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ORIGINAL ARTICLE

The effectiveness of zinc in alleviating salinity stress on pistachio seedlings

Vahid Tavallali*

Department of Agriculture Payame Noor University (PNU), P.O. Box, 19395-3697 Tehran, Iran

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Abstract – Introduction. Zinc (Zn) deficiency is a common nutritional disorder in Pistachio (*Pistacia vera*) which is grown on calcareous, saline and sodic soils. The effects of Zn amendment on the mineral nutrient uptake, the osmoregulation and the dynamics of phytohormones in pistachio under salt stress were investigated in the common conditions of Iran **Materials and methods.** A greenhouse study was conducted to evaluate the improving effects of zinc (0, 5, 10 and 20 mg Zn kg^{-1} soil) under saline (800, 1600, 2400 and 3200 mg NaCl kg^{-1} soil) conditions on pistachio 'Badami' seedlings. The concentrations of potassium, calcium, sodium, magnesium, and Zn were determined by atomic absorption spectroscopy, and chloride was measured by titration Specific absorption rates (SAR) and specific utilization rates (SUR) of the minerals were calculated. Abscisic acid (ABA), 3-indoleacetic acid (IAA) and cytokinin contents were measured by HPLC, and the concentrations of osmoregulators (proline, glycine betaine and choline) were determined by spectrophotometry **Results and discussion.** The K, Ca and Zn concentrations and the IAA and cytokinin contents were reduced under Zn deficiency and salt stress. Increasing salinity in soil under Zn-deficient conditions generally decreased the SAR and SUR of K, Ca, Zn and Mg and ABA contents. However, these adverse effects of salinity were alleviated by increasing Zn levels up to 10 mg kg^{-1} soil. The addition of Zn to the soil significantly decreased the proline and choline concentrations and increased the glycine betaine one of the pistachio seedlings exposed to salinity. Zinc treatments influenced the relationship between the relative growth rate (RGR) and the studied elements SARs and SURs. **Conclusion**. Overall, Zn improved plant growth under salt stress. According to the results obtained in this study, adequate Zn treatments can prevent the uptake and accumulation of sodium in the pistachio leaf and stem and improve both plant RGR and mineral nutrients absorption. Adequate Zn treatments also contribute to maintain the balance among seedling phytohormones even under salinity stress.

Keywords: Iran / pistachio / *Pistacia vera* / osmoregulation / phytohormone / growth rate / specific absorption rate / specific utilization rate

Résumé – Efficacité du zinc contre le stress salin sur semis de pistachier. Introduction. La carence Zinc (Zn) est un trouble nutritionnel commun des pistachiers (*Pistacia vera*) cultivés en sols calcaires, salins et sodiques. Les effets d'une fertilisation en Zn sur l'absorption des nutriments minéraux, sur l'osmorégulation et sur la dynamique des phytohormones du pistachier soumis au stress salin ont été étudiés dans des conditions couramment rencontrées en Iran. **Matériel et méthodes.** L'étude a été menée sous serre pour évaluer les effets positifs du zinc (0, 5, 10 et 20 mg Zn kg^{-1} de sol) sur la croissance des jeunes plants de pistachier cv. Badami en conditions de stress salin liées à une solution saline (800, 1600, 2400 ou 3200 mg NaCl kg^{-1} de sol). Les concentrations de potassium, calcium, sodium, magnésium et zinc ont été déterminées par spectroscopie d'absorption atomique, et le chlorure a été mesuré par titrage. Les taux spécifiques d'absorption (SAR) et les taux d'utilisation spécifiques (SUR) des minéraux ont été calculés. Les teneurs en acide abscissique (ABA), acide 3-indole acétique (IAA) et en cytokinines (CKs) ont été mesurées par HPLC, et les concentrations de divers régulateurs osmotiques (proline, glycine bétaïne et choline) ont été déterminées par spectrophotométrie. **Résultats et discussion.** Les concentrations en K, Ca Zn et les teneurs en IAA et CKs ont diminué en conditions de carence en Zn et de stress salin. L'augmentation de la salinité dans un sol carencé en Zn a généralement diminué les SAR et SUR en K, Ca, Zn et Mg ainsi que la teneur en ABA. Ces effets néfastes de la salinité ont été atténués par l'augmentation des teneurs en Zn jusqu'à 10 mg kg^{-1} de sol. L'addition de Zn au sol a diminué de façon significative les teneurs en proline et en choline et a augmenté la concentration en glycine bétaïne des jeunes plants de pistachier soumis au stress salin. Les traitements au zinc ont influencé la relation entre le taux relatif de croissance (RGR) ainsi que les taux SAR et SUR des éléments étudiés.

* Corresponding author: vtavallali@gmail.com

Conclusion. Dans l'ensemble, le zinc a amélioré la croissance des plantes sous stress salin. Selon les résultats obtenus dans cette étude, des traitements adéquats au Zn peuvent empêcher l'absorption et l'accumulation de sodium dans les feuilles et les tiges du pistachier et améliorer à la fois la croissance des plantes et l'absorption des minéraux nutritifs. Des traitements adéquats au Zn contribuent également à maintenir l'équilibre entre phytohormones des plantules soumises au stress salin.

Mots clés : Iran / pistachier / *Pistacia vera* / osmorégulation / régulateur de croissance végétale / taux de croissance / taux d'absorption spécifique / taux d'utilisation spécifique

1 Introduction

Soil salinity is one of the major abiotic stresses that adversely affect plant productivity and quality in both irrigated and non-irrigated areas of the world [1], due mainly to an excess of Cl and Na^+ ions in plants [2]. Salinity stress limits plant development by adversely affecting various biochemical reactions and physiological processes such as photosynthesis, antioxidant metabolism, mineral nutrients homeostasis, osmolytes accumulation and hormonal signaling [3, 4]. It is thought that the repressive effect of salinity on plant growth could be related to a decline in endogenous levels of phytohormones [5]. Furthermore high salt stress increases the deposition rate of Na in the growing zone of the root and hence decreases the selectivity for potassium (K) versus Na [6]. Uptake of essential ions (both cations and anions) including K^+, Ca^{+2}, magnesium (Mg^{+2}), ammonium (NH^{4+}), and nitrate (NO^{3-}) have been reported to be suppressed in various pistachio cultivars by high levels of sodium chloride (NaCl), especially in saline soil and irrigation water [7]. A large number of studies reported that the total nutrient uptake, accumulation, and nutrient partitioning within the plant were reduced by higher levels of salinity [8–10].

Poor quality of irrigation water in association with salt build-up soils has reduced the yields of pistachio (*Pistacia vera* L.) over recent years, especially in Kerman, and in central Iran, particularly in the Yazd and Qom regions. The symptoms of salinity in pistachio have been previously described [8, 10–12]. For example, saline stress can decrease growth, alter gas exchange, enzyme activity, and protein metabolism, and cause morphological change in the leaves [10, 11, 13].

Zinc deficiency is now recognized as one of the most critical micronutrient deficiency in plants grown on calcareous, saline and sodic soils with high pH values [14]. Zinc (Zn) is an essential nutrient element for plants and plays a role in several plant physiological processes *i.e.*, photosynthesis, respiration, and synthesis of protein, DNA, RNA, and plant hormones such as indole-3-acetic acid (IAA) metabolism [15]. Zinc is required for scavenging of reactive oxygen species (ROS) including superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) induced by salinity stress [16]. Zinc also alleviates the adverse effects of salinity on phytohormones levels [17]. Furthermore, Zn supply could mitigate the adverse effects of NaCl [18]. Welch *et al.* [19] stated that Zn is necessary for root cell membrane integrity. From this point of view, external Zn concentrations could mitigate the adverse effect of NaCl by inhibiting Na and/or Cl uptake or translocation. Alpaslan *et al.* [20] concluded that in the salt affected areas, Zn application could alleviate possible Na and Cl injury in plants. Fertilizer management can strongly affect plant growth and development under salinity condition by changing their nutritional status [21].

