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ASSESSMENT OF PHYTOBENEFICIAL RHIZOSPHERIC BACTERIA ISOLATED FROM TWO NIGERIAN RICE CULTIVARS FOR PLANT-GROWTH PROMOTING ABILITIES

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SUMMARY

Rhizospheric plant bacteria present a sustainable way to encourage cheap and environmental-friendly alternatives to inorganic agrochemicals. Bacteria were isolated from the rhizosphere of two Nigerian rice varieties (OFADA and ITA 150). Assays including indole acetic acid (IAA) production, phosphate solubilization, seed germination, hydrogen cyanide (HCN) and ammonia production, and antifungal assay were conducted to identify the phytobeneficial isolates. One hundred and fifty three (153) strains were isolated while ITA 150 had higher bacterial counts (5.18 logCFU/g) as compared to OFADA (4.12 logCFU/g). Major bacteria genera observed were *Bacillus*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus* and *Escherichia*. Nine isolates out of 153 were positive for the assays *in vitro*. Seeds coated with nine isolates had germination rate that ranged from 81.48% to 100%, while vigour index ranged from 682 to 140. Thus, the nine isolates possessed multiple plant beneficial traits and could be considered as promising plant growth promoting bacteria.

Keywords: Bacteria, Nigeria, Phytobeneficial, Rice, Rhizosphere,

RICE (*Oryza sativa* L.) is a common staple food in many homes throughout the world. In Africa, rice production has greatly increased as a result of numerous government initiatives taken by several countries through increased production area and/or using high-yielding varieties and fertilizers (17). However, the deleterious effect posed by chemical

fertilizers during rice production makes it unpleasant for continuous use.

Mader *et al*. (10) reported that rice growth is promoted and protected by using plant growth promoting rhizobacteria (PGPR) and can serve as alternative to pesticides, chemical nitrogen and phosphorus fertilizers during rice production. The mechanisms used by PGPR during plant growth, include, the enhancement of plant nutrition through associated nitrogen fixation, phosphate solubilization and phytosiderophore production (16). Plant growth promoting rhizobacteria can suppress the growth of phytopathogenic agents because of antagonism and competition mechanisms they elicit, and also able to induce systemic resistance thereby promoting plant defenses (4). They also help in rhizobial and mycorrhizal symbioses (22).

In Nigeria, the use of PGPR as biofertilizers and biopesticides towards improving rice production remain unutilized despite the potential benefits in crop production and protection. However, Abiala *et al*. (1) identified some phytobeneficial indigeneous rhizobacteria using molecular methods (1). Abiala *et al*. (1) further observed that molecular characterization of bacterial isolates using 16S rRNA genes was important in determining the taxonomy of beneficial rhizobacteria and the properties of related strains prior to field application. Thus, the present study was carried out to evaluate rhizobacteria associated with the rhizosphere of two Nigerian rice cultivars (Ofada and ITA 150) for their multiple phytobeneficial effects and also to determine the phylogenetic relatedness between identified isolates.

MATERIALS AND METHODS

Soil samples and bacterial isolation

Soil samples were collected from the Fadama farm rice field of the Federal University of Agriculture, Abeokuta, where two cultivars of rice (Ofada and ITA 150) were planted. The rhizosphere soils were collected from roots of 4 weeks old rice plants at 5-15 cm depth and placed in sterile plastic bags and stored at 4ºC till usage. A 10-fold serial dilution where aliquots (100 µL) of the 10^{-6} and 10^{-8} dilutions on Nutrient Agar (NA) supplemented with cycloheximide (100 µg/mL) to inhibit fungal growth was used. The colony forming units (CFU) for each plate was estimated after incubation and morphologically different isolates were selected, purified and the NA slants stored at 4°C.

Cultural characterization of bacterial isolates

Morphological and biochemical characteristics were used to characterize the bacterial isolates (2,3). Some of the biochemical tests carried out on the isolates includes; catalase, citrate utilization, capsule staining, Voges-Proskaeur, methyl red and sugar fermentation.

