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Antifungal activity of rice associated phyllosphere (RAP) communities against brown spot of rice (*Bipolaris oryzae*)

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Abstract
The brown spot of rice is a devastating disease in rice production. It is caused by the pathogen *Bipolaris oryzae* which reduces many quality and quantity impacts on the rice crop. It has been known well that some phylloplane bacterial antagonists are antagonistic to some foliar diseases. The research work was carried out with the aim to study the antagonistic property of phylloplane antagonists of rice against *B. oryzae*. Twenty phylloplane bacterial antagonists were isolated and characterized. The bacterial antagonists *Bacillus subitilis* and *Pseudomonas fluorescence* were identified by the biochemical tests. Among the bacterial isolates, the isolate PI (*Bs*) 5 recorded 52.96 per cent inhibition of the mycelial growth of the pathogen over control. It was followed by PI (*Pf*) 1, PI (*Pf*) 2 and PI (*Bs*) 8 which recorded 52.59, 52.22 and 49.26 respectively. The minimum inhibition was exerted by the isolate PI (*Bs*) 9 followed by PI (*Pf*) 17 which recorded 19.26 and 19.63 per cent inhibition respectively.

Keywords: *Bipolaris oryzae*, phylloplane, *Bacillus subtilis, Pseudomonas fluorescens*

Introduction
The brown spot of rice is incited by *Bipolaris oryzae* (Breda de Haan) Shoemaker. The pathogen causes various quality and quantity impacts on the rice crop variety by reducing the grains per panicle and also causes smaller and stained grains (Nunes *et al.*, 2004) [13] and sterility of the flowers (Ou, 1985) [14].

Use of resistant cultivars and application of contact or systemic fungicides are the effective management practices followed for the management of brown spot disease. The emergence of pathogen variability and the favorable environmental conditions (Nunes *et al.*, 2004) [13] and the fungicide resistant by the pathogen populations are the challenges (Prabhu and Filippi, 1997) [15]. In addition, use of fungicides inferred high production cost and also increases the environmental contamination and they are hazardous to health of the human beings by its toxic level.

The biological control, which is environmental friendly carried out by the antagonistic microorganisms involved an alternative way for the management of several crop diseases, including rice crop (Vidhyasekaran *et al.*, 2001; Chandler *et al.*, 2015) [21, 4]. Antagonistic bacteria belonging to the genus of *Bacillus* and *Pseudomonas* sp. have been widely used for the management of brown spot of rice (Kumar *et al.*, 2016) [11]. The bacterial colonization on the phylloplane, act against a wide range of the phytopathogens *in vitro* and *in vivo* (Jayaraj *et al.*, 2007; Sen *et al.*, 2009) [9, 19]. Phylloplane bioagents have been known to induce systemic resistance against several plant diseases (Ramamoorthy *et al.*, 2001; Radjacommare *et al.*, 2002) [18, 16]. The research work on the management of brown spot of rice by using phylloplane bacterial antagonists is meager. Therefore, the present study was undertaken to isolate the phylloplane bacterial antagonists from rice plants that could be used as potential biocontrol agents for the management of rice brown spot disease.

Materials and methods
Isolation of pathogen: The heavily infected paddy leaf showing typical symptoms of brown spot of rice disease collected from the field were used for isolation of the pathogen. The symptom showed minute brown dots, later becoming cylindrical or oval resembling sesame seed shape (Fig. 1). The leaves were made into small pieces of three mm size cut along the edges of the lesions using a sterilized scalpel. The ear head pieces were surface sterilized with 0.1 per cent mercuric chloride solution for 30 seconds. These bits were then washed 3-5 times separately in repeated changes of sterile distilled water.

