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In vitro salt stress tolerance of 'Sahand' cultivar grafted on two wild almond rootstocks: An evaluation of physiological and biochemical traits between rootstocks

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Abstract: The low salinity tolerance of almond cultivars can cause a significant setback in almond production. Therefore, selecting suitable cultivars and rootstocks in salinity-affected areas can facilitate sustainable crop production. In this research, the effects of two wild almond species, Badamkohiand Arjan as rootstocks on the salinity tolerance of 'Sahand' as a scion were investigated through *in vitro* culture. A factorial experiment of 2 (species) × 4 (levels of salinity) was conducted in a completely randomized design (CRD) with 4 replications. The results showed that 'Sahand' grafted on Badamkohi had the higher fresh and dry weight than grafted on Arjan in all level of salinity. The Na^+ and Cl^- ions contents inthe shoots and root of both micrografting combinations increased with increasing salinity. However, their amount in the shoot and the root of 'Sahand'/Arjan plants were significantly higher than those ions in 'Sahand'/Badamkohi plants at 80 and 120 mM NaCl. The amount of total chlorophyll in 'Sahand' grafted on Badamkohi was 0.68 mg g^{-1} FW which was significantly higher than the total chlorophyll of the same scion grafted on Arjan rootstock (0.51 mg g^{-1} FW) at 120 mM NaCl. The highest leaf cell electrical leakage occurred in 'Sahand' grafted on Arjan which was significantly higher than leaf electrical leakage of the same scion grafted on Badamkohi at 120 mM NaCl. The grafting combination of 'Sahand'/Badamkohi showed a higher proline and glycine betaine content, compared to the grafting combination of 'Sahand'/Arjan. The shoot and root antioxidant enzyme activities (SOD, POX and CAT) in micrografting combination of 'Sahand'/ Badamkohi were also significantly higher than those in 'Sahand'/Arjan. It can be concluded that 'Sahand/Badamkohi combination is a suitable choice for the regions with late spring frost and saline conditions.

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All relevant data are within the paper and its Supporting Information files.

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1. Introduction

Almond (*Prunus dulcis* Mill.) is one of the most important nut crops (Ansari and Gharaghan, 2019), with particular importance in the world. It

is characterized by a high storage capacity, low degree of waste, ease of processing and transportation. The feasibility of its economic production has led to an increase in the total area of almond orchards (Bybordi, 2013). Salinity still remains the major abiotic stress that limits agricultural production (Seleiman *et al.,* 2020). Almonds can be affected by salinity stress through osmotic mechanisms and by enhanced levels of osmotic potential in the soil solution (Shrivastava and Kumar, 2015). High salinity in root zone not only reduces water uptake and tree growth, but also can cause nutritional imbalances and toxicity effects of the major saline ions (Na^+ and Cl^-).

Meanwhile, the low salinity tolerance of almond trees can cause a significant setback in almond production (Kaundal *et al.*, 2019). Therefore, selecting suitable cultivars and rootstocks in salinity-affected areas can facilitate sustainable crop production.

The dynamic nature of salinity with respect to time and space, as well as limited experimental designs restrict the complete study of genotype-environment interactions (Sauvage *et al*., 2014). Therefore, the crop breeding program can be complemented with a suitable management option, such as grafting on appropriate salt tolerant rootstocks (Cuartero *et al*., 2006). Grafting has been reported as a rapid method for enhancing salt tolerance (Singh *et al*., 2020) that counteracts the salinity effects by maintaining low Na^+/K^+ ratios in the shoot and improves leaf stomatal conductance (Wei *et al*., 2017). The behavior of the rootstock in different plant species influences the metabolic processes of the scion leading to tolerance. Rootstocks are an essential component in modern fruit production (Shahkoomahally *et al*., 2020) and can provide several traits that may be absent in the scion, such as resistance to soil pest and disease, better root systems, enhanced nutritional uptake, better tolerance to soil salinity and water scarcity (Kumar *et al*., 2017).

The cultivation of grafted plants has gradually increased in recent years since grafting enables the plant system to control important agronomic traits and offers a flexible pattern to the growth of a particular scion (Kumar *et al.*, 2017). Grafting a scion on a suitable rootstock generally allows extensive use of the rootstocks (Gainza *et al*., 2015). While considering a wide range of salt-tolerant genotypes in the genus *Prunus*, the selection of more tolerant species as rootstock can lead to sustainable solutions in handling commercial cultivars of almond and peach (Najafian *et al*., 2008). The use of wild almond species as rootstocks, has been considered feasible especially in arid and semi-arid regions (Karimi *et al*., 2015). Using these rootstocks can highlight the strategy of allowing plants to overcome environmental stress, because of their adaptability and stimulated growth. Moreover, rootstocks affect the nutritional status of the scion and plant height (Aras and Eşitken, 2019).

