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ORIGINAL ARTICLE

Salt-tolerant plant growth-promoting bacteria enhanced salinity tolerance of salt-tolerant alfalfa (*Medicago sativa* L.) cultivars at high salinity

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Abstract

Alfalfa (*Medicago sativa* L.) plant growth decreases when cultivated under salinity or irrigated with salty water. Inoculation with plant growth-promoting bacteria (PGPB) is a method for mitigating the harmful effects of salinity on plants growth. To investigate salt-tolerant PGPB with salt-tolerant and salt-sensitive alfalfa cultivar interactions under salinity, some physiological and agronomical aspects were investigated. The inoculated plants of alfalfa cultivars with *Hartmannibacter. diazotrophicus and Pseudomonas* sp. bacteria were compared with non-inoculated plants. Plants were grown in growth room and irrigated with tap water until 6–7 weeks, and then, salinity stress imposed by irrigating with tap water (control), 10 dS m^{-1} and 20 dS m^{-1} NaCl. Salinity reduced relative water content (RWC), membrane stability index (MSI), K^+, photosynthesis rate (Pn) and stomatal conductance (gs), leaf number, height, and dry weight, and increased sodium in all cultivars. Inoculation of cultivars with both PGPB mitigated the negative effects of salinity on plants growth by increasing the root length and weight, nodule number, chlorophyll pigments, RWC, MSI, Pn, and gs. Chlorophyll pigments, plant height and leaf number, Na^+, K^+/Na^+, and nodule number improved more pronounced through inoculating with *Pseudomonas* sp., whereas K^+, carotenoids, and RWC improved more pronounced through *H. diazotrophicus* under salinity. The results showed inoculation with two bacteria improved growth performance in salt-tolerant and salt-sensitive cultivars under 10 dS m^{-1}, but at high salinity (20 dS m^{-1}), inoculation was successful only in salt-tolerant alfalfa cultivars.

Keywords Physiological aspects · Leaf number · Nodule number · Photosynthesis rate · PGPB · Stomatal conductance

Introduction

Soil salinity is one of the most important factors that restricts plants' productivity and quality. It also spreads out in the world year to year. All growth aspects and yield components decrease under salinity in many plants (Munns and Tester 2008; Shahbaz and Ashraf 2013). Photosynthesis, gas exchanges, and plant growth decrease due to the osmotic and ionic stress of salinity stress (Chaves et al. 2009). Two main strategies to overcome salinity stress include (i) technological strategy which is expensive; (ii) biological strategy which is economically feasible (Ashraf and Foolad 2013). One biological method for decreasing deleterious effects of salinity is application of plant growth-promoting bacteria (PGPB) (Bacilio et al. 2004; Shrivastava and Kumar 2015; Trdan et al. 2019). Alleviation of salt stress by PGPB has been reported in alfalfa (Noori et al. 2018; Wang et al. 2016), legume (Zahran 1999), barley (Suarez et al. 2015),

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okra (Habib et al. 2016), lettuce (Han and Lee 2005), and pepper (Del Amor and Cuadra-Crespo 2012). Recent studies emphasize on the mitigating effect of PGPB on plants growth by nutrient solubilization, nitrogen fixation, and phytohormone production mechanisms (Dodd, and Pérez-Alfocea 2012; Paul and Lade 2014).

Alfalfa (*Medicago sativa* L.) is a perennial plant which is cultivated as a forage in large areas of irrigated lands in the world. This plant has high economical and agronomical importance due to its high forage quality and N_2 fixation ability (Anower et al. 2013). Alfalfa is moderately saline tolerant plant among the legumes; however, its production decreases at salinity above 2 dS m^{-1} (Maas and Hoffman 1977). With regard to high water need of alfalfa production among plants, and also water shortages in many areas, the need to use saline or wastewater for alfalfa irrigation increases every day. Reports show that PGPB has improving effects on alfalfa germination indices and growth aspects under salinity (Bertrand et al. 2015; Ansari et al. 2017; Noori et al. 2018). Bertrand et al. (2015) showed that combining salt-tolerant rhizobial strains with salt-tolerant alfalfa cultivars was an effective strategy to improve alfalfa productivity under salinity. Noori et al. (2018) showed that nodule non-rhizobial strains in alfalfa plants had PGPB abilities and could use for improving salinity tolerance as a Rhizobium bio-fertilizers in salinity conditions.

In the previous study, we selected two salt-tolerant bacteria (two strains) among four commercial PGPB strains and we showed their significant positive influence on improving germination indices in nine alfalfa cultivars under salinity (Ansari et al. 2017). In this study, we investigated the hypothesis that salt-tolerant bacteria can improve salinity tolerance in alfalfa and this effect in salt-tolerant cultivars is more than salt-sensitive cultivars under saline conditions. The aims of this study were to investigate (i) whether inoculation with PGPB alleviates growth parameters and physiological properties in alfalfa cultivars, (ii) the interaction between salt-tolerant PGPB with salt-tolerant and salt-sensitive alfalfa cultivars, (iii) the differences between inoculated plants of salt-tolerant and salt-sensitive alfalfa cultivars under salinity, and (iv) evaluation of the effect of inoculation with PGPB on Na^+ and K^+ ions absorption.

Materials and methods

Salt-tolerant bacteria strains

This study is continuation of the previous experiments about the effects of alfalfa cultivar inoculation with salt-tolerant bacteria under salinity. To inoculate salt-tolerant and sensitive alfalfa cultivars seeds with salt-tolerant bacteria, two representative soil bacteria (*Pseudomonas* sp. DSMZ 13134 proradix and *Hartmannibacter diazotrophicus*) were prepared and used (Ansari et al. 2017). The *Pseudomonas* sp. bacteria came from an industrial product used in BIOFECTOR project (https://www.biofector.info) and *H. diazotrophicus* came from the Justus-Liebig-University, of Giessen in Germany from the collection of Prof. Sylvia Schnell and Stefan Ratering.

Alfalfa cultivars, inoculum procedure, and cultivation

Four alfalfa cultivars Hamadan, Hashtrod, Heris salt-tolerant, and Local 253 (salt-sensitive) were selected in germination test (Ansari et al. 2017). Alfalfa seeds were scarified for 2 min in 98% sulphuric acid and then surface-sterilized for 3 min in 5% sodium hypochloride. After washing, the seeds were placed in separated and sterile Petri dishes and 10 ml microbial solution cells (1.6×10^{13} CFU mL^{-1}) added to each of them. The Petri dishes were placed in room temperature for 1 h, and then, the seeds were dried. Forty seeds of alfalfa cultivars were planted in plastic pots (20 cm diameter) that were filled with mixture of farm soil and perlite (4, 1). The pots were placed in the growth room of the Soil Science and Water Management Department of Szent Istvan University under a 25/15 °C day/night temperatures, a 15 h photoperiod, and a photosynthetic photon flux density of 600–800 mmol photons m^{-2} s^{-1}. The Pots were irrigated with tap water after sowing and extra seedlings were cut after establishment and 20 plants retained in each pot.

