Scientia Horticulturae 237 (2018) 296–302

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Alleviating salt stress in almond rootstocks using of humic acid

Elnaz Hatami*, Ali Akbar Shokouhian, Ali Reza Ghanbari, Lotf Ali Naseri

Department of Horticulture, Faculty of Agriculture, University of Mohaghegh-E-Ardabili, Ardabil, Iran

ARTICLE INFO

Keywords:
Catalase
Electrolyte leakage
Humic acid
Leaf soluble protein
Peroxidase
Sodium chloride

ABSTRACT

Salinity is one of the most important factors that reduces the growth and production of plants in arid and semi-arid regions. In our study, a pot experiment has been conducted in a factorial based on a complete randomized block design with 3 replications at the Faculty of Agriculture, University of Urmia, Iran, in 2016. In this study after applying the humic acid for two-months in four levels: (A0): control (A1): 2.5, (A2): 5 and (A3): 7 kg ha^{-1}, salinity was applied at four levels: (B0): control, (B1): 60, (B2): 120 and (B3): 180 mM NaCl, for two months on 3 almond rootstocks: (C0): Sangi almond seedling, (C1): GF677 and (C2): GN15. Results showed that increasing the salinity increased the leaf soluble proteins synthesis and CAT and POX activity up to 60 mM NaCl, but reduced them at higher levels. Also, electrolyte leakage increased from control to 180 mM NaCl. Using humic acid, by contributing to the absorption of essential nutrients such as N and K, increases the soluble proteins and enzymes synthesis more, leading to reduction in the electrolyte leakage. So, the highest and lowest protein and enzymes synthesis were related to 60 mM NaCL, 7 kg ha^{-1} humic acid and 180 mM NaCL, control treatment of HA, respectively. The highest and lowest electrolyte leakage was related to 180 mM NaCL, 2.5 kg ha^{-1} humic acid and control treatment of salinity, 7 kg ha^{-1} humic acid. Finally, GF677 with the highest protein and enzyme synthesis and the lowest electrolyte leakage was better and Sangi seedling and GN15 were placed in the next positions, respectively.

1. Introduction

Almond (*Prunus dulcis* Mill.) is one of the oldest and most important dry fruits in the world and belongs to the *Rosacea* family, and its main homeland is attributed to the Middle East, especially Iran (Ladizinsky, 1999). According to the FAO (2008), Iran by producing 110,000 tons has the 5 t h rank among the top 5 almond producers in the world, after the United States, Spain, Syria and Italy. About 10 million hectares of agricultural lands under irrigation face the problem of salinity annually, which this problem limits the yield of 40 million hectares of these lands (Manaf and Zayed, 2015). Most of the stone fruit trees, including almonds, are susceptible to salinity stress, and their yield decreases in salinity higher than 1.5 ds m^{-1} (Ottman and Byrne, 1988). Synthesizing the proteins with compatibility properties and antioxidant effects (Ashraf and Harris, 2004) and increasing the hydrolytic enzymes activity such as SOD, APX, CAT, POX, etc. (Sorkheh et al., 2012) are the main plants mechanism for coping with osmotic stress. Measuring the amount of electrolyte leakage as a simple, repeatable, fast and inexpensive method is a suitable physiological indicator for assessing the membrane damage caused by environmental stresses (Bajji et al., 2001; Al Busaidi and Farag, 2015). Since the salinity tolerance in glycophyte plants depends on the roots ability to prevent the toxic ions transfer to the aerial parts, the role of rootstocks in identifying the trees behavior is an important issue (Grattan and Grieve, 1999). The use of pronus inter specific hybrids such as GF677, GF557, Titan, Hansen, GN15 or Garnem (*P.amygdalus* cv. Garfi and *P.persica* cv. Nemared), Cadaman (*P.persica* * *P.daviana*) and etc. is very useful in salinity and drought tolerance (Leifert and Casselles, 2001; Felipe, 2009; Dejampour et al., 2012). However, botanists need faster and more complete methods to cope with intense environmental stresses (Parvaiz and Satyawati, 2008). Using humic substances is one of these options that increases plant resistance to environmental stresses through increasing metabolism (Banks and Percival, 2014).

Humic substances which are the components of humus contain a wide variety of molecular components such as polysaccharides, fatty acids, polypeptides, lignins, etc- and they are brownish black in color. They play a vital role in soil fertility and plant nutrition such as, by increasing the production of adenosine triphosphate (ATP) within plant cells and increasing the permeability, resulting in an increased transport of various mineral nutrients to sites of metabolic need (Russo and Berlyn, 1990; Cimrin et al., 2010; Turan et al., 2011), increasing the chlorophyll content by increasing of Fe^{+} absorption, specialty in alkaline soils (Delfine et al., 2005), increasing the photosynthesis by increasing of Rubisco enzyme activity (Russo and Berlyn, 1990; Delfine

* Corresponding author.
E-mail address: e.hatami2010@gmail.com (E. Hatami).

https://doi.org/10.1016/j.scienta.2018.03.034
Received 24 December 2017; Received in revised form 9 March 2018; Accepted 16 March 2018

et al., 2005; Cimrin et al., 2010), increasing the enzyme synthesis and an increase in the protein contents of the leaves by increasing the N absorption (MacCarthy et al., 1990; Russo and Berlyn, 1990; Delfine et al., 2005), degradation or inactivation of toxic substances due to the chelation exchange reaction (MacCarthy et al., 1990), and changing the soil physical, chemical, and biological structure (Russo and Berlyn, 1990; Parandian and Samavat, 2012). Application of humic substances to saline soils results in reduction in the concentration of sodium salts which is not correlated with a leaching of the salt, yet it may be correlated with improving the root growth and accumulation of Na^+ in the root and less Na^+ transduction to plant aerial parts (Cimrin et al., 2010). Humic substances can be subdivided into three major fractions: (1) Humin, (2) Humic acids (HAs), and (3) Fulvic acids (FAs) (Mosley and Mosley, 1998), and 65–70% of humic substances are formed of humic acid and fulvic acid (Parandian and Samavat, 2012). The size of fulvic acid particles are smaller than humic acids, and because of this they can readily enter plant roots, stems, and leaves. Also, by binding to water molecules, they can reduce the amount of evapotranspiration and transpiration, and help to maintain water inside the plant (Bronick and Lai, 2005). Humic acids comprise a mixture of aliphatic (carbon chains) and aromatic (carbon rings) organic acids which are soluble in water under alkaline conditions. On average, 35% of the humic acid (HA) molecules are aromatic, while the remaining components are in the form of aliphatic molecules (Asgharzade and Babaeian, 2012). The biological activity of humic acid is related to its chemical structure and active groups (Russo and Berlyn, 1990). Humic acid as a macromolecule and one of the main branches of humic substances (Canellas et al., 2017) increases soil water-holding capacity via high water absorption groups (Fahramand et al., 2014). This substance was recognized as a hormone like in the early 1900s (Dell'Agnola and Nardi, 1987). Different mineral elements are bound to humic acid molecules, as a result, humic acids function as an important ion exchange and metal complexing (chelating) systems (Asgharzade and Babaeian, 2012) which release these elements at time of need of the plant (Russo and Berlyn, 1990).

