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Pseudomonas fluorescens mediated antifungal activity against *Rhizoctonia solani* causing sheath blight in rice

Rangaraj Nandakumar, Subramanian Babu, Ramalingam Radjacommare, Thiruvengadam Raguchander and Ramasamy Samiyappan

Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore 641003, India

Summary. Antifungal activity of *Pseudomonas fluorescens* strains PF1, FP7 and PB2 was tested against the rice sheath blight pathogen *Rhizoctonia solani*. The bacterial strains produced antibiotic 2,4-diacetyl phloroglucinol, iron chelating siderophore, hydrogen cyanide, lytic enzymes, such as chitinase and β-1,3-glucanase in culture, and induced chitinase in plants. Soaking *R. solani* sclerotia in either bacteria-free culture filtrates or in sap extracted from plants treated with *Pseudomonas* strains PF1 and FP7 or inoculated with *R. solani* significantly reduced the germination capacity of the sclerotia. The inability of treated sclerotia to cause sheath blight symptoms indicated loss of virulence. In all the experiments strain PF1 or FP7 always performed better than PB2. The 35 kDa chitinase induced by *Pseudomonas* in rice plants was purified and its antifungal activity against mycelium and sclerotia of *R. solani* was demonstrated. The production of antibiotics, antifungal compounds, bacterial lytic enzymes and the induction of plant chitinase were correlated with antifungal activity against *R. solani* sclerotia.

Key words: antifungal metabolites, chitinase, *P. fluorescens*, *R. solani*, sclerotia.

Introduction

Rhizoctonia solani (Kuhn), the sheath blight pathogen of rice, overwinters in soil as sclerotia or thickened hyphae in plant debris. Sclerotia form the primary source of inoculum and there is a positive correlation between the number of viable sclerotia in the soil and the incidence of sheath blight (Premalatha Dath, 1990). Sclerotia produced in one growing season become an additional source of inoculum for the following season or crop and thereby increase blight severity. Hence, the most effective and long-term strategy for the management of sheath blight is to reduce the concentration of initial inoculum by killing sclerotia, or to inhibit their germination. Several studies have examined the potential use of biocontrol agents for control of sheath blight through the application of antifungal bacteria isolated from the soil, sclerotia or other habitats (Mew and Rosales, 1986; Vasanthadevi *et al.,* 1989; Chatterjee *et al.,* 1996; Rabindran and Vidhyasekaran, 1996; Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al.,* 2001a, 2001b)

Production of antibiotics, iron chelating siderophores, cyanide and lytic enzymes by soil-residing bacteria are thought to be the major contributing factors in the biocontrol of pathogens by inhibiting both the germination and growth of their survival structures and mycelium in the rhizo-

Corresponding author: R. Samiyappan
Fax: 91 422 431672
E-mail: rsamiyappan@hotmail.com

sphere and spermosphere of plants (Thomashow and Weller, 1988; Voisard *et al.,* 1989; Rosales *et al.,* 1995; Nielsen *et al.,* 1998; Singh *et al.,* 1999). *Pseudomonas* spp. are known to produce one or an array of antifungal metabolites, and lytic enzymes such as chitinase and glucanase in culture (Friedlender *et al.,* 1993; Neilsen *et al.,* 1998; Viswanathan and Samiyappan, 2001). Although evidence for *in situ* production of metabolites and lytic enzymes is limited, examples of the suppression of soil-borne plant pathogens by both antibiotic-producing and chitinolytic strains have recently been described (Chatterjee *et al.,* 1996; Thomashow and Weller, 1996; Nielsen *et al.,* 1998). Purified forms of such metabolites and lytic enzymes inhibited the mycelial growth of certain fungi (Rosales *et al.,* 1995; Radhajeyalakshmi *et al.*, 2000). In addition, chitinase and glucanase activity was induced in plants previously treated with strains of some *Pseudomonas* spp. (Schneider and Ullrich, 1994; Nandakumar *et al.,* 2001a). The role of microbial as well as plant chitinase and glucanase against phytopathogenic fungi is well established since fungal cell walls contain chitin and glucan as major components (Ji and Kuc, 1996). Recently chitinases have taken center stage in the study of host-parasite interactions and in the control of fungal diseases (Neuhaus, 1999). The *Pseudomonas fluorescens* strains used in this study have already been shown to reduce sheath blight severity *in vitro* and under field conditions (Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al.,* 2001a; 2001b; Radjacommare *et al.,* 2002). Hence, the aim of the present study was to investigate the production of antifungal metabolites and lytic enzymes by these *P. fluorescens* strains, to purify the *Pseudomonas*-induced chitinase in rice and to determine its antifungal activity against *R. solani* as shown in the inhibition of sclerotia germination.

