ENZYME ACTIVITY IN POTATO CULTIVARS AND THEIR ROLE IN RESISTANCE OR SUSCEPTIBILITY AGAINST BACTERIAL WILT DISEASE.

Abdulsalam A. hajhamed[1], Mahmoud A.A. Sallam[2], Wafaa M. Abd El-Said[1] and Nagi Y. Abd El-Ghafar[1]

[1] *Department of Plant Pathology, Faculty of agriculture, Ain Shams Univ.*

[2] *Department of Genetic, Faculty of Agriculture, Ain Shams Univ.*

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ABSTRACT

Bacterial wilt disease of potato caused by Ralstonia solanacearum, (Smith, 1896) Yabuuchi *et al*. (1995) is one of the most important bacterial diseases over the world. This study was conducted to evaluate susceptibility/or resistance of six potato cultivars to bacterial wilt disease and their relation with host enzymatic activities. Potato cultivars, Nicola and Lady rosetta were highly susceptible to bacterial wilt disease, while Santana and Valor were moderately resistance to the disease. However, Diamont and Lady balfor cultivars appeared resistant. The enzymes phenylalanine ammonia-layse (PAL), pyroxidase (PO) and polyphenol oxidase (PPO) showed high activity in infected plants compared with healthy ones. Phenylalanine ammonia-layse and polyphenol oxidase activity have increased with increasing the period after *R. solanacearum* inoculation, On the contrary, activity of pyroxidase was decreased with increasing the period after inoculation. There was positive correlation between enzymatic activities and resistance of potato cultivars to bacterial wilt disease. Phenylalanine ammonia-layse showed high activity in Nicola and Lady rosetta (susceptible cultivars) but showed moderate activity in Santana and Valor (moderately resistance cultivars). Also, Lady balfor and Diamont (resistance cultivars) showed less activity with Phenylalanine ammonia-layse. There was no significant differerence in activity of peroxidaes and polyphenol oxidase in healthy potato cultivars, but significant differerence were found between potato cultivars in case of infected plants, However, peroxidase and

polyphenol oxidase showed high activity in Nicola compared with Lady rosetta, Santana, Diamont,Valor, and Lady balfor cultivars which showed moderate and/or lower activity in peroxidase and polyphenol oxidase.

Key words: Potato, *Solanum tuberosum* L., Bacterial wilt disease. *Ralstonia solanacearum,* Resistance, Susceptible, Cultivars, Enzyme activity. Phenylalanine ammonia-lyase (PAL), Peroxidase (PO) and Polyphenol oxidase (PPO).

INTRODUCTION

Potato (*Solanum tuberosum* L.), is the fourth most important commercial crop in the world. Potato plants are affected by many biotic and abiotic factors, including pathogens and environmental stresses (Juskiewicz *et al.,* 2005; Rauscher *et al.,* 2006). This is a serious economic problem in several countries where potatoes are cultivated over large areas and economical exportation crop like Egypt. *Ralstonia solanacearum* is the most devastating bacterial pathogens which infect more than 50 botanical families, some of which are economically important such as banana, potato, and tomato causing bacterial wilt disease. The bacterium is found world wide, mainly in tropical and subtropical areas, but also in warm-temperate countries and even in some cool-temperate regions (Hayward, *et al.*, 1998).

The natural resistance of plants to diseases is based not only on preformed defenses, but also on induced mechanisms. The induced mechanisms are associated with local changes at the site of pathogen infection, such as the hypersensitive response (HR), which is one of the most efficient forms of plant defenses. The HR also leads to an increase in the activity of peroxidases (Kortekamp and Zyprian, 2003) and polyphenol oxidases enzymes (Agrios, 1997) involved in defense responses (Thipyapong *et al.*, 2004).

Polyphenol oxidase (PPOs) enzymes are widely distributed within the plant, and act in the defense responses (Thipyapong *et al*., 2004). PPOs act in disease resistance by hydroxylizing monophenols to α-diphenols and oxidizing these compounds to quinones, which are often more toxic to the microorganisms than the original phenolic compounds (Gandia-Herrero *et al*., 2005). PPOs have been known to

biochemistry for century and several hypotheses regarding the function of PPO have been proposed, including roles in the phenylpropanoid pathway (Kojima and Takeuch, 1989).

Peroxidase enzymes are also related to plant defense, acting in strengthening the cell wall (Agrios, 1997; De Ascensão and Dubery, 2003), In addition, PPO and PO are multifunctional enzymes that can prevent biological and chemical attacks by raising physical barriers or by counter-attacking a pathogen with a high production of free radicals (Passardi *et al.,* 2005). Peroxidase oxidizes a vast array of compounds (hydrogen donors) in the presence of H_2O_2. Plant peroxidase are heme-containing glycoproteins and are usually classified as acidic, neutral or basic according to their isoelectric points. Several physiological functions for peroxidases in plants have been reported, such as removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls (Mader and Fussl, 1982 and Lagrimini, 1991).

