

## Impact of Salicylic Acid on Phenolic Metabolism and Antioxidant Activity in Four Grape Cultivars during Cold Storage

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### Abstract

Salicylic acid (SA) plays an important role in the regulation of plant ripening and responses to abiotic stresses. In this study, the protective effect of SA on cold stress-caused oxidative damage in grape (*Vitis vinifera* L.) bunches was investigated during cold storage. Grape bunches treated with 2 mM SA and stored at 0°C with 85-90% RH for 30 days. Samples were selected from each treatment for physical and chemical analysis before storage, midpoint of storage, end of storage and end of shelf life. The results showed that the cold storage induced lipid peroxidation, decay incidence and loss of fresh weight, while SA pretreatment mitigated these effects in “Bidaneh Sefid” and “Qzl uzum” cultivars. In these cultivars, SA-induced protection against cold damage was accompanied by decrease in malondialdehyde (MDA) content, and caused an increase in catalase (CAT) and superoxide dismutase (SOD) activity. Additionally, SA-induced protection against cold injury was mediated through promoting total phenolics concentration and phenylalanine ammonia-lyase (PAL) activity. The present findings suggest that although variability in the protective effect of SA against cold damage was observed between grape cultivars, SA could alleviate the cold-induced oxidative stress in grape bunches during cold storage.

**Keywords:** Cold stress, grape cultivars, lipid peroxidation, phenylalanine ammonia-lyase, salicylic acid.

### Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest and most important perennial, with about 14,000 numerous synonyms cultivars in the world (Alleweldt et al., 1991). “Bidaneh Sefid” is a seedless grape cultivar and “Qzl uzum”, “Sahebi” and “Dastrchin” are categorized as seeded grape cultivars. However, in Iran, the most cultivated variety is “Bidaneh Sefid”, which is mostly consumed as raisins and table grapes. The postharvest life of table

grapes is relatively short due to water loss and fungal decay (Ranjbarani et al., 2011).

Low temperature is an effective means for preserving quality of postharvest fruit. However, the use of chemicals is necessary to maintain the grape quality during a long-term storage (Zoffoli et al., 2009). Recently, application of chemicals such as SO<sub>2</sub> during cold storage has become restricted (Meng et al., 2008) because of the risk of its toxic and harmful residues for human health. Recent studies have shown that chemicals alternatively can be replaced with salicylic acid (SA), as a natural phenolic compound. Salicylic acid

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exhibits a high potential in controlling postharvest losses of horticultural crops (Luo et al., 2012). SA reduces fungal decay fruit in susceptible fruits and vegetables (Yao and Tian, 2005; Babalar et al., 2007; Habibi, 2015). Application of exogenous SA effectively induces resistance to chilling injury in post-harvest horticultural crops, including tomato (Ding et al., 2002), cucumber (Cao et al., 2009), peach (Cao et al., 2010) and pineapple (Lu et al., 2010). Therefore, SA treatment is potentially capable of commercial control of chilling injury in horticultural crops.

Abiotic stresses such as low temperature induce the generation of reactive oxygen species (ROS) (Panda et al., 2003). These ROS can cause oxidative damages to proteins, membrane lipids and other cellular components., plants possess enzymatic and nonenzymatic antioxidative defense systems to protect their cell against these damages (Creissen and Mullineaux, 2002). Previous works showed that antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) play pivotal role against oxidative stresses (Habibi and Hajiboland, 2012; Habibi, 2015). Exogenous SA with proper concentration enhances the efficiency of antioxidant system (Knorzer et al., 1999; Hayat et al., 2010). Furthermore, an increase in antioxidant enzymes activity following SA application was related to  $H_2O_2$  metabolism produced by chilling, which acts as a redox signaling message (Habibi, 2014), and thereby providing tolerance against the stress (Kang et al., 2003; Cai et al., 2015). Phenolic compounds are accompanied by colour and taste of grapes which positively influence the human health (Bonada and Sadras, 2014). SA stimulates the activity of phenylalanine ammonia lyase (PAL), an important enzyme in the biosynthesis of phenols in fruit and vegetables, during the storage period (Asghari and Aghdam, 2010). Therefore, SA could prevent postharvest oxidative stress and alleviate the injury caused by low temperature through the

alteration of antioxidant enzymes and phenylpropanoid enzymes activities (Karlidag et al., 2009; Cao et al., 2009; Tao et al. 2010; Asghari and Aghdam, 2010).

However, previous researches showed that postharvest treatment on table grapes could decrease total decay in fruits (Asghari et al., 2009). However, there is no published work concerning the performance of postharvest physiology in different cultivars belonging to *Vitis vinifera*. We hypothesized that postharvest application of SA in a given species is highly dependent on the cultivar. In the present study, we studied the effects of pre-harvest treatment of grape bunches with SA on antioxidant activity and phenylpropanoids metabolism in the skins of four grape cultivars at different time intervals after cold treatment.

