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EFFECT OF SALICYLIC ACID AND/OR *Pseudomonas fluorescens* ON POTATO STRESSED WITH *Ralstonia solanacearum*

Saker, M. T.* ; Heba M. Ibrahim* ; Nevein A. S. Messiha, and Shahenda M. Farag****

*** Agric. Botany Dept., Fac. Agric., Mans. Univ., 35516, El-Mansoura, Egypt.**

**** Potato Brown Rot Project (PBRP) Dokki, Egypt**

ABSTRACT

The present study was undertaken to unravel the biocontrol potential of *Pseudomonas fluorescens* (*Pf*) against potato bacterial wilt caused by *Ralstonia solanacearum* and the possible augmentative effect of salicylic acid (SA) in this respect. Pathogen-inoculated and uninoculated plants were treated with either *Pf*, SA or both, and the effects of the treatments were tested on the pathogen prevalence, management and some pathogenesis-related biochemical constituents as well as on vegetative growth parameters of the host. In addition, molecular analysis was performed to assess the antibiotic-producing ability of *Pf*. Results indicated that *Pf* harbors the phlD gene encoding the antibiotic 2, 4-diacetylphloroglucinol. Application of SA augmented the biocontrol effect of *Pf* hence, the suppressive effect of the combined treatment surpassed that of either *Pf* or SA alone. Besides the direct effect of the combined treatment on the pathogen, indirect effects manifested by increasing the accumulation of ascorbate, phenols, chlorophylls and carotenoids as well as by inducing activity of the antioxidant enzymes peroxidase and superoxide dismutase. Direct and indirect effects of the combined treatment were reflected in enhanced growth of *Pf*+SA-treated plants compared with untreated, pathogen-inoculated plants. It was concluded that SA could be utilized to boost the biocontrol action of *Pf* as an environment-friendly approach for controlling potato bacterial wilt disease.

Keywords: Potato bacterial wilt, *Pseudomonas fluorescens*, *Ralstonia solanacearum*, Salicylic acid, systemic acquired resistance.

Abbreviations

AsA, ascorbic acid; CAT, catalase; *Pf* , *Pseudomonas fluorescence*; POD, peroxidase; *Rs*, *Ralstonia solanacearum*,; SA, salicylic acid; SAR, systemic acquired resistance; SIR, systemic induced resistance; SOD, superoxide dismutase.

INTRODUCTION

Potato bacterial wilt disease caused by *Ralstonia solanacearum* (smith) Yabuuchi is a major constraint in the production of potato and many other crops in tropical, subtropical and temperate regions of the world (Hayward,1991). In Egypt the disease is endemic in some areas in Delta that forced potato production to move into what is called pest free areas (PFAs) which exist in the newly reclaimed areas. An effective control method is needed to gradually eradicate the pathogen from the Delta area along with protecting the PFAs from being infected. This method should consider the environmental safety.

Pseudomonas spp are a plant growth promoting rhizobacteria that are indigenous to soil and the plant rhizosphere and play a major role in the biocontrol of plant pathogens . Fluorescent pseudomonas was effective in biocontrolling bacterial wilt of tomato caused by *Rs* (Guo *et al.*, 2004). In greenhouse experiments, plants treated with *Pseudomonas* isolates EB9 and EB67 reduced the wilt incidence in eggplant by more than 70% (Ramesh *et al.*, 2009). The biocontrol effect of *Pseudomonas spp* is mediated through competition *via* secretion of siderophores which trap any available soil iron to starve the pathogens for the iron (Höfte and Bakker, 2007), production of antibiotics such as phenazine-1-carboxylic, phloroglucinol, 2,4-diacetylphloroglucinol (McSpadden Gardener *et al.*, 2001; Zhang *et al.*, 2005) or production of lytic enzymes (Bakker *et al.*, 2007). *Pseudomonas spp* may also contribute to biocontrol of plant pathogens indirectly through inducing systemic acquired resistance; SAR (Bakker *et al.*, 2007). Two *Pseudomonas* spp., *P. putida* (WCS358r) and *P. fluorescens* (WCS374r) triggered SAR in *Eucalyptus* when infiltrated into the two lower leaves 3–7 days before challenging the plant with the bacterial wilt pathogen (Ran *et al.*, 2005). *P.fluorescens*-induced SAR is mediated via inducing the biosynthesis of SA, iron-regulated metabolites, pseudobactin (Psb) and peudomonine (Psm), and a siderophore with a SA moiety under iron limitation (Leeman *et al.*, 1996 ; Ran *et al.*, 2005).

