Microbiol. Res. (2000) **155**, 233–242
http://www.urbanfischer.de/journals/microbiolres

Microbiological Research
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Antifungal characteristics of a fluorescent *Pseudomonas* strain involved in the biological control of *Rhizoctonia solani*

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Accepted: March 30, 2000

Abstract

A plant growth-promoting isolate of a fluorescent *Pseudomonas* spp. EM85 was found strongly antagonistic to *Rhizoctonia solani*, a causal agent of damping-off of cotton. The isolate produced HCN (HCN^+), siderophore (Sid^+), fluorescent pigments (Flu^+) and antifungal antibiotics (Afa^+). Tn5::*lacZ* mutagenesis of isolate EM85 resulted in the production of a series of mutants with altered production of HCN, siderophore, fluorescent pigments and antifungal antibiotics. Characterisation of these mutants revealed that the fluorescent pigment produced in PDA and the siderophore produced in CAS agar were not the same. Afa^- and Flu^- mutants had a smaller inhibition zone when grown with *Rhizoctonia solani* than the EM85 wild type. Sid^- and HCN^- mutants failed to inhibit the pathogen in vitro. In a pot experiment, mutants deficient in HCN and siderophore production could suppress the damping-off disease by 52%. However, mutants deficient in fluorescent pigments and antifungal antibiotics failed to reduce the disease severity. Treatments with mutants that produced enhanced amounts of fluorescent pigments and antibiotics compared with EM85 wild type, exhibited an increase in biocontrol efficiency. Monitoring of the mutants in the rhizosphere using the *lacZ* marker showed identical proliferation of mutants and wild type. Purified antifungal compounds (fluorescent pigment and antibiotic) also inhibited the fungus appreciably in a TLC bioassay. Thus, the results indicate that fluorescent pigment and antifungal antibiotic of the fluorescent *Pseudomonas* spp. EM85 might be involved in the biological suppression of *Rhizoctonia*-induced damping-off of cotton.

Key words: antibiotics – fluorescent pigment – biocontrol – Tn5::*lacZ* mutagenesis

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Introduction

Cotton seedling diseases, in particular damping-off caused by *Rhizoctonia solani*, are responsible for substantial damage (Hagedorn *et al.* 1993). Exposure risks, toxicity and residual persistence of available fungicides have led to serious consideration of the biological control approach to combat the menace of the pathogens (Gould 1990). Subsequently, binucleate *Rhizoctonia* spp. (Harris *et al.* 1994; Herr 1995), *Gliocladium virens* (Howell 1982), *Bacillus subtilis* isolate A13 (Turner and Backmann 1991), *Pseudomonas fluorescens* strain BL915 (Gaffney *et al.* 1994; Hill *et al.* 1994) and *Pseudomonas fluorescens* Pf-5 (Thompson *et al.* 1994) have been evaluated for their ability to suppress *R. solani*-induced damping-off of cotton.

Plant growth-promoting *Pseudomonas* species generally employ an array of mechanisms such as antibiosis, site competition, production of HCN, chitinase, siderophore and/or ammonia (Weller 1988; Thomashow and Weller 1988; Voisard *et al.* 1989; Thomashow *et al.* 1990; Bull *et al.* 1991; Thara and Gnanamanickam 1994; Cartwright *et al.* 1995) to antagonise pathogens. For genetic manipulation and improvement of biocontrol efficiency, it is essential to know which antifungal traits of biocontrol agents are crucial and involved in disease suppression. A plant growth-promoting rhizobacterium (PGPR) isolate of a fluorescent *Pseudomonas* EM85, obtained from maize rhizosphere, was found to suppress *R. solani* in cotton as well as maize root diseases caused by *Fusarium moniliforme*, *Fusarium graminearum* and *Macrophomina phaseolina* (Pal 1995). In the present study, we try to elucidate and identify the possible antifungal trait(s) of the fluorescent *Pseudomonas* isolate EM85 involved in the biological suppression of *R. solani*.

0944-5013/00/155/03-233 $15.00/0

Materials and methods

Strains. An isolate of *Rhizoctonia solani*, virulent on cotton, obtained from Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), was maintained on potato dextrose agar (PDA) slants at 4 °C, and subcultured at monthly intervals. A fluorescent *Pseudomonas* isolate, EM85, isolated from maize rhizosphere, exhibiting IAA production, P-solubilization, N_2-fixation, organic acid production and gelatinase activity, was maintained at 4 °C. The isolate had a generation time of 52 minutes at 28 °C (Pal 1995). The isolate was resistant to nalidixic acid (50 µg ml^{-1}) and sensitive to kanamycin (Km). The bacterium was grown at 28 °C in nutrient broth or potato dextrose broth. *E. coli* S17-1 (pSUP:Tn-B20) harbouring Tn5::*lacZ* was grown at 37 °C in tryptone-yeast extract (TY) broth amended with Km (50 µg ml^{-1}). It was sensitive to nalidixic acid.

