Ecotoxicology and Environmental Safety 162 (2018) 129–138

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Mining alfalfa (*Medicago sativa* L.) nodules for salinity tolerant non-rhizobial bacteria to improve growth of alfalfa under salinity stress

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ARTICLE INFO

Keywords:
Co-inoculation
Nodule salinity and drought tolerant bacteria
Rhizobia
Klebsiella sp.
Kosakonia cowanii
Legume

ABSTRACT

There are fewer reports on plant growth promoting (PGP) bacteria living in nodules as helper to tolerance to abiotic stress such as salinity and drought. The study was conducted to isolate rhizobial and non-rhizobial drought and salinity tolerant bacteria from the surface sterilized root nodules of alfalfa, grown in saline soils, and evaluate the effects of effective isolates on plant growth under salt stress. Based on drought and salinity tolerance of bacterial isolates and having multiple PGP traits, two non-rhizobial endophytic isolates and one rhizobial endophytic isolate were selected for further identification and characterization. Based on partial sequences of 16 S rRNA genes, non-rhizobial isolates and rhizobial isolate were closely related to *Klebsiella* sp., *Kosakonia cowanii,* and *Sinorhizobium meliloti*, respectively. None of the two non-rhizobial strains were able to form nodules on alfalfa roots under greenhouse and *in vitro* conditions. Co-inoculation of alfalfa plant with *Klebsiella* sp. A36, *K. cowanii* A37, and rhizobial strain *S. meliloti* ARh29 had a positive effect on plant growth indices under salinity stress. In addition, the single inoculation of non-rhizobial strains without rhizobial strain resulted in an increase in alfalfa growth indices compared to the plants non-inoculated and the ones inoculated with *S. meliloti* ARh29 alone under salinity stress, indicating that nodule non-rhizobial strains have PGP potentials and may be a promising way for improving effectiveness of *Rhizobium* bio-fertilizers in salt-affected soils.

1. Introduction

Salt stress is one of the major abiotic factors that negatively impacts crop growth and yield. Salt stress impairs several major processes in plants, such as photosynthesis, protein synthesis and lipid metabolism, due to both osmotic effects that result in water deficits and specific ion effects that can cause toxicity and nutrition imbalance (Munns and Tester, 2008). Worldwide, approximately 7% of the land on Earth and 20% of the total arable area are affected by salinity (Rizwan et al., 2015). There is a need to exploit the marginal lands, such as the salt-affected soil, for forage production. At the present time, leguminous forage plants have been part of the most critical components in sustainable agriculture and global food security by providing part of the feed requirement of ruminants for meat and milk production (Barth, 2012; Wang and Brummer, 2012). To meet the requests, leguminous forage cultivars with high salt tolerance are needed. Alfalfa (*Medicago sativa* L.) is a vital perennial leguminous forage crop for its high protein content, high biomass yield, excellent nutritive value and high digestibility. It is widely planted throughout the world, especially in the arid and semi-arid areas, for pasture, hay and silage making, and is also valued highly as a livestock feed directly (Zhang and Wang, 2015). This forage crop also helps nitrogen (N) incorporation to agricultural systems, which leads to a reduction in the application of chemical N fertilizers (Fernandez et al., 2017).

It has found that most of commercially available alfalfa cultivars are deficient in salt tolerance (Liu et al., 2011). Productivity and capacity of nodule formation and nitrogen-fixation of this plant could be severely affected by the saline stress as low as of 50 mM NaCl (Bruning and Rozema, 2013; Liu et al., 2011). In addition, it is known that N_2 fixing legumes are more susceptible to salt stress than N_2 non-fixing plants (N-fertilized plants) (Bouhmouch et al., 2005). Thus, development of alfalfa cultivars highly resistant to salinity stress is needed. In general, alfalfa is self-incompatible with inbreeding depression, and insect-assisted pollination. Its cultivars are thus heterogeneous and synthetic. Therefore, all these traits are difficult to carry out salt tolerance breeding directly (Zhang and Wang, 2015; Zhu et al., 2005).

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https://doi.org/10.1016/j.ecoenv.2018.06.092
Received 17 February 2018; Received in revised form 26 June 2018; Accepted 28 June 2018

An alternative strategy to improve crop salt tolerance may be to introduce salt-tolerant bacteria that enhance crop growth under salinity stress (Etesami and Beattie, 2018; Etesami and Maheshwari, 2018). Numerous studies have shown that plant growth promoting rhizobacteria (PGPR) have a beneficial impact on plant growth and development, especially under stress conditions (Etesami and Maheshwari, 2018). A lot of research has also indicated that plant growth and nodulation in legumes are higher in the presence of PGPR (Shaharoona et al., 2006; Tilak et al., 2006).

It is well known that the rhizobia are not the only nitrogen-fixing inhabitants of legume nodules (Lu et al., 2017; Martínez-Hidalgo and Hirsch, 2017). Previously, some endophytic bacteria were isolated from the nodules of legume plants (Martínez-Hidalgo and Hirsch, 2017). It has long been known that co-inoculation of rhizobia and endophytic non-rhizobial bacteria increases the growth and yield of plant, nodulation, and availability of nutrients (i.e., N) (Martínez-Hidalgo and Hirsch, 2017; Rajendran et al., 2012; Schwartz et al., 2013).

PGPR and endophytic bacteria can increase the plant growth by various mechanisms (Etesami and Maheshwari, 2018). However, it has been known that the performance of PGPR/endophytic bacteria is severely affected by environmental factors especially stress factors (Etesami and Beattie, 2018). For example, Upadhyay et al. (2009) found that PGPR loose plant growth promoting (PGP) traits with increasing salinity *in vitro*. Thus, the selection and use of salinity tolerant PGPR based on both high salt tolerance and efficiency in producing PGP compounds are potentially of enormous impact in facilitating the growth of crops in a wide range of saline environments, both natural salinity and induced salinity. It has been reported that the salinity resistant PGPR obtained from saline environments are more effective at improving the plant tolerance to salt than those isolated from salt non-affected habitats (Etesami and Beattie, 2018; Paul and Nair, 2008). So far, mostly soil (rhizosphere)-isolated microbes have been used for PGPR/PGPB formulations. However, studies of the nodule microbiome will better suggest which microbes are effective for developing inocula (Martínez-Hidalgo and Hirsch, 2017). Although many strategies have been adopted to improve the stress tolerance in legumes (Medeot et al., 2010), fewer reports have been published on salinity resistant-PGP bacteria living in nodules as helper to tolerance to abiotic stress such as salinity. In addition, few studies of the function of the nodule-associated non-rhizobial bacteria in nodules have been performed (Martínez-Hidalgo and Hirsch, 2017).

Keeping in view of the above, the objectives of this study were to: (i) isolate non-rhizobial and rhizobial bacteria from the root nodules of the alfalfa grown in saline soils; (ii) characterize these bacterial isolates in terms of tolerance to salinity and drought and PGP traits; and (iii) evaluate the effect of effective strains on some physiology and morphology features of alfalfa alone and its symbiosis with *Sinorhizobium* (*Ensifer*) *meliloti* under salinity stress.

We hypothesized that the root nodules of alfalfa grown in salt-affected soils harbor salinity tolerant PGP bacteria that might improve the alfalfa salt tolerance by affecting its physiological, morphological and biochemical processes during stress response.

