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Selection and identification of *Pseudomonas* and *Bacillus* Rhizobacteria with Bioinoculant potential for Sorghum cultivation

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Abstract

This study aimed to select and identify elite strains of plant growth-promoting rhizobacteria (PGPR) among phosphate-solubilizing bacteria (PSB) conserved at the Ethiopian Biodiversity Institute. In-vitro screening for plant growth-promoting traits and abiotic stress tolerance identified 12 promising PGPR isolates. These strains exhibited phosphate solubilization (index 1.8–3.2), auxin production, nitrogen fixation, and ammonia production. All isolates showed drought tolerance, and some exhibited salt tolerance (up to 10% NaCl). 16S rDNA sequencing identified *Pseudomonas frederiksbergensis*, *Pseudomonas fluorescens*, *Pseudomonas rhodesiae*, *Pseudomonas azotoformans*, and *Bacillus altitudinis*. These strains have strong potential as bioinoculants for sustainable sorghum cultivation. Future greenhouse and field trials are recommended to validate their effectiveness.

Keywords: *identification, Sorghum, plant growth, rhizobacteria*

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1. Introduction

Plant growth promoting rhizobacteria (PGPR) is collective name for bacteria which flourish in the rhizosphere of plant, that may grow in, on, or around plant tissues and exert beneficial effects on plant development (Akhtar et al., 2012). PGPR enhances plant growth and development in direct and indirect ways. Plant growth is promoted directly through synthesis of plant growth hormones by the rhizobacteria (E.g. Indole Acetic Acid), and improving nutrient acquisitions of phosphate, iron and nitrogen. Indirectly, plant growth is promoted by the production of antimicrobials against phytopathogens and inducing resistance which enhance the natural ability of plants to resist the diseases (Singh et al., 2019). The benefits that can be derived from plant–PGPR interactions can be improvements in seed germination rate, root development, shoot and root weights, yield, leaf area, chlorophyll content, hydraulic activity, protein content, and nutrient uptake (Kumar et al., 2017; Adesemoye & Kloepper, 2009). There is also increasing evidence that beneficial microbes can enhance plants' tolerance to adverse environmental stresses, which include salt stress (Egamberdieva, 2008), drought stress (Zahir et al., 2008; Ahmad et al, 2022), weed infestation (Babalola, 2010), nutrient deficiency, and heavy metal contaminations (Sheng, 2005).

The various species of PGPR that have been reported to enhance plant growth and elicit tolerance to salt and drought are *Pseudomonas, Azospirillum, Azotobacter, Flavobacterium, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* (Chiu-Chung & Bhagwath, 2003). They possess the capacity to stimulate 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. These PGPR also utilize osmoregulation, oligotrophic, endogenous metabolism, resistance to starvation, and efficient metabolic processes to adapt under dry and saline environments and can improve plant production in degraded sites (Lugtenberg et al., 2001; Egamberdiyeva & Islam, 2008; Maheshwari et al., 2012; Yang et al., 2009).

Agrochemicals (namely fertilizers and pesticides) have greatly influenced natural rhizosphere microbes in Agrosystems (Matson et al., 1997). Their toxic effects are devastating to the environment and cause great damage to the biodiversity. PGPR are considered as promising alternatives to replace these destructive chemicals and support ecofriendly crop production (Hart & Trevors, 2005; Rodríguez et al., 2006). The use of PGPR for the benefits

of agriculture and ecosystem functions is gaining worldwide importance and acceptance (Vessey, 2003; Lucy et al., 2004; Hart & Trevors, 2005; Rodríguez et al., 2006; Widawati & Suliasih, 2018). In the last few years, the use of PGPR in agriculture has shown a great increase in number. Currently, several PGPR inoculants are commercialized that promote growth through at least in one mechanism, suppression of plant disease (termed Bio-protectants), improved nutrient acquisition (Bio-fertilizers), or Phyto-hormone production (Bio-stimulants) (Saharan & Nehra, 2011).

PGPR result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants. They can be taken as a component in integrated pest management systems in which reduced rates of agrochemicals and cultural control practices are used as bio-control agents (Anith et al., 2017; Kloepper et al., 2004). Today, the economic and ecological problems 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 (Hart & Trevors, 2005; Rodríguez et *al.*, 2006).

