



IN VITRO BIOCONTROL TRAITS OF RHIZOBACTERIA ISOLATED FROM THE COASTAL SAND DUNE PLANTS IN CHENNAI, INDIA

M. Jayaprakashvel*

R. Primiya**

A. Jaffar Hussain*

Abstract: Coastal sand dunes (CSD) are one of the least explored marine ecosystems for beneficial microorganisms. As a small part of our ongoing continuous research on Bioprospecting of Microorganisms from the coastal sand dunes, a total of 40 rhizobacterial strains were isolated from the rhizosphere of coastal sand dune plant samples using Kings B Agar (KBA) medium. The isolated strains were screened for their antagonist activity against *Rhizoctonia solani* by using dual culture assay on Potato Dextrose Agar (PDA) medium. It has been found that among 40 bacterial strains, 14 exhibited antagonistic activity against *R. solani*. It has been found that a strain designated as *Pseudomonas* sp. AMET 6007 exhibited 32% control over Sheath blight pathogen of rice. These 14 antagonistic fluorescent pseudomonads were also characterized for their antagonistic mechanisms such as competition for iron (production of siderophores), production of volatile (hydrogen cyanide-HCN) and diffusible metabolites. They were also characterized for their ability to produce plant growth promoting hormone, indole acetic acid (IAA). The results of the present study indicates that research is to be intensified to study the effect of these antagonists on sheath blight suppression at controlled and field conditions to ascertain their efficacy as biocontrol agents. Thus the study on the bacterial community isolation from the rhizosphere helps in revealing all the significant activities which can be used for Agricultural Biotechnology applications.

Keywords: coastal sand dune, rhizobacteria, biocontrol, sheath blight, mechanisms

*Department of Marine Biotechnology, Academy of Maritime Education and Training (AMET), East Coast Road, Kanathur, Chennai, India

**Department of Biotechnology, Karpagam Academy of Higher Education (KAHE), Coimbatore, India



INTRODUCTION

Coastal sand dunes are important yet poorly studied marine ecosystems. The physical, chemical, nutritional and biological component of the coastal sand dunes makes them an interesting system for research. Because of their geomorphological and environmental heterogeneity, the coastal sand dunes support high ecological diversity and contain many endemic and endangered species (Chen et al., 2015). However, the studies on the bioprospecting of organisms especially microorganisms from the coastal sand dunes are scanty and need much attention (Jayaprakashvel et al., 2010; Muthezhilan et al., 2012; Jayaprakashvel et al., 2014 a&b; Vellasamy et al., 2014).

Studies were conducted world over to explore the possible benefits of microorganisms associated with coastal sand dunes. In order to develop the multi-functional rhizobacteria that can exert positive effect on the growth of plants growing in the coastal sand dune located along East Coast of Korea, 1330 rhizobacteria of 11 different plants from this area were isolated. Among these, 23 strains were able to produce auxin and had spectrum of antagonism toward various phytopathogenic microbes (Lim et al., 2008). Studies were done to find anti-fungal properties of chitinolytic dune soil bacteria and found that antibiotics were involved in the antagonistic activities of chitinolytic bacteria against fungi. Only growing fungi were antagonized by the chitinolytic bacteria; none of the chitinolytic bacteria were able to lyse existing mycelium of any of the fungi. The relevance of the results for the ecology of chitinolytic soil bacteria is discussed (De Boer et al., 1998). Siderophore-producing bacteria from rhizosphere and nonrhizosphere areas of coastal sand dunes were isolated using a culture-dependent approach. Studies on the ability of these isolates to grow on sodium benzoate revealed that a *Pseudomonas aeruginosa* produced yellowish fluorescent siderophore identified as pyoverdine. The study has concluded that increase in the requirement of iron for metabolism of aromatic compounds in ecosystems where the nutrient deficiencies occur naturally would be one of the regulating factors for the bioremediation process (Gaonkar et al., 2012). Phenotypic and phylogenetic studies were performed for the facultative alkalophile from the rhizosphere of *Ipomoea pes caprae*, a plant growing on coastal sand dunes. The 16S rDNA sequence of the *Microbacterium arborescens* isolate was deposited in the GenBank with an accession number DQ287961. The phylogenetic and phenotypic distinctiveness of the strain indicates it as a novel



Microbacterium sp., named as *M. arborescens* AGSB (Godinho and Bhosle, 2013a). Coastal sand dunes are a nutrient-limited ecosystem. The bacteria associated with the vegetation on the coastal sand dunes have adapted to this stressed environment by producing biologically active metabolites which have sustained the survival of the vegetation on the dunes. These bacteria produce important plant growth-promoting metabolites which have been harnessed for their use in agricultural crops (Godinho and Bhosle, 2013b). Hence, this study has concentrated on the bioprospecting of rhizobacteria from the coastal sand dune plants of Chennai coastal area to use them as biological control agents for the suppression of plant diseases.