Using humic acid reduced the petal membranes peroxidation of *Polianthes tuberose* and increased the total protein content by increasing the elements absorption, especially Ca and N (Amani Beni et al., 2013). Humic acid increased the proteins amount in banana tissue culture media, consequently improving root development and POX activity. This was related to activity of humic acid, which was the hormone like (Fernandez et al., 2013). Humic acid caused the plant to absorb different nutrient elements, especially K^+ by increasing the soil CEC in kiwi trees under salinity. K^+ as a main element for enzymes synthesis increases the water and nutrition absorption and improves photosynthesis by helping the stomata to open more (Mahmoudi et al., 2014). Humic acid reduced the sodium adsorption ratio (SAR) in pistachio cv. Akbari, under salinity (Javanshah and Aminian-Nasab, 2016).

This report presents the beneficial effects of humic acid on the plants salinity resistance and points out whether the soil application of this biological treatment can play an important role in reducing the effects of salinity stress on almond rootstocks. The measured factors included leaf soluble proteins, electrolyte leakage and catalase and peroxidase enzymes activity.

2. Materials and methods

2.1. Plant material and treatments

This research was carried out in the research farm of Faculty of Agriculture, University of Urmia, Iran, in 2016. Almond rootstocks, two years old, with id tags, healthy, and with the same growth ability, were provided by the seedling production institute of "Rooyan Pajoohesh-e-Azarbaijan", located on Urmia-Tabriz road. In March 2015, they were removed from polyethylene pots and transformed into 7 kg plastic pots containing equal amounts of surface soil and peat moss and each pot was considered as a repeat. The used soil texture was sandy loamy with pH = 6.8, EC = 1.63 ds m^{-1}, 379 ppm of K, 71 ppm of P, 0.17% of total N and 1.41% of C. The experimental treatments were concluded: Humic Acid (HA) in four levels: (A0): control (A1): 2.5, (A2): 5 and (A3): 7 kg ha^{-1} and salinity in four levels: (B0): control, (B1): 60, (B2): 120 and (B3): 180 mM NaCl, that were applied on almond rootstocks including: (C0): Sangi almond seedling, (C1): GF677 and (C2): GN15. From the 5th of May and for 2 months, humic acid (Sigma Aldrich, USA), prepared from Sahab Shimi Pasargad Co., has been applied once a week with irrigation water and soil application. The pots were irrigated with water containing various NaCl levels every 2 days, from the 5th of July and for 60 days. Some solution was removed from the bottom of the pot at each irrigation with saline water. The plant roots were thoroughly washed with ordinary water to minimize EC and pH changes due to salt accumulation in the planting bed each week. At the beginning of the experiment, in order to prevent the occurrence of sudden stress on plants, salinity stress increased by increasing the amount of 25 mM NaCl daily (Fisarakis et al., 2001).

2.2. Measurements

2.2.1. Leaf soluble proteins

The Bradford method (1976) was used to assay the amount of leaf soluble proteins. For this purpose, 0.5 g of fresh leaf was homogenized in 25.6 ml of extraction buffer solution (121.4 g Tris [1,3-dichloro-2-propyl-phosphate] dissolved in 1 L of distilled water, and pH set on 6.8) and kept for 24 h. After this time, the leaves were thoroughly chopped, and homogenate was centrifuged at 6000 rpm for 20 min (PRP JENUS TDLSO-2B Centrifuge). 0.1 ml of supernatant obtained was separated into another test tube and 5 ml of bio red agent was added to it and the absorbance was measured at 595 nm with a spectrophotometer (Unico 2100 UV). To determine the leaf soluble proteins of almond rootstocks, a standard curve was made using pure bovine serum albumin (BSA) and was expressed in units of μg gr FW^{-1}.

2.2.2. Electrolyte leakage

Salinity tolerance of almond rootstocks was assayed by measuring leakage of electrolytes of the youngest mature leaf membranes as described by Zhao et al. (1992). Ten leaf discs (0.5 cm diameter) from almonds were placed in individual glass tubes contains distilled water, and the electrical conductivity was measured after a short time using conductance meter (MARTINI EC/TDS/Temp wp, Italy) at 25 °C (EC_0). The tubes were incubated in a refrigerator at 4 °C, and the electrical conductivity of the samples was re-measured after 24 h (EC_1). The samples were placed in an autoclave for 15 min at 120 °C, and their electrical conductivity was recorded after cooling at room temperature (EC_2). Finally, the electrolyte leakage was measured via statement below:

$$RP(\%)=[(EC_1-EC_0)/(EC_2-EC_0)]\times 100 \quad (1)$$

2.2.3. Enzymes activity

To measure enzymes activity, leaf protein extract was made by 0.8 M potassium chloride and 0.5 M potassium phosphate buffer with pH = 7. 0.1 g of fresh weight tissue with 10 ml of the solution was crushed, and the slurry was centrifuged at 4000 rpm for 20 min at 4 °C. The supernatant, which contained enzyme activity, was used as the enzyme source for experiment.

The POX activity was assayed as Mac-Adam et al. (1992). The substrate mixture contained 100 ml of 50 mM potassium phosphate (pH = 7), 90 μL of 1% guaiacol as the substrate, and 90 μl of H_2O_2 0.3%, as the hydrogen donor. The reaction cuvette contained 2.87 ml substrate mixture, 20 μl of enzyme extract and 0.03 ml treatment solution, in total volume of 3 ml. Finally, this enzyme activity was determined at 25 °C with a spectrophotometer at 470 nm.

The CAT activity was assayed as Chance and Maehly (1995).

Reaction mixture consisted of 0.75 ml of 100 mM phosphate buffer (pH = 7), 1.0 ml of the enzyme extract, and 750 µl of 0.1 M H_2O_2. Changes in absorbance of the reaction solution at 240 nm were sequentially read every 15 s for 1 min with a spectrophotometer at 25 °C. The disappearance of H_2O_2 was detected by titrating the reaction mixture against 0.1 N potassium permanganate solution. The reaction mixture without enzymes was treated as blank. One unit of CAT activity was defined as the amount of enzyme which breaks down 1 mmol of H_2O_2 per minute.

