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Production of Gibberellic Acid by *Bacillus siamensis BE 76* Isolated from Banana Plant (*musa spp*)

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Abstract: ***Based on morphological, cultural, biochemical and 16s rRNA gene sequencing a newly isolated endophytic bacteria from the banana plant (musa spp) was identified as Bacillus siamensis and designated as BE 76. Fifteen bacterial isolates were screened out for their productivity of Gibberellic acid by spectrophotometeric method. Out of these isolates BE-76 isolate showed high amount of Gibberellic acid production supplemented with and without L tryptophan in medium(0.180 and 0.240 mg/mL, respectively). Gibberellic acid production of BE-76 was further confirmed by high performance liquid chromatography (198.16 and 254.29 ppm, respectively) with and without L-tryptophan in medium for more accuracy.***

Keywords: Banana, *Bacillus siamensis*, Gibberellic acid, 16s rRNA gene sequencing, HPLC

1. Introduction

Banana (*Musa paradisica* L.) is one of the leading tropical fruit crops. It ranks next to mango in both area and production in India [1]. Endophytes are defined as 'microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects'. Endophytes are diverse microorganisms inhabiting in internal plant tissues [13]. *Bacillus* and *Pseudomonas spp* were reported as endophytes [8]. Plant growth promoting bacteria (PGPB) are defined as free-living soil, rhizosphere, rhizoplane, endophytic, and phylosphere bacteria that, under certain conditions, are beneficial for plants [2]. The strains with PGPR activity, belonging to genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, and *Serratia*, have been reported [10]. Phytohormones (Indole acetic acid, Gibberellins) are plant growth regulators, which have stimulatory effects on plant growth [18]. It is also very likely that growth promoting effects of various PGPRs are due to bacterial production of plant growth regulators such as indole-3-acetic acid (IAA), gibberellins, and cytokinins [4], [3]. Gibberellic acid production was confirmed from *B. pumilus and B. licheniformis* [7]. The present investigation was conducted to demonstrate the Gibberellic acid production of *Bacillus siamensis BE 76* isolated from stem of banana (*musa spp*) and with and without l-tryptophan supplement in medium .Such bacteria are further useful for production biofertilizers to enhance growth and productivity of banana as well as to decrease use of harmful chemical fertilizers.

2. Materials and Methods

2.1. Collection of Sample

Banana plant sample (stems) was collected from village Zari, district of Parbhani, Maharashtra (India).

2.2. Isolation of endophytic bacteria from banana plant:

2.2.1. Surface disinfection: The banana stem samples were thoroughly washed in running tap water. They were then surface-disinfected using 70% ethanol for 2 min and immersed in 150 ml of 1.5% sodium hypochlorite plus a few drops of Tween 20 for 5 min with shaking. The samples were then rinsed thoroughly in five changes of sterile distilled water and dried in sterile paper towels [15].

2.2.2. Isolation of Endophytic bacterial isolates:
After Surface disinfection samples were macerated with a sterile mortar and pestle and then serially diluted in 12.5 mM potassium phosphate buffer at pH 7. For isolation of Gibberellin (GA_3) producing endophytic bacterial isolates several types of media were used such as nitrogen-free media - NFb [16], MacConkey's, Congo red [17], YEM agar [20] and nutrient agar [5]. Total 15 isolates were obtained from stems of banana plant. The isolates were further checked for Gibberellic acid production capability.

2.2.3. Morphological, cultural and biochemical characterization of isolates:
Morphological and cultural characterization was done on the basis of colony size, shape, color, margin, opacity, consistency, elevation, motility and gram staining, Endospore, capsule staining and based on the colony morphotypes selection of representative isolates was done. Biochemical tests performed were oxidase, amylase , gelatinase and catalase like enzyme production, citrate utilization, indole test, Vogus Proskauer test, methyl red test, H_2S production, sugars (Glucose, Sucrose, Lactose, Xylose and Mannitol) fermentation, Triple sugar iron (TSI) test, nitrate reduction, urease test etc. [9].

