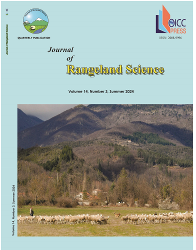

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Application of 24-epibrassinolide as an environmentally friendly strategy alleviating negative effects of salinity stress in *Satureja khuzistanica* Jamzad

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Abstract:

Satureja khuzistanica Jamzad is an endemic medicinal plant which grows naturally in arid rangelands of south west of Iran. It is also being cultivated in arid regions with salt problems. So, it is necessary to find a method to develop its cultivation under salinity condition. This study was aimed to determine the effects of 24-epibrassinolide (EBL) in three levels (0, 1 and 2 µM) on the reduction of the negative effects of salinity on morpho-physiological and biochemical traits in *S. khuzistanica* plant under four salt levels (0, 3, 6, 9 dS/m). A pot experiment was conducted using factorial experiment based on a completely randomized design with three replications in a greenhouse at Shahid Bahonar University of Kerman, Iran in 2021. The results of ANOVA showed that the values of all morphological traits including stem and root lengths, fresh and dry weights of aerial parts significantly reduced with increasing salt concentration ($P \leq 0.01$). The results showed that application of 1 and 2 µM EBL had better performance at all salt levels. The amounts of total chlorophyll, N, P^+, K^+ and K^+/Na^+ had significantly reduced by increasing the salt concentration alone and combined with EBL. The highest amounts of the latter traits were observed at 2 µM EBL without salt. The amounts of anthocyanin, proline, sugar and Na^+ significantly increased by rising salt levels. The highest amounts of anthocyanin with values of 38.64 and 38.21 mg/g were obtained at 9 dS/m salt coupled with 2 and 1 µM EBL, respectively. The lower amount of Na was observed under 2 µM EBL. The highest sugar value (2.02 mg/g) was observed in 9 dS/m salt coupled with 2 µM EBL. Similarly, for proline content, the highest values of 0.315 and 0.312 mg/g were obtained in 9 dS/m salt coupled with 1 and 2 µM EBL, respectively. There was no significant difference between 1 and 2 µM EBL levels in terms of proline. In overall, 2 µM EBL was the best treatment to alleviate the negative effects of salt at all treatments. According to our findings, 24-epibrassinolide application at the appropriate dose (2 µM) as an environmentally friendly strategy can be useful to improve *S. khuzistanica* tolerance and its production in arid rangelands with salt problems.

Keywords: Salinity; EBL; Physiological traits; Savory; Arid rangelands

1. Introduction

Saturja khuzistanica Jamzad (Lamiaceae family) is an endemic and valuable medicinal plant in Iran flora. It has various pharmacological properties including tranquillizing, appetizing, antimicrobial, anti-inflammatory and anti-cancerous (Shariat et al., 2013; Farsaraei et al., 2020). This plant grows naturally in arid rangelands and calcareous soils in the south west of Iran (Eskandari and Eskandari, 2013). It is also cultivated in the agricultural land in arid regions of Iran; most of them face with salinity problems.

Salt stress is one of the important environmental problems in arid and semiarid rangelands (Ouahzizi et al., 2021); in these regions, precipitation is not enough to leach the excess salts from the root zone. The same condition occurs in irrigated agricultural lands, especially when water with poor quality is used for irrigation (Khalil, 2020). Salinity can limit plant performance by decreasing growth, osmotic, turgor potentials and water absorption; it can also influence biochemical and physiological traits of plants and cause changes of secondary metabolite contents in aromatic and medicinal plants (Yazdanshenas et al., 2019; Farsaraei et al., 2020; Sharifian Bahraman et al., 2020). Since these traits have an important role in plant adaptation to their habitat, it is important to investigate their tolerance mechanisms against abiotic stresses (Khalil, 2020).

