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Phosphate solubilization by rhizobacteria isolated from the rhizosphere of *Mimosa pudica*: an investigation into microbial mobilization of phosphorus

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Abstract

Phosphorus (P), in addition to nitrogen (N) and potassium (K), makes up the essential macronutrients needed by plants for growth and development. P is also one of the most abundant elements present in the Earth's crust, and it occurs in both organic and inorganic forms. Although present in high concentrations, only a very minute amount is bioavailable to plants because of its poor solubility due to its high binding affinity to calcium, aluminium, and iron in the soil, forming insoluble calcium phosphate, aluminium phosphate, and ferrous phosphate, thereby becoming recalcitrant for plant utilization. In this study, bacteria isolated from the rhizosphere of *Mimosa pudica* from different locations at Nnamdi Azikiwe University, Awka, were screened for their ability to solubilize phosphate using point inoculation on Pikovskaya (PVK) media agar plates with tricalcium phosphate ($Ca_3(PO_4)_2$) as a source of phosphate. The result showed that of the twenty-one (21) rhizobacteria screened, four (4) were able to solubilize phosphate with varying phosphate solubilizing index (PSI). The degrees of PSI were in the following order: isolate SVM5 and UBG13 > isolate UBG14> isolate FEA6. Isolate SVM5 and UBG13 had the highest PSI of 4.0, while isolate FEA6 had the lowest PSI of 3.1. The biochemical tests of the isolates revealed that the phosphate-solubilizing bacteria were members of Klebsiella spp. (FEA6, SVM5, and UBG14) and Pseudomonas sp. (UBG13). This study, therefore, suggests the need to include rhizobacteria as part of biofertilizer formulations to improve plant yield via the solubilization of phosphate into forms that are bioavailable for plant growth.

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1. Introduction

Phosphorus (P) remains one of the essential macronutrients required by plants for proper development and wellbeing. It performs dedicated roles in signal transduction, energy production, and membrane stability, as well as aiding cell divisions in plants [1]. Phosphorus is second only to nitrogen in terms of abundance and can exist as both organic and inorganic phosphates in the soil. Sadly, these soil phosphates are largely insoluble due to fixation with metallic elements, thus they are not bioavailable for plant use. This fate of phosphate in the soil is mainly due to soil conditions such as moisture contents, pH, temperature, and other minerals

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present in the soil [2]. In order to remedy this challenge, there has been great dependence on chemical fertilizers to provide plants with the needed phosphate. However, chemical fertilizers not only come with environmental issues, such as the release of dangerous gases (fluorine) and toxic metals (cadmium) due to the mining of rock phosphates, but also incur both economic and energy burdens [3].

Plants can access only about 0.1% of soluble form of HPO_4^{-2} or H_2PO^{-4} in a P-rich soil, and this is made possible by certain groups of microorganisms [2]. The inaccessible insoluble ones are complexed with soil minerals like apatite and metal phosphates [4]. Modern plant cultivation requires sustainable approaches to promote eco-health [5]. However, this approach is faced with challenges such as unstable climatic factors, phytopathogens, pest attacks, and declining soil fertility. Sustainable agricultural practice entails the proper control, management, and mitigation of these challenges in order to promote high yield as well as continuous maintenance of environmental health [6].

Worldwide, there is an abundance of phosphorus in arable land, but the amount available to plant for growth is rarely up to Pan & Cai [7]. It has been estimated that 5.7 billion hectares of land globally contain insufficient amounts of available P for crop production [8]. This is because the organic phosphates are fixed in the soil as insoluble phosphate, while the inorganic phosphates are complexed with metal ions to also become insoluble phosphate that cannot be used by plants [7]. Microorganisms, especially root or rhizopheric microorganisms and phosphate solubilizing bacteria, provide beneficial services to plants by decomposition and production of organic acids that can chelate Fe and Al ions that bind to P, thus releasing them in a form they can be available in the soil [1]. They also play a key role in the soil phosphorus cycle by mineralizing organic phosphorus. Other ways of solubilization of phosphate are through secretion of enzymes and chelating activity by production of siderophores and extracellular polysaccharides [7]. The mechanisms of phosphate solubilization are explained using two theories: acid production theory and proton and enzyme theory [9].

