Journal of Fertilizers & Pesticides

Teshome et al., J Fertil Pestic 2017, 8:3
DOI: 10.4172/2471-2728.1000188

Research Article Open Access

Isolation, Screening and Biochemical Characterization of Phosphate-Solubilizing Rhizobacteria Associated with *Coffea arabica* L.

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Abstract

This study focused on screening, identifying and characterizing P-solubilizing rhizobacteria associated with *Coffea arabica* L. growing in Southwestern Ethiopia. Samples were collected from Kaffa and Jimma zones in the Southwestern part of Ethiopia which are not only the major coffee growing areas of Ethiopia but also the origin of *Coffea arabica* L. Natural forest, agroforestry-based and monoculture plantations were included. A total of 110 Coffee roots with adhering soil samples were collected in sterile plastic bags. At each sampling site, plant roots with adhering soil (approximately 50 g) were collected from each corner of a randomly selected square meter (One from each of 4 quadrants) which lied around coffee plant at a depth of 10-20 cm and placed into a sterile plastic bag to give a composite sample. One composite sample was taken at each coffee plant. Care was taken to keep rhizosphere soil intact around the root. The shade trees at each sampling site were also registered. A total of 169 bacteria were isolated on nutrient agar from root washing solutions (61 isolates), surface sterilized roots (45 isolates) and soil (63 isolates). These bacteria isolates were subjected to GEN III Micro-plate tests of Biolog bacterial identification system kits and evaluated for their ability to solubilize phosphates on Pikovskaya's agar plates. Members of the isolated rhizobacteria were dominated by the genus Pseudomonas (44.1%) followed by Bacillus (11.6%), Enterobacter (10.5%) and Stenotrophomonas (10.5%). Over 32.5% of the Rhizobacteria (both Gram-negative and Gram-positive) associated with the coffee rhizosphere could solubilize mineral P on Pikovskaya's agar, indicating a high proportion of such species is associated with coffee plants.

Keywords: P-solubilizing bacteria; *Coffea arabica*; Microbe; Rhizobacteria

Introduction

Rhizosphere is a thin layer of soil immediately surrounding plant roots which is an extremely important and active area for root activity and metabolism. The rhizosphere concept was first introduced by Hiltner to describe the narrow zone of soil surrounding the roots where microbe populations are stimulated by root activities. The original concept has now been extended to include the soil surrounding a root in which physical, chemical and biological properties have been changed by root growth and activity. Many microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere [1].

Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment where diversity is low. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology, especially considering their competitiveness in root colonization [1].

Bacteria which flourish in the rhizosphere of plant, but which may grow in, on, or around plant tissues and exert beneficial effects on plant development are collectively known as plant growth promoting rhizobacteria (PGPR) [2]. They possess the capacity to stimulate plant growth either directly or indirectly [3]. PGPR can affect plant growth by a wide range of mechanisms such as solubilization of inorganic phosphate, production of phytohormones, siderophores and organic acids, lowering of plant ethylene levels, N_2 fixation and biocontrol of plant diseases [4,5]. The use of such beneficial bacteria as bio-fertilisers and biocontrol agents has currently attracted increased interest world-wide in attempts to achieve sustainability, particularly in agriculture, forestry and horticulture [5].

Coffee has for centuries played an important role in the Ethiopian economy and represents the main cash crop cultivated by small scale farmers for social, economic, political and ecological sustainability [4]. Coffee production mainly involves agroforestry-based systems, although there are both natural coffee forests and monoculture plantations. The first two are well accredited in improving soil properties, where coffee grows beneath various shade trees (mainly tree legumes), and are well suited for sustainable production compared with conventional mono-cultural (un-shaded) coffee systems [6,7]. In addition, the presence of wild Arabica coffee at the centre of its origin is of paramount importance for genetic conservation of this global commodity [7,8]. The Southwestern part of Ethiopia is known as the genetic home of coffee. During a study between 1966 and 1984 more than 600 coffee species were found by the Ethiopian National Coffee Collection Programme. Twenty years later more coffee species have been found but not really documented [9].

