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ORIGINAL ARTICLE

Evaluation of the potential of multi-trait PGPR isolates as inoculants for maize (*Zea mays L.*) growth

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ABSTRACT

Background and Objectives: Plant growth-promoting rhizobacteria (PGPR) with a diverse set of traits can improve crop yield in agriculture. The current study aimed to evaluate the potential of multi-trait PGPR isolates as inoculants for maize *(Zea mays L.)* growth.

Materials and Methods: In this study, 23 bacterial isolates were initially screened from maize plant rhizosphere. Ten isolates (A1–A10) were selected based on N fixation, P and K solubilization and their in vitro specific PGPR traits, such as solubilization of Zn, and Mn, the production of IAA, siderophore, ammonia, and HCN were assayed. Finally, the potential of selected isolates in enhancing the germination, height, shoot collar diameter, shoot fresh and dry weight biomass, and root dry weight of maize were evaluated.

Results: Among the positive-PGPR colonies, the selected isolates demonstrated the better performance of PGPR traits such as highest nitrogen fixation, P, K, Mn, and Zn solubilization, and production of siderophore, HCN, NH3, and IAA. In addition, the maize seed germination and improvement of maize yield in a pot experiment were observed after their treatment by bacterial inoculants. Biochemical characteristics, 16S rDNA amplification, and sequencing demonstrated a high similarity of PGPR isolates to the strains of *Enterobacter, Pantoea*, *Kluyvera*, *Lelliottia*, *Klebsiella*, *Pectobacterium*, and *Stenotrophomonas*.

Conclusion: The findings demonstrated that these strains could prove effective PGPR inoculants for the improvement of maize crops as multiple bio-fertilizers.

Keywords: Bio-fertilizer; Plant growth regulators; Rhizobacteria; Siderophore; *Zea mays*

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INTRODUCTION

Microorganisms have a vital function in agriculture for stimulating the plant's nutrient uptake and growth (1). PGPR play a significant role in plant nutrition by absorption and increasing macro- and micro-nutrient uptake, and may be important for the bio-fertilization of crops (2, 3). The PGPR exerts beneficial effects on plant growth through one or more different mechanisms directly and/or indirectly (4). The direct mechanisms consist of the production of phytohormones (for instance indole acetic acid (IAA), cytokinin, and gibberellins), facilitating the uptake of nutrients by atmospheric nitrogen (N) fixation, solubilization of phosphorus (P), potassium (K), manganese (Mn), and Zinc (Zn), as well as the synthesis of siderophores for iron (Fe) sequestration (5, 6). On the contrary, indirect mechanisms include antagonism against soil-borne plant pathogens by the release of hydrogen cyanide (HCN), and antibiotics (4, 5).

In the recent decades, several bacterial strains like *Rhizobacteria, Pseudomonas, Azotobacter, Bacillus* etc., which are formulated as bio-fertilizers, have been recommended to enhance plant growth (4). Bio-fertilizers have become essential components of modern agriculture, which are developed for reduction of adverse effects of chemical pesticides and inorganic fertilizers as much as possible (7). Bacterial inoculants are able to improve plant germination, increase plant growth, enhance root uptake, and protect plants from abiotic stress and disease (8, 9). Among PGPR, bacteria with multifunctional abilities such as nitrogen fixing, solubilization of P, K, and micronutrients as well as root stimulation are considered to be the main constituents of bio-fertilizers (2).

N, P, and K are the most essential macro-nutrients for plant growth in comparison with other essential nutrients (10, 11). Most of the N is in the form of dinitrogen (N_2), which cannot be absorbed directly by plants and animals. The atmospheric N_2 must be converted into an available form such as ammonia by nitrogen fixing bacteria through a process called nitrogen fixation (12, 13). The PGPR have the capability to convert the unavailable form of P and K into available soluble form, which can be taken up by the plant's roots along with water (14). It is generally accepted that phosphate-solubilizing bacteria (PSB) and potassium-solubilizing bacteria (KSB) solubilize and mobilize the insoluble forms of P and K, respectively, via the production of low molecular weight organic acids or H^+ ions, and phosphatases secretion (14, 15). Iron and Zn are vital micronutrients, which play critical roles in various activities of plants (16). Microorganisms secrete siderophores in response to iron deficiency, which bind with ferric ion and enhance the growth of plants via Fe uptake (1, 17). Zn deficiencies in soils can be accomplished by rhizobacteria through Zn solubilization (18). In addition, the PGPR-induced release of phytohormones play a key role in the plants growth under stressful conditions (19). The IAA production is a relatively common property of the PGPR, which promotes plants root growth, and enhances nutrient uptake (3, 20).

Maize is one of the most important and widespread food crops in world especially in temperate to semi-arid regions. It is a primary source of many micronutrients, which is mostly used for food and livestock feed (21, 22). The enhancement of the number of lateral roots, the root biomass, and the root length in both pot and field trials have been demonstrated with inoculation of maize seeds with PGPR (18, 21, 23). However, few works have reported the bacterial inoculants with multifunctional abilities.

This study aimed to screen PGPR strains, which are capable of nitrogen fixing, and solubilization of P, K (NPK production), secreted siderophores secretion, HCN and IAA, and can effectively solubilize inorganic micronutrients such as Mn and Zn compounds. Then, the capability of improving maize growth was evaluated through PGPR isolates to exhibit the probable effects of these isolates on plant growth and improvement.

MATERIALS AND METHODS

Isolation of bacteria. Seventy-five different soil samples were collected in sterile tins from 5-20 cm around of rhizosphere of maize crops from six maize farm in Iran. The sampling location and chemical analysis of soils are shown in Table 1. The soil samples were conveyed to the laboratory. One gram from each soil sample was added into 100 ml distilled water. After precipitation of solid phase, 1 ml of the supernatant was added to test glass tubes containing 9 ml selective semi-solid N-free medium of the following composition; mannitol 20 g/l, K_2HPO_4 0.8 g/l, KH_2PO_4 0.2 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 6H_2O$ 0.10 g/l, $CaCl_2 \cdot 2H_2O$ 0.05 g/l, $CaCO_3$ 20 g/l, $NaMoO_4 \cdot 2H_2O$ 0.05 g/l, agar 5 g/l (Adjust to pH 7.2).

