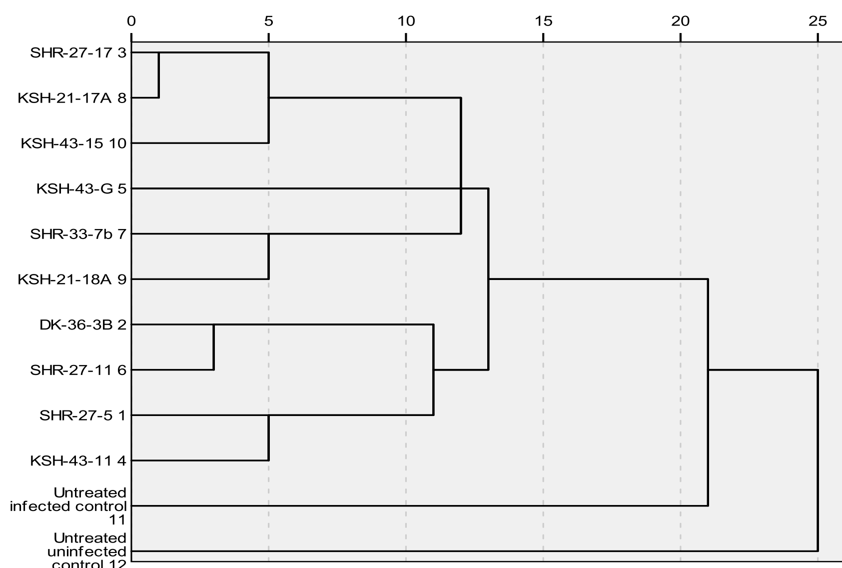

Fig.2: Dendrogram showing the combined data clustering of PR proteins (polyphenoloxidase (PPO) as well as peroxidase (PO), chitinolytic and β1,3-glucanase activity) recorded in greenhouse after two treatment with the selected *B. subtilis* and *B. pumilus* isolates before inoculation with the pathogen.

The PR proteins have become accepted markers of plant disease resistance and are differentially induced at the onset of various plant defense reactions (Van Loon *et al.* 1999). Chitinase belong to PR families 3,4,8 and 11 and have been characterized in tobacco Legrand *et al.* (1987), cucumber Metraux and Boller, (1986) and sugar beet Bargabus *et al.* (2002) and numerous of plant systems. The presence activity of chitinase control treated

sugar beet was not unusual, since the detection of these proteins in healthy, non-induced plant tissue is not uncommon.

Data presented in Table 3 showed that, most of the application with the selected bacterial isolates to sugar beet plants significantly increased the activity of the enzyme β 1, 3 glucanase compared to the activity detected in the untreated healthy and infected control. β -1-3-glucanase (PR-2) produced in sugar beet during systemic resistance responses were first isolated and characterized by Gottschalk *et al.* (2002). They reported the production of two distinct isoforms, 33 and 29 kDa in size. Once again the presence of active β -1-3 glucanase in negative control was not unexpected since the presence of β -1-3 glucanase in healthy plant tissue is common. Isolate KSH-21-18A recorded the highest β 1, 3 glucanase activity (131.5 µM of glucose released / ml /hr.) among the selected bacterial isolates while isolate KSH-43-G recorded the lowest activity (15.2 µM of glucose released / ml /hr.). It is worthy to mention that, the untreated healthy and infected control recorded (10.3 and 38.0 µM of glucose released / ml /hr.). The treatment with the other tested bacterial isolates rcorded enzyme activity ranged from 34.9 to 125.2 µM of glucose released / ml /hr.

The tested isolates of *B. subtilis* and *B. pumalis* elicited production of both β -1-3 glucanase and chitinase was significant since these PR proteins have a synergistic association leading to fungal pathogen control not evident when the two occur independently Jongedijk *et al.* (1995), Zu *et al.* (1994). It was hypothesized that together, these enzymes might be involved in reducing fungal disease severity by degrading the chitin and β - glucan components of fungal cell walls Abeles *et. al.,* (1970), Mauch *et al.* (1988). Although these enzymes might not be directly acting on *C. beticola*, the induction of both enzymes by the tested bacterial isolates of sugar beet is correlative with a significant control of CLS disease, agreeing with findings in tobacco *C nicotianeae* Neuhaus *et al.* (1992) and on sugar beet (Gottschalk *et al.* 2002).