Phosphorus (P) is one of the essential macronutrients, particularly second to nitrogen, that limit plant growth in tropical soils [10,11]. Most soils in tropical and subtropical areas are predominantly acidic and extremely P-deficient due to their strong fixation of P as insoluble phosphates of iron and aluminium [11,12]. This leads to wide P deficiency which is particularly the case for the large parts of Ethiopian soils [13,14]. To alleviate P deficiency, chemical phosphatic fertilizers are widely used. However, a large proportion of the soluble forms of

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Received September 18, 2017; **Accepted** September 26, 2017; **Published** October 01, 2017

Citation: Teshome B, Wassie M, Abatneh E (2017) Isolation, Screening and Biochemical Characterization of Phosphate-Solubilizing Rhizobacteria Associated with *Coffea arabica* L. J Fertil Pestic 8: 188. doi:10.4172/2471-2728.1000188

P fertilisers is precipitated in insoluble form soon after application and becomes unavailable to plants [15]. 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. These have been the major stresses that constrain the production of crops in the country.

Very little is known about microorganisms associated with *Coffea arabica* in Ethiopia regarding their functional characteristics towards plant growth promotion. However, several reports from other coffee growing areas have revealed that putative agriculturally beneficial bacteria are associated with *Coffea arabica* L [4,16-18]. In another study carried out, it is reported that *Coffea arabica* associated rhizobacteria (specifically phosphate solubilising bacteria) were screened that possess direct plant growth-promoting traits which intensifies the idea of extending the use of indigenous microbes as microbial bio-fertilisers [19]. Screening indigenous beneficial *Coffea arabica* phosphate solubilizing rhizobacteria resources at the centre of origin of the plant species with which they are associated are expected to display wide abundance and biodiversity. Since screening this microbial biodiversity and studying their functional characteristics towards plant growth promotion is a paramount importance, this study focused on screening, idintifying and characterizing P-solubilizing rhizobacteria associated with *Coffea arabica* from natural forest, agroforestry-based or monoculture plantations.

Materials and Methods

Study sites description

Sample collection was carried out in the major coffee growing areas of Ethiopia; Jimma zone and Kaffa zone; natural forest, agroforestry-based and monoculture plantations from June 09, 2016 to June 17, 2016. The included areas are thought as the origin of *Coffea arabica* L. The study sites are in southwestern Ethiopia. Kaffa zone is in Southern Nations and Nationalities Peoples' Regional State (S.N.N.P.R.S); and Jimma zone is found in Oromia Regional State, Ethiopia (Figure 1). Based on the GPS data recorded during sample collection, the sites of sample collection are located between 07-06' - 07-49'N and 35-42' - 36-43'E, with an altitude range of 1422-2195 m a.s.l. 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%). 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) [4].

Sample collection and transportation

A total of 110 Coffee roots with adhering soil samples were collected in sterile plastic bags. Eighty of them were collected from Kaffa zone and the rest 30 samples were collected from Jimma zone. Samples were collected based on altitude differences of coffee plant growing areas, cultivar types and plant age group. At each sampling site, plant roots with adhering soil (approximately 50 g) were collected from each corner of a randomly selected square meter (One from each of 4 quadrants) which lied around coffee plant at a depth of 10-20 cm and placed into a sterile plastic bag to give a composite sample. One composite sample was taken at each coffee plant. Care was taken to keep rhizosphere soil intact around the root. The shade trees at each sampled plant and/or sampling site were also registered. The collected samples were kept in ice-box and transported to EBI Bacteriology laboratory. All samples were kept at 4°C until use. Each of the collected soil samples were homogenously and separately mixed for further study [20-23].

Isolation of p-solubilizing rhizobacteria

The 110 coffee roots with adhering soils were merged into 25 composite samples separately based on similarity of cultivar type, plant age group and altitude category. The microbial fraction was dislodged from the merged roots and root washing solutions were prepared for the isolation of rhizoplane bacteria [24]. For the isolation of bacterial endophytes, merged roots were surface sterilised in 99% ethanol for 1 min, 3% NaOCl for 6 minutes, and 99% ethanol for 30 seconds and followed by rinsing with sterile distilled water for 6 times [21]. Before homogenisation, a root fragment was imprinted on nutrient agar to serve as a sterility check. Roots were homogenised and macerated with a sterile mortar and pestle [16].

