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Effect of Foliar Application of *Pseudomonas fluorescens* on Activities of Phenylalanine Ammonia-Lyase, Chitinase and ß-1,3-Glucanase and Accumulation of Phenolics in Rice

B. MEENA, R. RADHAJEYALAKSHMI,
P. VIDHYASEKARAN and R. VELAZHAHAN*

Department of Plant Pathology, Tamil Nadu Agricultural University
Coimbatore-641 003, Tamil Nadu, India

Changes in activities of phenylalanine ammonia-lyase, chitinase, ß-1,3-glucanase and phenolic content in rice leaves were measured at different times after treatment of leaves with *Pseudomonas fluorescens* strain Pf1. When rice leaves were sprayed with *P. fluorescens,* substantial increase in the phenylalanine ammonia-lyase activity was observed 1 day after treatment. Following increase of the first enzyme of the phenylpropanoid pathway, phenolic content of rice leaves also increased to a maximum at 4 days after *P. fluorescens* treatment. Chitinase activity increased in rice leaves in response to application of *P. fluorescens* and the maximum enzyme activity was observed 3 days after treatment. ß-1,3-Glucanase activity also increased significantly from 1 day after *P. fluorescens* treatment and continued to increase through 7 days. A five-fold increase in glucanase activity was observed 7 days after *P. fluorescens* treatment.

Key words: *Pseudomonas fluorescens,* phenolics, PAL, chitinase, glucanase.

Biological control of plant diseases using antagonistic bacteria is now considered as a promising alternative to the use of hazardous chemical fungicides or bactericides. Bio-pesticides are cheaper than synthetic pesticides by 50 per cent. They are ecofriendly, have a high cost-benefit ratio and do not pose risk of the pathogen developing resistance. They are easy to apply and are compatible with bio-fertilizers. Many root-colonizing bacteria are known to promote plant growth by producing gibberellins, cytokinins and indole acetic acid (Dubeikovsky et al., 1993) and hence they are called as "Plant Growth-Promoting Rhizobacteria" (PGPR) (Kloepper and Schroth, 1978). Several PGPR strains have been reported to control several fungal (Van Peer et al., 1991), bacterial (Liu et al., 1995) and viral (Raupach et al., 1996; Maurhofer et al., 1994) diseases. Mechanisms of biological control by PGPR strains generally involve production of siderophores (Kloepper et al., 1980; Becker and Cook, 1988), hydrogen cyanide (Ahl et al., 1986) and lytic enzymes (Sneh et al., 1984; Jones et al., 1986), biosynthesis of antibiotics (Howie and Suslow, 1991) and competition for substrates (Elad and Chet, 1987). PGPR are also known to act as inducers of systemic resistance in plants (Alstrom, 1991; Van Peer et al., 1991; Wei et al., 1991; Zhou and Paulitz, 1994; Raupach et al., 1996). Several studies have indicated that PGPR may stimulate the production of biochemical compounds associated with host defense (Albert and Anderson, 1987; Zdor and Anderson, 1992; M'Piga et al., 1997). A massive accumulation of phytoalexin (Van Peer et al., 1991), phenolic compounds (M'Piga et al., 1997), increases in the activities of PR-proteins (Hynes and Lazarovits, 1989), peroxidase (Albert and Anderson, 1987; Zdor and Anderson, 1992), increase in the levels of mRNAs encoding phenylalanine ammonia-lyase

*Corresponding author

(PAL), chalcone synthase and PR1a protein (Zdor and Anderson, 1992) and enhanced lignification (Anderson and Guerra, 1985) have been reported in plants following treatment with PGPR strains. Earlier studies from our laboratory indicated that rice blast could be controlled by foliar application of *Pseudomonas fluorescens* strain Pf1, a well-known PGPR (Vidhyasekaran et al., 1997a). The purpose of this study was to determine whether *P. fluorescens* strain Pf1 is able to induce accumulation of phenolics and activities of PAL, chitinase and ß-1,3-glucanase in rice.

Materials and Methods

Isolation of P. fluorescens

Fluorescent pseudomonads were isolated from the rhizosphere of rice (*Oryza sativa* L.) using King's medium B (KMB) (King et al., 1954) as described by Vidhyasekaran et al. (1997b). The strains were identified as fluorescent pseudomonads according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). One of the isolates of *P. fluorescens,* Pf1 which effectively inhibited the growth of *Pyricularia oryzae* Cavara *in vitro* and controlled blast disease under field conditions, was used in the present study.

Induction of defense mechanisms in rice by foliar application of P. fluorescens

Seeds of rice cultivar IR50 obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu was used in this experiment. Seeds were sown in pots containing field soil at the rate of 10 seeds/pot and grown under greenhouse conditions. Plants were sprayed with *P. fluorescens* strain Pf1 (10^9 Colony Forming Units (CFU)/ml) 30 days after sowing. At various times after application, leaf samples were collected and various analyses were made.