Detection of antifungal traits. The isolate EM 85 was screened to detect the production of chitinase, HCN, antifungal volatiles, siderophore, antifungal antibiotic(s) and fluorescent pigment(s). Each test was repeated three times with three replications.

Detection of chitinase activity was done according to Frandberg and Schnurer (1994). The basal medium was amended with 0.15% colloidal chitin from crab shell. The bacteria were spotted as phosphate buffer-washed, log-phase cells from nutrient broth on Petri dishes containing the medium and incubated for 3–4 days. Chitinase activity was visualised by lysed hyaline zones around the bacterial colonies. Production of antifungal volatiles was tested as described (Howell *et al.* 1988). Two sections of quadri-partitioned trypticase soya Agar (TSA) Petri dishes were inoculated with the EM85 isolate and incubated for 96 h. After incubation, the remaining two sections were inoculated with an agar plug with *Rhizoctonia solani* and covered with parafilm. One dish containing only the fungus was kept as control under identical conditions. The Petri dishes were incubated at 25 °C for 48 h. Production of antifungal volatiles was recorded as positive where radial growth of the fungus was inhibited compared to growth in control Petri dishes.

Production of cyanide was detected by the method of Bakker and Schipper (1987). King's B medium (King *et al.* 1954) with 4.4 g glycine l^{-1} was streak inoculated with EM85. A Whatman No. 1 filter paper disc (nine cm in diameter) soaked in 0.5% picric acid in 2% sodium carbonate was placed in the lid of the Petri dish. The dishes were sealed with parafilm and incubated for four days. An uninoculated control was also maintained. Positive reaction was detected by observing the colour change of the filter paper from deep yellow to orange and finally to orange brown to dark brown.

Siderophore production was detected by observing orange halos around bacterial growth on CAS agar (Schwyn and Neilands 1987) after 24–36 h of growth. Catechol type of siderophore was quantified by the method of Arnow (1937) as modified by Carson *et al.* (1992) in iron-free liquid medium. Absorbance was taken at 550 nm. Pyrocatechol was used as standard.

Production of antifungal antibiotic(s) was screened on PDA and nutrient agar (NA) as described by Hebbar *et al.* (1992). A two to three mm disc of *Rhizoctonia solani* was placed at the centre of the Petri dishes and EM85 isolate was spotted around the periphery. Inhibition zone(s) were measured after five days of incubation at 28 °C.

Production of fluorescent pigment was screened on PDA. Bacterial spots were incubated for three days and viewed on a UV-transilluminator. Uninoculated Petri dishes served as controls. Fluorescent pigments were detected by observing fluorescence around the bacterial spots.

Tn5::lac Z mutagenesis, selection and maintenance of mutants. Transposon (Tn5::*lacZ*) mutagenesis was carried out by biparental mating as described by Simon *et al.* (1983) for introduction of DNA from *E. coli* (donor) to the *Pseudomonas* isolate EM85 (recipient). Conjugation was carried out at 1:1 ratio (donor:recipient) on tryptone-yeast extract (TY) agar Petri dishes for 18–20 h at 28 °C. Transconjugants were selected on TY Petri dishes containing kanamycin and nalidixic acid (50 µg ml^{-1}) as well as IPTG (four µl of 200 mg ml^{-1} stock; 30 µg ml^{-1} in medium) and X-gal (40 µl of 20 mg ml^{-1} in dimethylformamide stock; 30 µg ml^{-1} in medium). Putative blue colonies were picked as mutants whereas colourless colonies were discarded as spontaneous mutants. All mutants were screened in the Voges-Proskauer (VP) test which was negative for EM85 and positive for *E. coli* in order to eliminate the spontaneous mutants of *E. coli*. Mutants were grown on TY Petri dishes in the presence of 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} nalidixic acid for five to six generations on single cell basis to provide stability and then maintained in TY slants amended with kanamycin (50 µg ml^{-1}) and nalidixic acid (50 µg ml^{-1}) at 4 °C.

Phenotypic characterisation of mutants. Mutants were screened for production or deficiencies in antifungal activities, production of HCN, siderophores, fluorescent pigments and antifungal antibiotics. All the characters mentioned were studied in presence of kanamycin and nalidixic acid (50 µg ml^{-1}). Inhibition zones were measured in mm against the pathogen on PDA and NA. Deficient mutants of antifungal antibiotic production were checked in NA. For antifungal antibiotics, cyanide and fluorescent pigments, one mutant was grown on one Petri dish whereas for siderophore pro-

duction several mutants were spotted. Each test was repeated three times with three replications for confirmation.

