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EFFECTS OF *Arthrobacter arilaitensis* AND *Pseudomonas putida* ON SALT STRESS TOLERANCE IN WHEAT

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Abstract

Soil salinity is a problem for agricultural production. Plant growth-promoting rhizobacteria (PGPR) can increase plant growth under salinity conditions by reducing the "stress ethylene" level by ACC deaminase activity. This study aimed to evaluate the effect of *Arthrobacter arilaitensis* and *Pseudomonas putida* on increasing the growth of wheat under different salt stress conditions. Pot experiments were conducted to determine the effectiveness of *A. arilaitensis*, and *P. putida* strains for plant growth of wheat under different salt conditions: 0.95 (control), 3.98, 7.80, and 11.05 dS m^{-1} with four replications. Inoculation of *A. arilaitensis* and *P. putida* increased membrane stability index (MSI) and carotenoid content of wheat, while malondialdehyde and proline content decreased under different salt stress conditions. *A. arilaitensis* increased MSI and carotenoid content by 10%, while *P. putida* increased by 16% and 12%, respectively. Similarly, *A. arilaitensis* and *P. putida* applications reduced leaf MDA content by 14% and 16%, respectively. Besides, wheat proline content decreased by 38% with inoculation of *A. arilaitensis* and 33% with *P. putida.* The results obtained show that *A. arilaitensis* and *P. putida* isolates reduce the harmful effects of salinity stress and can be used as biological inoculum to reduce the harmful effects caused by salinity.

*Key words:*coastal sediments, contamination factor, enrichment factor, sediment quality guidelines, trace metals

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1. Introduction

Climate change, water shortages, biotic and abiotic stresses, and decreases in soil fertility are among the main restrictions limiting the development and productivity of crops and threatening food safety. In agriculture, productivity losses occur 50% due to abiotic stresses and 30% due to biotic stresses worldwide (Kumar and Verma, 2018). Abiotic stresses such as salinity, drought, temperature, and heavy metals can be considered the leading causes of regression in plant development (Phour and Sindhu, 2020). Salt stress causes the yellowing of leaves and decreases plant growth and height. Salt stress not only prevents plant growth but also significantly changes plant physiology. Salt stress also leads to the production of reactive oxygen species (ROS)—such as hydrogen peroxide and superoxide radicals which induces lipid peroxidation and oxidative stress in plants (Zörb et al., 2019). Due to high salt stress conditions, the average yield of all crops such as sugar beet, sugar cane, sorghum, cotton, barley, and wheat has been reduced by 50% (Panta et al., 2014). Wheat (*Triticum aestivum* L.), Turkey's primary food, is often grown in semi-arid regions. Wheat is highly affected by salt stress at all stages of growth and development. Salt stress has a strong negative impact on yield during different developmental and growth stages of wheat production. Salinity causes lower biomass production and yield due to the adjustment of osmotic

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irregularities under stress conditions of plant metabolism (Borrelli et al., 2018). Salt stress causes the plant to reduce the amount of pigment involved in photosynthesis, such as chlorophyll and carotenoids (Yarsı et al., 2017). Under high salinity, increased sodium (Na^+) and chloride (Cl^-) concentrations in plant tissues cause ion imbalances in the plant, resulting in reduced nutrient intake (Parihar et al., 2015).

There are several strategies to overcome salt stress in plants. These strategies include the improvement of salt-tolerant plant species (Rabhi et al., 2009), washing the soil with water, the use of various chemicals, and the use of plant growth-promoting rhizobacteria (PGPR) (Yildirim et al., 2011). Among these various approaches, using salt-tolerant PGPR is the most ecologically appropriate and sustainable (Çakmakçı 2016; Forni et al., 2017). PGPR produces the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC (a precursor to stress ethylene) (Ali et al., 2014; Glick, 2014). High salinity causes increased production of ethylene. Ethylene is a plant growth hormone that increases plant development at a low (10 mg/L) concentration (Nadeem et al., 2010); however, increased ethylene levels restrict root and shoot growth, leaf growth, and plant growth and development (Glick, 2014). In this enzyme's presence, plants regulate the mobilization of nutrients and the intake of salts (Na^+, K^+, and Ca^{2+}),especially in saline conditions (Forni et al., 2017; Singh et al., 2017). The use of PGPRs increases plants growth and productivity in average soils and saline soils (Tahir et al., 2019). In this study, it was aimed to (1) determine the effects of inoculation with ACC deaminase producing *Arthrobacter arilaitensis*and *Pseudomonas putida* on the biochemical parameters of wheat under different salt conditions and (2) compare the effects of *Arthrobacter arilaitensis*and *Pseudomonas putida* on the growth of wheat under salt stress conditions.

