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Pseudomonas aeruginosa Inducing Rice Resistance Against *Rhizoctonia solani*: Production of Salicylic Acid and Peroxidases

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ABSTRACT. Three isolates of *Pseudomonas aeruginosa* were used for seed treatment of rice; all showed plant growth promoting activity and induced systemic resistance in rice against *Rhizoctonia solani* G5 and increased seed yield. Production of salicylic acid (Sal) by *P. aeruginosa* both *in vitro* and *in vivo* was quantified with high performance liquid chromatography. All three isolates produced more Sal in King's B broth than in induced roots. Using a split root system, more Sal accumulated in root tissues of bacterized site than in distant roots on the opposite site of the root system after 1 d, but this difference decreased after 3 d. Sal concentration 0–200 g/L showed no inhibition of mycelial growth of *R. solani in vitro*, while at ≥300 g/L it inhibited it. *P. aeruginosa*-pretreated rice plants challenged inoculation with *R. solani* (as pathogen), an additional increase in the accumulation of peroxidase was observed. Three pathogenesis-related peroxidases in induced rice plants were detected; molar mass of these purified peroxidases was 28, 36 and 47 kDa. Purified peroxidase showed antifungal activity against phytopathogenic fungi *R. solani*, *Pyricularia oryzae* and *Helminthosporium oryzae.*

Abbreviations

CD	critical difference	PR	pathogenesis-related
HPLC	high performance liquid chromatography	Sal	salicylic acid
IAA	3-indolylacetic acid	SAR	systemic acquired resistance
ISR	induced systemic resistance	ShB	sheath blight
PDA	potato-dextrose agar	TMV	tobacco mosaic virus
PGPR	plant growth promoting rhizobacteria		

ShB of rice caused by *Rhizoctonia solani* Kuhn, occurs almost in all the rice growing regions of the world. It causes considerable yield loss ranging from 5.2 to 50 % (Ou 1973). It is difficult to control the disease by cultural as well as chemical methods (Roy 1979). In recent years, PGPR-like *Pseudomonas* spp. have been extensively used for plant growth promotion and disease control (Park and Kloepper 2000; Saikia *et al.* 2003, 2005). *P. aeruginosa* is known to enhance plant growth and suppress many fungal diseases (Audenaert *et al.* 2002). Resistance can be induced on the aerial parts of plants by pre-inoculation with PGPR in the root system (Van Loon *et al.* 1998). This type of resistance is defined as ISR whose signaling pathway is different from the SAR (Knoester *et al.* 1999; Pieterse and Van Loon 1999). Many studies indicated that Sal accumulation was associated with plant physiological responses to pathogen infection. Malamy *et al.* (1990) reported that a Sal level was increased as much as 20× after TMV infection on resistant tobacco cultivar leaves. However, Vernooji *et al.* (1994) and Pieterse *et al.* (1996) reported that Sal has played a role in the translocation of primary signal for SAR in contrast to Sal playing only a regulatory role in the expression of SAR genes (Silverman *et al.* 1995). These experiments showed that Sal may not be involved in all cases of systemic resistance and may not act as exogenous inducers against all plant pathogens. Though, in general, scientists thought that Sal plays a more important role in SAR than in ISR; it is uncertain whether Sal works as a signal for SAR or ISR.

It is also not clear whether Sal is produced in infected plants or by PGPR strains and what role Sal plays in resistance to root disease. Production of PR proteins (chitinase, β-1,3-glucanase and peroxidase) by the several PGPR strains is considered as a major antagonistic property of these strains (Ramamoorthy and Samiyappan 2001; Saikia *et al.* 2004; Víteček *et al.* 2005). Knowledge of the molecular genetics of lignin-

