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Isolation and Identification of Rice Blast Disease - Suppressing Antagonistic Bacterial Strains from the Rhizosphere of Rice

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A total 60 bacterial strains from the rice rhizosphere plants grown under submerged conditions were isolated following serial dilution method. *Pseudomonas fluorescens* (*RPf1*) and *Bacillus subtilis* (*RBs1*) were used as reference culture were tested for their biocontrol activity against the devastating *M. oryzae* by *in vitro* and *in vivo*. Among these isolates eleven paddy rhizospheric isolates effectively inhibited the mycelial growth of *M. oryzae*. The isolates were identified and characterized based on morphological, biochemical and 16S rRNA gene sequencing. The presence of the antimicrobial peptide (AMP) biosynthetic genes were examined in 11 isolates and dominance of these particular genes in Bacillus strains associated with plants reinforces the competitive role of surfactin, bacyllomicin, and bacilysin in the fitness of strains in natural environments. The identification of the bacterial strains was done for isolates that showed high inhibition against *M. oryzae.* The results of *in-vitro* and *in vivo* revealed that the maximum percent inhibition and percent disease index, respectively against *M. oryzae* was observed in *Bacillus amyloliquefaciens* compared to control. From these studies, it was observed that the diversity of bacterial population in ecological niches of plant and their ability to suppress the blast of rice.

Keywords: Antimicrobial, biocontrol agents, Germination, Rhizosphere and Surfactin.

Rice is one of the most important staple foods for the increasing world population, especially in Asia. Diseases are among the most significant limiting factors that affect rice production, causing annual yield losses conservatively estimated at 5% (Song and Goodman, 2001). More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been recorded on rice, among which rice blast (*Magnaporthe oryzae*) is the most serious constraint on high productivity (Song and Goodman, 2001). Resistant cultivars and the application of pesticides have been used for blast control. However, the useful lifespan of many resistant cultivars is only a few years, due to the breakdown of the resistance in the face of the high pathogenic variability of the pathogen population. The use of pesticides is costly as well as environmentally undesirable. Thus, there is a need to develop strategies providing durable resistance that are useful over a broad geographic area. Among such new strategies, biocontrol agents appear to hold promise in blast management. Since biological control is a key component of integrated disease management, it is important to search for plant growth-promoting rhizobacteria (PGPR) active against blast and to evaluate these antagonists for application in field conditions. The mechanisms by which PGPR activate ISR are not yet understood, but it is clear that there is substantial variation between different microbes

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and plants. The ability to secrete siderophores into the environment is an important feature of many PGPR. Siderophores are iron-chelating compounds that many microbes use to acquire iron from the soil. Siderophore-producing bacteria are able to out-compete other soil microbes, providing one mechanism for their antagonistic properties (Duffy and De´fago, 1999).

Microbial biopesticides, which consist of microbial strains including bacterial or fungal species and bacteriophages by Montesinos and Bonaterra (2009), offer an alternative to or are able to complement chemical pesticides Fravel (2005). For example, several strains of the bacteria *Pantoea agglomerans* (Francés *et al*., 2006) in addition to *Pseudomonas fluorescens* (Temple *et al*., 2004), and *B. subtilis*, McSpadden and Gardener (2004), have been reported in the successful control of many plant diseases. *B. subtilis* and related species have been the object of particular interest because of their safety, their widespread distribution in very diverse habitats, their remarkable ability to survive adverse conditions due to the development of endospores, and their production of compounds that are beneficial for agronomical purposes. Several strains of Bacillus have been shown to control plant diseases by different mechanisms of action, including antibiosis, the induction of defense responses in the host plant, and competition for nutrient sources and space (Ahimou *et al*., 2000). Among these mechanisms, the dominance of antimicrobial peptide (AMP) biosynthetic genes *viz*., *surfactin, bacylisin, fengycin, bacyllomicin, subtilin* and *iturin* in *Bacillus* reinforces the competitive role for the fitness of strains in natural environments (Mora *et al*., 2011). These AMP gene markers assist in the selection of putative biocontrol agents against plant pathogens. The isolation of indigenous bacterial isolates from paddy ecological niche, its fitness as bioagent through its physiological properties, bioactivities and sustainability, including selection, screening, and characterization of strains for antagonism is studied here in managing the paddy blast disease caused by *Magnaporthe oryzae*. In future, it's prudent to develop a variety of options for disease management to sustain the food production, which may employ these bioagents or its pertinent method of application.