### Materials and Methods

Four grape cultivars (“Bidaneh Sefid”, “Qzl uzum”, “Sahebi” and “Dastrchin”) were picked on the 10 September 2014 at an experimental field station belonging to the Malekan region, Northwest Iran (latitude 37°45’ N, longitude 46°10’ E). The soil of this region was a sandy loam containing 0.3% of organic matter (OM), 48% sand with pH 6.97 and electrical conductivity (EC) of 1.2 ds m<sup>-1</sup>. The soil was fertilized with 170 kg N and 110 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>. Plants were subjected to three supplemental irrigations during different reproductive stages of growth. After harvesting, the selected bunches were randomly separated in two sections including i) SA-treated group immersed for 30 minutes in 2 mM SA solutions (Sigma-Aldrich) and ii) SA-treated samples as air-dried at room temperature. Control plants were immersed in water. After SA treatment, bunches were placed in cold storage at 0 °C with 85-90% RH for 30 days. Following 30 days of cold storage, bunches were placed to a controlled environment chamber maintained at 20°C for 3 days (simulating shelf life). Bunch

decay and bunch mass loss were estimated by using 35 berries per replicate. Symptoms of bunch decay were assessed according to Wang et al. (2006).

#### ***Assay of enzymes activity and related metabolites***

Metabolite measurements were performed in a temporal (before storage, midpoint of storage, end of storage and end of shelf life) manner. Activity of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were determined according to Habibi and Hajiboland (2012). For determination of SOD activity, the extract mixture consisted of 25 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture contained 0.1 mM EDTA, 50 mM Na<sub>2</sub>CO<sub>3</sub> pH 10.2, 13 mM methionine, 63 μM nitroblue tetrazolium chloride (NBT), 13 μM riboflavin. One unit of SOD was determined as the amount of enzyme which caused a 50% inhibition of NBT reduction under assay conditions. For determination of CAT activity, 50 mM phosphate buffer pH 7.0 was used for extraction and spectrophotometrically measurements was done by following the degradation of H<sub>2</sub>O<sub>2</sub> at 240 nm. Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid. Samples were homogenized in ice bath with 0.1% (w/v) TCA for determination of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) contents. After centrifugation at 12,000 × g for 15 min, 0.5 ml of the supernatant was used. The reaction mixture contained 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The reaction was dark-incubated for 1 h. The hydrogen peroxide contents were measured spectrophotometrically at 390 nm according to the method of Velikova et al. (2000).

#### ***Assay of PAL activity and phenolic content***

Samples were extracted in 50 mM sodium

phosphate buffer (pH 7.0) containing 2% (w/v) polyvinylpolypyrrolidon (PVPP), 2 mM EDTA, 18 mM β-mercaptoethanol and 0.1% (v/v) Triton X-100 and used for the determination of PAL activity. The homogenate was centrifuged at 15000 g for 15 min at 4 °C. Enzyme extracts were mixed with 5 mM L-phenylalanine in 60 mM sodium borate buffer (pH 8.8). PAL was assayed in the supernatant by measuring the formation of cinnamic acid at 290 nm according to modified method of Zucker (1965). One unit (U) of PAL activity was specified as the amount of the enzyme that produced 1 nmol cinnamic acid per h.

Total phenolic content was determined using the Folin-Ciocalteu method as modified by Velioglu et al. (1998). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight.

#### ***Free radical scavenging activity determination by DPPH method***

Antioxidant activity of the extracts of bunches was determined based on the radical scavenging ability in reacting with a stable DPPH free radical using an ultraviolet spectrophotometer. The amount of 750 μL of the extract was mixed with 750 μL of DPPH in absolute ethanol solution and incubated at 37 °C for 20 minutes. The absorbance of each solution was determined at 520 nm. IC<sub>50</sub> (concentration causing 50% inhibition) values of the methanolic extracts were assessed graphically (Lee et al., 1998). Radical scavenging activity was expressed as the percentage inhibition of DPPH radical and calculated by following equation:

$$\% \text{ Inhibition} = 100(\text{absorbance of blank} - \text{absorbance of extract}) / \text{absorbance of blank}$$

Experiment was conducted in a factorial experiment (including two factors: SA and storage time) based on completely randomized design (CRD) with 4 independent replications. Before the Analysis of Variance (ANOVA) was carried out, all data was examined graphically for the normality of distribution; and the

homogeneity of variance using Bartlett's test in Minitab software 17. Data were analyzed based on ANOVA test for normal distribution and homogeneity of variance. Two-way ANOVA test (mean of squares) has been done in order to determine the effect of SA and storage time and their interactions on various physiological parameters within each cultivar. Tukey's test, was used in Sigma Stat software for treatment groups with higher mean value as compare with random value ( $p = <0.05$ ).

