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Salicylic acid affects antioxidant system of some grape cultivar under cold stress conditions

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Abstract

Salicylic acid (SA) is a plant growth regulator mediates and ameliorates damages caused by cold stress in plant cells. To investigate the effects of SA foliar spray on soluble proteins content, antioxidants enzymes activity catalase (CAT), ascorbate peroxidase (APX), Guaiacol peroxidase (GPX), superoxide dismutase (SOD), Glutathion reductase (GR) and the effects on the amounts of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in two grapevine cultivars 'Thompson seedless' and 'Gizil uzum' an experiment was conducted with 4 SA level (Control and foliar application 1, 6 and 12 hrs before the cold stress treatment) and 5 thermal levels (Control of 22 ^oC and for 1, 3, 6 and 12 hrs subjection to 1^oC as cold treatments) factorial based on CRD with 4 replications under controlled growing condition. The results revealed that with the increase in cold stress subjection, the activity of APX, SOD and GR was decreased. In line, GPX activity with 'Gizil uzum' was increased with the increase in cold subjection treatment. CAT activity in 'Gizil uzum' was increased with cold treatment as well and the highest CAT activity was recorded with 3 hrs cold treatment exposure. SA treatment led to the amended activity of CAT enzyme in 'Gizil uzum' and also the increased activity of APX, SOD and GR in both the cultivars. Otherwise, SA treatment had no significant effect on the activity of GPX. SA treatment (12 hrs before than the cold treatment) led to a reasonable reduction in MDA content. Finally, SA due to its ameliorative effects and the induction of acquired tolerance. Improved the antioxidant enzymes activity and also increased the cold stress tolerance with the studied cultivars and especially with 'Gizil uzum'.

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Introduction

Grapevine is one of the main important cultivated fruits in the world. In 2012, something nearby 70 million tons of grapevines have been pinched up around the world ($FAOSTAT_{2012}$). *Vitis* genes with 60 species are under cultivation in 80 countries and *V.vinifera* is the most economical grapevine in the world (Fennell, 2004). Cold stress in vineyards; a major environmental problem with temperate region can cause great yield loss and the canopy section mainly the agrobacterium affected and weak organ of the canopy are more sensitive to frost and cold injury. *V.vinifera* cultivars may tolerate up to -25 ºC during the dormancy; however, during the growing season they are very sensitive to the cold exposure and the temperatures below zero to -4 ºC may affect and kill all the green parts and even the whole plant (Fennell, 2004; Mills *et al*., 2006). With sensitive cultivars, cold stress may cause wilting of the shoots slow growth response reduction and delay with stomata action (Wilkinson *et al*., 2001). Considering the several responses of the plants to several abiotic stresses it seems that the majority of the effects and the feedbacks are the same between the stressors but, at the same time some stresses have their own special effects and responses (Ferrandino and Lovisolo, 2014). Cold stress apart from the direct effects has some indirect impacts on the plants; the most highlighted ones are the increase in ROS molecules levels like increase superoxide and hydrogen peroxide which damage and deteriorates the main cellular components and also affects overall cellular activity, metabolism and function (Mittler, 2002; Chaves and Oliveira, 2004; Hola *et al.*, 2007).

Some of the predominant physiological effects of cold stress are; the reduction in respiration, variations in enzymes activity, and variations in growth regulators levels and disturbance in photosynthetic electron chain reactions as well as the production of reactive harmful radicals (Bracale and Coraggio, 2003). The plant responses to the low temperature is including the fluctuations in gene expression, proteins and metabolites profile and some profound variations in membrane lipids content and arrangements (Miura and Furmumoto, 2013). Plasma membrane is the first target affected by the cold stress and the main response is the deleterious effects on the membrane viscosity and this has been the primary feedback by the plants subjected to the cold stress (Sharma *et al*., 2005; Wathugala, 2012). Furthermore, sometimes, exposure to the cold stress induces some prominent responses by the plants; of them, variations in proteins concentration and the composition of the phospholipids bilayer are the most highlighted ones (Guy, 1999; Belintani *et al*., 2012). Any loss in the membrane integrity caused by the low temperature goes to the reduction in the ratios of the saturated fatty acids (Mahajan and Thleja, 2005). In rice, cold stress caused a significant increase in the sugar content and also stimulated the expression of some antioxidant enzymes like catalase (Morsy *et al*., 2006). Overall, the majority of the defensive mechanisms are the results of the integrated cooperation of some antioxidant enzymes and components. An appropriate equilibrium has to be established between the defensive mechanisms and, any great variation or reduction in any of the components may cause a great loss in the activity of the system (Scandalios, 1993). Antioxidants hold a tremendous role in the equilibrium maintenance since, these metabolites have the potential to directly react with the several types of ROS molecules and to efficiently scavenge them (Israr *et al*., 2006; Mutlu *et al.*, 2009).