2. Materials and methods

2.1. Sampling and isolation of nodule endophytic bacteria

To isolate and purify the non-symbiotic and symbiotic bacterial isolates of alfalfa (Cv, *Yazdi*) nodules, a number of healthy plants (13 samples) were randomly collected from alfalfa fields (N 34° 43', E 51° 06', and 854 m above sea level) in Qom Province in 2016 during the growing season (May 15th). Some of physical and chemical traits of field soil included: soil texture (20% clay, 36% silt, and 44% sand), loamy; pH, 8.2; electrical conductivity of saturated paste extract (EC), 10.94 dS m^{-1}; organic carbon, 5.7 g kg^{-1}; total nitrogen (N_{tot}), 5.6 g kg^{-1}; calcium carbonate equivalent (CCE), 89 g kg^{-1}; available potassium (K_{avail}), 270 mg kg^{-1}; and available phosphorus (P_{avail}), 7.6 mg kg^{-1}. These traits were measured according to previous methods (Okalebo et al., 2002). According to a previous standard procedure (Callow, 1971) and using yeast extract mannitol agar medium (YMA) supplemented with congo red, the nodule endophytic bacteria were isolated and purified (Vincent, 1970). To confirm the surface sterilization process, the aliquots of sterile distilled water used in the final wash were placed on YMA medium and the plates were incubated at 28 ± 2 °C for 3–5 days. Bacterial colonies phenotypically different from each other (*e.g.,* Gram-staining reaction, culture morphology, shape, color, rate of growth, and motility) were selected for the next experiment. In order to differentiate the symbiotic isolates with alfalfa from non-symbiotic isolates, the test of nodule formation for each isolate was checked by inoculation of alfalfa seedlings according to a previous method (Vincent, 1970). Finally, purified bacterial isolates (fast-growing colonies of bacteria-individual CFU (colony forming unit)) were selected, sub-cultured onto nutrient agar (NA), maintained on the respective slants, and stored in a refrigerator at 4 °C for further studies. In addition, these isolates were also maintained at −80 °C in nutrient broth (NB) that contained 20% glycerol for long-term storage. The bacterial isolates were coded (ARh1–ARh30 for rhizobial isolates and A31–A63 for non-rhizobial isolates).

2.2. Preparation of bacterial inocula

For preparing the bacterial culture, each non-rhizobial bacterial isolate was grown in 250-ml flasks containing 100 ml NB medium and shaken for 24 h at 28 ± 2 °C on a rotary shaker at 200 rpm until (turbid) when logarithmic phase is attained 5×10^8 cells ml^{-1}. For rhizobial isolates, each isolate was also grown in 250-ml flasks containing 100 ml YMB medium and shaken for 72–120 h at 28 ± 2 °C on a rotary shaker at 200 rpm until when logarithmic phase is attained 5×10^8 cells ml^{-1} (Woomer et al., 2011). The cell suspensions were used in all of the following assays.

2.3. Salinity and drought tolerance assay of nodule endophytic bacteria

To determine the tolerance level to NaCl salinity, each isolate was assayed by spotting 7 µl of cultures (5×10^8 CFU ml^{-1}) on NA plates amended with 0, 100, 200, 300, 400, 600, 800, 1000, and 1200 mM NaCl. After 7 days of incubation at 28 ± 2 °C, the plates were checked visually for bacterial growth (changes in diameter, state and appearance of colonies) (Khalifa et al., 2016). Since salinity stress results in osmotic stress, which limits water availability in plants and bacteria, the tolerance level to drought of these isolates was also assayed (Munns and Tester, 2008). In order to evaluate the tolerance of the endophytic isolates (5×10^8 CFU ml^{-1}) to different levels of drought stress, their growth potential in NB medium containing different concentrations of polyethylene glycol (PEG)-6000 (0, 202.2, 295.7, 367.7, and 428.4 g PEG-6000 l^{-1} NB medium) was assayed. These concentrations are equivalent to osmotic potential of 0, − 5, − 10, − 15, and − 20 bar, respectively. After being shaken at 120 rpm on a rotary shaker at 28 ± 2 °C for 72 h, the growth of isolates was determined by measuring the OD of the growth medium at a wavelength of 600 nm (Ali et al., 2014), which was determined by Absorbance Microplate Readers (Bio-Tek Elx800, USA). The experiments were done in triplicates.

2.4. Assay of PGP traits of nodule endophytic bacteria

The ability of hydrogen cyanide (HCN) production, the production of IAA, ACC deaminase activity, phosphate solubilization, and siderophore production of all bacterial isolates was determined according to the methods previously described by Bakker and Schippers (1987), Gordon and Weber (1951), Penrose and Glick (2003), Sperber (1958), and Schwyn and Neilands (1987), respectively. The experiments were done in triplicates. All isolates were also cultured for qualitative

assessment of nitrogen fixation in tubes containing N-free semi-solid medium (NFb) described by Döbereiner (1989). The presence of a halo of bacterial growth in the medium was considered as an indicator of nitrogen fixation. The bacterial growth halo in this medium was compared with a positive control (*Azosprillum brasilense*, a nitrogen fixing bacterium).

2.5. Identification of efficient endophytic bacteria

The genomic DNA of the isolates (one rhizobial isolate-ARh29, a rhizobial isolate with a symbiotic efficiency of more than 100%), and two non-rhizobial isolates-A36 and A37 grown in NB medium was extracted using isolation kit (Promega, Madison, WI, USA). The 16S rRNA gene amplification was performed pursuant to the conditions described by Edwards et al. (1989) by using general bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTA CGACTT-3′). Sequence of 16S rRNA genes was determined after purification of PCR products with purification kit (Promega, Madison, WI, USA) by DNA sequencer by the South Korean Macrogen Corporation using the 27F Primer. All information about the 16S rRNA gene nucleotide sequence was edited using the Edit sequence (version 5.1) software. The nucleotide sequences of PCR products were compared with the nucleotide sequences found in GenBank and the phylogenetic tree was constructed using the Neighbor Joining method using the Mega 5 software. The nucleotide sequences identified in this study were sent to GenBank databases and recorded with various accession numbers.

2.6. Effect of efficient strains on alfalfa growth under salinity stress

In this assay, effect of efficient non-rhizobial isolates on some physiology and morphology features of alfalfa (Cv, *Yazdi*) was studied under salinity stress. For this purpose, isolates A36 and A37 of non-rhizobial endophytic bacteria and isolate ARh29 as rhizobial isolate were selected and tested in this assay. This assay was performed as a completely randomized design (CRD) with factorial arrangement in three replications in potted soil (2 kg). The research was carried out in a research greenhouse located at Department of Soil Science, University of Tehran, Iran. The soil (a light textured soil) used in pots was a poor soil in terms of bacteria and micro-and macronutrients. The experimental treatments included five inoculation conditions, (i) the seedlings co-inoculated with non-rhizobial endophytic bacteria A36 and A37; (ii) the seedlings inoculated with rhizobial bacterium ARh29; (iii) the seedlings co-inoculated with isolates A36, A37, and ARh29; (iv) the seedlings non-inoculated with bacterial isolates (negative control); and (v) the seedlings non-inoculated with bacterial isolates and fertilized with N (positive control), as the first factor, and five salinity levels, (i) the alfalfa plants grown at 0 mM NaCl; (ii) the alfalfa plants grown at 50 mM NaCl; (iii) the alfalfa plants grown at 100 mM NaCl; (iv) the alfalfa plants grown at 150 mM NaCl; and (v) the alfalfa plants grown at 200 mM NaCl, as the second factor. Since these bacterial isolates were co-inoculated on alfalfa, the antagonistic assay was initially performed on these isolates as described by Etesami et al. (2014). None of the isolates had a negative effect on the growth of each other. In order to sow alfalfa, plastic pots (diameter, 15 cm and height, 14 cm) without drainage were used. Each pot was disinfected with sodium hypochlorite 5% solution and washed well with sterilized distilled water. To each pot, 2 kg of non-sterile soil passed through a 4 mm sieve was added. Depending on the experimental treatments, different saline solutions were prepared using NaCl to create different salinity conditions. In each pot, after being surface-sterilized (respectively for 7 s with 96% alcohol, 60 s with 5% sodium hypochlorite and 10 times washing with sterile distilled water), the 20 germinated healthy seeds of alfalfa (Cv, *Yazdi*), obtained from Seed and Plant Improvement Institute, were planted in a 1 cm depth of soil surface. After 14 days of sowing, each seedling was inoculated with one ml of bacterial suspension according to the relevant treatment. After 32 days of sowing (18 days after inoculation), salinity treatments were applied. To avoid salt shock, NaCl solutions were applied to pots in increments of 50 mM every 48 h, until final salt concentrations of 100, 150, and 200 mM were reached. The amount of 20 kg N per hectare of urea fertilizer was added to all pots as a starter. Nutritional need of the plants was provided by nitrogen-free Hoagland solution. Hoagland solution containing N was added to plants considered as positive controls. Soil moisture content was maintained by weight basis and daily at 80% of the field capacity. During the experiment, irrigation with sterile distilled water was performed. All pots were maintained in greenhouse with the light intensity of 15000 lx, at 25 ± 2 °C and light period of 12 h for 70 days.