There is limited work dealing with the interaction of Zn/salinity on pistachio. This experiment was, therefore, conducted on 'Badami' rootstock which is an important Iranian fruit producing cultivar for evaluation of responses to Zn fertilization and salinity on uptake and distribution of some macro and micronutrients in plant organs and phytohormone and osmolyte contents.

2 Materials and methods

2.1 Plant material and treatments

The experiments were conducted from February 2014 to December 2014 at the Agricultural research greenhouse of Shiraz Payame Noor University. Soil used in these studies was a loam taken from 0 to 30 cm depth of Chitgar series soils (Fine-loamy, carbonatic, thermic Typic Calcixerepts) located at Sarvestan township, 85 km southeast of Shiraz. Some physical and chemical properties of the soil are given in *table I*. The soil samples air-dried, crushed to pass through a 2-mm sieve and Zn treatments were combined thoroughly with soil and supplied at the rate of 0, 5, 10 and 20 mg kg^{-1} soil as $ZnSO_4.7H_2O$. Zinc treated soils were put in 8-L plastic pots.

Pistachio (*Pistacia vera* L. cv. Badami) seeds were placed in muslin sacks and soaked for 24 h in 0.4% captan solution. Seeds were then planted in sand and kept at 30 °C for one week. Four germinated seeds were planted in each pot and all 80 pots were irrigated with deionized water twice a week to keep the soil water content higher than the field capacity (on 20%, soil dry weight basis). Nitrogen and P at the rate of 50 mg kg^{-1} soil, and Cu and Mn at the rate of 5 mg kg^{-1} soil were applied uniformly to all pots each as NH_4NO_3, KH_2PO_4, $CuSO_4.5H_2O$ and $MnSO_4.H_2O$, respectively. After 25 days the 4-leaved seedlings were thinned to two uniform seedlings per pot. Seven days later, salt treatments (0, 800, 1600, 2400 and 3200 mg NaCl kg^{-1} soil) were added to the pots at 3-day intervals using 0.5 L irrigation water. After 50 and 100 days salt treatments, measurements were performed. Data are only shown for plants harvested after a 100-day salinization period. Twenty treatments were arranged in a factorial experiment based on completely randomized design (CRD) with 4 replications.

Table I. Some physical and chemical properties of the soil used in the experiment.

Water content (%, dry weight basis)								
Texture	Field capacity	Permanent wilting point	pH paste	EC_e (dS m^{-1})	$NaHCO_{3-}$ extractable P (mg kg^{-1} soil)	NH_4OAC-extractable K (mg kg $soil^{-1}$)	CEC (Cm_c kg^{-1})	DTPA-extractable Zn (mg kg $soil^{-1}$)
Loam	20	10	7.8	1.2	13.5	63	12	1.7

2.2 Elemental analysis

Concentrations of different elements were determined in roots, stems, and the leaves of seedlings. Oven dried plant materials were ground to a fine powder. A mass of 0.5 g dry samples was ashed at 500 °C heat for 8 h, and the ash was dissolved in 5 mL 2N hydrochloric acid (HCl). After digestion, the volume of the sample was made up to 100 mL with distilled deionized water [22]. The concentrations of Na and K were estimated by flame photometry (Model Jenway PFP7 ELE Instrument Co. Ltd.) and analyses of Zn, Ca and Mg were carried out with an atomic absorption spectrometer (Model Varian 220, Australia). Chloride (Cl^-) was extracted from 0.1 g of ground dried material with 50 mL of deionized water and measured by titration with silver nitrate [8]. The specific absorption rate, SAR (mg g^{-1} day^{-1}), an index of the element uptake efficiency of roots, was calculated using the formula

$$SAR = 1/RDW\partial M/\partial T$$

where RDW is the root dry weight (g), M is the element amount (mg) in the whole plant and T is the time of harvest in days. The specific utilization rate on a leaf basis, SUR_L (g mg^{-1} day^{-1}), an index of the efficiency of the element in producing biomass, was calculated as the rate of plant biomass production per unit of element in the leaves [23, 24]. The relationships between relative growth rate and SAR and SUR_L for the two harvests 50 and 100 days after salt treatment were evaluated by using regression equations.

2.3 Phytohormone quantification

2.3.1 Phytohormone extraction and purification

Phytohormones were extracted and purified according to Novakova *et al.* [25]. Frozen leaf samples (about 1 g FW) were ground in liquid nitrogen and extracted overnight with 10 cm^3 methanol/water/formic acid (15/4/1, by vol., pH ~ 2.5, –20 °C). For analyses of endogenous cytokinins (CKs), 50 pmol of each of the following 12 deuteriumlabelled standards were added: [$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z7G, [^{2}H5]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP7G,[$^2H_6$]iP9G, [$^2H_5$]DHZ, [$^2H_5$] DHZR (Apex Organics, Honington, UK). Tritiated internal standards were used for the determination of auxin (3[5(n)-^{3}H] 3-indoleacetic acid, Amersham, UK, specific activity 74 GBq $mmol^{-1}$, 5×10^3 Bq) and ABA (Amersham, UK, specific activity 1.74 TBq $mmol^{-1}$, 5×10^3 Bq). The extracts were purified using Si-C_{18} columns (SepPak Plus, Waters, Milford, MC, USA) and Oasis MCX mixed mode (cation exchange and reverse phase) columns (150 mg, Waters, USA) and evaporated.

2.3.2 HPLC of auxin and abscisic acid

The auxin (3-indolyeacetic acid, IAA) and abscisic acid (ABA) were determined using two-dimensional HPLC according to Dobrev *et al.* [26]. The instrumental set-up consisted of a series 200 autosampler (Perkin Elmer, Norwalk, CT, USA), two HPLC gradient pump systems (first pump: ConstaMetric 3500 and 3200 with 500 ll mixer, TSP, Riviera Beach, FL, USA; second pump: Series 200 Quaternary Pump, Perkin Elmer), two columns (first column: ACE-3CN, 150 × 4.6 mm, 3 mm, ACT, Aberdeen, Scotland, UK; second column: Luna C18(2), 150 × 4.6 mm, 3 mm, Phenomenex, Torrance, CA, USA), one 2-position, fluid processor SelectPRO with 1 ml loop (Alltech, Deerfield, IL, USA). The segment containing IAA and ABA obtained in the first dimension was collected in the loop of the fluid processor and redirected to the second HPLC dimension. IAA was quantified using fluorescence detector LC 240 (Perkin Elmer). Quantification of ABA was performed on the basis of UV detection using diode array-detector 235C (Perkin Elmer).

2.3.3 HPLC/mass spectrometry

LC-MS analysis was performed as described by Lexa *et al.* [27] using a Rheos 2000 HPLC gradient pump (Flux Instruments, Switzerland) and HTS PAL autosampler (CTC Analytics, Switzerland) coupled to an Ion Trap Mass Spectrometer Finnigan MAT LCQ-MS^n equipped with an electrospray interface. Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with deuterated cytokinins as internal standards. The detection limit was calculated for each compound as 3.3 σ/S, where σ is the standard deviation of the response and S the slope of the calibration curve. Each sample was injected at least twice.