Sorghum acts as a dietary staple for millions of people living in about 30 countries in the subtropical and semi-arid regions of Africa and Asia. It is a source of both food and fodder, mostly in the traditional, smallholder farming sector. It also finds a place in the high-input commercial farming sector as a feed crop and is fast emerging as a biofuel crop. In addition to its economic importance, sorghum, as a drought-resistant crop, assumes a great importance as a food and industrial crop under the currently changing climate regime and it is needed to ensure food and nutritional security in a sustainable manner in the coming years. However, more than 80% of the global sorghum area is characterized by low yield levels. This holds true in Ethiopia and other sorghum-growing East African countries that suffer with long seasons of drought and dry harsh environments.

The toxicity of agrochemicals to the environment and the damage it causes to the biodiversity, and the low yield level of sorghum in Ethiopia are the major challenges that need to be addressed by alternative strategies, like the adoption of PGPR bio-inoculant. There is a very limited information about the use of PGPR for sorghum production in Ethiopia. The current study aimed to select and identify elite strains of PGPR among phosphate-solubilizing bacteria (PSB) conserved at the Ethiopian Biodiversity Institute. It gave emphasis on *Pseudomonas* and *Bacillus* associated with cultivated Sorghum plants and their wild relatives. These bacteria displayed bio-fertilizer attributes and have the potential biotechnological

applications of native microbes in promotion of plant growth. It is basic for the long-term aim of enhancing sorghum plant growth within sustainable agriculture in the future.

2. Materials and Methods

2.1 Description of the Collected Samples and Bacteria Used as Study Materials

A total of 112 bacteria conserved at Microbial Gene bank of Ethiopian Biodiversity Institute were used for selection and *in-vitro* evaluation of PGPR traits. They were isolated from the rhizosphere soil and root samples by growing on Nutrient Agar plates and King's medium B, and incubated at 30 ± 2 ^{O}C for 48 h (Yamaoka-Yano *et al.*, 1988; Raj & Cherian, 2013). The isolated bacteria were first characterized for P-solubilization ability. Those bacteria with the ability of solubilizing phosphate were screened and conserved in Microbial gene bank at Ethiopian Biodiversity Institute. These P-solubilizing bacteria were used for this study. The sample collection, processing, and isolation methods employed previously for characterizing P-solubilizing bacteria are described.

Samples were collected from major Sorghum growing areas of Ethiopia locating at different agro-ecological zones having different altitudes categories, low lands (*Kolla*, 500 - 1,500m a.s.l) and mid-lands (*Woina Dega*, 1,500 - 2,300m a.s.l) where the sorghum crops are cultivated. In each agro-ecological zones of the Regions where the major sorghum growing farms are located, sample collection sites were stratified based on administration units and altitude differences. Samples were collected as appropriate by laying diagonal transects in the farm lands and collecting samples at equal distances where five sites were taken from a given land. The samples from the wild relatives were primarily collected alongside the main streets and farm areas.

The samples consisted of rhisosphere soil and roots with adhering soils from cultivated and wild relatives of Sorghum. At each sampling site, plant roots with adhering soil (approximately 50g) had been collected from each corner of a randomly selected square meter (one from each of 4 quadrants) which lied around the cultivated and wild relatives of Sorghum plant at a depth of 10-20cm. The samples had been kept in ice-box and transported to EBI Microbiology laboratory for the isolation processes (Gupta et al., 2014; Thanh & Diep, 2014; Monk et al., 2009; Yamaoka-Yano et al., 1988).

The 1.0 gram of soil were mixed in 9.0ml of saline solution (0.85% NaCl) [1/10 (wt/vol)]. The soil suspension was vortexed for 5 minutes in order to remove the bacterial cells

(Yamaoka-Yano et al., 1988; Gupta et al., 2014). For the isolation of bacterial endophytes, roots were surface sterilised in 2% NaOCl for 3 minutes, followed by rinsing with sterile water (Costa et al., 2005; Monk et al., 2009). Before homogenisation, a root fragment were imprinted on nutrient agar to serve as a sterility check. Roots were homogenised with a sterile mortar and pestle (Jimenez-Salgado et al., 1997). The soil suspensions, root washing solutions, and homogenised roots were serially diluted (10^{-1} to 10^{-9}) aseptically for inoculation. The isolation was carried out in two different media; 0.1ml samples were spread onto Nutrient agar plates and King's medium B and incubated at 28°C for 48 h (Yamaoka-Yano et al., 1988; Raj & Cherian, 2013). The screened P-solubilizing bacteria was conserved at microbial gene bank.