Efforts to expand plant resistance to stress and increase the yield and different secondary metabolites in medicinal plants are very important. For this purpose, there are various strategies such as using different elicitors and plant growth regulators, to decrease negative effects of salt. One of them is brassinosteroid. Brassinosteroids are a class of polyhydroxysteroids and a type of steroid hormones which are involved in a wide range of developmental, physiological and biochemical process (Saadatfar et al., 2021). Their exogenous application influences plant productivity and physiological responses like cell, expansion, reproductive development, seed germination, flowering, fruit set and secondary metabolite accumulations in plants. Eskandari and Eskandari (2013) found that the application of 28-homobrassinolide can improve growth and photosynthesis in *S. khuzistanica*. Akram et al. (2014) found that exogenous application of 24-epibrassinolide improved morph-physiological and biochemical attributes in *Jasminum sambac*. Among the 70 types of identifying brassinosteroids, 24-epibrassinolide (24-EBL), 28-homobrassinolide and brassinolide is well documented to have economic effects on plant growth, productivity, metabolism under different conditions (Coban and Baydar, 2016). They can protect plants under various stresses such as heat, drought and salt (Farsaraei et al., 2020; Saadatfar and Hossein Jafari, 2022). Tanveer et al. (2018) and Galal (2018) reported the positive effects of brassinosteroids on growth and physiological traits in lamiaceae family plants, squash and maize plants under saline conditions (Galal, 2018; Tanveer et al., 2018; Rattan et al., 2020). Indeed, brassinosteroids significantly alleviated negative salt effects in these plants.

Saturja khuzistanica (Lamiaceae family) plant is widely used in food, medicine and cosmetic industries. Wide application of this species caused to increase demand for its different products. Since this plant is cultivated in the regions with salt problems, it is necessary to find a method to develop its cultivation under salinity. This study aimed to determine the effects of 24-epibrassinolide on morpho-physiological and biochemical traits in *S. khuzistanica*plant under saline conditions. The results can be used to improve *S. khuzistanica* tolerance and production in arid rangelands with salt limits in Iran.

2. Materials and methods

2.1 Plant materials and growth conditions

A factorial experiment was conducted using a completely randomized design under greenhouse conditions at Shahid Bahonar University of Kerman, Iran. Two abiotic factors including four levels of NaCl (0, 3, 6 and 9 dS/m) and three concentrations of 24-epibrassinolide (EBL) (0, 1 and 2 μM)were applied on *S. khuzistanica* seedlings. *S. khuzistanica* seeds were provided from Pakanbazr Company, Esfahan, Iran. They were sterilized with 1 % sodium hypochlorite; after germination and radicle emergence, savory seedlings were transferred to 2 kg pots under greenhouse condition. The pots were filled with a mixture of garden soil, sand and manure (in a ratio of 2:1:1, respectively). Two months after cultivation, different levels of NaCl were applied at the growth stage of savory for one month. In order to prevent osmotic shock, every 10 days, the solution concentration was increased and it continued to reach the highest salt concentration (9 dS/m). The 24-epibrassinolide solution was made through the following method: EBL powder (Sigma-Aldrich, Saint Louis, USA) was gently mixed with 96 % ethanol in order to dissolve completely (Saadatfar et al., 2021). In the next stage, distilled water was added to solution to make desirable concentrations. Then, EBL solution was applied by spraying, aerial parts of *S. khuzistanica* plants. This process performed three times every other day for one week. Two weeks after EBL application, morphological traits were measured. Plants aerial parts from each pot were harvested for physiological and biochemical experiments. The first half of each pot (fresh plants) was used to measure several morphological and physiological traits. The second half was immediately frozen in liquid nitrogen and stored at −80 °C for other experiments, which needs fresh samples. The third half was dried in the shade in order to perform other analyses.

2.2 Morphological trait measurements

Morphological traits like stem and root lengths, fresh and dry weights of savory aerial parts were recorded.

2.3 Physiological trait measurements

Arnon (1967) method was used to measure chlorophyll. Savory fresh leaves (0.5 g) were ground in 5 mL acetone (80 % v/v). Its absorbance was recorded at 645 nm (Chl a) and 663 nm (Chl b). The blank contained 80 % acetone.

