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Exploring the Phosphate Solubilization Potential of *Rhizobia* Isolated from *Sesbania grandiflora*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Rhizobia, are known to exhibit the ability to solubilize phosphate (P) in the soil, apart from their primary function of nitrogen fixation. Hence this study was conducted to evaluate and characterize *rhizobia* isolates obtained from root nodules of *Sesbania grandiflora* for their potential in P solubilization. Two experiments were conducted under laboratory conditions arranged in a Complete Randomized Design (CRD) with three replicates. All data were analyzed using analysis of variance (ANOVA) and means were separated using the LSD test. Four bacterial strains (a,b,c,d) were isolated from root nodules using the trap plant method. They were initially identified as *Rhizobia* based on morphology and authenticated through gram staining, acid-alkaline tests, Congo Red Yeast Mannitol Agar (CRYMA), and Bromothymol blue Yeast Mannitol Agar (BRYMA) tests. The most effective *rhizobia* for P solubilization was identified based on the Phosphorus

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Solubilization Index (PSI) and solubilized P in Pikovaskya (PVK) solid and liquid media. Subsequently, optimization of PVK liquid media was conducted for effective P solubilizer, varying with carbon (C), nitrogen (N), and P sources. All isolates were gram-negative and exhibited acid production, authenticated as *rhizobia*. The significantly ($p<0.05$) highest PSI was recorded with isolate "c" and it also exhibited the highest ($p<0.05$) solubilized P. The isolate "c" showed potassium dihydrogen phosphate (35.1±0.46 ppm), glucose (25.47±0.49 ppm), and ammonium sulfate (3.20±0.17 ppm) as the optimum sources of P, C, and N, respectively, for achieving significantly higher phosphorus solubilization in PVK medium by thriving *rhizobia* "c" as an effective phosphorus solubilizer. However, further field studies are required to assess performance of *rhizobia* "c" before introducing it as a P solubilizing inoculum.

Keywords: Media optimization; P solubilization; P solubilizing inoculum; rhizobia.

1. INTRODUCTION

Phosphorus is one of the most essential nutrients for plant growth. It is undoubtedly clear that P is one of the most essential macro-elements required for growth and development of plants including photosynthesis, energy and sugar production. Moreover, it promotes nitrogen fixation in legumes (Saber *et al.* 2005; Xiao *et al.,* 2011). Only 0.1% of the total P (0.5%) is available to plants in soils, while rest of the total P is present in the insoluble form and therefore, cannot be taken up by plants [1]. In soils, phosphoric acid (H_3PO_4), dihydrogen phosphate ($H_2PO_4^-$) and hydrogen phosphate (HPO_4^{2-}) are the primary forms of P taken up by plants. The solubilization of P is important in various biological and environmental processes.

Soil microorganisms, particularly phosphate-solubilizing bacteria, play a crucial role in solubilizing P. The principal mechanism for mineral P solubilization is the production of organic acids, and acid phosphatases play a significant role in the mineralization of organic P in soil. It is generally accepted that the major mechanism of mineral P solubilization is the action of organic acids synthesized by soil microorganisms [2]. Apart from chemical fertilization, microbial P-solubilization and mineralization is the only possible way to increase plant-available P. Numerous microorganisms in the soil and rhizosphere are effective at releasing P from total soil P through solubilization and mineralization [3]. This group of microorganisms are referred to as Phosphorus Solubilizing Microorganisms (PSM).

Phosphate-solubilizing bacteria (PSB) are beneficial microorganisms capable of solubilizing inorganic P. Bacterial genera like *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium,* and *Pseudomonas* are reported as the most significant phosphate solubilizing bacteria [3]. Moreover, the ability to P-solubilization is found even among Rhizobiaceae, comprising *Rhizobium, Bradyrhizobium, Mesorhizobium* and other non-specified legume-nodulating bacteria (LNB) [4].

Rhizobia, belonging to the family Rhizobiaceae, are a group of soil bacteria known for their remarkable ability to form symbiotic relationships with certain leguminous plants, particularly legumes [5]. This unique association leads to the development of specialized structures called root nodules [6]. While their primary function is nitrogen fixation, some strains of *rhizobia* also exhibit the ability to solubilize P in the soil. The phosphate-solubilizing ability of *rhizobia* is crucial for plant nutrition and contributes significantly to plant growth when used as a component of biofertilizers for crops. This mutualistic interaction between *rhizobia* and leguminous plants has significant agricultural and ecological importance.

Sesbania grandiflora, a small, erect, fast-growing, and sparsely branched perennial tree belonging to the Leguminosae family, creates a favorable rhizosphere environment that supports the growth of *rhizobium.* This study was conducted to assess and characterize *rhizobia* isolates from *Sesbania grandiflora* root nodules for their potential in P Solubilization and optimization of the PVK media for effective P solubilization.

