Biological control of sheath blight of rice: Induction of systemic resistance in rice by plant-associated *Pseudomonas* spp.

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In previous studies on the biological suppression of fungal diseases of rice with plant-associated rhizosphere bacteria we had reported that production of antifungal antibiotic (afa) by the bacterium is the major mechanism involved. In this study we report that the induction of systemic resistance in rice by strains of pseudomonads leads to suppression of sheath blight. A molecular tracking system (*lacZY* gene) was used to engineer strains of *Pseudomonas fluorescens* 7-14 and *P. putida* V14i. We used the *lacZ* expression as tracking system to monitor the survival and migration of the biocontrol agents. In spite of the absence of bacteria on plant surfaces, the endophytic bacteria present in the stem led to suppression of rice sheath blight caused by *Rhizoctonia solani* up to 18%.

THE application of beneficial pseudomonads as agents for biological control of plant diseases in agriculture is emerging as a potential alternative to the use of chemical fungicides. Evidence for mechanisms of biological control of pathogens cannot be exemplified by a single factor. The known mechanisms include, competition, production of antibiotics, siderophores, hydrogen cyanide, phytohormones, enhancement of nutrient uptake by the plant, extracellular enzymes, nitrogen fixation, enzyme synthesis to regulate the level of plant ethylene, and other substances[1–8].

Recent investigations on mechanisms of biological control by plant growth-promoting rhizobacteria (PGPR) suggest that these strains protected plants from various pathogens by inducing a systemic resistance (ISR) in plants[9–14]. The evidence for involvement of pseudomonads in ISR was observed when lipopolysaccharides extracted from the outer membrane of *P. fluorescens* caused more accumulation of phytoalexins, increased amount of salicylic acid, phenolics and pathogenesis-related (PR) proteins[9,11,15–17]. Fluorescent pseudomonads have the capacity to exist inside plants endophytically in different host plants[12,18–20]. Previous research carried out also shows that pseudomonads have the potential for the suppression of rice diseases[5,21–24]. The objective of this study was to assess whether the rhizosphere pseudomonads could cause ISR to a fungal disease when applied as infiltration, and to monitor the migration and survivability of genetically engineered strains in the host plant.

Wild type strains of *Pseudomonas putida* V14i and *P. fluorescens* 7–14 used in this study were previously isolated from the rice rhizosphere[21,24]. *Escherichia coli* CE1 was obtained as a gift from R. Jefferson, CAMBIA, Australia. The sheath blight pathogen *Rhizoctonia solani* was isolated from infected rice tissues. LacZY gene cassette was inserted into the chromosome of recipients (7–14, V14i) by a disarmed Tn-7 lacZY transposon through triparental mating[25]. The genetically engineered strains of 7-14gal and V14igal and their constitutive expression of lacZY was stable over 50 generations and were recovered in M9 agar which contains 1% lactose, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and antibiotics (100µg/ml of rifampicin [rif] and ampicillin [amp] for 7-14gal strain and 100 µg/ml of streptomycin [stp] and amp for V14igal strain).

Bacterial strains of 7-14gal and V14igal were grown in M9 at 27°C for 24 h. *E. coli* CE1 strain (negative control) was grown in Lauria Bertani (LB) agar plates at 37°C. These were harvested separately, washed twice with sterile water by centrifugation at 5000 *g* for 10 min and the cell suspension was adjusted to 0.1 OD at 600 nm to obtain 10^9 cfu/ml. Twenty-day-old rice seedlings (cv. IR50) were bacterized by infiltration with 1,2 and 5 µl of bacterial cells with a microsyringe at approximately 2 cm above the soil surface. For *E. coli* CE1, only 2 µl was infiltrated about 2 mm into the stem. In another set of experiment, plants were inoculated by beading needles that were dipped in the bacterial suspension and were slightly brushed into the stem following the method reported earlier by Chen *et al.*[18]. Plants that received 2 µl of sterile water and also uninoculated plants served as controls. The experiments were conducted twice with three replications under greenhouse conditions.

The bacteria were also passively introduced by seed treatment, root-dip and foliar spray onto rice foliage with a bacterial suspension (10^9 cfu/ml) in 1% carboxymethylcellulose (cmc). For seed treatment, bacteria were applied to surface sterilized IR50 rice seeds and were incubated overnight. Excess amount of fluid was drained and it was observed that seeds had 10^9 cfu/g. The treated seeds were sown in pots containing sterile soil. Root dipping was carried out on 21-day-old rice seedlings that were uprooted, rinsed gently in sterile water, and roots were dipped for 30 min in the respective bacterial suspensions before transplanting into sterile soil. When the bacterial suspension was applied also as foliar spray on 15-day-old rice seedlings (IR50), the treatment was made until minute droplets appeared on both surfaces of the entire leaf. Rice seedlings that received plain cmc solution as spray served as control.

