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RESEARCH ARTICLE

PLANT GROWTH PROMOTING OF ENDOPHYTIC *BACILLUS CEREUS* ISOLATED FROM THE PNEUMATOPHORES OF *Avicennia marina*

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ABSTRACT

An endophytic bacterium was isolated from the surface sterilized pneumatophores of mangrove plant *Avicennia marina,* Vellar estuary, south east coast of India. Using 16S rRNA sequencing the bacterium was identified as *Bacillus cereus* –SjAM16104 with similarity of 99% and sequence has been deposited under accession number GU930360. It grew optimally at 26ºC, pH 7 and 5% of salinity. The major fatty acids of *B.cereus* were anteiso-15:0 (40.55%), anteiso-17:0 (17.39%), iso-16:0 (8.95%), iso-15:0 (7.82%), iso-17: 0 (6.62%), 16: 0 (5.23 %) and fatty acids iso-14:0 (2.03%), anteiso-13:0 (1.87%) were detected in small amounts. Experiments were conducted to evaluate the efficacy of endophytic *B.cereus* in improving crop growth of *Bacopa monnieri* under in vitro condition. The growth rate of endophytic *B.cereus* treated explants were highly significant than the control explants.

INTRODUCTION

Mangroves are highly productive ecosystems in tropical marine environment. *Avicennia marina* (Avicenniaceae), commonly known as grey or white mangrove occurs in the intertidal estuarine areas. It has aerial roots (pneumatophores); these grow to a height of about 20 centimetres, and a diameter of one centimetre. The only report stated that the genus *Rivularia* (heterocystous cyanobacterium) was commonly occurring on mangrove pneumatophores (Charles Lugomela and Birgitta Bergman 2002). But some nitrogen-fixing bacteria inhabiting barks of mangrove trees has been isolated and characterized (Uchino *et al.,* 1984). However, there were no published reports on endophytic bacteria isolated from the pneumatophores of *Avicennia marina*. Therefore, the present study was aimed to isolate and to identify the endophytic bacteria from the pneumatophores of *Avicennia marina*, south east coast of India.

Bacillus cereus is a gram-positive, facultatively anaerobic, endospore-forming, rod-shaped bacterium. *Bacillus cereus* spores and vegetative forms are frequent inhabitants of a wide range of environments, including soil, clay, sediment, dust, marine water and vegetation (Goepfert *et al.,* 1972; Norris *et al.,* 1981 and Johnson, 1984). All endophytic, aerobic, spore-forming bacteria described to date belong to species generally recognized as free-living soil organisms including *B. cereus* (Pleban *et al.,* 1997), *B. insolitus* (Bell *et al.,* 1995 and Sturz *et al.,* 1997), *B. megaterium* (McInroy and Kloepper, 1995), *B. pumilus* (McInroy and Kloepper, 1995; Benhamou *et al.,* 1998), *B. subtilis* (Misaghi and Donndelinger, 1990) and *Paenibacillus polymyxa* (Shishido *et al.,* 1999). Endophytic microorganisms play an important role in plant protection, they colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents and plant protection, enhance plant growth by the number of mechanisms as phosphate solubilization, fixation of carbon di oxide, sulphur reduction, nitrogen fixation, indole acetic acid production and the production of siderophore. Endophytic microbial inocula, primarily bacteria, are used as propagule priming agents, both as in vitro co-cultures and transplanting (Nowak and Pruski, 2002). It is an emerging trend in biotechnological approach aimed at reducing chemical input in plant production, while increasing plant fitness, productivity and resistance to diseases in the context of sustainable horticulture.

In the present study, endophytic bacterium was isolated from the pneumatophores of *Avicennia marina*. The isolate was identified phenotypically and genotypically as *Bacillus cereus*. This bacterial culture was inoculated into the explants of *Bacopa monnieri* to evaluate its efficacy in improving crop growth under invitro condition. *B.monnieri*, a medicinal plant traditionally used as a memory vitalizer, acts as a brain tonic and promotes longevity. It is a small annual creeping herb and contains the alkaloids brahmine and herpestine. Hence, this plant was used for this experiment as a host plant. Eventhough the endophytic *B.cereus* isolated from mangrove plant it shows better growth enhancement,

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which indicates that the endophytic *B.cereus* is not a host specific organism. This can be used as bioinoculant (as biofertilizers) in other plant species.

