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Rhizobacterial Culture Supernatant As A Promising Strategy For Banana Tissue Culture Improvement

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Abstract

Tissue culture propagation of Banana (*Musa* spp.) proved to be the alternative commercial tool for traditional vegetative propagation through suckers. The aim of this study was to explore the application of bacterial culture supernatant as a substitution for commercial indole acetic acid (IAA) for improving plant tissue culture production. Up to 200 bacterial isolates were obtained from different banana plant spheres (rhizosphere, endorhiza, and stem- endosphere) and screened for their *in vitro* ability to produce IAA. About 50% of isolates were able to produce IAA with different amounts; the most promising IAA producing isolates were further identified according to the 16S rRNA gene sequencing. The chemical composition of the culture supernatants of most promising IAA producing isolate; *Brevibacillus brevis*-R39 and *Klebsiella variicola*-R19 were determined using GC-MS analysis, then the culture supernatants of *Brevibacillus brevis*-R39 was selected as Murashige and Skoog (MS) medium supplement at different doses (5, 10, 20, and 40 ml/l), corresponding to 0.21, 0.42, 0.4, and 1.68 mg IAA/l, respectively) in comparison to commercial IAA (0.5, 1, 2, and 4 mg/l). The results demonstrated that the culture supernatant of *Brevibacillus brevis*-R39 at 10 ml/l significantly showed the highest average length of banana shoots (11.9% over the control), followed by its 5ml/l (7.8% over the control). The greatest width of leaves (24% over the control) was recorded with the bacterial extract at concentration of 5 ml/l, followed by 13.2% over the control with bacterial extract at 10 ml/l. The greatest length of roots (49% over the control) was recorded with the bacterial extract at 10 mg/l, followed by 22% over the control with IAA at 2 mg/l. Our results recommend the use of *Brevibacillus brevis*-R39 culture supernatant as a medium supplement as a substitution for commercial IAA to improve banana plantlet properties.

Keywords: Banana, tissue culture, PGPR, IAA, *Brevibacillus brevis*

Introduction

Traditional banana cultivation technique through suckers is a slow difficult process produces small amount of planting material that is likely to be contaminated with soil-borne pathogens. Plant tissue culture is a highly versatile technique which leads to micropropagation of thousands of plantlets from a small portion of mother plant [1], [2]. For commercialization, banana tissue culture technique produces high quality and healthy plants, which is especially important for avoiding the spread of soil borne pathogens and viruses [3].Plant growth-promoting rhizobacteria (PGPR) provide a wide range of services and benefits to plant such as playing a key role in nutrient acquisition and assimilation, improve soil texture, secreting, and modulating extracellular molecules such as hormones, secondary metabolites, antibiotics, and various signal compounds, all leading to enhancement of plant growth [4]. Furthermore, PGPR can suppress deleterious microorganisms along with promoting plant growth [5]. They also release bioactive

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compounds like vitamins, hormones and enzymes that stimulate plant growth and enhance biotic and abiotic stress tolerance [6]. The effect of PGPR on banana growth production through tissue culture was studied. Improving growth parameters (plant height, leaf number, leaf area, pseudo-stem thickness, root and shoot fresh weight, root and shoot dry weight) demonstrated that inoculation could partially replace the chemical fertilization [7]. On the other hand, several studies have approved the effects of rhizobacteria (*Azospirillum brasilense* Sp7, *Bacillus sphaericus* UPMB10, *Microbacterium oxydens* UPMB11, and *Agrobacterium rhizogenes* AR9402) inoculation, individually or mixed with MS basal medium on the increase in number, fresh and dry weight and total length of roots. In addition, they enhanced the total concentrations of the biochemical active compounds such as total soluble peroxidase, protein, proline, soluble nitrogen, nitrate reductase, phenolic nitrate, and chlorophyll of in *vitro* banana plantlets according to the type of the bacteria [8]. Moreover, using *Pseudomonas* sp., *Bacillus subtilis*, and *Pantoea* sp. with tissue explants stimulated various metabolisms in the resulting plantlets giving improved tolerance to abiotic and biotic stresses and exhibiting multiple plant growth-promoting actions with the *in vitro* increased weight of corm lets saffron and germination [9].

Experimental

Sampling and sample preparation for bacterial isolation

Three suckers of healthy adult banana plants (*Musa acuminata cv*. Grand Nain) were collected from the greenhouse of the National Research Centre, Giza, Egypt after 6 months of acclimatization in peat moss and sandy soil at ratio of (1:1 v/v). Five grams of rhizosphere samples were transferred aseptically into 50 ml sterile Falcon tubes, and then mixed with 45 ml of 0.85% NaCl saline solution by vortexing at maximum speed for 1 min. Five grams of roots and small pieces of corm, obtained from the same plants, were surface sterilized for 10 min in 0.5% sodium hypochlorite solution followed by three washing steps in sterile saline each for 10 min [10]. Sterilized roots and corm were grounded using sterile mortar and pestle. Tenfold serial dilutions of each sphere were plated onto nutrient agar medium (NA). After three days of incubation at 30 ºC, counts of colony forming units (CFU) were estimated. The bacterial numbers were determined as CFU/g and then expressed as a $\log_{10}$ CFU/g. Following visual differentiation, the distinct grown colonies were picked up, purified by striking and then kept at 5ºC for further analysis.