2.4 Quantification of proline

Quantification of proline was performed using the described method by Bates *et al.* [28]. Proline sample from pistachio leaf was extracted with 3% (w/v) sulfosalicylic acid, and the extractions was then added to the mixtures of ninhydrin reagent and glacial acetic acid and boiled at 100 °C for 30 min. After cooling to room temperature, 4 mL toluene was added and stirred well for 20–30 s. Later, the toluene layer was separated and allowed to retain room temperature. The proline concentration in red phase was determined at 520 nm of absorbance and expressed as μmol g^{-1} FW.

2.5 Choline and glycine betaine determination

To begin with, for total quaternary ammonium compound determination, dried finely-ground pistachio leaf (0.1 g) was mechanically shaken with 4 mL of deionized H_2O for 24 h at 25 °C Filtered extracts were diluted 1:1 with 2 N H_2SO_4. Aliquots (0.50 mL) were measured into heavy-walled glass centrifuge tubes and cooled in ice water for 1 h. Cold KI-I_2 reagent (0.20 mL), prepared by dissolving 15 g of iodine and 2.0 g of KI in 100 mL water was added and the reactants were gently stirred with a vortex mixer. The tubes were stored at 0–4 °C for 16 h and then centrifuged at 10 000 rpm for 15 min at 0 °C. The tubes were kept in ice and the supernatant which contained the periodide complex was carefully aspirated with a fine tipped glass tube. The periodide crystals were dissolved in 9.0 mL of 1,2-dichloroethane (reagent grade). Vigorous vortex mixing was frequently required to affect complete solution in the developing solvent. After 2.0–2.5 h, the absorbance was measured at 365 nm with a Jenway Spectrophotometer model 7305 Reference standards of glycine betaine (GB) (50–200 μg mL^{-1}) were prepared in 1 N H_2SO_4 [29].

For choline determination, sample extracts were diluted 1:1 with KPi buffer (0.2 M, pH 6.8). The choline periodides were precipitated and analyzed as previously described for total QAC. Glycine betaineperiodides do not precipitate at this pH.Total QAC, minus the concentration of choline, gave GB levels. The contents were expressed in μg g^{-1} DW [29].

2.6 Statistical analysis

Data were presented as the means for each treatment (n = 4). Data were subjected to analysis of variance (ANOVA) and means were compared using the Tukey's honest significant difference (HSD) test at the 5% probability level. Analysis of variance was performed using the SPSS software (ver. 22.0) SPSS Inc.

3 Results and discussion

Zinc (Zn) is an essential nutrient element for higher plants and is mainly absorbed in the form of Zn^{++}. It plays an important role in several plant physiological process *i.e.*, photosynthesis, respiration, and synthesis of protein, DNA, RNA, and plant hormones [21]. Zn also plays other indirect and significant roles as stabilizer of proteins, membranes, and DNA-binding proteins such as Zn-fingers [20] deficiency of Zn is one of the most common micronutrient deficiencies in plants worldwide [16]. The critical value of Zn deficiency for pistachios was determined to be about 7.0 mg kg^{-1} dry matter, while the adequate amount is determined to be about 1015 mg kg^{-1} dry matter [31, 32].

In the current study, salinity treatments led to a significant decrease in Zn concentration of leaf, stem and root tissues, however no such constant trend was found with increasing NaCl concentration. Zinc concentrations of the leaf, stem and stem tissues were lower at no Zn application treatments. At each salt treatment, increasing Zn accordingly increased Zn concentration of the seedling tissues (*table II*).

Table II. Effects of zinc (Zn) treatments on the Zn content of leaves, stems and roots of *Pistacia vera* L. cv. Badami seedlings under NaCl stress (DW: dry weight). Values are means of 4 replicates.

	Zn levels (mg kg^{-1})				
NaCl levels (mg kg^{-1})	0	5	10	20	Means
	Leaf Zn (mg kg^{-1} DW)				
0	8.8 efg†	9.1 efg	10.8 de	16.9 b	11.4 B
800	8.0 fg	8.0 fg	10.0 ef	10.8 de	9.2 D
1,600	4.5 j	7.7 gh	9.8 ef	12.5 d	8.6 D
2,400	5.5 ij	9.5 efg	12.7 cd	21.9 a	12.4 A
3,200	5.7 hij	7.5 ghi	12.8 cd	14.7 c	10.1 C
Mean	6.5 D	8.3 C	11.2 B	15.3 A	
	Stem Zn (mg kg^{-1} DW)				
0	8.5 h	9.9 gh	16.9 f	30.3 a	16.4 B
800	4.4 i	9.6 gh	11.4 g	16.9 f	10.5 D
1,600	8.7 h	11.0 g	17.8 f	26.5 b	16.0 BC
2,400	5.4 i	17.7 f	18.4 ef	20.3 de	15.4 C
3,200	10.0 gh	20.5 d	23.1 c	29.6 a	20.8 A
Mean	7.4 D	13.7 C	17.5 B	24.7 A	
	Root Zn (mg kg^{-1} DW)				
0	7.0 k	15.9 ef	22.4 c	26.3 b	17.9 B
800	12.1 hij	12.9 ghi	16.5 e	19.0 d	15.1 D
1,600	10.6 j	10.8 j	16.3 ef	29.1 a	16.7 C
2,400	6.1 k	13.5 gh	14.3 fg	24.0 c	14.4 D
3,200	11.0 ij	15.7 ef	23.1 c	26.1 b	18.9 A
Mean	9.3 D	13.7 C	18.5 B	24.9 A	

	Leaf Zn	Stem Zn	Root Zn
NaCl level	**	**	**
Zn level	**	**	**
NaCl × Zn	**	**	**

** Significant at $P \leq 0.01$.
† Means followed by the same letter (small letters for means and capital letters for means of main effects) are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Previous studies also reported the uptake and utilization of Zn as well as the absorption and utilization of K and Ca in wheat, rice, and pepper seedlings decreased with elevated soil salinity [32–34]. The relatively high concentrations of Na and/or limited water availability to plants caused by excess soluble salts were probably responsible for the decrease in mineral nutrients concentrations in tissues under saline condition. Genc *et al.* [35] stated that harmful effects of Zn deficiency under NaCl stress may act as a greater limiting factor than NaCl toxicity in reducing growth.

Table III. Effects of zinc (Zn) treatments on the Na content of leaves, stems and roots of *Pistacia vera* L. cv. Badami seedlings under NaCl stress (DW: dry weight). Values are means of 4 replicates.

	Zn levels (mg kg^{-1})				
NaCl levels (mg kg^{-1})	0	5	10	20	Means
	Leaf Na (mg kg^{-1} DW)				
0	1.10 gh†	0.60 i	0.60 i	0.60 i	0.72 E
800	2.00 e	1.40 fg	0.60 i	0.60 i	1.15 D
1,600	1.60 ef	1.40 fg	4.00 d	2.00 e	2.25 C
2,400	1.80 ef	0.90 hi	4.20 d	3.80 d	2.67 B
3,200	12.70 a	8.00 b	0.70 hi	5.20 e	6.65 A
Mean	3.84 A	2.46 B	2.02 C	2.44 B	
	Stem Na (g kg^{-1} DW)				
0	0.50 i	0.70 hi	0.90 fg	0.60 hi	0.67 D
800	0.60 hi	0.60 hi	0.60 hi	2.60 b	1.10 C
1,600	1.30 de	4.00 a	1.10 ef	0.90 fg	1.82 B
2,400	2.60 b	1.40 cd	1.40 cd	1.60 c	1.75 B
3,200	3.80 a	1.60 c	2.80 b	0.80 gh	2.25 A
Mean	1.76 A	1.66 B	1.36 C	1.30 C	
	Root Na (g kg^{-1} DW)				
0	1.10 i	2.20 gh	3.20 e	2.40 g	2.22 C
800	2.20 gh	0.70 j	2.00 h	2.40 g	1.82 D
1,600	2.80 f	4.50 b	4.20 c	3.20 e	3.67 B
2,400	2.40 g	4.50 b	4.00 c	3.50 d	3.60 B
3,200	0.50 j	4.50 b	2.80 f	10.40 a	4.55 A
Mean	1.80 C	3.28 B	3.24 B	4.38 A	

	Leaf Na	Stem Na	Root Na
NaCl level	**	**	**
Zn level	**	**	**
NaCl × Zn	**	**	**

** Significant at $P \leq 0.01$.
† Means followed by the same letter (small letters for means and capital letters for means of main effects) are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Table IV. Effects of zinc (Zn) treatments on the Cl content of leaves, stems and roots of *Pistacia vera* L. cv. Badami seedlings under NaCl stress (DW: dry weight). Values are means of 4 replicates.