Salinity treatments and experimental design

The pots irrigated with tap water until 6–7 weeks, and then, salinity treatments (tap water: 0 dS m^{-1} (control), 10 dS m^{-1}, and 20 dS m^{-1}) imposed by irrigating with salty water every 5 days. Irrigation was continued until the end of two harvests. The experimental design was factorial and comprised three levels of salt, four alfalfa cultivars (randomized complete block design), two bacteria, three replicates, and two harvests in 108 pots.

Measurement

Relative water content (RWC) and membrane stability index (MSI) were measured after the first and second week salinity treatment. *RWC*, six leaf samples were detached in each treatment and replication and weighed immediately to measure fresh weight (FW); after that, the samples were dipped in the distilled water for 24 h. The leaves were weighed to record fully turgid weight (TW) and were subjected to oven drying at 70 °C for 48 h to record the dry weight (DW). The RWC were calculated by the equation of $RWC = [FW - DW]/[TW - DW] \times 100$ (Smart and Bingham

1974). *MSI*, fresh leaf sample (0.1 g) was taken in 10 cm^3 of double distilled water in two sets. One set was subjected to 40 °C for 30 min and its conductivity was recorded using a conductivity meter (C1). The second set was kept in a boiling water bath (100 °C) for 10 min and its conductivity was also recorded (C2). MSI = [1 − (C1/C2)] × 100 (Sairam et al. 2005).

Photosynthesis rate (Pn) and stomatal conductance (gs) were measured using a portable infrared gas analyzer (two times) after the first week and second week salinity by LCi (ADC Bioscientific LTD. UK). Water-use efficiency (WUE) calculated by dividing photosynthesis rate to transpiration (T) $\left(\mathrm{WUE} = \frac{Pn}{T}\right)$.

Chlorophyll pigments were measured (one time) 2 weeks after salinity treatment. The content of chlorophyll *a* and chlorophyll *b* was extracted and quantified by the modified method of Arnon (1949). 500 mg (W) fresh leaf tissue was extracted using the volume (V) of 10 ml 80% Acetone. The extracted solutions were measured using a spectrophotometer (Biochrom Libra S22, UK) at wavelengths 470, 663, and 645 nm (Shivakrishna et al. 2018). The chlorophyll *a* (Chla) and chlorophyll *b* (Chlb) were calculated according to the following equations:

$$\mathrm{Chl}_a\ (\mathrm{mg/g}) = \left[12.7A_{663} - 2.69A_{645}\right](V/W)\ \mathrm{Chl}_b(\mathrm{mg/g}) = \left[22.9A_{645} - 4.68A_{663}\right](V/W)$$

$$\text{Total chlorophyll (mg/g)} = \left[(20.2A645 + 8.02A663)\,V/W\right]$$

$$\text{Carotenoids content (mg/g)} = \left(1000\ \mathrm{A}_{470} - 1.8\ \mathrm{Chl_a} - 85.02\ \mathrm{Chl_b}\right)/198,$$

where A is the optical density at specific wavelength (Arnon 1949).

Plants dry weight, height, and leaf number were measured at two harvests and after 10% starting flowering. Dry weight was measured by lab balance and expressed as a dry weight per plant after cutting the whole plants and drying in an oven at 55 °C for 72 h (Neres et al. 2010). The plant height was measured by ruler (cm) and the leaf number accounted per plant.

Root growth and *nodule number* were measured at the end of experiment. *Root length* was measured by ruler (cm), *root weight* was measured by lab balance and expressed as a root weight per plant, and *nodule number* was extracted at the end of experiment (after two harvest) and accounted after extracting the whole plants roots from pots and exact washing with distilled water.

Ion content

100 mg of alfalfa leaves samples were oven-dried and ashed at 600 °C for 5 h. Afterwards, samples were dissolved in 2 ml concentrated HNO_3 with gentle heating. The samples were adjusted to a volume of 50 ml with distilled water and filtered through a paper filter. Na^+ and K^+ contents were measured using flame photometry (Jenvey-PFP7 Flame Photometer, Japan) (Gao et al. 2016).

Statistical analysis

Data were subjected to analysis of variance (ANOVA), and means were compared using Duncan's range test and Fisher's protected least significant difference (LSD) test at $P < 0.05$. All calculations were performed using SAS software, version 9.4.

Results

Effect of inoculation with bacteria on dry weight, height, and leaf number

To understand the effect of inoculation with bacteria on alfalfa growth and dry mass, the plant height, leaf number, and dry weight were measured at two harvests. Data showed that in all cultivars, dry weight, height, and leaf number decreased under salinity at both harvests, and all of the mentioned aspects in inoculated plants were higher than non-inoculated plants (Fig. 2). Investigating the harvest, bacteria, and salinity interactions showed that dry weight, plant height and leaf number at second harvest were more than the first harvest at control. Dry weight, plant height, and leaf number at second harvest were less than the first harvest in all cultivars under salinity. Inoculated plants of Hashtrod at control and salinity had higher dry weight than non-inoculated plants at the first harvest. At second harvest, dry weight of inoculated plants of Hamadan and Local 253 was higher significantly than non-inoculated plants. The improvement effect of inoculation with bacteria was more pronounced in Heris and Local 253 at 10 dS m^{-1}. Inoculation with bacteria had no significant effect on the dry weight of alfalfa cultivars except Heris at the second harvest at 20 dS m^{-1}. The dry weight of inoculated plants of Heris was more than non-inoculated plants. Also, the inoculated plants with *Pseudomonas* sp. bacteria had higher height and leaf number than inoculated plants with *H. diazotrophicus* (Fig. 2). Plant height in inoculated plant improved 14% and 15% and leaf number improved 18% and 23% at control and salinity,

respectively, compared with non-inoculated plants. Study of interactions among harvest, salinity, and bacteria showed that at the first harvest, Heris and Local 253, at 10 dS m^{-1} and Local 253 at 20 dS m^{-1} had higher plant height and leaf number, but at second harvest, there was no significant difference among cultivars under salinity.

Effect of inoculation with bacteria on nodule number, root length, and root weight

Nodule number and root length decreased with increase in salinity in all cultivars; however, both of them in inoculated plants were more than non-inoculated at control and salinity. Study of the interactions between salinity and cultivar showed nodule number in inoculated plants with *Pseudomonas* sp. bacteria was more than inoculated plants with *H. diazotrophicus* and non-inoculated plants in control (Table 1). There was no significant difference in nodule number of inoculated plants with either bacterium at 10 dS m^{-1}. Also, the nodule number of inoculated plants of Heris and Hamadan was higher than non-inoculated plants, but in Local 253 and Hashtrod, there was no significant difference between inoculated and non-inoculated plants at 10 dS m^{-1}. At 20 dS m^{-1}, there was no significant difference in nodule number between inoculated and non-inoculated plants except Local 253. The interaction between salinity and cultivar showed at control Local 253, at 10 dS m^{-1} Hashtrod, and at 20 dS m^{-1} Hamadan had the highest root length among the cultivars. Root weight increased under salinity in all cultivars, and in inoculated plants, it was higher than non-inoculated. Investigating the interactions of salinity and cultivar showed that Heris and Hashtrod at control, Heris at 10 dS m^{-1}, and Local 253 at 20 dS m^{-1} had the highest root weight (Table 1).