2. METHODOLOGY

2.1 Seed Collection

Seeds of Sesbania grandiflora were collected in August 2023 from three locations in the *Anuradhapura, Puliyankulama* (DL1b) area of Sri Lanka.

2.2 Sample Preparation and Isolation of *Rhizobia* Using Trap Plant Method

The collected seeds were planted in pots containing soil samples known to have Reddish Brown Earth (RBE) [7] collected from the field (at the faculty of Agriculture, Rajarata University of Sri Lanka).

After approximately 45 days of planting, healthy pink root nodules were separated from the plants. Then, the separated nodules were placed on Yeast Manitol Agar (YMA) medium for isolated *rhizobia* strains. The plates were incubated at 28±2°C for 2-7 days. After incubation, four colors of single gummy colonies were successfully identified: (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish. To obtain pure cultures, the isolates were further purified by sub-culturing multiple times on the same medium.

2.3 Authentication of *Rhizobia* Isolates

All four isolated *rhizobia* cultures were subjected to check their authenticity using the following methods.

2.3.1 Acid alkaline production test

The production of acid and alkali was detected in this test by following the four treatments to grow on Yeast-extract-mannitol (YEM) broth supplemented with Bromothymol Blue (BTB). The change in color and the pH of the YEM broth was recorded after incubation at 28±2 ^{0}C for 24-48 hours [8].

2.3.2 CRYMA test

YMA media was prepared, the 2.5 ml Congo red was added to the solution, and it was put in Autoclave. The media was sterilized and it was poured into sterilized Petri plates and cultured isolated four treatments separately (Wijesundara *et al*., 2000).

2.3.3 BRYMA test

YMA media was prepared and the 1.25 ml BTB was added to the solution and it was put in Autoclave. The media was sterilized and it was poured into sterilized petri plates and cultured isolated four treatments separately (Wijesundara *et al*., 2000).

2.3.4 Microscopic observation

Four treatments were observed using a light microscope to identify morphological characteristics to trap the *rhizobia* colonies.

2.3.5 Gram staining test

Gram staining technique was used to differentiate gram-negative and gram-positive bacteria colonies using a microscope (Tripathi and Sapra, 2020).

2.4 Determination of P Solubilizing Index (PSI) on PVK Agar Medium

Four isolated Rhizobia strains produced a clear halo appearance in PVK agar solid media containing tricalcium phosphate [Ca_3 $(PO_4)_2$] as the phosphorus source. These media were incubated at 28±2°C for 3-7 days, and the strain with the highest PSI was selected as the most effective phosphorus solubilizer. After 7th day of incubation, a clear zone was observed around the colony of four isolates. Then zone diameter and colony diameter were measured in each four isolates. Finally, PSI was calculated using the following formula.

> Phosphate Solubilization Index (PSI)= (Colony diameter (mm)+Zone diameter(mm))/ (Colony diameter (mm))

(Saiyad *et al.,* 2015)

2.5 Evaluation of the Efficiency of Phosphate Solubilization in PVK Broth Medium

Quantification of solubilized P was assessed by measuring the available P content in PVK culture broths [9]. All samples were shaken at 1 rpm for 3-7 days at room temperature. After 3-7 days of culture, the media was filtered through the Whatman No. 42 filter paper (Kumari *et al*., 2010). Finally, supernatants were collected to estimate the available P concentration using the Murphy and Relay method (2002), and the remaining suspension was used to measure the pH values.

2.6 Identification of Effective P Solubilizer

The most effective P solubilizer among four isolates was determined based on the PSI and solubilized P.

2.7 Optimization of Media for Effective P Solubilizer

Different sources of N, P, C, were tested to optimize the media for higher solubilization of P with selected P solubilizer.

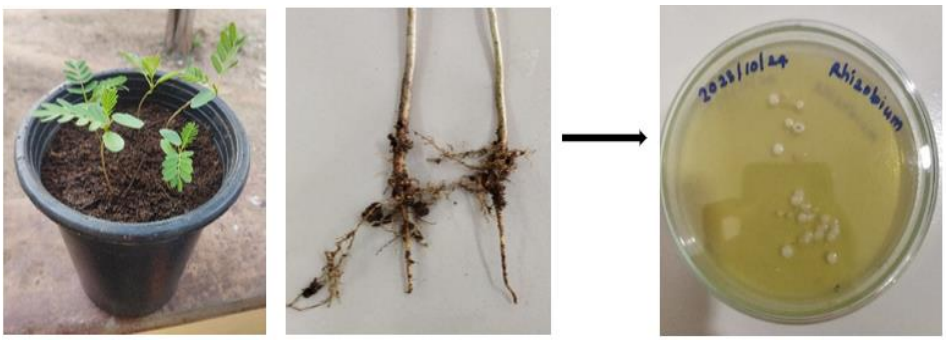

Fig. 1. Sesbania grandiflora nodules used for isolation of *rhizobia* using trap plants method