Assays for phytobeneficial abilities of isolates Indole Acetic Acid Production (IAA)

The detection of IAA production was carried out by inoculating pure bacterial isolates in nutrient broth enriched with tryptophan (200 µg/mL), and incubated at 28°C for 48 h in a shaker incubator. The resultant culture was centrifuged at 4°C for 10 mins at 15000 revolutions per minutes (rpm) and two drops of orthophosphoric acid and 4 ml of the Salkowski's reagent (50 ml, 35 % of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) were mixed 2 ml of the supernatant. The light absorption of the mixture was estimated at 540 nm using a spectrophotometer, after the mixture was kept in a dark room for 20 min. The quantity of IAA produced by each isolate in µg/mL was determined by comparing the light absorption estimates to a standard curve. This experiment was carried out in triplicates for each bacterial isolate (19).

Phosphate solubilization

The Pikovskaya (1948) method for determining phosphate solubilization of the bacterial isolates was used by spot-inoculating the isolates on Pikovskaya media containing tri-calcium phosphate in a plate and incubated at 28°C for 72 h. Halo zones surrounding the spotted colonies were determined, and the phosphate solubilization efficiency (PSE), expressed as a percentage using method according to Sharma *et al*. (18).

$$\text{PSE (\%)} = \frac{Halo\ zone\ diameter\ x\ 100}{Growth\ diameter}$$

Seed germination bioassay

A bacterial concentration of 10^6 cells/mL (10^6 CFU/mL) prepared from 24 h old bacteria cultures were used to inoculate surface sterilized seeds (Ofada variety) by dipping in the bacteria suspension for 30 mins and then air dried on a laminar flow cabinet for 1 to 2 h (5). Nine bacteria coated seeds were placed on a sterilized moistened filter paper in a 9 cm diameter Petri dishes and incubated for 7 days at 28°C. Germinated seeds were counted at day 7. Germination rate, average plumule and radical lengths as well as vigour index were calculated using the method described by the International Seed Testing Agency (9).

Ammonia production

The bacterial isolates ability to produce ammonia was determined by inoculating 24 h old bacterial cultures on 10 ml nutrient broth in a rotatory shaker and incubated at 30°C for 48 h. Nessler's reagent (0.5 ml) was added to each tube and a change in colour from yellow to brown indicated a positive reaction (3).

Hydrogen cyanide (HCN) production

The Hydrogen Cyanide producing abilities of the isolates was detected by streaking each bacterial isolate on Nutrient broth amended with 4.4 g/L glycine. A 2% Sodium carbonate in 0.5% picric acid moistened Whatman filter was placed directly streaked agar plate. Plates were incubated at 28°C for four days after being sealed with parafilm and HCN production was confirmed by the change in colour from orange to red (12).

Antagonism assay against phytopathogenic fungi

A 5 mm mycelia mat of *Fusarium oxysporum* and *Rhizoctonia solani* was placed in the centre of each Potato Dextrose Agar (PDA plate. Each bacterial isolate was streaked 3 cm away from the fungi on both sides of the fungus and the plates incubated for 5-7 days at 28°C (13). The zone of inhibition was observed and the inhibition index was estimated using the formula:

$$Inhibition\ index\ (\%) = \frac{(R1 - R2)\ x\ 100}{R1}$$

Where R1=Radial growth of *F. oxysporum* in control plate, and, R2=radial growth of *F. oxysporum* interacting with antagonistic bacteria. The control was a plate with the phytopathogenic fungus only. All experiments were done in triplicates.

Ribosomal sequencing and phylogenetic relatedness of isolates

Bacteria genomic DNA was extracted from bacterial isolates with PureLink Genomic DNA kits (Invitrogen Life Technologies, CA, USA) followed by amplification of 16S rRNA gene in 25 µL reaction premix using 27F (5'-AGAGTTTGATCMTGGCTCAG–3') and 1492R (5'–ACCTTGTTACGACTT–3') primers (Lane, 1991). The conditions of PCR amplification were as follows: 15 min at 96^{o}C followed by 35 cycles of denaturing at 95^{o}C for 30 sec, annealing at 51^{o}C for 30 sec, and extension at 72^{o}C for 2 min, and a final extension at 72^{o}C for 7 min. PCR amplification was performed in a Thermocycler (Agilent Surecycler 8800, Belgium). The electrophoresis was used to resolve the amplified 1500-bp fragments on a 1% agarose gel. Sequencing was done after the PCR amplicons were purified by using the ABI 3730 Genetic Analyzer

at STAB VIDA Technologies, Portugal.