The survival of bacteria was monitored by removing 2 cm sections of the rice stem (1 cm above and 1 cm below the point of infiltration). To assess the movement of bacteria from the point of infiltration, 2 cm long

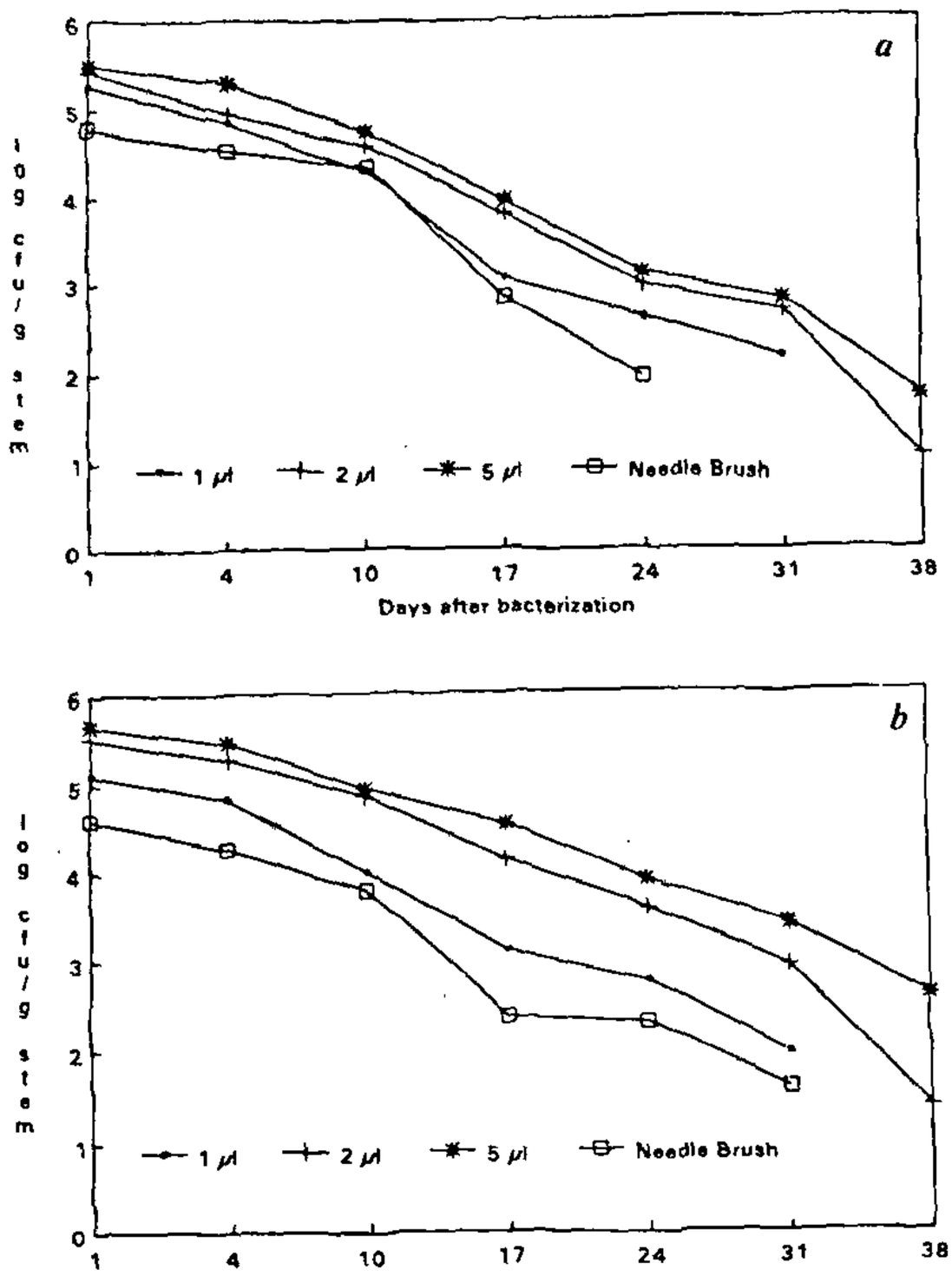
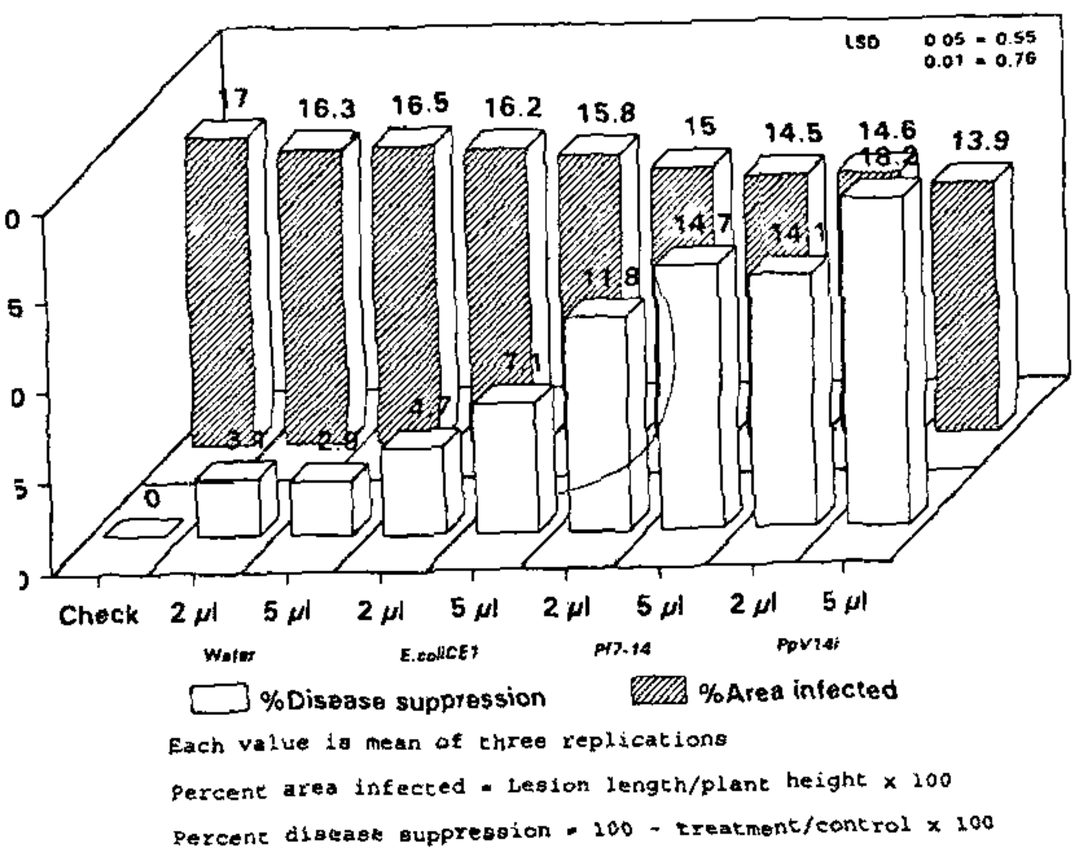