Salicylic acid (SA) has received much attention because of its contribution to plant defence response under biotic and abiotic stresses. It was considered as a signal during plant-microbe interaction as a chemical defense against the microbe infection (Malamy and Klessig, 1992; Raskin, 1992). SA, when applied exogenously, conferred resistance against various pathogens in Dicots (Abdel-Said *et al.*, 1996; Ryals *et al.*, 1996; Shah and Klessig, 1999) as well as Monocots (Pasquer *et al.*, 2005; Makandar *et al.*, 2006). SA–induced SAR is mediated through altering many aspects of plant metabolism which aid in plant's stress resistance. SA had a protective role against oxidative damage (Kusumi *et al.*, 2006) through its inhibiting effect on ROS production thereby maintaining membrane integrity (Cui *et al.*, 2010). In addition, SA was reported to induce a variety of metabolic defence responses in plants growing under stress conditions. It was found to increase the contents from chlorophylls (khodary, 2004; Hayat *et al.*, 2005; Kaydan *et al.*, 2007), carotenoids (Eraslan *et al.*, 2007; Kaydan *et al.*, 2007) and photosynthetic efficiency (khodary, 2004). In addition, SA-induced both the enzymatic (Szepesi *et al.*, 2008; Yusuf *et al.*, 2008) and non-enzymatic (Ali *et al.*, 2007) component of the plant antioxidant system. As part of the antioxidant activity of SA is its effect on inducing plant phenolics. SA was reported to induce phenolics content in stress-affected plants (Guleria *et al.*, 2005; Mandal, 2010; Radwan, 2012) with which antioxidant activity was positively correlated (Kim *et al.*, 2006). Nevertheless, when considering the protective role of SA against oxidative damage, it is worth to mention that application of SA, at suitable concentrations, may lead to transient oxidative stress in plants, which is required in generating the antioxidant processes cascade (Knörzeret *et al.*, 1999).

The objective of the present investigation was to test the hypothesis that the application of SA will augment the biocontrol effect of *P. fluorescens* against potato bacterial wilt caused by *R. solanacearum*.

MATERIALS AND METHODS

Antibiotic producing ability of *Pf* (PD 3339) isolate and its mutant

The antibiotic-producing ability of phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (Phl) of both the wild and the mutant isolate were studied according to Raaijmakers *et al.* (1997). Phl2a and Phl2b targeting *phlD* gene (745 bp) and PCA2a and PCA3b primers targeting *phzCD* gene(1400 bp) were utilized as recommended by Zhang *et al.*(2005). The sequences of the primers is shown in Table (1).

Table (1): Primers used for testing the Antibiotic producing ability of *Pf* (PD 3339) isolate and its mutant

Target gene	Primer	Sequence (5´-3´)	Amplicon size
phlD	Phl2a	GAGGACGTCGAAGACCACCA	745 bp
	Phl2b	ACCGCAGCATCGTGTATGAG	745 bp
phzCD	PCA2a	TTGCCAAGCCTCGCTCCAAC	1400 bp
	PCA3b	CCGCGTTGTTCCTCGTTCAT	1400 bp

DNA extraction and cell lysis: DNA extraction and PCR were conducted after Raaijmakers *et al* (1997) as follows:

The bacterial colonies (2-mm diameter) were suspended in 100 ml of lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate [SDS]) and incubated for 15 min at 100^{o}C. The suspension was centrifuged for 1 min at 12,000 rpm and diluted 50-fold in sterile distilled water. 5 µl of the diluted suspension was used in each reaction.

PCR amplification: was carried out in a 25-µl reaction mixture which contained 5 µl of a diluted heat-lysed cell suspension, 1x GeneAmp PCR buffer (Dream buffer), 200 µM each dATP, dTTP, dGTP, and dCTP (Promega), 20 pmol of each primer, and 2.0 U of Ampli*Taq*DNA polymerase (DreamTaq; fermentus)). Each mixture was covered with 1 drop of mineral oil. Amplifications was performed with a Biometra, T-Personal) thermal cycler. The PCR program consists of an initial denaturation at 94^{o}C for 2 min followed by 30 cycles of 94^{o}C for 60 s, 67^{o}C for 45 s, and 72^{o}C for 60 s and a final extension at 72^{o}C for 8 min. Samples (9 µl) of the PCR products was separated on a 1.2% agarose gel in 1x TBE buffer satined with ethidium bromide. The ladder used was 100bp (GelPilot). The separation was made at 75 V for 45 min. PCR products were visualized under a UV trans-illuminator.

***In vitro* inhibition bioassay of *Pf* (PD 3339) isolate and its mutant**

An antagonistic *Pf* strain (PD 3339), isolated from infected potato tubers and identified by biochemical test as well as fatty acid analysis according to Messiha (2001) was used in the present study. For easier tracing, a spontaneous rifampicin and chloramphenicol-resistant mutant (100

ppm) of the *P. fluorescens* strain were selected and adapted in the laboratory for testing its antagonistic potential *in vitro* and *in vivo.*

The antagonistic potential and the growth of the mutant strain was evaluated in *vitro* and compared with the original strain (PD 3339). Each strain was streaked separately over the middle on King's B medium plates (KB: proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4$, 1.5 g; agar, 20 g; glycerol, 15 ml; dist. water; 1000 ml). Plates were incubated at 28°C for 48 h. A mixture of 6 different isolates of *R. solanacearum*, previously isolated from infected potato tubers and identified according to Messiha (2001), was streaked in 6 lines perpendicular to the antagonist. The plates were incubated at 28°C for 48 hours. The distance from the antagonist that was free from the pathogen was determined (Messiha *et al.*, 2007) from at least 5 plates for each antagonistic strain.

