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Phytobeneficial traits of Rhizobacteria isolated from degraded soil and evaluate their effect in augmentation of Acacia (*Acacia abyssinica* Hochst. Ex Benth) seeds germination

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ABSTRACT: Microbes are an integral component of the soil ecosystem but degraded soil has few native beneficial microbes. This necessitates the characterization of phytobeneficial bacteria having numerous features. The study was initiated to evaluate rhizobacteria enhancing Acacia (*Acacia abyssinica*) seed germination. Isolates were selected based primarily on phosphate solubilization activity and other traits hydrogen cyanide (HCN), phytohormone, hydrolytic enzyme, siderophore, ammonia. Among isolates, 45% each was categorized as high and medium phosphate solubilizers and the amount was found to range from 195 to 373 µg/mL. The highest solubilization index (SI) 7 was recorded for *Acinetobacter* BS-27 and 6 for *Pantoea* BS-38. The maximum P and IAA were produced by *Pseudomonas* FB-49 (373 and 659.07µg/mL), respectively. Isolates with multiple traits were chosen for seed germination. Accordingly, *Agrobacterium* RS-79 and *Pseudomonas* BS-26 showed 100% activity. A 100% seed germination and vigor index (343.33 and 306.67) were observed in *Pseudomonas* BS-26 and FB-49. Degraded soil is considered a source for phosphate solubilizing and other beneficial bacteria with many traits to be used for seed germination assay.

Keywords: Enhancement of germination, Hydrolytic enzymes, Phosphate solubilization, Plant growth promotion

INTRODUCTION

The natural environment is a suitable component for the existence of all living organisms (Widawati, 2018). However, the rise in the human population and climate change disturbed the natural ecosystem and cause soil degradation, reduction in bacterial abundance and their activities. This in turn affects nutrient readiness, soil organic matter content, and plant growth and establishment (Gebhardt, 2015). Degraded soils are characterized by reduced soil fertility mainly phosphorus (Alemayehu Getahun *et al*., 2020a). Water-soluble P is limited due to the high tendency of complex formation in the soil particles (Bindraban *et al*., 2020). Since the earliest time, microbial utilization for plant improvements has been practiced with proven benefit (Bhattacharyya and Jha, 2012). Degraded areas call for nature helping hands via the re-introduction of potential microbes to maintain soil quality and fertility (Dastager *et al*., 2011). The use of soil microbes as biofertilizers helps to maintain soil fertility (Kesaulya *et al*., 2015). Plant growth- promoting rhizobacteria (PGPR) play a pivotal role in the revegetation of degraded habitats (Ansari and Ahmad, 2018). The interesting interaction between plants and PGPR is effective under nutrients deficient environment (Schillaci *et al*., 2019). For instance, PGPR inoculation can reduce the use of synthetic phosphorus fertilizer to 50% without affecting plant growth parameters (Yazdani *et al*., 2009). Land plants form interactions with the nearby microbiota is associated mainly with their ability to release root exudates (Schillaci *et al*., 2019).

Rhizobacteria use both direct (phytohormone production, N_2 fixation, phosphate solubilization, and iron sequestration) and indirect (siderophores production, competition, lytic enzymes, and HCN production) mechanisms (Mehnaz, 2017; Niu *et al*., 2018). These mechanisms can significantly be

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increasing seed germination, seedling growth, root length, and diameter of seedling (Antoun and Kloepper, 2001). The bacterial genera *Pseudomonas*, *Acinetobacter*, *Bacillus,* and others produce siderophores (iron-binding compounds) and make it accessible in usable forms and thus preventing the growth of potentially pathogenic microbes (Sah and Singh, 2015). Phosphate-solubilizing bacteria increase the bioavailability of phosphorus by releasing acid phosphatases and organic acids (Rodrıguez and Fraga, 1999; Schillaci *et al.*, 2019). Thus, applications of *Bacillus*, *Pseudomona*s, and *Actinobacteria* could play plant growth promotion in land plants by providing locked nutrients via their solubilization abilities (Sivasakthi *et al.*, 2014; Shivlata and Satyanarayana, 2017). *Azospirillum*, *Paenibacillus*, *Serratia*, *Klebsiella*, *Enterobacter*, and *Agrobacterium* have also been revealed to enhance crop production. Their ability to either mobilize mineral or organic bound nutrients from the pedosphere or to fix atmospheric N_2 and make it available to the plants, is a crucial feature in their application (Haghighi *et al.*, 2011; Saharan and Nehra, 2011; Benedetto *et al*., 2017). The application of beneficial bacteria increased root and shoot biomass in several agriculturally and ecologically essential plants (Khambani *et al*., 2019).

Seed dormancy affects both seed germination and subsequent growth of seedlings by causing a sizeable reduction in yield loss (Leubner-Metzger, 2006). One approach used to break dormancy in seeds and increase germination is inoculation with PGPR. PGPR application increase overall plant growth performance including grain, and fruit yields (Pliego *et al*., 2011). *Pseudomonas*, *Bacillus*, *Enterobacter*, *Azospirillum,* and *Acinetobacter* increased enormously seeds germination and seedlings vigor (Munees Ahemad and Mulugeta Kibret, 2014). *Pseudomonas* and *Bacillus* sp., are best for seed germination and growth promotion of multipurpose trees like *Eucalyptus cloeziana* and *E. grandis* (Mafia *et al*., 2009). They produce phytohormones that have a known positive effect on seed germination and seedlings growth in *Acacia senegal* and *Prosopis cineraria* and can fasten the rehabilitation of degraded habitats (Sunil *et al*., 2011). This indicates that PGPR exploitation as biofertilizers requires screening for multiple traits to enhance adaptation of acacia to nutrients and water-deficient habitats. There is a huge land degradation problem in Ethiopia. Hence, it needs urgent interventions to curb this problem. One strategy is rehabilitation via the inoculations of phytobeneficial soil bacteria with multiple plant growth promoting traits. Degraded soils are thought to be a possible source of phytobeneficial bacteria that could promote plant growth. The study was initiated to *in vitro* screening of PGPR and assess the application of rhizobacteria to improve seed dormancy and growth promotion in acacia seedlings.