2.2.4. 16s rRNA gene sequencing:
Bacterial genomic DNA was isolated using geneO-spin Microbial DNA isolation kit (geneOmbio technologies, Pune; India). Partially a 16S rRNA gene was sequenced. Bacterial 16S region gene was amplified using standard PCR (Machine by Applied Biosystems 2720) reaction. The primer pair 27F (AGAGTTTGATCMTGGCTCAG3) and 1492R (TACCTTGTTACGACTT) as a universal primer were used in a PCR reaction with an annealing temperature of 57°C. After amplification, products were purified by using a geneO-spin PCR product Purification kit (geneOmbio technologies, Pune; India) After PCR is completed, the PCR products were checked on 1% Agarose by Agarose Gel Electrophoresis and amplicon size was compared using reference Ladder. 1% agarose gel spiked with Ethidium bromide at a final concentration of 0.5 µg/ml was prepared using Agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5X TBE buffer. 5.0 µl of PCR product was mixed with 1 µl of 6X Gel tracking dye. 5µl of g Scale 100bp size standard *(geneOmbio technologies, Pune; India)* was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5V/cm until the tracking dye is 2/3 distance away from the lane within the gel.bands were detected under a UV Trans illuminator. Gel images were recorded using BIO-RAD Gel Doc XR gel documentation system. The PCR product of size 1450bp was generated through this reaction and directly sequenced using an ABI PRISM Big Dye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. DNA sequencing was performed using one of the PCR primers [11]. BLAST analysis was performed at Blast N site at NCBI server and matching of sequence was checked [http://ww.ncbi.nlm.nih.gov/BLAST], [geneOmbio technologies, Pune; India]

2.2.5. Estimation of Gibberellic acid by Spectrophotometeric Method:
The Gibberellic acid production by banana endophytes was determined by Borrow *et al.* Method [4].

2.2.6. Extraction, purification and quantification of Gibberellic acid:
Isolated bacterial strain was analyzed for Gibberellic acid production in pure culture. Bacterial strain was grown in nutrient broth medium. Growth media (100 ml) were inoculated with 24-h-old bacterial culture and placed on a rotary shaker (DBK – 7270/2) at 100 rpm, for 4 days. Thereafter, cultures were centrifuged (Remi, India) at 6000 rpm for 15 min, and supernatant was used for extraction of growth hormone excreted in the growth medium [16]. The pH of supernatant was adjusted to 2.8 with 1 N HCl. And Gibberellic acid was extracted with an equal volume of ethyl acetate, as described by [19]. This ethyl acetate extract was evaporated to dryness at 35°C, and the residue was dissolved in 1, 500µl of pure methanol (Fisher Scientific, India). The samples were analyzed on HPLC (Chemito 6600 isocratic) using UV detector and C18 column (39× 300 mm). For identification of hormones, a 100-µl sample was filtered through 0.45 Millipore filter and 20 µl of the filtered extract injected into a 5µm reverse phase column. Pure GA (SRL, India), dissolved in HPLC grade methanol, was used as standard for identification and quantification of bacterial hormone. Growth hormone was identified on the basis of retention time and peak area of the standard. The solvent system used to separate GA was water: acetonitrile [76:24 (v/v)] used as mobile phase. Flow rate was adjusted at 2 mL/min, with an average run time of 20 min/sample. The wavelength used for detection of GA was at 254 nm [12], [Department of chemistry, Fergusson College, Pune].

