Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Isolation and identification of antagonistic bacteria from phylloplane of rice as biocontrol agents for sheath blight

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Publication Info

Paper received:
25 May 2013

Revised received:
02 December 2013

Re-revised received:
15 December 2013

Accepted:
13 January 2014

Abstract

A total of 325 bacteria were isolated from both healthy and sheath blight infected leaf samples of rice plants, collected from different places of Malaysia, following dilution technique. Sheath blight pathogen was isolated from infected samples by tissue plating method. Out of 325, 14 isolates were found to be antagonist against the pathogen in pre evaluation test. All the 14 isolates were morphologically characterized. Antagonistic activity of these isolates was further confirmed by adopting the standard dual culture and extracellular metabolite tests. The best isolates were selected, based on the results. In dual culture test, the selected bacterial isolates KMB25, TMB33, PMB38, UMB20 and BMB42 showed 68.44%, 60.89%, 60.22%, 50.00% and 48.22% fungal growth inhibition, respectively and in extracellular metabolite test these bacterial isolates exhibited 93.33%, 84.26%, 69.82%, 67.96% and 39.26% of the same, respectively. Biochemical tests of selected isolates were performed following standard procedure. These bacterial isolates were tentatively identified as fluorescent pseudomonas by morphological and biochemical characterization. The identities were further confirmed by Biolog microstation system as *P. fluorescens* (UMB20), *P. aeruginosa* (KMB25, TMB33 and PMB38) and *P. asplenii* (BMB42) with similarity index ranging from 0.517 to 0.697. The effective bacterial isolates obtained from the present study can be used in the management of soil borne fungal pathogen *Rhizoctonia solani,* causing sheath blight of rice.

Key words

Bacterial antagonists, Differential characteristics, *Oryza sativa, Pseudomonas, Rhizoctonia solani*

Introduction

Sheath blight caused by *Rhizoctonia solani* Khun [*Thanatephorus cucumeris* (Frank Donk] is a global rice production constraint incurring economic loss to an extent of 40% annually (Zhong *et al*., 2007). The disease is soil borne and subsequently spreads up to foliar portion. The pathogen has wide host range and so far strong resistant source against the pathogen has not yet been found elsewhere (Ou, 1985). However, normally seed treatment; soil application and foliar spray with chemicals are adopted to combat the disease. It is well known that chemical fungicides cause hazardous effects on environment and beneficial microorganisms. In this context, biocontrol is an environmental friendly excellent option. Antagonistic fungi from *Trichoderma* sp. (Lin *et al*., 1994), bacteria belonging to the genus of *Bacillus* and *Pseudomonas* (Kumar *et al*., 2009) have been widely used in controlling sheath blight of rice. Among the genera, *Pseudomonas* has some good characteristics like capable of producing some secondary metabolites like siderophores, antibiotics, volatile compounds, hydrolytic enzymes and growth promoting hormones (Compant *et al*., 2005; Gupta *et al*., 2001). A good number of fluorescent pseudomonads have been reported as effective biocontrol agents all over the world against wide range of phytopathogens in vitro and in vivo (Jayraj *et al*., 2007; Sen *et al*., 2009) including *Rhizoctonia solani*. As a prodigious colonizer, *Pseudomonas* can survive in both rhizosplane and phylloplane. Bacteria inhabiting phylloplane are exposed to adverse environmental conditions

and can be a good source of diverse bacterial isolates having plant growth promotion potential (Kishore *et al*., 2005). But until now, great majority of them were isolated, identified from rhizoplane (Manjula *et al*., 2002; Sessitsch *et al*., 2004) and few of them were reported from phylloplane. Moreover, very little information on phylloplane bacteria for rice sheath blight management is available. Therefore, in mind the present study was undertaken to isolate, characterize and identify antagonistic bacteria from rice plants that could be used as potential biocontrol agents for the management of sheath blight disease.

