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Full Length Article

Isolation and Characterization of Free-living Nitrogen Fixing Bacteria from Balanites and Baobab Rhizosphere and their Effects on Millet Growth and Nutrient Uptake

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Abstract

The present study focused on the identification and characterization of free-living nitrogen-fixing bacteria isolated from the rhizosphere of *Balanites aegyptiaca* and *Adansonia digitata* trees as potential biofertilizer candidates. Nitrogen-fixing bacterial strains were isolated using Ashby's mannitol agar medium and characterized through morphological, biochemical and molecular analyses, including amplification and sequencing of the 16S rRNA gene. A total of thirty Composite soil samples were collected, yielding seven isolates on Ashby's medium. Morphological and biochemical evaluations identified two promising free-living nitrogen-fixing strains, one from each tree species, with potential as biofertilizer candidates. The selected strains exhibited circular morphology, milky-white pigmentation, smooth margins, motile rod-shaped structures and Gram-negative characteristics. Biochemical tests revealed positive results for catalase, urease, methyl red and nitrate reduction. Molecular characterization further identified the isolates as *Paenibacillus timonensis* and *Enterobacter hormaechei*. Experimental inoculation of millet plants with the isolates resulted in significant nutrient enhancements, with increases of 114.86% nitrogen, 165% phosphorus and 73.58% potassium in the millet shoots compared to the untreated control. Nitrogen uptake by millet was elevated by 208%, 108% and 420% for the experimental isolates and NPK fertilizer treatment respectively, relative to the negative control. Similarly, phosphorus uptake showed an increase of 257%, 100% and 171% for the isolates and NPK treatment, respectively. These findings suggest that the rhizospheres of trees found in the semi-arid and arid regions of Nigeria harbor nitrogen-fixing bacteria with considerable potential for biofertilizer development, thereby supporting sustainable agricultural practices.

Keywords: Millet; Nitrogen-fixing; Nutrient uptake; Sustainable agriculture

Introduction

Low agricultural yields and recent insecurity in North-East Nigeria are reported to have affected the livelihoods of over 30 million people (FAO 2023). This is particularly the case for inhabitants of the States of Adamawa, Borno and Yobe. It has, therefore, become necessary to seek ways not only to reduce insecurity but also to enhence crop yields. Improving yield has a wide range of dimensions. One of these is to improve the availability of soil nutrients such as nitrogen, phosphate and potassium; all of which play a crucial role in increasing soil productivity (Arsita *et al*. 2020).

Nitrogen is essential in plant cells for synthesis of enzymes, proteins, chlorophyll, DNA and RNA, thus essential for plant growth and production of food and feed (Matiru and Dakora 2004), its deficiency causes reduced growth, leaf yellowing, reduced branching and small trifoliate leaves in food crops (Wolfe *et al*. 1998; Ougham *et al*. 2005). Use of chemical fertilizers is reported to cause a decrease in the quality of agricultural land and is not eco-

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friendly (Arsita *et al*. 2020). One of the solutions that can be applied is the use of biofertilizers.

Bacteria that live freely arround the roots and in the tissues of plants, such as species of *Pseudomonas*, *Enterobacteriaceae, Bacillus*, *Azotobacter*, *Azospirillum* and *Herbaspirillum* have been reported to be capable of fixing nitrogen (Dang and Diep 2014; Aasfar *et al*. 2024; Lekhana *et al*. 2024). Free-living nitrogen fixing microorganisms have frequently been reported as plant growth promoters (Hamza *et al*. 2017; Aasfar *et al*. 2024).

Biofertilizers in the form of *Azotobacter* and other free-living nitrogen fixing bacteria increase yields with positive effects on height, weight, leaf index and corn yield (Peng *et al*. 2013). In field experiments, biofertilizer inoculation without the addition of inorganic fertilizers increased corn yields by 15–30% compared to plants that were uninoculated (Baral and Adhikari 2013). Positive effects on plants can also be due to a better balance of nutrients and improved absorption of nitrogen and other nutrients by plants (Peng *et al*. 2013). The role of nitrogen fixing bacteria in rice and maize indicates that these bacteria can be developed as biofertilizers for rice, maize, or plants which belong to the same family such as sorghum (Arsita *et al*. 2020).