Screening for indole-3-acetic acid (IAA) producing bacteria

Up to 200 bacterial isolates were obtained from the three sphere samples (rhizosphere, endorhiza, and stem-endosphere). All were screened for their *in vitro* ability to produce IAA as described by Ozdal *et al*. [11] using tryptone water medium supplemented with tryptophan (1 g/l). After 48 hr. of incubation on a rotary shaker (150 rpm at 30°C), bacterial cells of each culture were harvested by centrifugation at 4000 rpm for 10 min. Then, one ml of each culture supernatant was added to 2 ml of salkowski's reagent [12].This mixture was kept in the dark for 30 min as the formation of red-pink color, indicating the presence of IAA, was measured using spectrophotometer (Shimadzu UV 1700) at 530 nm. IAA concentrations were calculated using standard curve constructed after preparation of different concentrations of pure IAA (Sisco research laboratories pvt. ltd. India). IAA producing bacterial isolates were stored at -20°C in Luria-Bertani (LB) broth supplemented with 20% glycerol.

Bacterial DNA extraction

IAA producing bacteria were grown on LB medium for 48 hr. at 30°C then harvested by centrifugation at 12000 g for 5 min. After washing bacterial pellets three times with 0.85% NaCl saline solution, genomic DNA was extracted using the GeneJET genomic DNA purification kit (Thermo Scientific, Lithuania) following the manufacturer's standard protocol. DNA yields and purity were checked using both Nanodrop spectrophotometer and agarose gel electrophoresis.

16S rRNA gene sequencing and phylogenic analysis of bacterial isolates

The 16S rRNA gene fragments of bacterial isolates were amplified using the universal primers (F-27; AGAGTTTGATC(AC)TGGCTCAG and R1494; CTACGG(T/C)TACCTTGTTACGAC) [13], and Biorad T100 thermal cycler. The PCR products were tested via agarose gel electrophoresis and sequenced by Macrogen Korea. The phylogenic analysis was

inferred using the Neighbor-Joining method using the maximum composite Likelihood by comparing the sequences of 16S rRNA gene amplified from bacterial isolates of the current study with the most similar hits obtained from the NCBI Genbank database. Evolutionary analyses were conducted in MEGA 5 software [14].

Extraction of bacterial secondary metabolites

To obtain culture free supernatant, two promising IAA-producing isolates were inoculated in 250 ml of tryptone water media supplemented with filter-sterilized solution of L-tryptophan (1 g/l) and incubated for 48 hr. at 30ºC on a rotary shaker (120 rpm). Supernatant was obtained by centrifugation at 10,000 rpm for 10 min followed by filter sterilization using 0.22 micrometer filters.

Comparing the chemical composition of two rhizobacterial culture supernatant using GC-MS

The chemical composition of bacterial cultural supernatant of R19 and R39 were analyzed using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50ºC and then increased by 5°C /min to 230°C hold for 2 min. increased to the final temperature 290°C by 30°C /min and hold for 2 min. The injector and MS transfer line temperatures were kept at 250 and 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using auto-sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database [15].

Detection of IAA by High Performance Liquid Chromatography (HPLC)

IAA was detected using high performance liquid chromatography (HPLC Agilent 1200 infinity series). HPLC analysis was carried out at a flow rate of 1 ml/min and the multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the bacterial extract R39 and standard solutions IAA [16].

Investigating the effect of bacterial culture free supernatant on banana explants growth and development

To study this effect, banana shoots (11-12 cm in length) with (3-4) leaves, taken from vegetative stage, were grown on MS-medium supplemented with R39 culture supernatant (5, 10, 20, and 40 ml/l, corresponding to 0.21, 0.42, 0.84 and 1.68, respectively), commercial IAA (0.5, 1, 2, and 4 mg/l) and control MS medium (without additives, Table 4), four replicates of each. After that, explants were incubated at 25 ±2°C with light intensity of 2000 Lux and period of 16 hr. light and eight hr. dark. Banana cultures were incubated for 30 days under the previous conditions [17]. One month after culturing, the following measurements: shoot length, leaf numbers, leaf width, root numbers, root length and chlorophyll contents were recorded.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and tested by Bartlett's test at ($p \leq 0.05$) using the CoStat 6.303 software.

Results

Bacterial populations of banana plants

Different banana plant spheres showed different bacterial population densities, the average CFU counts were 4.49, 3.18, and 2.64 log CFU/ g for rhizosphere, root, and stem-endophytic compartments, respectively.

Isolation of IAA producing bacteria

A total of 200 bacterial isolates obtained from rhizosphere (60 isolates) and the endophytic compartments of banana plants (140 isolates), were screened for their IAA production ability. Seventy-seven of them showed positive reaction with salkowski's reagent and considered as IAA-producing bacteria (Fig. 1, Table 1).

