**R E S E A R C H   A R T I C L E**

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# INDUCED SYSTEMIC PROTECTION AGAINST TOMATO LEAF SPOT (EARLY LEAF BLIGHT) AND BACTERIAL SPECK BY RHIZOBACTERIAL ISOLATES

## ABSTRACT:

Two strains of growth promoting rhizobacteria (PGPR), *Pseudomonas putida* MG4, and *Pseudomonas fluorescens* MG18 selected as inducers of systemic resistance, were tested for biological control of leaf spot caused by *Alternaria solani* and bacterial speck caused by *Pseudomonas syringae* in tomato. The two bacterial isolates afforded reduced disease intensity and elicited systemic protection against the two studied pathogens. The two PGPR stimulated a systemic response in tomato by inducing high rates of enzyme activity of phenylalanine ammonialyase (PAL), peroxidase (PO), polyphenol oxidase (PPO) and chitinase as well as the accumulation of high level of phenolics. The combined effect of these factors induced drastic decrease in the degree of infection of the two pathogens.

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## INTRODUCTION:

Tomato (*Lycopersicon esculentum* Mill.) is a major contributor to the fruit vegetable diet of humans. It is cultivated in essentially all countries either in fields or in protected cultures. In Egypt, tomato is considered one of the most important vegetable crops. A destructive pathogen *Alternaria solani* infects aerial part of tomato and causes disease known as early leaf blight or leaf spot, which causes destruction of foliage and the fruits and can lead to complete loss of crop (Rotem, 1994; Vloutoglou and Kalogerakis, 2000). Another pathogen *Pseudomonas syringae* pv. tomato is the causal agent of bacterial speck disease. This disease causes moderate loss, which decreases production of tomato under greenhouse and field conditions (Yunis *et al.,* 1980). The overuse of chemical pesticides for crop protection causes development of fungicide resistance among the pathogens, and pollution of ground water and foodstuff. The utilization of potential microflora may help to develop an ecofriendly control strategy for disease management. The use of PGPR as an inducer of systemic resistance against different pathogens has been demonstrated under greenhouse and field conditions in crop plants such as cucumber (Press *et al.,* 2001); bean (Ongena *et al.,* 2004); tomato (Jayaraj *et al.,* 2007) and banana (Kavino *et al.,* 2007). Several bacterial strains belonging to the *Pseudomonas spp.* displaying biocontrol activities against fungal pathogens have already been isolated from rhizosphere of sugar beet (Bargabus *et al.,*2004); *Arabidopsis* (Wang *et al.,* 2005); green pepper (HaiMing *et al.,* 2007); banana (Ayyadurai *et al.,* 2006); rice (Choudhury and Kabi, 2006).

Fluorescent pseudomonas are non-pathogenic rhizobacteria and well known to colonize plant roots, promote plant growth, induce systemic resistance and suppress phytopathogens through production of antibiotics, siderophores, chitinase and HCN (Anitha and Rajendran, 2005; Egamberdieva *et al.,* 2008; Dutta *et al.,* 2008). *P. putida* WCS358r strains genetically engineered to produce phenazine and 2,4-diacetyl-

phloroglucinol (2,4-DAPG) displayed improved capacities to suppress plant diseases in field-grown wheat (Glandorf *et al.*, 2001). Systemic resistance is a mechanism operates through the activation of multiple defense compounds at sites distant from the point of pathogen attack (Dean and Kuc, 1985). The inducers include pathogens (Hammerschmidt, 1999), plant growth-promoting rhizobacteria (PGPR) (Vivekananthan *et al.,* 2004), and chemicals such as salycilic acid and acibenzolar-S-methyl (Michael *et al.,* 2001; YongHong *et al.,* 2008). Induced systemic resistance (ISR) involves production of oxidative enzymes such as peroxidase (PO) and polyphenol oxidase (PPO), which catalyze the formation of lignin, and other oxidative phenols that contribute to the formation of defense barriers for reinforcing the cell structure ( Meziane *et al.,* 2005; Jetiyanon, 2007). Also enzymes such as phenylalanine ammonia-lyase (PAL) which is a key enzyme concerned with the synthesis of salicylic acid and phenolic compounds which were proposed to reduce incidence of plant disease through antifungal activity and stimulation of plant defense responses against pathogens in several plants (Binutu and Cordell, 2000; Lavania *et al*., 2006). In addition, lytic enzymes like chitinases and β-1,4 glucosidases which act upon the fungal cell wall resulting in degradation and loss of inner contents of cells (Heil and Bostock, 2002; Kavino *et al.,* 2007).

The goals of this study are to evaluate the most promising *Pseudomonas* isolate for their effectiveness in controlling leaf spot and bacterial speck diseases in tomato, this including the application of selected isolates either individually or in mixture. Additionally, to evaluate the activity of some defensive enzymes that known to be involved in inducing systemic resistance.