Many enzymes are involved in the biosynthesis of lignin precursors. Phenylalanine ammonia-lyase (PAL) is one of the key enzymes which catalyzes the change of phenylalanine to cinnamic acid at the initial step in the biosynthesis of lignin precursors (Haddon and Northcote1975, Fukuda and Komamine 1982). On the other hand, phenylpropanoid metabolism in plants leads to the formation of numerous phenolic compounds that are believed to have important functions in plant defense responses to wounding and pathogen infection (Hahlbrock and Scheel, 1989; Nicholson and Hammershmitd, 1992). The phenylpropanoid pathway is initiated by the enzymatic conversion of phenylalanine to cinnamic acid, which is catalyzed by the enzyme phenylalanine ammonialyase (PAL) (Koukol and Conn, 1961).

In this study we aimed to evaluate response of six potato cultivars to elucidate response to bacterial wilt disease, by analyzing the changes in levels of activity of phenylalanine ammonia layse, polyphenol oxidases and peroxidases in plant roots and shoots, in addition relationship between enzyme activity levels and cultivars susceptibility or resistance to bacterial wilt disease.

MATERIALS AND METHODS

All experiments of this study were conducted in the laboratories and greenhouse of Plant Pathology Dept., Faculty of Agriculture, Ain Shams University, during the period 2008 to 2010. Six potato cultivars (Diamont , Lady rosetta, Valor, Santana, Nicola and Lady balfor), commonly cultivated in each of Syria and Egypt, were used throughout this study. These cultivars were planted in clay pots (30 cm diam.) each containing sterilized sand-clay (1:1 V/V). Two tubers were planted per pot and fifteen pots were used as replicates for each cultivar. The growing plants were maintained under greenhouse conditions during spring season.

Preparation of *Ralstonia solanacearum* inoculum:

Virulent isolate of *Ralstonia solanacearum* (AC20) was obtained from Bacterial Disease Laboratory, Plant Pathology Department, Faculty of Agriculture, Ain Shams University, Egypt. Bacterial cell suspensions of *R. solanacearum* was prepared from 48 hours old cultures on Tetrazolium medium (*Bacterial Selective Media TZC*) and adjusted to 10^8 colony forming units (cfu)/ml using spectrophotometer (0.3 as optical density at 600 nm). The suspension was immediately used for inoculation of potato plants (Poiatti, *et al.,* 2009).

Pathogen inoculation:

Two inoculation methods; stem injection and soil drench, according to **Bowman, and Sequeira, (1982) and McLaughlin and Sequeira (1988)** were used to evaluate resistance and/or susceptible of potato cultivars. Inoculation process was done 30 days after sowing potato tubers.

Disease assessment:

Disease incidence was recorded at 10 days intervals after 30 days of pathogen inoculation and determined according to a disease ratting scale where: 0 = no symptoms; l = up to 25% of the foliage wilted; 2 = 26-50% of the foliage wilted; 3 = 51-75% of the foliage wilted; and 4 = 76-100% of the foliage wilted. Disease severity was calculated per potato cultivar and percentage of disease index (DI) was calculated (Kempe and Sequeira, 1983) as follow: $DI = (\Sigma R.T/N\times4) \times100$, where: T = total number of plants with each category; R = disease ratting scale (R=0, 1, 2, 3 and 4); N = total number of tested plants.

Enzymes extraction and assay:

Enzyme activity of Phenylalanine ammonia-lyase (PAL), Peroxidase and polyphenol oxidase was evaluated at three intervals time after inoculation: 5, 10, 15 days.

Phenylalanine ammonia lays:

Phenylalanine ammonia-lyase (PAL) activity of shoots and roots of potato cultivars was measured following the method described by Solecka and Kacperska (2003) with slight modifications. Briefly, 1 g of each fresh sample was ground with 2 ml of 50 mM borate buffer (pH 8.8) using mortar and pestle at 4 °C, centrifuged at 12000 rpm for 10 min at 4 ºC. The supernatant was used as a source of crude enzyme for assaying PAL activity. The reaction mixture consisted of 1 ml of crude enzyme solution, 2 ml sodium borate buffer (pH 8.8) and 1 ml of 10^{-2} M L-phenylalanine. Incubation was performed at 37 °C for 1 h and the reaction was stopped by the addition of 500 µl of 6 M HCl. The reaction mixture was centrifuged for 10 min at 12000 rpm to pellet the denatured protein. The absorbance at wave length 290 nm was measured using spectrophotometer model Hitachi U-900.

Peroxidase and polyphenol oxidase:

Healthy and inoculated, Fresh samples of potato roots and shoots with *Ralstonia solanacearum* was collected, and were stored -8°C till use. Potato sample (1 g) was ground with 2 ml of 50 mM potassium phosphate buffer (pH 7.0) using mortar and pestle in ice-cold box. Samples were transferred to Eppendorf tubes, and then centrifuged for 20 min at 12000 rpm at 4 °C. Supernatant, containing water-soluble enzymes were stored at -8°C till use. Three replicates were prepared for each treatment**.** Peroxidase and polyphenol oxidase activities were determined according to the method of Biles and Martyn (1993) using catechol as a substrate and measuring was carried out every 25 second at wave length 495 nm using spectrophotometer model Hitachi U-900. Peroxidase and polyphenol oxidase activities were expressed as (Δ) change in absorbance of optical density (OD) per gram fresh weight.

