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Diversity of Rhizobacteria Associated with Sorghum Wild Relatives in Assosa Zone of Ethiopia and Their Ability to Solubilize Phosphate

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Abstract: Culture based diversity of Rhizobacteria associated with Sorghum wild relatives were identified by this study. Rhizobacteria, which include rhizosphere bacteria, rhizoplane bacteria and endophytic bacteria were isolated and identified. The isolates were also evaluated and screened for their ability to solubilize phosphates on Pikovskaya's agar plates. One hundred twenty-six rhizobacteria were isolated on nutrient agar from the samples. Out of these Rhizobacterial isolates, 21 of them were endophyte bacteria, 40 of them were rhizosphere bacteria and 65 of them were rhizoplane bacteria. The 126 isolated rhizobacteria were clustered into 36 similar representative Morphotype groups. And, they were identified using GEN III Biolog microbial identification system. Gram negative rhizobacteria were more predominant than Gram positive rhizobacteria. Genus Pseudomonas was found as the most dominant Rhizobacterial taxa among the identified P-solubilizing Rhizobacteria and Non-P-solubilizing Rhizobacteria isolates. Forty seven percent (47.2%) of the clustered rhizobacterial isolates showed clearly visible haloes (≥ 0.50 cm) around their colonies on Pikovskaya's agar after seven days of incubation at $30 \pm 2°C$. They showed a significant difference of solubilization index (SI) [$p<0.05$] and ranged from 1.3 to 5.3 with a mean value of 2.4. Gram positive bacteria (Bacillus cibi) produced the largest solubilisation index when compared with the Gram-negative isolates. This study indicated that P-solubilizing rhizobacteria associated with Sorghum Wild Relative have very large phosphate solubilization index. Thus, it is recommended to include them in research programs that intend to screen P-solubilizing bacteria for microbial inoculant development.

Keywords: Rhizobacterial Diversity, Sorghum Wild Relatives, P-solubilizing Rhizobacteria, Biolog

1. Introduction

Diverse microorganisms which exist around the plant rhizosphere modulate the physiology and morphology of plants and by that they improve plant growth through hormone production, nutrient uptake and protecting plants from pathogens [3, 19]. The plant rhizosphere is a small tiny zone of soil which encompasses the plant root, where high abundance and diversity of micro-organisms flourish [29]. The dominant bacteria which colonize this zone are known as rhizobacteria [8]. They are characterized by aggressive colonization and subsequent establishment on plant roots. Some of this diverse bacterial species are culturable and others are yet unculturable. Gram negative rhizobacteria predominate the rhizosphere [8, 24, 25].

Multiple interactions occur among the plant root, soil and bacteria in the dynamic environment of the rhizosphere. The soil is under the influence of plants through root exudates or rhizodeposits, plant litter, and nutrient exchanges [7]. Rhizodeposits are suitable for binding bacteria and such environments are richer in microflora and available nutrients than the bulk soil [3]. Plants absorb water and mineral nutrients from the soil and release rhizodeposits into the soil through the interface between root and soil. The different forms of the rhizodeposits facilitate below-ground interactions between plants and microorganisms and consequently affect the biodiversity of the rhizosphere. Root–microbial interactions involve myriads of microorganisms and are very

complex [21]. Most studies characterized rhizobacteria by isolating bacteria from the rhizosphere soil of cultivated plants. The most commonly characterized and identified Rhizobacterial genera are: *Acetobacter, Acinetobacter, Alcaligenes, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Derxia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Ochrobactrum, Pantoae, Pseudomonas, Rhodococcus, Serratia, Stenotrophomonas,* and *Zoogloea* [1].

Different factors determine the microbial diversity in the agricultural fields. Some of the important factors that directly affect the diversity of rhizobacteria are spatial and temporal variability, plant species, plant age, genotypes of plants, and root exudates. In addition, agricultural practices and ecological disturbances may affect the structure and diversity of soil microbial community [1]. For example, intensive land-use reduces metabolic diversity of rhizobacteria which by its turn may reduce the soils' resistance to stress or disturbance [8].

