Mycobiology 35(3): 129-134 (2007)

Foliar Application of Plant Growth-Promoting Rhizobacteria Increases Antifungal Compounds in Pea (*Pisum sativum*) Against *Erysiphe pisi*

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(*Received March 23, 2007*)

Systemic effect of two plant growth-promoting rhizobacterial (PGPR) strains, viz., *Pseudomonas fluorescens* (*Pf4*) and *P. aeruginosa* (*Pag*), was evaluated on pea (*Pisum sativum*) against the powdery mildew pathogen *Erysiphe pisi*. Foliar spray of the two PGPR strains was done on specific nodal leaves of pea and conidial germination of *E. pisi* was observed on other nodal leaves, distal to the treated ones. Conidial germination was reduced on distant leaves and at the same time, specific as well as total phenolic compounds increased in the leaves distal to those applied with PGPR strains, thereby indicating a positive correlation. The strains induced accumulation of phenolic compounds in pea leaves and the amount increased when such leaves were get inoculated with *E. pisi* conidia. Between the two strains, *Pag* was found to be more effective than *Pf4* as its effect was more persistent in pea leaves. Foliar application of PGPR strains for the control of powdery mildew of pea is demonstrated *in vitro* while correlating it with the increased accumulation of plant phenolics.

KEYWORDS: ***Erysiphe pisi*, Foliar spray, Induced resistance, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens***

Powdery mildew of pea (*Pisum sativum*) is incited by *Erysiphe pisi* and causes infection in all aerial green parts of the plant. Plant growth-promoting rhizobacteria (PGPR) are known to induce systemic resistance (ISR) in plants and restrict establishment of infection by the pathogens in the host (van Peer *et al.*, 1991; Wei *et al.*, 1991). *Pseudomonas* spp. are known to protect plants from pathogens through various mechanisms, viz., induced systemic resistance in the host (Maurhofer *et al.*, 1994; van Peer *et al.*, 1991), antibiotic production (Thomashaw and Weller, 1988; Maurhofer *et al.*, 1995), growth promotion (Schippers *et al.*, 1987) and competition for nutrients (Duijff *et al.*, 1993; Leeman *et al.*, 1996).

Phenolic compounds are natural constituents of all plants and antibiotic phenols have been implicated in plant defense mechanisms (Elgersma and Liem, 1989; Kuc, 1995; Nicholson and Hammerschmidt, 1992; Baker *et al.*, 2005). Among them, some occur constitutively and function as preformed inhibitors associated with non-host resistance (Millar and Higgins, 1970; Stoessl, 1983), while others are formed in response to pathogen ingress as part of an active defense response (Nicholson and Hammerschmidt, 1992). Matta *et al.* (1988) noted the activation of phenol metabolism in xylem vessels of tomato during pathogen attack. This observation was late supported by the results of Cooper *et al.* (1996) who found two phenolic compounds, a triterpenoid, and elemental sulfur in cells associated with vascular pathogens in resistance genotypes of *Theobroma cacao*. Accumulation of phenolic compounds in carnation by a *Pseudomonas* sp. and decrease in *Fusarium* wilt has been reported by van Peer *et al.* (1991). Similarly, successful protection of pea from *Erysiphe pisi* through foliar application of PGPR has been demonstrated by Singh *et al.* (2000). Looking into the antifungal activity of phenolic acids, the present study was conducted to investigate the systemic accumulation of phenolic compounds in pea plant parts following foliar application of two PGPR strains. Induction of resistance in the pea plants following PGPR application was assessed through inhibition of conidial germination of *E. pisi*.