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degrading enzymes has proceeded rapidly during the past decade (Maijala *et al.* 2003). Peroxidase has been implicated in phenol oxidation (Schmid and Feucht 1980), IAA oxidation (Beffa *et al.* 1990), lignification (Grisebach 1981; Saparrat and Guillén 2005), plant defense (Hammerschmidt *et al.* 1982) and regulation of plant cell elongation (Goldberg *et al.* 1986). Increase in peroxidase has been correlated with resistance in many species including rice and wheat (Young *et al.* 1995). Application of exogenous Sal has been long known to induce PR-gene expression and acquired resistance against a variety of microbial pathogens (Ward *et al.* 1991; Meena *et al.* 2001). The objectives of the present investigation were (*i*) to observe the plant-growth-promoting activity of three isolates of *P. aeruginosa*; (*ii*) whether Sal is produced *in vitro* and in root tissues of plant induced by *P. aeruginosa*; whether exogenous Sal could directly suppress mycelial growth of *R. solani*; (*iii*) to observe the peroxidase activity in induced rice plant; (*iv*) to analyze isozymes of peroxidase and (*v*) assessment of the antifungal property of peroxidase.

MATERIAL AND METHODS

Bacterial isolates and pathogens. *Pseudomonas aeruginosa* (*Pa*RsG18, *Pa*RsG27 and *Pa*RsG28) were isolated from rice rhizosphere soil of Guwahati, Assam (India). *Rhizoctonia solani* G5 (*Rs*G5), *Helminthosporium oryzae* and *Pyricularia oryzae* were obtained from *Culture Bank of Life Sciences Division, Institute of Advanced Study in Science and Technology*, Guwahati (India).

Soil preparation. Local rice field soils were collected and autoclaved 3× (1 h, 121 °C) at 12-h intervals and 5 kg of sterilized soil was taken per pot (diameter 230 mm).

Challenged inoculation and disease assessment. Seeds of rice cultivar IR 50 were surface-sterilized with 2 % sodium hypochlorite for 30 s, then rinsed in sterile distilled water and dried in a sterilized air stream. These seeds were sown in the above earthen pot (10 seeds per pot). After 40 d, 10 mL of bacterial suspension (10^8 CFU/mL) were applied in plants per pot. Two d after bacterization, one set of bacterized plants was challenged with *Rs*G5 by inoculation on sheath (10 mL per pot, 10^3 CFU/mL). Plants without bacteria or pathogen served as control. The experiment was carried out as a complete randomized design with 10 replications in a greenhouse. Disease severity was recorded by measuring the lesion height 12 d after inoculation. At harvest, shoot height, and grain yield per pot were recorded.

Sal produced in vitro. Each *P. aeruginosa* isolate was inoculated in a test tube containing 20 mL King's B broth and incubated (2 d, 28 ± 2 °C). The liquid culture was centrifuged (2800 ***g***, 20 min, 4 °C), and the supernatant was acidified to pH 2.0. The solution was filtered, resuspended in 1 mL of 23 % methanol in 20 mmol/L sodium acetate buffer (pH 5.0) and then it analyzed with HPLC (Yalpani *et al.* 1991).

Sal produced in vivo. A split-root technique was applied for induction of resistance. Sal was extracted after challenged inoculation (Chen *et al.* 1999) and stored at –80 °C. Sal was detected by HPLC at 280 nm with a Bondapak C 18 column (3.9 × 300 mm), with a mobile phase flow rate at 0.5 mL/min. Sal was separated isocratically with 23 % methanol (*V*/*V*) in 20 mmol/L sodium acetate buffer (pH 5) by injecting 10 μL of each sample into the column (Yalpani *et al.* 1991).

Effect of Sal on mycelial growth. Mycelial disc (diameter 5 mm) of 7-d-old actively growing *Rs*G5 was placed on the center of each Sal-amended Petri plates containing PDA medium (pH 5.5). The Sal concentrations in the medium were 0–2500 mg/L. The diameter of the colonies was measured after 5 d.

Extraction and *purification of peroxidases.* Plant samples (1 g each) were collected at 0–7 d after treatment with *P. aeruginosa* and *Rs*G5 was homogenized with 2 mL of sodium phosphate buffer (10 mmol/L, pH 6.5, 4 °C). The homogenate was filtered through 4 layers of muslin cloth and the filtrates were centrifuged (6000 ***g***, 4 °C, 20 min); the supernatant served as enzyme source.