Plant beneficial microbial bio-resources as an alternative solution for the many destructive and high intensity agricultural practices are being promised and considered to support ecofriendly crop production [23, 10]. Particularly, plant growth promoting rhizobacteria has gained worldwide importance and acceptance for benefiting agriculture and functioning ecosystem [10, 14, 23, 27]. To this end, a diverse array of rhizobacteria had been isolated from cultivated plants rhizosphere and has been used for maintaining soil fertility [8] but selecting and characterizing rhizobacteria isolated from wild relatives of crops which are grown naturally in relatively undisturbed environment has not that much been done before. But it is important to search and find novel microbial strains from such undisturbed natural environments that can be used for microbial inoculant development. In light of this assumption, this study had attempted to decipher the rhizobacterial diversity associated with sorghum wild relatives and evaluated phosphate solubilization ability of the isolated bacterial species.

2. Materials and Methods

2.1. Description of Study Area

Sorghum wild relative samples were collected from Assosa zone of Benishangul-Gumuz region of Ethiopia. The sample collection site is shown in Figure 1. It comprises three *woredas* (Administration units in a zone) which are found in Assosa zone. The names of the three woredas are *Assosa zuria, Bambasi* and *Oda*. The GPS data of sample collection sites are located between the latitudinal range: 009°44.344'N-10°05.4913'N and longitudinal range: 32°16.1941'E - 35°11.9250'E with an altitude range: 1005-1508 m a.s.l.
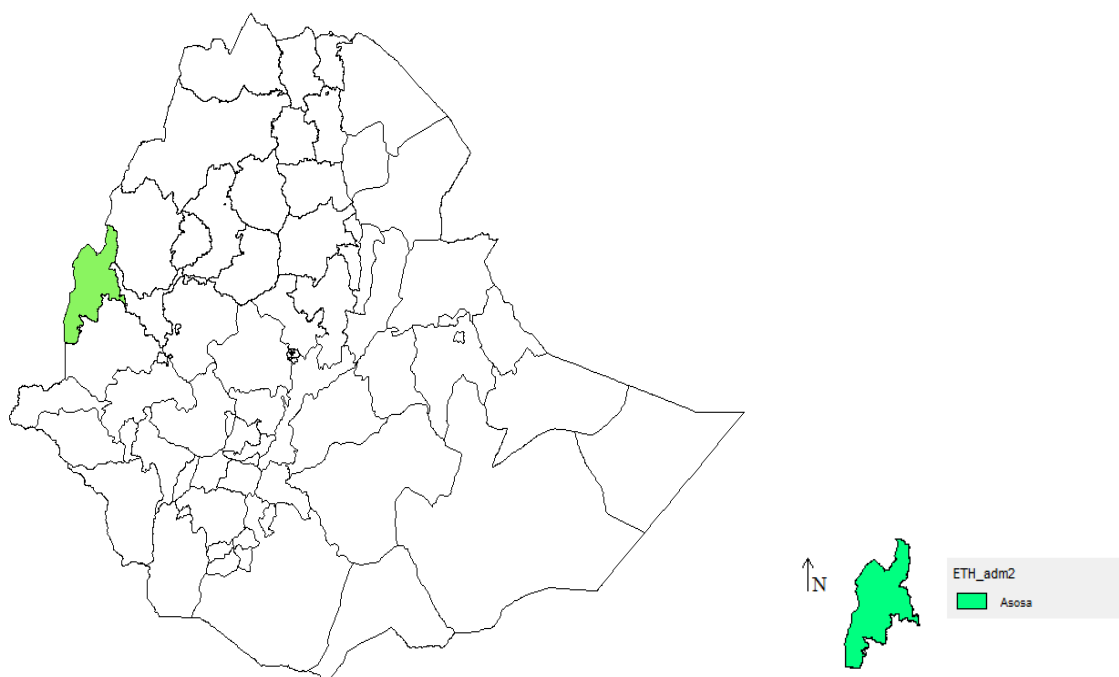

Figure 1. Map of study area:-Assosa zone.

