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Full Length Research Paper

Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon

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Phosphorus replenishment, particularly in smallholder agriculture, remains a challenge as it is mainly fertilizer dependent. While the use of soluble mineral phosphate fertilizers is the obvious best means to combat phosphate deficiency in Cameroon, their use is limited by their high cost and availability at farmer's level. This study was aimed at maintaining the fertility of Cameroon soils by biological means, in order to improve agricultural production, using low inputs technology. Isolates were obtained from oil palm tree (*Elaeis guineensis*) root fragments and rhizospheric soils and their activity in mobilizing phosphate from insoluble sources was evaluated on agar plates and liquid culture media containing sparingly soluble phosphates. At the end of incubation time, it appeared that, phosphate solubilization resulted from a combined effect of pH decrease of the media and organic acids production. Furthermore, each of the tested isolates was able to produce at least one of the most important organic acids such as citrate, malate and tartrate. Among the ten isolates tested, three were identified as *Pseudomonas fluorescens* and would be considered as potential biofertilizers.

Key words: Carboxylic acids, oil palm rhizosphere, phosphate solubilization, pH decrease, *Pseudomonas fluorescens*.

INTRODUCTION

Phosphorus is one of major limiting factors for crop production on many tropical and subtropical soils (Norman et al., 1995) as a result of high phosphorus fixation. A large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dey, 1988; Sanyal and De Datta, 1991; Yadav and Dadarwal, 1997). The concentration of soluble phosphorus (P) in tropical soil is usually very low. While most mineral nutrients in soil solution are present in millimolar amounts, phosphorus is only available in micromolar quantities or less (Ozanne, 1980, Goldstein, 1994). The majority of applied phosphorus is rapidly fixed in soil into fractions that are poorly available to plant roots (Sanyal and De Datta, 1991; Yadav and Dadarwal, 1997). Inorganic phosphates in acidic soils are associated with iron (Fe) and aluminium (Al) compounds where-as calcium (Ca)-phosphates are predominant form of inorganic phosphates in neutral or calcareous soils (Russel, 1973; Sample et al., 1980; McLaughlin et al., 1988; Gyaneshwar et al., 2002).

Chemical fertilizers have played a significant role in the green revolution, but unbalanced use of them, had led to reduction in soil fertility and to environmental degradation (Gyaneshwar et al., 2002). In addition, unfavourable pH and high reactivity of aluminium and iron in soils decrease P availability as well as P-fertilizer efficiency also with high total P contents (Börling et al., 2001; Hao et al.,

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2002). Emphasis has then been paid to the possibility of greater utilization of unavailable P forms by the action of phosphate mobibilizing micro-organisms. Micro-organisms involved in phosphorus acquisition include mycorrhizal fungi and phosphorus solubilizing mico-organisms. Previous works have shown the potential of local beneficial micro-organisms for nutrient uptake and crop production in central Africa (Nwaga et al, 2000). Micro-organisms are involved in a range of processes that affect the transformation of soil phosphorus and are thus an integral component of the soil P cycle (Deubel and Merbach, 2005). In particular, soil micro-organisms are effective in releasing P from inorganic P through solubilization (Subba Rao, 1982a,b; Goldstein, 1986; Tandon, 1987; Kucey et al., 1989; Richardson, 1994; Narula et al., 2000) and from organic pools of total soil P by mineralization (Greaves and Webley, 1965; Raghu and Mac Rae, 1966; Abd-Alla, 1994; Bishop et al., 1994). The microbial biomass in soil also contains a significant quantity of immobilized P that is potentially available to plants (Hedley and Steward, 1982; Brookes et al., 1984; Oberson et al., 2001).

Humid forest zones such as in southern Cameroon are constituted of ferrallitic red soils which are generally low in fertility, particularly phosphorus and nitrogen. Soil acidity is high and phosphorus replenishment, particularly in smallholder agriculture, remains a challenge as it is mainly fertilizer dependent. While the use of soluble mineral phosphate fertilizers is the best mean to combat phosphate deficiency in Cameroon, their use is limited by their high cost and availability at farmer's level. Given the limited access of most farmers to phosphate fertilizers, it was necessary to identify and incorporate into cropping systems some efficient strains of phosphate solubilizing micro-organisms that can mobilize unavailable phosphorrus from soil phosphate pools into forms that can be assimilated by plants.

MATERIALS AND METHODS

Sample collection

Seven soils samples with characteristics described in Table 1 and root fragment samples were collected on *E. guineensis* rhizosphere in four provinces (Centre, South, South-West and Littoral, Figure 1), located in two agro ecological zones of Cameroon, representing humid forest zones (volcanic, andosoil and oxisoil) and a wide range of various levels of acidity, aluminium and iron toxicity. The locations include soils with high total P contents (500-1700 $mg \cdot kg^{-1}$) in volcanic soils of South-West, but very low P availability as a result of high iron and aluminium contents. Furthermore, soils in the Centre and Littoral Provinces are extremely poor in total phos-phorus (ranging from 140-410 $mg \cdot kg^{-1}$). In general soils are acidic, with the most acidic ones in the red oxisol of South province (pH 3.69-4.12), while one is alkaline (pH 7.40) in sandy grey soils at Bokito in Centre province. Sampling was conducted on rhizosphere of young and old oil palm trees to obtain as much as possible variability in the targeted micro-organisms. Root fragment samples were also taken while collecting soil samples, but they were taken only on old oil palm tree rhizospheres. In each location, soil samples were collected in ten randomised oil palm tree rhizo-spheres, they were air dried, crushed to pass through 2 mm sieve and thoroughly mixed to represent one composite sample. In the same way, root fragments were collected in ten randomised oil palm tree rhizospheres while collecting soil samples and thoroughly mixed to represent one composite sample.

