Indole acetic acid (IAA) production by endophytic bacterial isolate *Agrobacterium tumefaciens BE-1* from roots of *Musa acuminata*

Ambawade Mukund S.[1*], Patil Dipak D.[1], Pathade Girish R.[2] and Mali Gajanan V.[3]
1. Haribhai V. Desai College, Pune (MS), INDIA
2. Krishna Institute of Allied Science, KVVDU, Karad (MS), INDIA
3. Bharati Vidyapeeth (Deemed to be University), Yashwantrao Mohite College, Erandwane, Pune, INDIA
*ambawade2014@gmail.com

Abstract
We have screened 23 endophytic isolates to estimate the production of indole acetic acid (IAA) from the root of the banana (Musa spp) by using Salkowski's reagent spectrophotometric method with and without L-tryptophan supplement. Amongst 23 isolates only eight isolates, showed the ability to produce IAA. It is observed that one isolates out of eight produced maximum amount of IAA supplemented with and without L-tryptophan in medium.

Based on morphological, cultural, biochemical and 16s rRNA gene sequencing method, this promising endophytic bacterial isolate (BE 1) was identified as Agrobacterium tumefaciens. IAA production of BE-1 was further confirmed by Thin-layer chromatography as well as by High-Performance Liquid Chromatography with and without L-tryptophan in medium. Statistical analysis was carried out using paired t-test to test whether the production of IAA significantly increased by using L-tryptophan in medium.

Keywords: *Musa acuminata*, 16s rRNA gene sequencing, IAA.

Introduction
Endophytic bacteria produce many components that stimulate plant growth and help them to respond more to the environment to sustain a healthy symbiosis[2]. The *Musa acuminata* (banana) is second major fruit crop in India in terms of acreage and production. It has an excellent socio-economic and religious significance and contributes to about 31% of the total food production in India. A perishable and nutritious fruit is grown mostly in tropical and subtropical regions of the world as reported by Dodo[4]. Nitrogen fixation, plant growth promotion and improved nutrient absorption are important criteria for achieving sustainable production.

During the last four decades, work on plant growth promoting rhizobacteria (PGPR) has brought together scientists in multiple disciplines endeavoring a wide range of topics including discovery of novel PGPR strains and traits, performance in greenhouse and field trials, production, formulation and delivery of inoculum, mechanisms of growth promotion and biocontrol and their molecular and biochemical basis, root colonization and rhizosphere competence traits, role in suppressive soils, plant, pathogen and rhizosphere community responses to PGPR and recombinant PGPR and risk assessment[10].

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host[15].There is an increasing commercial and scientific interest with endophytes due to their potential to improve quality and growth of plants through nitrogen fixation. Such bacteria show mutualistic relationships with plants and help them in alleviating several biotic and abiotic stresses without showing any apparent negative effect to the host plant. Depending upon the growth promoting competencies, all endophytic bacterial strains have ability to influence the growth of host plants and have potential to be used as effective growth promoting bioinoculant for *M. acuminata*[17].

IAA (indole-3-acetic acid), a member of the group of phytohormones is generally considered as the most important native auxin[1]. It is one of the most physiologically active auxins. It is a common product of L- tryptophan metabolism in several microorganisms including PGPR[9]. The present study aimed to isolate and characterize the plant growth-promoting potential of the endophytic bacterial isolates from the *Musa acuminata* plant.

Material and Methods
Collection of Sample: Banana plant root samples were collected from Jalgaon district of Maharashtra, India.

Isolation of endophytic bacteria from the roots
Surface sterilization: The banana roots samples were thoroughly washed in running tap water. They were then surface sterilized 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[13].

Isolation and characterization of Endophytic bacteria: Surface sterilized samples were macerated with a sterile mortar and pestle and then serially diluted in 12.5 mM potassium phosphate buffer of pH 7. The isolation of indole acetic acid producing endophytic bacteria was carried out on nitrogen free media[3] containing malic acid - 0.5g,

$MgSO_4.7H_2O$-0.2g, NaCl - 0.1g, $CaCl_2$ -0.02g, $Na_2\ MoO_4$ - 0.002g, $MnSO_4.H_2O$ -0.01g, EDTA 1.64% -4 ml, Bromothymol blue 0.5% (W/W in ethanol) -3 ml, KOH - 4.5g, Biotin - 0.1mg, distilled water -1000ml, pH-6.8.

