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Induction of Induced Systemic Resistance in Fodder Beet (*Beta vulgaris* L.) to Cercospora Leaf Spot Caused by (*Cercospora beticola* Sacc.)

Ehab Ali Deiaa Sarhan
Plant Pathol. Res. Inst., Agric. Res. Centre, Giza, Egypt.

Bacillus *subtilis, Paenibacillus polymyxa, Pseudomonas fluorescens* and *Pseudomonas putida* isolates were evaluated for their biocontrol activities against fodder beet Cercospora leaf spot disease under greenhouse and field conditions compared to the fungicide Topsin M-70. *β*-1,3-glucanase, peroxidase, polyphenoloxidase and phenylalanine ammonia lyase as well as indolacetic acid and total phenols content were determined in treated and untreated fodder beet plants. Under greenhouse conditions, the reduction in the disease severity of the treated plants with the aforementioned bioagents ranged between 58.82 - 88.24%. Under field conditions the reduction ranged between 46.67 to 80.00% and 58.33 to 83.33% in the two locations of the experiments *i.e.,* Nubaria and Sakha, respectively. The activities of defense-related enzymes *i.e., β*-1,3-glucanase, peroxidase, polyphenoloxidase and phenylalanine ammonia lyase were significantly increased in all treated plants with the tested bioagents. *P. fluorescens* resulted in the highest activity of oxidative enzymes activity. Meanwhile, the contents of indolacetic acid and total phenols were higher in treated plants than the untreated. Also crop parameters *i.e.,* root length, root diameter, fresh and dry weight and % dry matters were significantly increased in the treated fodder beet plants compared to the untreated control. The tested bioagents might be playing an important role in management of Cercospora leaf spot of fodder beet plants through induction of induced systemic resistance.

Keywords: *Cercospora beticola, Paenibacillus polymyxa, Pseudomonas fluorescens, Pseudomonas putida.*

Fodder beet (*Beta vulgaris*, L.) is one of the most promising winter forage crops under limited water and nutrient levels. The whole plant above and under-ground parts could be used in animal feeding directly (Abdallah and Yassen, 2008). The production of grown fodder beet plants under suitable conditions, can reached about 20 ton/ha dry matter (Anonymous, 1998).

Cercospora leaf spot caused by *Cercospora beticola*, is one of the most economically important and destructive foliar diseases of sugar and fodder beets (Holtschulte, 2000 and Harveson *et al.*, 2010). Severe epidemics of *C. beticola* are manifested by progressive destruction of leaves, followed by a continual replacement of leaves at the expense of stored reserves in the root and significant yield reduction (Shane and Teng, 1992).

Biological control is an important alternative method to avoid the application of chemical pesticides in controlling plant diseases and encouraging the organic production of the crops (Reddy *et al.,* 2014).

Epiphytic microbes have 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). Recently, the induction of plant resistance by application of several microorganisms or organic materials has emerged as a new strategy in the management of plant diseases (Rais *et al.,* 2017).

Understanding the mechanisms and behavior of a biocontrol agent (BCA) improve performance of BCA and result in better disease control. Also, it will allow researchers and industries to produce more reliable and predictable products (Upper, 1991 & Beattie and Lindow, 1994).

Biotic and abiotic inducers have potential in agriculture with regard to controlling plant diseases (Anand *et al.,* 2009 and Simonetti *et al.,* 2012). Biotic inducers are known to have eliciting activities leading to a variety of defense reactions in host plants in response to microbial infection, including the defense related enzymes and accumulation of phenolic compounds as well as specific flavonoids (Saikia *et al.,* 2005; Govindappa *et al.,* 2010; Esh *et al.,* 2011; Abd El-Rahman *et al.,* 2012 and Hussein *et al.,* 2018).

The activity of defence related enzyme β-1, 3 glucanase is known to be as an inducer of systemic resistance of many infected plants with fungal pathogens (Saikia *et al.,* 2005 and Govindappa *et al.,* 2010). Also, this enzyme acts synergistically in the partial degradation of fungal cell walls. Moreover, a parallel increase in the activities of these enzymes is important for optimal function in plant defense (Saikia *et al.,* 2005).

Also, peroxidase (PO), phenylalanine ammonia-lyase (PAL), and polyphenoloxidase (PPO) enzymes were mentioned as elicitors of the induced systemic resistance (ISR) in plant disease control (Yasmin *et al.,* 2016). These enzymes act as elicitors of phenylpropanoid pathway, resulting in the biosynthesis of a diverse array of plant metabolites such as, phenolic compounds, flavonoids, tannins and lignin. These products can provide defense in plants against pathogenic attack (Hahlbrock and Scheel, 1989). Many studies indicated to greater accumulation of phenolics as a result of increasing the activities of these oxidative enzymes which could be offer the protection against plant diseases (Singh *et al.,* 2003; Abd El-Rahman *et al.,* 2012 and Hussein *et al.,* 2018).

This investigation aimed to evaluate the capability of some biotic agents to induce resistance for fodder beet Cercospora leaf spot disease under greenhouse and field conditions. Also evaluation their effect on crop parameters, as well as the relationship between resistance and biochemical changes in the treated plants.

Materials and Methods

1. Biocontrol agents:

In this study, 4 bacterial bioagents *i.e. Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida* were kindly obtained from Biofertilizers Production Unit, Soils, Water & Environment Research Institute, Agricultural Research Center, Giza, Egypt.