Materials and Methods

Experimental set up. Ten pea seeds (var. Arkel) were sown in single plastic pots (20 cm diameter) containing sterilized sandy loam soil (pH 7.6) and allowed to grow in a glasshouse for 20 days. PGPR strains *Pseudomonas fluorescens* strain *Pf4* and *P. aeruginosa* strain *Pag* (Singh *et al.*, 2003) were grown on King's B (KB) agar (proteose peptone 20 g, $K_2HPO_4 \cdot 3H_2O$ 1.908 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, glycerol 15 *ml*, Bacto agar 15 g, distilled water 985 *ml*) medium for 48 h at 25 ± 2°C. Individual as well as mixed suspension of both the strains of approximately 10^8 cells per *ml* ($OD_{620\,nm}$ 0.8~0.9) in 0.1% carboxy methyl cellulose (CMC) was prepared separately. Five pots of pea plants comprised one set, and each set was sprayed with the bacterial suspensions with a hand atomizer separately. Another set of pea plants was inoculated with only *E. pisi* conidia by tapping severely infected pea leaves (Singh *et al.*, 2000a). For evaluation of the effect of PGPR in the

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presence of *E. pisi*, conidia of the pathogen were tapped on pea leaves after 24 h of foliar spray of the PGPR strains. A set of pea plants was left uninoculated (neither with PGPR nor with *E. pisi*) in a separate chamber in the glasshouse served as control. The above-mentioned treatments will be referred as T1 = Control; T2 = pea plants inoculated only with *E. pisi*; T3 = pea plants sprayed only with *Pf4* suspension; T4 = pea plants sprayed only with *Pag* suspension; T5 = pea plants sprayed with mixed suspension of *Pf4* + *Pag*; T6 = pea plants sprayed with suspension of *Pf4* followed by inoculation with *E. pisi* after 24 h; T7 = pea plants sprayed with suspension of *Pag* followed by inoculation with *E. pisi* after 24 h; T8 = pea plants sprayed with mixed suspension of *Pf4* and *Pag* followed by inoculation with *E. pisi* after 24 h.

Foliar spray of bacterial suspension. In a separate experiment, bacterial suspension of *Pf4*, prepared as above was sprayed with a hand atomizer on a set of 20-d-old pea plants. While spraying *Pf4* suspension on I and II nodal leaves, III and IV nodal leaves were protected by covering with polythene sheets. After 24 h *E. pisi* conidia were inoculated on III and IV nodal leaves by tapping over the pea plants as above. During conidial inoculation I and II nodal leaves were covered by polythene sheet. Similarly, a second set of pea plants was sprayed with *Pf4* suspension on III and IV nodal leaves while I and II nodal leaves were covered with polythene sheets. Conidia of *E. pisi* were inoculated on I and II nodal leaves as described earlier while III and IV nodal leaves were covered with polythene sheets. A third set of pea plants were treated only with *E. pisi* conidia and a fourth set of pea plants left un-inoculated (neither with PGPR nor *E. pisi*) in a separate chamber to serve as control. Percent conidial germination on pea leaves treated with *Pf4* and /or inoculated with *E. pisi* along with control were determined after 24 and 48 h of treatments (Singh *et al.*, 2000a). For estimation of phenolic compounds a fifth set of pea plants sprayed with *Pf4* suspension without inoculation of *E. pisi* were left as such.

The conidia of the pea powdery mildew were applied in such as way that each leaf received 200~300 conidia per mm^2. At least five leaves per treatment were excised and processed by the method of Carver and Adaigbe (1990). The leaves were placed on a pad of filter paper with adaxial side up containing fixative. Leaves were fixed for 48 h to remove chlorophyll completely and then placed on lacto-phenol cotton blue for 24 h. The germination of conidia were recorded at different time intervals after inoculation by counting 100 conidia per leaf using light microscope.

Extraction of phenolic compounds. Randomly selected 6 plants from 3 pots of a single treatment of the different treatments as described above were harvested and pooled together to make one sample each of leaves, collars and roots to extract phenolic compounds after 24, 48 and 96 h of treatments. At least three samples were prepared from each treatment. Phenolic compounds were extracted from leaves, stems and roots according to Sarma *et al.* (2002).

For the estimation of total phenolics, 1.0 g fresh plant part was extracted separately in 50% aqueous methanol thrice. The supernatant was evaporated to dryness and finally dissolved in 1.0 *ml* distilled water and samples prepared in triplicate were analyzed spectrophotometrically using Prussian blue method as modified by Graham (1992). Absorbance of the colour was recorded at 700 nm using an UV-VIS spectrophotometer (Bausch and Lomb, USA). Standard curve of gallic acid was prepared and TPC was calculated in terms of gallic acid equivalents. Analytical grade reagents were used throughout the experiment.