	Zn levels (mg kg^{-1})				
NaCl levels (mg kg^{-1})	0	5	10	20	Means
	Leaf Cl (mg kg^{-1} DW)				
0	17.00 l†	15.00 m	6.75 o	4.00 p	10.68 E
800	20.20 j	16.50 l	14.50 m	12.50 n	15.92 D
1,600	26.75 f	25.75 g	22.75 h	18.00 k	23.31 C
2,400	31.00 d	29.00 e	27.00 f	23.00 h	27.50 B
3,200	38.75 a	37.00 b	36.25 c	21.25 i	33.31 A
Mean	26.74 A	24.65 B	21.45 C	15.75 D	
	Stem Cl (g kg^{-1} DW)				
0	5.75 l	5.00 m	4.00 n	3.25 o	4.50 D
800	14.75 b	6.25 jkl	4.50 mn	3.25 o	7.18 C
1,600	9.50 c	8.00 e	6.25 jkl	6.00 kl	7.43 B
2,400	8.75 d	7.50 efg	6.75 hij	6.50 ijk	7.37 BC
3,200	26.25 a	7.75 ef	7.25 fgh	7.00 ghi	12.06 A
Mean	13.00 A	6.90 B	5.75 C	5.20 D	
	Root Cl (g kg^{-1} DW)				
0	8.75 ef	8.50 efg	7.75 h	4.25 k	7.31 E
800	9.50 cd	9.00 de	8.25 fgh	6.25 j	8.25 D
1,600	12.00 b	8.75 ef	8.00 gh	6.75 ij	8.87 C
2,400	11.50 b	10.00 c	9.75 c	9.50 cd	10.18 B
3,200	14.50 a	11.50 b	9.75 c	7.00 i	10.68 A
Mean	11.25 A	9.55 B	8.70 C	6.75 D	

	Leaf Cl	Stem Cl	Root Cl
NaCl level	**	**	**
Zn level	**	**	**
NaCl × Zn	**	**	**

** Significant at $P \leq 0.01$.
†Means followed by the same letter (small letters for means and capital letters for means of main effects) are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Furthermore, in the current study showed that leaf, stem and root concentrations of sodium (Na) increased with rising NaCl levels at each Zn treatment (*table III*). Application of Zn compared with control, decreased the Na concentration of leaf and stem of pistachio seedlings. However, at all salinity levels and control, plants supplied with Zn accumulated higher Na in root compared with the control (without Zn). Presented results in *table III* clearly show that in root, Na concentration was higher than in leaf and stem. In addition, chloride (Cl) concentration of leaf, stem and root of pistachio seedlings was significantly increased under salinity stress. Zinc application markedly decreased Cl concentration of leaf, stem and root, at levels of 20, 10 and 5 mg kg^{-1} soil, respectively. Results showed that under NaCl treatments, decreasing effect of Zn application at level of 20 mg kg^{-1} soil on Cl concentration was approximately 41% in leaf, 60% in stem and 40% in root tissues (*table IV*). The increase in Na and Cl uptake and accumulation in plant tissues causes cell damages and death, and the increases were

Table V. Effects of zinc (Zn) treatments on the Ca content of leaves, stems and roots of *Pistacia vera* L. cv. Badami seedlings under NaCl stress (DW: dry weight). Values are means of 4 replicates.

NaCl levels (mg kg^{-1})	Zn levels (mg kg^{-1}) 0	5	10	20	Means
	Leaf Ca (mg kg^{-1} DW)				
0	5.53 ab†	4.43 f	5.32 a-d	5.29 a-d	5.14 B
800	5.43 abc	5.75 ab	5.22 b-e	5.34 a-d	5.43 Ab
1,600	4.84 def	5.87 a	5.51 ab	5.38 a-d	5.39 A
2,400	4.66 ef	5.57 ab	5.57 ab	4.87 c-f	5.16 B
3,200	4.40 f	5.74 ab	5.43 abc	4.86 c-f	5.10 B
Mean	4.96 B	5.47 A	5.41 A	5.14 B	
	Stem Ca (g kg^{-1} DW)				
0	3.80 g	5.56 a	4.62 b-e	4.60 b-e	4.64 B
800	4.27 d-g	3.95 fg	4.22 efg	5.01 ab	4.36 C
1,600	4.15 efg	4.68 b-e	4.69 b-e	4.46 b-f	4.49 BC
2,400	4.14 efg	5.50 a	4.60 b-e	4.62 b-e	4.71 AB
3,200	4.32 c-g	5.49 a	4.86 bcd	4.88 bc	4.88 A
Mean	4.13 C	5.03 A	4.59 B	4.71 B	
	Root Ca (g kg^{-1} DW)				
0	4.22 e	5.50 a	5.00 abc	4.87 bc	4.89 A
800	4.76 b-e	4.71 b-e	4.75 b-e	4.78 b-e	4.75 A
1,600	5.03 abc	4.28 de	4.86 bcd	4.74 b-e	4.72 A
2,400	4.46 cde	4.60 b-e	4.59 b-e	5.12 ab	4.69 A
3,200	4.63 b-e	4.27 e	4.86 bcd	4.99 abc	4.68 A
Mean	4.62 C	4.67 BC	4.81 AB	4.90 A	

	Leaf Ca	Stem Ca	Root Ca
NaCl level	**	**	NS
Zn level	**	**	NS
NaCl × Zn	**	**	**

NS Non-significant
** Significant at $P \leq 0.01$.
†Means followed by the same letter (small letters for means and capital letters for means of main effects) are not signi?cantly different according to Tukey's HSD test at $P \leq 0.05$.

Table VI. Effects of zinc (Zn) treatments on the K content of leaves, stems and roots of *Pistacia vera* L. cv. Badami seedlings under NaCl stress (DW: dry weight). Values are means of 4 replicates.

