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ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC *BACILLUS* SPP. FROM PNEUMATOPHORE OF *AVICENNIA ALBA* WITH BIOCONTROL ACTIVITY AGAINST *FUSARIUM OXYSPORUM*

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ABSTRACT
The study was carried out to isolate, evaluate and characterize potential antibiotic producing bacteria from sediments of Bhitarkanika mangrove forest of India. A total of three strains of bacteria were isolated, identified and tested against rice pathogen *Fusarium oxysporum*. The isolates were characterized by using morphological, biochemical and molecular methods. All isolates were gram positive, endospore forming and most of the isolates were able to hydrolysis starch and urea; able to survive at 6% concentration of sodium chloride; optimum temperature for their growth was 37°C. PGPR activity assays were done among three *Bacillus subtilis* coded AN1, AN2 and AN11. All the isolates showed positive results for PGPR activity. Biocontrol activity was assayed against rice pathogen *Fusarium oxysporum* by well diffusion method and out of three isolates only AN11 demonstrated *in vitro* antagonistic activity. The partial 16S rDNA sequences of the isolated strains AN1, AN2 and AN11 showed 99% identity with *Bacillus subtilis* and were deposited in GenBank under accession numbers JX860846, JX860847 and JX860845 respectively.

INTRODUCTION

Endophytes are capable of invading inner tissues including xylem vessels and are of systemic spreading (Hurek, Reinhold-Hurek , Van Montagu , Kellenbberger , 1994; Olivares , James , Baldani, Dobereiner, 1997); however, their functions for the plants are still disputed (James and Olivares, 1998). They can promote the plant growth and yield and can act as a biocontrol agent (Hallmann, Quadt-Hallmann, Mahaffee, and Kloepper, 1997). Mangrove ecosystem is a bridge between terrestrial and marine environment and harbors unique microbial diversity. Recently, among the marine microorganisms, marine derived bacteria have been recognized as good resources for new biologically active secondary metabolites including antitumor, antibacterial, antiviral, antifungal and enzyme inhibitor compounds.

Rice (*Oryza sativa*) is an important food crop worldwide. Low productions due to disease effect have great impact on the producers. Therefore, it is necessary to recognize the pathogen and develop management practices for their control. In fact, it is essential to develop an effective control method to ensure good production and yield stability. Many methods such as chemical, cultural and biological techniques have been developed for the control of plant diseases by soil borne pathogens (Singleton, Mihail and Rush, 1992). The high costs of chemical control, failures in chemical control due to resistance development, and lack of other effective control measures have, therefore, generated considerable interest in BCAs that offers an effective and environmentally friendly alternative to control plant diseases. Achievement of biocontrol measure through microorganisms that occur naturally in the field has been made a reality via antibiotics and bacteriocins (Moore ,1983). *B. subtilis* have capacity to produce more than two dozen antibiotics and have been used widely in biotechnological applications as biological control agents (BCAs). Antibiosis against the phytopathogens has been associated mainly with production of secondary metabolites (Abee, Krockel and Hill, 1995). Co-cultures of fungi and *Bacillus* spp. can lead to increased bacteriocin production and also inhibitory to the growth of fungi (Cornea, Grebenisan, Mateescu, Vamanu and Campeanu, 2003; Pryor, Siebert, Gibson, Gossett and Walker, 2007). It has also been reported that *Bacillus* spp. can induce morphological changes in some fungi (Romero, De, Olmos, Davila and Perez-Garcia, 2007).

Fungi of the genus *Fusarium* are widespread pathogens on small-grain cereals in all areas of the world causing economic losses as high as 50%, depending on the crop (Webster and Gunnell, 1992). A natural characteristic of many *Fusarium* spp. is the production of dark cellular pigments, as a result of melanization process. Melanin has been linked to high virulence, resistance in adverse environmental conditions and low susceptibility to antifungal drugs (Shirokov, Loginov, Melent'ev and Aktuganov, 2002; Jacobson, 2000).