Anthocyanin was measured using the Wagner method ((Wagner, 1979)). 0.2 g of fresh leaf samples was extracted in acidified methanol (10 mL of methanol and HCl (99:1 v/v)); it was incubated for 24 h. After extracts filtrating, the anthocyanin content was measured by a spectrophotometer (550 nm).

Sugar content (mg/g FW) was measured using Hellubust and Craigie (1978) method. 70 % ethanol (10 mL) was added to the leaf powder and this solution was kept in the fridge for 1 week. Then, 5 mL H_2SO_4 was added to 1 mL of the solution + 1 mL of 5 % phenol. The solution was diluted 10 times and the absorbance was recorded at 485 nm.

Proline amount was measured using the Bates method

(Bates et al., 1973). Savory fresh leaves (0.5 g) were pulverized with 10 mL sulfosalicylic acid (3 %) by porcelain pestle and mortar. The mixture was centrifuged at speed 13000 g (10 min). 2 mL of extract, ninhydrin indicator (2 mL) and concentrated acetic acid (2 mL) were transferred to tubes; it was placed in boiling water at 100 °C (30 min). After cooling down the tubes (for 15 min), 4 mL of toluene was added to each tube and shaken by a vortex (15 s). The absorption of the red surface layer and standard samples were simultaneously determined at 520 nm by a spectrophotometer. The following formula was used to calculate proline amount using the standard curves:

$$Proline = (X \times 0.004)/g$$

where: X is wave length.

2.4 Mineral elements measurement

Nitrogen content was measured by Kjeldahl method after wet digestion using sulphuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). The following instruction was used to measure other mineral elements: 1 g of each dried leaf sample powder was burned in the oven at 550 °C for 4 h. 2 mol/L HCL (10 mL) was added to each sample to digest by a heater. After filtering the mixture, distilled water was added to obtain 50 mL. Calorimetry method was used to measure P (Chapman and Part, 1961). Na and K were measured using a flame photometer. Ca and Mg were determined by the titration method (Ryan et al., 2007).
After doing tests of normality (Kolmogorov-Smirnov test), the significant differences among treatments were determined by ANOVA test in SPSS software.

3. Results

The results of analysis of variance for morpho-physiological traits and mineral elements showed that salt × EBL interaction has a significant effect on all morpho-physiological traits and Na, Na/K and N elements ($P \leq 0.01$) (Table 1).

3.1 Morphological traits

All morphological traits including stem and root lengths, fresh and dry weights of *S. khuzistanica* aerial parts significantly reduced with increasing salt levels (Table 2). 1 and 2 µM EBL concentrations had better performance in comparison with others at all salinity levels. The highest amounts of all measured morphological traits were observed at 1 and 2 µM EBL without salinity. There was no significant difference between 1 and 2 µM EBL concentrations in terms of fresh and dry weights of aerial parts.

3.2 Physiological and biochemical traits

Total chlorophyll had a significant reduction with increasing salt concentration alone and with EBL interaction. At all treatments, 2 µM EBL caused a significant increase in the amount of chlorophyll. The highest total chlorophyll amount was observed at 2 µM EBL without salt (1.36 mg/g), respectively (Figure 1).
Anthocyanin content was positively influenced by salinity so that by increasing salt levels, anthocyanin amount enhanced. This enhancement was higher at 1 and 2 µM EBL concentrations. The highest anthocyanin amounts were observed at 9 dS/m salt 2 and 1 µM EBL with the amounts of 38.64 and 38.21 mg/g, respectively (Figure 1). According to Figure 1, there was significant increase in proline and sugar

Table 1. Analysis of variance for morpho-physiological traits and mineral elements.

