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Genetic diversity and plant growth-promoting activity of the dominant bacteria from canola plants in Western Iran

Azadeh Jamalzadeh¹, Mostafa Darvishnia^{1*}, Gholam Khodakaramian², Eydi Bazgir¹ and Doostmorad Zafari²

Abstract

Background: Plant growth-promoting bacteria (PGPB) can be used as a safe and sustainable agricultural measure to increase plant yield. In this study, cultivable rhizospheric, endophytic, and epiphytic bacteria associated with canola were isolated on nutrient agar medium. The SDS-PAGE method was used for grouping the bacterial strains. Bacterial strains with more than 80% similarity in their protein patterns were placed in the same species and selected as a representative for partial 16S rRNA encoding gene sequence analysis.

Results: It was found that the bacteria used in this study belonged to *Pseudomonas*, *Frigoribacterium*, *Sphingomonas*, *Sphingobacterium*, *Microbacterium*, *Bacillus*, and *Rhodococcus* genera. Three PGP bioactivities including siderophore production, nitrogen fixation ability, and phosphate solubilization were evaluated for 18 *Pseudomonas* representative strains. All tested strains were able to produce siderophore, although only 5 strains could fix nitrogen and none was able to solubilize phosphate. Greenhouse experiments showed that 4 bacterial strains (i.e., 8312, 642, 1313W, and 9421) significantly affected canola seed germination and seedling growth. Bacterial strain 8312, which was identified as *Pseudomonas* sp., showed the highest effect on the growth of the canola plant. These results indicated the potential of strain 8312 to be used as a biofertilizer in canola cultivation.

Conclusions: The results of this research indicated that canola-related bacteria were diverse during the flowering stage. One isolate had a significant effect on all canola plant growth factors in the greenhouse. Hence, it is recommended to investigate this strain further in field infestation experiments.

Keywords: SDS-PAGE, 16S rRNA, Gene sequence, Rapeseed, Bacteria, Growth

Background

Many efforts have been made to find a safe and secure way to increase agricultural production. In this regard, plant growth-promoting bacteria (PGPB) can be used as a safe and sustainable agricultural measure to increase plant growth and yield. This method is more economical and efficient compared to chemical fertilizers while being environmentally friendly (Pathania et al. 2020). PGP bacteria increase the growth and health of their host plants by different mechanisms. They can colonize both externally or internally in

their host plant tissues (Afzal et al. 2019). Epiphytic bacteria can establish and multiply on plant surfaces. The diversity of these bacteria is controlled by climate changes such as temperature, humidity, radiation, wind speed, and rainfall. The sizes and compositions of these bacteria are affected by the nutrient availability at a given site (Fenella and Joshi 2014). The rhizosphere is the soil volume around the plant root, which is directly impacted by the presence of plant roots. Rhizobacteria inhabit plant roots within the soil. In the rhizosphere, the diversity and behavior of microorganisms differ from those of the bulk soil (Fageria et al. 2002). Endophytic bacteria survive and thrive inside plants. A recently identified list of genes

* Correspondence: Darvishnia.m@lu.ac.ir; mdarvishnia44@yahoo.com

¹Department of Plant Protection, Lorestan University, Khorramabad, Iran
Full list of author information is available at the end of the article

showed that these bacteria are likely to be involved in endophytic behavior (Ali et al. 2014). Recent studies have highlighted the ability of endophytes to increase plant growth (Kandel et al. 2017). PGPB was increased the plant growth indirectly by suppression of phytopathogens (Hu et al. 2016) or directly. The most well-known direct mechanisms in this regard are nitrogen fixation, phosphate solubilization, production of siderophore, and phytohormones (El-Akhdar et al. 2020). For several decades, different *Pseudomonas* species have been considered plant growth-promoting bacteria. Numerous experiments have shown that siderophores produced by *Pseudomonas* species can boost plant growth. For example, Masalha et al. (2000) showed the important role of microbial siderophores in supplying iron for plants grown. Under sterile conditions, the plant showed very little growth and suffered from severe iron deficiency. Sharma et al. (2003) showed that siderophore-producing *Pseudomonas* strain GRP33 increased chlorophyll levels in plants grown under iron deficiency compared to un-inoculated plants. Vansuyt et al. (2007) demonstrated that *Pseudomonas fluorescent* C7 synthesized Fe-pyoverdine complex, which leads to an increase in the iron content inside the plant tissues and improves plant growth. Moreover, siderophores secreted by PGPB strains can protect microbial auxins from degradation and enable them to enhance plant growth (Tirry et al. 2018). Nitrogen, one of the most important and essential elements for plant nutrition and growth, is a signaling molecule. Levels of this ion influence roots development and finally the nitrogen uptake rate (De Silveira et al. 2016). Nitrogen fixation ability is an important feature of PGP bacteria that plays a key role in the bio-fertilization of crops in sustainable and nature-friendly agriculture by providing the main source of nitrogen for the plant (Zaidi et al. 2017). Many factors affect the genetic diversity of plant-associated bacterial populations such as the host plant, plant growth stage, and environmental conditions. The plant plays a key role in determining the diversity of rhizosphere and endo-rhizosphere bacteria (Farina et al. 2012).

