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The impact of salicylic acid on some physiological responses of *Artemisia aucheri* Boiss. under *in vitro* drought stress

Jalil ABBASPOUR[1], and Aliakbar EHSANPOUR[2]

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ABSTRACT

Salicylic acid (SA) is an important plant regulator which is involved in growth, development, and response to stress. This study was aimed to evaluate some physiological and biochemical responses of *Artemisia aucheri* Boiss. under drought stress after exogenous SA treatment. Experiment was performed *in vitro*. Polyethylene glycol (PEG/6000) with 0, 2 and 4 % (w/v) was used in MS medium to simulate drought stress and different concentrations of SA (0, 0.01 and 0.1mM) were added. After four weeks, SA alleviated the negative effects of PEG on dry and fresh mass as well as chlorophyll and carotenoid contents. Under drought stress, application of SA decreased storage polysaccharides and increased soluble carbohydrates respectively. Although PEG had no significant effect on flavonoid content, it increased significantly anthocyanin and total phenol content, total antioxidant capacity, PAL (phenylalanine ammonia-lyase) and TAL (tyrosine ammonia-lyase) activity and SA treatment improved these parameters significantly. According to the current data, it was concluded that SA increased drought tolerance of *Artemisia aucheri* by increasing biosynthesis of phenolic compounds, improvement of TAL and PAL activity as well as also by increased content of soluble carbohydrates.

Key words: *Artemisia aucheri* Boiss., salicylic acid, drought stress; polyethylene glycol, growth, phenylalanine ammonia-lyase, tyrosine ammonia-lyase

IZVLEČEK

VPLIV SALICILNE KISLINE NA NEKATERE FIZIOLOŠKE ODZIVE VRSTE PELINA (*Artemisia aucheri* Boiss.) NA SUŠNI STRES V IN VITRO RAZMERAH

Salicilna kislina je pomemben rastlinski hormon, ki je vključen v uravnavanje rasti, razvoja in odziva na stres. Cilj raziskave je bil ovrednotiti nekatere fiziološke in biokemične odzive vrste pelina *Artemisia aucheri* Boiss. v sušnem stresu po zunanjem dodajanju salicilne kisline. Poskus je potekal *in vitro,* v MS gojišču (Murashige and Skoog., 1962) z dodatkom polietilen glikola (PEG/6000, 0, 2 in 4 % (w/v)) za simulacijo sušnega stresa in dvema različnima koncentracijama salicilne kisline (SA) (0, 0.01 in 0.1 mM). Po štirih tednih je salicilna kislina zmanjšala negativne učinke polietilen glikola na suho in svežo maso kot tudi na vsebnosti klorofila in karotenoidov. V sušnem stresu je uporaba salicilne kisline zmanjšala vsebnost založnih polisaharidov in povečala vsebnost topnih ogljikovih hidratov. Čeprav polietilen glikol ni imel značilnega učinka na vsebnostn flavonoidov je značilno povečal vsebnost antocianinov, celokupnih fenolov, celokupno antioksidacijsko sposobnost, aktivnost PAL (fenilalanin amonium-liaza) in TAL (tirozin amonium-liaza), obravnavanje s salicilno kislino je te parametre značilno izboljšalo. Glede na te rezultate je bilo zaključeno, da salicilna kislina povečuje strpnost na sušo pri vrsti *Artemisia aucheri* s povečanjem biosinteze fenolnih snovi, z izboljšanjem aktivnosti TAL in PAL kot tudi s povečanjem vsebnosti topnih ogljikovih hidratov.

Ključne besede: *Artemisia aucheri* Boiss., salicilna kislina, sušni stres; polietilen glikol, rast, fenilalanin amonium-liaza, tirozin amoniium-liaza

[1] PhD student, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

[2] Prof., Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran; corresponding author: ehsanpou@sci.ui.ac.ir

1 INTRODUCTION

The genus *Artemisia* belongs to Asteraceae family. There are 500 species of *Artemisia* in Asia, Europe and North America. Thirty four species of this family are known as wild spicies all over Iran. One of these species is *Artemisia aucheri* Boiss which has limited ecological distribution, it is endemic to mountainous areas of Iran and surroundings (Mozaffarian et al., 2010). This plant has many medicinal properties. In traditional medicine it is used as astringent and disinfectant and has an antileishmanial, antiparasitic, and antioxidant activities (Asghari et al., 2012). Verbenone, camphor, 1, 8-cineole, trans-verbenol, chrysanthenone, mesitylene, α-pinene, acyclic monoterpenes, and monoterpene hydroperoxides are bioactive compounds extracted from this plant (Rustaiyan et al., 1987).

