Control of potato brown rot and wilt disease caused by *Ralstonia solanacearum* using some water plant extracts

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ABSTRACT:

Ten isolates of *Ralstonia solanacearum* were isolated from naturally infected potato plants, collected from different localities of Gharbia, El- Minia, Assiut and sohag governorates. The isolate GK5 exhibited the highest wilt severity. Antibacterial activity of aqueous plant extracts of *Hibsicus sabdariffa, Punica granatum* and *Eucalyptus, spp.* were tested against *Ralstonia solanacearum* the causal pathogen of bacterial wilt of potato *in vitro, vivo* and field experiments. *In vitro*, all concentrations 25%,50%, 75% and 100% of the tested plant extracts were used against of the pathogen. Among all tested concentrations of plant extracts only the concentration 100% and 75% from extract *Hibsicus sabdariffa* found to be able to inhibit the growth of bacterial pathogen *in vitro*. *In vivo* experiment, all plant extracts tested significantly reduced diseases severity by *Hibsicus sabdariffa* (13.2%) followed by *Punica granatum* (35.85%). In field experiment, application of plant extracts caused highest reduction in severity of bacterial wilt. These three plant extracts showed the increased activity of defences related enzymes viz, peroxidase (PO), polyphenoloxidase (PPO) and phenylalanine ammonia- layes (PAL) in treated potato plants challenged with *Ralstonia solanacearum*. Significant activities of PO, PPO and PAL was observed at 8days after the treatment. The maximum PO (4.5 unit/ mg potein) treatment with *Punica granatum*.
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INTRODUCTION

Potato (*Solanum tuberosum*) is the most important vegetable crops on the family Solanaceae (Khoso, 1994). Potato plants attacked by many diseases, among them soil-borne diseases are considered to cause loss of potato tuber yield was 10-90% annually (Hamedo and Makhlouf, 2016). Bacterial wilt caused by *Ralstonia solanacearum* is an important disease that spreads worldwide and infects hundreds of plant species, such as potato, tomato, banana, pepper and even trees. *R. solanacearum* is gram-negative, rod-shaped bacterium measuring 0.5-0.7 x 1.5-2.0 µl size. It grows well at 28-32 ^{o}C in the aerobic conditions (Hayward, 1991). In Egypt, it is considered as one of the limiting factors to potato production (Messiha *et al*., 2007). In the last few years, the disease has taken more attention as a serious problem for potato exportation to Europe and therefore plant quarantines in importing countries are quite alert for the Egyptian potatoes (El-Ariqi *et al*., 2005). Control of this bacterium is much difficult because it is a soil-borne pathogen, long survival period in the soil has a wide host range, and a wide biological variation. Control of bacterial wilt is difficult and no single methods recorded 100% effective the control of such bacterium could be possible through the use of a combination of diverse method (Champoiseau *et al*., 2010). The use of chemicals has not been effective in the control of bacterial wilt of the potato because the pathogen is a soil-borne and spreads systemically in plant tissues. The use of copper-based bactericides and antibiotics

seldom gave satisfactory control. Botanicals, because of their natural origin are biodegradable, and they do not leave toxic residues or by-products to accumulate in the environment (Abo-Elyousr and Asran, 2009). Natural plant products are important sources of new agrochemicals for the control of some plant diseases. Plant extracts are potentially environmentally safe alternatives and possible components of integrated disease management programs (Deberdt *et al.*, 2012). Several investigators reported the potential of aqueous extracts of flowers of roselle (*Hibiscus subdariffa*), peel of pomegranate (*Punica granatum*) and the leaves of Kafor (*Eucalyptus sp.*) to protect potato plants against bacterial wilt caused by *R. solanacearum* under greenhouse and field conditions (Hassan *et al*., 2008). Although certain plant extracts have demonstrated significant control of plant pathogens in laboratory, greenhouse and field studies. For many years, the role of oxidative enzymes and their metabolic products in the defense mechanisms of infected plants have been studied, results in the field have been inconsistent. Recent progress in our understanding of their diversity, colonizing ability and mechanism of action (Waheed and Tehmina, 2011). Peroxidase, poly-phenoloxidase and phenylalanine ammonia-lyase activity in diseased plants and its effects on resistance or susceptibility in many host-pathogen interactions has been studied (Hassan *et al*., 2009). Thus, the objectives of the present study are to evaluate the antimicrobial activity of plant extracts against bacterial wilt on potato caused *R. solanacearum* in laboratory, greenhouse and field as well as consider the involvement of some defence-related enzymes (PO, PPO and PAL).

MATERIALS AND METHODS

1- Isolation of the causal pathogen of potato brown rot and wilt disease:

Samples of diseased potato plants showing brown rot and wilt symptoms were collected during the 2014/2015 growing season from different localities of Gharbia, EL-Minia, Assiut, and Sohag governorates. Diseased plants were washed 2-3 times with tap water, followed by sterile water. Then the plant parts were cut off into small pieces and sterilized following the sequence of 3 min with 2% sodium hypochlorite solution, 30 s with 70% ethyl alcohol, and washing four times with sterile distilled water (SDW). Disinfected plant samples were homogenized in a sterilized mortar and pestle with 5 ml of sterile 0.05 M potassium phosphate buffer under aseptic conditions. Then a loop of the resulted suspension was streaked onto 2,3,5-triphenyl tetrazolium chloride (TZC) agar medium described by Kelman (1954) and nutrient sucrose agar (NSA) medium suggested by Dowson (1957) in 9.0 cm Petri plates. Plates were then incubated at 27 °C for 48 h and examined for bacterial growth. The single colony technique was used to obtain pure cultures of the isolated bacteria by growing on the same media used. Then the pure cultures of all bacterial isolates were maintained on TTC agar slants and in SDW at 4 °C for further studies.