HPLC analysis of phenolic acids. Quantitative analysis of the samples was performed as per the method of Singh *et al.* (2002). The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 AT*VP* reciprocating pumps, a variable Shimadzu SPD-10 A*VP* UV-VIS detector and a Rheodyne Model 7725 injector with a loop size of 20 μl. Peak area was calculated with Winchrom integrator. Reverse phase chromatographic analysis was carried out in isocratic conditions using C-18 reverse phase column (250 × 4.6 mm id, particle size 5 μm Luna 5 μ C-18 (2), Phenomenex, USA) at 25°C. Running conditions included injection volume 5 μl, mobile phase methanol: 0.4% acetic acid (80 : 20 v/v), flow rate 1 *ml*/min, and detection at 290 nm. Samples were filtered through membrane filter (pore size 0.45 μm, E-Merck, Germany) prior to injection in sample loop. Tannic, gallic, vanillic, caffeic, ferulic, o-coumaric, chlorogenic, cinnamic and synapic acids were used as internal and external standards. Phenolic acids present in the samples were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards.

Statistical analysis. Each experiment was conducted thrice and the data were subjected to statistical analysis using the software ORIGIN 5.0. In all the treatments, the data were subjected to ANOVA and results were subjected for statistical significance by Student's *t* test at $p \leq 0.05$.

Results

Foliar application of the two PGPR strains resulted in the induction of individual phenolic acids as well as total phenolics in different parts of pea. Based on retention time as

Table 1. Effect of foliar spray of plant growth-promoting rhizobacteria on the induction of phenolic compounds in pea (*Pisum sativum*) after 48 and 96 h

Treatments	Phenolic acids (μg/g fresh wt.)								
	Gallic			Cinnamic			Ferulic		
	Root	Stem	Leaves	Root	Stem	Leaves	Root	Stem	Leaves
					48 h				
T1 (Control)	5.4	5.5	30.5	1.4	0.2	0.3	0.3	0.0	0.1
T2 (*E. pisi*)	44.7	0.3	84.2	0.5	0.1	0.1	1.6	2.0	1.1
T3 (Pf4)	16.9	18.7	65.3	5.8	2.1	1.1	0.0	1.4	2.1
T4 (Pag)	20.4	22.6	172.5	1.6	0.4	0.1	3.4	0.8	0.0
T5 (Pf4 + Pag)	32.8	14.5	113.3	1.5	0.2	0.1	5.4	3.3	1.5
T6 (Pf4 + *E. pisi*)	18.7	5.6	40.9	0.4	0.1	2.0	4.4	4.4	2.0
T7 (Pag + *E. pisi*)	42.2	12.5	77.4	1.2	0.1	0.1	1.5	0.0	1.1
T8 (Pf4 + Pag + *E. pisi*)	59.2	16.4	65.9	2.5	0.3	0.1	7.1	2.4	2.0
					96 h				
T1 (Control)	56.3	14.8	97.5	0.2	0.1	0.1	0.0	0.0	0.0
T2 (*E. pisi*)	64.7	12.9	25.5	0.5	0.1	0.4	1.7	1.3	0.0
T3 (Pf4)	30.5	12.3	43.1	0.3	0.1	1.8	7.1	1.6	1.9
T4 (Pag)	62.5	2.9	81.5	0.7	1.0	0.1	9.9	0.0	1.0
T5 (Pf4 + Pag)	60.2	13.7	83.4	0.2	0.1	0.1	6.7	1.9	1.8
T6 (Pf4 + *E. pisi*)	65.4	14.2	36.6	0.5	0.1	0.2	4.7	2.7	1.7
T7 (Pag + *E. pisi*)	131.8	21.5	155.2	0.2	0.1	0.1	0.0	0.0	1.6
T8 (Pf4 + Pag + *E. pisi*)	56.0	10.16	86.7	0.2	0.1	0.1	2.1	2.2	1.5