Biofertilizers are products of microbial formulations that enhance crop yields and at the same time promote sustainable food production. Microorganisms that have been used in the formulation of biofertilizers include bacteria and fungi of soil origin. Primarily bacteria from the rhizospheres of plants, some of which are able to solubilize phosphate and provide other nutrients for plants. These beneficial bacteria are generally referred to as plant growth-promoting rhizobacteria (PGPR). Most researches focus on sourcing these rhizobacteria from different sources, such as the roots of halophytes and drought-resistant plants [10], seagrasses [11], mushroom residues [12], and soil [13]. Phosphate-solubilizing bacteria (PSB) help to cycle P in the rhizosphere, thus enhancing nutrient acquisition by plants and promoting their growth [14]. These PGPR colonize the rhizosphere and actively take part in the transformation and recycling of phosphorus in soil, thereby making it bioavailable to the plants. This modern approach to agriculture is encouraged because it promotes sustainability by offering a cost-effective method of improving yield via nutrient availability as well as promoting healthier crops by providing successful antagonizing activity against phytopathogens and then promoting an ecofriendly environment as a result of a decrease in phosphatic fertilizers that would have polluted the environment [5].

Mimosa pudica is a creeping plant that responds to touch by folding its leaves inward, which reopen within minutes. It has been found to have therapeutic properties [15]. *M. pudica* is an invasive plant and so has been regarded as a weed in some areas. However, the metalliferous properties of *M. pudica*, that is, their ability to hyperaccumulate elements, make their root nodules and the rhizosphere a hotspot for plant growth-promoting microorganisms capable of nitrogen fixation, siderophore production, phytohormone production, as well as solubilization of minerals such as phosphate, zinc, and potassium [16, 17]. There is therefore the need to explore this unique ecological niche and test for the ability of rhizobacteria inhabiting in the rhizosphere of *M. pudica* in order to assess their potential for solubilizing inorganic phosphate. In this study, rhizobacteria isolated from the rhizosphere of *Mimosa pudica* were screened for their ability to solubilize inorganic phosphate using plate halo zone assay.

2. Materials and methods

2.1. Collection of rhizosphere soil

The rhizosphere soil samples of *Mimosa pudica* (Shame-plant) were collected at 10 – 15cm depth from different locations of the university campus Nnamdi Azikiwe University, Awka. Non- rhizosphere soil were removed by gentle shaking, leaving behind only the rhizosphere soil [18]. Samples were collected in a sterile polythene bag and immediately transported to laboratory for further processing.

2.2. Isolation of rhizosphere bacteria

Ten grams of rhizosphere soil was added aseptically to 90ml of normal saline (0.85g NaCl + 100ml of distilled water). Mixture was vortexed for 30 mins, before serially diluted at a concentration of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Thereafter, 100µl of the aliquot from 10^{-4} and 10^{-5} were plated using spread plate technique on Luria Bertani (LB) agar and incubated at 35^0C for 24h. Pure isolates were obtain by subsequent subculturing on LB agar.

Table 1: Sample location and number of isolates.

Sample Location	Number of Isolates
Science Village/ MCTU Axis (SVM)	5
Faculty of Engineering and Agriculture Axis (FEA)	7
University Botanical Garden Axis (UBG)	9
Total	21

Table 2: Phosphate solubilizing index of the test isolates.

Isolate	DC (mm)	CZ (mm)	PSI
SVM5	4	12	4
UBG13	2	6	4
UBG 14	2	5	3.5
FEA6	13	27	3.1

DC= Diameter of Colony; CZ= Clear Zone; PSI= Phosphate Solubilizing Index.

Table 3: Biochemical tests of phosphate solubilizing rhizobacterial isolates.

Isolates	Sor	Glu	Lac	Suc	Mal	Ind	MR	VP	Ure	Cat	Oxi
SVM5	AG	AG	AG	AG	A	-	+	+	+	+	-
UBG13	-	-	-	-	-	-	-	-	-	+	+
UBG14	AG	AG	AG	AG	AG	-	+	+	+	+	-
FEA6	AG	AG	AG	AG	AG	-	+	ND	+	+	-

A= Acid; AG= Acid and Gas; -=Negative; +=Positive; ND=Non Detected
Sor = Sorbitol; Glu = Glucose; Lac = Lactose; Suc = Sucrose; Mal = Maltose; Ind = Indole;
MR = Methyl Red; VP = Voges Proskauer; Ure = Urease; Cat = Catalase; Oxi = Oxidase

2.3. *In vitro screening of phosphate solubilizing bacteria*

Twenty-one (21) Isolates from rhizosphere soil of *M. pudica* were screened in vitro to test their ability to solubilize phosphate by plating them on Pikovskaya (PVK) media agar plates The PVK medium contained (in g L^{-1}) 10 glucose, 0.5 yeast extract, 0.5 (NH4)2 SO4, 0.1 MgSO4 .7H2O, 5 Ca3(PO4)2, 0.2 KCl, 0.002 MnSO4 .2H2O, 0.002 FeSO4 .7H2O, and 15 agar [19]. All plates were incubated at 35 ^{0}C for up to 7 days to observe for halo zone of clearance.

