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Direct Antimicrobial Activity and Induction of Systemic Resistance in Potato Plants Against Bacterial Wilt Disease by Plant Extracts

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The potential of three plants extracts, to protect potato plants against bacterial wilt caused by *Ralstonia solanacearum* was determined under greenhouse and field conditions. All soil drenching treatments of aqueous plant extracts of *Hibsicus sabdariffa*, *Punica granatum* and *Eucalyptus globulus* significantly reduced the disease severity compared with inoculated control. Although the applications of all three plant extracts resulted in similar reductions of disease severity in field up 63.23 to 68.39%, treatment of *E. globulus* leaf extract was found greater in restricting the symptom development than other the two plant extracts in the greenhouse. More than 94% reduction in the bacterial wilt symptom was observed in potato plants. All tested plant extracts were effective in inhibiting the growth of bacterial pathogen, not only *in vitro*, but also in stem of potato plants as compared with the inoculated control. Potato plants treated with extract of *H. sabdariffa* reduced bacterial growth more effectively than treatment with *P. granatum* and *E. globulus*. Activity of defence-related enzymes, including peroxidase, polyphenoloxidase and phenylalanine ammonia lyase, were significantly increased in plants treated with the plant extracts compared to the control during the experimental period. In general, the higher enzymes activities were determined in both inoculated and non-inoculated treated potato plants after 8 days from plant extracts treatment. These results suggested that these plant extracts may be play an important role in controlling the potato bacterial wilt disease, through they have antimicrobial activity and induction of systemic resistance in potato plants.

Keywords : Enzymes activities, *Eucalyptus globulus*, *Hibsicus sabdariffa*, Potato plants, *Punica granatum*

Bacterial wilt caused by the soilborne *R. solanacearum* is probably one of the most devastating bacterial diseases (Hayward, 1991). Bacterial wilt is found worldwide, mainly in tropical and subtropical areas, but also in warm temperate countries and even in some cool temperate regions. More than fifty botanical families were concerned including some economically important species such as potatoes, tomatoes and bananas (Fock et al., 2001; Hayward, 1991). Such disease limits potato production worldwide in Asia, Africa, Europe and Central and South America, where causes severe crop losses (Fock et al., 2001; Williamson et al., 2002). Many trials have been carried out all over the world to control the disease without much success. No promising control of potato bacterial wilt was achieved using antibiotics (Habashy et al., 1993), soil fumigants (Weingartner and Shumaker, 1988), chemical control (Murakoshi and Takahashi, 1984) or breeding of resistant varieties (Fock et al., 2001).

Enhanced resistance to diseases in plants, both locally and systemically, can be achieved by treatment with different agents, such as virulent or avirulent pathogens, bacterial cell wall fragments, synthetic chemicals and plant extracts (Walters et al., 2005). The induced resistance (IR) is persistent and generally non-specific for a pathogen and one category of IR is systemic acquired resistance (SAR) (Cao et al., 2005). The resistance induced by SAR is generally effective against a broad range of pathogens and is mediated via a salicylic acid-dependent process. This type of resistance develops systemically in response to a pathogen and is associated with an increase in the activity of peroxidases (POX) (Van Loon, 1997), phenylalanine ammonia-lyase (PAL) (He et al., 2002) and polyphenol-oxidases (PPO) (Mayer and Staples, 2002).

Natural plant products are important sources of new agrochemicals for the control of plant diseases (Kagale et al., 2004). Furthermore, biocides of plant origin are non-phytotoxic, systemic and easily biodegradable (Qasem et al., 1966). It is now known that various natural plant products can reduce populations of soilborne plant pathogens and control disease development, and then these plant extracts have potential as environmentally safe alternatives and as components in integrated pest management programs (Bowers and Locke, 2004). In recent study by Wang et al. (2004) shown that extracts from leaves of *Inula viscosa* possess broad-spectrum protection activity against foliar diseases of crop plants. These findings may be significant to the agricultural industry when pathogen strains

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resistant to site-specific fungicides, as well as to organic farming where synthetic pesticides are prohibited. Additionally, several plant extracts have shown antimicrobial activity against bacteria and fungi pathogens *in vitro* and *in vivo* (Baysal and Zeller 2004; Kagale et al., 2004; Mosch et al., 1996). Evaluation of botanicals against diseases in several plants has been attempted earlier under both greenhouse and field conditions (Abd-El-Khair and Haggag 200; Wang et al., 2004). However, no attempts have been made to study the effect of plant extracts on enhancement of potato resistance against bacterial wilt disease and to understand the mechanisms of disease resistance induced by the plant extracts.