Peroxidase activity was determined (Hammerschmidt *et al.* 1982) and isozymes of peroxidase were analyzed by native PAGE (Pan *et al.* 1991). The enzyme was purified by fractionating the crude enzyme extract over a Sephadex G-50 column (15 × 300 mm) using 10 mmol/L sodium phosphate buffer (pH 6.0) as elution buffer (Ramanathan *et al.* 2001). The molar mass of each fraction was determined by SDS-PAGE with molar-mass standards.

Antifungal property of peroxidase was determined by agar diffusion test on PDA medium. Sterilized paper disc (diameter 5 mm) containing 15 μg of purified peroxidase was placed in the center of Petri plates containing sterilized PDA medium and mycelial discs (5 mm) of some actively growing pathogens, *viz*. *R. solani*, *P. oryzae* and *H. oryzae* were inoculated individually in 4 points of the Petri plates maintaining an equal distance from the center. The plates were incubated at 28 ± 2 °C and inhibition zone was recorded after 5 d.

RESULTS AND DISCUSSION

Plant growth promoting activity and suppression of *R. solani* infection was observed in rice by isolates of *P. aeruginosa* (Table I). Isolate *Pa*RsG18 increased plant growth by 14 and 128 % as compared to blank and *R. solani*-treated control, respectively. *P aeruginosa* suppressed ShB disease (11–33 %) and produced significantly ($p = 0.05$) higher yield over the controls (blank and *Rs*G5-treated control), isolate *Pa*RsG18 producing maximum yield (25.2 g per pot).

Table I. Plant growth promotion and induced resistance of rice by *Pseudomonas aeruginosa*[a]

Treatment	Plant height mm	Sheath blight lesion height mm	Grain yield g per pot
*Pa*RsG18	480 ± 23	0	25.2 ± 2.4
*Pa*RsG27	390 ± 26	0	21.0 ± 1.6
*Pa*RsG28	450 ± 20	0	24.0 ± 3.1
*Pa*RsG18 + *Rs*G5	350 ± 32	30.0 ± 2.0	16.5 ± 1.5
*Pa*RsG27 + *Rs*G5	280 ± 24	40.3 ± 3.6	12.7 ± 1.0
*Pa*RsG28 + *Rs*G5	330 ± 17	33.5 ± 2.5	15.0 ± 2.0
Blank control	420 ± 35	0	21.3 ± 2.3
*Rs*G5 control	210 ± 18	45.0 ± 4.0	11.0 ± 1.7

[a]CD = 4.6 for plant height, 1.66 for lesion height, 1.11 for grain yield; $p = 0.05$.

All three isolates could produce Sal *in vitro* conditions. Isolate *Pa*RsG18 produced more Sal than the other two isolates (Table II); however, isolate *Pa*RsG27 produced only 4.3 mg/L. *In vivo*, Sal level in *Pa*RsG18-treated root tissues was higher than in *Pa*RsG28-treated roots. Sal level was significantly higher on bacterized site than on the distant site (Fig. 1). The Sal level in bacterized site increased after 1 d, then it decreased; it was initially higher in bacterized site than in the distant induced site but the difference exists between the bacterized and distant sites.

Table II. *In vitro* salicylic acid production (mg/L) by *Pseudomonas aeruginosa*[a]

Treatment	mg/L
*Pa*RsG18	27.0 ± 3.3
*Pa*RsG27	4.3 ± 1.3
*Pa*RsG28	21.7 ± 2.7
Control	0

[a]CD = 1.37; $p = 0.05$.

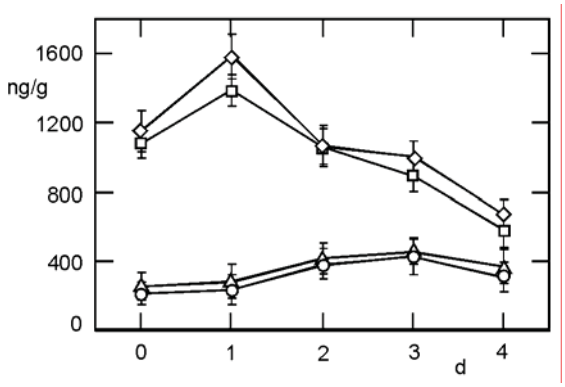

Fig. 1. Endogenous level of salicylic acid in bacterized and distant induced site of rice roots (ng/g fresh root); *diamonds* – *Pa*RsG18, *squares* – *Pa*RsG28 (bacterized site), *triangles* – *Pa*RsG18 (bacterized site), *circles* – *Pa*RsG28 (distant site).