Marine environment is the most dynamic and most variable among the natural environments present in the globe due to their continuous changing pattern of salinity, surface temperature, pH and pressure. Little work has been carried

out on the bacterial diversity of Bhitarkanika mangrove forest (Gupta, Mishra and Basak, 2007; Gupta, Das and Basak, 2007) but there is no encouraging report on endophytic marine bacteria from this environment. In the present study, endophytic bacteria were isolated from the surface sterilized pneumatophores of *A. alba* and characterized up to speices level through phenotypic and genotypic profile study. So the interaction of *B. subtilis* with *Fusarium* spp. was studied *in vitro*.

MATERIALS AND METHODS

Isolation of endophytic bacteria from pneumatophores of *A. alba*

Geographically Bhitarkanika is located between 20°4'-20°8'N Latitudes and 86° 45'-87° 50' Longitudes .It is the second largest mangrove ecosystem of India (Fig-1). Pneumatophores (6 to 8 cm in length) of *A. alba*. were collected from three intertidal zone of Bhitarkanika mangrove forest. The roots were washed properly with tap water and then distilled water to remove the sediments. They were subjected to three steps of surface sterilization procedure; Step 1: Washed with 70% ethanol for 1 minute followed by distilled water. Step 2: Soaked in 0.1% mercuric chloride solution for 3 minutes and washed with distilled water for 2 times. Step 3: Soaked in 70% ethanol for 30 seconds and washed for 5 to 7 times with distilled water. One additional step was followed in this sterilization procedure (Gagne, Richard, Roussean and Antoun, 1987). The surface sterilized roots were aseptically sectioned into small pieces (0.2 cm thickness) and placed on nutrient agar plates, followed by incubation at 37°C for 48 h. The colony forming units (CFU) were counted after 3days. The bacterial growths associated with root sections were purified by sub culturing to fresh medium. 20% Glycerol preservation technique was used for maintenance of pure culture for further study.

Collection and maintenance of rice fungal pathogens

Rice pathogen *Fusarium oxysporum* strains were collected from CRRI, Cuttack and maintained on SDA and PDA slants.

Bacterial DNA extraction and PCR amplification of ribosomal 16S gene

Total DNA was isolated by Kit method using HiPurA™ Bacterial Genomic DNA Purification Spin Kit – MB 505. The amplified 16S ribosomal DNA gene was obtained from bacterial strains, using PCR amplification, with the universal primers F (5'-AAG AGT TTG ATC CTG GCT CAG -3') and R (3'-GGT TAC CTT GTT ACG ACT T 5'). These primers are targeted to universally conserved regions and permit the amplification of an approximately 1500 bp fragment. PCR amplification was carried with a thermal program, which comprised 35 cycles at 95°C for 1min, 55°C for 40 sec and 72°C for 1min in a thermal cycler (Veriti 96-Well Thermal Cycler, Applied Biosystems).

Plant growth promoting activities (*in vitro*)

Bioassay for IAA production

IAA production was determined *in vitro* by the method described by Patten and Glick (1996). All the test strains were screened for IAA production. The test bacterial culture was inoculated in the nutrient broth with tryptophan (0.1 g/l) or without tryptophan incubated at 30° C. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of ortho-phosphoric acid and 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$).

Nitrogen Fixation

Fixation of atmospheric nitrogen by the bacterium was tested qualitatively using Burk's N_2-free medium (Subba, 1999). After overnight incubation halos around the colonies indicated positive result. The plating was done in triplicate.

Phosphate solubilization

Phosphate solubilization test was conducted qualitatively by inoculating the strains in agar containing precipitated tricalcium phosphate, modified Pikovskaya medium (Subba, 1999). Bacteria were streaked on the surface of replicated agar plates. The presence of clearing zone around bacterial colonies after overnight incubation indicated positive result of phosphate solubilization. The plating was done in triplicate.

Sulphur reduction

Reduction of sulphur by the bacterium was tested qualitatively by sulphate API agar (Subba, 1999).

Siderophore production

Siderophore production was tested qualitatively using chrome azurol S (CAS) agar. The plating was repeated for three times. Orange halos around the colonies after overnight incubation indicated siderophore production (Milagres, Machuca and Napoleao, 1999).