A loopful of the sample was spread on nitrogen free medium and kept for incubation for 48 to 72h at room temperature. Well isolated colonies were selected for morphological, cultural and biochemical characterization (VITEK-2 Compact Machine in the Joshi Lab., Pune) and identification (16s rRNA gene sequencing). A total number of 23 isolates were obtained and they were further checked for IAA production efficiency.

16s rRNA gene sequencing of potent isolates: The bacterial genomic DNA was isolated using gene O-spin Microbial DNA isolation kit (Gene Ombio Technologies, Pune, India). Bacterial 16S region gene was amplified using standard PCR (Machine by Applied Biosystems 2720) reaction. The primer pair 27F (AGAGTTTGATCMTGGCTCAG3) and 1492R (TACCTTGTTACGACTT) as universal primer were used in a PCR reaction with an annealing temperature of 57^0C. After amplification, products were purified by using a gene O-spin PCR product Purification kit (Gene Ombio 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 mg/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 1µL of 6X gel tracking dye. 5µL of g scale 100bp size standard 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 GelDocXR gel documentation system. The PCR product of size 1450bp was generated through this reaction and directly sequenced using an ABI PRISM BigDye 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[7]. Blast analysis was performed at BlastN site at NCBI server (http://ww.ncbi.nlm.nih.gov/BLAST) and similarity was checked.

Quantification of IAA production: To determine the amounts of IAA produced by each isolate, spectrophotometric technique was performed with Van Urk Salkowski's reagent using the Salkowski's method[5, 6].The isolates were grown in yeast malt dextrose broth ± 0.1% L-tryptophan (YMD± 0.1% L-tryptophan broth) (Himedia, India) at 28 °C for 4 days and the broth was then centrifuged at 3000 rpm for 30 min. 2 mL of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski's reagent (50 mL, 35% perchloric acid; 1 mL 0.5 FeCl3) and kept in the dark.

The optical density (OD) was recorded after red colour development on spectrophotometer (Systronics – 119) at 530 nm after 30 min and 120 min. The broths with high amount of IAA were selected for purification by thin layer chromatography. Further, its fractionation and quantification were carried out by HPLC method.

Extraction and purification of IAA: The isolates were grown in yeast malt dextrose broth (YMD + 0.1% tryptophan broth, Himedia, India) and incubated at 28°C for 4 days on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min**.** The supernatant was then acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate of the double volume of the supernatant. The extracted fraction was evaporated to dryness in a rotator evaporator at 40° C. It was then dissolved in 300 ml of methanol and kept in refrigerator[12].Thin layer chromatography (TLC) slide was prepared with silica gel G and calcium carbonate and propanol: water (8:2) was used as a solvent system. The extracted sample and standard IAA (10mg/100mL) were used for TLC. The spots with Rf values identical to authentic IAA were identified by spraying Salkowski's reagent[8].

Fractionation and Quantification of IAA by HPLC Method: The ethyl acetate extract was evaporated to dryness at 35°C and the residue was dissolved in 1,500µl of pure methanol. The samples were then analyzed on HPLC (Chemito 6600 isocratic) using UV detector and C18 column (39× 300 mm). For identification, 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 IAA (SiscoLaborarory, India), dissolved in HPLC grade methanol, was used as standard for identification and quantification of bacterial IAA. It was identified based on retention time and peak area of the standard. The solvent system used to separate IAA was water: acetonitrile [76:24 (v/v)] as a 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 IAA was 280 nm[16].

Results and Discussion

Isolation and characterization of isolates: The isolation of bacteria from surface sterilized roots of banana normally allows the recovery of putative endophytic bacteria. Growth in semisolid nitrogen free is a useful strategy to select for nitrogen fixing bacteria. In the present study, a total number of 23 nitrogen fixing endophytic bacterial isolates were obtained that showed different cultural characteristics. Among these, seven isolates were gram negative rods, six isolates showed gram positive rods while six were gram positive and four were negative cocci. Seven isolates were

spore formers, six were capsulated while thirteen were motile.

Estimation of IAA from isolates: All the 23 isolates were used to check and estimate the amount of IAA production. As revealed in table 1, these isolates were designated as endophytes BE-1, BE-2, BE-3, BE-4, BE-5, BE-6, BE-7, BE-8, BE-9, BE-10, BE-11, BE-12, BE-13, BE-14, BE-15, BE-16, BE-17, BE-18, BE-19, BE-20, BE-21, BE-22 and BE-23. Among these, eight isolates (BE-1, BE-4, BE-8, BE-10, BE-16, BE-17, BE-19 and BE-23) exhibited IAA production. The IAA was measured in absence and in presence of L-tryptophan supplement, after 30 minutes and after 120 minutes.