2.3. Experimental design and data analysis

Our experiment was arranged in a factorial based on a complete randomized block design with 3 replications for each treatment. The obtained data was analyzed using ANOVA to determine the effect of salinity stress and humic substances (HS) on the almond responses. Means comparison was conducted using the Duncan's multi range test at the significant level of $\alpha < 5\%$. The data was performed by Statistical Analysis System (SAS) version of 9.2.

3. Results

3.1. Leaf soluble proteins

According to the results of analysis of variance (Table 1), the effect of salt stress, rootstock and their interactions on the leaf soluble proteins amount was significant at the 1% probability level. The effect of HA, interactions of salt stress and HA, rootstock and HA and triple effects of salinity, rootstock and HA on the amount of leaf soluble proteins was significant at the significant level of $\alpha < 1\%$, also. The amount of leaf soluble proteins increased with increasing salinity stress, up to 60 mM of NaCl and then decreased. The leaf soluble proteins content increased by increasing levels of HA treatment (Table 2).

Interactions between salinity and HA on the leaf soluble proteins showed that 60 mM NaCl and 7 kg ha^{-1} HA, A3B1, with 2.06 µg g^{-1} FW^{-1} and 180 mM NaCl and control treatment of HA, A0B3, with 0.44 µg g^{-1} FW^{-1} had the highest and lowest amount, respectively. The interaction effects of salinity and rootstock showed that 60 mM NaCl and GF677, B1C1, with 1.89 µg g^{-1} FW^{-1} and 180 mM NaCl and GN15, B3C2, with 0.70 µg g^{-1} FW^{-1} were placed in the highest and lowest statistical groups, respectively. The interaction of the rootstock and HA on the leaf soluble proteins showed that the highest and lowest amount of this factor was related to the GF677 and 7 kg ha^{-1} of HA, C1A3, with 2.16 µg g^{-1} FW^{-1} and the GN15 and control treatment of HA, C2A0, with 0.67 µg g^{-1} FW^{-1} (Table 3).

Triple effects of Humic acid, salinity and rootstock showed that 60 mM NaCl, 7 kg ha^{-1} HA and GF677 rootstock, C1A3B1, with 2.63 µg g^{-1} FW^{-1} and 180 mM NaCl, control treatment of HA and GN15, C2A0B3, with 0.28 µg g^{-1} FW^{-1} were the highest and lowest levels, respectively (Fig. 1).

Table 2
The means comparison of almond rootstocks, salinity and humic acid (HA) on the leaf soluble proteins, electrolyte leakage and antioxidant enzymes.

Treatments	Leaf soluble proteins (µg gr FW^{-1})	Electrolyte leakage (%)	Catalase (U mg^{-1} protein)	Peroxidase (U mg^{-1} protein)
HA				
A0	0.74d	10.65c	0.00114d	0.00114d
A1	0.95c	11.07a	0.00144c	0.00131c
A2	1.33b	10.81b	0.00166b	0.00165b
A3	1.69a	10.50d	0.00182a	0.00195a
Salinity				
B0	1.14c	4.07d	0.00126d	0.00114d
B1	1.52a	5.33c	0.00178a	0.00184a
B2	1.17b	12.29b	0.00160b	0.00162b
B3	0.87d	21.34a	0.00143c	0.00146c
Rootstock				
C0	1.12b	10.09b	0.00131b	0.00129b
C1	1.44a	7.4 3c	0.00210a	0.00220a
C2	0.97c	14.75a	0.00113c	0.00104c

Similar letters to the averages indicate that there is no significant difference between them at the 1% probability level (Duncan test).
A (A0, A1, A2, A3): Humic acid (HA) levels (0, 2.5, 5 and 7 kg ha^{-1}).
B (B0, B1, B2, B3): Salinity stress levels (0, 60, 120 and 180 Mm NaCl).
C (C0, C1, C2): Root stocks (Sangi almond seedling, GF677 and GN15).

3.2. Electrolyte leakage

Based on the results of analysis of variance (Table 1), salinity stress had a significant effect on electrolyte leakage on all studied 3 almond rootstocks at the 1% probability level. The effect of rootstock and interaction between salinity and rootstock, the effect of HA and interactions between salinity and HA and between rootstock and HA and triple effects of salinity, rootstock and HA on this factor was significant at the 1% probability level. According to the results of the means comparison, salinity treatments were in different statistical groups. The highest and lowest amount of electrolyte leakage was related to 180 mM NaCl and control treatment of salinity, respectively. Increasing the concentration of humic acid in the GF677 decreased the electrolyte leakage, but in the Sangi seedling up to 2.5 kg ha^{-1}, it further increased the electrolyte leakage and then decreased. The level of electrolyte leakage in the GN15 also increased, even with the increase in the level of humic acid, and humic acid did not play a role in reducing the electrolyte leakage in this rootstock (Table 2). Investigating the interactions between salinity and HA on the electrolyte leakage showed that 180 mM NaCl and 2.5 kg ha^{-1} of HA, A1B3, with 21.69%, and control treatments of

Table 1
Analysis of variance of simple, interaction and triple effects of almond rootstocks, salinity and Humic acid (HA) on the leaf soluble proteins, electrolyte leakage and antioxidant enzymes.

S.O.V	df	Mean of squares			
		Leaf soluble proteins	Electrolyte leakage	Catalase	Peroxidase
Block	2	0.0039^{ns}	0.00097^{ns}	0.00000013^{ns}	0.00000017^{ns}
Rootstock	2	5.48**	659.22842**	0.00002579**	0.00001803**
HA	3	19.20**	2.10241**	0.00000949**	0.00000464**
Salinity	3	7.74**	2262.33228**	0.00000549**	0.00000312**
Rootstock*HA	6	1.64**	4.42403**	0.00000059**	0.00000027**
Rootstock*Salinity	6	0.31**	154.20142**	0.00000116**	0.00000135**
Salinity*HA	9	0.14**	5.93498**	0.00000046**	0.00000009**
Rootstock*HA*Salinity	18	0.30**	3.74074**	0.00000024**	0.00000003**
Error	94	0.0022	0.00079	0.00000002	0.00000001
CV (%)	–	4.02	1.26	2.33	2.26

** and $^{n.s}$: respectively significant at the 1% level probability and non-significant.

Table 3
The comparison of average of interaction effects of salinity, Humic acid (HA) and almond rootstocks on the leaf soluble proteins, electrolyte leakage and antioxidant enzymes.