The rhisosphere soil, root washing solutions (rhizoplane) and homogenised roots (endorhiza) were serially diluted (10^{-1} to 10^{-9}) asceptically for inoculation. 0.1 ml inoculums of the prepared samples

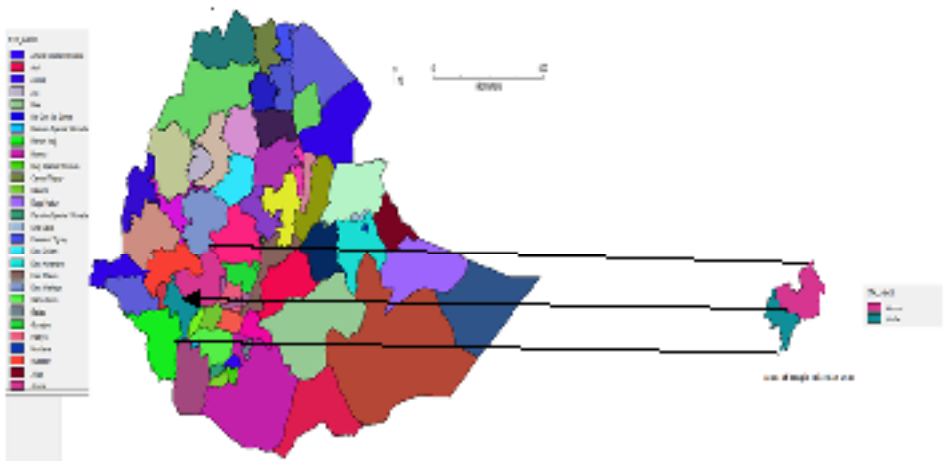

Figure 1: Map of zones in Ethiopia and map of study area.

were spread onto Nutrient agar plates and incubated at 28°C for 48 h [20,25]. Bacterial colonies ranging from 20 to 25 were selected and re-streaked to obtain pure colonies [22].

Biochemical characterization and identification of P-solubilizing rhizobacteria

The identification of the strains was performed according to their morphological, cultural, and biochemical characteristics by the procedures described in the [20,22,26]. Preliminary identification of P-solubilizing Rhizobacteria isolates were performed by examination for cell morphology using optical microscopy, Gram staining, and colony morphology [20,22]. Biochemical identification including the carbohydrate fermentation patterns and chemical sensitivity tests were determined by using GEN III Biolog bacterial identification system kit.

The Biolog GEN III Micro Plate analyzes a microorganism in 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity assays. The test panel provides a "Phenotypic Finger print" of the microorganism that can be used to identify it at the species level. The plates contained 96 wells, with a dehydrated panel of necessary nutrient medium (a carbon source), biochemical and tetrazolium violet. Tetrazolium violet is a purple formazan, a redox dye that turns purple when reduced, indicating use of the carbon source provided or resistance to inhibitory chemicals. Each plate contained a positive and negative control well.

Pure culture of bacteria isolates was grown on Biolog BUG agar plates at 28 ± 2°C for 20-24 hours. Single colonies were swabbed and suspended in inoculating fluid A. Cell suspensions (100 µl) adjusted at 90-98% transmittance was pipetted into 96 well Biolog Microplates for carbon utilization and chemical test. Panels were incubated at 28 ± 2°C for 20-24 hours. The microplates were inserted into the Biolog-Omnilog automatic system and the identification process was carried out using GEN III Biolog-Omnilog identification system software [27].

In vitro screening of bacteria for P-solubilization ability and determination of SI

Phosphate solubilization ability of the isolated bacteria was determined on Pikovskaya's agar. The isolates were spotted onto Pikovskaya's agar and incubated for 7 days at 28 ± 2°C. The presence of halo zone around the bacterial colony was considered as indicator for positive phosphate solubilization. Further, the solubilization index (SI) of the isolates was determined by the halo zone of clearance (HD) in the Pikovskaya's agar plates and the colony diameter (CD) [28]. SI was calculated with the formula: SI=(CD+HD)/CD Three replicate plates were used for each isolate [29].

Data analysis

Data were analyzed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). Coefficient of variation was calculated for the significances of differences within samples and ANOVA was employed for significances of differences between mean counts of microbial groups.

Results and Discussion

Isolation and identification of P-solubilizing rhizobacteria

A total of 169 bacteria were isolated on nutrient agar from root washing solutions (61 isolates), surface sterilized roots (45 isolates) and soil (63 isolates). Based on colony morphology on nutrient agar and Biolog Universal Growth (BUG) agar and Gram staining similarity, the 61 bacteria isolated from root washing solutions were clustered into 8 representative isolates. In the same manner, 10 and 7 representative bacteria isolates were screened from surface sterilized roots and soil, respectively. The inoculums of these 25 clustered representative isolates of morphological groups were transferred into GEN III Micro-plate kits and subjected to Biolog bacterial identification system test kits. Sixteen of the 25 clustered representative isolates were identified (Table 1). The 169 bacteria isolates were also evaluated for their ability to solubilize phosphates on PK agar plates.