2.6.1. Measurements

After the end of the growth period, alfalfa plants (five plants randomly from each pot) were cut off from the soil surface of the pots. Samples (shoot) were washed with distilled water, dried at 70 °C until they reached constant weight, and weighed and then milled. Also, the roots were removed from soil and placed in an oven for 72 h at 70 °C in order to stabilize their final dry weight and then the root dry weight was measured. Using an electric mill, these samples were then powdered. The total N content of the shoot was measured by the titration method after distillation using the Kjeldahl method (Okalebo et al., 2002). The digestion of the plant aerial parts was done by dry ashing method. In order to prepare the extracts necessary for the measurement of K, Na, and P, after the addition of 1N HCl, the extracts were filtered and analyzed by flame photometer (ELE) and spectrophotometer (Shimadzu UV3100), respectively, according to previous methods (Okalebo et al., 2002).

For measuring proline content, a 0.5 g frozen sample was crushed in a mortar with liquid N and then extracted with 5 ml of 3% (w/v) sulphosalycylic acid. After the homogenate was centrifuged at 18,000 × g for 10 min, 2 ml of the supernatant was transferred to a test tube. Afterward, 2 ml glacial acetic acid and 3 ml ninhydrin reagent were added to the test tube and kept for 40 min at 100 °C. Finally, toluene was added to the mixture reaction for extracting the product. After shaking, the tube was left for 15 min. The mixture gradually became two-phase and the upper phase absorbance was measured at 520 nm against toluene (Bates et al., 1973). The proline content was determined from calibration curve using L-proline as standard and expressed as $\mu g\, g^{-1}$ leaf fresh weight (FW).

Among antioxidant enzymes, activity of catalase (CAT) and superoxide dismutase (SOD) was measured in this study. Concisely, the samples of fresh leaf were homogenized with a mortar in 100 mM phosphate buffer (pH 6.8) containing 0.1 mM EDTA and 1% PVP (polyvinylpyrrolidone). After the homogenate was centrifuged at 15000 × g for 15 min, the supernatant was utilized as the source of enzymes. All the operations were carried out at 4 °C. For the activity of SOD, the reaction combination included 50 mM sodium carbonate (pH 10.2), 12 mM L-methionine, 50 mM HEPES-KOH buffer (pH 7.8) including 0.1 mM EDTA, 75 μM nitroblue tetrazolium (NBT), and 1 μM riboflavin. The activity of SOD was determined based on its ability to prevent the reduction of NBT by superoxide anion produced by the system of riboflavin below 4000 W (light intensity) at 25 °C (Giannopolitis and Ries, 1977). An SOD activity unit is considered an enzyme value that results in 50% inhibition of the reduction rate of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture for the CAT activity was contained 100 mM phosphate buffer (pH 7) and 15 mM H_2O_2. The decomposition of H_2O_2 (hydrogen peroxide) was absorbed at 240 nm for 3 min ($\varepsilon = 39.4\ mM\ cm^{-1}$) (Aebi, 1984). As well as, the soluble protein was calculated according to Bradford (1976) by using bovine serum albumin (BSA) as standard. The specific enzyme activity for these enzymes was expressed as unit mg^{-1} protein.

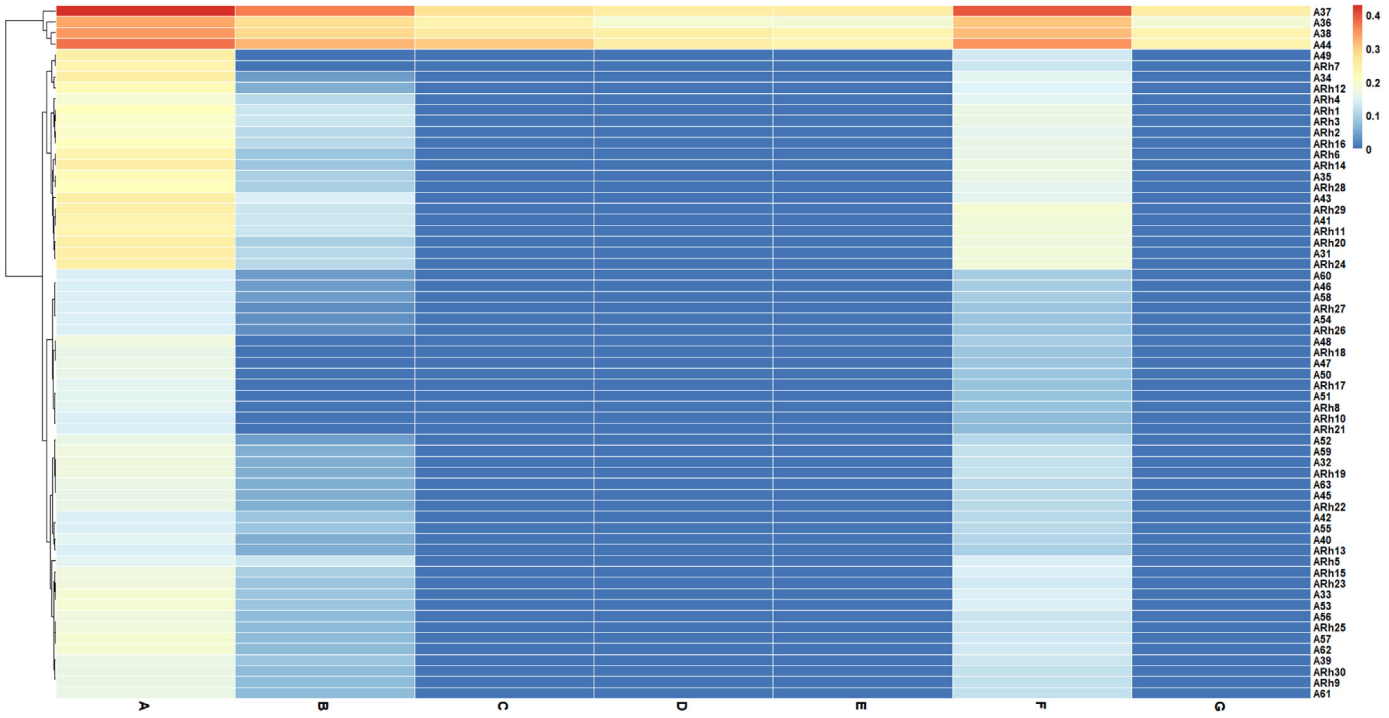

Fig. 1. A heatmap diagram of characteristics of salinity and drought tolerance of rhizobial isolates (ARh1-ARh30) and non-rhizobial isolates (A31–A63) isolated from root nodules of alfalfa grown in salt-affected-soils. The color bar on top shows the relative abundance of isolates tolerant to salinity and drought stress. Regarding the color scheme, red indicates higher tolerance of bacterial isolates to salinity and drought stress and blue indicates lower tolerance of bacterial isolates to salinity and drought stress or sensitivity of the bacterial isolates to salinity and drought stress. A, drought stress (osmotic potential of 0 bar); B, drought stress (osmotic potential of -5 bar); C, drought stress (osmotic potential of -10 bar); D, drought stress (osmotic potential of -15 bar); E, drought stress (osmotic potential of -20 bar); F, salinity stress (400 Mm NaCl); and G, salinity stress (1200 Mm NaCl) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