well as co-injection with standards, five phenolic compounds, viz., gallic (Rt 2.92 min), caffeic (Rt 3.18 min), vanillic (Rt 3.32 min), ferulic (Rt 3.56 min) and cinnamic (Rt 4.45 min) acids were identified. Among phenolic acids, gallic and cinnamic acids were consistently detected in varied amounts in all the parts of pea plants in all treatments including control. Maximum accumulation of gallic acid was observed in leaves that had received foliar application of *Pag* at 48 h while at 96 h its maximum accumulation was observed in T7 (*Pag* + *E. pisi*). The amount of gallic acid was maximum in leaves at 48 and 96 h but it gradually increased in roots as well in other treatments at 96 h. (Table 1). In contrast to gallic acid, maximum accumulation of cinnamic acid was observed in roots at 48 h (5.1 μg/g fresh wt.) in *Pf4* (T3) treated plants. Except T6, the amount of cinnamic acid was higher in roots as compared to stem and leaves at 48 h (Table 1). At 96 h maximum amount of cinnamic acid was observed in leaves of *Pf4* (T3) treated plants (1.8 μg/g fresh wt.) while almost in all other treatments its content was higher in roots (Table 1).

Consistent occurrence of ferulic acid was noticed in different parts of pea. In leaves, ferulic acid was detected at 48 and 96 h in most of the treatments, being maximum in *Pf4* (T3) treated plants followed by *Pf4* and *E. pisi* (T6) treated plants (Table 1). In stem maximum ferulic acid accumulation was observed in *Pf4* and *E. pisi* (T6) treated plants after 48 and 96 h. In roots, maximum accumulation of ferulic acid was found in *Pf4* + *Pag* + *E. pisi* (T8) treated plants after 48 h but after 96 h, root of only *Pag* (T4) treated plants showed maximum accumulation (Table 1).

The amount of total phenolics (TP) varied in different parts of pea plants in different treatments. Maximum amount of TP was observed in leaves at 48 and 96 h in *Pf4* (T3) treated plants (73.5 and 57.6 mg/g fresh wt., respectively) (Table 2). In stem and roots, *Pag* (T4) treated plants showed maximum accumulation of TP after 48 h. However, stem and roots of plants treated with *Pag* + *E. pisi* (T7) accumulated maximum TP after 96 h (23.6 and 51.3 mg/g fresh wt., respectively) (Table 2).

Table 2. Total phenolic content in pea (*Pisum sativum*) after foliar spray with plant growth-promoting rhizobacteria

Treatment	Phenolic acids (μg/g fresh wt.)‡					
	Time of sampling (h)					
	48			96		
	Leaf	Stem	Root	Leaf	Stem	Root
T1	18.5^{a}	12.0^{a}	14.5^{a}	19.2^{a}	14.6^{a}	14.9^{a}
T2	35.0^{b}	15.5^{ab}	22.5^{bc}	27.5^{b}	16.0^{a}	19.0^{a}
T3	73.5^{c}	21.0^{c}	20.0^{abc}	57.6^{c}	13.9^{a}	17.5^{a}
T4	55.0^{d}	21.5^{c}	26.3^{c}	36.3^{d}	21.0^{b}	24.3^{b}
T5	37.5^{b}	18.0^{bc}	15.4^{a}	27.5^{b}	18.5^{ab}	18.4^{a}
T6	24.9^{c}	20.2^{bc}	15.6^{a}	32.6^{bd}	22.6^{b}	31.5^{c}
T7	29.8^{e}	20.6^{bc}	17.5^{ab}	52.5^{c}	23.6^{b}	51.3^{d}
T8	25.8^{e}	17.3^{abc}	26.2^{c}	37.6^{d}	17.9^{ab}	45.0^{c}
CD=	(6.04)	(5.86)	(5.78)	(5.45)	(6.13)	(5.53)

T1 = Control; T2 = *Erysiphe pisi*; T3 = *Pf4*; T4 = *Pag*; T5 = *Pf4* + *Pag*; T6 = *Pf4* + *E. pisi*; T7 = *Pag* + *E. pisi*; T8 = *Pf4* + *Pag* + *E. pisi*.

