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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 $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ were tested on four different kinds of agar media. Solublization 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 $Ca_3(PO_4)_2$ 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 $Ca_3(PO_4)_2$ solubilization efficiency on the different culture media and ECE-21 (*Pseudomonas putida)* was versatile in solubilizing $Ca_3(PO_4)_2$ from the four test media. However, ECE-26A (*Bacillus subtilis)* showed the highest PS in broth media (140.8 µg 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±7.4µg ml^{-1}) solublizers 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 (Saida, 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.

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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 (Girmaye *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 $Ca_3(PO4)_2$ 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 2nd 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 $Ca_3(PO4)_2$ was estimated with spectrophotometer (Jenway, UV- 7804C, 470 nm) using a standard curve prepared with medium containing different concentrations of KH_2PO_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 solubilize 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 $Ca_3(PO4)_2$ 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, NBRIY and NBRIP) (Table 1). The number of isolates that solubilized $Ca_3(PO4)_2$ on the four culture media also varied. However, isolates that able to solubilize $Ca_3(PO4)_2$ 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 $Ca_3(PO_4)_2$ 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 $Ca_3(PO_4)_2$ were from the genera of *Bacillus, Bradyrhizobium, Brevibacillus, Enterobacter, Escherichia, Pseudomonas, Rhizobium, Serratia and Sphingobiu* (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	pH
			BSA	PVK	NBRIP	NBRIY	PVK medium	
ECR-9	*Rhizobium* sp.	*NI*	1.11±0.2	1.02±0.4	0	0	4.93±1.2^{ij}	6.01
ECE-10A	*Sphingobium yanoikuyae*	KY412529	0	1.36±0.4	0	1.12±0.2	8.06±2.1^{hi}	5.16
ECR-10	*Rhizobium* sp.	*NI*	1.12±0.3	1.21±0.1	1.23±0.2	0	3.66±1.0^{ij}	5.87
ECR-14	*Bradyrhizobium japonicum*	KY368580	1.21±0.6	0.97±0.2	1.5±0.2	0	2.71±1.1^{ij}	5.88
ECE -15A	*Brevibacillus borstelensis*	KY412531	0	1.15±0.5	1.32±0.2	0	90.55±8.3^{b}	4.41
ECE -15B	*Bacillus pumilus*	KY412532	1.07±0.2	1.15±0.4	1.30±0.1	0	9.58±3.2^{hi}	5.5
ECE -21	*Pseudomonas putida*	KY412533	1.45±0.6	2.67±0.1	2.20±0.2	1.22±0.2	87.74±7.4^{bc}	4.98
ECR-24	*Rhizobium rubi*	KY368581	0	0.88±0.1	0	0	2.17±0.9^{jk}	5.93
ECE -26A	*Bacillus subtilis*	KY412535	0	2.8±0.5	0	0	140.82±10.4^{a}	4.07
ECE -30	*Pseudomonas protegens*	KY412536	0	1.09±0.1	0	1.1±0.1	18.10±2.6^{g-i}	5.79
ECE -36B	*Pseudomonas putida*	KY412539	1.52±0.2	2.1±0.1	0	1.67±0.2	94.66±7.0^{b}	5.17
ECE -38	*Enterobacter* sp.	KY412540	0	1.5±0.7	0	1.40±0.1	5.69±1.2^{hi}	6.54
ECE -42	*Pseudomonas taenensis*	KY412542	0	0	0	1.7±0.2	78.45±9.2^{b-d}	5.36
ECR-45	*Rhizobium* sp.	*NI*	0	1.06±0.3	0	0	5.56±2.1^{ij}	5.97
ECR-55	*Rhizobium rubi*	KY368585	0	1.07±0.2	0	0	11.52±2.1^{hi}	5.99
ECR -62	*Bradyrhizobium japonicum*	KY368586	0	1.45±0.2	1.12±0.1	1.22±0.2	30.77±3.5^{fg}	6.13
ECR-68	*Bradyrhizobium* sp.	KY368589	0	0.73±0.2	0	0	2.03±0.7^{jk}	6.02
ECE -70	*Escherichia hermanni*	KY412545	0	2.12±0.7	0	0	21.32±2.1^{gh}	6.02
ECE -80B	*Pseudomonas protegens*	KY412546	0	1.09±0.3	1.07±0.1	0	15.74±2.1^{g-i}	5.46
ECE -82B	*Serratia proteamaculans*	KY412548	0	2.1±0.8	0	0	81.04±6.8^{b-d}	5.23
	Mean SI		0.37±0.6^{b}	1.37±0.7^{a}	0.48±0.7^{b}	0.47±0.7^{b}		

"BSA", Basal Sperber agar medium; "PVK", Pikovskaya agar medium; "NBRIP", National Botanical Research Institute's Phosphate agar medium; "NBRIY", NBRIP 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 isolates 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 japonicium, Rhizobium rubi*) showed significant variation ($p<0.05$) in mineral PS potential. For instance, PS potential of *Bradyrhizobium japonicium* ECR-62 was 15 folds higher than the PS of *Bradyrhizobium japonicium* 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 AlPO4 and FePO4. PS efficiency of the isolates was maximum on PVK 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 phosphorous fixed soils of Ethiopia after evaluation under field environment.

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