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Characterization of potential plant growth-promoting rhizobacteria isolated from sunflower (*Helianthus annuus* L.) in Iran

Nabi Khezrinejad[1], Gholam Khodakaramian[2] and Fatemeh Shahryari[1]

[1]Department of Plant Protection, Faculty of Agriculture, University of Zanjan, Zanjan, Iran
[2]Department of Plant Protection, Faculty of Agriculture, Buali Sina University, Hamedan, Iran

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Purpose: This study aims to characterize plant growth-promoting rhizobacteria (PGPR) in sunflowers growing in different locations at North West of Iran. *Materials and methods:* Sunflower plants were collected from different regions of West Azarbaijan, and rhizospheric bacterial strains were isolated and screened for PGP traits. Identification and characterization of the PGPR were conducted based on 16s rDNA sequences and phenotypic analysis, the strains clustered for genetic diversity by rep-PCR method. *Results:* Among the 80 bacterial isolates, 20 showed PGP traits and were selected for other potentials. All the selected isolates produced indole-3-acetic acid at the rate of 9.2–33.7 mg/ml. In addition, 13, 15, 12, and 16 were positive for phosphate solubilization, siderephore, hydrogen cyanide, and ammonia production, respectively. The results from a subsequent pot experiment indicated that PGPRs distinctly increased sun flower shoot and root length, shoot and root fresh weight, as well as shoot and root dry weight. Based on 16S rDNA sequences and biochemical and physiological characteristics, 20 PGPRs were identified as *Pseudomonas fluorescens* (five isolates), *Pseudomonas aeruginosa* (four isolates), *Pseudomonas geniculata* (one isolate), *Bacillus subtilis* (four isolates), *Bacillus pumilus* (two isolates), *Stenotrophomonas maltophilia* (two isolates), and *Brevibacterium frigoritolerans* (two isolates). In rep-PCR, PGPR isolates were differentiated into seven clusters (A–G) at 65% similarity level. These results demonstrated the existence of a considerable species richness and genetic diversity among PGPRs isolated from different regions of North West of Iran. *Conclusions:* To the best of our knowledge, this is first report for the identification and characterization of *B. frigoritolerans* as PGPR in sunflower plants.

Original Article

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Author for correspondence:
Gholam Khodakaramian
e-mail: Khodakaramian@yahoo.com

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the third most productive oleaginous plants in the world and has an increasing economic and agronomic importance. It is an important crop in Iran, which is grown 40,000 ha annually (FAO, 2016). West Azarbaijan province is one of the main sunflower growing centers in Iran with 15.6% of area under cultivation and 24% of total production (Ghaffari, 2006). The great adaptability to the various environmental conditions and high yield of oil in sunflower have made it to one of the most important plants producing oil in the world.

Intensive farming practices that achieve high yield require high inputs as well as continuous application of chemical fertilizers in agroecosystems. However, the concerns relative to the water and soil quality, the ever-increasing prices, and availability of these chemical fertilizers become the limiting factor for crop production especially in developing countries.

In the past decade, the use of chemical fertilizers had adverse environmental effects, such as water and soil pollution, and had negative effect on the health status of humans and other living organisms (Gerhardt et al., 2009). The use of microorganisms currently called plant growth-promoting rhizobacteria (PGPR) may provide alternative solutions (Dinesh et al., 2015). PGPR represents a wide variety of rhizosphere-inhabiting bacteria, which colonize the root systems of plants and can stimulate plant growth indirectly by prevention of the deleterious effects of phytopathogenic microorganisms, or directly by nutritional mechanisms (Rodríguez-Díaz et al., 2008; Yuan et al., 2013), so they are commonly used as biofertilizers in agriculture. However, studies regarding

the species diversity of PGPR in association with oilseed crops are scarce, with fewer reports performed on sunflower (Ambrosini et al., 2012).

Among the processes that contribute to increasing nutrient availability to plant roots, phosphorus solubilization (Kloepper et al., 1988), siderophore production (Dimkpa et al., 2008), indole-3-acetic acid (IAA) production (Glickmann & Dessaux, 1995), and nitrogen fixation (Roesch et al., 2007) by soil and rhizosphere microorganisms are the recognized mechanisms of plant growth promotion due to the importance of limiting factors for crop productivity. In recent years, species of *Agrobacterium*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Erwinia*, *Micrococcous*, *Pseudomonas*, and *Serratia* have been reported to dominate the PGPR population associated with different plant species (Dinesh et al., 2015; Sun et al., 2017).

Our objectives of this study were (a) to isolate and identify native PGPRs from the sunflower rhizosphere in different locations at west Azarbaijan province, based on morphological and physiological characteristics as well as data obtained from 16S rRNA sequences; (b) to assess the plant growth-promoting ability of isolates under greenhouse conditions; and (c) to determine genetic diversity of PGPR isolates by BOX, Enterobacterial Repetitive Intergenic Consensus (ERIC), and repetitive extragenic palindromic polymerase chain reaction (REP-PCR).