MATERIALS AND METHODS

Rhizobacteria culture preparations

Rhizobacteria were isolated from the highly degraded soil of North Shewa Zone, Oromia National Regional State, Ethiopia from September 2017 to September 2018. The reason of isolating the microbes from degraded land is for the purpose of rehabilitation using these microbes as a potential biofertilizer. Prior study on the physicochemical analysis soil in the study area (Alemayehu *et al*. (2020a) found that the nutritional status is below the standard. The site is located at 9˚08′ 52″ N and 38˚56′ 13" E with an altitude of 3100 meters above sea level. Composite soil samples were collected from dominant plants Acacia (50 rhizospheres) and Junipers (25 rhizospheres) and brought to Addis Ababa University Applied Microbiology Laboratory (Tang *et al*., 2020). Soil samples were processed within 24 h and a tenfold dilution was made. About 100 µL from appropriate dilution was spread on (Nutrient agar and King's B agar, HiMedia, India) Tang *et al.* (2020) and incubated for 24-48 h at 28±2˚C (Gorsuch *et al*., 2019). Totally 250 bacterial isolates were screened from the mentioned plant rhizospheres. The performances of (n=80) were critically evaluated for plant growth-promoting actions. Isolates were submitted and deposited to GenBank and accession numbers were obtained. Since phosphorus (P) is low (18.9 ppm) in the study area (Alemayehu Getahun *et al*., 2020a), potential phosphate solubilizing PGPR isolates were selected for multiple PGP traits analysis. For each strain and the subsequent activities to be performed, overnight broth cultures (100 µL inoculum with approximately 1 x 10^8 CFU/ mL) were used. All isolates were preserved in 40% glycerol at -20°C.

In vitro Screening of PGPR

Production of Ammonia

Production of ammonia by the isolates was done in peptone water (5 mL) and incubated at 30°C for 4 days. Then 1 mL of Nessler's reagent was added to each tube (Cappuccino and Sherman, 1992). Yellow to brown color development indicates ammonia production.

Tricalcium phosphate solubilization

Primary phosphate solubilizing activities of selected 73 bacteria isolates were carried out by allowing the bacteria to grow in selective medium i.e. Pikovskaya's agar. The advent of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria (Chakraborty *et al.*, 2013). Phosphate solubilization index (PSI) was calculated following a standard method Premono *et al.* (1996):

$$PSI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

The solubilization scale was formulated based on Silva Filho and Vidor (2000). Accordingly, PSI values less than 1.0 is very low (VLS), between 1.0 and 2.0 is low (LS), between 2.0 and 3.0 is medium (MS), whereas PSI greater than 3.0 is rated as high solubilizers (HS). The quantitative estimation of solubilized P was done using the solvent extraction of vanadomolybdophosphoric acid yellow color method with TCP (Pande *et al.*, 2017). The amount of phosphorus was deduced from the standard curve by plotting absorbance at 430 nm vs concentration of P (Pande *et al.*, 2017).

Indole-3-Acetic Acid (IAA) Production

Spectrophotometric estimation of IAA was performed as per the method of Goswami *et al.* (2014a). Forty-eight-hour old culture suspension in the presence of 500 µg/mL of L-tryptophan at 30 °C was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1mL 0.5M $FeCl_3$ solution). The development of pink color indicates IAA production, and the optical density (OD) at 530 nm was read by spectrophotometer. The concentration of IAA produced was estimated against a standard curve of IAA in the range of 0-100 µg/mL (Goswami *et al.*, 2014a).

HCN production

Production of HCN was measured by the qualitative method (Sadasivam and Manickam, 1992). Bacterial cultures of 25 µL were inoculated into 5 mL of nutrient broth supplemented with 4.4 g L^{-1} of glycine. Filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3 in 1 L distilled H_2O) were inserted in half of the vials and tightened with a screw cap. Vials were sealed with parafilm and incubated for 72 h in a mechanical shaker (Dinesh *et al.*, 2015). Production of HCN was indicated by the change in color of the filter paper strips from yellow to brown. The intensity of the color was recorded visually.

Starch hydrolysis and Gelatinase test

Starch hydrolysis was done on starch agar plates. The hydrolysis ability of the isolates was confirmed by clear halo zone around the culture after flooding with iodine solution (Shahzad *et al.*, 2012). Gelatinase test was performed on gelatin agar medium. Gelatin containing tubes were incubated for 4 to 7 days at 30°C (Goswami *et al.*, 2014b). Then the tubes were refrigerated at 4°C for half an hour and if gelatinase is present, the liquid medium was failed to solidify upon refrigeration (Shahzad *et al.*, 2012).

Protease, Cellulase, Lipase and Chitinase activity

Protease production was performed on skim milk agar (skim milk 15, yeast extract 0.5, agar 9.13) in g/L. The formation of clear zone indicates protease action (Geetha *et al.*, 2014). Each bacterial isolate was inoculated on carboxymethyl cellulose (CMC) agar plates. The clear zone formed by the isolates indicates their cellulase activity after flooded with iodine (Khianngam *et al.*, 2014). The bacterial isolates were spot inoculated on lipase medium. The precipitation of bacteria shows lipase action (Kumari *et al.*, 2010). The production of chitinase was performed on colloidal chitin agar (CCA) for 5 to7 days at 30°C (Bansode and Bajekal, 2006). The formation of halo zone following iodine flooding was considered as chitinase positive.

Siderophores production

Siderophore production ability of isolates was detected on Fiss Minimal Medium with the following compositions in g/L (5.03 KH_2PO_4, 5.03 L-asparagine, 5.0 glucose), 40 mg/L, $MgSO_4$ and

500µg/L $ZnCl_2$, 139µg/L $FeSO_4$ was added to the final medium containing 0.5 µM iron) (Goswami *et al.* (2014). After 48 h of incubation, the cultures were centrifuged at 1000xg for 15 min and the supernatant was examined for siderophore production using $FeCl_3$ test (Jalal and van der Helm, 2017). Thus, 0.5 mL of culture filtrate was mixed with 0.5 mL of 2% aqueous $FeCl_3$ solution. The appearance of reddish-brown or orange color was an indication for siderophore production.

Germination assays of Acacia seeds

The purpose of acacia seeds germination assay with PGPR is to break dormancy, determine seed quality, and fasten the tree growth for future rehabilitation purpose. Acacia seeds germination assay was done for 15 selected bacterial isolates before the pot experiment (Tang *et al.*, 2020). *A. abyssinica* seeds were collected from North Shewa Zone, Fiche. Seed sterilization was done following Oyebanji *et al.* (2009). Bacterial seed priming was done according to Shiferaw Demissie *et al.* (2013). Seven seeds of each treatment were kept equidistant in sterilized Petri plates containing moist filter paper and cotton. The treatments were arranged in Complete Randomized Design (CBD) in triplicate and then incubated at 28 ±2°C for 7 days. Seeds germination and percent seedlings emergence were calculated using the following formula Mia *et al.* (2012):

$$\text{Germination Percentage (GP) \%} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

Then, radicle and plumule length of germinated seeds were taken up to 7 days (Gholami *et al.*, 2009; Shiferaw Demissie *et al.*, 2013). The seed vigor index (VI) is calculated by determining the germination percentage and the total seedling length (mm) (radicle plus Plumule length) of the same seed lot.

$$\text{SeedVigor index (SVI)} = \text{\% germination} \times \text{Mean total plant length}$$

Genetic Characterization

DNA extraction and 16SrRNA Sequence Analysis

Eighty drought stress tolerant PGPR isolates (n=80) Alemayehu Getahun *et al.* (2020b) were selected and prepared for DNA extraction using DNeasy Blood & Tissue kit (QIAGEN®, Germany) (Chaiharn and Lumyong, 2009; Kasim *et al.*, 2016). The16S rRNA gene was amplified using fD1 (5′-AGAGTTTGA TCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) (Kasim *et al.*, 2016). The quality of purified (with PureLink® Quick PCR Purification Kit) PCR products was checked with 1kb size marker using electrophoresis on 1.5% agarose gel (Kasim *et al.*, 2016). The amplified samples were sequenced Paulitsch *et al.* (2019) and related by NCBI website (www.ncbi.nlm.nih.gov). Phylogenetic trees were produced using the neighbor-joining method with mega 7.0.2 software (Kumar *et al.*, 2016). GenBank and the accession numbers were received.