2- Pathogenicity tests:

Pathogenicity tests of the isolated bacteria were determined on potato (Berema cultivar) plants in the open greenhouse during the 2014/2015 growing season at the Department of Plant Pathology, Faculty of Agriculture, Assiut University, Assiut, Egypt. The pure culture of each bacterial isolate was grown in 250 ml flasks, each containing 100 ml of NSA broth and incubated at 27±2 ^{o}C for 48 h on a rotary shaker at 150 rpm. After incubation, the grown bacterial cells were centrifuged at 10.000 xg for 8 min. The cells were pooled and resuspended in tap water, and the cell density was then adjusted to 1×10^{8} CFU/ml using a spectrophotometer at a wavelength of 620 nm (Kelman, 1954). Healthy tubers were surface sterilized by soaking in 1% sodium hypochlorite for 5 min, washed thoroughly with SDW and planted directly in sterilized plastic pots (25 cm in diameter). Pots and soil were sterilized by 5% formalin and left for 15 days before planting. Pots were filled with 8 kg sterilized sandy-clay soil (3:1, w/w). The pots were kept in the open greenhouse during the growing season. Plants were fertilized with NPK as usual and

irrigated with water when necessary. Later, the potato plants at 7-weeks-old were used for all experiments conducted, according to Kelman and Winstead (1952). Stems of potato plants were inoculated with 100 µl bacterial suspensions using a syringe 10 cm above the soil (Kelman, 1954). Control plants were injected with 100 µl SDW. Four replicates (pots) were used for each tested isolate in a completely randomized experimental design. The disease severity (DS) was monitored six weeks after inoculation and recorded using five scales described by Kemp and Siqueira (1983) with some modifications as follows: 0 = no symptoms; 1 = 1-25% of leaves wilted; 2 = 25.1-50% of leaves wilted; 3 = 50.1-75% of leaves wilted; 4 = more than 75.1% and less than 80% of leaves wilted; 5 = more than 80.1% of leaves wilted and died. The following formula was used to calculate disease severity percent:

$$\textbf{DS \%= [}\Sigma\textbf{d/d (max} \times \textbf{n)]} \times \textbf{100}$$

Where, d= the disease rating on each plant; d max= the maximum disease rating possible; n= the total number of plants examined in each replicate. Also, the virulence of the ten isolates tested was scored as follows; less than 2 were considered low, between 3 and 4 were moderate (M), and more than 4 were considered high (H). Later, the pathogen (*R. solanacearum*) was recovered from wilted plants inoculated with each of the isolates on the TZC medium, as described before.

3- Identification of the causal pathogen of potato brown rot and wilt disease:

The isolated bacteria which were pathogenic to potato plants, and caused brown rot and wilt disease, were identified according to their morphological, cultural and physiological characteristics recommended by Schaad (1988), Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984), and (Holt *et al*., 1994).

4- Preparation of plant extracts:

To prepare the aqueous extracts from the flowers of roselle (*Hibiscus subdariffa*), peel of pomegranate (*Punica granatum*) and the leaves of (*Eucalyptus sp*.), each plant material (200 g) was ground with pestle and mortar in 200 ml sterile water, and the mixture was filtered through double-layered cheesecloth followed by centrifugation at 5.000 xg and room temperature for 10 min to obtain the stuck plant extract of 100% Conc. Then four concentrations of each plant extract were prepared by diluting the stock of each plant extract with sterile water in sterilized flasks plugged with sterile cotton to obtain final concentrations of 25, 50, 75, and 100%. Finally, flasks containing plant extracts were then kept in the dark at 5 °C until use (Kurucheve *et al.*, 1997).

4-1- Antibacterial activity of certain plant extracts against *R. solanacearum*:

The prepared concentrations of plant extracts were tested *in vitro* for their antibacterial activities against *R. solanacearum* using streptomycin and sterile water as positive control and negative control, respectively, according to the method of impregnated filter paper disc described by Sholberg *et al.* (2001). One ml cell suspension containing 1×10^8 cell/ml prepared from the 48-h-old culture of *R. solanacearum* (isolate GK1) was transferred to sterile Petri dishes (9 cm diameter) and mixed with the precooled NA medium. After the medium solidification, sterilized filter paper disks (9 mm diameter, 1 mm thick) were saturated with different concentrations of each plant extract and then placed in the middle of inoculated plates. Three plates were used for each treatment as replicates. The plates were then incubated at 27 °C for 48 h. After incubation, the inhibition zone around each disk was measured in mm.

4-2- Effect of certain plant extracts on the severity of potato wilt disease :

The aqueous extracts of flowers of roselle (*Hibiscus subdariffa*), peel of pomegranate (*Punica granatum*) and the leaves of Kafor (*Eucalyptus sp*.). that gave the highest inhibition of the growth of *R. solanacearum in vitro* were tested for their effect on the severity of wilt disease of potato in greenhouse and field trails.