P sources are potassium dihydrogen phosphate [KH_2PO_4], sodium phosphate [Na_2PO_4], tri-calcium phosphate [Ca_3 $(PO_4)_2$], N sources are ammonium sulphate [$(NH_4)_2SO_4$], urea, and sodium nitrate [$NaNO_3$], C sources are glucose, fructose and sucrose were tested in PVK Broth. Three replicates were arranged for each nutrient sources as treatments separately. Culture broths were shaken at 1 rpm in room temperature 3-7 days (Fasim *et al.,* 2002). After 7 days, pH was measured and media was filtered through Whatman No. 42 filter paper and 1 ml of each culture was taken out to estimate the amount of solubilized P concentration using the Murphy and Relay method (2002).

2.8 Statistical Analysis

The experiment data were analyzed using R software, followed by ANOVA, CRD. Mean separations were done using LSD mean comparison test.

3. RESULTS AND DISCUSSION

3.1 Microscopic Observation

Isolates were acquired from the root nodules of *Sesbania grandiflora* plants, demonstrated rapid growth within a temperature range of 25 to 28°C. The colonies of the isolates are individually cultured for purification, as illustrated in Fig. 2. During the initial stages, colony appearances exhibited variation, encompassing four isolates colorless (a), whitish (b), yellowish (c), and pinkish (d) morphologies.

3.2 Authentication of *Rhizobia* Isolates

3.2.1 Acid alkaline production test

Inoculation of these isolates in YEM broth supplemented with BTB changed the color of the broth to yellow after five days of growth, showing the production of acid, which is characteristic of *rhizobia* [4]. The pH of the culture broth was also decreased to 5.4-6.6 from an initial pH of 7.0 (Table 1).

Table 1. The pH of the isolated *rhizobia* strains at the end of the acid alkaline production test

***Rhizobia* isolates**	**Acid alkaline production test (pH)**
a	6.71
b	6.61
c	6.61
d	5.91

The YEMA medium incorporated with bromothymol blue was streaked with active culture of bacterial isolates, incubated 3-4 days and was observed either for yellow colour due to production of acids (fast growers) or blue colour due to production of alkali (slow growers) as per method described by Somasegaran and Hoben [10].

3.2.2 CRYMA test

Rhizobia isolates colony on YEMA medium did not absorb the supplemented Congo red dye and by this distinguished *Rhizobium* from other bacteria [4]. The isolates demonstrated the ability to grow and absorb the Congo red dye in the YEMA media plates, serving as an authentication test for *rhizobia* in Fig. 3.

The purity of the isolates was assessed by the addition of Congo red in YEMA medium. Most *rhizobia* produce white colonies, whereas many other bacteria take up the dye strongly. This characteristic differentiates Rhizobium from other bacterial contaminants [10].

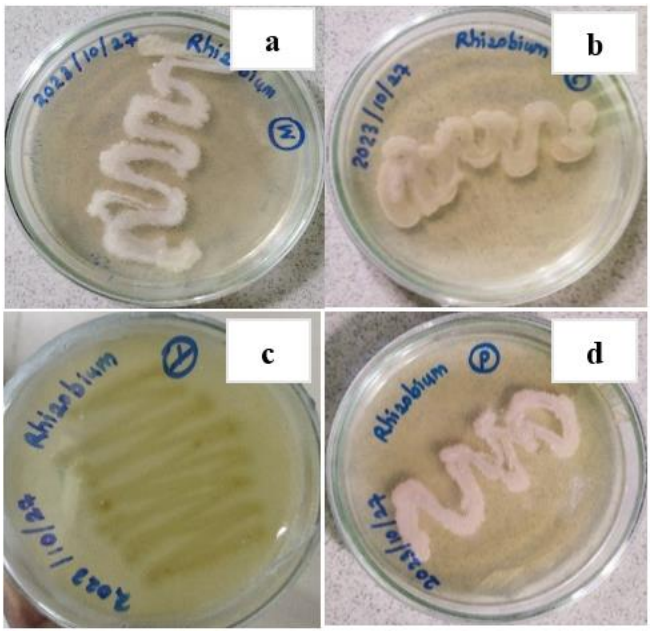

Fig. 2. Colony morphology of isolates from *Sesbania grandiflora* plant using trap plants method: (a) colorless, (b) whitish, (c) yellowish and (d) pinkish colonies