The sequenced data was cleaned on CLC 6.8.4 main workbench and aligned on ClustalX2 (21), followed by construction of Maximum Likelihood (ML) phylogenetic tree (20) using MEGA 6.0 molecular software. The 1000 replication bootstrap analyses were used to determine the confidence values for individual branches. The sequenced data were analyzed with the BLASTN program of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) to determine the genus and species of the isolates. GeneBank sequences used as reference were included in phylogeny study.

Statistical analysis

Descriptive statistics (mean and standard deviation) and Analysis of Variance (one-way) with was done using the statistical package for social sciences (SPSS) version 17.0 for Windows (SPSS, Chicago IL, U.S.A), while, significant means were separated using the Student-Newman-Keuls test.

RESULTS

Enumeration of bacterial isolates

One hundred and fifty three bacteria were isolated and identified from the rhizosphere of the two rice cultivars examined at 10^8 CFU/g. ITA 150 rice variety had significantly higher bacterial counts (5.18 $\log_{10}$CFU/g) as compared to OFADA (4.12 $\log_{10}$CFU/g) (Table 1).

Morphological and biochemical characteristics of bacterial isolates

There was significant variation in colony morphology. The colonies of the bacteria were round in shape with flat or raised elevation, undulating or smooth margin and cream to green pigmentation. Most bacterial isolates were flagellated with characteristics rod shape cell. All isolates reacted positively to Catalase and were Glucose fermenters while some of the isolates were Gram negative in reaction (Table 2).

Assays for phytobeneficial abilities of isolates IAA production ability

This assay was carried out on all 153 rhizosphere isolates. Different concentrations of pure IAA were used to prepare a standard curve and the curve was used to compute the IAA produced by the bacterial isolates assayed. Only nine isolates examined produced significant quantities of IAA ranging from 41 µg/mL to 72 µg/mL. Isolates L5 and ST1 produced the largest quantities of IAA at 72 µg/mL and 71 µg/mL respectively, while isolates. So11 and ST5 produced the least quantities of IAA at 52 µg/mL and 41µg/mL respectively (Table 3).

Phosphate solubilization

Screening of bacterial isolates for phosphate solubilization revealed variations among the identified isolates. Out of 153 isolates examined, 6 isolates solubilized tricalcium phosphate with efficiencies ranging between 128% and 200%, while 3 isolates did not solubilize the inorganic phosphate. Isolates ST1 and L7 produced the highest phosphate solubilization efficiency of 200% as compared to isolate ST7 with 128% solubilization efficiency. Isolates So14, So11 and R2 did not produce any halo zone on the Pikovskaya medium, and thus showed no phosphate solubilizing ability (Table 3).

HCN and Ammonia production

Five isolates out of 153 isolates examined produced HCN to varying degrees, while the four remaining isolates did not produce HCN *in vitro*. However, the degree of HCN production ranged from high intensity to weak intensity. Nine bacterial isolates assayed were positive for ammonia production (Table 3). Nine isolates examined inhibited *Fusarium oxysporum* and *Rhizoctonia solani*. *Fusarium oxysporum* had the highest inhibition index of 50% inhibition as observed in isolate L7, while isolate ST7 had 47.22% inhibition index. Isolate ST1 had the lowest inhibition index of 19.44%. On *R. solani*, isolate L5 had the greatest antifungal effect of 56.56% inhibition index, and isolate L7 had the lowest inhibition index of 18.18% (Table 3).

Seed germination bioassay

All nine isolates had varying degrees of influence on the germination rate of the Ofada rice variety used. While the untreated seeds of Ofada variety (Control) had the lowest germination rate of 70.37%, seeds coated with isolates So14 and ST1 had the least germination rate of 81.48%, while seeds treated with isolate R2 had the highest germination rate of 100%. The vigour index for each isolate was also computed from data obtained on the plumule and radicle lengths as well as the germination rate. It was observed that isolate R2 had the highest vigor index (682), while isolates ST5 and So14 had the least vigor index (241). All 9 isolates assayed had significantly higher vigor index values than the control (Table 4).