Isolation of micro-organisms from soil and roots

Ten gram (10 g) of soil sample was suspended in 90 ml of sterile distilled water and 10^{-1} dilution was obtained. Serial dilutions were prepared by mixing 1 ml of the suspension made into 9 ml sterile water blanks, until the 10^{-7} dilution was obtained. Each dilution was plated in modified Bunt and Rovira medium described by Abdel Hafez (1966) (0.4 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MgCl_2$, 0.1 g $FeCl_3$, 0.1 g $CaCl_2$, 1.0 g peptone, 1.0 g yeast extract, 5.0 g glucose, 250.0 ml soil extracts, 20.0 g agar, 750.0 ml tap water, pH 7.0), and left to incubate for three days at room temperature (28-30°C).

Extraction of micro-organisms from root fragments was made according to the method described by Mehta and Nautiyal (2001). Root fragments were thoroughly washed with tap water for 2 min to remove all the loosely adhering soil particles, followed by washing with sterile 0.85% NaCl solution. Ten grams (10 g) of roots were then macerated in 90 ml of 0.85% NaCl solution with a sterile mortar and pestle. Serial dilution of the root homogenate were then individually plated on the modified Bunt and Rovira medium as described above and left to incubate for three days at room temperature (28-30°C).

In both cases, colonies surrounded with halo zone were transferred three times by streaking in new Bunt and Rovira agar plates, and finally on sterile nutrient agar (3.0 g NaCl, 3.0 g beef extract, 5.0 g peptone, 15.0 g agar, 1000 ml distilled water, pH 7.0) until the pure cultures obtained.

Test of purity and stability

To estimate the capability of the obtained isolates in solubilizing sparingly soluble phosphates ($Ca_3(PO_4)_2$, $AlPO_4 \cdot H_2O$, $FePO_4 \cdot 2H_2O$), the modified nutrient agar medium was used plus dye as followed. Common components: 3.0 g NaCl, 3.0 g yeast extract, 5.0 g peptone, 5.0 ml 0.5% bromocresol green, 15.0 g agar, 1000.0 ml distilled water. Added phosphates: 4.7 g $Ca_3(PO_4)$, pH 7.0 or 4.2 g $AlPO_4 \cdot H_2O$, pH 7.5 or 5.6 g $FePO_4 \cdot 2H_2O$, pH 7.5. In these media, the bromocresol green (BCG) was used as pH indicators dye which, in pH greater than 6 is green but changes into yellow colour when the pH falls within 3.8-5.4. A stock solution of 0.5% dye was prepared as described Gadagi and Tongmin (2002) by dissolving a corresponding weight of BCG in 70% ethanol. The final pH was adjusted to 6.5 with 1 N KOH. A 0.5 ml aliquot of the stock solution was added to 100 ml of modified nutrient agar and plated separately in Petri-dishes formally divided into four parts. Ten microliter (10 µl) suspension with approximately 1 to $2 \cdot 10^7$ colony forming units (cfu) ml^{-1} of two days grown culture were used to inoculate the centre of each quarter part of the Petri-dish. Each isolate was inoculated in duplicate. Results were recorded five days later, the diameter of the colonies (n) and that of the halo zone (z) were measured and the ratio z/n evaluated.

Quantitative estimation of phosphate solubilization in liquid media

The phosphate solubilization activity of the tested isolates was recorded in Reyes basal medium (Reyes et al., 1999) described as followed: 0.1 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 1.56 mg $MnSO_4 \cdot H_2O$, 1.40 mg $ZnSO_4 \cdot 7H_2O$, 2.0 µg

Table 1. Soil chemical analysis of the investigated areas in Cameroon.