The effect of *Pf* and/ or SA in controlling potato bacterial wilt under greenhouse conditions

Layout of the experiment

The susceptible potato [*Solanum tuberosum* L.] cv. Lady Rosette was selected for conducting the experiment. Soil used was sandy soil from Ismalia governorate at the rate of 3.5 kg/pot (25-cm diameter). A mixture of 6 different isolates of *Rs*, previously isolated from infected tubers was added to the soil at density of 10^7cfu/g. *Pf* was added also to the soil at the same density. Salicylic acid was added at rate of 0.5 mM pre-planting by soaking seed tubers in SA solution for 30 min, in addition to a foliar spray at 30 and 45 d after sowing; DAS). The experiment comprised four treatments, each with four replicates [*Pf* (PD 3339) only, SA only*, Pf* + SA in addition to *Rs*-inoculated positive control. Watering of plants was made regularly throughout the growth period. Disease suppression was estimated by CFU count, in soil 21 DAS and again at the end of the experiment as well as in rhizosphere and crown area at the end of the experiment, on SMSA medium. In addition, area under disease progress curve (AUDPC) was determined according to Elphinstone *et al.*, (1996). The tubers were also tested using IFAS (immunofluorescent antibody staining) test (Janse 1988).

Plant growth parameters

At the end of the experiment, 67 DAS, vegetative growth was determined by random sampling of 3 plants from each replicate. Vegetative growth was determined as plant length (cm), plant fresh and dry weight (g), leaf weight (g), and leaf area (cm^2).

Chemical constituents

Photosynthetic pigments: Leaf photosynthetic pigments were determined in the terminal leaflet of the 4^{th} leaf from plant top 35 DAS. Total chlorophylls (chls) were determined as described by Arnon (1949) whereas carotenoids (carots) were estimated according to the method of Lichtenthaler and Wellburn (1983).

Ascorbic acid: ascorbic acid concentration in the leaves was determined according to the method of Cakmak and Marschner (1992). 0.2 ml of the plant extract was mixed with 0.5 ml phosphate buffer (150 mM, pH 7.4) containing 5 mM EDTA. For colour development, the following reagents were

added: 0.4 ml TCA (10%), 0.4 ml orthophosphoric acid (44%), 0.4 ml 1,2 bipyridine in 70% ethyl alcohol and 0.2 ml FeCl3 (3%). The mixture was incubated at 40 ^{o}C for 40 min. The absorbance was read at 525 nm. Ascorbic acid concentration was determined using a standard curve established with L-Ascorbic acid concentrations in the range of 10-100 µg ml-1.

Total Phenols: According to the method of Singleton (1999), **l**eaf sample of 1 g was homogenized in 10 times volume of 80% ethanol, then centrifuged at 10,000 rpm for 20 min. The residue was re-extracted with 5 times volume of 80% ethanol, centrifuged as previous and the supernatant was pooled and evaporated to dryness, then 0.5ml of folin ciocalteau reagent, was added. After 3 min, 2 ml of 20% Na_2CO_3, was added and mixed thoroughly. Tubes were kept in boiling water for one min, cooled and the absorbance was measured at 650 nm against a reagent blank.

Determination of the antioxidant enzymes activity

Preparation of the enzyme extract: Enzyme extract (EE) was prepared by grinding 1 g of the leaves in 10 ml of 0.1M K-P-buffer (pH 6.8) containing 1 mM Na-EDTA using a chilled mortar and pestle. The homogenate was then centrifuged at 3000 g for 15 min and the supernatant was used for the enzyme assays. All operations were performed at 4^{o}C**.**

Catalase: CAT activity (EC 1.11.1.6): was determined by the degradation of H_2O_2 according to Velikova *et al.* (2000). The reaction mixture (RM), 1 ml, contained 10 mM k-P-buffer (pH 7.0), 33 mM H_2O_2 and 50 µl of the EE. The decrease in H_2O_2 was followed as a decline in optical density at 240 nm.

Estimation of Superoxide dismutase (SOD) activity: SOD activity (EC 1.15.1.1) was determined according to the method of Van Rossun *et al.* (1997). 50 µl of the EE was added to a solution containing 13 mM L-methionine, 75 µM nitroblue tetrazolium chloride (NBT), 100 µM EDTA and 2 µM riboflavin in a 50 mM K-P-buffer (pH 7.8). The reaction took place in a chamber under illumination of a 30 W-fluorescent lamp, started by turning the lamp on and stopped 5 min later by turning it off. The blue formazane produced through NBT's photoreduction was measured as increase in absorbance at 560 nm. Control RM had no EE. The blank solution had the same components included in the complete RM but was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction in comparison with tubes lacking the EE.