MATERIALS AND METHODS

Rhizobacteria isolation

Samples of rhizosphere soil and roots were collected from commercial rice fields of cultivars Jaya, BR 2566 and HR-12 at ZARS, Mandya, Karnataka during 2014 rice growing season. The soil where the samples were collected and sampling was done mainly in the following two locations in India (1) Mandya, latitude 12.5200° N and longitude 76.9000° E (2) Bengaluru, GKVK, latitude 12.9667° N and latitude 77.5667° E. The soil samples from rhizosphere were collected from 40-day old rice plants showing five fully opened leaves and tillering under submerged conditions, following serial dilution method. Ten grams of rhizosphere soil in 90 mL of sterile saline (0.85%) was diluted to10^7 (Bharathi et al., 2004). 0.1 mL of each dilution was spread on Luria-Bertani (LB) agar and incubated at 28±2°C until colony development was observed.

Morphological, physiological and molecular characterization

Out of 110 rhizobacteria isolates 60 were randomly selected and purified bacterial strains were studied for colony and cell morphology using light microscopy and Gram staining (Vincent, 1970). And these bacterial strains were evaluated under *in vitro* and *in vivo* condition. Among these 11 isolates were selected on the basis of effectively inhibited the mycelial growth of *M. oryzae.* The isolates were identified and characterized based on morphological, biochemical and 16S rRNA gene sequencing. Biochemical tests for urease activity, nitrate reduction, hydrogen sulfide production, phosphate solubilization, and methyl red-Voges-Proskauer (Cappuccino and Sherman, 2002) and also done carbohydrate utilization and fermentation. All incubations of the bacterial cultures were done in a clean incubator at 30°C.

Urease Test

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using Christensen's urea broth (Oxoid ltd., Basingstoke, Hampshire, England) containing the pH indicator phenol red (Cappuccino and Sherman, 2002). A loopful of the bacteria were aseptically inoculated into sterile Christensen's urea broth using a sterile wire loop and incubated for 24 hours.

Starch hydrolysis

Iodine solution is used to detect the hydrolysis of starch. It gives a blue colour with starch, brown with erythrodextrins and no colour with maltose.

The starch medium is used for starch hydrolysis and its composition is as follows

Peptone	: 10 g
Beef extract	: 5 g
Starch (soluble)	: 2 g
Agar	: 2 g
Water	: 1000 ml
pH	: 7

The medium was sterilized by autoclaving and poured into sterilized Petri plate and then allowed to solidify, the test culture was spot inoculated on the sterilized medium. The plates were then incubated at 30 ºC, so as to the organism allow to grow for 48 hours. After the incubation period is over, the inoculated plates were flooded with lugol's iodine and left few minutes for its action.

Production of hydrogen sulphide

This test reveals the ability of the bacterium to liberate H_2S by dissimilation of sulphur containing amino acids like cystine and methionine. The usual bacteriological peptone contains cystine in enough concentration and it is used as a substrate. The following medium (peptone water) was used for the above study.

Peptone	: 10 g
NaC1	: 5 g
Water	: 1000 ml
pH	: 7

Five-milliliter medium was dispensed in tubes and autoclaved. To detect H_2S, the lead acetate test strips were prepared as follows, Whatman No.1 filter paper was cut into 5 x 50 mm strips, which were then soaked in a warm saturated solution of lead acetate. The strips were then dried, autoclaved and again dried at 60ºC. The medium in each tube was inoculated with a loopful of 48 hr slant growth of the bacterium. After inoculation, a test strip was inserted in between the plug and inner wall of the tube, so that, it hangs just above the broth but does not touch it. The tubes were incubated at 27 ºC and observations were recorded at regular intervals up to 14 days.

Catalase activity assay

CAT activity was estimated spectrophotometrically at 240 nm by the rate of decomposition of hydrogen peroxide (Wang *et al*., 2001). Five µl of supernatant was mixed with 500 µl of substrate (150 µL 3% hydrogen peroxide in 450 µL PBS). The mixture was incubated at 28 °! for 10 min. CAT activity is presented as U/mg protein.

Glutathione S-transferase activity assay

GST activity was determined spectrophotometrically at 340 nm by the change of the concentration of 5-(2, 4-dinitrophenyl)-glutathione, which is a conjugation product of DNCB and reduced glutathione catalyzed by GST (Habig *et al*., 1974).The reaction mixture contained 50 µL of 1 mM glutathione, 50 µL of 1 mM of DNCB and 1.35 ml of phosphate buffer in a total volume of 1.5 ml solution and allowed this mixture to equilibrate at 25ºC. Then 50 µL of gut homogenate was added to the reaction mixture and inverted for 3-5 sec, an increase in optical density was measured up to 3 min with the interval of 30 sec. GST activity is represented as U/mg protein.

