Chapter 5
Biological Control of Bacterial Blight of Rice

Pathogen: *Xanthomonas oryzae* pv. *oryzae*

Need for Biological Control

Bacterial blight (BB) of rice is among the most devastating rice diseases that occur globally, ranging in distribution from 20 °S in Queensland, Australia to 58 °N in Heilang Jiang, China (Mew, 1989). The disease is particularly destructive in the tropics, especially in Asia, where rice is grown throughout the year, during heavy rains of the monsoon season, and peaking at the flowering stage. Several disease-management strategies aimed at reducing crop losses and averting outbreaks of epidemics have been developed in the past. Effective chemical control for the management of rice BB is yet to be developed because of the extreme variability of the pathogen population in its sensitivity to antibiotics and chemicals used for control. Resurgence of drug-resistant populations also poses serious threats to efforts directed towards development of effective, long-lasting controls. Though the exploitation of host resistance appears feasible, breeding for BB-resistance with single major gene has proved unsuccessful due to rapid evolution of sub-populations that overcome these resistance genes. Biological control therefore appears to offer an ecology-conscious and cost-effective solution to this serious threat to rice cultivation.

Plant-Associated Bacteria as Biocontrol Agents

Pseudomonas, Bacillus Strains

In preliminary reports from this author's laboratory strains of *P. fluorescens* were shown to inhibit the growth of X. *oryzae* pv. *oryzae* in the laboratory (Sivamani, Anuratha, & Gnanamanickam, 1987). Over the years, however, there has not been a major study on the biological suppression of bacterial blight with bacterial agents. In this situation, twomajor studies were carried out in our laboratory during

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1997–2003. One of them involved *Bacillus* strains (Vasudevan, 2002) and the other involved *Pseudomonas fluorescens* strains that produced 2,4-diacetylphioglucinol (DAPG) (Velusamy, 2003; Velusamy & Gnanamanickam, 2003; Velusamy, Immanuel, Gnanamanickam, & Thomashow, 2006). These studies had the following objectives: 1. to identify efficient strains of *Bacillus and P. fluorescens*, and 2. to establish their role in (a) biological suppression of rice bacterial blight and (b) enhanced growth/yield of rice.

In these experiments, seeds of rice cultivar, IR24 (obtained from S. McCouch, Cornell U.) were used. This cultivar has no known resistance gene(s) for BB resistance and is a universal susceptible check for BB. Rice cultivar, Jyothi, an elite high-yielding variety grown extensively in the state of Kerala in southern India and susceptible to BB was also used. *Bacillus* and *Pseudomonas* strains were isolated from rice rhizosphere samples collected from different locations in southern India and on the basis of dual plate laboratory assays were short-listed if they showed consistent antibiosis to *X. oryzae* pv. *oryzae*.

Among 516 morphologically distinct rice-associated *Bacillus* strains isolated from rhizosphere samples, 42 strains were antagonistic to X. *oryzae* pv. *oryzae* in the dual-plate assays. The zone of inhibition caused by these strains varied from 1.2 to 4 cm in diameter (Fig. 5.1; Table 5.1). Among 637 strains of fluorescent bacteria, 278 strains (44%) showed inhibition of *X. o* pv. *oryzae* in laboratory assays. Twenty-seven of the antagonists produced 2,4-diacetylphloroglucinol (DAPG) and had the characteristic 745-bp fragment amplified by the PCR reaction when the sequence-specific primers developed from the *PhlD* sequence of *P. fluorescens* Q2-67 were used (Raaijmakers, Weller, & Thomashow, 1997; Velusamy & Gnanamanickam, 2003) (Fig. 5.2).