Treatments	Leaf soluble proteins (μg gr FW^{-1})	Electrolyte leakage (%)	Catalase (U mg^{-1} protein)	Peroxidase (U mg^{-1} protein)
A0*B0	0.74hi	4.44l	0.00078j	0.00073j
A0*B1	1.04fg	5.68i	0.00153f	0.00142fg
A0*B2	0.73hi	12.92e	0.00123h	0.00130g
A0*B3	0.44j	19.56d	0.00102i	0.00112h
A1*B0	0.95g	4.28m	0.00117h	0.00078ij
A1*B1	1.31e	5.60i	0.00170de	0.00166de
A1*B2	0.97g	12.70f	0.00152f	0.00146fg
A1*B3	0.59ij	21.69a	0.00135gh	0.00133g
A2*B0	1.28e	4.22m	0.00152f	0.00129g
A2*B1	1.69bc	5.12j	0.00186cd	0.00200b
A2*B2	1.28e	11.93g	0.00169de	0.00173cd
A2*B3	1.05fg	21.06c	0.00159e	0.00158e
A3*B0	1.60c	3.35n	0.00156ef	0.00175cd
A3*B1	2.06a	4.91k	0.00204ab	0.00226a
A3*B2	1.71bc	11.60h	0.00195bc	0.00197b
A3*B3	1.41de	21.15bc	0.00175d	0.00180c
B0*C0	1.08ef	4.32i	0.00113i	0.00118f
B0*C1	1.35cd	3.57j	0.00166de	0.00129ef
B0*C2	0.99fg	4.33i	0.00097j	0.00094i
B1*C0	1.45b	5.28h	0.00151e	0.00146de
B1*C1	1.89a	3.63j	0.00253a	0.00290a
B1*C2	1.23de	7.07g	0.00130gh	0.00115fg
B2*C0	1.13ef	11.69e	0.00137fg	0.00132ef
B2*C1	1.41bc	8.25f	0.00224b	0.00245b
B2*C2	0.97fg	16.92c	0.00118hi	0.00108gh
B3*C0	0.81gh	19.07b	0.00123hi	0.00120f
B3*C1	1.11ef	14.27d	0.00198c	0.00217c
B3*C2	0.70h	30.69a	0.00107ij	0.00100hi
C0*A0	0.73hi	10.03e	0.00094i	0.00101g
C0*A1	0.89fg	10.31d	0.00124gh	0.00116f
C0*A2	1.27cd	10.05e	0.00149ef	0.00138e
C0*A3	1.60b	9.97e	0.00157de	0.00161d
C1*A0	0.81gh	8.08f	0.00165d	0.00163d
C1*A1	1.17de	7.81g	0.00196c	0.00195c
C1*A2	1.62b	7.39h	0.00229b	0.00243b
C1*A3	2.16a	6.44i	0.00252a	0.00282a
C2*A0	0.67i	13.84c	0.00083j	0.00079h
C2*A1	0.81gh	14.99ab	0.00011h	0.00081h
C2*A2	1.09e	15.08a	0.00012gh	0.00111f
C2*A3	1.32cd	15.10a	0.00138fg	0.00142e

Similar letters to the averages indicate that there is no significant difference between them at the 1% probability level (Duncan test).
A (A0, A1, A2, A3): Humic acid (HA) levels (0, 2.5, 5 and 7 kg ha^{-1}).
B (B0, B1, B2, B3): Salinity stress levels (0, 60, 120 and 180 Mm NaCl).
C (C0, C1, C2): Root stocks (Sangi almond seedling, GF677 and GN15).

salinity and 7 kg ha^{-1} HA, A3B0, with 3.35%, had the highest and lowest amount of electrolyte leakage, respectively. The interaction effects between salinity and rootstock on this factor showed that 180 mM NaCl and GN15, B3C2, with 30.69%, and 60 mM NaCl and GF677, B1C1, with 3.57%, were placed in the highest and lowest statistical groups, respectively. The results of the interaction between the rootstock and HA on the electrolyte leakage rate showed that the GN15 and 2.5 kg ha^{-1} of HA, C2A1and 5 kg ha^{-1} of HS, C2A3, with 15.10%, and GF677 and 7 kg ha^{-1} of HA, C1A3, with 6.44%, had the highest and lowest electrolyte leakage (Table 3). The mean of triple effects showed that control treatment of salinity, 7 kg ha^{-1} of HA and GF677, C1A3B0, with 1.84% and 180 mM NaCl, 5 kg ha^{-1} of HA and GN15, C2A2B3, with 32.46% had the minimum and maximum of cell membrane leakage (Fig. 2).

3.3. Enzymes activity

Regarding the results of analysis of variance, simple and interaction between salinity and rootstock on the activity of CAT and POX enzymes was significant at the 1% probability level. Both enzymes activity increased by increasing salinity up to 60 mM, and then decreased (Table 1). Both enzymes activity, especially POX, in the GF677 was more than other rootstocks. The two enzymes activity have been increased by increasing HA concentration and the POX activity, especially at higher levels of humic acid, increased in GF677 (Table 2). Investigating the interaction between salt stress and HS showed that 60 mM NaCl and 7 kg ha^{-1} of HA, A3B1, and control treatment of salinity and HA, A0B0, had the highest and lowest amount of these two enzymes activity, respectively. The interaction between salinity and rootstock showed that 60 mM NaCl and GF677, B1C1 and control treatment of salinity and GN15, B0C2, were placed respectively in the highest and lowest statistical groups. The interaction between rootstock and HA showed that the highest and lowest enzymes activity was related to the GF677 and 7 kg ha^{-1} of HA, C1A3 and the GN15 and control treatment of HA, C2A0 (Table 3). Triple effects between salinity, Humic acid (HA) and rootstock on these characteristics showed that 60 mM NaCl, 7 kg ha^{-1} of HS and GF677, C1A3B1, and 180 mM NaCl, control treatment of HA, and GN15, C2A0B0, had the highest and lowest CAT and POX activity, respectively (Figs. 3 and 4).

According to the correlation table (Table 4), there was a significant positive correlation between the leaf soluble proteins and CAT and POX enzymes activity and between CAT and POX activity at the $P < 0.01$ probability level. There was a significant negative correlation between the leaf soluble proteins and electrolyte leakage at the $P < 0.01$, and there was a significant negative correlation between the electrolyte leakage and CAT and POX enzymes activity at the $P < 0.01$ and at the $P < 0.05$ probability levels, respectively.