Source	DF	MS						
		Stem length	Root length	Fresh weight	Dry weight	Total Chl	Sugar	Proline
Salt (S)	3	151.3**	60.1**	78.14**	11.41**	0.23**	0.25**	50.0**
EBL (E)	2	25.07**	8.57**	6.56**	1.22**	1.23**	9.55**	713300**
S X E	6	1.99*	1.95**	1.40**	0.16**	0.005*	0.043*	10.0*
Error	24	1.52	1.30	0.50	0.04	0.003	0.02	1.00
CV%	–	5.12	7.42	8.54	6.24	5.23	6.52	4.34

** and * = significant at 1 % and 5 % probability levels, respectively.

Source	DF	MS							
		Anthocyanin	Ca	Mg	Na	K	K/Na	Nitrogen	P
Salt	3	96.02**	2.00**	1.00**	2.00**	30.00**	8310**	0.38**	30.00**
EBL	2	85.49**	2.00**	1.00**	8.00**	5.00**	20.00**	0.41**	2.00**
S X E	6	0.85**	0.08	0.05	2.00**	8.00	600**	0.028**	3.00
Error	24	0.628	0.03	0.01	1.00	1.00	10.00	0.02	1.00
CV%	–	2.11	1.75	1.53	6.34	5.55	2.02	4.46	3.59

** and * = significant at 1 % and 5 % probability levels, respectively.

Table 2. Effects of salt by EBL bacteria interaction on morphological traits in *S. khuzistanica*.

Salinity (dS/m)	EBL (μM)	Stem (cm)	Root (cm)	Fresh weight (g)	Dry weight (g)
0 dS/m (control)	0 μM (control)	19.7 bc	15.45 bc	9.22 b	3.30 b
	1 μM	20.5 ab	16.0 ab	9.57 a	3.52 a
	2 μM	21.0 a	16.5 a	9.76 a	3.50 a
3 dS/m	0 μM (control)	19.3 c	14.7 cd	6.50 e	2.30 def
	1 μM	19.7 bc	14.8 cd	7.25 d	2.34 d
	2 μM	19.8 bc	14.0 d	7.57 c	2.78 c
6 dS/m	0 μM (control)	15.3 e	11.1 f	5.10 f	1.82 f
	1 μM	17.7 d	12.8 e	6.60 e	2.19 e
	2 μM	19.8 bc	13.0 e	6.57 e	2.36 d
9 dS/m	0 μM (control)	15.1 e	9.8 g	3.50 i	1.26 i
	1 μM	13.8 f	11.4 f	3.95 h	1.41 h
	2 μM	15.7 e	11.4 f	4.35 g	1.57 g

In each column means with similar letter are not significantly different using Duncan test (P $\leq$ 0.01).