2.2 Selection of PGPR

The conserved P-solubilizing bacteria were subjected to screening procedures designed for testing the production of plant growth promoting substances and abiotic stress tolerance properties.

2.2.1 In-vitro screening of bacterial isolates for plant growth-promoting traits

Phosphate solubilization ability assay. Phosphate-solubilizing ability of the bacteria was re-determined on Pikovskaya's agar (1948). The isolates were spotted onto Pikovskaya's agar and incubated for 3 days at 30 ± 2 ^{O}C. The presence of halo zone around the bacterial colony were considered as indicator for positive phosphate solubilization. Further, the solubilization index (SI) of the isolates were determined by measuring the halo zone of clearance (HD) in the Pikovskaya's agar plates and the colony diameter (CD).

SI was calculated with the given formula: SI = (CD+HD)/CD. Bacterial isolates were selected for further evaluation study based on the solubilization index they exhibited (Pikovskaya, 1948).

N_2 Fixation ability assay. The N_2 fixation ability was tested by growing the isolates on N-free solid Jensen medium. For further confirmation, the isolates were checked again for nitrogen fixation ability by growing on Burk'N free media (Thanh & Diep, 2014). Plates were incubated for 24hrs at 30 ± 2 ^{O}C for three days. The ability of the isolates to fix N_2 was observed by their growth on both media after the incubation period.

Auxin-production (IAA) ability assay. Indole Acetic Acid production was determined as described by Patten and Glick (2002). The isolates were grown in 100ml flasks containing 50ml Luria broth (LB) supplemented with L-tryptophan (100 μg ml^{-1}) for 48 hours on a rotary

shaker. Then, cultures were centrifuged at 10,000g for 15 minutes and the supernatants were collected. Two ml of Salkowsky's reagent (1ml of 0.5 M $FeCl_3$ in 50ml of 35% $HClO_4$) with one ml of the supernatant were allowed to react with at 30 ± 2 ^{O}C for 30 min. Development of pink color indicated the presence of IAA.

Detection of ammonia. Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in tubes and incubated for 48–72 h at 30 ± 2 ^{O}C. Nessler's reagent (0.5 ml) was added in each tube. The development of color from yellow to brown was taken as a positive test for ammonia production (Cappuccino & Sherman, 1992).

2.2.2 In-vitro screening of bacterial isolates for abiotic stress tolerance properties

Isolates were screened for their ability to tolerate different abiotic stresses; salinity and drought, using nutrient broth (Kumar et al., 2014).

Salt tolerance assay. The bacterial isolates were grown on nutrient agar amended with different concentrations of NaCl to create stress levels of 0%, 5%, 10%, 15% and 20%. The inoculated plates were incubated at 30 ± 2 ^{O}C for 3 days. The bacterial growth was observed and salt tolerant isolates were screened.

Drought tolerance (PEG Tolerance) assay. Bacterial isolates were grown on nutrient agar medium amended with different concentrations of Polyethylene glycol (PEG 6000) to create stress levels of 0%, 5%, 10%, 15% and 20%. The inoculated plates were incubated at 30 ± 2 ^{O}C for 3 days. Drought tolerant isolates were selected by observing their growth on the amended nutrient agar plates.

2.3 Identification of the Selected Indigenous Plant Growth Promoting Rhizobacteria

2.3.1 Phenotypic identification

The preliminary identification of the strains was performed according to their morphological, cultural, physiological and biochemical characteristics based on the procedures described by Krieg et al. (2010), Yamaoka-Yano et al. (1988) and Gupta et al. (2014). Cultures were grown on Nutrient Agar medium to obtain data on the microscopic and macroscopic identification of the bacterial isolates. Their cultural characteristics were observed. They were characterized for the following cultural traits: color pigment, form, elevation, margin,

diameter, surface, opacity and texture. Gram-staining was done and cell shapes were examined under a light compound microscope.

2.3.2 Genotypic identification of PGPR using 16S rDNA sequencing

Genomic DNA extraction, PCR amplification, and DNA sequencing. Genomic DNA was extracted from bacterial pure cultures using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. The primer pair rD1 and fD1 (Weisburg et al., 1991) were used to generate a 1500bp PCR product. The forward and reverse primers used were Fd1 (AGAGTTTGATCCTGGCTCAG) and Rd1 (AAGGAGGTGATCCAGCC), respectively. The IGS region between 16S and 23S rRNA was amplified using FGPS1490 and FGPS132 primers (Gise et al., 1996). The PCR condition was a 95-degree initial denaturation for 5 minutes followed by 35 cycles of denaturation at 95 degrees for 30 seconds, annealing at 55 degrees for 1 minute and extension at 72 degrees for 30 seconds with a final extension at 72 degrees for 5 minutes. The same primers were then used to sequence the PCR product on the ABI 3730XL genetic analyzer.