Estimation of phenolic content

For estimating phenolic content, rice leaves (1 g) were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 °C (Swain and Hillis, 1959). One ml of the methanolic extract was added to 5 ml of distilled water and 250 ml of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25 °C. After 3 min 1 ml of saturated solution of Na_2CO_3 and 1 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25 °C. The absorption of the developed blue colour was measured using a Beckman DU64 spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with phenol and expressed as phenol equivalents in μg g^{-1} fresh weight.

Determination of PAL activity

Leaves (1 g) were homogenized in 5 ml of 0.1 M sodium borate buffer, pH 7.0 containing 0.1 g insoluble polyvenylpolypyrrolidone (PVP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 20,000 g for 35 min. The

supernatant was used in the enzyme assay. PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm as described by Dickerson et al. (1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. In reference cell, 0.4 ml of enzyme extract was taken along with 1.0 ml of borate buffer. The amount of *trans*-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1} (Dickerson et al., 1984). Enzyme activity was expressed on a fresh weight basis (nmol min^{-1} g^{-1}).

Assay of chitinase

Leaves (1 g) were homogenized in 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 *g* at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared according to Berger and Reynolds (1958) from crab shell chitin (Sigma). For the colorimetric assay of chitinase 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml Eppendorf tube. After 2 h at 37 °C, the reaction was stopped by centrifugation at 1,000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme (Helicase, obtained from Sepracor, France) for 1 h. The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Reissig et al. (1959) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} fresh weight.

Assay of ß-1,3-glucanase activity

ß-1,3-Glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan et al., 1991). Leaves were extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4 °C using a pestle and mortar. The extract was then centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40 °C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. Enzyme activity was expressed as nmol min^{-1} mg^{-1} fresh weight.

Results

When rice leaves were sprayed with *P. fluorescens,* significant increase in the phenolic content was observed 1 day after treatment and maximum increase was observed 4 days after treatment (Table 1). Subsequently phenolic content started to decline although even at 7 days after treatment phenolic content was higher than in control leaves. Foliar application of *P. fluorescens* caused substantial increase in the activity of PAL in

Table 1

Effect of foliar application of *Pseudomonas fluorescens* on accumulation of phenolic in rice leaves

Days after *P. fluorescens* treatment	Phenolic content (µg/g fresh weight)		% increase over control
	Control	*P. fluorescens* treated	
0	434 ± 16	445 ± 14	2.5
1	448 ± 12	681 ± 17	52.0
2	466 ± 15	764 ± 16	63.9
3	482 ± 13	778 ± 15	61.4
4	462 ± 12	782 ± 18	69.2
5	434 ± 14	725 ± 9	67.0
6	443 ± 15	682 ± 12	53.9
7	445 ± 13	635 ± 12	42.6

The data are mean of 3 replications; ± indicates standard deviation.

rice leaves and the maximum enzyme activity was detected 1 day after treatment (Table 2). However, compared to control leaves significantly higher PAL activity was measured even at 7 days after treatment.

Table 2

Effect of foliar application of *Pseudomonas fluorescens* on phenylalanine ammonia-lyase (PAL) activity in rice leaves

Days after *P. fluorescens* treatment	PAL activity (nmol/min/g fresh weight)		% increase over control
	Control	*P. fluorescens* treated	
0	176 ± 6	192 ± 7	9.0
1	170 ± 8	485 ± 8	185.3
2	211 ± 6	515 ± 6	144.0
3	226 ± 9	519 ± 7	129.6
4	190 ± 7	409 ± 6	115.2
5	202 ± 6	390 ± 5	93.0
6	194 ± 7	373 ± 7	92.2
7	193 ± 6	333 ± 6	72.5

The data are mean of 3 replications; ± indicates standard deviation.

Foliar application of *P. fluorescens* increased the chitinase activity in rice leaves and the maximum increase was observed 3 days after treatment (Table 3). Subsequently the enzyme activity declined but remained significantly at higher levels than the control.

Table 3

Effect of foliar application of *Pseudomonas fluorescens* on activity of chitinase in rice leaves

Days after *P. fluorescens* treatment	Chitinase activity (nmol/min/g fresh weight)		% increase over control
	Control	*P. fluorescens* treated	
0	840 ± 20	860 ± 20	2.3
1	860 ± 50	2260 ± 30	162.7
2	860 ± 30	2350 ± 20	173.2
3	910 ± 20	2700 ± 60	196.7
4	940 ± 50	2410 ± 50	156.3
5	960 ± 30	2000 ± 40	108.3
6	900 ± 20	1840 ± 30	104.4
7	850 ± 20	1800 ± 20	111.7

The data are mean of 3 replications; ± indicates standard deviation.

Glucanase activity also increased from 1 day after *P. fluorescens* treatment and the enzyme activity consistently increased throughout the experimental period of 7 days (Table 4). A five-fold increase in glucanase activity was observed 7 days after *P. fluorescens* treatment.