54.7% of the identified bacteria were isolated from root, 27.9% from soil and the rest 17.4% were isolated from root washing solution (Table 2). Eighty-six out of total isolates were identified at genius and/or species level. Some isolates were left unidentified. This might be due to the limitations of using biochemical characteristics to identify the environmental isolates. Members of the identified rhizobacteria were dominated by the genus *Pseudomonas* (44.1%) followed by *Bacillus* (11.6%), *Enterobacter* (10.5%) and *Stenotrophomonas* (10.5%) (Table 2).

No.	Sample ID	Bacteria species	PROB	SIM	DIST
1	BRWS1c	*Stenotrophomonas maltophilia*	-	0.249	5.003
2	BRWS7a	*Citrobacter gillenii*	0.926	0.665	4.014
3	BRWS12a$_1$	*Enterobacter aerogenes (kleb.mobilis)*	0.367	0.704	4.208
4	BCR1a	*Pseudomonas tolaasii*	0.453	0.734	3.829
5	BCR2c	*Mycobacterium confluentis*	-	0.300	6.738
6	BCR5a	*Bacillus simplex*	0.272	0.660	4.901
7	BCR6b	*Pseudomonas tolaasii*	0.812	0.565	4.300
8	BCR6c	*Pseudomonas aeuroginosa*	0.568	0.689	4.456
9	BCR8a	*Corynibacterium freneyi*	0.318	0.646	5.061
10	BCR8c	*Enetrobacter cloacae ss dissolvens*	0.647	0.704	4.186
11	BCR12c$_1$	*Pseudomonas fluorescens*	0.696	0.617	5.608
12	BCS1b	*Rhodococcus rhodnii*	-	0.083	12.077
13	BCS2b	*Gordonia amicalis*	-	0.073	13.006
14	BCS3c	*Bacillus cereus/weihenstephanensis*	0.362	0.795	2.889
15	BCS9b	*Citrobacter gillenni*	0.926	0.666	4.016
16	JCS23a	*Pseudomonas cichorii*	0.640	0.631	5.336

Table 1: Biolog bacterial identification result of the 25 clustered representative isolates.

No.	Genus	Isolated from	Frequency (N=86)	Site (Zone)		Percentage
				Kaffa	Jimma	
1	*Stenotrophomonas*	RWS	9	8	1	10.5
		Root	-	-	-	
		Soil	-	-	-	
		Total	**9**	**8**	**1**	
2	*Citrobacter*	RWS	5	2	3	8.1
		Root	-	-	-	
		Soil	2	2	-	
		Total	**7**	**4**	**3**	
3	*Enterobacter*	RWS	1	1	-	10.5
		Root	8	7	1	
		Soil	-	-	-	
		Total	**9**	**8**	**1**	
4	*Rhodococcus*	RWS	-	-	-	9.3
		Root	-	-	-	
		Soil	8	6	2	
		Total	**8**	**6**	**2**	
5	*Gordonia*	RWS	-	-	-	2.3
		Root	-	-	-	
		Soil	2	2	-	
		Total	**2**	**2**	**-**	
6	*Bacillus*	RWS	-	-	-	11.6
		Root	1	1	-	
		Soil	9	6	3	
		Total	**10**	**7**	**3**	
7	*Pseudomonas*	RWS	-	-	-	44.1
		Root	35	23	12	
		Soil	3	-	3	
		Total	**38**	**23**	**15**	
8	*Mycobacterium*	RWS	-	-	-	2.3
		Root	2	2	-	
		Soil	-	-	-	
		Total	**2**	**2**	**-**	
9	*Corynibacterium*	RWS	-	-	-	1.2
		Root	1	1	-	
		Soil	-	-	-	
		Total	**1**	**1**	**-**	
10	**Sum of total**	**RWS**	**15**	**11**	**4**	**17.4**
		Root	**47**	**34**	**13**	**54.7**
		Soil	**24**	**16**	**8**	**27.9**
		Total	**86**	**61**	**25**	**100.0**

RWS: Root washing solution

Table 2: Frequency distribution of the identified bacteria genus.