Canola (*Brassica napus*) cultivation is increasing in different parts of Iran. Canola production is a highly valued strategy for agricultural policy-makers in Iran to decrease imports of vegetable oils (Zarafshani et al. 2017).

In this research, predominant cultivable bacteria associated with canola were isolated from different canola production fields in western Iran. Their diversity was estimated, some of the PGP properties of the selected isolates were investigated, and one of them was introduced as a bio-fertilizer.

Methods

Sampling and sample preparation

The samples were randomly collected between January 2015 and April 2016 from different canola fields (Hyola cultivar) of 5 provinces under the supervision of the agricultural organization in western Iran (Khuzestan, Ilam, Lorestan, Ardabil, and Hamedan). All samples (120 healthy plants with adhering soil that were at the flowering stage) were placed in paper bags, transported to the laboratory, and used for isolation. Five samples from each region were randomly grouped to obtain a representative composite sample. To extract epiphytic bacteria, composed samples (i.e., stems, leaves, and flowers) were separated and their dust was cleaned with cotton. Next, they were placed in 100-ml sterilized distilled water and kept on a 120-rpm shaker for 1 h. A loopful of the resulting suspension was plated on nutrient agar medium and was supplemented with sucrose (5 g l⁻¹) (EPP0 2011). To isolate endophytic bacteria, plant parts (roots, stems, leaves, and flowers) were surface sterilized and washed in running tap water, followed by washing with sodium hypochlorite solution (0.3 to 2%, v/v) for 1 to 3 min and 3 serial rinses in sterilized distilled water. Plant parts were sliced in 100 ml sterilized distilled water. The suspensions were placed on a 120-rpm shaker for 1 h to release bacteria from the plant tissues. A loopful of the resulting suspension was streaked on Petri plates containing the above-mentioned medium. To extract rhizosphere microorganisms, roots with adhering soil (rhizospheric soil) were placed in 100 ml sterile distilled water and placed on a 200-rpm shaker at 25 °C for 1 h. This solution was serially diluted, of which 0.1 to 1 ml was spread on a selective nutrient agar medium (Hammami et al. 2013). Petri dishes were incubated at 25–28 °C for several days to allow the bacterial growth. Different colony types in terms of shape, size, texture, pigmentation, margin, and elevation picked up were subcultured on fresh Petri plates. The purified colonies were individually stored at – 20 °C in 30% glycerol.

Protein extraction and SDS-PAGE

To extract protein of Gram-negative bacteria, bacterial strains grown on nutrient agar containing 0.5 g l⁻¹ NaCl for 24 h at 25–28 °C were suspended in 1 ml sample buffer (0.062 M Tris-HCl pH 6.8, 15% glycerol, 7.5% mercaptoethanol, 0.6% SDS) and then, they were placed in boiling water for 10 min. After centrifugation at 10,000 rpm for 10 min, 300 µl batches of supernatant containing soluble proteins were loaded into each well of the gel (Laemmli 1970). To extract the proteins of Gram-positive bacteria, bacterial isolates grown on nutrient agar were suspended in 1 ml of distilled water. Next, 50 µl of 1 M Tris-HCl pH 8.2 and then, 100 µl of

lysozyme solution (10 mg of lysozyme per ml of 0.05 M Tris-HCl buffer containing 0.02 M EDTA pH 8.2) were added to the vial and were thawed and frozen in triplicate. Subsequently, SDS was added to a final concentration of 2% and vials were placed in water at 60 °C for 10 to 15 min. After centrifugation at 12,000 rpm for 2 min, 300 µl batches of supernatant were transferred to new vials and loaded into each well of the gels (Chaasy and Giuffrida 1980). Electrophoresis was performed according to a modified Laemmli method in a constant current of 20 mA for 7 h using 5% stacking gel (pH 6.8) and a 12% running gel (pH 8.8). After electrophoresis, Coomassie brilliant blue solution was added to the mix for gel staining using 45% v/v methanol, 10% v/v glacial acetic acid, and 0.01% Coomassie brilliant blue R250. Afterward, it was kept for 24 h at room temperature and then, was destained in the same solution without Coomassie brilliant blue (45% v/v methanol and 10% v/v glacial acetic acid) until the gel was rehydrated (Laemmli 1970).

DNA extraction

The DNA extraction was prepared by the alkaline lysis method (Arabi et al. 2006). The suspension of bacterial cells was prepared in 1 ml of sterile distilled water (optical density at 600 nm, 1). Then, 30 µl of KOH 5% was added to the cells suspension, followed by placing it in boiling water for 10 min. Centrifugation was conducted at 12,000 rpm for 2 min and 60 µl of the supernatant was removed carefully (DNA extract). An appropriate amount of DNA of genomic was determined and used in PCR reactions.