Provinces	Location	Isolates code[1]	pH[2]	Total P[3] (mg kg^{-1})	Available P[4] (mg kg^{-1})	Total N[5] %	Total C[5] %	Fe[3] (mg kg^{-1})	Al[3] (mg kg^{-1})	Ca[3] (mg kg^{-1})	K[3] (mg kg^{-1})
Centre	Bokito	BOR_8	4.72	140	8.30	0.08	1.19	11 5	13 4	420	610
	Leboudi (Yaoundé)	LEJ_{14}	5.45	140	1.75	0.10	0.93	8 5	11 1	620	200
Littoral	Douala	DR_5 DR_9 EDJ_4 EDJ_6	4.51	210	49.90	0.10	1.16	8 1	9 9	480	390
	Edea	EDJ_8	4.06	170	4.45	0.21	2.69	21 3	31 40	490	220
South	Sangmelima	SR_7	4.04	370	8.50	0.27	2.98	45 9	53 0	450	460
South-West	Ekona (Mbassicam)	EMJ_5 EMJ_{14}	4.28	500	1.20	0.21	2.12	112 1	55.0	640	230
	Limbé	LR_7	5.95	1720	19.30	0.39	3.74	94 0	60 7	2 450	840

[1]Last letter indicates isolation from R: roots of 12-15 years old palms or J: rhizosphere of 3-5 years old palms
[2]20 g soil in 50 ml of 0.0125 M $CaCl_2$
[3]Aqua regia extract
[4]Bray 1 extract (Bray, 1945)
[5]Elementary Analysis system Hanau

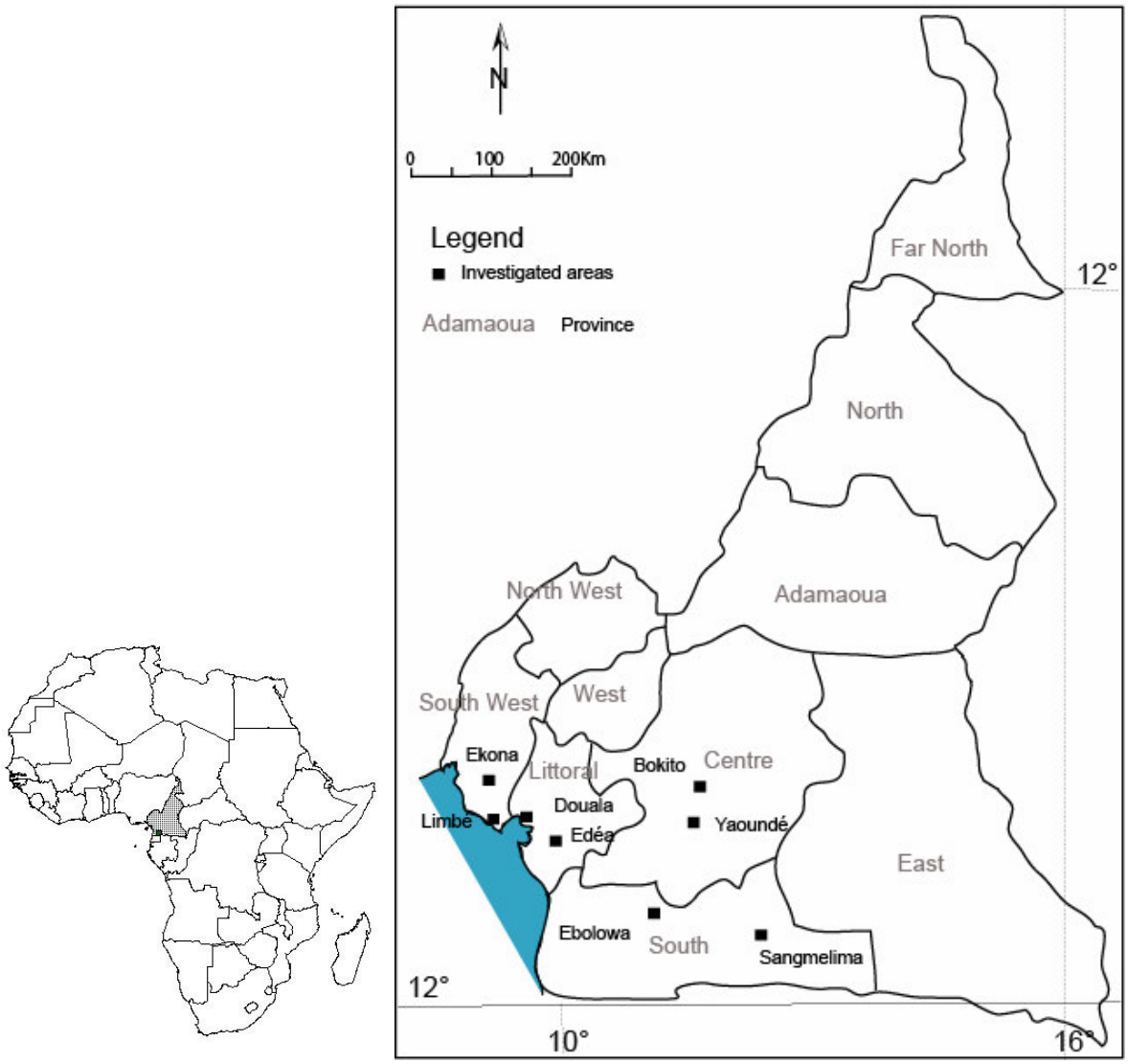

Figure 1. Localisation of Cameroon in Africa and distribution of the investigated sites in humid forest zones.

