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Differential Methods of Inoculation of Plant Growth-Promoting Rhizobacteria Induce Synthesis of Phenylalanine Ammonia-Lyase and Phenolic Compounds Differentially in Chickpea

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ABSTRACT. Foliar spray and micro-injection of plant growth-promoting rhizobacterial species, *viz. Pseudomonas fluorescens* and *P. aeruginosa* on chickpea induced synthesis of phenylalanine ammonia-lyase (PAL) when tested against *Sclerotinia sclerotiorum*. Induction of PAL was also associated with increased synthesis of phenolic compounds such as tannic, gallic, caffeic, chlorogenic and cinnamic acids. Treatment with *P. fluorescens* was found to be more effective in inducing phenolic compounds as compared to *P. aeruginosa*. However, persistence of PAL activity was observed more with *P. aeruginosa*. Although both the inoculation methods were effective, foliar application was found to be superior to micro-injection in terms of rapid PAL activity leading to the synthesis of phenolic compounds.

Abbreviations

HPLC	high performance liquid chromatography	*Pag*	*Pseudomonas aeruginosa*
IAA	3-indolylacetic acid	PAL	phenylalanine ammonia-lyase (EC 4.3.1.5)
ISR	induced systemic resistance	PDA	potato–dextrose agar
KB	King's B (agar)	PGPR	plant growth-promoting rhizobacteria
Pf4	*Pseudomonas fluorescens*	*Sst*	*Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (LIB.) DE BARY is an important pathogen distributed ubiquitously, attacking over 360 species of plants comprising 64 families (Purdy 1979). Diseases caused by *S. sclerotiorum* are difficult to control and may result in substantial yield losses. ISR occurs when the plant defense mechanisms are stimulated to resist infection by pathogens (Van Loon *et al*. 1998). PGPR-mediated ISR results in the reinforcement of plant cell wall by lignin, callose and phenolic compounds, alterations of the physiological and biochemical reactions of plant cells and production of antimicrobial substance, such as pathogenesis-related proteins and phytoalexins (Ramamoorthy *et al*. 2001). Currently, attempts to protect plants from pathogen attack through PGPR have gained worldwide attention because of their ecofriendly and sustainable nature in plant disease management.

Plants possess a wide array of defense responses to diseases by a combination of constitutive and inducible mechanisms. Several enzymes, such as peroxidases, lipooxidases, superoxide dismutases and PAL, are involved in the flow of carbon from primary to secondary metabolism in plants (Hahlbrock and Sheel 1989). These compounds play an important role in plants against fungal infection (Grey *et al*. 1997). PAL is the first committed enzyme in the phenylpropanoid pathway leading to the conversion of L-phenylalanine into cinnamic acid with the elimination of ammonia (Da Cunha 1987). Many plant-specific phenylpropanoid pathways and their corresponding functional diversity basically originate from the core phenylpropanoid metabolism initiated by PAL (Hahlbrock and Sheel 1989).

Early induction of PAL is more important as it is the first enzyme in the phenylpropanoid pathway, which leads to the production of phytoalexins and phenolic substances destined for lignin formation with the help of peroxidases. The presence of phenolic compounds in plants and their synthesis in response to infection is associated with resistance (Nicholson and Hammerschmidt 1992). Previous investigations reveal that the induction of phenolic compounds in PGPR-treated chickpea (*Cicer arietinum*) plants is increased when the plants are inoculated with *Sclerotium rolfsii* showing the role of PGPR-mediated induction of phenolic

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compounds in crop protection (Singh *et al*. 2003). However, the rapidity of induction of the phenylpropanoid metabolism by PGPR and their capacity to survive on leaf surfaces can function in a similar fashion to the endophytic association of the strains.

This study was conducted to investigate the PAL activity and status of phenolic compounds in chickpea after inoculation with two PGPR species individually as foliar spray and micro-injection. Their efficacy in imparting resistance to chickpea challenged with *S. sclerotiorum* was also investigated.