‡Column data superscript with similar letters vary significantly at $P \leq 0.05$ by Student-*t* test.

Table 3. Conidial germination of *Erysiphe pisi* on the leaves of 20 day old pea plants treated with rhizobacteria and inoculated with *E. pisi* after 24 h of inoculation

Treatment	Time of observation (hour)			
	% Conidial germination on III and IV nodal leaves with treatment on I and II nodal leaves		% Conidial germination on I and II nodal leaves with treatment on III and IV nodal leaves	
	24	48	24	48
T1	22	22	21	25
T2	8	6	17	13
T3	13	4	9	14

T1 = Control, T2 = *Pf4* treated plants, T3 = Whole plants sprayed with *Pf4* after 24 h of *Erysiphe pisi* inoculation; number of replications-three; 100 conidia were counted in each replication.

It is evident from Table 3 that germination of *E. pisi* conidia was severely affected in both the treatments as compared to control (conidial germination on healthy plants). Foliar application of *Pf4* on I and II nodal (lower) leaves of pea caused significant reduction in percent conidial germination on III and IV nodal (upper) leaves and vice-versa. In another set of conditions, where whole aerial part of the plants were sprayed with *Pf4* followed by *E. pisi* inoculation after 24 h, conidial germination reduced to several fold as compared to healthy plants (Table 3).

Tannic, gallic and ferulic acids were detected consistently in pea leaves treated with *Pf4* and/or inoculated with *E. pisi.* Treatment with *Pf4* on lower nodal leaves (I and II) followed by inoculation with *E. pisi* on upper leaves (T4) caused maximum accumulation of ferulic acid in upper (III and IV) and lower leaves (I and II) (3.8 and 17.9 μg/g fresh wt. after 24 h respectively). However, spraying with *Pf4* on the whole plants followed by inoculation with *E. pisi* (T5) caused maximum accumulation of ferulic acid in I and II (2.7 μg/g fresh wt.) and III and IV (20.8 μg/g fresh wt.) after 48 h (Table 4).

Table 4. Phenolic acid content in pea leaves after 24 and 48 h treatment with *Pseudomonas fluorescens* strain *Pf4* and *Erysiphe pisi*

Treatment	Phenolic acid content (μg/g fresh leaf tissues)					
	I and II nodal leaves			III and IV nodal leaves		
	TA	GA	FA	TA	GA	FA
	24 h					
T1	0.7	15.5	1.3	0.9	20.1	7.6
T2	0.6	15.1	1.0	0.7	23.6	4.8
T3	0.8	15.8	2.8	1.3	8.2	10.5
T4	1.1	6.8	3.8	2.5	20.5	17.9
T5	1.1	9.7	2.9	1.4	15.8	9.6
	48 h					
T1	1.6	5.8	1.0	3.8	6.6	17.8
T2	2.8	10.2	0.9	2.4	11.3	14.4
T3	1.7	4.8	1.6	4.2	10.9	10.7
T4	1.5	4.8	1.9	2.4	8.3	12.2
T5	1.9	9.5	2.7	3.9	17.1	20.8

T1 = Healthy, T2 = *Erysiphe pisi* inoculated, T3 = *Erysiphe pisi* inoculated I and II nodal leaves and *Pf4* treated III and IV nodal leaves, T4 = *Pf4* treated I, II nodal leaves and *Erysiphe pisi* inoculated III and IV nodal leaves, T5 = *Pf4* sprayed on whole plant followed by *E. pisi* inoculation after 24 h; TA-tannic, GA-gallic acid and FA-ferulic acid.