Materials and methods

Plant, pathogen and bacterial strains

The susceptible rice cv. IR50 and a virulent isolate of the sheath-blight pathogen, *R. solani* (RS7, anastomosis group AG1; Sriram *et al.,* 1997) were used in all experiments. The *P. fluorescens* strains used in this study were obtained from the *P. fluorescens* culture collections of the Department of Plant Pathology, TNAU, Coimbatore, India. The bacterial cultures were maintained in King's broth B (KBB) (King *et al.,* 1954) with 30% glycerol at -80°C.

Production of secondary metabolites by *Pseudomonas fluorescens*

Siderophore

Siderophore production was assayed by a plate method using the ternary complex chromeazurol S (CAS)/Fe^{3+}/hexadecyltrimethylammonium bromide as an indicator (Schwyn and Neilands, 1987). A 48-h-old culture of *P. fluorescens* was streaked onto succinate medium amended with the indicator and incubated at room temperature for three days. Formation of a bright zone with a yellowish fluorescence in the dark-blue medium indicated production of siderophore. Siderophore production was quantified according to Reeves *et al.* (1983). The bacterial strains were grown in KBB for 3 days and the supernatant from the cultures was collected by centrifugation at 2,000 rpm (all rpm indications in this paper were with a rotor radius of 8 cm) for 10 min. The pH was adjusted to 2.0 with 1 N HCl and extracted four times with equal volumes of ethyl acetate. The ethyl acetate fractions were pooled, evaporated to dryness and the residue dissolved in 5 ml of 50% ethanol. Five ml of Hathway's reagent (1.0 ml of 0.1 M $FeCl_3$ in 0.1 N HCl to 100 ml distilled water and 1.0 ml of 0.1 M potassium ferricyanide) was added to 5 ml of the ethyl acetate fraction in 50% ethanol, shaken thoroughly and the absorbance measured at 700 nm for dihydroxy phenols. A standard curve was prepared with dihydroxy benzoic acid and the quantity of siderophore produced was expressed as μg of benzoic acid equivalents ml^{-1}.

2, 4-diacetylphloroglucinol

Bacterial cultures were grown in 5 ml of pigment production medium (peptone, 20 g; glycerol, 20 ml; NaCl, 5 g; KNO_3, 1 g; distilled water, 1 l; pH 7.2) for 4 days on a rotary shaker at room temperature (28±2°C). The culture was centrifuged at 3,500 rpm for 5 min, the supernatant collected, acidified to pH 2 with 1 N HCl and then extracted with an equal volume of ethyl acetate (Rosales *et al.,* 1995). The ethyl acetate extract was reduced to dryness *in vacuo*, and the residue dissolved in methanol. Twenty microliter samples were applied to thin layer chromatography plates coated with a 250 μm layer of silica gel and developed in ace-

tonitrile/methanol/water (1:1:1). Spots were visualized by spraying with diazotized sulphanilic acid. R_f values of the spots were compared with synthetic 2,4-diacetyl phloroglucinol (50 mg ml^{-1} methanol).

Hydrogen cyanide

Bacterial cultures were streaked onto tryptic soy agar medium (Difco Laboratories, Detroit, MI, USA). A filter paper disc (1.5 cm diam.) was soaked in picric acid (picric acid, 2.5 g; Na_2CO_3 12.5 g; distilled water, 1 l) and placed in the lid of each Petri dish (Miller and Higgins, 1970). Dishes were sealed with Parafilm and incubated for four days. Hydrogen cyanide (HCN) production was assessed by the colour change observed in yellow filter paper, turning brown to reddish brown. Reactions were scored as weak (yellow to light red), moderate (brown) and strong (reddish brown).

Preparation of bacterial culture filtrate

From the glycerol stock, fresh cultures were prepared on plates of solid King's medium B (KMB). A single colony of each bacterial strain was inoculated and grown in KBB with constant shaking at 150 rpm for 48 h at room temperature. The culture was centrifuged at 6,000 rpm for 10 min to separate the bacterial cells. The supernatant was again centrifuged at 10,000 rpm for 10 min at 4°C and passed through a bacteriological filter (0.45 μm). This cell free culture filtrate was used to assess its antifungal activity against sclerotia of *R. solani*.

