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Isolation, Evaluation and Characterization of Phosphate Solubilizing Bacteria Associated with Soybean in Major Growing Agroecologies of Ethiopia

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ፎስፈረስ የተባለው ንጥረ-ነገር በብዛት በአፈር ውስጥ የሚገኙ ዕፅዋቶች በቀላሉ መጥጠው ሊጠቀመበት በማይችሉት ጠጣር መልክ የሚገኝ ነው። ይሄም የሚሆነው በአፈር ውስጥ ከሚገኙ ሌሎች ንጥረ-ነገሮች ጋር ጠንካራ ጥምረት በመፍጠር በቀላሉ በዉኃ የማይሟሟ ውህድ ስለሚፈጥር ነው። ከዚህም የተነሳ ተክሎች የሚገጥማቸውን የፎስፈረስ ንጥረ-ነገር እጥረት ለማሻሻል ከሚወሰዱ አማራጭ መፍትሄዎች ውስጥ የፎስፈረስ ውህድ አሟሚ ደቂቅ አካላት ለረጅም ጊዜ ትኩረት ተሰጥቶባቸው በመጠናት ላይ ይገኛሉ። ይሄም ጥናት ያተኮረው እነዚህን ደቂቅ አካላት በመጠቀም በኢትዮጵያ ውስጥ ሰፊ ኮምጣጣ አፈር ከመኖሩ ጋር ተያይዞ ያለውን ለዕፅዋቶች በቀላሉ ሊመጠጥ የማይችለውን የፎስፈረስ ንጥረ-ነገር በማሟት ለዕፅዋቶች እንዲቀርብ በማስቻል በአኩሪ አተር ምርት ላይ ያለውን ውጤታማነት ለመገምገም ነው። ለዚህም ጥናት ከአኩሪ አተር ስር ጋር ከተያያዘ አፈር አምስት የፎስፈረስ ውህድ አሟሚ ደቂቅ አካላት በቤተ ሙከራ ውስጥ ተለይተዋል። የነዚህም ደቂቅ አካላት ክላልሼም፣ ክብረት እና ከአልሙኒየም ጋር ውህድ የፈጠረን ፎስፈረስ የማሟት ብቃታቸው ተጠንቷል። በተጨማሪም የተወሰነ የዘረ-መላቸውን አካል (16S-23S rRNA region) በመጠቀም የደቂቅ አካላቱ የዝርያ ዓይነት ተለይቷል። ብሎም የእነዚህ ደቂቅ አካላት በምርት ላይ ሊያመጡት የሚችሉት ጭማሪ በመስክ ላይ በስድስት ቦታዎች ላይ ተግምግሟል። በጥናቱም መሠረት ደቂቅ አካላቱ ፎስፈረስን ክላልሺየም፣ ክብረትና ከአልሙኒየም ውህድ ውስጥ የማሟት ባህሪ እንዳላቸው ተረጋግጧል። እንዲሁም የደቂቅ አካላቱ ዘረመል ሲጠና አንዱ ሲደሞናስ ክሚባለው የደቂቅ አካላት ዝርያ የሚመደብ ሲሆን አራቱ ደግሞ ባሲለስ ተብለው ከሚጠሩት የዝርያ ዓይነቶች የሚመደቡ መሆናቸው ተለይቷል። የመስክ ላይ የምርት ግምገማ ጥናቱ እንዳሳየው ከተለዩት ደቂቅ አካላት መካከል የሲደሞናስ ምድብ የሆነው ደቂቅ አካል (EPS1 የሚል ስያሜ የተሰጠው) ናይትሮጅንን ከሚያክር ደቂቅ አካል (Bradyrhizobium, MAR 1495) ጋር በመሆን በአማካይ 17.2 በመቶ የአኩሪ አተር ምርት ጭማሪ አሳይቷል። ይህም የምርት ጭማሪ አካባቢው እንዲጠቀም ከተሰጠው የፎስፈረስ መጠን ምክረ-ሃሳብ ግማሹን ከላይ ከተጠቀሰው ናይትሮጅን ከሚያክር ደቂቅ አካል ጋር ቀላቅለን ብንጠቀም ከምናገኘው የምርታማነት መጠን በላይ ነው። ይሄም ጥናት የፎስፈረስ አሟሚ ደቂቅ አካላት የዕፅዋቶችን የፎስፈረስ አጠቃቀምን ለማሻሻል እንደምርጫ ተወስደው በቂ ጥናት ሊደረግባቸው እንደሚገባ ጠቋሚ ነው።