MATERIALS AND METHODS

Characterization of the PGPR species. Two strains of *Pseudomonas* sp., *viz*. *P. fluorescens* (*Pf4*) and *P. aeruginosa* (*Pag*), obtained from the *Center of Advanced Study in Botany, University of Madras*, Chennai (India) and *School of Biotechnology, Banaras Hindu University*, Varanasi (India), respectively, were screened for their plant growth promotion activity by assaying the following attributes: phosphate solubilization, and HCN, siderophore, and IAA production. The phosphate-solubilization test was done in Pikovskaya's medium (Pikovskaya 1948). The ability to produce HCN was assessed according to Bakker and Schipper (1987). Siderophore production was tested following the CAS plate assay (Schwyn and Neilands 1987). IAA production test was done according to Sarma *et al*. (2002).

Micro-injection and foliar spray with P. fluorescens *and* P. aeruginosa*.* Chickpea seeds (*Cicer arientinum* L., cv. Avrodhi) () were sown in earthen pots (diameter 100 mm) containing sterilized soil, 5 seeds per pot, in a glasshouse. Cultures of *Pf4* and *Pag* were grown on 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 1.5 %, distilled water 985 mL) medium at 25 ± 2 °C. Strains were maintained in 100-mL flasks containing 40 mL medium and after 2 d the medium was centrifuged (15 min); cell pellets obtained were suspended in sterile distilled water to give cell concentration of ≈100/nL (A_{620} 0.8–0.9). Bacterial suspensions (25 mL) of both strains were individually injected on the 3rd node of the 4-week-old chickpea plants raised in 3 pots each whereas control plants in 3 different pots were injected with sterilized distilled water only.

Similarly, the bacterial cultures grown on KB agar medium for 2 d at 25 ± 2 °C were scraped with a sterile spatula. A cell suspension (10^8 cells per mL) was made in 0.1 % carboxymethyl cellulose and sprayed on 4-week-old plants, in 3 pots with each strain. Control plants in 3 separate pots were sprayed with sterilized distilled water only.

Fungal material and inoculation method. *S. sclerotiorum* was isolated by picking up individual sclerotia formed on infected chickpea plants. Such sclerotia were surface-sterilized with 0.1 % $HgCl_2$ for a few seconds followed by 3 washings in sterilized distilled water. They were then singly placed on PDA (peeled potato 250 g, agar powder 15 g, dextrose 20 g, distilled water 1 L) medium in Petri dishes and incubated at 25 ± 2 °C. The cultures were purified on PDA slants from single sclerotia.

An isolate of *S. sclerotiorum* was grown on a PDA plate for 3 d. Mycelial suspension was prepared according to Hennin *et al.* (2002). Mycelial bits of 5 mm were cut from the plate and grown in PDA broth for 7 d. The liquid was suspended in water and mixed thoroughly for 1 min. The density of mycelial suspension was measured as A_{590}. This suspension was sprayed on chickpea plants grown in 3 pots after 3 h of micro-injection and foliar spray with *Pf4* and *Pag*. Similarly, mycelial suspension was sprayed on chickpea plants grown in 6 pots where 3 pots were micro-injected and 3 pots were sprayed with distilled water only.

The treatments used: H_2O (distilled), *Sst*, *Pag*, *Pag* + *Sst*, *Pf4*, *Pf4* + *Sst*.

PAL activity. The enzyme extract was prepared according to Havir *et al*. (1987) by extracting 0.5 g of fresh chickpea leaves in 4 mL of borate buffer (0.2 mol/L; pH 8.7) after 1, 2 and 3 d of application of mycelial suspension. Reaction mixture containing 1 mL enzyme extract, 0.5 or 2 mL borate buffer, and 0.2 mL 0.1 mol/L L-phenylalanine was incubated at 32 ± 1 °C for 30 min. The enzyme reaction was stopped by adding 0.5 mL of 1 mol/L trichloroacetic acid. The cinnamic acid formed was determined in a spectrophotometer by taking absorbance of the whole reaction mixture after incubation at room temperature; calculation was done with a calibration curve prepared by taking A_{290} of standard cinnamic acid. The whole experiment was repeated twice.