4. Discussion

4.1. Leaf soluble proteins

One of the important mechanisms of plants to deal with osmotic stress conditions is the synthesizing of proteins with osmotic regulation task (Ashraf and Harris, 2004). The results of this study, based on the increasing of the amount of leaf soluble proteins in low levels of salinity

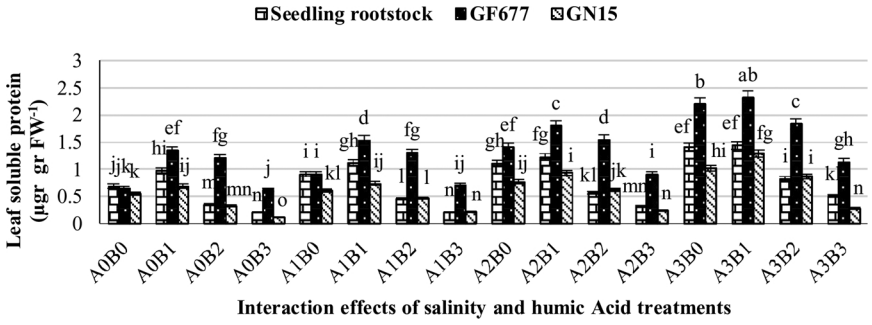

Fig. 1. The means comparison of triple effects of salinity, Humic acid (HA) and almond rootstocks on the leaf soluble proteins.

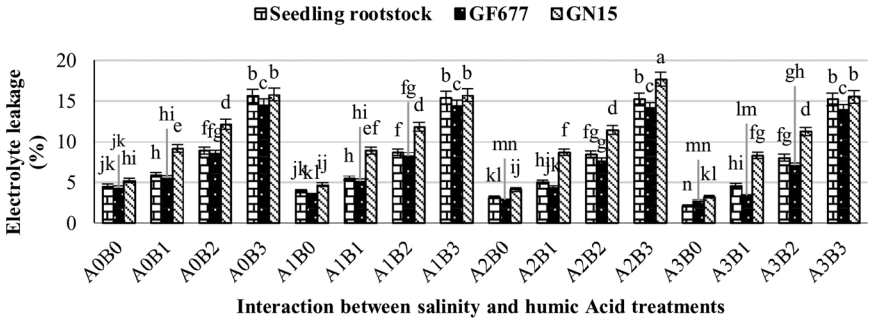

Fig. 2. The means comparison of triple effects of salinity, Humic acid (HA) and almond rootstocks on the electrolyte leakage.

and subsequent reduction in high level of salinity, was consistent with the results of Abdoli Nejad and Shekafandeh (2014) on "Shah Anjir" and "Anjir Shiraz" fig. In their research, the first increase in leaf soluble proteins was attributed to synthesizing the new stress proteins at low levels of salinity stress and subsequent reduction was attributed to photosynthesis reduction at higher levels of stress. Bano et al. (2014) also found that the content of leaf total soluble proteins in carrots under intense salt stress was reduced significantly. Reducing the amount of soluble proteins, especially at high levels of stress, in some researches was related to following factors: increasing the proline synthesis, protease enzyme activity, hydrolysis of the Robisco enzyme and other chloroplast and mitochondrial proteins and increasing of Na^+ concentration due to salinity and consequently, decreasing of K^+ concentration in the leaves, as an essential ion for protein synthesis (Blumwald et al., 2000). The results of this study contradicted the results of Zrig et al. (2015) in almond, Azevedo Neto et al. (2004) in maize and Brito et al (2003) in olive. They found that leaf soluble proteins increased under severe salinity condition especially in tolerant species, and by helping to better water absorbtion and leaf osmotic potential (Ψs), it provided photosynthesis and better plant growth. Ferreira-Silva et al. (2008) stated that increasing salinity did not significantly change the amount of soluble proteins in cashew leaves, but reduced the protein concentration in the root.

Humic acid increases plant growth and production by increasing protein synthesis (Russo and Berlyn, 1990; Delfine et al., 2005). The main mechanism of HA on improving plant growth may be related to the increasing of the effective nutrients absorption, such as N, P, K, etc. and decreasing the toxic elements absorption, such as Na and Cl (Aydin et al., 2012). The results of this study confirmed the results of Fernandez et al. (2013) research. They argued that humic acid application in the banana tissue culture medium caused a significant increase in the amount of soluble proteins due to hormone like effects. Balaket and Al-Himidawi (2014) have found that humic acid facilitates the nutrition transfer, especially K^+, which is an essential element in increasing the protein and enzymes synthesis in date palm cv. Berhee, under salinity. Increasing the amount of leaf soluble proteins in the pepper treated with humic acid is associated with B role on increasing the N uptake and consequent increasing of the protein synthesis (Manas et al., 2014).

4.2. Electrolyte leakage

The imbalance between the production and the removal of reactive oxygen species (ROS) due to environmental stresses such as salinity leads to membrane lipids peroxidation, and consequently cellular leakage through membranes and loss of water, damaging proteins, mutations in nucleic acids and ultimately cell death (Mittler, 2002). K^+ reduces the electrolyte leakage by attaching to the plasma membrane and maintaining its stability (Al Busaidi and Farag, 2015). Therefore, electrolyte leakage increases with reducing plant cells potassium (El-Sherkawy et al., 2017). The results of this study, based on the increasing of the electrolyte leakage in almond rootstocks leaf under salt stress especially GN15, were consistent with the results of Ferreira-Silva et al. (2008) in the cashew, Maia et al. (2010) in the pea, Aydin et al. (2012) and Talaat et al. (2015) in the bean and Wani et al. (2013) in the Cabbage. They noticed that the membrane damage and the electrolyte leakage percentage increased under salinity conditions especially in sensitive rootstocks.

Using humic acid reduced electrolyte leakage from leaf cell membranes of all 3 almond rootstocks and this result was consistent with the findings of Aydin et al. (2012) research on bean treated with humic acid, under salt stress. The role of humic acid on increasing the nutrient content of wheat leaf cells under salinity depends on the effect of these compounds on the increasing of the antioxidant enzymes and maintaining the stability and permeability of the cell membrane (AL-Erwy et al., 2016). Using the humic substances also reduced membrane damage and electrolyte leakage caused by ROS in barley, under salt stress and there was a significant negative correlation between the electrolyte leakage and the proline amount (El-Sherkawy et al., 2017).

