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Research Article

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Characterization of plant growth promoting rhizobacteria from rhizosphere of *Cajanus cajan* and their identification through 16s r DNA sequencing

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) found in the rhizosphere in association with roots are beneficial bacteria which can enrich the growth of plant directly or indirectly. Rhizobacteria were isolated using general microbiological media, from different locations of Karimganj District of Barak Valley Region, Assam, India and were characterized on the basis of morphological, biochemical and 16S rRNA gene sequencing analysis. All the isolates of were screened for their plant growth promoting traits such as Indole acetic acid production (IAA), Siderophore (Fe-III chelating agent), Hydrogen cyanide, Phosphate solubilization ability. IAA production was assayed colorimetrically using ferric chloride ($FeCl_3$) and perchloric acid. Siderophore and phosphate solubilization ability were tested qualitatively on Chrome azurolSulfonate agar and Pikovskaya's agar respectively. Among all, KD7showed highest IAA production. Phosphate solubilization was found to be positive for 61% isolates. Siderophore was produced by only 53% of the isolates and 1.6 cm was the highest zone diameter found. KD7 being the most promising isolate was identified at their strain level by 16srDNA sequencing and found to be Bacillus cereus.

Keywords: *Bacillus cereus, PGPR, Soil bacteria, Salinity.*

INTRODUCTION

Pigeon pea (*Cajanus cajan* L var. Manak) a member of the family Fabaceae is an economically important Kharif green legume crops a very popular food grain in India. It is a principal pigeon pea-growing country contributing nearly 90% of total world's production in India[11]. The benefits of incorporating pigeon pea into cropping systems include its role as a soil ameliorant, ability to fix nitrogen and extract phosphorous, and high drought tolerance.

The term rhizobacteria is used to describe a subset of rhizosphere bacteria capable of colonizing the root environment[19]. Microorganisms that colonize the rhizosphere can be classified according to their effects on plants and the way they interact with roots, some being pathogens whereas other trigger beneficial effects. To be an affective rhizobacteria, the bacteria must be able to colonize roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects[13].

Bacillus cereus is a Gram-positive, aerobic-to-facultative, spore-forming rod widely distributed environmentallybelongs to family *Bacillaceae*[8]. Multiple species of *Bacillus* and *Paenibacillus* are known to promote plant growth[20]. *Bacillus* strains have wide genetic heterogeneity in term of DNA G + C composition which ranges from 32-69% of known *Bacillus* spp. *Bacillus* spp. can resist and survive in a variety of environmental stresses and adverse conditions and considered as very important microbiota due to its functions such as uptake of nutrient, phytohormone production, nitrogen fixation and phosphate solubilizing[16].

MATERIALS AND METHODS

Collection of soil sample

Soil samples were first collected from the rhizospheric soil of *Cajanuscajan*(pigeon pea) from Karimganj district of Assam, India. The actively growing *Cajanuscajan*plant were selected and rhizosphere soil were dug out at a depth of 10 to 12 inch. Samples were collected in sterilized polythene bags after proper labeling and immediately bought to the laboratory so as to store at 4°C. Soil plating was done within 72 hours of sample collection.Isolation of *Bacillus* spp. was done by spreading the samples and incubatingat 37°C for 24 hours[17]. Pure cultures were finally obtained by repeated sub-culturing.

Characterization of the bacterial isolates

Initial identification was carried out by their morphological and biochemical characterization[7,15] and finally the isolate with highest PGPR activity as compared to other is further identified by 16S rDNA sequencing.

***Identification by 16S rDNA sequencing*:** Pure cultures were grown until log phase and genomic DNA was extracted from bacterial isolate[24]. The amplification of 16S rRNA gene was done by using forward primer and reverse primer. The ~1.4 kb-PCR products of 16S rRNA genes were used for DNA sequencing. After sequencing, the sequence was analysed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for finding the closest homologous sequence. The first ten homologous sequences were selected based on their maximum identity score. The sequences were than aligned and a distance matrix was constructed followed by the construction of a phylogenetic tree by Neighbour-Joining method.Finally the sequence was processed with the stand-alone software tool (sequin) developed by national centre for biotechnology information (NCBI). Sequin processed sequence was submitted to the GenBank.

