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FULL LENGTH ARTICLE

Phosphate-solubilising rhizobacteria associated with *Coffea arabica* L. in natural coffee forests of southwestern Ethiopia

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Abstract Phosphate-solubilising rhizobacteria associated with *Coffea arabica* L. in natural coffee forests of southwestern Ethiopia were investigated. The main purpose was to screen for potential microbial biofertilisers by assessing isolated strains for phosphate solubilisation efficiency and organic acid production in different media. Initial screening was performed on Pikovskaya's agar (PA). Quantitative colorimetric estimations of mobilised phosphate were made in different broth media in the presence of two phosphate sources. HPLC was employed for the detection of organic acids. From a total of 395 rhizobacterial isolates tested for P solubilisation, over 72% (mostly *Pseudomonas* spp.) formed visible dissolution haloes on PA. Two *Erwinia* species and a *P. chlororaphis* strain produced the largest solubilisation indices and also solubilised hydroxyapatite strongly in broth medium. Solubilisation of hydroxyapatite (HAP)/tricalcium phosphate (TCP) by all isolates coincided with a decrease in medium pH. HPLC analyses of culture supernatants confirmed the presence of several organic acids, with 2-ketogluconic acid dominating. The production of organic acids by these coffee-associated phosphobacteria could be considered the major mechanism involved in the solubilisation of insoluble HAP/TCP. Certain isolates deserve particular attention for bioinoculant development due to their remarkable efficiency of insoluble phosphate solubilisation. The present study could therefore be important with respect to screening of *Coffea arabica*-associated rhizobacteria that possess direct plant growth-promoting traits for extending the use of indigenous microbes as microbial biofertilisers.

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1. Introduction

Coffea arabica L., the most important world commodity, is the principal source of revenue for the agriculture-based Ethiopian economy (Gole et al., 2002), which greatly requires sustainable coffee production with healthier product quality to remain in the highly competitive current market.

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Coffea arabica is extensively cultivated in small gardens and large-scale plantations under a variety of shade trees in southwestern Ethiopia (Muleta et al., 2011). While the use of mineral fertilisers is considered the quickest and surest way of boosting crop production, their cost and other constraints (i.e. environmental hazards) potentially deter farmers in economically disadvantaged countries such as Ethiopia from using them in the recommended quantities. The current crisis in coffee prices on the world market due to overproduction (Albertin and Nair, 2004) and the progressive revival of interest in organically-grown coffee, which is closely coupled with fears about environmental health and biodiversity (van der Vossen, 2005), are additional problems to millions of coffee farmers in developing countries. These problems make it essential to look for alternative strategies that can ensure competitive coffee yields while protecting the health of soils. The pressing need for economically and ecologically acceptable fertiliser sources has prompted the search for a new approach to farming (sustainable agriculture). Sustainable agriculture constitutes a major strategy to counteract the rapid decline in environmental quality by maintaining the long-term ecological balance of ecosystems (Khan et al., 2006). In this context, the use of microbial inputs (microbial biofertilisers) represents an environmentally-friendly option to continued application of soluble mineral fertilisers.

Bacteria in the rhizosphere can affect plant growth either positively or negatively. The term 'plant growth-promoting rhizobacteria' (PGPR) is used to describe strains of naturally occurring root-colonising soil bacteria that possess the capacity to stimulate plant growth either directly or indirectly (Kloepper and Schroth, 1978). PGPR can affect plant growth by a wide range of mechanisms such as solubilisation of inorganic phosphate, production of phytohormones, siderophores and organic acids, lowering of plant ethylene levels, N_2 fixation and biocontrol of plant diseases (Muleta et al., 2007b; Datta et al., 2011). The use of such beneficial bacteria as biofertilisers and biocontrol agents has currently attracted increased interest world-wide in attempts to achieve sustainability, particularly in agriculture, forestry and horticulture (Datta et al., 2011).

Phosphorus (P) is second only to nitrogen as the major essential macronutrient restricting plant growth in highly weathered tropical soils (Vessey, 2003; Khan et al., 2006). Most natural ecosystems in tropical and subtropical areas are predominantly acidic and extremely P-deficient (Khan et al., 2006) due to their strong fixation of P as insoluble phosphates of iron and aluminium. To alleviate P deficiency, chemical phosphatic fertilisers are widely used. However, a large proportion of the soluble forms of P fertilisers is precipitated in insoluble form soon after application and becomes unavailable to plants (Mahantesh and Patil, 2011). This in turn leads to a need for excessive and repeated application of soluble P fertilisers, which in addition to the economic constraint can pose a serious threat to groundwater.

Supplying P through biological means is therefore a realistic alternative in order to lower the environmental risk and enhance the productivity of such ecosystems (Vessey, 2003). Evidence is increasing that phosphate-solubilising bacteria (PSB) and fungi (PSF) play a central role in conversion of insoluble P to bioavailable primary and secondary orthophosphate ions (Pal, 1998). Goldstein (1986) had previously demonstrated that various bacterial species are able to mobilise insoluble inorganic P compounds such as di- and tricalcium phosphate, hydroxyapatite and rock phosphate (RP). It is worth noting that hydroxyapatite (Ahn, 1993) and RP (Rodrìguez and Fraga, 1999) are the largest reserves of P.

The list of important genera of mineral P solubilisers is increasing over time as more research groups are engaged in screening potential rhizobacteria. The commonly reported genera include *Achromobacter*, *Aereobacter*, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Erwinia*, *Flavobacterium Micrococcus*, *Rhizobium* and *Pseudomonas* (Rodrìguez and Fraga, 1999). Accordingly, Tilak et al. (2005) emphasise that the most efficient phosphate-solubilising microorganisms (PSM) belong to the bacterial genera *Bacillus* and *Pseudomonas* and the fungal genera *Aspergillus* and *Penicillium*. These authors further note that the use of PSB as inoculants simultaneously increases P uptake by the plant and crop yield. Many PSB also exhibit other beneficial traits such as production of siderophores, phytohormones and 1-amino-cyclopropane-1-carboxylate deaminase activity (Poonguzhali et al., 2006; Muleta et al., 2007b). The possible positive effects of free-living PGPR on various crop types have been extensively reviewed (Vessey, 2003; Lucy et al., 2004).