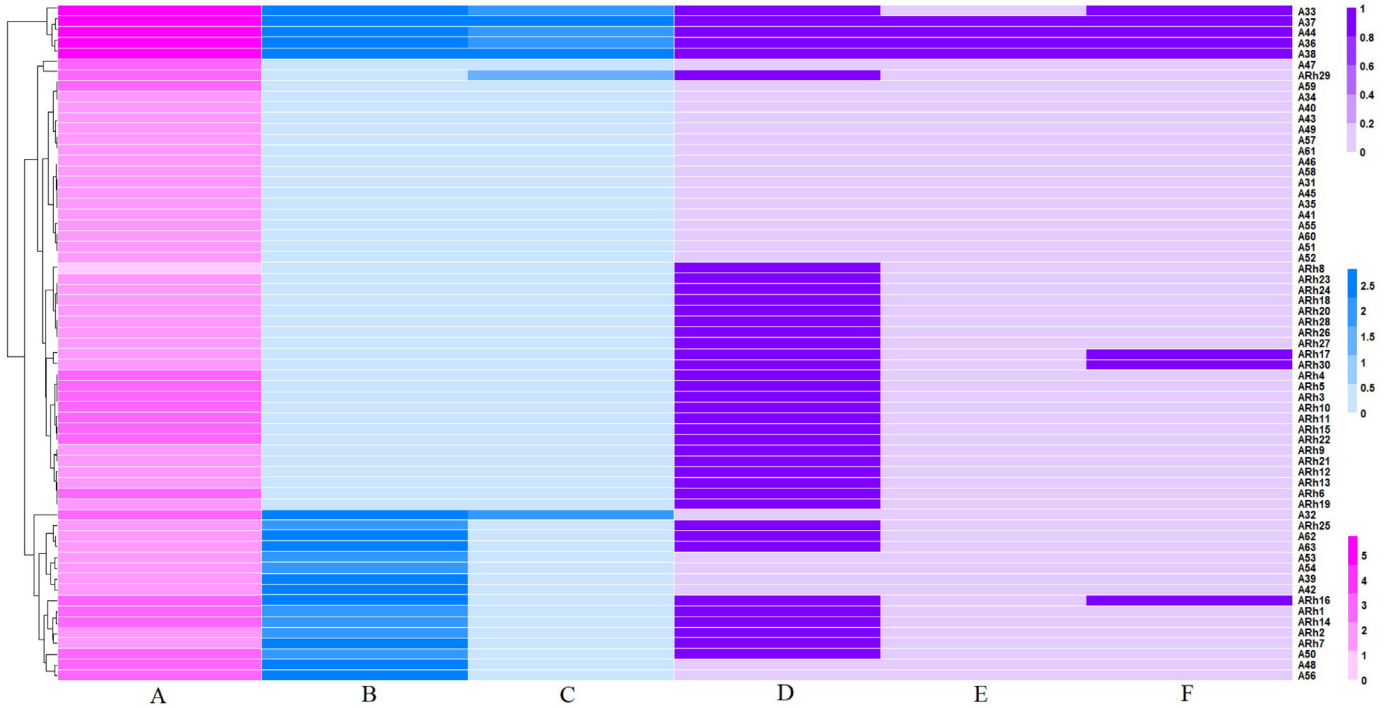

Fig. 2. A heatmap diagram of characteristics of promoting plant growth of rhizobial isolates (ARh1-ARh30) and non-rhizobial isolates (A31–A63) isolated from root nodules of alfalfa grown in salt affected-soils. The color bar on top shows the relative abundance of isolates having PGP traits. Regarding the color scheme, higher values indicate higher ability of bacterial isolates to produce plant growth promoting (PGP) traits and lower values indicate lower ability of bacterial isolates to produce PGP traits or without ability to produce PGP traits. A, IAA production; B, organic P solubilization; C, inorganic P solubilization; D, N_2 fixation; E, ACC deaminase activity; and F, HCN production.

2.7. Statistical analysis

Analysis of variance (ANOVA) of data and statistical calculations were performed using MSTAT and SAS computer programs (SAS Institute, Cary, NC, USA). Charts were plotted using prism 6 software. Comparison of mean of data was done by Duncan's multiple-range tests at 5% level. The data were reported as means ± the standard deviations.

3. Results

3.1. Isolation of nodule endophytic bacteria and their characterizations

A total of 63 distinct bacterial isolates from alfalfa surface-sterilized nodules were isolated. Thirty out of 63 isolates were rhizobial isolates that were able to form nodules on alfalfa roots (ARh1-ARh30). The ability of all 63 isolates to tolerate salinity and drought was evaluated (Fig. 1). All isolates were able to grow at salt concentrations up to 400 mM NaCl, while only four isolates (A36, A37, A38, and A44) could grow at salt concentrations up to 1200 mM NaCl. Most of the bacterial isolates were partially capable of growing at an osmotic potential to − 5 bar, but only four non-rhizobial isolates were able to tolerate osmotic potential to − 20 bar (Fig. 1). The non-rhizobial isolates A36, A37, A38, and A44 showed similar responses to salinity and drought stress and were more tolerant than other isolates. The ability to tolerate salinity of isolates using sodium chloride salt showed that with increasing salt concentration, the growth of bacteria decreased. In addition, with increasing concentration of PEG-6000 in culture media, optical density (OD_{600}) showed a decreasing trend as a criterion for bacterial growth. Among 63 bacterial isolates, non-rhizobial isolates A36, A37, A38, and A44 showed the highest drought and salinity tolerance (Fig. 1).

The potential of 63 isolates was also evaluated for the characteristics of promoting plant growth. The ability to produce PGP traits varied among isolates (Fig. 2). As shown in Fig. 2, all isolates were positive for IAA production. The amount of the IAA produced by these isolates varied from 2 to 63 $\mu g\,ml^{-1}$, with the highest production amount of this hormone among non-rhizobial isolates. Compared to the ability of the isolates to inorganic P solubilization, these bacterial isolates showed greater potential for solubilization of organic P. None of the rhizobial and non-rhizobial isolates were able to produce siderophore. All rhizobial isolates obtained from root nodules were able to grow in an N-free culture medium, while only eight non-rhizobial isolates were able to grow at this culture medium. None of the rhizobial isolates were able to use ACC as the only source of N, while only non-rhizobial isolates A36, A37, A38, and A44 were positive for the production of the enzyme ACC deaminase. Except for some non-rhizobial and rhizobial isolates, the isolates were not positive for HCN production.