Genome assembly and analysis. The forward sequence and reverse sequence of the sequencer output were read and the Genome assembly was performed using Bio-Edit alignment software. It helped generate the consensus sequence for each bacterium. The consensus sequences were analyzed and compared with others in the GenBank core nucleotide database using the NCBI BLASTn (Wen et al., 2015; Zahid et al., 2015).

Multiple sequence alignment and phylogeny. Sequences for both forward and reverse were trimmed off bad sequence reads and unambiguous bases. The reverse sequence was reverse compliment and aligned with the sequence for contig generation. This was done for all sequences of the 12 isolates. BLASTn on the NCBI database was used in blast search for similar sequences. In all about 60 sequences were selected from the database with Query coverage and percentage identity range of 98 to 100. Multiple sequence alignment of the 12 sequences of the isolates with the 60 from NCBI database was done using the CLustalW alignment in MEGA software version 11. Evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 10000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the

associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6124)). This analysis involved 57 nucleotide sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 281 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

2.4 Data Analysis

Results of measurements were subjected to analysis of variance (ANOVA) at a significance level of $P < 0.05$, and mean separation was performed using the LSD test (alpha = 0.05) when a significant difference was found in the ANOVA. SPSS software version 25 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

3. Results

In this study, 112 P-solubilizing bacteria were *invitro*-characterized for plant growth promoting traits. The screened 12 PGPR were identified using 16S rDNA sequencing technique.

3.1 Selected Plant Growth Promoting Rhizobacteria

The bioselection process was done in a step wise manner. Primarily, the bacteria were re-evaluated for their P-solubilizing ability and then they were tested for the N_2 fixation ability, NH_3 production capacity, and Auxin production ability. Based on their plant growth promoting efficiency at *in-vitro*, 12 PGPR were selected among them. The 12 PGPR were further undergone abiotic stress tolerance tests and DNA sequencing was performed for each isolate to identify their species.

The *in-vitro* screening result of the selected bacteria isolates is shown in table 1.

Table 1

In-vitro characterization result for PGPR isolated from Cultivated Sorghum (CS) and Sorghum Wild Relatives (SWR)

Isolate ID (PSB No.)	N_2 Fixation	NH_3 Production	Average PSI	Auxin Production	Plant source
Isolate 1 (PSB3)	√	-	2.2	++	SWR
Isolate 2 (PSB6)	√	-	1.8	++	SWR
Isolate 3 (PSB7)	√	-	3	++	SWR
Isolate 4 (PSB13)	√	-	2.3	+	SWR
Isolate 5 (PSB18)	√	++	1.9	+	SWR
Isolate 6 (PSB41)	√	++	3.2	++	CS
Isolate 7 (PSB80)	√	+	2.7	+	SWR
Isolate 8 (PSB81)	√	++	1.9	+	CS
Isolate 9 (PSB84)	√	-	2.7	+	CS
Isolate 10 (PSB104)	√	-	2	+	SWR
Isolate 11 (PSB183)	-	++	2.2	++	CS
Isolate 12 (PSB185)	√	-	2.3	+	CS

Key: +++: Very strong, ++: strong, +: moderate, -: Negative, SWR: Sorghum Wild Relative, CS: Cultivated Sorghum, PSI: Phosphate Solubilizing Index

Seven of the 12 selected bacteria were isolated from Sorghum Wild Relatives (SWR) and the rest five were isolated from Cultivated Sorghum (CS). The P-solubilizing index exhibited by the bacteria ranges between 1.8 to 3.2. All of the selected bacteria produced auxin. Five of them are strong auxin producers and produced ammonia. The rest 7 isolates were moderate auxin producers. All of the selected bacteria were capable of fixing nitrogen except isolate 11 (PSB183).

The selected 12 PGPR were tested for their properties of salt tolerance and drought tolerance (table 2). All of the bacteria were able to grow in a nutrient broth containing 5% salt. Only two isolates [Isolate 10 (PSB104) and Isolate 11 (PSB183)] were grown in 10% salt concentration. All of them did not grow in the 15% and 20% salt concentrations. All of them were drought tolerant which had grown on plates containing 5%, 10%, 15%, and 20% Polyethylene glycol (PEG).