Protease

Bacterial isolates were checked for the production of proteases by growing them on skimmed milk agar (SKM) as described by Kazempour (2004). An ability to clear the skimmed milk suspension in the agar was taken as evidence of the secretion of proteases.

Protein estimation by Lowry's method

The samples were electrophoresed on 10% native-PAGE. After electrophoresis, the gel was pre-soaked in 0.2 M Tris-HCl buffer, pH 7.1. Then the gels were incubated in a reaction mixture containing 0.5 ml of 7% α-napthylacetate in acetone, 12.5 mg Fast Blue RR salt; 1 ml of 0.2 M Tris-HCl buffer, pH 7.1 and 23.4 ml of water for 30 min and visualize the bands (Hunter and Burstone, 1960). Purified cultures of these inveterate strains are maintained in LB medium at -20ºC for future utility.

Partial 16S rRNA amplification and sequencing DNA

Molecular verification and confirmation of strains were carried out by amplification of 16S rRNA gene from the genomic DNA by using universal primers (Table 5; Isabel Mora *et al*., 2011).

Aliquot of each amplified product was electrophoresed on agarose gel (0.7%), TAE buffer (1X) at 50V for 45 min. and stained with ethidium bromide, visualized on a UV transilluminator (Liu *et*

al., 2005). Nucleotide sequencing of the PCR fragments was performed (Amnion Biotech Pvt. Ltd., Bangalore, India) to sequences corresponding to 16s rRNA gene of the strains were reverse complemented using software Bio edit and aligned using the clustlX software. The aligned sequences were compared utilizing NCBI database for bacteria homology and searches in 16S rDNA sequences in GenBank performed with the BLAST program. Based on maximum query coverage with reference sequences from EMBL database (http://www.ncbi.nlm.nih.gov/Genbank) the species were identified. The strains were identified based on the closest relative with 97-99 percent identity.

Use of AMP gene primers and PCR assays

Primers reported by Mora 2011, to detect the genes bmyB, ituC, srfAA bacA and spaS. PCR was carried out. The amplification products were analyzed on a 1.8 % agarose gel in 1× Tris-acetate-EDTA (TAE), run for 45 min at 90 V, and viewed after staining with ethidium bromide. Size comparisons were made with a 1-kb plus ladder. Gel images were captured with an imaging system. The presence of AMP biosynthetic genes in bacillus and non bacillus isolates were determined by PCR using the specific primers for these genes (bmyB, ituC, srfAA, bacA, and spaS).

Host

The seeds of the rice cv. HR-12, susceptible to blast obtained from ZARS., Mandya Karnataka state, India was used throughout the study.

Fungal pathogen

Identified and proven pathogens *M. oryzae* (blast disease) was obtained from the cultures deposited at C-CAMP, NGG, NCBS, Bengaluru.

Pathogenicity of bacteria

In order to test whether bacteria were pathogenic to rice plants, seeds were immersed in a suspension of the antagonistic bacteria ($5x10^9$ cfu mL^{-1}) for 5 min (Raupach and Kloepper, 1998). Sterile distilled water was used as a control. Once treated, seeds were germinated in autoclaved soil. Seedlings were maintained under glasshouse conditions at 25-28ºC with daily watering with the addition of fertilizers (Fig 6).

In vitro* antagonism against *M. oryzae

Bacterial isolates were streaked around the periphery of a petri dish (1 cm away from the edge) containing PDA. Five mm mycelial plug from seven day old PDA cultures of rice pathogen *M. oryzae* was placed at the centre of Petri dish. Four replicates were maintained for each bioagent. The Petri dishes were then incubated at 28±2ºC for 5 days. Petri dishes inoculated with fungal discs alone served as control. Observations on the width of inhibition zone and mycelial growth of test pathogens were recorded and percent inhibition of pathogen growth was calculated.

I =100(C-T)/C where, I= percentage inhibition of mycelial growth, C= growth of the pathogen in the control plate (mm) and T= growth of pathogen in dual culture (mm) plate.

***In vivo* assay of the antagonistic isolates under greenhouse**

Seeds were sown in pots (30 cm diameter) containing sterilized soil (5 kg) at a rate of five seeds/pot with five pots for each replicate. Plants were kept in the greenhouse at a temperature of 28 + 2ºC during the day and 25 + 2ºC at night for 40 days. For inoculation of plants, bacterial cultures were grown in nutrient broth for 24-48 h at 28 + 2ºC. Cell pellets were obtained by centrifugation at 6000 x g for 5 min, washed and resuspended in sterile water. *B. subtillis* strains (10^9 cfu/ml) suspension were individually sprayed on entire plants 2 days before inoculation with the pathogen and one control treatment sprayed with distilled water was maintained.