MATERIALS AND METHOD

Isolation of endophytic bacterium from Pneumatophores of *Avicennia marina*

The Pneumatophores (5 – 7 cm in length) of *Avicennia marina* were collected from the intertidal zone of Vellar estuary. The roots were washed with tap water and then distilled water to remove the soil particles. They were excised and subjected to three steps of surface sterilization procedure; *Step 1*: Washed with 70% ethanol for 1 minute and subsequently with distilled water. *Step 2*: Soaked in 0.1% mercuric chloride for 3 minutes and washed with distilled water for 2 times. *Step 3*: Soaked in 70% ethanol for 30 seconds and washed for 5 - 7 times with distilled water. The additional step was followed in this sterilization procedure, proposed by Gagne *et al.,* 1987. The surface sterilized roots were aseptically sectioned into small pieces (0.1 cm thickness). Totally 300 sections were made and placed on to the plates containing isolation medium (Nutrient agar), followed by incubation at 26˚C for 48 h. The bacterial growths were associated with root sections and were purified by repeated plating on nutrient agar. The cultures were maintained as spore suspension by freezing in 20% (v/v) glycerol.

Table 1. Phenotypic characteristics of *B.cereus*

Tests	Results
Morphological characteristics	
Shape	Rod
Motility	Positive
Gram's staining	Positive
Physiological characteristics	
Temperature(28ºC)	Positive
pH (7)	Positive
Salinity (5%)	Positive
Exoenzyme activities	
Starch hydrolysis	Positive
Gelatin hydrolysis	Positive
Casein hydrolysis	Positive
Endoenzyme activities	
Catalase production	Negative
Urease production	Negative
Oxidase production	Positive
Voges-proskauer	Positive
Plant growth promoting activities	
Sulphur reduction	Positive
Phosphate solubilization	Positive
Nitrogen fixation	Positive

Phenotypic characterization

The isolate was gram – stained and examined microscopically for its morphological characteristics. A set of 12 physiological characteristics, including acid production from sugars (TSI), sodium citrate utilization, urease production, starch, gelatin hydrolysis and voges-proskauer reaction was carried out using a prototype MICROBACT 36B *Bacillus* Identification System (Medvet science) which emulates the standard tests for *Bacillus* described by Gordon *et al.,* 1973. Casein hydrolysis was detected after incubation of strain for 3rd day on nutrient agar supplemented with 2% skimmed milk. Growth in the presence of NaCl (2%, 3%, 5%, 7% and 9%) was determined in nutrient broth as the basal medium during incubation at 28ºC for 3rd day. It was also estimated at selected temperatures (26, 32, 40 and 50ºC) and pH (4.5, 5.5, 7 and 9) in slants incubated at 28ºC for 3 days. The plant growth promoting activities (Sulphur reduction, Phosphate solubilization and Nitrogen fixation) of the isolate were determined by standard protocols (Subbarao, N.S.1982).

Determination of cellular fatty acid composition

Cellular fatty acid composition of endophytic bacterium was analyzed using the Sherlock system (MiDi Company, USA) and according to the manufacturer's instructions.

Sequence and analysis of 16S rRNA genes

Genomic DNA was isolated from pure culture (Sambrook *et al.,* 1989). A large fragment of 16S rRNA was amplified by PCR using primers 5' -TGA GGA AGA TAA TGA CGG -3' and 5' -CCT CTA TCC TCT TTC CAA CC -3'. The 50 µl PCR reaction mixtures contained 100 ng of DNA extract, 1×Taq reaction buffer, 20 pmol of primers, 200 µM dNTPs and 1.5 U of Taq DNA polymerase (Promega). The thermocycling conditions consisted of an initial denaturation at 94ºC for 3 minutes, 30 amplification cycles of 94ºC for 1 minute (denaturation), 57ºC for 1 minute (annealling), 72ºC for 2 minute (extension) and final polymerization at 72ºC for 4 minutes.PCR product was purified and sequenced. Searches in the Gen Bank/EMBL/DDBJ/PDB data libraries were performed using BLAST (blastn) search algorithm (Altschul, S.F.et al, 1997) in order to establish the identity of the isolate. Sequences of the close relatives were retrieved and aligned with the newly determined sequences. Multiple alignments were performed with CLUSTALX. The neighbour – joining program NEIGHBOR (Saitou and Nei, 1987) contained in the phylogenetic program package PHYLIP version 3.573 (Felsenstein, 1993) was used to infer phylogenetic relationships.