MATERIALS AND METHODS

Sampling and isolation of rhizosphere bacteria

The soil–root samples used in this study were collected from nine different sunflower growing regions of West Azarbaijan province (North West of Iran including Khoy, Miandoab, and Ourmieh regions). At each region, five different fields were selected, and at each field, three plant roots with surrounding soil (10 m apart from each other) were collected. The samples including plant roots and 300 g of rhizosphere soil were poured into a sterile stomacher bag, labeled, placed in an ice box, and transported to the laboratory. In the laboratory and after removing plant material and coarse roots, the soils were divided into two subsamples, one for estimation of moisture content and the other for microbial analysis.

Isolation of the bacteria was followed according to the method used by Speck (1976). For this, 10 g of each soil sample was added into an Erlenmeyer flask containing 90 ml of sterile distilled water and was vigorously shaked for about 30 s to obtain stock solution. Serial dilutions up to 10^{-8} were prepared. An aliquot (0.1 ml) from each dilution was streaked all over the Petri plates containing nutrient agar (NA) medium and incubated at 28 °C until the appearance of bacterial colonies.

Phenotypic identification of PGPR isolates

Identification of the isolated rhizobacteria was performed based on classical morphological and biochemical characteristics, such as colony morphology, pigmentation, Gram reaction, motility, cell shape, production of oxidase, catalase, indole and urease reactions, utilization of sole carbon and nitrogen sources, and tolerance to different concentrations of NaCl and pH ranges (Cappuccino & Sherman, 1992). In addition, *Pseudomonas fluorescens* ATCC 13525 was used as reference strain.

Growth-promoting properties of bacterial isolates

Indole-3-acetic acid production

For detection and quantification of IAA production, isolated bacteria were inoculated into Jensen's broth (20 g of sucrose, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$ $7H_2O$, 0.5 g of NaCl, 0.1 g of $FeSO_4$, 0.005 g of $NaMoO_4$, 2 g of $CaCO_3$, and 1 L of distilled water; Brick et al., 1991) containing 2 mg/ml L-tryptophan. The cultures were incubated at 27 °C with continuous shaking at 125 rpm for 48 hr (Rahman et al., 2010). Approximately 2 ml of culture solution was centrifuged at 15,000 rpm for 1 min, and a 1-ml aliquot of the supernatant was mixed with 2 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water, and 7.5 ml of 0.5 M $FeCl_3.6H_2O$) and incubated for 20 min in darkness at room temperature as described by Gordon and Weber (1951). IAA production was observed as the development of a pink–red color, and the absorbance was measured at 530 nm using a spectrophotometer. The concentration of IAA was determined using a standard curve prepared from pure IAA solutions (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, and 65 µg/ml).

NH3 production

NH_3 production was determined by the method described by Cappuccino and Sherman (1992). Briefly, 50 ml of bacterial cell suspension was added to 30 ml of peptone broth (4%) and incubated at 25 °C for 72 hr. Then, 1 ml of Nessler's reagent (50 g of potassium iodide, 35 ml of saturated mercuric chloride, 25 ml of distilled water, and 400 ml of 40% potassium hydroxide) was added. The formation of yellow to brown precipitate indicated the presence of NH_3.

Hydrogen cyanide production

Hydrogen cyanide production was determined following the method described by Lorck (1948). Each isolate was streaked on NA medium supplemented with glycine (4.4 g/L). The agar surface was covered with a Whatman number 1 filter paper, which was previously soaked in a specific solution (0.5% picric acid and 2% sodium carbonate w/v). Plates were sealed with parafilm and incubated at 36 ± 2 °C for 4 days. The appearance of orange or red color indicated the production of hydrogen cyanide (HCN).

Solubilization of phosphate (P)

Solubilization of P was tested using Pikovskaya's agar medium (10 g of glucose, 5 g of calcium phosphate, 0.5 g of ammonium sulfate, 0.2 g of potassium chloride, 0.1 g of magnesium sulfate, 0.5 g of yeast extract, 15 g of agar, and 1,000 ml of distilled water). After sterilization and pouring the medium in 8-cm Petri plates, one loop full of the 24 hr broth culture of each bacterial strain was spot inoculated onto the plates. Inoculated plates were incubated at

28 °C for 96 hr. The plates were observed for the zone of clearance around the bacterial colony, which indicated solubilization of P. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone (Gaur, 1990).

Siderophore production

An amount of 2 µl of overnight grown culture of each isolate (10^6 CFU/ml) was spot inoculated on chrome azurole S agar plates (Schwyn & Neilands, 1987) and incubated at 30 °C for 4–5 days. The appearance of an orange color around bacterial growth indicated siderophore production. All the experiments were conducted in triplicate.