Statistical analysis:

All experiments were set up in a complete randomized design. Data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Inc., 1996). Means were separated by Duncan's multiple range test at $P< 0.05$ level.

RESULTS AND DISCUSSION

RESULTS:

Reaction of potato cultivars to bacterial wilt disease:

Six potato cultivars; Diamont , Lady balfor, Lady rosetta, Nicola, Santana and Valor were evaluated against bacterial wilt disease, using virulent isolate of *Ralstonia solanacearum* and two inoculation methods. Results in Fig (1) clearly showed that disease symptoms were developed after 30 days from inoculation with *Ralstonia solanacearum* with Nicola cultivar. However symptoms on Lady rosetta, Santana and Valor cultivars, were developed after 40 days and after 50 days on Lady balfor and Diamont cultivars. Disease progress was developed with increasing the period after inoculation. After seventy days of inoculation, Nicola and Lady rosetta cultivars showed the highest infectious with disease, where the disease index was 85.1% – 87.5% and 73.0%- 80.0 % and mean disease ratting was 4.3 – 4.4 and 3.7- 4.0, respectively.

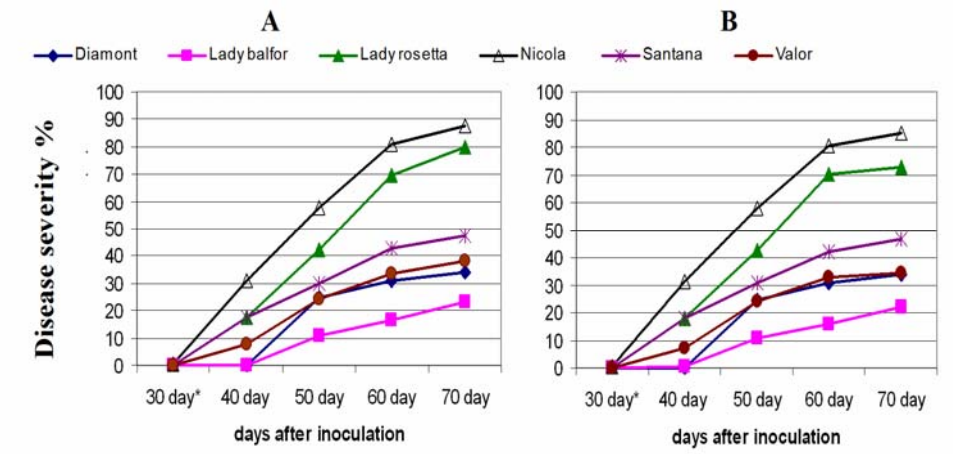

Fig (1): Severity of bacterial wilt disease on potato cultivars, at different intervals after inoculation with virulent isolate of *Ralstonia solanacearum* , using soil drench method (A)and stem injection method (B), under greenhouse conditions.

Meanwhile, Santana, Valor and Diamont cultivars were moderately infected with the disease; where the disease index was 46.8- 47.5, 34.5-38.5 and 34.0 % and mean disease ratting was 2.4, 1.8- 1.9 and 1.7, respectively. Also, Lady balfor cultivar was the least infected with the disease, where the disease index was 22.3- 23.0 %

and mean disease ratting was 1.2. According to disease development, the tested cultivars were divided into 3 groups: The first group contained Nicola and Lady rosetta which considered as susceptible cultivars, while the second group contained Santana and Valor as moderately susceptible and/or resistance cultivars and the third group contained Diamont and Lady balfor as resistance cultivar.

Enzymatic activity:

The activity of phenylalanine ammonia-layse (PAL), peroxidase (PO) and polyphenol oxidase (PPO) were assayed in shoots and roots of potato cultivars which inoculated or non-inoculated with *Ralstonia solanacearum*. Generally, Phenylalanine ammonia-layse (PAL) showed more activity in infected potato plants than healthy plants. As shown in Fig. (2) Phenylalanine ammonia-layse activity was increased with increasing the period after inoculation with *Ralstonia solanacearum.* Where, PAL show low activity, as optical density (OD) it was 3.302- 3.423 and 2.99- 3.316 in the shoots, and 2.684-2.980 and 2.906 – 2.981 in the roots of Nicola and lady rosetta cultivars respectively. Meanwhile, PAL showed moderate activity 3.490- 3.758, 3.450 – 3.500 and 3.637 – 3.882 in the shoots and 2.756 – 2.894, 2.89.4 – 2.709 and 3.541 -3.658 in the roots, respectively with Santana, Valor and Diamont cultivars as moderately susceptible cultivars. Also, PAL showed relatively high activity with Lady balfor cultivars which recorded 3.439- 3.879 in the shoots and 3.079-3.295 in the roots.