Plant growth promoting traits

IAA Production: Qualitative estimation of phytohormoneproduction by the isolates were carried out in nutrient broth supplemented with L- tryptophan and incubated for 48 hrs through constant shaking in rotary shaker at 120 rpmby the methods[5,22]. Cells were removed from culture medium by centrifugation at 8000rpm for 15 mins. 1ml aliquot of supernatant was mixed with 2 ml of Salkowski's reagent (1ml of 0.5M $FeCl_3$ in 35% $HClO_4^-$). The samples were incubated at roomtemparaturefor 25 mins.Development of pink colouration indicates positive result. The intensity of pink colour developed was read in a spectrophotometer at 535nm.

Siderophore production: Isolates were qualitatively detected using chrome azurol Sulfonate (CAS) agar[25]. After 24 hour of incubation, colonies were observed for orange halo formation.

HCN Production: Isolates were screened for hydrogen cyanide synthesisin nutrient agar medium supplemented with glycine (4.4 g/l). A Whatman filter paper no. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate)was placed inside the lid of the plate[21]. Plates were sealed with parafilm and incubated at 37°C. After incubation, plates were observed for the development of orange to brownish red color.

Ammonia Production: Bacterial isolates were tested for ammonia synthesis in peptone water[18]. After incubation for 24 hrs change in colour of the broth were observed for the development of brown yellow color.

Phosphate Solubilization: Phosphorus is second only to nitrogen in mineral nutrients which is most commonly limiting in the growth of plants[23]. All isolates were selectively screenedon Pikovskaya's agar medium containing 5 g of tri-calcium phosphate [$Ca_3(PO_4)_2$] as sole phosphorus source[14]. The isolates that have the ability to release inorganic phosphate from tri-calcium will formhalozone around the colonies indicating the solubilization of phosphate present in the media.The ability of the bacteria to solubilize insoluble phosphate was described by the phosphate solubilization index (PSI). The phosphate solubilization index was determined by measuring the halo diameter and the colony diameter, using the formula[12].

$$PSI = \frac{\text{colony diameter + halo zone}}{\text{colony diameter}}$$

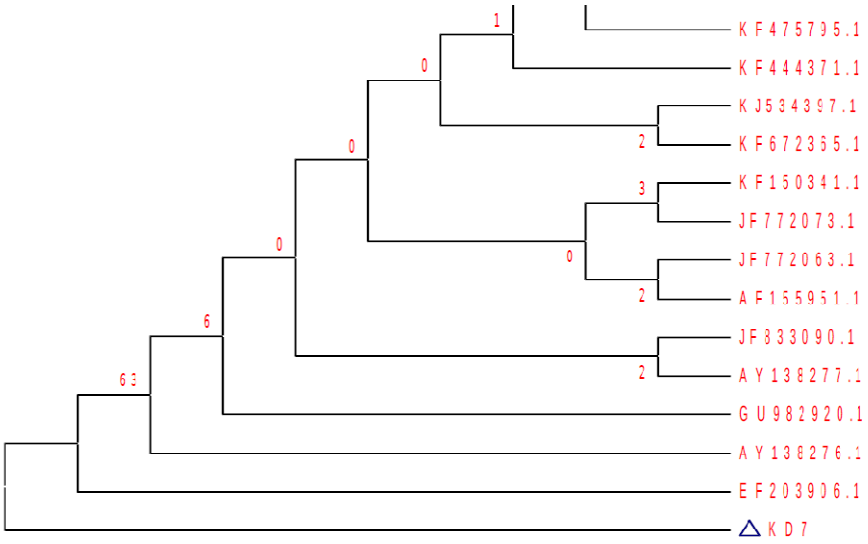
RESULTS AND DISCUSSION

Isolation and Enumeration of Bacteria

A total of thirteen rhizospheric bacteria from *Cajanuscajan*L var. Manak were isolated. Total viable counts ranges from 19 X 10^4 (CFU/g) to 58 X 10^4 (CFU/g). Colony morphology of all the isolates were examined. All the isolates were gram positive rod. Most of the isolates were found to be indole negative, methyl red and vogesproskauer negative, urease negative. Catalase tests, starch hydrolysis for *Bacillus* isolates were positive indicating them to be aerobic strains. Positive result for citrate test was observed, infers the ability of these organisms to utilize citrate as the sole source of carbon and energy. Isolates were also able to produce an enzyme "nitrate reductase" resulting in the reduction of nitrate (NO_3).