## Results

Salicylic acid decreased the berry weight

loss of "Qzl uzum" up to circa 30% at the end of shelf life (Table 1), whereas the effect of SA treatment on weight loss of the other cultivars was not significant during storage and shelf life. The incidences of fungal decay of cultivars "Bidaneh Sefid" and "Qzl uzum" were significantly reduced by SA treatment at 15 and 30+3 days of postharvest life in comparison to those of control (Table 1). In "Sahebi" and "Dastrchin", there was no difference in decay incidence between grapes treated with SA and those treated without SA during cold storage.

**Table 1. Effect of postharvest SA treatment and cold storage on fruit mass loss and the decay index in grape cultivars.**

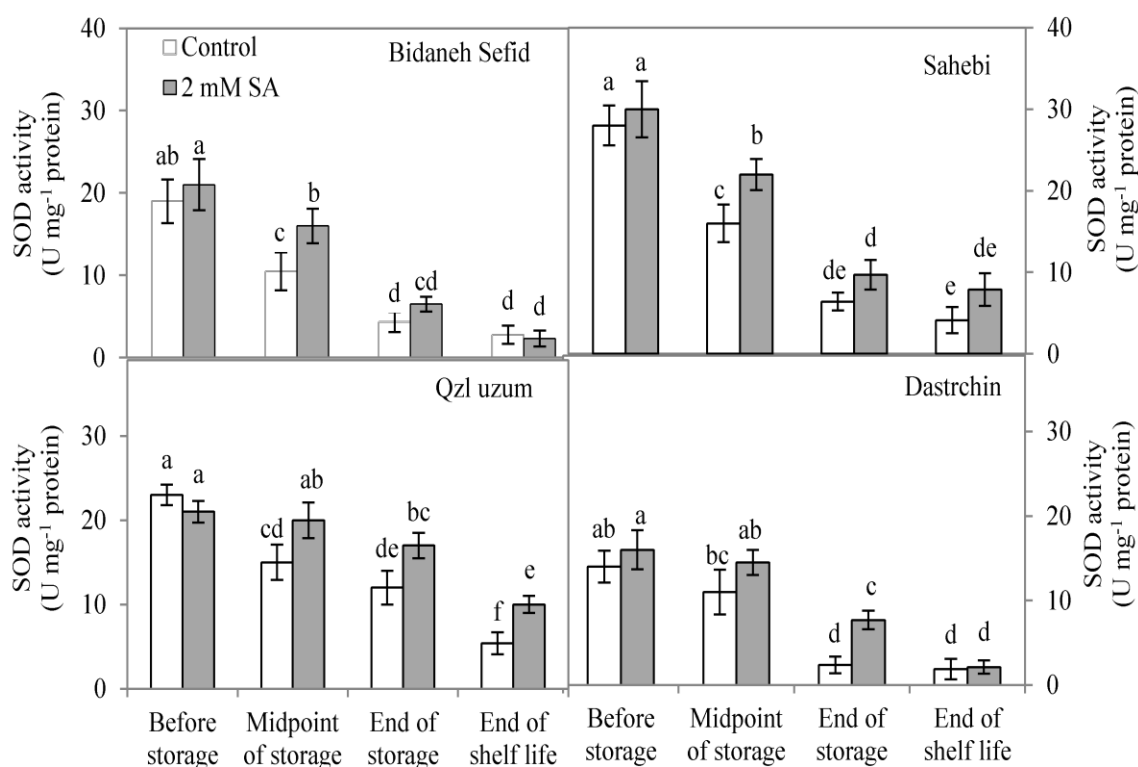
Cultivar	Time	Treatment	Water loss (%)	Decay index
Bidaneh Sefid	Midpoint of storage	Control	5.36 ± 1.30 <sup>a</sup>	1.75 ± 0.12 <sup>b</sup>
		2 mM SA	5.97 ± 0.42 <sup>a</sup>	1.65 ± 0.25 <sup>b</sup>
	End of shelf life	Control	8.29 ± 1.47 <sup>a</sup>	4.75 ± 0.32 <sup>a</sup>
		2 mM SA	7.26 ± 2.24 <sup>a</sup>	2.00 ± 0.18 <sup>b</sup>
Source of variation				
	SA		ns	*
	Storage duration		ns	**
	SA × Storage duration		ns	ns
Qzl uzum	Midpoint of storage	Control	4.76 ± 1.01 <sup>b</sup>	2.15 ± 0.08 <sup>b</sup>
		2 mM SA	4.03 ± 0.88 <sup>b</sup>	1.85 ± 0.23 <sup>b</sup>
	End of shelf life	Control	8.96 ± 1.33 <sup>a</sup>	3.48 ± 0.11 <sup>a</sup>
		2 mM SA	5.12 ± 1.08 <sup>b</sup>	2.06 ± 0.14 <sup>b</sup>
Source of variation				
	SA		*	*
	Storage duration		**	**
	SA × Storage duration		ns	ns
Sahebi	Midpoint of storage	Control	2.65 ± 0.62 <sup>a</sup>	3.85 ± 0.13 <sup>a</sup>
		2 mM SA	2.68 ± 0.37 <sup>a</sup>	2.48 ± 0.09 <sup>a</sup>
	End of shelf life	Control	3.65 ± 1.46 <sup>a</sup>	4.55 ± 1.52 <sup>a</sup>
		2 mM SA	4.01 ± 1.07 <sup>a</sup>	3.95 ± 1.44 <sup>a</sup>
Source of variation				
	SA		ns	ns
	Storage duration		ns	ns
	SA × Storage duration		ns	ns
Dastrchin	Midpoint of storage	Control	5.87 ± 1.65 <sup>a</sup>	2.75 ± 0.77 <sup>a</sup>
		2 mM SA	4.99 ± 0.72 <sup>a</sup>	2.65 ± 0.93 <sup>a</sup>
	End of shelf life	Control	7.66 ± 2.74 <sup>a</sup>	3.25 ± 1.87 <sup>a</sup>
		2 mM SA	6.24 ± 2.37 <sup>a</sup>	2.98 ± 0.76 <sup>a</sup>
Source of variation				
	SA		ns	ns
	Storage duration		ns	ns
	SA × Storage duration		ns	ns