Evaluation of PGP abilities in greenhouse conditions

For these experiments, 20 selected isolates based on *in vitro* tests along with un-inoculated controls and two levels of N and P fertilizer (1/2NP and full NP fertilizers) (Shemshad shimi company, Iran) were used. The seeds were surface sterilized and inoculated by immersing in PGPR suspensions for 1 hr. Clean earthenware pots (20-cm height and 15-cm depth) were filled with a sterilized soil (4:1 soil: sand). Four to five air-dried surface sterilized seeds were sown in each pot. The experiment was planned in a completely randomized design with 23 treatments, each with three replications and total of 69 pots. Treatments included: (a) Kh2 + 1/2NP, (b) Kh3 + 1/2NP, (c) Kh5 + 1/2NP, (d) Kh6 + 1/2NP, (e) Kh7 + 1/2NP, (f) Kh8 + 1/2NP, (g) Kh9 + 1/2NP, (h) Kh10 + 1/2NP, (i) Kh11 + 1/2NP, (j) Kh14 + 1/2NP, (k) Kh15 + 1/2NP, (l) Kh16 + 1/2NP, (m) Kh18 + 1/2NP, (n) Kh19 + 1/2NP, (o) Kh22 + 1/2NP, (p) Kh23 + 1/2NP, (q) Kh24 + 1/2NP, (r) Kh25 + 1/2NP, (s) Kh26 + 1/2NP, (t) Kh27 + 1/2NP, (u) control (without NP and inoculation), (v) 1/2NP, and (w) full NP. Nitrogen and phosphorus fertilizers were applied at the rate of 60 and 45 mg/kg (full dose) in the form of urea and single super phosphate, respectively. Pots were kept under greenhouse conditions and equally irrigated when needed. The plants were harvested at 30 and 60 days after germination, and shoot and root length, shoot and root fresh, as well as dry weights were measured.

Statistical analysis

The data were statistically analyzed using analysis of variance (ANOVA) for individual parameters on the basis of mean values to find out the significance at 5% statistical level. The standard error of the mean, variance, and ANOVA statistics were calculated using SPSS software, version 17.0 (IBM, Stanford, CA, USA).

rep-PCR pattern analysis

The genomic DNA from the selected PGPR isolates was extracted by alkaline lysis method (Rouhrazi & Rahimian, 2015). rep-PCR was performed under the BOX-A1R-based repetitive extragenic palindromic-PCR, ERIC-PCR, and REP-PCR conditions, using the BOX primer: BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′), ERIC primers: ERIC-1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′), REP primers: REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′; Versalovic et al., 1994). PCR was performed in a final volume of 25 µl containing 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of each primers, 2.5 µl of 10× buffer (100 mM of Tris–HCl, 500 mM of KCl, pH 8.4), 1.25 U of Taq DNA polymerase (CinnaGen, Iran), and 3 µl of template DNA. Temperature and

Table 1. Growth-promoting properties and clustering of 20 PGPR isolates

	Identification based on 16S rRNA gene sequencing	IAA production (mg/ml)[a]	HCN production	Phosphate solubilization	Ammonia production	Siderephore production	Rep-PCR cluster
Kh2	*S. maltophilia*	9.2 ± 0.8	−	+	+	−	G
Kh3	*P. aeruginosa*	18.5 ± 1.2	+	+	+	+	B
Kh5	*B. pumilus*	19.3 ± 0.12	−	+	+	−	E
Kh6	*B. frigoritolerans*	22.4 ± 0.5	−	+	+	+	F
Kh7	*P. geniculata*	20.1 ± 0.68	+	−	+	+	C
Kh8	*P. aeruginosa*	25.4 ± 2.1	+	−	+	+	B
Kh9	*B. subtilis*	29.62 ± 3.2	+	+	+	−	D
Kh10	*B. frigoritolerans*	23.5 ± 1.8	−	−	+	+	F
Kh11	*B. subtilis*	32.11 ± 0.05	−	+	−	+	D
Kh14	*P. fluorescens*	34.1 ± 0.3	+	+	+	+	A
Kh15	*B. subtilis*	28.2 ± 0.9	+	+	+	+	D
Kh16	*B. pumilus*	19.8 ± 2.14	−	+	−	+	E
Kh18	*S. maltophilia*	11.2 ± 1.3	−	+	+	+	G
Kh19	*P. fluorescens*	31.1 ± 2.3	+	−	+	−	A
Kh22	*P. fluorescens*	30.1 ± 0.3	+	+	+	+	A
Kh23	*P. fluorescens*	32.5 ± 1.5	+	−	+	+	A
Kh24	*P. fluorescens*	33.7 ± 1.6	+	−	−	+	A
Kh25	*P. aeruginosa*	27.5 ± 2.5	+	−	+	+	B
Kh26	*P. aeruginosa*	28.84 ± 0.2	+	+	+	−	B
Kh27	*B. subtilis*	18.2 ± 2.1	−	+	−	+	D

Note. HCN: hydrogen cyanide; PGPR: plant growth-promoting rhizobacteria; IAA: indole-3-acetic acid; PCR: polymerase chain reaction.
[a]Average values of three independent experiments for each isolate in triplicate ± standard deviation.

Table 2. Physiological and biochemical characteristics of PGPR isolates and reference strain

