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Biological Control of Sheath Blight of Rice using Marine Associated *Fluorescent pseudomonads*

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Rice, the crop that gives staple food for India, is being severely affected by several diseases and insect pests. Of these "Sheath Blight" (ShB) disease is one of the economically significant disease that gains serious concern. It is one of the most destructive fungal diseases of rice caused by *Rhioctonia solani.* It recorded a very high yield loss. Biological control is an efficient disease management strategy. Several microorganisms belong to the genera *Bacillus, Pseudomonas, Streptomyces* etc are used as "Biological Control Agents" (BCAs) for the management of sheath blight disease in rice. In this scenario, the present work has been aimed to isolate antagonistic rhizobacteria from the least explored coastal sand dune ecosystem, characterize their biological control potential for the suppression of ShB pathogen *R. solani* and evaluate them *in vivo*. A total of 46 rhizobacterial strains were isolated from the root hairs of sand dune plant samples using Kings B Agar (KBA) medium. The isolated strains were screened for their antagonist activity against *R. solani* by using dual culture assay on Potato Dextrose Agar (PDA) medium. About 10 strains were found to have higher *in vitro* antifungal activity against *R. solani i.e*., they exhibited zone of inhibition around them. These 10 strains were evaluated for their biocontrol potential in suppressing sheath blight disease in rice using detached leaf bits assay. Five marine associated fluorescent pseudomonad strains such as AMET1102, AMET1104, AMET1133, AMET1133 and AMET1140 have noticeably reduced the ShD incidence in rice leaves.

Key words: Coastal sand dunes, rhizobacteria, fluorescent pseudomonads, Biological control, sheath blight..

Sheath blight of rice caused by a soil borne necrotrophic fungus *Rhizoctonia solani* Kuhn. It is regarded as one of the most widely distributed diseases of rice. Its occurrence in India was reported by Paracer and Chahal (1963)[1] from Gurdaspur in the Punjab. Presently, rice sheath blight is widely distributed in most rice-growing area, including temperate, tropical, and subtropical regions, and in diverse rice production systems. It appears throughout the rice growing area of India in varying degree. The disease appears both on sheath and laminar portion of leaf. Disease lesions may coalesce to form bigger lesions and the

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disease can spread to adjacent plants in the field. Rice sheath blight disease has been reported to cause approximately 50% yield reduction in test plots of susceptible cultivars. Depending upon the age of the plant, time of infection and severity, it causes yield loss to the extent of 5.9 to 69 per cent[2, 3]. In the absence of suitable resistance donor, chemical control is the only alternative to check this disease. Since the seriousness of disease warrants chemical protection, it is important to explore alternative chemical molecule to avoid build up of resistance in the pathogen. Several new fungicides have been recently developed which are known to possess good control against *R. solani*. Keeping in view of public health and environmental safety, the ill effects of chemical pesticides have made researchers to look for other disease control strategies of which biological control by antagonistic microorganisms is gaining momentum [4].

Biological control can be defined as a population-leveling process, in which the population of one species lowers the number of another species by mechanisms such as predation, parasitism, pathogenicity or competition [5]. Biological control is an efficient disease management strategy gaining momentum in recent times [6, 7]. Several microorganisms belonging to the genera *Bacillus*, *Pseudomonas*, *Streptomyces*, *Trichoderma* and *Trichotheceum* were used as biocontrol agents (BCAs) for the management of ShB disease in rice [8-14]. Several marine bacteria isolated from coastal sea water were also found to have antagonistic activity against *R. solani* [15]. In this context, the present study has been aimed to isolate rhizobacteria from coastal sand dune plants. The coastal sand dunes are one of the neglected marine ecosystems. The microbiology of the rhizosphere of the plants in coastal sand dunes is yet to be given much importance [16]. In the present study, several rhizobacteria, mostly fluorescent pseudomonads (FPs) were isolated and screened for their antifungal activity against *R. solani.* There *in vivo* potential to suppress the sheath blight disease has also been demonstrated. The study has primarily concluded that marine associated fluorescent pseudomonads could be potential candidates as biocontrol agents against plant diseases.