3.2. Identification of effective endophytic isolates

Based on their tolerance to salinity and drought and having the highest potential in terms of PGP characteristics (Table 1), two non-rhizobial isolates A36 and A37 were selected and identified. Among rhizobial isolates, rhizobial isolate ARh29 was identified. This rhizobial isolate indicated a symbiotic efficiency of more than 100% (data not shown). For finding sequences similar to the 16S rRNA gene sequence of these isolates, databases in the GeneBank were checked. Identification of the 16S rRNA gene of the isolates A36, A37, and ARh29 was shown to be closely related to *Klebsiella* sp., *Kosakonia cowanii*, and *Sinorhizobium meliloti*, respectively. The 16S rRNA gene sequences of A36, A37, and ARh29 were closely similar to strains *Klebsiella* sp. M5al (accession number CP020657), *K. cowanii* Esp_Z (accession number CP022690), and *S. meliloti* B399 (accession number CP019488) on the BLAST search in the NCBI, respectively. The corresponding dendrogram was drawn using the sequences of the strains and representative sequences from the databases (Fig. 3). The nucleotide sequences assigned to this study were sent to the GeneBank database and registered with the accession numbers MH201369, MH201368, and MG586242 for strains A36, A37, and ARh29, respectively.

3.3. Effect of effective bacterial strains on shoot N concentration and root dry weight

Analysis of variance of the interaction effect between salinity levels and bacterial treatment on N concentration and root dry weight was not significant in this study (ANOVA table not shown). However, the average N concentration of the shoot and the average root dry weight decreased significantly with increasing salinity level (Fig. 4A and C). In this study, the use of plant growth promoting bacteria (both rhizobial strains and non-rhizobial ones) significantly increased N concentration and root dry weight and the highest amount of shoot N and root dry weight was observed in plants co-inoculated with non-rhizobial strains *Klebsiella* sp. A36 and *K. cowanii* A37 and rhizobial strain *S. meliloti* ARh29 (a 31.54% increase and a 14.92% increase in N concentration compared to negative and positive controls, respectively), while plants co-inoculated with non-rhizobial strains A36 and A37 and the plants inoculated with *S. meliloti* ARh29 were in the next ranks in terms of N content and root dry weight compared with positive and negative controls, respectively (Fig. 4B and D). An interesting result of this assessment was that the plants inoculated with non-rhizobial strains (A36 + A37) were able to take up the amount of N statistically equivalent to the plants co-inoculated with non-rhizobial strains and rhizobial strain (A36 + A37 + ARh29). This indicates that non-rhizobial bacteria had a considerable role in the fixation of N_2 in the absence of *S. meliloti* ARh29. On roots of alfalfa plants inoculated with non-rhizobial strains, no nodule was observed, indicating that the soil used in the pots did not contain any indigenous rhizobial bacteria symbiotic with the alfalfa.

Table 1
Characteristics of promoting plant growth and salinity and drought tolerance of effective non-rhizobial isolates A36 and A37 and rhizobial isolate ARh29 isolated from root nodules of alfalfa selected in this study.

Strain	IAA production ($\mu g\,ml^{-1}$)	Inorganic P solubilization	Organic P solubilization	Siderophore production	ACC deaminase	HCN production	N_2 fixation	Tolerance to salinity		Tolerance to drought (Osmotic potential of − 20 bar)
								400 mM NaCl	1200 mM NaCl	
ARh29	7.50	+	–	–	–	–	+	+	–	–
A36	62.16	+	+	–	+	+	+	+	+	+
A37	35.16	+	+	–	+	+	+	+	+	+

+ , positive activity; -, negative activity.

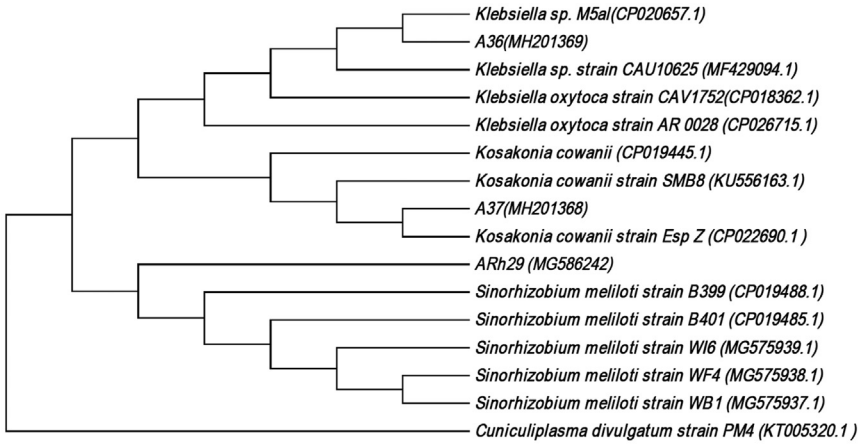

Fig. 3. Phylogenetic tree constructed using 16S rRNA gene sequences, available in the GenBank database, employing the Neighbor-joining method. Bootstrap values based on 1000 replications were listed as percentages at the nodes. The scale bar indicates genetic distance. The GenBank accession number is given in parentheses for each organism. The bacterium *Cuniculiplasma divulgatum* was considered as outgroup.

3.4. Effect of superior bacterial strains on shoot dry weight

Analysis of variance of the interaction effect between salinity levels and bacterial treatment on shoot dry weight was significant ($p < 0.01$) in this study (Table 2). At all levels of salinity, bacterial strains could augment the dry weight of the plant compared with non-inoculated control. However, the highest dry weight of shoot was recorded in plants co-inoculated with *S. meliloti* ARh29 and non-rhizobial strains

