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Isolation, characterization and assessment of phosphate and potassium solubilizing microbes from agricultural soil in Imo state Nigeria.

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Abstract

The environmental consequences of chemically applying nitrogen, phosphorus, and potassium necessitate the development of biological alternatives. Garden soil isolates were screened for phosphate and potassium solubilizing using Pikovskaya's agar and Aleksandrov medium respectively. The promising positive isolates were identified to molecular level. The results obtained were as follows: 145 discrete colonies were obtained The total bacterial isolates were 111 while 34 were fungal. Potassium solubilizing (KSM) isolates were 20 (13.8%) while 39 (26.8%) were phosphate solubilizing (PSM). Phosphate solubilizing index (PSI) and potassium solubilizing index (KS1) range from 109.1 to 190 and 115.4 to 180 respectively. Isolates with PSI and KSI ≥140 were preliminarily identified as *Pseudomonas* sp, *Bacillus* sp, *Micrococcus* sp. *Aspergillus* sp and *Penicillium* sp. The use of these isolates biofertilizers may successfully substitute the use of chemical fertilizers and ensure environmental conservation.

Keywords: phosphate, potassium, solubilizing microbes, KSM, PSM, PSI.

1. Introduction

The introduction of the use chemical fertilizer in agriculture came as a result of the Green Revolution (Pingali 2012). The concept of chemical fertilizer is the chemically compounding of nitrogen, phosphorus, and potassium in various proportions for application in agriculture.

Phosphorus (P) is the second limiting nutrient required in right amount for proper plant growth and development (Nesme *et al.*, 2018). Phosphorus plays essential biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant (Nesme *et al.*, 2018). An adequate supply of phosphorus in the early stages of plant growth help to promote physiological functions including early root formation; it is important in the development of reproductive parts of plants and the formation of seed. It is important for plants to survive stressors (Balamurugan *et al.*, 2010). The soil P reserves are largely insoluble and unavailable to plants. Phosphorus solubilizing microbes (PSM) can promote the level of plant-absorbable phosphorus (P) in agro-ecosystems.

Potassium (K) is the most abundant cation in plant cells. it is the second most abundant nutrient after nitrogen in the leaves (Basak and Biswas, 2010). Most of the potassium in soils exists as insoluble rocks and minerals such as micas, illite, feldspar, and orthoclases. K participates in nutrient transportation and uptake and also confers resistance to both abiotic and biotic stresses. It leads to enhanced production of crops of quality and aids in disease resistance in plants.

Potassium is the third essential nutrient required by plants. It is involved in numerous biochemical and physiological processes in plants like stomatal regulation for plants depend upon K to regulate the opening and closing of stomata (Nwankwo and Onyewenjo, 2005). Proper functioning of stomata is essential for photosynthesis. The activation of enzymes by K and its involvement in adenosine triphosphate (ATP) production is important in regulating the rate of photosynthesis, sugars produced in photosynthesis must be transported through the phloem to other parts of the plant for utilization and storage.

The aim of this work is to isolate, characterize and assess the phosphate and potassium solubilizing and nitrogen fixing microbes from agricultural soil in Imo state Nigeria.

2. Materials and Methods

2.1 Sample Collection

Forty rhizospheric soil and 40 roots sampled, 10 each from *Hibiscus esculentus*(okro), *Manihot esculenta*(cassava), *Musa paradisiaca* (plantain), and *Zea mays*(maize) plants. from farm lands located at Ihiagwa-Owerri, latitude $5^{o}25'26"$N, longitude $7^{o}1'31"$E. The samples were aseptically dug out from the depths 10-30 cm into sterile polythene bags (Philippot et al., 2012). The samples were transported to the laboratory at 4^{0}C temperature.

2.2 Sample Preparation and Microbial Isolation

Ten soil samples of each crop were thoroughly mixed to form a composite sample. Ten grams of each composite sample were transferred to 250 ml Erlenmeyer flask containing 90ml sterile distilled water and shaken at 150 rpm in an orbital shaker incubator for 30 min. The dilution was used for ten-fold serial dilution and an aliquot (0.1ml) of dilution 10^{-2} was utilized for spread plate inoculation of nutrient agar (NA) and sabrourd dextrose agar (SDA) plates, which were incubated at ambient warmth for 2-7days. Discrete colonies were stored in slants afterwards (Philippot et al., 2012).