Among these eight isolates, isolate BE-1 produced maximum IAA, followed by isolates BE-8 and BE-10 in medium supplemented with and without L-tryptophan (Table 1). As shown in fig. 1 and fig. 2, the IAA production is, on an average, more at 120 minutes than at 30 minutes, in both with and without supplement of L-tryptophan. The isolate BE-23 produced least IAA without L-tryptophan supplement at 30 minutes and improved to great extent at 120 minutes. As demonstrated in fig. 3 and fig. 4, the IAA production with use of L-tryptophan is more than that of its without use at 30 minutes and 120 minutes. Further, to check the effectiveness of use of 0.1% tryptophan as a catalyst statistically, the pair t-test was carried out[14]. The p-value was found to be 1.86×10^{-06} in the production at 30 minutes while it was 0.010840093 at 120 minutes, which is less than level of significance 0.05. Therefore, it was concluded that the supplement of L-tryptophan improves the production of IAA significantly in the medium.

The maximum IAA production was exhibited by isolate BE-1. Therefore, it was considered as a potent isolate and used for its biochemical characterization (Table 2). Bergey's manual of determinative of bacteriology was used as a reference to identify the isolate[11].

Molecular characterization (16s rRNA gene sequencing) of isolate BE-1: After NCBI BLAST analysis, these 99% sequences were matched. *Agrobacterium tumefaciens* belonged the family *Rhizobiaceae* and hence this isolate was identified and confirmed as *Agrobacterium tumefaciens* basedon morphological, cultural, biochemical analysis and 16s rRNA gene sequencing and was designated as BE 1.

Extraction and purification of Indole acetic acid (IAA): Based on IAA production level, centrifuged culture of *Agrobacterium tumefaciens* BE 1 was used to extract IAA for characterization by TLC. The spots of ethyl acetate extracts of the respective culture broth and standard IAA were tested in solvent systems propanol: water (8:2). Chromatograms of culture spots and standard IAA, sprayed with Salkowski's reagent, showed almost same Rf values. This indicated that our TLC findings are in agreement with reports by other researchers[18].

Table 1
IAA produced by bacterial isolates from roots of *Musa acuminata*:

S.N.	Isolates	IAA in ppm (YEM + without tryptophan)		IAA in ppm (YEM + 0.1% tryptophan)	
		30 min	120 min	30 min	120 min
1	BE – 1	**33.111**	**34.905**	**72.042**	**78.145**
2	BE – 2	-	-		
3	BE – 3	-	-		
4	BE – 4	26.429	35.730	36.467	47.506
5	BE – 5	-	-		
6	BE – 6	-	-		
7	BE – 7	-	-		
8	BE – 8	30.602	32.667	40.116	61.001
9	BE – 9	-	-		
10	BE – 10	29.211	33.348	38.13	45.102
11	BE – 11	-	-		
12	BE – 12	-	-		
13	BE – 13	-	-		
14	BE – 14	-	-		
15	BE – 15	-	-		
16	BE – 16	20.865	25.408	32.152	36.204
17	BE – 17	11.116	19.224	22.131	34.215
18	BE – 18	-	-		
19	BE – 19	25.038	33.348	33.564	48.987
20	BE – 20	-	-		
21	BE – 21	-	-		
22	BE – 22	-	-		
23	BE – 23	2.782	32.554	15.623	29.152

