In vitro Inorganic Phosphate Solubilization Tests of Cowpea Root Nodule Bacteria from Ethiopia

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ABSTRACT

Background: Phosphorus is one of the limiting plant nutrients in most soil due to its fixation with metal ions both in acidic and alkaline soil. However, some soil bacteria have potential to solubilize the cation-fixed phosphorus and make it available to plants.

Methods: Sixty-six cowpea root nodule bacterial species were tested for inorganic phosphate solubilization (PS). PS potential of the isolates from Ca\(_4\)\((PO_4)\)\(_2\), AlPO\(_4\) and FePO\(_4\) were tested on four different kinds of agar media. Solubilization efficiency was determined as Solubilization Index (SI) on agar media and quantity of bioavailable phosphorous on broth media.

Result: About 30% of the bacterial isolates, out of which 60% endophytes were Ca\(_4\)\((PO_4)\)\(_2\) solubilizers on Pikovskaya medium but did not grow on media containing AlPO\(_4\) and FePO\(_4\) as sole P sources. Isolates showed significant variation (p<0.05) in Ca\(_4\)\((PO_4)\)\(_2\) solubilization efficiency on the different culture media and ECE-21 (Pseudomonas putida) was versatile in solubilizing Ca\(_4\)\((PO_4)\)\(_2\) from the four test media. However, ECE-26A (Bacillus subtilis) showed the highest PS in broth media (140.8 mg ml\(^{-1}\)) indicating the moderate correlation (r=0.5578; p<0.05) of PS between solid and liquid media. Amounts of P solubilized and pH change of the culture filtrate was inversely correlated (r=−0.731; p<0.01). In general, ECE-21 was the most efficient P solubilizer (SI=2.67±0.1; P=87.74±7.4 mg ml\(^{-1}\)) solubilizers and hence can be an ideal candidate as inoculants for the crop production in Ethiopia after field evaluation.

Key words: Endophytes, Nodule bacteria, Phosphate solubilization, Rhizobia, Solubilization Index.

INTRODUCTION

On average, only 0.1% of phosphorus existing in the soil is supposed to be available for the plant use due to its fixation with soil cations (Zhu et al. 2011). To alleviate soil phosphorus deficiency, excessive amount of phosphorus fertilizers is applied to soil which can lead to the groundwater contamination and waterway eutrophication. Therefore, there is great interest to investigate the management strategies that are capable of improving the phosphorus fertilization efficiency which can increase crop yields and reduce environmental pollution. Some soil microorganisms are reported to undergo solubilization and mineralization processes and release immobilized P for the plant use. Bacteria are common microorganisms that can solubilize the cation-fixed phosphate for plant use (Sengupta et al., 2018).

Some species of nodulating, non-nodulating and rhizospheric bacteria from the genera of Bacillus, Bradyrhizobium, Burkholderia, Gluconacetobacter, Mesorhizobium, Microbacterium, Paenibacillus, Pseudomonas and Rhizobium are known for their solubilization properties of metal fixed phosphate in the soil (Marra et al., 2012; Sengupta et al., 2018). Phosphate solubilizing (PS) microorganisms have been screened by a plate assay method on Pikovskaya (PVK) agar (Pikovskaya, 1948) depending on halo/clear zone formation because of organic acids production into the surrounding medium (Tyagi et al., 2002). However, the reliability of this halo-based technique is questioned as many isolates which did not produce any visible halo/zone on agar plates could solubilize the various types of insoluble inorganic phosphates in liquid medium (Gupta, et al., 1994). Besides, the PS potential of soil bacteria from different metal fixed phosphate also varies (Marra et al., 2011; Thakur et al., 2014). However, the ability of microbial isolates to solubilize the phosphate from different metals on different culture media could show the suitability of the isolates as inoculants under field condition (Saída, 2015). In general, inoculation of legumes with PS bacteria improves the growth and yield of crop (Sengupta et al., 2018). However, PS properties of cowpea root nodule bacteria from soil of Ethiopia are yet not studied. Therefore, this experiment was aimed to test the PS properties of cowpea root nodule bacterial isolates as inoculant for the crop production in Ethiopia.