Discussion

Foliar application of PGPR strains namely *P. fluorescens* (Pf4) and *P. aeruginosa* (Pag) either alone or in different combinations, on different nodal leaves of pea plants, greatly affected the accumulation of specific as well as total phenolics systemically in leaves distal to the application as well as in other parts of pea plants. Observations in the present investigation that the induced distal accumulation of phenolic compounds, away from the site of application following foliar application of PGPR strains and subsequent reduction in conidial germination of the pathogen at those sites confirms systemic nature of the effect caused due to these rhizobacteria. It is interesting to note that whole plants when sprayed with PF4 following inoculation of *E. pisi* after 24 h (treatment T3 in Table 3 and T5 in Table 4) not only caused reduced conidial germination as compared to control, but also showed greater accumulation of antifungal phenolics namely ferulic and gallic acids. In general, among individual phenolic acids, gallic acid content was vary high as compared to the ferulic and cinnamic acids, indicating that these compounds are minor constituents of the phenolic cascade. Gallic acid accumulation was found to be maximum in leaves after 48 and 96 h of treatment. Cinnamic acid accumulation was not very consistent although it increased after 96 h in several treatments. Lesser content of ferulic acid in leaves after 96 h and increased accumulation in roots at the same time, in almost all treatments may result from the downward transmission of the compound from leaves to roots. However, further experiments are required to verify these data.

These results are further supported by several workers who have successfully demonstrated rhizobacteria-medi-

ated induction of phenolic compounds against phytopathogens. van Peer *et al.* (1991) showed that *Pseudomonas* sp. strain WCS417r protected carnation from *Fusarium oxysporum* f. sp. *dianthi* infection through induction of phenolic compounds. Similarly, Benhamou *et al.* (2000) recently demonstrated that phenolic compounds were key components of the structural resistance response induced by *Serratia plymuthica*, an endophytic bacteria, in cucumber plants against *Pythium ultimum*. Daaye *et al.* (2003) also showed that induction of phenolic acids in cucumber plants formed the basis of resistance of host against powdery mildew infection. Antifungal activity of ferulic acid was reported by several workers (Demyttenaere *et al.*, 1997; Sarma and Singh, 2003). Similarly, gallic acid not being antifungal, is converted into gallotannins, which along with other tannins, is also known to provide protection to the hosts from bacterial and fungal infections (Salisbury and Ross, 1986). Singh *et al.* (2000b) have shown successful control of powdery mildew of pea through the application of *P. fluorescens* (strains Pf1, Pf3, Pf5) and *P. aeruginosa* (Pag). Hence, the report of the induction of antifungal phenolic acids in pea in the present investigation following foliar application of the two PGPR strains provides a biochemical basis of resistance in pea against powdery mildew by PGPR. Reduction of conidial germination in both upper and lower leaves further shows that the systemic effect progresses in both upward and downward directions. However, involvement of more than one mechanism in inducing resistance in pea in the present investigation may not be ruled out.

Similarly, ferulic acid is highly antifungal and its higher accumulation in the plant is an important correlation with host resistance. The synthesis of ferulic acid does not always take place through the usual phenyl propanoid pathway as it is also reported to be synthesized in the host following pathogen ingress through some alternative pathway (Nicholson and Hammerschmidt, 1992). Its higher accumulation within 48 h in the present investigation suggests involvement of such a phenomenon. The amount of TPC at 96 h decreased after an initial rise at 48 h in the plants following treatments with either PGPR or *E. pisi* alone. But, interestingly, combined application of PGPR strains with *E. pisi* resulted into higher accumulation of TPC at 96 h than from their corresponding amounts at 48 h. Although activation of the phenyl propanoid pathway is evident following inoculation with only *E. pisi* conidia, corresponding higher amount of the same products as well as TPC in the host following co-inoculation of *E. pisi* with PGPR, further supports the efficacy of the PGPR strains in inducing synthesis of the phenolics in presence of *E. pisi*.

Both the PGPR strains used in this investigation are effective in inducing phenolic acids in the host applied either singly or in combination. However, the effect of *Pag* was found to be more persistent as it performed well in presence of *E. pisi* at 96 h. Survival of PGPR in the phylloplane of pea for a significant time period (Singh *et al.*, 2000b) and their ability to induce synthesis of defence-related compounds systemically in the host further strengthens their use as foliar spray to control powdery mildew of pea.

Acknowledgements

The Department of Science and Technology, New Delhi is gratefully acknowledged for financial support in this work.

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