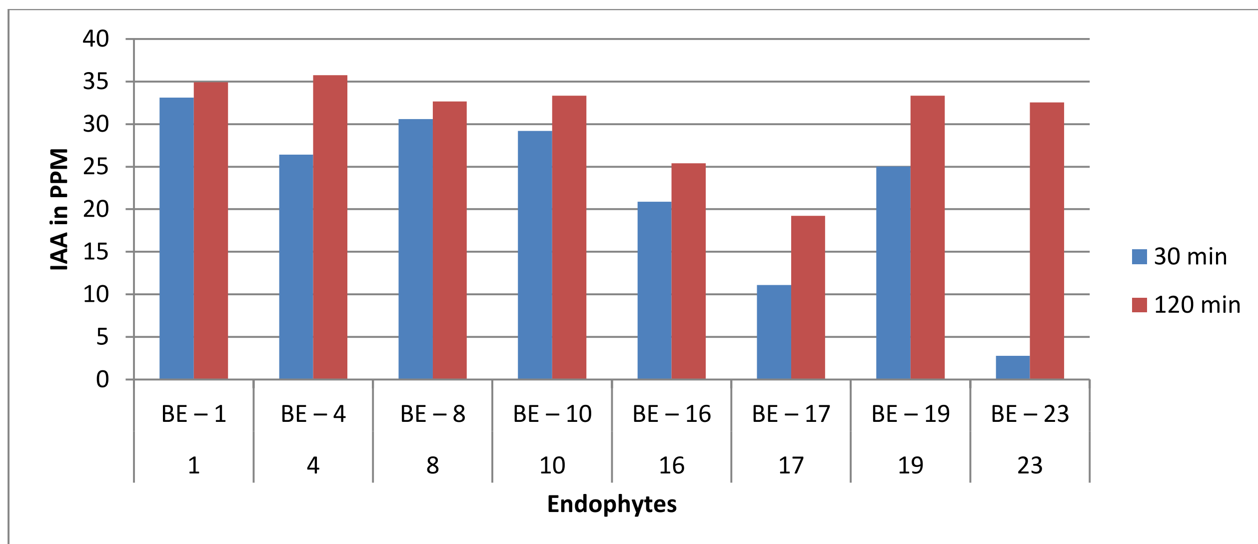

Figure 1: IAA in ppm (YEM + without tryptophan) at 30 and 120 min.

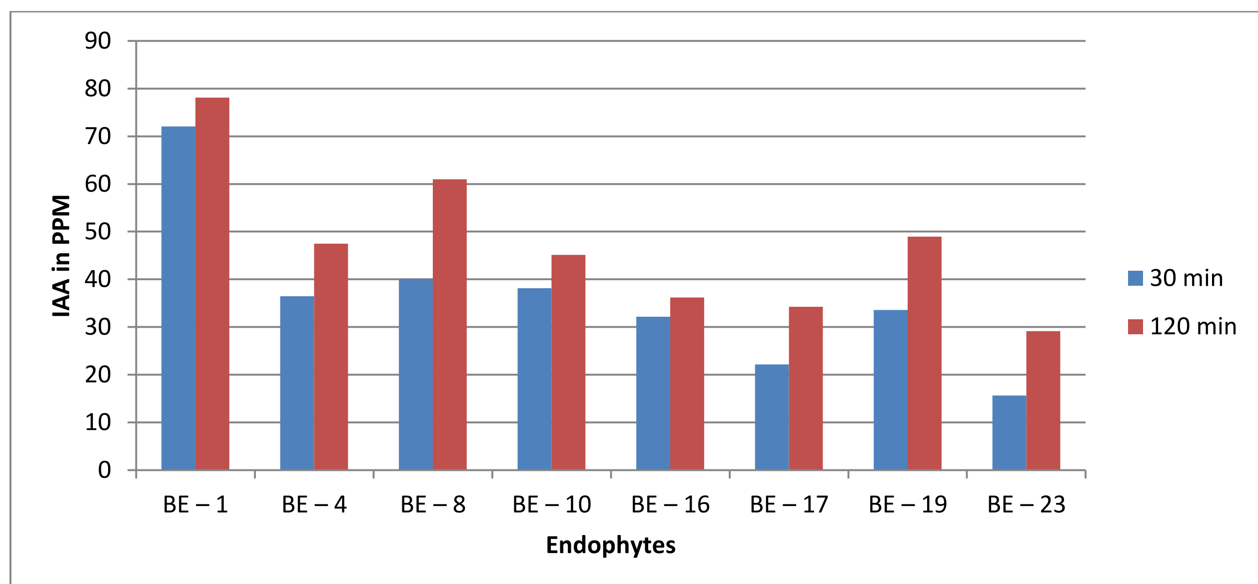

Figure 2: IAA in ppm (YEM + 0.1% tryptophan) at 30 and 120 min.

