Identification of 2,4-diacetylphloroglucinol production by plant-associated bacteria and its role in suppression of rice bacterial blight in India

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* is one of the most important and oldest known rice diseases. It was first noticed by the farmers of Japan[1] in 1884. Severe crop losses of 10–20% in moderate conditions and up to 50% in highly conducive conditions have been recorded in several parts of Asian and South-East Asian countries[2,3]. Globally, the occurrence of the disease has been reported from Northern Australia, Africa, USA and parts of Asia. In India, BB disease is known to routinely occur in rice-growing states like Andhra Pradesh, Bihar, Haryana, Kerala, Orissa, Punjab and Uttar Pradesh. The disease occurred in an epidemic form during 1998 in Palakkad district of Kerala[4] and since then it has been observed almost every year. BB management has been carried out using chemicals and resistant cultivars. Breeding for bacterial blight resistance is the most economic strategy for disease management and this has only been partially successful because of the enormous diversity in the pathogen[5]. More recently, varieties of transgenic indica rice resistant to bacterial blight have been generated[6] but are still to be field-evaluated in India.

Biological control, therefore, assumes special significance in being an ecology-conscious, cost-effective alternative strategy for bacterial blight management. Recent studies from our laboratory have identified rice-associated rhizosphere *P. fluorescens* and *Bacillus* strains, which could provide significant levels of BB suppression and, in addition, could also substantially enhance plant growth and grain yield[7–11]. Antagonistic bacteria are considered ideal biological control agents owing to their rapid growth, easy handling and aggressive colonization of rhizosphere[12]. These bacteria may mediate biocontrol by one or more of the several mechanisms of disease suppression. A primary mechanism of pathogen inhibition is by the production of antibiotics[13–16]. 2,4-diacetylphloroglucinol (DAPG) is produced by fluorescent *Pseudomonas* spp. of diverse geographic origin that have in common the ability to suppress one or more root and seedling diseases of

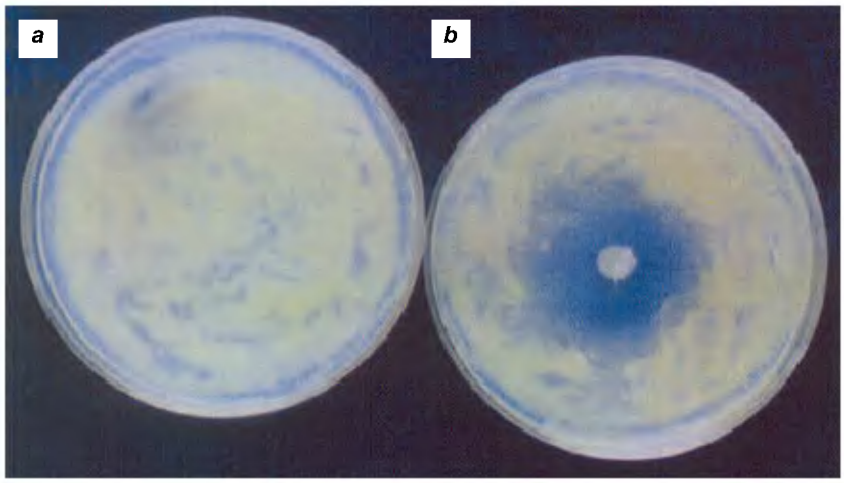

Figure 1. ***a***, Inhibition of the growth of *Xanthomonas oryzae* pv. *oryzae* induced by *P. fluorescens* strain PTB9. ***b***, Control plate of peptone-sucrose agar.

Table 1. Plant-associated fluorescent pseudomonads that inhibited the growth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and produced 2,4-diacetylphloroglucinol

