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Evaluation of antagonistic fluorescent pseudomonads for the suppression of bacterial blight of rice

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Abstract

Rice (*Oryza sativa*) is the most widely cultivated food crop in the world. India has the largest area under rice crop and ranks second in production after the China. In India, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is considered as a serious production limiting factor especially in irrigated and rainfed lowland ecosystem. In the absence of highly effective chemicals against BB, biological management provides an alternative approach. In the present study, we isolated 21 strains of antagonistic fluorescent pseudomonads (FP) from rice rhizospheric soils, farm yard manure and vermicompost. All the isolates were biochemically tested and identity confirmed. All the FP strains except FP-3, FP-13, FP-14 and FP-15 tested positive for HCN production. Among the positive isolates, FP-18, FP-19, FP-20 and FP-21 were comparatively strong producers of HCN. Volatile metabolites produced by different FP isolates strongly inhibited the growth of *Xoo*. The strains like FP-2, FP-4, FP-5 and FP-14 were most effective in inhibiting the growth of *Xoo*. *In vitro* antagonistic activity of FP strains showed positive zone of inhibition ranging from 1.47 cm with FP-14 to 6.80 cm with FP-8. The FP strains like FP-6, FP-8, FP-13, FP-17, FP-19 and FP-21 were highly promising with inhibition zone ranging from 3.83-6.8 cm. These selected five strains significantly suppressed the BB disease severity under glasshouse conditions. Among the strains, FP-13 was the most effective and reduced the BB lesion length by 57%.

Key words: Rice, bacterial blight, biological control and fluorescent pseudomonads

Introduction

Rice (*Oryza sativa*) is the most widely cultivated food crop in the world, feeding about half of humanity with a worldwide production of 470.63 million metric tons from an area of 157.46 million hectares (Annonymous, 2016). India has the largest area under rice crop and ranks second in production after China. Rice forms an important part of diet of Indian population. Rice contributes 43 percent of total food grain production and 46 per cent of total cereal production. Rice is highly vulnerable to different biotic stresses that affect its quality and yield. Among different biotic stresses, bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most important one in different rice producing countries in Asia (Nayak *et al*., 2008; Sudir and Yuliani, 2016). BB is known to occur in epidemic proportions in many parts of the world, causing 6-81% yield loss in some rice varieties (Win *et al*., 2013; Yugander *et al*., 2017). In India, BB is considered a serious production limiting factor especially in irrigated and rainfed lowland ecosystem. Generally, the stage between maximum tillering and booting is highly sensitive to disease infection, as it significantly affects grain filling and total yield. BB management strategy, such as use of resistant cultivars, is the most economical strategy for disease management, although there has been only partial success because of an enormous diversity in the pathogen. In spite of extensive trials conducted, chemical management of the disease has not been very successful.

Bio-control assumes a special significance in being an ecologically conscious, environmentally safe and cost-effective alternative strategy for management of plant diseases, without the negative effect of synthetic chemicals that can cause environmental pollution and induce pathogen resistance in some cases. This approach can be integrated with other strategies to afford greater levels of protection and sustain rice yields. Fluorescent pseudomonads (FPs) are considered as potential biological control agents because of production of diverse secondary metabolites

and their ability to show 'induced systemic resistance (ISR) (Whipps *et al*., 2001; Velusamy *et al*., 2006). FPs exhibit diverse mechanisms of bio-control which include antibiosis, cyanide production, siderophore production, competition for space and nutrient and induced systemic resistance. Certain strains of FPs have been used as bio-control agents to suppress rice BB (Vasudevan *et al*., 2002). In the present study, we studied the efficacy of FPs isolated from rice rhizosphere and other sources against BB disease of rice.

Material and methods

The study was carried out at ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad, India. Rice rhizosphere soil samples were collected from different rice growing fields from Telangana. In addition to rice rhizosphere, farm yard manure and vermicompost was also used for isolation of FPs. All samples were properly labeled after collection and stored at 4°C till bacterial strains were isolated.