Trees growing in nutrient-poor soils are reported to use various mechanisms to allow them to survive the environment. Such mechanisms include forming symbiotic relationships with microorganisms to provide nutrients like nitrogen (Högberg 2009). In this study, we hypothesized the presence of nitrogen fixing bacteria from the rhizosphere soils of *Balanites aegyptiaca* (Balanites) and *Adansonia digitata* (Baobab) growing in the arid and semi-arid soils of North-East Nigeria. The objective of this study was isolation and characterization of nitrogen fixing rhizobacteria from balanites and baobab trees and to assess the effect of the isolates on millet growth and nutrient uptake.

Materials and Methods

Nitrogen-fixing bacteria isolation

Rhizosphere soils samples were collected from balanites and baobab trees using a soil auger into sterile Ziplock bags from the North-East Nigeria states of Borno, Yobe, Bauchi, Gombe and Adamawa. A total of 15 composite samples for each of the trees from discrete locations across the States were collected and transported to the University of Maiduguri, Centre for Biotechnology for storage and analysis. The GPS coordinates of all the sites where samples were collected were recorded.

Standard microbiological techniques were used for the isolation of the total viable bacteria. Briefly, a 100 µL aliquot of each dilution (ranging from 10^{-4} – 10^{-6}) was transferred into nutrient agar medium using the pour plate method. The plates were then incubated at 28 ± 2°C for 48 h. The total bacterial count was calculated by multiplying the number of colonies per plate by the dilution factor, which is the reciprocal of the dilution.

Semi-solid Norris nitrogen-free medium (HIMEDIA) was used for the isolation of the nitrogen-fixing bacterial strains, which was indicated by the formation of a subsurface pellicle. A 100 μL aliquot of each serially diluted soil sample (10^{-4}) was transferred into the semi-solid nitrogen-free medium and incubated at 30°C for five days. The formed pellicle was sub-cultured twice to obtain a pure culture. The subsurface pellicles formed in the nitrogen-free semi-solid medium were aseptically inoculated onto Ashby's mannitol agar medium plates and incubated at 28 ± 2°C for 48 h. Pure colonies of nitrogen fixing species were obtained by sub-culturing 2-3 times on the same medium.

Characteristics of the isolates

Ashby's mannitol agar medium was used for the determination of the strain's morphology and gram staining, the morphological characteristics considered were colony shape, size, color, margin, nature of the colony and texture (Holt *et al*. 1994). The method described by Bradshaw (1992) was utilized for the Gram staining. Briefly, a clean slide and sterile wire loop were used to place a drop of distilled water at the center of the slide, followed by adding a loopful of a discrete pure colony of the isolates to create a smear. Once dried, the smear was heat-fixed by gently passing it over a Bunsen flame. The smear was then flooded with crystal violet for 30s and rinsed off. Iodine solution was applied to the slide and left for one minute before being washed off with distilled water. Acetone was then used to decolorize the smear for 20s. Safranin was added as a counterstain for one minute and then washed off. After air drying, the stained slide was examined using a Zeiss compound microscope with a x100 oil immersion objective lens.

Gillies and Dodds (1968) method was employed to determine the presence of motile organisms. Using a sterile bacteriological needle, a single pure discrete colony of each isolate was picked and horizontally stabbed two-thirds into semi-solid motility medium in a tube. The culture tubes were then incubated at 28 ± 2°C for 24 h. Gram-negative and motile isolates were subjected to further biochemical characterization. The tests were catalase, oxidase, urease, citrate, indole, nitrate reduction, gelatin liquefaction and starch hydrolysis (Harley and Prescott 2002).