Place of collection	Code number of strain	Diameter (in cm) of inhibition zone of *Xoo*	Production of 2,4-DAPG[1]	Per cent of rice BB suppression in field expt[2]
Karnataka	KAD7	1.8	+	53.93
Karnataka	IMV14	2.8	+	56.74
Karnataka	IMV2	2.8	+	33.18
Karnataka	BGR19	0.5	+	42.71
Kerala	PTB9	2.1	+	64.46
Kerala	MON1	3.3	+	37.13
Tamil Nadu	TVM8	1.2	+	23.51
Tamil Nadu	VEL17	2.3	+	54.08
Tamil Nadu	VEL10	0.7	+	17.52
Tamil Nadu	GDY4	2.1	+	41.44
Tamil Nadu	GDY7	2.3	+	45.30
Tamil Nadu	TRP5	1.7	+	34.54
Tamil Nadu	TRP18	2.3	+	48.34
Tamil Nadu	MDR9	2.1	+	23.29
Tamil Nadu	MDR7	1.3	+	54.43
Tamil Nadu	STR7	1.8	+	36.04
Tamil Nadu	VGP13	2.5	+	51.62
Tamil Nadu	MDR16	1.4	+	46.12
Tamil Nadu	PDY5	1.8	+	56.88
Tamil Nadu	VLB7	1.7	+	9.08
Tamil Nadu	KVR5	2.5	+	49.75
Tamil Nadu	TNI13	2.2	+	28.19
Tamil Nadu	KOV8	3.0	+	9.71
Tamil Nadu	RJP31	2.4	+	0.50
Tamil Nadu	KOV3	1.8	+	49.21
Tamil Nadu	PDU1	2.9	+	11.80
Tamil Nadu	PDU9	2.7	+	2.04
Untreated control		–	–	22.03

[1]Production of 2,4-DAPG was identified through a PCR-based screening procedure which amplified a 745 bp DNA fragment in these strains.
[2]Results of a replicated field experiment (RBD) conducted at the Regional Agricultural Research Station, Pattambi, Kerala. Each figure is a mean of three replications.

crop plants caused by soil-borne pathogens. These include take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*[17], root rot of wheat caused by *Fusarium oxysporum*[18,19], black root of tobacco caused by *Thielaviopsis basicola*[20,21] and damping-off of sugar beet caused by *Pythium ultimum* Trow[22,23]. In addition to its antifungal activity, it has some antiviral properties[24] and also inhibits the growth of soft-rotting bacteria and cyst nematodes of potato[25].

Bangera and Thomashow[26] reported four genes (*PhlACBD*) that are involved in the synthesis of 2,4-DAPG. These genes were localized in a 6.5 kb DNA fragment of a *P. fluorescens* strain. Protein products of all four genes are required for the synthesis of both monoacetyl phloroglucinol (MAPG) and 2,4-DAPG. Of these, only the product of *PhlD* has a striking homology to a plant chalcone synthase of a novel kind of polyketide synthase (PKS) not previously described in microorganisms.

Rhizosphere samples from different crops like rice, sorghum, sunhemp, finger millet, black gram and green gram were collected from various places of Karnataka, Maharashtra and Tamil Nadu. Bacteria were isolated from the soil suspensions of these samples after serially diluting them and plating onto King's B agar medium. Fluorescent bacteria were selected under UV light ($\lambda = 365$ nm) and further purified on the same medium. A total of 637 fluorescent bacterial strains were obtained from the rice rhizosphere samples. Out of these, 278 strains showed antibiosis towards *Xoo* in laboratory assays (Figure 1), and only 27 of these strains produced 2,4-DAPG, as confirmed by PCR analysis (Table 1). Their identification as strains of *P. fluorescens* was confirmed on the basis of their biochemical profile.

Bacteria were grown on King's B medium for 48 h at 27°C. Two bacterial colonies (2 mm diameter) were suspended in 100 µl lysis solution and incubated for 10 min at 99°C. The suspension was centrifuged for 1 min at 5000 rpm, then it was frozen (– 20°C) for 30 min and after thawing, 4 µl of the supernatant was carefully taken and used for PCR reaction. Production of 2,4-DAPG, intervening sequence-specific primers *Phl*2a 20-mer (5′-GAGGACGTCGAAGACCACCA-3′) and *Phl*2b 20-mer (5′-ACCGCAGCATCGTGTATGAG-3′) confirmed by the method of Raaijmakers *et al.*[27] also developed the *PhlD* sequence of *P. fluo-*