NaCl levels (mg kg^{-1})	Zn levels (mg kg^{-1}) 0	5	10	20	Means
	Leaf K (mg kg^{-1} DW)				
0	12.40 def†	18.00 b	15.50 bc	23.00 a	17.22 A
800	11.60 ef	17.50 b	12.75 cde	12.50 def	13.58 B
1,600	6.80 g	18.00 b	15.50 bc	13.25 cde	13.38 B
2,400	13.20 cde	15.40 bc	13.00 cde	13.25 cde	13.71 B
3,200	14.20 cde	14.50 cd	9.80 f	6.00 g	11.12 C
Mean	11.64 C	16.68 A	13.31 B	13.60 B	
	Stem K (g kg^{-1} DW)				
0	9.80 def	15.50 a	11.25 b-e	10.50 cde	11.76 A
800	10.00 def	13.00 b	11.25 b-e	12.00 bcd	11.56 AB
1,600	11.60 b-e	12.00 bcd	11.30 b-e	12.00 bcd	11.72 A
2,400	7.75 fg	12.40 bc	10.50 cde	12.40 bc	10.76 B
3,200	9.30 efg	7.00 g	13.00 b	10.00 def	9.82 C
Mean	9.69 B	11.98 A	11.46 A	11.38 A	
	Root K (g kg^{-1} DW)				
0	7.00 ef	9.40 cde	14.20 a	8.00 def	9.65 A
800	7.00 ef	12.40 ab	12.00 abc	6.75 ef	9.53 A
1,600	7.40 def	10.00 bcd	8.00 def	7.75 def	8.28 B
2,400	7.60 def	7.80 def	8.00 def	7.50 def	7.72 B
3,200	7.60 def	7.50 def	7.75 def	6.25 f	7.27 B
Mean	7.32 B	9.42 A	9.99 A	7.25 B	

	Leaf K	Stem K	Root K
NaCl level	**	**	**
Zn level	**	**	**
NaCl × Zn	**	**	**

** Significant at $P \leq 0.01$.
†Means followed by the same letter (small letters for means and capital letters for means of main effects) are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

paralleled by decreases in RGR [36]. This salt-induced growth inhibition was associated with the accumulation of salt ions (Na and Cl) in plant tissues and with a nutrient imbalance. Salinity up to 1,600 mg kg^{-1} soil significantly increased Ca levels in leaf of pistachio seedlings, however, in stem tissues, higher salinity concentrations show significant Ca concentration compared with control. Salinity had no significant effect on root calcium concentration. Zinc application significantly increased the calcium concentration of leaf, stem and root tissues. At all salinity levels, Ca concentration of leaf significantly increased under Zn application at 5 and 10 mg kg^{-1} soil. The Ca concentration of stem and root significantly was affected by Zn application under salinity stress (*table V*). Under salinity stress, potassium concentrations of leaf, stem and root significantly decreased compared with control treatment.The K concentration was more pronounced in stem and leaf than in root. Increasing Zn concentration in soil up to 10 mg kg^{-1} soi,

led to a significant increase in K concentration of leaf, stem and root tissues. Under Zn level of 20 mg kg^{-1} soil, the K concentration of root decreased (*table VI*). NaCl and Zn applications had no significant effect on magnesium (Mg) concentration of leaf, stem and root of pistachio seedlings (Data not shown).

The specific absorption rates (SAR) of K^+, Ca^{+2} and Zn were relatively high in non-saline conditions. Salinity severely reduced the SARs of these elements. Differences in SARs among Zn treatments were evident after 100 days of salt treatment. Zinc application increased the specific absorption rate (SAR) for these elements under salinity stress. The SAR values of $^+$ and Ca^{+2} were higher in 5 and 10 mg Zn kg^{-1} soil treatments than in 20 mg Zn kg^{-1} soil treatment (*figure 1*). Clearly, the maximum SAR of Zn was observed in the 20 mg Zn kg^{-1} soil treatment. Saline inhibition on the uptake rate of Zn was evident in low salinity levels. In contrast to these elements, there were no significant salt and Zn treatment effects on the rate of Mg^{2+} uptake (SAR_{Mg}). The SAR of salt ions (Na and Cl) were very low in non-saline conditions, but salinity increased the absorption rates of Na^+ and Cl^- as ranged from 0.03 to 0.1 mg g^{-1} root day^{-1} and from 0.6 to 1.2 mg g^{-1} root day^{-1} respectively. These corresponding values decreased to about half amount by Zn application (*figure 1*).

Specific utilization rate on leaf basis (SUR_L) showed similar trends for all of the elements studied. There was a significant decrease in SUR_L with increasing salinity in all Zn treatments. Zinc application especially in 10 mg kg^{-1} soil increased the SUR_L for all elements. The SUR_L for Mg^{+2} was higher than K^+ and Ca^{+2}, and the highest SUR_L was for Zn. Zinc application showed a significant decrease in the utilization rate of Na^+ and Cl^-, however under salinity stress higher SU_{RL} for the other nutrients were sustained by Zn treatments. For all elements, the 5- and 10-mg Zn kg^{-1} soil treatments exhibited higher SUR_L values than the 20 mg Zn kg^{-1} soil treatment (*figure 2*).

Regression equations of relative growth rate (RGR) with SAR and with SUR_L of all elements studied were calculated to evaluate the relative importance of these parameters for each nutrient with respect to their effects on RGR. The correlation coefficients are presented in *table VII*. The RGR was significantly correlated with the SARs of K and Ca. No significant correlation was observed for Mg and Zn. The SARs of salt ions (Na and Cl) had a significant negative correlation with RGR. Relative growth rate also showed a significant correlation with the SURs of all elements studied (*table VII*).

The results emphasize the lower absorption rates of K, Ca and Zn (*figure 1*) and the lower utilization rates of K, Ca, Zn and Mg (*figure 2*). Similar findings were achieved in annual sweet clover (*Melilotus officinalis*) and citrus rootstocks [24, 37]. Similarly, Alpaslan *et al.* [2] found that a sufficient Zn supply could reduce Na and Cl accumulation and contribute to salt tolerance in tomato plants. Elevated K and Ca contents in roots and stems of lettuce [38] and pepper [39] supplied with Zn at saline conditions is confirmed. In Zn-deficient plants, the loss of membrane integrity and the increase in membrane permeability are very common in different plant species [16]. The loss of membrane integrity under Zn deficiency may affect the uptake and accumulation of Na and Cl at toxic levels in plants. Previously, Norvell and Welch [4] reported that adequate supply of Zn is important in controlling root uptake and stem accumulation of Na and Cl. The other role of Zn is its function as inhibitor on the anion/Cl channels. Zn, acting as an inhibitor on hyperpolarization-activated inward anion/Cl channels, may be beneficial for reducing the Cl absorption and enhancing the NO_3 uptake to the pistachio seedlings exposed to salt stress. This role of Zn has also been reported by other researchers [6].

Table VII. Correlation coefficients of the regression equations for the relative growth rates (RGR) with the specific absorption rates (SAR) and the utilization rates on a leaf basis (SUR_L) of mineral elements in *Pistacia vera* L. cv. Badami seedlings under NaCl stress and Zn treatments.

Elements		
	SAR	SUR_L
Na	–0.90**	0.76**
Cl	–0.83**	0.73**
K	0.92**	0.81**
Ca	0.77**	0.86**
Mg	0.44NS	0.87**
Zn	0.39NS	0.88**

NS Non-significant.
** Significant at $P \leq 0.01$.

Unfavorable environmental factors such as salinity lead to sharp changes in the balance of phytohormones associated with not only the accumulation of ABA, but also with a decline in the level of the growth activating hormones IAA and cytokinins [6]. Zinc might be effective in alleviating salt stress damage on plant growth *via* maintaining hormones balance within plant tissues. In the current study, increasing salinity resulted in a significant accumulation of ABA, a progressive decline in IAA and a significant decrease in the level of cytokinins. Treatment with Zn prevented the salinity-induced decline in concentration of IAA and cytokinins in seedlings and reduced the accumulation of ABA (*figures 3A–3C*). Zinc deficiency reduced the IAA and cytokinins regardless of NaCl levels. As Zn levels increased, IAA and cytokinins concentrations significantly developed (*figures 3A* and *3C*). Zinc at the concentration of 20 mg kg^{-1} soil significantly increased cytokinins compared to the control, but its effect was smaller than 5 and/or 10 mg Zn kg^{-1} soil. The reverse trend was observed in the case of ABA. Abscisic acid level of the Zn deficient seedlings was higher than those supplied with Zn, particularly at higher NaCl levels. Seedlings supplied with 10 mg Zn kg^{-1} maintained ABA of approximately 272.92 ng g^{-1} FW, followed a slight rise with 5 and 20 mg Zn kg^{-1} soil (*figure 3B*).