Effect of inoculation with bacteria on relative water content (RWC) and membrane stability (MSI)

To understand the inoculation effect on RWC and MSI in alfalfa plants under salinity, both of them were measured at the first and the second week after salinity. Data showed that RWC and MSI decreased under salinity stress during 2 weeks after salinity, and in inoculated plants, RWC and MSI were higher than non-inoculated plants (Fig. 1). Investigating the interactions among harvest, salinity, bacteria, and cultivar showed that there was no significant difference between inoculated and non-inoculated plants at 20 dS m^{-1} a week after salinity; however, inoculated plants had relatively higher RWC compared with non-inoculated plants. Inoculated plants of the Local 253 and Heris had higher RWC compared with other cultivars at 10 dS m^{-1} a week after salinity. At the second week, the inoculated plants of Heris and Hashtrod had a high RWC among the cultivars at 10 dS m^{-1} and there was no significant difference among cultivars at 20 dS m^{-1}. Inoculated plants with *H. diazotrophicus* had higher RWC than inoculated plants with *Pseudomonas* sp. bacteria. Investigating the interaction harvest with bacteria and salinity with bacteria showed that the inoculated plants of Heris and Hashtrod had a higher MSI than other cultivars at 20 dS m^{-1}, but there was no significant difference in MSI between inoculated and non-inoculated plants at 10 dS m^{-1} a week after salinity treatment (Fig. 1). At the second week, the inoculated plants of Hamadan and Local 253 had a higher MSI than other cultivars at 10 dS m^{-1}, but there was no significant difference among cultivars at 20 dS m^{-1}.

Chlorophyll *a* (Chla), chlorophyll *b* (Chlb), total chlorophyll (Chlab), and carotenoids content (Car)

To understand the effects of inoculation with bacteria on chlorophyll pigments in alfalfa plants under salinity, *Chla*, *Chlb*, *Chlab*, and *Car* were measured 2 weeks after salinity treatment. Data showed that *Chla*, *Chlb*, *Chlab*, and *Car* decreased under salinity, but the rate of reduction in inoculated plants was less than non-inoculated plants (Table 1). Moreover, inoculated plants with *Pseudomonas* sp. had a higher *Chla*, *Chlb*, and *Chlab* than the inoculated plants with *H. diazotrophicus*. The study of interactions among salinity, bacteria, and cultivar showed that *Chla* in inoculated plants of all cultivars except Local 253 was more than non-inoculated under salinity. The study of the interaction between salinity and cultivar showed Heris and Hamadan had higher Chlb than other cultivars at 10 dS m^{-1}, but there was no significant difference among cultivars at 20 dS m^{-1}. And also, the study of the interactions between salinity and cultivars showed Heris had higher *Chlab* and *Car* content compared with other cultivars at 10 dS m^{-1} and 20 dS m^{-1}. Inoculated plants with *H. diazotrophicus* had higher *Car* than the inoculated plants with *Pseudomonas* sp. (Table 1).

Photosynthesis rate (Pn), stomatal conductance (gs), and water-use efficiency (WUE)

Pn, *gs*, and *WUE* were measured to understand the effect of inoculation with bacteria on the photosynthesis process in alfalfa plants under salinity at the first week and the second week after salinity treatment. Data showed that *Pn*, *gs*, and WUE in inoculated plants with bacteria were higher than non-inoculated plants under control and salinity. Pn and gs decreased, but WUE increased in all cultivars with the increase in salinity. There was no significant difference between the inoculated plants of *Pseudomonas* sp. and *H. diazotrophicus* bacteria in Pn and gs (Fig. 1). The interactions between salinity and cultivar showed that inoculated plants of Hamadan at control and Hashtrod at 10 dS m^{-1} had the highest gs among the cultivars, and there was no significant difference among

Table 1 Mean value and standard deviations of chlorophyll a, b, and total chlorophyll (Chla, Chlb, and Chlab), carotenoid content (Car), root length, root weight, nodule number, potassium (K^+), sodium (Na^+), and K^+/Na^+ inoculated with *Pseudomonas* sp. Proradix (P. sp. Proradix) and *Hartmannibacter diazotrophicus* (*H. diazotrophicus*) strains and non-inoculated irrigated at 0, 10, and 20 dS m^{-1}