Table 2

Abiotic stress tolerance test result of the selected PGPR

Isolate ID (PSB No.)	Salt Tolerance (NaCl)				Drought Tolerance (PEG Tolerance)			
	5%	10%	15%	20%	5%	10%	15%	20%
Isolate 1 (PSB3)	√	-	-	-	√	√	√	√
Isolate 2 (PSB6)	√	-	-	-	√	√	√	√
Isolate 3 (PSB7)	√	-	-	-	√	√	√	√
Isolate 4 (PSB13)	√	-	-	-	√	√	√	√
Isolate 5 (PSB18)	√	-	-	-	√	√	√	√
Isolate 6 (PSB41)	√	-	-	-	√	√	√	√
Isolate 7 (PSB80)	√	-	-	-	√	√	√	√
Isolate 8 (PSB81)	√	-	-	-	√	√	√	√
Isolate 9 (PSB84)	√	-	-	-	√	√	√	√
Isolate 10 (PSB104)	√	√	-	-	√	√	√	√
Isolate 11 (PSB183)	√	√	-	-	√	√	√	√
Isolate 12 (PSB185)	√	-	-	-	√	√	√	√

3.2 Identification of the Selected Indigenous Plant Growth Promoting Rhizobacteria

The selected indigenous plant growth promoting rhizobacteria were identified using both phenotypic and genotypic identification techniques.

Phenotypic identification of the isolated PGPR. The colony characteristics of the selected bacteria with the plant growth promoting characteristics are shown in table 3. Ten of them had medium size and two were large. All of them were circular in shape. Nine of them had convex elevations, two flats and one raised elevation. All of them had an entire margin and smooth surface. Most of them were translucent and have different colony colors including light green, light yellow, light brown, gray and cream.

Table 3

Colony characteristics of the selected PGPR isolated from the cultivated sorghum and sorghum wild relatives

Isolate ID	Colony Characteristics						
(PSB No.)	Size	Shape	Elevation	Margin	Surface	Color	Transparency
Isolate 1 (PSB3)	Medium	Circular	Convex	Entire	Smooth	Light green	Translucent
Isolate 2 (PSB6)	Medium	Circular	Convex	Entire	Smooth	Light green	Translucent

Isolate ID (PSB No.)	Colony Characteristics						
	Size	Shape	Elevation	Margin	Surface	Color	Transparency
Isolate 3 (PSB7)	Medium	Circular	Convex	Entire	Smooth	Cream	Opaque
Isolate 4 (PSB13)	Medium	Circular	Raised	Entire	Smooth	Light yellow	Translucent
Isolate 5 (PSB18)	Large	Circular	Convex	Entire	Smooth	Cream	Opaque
Isolate 6 (PSB41)	Medium	Circular	Flat	Entire	Smooth	Gray	Translucent
Isolate 7 (PSB80)	Medium	Circular	Convex	Entire	Smooth	Light brown	Translucent
Isolate 8 (PSB81)	Medium	Circular	Convex	Entire	Smooth	Light green	Translucent
Isolate 9 (PSB84)	Medium	Circular	Convex	Entire	Smooth	Light green	Translucent
Isolate 10 (PSB104)	Large	Circular	Flat	Lobate	Smooth	Cream	Opaque
Isolate 11 (PSB183)	Medium	Circular	Convex	Entire	Smooth	Light yellow	Translucent
Isolate 12 (PSB185)	Medium	Circular	Convex	Entire	Smooth	Cream	Opaque

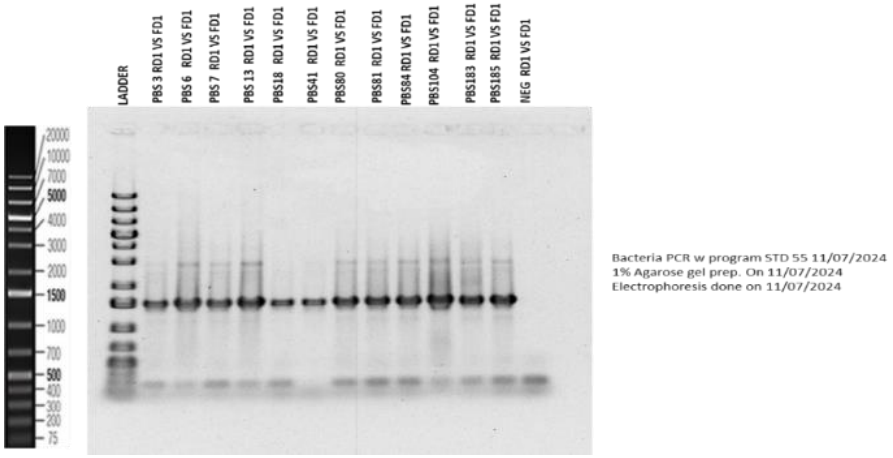
Key: PSB: Phosphate Solubilizing Bacteria