Collection of cell-free extracts from fluorescent Pseudomonas sp. EM85. For the preparation of cell-free extracts containing fluorescent pigments, fluorescent *Pseudomonas* isolate EM85 was grown on PDA. After three days, the bacteria were scraped off the agar with the edge of a sterile glass slide. The agar was minced, and fluorescent pigment was extracted using sterile distilled water. It was found that antibiotic produced by the organism was not soluble in water but pigment was soluble in water. For collection of antibiotics, EM85 was grown in nutrient broth (since the organism failed to produce fluorescent pigment in nutrient broth) for seven days on a rotary shaker (240 rpm), centrifuged at 15,000 rpm for 10 min and then supernatant was collected. The supernatant from nutrient broth and water extract from PDA were filter sterilized by passing through sterile membrane filter of 0.22 µm.

Preparation of fungal inoculum, fluorescent Pseudomonas EM85 and its Tn5:: lacZ mutants. Fungal inoculum was grown in 250 ml Erlenmeyer flasks containing sand and maize meal mixed in a 3:1 ratio, i.e. 75 g dry sand and 25 g of maize meal, with 30 ml of water to moisten the mixture. Each flask was sterilised at 121°C for one hour on two consecutive days. Each flask was then inoculated with one agar bead containing *R. solani* (obtained from two days growth of *R. solani* on PDA and the beads were made using Pasteur pipettes) and incubated at 28 °C for five to seven days for uniform mycelial growth. The inocula of fluorescent *Pseudomonas* sp. EM85 and its Tn5::*lacZ* mutants were grown in nutrient broth and nutrient broth containing kanamycin and nalidixic acid (50 $\mu g\ ml^{-1}$, each). The broth was centrifuged at 12,000 rpm, washed with phopshate buffer three times, the pellets were dissolved in phosphate saline buffer, and the OD was adjusted to 1.2.

Isolation, purification and characterisation of antifungal compounds from EM85 isolate. Antifungal compounds were isolated from EM85 by the procedure of Douglas and Gutterson (1986). EM85 was grown on TY Petri dishes and incubated at 28 ± 2 °C for five days. The minced agar, after five days, was shaken overnight in 80% acetone (v/v) in equal proportion. The acetone fraction was decanted and filtered through Whatman no. 44 filter paper. The liquid volume was reduced by flash evaporation at 55 °C to 80 ml and ethanol was added to it to make the final volume of the compound 80% (v/v), the volume of ethanol being 20%. The mixture was incubated overnight at 4 °C. The mixture was then filtered and its volume was reduced to 25 ml and extracted once with an equal volume of methanol:chloroform (1:1, v/v) after adjusting the pH of the filtered mixture to 4.55. The filtered mixture was again extracted with an equal volume of chloroform three times. The organic phases were combined, evaporated to dryness, and dissolved in 5 ml of chloroform-methanol (1:1, v/v). Bioassay was done against *R. solani* in PDA. Fluorescent pigment was isolated from PDA Petri dishes by extracting with water.

Antifungal compounds were purified as described by Homma *et al.* (1989). Purification was done on TLC plates (Silica gel GF_{254} and Silica gel G_{60}, Merck) using different solvent systems viz., ethyl acetate:methanol (1:1) for antibiotics and ethylacetate:methanol:water (1:1:1) for fluorescent pigments. Spots were developed by I_2 vapour and observed under UV (254 nm). Bioassays on TLC plates were done using *Macrophomina phaseolina* following the procedure of Homma *et al.* (1989) as isolate EM85 was also antagonistic to *M. phaseolina*. Active spots were eluted from the preparative TLC plates after optimising the Rf values in the previously mentioned solvent systems. The eluted samples were dissolved in the same solvents in which they were originally dissolved. Purity was repeatedly checked until a single spot developed. The purified fraction was dried in vacuo, crystallized at low temperature and stored at low temperature for further studies.

Purified antifungal compounds were analysed by UV-spectrum analysis. UV spectra were taken by a Hitachi Mode double-beam UV-VIS spectrophotometer in methanol and distilled water using a quartz cuvette (1 cm path length). IR analyses were also carried out.

In situ biological control assay. For the pot experiment, *R. solani* was grown on sand-maize meal. Potting mixture was prepared by mixing 100 g of sand-maize meal containing *Rhizoctonia solani* with 900 g sterile soil (clay loam, pH 6.8) in polythene bags and then this mixture was mixed thoroughly with soil in 18″ earthen pots containing 30 kg unsterile soil. The soil was amended with nitrogen (at the rate of 40 kg N ha^{-1}) in the form of urea and phosphorus (at the rate of 60 kg P_2O_5 ha^{-1}) in the form of single super phosphate. The control treatment received only unsterile soil with above mentioned fertilizer doses. The fungal inoculum was applied five days prior to sowing to facilitate its multiplication. Cotton seeds, mechanically delinted (variety Pusa 8–6) were imbibed for 12 h and 20 seeds were sown per pot at a depth of 2.5 cm. Four ml of the bacterial inoculum in phosphate saline buffer was placed onto the surface of each seed after adjusting the O.D. to 1.2 (660 nm) for all the cultures. In case of cell-free extracts, four ml of the extract was applied in a similar way. The experiment was conducted during the rainy season to ensure that the humidity was conducive for disease expression. Pots were kept in the open with average day and night temperatures of 30 °C and 22 °C, respectively. Enough