1.1. Preparation of tested bacterial inocula.

The four tested bacterial isolates were cultured individually in nutrient broth medium in 250-mL conical flasks and incubated at 28 ±1°C for 48 h. on a rotary shaker then a cell suspension of each isolate was diluted by sterilized distilled water with adding 0.1 mL Tween-80 as described by Vereijssen *et al.* (2003) and Esh, (2005) and then adjusted to $1x10^6$ cfu/mL prior to spraying them on fodder beet plants.

1.2. The tested fungicide Topsin M 70 WP:

Common name: Thiophanate-methyl

Chemical name: dimethyl [1,2-phenylenebis (iminocarbonothioyl)] bis [carbamate]

2. Production of β-1,3-glucanase and indoleacetic acid (IAA) in vitro:

2.1. β-1,3-glucanase:

β-1,3-glucanase was assayed by incubating the tested isolates on King's B medium (KB medium) containing 1 mL 0.2% laminarin (w/v) in 50mM sodium acetate buffer (pH = 4.8) with 1ml enzyme solution at 50°C for 1 h and by determining the reduced sugars with dinitrosalicylic acid (DNS) (Nelson, 1944). The amount of reduced sugars released was calculated from standard curve for glucose. One unit of *β*-1,3- glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of glucose equivalents per min. Protease activity (casein degradation) was determined from clearing zone in skimmed milk agar (SMA) according to Nielsen *et al.* (1998).

2.2. Indoleacetic acid (IAA):

Production of IAA was determined according to the method of Bano and Musarrat (2003). Isolates were grown on King's B medium and incubated at 28±1°C for 5 d, then transferred to 5 mL KB broth containing 2 mg/mL L-tryptophan. Cultures were incubated at 28±1°C with shaking at 125 rpm for 7 d then harvested by centrifugation at 11,000xg for 15 min. One milliliter of the supernatant was mixed with 2 mL of Salkowski reagent; the appearance of a pink color indicated IAA production. Optical density (OD) was read at 530 nm. The level of IAA produced was estimated according the IAA standard.

3. Preparation of C. beticola inoculum:

C. beticola of 30-days old cultures were flooded with 10 mL sterile distilled water and rubbed with a glass rod. Five hundred µl of this suspension were used to inoculate fodder beet leaf broth medium (FBLB) then incubated at 28 ±1°C under a 16-hr photoperiod (fluorescent light) for 30 days. After incubation, cultures were blinded separately in a partial sterilized (by ethanol 70%) electrical blinder for 5

min. The fungal suspension was diluted by distilled water to reach $3x10^4$ cfu/mL to spray the experimental plants (Vereijssen *et al.,* 2003 and Esh, 2005).

4. Preparation of the bacterial bioagents inoculum:

The four tested bacterial isolates *i.e. B. subtilis*, *P. polymyxa*, *P. fluorescens* and *P. putida* were grown in 250 ml nutrient broth at 28 ±1°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.

5. Greenhouse trials:

Fodder beet plants cv. Voroshenger 8 weeks old (grown each alone in 30 cm diameter pots) were sprayed with inoculum of the four tested bacteria each alone two times 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 fodder beet leaves from all directions until run off. After inoculation, plants were irrigated and covered with transparent plastic bags to raise relative humidity responsible for infection by the causal pathogen. After 5 days, the plastic sheet was removed, and the plants were 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).

6. Determination of defense related enzymes activity and biochemical changes in treated fodder beet leaves with tested bacterial bioagents:

The activities of *β*-1,3-glucanase, peroxidase, polyphenoloxidase and phenylalanine ammonia lyase in additional to total phenol content and indoleacetic acid were determined in tissues of treated fodder beet leaves with the tested bioagents as well as in untreated healthy and untreated infected leaves as control. All treatments were inoculated individually with *C. beticola* inoculum.

6.1. Sample collection:

From the greenhouse experiment, samples of treated fodder beet leaves with the tested bioagents as well as the untreated healthy and infected plants were collected at 6 days after inoculation with the pathogen, then were grounded with liquid nitrogen ($L\text{-}N_2$) as fine powder with a mortar. One gram of the grounded tissues was mixed with one mL of extraction buffer phosphate, pH 6.0 according to Bollage *et al.* (1996). Samples were vortexed and centrifuged at 8000 rpm for 25 min. under 4°C to remove cell debris. The clear supernatant (crude enzyme source) was collected and kept at -20°C for further studies (Biles and Martyn, 1993).

6.2. β-1, 3 glucanase assay:

β-1,3 glucanase activity was determined according to the method of Abeles *et al.* (1970). Laminarin was used as the substrate and dinitrosalicylic acid as reagent. The optical density was read at 500 nm. *β*-1,3 glucanase activity was expressed as mM glucose equivalents released/g fresh weight tissue/60 min.

6.3. Peroxidase activity (PO):

Peroxidase activity was determined directly using a spectrophotometrical method of Hammerschmidt *et al.* (1982) using guaiacol as common substrate. The reaction

mixture consisted of 0.2 mL crude enzyme extract and 1.40 mL of a solution containing guaiacol, hydrogen peroxide (H_2O_2) and sodium phosphate buffer (0.2 mL 1% guaiacol+0.2 mL 1% H_2O_2 + 1 mL of 10 mM potassium phosphate buffer). The mixture was incubated at 25±1°C for 5 min and the initial rate of increase in absorbance was measured over 1 min at 470 nm. Activity was expressed as units of PO/mg protein (Urbanek *et al.,* 1991).