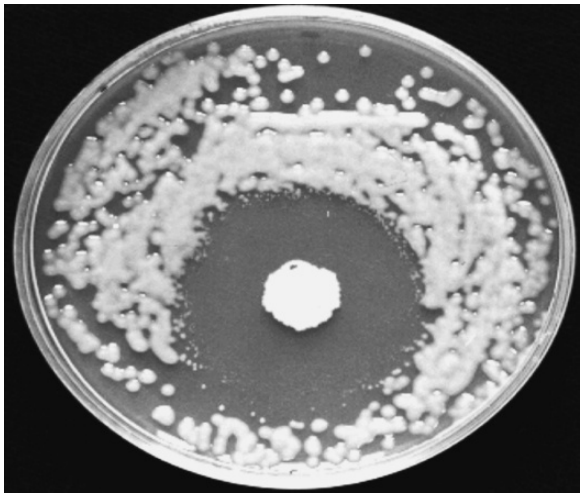

Fig. 5.1 Inhibition of *Xanthomonas oryzae* pv. *oryzae* by *Bacillus lentus* ALP18 in laboratory assays (Vasudevan, 2002)

Table 5.1 Inhibition of growth of *Xanthomonas oryzae* pv. *oryzae* in the laboratory and reduction of bacterial blight (BB) in rice cultivars, IR24 and Jyothi by *Bacillus* strains (Gnanamanickam, Vasudevan, & Velusamy, 2004; Vasudevan, 2002)

*Bacillus*strain	Diameter (in cm) of inhibition zone of *Xanthomonas oryzae* pv *oryzae*	BB reduction in rice (%) from the untreated control			
		1	2	3	4
1. *B. lentus* (ALP 18)	1.2	37.4	55.3	31.4	32.9
2. *B. cereus* (NGC I 15)	4.0	58.8	54.2	21.4	NT
3. *B. circulans* (VY I 18)	2.2	57.7	52.0	56.8	18.3
4. *Bacillus sp*. (CAL 9)	3.0	36.1	52.9	54.6	34.9
5. *Bacillus sp*. (MON 2–17)	2.0	38.5	54.9	45.2	53.9

1 and 2 – Disease reduction in rice cv. IR24 in the net-house and field experiment, respectively. 3 and 4 – cv. Jyothi (net-house and field).

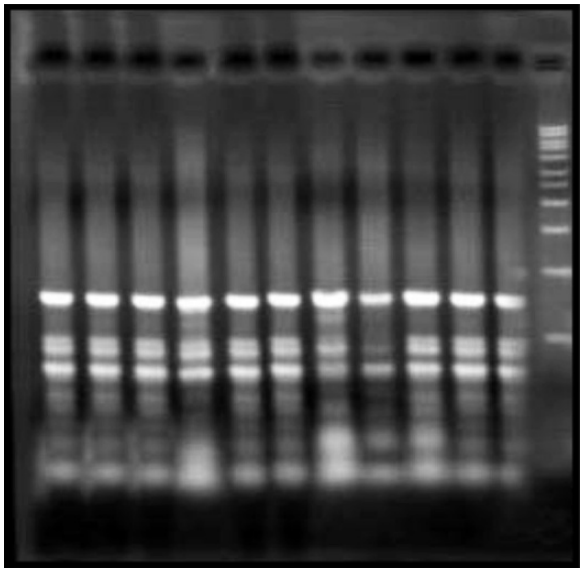

Fig. 5.2 PCR-based detection of 2,4-diacetylphloroglucinol (DAPG) production by *Pseudomonas fluorescens* from the rice rhizosphere of southern India (Velusamy & Gnanamanickam, 2003)

Net-House and Field Experiments

Identification of efficient *Bacillus* strains for bacterial blight suppression.

Bacterial strains were prepared at 10^8 cfu/ml in 1% carboxymethylcellulose and applied as seed treatment before sowing. Each bacterium was also applied as two foliar spray applications on the 35th and 45th day after planting in field plots. Application of 42 bacterial strains to rice cv. JR24 and Jyothi resulted in significant reductions in the mean length of BB lesions in bacteria-treated plants compared to the untreated control in both the net-house and field experiments (Table 5.1).

Disease suppression on cv. 1R24 ranged from 36 to 59% in the net-house experiment, while on cv. Jyothi, disease reductions ranged from 21 to 57%. Disease

suppression of more than 50% was observed in the field experiment with the cultivar 1R24 (Table 5.1). The levels of BB suppression in cv. Jyothi ranged from 18 to 54%.