4-2-1- Greenhouse trials:

In the open greenhouse, tubers of Berema potato cultivar were sown in pots during the 2015/2016 growing season at Plant Pathology Department, Fac. of Agric., Assiut Univ., Assiut as mentioned before in the pathogenicity tests. Forty-five days after planting, stems of the growing plant were injected with 100 µl bacterial suspension of *R. solanacearum* (isolate GK1), contacting 1×10^8 CFU/ml using a syringe 10 cm above the soil as mentioned before. After the inoculation, the plants were kept in a moist chamber at 25 °C for 2 days before being transferred to the greenhouse. Then 50 ml of each plant extract (at 100% conc.) was added to the pots 42 days after inoculation and the development of bacterial wilt symptoms was then observed. The inoculated plants in pots treated with 50 ml SDW of each served as control. Four replicates (pots) were used for each treatment tested in a completely randomized design of the twice repeated experiment. The disease severity was estimated, as mentioned before.

4-2-2- Field trails:

These experiments were conducted in the two successive seasons 2016/2017, and 2017/2018, at the Experimental Farm, Faculty of Agriculture, Sohag University, Sohag governorate. The sowing date in both experiments was 4 and 5 October.

The treatments were distributed in a complete randomized block design with four plots (replicates). The plot area was 25 m^2 containing nine rows, each row was the 4.5-meter length, and distance between rows was 50 cm. Seed tubers of Berema potato cultivar were sown in the middle of the ridge at 40 cm apart.

The treatments of plant extracts were added singly into the soil around the plants before 48 h of inoculation with *R. solanacearum* (isolate GK1). Also, 50 ml of each plant extract at 100% Conc. were added into the soil around the basis of each plant. Control plants were treated with distilled water. The other agricultural practices were carried out as the recommended program of the Egyptian Ministry of Agriculture for Potato Production. The disease severity was recorded and calculated after six weeks of inoculation.

4-4- Effect of certain plant extracts on the biochemical changes of potato:

The effect of certain plant extracts on the biochemical changes of potato plants inoculated with *R. solanacearum* were studied. Following greenhouse trails, plant samples were taken two days before inoculation, at zero time and 2, 4, and 6 days after inoculation for determining total contents of phenolic and salicylic acid and some important enzymatic activities.

4-4-1- Determination of total phenolic and salicylic acid contents:

The method described by Rapp and Zeigler (1973) was used for the preparation of plant samples. Plant leaves (1.0 g) of potato were ground in liquid nitrogen and homogenized in 10 ml of 80% methanol. The homogenate was centrifuged at 1.000 xg and 4 °C for 30 min. The pellet was discarded after the addition of ascorbic acid (0.1 g/5 ml). Then the homogenate was evaporated in a rotary evaporator at 65 °C, and the process was repeated three times each for 5 min. The residues were then dissolved in 5 ml of 80% methanol. Four samples as replicates were used for each treatment.

4-4-1- Total phenolic content:

The phenolic content was determined according to the method described by Sahin *et al.* (2004). The reaction mixture was composed of 0.02 ml methanol extract, 0.5 ml Folin reagent, 0.75 ml of 20% Na_2CO_3 solution, and 8 ml water. The mixture was incubated at 37 °C in a water bath for 60 min. Methanol was used as a negative control. Total phenolic content was assayed spectrophotometrically at 767 nm as mg/g plant fresh weight using gallic acid as standard.

Total phenolic content = mg gallic acid/g plant material

4-4-2- Salicylic acid content:

Salicylic acid content was estimated by the method described by Dat *et al.* (1998) with some modifications. A 500 µl of

homogenate sample was mixed with 250 µl of 10 N HCl and 1.000 µl methanol. The sample was incubated in a water bath at 80 °C for 2 h. The sample was then neutralized with 4-5 drops of 1M $NaHCO_3$, and 1.000 µl methanol was added to the mixture. The Optical Density (OD) was measured at 254 nm, and the salicylic acid content was calculated as µg salicylic acid per g plant fresh weight.

4-4-3- Determination of total protein:

The total protein content of potato plants was determined using Bradford reagent according to the method described by Bradford (1976). 100 mg of Coomassie Brilliant Blue G-250 was gently dissolved in 50 ml ethanol (95 %), then 100 ml of 85% H_3PO_4 were added, and the mixture was completed to 1.000 ml by distilled water. The reagent was filtered and preserved at 4 °C until use. For the assay, sample of 100 µl was shaken gently with 900 µl Bradford reagent and incubated for 15 min at room temperature. The protein content was assayed spectrophotometrically at 595 nm using Bovine serum albumin (BSA) as standard. Total protein content in each sample was calculated as mg Gallic acid g^{-1} fresh weight from the standard curve of BSA.

4-4-4- Enzymes activity:

For activity determination of peroxidase (PO), Polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), Catalase (CAT) and Lipoxygenase (LO), 1 g fresh weight of potato plant leaves were treated with liquid nitrogen and homogenized with 10 ml of 0.1 M Na-acetate buffer (pH= 5.2). The mixture was centrifuged at 1.000 xg for 30 min at 4 °C, and the activities of the enzymes were determined in the supernatants. Four replicates were used for each treatment.

