جداسازی باکتریهای اندوفیت از ریشه گیاهان علفی و ارزیابی توانایی انحلال فسفات، آزادکنندگی پتاسیم و تولید اکسین باکتریهای جداشده

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چکیده

مقدمه: برخی از باکتری‌های محرک رشد گیاه (PGPR)، باکتری‌های اندوفیت هستند که در اندوریزوسفر مستقر و با سازوکارهای مختلف رشد گیاه را تحریک می‌کنند.

مواد و روش‌ها: در مطالعۀ حاضر، ۲۴ جدایه باکتری اندوفیت از ریشۀ گیاهان گرامینه مانند گندم، جو و برنج با استفاده از محیط‌کشت نیمه‌جامد NFB جداسازی شدند. ویژگی‌های محرک رشدی شامل حل‌کنندگی فسفات، تولید ایندول‌استیک‌اسید (IAA)، رهاسازی پتاسیم از کانی‌های میکا و تولید سیدروفور بر اساس روش‌های استاندارد اندازه‌گیری شدند.

نتایج: انحلال فسفات از منبع تری‌کلسیم‌فسفات ۱/۶۷ برابر بیشتر از سنگ فسفات و رهاسازی پتاسیم از بیوتیت نسبت به موسکویت ۴/۷ برابر بیشتر بود. همچنین میزان تولید IAA در حضور تریپتوفان در مقایسه با نبود تریپتوفان بیشتر بود (۳۰ درصد). بیشترین مقدار تولید IAA به‌ترتیب در جدایه‌های Az-3 (۲۸/۲ میلی‌گرم‌برلیتر) و Az-48 (۷۵/۰ میلی‌گرم‌برلیتر) مشاهده شد. بیشترین انحلال فسفات در جدایۀ Az-52 (۱۳/۳۴ میلی‌گرم‌برلیتر) و کمترین آن در جدایۀ Az-13 (۱۳/۹۸ میلی‌گرم‌برلیتر) به دست آمد. جدایۀ Az-65 بیشترین رهاسازی پتاسیم (۶/۶۶ میلی‌گرم‌برلیتر) و Az-3 (۷۸/۴ میلی‌گرم‌برلیتر) کمترین مقدار را به خود اختصاص دادند؛ درنهایت، جدایه‌های کارآمد به روش توالی‌یابی *16S rRNA* شناسایی شدند و Az-8، Az-19 و Az-50 به جنس *سودوموناس*، Az-3 به *اسینتوباکتر*، Az-18 به *ریزوبیوم*، Az-48 به *انتروباکتر* و Az-63 به جنس *ادونلا* تعلق داشتند.

بحث و نتیجه‌گیری: نتایج نشان دادند جنس‌های مختلف باکتریایی با صفت‌های مختلف محرک رشدی به‌شکل باکتری‌های اندوفیتی در ریشه‌های گیاهان وجود دارند. این باکتری‌ها قادر به انحلال فسفات بیشتری از منبع تری‌کلسیم‌فسفات نسبت به سنگ فسفات بودند و پتاسیم بیشتری را از منبع بیوتیت در مقایسه با موسکویت آزاد کردند و تولید IAA در حضور تریپتوفان بیشتر بود. پژوهش‌های بیشتر به‌ویژه در کشت گلدانی و در سطح مزرعه برای بررسی آثار این باکتری‌ها لازم است.

واژه‌های کلیدی: PGPR، فسفات، IAA، پتاسیم، باکتری‌های اندوفیت

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Isolation of Endophytic Bacteria from Grasses Root and Assessing Phosphate Solubilization, Potassium Releasing and Auxin Production Abilities of Isolated Bacteria

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Abstract

Introduction: Some of plant growth-promoting rhizobacteria (PGPR) are endophytic bacteria that exist in the endo-rhizosphere and are able to promote plant growth via different mechanisms.

Materials and methods: Twenty four endophytic bacteria were isolated from the roots of grasses such as rice, wheat and barley using semi-solid nitrogen-free bromothymol blue (NFB) medium. PGP features including phosphate solubilization, indole acetic acid (IAA) production, potassium releasing, and siderophore production were measured according to the standard methods.

Results: The dissolution of phosphate 1.67 times was higher in the presence of tri-calcium phosphate (TCP) than rock phosphate (RP), and K release from biotite was more than muscovite (4.7 times); furthermore more IAA was produced in the presence of tryptophan, and this increment was by 30%. The highest and lowest amount of IAA were recorded in Az-3 (2.28 mg/l) and Az-48 (0.75 mg/l), respectively; while the maximum dissolution of phosphate was obtained with Az-52 (340.13 mg/l) and the least dissolution of phosphate occurred in Az-13 (98.13 mg/l). The highest K release was accomplished by Az-65 (6.66 mg/l) and the lowest was in Az-3 (4.78 mg/l). Finally, identification of robust isolates by 16S rDNA sequencing showed that Az-8, Az-19, and Az-50 are belonging to the *Pseudomonas*, Az-3 to *Acinetobacter*, Az-18 to *Rhizobium*, Az-48 to *Enterobacter*, and Az-63 to *Advenella*.