Materials and Methods

Healthy and sheath blight infected rice plant samples, at ripening stage, were collected from irrigated paddy fields in different rice growing areas of Peninsular Malaysia. For isolation of bacteria, two grams of each sample were surface sterilized and blot dried. Cut pieces of samples ($5mm^2$) were taken into 250 ml Erlenmeyer flask with 20 ml of sterile distilled water (SDW). After 24 hr of shaking with an orbital shaker at 150 rpm at 28±2°C, eight fold (10^{-1} to 10^{-8}) serial dilutions of the suspension was made. Small aliquots (50 µl) from dilutes of 10^{-7} and 10^{-8} were lawned onto Kings B agar (KBA) and nutrient agar (NA) media in petri dish. The plates were incubated at 28±2°C for 24-48 hr or until colony formation. Selection of single bacterial colonies was done based on morphological variation and after purification they were preserved in refrigerator. Tissue plating technique was followed to isolate *Rhizoctonia solani* from sheath blight infected samples. Single tip culture was made and stock culture was preserved on potato dextrose agar (PDA) in refrigerator at 4°C for further use.

The obtained bacterial isolates were pre-evaluated for inhibitory activity against pathogen. Four millimeter diameter of 3 day-old *Rhizoctonia solani* plug was placed on PDA plate in the centre. Bacterial isolates (48 hr old) grown on NA was inoculated to the plate at 4 places at equal distances. Each spot was 3 cm apart from the centre of fungal plug. The plates were incubated for 72 hrs at room temperature (28°C±2) and the growth of mycelia was intermittently observed. Control petri dishes were maintained, inoculated with pathogen only.

Bacterial isolates were tested for hypersensitivity reaction (HR) on tobacco leaves, according to Lelliott and Stead (1987). About 100 µl of each bacterial suspension (grown on NA for 48 hr at room temperature) was injected into the intercellular space between the veins vessel of 4 weeks old tobacco leaves by 1 ml hypodermal syringe. Three tobacco plants and 3 leaves from each plant were inoculated with each bacterial isolate and for check sterilized aquadest was inoculated in the same manner. Observation was done up to 5 days of incubation.

Morphological and cultural characteristics of the bacteria: Gross morphology was observed visually grown on KBA medium. Pure cultures of bacteria were streaked on KBA petri plates separately and incubated at room temperature until single colony developed. Individual colony was examined for shape, size, colour, elevation and texture. Micro-morphological study was done under light microscope. Production of UV-fluorescent pigments was also observed during growth time for colony morphology.

Dual culture test for selection of bacterial strains : In-vitro antagonism test was done by dual culture method (Gupta *et al*., 2001) on PDA. One loop of 48 hr old culture of bacterial strains was streaked at 1 cm from the outer side of 9 cm PDA petri plates. Four millimeter disks of actively growing 3 day-old fungus were placed at the centre of plates, 2.5 cm apart from the bacteria. Bacteria were inoculated 24 hr before inoculating the fungus. Plates inoculated with fungus without bacterial isolates served as control. The plates were incubated at 28±2 ºC for 3 days and the growth of *R. solani* in the presence or absence of any bacterial isolates was measured. The experiment was done following complete randomized design (CRD) with 10 replications.

Extracellular metabolites assay : This assay was done according to the method described by Intana *et al.* (2008) with slight modifications. One ml of cell suspension (OD=1 at 600 nm) of each strain was added to 250 ml Erlenmeyer flask containing 100 ml of sterilized NB and incubated at 28±2°C on a rotary shaker (150 rpm) for 4 days. Then the culture was centrifuged (ALC Multispeed Centrifuge, PK 121) at 10000 rpm for 8 min. The supernatant was filtrated with germ filter (d=0.22µm). From each isolate culture filtrate was mixed with PDA medium at the rate of 25% (v/v) and poured on petri dishes. Four millimeter diameter of 3 day-old culture of *R. solani* plug was transplanted directly to the mixed medium. After 3 days of incubation at room temperature, diameter of fungal colony was measured. Sterilized water mixed with PDA at the same proportion, served as control. The experiment was done following CRD with 6 replications and repeated once. Results from two experiments were averaged.

Physiological and biochemical tests of bacteria : Five bacterial isolates showing consistent efficacy in inhibition of fungal growth were selected for physiological and biochemical characterization. Physiological characterization was done on NA and KBA medium. Some routine biochemical tests such as catalase, indole, methyl red (MR), voges proskaeur (VP), nitrate reduction (NR), gelatin liquefaction, oxidase and urease production were done following standard methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Identification of bacteria : After tentative identification, isolates were confirmed their identities by Biolog identification system. Biolog universal growth medium was inoculated by a single colony of bacteria separately. Approximate bacterial concentration was quantified (96%) with turbididimeter and 100 ml of bacterial solution were pipetted into each of the 96 wells in the Biolog microplates. After incubation for 24 hr at 33ºC, reading

of the plates was taken, inserting microplates into Biolog's reader apparatus.