Table 1. The sampling location and chemical analysis of soils

No.	Sampling location (Iran)	GPS position	Soil Type	pH	Electrical Conductivity (Ds/m)	Elemental composition (mean values of three replicates)			
						N	P	K	Organic carbon %
1	Hamadan Province	34°55'26.0"N 48°31'28.9"E	Loam	8.04	0.9	0.13	9.8	616	1.32
2	Markazi Province	34°09'07.2"N 50°04'39.7"E	Loam	7.43	4.9	0.04	8.9	150	0.41
3	Markazi Province	34°28'36.9"N 50°25'08.8"E	Sandy loam	7.7	4.6	0.02	4	650	0.23
4	Isfahan Province	34°04'28.9"N 51°19'46.7"E	Loam	7.56	5.93	0.11	3.2	393	0.57
5	Isfahan Province	33°20'48.7"N 51°11'13.0"E	Loam	7.73	6.0	0.036	8.14	185	0.42
6	Yazd Province	31°06'32.5"N 53°16'59.0"E	Clay loam	7.49	7.08	0.14	11	168	1.44

Tubes were incubated in shaking incubators at 100 rpm at 28°C for 72 h. Then, the pellicle was sub-cultured on solid N-free medium and incubated for 72-96 h at 28°C and then the isolates were purified.

***In vitro* assessment of PGPR properties: Nitrogen fixation and Nitrogenase activity.** The nitrogen fixation ability of isolates was first assessed by the growth of isolates on solid N-free media. Then, the presence of the *nif* gene in these isolates was determined by a PCR molecular identification using IGK3/DVV primers GCIWTHTAYGGIAARGGIGGIATHGGIAA (24). The acetylene reduction assay (ARA) was applied to evaluate the nitrogenase activity of isolates. Briefly, 100 µl of each bacterial suspension (~10^4 CFU/ml) was inoculated into 5 ml of the N-free broth media in 12 ml vials and incubated at 28ºC for 72 h. Then, 10% of the air of the headspace (7 ml) was changed with pure acetylene gas (C_2H_2) employing a disposable plastic syringe. After 24 h, the rate of ethylene production was assayed with a gas chromatograph (GOW MAC - GM816 model) consisting of a column Porapak N and an H_2-FID detector. The nitrogen fixation rate was expressed as nmoles C_2H_4 h^{-1} $vial^{-1}$ (25).

Phosphate solubilization and phosphatase activity. 20 µl of the bacterial suspensions (~10^4 CFU/ml) as a spot was located on the center of a solid Sperber medium plate composed of 10 g glucose, 0.1 g $CaCl_2$, 0.25 g $MgSO_4.7H_2O$, 0.5 g yeast extract, 15 g agar per liter supplemented with 2.5 g $Ca_3(PO_4)_2$ (TCP/insoluble Pi) at pH: 7.2-7.3 (26). After 2, 4, and 7 days of incubation at 28°C, the P_i solubilizing index (PSI) was determined as the ratio of the diameter of clear halo surrounding the bacterial colony to the diameter of colony. To evaluate the quantitative assessment of the amounts of solubilized P_i from TCP substrate, 100 µl of bacterial suspension (~10^4 CFU/ml) were inoculated into 25 ml Sperber broth medium and incubated at 28°C and 120 rpm. The uninoculated medium was used as a control in each case. At 0, 48, and 96 h, 1000 µl of the medium was centrifuged for 5 min at 13000×g and 290 µl from the supernatant was mixed with 570 µl molybdovanadate reagent and adjusted to 2 ml with ddH_2O. The absorbance of the samples were determined after 10 min at 420 nm in triplicates and the amount of hydrolyzed P_i was calculated by a standard curve (27).

The phosphatase activity of the isolates was also assayed based on intensities of blue-stained colonies on the solid Sperber medium consisting of 50 mg/l 5-bromo-4-choloro-3-indolyl phosphate (BCIP) after 72 h (26).

Potassium solubilization. A spot of the bacterial suspensions (20 µl / ~10^4 CFU/ml) was added on the center of solid Aleksandrov medium including 0.5% glucose, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCO_3$, 0.0005% $FeCl_3$, 0.2% $Ca_3(PO_4)_2$, 0.2% Mica powder,

and 15 g agar (pH: 7.0 to 7.5) and were incubated at 28°C for 7 days. The potassium solubilization index (PSI) including the ratio of the transparent halo diameter to the colony diameter was determined at 2, 4, and 7 days in triplicates. The K-solubilized concentration by isolates was quantified by inoculation of 100 µl of bacterial suspension (~10^4 CFU/ml) into 25 ml of broth Aleksandrov medium and incubation at 28°C at 100 rpm. At 0, 48, and 96 h, 1000 µl of each sample was centrifuged for 25 min at 4500 rpm and the supernatant K was evaluated using the Flame Photometer method (28).

Following the aforementioned steps (NPK assessment), 10 isolates (A1 to A10) with the best performance were selected for further tests.

Auxin and root stimulator production. To determine IAA, bacteria were cultured in 25 ml LB culture medium containing 0.1% (w/v) L-tryptophan and were incubated in shaking incubator at 150 rpm at 28°C for 24 h. Then, samples were centrifuged at 8000×g for 2 min and 1 ml of samples' supernatants were mixed with 2 ml Salkowski reagent (2 ml of 0.5 M $FeCl_3$, and 98 ml of 35% $HClO_4$) and incubated for 30 min at 28 °C. The absorbance of each sample was ultimately determined at 530 nm using a spectrophotometer and the IAA content was determined according to the standard curve of auxin different concentrations (29).

Siderophore production. Siderophore-producing isolates were selected on Chrome azurol S (CAS) agar media by adding 20 µl bacterial suspensions (~10^4 CFU ml^{-1}) on the center of this medium and incubation at 28°C for 72 h. The orange color zones surrounding the colonies were considered as siderophore producing ability of bacteria (18).

Assessments of Zn solubilization. To examine the efficiency of Zn solubilization, a bacterial suspension spot (~10^4 CFU ml^{-1}) was placed on the center of solid mineral salt medium (MSM) containing (g/l) glucose 10 g, $(NH_4)_2SO_4$ 1.0 g, KCl 0.2 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.2 g, and 0.1% insoluble zinc oxide (ZnO) and incubated at 28°C. Then, Zn solubilizing index (ZSI) of each isolate was determined after 2, 4, and 7 days in triplicates based on the ratio of (colony+halo)/colony diameters (18). To determine the hydrolyzed soluble Zn, 100 µl of bacterial suspension (~10^4 CFU ml^{-1}) was inoculated into 100 ml broth MSM medium consist of 0.1% ZnO and incubated at 28°C at 120 rpm shaking speed. After 0, 48, and 96 h, 400 µl of samples were centrifuged for 20 min at 2,000 rpm. Then, the released Zn in the supernatant (ppm Zn) was assayed using atomic absorption spectrophotometry (AAS) (30). A medium without bacterial isolate was used as a control.