Inoculation of plants with endophytic bacterium

Nodal segments (length: 0.5 cm) of *Bacopa monnieri* were disinfected by sonicating in water for 20 min and dipping in 70% ethanol for 1 min, followed by 25 min of sodium hypochlorite (25%) / Tween 80 (0.01%) solution and rinsed three times with distilled and sterile water (Alderete *et al.,* 2006). The explants were cultured onto a hormone free Murashige– Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 20 gl^{-1} sucrose, 7 gl^{-1} agar, and the pH was adjusted to 5.7 with KOH with the addition of 200µl of endophytic *B.cereus*. Then, the explants were cultured under a photoperiod of 16 h of light and 8 h of dark under an irradiance of 52 mmol m^{-2} seg^{-1}. The explants without endophytic *B.cereus* were marked as control explants. This experimental setup was repeated for 10 times. All the data collected from these experiments were subjected to an analysis of variance (ANOVA) using Microsoft Excel 2000.

Table 2. Nucleotide sequence of endophytic *B.cereus*

GCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCCATAAGACTGGGATAACTCCGGGAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGG
TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGC
TGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATG
TGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGAC
ACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GCCGCAAGGCTGAAACTCAAAGGAATTGACGGG

RESULTS

The isolate was identified as Gram – positive rods, 1.5 - 2.5 × 1.0 µm and occurred in chain forms. Spores were ellipsoidal. Occasionally the isolate produced an intense yellow - brown colour pigment that did not diffuse into solid or liquid medium. Both pigmented and white colonies could sometimes be observed on the same plate, but lines with constant pigmentation could not be isolated and the culture conditions necessary to consistently induce pigmentation could not be defined. The optimum growth was occurred at 26ºC, pH 7 and in 5 % salinity. The distinguishing phenotypic characteristics of endophytic bacterium are listed in Table 1.

Using BLAST (blastn) the endophytic bacterium was identified as *Bacillus cereus* – SjAM16104 with similarity value of 99%. Nucleotide sequence is given in Table 2. The nucleotide sequence of *B.cereus* has been deposited in the Genbank/EMBL/DDBJ/PDB under accession number GU930360.

The major fatty acids of *B.cereus* were anteiso-15:0 (40.55%), anteiso-17:0 (17.39%), iso-16:0 (8.95%), iso-15:0 (7.82%), iso-17: 0 (6.62%), 16: 0 (5.23 %) and fatty acids iso-14:0 (2.03%), anteiso-13:0 (1.87%) were detected in small amounts (Fig 1).

Inoculation of plants with endophytic bacterium

Growth parameters were measured to assess the growth promotion capability of endophytic *B.cereus*. The number of roots was counted. Shoot length and root length was measured in centimeters. Early roots were obtained in the explants inoculated with endophytic *B.cereus*. The growth of the explants treated with the endophytic bacterium was higher than the control explants (Fig 3 – A & B). Root curling was obtained only in the explants inoculated with the endophytic *B.cereus* (Fig 3 – C). Differences in the growth parameters of treated explants and control explants were analysed by Two – Way ANOVA.

Number of roots

The value of treated explants ($p<0.05$) were significant than the control explants (Table 3 & Fig 2 A). The explants treated with endophytic *B.cereus* promoted the formation of more roots. This may be due to the endophytic bacterium attributed to solubilizing activity of the inorganic substances to organic substances in the host plant root.

Root length

The value of treated explants ($p<0.05$) were highly significant than the control explants (Table 3 & Fig 2 B).

Table 3. Two – way ANOVA for the differences in number of roots, root length and shoot length of treated and control explants

S.No	Samples	F value	F crit value	Significance
		Number of roots		
1.	Treated explants	455.1952	2.35926	< 0.05 **
2.	Control explants	1.58963	2.35926	NS
		Root length		
1.	Treated explants	1924.825	2.35926	< 0.05 **
2.	Control explants	1.324568	2.35926	NS
		Shoot length		
1.	Treated explants	1715.19	2.35926	> 0.05 *
2.	Control explants	2.01254	2.35926	NS

*- Significant; ** - Highly significant; NS – Non- significant.