Abstract

Phosphorus (P) is often found in forms that are inaccessible to plants, as it forms precipitates with cations or is locked in phosphorylated organic compounds. Phosphate solubilizing microorganisms have been considered as options to alleviate a deficiency of plant-available P in soils, and this experiment is conducted to make use of this microbial potential in Ethiopia where acid soils are rampant. Five phosphate solubilizing bacteria associated with soybean rhizosphere were isolated on culture media and their P dissolution efficiencies were quantified on solid and liquid media containing insoluble Ca, Fe, and Al. The isolates were genetically characterized using their 16S-23S rRNA region. Three of the best P dissolving strains were field evaluated in six different areas of Ethiopia. The isolates demonstrated P dissolving capacities. One of the isolates was from Pseudomonas genera while the rest were from Bacillus. Inoculation with EPS1, Pseudomonas fluorescens, in combination with Bradyrhizobium (MAR 1495), led to an average of 17.2% yield increase across 6 test locations. This was greater than the yield obtained with the application of half of the recommended inorganic phosphorus fertilizer rate plus Bradyrhizobium, MAR 1495 (average 10.4% yield increase). Phosphorus solubilizing microbes appear to provide an option for improving plant P uptake.

Keywords*:* Phosphorus, phosphate solubilising bacteria, soybean

Introduction

Phosphorus is the most important key element in the nutrition of plants, next to nitrogen (N). It plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan *et al.,* 2010) and nitrogen fixation in legumes (Saber *et al.*, 2005). Despite its abundance in the soil, phosphorus (P) is often found in forms that are not soluble or accessible to plants, as it precipitates with inorganic cations (Richardson, 2001) or is covalently combined in organic compounds (Goldstein, 1986). In acidic soils, P is usually bound with iron (Fe) or Aluminium (Al); in alkaline soils, P is predominantly associated with calcium (Richardson, 2001; Khan *et al.*, 2009).

Phosphatic fertilizers are often applied to meet plant demands for available P. However, the cost of such fertilizers is continually increasing, as they require high-energy in production and the availability of raw P-containing materials for fertiliser production is in conspicuous decline (Neset and Cordell, 2012). Due to the binding of P in soils, P use efficiency is generally low and it is estimated that plants only use up to 25% of applied fertilizers (Rodríguez and Fraga, 1999; Stevenson, 1999; Turan *et al*., 2006). In addition, the fertilizers can have negative

environmental impacts through run-off as particulate matter and occasional leaching (Richardson, 2001; Khan *et al.,* 2009; Vitorino *et al.,* 2012).
Phosphate solubilising microorganisms (PSM) have been considered as an option to alleviate a deficiency of plant-available P in soils. PSMs have the capability to dissolve the plant-inaccessible P pool in the soil (Richardson, 2001; Khan *et al.*, 2007; Saharan, 2011; Vitorino *et al.,* 2012). In laboratory and greenhouse experiments, phosphate solubilisation traits of microorganisms can promote plant growth when plants are under P deficiency (Cattelan *et al.,* 1999; Vikram *et al.*, 2007; Taurian *et al.,* 2010; Viruel *et al.,* 2011). Secretion of low molecular weight organic compounds is reported to be the major mechanism for releasing cation-bound P into soil solution (Richardson 2001).