Interestingly, a high proportion of PSB is concentrated in the rhizosphere and such bacteria are more metabolically active than those isolated from other sources (Vazquez et al., 2000), which is of great relevance to plants, particularly in P-deficient soils (Khan et al., 2006). Although there have been a few efforts to understand the P solubilisation process at the molecular genetics level, the precise mechanism utilised by different PSB still remains largely unidentified (Babu-Khan et al., 1995). Nevertheless, the production of organic acids seems to be the main mechanism for solubilisation of insoluble P (Illmer et al., 1995).

Very little is known about microorganisms associated with *Coffea arabica* in Ethiopia regarding their functional characteristics towards plant growth promotion. However, reports from other coffee growing areas have revealed the presence of nitrogen-fixing bacterial endophytes such as *Acetobacter diazotrophicus* in coffee roots (Jiménez-Salgado et al., 1997) and *Paenibacillus* spp. in coffee berries (Sakiyama et al., 2001). Likewise, 87 cultivable endophytic bacterial isolates in 19 genera including *Bacillus*, *Burkholderia*, *Pseudomonas* and *Serratia* were obtained more recently from coffee tissues collected from Colombia, Hawaii and Mexico (Vega et al., 2005).

As a complement to our present investigation of coffee rhizosphere bacteria, we also quantified arbuscular mycorrhizal fungi (AMF) spore density in the coffee rhizosphere (Muleta et al., 2007a; Muleta et al., 2008) and investigated coffee seedling root colonisation by AMF (Muleta et al., unpubl.), as well as conducting antagonistic studies of rhizobacteria against some emerging fungal coffee pathogens (Muleta et al., 2007b) in southwestern Ethiopia. Special emphasis has been placed on this region because it is the indigenous source of *Coffea arabica*, where coffee bushes still grow wild as an important Arabica coffee gene pool (Aga et al., 2003) and we considered it advantageous to screen indigenous beneficial microbes from this centre of origin. It has been strongly suggested that the greatest benefit to the host arises from the use of native, and not introduced, strains of PGPR and AMF (Requena et al., 1997). Furthermore, Pandey et al. (2006) have demonstrated the potential biotechnological applications of native microbes in promotion of plant growth. The present study therefore aimed to screen coffee-associated phosphate-solubilising

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rhizobacteria from our own collection and to evaluate their efficiency in mobilising insoluble P in different media in the presence of different P sources. In addition, acidification of the media and organic acid production by selected isolates were assessed in the presence of different P sources.

2. Materials and methods

2.1. Description of isolates locations and sources

The rhizobacterial isolates originated from two locations in southwestern Ethiopia: (i) Bonga district (Kaffa zone) in Southern Nations and Nationalities Peoples' Regional State (S.N.N.P.R.S); and (ii) Yayu district (Illubabor zone) in Oromia Regional State, Ethiopia (Fig. 1). The sites are located between 07°28'–08°28'N and 35°50'–36°45'E, with an altitude range of 1376–1890 m asl. The sites have diurnal and seasonal fluctuations in temperature (14–30 °C) and relative humidity (43–85%) and heavy rainfall (1000–2000 mm per year).

In southwestern Ethiopia, the dominant coffee growing soil types are of volcanic origin, with Nitosols (25%), Acrisols (17%), and Luvisols (14%) (Höfner, 1987). The soil composition is clay (13%), loamy clay (29%), silty clay (29%), and sandy clay (22%) in relative proportions, with pH ranging mostly from 5 to 6.8 (water extract). Some soil chemical parameters such as available P, total organic C and N have been described previously for both Bonga (Muleta et al., 2007a) and Yayu natural coffee forests (Taye, 2001).

The rhizobacteria (395 strains) used in this study were from the culture collection at the Department of Biology, Addis Ababa University, and were originally isolated from the rhizosphere of *Coffea arabica* L. growing in natural forests in southwestern Ethiopia (Muleta et al., 2009). The strains were characterised by conventional (API kits) methods and 16S rDNA analysis (Muleta et al., 2009). The bacterial isolates in this study were tested for phosphate solubilisation ability. The genera present according to API kit tests (Table 1) included *Bacillus* (n = 147), *Pseudomonas* (n = 134), *Burkholderia* (n = 51), *Chryseomonas* (n = 20), *Aeromonas* (n = 13),