Drought stress is one of the major environmental factors limiting plant growth and productivity (Nazar et al., 2015). Drought stress induces the massive generation of reactive oxygen species (ROS). The accumulation of ROS inhibits normal function of lipids, proteins and DNA and finally reduces plant growth and development (Asada, 1999). Plants expose to drought display several morphological, physiological and molecular responses (Jiménez et al., 2013).

Salicylic acid (SA) is a phenolic compound that acts as important phytohormone. Several studies have demonstrated that SA participates in many physiological processes such as growth and development, respiration, stomatal aperture, senescence, seed germination, seedling growth and thermo-tolerance (Vicente and Plasencia, 2011). Moreover, many previous studies have shown that SA plays a role in many biotic and abiotic stresses (Vicente and Plasencia, 2011). This phytohormone can regulate responses to salinity (Idrees et al., 2012) and cold (Siboza et al., 2014), drought (Shen et al., 2014) and the toxicity of heavy metals (Tamás et al., 2015). Moreover, research has shown that SA levels and/or SA signaling played a positive regulatory role in plant response to polyethylene glycol (PEG)-simulated drought stress (He et al., 2014). It has been found that plants treated with SA generally exhibited more tolerance to water deficiency, SA alleviates the negative effects of drought stress on growth of Zoysiagrass (*Zoysia* Willd.)(Chen ret al., 2014). Singh and Usha (2003) showed that SA increases dry weight and chlorophyll content in wheat seedlings in wheat seedlings under water stress. In addition, SA improves photosynthesis and growth of mustard under drought stress (Nazar et al., 2015). Foliar spray with SA positively affected physiological characteristics of fennel genotypes such as chlorophyll, carotenoid contents and soluble carbohydrate and increased drought tolerance (Askari and Ehsanzadeh, 2015).

Biotic and abiotic stresses induce the production of secondary metabolites, which are involved in the defense against harsh environmental conditions and enhance significantly the antioxidant activity of plant tissues. The phenylpropanoid pathway is one of the important pathways in plant secondary metabolite production including, phenolics and among them flavonoids (flavanols, anthocyanins and flavan-3-ols) compounds. These are also considered as antioxidant molecules because they are involved in scavenging of free radical (Pourcel et al., 2007). Key enzymes in the phenylpropanoid pathway are phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL). There has been few reports on activity of TAL in plants treated with SA under drought stress, while PAL activity has frequently been studied. Increase in PAL activity in wheat seedlings treated with SA under salinity stress leading to increase in phenolics and flavonoids and subsequently caused an improvement in antioxidant defense system (Saleh and Madany, 2015). On the other hand, Bandurska and Cies´lak (2013) showed a positive correlation between increase of SA content and PAL activity in leaves and roots of barley, under drought stress and UV-B radiation. Hence, there has been a positive relationship between the key enzymes activity in the phenylpropanoid pathway and production of flavonoids, among them anthocyanines and generally all phenolic compounds.

Artemisia aucheri has always been of great interest for botanical and pharmaceutical aspects. The effect of drought stress on physiological and biochemical responses in *Artemisia aucheri* has not been studied yet. Since, tissue culture technology is a rapid and fast method in assessment of

physiological responses of the plant consequently; the present study was aimed to evaluate the mechanism of responses of *A. aucheri* to *in vitro* drought stress treated with salicylic acid (SA).

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

The *Artemisia aucheri* Boiss. plants were obtained from stock shoot culture of Department of Biology, University of Isfahan. Isfahan, Iran. Single node stem sections were propagated on MS (Murashige and Skoog., 1962) medium supplemented with 30 g/l sucrose and 8 g/l agar with adjusted pH, to 5.8. Plants were grown in the culture room at 25±1 °C with 16/8 h photoperiod under 44 µmol phot.m^{-2}.s^{-1} light.

In treatment experiment SA was added to the media at following concentrations: 0, 0.01 and 0.1 mM. For drought stress treatments, PEG (polyethylene glycol, MW 6000) was used in 0, 2 % and 4 % (w/v) concentrations. PEG was added to MS medium according to diffusion based method described by Girma and Kreig (199). Based on the Factorial Design of the experiments, three explants were cultured per jar (each jar was one replicate) and after 4 weeks, explants were harvested for analysis of different physiological and biochemical parameters. Plants were grown in three different medium, 1) medium without SA and PEG (control), 2) medium with SA and PEG separately, 3) medium with combination of PEG and SA.

2.2 Growth measurement

Fresh mass was measured directly after harvesting of plants and dry mass was measured after drying the plant materials at 70 °C for 24 hours. Shoot relative water content was measured according to Weatherly (1950) as described by Bandurska (2000) and it was calculated by the following formula: RWC = [(fresh mass – dry mass)/ (fresh matter at full turgor – dry mass)] × 100. TW is turgid mass after saturating the fresh sample with water for 4 h.