Discussion and conclusion: Our results revealed that different genera of bacteria with varied PGP traits are existing as endophytic bacteria in the plant roots. In total, these bacteria were able to solubilize more phosphate from TCP than RP; and higher K from biotite than muscovite. It seems that the production of organic acids is the main mechanism of P and K solubilization. Moreover, higher production of IAA was measured in the presence of tryptophan. Further research, especially in pot or farm, is necessary to assess the effects of these bacteria**.**

Key words: PGPR; Phosphate; Auxin; Potassium; Endophytic Bacteria.

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Introduction

The rhizosphere is the narrow zone of soil which is influenced by the root system. It is a nutrient-rich habitat for microorganisms due to the accumulation of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (1). Plant growth-promoting rhizobacteria (PGPR) are a group of bacteria that exist in the rhizosphere, at root surfaces and in association with roots, and are able to promote plant growth directly and/or indirectly. In recent decades some bacterial genus including *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported as PGPR (2). The direct growth-promoting activities are as follows; facilitating nutrient uptake or increase nutrient availability by nitrogen fixation, solubilizing mineral nutrients, mineralizing organic compounds and production of phytohormones (3). The indirect promotion of plant growth occurs when PGPR minimizes or prevents the deleterious effects of phytopathogens by producing antagonistic substances and/or by inducing resistance to pathogens (2).

Phosphate solubilization activity of microorganisms is considered as one of the most important characteristics of PGPRs associated with phosphorus nutrition (4). Plant growth-promoting rhizobacteria have many mechanisms for the dissolution of unavailable forms of phosphorus and help in making phosphorus available for plants. The main phosphate solubilization mechanisms include (a) release of complexing compounds e.g. organic ligands (b), proton, hydroxyl ions, CO_2, (c) liberation of extracellular enzymes and the release of phosphate during substrate degradation (biological phosphate mineralization) (4,5). Different bacterial genera such as *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, and *Serratia* have been noticed to improve plant growth and yield (6).

Indole-3-acetic acid affects plant growth through cell division, extension and differentiation; stimulates seed and tuber germination, root development, controls vegetative growth and the formation of lateral roots, photosynthesis rate, pigment formation, biosynthesis of various metabolites and resistance to stressful conditions (7). About 80% of rhizobacteria are able to synthesize IAA through different biosynthetic pathways. These microorganisms have the power to affect the endogenous levels of this regulator and therefore have remarkable effects on plant growth (8). The production of IAA by PGPR involves formation via indole-3-pyruvic acid and indole-3-acetic aldehyde, which is the most common mechanism in bacteria like *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Enterobacter* and *Klebsiella* (9).

Some soil microorganisms are able to dissolve K-bearing minerals such as mica, illite, and orthoclase which lead to releasing their potassium (10,11). Some PGPRs such as *Pseudomonas* spp., *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *B. edaphicus* and *B. megaterium* have been reported for release of potassium from K-bearing minerals (12). Siderophores can also improve plant growth through both direct and indirect pathways. Direct beneficial effects of bacterial siderophores on plant growth have been documented by using radiolabeled ferric siderophores as the only source of iron. A large number of PGPRs such as *Aeromonas*, *Azadirachta*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Streptomyces* sp. are capable of producing

siderophores (13).

The main objectives of this study were isolation and identification of endophytic bacteria from some grasses root and determination of their growth-promoting properties including phosphate solubilization, IAA and siderophore production and potassium releasing from K-bearing minerals.

Material and Methods

Isolation and Purification of Endophytic Bacteria: The rhizosphere soil samples were collected from three provinces including East Azerbaijan, Ardabil, and Gilan. Roots were separated, washed and disinfected by 1% sodium hypochlorite and 70% ethanol (14), then were crashed in sterilized water and diluted up to 10^{-4}. Then, 100 µL of the dilution was added to the tubes containing semi-solid Nitrogen Free Bromothymol blue (NFB) medium and incubated at 28 °C for 48-72 hours. After incubating and observing the rising pellicle formation, a part of this pellicle was transferred to the solid NFB medium by performing streak culture. NFB medium containing per liter of distilled water: 5 g L-Malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.02 g NaCl, 4 g KOH, 2 mL micronutrients solution (0.2 g $Na_2MoO_4.2H_2O$, 0.235 g $MnSO_4.H_2O$, 0.28 g H_3BO_3, 0.008 g $CuSO_4.5H_2O$, 0.24 g $ZnSO_4.7H_2O$ and 1000 mL distilled water), 2 mL bromothymol blue, 4 mL Fe-EDTA (1.64%), 1 mL vitamin solution (0.01 g biotin, 0.02 g pyridoxine and 1000 mL distilled water) and 1.75 g agar. The pH of the medium was adjusted to 6.8 (15). Once more, white and fine colonies were transferred to semi-solid NFB medium to check the pellicle formation. For final purification, pellicles were transferred to the congo red medium containing per liter of distilled water: 5 g L-Malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.02 g NaCl, 1 mL vitamin solution, 4.8 g KOH, 0.02 g $CaCl_2.2H_2O$, 15 mL congo red (1:400), 0.5 g NH_4Cl, 0.5 g yeast extract, 15-20 g agar and pH 7. Light-pink and colorless colonies were observed after 48 h. After 72 h, the light-pink colonies became scarlet. Pink and often wrinkled colonies were picked out. Purified isolates were frozen and kept at -20 °C in the presence of 15% (v/v) glycerol (16).

