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Isolation and Characterization of Phylloplane Associated Bacteria and its *in vitro* Antagonistic Activity against *Bipolaris oryzae*

R. Karan, D. Kalaimathi, P. Renganathan, P. Balabaskar

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ABSTRACT

Background: Rice brown leaf spot is a major fungal disease caused by *Bipolaris oryzae.* Biological management of brown leaf spot can be achieved by bacteria associated with phyllosphere region of rice crop.

Methods: Antagonistic bacteria were isolated from phylloplane of the rice crop using dilution method. Phylloplane bacteria were morphologically and biochemically characterized. Antagonistic activity of bacteria was tested against *Bipolaris oryzae* using Dual culture technique.

Result: Among the isolates, PB1, PB3, PB6, PB7, PB9 were identified as *Pseudomonas fluorescens* and PB2, PB4, PB5, PB8, PB10 were identified as *Bacillus subtilis* by morphological and biochemical characterization. Phylloplane bacteria PB5 showed the maximum inhibition of *B. oryzae* mycelium (70.00%) followed by PB1 (67.77%). Minimum inhibition of mycelial growth (31.11) was found in PB9.

Key words: Bacteria, *Bipolaris oryzae*, Brown leaf spot, Phylloplane, Rice.

INTRODUCTION

Rice brown leaf spot caused by *Bipolaris oryzae* is a serious disease which affects yield of rice crop. Rice brown spot have been reported in almost all rice growing countries. Application and use of antagonistic microorganisms has now becoming an effective strategy for management of brown leaf spot of rice. Moreover, biological control is eco-friendly method which can be alternate to fungicides for the management of several crop diseases, including rice crop (Vidhyasekaran *et al*. 2001; Chandler *et al*. 2015). Several bacteria and fungi having bio control properties are well known, among them, *Pseudomonas fluorescens* and *Bacillus subtilis* are widely used bacteria for the management of crop diseases (Kumar *et al*. 2016). Phylloplane having wide diversity of microbes. These microbes play an important role in disease suppression, growth promotion activities and biogeography of plant. They also induce systemic resistance in plant (Qin *et al*. 2019). Understanding the importance of phylloplane microbes, this study aimed isolation and characterization of antagonistic bacteria from phylloplane region of rice crop.

MATERIALS AND METHODS

Isolation of Phylloplane bacteria (PB) by Dilution method

The entire experiment was performed in Department of Plant Pathology, Faculty of Agriculture, Annamalai University during 2021. Healthy leaf from the paddy fields collected and cut into small pieces. Leaf pieces were put in the conical flask containing sterile distilled water. Flask containing leaf pieces were kept in the shaker at 60 rpm for 24 hrs. This suspension was serially diluted upto 10^{-6} and 50 microlitre from 10^{-5} and 10^{-6} dilutes were taken and poured on the petri dishes containing Nutrient Agar Medium. The plates were incubated for 24 hrs. under room temperature. After incubation single bacterial colonies were streaked on the slants containing Nutrient Agar. These agar slants were used for the further studies (Rajasekar *et al*. 2019).

Morphological and Biochemical characterization of Phylloplane bacteria (PB)

Pure cultures were streaked on Nutrient Agar medium and Incubated for 48 hrs. Individual colonies were observed for size, shape, texture, elevation and pigmentation of the colonies. Biochemical test such as gram staining, starch hydrolysis, gelatin liquefaction, KOH test, Methyl red test, H_2S test, catalase test, urease activity and different carbon source (Glucose, sucrose, Lactose, Maltose) utilization test was performed (Pranaya *et al*. 2020).

Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, Chidambaram-608 002, Tamil Nadu, India.

Corresponding Author: R. Karan, Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, Chidambaram-608 002, Tamil Nadu, India.
Email: karanrajumari7@gmail.com

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Indole acetic acid (IAA) production test

All the test bacteria were grown on Nutrient broth amended with 0.2% tryptophan and without tryptophan. They were incubated for 2 days at 28±2°C. The resulted bacterial cultures were centrifuged at 3000 rpm for 30 min. The 2 ml of resultant supernatant was added with 2 drops of orthophosphoric acid and 4 ml of Solawaski's reagent (50 ml of 35% perchloric acid and 1ml of 0.5% $FeCl_3$). Development of pink colour results IAA production by the bacteria (Ahmad *et al*. 2005).

Hydrogen Cyanide (HCN) Production test

Tryptic Soy Agar (TSA) were supplemented with 4.4 g/l of glycine. All the test bacteria were streaked on the media in lower plate. White filter paper strips dipped in picric acid solution (2.5 g of Na2CO3 and 1 lit. of water) were placed in the upper lid of each Petri dishes. Petri dishes were sealed with paraffin film and incubated for two to three days at 28±2°C. Presence of red coloured zone indicated the production of HCN (Wei *et al*. 1996).