Trees have lengthy biological cycles, which implies prolonged time lapses until plants are produced for study, as well as evaluations that can last for the entire growing season or even more than one season (Bado *et al*., 2015). This is of particular importance in plant breeding programs where thousands of plants are handled yearly and the staff and land are restricted. However, the long juvenile periods and cost of maintaining all the seedlings until they are grown trees have encouraged researchers to develop early studies in young plants to discard those genotypes which do not fit in the breeding goals and minimize the cost and time of field evaluations (Vives-Peris *et al*., 2017).

In recent years, tissue culture and *in vitro* selection have emerged as an effective tool in the furtherance of efforts to develop stress-tolerant plants. *In vitro* cultivation techniques can largely assist with the study and selection of plant species, because more control is exerted on plant growth compared to the outside environment, and evaluations are usually conducive to good results in a confined space (Ghaleb *et al*., 2010; Rai *et al*., 2011). When the stability of medium culture enables a controlled condition, a uniform application of stress to all explants can create reliable results, because other intervene factors are eliminated (Seth and Kendurkar, 2015). The *in vitro* system can characterize the degree of salt tolerance of different genotypes at their primary growth phase within a short time, limited space and low cost (Ghaleb *et al*., 2010). This method has been applied for screening salt tolerant genotypes of some fruit species including cherry rootstocks (Erturk *et al*., 2007), citrus rootstocks (Ghaleb *et al*., 2010), grape rootstocks (Alizadeh *et al.,* 2010), fig cultivars (Abdoli Nejad and Shekafandeh, 2014), apple varieties (Shibli *et al.,* 2000), kiwifruit (Sotiropoulos and Dimassi, 2004) and pear (Sotiropoulos *et al*., 2006). The aim of this research was to study the influence of Badamkohi and Arjan as rootstocks on the salinity tolerance of 'Sahand' (a late-bloom almond cultivar) as a scion through certain morphological and bio-

chemical responses *in vitro* condition.

2. Materials and Methods

Establishment of in vitro *micrografting*

According to Asadi and Shekafandeh (2021) procedure, the mature seeds of naturally grown wild almond trees, *Prunus scoparia* (C. Schneider) and *Prunus elaengnifolia* (E. Murrary) named Badamkohi and Arjun respectively, grow in arid and semi-arid regions were grown *in vitro* to produce seedlings. After removing the endocarps, they were surface-sterilized by immersion in 70% alcohol for 1 min and then in 20% Whitex solution (sodium hypochlorite 5%) for 10 min. Subsequently, they were rinsed three times with sterile distilled water. The sterilized seeds were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar as gelling agent (Fig. 1 a, b). For preparation of the micro-scion, young shoots of the late-blooming 'Sahand' were disinfected and cultured on MS medium supplemented with 2.2 Mm benzyladenine (BA) and 0.54 µM naphthaleneacetic acid (NAA). After two weeks, the young offshoots have reached a suitable size to be used as scions (Fig. 1 c). Then, the two weeks old *in vitro* produced seedlings were decapitated and by the help of a sharp scalpel a vertical slit (0.5 cm) was created on top of the stump (Fig. 1 d). The scion was cut into a "V" shaped wedge (Fig. 1 e) and inserted into the rootstock to form a micrograft (Fig. 1 f).

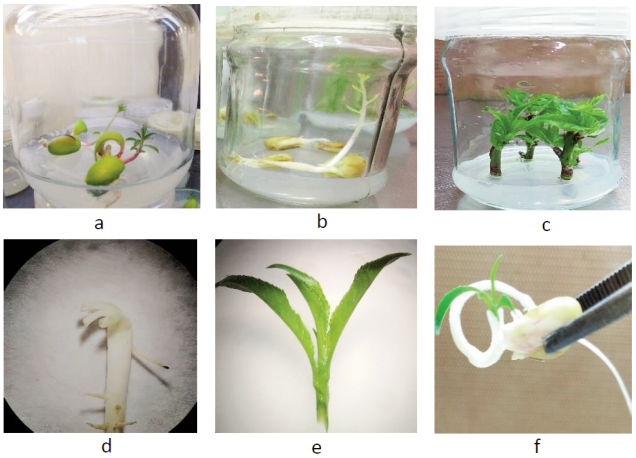

Fig. 1 - Performing *in vitro* micrograft. Germinated seeds of Arjan (a) and Badamkohi (b) using as rootstock. Proliferated shoots of 'Sahand' cv. on MS medium supplemented with 2.2 µM BA and 0.54 µM NAA using as scion (c). An Arjan rootstock ready to be grafted (d). A scion ready for grafting (e). A micrograft combination of 'Sahand'/Arjan (f).

The jars containing explants were maintained at 25±1°C under a 16 h photoperiod (light intensity of 4000 lux).

Salinity treatments

The graft combinations were allowed to grow for 4 weeks, and then they were cultured on agar free MS medium with a bridge paper and different concentrations of NaCl (0, 40, 80 and 120 mM). After 4 weeks of culture, certain morpho-physiological and biochemical characteristics of graft combinations were measured as follow.

Fresh and dry weight of scion and rootstock

After measuring the fresh weight of the shoot (scion) and the root (rootstock), they were dried in an oven for 24 h at 60°C and then re-measured for their dry weight (in mg).