4.3. Enzymes activity

ROS molecules are continuously produced at the lower level, mainly in chloroplasts, mitochondria, and peroxisomes, under favorable environmental conditions, and there is a balance between ROS production and elimination (Gill and Tuteja, 2010). Increasing the content of ROS

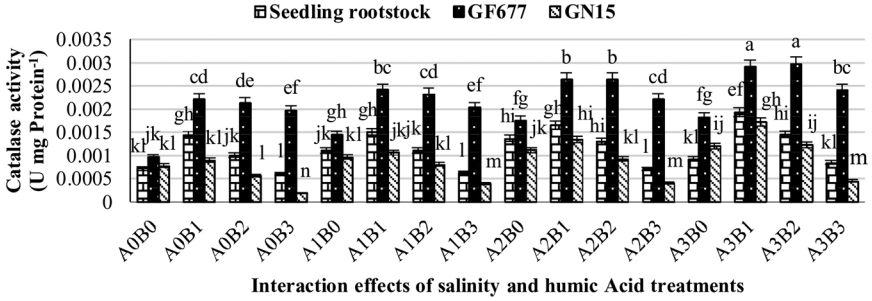

Fig. 3. The means comparison of triple effects of salinity stress, Humic acid (HA) and almond rootstocks on the CAT activity.

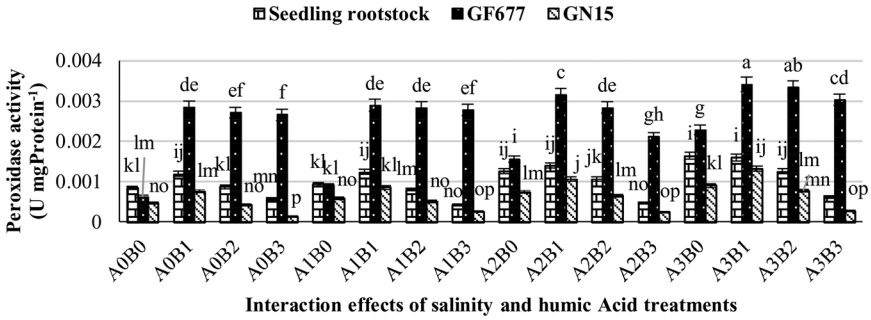

Fig. 4. The means comparison of triple effects of salinity, Humic acid (HA) and almond rootstocks on the POX activity.

Table 4
Correlation coefficients between leaf soluble proteins, electrolyte leakage and antioxidant enzymes activity.

	Leaf soluble proteins	Electrolyte leakage	Catalase	Peroxidase
Leaf soluble proteins	1.00	–	–	–
Electrolyte leakage	−0.45**	1.00	–	–
Catalase	+0.79**	−0.29**	1.00	–
Peroxidase	+0.76**	−0.26*	+0.95**	1.00

* and **: respectively significant at the 1% level and significant at the 5% level probability.

in some species can occur as a result of the closure of stomata and the reduction of carbon dioxide concentration within the chloroplasts (Cavalcanti et al., 2004). The enzymes play an important role in this condition (Wani et al., 2013) and convert the cells accumulated ROS to water and oxygen molecules (Mittler, 2002). The pattern of CAT and POX enzymes activity in each of the 3 studied almonds was similar in this study. Both enzymes activity increased with increasing the salinity level up to 60 mM, and then decreased. Although the CAT activity increased with increasing salinity, it was not enough to remove H_2O_2, and increasing the POX activity, especially in the GF677, was responsible for the greater stability of the membrane in this rootstock than the Sangi seedling and GN15. Therefore, there was a negative correlation between the enzymes activity, especially POX, and membrane lipids peroxidation and more increase in the POX activity at GF677 caused this enzyme to be as the main enzyme for the removing of peroxide toxicity under salinity. The results of this study was in line with the results of Cavalcanti et al. (2004) on chickpea. They found that the POX activity increased and the CAT and SOD activity did not chang, under salt stress. SOD, CAT and POX activity in grape cuttings increased under osmotic stress conditions (Pinheiro et al., 2004). Sahu et al. (2010) stated that osmolytes such as sucrose was the CAT regulating agent in response to salinity in rice and not NaCl. However, reducing the SOD and CAT activity and increasing the POX activity at chickpea caused this enzyme to be as the main enzyme for the removing of H_2O_2 toxicity under salinity (Maia et al., 2010). Highest increase in the antioxidant enzymes activity such as POX, SOD and CAT was due to citrus tolerated rootstocks, under salt stress (Balal et al., 2010). Bano et al. (2014) concluded that decreasing the CAT, POX and SOD activity increased the lipid peroxidation index in *Daucus carota* L. under salinity, and carrot was introduced as a susceptible plant and its nutritional value was reduced by salinity. By increasing salinity level on almond, CAT and POX activity decreased and increased, respectively (Zrig et al., 2015). Abdoli Nejad and Shekafandeh (2014) stated that all 3 enzymes activity increased in fig, under salt stress. Wani et al. (2013) concluded that SOD, POX and CAT activity increased by 96.8, 57 and 39.8% respectively, by increasing of salinity on cabbage.

Humic acid activates the several biochemical processes results in an increasing the enzyme such as CAT, POX, SOD synthesis and these enzymes activate the formation of both carrier and structural proteins. Fernandez et al. (2013) stated that the use of humic acid in banana tissue culture media increased the POX activity and reduced the H_2O_2 content. The antioxidant enzymes activity have been increased by humic acid treatment in the research of Balaket and Al-Himidawi (2014) on data palm, Mahmoudi et al. (2014) on Kiwi and El Ervy et al. (2016) on wheat, under salinity stress condition. This was due to the role of this compound on increasing the roots ability to absorb water and nutrients such as N, Fe, P and especially K, which is a major element in the synthesis of proteins and enzymes, and it was effective in coping with stress and also improving photosynthesis by helping the stomata to open more.

5. Conclusion

Increasing the salinity destructed the cell membrane and increased electrolyte leakage at all 3 studied almond rootstocks. The rootstocks under salinity stress for one through synthesizing nitrogen compounds such as leaf soluble proteins regulate osmotic potentials in their parts and for the other by increasing antioxidant enzyme activity such as CAT and POX stand against free radicals caused by stress, as a result, GF677 with synthesizing more the leaf soluble proteins and greater enzymatic activity, showed lower electrolyte leakage and more tolerance to salinity. The use of HA in these conditions, by helping to release essential nutrients such as K and N, increased the protein and antioxidant enzymes synthesis and, resulted in inhibiting the ROS molecules activity and reducing the electrolyte leakage specially at GF677. The highest electrolyte leakage, even with HA application, was related to the GN15. The most antioxidant enzymes activity, especially POX, the most leaf soluble proteins synthesis and the lowest electrolyte leakage was related to GF677 and 7 kg ha^{-1} HA. Therefore, since salinity is one of the increasing problems in the world and covers a large area of Iran, it is necessary to study and develop physiological techniques to increase plant resistance to stress. With the gradual replacement of chemical fertilizers, especially phosphorus and nitrogenous fertilizers with biological fertilizers, such as humic acid, it can be used for sustainable agriculture in order to reduce the cost of production and to help protect the environment.