Estimation of lytic enzyme activity

Chitinase activity of bacterial culture filtrates was determined using colloidal chitin as a substrate. The release of N-acetyl glucosamine (GlcNAc) was determined colorimetrically at 585 nm using GlcNAc (Sigma, St. Louis, MO, USA) as a standard and expressed as ηmol of GlcNAc released min^{-1} ml^{-1} of culture filtrate (Boller and Mauch, 1988). β-1,3-glucanase activity was colorimetrically assayed following the method given by Pan *et al.* (1991) using glucose as a standard and expressed as μg of glucose equivalents min^{-1} ml^{-1} of culture filtrate

Preparation of bacterial inoculum for glasshouse study

A single colony of bacteria was grown as described above. The cells were harvested after 48 h of growth. All bacterial cell suspensions in this study were adjusted to 9×10^8 cfu ml^{-1}. Two percent carboxy methyl cellulose was added to the suspension as a sticker and this mixture was used as bacterial inoculum (Vidhyasekaran and Muthamilan, 1999).

***Pseudomonas* treatment and challenge inoculation in the glasshouse**

The *Pseudomonas* strains were tested for their efficiency in controlling sheath blight under glasshouse conditions. All four treatments: seed treatment, seedling root dip, soil application and foliar spray, were tested on the same material. For seed treatment, rice seeds were surface-sterilised with 1% sodium hyphochlorite for 1 h, washed with distilled water, and soaked in double the volume of bacterial suspension. After 24 h, the bacterial suspension was drained off and the seeds were dried in the shade for 30 min. The seeds were then allowed to sprout for another 24 h before sowing (Vidhyasekaran and Muthamilan, 1999). Twenty-five days after sowing, the seedlings were transplanted at the rate of four seedlings per hill and five hills per pot (30 cm diam. containing 10 kg of clay soil). For root dipping, bundles of rice seedling (approximately 200 seedlings) were dipped in a bacterial suspension for 2 h, ensuring that the roots alone were immersed in the inoculum, and then replanted in plastic pots. For soil applications, 25 ml of bacterial suspension was poured into each pot 30 days after planting, and for foliar application 25 ml of bacterial suspension per pot was sprayed onto the leaves 30 days after planting. Plants raised from seeds treated with carbendazim (4 g kg^{-1} of seeds) and seedlings sprayed with 0.1% carbendazim at 30 days after planting served as the fungicide checks, and wherever needed foliar sprays with distilled water were carried out in the control treatment. Control plants without *Pseudomonas* treatment were also maintained. All treatments were replicated three times in a factorial randomized complete block design. When the plants were 40 days old, sclerotia of *R. solani* were placed (2 per tiller) between the stem and sheath of the rice plants. The inoculated part of the plants was covered with absorbent cotton held in place with Parafilm. The inoculated area was regularly moistened with sterile distilled water to maintain high humidity. Development of symptoms was observed 7 days after inoculation and samples were collected for sap extraction.

Extraction of sap from rice plants

The sheath of rice plants treated with *P. fluorescens* strains PF1 and FP7, and with or without inoculation of *R. solani*, was cut and powdered in liquid nitrogen. Two grams of powdered sample were homogenised in 5 ml of 0.1 M sodium phosphate buffer (pH 7) and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was retained and total protein content was determined (Bradford, 1976). Protein content of plant extract from different treatments was adjusted uniformly with 0.1 M sodium phosphate buffer (pH 7), passed through a bacteriological filter to remove the bacteria from the plant extract and used for studying the antifungal activity against sclerotia of *R. solani*.

Antifungal activity of bacterial culture filtrate and plant extract

One ml of bacteria-free culture filtrate and an equal amount of plant extract were dispensed each into three microfuge tubes containing ten sclerotia (10-day-old) per tube and incubated at room temperature. There were three replicates for each treatment. After soaking for 24, 48 and 72 h in either bacterial culture filtrate or plant extract, the sclerotia were blotted dry under sterile conditions and plated on potato dextrose agar (PDA) (Difco). Sclerotia soaked in sterile water, carbendazim (0.1% solution) and without soaking served as controls. Sclerotial germination was checked with a dissecting microscope after incubation at room temperature (28±2°C) for 48 h (Vasanthadevi *et al.,* 1989).

Virulence of sclerotia

To test the virulence of ungerminated and germinated sclerotia soaked for 72 h in bacterial culture filtrate or plant sap, they were inoculated individually onto 40-day-old rice plants as described above. Development of symptoms was recorded 7 days after inoculation on a scale of 0–5 (Sriram *et al.,* 1997).