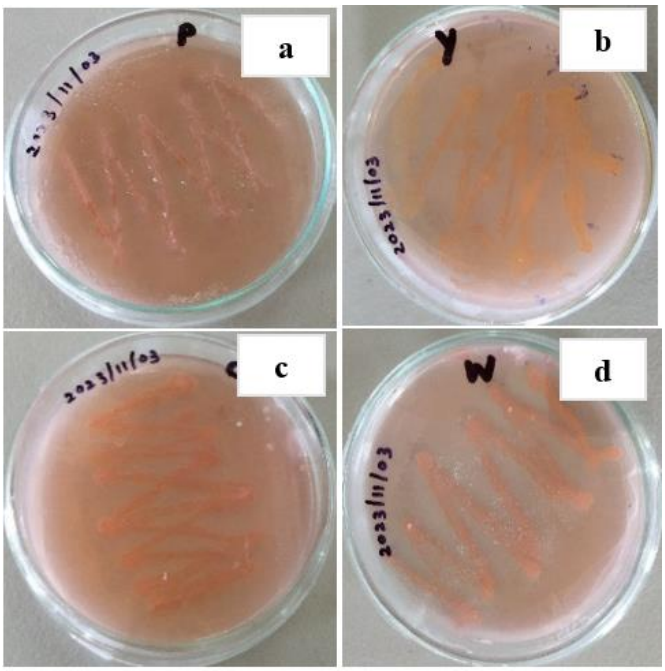

Fig. 3. Congo Red YEMA plates for CRYMA test authentication of *Rhizobium* from four isolated rhizobia strains, showing (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish colonies

3.2.3 BRYMA test

The isolates were able to produce a yellow color change in BTB added YEM media plates, which is an indicator of fast growth and serves as an authentication test for *Rhizobium*. The yellow color change occurs because acid producers react with the pH indicator BTB dye in an acidic reaction (Dhiman et al., 2022). In this study, all four isolates formed colonies on YEMA medium containing BTB. The isolates exhibited the ability to generate a yellow color signifying rapid growth and serving as an authentication test for *Rhizobia.*

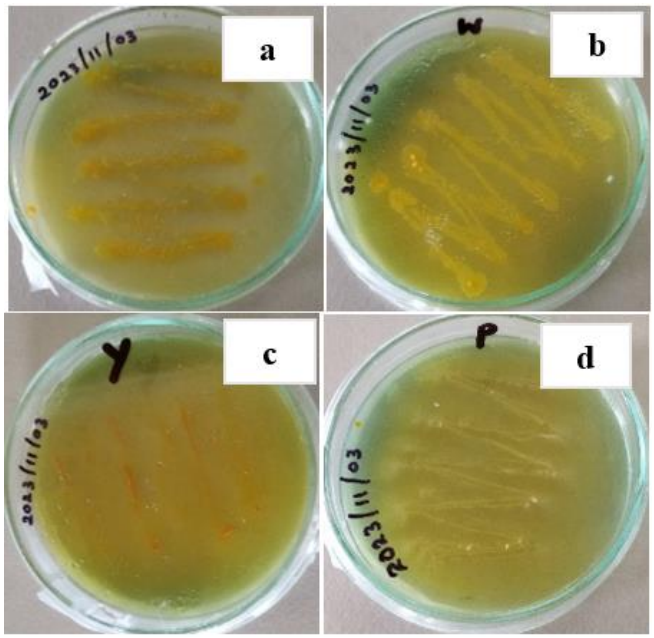

Fig. 4. BRYMA test as an authentication test of *Rhizobium* from four isolated rhizobia strains, showing (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish colonies

3.2.4 Microscopic observation

Four isolates were evaluated for their morphology under the microscope to authenticate *rhizobia*. The isolate “a” showed cocci-shaped cells, while the others (b, c, and d) exhibited rod-shaped structures.

Rhizobia typically exhibit a rod-shaped morphology, are aerobic, and possess motility [10]. The genus encompasses various species including *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium*, and *Sinorhizobium* (Berrada, 2014). Through a chemotactic response to flavonoid molecules released by the legume host, these bacteria form symbiotic relationships with legumes (Poonia, 2011).

3.2.5 Gram staining test

All isolates exhibited pink-colored cells in the gram staining test under microscopic observation. Gram-negative organisms typically appear pink or red in color (Tripathi and Sapra, 2020). Most rhizobia are gram-negative bacteria capable of inducing the formation of specialized organs called root nodules on leguminous host plants (Fauvart and Michiels, 2008). Accordingly, these gram staining results indicated that all isolates were as *rhizobia*.

3.3 Efficiency of Phosphate Solubilization

Four isolated *rhizobia* strains evaluated for their phosphate solubilization efficiency using PSI (Table 2)

Table 2. PSI for isolated *rhizobia* strains

***Rhizobia* isolates**	**PSI ± SE**
a	2.23±0.03
b	1.8±0.003
c	2.53±0.04
d	2.09±0.04

Among the isolated *rhizobia* strains, *rhizobia* "c" displayed the highest average PSI (2.53±0.04), indicating excellent P solubilization compared to the other *rhizobia* strains. These isolates hold tremendous potential in the near future for utilization as biofertilizers, not only improving phosphorus solubilization but also enhancing the overall plant growth of *Sesbania grandiflora.*

3.4 Efficiency of Phosphorus Solubilization in PVK Broth Medium

The significantly highest solubilization (36.90±4.75 ppm), was observed in isolate “c” compared to the other isolates. In this study, the reduction of pH in the culture media was synchronized with the process of P solubilization, as evidenced by the lowest pH value (4.97)

recorded in the medium containing *rhizobia* “c”, which also exhibited the highest solubilized P.