Treatment	Cultivars	Bacteria	Chla	Chlb	Chlab	Car	Root length	Root weight	Nodule number	K^+ (mg/g)	Na^+ (mg/g)	K^+/Na^+
Control	Hamadan	Non-inoculated	2.84 ± 0.14	0.67 ± 0.03	3.9 ± 0.21	10.4 ± 1.2	15.67 ± 2.1	2.35 ± 0.2	30 ± 4.4	11.43 ± 2.7	0.7 ± 0.2	15.74 ± 1.0
		H. diazotrophicus	3.03 ± 0.01	0.71 ± 0.04	4.2 ± 0.04	10.9 ± 0.4	15.67 ± 0.6	2.47 ± 0.2	34.67 ± 3.2	12.46 ± 3.0	0.81 ± 0.2	15.44 ± 0.3
		P. sp. Proradix	3.03 ± 0.04	0.74 ± 0.04	4.2 ± 0.08	12.1 ± 1.0	17.33 ± 1.5	2.4 ± 0.1	39.33 ± 4.0	12.51 ± 1.6	0.68 ± 0.1	18.57 ± 0.9
	Hashtrod	Non-inoculated	2.64 ± 0.15	0.54 ± 0.03	3.7 ± 0.21	7.5 ± 0.8	15.33 ± 0.6	2.47 ± 0.2	24.33 ± 2.1	12.62 ± 2.0	0.6 ± 0.1	20.33 ± 4.3
		H. diazotrophicus	2.71 ± 0.10	0.58 ± 0.01	3.8 ± 0.16	10.7 ± 3.5	16.00 ± 1.0	2.78 ± 0.1	35 ± 5.0	13.9 ± 1.7	0.88 ± 0.4	17.41 ± 5.5
		P. sp. Proradix	2.82 ± 0.14	0.63 ± 0.02	3.9 ± 0.19	8.2 ± 1.8	16.00 ± 1.0	2.7 ± 0.2	34.67 ± 6.1	13.68 ± 1.0	0.86 ± 0.2	16.27 ± 2.7
	Heris	Non-inoculated	2.79 ± 0.12	0.63 ± 0.08	3.9 ± 0.22	10.8 ± 2.3	14.00 ± 1.7	2.69 ± 0.4	22 ± 2.0	14.1 ± 1.6	0.78 ± 0.1	18.08 ± 1.2
		H. diazotrophicus	2.84 ± 0.16	0.64 ± 0.07	3.9 ± 0.26	13.2 ± 1.7	16.00 ± 3.6	2.98 ± 0.1	21 ± 4.4	13.44 ± 3.3	0.75 ± 0.1	18.86 ± 7.8
		P. sp. Proradix	2.9 ± 0.10	0.68 ± 0.07	4 ± 0.19	11.1 ± 1.6	18.00 ± 2.0	2.91 ± 0.3	34.33 ± 4.0	13.56 ± 3.2	0.72 ± 0.1	19.62 ± 7.3
	Local 253	Non-inoculated	2.58 ± 0.11	0.58 ± 0.07	3.6 ± 0.12	6.0 ± 1.5	18.33 ± 3.5	1.96 ± 0.1	26.67 ± 3.1	13.71 ± 1.1	0.8 ± 0.2	17.69 ± 3.3
		H. diazotrophicus	2.75 ± 0.08	0.62 ± 0.04	3.8 ± 0.10	7.1 ± 0.8	21.67 ± 4.0	2.11 ± 0.1	39 ± 1.0	14.35 ± 2.2	0.69 ± 0.2	22.19 ± 8.3
		P. sp. Proradix	2.86 ± 0.14	0.67 ± 0.05	3.9 ± 0.19	8.4 ± 1.2	18.67 ± 3.1	2.13 ± 0.2	44.33 ± 3.2	14.49 ± 1.9	0.72 ± 0.1	20.09 ± 1.3
EC = 10 dS m^{-1}	Hamadan	Non-inoculated	2.25 ± 0.23	0.51 ± 0.02	3.1 ± 0.33	8.0 ± 0.8	14.33 ± 1.2	2.49 ± 0.4	5.33 ± 0.6	10.54 ± 0.8	9.95 ± 0.4	1.06 ± 0.1
		H. diazotrophicus	2.77 ± 0.33	0.64 ± 0.04	3.8 ± 0.48	9.3 ± 2.7	14.67 ± 1.5	2.72 ± 0.3	8.67 ± 3.2	11.17 ± 1.5	10.89 ± 1.3	1.03 ± 0.1
		P. sp. Proradix	2.74 ± 0.29	0.65 ± 0.11	3.8 ± 0.46	8.0 ± 2.0	15.67 ± 1.2	3.13 ± 0.4	9 ± 1.0	10.91 ± 1.3	6.52 ± 0.3	1.68 ± 0.3
	Hashtrod	Non-inoculated	1.88 ± 0.42	0.36 ± 0.08	2.6 ± 0.64	4.7 ± 1.1	13.33 ± 0.6	2.63 ± 0.3	6.33 ± 1.2	9.01 ± 0.7	9.14 ± 0.7	0.99 ± 0.1
		H. diazotrophicus	2.45 ± 0.11	0.44 ± 0.05	3.5 ± 0.18	7.2 ± 1.9	13.67 ± 0.6	2.72 ± 0.2	6.67 ± 0.6	10.34 ± 1.8	9.18 ± 0.7	1.13 ± 0.2
		P. sp. Proradix	2.55 ± 0.19	0.48 ± 0.05	3.6 ± 0.26	7.7 ± 1.2	14.00 ± 1.7	2.79 ± 0.2	6.33 ± 0.06	11.11 ± 0.9	7.13 ± 0.7	1.58 ± 0.3
	Heris	Non-inoculated	2.62 ± 0.15	0.52 ± 0.02	3.7 ± 0.24	10.7 ± 3.1	15.33 ± 0.6	3.14 ± 0.1	10 ± 2.0	10.26 ± 0.4	7.85 ± 0.2	1.31 ± 0.1
		H. diazotrophicus	2.92 ± 0.14	0.63 ± 0.05	4.1 ± 0.23	11.8 ± 2.5	17 ± 1.0	3.3 ± 0.2	17 ± 4.6	11.36 ± 0.9	8.82 ± 0.6	1.29 ± 0.1
		P. sp. Proradix	3.08 ± 0.13	0.66 ± 0.05	4.3 ± 0.18	12.4 ± 1.2	16 ± 0.2	3.22 ± 0.2	18.33 ± 5.8	10.8 ± 1.5	7.32 ± 0.4	1.49 ± 0.3
	Local 253	Non-inoculated	1.75 ± 0.27	0.4 ± 0.04	2.4 ± 0.40	6.4 ± 2.0	15.33 ± 0.6	1.98 ± 0.1	4 ± 1.0	10.09 ± 0.6	8.45 ± 0.8	1.21 ± 0.2
		H. diazotrophicus	2.02 ± 0.08	0.49 ± 0.01	2.8 ± 0.11	6.7 ± 0.4	15.67 ± 0.6	2.14 ± 0.2	6.33 ± 3.1	11.52 ± 0.8	8.47 ± 0.7	1.36 ± 0.1
		P. sp. Proradix	2.2 ± 0.24	0.52 ± 0.03	3 ± 0.35	6.3 ± 0.8	15.17 ± 0.8	2.21 ± 0.2	6.67 ± 01.2	12.13 ± 0.9	4.97 ± 0.4	2.45 ± 0.2
EC = 20 dS m^{-1}	Hamadan	Non-inoculated	1.65 ± 0.51	0.2 ± 0.03	2.4 ± 0.77	4.4 ± 0.6	16.33 ± 0.6	2.58 ± 0.1	5.67 ± 2.1	8.91 ± 1.1	21.12 ± 0.8	0.42 ± 0.1
		H. diazotrophicus	2.24 ± 0.08	0.32 ± 0.03	3.2 ± 0.10	10.5 ± 2.5	17 ± 1.0	2.68 ± 0.2	6.67 ± 1.5	9.74 ± 0.5	17.13 ± 0.5	0.57 ± 0.1
		P. sp. Proradix	2.37 ± 0.24	0.4 ± 0.07	3.4 ± 0.38	7.6 ± 1.4	16.67 ± 1.5	2.67 ± 0.5	6 ± 1.7	9.2 ± 1.1	19.88 ± 0.8	0.46 ± 0.1
	Hashtrod	Non-inoculated	1.31 ± 0.28	0.19 ± 0.02	1.9 ± 0.43	3.3 ± 0.5	13.33 ± 1.2	2.62 ± 0.1	2 ± 1.0	7.7 ± 0.8	22.81 ± 3.2	0.34 ± 0.1
		H. diazotrophicus	1.61 ± 0.11	0.25 ± 0.01	2.3 ± 0.17	5.5 ± 1.5	15.00 ± 1.0	2.76 ± 0.4	3.33 ± 0.6	10.17 ± 0.9	17.44 ± 1.1	0.58 ± 0.1
		P. sp. Proradix	1.72 ± 0.31	0.31 ± 0.06	2.4 ± 0.44	5.7 ± 0.8	16.00 ± 1.0	2.86 ± 0.6	4.33 ± 1.2	7.18 ± 1.1	18.85 ± 0.6	0.36 ± 0.1
	Heris	Non-inoculated	2.08 ± 0.16	0.25 ± 0.03	3 ± 0.23	7.2 ± 0.6	13.33 ± 1.2	2.02 ± 0.5	5.67 ± 0.6	8.54 ± 0.7	21.77 ± 1.3	0.39 ± 0.1
		H. diazotrophicus	2.23 ± 0.16	0.33 ± 0.04	3.2 ± 0.22	10.4 ± 3.6	15.33 ± 0.6	2.37 ± 0.1	6.33 ± 2.5	8.97 ± 0.7	19.86 ± 0.9	0.45 ± 0.1
		P. sp. Proradix	2.51 ± 0.13	0.39 ± 0.05	3.6 ± 0.17	8.9 ± 0.2	15.33 ± 0.6	2.25 ± 0.1	7.67 ± 2.5	8.75 ± 0.8	19.46 ± 0.9	0.38 ± 0.1
	Local 253	Non-inoculated	1.36 ± 0.13	0.23 ± 0.03	1.9 ± 0.18	3.9 ± 0.6	13.33 ± 1.5	3.14 ± 0.7	3.67 ± 2.1	10.13 ± 1.7	18.13 ± 1.6	0.57 ± 0.2
		H. diazotrophicus	1.61 ± 0.15	0.28 ± 0.01	2.3 ± 0.22	6.1 ± 0.4	15.33 ± 0.6	3.7 ± 0.4	6.33 ± 1.5	10.87 ± 0.2	18.62 ± 1.1	0.58 ± 0.1
		P. sp. Proradix	1.74 ± 0.16	0.35 ± 0.08	2.4 ± 0.20	5.6 ± 2.0	15.00 ± 1.0	3.45 ± 0.4	7.67 ± 2.5	11.44 ± 1.0	13.25 ± 0.7	0.87 ± 0.1