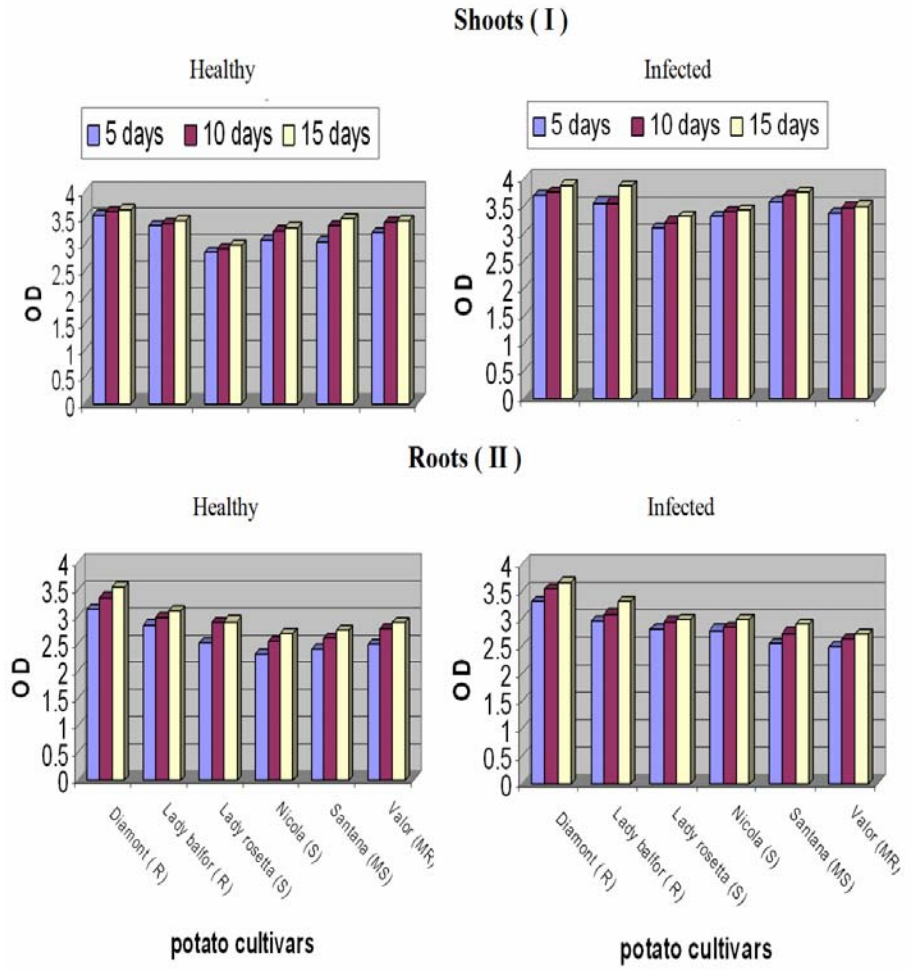

Fig (2): Phenylalanine ammonia-lyase (PAL) activity (as optical density (OD)) in shoots and roots of potato cultivars in response to infection with *Ralstonia solanacearum* by stem injection method (I) and soil drench method (II), at 5, 10, and 15 days after inoculation, under greenhouse conditions.

The activity Peroxidase (PO) and polyphenol oxidase (PPO) activity were higher in infected plants than healthy ones (Figs. 3 - 6). Activity of PO has decreased with increasing the period after inoculation with *Ralstonia solanacearum,* but, the activity of PPO has increased with increasing the period after inoculation. There was no significant difference in peroxidase or polyphenol oxidase activity

between different potato cultivars in case of healthy plants, but significant difference were recorded among infected potato cultivars. Where, PO and PPO showed high activity in Nicola cultivar compared with Lady rosetta, Santana, Diamont, Valor and Lady balfor cultivars which showed moderate in PO and PPO activity.