SDS-PAGE and Western analysis for chitinase isoforms

Protein samples (50 μg) were separated in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). A medium-range protein marker (Genei, Bangalore, India) was used as molecular mass standard. After electrophoresis, proteins were electrotransferred from the gel to nitrocellulose membranes using a Biorad Trans blot semi-dry transfer cell (Bio-rad, Hercules, CA, USA) in accordance with manufacturer's instructions. Western analysis was carried out as described by Gallagher *et al.* (1995) with 28 kDa barley chitinase polyclonal antibody (kindly provided by S. Muthukrishnan, Kansas State University, KA, USA). The bands were visualised in an alkaline phosphatase reagent containing nitroblue tetrazolium and 5 bromo, 4 chloro, 3 indolyl phosphate (Sigma, USA).

Purification of chitinase

Tissue collection and enzyme extraction

In our earlier study, the induction of 35 kDa chitinase in *Pseudomonas*-treated plants inoculated with *R. solani* was well demonstrated through a chitinase assay procedure and Western analysis (Nandakumar *et al.,* 2001a). Hence, in this study, the 35 kDa rice chitinase was purified from the sheath portions of rice plants treated with *Pseudomonas* strain FP7 (one week after pathogen inoculation). Samples were collected from the glasshouse-grown plants and immediately homogenised with liquid nitrogen. Ten g of powdered sample was extracted with 100 ml of 100 mM sodium citrate buffer (pH 5) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. Proteins in the supernatant were precipitated with 60% $(NH_4)_2SO_4$. The pellets obtained after centrifugation at 12,000 rpm for 20 min at 4°C were resuspended in a minimal amount of sterile distilled water and dialysed overnight at 4°C against 25 mM sodium phosphate buffer pH 7.0 (SPB). The dialysed enzyme solution was mixed in a beaker with 5 g of colloidal chitin equilibrated with SPB. After 4 h of mixing, the chitin was washed three times with 30 ml of SPB, followed by three more washes with 30 ml of 25 mM sodium citrate, pH 4.0 in a beaker. The chitin was then placed in an affinity column (1.5×20 cm, Pharmacia, Uppsala, Sweden) and bound material was eluted with 20 mM acetic acid, pH 3.2 (Swegle *et al.,* 1992). Fractions of acetic acid eluate (10 ml) were collected. Protein content of all fractions was measured, pooled peak fractions were lyophilised and further purified by gel filtration. The lyophilised protein was dissolved in 2 ml of 25 mM SPB (pH 7.0) and chromatographed on a Sephadex G-75 column previously equilibrat-

ed with 50 mM SPB pH 6.8 containing 100 mM of NaCl. Series of five-ml fractions were collected. The peak fractions were pooled, separated by SDS-PAGE and the gel stained with Coomassie brilliant blue. Chitinase activity in the peak fraction was assayed as described earlier. The peak fractions were then analysed by Western blotting with barley chitinase antibody. The purified chitinase solution was filter-sterilized and used for assay of its antifungal activity.

Antifungal activity of purified chitinase against mycelium and sclerotia of *R. solani*

The assay was carried out in Petri plates (9 cm diam.) containing 15 ml of PDA medium. A mycelial disc of *R. solani* was placed at the centre and three sterile filter paper discs (6 mm diam.) were placed on the agar surface 3 cm from the centre. The purified chitinase and the crude dialysed fraction (20 µl aliquots containing 50 µg of protein), and buffer were applied on the filter paper and incubated at room temperature. For sclerotia, 10 sclerotia were separately soaked in 500 µl of purified chitinase solution (500 µg ml^{-1}), 50 mM SPB and incubated at room temperature for 72 h. The germination and virulence tests were carried out as described above.

Results

Antifungal compounds and lytic enzymes of bacterial strains

All three isolates had a positive reaction to siderophore production on succinate medium. Strains PF1 and FP7 produced the greatest amount of siderophore in culture. A strong reaction for HCN production was observed for PF1 while FP7 and PB2 had moderate and weak reactions respectively. Although all three strains produced 2-4- diacetylphloroglucinol, FP7 had the same R_f value as synthetic phloroglucinol (Table 1). More chitinolytic activity was detected in the culture inoculated with FP7 than in cultures with other strains. PF1 showed higher glucanase activity than the other strains.