Molecular characterization

The genomic DNA of the nine bacterial isolates were amplified at 1500 base pairs when tested using polymerase chain reaction (Figure 1). Based on the 16S RNA gene sequencing and phylogenetic analyses, isolate So24 was identified as *Acinetobacter calcoaceticus*, So14 as *Staphylococcus saprophyticus*,

So11 as *Escherichia coli*, R2 as *Staphylococcus aureus*, ST1 as *Bacillus subtilis*, ST5 as *Pseudomonas aeruginosa*, ST7 as *Klebsiella pneumoniae*, L7 as *Citrobacter freundii* and L5 as *Enterobacter cloacae*. The percentage sequence similarity ranged between 96% and 99%. The phylogenetic tree constructed showed that the isolates were clustered into two main groups; the firmicutes and the γ-proteobacteria (Figure 2).

Table 1: Mean total heterotrophic bacterial count of the rhizosphere of different rice cultivars

Cultivar	Total heterotrophic bacterial count (10^6 log CFU/g)	Total heterotrophic bacterial count (10^8 log CFU/g)
OFADA	7.20±3.07[a]	4.12±2.44[a]
ITA 150	9.33±3.07[b]	5.18±2.44[b]

Values are mean ± standard error of mean.

Table 2: Biochemical characteristics of bacterial isolates from rice varieties.

ID	GR	CA	CP	CO	MO	IN	CI	UR	MR	VP	G	L	M	SUSPECTED ORG
So24	-	+	-	-	-	-	+	-	-	-	A	A	-	*Acinetobacter sp*
So14	+	+	-	-	-	-	-	-	-	+	A	-	-	*Staphylococcus saprophyticus*
So11	-	+	-	-	+	_-	-	+	+	-	A	A	A	*Escherichia coli*
R2	+	+	-	+	-	-	-	-	-	+	A	A	A	*Staphylococcus aureus*
ST1	+	+	+	-	+	-	+	-	-	+	A	-	A	*Bacillus subtilis*
ST5	-	+	-	-	+	-	+	+	+	-	-	-	-	*Pseudomonas aeruginosa*
ST7	-	+	+	-	-	-	+	+	-	+	A	A	A	*Klebsiella sp*
L5	-	+	-	-	+	-	+	+	+	+	A	A	A	*Enterobacter sp*
L7	-	+	-	-	+	+	+	-	+	-	A	A	A	*Citrobacter sp*

KEY: GR= Gram stain, CA= Catalase, CP= Capsule stain, CO= Coagulase, MO= Motility, 498 IN= Indole, CI= Citrate, UR= Urease, MR= Methyl-red, VP= Voges proskeur, G= Glucose, L= Lactose, M= Mannitol, A= Acid production, + = POSITIVE, - = NEGATIVE.

Table 3: Determination of characteristics associated with plant growth promotion of bacterial strains isolated from rice rhizosphere.

Isolate	IAA (µg/mL)	Phosphate solubilization efficiency (%)	HCN production	Ammonia production	*F. oxysporum* inhibition index (%)	*R. solani* inhibition index (%)
So24	65.00±0.58^{b}	138.90±9.30^{c}	++	+	22.22±0.52^{h}	45.45±0.52^{b}
So14	56.00±0.58^{e}	-	-	++	38.89±0.52^{d}	36.36±0.52^{c}
So11	52.00±0.58^{f}	-	+++	++	30.55±0.52^{g}	34.55±0.52^{d}
R2	60.00±0.58^{d}	-	-	+	36.11±0.52^{e}	29.09±0.52^{h}
ST1	71.00±0.58^{a}	200.00±15.00^{a,b}	-	+++	19.44±0.52^{i}	34.55±0.52^{e}
ST5	41.00±0.58^{g}	157.10±44.80^{b}	+	++	41.67±0.52^{c}	32.73±0.52^{f}
ST7	62.00±0.58^{c}	128.60±3.50^{c}	+++	++	47.22±0.52^{b}	30.91±0.52^{g}
L7	57.00±0.58^{e}	200.00±10.00^{a,b}	-	+	50.00±0.52^{a}	18.18±0.52^{i}
L5	72.00±0.58^{a}	183.30±27.80^{b}	+++	+	33.33±0.52^{f}	56.36±0.52^{a}