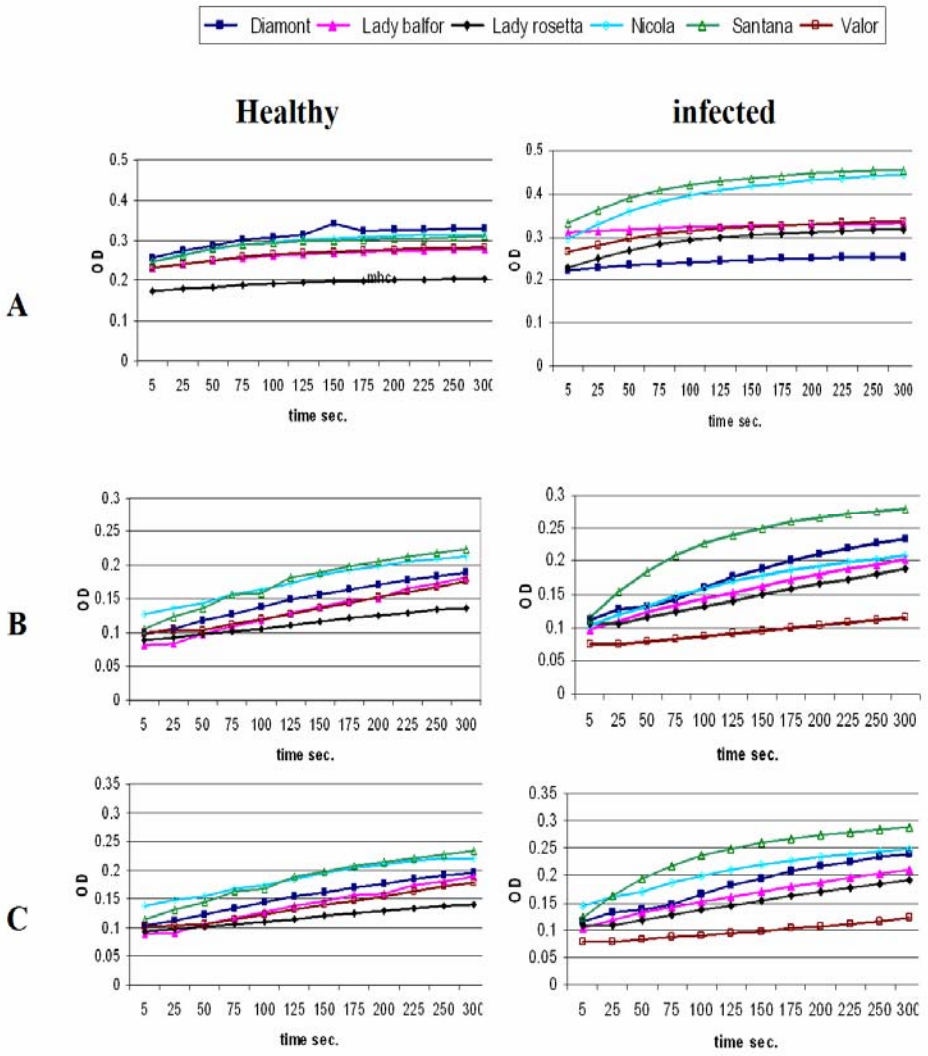

Fig (3): Pyroxidase (PO) activity (as optical density (OD)) in potato cultivars shoots in response to infection with *Ralstonia solanacearum* using stem injection method, after 5 (A), 10 (B) and 15(C) days from inoculation, under greenhouse conditions.