MATERIALS AND METHODS

Sources of the root nodule bacterial isolates

The cowpea root nodule bacterial isolates were previously
collected from different parts of Ethiopia and stored with 30% glycerol (HIMEDIA, India) at -20 °C temperature in Addis Ababa University, Ethiopia. Applied Microbiology Laboratory for further studies. Totally, 66 cowpea root nodule bacterial isolates were included in this study of which 26 isolates were endophytes (non-nodule inducing bacteria during authentication). The rhizobial isolates were previously characterized for phenotypic and genotypic properties (Girma et al., 2019).

Inorganic phosphate solubilization (PS) tests

The isolates were tested for inorganic PS from Ca_{3}(PO_{4})_{2}, AlPO_{4} and FePO_{4} on solid Pikovskaya’s (PVK) medium in the duration of September, 2019 to March, 2020 in Wollega University, Ethiopia. In the medium containing AlPO_{4}, the pH was adjusted to 5, while in the medium containing Ca_{3}(PO_{4})_{2} and FePO_{4} the pH was adjusted to 7.0. Culture media preferences of the isolates for Ca_{3}(PO_{4})_{2} solubilization was checked on solid PVK medium, Basal Sperber (BS) medium, National Botanical Research Institute’s Phosphate (NBRIP) medium and NBRIP devoid of yeast extract (NBRiY) medium (Pikovskaya, 1948; Nautiyal, 1999). Precipitation of the insoluble phosphate in the three different media was verified by a “milky” appearance. Care was taken to spread the insoluble phosphate in the medium throughout the plate by agitating it carefully during pouring while it was still liquid at a temperature around 60 °C. Solubilization index (SI) on solid medium was calculated as

\[ SI = \frac{\text{Halo diameter (mm)}}{\text{Colony diameter (mm)}} \]

Halo diameter was obtained by subtracting colony diameter from total diameter (Marra et al., 2011).

PS was also quantitatively determined on PVK liquid medium. 50 ml of PVK medium containing Ca_{3}(PO_{4})_{2} was adjusted at pH 7 and inoculated with 25 μl of bacterial suspension (10^9 cells ml^-1) in 125 ml flask. The culture was incubated on rotary shaker at 120 rpm for 15 days. Starting from the second day of incubation every three days, 8 ml of the aliquots was transferred into test tubes and centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred into other test tubes, autoclaved and filtered through 0.22 μm membrane. The pH changes associated with PS was measured using pH meter (DELTA OHM, HD 8602). One milliliter of cell-free supernatant was mixed with 3 ml of distilled water and 1 ml of ammonium molybdate-vanadat (Barton’s Reagent) and the mixture was incubated for 20 min in dark room (Islam et al., 2007). Quantity of bioavailable phosphorous as microgram of P from 1ml of PVK medium containing Ca_{3}(PO_{4})_{2} was estimated with spectrophotometer (Jenway, UV-7804C, 470 nm) using a standard curve prepared with medium containing different concentrations of KH_{2}PO_{4}. The inoculated culture media that has no the phosphate sources were used as control.

Data analysis

Data were analyzed using a statistical software program version 8.0. A univalent general linear model was used to test the effect of culture media on PS potential of the isolates at α=0.05. Comparison of PS potential of the isolates was analyzed by one-way ANOVA at α=0.05. Pearson correlations were tested for PS of the isolates on solid media (SI), on liquid media (P, μg/ml) and pH change of the culture filtrate associated with the PS.

