



Research Article

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# In vitro Screening and Identification of P-Solubilizing Rhizobacteria Associated with *Sorghum bicolor* L.



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## Abstract

In the present study, P-solubilizing rhizobacteria were screened and identified from *Sorghum bicolor* L root adhering soil and root which were collected from sorghum growing zones of Tigray, Ethiopia. A total of 94 bacteria were isolated from root washing solutions and surface sterilized roots. These isolates were evaluated for their ability to solubilize phosphates on Pikovskaya's agar plates. The P-solubilizing bacterial isolates were identified by GEN III Biolog bacterial identification system. Fifty four of the 94 (57.5%) rhizobacterial isolates showed clearly visible haloes (>0.50cm) around their colonies on Pikovskaya's agar after seven days of incubation. The solubilization index (SI) of the potential P-solubilizing rhizobacterial isolates differed significantly ( $p < 0.05$ ) and ranged from 0.5 to 4.83. Gram negative rhizobacteria dominated the identified P-solubilizing Rhizobacteria isolates and produced larger solubilization indices when compared with the Gram-positive isolates. Members of the phosphobacteria were dominated by the genus *Pseudomonas* (35.71%). Some of the isolates lost their capacity for phosphate solubilization on repeated sub-culturing. Overall, this finding indicated that there is a great number of rhizobacterial potential associated with *Sorghum bicolor* L which can be utilized for development of P-solubilizing bio-fertilizers.

**Keywords:** P-solubilizing rhizobacteria; *Sorghum bicolor* L.; Biolog bacterial identification

**Abbreviations:** PGPR: Plant Growth Promoting Rhizobacteria; PSM: Phosphate Solubilizing Microbes; PSB: Phosphate Solubilizing Bacteria; CD: Colony Diameter; SI: Solubilization Index; BUG: Biolog Universal Growth

## Introduction

Plant growth promoting rhizobacteria (PGPR) flourish in the rhizosphere of plant, which may grow in, on, or around plant tissues and exert beneficial effects on plant development [1,2]. 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 phyto-hormones, siderophores and organic acids, lowering of plant ethylene levels, N<sub>2</sub> fixation and bio-control of plant diseases [4,5]. The use of such beneficial bacteria as bio-fertilisers and bio-control agents has currently attracted increased interest world-wide in attempts to achieve sustainability, particularly in agriculture, forestry and horticulture [5].

The number of PGPR that have been identified has seen a great increase in the last few years, mainly because of the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere. Various species of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance plant growth. There are several

PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism; suppression of plant disease (termed Bio-protectants), improved nutrient acquisition (Bio-fertilizers), or phyto-hormone production (Bio-stimulants) [2].

The use of PGPR offers an attractive way to replace chemical fertilizer, pesticides, and supplements; most of the isolates result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants. The economic and ecological problems of today have re-invigorated the idea of using bio-fertilizers and bio-control agents in order to reduce the application of costly and environmentally-polluting agrochemicals to a minimum [6,7]. Agrochemicals (namely fertilizers and pesticides) have greatly influenced natural rhizosphere microbes in agro-systems [8]. Plant beneficial microbial bio-resources promise to replace or supplement many such destructive, high intensity practices and support ecofriendly crop production [6,7]. In particular, plant growth promoting rhizobacteria (PGPR) for the benefits of agriculture and ecosystem functions is gaining worldwide importance and acceptance [6,7,9,10].

Phosphorus is the second most important nutrient for plants, after nitrogen. It exists in soil as mineral salts or incorporated into organic compounds. Despite these phosphorus compounds being abundant in agricultural soils, the majority of them occur in an insoluble form. Plants require approximately 30 μmol l<sup>-1</sup> of phosphorus for maximum productivity, but only about 1 μmol l<sup>-1</sup> is available in many soils. Therefore, the unavailability of phosphorus in many soils has been recognized as a major growth limiting factor in agricultural and horticultural systems. This necessitates the application of soluble forms of phosphorus in the form of phosphate fertilizers, which in itself has constraints in that it too is rapidly immobilized (fixed) to insoluble forms upon its application in the soil due to its reaction with aluminum and iron minerals. The efficiency of applied phosphorus rarely exceeds 30% due to fixation in soil. It is also lost as a result of run-off and leaching, leaving as little as 10-20% available for plant utilization. Phosphate fertilizers are dependent on phosphorus derived from phosphate rock, which is a non-renewable resource and current global reserves may be depleted in 50-100 years. Therefore, exploring alternative forms of agriculture, where nutrient conservation is key, is of vital importance [11].