Fig 1: Symptom of brown spot of rice

The sterilized medium (20 ml) was poured in to sterile Petri plates (90mm) and allowed to solidify. The surface sterilized plant tissue bits were placed individually at equidistance at the rate of 3 bits per plate. The plates were incubated at room temperature (28 ± 2ºC) for 5 days and observed for fungal growth. Identification of cultures was done by microscopic examination based on their unique hyphal and conidial characters of *B. oryzae*. The growing fungal colony of each plant piece was sub cultured and purified by single hyphal tip method (Tuite, 1969) [20]. The pure culture of the pathogen was maintained on PDA slants for further use in this study.

Isolation of Phylloplane antagonist: Healthy leaves from brown spot of rice infected area were collected from paddy fields. For isolation of phylloplane bacteria, each sample were surface sterilized and blot dried. Samples were cut into 5mm^2 and put in to 250 ml Erlenmeyer flask with 20 ml of sterile distilled water. After 24 hours of continuous shaking with shaker at 60rpm at 28±2 °C, serial dilutions (10^{-1} to 10^{-8}) of the suspension was made. 50 µl from dilutes of 10^{-7} and 10^{-8} were streaked onto King's B agar (KBA) and nutrient agar (NA) media in petri dish. The plates were incubated at 28±2 °C for 24-48 hours (Akter *et al.*, 2014) [1]. After incubation single bacterial colonies was isolated based on the morphological resemblance and they were preserved under refrigerator for further use. The name of the isolates designated as PI (*Pf*) and PI (*Bs*) for *Pseudomonas* and *Bacillus* sp. respectively.

Physiological and biochemical tests for bacteria: Physiological characterization was done at NA and KBA medium based on the morphological resemblance of typical *Bacillus* and *Pseudomonas* sp. Whereas, routine biochemical tests such as Gram staining, pigment production, KOH, growth @ 4 °C, growth @ 45 °C, starch hydrolysis, catalase, citrate utilization (CU), methyl red (MR), voges proskaeur (VP), nitrate reduction (NR), gelatin liquefaction (GL) were done following standard methods described in Bergey's Manual of Determinative Bacteriology (Brown, 1939; Holt *et al.*, 1994) [3, 8].

Efficacy of phylloplane bacterial antagonists (RAP): The antagonistic effect against the pathogen was tested by dual culture technique (Dennis and Webster, 1971) [6]. A five mm actively growing 5 days old culture disc of *B. Oryzae* was placed at one end of the Petri plate containing PDA medium. A gentle three cm long streak was made with a loopful of the actively growing culture of the test bacterium, at the opposite end by means of a sterilized inoculation needle. The plates were incubated for 10-15 days at room temperature (28±2 °C) and the growth of mycelia was intermittently observed. The control petri plates were maintained by inoculated with pathogen only.

$$PI = \frac{Dc - Dt}{Dc} \times 100$$

Were,
Dc= Average diameter of fungal growth (cm) in control
Dt= Average diameter of fungal growth (cm) in treatment
PI=Per cent inhibition over control

Statistical analysis
The data were statistically analysed using the SPSS (Statistical Package for the Social Sciences) version 16.0. Data were subjected to analysis of variance (ANOVA) at significant levels (P< 0.05) and means were compared by Duncan's Multiple Range Test (DMRT).

Results
Morphological confirmation of *B. oryzae*: The causal agent of brown spot disease *B. oryzae* was isolated from the spotted leaves using PDA medium and sub cultured by the single hyphal tip method. The mycelium of the pathogen initially appeared white colour. Later, mycelium turned to greyish to dark grey on the PDA medium (Fig. 2).

Fig 2: Colony growth of *B. oryzae*

Microscopic observation of the mycelia showed light brown colored septate mycelia of brown spot pathogen was observed. The conidia were dark brown to olivaceous brown, fusiform, slightly curved with 6 to 14 transverse cell walls (Fig. 3).

