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ISOLATION, IDENTIFICATION AND PHYLOGENETIC CHARACTERIZATION OF POTASSIUM-SOLUBILIZING RHIZOBACTERIA ISOLATED FROM THE ROOTS OF *Mimosa indica* WEED

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ABSTRACT

Most microorganisms residing within plant rhizosphere are of great importance to soil fertility. The either release plant nutrient into soil solution and make it available for plant absorption directly or indirectly, excrete enzymes or organic acids that facilitate the solubilization of insoluble minerals, such as potassium (K). A laboratory analysis was carried out at the Soil Science Laboratory in Michael Okpara University of Agriculture, Umudike, to isolate potassium-solubilizing rhizobacteria from *Mimosa indica* a weed that have invaded most agricultural land in the southeastern Nigeria. It was observed that the roots of *Mimosaindica* had nodules, which is peculiar to legume plants. Two cultivable isolates possessed the ability to solubilize K. DNA extraction was done and the two bacteria were identified as: *Ochrobactrum anthropi* and *Comamonas testosteroni*. Biochemical tests and Phylogenetic characterization were carried out. *Comamonas testosterone* gave the highest solubilization efficiency of 650 compared to *Ochrobactrum anthropi* (325).

Keywords: DNA Extraction, *Mimosa indica*, Plant-growth promoting rhizobacteria, Potassium-solubilizing rhizobacteria, Solubilization efficiency

INTRODUCTION

Soil fertility issues are drawing considerable attention to farmers and soil science experts all over the world. It has become a known fact that the pressure mounted on agricultural soils to increase crop production has resulted to deficiencies of plant nutrient elements. The trend is so challenging especially now, that human and animal feeds are in high demand. As farmers are faced with the challenge to produce enough food for man, on the other hand the same resources are used to provide feed for animals. Due to the important roles potassium play in crop production, research is still on-going to find out way of harnessing the imbedded insoluble potassium stored in the soil that are not accessible by plants (Zhang and Kong, 2014). It has become needful to access biological approaches that have the potential to restore a vibrant soil ecosystem and at the same time increase nutrient availability in the soil solution. Most researches are geared towards utilization of beneficial microbes as biofertilizers. This has also gained much attention in the present time. For instance, it has been revealed that some soil bacteria can solubilize insoluble potassium and make it available for plant roots to take in (Zhang and Kong, 2014). The mechanism of solubilization is fairly complex. One mechanism these bacteria adopt is by excreting organic acids that can dissolve the insoluble K stored in soil (Friedrich *et al*., 2004).

A quick attention is drawn to some weeds that thrive well in arable lands and have their external feature well-flourished without application of chemical fertilizer. It is suspected that specialized bacteria may be residing within their rhizosphere (Baber *et al*., 2018 and Nwokeh *et al*., 2022) and these weeds might be housing microbes which possess the capability to harvest K for their growth and development. These potassium-solubilizing bacteria can be isolated from plant rhizosphere, where organic acids or substance are released to facilitate K-solubilization. They are specific in their activities. In order to exploit the K-reservoir in soil, many bacteria have been isolated from specific crops to test their ability for K-solubilization.

Mimosa sp. is one weed that easily invades many cultivated lands where some crops have not produced bomber yield. It is a leguminous weed with shallow root system. It grows in many soils in the southeastern Nigeria and has become quite difficult to eliminate completely. Like other legumes, it has the capacity to fix nitrogen in the soil, even for its growth and development. The need to explore the rhizosphere of *Mimosa* weeds becomes imperative. It is suspected that some strains of bacteria species, residing within the rhizosphere of such weeds may be responsible for the mining ability insoluble K already stored in the soil. Based on this, the objective of this work is to isolate and identify potassium-solubilizing bacteria resident in the rhizosphere of *Mimosa* plant.

MATERIALS AND METHODS

Collection of root samples

Mimosa indica plant was carefully harvested from the research farm of the College of Crop and Soil Sciences in Michael Okpara University of Agriculture Umudike. The roots were aseptically cut out using a sterilized razor blade. 1 gram of the root sample placed in a sterile bottle and 10 ml of sterile water was measured into the bottle containing the root sample. After shaking the mixture properly, 1 ml of the stock solution was pipette into 9 ml of sterile water into the first test tube labeled 10^{-1}. Another 1 ml, taken from the first test tube was transferred into a second test tube (10^{-2}) containing 9 ml of water. This was repeated until the fifth test tube.