Antifungal activity of bacterial culture filtrate and plant sap

Soaking sclerotia in bacteria-free culture filtrate of PF1, FP7 and PB2 either significantly reduced the germination of sclerotia or retarded mycelial growth from germinated sclerotia. When comparing culture filtrates of the three strains, the culture filtrate of FP7 showed the greatest level of antifungal activity. Only 33.3% of the sclerotia soaked in FP7 culture filtrate for 72 h germinated, compared with 100% of those soaked in sterile water or unsoaked (Table 2). The sap extracted from rice plants treated with the two *Pseudomonas* strains PF1 and FP7 was also tested for antifungal activity. Sap from both strains showed statistically equal levels of antifungal activity; germination of sclerotia soaked for 72 h in sap from plants treated with PF1 and FP7 was 30.0 and 33.3% respectively. Antifungal activity increased when the soaking time increased from 24 h to 72 h. The sap from plants treated with *Pseudomonas* alone ex-

Table 1. Secondary metabolites and lytic enzymes of *Pseudomonas fluorescens* strains.

P. fluorescens strain	2,4-diacetyl phloroglucinol[a]	Siderophore[b]	HCN[c]	Chitinase activity[d]	β-1,3-glucanase activity[e]
PF1	+ (0. 73)	+ (24.3) a[f]	Strong	5.45 b	30.01 a
FP7	+ (0. 76)	+ (23.9) a	Moderate	6.33 a	26.34 b
PB2	+ (0. 73)	+ (14.5) b	Weak	3.82 c	21.20 c

+ or - , Positive or negative to the test.
[a] Values in parentheses indicate the R_f value. Synthetic phloroglucinol has an R_f of 0.76.
[b] Values in parentheses are siderophore produced by bacterial isolates expressed as µg of benzoic acid equivalents per ml of culture filtrate.
[c] HCN production is scored as: weak, light brown; moderate, brown; strong, reddish-brown; none, yellow.
[d] Chitinase activity is expressed as ηmol of GlcNAc released min^{-1} ml^{-1} of culture filtrate.
[e] β-1,3-glucanase activity is expressed as µg of glucose equivalents min^{-1} ml^{-1} of culture filtrate.
[f] Means followed by a common letter within a column are not significantly different (P=0.05) by Duncan's Multiple Range Test.

hibited a significant level of antifungal activity compared to untreated plants (Table 3).

Virulence of sclerotia

The virulence of ungerminated and germinated sclerotia were tested in inoculation experiments and compared on the basis of the severity of the necrosis they produced in the leaf sheaths. When 53 and 51 ungerminated sclerotia (soaked in bacterial culture filtrate and plant sap respectively) inoculated in rice plants, 38 and 36 were unable to germinate and cause any sheath blight symptoms. Inoculation with germinated sclerotia always produced typical sheath blight identical to that seen in plants inoculated with either unsoaked or sterile water soaked sclerotia, with maximum disease scores of 4 or 5. On the other hand, all the carbendazim-soaked sclerotia failed to produce blight symptoms (Table 4).

Purification of chitinase

In earlier studies it was observed that inoculation of rice plants with *R. solani* induced two isoforms of chitinase with molecular weights of 35 and 28 kDa. These isoforms were also induced in *Pseudomonas*-treated plants (Fig. 1). Hence, in this

Table 2. Antifungal activity of bacterial culture filtrate against sclerotia of *Rhizoctonia solani* with different soaking times (24, 48, and 72 h).

Culture filtrate	Percentage of sclerotial germination[a]		
	24	48	72
PF1	66.7 c[b]	46.7 c	43.3 b
FP7	66.7 c	43.3 c	33.3 c
PB2	75 b	56.7 b	46.7 b
Sterile water	100 a	100 a	100 a
Unsoaked	100 a	100 a	100 a
Carbendazim (0.1% solution)	3.3 d	0 d	0 d

[a] Data were arcsine transformed before analysis. Values are the mean of three replications.
[b] Means followed by a common letter within a column are not significantly different (P=0.05) by Duncan's Multiple Range Test; LSD for comparison of any two means: 5.48 (P=0.05).

Table 3. Antifungal activity of plant sap extracted from rice plants treated with *Pseudomonas fluorescens* strains PF1 and FP7. Germination of *Rhizoctonia solani* sclerotia was recorded after soaking in the sap for 24, 48 or 72 h.