2.4. *Determination phosphate solubilizing index (PSI)*

The phosphate solubilizing index of isolates that showed halo clearance were calculated using the following equation [20, 21]:

$$\text{Phosphate Solubilizing Index} = \frac{\text{Diameter of Colony + Clear Zone}}{\text{Diameter of Colony}}.$$

2.5. *Characterization of rhizobacteria isolates*

Isolates were subjected to microscopic analysis to determine cell shapes and arrangements and also Gram's stain was done [22]. Also, biochemical tests such as catalase test, urease test, indole test, methyl red test [23], voges proskauer test, acid production test [24] were done to aid identification of isolates using Bergey's manual of determinative bacteriology.

3. Results

A total of twenty one (21) rhizobacteria were isolated from the rhizospheres of *M. pudica* obtained from various sampling sites from Nnamdi Azikiwe University, Awka (Table 1).

Table 2 is showing the phosphate solubilizing index of the phosphate solubilizing rhizobacteria derived from the diameter of their colony and clearing zone.

Tables 3 and 4 present the biochemical features and tentative names of the phosphate solubilizing rhizobacterial isolates as identified by Bergey's manual of determinative bacteriology:

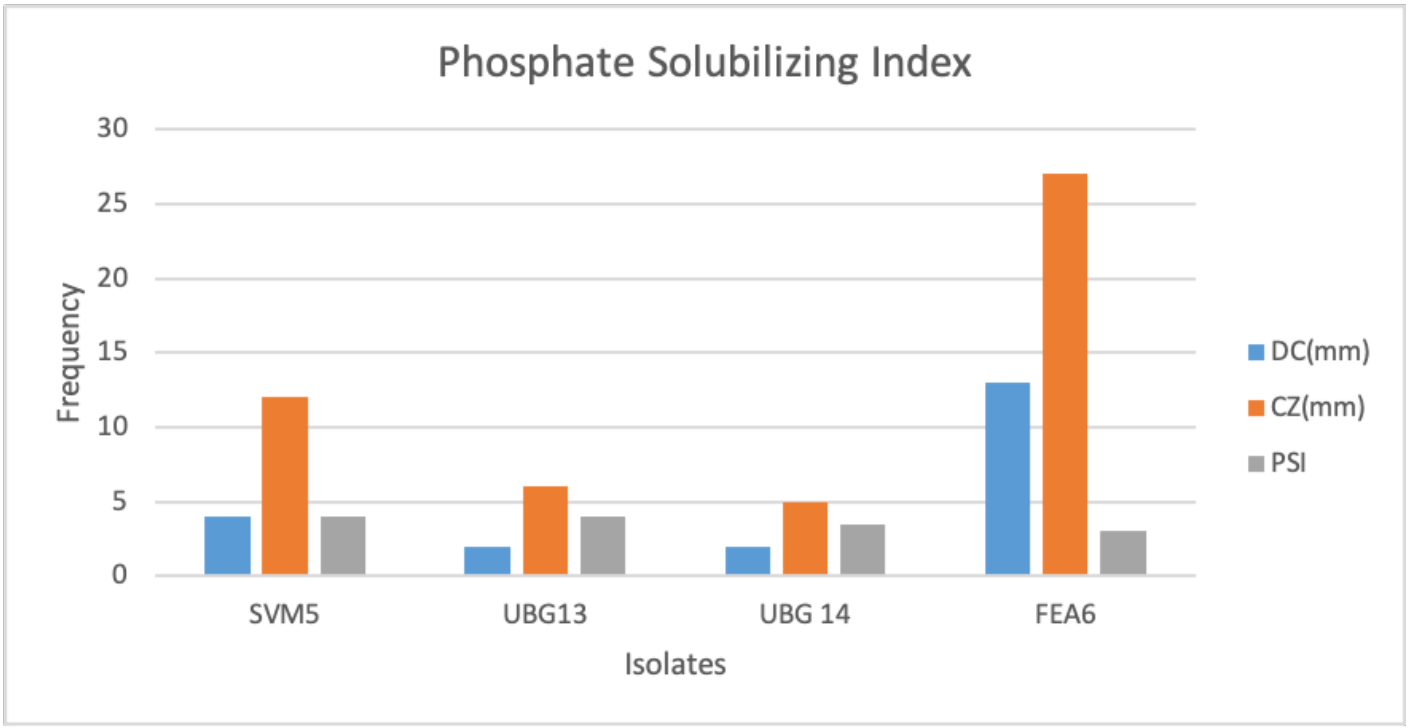

Figure 1: Phosphate solubilizing index of isolates.