Members of the genus *Pseudomonas* were dominated by *Pseudomonas tolassii* while the genus *Bacillus* was dominated by *B. cereus/weihenstephanensis*. *Bacillus* and *Pseudomonas* are the most dominant genera commonly reported in many plant studies [30]. The biochemical analysis revealed the presence of a wide array of Gram negative and Gram-positive bacteria associated with Coffee plants. Gram negative rhizobacteria dominated the system accounting for 73.2% of isolates (Table 2). Previous observation showed that the rhizosphere of many agriculturally important plants favour more Gram negative rhizobacteria than the Gram positives [31,32]. In the studies done, several bacteria from the rhizosphere of *C. arabica* L. were also isolated in Ethiopia which showed multiple plant growth promoting activity and potent bacteria having antagonistic characteristics to coffee pathogens [19,33].

In vitro screening of bacteria for P-solubilization ability and determination of SI

Thirty-two-point five percent (55/169) of the rhizobacterial isolates showed clearly visible haloes (>0.50 cm) around their colonies on PA after seven days of incubation. Members of the phosphobacteria were dominated by the genus *Pseudomonas* (14.5%) followed by *Citrobacter* (3.6%), *Rhodococcus* (9.1%), *Stenotrophomonas* (7.3%), *Gordonia* and *Bacillus* (3.6%) each respectively (Table 3). Some of the Gram-negative isolates (n=5/19) and Gram-positive isolates (n=1/9) lost their capacity for phosphate solubilisation on repeated sub-culturing like in many other studies [19,34]. The largest solubilisation indices were produced by Gram-negative isolates compared with the Gram-positive isolates.

No.	*Bacteria genus*	Gram	% (N=55)
1	*Pseudomonas sp*	Gm-	14.5 (8)
2	*Citrobacter sp*	Gm-	10.9 (6)
3	*Rhodococcus sp*	Gm+	9.1 (5)
4	*Stenotrophomonas sp*	Gm-	7.3 (4)
5	*Bacillus sp*	Gm+	3.6 (2)
6	*Gordonia sp*	Gm+	3.6 (2)
7	*Enterobacter sp*	Gm-	1.8 (1)
8	Not identified PSB	---	49.1 (27)

PSB: Phosphate solubilizing bacteria

Table 3: Percentage distribution of the phosphate solubilizing bacteria genus.

The SI of the potential P solubilising rhizobacterial isolates differed significantly ($p<0.05$) and ranged from 0.53 to 6.1. The bacterial strain BCR17b (*Pseudomonas tolassii*) produced the largest zone of solubilisation, followed by BRWS25b (*Citrobacter gillenii*).

Conclusion

Some studies in Ethiopia showed the existence of PSB in the rhisosphere soil of *Coffee arabica* L. but the present study included not only the rhisosphere soil bacteria but also the rhizoplane and the endophytic PSB associated with *Coffea arabica* L. Thus, this study can be assumed as the first reported analysis of particularly coffee root endophytic PSB in Ethiopia. It revealed the existence of PSB in and on coffee roots and rhizosphere soils.

In the current study, several bacteria from the root, root washing solution and rhizosphere of *C. arabica L.* were isolated and evaluated for their capacity of phosphate solubilization. They belong to different bacterial genera: *Pseudomonas, Bacillus, Enterobacter, Citrobacter, Stenotrophomonas, Gordonia, Mycobacterium, Corynibacterium* and *Rhodococcus.* Members of the identified rhizobacteria were dominated by the genus *Pseudomonas* followed by *Bacillus*, *Enterobacter* and *Stenotrophomonas.*

Even though the biochemical analysis in this study revealed the presence of a wide array of Gram negative and Gram-positive bacteria associated with Coffee plants, it is not enough to show the existing diversity of bacteria strains. Thus, further studies should focus on the use of molecular techniques in the identification of isolates so that the complete diversity of the existing PSB associated with Coffee plants would be revealed and used for bio-inoculant preparations.

Conflict of Interest

Authors did not declare any conflict of interest.

Acknowledgements

We are very much indebted to agricultural experts working at Kaffa and Jimma zone and woreda offices, and extension workers at each kebeles where sample collection was undertaken in Southwestern Ethiopia.

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