Root samples were washed against running tap water for three minutes and then washed with 0.85% saline water for one minute. The washed root was macerated and subjected to ten-fold serial dilution (Philippot et al., 2012). Nutrient agar (NA) and sabrourd dextrose agar (SDA) plates were inoculated with 0.1ml of dilution10^{-2} using for spread plate method and incubated at ambient warmth for 2-7days. Discrete colonies were stored in slants afterwards.

2.3. Screening of potassium solubilizing microorganisms (KSM) and phosphate solubilizing microorganisms (PSM)

Aleksandrov medium was utilized for the screening KSM, and pikovskaya's agar (PVK) for PSM. After sterilization of the media, 0.2mg of streptomycin was then added to 100ml of the cooled media in conical flasks to inhibit bacteria and isolate fungi, while 500IU of nystatin was added to 100ml of the media to inhibit fungi and isolate bacteria. Loopful of 48 hours old bacterial isolates and 3 days needle scrap of fungal isolates were utilized to inoculate sterile solidified Aleksandrov agar and PVK media using spot test method, which involves placing a loopful of pure culture on the center of the same agar plates. Incubation was for seven days at ambient warmth (Prajapati & Modi, 2012). Colonies showing halo zones were taken as evidence of potassium and phosphate solubilization. The bacterial and fungal colonies with clear halo zone were purified three times on same medium and maintained on NA and SDA slants.

2.4 Determination of potassium solubilization index (KSI) and phosphate solubilization index (PSI)

Qualitative estimation of K-solubilization and P-solubilization were done by measuring the K-solubilizing index (KSI) and P-solubilizing index (PSI) respectively. Loopful of isolate (48hours for bacterial and 3 days fungal) was smudged on the Aleksandrov and PVK media, and incubated at 30 °C for 14 days. This was done in three replicates with the sterile medium served as a control. KSI formula, KSI = C+H/C, (C = Colony diameter; H =Halo zone diameter) (Pathak et al., 2017). Isolates with KSI and PSI ≥140 were preserved for preliminary identification and further studies.

2.5. Preliminary Identification of PSM and KSM

Preliminary identification was carried using colonial morphology (the colony colour, colony shape, and elevation aided by hand magnifying glass), gram staining, and biochemical test (citrate utilization, catalase, urease, indole, methyl red, vogues Proskauer, H_2S, sugar fermentation, and nitrate reduction test) for bacteria; and cultural and microscopic characteristics for fungi. The outcome was matched against Bergey's Manual, 9th edition, and atlas of fungi.

3. Results and Discussion

3.1 Results

3.1.1. Isolates' potassium and phosphate solubilization potential

The result of the potassium and phosphate solubilization test is presented in Table 1. The diameter of the isolates zone of clearance on Aleksandrov and Pikovskaya's agar medium were in millimeter. A total of 145 discrete isolates were obtained from the eighty samples (forty soil samples, forty root samples). Twenty (13.8%) were potassium solubilizers. Out of the 20 isolates able to solubilize potassium, 8(40%) solubilization ability was represented by one (+), 6(30%) solubilization ability was represented by two (++), 4(20%) was represented by three (+++) and 2(10%) was represented by four (++++).

Thirty nine (26.9%) were phosphate solubilizers. Out of the 39 isolates able to solubilize phosphate, 18(46.1%) solubilization ability was represented by one (+), 12(30.8%) solubilization ability was represented by two (++), 6(15.4%) was represented by three (+++) and 3(7.7%) was represented by four (++++).

Table 1: Isolates Zone of Potassium and Phosphate Solubilization

No of Isolates	Solubilization Sign/Range (mm)	No of P solubilizers	No of K solubilizers
145	+ (0-1.5)	18	8
	++ (1.5-3.0)	12	6
	+++ (3.0 – 4.5)	6	4
	++++ (>4.5)	3	2
		39 (26.9%)	20 (13.8%)
	- (0)		

3.1.2 Solubilization Indexes of Isolates

The result of the potassium and phosphate solubilization indexes of positive isolates ranged from 115.4 to 180 and 109.1 to 190 respectively. The index is the ratio of the diameter of the zone of clearance to the diameter of colonial growth by a hundred. Table 2. Contain the isolates with KSI and PSI ≥ 140.