Fig 3: Conidia of *B. oryzae*

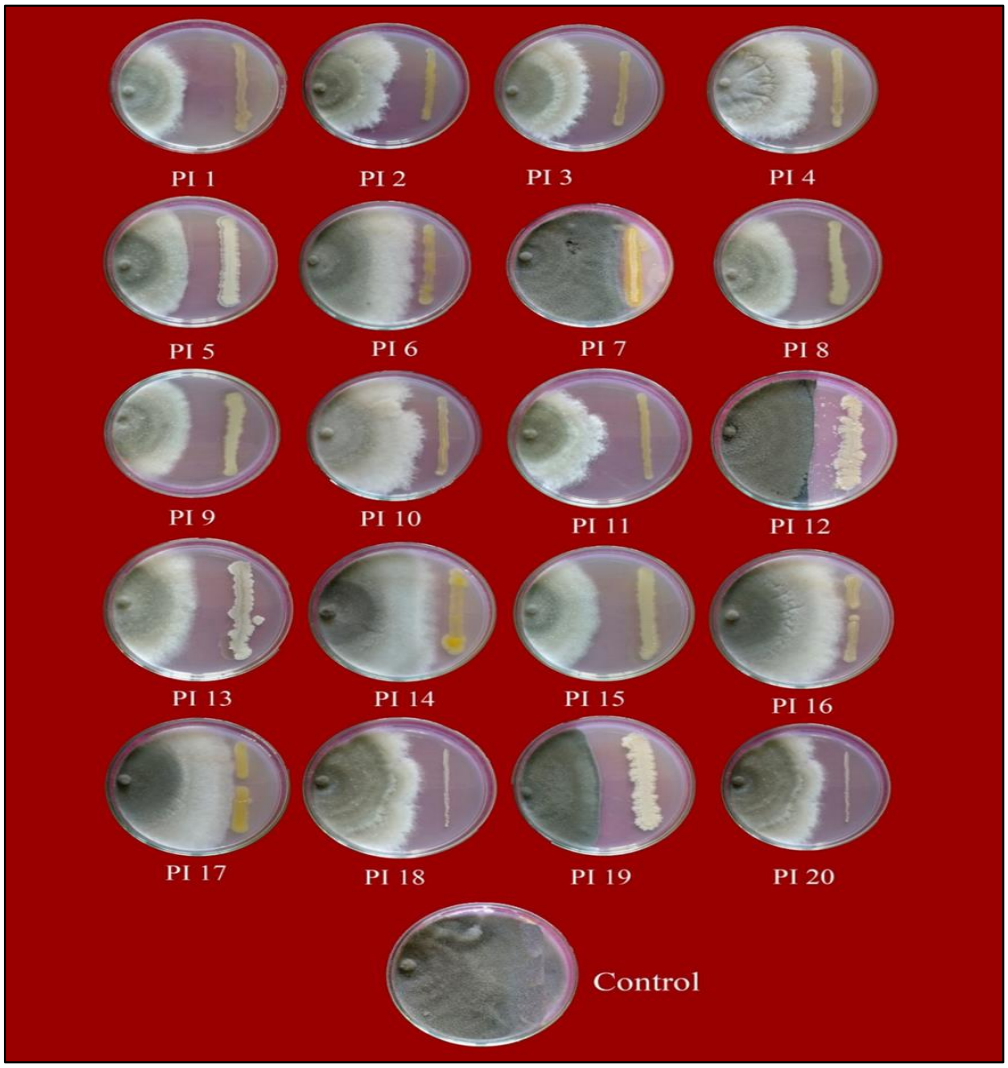

Fig 4: Effect of phylloplane antagonists against *B. oryzae in vitro*

Biochemical characterization of *Pseudomonas* spp. isolates

All the ten isolates of *Pseudomonas* spp. gave positive reaction to fluorescent pigment production, KOH test, growth at 4 °C, growth at 45 °C, catalase, citrate utilization, nitrate reductase and gelatin liquefaction. These isolates gave negative reaction to Gram staining reaction, starch hydrolysis, methyl red and voges proskauer (Table 1). Thus, these isolates were identified as *Pseudomonas fluorescens*.