## MATERIAL AND METHODS:
### Plant materials:
Tomato seedlings (*Lycopersicon esculentum* Mill. Cv. Castlerock) of 30 days old were kindly provided from Agriculture Research Center, Egypt. Pathogens isolates *A. solani, P. syringae* were previously isolated from infected tomato plants showing leaf spot and bacterial speck symptoms, respectively (Farahat, 2009).

### Bacterial strains and inoculums preparation:
The two PGPR, *P. putida* isolate MG4 and *P. fluorescens* isolate MG18 that previously isolated from roots of healthy tomato plants collected from different agriculture areas in Egypt showed significant antagonistic activity against fungal and bacterial plant pathogens (Farahat, 2009) were used in this study. Bacteria were grown on King's B plates for 24 h at 28°C and the cells were harvested by centrifuging at 10,000 g.

The cells were re -suspended in 10 mM $MgSO_4$ and adjusted to $10^{10}$ $CFU^*$ $ml^{-1}$.

Inoculum of *A. solani* was prepared according to Beshir, 1990; concerning that suspension of mycelial fragments was adjusted to $10^5$ $CFU^*$ $ml^{-1}$. Inoculum of *P. syringae was prepared* according to Romeiro, 2001; concerning that the cell suspension at $OD_{540} = 0.1$.

### Effectiveness of PGPR for controlling leaf spot and bacterial speck diseases:
### Application of PGPR to tomato plants:
The roots of 30 days old tomato seedlings were washed several times with sterile distilled water and dipped in cell suspension of *P. putida,* or *P. fluorescens* individually or in a mixture for one min. Samples with roots dipped in sterile distilled water were used as controls. Plastic pots (20 cm diameter x 15 cm height) were used for planting these seedlings. Each pot contained 2 kg of a sterilized mixture of clay and sand (1:1 w/w) provided from Agricultural Research Center, Cairo, Egypt. Each pot was transplanted with one seedling. Each treatment was run with ten replicates. The experiment lasted for 7 days before pathogens application. Pots kept in green house and irrigated with sterilized distilled water after 24 hrs of planting. The experiment was carried out during the summer season from June to August of two successive growth seasons.

### Foliar application of pathogens:
Suspension of *A. solani* (prepared as previously mentioned) was applied to tomato plant leaves one week after transplanting by spraying tomato leaves using hand automizer according to Schilder and Bergstrom (1990). The infected plants were covered with polyethylene bags for 48 hours to provide enough moisture for conidiae germination; control plants were sprayed with sterile water. Disease assessment was carried out 7- days after inoculation; according to zero - four scale; 0 = No infection, 1 = 20% infection, 2 = 20-40% infection, 3 = 40-60% infection, 4 = 60-80% infection

Disease severity index (DSI) = (Σ n x v  x 100) / (N x S),

where: n = numerical value of each category, v = number of leaves in each category, S = the highest number in the scale, N = total number of leaves in the sample.

One week after transplanting and bioagent treatment, another set of plants was challenged with cell suspension of *P. syringae* following the procedure described by Romeiro (2001). Disease severity was evaluated visually when the typical symptoms became evident and scored using a disease index with a range of 0 to 3 (0 signifies a healthy-looking plant; 1 signifies 2 to 5 specks together or spread over each leaf; 2 signifies 6 to 10

specks; and 3 signifies more than 10 specks), as described by Yunis *et al.,* (1980).

## Evaluation of pathogenecity related enzymes and phenolic:

Thirty days old seedlings were bacterized and planted as previously mentioned. The cultivated pots were divided into three sets. The first set was used to evaluated pathogenecity related enzymes (PAL, PO, PPO and chitinase) and phenolic compounds daily for 7 days. The second set was challenged with *A. solani,* 7 days after bacterization. The third set was challenged with *P. syringae,* 7 days after bacterization. Pathogenecity related enzymes and phenolics were evaluated in the second and the third sets three days after challenge with the mentioned pathogens.

## Preparation of plant extract:

Tomato leaves of 3-5 cm in length were collected and stored at − 80°C until plant extracts were prepared, following method of Lanna *et al.* (1996). Three replicates per treatment were used for each enzyme analyzed. Leaf tissue was ground in a mortar using liquid nitrogen, the resulting powder was macerated for 30 s in 3mL of 50 mM sodium phosphate buffer, pH 6.5, containing 1% polyvinylpyrrolidone (w/v) and 1mM phenylmethylsulfonyl (PMSF), and then centrifuged at 20,000g for 25 min at 4°C. The supernatants kept at 4°C and used for determination of enzyme activities.