4-4-4-1- Peroxidase activity:

Peroxidase activity was determined using the method described by Putter (1974). PO activity was determined spectro-photometrically using Guaiacol as a substrate. The reaction mixture composed of 0.2 ml supernatant, 1 ml of 0.1 M Na-acetate-buffer (pH= 5.2), 0.2 ml of 1% guaiacol and 0.2 ml of 1% H_2O_2. The mixture was incubated at 25 °C for 5 min and then measured at 436 nm. The extraction buffer was used as a blank. Enzyme activity was calculated according to the change in absorbance and expressed as enzyme per 1 mg protein.

4-4-4-2- Polyphenol oxidase activity:

Polyphenol oxidase activity was determined using the method described by Batra and Kuhn (1975). The reaction mixture was 0.5 ml of the supernatant, 2 ml of 50 mM Sorensen phosphate buffer (pH= 6.5), and 0.5 ml of the substrate Bren catechol (Sigma Aldrich). Sorensen phosphate buffer (g/L) consisted of 6.8 g KH_2PO_4, 8.99 g Na_2HPO_4 $2H_2O$, 0.372 g EDTA, and distilled water up to 1000 ml. The reaction mixture was incubated in a water bath at 37 °C for 2 h and measured at 410 nm.

Activity of PPO = OD at 410 nm/mg protein

4-4-4-3- Phenylalanine ammonia-lyase activity:

Phenylalanine ammonia-lyase activity was determined using the method described by Silva *et al.* (2004). The reaction mixture was 0.5 ml of the supernatant, 2 ml of 50 mM Na-borate/HCl buffer (pH= 8.8), Mercapto ethanol, and 1 ml of 60 mM phenylalanine. Sodium borate buffer consisted of 25 ml of 1N NaOH, 3.09 g H_3BO_3, and 349 µl Mercaptoethanol dissolved in 1.000 ml distilled water. The mixture was incubated at 37 °C for 2 h. The PAL activity was determined spectrophotometrically at 290 nm using spectrophotometer unican -UV. Cinnamic acid was used as a standard.

PAL activity = mM cinnamic acid/mg protein

Statistical analysis:

The complete randomized experimental design with four replicates per treatment was used for all twice performed experiments. All data obtained were analyzed using the statistical analysis system (SAS Institute Inc., 1996). To assess the statistical significance of treatment differences, a one-way analysis of variance (ANOVA) followed by Tukey′s multiple comparison test with L.S.D. test at $P \leq 0.05$ level were employed

(Gomez and Gomez, 1984). Means of standard deviation for four plants per treatment were also shown.

RESULTS

1- Isolation of the causal pathogen of potato wilt disease:

Ten bacterial isolates were obtained from diseased potato plants showing typical symptoms of bacterial wilt collected from different counties of Gharbia, El-Minia Assiut, and Sohag governorate. The bacteria obtained were 4 isolates from Kafar Alziyat, Tanta, Basyoun and Zefta of Gharbia governorate, 2 isolates from El-Minia, and Samalut of El-Minia governorate, 2 isolates from Dairout and Manfalout of Assiut governorate and 2 isolates from Akhmem and El-Osayrat of Sohag governorate.

2- Pathogenicity tests:

Results presented in Table 1 showed that all tested bacterial isolates were pathogenic to potato plants and produced typical symptoms of the wilting disease on infected plants. However, the bacterial isolates significantly varied in their virulence to potato plants. The isolates No.1 (GK1) and No.2 (GT2) were the highest ones with no different , where they exhibited 100 and 96.7% of wilt disease severity, respectively. While the isolates No.9 (SA9) and No.10 (SE10) were the lowest ones and caused 46.4 and 50% of wilt disease severity, respectively. The other bacterial isolates ranged from 56.6-83.3% of wilt disease severity. Based on the data obtained, the ten bacterial isolates could be characterized as high virulent [No.1 (GK1), No.2 (GT2) and No.8 (AM8)], moderate virulent [No.6 (ES6), No. 3 (GB3), No.5 (EM5), and No.7 (AD7),], and low virulent [No.9 (SA9), No.10 (SE10) and No.4 (GZ4)].

Table(1): Source and pathogenicity tests of 10 isolates of *R. solanacearum* obtained from different regions of Gharbia, El-Minia Assiut, and Sohag governorate performed on potato Berema cv. in the open greenhouse during the 2015/2016 growing season.

Isolate		Source		Wilt disease severity (%)
Number	Code	Governorate	County	
1	GK1	Gharbia	Kafar Alziyat	100.0
2	GT2		Tanta	96.7
3	GB3		Basyoun	63.4
4	GZ4		Zefta	56.6
5	EM5	El-Minia	El-Minia	74.0
6	ES6		Samalut	63.3
7	AD7	Assiut	Dairout	76.0
8	AM8		Manfalout	83.3
9	SA9	Sohag	Akhmem	46.4
10	SE10		El-Osayrat	50.0
Control, plants inoculated with sterile distilled water				**0.00**
L.S.D. 0.05:				9.36

3- Identification of the causal pathogen of potato wilt disease:

The identification of the pathogenic isolate bacteria in the current study was carried out using morphological and physiological characteristics. The tests of cell shape, motility, gram staining reaction, hydrolysis of casein, gelatin liquefaction, hydrolysis of aesculin, urease test, starch hydrolysis, levan production, catalase test, hydrogen sulphide production, tolerance of tetrazolium chloride, phenylalanine deaminase, growth in litmus milk, production of reduced substances from sucrose, potato soft rot, growth in 4 °C and 40 °C, growth in NaCl, production of oxidase, acetyl-methyl carbinol production (V.P. test) and reduction of methyl red (M.R. test).