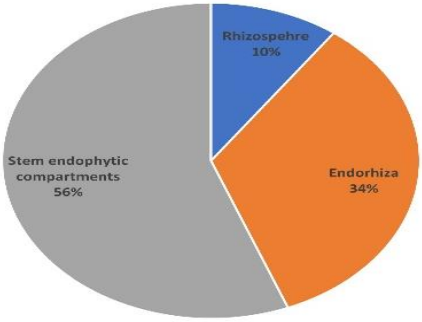


Fig. 1 Percentages of IAA producing bacteria obtained from the rhizosphere and the root and stem endophytic compartments of banana (*Musa* sp.)

Table 1 IAA producing bacterial isolates

Isolates	Sphere	IAA	Isolates	Sphere	IAA
Er4	Endorhiza	+	Er61	Endorhiza	+
Er46	Endorhiza	+++	Er62	Endorhiza	+
Er49	Endorhiza	+	Er63	Endorhiza	+
Er50	Endorhiza	+	Er64	Endorhiza	+
Er51	Endorhiza	+	Er 66	Endorhiza	+
Er52	Endorhiza	+	Er67	Endorhiza	+
Er53	Endorhiza	+	Er70	Endorhiza	+
Er57	Endorhiza	++	Er72	Endorhiza	+
Er58	Endorhiza	+	Er73	Endorhiza	+
Er 59	Endorhiza	++	Er74	Endorhiza	+++
Er60	Endorhiza	+	Er75	Endorhiza	+
Er77	Endorhiza	+	C26	Corm (stem endosphere)	+
Er78	Endorhiza	+	C27	Corm (stem endosphere)	+
Er79	Endorhiza	+	C30	Corm (stem endosphere)	+
Er80	Endorhiza	++	C31	Corm (stem endosphere)	+
C3	Corm (stem endosphere)	+	C32	Corm (stem endosphere)	+
C5	Corm (stem endosphere)	+	C34	Corm (stem endosphere)	+
C7	Corm (stem endosphere)	+	C36	Corm (stem endosphere)	+
C8	Corm (stem endosphere)	+	C37	Corm (stem endosphere)	+
C9	Corm (stem endosphere)	+	C38	Corm (stem endosphere)	+
C10	Corm (stem endosphere)	+	C40	Corm (stem endosphere)	+
C11	Corm (stem endosphere)	+	C41	Corm (stem endosphere)	+
C12	Corm (stem endosphere)	+	C42	Corm (stem endosphere)	+
C13a	Corm (stem	+++	C43	Corm (stem	+++

+++ Very good activity, ++ good activity, + low activity

Plant growth promoting properties of IAA-producing bacteria

Six bacterial isolates out of 77 (R19, R39, C13a, C43, Er46, and Er74) were selected based on their high IAA production ability and were further tested for other plant growth promoting properties such as nitrogen fixation and phosphate solubilization. R19 and Er46 showed positive results for both *in vitro* nitrogen fixation and phosphate solubilization in addition to the production of IAA (Table 2).

Molecular identification of plant growth promoting rhizobacteria

16S rRNA gene sequencing and phylogenetic analysis

The analysis of 16S rRNA gene sequence of 5 bacterial isolates revealed that R19 had maximum similarity of 99% with *Klebsiella variicola*, R39 also showed 99% similarity to *Brevibacillus brevis*, both C13 and Er46 showed 99% to *Enterobacter cloacae*, C43 had maximum similarity of 99% to *Brevibacterium epidermidis* (Table 2). Fig. 2 shows the phylogenetic affiliation of the IAA producing bacterial isolates to the closest hits obtained from the NCBI GenBank. All sequences were submitted to the NCBI GenBank under the accession numbers of (MZ411463 to MZ411467)

Comparison of the chemical composition of culture supernatants of *Klebsiella variicola*-R19 and *Brevibacillus brevis*-R39

From the above-mentioned isolates, *Klebsiella variicola*-R19 and *Brevibacillus brevis*-R39 bacterial culture supernatants were analyzed through GC-MS analysis to reveal their chemical composition (Table 3, Fig. 3). A total of 17 compounds were identified from the two bacterial extracts. Six compounds were identified in bacterial extract of *Klebsiella variicola*-R19 such as ethyl benzene (5.16%), 3-ethyltoluene (9.98 %), undecane (5.81%), bisabolol oxide B (37.16%), palmitic acid, methyl ester (1.88%), oleic acid, methyl ester (Z)-9-octadecenamide (10.23%). While, 11 compounds were identified in bacterial extract of R39 such as carvone (3.90%), dodecanoic acid, 3-hydroxy- (3.38%), (E)-β-farnesene (6.96%), α-copaene (2.61%), β -eudesmol (2.05%), methyldihydrojasmonate (12.48%), 1-(4-isopropylphenyl)-2-methylpropyl acetate (3.63%), retinal (3.93%), bisabolol oxide B (6.69%) dodecanoic acid (lauric acid) (4.41%), oleic acid (6.26%), octadecanoic acid trimethylsilyl ester (stearic acid TMS derivative) (5.04%).