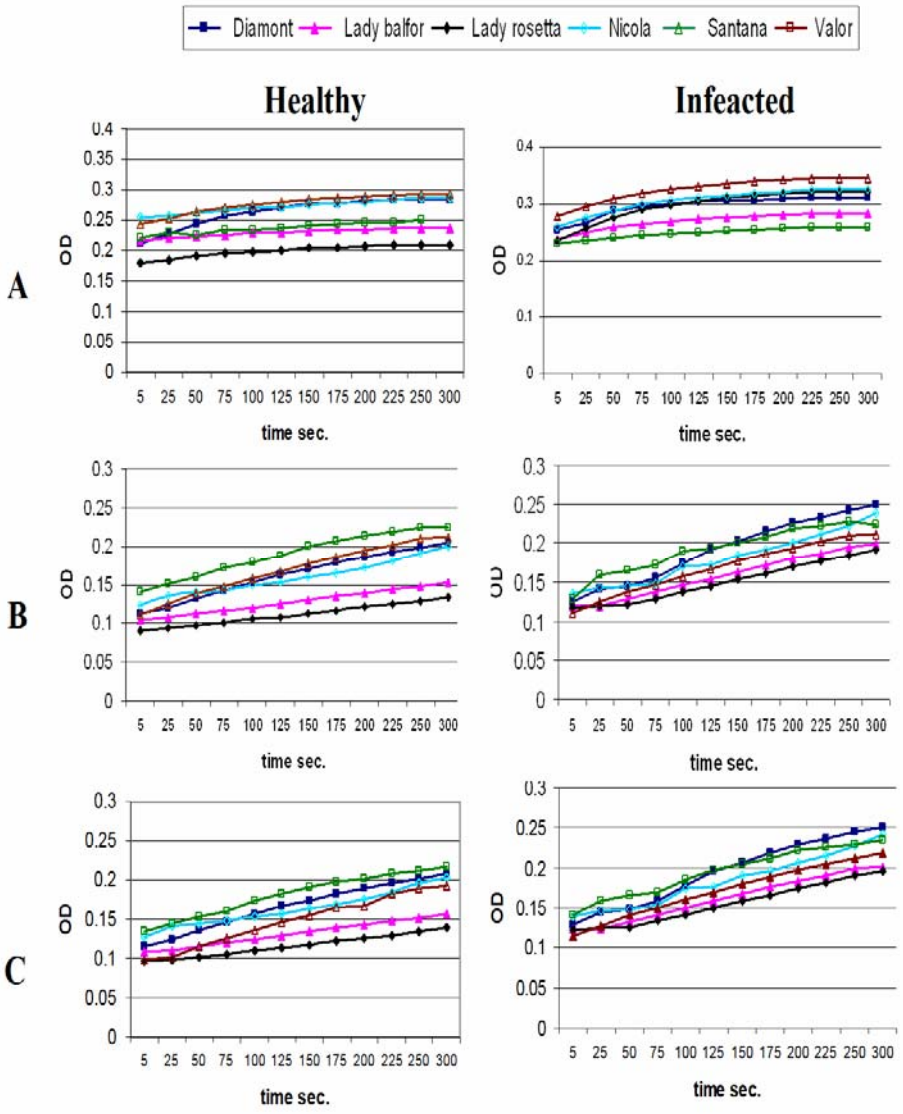

Fig (4):Pyroxidaes (PO) activity (as optical density (OD)) in potato cultivars roots as response to infection with *Ralstonia solanacearum* using soil drench method, after 5 (A), 10 (B) and 15(C) days from inoculation, under greenhouse condition.

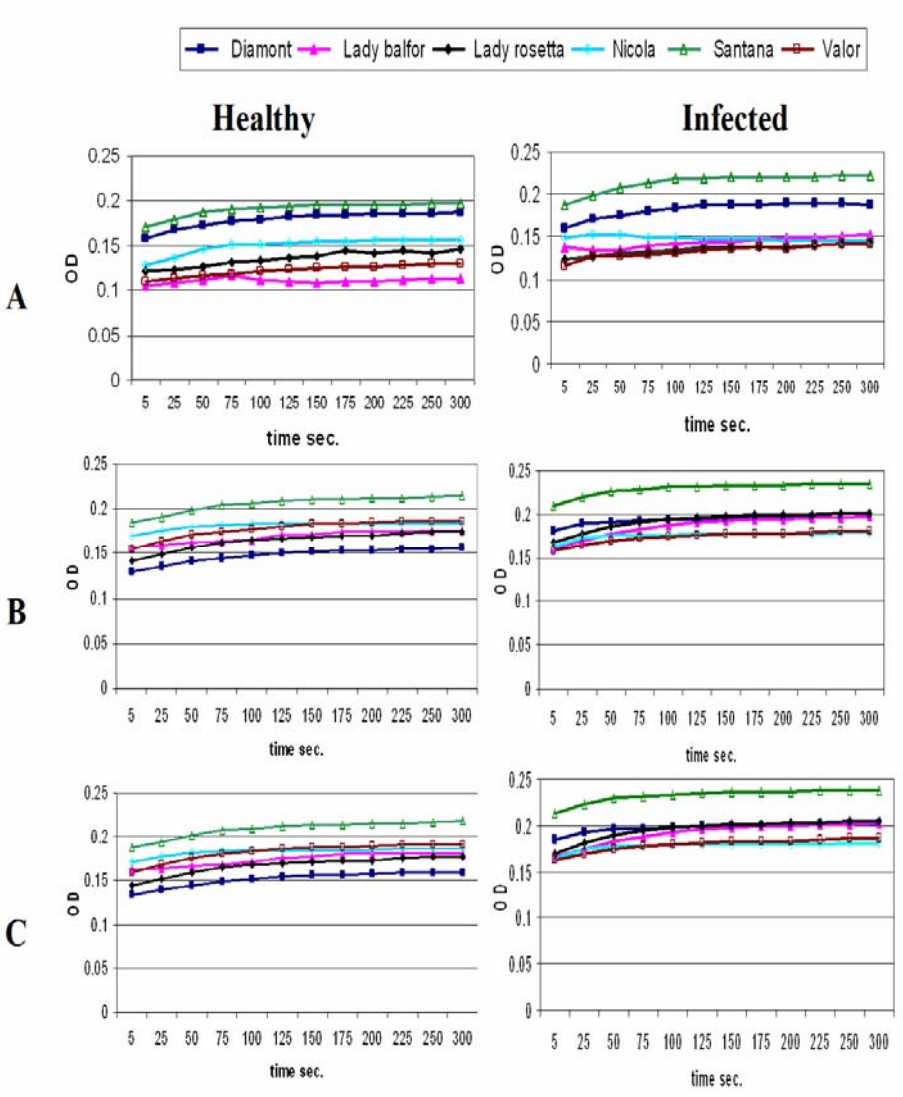

Fig (5): Polyphenol oxidase (PPO) activity (as optical density (OD) of potato cultivars shoots, in response to infection with *Ralstonia solanacearum* using stem injection method, at (A), 10 (B) and 15(C)days after inoculation, under greenhouse conditions.