16S rRNA gene amplification and sequencing

Bacterial DNA was isolated using the GDSBio Quick Bacteria Genomic DNA Extraction Kit (GDSBio, Guangzhou, China) following the manufacturer's instructions. The DNA was eluted with TE Buffer (pH 6.8) and quantified using a NanoDrop spectrophotometer (NanoDrop200C, Thermo Scientific, CA, USA). The concentration and purity of the DNA were determined based on absorbance at 260 nm and the A260:A280 ratio.

The 16S rRNA gene was amplified using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'), targeting a 1465 bp segment of the 1500 bp gene. The polymerase chain reaction (PCR) was performed with FIREPol Master Mix (Solis BioDyne, Estonia). The reaction mixture contained 0.08 M Tris HCl, 0.02 M (NH4)4SO4, 2.5 mM MgCl2, 200 mM each of dATP, dCTP, dGTP, dTTP and FIREPol DNA polymerase. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 48°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min.

Aliquots (10 μL) of PCR products were electrophoresed and visualized in 1.8% agarose gels using standard electrophoresis procedures. Partial 16S rRNA genes of isolates were sequenced on an ABI 3500Xl at Inqaba Biotech, West Africa. Finally, the 16S rRNA sequence of the isolates were compared with that of other microorganisms by way of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

Pot trial using isolated biofertilizer candidates

Soil (depth of 0-15 cm) from the Faculty of Agriculture Teaching and Research Farm at the University of Maiduguri was used for the trial. The soil characteristics included pH of 6.39, organic carbon content of 0.43%, total nitrogen of 1.30 g kg^{-1}, available phosphorus of 3.50 mg kg^{-1} and potassium of 0.14 cmol kg^{-1}. The soil sample was sieved (through a 4.0 mm) and steam sterilized at 121°C for 2 h.

Five kilograms of the sterilized soil were weighed into individual plastic pots with dimensions of 15 cm in diameter and 20 cm in height. The treatments included the isolates (T1 and T2), a positive control (NPK) and a negative control (C1), arranged in a completely randomized block design (CRD) with six replications. The test treatments were inoculated with 1×10^9 viable cells mL^{-1} of the isolates, except for the negative control (C1) and the positive control (NPK). The positive control received 200 kg N ha^{-1} in the form of urea, 60 kg ha^{-1} P in the form of single superphosphate and 60 kg ha^{-1} K in the form of muriate of potash.

Six viable millet seeds were planted in each pot at a depth of 2.5 cm according to the treatments and later thinned to four plants per pot. The pots were arranged on a bench in the screen house and watered as needed. Growth parameters, plant height and the number of leaves, were measured at two-week intervals and stem girth was measured at harvest. The experiment was concluded six weeks after sowing. After harvesting, fresh and dry weight biomass and nutrient uptake were evaluated.

Statistical analysis

The data generated from the effects of biofertilizer candidates' inoculation on millet plants were analyzed using analysis of variance (ANOVA). Treatment means were presented as Mean ± Standard Error (SE) and differences were considered significant at ($P < 0.05$). BioEdit software was used for sequence editing while alignment and phylogenetic analysis were carried out using Clustal X and MEGA 11 respectively.

Results

Free-living nitrogen-fixing bacteria isolation

A total of fifteen rhizosphere soils each of balanites and baobab from five states of North-East Nigeria were collected and screened for bacteria with nitrogen-fixing potentials on Ashby's Mannitol agar medium. The results indicate that five potential nitrogen-fixing bacteria were isolated from *B. aegyptiaca* and two from *A. digitata*. One each was isolated from Bauchi, Gombe and Borno States while Adamawa had two isolates for the balanites. The two baobab isolates were one each from Adamawa and Gombe states (Table 1).

