Original scientific paper
DOI: /10.5513/JCEA01/23.4.3636
Journal of Central European Agriculture, 2022, 23(4), p.817-831

Minjingu phosphate rock solubilization and potential for use of *Klebsiella variicola*-MdE4 and *Klebsiella variicola*-MdG1 as biofertilizer for maize production

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Received: May 9, 2022; accepted: September 6, 2022

ABSTRACT

Limited solubility of the Tanzania's Minjingu phosphate rock (MPR) in non-acidic soil conditions has held back its potential for widespread use in agricultural production. This study was designed to isolate, characterize and test phosphate-solubilizing bacteria for their potential to increase solubility of MPR and enhance maize plant growth under field conditions. Ten out of 19 isolates showing greatest phosphate solubilization indices on a Pikovskaya agar medium were further characterized for other plant-growth promoting traits including production of IAA, siderophores and ammonia. Two of them, namely isolates-MdE4 and MdG1 substantially outperformed other isolates in phosphate solubilization and production of IAA, ammonia and siderophores. The two isolates molecularly identified as *Klebsiella variicola*-MdE4 and *K. variicola*-MdG1 produced up 701, 699 and 750, 680 µg/ml of soluble phosphate from tricalcium phosphate and hard Minjungu rock phosphate, respectively. Additionally, Biorock P- a biofertilizer formulation containing MdE4 and MdG1 co-cultured in a molasses-based modified broth medium retained most of the phosphate solubilizing potential and other plant-growth promoting traits of MdE4 and MdG1. Co-application of MdE1 and MdG1-containing "bio-rock P" and inorganic phosphate at 20 kg P/ha resulted in higher maize grain yield than that of positive control (40 kg P/ha) under field conditions. The two isolates- *Klebsiella variicola*-MdE4 and *K. variicola*-MdG1 have the potential for use in a biofertilizer formulation for commercial field applications.

Keywords: rock phosphate biofertilizer, inoculant, plant growth promoting bacteria, biofertilizer formulation

INTRODUCTION

Despite its abundance in soils, phosphorus availability to plants in most tropical soils, including agricultural soils of Tanzania, is limited mainly due to fixation by kaolinitic clays and sesquioxides (Msolla et al., 2005; Mtama, 2018). Its replenishment through water-soluble fertilizers is not always cost-effective for resource-poor farmers. One of the suggested approaches to address this challenge is the direct application of phosphate rocks as a source of phosphorus to plants growing on such soils (Savini et al., 2016; Tapiwa et al., 2018). However, the practice of directly applying phosphate rocks into soils is characterized by insolubility of the phosphate rocks in non-acidic soils (Savini et al., 2015). This is because, most of the world's phosphate rocks such as the Minjingu phosphate rock (MPR) locally available in Tanzania, are extremely water-insoluble and may remain unavailable to plants in non-acidic soil conditions. Solubility of phosphate rocks in soils can be enhanced through various techniques which may be either microbial or non-microbial (Sumner, 2000). Non-microbial techniques include use of acidifying agents such as lemon and pineapple juices (Mwangi et al., 2020) or buttermilk (Cicek et al., 2020). These approaches are, however, only feasible on small-scale field applications. On the other hand, microbial approaches involve the

use of phosphate solubilizing microorganisms (PSMs) (Sumner, 2000; Sharma et al., 2013; Alori et al., 2017). The PSMs solubilize phosphate by producing organic acids and metal chelating agents, leading to (1) lowering the pH, or (2) chelation of the cations responsible for precipitation of P, (3) competing with P for sorption sites on the soil, and (4) forming soluble complexes with the metal ions associated with insoluble P compounds (phosphates of Ca, Al, and Fe). Several microbial species are known for their P solubilisation abilities. They include bacteria from genus of *Pseudomonas*, *Klebsiella*, *Bacillus*, and *Proteus*, fungi from genus *Aspergillus*, *Penicillium*, *Rhizopus*, and actinomycetes from genus *Streptomyces* (Khan et al., 2014). Other strong phosphate solubilizing microorganisms include arbuscular mycorrhizal fungi (Sharma et al., 2013; Etesami, 2020; Etesami et al., 2021) and ectomycorrhizal fungi (Lapeyrie, et al., 1991).

When such PSMs have additional plant-growth promoting traits, their use in developing phosphate rock-based multi-purpose bio fertilizer products is more rational and urgently needed now than ever before. PSMs with additional plant growth-promoting traits, including those possessing antifungal properties have been widely documented (Singh, 2013; Timmusk et al., 2017; Mącik et al., 2020). Ironically, there are no known attempts in literature to enhance solubility of Tanzania's locally available Minjingu Phosphate Rock PSMs.

There are reports, however, that quality and quantity of the carbon substrate influences the ability of microorganism to solubilize phosphates resources (Mardad et al., 2014; Rathore, 2014; Ibrahim et al., 2017; Mącik et al., 2020; Vassilev et al., 2020). We hypothesized that replacing laboratory-grade glucose with locally available molasses as alternative carbon sources can enhance phosphate solubilization at a reduced cost without negatively affecting other plant-growth promoting traits such as IAA production, siderophore production and antifungal activity of isolates.

MATERIALS AND METHODS

Isolation and selection of phosphate solubilizing microorganisms

Soil samples were collected at depth of 0 – 30 cm from randomly selected agricultural fields in the central and southern highlands of Tanzania and transported to the University laboratory. One gram of each soil sample was placed into an Erlemeyer flask containing 0.9% NaCl solution and vortexed prior to performing serial dilution. Duplicate serial dilutions of 10^{-4} - 10^{-8} were spread-plated on Pikovskaya (PVK) agar medium (Khan et al., 2014 ; Simfukwe and Tindwa, 2018) and incubated at 28 °C for 8 days. At the end of incubation period the phosphate solubilization index (PSI) was calculated as a ratio of the total diameter (colony + halo zone) to the colony diameter, i.e., PSI (cm) = (diameter of colony + halo zone / diameter of colony).

Ten bacterial isolates with greatest PSI values were further selected and individually inoculated either on (a) Pikovskaya broth (glucose, 10 g; Calcium phosphate 5 g; Ammonium sulphate 0.5 g; Potassium chloride 0.2 g; Magnesium sulphate 0.1; Manganese sulphate 0.0001 g; Ferrous sulphate 0.0001 g) or (b) modified Pikovskaya broth (Fe-PVK) or (c) hard Minjingu phosphate rock (HMPR) broth. The Fe-PVK and HMPR broths were developed by replacing the original calcium phosphate in PVK with either Ferric phosphate (FePO4) or finely ground powder of hard Minjingu phosphate rock, respectively. All preparations were incubated for up to 14 days at 28°C. The final acidification of the growth medium was measured using a pH meter and the concentration of soluble P in the broth was measured spectrophotometrically by the chlorostannous reduced molybdo-phosphoric acid blue colour method (Olsen and Sommers 1982; Bashan et al.2013). Results were compared and two best performing isolates- MdE4 and MdG1 were selected for further tests and field studies.