Thus, the objectives of the present study are to evaluate the antimicrobial activity of plant extracts and induction of systemic resistance against bacterial wilt on potato caused by *R. solanacearum in vitro* and under greenhouse and field conditions. In addition, we considered the involvement of some defence-related enzymes (POX, PAL and PPO) with the SAR.

Materials and Methods

Potato plants. Healthy tubers of potato plants (*Solanum tuberosum* L.) cv. Diamont were surface sterilized by soaking 1% sodium hypochlorite for 5 min., washed thoroughly with sterilized distilled water and one tuber was planted directly in every sterilized pot (diameter 25 cm). Pots and soil were sterilized by 5% formalin and then left for 15 days before planting. They filled with 4 kg of clay and sand mixture (3:1 v/v). The plants were grown in greenhouse under natural temperature and photoperiods during the growing season. Plants were fertilized every 15 days with urea 46% (20 g/pot) and irrigated with water when necessary. Six weeks old potato plants were used in all greenhouse experiments.

Bacterial pathogen and inoculum preparation. The highly virulent isolate M4 of *R. solanacearum* was obtained from the culture collection of Plant Pathology Department, Faculty of Agriculture, Assiut University, Assiut, Egypt. Stored stock cultures of the isolate was streaked on 2,3,5-triphenyltetrazolium chloride agar medium (TTC) in Petri dishes and incubated at 27°C for 48 h (Kelman, 1954). A single colony of the isolate was selected and grown in 250 ml Erlenmeyer flasks containing 100 ml of nutrient sucrose broth (NSB) and incubated at 27±2°C for 48 h on a rotary shaker at 150 rpm. Bacterial suspension culture was centrifuged for 8 min. at 10.000 g, the cells resuspended in tap water and cell density adjusted to be 1×10^8 cfu/ml using a spectrophotometer at wavelength of 620 nm.

Preparation of plant extracts. Aqueous extracts of *H. sabdariffa*, *P. granatum* and *E. globulus*, were obtained from 100 g fresh leaves of each plant species, collected from organic farm system of Assiut University, Egypt. Leaves material were washed separately with distilled water. They were ground with 100 ml of sterile distilled water (1:1 w/v), with pestle in mortar and filtered through double-layered cheese cloth, followed by centrifugation at 5000 rpm for 10 min (Kurucheve et al., 1997). This concentration (100%), as an aqueous extract, was used in the laboratory test and greenhouse and field experiments.

Inhibition assay. To evaluate whether the protective effect of plant extracts on potato plants against *R. solanacearum* was due to direct antibacterial activities of these extracts, their *in vitro* growth inhibition activity against the virulent isolate M4 of *R. solanacearum* was assessed in TTC agar medium using the impregnated filter paper disk method according to Sholberg et al. (2001). Aqueous plant extracts (1:1 w/v) were filtered through a 0.22 μm Millipore membrane. Sterilized water was used as controls. Four replicates were used for each treatment. After incubation, the inhibition zone around each disk was measured and the area of inhibition zone was expressed in cm^2.

Greenhouse experiments. Six week old potato plants were treated by soil drenching with 50 ml/pot of each aqueous plant extract. Inoculated and non-inoculated control plants were treated with an equal volume of distilled water. Two days after treatment, potato plant stem was injected with 100 μl bacterial suspension by syringe 10 cm above the soil (Kelman, 1954). The non-inoculated control plants were injected with 100 μl sterilized distilled water. Six replicates were used for each plant extract. Six weeks after inoculation, observations for development of bacterial wilt symptoms were recorded as disease severity (DS%) for each treatment using the scale of Kempe and Sequeira (1983) as follow: 0 = no symptoms; 1 = slightly to 25%, leaves wilted; 2 = 26-50% leaves wilted; 3 = 51-75% leaves wilted; 4 = more than 75%, but less than 100% of leaves wilted; 5 = all leaves wilted and died.

Disease severity index was calculated by following equation:

$$DS\% = [\Sigma d/m \times n] \times 100$$

Where: d = the disease rating on each plant
m = the maximum disease rating possible
n = the total number of plants examined in each replicate.