Characteristic	Reaction of PGPR isolates																				
	Kh2	Kh3	Kh5	Kh6	Kh7	Kh8	Kh9	Kh10	Kh11	Kh14	Kh15	Kh16	Kh18	Kh19	Kh22	Kh23	Kh24	Kh25	Kh26	Kh27	*P. fluorescens* ATCC 13525
Bacteria shape	Rod	Rod	Rod	Long rods	Short rods	Rod	Rod	Long rods	Rod	Short rods	Rod	Rod	Rod	Short rods	Short rods	Short rods	Short rods	Rod	Rod	Rod	Short rods
Gram reaction	−	−	+	+	−	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−
Catalase	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	−	+	−	−	+	+	−	−	−	+	−	−	−	+	+	+	+	+	+	−	+
Starch hydrolysis	+	+	−	+	+	+	−	+	−	+	−	−	+	+	+	+	+	+	−	−	+
Casein hydrolysis	+	+	+	+	−	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−
Methyl red	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Indole	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tolerance to pH																					
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	+	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	−	−	+	−	−	+	+	−	−	+	−	+	−	−	−	−	−	−	+	−
10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NaCl tolerance																					
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
7	−	−	+	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	+	−
10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Temperature growth (°C)																					
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40	−	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	+	+	+	−
45	−	−	+	+	−	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−
55	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Utilization of																					
Xylose	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−
Ribose	+	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+
Fructose	+	−		+	−	−	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−
Sorbitol	−	+	−	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Raffinose	+	+	−	−	−	+	−	−	−	+	−	−	+	+	+	+	+	+	+	−	+

(Continued)

Table 2. (Continued)

Characteristic	Reaction of PGPR isolates																				*P. fluorescens* ATCC 13525
	Kh2	Kh3	Kh5	Kh6	Kh7	Kh8	Kh9	Kh10	Kh11	Kh14	Kh15	Kh16	Kh18	Kh19	Kh22	Kh23	Kh24	Kh25	Kh26	Kh27	
D-Maltose	+	−	+	+	−	−	+	+	−	+	−	−	−	+	+	+	+	−	−	−	+
Trehalose	−	−	−	−	+	−	−	−	+	+	+	+	−	+	+	+	+	−	−	+	+
Sucrose	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arginine	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Phenylalanine	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
L-Histidine	+	−	+	+	−	−	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−
Protease	−	+	+	+	−	−	−	+	+	+	−	+	−	+	+	+	+	+	+	−	−
Cellulase	+	−	+	+	−	−	+	+	+	−	+	+	−	+	−	−	−	−	−	+	+
Chitinase	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	−	−	+	+

Note. PGPR: plant growth-promoting rhizobacteria.

time profiles of the amplification reactions were as: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 40, 52, or 50 °C for 1 min with REP, ERIC, or BOX primers, respectively, extension at 72 °C for 1 min and final extension step for 10 min at 72 °C. PCR products were electrophoresed in 2% agarose gels in TEB buffer (90 mM of Tris base, 2 mM of EDTA, 90 mM of boric acid, pH 8.3) at 85 V and stained with ethidium bromide (0.5 μg/ml). Gels were photographed under UV light.

For each individual isolate, the presence or absence of each band was determined and designated 1 if present or 0 if absent to obtain binary banding data. Similarity matrices from binary banding data of each of the five primer combinations were calculated with the similarity for qualitative data program (SIMQUAL) in the Numerical Taxonomy and Multivariate analysis System for personal computer (NTSYS-pc, IBM, Kansas, USA) version 2.0 (Rohlf, 1993). Estimates for similarity were based on Jaccard's coefficient. Matrices of similarity were analyzed using unweighted pair group method with arithmetic averages (UPGMA) clustering method. Dendograms were generated with the tree option of NTSYS-pc and goodness of fit was calculated using COPH and MXCOPH programs (Rohlf, 1993).

16S rRNA sequence analysis

Eubacterial primer pairs, fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′), which correspond to *Escherichia coli* 16S rRNA gene region, were used for PCR amplification as described by Weisburg et al. (1991). Amplified PCR products were resolved on 1% agarose gel. The PCR products were sent to Takapouzist Ltd., Iran for sequencing. The sequences obtained were compared with those from GenBank, using the BLASTn program and EzTaxon-e server (Kim et al., 2012). These sequences and those of related bacteria were aligned using the Clustal W program. The distances were calculated according to Kimura's two-parameter model (Kimura, 1980). The phylogenetic trees were inferred using the neighbor-joining model (Saitou & Nei, 1987) and MEGA5.0 (Mega, Molecular Evolutionary Genetics Analysis, PA, USA; Tamura et al., 2011) was used for all the phylogenetic analyses. All the sequences obtained in this study were deposited in GenBank and their accession numbers are provided.

RESULTS

Isolation and identification of PGPR isolates

In this study, 80 bacterial isolates were isolated from the rhizosphere of sunflower plants. Among the isolated bacteria, 20 had plant growth-promoting potentials (Table 1). These isolates produced IAA at the rate of 9.2–34.1 mg/ml, with isolates kh2 and kh24 that produced the lowest and highest amounts, respectively. Thirteen isolates showed clear zone of phosphate solubilization on Pikovskaya's agar plate amended with bromophenol blue. In addition, 12, 15, and 16 isolates were positive for HCN, siderophore, and ammonia production, respectively (Table 1). Some phenotypic characteristics of the PGPR isolates are listed in Table 2.