In vitro assay of antagonistic effect

The well diffusion assay was used for *in vitro* test of antagonistic activities of the bacterial isolates (Schillinger, and Lucke, 1989). The potential bacterial isolates were grown on NA at 37°C for 24 hr to obtain fresh culture for plate assay. Rice pathogen *Fusarium* strains were cultivated on PDB at 28°C for 7–10 days until sporulation. The fungal culture was spread over SDA and PDA plates and was inoculated with 0.1ml broth culture of *B. subtilis* in the well, without antibiotic. After an incubation of 7 days at 28°C, antagonistic activities were evaluated by measuring (in mm) the inhibition zones between pathogens and tested bacteria (Table-4).

RESULTS

Biochemical characterization

Out of 23 endophytic bacterial isolates only 3 i.e. AN1, AN2, AN11 were characterized as PGPR. They were found to be gram positive, motile rod having terminal endospores (Table-1). The biochemical tests were carried out (Table-2) and out of 3 isolates, only AN1 was found to be negative for casein hydrolysis. The tests for methyl red, urease, amylase, gelatinase, oxidase production, nitrate reduction and citrate utilization

Table 1: Phenotypic characteristics of bacterial isolates

Tests	Physiological characteristics		
	AN 1	AN 2	AN 11
Temperature	26°C +	28°C +	28°C +
pH (7)	+	+	+
Salinity (6%)	+	+	+
	Morphological characteristics		
Shape	Rod	Rod	Rod
Gram's staining	+	+	+
Motility	+	+	+
Endospore formation	+Central	+Central	+Central

Table 2: Biochemical Tests

Tests	AN 1	AN 2	AN 11
Anaerobic growth	-	-	-
Methyl Red	+	+	+
Voges Proskauer's	-	-	-
ONPG	-	-	-
Urease	+	+	+
Nitrate Reduction	+	+	+
Citrate Utilization	+	+	+
Indole	-	-	-
Oxidase	+	+	+
Casein	-	+	+
Amylase	+	+	+
Gelatinase	+	+	+

was found to be positive while indole, Voges Proskauer's and ONPG tests were found to be negative for three tested strains

Plant growth promoting activities (*in vitro*) of the isolates

The plant growth promoting activities were tested (Table-3) and all three isolates showed positive results for phosphate solubilization and nitrogen fixation. AN2 and AN11 showed sulphur reduction while AN1 and AN11 showed siderophore production. Out of all the three isolates studied, only AN11 was positive for all the tests.

Table 3: Plant growth promoting activities of the isolates (*in vitro*)

Strains	IAAproduction (μmol/ml)	Psolubilization	Nfixation	Sreduction	Siderophore production
AN 1	2.21	+	+	-	+
AN 2	2.95	+	+	+	-
AN 11	3.77	+	+	+	+

Table 4: *In vitro* antagonistic study of AN11 against *Fusarium oxysporum*

Fusarium oxysporum strains	Zone of inhibition (mm) *	Decay diameter (mm)**
F-37	29	44
F-40	40	45
F-44	34	39
F-49	21	33

* The mean zone of inhibition measured in mm between pathogens and tested bacteria on PDA medium after an incubation of seven days at 28°C. The data presented are the mean of three experiments.

** The diameters of decayed area on Petri plates were measured after two weeks incubation at 28°C. The data presented are the mean of three experiments.

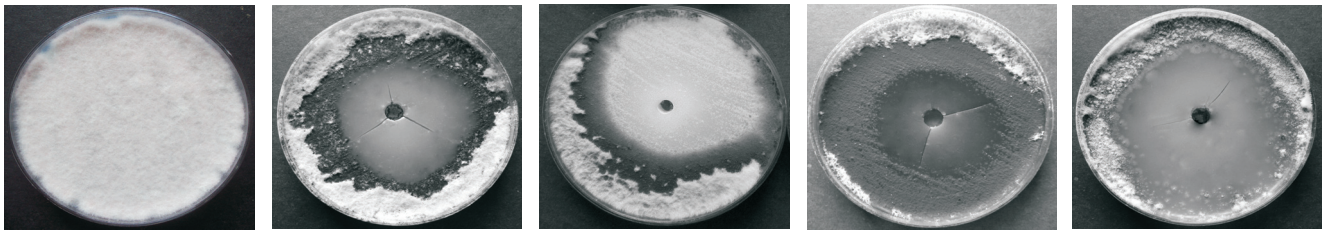
Molecular characterization

DNA fragment containing the 16S rRNA gene of all the three isolates were PCR-amplified (Fig-3), and sequenced. This sequence information was deposited to Gen-Bank of NCBI with accession numbers of JX860845, JX860846 and JX860847 for isolates AN11, AN1 and AN2 respectively. The 16S rDNA sequence exhibited maximum identity to the *Bacillus* genus in all the bank databases for the 3 isolates. However, a crossed identity of ribosomal genes over 99% was detected among the species of *B. subtilis*.