The blast fungus cultured on oatmeal agar and incubated at 25ºC under fluorescent lights with a 12-hour photoperiod for 2-3 weeks. Spores of the fungus were harvested by flooding the cultured agar plates with 5-7 mL sterile water containing 0.5% gelatin then filtered through 0.2 µm nylon meshes and transferred immediately to a container containing ice to prevent spore germination. Spore concentration was adjusted to $1x10^5$ spores mL^{-1} and sprayed on 20 days old rice plants 100 ml/plant that was transferred to the inoculation chamber 1 day before the inoculation for acclimatization during the morning (28 ± 5ºC day and 25 ± 5ºC night temperature and relative humidity above 90%). Immediately after inoculation, the plants were covered with a polythene hood and black polythene sheets for 24 hours to stimulate infection in the dark. Disease assessment was done of six days after fungal inoculation, each plant was assessed individually

for blast infection and each leaf was scored for number and size of lesions. Disease incidence was evaluated according to a lesion index (Someya *et al*., 2002).

RESULTS AND DISCUSSION

Isolation and Morphological characterization of bio control strains

(Total 60 bacterial strains were selected from paddy rhizosphere soils (Betelho *et al*., 1998; Naureen *et al*., 2005) among these 11 bacterial strains of maximum inhibited against blast fungus were selected for detailed investigation. Species diversity investigation among these indicated that they likely belong to a range of different bacterial genera, Bacillus, and Pseudomonas, as they showed morphological characteristics most closely resembling these genera (Grimont and Grimont 1992). Different strains exhibited different combinations of properties for nitrogenase activity, esterases, proteases, catalases and H_2S production. The bacterial strains isolated from rice rhizosphere soils along with two reference strains were used. Colony morphology was observed after 24 hrs of inoculation, different (individual) colonies were picked from inoculated plates. Gram's staining and other morphological studies were done (Table 1). Out of 11 bacterial isolates, 7 isolates were gram-positive and 3 isolates were gram-negative. For carbohydrate utilization, all were able to utilize maltose except strain UASBR11. All-Strain were able to produce acid from lactose, sorbitol, glucose, dulcitol, sucrose and mannitol, but strains UASBR2, UASBR4 and UASBR11 did not utilize any of these whereas for phenyl alanine test all isolates showed negative and all isolates showed positive for urease test. The data were summarized in Table 1.

Almost all the isolates fermented glucose,

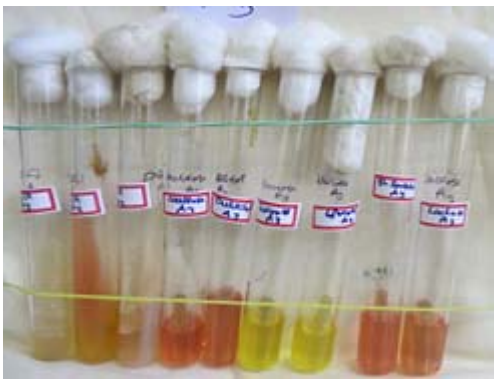


Fig. 1a. Carbohydrate utilization test

Fig. 1b. Amylase activity

Fig. 1c. Production of H_2S

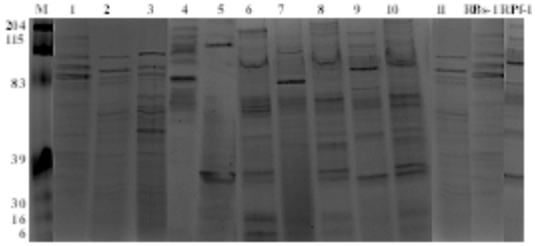
Fig. 1d. Skim milk agar test

Fig. 1. Enzyme producing attributes of bacterial isolates

lactose, sucrose with the production of both by Ali *et. al*., (1998) also studied the biochemical characteristics of the different bacterial isolates isolated from different sources. They reported a little or no difference in these biochemical characters and stated that such similarity among the isolates might be due to the presence of some common genetic materials. Bacterial isolates from rhizosphere soil sample all the biochemical characteristics gives starch, catalase, urease gives positive results which are reports of Musliu Abdulkadir *et al*. (2012).