2.3 Determination of chlorophyll and carotenoid content

For photosynthetic pigment measurements, leaves (0.1 g) of the plants were grounded in 80 % cold acetone and centrifuged at 5000 g for 10 min. The absorbance of the purified chlorophyll samples were measured at 470, 646, and 663 nm (Shimadzu, Japan). Chlorophylls and carotenoid contents were calculated according to Lichtenthaler and Wellburn (1983).

2.4 Soluble carbohydrates and storage polysaccharides

Water soluble carbohydrates were determined based on the phenol-sulfuric-acid method (Dubois et al., 1956). To prepare carbohydrate extract, 10 mg of dry leaf and stem was homogenized with 10 ml deionized water. The samples were centrifuged and supernatant used to determine soluble sugars. Sample (0.5 ml) was mixed with 0.5 ml of phenol (5 %) and then mixed with 2.5 ml sulfuric acid (96 %). The samples were vortexed slowly for 30 min then soluble carbohydrates were measured at 490 nm. To determine the storage polysaccharides, sediment of carbohydrate extract was weighted and again homogenized with deionized water and boiled for one hour. Finally, storage polysaccharides were measured at 490 nm.

2.5 Anthocyanin content

Total anthocyanins were extracted and determined based on the method described by Laby et al (Laby et al., 2000) with minor modifications. Leaves (0.1 g) were grounded in 99:1 methanol:HCl (v/v) and incubated at 4º C for 16 h. Then, samples were centrifuged at 4º C and the absorbance of the supernatants were measured at 530 and 657 nm. Total anthocyanin content was expressed as A_{530} g^{-1} FW.

2.6 Phenolic content

The Folin-Denis method was applied to estimate total phenols contents in the supernatant (Singleton et al., 1999). Leaf samples (0.1 g) were homogenized with 10 ml of methanol 80 %. The homogenates were centrifuged at 12,000 g for 10 min then 1/5 ml Folin–Ciocalteu reagent (10 %) and 1 ml Na_2CO_3 (7.5 %) was added to 0.5 ml methanol extract. The absorbance of samples was measured at 760 nm. The total phenolic

compounds were calculated from the standard curve, using gallic acid as a standard and expressed as mg gallic acid (Sigma, USA) g^{-1} FW.

2.7 Flavonoid content

To determine the flavonoid content, 0.1 g of fresh leaf tissue homogenized in 80 % methanol and centrifuged at 10000 g for 10 min. The reaction mixture containing 0.2 ml of 80 % methanol, 0.2 ml of aluminum chloride (10 %), 0.2 ml of sodium acetate and 0.1 ml leaf extract. After 30 min, the absorbance of the samples was measured at 415 nm. The quercetin (Sigma, USA) was used for the standard curve and results were expressed as mg g^{-1} FW (Chang et al., 2002).

2.8 PAL and TAL assay

To estimate the PAL and TAL activity, leaf samples (300 mg) were grounded in a mortar at 4 ºC with 4 mL buffer (50 mmol/l Tris pH 8.5, 14.4 mmol/l 2-mercaptoethanol, 5 % w/v polyvinylpolypyrrorolidone) and was centrifuged at 6,000 g for 10 min at 4 ˚C. The supernatant was collected and centrifuged at 10,000 g for 10 min at 4 ˚C and was used to assay enzyme activity. The total protein concentration in soluble enzyme extracts was determined using the Bradford (1976) method. The reaction mixture contained 500 µmol of Tris- HCl buffer (pH 8), 100 µl of enzyme extraction and either 6 µmol of L-phenylalanine for measuring PAL activity (EC 4.3.1.5) or 5.5 µmol of L-tyrosine (Sigma, USA) for measuring TAL activity (EC 4.3.1). After 60 min at 40°C, the reaction was stopped by the addition of 0.05 ml 5 N HCl. The amounts of trans-cinnamic and p-coumaric acids were determined by measuring absorbance at 290 and 333 nm, respectively. The PAL and TAL activities were expressed as nmoles (cinnamic or coumaric acid) h^{-1} mg^{-1} protein (Beaudoin-Eagan and Thorpe., 1985).

2.9 Total antioxidant capacity

Leaf samples (0.1 g) were homogenized with 80 % methanol in cold mortar and pestle and centrifuged at 18000 g for 10 min. The extrications were used to measure total antioxidant capacity by ferric reducing antioxidant power (FRAP) method (Benzie and Strain., 1996) This method is based on the reduction of ferric tripyridyltriazine (FeIII_TPTZ) complex to ferrous (FeII) form that makes blue color with maximum absorption at 593 nm. FRAP working solution consisted with 25 ml of acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ (Sigma, USA) solution (10 Mm in 40 mM HCl) and 2.5 ml of $FeCl_3$ (20 mM) solution.1.5 ml of FRAP reagent was added to 50 µl plant extract and mixed well. The absorbance was measured at 593 nm after 5 min. Standard curve was prepared using the similar procedure with ascorbic acid as standard.