In vitro assays

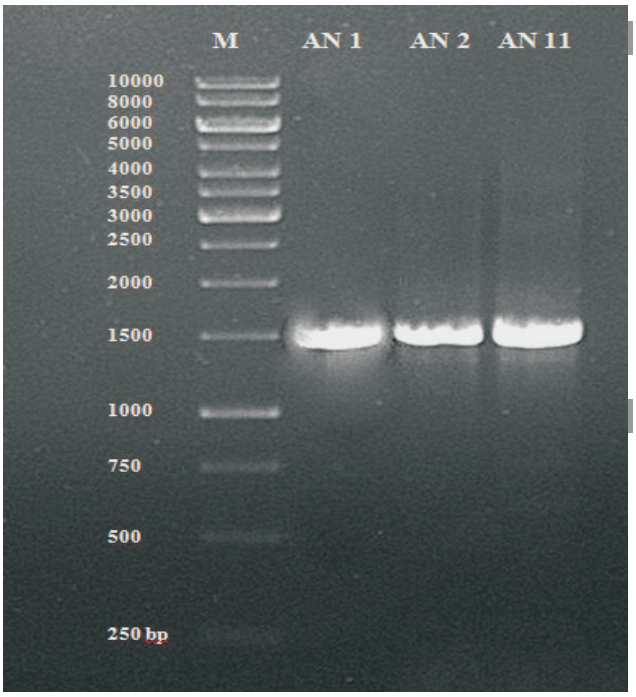
Among 3 endophytic PGPRs isolated, only AN11 showed antibiosis on co-culture method against rice pathogen of *F.oxysporum* (Table-4). Antifungal activity was more pronounced on SDA medium in comparison to PDA medium. In many instances, a line of precipitation was observed in co-cultures plates near the bacterial colony (Fig-2).

Figure 1- Three sample collection sites of Bhitarkanika mangrove forest (black dots)



Control F-37 F-40 F-44 F-49

Figure 2- Antagonistic activity of *Bacillus subtilis* AN11 against *Fusarium oxysporum* strain (7d old)



Lane 1: Kb Marker DNA, Lane 2: AN 1 sample, Lane 3: AN 2 sample, Lane 4: AN 11 sample

Figure 3- Gel showing the PCR products of 16S rDNA primer that amplifies around 1.4Kb of *Bacillus* spp.

The diffusion of red and red-ochre pigments, on agar plates by all fungal colonies were observed after one week of co-culture with *B. subtilis*.

DISCUSSION

Twenty three endophytic bacteria were isolated from pneumatophores of *A. alba* and three out of them were characterized as PGPR i.e. AN1, AN2, AN11 on the basis of IAA production, phosphorus solubilization, sulphur reduction and nitrogen fixation capacity. Out of three PGPR isolates, AN1 showed antagonistic activity against rice pathogen *Fusarium* spp. causing rice seedling mortality. The PGPR promote plant growth through more than one mechanism that includes secretion of variety of growth stimulating hormones and suppression of plant growth retarding agents, like pathogens. Production of growth hormone such as IAA by PGPRs has also been reported by Dilfuza (2008).

The strain AN11 had significant effect on controlling all fungal species *in vitro* and was identified as *Bacillus subtilis* which correlates with the results of previous workers (Benhamou, Kloepper, and Tuzun, 1998). There is a lot of literature indicating that strains of *Bacillus subtilis* may be used as BCAs against rice diseases. Gram-positive bacilli and fungi are important bio-regulators and widespread in soil (Boer, Folman, Summerbell and Boddy, 2005).

It can be concluded that endophytic bacteria isolated from pneumatophores of *A. alba* have potential to be used successfully as PGPRs. Among them AN11 showed antagonistic activity against rice pathogen *Fusarium* spp *in vitro* which was identified as *Bacillus subtilis*.

The above study needs further experiment for isolation of the active molecule working against the rice pathogens.

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