Extraction of phenolic acids from chickpea leaves. Fresh leaves from plants receiving individual treatment were collected randomly after 1, 2 and 3 d after foliar spray of mycelial suspension. A 1-g sample was taken out from each treatment and the leaves were macerated in a pestle-mortar followed by suspension of the fine crushed samples in 10 mL of ethanol–water (4 : 1, *V*/*V*). Samples were collected in screw-capped tubes and subjected to ultrasonication for 15 min. The clear greenish supernatant kept in glass tubes was mixed with charcoal for removal of pigments. After 3 h the clear white supernatant was filtered through *Whatman* no. 1 filter paper and collected into glass tubes. The whole extraction process was repeated 2× and

the extracts from the same treatment were pooled. The supernatant was evaporated *in vacuo*. Dried samples were resuspended in 1 mL methanol (HPLC grade) by vortexing and stored at 4 °C.

HPLC analysis of the samples was performed according to Singh *et al.* (2002). The HPLC system (*Shimadzu*, Japan) was equipped with a variable UV-VIS detector (WinChrom®). Reverse-phase chromatographic analysis was carried out under isocratic conditions (C-18 reverse phase column, 25 °C); injection volume 5 mL, mobile phase methanol–0.4 % acetic acid (4 : 1), flow rate 1 mL/min, detection at 290 nm. Samples were filtered through a membrane filter (pore size 0.45 μm, *Merck*) prior to injection. Tannic, gallic, caffeic, vanillic, chlorogenic, ferulic, *o*-coumaric, cinnamic and salicylic acids were used as internal and external standards. Phenolic compounds were identified by comparing the retention time of a standard compound and by co-injection. Concentrations were calculated by comparing peak areas of reference compounds with those in the samples. The experiment was duplicated.

RESULTS

Characterization of the bacterial strains. Of the *Pseudomonas* strains only *Pf4* solubilized phosphate and produced siderophore and IAA but did not produce HCN (*data not shown*). The *Pag* strain did not give positive result in any of the tests performed.

PAL activity. Increase was observed in both foliar and micro-injection treatments with both strains following inoculation with *S. sclerotiorum*. PAL activity was more pronounced in foliar spray, increasing up to 2 d and then declined in most of the treatments. Activity in foliar spray was maximum after 2 d in *Pag* + *Sst* and minimum in *Sst* after 1 d. The strain *Pag* showed slightly better PAL induction compared to *Pf4*. A similar trend was also observed in micro-injection treatment. PAL activity was maximum after 2 d in most of the treatments, declining after 3 d. Maximum activity was observed in *Pag* + *Sst* and minimum after 1 d in H_2O (Table I).

Table I. PAL activity in chickpea infected with *S. sclerotiorum* following foliar application and micro-injection of PGPR (sampling time 1, 2, and 3 d)

Treatment	μmol/h cinnamic acid per g of leaf fresh mass		
	1	2	3
Foliar application			
H_2O	12.3 ± 0.6	23.5 ± 1.2	19.4 ± 0.9
Sst	22.1 ± 1.1	18.3 ± 0.9	11.8 ± 0.6
Pag	23.3 ± 1.2	27.1 ± 1.4	24.5 ± 1.2
Pag+Sst	28.6 ± 1.4	42.3 ± 2.1	30.2 ± 1.5
Pf4	24.1 ± 1.2	27.5 ± 1.4	16.7 ± 0.8
Pf4+Sst	32.8 ± 1.6	42.3 ± 2.1	19.2 ± 0.9
Micro-injection			
H_2O	12.3 ± 0.6	23.5 ± 1.2	19.4 ± 0.9
Sst	22.1 ± 1.1	18.3 ± 0.9	11.8 ± 0.6
Pag	17.5 ± 0.9	28.1 ± 0.3	24.9 ± 1.3
Pag+Sst	26.6 ± 1.3	30.0 ± 1.5	30.8 ± 0.3
Pf4	26.6 ± 1.3	26.3 ± 1.1	22.6 ± 1.1
Pf4+Sst	30.2 ± 1.5	28.6 ± 1.4	21.5 ± 1.1