moisture was maintained by watering the pots regularly with 500 ml pot^{-1}. There was a total of 14 treatments, which included: a) soil control; b) pathogen control; c) pathogen with EM85; d) pathogen with nine mutants of *Pseudomonas*, and e) pathogen with cell-free extract, either containing fluorescent pigments or antifungal antibiotics. Each treatment was replicated five times. The experiment was repeated thrice. A total of 100 plants per treatment were screened for disease studies. After 14 days, cotton seedlings were observed for appearance of brown lesions characteristic of post-emergence damping-off of cotton caused by *R. solani*. The number of dead or unemerged plants as well as toppled and infected plants were counted. Numerical disease rating was assigned (modification of the rating system used by Gaffney *et al.* (1994), as follows: 0, healthy seedlings; 1, minor lesion; 2, moderately severe lesions; 3, severe lesions with girdling of the stem at collar region with brown patches; 4, toppled, dead and unemerged plants.

Mean disease rating (MDR)

$$= \frac{a \times 0 + b \times 1 + c \times 2 + d \times 3 + e \times 4}{a + b + c + d + e}$$

Where a, b, c, d, e are the number of plants with the disease rating of 0, 1, 2, 3, 4, respectively. Percent disease reduction (PDR) with respect to pathogen control was calculated as follows:

$$\text{PDR} = \left(\frac{\text{MDR of pathogen control treatment}}{\text{MDR of pathogen control}} - \frac{\text{MDR of treatment with biocontrol agent}}{\text{MDR of pathogen control}} \right) \times 100$$

Monitoring of introduced organisms. Ten grams of the soil samples were taken for enumeration of rhizosphere population of the introduced organism. Potato dextrose agar (PDA) supplemented with antibiotics ($\mu g\ ml^{-1}$): Km^{50}, Nal^{50}, Cm^{100}, and $cycloheximide^{100}$ along with IPTG (4 µl of 200 mg ml^{-1} stock; 30 µg ml^{-1} in medium) and X-Gal (40 µl of 20 mg ml^{-1} stock; 30 µg ml^{-1} in medium) was used for enumeration of Tn5::*lacZ* mutants and expressed as log no. of cells g^{-1} soil. Wild type was enumerated in the same medium without kanamycin, IPTG and X-Gal.

Statistical analyses. Disease severity ratings were analysed according to Dunnett's test. Seedling mortality and percent disease reductions were analysed following Duncan's multiple range test after arc sine transformations.

Results

In vitro antagonism

Fluorescent *Pseudomonas* isolate EM85 (Fig. 1) strongly inhibited the fungus, *Rhizoctonia solani*, both on PDA (Fig. 2) and NA. It produced 10 mm and 12 mm inhibition zones on PDA and NA, respectively. It was found that fluorescent *Pseudomonas* isolate EM85 prevented the formation of sclerotia of *R. solani* upto an incubation period of 12 days whereas under normal conditions the pathogen was able to produce sclerotia on PDA within five days of incubation. This is very important as the pathogen may not be able to overwinter in the soil by forming sclerotia in the presence of the biocontrol agent.

Antifungal traits of fluorescent Pseudomonas EM85

The fluorescent *Pseudomonas* isolate EM85 was found to produce antifungal antibiotics (Afa^+), siderophore (Sid^+), cyanide (HCN^+) and fluorescent pigments (Flu^+) (Table 1). Production of ammonia, chitinase and antifungal volatiles were not detected. The fluorescent pigment produced in PDA was yellow-green in colour and a typical character of this isolate.

Isolation and phenotypic characterisation of Tn5::lacZ insertion mutants of isolate EM85

To define the role of antifungal characteristics in suppression of *R. solani*, both in vitro and in vivo, transposon mutagenesis was carried out.

Transposon insertion into the genome of the fluorescent *Pseudomonas* isolate EM 85 yielded mutants. One hundred and sixty mutants were obtained and screened

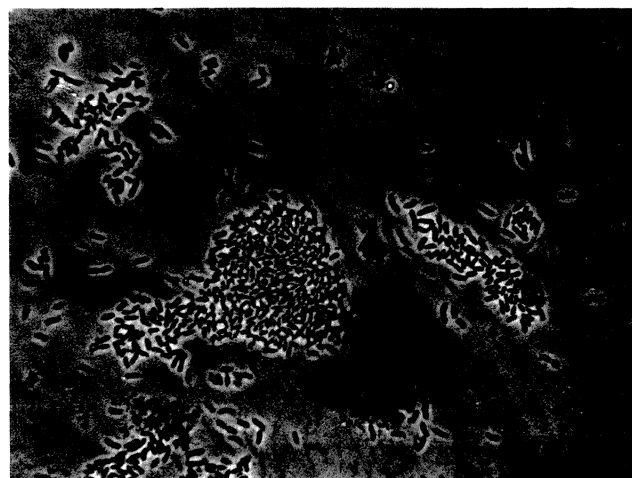

Fig. 1. Phase-contrast micrograph of the fluorescent *Pseudomonas* isolate EM85 (× 2765).