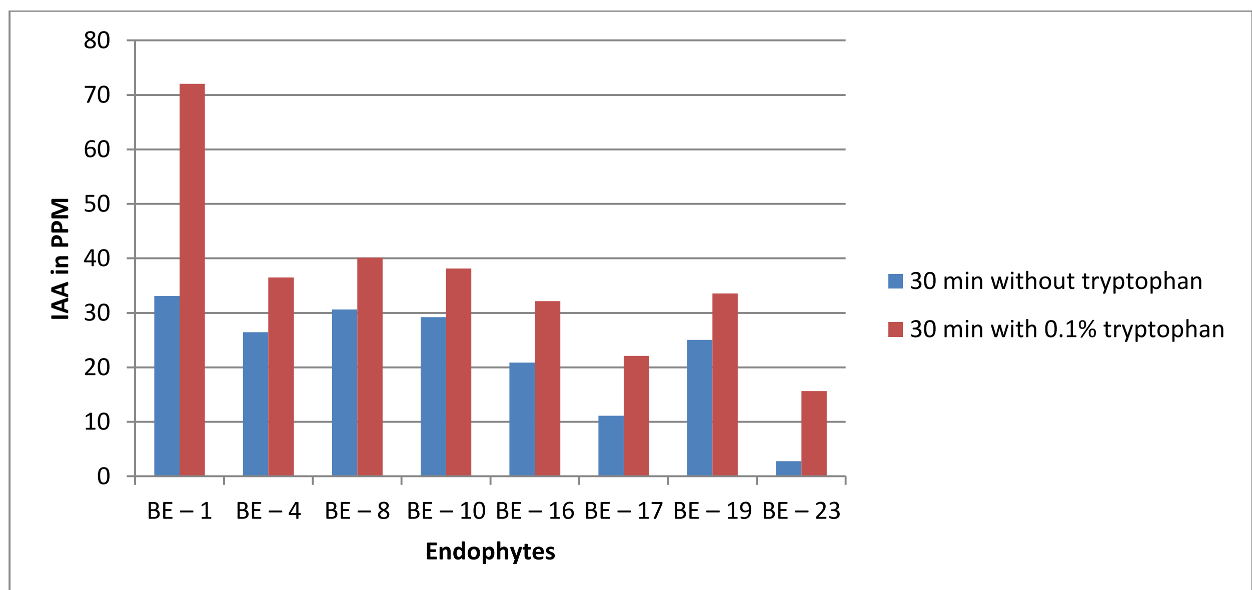

Figure 3: IAA in ppm (YEM + without tryptophan) and IAA in ppm (YEM + 0.1% tryptophan) at 30 min.

Table 2
Biochemical characterization of bacterial isolate BE 1

S. N.	Biochemical Tests	Isolates
		BE- 1
1	Ala-Phe-Pro Arylamidase (Appa)	-
2	Adonitol (Ado)	-
3	L-Pyrrolydonyl-Arylamidase (Pyra)	-
4	L-Arabitol (Iarl)	-
5	D-Cellobiose (Dcel)	+
6	Beta-Galactosidase (Bgal)	+
7	H_2s Production (H_2s)	-
8	Beta-N-Acetyl-Glucosaminidase (Bnag)	-
9	GlutamylArylamidasePna (Agltp)	-
10	D-Glucose (Dglu)	+
11	Gamma-Glutamyl-Transferase (Ggt)	-
12	Fermentation/Glucose (Off)	-
13	Beta-Glucosidase (Bglu)	+
14	D-Maltose (Dmal)	+
15	D-Mannitol (Dman)	+
16	D-Mannose (Dmne)	+
17	Beta-Xylosidase (Bxyl)	-
18	Beta-Alanine ArylamidasePna (Balap)	-
19	L-ProlineArylamidase (Proa)	+
20	Lipase (Lip)	-
21	Palatinise (Ple)	-
22	Tyrosine Arylamidase (Trya)	+
23	Urease (Ure)	-
24	D-Sorbitol (Dsor)	-
25	Saccharose/Sucrose (Sac)	+
26	D-Tagatose (Dtag)	-
27	D-Trehalose (Dtre)	-
28	Citrate (Cit)	-
29	Malonate (Mnt)	-
30	5-Keto-D-Gluconase (5kg)	-
31	L-Lactate-Alkalinisation (Ilatk)	-
32	Alpha-Glucosidase (Aglu)	+
33	Succinate (Suct)	-
34	Beta-N-Acetyl-Galactominidase (Naga)	-
35	Alpha-Galactosidase (Agal)	-
36	Phosphate (Phos)	-
37	Glycine-Arylamidase (Glya)	+
38	Ornithine Decarboxylase (Odc)	-
39	Lysine Decarboxylase (Ldc)	-
40	L-Histidine Assimilation (Ihisa)	-
41	Coumarate (Cmt)	-
42	Beta-Glucuronidase (Bgur)	-
43	Resistance Comp.Vibrio (O129r)	-
44	Glu-Gly-Arylamidase (Ggaa)	-
45	L-Malate-Acetyl-Galactominidase (Imlta)	-
46	Ellman (Ellm)	-
47	L-Lactate Assimilation (Ilata)	-