Table 3. Effect of inoculation with PGPR on the growth of sunflower grown in pots under greenhouse conditions

Treatments	Root length (cm)	Shoot length (cm)	Root fresh weight (g/plant)	Shoot fresh weight (g/plant)	Root dry weight (g/plant)	Shoot dry weight (g/plant)
Kh2 + 1/2NP	34.6^{e}	63.8^{j}	41.9^{c}	41.8^{h,i}	7.6^{e}	24.3^{d}
Kh3 + 1/2NP	29.8^{f}	64.3^{j}	35.5^{g}	47.3^{e}	10.2^{b}	23.3^{e}
Kh5 + 1/2NP	27.4^{g}	67.6^{i}	39.6^{de}	50.5^{c}	9.9^{c,d}	21.6^{g}
Kh6 + 1/2NP	38.1^{b}	75.3^{h}	43.4^{c}	53.1^{b}	10.4^{b}	26.7^{b}
Kh7 + 1/2NP	37.5^{c}	80.8^{f}	39.2^{de}	47.5^{e}	8.7^{d}	24.3^{d}
Kh8 + 1/2NP	31.6^{f}	71.3^{g}	44.8^{b}	45.8^{f}	7.9^{e}	25.4^{c}
Kh9 + 1/2NP	39.2^{b}	64.6^{j}	40.7^{d}	42.1^{h,i}	9.3^{c}	20.5^{h}
Kh10 + 1/2NP	37.4^{c}	79.3^{f}	37.6^{f}	52.4^{b}	7.5^{e}	23.4^{e}
Kh11 + 1/2NP	36.4^{cd}	82.5^{e}	44.1^{b}	50.3^{c}	10.5^{b}	22.9^{ef}
Kh14 + 1/2NP	27.9^{g}	89.6^{c}	37.2^{f}	45.3^{f}	7.7^{e}	17.6^{j}
Kh15 + 1/2NP	33.2^{e}	90.3^{c}	30.7^{h}	44.3^{g}	8.6^{d}	23.2^{e}
Kh16 + 1/2NP	39.9^{b}	84.7^{d}	34.8^{g}	45.1^{f}	9.4^{c}	20.1^{h}
Kh18 + 1/2NP	35.6^{cd}	93.4^{b}	42.9^{c}	52.1^{b}	8.2^{d}	24.5^{d}
Kh19 + 1/2NP	40.3^{b}	79.4^{f}	33.7^{g}	47.2^{e}	5.9^{g}	18.7^{ij}
Kh22 + 1/2NP	38.5^{b}	82.1^{e}	37.1^{f}	50.7^{c}	7.9^{e}	20.3^{h}
Kh23 + 1/2NP	29.8^{f}	80.0^{f}	34.2^{g}	48.3^{d}	10.1^{b}	25.7^{c}
Kh24 + 1/2NP	47.1^{a}	97.6^{a}	48.3^{a}	56.9^{a}	11.1^{a}	27.2^{a}
Kh25 + 1/2NP	36.7^{c,d}	93.8^{b}	39.3^{de}	45.5^{f}	8.9^{d}	22.3^{f}
Kh26 + 1/2NP	33.5^{e}	82.6^{e}	38.4^{e}	43.2^{h}	7.8^{e}	20.1^{h}
Kh27 + 1/2NP	29.7^{f}	77.2^{g}	42.1^{c}	40.5^{j}	6.9^{f}	21.5^{g}
Zero control	23.6^{h}	50.8^{k}	28.6^{i}	35.3^{k}	4.1^{h}	10.2^{k}
1/2NP control	30.1^{f}	68.9^{i}	34.2^{g}	42.9^{h,i}	7.5^{e}	19.5^{i}
Full NP control	49.2^{a}	99.8^{a}	49.7^{a}	57.3^{a}	11.4^{a}	27.6^{a}
LSD ($p \leq .05$)	1.92	2.70	1.84	1.62	1.1	1.56

Note. Samplings for the above characteristics were taken twice, i.e., 30 and 60 days after germination and the values presented are the average of the two samplings. The values with different superscript letters in the columns are significantly different ($p < .05$). PGPR: plant growth-promoting rhizobacteria; LSD: least significant difference.

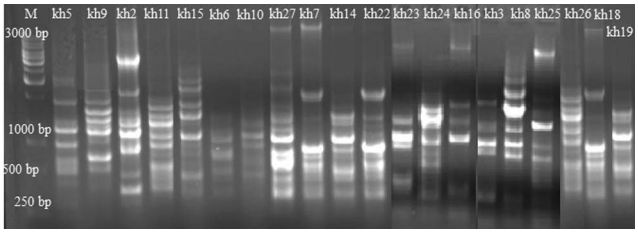

Fig. 1. PCR fingerprinting patterns of genomic DNA of PGPR isolates from sunflower in Iran generated by ERIC (ERIC-1R and ERIC-2) primers in 2% agarose gel. M: bp molecular size marker (1-kb ladder, Fermentas)

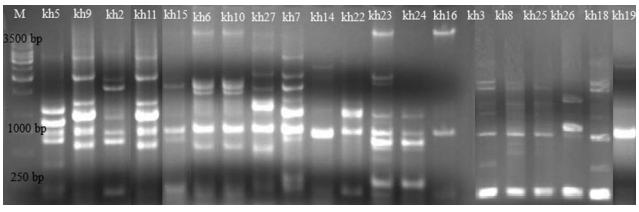

Fig. 2. PCR fingerprinting patterns of genomic DNA of PGPR isolates from sunflower in Iran generated by REP (REP1R-I and REP2-I) primers in 2% agarose gel. M: bp molecular size marker (1-kb ladder, Fermentas)

Evaluation of PGP abilities in greenhouse conditions

The results indicated that all isolates significantly ($p \leq .05$) increased the growth of maize compared to the control and in some cases to that recorded under 1/2NP treatment (Table 3). Co-inoculation of selected isolates along with 50% reduced fertilizer dose (1/2NP) in comparison with control (un-inoculated) and two levels of NP fertilizer (1/2NP and full NP fertilizer) showed plant growth-promoting potential (Table 3). Different bacterial isolates had different PGP activities and the measured parameters were significantly ($p \leq .05$) different. The shoot/root length as well as shoot/root fresh and dry weights were highest in Kh24 + 1/2NP and full NP treatments. Most of the isolates when combined with 1/2NP showed significantly higher growth characteristics compared to the treatments supplemented with 1/2NP.