PCR amplification of the 16S rRNA gene and analysis

Two conserved primers fD1 (5-AGA GTT TGA TCC TGG CTC AG-3) and rP2 (5-ACG GCT ACC TTG TTA CGA CTT-3) were used to amplify the almost complete 16S rRNA gene sequence (1506 bp). The polymerase chain reaction (PCR) was performed using a PCR thermocycler (Techno-TC-512; UK). About 25 µl of reaction volume contained 1 µl of bacterial DNA as a template for PCR procedures, 0.1 mM of primers, 1 mM MgCl₂, 20 mM of each dNTP, and 0.5 U Taq DNA polymerase (Cinna Gen, Iran). The thermal cycling used for this reaction was as follows: initial denaturation at 94 °C for 5 min, 94 °C for 45 s followed by 30 cycles of 58 °C for 1 min, 72 °C for 90 min, and a final extension at 72 °C for 10 min. PCR products were sent to a sequencing service (Macrogen, Seoul, South Korea). The nucleotide sequences were aligned in the NCBI database using nucleotide BLAST software (NCBI BLAST® homepage). Phylogenetic analysis was performed using MEGA 7.0.26 software and neighbor-joining method with 1000 repetitions bootstrap to confirm the stability of the

relationships (Kumar et al. 2016). The nucleotide sequences of 16S rRNA gene segments from different bacterial samples determined in this research have been deposited in the GenBank database under accession numbers (MW703484 and MW712709 to MW712715).

In vitro screening for antagonism (dual test)

The antagonistic effects of all 362 isolates were evaluated against the causative agent of rapeseed blackleg disease (*Leptosphaeria maculans*) using the dual-culture method and the inhibition percentage was calculated using the following formula (Vincent 1947):

$$\% \text{Inhibition of mycelial growth} = [(C - D)/C] \times 100 \quad (1)$$

Where *C* is mycelial growth of pathogen in absence of antagonists and *D* is mycelial growth of pathogen in presence of antagonists. The experiments were conducted in a completely randomized design with three replicates.

Evaluation of plant growth-promotion properties

Regarding the ability of some *Pseudomonas* isolates (18 strains) to biologically control the blackleg disease, these isolates (13 leaf epiphytes, 1 stem epiphyte, 1 flower epiphyte, 1 root endophyte, and 2 leaf endophyte) were selected for the next laboratory experiments (plant growth-promotion properties and greenhouse assays). Siderophore production (Schwyn and Neilands 1987), phosphate solubilization (Lynn et al. 2013), and nitrogen fixation (Döbereiner 1998) activities were performed for the selected isolates.

Effect of bacterial seed priming on germination and growth in greenhouse

For greenhouse assays, seeds of *B. napus* (cv. Hyola) obtained from Agriculture and Natural Resources Research and Education Center of Hamedan were surface sterilized by 1% sodium hypochlorite for 1 min and then were washed in triplicate in sterile distilled water (Nezaret and Gholami 2009). Canola seeds were then inoculated with each bacterial suspension to a final concentration of 10⁶ cfu ml⁻¹ containing 1% Carboxymethylcellulose in 170 rpm for 12 h at 28 °C. In the control treatment, seeds were inoculated with distilled water. The treated seeds were exposed to sterile air for about 6 h to dry completely (Kumar et al. 2011) and then were sown in pots with 10 seeds per pot. The soil used for the experiment was made by combining sand and compost (1:1 v/v) and was sterilized by autoclaving. Seedlings were grown in a greenhouse with 12 h of light at 25 °C and were irrigated with distilled water. Thirty days after sprouting, plants were harvested and vegetative

parameters were measured according to Orozco-Mosqueda et al. (2013) as follows:

$$VI = GR \times L \quad (2)$$

$$GR = (SG/NS) \times 100 \quad GR = \frac{SG}{NS} \times 100 \quad (3)$$

Where *VI* is Vigour index, *GR* is Germination rate (%), *L* is the total plant length, *SG* is the number of seeds germinated from total number of seeds, and *NS* is the total number of seeds.

A completely randomized block design with three replicates was used to perform this experiment. The data were analyzed using SAS9 software and the mean data were compared using Duncan's multiple range tests at 5% level ($P \leq 0.05$).

Results

Isolation, screening, identification, and diversity of bacteria

A total of 362 bacterial isolates associated with canola plants based on their growth on nutrient agar medium was obtained. Of these 362 isolates, 160 were from roots (94 rhizobacteria and 66 endophytes), 74 from stems (90 epiphytes and 32 endophytes), 98 from leaves (12 epiphytes and 42 endophytes), and 30 from flowers (22 epiphytes and 4 endophytes). These isolates represented 25.9, 18.2, 20.4, 27, and 8.2% of the bacteria, respectively. Samples were collected at the flowering stage.

In this study, SDS-PAGE was used to screen the isolates and to prove that this method is of high efficiency to screen and group the isolates. Isolates with more than 80% similarity in their protein patterns were placed in the same species (Fig. 1). From each group of protein electrophoresis patterns obtained, a representative bacterium belonging to the same cluster was selected for the partial 16S rRNA gene sequence analysis. The partial sequencing of the 16S rRNA gene of representative strains showed that isolates belonged to the *Pseudomonas*, *Frigoribacterium*, *Sphingomonas*, *Sphingobacterium*, *Microbacterium*, *Bacillus*, and *Rhodococcus* genera (Fig. 2).