KEY: - = not detected. + = low. ++ = medium. +++ = high. Values are mean ± standard error of mean. Values followed by different letters within a column indicates significant differences according to the Student-Newman-Keuls multiple-range test ($\alpha = 0.05$)

Table 4: Beneficial effects of identified bacterial isolates on Ofada seed germination and vigour index.

Isolate	Germination rate (%)	Mean plumule (cm)	Mean radicle (cm)	Vigour index
Control	70.37±1.67^{d}	1.1±0.55^{d}	0.9±0.57^{c}	140.74±27.35^{c}
So24	81.48±2.94^{c}	1.63±0.12^{c}	1.45±0.13^{d}	250.96±10.84^{d}
So14	96.29±4.00^{b}	2.3±0.85^{b}	2.61±0.18^{b}	472.78±41.31^{c}
So11	96.29±4.00^{b}	2.3±0.85^{b}	2.61±0.18^{b}	472.78±41.31^{c}
R2	100.00±5.00^{a}	3.91±0.20^{a}	2.91±0.51^{b}	682.00±71.53^{a}
ST5	85.19±8.01^{c}	1.38±0.16^{d}	1.45±0.00^{d}	241.09±4.81^{d}
ST1	81.48±2.94^{c}	1.72±0.04^{c}	1.58±0.16^{d}	268.88±8.03^{d}
ST7	96.29±4.00^{b}	2.34±0.09^{b}	2.61±0.18^{b}	476.64±30.51^{c}
L7	96.29±4.00^{b}	2.63±0.04^{b}	3.78±0.38^{a}	617.22±22.13^{b}
L5	81.48±2.94^{c}	1.8±0.30^{c}	1.85±0.85^{c}	297.40±49.37^{d}

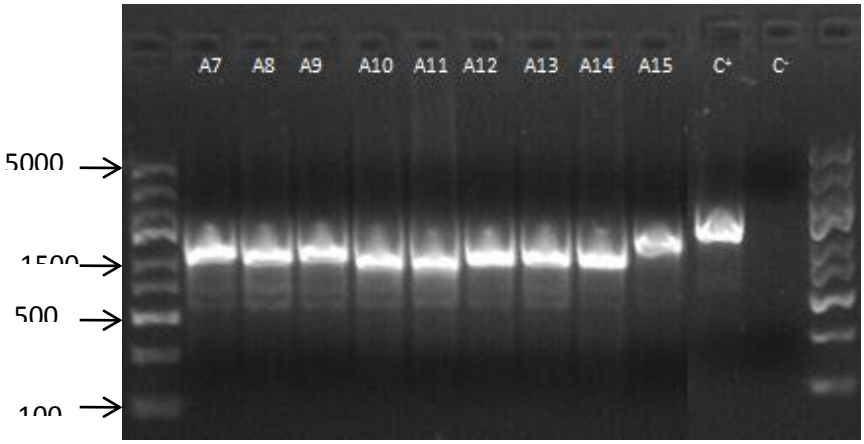

Plate 1: Gel electrophoresis of the amplified PCR profiles of bacterial isolates run in a 1% agarose gel using 2 µL of PCR product and 5 µL of DNA ladder. A7 represents isolate L5, A8 represents isolate L7, A9 represent isolate So14, A10 represents isolate R2, A11 represents isolate ST5, A12 represents isolate So24, A13 represents isolate ST1, A14 represents isolate So11, and A15 represents isolate ST7. C^+ represents positive control and C^- represents negative control (Distilled water).