2.2. Sample Collection

Eight sorghum wild relative (SWR) varieties found in the study area were included during sample collection. These varieties are known by their local names: *Bundule, Abendu, Ereregn, Abirbid, Aduhen, Gelabro, Agamo, and Bokona*. Plant roots with approximately 50 g of adhering soil were uprooted and collected in a sterile plastic bag. Root adhering soil samples were collected carefully to keep the soil intact around the root. On top of it, rhizosphere soil was also collected from each SWR plants. The samples were transported to Ethiopian Biodiversity Institute, Microbiology Laboratory under ice-box and were kept at 4°C until use [9, 16, 26].

2.3. Isolation of Rhizobacteria

Three different sample types: root, root washing solution

and rhizosphere soil were employed to isolate rhizobacteria. In order to homogenize and remove bacterial cells from the rhizosphere soil, 1.0 gram of soil were mixed in 9.0 ml of saline solution (0.85% NaCl) [1/10 (wt./vol)] and vortexed for 5 min. [9, 28]. The root adhering soil were washed and dislodged from the roots using sterile distilled water by shaking at 250 rpm for 20 minutes. The rhizoplane bacteria were isolated from the root washing solutions [5]. Washed roots were surface sterilized using three steps surface sterilization technique 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 [16]. These washed and sterilized roots were used to isolate bacterial endophytes. Roots were homogenised and macerated with a sterile mortar and pestle after checking the sterility of the roots [13].

Serial dilutions (10^{-2} to 10^{-4}) were prepared for the homogenized rhizosphere soil, root washing solutions and homogenised roots for aseptic inoculation. Inoculums (0.1 ml each) of the diluted samples were spread onto Nutrient agar plates and incubated at $30\pm2°C$ for 48 h [22, 28]. Purification of bacteria was done by selecting and re-streaking bacterial colonies with distinct and peculiar morphologies [9].

2.4. Screening of P-solubilizing Rhizobacteria

Phosphate solubilization ability of the isolated bacteria was determined on Pikovskaya's agar. Bacterial isolates were spotted onto Pikovskaya's agar and incubated for 7 days at 30 ± 2°C. Positive phosphate solubilization was detected by the presence of halo zone around the bacterial colony. The halo zone of clearance (HD) and the colony diameter (CD) of bacteria in the Pikovskaya's agar plate were measured using caliper [20]. The solubilization index (SI) of the isolates were calculated using the formula: $SI=(CD+HD)/CD$ [6]. Triplicate plates were used for each isolate.

2.5. Identification of Rhizobacteria

Biochemical identification including the carbohydrate fermentation patterns and chemical sensitivity tests were determined using GEN III Biolog bacterial identification system kit. Pure culture of bacterial 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 (100 μl) adjusted at 90-98% transmittance was pipetted into 96 well Biolog Micro-plates for 71 carbon source utilization assays and 23 chemical sensitivity assays.

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 [4].

2.6. Data Analysis

SPSS software version 25 was used for data analysis [12]. Descriptive statistics was performed to organize and describe data. Analysis of Variance (ANOVA) was employed as a statistical test for measuring significances of differences between mean counts of microbial groups. P-value less than 0.05 ($p<0.05$) was taken as significant. For mapping study area, DIVA_GIS 7.5.0 [11] was used.