In vitro antagonism of *Leptosphaeria maculans*

According to the results of dual-cultures tests of bacterial isolates against *Leptosphaeria maculans*, 18 *Pseudomonas* isolates exhibited significant antagonistic activity against *Leptosphaeria maculans*.

PGP traits of bacterial isolates

All the 18 selected *Pseudomonas* isolates were evaluated for plant growth-promoting characteristics viz siderophore production, nitrogen fixation, and phosphate solubilization. The isolates were able to produce siderophore, which appeared by an orange halo formation around the bacterial culture site (Fig. 3). Five isolates could fix nitrogen (Fig. 4). In this research, the selected isolates were unable to solubilize phosphate. Overall, the

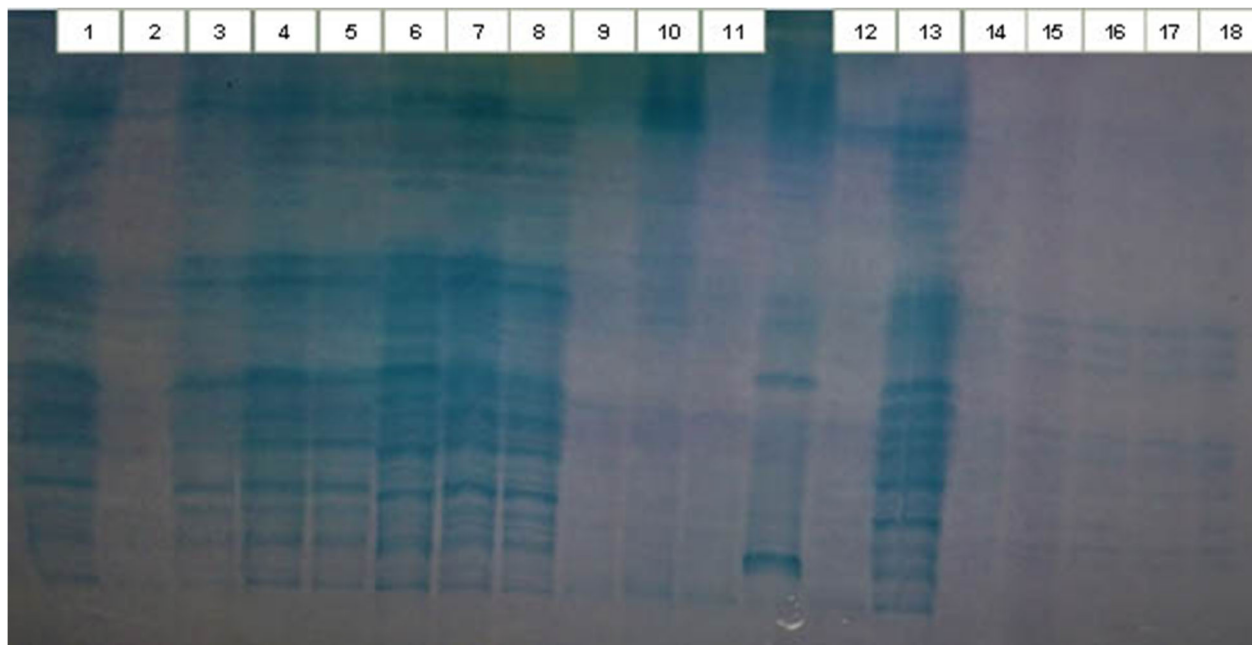
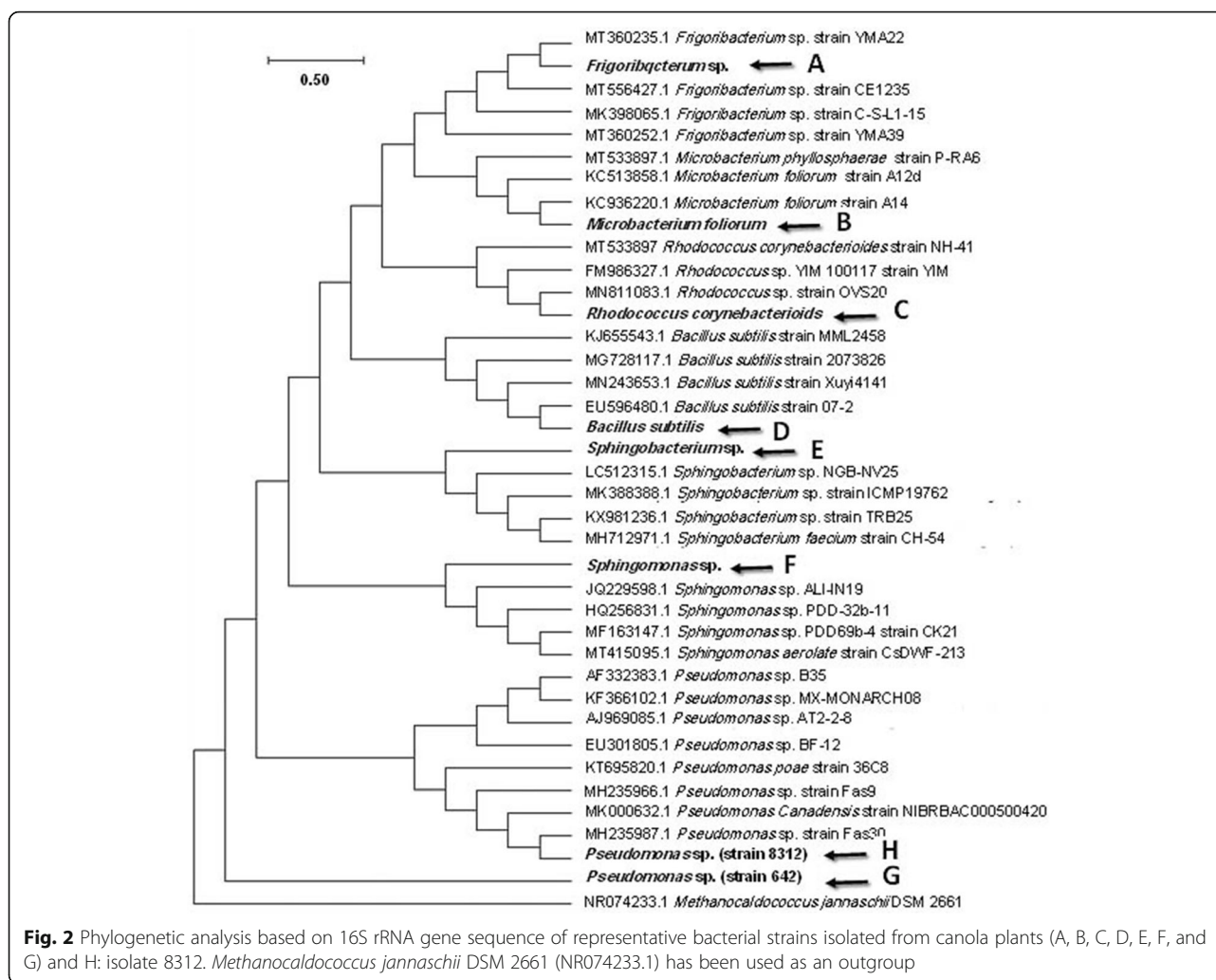