Estimation of peroxidase (EC 1.11.1.7) activity: The RM contained 1.8 ml 50 mM sodium phosphate buffer (pH 7.0), 0.1 ml phenol (1.0 mg/ml), 0.1 ml 4-aminophenazone (0.5 mg/ml) and 0.1 ml enzyme extract. The mixture was incubated at 40^{o}C for 5 min. Then 1.0 mM H_2O_2 was added and the mixture was incubated at 40^{o}C for 10 min. The absorbance was measured at 520 nm. One unit of enzyme activity is defined as the amount of H_2O_2 decomposed per min per mg protein (Pundir *et al.,* 1999; Verma and Mishra, 2005).

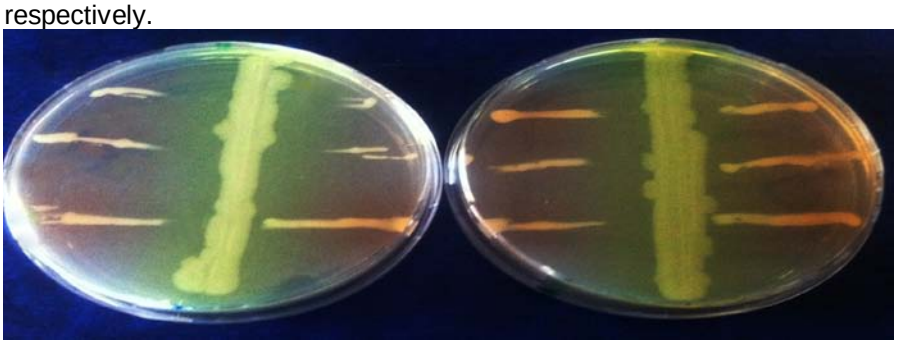
Statistical analysis

All analysis was conducted using SPSS v16. When data was not normally distributed, Kruskal-Wallis, non-parametric analysis was conducted to compare different parameters expressing disease incidence.

RESULTS

The antagonistic ability of Pf (PD 3339) versus its derived mutant

There was no significant difference in antagonistic potential in both the wild strain (PD 3339) and the mutant (Fig 1). The mean clear distance between the antagonist and the pathogen (distance free from the pathogen) was equal to 1.00 and 1.05 cm, for the wild strain and its derived mutant respectively.



Wild strain **Mutant strain**

Fig (1): The antagonistic ability of *Pf* (PD 3339) compared with its derived mutant

Antibiotic producing ability of *Pf* (PD 3339) versus its drived mutant

The two *P. fluorescens*, the wild and mutant strains showed similar bands developed in the gel for the two primers used, Phl2a (forward), Phl2b (reverse) targeting *phlD* gene responsible for producing the antibiotic 2,4-diacetylphloroglucinol (Fig 2). The two strains didn't found to have *phzCD* gene using PCA2a (forward) and PCA3b (reverse) primers (data not shown).

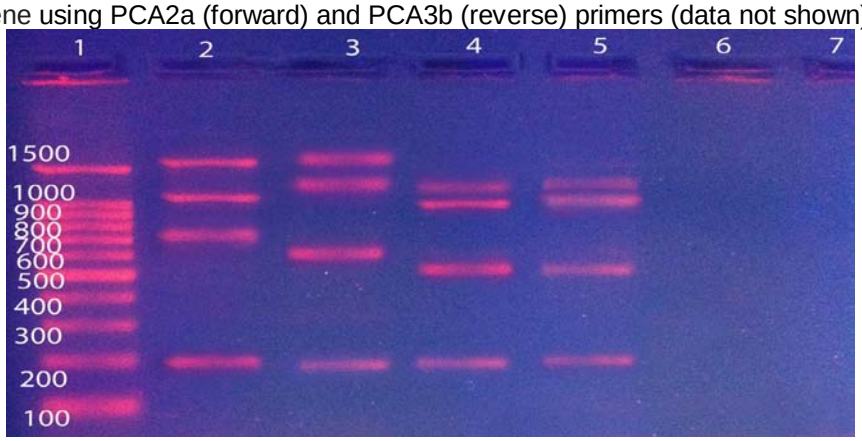


FIG (2): Agarose gel electrophoresis of PCR product amplified from genomic DNA of different fluorescent pseudomonas strains, using Phl2a (forward), Phl2b (reverse) primers targeting *phlD* gene. Lanes (1=marker, 2=*P. putida* (PD 3142), 3= *P. fluorescens* (PD 3340), 4= *P. fluorescens* wild strain (used in this research) (PD 3339), 5=mutant strain (used in this research), 6=negative control,7= negative control)