RESULTS AND DISCUSSION

Phosphate solubilization (PS) properties of nodule bacterial isolates

Isolates failed to solubilize phosphate in AlPO_{4} and FePO_{4} sources and poorly grew on the culture media containing the phosphate sources (data not shown). Despite differences in bacterial isolates, previous reports also showed the poor PS potential of cowpea nodule bacteria from AlPO_{4} and FePO_{4} sources (Marra et al., 2011). However, later Marra (2012) found about 21% of AlPO_{4} solubilizing cowpea nodule bacteria, while none displayed the ability to solublize from FePO_{4}. Therefore, AlPO_{4} and FePO_{4} solubilizers are uncommon among cowpea nodule bacteria that could be associated with sensitivity of the isolates to toxicity of the metals (Rogers, 2001) which increase along with the solubilization. The data showed that 20% of the rhizobial isolates and 46% of the endophytes solubilized Ca_{3}(PO_{4})_{2} on at least one of the four kinds of the culture media used for the experiment. The isolates showed highly significant variation (p<0.05) in PS on the growth media. The highest mean SI was 1.37 on PVK medium which was significantly higher (p<0.05) than mean SI on the other three culture media (BSA/NBRIP and NBRIP) (Table 1). The number of isolates that solubilized Ca_{3}(PO_{4})_{2} on the four culture media also varied. However, isolates that able to solubilize Ca_{3}(PO_{4})_{2} on either of the three growth media (BSA/NBRIP/ NBRiY) also solubilized the phosphate source in PVK medium except ECE-42 (Table 1). This might show the suitability of PVK medium for evaluating PS in cowpea nodule bacteria under in vitro conditions that stated the high PS efficiency of soil bacteria on NBRIP and PVK (Nautiyal, 1999; Tyagi et al., 2002).

In this experiment, ECE-21 (Pseudomonas putida) was the most versatile phosphate solubilizing isolates as it solubilized Ca_{3}(PO_{4})_{2} on the four kinds of culture media. In addition, the rhizobial isolates like ECR-10, 14 and 62 also solubilized Ca_{3}(PO_{4})_{2} on at least three of the four kinds of the solid culture media including in PVK liquid medium (Table 1). Isolates that are versatile in PS from different culture media are supposed to be competitive and persistent under varied edaphic conditions, which are an important trait for bioinoculant selection (Saida, 2015. The maximum phosphate SI on PVK medium was 2.8±0.45 by ECE-26A that was identified as species of Bacillus subtilis (Table 1). In addition, phosphate SI of the endophytic bacterial isolates tested in this experiment was higher than the maximum phosphate SI of rhizobial isolates (1.07±0.15). Cowpea nodule bacteria from Brazil and India showed similar
phosphate SI that ranged in 1.04 - 3.55, of which 78% showed phosphate SI < 2.0 (Marra et al., 2012; Sengupta et al., 2018). According to the categories of Marra et al. (2012), PS of cowpea rhizobia from soils of Ethiopia is grouped as low solubilizer (SI<2) and 42% of the endophytic bacterial isolates was intermediate phosphate solubilizers (2<SI<4).

Cowpea nodule bacteria isolated from soils of Ethiopia showed variation in PS of Ca\(_2\)(PO\(_4\))\(_2\) in liquid medium that ranged in 2.17-140.83 µg ml\(^{-1}\) and ECE-26A was the highest in P solubilization (Table 1). Similar to SI on solid media, quantity of P solubilized by the endophytic isolates was higher than P solubilized by rhizobial isolates in liquid medium (Table 1). Quantity of P solubilized by the present isolates is comparable to the one reported by Marra et al. (2011) (a maximum of 95 µg/ml) for the same crop although there is difference in bacterial species. The amount of bioavailable P in liquid culture medium was inversely correlated (r=-0.731; p<0.01) to pH of culture filtrate, indicating the importance of acid production during the solubilization process that is the characteristics of fast-growing rhizobacterial strains (Abbaszadeh et al., 2012). Marra et al. (2011) also reported inorganic PS as common properties of acid producing which are fast-growing cowpea rhizobacterial strains. However, in this study, non-acid producing isolates were also inorganic phosphate solubilizer under in vitro conditions, similar to the previous reports (Jadhav, 2013). Particularly, the slow-growing bradyrhizobial isolates such as ECR-14 and ECR-68 were inorganic phosphate solubilizers (Table 1). Therefore, PS among non-acid producing isolates could be associated with the release of specific ions or compounds in the culture that can substitute metal ion of the mineral phosphate (Park et al., 2009).