***In-vitro* evaluation of antagonistic effect of Phylloplane bacteria (PB) against *B. oryzae* (Dual culture technique)**

The antagonistic effect of PB communities were tested against *B. oryzae* using standard Dual culture technique (Dennis and webster 1971). A 9mm disc of actively growing fungal culture was taken and placed near the periphery of the petri dishes containing Potato Dextrose Agar Medium. On the other side of the plate actively growing bacterial cultures were streaked. The control plates were inoculated with fungal disc and streaked with distilled water only. Incubate the plates at room temperature until the mycelium of the fungal culture in control covers full plate. Distance between the fungal growth and bacterial colonies were recorded as inhibition zone.

Inhibition of mycelial growth over control was calculated using following formulae proposed by Vincent (1927).

$$I = \frac{C-T}{C}$$

I= Inhibition of mycelial growth over control.
C= Mycelial growth in control.
T= Mycelial growth in treatment.

RESULTS AND DISCUSSION

Morphological characterization of Phylloplane bacteria

Ten isolates collected from Cauvery Delta Region of Tamil Nadu showed two different morphological characters. Circular, Pale yellow, Convex, Slimy and Glistening colony Produced by PB1, PB3, PB6, PB7, PB9 bacteria. In further studies they are identified as a *Pseudomonas fluorescens.* Round, white, Opaque, flat medium sized colony with serrated margin produced by PB2, PB4, PB5, PB8, PB10 are identified as a *Bacillus subtilis* (Table 1).

The results are accordance with Manasa *et al*. (2017). They reported that all *P. fluorescens* developed small to medium, smooth, glistening colonies, out of the 15 isolates 6 isolates showed yellowish green colour with light green pigmentation and the remaining isolates showed dull white colonies with no pigmentation. Meera and Balabaskar (2012) and Suman (2015) also found similar results, yellowish slimy colonies with fluorescent pigment production by *P. fluorescens.* The results are further confirmed with Priyanka *et al*. (2017). They reported round to irregular yellow fluorescent coloniesproduced by *P. fluorescens.*

Lu *et al*. (2018) reported that *B. subtilis* produce gray-white, round, opaque, flat and dry with medium-size colonies on nutrient agar medium. The results are accordance with Joo *et al*. (2007) which confirms that white coloured, circular and flat appearance colonies produced by *B. subtilis.*

Biochemical characterization of Phylloplane bacteria

Results obtained from various biochemical test was presented in Table 2. Phylloplane bacterial isolates PB1, PB3, PB6, PB7, PB9 produced positive results in pigment production, gelatin liquefaction, KOH test, H_2S production test, casein hydrolysis, IAA production test, HCN production test, catalase test, glucose utilization, sucrose utilization, urease activity. They produce negative result in gram staining, starch hydrolysis, methyl red test, lactose utilization, maltose utilization.

Phylloplane bacterial isolates PB2, PB4, PB5, PB8, PB10 Produced positive result in gram staining, starch hydrolysis, gelatin liquefaction, casein hydrolysis, IAA production test, HCN production test, Catalase test, glucose utilization, sucrose utilization. Negative results were obtained

Table 1: Morphological and cultural characterization of Phylloplane bacteria.

Place	Isolate No	Colony character	Identified Organism
Valayappatti	PB1	Circular, Pale yellow, convex, Slimy and Glistening colony	*Pseudomonas fluorescens*
Kumaramangalam	PB2	Round, white, Opaque, flat medium sized colony with serrated margin	*Bacillus subtilis*
Thiruparaithurai	PB3	Circular, Pale yellow, Slimy and Glistening colony	*Pseudomonas fluorescens*
Thiruverumboor	PB4	Round, white, Opaque, flat medium sized colony with serrated margin	*Bacillus subtilis*
Kathiramangalam	PB5	Round, white, Opaque, flat medium sized colony with serrated margin	*Bacillus subtilis*
Sirkazhi	PB6	Circular, Pale yellow, Slimy and Glistening colony	*Pseudomonas fluorescens*
Kollidam	PB7	Circular, Pale yellow, Slimy and Glistening colony	*Pseudomonas fluorescens*
Annamalai nagar	PB8	Round, white, Opaque, flat medium sized colony with serrated margin	*Bacillus subtilis*
Sivapuri	PB9	Circular, Pale yellow, Slimy and Glistening colony	*Pseudomonas fluorescens*
C Mutlur	PB10	Round, white, Opaque, flat medium sized colony with serrated margin	*Bacillus subtilis*

Table 2: Biochemical characterization of phylloplane bacteria.