2.10 Statistical analysis

All experiments were carried out with at least three replicates and the results were expressed as mean ± standard deviation (SD). ANOVA was performe to determine the significance of differences between means by Duncan's test ($p < 0.05$).

3 RESULTS AND DISCUSSION

Plants have developed mechanisms to alleviate negative effects of drought stress to increase their chance for survival. SA as a phytohormone can be effective in modulating physiological and biochemical responses leading to adaptation of plants to unfavorable environments such as drought stress (Kang et al., 2013; Nazar et al., 2015).

The obtained data indicate that fresh and dry mass of control (without SA and PEG) plants supplemented with 0.01 mM SA were increased significantly, while fresh and dry mass of 0.1 mM SA treated plants was comparable to untreated controls. Fresh mass decreased dramatically by 57 % and 76 % when control plant treated with 2 and 4 % PEG respectively, and 2 and 4 % PEG decreased dry mass by 53 % and 72.5 % comparing to the control respectively. Application of SA (0.01 and 0.1 mM SA) significantly improved both parameters when compared to PEG treated controls without SA treatment. The maximum increase of fresh and dry mass was observed at 0.01 mM SA (56 and 59 % in fresh mass under 2 % PEG and 51.5 and 43 % in dry mass under 4 % PEG) (Figure. 1). Furthermore, as

it is shown in the Figure. 2, SA (0.01 mM) increased photosynthetic pigments in control plant without PEG treatment. On the other hand, treatment of *A.aucheri* with PEG reduced chlorophyll and carotenoid contents compared with untreated plants. Under 2 % PEG treatment, a similar increase in total chlorophyll and carotenoid concentrations was measured when plants were treated with 0.01 and 0.1 mM SA, respectively. However, the effect of SA treatment on pigment contents was insignificant when plants were pretreated with 4 % PEG. The results in our study are in agreement with those reported by other authors. It is known that exogenous SA application enhanced the growth and photosynthetic pigments in several plant species treated under water stress such as wheat (Singh and Usha, 2003), *Nigella sativa* (Kabiri et al., 2014), Zoysiagrass (Chen et al., 2014), mustard (Nazar et al., 2015). Increase in fresh and dry mass by SA application under drought stress can be related to the positive effect of SA on photosynthetic pigments which led to the improvement in growth. In other words, the low water availability reduces photosynthesis, resulting in a reduction of carbohydrate accumulation, limiting overall plant growth (Chaves and Oliveira, 2004). In the control plant, the RWC remained at similar levels when plant was treared with SA. RWC decreased progressively under water deficit as compared with control plants, both concentration of salicylic acid increased RWC as compared to plant without application of SA under drought conditions (Fig. 3). Similar result were observed in pot experiments with barley (Habibi, 2012) and *Arabidopsis* (Khokon et al., 2011). It was suggested that the observed increase in RWC may be due to SA induced stomatal closure which reduces water loss. However, these results cannot be directly compared to our observations, since *in vitro* cultivated plants have quite unique water balance regulation. Their control of transpiration is poor due to very thin cuticle and malfunctioning of the stomata (Sutter, 1988). In this respect the results of our study only prove the involment of SA in plant response to drought, but have limited direct ecological relevance.