Five superior strains, namely, ALP 18 *(B. lentus)*, NGC 115 *(B. cereus)*, VY 118 *(B. circutans)*, CAL 9 *(Bacillus* sp.), and MON 2-17 *(Bacillus* sp.) were identified at species level and were selected for further evaluation (Table 5.1).

Evaluation of DAPG-Producing *P. fluorescens* for Suppression of BB

Twenty-seven of the DAPG-producer strains of *P. fluorescens*, also identified as efficient antagonists of *X. oryzae* pv. *oryzae* were evaluated in a field experiment planted with rice cultivar IR24 in Pattambi, Kerala (Table 5.2). Application protocols used were the same as in the earlier experiment conducted with *Bacillus* strains. In addition to the seed treatment and two foliar spray applications, the rice seedlings also received a root-dip in the respective bacterium at 10^8 cfu/ml for 1 hour at transplanting. Mean BB lesion length on rice leaves in bacteria-treated plants ranged from 7.8 to 21.9 cm while in the untreated plants, the average BB lesion length was 22.0 cm (Table 5.2). The untreated plants showed severe BB development with long and spreading blight lesions while the bacteria-treated plants had relatively healthy leaves with shorter BB lesions. The following seven strains that included, IMV14, PTB9, MDR7, KAD7, VEL17, VGP13, and PDY7 reduced BB lesion length by 50-to-64% and were identified as strains of *P. fluorescens*. *P. fluorescens* strain PTB9 afforded the maximum protection of 64%.

Mechanism(s) of BB Suppression

Bacillus

One of the Bacillus strains, ALP 18 produced a heat-resistant (at 121 °C) and pronase-resistant metabolite in culture fluids. The crystalline product of this substance, was produced at the rate of 1.6 mg/ml, and when amended in to peptone-sucrose agar (PSA), inhibited the growth of *X. oryzae* pv. *oryzae*. A careful analysis of its physical properties through FTIR, ^{1}H NMR, and ^{13}C NMR analyses suggested that the heat-resistant antibacterial metabolite is kanosamine (Vasudevan, 2002). Kanosamine production has been reported earlier from other *Bacillus* spp. (Cron et al., 1958; Milner et al., 1996; Umezawa, Umnio, Shibahara, Hamada, & Hashimoto, 1967).

Pseudomonas

To study the relationship between DAPG production and BB suppression by *P. fluorescens* PTB *9*, the most efficient strain and a producer of DAPG (identification made by the PCR method developed by Raaijmakers et al., 1997) (Fig. 5.2).

Table 5.2 Bacterial blight suppression by 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains. Field experiment, Pattambi, Kerala, India (Velusamy, 2003)

S. No	Name of bacterial treatment	Mean of BB lesion length (cm)[a, b]	Difference in lesion length from control (cm)	Percent of disease suppression	LSD value	
					5%	1%
1	KAD7	10.15	11.88**	53.93	3.4	4.5
2	IMV14	9.53	12.50**	56.74	3.4	4.5
3	IMV2	14.72	7.31**	33.18	3.4	4.5
4	BGR19	12.62	9.41**	42.71	3.4	4.5
5	PTB9	7.83	14.20**	64.46	3.4	4.5
6	MON1	13.85	8.18**	37.13	3.4	4.5
7	TVM8	16.85	5.18**	23.51	3.4	4.5
8	VEL17	11.07	10.96**	50.75	3.4	4.5
9	VEL10	18.17	3.86*	17.52	3.4	4.5
10	GDY4	12.90	9.13**	41.44	3.4	4.5
11	GDY7	12.05	9.98**	45.30	3.4	4.5
12	TRP5	14.42	7.61**	34.54	3.4	4.5
13	TRP18	11.38	10.65**	48.34	3.4	4.5
14	MDR9	16.90	5.13**	23.29	3.4	4.5
15	MDR7	10.04	11.99**	54.43	3.4	4.5
16	STR7	14.09	7.94**	36.04	3.4	4.5
17	VGP13	11.19	10.84**	51.21	3.4	4.5
18	MDR16	11.87	10.16**	46.12	3.4	4.5
19	PDY7	9.50	12.53**	51.88	3.4	4.5
20	VLB7	20.03	2.00	9.08	3.4	4.5
21	KVR5	13.42	8.61**	39.08	3.4	4.5
22	TNI13	15.82	6.21**	28.19	3.4	4.5
23	KOV8	19.89	2.14	9.71	3.4	4.5
24	RJP31	21.92	0.11	0.50	3.4	4.5
25	KOV3	20.13	1.90	8.62	3.4	4.5
26	PDU1	19.43	2.60	11.80	3.4	4.5
27	PDU9	21.58	0.45	2.04	3.4	4.5
28	Control	22.03	0.00	0.00	0.0	0.0