Immediately (six weeks after inoculation), the same treated potato plants were used for the determination of bacterial multiplication in plants. One gram of the lower stem inter-

nodes (15 to 20 cm above the soil) of each treatment was washed with tap water, surface sterilized with 3% sodium hypochloride and washed with sterile water. Stem tissues were homogenized in a sterile mortar and pestle with 10 ml of 0.1 M potassium phosphate buffer (pH 7.0). Stem homogenates were serially diluted from 10^{-1} to 10^{-9} with 0.1 M potassium phosphate buffer. The 200 µl of each dilution were transferred on a TTC medium and spread by using a glass rod. Plates were incubated at 27°C for 48 hr and the number of bacterial colonies was counted (Roberto et al., 2002).

Field experiments. The experiment was carried out in the Experimental Farm of Plant Pathology Department, Faculty of Agriculture, Assiut University, Assiut, Egypt. Tested treatments were distributed in a complete randomized block design with six replicates, the experimental plot area was 24.75 m^2 (4.5×5.5 meter) containing four rows, each row was 4.5-meter length and distance between rows was 50 cm. Potato seed tubers were sown on the middle of the ridge at 40 cm apart. After two months from planting, 100 ml of the each plant extracts was added singly around potato plants 2 days before the inoculation. The plants were injected with 100 µl bacterial suspension of *R. solanacearum* by syringe 10 cm above the soil. A control plants were injected with 100 µl sterilized distilled water. Disease severity index was recorded after 6 weeks from inoculation as described before.

Preparation of samples for determining enzyme activities. For determination peroxidase (POX), phenylalanine ammonialyase (PAL) and Polyphenenoloxidase (PPO) activities in potato leaves, plants were treated separately by soil drenching with 50 ml/pot of aqueous leaf plant extracts (1:1 w/v) of *H. sabdariffa*, *P. granatum* and *E. globulus*. For each treatment 12 plants were used as replicates (one plant per pot). Two days after the treatment of each variant one half of the treated potato plants was inoculated with *R. solanacearum* as described before; the other half of treated plants was not inoculated. Inoculated and non-inoculated control plants were treated with an equal volume of water.

Leaves of potato plants were separately sampled for enzyme assay immediately before treatment (0 days after treatment) as well as 2, 4, 6 and 8 days after treatment with each plant extract. One gram of leaf tissue was immersed in liquid nitrogen in a prechilled mortar and pestle, and then homogenized in 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.8) containing 1 M NaCl, 1% polyvinylpyrrolidone, 1 mM EDTA and 10 mM β-mercaptoethanol. After filtration through cheesecloth, the homogenates were centrifuged at 15000 g at 4°C for 30 min. The supernatants (crude enzyme extract) were stored at –20°C or immediately used for determination enzymes activities and total protein. In the case of every enzyme under investigation, each treatment consisted in six replicates (plants) and two spectrophotometric readings using spectrophotometer (spectronic® 20 Genesys™, Schutt Labortechnik) were taken per replicate. The experiment for bioassays was repeated twice in time.

Peroxidase activity. The enzyme activity of POX was determined a direct spectrophotometrically method (Hammerschmidt et al., 1982) using guaiacol as common substrate for peroxidases. 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 10 mM potassium phosphate buffer), was incubated at 25°C for 5 min and the initial rate of increase in absorbance was measured over 1 min at 470 nm using spectrophotometer. POX activity was expressed as units of POX/mg protein (Urbanek et al., 1991).

Phenylalanine ammonia-layse activity. Phenylalanine ammonia-layse (PAL) activity was determined following the direct spectrophotometric method adapted by Cavalcanti et al. (2007). Two hundred microlitres of the crude leaf extract previously dialysed overnight with 100 mM Tris-HCl buffer, pH 8.8, were mixed to obtain a solution containing 200 µl 40 mM phenylalanine, 20 µl 50 mM β-mercaptoethanol and 480 µl 100 mM Tris-HCl buffer, pH 8.8. After incubation at 30°C for 1 h, the reaction was stopped by adding 100 µl 6 N HCl. Absorbance at 290 nm was measured and the amount of trans-cinnamic acid formed was evaluated by comparison with a standard curve (0.1-2 mg trans-cinnamic acid/ml) and expressed as mMol cinammic acid min^{-1} mg $protein^{-1}$.

Polyphenoloxidase activity. The activity of PPO was determined by adding 50 µl of the crude extract to 3 ml of a solution containing 100 mM potassium phosphate buffer, pH 6.5 and 25 mM pyrocatechol. The increase of absorbance at 410 nm, for 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 milligram of soluble protein per minute.

Protein concentration. Total protein content of the samples was quantified according to the method described by Bradford (1976).