Table 2 Molecular identification and growth promoting properties of the most promising bacterial isolates.

Isolates	Source	Identification	Percent identity	Nitrogen fixation	Phosphate solubilizing activity	IAA production (µg/ml)	Accession number
R19	Rhizosphere	*Klebsiella variicola*	99	+	+	82.49	MZ411463
R39	Rhizosphere	*Brevibacillus brevis*	99	+	-	42.4	MZ411464
C13	Stem endosphere	*Enterobacter cloacae*	99	+	-	17.81	MZ411465
Er46	Endorhiza	*Enterobacter cloacae*	99	+	+	18.61	MZ411466
C43	Stem endosphere	*Brevibacterium epidermidis*	99	+	-	5.49	MZ411467

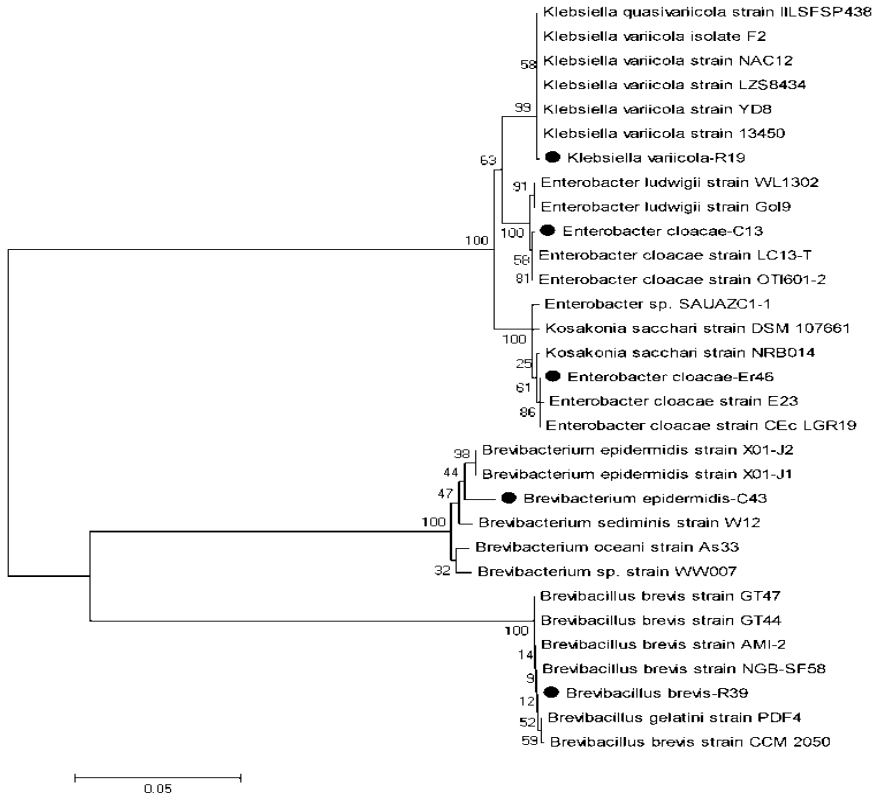


Fig. 2 A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of IAA producing bacterial isolates obtained in this study (Dark circles) with the closest hits obtained from the NCBI GenBank.

Results exhibited that, extract of *Brevibacillus brevis*-R39 had many compounds which considered as anti-bacterial and anti-fungal such as carvone, dodecanoic acid, 3-hydroxy, (E)-β-farnesene, α-copaene, β –eudesmol, dodecanoic acid (lauric acid), oleic acid, trimethylsilyl ester, octadecanoic acid, trimethylsilyl ester (stearic acid TMS derivative) and alpha-bisabolol oxide B antibiotic, in addition to methyl di hydrojasmonate which plays a role in the regulation of the fruit ripening and in defense mechanism against pathogen. The biological functions of these compounds were identified in both extracts of rhizobacterial *Klebsiella variicola*-R19 and *Brevibacillus brevis*-R39 as shown in Table 3, Fig. 3. The amount of IAA in the culture supernatant of *Brevibacillus brevis*-R39 was 42 µg/ml as analyzed by high performance liquid chromatography (HPLC) showed in Fig. 4.

Table 3 Comparison of the chemical constitutes of the culture supernatants of both *Klebsiella variicola*-R19 and *Brevibacillus brevis*-R39.