The correlation between amount of P released in liquid media and SI on agar media was moderately positive (r=0.5578; p<0.05). However, Mihalache et al. (2015) reported the absence of correlation between PS of rhizobacteria on solid media and in liquid media. Therefore, testing PS both under liquid and solid media helps to select efficient phosphate solubilizing isolates, although studies have indicated the higher PS efficiency of isolates in liquid medium than on solid medium (Nautiyal, 1999). Cowpea nodule bacterial isolates that solubilized Ca\(_2\)(PO\(_4\))\(_2\) were from the genera of Bacillus, Bradyrhizobium, Brevibacillus, Enterobacter, Escherichia, Pseudomonas, Rhizobium, Serratia and Sphingobium (Girmaye et al., 2019). The most efficient phosphate solubilizing endophytes in liquid medium

### Table 1: Phosphate solubilization properties of cowpea nodule bacterial isolates on solid media of BSA, PVK, NBRIP and NBRIY and liquid medium of PVK and pH change.

| Isolates | Most similar species | Accession | Solubilization Index (SI) on solid media | P (µg/ml) in liquid medium | pH |
|----------|---------------------|-----------|-----------------------------------------|---------------------------|-----|
| ECR-9    | Rhizobium sp.       | NH        | 1.11±0.2                                | 4.93±1.2\(^b\)            | 6.01|
| ECE-10A  | Sphingobium yanoikuyae | KY412529  | 0.13±0.4                                | 8.06±2.1\(^b\)            | 5.16|
| ECR-10   | Rhizobium sp.       | NH        | 1.13±0.3                                | 3.66±1.0\(^b\)            | 5.87|
| ECR-14   | Bradyrhizobium japonicum | KY368580  | 1.21±0.6                                | 2.71±1.1\(^b\)            | 5.88|
| ECE-15A  | Brevibacillus borstelensis | KY412531  | 1.15±0.5                                | 90.55±8.3\(^b\)           | 4.41|
| ECE-15B  | Bacillus pumilus    | KY412532  | 1.07±0.2                                | 9.58±3.2\(^b\)            | 5.5 |
| ECE-21   | Pseudomonas putida  | KY412533  | 1.45±0.6                                | 87.74±7.4\(^c\)           | 4.98|
| ECE-24   | Rhizobium rubi      | KY368581  | 0.88±0.1                                | 2.17±0.9\(^b\)            | 5.93|
| ECE-26A  | Bacillus subtilis   | KY412535  | 2.8±0.5                                 | 140.82±10.4\(^b\)         | 4.07|
| ECE-30   | Pseudomonas protegens | KY412536  | 0.10±0.1                                | 18.10±2.6\(^b\)           | 5.79|
| ECE-36B  | Pseudomonas putida  | KY412539  | 1.52±0.2                                | 94.66±7.0\(^d\)           | 5.17|
| ECE-38   | Enterobacter sp.    | KY412540  | 1.5±0.7                                 | 5.69±1.2\(^b\)            | 6.54|
| ECE-42   | Pseudomonas taenensis | KY412542  | 0.06±0.3                                | 78.45±9.2\(^d\)           | 5.36|
| ECR-45   | Rhizobium sp.       | NH        | 0.06±0.3                                | 5.56±2.1\(^b\)            | 5.97|
| ECR-55   | Rhizobium rubi      | KY368585  | 0.10±0.2                                | 11.52±2.1\(^b\)           | 5.99|
| ECR-62   | Bradyrhizobium japonicum | KY368586  | 0.45±0.2                                | 30.77±3.5\(^c\)           | 6.13|
| ECR-68   | Bradyrhizobium sp.  | KY368589  | 0.73±0.2                                | 2.03±0.7\(^b\)            | 6.02|
| ECE-70   | Escherichia hermanni | KY412545  | 0.21±0.7                                | 21.32±2.1\(^d\)           | 6.02|
| ECE-80B  | Pseudomonas protegens | KY412546  | 0.10±0.1                                | 15.74±2.1\(^d\)           | 5.46|
| ECE-82B  | Serratia proteamaculans | KY412548  | 0.21±0.8                                | 81.04±6.8\(^d\)           | 5.23|