3-1- Morphological characteristics:

The results revealed that all bacterial isolates were rod-shaped, motile, gram-negative, and non-spore forming cells. On the CPG medium, all 48-h-old cultures of the bacterial isolates were smooth, opaque, and highly fluid. On the TZC agar medium, the bacterial colonies were creamy white with small pink or light red centers.

3-2- Physiological characteristics:

Results show the physiological characteristics of the ten bacterial isolates tested. The results showed that all isolates were positive in the oxidase, urease, catalase, and M.R. test. However, they had no abilities to hydrolyze starch and levan, produce hydrogen sulphide, and hydrolyze aesculin and casein and liquefy gelatin. They could grow at 1% NaCl and could not grow at 4 °C, 40 °C and 2% NaCl. They were negative V.P., could tolerate 0.1% and 0.02% tetrazolium salt and could not produce phenylalanine deaminase. Results of the sugars fermentation showed that all bacterial isolates produced acid from sucrose, glucose, maltose, fructose, lactose, and mannose, did not produce acid from sorbitol and mannitol. Based on the morphological and physiological characteristics of the isolated pathogenic bacteria are isolated. Therefore, all bacterial isolates causing wilt disease of potato were identified as *R. solanacearum* race 3 (biovar II).

4 - Plant extracts:

4-1- Antibacterial activity of certain plant extracts against *R. solanacearum*:

Results presented in Table 2 showed that all plant extracts tested significantly inhibited the growth of *R. solanacearum* at the high concentrations of 75 and 100%. The highest inhibition zone was recorded by 100% extract of *H. subdariffa*, (3.5 cm). While the lowest inhibition zone was recorded by 75% extract of *Eucalyptus* sp., (0.5 cm). Moreover, 100% extracts of *Eucalyptus* sp. and *P. granatum* exhibited inhibition zones of 1.1 and 1.5 cm, respectively.

Table 2: Antibacterial activity of the aqueous plant extracts of *H. subdariffa, Eucalyptus* sp., and *P. granatum* at different concentrations on the growth of *R. solanacearum in vitro*.

Aqueous plant extract	Concentration (%)	Inhibition zone (cm)
H. subdariffa	0.0	0.0
	25	0.0
	50	0.0
	75	1.1
	100	3.5
Mean		**0.92**
Eucalyptus sp.	0.0	0.0
	25	0.0
	50	0.0
	75	0.5
	100	1.1
Mean		**0.22**
P. granatum	0.0	0.0
	25	0.0
	50	0.0
	75	0.7
	100	1.5
Mean		**0.30**

L.S.D. at 5% for plant extrcats (PE) = 0.02 Concentrations (C) = 0.05 PE × C = 0.08

4-2- Effect of certain plant extracts on the severity of wilt disease of potato:

4-2-1- In greenhouse trials:

Results presented in Table 3 showed that all tested bacterial bioagents and plant extracts significantly reduced disease severity of potato bacterial wilt compared to infected control, except in the case of bacteria *L. macroides* did not provide such effect. The highest reduction of disease severity was recorded by extract of *H. subdariffa* (85.82%). While the lowest reduction of disease severity was recorded by the treatment of *H. subdariffa*, (6.16%). Moreover, the remaining treatments exhibited a reduction of disease severity ranging from 35.36 to 67.02%.

Table 3: Effect of some plant extracts on the severity of wilt disease of potato caused by *R. solanacearum* in the open greenhouse during the 2015/2016 growing season.

Treatments	Disease severity (%)	Disease severity reduction (%)
H. subdariffa	13.22	85.82
P.granatum	35.8	61.60
Eucalyptus sp.	57.35	38.49
Infected control	93.25	0.0
Healthy control	0.0	100

L.S.D. 0.05: **8.05**

4-2-2- In field trials:

Under field conditions, results in Table 4 revealed that treatments with the plant extracts significantly reduced the disease severity of wilt disease of potato compared to infected control. Treatment of soil drenching with 100 ml of plant extracts *H. subdariffa,* and *P. granatum* reduced the disease severity by 36.7 and 26.7%, respectively.

Table 4: Effect of plant extracts on the severity of wilt disease of potato caused by *R. solanacearum* under field conditions during the 2016/2017 and 2017/2018 growing seasons.

Treatments	Season 2016/2017		Season 2017/2018		Mean	
	Disease severity (%)	Disease severity reduction (%)	Disease severity (%)	Disease severity reduction (%)	Disease severity (%)	Disease severity reduction (%)
H. subdariffa	36.7	38.83	48.0	22.95	42.33	30.6
P. granatum	26.7	55.50	46.0	26.16	29.67	51.3
Eucalyptus sp.	40.0	33.33	32.7	47.51	43.00	29.5
Infected control	60.0	0.0	62.3	0.0	61.00	0.0
Healthy control	0.0	100	0.0	100	00.00	100
L.S.D.0.05%:	21.87		7.29		11.01	

5- Effect of plant extracts on some host biochemical changes of potato plants inoculated with *R. solanacearum*:

5-1- Salicylic acid:

The results in Table 5 showed a significant increase in the salicylic acid content of potato plants in all tested treatments. Also, no significant differences were to and between 2 and 4 days after the application, but significant differences between 0, 6, and 8 days compared to two days after the application. Data also showed that the treatment with *Eucalyptus* sp. extract caused an increase of SA reached to 3.940 µg/g plant material after two days of application.