Statistical analysis : Quantitative data of percent inhibition of fungal growth from dual culture test and extracellular metabolites test were subjected to analysis of variance (ANOVA) with SAS software version 9.2 (SAS, Institute Cary, NC, 1987), by analysis of variance and significant differences among the treatments were determined using least significant difference (LSD) at $P \leq 0.05$.

Results and Discussion

A total of 325 bacterial isolates, yielded from 100 samples including healthy and infected ones, were tested against *R. solani* for their antagonistic potential. Among them, 14 isolates showed more than 20% fungal growth inhibition (data are not shown) and were designated as per described in the Table 1. The isolates were tested for hypersensitivity reaction on tobacco leaves. None of them were found to produce necrotic lesion on the leaves after 2 days of inoculation and even after 5 days, indicated that all of the bacterial isolates were negative to hypersensitivity reaction on tobacco leaves.

Morphological and cultural characteristics of all 14 isolates are summarized in Table 1. Six bacterial strains UMB20, UMB22, KMB27, KMB28, BMB42 and BMB59 formed small to medium round, smooth, cream colour with entire margin convex colonies. The colonies of isolate, KMB25, KMB26, KMB29 TMB32, TMB33, PMB35, PMB38 and BMB39, were found as large round, yellow, rough and flat. Micro morphological study under microscope revealed that the cells of all bacteria were rod shaped and their arrangement was mostly single or paired. They were motile in nature and non-spore formers. After 24 hr of incubation, isolates UMB20, UMB22, KMB27, BMB42 and BMB59 produced blue-green and the rest of them showed yellow-green pigment on KBA medium under UV light. Isolate KMB28 did not produce any pigment under UV light even after 48 hr of incubation. In *Pseudomonas*, pigmentation of colony is one of the most distinctive cultural characteristics. In different media they can produce several kinds of pigments. Of them yellow and water soluble fluorescent pigments (pyoverdines) are produced in iron deficient medium (Chaudhry and Rashid, 2011). It is reported that more than half of the *Pseudomonas* bacteria produce pyocyanin which is a blue-green pigment, while the nonpathogenic saprophyte *P. fluorescens* produce yellow-green fluorescent pigment (Todar, 2004). In this study, all the bacterial isolates produced yellow-green pigments on KBA medium under UV light. From the above observations, they (except KMB28) can be identified as *Pseudomonas fluorescens*.

Bacterial isolates were separated into two groups, based on the pattern of their fungal growth inhibition in dual culture. Group A comprised of 6 isolates like UMB20, UMB22, KMB27, KMB28, BMB42 and BMB59 showing clear inhibition zone during interaction with fungus (Table 2). Among the isolates in this group, BMB42 produced 10 mm zone which was significantly the highest, followed by UMB20 (9.5 mm), KMB28 (9.0 mm), KMB27 (8.8 mm). The lowest inhibition zone was obtained by BMB59 (8.0 mm). Regarding fungal growth inhibition, isolate UMB20 was found best in reducing (50%) fungal growth. The second highest (48.22%) inhibition was obtained by BMB42. Group B comprising of 8 isolates did not produce clear inhibition zone (Table 2). All bacterial isolates from this group significantly reduced the growth of *R. solani* as compared to control. The highest reduction (68.44%) over control was obtained by isolate KMB25. The second highest (63.77%) was by KMB26 followed by TMB 33