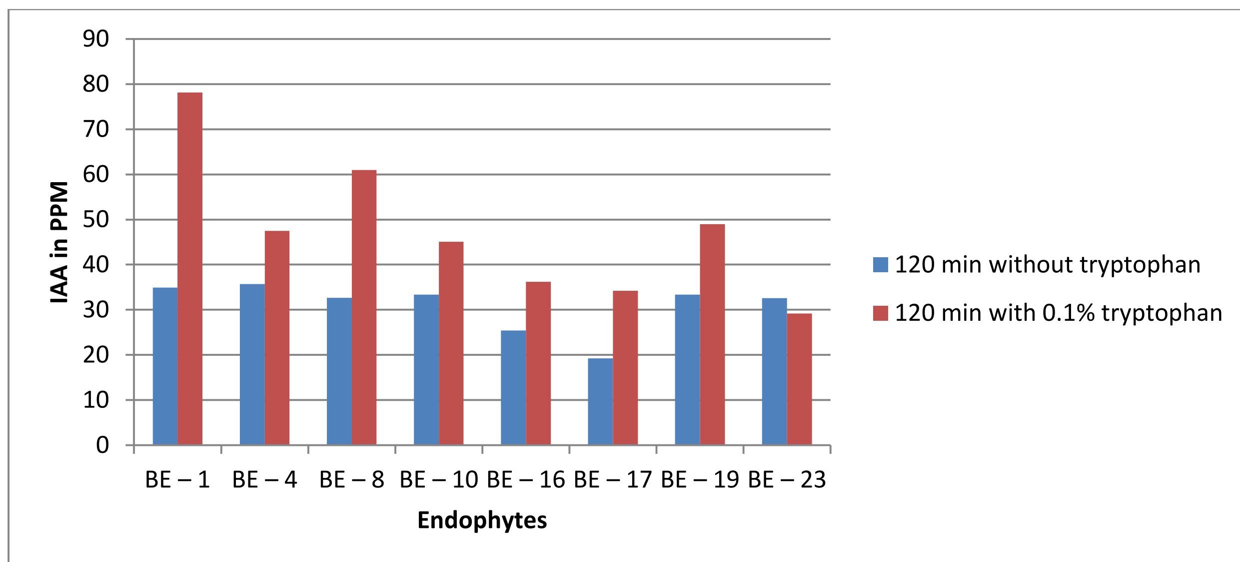

Figure 4: IAA in ppm (YEM + without tryptophan) and IAA in ppm (YEM + 0.1% tryptophan) at 120 min.

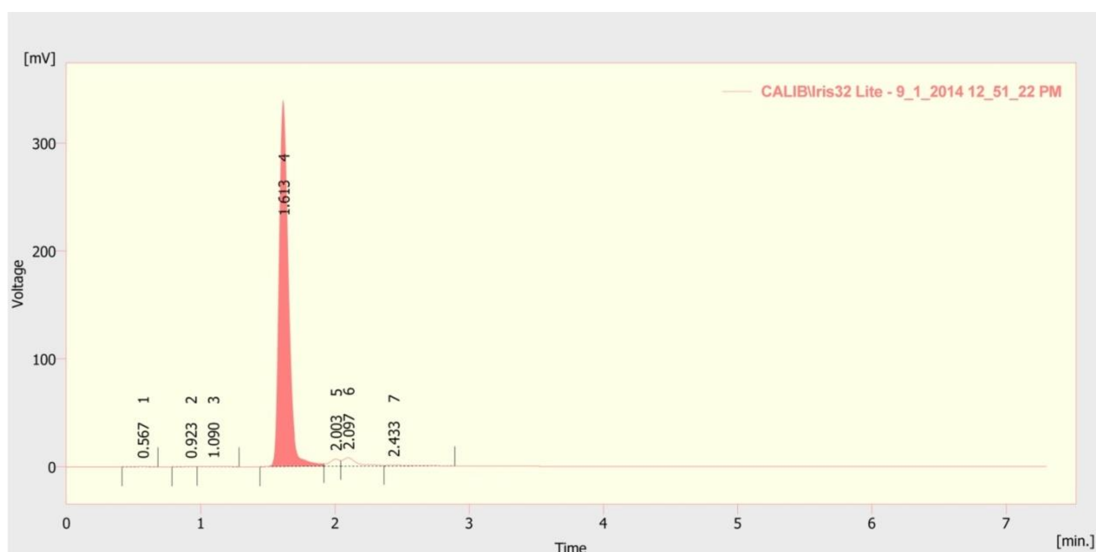

Figure 5: Chromatogram of IAA produced by endophytic bacterial isolate BE-1, with 0.1% tryptophan