Table 3. pH changes during the P solubilization process

***Rhizobia* isolates**	**pH value**
a	5.17
b	5.12
c	4.97
d	5.44

The pH is a vital factor in solubilization, with P solubilization being the result of organic acid production. The pH of the media was adjusted to pH 7 using NaOH or HCl, and growth was recorded as described above. The results showed that the lowest pH value recorded, with *rhizobia* “c”, also exhibited the highest concentration of solubilized phosphorus.

3.5 Effective P Solubilizer

The rhizobia isolated from Sesbania grandiflora root nodules, particularly *rhizobia* "c," were identified as the most effective phosphorus solubilizers. This determination was made based on the highest PSI and its capacity to solubilize the highest phosphorus in PVK broth medium.

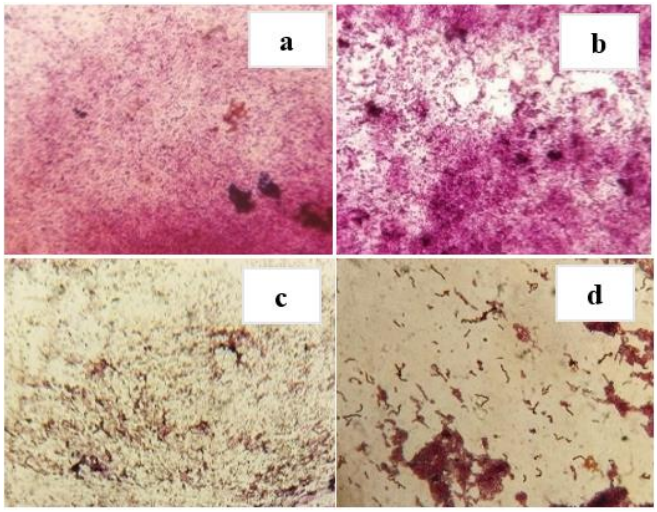

Fig. 5. Gram-negative colonies of *rhizobia* isolates authenticated by gram staining test, showing (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish

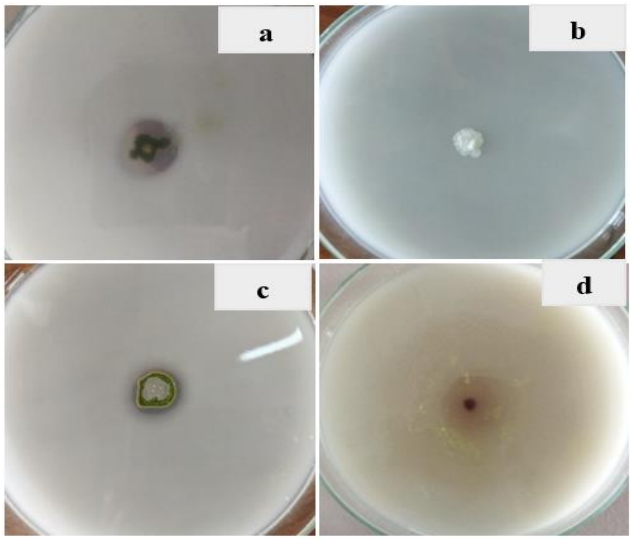

Fig. 6. P solubilization zones exhibited by four isolated *rhizobia* strains from Sesbania grandiflora on PVK agar medium plates; (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish

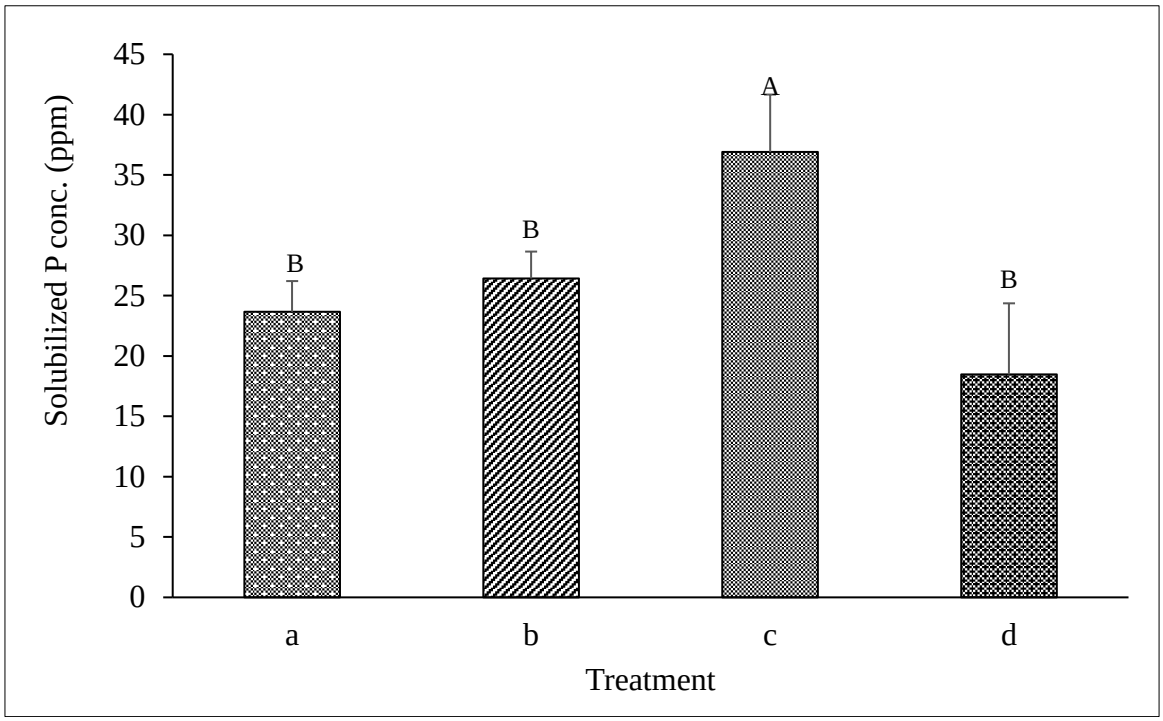

Fig. 7. Average solubilized P in PVK broth for four isolates; (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish. Vertical bars with different letters indicate statistically significant differences at the p ≤ 0.05 probability level according to the Least Mean Comparison test

3.6 Media Optimization for Effective P Solubilizer

Different sources of P, C, N were tested to optimize the PVK broth media for the highest solubilization of P with *rhizobia* "c".

The culture was grown in PVK liquid media with different P sources. (Fig. 8) The results showed that potassium dihydrogen phosphate was the best P source, with a solubilization rate of 35.1±0.46 ppm. Sodium phosphate exhibited moderate P solubilization, while the minimal solubilized P was recorded with tricalcium phosphate. *Rhizobia* "c" showed higher P solubilization with potassium dihydrogen phosphate than with other P sources. Potassium dihydrogen phosphate is an acidic source that directly provides $H_2PO_4^-$ ions, which are readily available for uptake by plants or microorganisms. This immediate availability of P can promote plant growth or support microbial activity. Potassium dihydrogen phosphate dissolves phosphorus more efficiently due to its high solubility in water (Nguyen et al., 1992).

The amount of glucose as a C source played an important role in the P solubilization. In this study, the effect of various C sources was investigated (Fig. 9). Glucose displayed the highest P solubilization at 25.47±0.49 ppm, while fructose showed the lowest P solubilization. PSB isolated from different C sources revealed that glucose resulted in the highest P solubilization accompanied by a decrease in pH [11].

While studying the effect of various N sources on the P solubilization (Fig. 10) it was found that ammonium sulphate recorded the significantly highest solubilized P at a rate of 3.2 ± 0.17 ppm, while the lowest solubilized P was observed with urea at 0.79 ± 0.61 ppm. In a previous study, the impact of different N sources solubilization was investigated by substituting five sources in the PVK medium: ammonium sulphate, sodium nitrate, potassium nitrate, calcium nitrate, and urea. It was found that ammonium sulphate resulted in the highest P solubilization (Sridevi and Mallaiah, 2009). Ammonium as the N source of several bacteria and fungi only have been reported to be able to solubilize phosphate (Illmer and Schinner, 1992; Lapeyrie, 1991). This finding aligns with previous reports indicating that many fungi and bacteria can solubilize phosphate effectively only in the presence of ammonium as the nitrogen source (Illmer *et al.,* 1995).