## Determination of enzymes activity and phenolics:

PAL (EC 4.3.1.5) activity was determined by the direct spectrophotometric method described by Pascholati *et al.* (1986) PAL activities were determined from a standard curve of cinammic acid vs. absorbance (290 nm) and expressed as nmol cinammic acid $min^{-1}$ $g^{-1}$ fresh wt. PO (EC 1.11.1.7) activity was determined at 30°C by a direct spectrophotometric method (Hammerschmidt *et al.,* 1982). The enzyme activity expressed as change in the absorbance of the reaction mixture $min^{-1}$ $g^{-1}$ of fresh weight. PPO (EC 1.14.18.1) activity was determined at 30°C by a direct spectrophotometric method. The enzyme activity expressed as change in the absorbance of the reaction mixture $min^{-1}$ $g^{-1}$ of fresh weight (Mayer *et al.,* 1965). Chitinase activity assayed by a colorimetric method using colloidal chitin as a substrate (Reissig *et al.* 1955). One unit of chitinase activity was defined as the amount of enzyme that released 1 µmol N-acetylglucosamine (GlcNAc). $min^{-1}$ .$g^{-1}$ of fresh weight. Phenolic compounds was determined as described by Swain and Hillis (1959) and expressed as µg phenol. $g^{-1}$ of fresh weight.

## Statistical analysis:

The experiment followed a completely randomized design. The measured data were subjected to the analysis of variance (ANOVA). The significant differences between treatments were compared at 5% level of probability by the Duncan's test using SPSS.

## RESULTS:

Figure 1 presents the effect of different treatments of tomato roots with PGPR either individually or in a mixture on leaf spot disease incidence after infection with *A. solani.* The disease incidence was 70 % in case of non- bacterized plants (control), while it is reduced to about 30 % by treatment with *P. putida* and to about 15 % by treatment with *P. fluorescens* or mixture of both.
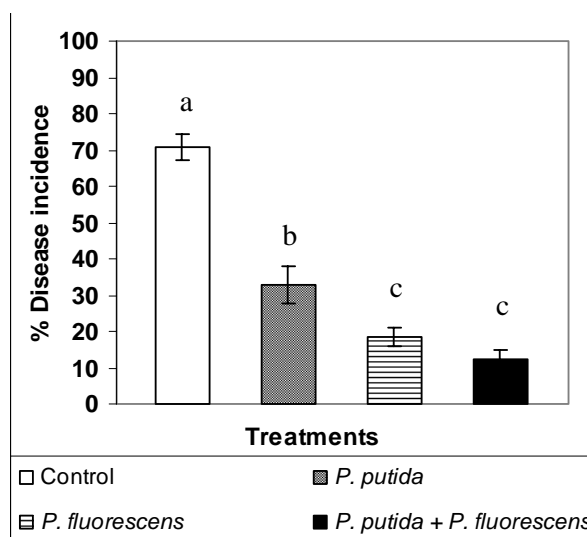


Fig. 1.   Effect of different treatments of tomato roots with PGPR either individually or in a mixture on leaf spot disease incidence. Bars represent ± SD.

Figure 2 presents the effect of different treatments of tomato roots with *P. putida* or *P. fluorescens,* separately or in a mixture on bacterial speck disease index. The disease index was about three in case of non-bacterized plants (control). Treatment with *P. fluorescens* or mixture of both bacteria decreases the disease index to about one.
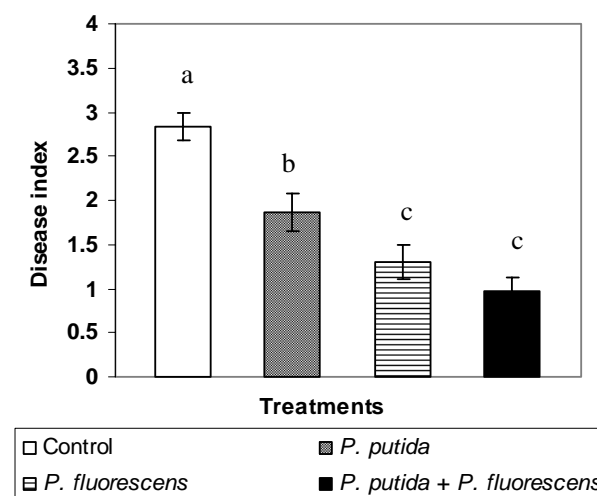


Fig. 2.   Effect of different treatments of tomato roots with PGPR either individually or in a mixture on bacterial speck disease index.  Bars represent ± SD.

As shown from figure 3A-D the control samples (non-bacterized) in all cases were more or less constant values. The activities of all enzymes extracted from tomato leaves (PAL, PO, PPO and chitinase) were increased in response to treatments with *P. putida* or *P. fluorescens* individually or in a mixture as compared with control.