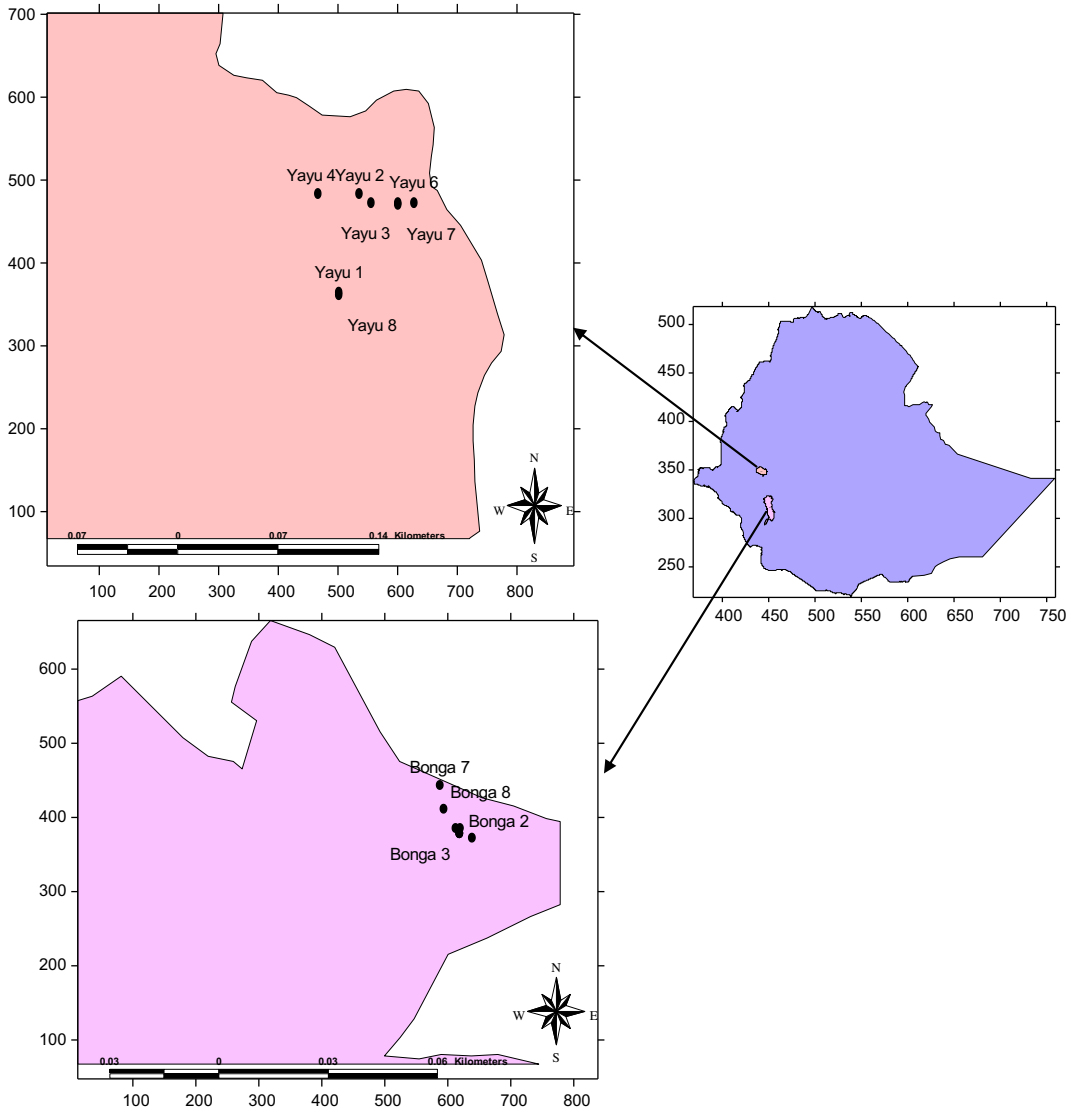

Figure 1 Map of the study sites.

Table 1 Frequency distribution of P solubilising rhizobacteria associated with *Coffea arabica* in natural coffee forests, southwestern Ethiopia.

Rhizobacterial genus (API kit-based identification)[a]	Gram reaction	Site		% P mobilisers on PA
		Bonga	Yayu	
Pseudomonas spp.	–	60	74	33.92
Burkholderia spp.	–	18	33	12.91
Bacillus spp.	+	75	72	11.39
Chryseomonas sp.	–	9	11	5.06
Aeromonas spp.	–	8	5	3.29
Acinetobacter sp.	–	4	9	3.29
Vibrio spp.	–	1	3	1.01
Pasteurella sp.	–		2	0.51
Alcaligenes sp.	–		1	0.25
Chromobacterium sp.	–		3	0.25
Chryseobacterium sp.	–	1		0.25
Agrobacterium sp.	–	1		0
Stenotrophomonas sp.	–	2	3	0
Total		179	216	72.13

[a] Muleta et al. (2009).

Acinetobacter ($n = 13$), *Stenotrophomonas* ($n = 5$), *Vibrio* ($n = 4$), *Chromobacterium* ($n = 3$), *Pasteurella*, *Agrobacterium*, *Alcaligenes* and *Chryseobacterium* (one isolate each). Nutrient agar (Oxoid) was used for reviving the cultures.

2.2. Screening for P-solubilising capacity

Pikovskaya's agar (PA; Pikovskaya, 1948) was employed for screening the rhizobacterial isolates for P-solubilising capacity. The inoculated plates were incubated for 7–14 days at 30 °C. Isolates showing zones of clearance on PA were stored at −70 °C for subsequent analyses. P-solubilising rhizobacterial isolates (PSB; $n = 135$) were subjected to repeated subculturing on PA to confirm their consistency in dissolution of mineral P before other subsequent tests.

2.3. Determination of solubilisation index

The ability of the rhizobacterial isolates to solubilise insoluble phosphate was primarily described by the solubilisation index (SI): Ratio of total diameter (colony + halo zone) to colony diameter on PA incubated at 30 °C (Edi-Premono et al., 1996). Measurement was carried out on Day 7 of incubation.

2.4. Mineral P-solubilisation efficiency in liquid media

The efficiency of the selected rhizobacterial isolates for P-solubilisation was quantitatively measured in National Botanical Research Institute's Phosphate growth medium (NBRIP; Nautiyal, 1999) containing ($g\,l^{-1}$) 10 glucose, 5 $Ca_3(PO_4)_2$; 5 $MgCl_2{\cdot}6H_2O$; 0.25 $MgSO_4{\cdot}7H_2O$; 0.2 KCl and 0.1 $(NH_4)_2SO_4$. Since tricalcium phosphate (TCP) suffers from inherent self-solubility (M. Habte, pers. comm. 2010), a more insoluble phosphate mineral, hydroxyapatite ($HAP \approx Ca_{10}(OH_2)(PO_4)_6$, Aldrich Chemical Co.) was used in NBRIP (NBRIP + HAP) at a rate of $4\,g\,l^{-1}$ to evaluate the efficiency of some selected rhizobacterial isolates. The pH of each medium was adjusted to 7.0 before autoclaving. Quantitative spectrophotometric (UV-160A Shimadzu, Stockholm, SWE) analysis of the soluble phosphate was performed according to the method of Murphy and Riley (1962) at different time points (Days 0, 3, 5, 7 and 10 in the presence of TCP or Days 0, 3, 5, 7, 10 and 20 in the presence of HAP). Values obtained with uninoculated controls were always subtracted from their respective treatments. The pH of the cultures was also measured using a pH meter (PHM93 Reference pH meter, Copenhagen, DE). All experiments were performed in duplicates.

2.5. Determination of organic acid production

For the evaluation of organic acid production, rhizobacterial isolates were grown in NBRIP using two P sources, namely HAP or TCP, on a shaker (150 rpm) at 30 °C. Sampling for organic acid detection and pH measurement was made on the third day. Cultures were centrifuged at 3000 rpm for 10 min before analysis of organic acids by high performance liquid chromatography (HPLC). The HPLC system was an Agilent 1100 (Agilent Technologies, Stockholm, Sweden) containing an automatic injector, quaternary pumps, a Rezex-ROA-Organic Acid H+ column (Skandinaviska Genetec AB, Västra Frölunda, Sweden) and a refractive index detector. Five microliter of undiluted culture supernatant was injected and the eluent was 5 mM H_2SO_4 with a flow rate of $0.6\,ml\,min^{-1}$. The identity of the organic acids was determined by comparing the retention times and peak areas of chromatograms with pure standards of acetic, citric, formic, fumaric, gluconic, 2-keto-gluconic, iso-valeric, lactic, oxalic, propionic, succinic and n-valeric acids.