MATERIALS AND METHODS

Collection of rhizosphere samples and isolation of rhizobacteria from coastal sand dune plants

Rhizosphere samples of coastal sand dune plants of three commonly occurring species namely *Canavalia rosea*, *Spinifex* sp. and *Ipomoea* sp. were collected from seven different locations of Chennai coastal area for isolating Rhizobacteria. Rhizobacteria were isolated from the samples using serial dilution plating technique on Kings B Agar Medium. The morphological nature of the bacterial colonies was characterized by observing the colonies in pure culture form on nutrient agar growth medium. The isolated pure cultures of rhizobacteria were identified and the results are presented in our previous publication (Jayaprakashvel *et al.*, 2015)

Detection of beneficial bioactivities of the bacterial isolates

a. Screening for antifungal activity

The antifungal activity of the isolated bacterial colonies was tested in vitro by Dual plate assay against *Rhizoctonia solani*. The medium used for this assay is PDA. The fungal discs measuring 8 mm were cut out from 4 days old culture of *R. solani* on PDA and inoculated at the center of a fresh PDA plate. The bacterial cultures are placed on the medium in the form of circular patches. By this method all the isolated bacterial strains were patched on the plates, by placing 4 cultures in each plate and incubated at room temperature. The growth of *R. solani* and bacteria were observed periodically and the inhibition zone was measured. Efficient antagonists were identified by their ability to produce a zone of inhibition of the pathogen after three days of incubation.



b. Production of iron chelating substances

Role of iron chelating substances in the inhibition of test pathogen was tested by conducting dual plate assay simultaneously in PDA plates amended with 1Mm FeCl₃ and without Fe. The principle behind this experiment is that the siderophores are produced in iron limiting conditions only and in iron abundant conditions, the siderophore production is not supported. Increased antagonistic zone by an antagonist against *Rhizoctonia solani* in iron free medium than in the iron supplied medium, indicated the role of iron competition as there can be no siderophore production under iron abundant conditions. Two sets of PDA plates were prepared. One set contained 1 mM FeCl₃ and the second set was iron deficient. The selected bacterial strains, two in each plate were streaked on the plates leaving 1 cm gap from the periphery. Mycelial disc of *R. solani* measuring 8 mm diameter was placed in the center of the PDA plate. The plates were incubated for 2 days at room temperature (30±20°C). A control plate with the fungi alone was maintained for each set. The zone of inhibition in both iron amended and iron deficient media were measured and recorded.

c. Screening for antibacterial activity

All the test bacteria were grown in nutrient broth medium for two days at room temperature. The cell free culture filtrate was obtained by centrifugation at 8000 rpm. The agar well diffusion assay was performed for detecting the antibacterial activity of thus obtained culture filtrate against the bacterial pathogens. Four bacterial pathogens were inoculated on NA as a lawn culture and wells were made on the agar plate. The cell free culture filtrate at 100 µl concentration was placed in each well and antibacterial as a zone of inhibition was recorded if any after two days of incubation at room temperature.

d. Production of phytohormone

The plant growth promoting ability of the bacterial isolates was determined by identifying the capability of the strains to produce the growth hormone IAA and also by estimating the amount of IAA that are produced.

1. Qualitative analysis of IAA

The indole-3-acetic acid (IAA) bacterial strains were tested by the method reported by Brick *et al.* (1991). The bacterial strains that produce the plant growth promoting hormones like Indole acetic acid were determined by the inoculating the bacterial cultures in the nutrient broth containing 0.3% of tryptone. The supernatant for each culture was collected by



centrifuging at 8000 rpm for 10 minutes. 1.5 ml of the culture filtrate was taken and 2 drops of orthophosphoric acid was added. The tubes were left undisturbed for 10 minutes. To this mixture 1.5 ml of Salkowski reagent was added and were kept static at room temperature for 10 minutes. The formation of pink or brown colour indicates the production of IAA by the bacterial culture (Brick *et al.*,1991).