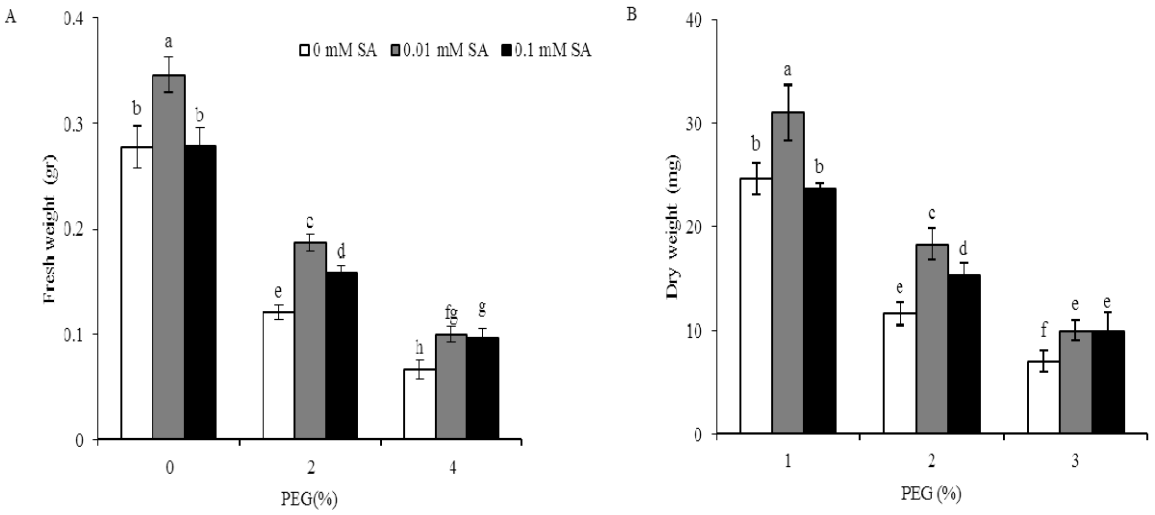

Figure 1: Effects of SA and PEG on (A) fresh and (B) dry mass of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

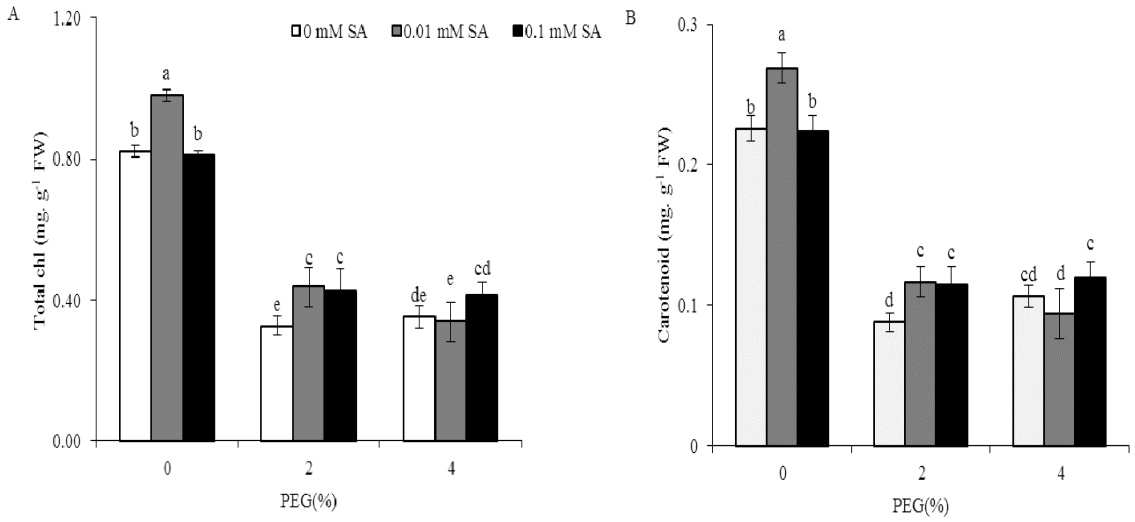

Figure 2: Effects of SA and PEG on (A) chlorophyll and (B) carotenoid of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

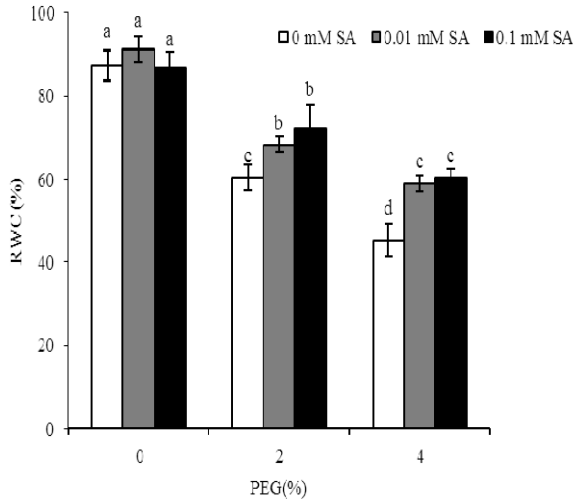

Figure 3: Effects of SA and PEG on relative water content (RWC) of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

Changes of carbohydrate and storage polysaccharides are presented in Figure 4. Drought stress (2 and 4 % PEG) declined the content of storage polysaccharides compared with untreated plants, and maximum decreased was observed at 4 % PEG. However it had no significant effect on soluble carbohydrates. Under 2 % and 4 % PEG, SA treatments decreased storage polysaccharides in the same manner (Figure. 4B), while soluble carbohydrates were increased (Figure. 4A). Moreover, both forms of carbohydrates in control plant were enhanced with 0.01 mM SA treatment. Soluble carbohydrates act as compatible solutes which support osmoregulation and are the main source of energy when plants are exposed to unfavorable environmental conditions (Patakas and Noitsakis, 2001). Increase of total soluble carbohydrates and storage polysaccharide content with 0.01 mM SA in control plants reflects stimulating role of SA in plant growth.