Characterization of phosphate solubilising isolates for other plant growth-promoting traits

Siderophore production assay

Siderophore production by isolates was detected on crome azurol S (CAS) agar assay. CAS agar was prepared according to protocol described by Louden et al., (2011). Each isolate was innoculated on the a CAS agar plate and incubated at 28 °C for 24 h. Development of a halo zone around the colony with a distinct colour change of the medium from deep blue to purplish-red or orange was taken to signify production of siderophore by the involved isolate.

IAA production and quantification

Pure cultures of bacterial isolates were grown in nutrient broth amended with (or without) tryptophan and incubated at 30 ± 2 °C for five days. The cultures were then centrifuged at 7500 x g for 30 min. Then, 2 ml of the supernatant were mixed with two drops of 10 mM orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M ferric chloride solution) and incubated at 28 ± 2 in darkness by wrapping the container with aluminum foil (Khan et al., 2014; Gang et al., 2019). A change of colour of the mixture from a clear to pink or reddish orange solution was taken to represent ability of the isolate to produce IAA *in vitro* (Gang et al., 2019). IAA quantification was done spectrophotometrically by comparing absorbance values of culture supernatants to those of standard solutions read at 530 nm on an iCE 3300

Ammonia production and quantification

The modified Nesslerization method (Heonsang et al., 2013) was used to quantify ammonia production by the isolates. Accordingly, bacterial isolates were grown in peptone water (g/l: peptone 10; NaCl 5; pH 7) and incubated at 28 ±2 °C for 4 days. After incubation, the cultures were centrifuged at 2000 × *g* for 10 minutes. Separately, standard solutions (0, 10, 20, 40, and 80 mg/L of ammonia) were prepared from a 1000 mg/L NH_4Cl stock solution. Then, 0.2 ml of Ethylenediaminetetraacetic acid (EDTA) were added to each of the supernatants as well as standard solutions in order to eliminate calcium and magnesium interference. Then, 0.4 ml of Nessler's (K_2HgI_4) reagent was added to each standard and supernatants and the intensity of yellow colour that developed was read by iCE 3300 atomic absorption spectrophotometer (Thermo-scientific) at 420 nm.

Sugar fermentation and starch hydrolysis test

Fermentation of glucose, lactose and sucrose were tested using the Klingler's Iron agar (KIA) and Triple Sugar Iron (TSI) test methods. KIA medium contained in g/L: lactose -10, glucose- 1, and peptone. TSI contained in addition to KIA's ingredients, 10 g of sucrose per litter. In each medium, 0.024 g of phenol red and 0.2 g of ferrous sulphate were added as indicators of acidification and H_2S formation, respectively. Accordingly, a loop-full of each of the bacterial isolates was inoculated separately into sterile slant tubed TSI or KIA medium and incubated at room temperature for 48 hours after which the results were observed. A yellow butt was taken to indicate acid production, a red pink slope indicated the fermentation of glucose only while a red pink slope and butt was taken to indicate lack of fermentation of both sugars. To test starch hydrolysis, overnight bacterial cultures in nutrient broth were streaked onto the surfaces of starch agar medium (peptone 5 g, sodium chloride 5 g, yeast extract 1.5 g, beef extract 1.5 g, starch soluble 2 g, and agar 15 g into 1 L of distilled water, pH 7.4) and incubated for 48 hours at 28 °C. Presence of a clear zone around the line of bacterial colonies after the addition of iodine solution was considered a positive test for starch hydrolysis.

Antibiotic Sensitivity test

The agar well diffusion assay (Balouiri et al., 2016) was used to test isolates' antibiotic sensitivity. 100 µL of overnight bacterial cultures were spread-plated on Mueller Hinton agar plates after which 8 mm holes were punched on the inoculated plates by using sterile tips, and then 100 µL of either penicillin and streptomycin solution at a concentration of 50 µM were added to the wells, separately. The plates were incubated at 28 °C for 24 hours before making observations of inhibition zones. The interpretation of inhibition zones was done based on standards described in (CLSI, 2020).

Molecular identification of the isolates

All ten bacterial isolates were identified by using partial sequencing of the 16S rRNA gene fragments. For each of the bacterial isolates, total DNA was prepared by using a commercial DNA extraction kit (ZymoBiomics Tm DNA Miniprep Kit, Irvine, CA, U.S.A) by following manufacturer's instructions. The 16S rRNA gene fragments were amplified using universal primers (27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3') (Frank et al., 2008). The sq-PCR reaction mixture comprised of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer, primers and the genomic DNA. The mixture was incubated at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 15 s, and extension at 72 °C for 45 s and a final extension at 72 °C for 10 minutes. Amplification of the fungal ITS1-5.8S-ITS2 rDNA was done using universal primers ITS-1F and ITS-4R primers and the sq-PCR reaction. The reaction included an initial denaturation at 94 °C for 10 minutes followed by 35 cycles each for 45 seconds at 94 °C, 30 seconds at 55 °C, and 1 minute at 72 °C, and a final extension at 72 °C for 10 minutes. The resultant bacterial PCR products were purified and the DNA sequenced and directly determined using a BrilliantDye™ Terminator v3.1 and ABI 3500XL Genetic Analyzer, POP7™ (Inqaba Biotechnical Industries Ltd, SA). The gene sequences were aligned using CLUSTALW software version 2.1 and the homology trees were constructed using Mega5 software (Tamura et al., 2011). Strain MdE4 and MdG1 were, respectively, identified as *Klebsiella variicola* and *Klebsiella variicola*, with 99.8 and 100 % similarity, respectively.

Effect of replacing glucose for molasses on phosphate solubilization by MdE4 and MdG1 isolates

Molasses was initially evaluated for its physicochemical properties according to methods described by Peters et al. (2003). From the standard composition of PVK, 10 g of glucose were replaced by either, 30, 20, 10 or 5 ml of molasses per liter of the PVK broth. The pH of each of the preparations was adjusted to 7.0 and autoclaved at 121 °C for 15 minutes. After cooling, MdG1 or MdE4 isolate was inoculated to each preparation and the resultant cultures were incubated at 28 °C and 150 rpm for 10 days in an orbital shaking incubator. The amount of soluble P in the culture supernatants were -quantified using the chlorostannous reduced molybdo-phosphoric acid blue -colour method (Olsen and Sommers 1982) and their values compared to both the positive control (inoculated standard PVK broth).