2. Materials and methods

2.1. Bacterial strains

Previously isolated ACC deaminase-producing *Arthrobacter arilaitensis* (CP012750.1) and *Pseudomonas putida* (GQ2008822.1) were used in pot experiments. Phosphate solubilization, indole acetic acid (IAA) production, nitrogen fixation, and hydrogen cyanide production properties of bacteria used in the experiment were each determined.

2.2. Phosphate solubilization of strain

National Botanical Research Institute's Phosphate (NBRIP) medium was used to determine bacterial strains' phosphorus solubilization. 0.2 mL of 24-hour bacterial culture was added to 20 mL of NBRIP medium and incubated at 150 rpm for three days at 28.5±1ºC. At the end of incubation, the medium was centrifuged at 5000 rpm for 10 min. The amount of phosphorus in the supernatant was determined by an inductive coupled plasma-optical emission spectrometer (ICP-OES). ICP OES specifications were RF power (KW): 1400, Auxiliary gas flow rate (L/min): 0.5, Atomizer flow rate (L/min):0.5, Plasma flow rate (L/min): 18 and gas: argon.

2.3. Indole acetic acid (IAA) production of strain

Indole acetic acid production of strains was determined using the method described by Bharucha et al. (2013). 0.2 mL of 24-hour bacterial culture was added to the 20 mL medium and incubated at 150 rpm for four days at 28.5 ± 1ºC. At the end of incubation, 2 mL of supernatant was taken by centrifuging the medium at 5000 rpm for 10 min. 2 drops of phosphoric acid and 4 mL of Salkowski solution (50 mL of 35% $HClO_4$ containing 1 mL of 0.5 M $FeCl_3$) were added and incubated in the dark for 1 hour. The color formed after incubation was calculated by measuring 535 nm in the spectrophotometer with the standard IAA graph's help.

2.4. Nitrogen fixation

A nitrogen-free medium development (Döbereiner, 1988) was used to determine the growth of bacterial strains. 24-hour bacterial culture was streaked on a nitrogen-free medium and incubated at 28.5±1ºC for seven days bacteria growing in the medium after incubation were evaluated as positive.

2.5. Hydrogen cyanide production of strain

Hydrogen cyanide (HCN) production strains were determined according to Ahmad *et al.* (2008). 200 µl in a 24-hour bacterial culture was streaked on nutrient agar containing 4.4 g/L glycine. The filter paper was wetted with 1 mL of 2% sodium bicarbonate dissolved in a 0.5% picric acid solution and placed on the lids of petri dishes. The petri dishes were wrapped with parafilm and incubated at 28.5 ± 1 ºC for five days. The brown-red development at the end of incubation was indicated as HCN production.

2.6. Inoculation of seeds with bacteria

Wheat seeds were immersed in 95% ethanol for 5 seconds, then kept in 2% $HgCI_2$ solution for 3 min. to surface sterilization. The seeds were washed with sterilized distilled water and kept for 30 min in 24-h bacterial culture (1×10^8 cfu mL^{-1}) (Nadeem et al., 2010).