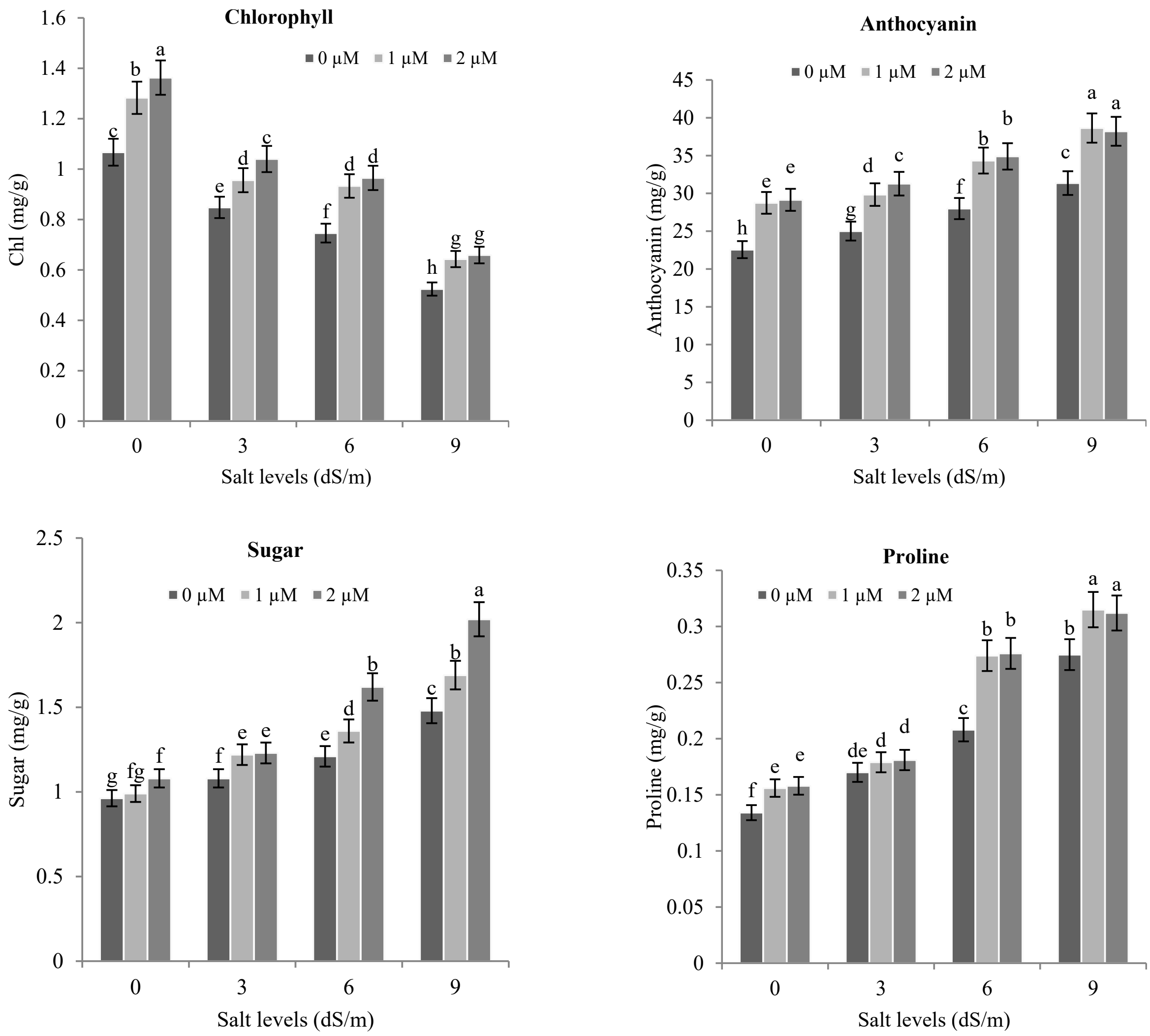

Figure 1. Effects of salt*EBL interaction on physiological and biochemical traits in savory. According to Duncan test (P $\leq$ 0.01). Means of column with similar letter are not significantly different.

amounts with rising salt concentration. Proline and sugar contents also increased in plants under EBL application at 2 µM EBL than that for control. The highest value of sugar (2.02 mg/g) was obtained in 9 dS/m salt coupled with 2 µM EBL; in terms of proline, the highest values of 0.315 and 0.312 mg/g were obtained in 9 dS/m salt coupled with 1 and 2 µM EBL, respectively; there was no significant difference between 1 and 2 µM EBL levels in terms of proline.

3.3 Mineral elements

The findings showed that there was a significant reduction in nitrogen content and increasing salt levels. Higher N contents were always observed at 2 µM EBL treated plants. The highest N^+ was for 3 dS/m salt coupled with 2 µM EBL (2.34 %) (Figure 2).

Higher salt levels showed lower phosphorus content so that 9 dS/m salt had the lowest amount of phosphorus (0.119 mg/g). In most cases, 2 µM EBL treated plants had higher phosphorus content compared to others. The highest amount of phosphorus (0.19 mg/g) was obtained in 2 µM EBL without salinity (Figure 2).