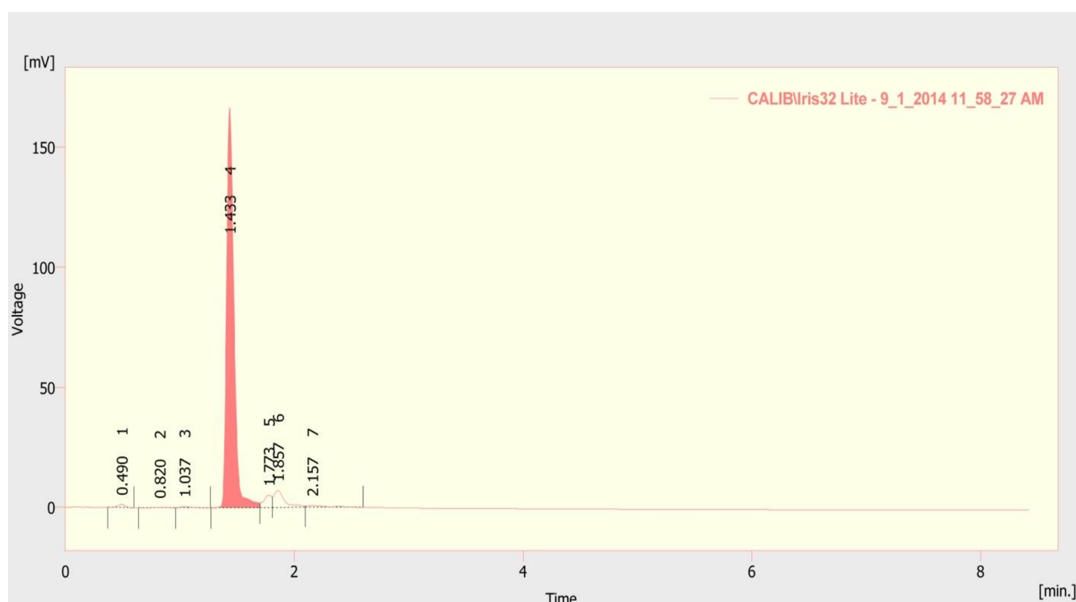

Figure 6: Chromatogram of IAA produced by endophytic bacterial isolate BE-1, without L- tryptophan

The purified IAA sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the Rf corresponding to the authentic IAA (0.54cm). This confirmed the IAA producing potential of endophytic bacterial isolate BE 1.

Fractionation and Quantification of IAA by HPLC Method: The estimation of IAA by promising bacterial isolate was also tested by High Performance Liquid Chromatography (HPLC). The results of HPLC chromatogram of isolate and standard chromatogram of IAA, are presented in fig. 5 and fig. 6. The isolate BE-1 produced IAA (36.22, 79.02ppm) with and without L-tryptophan supplement in the liquid medium. The use of the technique for the detection of IAA using the Salkowski's reagent is an important option for qualitative and semi-qualitative determination that assured the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. The amount of IAA produced by the bacteria was within the detection limits of Salkowski's reagent[5]. As per HPLC analysis in this study, *Agrobacterium tumefaciens* BE 1 produced a high amount of IAA with 0.1 % L-tryptophan supplement in medium comparative to previous studies found in literature.

Conclusion

From the present study, it is concluded that the endophytic bacteria isolated from roots of banana can provide a good source of IAA and can increase the production significantly in presence of L-tryptophan-supplemented medium. Furthermore, the ability of more IAA production suggested supplementary beneficial role in plant growth promotion (PGPR) even under stressed environmental conditions. Out of the 23 isolates studied, only eight isolates showed ability to produce IAA without L-tryptophan supplement as well as with 0.1% L-tryptophan supplement in the medium. Thus, the endophytic bacterium *Agrobacterium tumefaciens* BE-1 gives high amount of IAA estimated by spectrophotometer, HPLC and TLC. It was followed by BE-8 and BE-10.

The current information on PGPR, its application on bananas, as well as different crop plants indicates the potential of PGPR as a bioenhancer and biofertilizer for banana cultivation. The potential of the IAA producing isolate will flourish the growth and ultimately IAA production in the field and will prevent environmental pollution by avoiding excess applications of chemical fertilizers. These endophytic bacteria are also helpful for the development of liquid bioinoculant for economically convenient and sustainable agriculture. The statistical analysis is carried out using paired t-test which strengthens the result of effectiveness of *Agrobacterium tumefaciens* BE-1 isolate.