Sodium (Na^+) and Chlorine (Cl^-)

The samples (1 g) were dried in an oven at 500 to 550°C for 5 h, then reduced to ash. To each sample in the crucible was added 5 ml of 2 normal HCl. After passing the solution through filter paper, the filtered solution was transferred to a jug balloon. The volume of each sample was made up to 50 ml with hot distilled water and then sodium was measured using a flame photometer (Model Jenway PFP7 Bibby Scientific Ltd, Staffordshire, UK) and calculated in mg g^{-1} dry weight.

To determine the chlorine of each sample, according to the method of Chapman and Pratt (1961), one gram of sample was poured into a Chinese mortar and 250 mg of calcium oxide was added to each and was kneaded with distilled water. They were then placed in a kiln at 250°C for one h to remove all the soot from the initial burning. After, the kiln temperature was slowly raised to 550°C to reduce the samples to ashes. Then 15 mL of hot distilled water was added to the samples. After cooling, 5 drops of 5% potassium chromate were added to the solution and titrated with 0.05 N silver nitrate (2.12 g of silver nitrate in 250 mL of distilled water) to observe a red brick-colored precipitate. Finally, chlorine was calculated as mg g^{-1} dry weight.

Total chlorophyll (Chl)

Fresh leaf samples (0.1 g) were placed in test tubes and added 7 mL of dimethyl sulfoxide, then they were placed in an incubator for 30 minutes at 65°C. After extraction, the volume of extracts was made up to 10 mL by adding dimethyl sulfoxide.

Finally, the absorbance of the extracts at wavelengths of 645 and 663 nm was read using a spectrophotometer (USA Epoch Microplate, BioTek instruments, Inc) (Gross, 1991). Chlorophyll content was determined as follows:

$$\text{Chl (mg g}^{-1}\text{) FW} = [20.2\ (OD645nm) + 8.02\ (OD663nm)] \times V/FW \times 1000$$

Where V is final volume of solution (mL), FW the leaf fresh weight (mg), and OD the optical density.

Electrolyte leakage(EL)

EL was determined according to the method of Gulen and Eris (2004). Ten discs were cut from the fully developed leaves of the plants in each replication. Then, they were transferred to vials containing 5 mL deionized water and kept at 10°C for 24 h. After measuring their electrical conductivity (EC1) using a conductometer (Metrohm 644, Awess), the samples were then placed in a water bath at 95°C for 20 min, and after cooling at 25°C the electrical conductivity (EC2) was re-measured. The EL was calculated with the following formula:

$$EL\ (\%) = (EC1/EC2) \times 100$$

Proline contents

According to the modified method of Bates (1973), the proline content was determined. The leaf sample (0.5 g) was grinded in 10 mL sulfosalicylic acid (3%). The mixture was then centrifuged at 10,000×*g* for 10 min. Two ml of the supernatant was added to each test tube which contained freshly prepared acid-ninhydrinas a diluted solution (2 ml). The tubes were incubated in a water bath at 90°C for 30 min. Ultimately, the reaction ended in an ice bath. The reaction mixture was extracted using toluene (5 mL) and was vortexed for 15 s. The tubes were stored in darkness at room temperature for 20 min, thereby allowing the separation of toluene from the aqueous phase. The toluene phase was then carefully collected and the absorbance was measured at 520 nm by a spectrophotometer (model T60 USA).

Glycine betaine

Glycine betaine was measured by the method of Grattan and Griere (1985). The powdered sample of leaf (0.5 g) was mixed in a mortar with 20 mL of ionized water. The specimens were placed on a shaker for 48 h at 25°C. They were passed through a Whatman filter paper 'G42' and diluted in a 1:1 ratio with two-molar sulfuric acid. Then, 0.5 mL of this solution was removed and poured into the Eppendorf tube. After cooling the samples for 2 h, 0.2 ml of potassium tri-iodide solution was added to each. Then, they were centrifuged for 20 min at 15,000 rpm at 0°C. The top phase discarded and the periodontal crystals were dissolved in the bottom of the container in 9 mL of 1-2 dichloroethane. Then, the absorption of the samples was measured at 365 nm with a spectrophotometer.

Enzyme activity

In order to estimate the enzymes activities, the leaf samples (0.5 g) were first homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 3 mM 2-mercaptoethanol, and 2% (w/v) polyvinyl polypyrrolidone (PVPP) in a chilled mortar. The homogenate was then centrifuged at 16000 *g* for 30 min at 4°C and the supernatant was used for enzyme assays.

Superoxide dismutase (SOD)

The SOD (EC 1.15.1.1) can be measured by determining its ability to halt the photochemical reduction of nitro blue tetrazolium chloride (NBT) in the presence of light. In this method, the reaction mixture (3 mL) contained 50 µL enzyme extract, 50 mM potassium phosphate buffer, 13 mM l-methionine, 75 µM NBT, 0.1 mM EDTA and 4 µM riboflavin. The reaction mixture was shaken and placed in a light chamber for 15 min to allow the reaction to take place. Eventually, the absorption rate of each specimen was recorded at 560 nm using a spectrophotometer (Biochrom WPA Biowave II UV/Visible Spectrophotometer, England) against the non-irradiated blank (Dhindsa and Motowe, 1981).