It should be mentioned that in this experiment two isolates namely AC46-I and C162-O belonging to *Azospirillum* sp. and *Pseudomonas* sp. were used as control bacteria to perform in-vitro PGP assessments (these bacteria were prepared from Soil Biology Laboratory of Department of Soil Science, University of Tabriz).

Phosphate Solubilization Activity: In order to evaluate the phosphate solubilization of bacterial isolates in the presence of tri-calcium phosphate (TCP) and rock phosphate (RP), a factorial experiment based on the completely randomized design with three replications was conducted. Phosphate solubilization ability of 24 bacterial isolates was determined using Pikovskaya medium (17) containing TCP and RP as sole sources of phosphorus (containing per liter of distilled water: 10 g glucose, 5 g TCP, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.0001 g $MnSO_4 \cdot H_2O$, 0.0001 g $Fe_2(SO_4)_3$ and 0.1 g $MgSO_4.7H_2O$). Overnight culture of the isolates was prepared in the nutrient broth (NB) and 0.5 mL was added to 100 mL Erlenmeyer flasks containing 30 mL Pikovskaya medium. In control treatment, only 0.5 mL of sterile NB was added. The flasks were incubated for 7 days at 26 °C on shaker incubator with 150 rpm. The contents of Erlenmeyer flasks were centrifuged at 5000 rpm for 5 min. The concentration of P was determined by vanadate–molybdate method using spectrophotometer (Hach DR/2000) (18).

K Releasing Ability: The assessment of potassium solubilization was examined based on the bacterial abilities to release K from biotite and muscovite as insoluble sources of potassium. This experiment was conducted as a completely randomized

factorial design with three replications. The factors included isolates of bacteria and sources of potassium (biotite and muscovite). Acid washed pretreated minerals (19) as a source of potassium and 0.5 mL of bacterial inoculum were added to 30 mL of Aleksandrov medium containing per liter of distilled water: 5 g glucose, 2 g TCP, 2 g mica (biotite or muscovite), 0.5 g $MgSO_4.7H_2O$, 0.005 g $FeCl_3$ and 0.1 g $CaCl_2$ (19,20). After incubation for one week at 26 °C and shaking at 120 rpm, released K in the supernatant was measured by using a flame photometer (Corning Model 410).

IAA Production: Measurement of IAA was performed by colorimetric method as described by Gordon and Weber (21). For this purpose, NF medium (NFB without bromothymol blue) containing 100 mg/l L-Tryptophan was used. This experiment was performed in a completely randomized factorial design with three replications. The factors included two levels of L-Tryptophan (0 and 100 mg/l) and bacterial isolates. Estimation of IAA was carried out in 100 mL Erlenmeyer flasks containing 30 mL NF medium. Each flask was inoculated with 0.5 mL of bacterial inoculums (overnight cultures of bacteria in nutrient broth) and the flasks were incubated for 72 h at 26 °C on shaker incubator with 120 rpm. The contents of flasks were centrifuged at 5000 rpm for 5 min. Then, 2 mL supernatant of each particular isolates was mixed with 4 mL of Salkowski reagent (H_2SO_4 (12M) + $FeCl_3$ (0.5M)) and kept for 30 minutes (21) and development of pink color indicated IAA production. Absorbance was recorded at 530 nm by spectrophotometer and the concentration of IAA was determined by using a standard graph (22).

Siderophore Production: Bacterial isolates were assayed for siderophore production by the method of Schwyn and Neilands (23) using chrome azurol S (CAS) agar. Siderophore was detected by the formation of orange halos surrounding bacterial colonies on CAS agar plates. Spot inoculation of the bacterial isolates was performed on the CAS agars plates and incubated at 26°C for 48–72 h. The appearance of yellow-orange halo around the colony was considered positive for siderophore production.

Identification of Bacterial Isolates

Molecular Identification: After assessing the growth-promoting properties of the isolates, molecular identification was performed based on the amplification of 16S rDNA gene. Molecular identification of the bacteria was done without genomic DNA extraction through the application of colony-PCR method. A part of a bacterial single colony was used as a source of DNAg in PCR reaction. Bacterial 16S rDNA was amplified by using the universal bacterial 16S rDNA primers, 24F (5’ AGAGTTTGATCCTGGCTCAG 3’) and 1525R (5’ AAGGAGGTGATCCAGCCGCA 3’). PCR was performed with a 20 µL reaction mixture containing bacterial colony as a template of DNA, 0.1 pmol of each primer, 2 mM $MgCl_2$ and dNTPs at a concentration of 0.2 mM, as well as 0.2 U of Taq-DNA polymerase and buffer was used as recommended by the manufacturer (Fermentas, Hanover, Germany). The instruction for amplification by PCR was: 94 °C for 5 min (first DNA denaturation), 94 °C for 1 min (denaturation), 53 °C for 1 min (annealing), and 72 °C for 1 min (extension). The three stages of PCR adjusted at 35 cycles and final extension was 72 °C for 10 min. PCR was carried out in a Flexigenethermocycler. PCR products were analyzed by 1% (w/v) agarose gel electrophoresis in 1X TBE buffer. A DNA fragment (approximately 1.5 kb) was eluted by using Qiagen Gel Extraction Kit. PCR products were sequenced and sequences were matched with previously published bacterial 16S rDNA sequences in the NCBI databases using BLASTn (24).