Though Sal did not inhibit mycelial growth of *R. solani* at ≤200 mg/L in Petri plate assay it inhibited at 500–2500 mg/L, complete inhibition being observed at 2500 mg/L (Fig. 2).

Rice plants inoculated with *R. solani* alone and *P. aeruginosa* alone, peroxidase activity was increased significantly ($p = 0.05$) whereas in plants pre-inoculated with *P. aeruginosa* when challenged with *R. solani*, an additional increase was observed (Fig. 3). Up to a 3-fold increase was exhibited after 2 d, then it decreased progressively.

Under laboratory conditions, in induced rice plants three PR peroxidase isozymes (PR1, PR2, PR3) were detected (*data not shown*). They were not detected in control plants. The highest expression of them was observed in *P. aeruginosa* pretreated plant challenge inoculated with *R. solani.* Purified peroxidases

showed similar migration on native and denaturing acrylamide, molar mass of them was 28, 36 and 47 kDa (Fig. 4).

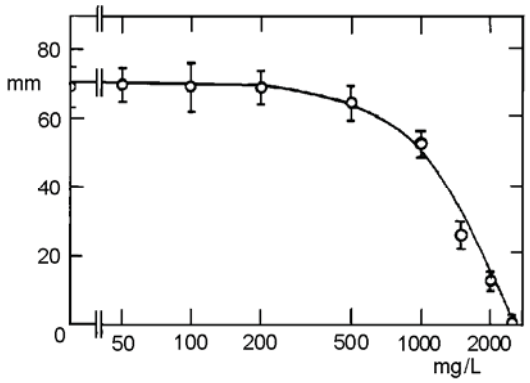

Fig. 2. Inhibition of mycelial growth (mm) of *R. solani* G5 by salicylic acid (mg/L) in Petri dish assay.

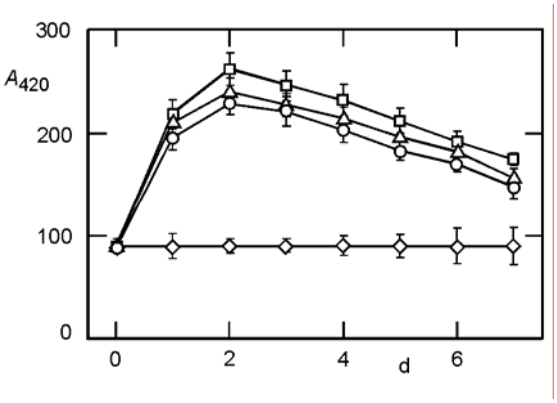

Fig. 3. Change in peroxidase activity (A_{420} per min per g sample) in induced rice plant; *diamonds* – control, *circles* – *Pa*RsG18, *triangles* – *Rs*G5, *squares* – *Pa*RsG18 + *Rs*G5.

The purified peroxidase exhibited antifungal activity by inhibiting the redial growth of *R. solani*, *P. oryzae* and *H. oryzae* (Table III). The enzyme extracted from *P. aeruginosa*-pretreated plant challenge inoculated with *R. solani* exhibited the highest activity with *R. solani* (inhibitory zone 7.0 mm), lowest inhibition being observed with the peroxidase from *P. aeruginosa.*