The isolate which delivered maximum points while screening for plant growth promoting trait was selected for 16S rDNA sequencing. A neighbour-joining tree was generated using the sequence from KD7 (1445bp) and representative sequences from databases. It has been observed that the strain code KD7 had maximum sequence similarity with the species of *Bacillus cereus* strain Js16(GenBank Accession No. JF833090.1; figure 1). The sequence has been identified as *Bacillus cereus* KD7 and submitted to NCBI-GenBank database with accession number KM555167.

Fig. 1:



Phylogenetic relationship between studied sample (KD7) and representative species based on partial 16S rDNA sequences constructed using the neighbour-joining method. Studied sample (KD7) has been submitted to NCBI-Genbank and the accession number thus obtained is KM555167 (*Bacillus cereus* strain KD7).

Plant Growth promoting (PGP) traits of the test isolates

The bacterial isolates were screened for multiple plant growth promoting activities which are listed in the table 3. Three among thirteen showed ammonia production. KD7 is best among others as it showed positive result for all the attributes. IAA i.e. indole-3-acetic acid is considered to be the best categorized auxin found in plants. IAA is known to enhance cell elongation, cell division and differentiation in plants[27]. *Bacillus cereus* KD7 exhibited highest IAA productionwith 0.189 concentration spectrophotometric reading at 535nm (Table 1). High amounts of IAA (99.7 µg/ ml) were produced by *Rhizobium* isolates from root nodules of *Cajanuscajan* in L-tryptophan supplemented basal medium[9].

IAA production by *Bacillus* sp. was deprived as reported by Vessey, (2003) whereas other researchers found it to be proficient in producing IAA and also increased wheat, spinach and rice growth[6,3]. Besides IAA production, microorganisms also enhance plant growth by scavenging available iron (Fe^{3+}), which involves secretion of high affinity, low molecular weight iron chelating ligands called siderophores[1]. In this investigation, only 53% isolates were able to form halo zone CAS agar symbolizing siderophore production, isolate KD7 being the highest producer with zone diameter (1.6 cm).

The genus *Bacillus* when applied to soil not only helps in proliferation of phosphorus uptake in crops but also improvethe plant's availability to phosphorus by solubilizing fixed phosphates in soil and utilizing rock phosphates[10]. In our studies, 61 % of the isolates were able to solubilize tricalcium phosphate in the PKV media. The PSI ranged from 1.33 to 2.25. *Bacillus* can be found in adverse soil environment and behaved as PGPR even in acidic soils[29].

Table 1: Screening of multiple PGP traits of the bacterial isolates

Isolates	IAA Production		Siderophore Production		Ammonia Production	HCN Production	Phosphate Solubilization		
	Pink Colouration	Conc. At 535 nm	CAS assay (halo formation in blue agar)	Halo size (cm)			Diameter of Halo zone (cm)	Diameter of colony	Phosphate Solubilization Index
KD1	+	0.101	+	0.2	+	+	-	-	-
KD2	+	0.134	+	0.5	-	+	0.2	0.2	2
KD3	+	0.135	+	0.7	-	+	0.1	0.3	1.33
KD4	+	0.129	-	-	-	+	-	-	-
KD5	+	0.156	-	-	-	+	-	-	-
KD6	+	0.092	-	-	+	+	0.3	0.4	1.75
KD7	+	0.189	+	1.6	+	+	0.5	0.4	2.25
KD8	+	0.107	-	-	-	+	0.2	0.2	2
KD9	+	0.105	+	0.3	-	+	0.2	0.3	1.66
KD10	+	0.124	+	0.6	-	+	0.2	0.3	1.66
KD11	+	0.135	-	-	-	+	0.2	0.4	1.5
KD12	+	0.106	-	-	-	+	-	-	-
KD13	+	0.136	+	0.6	-	+	-	-	-

Fig. 2: Production of IAA by isolates supplemented with 0.2% L-Trytophan in the media