Manganese solubilization. To determine Mn solubilization capacity, 20 µl of the bacterial suspensions (~10^4 CFU ml^{-1}) were inoculated at the center of modified Aleksandrov solid medium containing 0.2% MnO_2 and were incubated at 28°C for up to one weeks. The clear halo around bacteria was measured on 2, 4, and 7 days and the Mn solubilization index (MSI) was determined as the ratio of the halo diameter to the colony diameter. To evaluate the solubilized Mn in liquid media, 100 µl of isolates suspension (~10^4 CFU/ml) were added to 100 ml of modified Aleksandrov medium and were incubated in a shaking incubator at 100 rpm at 28°C. In this assay, control was a non-inoculated medium. At 0, 48, and 96 h, 500 µl of this medium were centrifuged at 4500 rpm for 25 min and the Mn content (ppm) in the supernatant was determined using AAS (29).

Ammonia production. The ammonia production capacity was evaluated using the Cappuccino-Sherman method. The appearance of a yellow to dark brown color after addition of Nessler indicator to the bacterial suspensions indicates ammonia production (31). Nessler spectrophotometric method was also employed to estimate the quantitative amount of ammonia. 50 µl of bacterial suspension in liquid LB were inoculated in 10 ml of liquid peptone and the tubes were incubated at 28°C in a shaking incubator at 120 rpm for 72 h. 2 ml of each sample was centrifuged at 10000 rpm for 5 min. 250 µl of the supernatant were mixed with 1670 µl ddH_2O, 40 µl sodium potassium tartrate (0.5 gr/ml), and 40 µl Nessler reagent. The absorbance of each sample was determined at 450 nm and the amount of ammonia was determined based on a standard curve of various concentrations of ammonium sulfate in triplicate.

Hydrogen Cyanide production. To measure HCN production, isolates were firstly cultured on glycine-enriched TSA solid medium (4.4 grams per liter). Filter paper soaked in sodium picrate (picric acid 0.5% and Na_2CO_3 2%) was then placed on the interi-

or surface of the plate lid and parafilm was wrapped around its lid. The plates were incubated for 120 h at 28°C. HCN production capacity was assessed based on alteration in filter paper color from red to dark brown (32).

Molecular identification of the selected isolates. Ten selected isolates with the best performance were recognized by biochemical tests, including Gram staining, H_2S production, citrate utilization, lactose, glucose, and mannitol, as well as the oxidase, catalase, and urease activities. The molecular identification was performed based on 16S rDNA amplification and blasting by universal bacterial primers 27F; (AGAGTTTGATCCTGGCTCAG) and 1492R; (GGTTACCTTGTTACGACTT). Sequences were aligned by BLAST program at http://www.ncbi.nih.gov/blast at the NCBI GenBank database to identify the similarity of the isolates (33).

***In vitro* maize seed germination assay by the selected strains.** The maize seeds were sterilized with 2% sodium hypochlorite solution (2 min) and 70% ethanol (1 min), and then washed with sterile ddH_2O. The seeds were placed under a laminar hood for 30 min to dry. Then, the sterilized seeds were suspended in bacterial suspension (10^7 CFU/mL) for 2 h, whereas the control seeds were soaked in ddH_2O. After soaking, the seeds were placed on autoclaved Whatman filter paper inside a petri dish containing 3 seeds in triplicates and incubated at the growth room for 12 days. The filter paper was daily wet to provide the necessary moisture for seed germination. After incubation, the germination index (number of germinated seeds/total seeds×100) of seeds was evaluated. In addition, root fresh weight, root dry weight, root length, and leaf length were determined after 11 days.

Evaluation of inoculation of maize with PGPR strains at greenhouse experiment. Pot culture experiments were piloted from August 2019 to October 2020 in 4 liters (2.5 Kg) of plastic pots packed with a sterilized mixture of equal volumes of sand and soil. The physico-chemical properties of pre-sowing soil used in the pot experiment are shown in Table 2. A total of fifteen seeds of hybrid maize (*Zea mays* L. CV. single-cross 704) were inoculated with 50 ml of the bacterial suspension containing 10^7-10^8 CFU/ml (1×10^4-10^5 per gram soil) and sown at the same depth in all pots. After the maize germination, three maize seedlings were kept in each pot. The water-treated maize seeds without bacteria inoculum were used for the control treatment. The pots arrangement was performed based on randomized complete block design having six replicates with thirty-one treatments (Table 3). Treatments consisted of three controls in which 0, 50, and 100% chemical fertilizers and no bacteria were used; The other treatment were inoculated with two, three, four, and five bacterial strains as shown in Table 3. Equal doses of N, P, and K (50%) were applied in these pots in the form of urea, superphosphate, and potassium sulfate, respectively. Plants were grown for 2 months under optimum greenhouse conditions: 25 ± 5°C, 8 h of a dark time and 16 h of light, and a humidity about 40-60% and were daily watered with 100 ml of good-quality fresh water. Growth parameters of the plants including, plant germination, plant height, shoot collar diameter, shoot fresh and dry weight biomass, and root dry weight were analyzed.

Statistical analysis. The statistical analysis was performed by using GraphPad Prism 8 (GraphPad Software, Inc.) by one-way analysis of variance (ANOVA) and Tukey's test to determine the significant difference between groups ($P < 0.05$). The ANOVA and Duncan's means comparison was also used to compare the plant growth parameters.

RESULTS

Isolation of PGPR. Out of 6 soil samples, a total of 23 isolates were first screened by growing a semi-solid N-free medium and were coded A1 to A37. Then, in a series of subsequent experiments, 10 isolates showing nitrogen fixing and solubilization of P and K were selected for further PGPR properties investigation.