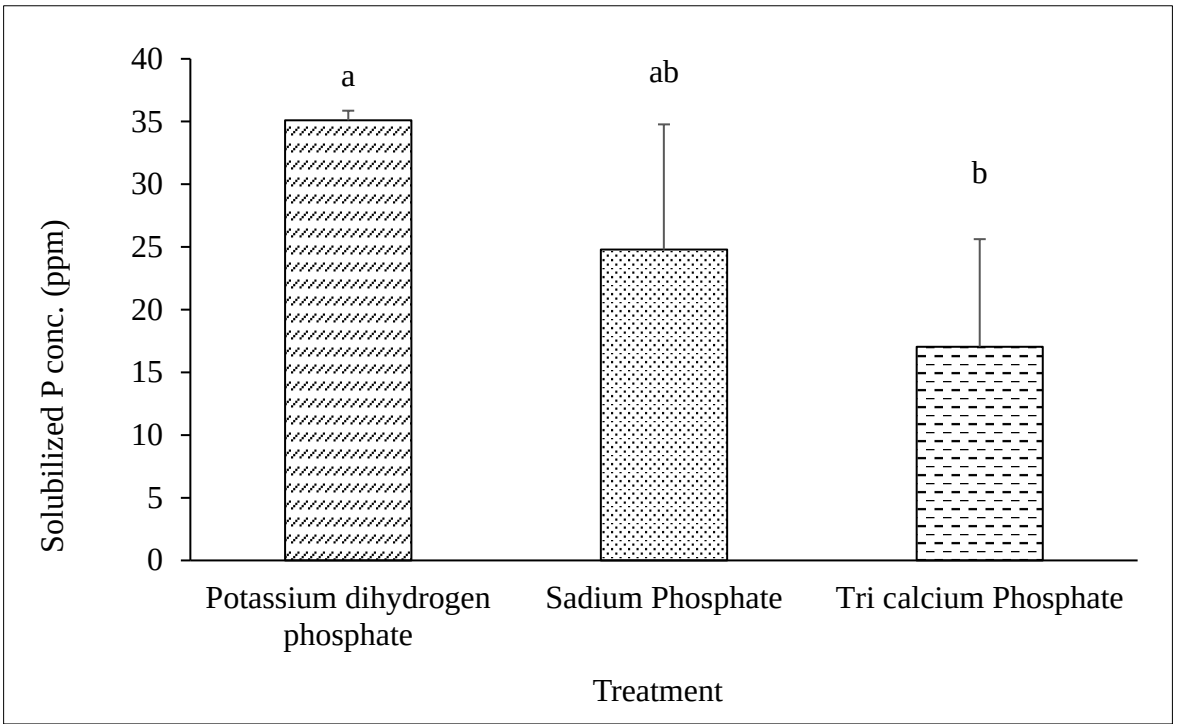

Fig. 8. Effect of various P sources on the efficiency of P solubilization with rhizobia "c". Vertical bars with different letters indicate statistically significant differences at p≤ 0.05 probability level to the Least Mean Comparison test

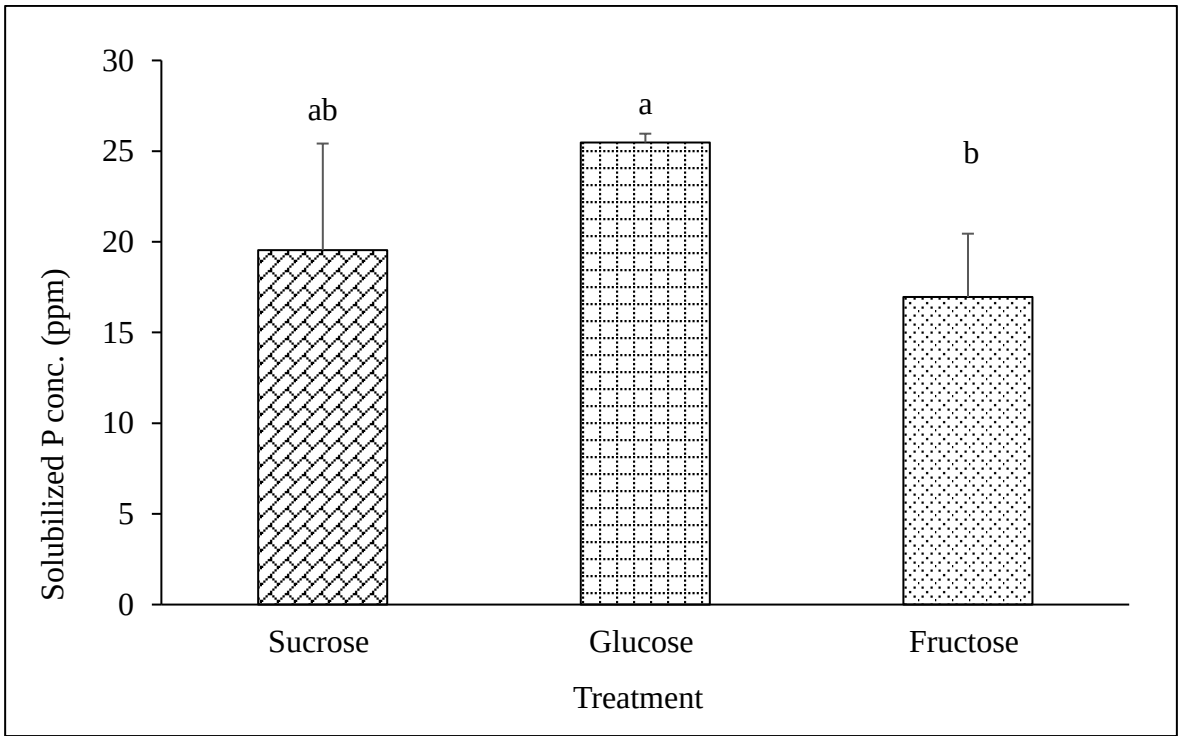

Fig. 9. Effect of various Carbon sources on the efficiency of P solubilization with *rhizobia* "c". Different letters indicate statistically significant differences at p≤ 0.05 probability level to the Least Mean Comparison test