Preparation of culture substrate (Aleksandrov medium)

Aleksandrov medium was prepared using the following composition: Magnesium sulphate ($MgSO_4.7H_2O$) (0.50g), Calcium carbonate ($CaCO_3$) (0.10g), Potassium alumino silicate (3.00g), Glucose (5.00g), Ferric chloride (FeCl) (0.006g), Calcium phosphate ($Ca_3(PO_4)_2$) (2.00g), Agar (20.00g). One liter (1000 ml) of distilled water was added to the chemicals measured and the final pH will be adjusted to about 7.2±0.2 by adding 1N NaOH (25°C). The composition was heated to boiling to dissolve the mixture completely. The mixture was sterilized by autoclaving at 121°C for 15

minutes. It was then allowed to cool at about 45°C, before dispensing into sterile Petri dishes containing the serially diluted samples.

Isolation of KSR from *Mimosa indica* roots

The pure cultivable rhizobacteria cultures were spot-inoculated on Aleksandrov media containing potassium aluminosilicate as sole source of K (Sugumaran and Janartham, 2007). The cultures were incubated at 28°C for 72 hours. After incubation, the plates were checked for formation of clear zone which is an indication of K-solubilization. The diameters of the solubilization zone (clear zone) were measured in millimeter (mm) and the values recorded.

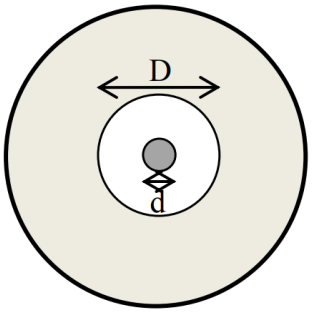

Figure 1: Diagram illustrating clear zone of solubilization by bacteria isolate

Potassium Solubilizing Indices

Solubilization efficiency was calculated using the formular below:

$$\text{Solubilization Efficiency} = \frac{Solubilizationdiameter\ (mm)}{Growthdiameter\ (mm)} \times 100 \quad (1)$$

Secondary screening was carried out on the basis of zone activity of the different isolates by using Khandeparkar's selection ratio.

$$\text{Khandeparkar's selection} Ratio = \frac{D}{d} = \frac{Diameterofzoneofclearance}{Diameterofgrowth} \quad (2)$$

Where, *D* = diameter of zone of clearance and

d = diameter of growth of colony

$$\text{Potassium solubilization index (KSI)} = \frac{Colonydiameter+Halozonediameter}{Colonydiameter} \quad (3)$$

Biochemical tests conducted on KSR isolates from roots of *Mimosa indica*

Cultural, morphological and biochemical tests were conducted on the two isolates. The following selected biochemical tests were conducted; Mannitol, Triple Sugar Iron, Fructose, Galactose, Raffinose, Glucose, Sorbitol, Arginine, Mannose, Arabinose, Lactose and Salign were determined for both isolates.

Molecular identification and characterization of isolates

Genomic DNA of the isolates were extracted using column-based JENA Bioscience Bacteria DNA Preparation Kit. Bacteria cells were harvested from 500µl aliquot of bacteria broth culture using a microcentrifuge at 10,000 g for 1min. The residual pellet was resuspended in 300µl of Resuspension Buffer and 2µl of Lysozyme Solution. The mixture was homogenized by inverting several times thereafter incubated at 37°C for 1 hour.

Re-suspended cells were recovered by centrifugation and lysed by adding 300µl of Lysis Buffer after which 2µl RNase A and 8µl proteinase K solution were added; followed by incubation at 60°C for 10mins. The tube was cooled on ice for 5 minutes. 300µl binding buffer was added to the mixture and vortexed briefly; the mixture was cooled on 5 minutes and thereafter centrifuged at 10,000g for 5 minutes.

The supernatant was transferred directly into the spin column and centrifuged at 10,000g for 1 minute to trap the DNA. The trapped DNA was washed twice with washing buffer after which it was eluted with 50µl elution buffer into a clean eppendorf tube.

Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start mastermix), 1µl (10pmol) each of forward primer 27F 5'AGA GTT TGA TCM TGG CTC AG3' and a reverse primer 1492R-5' TAC GGY TAC CTT GTT ACG ACT T 3', 1 µl DNA template and 9.5 µl sterile nuclease free water to make up a total reaction volume of 25 µl.

PCR amplification was carried out in an Applied Biosystem 2720 Thermocycler. The mixture was subjected to an initial denaturation at 94°C for 3min; followed by 35 cycles denaturation of 94°C for 45s , annealing at 55°C for 60s and extension at 72°C for 60 seconds; and a final extension of 72°C for 10 minutes.