Plant treatment	Percentage of sclerotial germination[a]		
	Soaking time (h)		
	24	48	72
PF1 alone	46.7 de[b]	43.3 cd	41.7 c
PF1+*R. solani*	40 ef	40 cd	30 d
FP7 alone	50 d	46.7 c	36.7 cd
FP7+*R. solani*	36.7 f	36.7 d	33.3 d
Untreated rice plants	76.7 b	63.3 b	66.7 b
Untreated rice plants + *R. solani*	70 c	66.7 b	60 b
Sterile water	100 a	100 a	100 a
Unsoaked	100 a	100 a	100 a
Carbendazim (0.1% solution)	0 g	0 e	0 e

[a] Data were arcsine transformed before analysis. Values are the mean of three replications.
[b] Means followed by a common letter within a column are not significantly different (P=0.05) by Duncan's Multiple Range Test; LSD for comparison of any two means: 4.09 (P=0.05).

study, the 35 kDa chitinase was purified by ammonium sulphate precipitation, chitin affinity chromatography and gel filtration. The purified 35 kDa protein is shown in Fig. 2a; it strongly reacted with barley chitinase antibody in Western analysis (Fig. 2b).

Antifungal activity of purified chitinase

Purified chitinase significantly reduced mycelial growth of *R. solani* (Fig. 3). Out of 10 sclerotia soaked in purified chitinase solution, only four germinated on PDA. The ungerminated sclerotia did not cause sheath blight symptoms in rice plants on further inoculation (Table 4).

Discussion

The biological control of soil-borne pathogens with antagonistic bacteria, particularly *Pseudomonas* spp. belonging to Plant Growth Promoting Rhizobacteria, has received prominent attention because of the dual role of these bacteria in plant-growth promotion and disease control (Zehnder *et al*., 2001). When identifying potential biocontrol agents, antifungal metabolites produced by them or the ability of these agents to induce antifungal compounds in plants are important factors to be taken into account. Many research groups are actively trying to find metabolite(s) produced by biocontrol agents or induced by them in plants, and that will suppress particular diseases (Dowling and O'Gara, 1994). In earlier studies (Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al.,* 2001a; 2001b; Radjacommare *et al.,* 2002) a talc-based formulation of the *P. fluorescens* strains also used here reduced sheath-blight incidence under field conditions in different zones of Tamil Nadu state, India. In this study we studied the various antifungal compounds and lytic enzymes produced by strains PF1, FP7 and PB2. Induction of plant chitinase production was found to be a mechanism of disease control by inhibiting sclerotia germination of the blight pathogen.

Considerable attention has been paid to the ability of bacterial antagonists to reduce the number of sclerotia in infested soil (Bin *et al.,* 1991), because this has a practical bearing on the control of sclerotial fungi. Bacteria can weaken or kill sclerotia and thereby reduce the amount of initial inoculum available in the following crop (Epert and

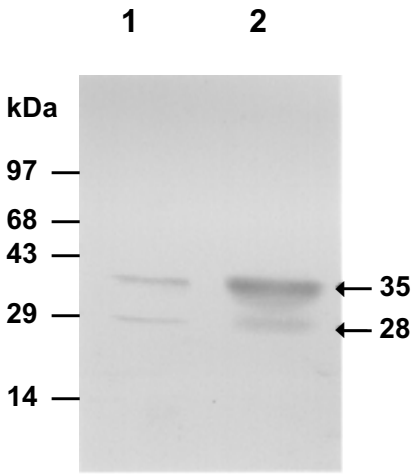

Fig. 1. Induction of chitinase in *Pseudomonas fluorescens* treated rice plants. Lane 1, rice plants inoculated with *Rhizoctonia solani*; lane 2, *Pseudomonas fluorescens* treated rice plants inoculated with *R. solani*.

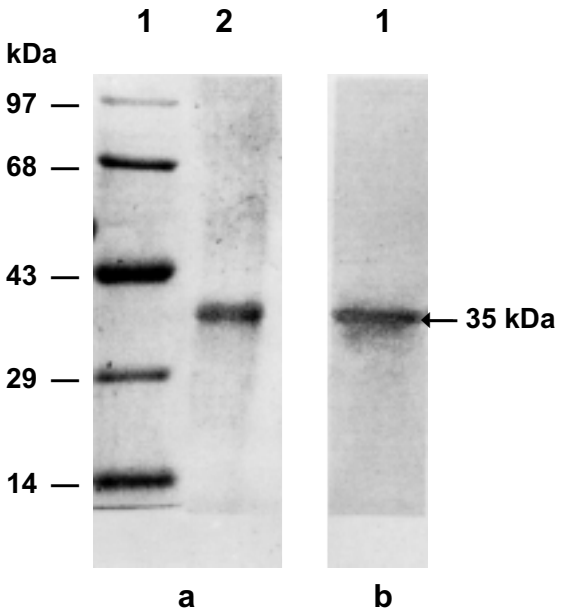

Fig. 2. SDS-PAGE analysis (a) and Western analysis (b) of purified protein from rice sheath treated with *Pseudomonas fluorescens* strain FP7. Lane 1, protein marker; lane 2, purified 35 kDa protein.