Data of each parameter within each cultivar followed by the same letter are not significantly different ( $p < 0.05$ ). Values are the mean ± SD (n=20); ns: non-significant, according to Tukey test, \*\* P<0.01, \* P<0.05, respectively (two-way ANOVA).

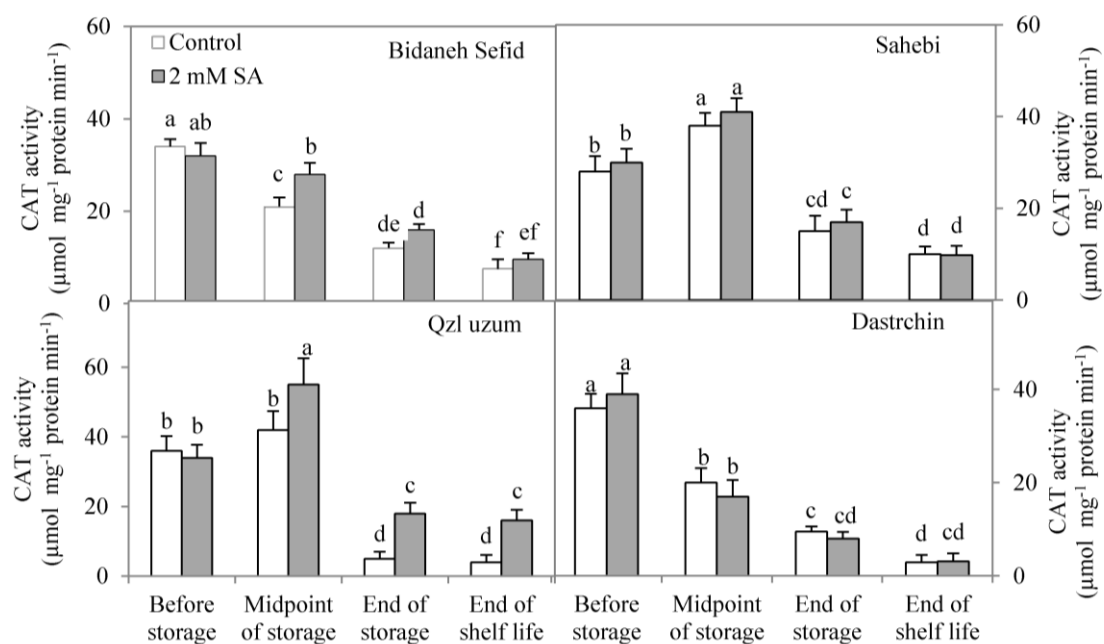
By 30 days of cold storage and the end of shelf life, SOD activity gradually decreased in all cultivars, but SOD activity in SA-treated “Qzl uzum” was significantly higher than that of control (Fig. 1). In “Sahebi” and “Qzl uzum”, CAT activity significantly increased within the first 15 days, followed by a remarkable decrease during 15 to 30+3 days. In “Sahebi” and “Dastrchin”, no difference in CAT activities between SA-pretreated and control bunch was observed during the storage period, whereas CAT activity in SA-pretreated “Qzl uzum” was significantly higher than that in the controls at 15 and 30+2 days of postharvest life (Fig. 2). Overall, the reduction of SOD and CAT activities in SA-pretreated bunch was less

than their activities in the controls at the same time.