There are some reports on the role of SA on the signal transduction processes in tolerance to biotic and abiotic stresses (Miura and Tada, 2013). Exogenous SA application induced the production of the proteins related to the diverse type of stressors (Yuan and Lin, 2008; Miura and Tada, 2013). SA is a prerequisite component for the systemic acquired resistance (SAR) development. Low temperature is one of main factors limits the production and the quality of the produce (Janda *et al*., 2003; Zhang *et al*., 2011). Recent studies have also reported the effects of SA on cold tolerance, that SA treatment increased the chilling tolerance in maize (Horvath *et al*., 2002),

tomato (Ding *et al*., 2002), banana (Kang *et al*., 2003), grape (Li *et al*., 2005), rice (Wang *et al*., 2009), eggplant (Chen *et al*., 2011) and barely (Mutlu *et al*., 2013). However, the molecular signals involved in SA responses are still waiting to be disclosed. The current study was planned to evaluate the effects of SA on the content and the activity of some antioxidant enzymes in response to cold stress.

Material and methods

Plant material and growing condition

The homogenous and same sized cuttings of *Vitis vinifera* cultivars 'Thompson seedless' and 'Gizil uzum' were prepared from the mother plants growing under controlled condition in greenhouse with regular irrigation and nutrition program. The cuttings were rooted in prelite and later the rooted cuttings were planted in the pots in a greenhouse with 22-25 ºC and 18-20 ºC during the day and night, respectively. The experiment was conducted on two years old cutting. During the cutting maintenance we tried to feed the plants with *Cramer* and *Läuchli (1986)* hydroponic nutrient solution.

Treatments

For running the experiment, the cuttings were trimmed and two shoots, each with two nods were remained. SA treatments were applied when the plants had 10-12 complete leaves. For this, SA (100 $\mu molL^{-1}$) was sprayed on the plants as control (without foliar spry) and 1, 6 and 12 hrs before the cold stress exposure. After each SA treatment, the plants were transferred to the cold room (for the cold treatment exposure) with +1±0.5 ºC for 1, 3, 6 and 12 hrs and the control of 22 ºC. After the treatments (Cold exposure) the young leaves were immediately harvested and were breezed in liquid nitrogen and kept in -80 ºC freezer until use for analysis.

Hydrogen peroxide measurements

0.2 g of the plant material was homogenized in 2 ml of 0.1% Tricloroacetic acid and the centrifuged at 12000 g for 15 minutes. 0.5 ml supernatant was added to 0.5 ml of phosphate buffer (10 mmol, pH=7) and 1 ml of Iodide potassium (1 mol). The samples absorbance was measured at 390 nm. Standard curves were established with the different concentrations of Hydrogen peroxide (Sergiev *et al*., 1997).

MDA measurements

0.2 g of the plant sample was homogenized in 2 ml of 20% Tricloroacetic acid containing 0.05% TBA. The samples later were incubated in 95 ºC for 30 minutes and they were transferred to the ice. The samples were then centrifuge at 10000 rpm for 10 minutes and the absorbance was measured at 532 and 600 nm (Steward and Bewleyl, 1980).

Antioxidant enzymes assay

For the extraction of catalase (CAT), Guaiacol peroxidase (GPX), superoxide dismutase (SOD), Glutathion reductase (GR) and soluble proteins, 0.2 g of the sample was homogenized in liquid nitrogen. 2 ml phosphate buffer (pH=7.5) containing, EDTA (0.5 mol) was added. The samples were incubated in 4 ºC for 15 minutes and were centrifuged at 15 rpm (Sairam *et al*., 2002).

Due to the instability and very low half-life of ascorbate peroxidase with ex-vivo conditions and for the keeping structure of the compound, we tried to use polyvinylpyrrolidone 5% and ascorbat at 2 ml to the respected enzyme solution (Yoshimura *et al*., 2000).