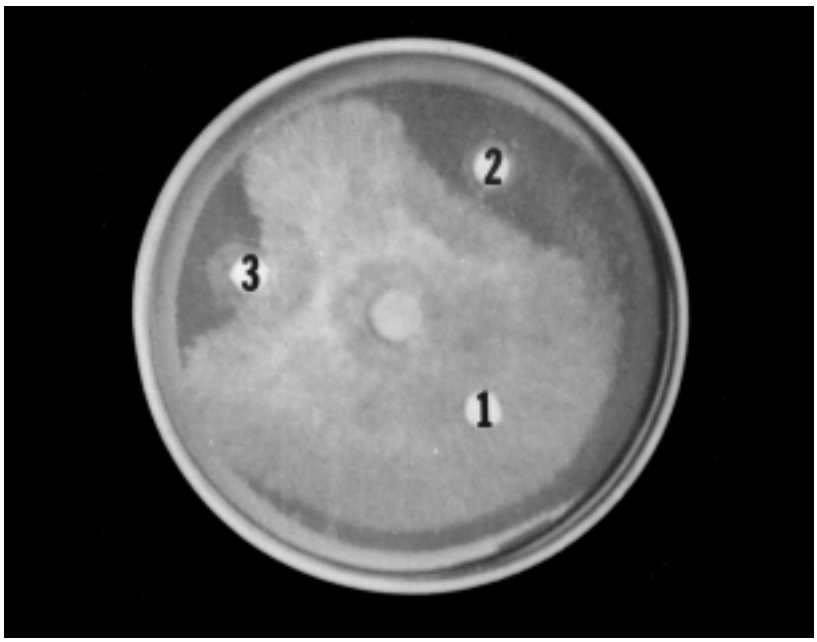

Fig. 3. Antifungal activity of purified rice chitinase against mycelium of *Rhizoctonia solani*. 1, buffer; 2, crude dialysed fraction; 3, purified fraction.

Digat, 1995). In the present study, soaking sclerotia of *R. solani* either directly in bacteria-free culture filtrates of *P. fluorescens* strains or in the sap extracted from *Pseudomonas*-treated rice plants, caused a reduction in sclerotial germination. Many workers have reported similar results. Savithri and Gnanamanickam (1987) found that soaking sclerotia in a bacterial cell suspension reduced sclerotial germination by 47%. In another study, sclerotial germination decreased with prolonged incubation in bacterial suspension, with most bacterial strains causing 50–100% inhibition of sclerotial germination after 4 weeks (Vasanthadevi *et al.,* 1989). However in all these studies sclerotia were immersed in a bacterial suspension. In our studies, we separated the bacteria from the culture filtrate and from the plant sap and studied the effect of the antifungal metabolites produced by these bacteria in a bacterial-free filtrate. In these circumstances, antifungal substances such as 2-4 diacetyl phloroglucinol, siderophore and hydrogen cyanide, and lytic enzymes such as chitinase and glucanase, produced by the bacteria in culture, or the chitinase induced in the plant sap acted on the sclerotia and inhibited their germination. Phloroglucinol, one of the antibiotics produced by *Pseudomonas* spp., has been shown to have antiviral, antibacterial, antifungal and anthelminthic properties (Tada *et al.,* 1990; Keel *et al.,* 1992). In an earlier study, purified pyrrolnitrin from a strain of *Pseudomonas cepacia* isolated from a rice ecosystem inhibited mycelial growth of *R. solani* (Rosales *et al.,* 1995).

Defence systems induced by rhizobacteria against soil-borne pathogens have been reported in the literature. Of the substances induced, chitinase and glucanase are the most important because of their strong antifungal activity. Expression of the genes encoding chitinase and glucanase was further increased when the plants were challenged with a corresponding pathogen (Schneider and Ullrich, 1994; Dalisay and Kuc, 1995; Nandakumar *et al.,* 2001a). The present study showed that sap extracted from rice plants inoculated with *R. solani* exhibited more antifungal activity against sclerotia than sap from uninoculated plants. This may have been due to the higher activity of chitinase in plants inoculated with *R. solani* previous-

Table 4. Viability of ungerminated and germinated[a] sclerotia upon inoculation in rice plants. Individual experiments are separated by horizontal lines.