PCR products were visualized on a 2 % agarose gel containing ethidium bromide in 0.5x Tris-borate buffer (pH 8.0) using blue led transilluminator (image attached). A molecular ladder marker (Jena Bioscience, 200bp) was run simultaneously to determine the size of the amplicons.PCR products were purified and sequenced by Sanger sequencing method using AB1 3730XL sequencer and done by Inqaba biotec, Pretoria, South Africa.

RESULTS

Agarose Gel Images

The identities of the isolates were confirmed using PCR parameters. Ladder marker was ran simultaneously with the samples to determine the length of the amplicons. The obtained sequences were submitted to BLAST to find similar 16S rDNA homologous sequences. The sequences in the GenBank were compared with the obtained sequences of 16S rDNA gene of the isolates. The results revealed that M1 and M4 possessed 75.85% and 82.84% similarities to *Ochrobactrum anthropi* and *Comamonas testosteroni* strains respectively (See Table 1).The sizes of the bands of both isolates on agarose DNA gel fall within 1500pb (See Figure 2).

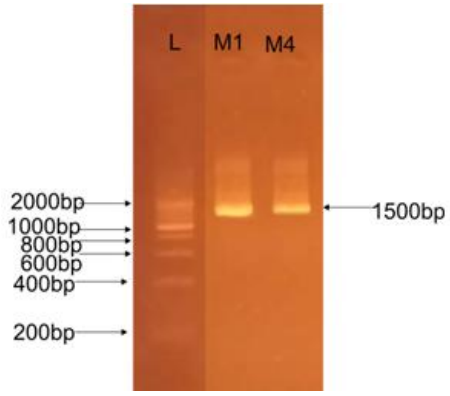

Figure 2: Electrophoresis results of PCR products from M1 andM4isolates

Table 1 shows the identification of isolated KSR by 16S rDNA partial sequencing. The length of the M1 gene sequenced had a value of 1479 while that of M4 gave a value of 1533. The percent identity and length of 16s rDNA gene sequenced was higher in M4 (See Table 1).

Table 1: Identification of Isolated KSR by 16s rDNA Partial Sequencing

Isolates	Length of 16s rDNA gene sequenced	Most closely related bacteria strain	Percent Identity (%)
M1	1476	*Ochrobactrum anthropi*	75.85
M4	1533	*Comamonas testosteroni*	82.84

Assessment of the solubilization ability

Plate assays were carried out, and based on observations of the zones of solubilization around the colonies in Aleksandrov medium (which contains potassium aluminosilicate as a source of insoluble potassium), the qualitative estimations of solubilized K was determined. The isolated rhizobacteria were tested for their abilities to dissolve K in Aleksandrov medium (potassium aluminosilicate is a source of insoluble K).

From the bacteria culture plates, the two colonies (isolate *Ochrobactrum anthropi* and isolate *Comamonas testosteroni*) showed the formation of clear zones after 72 hours indicating potassium solubilization. The clear zones were measured in millimeters. The solubilization diameters and growth diameters (colony diameter) were determined as shown in Table 2. Figure 3 shows the images of K-solubilization in Aleksandrov agar medium.

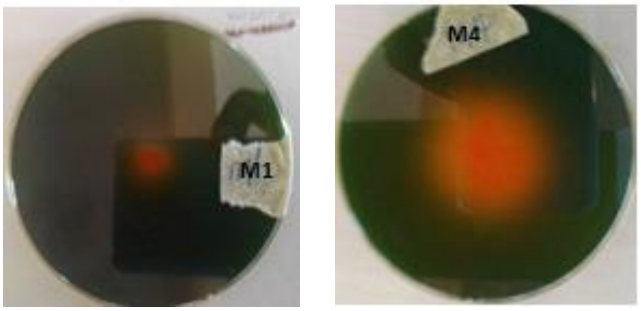

Figure 3: Zones of Solubilization of Potassium-Solubilizing Rhizobacteria isolated from *Mimosaindica* weed

Comamonas testosteroni strain gave a higher solubilization diameter of 26.00 mm which was double the solubilization diameter of *Ochrobactrum anthropi*strain (13.00 mm). However, the colony diameters of both strains were the same (4.00 mm).

Following the assessment of solubilization ability, solubilization efficiency was observed to be higher at isolate *Comamonas testosteroni*, with the value of 650 as against isolate *Ochrobactrum anthropi* (350). KSR was also observed to be higher at isolate *Comamonas testosteroni* (6.50) as against isolate *Ochrobactrum anthropi* (3.25). However, isolate *Ochrobactrum anthropi* demonstrated a higher potassium solubilization index (KSI) of 1.30 than isolate *Comamonas testosteroni* (1.15).

