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Characterization and evaluation of actinomycete isolates for traits associated with plant growth promotion

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Abstract
Eighty five isolates of actinomycetes were isolated from different sources *viz*., decayed wood, compost dumped soil, and mangroves of North Karnataka and were tested for the typical traits of plant growth promotion. The isolates exhibited varied potential of siderophorogenesis and cyanogenesis. Indole 3-acetic acid (IAA) produced by the isolates ranged from 6.88 to 20.22 µg ml^{-1}.The potent isolates A-34 and A-57 which exhibited maximum ability to produce siderophore, hydrogen cyanide (HCN) and Indole-3-acetic acid were characterized by 16S rDNA sequencing and revealed maximum similarity to the genus *Streptomyces*. Thus the isolated *Streptomyces* strains with plant beneficial traits have potential for use in sustainable agriculture.

Keywords: Siderophore, IAA, 16S rDNA, actinomycetes, cyanogenesis, PGPR

Introduction
Plant growth promoting rhizobacteria (PGPR) are beneficial soil microbes which facilitate plant growth either directly by providing plants with fixed nitrogen, soluble phosphate, iron chelators and Phytohormones, or indirectly by inhibiting phytopathogens, thereby promoting plant growth and development [1]. From the standpoint of sustainable agriculture, under changing climate conditions and degrading soil health, it is important to explore and identify soil microorganisms capable of enhancing plant nutrition, health and improve soil quality. Among the diverse soil microflora, actinomycetes bear tremendous potential to improve plant nutrition by maintaining soil health and inducing systemic resistance, consequently gaining importance among agricultural scientists.

Actinomycetes are one of the major components of the microbial populations present in soil. They belong to an extensive and diverse group of Gram-positive, aerobic, mycelial bacteria that play important ecological roles in soil nutrient cycling and plant growth promoting activities. They are well known source of natural bioactive compounds used in pharmaceuticals and agrochemicals. Actinomycetes, particularly *Streptomyces* sp. have been widely exploited group of microorganisms in the promotion of plant growth activities, production of secondary metabolites and enzymes of commercial importance in medical and agricultural applications [2]. Iron is essential element required by bacteria for physiological processes necessary for survival. Production of siderophore is one strategy employed by bacteria to overcome iron limitation and it is seen that the survivors of iron limitation are largely actinomycetes [3].

In the last decade research has been focused on minor groups of actinomycetes, including species that are difficult to isolate and cultivate and those that grow under extreme conditions, i.e. alkaline and acidic conditions with potential plant growth promoting traits [4, 5]. The aim of the present study was to isolate actinomycetes from different sources of North Karnataka and evaluate them for typical plant growth promoting characters, with an objective of using them for *in* planta studies in future.

Materials and Methods
Isolation of actinomycetes
Actinomycetes were isolated from various sources like decayed wood, compost dumped soil and mangroves of North Karnataka by following serial dilution plate count technique using Starch Casein Agar (SCA) medium and plates were incubated at 28±2°C for 6-7 days. After incubation, the single, isolated colonies with powdery growth were selected and maintained on SCA slants and used for the further investigation.

Screening of actinomycetes for plant growth promoting traits
Cyanogenesis
The actinomycetes were screened for the production of hydrogen cyanide by the following method. Isolates were grown in Soyabean casein digest agar amended with glycine (4.4 g l^{-1}). A Whatman filter paper no. 1 was soaked in 1% Picric acid solution (in 10% sodium carbonate) for a minute and placed underneath the Petridish lids. The plates were sealed with Para film and incubated at 28 + 20°C for four days. Development of the reddish brown colour on the filter paper indicated positive for HCN production. Observations were recorded (by a panel of 3 observers) on a 0-3 rating scale (they were rated based on the intensity of the reddish brown color) as follows: 0 = no color change; 1 = light reddish brown; 2 = medium reddish brown and 3 = dark reddish brown.