Biochemical tests such as oxidase, catalase, nitrate reductase, citratase, urease,

amylase, gelatinase, tryptophanase, and indole and hydrogen sulfide production (25) were carried out.

Statistical Analysis: The statistical analysis was performed using MSTATC software. The statistical variation in IAA production, potassium release and phosphate solubilization by isolates were analyzed using an analysis of variance (ANOVA). Means were compared using Duncan's multiple range test at $p < 0.01$.

Results

Isolation and Screening of the Endophytic Bacteria: Bacteria were isolated and identified from rhizospheric soil samples as described in Bergey's Manual of Determinative Bacteriology. For the isolation of endophytic bacteria, NFB semi-solid medium was used. After 24-48 h incubation, white-colored pellicles were appeared in the NFB semi-solid medium. Subsurface pellicles formed 3-20 mm below the surface of the medium (Figure 1). In this study, the appearance of pellicle formation in the NFB semi-solid medium indicated successful isolation of endophytic bacteria. A total number of 24 isolates were isolated from the root samples. The pellicles were transferred into NFB plates via streak culture preparation by a sterile loop. After 48-72 h the colonies with white and merged appearance were observed on the medium. Typical white or pink, often wrinkled colonies were picked out and transferred into CR medium for purification. Light-pink and colorless colonies were observed after 48 h in the RC medium. Colonies were picked from these plates and maintained as pure cultures in respective media with periodic transfer to fresh media and stocked for further use.

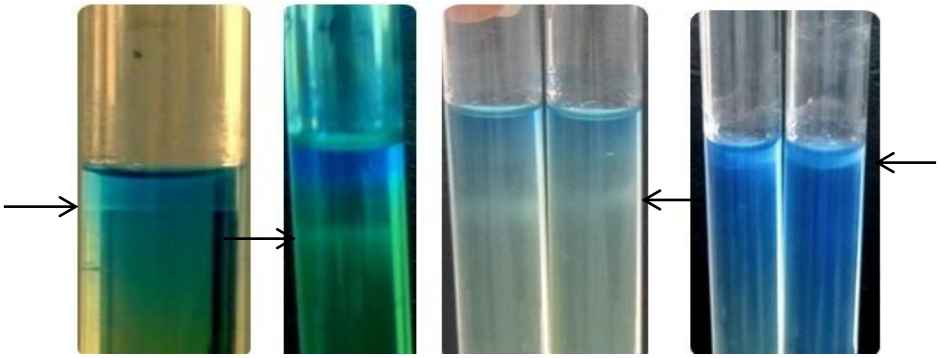

Fig. 1- Pellicle formation due to growth of bacterial isolates in the NFB medium. Some bacteria produced pellicles close to the surface and the others were deeper.

Phosphate Solubilization: Phosphate solubilization was significantly ($p < 0.01$) influenced by bacterial isolates, P sources and, bacteria × phosphorus sources. The results showed a considerable enhancement of phosphate solubilization in the inoculated treatments compared with the control. The soluble-P concentration ranged from 98.13 to 340.13 mg/l with variations among different isolates. All bacterial isolates had higher (9.1 times) P solubility activity in the presence of tri-calcium phosphate compared with rock phosphate (Table 1). The greatest P solubilization was obtained by Az-52 (340.13 mg/l) with TCP that was accompanied by a significant increase (5.5 times) (Figure 2). The lowest value was observed in Az-13 (98.13 mg/l) that showed a 61% increase compared to the control whereas it was in the same statistical group. Other isolates including Az-6, Az-19, Az-50, Az-59, Az-65 and, Az-71 indicated relatively higher abilities in phosphate solubilization with a range of 261-329.38 mg/l (Figure 2).