3. Results and Discussions

3.1. Rhizobacterial Isolation

One hundred twenty-six rhizobacteria were isolated on nutrient agar from the composite samples. Out of these Rhizobacterial isolates, 21 of them were endophyte bacteria which were isolated from root, 40 of them were rhizosphere bacteria which were isolated from rhizosphere soil and 65 of them were rhizoplane bacteria which were isolated from the root washing solutions. Due to lack of sufficient GEN III microplate necessary for identification of the 126 isolated rhizobacteria, they were clustered into 36 similar representative Morphotype groups. Similarity of colony morphology and Gram staining result were the basis for clustering the bacterial isolates. Eight of the clustered Rhizobacterial isolates were endophyte bacteria, 14 of them were rhizosphere bacteria and the rest 14 bacterial isolates were rhizoplane bacteria (Table 1). The result indicated that a greater number of rhizobacteria is isolated from the soil and root washing solutions than the rhizobacteria isolated from the root.

3.2. Screening of P-solubilizing Rhizobacteria

Evaluation of phosphate solubilization ability for the 36 bacterial isolates were done on Pikovskaya's agar plates. Forty seven percent of them showed clearly visible haloes (≥0.50 cm) around their colonies after seven days of incubation (Table 1). They showed a significant difference of solubilization index (SI) [($p<0.05$] and ranged from 1.3 to 5.3 with a mean value of 2.4.

Table 1. Frequency distribution of clustered rhizobacteria with p-solubilization ability.

Sample type	No of Rhizobacterial isolates	No of P-solubilizers	No of Non P-solubilizers
Root	8	5	3
Soil	14	9	5
RWS	14	3	11
Total	36	17 (47.2%)	19 (52.8%)

Key: RWS: Root Washing Solution.

3.3. Identification of Rhizobacteria

Twenty three of the 36 Rhizobacterial isolates were identified using Biolog-Omnilog bacterial identification system (Table 2).

Table 2. Biolog identification result of rhizobacteria isolated from sorghum wild relatives' root, soil and root washing solution.

| No.	Isolate ID	PSI	Identified bacteria species	Gram staining
1	AWSR RWS1A	2.1	*Bacillus cibi*	GM$^+$ Rods
2	AWSR RWS2A	Non	*Pseudomonas viridilivida*	GM$^-$ Rods
3	AWSR RWS2B	Non	*Bacillus amyloliquefaciens*	GM$^+$ Rods
4	AWSR RWS3B	Non	*Pseudomonas viridilivida*	GM$^-$ Rods
5	AWSR RWS4A	1.6	*Pseudomonas putida biotype B*	GM$^-$ Rods
6	AWSR RWS4C	Non	*Stenotrophomonas rhizophila*	GM$^-$ Rods
7	AWSR RWS6A	1.5	*Serratia marcescens ss marcescens*	GM$^-$ Rods
8	AWSR RWS15D	Non	*Enterobacter aerogenes (kleb. mobilis)*	GM$^-$ Rods
9	AWSR R1A	3.4	*Pseudomonas viridilivida*	GM$^-$ Rods
10	AWSR R2A	5.3	*Bacillus cibi*	GM$^+$ Rods
11	AWSR R4A	1.7	*Pseudomonas putida biotype B*	GM$^-$ Rods
12	AWSR R4B	Non	*Pseudomonas tolaasii*	GM$^-$ Rods
13	AWSR R11A	2.6	*Pseudomonas putida biotype B*	GM$^-$ Rods
14	AWSR R12A	Non	*Pseudomonas tolaasii*	GM$^-$ Rods
15	AWSR R13A	2	*Pseudomonas viridilivida*	GM$^-$ Rods
16	AWSR R13B	Non	*Pseudomonas synxantha*	GM$^-$ Rods
17	AWSR S6C	2.3	*Ochrobactrum tritici*	GM$^-$ Rods
18	AWSR S7A	Non	*Lactobacillus mali*	GM$^-$ Rods
19	AWSR S10 C2	2.9	*Pseudomonas tolaasii*	GM$^-$ Rods
20	AWSR S14A	1.6	*Pseudomonas putida biotype B*	GM$^-$ Rods
21	AWSR S15B	1.8	*Chryseobacrerium gleum/indologenes*	GM$^-$ Rods
22	AWSR S15C	Non	*Pseudomonas viridilivida*	GM$^-$ Rods
23	AWSR S17A	3.2	*Pseudomonas synxantha*	GM$^-$ Rods

Key: ASWR: Assosa Sorghum Wild Relative, RWS: Root Washing Solution, S: Soil, R: root, PSI: Phosphate solubilization index and GM: Gram's staining.