Phenolic acid production with micro-injection of PGPR. HPLC analysis of leaves from plants treated through micro-injection with *Pag* and *Pf4* strains and challenged with *S. sclerotiorum* revealed 6–8 peaks. Out of these, 6 peaks could be identified as tannic, gallic, caffeic, ferulic, *o*-coumaric, and chlorogenic acids. Among them, tannic acid concentration was maximum in *Pf4* + *Sst* treatment after 1 d. Gallic acid was also found in all treatments after 1 d except in *Pf4*, having maximum in *Pag* + *Sst* treatment. Similarly, caffeic acid in leaves was observed in all treatments after 1 d, having maximum in *Pag* + *Sst* and minimum in *Sst* after 1 d. Ferulic acid was induced in *Pag*, *Pag* + *Sst* and *Pf4* + *Sst* after 1 d while *o*-coumaric acid was found in *Pf4* and chlorogenic acid in H_2O (Table II).

After 2 d, 5 peaks were identified. Cinnamic acid (not observed after 1 d) was in the treated plants challenged by the pathogen after 2 d. Nearly equal amounts were observed in plants treated with both strains. Tannic acid was maximum in *Pf4* + *Sst* while being absent in *Sst*. Gallic acid was slightly increased in all treatments as compared with 1 d. Similarly, caffeic acid also showed the same trend after 2 d, its maximum accumulation was seen in *Pag* + *Sst* and minimum in H_2O after 2 d. However, it was not detected in *Sst*. Accumulation of ferulic acid after 2 d was found only in PGPR-treated plants.

Table II. Phenolic acid content in chickpea leaves (µg/g fresh mass) after 1, 2 and 3 d of micro-injection of PGPR and infected with *S. sclerotiorum*

Treatment	Tannic	Gallic	Caffeic	Ferulic	*o*-Coumaric	Chlorogenic[a]
1 day						
H_2O	7.7 ± 0.4	20.6 ± 1.0	2.2 ± 0.1	0	0	2.4 ± 0.1
Sst	0	16.6 ± 0.8	0.8 ± 0.4	0	0	0
Pag	0	10.5 ± 0.5	6.0 ± 0.3	1.7 ± 0.1	0	0
Pag+Sst	0	22.2 ± 1.1	21.4 ± 1.1	4.3 ± 0.2	0	0
Pf4	5.4 ± 0.3	0	12.8 ± 0.6	0	0.9 ± 0.1	0
Pf4+Sst	23.3 ± 1.2	18.3 ± 0.9	17.8 ± 0.9	9.9 ± 0.5	0	0
2 days						
H_2O	13.7 ± 0.7	31.7 ± 1.6	6.1 ± 0.3	0	0	0
Sst	0	10.4 ± 0.5	0	0	0	0
Pag	5.8 ± 0.3	12.8 ± 0.6	8.3 ± 0.4	2.1 ± 0.1	0	0.1 ± 0.0
Pag+Sst	3.3 ± 0.2	26.6 ± 1.3	29.2 ± 1.5	11.6 ± 0.6	0	1.2 ± 0.1
Pf4	7.2 ± 0.4	2.1 ± 0.1	14.6 ± 0.7	4.7 ± 0.2	0	0
Pf4+Sst	18.6 ± 0.9	23.2 ± 1.2	21.7 ± 1.1	15.6 ± 0.8	0	1.1 ± 0.1
3 days						
H_2O	16.7 ± 0.8	56.7 ± 2.8	2.2 ± 0.1	0	0	0
Sst	2.6 ± 0.1	13.3 ± 0.6	0	0	0	0
Pag	4.8 ± 0.2	16.5 ± 0.8	11.5 ± 0.6	1.6 ± 0.1	0.8 ± 0.0	3.4 ± 0.12
Pag+Sst	7.5 ± 0.4	14.6 ± 0.7	14.7 ± 0.7	3.9 ± 0.2	0	0
Pf4	9.0 ± 0.5	10.1 ± 0.5	0.9 ± 0.0	2.7 ± 0.1	0	0
Pf4+Sst	10.3 ± 0.5	30.3 ± 1.5	3.5 ± 0.2	9.6 ± 0.5	0.7 ± 0.0	0

[a]For 2 days cinnamic acid.