6.4. Polyphenoloxidase activity (PPO):

The activity of PPO was determined by adding 50 µL of the crude extract to 3 mL of a solution containing 100 mM of potassium phosphate buffer, pH 6.5 and 25 mM of pyrocatechol. The increase of absorbance at 410 nm during 10 min at 30°C, was measured (Gauillard *et al.,* 1993). One PPO unit was expressed as the variation of absorbance at 410 nm per mg soluble protein per min.

6.5. Phenylalanine ammonia-lyase activity (PAL):

PAL activity was determined following a previously-described direct spectrophotometric method of Gauillard *et al.* (1993). Two hundred microlitres of the crude enzyme extract previously dialyzed overnight with 100 mM of Tris-HCl buffer, (pH 8.8), were mixed to obtain a solution containing 200 µL of 40 mM phenylalanine, 20 µL of 50 mM *β*-mercaptoethanol, and 480 µL of 100 mM Tris-HCl buffer, (pH 8.8). After incubation at 30±1°C for 1 h. the reaction was stopped by adding 100 µL of 6 N HCl. Absorbance at 290 nm was measured and the amount of formed trans-cinnamic acid was evaluated by comparison with a standard curve (0.1~2 mg/mL trans-cinnamic acid) and expressed as units of PAL/min/mg protein.

6.6. Total phenols content (TPC):

To assess total phenols content, 1 g fresh plant sample was homogenized in 10 mL of 80% methanol and agitated for 15 min at 70°C. One milliliter of the extract was added to 5 mL of distilled water and 250 µL of 1 N Folin-Ciocalteau reagent and the solution was kept at 25±1°C. The absorbance was measured using a spectrophotometer at 725 nm. Catechol was used as a standard. The amount of phenolic content was expressed as phenol equivalents in mg/g fresh tissue (Velioglu *et al.,* 1998).

6.7. Determination of indoleacetic 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).

7. Field trials:

Field experiments were carried out during the season of 2017/2018 in fields naturally infested with *Cercospora beticola*, the causal organism of Cercospora leaf spot of fodder beet at the experimental farms of Sakha Agric. Res. Stat., Kafrelsheikh governorate and Nubaria Agric. Res. Stat., El-Beheira governorate at sowing date of 1st and 3rd November, respectively. The field was divided to (3x3.5 meter) plots and each plot consisted of five rows, 50 cm row spacing, seeds were sown in hills (2 seeds/hill-1 and 25 cm apart), fertilizers application at the rate of

recommended doses. The crop was irrigated at 12-15 days intervals, hand thinned to one plant per hill after 5 weeks from planting (Abdel-Naby *et al.,* 2014). The used experimental design was as complete randomized design with three replicates (plots) for each treatment. The same used procedures and fodder beet cv. used under greenhouse conditions were used in the field trails. Cercospora severity was assessed according to Battilani *et al.* (1990). At the end of the experiment (harvest time) 10 plants from the central ridges were pulled up to determine the following growth traits and forage yield:

1. Root length (cm) = distance between the beginning of the root to its end.
2. Root diameter (cm) = Circumference of circle when the maximum width of root divided on 2.14.
3. Fresh and dry weights of roots (ton/fed.).
4. Dry matters (%) = Dry weight of roots/Fresh weight of roots ×100

8. Statistical analysis:

Data of the present work were statistically analyzed by analysis of variance according to Snedecore and Cochran (1989) using the ANOVA on Computer package MSTATC (Anonymous, 1986).

Results

1. Production of β-1, 3- glucanase and indoleacetic acid (IAA) by tested bacteria in vitro:

Data in Table 1 show the capability of the three tested bacteria as bioagents (*B. subtilis*, *P. fluorescens* and *P. putida*) to produce *β*-1,3 glucanase, with highest amount in case of *P. fluorescens* followed by *B. subtilis* and *P. Putida* whereas *P. polymyxa* was not able to produce *β*-1,3 glucanase. Meanwhile, the four tested bacterial bioagents produced indoleacetic acid (IAA), which evidenced by development of pink color with and without the addition of tryptophan into the culture medium.

Table 1. Capability of the tested bacterial bioagents to produce indoleacetic acid (IAA) and *β*-1,3-glucanase *in vitro*

Bioagent	Indoleacetic acid	*β*-1,3 glucanase
B. subtilis	+*	+
P. polymyxa	+	-
P. fluorescens	+	+
P. putida	+	+

* - , Negative; +, Positive.

2. Effect of the tested bacterial bioagents on Cercospora leaf spot disease under the greenhouse conditions:

Table 2 shows that all the four tested bioagents decreased significantly the severity of infection by Cercospora leaf spot under greenhouse conditions compared to the control treatment. *P. fluorescens* resulted in the lowest disease severity (0.4) compared to the untreated control treatment which recorded 3.4 and caused a percentage of 88.24% disease severity inhibition followed by *B. subtilis*, *P. putida* and *P. polymyxa*, being 82.35, 64.71 and 58.82% respectively. Whereas, there were

no significant differences among *P. fluorescens* and *B. subtilis* treatment and the fungicide Topsin M-70, which recorded disease inhibition of 94.12%.