Research on microbial phosphate dissolution has focused on the P solubilising properties of microorganisms *in vitro* either singly or with other microorganisms. However, field evaluation of their effect has not been widely reported and consistent results have not been obtained (Richardson 2001; Khan *et al.,* 2009). Accordingly, this study investigated the potential for P-solubilising microorganisms isolated from Ethiopian soils to increase the yield of soybean at field condition in order to provide alternative and cheap P-management options.

Materials and Methods

Initial invitro-screening for phosphate dissolution

Five soil samples from a set of 54 previously collected samples (Daniel Muleta *et al.,* 2017) were used for the isolation of P-solubilizing bacteria. The 5 soil samples were collected from four soybean growing areas of Ethiopia (South Ethiopia, Lat= 05°50.260` & Long= 37°55.638`; South Western, Lat=08°50.243` & Long= 036° 12.002` and Western part of Oromia region, where the first sampling point is at Lat= 09°00.074 & Long= 036°39.084` and the second is at Lat= 09°05.453` & Long= 037°04.179`; and Benishangul Gumuz, Lat= 10°00.785` & Long= 034° 86.287`). The soil samples were taken from the rhizosphere of soybean plants. Serially diluted soils (1g each) were streaked on plates (dilution levels from 10^{-6} to 10^{-8}) containing 5g L^{-1} tricalcium phosphate (TCP) (Pikovskaya, 1948), and on plates containing 5 g L^{-1} $AlPO_4$ or 2 g L^{-1} $FePO_4$ in Reyes basal media (Reyes *et al.,* 1999). The plates were incubated at 28°C for 10 days. The isolates that showed a halo (clearing zone) on the TCP containing media were spot inoculated onto the above two types of agar plates and incubated for 10d at 28°C. Total diameter (TD) of colony plus halo zone (solubilisation zone), halo zone diameter (HD) and colony diameter (CD) were measured and their phosphate solubilisation index (SI) was determined. The phosphate solubilisation index was calculated according to Kumar and Narula (1999) using the formula:

SI= TD/CD, where TD= CD+HD

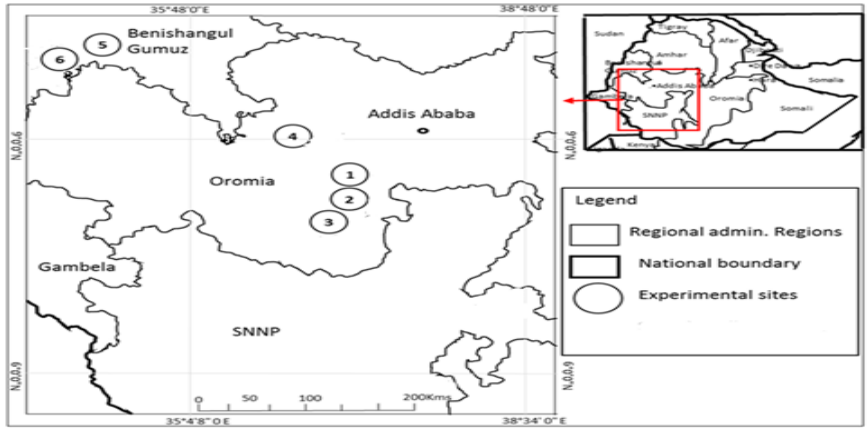

Figure 1. Experimental sites in South Western and West Ethiopia. site1) Ababiya farm, site 2) Seifu farm, site 3) research station in the Jimma area, site 4) Bako farm, site 5) Assossa research Centre and site 6) Assossa farm (Modified from Daniel Muleta et al. 2017).

Determination of phosphate dissolution efficiency in liquid culture

Three replicate solutions of the above three media (Ca in TCP of Pikovskaya's medium, Al and Fe in Reyes salts as P sources) were prepared and inoculated with 0.2 mL of a 10^8 cfu mL^{-1} suspension of each of the bacterial isolates that were pre-grown on 20 mL of trypticase soy broth. Isolates chosen were those with wider halo diameters when grown on Pikovskaya's (PKV) medium. Un-inoculated controls were used for each P-source. After 10 d of growth at 28 ^oC and a shaking speed of 120 rpm, the pH of each of the solutions was determined and 1 mL of each culture solution was taken and centrifuged at 10,000 xg for 5 min. The available P was quantified from the supernatant solution using a spectrophotometer (Jenway, 6300, UK) following the Bray-II procedure (Bray and Kurtz, 1945).