2. Quantitative analysis of IAA:

The quantification of IAA was performed according to Sarwar *et al.*, (1992). For this purpose, nutrient broth with 0.3% tryptone was prepared. Each of the bacterial cultures was inoculated in 5ml of the sterilized nutrient broth. These tubes were incubated at room temperature for 3 days. After incubation, the cultures were centrifuged at 3000 rpm for 30 minutes. For measuring the IAA equivalents, 1.5 ml of the fluid supernatant were pipetted into test tubes and 4 ml of Salkowski reagent (0.5 M FeCl₃ + 98 ml 35% HClO₄) were added as colouring agent. The tubes containing the mixture were left for 30 minutes. The IAA produced by the strains was measured spectrophotometrically at 530 nm.

e. Production of volatile metabolite: hydrogen cyanide (HCN)

Soil bacterial biocontrol agents (BCAs) can produce volatile metabolites such as hydrogen cyanide (HCN). The production of the HCN by the antagonistic bacteria can be detected using the Nutrient sucrose agar (NSA) medium (Lorck,1948). The rhizobacteria have the ability to synthesis the HCN which can be used for beneficial processes.

Nutrient sucrose agar (NSA) medium (Sucrose 5 g; Yeast extract 4 g; Beef extract 2 g; Agar 18 g; Distilled water 950 mL; pH 7 and 4.4 g filter sterilized glycine in 50 mL distilled water at the time of plating) was used to detect the production of HCN by antagonistic bacteria as described by Lorck (1948). Filter paper strips saturated with 0.5% picric acid in 2% aqueous sodium carbonate solution were placed on the lid of test isolate inoculated NSA plates. The plates were sealed with the help of parafilm to trap the HCN produced, if any. After two days of incubation, the production of HCN was determined by the change of colour of the filter paper from yellow to reddish brown.

Preparation of Na₂CO₃. picric acid solution

2% of aqueous sodium carbonate solution was prepared in 100ml of sterilized distilled water. To it 0.5g of picric acid was added and mixed well under aseptic condition.



Saturation of filter paper strips

The filter paper strips were prepared by having the measurement as 2 x 6cm. These strips were sterilized and were immersed in the Na₂CO₃ - picric acid solution till the strips get saturated.

f. Screening for thermo-stability

The bacterial cultures isolated from the rhizosphere may have the ability to withstand the extreme heat condition. This nature of the organism to tolerate the heat can be detected by screening them for thermostability. In this method, the PDA medium was prepared. The bacterial culture that was grown in the nutrient broth was taken and was centrifuged at 10000 rpm for 10 minutes. The supernatant was collected and autoclaved. Wells were cut in the medium by using sterilized 8mm cork borer. 100µl of the autoclaved culture filtrate was added to the wells. The fungal disc of *Rhizoctonia solani* was placed in the middle of the plate. If the organism has the ability to withstand the high temperature, they will inhibit the growth of the fungi and thus will produce a clear zone.

RESULTS AND DISCUSSION

Plants surviving on coastal sand dunes are subjected to numerous environmental fluctuations such as high solar radiation, nutrient deficiency, drought, salt spray and high winds which affect their growth, survival and community structure (Oosting and Billings, 1942; Arun et al., 1999). The microbial communities associated with these plants are also likely to be adapted to the different habitats available (Rosa et al., 1995). However, exploration of coastal sand dune associated bacteria for beneficial activities are very scarce despite of their potential. The present study has concentrated on bioprospecting of beneficial bioactivities of rhizobacteria isolated from coastal sand dunes.

Screening for antifungal activity

The rhizobacteria isolated from coastal sand dune plants were screened for their antagonist activity against *Rhizoctonia solani* by using dual culture assay on Potato Dextrose Agar (PDA) medium. It has been found that among 40 bacterial strains, 14 exhibited antagonistic activity against *R. solani*. It has been found that a strain designated as *Pseudomonas* sp. AMET 6007 exhibited 32% control over Sheath blight pathogen of rice.