High salt levels markedly enhanced Na^+ accumulation in savory plants. According to the results, the highest Na^+ content (0.23 mg/g) was obtained at 9 dS/m salt treatments without EBL application. In most cases, EBL performance was effective to reduce Na amount. The lower amounts of Na^+ were observed under 2 µM EBL (Figure 2).

On the other hand, by increasing salt concentrations, the K^+ value and K^+/Na^+ ratio were significantly decreased. EBL application in both concentrations, especially 2 µM EBL resulted in a significant enhancement in K^+ and K^+/Na^+. The highest amounts of K^+ and K^+/Na^+ ratio were observed at 2 µM EBL with no salt stress (0.19 mg/g and 1.52, respectively) (Figure 2).

According to Figure 2, the enhancement of salt concentration negatively affected Ca^{2+} and Mg amounts; this reduction is sharper in terms of Ca^{2+} content. The highest amounts of Ca^{2+} and Mg^{2+} were observed at 2 µM EBL without salinity (0.096 and 0.067 mg/g).

4. Discussion

4.1 Morphological traits

Salt stress influences different mechanisms and decreases plant productivity and yield. Several elicitors like EBL can increase plant tolerance under salt negative effects (Etesami and Glick, 2020). The results of this research showed that salt negatively influenced growth parameters of *S. khuzistanica*. Osmotic stress can reduce cell turgor pressure, which affects the sizes of root and stem cells and prevent their development (Hossein Jafari et al., 2022). EBL application improved all morphological traits in savory plants under salinity. It has been proved that plant hormone such as brassinosteroids can help to alleviate the negative effects of various stresses. Ozdemir et al. (2004) found that 24-epibrassinolide increase seedling growth of *Oriza sativa* under salinity. The result of this study indicated more positive changes at 2 µM EBL concentration compared to others. According to several researches, EBL may prevent or activate plant growth under different stresses; it depends on some factors such as EBL concentration, plant species and time duration that plants exposed to EBL. In agreement to this explanation, Galal (2018) reported that 24-epibrassinolide low concentrations promote the root formation while the higher concentrations prevent its formation in *Lagenaria vulgaris*. In another study, Li et al. (2005) found that EBL higher concentrations can inhibit indole-3-acetic acid (IAA) expression and modulate polar auxin transport in Brassica and Arabidopsis plants.

4.2 Physiological and biochemical traits

In this research, total chlorophyll amount reduced with increasing salt levels This reduction can be due to lower K^+, Mn^{2+}, Fe^+, P^+ absorption which causes for the 5-aminolaevulinic acid reduction and increasing chlorophyllase enzyme activity (Khalvandi et al., 2019). EBL application can help to enhance chlorophyll amount. Different studies reported enhancement of chlorophyll contents after brassinosteroids application in several Lamiaceae family plants (Eskandari and Eskandari, 2013). In this case, it was also observed that 2 µM EBL can be more effective to ameliorate destructive salt effects at different salt concentrations. Yu et al. (2004) reported that spraying EBL caused a significant increase in photosynthetic components on cucumber including net CO_2 assimilation, rubisco activity and other enzymes involve in photosynthesis for example acid invertase activities, sucrose synthase and sucrose phosphate synthase. They found the most effective EBL concentration was 2 µM which improved Calvin cycle capacity. The effect of EBL at 2 µM on *Brassica oleracea* revealed that chlorophyll amount was increased significantly; chloroplast ultrastructure was preserved much better compared to the control. Cai et al. (2019) found that EBL maintains the integrity of grain thylakoids in the chloroplast. It was also found that 2 µM EBL can protect chlorophyll by degrading the expression of chlorophyll genes such as *BoACS4* and *BoACO3*.