Table 1 : Cultural and morphological characteristics of bacterial isolates

Isolate	Colony morphology					Cell morphology				
	Shape	Size	Elevation	Density	Texture	Colour	Shape	Motility	Fluorescence	Growth
UMB20	Ro	Me	Ra	O	Sm	C	R	M	+	Me
UMB22	Ro	M	Ra	O	Sm	C	R	M	+	Me
KMB25	Ro	L	Fl	O	Rg	Y	SR	M	+	F
KMB26	Ro	L	Fl	O	Rg	Y	SR	M	+	F
KMB27	Ro	M	Ra	O	Sm	C	R	M	+	Me
KMB28	Ro	S	Ra	O	Sm	C	R	M	-	SL
KMB29	Ro	L	Fl	O	Sm	Y	SR	M	+	F
TMB32	Ro	L	Fl	O	Rg	Y	SR	M	+	F
TMB33	Ro	L	Fl	O	Rg	Y	SR	M	+	F
PMB35	Ro	L	Fl	O	Rg	Y	SR	M	+	F
PMB38	Ro	L	Fl	O	Rg	Y	SR	M	+	F
BMB39	Ro	L	Fl	O	Rg	Y	SR	M	+	F
BMB42	Ro	S	Ra	O	Sm	C	R	M	+	Me
BMB59	Ro	M	Ra	O	Sm	C	R	M	+	Me

Note: Ro=Round; Me =Medium; L= large; S= Small; Ra=raised; Fl= Flat; O=Opaque; SM=Smooth; Rg=Rough; C= Cream; Y=Yellow; R= Rod; SR= Short rod; M= Motile; Yellow; F= Fast; SL= Slow

Table 2 : Antagonistic activity of bacterial isolates in dual culture and effect of extracellular metabolites on *Rhizoctonia solani*

Bacterial isolate	Inhibition zone (mm)	Growth inhibition (%)	
		Dual culture test	Culture filtrate test
Group A			
UMB20	9.5ab	50.00a	67.96a
UMB22	8.5bc	45.11b	67.96a
KMB27	8.8abc	46.44b	31.11c
KMB28	9.0abc	45.77b	41.66b
BMB42	10.00a	48.22ab	39.26b
BMB59	8.0c	45.11b	31.85c
Control	0.00	0.00	0.00
Group B			
KMB25	-	68.44a	93.33a
KMB26	-	63.77ab	90.75a
KMB29	-	52.66de	59.63d
TMB32	-	57.33bcd	60.55d
TMB33	-	60.89bc	84.25b
PMB35	-	49.55e	33.33e
PMB38	-	60.22bc	69.81c
BMB39	-	55.77cde	60.74d
Control	-	0.00	0.00

Means followed by a common letter are not significantly different (P=0.05)

(60.89%), PMB38 (60.22%). Efficient antagonistic bacteria from both groups i.e., KMB25, TMB33 and PMB38, UMB20 and BMB42 were selected for further studies (Table 2). KMB26 was not selected as it was collected from the same location as KMB25.

Comparatively the higher inhibition rates were obtained in extracellular metabolites assay (Table 2). Isolates KMB25, KMB26, TBM33 and PMB38 were found to inhibit fungal growth of 93.33%, 90.77%, 84.26% and 69.82% respectively. From group A, UMB20 and UMB22 were the most promising isolates to inhibit fungal growth. Second highest inhibition was obtained by KMB28 (41.47%) followed by BMB42 (39.26%).

In both antagonism tests, all the bacterial isolates showed a range of inhibition against the growth of *R. solani*. Dual culture assay can be used as the standard test for selection of biocontrol agent and many reports have shown the cumulative effect of all mechanisms undergoing for biocontrol due to the production of antibiotics, toxic metabolite including diffusible and volatile metabolites or siderophore or lytic enzymes (Compant *et al.,* 2005). These compounds may dissolve the cell wall of the pathogen mycelium and block normal growth. There are other modes of inhibition that may be involved. Bacterial isolates were selected, based on the results of dual culture test. Extracellular metabolite efficacy test is also considered as an effective antagonism test. According to Tariq *et al.* (2010), this test result did not represent the cumulative effect of all biocontrol mechanisms.

From both the groups, selected 5 isolates UMB20, KMB25, TMB33, PMB38 and BMB42 were subjected for identification based on the results of physiological and biochemical tests. These tests were performed using conventional methods as well as commercial kit. Test results are shown in Table 3. All of them produced similar results with little exceptions. They could tolerate 1% NaCl and pH level 5-6 (Biolog). All the isolates were positive to catalase, urease production and nitrate reduction and negative to gram staining, starch hydrolysis, MR, VP, oxidase and indole production. Isolates UMB20 and BMB42 were negative to gelatin hydrolysis while others were positive. By adopting biochemical approaches, many researchers indentified soil bacteria up to genus level using the results of these approaches (Da Silva and Nahas, 2002; Phillips *et al*., 2012). In the present study, all the isolates were catalase positive, indicating that these were aerobic and facultative anaerobic bacteria (Schaad, 2001). *Pseudomonas* bacteria were classified as nitrate reducer. All the isolates responded positively to nitrate reduction test. This result was supported by the findings of Roussel-Delif *et al.* (2005). Earlier workers (Nathan *et al*., 2011) confirmed the identification of *P. fluorescens* using biochemical test results.