PGPR traits of selected isolates. Screening results of PGPR traits are represented in Tables 4 and 5, and Figs. 1 and 2. Among 10 selected isolates that were positive for the *nif* gene, five isolates showed nitrogenase activities based on the acetylene reduction method in the range of 1.31 to 16.41 nmol C_2H_4/h/vial while cell numbers were adjusted to 10^7 CFU/ml (Table 4 and Fig. 1a). A qualitative colorimetric assay for phosphatase secretion exhibited that all the selected isolates were positive for phosphatase activities in the

Table 2. Pre-sowing analysis of different parameters of soil used in pot experiment.

Parameter	value	Critical rate	Recommended fertilizer	Amount of fertilizer used (grams/2.5 kg pot)
pH	7.4	-	-	-
Texture	Clay sandy loam	-	-	-
Ec	4.5 dS m^{-1}	-	-	-
Organic matter	0.15 %	2-3%	-	-
Total N	0.013 %	0-2.3%	Urea	1.1 g
P (extractable)	8.6 ppm	<10 ppm	Superphosphate	0.125 g
K (extractable)	76.0	<250 ppm	Potassium sulfate	0.25 g

Table 3. The treatment attributes of pot culture experiments of maize

Treatments	Superphosphate	Urea	Potassium	Selected bacteria
T1	50%	50%	50%	A1+A2+A5+A10
T2	50%	50%	50%	A1+A4+A6
T3	50%	50%	50%	A1+A4+A6+A10
T4	50%	50%	50%	A1+A6
T5	50%	50%	50%	A1+A6+A8+A10
T6	50%	50%	50%	A2+A4+A6
T7	50%	50%	50%	A2+A4+A6+A10
T8	50%	50%	50%	A2+A6+A9
T9	50%	50%	50%	A2+A6+A10
T10	50%	50%	50%	A1+A2+A4+A6+A10
T11	50%	50%	50%	A1+A4+A5+A6
T12	50%	50%	50%	A1+A3
T13	50%	50%	50%	A1+A3+A10
T14	50%	50%	50%	A1+A2+A6
T15	50%	50%	50%	A1+A2+A6+A10
T16	50%	50%	50%	A2+A7
T17	50%	50%	50%	A2+A7+A10
T18	50%	50%	50%	A1+A7
T19	50%	50%	50%	A1+A7+A10
T20	50%	50%	50%	A3+A7
T21	50%	50%	50%	A3+A7+A10
T22	50%	50%	50%	A2+A5
T23	50%	50%	50%	A5+A8+A9+A10
T24	50%	50%	50%	A1+A5
T25	50%	50%	50%	A1+A5+A10
T26	50%	50%	50%	A3+A5
T27	50%	50%	50%	A3+A5+A10
T28	0%	0%	0%	-
T29	50%	50%	50%	-
T30	100%	100%	100%	-
T31	50%	50%	50%	A1+A2+A5

Table 4. Nitrogenase activity and production of HCN, phosphatase, siderophore, IAA, and NH_3 by selected bacterial isolates grown on their respective medium.

Isolates	Growth in the N-free media	Nitrogenase activity (nmol C_2H_4 /h/vial)	HCN (96 h)	Phosphatase (72 h)	Siderophore (72 h)	NH_3 (72 h/ µmol/ml)	IAA (24 h/ µg/ml)
A1	+	-	-	+	+	0.569	8.99 ± 0.2
A2	++	3.339	-	++	-	0.545	15.18 ± 0.07
A3	++	-	-	+	-	0.554	14.03 ± 0.5
A4	+	-	-	+	+	0.589	13.58 ± 0.06
A5	++	1.489	+++	+	+	0.542	13.81 ± 0.05
A6	+	-	-	+	+	0.501	12.23 ± 0.07
A7	++	16.414	-	+	+	0.432	23.66 ± 0.06
A8	+	1.315	+	+	-	0.569	79.19 ± 0.04
A9	++	10.754	+	+	+	0.513	18.09 ± 0.07
A10	+	-	+	+++	+	0.285	11.33 ± 0.05

HCN: hydrogen cyanide; IAA: indole acetic acid

Table 5. The solubilization efficiency of insoluble mineral by isolates in plate assay after 7 days and released soluble form in liquid media (96 h)

Isolates	Solubilization in solid plate assay (7 days)				Released soluble form in liquid media (96 h)			
	Phosphate solubilization index (PSI)	Potassium solubilization index (KSI)	Zinc solubilization index (ZSI)	Manganese solubilization index (MSI)	P (mg/l)	K (mg/l)	Zn (ppm)	Mn (ppm)
A1	2.91 ± 0.07	2.91 ± 0.19	4.22 ± 0	2.61 ± 0.05	2016.41	43.17	112.4	153.36
A2	2.48 ± 0.27	3.09 ± 0.09	3.85 ± 0.05	2.45 ± 0.1	1751.56	12.3	54.70	59.86
A3	3.8 ± 0.07	2.83 ± 0.40	4.03 ± 0.06	2.73 ± 0.13	1467.68	15.38	115.24	143.63
A4	1.12 ± 0.03	1.11 ± 0.01	2.04 ± 0.04	1.11 ± 0.02	345.18	3.08	10.43	17.65
A5	1.42 ± 0.08	1.21 ± 0.04	2.16 ± 0.02	1.25 ± 0.02	571.06	4.92	8.41	26.02
A6	0.95 ± 0.05	1.2 ± 0.09	2.57 ± 0.06	1.11 ± 0.03	343.09	3.69	14.25	23.1
A7	1.21 ± 0.006	1.16 ± 0	2.96 ± 0.05	1.21 ± 0.06	477.51	7.15	25.6	26.07
A8	2.15 ± 0.19	2.57 ± 0.24	3.36 ± 0.16	1.97 ± 0.06	1786.91	14.35	197.42	241.78
A9	3.58 ± 0.38	3.03 ± 0.37	4.27 ± 0.24	2.71 ± 0.22	1859.17	15.38	210.21	247.51
A10	1.14 ± 0.03	1.1 ± 0.03	1.52 ± 0.08	1.02 ± 0.02	51.23	2.23	12.01	12.3
Control	-	-	-	-	5.6	2.12	2.8	6.29

[a] Data are offered as means ± SD of triplicates.

same time (Table 4 and Fig. 1b). The results revealed that seven bacterial isolates (A1, A4, A5, A6, A7, A9, and A10) out of 10 selected isolates showed siderophore secretion ability on CAS agar medium within 72 h (Table 4, Fig. 1c). In addition, the isolates A5, A8, A9, and A10 were identified positive for HCN production at 96 h (Table 4, Fig. 1e). Therefore, HCN production was not a more common trait of all isolates. IAA and NH3 production were shown in all the isolates (Table 4, Fig. 1d, e). The highest IAA production was observed in isolates A8 (79.19 µg/ml), followed by A7 (23.66 µg/ml) and A9 (18.09 µg/ml), respectively. While the lowest IAA production was observed in A1 isolate with 8.99 µg/ml as depicted in Table 4. The results of NH3 traits revealed a range of 0.285 – 0.589 µmol/ml ammonia production at 72 h (Table 4).