[a]Each value is a mean of 20 measurements. [b]Mean of normalized lesion length (mean of lesion length in control-lesion length in treatment). *Reduction in lesion length significant at 1% and **Reduction in lesion length significant at 5%.

Extracts of 72 h-grown culture fluids of *P. fluorescens* PTB 9 were analyzed by hplc and the extracts yielded 40 μg of the antibiotic in 1 ml of the culture. When this material dissolved in 65% methanol was assayed for antibiosis towards *X. oryzae* pv *oryzae* in agar (PSA) well diffusion assays, 50–75 μg/ml inhibited the growth of the rice BB pathogen. In control plates which had 65% methanol, there was no inhibition.

Phl-Negative Mutants

In a net-house experiment five Phl$^-$ mutants of *P. fluorescens* PTB9 generated through transpositional mutagenesis-(*Tn5*-Km) were less effective in protecting

Fig. 5.3 Protection of IR24 rice plants from BB infection by *Pseudomonas fluorescens* PTB9 (first leaf from the *left*) and less protection by its Phl⁻ mutants (a,b,c,d, and e) (represented by leaf number 2–6 from the *left*). The last leaf (7th) is from an untreated check and has spreading BB lesion for the entire length of the leaf

IR24 rice plants against BB (Fig. 5.3, Table 5.3). While the wild type strain suppressed BB by 59.5%, the four phl⁻ mutants afforded 19.7, 17.1, 23.8 and 20.8% reductions of BB, respectively.

It can be mentioned that this was the first record of any detailed study on the biological control of rice bacterial blight. *Bacillus* and *P. fluorescens* strains applied as seed treatment and foliar applications afforded significant levels (> 50%) of disease suppression. It is also the first time when 2,4-diacetylphloroglucinol-producing tropical strains of *P. fluorescens* were identified in rice rhizospheres (Velusamy, 2003).

DAPG is a polyketide antibiotic which was known for its key role in "take-all decline" in the United States and for suppression of diseases of tobacco and other crops, has been shown to play a definite role in BB suppression in rice (Bangera & Thomashow, 1999; Defago et al., 1990; Gnanamanickam et al., 2004; Raaijmakers & Weller, 1998; Velusamy et al., 2006).

Enhancement of Rice Growth due to *Bacillus* Treatments

In addition to BB suppression, increases in average plant height were observed in *Bacillus*-treated rice plants against untreated check. Also, mixtures of three and four *Bacillus* strains resulted greater enhancement of plant height than the respective treatments of single strains. Increases in numbers of tillers per hill (as much as 3 fold as that of untreated control) (Fig. 5.4) and grain yields were also observed (Gnanamanickam et al., 2004). Isolation and purification of the

Table 5.3 Evaluation of *Pseudomonas fluorescens* PTB9 and its *Phl*$^-$ mutants for suppression of bacterial blight (BB) in IR24 rice. Greenhouse experiment, Chennai, southern India, July–November, 2003 (Velusamy, 2003; Velusamy et al., 2006)