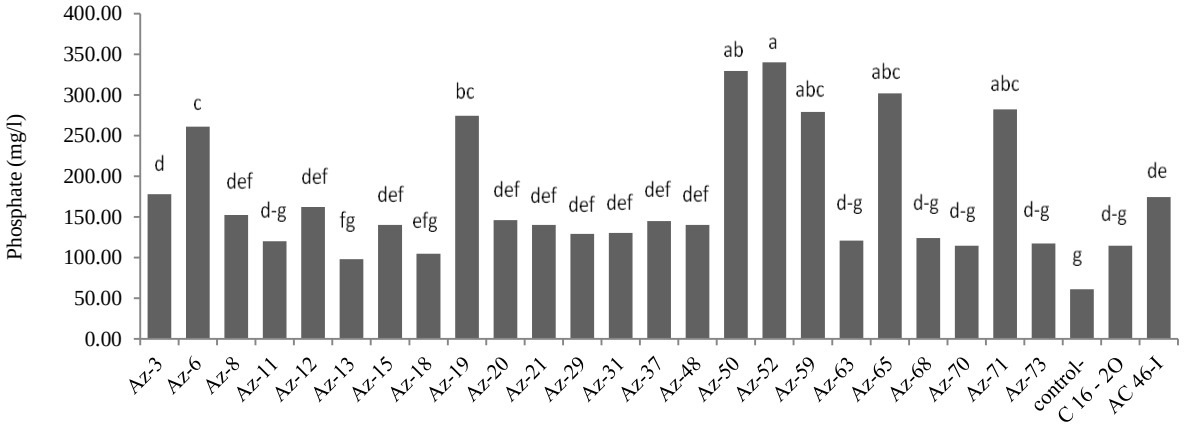

Fig. 2- Phosphate solubilization by bacterial isolates. Bars with a similar letter are not significantly different at $p < 0.01$ according to Duncan's multiple range test.

IAA production: Analysis of variance showed that the effect of bacterial isolates, tryptophan (two levels; 0 and 100 mg/l), and their interaction were significant ($P < 0.01$) on IAA production. A total of 24 isolates were tested for the quantitative estimation of IAA in the presence and absence of tryptophan. The average production of auxin by bacteria is shown in Figure 3 and Table 1. Results showed that IAA-production by the isolates in the NF medium containing 0 and 100 mg/l of tryptophan varied from 0.75 to 2.28 mg/l. With the increasing of tryptophan from 0 to 100 mg/l, the production of IAA increased. The highest amount of IAA was obtained in C16-2O and Az-3 and the minimum amount of IAA was obtained by Az-48. Moreover, Az-3 caused an increase in IAA production (5 times) as compared to the control. When tryptophan was used as a precursor, the maximal amount of IAA was produced by isolates after 72 h and caused an average increment by 30% when compared with the absence of tryptophan. Statistical analysis showed a significant difference between treatments concerning the presence and absence of tryptophan. Some isolates including Az-12, Az-37, Az-50, Az-63, and Az-73 indicated the ability to produce auxin at a range of 1.52-2.01 mg/l (Figure 3).

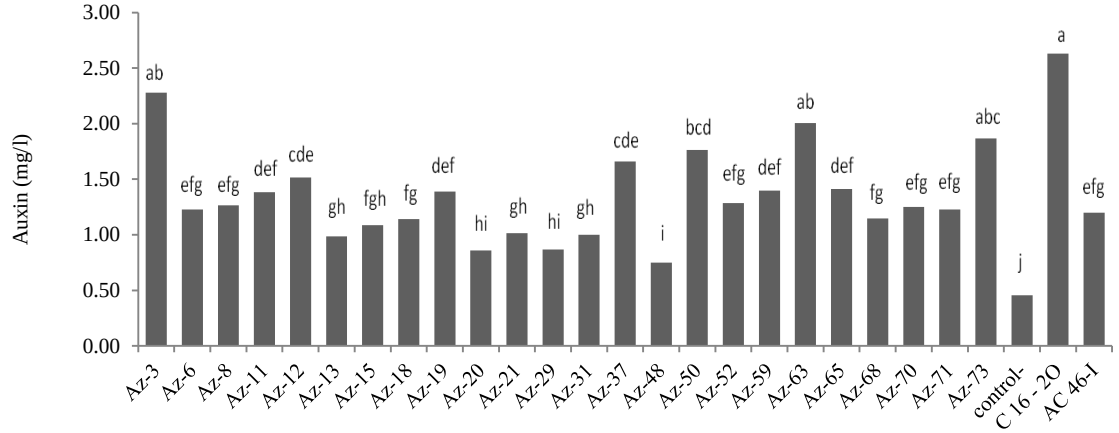

Fig. 3- Auxin-production by bacterial isolates in the NF medium. Bars with a similar letter are not significantly different at $p < 0.01$ according to Duncan's multiple range test.

K Release: The effect of 24 bacterial isolates on K release from micas was shown in Table 1. The results showed a considerable enhancement of K release by Az-21, Az-59, and Az-65 compared to the control, whereas most bacterial isolates did not show a significant increase in K release (Figure 4).

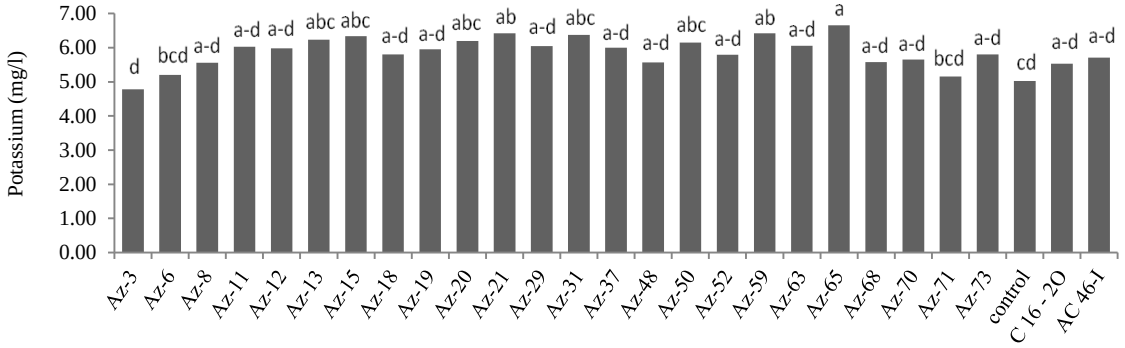

Fig. 4- K release from micas by bacterial isolates. Bars with a similar letter are not significantly different at $p < 0.01$ according to Duncan's multiple range test.