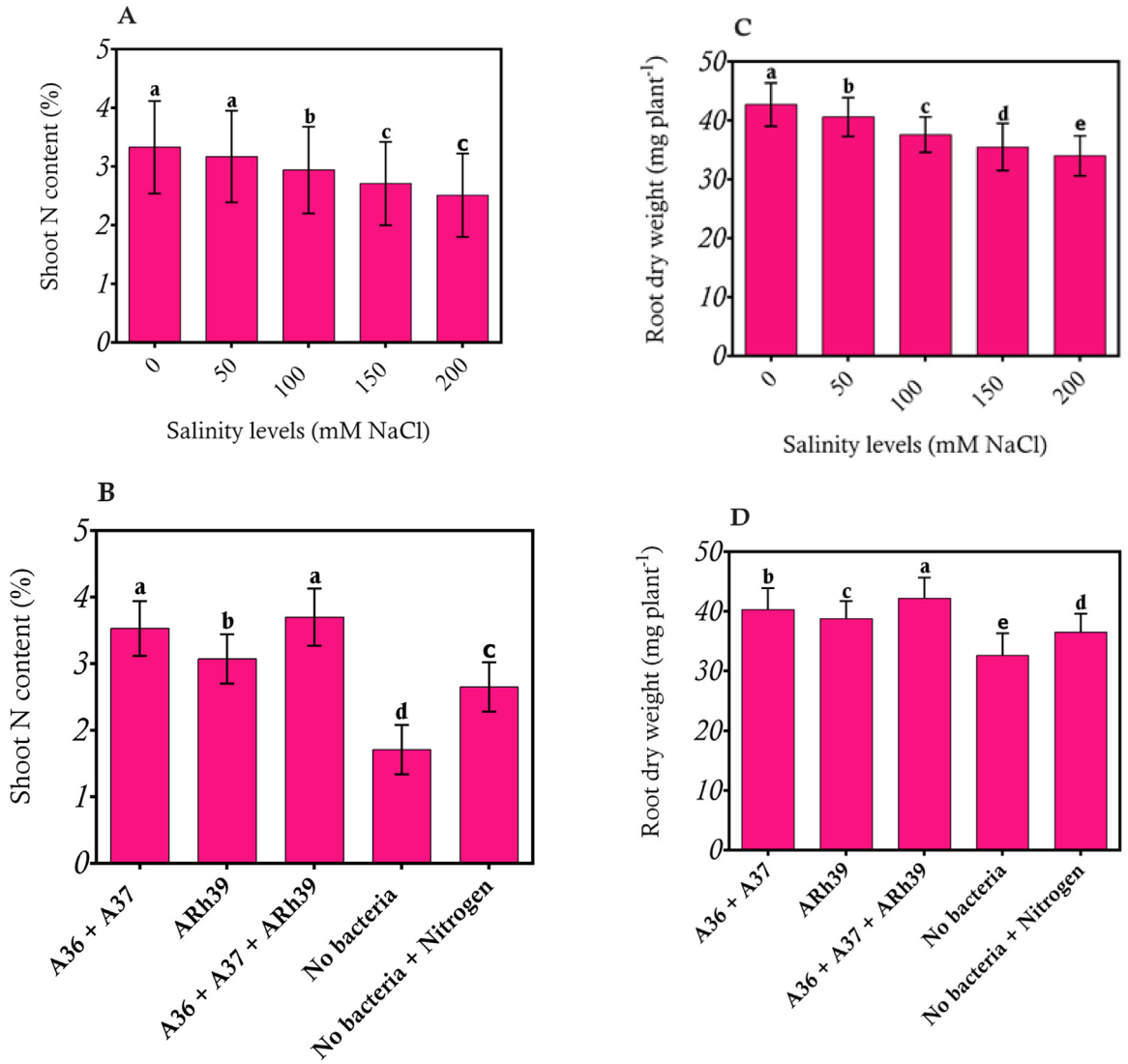

Fig. 4. Effect of non-rhizobial strains and rhizobial strain on the N content of shoot and root dry weight of alfalfa plant grown under salinity stress in greenhouse conditions for 70 days. Means ± SD with different letters are significantly different at the 5% level according to Duncan's multiple-range tests. A36, *Klebsiella* sp.; A37, *K. cowanii*; and ARh29, *S. meliloti*.

Table 2
Interaction effect of non-rhizobial strains *Klebsiella* sp. A36 and *K. cowanii* A37 and rhizobial strain *S. meliloti* ARh29 on some characteristics of physiology and morphology of alfalfa plant grown under salinity stress in greenhouse conditions for 70 days. Means ± SD with different letters are significantly different at the 5% level according to Duncan's multiple-range tests.

Treatments		Shoot DW (mg plant^{-1})	Na^+ (mg g^{-1} leaf DW)	K^+ (mg g^{-1} leaf DW)	Leaf K^+/Na^+	Shoot P content (%)	Proline (μg g^{-1} leaf FW)	SOD activity (unit mg^{-1} protein)	CAT activity (unit mg^{-1} protein)
0 mM NaCl	Strains A36 + A37	138.9 ± 3.2 bc	2.46o ± 0.22 p	53.7 ± 0.79 a	21.97 ± 1.72 c	0.76 ± 0.02 b	3.13 ± 0.21 mno	12.7 ± 0.29 kl	1.63 ± 0.14 kl
	Strain ARh29	142.40 ± 5.8 b	1.31 ± 0.25 q	52.3 ± 0.88 b	41.20 ± 7.02 a	0.53 ± 0.01 e	2.67 ± 0.21 n-q	11.6 ± 0.31 m	1.44 ± 0.07 kl
	Strains A36 + A37 + ARh29	152.20 ± 2.9 a	1.83 ± 0.33 pq	52.3 ± 0.41 b	29.60 ± 5.75 b	0.87 ± 0.02 a	3.40 ± 0.22 lmn	13.6 ± 0.42 j	1.82 ± 0.09 kl
	No bacteria	89.80 ± 2.5 lm	2.71 ± 0.20 o	47.0 ± 1.00 fg	17.40 ± 1.04 d	0.25 ± 0.02 jk	1.94 ± 0.12 q	8.20 ± 0.15 p	0.84 ± 0.07 m
	No bacteria + Nitrogen	127.80 ± 2.6 ef	1.96 ± 0.16 pq	49.3 ± 0.78 cd	25.40 ± 2.25 c	0.30 ± 0.01 i	2.27 ± 0.21 pq	9.9 ± 0.48 o	1.51 ± 0.12 kl
50 mM NaCl	Strains A36 + A37	132.20 ± 1.7 de	8.50 ± 0.26 m	49.9 ± 0.43 c	5.87 ± 0.19 efg	0.56 ± 0.01 e	3.93 ± 0.21 klm	13.7 ± 0.37 ij	2.37 ± 0.21 j
	Strain ARh29	135.10 ± 1.0 cd	7.45 ± 0.05 n	48.5 ± 1.04 de	6.50 ± 0.12 ef	0.44 ± 0.02 g	3.83 ± 0.21 klm	13.1 ± 0.29 jk	2.40 ± 0.08 j
	Strains A36 + A37 + ARh29	136.90 ± 1.2 cd	7.05 ± 0.21 n	50.0 ± 0.61 c	7.11 ± 0.29 e	0.70 ± 0.02 c	4.13 ± 0.33 kl	15.7 ± 0.24 h	3.17 ± 0.17 gh
	No bacteria	80.40 ± 0.8 n	10.20 ± 0.21 l	42.3 ± 0.49 k	4.17 ± 0.10 e-h	0.19 ± 0.01 mn	2.37 ± 0.12 opq	9.60 ± 0.29 o	1.30 ± 0.22 lm
	No bacteria + Nitrogen	124.90 ± 2.3 f	9.55 ± 0.40 l	44.7 ± 0.30 j	4.69 ± 0.17 e-h	0.20 ± 0.0 lm	2.93 ± 0.12 nop	12.3 ± 0.29 l	1.86 ± 0.13 k
100 mM NaCl	Strains A36 + A37	123.80 ± 1.8 fg	14.70 ± 0.31 j	47.6 ± 0.71 ef	3.24 ± 0.12 e-h	0.44 ± 0.02 g	7.13 ± 0.25 ij	17.7 ± 0.30 g	3.47 ± 021 g
	Strain ARh29	119.10 ± 1.3 g	13.40 ± 0.30 k	47.1 ± 0.73 fg	3.52 ± 0.13 e-h	0.36 ± 0.01 h	7.27 ± 0.58 i	16.1 ± 0.25 h	3.47 ± 0.25 g
	Strains A36 + A37 + ARh29	124.70 ± 1.0 f	14.40 ± 0.31 j	48.6 ± 0.27 cde	3.37 ± 0.09 e-h	0.60 ± 0.01 d	8.43 ± 0.46 h	19.7 ± 0.29 f	4.23 ± 0.29 f
	No bacteria	72.97 ± 2.3 o	19.00 ± 0.42 g	38.9 ± 0.78 m	2.05 ± 0.07 fgh	0.13 ± 0.01 o	4.53 ± 0.17 k	10.8 ± 0.33 n	1.63 ± 0.17 kl
	No bacteria + Nitrogen	107.70 ± 1.6 h	17.10 ± 0.50 h	42.2 ± 1.18 k	2.47 ± 0.13 fgh	0.18 ± 0.01 n	6.37 ± 0.25 j	14.4 ± 0.34 i	2.96 ± 0.24 ghi
150 mM NaCl	Strains A36 + A37	101.40 ± 2.1 ij	17.70 ± 0.42 h	46.1 ± 0.22 ghi	2.61 ± 0.07 e-h	0.36 ± 0.01 h	12.60 ± 0.74 e	24.1 ± 0.25 d	4.86 ± 0.18 de
	Strain ARh29	100.40 ± 2.4 ij	16.20 ± 0.18 i	46.4 ± 0.30 fgh	2.87 ± 0.02 e-h	0.31 ± 0.00 i	11.30 ± 0.60 f	23.2 ± 0.60 e	4.78 ± 0.13 e
	Strains A36 + A37 + ARh29	104.20 ± 2.2 hi	16.20 ± 0.35 i	46.7 ± 0.56 fg	2.89 ± 0.04 e-h	0.47 ± 0.01 f	14.30 ± 0.33 d	26.8 ± 0.27 c	6.07 ± 0.21 c
	No bacteria	65.93 ± 3.3 p	23.30 ± 0.38 c	36.2 ± 0.20 n	1.55 ± 0.02 gh	0.10 ± 0.00 po	7.03 ± 0.21 ij	13.8 ± 0.50 ij	2.53 ± 0.21 ij
	No bacteria + Nitrogen	86.77 ± 3.3 m	20.30 ± 0.25 f	40.8 ± 0.46 l	2.01 ± 0.02 fgh	0.14 ± 0.01 o	9.64 ± 0.45 g	18.2 ± 0.21 g	4.20 ± 0.41 f
200 mM NaCl	Strains A36 + A37	94.37 ± 2.1 kl	22.10 ± 0.20 d	44.0 ± 0.35 j	1.99 ± 0.01 fgh	0.26 ± 0.01 j	18.20 ± 0.69 b	27.6 ± 0.29 b	7.27 ± 0.33 b
	Strain ARh29	90.37 l ± 1.6 m	20.0 ± 0.42 f	45.0 ± 0.53 ij	2.25 ± 0.06 fgh	0.23 ± 0.01 kl	17.30 ± 0.26 c	26.4 ± 0.37 c	6.83 ± 0.57b
	Strains A36 + A37 + ARh29	97.27 ± 2.3 jk	21.20 ± 0.27 e	45.2 ± 9.26 hij	2.14 ± 0.04 fgh	0.32 ± 0.01 i	19.70 ± 0.44 a	30.6 ± 0.58 a	8.57 ± 0.30 a
	No bacteria	61.33 ± 2.5 p	27.70 ± 0.48 a	33.1 ± 0.66 o	1.20 ± 0.04 h	0.09 ± 0.00 q	9.20 ± 0.45 g	17.7 ± 0.27 g	2.92 ± 0.09 hi
	No bacteria + Nitrogen	74.87 ± 2.9 o	24.90 ± 0.08 b	38.3 ± 0.55 m	1.53 ± 0.02 gh	0.13 ± 0.00 op	13.90 ± 0.25 d	22.8 ± 0.29 e	5.30 ± 0.43 d
	Source of variation	Shoot DW	Na^+	K^+	K^+/Na^+	P	Proline	SOD activity	CAT activity
	Salinity levels (**S**)	**	**	**	**	**	**	**	**
	Bacterial strains (**B**)	**	**	**	**	**	**	**	**
	S × B (**Interaction**)	**	**	**	**	**	**	**	**