Characterization of phosphate solubilising bacteria using internal transcribed region (ITS) sequences of 16S-23S rRNA. PCR amplification

The 16S-23S rRNA region of the isolates was amplified based on the method of Jaiswal et al. (2016) using the primers FGPS1490-72 (5′TGCGGCTGGATCACCTCCT3′) and FGPL132-38 (5′ CCGGGTTTCCCCATTCGG3′) (Bioline, Australia) with a thermal cycler (GeneTouch, BIOER). Polymerase chain reaction (PCR) was carried out in a 23-µl reaction volume containing 5 µl (5×) MyFi Reaction Buffer, 1 µl (5 U $µl^{-1}$) MyFi DNA polymerase (Bioline, Australia), 1 µl (10 pM) of each of the primers and double distilled water. Bacterial cells were transferred to the appropriate tubes

by lightly touching a freshly grown culture with a sterile pipette tip and swirling in the PCR solution to transfer cells; tubes were briefly centrifuged before the reaction was carried out. The PCR reaction conditions were set for lysis (and initial denaturation) at 95°C for 5 min, followed by 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 56°C, 2 min of extension at 72°C. The final step involved an extension for 5 min at 72°C. The presence of amplicons were checked by horizontal gel electrophoresis on 1.5 % agarose gel stained with SYBR safe with a 2 kb DNA marker (EASY ladder I, Bioline, Australia).

DNA purification and Sequencing

Amplicons were purified with Isolate II PCR and Gel purification kit (Bioline, Australia) according to the manufacturer's instructions. The purified samples were sequenced using Sanger sequencing (Applied Biosystems genetic analysis systems, Thermo Fisher Scientific) at AGRF (Adelaide, South Australia).

Field experiment using bacterial isolates

Preparation of bacterial inoculant

Three isolates with the highest measured TCP dissolution, based on the liquid dissolution experiment (designated as EPS1, EPS2, and EPS3), were prepared for field inoculation. The carrier material used was filter mud, a by-product of sugar cane processing, which has been used previously to formulate microbial inoculants (Philpotts, 1976). The filter mud was ground, passed through a 200-mesh sieve (0.09 mm) and neutralised by addition of $CaCO_3$. The carrier material was sealed in polyethylene bags (125 g per bag) and autoclaved. The isolates (EPS1, EPS2, and EPS3) were grown in 25 mL nutrient broth (beef extract 1g, yeast extract 2 g, peptone 5 g and NaCl 5 g in 1 L of distilled water) in 50 mL flasks. After 5 d of growth, shaking at 28 ℃, the broths were transferred to 1 L Nutrient broth in sterilized 2 L flasks and grown with shaking for 5 d to achieve 10^9 cfu mL^{-1} before being used to aseptically inoculate the prepared carrier. Sachets containing125 g of carrier were inoculated with 45 ml of the nutrient broth containing the bacterial isolates in a laminar flow cabinet.

Experimental design

Experiments were established in six farmers' fields using randomized complete block design (RCBD) with six treatments replicated four times. Three treatments consisted of the bacterial strains; another two treatments were full and half dose of phosphorus fertilizers (20 kg P ha^{-1} and 10 kg P ha^{-1}) applied as Triple Super Phosphate (TSP), $Ca(H_2PO_4)_2.H_2O$ without bacterial inoculation; the final treatment was a negative control that received neither bacterial inoculant nor P fertilizer. Soybean cv. Clark was used for all treatments and the seed were inoculated with a commercial N fixing strain of *Bradyrhizobia* (MAR 1495, TSBF-Nairobi) at approximately log 4.5 rhizobia $seed^{-1}$ while the phosphate

dissolving strains were applied (500 g ha^{-1} at 10^9 cfu g^{-1} carrier) in furrow at 5 cm depth before seeding the inoculated soybean seeds in the same day. Then after, the soybean seeds were hand sown in rows 60 cm apart. The seeds in rows were 5 cm apart with row length of 4 m and 4 rows per plot. Blocks and plots were separated by 1 m to reduce the chance of contamination from one plot to another. The variability in rainfall distribution of the test sites is indicated in Daniel Muleta *et al.*, 2017.