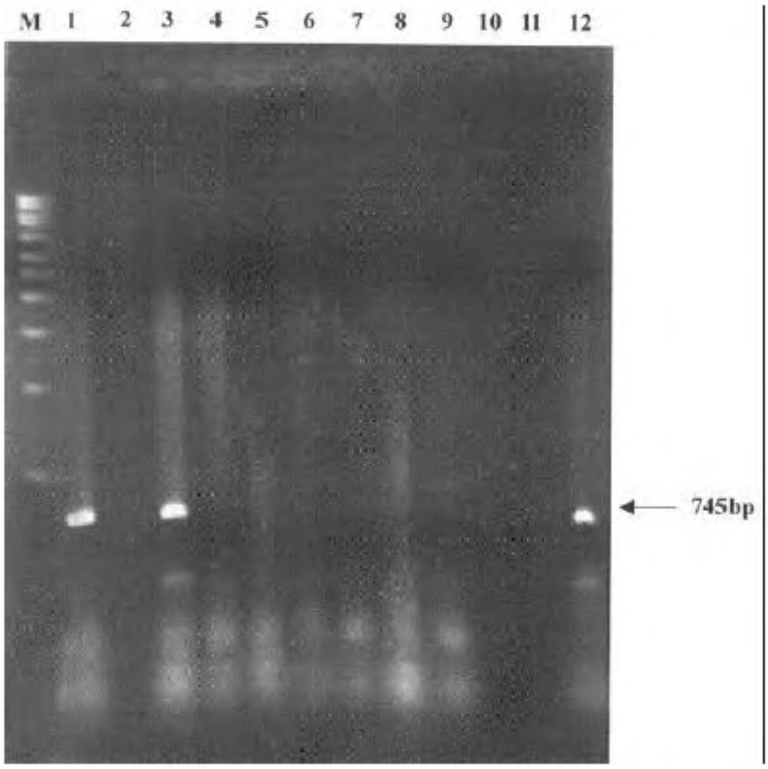

Figure 2. A PCR-based screening method for the detection of 2,4-DAPG in plant-associated *P. fluorescens* strains. Primers for *PhlD* gene amplified a 745 bp fragment characteristic of 2,4-DAPG (lanes 3, 12). Lane M has a 1 kb ladder (molecular weight marker) and lane 1 has a reference strain positive for 2,4-DAPG production.

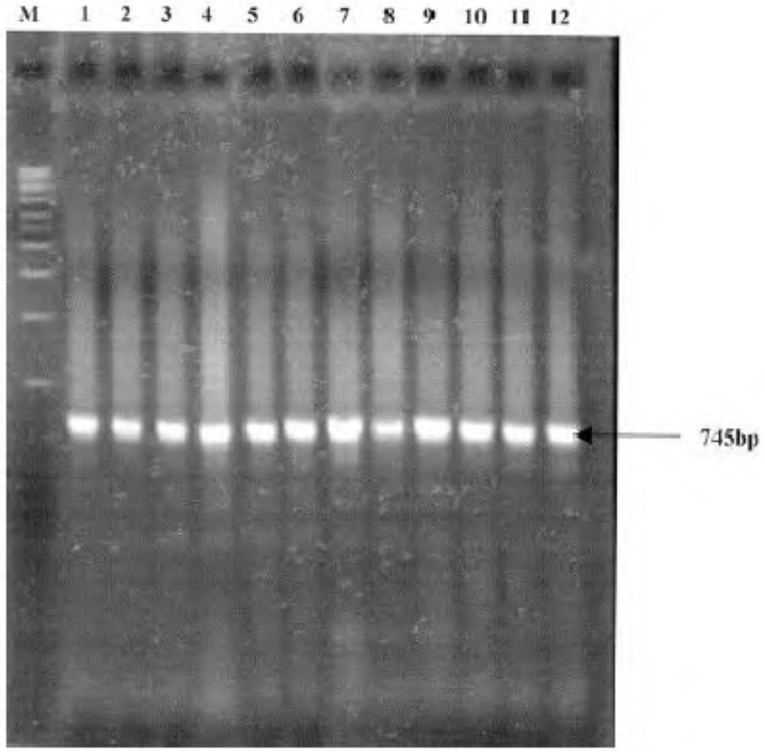

Figure 3. Amplification of a 745 bp DNA fragment in 2,4-DAPG-producing plant-associated *P. fluorescens* strains. Lane M: 1 kb ladder (molecular weight marker); lanes 1–12: DNA samples of 2,4-DAPG producer strains.