vitamin B_{12}, 30.0 g sucrose. The basal medium was prepared, the pH adjusted, and the following inorganic phosphate sources were added at the concentration of 30 mM P of $Ca_3(PO_4)_2$, $AlPO_4 \cdot H_2O$ or $FePO_4 \cdot 2H_2O$. For each inorganic phosphate tested, triplicate 250-ml flasks containing 50 ml basal medium were used. Each flask received 200 µl of a two days culture containing approximately 1 to $2 \cdot 10^7$ cfu ml^{-1}. Inoculated flasks and un-inoculated controls were incubated at 28°C on a rotary shaker at 150 rev min^{-1} in the dark. At each sampling date (3, 5 and 7 days), a 5 ml sub-sample of the culture supernatant was aseptically withdrawn from each flask. 3 ml were used for pH measurement and the remaining 2 ml were centrifuged and used for colorimetric determination of solved phosphate (P) by Murphy and Riley method (Murphy and Riley, 1962).

Determination of carboxylic acid production in microbial cultures

At the end of incubation time, nutrient solution was centrifuged (10 000 × *g*, 4°C, 10 min). An aliquot of 2.5 ml of the supernatant was diluted with 2.5 ml 96% ethanol and stored in a freezer to prevent further conversions. Acidic compounds were separated by ion chromatography using Bond Elut cartridges (Varian, Harbor City, CA, USA) (Johnson et al., 1996). Anion exchange cartridge (SAX, capacity 2.8 ml; 500 mg LRC) was activated by rinsing with 2 ml pure methanol and conditioned with 4 ml of a mixture of 96% ethanol and 0.001 M NH_4OH (1/1; vol/vol) before admitting the sample. Neutral and alkaline substances go to the effluent by rinsing with 50 % ethanol. The cartridge was eluted with 2% conc. HCl in methanol to desorb acidic compounds. After concentration using rotation evaporator (35°C) single compounds were separated by a HPLC (high performance liquid chromatography) system equipped with a L-6200 intelligent pump, D-6000 interface (Merck-Hitachi, Darmstadt) and Aminex HPX-87H column (300 mm × 7.8 mm) with flow rate of 0.6 ml min^{-1} and column temperature of 30°C with 10 mM perchloric acid as eluant (Deubel et al., 2000).

Individual acidic compounds were detected at 215 nm by an L-4000 UV detector (Merck-Hitachi).

Table 2. Conditions in which PCR of the identified strains were made.

PCR cycles	Number of cycles	Temperature (°C)	Time
Denaturation	1	94	5 min
Denaturation	20	94	45 s
Hybridization		65*	45 s
Synthesis		72	1 min
Denaturation	10	94	45 s
Hybridization		55	45 s
Synthesis		72	1 min
End synthesis	1	72	10 min

* -0.5°C per cycle

Identification of some strains

Identification of strains was made from pure culture obtained by washing a 48 h colony culture from nutrient agar plates with 10 ml of liquid nutrient broth. The mixture was then incubated at 28°C on a rotary shaker for 48 h and then kept at 4°C until used. They were subjected to polymerase chain reaction (PCR) using two primers with following characteristics: current name 341f, length 17 base pairs (bp), 70% C-G bounds, sequence 5' CCT ACG GGA GGC AGC AG 3' and current name 907r, length 20 bp, 40% C-G bounds, sequence 5' CCG TCA ATT CCT TTG AGT TT 3' for amplification of 16S ribosomal RNA. Both are used for a wide range of bacteria. Conditions for PCR are described in Table 2. Subsequently, a partial sequencing of bacterial genes was accomplished.

RESULTS

Qualitative screening of micro-organisms

The data in Table 3 indicate the values of diameter of the colony (n), that of the halo zone (z) and the ratio z/n of the different isolates obtained on agar plates containing different phosphate sources ($Ca_3(PO_4)_2$, $AlPO_4{\cdot}H_2O$ or $FePO_4{\cdot}2H_2O$). The use of the ratio z/n helps to evaluate the activity of a given micro-organism. The change of the colour in the zone surrounding the colony into yellow clearly indicates the drop of pH at this place, and that acidification of the medium seems to be directly associated to the process of phosphate solubilization. All the tested isolates were able to show halo zone on plates containing $FePO_4{\cdot}2H_2O$ Fe–phosphate (see letter c) while five were able to show halo zone in plates containing either Ca- or Al- or Fe-phosphate (see a, b, c in Table 3).

Phosphate solubilization in liquid media

Table 4 summarizes the values of P (mg l^{-1}) solubilized in liquid culture and the pH of the corresponding media after seven days of incubation. It clearly appears that in media with $Ca_3(PO_4)_2$, the values of solved P obtained with all the isolates were significantly different from those of control, showing that the tested isolates have effectively converted the inorganic insoluble phosphate into soluble form. Also, a decrease of pH values was observed in the tested isolates compared to control. According to their efficiency, those isolates can be classified into groups from the least efficient (letter b) to the most efficient ones (letter g). In that medium, the most efficient is represented by strain EDJ_6 (308 mg P l^{-1}) isolated from young *E. guineensis* rhizospheric soil, collected at Edea in the Littoral province, even though this strain showed no clear zone on $Ca_3(PO_4)_2$ containing agar plates (Table 3). It was followed by DR_5 (191 mg P l^{-1}) isolated of phosphate solubilized in these media was significantly reduced compared to the values obtained in media with $Ca_3(PO_4)_2$. Also here, phosphate solubilization seems to be link with pH decrease of the medium but this pH decrease was not strictly proportional to the amount of phosphate solubilized.