Data Analysis

The means and standard deviations of the data were calculated. Three replicates were used for each experiment. Mean significant variations among experimental measurements were done by using ANOVA. Mean separation between treatments were done by Duncan's Multiple Range (DMR) at $p \leq 0.05$ using SAS Ver. 9.0 software for the analysis. Phylogenetic tree was constructed by using Mega 7 software version 7.0.2 (Kasim *et al.*, 2016; Kumar *et al.*, 2016).

RESULTS

In vitro Growth Promotion (PGP) features

Totally 73 rhizobacteria showed growth promoting activities. All the isolates had 49-100% plant growth-promoting traits (Table 1). Remarkably, 38(52%) of PGPR solubilized phosphate with different solubilization index, and these isolates were further screened for other PGP traits.

Phosphate Solubilization

A total of 38 isolates solubilized phosphate as indicated by a clear zone around bacterial colonies (Table 1). Based on the solubilization index (SI), four distinct groups of PS isolates were identified as high solubilizers (HS), medium solubilizers (ms), low solubilizers (LS), and very low solubilizers (VLS) (Figure 1).

Table 1. Qualitative and quantitative phosphate solubilizing (PS) potential of PGPR strains isolated from degraded soil after 8 days of incubation. HS- high solubilizers, MS- medium solubilizers, LS- low solubilizers and VLS- very low solubilizers.

S. No.	Isolates	SI	Scale of PS	Concentration of P µg/mL
1	*Bacillus* sp. Strain BS-22	3.6±0.68^{c-f}	HS	354.85±4^{d}
2	*Bacillus* sp. Strain BS-40	2.7±0.36^{h-k}	MS	323.03±3.5^{e}
3	*Bacillus* sp. Strain BS-47	3±0.36^{f-k}	MS	303.33±2^{f}
4	*Enterococcus* sp. Strain PS-4	2.8±0.53^{d-i}	MS	301.82±4^{f}
5	*Enterococcus* sp. Strain PS-5	3.33±0.46^{d-i}	HS	353.33±3.5^{d}
6	*Enterococcus* sp. Strain PS-9	2.6±0.3^{i-k}	MS	204.85±3^{e}
7	*Paenibacillus* sp. Strain BS-30	2.6±0.53^{i-k}	MS	321.52±4^{e}
8	*Agrobacterium* sp. Strain RS-79	3±0.2^{e-i}	MS	326.06±3^{e}
9	*Ochrobactrum* sp. Strain RS-67	3±0.36^{e-i}	MS	359.39±1^{cd}
10	*Ochrobactrum* sp. Strain RS-70	3±0.17^{e-i}	MS	320.00±2^{e}
11	*Acinetobacter* sp. Stain BS-27	7±0.35^{a}	HS	350.30±2.5^{d}
12	*Pseudomonas* sp. Strain BS-19	3.34±0.4^{d-i}	HS	368.48±2.5^{ab}
13	*Pseudomonas* sp. Strain BS-21	3.33±0.55^{d-i}	HS	351.82±6^{d}
14	*Pseudomonas* sp. Strain BS-23	3.4±0.53^{e-h}	HS	323.03±4^{e}
15	*Pseudomonas* sp. Strain BS-24	3.66±0.38^{c-f}	HS	195.76±2^{n}
16	*Pseudomonas* sp. Strain BS-26	3±0.62^{e-k}	MS	213.94±4^{l}
17	*Pseudomonas* sp. Strain BS-28	4.25±0.2^{c}	HS	353.33±2.5^{d}
18	*Pseudomonas* sp. Strain BS-52	3.2±0.46^{e-j}	HS	368.48±2^{ab}
19	*Pseudomonas* sp. Strain BS-53	3±0.45^{e-i}	MS	288.18±2^{g}
20	*Pseudomonas* sp. Strain RS-75	2.86±0.36^{f-k}	MS	262.42±2.5^{i}
21	*Pseudomonas* sp. Strain FB-49	3.4±0.2^{c-g}	HS	373.03±3^{a}
22	*Klebsiella* sp. Strain PS-1	3.66±0.15^{c-e}	HS	201.82±1^{mn}
23	*Klebsiella* sp. Strain PS-3	3±0.36^{e-k}	MS	198.79±2^{mn}
24	*Morganella* sp. Strain PS-12	4±0.36^{cd}	HS	363.94±3^{bc}
25	*Pantoea* sp. Strain BS-48	3.4±0.36^{e-h}	HS	303.33±2^{f}
26	*Pantoea* sp. Strain BS-35	2.5±0.2^{jk}	MS	257.88±3^{ij}
27	*Pantoea* sp. Strain BS-38	6±0.2^{b}	HS	363.94±2.5^{bc}
28	*Serratia* sp. Strain BS-20	3±0.3^{e-k}	MS	329.09±2.5^{k}
29	*Serratia* sp. Strain BS-42	2.4±0.1^{k}	MS	303.33±2^{f}
30	*Serratia* sp. Strain PS-54	3.5±0.5^{c-g}	HS	289.70±3^{g}
31	*Serratia* sp. Strain RS-65	2.5±0.2^{jk}	MS	285.15±2^{g}
32	*Serratia* sp. Strain RS-73	3.66±0.12^{c-f}	HS	273.03±3^{h}
33	*Serratia* sp. Strain RS- 57	4±0.46^{cd}	HS	351.82±2.5^{j}
34	*Serratia* sp. Strain RS-64	3±0.23^{e-j}	MS	300.30±2.5^{f}

Values are tabulated as means ± the standard deviation (SD), n=3. The same letters within row are not significant at (p ≤0.05). SI= solubilization index

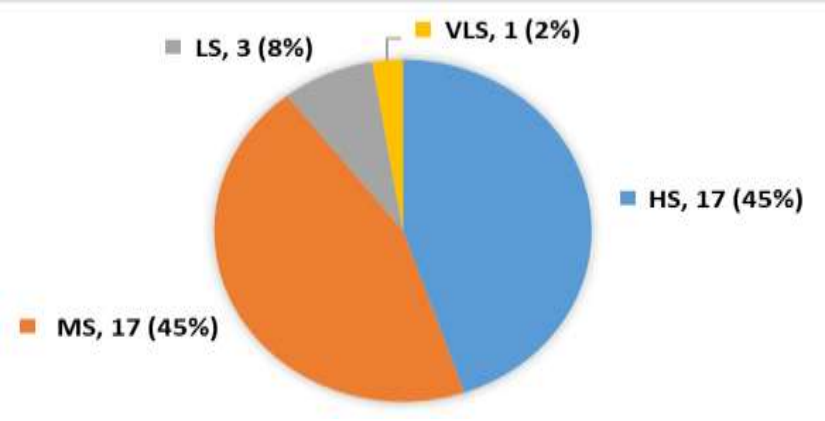

Figure 1. Percentage phosphate solubilization categories of PGPR strains based on their SI values.LS- Low solubilizers, VLS- very low solubilizers, MS- medium solubilizers, HS- high solubilizers.