**BSA**, Basal Sperber agar medium; **PVK**, Pikovskaya agar medium; **NBRIY**, National Botanical Research Institute’s Phosphate agar medium; **NBRIY**, NBRIY devoid of yeast extract agar culture medium; **NI**, Not identified. Quantity of soluble P in liquid medium along a column designated by the same latter were not significantly different from each other at α=0.05; similarly, mean SI on different culture media across a row (at the end of the row) designated by the same latter were not significantly different from each other at α=0.05.
(in decreasing order from left to right) were isolated from the genera of *Bacillus*, *Pseudomonas*, *Brevibacillus* and *Serratia* (Table 1). PS rhizobial isolates showed closest similarity to the species of *Bradyrhizobium* sp. (ECR-68), *Bradyrhizobium japonicum* (ECR-14, 62) and *Rhizobium rubi* (ECR-24) but not from the genera of *Mesorhizobium*, *Paenibacillus* and *Agrobacterium*. However, the PS properties of these genera were previously reported from the rhizosphere of different hosts (Stajkovic et al., 2009). In this experiment, nodule bacterial isolates identified as the same species (*Bradyrhizobium japonicum*, *Rhizobium rubi*) showed significant variation (p<0.05) in mineral PS potential. For instance, PS potential of *Bradyrhizobium japonicum* ECR-62 was 15 folds higher than the PS of *Bradyrhizobium japonicum* ECR-14 in liquid media. Similarly, quantity of P released by *Rhizobium rubi* ECR-55 was fivefold higher than *Rhizobium rubi* ECR-24 (Table 1). Inorganic phosphate solubilizing rhizobial isolates are presumed to be effective in biological nitrogen fixation, given that nodulated plants require more phosphorus than non-nodulating plants (Sengupta et al., 2018). Therefore, rhizobial isolates such as ECR-9, 10, 14, 24, 45, 55, 62 and 68 could be potential inoculants for cowpea production under limited availability of soil phosphorus and nitrogen in Ethiopia.

**CONCLUSION**

Cowpea nodule bacteria can use phosphate of Ca$_3$(PO$_4$)$_2$ but not in the form of AlPO$_4$ and FePO$_4$. PS efficiency of the isolates was maximum on PKV solid medium. SI of the isolates on solid medium also showed similar pattern with their performance in the liquid medium. Quantity of phosphate solubilized was inversely correlated to pH of the culture filtrate. Root nodule bacteria like *Pseudomonas putida* ECE-21 was the most efficient phosphate solubilizer which can be presumed as candidate of inoculant in phosphorized fixed soils of Ethiopia after evaluation under field environment.

**REFERENCES**

Abbaszadeh, L.P., Savaghebi, R., Asadi-rahmani, H., Rejali, F., Farahbakhsh, M., Moteshareh-zadeh, B., Omidvari, M. and Lindstrom, K. (2012). Symbiotic effectiveness and plant growth promoting traits in some *Rhizobium* strains isolated from *Phaseolus vulgaris*. Plant Growth Regul. 68: 361-370.

Girmaye, K., Fassil, A. and Mussie, H.Y. (2019). Phenotypic and genotypic characteristics of cowpea rhizobia from soils of Ethiopia. African Journal of Biotechnology. 17(42): 1299-1312.