According to the medium with $FePO_4{\cdot}2H_2O$, all the tested isolates have effectively converted the sparingly insoluble phosphate into the soluble form. According to their efficiency, those isolates can be classified into groups from less efficient (letter b) to the most efficient ones (letter f). In these media, the most efficient is represented by strain EDJ_8 (65 mg P l^{-1}) isolated from from an old *E. guineensis* root fragments collected at Douala in the Littoral province. In general, Ca-phosphate solubilization seems to be link with pH decrease of the medium but this pH decrease was not strictly proportional to the amount of the phosphate solubilized.

As far as the medium with $AlPO_4{\cdot}H_2O$ is concern, the values of P obtained with all the isolates were significantly different from that of control, showing that the tested isolates have effectively converted the inorganic insoluble phosphate into the soluble one. Great differences were also observed with pH values. According to their efficiency, those isolates can be gathering from the least efficient (letter b) to the most efficient ones (letter i). In this medium, the most efficient is represented by strain EMJ_5 (94 mg P l^{-1}) isolated from young *E. guineensis* rhizosphere collected at Ekona in the South-West province, followed by DR_9 (92 mg P l^{-1}) isolated from old *E. guineensis* root fragments collected at Douala in the Littoral province. In general, the amount young *E. guineensis* rhizosphere collected at Edea in the Littoral province, followed

Table 3. Phosphate solubilization on nutrient agar plates containing sparingly soluble $Ca_3(PO_4)_2$, $AlPO_4 \cdot H_2O$ or $FePO_4 \cdot 2H_2O$.

Strain	Modified Nutrient Agar with $Ca_3(PO_4)_2$			Modified Nutrient Agar with $AlPO_4 \cdot H_2O$			Modified Nutrient Agar with $FePO_4 \cdot 2H_2O$			
	z(mm)	n(mm)	z/n	z(mm)	n(mm)	z/n	z(mm)	n(mm)	z/n	
BOR_8	21.63	13.00	1.66	20.00	15.00	1.33	19.38	10.44	1.87	abc
LEJ_{14}	0.00	0.00	—	0.00	9.50	—	15.00	8.00	1.88	c
DR_5	23.00	10.00	2.30	20.50	8.25	2.48	15.00	8.00	1.86	abc
DR_9	0.00	24.88	—	0.00	17.75	—	24.00	15.63	1.54	c
EDJ_4	29.75	12.75	2.33	17.63	10.38	1.70	23.50	12.38	1.90	abc
EDJ_6	0.00	14.00	—	14.25	12.75	1.12	18.00	10.75	1.67	bc
EDJ_8	41.63	30.50	1.36	0.00	18.63	—	42.75	31.50	1.36	ac
SR_7	21.00	11.00	1.91	20.50	9.50	2.12	18.00	7.50	2.40	abc
EMJ_5	0.00	11.00	—	0.00	11.50	—	19.25	11.00	1.75	c
LR_7	35.13	20.00	1.76	33.75	18.50	1.82	24.25	15.50	1.54	abc

z) Diameter of halo zone
n) Diameter of colony
z/n) ratio between halo zone and colony diameter
a) Solubilization of $Ca_3(PO_4)_2$
b) Solubilization of $AlPO_4 \cdot H_2O$
c) Solubilization of $FePO_4 \cdot 2H_2O$

Table 4. Phosphate solubilization activity in liquid media at the end of the incubation time.

Treatment	Medium with $Ca_3(PO_4)_2$		Medium with $AlPO_4 \cdot H_2O$		Medium with $FePO_4 \cdot 2H_2O$	
	Pi (mg l^{-1})	pH	Pi (mg l^{-1})	pH	Pi (mg l^{-1})	pH
Control	15.29 a	6.27 a	29.88 a	7.06 a	29.73 a	6.72 a
BOR_8	99.80 d	5.00 c	37.88 b	4.02 e	44.65 c	4.32 e
LEJ_{14}	97.41 d	4.91 d	71.27 g	4.12 e	38.34 b	4.17 f
DR_5	190.57 f	4.08 e	45.51 d	3.91 e	38.09 b	3.88 g
DR_9	59.46 b	5.40 b	92.15 i	5.11 b	47.59 d	4.92 c
EDJ_4	58.56 b	4.87 d	56.94 e	4.69 c	51.01 e	5.02 b
EDJ_6	308.38 g	4.27 e	80.14 h	3.35 f	44.14 c	3.65 g
EDJ_8	66.15 c	5.10 c	60.72 f	4.34 d	64.83 f	4.53 d
SR_7	97.07 d	4.28 e	43.94 c	3.98 e	45.57 c	3.95 g
EMJ_5	96.57 d	5.06 c	93.96 i	4.33 d	50.73 e	4.31 e
LR_7	103.41 e	5.20 c	60.78 f	4.34 d	42.78 c	4.21 f