The laboratory experiment was checked for the presence and absence of halo zones, the diameters of the halo zones and the diameter of the colony were recorded for phosphate dissolution activity of the microorganisms grown on the three P sources contained in agar plates. The concentration of available P and the pH were determined for the phosphate dissolution experiment. In the field experiments, plants of the entire plots were harvested and grain yield was recorded after adjusted at 12% seed moisture level.

Statistical analysis

The data from phosphate dissolution experiments in liquid culture and yield data from field experiments were subjected to analysis of variance (ANOVA) using the General Linear Model Procedure of GenStat (VSN International, 2014) and considered significant at the probability level, $P<0.05$. Means of all the treatments were separated using an LSD test.

Results and Discussions

Halo (clearing) zones, indicating phosphate dissolution by bacterial isolates, were measured on PKV media. Five isolates that formed halo zones were selected (Table 1). The solubilization indices of the five isolates were between 3.3 and 1.5 while their colony diameters were between 2.9 mm and 3.2 mm at day 10. All five strains changed the color of growth media containing both $AlPO_4$ and $FePO_4$ to yellow, indicating a reduction of pH but there was no clear halo zone formation as seen on $Ca_3(PO_4)_2$.

Inoculations with phosphate solubilising microorganisms can improve the phosphorus nutrition of plants. Al-P and/or Fe-P in acid soils or Ca-bound P in alkaline soils are released into the plant available P pool of the rhizosphere due to the action of the PSMs (Goldstein 1986). The bacterial isolates in this study increased the concentration of available P in the three tested P sources under laboratory conditions compared with the un-inoculated controls. The highest concentrations of P released, compared to the controls were 86%, 73% and 216% for Al, Fe and Ca sources, respectively.

Table 1. Taxonomy and *in vitro* colony and clearing zone sizes of 5 phosphate solubilising test isolates, and concentration of dissolved P, 10 d after inoculation of the isolates into media containing three different P sources

			Size			Pi concentrations liberated from three P sources					
			Initial *in vitro* screening*			$AlPO_4$		$FePO_4$		$Ca_3(PO_4)_2$	
Test Strain	**Confirmed Genus**	**Taxonomic affiliation, ITS (99% similarity)**	*CD	TD	SI	pH**	P (mg L^{-1})	pH	P (mg L^{-1})	pH	P (mg L^{-1})
EPS1	*Pseudomonas*	*P. fluorescens*	2.9	9.5	3.3	5.1	11.3 ± 0.06b	5.4	32.4 ± 0.5a	5.2	187.4 ± 6.8a
EPS2	*Bacillus*	*B. subtilis*	3.2	9.4	2.9	4.2	12.9 ± 0.2a	4.7	24.9± 0.5b	5.8	68.2 ± 0.4c
EPS3	*Bacillus*	*B. safensis*	3.0	8.8	2.9	5.6	9.6 ± 0.3c	4.3	24.9 ± 0.8b	5.7	81 ± 0.6b
EPS4	*Bacillus*	*B. velezensis*	3.2	6.6	2.1	5.7	9.3 ± 0.2c	5.8	20.7 ± 0.6c	6.2	62.53 ± 1.3cd
EPS5	*Bacillus*	*B. velezensis****	3.0	4.5	1.5	5.8	8.37 ± 0.6cd	5.9	22.5 ± 0.06d	5.8	62.17 ± 1cd
Control						5.9	6.93 ± 0.2d	6.0	18.7 ± 0.5e	6.5	59.27 ± 0.2d
CV (%)							2		2		3
$LSD_{0.05}$							0.4		1.67		8.8

* CD = colony diameter; TD = total diameter of colony plus clearing zone; SI = solubilisation index.