2.7. Pot experiments

Pot experiments were conducted in a greenhouse to determine the effectiveness of *A. arilaitensis*, and *P. putida* strains for plant growth of wheat under different salt conditions. Soils were taken from the land and sieved through 2 mm. Pot soils were

analyzed for physicochemical properties. The properties of the soils are: saturation: 56%, pH: 7.82, EC: 0.95 dS m^{-1}, organic matter: 1.44%, extractable P: 4.12 mg kg^{-1} and extractable K: 120.38 mg kg^{-1}. Pot trials were conducted at four different salt levels (0.95, 3.98, 7.80, and 11.05 dS m^{-1}) with four replications. The amount of NaCl was calculated, dissolved in water, and applied to the pots soils. *A. arilaitensis* and *P. putida* inoculated wheat seeds were planted in the pots.

Four wheat seeds were sown in each pot and seeds without bacterial inoculation were used as a control. Bezostaja wheat variety was used in this study.The experiment was carried out according to the factorial experiment design in randomized plots. Lipid peroxidation, proline, carotenoid, membrane stability index, Ca^{2+}, Na^{+}, K^{+} content of wheat leafs were determined and analyzed. Wheat leaves were collected in the earing period.Data were analyzed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Differences among means were compared with Duncan's multiple comparison tests.

2.8. Lipid peroxidation

Oxidative damage occurring in lipids due to salt stress was determined by measuring malondialdehyde content(MDA). To determine the amount of MDA, 0.5 g of leaf sample was homogenized in 10 mL of 0.1% trichloroacetic acid. The homogenate was centrifuged for 7500 g for 10 minutes, and, after centrifugation, 1 mL of supernatant was placed into glass tubes, and 4 mL of thiobarbituric acid solution containing 20% trichloroacetic acid was added. After the glass tubes were heated at 95 ^{0}C for 30 minutes, the reaction was terminated by a cold water bath. After the second centrifugation of 7500 g for 10 min, the samples' absorbance was determined in the spectrophotometer at wavelengths of 532 and 600 nm (Erdogan et al., 2016).

2.9.Determination of membrane stability index (MSI)

To determine the membrane stability ındex, 1 cm diameter discs were removed from leaf samples. The removed discs were placed in glass tubes containing 10 mL of distilled water, and the tubes were kept in a 30^{0}C water bath for 3 hours. Later, the electrical conductivity of the tubes was measured with an EC meter (*C1*). Tubes were autoclaved at 121^{o}C for 15 minutes, and electrical conductivity was measured for the second (C2) (Sairam et al., 2002). The membrane stability index was calculated by following the formula (Eq.1).

$$MSI(\%) = 1 - (C1/C2) \times 100 \quad (1)$$

2.10. Determination of proline content

Bates (1973)method was used to determine the proline content of fresh wheat leaves. A 0.5 g fresh leaf sample was homogenized in a solution containing 10 mL of 3% sulfosalicylic acid. The homogenate was centrifuged at 5000 g for 10 min. 2 mL of supernatant was placed into glass tubes, and 2 mL of ninhydrin and 2 mL of acetic acid were added to the tubes. After the glass tubes were kept in a hot water bath at 95°C for 1 hour, the reaction was terminated using cold water. The amount of proline was determined using a standard proline graph.

2.11.Determination of carotenoids content

The amount of carotenoid in wheat leaves was calculated, as Lichtenthaler (1987) reported. In the carotenoid determination, 0.5 g of fresh leaves were taken and crushed in 25 mL 90% acetone in a porcelain mortar. The crushed samples were then filtered to make up 50 mL of the final volume with acetone. Chla, Chl b and carotenoids were calculated using(Eqs. 2-4):

Chlorophyll a (mg g^{-1} FW) = (11.75 x A663 – 2.35 x A645) x 50/500 (2)

Chlorophyll b (mg g^{-1} FW) =(18.61 x A645 – 3.96 x A663) x 50/500 (3)

Carotenoid (mg/gFW) = ((1000xA470) - (2.27 xChlA) - (81.4xChlB)/227)x50/500 (4)

2.12.Determination of leaf Ca^{2+}/Na^{+} and K^{+}/Na^{+} contents

The leaf samples were washed with distilled water and then dried at 65^{o}C for 48 hours. The dried samples were ground and then weighed into 0.25 g microwave tubes. 10 mL of HNO_3 containing 20% $HClO_4$ was added and digested for 30 min. The liquid resulting from digestion was completed to 50 mL, and then Na^{+}, K^{+}, and Ca^{+2} were determined as mg g^{-1} DW by ICP-OES. ICP OES specifications were RF power (KW): 1400, Auxiliary gas flow rate (L/min): 0.5, Atomizer flow rate (L/min):0.5, Plasma flow rate (L/min) : 18 and gas: argon.