Table 2. Effect of the tested bacterial bioagents on the severity of Cercospora leaf spot of fodder beet (cv. Voroshenger) under greenhouse conditions

Treatment	Disease severity	% Reduction
B. subtilis	0.60cd	82.35
P. polymyxa	1.40b	58.82
P. fluorescens	0.40d	88.24
P. putida	1.20bc	64.71
Topsin M-70	0.20d	94.12
Infected control	3.40a	-
L.S.D. at 0.05	0.67	-

Values in the column followed by different letters indicate significant differences among treatments according to L.S.D. at 0.05.

3. Effect of application the tested bioagents on some compounds related to induction of resistance in fodder beet plants:

3.1. Determination the amount of indoleacetic acid (IAA) in the treated fodder beet leaves:

Results presented in Table 3 reveal that the treated fodder beet plants with the tested bioagents secreted IAA with different levels. There were significant difference among IAA levels in the treated plants compared with the untreated artificially infected control and the untreated healthy control. The highest IAA level was recorded when fodder beet plants treated with *B. subtilis* (3.19 mg/ml), followed by *P. fluorescens* (3.02 mg/mL), then *P. polymyxa* (2.74 mg/mL) and *P. putida* (1.65 mg/mL). The value of IAA in the untreated healthy control plants recorded 0.99 mg/mL which was lower than the determined level in the untreated infected plants (1.12 mg/mL).

Table 3. Determination the amount of indoleacetic acid (IAA) in the treated fodder beet plants (cv. Voroshenger) with the tested bacterial bioagents two weeks before inoculation with *C. beticola*

Treatment	Indoleacetic acid (IAA)	
	Amount of IAA (mg/mL)	Increase over control %
B. subtilis	3.19a	184.27
P. polymyxa	2.74a	143.92
P. fluorescens	3.02a	168.55
P. putida	1.65b	46.88
Infected control	1.12b	-
Healthy control	0.99b	-
L.S.D. at 0.05	0.71	

Values in the column followed by different letters indicate significant differences among treatments according to L.S.D. at 0.05.

3.2. Activity of defence related enzymes in the treated leaves of fodder beet plants (cv. Voroshenger) with four bacterial bioagents, two weeks before inoculation with C. Beticola:

The effect of the four tested bacterial bioagents (*B. subtilis*, *P. polymyxa*, *P. fluorescens* and *P. putida*) as biotic inducers on the activity of defence enzymes [*β*-1,3-glucanase, peroxidase (PO), polyphenoloxidase (PPO) and phenylalanine ammonia lyase (PAL)] in fodder beet leaves infected with *C. beticola* disease was studied. Data in Table 4 show that all the tested bacterial bioagents increased the activity of *β*-1,3-glucanase, PO, PPO and PAL in the treated fodder beet leaves compared with the untreated artificially infected control and the untreated healthy control. *P. fluorescens* recorded the highest level of the activity of oxidative enzymes followed by *B. subtilis* then *P. putida*. Whereas, the least enzymes activity was recorded with *P. Polymyxa.*

Table 4. Activity of defence related enzymes in the treated fodder beet plants (cv. Voroshenger) with the tested bacterial bioagents two weeks before inoculation with *C. beticola*

Treatment	*β*-1,3 glucanase*		PO		PPO		PAL	
	Activity	Increase over control %	Activity	Increase over control %	Activity	Increase over control %	Activity	Increase over control %
B. subtilis	75.67b	94.68	2.08a	133.71	0.49b	177.36	1.06a	78.65
P. polymyxa	45.57d	17.24	1.23b	38.58	0.25d	41.51	0.85b	43.26
P. fluorescens	96.30a	147.77	2.30a	158.80	0.64a	260.38	1.17a	96.63
P. putida	59.40c	52.83	1.52b	70.79	0.34c	92.45	0.91b	52.81
Infected control	38.87de	-	0.89c	-	0.18de	-	0.59c	-
Healthy control	34.95e	-	0.77c	-	0.13e	-	0.46c	-
L.S.D. at 0.05	8.85		0.30		0.08		0.13	

**β*-1,3 glucanase (enzyme activity as µM of glucose released/mL/h.); PO, peroxidase (enzyme unit/mg protein/min); PPO, polyphenoloxidase (enzyme unit/mg protein/min); PAL, phenylalanine ammonia lyase (enzyme unit/mg protein/min). Values in the column followed by different letters indicate significant differences among treatments according to L.S.D. at 0.05.

3.3. Effect of treatment with the tested bacterial bioagents on total phenols content in fodder beet leaves:

Data in Table 5 indicate that total phenolic compounds were significantly higher in fodder beet plants treated with the tested bacterial bioagents than those of untreated infected and untreated healthy control plants. The highest total phenolic contents were recorded in plants treated with *P. fluorescens* (8.03 mg/g) followed by *B. subtilis* (7.53 mg/g) then *P. putida* (6.67 mg/g). While, the lowest content of total phenolic compounds were recorded in plants treated with *P. polymyxa* (5.27 mg/g). The higher total phenolic content in the untreated healthy control plants (1.23 mg/g) was lower than determined level in the untreated infected plants (2.87 mg/g).