Seventeen isolates were classified as HS with SI values >3 and 17 isolates were classified as MS with SI values between 2.1 and 3. Of the remaining, 3 strains were categorized as LS with SI values between 1 and 1.9, while 1 strain showed VLS <1 SI. *Acinetobacter* isolate BS-27 showed the highest SI (7) followed by *Pantoea* isolate BS-38 with SI (6), while *Morganella* strain PS-6 had the lowest solubilization (0.82) SI. The amount of solubilized phosphate was found to range from 195.76 - 373.03 µg/mL. The highest P solubilization was measured in strain FB-49 (373.76 µg/ml) followed by BS-19 (368.48 µg/mL) and BS-52 (368.48 µg/mL) Table 1.

Of 73 isolates screened, 49 produced IAA in the medium amended with tryptophan. Maximum IAA production was recorded in *Pseudomonas* strain FB-49 (659.07µg/mL; Table 2). *Morganella* strain PS-6 and ps-18 also produced a significant amount of IAA (533.15 µg/mL and 514.63 µg/mL, respectively) but could not solubilize phosphorus. The lowest quantity of IAA was observed in *Serratia* strain RS- 62 (18.33 µg/mL; Table 2). A positive correlation is observed between absorbance reading and the quantity of IAA produced among each isolate i.e., the highest OD at 530 nm in FB-49 is 1.78 and the quantity of IAA produced is (659.07µg/mL.

Indole-3-Acetic Acid (IAA) Production

Table 2. Concentration of IAA produced by top twelve selected potential PGPR strains isolated from degraded soil.

S. No.	Isolates	Absorbance (OD) 530 nm	Concentration of IAA (µg/ml)
1	*Bacillus* sp. Strain BS-22	0.63	$233.15\pm0.033^{l\text{-}p}$
2	*Bacillus* sp. Strain BS-40	-	-
3	*Bacillus* sp. Strain BS-47	-	-
4	*Enterococcus* sp. Strain PS-4	1.15	425.74 ± 0.028^{d}
5	*Enterococcus* sp. Strain PS-5	-	-
6	*Enterococcus* sp. Strain PS-9	-	-
7	*Paenibacillus* sp. Strain BS-30	0.17	62.78 ± 0.021^{t}
8	*Agrobacterium* sp. Strain RS-79	0.78	$288.70\pm0.023^{f\text{-}i}$
9	*Ochrobactrum* sp. Strain RS-67	-	-
10	*Ochrobactrum* sp. Strain RS-70	0.25	92.41 ± 0.015^{st}
11	*Acinetobacter* sp. Stain BS-27	0.73	$270.19\pm0.032^{f\text{-}k}$
12	*Pseudomonas* sp. Strain BS-19	0.97	359.07 ± 0.021^{e}
13	*Pseudomonas* sp. Strain BS-21	-	-
14	*Pseudomonas* sp. Strain BS-23	0.95	351.67 ± 0.020^{e}
15	*Pseudomonas* sp. Strain BS-24	0.57	$210.93\pm0.018^{n\text{-}q}$
16	*Pseudomonas* sp. Strain BS-26	0.69	$255.37\pm0.028^{i\text{-}m}$
17	*Pseudomonas* sp. Strain BS-28	0.75	$277.59\pm0.020^{f\text{-}j}$
18	*Pseudomonas* sp. Strain BS-52	-	-
19	*Pseudomonas* sp. Strain BS-53	1.18	436.85 ± 0.031^{d}
20	*Pseudomonas* sp. Strain RS-75	0.71	$262.78\pm0.026^{f\text{-}l}$
21	*Pseudomonas* sp. Strain FB-49	1.78	659.07 ± 0.052^{a}
22	*Klebsiella* sp. Strain PS-1	0.63	$233.15\pm0.028^{k\text{-}o}$
23	*Klebsiella* sp. Strain PS-3	-	-
24	*Morganella* sp. Strain PS-12	0.65	$240.56\pm0.040^{i\text{-}n}$
25	*Pantoea* sp. Strain BS-48	0.81	299.81 ± 0.023^{f}
26	*Pantoea* sp. Strain BS-35	0.48	177.59 ± 0.015^{rq}
27	*Pantoea* sp. Strain BS-38	0.51	$188.70\pm0.018^{p\text{-}q}$
28	*Serratia* sp. Strain BS-20	0.59	$218.33\pm0.025^{m\text{-}p}$
29	*Serratia* sp. Strain BS-42	-	-
30	*Serratia* sp. Strain PS-54	-	-
31	*Serratia* sp. Strain RS-65	0.24	88.70 ± 0.026^{st}
32	*Serratia* sp. Strain RS-73	0.8	296.11 ± 0.034^{fg}
33	*Serratia* sp. Strain RS- 57	-	-
34	*Serratia* sp. Strain RS-64	0.29	107.22 ± 0.058^{s}

Values are tabulated as means ± SD), n=3. The same letter within row are not significant. - = Means the isolates never produce pink color.

Screening for Biocontrol traits

The formation of reddish-brown or orange color indicated that 47 (64.38%) of the 73 isolates could manufacture siderophore (data not shown). However, only 23 (67.64%) of the 34 phosphate-solubilizing bacteria have the capacity to produce siderophore. Similarly, 68% of the isolates produced HCN (Table 3). Ammonia was produced by each and every isolates (100%) (Table 3).

Table 3. Phytobeneficial traits (HCN, ammonia and siderophore production) of PGPR isolates.