Generally, in the present study, foliar treatment with bacterial isolates increased the activities of various defense enzymes when the plants were inoculated with the pathogen. The activity of phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenoleoxidase (PPO) declined rapidly in tomato after 4 days of inoculation with *Fusarium oxysporum* f.sp. *lycopersici* compared to bacterized plants challenged with the pathogen Ramamoorthy *et al.* (2002a&b). PO and PPO catalyze the last step in the biosynthesis of lignin and other oxidized phenols. The higher PO activity was noticed in cucumber roots treated with *P. corrugata* challenged with *P. aphanidermatum* (Chen *et al.* 2000). Enhanced PO activity is very often associated with resistance and lignin production (Reuveni *et al.* 1992). In the present study, foliar treatment of *B. subtilis* selected isolates showed a higher activity in PO and PPO in plants which might contribute to cross linking of hydroxyproline rich glycoproteins (HRGPs), lignifications that will act as barriers against pathogen entry. PO-generated hydrogen peroxide might function as an anti-fungal agent in disease resistance. Hydrogen peroxide inhibits pathogens directly or it might generate other free radicals that are antimicrobial in nature Chen *et*

al. (2000). Phenolic compounds are fungitoxic. In the present study, foliar treatment the selected bacterial isolates resulted in increased accumulation of phenolic substances in response to infection by the pathogen.

In addition, it also increased the activity of PAL PO, PPO, chitinase and β -1,3- glucanase. Bacterized tomato plants also induced the expression of PR protein chitinase and prevented the establishment of *F. oxysporum.* f. sp. *Lycopersici* Ramamoorthy *et al.* (2002b). *P. fluorescens* isolate 63-28 induced the accumulation of phenolics in tomato root tissues. The hyphae of *F. oxysporum.* f. sp. *radicis lycopersici* surrounded by phenolic substances exhibited cytoplasmic disorganization and loss of protoplasmic content M'Piga *et al.* (1997). Benhamou *et al.* (2000) reported that an endophytic bacterium *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots following infection by *Pythium ultimum*. Similarly, Ramamoorthy *et al.* (2002a) reported that, pretreatment of tomato and hot pepper plants with *P. fluorescens* isolate Pf1 challenged with *Pythium aphanidermatum* induced earlier and higher accumulation of PAL, PO, PPO, and phenolics compared to non-inoculated control.

Effect the selected bioagents treatments on the sucrose contents in sugar beet root:

The treated sugar beet plants with the tested bacterial isolates significantly increased the sucrose contents in the roots compared to the untreated infected control 14.1% (Table 4). The calculated losses in sucrose per ton of fresh roots show that, all the tested biocontrol active isolates used in this study reduced the losses in sucrose significantly compared to the untreated infected control. On the other hand, sugar beet plants treated with the selected bioagents against CLS disease showed a significant effect on increasing sugar contents in sugar beet roots than in the infected untreated plants in other words they reduced the losses in sugar contents.

Table 4: Effect of two sprays of the selected bacterial biocontrol active isolates on sugar beet plants before infection with *C. beticola* on sucrose contents in the roots under field conditions

	Bioagent	Sucrose (%)	Sucrose losses %	Gain of sucrose over the infected control kg/ton(roots)
B. subtilis	SHR-27-5	16.5	2.37	24
	DK-36-3B	15.6	7.69	15
	SHR-27-17	15.6	7.69	15
	KSH-43-11	15.8	6.51	17
	KSH-43-G	15.6	7.69	15
	SHR-27-11	15.7	7.1	16
	SHR-33-7b	16.4	2.96	23
B. pumilus	KSH-21-17A	16.4	2.96	23
	KSH-21-18A	16.7	1.18	26
	KSH-43-15	15.6	7.69	15
	Infected control	14.1	16.570	28
	uninfecTed control	16.9	0	-
LSD at 0.05		0.316		

The tested bacterial isolates KSH-21-18, SHR-27-5, KSH-21-17A and SHR-33-7b significantly increased the sucrose contents in the roots (16.7, 16.5, 16.4 and 16.4% respectively) compared to the untreated infected control (14.1%) with no significant difference with the healthy control (16.6%) followed the other selected bacterial isolates that recorded a sugar percentage ranged from 15.6% to 15.7%. The calculated gain in sucrose (Kg/ ton roots) over the infected control contents indicated that the tested bacterial isolates KSH-21-18A, SHR-27-5, KSH-21-17A and SHR-33-7b increased the sucrose contents to 23-26 kg/ton.