Isolation of FPs and culture conditions: Isolation of FPs was carried out by serial dilution method. One gram of each sample was added in a 100 ml capacity conical flask containing 10 ml of sterile distilled water and constantly shaken in an orbital shaker. Each sample suspension was then serially diluted up to 10^{-4} and an aliquot of 100 µl of each sample of the highest dilution was spread on a King's B (KB) medium plate using a sterilized glass spreader and the plates were incubated at 28±2°C for 3-4 days. Well separated colonies of FPs showing typical yellowish green pigment around the colony were picked up and further purified and then sub-cultured on King's B agar slants. A total of 21 FPs colonies were picked up from different soil/FYM/vermicompost samples and were coded by putting a prefix FP-1 to FP-21 (Table-1). The isolates were maintained in 15% glycerol at -80°C for long term storage and in the King's B agar slants at 4°C for short term storage.

In-vitro screening of FPs for antagonistic activity: All the FP isolates were screened for their antagonistic activity against *Xoo* by double layer technique. Each FP isolate was spot inoculated in the centre of freshly prepared King's B agar plate and the plates were incubated at 28±2°C for 3 days. The bacteria were then killed by chloroform vapor (by adding 1 ml chloroform in the upper lid after inverting the plate) in a laminar air flow for 2 hours. The plates were then kept open in the laminar air flow for 10 minutes to allow the chloroform vapor to evaporate completely. A suspension (~10^6 cfu/ml) was made by mixing a 3-day old culture of *Xoo* (strain IX-133) with 5 ml sterile distilled water under a laminar air flow chamber and then this suspension was mixed with melted and cool soft modified Wakimoto's agar (MWA) medium (0.4% agar). This soft medium with *Xoo* culture was then gently poured on the King's B plate having the dead FPs colony. The plates were allowed to solidify under the laminar air flow chamber and then incubated at 28±2°C. After two days of incubation, zone of growth inhibition of *Xoo* around the FPs colony was measured. The data were analyzed statistically following Gomez and Gomez (1984).

Biochemical characterization of FPs: The FP isolates were characterized by performing standard biochemical tests following Schaad *et al*. (2003). Different biochemical testes viz., Gram test/KOH test, production of green fluorescent pigment on King's B medium (King *et al*.1954), gelatin liquefaction, arginine dihydrolase, oxidase, and growth at 45°C were conducted following Fahy and Persley (1983) and Schaad (2003).

Hydrogen Cyanide (HCN) production by FPs: HCN production by FP isolates was tested following the method described by Lorck (1948). FP isolates were inoculated by streaking on freshly prepared King's B agar medium plates supplemented with the glycine amino acid (4.4 g/liter of medium). Simultaneously, a strip of sterilized Whatman filter paper No.1 impregnated with alkaline Picric acid (yellow) solution [0.5% Picric acid (w/v) in 2.0% Sodium carbonate] was placed in the upper lid of the inoculated petri plates under aseptic condition. Petri plates were sealed with petri seal or parafilm and incubated at 28±2°C for four days. The un-inoculated plates served as control. A change in strip color from yellow to light brown (+), moderate brown (++) or strong reddish brown (+++) was taken as indication of HCN production.

Effect of volatiles produced by FPs on the growth of *Xoo:* The inhibitory effect of volatile compounds produced by FP isolates on the growth of *Xoo* was performed by the paired petri plate technique using two different culture media. Two culture media *viz*., King's B medium (with and without glycine) and MWA was separately poured in sterile petri plates in a laminar air flow chamber. When the culture media got solidified, the MWA plate was streak inoculated with *Xoo* (strain IX-133) and the King's B medium plates with respective FP isolates. Then bottom Petri plate half with *Xoo* (upper) was face to face paired with bottom Petri

plate half with FP isolate (lower) and then sealed with petri seal or parafilm to prevent the leakage of volatiles from the plates and incubated at 28 ± 2°C for four days. Observations were taken visually on the suppression of growth of *Xoo*.