Table 1 (continued)

Treatment	Cultivars	Bacteria	Chla	Chlb	Chlab	Car	Root length	Root weight	Nodule number	K^+ (mg/g)	Na^+ (mg/g)	K^+/Na^+
LSD = 0.05			0.35	0.08	0.5	2.9	2.6	0.41	5.2	1.58	1.45	1.2

Each value represents the mean of three replicates

Significant differences according to LSD test ($P < 0.05$)

the cultivars at 20 dS m^{-1}. The study of the interactions harvest with salinity and salinity with cultivar showed that *Pn* in the second week significantly was less than the first week at 10 dS m^{-1}. And there was no significant difference in *Pn* between the first week and the second week at control and 20 dS m^{-1} treatments. Hamadan at control and 20 dS m^{-1} and Local 253 at 10 dS m^{-1} had the highest *Pn* among the cultivars. Study of the interactions among harvest, salinity and cultivar showed that WUE increased in the second week compared with the first week also WUE at 10 dS m^{-1} was more than control and 20 dS m^{-1}. Moreover, Hamadan at control and Local 253 at 20 dS m^{-1} had the lowest WUE, and there was no significant difference among other cultivars; besides, Local 253 at 10 dS m^{-1} had the highest WUE (Fig. 2).

Ion content

Potassium (K^+) and K^+/Na^+ ratio decreased and sodium (Na^+) increased by increasing salinity in all cultivars. The interactions among salinity, bacteria, and cultivar showed that Na^+ in inoculated plants with *Pseudomonas* sp. bacteria was less than inoculated plants with *H. diazotrophicus* and non-inoculated plants at 10 dS m^{-1}. Also, the inoculated plants with both bacteria had fewer Na^+ at 20 dS m^{-1}. The inoculated plants of Local 253 had fewer Na^+ than other cultivars under salinity (Table 1). Investigating the interaction among salinity and cultivar showed that K^+ in Local 253 which inoculated with *Pseudomonas* sp. at 10 dS m^{-1} and Hashtrod inoculated with *H. diazotrophicus* at 20 dS m^{-1} was higher than other cultivars. The inoculated cultivars by *H. diazotrophicus* had higher K^+ than inoculated by *Pseudomonas* sp. (Table 1). The interaction among salinity, cultivar, and bacteria showed that K^+/Na^+ in Local 253 and Heris was higher than other cultivars at 10 dS m^{-1}, but there was no significant difference among cultivars at 20 dS m^{-1}. Moreover, the K^+/Na^+ ratio in inoculated plants with *Pseudomonas* sp. was higher than inoculated plants with *H. diazotrophicus* at 10 dS m^{-1}. Local 253 which inoculated with *Pseudomonas* sp. had higher K^+/Na^+ ratio compared with other inoculated cultivars at 10 dS m^{-1}.

Discussion

Salinity reduced significantly alfalfa dry weight production and growth due to increase in Na^+ and reduction in RWC, chlorophyll pigments, photosynthesis rate, nodule number, and K^+. The same results were reported by Munns and Tester (2008), Chaves et al. (2009), Ashraf and Foolad (2013) and Acosta-Motos et al. (2017). The results showed that inoculation by bacteria reduced the negative effects of salinity on alfalfa growth and dry weight, because the inoculated plants had high gs, Pn, RWC, K^+, leaf number, and height under salinity compared with non-inoculated plants.