4. Effect of treatment with the tested bacterial bioagents on Cercospora leaf spot infection under field conditions:

Results in Table 6 show that field experiments at two growing locations *i.e.* Nubaria and Sakha Agri. Res. Stat. during 2017/2018 growing season showed that sprayed fodder beet plants twice (two weeks intervals) with the tested bacterial bioagents (biotic inducers) *i.e., B. subtilis, P. polymyxa, P. fluorescens, P. putida* and the fungicide Topsin M-70 significantly reduced the severity of Cercospora leaf spot compared with the untreated control. The highest reduction in the severity of Cercospora leaf spot disease severity was obtained with *P. fluorescens* (75.0%) followed by *B. subtilis* (74.17%) and *P. putida* (70%), whereas, *P. polymyxa* was the lowest effective one (52.5%). These results were, to somewhat confirmed in the two locations, Nubaria and Sakha Agri. Res. Stat., with a slight increase in Cercospora leaf spot reduction in Sakha compared to Nubaria location. It is worthy to mention that there were no significant differences among the averages of the values of disease severity due to the treatment with *P. fluorescens* and *B. subtilis, P. putida* and the fungicide Topsin M-70.

Table 5. Effect of the treatment with the tested bioagents bacteria on total phenol content (TPC) in fodder beet plants (cv. Voroshenger) two weeks before the inoculation with *C. beticola*

Bioagent	Total phenols content (mg/g)
B. subtilis	7.53a
P. polymyxa	5.27b
P. fluorescens	8.03a
P. putida	6.67b
Infected control	2.87c
Healthy control	1.23d
L.S.D. at 5% for	0.65

Values in the column followed by different letters indicate significant differences among treatments according to L.S.D. at 0.05.

5. Effect the treatment with the tested bacterial bioagents on fodder beet crop parameters under field conditions

Data presented in Table 7 reveal that spraying fodder beet plants twice (two weeks intervals) with the tested bacterial bioagents (biotic inducers) and the fungicide Topsin M-70 significantly increased crop parameters *i.e.,* root length, root diameter, fresh and dry weights and % dry matters in the two locations. Application of *P. fluorescens* scored the highest increase in the average of estimated crop parameters in this regard comparing with the other three bioagents (*B. subtilis, P. putida* and *P. polymyxa*) and the control in the two locations. The respective averages were 45.35 cm root length, 21.72 cm root diameter, 58.11 and 8.30 ton/fed fresh and dry weights and 14.28% dry mater compared with 29.86 cm, 13.07 cm, 38.83 and 5.02 ton/fed, 11.77% in the control treatment. On the other hand, fodder beet plants treated with *P. polymyxa*, recorded the lowest averages of these parameters, being 35.73 cm root length, 15.83 cm root diameter, 44.77 and 6.28 ton/fed fresh and dry weights and 14.02 % dry matter.

Table 6. Effect of spraying four bacterial bioagents on severity of Cercospora leaf spot disease of fodder beet (cv. Voroshenger) two sprays in field experiments at Nubaria and Sakha Agric. Res. Stat. during the season of 2017/2018

Treatment	Nubaria		Sakha		Average of the two locations	
	Disease severity	Reduction %	Disease severity	Reduction %	Disease severity	Reduction %
B. subtilis	0.8	73.33	0.6	75.00	0.7	74.17
P. polymyxa	1.6	46.67	1.0	58.33	1.3	52.50
P. fluorescens	1.0	66.67	0.4	83.33	0.7	75.00
P. putida	0.8	73.33	0.8	66.67	0.8	70.00
Topsin M-70	0.6	80.00	0.4	83.33	0.5	81.67
Control	3.0	-	2.4	-	2.7	-
Mean	1.3	68.00	0.9	73.33	1.1	70.67
L.S.D. at 5% for:						
Treatment (T)	0.54					
Location (L)	*					
T×L	n.s.					

Table 7. Effect of the tested bacterial bioagent treatments on growth and yield parameters of fodder beet cv. Voroshenger cultivated in Nubaria and Sakha locations under field conditions during the winter season 2017/2018

Treatment	Root length (cm)	Root Diam. (cm)	Fresh weight (ton/fed.)	Dry weight (ton/fed.)	% Dry matters
Nubaria					
B. subtilis	41.27	19.17	49.31	6.94	14.09
P. polymyxa	36.57	16.56	45.19	6.35	14.05
P. fluorescens	46.54	21.66	58.98	8.34	14.13
P. putida	38.69	18.62	47.05	6.64	14.10
Topsin M-70	49.67	24.47	63.85	9.12	14.28
control	30.23	13.56	38.86	5.35	12.05
Mean	40.49	19.01	50.54	7.12	13.79
Sakha					
B. subtilis	39.59	19.00	48.83	6.90	14.14
P. polymyxa	34.90	15.10	44.36	6.21	13.99
P. fluorescens	44.17	21.77	57.23	8.26	14.42
P. putida	36.62	17.54	45.82	6.49	14.16
Topsin M-70	48.02	22.62	65.12	9.19	14.11
control	29.50	12.57	38.80	4.68	11.48
Mean	38.80	18.10	50.03	6.95	13.72
Mean					
B. subtilis	40.43	19.09	49.07	6.92	14.11
P. polymyxa	35.73	15.83	44.77	6.28	14.02
P. fluorescens	45.35	21.72	58.11	8.30	14.28
P. putida	37.65	18.08	46.44	6.56	14.13
Topsin M-70	48.84	23.55	64.49	9.15	14.20
control	29.86	13.07	38.83	5.02	11.77
Mean	39.64	18.56	50.29	7.04	13.75
L.S.D. at 0.05 for:					
Location (L)	n.s.	n.s.	n.s.	n.s.	n.s.
Treatment (T)	1.05	1.17	2.32	0.28	0.16
L×T	n.s.	n.s.	n.s.	n.s.	0.22