Table 5: Effect of the plant extracts on salicylic acid contents in potato plants inoculated with *R. solanacearum*.

Treatments	Salicylic acid contents (µg/g plant material)					
	Days after application					Mean
	0	2	4	6	8	
H. subdariffa	2.600	2.640	3.899	3.205	3.133	3.096
P. granatum		3.699	3.242	2.960	3.741	3.248
Eucalyptus sp.		3.940	3.566	3.001	3.566	3.335
Infected control		2.799	2.566	2.393	2.385	2.549
Healthy control		2.799	2.240	2.130	1.941	2.342
Mean		3.175	3.103	2.738	2.953	-

L.S.D. at 5% Treatments (T)= 0.21 Days after application (D) = 0.17 T × D= 0.48

5-2- Total phenolic contents:

Results in Table 6 showed that the total phenolic content of potato plants treated with the plant extracts was significantly higher than that of infected and healthy control. The lowest total phenolic content was 4.60 mg gallic acid/g fresh weight of plants treated with *H.Subdarida*.. The highest phenolic contents 5.27, and 5.30 mg gallic acid/g fresh weight was recorded after four days of treatment with *P. granatum* and the extract of *Eucalyptus* sp., respectively.

Table 6: Effect of the plant extracts on total phenolic contents in potato plants inoculated with *R. solanacearum.*

Treatments	Total phenol contents (mg gallic acid/g fresh weight)					
	Days after application					Mean
	0	2	4	6	8	
H. subdariffa	3.23	4.65	5.00	5.03	5.10	4.60
P. granatum		4.63	5.27	4.76	5.18	4.61
Eucalyptus sp.		4.39	5.30	5.13	5.11	4.63
Infected control		3.67	4.05	4.27	3.77	3.8
Healthy control		3.67	3.5	3.24	3.68	3.46
Mean		4.21	4.62	4.49	4.56	-

L.S.D. at 5% Treatments (T) = 0.56 Days after application (D) = 0.44 T × D =1.24

5-3- Effect of some plant extracts on the enzymatic activities of potato plants inoculated with *R. solanacearum*:

5-3-1- Peroxidase activity (PO):

Data presented in Table 7 showed that all tested plant extracts showed higher PO activity in potato plants compared to infected and healthy control except in the case of *H. subdariffa* extract. Also, data showed that potato plants treated with *P. granatum* exhibited the highest increase in PO activity (4.51 unit/mg protein) followed by those treated with *Eucalyptus* sp. (3.45 unit/mg protein). No significant differences in enzyme activity were found between 0, 2, and 4 days from the application but significant differences between 6 and 8 days from application. In general, the activity of the enzymes was higher 6 days after application, followed by that determined after 4 and 8 days.

Table 7: Effect of the plant extracts on peroxidase activity in potato plants inoculated with *R. solanacearum.*

Treatments	Peroxidase activity (unit/ mg protein)					
	Days after application					Mean
	0	2	4	6	8	
H. subdariffa	1.37	0.8	1.92	2.69	2.22	1.8
P. granatum		2.46	2.31	14.51	1.92	4.51
Eucalyptus sp.		3.03	2.99	5.02	4.86	3.45
Infected control		1.14	2.42	2.50	2.80	2.04
Healthy control		1.14	1.78	2.23	2.14	1.73
Mean		1.71	2.28	5.39	2.78	-

L.S.D. at 5% Treatments (T) = 0.75 Days after application (D) = 0.95 T × D = 1.67

5-3-2- Polyphenol oxidase activity (PPO):

Results in Table 8 revealed that potato plants treated with the plant extracts exhibited the highest levels of PPO activity, mainly

after 2, 4, 6, and 8 days of the application as compared to the infected and healthy control. Also, results showed that the water extract of *Eucalyptus* sp. exhibited the highest increase in activity of PPO (0.58 unit/mg protein), followed by the extract of *H. subdariffa* (0.51 unit/mg protein). In general, all treatments of the plant extracts highly increased the activity of PPO enzyme.

Table 8: Effect of the plant extracts on polyphenol oxidase activity in potato plants inoculated with *R. solanacearum.*

Treatments	Polyphenol oxidase activity (unit/ mg protein)					
	Days of application					Mean
	0	2	4	6	8	
H. subdariffa	0.11	0.14	0.34	0.34	1.61	0.51
P. granatum		0.32	0.30	0.51	1.00	0.45
Eucalyptus sp.		0.44	0.44	1.66	0.25	0.58
Infected control		0.09	0.32	0.62	0.71	0.37
Healthy control		0.09	0.20	0.26	0.24	0.18
Mean		0.22	0.32	0.68	0.76	

L.S.D. at 5% Treatments (T) = 0.12 Days after application (D) = 0.09 T × D = 0.27

5-3-3- Phenylalanine ammonia-lyase activity (PAL):

Results in Table 9 show that potato plants treated with the water extracts of *Eucalyptus* sp. and *P. granatum* exhibited the highest increase in PAL activity (16.0 and 151.4 mg cinnamic acid/mg protein, respectively). Results also showed that the plant extracts caused a significant increase in the activity of PAL enzyme except for the treatments of *H. subdariffa.* Moreover, extracts of *P. granatum* and *Eucalyptus* sp. caused the highest enzyme activity (539.8 and 343.0 mg cinnamic acid/mg protein, respectively) after 6 days of the application. Results obtained also showed that the lowest enzyme activity was recorded at the same time of application (0 days), where it was 34.7 mg of cinnamic acid/mg protein. In general, PAL activity was the highest (260.2 mg cinnamic acid/mg protein) after 6 days of application, followed by those of 8, 4, and 2 days after application.