A preparation (20 ml molasses-amended PVK broth plus inoculant) that produced the highest amount of soluble P was amended, further, by replacing the 5 g of calcium phosphate with 5 g of sterile powder of Minjingu phosphate rock as source of P. This formulation was labelled "Bio-rock P" in this report. Therefore, the Biorock P medium contained per litre of solution:- 20 ml molasses; 0.5 g, sulphate of ammonia; 10 g, hard Minjingu phosphate rock powder; 0.1 g, $MgSO_4$; 0.2 g, KCl; 0.00001 g, $FeSO_4$ and 0.00001 g $MnSO_4$. The pH of this medium was adjusted to 7 ± 0.2 prior to sterilization by autoclaving at 115 °C for 15 minutes. After cooling, a loop-full of the starter culture of each isolate was aseptically transferred into a 5 L bottle and incubated for 72 h to produce a co-culture of the two isolates (Bio-rock P inoculum) for field application.

Performance of Biorock P inoculum containing MdE4 and MdG1 isolates as phosphate solubilizers

Study sites and soils

Two agro-ecologically distinct study sites were selected for the field trials. Magadu site sits on the foot slopes of the Uluguru Mountain range in the Eastern Plateau and Mountain blocks of Tanzania and characterized by soils formed from gneiss parent material overlain with coastal sands on undulating to rolling topography at an altitude of about 550 m.a.s.l. (De Pauw, 1984). Magadu's climate is a warm sub-humid tropical type with bimodal rainfall distribution and receives an annual rainfall of 950 mm, and its mean annual air temperature is about 24°C. Madaba site is located over 650 km away from Magadu in the southern highlands of Tanzania and has soils formed from gneiss parent material on hilly topography at around

2000 m.a.s.l. (De Pauw, 1984). The climate in Madaba district warm sub-humid tropical with unimodal rainfall between late November and early May with around 800 to 1,200 mm per annum and a mean 15 °C.

Selected properties of the soils at study sites

From each site, ten soil sub-sample portions were collected randomly from a 1 acre area at the rooting depth of 0-30 cm and mixed to make 1 kg composite sample which was then transported to the laboratory. Each of the compound samples was air-dried, ground and sieved through a 2 mm sieve. Soil pH, was measured electromagnetically in 1:2.5 (weight/volume) soil: water suspensions (Okalebo, 2006). Phosphorus was extracted by Bray and Kurtz-1 method (Bray and Kurtz, 1945) and determined spectrophotometrically (Murphy and Riley, 1962). Total nitrogen was determined by micro-kjeldah method. Particle size distribution was determined by the hydrometer method after dispersion with 5% sodium hexametaphosphate (Gee and Bauder, 1986) and soil textural class was determined using the USDA soil textural class triangle (United State Department of Agriculture, 1975). Cation Exchange Capacity (CEC) and exchangeable bases were determined by 1 M (pH 7) NH_4 – acetate saturation method followed by displacing the adsorbed NH_4^+ using 1 M KCl. CEC was then determined by quantifying the NH_4^+ displaced by 1 M KCl whereas the Exchangeable Ca^{2+} and Mg^{2+} were quantified using an atomic absorption spectrophotometer (AAS) and exchangeable K^+ and Na by the use of a flame photometer (Thomas, 1996). Organic carbon was determined by the Walkely and Black wet oxidation method (Nelson and Sommers, 1996).

Similarly, the soil's phosphorus adsorption-desorption behaviour was determined by following a procedure developed by Nair et al. (1984). Eventually, the quantity of sorbed phosphate was calculated as the differences between quantities of P initially present in the solution and the P concentration in equilibrated solution. The adsorption data were fitted to the Langmuir equation and adsorption parameters, b (sorption maxima) and K_L (Affinity parameter) were estimated by using the linear least-squares regression, as described in (Essington, 2005).

Field experiments

The experiments at both Magadu and Madaba sites were laid out in a randomized complete block design (RCBD) replicated four times. As informed by a prior soil fertility assessment, all nutrient deficiencies except phosphorus were corrected by applying them to recommended rates. The source of P used in the field treatments was hard MPR powder and the Biorock P inoculum used had an average of 109 cells/ml and was applied at the rate of 10 L/ha. The seven treatments for the experiment were 0 kg P/ha (absolute control), 0 kg P/ha + Biorock P inoculum, 20 P/ha + Biorock P inoculum, 40 kg P/ha (Recommended rate), 40 kg P/ha + Biorock P inoculum; 60 P/ha + Biorock P inoculum, and 80 P/ha + Biorock P inoculum. Treatment and fertilizer application was by placement to the planting holes 2 cm deeper than the sowing depth. The inoculum was first diluted in 1:20 (inoculum: water ratio), and then five millilitres of the mixture was applied to each planting hole. Test crop was maize variety Aminika planted at a spacing of 75 cm × 30 cm. Parameters monitored included available phosphorus content of the soil, total P uptake by the maize plants, maize grain yield, and biomass yield phosphorus use efficiency (PUE) of maize crop under each treatment (Syers et al., 2008; van de Wiel et al., 2016).

Statistical analysis

The GenStat statistical package was used for the Analysis of Variance (ANOVA) and comparison of means for soil available P, plant P uptake, and yields was done by Duncan's New Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Isolation, biochemical characterization and molecular identification of isolates

19 bacterial strains with ability to solubilize inorganic phosphate on Pikovskaya (PVK) agar medium were isolated and subjected to series of sub-culturing to obtain pure colonies. Only 10 of the isolates with substantially

larger halo zones around their colonies and significant amounts of soluble P in PVK broth were selected for further characterization on their potential plant-growth promoting traits. Of these 10, isolates MdE1 and MdG1 were substantially superior in all the four major plant-growth promoting traits tested- phosphate solubilization, IAA, ammonia and siderophore production (Table 1). Further tests on phosphate solubilization in liquid cultures affirmed that isolate MdE1 and MdG1 were distinct from the rest as they produced higher (P<0.05) quantities of soluble P in regardless of the source of inorganic phosphate. In this study, we used the PVK agar plate assay to detect isolates abilities to solubilize phosphate resources. Similar approaches on isolation and characterization of phosphate solubilising bacteria have been reported by other researchers (Mendoza-Arroyo et al., 2020) although other different approaches using different culture media have also been successfully used before (Singh et al., 2016). The ten isolates characterized for additional plant-growth promoting traits exhibited varying abilities in IAA, ammonia and siderophore production (Table 1). Overall, two isolates- *Klebsiella variicola*-MdE4 and *K. variicola* MdG1 showed high overall plant growth promoting potential compared to the rest of the studied isolates

Phosphate solubilizing rhizobacteria with additional plant-growth promoting traits may exhibit greater potential to increase yields of target crops by promoting general plant growth. This may be through the produced IAA (Simfukwe and Tindwa, 2018; Myo et al., 2019) or improvement of iron nutrition of crops and tolerance to iron deficiency chlorosis through produced siderophores (Lurthy et al., 2020) or protection of the crop plants from damage by pathogenic bacteria through their antifungal activities (Panda et al., 2016; Ibáñez et al., 2021).