Data are means from experiments performed in triplicate. For each isolate, means in each column followed by different letters are significantly different ($P<0.05$) according to ANOVA test performed with SPSS 10.1 Software.

by EDJ_4 (51 mg P l^{-1}) from the same origin and EMJ_5 (51 mg P l^{-1}) isolated from young *E. guineensis* rhizosphere collected at Ekona in the South-West province. In general, the amount of phosphate solubilized in these media was much more reduced compared to the values obtained in media with $Ca_3(PO_4)_2$ and $AlPO_4.H_2O$ respecttively. Also here, phosphate solubilization seems to be link with pH decrease of the medium but this pH decrease was not strictly proportional to the amount of the solubilized phosphate.

Carboxylic acid production of the tested strains

At the end of the incubation period, various molecules such as oxalate, tartrate, succinate, transaconitate, gluconate, lactate, oxaloacetate, xylonate, fumarate, citrate and malate were purified in media containing different sparingly soluble phosphates. The type and concentration of each carboxylic anion relatively varied from one strain to another and seems to be independent to the phosphate source. The main carboxylic anion (citrate, tartrate, malate and gluconate) patterns of the tested isolates are described in Figure 2. When the patterns of those strains are compared, it clearly appears that citrate was produced only by three strains (EDJ_6, EMJ_5 and DR_9) and the amount was important with strain EDJ_6 followed by strain DR_9 and then by strain EMJ_5. Furthermore, citrate was found in all the media with EDJ_6 and DR_9 while with EMJ_5, it was found only in medium containing $FePO_4 \cdot 2H_2O$ as phosphate source. Malate

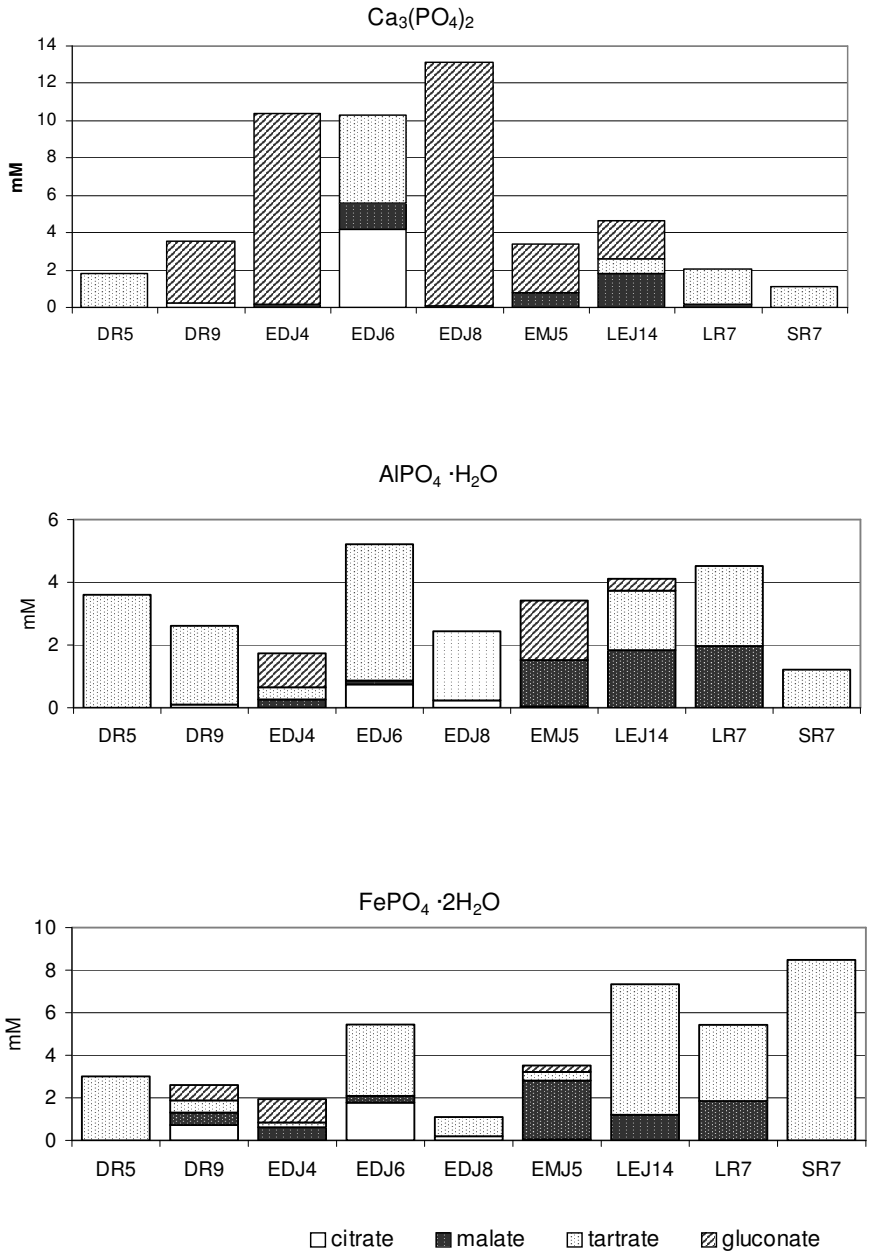

Figure 2. Main carboxylic anions patterns of nine of the tested isolates growing with $Ca_3(PO_4)_2$, $AlPO_4{\cdot}H_2O$ or $FePO_4{\cdot}2H_2O$ as single P source. Those of BOR_8 were not determined.