rescens Q2-8. PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl lysates of bacterial suspension, 1X PCR buffer, 0.5 g/l bovine serum albumin, 5% dimethyl sulphoxide, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.4 µM of each primer and 1.4 U of *Taq* DNA polymerase. Amplification was performed with a PTC-100™ thermal cycler (MJ Research Inc., Watertown, MA), using the following PCR conditions: initial denaturation for 2 min at 94°C, followed by 30 PCR cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 10 min. Samples (9 µl) of the PCR products were separated on a 1.2% agarose gel in 1X TBE buffer at 75 V for 3 h. The gel was stained with ethidium bromide for 30 min, and the PCR products were visualized with UV transilluminator. By this method, a 745 bp DNA fragment was amplified from the PCR products of a 2,4-DAPG-producing reference strain *P. fluorescence* CHAO (obtained from G. Defago, Switzerland), whereas no PCR products were amplified from DNA of the non-producing strains (Figure 2). The predicted 745-bp fragment of the same size was amplified from DNA of all other 2,4-DAPG producing strains (Figure 3).

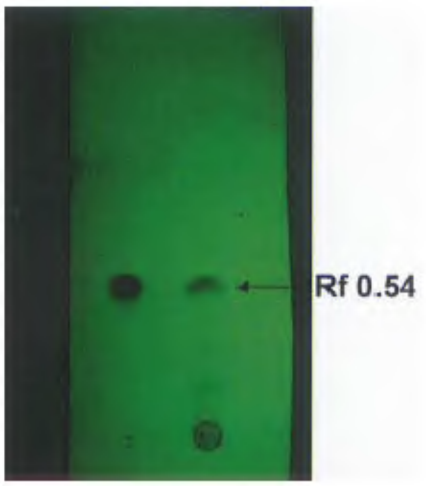

Figure 4. Thin layer chromatogram (pre-coated silica gel) shows matching Rf values of 0.54 for 2,4-DAPG extracted from a *P. fluorescens* strain (right lane) and a reference sample of DAPG (left lane).

2,4-DAPG production by this sub-set of 27 strains was further confirmed by co-chromatography of ethyl acetate extracts of their culture fluids with an authentic sample of 2,4-DAPG (gift sample provided by G. Defago, Switzerland) using standard protocols and previously described methods. Figure 4 shows a thin layer chromatogram with identical Rf values of 0.54 for the 2,4-DAPG extracts from the Indian strains and for the reference DAPG. The purified antibiotic 2,4-DAPG inhibited the growth of *Xoo* on peptone-sucrose agar (PSA) plates in laboratory assays (Figure 5).

In a field experiment, 2,4-DAPG producer strains of *P. fluorescens* (Table 1) were applied as a seed coat to rice seeds of bacterial blight-susceptible cv. IR24. The seed coat contained 10^8 cfu/ml in 0.1% carboxymethylcellulose (cmc) which served as an adhesive when the bacterial treatments were given overnight (12–14 h). Subsequently, the seeds were sown in seed beds and the rice seedlings raised from bacterial treatments and a non-treated control was transplanted when they were 21 days old in 1 × 1 m sized field plots at the Regional Agricultural Research Station, Pattambi, Kerala. At the time of transplanting, the seedlings were given a root-dip in the bacterial cell suspension (containing 10^8 cfu/ml) (or in sterile distilled water in the case of untreated control). When the seedlings were 35 and 45 days old, two additional foliar sprays of the respective bacteria at 10^8 cfu/ml were made. A day after the second foliar spray application or when the seedlings were 46 days old, they were inoculated with a cell suspension (10^6 cfu/ml) of rice bacterial blight pathogen, *Xoo* by the clipping method. The inoculated plants (both bacterial treatments and control) were evaluated for the development of bacterial blight lesions by measuring the lesion length (in cm) 15 days after the inoculation. Table 1 shows reductions in bacterial blight lesion length, compared with lesions of those in untreated control plants. While 6 of the 17 strains recorded more than 50% reduction in bacterial blight incidence, application of strain PTB9, a rice-associated bacterium, reduced bacterial blight by 64.5% (Table 1).

Thus the results suggest that DAPG production in crop rhizosphere is yet another important mechanism that reduces the severity of this important rice disease. This could be further exploited or accelerated by developing superior 2,4-DAPG-producing strains as microbial inoculants for rice. We have initiated some research on the genetic improvement of 2,4-DAPG-producing strain to combine other traits in them.