In addition, the isolates exhibited varying abilities to starch hydrolysis and sugar fermentation. Majority of the isolates were more susceptible to Streptomycin than Penicillin. Only one isolate *Klebsiella variicola*-NA5 was 100 % susceptible and thus its growth on solid agar completely inhibited by both Penicillin and Streptomycin (Table 2).

Table 1. Selected plant growth-promoting traits possessed by PSB isolates

Isolate	Solubilization index in: -			IAA production (µg/ml)				
	$FePO_4$	$Ca_2(HPO_4)_3$	$ZnCO_3$	1% Tryp[a].	Without Tryp.	NH_3 prodution (µg/ml)	Antifungal efficiency (%)	Siderophore production ability test
contrl	0	0	0	1.4^{l}	1.3^{l}	9.9^{d}	-	-
SLSp1	2.4	2.7	7.3	8.3^{fgh}	1.680^{l}	147.9^{b}	38.5^{b}	-
NA19a	2.9	3.3	8.6	8.6^{fg}	6.0^{ghijk}	168.3^{ab}	32.3^{c}	+
NA4b	2.3	2.7	8.4	5.2^{ghijkl}	2.2^{ijkl}	114.5^{c}	39.1^{b}	+
SUApp3	1.7	2.7	7.3	14.1^{cd}	6.2^{ghi}	119.0^{c}	34.5^{c}	-
MdG1	2.5	3.6	10.1	29.5^{ab}	10.6^{ef}	179.3^{a}	27.2^{d}	+
Mk10	1.9	2.6	5.8	17.3^{bc}	6.0^{ghij}	15.9^{d}	47.7^{a}	+
MbMz1	2.4	3.5	6.0	13.2^{de}	3.2^{ijkl}	143.4^{ab}	22.7^{e}	+
NA5	3.1	2.9	1.0	nd	nd	140.6^{bc}	27.4^{d}	+
MdE4	1.8	3.8	9.9	32.8^{a}	11.8^{jl}	163.7^{ab}	38.5^{b}	+++
Kjm3	2.2	2.5	7.9	3.6^{ijkl}	4.4^{hijkl}	134.1^{ab}	32.8^{c}	+

Tryp[a] = Tryptophan; nd = Not detected; - = negative for the test + = Positive for the test; +++ = Highly positive for the test. Means followed by the same letter or set of letters within a row are not significntly different at (P<0.05) according to the Duncan's New Multiple Range Test (DNMR)

Two of the 10 isolates were identified as species of *Burkholderia* while the remainder are members of the genus *Klebsiella* (Table 2; Figure 1). Species of *Klebsiella* as well as those of *Burkholderia* are known to solubilise phosphates and produce other plant growth promoting substances (Singh et al., 2015; Trinetra et al., 2020).

Table 2. Molecular Identity and selected biochemical properties of studied isolates

Isolate	Species	Accession Number	Selected biochemical properties							
			Nucleotide Identity	Catalase activity	Starch hydrolysis	Glucose fermentation	Lactose fermentation	Sucrose fermentation	Penicillin inhibition zone	Streptomycin inhibition
SLSp1	*Klebsiella* sp.	MZ502674	99.8	+	-	+	-	-	23	0
0Mk10	*Burkholderia* sp.	MZ502221	99.9	+	-	-	-	-	18	0
NA19a	*Klebsiella* sp.	MZ502673	99.8	+	-	+	+	+	47	38
NA4b	*Klebsiella* sp.	MZ502671	99.8	+	+	+	-	-	23	41
SUApp3	*Klebsiella* sp.	MZ502675	99.7	+	+	+	-	+	25	0
MdG1	*Klebsiella variicola*	MZ502670	99.8	+	+	+	+	+	12	0
Kjm3	*Burkholderia* sp.	MZ502220	99.9	+	-	-	-	-	18	47
MbMz1	*Klebsiella* sp.	MZ502668	99.8	+	-	+	+	+	25	0
NA5	*Klebsiella variicola*	MZ502672	99.8	+	-	+	-	+	0	0
MdE4	*Klebsiella variicola*	MZ502669	100	+	-	-	-	-	25	44

+/- Isolate is positve/ negative for the indicated test. Values are means of three replicate trials