was produced by all the strains excepted DR_5 and SR_7, and high quantities were obtained with strains EMJ_5, LR_7 and LEJ_{14}. With strain DR_9, it was produced only in medium with $FePO_4{\cdot}2H_2O$ as phosphate source. Tartrate was almost produced by all the strains and with strains LR_7, SR_7, EDJ_6, LEJ_{14} and DR_5, it appe-ars in all the media with different phosphate sources. With strains EDJ_4 and DR_9, it appears only in media cont-aining $AlPO_4{\cdot}H_2O$ and $FePO_4{\cdot}2H_2O$, while with strain EMJ_5, it was found only in medium containing $FePO_4{\cdot}2H_2O$ as phosphate source. Gluconate was pro-duced by four strains (EDJ_4, EMJ_5, LEJ_{14} and DR_9). It was found in all the media with strains EDJ_4 and EMJ_5. With strain LEJ_{14}, it was produced only in media with $Ca_3(PO_4)_2$ and $AlPO_4{\cdot}H_2O$, while with strain DR_9 it was produced in media containing $Ca_3(PO_4)_2$ and $FePO_4{\cdot}2H_2O$ respectively. High quantities were generally obtained in medium with $Ca_3(PO_4)_2$ as phosphate source.

Identification of strains

Only three of the ten tested strains were identified by partial sequencing. All those three strains have been identi-

fied as *Pseudomonas fluorescens* with different lengths and with different sequences (Table 5). Strain BOR_8 was isolated from *E. guineensis* root fragments collected at Bokito in the Centre province, with a length of 501 bp and a sequence homology of 99.4%. Strain DR_9 was isolated from root fragments collected from an old oil palm tree at Douala in the Littoral province, with a length of 511 bp and an identity of 100%. Strain EDJ_4 was isolated from soil samples of young *E. guineensis* rhizo-sphere at Edea in the Littoral province, with a length of 509 bp and 100% sequence homology.

DISCUSSION

The objective assigned to the present study could only be achieved through several methods starting from isolation of micro-organisms to identification of the most efficient ones which could be proposed as potential biofertilizers.

Numerous articles have described several methods to undertake such a study. The ones used in the present study were those adapted to our equipments and to our environmental constraints. The use of Bunt and Rovira medium for isolating micro-organisms was a simple way to detect phosphate solubilizing micro-organisms through the formation of halo zone on agar plates containing mineral phosphate (KH_2PO_4) freshly precipitated with calcium chloride ($CaCl_2$). But with the test of purity and stability, we wanted to appreciate a direct way to test the efficiency of theses strains in solubilizing sparingly soluble phosphate associated to aluminium or iron as the case in Cameroonian acidic soils. For that purpose, we used the simple nutrient agar medium with bromocresol green dye for a better observation and as pH indicator (Mehta and Nautiyal, 2001; Gadagi and Tongmin, 2002). All our tested isolates could be able to show a drop of pH in the zone surrounding the colony at least in the media containing sparingly soluble iron-phosphate. Although the colour change was directly linked to pH decrease of the medium, the activity of isolates was evaluated by determining the ratio diameter of halo zone (z) over the diameter of the colony (n), taking into consideration the value of the ratio (z/n) as an indicator for the isolate efficiency. The higher the value of the ratio, the greater the activity of the tested isolate was. But with that method we could not be able to quantify the amount of phosphate solubilized at the end of the incubation time. So, liquid culture experiments involved evaluation of the amount of phosphate solubilized and evolution of the pH with time (3, 5 and 7 days), purification and identification of carboxylic acids at the end of incubation time in liquid media containing different insoluble phosphate sources ($Ca_3(PO_4)_2$, $FePO_4{\cdot}2H_2O$ or $AlPO_4{\cdot}H_2O$).