Table 9: Effect of some plant extracts on phenylalanine ammonia-lyase activity in potato plants inoculated with *R. solanacearum.*

Treatments	Phenylalanine ammonia-lyase activity (mg cinnamic acid/mg protein)					
	Days after application					Mean
	0	2	4	6	8	
H. subdariffa	27.0	34.7	102.9	224.9	80.2	93.9
P. granatum		71.0	63.9	539.8	55.3	151.4
Eucalyptus sp.		57.8	71.57	343.0	305.4	161.0
Infected control		37.0	82.4	121.3	143.4	82.2
Healthy control		37.0	70.0	71.8	74.7	56.1
Mean		47.5	78.2	260.2	131.8	-

L.S.D. at 5% Treatments (T) = 16.50 Days after application (D) = 13.04 T × D = 36.90

DISCUSSION

Bacterial wilt and brown rot of potato crop caused by *Ralstonia solanacearum* is a

destroying disease worldwide, where it spreads in tropical, subtropical, and temperate regions. In many undeveloped countries as well as Egypt, it is a limiting factor for the production of potato tubers (Abd El-Kareem *et al.*, 2001). Also, the pathogen has an extensive host range of 200 plant species in over 50 families (Hayward, 1991), and the family Solanaceae is one of the most economically essential host plants. However, the specific host range and distribution of *R. solanacearum* depend on the race and, to some degree, the biovars (Hayward, 1991 and McGarvey *et al.*, 1999). In this study, ten bacterial isolates were obtained from wilted potato plants in Gharbia, El-Minia, Assiut, and Sohag governorates, Egypt. Later, they were identified as *R. solanacearum* race 3 (biovar II) according to their morphological, cultural and physiological characteristics described by Schaad (1988), Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984 and Holt *et al.*, 1994). Also, these isolates were similar to those of race 3 (biovar II) of *R. solanacearum* described by Saad, Maryan (2011), and Mikhail *et al.* (2012). The biovars of *R. solanacearum*, which are mainly based on acidification of medium during the metabolism of sex sugars, are well defined than their races (Singh *et al.*, 2016). In Egypt, the biovar II of race 3 is commonly found (Mikhail *et al.*, 2012), which infect potato plants and cause wilt symptoms in different regions (Ashmawy, 2015).Pathogenicity tests proved that all ten isolates of *R. solanacearum* were pathogenic, induced wilt symptoms typically, and re-isolated from infected potato plants. However, these isolates significantly varied in their virulence and characterized as high virulent (GK1, GT2, and AM8), moderately virulent (ES6, GB3, EM5 and AD7), and low virulent (SA9, SE10, and GZ4). Such results are in agreement with those reported by Mikhail *et al.* (2012), Ashmawy (2015), and Karim and Hossain (2018). In this study, the low virulent isolate produced symptoms comparable to those produced by the moderate or high virulent isolate when getting access to the host potato plant. However, no differences between these isolates could be observed when they were cultured on the TZC agar medium described by (Kelman, 1954), where the bacterial colonies were creamy white with small pink or light red centers. Also, no differences in colonies' morphology were proved when grown on the CPG agar medium supplemented with TZC (Kelman, 1954), where the bacterial colonies were smooth, opaque, and highly fluid.Various control measures of bacterial wilt have been documented. However, it is still complicated to control because of the wide host range and long survival period of the pathogen in the soil, especially in deeper layers (Hsu, 1991). As per literature, to date, no single control method is 100% effective. In this regard, control of bacterial wilt has been possible through the use of a combination of diverse approaches, and these methods include Phyto-sanitation and cultural practices (EPPO, 2004; Champoiseau *et al.*, 2010). Chemical control is also intricate to manage the disease and recently becomes much less of an option, where it has adverse effects on the environment and the non-target organisms (Lemessa and Zeller, 2007). Therefore, integrated and sustainable disease management options are needed to control this highly destructive challenged disease (Aguk *et al.*, 2018). Recently, it has become necessary and highly relevant to explore the plant growth-promoting rhizobacteria that exhibit antagonistic activities to the pathogen and incorporate them into successful disease management as biocontrol agents. Also, a key feature of such organisms is their ability to adjust to the rhizosphere and to aggressively colonize the host plant roots (Maji and Chakrabartty, 2014). Natural plant products are essential sources of new agrochemicals for the control of some plant diseases. In this regard, plant-derived preparations based on plant extracts are environmentally safe alternatives and possible components of integrated disease management (Deberdt *et al.*, 2012). *In vitro*, the inhibitory effect of plant extracts on the growth of *R. solanacearum* were investigated. Data obtained revealed that the tested concentrations 25, 50, and 75% of water extracts prepared from *H. subdariffa*, *Eucalyptus* sp., and *P. granatum* did not inhibit the bacterial growth at low