The twenty three identified Rhizobacterial isolates belong to 8 bacterial genera. These are *Pseudomonas* (60.9%), *Bacillus* (13.3%), *Stenotrophomonas* (4.3%), *Serratia* (4.3%), *Enterobacter* (4.3%), *Ochrobactrum* (4.3%), *Lactobacillus* (4.3%), and *Chryseobacrerium* (4.3%).

3.3.1. Diversity of P-solubilizing Rhizobacteria

Thirteen of the twenty-three identified bacteria were P-solubilizing rhizobacteria and belong to 5 bacterial genera. These are *Pseudomonas* (61.5%), *Bacillus* (15.4%), *Serratia* (7.7%), *Ochrobactrum* (7.7%) and *Chryseobacrerium* (7.7%). The most predominant bacterial species are *Pseudomonas putida biotype B* (30.8%), followed by *Pseudomonas viridilivida* (15.4%) and *Bacillus cibi* (15.4%)

[Figure 2]. The identified P-solubilizing Rhizobacteria isolates were dominated by Gram negative rhizobacteria accounting for 80.4% (11/13) [Figure 2, Table 2]). But Gram-positive isolate showed the largest solubilisation index (*Bacillus cibi)* when compared with Gram negative isolate. Previous studies by other researchers showed that Gram negative rhizobacteria are favored more by the rhizosphere of many agriculturally important cultivated plants than the Gram positives [15, 17, 18]. The largest solubilisation index was also produced by Gram negative isolate than the Gram-positive isolates. Repeated sub-culturing caused some of the isolates to lose their capacity for phosphate solubilisation as previously reported in many other studies [17].
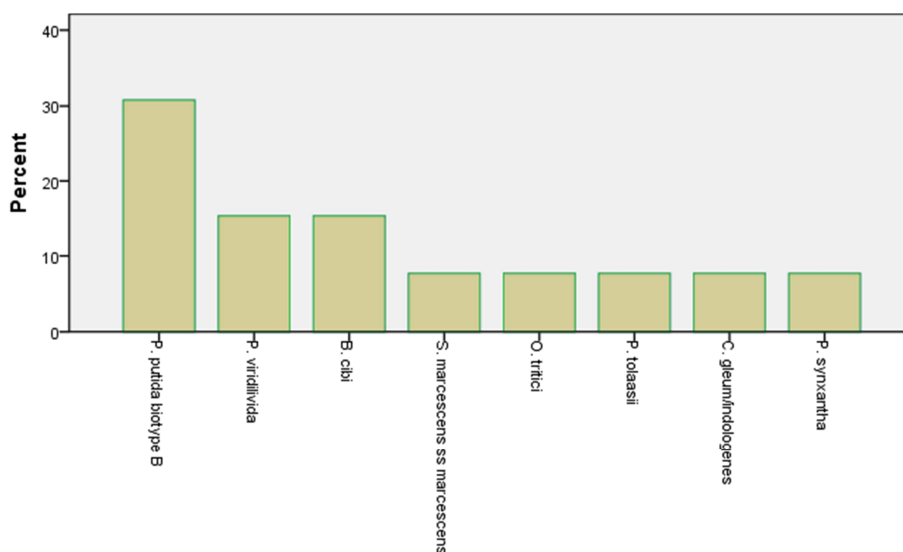
Figure 2. Percent distribution of identified phosphate solubilizing rhizobacteria.