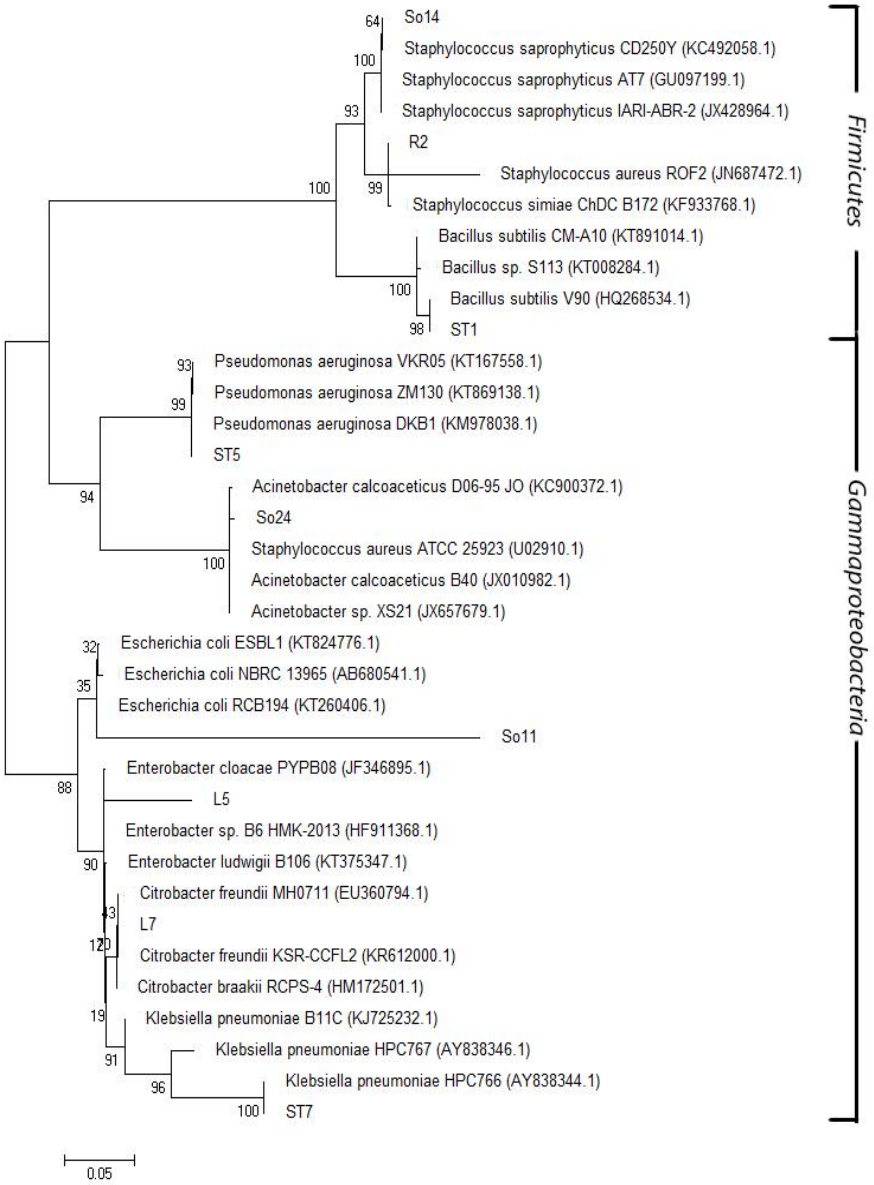

Figure 1: Phylogenetic relationship of bacterial isolates based on 16S rRNA genes and inferred using the maximum likelihood method. Type strains used for comparison are given. Numbers above each node are bootstrap confidence levels (expressed as percentages) generated from 1000 bootstrap trees. The GeneBank accession number is given in parentheses for each organism.

DISCUSSION

The evaluation of rhizosphere bacteria of rice plant with potential of improving its growth and overall cultivation was undertaken in this study. The total heterotrophic bacterial counts of the rice cultivars examined showed significant differences as ITA 150 possessed the higher bacterial counts as compared to OFADA. This difference could be due to the quality and quantity of the metabolites from plant roots. Haichar *et al*. (8) reported that the constituents of a metabolite depend on the varieties, the length at which it is stressed, the development stage and possibility of differences on the root structure which could be as a result of differences in the bacterial community composition.