3. Results and discussion

Plant growthpromoting properties (phosphorus solubilizing, IAA production nitrogen fixation, and HCN production) of *A. arilaitensis* and *P. putida* were determined (Table 1). *A. arilaitensis* produced 25.42 mg L^{-1} IAA, and HCN. *P. putida* produced 14.02 mg L^{-1} IAA, 68.22 mg L^{-1} phosphate solubilized and developed in a nitrogen-free medium. *A. arilaitensis* andP. putida were selected and used according to their growing status in the ACC minimal agar medium, indicating the presence of ACC deaminase enzyme. Due to the bacterial strain, there is wide variability in ACC deaminase production. ACC deaminase activity has reported *Bacillus* sp (Misra and Chauhan, 2020), *Serratia* sp. (Turhan et al., 2020), *Enterobacter* sp.

(Nadeem et al., 2010; Singh et al., 2017; Singh and Jha, 2016), *Arthobacter* sp. (Barnawal et al., 2014), *Streptomyces* sp. (Palaniyandi et al., 2014), and *Pseudomonas* sp. (Zahir et al., 2009). *A. arilaitensis* and *P. putida* produced indol acetic acid (IAA) besides the ACC deaminase. Recent studies have shown that rhizobacteria can produce auxin and ACC deaminase, are considered one of the main factors promoting plant growth attributes (Glick, 2014). In addition to auxin and ACC deaminase activities, *A. arilaitensis* exhibited hydrogen cyanide production, while *P. putida* showed mineral phosphate solubility and nitrogen fixation. Hydrogen cyanide production, mineral phosphate solubility, and nitrogen fixation have been accepted as the basic mechanisms of PGPR (Çakmakçı et al., 2007).

Salinity stress causes nutritional and hormonal imbalances in affected plants due to increased ethylene levels, causing various physiological damage. Malondialdehyde (MDA) is an indicator of oxidative damage occurring in cells under abiotic stress conditions and occurs due to lipid peroxidation. Therefore, increased MDA content is a cellular indicator of damage occurring in stress-induced lipid membranes (Arbona et al., 2008). In this study, the MDA content of wheat increased due to increased salt concentrations (Table 2). However, the application of *A. arilaitensis* and *P. putida* reduced MDA content compared to the uninoculated control. A. *arilaitensis* application 14% and *P. putida*application decreased MDA content by 16% compared to the uninoculated control. A recent study (Singh et al., 2017) showed that the application of PGPRs producing ACC deaminase reduces MDA content as a result of lipid peroxidation in wheat under salt stress conditions. Similarly, Singh and Jha (2016) and Koç (2015) reported that inoculated with ACC deaminase-producing bacteria caused lower MDA content in plants than controls of wheat and strawberry, respectively.

Salt applications caused a decrease in the membrane stability index of wheat (Table 3). Bacteria applications increased membrane stability compared to the uninoculated control. *A. arilaitensis* and *P. putida* applications increased MSI of wheat by 10% and 6%, respectively. Due to the increased salt stress, decreases were observed in MSI. MSI is closely related to reactive oxygen species (ROS) production that produces stressful conditions, leading to electrolyte leakage (Kumar et al., 2017).

In this study, inoculation with *A.arilaitensis* and *P. putida* increased the MSI of wheat under salt stress conditions. These results are supported by Garg and Pandey (2015), Singh et al., (2017), and Talaat et al., (2015), who report that plants inoculated with ACC deaminase-producing bacteria have a higher membrane stability index. Low MDA and high MSI can be considered to indicate that plants are less affected by salt stress conditions.