Table 4

Effect of foliar application of *Pseudomonas fluorescens* on activity of ß-1,3-glucanase in rice leaves

Days after *P. fluorescens* treatment	ß-1,3-glucanase activity (nmol/min/mg fresh weight)		% increase over control
	Control	*P. fluorescens* treated	
0	5.2 ± 0.2	5.3 ± 0.3	1.9
1	5.6 ± 0.3	12.8 ± 0.8	128.5
2	6.0 ± 0.6	15.8 ± 1.2	163.3
3	6.2 ± 0.4	19.0 ± 1.2	206.4
4	5.9 ± 0.2	19.6 ± 1.4	232.2
5	6.8 ± 0.3	20.6 ± 1.6	202.9
6	5.7 ± 0.2	24.9 ± 1.7	336.8
7	4.6 ± 0.2	25.0 ± 1.9	443.4

The data are mean of 3 replications; ± indicates standard deviation.

Discussion

Induction of systemic resistance is one of the mechanisms by which PGPR could protect the plants against diseases (Zhou and Paulitz, 1994; Van Wees et al., 1997). The induction of systemic resistance is associated with rapid activation of multiple defense mechanisms which are latent in control plants. These defense mechanisms include accumulation of phenolics, phytoalexins, lignins, hydroxyproline-rich glycoproteins, increases in the activities of chitinase, ß-1,3-glucanase, peroxidases several antioxidants and other PR-proteins. Fodor et al. (1997), Liu et al. (1995) and Hoffland et al. (1995) demonstrated that leaf application of PGPR strain is effectively inducing resistance against leaf pathogens. Our present experiments demonstrated that the foliar application with *P. fluorescens* induced the PAL enzyme activity and accumulation of phenolics in the leaves of rice plants. Maximum accumulation of phenolics occurred by 4 days after *P. fluorescens* treatment, concomitant with a marked increase of PAL activity. Phenolic compounds produced by plants are formed through phenylpropanoid metabolism. Phenylpropanoid metabolism starts with the conversion of L-phenylalanine into *trans*-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) (Massala et al., 1980). The presence of phenolic compounds in plants or their synthesis in response to infection by pathogens has been associated with resistance of the host (Nicholson and Hammerschmidt, 1992). Zdor and Anderson (1992) observed an increase in the level of mRNAs encoding for PAL and chalcone synthase in the early stages of interaction between bean roots and various bacterial endophytes. Since the production of phenolic compounds depends upon PAL activity (Graham and Graham 1991), increased phenolic synthesis in *P. fluorescens*-sprayed rice plants may be due to increased activity of PAL.

The present study also indicated that foliar application of *P. fluorescens* caused increases in the chitinase and ß-1,3-glucanase activities in rice. Several PR-proteins, either alone or in combination have been shown to have antifungal activity (Datta and Muthukrishnan, 1999). PR-proteins like chitinase and ß-1,3-glucanase have the potential to hydrolyze chitin and ß-1,3-glucan, respectively, which are major components of fungal cell walls. Moreover the chitinase and glucanase release elicitors from the walls of fungi which, in turn, stimulate various defense responses in plants (Ham et al., 1991; Ren and West, 1992). Several studies have shown that PR-proteins are induced in plants upon treatment with *P. fluorescens* (Hynes and Lazarovits, 1989; Maurhofer et al., 1994; M'Piga et al., 1997). Hynes and Lazarovits (1989) reported that the levels of a PR-protein increased in bean leaves following seed treatment with PGPR strains. Maurhofer et al. (1994) reported that PR-proteins viz., PR-1a,1b,1c, endochitinase and b-1,3-glucanases were induced in the intercellular fluid of tobacco leaves of plants grown in the presence of *P. fluorescens* strain CHA0. In contrast, Hoffland et al. (1995) reported that resistance induced in radish by *P. fluorescens* strain WCS417r was not associated with induction of PR-proteins.

Several strains of fluorescent pseudomonads are known to produce signal molecules. The lipopolysaccharides (LPS), one of the constituents of outer membrane of *P. fluorescens* WCS417 induced systemic resistance in carnation to *Fusarium oxysporum*

f. sp. *dianthi* (Van Peer and Schippers, 1992). Fluorescent pseudomonads are also known to produce salicylic acid which acts as local and systemic signal molecules in inducing resistance in plants (Dowling and O'Gara, 1994; De Meyer and Hofte, 1997). Leeman et al. (1996) demonstrated that the siderophore of *P. fluorescens* strain WCS374r acts as an elicitor of ISR in radish. The increased activities of PAL, chitinase and ß-1,3-glucanase and accumulation of phenolics in rice may be due to the release of signal molecules by *P. fluorescens.*

Vidhyasekaran et al. (1997a) reported that treatment of rice leaves with *P. fluorescens* strain Pf1 4 days prior to leaf inoculation with *P. oryzae* resulted in a significant reduction in blast disease intensity. *Pyricularia oryzae* conidium germinates rapidly and produces a germ tube within an hour after landing on the host surface. The fungus proliferates rapidly after colonization and spread throughout young rice leaves within 72–96 h (Talbot, 1995). The enhanced activities of PAL, chitinase and ß-1,3-glucanase and accumulation of phenolics in rice in response to application of *P. fluorescens* corresponded with the time of attempted penetration and colonization by the *P. oryzae.* Hence these defense-related activities might have also contributed for increased resistance against *P. oryzae* besides antagonism.

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