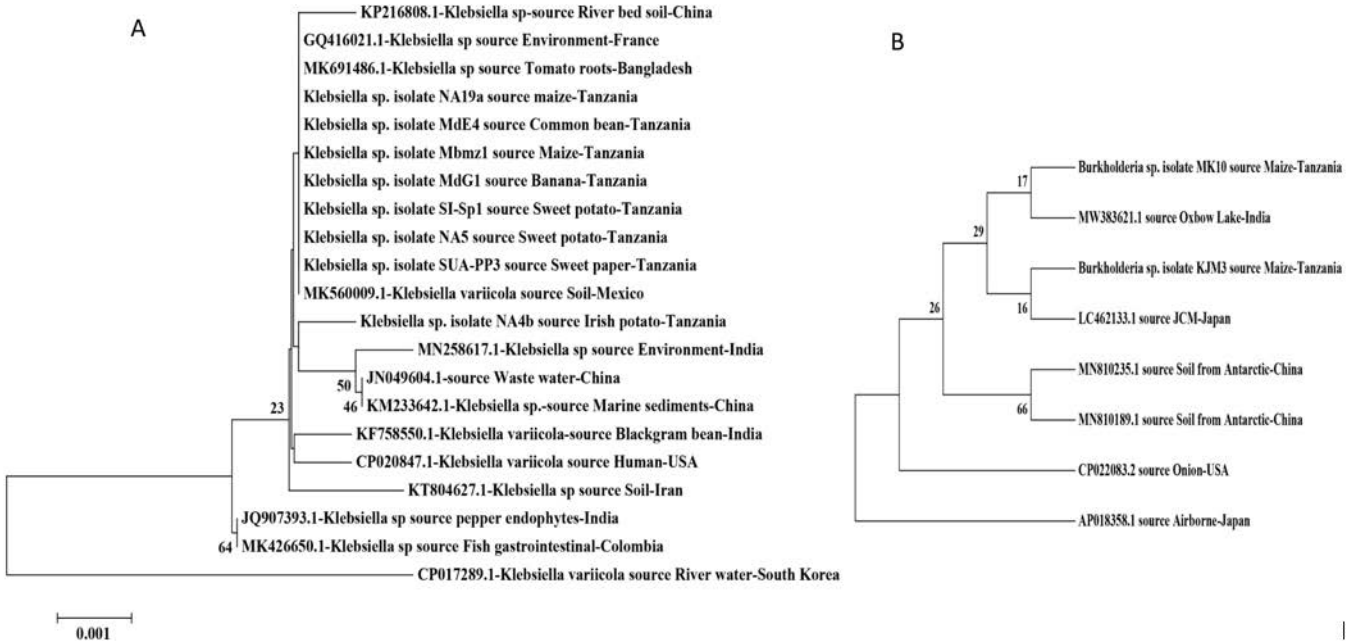

Figure 1. (A) Molecular phylogenetics of *Klebsiela* species isolated from Tanzanian agricultural soils. *Klebsiella variicola* sequence sourced from river water in Korea was used as an outgroup (B): Molecular phylogenetics of various *Burkholderia* species isolated from rhizosphere soils in Tanzania. Sequence no. AP018358.1 sourced from air in Japan was used as an outgroup. A bootstrap con-census tree is shown based on the Jones-Taylor-Thornton matrix model.

Performance of isolates on phosphate solubilization from various inorganic P resources

Studied isolates exhibited differing abilities to solubilize inorganic phosphate from three different forms namely ferric phosphate (FeP), tricalcium phosphate (TCP) and the powdered Hard Minjingu phosphate rock (HMPR). Amounts of soluble phosphate after 10 days of incubation in liquid cultures were numerically higher in TCP and HMRP broths than in FeP for all the ten isolates (Figure 2). Irrespective of the inorganic phosphate resource used, *Klebsiella variicola*-MdE4 and *K. variicola*-MdG1 outperformed the rest of the isolates by producing up 701, 699 and 750, 680 µg/ml of soluble phosphate from TCP and HMRMP, respectively. Ironically, no differences (P<0.05) were observed in the amounts of soluble P from FeP for all the ten isolates tested. These results reaffirmed the selection of *Klebsiella variicola*-MdE4 and *K. variicola*-MdG1 for further tests as potential ingredients in an inoculant formulation.

Effect of replacing glucose for molasses as a carbon source in growth medium

Replacing the 10 g glucose with 20 ml of molasses in the original PVK medium led to a decrease (P<0.05) in the amounts of inorganic phosphate solubilized by both isolates- *Klebsiella variicola*-MdE4 and *K. variicola*-MdG1. However, the amounts of soluble phosphate in the 20 ml molasses-based preparations were high enough to support a bio-inoculant formulation (Figure 3). A further increase in amount of molasses used in the growth medium from 20 to 30 ml did not result in any increment in amounts of soluble phosphate by either isolate. So the 20 ml molasses-containing preparation was used to develop a modified medium for the development of the Biorock P inoculum.

The two isolates also showed abilities to solubilize phosphate while using molasses as alternative carbon sources without reductions in amounts of P solubilized. A recent report has indicated molasses as a good carbon source to support bacterial growth and phosphate solubilization in liquid cultures (Mažylyte et al., 2022). Use of molasses (cane or beet molasses) as alternative carbon source in microbial fermentation cultures is advocated due to low cost and high (40-50%) sugar content (Vandenberghe et al., 1999; Wang et al., 2020). We therefore, used the 20 ml molasses and powdered hard Minjingu phosphate rock (HMPR) to amend the original PVK medium into MdG1 and MdE4-based Biorock P inoculum for field application studies.

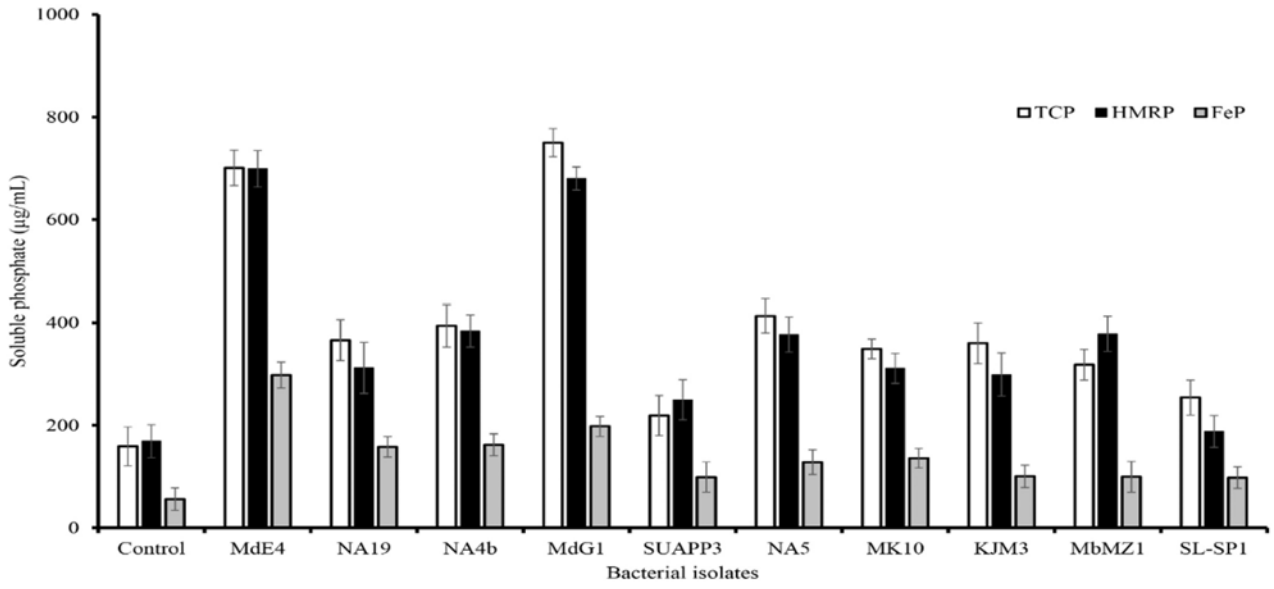

Figure 2. Performance of various bacterial isolates on different types of inorganic phosphate resources as sole sources of P in the growth medium. Error bars indicate standard error of the mean for at least three replications

TCP – Tricalcium phosphate, HMRP = Hard Minjingu Phosphate rock, FeP = Ferric phosphate as a sole source of P.