Several reports have indicated that different bacterial species, particularly rhizosphere colonizing bacteria, have the ability to liberate organic phosphates or to solubilize insoluble inorganic phosphate compounds such as tri-calcium phosphate, di-calcium phosphate, hydroxyapatite, and rock phosphate. These bacteria make available the soluble phosphates to the plants, and in return gain root borne carbon compounds, mainly sugars and organic acids, necessary for bacterial growth [12]. Current research suggests that the inoculation of crops with Phosphate Solubilizing Microbes (PSM) has the potential to reduce application rates of phosphate fertilizer by 50% without significantly reducing crop yield [13,14]. Phosphate Solubilizing Bacteria (PSB) may also

be useful in the phyto-remediation of heavy metal impacted soil [15,16] or for bioleaching of rare Earth elements for mined ores [17].

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 aluminum [9,12,18]. This leads to wide P deficiency which is particularly the case for the large parts of Ethiopian soils [19,20]. To alleviate P deficiency, chemical phosphate fertilizers are widely used. However, a large proportion of the soluble forms of P fertilizers is precipitated in insoluble form soon after application and becomes unavailable to plants [21]. This in turn leads to a need for excessive and repeated application of soluble P fertilizers, 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.

Thus, in relation to this fact, P-solubilizing Rhizobacteria associated with cultivated Sorghum plant roots that displayed bio-fertilizer characteristics and have potential applications as native P-solubilizing bacterial bio-fertilizers were screened and identified in this study.

## Materials and Methods

### Description of sample collection areas

Sample collection was carried out in two major sorghum producing zones of Tigray region in Ethiopia. The sample collection site is shown in Figure 1. It comprises Central Tigray and South Tigray zones which are found in the northern part of Ethiopia. Based on the GPS data recorded during sample collection, the sample collection sites are located between 12028.0988'-13019.9522'N and 38053.1815'-39040.9870'E with an altitude range of 1342-1822m a.s.l.

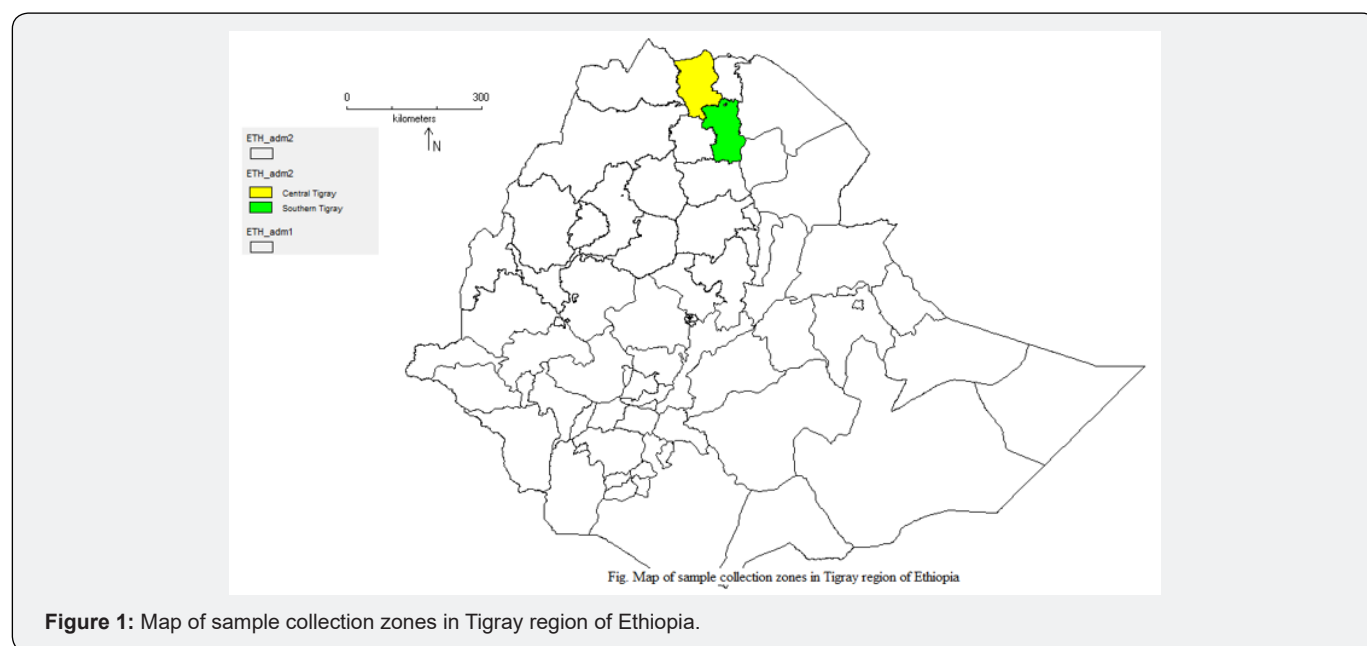


Figure 1: Map of sample collection zones in Tigray region of Ethiopia.