Table 1: Biochemical characterization of phylloplane bacterial antagonists

S. No.	Isolate	Gram Staining	Pigment production	KoH	Growth @ 4 °C	Growth @ 45 °C	Starch hydrolysis	Catalase	CU	MR	VP	NR	GL
1.	PI (*Pf*) 1	-	+	+	+	+	-	+	+	-	-	+	+
2.	PI (*Pf*) 2	-	+	+	+	+	-	+	+	-	-	+	+
3.	PI (*Pf*) 3	-	+	+	+	+	-	+	+	-	-	+	+
4.	PI (*Bs*) 4	+	-	-	-	-	+	+	+	-	+	+	+
5.	PI (*Bs*) 5	+	-	-	-	-	+	+	+	-	+	+	+
6.	PI (*Pf*) 6	-	+	+	+	+	-	+	+	-	-	+	+
7.	PI (*Pf*) 7	-	+	+	+	+	-	+	+	-	-	+	+
8.	PI (*Bs*) 8	+	-	-	-	-	+	+	+	-	+	+	+
9.	PI (*Bs*) 9	+	-	-	-	-	+	+	+	-	+	+	+
10.	PI (*Pf*) 10	-	+	+	+	+	-	+	+	-	-	+	+
11.	PI (*Pf*) 11	-	+	+	+	+	-	+	+	-	-	+	+
12.	PI (*Bs*) 12	+	-	-	-	-	+	+	+	-	+	+	+
13.	PI (*Bs*) 13	+	-	-	-	-	+	+	+	-	+	+	+
14.	PI (*Pf*) 14	-	+	+	+	+	-	+	+	-	-	+	+
15.	PI (*Bs*) 15	+	-	-	-	-	+	+	+	-	+	+	+
16.	PI (*Pf*) 16	-	+	+	+	+	-	+	+	-	-	+	+
17.	PI (*Pf*) 17	-	+	+	+	+	-	+	+	-	-	+	+
18.	PI (*Bs*) 18	+	-	-	-	-	+	+	+	-	+	+	+
19.	PI (*Bs*) 19	+	-	-	-	-	+	+	+	-	+	+	+
20.	PI (*Bs*) 20	+	-	-	-	-	+	+	+	-	+	+	+

+ positive – negative (CU- Citrate Utilization MR-Methyl Red VP-Voges Prausker's NR-Nitrate Reductase GL-Gelatin Liquefaction

Biochemical characterization of *Bacillus* spp. isolates

All the ten isolates of *Bacillus* spp. showed positive reaction to gram staining, starch hydrolysis, catalase, citrate utilization, voges proskauer, nitrate reductase and gelatin liquefaction. These isolates gave negative reaction to pigment production, KOH, growth at 4 °C, growth at 45 °C and methyl red (Table 1). All these isolates produced cauliflower like colonies. Thus, these isolates were identified as *Bacillus subtilis*.

Efficacy of bacterial antagonists against *B. oryzae in vitro*:

Twenty phyllosphere bacterial isolates were tested against *B. oryzae* for their antagonistic potential. Among the isolated phylloplane antagonist, the isolate PI (*Bs*) 5 recorded 52.96 per cent inhibition of mycelial growth over the control followed by PI (*Pf*) 1, PI (*Pf*) 2 and PI (*Bs*) 8 which recorded 52.59, 52.22 and 49.26 percent inhibition respectively. The minimum inhibition was exerted by the isolate PI (*Bs*) 9 which was followed by PI (*Pf*) 17 which recorded 19.26 and 19.63 per cent inhibition respectively (Table 2).