Table 1. Characterisation of wild type and Tn5::lac Z mutants of fluorescent *Pseudomonas* isolate EM85 and in vitro inhibition of *Rhizoctonia solani*.

S. No.	Phenotypes	Mutants	No of Mutants	Average inhibition zone in mm*	Source
1	Afa^+ HCN^+ Sid^+Flu^-	M54, M82	2	6.0	This study
2	Afa^+ HCN^+ Sid^-Flu^-	M79, M87	2	6.5	This study
3	Afa^+ HCN^- Sid^-Flu^-	M4, M58	2	9.0	This study
4	Afa^+ HCN^+ Sid^-Flu^+	M3	1	9.5	This study
5	Afa^- HCN^- Sid^-Flu^+	M86	1	6.0	This study
6	Afa^+ HCN^- Sid^+Flu^-	M8	1	9.5	This study
7	Afa^- HCN^+ Sid^+Flu^-	M18	1	0.0	This study
8	Afa^o HCN^+ Sid^+Flu^+	M42	1	16.0	This study
9	Afa^+ HCN^o Sid^+Flu^+	M30	1	11.0	This study
10	Afa^+ HCN^+ Sid^oFlu^+	M55, M83, M88, M90	4	11.5	This study
11	Afa^+ HCN^+ Sid^+Flu^o	M43	1	13.0	This study
12	Afa^+ HCN^+ Sid^+Flu^+	isogenic, M23 and rest	143	9.5	This study
13	Fluorescent *Pseudomonas* isolate EM85 (Nal^rKan^s) (Afa^+ HCN^+ Sid^+Flu^+)	Wild type	1	10.0	This study
14	*E. coli* S17-1(Kan^r)	harbouring Tn5::*lacZ*	1	00	Simon *et al.* 1983

° indicates enhanced production of the traits
* average of three replications

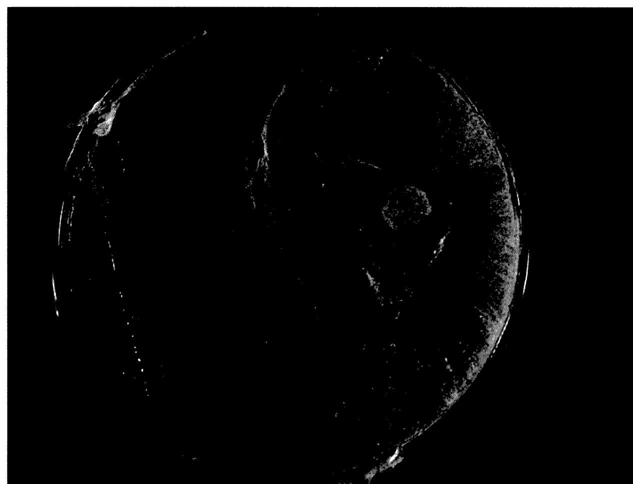

Fig. 2. In vitro antifungal activity of a fluorescent *Pseudomonas* isolate EM85 against *Rhizoctonia solani*.

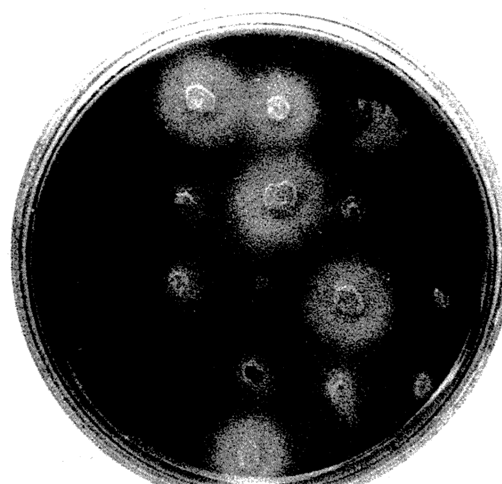

Fig. 3. Screening of Tn5::*lacZ* mutants for siderophore production on CAS agar plate (arrow shows a mutant deficient in siderophore production).

for the production of antifungal antibiotics, siderophore, HCN and fluorescent pigments. Different classes of mutants were obtained (Table 1): i) Two mutants were defective in fluorescent pigment production; ii) two mutants were defective in both siderophore and fluorescent pigment production; iii) two mutants failed to produce cyanide, siderophore and fluorescent pigments; iv) one mutant was defective in siderophore production (Fig. 3); v) one mutant produced only fluorescent pigment another one produced antibiotics and siderophore; vi) one mutant failed to produce both antibiotics and fluorescent pigments; vii) seven mutants were obtained with enhanced production of antifungal products out of which one, one, four and one mutants produced enhanced amount of antibiotics, cyanide, siderophore and fluorescent pigment, respectively; viii) one hundred and forty three mutants were isogenic so far as antifungal traits were concerned.