No.	RT	Percentage area		Compound name	Molecular formula	Molecular weight	Proposed function
		R19	R39				
1	4.08	5.16	-	p-Xylene Ethyl benzene	C_8H_{10}	106	Antibacterial [43]
2	5.41	9.98	-	3-Ethyltoluene	C_9H_{12}	120	Antifungal (*Fusarium oxysporum* f.sp. *cubense*) [44]
3	8.84	5.81	-	Undecane	$C_{11}H_{24}$	156	Antifungal [35]
4	12.78	-	3.90	Carvone	$C_{10}H1_{4O}$	150	Antifungal [45]
5	16.74	-	3.38	Dodecanoic acid, 3-hydroxy-	$C_{12}H_{24}O_3$	216	Antibacterial and fungal [46]
6	19.71	-	6.96	(E)-β-farnesene	$C_{15}H_{24}O$	220	Antibacterial [47], [48]
7	22.77	-	2.61	α-copaene	$C_{15}H_{24}$	204	Antioxidant and antigenotoxic, anticancer agent [49], [50]
8	22.97	-	2.05	Beta-Eudesmol	$C_{15}H_{26}O$	222	Antifungal [51]
9	23.09	-	12.48	Methyl di hydrojasmonate	$C_{13}H_{22}O_3$	226	Phyto-hormones [52] Virus resistance [53] Play a role in fruit ripening [54], [55] Defense mechanism against pathogen infection [56] Antibacterial [39]
10	23.27	-	3.63	1-(4-Isopropylphenyl)-2-Methylpropyl acetate	$C_{15}H_{22}O_2$	234	Anti-inflammatory, anti-heistamanial and anti-trypanosoma [57]
11	23.72	-	3.93	Retinal Vitamin A aldehyde	$C_{20}H_{28}O$	284	Antioxidant [58] Play a role in human nutrition and health [59]
12	25.08	37.16	6.69	Bisabolol oxide B alpha-Bisabolol oxide B	$C_{15}H_{26}O_2$	238	Anti-inflammatory [60], [61] Analgesic, antibiotic and anticancer activities [61].
13	27.37	-	3.30	1,25-Dihydroxyvitamin D3, TMS derivative	$C_{30}H_{52}O_3Si$	488	Antioxidants [58]
14	27.37	-	3.30	1,25-Dihydroxyvitamin D2, TMS derivative	$C_{37}H_{68}O_3Si_3$	644	

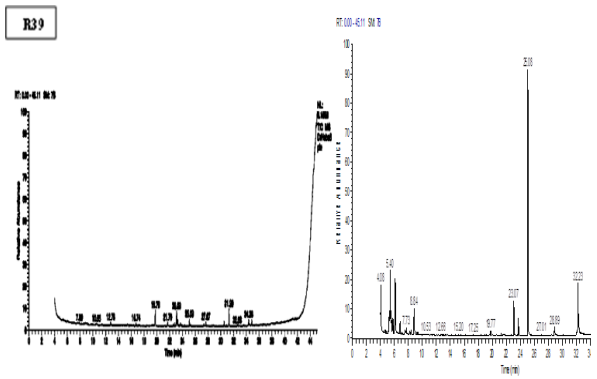


Fig. 3 GC-MS analysis of the two bacterial supernatants: *Klebsiella variicola*-R19 and *Brevibacillus brevis*-R39

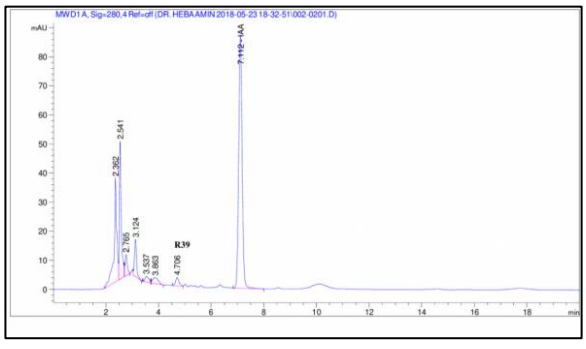


Fig. 4 IAA detection of *Brevibacillus brevis*-R39 bacterial supernatant as analyzed by HPLC.

Effect of bacterial supernatant on banana tissue culture *in vitro* propagation

In this experiment *in vitro* culture technique was applied to study the effect of *Brevibacillus brevis*-R39 bacterial supernatant (Table 4) on the micropropagation of *Musa* sp. (Grand Nain**)** in comparison to the commercial IAA.

According to data illustrated in Fig. 5, Fig. 12 the highest length of the shoot 13.67cm (11.9% over the control) was recorded with the bacterial extract at 10 ml/l (corresponding to 0.42 mg IAA), followed by 13.17 cm (7.8% over the control) with bacterial extract at 5ml/l (corresponding to 0.21 mg IAA). Meanwhile, the lowest length of shoot 11.92 cm (2.5% less than the control) was recorded in commercial IAA (0.5 mg/l). Data in Fig. 6, Fig. 12 revealed that number of leaves was significantly higher with bacterial extract treatment (at 10 ml/l) and commercial IAA (at 2 mg/l) and the lowest number of leaves was in treatment of IAA (at 4 mg/l). The effect of different treatments of bacterial supernatant and the commercial IAA on banana plantlets leaves width is illustrated in Fig. 7, Fig. 12. The greatest width of leaves of 2.83cm (24% over the control) was recorded with the bacterial extract at concentration of 5 ml/l, followed by 2.58 cm (13.2% over the control) with bacterial extract at 10 ml/l. While, the lowest width of leaves of 1.83 cm (19.7% less than the control) was recorded with bacterial extract at concentration of 40 ml/l. The highest content of chlorophyll 23.27 SPAD value (23.2% over the control) was recorded with commercial IAA at 1 mg/l, followed by bacterial supernatant at 40 ml/l (20.75 SPAD value as 9.8% over the control). However, the lowest content of chlorophyll (16.18 SPAD value as 14% less than the control) was recorded with commercial IAA at 0.5 mg/l. The effect of different treatments of bacterial supernatant and commercial IAA on the chlorophyll content of banana plantlets is shown in Fig. 8, Fig. 12.