S. No.	Isolates	HCN	Ammonia	Siderophore
1	*Bacillus* sp. Strain BS-22	+	+	-
2	*Bacillus* sp. Strain BS-40	-	+	+
3	*Bacillus* sp. Strain BS-47	-	+	+
4	*Enterococcus* sp. Strain PS-4	+	+	+
5	*Enterococcus* sp. Strain PS-5	+	+	-
6	*Enterococcus* sp. Strain PS-9	+	+	+
7	*Paenibacillus* sp. Strain BS-30	-	+	+
8	*Agrobacterium* sp. Strain RS-79	+	+	+
9	*Ochrobactrum* sp. Strain RS-67	+	+	+
10	*Ochrobactrum* sp. Strain RS-70	+	+	+
11	*Acinetobacter* sp. Stain BS-27	+	+	+
12	*Pseudomonas* sp. Strain BS-19	+	+	+
13	*Pseudomonas* sp. Strain BS-21	+	+	+
14	*Pseudomonas* sp. Strain BS-23	+	+	-
15	*Pseudomonas* sp. Strain BS-24	+	+	-
16	*Pseudomonas* sp. Strain BS-26	+	+	+
17	*Pseudomonas* sp. Strain BS-28	-	+	+
18	*Pseudomonas* sp. Strain BS-52	-	+	+
19	*Pseudomonas* sp. Strain BS-53	-	+	-
20	*Pseudomonas* sp. Strain RS-75	-	+	+
21	*Pseudomonas* sp. Strain FB-49	+	+	+
22	*Klebsiella* sp. Strain PS-1	+	+	-
23	*Klebsiella* sp. Strain PS-3	-	+	-
24	*Morganella* sp. Strain PS-12	-	+	+
25	*Pantoea* sp. Strain BS-48	+	+	+
26	*Pantoea* sp. Strain BS-35	+	+	+
27	*Pantoea* sp. Strain BS-38	+	+	-
28	*Serratia* sp. Strain BS-20	+	+	-
29	*Serratia* sp. Strain BS-42	-	+	+
30	*Serratia* sp. Strain PS-54	+	+	-
31	*Serratia* sp. Strain RS-65	+	+	+
32	*Serratia* sp. Strain RS-73	+	+	+
33	*Serratia* sp. Strain RS- 57	+	+	-
34	*Serratia* sp. Strain RS-64	+	+	+

HCN = hydrogen cyanide, – = non producers, + = producers for the tested traits.

Screening for potential lytic enzymes production

Of the 34 PS isolates, 14 (41.18%) produced chitinase (Table 4). *Enterococcus* sp. PS -5 exhibits a 4.75 mm maximal chitin degradation halo zone diameter while the lowest halo zone 1.2 mm is found in *Enterococcus* sp. PS -4. Similar to this, 30 (88.23%) isolates developed halo zones on skim milk agar, with *Bacillus* sp. BS-22 forming the largest (4.52 mm) and *Acinetobacter* sp. BS-27 forming the smallest (2.04 mm) protein degradation halo zones. Also, 27 (79.41%) and 26 (76.47%) isolates were found to produce cellulase and lipase, respectively (Table 4). Additionally, gelatinase and amylase were both produced similarly by 19 (55.88%) isolates.

Table 4. Hydrolytic enzymes activities of the selected rhizobacteria isolated from degraded soil.

S. No	Strains	Chitinase activity halo zone	Proteinase activity	Cellulase activity	Lipase activity	Gelatin liquefaction	starch hydrolysis
1	*Bacillus* sp. BS-22	3.73±0.42^{c-f}	4.52±0.12	+	+	-	+
2	*Bacillus* sp. BS-40	2.5±0.5^{jk}	3.36±0.09	-	-	-	-
3	*Bacillus* sp. BS-47	-	-	+	+	+	-
4	*Enterococcus* sp. PS-4	1.2±0.21^{l}	2.65±0.17	+	-	+	-
5	*Enterococcus* sp. PS-5	4.75±0.32^{a}	3.9±0.50	+	+	+	+
6	*Enterococcus* sp. PS-9	-	2.44±0.72	+	+	+	+
7	*Paenibacillus* sp. BS-30	-	2.61±0.44	-	-	-	-
8	*Agrobacterium* sp. RS-79	3±0.36^{g-j}	2.47±0.21	+	+	+	+
9	*Ochrobactrum* sp. RS-67	3.67±0.40^{c-g}	3.34±0.13	-	+	+	-
10	*Ochrobactrum* sp. RS-70	-	2.47±0.11	+	+	+	+
11	*Acinetobacter* sp. BS-27	3.67±0.32^{c-g}	2.04±0.14	+	-	-	+
12	*Pseudomonas* sp. BS-19	-	2.33±0.53	+	+	-	+
13	*Pseudomonas* sp. BS-21	-	2.68±0.65	+	+	-	-
14	*Pseudomonas* sp. BS-23	-	2.61±0.27	+	+	-	-
15	*Pseudomonas* sp. BS-24	-	2.53±0.16	+	+	+	-
16	*Pseudomonas* sp. BS-26	3±0.3^{g-j}	2.15±0.91	+	+	+	+
17	*Pseudomonas* sp. BS-28	3.87±0.31^{b-e}	2.74±0.18	+	+	-	+
18	*Pseudomonas* sp. BS-52	-	2.66±0.77	+	-	+	+
19	*Pseudomonas* sp. BS-53	-	2.26±0.54	-	+	+	-
20	*Pseudomonas* sp. RS-75	-	3.07±0.04	+	+	+	-
21	*Pseudomonas* sp. FB-49	3.9±0.2^{b-d}	3.45±0.29	+	+	+	-
22	*Klebsiella* sp. PS-1	-	-	+	+	+	+
23	*Klebsiella* sp. PS-3	-	-	+	+	-	+
24	*Morganella* sp. PS-12	-	2.49±0.34	+	+	-	-
25	*Pantoea* sp. BS-48	3.5±0.36^{d-h}	3.31±0.03	-	-	-	+
26	*Pantoea* sp. BS-35	-	2.68±0.41	+	+	-	+
27	*Pantoea* sp. BS-38	-	2.71±0.98	-	-	+	-
28	*Serratia* sp. BS-20	3.5±0.56^{d-h}	2.38±0.07	+	+	+	+
29	*Serratia* sp. BS-42	3.5±0.58^{d-h}	2.57±0.35	-	+	-	-
30	*Serratia* sp. PS-54	-	2.35±0.51	+	+	-	+
31	*Serratia* sp. RS-65	-	2.27±0.82	+	+	+	+
32	*Serratia* sp. RS-73	2.25±0.21^{k}	2.52±0.66	+	+	+	-
33	*Serratia* sp. RS- 57	-	-	+	-	-	+
34	*Serratia* sp. RS-64	-	2.79±0.22	+	+	+	+

Data are mean ± SD, (n =3). The same letter(s) within a row is not significant. - = non producers, + = producers for the tested traits

Multiple Plant Growth Promoting Traits

Isolates RS-79 and BS-26 were found to be the two most efficient PGPR which responded to all the 12 (100%) PGP traits (Table 5). Next to this, RS-64, rs-73, RS-65, RS-70, FB-49 and BS-20 isolates showed better MPGP traits (Table 5). Four (11.76%) isolates BS-40, BS-30, PS-3 and RS-57 each possess the smallest (50%) MPGP traits.