Under salt stress an enhancement of IAA and CKs by decreasing ABA concentration in the experimental pistachio seedlings were observed after Zn amendment. In line with this result, such a pattern is well documented for IAA in bean (*Phaseolus vulgaris*) [17], cytokinins in lupin (*Lupinus albus*) [41] and ABA in *Ricinus communis* and *Xanthium strumarium* [42]. Cakmak *et al.* [17] confirmed the role of Zn in protein synthesis and demonstrated that the decrease in IAA level in Zn-deficient plants, also that in Zn-deficient plants the

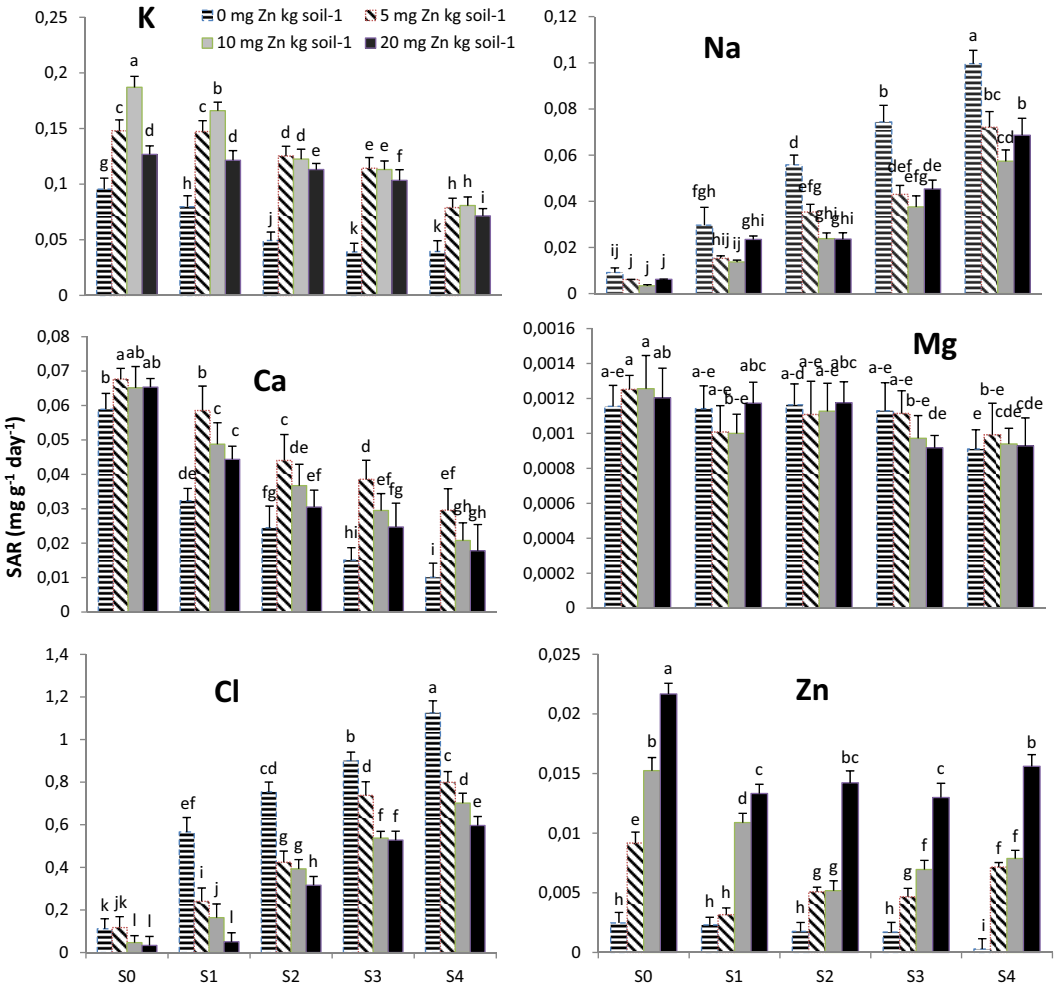

Figure 1. The effects of soil application of zinc (Zn) on specific absorption rates (SAR) of K, Na, Ca, Mg, Cl and Zn in seedlings of *Pistacia vera* L. cv. Badami under NaCl stress. Bars represent means and error bars represent standard error (n = 4). Bars having different letters are significantly different at the 5% level by Tukey HSD. S0, S1, S2, S3 and S4 refer to 0, 800, 1,600, 2,400, and 3,200 mg NaCl kg^{-1} soil, respectively.

conversion of tryptophan to IAA specifically inhibited by enhanced oxidation. Zn deficiency and/or saline conditions induced oxidative depredation of IAA and cytokinins [43, 44] Iron, by catalyzing the Haber-Weiss reaction, is responsible for the $OH^{\bullet}$ production. IAA is extremely sensitive to $OH^{\bullet}$, and can be oxidized rapidly upon exposure to high concentrations of $OH^{\bullet}$ [43, 44]. Besides nonenzymic oxidation, IAA and also cytokinins are also oxidized by H_2O_2 dependent peroxidases [45, 46], and $O_2^{\bullet}$ and $^1O_2$ are involved in this degradation pathway [47]. Higher peroxidase activity, $O_2^{\bullet}$ in Zn-deficient plants under salinity stress may result in enhanced oxidative degradation of IAA and cytokinins. ROS-scavenging antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), play a vital role in removing these destructive oxidant species. The positive effects of Zn on antioxidant enzyme activity scavenging the reactive oxygen species (ROS) produced in response to salt stress and/or Zn deficiency are well demonstrated [16]. Tavallali *et al.* [48] reported that pistachio seedlings treated with Zn had very high SOD, CAT and APX activity, indicating that there was efficient ROS scavenging activity in the system. This could be another relevant reason why Zn-deficient plants become very sensitive to ROS when grown under saline conditions.

Osmolytes accumulation is one of the most important responses of cell structures to environmental stress and could be an adaptive mechanism in this condition [49]. In this study, proline significantly increased in leaves of salt stressed plants compared to the control treatment. Regardless of salt concentration, proline was significantly reduced by Zn addition. Interaction of 10 mg Zn kg^{-1} regardless of NaCl levels caused a significant decrease in proline as compared with other Zn levels (*figure 4A*). Thus, increased proline level in this experiment could be considered as a response to stress induction which was decreased by Zn (*figure 4A*). In line with this result, Saleh and Maftoon [50] found that increased Zn concentration combined with salinity stress leads to a reduction in proline accumulation in rice. The interaction between soil salinity