3. Results and Discussion

3.1. Morphological, cultural and biochemical characterization of isolates:
The isolation of bacteria from surface disinfected stems of banana normally allows the recovery of putative endophytic bacteria. All isolates obtained from banana and selected by these combined criteria were grouped by morphological similarities and phenotypic characteristics [14]. In the present study total 15 N_2 fixing Endophytic bacterial isolates were isolated from stems of banana plant which showed different culture and morphological characteristics in which eleven isolates showed gram negative rods in nature, three were gram positive rods and remaining gram positive cocci. The sporulation, capsulation and motility as well as biochemical characteristics were also studied (Table 3.1.1)

Table 3.1.1: Biochemical characterization of promising isolate:

S.N	Biochemical Tests	Results of Isolate BE- 76
1	Oxidase test	+
2	Amylase test	+
3	Gelatinase liquification test	+
4	Catalase test	-
5	indole test	+
6	methyl red test	-
7	Vogus Proskaur test	-
8	citrate utilization	-
9	H_2S production	+
10	Glucose	+
11	Sucrose	+
12	Xylose	+
13	Mannitol	+
14	Lactose	+
15	Triple sugar iron (TSI) test	+
16	nitrate reduction	+
17	Urease test	-

3.2.16s rRNA gene sequencing of isolate BE 76: As described in more detail in methods we devised and implemented a method to extract the bacterial SSU rRNA 27f and 1492r primer-binding site sequences from the data in the RDP and the Sargasso Sea metagenomic data. A key point of the method is that it assumes only a 50% sequence identity between the region containing the primer-binding site in the sequence being analyzed and at least one member of a diverse set of "reference" sequences. The method is general and can be used to extract any portion of a sequence that is sufficiently conserved or at least is flanked by conserved sequences [11]. As per partially 16s rRNA gene sequencing of the isolate 575 bases sequenced are as follows:

(GTCTGAACCGCATGGTTCAGACATAAAAGGTGGC
TTCGGCTACCACTTACAGATGGACCCGCGGCGCAT
TAGTAGTTGGTGAGGTAACGGCTCACCAAGGCGA
CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
GGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAG
AACAAGTGCCGTTCAAATAGGGCGGCACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGC
GGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA
ACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGT
GGCGAAGGCGACTCTCTGGTCTGTAA) After NCBI BLAST analysis these bases 99% matched with *Bacillus siamensis* belong the family *Bacillaceae* and hence this isolate was identified as *Bacillus siamensis* based on Bergey's manual, and 16s rRNA gene sequencing and was designated as *BE 76.*

3.3. Gibberellic acid production potential of banana endophytic bacterial isolates:

Fifteen isolates were screened out for their productivity of gibberellic acid on spectrophotometer. Out of these 15 isolates six isolates (BE-63, BE-66, BE 79, BE-71, BE-74 and BE-76) showed ability to produce gibberellic acid (GA_3) without L-tryptophan supplement in medium at 75 minutes. From these gibberellic acid producing isolates, BE-76 produced high amount of gibberellic acid (0.180 and 0.240 mg/ml) supplemented with and without L-tryptophan in medium at 254nm (Fig.3.3.1). Productivity of gibberellic acid of BE-76 isolate was further estimated and confirmed on high-performance liquid chromatography (HPLC).

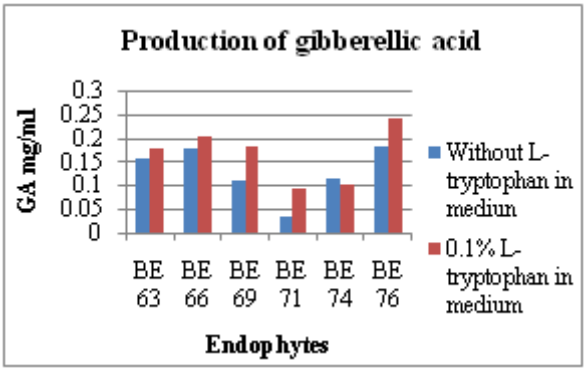

Figure. 3.4.1: Chromatogram of Gibberellic acid produced by banana Endophytic bacterial isolate (BE-76) without tryptophan in medium