For the activity measurement of GPX the reaction mixture was contained 1 ml phosphate buffer (100mmol, pH=7) along with EDTA (0.1 mmol), 1 mL guaiacol (15 mmol), 1 ml H_2O_2 (3 mmol) and 50 µL of the extracted enzyme solution. The reaction response was measured at 470 nm for 1 min (Yoshimura *et al*., 2000). For CAT, the reaction mixture was containing 1.5 ml phosphate buffer (100 mmol, pH=7), 0.5 ml H_2O_2 (7.5 mmol) and 50 µL of extracted enzyme solution. The absorbance at 240 nm during 1 minute was measured (Aebi, 1984). APX was assayed as; the reaction mixture was containing 250 µL phosphate buffer (pH=7) along with EDTA, 10 µL H_2O_2 (1 mmol) , 250 µL sodium ascorbate (0.25 mmol) and 50 µL

enzyme solution. The absorbance was measured at 290 nm (Yoshimura *et al*., 2000). GR assay was in a reaction mixture containing 1 ml phosphate buffer (200 mmol, pH=7.5) containing EDTA, 0.5 ml 5, 5-ditiobis-2-benzoic acid (3 mmol) dissolved in phosphate buffer. 0.1 ml of NADPH (2 mmol) and 0.1 ml of enzyme solution. Reaction was began with the addition of glutathione oxide (2 mmol) and the absorbance was recorded at 412 nm within 1 minute (Sairam *et al*., 2002). SOD activity was evaluated in a reaction mixture having 1.6 ml phosphate buffer (100 mmol) containing 0.1 ml EDTA, and sodium carbonate (1.5 M), 0.2 ml of L-methionine (0.2 M), 1 ml of distilled water, 0.1 ml of NBT (2.25 mM) and 50 µL of enzyme solution. The enzyme reaction and activity was initiated with the addition of 0.1 ml riboflavin (60 µL) and the samples absorbance was recorded at 560 nm (Sengupta *et al*., 1993).

Soluble protein content

Reaction solution was contained 100 µL of enzyme solution, 200 µL of Bradford regent and 700 µL of deionizer water. 2 minutes after the complex formation, Bradford regent shows the highest integration with the amino acids. Absorbance was evaluated at 535 nm. Protein content of the samples was calculated based on standard curve obtained from the defined amounts of bovine serum albumin (Bradford, 1976).

The data were subjected for statistical analysis by SPSS software and the mean compansons were conducted by Duncan's multiple range tests.

Results

ANOVA result for MDA content revealed that the main and the interaction effects of low temperature, SA treatment and cultivar were significant (P≤0.01) (Table 1). Furthermore, the fig.1 depicts that with any increase in cold treatment exposure, MDA content in the leaves dramatically increased. This results says that cold treatment caused the oxidative stress and hence the increase in the MDA production. SA treatment (12 hours before than cold treatment) statistically decreased the MDA concentration in the leaves and so reduced the incidence and the deterioration of the membranes and caused severe lipids Peroxidation.

Table 1. Effect of exogenous SA pretreatment on antioxidant enzymes content and protein, H_2O_2 and Malondialdehyde content in grape leaves under cold (1 °C) stress.

Value source	df	CAT	APX	Pro	GPX	SOD	GR	MDA	H_2O_2
Factor A	4	1.02**	0.163**	0.033**	0.201ns	1195.401**	0.192**	787696.412**	0.007*
Factor B	3	0.583**	0.308**	0.015**	0.38ns	86.1**	0.142**	208709.06**	0.002ns
Factor C	1	1.626**	0.308**	0.078**	1.73**	420.034**	0.289**	372765.071**	0.02**
AB	12	0.067**	0.025**	0.007**	0.304ns	47.905**	0.012ns	58155.428**	0.004ns
AC	4	0.398**	0.008ns	0.024**	1.981**	737.98**	0.038*	47935.947**	0.012**
BC	3	0.017ns	0.043**	0.006**	0.368ns	169.306**	0.047*	17142.906**	0.002ns
ABC	12	0.026ns	0.013ns	0.004**	0.227ns	84.573**	0.004ns	8172.425**	0.004ns
error	120	0.022	0.008	0.001	0.169	17.788	0.013	788.43	0.003

Factor A: temperature treatment; Factor B: salicylic acid treatment; Factor C: cultivar; ns, *, ** nonsignificant, significant at p≥0.05 or 0.01 respectively