In Biolog system substrate utilized by 5 isolates (UMB20, KMB25, TMB33, PMB38 and BMB42) included Fusidic acid, 1% Sodium lactate (except KMB25), Troleancomycin, Rifamycin-SV, L-arginin, L-aspertic acid, L-glutamic acid, Lincomycin, Guanidine hydrochloride, Niaproof-4, D-Galacturonic acid, Quinic acid, L-Lactic acid, Citric acid, α-Keto glutamic acid, L malic acid and Potassium tellurite but did not utilize Maltose, D-cellobiose, Gentibiose, Sucrose, D-turanose, Stachynose, D-Raffinose, α-D-Lactose, D- melibiose, 3-methyl-D-glucosid, Salicin, N-acetyl-D-Glucosamine, N-acetyl-D-Manosamine, N-acetyl-D-Galactosamine, N-acetyl-Neuraminic acid, D-sorbitol, Myo-inositol, Pectin, L-Galactonic acid-g-Lactone, D-Lactic acid methyl ester. Reactions with other carbon sources were borderless and are not mentioned here. Results of each strain yielded characteristic patterns using the substrate which was compared with the database of the Biolog system. Based on the reactions with carbon sources, isolates were identified as *P. fluorescens* (UMB20), *P. aeruginosa* (KMB25, TMB33, PMB38) and *P. asplenii* (BMB42) with the similarity index ranging from 0.517 to 0.697 (Table 5). Our results corroborated with the findings of previous workers (Yang *et al*., 2011). They identified bacteria with similarity index (SIM) greater than 0.50. Here all the identified bacterial isolates showed similarity index greater than 0.50. Further confirmation of their identification should be done with molecular approach.

Phylloplane *Pseudomonas* bacteria were isolated, characterized and identified. The present investigation confirmed their antagonistic activity against sheath blight pathogen. Selected bacterial isolates were found promising in reducing *in-vitro* fungal growth. Higher percentage of growth inhibition obtained by these isolates in extra cellular metabolites test

Table 3 : Biochemical and physiological characteristics of selected bacterial isolates

Characteristics	Response				
	UMB20	KMB25	TMB33	PMB38	BMB42
Indole production	-	-	-	-	-
Methyl Red (MR)	-	-	-	-	-
Voges Proskaeur (VP)	-	-	-	-	-
Hydrolysis of					
Gelatin	-	+	+	+	-
Starch	-	-	-	-	-
Urease	+	+	+	+	+
Catalase	+	+	+	+	+
Nitrate reduction (NR)	+	+	+	+	+
Oxidase	-	-	-	-	-
Tolerance of					
1% NaCl (Biolog)	+	+	+	+	+
pH^5 (Biolog)	+	+	+	+	+
pH^6 (Biolog)	+	+	+	+	+

Table 4 : Identification of selected Pseudomonas spp. by means of Biolog system based on carbon source utilization

Strain	Level of ID	Identified as	Probability index	Similarity index	Distance	Identification type
UMB20	Species	P. fluorescens	0.646	0.646	5.166	GN-N ENt
KMB25	"	P. aeruginosa	0.858	0.542	5.355	"
TMB33	"	P. aeruginosa	0.766	0.517	4.654	"
PMB38	"	P. aeruginosa	0.846	0.565	4.794	"
BMB42	"	P. asplenii	0.697	0.697	4.373	"

indicated that they had potential to produce essential metabolites to control the pathogen. Therefore, these isolates can be used as efficient biocontrol agents against sheath blight disease. Further evaluation of their antagonistic ability with rice plants in field condition is necessary.

Acknowledgments

We are thankful to the National Agricultural Technology Project (NATP) under the Ministry of Agriculture, Bangladesh and Universiti Putra Malaysia (UPM) for their logistic and financial support to conduct this research.

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