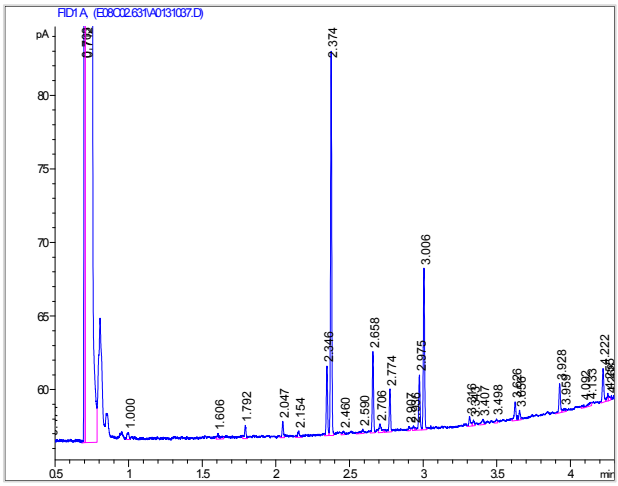

Fig 1: Gas chromatographic methyl ester profiles of endophytic *B.cereus*

Endophytic *B.cereus* promoted number of roots along with its length in treated explants. (Maximum length measured was about 4cm). This confirms that the endophytic bacterium can promote the roots and root length even in the soilless environment.

Shoot length

The value of treated explants ($p>0.05$) were significant than the control explants (Table 3 and Fig 2C). Explants treated with endophytic *B.cereus* improved the growth and length of shoots (Maximum length measured was about

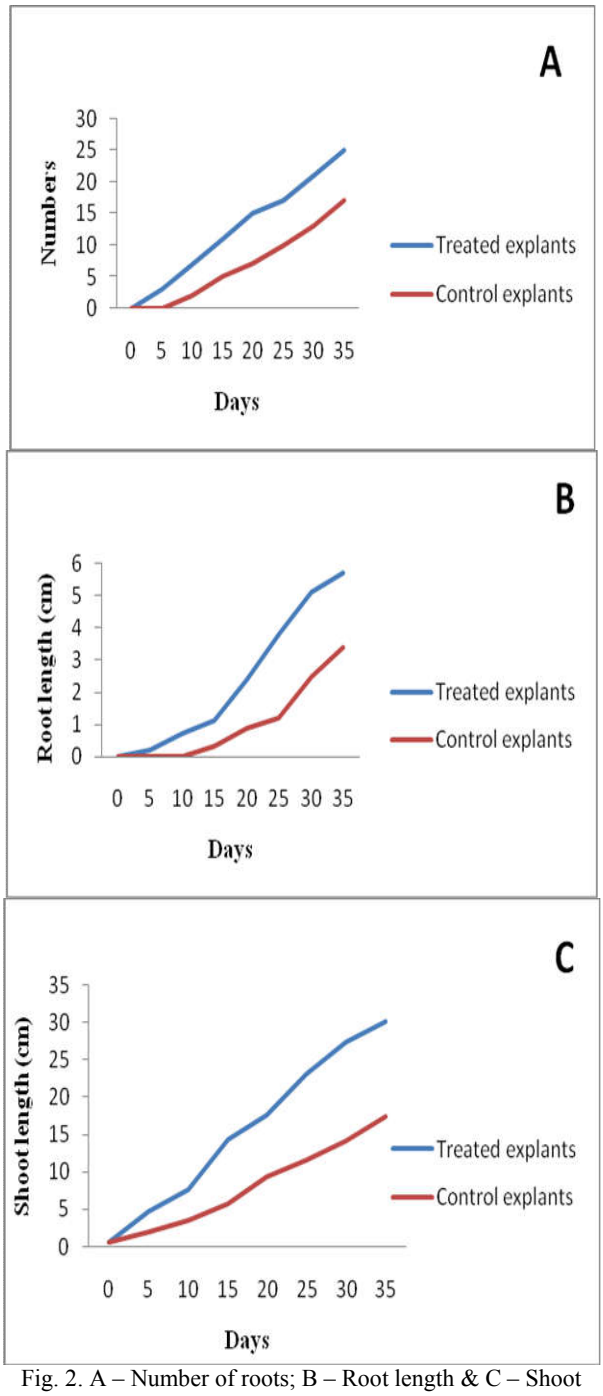

Fig. 2. A – Number of roots; B – Root length & C – Shoot length

25cm). This indicates the endophytic *B.cereus* enhances the plant growth.