Interestingly, when SA treated plants have been exposed to drought stress, it has shown reverse trends in soluble sugars and polysaccharide contents as a result of graduate increase in SA treatment. It seemed that SA has facilitated conversion of polysaccharide to soluble carbohydrates and resulted in osmotic adjustment and resistance under drought. Accumulation of compatible solution is as an effective response to dehydration (Patakas and Noitsakis, 2001).

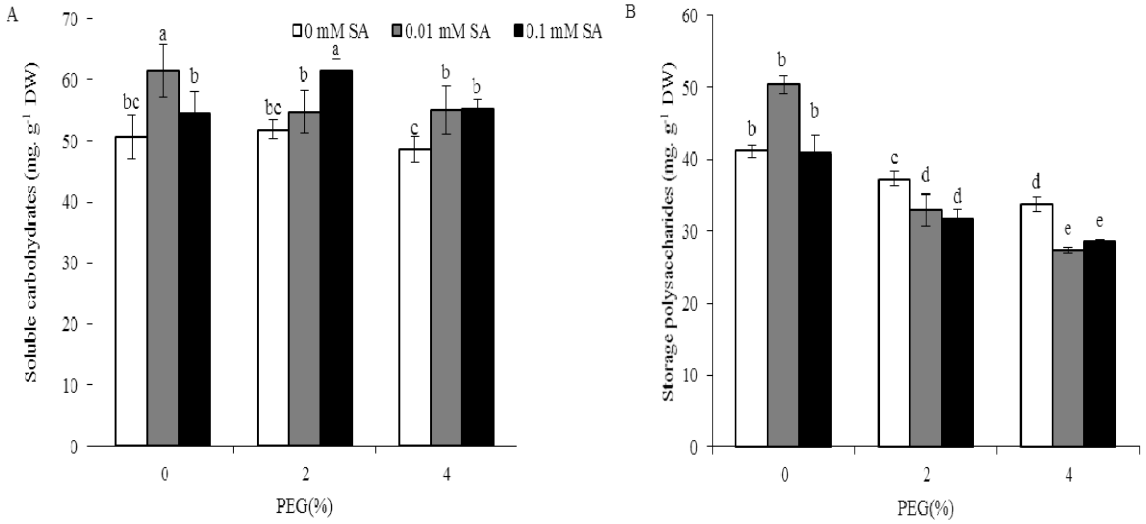

Figure 4: Effects of SA and PEG on (A) soluble carbohydrates and (B) storage polysaccharides content of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

According to our results, SA as a single treatment is able to increase phenolic compounds. Drought stress significantly elevated total phenolic contents compared with untreated plants. Moreover, both concentrations of SA (0.01 and 0.1 mM) increased the total phenol in 2 % PEG treated plants while SA with 0.1 mM increased phenol content in 4 % PEG (Figure. 5A). Drought stress induced by PEG had no significant effect on flavonoid levels compared with untreated plants. SA treatments (0.01 and 0.1mM SA) were remarkably effective on increasing of flavonoid content under 4 % PEG. In addition, flavonoid content was enhanced in response to 0.1 mM SA compared to control plants (Figure. 5B). Application of SA in the culture medium increased anthocyanin content in both PEG-treated and untreated plants. SA treatments in SA (0.1 Mm) + PEG (2 %) and SA (0.01 mM) + PEG (4 %) showed significant difference compared with the same PEG levels without SA (Figure. 5C).