Enzyme producing attributes of bacterial isolates

Protease activity of bacterial isolates was carried out on skim milk agar medium, out of 11



1: UASBR1 6: UASBR6 11: UASBR11
2: UASBR2 7: UASBR7 12: RBs-1
3: UASBR3 8: UASBR8 13: RPf-1
4: UASBR4 9: UASBR9
5: UASBR5 10: UASBR10

Fig. 2. Protein profile on 10 % SDS-PAGE

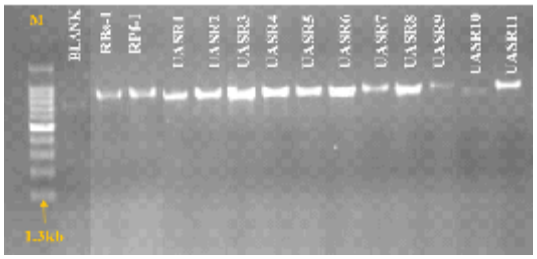


Fig. 3. Amplified PCR products of 13 isolates run in 1 % (W/V) agarose gel. M- 1300 bp marker

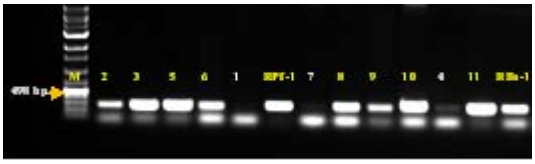


Fig. 4a. Amplification of PCR products of bacterial isolates run in 1 % (W/V) agarose gel. M- 498 bp marker for *bacA gene*

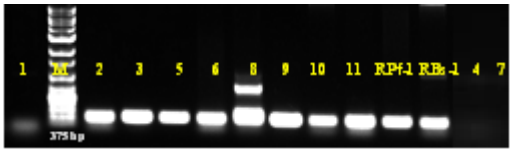


Fig. 4b. Amplified PCR products of bacterial isolates run in 1 % (W/V) agarose gel. M- 375 bp marker for *spaS gene*

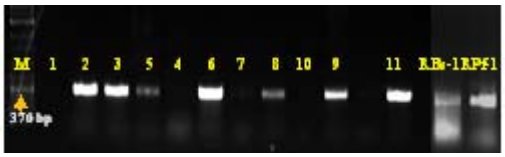


Fig. 4c. Amplified PCR products of bacterial isolates run in 1 % (W/V) agarose gel. M- 423 bp marker for *ituC gene*

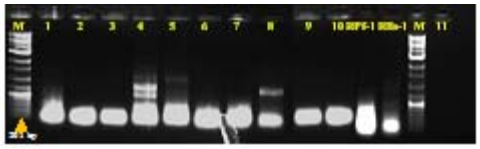
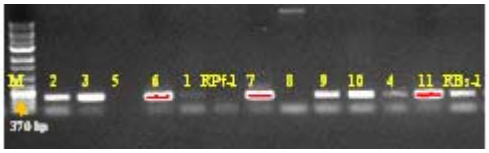


Fig. 4d. Amplified PCR products of bacterial isolates run in 1 % (W/V) agarose gel. M- 423 bp marker for *Surfactin - srfAA gene*



1: UASBR1 6: UASBR6 11: UASBR11
2: UASBR2 7: UASBR7 12: RBs-1
3: UASBR3 8: UASBR8 13: RPf-1
4: UASBR4 9: UASBR9
5: UASBR5 10: UASBR10

Fig. 4e. Amplified PCR products of bacterial isolates run in 1 % (W/V) agarose gel. M- 423 bp marker for *Bacyllomicin - bmyB gene*

Fig. 4. Representative electrophorograms of AMP biosynthetic genes amplification products in different bacterial isolates

isolates, 2 isolates; UASBR1 and UASBR7 showed positive for the test (Fig. 1d) and remaining 9 isolates showed negative for the test. In these isolates UASBR1 showed a zone of clearance of 1mm and these results showed UASBR1 have the ability to produce more enzyme hence strong protease activity compared to isolate UASBR7.

A starch agar plate assay with iodine added to detect amylase activity. Strains UASBR9 exhibiting amylase activity with the zone of clearance of 4mm. Out of 11 isolates, 8 isolates showed positive for the test (Fig. 1b) and remaining 3 isolates showed negative for the test. Colonies are white to cream in colour with a rough surface (often wrinkled) and capable of starch hydrolysis, which matches with characters of *B. subtilis* (Claus and Berkeley, 1986)..

Note: Fungal culture in the center of the plate; BCA around (perimeter)

Fig. 5. *In vitro* screening of bacterial strains against *M.oryzae*.

H_2S production (Fig. 1c) out of 11 isolates 3 isolates showed positive for H_2S production and remaining isolates were negative and all isolates were positive for GST activity ranged from 4.69 to 7.59 U/ml of enzyme and Catalase activity between 3.15 to 7.52 (Table. 3).