Suppression of BB under glass house condition: Five selected strains of FP which showed maximum inhibition zone against *Xoo* under *in vitro* tests, were evaluated for their disease suppression ability under glasshouse condition. A combination of seed treatment and foliar spraying with respective FPs was done to study the disease suppressing ability of the FPs. Selected antagonistic FPs (FP # -8, 13, 17, 19 and 21) were mass multiplied in King's B broth at 28 ± 2°C for 48 hrs in an incubator shaker. In a sterilized glass beaker, 10 ml bacterial culture broth, 2 g commercial talcum powder, 0.5 g gum acacia and 0.5 g carboxy methyl cellulose (Sodium salt) were added and mixed thoroughly. About 15 g seeds of BB susceptible rice variety, TN1 was added to this mixture and seeds were thoroughly coated with bacterial culture. The bacterized seeds were then dried under shade for 12 hours and then used for sowing. TN1 seeds coated with selected FP isolates were directly sown in earthen pots filled with a mixture of field soil and farm yard manure (3:1). For each isolate, three replications were maintained. Standard agronomic practices were followed to raise the plants. The plants at 35 days old stage were sprayed with respective FP isolate (0.5×10^7 cfu/ml). *Xoo* (strain IX-133) was multiplied in MWA culture medium as described by Yugander *et al*. (2017). The plants were clip-inoculated with a freshly grown *Xoo* culture suspension (~10^8 cfu/ml), 3 days after antagonist spray. The plants were again sprayed with the respective FP isolate, 3 days after inoculation with BB pathogen. Observations were recorded by measuring the lesion length of bacterial blight disease after 15 days of inoculation.

Results and discussion

Twenty one FP strains were isolated from various sources. These included 12 isolates from farm yard manure, 6 isolates from rice rhizosphere and 3 isolates from vermicompost. The isolates were designated as FP # 1-21. All the FP isolates were gram negative, produced typical yellowish green fluorescent pigment on King's B agar medium and were positive for oxidase, arginine dihydrolase (except one strain) and gelatin liquefaction. None of the FP strains could grow at 45°C. The strain FP-3 showed negative reaction for gelatin liquefaction. Among the common plant associated FPs, *Pseudomonas fluorescens* is known to be positive for gelatin liquefaction while *Pseudomonas putida* shows negative for gelatin liquefaction (Schaad *et al*. 2003). Five FP strains (FP # 17-21) showed comparatively higher gelatin liquefaction ability. The results of various biochemical tests conducted confirmed the identity of these bacteria as fluorescent *Pseudomonas* spp. strains.

In vitro antagonistic activity of FP strains

All the FP strains produced typical inhibition zone in dual culture plate against *Xoo* under *in vitro* condition. The diameter of inhibition zone ranged from 1.47 cm with FP-14 to 6.80 cm with FP-8 (Table 1). *In vitro* growth inhibition (%) ranged from 2.82 % (FP-14) to 57.22 % (FP-8) (Table 1). The FP strains like FP-6, FP-8, FP-13, FP-17, FP-19 and FP-21 were highly promising (inhibition zone ranging from 3.83-6.8 cm) with percentage of growth inhibition ranging from 18.15% to 57.22% (Table 1). The study revealed that different FP isolates have different capacities as biological weapons in inhibiting the *Xoo* strains. Use of bacterial bio-control agents to suppress plant diseases is very common. Jambhulkar and Sharma (2014) evaluated potential of *P. fluorescens* isolate RRb-11 against BB pathogen *Xoo* under *in vitro* and *in vivo* conditions. Similarly, Velusamy *et al*. (2013) and Yasmin *et al.* (2016) evaluated different rice rhizosphere associated antagonistic bacteria for plant growth promotion and BB disease suppression. Salaheddin *et al.* (2010) evaluated several fluorescent *Pseudomonas* spp. against *Xanthomonas axonopodis* pv. *malvacearum* causing cotton leaf blight under *in vitro* condition.