Table 2: Isolates with Solubilization Indexes ≥ 140.

Isolates	KSI	PSI
PRZS-14	145.5	180..0
MRZS-14	-	154.5
CRZS-23	180.0	190.0
CRZS-20	166.7	161.5
PRTS-5	146.0	150.0
ORZS-18	171..4	164.3
PRZS-21	-	180.0
MRTS-3	144.0	145.5
MRZS-18	150.0	146.2
ORZS-3	-	150.0
ORZS-13	166.7	166.7
PRZS-38	144.4	142.9

3.1.3 Identification of Bacterial Isolates with KSI and PSI ≥140 Using Colonial, Morphological and Biosynthetate Characteristics

The morphological and biochemical characteristics of the bacterial isolates with KSI and PSI≥ 140 are presented in Table 3. The isolates with were *Pseudomonas* sp. and *Bacillus* sp. Their morphological characteristics were recorded based on their size, shape, margin, elevation, pigmentation, and color. Gram stain test revealed that *Pseudomonas* sp is gram-negative as it retained the color of the counterstain used. Oxidase test was used to differentiate *Pseudomonas* from other gram-negative bacilli. Catalase test was used to test the ability of *Pseudomonas* to grow in the presence of oxygen. Other biochemical tests carried out showed that *Pseudomonas* was positive for the following test: “citrate, gelatin hydrolysis, nitrate oxidase, and test”. On the other hand, *Pseudomonas* was negative for indole, methyred urease, voges proskauer, maltose, glucose and sucrose. *Pseudomonas* does not produce gas and does not hydrolyze starch. The spore test revealed that *Pseudomonas* is a non-spore former.

The result of the gram stain revealed that *Bacillus* sp was a gram-positive organism because it retained the color of the primary stain (crystal violet) and was not decolorized by alcohol. It was found to be catalase, citrate, nitrate, voges proskauer, mannitol, maltose, glucose, sucrose and gelatin hydrolysis positive. *Bacillus* sp. showed a negative result for indole, methyl red, oxidase, and urease. The result of the spore stain revealed that it is a spore former.

3.1.4 Identification of Fungal Isolates with KSI and PSI ≥140 Using Cultural and Microscopic Characteristics

The result of the identification of fungal isolates with PSI≥ 140 is as presented in Table 4. The isolates were *Aspergillus* sp.

and *Penicillium* sp. *Aspergillus* sp. was found to be a powdery colony, with dark brown front colour. The reverse colour was also brown. It had a flatty spread on the surface of the solid medium. Microscopically, *Aspergillus* sp. had septate and branched hyphae with conidia that appeared in chains. *Penicillium*s sp. front colour was found to be grey with a large white border and white reverse. Microscopically, *Penicillium* sp. has long branched septate conidiophores consisting of brown-like conidia in chains at the tips of the phialides.

TABLE 3: Identification of Bacterial Isolates with PSI ≥ 140 using Colonial, Morphological and Biochemical Characteristics

Isolates	**PRZS-14**	**MRZS-14**	**CRZS-23**	**CRZS-20**	**PRZS-21**	**PRZS-5**	**ORZS-18**	**ORZS-13**
Colonial and Morphological Characteristics								
Size	2mm	3mm	3mm	3mm	3mm	2mm	3mm	3mm
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Margin	Smooth edge	Entire	Irregular egde	Irregular egde	Entire	Smooth edge	Irregular egde	Irregular egde
Elevation	Convex	Convex	Flat	Flat	Convex	Convex	Flat	Flat
Colour	Bluish green	Yellow	Glassy Appearance	Glassy Appearance	Pale orange	Bluish green	Glassy Appearance	Glassy Appearance
Pigmentation	+	-	-	-	-	+	-	-
Gram reaction	-	+	+	+	+	-	+	+
Biochemical Test								
Catalase	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+
Gas	+	-	-	-	-	+	-	-
Gelatin hydrolysis	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-
Methyle red	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+
Oxidase	+	-	+	-	+	+	-	-
Spore	-	+	+	+	+	-	+	+
Urease	-	-	-	-	-	-	-	-
Voges Proskauer	-	+	+	+	+	-	+	+
Mannitol	+	+	+	+	+	+	+	
Maltose	-	+	+	+	+	-	+	+
Glucose	-	+	+	+	+	-	+	+
Sucrose	-	+	+	+	+	-	+	+
Starch	-	+	+	+	+	-	+	+
Preliminary Identification	***Pseudomonas* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Pseudomonas* sp.**	***Bacillus* sp.**	***Bacillus* sp.**