2.6. Data analysis

Pearson correlation analysis was used to explore the relationships between solubilised P, SI, pH values, organic acids and incubation periods of selected rhizobacterial isolates. ANOVA was employed to test differences in solubilisation efficiency, solubilisation index and ability to acidify the growth medium, using SPSS version 13 and Tukey's HSD post hoc test to assess for mean separation ($p < 0.05$).

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3. Results

3.1. Screening for P-solubilising capacity and determination of SI

Over 72% of the rhizobacterial isolates showed clearly visible haloes (>0.50 cm) around their colonies (Fig. 2a,b) on PA after five days of incubation. Members of the phosphobacteria were dominated by the genus *Pseudomonas* (33.9%) followed by *Burkholderia* (12.9%), and *Bacillus* (Table 1). The majority of Gram-positive isolates (n = 36/45) and some of the Gram-negative isolates (n = 6/90) lost their capacity for phosphate solubilisation on repeated subculturing but the majority of the latter group retained this property with good clear zones (Fig. 2a,b).

The isolation sites and identity (Muleta et al., 2009) of 18 consistent and efficient rhizobacterial isolates used for evaluation of solubilisation indices (SI) and P solubilisation ability in liquid media are presented in Table 2. The SI of the potential P solubilising rhizobacterial isolates differed significantly ($p < 0.05$) and ranged from 2.05 to 5.82. The bacterial strain AUEY28 (*Erwinia rhapontici*) produced the largest zone of solubilisation, followed by AUPY10 (*P. chlororaphis*) and AUEY29 (*E. rhapontici*). In general, the largest solubilisation indices were produced by Gram-negative isolates compared with the *Bacillus* species. SI and solubilised *P* values in liquid media (see below) were strongly (r = 0.71, $p < 0.01$) correlated.

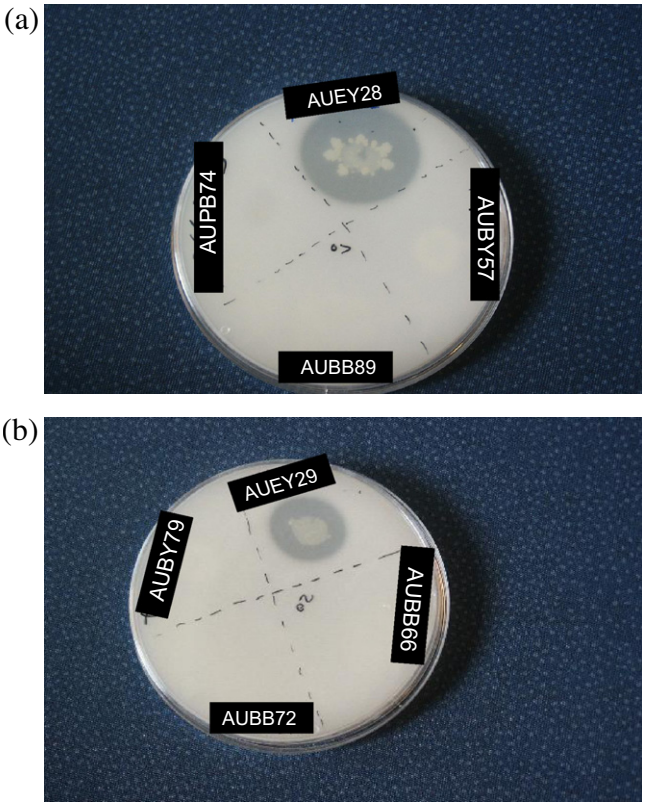

Figure 2 Insoluble phosphate solubilisation studies on Pikovskaya's agar (PA): (a) and (b) show two consistent and efficient phosphate-solubilising isolates (large haloes), whereas six others lost their activity (no visible halo) during repeated subculturing on PA.

3.2. P-solubilising efficiency of selected phosphobacteria in NBRIP

The activity of 11 test isolates (SI = 2.2–5.8; Table 2) was measured in NBRIP. Almost all these isolates showed a higher inherent potential for dissolution of inorganic P as indicated by a gradual increase in the amount of phosphates in the medium mostly until Day 5 (Fig. 3a). These isolates (Fig. 3a) showed significant differences (F = 4.38, $p < 0.01$) in the amount of solubilised P. The highest dissolved P value was exhibited by isolate AUPY10 (*P. chlororaphis*), followed by AUEY29 (*E. rhapontici*) and AUEY28 (*E. rhapontici*), all from Yayu natural coffee forest, with a corresponding decrease in the pH of the medium (Fig. 3b). Isolate AUPB01 (*P. chlororaphis)* from Bonga solubilised P effectively and progressively until Day 7 of sampling, but the amount of mobilised P thereafter declined sharply (Fig. 3a). Most of the tested microbes, however, maintained their levels of solubilised P until the last day of sampling (Day 10; Fig. 3a).

The rhizobacterial isolates showed significant differences (F = 3.66, $P < 0.01$) in decreasing the pH value of the growth medium from the initial value of 7 to a pH value between 4.83 and 3.93 during Day 3 of sampling (Fig. 3b). Beyond this time, no further significant fall in pH value was noted. However, the sharp decline in pH values observed on Day 3 was maintained by most isolates until Day 10 of incubation except for isolates AUSB14 (*Serratia marcescens*) and AUEY28 (*E. rhapontici*), which showed some pH increases during later sampling days (Fig. 3b). On the final day of sampling, the highest (5.20) and lowest (4.00) pH values were recorded in the growth medium of isolates AUSB14 (*S. marcescens*) and AUPY10 (*P. chlororaphis)*, respectively (Fig. 3b). The solubilised P (total) was significantly negatively correlated with recorded pH values (r = −0.73, $p < 0.01$) and positively correlated (r = 0.67, $p < 0.01$) with the incubation time. Longer incubation periods (10 days), however, did not yield additional P solubilisation for most isolates.