Experimental design and statistical analyses. A completely randomized design with six replicates per treatment was used for greenhouse and field experiments. The data

were analyzed using the one-way analysis of variance (ANOVA). In the case of every enzyme under investigation, the experiment was carried out in spilt-plot design with six replicates (plants) and two spectrophotometric readings were taken per replicate. Plant extracts were arranged in main plot and the time of enzyme activity determination in sub-plot. The data were analyzed by the statistical analysis system MSTAT-C (Version 2.1). Means were compared with Least Significant Difference (LSD) test at $P \leq 0.05$ levels.

Results

Effect of plant extracts on severity of bacterial wilt of potato plants. Soil drenching with three aqueous leaf extracts significantly reduced the disease severity (DS) of potato bacterial wilt disease compared to inoculated control under both greenhouse and field conditions (Fig. 1). Although the applications of all the three plant extracts resulted in similar reductions of DS in the field, treatment of *E. globulus* leaf extract was found better in restricting the symptom development disease than other the two plants extracts under in greenhouse condition. More than 94% reduction in the severity of bacterial wilt was observed in potato plants treated with leaf extract of *E. globulus* in the greenhouse experiment. Under field condition, the reduction of DS were 68.39, 64.06 and 63.23% compared to infected control after treatments by leaf extract of *P. granatum*, *E. globulus* and *H. sabdariffa*, respectively.

Effect of plants extracts on the growth of *R. solanacearum in vitro.* All the three plant extracts exhibited antimicrobial activity against *R. solanacearum in vitro*. The leaf extracts of *H. sabdariffa* and *P. granatum* showed higher effect in inhibiting the growth of *R. solanacearum* than extract of *E. globulus*. They displayed the similar highest antagonistic activity against the bacterial pathogen (inhibition zone area is 3.14 cm^2), while the extract of *E. globulus* came next (inhibition zone area is 2.01 cm^2).

Population of the bacterial pathogen *in planta.* Bacterial proliferation of *R. solanacearum* drastically increased in stem sections of inoculated control plants. A high number of *R. solanacearum* cells in lower stem sections of inoculated control plants was determined (2.8×10^{10} cfu/g). Data in Table 1 showed that all tested plant extracts significantly reduced the numbers of *R. solanacearum* cells in stem of

Table 1. Effect of plant extracts on growth of *Ralstonia solanacearum in vitro* and in stem tissues of potato plants under greenhouse conditions (*in vivo*).

Treatments	*in vitro*	*In vivo*
	Inhibition zone area (cm^2)	Bacterial pathogen population (cfu/g stem tissue)
Sterile distilled water	0.0	2.80×10^{10}
Hibsicus sabdariffa	3.14	4.40×10^{5}*
Punica granatum	3.14	5.10×10^{7}*
Eucalyptus globulus	2.01	2.23×10^{6}*

Means indicated with asterisk (*) differ statistically from the infected control variant.