Table 2. Properties of some antifungal compounds of the fluorescent *Pseudomonas* isolate EM85.

Source	Soluble in	Solvent system	R_f of active spot	UV absorption λ_{max}	Physical state	Inhibitory against	Molecular weight
EM85	Methanol	Ethylacetate: Methanol (1:1)	0.85	223.5 nm	Viscous	*R. solani*	–
EM85	Water	Ethylacetate: Methanol: water (1:1:1)	0.71	220.5 nm	Solid	*R. solani*	567

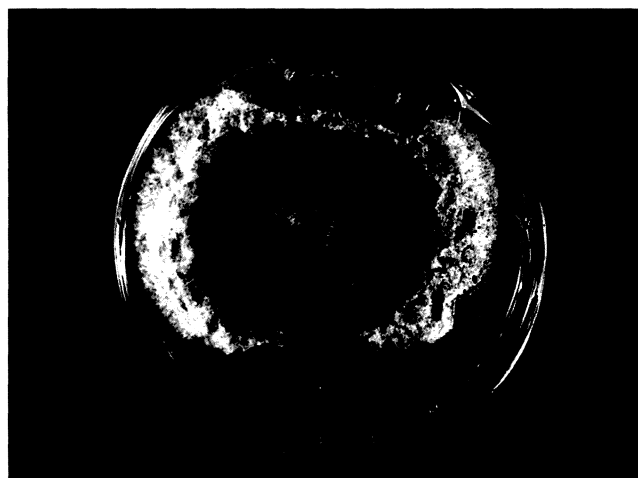

Fig. 4. Petri dish bioassay of the purified antifungal antibiotics and fluorescent pigment. Inhibitory effect against *R. solani*. Left trough: fluorescent pigment, right trough: antifungal antibiotic.

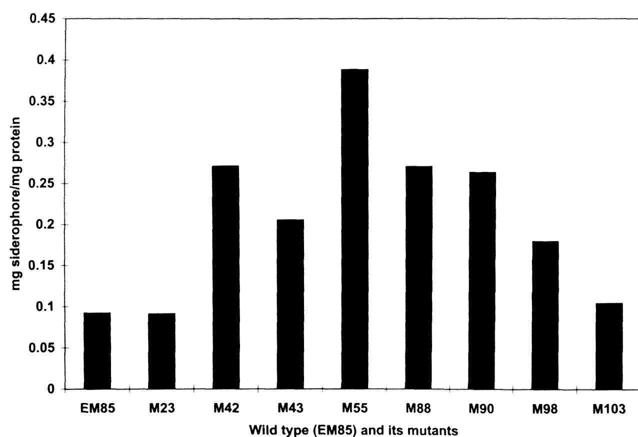

Fig. 5. Differential catechol siderophore production by a fluorescent *Pseudomonas* isolate EM85 and its mutants (average of three replications).

In vitro antagonism of mutants against R. solani

Mutants, deficient as well as with enhanced production of different antifungal traits, were tested in vitro for extent of inhibition against *R. solani*. Mutants deficient in both antifungal antibiotics and fluorescent pigments failed to inhibit the pathogen in vitro (Table 1). Mutants deficient in the production of either antibiotics or fluorescent pigments could inhibit the pathogen to a lesser extent. Mutants with enhanced production of fluorescent pigments and antibiotics produced larger inhibition zones (13 mm and 16 mm, respectively) as compared to wild type (10 mm).

Inhibition studies with the deficient mutants of cyanide (M 4 and M 58) and siderophores (M 3) in King's B (amended with glycine) and CAS agar plates, respectively, showed that the mutants could not inhibit *R. solani* while the mutants with enhanced production of these traits exhibited better inhibition compared to wild type isolate EM85. The observation indicates that both siderophore and cyanide could be the factors involved in the in vivo suppression of the pathogen.

Isolation, purification and bioassay with the antifungal compounds of EM85

Both fluorescent pigment and antifungal antibiotic were isolated and purified. The fluorescent pigment was a water-soluble solid (Table 2) with an UV absorption of 220.5 nm (λ_{max}) and an R_f of 0.71 (ethylacetate: methanol: water, 1:1:1 solvent system). In case of antibiotic, the compound was viscous and soluble in methanol (Table 2) with an UV absorption of 223.5 nm (λ_{max}) and an Rf of 0.85 (ethylacetate: methanol, 1:1 solvent system). The molecular weight of the fluorescent pigment was worked out to be 567. Bioassay was done with the purified pigment and antibiotic, both strongly inhibiting *R. solani* (Fig. 4) in vitro. The chemical nature and structure of these unique compounds are under investigation.