3.3. P-solubilising efficiency of selected Gram-negative and -positive isolates in NBRIP + HAP

Seven isolates with consistent solubilisation (without any decrease in phosphate value) at least until Day 7 of sampling and with dissolved P values greater than 60 μg ml^{-1} (Fig. 4a) were selected to test their potential for solubilisation of highly insoluble hydroxyapatite (HAP) in NBRIP (NBRIP + HAP). Significant differences ($p < 0.05$) were observed between isolates in their P-solubilisation efficiency on Days 3, 10 and 20 of sampling (Fig. 4a). The amount of phosphate released in the medium of isolate AUPB01 (*P. chlororaphis*) was low compared with that in the other isolates tested (Fig. 4a). Although the dissolved P values were lower than the rest of the isolates, AUPB01 (*P. chlororaphis*) retained its ability to solubilise HAP until the final day of incubation (Fig. 4a). The highest amount of P was released by isolates AUPB09 (*Pseudomonas* sp.; on Day 3), AUEY29 (*E. rhapontici*; on Day 10) and AUEY28 (*E. rhapontici*; on Day 20; Fig. 4a). Among these isolates, AUEY28 showed remarkable consistency of solubilisation,

Table 2 Solubilisation index (SI) of the selected consistent rhizobacterial isolates associated with forest-grown *Coffea arabica* from southwestern Ethiopia.

Isolate	Identity (16S rDNA analysis)[a]	Gram reaction	Site		SI[b]
			Bonga	Yayu	
AUEY28	*Erwinia rhapontici*	−		X	5.82 ± 0.45a
AUPY10	*Pseudomonas chlororaphis*	−		X	4.93 ± 0.1ab
AUEY29	*Erwinia rhapontici*	−		X	4.79 ± 0.23abc
AUPB09	*Pseudomonas* sp.	−	X		3.93 ± 0.72bcd
AUPB08	*Pseudomonas* sp.	−	X		3.60 ± 0.33bcde
AUPB07	*Pseudomonas* sp.	−	X		3.50 ± 0.71cdef
AUPY26	*Pseudomonas plecoglossicida*	−		X	3.22 ± 0.02defg
AUPB25	*Pseudomonas* sp.	−	X		3.15 ± 0.21defg
AUSB14	*Serratia marcescens*	−	X		3.15 ± 0.64defg
AUPB04	*Pseudomonas koreensis*	−	X		2.99 ± 0.22defg
AUPB13	*Pseudomonas sp.*	−	X		2.99 ± 0.41defg
AUPB01	*Pseudomonas chlororaphis*	−	X		2.74 ± 0.17defg
AUPB49	*Pseudomonas* sp.	−	X		2.65 ± 0.21defg
AUPY11	*Pseudomonas plecoglossicida*	−		X	2.51 ± 0.1efg
AUPY27	*Pseudomonas* sp.	−		X	2.37 ± 0.19efg
AUBB20	*Bacillus subtilis*	+	X		2.36 ± 0.45efg
AUPB03	*Pseudomonas* sp.	−	X		2.19 ± 0.07 fg
AUBB19	*Bacillus subtilis*	+	X		2.05 ± 0.09 g

[a] = Muleta et al., 2009,
[b] = Data are mean ± SD from two replications and values followed by the same letter(s) indicate no significant difference ($p > 0.05$) at 95% confidence interval.

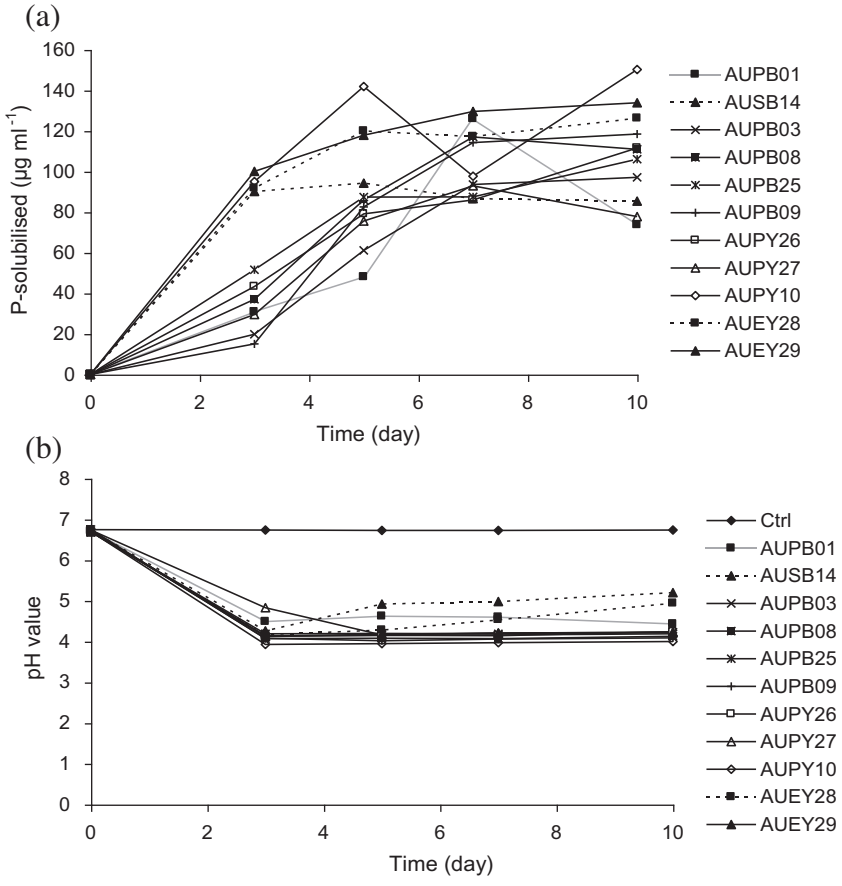

Figure 3 Change in phosphate concentrations and pH values in NBRIP liquid medium by eleven selected Gram-negative phosphobacteria (cf. Table 2) during ten days of incubation.