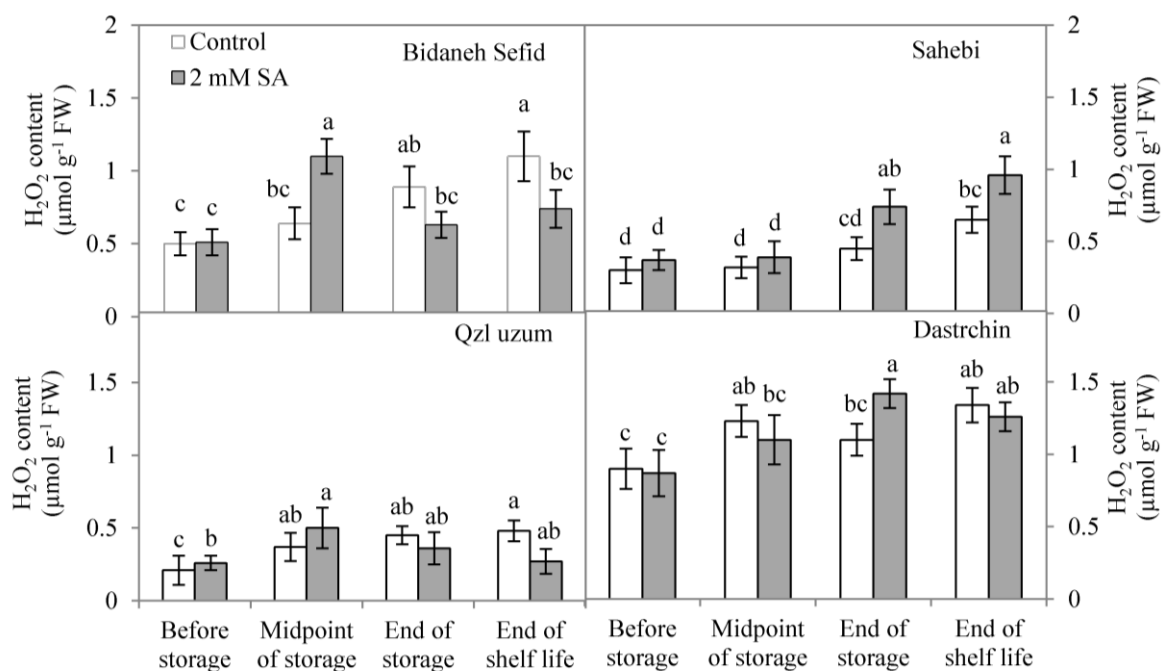
Minor changes in H<sub>2</sub>O<sub>2</sub> content were observed when bunches were stored at 0 °C in the first 15 days, but contents continuously increased to the end of storage (Fig. 3). H<sub>2</sub>O<sub>2</sub> content in the SA-pretreated bunch remained significantly higher during late stages of cold stress in cultivars “Sahebi” and “Dastrchin” than in cultivars “Bidaneh Sefid” and “Qzl uzum”. Lipid peroxidation, measured on the basis of MDA content, was investigated during storage (Fig. 4). The MDA content in all cultivars showed a notable increase during storage, however, MDA content in SA-pretreated “Bidaneh Sefid” and “Qzl uzum” bunches was significantly lower than that in the controls at the end of storage.



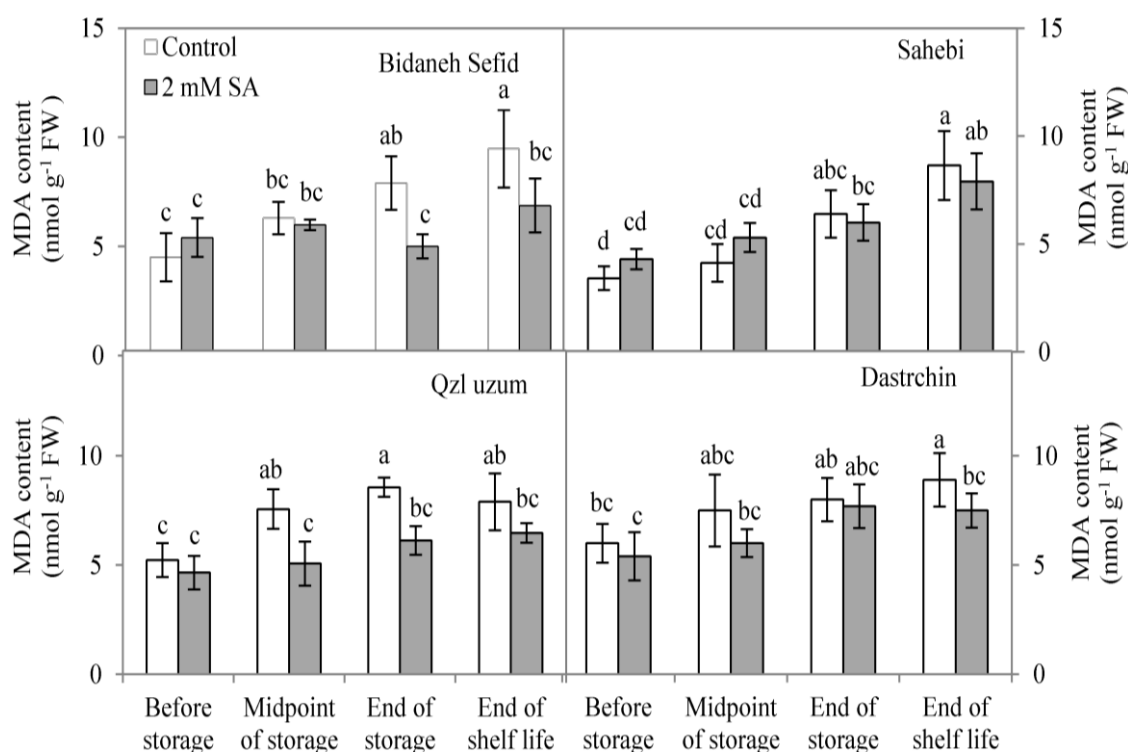
**Fig. 1.** Effect of postharvest SA treatment on specific activity of superoxide dismutase (SOD) in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD ( $n=4$ ).