Hydrogen Cyanide (HCN) production by FPs

All the FP strains except FP # -3, 13, 14 and 15 tested positive for HCN production. Among the positive isolates, FP-18, FP-19, FP-20 and FP-21 were comparatively strong producer of HCN as indicated by change in color of the strips from yellow to reddish brown. Production of HCN is an important mechanism of many antagonistic fluorescent *Pseudomonas* spp. (Kumar *et al.,* 2012). HCN producing *Pseudomonas* strains effectively reduce or kill the plant pathogenic microorganism. Microbial cyanogenesis has been demonstrated only in a few species of bacteria in the genera *Chromobacterium* and *Pseudomonas* (Patty, 1921; Michaels and Corpe, 1965).

Effect of volatiles produced by FPs on the growth of *Xoo*

Volatile metabolites produced by different FP isolates strongly inhibited the growth of *Xoo* (Table 1). *Xoo* growth suppression was more when the King's B medium

was supplemented with glycine (4.4 g/l) indicating the enhanced production of HCN had more detrimental effect on the growth of *Xoo*. The strains like FP # 1, 6, 8, 13, 16, 17, 18, 19 and 20 showed stronger growth suppression when the King's B medium was supplemented with glycine (Table 1). On the other hand, strains like FP # 5 and 14 showed stronger growth suppression when cultured in King's B medium without glycine. Some of the strains like FP # 13, 14 and 15 which did not show the production of HCN, suppressed the *Xoo* growth to some extent indicating production of volatiles other than HCN (Table 1). The antagonistic activity of the selected bacterial isolates might be due to the production of HCN or synergistic interaction with other metabolites and it has been documented earlier that microorganisms showing the ability to produce HCN can be used as bio-control agents for the suppression of plant pathogens (Ramette *et al.*, 2003). Sarangi *et al.* (2010) reported production of different volatile compounds like nonanal, benzothiazole and 2-ethyl-1-hexanol as the primary mechanism of bio-control of *Sclerotinia sclerotiorum* in canola by *Pseudomonas chlororaphis* strain PA23.

Table 1: *In vitro* antagonistic effect of fluorescent Pseudomonad strains against *Xanthomonas oryzae* pv. *oryzae*

FP Isolates	Source	Mean Inhibition zone (cm) ± SE	Growth inhibition (%)	HCN production	*Xoo* growth suppression by volatiles of FPS (w/o glycine)	*Xoo* growth suppression by volatiles of FPS (with glycine)
FP-1	Rice rhizosphere	3.17 ± 0.18^{gh}	12.46(20.60) ^{gh}	+	-	+++
FP-2	Rice rhizosphere	3.40 ± 0.10 ^{fg}	14.30 (22.19) ^{fg}	+	+++	++
FP-3	Rice rhizosphere	3.03 ± 0.03 ^{h}	11.36 (19.69)^{h}	-	-	-
FP-4	Rice rhizosphere	1.83 ± 0.09 ^{j}	4.17 (11.75) ^{k}	+	+++	++
FP-5	Rice rhizosphere	4.17 (11.75)	8.81 (17.23) ^{ij}	+	+++	+
FP-6	Rice rhizosphere	3.83 ± 0.07 ^{de}	18.15 (25.20) ^{de}	+	+	+++
FP-7	FYM	3.63 ± 0.07 ^{ef}	16.31 (23.80) ^{ef}	+	+	+
FP-8	FYM	6.80 ± 0.23^{a}	57.22 (49.16) ^{a}	+	-	+++
FP-9	FYM	3.27 ± 0.03 ^{gh}	13.18 (21.27) ^{gh}	+	+	+
FP-10	Vermicompost	3.10 ± 0.12 ^{gh}	11.90 (20.14) ^{gh}	+	+	-
FP-11	Vermicompost	3.40 ± 0.06 ^{fg}	14.28 (22.19) ^{fg}	+	-	+
FP-12	Vermicompost	3.23 ± 0.03 ^{gh}	12.91 (21.05) ^{gh}	+	-	-
FP-13	FYM	4.03 ± 0.03 ^{D}	20.09 (26.62) ^{d}	-	+	++
FP-14	FYM	1.47 ± 0.26 ^{K}	2.82 (9.38) ^{k}	-	+++	-
FP-15	FYM	2.63 ± 0.07 ^{I}	8.57 (17.01) ^{j}	-	+	-
FP-16	FYM	3.00 ± 0.12 ^{h}	11.14 (19.47) ^{hi}	+	-	++
FP-17	FYM	4.47 ± 0.03 ^{C}	24.63 (29.74) ^{c}	++	+	+++
FP-18	FYM	2.67 ± 0.12 ^{i}	8.81 (17.23) ^{ij}	+++	-	+++
FP-19	FYM	3.83 ± 0.17 ^{de}	18.21 (25.21) ^{de}	+++	-	++
FP-20	FYM	2.47 ± 0.03 ^{I}	7.51 (15.90) ^{j}	+++	-	+++
FP-21	FYM	5.03 ± 0.03 ^{b}	31.28 (33.99) ^{b}	+++	-	+
CV (%)		6.60	5.89			
LSD (*P=0.05*)		2.43	0.33			