Fig. 1 Protein pattern of selected *Pseudomonas* isolates. 1: 4532, 2: 1223, 6424, 425, 4531, 6: LN1112, 7: 9421, 8: S1121W, 9: 8312, 10: 1111, 11: 12122, 12: 1RN2, 13: 12112, 14: F7122, 15: LN1111, 16: 8311, 17: 943, 18: .1313W



isolates represented some properties associated with plant growth promotion.

In vivo seed priming effect on seed germination and plant growth factors

The ability of 18 *pseudomonas* isolates to increase seed germination and growth of canola plants was investigated by the priming method (Figs. 5 and 6). The test results showed that bacteria had a positive effect on seed germination and plant growth. Strains 8312 and 642 significantly increased root length than the control and the other treatments did not show significant difference than the control. Treatments 8312, 642, 1313W, and 9421 had the maximum wet weights compared to the control. The greatest effect on germination was related to 8312 and 1313W treatments. Treatment 8312 showed a significant difference with the control, in other factors. Treatment 8312 indicated a significant difference in all factors compared to the control, and it was recognized

as the best effective isolate on canola seed growth factors. These isolates were leaf epiphyte bacteria.

Discussion

In this research, PGP bacteria associated with canola (*B. napus*) isolated on nutrient agar medium were supplemented with sucrose for investigating genetic diversity and the presence of plant growth-promoting bacteria. There is a little information about bacteria associated with canola in western Iran. In this study, the flowering stage was selected to isolate all canola-related bacteria because the plant has the highest level of physiological activity during the flowering stage (Idris et al. 2004). Houlden et al. (2008) reported an increase in the population of *Pseudomonas* in the flowering stage of pea. However, Farina et al. (2012) showed that canola root-related bacteria in the rosette stage were more diverse than at the time of flowering.