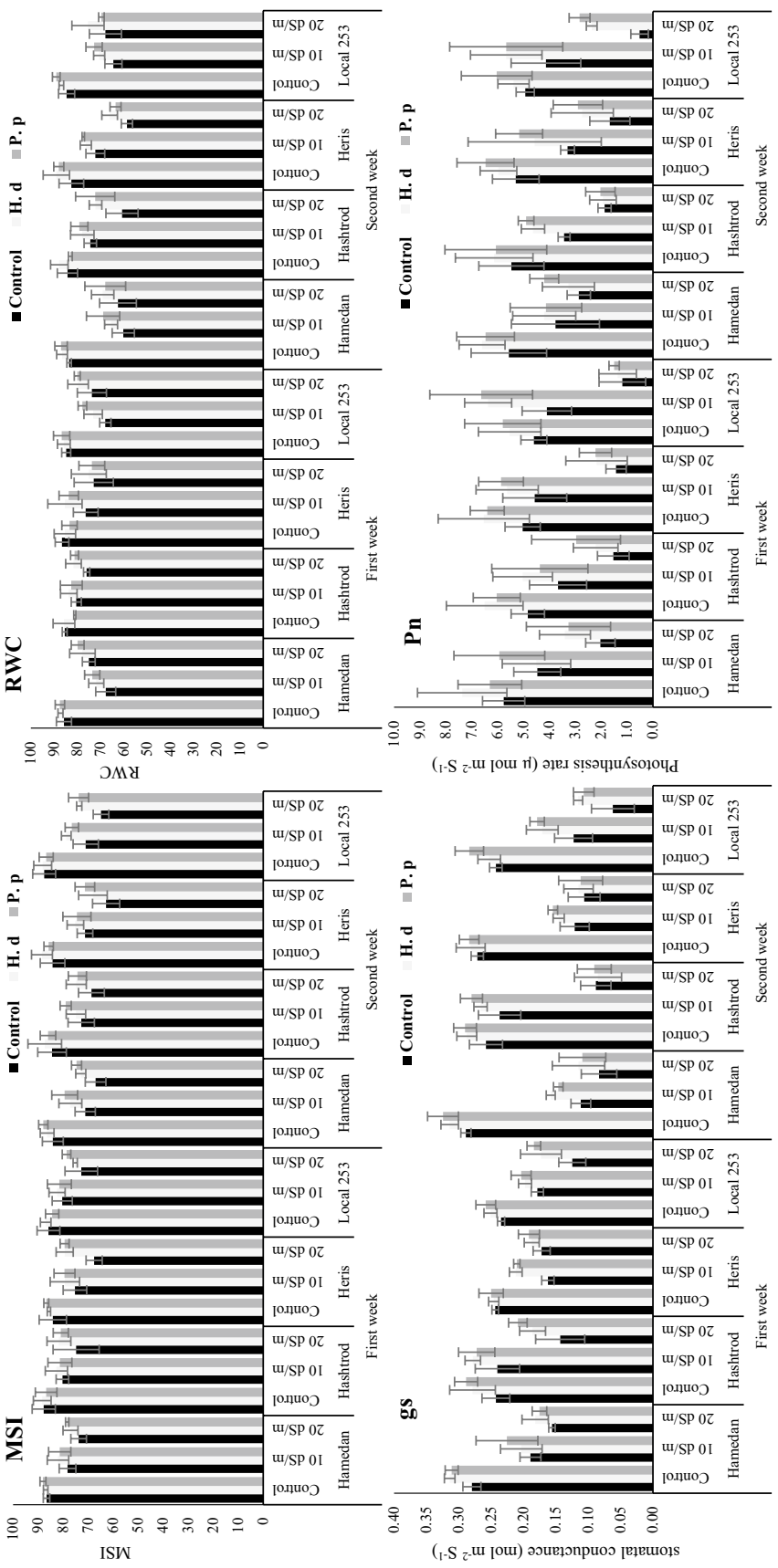

Fig. 1 *Pseudomonas sp. Proradix* (*P. p*) and *Hartmannbacter diazotrophicus* (*H. d*) effects on means of MSI, RWC, Pn, and gs in alfalfa cultivars. Error bars represent standard deviation * ($P<0.05$) (three replicates)

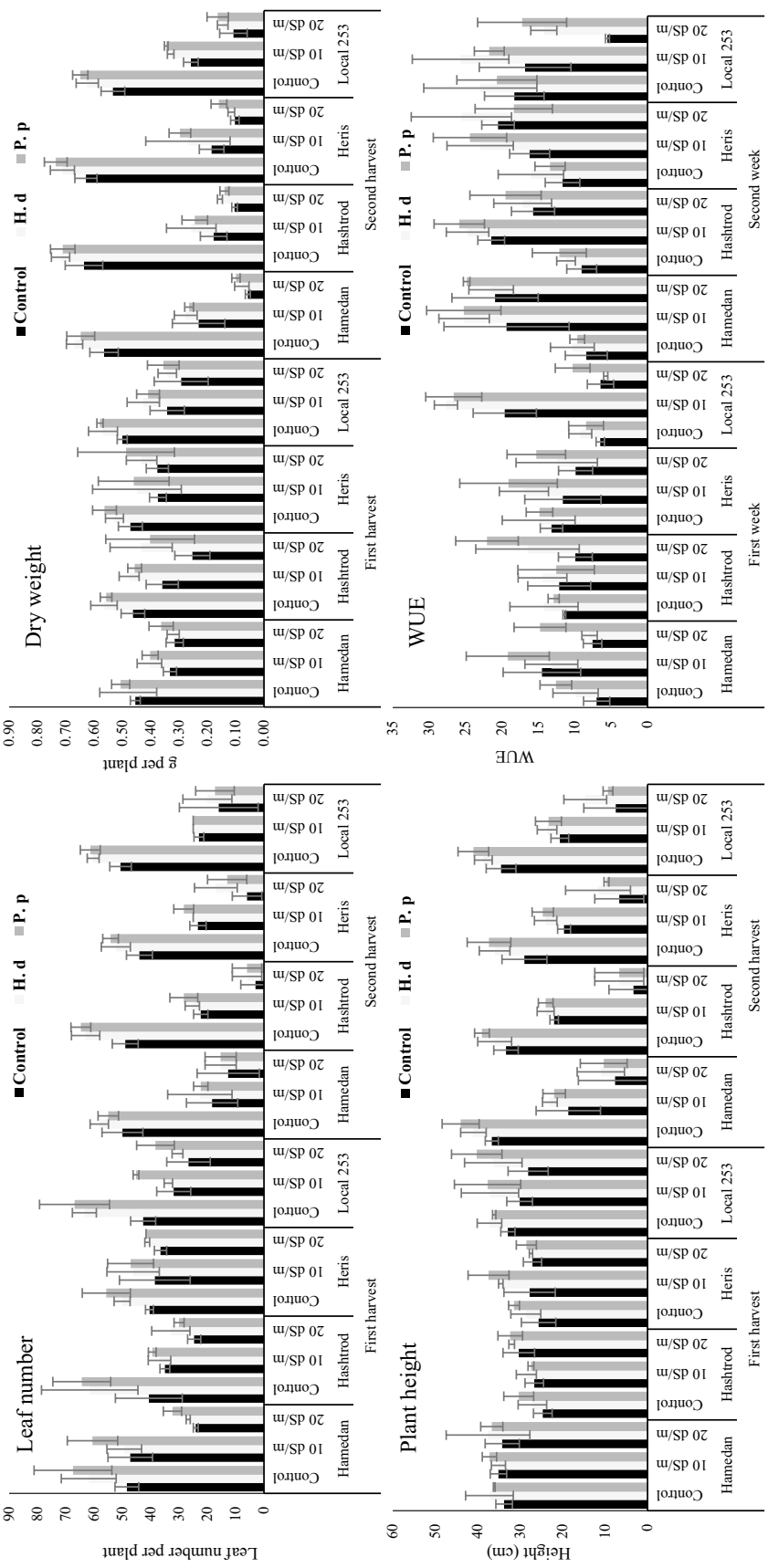

Fig. 2 *Pseudomonas sp. Proradix* (*P. p*) and *Hartmannbacteria diazotrophicus* (*H. d*) effects on means of WUE, dry weight, leaf number, and plant height in alfalfa cultivars. Error bars represent standard deviation * ($P < 0.05$) (three replicates)