The esterase activity three isolates (UASBR8, UASBR3 and UASBR6) were showed esterase isozymes (4 isozymes around 110 kDa, 75 kDa, 74 kDa and 68 kDa; while other isolates were not showing esterase activity and protein extract was run on 10 % SDS-PAGE to know the bands of different bacterial isolates were observed as shown in Fig 2. it was found that there was multiple banding patterns was observed among the isolates and protein concentration ranged from 1.99 to 4.45 mg/ ml (Table. 3) There are several phenotypic similarities among isolated strains and their ability to produce acid from carbon sources is the same. If showed that the presence of multiple bands because of all the causes the total protein content of the samples was moderately high. Similar work was done by Xu *et al.*, 2009. Protein studies, the ranges of proteins and variations in their molecular weight suggest the diversity and probability of getting bacterial isolates from paddy rhizosphere soils.

Pathogenicity and effects of bacteria on plant growth

No signs of pathogenicity, such as lesion formation or wilting were observed in the seedlings that had been incubated with bacterial cultures (Table 6).

Species identification by partial 16S rRNA sequencing

Bacterial isolates that were found to be most effective in controlling rice blast both *in vitro*

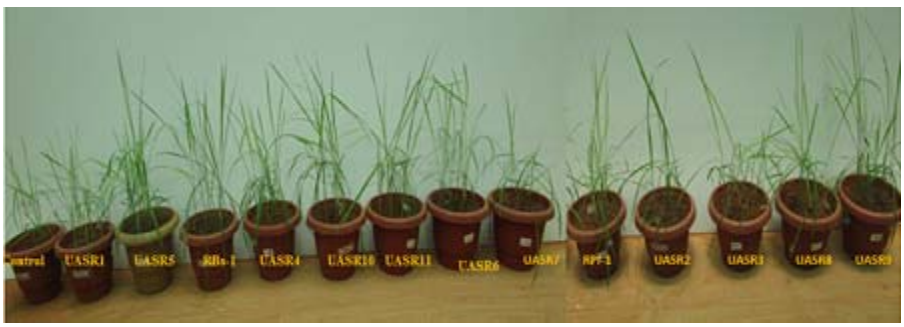


Fig. 6. Effect of bacterial isolates treatment compared to untreated on the growth at 60 days old paddy seedlings

and *in vivo* were identified using partial 16S rRNA gene sequencing. The 16S rRNA primers were used and the excised DNA bands of the ladder and sample isolates amplified without any problems. Amplification products were of the expected 1300bp size (Fig. 3). All 11bacterial isolates were amplified and showed the presence of 16S rRNA genes. The sequences obtained were compared with those in public databanks, and on the basis of similarities with existing sequences, we were able to identify most isolates at least to the genus level, as shown in Table 4, PCR amplicons of 16S rDNA of about 1300 bp was observed as discrete bands on an agarose gel. The sequences were submitted to NCBI data Bank having accession nos. The cultures were identified. It has been reported that the bacteria from genera *Bacillus*, *pseudomonas*, *Serratia*, *Alcaligenes* and *Proteus* promoted growth and yield of different non-leguminous plants were also reported by Selva Kumar *et al.* (2008). Indeed, the root associated bacterium showed the potential capacity to promote the growth of the rice plantlets and very probably in another plant also, a regulatory function play role on the interaction of the plant with other components of rhizosphere (Hallmann *et al.*, 1997).

Table 1. Morphological characters of bacterial isolates from rice rhizosphere soils

Sl. No.	Isolate	NCBI, BLAST results	Gram staining	Shape	Colony size	Pigmentation	Mucoidness
1	RBs-1	*Bacillus subtilis*	+	Serrated	20-25 mm	White	Absent
2	RPf-1	*Pseudomonas fluorescens*	-	Round	5-10 mm	Creamy	Absent
3	UASBR1	*Alcaligenes* sp.	+	Round	4-3 mm	Creamy yellow	Absent
4	UASBR2	*Pseudomonas fluorescens*	-	Round	5-10 mm	Creamy	Absent
5	UASBR3	*Bacillus cereus*	+	Round	15-20 mm	White	Absent
6	UASBR4	*Serratia marcescens*	-	Round	5-10 mm	Red	Present
7	UASBR5	*Bacillus subtillis*	+	Round	10-15 mm	White	Present
8	UASBR6	*Bacillus cereus*	+	Round	15-20 mm	White	Absent
9	UASBR7	*Alcaligenes faecalis*	-	Irregular	4-6 mm	Creamy yellow	Absent
10	UASBR8	*Bacillus pumilus*	+	Round	10-15 mm	White	Absent
11	UASBR9	*Bacillus amyloliquefaciens*	+	Round	15-17 mm	White	Present
12	UASBR10	*Proteus mirabilis*	-	Round	5-9 mm	Creamy yellow	Present
13	UASBR11	*Lysinibacillus sphaericus*	+	Round	10-12 mm	White	Present