Acknowledgments

The authors of this article would like to extend their gratitude and thanks to the compassionate cooperation of the distinguished officials of the seedlings production institute of “Royan-Pajoohesh-e-Azarbaijan” and the Biochemical Laboratory of “Iqbal” in supporting this research.

References

Abdoli Nejad, R., Shekafendeh, A., 2014. Salt stress-induced changes in leaf antioxidant activity, proline and protein content in Shah Anjir and Anjir Shiraz fig seedlings. Int.

J. Hortic. Sci. Technol. 1 (2), 121–129.
Al Busaidi, K.T.S., Farag, K.M., 2015. The use of electrolyte leakage procedure in assessing heat and salt tolerance of Ruzaiz date palm (*Pheonix dactylifera* L.) cultivar regenerated by tissue culture and offshoots and treatments to alleviate the stressful injury. J. Hortic. For. 7 (4), 104–111.
Al-Erwy, A.S., Al-Toukhy, A., Bafeel, S.O., 2016. Effect of chemical, organic and bio fertilizers on photosynthetic pigments, carbohydrates and minerals of wheat (*Triticum aestivum* L.) irrigated with sea water. Int. J. Adv. Res. Biol. Sci. 3 (2), 296–310.
Amani Beni, M., Hatamzadeh, A., Nikbakht, A., Ghasemnezhad, M., Zarchini, M., 2013. Improving physiological quality of cut tuberose (*Polianthes tuberose* cv. Single) flowers by continues treatment with humic acid and nano-silver particles. J. Ornam. Hortic. Plants 3 (3), 133–141.
Asgharzade, A., Babaeian, M., 2012. Investigation the effects of humic acid and acetic acid foliar application on yield and leaves nutrient content of grape (*Vitis vinifera*). Afr. J. Microbiol. Res. 6 (31), 6049–6054.
Ashraf, M., Harris, P.J.C., 2004. Potential biochemical indicators of salinity tolerance in plants. Plant Sci. 166 (1), 3–16.
Aydin, A., Kant, C., Turan, M., 2012. Humic acid application alleviate salinity stress of bean (*Phaseolus vulgaris* L.) plants decreasing membrane leakage. Afr. J. Agric. Res. 7 (7), 1073–1086.
Azevedo Neto, A.D., Prisco, J.T., Enas-Filho, J., de Lacerda, C.F., Silva, J.V., da Costa, P.H.A., Gomes-Filho, E., 2004. Effects of salt stress on plant growth, stomatal response and solute accumulation of different maize genotypes. Braz. J. Plant Physiol. 16 (1), 31–38.
Bajji, M., Kinet, J.M., Lutts, S., 2001. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul. 36 (1), 61–70.
Balaket, R.T.M.A., Al-Himidawi, S.A.M., 2014. Effect of humic acid and water quality on peroxidase and catalase enzymes activity in leaves of date palms cv. Barhee. Glob. J. Biosci. Biotechnol. 3 (4), 402–405.
Balal, R.M., Khan, M.M., Shahid, M., Mattson, N.S., Abbas, T., Ashfagh, M., Garsia Sanchez, F., Ghazanfer, U., Gimeno, V., Iqbal, Z., 2010. Comprative studies on the physiochemical, enzymatic and ionic modifications in salt-tolerant and salt-sensitive citrus rootstocks under NaCl stress. J. Am. Soc. Hortic. Sci. 137 (1), 86–95.
Banks, J.M., Percival, G.C., 2014. Failure of foliar-applied bio stimulants to enhance drought and salt tolerance in urban trees. Agric. Urban For. 40 (2), 78–83.
Bano, S., Ashraf, M., Akram, N.A., 2014. Salt stress regulates enzymatic and no enzymatic anti oxidative defense system in the edible part of carrot (*Daucus carota* L.). J. Plant Interact. 9 (1), 324–329.
Blumwald, E., Aharon, G.S., Apse, M.P., 2000. Sodium transport in plant cells. Biochim. Biophys. Acta 1465 (1), 140–151.
Bradford, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 7 (72), 248–254.
Brito, G., Costa, A., Fonceca, H.M.A.C., Santos, C.V., 2003. Response of *Olea europaea* ssp. Maderensis in vitro shoot exposed to osmotic stress. Sci. Hortic. 97 (1), 411–417.
Bronick, E.J., Lai, R., 2005. Soil structure and management: a review. Geoderma 124 (1), 3–22.
Canellas, L.P., Olivers, F.L., Okorokova-Facanha, A.L., Facanha, A.R., 2017. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence and plasma membrane H^+-ATPase activity in maize roots. Plant Physiol. 130 (1), 1951–1957.
Cavalcanti, E.R., Oliveria, J.T.A., Martins-Miranda, A.S., Viegas, R.A., Silveria, J.A.G., 2004. Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. New Phytol. 163 (3), 563–571.
Chance, B., Mahley, A.C., 1995. Assay of catalase and peroxidase. In: Colowick, S.P., Kaplan, N.D. (Eds.), Methods in Enzymology. Academic Press, New York (NY, pp. 764–791.
Cimrin, K.M., Turkmen, O., Turan, M., Tuncer, B., 2010. Phosphorus and humic acid application alleviate salinity stress of pepper seedling. Afr. J. Biotechnol. 9 (36), 5845–5851.
Dejampour, J., Aliasgharzad, N., Zeinalabedini, M., Niya, M.R., Hervan, E.M., 2012. Evaluation of salt tolerance in almond (*Prunus dulcis* L. Batsch) rootstocks. Afr. J. Biotechnol. 11 (56), 11907–11912.
Delfine, S.R., Tognetti, R., Desiderio, E., Alvino, A., 2005. Effect of foliar application of N and humic acids on growth and yield of durum wheat. Agron. Sustain. Dev. 25 (1), 183–191.
Dell'Agnola, G., Nardi, S., 1987. Hormone-like effect and enhanced nitrate uptake induced by de poly condensed humic fractions obtained from *Allobofora* and *A. caliginosa* faeces. Biol. Fertile Soils 4 (1), 115–118.
El-Sherkawy, M.S., El-Beshbeshy, T.R., Hassan, S.M., Mahmoud, E.K., Abdelkade, N.I., Al-Shal, R.M., Missaoui, A.M., 2017. Alleviating salt stress in barley by use of plant growth stimulants and potassium sulfate. J. Agric. Sci. 9 (4), 136–154.
Fahramand, M., Moradi, H., Noori, M., Sobhkhizi, A.R., Adibian, M., Abdollahi, S., Rigi, K., 2014. Influence of humic acid on increase yield of plants and soil properties. Int. J. Farm. Allied Sci. 3 (3), 339–341.