Siderophorogenesis
For the siderophore production assay, all the glassware were soaked in 2N HCl solution for 24h to avoid contamination of iron from the glassware. The chrome azurol S (CAS) solution was prepared by dissolving 0.06 g dehydrated chrome azurol S in 50 ml double distilled water and further mixing with 10 ml of iron solution (1 mM $FeCl_3$. $6H_2O$ in 10 mM HCl). This was then slowly added to the 40 ml aqueous solution containing 0.0729 g cetyltrimethyl ammonium bromide with continuous stirring and the final solution was autoclaved. Starch Casein Agar media was prepared by using PIPES buffer (30.2 g) and by the addition of 0.1 N NaOH, the pH was adjusted to 6.8 before autoclaving. After cooling the CAS solution (100 ml) was added along the wall of flask with gentle agitation to mix to avoid formation of foam. The CAS agar thus prepared was poured in to the plates. After solidification the plates were kept in the refrigerator (4° C) for 24 h. The disc of grown cultures of actinomycetes were inserted into the wells made with the help of Cork borer on these CAS agar plates and incubated at 28 $\pm$ 2° C for 4 days. Formation of orange coloured zone around the colony was taken as positive for the siderophore production. The diameter of orange coloured zone was recorded.

Indole-3-acetic acid production
Estimation of IAA was done as per the protocols [6]. The actinomycetes were grown in starch casein broth supplemented with L-tryptophan (1µg ml^{-1}) for four days. One ml of the centrifuged culture filtrate was then allowed to react with 2ml of Salkow sky reagent (1ml of 0.5 M $FeCl_3$ in 50ml of 35% $HClO_4$) for 30 min. at 28 $\pm$ 2^0 C. The development of pink color indicated the presence of IAA. The IAA was extracted using methanol and quantified using the method [7].

Molecular characterization of efficient Actinomycete isolates
The potent isolates A-34 and A-57 exhibiting PGPR traits were selected for 16S rDNA based molecular characterization followed by BLASTn analysis. The genomic DNA was isolated by the Sambrook and Russell method [8] and the16S rDNA gene was amplified with a set of universal primers: 5`AGAGTTTGATCCTGGCTCAG3` and 1492R 5`TACGGYTACCTTGTTACGACTT3`.PCR was performed with 100 ng template DNA, 5U Taq DNA polymerase, 1 mMdNTP, 10pM in an Eppendorff Thermal Cycler under the following conditions: 5 min of denaturation at 94°C followed by 35 cycles of amplification with 1 min denaturation at 94°C, 1 min of annealing at 55°C, 2 min of extension at 72°C, final extension step of 90 min at 72°C.
The PCR products were purified using a QIAgen gel extraction kit (QIAgen, Germany) and sequenced with an ABI 3730 xl sequencer (Applied Biosystems, USA) with Ampli Taq® DNA polymerase (FS enzyme) (Applied Bio systems, USA). The forward and reverse 16S rRNA gene sequences A-34 and A-57 were assembled individually in BTI Gene Tool Lite software v. 1.0 to produce contiguous DNA sequences.