In vitro mineral solubilization assay

Qualitative assessments of mineral solubilization. In plate-based assays, a qualitative P, K, Zn, and Mn

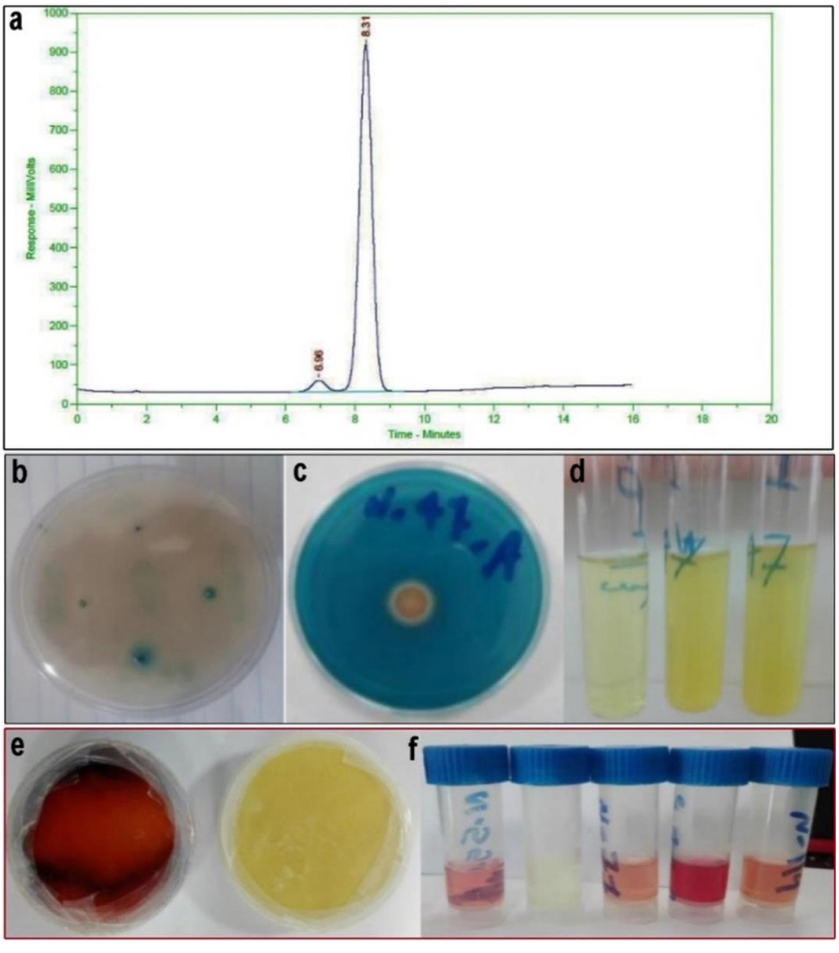

Fig. 1. Screening results of PGPR traits, a) nitrogenase activities based on the acetylene reduction method, b) qualitative colorimetric assay for phosphatase activity, c) siderophore secretion based on the appearance of orange halo in CAS agar medium, d) NH_3 production, e) a positive HCN production capacity based on alteration in filter paper color from red to dark brown, and f) the production of IAA in comparison with control.

solubilization assay revealed a clear halo surrounding the colonies of all of selected isolates, suggesting their mineral solubilization capability (Fig. 2). The solubilizing clear zone diameter of all isolates was significantly enhanced after 7 days in comparison with 2 days ($p \leq 0.05$). The largest P, K, Zn, and Mn solubilizing clear zone diameter was observed for A8, A2, A9, and A8 about 29 mm, 34 mm, 47 mm, 30 mm, respectively within 7 days (Table 5). The results of qualitative mineral solubilization were standardized based on PSI, KSI, ZSI, and MSI indices. In all case, the PSI, KSI, ZSI, and MSI values were significantly increased during the experimental assays time ($p \leq 0.05$).

As shown in Table 5, the PSI value of A3 (3.8 ± 0.07) and A9 (3.58 ± 0.38) was remarkably higher than that of the lowest level that belonged to A6 (0.95 ± 0.05) ($p < 0.01$). Isolates A2, and A9 also showed maximum KSI (3.09 ± 0.09 and 3.03 ± 0.37, respectively), which is significant compared to the minimum KSI obtained from isolates A4 and A10 (~1.1), in 7 days ($p < 0.01$). For ZSI values, A9 was the leading isolate having maximum values of 4.27 ± 0.24 followed by isolate A1, having 4.22 ± 0 in 7 days. In contrast, isolate A10 significantly showed the lowest ZSI value (1.52 ± 0.08) ($p < 0.01$). The results also revealed significant MSI value for A3 and A9 (~2.7) in 7 days while the A10 presented the lowest value (1.02 ± 0.02) in the same time (Table 5).

Quantitative assessments for mineral solubilization. As shown in Table 5, the highest values of soluble P were 2016.41 mg/l, 1859.17 mg/l, and 1786.91 mg/l after 96 h for isolates A1, A9, and A8, respec-

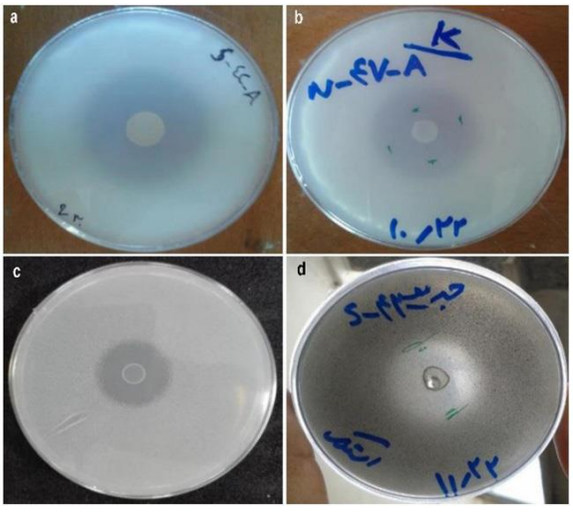

Fig. 2. A plate-based demonstration of minerals solubilization by isolated PGPR strains based on appearance of solubilization zones in respective media incubated for 7 days. The clear zone with respect to solubilization assays of P, K, Zn, and Mn after inoculation of isolates on TCP-supplemented Sperber agar (a), mica-amended Aleksandrov agar (b), ZnO-amended MSM (c), and MnO_2 containing Aleksandrov solid medium (d), respectively.