Figure 3A shows that PAL activity increased by about three folds, after three days when treated with *P. putida*. Treatment with *P. fluorescens* or mixture of both (PGPR) showed further increase in the activity especially after two to four days. Figure 3B shows that the activity of PO was doubled by treatment with the mixture of PGPR; no remarkable difference was observed by other treatment. Maximum activity of PPO observed in two days after treatment with *P. putida*; and in 4 days after treatment with *P. fluorescens*. The mixture of both shows maximum activity (about two folds) in three days (Fig. 3C). Figure 3D shows that treatment with *P. fluorescens* or mixture of both (PGPR) increased chitinase activity as compared with that treated with *P. putida* especially after four days.
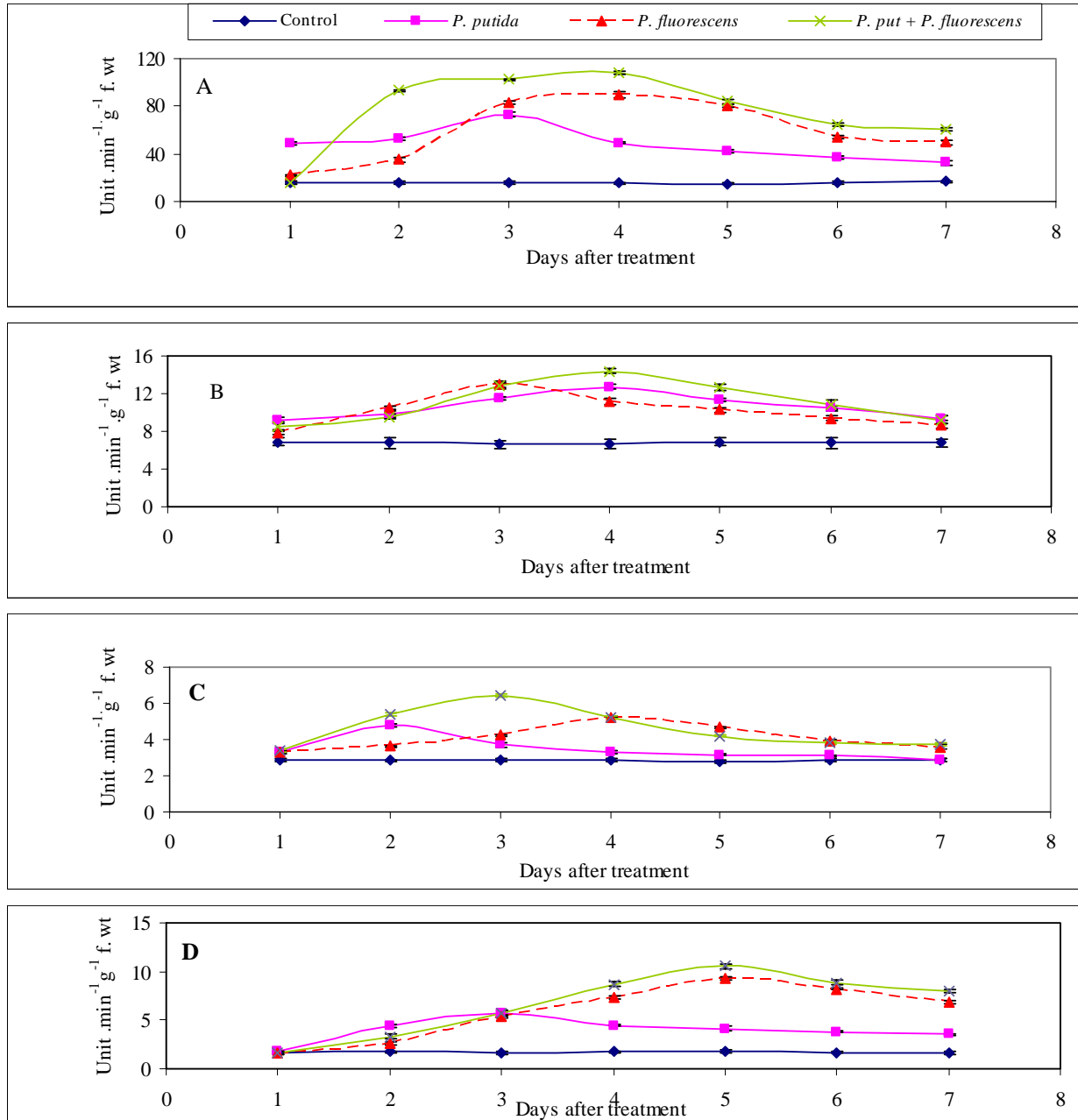


Fig. 3. Evaluation of PAL (A), PO (B), PPO (C) and chitinase (D) in tomato leaves after root treatment with PGPR either individually or in a mixture. Bars represent + SD.