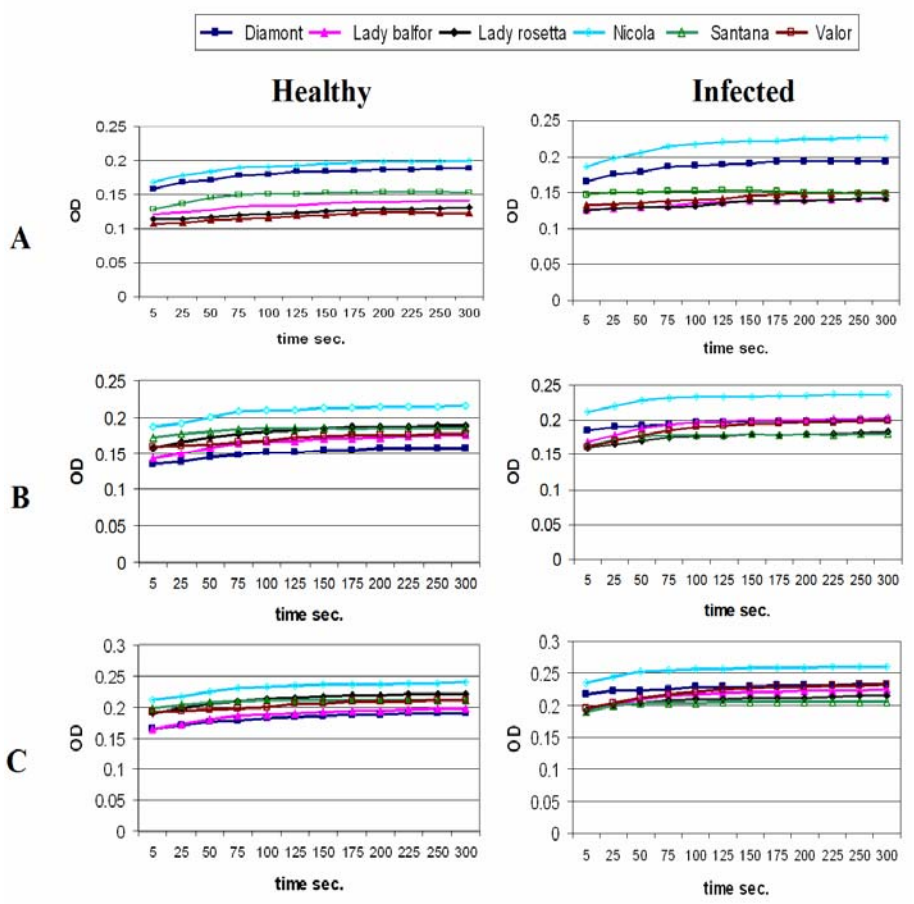

Fig (6): Polyphenol oxidaes (PPO) activity (as optical density (OD)) in potato cultivars roots in response to infection with *Ralstonia solanacearum*, using soil drench inoculation method, at (A), 10 (B) and 15(C)days after inoculation, under greenhouse conditions.

DISCUSSION

The present study demonstrated that, Lady balfor cultivar appeared to be resistance to bacterial wilt disease, but Santana, Valor and Diamont cultivars were moderately resistance, and Nicola and Lady rosetta cultivars were highly susceptible to bacterial wilt disease, under artificial inoculation in greenhouse conditions.

Resistance or susceptibility in the potato (Agata, cv.) resulted in defense response that differed qualitatively and quantitatively depending on leaf age, type of inoculation performed and on the interaction between the plant and the pathogen (Poiatti *et al.,* 2009).

In the present study, Phenylalanine ammonia-lyase (PAL), peroxidase (PO) and polyphenol oxidase PPO showed more activity in infected plants compared with healthy plants. There was positively correlated between activity of PAL, PO and PPO and susceptibly or resistance of potato cultivars against bacterial wilt disease.

Many defense enzymes are involved in defense reaction against plant pathogens. These include oxidative enzymes such as peroxidase (PO) and polyphenoloxidase (PPO), which catalyze the formation of lignin, and other oxidative phenols that contribute to the formation of defense barriers for reinforcing the cell structure (Avdiushko *et al.,* 1993). Enzymes such as phenylalanine ammonia-lyase (PAL) are involved in phytoalexin or phenolic compound biosynthesis. Such enzymes have been correlated with defense against pathogens in several plants (Binutu and Cordell, 2000).

Phenylalanine ammonia-lyase (PAL), peroxidase (PO), and polyphenol oxidase (PPO) are based as the key enzymes in the plant-defense mechanism, and they exert their effect through different pathways as explained by different investigators (Lavania *et al*., 2006). Daayf *et al.* (1997) postulated the production of phenolics and phytoalexins in cucumber mediated through the PAL pathway. *Ralstonia solanacearum* mainly attacks and moves through vascular system of tomato, where accumulation of polyphenol oxidase is limited to phloem (Thipyapong *et al.,* 1997).

Peroxidase (PO) is an oxido-reductive enzyme that involve in wall binding process (May *et al*., 1996). It represents another component of an early response in plant to pathogen attack and plays a key role in biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West, 1989). The products of PO in the presence of a hydrogen donor and hydrogen peroxide have antimicrobial activity and even antiviral activity (Van Loon and Callow, 1983). PO also catalyses the condensation of phenolic compounds into lignin and is associated with disease resistance in plants (Hammerschmidt *et al*., 1981).