Catalase (CAT)

The determination of the activities of CAT (EC 1.11.1.6) was based on the rate of H_2O_2 decomposition as measured by decreasing the absorbance at 240 nm (Dhindsa and Motowe, 1981). While the reaction mixture contained 50 mL potassium phosphate buffer (pH 7) and 15 mM hydrogen peroxide (H_2O_2), the reaction started by adding 1000 µL of the enzyme extract. One unit of activity is the amount of enzyme that could decompose 1 mM of H_2O_2 in 1 min.

Peroxidase (POX)

Peroxidase enzyme activity was read at 470 nm, based on an enhanced degree of light absorption as a result ofguaiacol oxidation in the presence of peroxidase hydrogen. This was carried out by a spectropho-

tometer (JENWAY model 7315 UK) in 1 min with a time interval of 10 s (Ozden *et al*., 2009). The activity of the enzyme was calculated based on the oxidized µmol of guaiacol per min and per g of fresh leaf weight.

Statistical analyses

The experiment was carried out as a factorial 2 (species) × 4 (levels of salinity) in a completely randomized design (CRD) with 4 replications and 4 micrografted plants per replicate. A total of 122 micro-grafting combination of 'Sahand'/Argan and 'Sahand'/Badamkohi were used in this experiment. Data were analyzed using SAS 9.4 software and mean values were compared using LSD test (P≤0.05).

3. Results

The results of analysis of variance showed that the interaction between species and salinity was significant at 5% or 1% level in all measured traits. So, all the results were presented by the interactions.

Effects of salinity on scion-rootstock combination growth

The results showed that, both rootstocks (Badamkohi and Argan) had significant difference in the root length of control (free salt medium). Badamkohi showed the highest root length of 263 mm that was significantly higher than Arjan (122 mm) (Table 1).

This showed that they have different growth habit. However, in both rootstocks with increasing salinity to 120 mM in culture medium the root length decreased significantly. This reduction was 50% and 18% for Badamkohi and Arjan respectively compared to their controls. The highest length of scion (64 mm) was related to the salt-free treatment on Badamkohi rootstock which showed a significant difference with the same scion on Arjan rootstock (41 mm) in the same treatment.

With increasing the concentration of sodium chloride from 0 (control) to 120 mM, the scion ('Sahand') length on both rootstocks was significantly reduced (Fig. 2 b, c, e, f), this reduction was 50.6% and 49% on Badamkohi and Arjan respectively.

Table 1 - Effect of sodium chloride on scion and root length in micrografting combinations of 'Sahand'/Badamkohi and 'Sahand' /Arjan

Micrografting combination	NaCl mM	Root length (mm)	Shoot length (mm)
'Sahand'/Badam kohi	0	263 a	64 a
	40	202 b	44 b
	80	148 c	38 bc
	120	130 d	30 d
'Sahand'/Arjan	0	122 ed	41 b
	40	114 edf	33 cd
	80	106 ef	29 d
	120	100 f	21 e

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

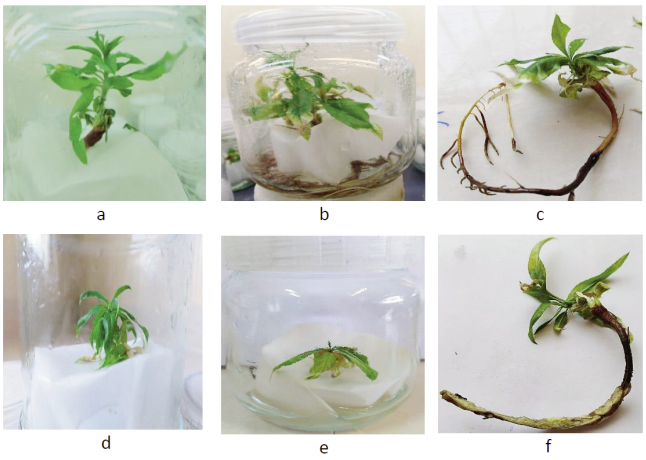

Fig. 2 - Growth of a micrografted combination of 'Sahand'/Badamkohi in control (a) and in 120 mM salinity (b and c); a micrografted combination of 'Sahand'/Arjan in control, (d) and 120 mM salinity (e and f).

In both rootstocks, root fresh and dry weight decreased with increasing NaCl concentrations, however fresh weight loss in Badmkohi and Arjan was 62% and 76%, respectively, and also dry weight loss was 55% in Badamkohi and 51% in Arjan (Fig. 3 a and c). Fresh and dry weight of 'Sahand' grafted on Badamkohi at all salinity levels (except scion dry weight in 120 mM salt) was significantly higher than fresh and dry weight of 'Sahand' grafted on Arjan (Fig. 3 b and d). In both rootstocks, increasing the salt from 0 to 120 mM reduced the fresh weight of the scion by about 60% while this reduction in the dry weight of scion was 62% on Badamkohi rootstock and 55% on Arjan rootstock (Fig. 3 b and d).