The effect of *Pf* and / or salicylic acid in controlling potato bacterial wilt under greenhouse conditions

The effects of *P. fluorescens* and SA separately and in combination in bacterial wilt suppressiveness are shown in Table (2). Disease incidence was expressed by different parameters including, count of the pathogen in 1 g soil 21 DAS as well as AUDPC and count of the pathogen in soil, rhizosphere and crown area at the end of the experiment (67 DAS). The most effective treatment was *P. fluorescens* in combination with salicylic acid compared to the positive control in all tested parameters (p=0.008). The count of the pathogen in soil at the end of the experiment decreased from about 10^5cfu/g dry soil to less than 10 cfu/g dry soil, the pathogen disappeared from the plant rhizosphere and crown area and the AUDPC decreased from 34 into 0 (Table 2). The second effective treatment was salicylic acid only, which eliminated the pathogen from soil and rhizosphere at the end of the experiment, and decreased the count of the pathogen in the crown area from about 0.5 x 10^7 for the positive control to about 10^4 . AUDPC was not significantly affected due to SA treatment. The least effective treatment was *Pf* which slightly decreased the count of the pathogen in the soil after 21 days (p=0.008) and noticeably at the end of the experiment from about 10^5 cfu/g dry soil to 10^2 cfu/g dry soil (p=0.016).

Table (2)*: Effect of *Pf* and/ or SA on potato bacterial wilt disease as measured by CFU of *Rs* per g dry soil, plant crown, rhizosphere and AUDPC (log transformed +1).

Treatments	Rs count in soil 21 DAS	Rs count in soil at the end of experiment	Rs count in crown at the end of experiment	Rs count in rhizosphere at the end of experiment	AUDPC
PC**	6.45± 0.06	5.42± 0.16	7.52± 0.09	8.37± 0.2	34.02± 1.95
Pf	6.00± 0.07	2.04± 0.13	7.45± 0.03	7.90± 0.8	1.32± 0.83
SA	5.89± 0.08	0	3.82± 0.15	0	87.00 ±5.80
Pf+SA	5.60± 0.11	0.9±0.09	0	0	0

***, Mean of four replicates ± standard error; **, Positive control (pathogen only)**

Detection of *Rs* in potato tubers after different treatments using IFAS (immunofluorescent antibody staining)

Potato tubers were collected from different replicates for each treatment separately.The results showed difference in number of pathogen cells/ ml between different treatments (Table 3).

Table (3): IFAS (immunofluorescent antibody staining) for treated potato tubers

Treatments	Number of cell count/ml
Negative control	0
Positive control	8×10^7
Pf only	8×10^6
SA only	4×10^6
Pf +SA	4×10^5

Effect of *Pf* and/ or SA on vegetative growth under greenhouse conditions

a) Rs-unstressed plants

Vegetative growth as expressed by plant height, leaf weight, leaf area, plant fresh and dry weight as affected by different treatments as compared with negative control are shown in Table (4).

Data revealed that, generally, *Pf* enhanced vegetative growth where a significant increase in fresh weight and dry weight was recorded. On the other hand, SA inhibited vegetative growth parameters, notably leaf area. The combined treatment increased dry weight. Plant height didn't affected significantly.

Table (4): Effect of *Pf* and / or SA on vegetative growth characteristics of *Rs*-free plants

Treatments	plant height (cm)	leaf weight (gm)	leaf area (cm^2)	Plant fresh weight (gm)	Plant dry weight (gm)
negative control[1]	52.67	12.00	333.33	21.87	3.4
Pf	58.84	15.30*	365.00	28.53*	4.6*
SA	63.67	8.47*	173.33*	18.17	2.8*
Pf +SA	61.00	12.77	338.34	22.57	3.7*

[1] = Rs un-inoculated plants; *, significant ± relative to negative control at p≤ 0.05

b) *Rs*-stressed plants:

Vegetative growth as expressed by plant height, leaf weight, leaf area, plant fresh and dry weight as affected by different treatments are shown in Table (5).

Results showed that soil inoculation with the pathogen significantly decreased all vegetative growth parameters compared to uninoculated plants. Inoculation with *Pf* increased plant height as well as plant fresh and dry weights relative to positive control. SA treatment alone did not restore the damaging effect of *Rs* on vegetative growth and further decreased the tested parameters. When the action of *Pf* was combined with that of SA, plant fresh and dry weights were restored and even increased relative to positive control.