**Final pH of the replicates of the solutions for each strain was similar for the respective $AlPO_4$, $FePO_4$ and $Ca_3(PO_4)$ in PKV media.

*** 92% similarity

The ITS sequences of the five-phosphate solubilising bacterial isolates were compared with microbial taxonomic databases *in silico* and the isolates were identified based on their similarity with known species. Accordingly, the first isolate (EPS1) was found to belong to the genus *Pseudomonas* while the remaining isolates (EPS2 to EPS5) were assigned to the genus *Bacillus* (Table 1).

Plant growth promotion by similar species of this study, *B.subtilis*, *B. velezensis*, and *P. fluorescens*, were previously reported from acidic soils of coffee growing areas of Ethiopia (Diriba Muleta *et al*., 2009). Strains of *B. safensis* were also found in diverse terrestrial and marine environments and are known for their plant growth promoting properties (Lateef *et al*., 2015).

In the laboratory experiment, the amount of available P released into solution differed among the bacterial isolates and the P sources used (Table 1). The largest concentration of available P in liquid culture was recorded for $Ca_3(PO_4)_2$ (187.4 mg L^{-1}) with *P. fluorescens* EPS1. This strain released the highest concentration of inorganic P both from $Ca_3(PO_4)_2$ and from $FePO_4$ (32.4 mg L^{-1}), which were 216% and 73% larger than the P concentrations of the respective controls. Isolate EPS1 and *Bacillus* isolates EPS2, and EPS3 appeared to dissolve larger concentrations of P from $AlPO_4$ and the increase in soluble P ranged between 34-86% compared with the un-inoculated control. Among these three isolates, EPS2 released the highest concentration of P (12.9 mg L^{-1}) from $AlPO_4$, which was 86% larger than the P concentration of the control. EPS3 increased the P concentration of $Ca_3(PO_4)_2$ solution by 37% compared to the control. The rest of the isolates increased soluble P from $AlPO_4$ by 20-73%.

The increase in P concentration in the solution was accompanied by a decrease in pH of the media relative to the respective controls, and the correlation coefficients between pH and available P were negative. The correlation coefficients between solution pH and inorganic P were -0.8, -0.7 and -0.93 for $Ca_3(PO4)_2$, $FePO_4$ and $AlPO_4$, respectively. The highest soluble P concentrations from both $AlPO_4$ and Ca_3 $(PO_4)_2$ were recorded from the lowest numerical pH values, while for $FePO_4$ it was from an intermediate pH value of 5.4 (Table 1). Incubation of isolates in both $AlPO_4$ and $FePO_4$ resulted in a maximum pH decrease of 1.7 units while growth on $Ca_3(PO_4)_2$ was accompanied by a maximum pH decrease of 1.3 pH units (Table 1).

Three of the PSB isolates were selected for field tests on soybean grown on acidic soils in Ethiopia (Table 3). The metrological conditions of the growing season are explained in Daniel Muleta *et al.,* (2017). For the field experiments, a significant difference in grain yield among treatments (controls, P fertilizer applications and inoculation treatments) was observed at Site 2 but not at the other sites (Table 2). Even though the seed yield data obtained from five sites did not show statistically

significant differences, there were consistent yield increases for treated treatments against the untreated controls. Yield increase due to the application of the full dose of P (20 kg ha^{-1}) ranged between 5-50% (site 1 and site 6, respectively) for the six experimental sites, with a mean increase of 22%. Yield increases due to the application of a half dose of P over the control ranged from 2.6 to 31.7% with a mean of 10.4% across the six experimental sites. Inoculation with *P. fluorescens* EPS1 increased yield between 3.5-54% with a mean increase of 13.8%. The yield increase with EPS 3 compared to control ranged between 12.5 to 26.7% with a mean increase of 12.2%. The lowest yield increase among the treatments was observed for *Bacillus* EPS2, ranging from 12.5 to 27% with a mean increase of 5.7%.