Table 4: Gram Result and Probable Organisms.

Isolates	Gram	Probable Organisms
SVM5	-Rod	*Klebsiella* species
UBG13	-Rod	*Pseudomonas* species
UBG14	-Rod	*Klebsiella* species
FEA6	-Rod	*Klebsiella* species

4. Discussion

The use of a halo zone plate assay to determine the ability of microorganisms to solubilize phosphate is a qualitative method that is used by researchers to demonstrate solubility of phosphate using tricalcium phosphate as a source of phosphorus in a plate medium called Pikovskaya (PVK) agar. The production of organic acid by the organisms facilitated the dissolution of phosphate to produce a visible halo zone [25]. Measurement of the halo zone provides qualitative evidence of the solubilization ability of the tested isolates by providing information on the phosphate solubilizing index of the dissolution. Phosphate solubilizing index is used to explain the ability of microorganisms to solubilize inorganic phosphate [20].

In this study, only four rhizobacteria out of the twenty-one rhizobacteria isolated from *M. pudica* showed indication of solubilizing tricalcium phosphate by producing halo zone on PVK agar. The decision to use PVK agar plates stemmed from the fact that PVK has been reported and accepted by researchers to qualitatively determine the phosphate solubilizing ability of microorganisms. Microorganisms that can solubilize phosphate produce a visual halo zone on PVK medium, making it an easy and rapid method of accessing phosphate solubilizers, especially when it involves large numbers of microorganisms. Also, PVK can support the growth of a greater number of bacteria on a solid agar plate than most media for phosphate solubilization. Ghosh *et al*. [26] in their work on the role of phosphate solubilizing bacteria, compared three media for phosphate solubilization and reported that PVK outperformed the National Botanical Research Institute's phosphate growth medium (NBRIP) but was only second to the National Botanical Research Institute's phosphate growth medium devoid of yeast (NBRIY). In this study, of the four isolates coded in their sample location names (Table 1), SVM5 and UBG13 had the highest phosphate solubilizing index (PSI) of 4, with FEA6 having the least, that is, PSI of 3.1 (Figure 1 and Table 2 showing diameter of colony and also zone of halo clearance).

Tentatively, the test isolates were identified as SVM5, FEA6, and UBG14 as members of *Klebsiella* spp., while UBG13 as Pseudomonas sp. In this study, *Klebsiella* sp was reported to produce a PSI of 4 on a PVK agar; this result obtained is similar to the work of Bhardwaj *et al*. [27] that reported a PSI of 3.95 for *Klebsiella pneumoniae* assayed on a PVK. Some authors have reported varying ranges of PSI; for instance, Islam *et al*. [28] reported in their work to have PSI from 1.2 to 6.7, and it was reported in their

work that *Klebsiella* sp. assessed for PSI gave 4.8, which is close to what was recorded for *Klebsiella* sp.; however, the assay medium used in their work was NBRIP. Both media are the prominent media used for assaying phosphate solubilization. Biswas *et al*. [29] in their study, also validated the ability of *Klebsiella* sp. to solubilize phosphate in their study of three endophytic bacteria. *Klebsiella pneumonia* was reported to have the highest PSI of 1.6 compared to 1.4 and 1.3, respectively, by *Bacillus amyloliquefaciens* and *B. subtilis*.

Pseudomonas sp. has also been known to be a good phosphate solubilizing bacteria. Kumar *et al*. [30] reported that *Pseudomonas* sp. gave the highest phosphate solubilization ability compared to the other twelve bacteria isolated from French beans; the result of their work was presented in millimeters of the zone of clearance. The work of Paul & Sinha [31] also validated the ability of *Pseudomonas* sp. to solubilize phosphate. *Pseudomonas aeruginosa* KUPSB12 was reported to produce a PSI of 2.85. In this study, *Pseudomonas* sp. produced a PSI of 4. The ability of an organism to solubilize phosphate is dependent on the amount of organic acid they can produce or if they can produce extracellular polysaccharides or enzymes (phosphatases) [31].