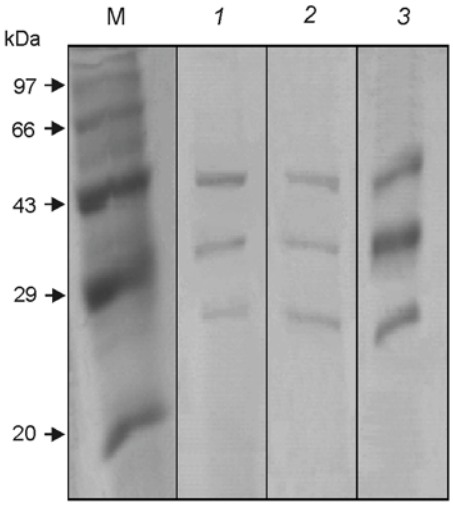

Fig. 4. SDS-PAGE and molar mass of purified peroxidases in rice plant induced by *R. solani* G5 (*1*), *P. aeruginosa Rs*G18 (*2*) and *P. aeruginosa Rs*G18 + *R. solani* G5 (*3*); M – molar-mass markers.

P. aeruginosa has been widely used for plant growth promotion and control of plant diseases (Buysens *et al.* 1996; Audenaert *et al.* 2002). Our results indicated that *P. aeruginosa* promoted the growth of rice plants as well as suppressed the disease caused by *R. solani.*

Sal (as an important endogenous signal molecule involved in the transduction pathway) is required for the establishment of SAR (Benhamou *et al.* 2000). Rhizobacterially mediated ISR does not require Sal (Van Loon 2000), however, other workers have shown a role of Sal in ISR (Meyer and Hofte 1997; Audenaert *et al.* 2002). Though the ability of PGPR to produce Sal may not be correlated with its ISR activity (Chen *et al.* 1999) our results indicated that *P. aeruginosa* could stimulate rice plants to accumulate Sal in their roots. It is not clear whether Sal detected in bacterized rice root was produced by plant itself or by bacteria; whether the increased amount of Sal is locally or systematically generated or transferred from bacterized sites or other tissues because of free Sal translocated in plant phloem (Metraux *et al.* 1990; Yalpani *et al.* 1991).

Our results on mycelial growth inhibition are in agreement with Chen *et al.* (1999), who reported that Sal inhibited the mycelial growth of *P. aphanidermatum* only at high concentration (300–3000 mg/L); at 3000 mg/L it was completely inhibited.

Table III. Antifungal activity of purified peroxidase (growth inhibition zone, mm)[a]

Source of peroxidase[b]	*Rhizoctonia solani*	*Pyricularia oryzae*	*Helminthosporium oryzae*
R. solani G5 (*Rs*G5)	7.0 ± 0.34	3.0 ± 0.13	5.1 ± 0.32
P. aeruginosa G18 (*Pa*G18)	4.3 ± 0.22	2.4 ± 0.10	4.2 ± 0.11
*Pa*RsG18 + *Rs*G5	5.3 ± 0.12	3.6 ± 0.23	6.2 ± 0.23

[a]CD = 2.43 for peroxidase source, 2.87 for fungus, 1.72 for peroxidase source *vs*. fungus; $p = 0.05$.
[b]Treatment of rice plant.

Peroxidases are important PR proteins (Van Loon *et al.* 1994) and plant expresses peroxidase activity during host–pathogen interaction (Young *et al.* 1995; Saikia *et al.* 2004). Several peroxidase isozymes were detected in tobacco cell wall (Ye *et al.* 1990), greengram (Ramanathan *et al.* 2001) and rice (Chittoor *et al.* 1997). Accumulation of peroxidase in xylem vessels during incompatible interactions with *Xanthomonas oryzae* pv. oryzae was also reported (Young *et al.* 1995). Manandhar *et al.* (1999) has detected peroxidase activity in rice plants triggered by *P. oryzae*, *Bipolaris sorokiniana* and UV light. Ramanathan *et al.* (2001) detected two pathogenesis-related peroxidases in greengram leaves and cultured cells induced by *M. phaseolina* and its elicitor; the molar mass of them was 27 and 38 kDa.

Our results demonstrated that (*i*) *P. aeruginosa* can enhance growth of rice plants and suppress ShB caused by *R. solani*, (*ii*) *P. aeruginosa* can produce Sal both *in vitro* and *in vivo*, (*iii*) induced rice plants produced peroxidases with molar mass 28, 36 and 47 kDa, and (*iv*) purified peroxidases showed antifungal activity against three phytopathogenic fungi.

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