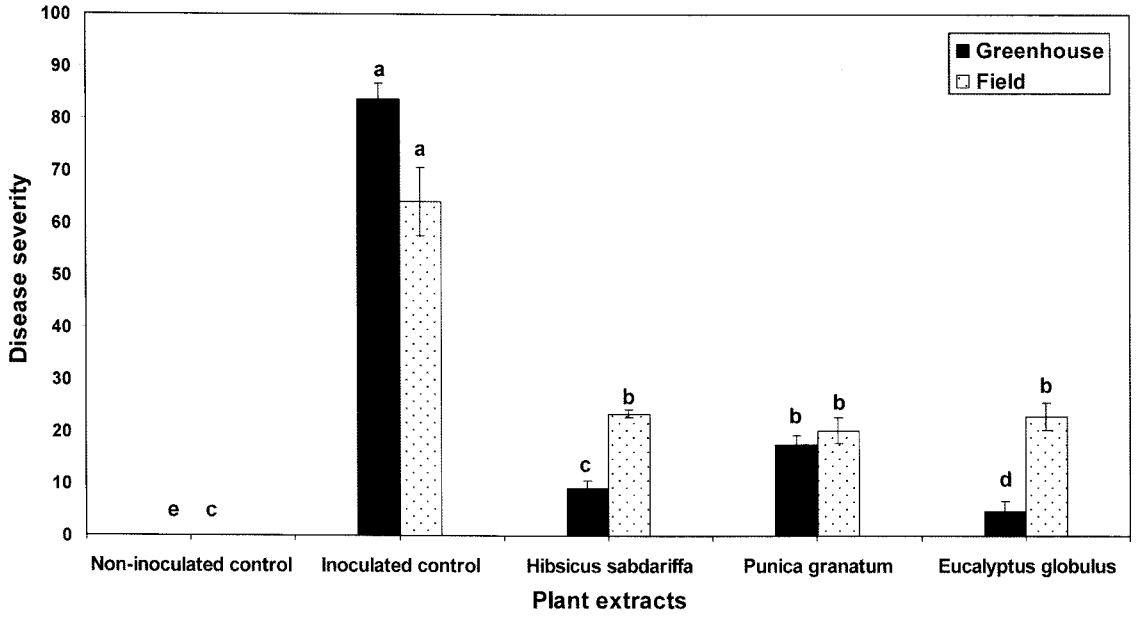

Fig. 1. Effect of three plant extracts treatment on the diseases severity of bacterial wilt of potato. Different letters in the same column indicate significant differences between treatments according to L.S.D. test (P=0.05).

potato plants as compared with the inoculated control. The population of *R. solanacearum* was lowest in potato plants treated with plant extracts of *H. sabdariffa* (4.4×10^5 cfu/g), *E. globulus* (2.23×10^6 cfu/g) and *P. granatum* (5.1×10^7 cfu/g).

Peroxidase activity. Data in Table 2 showed that peroxidase (POX) activity of non-inoculated potato plants treated with all tested plant extracts was significantly higher than that of untreated potato plants after all time from application. No significant differences were found between treated plants with extracts in enzymes activity after 2 and 6 days after treatment. Extracts of *H. sabdariffa* caused the highest enzyme activity followed by the extracts of *P. gramatum* and *E. globulus*. The highest levels of POX were determined 4 days after extract of *H. sabdariffa* treatment (13.0 unit/mg protein). In general, the enzyme activity was higher after 4 days from application followed that determined after 8 days.

In inoculated potato plants treated with the plant extracts, results presented in Table 2 indicated that *E. globulus* extract caused the highest increase in POX activity followed those of *H. sabdariffa* and *P. gramatum*. Data also showed that POX activity in potato plants treated with *H. sabdariffa* extracts was higher than those of both untreated and treated plants with other extracts 2 and 4 days after treatment. The highest levels of POX were determined 6 and 8 days after extract of *E. globulus* treatment (13.01 and 12.89 unit/mg protein, respectively) and 8 days after *P. gramatum* treatment (11.65 unit/mg protein). After *H. sabdariffa* extract treatment, the POX levels increased slowly and reached to 5.94 and 8.45 unit/mg protein after 2 and 4 days from treatment, respectively. In the same treatment, the POX levels decreased slowly at the two later sampling times 6 and 8 days after treatment (7.19 and 4.03 unit/mg protein, respectively).

Phenylalanine ammonia-layse activity. In general, a significant increase in the activity of PAL was observed in both non-inoculated and inoculated potato plants following treatment of all tested leaf extracts Table 3. PAL accumulated more markedly in leaf tissues treated with leaf extract of *H. sabdariffa* than other two extracts in inoculated and non-inoculated plants. Data also show that PAL activity increase as days after application of treatment increased. Changes in the levels of PAL activity in potato plants were observed 2 days after treatment of *H. sabdariffa* and *P. granatum*, compared to plants before treatment. While, the leaf extract of *E. globulus* increased the PAL activity after 4 days from treatment. After that, in case of non-inoculated plants, the level of PAL was slightly increased in potato plants treated with all tested extracts and reached to a maximum after 6 and 8 days from treatment of *H. sabdariffa* and *P. granatum*, respectively. In inoculated control plants, infection with *R. solanacearum* only slightly induced PAL activity in leaves of potato plants. The PAL levels increased to 0.91 unit/mg protein 2 days after inoculation and remained at the same level during the experimental periods. The highest level of PAL in treated inoculated potato plants was determined 8

Table 2. Effect of aqueous plant extracts of *Hibsicus sabdariffa*, *Punica granatum* and *Eucalyptus globulus* on peroxidase activities in potato plants.