Discussion

Application of biotic and abiotic inducers has a good potential in controlling plant diseases. They elicit activities leading to a variety of defence reactions in host plants in response to microbial infection, including the accumulation of pathogenesis related PR- proteins, defence related enzymes, lignin synthesis, accumulation of phenolic compounds and specific flavonoids. (Biles and Martyn 1993; Bargabus *et al.,* 2002; Sarwar *et al.,* 2010; Esh *et al.,* 2011; Abd El-Rahman *et al.,* 2012 and Hussein *et al.,* 2018). Resistance inducers are considered one of the alternative methods to decrease the use of fungicides to manage plant diseases and maintaining sustainable production (Da Rocha and Hammerschmidt 2005 and Walters *et al.,* 2005).

Recently, several researches investigated different bioagents for protecting plants against airborne pathogens such as *Bacillus licheniformis*, to control tomato gray mold disease caused by *Botrytis cinerea* (Lee *et al.,* 2006); *P. fluorescens* and *P. aeruginosa* were used as foliar spray on chickpea against *Sclerotinia sclerotiorum* (Basha *et al.,* 2006), *Trichoderma* sp for controlling Cercospora leaf spot of sugar beet caused by *C. beticola* (Galletti *et al.,* 2008). *Serratia proteamaculans* against tomato early blight caused by *Alternaria solani* (Youssef *et al.,* 2018). Also, *Bacillus* spp were used against Cercospora leaf spot (CLS) of sugar beet caused by *Cercospora beticola* (Esh *et al.,* 2011).

Cercospora leaf spot (CLS) caused by the fungus *C. beticola* is the most widespread and most damaging foliar disease to fodder beet, sugar beet and *Beta vulgaris*. worldwide (Shane and Teng 1992 and Harveson *et al.,* 2010).

Thus, the bacterial abilities to suppress Cercospora leaf spot of fodder beet are depending on the direct and indirect modes of action via the application as a foliar spray were investigated.

The capability of the tested bacterial bioagents *i.e., B. subtilis, P. polymyxa, P. fluorescens* and *P. putida* to produce *β*-1,3 glucanase indoleacetic acid (IAA) was determined *in vitro*, to confirm their antifungal activity against the air borne pathogen *C. beticola*. These results are in harmony with those obtained by Gottschalk *et al.* (1998); Karnwal (2009); Sarhan and Shehata (2014) and Prasad *et al.* (2017) who showed the ability of the bioagents to inhibit pathogenic fungi by producing one or more of inhibitory substances such as antibiotic(s), hydrolytic enzymes, indoleacetic acid, siderophore or hydrogen cyanid.

In the present work, the ability of the tested bioagents *i.e., B. subtilis, P. polymyxa, P. fluorescens, P. putida* to suppress Cercospora leaf spot infection on fodder beet leaves is reliant to the induction of systemic resistance as the main mechanism of action, which was illustrated via reduction of the disease severity.

Results indicated that application of the tested bioagents and the fungicide Topsin M-70, as foliar treatment significantly reduced the severity of Cercospora leaf spot disease on fodder beet under greenhouse and field conditions compared to untreated control. Moreover, the treated plants with *P. fluorescens* resulted in the

highest reduction in the disease with high figures of the assessed crop parameters compared with the other bioagents and the control.

Also, under field conditions, all the tested bioagents improved crop components of fodder beet in both locations. *P. fluorescens* treatment scored the highest increase of crop parameters (root length, root diameter, fresh and dry weights and % dry matters) comparing with the other three tested bioagents in both locations and the control. The mechanisms by which these bacteria affect plants involve the production of phytohormones (indole acetic acid, gibberellin and cytokinin) and other associated activities which include phosphate solubilization in soil, resulting in stimulation of sunflower plant growth (Bhatia *et al.,* 2005). Also, the results could be discussed in the light of the findings of Basha *et al.* (2006); Govindappa *et al.* (2010) and Abd El-Rahman *et al.* (2012) who found that application of biotic inducers was accompanied by pronounced increases in crop parameters.

The obtained results are in agreement with those reported by Gottschalk *et al.* (1998); Collinsa and Jacobsenb (2003); Larson (2004); Galletti *et al.* (2008) and Esh *et al.* (2011) who found that, some of the biological control treatments reduced Cercospora leaf spot (CLS) caused by *C. beticola*.

In general, plants defend themselves against a wide array of pathogenic microorganisms via strategies such as structural mechanism and biochemical reactions. The biochemical defense mechanisms can be subdivided in pre-existing factors generally present in healthy plants and a defense response that is induced in plants in response to external factors. Mostly, the activation of plant defense responses is initiated by host recognition of pathogen elicitors or through induction via application of other microorganisms as approach of biological control (Biles and Martyn, 1993; Yang *et al.,* 1997 and Lee *et al.,* 2006).

Induced systemic defense reaction in plants using plant growth promoting rhizobacteria (PGPR) is considered one important means to suppress plant disease' symptoms in foliar plant organs. As well several biocontrol agents such as *Bacillus* spp. and *Pseudomonas* spp. can induce plant defense responses that are directly linked with induction of defense enzymes and pathogenesis related proteins (PR) such as β-1,3-glucanase, peroxidase, polyphenoloxidase, phenylalanine ammonia lyase and indoleacetic acid (IAA) and accumulation of phenolic compounds (Sarwar *et al.,* 2010; Abd El-Rahman *et al.,* 2012; Prasad *et al.,* 2017 and Youssef *et al.,* 2018).