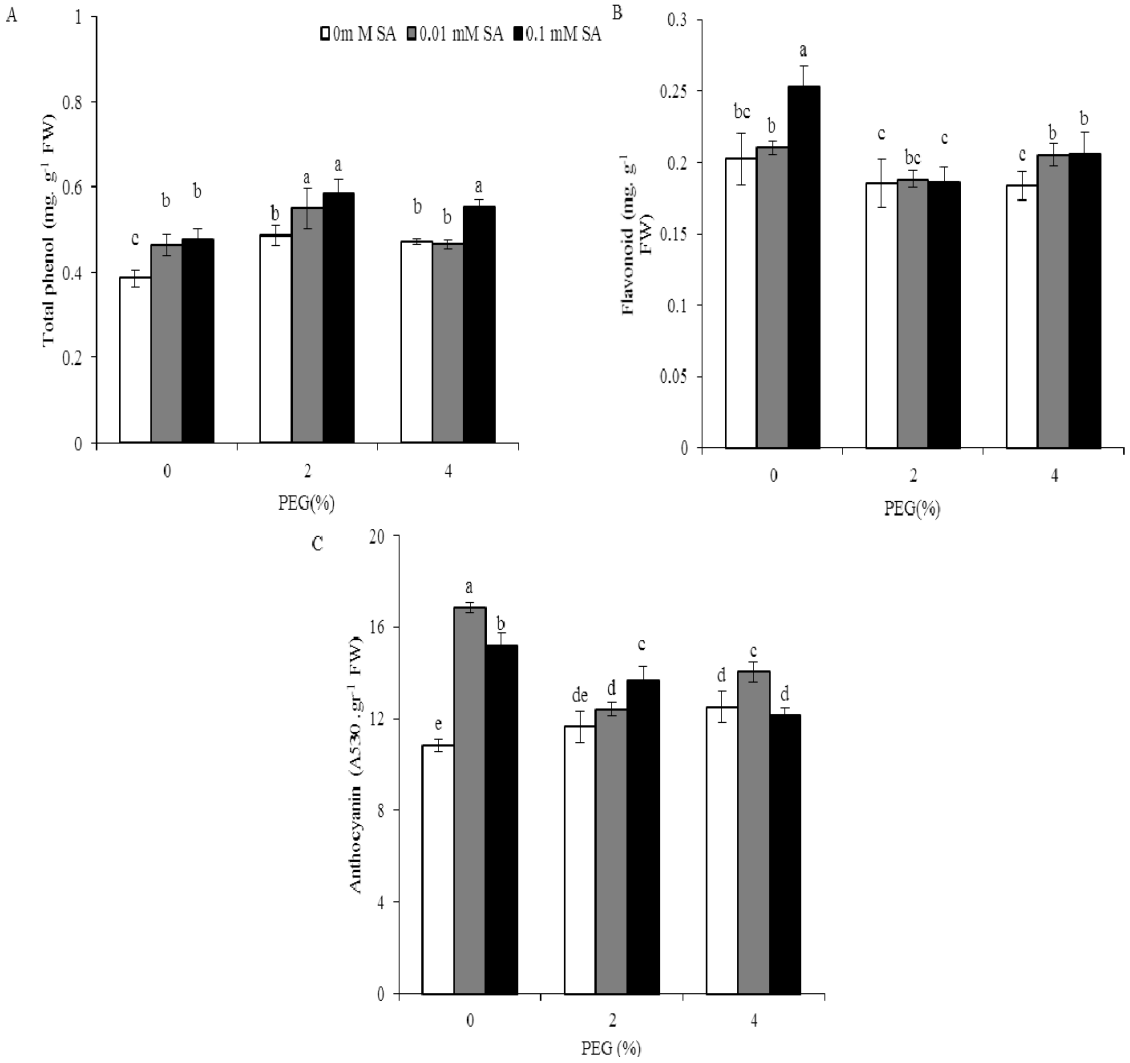

Figure 5: Effects of SA and PEG on (A) total phenol and (B) flavonoid and (C) anthocyanin content of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

PAL and TAL are two key enzymes in the phenylpropanoid pathway that catalyse the conversion of L-phenylalanine and L-tyrosine to trans-cinnamic acid and p-coumaric acids, respectively (Schroeder et al., 2008). Drought stress induced by PEG in combination with SA treatments remarkably enhanced PAL and TAL activity (Fig. 6). Notably, the increase of PAL activity in SA (0.01 and 0.1 mM) treated plants was also observed. The plants treated with 2 % and 4 % PEG showed an increase in PAL activity in 0.1 mM SA (Figure. 6A). Moreover, SA (0.1 mM) elevated TAL activity under 2 % PEG treatment, while SA treatments had no significant effect on TAL activity when plants were exposed to 4 % PEG (Figure. 6B). Our results are in agreement with the previous findings, that the increase in total phenolic content, such as phenol and flavonoids is accompanied by the induction of PAL and TAL activities that are involved in the defense system against biotic and abiotic stresses (S′wieca, 2015). The increase in level of anthocyanin, flavonoid and total phenol content by SA treatment was associated with TAL and PAL activity. Similar observation was reported by Dogbo et al (2012). They also showed exogenous salicylic acid induced PAL and TAL activity in cassava cell suspensions. Furthermore, elicitation using hydrogen peroxide in lentil sprouts enhanced PAL and TAL activity and subsequently elevated phenolic levels (S′wieca, 2015). Several studies have demonstrated that the production of ROS

especially hydrogen peroxide is induced by SA (Miura and Tada, 2013). Although under PEG treatments flavonoid contents did not change significantly, but anthocyanin as a phenolic component and total phenol as well as TAL and PAL activity elevated significantly. Similar findings were observed in the drought-tolerant barley cultivars under drought and salinity stress which confirms the role of antioxidant activity in this components and enzymes (Ahmed et al., 2015). Generally, our results showed that combination of SA with PEG has improved phenolic compounds production and two key enzymes of phenolic biosynthesis pathway. Kabiri et al (2014) reported that SA increased total phenol, anthocyanin, and flavonoid content under drought stress and drought damages were reduced. In fact SA could induce activity of PAL and TAL activity and consequently phenolic compounds were increased (Bandurska and Cie´slak, 2013).