Phylogenetic Tree Analysis

Of the 34 phosphate solubilizing isolates, 16 isolates showed high phosphate solubilizing (HS) abilities. Their phylogenetic relationships with other closely related species was indicated in a phylogenetic tree made for 4 gram-positive and 12 gram-negative potential rhizobacteria (Figure 2 and Figure 3).

Table 5. Multiple PGP traits of the 34 phosphate solubilizing bacteria.

Isolates	Multiple PGP traits												
	PS.	IAA	HCN	SP	Chitinase	Protease	CMC	Lipase	Gelatinase	Starch	Ammonia	N_2 fixation	Total %
BS-22	+	+	+	-	+	+	+	+	-	+	+	+	83
BS-40	+	-	-	+	+	+	-	-	-	-	+	+	50
BS-47	+	-	-	+	-	-	+	+	+	-	+	+	58
PS-4	+	+	+	+	+	+	+	-	+	-	+	+	83
PS-5	+	-	+	-	+	+	+	+	+	+	+	+	83
PS-9	+	-	+	+	-	+	+	+	+	+	+	+	83
BS-30	+	+	-	+	-	+	-	-	-	-	+	+	50
RS-79	+	+	+	+	+	+	+	+	+	+	+	+	100
RS-67	+	-	+	+	+	+	-	+	+	-	+	+	75
RS-70	+	+	+	+	-	+	+	+	+	+	+	+	92
BS-27	+	+	+	+	+	+	+	-	-	+	+	+	83
BS-19	+	+	+	+	-	+	+	+	-	+	+	+	83
BS-21	+	-	+	+	-	+	+	+	-	-	+	+	67
BS-23	+	+	+	-	-	+	+	+	-	-	+	+	67
BS-24	+	+	+	-	-	+	+	+	+	-	+	+	75
BS-26	+	+	+	+	+	+	+	+	+	+	+	+	100
BS-28	+	+	-	+	+	+	+	+	-	+	+	+	83
BS-52	+	-	-	+	-	+	+	-	+	+	+	+	67
BS-53	+	+	-	-	-	+	-	+	+	-	+	+	58
RS-75	+	+	+	+	-	+	+	+	+	-	+	+	75
FB-49	+	+	+	+	+	+	+	+	+	-	+	+	92
PS-1	+	+	-	-	-	-	+	+	+	+	+	+	75
PS-3	+	-	-	-	-	-	+	+	-	+	+	+	50
PS-12	+	+	+	+	-	+	+	+	-	-	+	+	67
BS-48	+	+	+	+	+	+	-	-	-	+	+	+	75
BS-35	+	+	+	+	-	+	+	+	-	+	+	+	83
BS-38	+	+	+	-	-	+	-	-	+	-	+	+	58
BS-20	+	+	-	-	+	+	+	+	+	+	+	+	92
BS-42	+	-	+	+	+	+	-	+	-	-	+	+	58
PS-54	+	-	+	-	-	+	+	+	-	+	+	+	67
RS-65	+	+	+	+	-	+	+	+	+	+	+	+	92
RS-73	+	+	+	+	+	+	+	+	+	-	+	+	92
RS- 57	+	-	+	-	-	-	+	-	-	+	+	+	50
RS-64	+	+	+	+	-	+	+	+	+	+	+	+	92
%	100	67.6	70.6	67.6	41.2	88.2	79.4	76.5	55.9	52.9	100	100	

PS- phosphate solubilizer, SP- siderophore production, CMC- carboxymethyl cellulose, - = negative (non-producers), + = positive (producers), % = isolates showed positive response

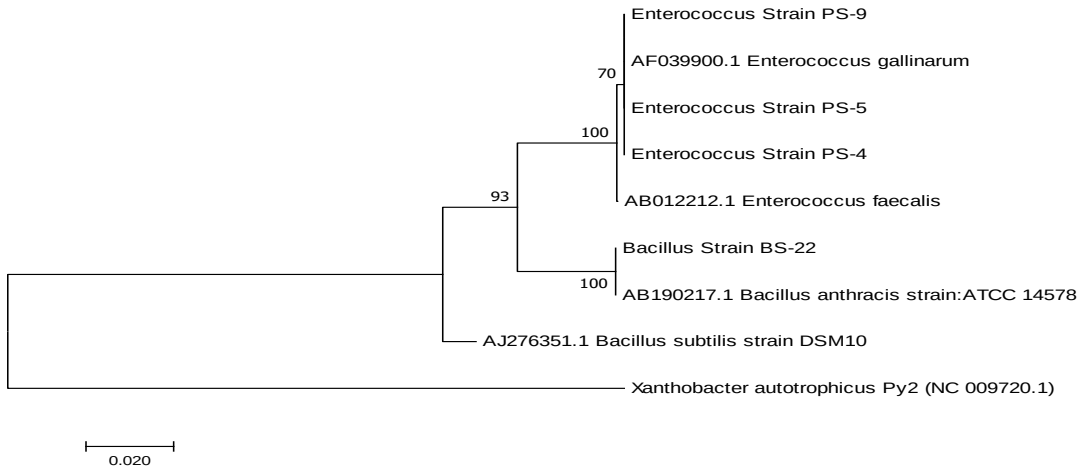

Figure 2. Evolutionary relationships of taxa for gram positive rhizobacteria.

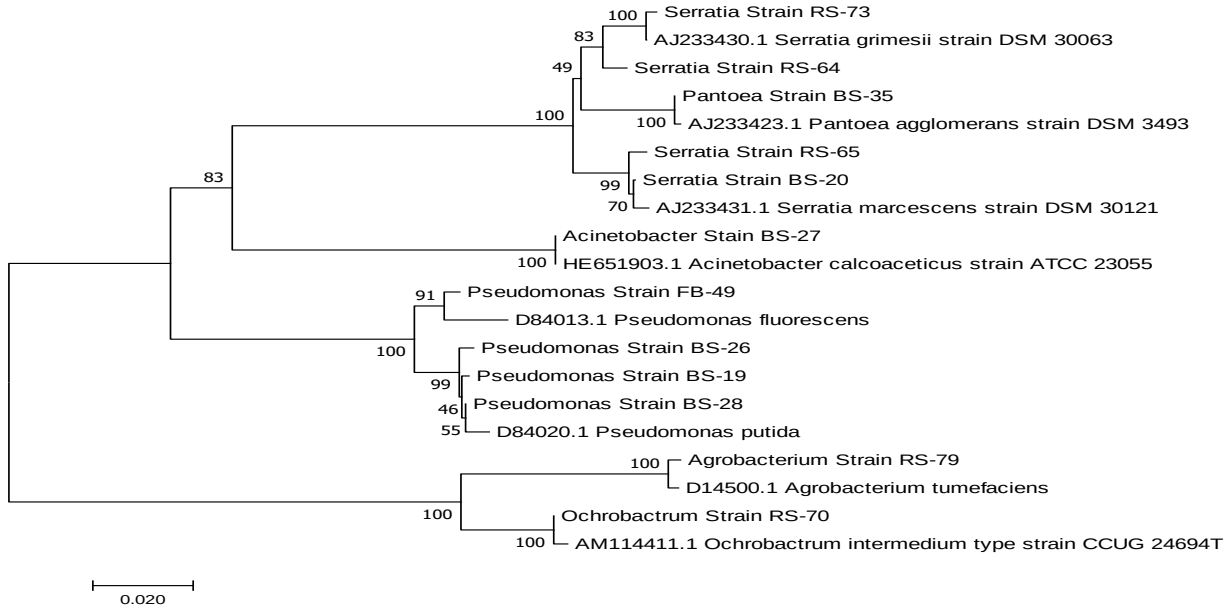

Figure 3. Evolutionary relationships of taxa for gram negative rhizobacteria.