Table (5): Effect of *Pf* and/ or SA on vegetative growth of pathogen-inoculated plants

Treatments	plant height (cm)	leaf weight (gm)	leaf area (cm^2)	Plant fresh weight (gm)	dry weight (gm)
Negative control[1]	59.67	8.57	428.33	19.67	2.27
Positive control[2]	53.66 ^{*n}	6.67 ^{*n}	333.33 ^{*n}	16.93 ^{*n}	1.766 ^{*n}
Pf	58.33 ^{*p}	6.53	326.67	20.23 ^{*p}	2.06 ^{*p}
SA	47 .00 ^{*p}	4.00	200.00 ^{*p}	12.90 ^{*p}	1.43 ^{*p}
Pf+SA	51.33	5.73	286.67	19.07 ^{*p}	2.03 ^{*p}

[1] , Rs-uninoculated plants; 2, Rs-inoculated plants; *n and *p, significant ± relative to negative and positive control, respectively at p≤ 0.05 .

The effect of *Pf* and/ or SA on chemical constituents and enzyme activities.

a): Rs-uninoculated plants

Data presented in Table (6) illustrate the effects of different treatments on the estimated biochemical constituents of *Rs*-uninoculated plants. Data show that treatment with *Pf* increased both chlorophylls (chls) and carotenoids (carots) content and enhanced the activities of CAT and POD. AsA and phenols content as well as the activity of SOD were not significantly affected. SA treatment increased AsA and phenols content and enhanced the activity of SOD and did not affect other parameters significantly. In plants received the combined treatment all tested parameters were increased except phenols content.

Table (6): Effect of *Pf* and/ or SA on Chemical constituents of *Rs*-uninoculated plants.

Treatments	AsA µg g^{-1} FW	Total phenols mg g^{-1} FW	Total chlorophylls mg g^{-1} FW	Carotenoids mg g^{-1} FW	Enzymes		
					SOD U g^{-1} FW	POD U g^{-1} FW	Catalase**
Negative control[1]	6.7	1.3	2.9	0.9	111	20.8	0.19
Pf	7.2	1.2	3.6*	1.4*	133	27.2*	0.33*
SA	7.7*	1.7*	3.1	1.1	223*	19.4	0.18
Pf+SA	7.4*	1.5	3.8*	1.5*	235*	28.8*	0.34*

[1] = Rs-uninoculated plants; *, significant ± relative to negative control at 0.05 ; **, estimated as µ mol H_2O_2 reduced g^{-1} DW S^{-1}

***b): Rs*-stressed plants:**

Data presented in Table (7) illustrate the effects of different treatments on the estimated biochemical constituents of *Rs*-inoculated plants**.** Data show that inoculation with *Rs* elicited the accumulation of phenols and also increased AsA content. In addition, catalase activity was enhanced in response to pathogen infection. On the other hand, chls and carots contents as well as the activities of POD and SOD were decreased in *Rs-stressed plants.* When the effects of *Pf*, SA or their combination on *Rs*-infected plants were considered, data indicate that *Pf* increased chls , carots and the activity of SOD, whereas reduced total phenols and catalase activity. SA treatment increased phenols, chls and carots contents as well as the activities of both POD and SOD. On the other hand, AsA content was not affected significantly relative to positive control in response to SA. In plants treated with both *Pf* and SA, all tested parameters were increased, CAT activity was reduced and phenols content was not affected significantly compared with untreated, pathogen-affected plants

Table (7): Effect of *Pf* and/ or SA on Chemical constituents of *Rs*-uninoculated plants.

Treatments	AsA µg g^{-1} FW	Total phenols mg g^{-1} FW	Total chlorophylls mg g^{-1} FW	Carotenoids mg g^{-1} FW	Enzymes		
					SOD U g^{-1} FW	POD U g^{-1} FW	Catalase**
Negative control[1]	6.8	1.0	4.4	1.2	121	17	0.19
Positive control[2]	9.4*n	2.3*n	3.1*n	0.8*n	96*p	10*p	0.63*n
Pf	10.1	1.7*p	4.9*p	1.8*p	361*p	10	0.11*p
SA	10.4	2.6*p	3.8*p	1.6*p	260*p	18*p	0.15*p
Pf + SA	12.4*p	2.2p	3.7*p	1.5*p	250*p	16*p	0.11*p

[1], Rs-uninoculated plants; 2, Rs-inoculated plants; *n and *p, significant ± relative to negative and positive control, respectively at p0.05 ; **, estimated as µ mol H_2O_2 reduced g^{-1} DW S^{-1}

DISCUSSION

The efficiency of a biocontrol agent may be related to antibiotic and proteolytic enzyme secretion,nutrient competition, colonization activities and plant growth promoting activities as well as inducing plant resistance (IR). *Pf* is considered as a biocontrol module as different mechanisms are involved in its effectiveness as a biocontrol agent.

Antibiosis:

Results of the present investigation confirmed the harborage of the *phlD* gene which is responsible for producing the phenazine derivative, 2,4-diacetylphloroglucinol antibiotic (DAPG) by the adopted biocontrol agent *Pf* (strains PD 3339) as well as its rifampicin and chloramphenicol resistant mutant. Phenazine derivatives are the first antibiotics found to be involved in the biocontrol activities of fluorescent pseudomonas in general and particularly *Pf which* produce 2,4-diacetylphloroglucinol (Handelsman and Stabb, 1997). DAPG is known to have a broad-spectrum antibacterial and antifungal activities (Marchand *et al.*, 2000).