The morphology and biochemical characteristics of the seven isolates from the two trees were studied (data not shown) and the morphology for most of them revealed circular shape, medium to small size, white milky in colour and gram negative. The isolates were biochemically positive for catalase, urease and nitrate reduction, but negative for indole and gelatin liquefaction tests (data also not shown). The morphology and biochemical tests of the isolates resulted in the selection of two potential strains one each from the two trees with biofertilizer potentials. Table 2 presents the morphology and biochemical tests results of the two biofertilizer candidates. The two candidates (Fig. 1) were all circular in shape, milky white in colour, smooth margin, motile rod bacterium shape gram negative and are likely to solubilize phosphate. The two candidates showed positive biochemical tests for catalase, urease, methyl red and nitrate reduction (Table 2; Fig. 2).

Molecular characterization of biofertilizer candidates

Table 3 shows the closest relatives of isolates BOR/BLN/002 (40) and AD/BB/005 (71) as *Paenibacillus timonensis* and *Enterobacter hormaechei* respectively based on values obtained from BLAST with NCBI against all bacterial taxid using the *16S rRNA* gene sequence. Sample BOR/BLN/002 is 91.46% identical with its closest ancestor P*aenibacillus timonensis* strain (KY286402.1), while sample AD/BB/005 is 99.90% is identical to *Enterobacter hormaechei*.

The evolutionary history of the isolate BOR/BLN/002 (Fig. 3) was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-611.97) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Heuristic searches were obtained automatically by applying Neighbor-Join and BioNJ

Table 1: Number of bacteria isolated from *B. aegyptiaca* and *A. digitata* across the States on Ashby's Manitol agar medium

Sr. No.	States	Balanites	Baobab
1	Adamawa	2	1
2	Bauchi	1	0
3	Borno	1	0
4	Gombe	1	1
5	Yobe	0	0

Table 2: Morphological and biochemical characterisation of isolates BO/BLN/002(40) and AD/BB/005(71)

Sr. No.	Test	BO/BLN/002	AD/BB/005
Morphological characterization			
1	Shape of colony	circular	circular
2	Size	medium	small
3	Elevation/surface texture	Raised/mucoid	Flat/mucoid
4	Colour	Milky white	Milky white
5	Motility	Motile	motile
6	Bacterium shape	Rod	rod
7	Gram reaction	-	-
8	Margin	smooth	smooth
9	Phosphate solubilization	+	+
Biochemical Characterization			
10	Catalase test	+	+
11	Urease test	+	+
12	Gelatin liquefaction test	-	-
13	Indole test	-	-
14	Nitrate reduction test	+	+
15	Methyl red	+	+
16	Voges proskauer	-	-
17	Citrate utilization test	-	+
18	Oxidase test	+	-

Note: + for positivity and – for negativity

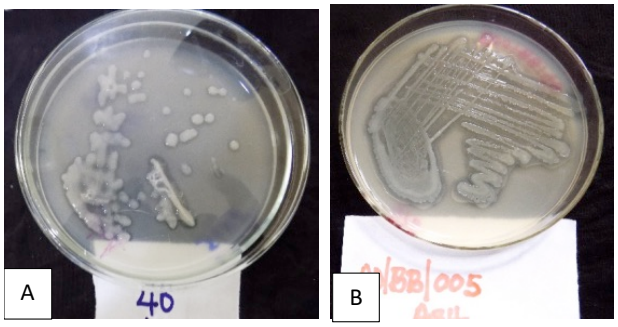

Fig. 1: Pure Free-living bacterial species from samples 40 (**A**) and 71(**B**) on Ashby's Mannitol Agar medium

algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. This analysis involved 11 nucleotide sequences obtained from the first 11 datasets with the closest ancestry with the sequence BOR/BLN/002. There was a total of 322 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

The evolutionary history of the second isolate AD/BB/005 was also inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2825.92) is shown (Fig. 4). The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree for the