Table 2. Yield response of soybean at harvest following the application of 3 different phosphate dissolving inoculants and P fertilizer at six experimental field sites

	Grain yield (kg ha^{-1})					
Treatment	Site1 (Ababiya)	Site 2 (Seifu)	Site 3 (JARC)	Site 4 (Bako)	Site 5 (AARC)	Site 6 (Assossa on farm)
Control	2160a	1389ab	856.0a	757.7a	758.4a	420.5a
EPS1	2299a	1458ab	885.5a	1167.8a	961.9a	451.1a
EPS2	2257a	1215b	758.7a	881.0a	961.9a	463.6a
EPS3	2247a	1215b	941.0a	960.5a	959.1a	498.3a
½ rate of P*	2254a	1458ab	878.5a	790.7a	869.2a	553.9a
P**	2268a	1667a	928.9a	935.5a	938.3a	630.3a

JARC= Jimma Agricultural Research Centre, AARC=Assossa Agricultural Research Centre
* 1/2P = 10 kg P ha^{-1}; P**= 20 kg P ha^{-1}
Data followed by the same letter are not significantly different at P = 0.05.

Table 3. Location and selected physical, chemical and biological properties of soils at the experimental sites (Adapted from Daniel Muleta *et al.,* 2017)

	Jimma, Ababiya farm	**Jimma, Seifu farm**	**Jimma, Research Centre**	**Bako, farm**	**Assossa, Research Centre**	**Assossa, farm**
Designation	**Site-1**	**Site-2**	**Site-3**	**Site-4**	**Site-5**	**Site-6**
Latitude	0.7°42.770'	0.7°42.633'	0.7°06.668'	9°04.503'	10°03.251'	10°01.237'
Longitude	37°00.461'	37°00.305'	36°07.867'	36°59.620'	34°59.412'	34°45.613'
Altitude (m)	1781	1767	1753	1755	1588	1578
%Clay	64	71	44	61	71	64
%Silt	24	19	12	19	15	10
%Sand	12	10	44	27	14	26
pH H_20	4.37	4.26	4.53	4.39	4.81	4.62
P mg/kg	4.92	4.72	2.12	4.94	5.28	8.21
CEC meq/100g	17.19	17.92	19.6	14.55	34.7	31.6
%OC	2.05	1.98	2.07	1.56	2.96	2.61
Ex. Acidity	0.46	0.81	1.74	0.78	0.18	0.42
Total N %	0.16	0.15	0.45	0.1	0.18	0.14
Rhizobia MPN cfu/g soil	250	16	$>10^6$	ND*	$1.4x10^3$	$>10^6$

The major soybean growing areas of Ethiopia are characterized by acidic soils, pH (H_2O) < 5 (Daniel Muleta *et al*., 2017). The major P forms in acidic nitisols of Ethiopia are comprised of Fe-P and smaller amounts of Al-P and Ca-P (Piccolo and Huluka, 1986, Tekalign Mamo and Haque, 1987). Hence, testing microbial isolates that have the capacity to dissolve Fe-P, together with Al- and Ca-bound P is desirable, as an effort to increase plant available P uptake improve crop growth and yield of soybean. *P. fluorescens* strain EPS1, which increased the available P from Fe-P by 73% *in vitro* was considered the best candidate, followed by *Bacillus* isolates EPS2 and EPS3. Although the effects of inoculation with these isolates were not significantly different from the unfertilized control treatments under field conditions, substantial increases in average yield were recorded, relative to uninoculated controls.