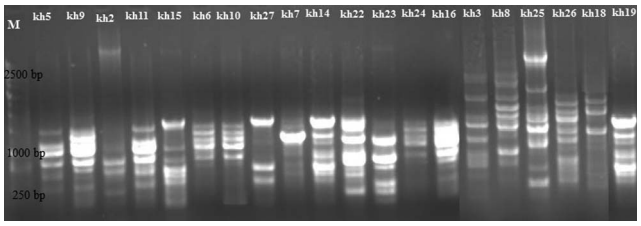

Fig. 3. PCR fingerprinting patterns of genomic DNA of PGPR isolates from sunflower in Iran generated by BOX (BOXA1R) primer in 2% agarose gel. M: bp molecular size marker (1-kb ladder, Fermentas)

rep-PCR analysis

Banding patterns of PGPR isolates were generated using the BOXA1R, ERIC, and REP primers. The amplified bands in

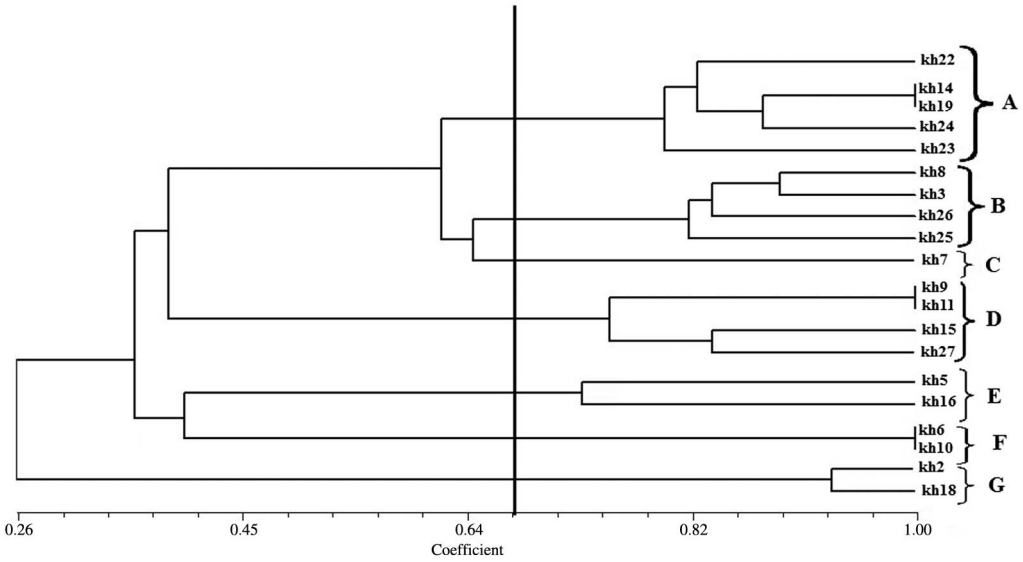

Fig. 4. Dendrograms illustrating the relationship between 20 PGPR isolates from sunflower in Iran based on combined REP, BOX, ERIC amplification patterns and using UPGMA clustering and Jaccard's coefficient

ERIC ranged in size from 150 to 3,000 bp (Fig. 1), whereas for the REP and BOX, they ranged between 100 to 3,500 bp and 250–2,500 bp, respectively (Figs 2 and 3). A dendrogram calculated with Jaccard's similarity coefficients with UPGMA clustering for the combination of rep-PCR results grouped PGPR isolates into seven clusters (A–G) at 65% similarity level (Fig. 4). Cluster A includes five isolates from the Khoy and Urmia regions, cluster B includes four isolates from the Maku region, cluster C includes one isolate from the Urmia region, cluster D consists of four isolates from the Bukan region, cluster E includes two isolates from the Piranshahr region, and cluster F includes two isolates from the Mahabad region.

The cophenetic correlation coefficient for the dendrogram obtained from the combined data set of all primers was 0.949. The percentage of polymorphic loci was 75.21%, 68.4%, and 78.9% using the BOX, ERIC, and rep-PCR primers, respectively. These results demonstrated the existence of a considerable genetic diversity among PGPR isolates, isolated from different regions of West Azarbaijan province in Iran.