Figure 1. Survival of lacZY-marked bacterial strains after infiltration and needle brush application into the rice (cv. IR50) stem. (*a*), *P. putida* V14igal; (*b*), *P. fluorescens* 7-14gal.



gure 2. Suppression of rice sheath blight caused by *Rhizoctonia lani* in plants by plant associated *Pseudomonas* strains of bacteria the induction of systemic resistance. Disease incidence in *R. solani* fected controls is assigned a value of 100%.

section was also sampled (1.5–3.5 cm [I stage] and 4–6 cm [II stage]) above the point of infiltration. The samples were weighed, rinsed for 5 min in running tap water and then were soaked for 2 min in 2% sodium hypochlorite solution. Subsequently, they were washed thrice in sterile water and were triturated in 1 ml of 0.01 M phosphate buffer (pH 7.1). Plants were sampled at 1, 4, 10, 17, 24, 31 and 38 days after the bacterial infiltration. Endophytic presence of bacteria was assessed in 1 g of rice roots removed from 10 and 15-day-old seedlings that received seed treatment or root-dip treatment or from 1 g of leaf sample in the case of foliar spray (5 and 10 days after bacterization) treatment. Appropriate serial dilutions of the triturated samples were plated on to triplicate agar plates of KB (King's B) with X-Gal with or without cycloheximide (40 µg/ml) for 7-14gal and V14igal strains. Samples that received *E. coli* CE1 strain were plated on LB agar with X-Gal and samples of untreated checks were plated on KB/LB agar plates. After 48 h of incubation, the blue coloured bacterial colonies were counted (7-14gal and V14igal were also observed under UV lamp at 365 nm for their fluorescent character) and their cfu/g of tissue was determined.