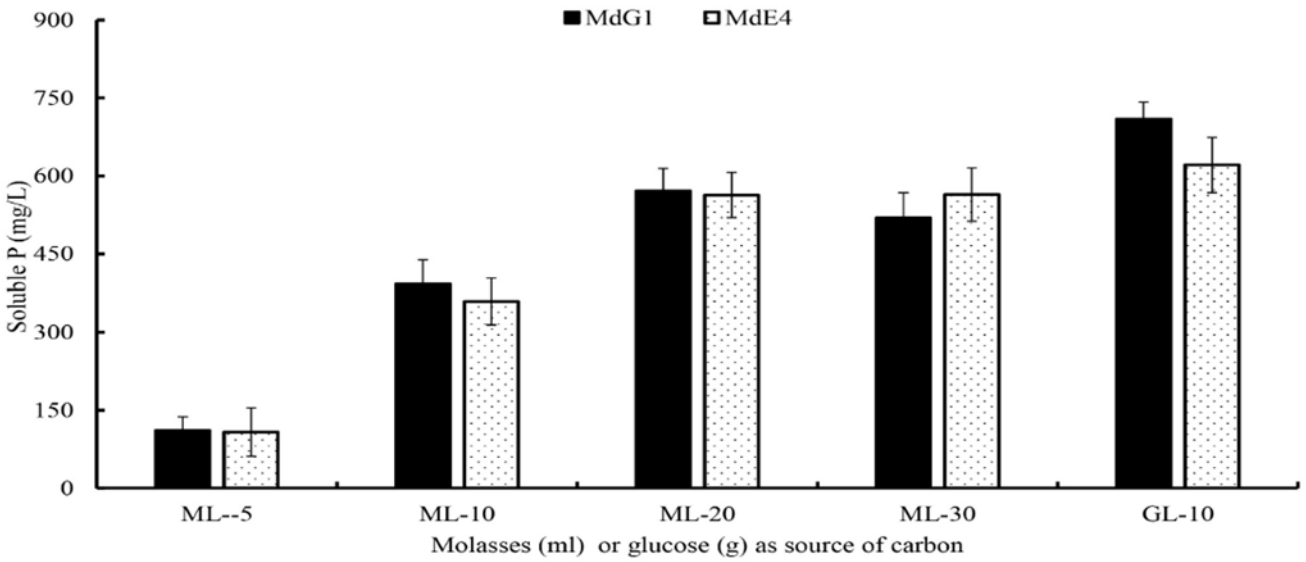

Figure 3. Effect of replacing glucose with molasses as a source of carbon on phosphate solubilization of isolates MdG1 and MdE4. Error bars indicate standard error of the mean for at least three replications

Field performance of the Biorock P inoculum application with maize as a test crop

The selected physicochemical properties of the soils at study site

Main physical chemical properties of the soils at the study sites are presented in Table 3. The soils' textural classes are clay and Sandy clay for Magadu and Madaba sites, respectively, based on soil textural triangle of the United State Department of Agriculture (1975). The soils were characterized as very strongly acidic and strongly acidic for Magadu and Madaba sites, respectively. Bray-1 extractable soil P for the soils of both site were very low. Both soil had also very low total nitrogen, and low to medium organic carbon content (Table 3). The two soils were poorly suited for maize production and needed corrective measures. Soils of the two sites exhibited different P adsorption properties with that of Magadu having a higher adsorption maxima (769.23 mg P/kg) as compared to that of Madaba (185.185 mg P/kg) (Figure 4). Consequently, Magadu soil is considered a high phosphorus fixing soil since it removes more than 700 mg P/kg of soil (adsorption maxima = 769.23 kg P/ha) while Madaba soil are considered low P-fixing (Figure 4).

Effect of Bio-rock P inoculation on available phosphorus, maize P uptake, Phosphorus Use Efficiency (PUE)

There has been a general increase in soil available P as the applied inorganic phosphate + Biorock P inoculant increased at both Madaba and Magadu sites (Figure 5). Irrespective of the differences in physico-chemical properties, and especially the P-fixing potentials of the soils of the two study sites, inclusion of biorock P inoculant during the inorganic P fertilizer application showed an increase ($P<0.05$) in the amount of available P. (c.f. values for 0P vs 0P+I and values for 40P vs 40P + I) at both site sites.

Table 3. Selected physicochemical properties of Magadu and Mabada site soils used in this study

Site	Texture	pH H_2O (1:1.25)	OC (%)	*Avail P (mg/kg)	TN (%)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	CEC (Cmolc / kg)
Magadu	Clayey	4.9	0.9	3.34	0.08	1.41	1.29	1.2	10.7
Madaba	Sandy Clay	5.1	1.4	3.9	0.09	2.745	1.8	1.08	17.4

*Available P based on Bray 1 extraction method, TN = Total Nitrogen. Values are means of three replicate trials

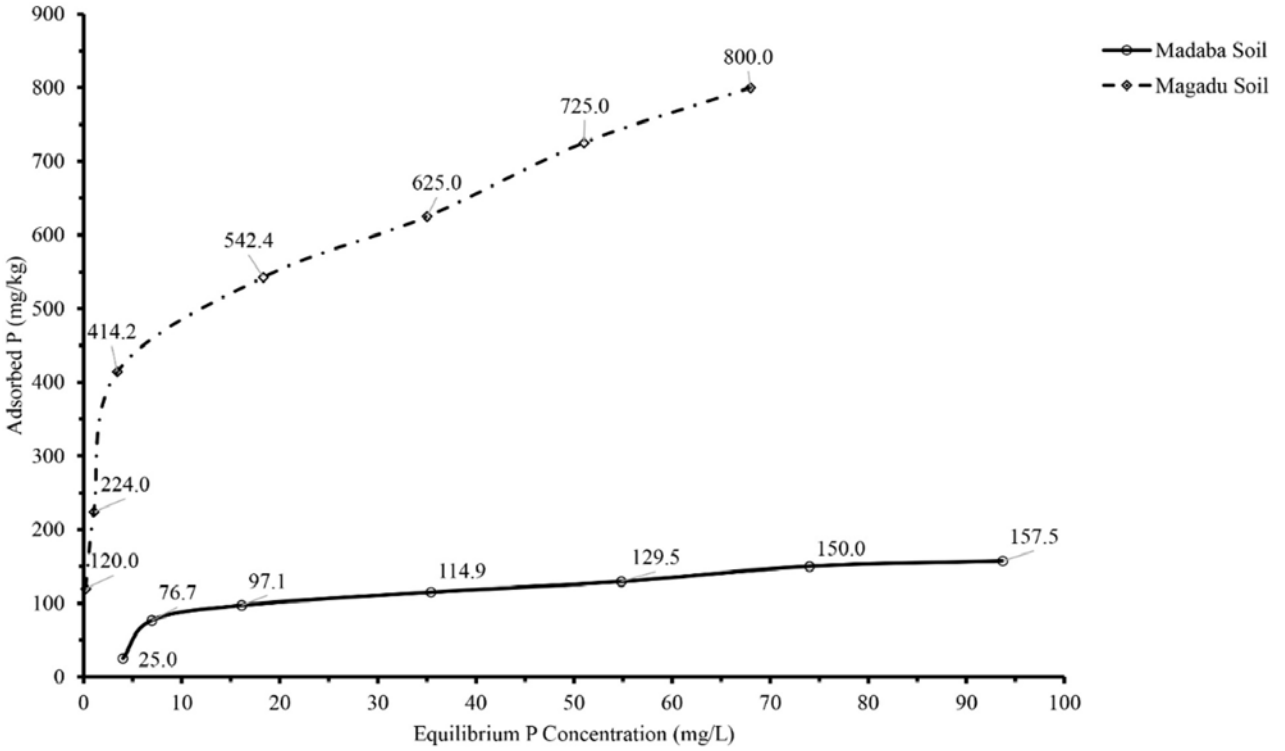

Figure 4. Phosphorus adsorption properties of soils in Magadu and Madaba experimental sites