MATERIALS AND METHODS

Culture and culture conditions

The test strain of fungal phytopathogen *viz.,Rhizoctonia solani* MML4001 (causes sheath blight in rice) was obtained from the Biocontrol and Microbial Metabolites Lab, Centre for Advanced Studies in Botany, University of Madras, Chennai India. All the experiments were carried out at room temperature (35 ± 2°C), unless otherwise stated.

Isolation of fluorescent pseudomonads (FPs)

Ten gram of rhizosphere soil along with root bits of coastal sand dune plants were added to 95 ml of sterile distilled water in a conical flask. This suspension was serially diluted and spread plated on King's B agar (KBA) medium (Peptone 20 g; glycerol 10 mL; K_2HPO_4 1.5 g; $MgSO_4$ 1.5 g; Agar 20 g; Distilled water 100 mL; pH 7) [17]. The plates were incubated at room temperature (30 ± 2ºC) for 48 h. The colonies that fluoresced under UV light were identified as FPs and distinct single colonies of FPs were picked, sub-cultured for purity on KBA [16].

In vitro screening of FPs for anti fungal activity

The antifungal activity of the FPs was tested *in vitro* by dual plate assay against *Rhizoctonia solani*. Mycelial disc of *R. solani* measuring 8 mm was placed in the center of a Potato Dextrose Agar (Potato 200 g; Dextrose 20 g; Agar 18 g; Distilled water 1L; pH 6.5) plate and six/four different rhizobacteria were placed at equidistance in the periphery of the plate leaving 1 cm gap from the plate rim. The plates were incubated for two days at room temperature (30 ± 2° C) after which, the antagonistic activity of rhizobacteria against *R. solani* was observed as zone of inhibition (ZOI), if any [16].

Identification of selected antagonistic bacteria

The ten selected bacterial antagonists were subjected to various biochemical and staining techniques as described by Cappuccino and Sherman (2004) [18] and the results were interpreted with the key provided in the Bergy's Manual of Determinative Bacteriology [19].

Evaluation of the selected antagonists against sheath blight in rice: detached leaf assay

Fresh paddy leaves were collected and were cut uniformly into bits measuring 6 cm in length. The leaf was protected from drying off by

attaching a wick made up of moistened blotting paper at the ends of each leaf bits. A wound was created at the centre of all the leaf bits. A small *R. solani* fungal disc (6 mm diameter) was placed over the wound. Two control were maintained. In pathogen control, on top of the wound a disc of *R. solani* was kept and received water spray only. In wound control to ascertain the effect of physical damage on leaf bits, a wound alone was maintained with water spray without any pathogen or bacterial treatment. The other leaf bits were treated with the selected bacterial cultures individually as a spray according to their treatments. This set up was under constant observation for 5 days. The antagonistic bacteria would prevent the occurrence and spread of lesions on the leaf bits [20].

Percentage of disease incidence was calculated using the formula,

Percent disease incidence (PDI) = lesion length/ leaf bit length × 100

Percentage of disease reduction was calculated using the formula,

% disease reduction = PDI in control – PDI in treated/control ×100.

RESULTS AND DISCUSSION

Biological control of plant diseases using bacterial antagonists has always attracted researchers across the world. The bacterial BCAs have many advantages such as faster growth rate, easy to handle, amenable for genetic manipulation, rhizosphere competence, etc. Several attempts were made in India and other countries to control ShB of rice using biocontrol agents [4]. Several microbial antagonists, some of which are available in commercial formulations, have shown potential for control of *R. solani* in rice and other host crops. Faltin *et al*. (2004)[21] have also suggested that isolation of rhizobacteria is the foremost essential step in developing biocontrrol agents for sheath blight suppression in rice. Here, in the present study a relatively unexplored ecosystem is approached for the isolation of rhizobacteria. The ultimate success of biocontrol is depending on how well the searching and screening processes were done. There is no single, distinct way to search or screen the BCAs. The searching and screening for BCAs depend on the target pathogen, the crop and the cropping system [22].