Table 2. Biochemical reactions of bacterial isolates from rice rhizosphere soils

Sl. No.	Isolate	NCBI, BLAST results	LA	GLU	SU	MA	DS	DU	SA	NA	UA	PA	CA	H_2S	H_2O_2
1	RBs-1	*Bacillus subtilis*	+	+	+	+	-	-	+	+	+	-	+	-	-
2	RPf-1	*Pseudomonas fluorescens*	-	-	-	+	+	+	+	+	-	-	+	-	+
3	UASBR1	*Alcaligenes* sp.	+	+	+	+	+	+	+	+	-	-	+	-	-
4	UASBR2	*Pseudomonas fluorescens*	-	-	-	+	+	+	+	+	-	-	+	-	+
5	UASBR3	*Bacillus cereus*	+	+	+	+	NR	-	+	+	+	-	+	-	-
6	UASBR4	*Serratia marcescens*	-	-	-	+	+	+	-	+	+	-	+	-	+
7	UASBR5	*Bacillus subtillis*	+	+	+	+	NR	-	+	+	+	-	+	-	-
8	UASBR6	*Bacillus cereus*	+	+	+	+	NR	-	+	+	+	-	+	-	-
9	UASBR7	*Alcaligenes faecalis*	+	+	+	+	+	+	+	+	V	-	+	-	+
10	UASBR8	*Bacillus pumilus*	+	+	+	+	-	-	+	+	-	-	+	-	+
11	UASBR9	*Bacillus amyloliquefaciens*	+	+	+	+	NR	-	+	+	+	-	+	-	-
12	UASBR10	*Proteus mirabilis*	+	+	+	+	+	+	+	+	+	-	-	+	-
13	UASBR11	*Lysinibacillus sphaericus*	-	-	-	-	-	-	-	v	v	-	+	-	

Note: LA-Lactose, MA-Maltose, DS- D-Sorbitol, GLU-Glucose, DU-Dulcitol, SU-Sucrose, SA-Starch, NA-Nitrate, UA-Urease, PA-Phenyl alanine, CA-catalase, SU-Sucrose, NR- No results, V- Varied reaction

Table3. Protein and enzyme activity in different bacterial isolates

Isolates	Protein (mg/ ml)	GST activity U/ml of enzyme	Catalase activity U/mg of protein
UASBR1	3.01	5.47	6.31
UASBR2	2.92	6.37	6.43
UASBR3	2.65	5.03	3.52
UASBR4	3.03	4.69	3.41
UASBR5	3.18	6.41	4.42
UASBR6	3.12	4.96	3.49
UASBR7	3.10	5.31	3.45
UASBR8	3.38	6.81	4.42
UASBR9	4.45	7.59	7.52
UASBR10	1.99	6.8	4.13
UASBR11	2.35	7.09	3.15
RBs-1	3.28	6.45	4.32
RPf-1	2.82	6.24	6.40

Table 4. List of rhizospheric bacteria isolated from rhizosphere of *O. sativa* cultivar Jaya and identified by 16S rRNA gene sequencing

Strain Label	NCBI, BLAST results	Closest sequence similarity	Percentidentity	Accessionnumber
RBs-1	*Bacillus subtilis*	*Bacillus sp.* HP-Z73-B1	99	*KJ526902.1*
RPf-1	*Pseudomonas fluorescens*	*Pseudomonas fluorescens*	87	L49465.1
UASBR1	*Alcaligenes* sp.	*Alcaligenes faecalis* strain B18	99	KF641843.1
UASBR2	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* LMG5167	97	GU198104.1
UASBR3	*Bacillus cereus*	*Bacillus altitudins strain 5S6*	90	KM374744.1
UASBR4	*Serratia marcescens*	*Serratia marcescens strain D*	92	JF681182.1
UASBR5	*Bacillus subtillis*	*Bacillus subtillis* strain Bs 11	99	HQ234327.1

Table 5. The effects of bacterial isolates on fungal growth *in vitro* and *in vivo* (percent inhibition)