Table 2: Effect of phylloplane isolates against the growth of *B. oryzae*

S. No.	Isolate	Mycelial growth	Per cent inhibition over control
1.	PI (*Pf*) 1	4.27^{g}	52.59
2.	PI (*Pf*) 2	4.30^{g}	52.22
3.	PI (*Pf*) 3	6.27^{d}	30.37
4.	PI (*Bs*) 4	6.10^{d}	32.22
5.	PI (*Bs*) 5	4.23^{g}	52.96
6.	PI (*Pf*) 6	6.23^{d}	30.74
7.	PI (*Pf*) 7	6.83^{bc}	24.07
8.	PI (*Bs*) 8	4.57^{efg}	49.26
9.	PI (*Bs*) 9	7.27^{b}	19.26
10.	PI (*Pf*) 10	6.27^{d}	30.37
11.	PI (*Pf*) 11	6.23^{d}	30.74
12.	PI (*Bs*) 12	5.07^{e}	43.70
13.	PI (*Bs*) 13	6.13^{d}	31.85
14.	PI (*Pf*) 14	4.47^{fg}	50.37
15.	PI (*Bs*) 15	6.47^{cd}	28.15
16.	PI (*Pf*) 16	4.83^{ef}	46.30
17.	PI (*Pf*) 17	7.23^{b}	19.63
18.	PI (*Bs*) 18	4.90^{ef}	45.56
19.	PI (*Bs*) 19	6.57^{cd}	27.04
20.	PI (*Bs*) 20	6.53^{cd}	27.41
21.	Control	9.00^{a}	0.00
	CD (P=0.05)	0.51	

Discussion

The brown spot disease lesions appear on the leaves as light brown to dark brown colored spots. Lesions may vary between 1mm to 14 mm depending on the cultivar and virulence of the pathogen. During the experimental period twenty phyllosphere bacterial isolates were isolated. Among the isolates, Isolate PI (*Bs*) 5, PI (*Pf*) 1, PI (*Pf*) 2 and PI (*Bs*) 8 recorded 52.96, 52.59, 52.22 and 49.26 per cent inhibition of the mycelial growth of the brown spot pathogen over control.

The similar results were obtained by Nayak and Hiremath (2019) [12] who have evaluated biocontrol agents, They reported that maximum mycelial growth was observed against *B. oryzae* by the *Pseudomonas fluorescens* with 62.75 per cent inhibition followed by *Bacillus subtilis* 51.76 per cent over untreated control. Kamei and Simon (2018) [10] reported that *P. fluorescence* which was isolated from the phyllosphere at cell concentration of 1.3×10^{8}/ml was found to be the most effective and showed mycelial growth reduction of *H. oryzae* at 52.23 per cent inhibition over the control

Ramachandra Naik *et al.* (2016) [17] isolated 39 endophytic bacteria, among them 23 were *Bacillus* spp and 16 were *Pseudomonas* spp which were confirmed by the standard biochemical tests. *Bacillus* strain of BA_2 showed reduction of maximum mycelial growth of 52.22% and *Pseudomonas* strain of PS_1 showed reduction of maximum mycelial growth of 53.33% against the brown spot of rice pathogen. Balabaskar *et al.* (2016) [2], tested four bacterial antagonists against the brown spot pathogen *B. oryzae.* Among the tested bacterial antagonists, *Pseudomonas fluorescens* showed maximum inhibition of 75.22 per cent, which was followed by *Serratia marcescens* 72.78 per cent and *Bacillus subtilis* 70.56 per cent inhibition in the decreasing order of efficiency.

Chung *et al.* (2015) [5] isolated endophytic bacteria from the rice roots of paddy. Among the isolates, the endophytic strain *Bacillus* sp. YC7007 showed the mycelial inhibition activity against *B. oryzae* KACC 40853 with the inhibition of 27.6 ±0.3 mm between mycelia of the pathogen and the border line of YC7007 bacterial strain. Harish *et al.* (2007) [7] tested Phylloplane microorganisms such as *Cladosporium* sp., *Penicillium* sp., *Aspergillus* sp., *P. fluorescence, A. flavus, Curvularia* sp., *Fusarium* sp., *and Bacillus subtilis* from the rice phylloplane region. Among the isolates tested against *H.oryzae Pseudomonas* sp. recorded 47.24% inhibition and *Bacillus* sp. recorded 34.97% inhibition under *in vitro*.

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