Treatments	POX activity (Δ absorbance units/mg protein) in non-inoculated plants					
	Days after treatment					
	0	2	4	6	8	Mean
Water (non-inoculated control)	2.91 ijk	2.72 k	2.94 hijk	2.97 hijk	3.13 hijk	2.93 d
H. sabdariffa	2.91 ijk	5.94 c	13.00 a	4.58 ef	4.94 de	6.27 a
P. granatum	2.91 ijk	3.55 gh	4.38 ef	8.06 fg	8.16 b	4.61 b
E. globulus	2.91 ijk	3.39 hij	3.46 ghi	3.43 ghij	5.51 cd	3.72 c
Mean	2.91 ijk	3.90 c	5.95 a	3.76 c	5.44 b	
	POX activity (Δ absorbance units/mg protein) in inoculated plants					
	0	2	4	6	8	Mean
Water (inoculated control)	2.91 ij	2.72 j	5.16 f	5.33 ef	5.48 ef	4.32 d
H. sabdariffa	2.91 ij	5.94 e	8.45 c	7.19 d	4.03 g	5.70 b
P. granatum	2.91 ij	3.55 gh	3.60 gh	4.00 gh	11.65 b	5.14 c
E. globulus	2.91 ij	3.39 hi	5.65 ef	13.01 a	12.89 a	7.57 a
Mean	2.91 e	3.90 d	5.72 c	7.38 b	8.51 a	

The samples were collected from both non-inoculated as well as inoculated plants after specified times (immediately before treatment (0), 2, 4, 6 and 8 days after treatment). The soil drenching of leaf extract was taken 2 days prior to inoculation with *Ralstonia solanacearum*.
Within each inoculated or non-inoculated experiment, values with the same letters are not significantly different at $P = 0.05$

Table 3. Effect of aqueous plant extracts of *Hibsicus sabdariffa*, *Punica granatum* and *Eucalyptus globulus* on phenylalanine ammonia lyase, (PAL) activities in potato plants.

Treatments	PAL activity (mmol of transcinnammic acid/min/mg protein) in non-inoculated plants					
	Days after treatment					
	0	2	4	6	8	Mean
Water (non-inoculated control)	0.604 k	0.624 k	0.642 k	0.703 j	0.743 hi	0.663 d
H. sabdariffa	0.604 k	0.893 e	0.928 e	1.192 b	1.067 c	0.936 a
P. granatum	0.604 k	0.775 h	0.803 fg	0.8160 f	1.320 a	0.864 b
E. globulus	0.604 k	0.634 k	0.709 ij	0.788 ij	0.981d	0.743 c
Mean	0.604 e	0.732 d	0.772 c	0.875 b	1.027 a	
	PAL activity (mmol of transcinnammic acid/min/mg protein) in inoculated plants					
	0	2	4	6	8	Mean
Water (inoculated control)	0.604 i	0.624 i	0.908 efg	0.946 efg	0.932 efg	0.803 d
H. sabdariffa	0.604 i	0.893 fg	1.189 d	1.516 b	1.632 a	1.167 a
P. granatum	0.604 i	0.775 h	0.955 ef	1.103 d	1.469 bc	0.981 b
E. globulus	0.604 i	0.634 i	0.850 gh	0.999 e	1.383 c	0.894 c
Mean	0.604 e	0.733 d	0.976 c	1.141 b	1.354 a	

The samples were collected from both non-inoculated as well as inoculated plants after specified times (immediately before treatment (0), 2, 4, 6 and 8 days after treatment). The soil drenching of leaf extract was taken 2 days prior to inoculation with *Ralstonia solanacearum*.
Within each inoculated or non-inoculated experiment, values with the same letters are not significantly different at $P = 0.05$

days after treatment of extract of *H. sabdariffa* fowled by extract of *P. granatum* and *E. globulus*.

Polyphenoloxidase activity. The levels of PPO activity in inoculated control plants were different from that in non-inoculated control plants. In leaves of non-inoculated control potato plants, PPO levels were determined at certain times during the experimental period, the levels varied between 3.92 and 5.16 unit/mg protein. In untreated inoculated plants, PPO levels slightly increased to 7.19 unit/mg protein 4 days after inoculation and remained at the same level during the experimental periods. In both non-

Table 4. Effect of aqueous plant extracts of *Hibsicus sabdariffa*, *Punica granatum* and *Eucalyptus globulus* on polyphenoloxidase (PPO) activities in potato plants.