tively. The highest solubilized K content was recorded 43.17 mg/l for A1 at 96 h. However, the solubilized K content of isolates A10, A4, and A6 respective to 2.23 mg/l, 3.08 mg/l, and 3.69 mg/l were non-significant and similar to the un-inoculated control composed of 2.12 mg/l K ($p > 0.05$). A wide range of 8.41 to ~210 ppm soluble Zn was found for selected isolates after 96 h. The isolates A9 and A8 showed the highest soluble Zn, with activities of 210.21 and 197.42 ppm, respectively (Table 5). Quantitative Mn solubilization exhibited the maximum solubilized Mn concentration for isolate A9 (247.51 ppm), followed by isolate A8, which solubilized Mn concentrations up to 241.78 ppm. In addition, in liquid media, in all cases, a shift in pH after 96 h from neutral to acidic pH, and a close association between growth rates and mineral solubilization were noticeable ($p < 0.05$).

Identification of the isolates. The selected strains were firstly characterized by morphological and biochemical tests. Based on the biochemical tests, all of the selected isolates were gram negative strains, which were positive for lactose, glucose, and mannitol utilization and negative for urea and H_2S test. Regarding catalase and oxidase, all the isolates exhibited positive and negative results, respectively, except A10. The general features of the selected isolates are illustrated in Table 6. In addition, the selected isolates were further characterized based on 16S rDNA sequencing to clarify their similarity. Alignments of the 16SrDNA sequences of A1 to A10 isolates revealed 99% and 100% similarity with the identified strain with specific GenBank Accession numbers (Table 6).

***In vitro* maize growth promotion by the selected strains.** In a plate assay inoculation of maize seeds with selected isolates, an increase in root length, leaf length, root fresh and dry weight of maize was obtained after 12 days in comparison with the un-inoculated control. Table 7 shows the difference in maize seed germination among treatments and the un-inoculated control. The results also indicated that the germination rate of the seeds treated with PGPR were significantly higher than that of the control. The highest average root length, leaf length, root fresh and dry weight belonged to isolate A8 compared to the other treatments. A morphological difference of maize seeds germination with and without PGPR inoculation is shown in Fig. 3.

Promotion of maize growth by the selected PGPR isolates at greenhouse. The treatment of maize seeds with selected isolates inoculant remarkably stimulated maize growth after 20 days of germination. Fig. 3 shows the difference in plant height and root growth among treatments and the un-inoculated control.

According to the variance analysis (Table 8), a statistical difference ($P< 0.05$) was revealed between the treatments in plant growth parameters including seed germination on the 10^{th} day, plant height, collar diameter, shoot fresh and dry weight, and root dry weight on the second month (Fig. 4). Under controlled conditions, the highest average germination of seeds belonged to treatments T1 and T21 (Fig. 4a). Among treatments inoculated with bacteria, the highest average shoot height belonged to treatments T1, T9, T11, T15, and T27 compared to the other treatments. On the other hand, the inoculation of plants with multiple isolates A1+A2+A5+A10, A2+A6+A10, A1+A4+A5+A6, A1+A2+A6+A10, and A3+A5+A10 increased the shoot height (Fig. 4b).

Fig. 4c demonstrates a significant increase in average collar diameter of plants after treatment with T9, T11, T15, T16, T17, and T27, which were inoculated with A2+A6+A10, A1+A4+A5+A6, A2+A7, A2+A7+A10, and A3+A5+A10, respec-

Table 6. The characterization of the selected PGPR isolates

Isolates	Biochemical test									Identified species	Accession number	Similarity (%)
	Gram staining	Catalase	Oxidase	Simmon Citrate	Urea	H_2S	Lactose	Glucose	Mannitol			
A1	-	+	-	-	-	-	+	+	+	*Enterobacteriaceae* bacterium	AB703079.1	100
A2	-	+	-	+	-	-	+	+	+	*Pantoea* sp. strain SPC23	MK053631.1	100
A3	-	+	-	+	-	-	+	+	+	*Kluyvera* sp. strain PS20	MH883998.1	99
A4	-	+	-	+	-	-	+	+	+	*Enterobacter ludwigii* strain SNU502	MK424123.1	100
A5	-	+	-	+	-	-	+	+	+	*Enterobacter ludwigii* strain SNU502	MK424123.1	100
A6	-	+	-	-	-	-	+	+	+	*Lelliottia amnigena* strain NCTC12124	LR134135.1	100
A7	-	+	-	+	-	-	+	+	+	*Klebsiella* sp. strain MWX2	MH105765.1	99
A8	-	+	-	-	-	-	+	+	+	*Enterobacter ludwigii* strain DM27	MK370572.1	99
A9	-	+	-	+	-	-	+	+	+	*Pectobacterium cypripedii* strain ICMP 1591	EF122434.1	99
A10	-	-	+/ -	+	-	-	+	+	+	*Stenotrophomonas* sp. G4	CP031741.1	99

Table 7. ***In vitro*** effect of the selected isolates on maize seed germination indicators

Isolates	Root length (mm)	Germination rate (%)	Leaf length (mm)	Root fresh weight	Root dry weight
A1	120^{d}	88.88^{ab}	30.55^{e}	0.43^{d}	0.19^{d}
A2	131.11^{c}	100^{a}	33.33^{c}	0.48^{c}	0.23^{bcd}
A3	131.77^{c}	100^{a}	33.11^{c}	0.49^{c}	0.22^{bcd}
A4	130.33^{c}	100^{a}	32.22^{cd}	0.48^{c}	0.22^{cd}
A5	130.55^{c}	100^{a}	31.77^{de}	0.49^{c}	0.22^{cd}
A6	129.88^{c}	88.88^{ab}	31.33^{de}	0.48^{c}	0.21^{d}
A7	138.22^{b}	100^{a}	35.88^{b}	0.54^{b}	0.26^{b}
A8	150.66^{a}	100^{a}	42^{a}	0.76^{a}	0.31^{a}
A9	137.22^{b}	100^{a}	35.22^{b}	0.52^{bc}	0.25^{bc}
A10	129.33^{c}	88.88^{ab}	30.88^{de}	0.48^{c}	0.21^{d}
control	113.22^{e}	66.66^{b}	25.55^{f}	0.37^{e}	0.16^{e}