In the present study, it has been found that all the samples had the bacterial population not less than 10^7 cfu/g of soil fresh weight. The distinct colonies with diverse morphology were subcultured on nutrient agar. Thus, 46 pure cultures of rhizobacteria were obtained and were designated as AMET1101-AMET1146. These cultures were preserved in separate Eppendorf tubes for further study. Recently, our research group has isolated a total of 32 fluorescent pseudomonads from the rhizosphere of coastal sand dune plants and found that 24 strains were having antifungal activity against *R. solani* and their antagonistic mechanism is through the production of volatile antibiotic, hydrogen cyanide[14].

The ultimate success of biocontrol depends on how well the searching and screening processes were done. There is no single, distinct way to search or screen the BCAs. The searching and screening for BCAs depend on the target pathogen, the crop and the cropping system [22]. Characterization of bacterial biocontrol agents are considered as one of the important strategies in the disease management programs[23]. Nguyen *et al*. (2011) [24] have screened various bacterial antagonists such as *Bacillus megaterium*, *Enterobacter cloacae, Pichia guilliermondii* and *Candida ethanolica* to ascertain their antagonistic activity against wilt disease of tomato. So, all the 46 strains isolated in the present study were screened for antifungal activity against the target pathogen *R. solani* (Fig. 1). A total of 10 strains were found to have *in vitro* anti fungal activity,

Table 1. Screening of rhizobacteria from coastal sand dune vegetation for antimicrobial activity

S. No.	Strain code	Zone of inhibition against *R. solani* MML4001 (mm)
1	AMET1101	08.33 ± 1.03
2	AMET1102	08.00 ± 089
3	AMET1103	11.33 ± 0.51
4	AMET1104	11.33 ± 1.03
5	AMET1105	12.00 ± 0.00
6	AMET1131	11.00 ± 0.89
7	AMET1133	10.30 ± 1.03
8	AMET1140	17.66 ± 1.36
9	AMET1142	06.67 ± 0.51
10	AMET1143	08.33 ± 0.51

i.e., they exhibited a zone of inhibition around them against the test pathogen *R. solani* MML4001 (Table 1). The zone of inhibition was measured after two days of incubation. Adesina *et al.*, (2007)[25] have isolated and screened bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum.* They have concluded that the diversity and antagonistic activity of bacteria is site-dependent. Several workers have considered in vtro studies to select antagonistic microorganisms. Amin et al. (2010)[26] has studied the effect of volatile metabolites of *Trichoderma* species against seven fungal plant pathogens including *Rhizoctonia solani*. Thus, our expectation that the much competitive coastal sand dunes may harbour potent microorganisms with superior bioactivities has been proved.

Identification of bacterial antagonists is an essential step in developing bacterial biocontrol agents. There are different ways followed to identify a bacterium such as molecular taxonomy, numerical taxonomy etc. However, in the present study we have identified the newly isolated bacteria through various biochemical and staining techniques by following standard procedures. Two staining techniques and five biochemical tests were done for ten selected bacterial antagonists and the results are presented in table 2. All of the isolates were gram negative shorts rods which produced fluorescence in KBA when observed under UV illumination (Fig. 2). This has clearly indicates that all the bacteria belongs to the genera *Pseudomonas*. Biochemcial tests were already considered for the identification of much studied genera such as *Pseudomonas*[15, 16].

The ultimate success of bacterial biocontrol agents against fungal diseases is relies

Table 2. Biochemical characterization of selected antagonistic rhizobacteria

S. No.	Strain code	Grams reaction	Cell morphology	Motility	Catalase hydrolysis	Starch hydrolysis	Gelatin	Fluorescence in KBA
1	AMET1101	Negative	Short rods	Motile	+	-	+	+
2	AMET1102	Negative	Short rods	Motile	-	-	+	+
3	AMET1103	Negative	Short rods	Motile	-	-	+	+
4	AMET1104	Negative	Short rods	Motile	+	-	+	+
5	AMET1105	Negative	Short rods	Motile	+	-	+	+
6	AMET1131	Negative	Short rods	Motile	-	-	+	+
7	AMET1133	Negative	Short rods	Motile	+	-	+	+
8	AMET1140	Negative	Short rods	Motile	+	-	+	+
9	AMET1142	Negative	Short rods	Motile	+	-	+	+
10	AMET1143	Negative	Short rods	Motile	+	-	+	+