Genotypic identification of PGPR using 16S rDNA sequencing

Gel-electrophoresis result. 16S rDNA was extracted from the 12 selected PGPR, and multiplied by Polymerase chain reactions (PCR). The PCR products were used for gel electrophoresis and documentation and, DNA sequencing purposes. 1% agarose was used for gel electrophoresis. The gel documentation result is shown in figure 1.

Figure 1

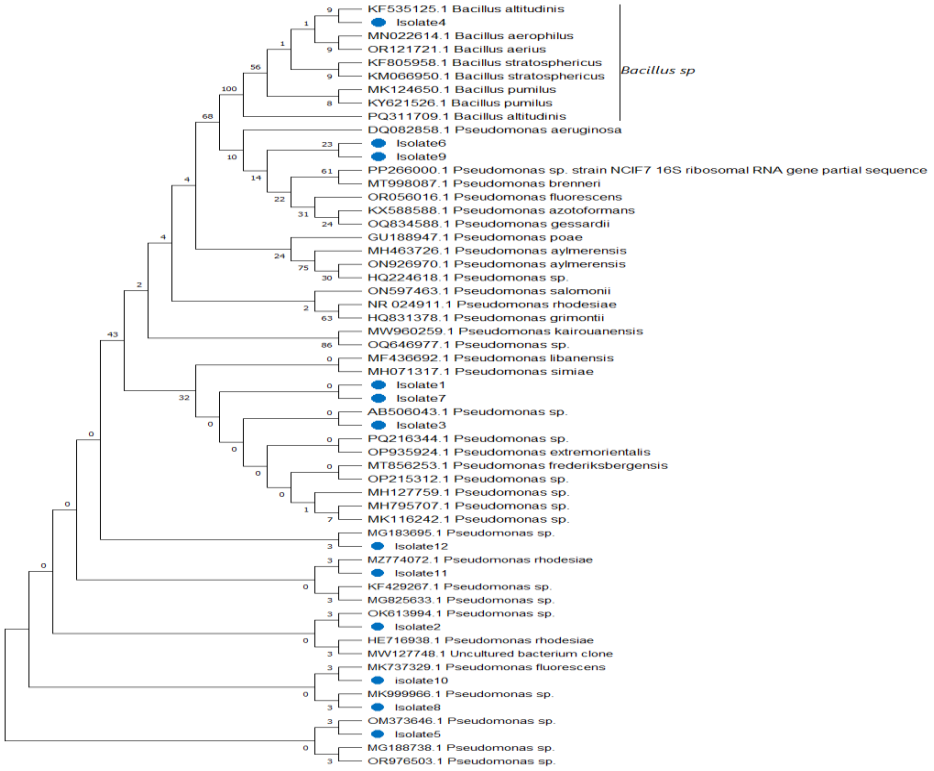
Gel electrophoresis result on 1% Agarose Gel



Species identification (multiple sequence alignment and phylogeny. The NCBI BLASTn nucleotide analysis result of the contigs derived from the 12 PGPR are shown in figure 2 and table 4. The phylogenetic tree which was constructed based on the DNA sequence is indicated in figure 2.

Figure 2

Phylogenetic tree based on the consensus DNA Sequence



The identified PGPR belonged to two genera; *Pseudomonas,* and *Bacillus.* Eleven of the isolates were identified to be *Pseudomonas sp.* While the remaining one, that is isolate 4 was identified as *Bacillus sp.* Isolate 1 (PSB3) was identified as *Pseudomonas frederiksbergensis*. Two isolates; Isolate 2 (PSB6) and Isolate 5 (PSB18) were identified as *Pseudomonas rhodesiae*. Two isolates; Isolate 9 (PSB84) and Isolate 10 (PSB104) were identified as *Pseudomonas fluorescence*. One isolate was identified as *Pseudomonas azotoformans.* Five isolates; Isolate 3 (PSB7), Isolate 6 (PSB41), Isolate 7 (PSB80), Isolate 11 (PSB183), and Isolate 12 (PSB185) were identified as genus *Pseudomonas sp*. The rest isolate; Isolate 4 (PSB13) was identified as *Bacillus altitudinis* which falls into the genus *Bacillus*.