Treatments	PPO activity (Δ absorbance units/min/mg protein) in non-inoculated plants					
	Days after treatment					
	0	2	4	6	8	Mean
Water (non-inoculated control)	4.53 gh	4.76 gh	3.92 h	4.16 h	5.17 g	4.49 c
H. sabdariffa	4.53 gh	6.43 f	8.59 d	8.44 d	15.80 b	8.76 a
P. granatum	4.53 gh	7.75 de	7.20 ef	8.55 de	17.67 a	9.14 a
E. globulus	4.53 gh	7.75 de	6.89 ef	7.82 de	10.59 c	7.51 b
Mean	4.53 c	6.67 b	6.65 b	7.22 b	12.31 a	
	PPO activity (Δ absorbance units/min/mg protein) in inoculated plants					
	0	2	4	6	8	Mean
Water (inoculated control)	4.53 h	4.76 h	6.30 g	7.20 fg	7.84 ef	6.13 c
H. sabdariffa	4.53 h	6.43 g	9.57 cd	9.56 cd	17.73 b	9.48 b
P. granatum	4.53 h	7.75 f	7,84 ef	9.24 cd	21.93 a	10.26 a
E. globulus	4.53 h	7.74 f	8.28 def	8.92 cde	17.13 b	9.32 b
Mean	4.53 e	6.67 d	7.90 c	8.730 b	16.16 a	

The samples were collected from both non-inoculated as well as inoculated plants after specified times (immediately before treatment (0), 2, 4, 6 and 8 days after treatment). The soil drenching of leaf extract was taken 2 days prior to inoculation with *Ralstonia solanacearum*.
Within each inoculated or non-inoculated experiment, values with the same letters are not significantly different at $P = 0.05$

inoculated and inoculated treated potato plants, over the entire experimental period, PPO activity was markedly increased in all plant extracts treatments (Table 4). The Application of *P. granatum* extract caused the highest enzymes activity followed by that of *H. sabdariffa* extract and then *E. globulus* extract. Data also showed that after 8 days from application, all extracts treatments exhibited highest enzyme activity than those of the other tested periods of enzymes determination. Focusly, the higher activities of PPO were determined in both inoculated and non-inoculated potato plants 8 days after treatment with extract of *P. granatum* (21.9 and 17.6 unit/mg protein, respectively).

Discussion

The effectiveness of plant extracts in controlling the bacterial wilt disease caused by *R. solanacearum* on potato cv. Diamont under greenhouse and field conditions was tested. In our experiments, the plant extracts of *H. sabdariffa*, *P. granatum* and *E. globulus* gave significant reduction in disease severity of bacterial wilt in potato plants. These results are in agreement with previous reports (Basim et al., 2006; Bowers and Locke, 2004; Kagale et al., 2004). They found that the foliar or soil applications of leaf plant extracts effectively reduced the incidence of fungal and bacterial diseases in host plants under field and greenhouse condition. To evaluate whether the protective effect of these plant extracts on potato plants against bacterial wilt caused by *R. solanacreaum* was due to direct antibacterial activities or indirect (induction of resistance) of these extracts, antagonistic capability of the plant extracts was investigated *in vitro*.

Data revealed that plant extracts of *H. sabdariffa*, *P. granatum* and *E. globulus* were able to inhibit the growth of the causal pathogen (*R. solanacearum*) *in vitro*. Plant extracts of many plant species have been reported to have antibacterial effect against certain plant pathogenic bacteria and this property can utilized for management of bacterial diseases (Basim et al., 2006; Kagale et al., 2004).

In the current study, colonization of xylem of lower stem sections of treated potato plants with plant extracts was determined after 6 weeks from inoculation by the plating method. The populations of *R. solanacearum* were lower in potato plants treated with plant extracts of *H. sabdariffa*, *E. globulus* and *P. granatum* than in untreated inoculated control plants. Such results indicated that the reduction in bacterial wilt severity of potato plants was correlated with lower bacterial growth in treated plants by about one third as compared to control plants (Lawton et al., 1996). Similarly, Hassan and Buchenauer (2007) reported that the combination of BABA with ASM application reduced bacterial pathogen population in apple seedlings. On the basis of our results, it may be assumed that reduction of bacterial multiplication in treated plants was accompanied by accumulation of defense components in plant tissues especially in the xylem as a result of resistance induction already before inoculation or the plant respond quickly to inoculation by production of bacterial growth inhibitors after inoculation. Antimicrobial compounds, for example phenolic acids, peroxidases, lignin and other defense molecules may be accumulated in plant tissue treated with plant extracts. These compounds might be involved in retardation of multiplication of bacterial cells. We have shown that natural plant extracts can reduce pathogen populations and disease severity.