The highest number of p-solubilizing rhizobacteria was obtained from the rhizosphere soil than the root and root washing solutions. But, the highest solubilization index was exhibited by endophytic bacteria. The p-solubilization index ranges between 1.5 and 2.1 for rhizoplane bacteria, 1.7 and 5.3 for endophytic rhizobacteria, and 1.3 to 3.6 for rhizosphere rhizobacteria with a mean of 1.7, 3, and 2.3, respectively (Table 3).

Table 3. *P-solubilization index of rhizobacteria: rhizoplane, endophyte and rhizosphere bacteria.*

Statistics	P-solubilization index of rhizoplane bacteria	P-solubilization index of endophytes	P-solubilization index of rhizosphere bacteria
Number	3	5	9
Mean	1.7	3.0	2.3
Range	.60	3.60	2.30
Minimum	1.50	1.70	1.30
Maximum	2.10	5.30	3.60

3.3.2. Diversity of Non P-solubilizing Rhizobacteria

Ten of the twenty three identified bacteria were Non P-solubilizing rhizobacteria which belong to 5 bacterial genera. These are *Pseudomonas* (60%), *Bacillus* (10%), *Stenotrophomonas* (10%), *Enterobacter* (10%), and *Lactobacillus* (10%). The most predominant bacterial species are *Pseudomonas viridilivida* (30%) followed by *Pseudomonas tolaasii* (20%). Gram negative rhizobacteria dominated the system accounting for 90% (9/10) of the identified non P-solubilizing Rhizobacteria isolates (Figure 3, Table 2).
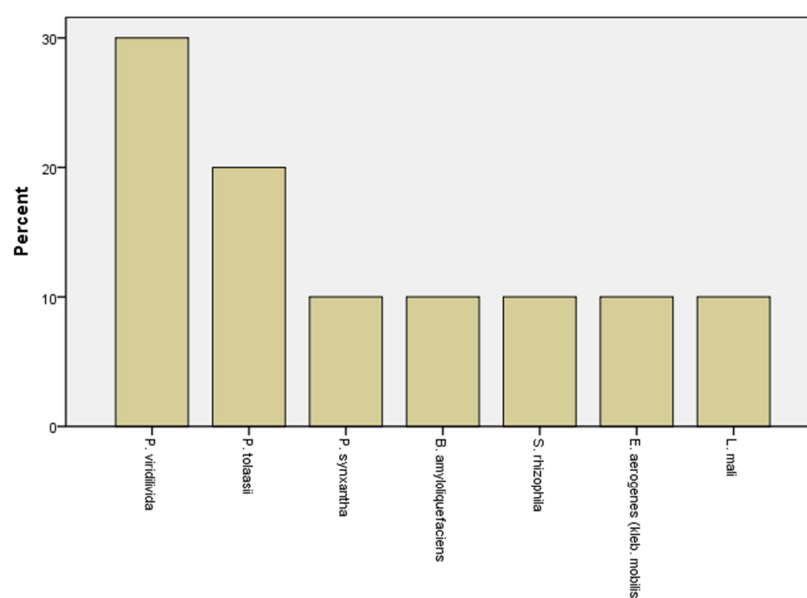
Figure 3. *Percent distribution of identified Non-p-solubilizing rhizobacteria species.*

4. Conclusion

The Rhizobacterial species diversity associated with Sorghum Wild Relative plants are identified in this study. Gram negative bacteria are more predominant than Gram positive bacteria where *Pseudomonas* is the most dominant Rhizobacterial genus. This study also indicated that there are P-solubilizing rhizobacteria associated with Sorghum Wild Relative which have very large phosphate solubilization index. Thus, it is recommended to include bacteria associated with the wild relatives of sorghum in research program that intends to screen p-solubilizing bacteria for bio-fertilizer development.

Declaration on Conflict of Interest

There is no conflict of interest.

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