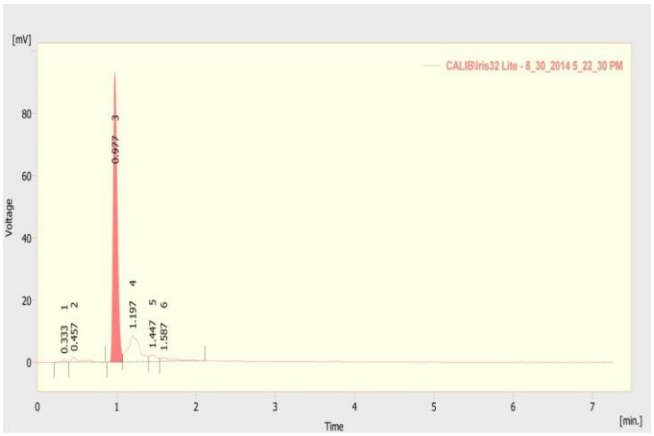

Figure. 3.3.1: Production of gibberellic acid by banana Endophytic bacterial isolates with and without L-tryptophan supplement in medium

3.4. Fractionation and Quantification of Gibberellic acid by high-performance liquid chromatography (HPLC)

The concentration of gibberellic acid produced by Endophytic bacterial isolate (BE-76) without and 0.1% L-tryptophan supplement in the medium was also analyzed by HPLC. The promising isolate produced gibberellic acid **198.16** ppm without L-tryptophan supplement as well as **254.29** ppm with 0.1% L-tryptophan supplement in the medium (Figure 3.4.1, 2). The findings of the present investigation highlighted that Gibberellic acid producing endophytes from stems of banana could be easily isolated and may be exploited after strain improvement for research. However, further studies using PGPR strains of these isolates are needed to be explored for the exact contribution of Gibberellic acid production in the promotion of plant growth as well as the contribution of other PGPR traits and development of biofertilizers.

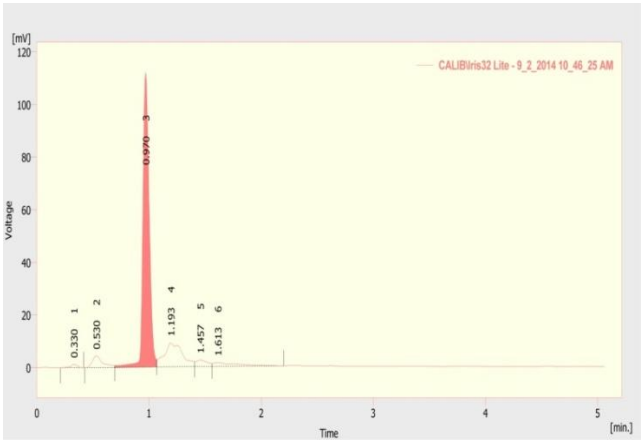

Figure 3.4.2: Chromatogram of Gibberellic acid produced by banana Endophytic bacterial isolate (BE-76) with 0.1% tryptophan in medium

4. Conclusions

From the present investigation, it is clear that endophytic bacteria isolated from stems of banana can provide a rich source of Gibberellic acid and has the ability to produce a significant amount of Gibberellic acid with and without tryptophan-supplemented in media. Out of 15 isolates six

isolates showed ability to produce Gibberellic acid with and without L-tryptophan supplement in the medium. BE-76 showed high amount of Gibberellic acid production which was estimated on Spectrophotometer and for more accuracy the productivity of Gibberellic acid of BE 76 isolates HPLC was used. Based on morphological, cultural, biochemical and 16s rRNA gene sequencing this isolate was identified as *Bacillus siamensis* and designated as *BE 76*. It is concluded that presence of such growth promoting Endophytic bacteria (*Bacillus siamensis*) were accountable for the beneficial effects on crop growth and yield. Nitrogen fixation, plant growth promotion and improved nutrient absorption are important criteria for achieving a sustainable banana production system. The Gibberellic acid producing *Bacillus siamensis BE 76* will promote the growth at the field level and prevent environmental pollution by avoiding excessive applications of chemical fertilizers and add to development of liquid bioinoculant for sustainable agriculture.

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