Table 4: Identification of Fungal Isolates with PSI ≥140 Using cultural and Microscopic characteristics

Isolates	Cultural Characteristics	Microscopic Characteristics	Preliminary Identification
MRTS-3	Powdery, dark brown, flatty spread and brown reverse.	Septate and branched hyphae with conidia in chains.	*Aspergillus* sp.
MRZS-18			*Penicillium* sp.
ORZS-3	Grey colony with large white border and white reverse.	long conidiophores consisting of brown like conidia in chains.	*Penicillium* sp.
PRZS-38	Grey colony with large white border and white reverse.	long conidiophores consisting of brown like conidia in chains	*Aspergillus* sp.
	Powdery, dark brown, flatty spread on the surface of the solid medium and brown reverse.	Septate and branched hyphae with conidia in chains.	

3.2 Discussion

The intensification of agriculture through the espousal of agro-technologies owing to this revolutionary agenda of which the utilization of synthetate fertilizer was one, heralded the substantial degradation of the fragile agroecosystems with erosion, ecological imbalances, and pollution as upshots (Pingali 2012). Continual utilization of NPK fertilizer will amplify the pace of diminution of the stock of non-renewable resources used for their production (Cordell et al., 2009). Most used N-fertilizer is lost to the environment, causing increased levels of freshwater nitrate and nitrous oxide production which contributes to global climate change (Duce et al., 2008).

The study: "Isolation, Characterization and Assessment of Phosphate and Potassium Solubilizing and Nitrogen Fixing Microbes from Agricultural Soil in Imo State Nigeria." inclining with the trend of developing the technology of the exploit of microbial-inoculants as a real replacement to synthetic agrosynthetates. Rhizospheric and root isolates of okro, cassava, plantain, and maize displayed solubilization ability for K and PO_4. According to Sarkar et al., (2017) "rhizospheric microbes help in the solubilization of nutrient-bearing minerals which are indigenous sources of phosphates, potassium, and other nutrients". Sheng et al. (2003) reported that organic compounds produced by micro-organisms such as acetate, citrate, and oxalate can increase mineral dissolution in soil, including solubilization of potassium. This solubilization Styriakova et al. (2003) reports is an intricate construct betwixt organic acids and Fe^{2+}, Al^{3+}, and Ca^{2+}.

The actualizing of the utility of soil microbes to boost up crop productivity inclines towards the isolation and deploying the microorganisms that can solubilize potassium and phosphate and fix nitrogen which is ingenious to the location of interest.

Potassium (K) is the most abundant cation in plant cells. it is the second most abundant nutrient after nitrogen in the leaves (Basak & Biswas, 2010). Most of the potassium in soils exists as insoluble rocks and minerals such as micas, illite, feldspar, and orthoclases. K participates in nutrient transportation and uptake and also confers resistance to both abiotic and biotic stresses. It leads to enhanced production of crops of quality and aids in disease resistance in plants.

Potassium is the third essential nutrient required by plants. It is involved in numerous biochemical and physiological processes in plants like stomatal regulation which is essential for photosynthesis; activation of enzymes involved in adenosine triphosphate (ATP) production; transportation of sugars produced in photosynthesis, through the phloem, to other parts of the plant for utilization and storage. The plant's transport system uses energy in the form of ATP. If K is inadequate, less ATP is available, and the transport system breaks down.

Potassium also plays a major role in the transport of water and nutrients in the plant through the xylem. The enzyme responsible for the synthesis of starch (starch synthetase) is activated by K, hence it plays a crucial role in water and nutrient transport.