Type of inoculatum[b]	Sheath blight score[c]					
	0	1	2	3	4	5
Ungerminated sclerotia						
Sclerotia soaked in PF1 culture filtrate (17)	12	5	-	-	-	-
Sclerotia soaked in FP7 culture filtrate (20)	16	3	1	-	-	-
Sclerotia soaked in PB2 culture filtrate (16)	10	3	2	1		
Sclerotia soaked in carbendazim (10)	10	-	-	-	-	-
Germinated sclerotia						
Sclerotia soaked in culture filtrate (10)	-	-	-	-	4	6
Sclerotia soaked in sterile water/unsoaked (10)	-	-	-	-	3	7
Ungerminated sclerotia						
Sclerotia soaked in PF1 treated + *R. solani*-inoculated plant sap (21)	15	4	2	-	-	-
Sclerotia soaked in FP7 treated + *R. solani*-inoculated plant sap (20)	16	4	-	-	-	-
Sclerotia soaked in untreated + *R. solani*-inoculated plant sap (10)	5	1	3	1	-	-
Inoculation of germinated sclerotia soaked in plant sap (10)	-	-	1	2	5	2
Ungerminated sclerotia soaked in purified chitinase solution (6)	5	1	-	-	-	-
Germinated sclerotia soaked in purified chitinase solution (4)	-	-	1	1	2	-

[a] PDA plate derived ungerminated/germinated sclerotia (soaked either in bacterial cultural filtrate or in plant sap for 72 h) used for inoculation study.
[b] All ungerminated sclerotia and randomly selected germinated sclerotia were inoculated in a separate tiller of rice plants individually. Number of sclerotia inoculated are shown in parentheses.
[c] Disease incidence was scored on a 0–5 scale (Sriram *et al.,* 1997) for each tiller at seven days after inoculation.

ly treated with *Pseudomonas* strains, as was observed in our earlier studies (Nandakumar *et al.,* 2001a), and also to the inhibition of sclerotial germination by purified chitinase as seen in this study. Several studies have demonstrated the antifungal activity of purified chitinase against a wide range of pathogens (Neuhaus, 1999; Radhajeyalakshmi *et al.,* 2000).

Numerous reports state that bacterial strains produce antibiotic substances and lytic enzymes in culture but not in the soil. However, published information has shown that the same antibiotic and lytic enzymes were also produced in the soil and exerted the same effect as that observed *in vitro* (Rodriguez-Kabana *et al.,* 1983; Thomashow and Weller, 1996). Thus, production of phenazine-1-carboxylic acid by *P. fluorescens* in the rhizosphere of wheat was correlated with take-all disease control (Thomashow *et al.,* 1990). Antibiotics induced by *P. fluorescens* inhibit *P. ultimum* in the cotton spermosphere and rhizosphere (Howie and Suslow, 1991). Nielsen *et al.* (1998) reported that both secondary metabolite and endochitinase-producing bacterial isolates effectively inhibited the growth of *R. solani* and *P. ultimum in vitro* and in pot culture and a mutant of *P. fluorescens* (Pf7-14) which did not produce antibiotics was also unable to control sheath blight and rice blast under field conditions unlike the antibiotic-producing strain (Chatterjee *et al.,* 1996). In our study, we do not shown any evidence for *in vivo* production of antibiotics or lytic enzymes: we assume that the bacterial strain produced antibiotics and lytic enzymes either in the rhizosphere or in the spermosphere of the rice plants, and thereby suppressed the blight pathogen, leading to a reduction in sheath blight under both glasshouse and field conditions.

Since a sclerotium is a dormant mass of myc-

elium, the reduction in germination on PDA could also be due to its dormancy. Inoculation of ungerminated sclerotia (from PDA plates) in rice plants revealed that most of these sclerotia, like the sclerotia soaked in carbendazim, did not cause lesions on rice plant, indicating their loss of virulence. This suggests that killing the sclerotia initially or arresting their germination has an important role in the control of sheath blight. The present study provides evidence that *P. fluorescens* applied through seed treatment, seedling root dip, soil application or foliar spray controls sheat blight by killing or arresting the germination and growth of soil-residing *R. solani* sclerotia.

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