PSB have been recorded among different domains of microorganisms isolated from diverse environments, both in perturbed and unperturbed conditions [32]. The genera that have most been reported as PSB include *Pseudomonas, Bacillus, Enterobacter*, and *Azotobacter* [33]. Panda *et al*. [34] in their study on phosphate solubilizing bacteria from the acidic soils of the Eastern Himalayan region and their antagonistic effect on fungal pathogens, reported that *Pseudomonas* spp. were among the bacteria that were able to solubilize phosphate together with *Bacillus* spp. Their studies underscore the importance of *Pseudomonas* and others in promoting plant growth and the additional role played in controlling phytopathogens. Joshi *et al*. [35] worked with isolates from the rhizosphere of *Dalbergia sissoo Roxb*. (Shisham) and reported that all eighteen (18) isolates tested were able to solubilize phosphate, and among the identified bacteria were members of *Pseudomonas*, *Klebsiella*, *Streptomyces*, *Pantoea, Kitasatospora, Micrococcus*, and *Staphylococcus*. Similar to this study, *Klebsiella* and *Pseudomonas* isolated from the rhizosphere of *M. pudica* were able to solubilize phosphate. It has also been noted that a greater amount of PSB is cultured from rhizospheric soil than non-rhizospheric soil [36].

Rhizospheric environments that have been reported as sources of PSB include tea garden soil [37], grapevine rhizosphere [38], rice [39], and oil palm [40]. A more modern approach to tackling the challenges facing agriculture is the incorporation of phosphate solubilizing bacteria as biofertilizers to enhance crop yield and reduce environmental pollution that may arise as a result of dependence on phosphatic fertilizers. The addition of these fertilizers does not promote sustainability because the added fertilizers are fixed to the soil, thereby becoming inaccessible to plants [41]. But they rather impact the environment by leading to pollution of water bodies, including ground water. PSB greatly affect soil characteristics and play a vital role in transforming poor quality soil into cultivable soil. One way of achieving this is supplying P to legumes through phosphate solubilizing bacteria [5]. However, the factors that limit the prolific application of these sustainable farming methods are soil types (acidic or alkaline), interactions with other microorganisms, agronomic activities, and ecological conditions [42].

Very worthy of noting is the role of phosphorus in aiding leguminous plants in fixing nitrogen [32]. The plant-microbe interactions between microorganisms and legumes through biomineralization and synergistic co-evolution have great potential for improving soil quality and fertility [43]. Inoculation of nitrogen-fixing bacteria generally enhances the root architecture of legumes via colonization of roots to form nodules and fixation of atmospheric nitrogen. Therefore, the synergistic association between them and PSB bacteria will further improve the nutrient pool and availability of phosphorus to plants. This validates the necessity of this study in screening for phosphate solubilizing bacteria from the rhizosphere of leguminous plants. Most studies had centred on the isolation of phosphate solubilizing bacteria from the rhizospheres of edible (food crop) legumes such as cowpea, groundnut, soy bean [44], pigeon pea, sword bean, and black-eyed pea, and only a few instances in non-edible legumes such as sun hemp [45]. Ejeagba *et al*. [46] reported a PSI of 2.0 from *Pseudomonas* sp., isolated from cowpea. In this study, the *Pseudomonas* sp. isolated from *M. indica* gave a PSI of 4. This high PSI may equally have been the ability of the isolate in this study to produce more organic acid or enzymes responsible for phosphate solubilization.

5. Conclusion and recommendations

The rhizosphere contains diverse group of microorganisms whose activities either directly or indirectly affect positively or negatively the host plants. Amongst the beneficial role is solubilization of fixed phosphate in the soil that plant can utilize for their growth. In this study it was demonstrated that the rhizosphere of *M. pudica* a sensitive creeping plant with therapeutic and aesthetic functions as well as phytoremediation ability .can be a source of rhizobacteria with the potential to solubilize inorganic phosphate by producing halo zone during the assay. Exploration of related plants for phosphate solubilizing prowess could be an untapped resources to advance agricultural sustainability. Future studies should focus on quantifying the amount of phosphorus being released as a result of solubilization activity. Also field trials on different soil types with the isolates as biofertilizers to see how they fair in promoting the growth of crops. Finally molecular techniques to understand the plant-microbe interactions should be explored.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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