Table 3. *In vivo* evaluation of selected antagonists for the suppression of sheath blight disease of rice

S. No.	Treatment	Disease incidence (%)	Disease reduction (%)
1	Pathogen control	83.3	0
2	Universal control	0	100
3	AMET1101	75	9.9
4	AMET1102	63.3	24
5	AMET1103	66.7	19.9
6	AMET1104	58.3	30
7	AMET1105	68.3	18
8	AMET1131	70.8	15
9	AMET1133	62.5	24.9
10	AMET1140	56.7	31.9
11	AMET1142	70	15.9
12	AMET1143	70.8	15

on the performance of the antagonists against pathogen in *in vivo* conditions. Several workers have studied the in vivo biocontrol potential of rhizobacteria. A pot experiment was performed to assess the in vivo disease-control efficiency of *B. pumilus* SQR-N43 and its bio-organic fertilizer. Results indicate that *B. pumilus* SQR-N43 induced

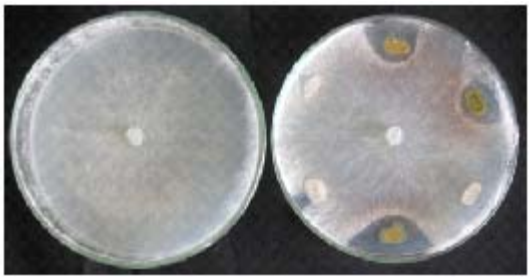

Fig. 1. Screening of rhizobacteria for antifungal activity against *R. solani* MML4001

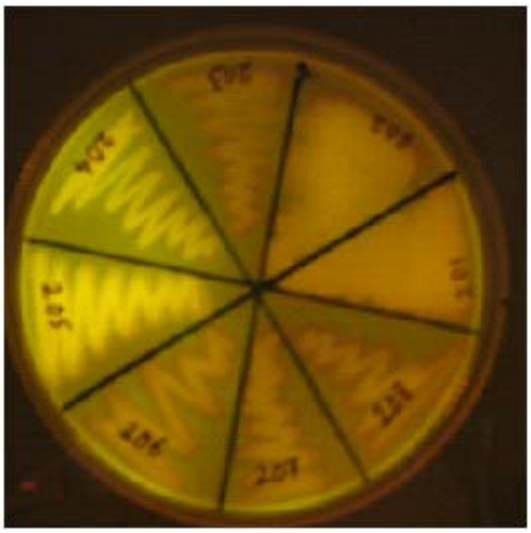

Fig. 2. Fluorescence of some rhizoabcteria in KBA

Fig. 3. Detached Leaf Assay for the evaluation of selected antagonists against sheath blight of rice

hyphal deformation, enlargement of cytoplasmic vacuoles and cytoplasmic leakage in *R. solani* Q1 mycelia[27]. Prabavathy *et al.* (2006)[10] have also tested the disease control potential of partially purified microbial metabolites against sheath blight of rice using pot experiment. However, in the present study, we have adopted detached leaf assay for the *in vivo* evaluation of biocontrol potential of FPs in the suppression of sheath blight of rice (Fig. 3). The occurrence and the spread of lesion were observed in the leaf bits. The distance of spread of lesion was measured in cm and its control by the different samples was calculated. The lesion spread has varied with the treatments. It has been found that an antagonist designated as *Pseudomonas* sp. AMET1140 has reduced the sheath blight incidence up to 31.9% than that of the control. The leaf bits treated with this bacterium were recorded a very less disease incidence of about 56.7% which is the lowest among all other treatments. *Pseudomonas* sp. AMET1104 is the next best biocontol agent against the sheath blight disease of rice (Table 3).

CONCLUSION

The present study has successfully demonstrated that the least explored marine ecosystem, the rhizosphere of coastal sand dune plants contains beneficial bacteria that are having the potential to be used as biocontrol agents against the plant diseases. They are found to reduce the incidence of sheath blight disease of rice.

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