Nutrient competition (iron competition):

Fluorescent pseudomonas is known to produce a class of siderophores which are able to harbor ferric ions making complex iron-binding molecules and hence starve the pathogen to the iron (Mirleau *et al.*, 2000).The used wild strain of *P. fluorescens* (PD 3339) was able to produce the siderophore pyoverdine compound, as ferric ions availability in vitro decreased the efficiency of this biocontrol agent (Messiha 2001).

Colonization activities:

The ability of the biocontrol agent to colonize in the rhizosphere of the host crop is an important determinant of the efficiency of the biocontrol agent. Tracing the rifampicin and chloramphenicol mutant strain in the rhizosphere of potato by plating on media amended with the two antibiotics showed a great tendency of the strain to colonize the rhizosphere of potato

plants. The density of the biocontrol agent in the rhizosphere area was more than 10^8cfu/g dry rhizosphere soil compared to 10^2cfu/g in soil part (data not presented).

Plant growth promotion:

In general *Pf* increased the vegetative growth of *Rs*-stressed or unstressed potato plants (Tables 4, 5). As a plant growth promoting rhizobacterium, *Pf* may induce plant growth via either direct or indirect approaches. The direct one involves the production of plant growth substances (auxins, cytokinins and gibberellins) and facilitation of the uptake of nutrients. Indirectly, *Pf* may enhance plant growth through attenuating plant pathogens or by increasing the systemic resistance of the host (Seleim *et al.*, 2011).

Induced resistance:

Plants develop a variety of metabolic defence responses against biotic and abiotic stresses. Defencive responses induce the production of several secondary metabolites such as phenolics, flavonoids and other low molecular weight substances (Ali *et al.*, 2007). In this study, application of SA increased the potential of *P. fluorescens* (PD 3339) to suppress *Rs*, the causative agent of potato bacterial wilt. This effect may be due to its effect in inducing SAR. There are two types of induced resistance, systemic acquired resistance (SAR) which depends on transcriptional activation of genes encoding PRs (Van Loon 1997). Chemicals such as SA activates this SAR response as well as inducing the PRs genes (Djavaheri, 2007). SA-induced expression of PRs genes was accompanied with conferred resistance against various pathogens in Dicots (Shah and Klessig, 1999) and Monocots (Makandar *et al.*, 2006). Accumulation of various products as a result of SA-induced expression of PRs genes were reported and were considered components of SA-enhanced SAR. These products include callose plugs (Kohler *et al.*, 2002), H_2O_2 (Lamb and Dixon, 1997), carotenoids (Moharecar *et al.*, 2003); Phenolics (Ali *et al.*, 2006; Mandal , 2010), lignin (Mandal and Mitra, 2007; Mandal , 2010). Our results indicated that SA enhanced phenolics formation in pathogen-free plants and exacerbated pathogen-induced phenolics. SA-induced phenolics was regarded as an important part of the SA-antioxidant activity due to the concomitant increase of the DPPH radical scavenging activity (Ali *et al.*, 2007). In addition, Kim *et al.* (2006) reported a linear correlation between phenolics content and antioxidant activity. SA-induced phenolics may be mediated through activating the enzymes responsible for phenolics biosynthesis especially glucose 6-phosphate dehydrogenase, phenylalanine ammonia lyase, shikmate dehydrogenase and cinnamyl alcohol dehydrogenase (Ali *et al.*, 2007).

Another important part of the effect of SA on inducing resistance in plants under stressful conditions is its effect on both the enzymatic and non-enzymatic components of the plant defense system. In the present study, SA enhanced theactivities of POD and SOD in unstressed and pathogen-stressed plants whereas it didn't affect and reduced CAT activity in unstressed and pathogen-stressed plants, respectively. In this regard, He *et al.* (2002) reported that when SA was applied exogenously at suitable

concentrations, it led to an enhancement in the efficiency of the plant's antioxidant system. With regard to the enzymatic antioxidant system, activities of POD and SOD were reported to be enhanced (Yusuf *et al.*, 2008; Noreen *et al.*, 2009). On the other hand, CAT was reported to either decreased (Janda *et al.*, 2003; Krantev *et al.*, 2008) or remain unchanged (Noreen *et al.*, 2009). According to Janda *et al.* (2003), SA-reduced CAT activity led to an increase in H_2O_2 in plant tissues which may play a key role in providing the SAR and the capacity of oxidative stress tolerance (Gechev *et al.*, 2002; Nemeth *et al.*, 2002). In this context, Baby-Joseph and Sujatha (2010) concluded that moderate doses of SA can increase H_2O_2 concentration in plant tissues thereby activate the antioxdative mechanisms. According to Lamb and Dixon (1997), SA-induced H_2O_2 plays a key role in initiating a hypersensitive response and providing SAR against pathogenic microbes. In addition, the recorded enhancement of SA on POD activity may aid lignifications and rigidification of plant cells, which protect plant tissues from pathogen's attack (Kobayashi *et al.*, 1994). Cell wall strengthening, through the deposition of lignin, that was preceded by induction of lignin biosynthesis enzymes, played an important role in the defence response of tomato that have been treated with resistance inducers (Mandal and Mitra, 2007).