Phenolic content increased in response to treatment with *P. putida* or *P. fluorescens* individually or in a mixture. The maximum increase was in case of treatment with mixture of bacteria after three days of bacterization. This increase amounted to four folds compared with control (Fig. 4).
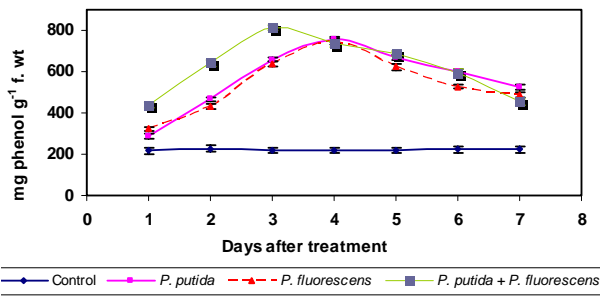
Fig.4. Evaluation of phenolics content of tomato leaves in plants treated with PGPR either individually or in a mixture. Bars represent ± SD

Figure 5A-D shows the activity of different enzymes (PAL, PO, PPO and chitinase) of the different treatments. In general, inoculation with both pathogens (*A. solani* and *P. syringae*) increased the activity of the previously mentioned enzymes as compared with their corresponding controls (non- pathogenized). In case of PAL (Fig. 5A) inoculation with *A. solani* was more effective in increasing the enzyme activity than the inoculation of the other pathogen, *P. syringae.* Using the mixture of the two PGPR and *A. solani* induced highest activity of PAL as compared with samples treated with separate strains. The activity of PO showed maximum value when the plants were treated with mixture of *P. putida* and *P. fluorescens* and challenged with *P. syringae* (Fig. 5B,)*.* As in case of PAL, the activity of PPO (Fig. 5C) and chitinase (Fig. 5D) the infection with *A. solani* in bacterized plant with *P. putida* or *P. fluorescens* individually or in a mixture were higher as compared with the corresponding samples with the other pathogen *P. syringae*. It is clear that, phenolic content of tomato plants challenged with *A. solani* and bacterized with *P. putida* or *P. fluorescens* individually or in mixture, were higher than the phenolic contents of the corresponding samples challenged with *P. syringae* (Fig. 6).
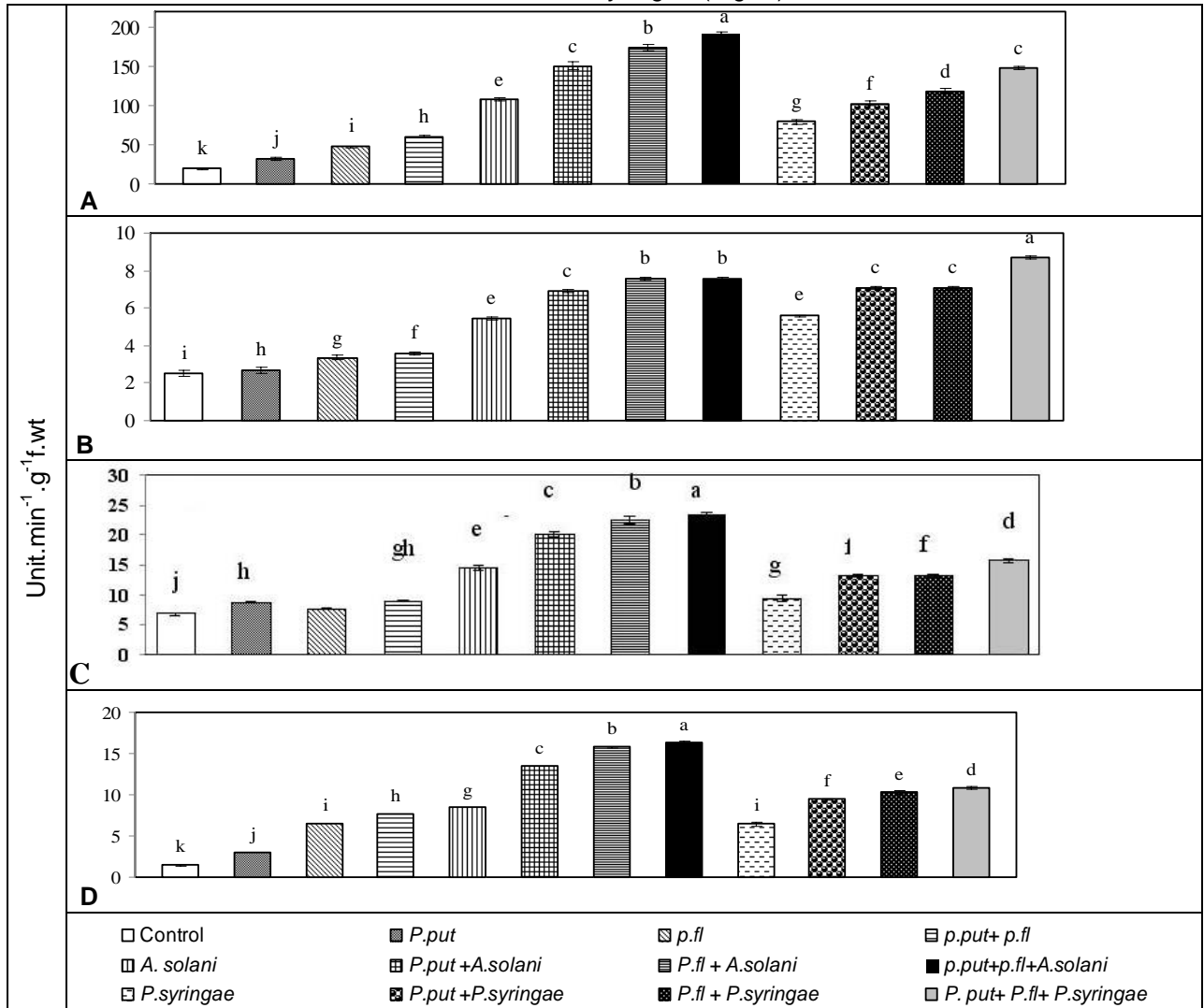