concentrations except concentration 75% of *H. subdariffa*. However, all plant extracts were able to inhibit bacterial growth at a concentration of 100%. Results reported herein indicate that significant inhibitory effect of plant extracts on the bacterial pathogen growth, in *vitro*, similarly to those obtained by Abo-Elyousr and Asran (2009), Hassan *et al.* (2009), Wagura *et al.* (2011) and Abo-Elyousr and Khalil-Bagy (2018). In greenhouse trials, the most plant extracts selected to control the bacterial wilt provided a significant reduction in disease severity. In this respect, plant extract treatments gave the highest reduction of disease severity. Such results are in agreement with those reported by several researchers who have controlled the bacterial wilt disease by plant extracts (El-Ariqi *et al*., 2005; Hassan *et al.*, 2008 and Abo-Elyousr and Asran, 2009).In the field trials, all tested treatments gave a significant reduction in disease severity. Results reported herein showed that the application with plant extracts significantly increased tuber yield of potato plants as compared to the control of infected plants during the two seasons of growth. These results are in agreement with those obtained by El-Ariqi *et al*. (2005) and Xianling *et al.* (2008). This effect could be explained by the reduction of the disease incidence that leading to an increase in vegetative characteristics. Several biochemical studies such as the production of salicylic acid (SA) and phenolic compounds, and some the enzymatic activities of peroxidase polyphenol oxidase, phenylalanine ammonia layse, catalase and lipoxygenase in infected leaves of potato plants were investigated. The biological role of SA inhibition of catalase is unknown. However, several reports have shown that at high concentrations of SA (1 mM), it can bind to and inhibit several heme-containing enzymes such as catalase, ascorbate peroxidase, or aconitase under its affinity for iron. At the site of infection, once produced, the level of SA is more likely to be in the correct range for inhibition of catalase or other related enzymes. SA may have different actions at the local and at the systemic levels. Other known actions of SA include a direct antimicrobial effect was reported (Sticher *et al*., 1997).In the current study, SA contents were significantly increased more rapidly by the application of aqueous extract of *Eucalyptus* sp. after two days to six-day of treatment. Results obtained are in agreement with the reported studies by Malamy *et al.* (1990), Becker *et al.* (1998), De Meyer *et al.* (1999), and Hassan and Abo-Elyousr (2013). They suggested that SA accumulation is essential for the expression of multiple modes of plant disease resistance. Also, SA is a mediates plant defense against pathogens, accumulating in both infected and distal leaves in response to pathogen attack. The enhanced SA contents are, thus, a prerequisite for expression systemic acquired resistance against *R. solanacearum* in potato plants (Zimmerli *et al*., 2000 and 2001). Total phenolic contents were higher in treated potato plants than the infected and healthy control plants and reached the maximum value at 8 days after application. It is known that the accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development since such compounds are toxic substances to pathogen. Also, the resistance may be increased by a change in the pH of plant cell cytoplasm due to the increase in phenolic acid content resulting in inhibition of pathogen development (Abo-Elyousr *et al*., 2005; Bereika, 2008; Hassan and Abo-Elyousr, 2013). In the present study, tuber treatment and soil application with plant extracts resulted in increasing the accumulation of phenolic substances in response to infection by the pathogen. Peroxidase (PO) activity significantly increased in the infected plants treated with all treatments of plant extracts except with the extract of *H. subdariffa.* However, *P. granatum* extract was superior in increasing PO activity than the other tested treatments followed by *Eucalyptus* sp. extract (Miyazawa *et al*., 1998; Kagale *et al*., 2004; Hassan *et al.*, 2009 and Seleim *et al.*, 2014). Several investigators have reported that the enhancement of PO activity is associated with plants to fungal, bacterial, and viral pathogens (Baysal *et al.*, 2003). Polyphenol oxidase (PPO) activity did not significantly increase in infected potato plants except extracts of *H. subdariffa*, and *Eucalyptus* sp. However, the

highest value of PPO activity was obtained by *Eucalyptus* sp. extract 6 days after treatment. Such results are in agreement with those reported by Bereika (2008) and Hassan *et al.* (2009). The importance of PPO activity in plant disease resistance probably seems from its property to oxidize phenolic compounds to quinines, which are often more toxic to microorganisms than the original phenols (Mayer, 2006). Phenylalanine ammonia-lyase (PAL) activity significantly increased in the infected plants treated with all treatments of plant extracts except *H. subdariffa*. The extract of *P. granatum* caused the highest value of PAL activity after 6 days. Such results are in agreement with those reported by Girish and Umesha (2005) and Hassan *et al*. (2009). It is known that PAL plays a vital role in the biosynthesis of phenolics and phytoalexins, and the increase in PAL activity indicates the activation of the phenyl propanoic pathway. In several host-pathogen interactions, increased levels of PAL are correlated with incompatibility (Silva *et al*., 2004 and Umesha *et al*., 2005). In the present investigation, the catalase (CAT) activity was significantly decreased to lower levels in healthy and infected plants with all treatments. The CAT was lowest in the treated plants than the healthy and infected control. The highest value was recorded after 2 days until 8 days from treatments except for 4 days. Such results also are similar to those obtained by Baysal *et al*. (2006), Nunez *et al*. (2006), Bereika (2008), and Hassan and Abo-Elyousr (2013).

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