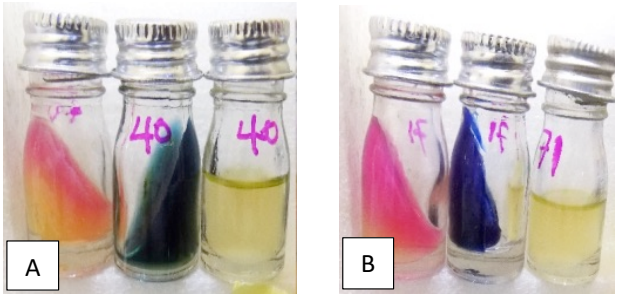

Fig. 2: Biochemical tests for isolates 40 and 71 showing urease positive, citrate negative and indole negative reactions (**A**), urease positive, citrate positive and indole negative reactions (**B**)

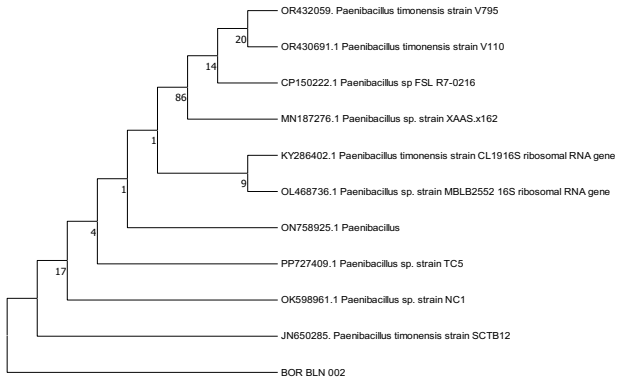

Fig. 3: Evolutionary analysis of closely related sequences obtained from NCBI the database with sequence of bacterial isolate BOR/BLN/002 isolated from *B. aegyptiaca*

heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. This analysis involved 15 nucleotide sequences, which showed the closest ancestry with isolate AD/BB/005 obtained from BLAST on NCBI. There were a total of 1044 positions in the final dataset with evolutionary analyses conducted in MEGA11 software using the 16s rRNA gene sequences.

Pot trial using isolated biofertilizer candidates

Plant growth characteristics: Table 4 presents the plant growths characteristics *viz*., plant height, number of leaves/plants, stem girth and shoot dry biomass of millet inoculated with the biofertilizer candidates (BO/BLN/002, 40 and AD/BB/005, 71) six weeks after sowing. The millets growth characteristics were not significantly affected with the biofertilizer inoculation when compared with the normal control. The plants height, number of leaves/plant and stem girth values were all statistically similar with the values obtained with those of the normal control. The dry shoot biomass of the BO/BLN/002 isolate was 4.62 g and was significantly ($P < 0.5$) higher than the weight of the dry shoot biomass seen in the control (3.25 g).

Table 3: Comparison of isolates sequences against sequences deposited in the NCBI database using BLAST

Sr. N		Accession no. of Closest relative	ID (%)	E Value	Name	Querry cover (%)
40	BOR/BLN/002	KY286402.1	91.46	3e-117	*Paenibacillus timonensis*	98%
71	AD/BB/005	PQ416037.1	99.90	0.00	*Enterobacter hormaechei*	100

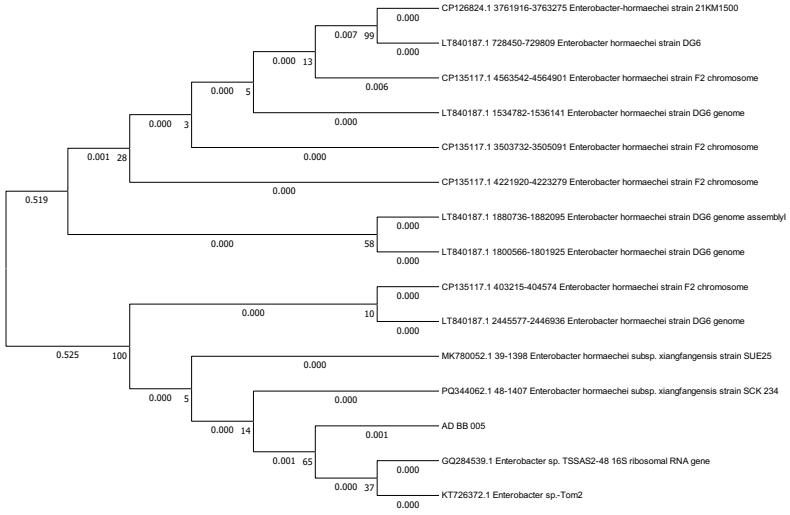

Fig. 4: Evolutionary analysis of closely related sequences obtained from the NCBI database with sequence of bacterial isolate AD/BB/005 isolated from *A. Digitata*