SDS-PAGE method is a very useful method to prove to be extremely reliable for the identification of bacteria

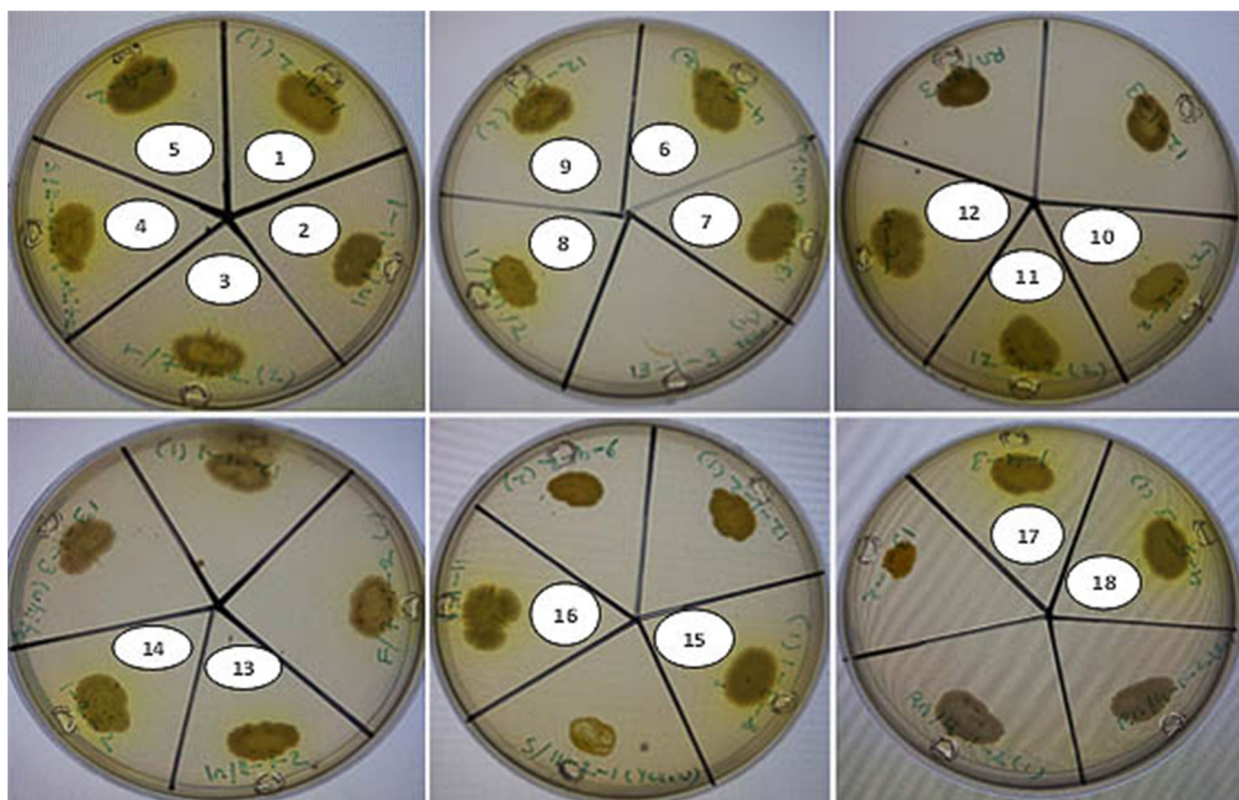


Fig. 3 Ability of the selected *Pseudomonas* isolates to produce siderophore. 1: 9421, 2: LN1111, 3: F7122, 4: S1121W, 5: 642, 6: 4532, 7: 1313W, 8: 1RN2, 9: 12112, 10: 8312, 11: 12122, 12: 42, 13: LN1112, 14: 122, 15: 8311, 16: 1111, 17: 943, 18: 4531

on the species level. Also, the technique allowed estimating molecular mass (Thierry et al. 2002). Strains belonging to a single species have qualitatively highly similar protein patterns. Visual inspection of the protein patterns was sufficient to distinguish among different species (Esteban et al. 2003). SDS-PAGE was a fast method to compare large numbers of strains to screen them.

Based on 16S rRNA sequences, isolated bacteria belonged to the *Pseudomonas*, *Frigoribacterium*, *Sphingomonas*, *Sphingobacterium*, *Microbacterium*, *Bacillus*, and *Rhodococcus* genera. Similar to obtained results, bacteria belonging to the genus *Pseudomonas* were one of the most abundant genera isolated from canola in

some other studies. In the study conducted by Siciliano and Germida (1999), a large number of *Pseudomonas* was isolated from rapeseed. Bertrand et al. (2001) identified the *Xanthomonas*, *Escherichia*, *Burkholderia*, *Azospirillum*, *Agrobacterium*, *Rhizobium*, and *Pseudomonas* genera from the endorhizosphere and rhizoplane of canola plants. Misko and Germida (2002) reported that *Pseudomonas* was one of the most abundant genus (35%) in bacterial populations isolated from rapeseed. Also, *Stenotrophomonas*, *Flavobacterium*, and *Arthrobacter* had a high abundance. Farina et al. (2012) reported that bacteria belonging to the *Agrobacterium*, *Burkholderia*, *Enterobacter*, and *Pseudomonas* genera