Effect of salinity on Na^+ and Cl^- contents in micrografting combinations

The results showed that the Na^+ and Cl^- contents in the roots and the shoots of both micrografting combinations increased with increasing salinity (Fig. 4 a, b, c, d,). However, the amount of Na^+ and Cl^- in the shoot and the root of 'Sahand'/Arjan plants were sig-

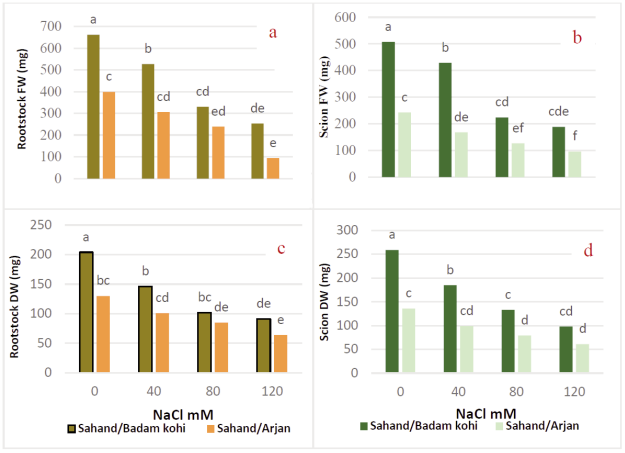

Fig. 3 - The effect of different concentrations of NaCl on fresh and dry weight of micrografting combinations of 'Sahand'/ Badamkohi and 'Sahand'/Arjan. Different letters indicate significant difference at P ≤ 0.05 level of probability using LSD test.

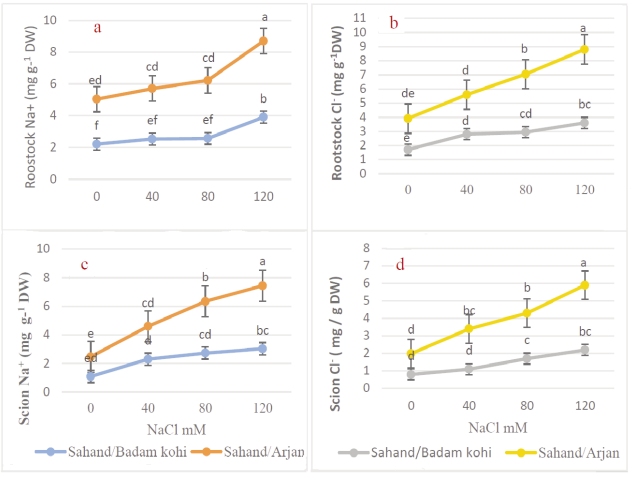

Fig. 4 - The Na+ and Cl- contents of both rootstocks (a, b) and scion (c, d) in grafting combinations of 'Sahand' /Badamkohi and 'Sahand'/Arjan in different concentration of NaCl. Different letters indicate significant difference at P ≤ 0.05 level of probability using LSD test.

nificantly higher than these ions in the shoot and the root of 'Sahand'/Badamkohi plants at 80 and 120 mM NaCl.

Total chlorophyll (Chl)

The Chl of 'Sahand' grafted on both rootstocks decreased with increasing salt concentration. The highest amount of Chl was obtained in 'Sahand' (as scion) leaf grafted on Badamkohi rootstock in unsalted medium (control) which showed a significant difference compared to the same scion grafted on Arjan rootstock. The amount of Chlin 'Sahand' grafted on Badamkohi was 0.68 mg g^{-1} FW which was significantly higher than the Chl of the same scion grafted on Arjan rootstock (0.51 mg g^{-1} FW) at 120 mM NaCl (Table 2). At the highest salinity level, the leaf chlorophyll content of 'Sahand' either grafted on Badamkohior Arjan was decreased by 51% and 63% respectively compared to their controls.

Electrolyte leakage (EL)

In all levels of salinity, the EL of 'Sahand' grafted on Arjan rootstock was significantly greater than the same cultivar grafted on Badamkohi. The highest leaf cell EL (72.29%) occurred in 'Sahand' grafted on Arjan rootstock which was significantly higher than leaf EL of the same scion grafted on Badamkohi rootstock at 120 mM NaCl (Table 2).

Table 2 - Effects of sodium chloride on leaf total chlorophyll and electrolyte leakage in micrografting combinations

Micrografting combination	NaCl mM	Total chlorophyll (Mg g^{-1} FW)	Electrolyte leakage (%)
'Sahand'/Badam kohi	0	1.45 a	23.28 e
	40	1.42 ab	31.32 de
	80	0.95 bc	38.05 cd
	120	0.68 d	43.86 c
'Sahand'/Arjan	0	1.33 b	38.15 cd
	40	1.25 bc	43.77 c
	80	0.77 d	55.15 b
	120	0.51 e	72.29 a

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

Proline and glycine betaine (GB) in micrografting combination

Increasing the level of salinity caused a significant rise in the proline content of both grafting combinations ('Sahand'/Badamkohi and 'Sahand'/Arjan) (Table 3). The grafting combination of 'Sahand'/ Badamkohishowed a higher proline content (in both parts, scion and rootstock) compared to the grafting combination of 'Sahand'/Arjan in all salinity levels.