Germination assay

Of the 34 PS isolates, 16 isolates with MPGP traits were examined for seeds germination assay and showed a significant difference in germination percentage, radicle and plumule growth, and seed potency index related to control (Table 6). A 100% seed germination was observed in *Pseudomonas* BS-26 and *Pseudomonas* FB-49 with a similar trend in vigor index (343.33 and 306.67) for these strains, respectively. The vigor index of *Acinetobacter* BS-27 is 176.17 while that of *Pseudomonas* BS-28 is 104.76 ($p \leq 0.05$) compared to the un-inoculated control (38.31). Similarly, the highest plumule and radicle length increments were observed in the inoculated seeds with *Pseudomonas* BS-26 (2.37 and 1.07 cm) compared to un-inoculated once (0.57 and 0.33 cm; Table 6).

Table 6. Effects of bacteria inoculation on acacia seed germination assay.

Isolate	Plumule length (cm)	Radicle length (cm)	Total length (cm)	% Germination	SVI
Pseudomonas BS-19	1.27±0.33^{bcde}	0.50±0.06^{bcde}	1.77^{bcd}	57.14	100.95
Acinetobacter BS-27	1.73±0.15^{abcd}	0.73±0.15^{abc}	2.47^{abc}	71.42	176.17
Ochrobactrum RS-72	1.13±0.44^{cde}	0.33±0.09^{cde}	1.47^{cd}	42.86	62.86
Bacillus BS-22	0.40±0.40^{ef}	0.17±0.17^{de}	0.57^{ef}	14.29	8.10
Serratia RS-65	0.60±0.32^{ef}	0.20±0.12^{de}	0.80^{ef}	28.57	22.86
Enterococcus PS-5	0.60±0.60^{ef}	0.17±0.17^{de}	0.77^{ef}	14.29	10.96
Pseudomonas BS-26	2.37±0.45^{a}	1.07±0.18^{a}	3.43^{a}	100.00	343.33
Pantoea BS-35	0.84±0.09^{def}	0.31±0.14^{de}	1.15^{def}	28.57	32.95
*Serratia*RS-73	0.63±0.35^{ef}	0.23±0.15^{de}	0.87^{ef}	28.57	24.76
Pseudomonas BS-28	1.27±0.38^{bcde}	0.57±0.22^{bcd}	1.83^{bc}	57.14	104.76
Serratia BS-20	1.23±0.15^{bcde}	0.53±0.29^{bcd}	1.77^{bcd}	57.14	100.95
Agrobacterium RS-79	0.93±0.47^{cdef}	0.50±0.26^{bcde}	1.43^{cd}	14.29	20.48
Enterococcus PS-9	0.77±0.23^{ef}	0.43±0.12^{cde}	1.20^{de}	42.86	51.43
Enterococcus PS-4	0.47±0.26^{ef}	0.17±0.09^{de}	0.63^{ef}	28.57	18.09
Pseudomonas FB-49	2.10±0.15^{ab}	0.97±0.15^{a}	3.07^{ab}	100.00	306.67
Serratia RS-64	1.10±0.21^{cdef}	0.37±0.09^{cde}	1.47^{cde}	42.57	62.44
Control	0.57±0.35^{ef}	0.33±0.18^{cde}	0.90^{ef}	42.57	38.31

Values are Mean ± SD, n = 3. The same letters within the row are not significant. SVI = Seed vigor index

The application of PGPR visibly increased the plumule height and radicle length in the germination assay of acacia seeds compared to un-inoculated one (Figure 4).

Figure 4. Effect of PGPR inoculation on acacia seeds germination in inoculated (left) and control (right).

DISCUSSION

This study revealed that *Pseudomonas* and *Serratia* spp. were the dominant genera having high and medium phosphate solubilization potential. There is a significant ($p \leq 0.05$) variation in microbial PSI. These genera might have hydroxyl and carbonyl groups that could chelate the cations bound to phosphate, thereby converting into soluble forms. The production of microbial metabolites and organic acids may result in a clear zone formation an indication of P solubilization (Bashan *et al.*, 2013). A comparable result to our finding has been reported for P solubilization with SI 4 to 7 (Batool and Iqbal, 2018). Availability of phosphorus to the plant for its yield increment is done by phosphate solubilizing rhizobacteria via organic acid production (Karthik *et al.*, 2017).

Our study revealed that 66% of the rhizobacterial isolates produced IAA and the quantity produced showed significant differences ($p \leq 0.05$). The higher IAA production was observed in *Pseudomonas fluorescens* strains FB-49. Variation in IAA production is most probably due to culture condition, growth stage, and substrate availability (Bessai *et al.*, 2022). Differences in the production of IAA among bacterial isolates can be attributed to biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions (Pant and Agrawal, 2014). Previous studies also revealed the production of IAA by *Azotobacter* and *Pseudomonas*

species (Aly *et al*., 2012). The maximum production in *Pseudomonas* species is due to the biochemical mechanism and pathway of IAA synthesis that has remained elusive and versatile (Saharan and Nehra, 2011). Nearly, 50% of IAA producing bacterial isolates were reported by Sahay *et al.* (2012). In another study, strains of *B. pumilus* and *B. licheniformis* produced 445.5 and 335 µg/ml of IAA, respectively (Singh *et al*., 2011). IAA producers are potential plant growth stimulators provided that tryptophan, the precursor of IAA is available and the amount of IAA meets the plant requirement (Cruz and Cadiente, 2016). Different bacterial groups were reported to produce IAA and involve in cell expansion, gene regulation, nutrient uptake, and root proliferation, improved seed germination, and stimulated orchid development (Francis *et al*., 2010; Pandey *et al*., 2015). IAA production facilitates effective nutrient uptake by highly organized root systems (Khambani *et al*., 2019). Qualitative analysis of HCN production is secondary metabolite implicated in plant protection and an indication of cyanogenic bacteria. Thus, the ability to produce HCN by rhizobacteria is considered as a desirable trait to promote plant growth to induce plant resistance to stresses (Ngoma *et al*., 2013).