Table 4

PGPR species identified using 16S rDNA sequencing along with the in-vitro characterization results

Isolate ID (PSB No.)	Bacteria Species	N_2 Fixation	NH_3 Production	Average PSI	Auxin Production	Plant source
Isolate 1 (PSB3)	*Pseudomonas frederiksbergensis*	√	-	2.2	++	SWR
Isolate 2 (PSB6)	*Pseudomonas rhodesiae*	√	-	1.8	++	SWR
Isolate 3 (PSB7)	*Pseudomonas sp.*	√	-	3	++	SWR
Isolate 4 (PSB13)	*Bacillus altitudinis*	√	-	2.3	+	SWR
Isolate 5 (PSB18)	*Pseudomonas rhodesiae*	√	++	1.9	+	SWR
Isolate 6 (PSB41)	*Pseudomonas sp.*	√	++	3.2	++	CS
Isolate 7 (PSB80)	*Pseudomonas sp.*	√	+	2.7	+	SWR
Isolate 8 (PSB81)	*Pseudomonas azotoformans*	√	++	1.9	+	CS
Isolate 9 (PSB84)	*Pseudomonas fluorescens*	√	-	2.7	+	CS
Isolate 10 (PSB104)	*Pseudomonas fluorescens*	√	-	2	+	SWR
Isolate 11 (PSB183)	*Pseudomonas sp.*	-	++	2.2	++	CS
Isolate 12 (PSB185)	*Pseudomonas sp.*	√	-	2.3	+	CS

***Key**:* +++: Very strong, ++: strong, +: moderate, -: Negative, SWR: Sorghum Wild Relative, CS: Cultivated Sorghum, PSI: Phosphate solubilizing Index

4. Discussion

4.1 Source of PGPR

The bacteria used for this study were sourced from cultivated and wild sorghum varieties. More than half of the isolates (58%) were sourced from sorghum wild relatives and the rest (42%) were isolated from cultivated sorghum. This enhanced the isolation of diversified bacteria with strong plant growth promoting traits. This is in line with the review made by Barriuso et al. (2008), who stated that the rhizosphere of wild populations of plants is optimal source to isolate PGPR.

4.2 Plant Growth Promoting Activities of the Isolates

The P-solubilizing bacteria were thawed, recultured and retested for their P-solubilization ability. Some P-solubilizing bacteria lose their ability of P-solubilization due to the repeated sub-culturing (Rashid et al., 2004; Muleta et al., 2013). Overall, the characterized PGPR identified with diverse growth-promoting traits showed phenotypic diversity in colony characteristics and demonstrated strong abiotic stress tolerance.

The two identified PGPR genera; *Pseudomonas* and *Bacillus,* are well known in enhancing plant growth (Dardanelli et al., 2010). *Pseudomonas* is the most abundant genus in the rhizosphere among the Gram-negative soil bacteria. Some strains of the *Pseudomonas* have known for many years for their PGPR activity (Barriuso et al., 2008; Kumar et al., 2012). Barriuso et al. (2008) stated in their review that ninety-five percent of Gram-positive soil bacteria belong to the genus *Bacillus*. Members of *Bacillus* species are able to form endospores and hence survive under adverse conditions and many reported on their growth promoting activity.

Pseudomonas and *Bacillus* sps show high versatility in their metabolic capacity. These metabolites inhibit growth of other deleterious microorganisms and increase nutrient availability for the plant. A study done by Idris et al. (2009) generated valuable information towards application of plant growth promoting rhizobacteria as alternatives to chemical fertilizers in Ethiopia and South Africa. They also identified *Pseudomonas* and *Bacillus* species that were isolated from cultivated sorghum and grasses.

Phosphate solubilization. Phosphate solubilization was observed in all the 12 isolates which was confirmed by halo zone formation in Pikovskaya's (PKV) medium (Verma & Pal, 2020). The range of P-solubilization index exhibited by the bacteria were between 1.8 to 3.2. The largest P-solubilization index (3.2) was exhibited by isolate 6 (PSB41) which was identified as *Pseudomonas* sp and followed by Isolate 7 (PSB80) and Isolate 9 (PSB84) which were identified as *Pseudomonas* sp. and *Pseudomonas fluorescens,* respectively. They exhibited 2.7 P-solubilization index. The present study also showed that [Isolate 4 (PSB13) [*Bacillus altitudinis*], which was isolated from Sorghum Wild Relative has a P-solubilization index of 2.3. Elfira et al. (2020) showed *Bacillus altitudinis* has biofertilizer and biopesticide activities and its seed quality enhancing effect on Corn (*Zea mays* L).