Results and Discussion
A total of eighty five isolates of actinomycetes were obtained from different sources *viz*., decayed wood, compost dumped soil, mangroves of North Karnataka by soil dilution agar technique. All the isolates developed well grown colonies, following 7 days incubation at 28 °C. The developed colonies were observed as smooth with aerial and substrate mycelia with entire margins and the optimum temperature for growth of all the isolates was 28°C.
In the recent past, several studies have targeted plant growth promoting actinomycetes from different sources *viz.,* herbal vermicomposts [9, 10], rhizosphere [11], mangrove soils [12], etc. Endophytic actinomycetes associated with medicinal plants are also shown to possess unique plant growth promoting as well as biocontrol traits *viz*., phosphate solubilisation, siderophores, HCN, ammonia, chitinase, indole-3-acetic acid production and antifungal activities [3]. Moreover, PGPR activities of actinomycetes also extend to helping Arbuscular Mycorrhizal fungal development in the host roots [13]. In another study by Damam *et al*., 2016 [14], of the total acinomycete isolates from rhizosphere of forest medicinal plants75% isolates showed Ammonia production, 72% isolates for indole acetic acid production, 51% for HCN production and (29%) for phosphate solubilization.
In the present study, eleven strains scored positive for cyanogenesis, differing in their ability to produce HCN as shown in the table 1. Fifteen isolates scored positive for siderophorogenesis. The differential ability of isolates to produce siderophores as determined by the orange halo around the colonies is outlined in table 2. Highest siderophore production was observed for the isolate A-34 with zone of 25 mm followed by A-57 which showed an orange halo of 14mm diameter around the colony. Siderophores are usually produced by the various soil microbes to bind Fe^{3+} from the surrounding environment and make it available for its own growth and for plant. The production of siderophores is also a mechanism used by PGPR for rhizosphere colonization competence. Besides, competition for iron plays a vital role in controlling the phytopathogens [15].
As seen in table 3, Only 14 actinomycetes were able to produce IAA ranging from 6.88 to 20.22 µg ml^{-1}. Isolate A-34 produced the highest amount of 20.22 µg of IAA per ml of culture filtrate followed by A-57 which produced 17.33 µg ml^{-1}. Microorganisms which produce IAA are known to promote plant growth and root elongation [16]. Tryptophan is believed to be the primary precursor for the formation of IAA in plants and microorganisms. By the production of plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial for their growth. Diverse Actinomycete species possess the ability to produce the auxin phytohormone IAA, these actinomycetes produce auxins in the presence of a suitable precursor such as L-tryptophan [17].
Our study relied mainly on phenotypic and16S rDNA based molecular characterization for the strain identification. The isolate A34 showed maximum similarity to *S. wuyuanensis*

strain FX61 (NR_118447) followed by *S. rectivirticillatus* strain NBRC 13079 (NR_118284). Similarly, the isolate A57 found closely related to *S. toxyticini* strain NRRL-B-5426 (NR_043839) followed by *S. toxyticini* strain NBRC 12823 (11229). The NCBI Genbank accession numbers obtained for these two promising strains A34 and A57 are KP826717 and KP826718 respectively. However it is to be noted that antibiotic resistance data can also offer valuable information particularly for actinomycete characterization and identification [18].

Thus it can be concluded that the putative Actinomycete strains A-34 and A-57 can be used as bioinoculants for plant growth promotion tests.

Table 1: HCN production by actinomycete isolates

S. No	Isolates no.	Light Reddish brown	Medium reddish brown	Dark reddish brown
1	A2	+ve		
2	A4		+ve	
3	A5		+ve	
4	A7		+ve	
5	A8		+ve	
6	A-34			+ve
7	LG101		+ve	
8	LG18			+ve
9	A-57			+ve
10	LG20		+ve	
11	LG125		+ve	

Table 2: Siderophore producing ability of Actinomycete isolates

S. No	Sample	0 (no change)	1 (Positive)	2 (Halo zone of 1-3mm)	3 (Halo zone of 4-6mm)	4 (Halo zone of 7mm and above)
1	A2	-	+ve	1mm	-	-
2	A3	-	+ve	-	-	8mm
3	A4	-	+ve	-	-	9mm
4	A5	-	+ve	-	6mm	-
5	LGM	-	+ve	-	6mm	-
6	A7	-	+ve	-	-	7mm
7	LG117	-	+ve	-	4mm	-
8	LG125	-	+ve	-	-	8mm
9	A11	-	+ve	-	4mm	-
10	A-34	-	+ve	-	-	25mm
11	A-57	-	+ve	-	-	14mm
12	A14	-	+ve	-	-	14mm
13	LG3	-	+ve	-	-	12mm
14	A18	-	+ve	-	-	8mm
15	A19	-	+ve	-	-	14mm

Table 3: Production of IAA by actinomycete isolates

S. No.	Isolates no.	IAA Concentration of IAA (µg ml^{-1})
1	A28	12.00
2	A41	14.66
3	A46	09.77
4	A47	18.66
5	A-57	17.33
6	A50	08.44
7	A54	07.77
8	A55	10.66
9	A56	08.88
10	LG1	16.00
11	LGM	07.77
12	LG117	09.33
13	A-34	20.22
14	LG3	06.88

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