A36, *Klebsiella* sp.; **A37**, *Kosakonia cowanii;* **ARh29,** *Sinorhizobium meliloti*; **DW**, dry wright; **FW**, fresh weight; **CAT**, activity of catalase; **SOD**, activity of superoxide dismutase; and **, Significant at $p < 0.01$.

(A36 + A37 + ARh29) at all salinity levels. In addition, there were not any symptoms of a disease or other specific signs on plants inoculated with these strains.

3.5. Effect of effective bacterial strains on Na^+ and K^+ concentration in leaves

The results of mean comparison showed that the highest amount of Na^+ and K^+ was observed in the negative control (No bacteria + 200 mM NaCl) and in the plants inoculated with non-rhizobial strains (A36 + A37) and without salinity (0 mM NaCl), respectively (Table 2). Compared to non-inoculated plants, inoculated plants absorbed the lower amount of Na^+. An increase in Na^+ concentration in inoculated and non-inoculated plants can also be due to the abundance of Na^+ ions in the root environment and the sodium ion toxicity-mediated retardation of plant growth in higher salinity (contrary to dilution effect). At all salinity levels, bacterial strains increased K^+ uptake in plants, but the highest K^+ absorption was observed in plants co-inoculated with *Klebsiella* sp. A36, *K. cowanii* A37, and rhizobial strain *S. meliloti* ARh29 (A36 + A37 + ARh29), followed by plants inoculated with non-rhizobial strains *Klebsiella* sp. A36 and K. *cowanii* A37 (A36 + A37). Although K^+ concentration increased under salt stress, this increase was negligible as compared to Na^+ concentration. The highest ratio of K^+/Na^+ was observed in non-stressed plants with *S. meliloti* ARh29. As the salinity levels increased, the K^+/Na^+ ratio decreased in inoculated and non-inoculated plants.

3.6. Effect of effective bacterial strains on P concentration in shoot

The average concentration of P decreased significantly with increasing salinity level. The results of mean comparison showed that the effect of bacterial treatments on P concentration was significantly higher than the control (plants non-inoculated with bacterial strains), and its highest concentration was observed in the plants co-inoculated with rhizobial strain and non-rhizobial strains (A36 + A37 + ARh29) and without salinity stress (Table 2). In addition, in the higher salinity levels (50, 100, 150, and 200 mM NaCl), the highest concentration of P was also observed in bacterial treatment of A36 + A37 + ARh29, and treatment of A36 + A37 was in the next rank.

3.7. Effect of effective bacterial strains on proline concentration and antioxidant enzymes

Analysis of variance of data showed that the effect of salinity stress and bacterial levels on proline concentration and antioxidant enzymes was significant ($p < 0.01$) (Table 2). Activity of antioxidant enzymes augmented with enhancing salinity stress initially and indicated a diminishing trend in high salinity. At all salinity levels, plants inoculated with bacterial strains had higher proline concentration and enzymatic activity (CAT and SOD) compared with non-inoculated plants. Compared to low salinity, proline concentration and activity of the enzymes CAT and SOD were higher in plants inoculated and grown under high salt concentrations. The highest concentration of proline (19.70 $\mu g\ g^{-1}$ leaf FW) and activity of enzymes of CAT (8.60 unit mg^{-1} protein) and SOD (30.60 unit mg^{-1} protein) were observed in plants co-inoculated with *Klebsiella* sp. A36, *K. cowanii* A37, and rhizobial strain *S. meliloti* ARh29 (A36 + A37 + ARh29) and subsequently in plants inoculated with non-rhizobial strains of *Klebsiella* sp. A36 and *K. cowanii* A37 (A36 + A37), compared to controls (no bacteria and no bacteria + nitrogen).