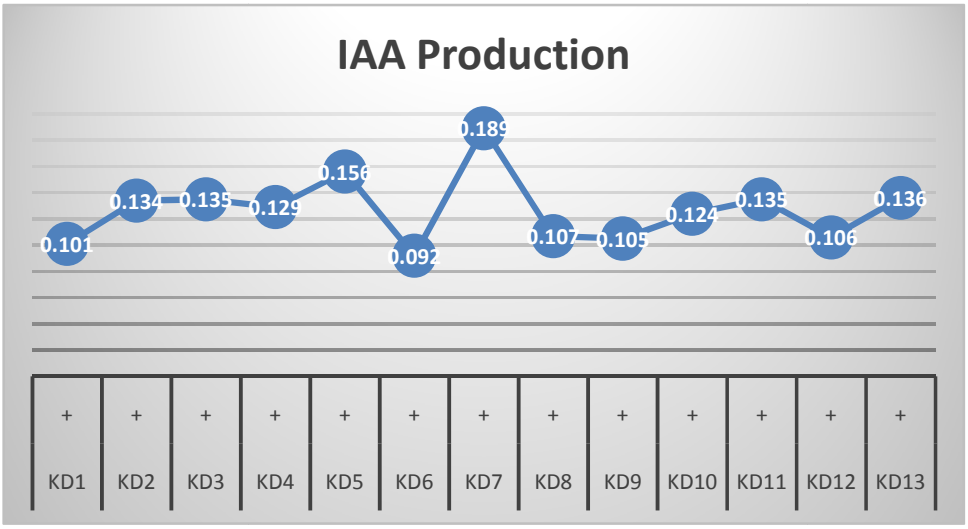


Fig. 3: Siderophore production by isolates and the amount of production is being denoted by the size of their halozone formation

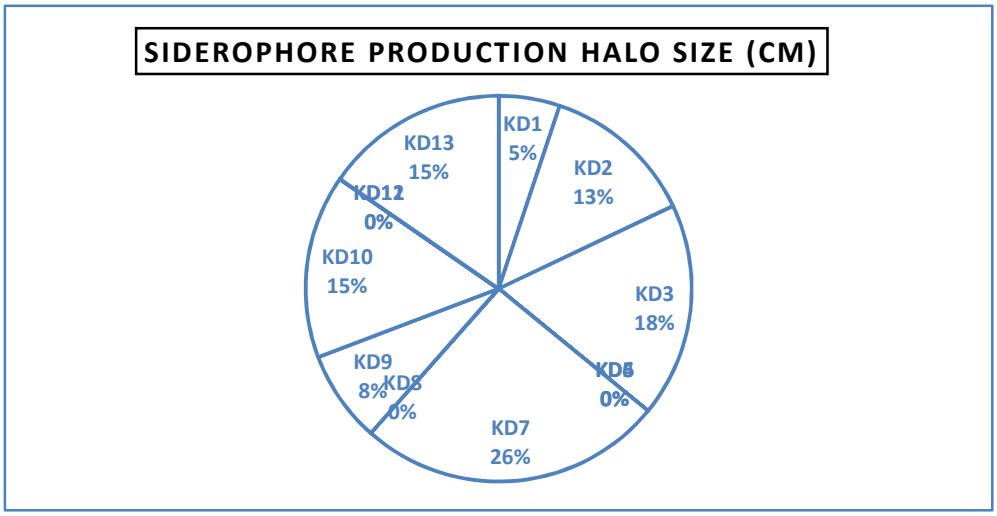


Fig. 4: Phosphate solubilizationability and their PSI values

Some reports have been published that species of *Bacillus*play an important role in plant protection and growth promotion and are common dwellers of various species of plants, including cotton, grape, peas, spruce, and sweet corn[4,26,2].

Finally, it can be concluded that bacterial isolates obtained from rhizospheric region of *Cajanuscajan* were performing Plant Growth Promoting activities, which are essential for plant growth and nutrition.To enhance sustainable agriculture, the use of such approaches i.e. PGPRs can be reliable, and have to be made assessable for the farmers. Subsequently, researches needs to be continued so as to develop new methodologies to enrich the efficacy of PGPRs and for better understanding of their ecological, genetic and biochemical interactions in their environment.

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