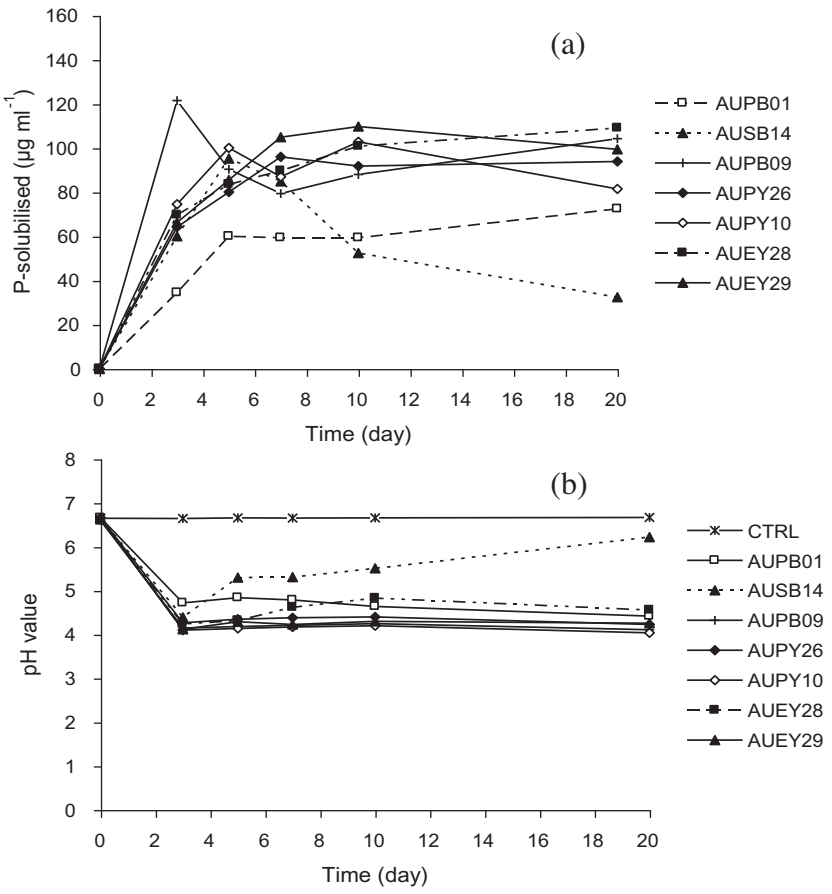

Figure 4 Change in phosphate concentrations and pH values in NBRIP + HA liquid medium by seven selected Gram-negative phosphobacteria (cf. Table 2) during twenty days of incubation.

with progressively increasing values (Fig. 4a). The P-solubilisation activity of isolate AUPB09 (*Pseudomonas* sp.) was quite different from that of the rest of the isolates because it attained its highest value on Day 3 of incubation and started to decrease thereafter. However, during the course of incubation, there was again a tendency for an increase until the final day of sampling, where it attained a significant amount of P released, second only to the most efficient mobiliser (AUEY28; *E. rhapontici*). There were significant differences ($F = 13.81$, $p < 0.01$) between days of incubation in the amount of solubilised *P* values. In general, a gradual increase in *P* values started from Day 3 of the incubation period (Fig. 4a). Solubilised *P* values were positively correlated ($r = 0.41$, $p < 0.01$) with the day of incubation but significantly negatively correlated ($r = -0.85$, $p < 0.01$) with pH of the growth medium. The majority of the isolates maintained their levels of solubilised *P* above 60 µg ml^{-1} until the final day of sampling. Strain AUSB14 (*S. marcescens*) was an exception.

The pH values of the growth medium were greatly decreased, starting from Day 3 of sampling (Fig. 4b). Almost all the isolates maintained the decreased pH values until the final day of incubation (Day 20) except isolate AUSB14 (*S. marcescens*) (Fig. 4b). There were significant differences ($p < 0.05$) between isolates in the decline in pH values. The highest and lowest pH values were produced by isolate AUSB14 (*S. marcescens*; from Day 5 onwards) and AUPY10 (*P. chlororaphis*), respectively (Fig. 4b).

Isolate AUBB19 (*Bacillus subtilis*) showed the best activity (43.19 µg phosphate ml^{-1}) on Day 3 of sampling but performed less well thereafter (27.31–9.37 µg phosphate ml^{-1}, data not shown). However, isolate AUBB20 (*B. subtilis*) showed the best performance in solubilising HAP (43.18 µg phosphate ml^{-1}) on Day 10 of incubation. Both isolates were poor at decreasing the pH value of their respective growth medium (data not shown).

3.4. Organic acid production

Production of organic acids and changes in pH values of the growth media by coffee-associated rhizobacterial isolates are presented in Table 3. All the isolates (both Gram-negative and Gram-positive) acidified their respective growth medium to a range from pH 6.00 to 3.95 (ΔpH = 0.74–2.79) in HAP and 6.06 to 3.93 (ΔpH = 0.68–2.81) in TCP. The concentrations of the total organic acids excreted and pH values were significantly negatively ($r = -0.65$ to -0.73, $p < 0.01$) correlated, but not related, to mobilised *P* values ($r = 0.12$ to 0.14, $p > 0.05$) with both P sources.

HPLC testing of the 18 efficient phosphobacteria revealed that all the isolates excreted one or more organic acids with both P sources. The type of P source had an effect on the type and amount of organic acids excreted into the respective growth medium. Thus, three isolates excreted trace amounts of acetic acid in the presence of HAP but only one isolate in

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Table 3 Organic acid production by coffee-associated phosphobacteria.

Organism[a]	Organic acids identified in undiluted culture supernatant (m*M*)									
	HA					TCP				
	pH	AA	GA	2-KG	PA	pH	AA	GA	2-KG	PA
AUEY28	4.47		29.6	68.4		4.24		28.7	55.4	
AUPY10	3.95		35.9	120		3.98		22.8	81.8	
AUEY29	4.10	10.0	17.2	84.0		4.10	11.5	16.7	75.8	
AUPB09	4.03		9.6	103.3		3.96		12.2	73.5	
AUPB08	4.06			62.0		4.00		4.7	83.2	
AUPB07	4.08		8.3	120.0		3.93		7.3	83.5	
AUPY26	4.49		1.4	91.2		4.92		1.0	81.0	
AUPB25	4.51		2.1	109.3		4.79			72.3	
AUSB14	4.21	2.2	1.6	75.3		4.22		5.0	62.7	
AUPB04	4.22	3.2	8.6	79.4		4.25		1.3	63.0	
AUPB13	4.36		1.1	110.0		4.29			59.3	
AUPB01	4.28		23.2	96.4		4.24		15.4	105.2	
AUPB49	4.15		7.2	127.3		4.80		1.9	67.9	
AUPY11	4.13		10.3	71.1		4.19		108.1	10.9	
AUPY27	4.42		2.0	109.4		4.40		1.8	83.7	
AUBB20	5.69				4.7	6.06				4.3
AUPB03	4.29		21.1	200.8		4.22		14.5	147.7	
AUBB19	6.00				2.7	5.59				2.9
Control	6.74					6.57				