16S rRNA sequence analysis

The sequence analysis of a 1.5-kb fragment of 16S rRNA gene of PGPR isolates grouped them with high-similarity indices with reference isolates of several bacterial species (Fig. 5). Isolates Kh14 (GenBank accession no. MG817405), Kh19 (MG817406), Kh22 (MG982452), Kh23 (MG982453), and Kh24 (MG982454) had 99%–99.4% sequence similarity with that of *P. fluorescens*. Isolates Kh3 (MG817457), Kh8 (MG817458), Kh25 (MG982485), and Kh26 (MG982486) showed 99.1%–99.6% similarity with *Pseudomonas aeruginosa*. Isolates Kh2 (MG819159) and Kh18 (MG819160) were closely related to *Stenotrophomonas maltophilia* with 99% sequence similarity. Isolate Kh7 (MG817388) was closely related to *P. geniculata* with 99.8% sequence similarity. The isolates Kh6 (MG818473) and Kh10 (MG818473) showed 99.2% similarity with *Brevibacterium frigoritolerans*. Isolates KH5 (MG818473) and Kh16 (MG818474) were related to *Bacillus pumilus* with 99.4% sequence similarity. Isolates Kh9 (MG817385), Kh11 (MG817386), Kh15 (MG817387), and Kh27 (MG982489) were closely related to *Bacillus subtilis* with 99.2%–99.6% sequence similarity.

DISCUSSION

It is shown that PGPRs which are colonizing the surface or inner parts of plant roots generally use one or more indirect or direct mechanisms to promote the health and growth of plants. Our results showed that a high proportion of rhizobateria with plant growth-promoting potentials are present in the studied area. Similar results have been reported in maize (Shaharoona et al., 2006; Sun et al., 2017), common bean (Egamberdieva, 2011), tomato (Kurabachew & Wydra, 2013), and canola (Bertrand et al., 2001).

The greenhouse results indicated that the selected isolates could promote sunflower growth by significantly ($p \leq .05$) increasing plant growth parameters, such as root/shoot fresh weight, root/shoot dry weight, and root/shoot length (Table 3). Plant growth promotion in response to PGPRs applied alone or with N or P fertilizers has been reported recently for different crops under different ecological and environmental conditions (Mehta et al., 2014).

Indirect mechanisms may involve biologically controlling plant pathogens and deleterious microorganisms, through producing antibiotics, HCN, lytic enzymes, catalase, and siderophore (Roesch et al., 2007). In this study, the tested isolates possessed multiple plant growth-promoting traits, such as P-solubilization capability, production of IAA, siderephores, HCN, and ammonia. IAA production by microbes promoted the root growth by directly stimulating plant cell elongation or cell division (Glickmann & Dessaux, 1995). The effectiveness of PGPR isolates with NP fertilizers clearly indicates that the chemical fertilizers rate or dose could be reduced