The calculated gain in sucrose (Kg/ ton roots) over the infected control contents indicated that the tested bacterial isolates KSH-21-18A, SHR-27-5, KSH-21-17A and SHR-33-7b increased the sucrose contents to 23-26 kg/ton. The sugar determination results showed the effect of biocontrol agents used in this study on the increasing of sugar percentage in the treated plants than in the untreated infected control which reflect the reduction of disease severity that lead to increase in photosynthesis and sugar synthesis, these results found to be in harmony with those obtained by El-Kholi, (1995).

REFERENCES

Abeles, F. B.; R .P. Bosshart and L. E. Forrence (1970). Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiology 47: 129-134.

Anonymous (1986). MSTATC. Microcomputer Statistical Programme. Michigan State University, Michigan, Lansing, U.S.A.

Arkhipova, T. N.; S.U. Veselov; A. I. Melentiev; E. V. Martynenko and G. R. Kudoyarova (2005). Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. Plant and Soil 272: 201–209.

Bargabus, R. L.; N. K. Zidack; J. W. Sherwood and B. J. Jacobsen (2002). Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol. Mol. Plant Pathol. 61:289-298.

Battilani, P. ; G. Beltrami; P. Meriggi; I. Ponti; A. Rossi; V. Rossi; F. Rosso; V. Tugnoli and A. Zocca (1990). Nuovi indrizzi di difesa anticercosporica. L'Informatore Agrario 46: 53-70 (cf. Vereijssen ; 2003).

Benhamou, N.; S. Gagne; D. L. Quere and L. Dehbi (2000). Bacterial mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum*. Phytopathology 90:45-56.

Bollage, M.D.; D. M. Rozycki and J. S. Edelstein (1996). Protein Methods. Wiley-Liss;Inc.; New York. 413pp.

Braun-Kiewnick, A.; S. Kiewnick; B.J. Jacobsen (1998). Induction of systemic resistance by antagonistic *Bacillus* sp. and the chemical inducer benzothiadiazole controls Cercospora leaf spot of sugar beet. Phytopathology 88: S10 (Abstract).

Brewer, M. T. and R. P. Larkin (2005). Efficiency of several potential biocontrol organisms against *Rhizoctonia solani* on potato. Crop Protection Journal 24: 939-950.

Bric, J. M.; R. M. Bostock and S. E. Silverstone (1991). Rapid *in situ* assay for indol acetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl. Eniron. Microbiol. 57: 535-538.

Bugbee, W. M. (1995). *Cercospora beticola* tolerant to triphenyltin hydroxide. Journal. Sugar Beet Res. 32: 167-174.

Chen, C.; R. R. Belanger; N. Benhamou and T.C. Paulitz (2000). Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *P. aphanidermatum*. Physiological and Molecular Plant Pathology 56:13-23.

El-Kholi, M. M. A. (1995). *Cercospora beticola* Sacc. and its effect on sugar beet plants. Egypt. J. Agric. Res. 73:1035-1045.

Esh, A. M. H. (2005). Controlling sugar beet cercospora leaf spot disease using environment friendly calcium salts. Zagazig J. Agric. Res. 32:1517-1535.

Evans, M. L. (1984) Function of hormones at the cellular level of organization. In Encyclopedia Plant Physiology 10: 23–79.

Gottschalk, K. E.; P. D. Adams; A. T. Brunger and H. Kessler (2002) Trans-membrane signal transduction of the integrin Protein Sci. 11:1800–1812.

Grant, J.J. and G.J. Loake (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol. 124: 21–29.

Jacobsen, B. J.; B. Larson; N. K. Zidack; J. Ansley; J. L. A. Eckhoff and J. Bergman (2003). Integrated management of CLS. 2002 Sugar beet Res. Ext. Rep. 33: 235-240.

Jongedijk, E.; H. Tigelaar; P. J. M. Van Roeckl and L. S. Melchers (1995). Synergistic activity of chitinases and β-1;3 glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173-180.