The amount of tannic acid varied while gallic acid increased after 3 d with both strains in all treatments except in *Pag* + *Sst*. Most of the acids (caffeic, ferulic, *o*-coumaric, chlorogenic) declined after 3 d. Ferulic acid was maximum in *Pf4* + *Sst* and minimum in *Pag* after 3 d.

Phenolic acid production in foliar spray of PGPR. After 1 d of foliar spray with both strains, 5 phenolic acids (tannic, gallic, caffeic, chlorogenic, cinnamic) were detected. Their higher accumulation was observed in plants receiving foliar spray compared to micro-injection. Accumulation of tannic acid was maximum in *Pf4* + *Sst*, but absent in *Sst* after 1 d while gallic acid was maximum in *Pf4* + *Sst*. Similarly, caffeic acid content also varied in different treatments (minimum in *Sst* and maximum in *Pag* + *Sst*) while chlorogenic acid content varied from minimum in *Sst* to maximum in *Pag*. Maximum amount of cinnamic acid was found in *Pf4* after 1 d (Table III).

Accumulation of tannic, gallic, caffeic, chlorogenic and cinnamic acid was increased after 2 d in most treatments. Maximum amount of tannic and gallic acids was observed in *Pf4* + *Sst* and minimum in *Sst*. However, in *Sst*, caffeic, chlorogenic and cinnamic acids were not present after 2 d. Their accumulation was higher in treated plants especially when challenged against the pathogen.

Concentration of tannic acid was reduced in all treatments except in H_2O after 3 d. However, gallic acid accumulated more after 3 d. Its maximum accumulation was found in *Pf4* + *Sst* and minimum in *Sst*. Caffeic acid accumulation was reduced from the amount after 2 d in most of the treatments. Similarly, the amount of chlorogenic and cinnamic acids also declined after 3 d in all treatments.

DISCUSSION

The mechanisms for ISR by PGPR have been extensively studied for over a decade. In *Arabidopsis thaliana* specific strains of nonpathogenic fluorescent pseudomonads induced ISR through jasmonic acid and ethylene-mediated pathways. Different bacterial determinants appear to be involved in triggering ISR, *e.g.*, lipopolysaccharides, siderophores, salicylic acid and flagella. Following characterization of the two bacterial strains used by us, the strain *Pf4* possesses several characteristics of a PGPR.

Table III. Phenolic acid content in chickpea leaves (µg/g fresh mass) after 1, 2 and 3 d of foliar application of PGPR and infected with *S. sclerotiorum*

Treatment	Tannic	Gallic	Caffeic	Chlorogenic	Cinnamic
1 day					
H_2O	11.5 ± 0.6	45.3 ± 2.3	2.9 ± 0.1	2.7 ± 0.1	0
Sst	0	30.1 ± 1.5	0.8 ± 0.0	0.2 ± 0.0	0
Pag	9.7 ± 0.5	65.2 ± 3.3	5.4 ± 0.3	6.2 ± 0.3	2.3 ± 0.1
Pag+Sst	16.3 ± 0.8	84.4 ± 4.2	9.2 ± 0.3	2.7 ± 0.1	0.7 ± 0
Pf4	20.1 ± 1.0	80.2 ± 4.0	2.4 ± 0.1	5.4 ± 0.3	3.2 ± 0.2
Pf4+Sst	27.2 ± 1.4	100 ± 5.0	6.3 ± 0.3	1.2 ± 0.1	1.5 ± 0.1
2 days					
H_2O	13.2 ± 0.7	50.2 ± 2.5	2.1 ± 0.1	3.1 ± 0.2	2.5 ± 0.1
Sst	2.2 ± 0.1	26.8 ± 1.3	0	0	0
Pag	13.5 ± 0.7	79.1 ± 3.9	6.8 ± 0.3	4.8 ± 0.2	4.2 ± 0.2
Pag+Sst	20.7 ± 1.0	92.2 ± 4.6	20.5 ± 1.0	9.5 ± 0.5	11.1 ± 0.5
Pf4	27.9 ± 1.4	70.3 ± 3.5	4.8 ± 0.2	6.2 ± 0.3	3.1 ± 0.2
Pf4+Sst	36.3 ± 1.8	100 ± 5.6	13.6 ± 0.7	18.3 ± 0.9	8.8 ± 0.4
3 days					
H_2O	18.5 ± 0.9	65.3 ± 3.2	2.1 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
Sst	0	13.2 ± 0.6	0	0	0
Pag	2.7 ± 0.1	99.5 ± 4.9	2.1 ± 0.1	0	3.1 ± 0.1
Pag+Sst	11.9 ± 0.5	119 ± 5.9	12.2 ± 0.6	0	7.6 ± 0.3
Pf4	10.2 ± 0.5	80.7 ± 3.0	1.6 ± 0	2.9 ± 0.1	2.5 ± 0.1
Pf4+Sst	14.6 ± 0.7	128 ± 6.3	3.2 ± 0.1	10.7 ± 0.5	3.2 ± 0.1