Generally, however, at any inorganic phosphate + inoculant application rate, there were numerically higher amounts of available P at Madaba than Magadu study site (Figure 5) which is attributable to the lower fixing capacity of the Madaba than Magadu site.

While there is plenty of information on the ability of soil microorganisms to solubilise inorganic phosphate resources under laboratory conditions, only a few studies, demonstrated effects of such organisms on plant growth and development under field conditions (Batool, and Iqbal, 2020; Wang et al., 2021). We have demonstrated in the current study that field application of Biorock P- a molasses-based biofertilizer formulation containing *Klebsiella variicola*-MdE4 and *K. variicola*-MdG1 can enhance phosphate solubilisation and promote maize plant growth and yield. We have shown further that the effects of Biorock P inoculant formulation were numerically more pronounced in soils with low P absorption maxima (Madaba site) than higher P-fixing soils of Magadu site.

Total phosphorus accumulation in the whole plant was taken to reflect P uptake by plants. There were differences (P<0.001) in the total P uptake among treatments at both experimental sites (Figure 6). Although all levels of inorganic phosphate application (with or without biorock P inoculant) resulted into an increase in total P uptake by plants, addition of the biorock P inoculant did result into increment (≤ 0.001) in total P uptake compared to the absolute control (0 kg P /ha). Respectively, the highest P uptake values by maize plants were recorded in plots treated with 80 kg P ha + biorock P inoculant (Figure 6). Furthermore, the influence of biorock P inoculant/ on total P content of plants was so significant that applying 20 kg P/ha + biorock P inoculant resulted into higher plant P uptake than where inorganic phosphate alone was applied at a rate of 40 kg P/ha (Figure 6).

Phosphorus use efficiency varied (P<0.05) with different inorganic phosphorus plus biorock P inoculant application rates. At the recommended P application rates (40 kg P/ha) addition of biorock P inoculant resulted into the higher PUE values than when inorganic P was applied without the inoculant.

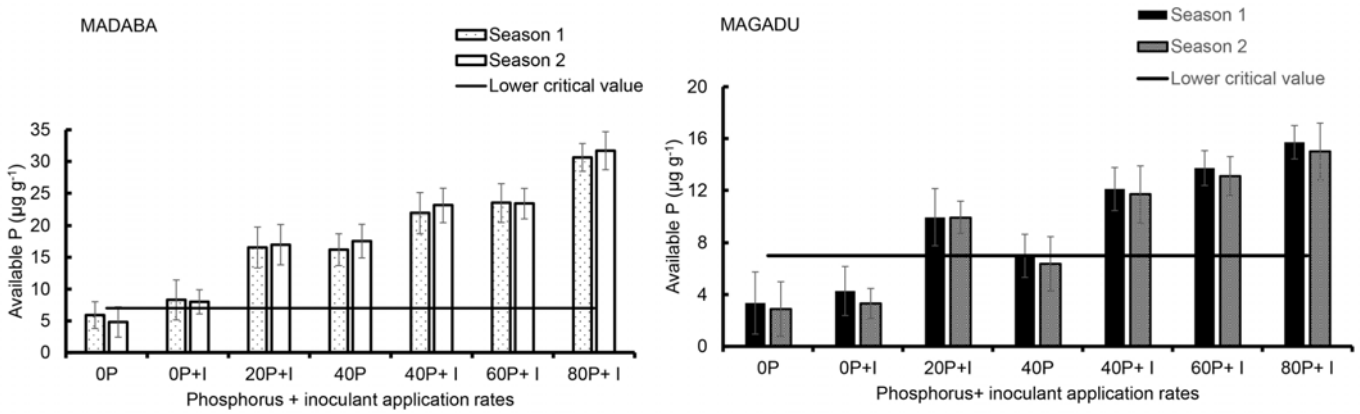

Figure 5. Effects of Bio-rock P application rates on available phosphorus under field conditions after two growing seasons at Madaba and Magadu sites. Error bars indicate standard error of the mean for at least three replications