Proline accumulation in plants is an important process that plays a critical role in maintaining intracellular osmotic balance and minimizing the damage caused by salt stress. The amount of proline in plants increases under salt stress, and the salt has a strong correlation with the duration and degree of stress. Proline accumulation allows plants to maintain proper osmotic balance under their low water potential (Lei et al., 2016).

Table 1. Plant growth-promoting traits of *A. arilaitensis* and *P. Putida*

Strains	***P Solubilization (mg L^{-1})***	***I.A.A Production (mg L^{-1})***	***Nitrogen Fixation***	***HCN Production***
A. arilaitensis	-	25.72	-	+
P. putida	68.72	14.02	+	-

Table 2. Effects of *A. arilaitensis* and *P. putida* inoculation on malondialdehyde (MDA) content of wheat (nmol mL^{-1})

	0.95 dS m^{-1}	***3.98 dS m^{-1}***	***7.80 dS m^{-1}***	***11.05 dS m^{-1}***	***Average***
Control	0.58±0.04 e	0.63±0.02 cde	0.76±0.03 b	1.08±0.05 a	0.76±0.20 a
A. arilaitensis	0.58±0.04 e	0.68±0.01 c	0.64±0.03 cde	0.67±0.03 cd	0.65±0.05 b
P. putida	0.58±0.08 e	0.62±0.08 de	0.67±0.01 cd	0.81±0.03 c	0.64±0.07 b
Avarage	0.58±0.05 d	0.64±0.05 c	0.69±0.06 b	0.81±0.02 a	

p<0.05, salinity*, bacteria*, salinity x bacteria*, Different letters indicate significant differences between applications

Table 3. Effects of *A. arilaitensis* and *P. putida* inoculation on membrane stability index (MSI) of wheat (%)

	0.95 dS m^{-1}	***3.98 dS m^{-1}***	***7.80 dS m^{-1}***	***11.05 dS m^{-1}***	***Average***
Control	82.51±1.69 a	75.47±2.90 b	59.79±1.59 d	50.09±1.94 f	66.96±7.44 c
A. arilaitensis	83.96±0.79 a	80.28±0.40 a	64.92±2.25 c	64.15±4.35 c	73.44±6.43 a
P. putida	83.89±3.39 a	81.42±2.26 a	61.23±4.63 cd	55.23±2.37 e	70.35±8.42 b
Average	83.47±2.18 a	79.06±3.39 b	61.87±3.48 c	56.62±7.15 d	

p<0.05 salinity*, bacteria*, salinity x bacteria*, Different letters indicate significant differences between applications

In this study, we determined that the proline content of wheat increased depending on increasing salt levels (Table 4). Wheat proline content increased by 21% at 3.98 dS m^{-1}, 65% at 7.80 dS m^{-1}, and 140% at 11.05 dS m^{-1} compared to 0.95 dS m^{-1}. *A. arilaitensis* application reduced the proline content of wheat an average of 38% compared to the uninoculated control. Similarly, *P. putida* application reduced proline content by 33% compared to the uninoculated control. This may be that salt stress in wheat plants is reduced by a different mechanism from the accumulation of proline. A lower proline level in plants incubated with *A.arilaitensis* and *P. putida* under salinity stress showed that plants incubated with ACC deaminase-producing bacteria were less affected by salinity. The results of this study are similar to Afridi et al. (2019), Akbari et al.(2020), Misra and Chauhan (2020), Singh and Jha (2016),Singh etal.(2017), and Barnawal et al. (2014).

The carotenoid content of wheat leaves was negatively affected by salt conditions (Table 5). However, bacterial applications producing ACC deaminase caused an increase in carotenoids compared to the uninoculated control. *A. arilaitensis* and *P. putida* applications increased the carotenoid content of wheat by 10% and 12%, respectively. Previous studies have shown that the carotenoid content of wheat decreases due to salt stress (Dugasa et al., 2018).

In this study, the number of carotenoids was higher in plants inoculated with *A. arilaitensis* and *P. putida* compare to uninoculated plants under different salt stress conditions. Akbari et al., (2020), Misra et al., (2019), Misra and Chauhan, (2020) have reported that inoculation with ACC deaminase-producing bacteria increases the amount of carotenoids under salt stress conditions.The wheat Ca^{2+}/Na^{+} ratio decreased due to salt applications (Fig. 1).