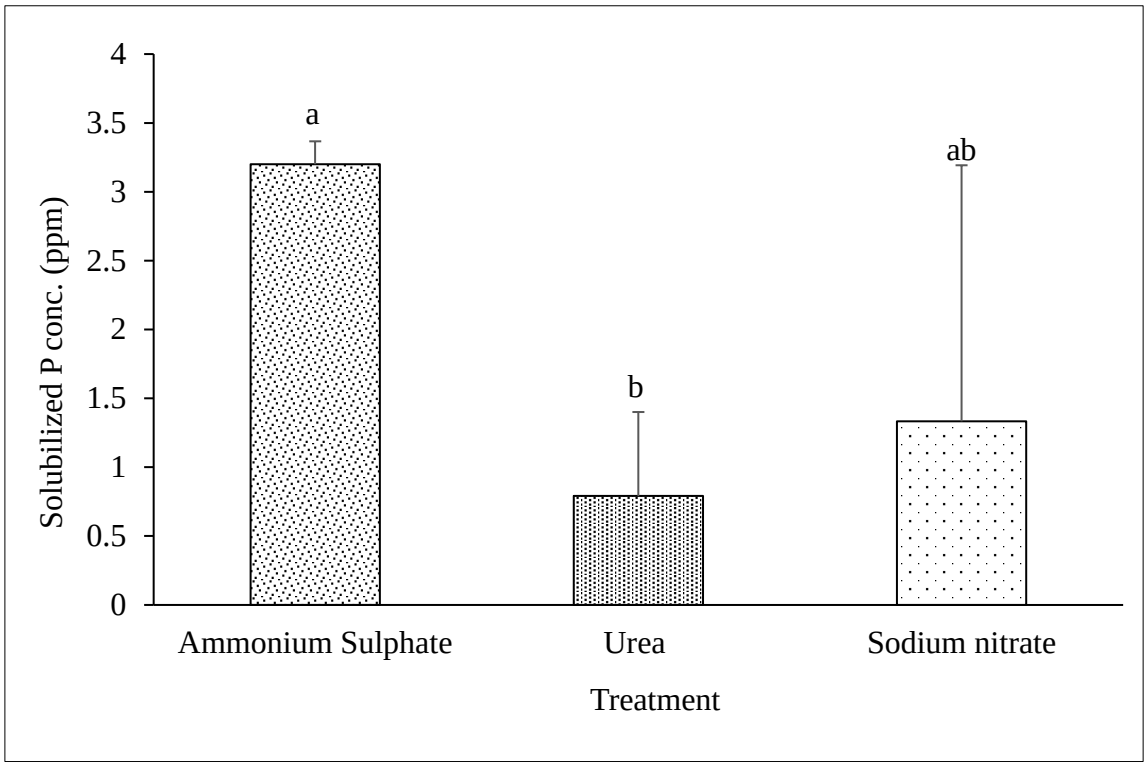

Fig. 10. Effect of various N sources on the efficiency of P solubilization with *rhizobia* "c". Different letters indicate statistically significant differences at p≤ 0.05 probability level to the Least Mean Comparison test

In another literature investigating the effect of various N sources on P solubilization, ammonium sulphate exhibited the highest P solubilization, followed by casein. Urea and sodium nitrate showed very low P solubilization [12,13]. In the control group with no N source, substantial growth and a decrease in pH were observed, along with slight P solubilization, likely due to yeast extract and glucose in the medium, which were utilized by bacteria as nitrogen sources [11].

4. CONCLUSION

Four bacterial strains (a,b,c,d) were isolated from *Sesbania grandiflora* root nodules and they were initially identified as *rhizobia* based on morphology and authenticated through gram-staining, acid-alkaline tests, CRYMA and BRYMA tests. Out of the four isolates tested, isolated *rhizobia* “c” was identified as effective P solubilizers based on PSI in PVK solid media and efficacy on PVK liquid media. Results of media optimization tests revealed that potassium dihydrogen phosphate as P source, glucose as C source and as N source performed better in the highest solubilization of P with modified PVK media with different nutrient sources. Moreover, this effective solubilizer *rhizobia* “c” showed good potential for developing an inoculum for soil with its optimized media.

However, further improvements would be essential prior introducing *Rhizobia* “c” as a P solubilizing inoculum.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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