It has been found significant increase in the levels of indoleacetic acid (IAA) in the treated fodder plants with the tested bioagents compared with the untreated healthy control and the untreated artificially infected control. The increase in IAA levels resulted by the treatment of fodder beet plants were at least two folds higher than the levels recorded in the untreated healthy and infected control. Changes in the content of IAA in inoculated plants may attribute 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 be modified by accumulation of cytokinins in inoculated plants. The presence of cytokinin produced by microorganisms can therefore be expected to influence not only cytokinin content in treated plants but also other hormones (Arkhipova *et al.,* 2005). Moreover, the obtained results are in agreement with earlier reports on the production of different antifungal metabolites (hydrolytic enzymes) including siderophore, hydrogen cyanide, organic acids, IAA, solubilized insoluble phosphate, by the PGPRs *Bacillus* spp. and *Pseudomonas* spp. suggests the plant growth promotion and broad spectrum biocontrol potential of this isolate and confirm the ability of indirect mechanism of PGPR to suppress plant diseases like wilt (*Fusarium oxysporum*) and root rot (*Rhizoctonia solani*) by *Bacillus* spp. and *Pseudomonas* spp. (Kumar *et al.,* 2010; Singh *et al.,* 2010; Esh *et al.,* 2011 and Sarhan and Shehata, 2014).

In the current study, data showed that spraying with the tested bioagents on fodder beet plants increased the activity of *β*-1,3-glucanase, it is worthy to mention that it reached two folds or higher than the activity recorded in the untreated healthy and infected controls, suggesting induction of PR proteins that enhanced fodder beet plant resistance against Cercospora leaf spot (*C. beticola*). Pathogenesis-related proteins (PR) are a large group of proteins with multiple functions induced in cells upon pathogens attack, the enzyme *β*-1,3-glucanase is a key enzyme that hydrolyzes the major pathogen cell wall component *β*-1,3-glucan (Schlumbaum *et al.,* 1986). Consequently, several reports have documented such stimulation as *β*-1,3-glucanase has been reported earlier with induction of systemic resistance in plants infected with *C. beticola* after application the bioagents'. For instance, induction of *β* -1,3-glucanase was observed rather late after infection by *C. beticola* resulting in the inability to inhibit the propagation of the pathogen, the local accumulation proximal to the necrosis suggests that this enzyme may play a role in the disease resistance by limiting the extension of the fungal hyphae within the necrotic tissue (Gottschalk *et al.,* 1998). An increase in the activity of *β*-1,3-glucanase was observed in sugar beet leaves after treatment with the bioagent *Bacillus mycoides* (Bargabus, *et al.,* 2002). *β*-1,3-glucanase, produced in sugar beet during systemic resistance responses was first isolated and characterized by Gottschalk *et al.* (2002). The tested isolates of *B. subtilis* and *B. pumalis* elicited production of both *β* -1-3 glucanase and chitinase were significant since these PR proteins have a synergistic association leading to *Cercospora beticola* control (Esh *et al.,* 2011).

Foliar treatment with the tested bioagents resulted in markedly increase in peroxidase activity than that recorded in the untreated healthy and infected control. Peroxidases are monomeric proteins commonly dispersed in both the intra- and extracellular natural environment (Rathmell and Sequeira, 1974). Consequently, reinforcing the plant cell wall which decreases cell vulnerability to fungal wall-degrading enzymes, constrains diffusion of pathogen toxins, and act as a mechanical barrier against fungal physical penetration force (Brisson *et al.,* 1994). Furthermore, peroxidase is involved in the degradation of excessive levels of hydrogen peroxide (H_2O_2) that is generated in the plant tissues immediately after pathogen attack and induced several plant defense mechanisms, such as lignin biosynthesis and oxidative cross-linking of plant cell walls, as well as the generation of oxygen species (Bestwick *et al.,* 1998). The enhanced peroxidase activity was reported to be

associated with the induced systemic resistance in plants against several pathogens (Baysal *et al.,* 2005).

Application of the tested bioagents showed considerable increase in polyphenoloxidase activity in fodder beet plants, compared to the untreated healthy and infected controls. However, the highest PPO activity was achieved by *P. fluorescens*. PPO catalysis is the last step in the biosynthesis of lignin and other oxidative phenols. The mechanisms of PPO depend on two ways: firstly, by a direct action of PPO on the pathogen inhibition and suppression of its life cycle and secondly, induces mediated phenolic compounds which restrict the pathogen and enhance the biocontrol action (Mayer 2006 and Seleim *et al.,* 2014). Esh *et al.* (2011) found that the higher PPO activity was noticed in sugar beet plants treated with *Bacillus pumilus* and *B. subtilus* challenged with *Cercospora beticola*. In the present study, foliar treatment of bioagents showed a higher activity in PO and PPO in fodder beet plants which might be contribute to lignifications that will act as barriers against pathogen entry.

Due to application of biotic inducers as foliar treatments, substantial increase in phenylalanine ammonia lyase (PAL) activity was found in fodder beet plants inoculated with *C. beticola*. *P. fluorescens* (biotic inducer) provided the best protection against the pathogen, reflecting the maximum increase in PAL activity. Early induction of PAL is important, as it is the first enzyme in the phenylpropanoid pathway, which leads to the production of phytoalexins and phenolic substances destined for lignin formation, with the help of peroxidase (Nicholson and Hammerschmid, 1992).