A. arilaitensis and *P. putida* applications increased the Ca^{2+}/Na^{+} ratio compared to controls at all salt levels. *A. arilaitensis* application increased the Ca^{2+}/Na^{+} ratio of wheat average by 41% compared to the uninoculated control. Likewise, *P. putida* increased the Ca^{2+}/Na^{+} ratio of wheat by 35% compared to the control. Salt applications caused a decrease in wheat K^{+}/Na^{+} ratio (Fig. 2).

Table 4. Effects of *A. arilaitensis* and *P. putida* inoculation on proline content of wheat (µmol g^{-1})

	0.95 dS m^{-1}	***3.98 dS m^{-1}***	***7.80 dS m^{-1}***	***11.05 dS m^{-1}***	***Average***
Control	0.76±0.04 f	1.07±0.02 de	1.68±0.08 b	2.86±0.18 a	1.60±0.84 a
A. arilaitensis	0.79±0.20 f	0.88±0.10 ef	0.92±0.22 ef	1.42±0.12 c	1.00±0.29 b
P. putida	0.80±0.07 f	0.89±0.22 ef	1.26±0.08 cd	1.32±0.12 c	1.06±0.12 b
Avarage	0.78±0.12d	0.95±0.16c	1.29±0.07b	1.87±0.12a	

p<0.05 salinity*, bacteria*, salinity x bacteria*, Different letters indicate significant differences between applications

Table 5. Effects of *A. arilaitensis* and *P. putida* inoculation on carotenoid content of wheat (mg g FW^{-1})

	0.95 dS m^{-1}	***3.98 dS m^{-1}***	***7.80 dS m^{-1}***	***11.05 dS m^{-1}***	***Average***
Control	0.84±0.04 a	0.72±0.06 abc	0.65±0.03 bcd	0.46±0.02 d	0.66±0.15b
A. arilaitensis	0.87±0.11 a	0.74±0.03 ab	0.69±0.03 abc	0.63±0.10 bcd	0.73±0.12a
P. putida	0.85±0.05 a	0.80±0.09 ab	0.75±0.02 ab	0.69±0.11 abc	0.74±0.14a
Avarage	0.85±0.06a	0.75±0.07b	0.69±0.06c	0.55±0.09d	

p<0.05 salinity*, bacteria*, salinityxbacteria*, Different letters indicate significant differences between applications

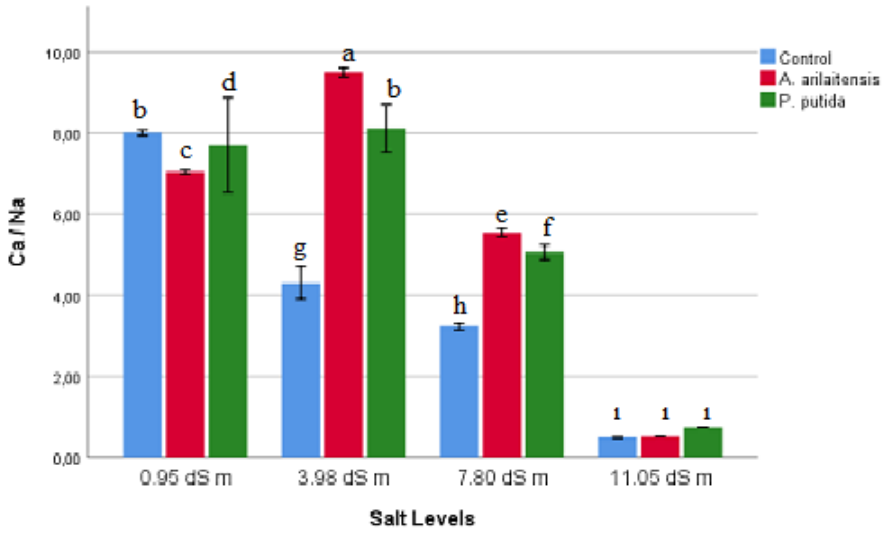

Fig. 1. Ca^{2+} / Na^{+} ratio of wheat (p<0.05 salinity*, bacteria*, salinity x bacteria*, Different letters indicate significant differences between applications)