2-KG = 2-keto-gluconic acid. GA = gluconic acid. AA = acetic acid. PA = propionic acid. All values are average of two replications.
[a] Identity of the isolates as in Table 2.

the presence of TCP. Likewise, over 82% of the phosphobacteria (all Gram-negative) exuded more 2-ketogluconic acid in the presence of HAP than TCP. The results indicate that 2-ketogluconic acid was the major organic acid produced with both P sources (Table 3). Similarly, gluconic acid was the second major acid produced by most tested isolates. The two Gram-positive isolates (*Bacillus subtilis*) were exceptional in forming neither 2-ketogluconic acid nor gluconic acid in any of the media. The mean concentrations of 2-ketogluconic acid produced ranged from 12.9–200.8 mM in the presence of HAP and 1.1–147.7 mM in the presence of TCP (Table 3). The highest concentrations of 2-ketogluconic acid (200.8 mM in HAP and 147.7 mM in TCP) were both detected in the growth media of isolate AUPB03 (*Pseudomonas* sp.), with corresponding pH values of 4.29 and 4.22, respectively. However, the top mineral P mobilisers AUEY28, AUEY29 (both *Erwinia* sp), AUPB09 (*Pseudomonas sp.*) and AUPY10 (*P. chlororaphis*) showed 2-ketogluconic acid concentrations ranging from 68.4 to 120 mM in HAP and 55.4 to 81.8 mM in TCP, respectively, with corresponding pH values less than 4.48. The concentration of 2-ketogluconic acid and pH values were significantly negatively correlated ($r = -0.68$, $p < 0.01$ in HAP and $r = -0.71$, $p < 0.01$ in TCP) i.e. quite similar to the sum of all acids. However, for gluconic acid, a significantly negative ($r = -0.52$, $p < 0.05$) correlation was only observed in the presence of TCP.

4. Discussion

4.1. Screening for P-solubilising capacity and determination of SI

We studied phosphate-solubilising rhizobacteria associated with the rhizosphere of *Coffea arabica* at its very centre of origin, southwestern Ethiopia. This study is the first report on coffee-associated phytobeneficial rhizobacteria of Ethiopian natural coffee forests. Over 72% of the rhizobacteria (both Gram-negative and Gram-positive) associated with the coffee rhizosphere were able to solubilise mineral P on PA, indicating a high proportion of such species as demonstrated by Kundu et al. (2009), who reported 193 PSB from chickpea rhizosphere.

Isolates that showed superior SI on solid medium (PA) also performed better in mobilising P in liquid growth media (Figs. 3a; 4a; Table 2). We found a strong positive correlation between the size of halo zones (SI) and mobilised *P* values in liquid media, in contrast to an earlier report (Rashid et al., 2004). The majority of Gram-positive and a few Gram-negative phosphobacteria, however, failed to show clear visible haloes during subsequent subculturing. Similar investigations indicate that some PSB tend to lose this trait after several subcultures on solid medium (Rashid et al., 2004; Babana and Antoun, 2006).

4.2. P-solubilising efficiency of selected phosphobacteria in NBRIP

In the presence of TCP, the majority of the selected isolates (9/11) mobilised maximum amounts of P on Day 7 of sampling and six of these maintained the highest values until the final day of the incubation period (Day 10; Fig. 3a). Rodrìguez and Fraga (1999) have demonstrated that microorganisms mobilise P better from insoluble inorganic P salts than from naturally occurring phosphate rocks, e.g. apatite. Thus, the idea of changing the P source to hydroxyapatite (HAP) and extending the incubation period to further screen the most efficient P mobilisers among the test isolates stemmed from such observations.

4.3. P-solubilising efficiency of selected Gram-negative and -positive isolates in NBRIP + HAP

All the above test isolates ($n = 9$) mobilised P in the presence of HAP in NBRIP. Seven of our nine isolates gave the highest mobilised *P* values (>60 μg ml^{-1}; Fig. 4a) in NBRIP + HAP on Day 5 of sampling and only isolate AUEY28 (*E. rhapontici*.) exhibited a progressive increase in solubilised P values until the final day of sampling (Day 20; Fig. 4a). In terms of solubilisation of HAP, Gram-negative isolates exhibited superior consistency and efficiency compared with Gram-positive phosphobacteria, as also observed by Tripura et al. (2007). The released phosphate values measured by us in growth medium of the latter group were, however, far higher than in a previous report (de Freitas et al., 1997).

Many isolates from both groups showed inconsistency in P dissolution with respect to incubation time, particularly in the presence of HAP (Fig. 4a), with more or less stable corresponding pH values (Fig. 4b). This also occurred in NBRIP + TCP (Fig. 3a) and could be attributed to differences among the test isolates in the amount of immobilised P. When cells in the culture immobilise phosphate for microbial biomass production, the corresponding values in the medium decrease, creating such fluctuations.

The observation of a decrease in soluble P by the isolate AUSB14 (*S. marcescens*; Fig. 3a) and a concomitant increase in the pH values of the growth medium (Fig. 3b), particularly towards the end of incubation, strongly indicates re-utilisation of available P (cf. Rashid et al., 2004; Tripura et al., 2007). In such cases the organic acids excreted are reused by the cultures for their own metabolism (Tripura et al., 2007), which is linked to an exhaustion of the original carbon source (Rashid et al., 2004). Nautiyal (1999) has in fact demonstrated that the rate of phosphate solubilisation increases with increasing concentrations of glucose.

4.4. Organic acid production

In both growth media studied here (NBRIP + TCP and NBRIP + HAP), pH declined significantly for most isolates and remained at low values indicating the production of strong acids. The R^2 value of P concentrations as a function of pH ranged from 0.45 to 0.68. We found that the amounts of soluble P and concentrations of total acids (2-ketogluconic acid in particular) were inversely correlated with pH, as observed by Kim et al. (2003). This indicates that organic acid production played a central role in the solubilisation of insoluble phosphate sources, although acid production and P release were not always in phase (not significantly correlated).