The increase in available P in solution following inoculation of the isolates was negatively correlated with pH of the media. Such associations between decreasing pH and increasing available P concentrations that have been reported previously (Illmer *et al*., 1995; Whitelaw *et al*., 1999; Chen *et al*., 2006; Yu *et al*., 2012) and secretion of carboxylates is considered as one of the main mechanisms of dissociation of cations and P (Whitelaw *et al*., 1999; Richardson, 2001). In one study, HPLC analysis of the media in which phosphate-dissolving bacteria had grown indicated the secretion of organic acids, including citric, gluconic, lactic, oxalic and propionic acids, with the most common forms of organic acids being gluconic, oxalic and citric acids (Richardson, 2001).

There is increasing interest in the application of phosphate solubilising microorganisms to improve P nutrition and yield. Promising results have been demonstrated in the field and greenhouse for plant growth promotion through inoculation of these microorganisms on several crops including rice (Estrada *et al*., 2013), canola (De Freitas *et al*., 1997), maize (Hameeda *et al*., 2008), and sunflower (Ekin, 2010). However, it is not uncommon that microorganisms displaying phosphate solubilising traits in a laboratory fail to demonstrate significant plant growth promotion in field conditions (Richardson 2001). Further investigation is required on methodologies of selecting these microorganisms, carrier material formulation for inoculations, and interaction of the organisms with the host crops and the environment (Richardson, 2001).

In this study, the isolates demonstrated phosphate solubilising capacity, especially in liquid culture. When inoculated in furrow at sowing in the field, yield increases compared to control treatments were not statistically significant differences. However, the grain yield of untreated control plots was the lowest or near the lowest among the treatments across all six field sites. Application of 20 kg/ha P (full P) resulted in an average yield increase of 22% across the six sites (range 5% at site 1 to 49.9% at site 6). Responses to inoculation with the bacterial isolates

varied with location. Inoculation with *P. Fluorescens* EPS1 resulted in an average yield increase of 17.2% across locations (range 3% to 54%), which was greater than the response to the application of 10 kg/ha P ("half of the recommended P"). Isolate EPS1 can be further investigated for possible growth promotion and effects on plant P content.

Lack of significant yield increase of soybean after application of 20 kg P ha^{-1} at Assossa, near to sites 5 and 6, has also been reported previously (Anteneh Argaw, 2011). A lack of P response could be due to the high phosphorus buffering index of the soil at the experimental sites such that 20 kg P ha^{-1} did not have a significant effect, since the P might be adsorbed due to high phosphorus buffering index (PBI) as mentioned in Daniel Muleta *et al*., 2019. The local recommendation of applying 20 kg P ha^{-1} might not consider the possibility of high PBI values at some sites with low soil pH. Higher rates of P application (30 kg P ha^{-1}) were required to increase the production of soybean in a previous study in Alabama, USA in acidic, and kaolinitic soils with low organic content (Cope, 1981). In other circumstances the application of 60 kg P ha^{-1} did not show significant soybean yield increases above controls, unless 112 kg K $ha^{-1}$was also added (Jones *et al.*, 1977). Clearly, an understanding of soil nutrition, including phosphorus chemistry in acidic soils are an important factor in the assessment of potential for responses to inoculants.

Conclusion

The inoculation of P solubilizing bacteria in acidic field soils indicated some potential to increase yield of soybean. However, the increases were not statistically significant and the identification of responses was constrained due to the high PBI of the soils, and the inability of the most common P fertilizer recommendation (20 kg ha^{-1}) to increase yield significantly. To demonstrate the benefit of P solubilizing microorganisms, soils in which there is a response to applied P or the interaction of P with other nutrients need to be identified, as observed for K and Mg in Kenyan soils (Keino *et al.*, 2015). Efforts will be made to evaluate these P solubilising strains in low PBI soils. Application of the inoculants to seed may provide a better opportunity for improving yield responses compared with furrow application, as has been observed with N fixing inoculants. The current study demonstrated the potential of phosphate dissolving microorganisms to improve soybean yield in acid soils, however, further work on improved application methods for inoculation of P solubilizing organisms may assist in exploiting these microorganisms.

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