Enhanced production of catechol siderophore in liquid medium

Quantification of the catechol type of siderophore produced by the wild type as well as the mutants in iron-free liquid medium revealed that transposon mutagenesis could also enhance the efficiency of siderophore production severalfold in different mutants like M55, M88 etc. (Fig. 5). In vitro antagonism studies with these siderophore-overproducing mutants resulted in the enhancement of inhibition zones against *R. solani*.

Fig. 6. In situ suppression of damping-off of cotton. Numbers show: 1, treatment with *Rhizoctonia solani*; 2, treatment with *R. solani* and Afu^{o} (M 42) mutant of EM85; 3, treatment with cell-free extract containing fluorescent pigments; 4, treatment with mutant M 18 (Afu$^-$Flu$^-$).

Suppression of damping-off of cotton by wild type and mutants of the fluorescent Pseudomonas isolate EM85

Treatment with *R. solani* caused severe damping-off of cotton seedlings (Fig. 6) and mortality was as high as 50.83% (Table 3). Typical symptoms of toppling off of seedlings at collar region was noticed. Soil treatment with the fluorescent *Pseudomonas* isolate EM 85, protected the seedlings from pathogen invasion and produced healthy seedlings (Fig. 6) compared to the pathogen control treatment.

Results of the pot experiment indicated that the Flu$^-$ mutant, M54, and Afa$^-$ Flu$^-$ mutant, M18, did not provide protection from damping-off disease expression (Table 3). However, HCN$^-$ Sid$^-$ Flu$^-$ mutant (M58) and Sid$^-$ mutant (M3) significantly reduced the disease severity caused by *R. solani*. Treatments with cell-free extracts containing fluorescent pigments and antibiotics, separately, could control the disease and reduce seedling mortality (Fig. 6) like that of fluorescent *Pseudomonas* isolate EM 85 and M 58 mutant.

Soil treatment with mutants producing fluorescent pigments (M43) and antibiotics (M42) at an enhanced rate significantly suppressed the disease, while mutants which produced enhanced amount of siderophore (M55) and cyanide (M30) were ineffective in controlling *R. solani*. M23 was equally competent in controlling the disease like fluorescent *Pseudomonas* isolate EM85.

Reduction of disease severity was consistent with the seedling stand after 14 days. Mutant, M58, reduced the mortality rate from 50.83% (in pathogen control) to 20.52%, while treatments with cell-free extracts containing fluorescent pigments and antifungal antibiotics, separately, reduced the mortality to 26.26% and 24.58%, respectively. Mutants like M18, M54, M30 and M55 failed to reduce the mortality rate significantly (Table 3).

Table 3. In situ disease suppression of cotton caused by *Rhizoctonia solani***

S. No.	Treatments	Mean disease rating/ plant at 14 days after sowing*	Percent disease reduction	Mortality (%) at 14 days after sowing
1	Soil control	00 h	–	1.25 d
2	Pathogen control (P)	2.97 a	–	50.83 a
3	P+EM85	1.80 e	39.15 abc	33.50 bc
4	P+M18	2.56 bc	20.71 cd	44.40 ab
5	P+M58	1.10 g	52.48 a	20.52 c
6	P+M3	1.68 ef	39.45 abc	33.48 bc
7	P+M54	2.69 ab	13.21 d	40.91 ab
8	P+CFE containing fluorescent pigments	1.27 g	48.80 ab	26.26 c
9	P+CFE containing antifungal antibiotics	1.15 g	51.63 a	24.58 c
10	P+M42	1.35 fg	48.26 ab	26.17 c
11	P+M30	2.24 cd	29.08 bcd	40.90 ab
12	P+M55	2.55 bc	11.31 d	43.46 ab
13	P+M43	1.28 g	49.00 ab	26.20 c
14	P+M23	1.95 de	35.70 abc	34.90 bc

** Means within the same column and followed by the same letter(s) are not significantly different at P = 0.05 according to Dunnett and Duncan multiple range tests.
* Results based on 100 plants in each treatment with five replications, having 20 plants in each replication. The experiment was repeated thrice.

Transposon mutagenesis also increased the biocontrol efficiency in case of M58, M3 and M43 compared to wild type and other mutants (Table 3).

Monitoring of the inoculants for ecological competence

The ability of the wild-type isolate EM 85 and its Tn5::*lac Z* mutants to colonise the rhizosphere of cotton was evaluated and expressed as log no. of cells g^{-1} soil. The results indicated that wild-type isolate EM85, which was originally isolated from maize rhizosphere could also colonise the root zones of cotton efficiently. In the rhizosphere, mutants M30 (6.9), M3 (6.82) and M18 (6.7) each could build up a higher population than the wild type EM85 (6.58) whereas mutants M55 (6.53), M43 (6.43) and M58 (6.30) built up smaller populations than the wild type EM85. Rhizosphere colonisation of two mutants, M54 (5.57) and M42 (6.57), was similar to that of the wild type EM85.