It is proposed that bound PO is attached to the cell wall, where they are involved in constructing a physical barrier (Gaspar *et al*.,

1985; Hiraga *et al.,* 2001). Phenylalanine ammonia-lyase (PAL) was found to be the initial gateway enzyme in phenolic compound biosynthesis, and playing an important role for salicylate synthesis (Pallas *et al*., 1996). Obtained results of the present study are in agreement with those reported by other workers. Raju *et al.,* (2008) indicates a rapid increase of PAL activity in chemical and pathogen treated chickpea seedlings as well as invasion of root tissues by the pathogen might have resulted in decreased activity of PAL in susceptible cultivar. Whereas, increased activity of PAL in resistant cultivar might be due to prevent the fungal invasion and thus the activity maintained at higher levels during the experimental period. Therefore, these results suggests that increased accumulation of PAL with pathogen could re-established the notion that in response to invasion of pathogen, PAL is synthesized more rapidly in resistant cultivar than that in susceptible cultivar. Increased PAL activity level in response to pathogen or elicitor spray has been reported by Song *et al.,* (1993).

Similar findings to our results were reported in other studies; Stout *et al.,* (1999) mentioned that, localized inoculation of tomato leaflets with *Pseudomonas. syringae* induces a significant increase in PPO activity and leads to systemic resistance to the subsequent infection by *P. syringae*. As well as Raju *et al.,* (2008) mentioned that higher activities of PPO in the resistant cultivar than that of susceptible cultivar, in all the treatments. The role of PPO in *P. syringae* resistance was further substantiated when transgenic tomato plants with 5-10 fold increase in PPO activity levels showed over 15-fold fewer lesions and over 100 fold reduction in *P. syrinagae* growth compared to control at a high inoculum concentration (Li, and Steffens, 2002). Thipyapong, *et al.*, (2007) mentioned that, although the mechanism by which PPO contributes to disease limitation is still unclear, it is evident that PPO may participate in plant defense as a component of both the response and signaling process that ultimately limits disease progression. In contrast, when treated and control seedlings were evaluated for resistance against *R. solanacearum,* the causal agent of bacterial wilt in tomato, no significant differences in susceptibility were observed between the 2 genotypes. *P. syringae* infects epidermal, mesophyll and cortical cells of tomato foliage and stems in which PPO has been shown to accumulate abundantly, particularly in young tissues (Thipyapong *et al.*, 1997).

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النشاط الأنزيمي في أصناف البطاطس ودوره فى مقاومة أو قابلية الإصابة بمرض الذبول البكتيري

عبدالسلام عبدالله الحاج حامد [1]، محمود عبدالحفيظ سلام [2] ، فاء محمد عبدالسيد [1] ، ناجي يسين عبدالفغار [1]

[1] قسم أمراض النبات، كلية الزراعة، جامعة عين شمس.

[2] قسم الوراثة ، كلية الزراعة، جامعة عين شمس.

يعتبر مرض الذبول البكتيري الذي يتسبب عن بكتريا *Ralstonia solanacearum* من الأمراض البكتيرية الخطيرة جداً على مستوى العالم. هدفت هذه الدراسة تقييم إستجابة النشاط الأنزيمي لإنزيمات الفينيل آلانين أمونيا لياز (PAL) ، البولي فينول أوكسيداز(PPO) والبيروكسيداز (PO) في ستة أصناف من البطاطس عن الإصابة بمرض الذبول البكتيري. أوضحت النتائج أن صنفي نيكولا و ليدي روزيتا كانا من أكثر الأصناف قابلية للإصابة بالمرض، بينما كان صنفي سانتانا و فالور من الأصناف المتوسطة القابلية للإصابة بالمرض، أما صنفي دايمنت وليدي بالفور كانا أكثر الأصناف مقاومة. كان هناك زيادة في نشاط أنزيمات الفينيل آلانين أمونيا لياز، البيروكسيداز و البولي فينول أوكسيداز في حالة النباتات المصابة مقارنة بالنباتات السليمة، بينما لوحظ إزدياد نشاط أنزيمي الفينيل آلانين أمونيا لياز والبولي فينول أوكسيداز مع زيادة الفترة الزمنية بعد العدوى بالبكتيريا الممرضة، في حين كان العكس في حالة أنزيم البيروكسيداز. كان النشاط الأنزيمي للأنزيم الفينيل آلانين أمونيا لياز مرتفعاً في حالة الصنفين نيكولا وليدي روزيتا بإعتبارهما من أكثر الأصناف قابلية للإصابة بالمرض في حين كان النشاط الأنزيمي متوسطاً في حالة الصنفين سانتانا وفالور باعتبارهما متوسطا القابلية للإصابة وعلى العكس من ذلك فقد كان النشاط الأنزيمي منخفض في الصنفين دايمنت وليدي بالفور باعتبارهما أكثر الأصناف مقاومة للمرض. كما لم يكن هناك إختلافات جوهرية لنشاط أنزيم البيروكسيداز والبولي فينول أوكسيداز بين أصناف البطاطس في حالة النباتات السليمة ولكن هناك أختلافات معنوية لنشاطهما بين الأصناف المختلفة في حالة الإصابة بالمرض ووجد أن نشاطهما كان أكثر في حالة الصنف نيكولا في حين كان متوسطاً إلى منخفض نسبياً في باقي الأصناف .