**Fig. 2.** Effect of postharvest SA treatment on the specific activity of catalase (CAT) in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD ( $n=4$ ).



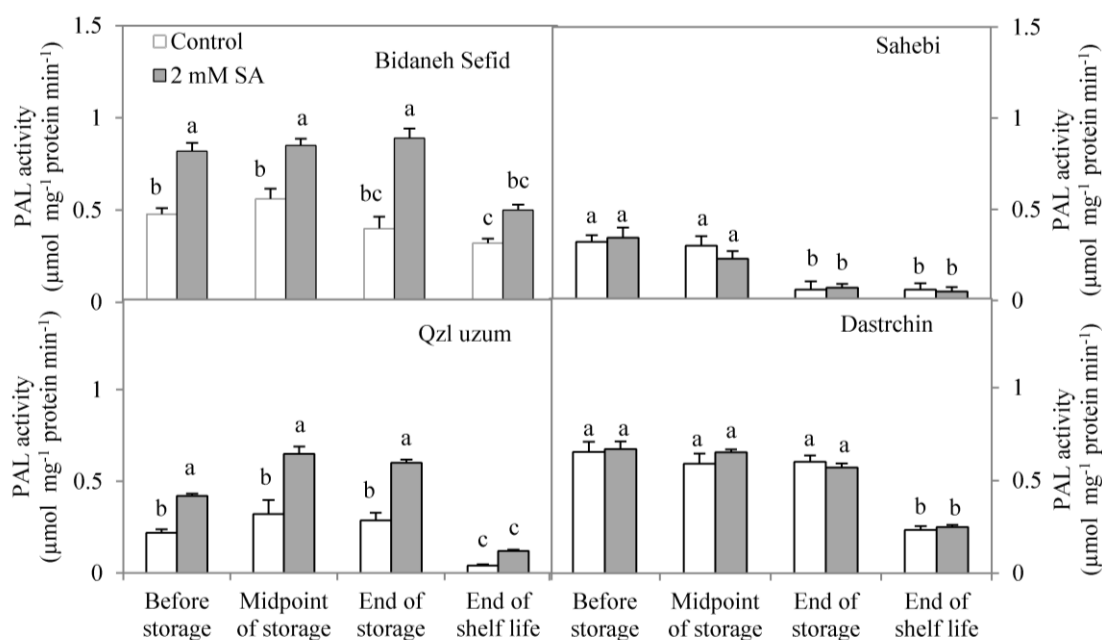
**Fig. 3.** Effect of postharvest SA treatment on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD ( $n=4$ ).



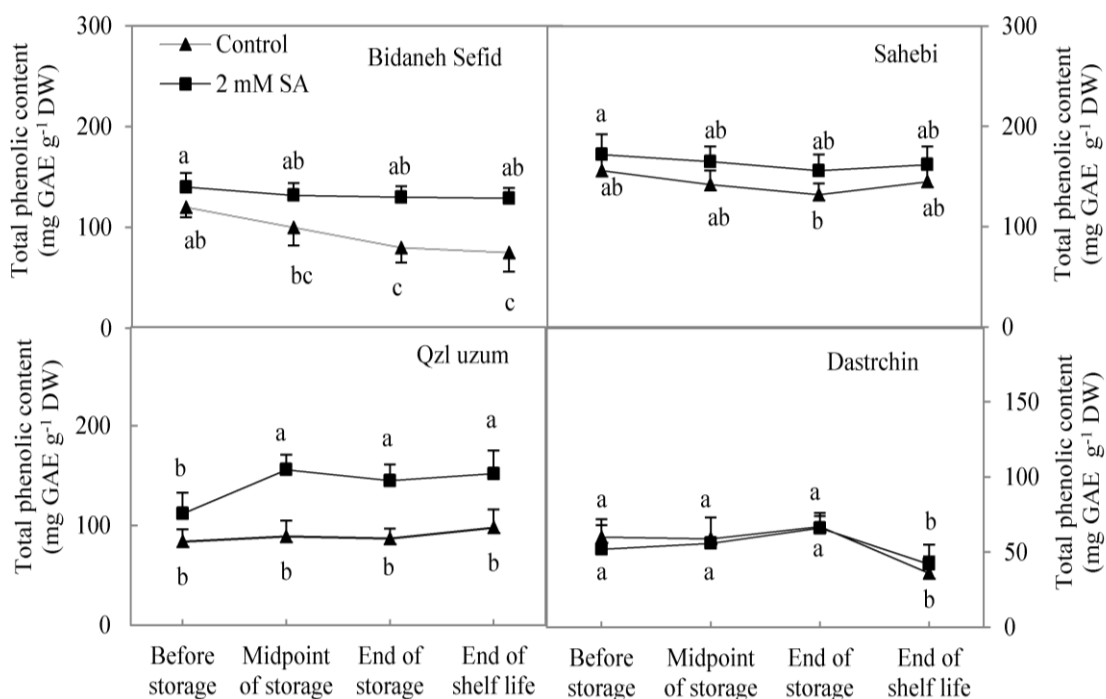
**Fig. 4.** Effect of postharvest SA treatment on the malondialdehyde (MDA) content in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD ( $n=4$ ).