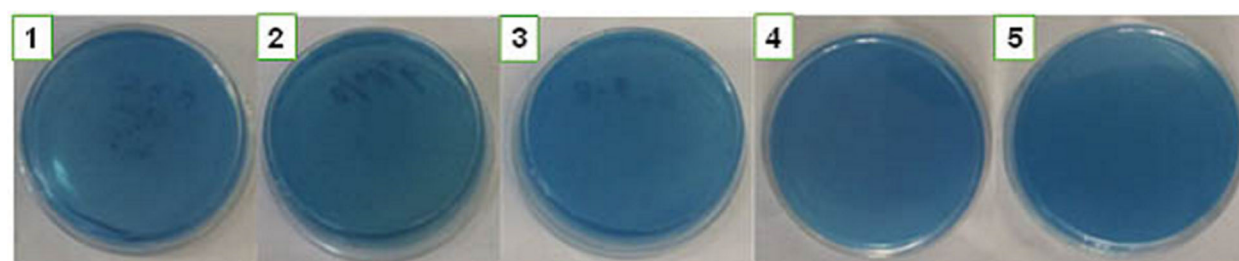


Fig. 4 Nitrogen fixation capacity of the selected *Pseudomonas* isolates. 1: 8312, 2: 642, 3: 4532, 4: 1111, 5: 1RN2

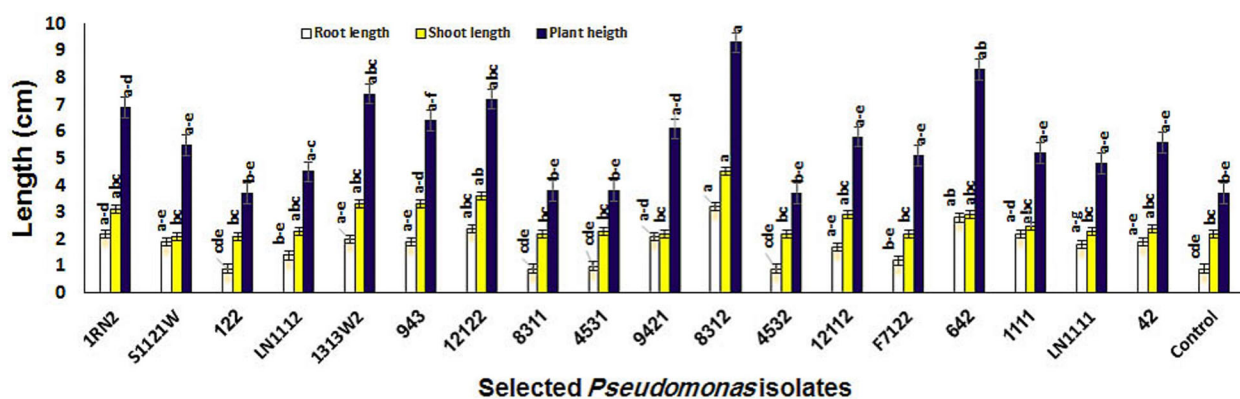


Fig. 5 Effect of canola seed priming by selected *Pseudomonas* isolates on root, shoot and plant length of canola in greenhouse conditions. Each treatment had three replications and treatments with common letters in Duncan test at 5% level were not significantly different from each other

had the highest abundance among all bacteria isolated from the rhizosphere and roots, as well as the isolated strains belonging to the *Sphingomonas* in roots of canola at the flowering stage. In the present research, *Sphingomonas* was identified in all samples in the flowering stage. Besides, Gram-positive *Bacillus* and *Microbacterium* bacteria were isolated and identified from canola plants that had been isolated from canola plants several times before (Card et al. 2015).

Plant growth-promoting bacteria use different methods to increase plant growth and yield. The role of these bacteria in plant nutrition is one of the direct mechanisms used to promote plant growth and development. They can facilitate the uptake of plant nutrients or provide plant growth-promoting nutrition elements that synthesize by the bacterium (Pandya et al. 2015). A bacterium may use one or more mechanisms during the time of plant growth. In this study, 3 plant growth-promoting characteristics (i.e., siderophore production, nitrogen fixation, and phosphate solubilization) were tested on selected isolates.

In the present study, all the selected isolates were able to produce siderophore on CAS agar plate. Siderophores are low-molecular-mass (~ 400–1500 Da) compounds that bind to Fe³⁺ (an available form of iron). This form of iron is produced by microorganisms such as bacteria under iron-limiting conditions to keep it out of the reach of plant pathogens and protect the plant (Beneduzi et al. 2013). This mechanism was reported to play a role in enhancing the growth of seedlings (Rudolph et al. 2015) and increasing their yield by enhancing the absorption of iron by the plants (Saha et al. 2015).

In this study, the nitrogen-fixing ability was reported in the strains 8312, 642, 1111, 4532, and 1RN2. N-fixing bacteria had a significant effect on the biofertilization of crops (Farina et al. 2012). Ke et al. (2019) stated that nitrogen-fixing bacterium *P. stutzeri* A1501 improved the growth of maize under experimental greenhouse conditions. They suggested that biologically fixed nitrogen transfer is maybe the main reason for maize growth promotion.