The dominant bacteria genera identified using morphological and biochemical tests were *Acinetobacter, Bacillus, Pseudomonas, Klebsiella, Enterobacter, Staphylococcus, Citrobacter* and *Escherichia* and this is in agreement with studies of Mwajita *et al*. (11) and Gopalakrishnan *et al*. (7). Gram negative bacteria were in greater abundance in the rhizosphere of the rice varieties examined as compared to Gram positive bacteria and this confirmed the earlier report of Elbeltagy *et al*. (6) and that of Ngoma *et al*. (12).

In this study, all bacteria tested produced IAA in the presence of tryptophan, although at different concentrations and suggested to be IAA producing rhizobacteria and this is in agreement with earlier report that root elongation and plant growth are promoted by IAA-producing bacteria (15). Isolates ST5 and L5, corresponding to *Pseudomonas aeruginosa* and *Enterobacter* sp. produced the largest concentration of IAA (72 µg/mL and 71 µg/mL respectively) while *Bacillus* sp. produced the lowest concentration of IAA at 41 µg/mL. This result has implication on the growth of rice on lateral and adventitious root formation, primary root growth and primary root elongation as reported by Xie *et al*. (28).

This study also identified 6 bacteria; *Pseudomonas aeruginosa*, *Citrobacter* sp. *Klebsiella* sp. *Enterobacter* sp., *Bacillus* sp. and *Acinetobacter* sp. that were able to solubilize tri calcium phosphate (Ca_3PO_4) *in vitro* (23,24) with solubilization efficiencies ranging from 136 % to200 %. Earlier report suggested that many agricultural soils were low in phosphorus which is an essential plant macronutrient which these bacteria would be able to release through solubilization (24). On the other hand, *Staphylococcus saprophyticus, Escherichia coli* and *Staphylococcus aureus* showed no

halo zone on the Pikovskaya agar plates and thus no phosphate solubilization efficiency.

This study observed that all bacteria improved seed germination rate when compared with the control. The nine bacterial improved the growth parameters (plumule, radicle and vigour index) of the inoculated rice seeds as compared to the uncoated control. The ability of certain bacterial to increase seed emergence, vigour index and other plant growth parameters suggests an increased production of IAA and other phytohormones and were implicated in nutrient acquisition by plants. It was also observed IAA could promote the activity of specific enzymes that ensures early germination with increased plumule and radicle length. Early seedling establishment is possible through seed inoculation with IAA-producing rhizobacteria.

Production of HCN was also observed with *Citrobacter* sp. and *Klebsiella* sp. while *Staphylococcus aureus, Staphylococcus saprophyticus, Pseudomonas aeruginosa* did not produce it. The production of ammonia by some of the bacteria is also an important trait of plant growth-promoting bacteria and this strengthens plant disease resistance mechanism (26,29). The ability of these bacteria to produce HCN has indirect influence on plant growth (25).

The inhibition index of *R. solani* ranged from 18% to 56% as *Enterobacter* sp. was highly antagonistic to *R. solani*, while *Citrobacter* sp. was not least effective. Inhibition index against Fusarium ranged from 19% to 50% as *Citrobacter* sp. was also highly antagonistic to this pathogen. The differential rates of fungal inhibition by the bacteria species indicates that rhizobacteria differ from one another through the effects on hosts and kinds of antifungal substances produced (27). This is also in agreement with Noumavo *et al*., (14) that antifungal metabolites and or lytic enzymes produced by PGPB might be responsible for the *in vitro* inhibition zones and reduced fungal growth by certain PGPR.

In conclusion, this study was successful in screening bacterial isolates from rice rhizosphere possessing multiple plant growth-promoting traits, However, of the nine isolates, isolates L7, ST7 and R2 corresponding to *Citrobacter freundii, Klebsiella pneumoniae* and *Staphylococcus aureus* showed the best abilities to promote plant growth *in vitro* as seen by the results of the various assays conducted, and this

suggests the possibility of being used as inoculant for plant growth development. Further studies are needed *in vivo* to ascertain the phytobeneficial abilities of some of the bacteria used in this study.

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