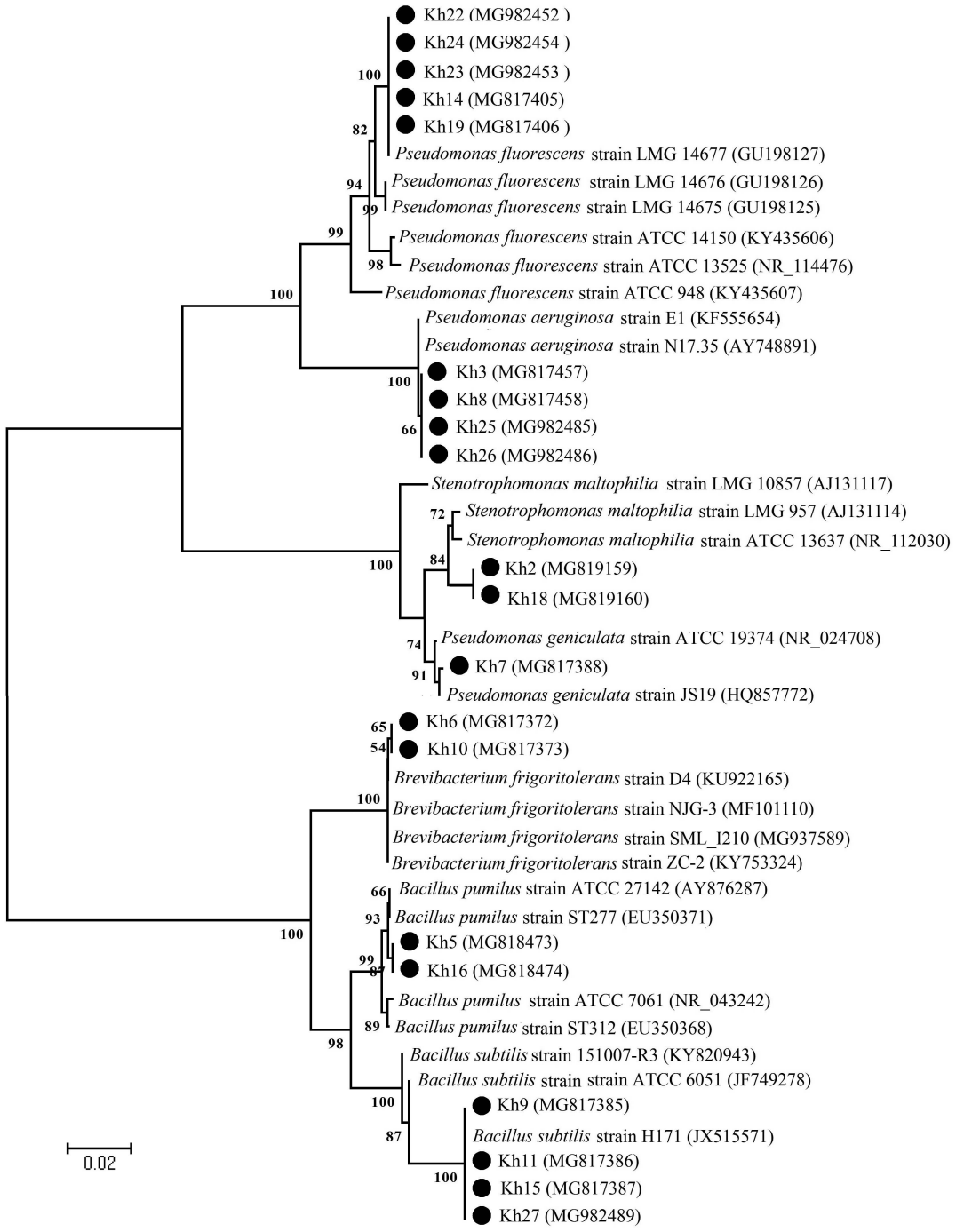

Fig. 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the PGPR isolates from sunflower in Iran, their phylogenetically. Bootstrap values calculated from 1,000 replications are indicated above the nodes. Bar: 2 nt substitution per 100 nt. Accession numbers from GenBank are given in brackets

through combination of PGPR isolates with reduced rate of fertilizers, which might be an eco-friendly and cost-effective management strategy. Therefore, these isolates might have potential in future field applications as plant growth promoters.

The results of this study demonstrated that the genera *Pseudomonas* and *Bacillus* were the prominent bacterial genera in sunflower rhizosphere in the studied area. In contrast, *Enterobacter* and *Burkholderia* were the dominant rhizospheric bacterial genera associated with sunflower plants in Brazil (Ambrosini et al., 2012). In this study, all the six isolates that were identified as two species of *Bacillus* were found to have the potential to PGP traits, since they tested positive in phosphate solubilization and IAA production assays, which together with the effect of ammonia production boost the potential to enhance plant growth. *Bacillus* species that have been used as biofertilizers have positive effects on the growth of plants as they synthesize plant growth hormones, fix nitrogen, solubilize phosphates (Cakmakci et al., 2001; Sahin et al., 2004), and increase the uptake of nitrogen, potassium, phosphorous, and iron (Biswas et al., 2000).

Morphological, biochemical, and phylogenetic analyses of other PGPR isolates indicated that they are the members of the genera *Stenotrophomonas* and *Brevibacterium*. In particular, strains of *Stenotrophomonas* have been successfully used in attempts to control plant pathogens and increased plant growth (Berg et al., 2010; Ryan et al., 2009). The two *B. frigoritolerans* (kh6 and kh10) isolates showed *in vitro* characteristics of PGPR such as phosphate solubilization, IAA, ammonia, and siderophore production. To the best of our knowledge, this is first report of the identification and characterization of *B. frigoritolerans* as PGPR.

One of the major aims of this study was to assess the applicability of the ERIC, REP, and BOX-PCR methods for the characterization of PGPRs isolated from rhizoshphere of sunflower plants. The tested primers were found to be very useful for the differentiation of the PGPR isolates mostly according to their site of origin. Although more complex DNA fingerprints should generate more reliable results, this study showed that BOX, REP, or ERIC-PCR, used alone or in combination, generated reliable and similar clusters with a high cophenetic correlation coefficient. The banding patterns of all 20 PGPR isolates showed the presence of 12, 14, and 17 polymorphic bands by the ERIC, REP, and BOX-PCR methods, respectively.

CONCLUSION FOR FUTURE BIOLOGY

This is the first report of isolation, characterization, and evaluating plant growth-promoting bacteria diversity and population on sunflower in Iran. The future work on these isolates should focus on evaluating plant growth promotion in other oilseed crops such as corn, soybean, and rapeseed.

The need of today's world is high output yield and enhanced production of the crop as well as fertility of soil to get in an eco-friendly manner. Hence, the research has to be focused on the new concept of rhizoengineering based on favorably partitioning of the exotic biomolecules, which create a unique setting for the interaction between plant and microbes. Research on nitrogen fixation and phosphate solubilization by PGPR is in progress but little research can be carried out on potassium solubilization, which is the third major essential macronutrient for plant growth. This will not only increase the field of the inoculants but also create confidence among the farmers for their use. In addition, future marketing of bioinoculant products and release of these transgenics into the environment as eco-friendly alternations to agrochemicals will depend on the generation of biosafety data required for the registration of PGPR agents. A part from that future research in optimizing growth condition and increased PGPR products, not phytotoxic to crop plants, tolerate adverse environmental condition, higher yield, and cost-effective PGPR products for use of agricultural farmer will be also helpful.

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Data Accessibility: The gene sequences were deposited in GenBank.

Competing Interests: The authors declare no conflict of interest.

Authors' Contributions: NK performed experiments, analyzed data, performed bioinformatic analyses, and cowrote the paper. GK supervised the research, designed experiments, and cowrote the paper. FS advised the research and cowrote the paper.

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