Also, there was no significant difference in dry weight of salt-tolerant cultivars with salt-sensitive cultivar (Local 253) at 10 dS m^{-1}. However, at 20 dS m^{-1}, inoculated plants of salt-tolerant cultivar (Heris) had higher dry weight than salt-sensitive cultivar and non-inoculated plants which showed under 10 dS m^{-1}, salinity tolerance in both salt-tolerant and sensitive cultivars improved by inoculation. However, at high salinity, salinity tolerance improved by inoculation only in salt-tolerant cultivars. Moreover, no difference between the inoculated plants with both bacteria showed that either bacterium could increase dry weight, height, and leaf number under salinity. The reports showed in inoculated plants with bacteria, root length and nutrient absorption such as phosphor, potassium, and nitrogen (Babalola 2010), synthesis of auxins, cytokinins, and gibberellins (Glick et al. 2007), growth rate, and dry mass production were higher than non-inoculated plant under stress and control conditions (Gupta et al. 2015; Xiao et al. 2018; Liu et al. 2019; Trdan et al. 2019). Liu et al. (2019) and Xiao et al. (2018) suggested that inoculation with rhizomicrobiome increased significantly plant growth in alfalfa plants and also plant biomass was affected by the composition of the rhizomicrobiome, soil pH, N, P, and plant growth stage and species. Also, Liu et al. (2019) showed that shoot height, fresh and dry weights, yield, crude protein, and antioxidant enzyme activity of alfalfa-inoculated plants were higher than non-inoculated plants, but ethylene content was lower in inoculated plants. Trdan et al. (2019) reported that mixture of two PGPB (*Pseudomonas fluorescens* and *Azospirillum brasilense*) increased the potato yield 17–31% under dry conditions. Also, investigating data and literature suggested that the reducing effect of salinity on growth aspects of inoculated plants at high salinity was related to root number reduction, root hair deformation (Gopalakrishnan et al. 2015), K^+ ion depletion, carbohydrate composition alteration of bacterial cell surface, bacterial mobility inhibition (Paul and Lade 2014), nodule functions suppress, and photosynthesis rate decrease (Wang et al. 2016).

Reduction in nodule number, root length, and weight of alfalfa plants under salinity was similar to other plants (Zahran 1991; Manchanda, and Garg 2008; Zahaf et al. 2012). The previous studies on root growth under salinity showed that increased synthesis of ethylene and reactive oxygen species under salinity could decrease roots length and weight (Steffens 2014; Habib et al. 2016). Also, reduction in the root hair number, the formation of infection threads, the nutrient availability via photosynthesis products, nodule metabolism, atmospheric nitrogen diffusion, and deformation of root hairs could reduce nodule number under salinity (Gopalakrishnan et al. 2015; Egamberdieva et al. 2017). Our study showed inoculation with bacteria alleviated the harmful effects of salinity on root length, weight, and nodule number at 10 dS m^{-1}, but it had no significant effect at 20 dS m^{-1}. Inoculated plants of Heris, the salt tolerant, had a higher root growth and nodule number under salinity which showed the interaction between salt-tolerant cultivar and salt-tolerant bacteria was stronger than the interaction between salt-sensitive cultivar and salt-tolerant bacteria. Our results were similar to Noori et al. (2018)'s results. Noori et al. (2018) showed that PGPB significantly increased alfalfa plant growth indices in the absence of rhizobial strains. Moreover, PGPB could even provide plant nitrogen in the absence of rhizobial strains and nitrogen in the soil. The previous studies demonstrated that inoculated plants had higher RWC and less ethylene content (Babalola 2010; Arora et al. 2012; Cedeno-Garcia et al. 2018). Also, Cedeno-Garcia et al. (2018) reported that nodulation in inoculated alfalfa plants improved earlier than non-inoculated plants under greenhouse conditions. Besides, it was known that bacteria could produce a higher content of phytohormones such as auxin that could increase nodule number, and root and shoot growth under salinity (Gupta et al. 2015; Gopalakrishnan et al. 2015). Auxin had an important role in xylem and root development, nodule formation, and pigment formation (Gupta et al. 2015; Cedeno-Garcia et al. 2018). Therefore, the increase in auxin content and the decrease in ethylene content are the main reasons for increasing the nodule number, and root and shoot growth under salinity in inoculated plants.

Salinity stress decreased RWC and MSI in alfalfa cultivars similar to other plants (Munns and Tester 2008). RWC reduction during 2 weeks after salinity was related to the reduction of soil water potential and water absorption by plants roots. Salinity disturbed the balance between transpiration and water uptake by plants and reduces RWC. Inoculation plants with bacteria increased water absorption and RWC compared with non-inoculated under salinity, because inoculated plants had higher root length and root weight than non-inoculated plants. It appeared that inoculation plant with bacteria could change lateral root system architecture and increase RWC. Similar results were reported in alfalfa (Bertrand et al. 2015), Zea mays (Bano and Fatima 2009), and pea plants (Ali et al. 2015). Na^+ ions could disturb MSI as soon as they enter the cells (Volkov 2015). Water shortage and high level of reactive oxygen species under salinity stress could decrease MSI (Parida and Das 2005; Ashraf and Foolad 2013). MSI in inoculated plants was higher than non-inoculated plants because of less absorption of Na^+ ions and high RWC. Similarly, Werner and Newton (2005) reported that the inoculated plants had fewer symptoms of oxidative damage and high membrane stability under salinity due to high water and nutrient absorption. Inoculated plants of Heris and Hashtrod, the salt-tolerant cultivars, had the highest RWC and MSI at 10 dS m^{-1}, but inoculation had no significant effect at 20 dS m^{-1} during 2 weeks after salinity.

Salinity stress by increasing chlorophyllase activity, decreasing chlorophyll synthesis, and destroying

pigments proteins caused to decrease chlorophyll pigments in alfalfa and other plants (Santos 2004; Jaleel et al. 2008; Anower et al. 2013). Our results showed inoculation with bacteria saved alfalfa chlorophyll pigments from the harmful effects of salinity. Inoculation with bacteria had no significant effect on chlorophyll pigments in Local 253, the salt-sensitive cultivar. It appeared that the interaction between salt-tolerant cultivar and salt-tolerant bacteria was more effective in saving chlorophyll pigments than the interaction between salt-sensitive cultivar and salt-tolerant bacteria. Similar results reported in inoculated plants of lettuce (Han and Lee 2005), wheat (Bashan et al. 2006), and basil (Heidari and Golpayegani 2012). Investigating the studies showed that higher content of chlorophyll pigments in inoculated plants was related to higher absorption of iron, magnesium, nitrogen (Hosseinzadah et al. 2011), and less ethylene synthesis (Nadeem et al. 2010; Habib et al. 2016).