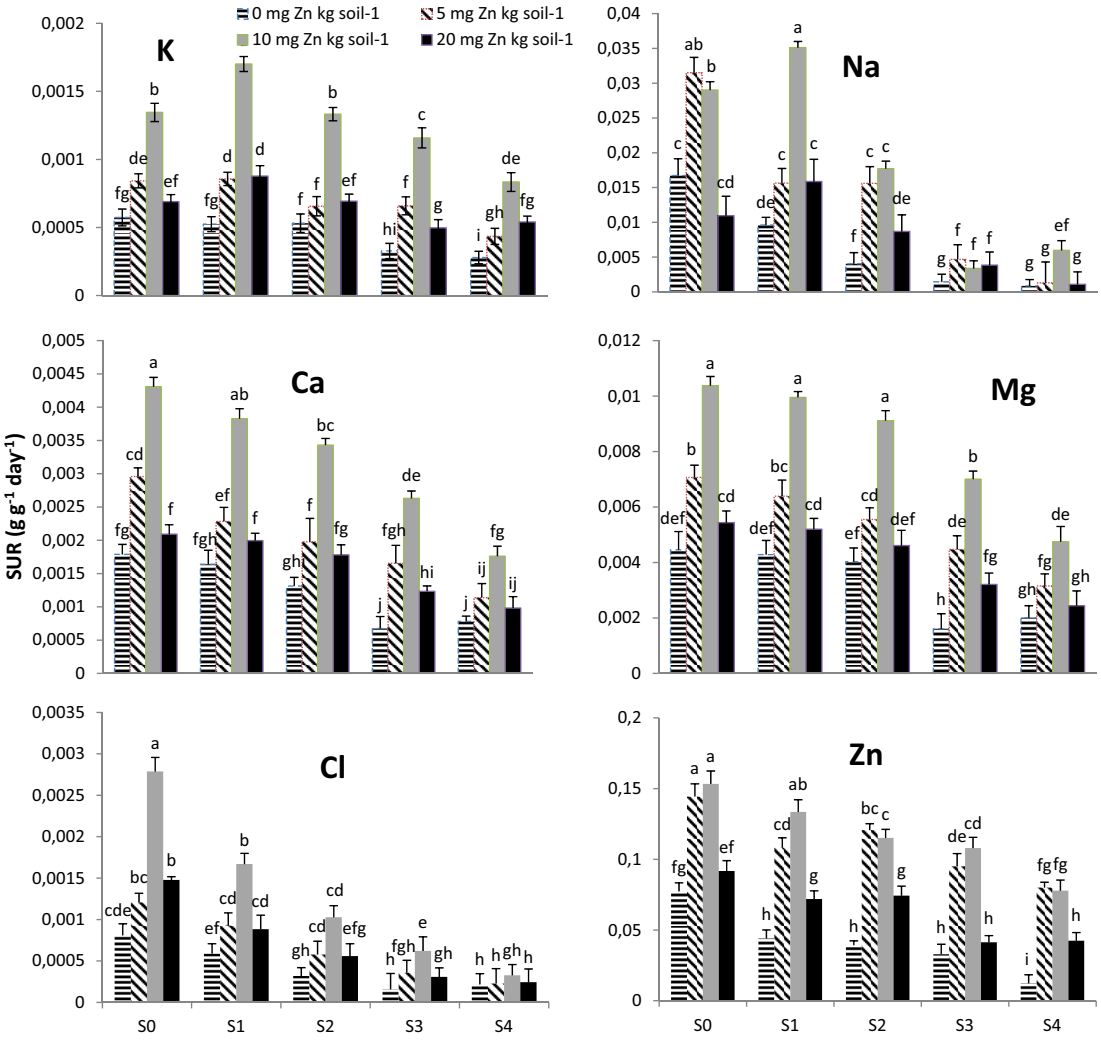

Figure 2. The effects of soil application of zinc (Zn) on specific utilization rates (SUR) of K, Na, Ca, Mg, Cl and Zn in seedling leaves of pistachio 'Badami' under NaCl stress. Bars represent means and error bars represent standard error (n = 4). Bars having different letters are significantly different at the 5% level by Tukey HSD. S0, S1, S2, S3 and S4 refer to 0, 800, 1,600, 2,400, and 3,200 mg NaCl kg^{-1} soil, respectively.

and Zn application resulted in a marked decrease in proline content in *Salvia officinalis*, which also has been observed by Hendawy and Khalid [51]. It seems that this may be a result of this fact that proline accumulation in plant is mainly known as a symptom of stress damage, not as a salinity resistance indicator [52]. Under stress conditions, the great amount of proline forms during the oxidation of carbohydrates and proteins [53]. Cakmak [16] suggested Zn as an excellent protective antioxidant may participate in proline production due to its role in alleviating stress.

Increasing NaCl without Zn significantly enhanced the concentration of other osmolytes, choline and glycine betaine compared with the control (*figures 4B* and *4C*). The interaction between soil salinity and Zn application especially at 20 mg kg^{-1} soil resulted in a significant decrease in choline contents in comparison with no Zn treatment. Choline contents were the lowest at 20 mg Zn kg^{-1} soil (*figure 4B*). Zinc application without NaCl significantly increased the glycine betaine contents compared with control, however no significant differences were observed among Zn levels (*figure 4C*). A decrease in the proline and choline contents in leaves under non-saline condition amended with 10 and 20 mg Zn kg^{-1} soil were considerably lower than those under control and/or 5 mg Zn kg^{-1} soil (*figures 4A* and *4B*).

Glycine-betaine (GB) is a quaternary nitrogenous compound which plays an important role in osmoregulation in various organisms and plants under salinity stress [54]. Glycine-betaine is synthesized from choline through two steps:

Choline → betainealdehyde → glycinebetaine [55]

The activity of GB synthesizing enzymes (*e.g.*, betaine aldehydehydrogenase) increased in several plant species at high NaCl concentration [56]. Exogenous application of GB enhanced the growth of plants under different stresses [57]. The addition of choline, GB precursor, to the growth medium increases acclimation of salt sensitive wheat genotype to NaCl stress [58].