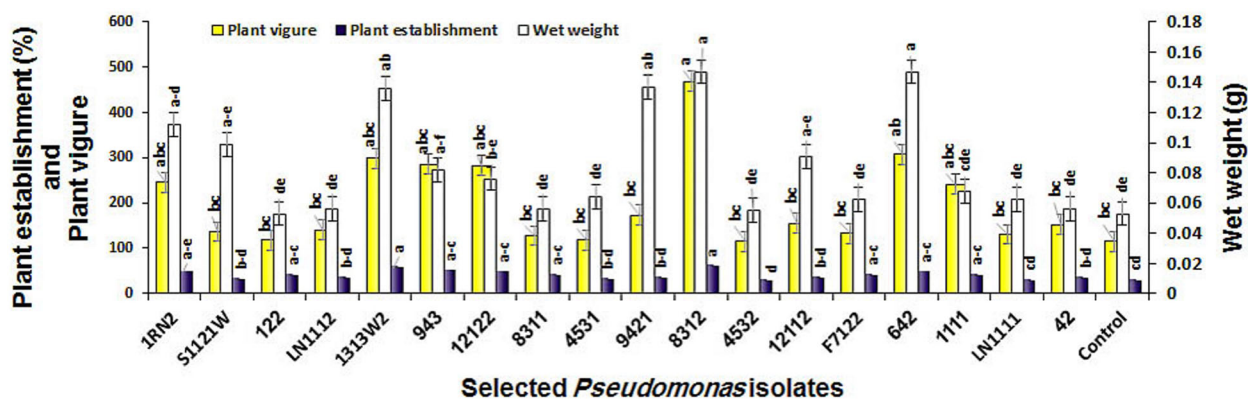


Fig. 6 Effect of canola seed priming by selected *Pseudomonas* isolates on wet weight, plant establishment and vigor of canola in greenhouse conditions. Each treatment had three replications and treatments with common letters in Duncan test at 5% level were not significantly different from each other

In this research, the selected isolates were unable to solubilize phosphate. According to De Freitas et al. (1997) experiments, phosphate-solubilizing rhizobacteria without increasing phosphate uptake significantly increased the growth of canola plant in P-deficient soil amended with rock phosphate in potting soil. Hence, they concluded that P-solubilization was not the main mechanism for enhancing plant growth. Meyer et al. (2019) showed that plant growth and phosphate uptake were significantly reduced in sterilized soil.

Greenhouse observations revealed that inoculation of canola seeds (cv. Hyola) with 4 PGPR isolates (8312, 642, 1313W, and 9421) among 18 bacterial isolates led to a significant increase in germination percentage and growth of rapeseed. Isolates 8312 and 642 had two characteristics related to plant growth enhancement (siderophore production and nitrogen-fixing ability) that were studied in this research. Isolates 1313W and 9421, like other isolates, were only capable of producing siderophore. However, they had a significant impact on some plant growth factors. In comparison, isolates 1111, 4532, and 1RN2, which had 2 characteristics of siderophore production and nitrogen fixation ability, did not have a significant effect on growth factors. Therefore, a PGP bacterial isolate used a specific mechanism or several mechanisms simultaneously to increase plant growth. The presence of several characteristics cannot be a definite reason for the effect on increasing plant growth. Moreover, properties that were effective in increasing plant growth in one isolate may not be effective or be less effective in the other isolates. In this research, 642 and 1313W isolates showed a significant increase than the control in 2 factors and strain 8312 in all the studied factors. This probably could be related to other plant growth-promoting traits that were not investigated in this study. Strain 8312, confirmed as *Pseudomonas* sp. showed the best effect on canola plant growth. This result suggested the potential of this isolate for using as a biofertilizer. Nevertheless, many steps have been taken, for estimating a PGPR-potential to a biofertilizer application to find a suitable formulation for field use.

Conclusions

The results indicated that canola-related bacteria were diverse during the flowering stage. *Pseudomonas*, *Frigoribacterium*, *Sphingomonas*, *Sphingobacterium*, *Microbacterium*, *Bacillus*, and *Rhodococcus* genera isolated from all the plant parts were analyzed. Several of them were able to produce siderophore and fix nitrogen. These bacteria had a significant effect on canola plant growth factors in the greenhouse. Isolate 8312 (confirmed as *Pseudomonas* sp.) had a significant effect on all plant growth factors. Hence, it is recommended to investigate this strain further in field infestation experiments.

Abbreviations

16S rRNA: 16 subunit ribosomal ribonucleic acid; CAS-Agar: Chrome azurol S-agar; cv: Cultivar; DNA: Deoxynucleotide acid; NCBI: National Center for Biotechnology Information; NJ: Neighbor-joining; OD: Optical density; PCR: Polymerase chain reaction; PGPB: Plant growth-promotion bacteria; P-solubilizing: Phosphate solubilizing; SDS: Sodium dodecyl sulfate; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sp: Species

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Authors' contributions

JA, DM, KG, ZD, and BE conceptualized the project. JA isolated bacteria, carried out in vitro, biochemical, and greenhouse experiments, data analyses, final interpretation of data, and original manuscript draft preparation. DM and KG was involved in the conceptualization, supervision, final interpretation of data, and editing of the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

All data are available in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Plant Protection, Lorestan University, Khorramabad, Iran.

²Department of Plant Protection, Bu-Ali Sina University, Hamedan, Iran.

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