FAO STAT data. 2008. Last Accessed by Top 5 of Anything, August 2010.
Felipe, A.J., 2009. Felinem, Garnem and Monegro almond *peach hybrid rootstocks. Hort Sci. 44 (1), 196–197.
Fernandez, M.B.M., Cosio-Vargas, L.E., Montero, D.C., Garcia, A.C., Lopez, D.M., Alvarez, S.P., 2013. Potentiality of vermicomposting humic acids in banana in vitro micro propagation clone: Enano Guantanamero. J. Environ. Sci. Eng. 2 (1), 677–685.
Ferreira-Silva, S.L., Silveria, J.A.G., Voigt, E.L., Soares, L.S.P., Viegas, R.A., 2008. Changes in physiological indicators associated with salt tolerance in two contrasting cashew rootstocks. Braz. J. Plant Physiol. 20 (1), 51–59.
Fisarakis, I., Chartzoulakis, K., Stavrakas, D., 2001. Response of Sultana vines (*V. vinifera* L.) on six rootstocks to NaCl salinity exposure and recovery. Agric. Water Manage. 51 (1), 13–27.
Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biotechnol. 48 (12), 909–930.
Grattan, S.R., Grieve, C.M., 1999. Salinity-mineral nutrient relation in horticultural crops. Sci. Hortic. 78 (1), 127–157.
Javansha, A., Aminian Nsab, S., 2016. The effects of humic acid and calcium on morpho-physiological traits and mineral nutrient uptake of pistachio seedling under salinity stress. J. Nuts 7 (2), 125–135.
Ladizinsky, G., 1999. On the origin of almond. Genet. Resour. Crop Evol. 46 (2), 143–147.
Leifert, C., Casselles, A.C., 2001. Microbial hazards in plant tissue and cell cultures. In Vitro Cell. Dev. Biol. Plant 37 (2), 133–138.
Mac-Adam, J.W., Nelson, C.J., Sharp, R.G., 1992. Peroxidase activity in the leaf elongation zone of tall fescue. Plant Physiol. 99 (1), 872–878.
MacCarthy, P., Clapp, C.E., Malcom, R.L., Bloom, P.R., 1990. An Introduction to Soil Humic Substances. ASA-CSSA, Madison, WI.
Mahmoudi, M., Samavat, S., Mostafavi, M., Khalighi, A., Cherati, A., 2014. The effect of humic acid and proline on morphological properties of *Actinidia deliciosa* cv. Hayward under salinity. J. Appl. Sci. Agric. 9 (1), 261–267.
Maia, J.M., Voigt, E.L., Macedo, C.E.C., Ferreira-Silva, S.L., Silveria, J.A.G., 2010. Salt-induced changes in anti-oxidative enzyme activities in root tissues do not account for the differential salt tolerance of two cowpea cultivars. Braz. J. Plant Physiol. 22 (2), 113–122.
Manaf, H.H., Zayed, M.S., 2015. Productivity of cowpea as affected by salt stress in presence of endo mycorrhizae and *Pseudomonas fluorescens*. Ann. Agric. Sci. 60 (2), 219–226.
Manas, D., Bandopadhyay, P.K., Chakravarty, A., Pal, S., Bhattacharya, A., 2014. Effect of foliar application of humic acid, zinc and boron on biochemical changes related to productivity of pungent pepper. Afr. J. Plant Sci. 8 (6), 320–335.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7 (1), 405–410.
Mosley, R., Mosley, M., 1998. The effects of humates on remediation of hydrocarbon and salt contaminated soils. In: Paper Presented at 5th International Petroleum Environmental Conference. Nov. 7–10, Albuquerque, New Mexico.
Ottman, Y., Byrne, D.H., 1988. Screening rootstocks of pronus for relative salt tolerance. Hortic. Sci. 23 (2), 375–378.
Parandian, F., Samavat, S., 2012. Effects of fulvic acid and humic acid on anthocyanin, soluble sugar, α-amylase enzyme and some micronutrients in lilium. Int. Res. J. Appl. Basic Sci. 3 (5), 924–929.
Parvaiz, A., Satyawati, S., 2008. Salt stress and phyto-biochemical responses of plants—a review. Plant Soil Environ. 54 (3), 89–99.
Pinheiro, H.A., DaMatta, F.M., Chaves, A.R.M., Fontes, E.P.B., Loureiro, M.E., 2004. Drought tolerance in relation to protection against oxidative stress in clones of *Coffe canephora* subjected to long-term drought. Plant Sci. 167 (1), 1307–1314.
Russo, R.O., Berlyn, G.P., 1990. The use of organic bio stimulants to help low input sustainable agriculture. J. Sustain. Agric. 1 (1), 19–42.
Sahu, S., Das, P., Ray, M., Sabat, S.C., 2010. Osmolyte modulated enhanced rice leaf catalase activity under salt-stress. Adv. Biosci. Biotechnol. 1 (1), 39–46.
Sorkheh, K., Shiran, B., Rouhi, V., Khodambashi, M., Sifo, A., 2012. Salt stress induction of some key antioxidant enzymes and metabolites in English Iranian wild almond species. Acta Physiol. Plant 34 (1), 203–213.
Talaat, N.B., Ghoniem, A.E., Abdelhamid, M.T., Shawky, B.T., 2015. Effective microorganisms improve growth performance, alter nutrients acquisition and induce compatible solutes accumulation in common bean (*Phaseolus vulgaris* L.) plants subjected to salinity stress. Plant Growth Regul. 75, 281–295.
Turan, M.A., Ashik, B.B., Katat, A.V., Chelik, H., 2011. The effects of soil-applied humic substances to the dry weight and mineral nutrient uptake of maize plants under soil-salinity conditions. Not Bot Hort Agrobot Cluj 39 (1), 171–177.
Wani, A.S., Ahmad, A., Sh, Hayat, Fariduddin, Q., 2013. Salt-induced modulation in growth, photosynthesis and antioxidant system in two varieties of *Brassica junica*. Saudi J. Biol. Sci. 20 (2), 183–193.
Zhao, Y., Aspinall, D., Paleg, L.G., 1992. Protection of membrane integrity in *Medicago sativa* L. by glysinebetaine against the effects of freezing. J. Plant Physiol. 140 (5), 541–543.
Zrig, A., Ben Mohammed, H., Tounekti, T., Ahmed, S.O., Khemira, H., 2015. Differential responses of antioxidant enzymes in salt-stressed almond tree grown under sun and shade condition. Plant Sci. Res. 1 (4), 1–12.