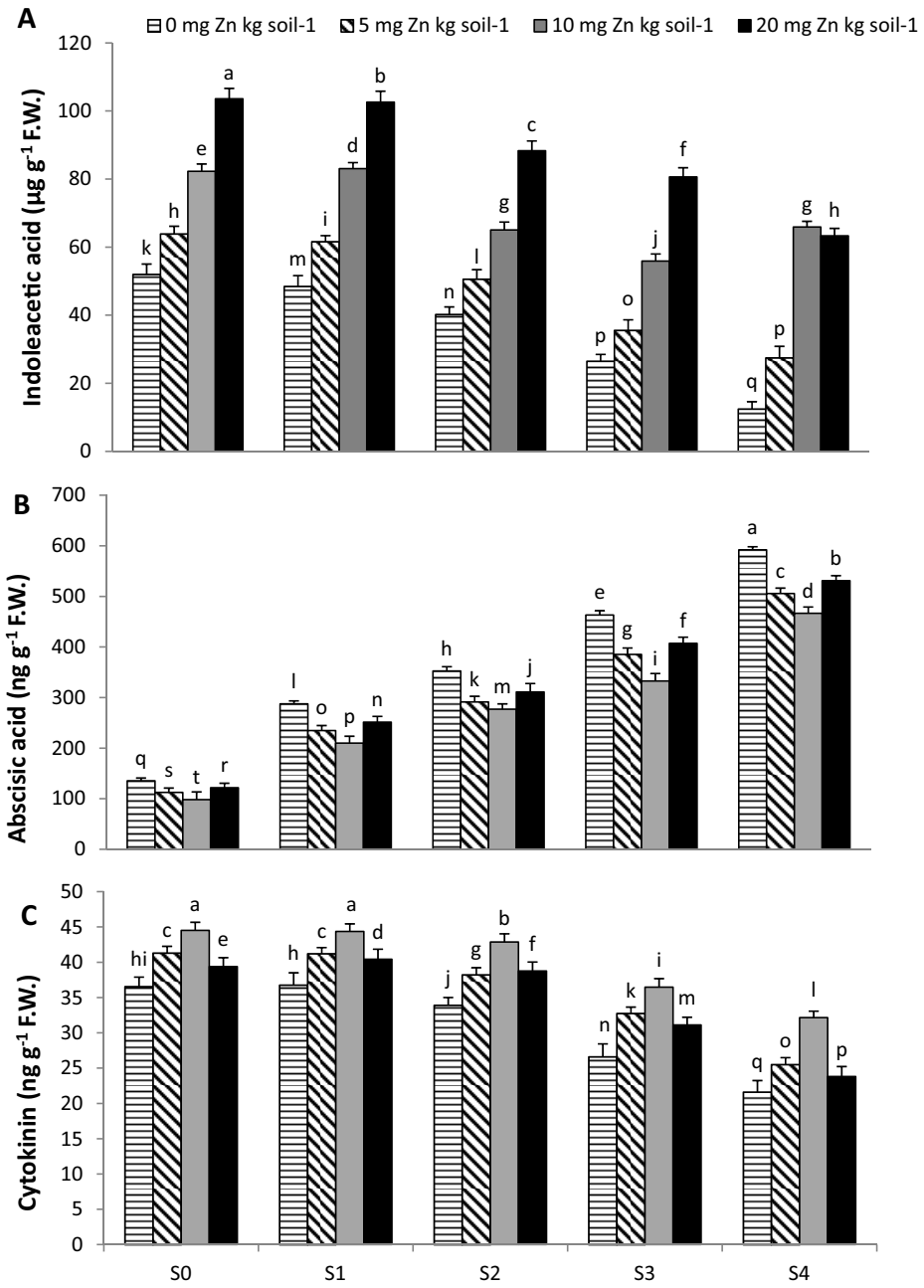

Figure 3. The effects of soil application of zinc (Zn) on indoleacetic acid (IAA) (A), abscisic acid (ABA) (B) and cytokinin (C) contents in seedling leaves of pistachio 'Badami' under NaCl stress. Bars represent means and error bars represent standard error (n = 4). Bars having different letters are significantly different at the 5% level by Tukey HSD. S0, S1, S2, S3 and S4 refer to 0, 800, 1,600, 2,400, and 3,200 mg NaCl kg^{-1} soil, respectively (F.W.: fresh weight).

Glycine betaine is mainly localized in chloroplasts [55] and its accumulation contributes to chloroplast adjustment and protection of thylakoid membrane thus maintaining photosynthetic activity [59]. Glycine betaine application increased net photosynthetic rate of salt stressed tomato and turnip which was due to increased stomatal conductance and decreased photorespiration [60]. A Zn-enhancement of GB is very beneficial for plants in order to provide osmotic adjustment and participate in stomatal regulation and facilitate the photosynthetic activity [57].

4 Conclusion

This study demonstrated that a Zn deficiency reduces the nutrient uptake and utilization and the phytohormone balance especially under saline conditions. Adequate Zn treatments may improve salt tolerance of *Pistacia. vera* 'Badami' seedlings by enhancing the accumulation of osmolytes in plant tissues and improving supplementation on K, Ca and Zn absorption and utilizing efficiency. Adequate Zn supply was suggested to limit uptake and accumulation of Na in leaf and stem.

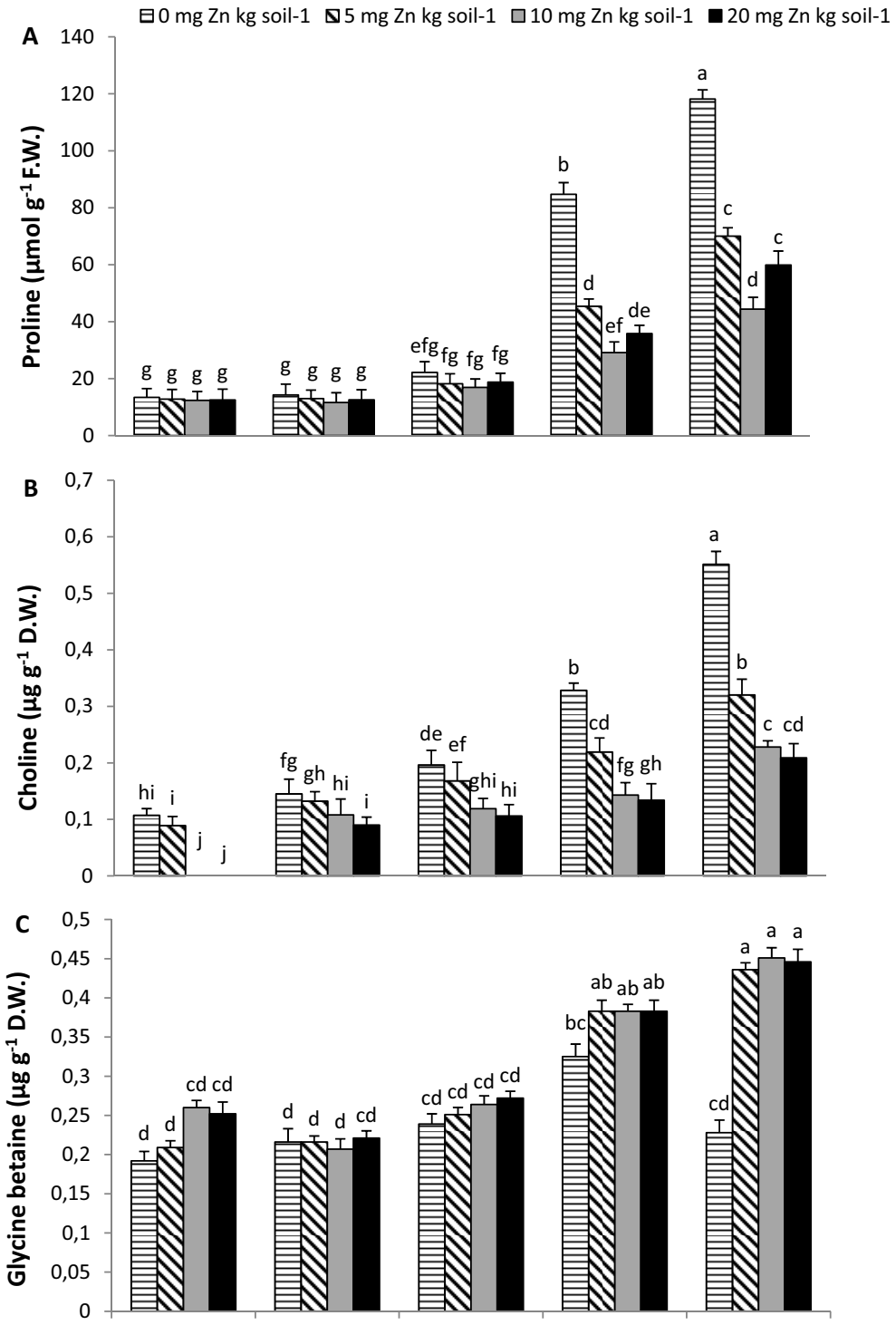

Figure 4. Effects of Zn on proline (A), choline (B) and glycine betaine (C) contents in seedling leaves of pistachio 'Badami' under NaCl stress. Bars represent means and error bars represent standard error (n = 4). Bars having different letters are significantly different at the 5% level by Tukey HSD. S0, S1, S2, S3 and S4 refer to 0, 800, 1,600, 2,400, and 3,200 mg NaCl kg^{-1} soil, respectively (F.W./D.W.: fresh/dry weight).

The addition of Zn may enhance pistachio salt tolerance by reducing ABA level and enhancing IAA and cytokinin concentrations in the plants. Therefore, the application of 10 mg Zn kg^{-1} soil is recommended for alleviating salt-induced damages on pistachio seedlings.

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