In general, all the tested isolates showed good aptitude in mobilizing phosphorus from insoluble sources, independently to phosphate type contrary as the case on agar plates. This agrees with former studies made by Alikhani et al. (in press) who found similar contradictory results between plate halo detection and phosphate solubilization in liquid cultures. In previous studies, Deubel and Merbach (2005) tested eight strains on calcium-phosphate agar plates and found that only two of them showed clear zone around their colony and would be identified as phosphate solubilizers. Moreover, they realized that their best strain in solubilizing the same phosphate source in liquid media was one of the strains which could not show clear zone on agar plates. Also the most efficient calcium phosphate mobilizing strain in this study EDJ_6 produced no halo zones on the corresponding agar plates. This was the strain with the highest citrate production, the only carboxylate that can effectively solubilize $Ca_3(PO_4)_2$ under neutral conditions. Probably the precipitation of calcium citrate prevents the detection of halo zones despite of P solubilization. This indicates that the halo zone as criteria is not enough for phosphate solubilizing micro-organisms selection, as many isolates that did not produce any visible halo zone on agar plates could conversely mobilized significant amount phosphate in liquid media (Louw and Webley, 1959; Gupta et al., 1994). However, while screening a large number of micro-organisms, this method can be regarded as generally reliable for isolation and preliminary characterization of phosphate solubilizing micro-organisms (Rodriguez and Fraga, 1999). However, additional tests should examine, if they can be improved by the use of iron phosphate instead of calcium phosphate. Iron phosphate can only be solubilized by efficient carboxylates, not by acidification. From theses results, it appears that the solubilization rate varied from one medium to another. Aluminium and iron-phosphates were less solubilized than calcium phosphate and the solubility of aluminium phosphate was greater than that of iron phosphate. This correlates with Ahn (1993) who stated that in tropical soils, the solubility of calcium, iron and aluminium phosphates (Ca-P, Al-P and Fe-P) with respect to soil acidity decreases in the following order: Ca-P > Al-P > Fe-P. Similar results were also obtained by Gadagi and Tongmin (2002) working with *Penicillium oxalicum*. In general the phosphate solubilization was associated to pH decrease and to carboxylic acids production. These results indicate that strains which regularly produced citrate, malate, tartarate and gluconate were also those that mobilized a great amount of phosphate in liquid media. It apparently appears that, the wider the production scales of those acids, the greater the mobilization rate of phosphate in the media. This agrees with the tests for P-solubilizing efficiency of identified carboxylic acids (data not shown). Some former studies have shown the importance of those carboxylic acids in the process of phosphate solubilization. Ryan et al. (2001) stated that, among the carboxylic acids identified, dicarboxylic (oxalic, tartaric, malic, fumaric, malonic acids) and tricarboxylic (citric) acids were more effective for phosphorus mobilization. Illmer and Schinner (1992) realized that gluconic acid may be the most frequent agent for mineral phosphate solubilization.

Table 5. Partial 16S RNA sequence of identified strains.

Strain	Partial 16S RNA sequence
BOR_8	TGTGAAGAAGGCCTTCGGGGTTGTAAAGCACTTTCCGCGAGGAGG AAGGTGGTGAGCTTAATACGCTCATCAATTGACGTTACTCGCAGA AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG CGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA CTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGA ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC GGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCG AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC TGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGTCTT CCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCA AGGTTA
DR_9	CATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTC AGCGAGGAGGAAGGTGGTGAGCTTAATACGCTCATCAATTGACGT TACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG CGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTC AACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGA GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACG CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG TAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGA GGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAG TACGGCCGCAAGGTTA
EDJ_4	TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAG CGAGGAGGAAGGTGGTGAGCTTAATACGCTCATCAATTGACGTTA CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAA CCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGG GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA GGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA GTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGG CGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTA CGGCCGCAAGGTTA

Three of our tested strains were identified as *P. fluorescens*, one of the famous and well-known plant growth-promoting rhizobacteria and non human pathogen. Besides the results observed in these experiments, other roles are attributed to *P. fluorescens*. Strains of *P. fluorescens* are already registered for use in controlling bacterial blotch and seedlings diseases (Powell et al., 1990). Thomashaw and Weller (1988) identified a strain of *P. fluorescens* that produced a phenazine antibiotic capable of inhibiting growth of *Gaeumannomyces graminis* var. *triciti*, the causal agent of take-all, a major root disease of wheat worldwide. These obtained results are promising and when identified, all the ten tested isolates can be considered as potential biofertilizers.

The present investigation has been carried out mainly to find out some phosphate solubilizing micro-organisms able to promote plant growth and yield through mineral nutrition. Micro-organisms were isolated from root and soil samples and were subjected to test for purity and stability on agar plates in which precipitated phosphate was replaced by inorganic sparingly soluble phosphates. In general, all the selected isolates showed good aptitude in mobilizing phosphorus from insoluble sources, independent of phosphate type. *In vitro* phosphate mobilization in liquid culture was caused by acidification of the nutrient

medium and the production of different carboxylic anions. Strong pH decrease in bacterial cultures with these phosphates is a combined effect of microbial carboxylic acid production and the acidification as consequence of calcium, aluminium or iron mobilization. After identification of the remained strains, field trials will be conduct on oil palm tree, on maize and other P-demanding crops cultivated in Cameroon under nursery or farm conditions such as cocoa, legumes crops, fruit trees or garden crops, in order to assess the impact of inoculation on plant growth/promotion. As far as the micro-organisms obtain-ned from root fragments are concerned, it will be also necessary to determine whether they are really indige-nous or random contaminants. It will be also useful to assess the interaction of theses rhizospheric micro-organisms with other soil organisms such as arbuscular mycorrhiza as 'helper bacteria' for plant benefits.

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