Kar, M. and D. Mishra (1976). Catalase; peroxidase and polyphenoloxidase activities during rice leaf senescence. Plant Physiology 57:315-319.

Karaoglanidis, G. S.; P.M. Ioannidis and C.C. Thanassoulopoulos (2000). Reduced sensitivity of *Cercospora beticola* to sterol-demethylation inhibiting fungicides. Plant Pathology. 49:567–572.

Karaoglanidis, G. S.; P. M. Ioannidis and C. C. Thanassoulopoulos (2001). Influence of fungicide spray schedules on the sensitivity to the sterol demethylation-inhibiting fungicides in *Cercospora beticola*. Crop Protection. 20: 941-947.

Kloepper, J.W.; C.M. Ryu and S.A. Zhang (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94: 1259-1266.

Köller, W. and W.F. Wilcox (1999). Evaluation of tactics for managing resistance of *Venturia inaequalis* to sterol demethylation inhibitors. Plant Disease 83: 857-863.

Le-Docte, A. (1927). Commercial determination of sugar in beet root using the Saches-Le Docte Process. Int. Sug. J. 29:488-492.

Legrand, M.; S. Kauffmannn; P. Geoffroy and B. Fritig (1987). Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinase. Proceedings of the National Academy of Sciences; USA 84:6750-6754.

Lim, H.; Y. Kim and S. Kim (1991). *Pseudomonas stutzeri* YLP-I genetic transformation and antifungal mechanism against *Fusarium solani;* an agent of plant root rot .Appl. Environ. Microbiol. 57:510-516.

M'Piga, P.; R. R. Belanger; T. C. Paulitz and N. Benhamou (1997). Increased resistance to *Fusarium oxysporum* f sp*. radicis lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiological and Molecular Plant Pathology 50:301-320.

Malik, C. P. and M. B. Singh (1990). Extraction and estimation of amino acids and kito acids.In:Plant Enzymology and histo-Enzymology.New Delhi-Lud Hana; India.

Matta, A. and A.E. Dimond (1963). Symptoms of Fusarium Wilt in relation to quantity of fungus and enzyme activity in tomato stems. Phytopathology; 53:574-587.

Mauch, F.; B. M. Mani and T. Boller (1988). Antifungal hydrolases proteins in pea tissue. Plant Physiology 88:936-942.

Metraux, J. and T. Boller (1986). Local and systemic induction of chitinase in cucumber plants in response to viral*;* bacterial and fungal infections*.* Physiological and Molecular Plant Pathology 28; 161-169.

Naguib, M.I. (1964). Effect of Seivn on the carbohydrate and nitrogen metabolism during the germination of cotton seeds. Ind. J. Agric. Sci. 35:179-185.

Naguib, M.I. (1965). Effect of maleic hydrazine on the nitrogen metabolism during the germination of cotton seed. Ind. J. Exp. Biol. 2:149-152.

Nelson, N. (1944). A photometric adaptation of the Somogy method for the determination of glucose. J. Biol. Chem. 152:375-380.

Neuhaus, J. M.; S. Flores; D. Keefe and J. R. F. Meins (1992). The function of vascular B-1;3 glucanase investigated by antisense transformation. Susceptibility of transgenic *Nicotiana sylvetris* plants to *Cercospora nicotianae* infection. Plant Molecular Biology 19:803-813.

Ongena, M.; A. Adam; E. Jourdan; M. Paquot; A. Brans; B. Joris; J.L. Arpigny and P. Thonart (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ. Microbiol. 9: 1084-1090.

Ongena, M.; F. Duby; E. Jourdan; T. Beaudry; V. Jadin; J. Dommes and P. Thonart (2005). *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. Appl. Microbiol. Biotechnol. 67: 692-698.

Ramamoorthy, V.; T. Raguchander and R. Samiyappan (2002a). Enhancing resistance of tomato and hot pepper to Pythium diseases by seed treatment with fluorescent Pseudomonads. European Journal of Plant Pathology 108:429-441.

Ramamoorthy, V.; T. Raguchander and R. Samiyappan (2002b). Induction of defense related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici* Plant and Soil 239:55-68.

Reissig, J. L.; J. L. Stringer and L. F. Leloir (1955). A modified colorimetric method for the estimation of *N*-acetylaminosugars. J. Biol. Chem. 217: 959–966.