NPK nutrient elements content in millet plant shoot

Significantly ($P < 0.5$) higher increases in N, P and K contents in millet shoot were observed in isolates inoculated plants compared to control (Table 5). The highest values for N, P and K contents were recorded in isolate strain 40 and the lowest in the control. In all the treatments, inoculation with isolate 40 strain had an increase of 114.86% N; 165% P and 73.58% K over the control plants.

NPK uptake by millet plant in shoot

N, P and K uptake were significantly different among all the treatments (Table 6). The uptake in the test plants were significantly ($P < 0.5$) higher than the uptake recorded in the control plants. NPK chemical fertilizer treated plants had the maximum uptake of N and K compared to all the treatments. On the contrary, uptake value of P (0.025 g pot^{-1}) for 40 treated plants was significantly higher compared to all the treatments. Nutrients uptake (N, P, & K) of strain 40 inoculated plants were significantly ($P < 0.5$) higher than the uptake observed in millet plants inoculated with strain 71 plants.

Discussion

Majority of isolates examined in this study exhibited a circular morphology, medium to small size, milky-white coloration and were classified as Gram-negative. The two isolates selected for the pot trial demonstrated a circular shape with milky-white coloration, smooth margins and a motile, rod-shaped bacterial structure. These isolates were also identified as Gram-negative and showed potential for phosphate solubilization. Additionally, both isolates tested positive in biochemical assays for catalase, urease, methyl red and nitrate reduction activities.

The morphological features of circular shape, milky white colour, smooth margin, motile rod bacterium and Gram negative are features reported for many free living nitrogen-fixing bacteria (Mujahidy *et al*. 2013; Rosemary *et al*. 2013; Hamza *et al*. 2017; Ujah 2021) and they play beneficial roles of producing plant-growth promoting secondary metabolites that influence the growth of shoots, roots and seed germination of many agricultural plants (Abhang *et al*. 2024). The positive biochemical catalase tests confirm the ability of the bacteria to convert hydrogen peroxide to oxygen and water. Methyl red test showed the ability of the organisms to produce stable acids, another important feature of nitrogen fixing bacteria (Abhang *et al*. 2024). The isolates also produced positive results for both urease and nitrate reduction. A positive urease test indicates that the bacteria can produce the enzyme urease, which hydrolyses urea into ammonia and carbon dioxide while a positive nitrate reduction test shows that the bacteria can reduce nitrate (NO_3^{-1}) to nitrite (NO_2^{-1}) or other nitrogenous compounds, such as nitrogen gas (N2), a characteristic also reported for nitrogen-fixing bacteria (Arsita *et al*. 2020).

The closest relatives of isolates BOR/BLN/002 (40) and AD/BB/005 (71) are *Paenibacillus timonensis* and *Enterobacter hormaechei* respectively based on values obtained from BLAST with NCBI against all bacterial taxid

Table 4: Effect of biofertilizer on growth characteristics of millet six weeks after sowing

Treatments	Plant Height (cm)	Number of Leaves	Stem Girth (cm)	Dry Shoot Biomass (g)
Control	81.83[b]	6.17[b]	1.33[b]	3.25[c]
40	80.50[b]	6.50[b]	1.48[b]	4.62[b]
71	84.33[b]	6.50[b]	1.49[b]	3.60[c]
NPK	109.17[a]	10.17[a]	2.29[a]	11.47[a]
SE ±	5.5268	0.3944	0.1250	0.5628

Means followed by same letter within a column are not significantly different according to LSD at 5% level of probability