Production of iron chelating substances in low iron medium



Siderophores, the iron binding molecules produced by many rhizobacteria were responsible for inhibition of many phytopathogens. Mercado-Blanco et al. (2004) reported the siderophore mediated suppression of *Verticillium* wilt by root associated *Pseudomonas* sp. The bacterial strains that were isolated from the locations such as Kanathur, Kalpakkam and Sadras were found to inhibit the growth and development of the plant pathogenic fungi. Totally 6 bacterial strains were found to have more antagonistic activity against *R. solani* in the iron deficient medium which indicates that they are producing siderophores (Table 1).

Antibacterial activity of the isolates

The screening for the antagonistic activity of the rhizobacterial strains against the pathogenic bacteria showed that the bacterial culture that were isolated from the rhizosphere were devoid of the inhibition activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus*, and *Escherichia coli*.

Production of phytohormones

The screening for the production of the plant growth promoting hormone showed that the bacterial strains were able to produce the growth hormone known as Indole acetic acid. The quantification of the IAA was measured at 530 nm. IAA (Table 2). Among the 40 strains tested, 17 have produced IAA in their culture filtrates. A strain designated as AMET 6009 has produced highest amount of absorbance (indirect estimation for IAA). The production of IAA by microorganisms such as *Bacillus* spp., *Pseudomonas* spp. and *Rhizobium* spp., was commonly observed in the rhizosphere of crop plants and often associated with plant growth (Patten and Glick, 2002; Jayaprakashvel and Mathivanan, 2011).

Production of HCN

The isolates were screened for their ability to produce volatile metabolite like hydrogen cyanide. It was identified that the isolated bacterial strains were not found to produce the HCN in the present study. Production of volatile antibiotics is still less explored biocontrol mechanism of BCAs. Hydrogen cyanide (HCN) is a volatile secondary metabolite produced by some gram-negative bacteria. Earlier our group has isolated 24 hydrogen cyanide (HCN) producing fluorescent pseudomonads (FPs) from the rhizosphere of sand dune vegetation from Chennai coastal area. Five FP strains designated as AMET1039, AMET1041, AMET1042, AMET1055 and AMET1064 produced more amount of HCN in their volatile fraction. In dual



bottom plates assay, all these five isolates exhibited maximum mycelial growth inhibition of *Rhizoctonia solani* MML4001 due to the production of HCN (Jayaprakashvel et al., 2010a)

Results for thermo stability

Thermal stability is very much required characteristic of a metabolite to be used for crop protection practices in agriculture (Jayaprakashvel et al., 2010b). Hence, all the 40 bacterial isolates were tested for their thermostability by selecting their culture filtrate. This screening showed that the strains were not thermostable and thus could not withstand high heat conditions. This may be due to the absence or inactivation of the proteins or peptides that provide the resistance to the high temperature.

CONCLUSION

The study has concluded that coastal sand dune plants and their rhizosphere could be a potential source for beneficial microorganisms to be used for agricultural biotechnological applications.

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Table 1. Production of iron chelating substances in low iron medium

SAMPLE CODE	Zone of Inhibition (mm) against <i>R. solani</i>	
	Medium with 1mM FeCl ₃	Iron deficient medium
AMET 6001	8	12
AMET 6002	12	15
AMET 6004	7	12
AMET 6005	7	8
AMET 6007	6	7
AMET 6008	9	10
AMET 6009	6	9

Table 2. Qualitative and Quantitative detection of IAA production by the rhizobacteria of coastal sand dune plants

Sl. No.	Sample Code	IAA Production	A ₅₃₀
1	AMET 6001	POSITIVE	0.127
2	AMET 6002	POSITIVE	0.109
3	AMET 6004	POSITIVE	0.077
4	AMET 6005	POSITIVE	0.108
5	AMET 6009	POSITIVE	0.141
6	AMET 6011	POSITIVE	0.110
7	AMET 6012	POSITIVE	0.092
8	AMET 6013	POSITIVE	0.105
9	AMET 6015	POSITIVE	0.119
10	AMET 6016	POSITIVE	0.104
11	AMET 6017	POSITIVE	0.099
12	AMET 6018	POSITIVE	0.105
13	AMET 6023	POSITIVE	0.114
14	AMET 6024	POSITIVE	0.137
15	AMET 6025	POSITIVE	0.121
16	AMET 6027	POSITIVE	0.124
17	AMET 6030	POSITIVE	0.133