4. Discussion

It is well known that the bacteria isolated from saline environments are more efficient to improve the plant tolerance to salt compared to the bacteria isolated from non-saline environments (Etesami and Beattie, 2018). It has also been found that the rhizobia are not the only nitrogen-fixing inhabitants of legume nodules (Lu et al., 2017; Martínez-Hidalgo and Hirsch, 2017). In the present study, we isolated a set of salinity-tolerant rhizobial and non-rhizobial endophytic bacteria from root nodules of alfalfa grown in saline soils (EC, 10.94 dS cm^{-1}). Isolation of non-rhizobial bacteria from root nodules of alfalfa (Lai et al., 2015) and other legumes (Korir et al., 2017; Leite et al., 2017) has also been reported in previous studies. The tolerance of these isolates to salinity and drought was different. According to previous findings, these differences may be related to the status of ion pumps, including sodium pumps, and the regulation of ions by different bacteria (Preiss et al., 2015). In addition to their tolerance to salinity and drought, these endophytic isolates were also able to produce PGP characteristics. In this study, all isolates were positive for IAA production. Dilfuza (2011) reported that 90% of the bacteria associated with plants were able to produce the IAA hormone. It is known that bacterial metabolite of IAA is able to elevate protection of bacterial cells against different abiotic stresses such as high salt concentration (Bianco et al., 2006). The ability to produce PGP properties like N_2 fixing activity, IAA, ACC deaminase, and phosphate solubilization in endophytic bacteria has been reported in previous studies (Bulgarelli et al., 2013; Etesami and Alikhani, 2016). In this study, the best non-rhizobial isolates and rhizobial isolate were identified, which were similar to *Klebsiella* sp., *K. cowanii*, and *S. meliloti*, respectively. To our knowledge, this is the first study showing *Klebsiella* sp. and *K. cowanii* as salinity and drought endophytic bacteria isolated from alfalfa root nodules with multiple PGP traits (Table 1). In previous studies, Chimwamurombe et al. (2016) and Almeida Lopes et al. (2016) also isolated endophytic strains *Kosakonia cowanii* from bean and soybean plant. The strain A37 identified in this study was positive for production of IAA and ACC deaminase and phosphate solubilization. The ability to fix nitrogen in this genus has also been reported (Chen et al., 2014). Martínez-Hidalgo and Hirsch (2017) also stated that some nodule non-rhizobial bacteria can fix nitrogen. *Klebsiella* sp., as an endophytic bacterium, has also been isolated from *Glycine* and *Vicia* (Martínez-Hidalgo and Hirsch, 2017). This bacterium has increased plant growth by fixing nitrogen, IAA production, and phosphate solubilization (Martínez-Hidalgo and Hirsch, 2017).

Co-inoculation of alfalfa with *Klebsiella* sp. A36, *K. cowanii* A37, and *S. meliloti* ARh29 increased the growth indices of the alfalfa plant at all levels of salinity compared with the inoculation of these isolates alone. Non-rhizobial strains *Klebsiella* sp. A36 and *K. cowanii* A37 significantly also increased plant growth in the absence of rhizobial strain *S. meliloti* ARh29. These bacteria could even provide plant N in the absence of *S. meliloti* ARh29 and nitrogen (Fig. 4B), which was an interesting result of this research. Stajković et al. (2009) also isolated non-rhizobial endophytic strains (*i.e., Brevibacillus chosinensis* and *Microbacterium trichothecenolyticum*) from alfalfa nodules that were able to stimulate alfalfa growth in the presence and absence of rhizobial strain *S. meliloti*. Further increase in the growth of alfalfa plants inoculated with non-rhizobial strains of *Klebsiella* sp. A36 and *K. cowanii* A37compared to that of the alfalfa plants inoculated with rhizobial strain *S. meliloti* ARh29 may be attributed to the higher tolerance of the non-rhizobial strains (A36 and A37) to salinity and drought and ACC deaminase production capacity of these strains as well as to their higher ability to produce IAA hormone (Table 1). In previous studies, *Klebsiella* sp. could also confer enhanced tolerance to salinity and plant growth promotion in oat seedlings (*Avena sativa* L.) (Sapre et al., 2018) and in wheat (*Triticum aestivum* L.) (Singh et al., 2015).

Under salinity stress, absorption of nutrients is decreased due to reduced root growth caused by stress ethylene production (Siddikee et al., 2011) and reduction of the activity of ion transporter by sodium (Yao et al., 2010). It has been proven that the bacteria capable of producing ACC-deaminase enzyme (by decreasing stress ethylene) and IAA can increase the growth of plant cells and/or plant tissue elongation (increased root growth and root length), which increases greater root surface area and enables the plant to get more nutrients (*i.e.,* P and

K) and water from the soil (Etesami and Maheshwari, 2018). According to this finding, the alfalfa plants inoculated with these non-rhizobial strains (A36 + A37) showed a better rooting system compared with the plants non-inoculated with these strains (Fig. 4D).

The generation of reactive oxygen species (ROS) is one of the adverse effects of salinity. It has been reported that plant-associated bacteria can increase the plant tolerance by improving the activity of antioxidant enzymes and osmotic adjustment capacity (Etesami and Maheshwari, 2018; Grover et al., 2011; Shrivastava and Kumar, 2015). In the present study, proline concentration and activity of SOD and CAT were significantly increased by co-inoculation of *Klebsiella* sp. A36 + *K. cowanii* A37 + *S. meliloti* ARh29 at all salinity levels compared to control. It was found that *Rhizobium* symbiosis also increased alfalfa salt tolerance by improving the activity of antioxidant enzymes and osmotic adjustment capacity (Wang et al., 2016).

Studies indicate that salinity reduces the uptake/accumulation of plant nutrients (*e.g.,* N, P, and K) and the translocation of water in stressed plants (Etesami and Maheshwari, 2018). It is known that salinity-tolerant-PGPR are able to enhance the uptake of K^+ ion by mediating the expression of an ion high-affinity K^+ transporter (AtHKT1) in plant under saline conditions and, in turn, a higher K^+/Na^+ ratio that favors salinity tolerance (Etesami and Beattie, 2018; Rojas-Tapias et al., 2012). In this study, the highest content of P and K was observed in the plant inoculated with non-rhizobial bacteria (Table 2), which can probably be due to synergistic effects of IAA and ACC deaminase on root length, and finally P and K uptake of soil by root and their transfer to plant aerial parts.

In most previous studies (Elkoca et al., 2010; Figueiredo et al., 2008; Korir et al., 2017; Medeot et al., 2010; Tsigie et al., 2011), it has been reported that the efficiency of N_2 fixation and the growth indices of legume plants by rhizobial bacteria increase in the presence of PGPR. For the first time in this study, we showed that N_2 fixing-non rhizobial bacteria (*Klebsiella* sp. A36 + *K. cowanii* A37) could provide plant N and increase plant growth indices without rhizobial bacteria and nitrogen (Fig. 4B). This indicates that the non-rhizobial bacteria identified in this study were able to fix N_2. According to the results obtained from this study, we confirmed the hypothesis that salinity tolerant bacteria, isolated from the root nodules of alfalfa grown in saline soils, can have a significant role in improving plant growth and alfalfa tolerance to salinity stress in the presence and absence of rhizobial bacterium symbiotic to alfalfa plant (*S. meliloti*).

5. Conclusions

The results of this study clearly indicate that the root nodules of alfalfa plants grown in saline areas can be a useful source of drought and salinity tolerant bacteria with multiple PGP traits. Although many of these nodule non-rhizobial bacteria were not capable of fixing nitrogen, they had the potential to increased alfalfa growth under salinity conditions both in the presence and in absence of rhizobial bacterium symbiotic to alfalfa plant (*S. meliloti*). This knowledge will be useful in defining strategies to apply these bacteria as bio-inoculants by themselves or combined with rhizobial bacteria. Such an approach will enhance rhizobial performance or persistence as well as diminish the usage of chemical fertilizers. According to the findings of this study, PGP bacteria of *Klebsiella* sp. A36 and *K. cowanii* A37 may be suitable candidates as tolerant bacteria in different degrees to salinity and can be used in the formulation of agricultural products (as a cheaper alternative to farmers in production of alfalfa in saline soils) after being tested under field conditions.

Acknowledgments

We wish to thank University of Tehran and Sari Agricultural Sciences and Natural Resources University for providing the necessary facilities for this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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