Table 5: NPK content in millet shoot six weeks after sowing

Treatments	N (%)	P (%)	K (%)
Control	0.74[d]	0.20[c]	0.53[d]
40	1.59[a]	0.53[a]	0.92[a]
71	1.38[b]	0.38[b]	0.80[b]
NPK	1.09[c]	0.17[c]	0.67[c]
SE ±	0.1067	0.1228	0.0527

Means followed by same letter within a column are not significantly different according to LSD at 5% level of probability

Table 6: NPK uptake in millet shoot six weeks after sowing

Treatments	N (g/pot)	P (g/pot)	K (g/pot)
Control	0.024[d]	0.007[d]	0.017[d]
40	0.074[b]	0.025[a]	0.042[b]
71	0.050[c]	0.014[c]	0.029[c]
NPK	0.125[a]	0.019[b]	0.078[a]
SE ±	0.0237	0.0053	0.0052

Means followed by same letter within a column are not significantly different according to LSD at 5% level of probability

using the *16S rRNA* gene sequence. Sample BOR/BLN/002 is 91.46% identical with its closest ancestor P*aenibacillus* timonensis strain while sample AD/BB/005 is 99.90% identical to Enterobacter *hormaechei*. Isolation and characterization of *Paenibacillus timonensis* and *Enterobacter hormaechei* as plant growth promoting rhizobacteria was previously reported (Katiyar *et al.* 2017; Lindang *et al.* 2021; Irawati *et al.* 2022) and the microbes were reportedly used in the global agriculture industry as promising biofertilizers (Yahiaoui *et al.* 2021).

After six weeks of pot trial with microbial inoculation of free-living nitrogen-fixing strains isolated from *A. aegyptiaca* and *A. digitata* the results indicated improvement of plant growth characteristics, shoot nutrient uptake and content when compared to the control. Significant increases in N, P and K contents in millet shoot were observed in the two tests inoculated plants compared to the normal and positive controls. The isolate 40 produced 114.86% N; 165% P and 73.58% K percent increases in nutrients contents over the control plants. This nutrient content increase can be attributed to the isolates ability to fix nitrogen without the requirement of any association since millet is non leguminous plant and isolates characterization indicated that the isolates maybe free-living nitrogen fixers. The percent increases in N uptake seen in the present study are 208, 108 and 420%, respectively for isolates 40, 71 and the NPK control when compared to the normal controls. The P uptake was also increased following the isolates inoculation (257% isolate 40, 100% isolate 71 and 171% NPK control). This observation suggests an enhanced nitrogen fixation and phosphorus solubilization in response to the inoculant's application, leading to improved plant growth. Similar results were reported by many researchers (Deshwal *et al*. 2003; Sadik *et al*. 2016; Hamza *et al*. 2017; Li *et al*. 2023; Aasfar *et al*. 2024; Lekhana *et al*. 2024; Zhiyong *et al*. 2024). Nitrogen fixation by means of microorganism which exist naturally in the soil plays a great role through minimizing the input and effect of chemical fertilizer in the field, positively impacting on sustainable agriculture development particularly for countries like Nigeria whose agriculture largely depend on the use of chemical fertilizers. Considering this, the present investigation revealed that nitrogen-fixing bacterial strains native to Nigeria are present in rhizospheres of trees in the country.

Conclusion

Rhizobacteria from soils of *B. aegyptiaca* and *A. digitata* were successfully isolated, characterized and tested as biofertilizers on millet growth and nutrient uptake. The increased N and P uptakes observed following isolates treatments strongly suggests the biofertilizer potentials of the rhizobacteria and has the potential to increase the efficiency and production of food crops in a sustainable manner.

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Author Contributions

AG, MPS, AA, ABM, AR, MB and KTJ planned the experiments. MB, KJ, DAI and DMA carried out the conventional microbial isolation and characterization. MB, DAI, DMA and HM run the molecular aspects. AR and KJ conducted the pot trials. AG, MPS, AR, MB and KJ wrote the manuscript and MB did the sequence analysis.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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