It is clearly observed from Fig. 9, Fig. 12, that treatments of the pure IAA with concentration of 0.5 mg/l caused significant effect of number of roots. The greatest number of roots (6 as 49% over the control) was recorded with IAA at 0.5 mg/l, followed by bacterial extract at 5 ml/l (5.25 as 24% over the control) then IAA at 1 mg/l (5 as 19% over the control). On the other hand, the lowest number of roots (4 as 8% less than the control) was recorded with bacterial extract at 20 ml/l. According to data illustrated in Fig. 10, Fig. 12, the various treatments of bacterial extract caused a significant effect on root length. The greatest root length (8.29 cm, as 49% over the control) was recorded with the bacterial supernatant at 10 mg/l, followed by (6.79 cm as 22% over the control) with commercial IAA at 2 mg/l. On the other hand, the lowest root length (3.29 cm as 41% less than the control) was recorded in the bacterial supernatant with concentration of 40 mg/l. According to data exhibited in Fig. 11, Fig. 12, treatment with bacterial supernatant and the commercial IAA caused significant effect on hairy roots. The greatest score of hairy roots (3.33 as 50% over the control) was recorded with bacterial supernatant at 5 ml/l as well as commercial IAA at 0.5 mg/l. On the other hand, the lowest hairy roots score (1.44 as 35% less than the control) was recorded with bacterial supernatant at 40 mg/l and commercial IAA with concentration of 4 mg/l.

Table 4 Experimental treatments of *Brevibacillus brevis* supernatant -R39 and commercial IAA for propagation of banana tissue cultures.

Treatment	Different concentration ml/l from treatments			
	(1)	(2)	(3)	(4)
Bacterial supernatant (R39)	5 ml/l	10 ml/l	20 ml/l	40 ml/l
Concentration of IAA in bacterial supernatant (R39)	0.21 mg /l	0.42 mg /l	0.84 mg /l	1.68 mg /l
Commercial IAA	0.5 mg/l	1 mg/l	2 mg/l	4 mg/l

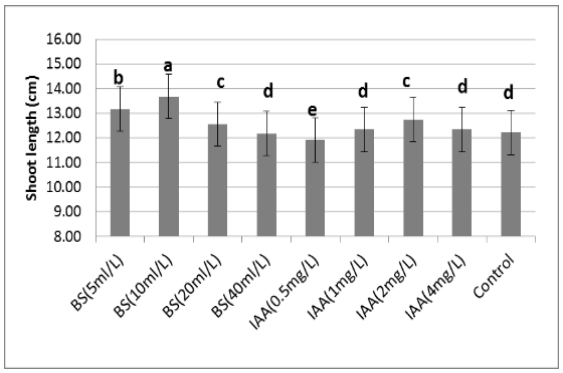


Fig .5 Effect of Brevibacillus brevis-R39 supernatant (BS) and the commercial IAA on shoot length of in vitro banana plantlets.

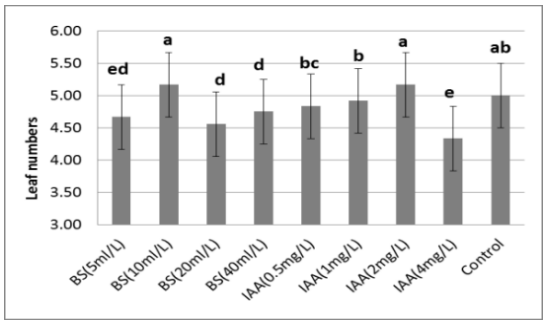


Fig.6. Effect of Brevibacillus brevis-R39 supernatant (BS) and the commercial IAA on leaf numbers of in vitro banana plantlets

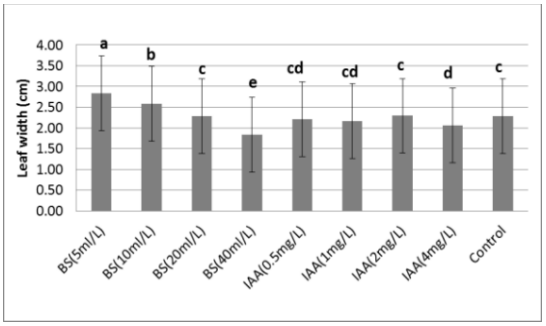


Fig. 7. Effect of *Brevibacillus brevis*-R39 supernatant (BS) and the commercial IAA on leaf width of *in vitro* banana plantlets.