DISCUSSION

Research performed so far has been mostly related to plant growth promotion and/or to rhizosphere or root endophytic colonization. Although novel root colonizers are being detected (Andreote *et al.,* 2009), the functioning and contribution to plant growth of endophytes localized in aerial parts is rather poorly understood. Correlation between colonization and beneficial effects as well as genomic comparison of bacteria colonizing different plant tissues will help to better understand the role of these endophytes.

There were some reports on *Phomopsis* and *Phyllosticta* fungal species isolated from the mangrove plants in South India (Suryanarayanan et al. in 1998). But

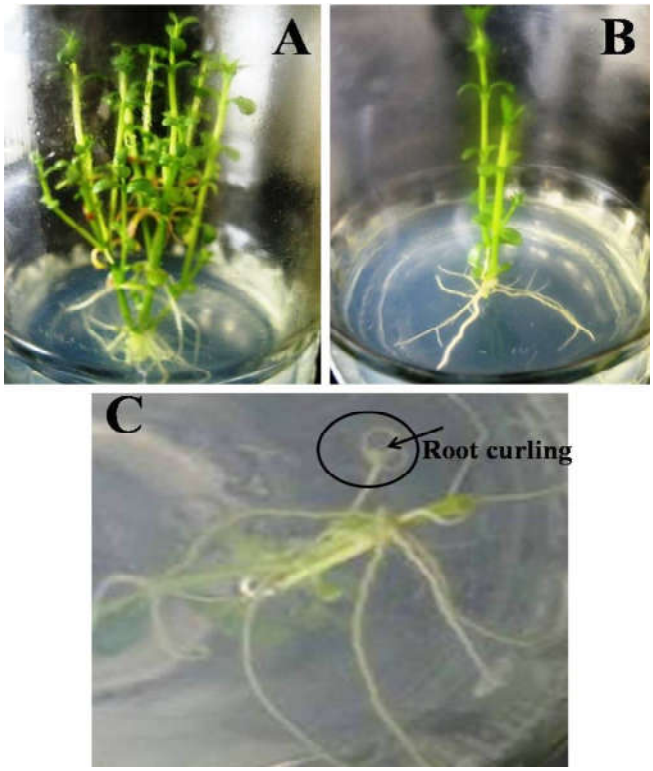

Fig. 3. A - Treated explant (inoculated with Endophytic *Bacillus cereus*) showing healthy growth of shoots, leaves and formation of roots;
B - Control explant, number of roots and shoot growth was minimal than the treated explant;
C - Formation of root curling in treated explant.

there were no published reports on endophytic bacteria isolated from the mangrove plant *Avicennia marina*. However this is the first report on endophytic *Bacillus cereus* isolated from the pneumatophores of *Avicennia marina* in South India. *Bacillus* species are included one among the plant growth promoting rhizobacteria (PGPR), in which this strain has been marketed for its biocontrol products.

Addition of a crude endophytic inoculum to tissue culture *B.monnieri* explants resulted in increases of plant growth parameters (Number of roots, Roots length and Shoot length). These results indicate that re-introduction of naturally-occurring endophytes into tissue culture *B.monnieri* can lead to improve plant growth and yield. In the present study revealed that the endophytic *B.cereus* isolated from the pneumatophores of *A.marina* was not host specific. Some endophytic genera, however, exhibit no host specificity and are invariably recovered from plants belonging to different groups and growing in different geographical locations. In future this can be used as a biofertilizer in the agricultural crops. Plant growth promoting bacteria were environmentally friendly alternative to chemical fertilizers and pesticides, the use of which was regulated and sometimes forbidden, the market for bioinoculants is still expanding.

In this investigation root curling was observed in the explants inoculated with endophytic *B.cereus*. This result strongly support the model of Van Batenburg *et al.,* (1986), root hair curling is continuous reorientation of tip growth. The reason for root curling may be because of (a) the attachment of one inducing principle (e.g. the NF droplet), (b) within the growth area of the root hair; (c) translocation of the inductor along the growing root hair

tip (e.g. iterative spot application); and (d) redirection of the original plant-driven tip growth (Van Batenburg *et al.,* 1986).

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