Discussion

Inspite of many studies showing the antagonism of *Pseudomonas fluorescens* BL 915 (Gaffney *et al.* 1994; Hill *et al.* 1994), *Pseudomonas fluorescens* Pf-5 (Thompson *et al.* 1994) and *Bacillus subtilis* A 13 (Turner and Backmann 1991) on *Rhizoctonia solani*, no studies on the biocontrol of this pathogen with fluorescent *Pseudomonas* isolate EM85 (which could produce fluorescent pigment in PDA) has been undertaken.

The fluorescent *Pseudomonas* isolate EM85 exhibited antifungal characteristics such as production of siderophore, HCN, antibiotics and fluorescent pigment. Different types and number of compounds that contribute to the biocontrol activity of pseudomonads have been reported (Weller 1988; Voisard *et al.* 1989; Bull *et al.* 1991; Cartwright *et al.* 1995).

Transposon mutagenesis was carried out to define the role of antifungal traits in suppression of *R. solani* both in vitro and in vivo.

Characterisation of these insertional mutants revealed interesting information about the involvement and function of the antifungal characteristics of the fluorescent *Pseudomonas* isolate EM85. Analysis of Flu^- mutants conclusively showed that the mutants were less effective in suppressing the pathogen (Table 1). The yellow green fluorescent pigments produced in PDA are unique for this isolate and appear to be different from siderophores or pyoverdin as they are produced in iron-rich environment. This was further substantiated by the analysis of Sid^- mutant (M 3). This mutant did not produce an orange halo in CAS agar Petri dishes after initial growth (Fig. 3) but produced fluorescent pigments in PDA and inhibited fungal growth.

In addition to fluorescent pigment, antifungal antibiotic appears to have a role in pathogen control as it is evident from the total failure of the Afu^- Flu^- mutants (M 18, M 80) in suppressing *R. solani* in vitro. The very low frequency of mutants defective in antifungal anti-

biotic production indicated the involvement of several genes or more than one antibiotics for total inhibition of the pathogen by the fluorescent *Pseudomonas*. Similar observations were reported by different workers (Thomashow *et al.* 1990; Cartwright *et al.* 1995; Fenton *et al.* 1992).

The fungal inhibition assay using mutants of different phenotypic classes suggested that all the four traits (siderophore, HCN, antibiotics and fluorescent pigments) could be involved in the biocontrol of the pathogen. However, the results of the pot culture experiment gave insight into the factors responsible for the suppression of the development of disease symptoms on the host plant. The results of the pot experiments clearly suggested that antifungal antibiotics and fluorescent pigments are the factors which are chemically active in soil in suppressing the disease. Three lines of evidence substantiate this fact. First, both Flu^- (M54) and Afu^- Flu^- (M18) mutants failed to control seedling mortality and disease incidence in cotton, whereas Sid^-(M3) and HCN^- Sid^- Flu^- (M58) mutants significantly suppressed the disease. Secondly, the mutants which produced antibiotics (M42) and fluorescent pigments (M43) in enhanced amounts exhibited a significantly greater degree of control than the wild type. Third, the soil treatment with sterile cell-free extracts containing either fluorescent pigments or antibiotics was equally effective in the suppression of disease like those of the mutants producing enhanced amounts of antibiotics or fluorescent pigments. Again, strong inhibition of *R. solani* by the purified fluorescent pigment and antibiotic could substantiate the results obtained in the pot experiment. Proper establishment of the mutants as well as the wild type in the rhizosphere of cotton also helped in the expression of the performance of the inoculated cultures against the pathogen, *R. solani*.

Production of HCN and siderophore could suppress the mycelial growth in Petri dish assay but was found to have little role in disease suppression in pot experiment. High degree of disease suppression by HCN^- and Sid^- mutants as well as failure in reducing the disease severity by the mutants producing HCN (M30) and siderophore (M55) in enhanced amount, gave evidence for this fact.

Availability of sufficient iron in early stages of plant growth could have hindered the production of siderophores. In the same way, insufficient amount of cyanogenic glucosides in the root exudates at early stages of plant growth could have prevented the cyanide production. In several studies, it has been shown that siderophore has little or no role in disease suppression (Hamdan *et al.* 1991) while antibiotics were involved in *R. solani* suppression (Hill *et al.* 1994; Cartwright *et al.* 1995; Howell and Stipanovic 1980).

As far as we know, this is the first report showing the involvement of non-siderophore fluorescent pigment in the biological suppression of *R. solani* by a fluorescent *Pseudomonas* isolate (EM85). This is a new biocontrol agent for controlling *Rhizoctonia*-induced damping-off of cotton.

Acknowledgements

The work was supported by Senior Research Fellowship of Indian Agricultural Research Institute, New Delhi-110012, India. Authors are thankful to T. Dasgupta and Dr. P. Paria for photography and Amrit Pal for helping in statistical analyses.

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