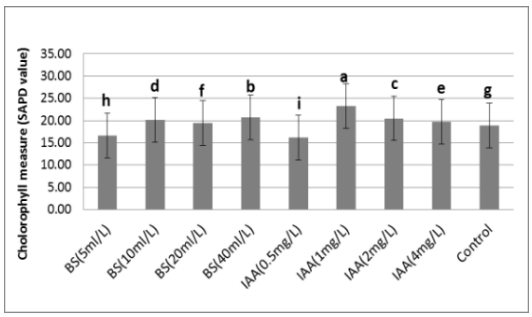


Fig. 8 . Effect of *Brevibacillus brevis*-R39 supernatant (BS) and the commercial IAA on the concentration of chlorophyll of *in vitro* banana plantlets.

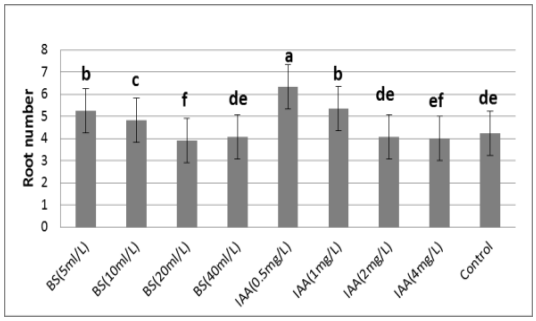


Fig.9. Effect of *Brevibacillus brevis*-R39 supernatant (BS) and the commercial IAA on root numbers of *in vitro* banana plantlets.

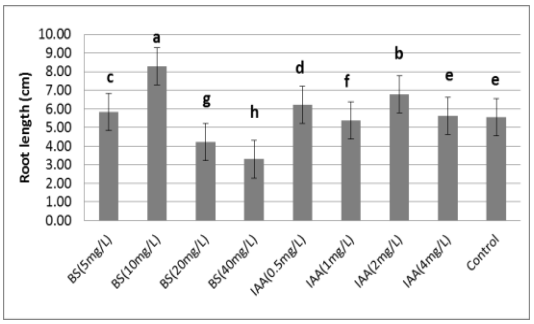


Fig.10. Effect of Brevibacillus brevis-R39 supernatant (BS) and the commercial IAA on root length of in vitro banana plantlets.

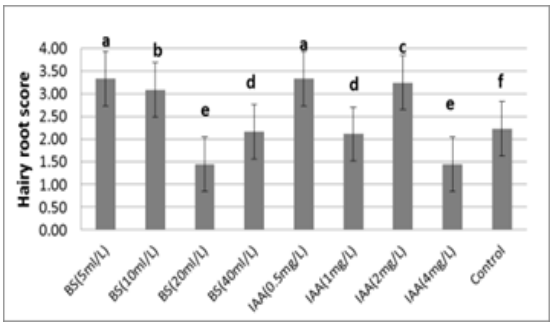
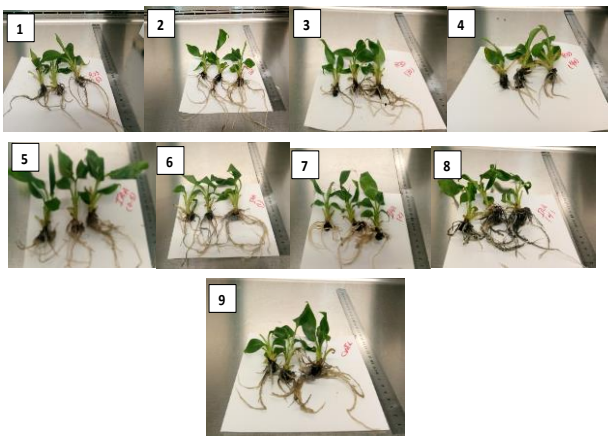


Fig. 11. Effect of *Brevibacillus brevis*-R39 supernatant (BS) and the commercial IAA on the *in vitro* hairy roots of banana plantlets.



1) 5 ml/l supernatant of *Brevibacillus brevis*-R39 **5)** 0.5 mg/l commercial IAA
2) 10 ml/l supernatant of *Brevibacillus brevis*-R39 **6)** 1 mg/l commercial IAA
3) 20 ml/l supernatant of *Brevibacillus brevis*-R39 **7)** 2 mg/l commercial IAA
4) R39 - supernatant of *Brevibacillus brevis*-R39 **8)** 4 mg/l commercial IAA
9) Control MS medium without additives

Fig.12 Effect of various treatments of bacterial supernatant of *Brevibacillus brevis*-R39 in comparison to the commercial IAA and control MS medium without additives.

Discussion

Rhizobacteria are root-associated bacteria that form symbiotic relationships with many plants. In the present study, the recorded microbial populations were found to be 4.49, 3.18, and 2.64 log CFU/g for rhizosphere, and the endophytic compartments of banana root and stem. However, other study reported that the bacterial rhizosphere population can range between 10^7-10^9 CFU/g of the rhizosphere soil [18]. After root penetration, endophytes can spread systemically to colonize above ground tissues. They can establish stem and leaf population densities between 10^3–10^4 CFU/g fresh weights under natural conditions [19].