HPLC analyses of the culture supernatants showed that the majority of the isolates produced more than one organic acid, which is in line with other studies (Rashid et al., 2004). For all Gram-negative isolates in this study, the major products with both P sources were 2-ketogluconic and gluconic acids. The production of gluconic and 2-ketogluconic acids by various PSB has been well reviewed (Khan et al., 2006). The effect of the P sources was apparent in the concentrations of organic acids, however, with over 82% of isolates producing considerably higher amounts of 2-ketogluconic acid in HAP than in TCP. The greater insolubility of HAP may thus have triggered a larger production of acids. It has been shown that P starvation results in induction of glucose dehydrogenase (GDH), an enzyme involved in oxidative glucose metabolism, which is commonly believed to drive the P solubilisation processes in Gram-negative bacteria (Gyaneshwar et al., 1999). These authors reported that the activity of GDH increased fivefold upon phosphate starvation and this correlated with a decrease in medium pH. Furthermore, in both growth media in the present study, the amount of excreted 2-ketogluconic acid was far higher than that of its physiological precursor, gluconic acid, which could be linked to extended time of incubation (i.e. three days; cf. Kim et al., 2003). The frequent detection of 2-ketogluconic acid in culture media of our isolates is quite advantageous because 2-ketogluconic acid ($pK_a = 2.66$) has a more powerful P solubilisation ability than gluconic acid ($pK_a = 3.41$; Kim et al., 2002).

In the present investigation, one isolate, AUPB03 (*Pseudomonas* sp.) exuded two to three times the amount of 2-ketogluconic acid in both media compared to the efficient and consistent mineral P mobilisers AUEY28 and AUEY29 (both *Erwinia* sp; Table 3) under similar pH ranges. This discrepancy cannot be readily explained. However, the possible contribution of some additional strong organic acids (unidentified peaks) causing further release of phosphate by the latter isolates cannot be excluded. Furthermore, isolate AUEY29 (but not AUEY28) in addition to 2-ketogluconic acid, produced significant amounts of acetic acid, a commonly reported organic acid in P biosolubilisation studies (Vazquez et al., 2000), which could augment the mobilisation process. The above observations indicate that acid production is an important way, but not the only mechanism, of mineral phosphate solubilisation by bacteria (cf. Illmer and Schinner, 1995).

The mobilisation of HAP by some of the potent isolates (this study) indicates the remarkable agronomic importance of these microbes in solubilising the sparingly soluble HAP since soluble phosphate fertilisers easily turn to hydroxyapatite when they are applied to calcareous soils (Kim et al., 1997). Moreover, the dissolution of HAP to a significant extent holds great promise for the utilisation of the deposits of rock phosphate (RP) in the study area (Yayu, Illubabor zone; G. Debela, pers. commun.) and in other parts of the country (Assefa, 1991), possibly minimising the use of costly commercial soluble P fertilisers. In recent years various techniques for RP solubilisation have been proposed, with increasing emphasis on the application of PSM (Vassilev et al., 2001; Reyes et al., 2002) to utilise such cheap domestic resources for agricultural development. In addition, it has been verified that the original P source for plants in most soils is apatite (Ahn, 1993).

In Ethiopia, successful use of RP has been reported in greenhouse experiments for some agricultural crops (Bekele and Höfner, 1993). Very recently, Babana and Antoun (2006) have reported promising results on wheat performance in a field trial by co-challenging of AMF and an efficient indigenous P mobiliser and known AMF helper (*Pseudomonas* sp.) in the presence of naturally occurring RP as the main P source. The authors further verified that rock P does not reduce the level of root infection with AMF compared with the addition of soluble P. Interestingly, many of our potent isolates from coffee rhizospheres in natural forests were identified as *Pseudomonas* spp. using both conventional (API kits; Table 1) and molecular (16S rDNA analysis; Table 2) identification techniques (Muleta et al., 2009).

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The presence of the large number of coffee-associated phosphobacteria found in the present study could be assumed to be highly important for the mycorrhiza-dependent coffee bushes (Habte and Bittenbender, 1999) at the P-poor study sites, particularly since many PSB can also promote mycorrhizal functioning (i.e. increase mycorrhizal root colonisation) by production of specific metabolites such as vitamins, amino acids and hormones (Villegas and Fortin, 2001), thus acting as mycorrhizal helper bacteria (MHB). Vigorous microbial activities in the soil optimise nutrient cycling and maximise the efficiency of their use in agronomy (Sashidhar and Podile, 2010). A recent extensive review (Muleta, 2009) from both greenhouse and field trials has demonstrated a remarkable improvement in growth responses of various crops to phosphate solubilising microorganisms' inoculations. This necessitates the need for further comprehensive screening and inoculum development after testing rhizosphere competence of the microbes which demonstrated phytobeneficial traits. Consequently, the impression of utilising phosphate-solubilising microbes in low inputs for sustainable agriculture presents a cheap and environmentally promising strategy. By and large, native efficient P-mobilisers with additional desirable features could be suitable indigenous bioinoculants for production of *Coffea arabica*. Studies have revealed that indigenous/local isolates are superior to exotic inoculants in terms of functional diversity and efficiency for biotechnological applications (Requena et al., 1997) as well as overall biodiversity status (K. Lindström. Pers. commun.).

4.5. Conclusions

The lowering of pH values coincided with an increase in the P solubilisation. In general, Gram-negative phosphobacteria were found to be more active in lowering the pH and releasing P into the growth medium than Gram-positive isolates.

All Gram-negative isolates tested demonstrated a concomitant release of organic acids, mainly 2-ketogluconic and gluconic acids, and steep declines in pH values, which indicate the main mechanism used by these rhizobacteria to mobilise insoluble P sources. Higher concentrations of 2-ketogluconic acid were measured in the presence of the most insoluble P source, HAP, suggesting enhanced induction of GDH in this particular medium.

The P-solubilising abilities of isolates AUEY28 and AUEY29 (both *Erwinia* sp.) were maintained throughout the incubation period and these isolates thus seemed quite tolerant to excreted organic acids in the medium, making them possible bioinoculant candidates for acidic soils.

The current study identified the presence of many potent phosphobacteria in the rhizosphere of coffee plants. This calls for thorough and continuous studies of their field applicability as inoculants, after adequate formulation, in establishing a potentially important adjuvant for agricultural practices, particularly on sites where P is a main constraint. Inoculations with potent indigenous microorganisms are in accord with contemporary views on the possible future role of plant growth-promoting and soil-supporting bacteria in enhancing plant yields.

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