This study publicized that, degraded soil harbors potential ammonia producing rhizobacteria. All the 34 (100%) phosphate solubilizing isolates showed ammonia producing capabilities. Color development from deep yellow to brown indicates ammonia production. Ammonia is a secondary metabolite produced by rhizobacteria that influences plant growth indirectly and suppress spore germination. PGPR strains that exhibit a strong production of ammonia play a key role in plant growth (Karthik *et al*., 2017). Nitrogen in the form of ammonia is an essential plant nutrient (Karthik *et al*., 2017). *Z. mays* biomass enhancement was previously reported (Marques *et al*., 2010). Plants better able to achieve optimum physical growth when they receive enough nutrients such as fixed nitrogen via nitrogen fixers (Ai'shah *et al*., 2009). Rhizobacteria can convert gaseous nitrogen (N_2) to ammonia (NH_3) to make it available to the host plants and thereby enhance plant growth (Abd El-Aal and Salem, 2018).

Another beneficial trait obtained in this study is siderophores production. The isolate could produce nearly 64%, which was verified by the development of an orange color in the tetrazolium assay. Siderophore secreting bacteria help in the transport of Fe^{3+} (Calvo *et al*., 2014). Siderophore production was previously testified in *P. fluorescens*, *Magnetospirillum magneticum,* and root nodulating bacteria *Rhizobium* (Calugay *et al*., 2003; Ali and Vidhale, 2013;). Siderophores production is important in nitrogen fixation and assimilation processes (Pandey *et al*., 2015). As a chelating agent, siderophore producers deprived off iron from pathogenic fungi because of the higher affinity of bacteria to iron and this facilitates plant growth promotion indirectly (Compant *et al*., 2010). Rhizobacterial strains produce a diverse range of siderophores like catechol, carboxylate, and hydroxamate types that have a higher affinity for iron (Ashish *et al*., 2016; Kumar *et al*., 2017). Siderophore producing *Bacillus* and *Pseudomonas* spp. are described as a possible source for biofertilizer (Khambani *et al*., 2019).

The hydrolytic activities of our isolates were indicated in gelatinase, amylase, protease, chitinase, cellulase, and lipase. The present study revealed that most of the PGPR could involve at least one of the mentioned lytic enzyme activities. The production of the lytic enzyme is a very important mechanism for fungal cell wall lysis. Our rhizobacteria possess multiple plant growth-promoting traits. These potential bacteria could destruct plant pathogens by producing lytic enzymes, and siderophores oriented iron chelation that ultimately exclude the pathogen from the niche (Tariq *et al*., 2017). As previously reported, the production of fungal cell wall degrading enzymes were analyzed, because of lytic enzymes for fungal inhibition. In *P. fluorescens* protease, gelatinase, and cellulase production was recorded more than other enzymes such as amylase and pectinase (Velu, 2013). Several bacterial genera including *Bacillus*, *Enterobacter, Ochrobactrum, Pseudomonas, Serratia*, *Klebsiella,* and *Acinetobacter* are well known antagonistic bacteria to combat plant pathogens (Berg, 2009; Tariq *et al*., 2010). *Pseudomonas* spp. produces lytic enzymes with pronounced antifungal activity, especially proteolytic enzymes that enhance apple and pearl growth (Ruchi *et al*., 2012). *Bacillus* species are also found to secret several hydrolytic enzymes such as protease, chitinase, and cellulase which have a

vital role in plant growth promotion and plant disease management (Kumar and Dap, 2012). Antagonistic bacteria such as *Serratia marcescens* reduces the mycelial network of *Sclerotium rolfsii* by expressing chitinase. The consortia of lytic enzyme-producing rhizobacteria were used in the field by expressing a potent synergistic inhibitory effect against pathogens and promote plant growth (Someya *et al*., 2007). Generally, lytic enzymes could enhance plant growth by reducing deadly pathogens (Tariq *et al*., 2017).

Multiple plant growth promotion PGP traits were confirmed in some of our PGPR under *in vitro* conditions. A single rhizobacteria has been found to show multiple modes of action (Dinesh *et al*., 2015). Of the 34 phosphate solubilizers, 16 of them exhibited greater than 83% multiple PGP traits by revealing at least ten and/or more positive results for the tested traits. These features are supposed to be a crucial reason that the action required for the growth improvement in plants may be a result of synergistic effects (Chaiharn and Lumyong, 2009; Dinesh *et al*., 2015). The production of hydrolytic enzymes by rhizobacteria are a prominent trait of antagonism against many plant pathogens (Karthik *et al*., 2017). The production of lytic enzymes by phytobeneficial bacteria induce exploitation of the cell walls of pathogenic fungi as a carbon source (Brzezinska *et al*., 2014; Karthik *et al*., 2017).

In this study, *Pseudomonas* BS-26 and FB-49 were considered as potential strains in increasing acacia seed germination and vigor index. These might be due to the release of specific enzymes (Nezarat and Gholami, 2009). In another study, the highest seed germination percentage of *Eucalyptus grandis* was caused when inoculated with *Pseudomonas fulva* compared to the control treatment (Mafia *et al*., 2009). Seed germination increment was stated in *Crataegus pseudoheterophylla* Fatemeh *et al*. (2014), *Acacia Senegal* Sunil *et al*. (2011), *Rosa damascene* Kazaz *et al*. (2010) and *Abies* spp. (Zulueta-Rodríguez *et al*., 2015). Moreover, inoculation of *Acacia gerrardii* with *Bacillus subtilis* enhanced germination and resulting in stress adaptation (Egamberdieva *et al*., 2017;). Also, inoculations of nodule forming bacteria play a role in breaking dormancy in acacia (Thrall *et al*., 2008). The germination increment may be due to better synthesis of auxins (Ahemad and Khan, 2012). A similar finding was reported by Patten and Glick (2002). Also, significant promotion in root and shoot vigor could be occurred by the better synthesis of auxins. Vigor index reflects the health of the seedlings and accounts the germination status (Patel and Saraf, 2013). Accordingly, the higher the vigor index the better seedlings quality (Patel and Saraf, 2013). Generally, phytobeneficial rhizobacteria can be considered as an outstanding solution to break seeds dormancy in acacia.

CONCLUSION

This study concluded that plant rhizospheres from degraded soil harbor rhizobacteria with adaptation mechanisms (P-solubilization, HCN and IAA production, ammonia production, hydrolytic enzyme and siderophore production). *Pseudomonas* species are the dominant phosphate solubilizers followed by *Serratia* species. *Klebsiella* PS-2 and *Pseudomonas* FB-49 had the highest seed germination, shoot, and root length, and seed vigor index. There is no previous study on the potential roles of PGPR on acacia seed germination assay. The presence of various PGP traits in the strains may be the possible reason to protect the plant from various stresses.

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