Strain	*M. oryzae* growth inhibition (%)	*M. oryzae* (PDI)
UASBR1	52.44±1.2	1.23±0.0
UASBR2	63.41±3.3	1.23±0.0
UASBR3	76.83±5.8	1.00±0.0
UASBR4	76.83±0.9	1.13±0.4
UASBR5	76.83±4.2	1.10±0.3
UASBR6	74.39±5.3	1.23±0.4
UASBR7	63.50±1.2	1.23±0.0
UASBR8	75.00±5.6	1.23±0.0
UASBR9	84.15±3.4	1.23±0.5
UASBR10	63.41±3.5	2.47±0.0
UASBR11	52.44±4.3	1.23±0.0
RBs-1	71.95±4.2	1.78±0.3
RPf-1	56.1±4.9	1.21±0.3
Control	0	3.7±0.9

Biosynthesis of AMPs and evaluation of PCR assays

PCR using the generalise primers for Bacillus directed at the 16S rRNA genes resulted in amplifications for the five *Bacillus* strains tested. However, primers bmyB and bacA also amplified two other bacterial isolates ($UASBR_2$ and $UASBR_4$). For the biosynthetic genes (bmyB, ituC, srfAA, bacA, and spaS), the sensitivity of primer detection was highly variable, also among the strains. Generally, unspecific amplifications of the AMPs genes were not observed in the strains of the other bacterial species, except for *P. fluorescens* (UASBR2), which amplified for spoVG, bmyB and bacA primers (Fig 4a to 4e) In Studies on AMP biosynthetic genes in natural populations of *Bacillus* may be useful for discovering new bacterial isolates with broad-ranging and better

efficacy of pathogen control as well as improved fitness in plant environments. Finding of relevant genes in other strains has been applied to the identification of functional molecular markers related to secondary metabolite production (e.g. AMPs) and to their beneficial effects in plants (Chen *et al*.,2011). The study of these markers in a reference collection of Bacillus sp. confirmed the presence of bmyB, ituC, srfAA, bacA, and spaS in all bacillus strains and of bmyB and bacA genes in other than Bacillus strains, in agreement with previous reports (Koumoutsi *et al*., 2004). Bacyllomicin, a member of the iturin family, has been reported to have strong antifungal activity being responsible for the main antagonistic activity of *B. amyloliquefaciens* FZB42 against *Fusarium oxysporum* (Koumoutsi *et al*., 2007).

***In vitro* screening of bacterial isolates against blast of paddy**

In the dual culture assays (Fig. 5) many of the bacterial strains significantly inhibited fungal growth over a (the incubation) period of 28 days (Table 5). Bacterial strains UASBR9 and UASBR3&4 remained antagonistic at a level of 84.14 and 76.83 %, respectively. No physical contact was observed between any of the bacteria tested and *M. oryzae*; moreover, an inhibitory halo was observed in the case of few bacterial strains, suggesting the presence of fungistatic metabolites secreted by the bacteria. With bacterial strain UASBR9, production of spores around the fungus causing complete fungal lysis was observed (Fig. 5). There appeared to be a strong association between siderophore productions. *B. subtilis* (NSRS 89-24) resulted in approximately 60 % inhibition of *P. grisea* in dual culture assay (Harman, 2000), also, it was reported that *B. subtilis* produced more than 50 percent inhibition of radial growth of pathogens where in this inhibition was experimentally attributed to the release of several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin (Moubarak and Abdel-Monaim 2011).

***In vivo* screening of strains against blast of paddy**

In vivo assay revealed highest reduction in the percentage of disease index of paddy blast (1.00) was recorded by the strain UASBR3 followed by UASBR5 (1.10), UASBR4 (1.13) and UASBR6 (1.23) as compared to positive controls RBs-1 and RPf-1 (1.78 and 1.21, respectively) and negative control 3.70 (Table 5 & Figure 5). The antagonistic effect of these strains against *M. oryzae*, individually with *Bacillus* strains were more evident when compared to uncontrolled conditions, this could be due to the Bacillus effect on plant pathogens attacking the causal agents by sugar linkages and release of extracellular enzymes such as proteases, cell wall degrading enzymes (CDWEs) and lipase. They also produced and secreted siderophores and hydrogen cyanide that are toxic to microorganisms (Wang *et al*., 2009).

Based on these criteria bacterial isolates selected to assess its performance for the management of rice disease both under lab and glasshouse conditions. Van Wees *et al*. 2000 suggested that a single bacterium may induce systemic resistance through more than a single mechanism. The PGPR are ideal organisms for crop protection because they can be used in various forms to seed or mixed with soil during seedling stage or transplanting. Our strains exhibited properties found in good colonizers and showed biofertilizer traits *in vitro*. A further contribution of these factors to the *in vivo* biocontrol activity would be of interest.

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