## Sample collection

A total of 93 sorghum roots with adhering soil samples were collected in sterile plastic bags. Samples were collected based on altitude differences of sorghum plant growing areas, cultivar types and plant age group. At each sampling site, plant roots with adhering soil (approximately 50g) were uprooted and placed into a sterile plastic bag. Care was taken to keep rhizosphere soil intact around the root. The collected samples were kept in ice-box and transported to Ethiopian Biodiversity Institute Microbiology Laboratory. All samples were kept at 4 °C until use [22-24].

## Isolation of Rhizobacteria

Sorghum roots with adhering soils were merged into 17 composite samples separately based on similarity of cultivar type, plant and age group. The root adhering soils were dislodged from the roots using sterile distilled water by shaking at 250rpm for 20 minute and the root washing solutions were used for the isolation of rhizoplane bacteria [25]. For the isolation of bacterial endophytes, merged and washed roots were surface sterilized in 99% ethanol for 1min, 3% NaOCl for 6 minutes, and 99% ethanol for 30 seconds and followed by rinsing with sterile distilled water for 6 times [23]. Before homogenization, a root fragment was imprinted on nutrient agar to serve as a sterility check. Roots were homogenized and macerated with a sterile mortar and pestle [26]. The root washing solutions and homogenized roots were serially diluted (10<sup>-2</sup> to 10<sup>-4</sup>) aseptically for inoculation. 0.1ml inoculums of the prepared samples were spread onto Nutrient agar plates and incubated at 30±2 °C for 48h [27,28]. Bacterial colonies with distinct and peculiar morphologies were selected and re-streaked to obtain pure colonies [24].

## In vitro screening of bacteria for P-solubilization potential

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 30 ± 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 measuring the halo zone of clearance (HD) in the Pikovskaya's agar plates and the colony diameter (CD) [29]. SI was calculated with the formula:  $SI = (CD+HD)/CD$ . Three replicate plates were used for each isolate [30].

## Identification of P-solubilizing rhizobacteria

Preliminary identification of P-solubilizing Rhizobacteria isolates were performed by examination for cell morphology using optical microscopy, Gram staining, and colony morphology [27,24]. Biochemical identification including the carbohydrate fermentation patterns and chemical sensitivity tests were determined 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 30 ± 2 °C for 20-24 hours. Single colonies were swabbed and suspended in inoculating fluid A. Cell suspensions (100µl) adjusted at 90-98% transmittance was pipetted into 96 well Biolog Micro-plates for carbon utilization and chemical test. Panels were incubated at 30 ± 2 °C for 20-24 hours. The micro-plates were inserted into the Omnilog automatic system and the identification process was carried out using GEN III Biolog-Omnilog identification system software [31].

## 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. DIVA\_GIS 7.5.0 was used for mapping study areas.

## Results and Discussion

### In vitro screening of P-solubilizing rhizobacteria

Ninety-four bacteria were isolated from root washing solutions and surface sterilized roots on nutrient agar. Fifty-one bacteria were isolated from sorghum root washing solutions which were prepared from the root adhering soils and the rest 43 were endophyte bacteria isolated from sorghum roots. These 94 bacterial isolates were evaluated for their ability to solubilize phosphates on Pikovskaya's agar plates (Table 1). Fifty four of the 94 (57.5%) rhizobacterial isolates showed clearly visible haloes (>0.50cm) around their colonies on Pikovskaya's agar after seven days of incubation. The solubilization index (SI) of the potential P-solubilising rhizobacterial isolates differed significantly ( $p < 0.05$ ) and ranged from 0.5 to 4.83. Bacterial strain TS RWS7b produced the largest zone of solubilisation, followed by TS RWS 1b.

### Identification of P-solubilizing rhizobacteria

Based on colony morphology shown on nutrient agar and Biolog Universal Growth (BUG) agar, and Gram staining similarity, the 54 P-solubilizing Rhizobacteria screened from root washing solutions and sorghum roots were clustered into 17 representative isolate morphological groups. Inoculums of the 17 clustered representative isolates were prepared and transferred into GEN III Micro-plates. After 24 hours of incubation at 30±2 °C, the micro-plates were subjected to Biolog-Omnilog bacterial identification system test. Fourteen of the 17 clustered representative P-solubilizing Rhizobacteria isolates were identified (Table 1).