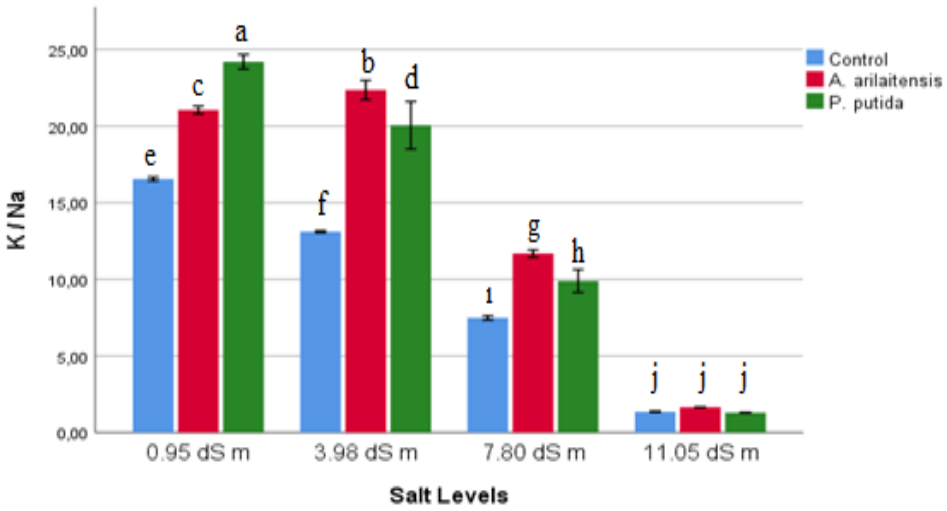

Fig. 2. K^+ / Na^+ ratio of wheat ($p<0.05$ salinity*, bacteria*, salinity x bacteria*, Different letters indicate significant differences between applications)

A. arilaitensis and *P. putida* increased the K^+/Na^+ ratio compared to the uninoculated control. *P. putida* application increased the K^+/Na^+ ratio an average of 41% compared to the uninoculated control. Similarly, *A. arilaitensis* increased the K^+/Na^+ ratio by an average of 45% . Salt soil contains various dominant cation-anion ionic pairs. Depending on salt stress, imbalances can occur in plant nutrients. Increasing the uptake of elements in salt stress conditions is essential for plant growth. The decline of plant growth depending on soil salinity is generally associated with higher Na^+ content and lower Ca^{2+}/Na^+ and K^+/Na^+ ratio accumulation (Etesami and Beattie, 2018).

In this study, the Ca^{2+}/Na^+ and K^+/Na^+ ratios were higher in plants inoculated with *A. arilaitensis and P. putida* than uninoculated controls. Similar results have been obtained in studies using ACC deaminase-containing bacteria (Afridi et al., 2019;Singh et al., 2017; Singh and Jha, 2016). Wheat yield under saline conditions was inversely proportional to increased Na^+ concentration. In contrast, wheat yield increased as K^+ concentration increased (Nio et al., 2018). These results indicate that ACC deaminase-producing PGPR is vital to maintain the balance of essential ions necessary for wheat growth.

4. Conclusion

Rhizobacteria that affect plant growth with more than one mechanism are more advantageous than rhizobacteria with a single mechanism. This study revealed that *A. arilaitensis* and *P. putida* with ACC deaminase activity exhibited different plant growth-promoting properties in wheat plants, which reduced salinity-dependent growth inhibition. In this study, inoculation with *A. arilaitensis* and *P. putida* promoted wheat growth under salt stress.

Besides, inoculation with *A. arilaitensis* and *P. putida* has been shown to improve plant growth by improving the physiological and biochemical parameters of the plant. Therefore, the use of bacteria that promote plant growth with multiple mechanisms promises hope for sustainable cultivation of wheat in saline soils. In future studies, our studies results should be tested under field conditions, and bacteria formulation studies should be conducted.

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