Results obtained here are in agreement with those reported by Basha *et al.* (2006), who observed early and rapid induction of PAL activity in chickpea plants pretreated with *P. fluorescens* or *P. aeruginosa* challenge inoculated with *Sclerotinia sclerotiorum*. In addition, foliar treatment with *Bacillus pumilus* and *B. subtilus* led to a marked increase in PAL activity in induced sugar beet plants challenged with *C. beticola* (Esh *et al.,* 2011). Youssef *et al.* (2018), recorded an increase in PAL activity in tomato seedlings treated with *S. proteamaculans* challenged or not with *Alternaria solani*, as well as the increase in PAL activity reached 2 fold.

Furthermore, the biotic inducers as foliar treatments led to increase the phenolic compounds content compared with the untreated control. In this respect, the role of phenolic compounds in disease resistance was postulated by many authors like Nicholson and Hammerschmidt (1992) who indicated that phenols are oxidized to quinones or semi-quinones which are more toxic and play a great role as antimicrobial substances on the invaded pathogen. In addition, phenolic compounds may impede pathogen infection by increasing the mechanical strength of the host cell wall (Benhamou *et al.,* 2000). Accumulation of phenolic compounds at the infection sites showed a correlation with the restriction of pathogen development, since such compounds are toxic substances to pathogens. Also, the resistance may be increased by change of pH of plant cell cytoplasm, due to the increase in phenolic acid content, resulting in inhibition of pathogen development (Khaledi *et al.,* 2015).

Results obtained in the present work are in agreement with the previous observations of Basha *et al.* (2006) who found that foliar spray and micro-injection of plant growth-promoting rhizobacterial, viz. *Pseudomonas fluorescens* and *P. aeruginosa* on chickpea, induced synthesis of phenolic compounds. Also, Sangeetha *et al.* (2010) reported higher accumulation of total phenolic compounds in plants treated with *Pseudomonas* spp. strains, especially in presence of pathogens.

Conclusions

The present study indicated that application of bacterial bioagents *i.e., B. subtilis, P. polymyxa, P. fluorescens, P. putida* could play a significant role in the protection of fodder beet plants against Cercospora leaf spot, mainly through the induction of systemic resistance. In addition, *in vitro* and *in vivo* results, the application of such bio-products in the control of Cercospora leaf spot on the field scale might provide a practical supplement to environmentally friendly disease management of the pathogen when they are combined with appropriate integrated disease management.

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إحداث المقاومة المُستحثة في بنجر العلف (*Beta vulgaris* L.) لمرض تبقع الأوراق السركسبوري المتسبب عن الفطر (*Cercospora beticola* Sacc.)

إيهاب علي ضياء سرحان
معهد بحوث امراض النباتات - مركز البحوث الزراعية – الجيزة

تم تقييم نشاط المقاومة الحيوية لعزلات البكتيريا *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida* ضد مرض تبقع الأوراق السركوسبوري في بنجر العلف تحت ظروف الصوبة والحقل مقارنة بالمبيد الفطري توبسين م-٧٠، وتم دراسة نشاط إنزيمات بيتا ١-٣ جلوكانيز، وبيروكسيديز، وبولي فينول اكسيديز، وفينيل الانالين امونيا ليز، وكذلك حمض الإندول أسيتيك، ومحتوى الفينولات الكلية في أوراق نباتات بنجر العلف المعاملة بالبكتيريا. وقد تراوح انخفاض شدة الإصابة تحت ظروف الصوبة في النباتات المعاملة بالبكتيريا المختبرة السابق ذكرها مابين ٥٨.٨٢ الي ٨٨.٢٤% ، وتحت ظروف الحقل تراوح انخفاض شدة الإصابة في النباتات المعاملة بالبكتيريا مابين ٤٦.٦٧ إلى ٨٠.٠٠% ، و٥٨.٣٣ إلى ٨٣.٣٣% في منطقتي التجريب في النوبارية وسخا على التوالي. ارتفع مستوى الإنزيمات المرتبطة بالدفاع النشط عن النبات وهي بيتا ١-٣ جلوكانيز، وبيروكسيديز، وبوليفينول اكسيديز، وفينيل الانالين أمونيا ليز بشكل معنوي حيث سجلت المعاملة *P. fluorescens* أعلى مستوى في النشاط الإنزيمي وكذلك كانت مستويات حمض الإندول أسيتيك، ومحتوى الفينولات الكلية في أوراق نباتات بنجر العلف المعاملة بالبكتيريا بدرجة أكبر من مستوياتها في النباتات غير المعاملة وبشكل معنوي. كما زادت معدلات النمو والإنتاجية، مثل طول الجذر، وأقطار الجذور، والأوزان الطازجة والجافة والنسبة المئوية للمواد الجافة لتحقق زيادة كبيرة في نباتات بنجر العلف المعاملة مقارنة بغير المعاملة. وتشير هذه النتائج إلى أن عوامل المكافحة الحيوية المستخدمة لعبت دوراً هاما في مكافحة مرض تبقع الأوراق السركسبوري في بنجر العلف من خلال تعزيز المقاومة الجهازية المستحثة في نباتات بنجر العلف.