Crop production is usually severely affected by the deficiency of potassium K. Soil potassium content is huge but largely unavailable to plants. They exist mainly in insoluble forms like mica, feldspar, and others. Potassium solubilizing microorganisms play a vital role in making available insoluble forms of potassium by mineralization. The exploitation of this microbial ability to ensure sustainable agro development void of chemicalization was the reason for the research.

Microbial identification protocol carried out on these isolates revealed that 66.7% were bacterial while 33.3% were fungal. The genera of microbes were *Bacillus* sp. were 66.7% of the bacterial isolates while *Pseudomonas* sp. was 33.3%. Fungal isolates were *Aspergillus* sp and *Penicillium* sp, occurring in the frequency of 66.7% and 33.3% respectively.
Prajapati & Modi, (2012) Reported *Cladosporoides*, *Cladosporium*, and *Penicillium* sp. as able to solubilize potassium They have also characterized potassium solubilizing fungi as *Aspergillus terreus* and *Aspergillus niger* based on their colonies and morphology characters.. This was in agreement with our result of isolating 66.7% *Aspergillus* sp and 33.3% *Penicillium* sp. In the study of Archana et al., (2013) on rhizosphere soil of different crops from Dharwad and Belgaum districts, a total of 30 bacteria isolates were tested for K solubilization and characterized up to genus level based on morphological and biochemical characters. Out of them, 26 were gram-positive rods belongs to genera *Bacillus* and four were gram-negative rods belongs to genera *Pseudomonas*. This corroborates with our result of having 66.7% frequency of occurrence *Bacillus* sp. to 33.3% of *Pseudomonas* sp. isolates as potassium solubilizers.

Some isolates from the root and rhizosphere of indigenous corps were found to display a varying degree of phosphate solubilization. This study, which isolated and characterized Phosphate solubilizing microorganisms from the rhizosphere and the roots of crops indigenous to Ihiagw-Owerri , Imo State Nigeria, was inclining to the new trend of sourcing P biologically. The mechanisms used by these microbes include the excretion of organic acids or the production of phosphatase enzymes (Kucey, 1983). The medium used for the screening of the isolates for phosphate solubilizing ability, Pikovskaya's agar, contains tricalcium phosphate ($Ca_3(PO_4)_2$) an insoluble phosphate. The ability of the isolates to solubilize it produces a positive result which manifests as the zone of clearance by 26.9% (39) of the 145 isolates screened. The production of organic acids by these solubilizers was the reason for the zone of clearance. Microbial identification protocol carried out on these isolates revealed that 66.7% were bacterial while 33.3% were fungal. The genera of microbes were *Bacillus* sp. were 75% of the bacterial isolates while *Pseudomonas* sp. was 25%. Fungal isolates were *Aspergillus* sp and *Penicillium* sp with an equal frequency of occurrence.

Bhattacharyya and Jha (2012) report of most significant bacterial solubilizers as Azotobacter, *Bacillus (B. megaterium, B. circulans, B. subtilis, B. polymyxa*, *Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas (P. striata),* and *Serratia. Bacillus* sp. are among their report. The genera were 75% of the bacterial isolated as phosphate solubilizer. In the report of Oteino et al., (2015) *Pseudomonas*, *Acinetobacter, Pantoea,* and *Ent erobacter*, and *Bacillus* were among the major solubilizers of phosphate. The two bacterial genera isolated in this work, *Pseudomonas* and *Bacillus*, were commonly reported as been among effective phosphate solubilizing bacteria. The report of Krishnananda and Dipika (2017) is in line with the result that the fungal isolates of the genera *Aspergillus* sp. and *Penicillium* sp. solubilize phosphate. The application of phosphorus solubilizing microbes provides a new approach to improve soil quality, this will help in achieving sustainability in agriculture.

4. Conclusion

Some isolates from the rhizosphere and roots of crops indigenous to Ihiagwa-Owerri exhibit phosphate and potassium solubilization and nitrogen fixation. The isolates were identified as *Penicillium citrinm, Pichia cecembensis, Aspergillus niger, Pseudomonas aeruginosa, Acinetobacter venetianus, Bacillus flexus, Bacillus subtilis,* and *Azotobacter chroococum.* The use of these organisms as biofertilizer will boost agro yield and at the same time conserve the environment.

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