Investigations on mechanisms of disease suppression by plant products have suggested that the active principles present in them may either act on the pathogen directly (Amadioha, 2000), or induced systemic resistance in host plants resulting in reduction of disease development (Kagale et al., 2004; Narwal et al., 2000; Paul and Sharma, 2002). Induced resistance has been demonstrated to protect plants against pathogen infections and may be a new approach in control of plant diseases, following intensive screening studies we have selected three aqueous plant extracts to study their inducing resistance activity and effectiveness against the disease. These studies will provide a more comprehensive evaluation of the impact of plant extracts in the production of potato and may facilitate the feasibility for its practical application in controlling bacterial wilt of potato. In the greenhouse experiments, soil drenching with plant extracts of *H. sabdariffa*, *P. granatum* and *E. globulus* (50 ml/pot) reduced profoundly the disease severity compared to the untreated inoculated control. Our results agree with those reported by Abd-EL-Khair and Haggag, 2007 and Krebs et al., 2006. They mentioned that the water extracts of medicinal plants reduced the disease severity of both early and late blight of potato plants. The efficacy of medicinal plant species may be due to induction of the resistance system in treated plants (Jayaraj et al., 2008; Mosch et al., 1996) or cause a delay in the development of infection in early growth stage by inhibition the mycelial growth of pathogen (Abd-EL-Khair and Haggag, 2007).

Biochemical studies on the mode of action of the plants extracts as inducers of resistance against bacterial wilt of potato plants were performed. In this study, the enzymatic activities of peroxidase (POX), phenylalanine ammonia-layse (PAL) and polyphenoloxidase (PPO) were determined in leaves of non-inoculated and inoculated potato plants with *R. solanacearum*. In case of POX activity, the present results showed that all tested plant extracts showed higher POX activity in inoculated potato plants than that of the untreated inoculated plants after 4, 6 and 8 days from

application. These results suggested that the plant extracts promotes induced systemic resistance in potato plants by increasing defense-related peroxidase enzymes. Several investigators have reported that enhanced peroxidase activity was associated with induced systemic resistance in plants to fungal, bacterial and viral pathogens (Hammerschmidt et al., 1982; Hassan and Buchenauer 2007; Reuveni et al., 1990). It was involved in several plant defense mechanisms, such as lignin biosynthesis, oxidative cross-linking of plant cell walls, and also generation of active oxygen species as reported by Lam and Dixon (1997) and Mehdy (1994). From our data, it may conclude protection against bacterial wilt was generally obtained when plant extracts of *H. sabdariffa*, *P. granatum* and *E. globulus* were applied two days before inoculation, and enzyme analysis in identically treated stem tissues indicated that bacteria would be exposed to tissues that already express an increase in defense reactions. The data also indicated that level of POX activity remained at high concentration for eight days in potato plants treated with plant extracts. According to our results, peroxidase can be recommended as typical marker of SAR-mediated defense reaction in potato plants in greenhouse.

Data reported herein indicated that all tested treatments of plant extracts caused significant increase in activity of PAL and PPO enzymes in leaves of non-inoculated and inoculated potato plants. These results were in accordance with those reported by Jayaraj et al. (2008) and Kagale et al. (2004), they found that a significant increase in the activity of PAL and PPO was observed in rice and carrot plants following treatment of seaweed and *Datura metel* leaf extract and enhanced disease resistance in carrot against *Alternaria radicina* and *Botrytis cinerea* and in rice against *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae*. Accumulation of phytoalexins and reinforcement of cell wall polymers resulting from the deposition of lignin derivatives (Thangavelu et al., 2003) are also found, as there is an increase in enzyme activities related to these metabolic pathways such as PAL (He et al., 2002) and PPO (Mayer and Staples, 2002). This process has been associated with local and systemic induced resistance. From our results, the induction of resistance against bacterial wilt of potato plants caused by application of tested plant extracts was associated with the increase of PPO and PAL activities. Here, it was evidenced that plant extract were efficient in reduction of bacterial wilt severity. In plants, PPO has been associated as well with lignifcation of cell walls, thus playing a protective role in injured plants against other organisms, due to reactive quinones produced from phenolic compound catalysis (Mayer and Staples, 2002). PAL is a key enzyme of the phenylpropanoid biosynthesis pathway involved in the production of plant defence-related secondary metabolites including salicylic acid, phytoalexins and lignin-based polymers (La Camera et al., 2004).

In conclusion, the results from our study suggest that the plant extracts of *H. sabdariffa*, *P. granatum* and *E. globulus* have strong inhibitory effect against *R. solanacearum* under both *in vitro* and *in vivo* conditions, and have the potential to induce systemic resistance in potato against this pathogen. Antimicrobial activity and induction of systemic resistance in potato plants by leaf extracts of certain plants against the disease may have a key role in the concept of new control strategy of this disease.

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