Table 1. P Solubilization, Auxin Production, and K Releasing Ability of Bacterial Isolates

	Phosphate solubilization (mg/l)			Auxin production (mg/l)			K release (mg/l)		
Isolates	TCP		Rock Phosphate	Tryptophan^{+}		Tryptophan^{-}	Biotite		Muscovite
Az-3	341.00^{bc}		15.00^{ijk}	2.96^{ab}		1.60^{c-j}	7.21^{e}		2.35^{f}
Az-6	497.25^{ab}		24.75^{i}	1.27^{g-r}		1.19^{h-t}	8.32^{de}		2.10^{f}
Az-8	290.00^{cd}		15.00^{ijk}	1.54^{c-k}		0.99^{l-t}	8.67^{b-e}		2.45^{f}
Az-11	104.00^{gh}		136.50^{efg}	1.34^{f-n}		1.43^{d-m}	9.72^{a-d}		2.35^{f}
Az-12	198.00^{def}		126.25^{fg}	1.65^{c-i}		1.38^{f-n}	9.27^{bcd}		2.70^{f}
Az-13	95.25^{gh}		101.00^{gh}	1.02^{k-t}		0.96^{m-u}	10.02^{a-d}		2.45^{f}
Az-15	201.25^{def}		79.00^{h}	1.12^{i-t}		1.06^{j-t}	10.22^{a-d}		2.45^{f}
Az-18	193.5^{def}		16.25^{ijk}	1.21^{h-s}		1.07^{j-t}	9.32^{bcd}		2.30^{f}
Az-19	526.00^{ab}		22.50^{i}	1.52^{c-k}		1.27^{g-r}	9.62^{bcd}		2.30^{f}
Az-20	276.75^{cd}		15.00^{ijk}	0.93^{n-u}		0.79^{tu}	10.26^{abc}		2.14^{f}
Az-21	265.25^{cd}		15.00^{ijk}	1.02^{k-t}		1.02^{k-t}	10.50^{1ab}		2.24^{f}
Az-29	244.75^{cd}		13.50^{jk}	0.85^{r-u}		0.88^{o-u}	9.86^{a-d}		2.24^{f}
Az-31	250.50^{cd}		10.50^{k}	1.15^{h-t}		0.85^{q-u}	10.5^{1ab}		2.24^{f}
Az-37	274.00^{cd}		15.75^{ijk}	2.13^{bcd}		1.19^{i-t}	9.71^{a-d}		2.29^{f}
Az-48	270.00^{cd}		10.25^{k}	0.84^{stu}		0.66^{uv}	9.26^{bcd}		1.89^{f}
Az-50	645.50^{a}		13.25^{jk}	2.07^{b-e}		1.46^{d-l}	10.31^{abc}		1.99^{f}
Az-52	656.00^{a}		24.25^{i}	1.71^{c-h}		0.87^{p-u}	9.61^{bcd}		1.99^{f}
Az-59	540.00^{ab}		17.75^{ij}	1.44^{d-l}		1.35^{f-o}	10.51^{ab}		2.34^{f}
Az-63	229.25^{cd}		12.75^{jk}	2.16^{bc}		1.85^{c-g}	10.22^{a-d}		1.90^{f}
Az-65	580.50^{a}		23.50^{i}	1.41^{e-n}		1.41^{d-m}	11.57^{a}		1.75^{f}
Az-68	235.50^{cd}		12.50^{jk}	1.31^{f-p}		0.99^{l-t}	9.97^{a-d}		1.19^{f}
Az-70	216.50^{cde}		12.50^{jk}	1.44^{d-m}		1.06^{j-t}	9.87^{a-d}		1.44^{f}
Az-71	545.75^{ab}		18.75^{ij}	1.28^{f-q}		1.18^{h-t}	8.97^{bcd}		1.34^{f}
Az-73	220.25^{cde}		14.75^{ijk}	1.91^{c-f}		1.82^{c-g}	10.07^{a-d}		1.54^{f}
control	106.25^{gh}		15.50^{ijk}	0.52^{vw}		0.40^{w}	8.43^{cde}		1.62^{f}
C 16 - 2O	217.25^{cde}		12.25^{jk}	3.88^{a}		1.38^{f-n}	9.47^{bcd}		1.60^{f}
AC 46-I	222.00^{cde}		127.00^{fg}	1.27^{g-r}		1.13^{h-t}	9.87^{a-d}		1.54^{f}

In the presence of biotite, the concentration of potassium in the inoculated treatments was approximately 4.7 times greater than that of muscovite. The total K contents ranged from 4.78 to 6.66 mg/l. The interaction effects of bacterial isolates and the source of potassium showed that all isolates were able to release greater potassium from biotite than muscovite (Table 1). The greatest potassium released was obtained by biotite inoculated with Az-65 (32% higher than control). The lowest increase was achieved by Az-3 which had no significant difference with control.