Reuveni, R.; M. Shimoni and I. R. Crute (1992). Peroxidase activity as a biocontrol marker for resistance of muskmelon to *Pseudopernospora cubensis* . Phytopathology 82:749-753.

Snedecore, G. W. and W. G. Cochran (1982). Statistical methods 7th Ed. Iowa state University; Pres Ames USA.

Soltis, D. E. and P. S. Soltis (1990). Isozymes in Plant Biology. Dioscorides press; Portland; Oregon pp.259.

Taghian, Shadia A.; A.M.H. Esh; A.Z. Aly; and M.R.A. Tohamy (2008). *Bacillus subtilis* as bioagent used to control *cercospora* sugar beet leaf spot disease. Zagazig J. Agric. Res.; 35

Van Loon, L.C. and B.R. Glick (2004). Increased plant fitness by Rhizobacteria. In: H Sandermann (ed.) Molecular ecotoxicology of plants. Springer; Berlin.

Van Loon, L.C.; H. M. Bakker and C. M. J. Pieterse (1999). Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol.; 36:453-483.

Van Loon, L.C.; P.A.H.M. Bakker and C.M.J. Pieterse (1998). Systemic resistance induced by rhizosphere bacteria. Ann. Rev. Phytopathol. 36: 453- 483.

Van Peer, R.; G.J. Niemann; B. Schippers; J. Wolfe; C.J. Hutcheon; V.J. Higgins and R.K. Cameron; (2000). A functional gene-for-gene interaction is required for the production of an oxidative burst in response to infection with avirulent *Pseudomonas syringae* pv. tomato in *Arabidopsis thaliana*. Physiol. Mol. Plant Pathol. 56; 253–261.

Vereijssen, J.; J. H. M. Schneider; A. J. Termorshuizen and M. J. Jeger (2003). Comparison of two disease assessments keys to assess *Cercospora beticola* in sugar beet. Crop Protection 22: 201-209.

Zu; Q., S. Masoud; R. A. Dixon and C. Lamb (1994). Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. Bio Technology 12:807-812.

المقاومه الجهازيه في بنجر السكر المحفزه بواسطة البكتريات باسيلوس ساتليس و باسيلوس بيوميليس الغير ممرضه المصاحبه لسطح الاوراق ضد المسبب المرضي سيركوسبورا بتيكولا

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تم تقييم نشاط المقاومه الحيويه لعشرة عزلات من البكتريات *باسيلوس ساتليس* (٧) و *باسيلوس بيوميليس* (٣) المعزوله من أسطح أوراق بنجر السكر علي مرض التبقع السيركوسبوري تحت ظروف الصوبه و الحقل مقارنه بالمبيد الفطري توبسين إم ٧٠. تم دراسة البروتينات المرضيه في النبات - كايتينيز و بيتا ١-٣ جلوكانيز و بيروكسيديز و بوليفينول اكسيديز - و كذلك حمض الإندول أسيتيك في نباتات بنجر السكر المعامله بالبكتيريا تحت ظروف الصوبه. تراوح إنخفاض شدة الاصابه في النباتات المعامله بالبكتيريات التابعه لـ *باسيلوس ساتليس* ٧٣.٣٣ و ٨٠% في حين انه تراوح بين ٨٠% و ٨٣% في النباتات المعامله بالبكتيريات التابعه لـ *باسيلوس بيوميليس*. و تحت ظروف الحقل تراوح إنخفاض شدة الاصابه في النباتات المعامله بالبكتيريات التابعه لـ *باسيلوس ساتليس*٥٦.١٨ و ٨١.٢٧% في حين انه تراوح بين 62.55-81.27% في النباتات المعامله بالبكتيريات التابعه لـ *باسيلوس بيوميليس*. ارتفع مستوي البروتينات المرضيه بشكل معنوي في النباتات المعامله بكلا النوعين من البكتيريا و كذلك زاد فيها معدل حمض الإندول أسيتيك مقارنة بالنباتات غير المعامله.

و من ناحيه أخري أدت المعامله بكلا البكتيريتين الي زيادة نسبة السكروز في الجذور مقارنة بالنباتات المعديه بالفطر و الغير معامله. تظهر هذه الدراسه دور البكتيريا المختبره في حث عوامل المقاومه المرضيه في بنجر السكر و كذلك تأثيرها الوقائي .

قام بتحكيم البحث

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