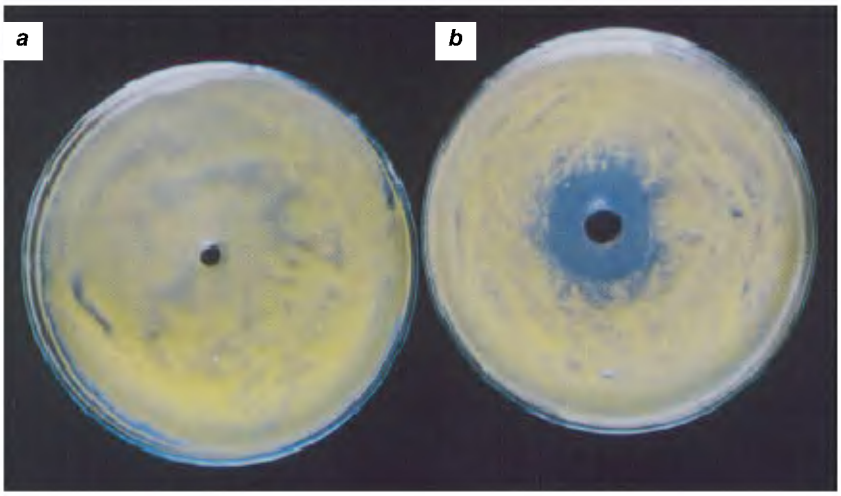

Figure 5. Biological activity of 2,4-DAPG purified from culture fluids of a *P. fluorescens* strain. ***a***, Growth of the rice bacterial blight pathogen. ***b***, Control.

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Mechanism of adhesion in a hillstream fish, *Glyptothorax garhwali* Tilak, as revealed by scanning electron microscopy of adhesive apparatus

The Western Himalayan hillstreams pose harsh living conditions to the fish communities because of varied topography, torrential water currents coupled with a variety of substratum. The most important characteristics in response to these conditions are the integumentary modifications in the form of an adhesive disc, which has become a life-saving kit for most of the hillstream fishes. Though investigations have been carried out using light microscope[1,2], the exact mechanism of adhesion and detailed structure of the adhesive apparatus are still not well-known. Hence, an attempt has been made to study the details of the adhesive mechanism of a typical hillstream fish, *Glyptothorax garhwali* Tilak using scanning electron microscopy (SEM).

G. garhwali is an extremely specialized fish inhabiting the fast-flowing upper reaches of Western Himalayas. It has a well-developed adhesive apparatus, unlike *Schizothorax richardsonii* in which the lower lip is modified to form a suctorial disc. In the present case, the lateral folds of the skin just above the adhesive disc and a portion of the adhesive disc were subjected to SEM investigation. The adhesive apparatus was removed with the help of a sharp blade and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2–7.4 for 24 h. After several washings in the rinsing buffer, 0.1 M sodium cacodylate buffer containing 7% sucrose was added and further dehydration was carried out in various grades of acetone. The specimens after acetone treatment were transferred into emylacetate solution, dried in a Polaram Critical Point Dryer (CPP), mounted on metal stubs and then coated with 100 Å thick layer of gold in JEOL sputter ion coater. The specimens were examined with JEOL TSM 6100 SEM at 20 kV and the images were observed on the screen. Negatives were prepared for photography.

The studies indicated the presence of numerous mucous pores (MP) over the lateral folds and the adhesive disc. The primary function of these is the secretion of mucus (Figure 1 *a*). Tiny hook-shaped projections arising out of the epithelial cells (Figure 1 *a*) are present alongside. The adhesive disc has numerous long, hook-shaped, spine-like structures (LHSs) (Figure 1 *c* and *d*), which are the epidermal growths (EG) (Figure 1 *b*). These epidermal growths are present all over the central pit, which aids in the process of adhesion. For the purpose of attachment, the LHSs get entangled with the rough surface of the substratum, forming a sort of interlocking mechanism. Along with the mucus openings, these hook-like, spiny structures present the most advanced case of morphological adaptation amongst the hillstream fishes. What seems to be the case here is that mucus is secreted on receiving the necessary stimuli from the surrounding environment, providing a sort of platform in the form of a feeble adhesion for the secondary adhesion of spines with the rough surface of the substratum. There is