Indole-3-acetic acid (IAA) is a key hormone for many aspects of plant growth that can regulate many physiological processes [20]. Endophytic bacteria isolated from banana plants can provide a rich source of IAA production [21]. Kafrawi et al. [22] reported that a total of rhizobacterial isolates were screened for the production of IAA and it gave about (40.8 %) by colorimetric method, while endophytic bacteria produced 57 % IAA. Padder et al. [23] reported that screening for IAA production in bacterial root endophytes were about 54.3%.

Plant growth promoting rhizobacteria (PGPR) can promote plant growth by regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients and inducing resistance against plant pathogens [24]. PGPR can interact with plants directly by increasing the availability of essential nutrients (*e.g.* nitrogen, phosphorus, and iron), producing and regulating compounds involved in plant growth (*e.g.* phytohormones), and regulate stress hormonal (*e.g.* ethylene levels by the production of ACC-deaminase) [25]. However, endophytic bacteria can prevent phosphate adsorption and fixation under phosphate-limiting conditions by assimilating solubilized phosphorus [26]. Tsavkelova et al. [27] reported that endophytic bacteria that were isolated from terrestrial orchids produced IAA.

The isolates tested for plant growth promotion belonging to the *Enterobacteriaceae* family; *Klebsiella*, *Enterobacter* and *Pantoea* genera promoted plant growth and were potential as biofertilizers [28]. Otherwise, *Paenibacillaceae* have significant potential for practical application in agriculture [29]. Some *Brevibacillus* spp. were able to *in vitro* synthesis of 3.8 mg/l IAA and this might have contributed to the beneficial effects noticed, since the production of IAA or ethylene has been suggested as a mechanism for plant growth promotion under heavy metal stress [30], *Brevibacillus brevis* produce IAA 38.16 μg/ ml with 500 μg/ ml L-tryptophan [31], our study showed that the *Brevibacillus brevis* produced 42 μg/ ml IAA. The isolate *Brevibacillus brevis* was found positive for many of the plant growth promoting like IAA, nitrogen fixation, anti-fungal activity, and ammonia production [32].

Volatile compounds produced by plant-associated microorganisms represent a diverse source to promote plant growth and health [33]. Volatile organic compounds (VOCs) produced by PGPR have recently been investigated due to their role in plant growth promotion and defense [34]. It was found that rhizobacteria resulted in producing volatile metabolites which inhibited the growth of the majority of the pathogens and fungal growth [35]**.** Many VOCs with nematicidal activity also have fungicidal activity [36], and may promote plant growth directly, through induced resistance systemic [37].

Several bioactive metabolites have been detected in the current study, as produced by biocontrol agents such as *Brevibacillus* [30]. *Brevibacillus* strains with antifungal properties are potentially valuable biocontrol agents [38]. GC-MS analysis of *Brevibacillus brevis* (R39) bacterial extract had many compounds acting as antibacterial and antifungal metabolites such as carvone, dodecanoic acid, 3-hydroxy, (E)-β-farnesene, α-copaene, β –eudesmol, dodecanoic acid (lauric acid), oleic acid, trimethylsilyl ester, octadecanoic acid, trimethylsilyl ester (stearic acid TMS derivative) and alpha-bisabolol oxide B antibiotic, methyl di hydrojasmonate act to protect the plants as antibacteria [39]. Furthermore, it has vitamins like 1,25-dihydroxyvitamin D3, TMS derivative, 1,25-dihydroxyvitamin D2, TMS derivative, vitamin A aldehyde. Vitamins can be produced by plants and bacteria and also by PGPB. The main function of vitamins is to (1) act as a cofactor in diverse metabolic pathways, (2) facilitate production of essential compounds for plants and bacteria, (3) induce resistance against pathogens, (4) directly promote plant growth, and (5) participate in energy conversion in the plant from stored compounds [40].

Studying the effect of inoculation of *Brevibacillus brevis* on shoot length and root length of cotton plant showed that root growth was positively affected

more than shoot growth. So it is suggested *Brevibacillus brevis* to be working on as a potent PGPR in cotton crop for the first time [31]. *B. brevis* may be related to its ability for improving root growth, nodule production [41]. Girish and Umesha [42] showed that *B. brevis* enhances the seed quality parameters like seed germination and seedling vigor. It promotes phenylalanine ammonia lyase (PAL) with total phenol contents, recorded for maximum disease protection under greenhouse conditions.

Conclusion

It could be concluded that the bacterial isolate *Brevibacillus brevis* has showed various direct and indirect mechanisms for plant growth promotion, including IAA production, nitrogen fixation, and extracellular many compounds act as antibacterial and antifungal. Therefore, the inoculation of banana tissue culture with culture supernatant of *Brevibacillus brevis* enhanced shoots and roots length, number of leaves, width of leaves and hairy roots compared to un-inoculated plants.

Thus, we recommend the supplementing of MS medium with the supernatant of *Brevibacillus brevis* for banana tissue culture improvement instead of the use of commercial IAA.

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