Auxin production. All of the selected bacteria produced auxin. Five of them were strong auxin producers and the rest (7 isolates) were moderate auxin producers. Most of the

strong auxin producing bacteria were isolated from sorghum wild relatives and belonged to *Pseudomonas* sp. Many studies showed that IAA production by PGPR can vary among different bacterial species and strains (Gusain and Bhandari, 2019).

Nitrogen fixation. All of the selected bacteria were capable of fixing nitrogen except isolate 11 (PSB183). Nitrogen fixing activity is an important criterion for the selection of potential PGPR. Plants achieve their optimal growth when they obtain enough amount of the required nitrogen nutrient which can be available to them by the presence of N-fixing PGPRs (Oo et al., 2020). Isolate 9 (PSB84) and isolate 10 (PSB104)] which were identified as *Pseudomonas fluorescens* showed nitrogen fixing plant growth promoting characteristics. As stated by Manasa et al. (2017), most *Pseudomonas fluorescens* strains show positive PGPR activity and is an effective plant growth promoting bacterium which is comparable to this study.

Ammonia production. The isolates showed diversified ammonia production abilities. Five of them were able to produce ammonia. Two of them showed strong production of ammonia where they converted peptone water from yellow color to brown (Cappuccino & Sherman, 1992). Isolate 8 (PSB81) [*Pseudomonas azotoformans] and* isolate 5 (PSB18) [*Pseudomonas rhodesiae]* were strong ammonia producers. Ye et al. (2022) mentioned that *Pseudomonas rhodesiae* is a strain that promotes plant growth and biocontrol of plants' pathogens.

4.3 Abiotic Stress Tolerance Activities of the Isolates

PGPR plays an important role in alleviating the plants response to stressful conditions (Lone et al., 2023). The identified strains in this study have very strong salinity and drought stress tolerance characteristics. All of the bacteria were able to grow in a nutrient broth containing 5% salt. Two isolates [Isolate 10 (PSB104) and Isolate 11 (PSB183)] were grown in 10% salt concentration. All of them were drought tolerant which had grown on plates containing 5%, 10%, 15%, and 20% Polyethylene glycol (PEG). Example, isolate 1 (PSB3) which was identified as *Pseudomonas frederiksbergensis* has already been proved to possess stress mitigating property which can enhance plant growth under high soil salinity by reducing the emission of ethylene and regulating antioxidant enzymes (Chatterjee et al., 2017). Moreover, isolate 8 (PSB81) which was identified as *Pseudomonas azotoformans* was capable of growing in 5% salinity and drought stress tolerant strain. Ansari et al. (2021) obtained

Pseudomonas azotoformans with multifunctional traits from screening of 50 indigenous isolates. It was drought stress-tolerant PGPR.

5. Conclusion

This study showed that wild relative plants of sorghum, in addition to cultivated sorghum, are good sources of PGPR with a very good potential for bioinoculant development. The two main genera, *Pseudomonas and Bacillus,* identified by 16S rDNA sequencing are well known for possessing plant growth promoting traits. The identified species were *Pseudomonas frederiksbergensis, Pseudomonas fluorescens, Pseudomonas rhodesiae, Pseudomonas azotoformans* and *Bacillus altitudinis.* They showed moderate to strong auxin producing capability, P-solubilizing index (1.8-3.2) and nitrogen fixing ability. All of them were drought tolerant and were also able to grow in 5% salinity. Overall, the identified PGPR exhibited diverse plant growth-promoting traits, showed phenotypic diversity in colony characteristics and demonstrated strong abiotic stress tolerances. These characteristics make them potential candidates for bioinoculant development which is very important in replacing chemical fertilizers that have negative effects on the environment and biodiversity. Particularly, isolate 1 (PSB3), 6 (PSB41), 7 (PSB80) and 9 (PSB84) are very promising and can be potentially used to promote plant growth and sustainable agriculture. Thus, testing them in greenhouse and field trials on various sorghum varieties and agroecology are highly recommended.

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