Gupta, M., Chyi, Y.S., Romero-Severson, J. and Owen, J.L. (1994). Amplification of DNA markers from evolutionarily diverse genomes using simple primers of simple-sequence repeats. Theor. Appl. Genet. 89: 998-1006.

Islam, M.T., Deora, A., Hashidoko, Y., Rahman, A., Ito, T. and Tahara, S. (2007). Isolation and identification of potential phosphate solubilizing bacteria from the rhizoplane of *Oryza sativa* L. cv. BR29 of Bangladesh. Z. Naturforsch. 62: 103-110.

Jadhav, R.N. (2013). Isolation of rhizobia from soybean cultivated in Latur area and study of its phosphate solubilization activity. Biosci. Discovery. 4: 100-103.

Tyagi, M.K., Singh, C.P. and Sharma N.L. (2002). Interactive effect of *Rhizobium* and Phosphate Solubilizing Micro-organisms on the Solubilization of Insoluble Phosphate. Agric. Sci. Digest. 22: 71-74.

Marra L.M., Soares, C.R., Oliveira, S.M., Ferreira, P.M., Soares, B.L., Carvalho, R.F., Lima, J.M. and Moreira, F.M. (2012). Biological nitrogen fixation and phosphate solubilization by bacteria isolated from tropical soils. Plant and Soil. 35(7): 289-307.

Marra, L.M., de Oliveira, S.M., Soares, C.R. and Moreira, F.M. (2011). Solubilisation of inorganic phosphates by inoculant strains from tropical legumes. Scientia Agricola. 68: 603-609.

Mihalache, G., Zamfirache, M., Mihasan, M., Ivanov, I., Stefan, M. and Raus, L. (2015). Phosphate-solubilizing bacteria associated with runner bean rhizosphere. Arch. Biol. Sci. Belgrade. 67: 793-800.

Nautiyal, C.S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170: 265-270.

Park, K.H., Lee, C.Y and Son, H.J. (2009). Mechanism of insoluble phosphate solubilization by *Pseudomonas fluorescens* RAF15 isolated from ginseng rhizosphere and its plant growth-promoting activities. Lett. Appl. Microbiol. 49: 222-228.

Thakur, D., Kaushal, R. and Shyam, V. (2014). Phosphate solubilising microorganisms: Role in phosphorus nutrition of crop plants. Agri. Review. 35(3): 159-171.

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiologiya. 17: 362-370.

Rogers, N.J., Carson, K.C., Glenn, A.R., Dilworth, M.J., Hughes, M.N. and Poole, R.K. (2001). Alleviation of aluminum toxicity to *Rhizobium leguminosarum* bv. vicieae by the hydroxamate siderophore vicibactin. BioMetals. 14: 59-66.

Salea, A., Francisco J.O., Manuel, M., Amin, L., Mohammed, B. and Abdelhay, A. (2015). Isolation and screening of bacteria from rhizospheric soils of rice fields in Northwestern Morocco for different plant growth promotion (PGP) activities: An *in vitro* study. Int. J. Curr. Microbiol. Appl. Sci. 4(1): 260-269.

Sengupta, A., Gunri, S.K. Biswas, T. and Saha, J. (2018). Efficacies of freshly isolated phosphate solubilising bacteria (PSB) on growth promotion in groundnut (*Arachis hypogaea* L.) upon commonly used PSB biofertilizers in eastern India. Legume Research-An International Journal. DOI: 10.18805/LR-4020.

Stajkovic, O., Meyer, S.D., Milicić, B., Willems, A. and Delić, D. (2009). Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa*). Botanica Serbica. 33: 107-114.

Zhu, F., Qu, L., Hong, X. and Sun, X. (2011). Isolation and characterization of a phosphate solubilizing halophilic bacterium *Kushneria* sp. YCWA18 from Daqiao Saltern on the coast of Yellow Sea of China. Evid. Based Complement. Alternat. Med. 61: 32-50.