References

1. Ashrafuzzaman M., Hossen F.A., Ismail M.R., Hoque M.A., Islam M.Z., Shahidullah S.M. and Meon S., Efficiency of plant growth promoting Rhizobacteria (PGPR) for the enhancement of rice growth, *African Journal of Biotechnology*, **8(7)**, 1247- 1252 **(2009)**

2. Das A. and Varma A., Symbiosis: The art of living, In Varma A. and Kharkwal A.C., Symbiotic Fungi Principles and Practice, Berlin, Germany, Springer, 1-28 **(2009)**

3. Dobereiner J., Marriel I.E. and Nery M., Ecological distribution of Spirillum lipoferum, *Can. J. Microbiol.*, **22**, 1464-1473 **(1976)**

4. Dodo M.K., Multinational Companies in Global Banana Trade Policies, *J Food Process Technol*, **5**, 351 **(2014)**

5. Ehmann A., The Van Urk-Salkowski reagent-a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives, *Journal of Chromatography*, **132**, 267-276 **(1977)**

6. Farah Ahmad, Iqbal Ahmad and Mohd Saghir Khan, Indole Acetic Acid Production by the Indigenous Isolates of Azotobacter and Fluorescent Pseudomonas in the Presence and Absence of Tryptophan, *Turk J Biol*, **29**, 29-34 **(2005)**

7. Frank Jeremy A. et al, Critical evaluation of two primers commonly used for amplification of bacterial 16s rRNA genes, *Applied and Environmental Microbiology*, **74(8)**, 2461–2470 **(2008)**

8. Kuang-Ren C., Turksen S., Umran E., Timmer L.W. and Peter P.U., Indole derivatives produced by the fungus Colletotrichumacutatum causing lime anthracnose and postbloom fruit drop of citrus, *FEMS Microbiology Letters*, **226**, 23-30**(2003)**

9. Lynch J.M., Origin, nature and biological activity of aliphatic substances and growth hormones found in soil, In Vaughan D. and Malcom R.E., eds., Soil Organic Matter and Biological Activity, MartinusNijhoff /Dr. W. Junk Publishers. Dordrecht, Boston, Lancaster, 151-174 **(1985)**

10. Mairaj Samya, Nagar Richa Dave, Rehman F. and Jindal Tanu, Effects of arsenic-induced toxicosis (Arsenicosis) on human health and its prevention, *Res. J. Chem. Environ.*, **27(3)**, 116–123 **(2023)**

11. MacFaddin J.F., Biochemical tests for identification of medical bacteria, Williams and Wilkins, London **(2000)**

12. Mohite B., Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth, *Journal of Soil Science and Plant Nutrition*, **13(3)**, 638-649 **(2013)**

13. Ngamau C.N., Viviene N.M., Akio T. and Catherine W.M., "Isolation and identification of endophytic bacteria of bananas (Musa spp.) in Kenya and their potential as biofertilizers for sustainable banana production, *African Journal of Microbiology Research*, **6(34)**, 6414-6422 **(2012)**

14. Ross Amanda and Victor L. Willson, Basic and Advanced Statistical Tests, Springer, USA **(2017)**

15. Ryan R.P., Germaine K., Franks A., Ryan D. and Dowling D., Bacterial endophytes: recent developments and applications, *FEMS Microbial. Letter*, **278**, 1-9 **(2008)**

16. Sarwar M., Arshad M., Martens D.A. and Frankenberger W.T. Jr., Trypthtophan-dependent biosynthesis of auxins in soil, *Plant Soil*, **147**, 207-215 **(1992)**

17. Singh Shilpi, Kamlesh Choure, Rai Piyush Kant, Gour Sourabh Singh and Agnihotri Vivek Kumar, Evaluation of plant growth-promoting activities of endophytic bacteria of Musa acuminata and their characterization, *Journal of Applied Biology & Biotechnology*, **10(5)**, 94-101 **(2022)**

18. Xie H., Pasternak J.J. and Glick B.R., Isolation and characterization of mutants of the plant growth promoting rhizobacterium Pseudomonas putida GR-122 that overproduce indoleacetic acid, *Curr. Microbiol*, **32**, 67-71 **(1996)**.

(Received 28th November 2023, accepted 02nd January 2024)