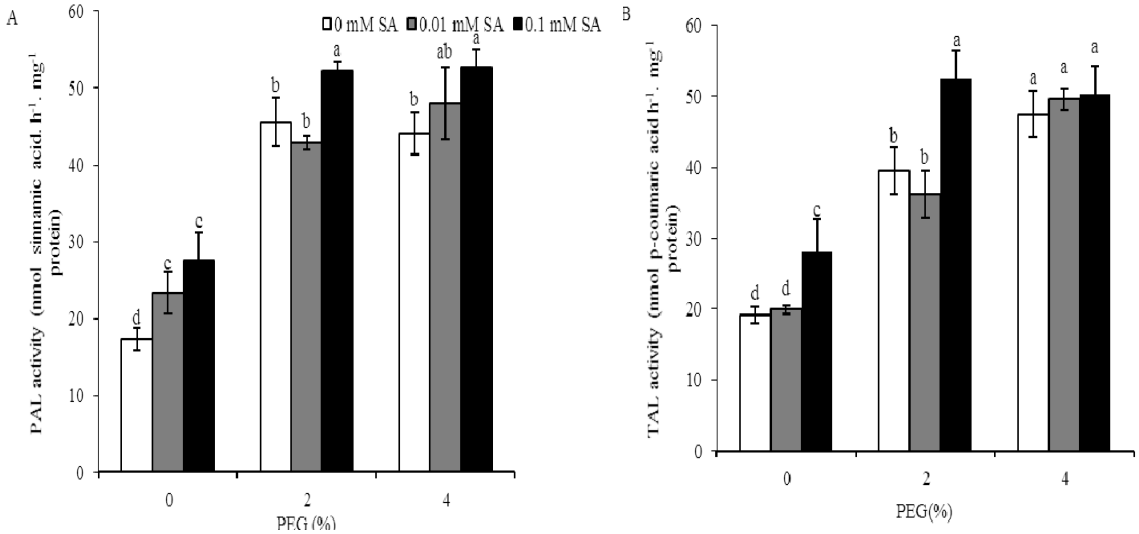

Figure 6: Effects of SA and PEG on (A) PAL and (B) TAL activity of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

Plants subjected to drought stress demonstrated higher total antioxidant capacity when exposed to higher PEG concentration. Total antioxidant capacity increased significantly in 0.01 mM SA treated controls and 0.01mM SA + 2 % PEG treated plants, the last showing a maximum level of antioxidant capacity. Moreover, plants subjected to drought and 0.1mM SA showed higher total antioxidant capacity compared to the same PEG level without SA (Fig. 7). It seemed that antioxidant property of phenolic compounds increased drought tolerance of *A. aucheri* as previously reported in barley (Saleh and Madany, 2015) and olive (Hashempour et al., 2014).

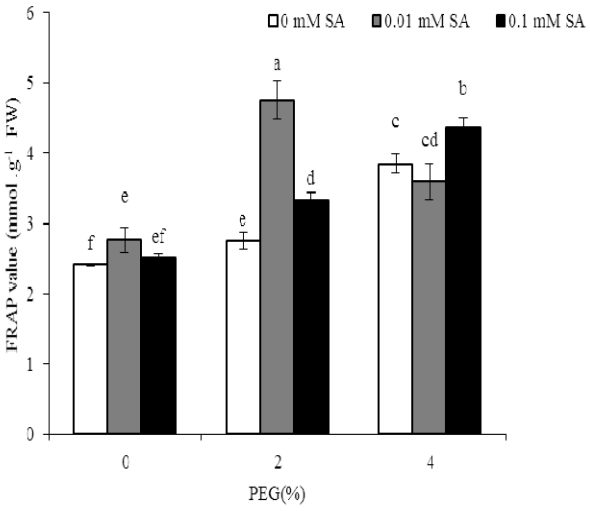

Figure 7: Effects of SA and PEG on total antioxidant capacity of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

4 CONCLUSION

Based on the present data, SA treatment alleviates drought stress induced by PEG in *A. aucheri* plants. This enhanced tolerance could be related to the improvement of antioxidant capacity via increase in PAL and TAL activities and the subsequent increase in anthocyanin, flavonoid and total phenol content. Furthermore, SA improves osmotic adjustment by conversion of storage polysaccharide to soluble carbohydrate.

5 ACKNOWLEDGEMENTS

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