Different letters indicate statistically significant differences ($p < 0.05$).

tively. The inoculants of treatments T9, T11, T15 and T27, including A2+A6+A10, A1+A4+A5+A6, A1+A2+A6+A10, and A3+A5+A10 had the highest shoot fresh weight compared to other treatments (Fig. 4d). Regarding the shoot dry weight, treatments T9 (A2+A6+A10), T11 (A1+A4+A5+A6), T12 (A1+A3), T15 (A1+A2+A6+A10), T20 (A3+A7), and T27 (A3+A5+A10) exhibited a better increase in shoot dry weight compared to other treatments (Fig. 4e). In addition, the maximum root dry weight was reported for T9 (A2+A6+A10), T11 (A1+A4+A5+A6), T12 (A1+A3), and T15 (A1+A2+A6+A10) treat-

Fig. 3. Morphological differences of maize seeds germination, maize growth, and root growth with (a) PGPR inoculation (treatment T27: A3+A5+A10) compared to (b) treatment T30 (chemical NPK fertilizer without PGPR inoculation).

Table 8. Analysis of variance of seed germination, plant height, collar diameter, shoot dry and fresh weight, and root dry weight of maize inoculated with the selected PGPR.

S.O.V	DF	Seed germination	Plant height (cm)	Collar diameter (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Block	30	9.733	187.926^{ns}	0.146	2788.502	170.313	70.326
Treatment	155	3.298	124.479**	0.018	615.726	31.048	25.729
Error	-	0.153	0.851	0.014	2.281	0.536	0.420
CV	-	2.084	11.60	0.197	31.113	7.323	5.741

ns, non-significant, ** significant compared to the control group ($P< 0.01$).

ments (Fig. 4f). According to the obtained results, a mixture of bacteria A2+A6+A10, A1+A4+A5+A6, A1+A2+A6+A10, A3+A5+A10, A1+A2+A5+A10, and A1+A3 had the highest average among all parameters.

The growth rates between plants receiving chemical fertilizer and inoculated plants with selected PGPR isolates were noticeable. Regarding, treatment T28 exhibited (without bacterial isolates) the lowest average germination, plant height, collar diameter, plant fresh weight, and dry weight root (Fig. 4).

DISCUSSION

Given that the ability of diverse groups of rhizobacteria, named as PGPR, to enhance directly and/or indirectly the plant growth and crop production (4), the isolation and investigation of multi-trait PGPR isolates was favored in this research. We isolated the numerus rhizobacteria from divers region due to it has been revealed that the isolation of an effective PGPR from various region making them adaptable to diverse soil types and climatic conditions and would functionally be active in most cultured lands (34). The current study then evaluated their PGPR traits such as N fixation, P, K, Zn, Mn solubilization, and the production of IAA, siderophore, ammonia, HCN. Finally, potential of selected isolates in plant growth promotion of maze seedlings were evaluated. In this line, ten PGPR isolates were firstly selected based on NPK performance of PGPR.

N is the most important nutrient for plant compared to the other essential nutrients (35). P and K are known to be the second most limiting nutrients after N but their low availability for plants is one of the main challenge (11, 36). It has been demonstrated that a mixture of bacteria with high potency in N_2