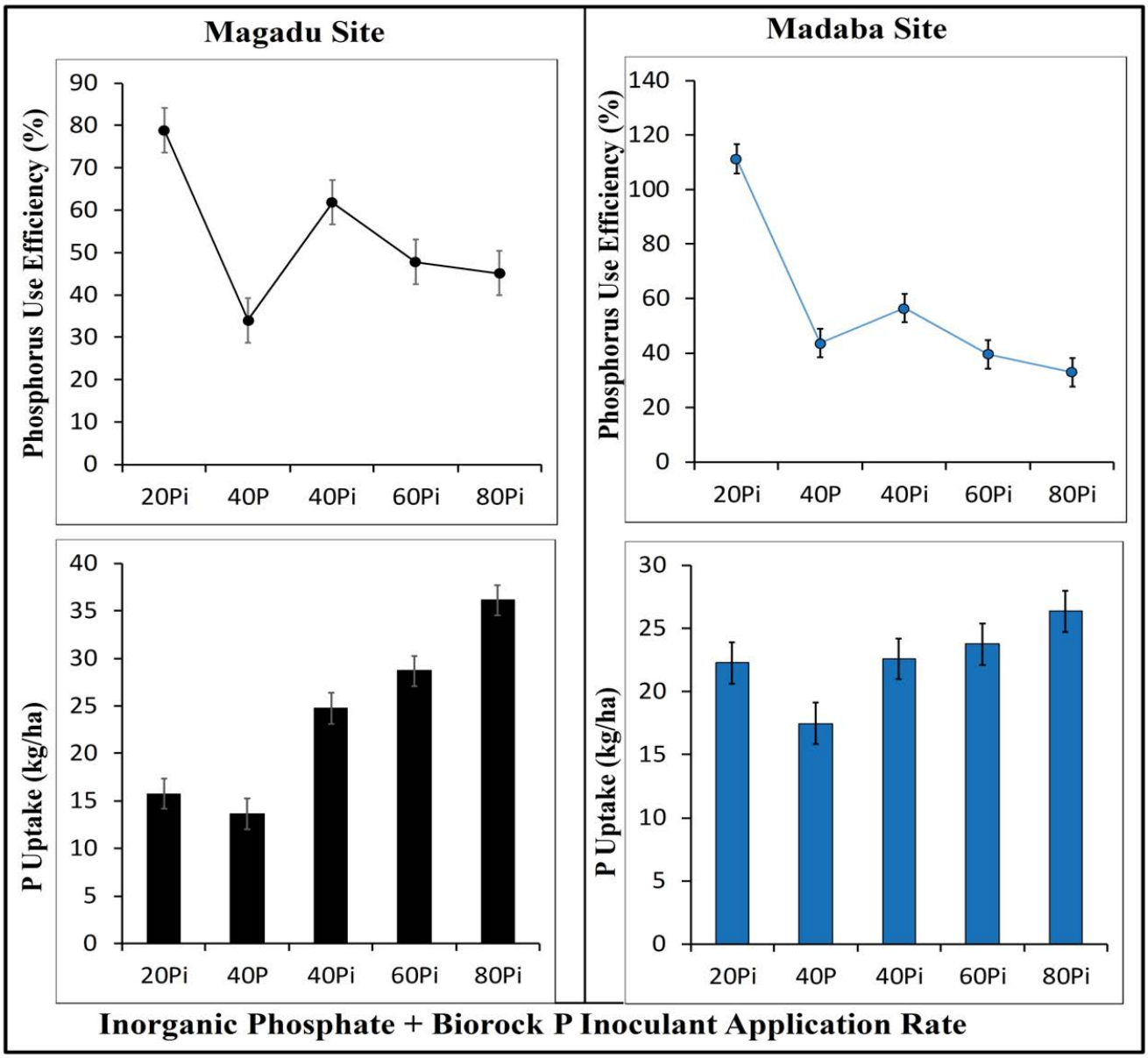

Figure 6. Effect of Biorock P inoculant application on maize P uptake and P use efficiency in second season. Error bars indicate standard error of the mean for at least three replications

Using biorock P at a lower inorganic P application rate (20 kg P/ha) gave even higher PUE values than at higher application rates (>40 kg P/ha)

The observed numerically lower values of available phosphorus, lower P concentration in plants and lower P use efficiency at the Magadu site compared to those of Madaba site could be directly attributable to a higher P adsorption maximum of the soils at the Magadu site. The observed differences between sites may also be due to unsorted conditions such as soil moisture availability, temperature, soil reaction, and soil texture.

The higher P adsorption tendencies of the Magadu soils may have rendered most of the microbially solubilized phosphate unavailable to the plant due to transformation of P into less available forms including adsorption by soil colloids. The differences in the extent of P adsorption in soils and consequently availability of P to plants at the two study sites could be a result of their differences in physicochemical properties and management (Muindi et al., 2015; Ayenew et al., 2018).

Effects of Bio-rock P inoculation on maize grain yield

Co-application of inorganic phosphate and bio-rock P inoculant under field conditions resulted into higher (P<0.05) maize grain yields compared to non-inclusion of bio-rock P. Accordingly, application of the bio-rock P inoculant without additional inorganic P (0P +I) resulted into nearly doubling the maize grain yield at both sites for two consecutive growing seasons (Figure 7). Co-application of bio-rock P and inorganic phosphate at 20 kg P/ha gave higher (P<0.05) maize grain yield than that of positive control (40 kg P/ha). Bio-rock p inoculant in the 40 kg P/ha treatment or higher did not result into any higher grain yield compared to that of 20 kg P/ha plus bio-rock P (Figure 7).

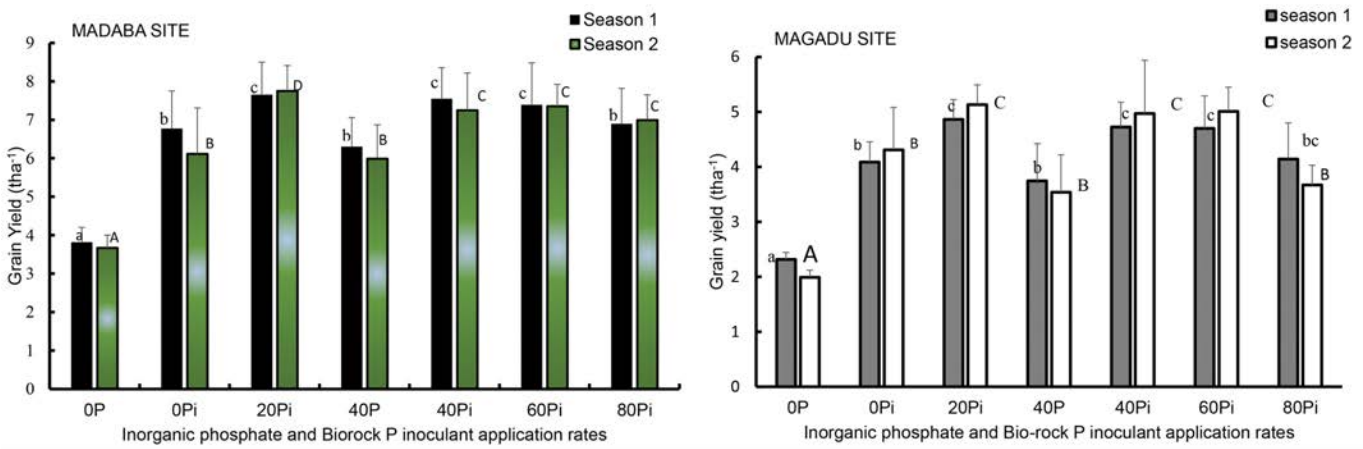

Figure 7. Effects of Bio-rock P application rates on maize grain yield under field conditions after two growing seasons at Madaba and Magadu sites. Bars of the same category followed by the same type of letters are not different according to the DNMRT at P=0.05. Error bars indicate standard error of the mean for at least three replications

CONCLUSION

In conclusion, the present study has demonstrated that the two isolates *Klebsiella variicola*-MdE4 and *K. variicola* MdG1 can solubilize Minjingu Phosphate Rock (MPR) in concentrations high enough to constitute a reliable bio fertilizer inoculant. These strains exhibited additional qualities through production of siderophores, IAA and ammonia. All of these qualities are important in enhancing plant growth and development and ultimately better yields. Their compatibility when grown in a single culture formulation that uses molasses as an alternative and cheaper source of carbon make them a potent bio fertilizer formulation for commercial application.

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