Siderophore Production: The results showed that none of the isolates were able to form an orange halo on the CAS agar medium indicating no siderophore production ability in all 24 bacterial isolates.

Identification of Bacterial Isolates: Among the bacterial isolates, those showed great potential in phosphate solubilization (e.g. Az-19, Az-50), IAA production (e.g. Az-3) and K release (e.g. Az-63), were selected for molecular and biochemical identification. The results of Gram staining indicated that all isolates were Gram-negative. In the molecular identification of bacterial isolates, 700-900 nucleotides of the total length of 16SrDNA were used for sequencing. Sequence comparison of the 16S rDNA gene corresponding to the isolates with the sequences in GenBank showed that Az-8, Az-19, and Az-50 belonged to the *Pseudomonas*, Az-3 to the *Acinetobacter*, Az-18 to the *Rhizobium*, Az-48 to the *Enterobacter* and Az-63 to the *Advenella*. The result of molecular identification and biochemical tests are illustrated in Table 2.

Table 2. Results of Molecular Identification for the Selected Isolates

Isolates	Results of Blast-n for obtained sequences from 16SrDNA
Az-3	*Acinetobacter* sp./ *A. calcoaceticus*
Az-8	*Pseudomonas* sp./ *P. fluorescens*
Az-18	*Rhizobium* sp.
Az-19	*Pseudomonas* sp.
Az-48	*Enterobacter* sp.
Az-50	*Pseudomonas* sp.
Az-63	*Advenella* sp.

Table 3. Results of some Biochemical Tests for the Selected Isolates

Isolates	Urease	Nitrate reductase	Oxidase	Catalase	Amylase	Hydrogen sulfide	Citratase	Indole	Glucose	Lactose	Sucrose	Glycerol	Gelatinase	Tryptophanase	Proline	Mannitol
Az-3	-	-	-	+	-	-	+	-	+	-	-	-	-	+	+	-
Az-6	-	+	-	+	-	-	+	-	+	-	+	+	-	+	+	+
Az-8	-	+	+	+	-	-	+	-	+	+	-	-	+	+	+	-
Az-12	-	+	+	+	-	-	+	-	-	-	-	-	-	+	+	-
Az-13	-	+	-	+	-	-	-	-	+	-	-	-	-	+	+	-
Az-15	+	+	-	+	-	-	-	-	+	-	-	-	-	+	+	-
Az-18	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Az-19	-	+	+	+	-	-	+	-	+	-	-	-	-	+	+	-
Az-21	-	+	-	+	-	-	+	-	+	-	+	+	-	+	+	+
Az-37	-	-	+	+	-	-	+	-	+	+	-	-	-	-	+	-
Az-48	-	+	-	+	-	-	+	-	+	+	+	+	-	+	+	+
Az-50	-	+	+	+	-	-	+	-	+	-	+	-	+	+	+	-
Az-63	-	-	+	+	-	-	+	-	-	-	-	-	-	-	+	-
Az-65	-	-	+	+	-	-	+	-	+	-	-	-	-	-	+	-
Az-73	-	-	-	+	-	-	+	-	+	-	-	-	-	-	+	-

Discussion and Conclusions

Phosphate Solubilization: The ability of a few soil microorganisms to transform insoluble forms of phosphorus to an accessible form is an important trait in PGPs for increasing plant yields (26). In the present study, phosphate-solubilizing activities of the isolates against two types of insoluble phosphate were quantitatively determined and the amount of P-solubilization by isolates varied from 98.13 to 340.13 mg/l (Figure 2). Maximum P solubilization was observed by Az-65 (340.13 mg/l) followed by Az-50 (329.38 mg/l), Az-65 (302.00 mg/l), and Az-71 (282.25 mg/l) (Figure 2). P solubilization by the bacterial isolates was significantly influenced by the sources of P used in the broth medium. Except for Az-11 and Az-13, all other bacterial isolates showed higher P solubilizing activity in the presence of TCP compared to RP (Table 1). The results showed that there were no significant differences in phosphate solubilization of RP and TCP among Az-11, Az-12, and Az-13 (Table 1). It seems that these isolates had a higher potential for P solubilization, however, further research is required to study the P solubilization in the presence of different sources of phosphorus. The plant growth-promoting rhizobacteria were able to solubilize insoluble phosphate compounds such as tri-calcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (27). Kumari et al. (28) reported that the TCP was higher soluble than RP. The presence of free carbonates in rock phosphate led to decreasing in P solubility. Rock phosphate had a crystalline structure containing apatite that is solubilized slowly, whereas TCP with simple structure was solubilized easily (28).

It is generally believed that the main mechanism of the mineral phosphate solubilization is the release of microbial metabolites such as organic acids like gluconic, 2-Ketogluconic, oxalic, succinic acids (29). There are few reports on the effect of PSB on the decreasing pH of the medium. This may be due to the varied ability of bacterial isolates in the production of organic acids. Organic acids are mainly involved in the P solubilization process (30). Similar observations have been reported on the P solubilization by different bacterial genera in the presence of different sources of phosphorus (31).