Bacterial strain	Mean BB lesion length (cm)[a]	Difference in lesion length from control (cm)[b]	Percent BB suppression
Wild type *Pseudomonas fluorescens* PTB9	7.66**	11.27**	59.52
Phl$^-$ mutants	15.21^{ns}	3.72^{ns}	19.65
PTB9a	15.69^{ns}	3.24^{ns}	17.11
PTB9b	14.43^{ns}	4.50^{ns}	23.77
PTB9c	14.99^{ns}	3.94^{ns}	20.81
PTB9d	15.50^{ns}	3.43^{ns}	18.11
PTB9e			
Check	18.93	0.00	0.00

**Reduction in lesion length significant at 1% by LSD method of analysis; ^{ns} = not significant. [a]Mean of three replications. [b]Mean of normalized lesion lengths (mean lesion length in untreated control/check deducted from mean lesion length in bacteria-treated plants).

metabolites produced by the *Bacillus* strains showed production of 3.9–5.5 μg/ml of IAA by the different *Bacillus* strains (Vasudevan, 2002). Some of them also produced GA_3-like substance (Lindow et al., 1998; Tien, Qaskis, & Hubbell, 1979).

Fig. 5.4 Increased tillers in rice cv. IR24 due to *Bacillus* strain Mon2-17 treatment (Gnanamanickam et al., 2004; Vasudevan, 2002)

Lysobacter

Ji, Wei, He, Wu, & Bai (2008) used whole cells or dilutions of culture fluids of a novel strain of *Lysobacter antibioticus* 13-1 isolated from the rice rhizosphere in Yunnan province of China for biological suppression of rice bacterial blight (BB) both in greenhouse and multi-location field trials. In greenhouse experiments, whole bacterial broth culture (WBC) of strain 13-1 afforded up to 69.7% BB suppression. In three field trials, strain 13-1 reduced BB incidence by 73.5%, 78.3%, and 59.1%, respectively. However, disease suppression by strain 13-1 varied significantly among different rice cultivars and also showed variations with pathogen (*Xoo*) strains used. The biocontrol agent outperformed the chemical/antibiotic, zhongshengmycin (1%) that was used as the chemical standard. This is the first report on the use of *L. antibioticus* for rice BB control (Ji et al., 2008). It may be mentioned that *Lysobacter* are gliding bacteria of the family *Xanthomonadaceae* within the gamma *proteobacteria* and the genus has 13 known species. *Lysobacter enzymogenes* (synonym: *Stenotrophomonas maltophilia*) strainC3 is a well known biocontrol agent for several of the fungal pathogens (Giesler & Yuen, 1998; Jochum, Osborne, & Yuen, 2006; Kobayashi, Reedy, Palumbo, Zhou, & Yuen, 2005).

Bacteriocinogenic Strains of X. oryzae pv. oryzae

The avirulent or less virulent strains of the BB pathogen that produce bacteriocins are antagonists of the pathogen *Xoo*. Bacteriocin production was reported to have a role in BB suppression (Sakthivel & Mew, 1991). However, more recent reports do not agree with this claim. Dardick, de Silva, Shen, and Ronald, (2003) found no correlation between in vitro bacteriocin activity and in planta inhibition of the BB pathogen.

Epiphytic Erwinia herbicola

Hsieh and Buddenhagen (1974) and Santhi, Unnamalai, and Gnanamanickam, (1987) observed that epiphytic populations of *E. herbicola* present in rice leaf surfaces lowered the pH of rice leaves by producing an acid and thus suppressed the growth of *X. oryzae* pv. *oryzae*. This appears to be a natural method of BB suppression and has been verified recently by Babu and Thind (2005)

Transgenic Rices for BB Management

The cloning of two of the major genes for BB resistance, *Xa*21 and *Xa*1 are major achievements in plant pathology. *Xa*21 was transferred by IRRI scientists from the wild rice species, *Oryza longistaminata* into a cultivated indica variety IR24.