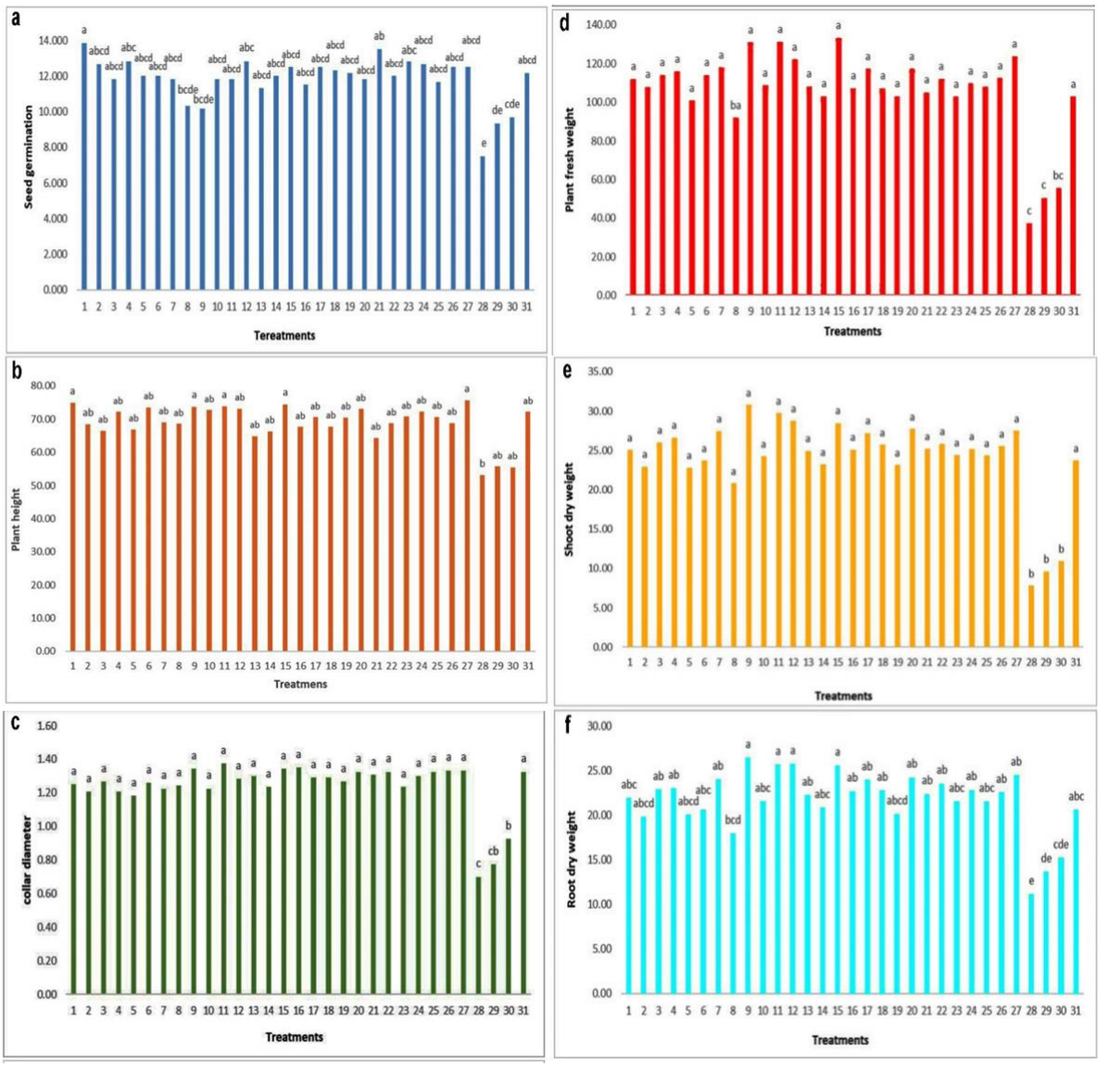

Fig. 4. Effect of the PGPR inoculant (T1 to T31) on maize growth indicators such as seed germination (a), shoot length (b), collar diameters (c), shoot fresh weight (d), shoot dry weight (e), and root dry weight (f) in greenhouse conditions after 60 days. Means comparison carried out with Duncan's method. Means with the same letters are categorized in the same groups.

fixation and solubilizing of macronutrients (P and K) as NPK biofertilizer is one of the most efficient strategies to access them for plants (27, 37). Herein, the isolates that could grow in N-free media, were able to solubilize the P and K in the sufficient level (Table 5). In addition, the phosphatase activity was observed in all of isolates. This phenomena confirm the solubilization of P via simultaneous excretion of organic acids and phosphatase activity (26).

Generally, solubilization of insoluble mineral nutrients, for instance P, K, Zn, Mn into available form for plant assimilation is a famous PGPR trait displayed by several bacterial strains (38). Major of released form of mineral nutrients in the liquid medium exist as free ions, which are readily absorbed by plants root (39, 40). The findings demonstrated isolates A1, A9, and A8 as the efficient isolates for mineral solubilization after 96 h in liquid media. A1 and A9 displayed the significant value of soluble P and K. The isolates A9 and A8 also showed the maximum soluble Zn (210.21 and 197.42 ppm), and Mn (247.51 and 241.78 ppm). The PSI, KSI, ZSI, and MSI data obtained in the solid medium also confirmed the performance of these isolates in the solubilization of these minerals.

These findings recommend that these isolates could produce remarkable values of organic acids or H+ ions (14, 15). These isolates can apply as effective biofertilizer nominees for improving the macro- and micro- nutrition of plants simultaneously.

Although the availability of nutrients for the plant is considered as the major mode of action of many PGPRs, but it emphasizes that the elevated concentrations of ions especially P and Zn can also stimulate the production of siderophore (18, 41). Siderophore producing bacteria are one of the most well-known groups of PGPR, which can promote the plant growth by the secretion of various siderophores under iron-depleted conditions (1, 17). Regarding, the siderophore production was observed in 7 out of 10 selected isolates, confirming the Fe availability effects of higher isolates for crop growth in the iron deficient soil. In the current study, selected isolates were also positive for production of IAA (Table 4). The production of phytohormones like IAA as a part of PGPR strains metabolism played an important role in the promotion of plant growth in stress conditions (42). The selected isolates could also exhibit more PGPR traits such as HCN and ammonia production, which may stimulate growth of plant directly, indirectly or synergistically. The similar outcomes for multiple PGPR traits have been reported from some other studies (29, 33, 34, 43). Our selected isolates showed a high similarity to strains of *Enterobacter, Pantoea, Kluyvera, Lelliottia, Klebsiella, Pectobacterium,* and *Stenotrophomonas.*

Although our isolates displayed multiple PGPR traits in vitro, their effect on the stimulation of plants (such as maize) growth was necessary. Inoculation of maize with PGPR has previously been shown to improve seed germination, growth, and yield of maize (33, 44). In our research, the selected strain significantly improved maize growth inducing on six parameters (seed germination, plant length, collar diameter, shoot fresh and dry weight, and root dry weight) under controlled conditions in comparison with un-inoculated plants (Fig. 4). It indicates the beneficial role of these rhizobacteria, which might be attributed to nitrogen-fixing capacity and mineral nutrients solubilization, production of HCN, siderophore, and IAA, and any other PGPR activity. In addition, similar to our findings, enhancement of maize growth with PGPR strains has been reported (33). Ijaz et al. indicated that the inoculation of maize with Mn solubilizing bacteria with multiple PGP attributes significantly enhanced the maize growth parameters after 60 days (29). Similarly, significant improvement of wheat plant parameters such as plant height (40.91%), root weight (85.71%), and panicle weight (37%) were observed in three isolates (AW1, AW5 and AW7) as PGPR inoculants (34). In our study, inoculations with a mixture of strains A1+A2+A5+A10 (T1), A2+A6+A10 (T9), A1+A4+A5+A6 (T11), A1+A3 (T12), A1+A2+A6+A10 (T15), and A3+A5+A10 (T25) showed a remarkable increase in maize growth parameters means compared to other treatments and chemical fertilizers (Fig. 4). The combined use of PGPR inoculants and chemical fertilizers has been revealed as a sustainable approach to increase the maize yield together with decreasing of unnecessary use of chemical fertilizers. In our study, the commercial NPK fertilizer did not cause a significant yield increase.

CONCLUSION

In conclusion, we isolated several PGPR strains, some of which demonstrated the better performance of PGPR traits such as nitrogen fixation, P, K, Mn, and Zn solubilization, and production of siderophore, HCN, NH_3, and IAA. In addition, our data indicated the ability of bacterial inoculation, applied as seed treatments, in the improvement of maize yield in a pot experiment. The molecular characteristics and alignment process confirmed the high similarity of our selected isolates to the strains of *Enterobacter, Pantoea, Kluyvera, Lelliottia, Klebsiella, Pectobacterium,* and *Stenotrophomonas.* These findings demonstrated the positive role of these strains as PGPR in macro- and micro-nutrients deficient soils. Moreover, these strains may have the potential to be used as multiple bio-fertilizers, in the future.

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