Isolate no	Gram staining	Pigment	Starch hydrolysis	Gelatin liquefaction	KOH test	Methyl red test	H_2S test	Casein hydrolysis	IAA production	HCN production	Catalase test	Glucose utilization	Lactose utilization	Sucrose utilization	Maltose utilization	Urease activity	Identified organism
PB1	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*
PB2	+	-	+	+	-	-	-	+	+	+	+	+	-	+	-	-	*B. subtilis*
PB3	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*
PB4	+	-	+	+	-	-	-	+	+	+	+	+	-	+	-	-	*B. subtilis*
PB5	+	-	+	+	-	-	-	+	+	+	+	+	-	+	-	-	*B. subtilis*
PB6	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*
PB7	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*
PB8	+	-	+	+	-	-	-	+	+	+	+	+	-	+	-	-	*B. subtilis*
PB9	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	*P. fluorescens*
PB10	+	-	+	+	-	-	-	+	+	+	+	+	-	+	-	-	*B. subtilis*

in pigment production, Methyl red test, KOH test, H_2S test, Urease activity, Lactose utilization and Maltose utilization. The results of biochemical tests compared with Bergey's manual of Determinative Bacteriology. PB1, PB3, PB6, PB7, PB9 were identified as *P. fluorescens* and PB2, PB4, PB5, PB8, PB10 were identified as *B. subtilis.* Several earlier workers *viz*., Thalhun *et al*. (2017), Prabhukarthikeyan *et al*. (2015) , Kumar *et al*. (2014), Rajashekhar *et al*. (2017)and Rajasekar *et al*. (2019) also reported similar such results in their respective experiments.

In vitro effect of Phylloplane Bacteria (PB) against the growth of *B. oryzae* (Dual culture)

In the present investigation, among ten isolates of Phylloplane bacteria PB 5 (*B. subtilis)* showed the maximum inhibition of mycelial growth of *B. oryzae* (70.00%) with a inhibition zone of 13 mm followed by PB1 (*P. fluorescens)*. The isolate PB1 inhibited the mycelial growth of *B. oryzae* upto 67.77 % with a inhibition zone of 11.50 mm. The lowest percentage of inhibition (31.11%) was observed in PB9 (*P. fluorescens)* (Table 3). Similarly, Rajasekar *et al*. (2019) reported the antifungal activity of Rice Associated Phyllosphere (RAP) communities with twenty phylloplane bacterial isolates tested against *B. oryzae.* Among the twenty isolates *B. subtilis* (PI5) showed the maximum inhibition (52.96%) followed by *P. fluorescens* (PI1) with 52.59 % inhibition over control.

Balabaskar *et al*. (2016) also reported that *P. fluorescens* was found to be more antagonistic to *B. oryzae* as it recorded the maximum percent inhibition (75.22%) which was followed by *S. marcescens* (72.78%) and *B. subtilis* (70.56%) in the decreasing order of merit. Similarly, Harish *et al*. (2015) recorded that in dual culture technique *P. fluorescens* inhibited the radial growth of *H. oryzae*. Nayak and Hiremath (2019) also reported *P. fluorescens* registered significantly maximum mycelial inhibition (62.75%) followed by *B. subtilis* (51.76%). The bacteria *P. fluorescens* and *B.*

Table 3: Antagonistic activity of PB isolates against the growth of *B. oryzae.*

PB isolate no	Mycelial Growth (mm)*	Percent Inhibition over control	Inhibition zone (mm)*
PB1	29.00^{f}	67.77	11.50^{b}
PB2	31.00^{e}	65.55	11.00^{c}
PB3	37.00^{c}	58.88	8.00^{f}
PB4	44.00^{b}	51.11	6.00^{h}
PB5	27.00^{g}	70.00	13.00^{a}
PB6	35.00^{d}	61.11	8.50^{e}
PB7	30.00^{ef}	66.66	11.50^{b}
PB8	38.00^{c}	57.77	7.50^{g}
PB9	62.00^{a}	31.11	2.00^{i}
PB10	35.00^{d}	61.11	9.00^{d}

*Mean of three replications.

*Values in each column followed by the same letter are not significantly different according to the DMRT method (p=0.05).

subtilis inhibited the pathogen growth by competing with them for nutrient and space and produced antibiotic substances like DAPG, iturin which proved pathogen growth inhibition. Cellulolytic and chitinolytic enzymes produced by phylloplane bacteria may also acted against the pathogen (Kohl *et al*. 2019).

CONCLUSION

Rice brown spot is one of the serious foliar diseases of rice. Bacteria isolated from phylloplane region of rice have been tested against brown spot disease in laboratory condition. These bacterial isolates were morphologically and biochemically characterized. Among the bacterial agents, PB 5 (*Bacillus subtilis*) performed well followed by PB 1 (*Pseudomonas fluorescens*). The least inhibition was observed in PB9 (*Pseudomonas fluorescens*).

Conflict of interest: None.

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