In “Bidaneh Sefid” and “Qzl uzum”, a significant increase in PAL activity was observed at 15 and 30 days of cold storage (Fig. 5). In cultivars “Sahebi” and “Dastrchin”, the activity of PAL showed no significant difference in SA-pretreated bunch compared with that in controls during cold storage. In “Bidaneh Sefid” and “Qzl uzum”, phenolic content in SA-treated bunches was higher than that in the control during cold storage. In cultivars “Sahebi” and “Dastrchin”, however, the changes of phenol levels in the SA-treated bunch and control bunch were similar during cold storage (Fig. 6). In “Bidaneh Sefid” cultivar, there was a significant negative correlation between decay

incidence and PAL activity at 15 and 30+3 days of postharvest life (Fig. 7). Before cold storage, DPPH free radical scavenging activities of bunches extracts were significantly greater than that of fruits at the end of cold storage (Fig. 8). In cultivars “Sahebi” and “Dastrchin”, at each sampling time, there were no significant differences in DPPH free radical scavenging activity in bunches between the SA treatment and the control. In “Bidaneh Sefid” and “Qzl uzum”, however, an increase in DPPH free radical scavenging activities of bunches extracts was observed in SA-treated bunches compared with non-SA-treated bunches during cold storage.

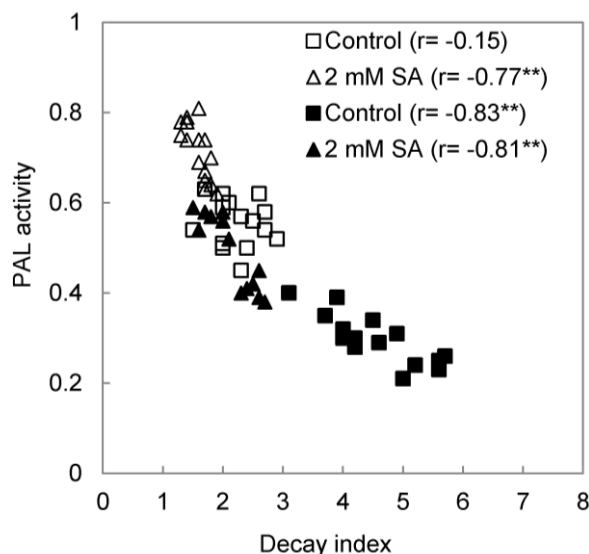


**Fig. 5. Effect of postharvest SA treatment on the on the specific activity of phenylalanine ammonia-lyase (PAL) in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD (n=4).**