Fig. 5. Evaluation of PAL (A), PO (B), PPO  (C) and chitinase (D) activities in tomato leaves infected with with *P. syringae* or *A. solani fter* root treatment with  *P. putida* and *P. fluorescens* either individually or in a mixture. Columns headed by the same letter are not significantly according to Duncan's multiple range test (p<0.05). Bars represent ± SD.
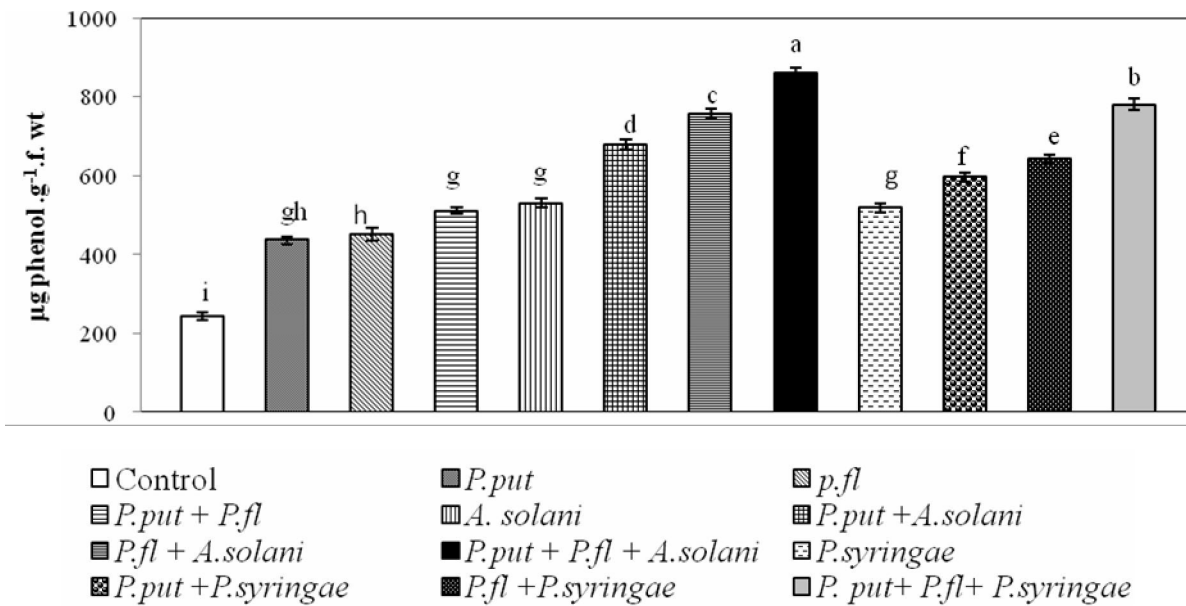
Fig. 6. Evaluation of phenolic content in tomato leaves infected *with P.syringae* or *A. solani fter* root treatment with *P. putida* and *P. fluorescens* either individually or in a mixture. Columns headed by the same letter are not significantly according to Duncan's multiple range test (p<0.05).     Bars represent ± SD.