PAL is the primary entry enzyme that leads to phenylpropanoid pathway resulting in the biosynthesis of a diverse array of plant metabolites, such as cinnamic, coumaric, ferulic and caffeic acids, flavonoids, tannins and lignin (Hahlbrock and Sheel 1989). These products consequently protect plants against various abiotic stresses and pathogenic attacks (Jones 1984). Plants may accumulate phenolics through the phenylpropanoid pathway on activation of PAL as a means of passive defense (Barry and Manley 1986) and the magnitude of the accumulation primarily depends on the supply of the primary precursor, L-phenylalanine (Da Cunha 1987). Our results indicate that micro-injection and foliar treatment with PGPR strains caused greater accumulation of phenolic compounds and PAL activity as compared to untreated control; they confirm the observations of Da Cunha (1987). Moreover, low PAL activity as well as phenolic compounds in micro-injection method as compared in foliar spray (as observed by us) suggests that foliar spray is an effective method and could be used to manage diseases under field conditions. Maximum activity of PAL after 2 d in both foliar application and micro-injection of *Pseudomonas* strains further indicates the efficiency of the strains in inducing resistance in the host. Besides, shooting up of PAL activity in PGPR-treated plants in the presence of *S. sclerotiorum* further supports the role of PGPR as ISR inducer. However, between the two strains, *Pf4* was found to be relatively superior to *Pag* in inducing PAL activity initially whereas the effect of *Pag* was more persistent.

Phenolic compounds that occur constitutively are thought to function as preformed inhibitors associated basically with non-host resistance (Millar and Higgins 1970; Stoessl 1983) whereas others formed in response to the ingress of pathogens are considered as part of an active defense response (Nicholson and Hammerschmidt 1992). Antibiotic phenols have the ability to bind to some proteins *in vitro*, forming soluble and insoluble complexes (Hagerman and Buttler 1989; Hagerman and Klucher 1986; Hagerman and Rob-

bins 1987; Mc Manus *et al*. 1981). These phenolic–protein interactions are thought to be, in part, responsible for the putative function of phenolics as plant defence compounds (Millar and Higgins 1970; Feenly 1976; Coley 1983). However, several phenolic compounds are directly antifungal in their free state as well. Ferulic acid is one among them, which is highly inhibitory to *Sclerotium rolfsii* growth when accumulated in chickpea plants in high amount (Sarma and Singh 2003). Higher accumulation of phenolic compounds in chickpea plants treated with PGPR strains (especially in the presence of pathogens) in our investigation can be attributed to ISR by the bacterial strains. Higher accumulation of the phenolic compounds in plants treated with PGPR strains as foliar spray compared to micro-injection can further be correlated with the increased PAL activity in foliar sprayed plants.

Many studies indicate that greater accumulation of phenolics due to increased PAL activity offered protection against diseases and herbivory (Grey *et al*. 1997; Singh *et al*. 2002, 2003). Since it is assumed that different kinds of pathways may lead to the activation of plant defence reaction (Hahlbrock and Sheel 1989), the results presented here may only be a part of a more complex regulatory network and the induction of PAL activity may be one of the complex pathways. However, our investigation opens the way for wider application of PGPR as foliar spray to manage fungal diseases of crop plants.

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