Table 2: Potassium Solubilizing Indices of Isolated Potassium-Solubilization Rhizobacteria

Isolates	Solubilization diameter (mm)	Growth diameter (mm)	SE	KhSR	KSI
Ochrobactrumanthropi	13.00	4.00	325	3.25	1.31
Comamonastestosteroni	26.00	4.00	650	6.50	1.15

Note: SE=Solubilization efficiency, KhSR= Khandeparkar's selection ratio, KSI= Potassium solubilizing index

Biochemical Tests of Potassium-solubilizing rhizobacteria isolated from the rhizosphere of *Mimosa indica*

The biochemical tests carried out included mannitol, triple sugar iron, fructose, galactose, raffinose, glucose, sorbitol, arginine, mannose, arabinose, lactose and salign tests (Table 3). *Ochrobactrum anthropi* showed positive in mannitol, glucose and mannose tests. While the other biochemical tests came out negative. *Comamonas testosteroni* showed positive in Arginine test alone.

Table 3: Biochemical Tests of Potassium-solubilizing rhizobacteria isolated from the rhizosphere of *Mimosa indica*

Biochemical Test	*Ochrobactrum anthropi*	*Comamonas testosterone*
Mannitol	+	–
Triple Sugar Iron	–	–
Fructose	–	–
Galactose	–	–
Raffinose	–	–
Glucose	+	–
Sorbitol	–	–
Arginine	–	+
Mannose	+	–
Arabinose	–	–
Lactose	–	–
Salign	–	–

Phylogenetic analysis of KSR isolates from *Mimosa indica*

The evolutionary histories of the isolates were inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Jukes *et al*., 1969). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Evolutionary analyses were conducted using MEGA X (Kumar *et al*., 2018). The 16S rDNA sequences of the two strains were compared to those of known 16S rDNA sequences using BLAST and GenBank database. These analyses involved 23 nucleotides (*Ochrobactrum anthropi*) in Figure 4, and 22 nucleotide (*Comamonas testosteroni*) sequences in Figure 5. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 690 positions for *Ochrobactrum anthropi* and total of 759 positions for *Comamonas testosteroni* in the final datasets.

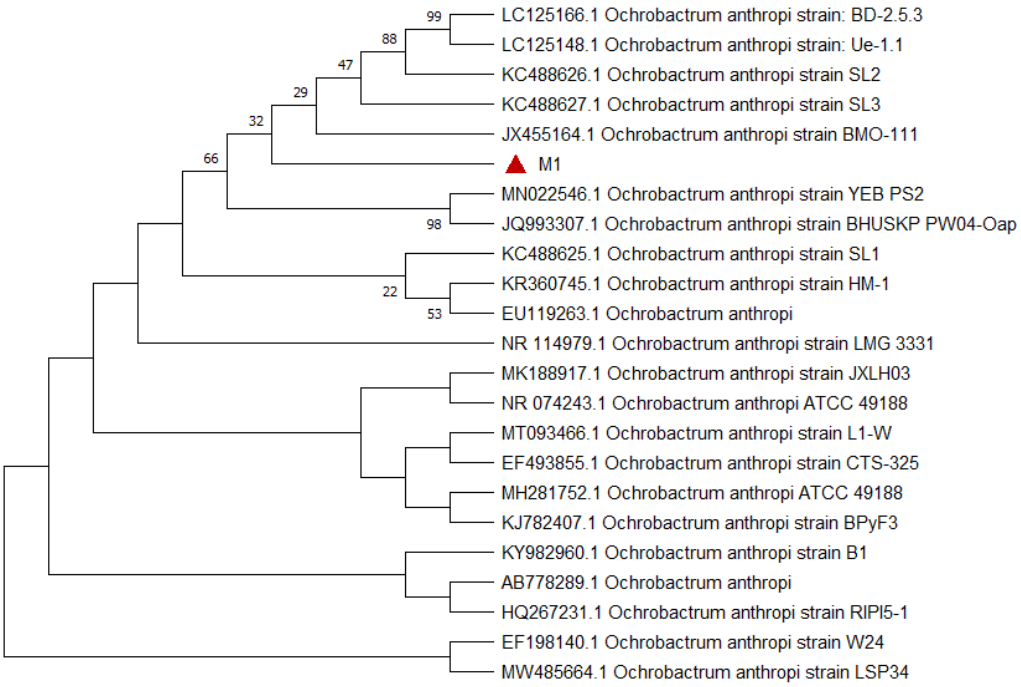

Figure 4: Genetic relationship of selected 16s ribosomal RNA nucleotide sequences of bacteria genus *Ochrobactrum*.