## DISCUSSION:

PGPR play a vital role in management of various fungal and bacterial diseases.The results obtained from greenhouse experiment demonstrated significant suppression of leaf spot and bacterial speck diseases in tomato plants previously treated with the two selected isolates either individually or in mixture. For leaf spot disease, the mixture of *P. putida* and *P. fluorescens* reduced disease severity to 15% whereas it was 70% in case of control. Application of mixture of *P. putida* and *P. fluorescens* caused the highest significantly suppression of both disease compared to control. Similarly, Bashan and de-Bashan (2002) reported significant protection against bacterial speck disease in tomato after application of *Azospirillum brasilense*. Silva *et al.* (2004) evaluated five rhizobacterial strains for biological control of multiple pathogens causing foliar diseases in tomato plants including *P. syringae* and *A. solani* and observed reduced disease intensity in plants microbiolized with rhizobacteria. Wilson *et al.* (2002) reported that the non-pathogenic bacteria *P. syringae* strains TLP2 and Cit7, *P. fluorescens* strain A506, and *P. syringae* pv. *tomato* DC3000 *hrp* mutants found to reduce foliar bacterial speck disease severity in tomato. The current results proved that, bacterization of tomato by mixture of *P. putida* and *P. fluorescens* gave the highest disease suppression compared to single treatments as reported in previous studies by Meziane *et al.*, 2005. The main mode of action includes combining biological control agents with antagonistic properties with that those induce systemic resistance (Bargabus *et al.*, 2004) in addition to the production of siderophores

which contributed to suppression of pathogens (Mathiyazhagan *et al.,* 2004). In this study, treatment of tomato roots with rhizobacteria induced significant protection against the phylospheric pathogens *A.solani* and *P.syringae* the causal agents of leaf spot and bacterial speck diseases, respectively that support the suggestion of systemic resistance and excluding the possibility of direct antagonism because of the spatial separation between rhizobacteria in the rhizosphere and pathogens in the phyloplane. Application of PGPR induced increase in the activity of defense enzymes such as PAL, PO, PPO and chitinase over the control (Fig. 3). The mixture of the two-used PGPR was more effective than each of them when used separately. It is clear that these bacteria stimulated the activity of the defensive enzymes when applied individually or in a mixture. In all cases, the mixture induced higher activity as compared with the separate treatments. Figure 4 shows that the phenolic contents of tomato leaves of bacterized plants were higher than that of control. When the mixture of bacteria was applied, the phenolic content of leaves was higher than the separate samples. Comparison between the degree of infection by the two pathogens and the corresponding activity of the defensive enzymes revealed a reverse relationship i.e. when the enzyme activity is high the degree of infection is low. This observation was noticed in all tested enzyme (PAL, PO, PPO and chitinase) as well as the phenolic content. The activity in case of the mixture of PGPR coincides with the minimum degree of infection. Such decrease in the degree of infection of the two pathogens may be attributed to the companied influence of the

previously mentioned defensive enzymes. The enzyme PAL, is a key enzyme for the production of phenolics which inhibit the fungal growth. Transcinnamic acid, which is the product of PAL, is an immediate precursor for the biosynthesis of salicylic acid, a signal molecule in systemic acquired resistance (Klessig and Malamy, 1994; Daayf *et al.*, 1997). The present results are in agreement with those reported by Kumar *et al.* (2007) who found that *P. fluorescens* Pf4-99 induced maximum increase in PAL activity on 4th day after challenge inoculation with *Macrophomina phaseolina* the causal agent of dry root rot of chickpea. PO, is a key enzyme in biosynthesis of lignin and other oxidative phenols. In addition, PO acts as a modulator of active oxygen species, which may play various roles directly or indirectly in reducing pathogen viability and spread (Lamb and Dixon, 1997; van Loon *et al.*, 1998; Saikia *et al.*, 2006). In this context, it is noteworthy to mention that PO activity increased when using biocontrol of *Rhizoctonia solani* AG-4 with *P. aureofaciens* in soyabean (WooJin *et al.*, 2007), and with *P. aeruginosa* in rice (Saikia *et al.* 2006). The enzyme PPO, is effective in systemic resistance by catalyzing the formation of lignin and other oxidative phenols, contributes to the formation of defense barriers for reinforcing the cell structure (Audenaert *et al.,* 2002; Bull *et al.,* 2002; Meziane *et al.,* 2005). Similarly, (Ramamoorthy *et al.*, 2002) reported the increase of activity of PPO in biocontrol of Pythium disease by *P. fluorescens* on tomato and hot pepper.       Pathogenesis-related

proteins (PR) such as chitinase are host-coded proteins with direct action against the major fungal cell wall compounds, chitin, thereby they could reduce pathogen viability. This high chitinase activity might have resulted in lyses of invading fungal pathogens. Chitinase activity reported in this study agree with those reported by Bharathi *et al.* (2004) who found multifold increase in induced chitinase by mixed bioformulation of *P. fluorescens* (Pf1), *Bacillus subtilis*, neem and chitin that reduced the fruit rot incidence of chillies caused by *Colletotrichum capsici*. The phenolic compounds contribute to enhance the mechanical strength of host cell wall and inhibit the fungal growth; it is acting as fungitoxic. In this context, Nandakumar *et al.* (2001) found that, a mixture of PGPR acted as biocontrol of sheath blight in rice. Application of *Pseudomonas chlororaphis (PA-23)* on canola plants challenged with the ascospores of *S. sclerotiorum* triggered increased levels of hydrolytic enzymes including chitinase (Fernando *et al.*, 2007).