The results of this study caused a significant reduction in nitrogen content and an increase in the proline amount by rising salt levels. When plants are subjected to salt stress, nutritional imbalance, osmotic stress, nitrogen and chloride a competition for transporters was occurring; therefore, nitrogen uptake is difficult for the plants. The plants under salt stress also enhance proline content to regulate osmotic pressure, cytosolic acidity and maintain protein (Khalil, 2020). In the current study, EBL treatment application increased the amount of proline and helped to preserve osmotic balance. In agreement with the results, Rattan et al. (2020) reported that EBL can help to osmotic adjustment by enhancement of the osmolytes (like proline content) in plants. On the other hand, it has been reported that EBL may activate the expression of several genes involved in proline biosynthesis in metabolite profiling; this process can lead to osmotic balance through increasing osmolytes (Rattan et al., 2020).

Another osmolyte which is affected under stressful conditions is sugar content. The findings of this study indicated an increase in total sugar with increasing salt concentrations. This may reduce the osmotic potential and accelerate water

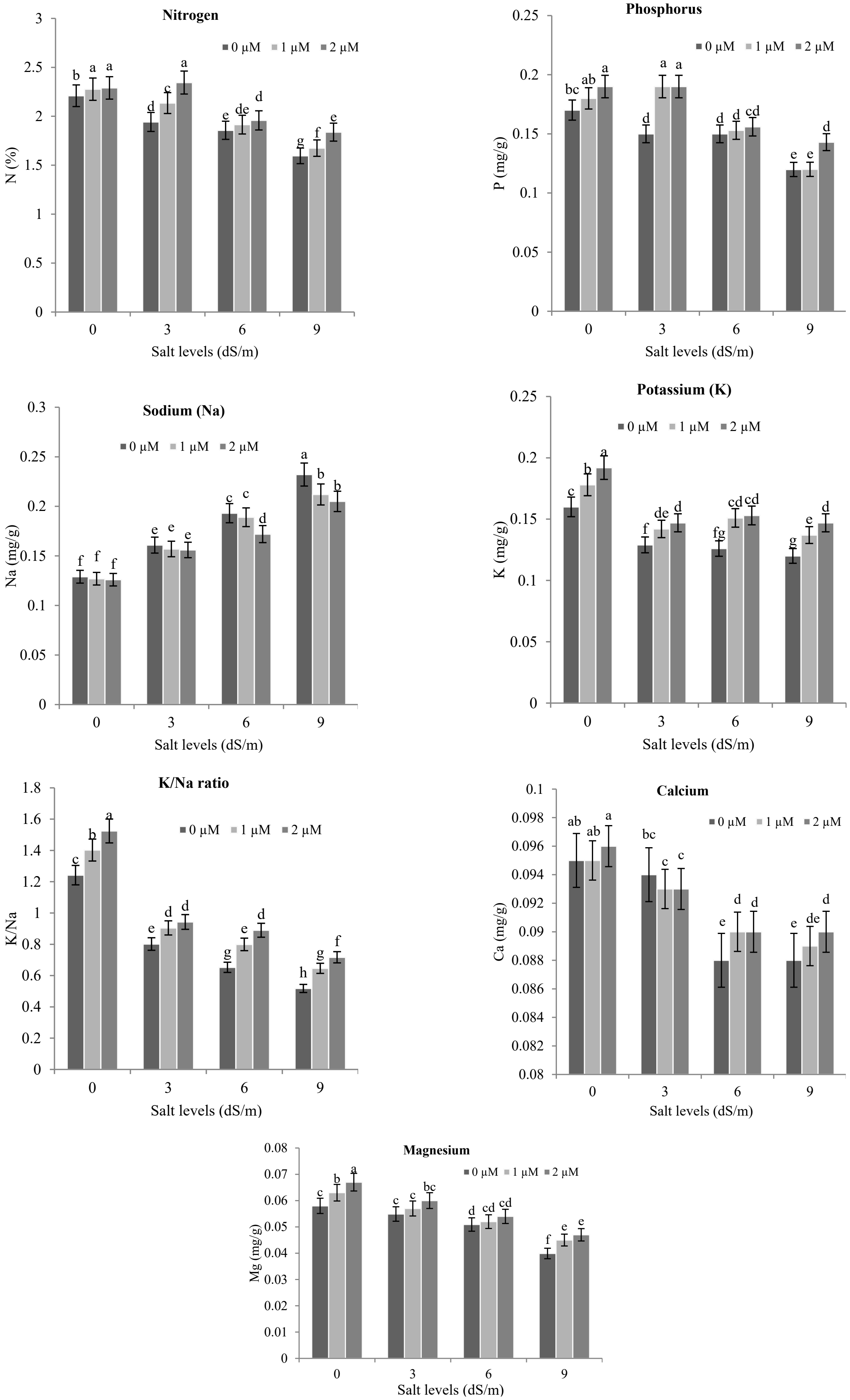

Figure 2. Effects of salt*EBL interaction on mineral elements in *S. khuzistanica*. According to Duncan test (P ≤ 0.01), means with similar letter are not significantly different.

absorption (Torabi and Halim, 2010; Khalil, 2020). This will lead to enhancing photosynthesis or breaking down Polysaccharide carbohydrates to monosaccharide (Bohnert et al., 1995). In this research, EBL treatment caused higher amounts of sugar, especially 2 µM concentrations at all salt levels. Tanveer et al. (2018) stated that EBL has the ability to modulate photosynthesis and osmolytes like sugar under salt stress. Sugars cause different factors including osmotic regulation, carbohydrate storage and stabilizes cell membranes and protein. EBL contributes in carbohydrate metabolism through increasing several enzyme activities like sucrose synthase, acid invertase and sucrose phosphate synthase (Galal, 2018).

4.3 Mineral elements