Another kind of induced resistant not depending on *PRs* genes occurred by colonization of plant roots by certain strains of non-pathogenic rhizobacteria results in systemically induced resistance (SIR) which enhanced plant defense mechanism (Van Peer *et al.* 1991; Wei *et al.* 1991). Fluorescent pseudomonas is examples for these rhizobacteria which Induced systemic resistance by root colonization ((Bakker *et al.* 2007; Pieterse *et al.* 1996). *P. aeruginosa* produces pyoverdin, pyochelin and SA under iron limited conditions (Buysens *et al.*, 1996). Production of the phenazine compound pyocyanin together with the SA-containing siderophore, pyochelin, are required for SIR against *Botrytiscinerea* in tomato (Audenaert *et al.* 2002). Involvement of the two different induced resistances may explain the increase of disease suppression after combining the antagonist with salicylic acid.

Based on the obtained results, It could be concluded that SA may be utilized to boost the biocontrol action of *P.fluroscence* as an environment-friendly approach for controlling potato bacterial wilt disease.

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تأثير المعاملة بحامض السالسيلك و/ أو بكتريا *Pseudomonas fluorescence* على نباتات البطاطس المعرضة للإجهاد الناشئ عن الإصابة بالكائن الممرض *Ralstonia solanacearum*

محب طة صقر* ، هبة محمد إبراهيم * ، نيفين انور شحاته مسيحة و شاهندة محمد فرج****

*** قسم النبات الزراعى، كلية الزراعة، جامعة المنصورة، 35516 ، المنصورة، ج.م.ع**

**** مشروع العفن البنى فى البطاطس (PBRP) ، وزارة الزراعة، الدقى، ج.م.ع**

أجريت الدراسة بهدف إيضاح مدى فاعلية النوع البكتيرى *Pseudomonas fluorescence* (*Pf*) فى المقاومة الحيوية للنوع البكتيرى *Ralstonia solanacearum* المسبب لمرض الذبول البكتيرى فى نبات البطاطس، وما إذا كانت إضافة حامض السالسيلك سوف تعزز فعل بكتريا *Pf* / فى مقاومة الكائن الممرض. تمثلت معاملات الدراسة فى التلقيح ببكتريا *Pf* ، إضافة حامض السالسيلك، أو كلاهما معا للنباتات التى لقحت بالكائن الممرض والتى لم تلقح، وأختبر تأثير المعاملات على مدى إنتشار الكائن الممرض والفعل المقاوم لة وكذلك على بعض المكونات البيوكيماوية التى لها علاقة بتحمل الإجهاد الناشئ عن الإصابة المرضية ومدى إنعكاس ذلك على نمو النبات العائل. وبالإضافة لذلك فلقد أختبرت قدرة بكتريا *Pf* على إنتاج المضادت الحيوية من خلال عمل تحليل جزيئ Molecular analysis. ولقد أوضحت نتائج الدراسة أن بكتريا *Pf* تحتوى على الجين phlD المنتج للمضاد الحيوى 2, 4-diacetylphloroglucinol. ولقد كان لإضافة حامض السالسيلك تأثير مؤازر لفعل بكتريا *Pf* حيث كان الأثر المقاوم للكائن الممرض للمعاملة المشتركة أكبر من أثركل منهما على حدة. وبالإضافة للتأثيرات المباشرة، فلقد أدت المعاملة المشتركة إلى إستحثاث مقاومة العائل من خلال زيادة المحتوى من حامض الأسكوربيك، الفينولات، الكلوروفيلات، الكاروتنيدات، ونشاط إنزيمات Peroxidase و Superoxide dismutase وكانت محصلة هذة التأثيرات زيادة نمو النباتات التى تأثرت بالفعل المشترك لكل من حامض السالسيلك و *Pf* ، مقارنة بالنباتات الغير معاملة والمعرضة لإجهاد الكائن الممرض.

وبناء على نتائج الدراسة، فإنة يمكن إستنتاج أن المعاملة بحامض السالسيلك يمكن أن تستخدم لمؤازرة الفعل المضاد للبكتريا Pf لمقاومة مرض الذبول البكتيرى فى البطاطس لتلافى أو تقليل إستخدام المبيدات الملوثة للبيئة

قام بتحكيم البحث

أ.د / محمد نصر الدين هلالى	**كلية الزراعة – جامعة المنصورة**
أ.د / سعيد عواد شحاته	**كلية الزراعة – جامعة عين شمس**