The results also indicated that by increasing the salt concentration from 0 to 120 mM, the amount of GB increased in both micrografting combinations. The shoot and the root in micrografting combination of 'Sahand'/Badamkohi showed significantly greater GB than those in micrografting combination of 'Sahand'/Arjan in all level of salinity (except for root GB in salt free medium of both rootstocks).

Table 3 - Effects of sodium chloride on the amounts of proline and glycine betaine (GB) in the shoots and roots of micrografting combinations

Micrografting combination	NaCl levels (mM)	Root GB µmol g^{-1} DW	Shoot GB µmol g^{-1} DW	Root proline µmol g^{-1} FW	Shoot proline µmol g^{-1} FW
'Sahand'/Bdam kohi	0	10.55 ef	6.88 cd	24.93 e	20.64 d
	40	14.60 d	7.48 c	38.03 c	27.73 c
	80	19.00 b	8.90 b	46.70 b	40.88 b
	120	22.60 a	11.00 a	62.40 a	55.38 a
'Sahand'/Arjan	0	9.30 f	5.70 e	14.80 f	13.22 e
	40	11.47 e	6.30 de	21.70 ef	18.97 de
	80	14.02 d	6.80 cd	28.9 de	24.06 cd
	120	16.50 c	7.60 c	34.50 cd	30.40 c

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

Superoxide dismutase (SOD)

SOD activity in both'Sahand'/Badamkohi and 'Sahand/Arjan plants increased with increasing the levels of salinity (Table 4). The highest increase in the activity of SOD was obtained in the 'Sahand' /Badamkohi, combination with 101.27 and 103.30 (U g^{-1} FW min^{-1}) in shoot and root respectively at 120 mM salt, which was significantly higher than those in 'Sahand/Arjan plants.

Peroxidase (POX)

In the shoots and in the roots of both micrograft combinations, POX activity increased with increasing salt concentrations in the medium. Although, at all salinity levels, POX activity was higher in the shoot and the root of micrografting combination of 'Sahand'/Bada Kohi than 'Sahand'/Ajan, however the difference was only significant at 120 mM NaCl.

Catalase (CAT)

Both micrografting combinations showed the enhancement CAT activity in response to increasesalt concentrationfrom 0 to 120 mM NaCl. The highest CAT activity occurred in the shoot (63.70 U g^{-1} FW min^{-1}) androots (59.70 U g^{-1} FW min^{-1}) of 'Sahand'/ Badamkohi plants at 120 mM NaCl which was significantly higher than the activity of this enzyme in the 'Sahand'/Arjan plants (Table 4).

Table 4 - Effects of sodium chloride on the shoot and root enzymes activities (SOD, POX and CAT) in micrografting combinations of 'Sahand'/ Badamkohi and 'Sahand'/Arjan

Micrografting combination	NaCl (mM)	SOD shoot U g^{-1} FW min^{-1}	SOD root U g^{-1} FW min^{-1}	POX U g^{-1} FW min^{-1}shoot	POX root U g^{-1} FW min^{-1}	CAT shoot U g^{-1} FW min^{-1}	CAT root U g^{-1} FW min^{-1}
'Sahand'/Badam kohi	0	90.10 e	76.60 c	31.62 ed	51.60 fe	40.60 cd	32.50 ef
	40	92.90 d	77.30 bc	48.81 bc	60.30 cde	41.80 cd	44.90 bc
	80	96.10 c	80.00 bc	50.70 b	69.80 bc	46.90 bc	48.70 b
	120	101.27 a	103.30 a	76.99 a	95.00 a	63.70 a	59.70 a
'Sahand'/Arjan	0	87.60 f	72.10 c	23.40 e	48.10 f	39.60 d	31.30 f
	40	92.20 d	75.20 c	36.60 cd	58.80 de	41.10 cd	38.60 de
	80	95.20 c	78.00 c	45.60 bc	67.20 bcd	43.60 bcd	42.40 cd
	120	97.60 b	85.60 b	47.80 bc	73.50 b	50.50 b	46.20 bc

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

4. Discussion and Conclusions

Performance of grafted plants compared to non-grafted or self-grafted plants under a stressful condition is often dependent on the rootstock's root system characteristics. A vigorous root system could be the most important criterion for increasing salt tolerance (Balliu *et al*., 2007). In this research, Badamkohi showed the highest root length of 263 mm that was significantly higher than Arjan (122 mm). The rootstock's root systems architecture specified by root length and density, root hairs and root surface area play a critical role in ion and water uptake, thus determining salt tolerance of grafted plants (Colla *et al*., 2010). A vigorous root system, for instance, produced more cytokinins and transported water to the shoot system by xylem sap, which positively affected plant growth and crop yield (Oztekin and Tuzel, 2011). Furthermore, hydraulic conductivity of the roots may control plant growth by manipulation of the water supply to epigeous plant parts (Gregory *et al*., 2013).