IAA Production: In this study, the ability of auxin production by bacterial isolates in two levels of tryptophan (presence and absence of tryptophan) was examined. Although bacterial isolates were not able to produce high amounts of IAA, there was an increase in the IAA production with the addition of tryptophan. The largest amount of IAA was for Az-3 (2.28 mg/l) and the lowest IAA was produced by Az-48 (0.75 mg/l) (Figure 3). The results illustrated the significant effect of tryptophan on IAA production, as shown in Table 1. The presence of tryptophan resulted in significantly higher IAA (30%) compared to the absence of tryptophan. All of the bacterial isolates showed the significant capability of IAA production, compared with the control (Table 1). Bacterial IAA biosynthesis perhaps could be a tryptophan dependent or independent process (32). Tryptophan is an amino acid that is commonly found in plant root exudates and it is generally considered as the IAA precursor (33). Many studies have shown that the amount of auxin produced by bacterial isolates generally increases by an increment of tryptophan concentration (32). The presence of environmental factors including tryptophan, vitamins, salt, oxygen, pH, temperature, carbon source, nitrogen source, and bacterial growth phase are all contributing factors in the regulation of IAA biosynthesis (32). Shokri and Emtiazi (34) reported that the

most important factor that had impacts on IAA production was the nitrogen source, although the production of IAA by bacterial isolates varies among different species and strains of the same species.

K Release: The ability of 24 bacterial strains was assessed in Aleksandrov medium containing biotite and muscovite as sources of insoluble potassium. The ability of potassium release from biotite and muscovite minerals in Aleksandrov medium has been evaluated by some researchers (19). In this experiment, the greatest K released content was obtained by biotite inoculated with the Az-65 (Figure 4). Results indicated that the studied isolates could potentially promote the solubilization of K when biotite was used as the source of potassium (Table 1). The amount of K released by isolates was 4.7 times higher than when muscovite was used. It could be attributed to the tri-octahedral structure of biotite (27). Except for Az-21, Az-59, and Az-65, there was not any significant increase in K release by bacterial isolates (Figure 4). Potassium release from minerals was affected by various environmental factors. Mechanism of K-solubilization by microorganisms could be mainly attributed to excrete organic acids which are able to solubilize unavailable forms of K-bearing minerals, such as micas, illite, and orthoclases (27) and the production of protons, organic acids, siderophores, exopolysaccharides, and organic ligands.

Recent studies have shown that organic exudates of few bacteria also have a key role in the release of potassium from K-bearing minerals (12). Microorganisms were able to solubilize K minerals such as illite and feldspar through the production and excretion of organic acids. Studies on the release of interlayer potassium of pure minerals have shown that interlayer potassium in tri-octahedral micas released easier than dioctahedral micas (35). Dioctahedral micas were normally more resistant to weathering than tri-octahedral micas (35).

Siderophore Production: Another growth promoting property of bacteria that indirectly influenced the plant growth was the production of siderophore. In the quality assessment of siderophore production in our study, none of the bacterial isolates produced an orange halo on CAS-agar. Many researchers reported positive and negative results in assessing the siderophore production by different bacterial strains (36). On the other hand, siderophore biosynthesis by bacteria was a complex biosynthetic pathway. Both the amount of iron present in the environment and iron acquired by the bacteria were major factors affecting siderophore production (37). Many factors affected siderophore production including iron, nitrogen and carbon sources and other metal ions concentrations (38).

Identification of Selected Endophytic Bacteria: Molecular identification of selected isolates was done using sequencing of amplified 16S rDNA. The BLAST-n of sequences in GenBank revealed that Az-8, Az-19, and Az-50 belonged to the *Pseudomonas* genus, Az-3 belonged to the *Acinetobacter* genus, Az-18 belonged to the *Rhizobium* genus, Az-48 belonged to the *Enterobacter* genus and Az-63 belonged to the *Advenella* genus (Table 2). Furthermore, bacterial isolates were biochemically characterized by some biochemical tests and were identified as summarized in (Table 3). All the isolated bacteria were Gram-negative.

The bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Flavobacterium*, *Pseudomonas* and *Rhizobium* belonged to the plant growth-promoting rhizobacteria. In similar studies, different bacterial genera and species have been isolated (39).

In this study, we used some grassroots to isolate endophytic bacteria. A method of isolation according to the formation of a rising pellicle in semisolid NFB media (a trait of nitrogen-fixing bacteria) was the main indicator to isolate endophytic bacteria. Our results revealed that broad ranges of bacteria from the different genus (such as *Pseudomonas*, *Acinetobacter*, *Rhizobium*, *Enterobacter*, and *Advenella*) were able to grow in these conditions. *In vitro* assessment of PGP properties including phosphate solubilization, IAA production, releasing of potassium and siderophore production showed that although none of these bacteria produced siderophore, but P solubilization, K releasing and IAA production of some of these bacteria could be concerned. However, the PGP potentials of selected endophytic bacteria should be examined in the natural condition. According to these findings, we suggest its application for pot and field experiments to evaluate its efficiency under field conditions.

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