A field experiment for the systemic suppression of rice sheath blight (ShB) was conducted by planting surface sterilized rice (IR50) seeds in a randomized complete block design. Rice seedlings of 20–22-day-old were infiltrated with 2 µl and 5 µl of 10^9 cfu/ml of 24 h grown wild type 7-14, V14i and CE1 strains. Control plants received plain sterile water or did not receive any treatment (untreated checks). One day after the bacterial treatment, the plants were inoculated with *R. solani*. Inoculum of *R. solani* has been described previously[23] and was applied at the rate of half a teaspoon per hill close to the sheath portion of the rice plants. ShB severity rating was recorded on the 10th day after *R. solani* inoculation from randomly selected 10 rice plants per plot (0.5 × 0.5 m)/treatment. From these assessments, per cent disease incidence and per cent disease control were determined by statistical analysis.

The data on the survival of the two marked strains of *P. putida* V14igal and *P. fluorescens* 7-14gal are presented in Figure 1 *a*, *b*. These data show that bacteria survived up to day 38 when 2 µl or 5 µl aliquots were infiltrated. Whereas bacteria applied in a needle brush did not survive beyond 24 days (V14igal) or 31 days (7-14gal) after the treatment. The *E. coli* CE1 was detected up to 10 days after infiltration or needle brush treatment. In water treatment or nontreated control tissues, blue-coloured colonies did not develop.

Upward migration of the marked strains of V14igal and 7-14gal occurred up to 6 cm (stage II) away from the point of infiltration in the rice sheath or stem (Table 1). Beyond this point the stem and the leaves did not

Figure 3. Reduction of sheath blight in IR50 rice plants due to the induction of systemic resistance by *Pseudomonas putida* strain. *Right*: Plants infiltrated with 5 µl of PpV14i strain; *Left*: Nontreated control plants infected with *R. solani*.

Table 1. Migration of *Pseudomonas putida* V14igal, *P. fluorescens* 7-14gal and *Escherichia coli* CE1 in the rice stem

Bacterial strain	Aliquot of inoculation used (µl)	Sampling stage	Mean log cfu/g of stem tissue recovered after (days)				
			1	4	10	17	24
V14igal	1	I	4.90[a]	3.89	3.11	ND[b]	ND
	1	II	4.71	3.67	2.38	ND	ND
	2	I	4.95	4.08	3.45	1.94	ND
	2	II	4.79	3.69	2.57	ND	ND
	5	I	4.98	4.58	4.08	1.36	ND
	5	II	4.86	3.99	3.11	1.04	ND
	NB[c]	I	3.94	3.23	1.68	ND	ND
	NB	II	2.94	1.72	ND	ND	ND
7-14gal	1	I	4.88	3.73	2.91	ND	ND
	1	II	4.71	3.51	1.95	ND	ND
	2	I	4.96	3.74	3	1.43	ND
	2	II	4.78	3.59	2.46	ND	ND
	5	I	5.08	4.58	3.43	2.92	2.54
	5	II	4.86	3.99	2.18	ND	ND
	NB	I	3.96	3.32	ND	ND	ND
	NB	II	3.04	1.85	ND	ND	ND
CE1	2	I	3.96	3.15	1.66	ND	ND
	2	II	2.99	1.11	ND	ND	ND
	NB	I	1.83	ND	ND	ND	ND
	NB	II	ND	ND	ND	ND	ND

[a] Each figure is a mean of 3 replications.
[b] Not detected.
[c] Needle brush application.

have the cells of the marked strains. However, below the point of infiltration these bacteria were detected at 57 cfu/g tissue (V14igal) and 39 cfu/g tissue (7-14gal) from day 10 after infiltration.

From the results there is no indication for the endophytic presence of the marked strains of bacteria in roots of seedlings that were raised from bacteria-treated seeds. There was also no endophytic presence of bacteria in leaf sample of rice seedlings that received foliar sprays of V14igal and 7-14gal. Bacteria applied as a root-dip were detected at 23 cfu/g root in V14igal treatment.

Figure 2 shows that *Pseudomonas* strains (V14i and 7-14) present in the rice stem reduced the sheath blight incidence by 18 and 15% in comparison with infected control, which is taken as 100% infection. Yet these bacteria were not present on the surface of the sheath or pseudostem. In spite of the absence of bacteria on surface of the rice plant there is evidence for pathogen suppression by bacteria (Figure 3). This is most likely due to the induced systemic resistance triggered by the endophytic bacteria introduced into the rice stem. The results indicate that there is a minimal reduction of ShB even with *E. coli* CE1 or by water infiltration. This might be due to the response of plants to lipopolysaccharides or other defence-inducing signal factors. This is the first report on the induction of induced systemic resistance (ISR) in rice by the rhizosphere-bacteria. Our future studies will be directed to analysing the nature of the defence substances (phytoalexins, salicylic acid or other PR proteins) involved in the induced systemic resistance in rice.

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