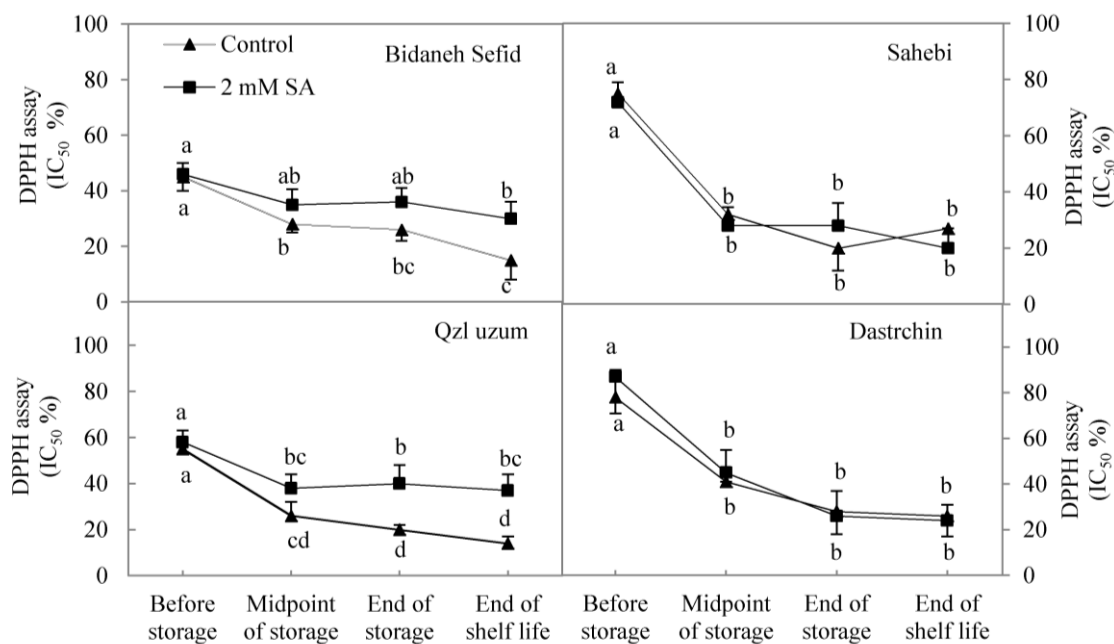


**Fig. 6. Effect of postharvest SA treatment on the total phenol content in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD (n=4).**





**Fig. 7.** Correlations between phenylalanine ammonia-lyase (PAL) activity and decay index in “Bidaneh Sefid” cultivar at midpoint of storage (*open symbols*) and end of shelf life (*closed symbols*). ns: \*, and \*\*: non-significant and significant, at the 5% and 1% levels of probability, respectively.



**Fig. 8.** Effect of postharvest SA treatment on DPPH radical scavenging activity in four grape cultivars at different time intervals before and during cold storage. Bars indicated with the same letter are not significantly different ( $p < 0.05$ ). Values are the mean  $\pm$  SD (n=4).

**Discussion**

Grapes are susceptible to chilling injury when they are stored at low temperatures after harvest (Ranjbarani et al., 2011). In the present study, it was shown that treatment with SA at 2 mM was effective in alleviating fungal decay of “Qzl uzum” and “Bidaneh Sefid”. SA often acts as a

small signaling molecule in plants and triggers the defense mechanisms against pathogens (Wang et al., 2006). The beneficial effect of SA treatment in reducing fungal decay is consistent with previous studies (Asghari et al., 2009; Ranjbarani et al., 2011; Habibi, 2015).

In this study, postharvest treatment of

“Bidaneh Sefid” bunches with SA significantly stimulated SOD and CAT activities only at the midpoint of storage, indicating that SA directly or indirectly activates antioxidant enzymes (Tian et al., 2007). The effect of SA in alleviating decay of “Qzl uzum” and “Bidaneh Sefid” is probably due to a greater activation of antioxidant enzymes. These results are in agreement with the findings of some authors that SA could increase cold tolerance and alleviate the injury caused by low temperature in maize (reference??), strawberry (Karlidag et al., 2009), cucumber (Cao et al., 2009, Tao et al., 2010) and anthurium (Promyou et al., 2012) through the alteration of antioxidant enzyme activities. Lipid peroxidation is one of the adverse effects of chilling injury on plant cells leading to MDA accumulation (Posmyk et al., 2005, Luo et al., 2012). In our experiments SA considerably reduced the rise in H<sub>2</sub>O<sub>2</sub> and MDA in cultivars “Bidaneh Sefid” and “Qzl uzum” during storage, obviously because of an efficient scavenging following significant enhancement of SOD and CAT activities. Lowering of ROS levels through SA treatment was reported by Cai et al. (2015) for barley and Wang et al. (2009) for *Jasminum sambac* under cold stress. These data clearly indicated that damage caused by MDA accumulation could be alleviated by SA treatment in cultivars “Bidaneh Sefid” and “Qzl uzum” under cold stress.

In cultivars “Bidaneh Sefid” and “Qzl uzum”, enhanced phenolic accumulation was correlated to the increase in PAL activity in SA-treated bunches. This is in agreement with Luo et al. (2012) who reported that SA increased cold tolerance of postharvest bamboo shoot by promoting the pool of total phenolic content. Vatterm et al. (2005) reported that phenols can play a role in modulating cellular homeostasis because of their physiochemical properties. However, the involvement of phenolics as regulators of postharvest states and in other

cold-response mechanisms in bunches remains to be established. Since phenolic compounds are natural antioxidants that have a remarkably high scavenging activity against free radicals (Cao et al., 2009), they have an important role in preventing a variety of diseases and decay because these are closely related to the active oxygen and lipid peroxidation. Accordingly, this study showed that the increase of PAL activity was observed to be concomitant with the accumulation of the total phenolic content. There was a significant negative correlation between decay incidence and PAL activity in “Bidaneh Sefid” cultivar. In this study, the analysis of antioxidant activity of bunches by the DPPH method showed that exposure to the SA significantly enlarged the antioxidant potential of the extract. In this work, the increase in antioxidant potential of the bunch extract was connected to the increased synthesis of phenolic compounds, since these compounds exhibit high antioxidant activity (Heim, 2002, Lachowicz et al., 2017).

### Conclusion

The results showed that the cold storage induced lipid peroxidation and decay incidence, while postharvest SA application increased antioxidant activity and total phenolic, reduced malondialdehyde content and lipid peroxidation, and led to increase the bunch's ability to better withstand stressful cold impacts.

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