Reduction in stomatal conductance (*gs*) and photosynthesis (*Pn*) is normal under salinity (Li et al. 2010; Torabi et al. 2014). Inoculation with bacteria could improve the negative effects of salinity on gs and Pn in all cultivars at 10 dS m^{-1}, but it had less effect at 20 dS m^{-1}. Inoculated plants of Local 253, the salt-sensitive cultivar, had the highest Pn and WUE at 10 dS m^{-1}, but they had the lowest at 20 dS m^{-1}. Inoculated plants of Hamadan and Hashtrod, the salt-tolerant cultivars, had the highest *Pn* and gs, respectively, among the cultivars at 20 dS m^{-1}. It is accepted that *gs* reduction under salinity is related to decreasing water absorption by roots, increasing abscisic acid, and closing stomata that lead to decrease CO_2 availability for *Pn* (Chaves et al. 2009; Li et al. 2010). Also, stomatal closure, chlorophyll pigments, and photosynthesis enzyme activities reduction, electron-transport chain activity inhibition, and chloroplast structure change could lead to *Pn* and *gs* reduction under salinity (Chaves et al. 2009; Ashraf and Harris 2013). Reduction in stomatal conductance saved water in plant leaves and increased water-use efficiency (*WUE*) in mild stress (Chaves et al. 2009). The higher rate of gs in inoculated plants compared with non-inoculated plants may relate to less absorption of chloride ions (Cl^-) (del Amor and Cuadra-Crespo 2012), cytokinin increase, and abscisic acid reduction and better hormonal balance alteration (Dodd and Pérez-Alfocea 2012). It appeared that high Pn of inoculated plants in this study was related to higher gs, RWC, and chlorophyll pigments. Similar results were reported in lettuce (Han and Lee 2005), legume (Zahran 1999), sweet pepper (del Amor and Cuadra-Crespo 2012), and other plants (Babalola 2010) under salinity. These results suggested that all photosynthesis aspects improved by inoculation with bacteria in salt-tolerant and salt-sensitive cultivars.

Na^+ in alfalfa cultivars increased and K^+ and K^+/Na^+ ratio decreased under salinity, and these results are

Table 2 Mean value and standard deviations of stomata conductance (gs), photosynthesis rate (Pn), water–use efficiency (WUE), chlorophyll b (Chlb) and total chlorophyll (Chlab), carotenoid content (Car), height, dry weight, root length, root weight, nodule number, potassium inoculated with *Pseudomonas* sp. Proradix (P. sp. Proradix) and *Hartmannibacter diazotrophicus* (*H. diazotrophicus*) strains, and non-inoculated irrigated at 0, 10, and 20 dS m^{-1}

Inoculation	Sample number	gs	Pn	WUE	Chlb	Chlab	Car	Height	Dry weight	Sample number	Potassium	Nodule number	Root length	Root weight
Non-inoculated	72	0.182 ± 0.1b	3.58 ± 1.9b	13.08 ± 5.8b	0.422 ± 0.19c	3.0 ± 0.84 c	6.94 ± 2.6 b	24.84 ± 12.6b	0.34 ± 0.22b	36	10.5 ± 2.0b	13.11 ± 15c	14.83 ± 1.5 b	2.51 ± 0.36b
P. sp. Proradix	72	0.214 ± 0.1a	4.76 ± 2.0a	17.57 ± 6.4a	0.539 ± 0.17a	3.54 ± 0.64a	8.39 ± 2.3a	29.29 ± 13.8a	0.42 ± 0.23a	36	11.18 ± 2.4ab	18.36 ± 11a	16.14 ± 1.5a	2.71 ± 0.236a
H. diazotrophicus	72	0.209 ± 0.1a	4.59 ± 1.8a	16.71 ± 6.9a	0.494 ± 0.18b	3.4 ± 0.65b	9.03 ± 2.8a	28.72 ± 11.9a	0.41 ± 0.22a	36	11.5 ± 2.1a	16.58 ± 14b	16.08 ± 1.4a	2.73 ± 0.25a

Each value represents the mean of three replicates

Different letters represent significant differences according to Duncan test ($P < 0.05$)

common (Munns and Tester 2008; Acosta-Motos et al. 2017). The results also indicated that inoculation with both bacteria increased roots growth and contact with soil and K^+ uptake, but decreased Na^+ uptake under salinity. In addition, inoculated plants had higher K^+/Na^+ ratio at 10 dS m^{-1}, but there was no significant difference between inoculated and non-inoculated plants at 20 dS m^{-1}. Recent studies of inoculated plants with different bacteria demonstrated that bacteria change the selectivity of Na^+ and K^+ by plant roots and decrease Na^+ uptake and transport in the whole of the plants under salinity (Volkov 2015). Moreover, the reports showed that inoculation with bacteria could increase root growth, macro–micronutrient absorption, organic acid production, pH reduction, and siderophore exudation in the rhizosphere of inoculated plants (Baset et al. 2010; Dodd and Pérez-Alfocea 2012). Also, Etesami and Beattie (2018) showed that salt-tolerant bacteria could enhance K^+ absorption by mediating the expression of an ion high-affinity K^+ transporter (AtHKT1) in plants under salinity. Local 253, the salt-sensitive cultivar, which inoculated with *Pseudomonas* sp. bacteria had less Na^+ that showed high dry weight production under salinity was related to less Na^+ absorption.

Comparing inoculated and non-inoculated plants showed that all measured aspects in inoculated plants were higher than non-inoculated plants (Table 2). Inoculation with both bacteria could improve all aspects of plants growth and performance under control and salinity. The improving effect of inoculation with bacteria was less under high salinity (20 dS m^{-1}), but it was higher at salinity levels below 10 dS m^{-1}. Comparing the performance of salt-tolerant bacteria showed chlorophyll pigments (*Chla*, *Chlb*, and *Chlab*), plant height, and leaf number, Na^+, K^+/Na^+ ratio, and nodule number were improved highly by *Pseudomonas* sp., while K^+, carotenoids, and RWC were improved highly by *H. diazotrophicus* under salinity (Table 2). Comparing inoculated plants of salt-tolerant cultivars and salt-sensitive cultivar showed that, in most measured aspects, Local 253, the salt-sensitive cultivar, was the same or similar to other salt-tolerant cultivars at 10 dS m^{-1}. The inoculated plants of Local 253 had high membrane stability, photosynthesis rate and water-use efficiency, dry weight and plant height, root weight, Na^+ and K^+, and K^+/Na^+ ratio among the cultivars at 10 dS m^{-1}, but, at 20 dS m^{-1}, Heris, the salt-tolerant cultivar, had the highest dry weight. These results suggested that inoculation with both salt-tolerant bacteria improved growth and dry weight in the salt-tolerant and salt-sensitive cultivars under low salinity, but at high salinity, inoculation improved growth aspects only in salt-tolerant cultivars.

Conclusion

Inoculation alfalfa seeds with two salt-tolerant bacteria improved growth aspects such as dry weight, plant height, leaf number, photosynthesis performance (Pn, gs, and WUE), and chlorophyll pigments (Chla, Chlb, Chlab) under salinity and non-salinity. Also, inoculation was successful under salinity blew 10 dS m^{-1} in all cultivars, but inoculation was successful at 20 dS m^{-1} only in salt-tolerant cultivars. The results of this study emphasized alleviated effects of inoculation with salt-tolerant bacteria on growth aspects under salinity in alfalfa cultivars under salinity.

Author contribution statement MA (PhD Student of university of Zanjan and research visitor in Department of Soil Science and Water Management, Szent István University, Budapest, Hungary), FS, and MH M (supervisor of PhD thesis) help MA to design, perform, and data analysis of the research, and write and revise the article. GV and BB prepared experiment condition (soil, growth room, pots, and bacteria media) and supervisor during scholarship in Hungary also help to perform, write, and revise the article, and KJ measured potassium and sodium content.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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