The most immediate effect of salinity on plants is the inhibition of root and shoot development. Due to the imbalance of water potential between the apoplast and simplast, the osmotic potential decreases and the absorption of water is hampered. Ultimately, this reduces plant growth by closing the stomata and weakening photosynthesis (Dustgeer *et al*., 2021). Fresh and dry weight of plants decrease with increasing salinity, which is usually due to ion toxicity and water stress (Arif *et al.*, 2020; Corell *et al*., 2020). Salinity has the effects on metabolic activity and reduces the division of new cells and disrupt cellular processes, thus reduces plant growth compared to normal conditions (Carillo *et al*., 2019). In this study, as mentioned before, the root system of Badamkohi was stronger than Arjan. However, in high level of salinity, the shoot length of the same scion ('Sahand') on Badamkohi was greater than on Arjan. It seems that the Arjan rootstock has a dwarfing effect on the scion. On the other hand, at all salinity levels, the fresh and dry weight of the scion on Badamkohi was more pronounced than on Arjan. This means that Badamkohi supports scion growth better than Arjan.

It has been also reported some rootstocks are more capable of inducing tolerance to the scion against salt stress (Zrig *et al*., 2016; Aras and Eşitken, 2018). In this regard, adding sodium chloride to the growth medium of two graft combinations ('Sahand'/Badamkohi and 'Sahand'/Arjan) led to a decrease in scion growth, however this growth reduction of 'Sahand' subjected to increasing concentrations of NaCl was more acute when the rootstock was Arjan. This result is in consistence with the results of sweet almond grafted on different rootstocks (Zrig *et al.,* 2016).

Sodium ion (Na^+) is toxic to cellular metabolism and affects the activity of some enzymes, and high concentrations of Na^+ causes ion imbalance (Roy *et al.,* 2014).

The ability of almond rootstocks varies in absorbing or transferring sodium to the scion and there is a very close relationship between tolerance to salinity and the amount of sodium transferred to the leaves (Mickelbart and Arpaia, 2002). In the present research, Na^+ concentration in the aerial parts was lower in 'Sahand'/ Badamkohi compared to 'Sahand'/Arjan. Such a prohibiting mechanism may explain, the higher shoot length and biomass of 'Sahand'/Badamkohithat observed in our study. A higher Na^+ concentration in the environment of root can depress K^+/Na^+ ratios in the plant, thereby, the plant becomes susceptible to specific ion injury as well as to nutritional disorders which may affect growth and yield. The exclusion of Cl^- from shoots is related to the ability of cell membranes to restrict the movement of Cl^- through the root to vascular tissue and the degree of Cl^- accumulation in the roots (Walker and Douglas, 1983). In this experiment, Badamkohi rootstock was able to slow the accumulation of Cl^- in the leaves. Similar results have been reported by García-Sánchez *et al*. (2002) in which the Cleopatra rootstock reduced the accumulation of Cl^- in the scion compared to the Carrizo rootstock.

The finding of this experiment showed that Badamkohi rootstock hold up more the chloroplast integrity of 'Sahand' than Arjan rootstock. Chlorophyll depletion in salt stress can be linked to factors such as structural damage of chloroplasts due to the formation of reactive oxygen species and photo oxidation of chlorophyll (Taïbi *et al.,* 2016), the destruction of chlorophyll synthesis precursors, the inhibition of biosynthesis of new chlorophylls, and hormone disorders (Sabzmeydani *et al.,* 2020). This reduction could also be due to the increase in the activity of the enzyme chlorophyllase or to the instability of the protein pigment complexes by the ions (Saha *et al.,* 2010). Surendar *et al.* (2013) reported a decrease in chlorophyll content under stress was caused to the destruction of the chloroplast mem-

brane with increasing phosphatase activity, which is located on the membrane.

Plasma membranes are the primary site of ion-specific salt injury. Undesirable performance of the cell's metabolism during periods of abiotic stress leads to the stimulation of reactive oxygen species which would damage the cell membrane and increase electrolyte leakage. Therefore, electrolyte leakage from plasma membranes is reported as one of the most important selection criteria for identification of salt-tolerant plants (Besma and Denden, 2012). In this study, especially at higher levels of salinity, the 'Sahand' grafted on Arjan experienced a greater damage to the cell membrane of its leaves, than grafted on Badamkohi. This indicates Badamkohi rootstock's ability to maintain the integrity of scion cell membrane in salt stress conditions. In accordance with our finding, Colla *et al.* (2012) reported that in cucumber grafted plants, the amount of ion leakage in salinity stress is reduced compared to non-grafted plants and rootstock helps the maintenance of membrane function.