Eleven of the 14 identified P-solubilizing Rhizobacteria were isolated from root washing solution and the rest 3 were isolated from sorghum root. Gram negative rhizobacteria dominated the system accounting for 78.57% (11/14) of the identified P-solubilizing Rhizobacteria isolates (Table 1,2). Previous observation showed that the rhizosphere of many agriculturally

important plants favors more Gram negative rhizobacteria than the Gram positives [4,32]. The largest solubilization index was also produced by Gram negative isolate when compared with Gram-positive isolate. Some of the isolates lost their capacity for phosphate solubilization on repeated sub-culturing as previously reported in many other studies [33,34].

**Table 1:** Biolog-Omnilog identification result of P-solubilizing Rhizobacteria.

No.	Isolate ID	Bacteria Species	PROB	SIM	DIST	Organism Type
1	TS RWS1b	<i>Bacillus marisflavi</i>	0.925	0.672	3.938	GP-Rod
2	TS RWS2b	<i>Cupriavidus pauculus</i>	0.469	0.617	5.595	GN-Nent
3	TS RWS3b	<i>Staphylococcus gallinarum</i>	0.397	0.61	5.676	GP-Coccus
4	TS RWS3d	<i>Rhizobium radiobacter</i>	-----	0.292	6.174	GN-Nent
5	TS RWS4c	<i>Pantoea agglomerans</i>	0.961	0.684	4.065	GN-Ent
6	TS RWS5a	<i>Pseudomonas viridilivida</i>	-----	0.129	8.152	GN-Nent
7	TS RWS6a	<i>Pseudomonas citronellolis</i>	----	0.188	8.158	GN-Nent
8	TS RWS7c	<i>Pseudomonas putida</i> biotype B	0.478	0.624	5.468	GN-Nent
9	TS RWS9a	<i>Kluyvera ascorbate</i>	0.527	0.535	6.875	GN-Ent
10	TS RWS9b	<i>Serratia marcescens</i> ss <i>marcescens</i>	0.6	0.617	5.573	GN-Ent
11	TS RWS9c	<i>Pseudomonas marginalis</i>	0.466	0.589	5.996	GN-Nent
12	TS R4c	<i>Enterococcus mundtii</i>	0.309	0.734	3.749	GP-Coccus
13	TS R7c	<i>Stenotrophomonas maltophilia</i>	----	0.236	5.932	GN-Nent
14	TS R8a	<i>Pseudomonas fluorescens</i>	0.328	0.535	6.89	GN-Nent

**Table 2:** Frequency distribution of identified P-solubilizing Rhizobacteria genus.

No.	Genus	Isolated from	Frequency (%)
1	<i>Pseudomonas</i>	Root washing solution	4(28.57)
		Root	1(7.14)
2	<i>Bacillus</i>	Root washing solution	1(7.14)
3	<i>Cupriavidus</i>	Root washing solution	1(7.14)
4	<i>Rhizobium</i>	Root washing solution	1(7.14)
5	<i>Staphylococcus</i>	Root washing solution	1(7.14)
6	<i>Pantoea</i>	Root washing solution	1(7.14)
7	<i>Kluyvera</i>	Root washing solution	1(7.14)
8	<i>Serratia</i>	Root washing solution	1(7.14)
9	<i>Enterococcus</i>	Root washing solution	1(7.14)
10	<i>Stenotrophomonas</i>	Root	1(7.14)

Ten different genera of Rhizobacteria were identified. Most of them were isolated from root washing solutions. Eight of the 10 identified Rhizobacteria genera were isolated only from sorghum root washing solutions. But, only *Stenotrophomonas* species was isolated from root. Meanwhile, *Pseudomonas* species was isolated from both root washing solutions and root. Members of the phosphobacteria were dominated by the genus *Pseudomonas* (35.71%) (Table 2). *Pseudomonas* are the most dominant genera commonly reported in many plant studies [35].

### Conclusion

This study showed that there are a large proportion of P-solubilizing rhizoplane and endophytes rhizobacteria associated with *Sorghum bicolor* L. *Pseudomonas* is the most dominant rhizobacteria both in the root adhering soil and roots of sorghum. In general, Gram negative bacteria were not only more predominant than Gram positive bacteria but also, they produced the largest solubilization index. This finding indicated that there is a great number of

rhizobacterial potential associated with *Sorghum bicolor* L. which can be utilized for development of P-solubilizing bio-fertilizers.

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### Conflict of Interest

Authors did not declare any conflict of interest.

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