Thus, it is concluded that application of a mixture of *P. fluorescens* and *P. putida* could be promising approach for biological control of the two tomato pathogens, *A. solani* or *P. syringae*, which plays an important role in sustainable agriculture. The suppression of symptoms of the fungal pathogen *A. solani* and the bacterial pathogen *P. syringae* in plants that treated with rhizobacteria confirm the condition of non-specific protection proposed by Schoonbeck (2001) as a criterion of induced systemic resistance.

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## إستحثاث المقاومة الجهازية ضد أمراض الطماطم بواسطة البكتيريا الجذرمحيطية المنشطة للنمو

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إن الاســتخدام المفـرط للمبيـدات الكيميائيـة فـي المجـال الزراعـي يسـبب مشـاكل بيئيـة جسـيمة حيـث أن العديد من هذه المركبات لها سـمية شـديدة و يـؤدي إلـى أخطـار بالغـة للإنسـان و الكائنـات الغيـر مسـتهدفة بمـا فيهـا الأعداء الطبيعية لمسـببات الأمراض النباتية، كما يؤدي إلـى تلوث المياه الجوفية و كذلك المواد الغذائية مما يتسـبب في كثيـر مـن الأمـراض السـرطانية. و لـذلك اتجـه التفكيـر فـي السـنوات الأخيـرة إلـى البحـث عـن طـرق بديلـة أكثـر أمانـا لمكافحـة الآفـات النباتيـة و مـن أهـم هـذه الطـرق هو ما يعرف بالمقاومـة الحيويـة باسـتخدام كائنـات دقيقـة غيـر ممرضـة ومتواجـدة طبيعيـا فـي التربـة. و تعتيـر البكتيـريا الجـذر محيطيـة مـن أكثـر الكائنـات نشـاطا فـي تثبيـط الأمـراض النباتيـة حيـث أنهـا تسـتوطن الجـذور و تقـاوم مسـببات الأمـراض النباتيـة بطـرق مباشـرة- عـن طريـق إنتـاج المضـادات الحيويـة و غيرهـا من مركبـات الأيـض الثانويـة – و بطـرق غيـر مباشـرة مـن خـلال إسـتحثاث المقاومـة الجهازيـة للنباتـات عـن طريـق تنـشيط وسـائل الدفـاع الكامنـة للنبـات نفسـه و جعلـه أكثـر مقاومة. وتم فى هذا البحث عزل البكتيريا الجذرمحيطية مـن جنـس ســودوموناس (*Pseudomonas* *spp.*) واختـار قـدرتها التضـادية ضـد مسـببات الأمراض النباتيـة ، و مـن بـين هـذة العزلات تم اختيار أكثر عزلتين كفاءة في النـشاط التضادي وسـميت MG4 و MG18 و تثبيط مرض النـدوة الـورقي الـذي يسـببه فطـر الترناريـا سـولاني (*Alternaria solani*) و مـرض التبقع البكتيري الذي تسببة بكتريا سودوموناس سيرينجي العزلات علـى إسـتحثاث المقاومـة الجهازيـة لنبات الطمـاطم. و قـد تـم تقيـيم كفـاءة العـزلتين المختـارتين فـرادى أو مجتمعتـين علـى إسـتحثاث المقاومـة الجهازيـة لنبـات الطمـاطم و ذلك بمتابعة التغيرات الناتجة عن معاملـة نبات الطمـاطم بالبكتيريا الجذرمحيطية مثل تقدير كميـة المـواد الفينوليـة. كمـا تـم تقدير التغيـرات فـي إنزيمـات الـدفاع والمحتـوى الفينولي في نبات الطماطم بعد تعريض النبـات لكـل مـن الترناريـا سـولاني و سـودوموناس سـيرينجي الممرضـين. و أوضحـت النتائـج أن معاملـة نبـات الطمـاطم بواسطة سـودوموناس بوتيـدا و سـودوموناس فلورسـينس أدى إلـى حـدوث زيـادات كبيـرة في إنزيمـات الـدفاع مثـل الفينيل الانين امونيا ليبز و البيروكسيديز والبولي فينـول اوكسـيديز و الكتينيـز و أيضا أدى إلى زيادة المواد الفينولية في نبـات الطمـاطم إذ مـا قورنـت بالنباتـات الغيـر معاملـة. وأظهــرت النتائـج أن كـل النباتـات المعاملـة بالبكتيريـا (*Pseudomonas syringae*) ، و كـذلك تقيـيم قـدرة هـذه الجـذر محيطيـة أظهـرت نشـاط كبيـر لإنزيمـات الدفاع و المـواد الفينوليـة عندمـا تعرضـت لمسـببات الأمـراض إذ مـا قورنـت بالنباتات الغير معاملة.

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