In this study, the grafting combination of 'Sahand'/Badamkohi accumulated more proline and glycine betaine in the root and shoot, compared to the same scion grafted on Arjan. Proline accumulation in salinity condition can play a role in stress tolerance mechanismsby stabilizing proteins at high ionic concentrations (Krasensky and Jonak, 2012). It also reduces damages caused by salinity via the preservation of water in cells and by diluting salts in the plant (Gulen *et al*., 2018). Proline has essential functions by osmoregulation, reducing the undesirable effects of ROSs under salinity stress.Therefore, higher proline accumulation can induce higher tolerance against salinity by the plant (Akbari *et al.,* 2018). The proline content of micrografting combination significantly increased in response to an increase in the salinity level, in this regards 'Sahand'/Badamkohi micrograft combination was more prominent than 'Sahand'/Arjan. Our finding is in agreement with other reports on *in vitro* salt tolerance of pistachio (Raoufi *et al.*, 2020) and fig (Abdoli Nejad and Shekafandeh, 2014). Glycine betaine was also found to play significant roles in enhancing salt tolerance (Wei *et al*., 2017). It can maintain the osmotic regulation, improve the production, nutrients and water absorption, thereby photosynthetic proteins are produced and membrane peroxidation is reduced (Dustgeer *et al.,* 2021). In this research, the grafting combination of 'Sahand'/Badamkohi accumulated more glycine betaine in the root and shoot, compared to the grafting combination of 'Sahand'/Arjan, that means Badamkohi protects cell osmotic pressure, enhancing cell membrane integrity as well as maintaining photosynthetic apparatus (Niazian *et al*., 2021).

As mentioned above, with increasing salinity levels, Badamkohi rootstock enhanced SOD, POX and CAT activity in 'Sahand' Scion more than Arjan rootstock. SOD plays a major role in ROS scavenging in plants and is considered as the first line of defense against the toxic effects of elevated ROS (Hou *et al.,* 2019). SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2 (Feng *et al.,* 2015). Increasing the level of superoxide dismutase activity is important for protecting chloroplasts and mitochondria from the stress of reactive oxygen species. In fact, under stress conditions, the chloroplast is where the majority of active oxygen species are produced and where is caused the highest degree of damage (Sofo *et al.,* 2005; Kuşvuran *et al.,* 2016).

According to the results of the present study, salinity stress caused the SOD activity to increase significantly in both micrograft combinations ('Sahand'/Badamkohi and 'Sahand'/Arjan). Nonetheless, Badamkohi rootstock enhanced the SOD activity in 'Sahand' more than Arjan rootstock. Enhanced activities of SOD enzyme usually reflect defensive responses to cellular damage induced by higher NaCl concentrations in the culture medium (Akbari *et al.,* 2018; Kuşvuran *et al.,* 2021).

The enzyme hydrogen peroxidase (POX) also reduces oxidative stressby protecting the metabolic enhancers that sustain cell and plant survival (Aliakbarkhani *et al*, 2017). The results of this study showed that by increasing salinity levels in the growth medium of the two grafted combinations, there was an increase in the activity of peroxidase enzyme and this increment was more obvious in 'Sahand' grafted on Badamkohi rootstock. According to Fayek *et al*. (2018), the activity of POX enzyme changes with different scions graftedon different rootstocks. An increase in POX activity in grafted plants could be an indicator that the grafting process can rapidly induce a higher capacity to breakdown H_2O_2 in plant cells (Elsheery *et al*., 2020).

Catalase is one of the most important enzymes that can inhibit ROS activity. It converts hydrogen peroxide to water and oxygen in the mitochondria, peroxisomes and cytosol (Acosta-Motos *et al.,* 2017). Based on the results of this study, with increasing salinity levels, the activity of catalase increased in

both grafted combinations showing that tolerance to salinity corresponds with an increase in catalase enzyme of the plants, although Badamkohi rootstock was more tolerant than Arjan. The greater availability of CAT can enable the plant defense mechanism to increase the capability of eliminating reactive oxygen species (Madadkhah *et al.,* 2018). Our finding corresponds with those reported previously in pistachio (Akbari *et al.,* 2018) and cherry (Chatzissavvidis *et al.,* 2008).

In all levels of salinity 'Sahand'as scion grafted on Badamkohi had more shoot length, fresh and dry weight than grafted on Arjan. Badamkohi restricted the absorption of Na^+ and Cl^- ions from the root medium and reduced their transportation to aerial parts. The 'Sahand' leaf chlorophyll depletion and EL were higher on Arjan rootstock than on Badamkohi in all level of salinity condition. Badamkohi protected cell osmotic pressure, enhancing cell membrane integrity than Arjan by inducing more proline and GB as osmoprotectants in the shoots. Badamkohi rootstock also enhanced the activities of antioxidant enzymes in 'Sahand' more than Arjan rootstock which reflect defensive responses to cellular damage induced by reactive oxygene species in higher NaCl concentrations in the culture medium. In conclusion, Badamkohi could be a more suitable rootstock than Arjan for 'Sahand' scion under salinity conditions.

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