Pam Ronald and her colleagues at University of California-Davis used map-based cloning method to clone *Xa*21 (Song et al., 1995). These researches showed that its molecular structure represented an uncharacteristic class of plant disease-resistance genes as it coded for a receptor-kinase like protein. Yoshimura et al. (1998) cloned the second BB resistance gene, *Xa*-1 also by using map-based cloning method. Wang, Song, Ruan, Sideris, and Ronald (1996) constructed the first set of japonica transgenic rices of T-309 by incorporating *Xa*21 and showed that it conferred resistance to all pathotypes of *X. oryzae* pv. *oryzae*. Since T-309 was not a commercial variety, Datta and his co-workers at IRRI, Philippines introduced *Xa*21 into several japonica and indica varieties, such as IR72, MH63, and IR51500 (Datta, 2002; Tu et al., 1998; Tu, Datta, Khush, Zhang, & Datta, 2000). The transgenic plants carried a 3.8 kb *EcoRV*-digested DNA fragment corresponding to most of the coding region of *Xa*21 gene. Detailed protocols for generation and assay of the transgenic rices were described by Datta (2002).

In collaboration with Datta (2002) and Narayanan et al. (2004) generated blast and BB-resistant indica varieties, CO39 and IR50. For protocols, please see Chapter 4 of this volume.

Bioassay for Bacterial Blight Resistance

The set of test cultivars IR50, IR24 (susceptible controls), IRBB21 (near isogenic line for *Xa21*), IRBB4 (near isogenic line for *Xa4*), and 20 lines from each transgenic line were sown in plastic trays. Plants were tested against three different races of *Xanthomonas oryzae* pv. *oryzae (Xoo)* to differentiate the genes *Xa21* and endogenous *Xa4* such as *PXO86* (race 2), *PXO99* (race 6) and *PXO341* (race 10). The inoculum of each strain was prepared by incubating the bacteria on Wakimoto's medium (Medium composition: Modified Wakimoto's medium (MF-P): sucrose – 30g, bacteriological peptone – 5g, calcium nitrate – 0.5g, sodium phosphate (dibasic) – 0.82g, ferrous sulphate – 0.05g, agar – 15–17g, pH – 6.0) for 72h at 30 °C, then suspending each pure culture in sterile distilled water and adjusting the inoculum to about 10^9 cells per milliliter. At the maximum tillering stage, each plant was inoculated with the above three strains of *Xoo* using the leaf clipping method at the transgenic greenhouse, IRRI. Reaction of rice plants to each race of *Xoo* was scored 14 days after inoculation.

Resistance to bacterial blight was observed in T_1 plants of IR50 and CO39 (Table 5.4). Bacterial blight lesions of < 2.0 cm length observed in transgenic plants were characteristic resistance reactions. The non-transformed BB-susceptible parent plants showed bacterial blight lesions of > 10.0 cm length.

These transgenic plants will be evaluated in the rice fields in southern India under strict biosafety precautions and their field performances will be monitored carefully. This has not happened yet and efforts are in progress.

In recent years, several transgenic lines/varieties of rices, including Pusa Basmati 1, the aromatic rice, have been constructed and evaluated for BB resistance (Swamy et al., 2006). They are all not described in this volume.

Table 5.4 Reactions of transgenic IR50 plants carrying *Xa21* to races 2 and 6 of *Xanthomonas oryzae* pv. *oryzae* (Narayanan et al., 2002, 2004)

Genotype	Race 2 (PXO86)				Race 6 (PXO99)			
	7d		14d		7d		14d	
	MLL (cm)	Reaction	MLL (cm)	Reaction	MLL (cm)	Reaction	MLL (cm)	Reaction
IR50 (C)	5.3	S	7.8	S	5.8	S	9.4	S
IR24 (C)	13.5	S	15.8	S	14.5	S	17.8	S
IRBB21 (C)	1.3	R	2.4	R	3.4	R	4.7	R
IRBB4 (C)	4.6	R	6.8	S	6.4	S	7.9	S
T 13 R-1[a]	2.6	R	2.6	R	2.8	R	3.8	R
T 13 S-1[b]	5.6	S	7.8	S	12.8	S	15.3	S

[a]Average of 15/20 T_1 progenies showing resistance to BB pathogen. [b]Average of 5/20 T_1 progenies showing susceptible to BB pathogen.
C – non-transformed parental lines; R – Resistant; S – Susceptible; MLL – Mean Lesion Length.

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