The findings demonstrated that with increasing salt levels, Na^+ increased and the amounts of K^+ and K^+/Na^+ decreased. The Na^+ and K^+ ions are similar in physiochemical traits and they compete with each other to go into the cells. Therefore, when Na^+ amount is high in the rhizosphere, the absorption of K^+ ion faces with problem; following this process, K^+/Na^+ ratio decreases in the plant. So, there will be an interruption in several processes like protein synthesis, enzyme activities, stomatal protecting cells and photosynthesis. This will lead to plant growth prevention. In the present research, EBL application decreased Na^+ and increased K^+ accumulation under salt stress. Liaqat et al. (2020) emphasized the importance of EBL in salt stress in *Alliaria petiolate* plants.

The results showed that phosphorus reduced below different salt levels. Enhancement of salt concentrations caused a decrease in phosphorus uptake by plants; it may be due to competition among P, H_2PO_{4-} and CL- ions (Maksimovic and Ilin, 2012). Totally, N, P^+, Ca^{2+} and Mg amounts in *S. khuistanica* plants had a significant reduction in high salt levels. These elements have important activities in plants; for example, several activities of Ca^{2+} and Mg^{2+} are: molecular grafting forming, balancing some cellular activities, activating several enzymes and biosynthesis of secondary products (Pessarakli et al., 2015). Our findings showed that the mentioned elements increased after 24-EBL application under salinity. It has been proved that 24-EBL is involved in ion homeostasis, which is necessary for various biochemical and physiological processes and it can control plant growth (Ghasemi-Golezani et al., 2020). Song et al. (2016) also reported that EBL can induce H+-ATPase activity; the reason that they mentioned was enhancement of Ca^{2+} and Mg^{2+} absorption under salinity. In accordance with our findings, Fariduddin et al. (2014) also stated that EBL increases nitrogen metabolism in plants under different stresses.

5. Conclusion

Salinity is one of the effective abiotic stresses on natural ecosystems which has restricting impact on plants growth and production in rangeland ecosystems. In this study, the role of 24-brassinosteroid was investigated on morphophysiological and biochemical traits of *S. khuzistanica*. The results showed that EBL application, especially at the appropriate dose, especially 2 µM EBL can be beneficial and alleviate the negative effects of salt. So, EBL application, as an environmentally friendly strategy, has positive effects to improve *S. khuzistanica* tolerance and its quality production in arid rangelands with salt problems.

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Authors Contributions

All authors have contributed equally to prepare the paper.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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