Data in parentheses are arc sine transformed values; means followed by a common letter in a column are not significantly different at 5% level by DMRT.

Suppression of BB under glasshouse condition

Based on the consistent performance in the laboratory tests, five FP isolates were selected for their efficacy to reduce BB disease severity under glasshouse condition. The results indicated that combined application of seed treatment as well as foliar spray with respective FP isolates significantly reduced the BB lesion length (5.33-7.00 cm) when compared to untreated control (12.33 cm) (Table 2).

Table 2: Efficacy of selected fluorescent pseudomonad strains against bacterial blight of rice under glasshouse condition

FP isolate	Mean lesion length (cm) ± SE
FP-13	5.33 ± 0.33
FP-8	7.00 ± 0.58
FP-21	5.67 ± 0.33
FP-17	6.33 ± 0.88
FP-19	5.67 ± 0.33
Un-inoculated control	12.33 ± 1.45
CV (%)	19.99
LSD (*P=0.05*)	2.57

Among the strains, FP-13 was most effective and reduced the lesion length by 57%. Seed treatment with some of these FP isolates, showed significant improvement in different plant growth parameters like germination (%), shoot length, root length and fresh plant weight when compared with the control treatment. Some of the promising FP strains which significantly improved different plant growth parameters were FP-8, FP-13, FP-17, FP-19 and FP-21. Management of plant diseases using antagonistic micro-organisms offers the best alternative to chemical control. Timely and augmented application of bio-agent is required for proper establishment on the plant surface. The combination of different application methods and different bio-agents has been advocated for better bio-control of plant diseases (Van Loon, 1998). As chemical control is not successful in managing bacterial blight of rice, bio-management of the disease offers an alternative approach. Many workers have used different bio-control agents for management of BB in rice. Vidhyasekaran *et al*. (2001) reported the effectiveness of selected *P. fluorescens* in the management of BB in rice. Kaur and Thind (2002) reported that seed bacteriazation followed by two foliar applications of antagonistic strains of *Pseudomonas fluorescens* can significantly reduce BB severity in both glasshouse and fields. Reduction of BB severity through seed bacterization and foliar spray has been reported by several workers (Shivalingaiah and Sateesh, 2012; Singh and Sinha, 2005; Sivamani *et al*., 1987). It can be concluded from this study that combined application of seed treatment and foliar application of selected fluorescent pseudomonads can significantly reduce the bacterial blight severity.

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