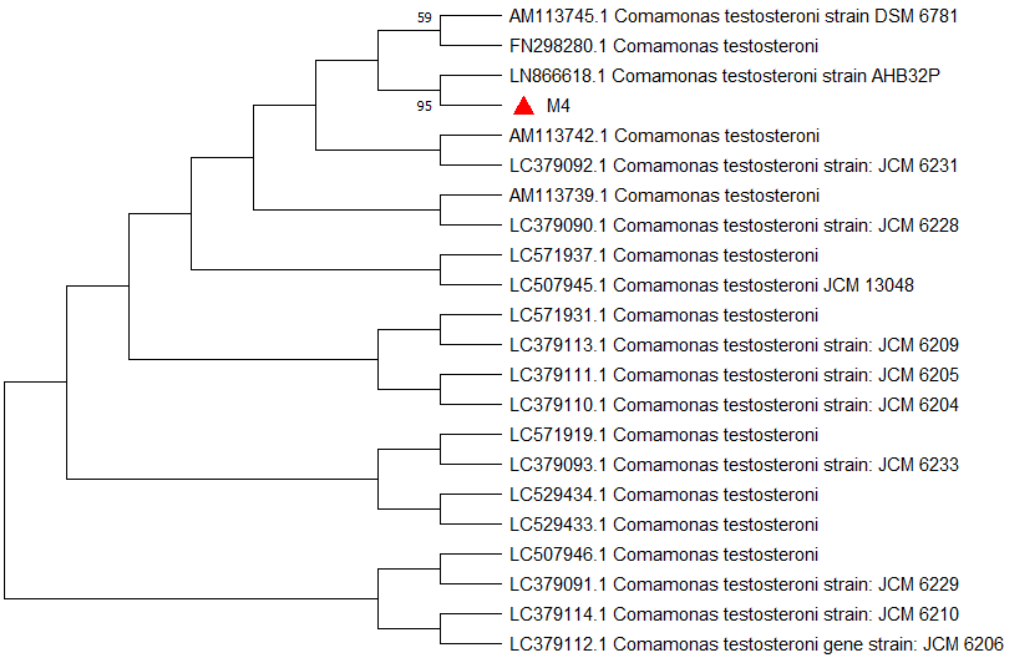

Figure 5: Genetic relationship of selected 16s ribosomal RNA nucleotide sequences of bacteria genus *Comamonas*

DISCUSSION

The use of some beneficial soil bacteria as biofertilizers is generating interest as a strategy against rampant application of chemical fertilizers, this is because of the consequential effect on soil health. In this study, the rhizobacteria isolated from roots of *Mimosa indica* is an indication that some weeds could be a source of biofertilizer production for certain plant element. Pramanik *et al*. (2018) reported that potassium-solubilizing bacteria could be adopted as a strategy for effective soil management. Some species of *Bacillius* strains have been identified as potassium solubilizers (Meena *et al.,* 2016). As biofertilizers, Meena *et al*. (2014) mentioned that the functions of most KSR may not be just the dissolution of insoluble K, they can also produce hormones such as gibberellins, cytokinin and some organic acids which can affect the performance of plants and as well enhance nutrient absorption (Pastor-Bueis *et al*., 2017)

Saveli *et al*. (2010) and Ashraf (2016) documented that *Ochrobacterum anthropi*, a gram-negative bacterium and a non-fermenting, obligate aerobe flourish in the rhizosphere and the soil. They also reported that *Ochrobactrum* spp, bacteria are flagellate, oxidase-positive and indole-negative. They can also be isolated from contaminated biological products, such as human wastes, fluids and medical devices.

The mechanism of solubilization is an important aspect of this biotechnology. The amount of organic acid and other metabolites may affect the solubilization diameter of each zone of solubilization.

CONCLUSION

Modern techniques in biotechnology are recently been employed to address the issues surrounding nutrient availability in soil solutions. These approaches are aimed at minimizing or eliminating the frequent application of chemical fertilizers for increased crop production as well environmental and soil sustainability. Generally weeds are referred to as unwanted plants, but this study reveals that rhizosphere of certain weeds might be effective in restoring biological systems of the soil. They may be a very efficient source where beneficial bacteria can be harvested and produced as biofertilizers; since rhizospheres of such plants accommodate the plant-growth promoting microbes. If the composition of their rhizosphere is well explored, may aid in the production of efficient biofertilizers. It has been noted in several works, that about 98% of potassium in soil is not accessible by plants. This makes it difficult for plant roots to assimilate. The amount of insoluble K that can be converted to soluble K fraction within short period of microbial activities needs to be properly understood. It might be possible to manipulate the internal properties of potassium-solubilizing bacteria, for effective and efficient K-solubilization

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