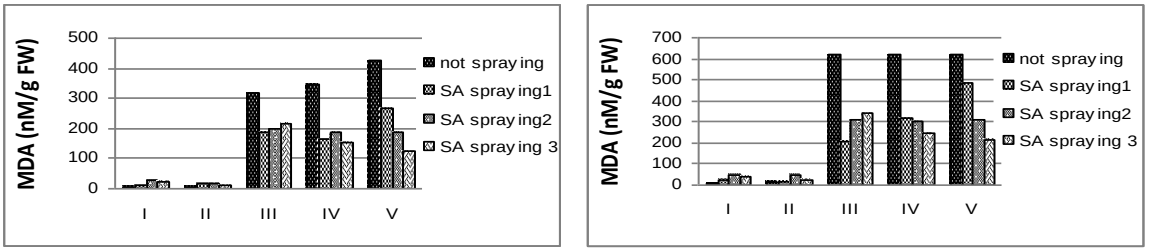

Fig. 1. Effect of exogenous SA pretreatment on MDA in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 °C); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

Data analysis for SOD showed that the main and the interaction effects of cold stress treatment, SA treatment and the cultivar were significant (P≤0.01) as well (Table 1). In 'Gizil uzum' with increase in the cold stress treatment, the SOD activity increased was compared to the control. However, in 'Thompson seedless' SOD activity statistically reduced by any increase in the cold treatment exposure (Fig. 2).

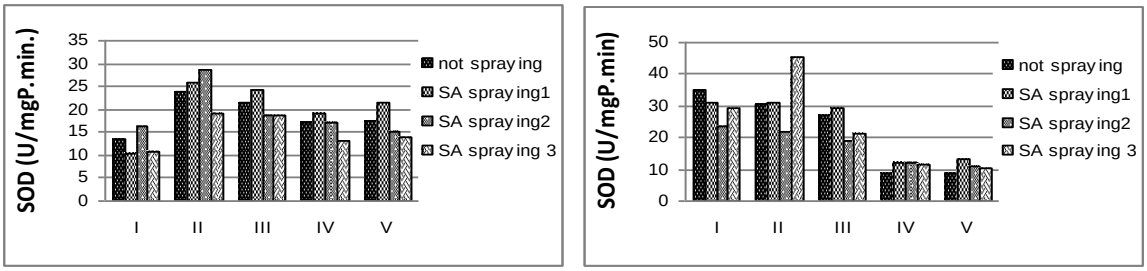

Fig. 2. Effect of exogenous SA pretreatment on SOD in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 ºC); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

ANOVA result revealed that for soluble protein content, the interaction effects of all the three factor were signification (P≤0.01) as well. The lowest content for soluble proteins in both cultivars were belonged to the stress treatment (12 horses in 1 ºC). SA treatment (1 horse before the cold exposure) meaningfully increased the protein content in the leaves (Fig. 3).

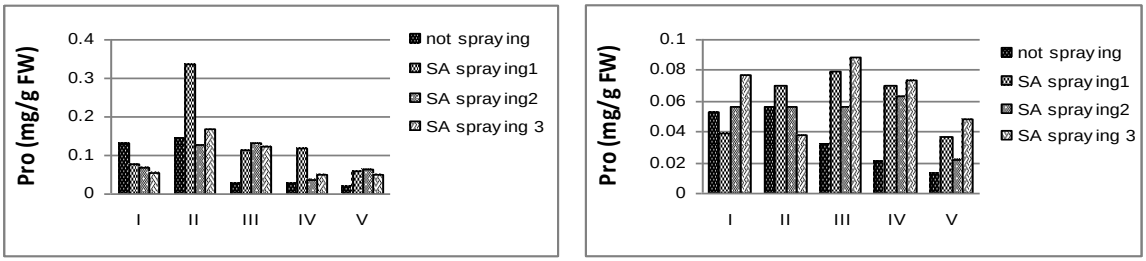

Fig. 3. Effect of exogenous SA pretreatment on Protein in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 ºC); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

The remaining results demonstrate the significant (P≤0.1) effects of cold stress treatment × SA and cold stress treatment × cultivar for CAT activity, but the interaction effects of the SA × cultivar and the triple interaction effects were not significant (table 1). CAT activity in 'Gizil uzum' was significantly increased with prolonged cold exposure compared to 'Thompson seedless'. The highest amount for this trait was recorded in cold treatment of 3 hours exposure at 1°C (Fig. 4).

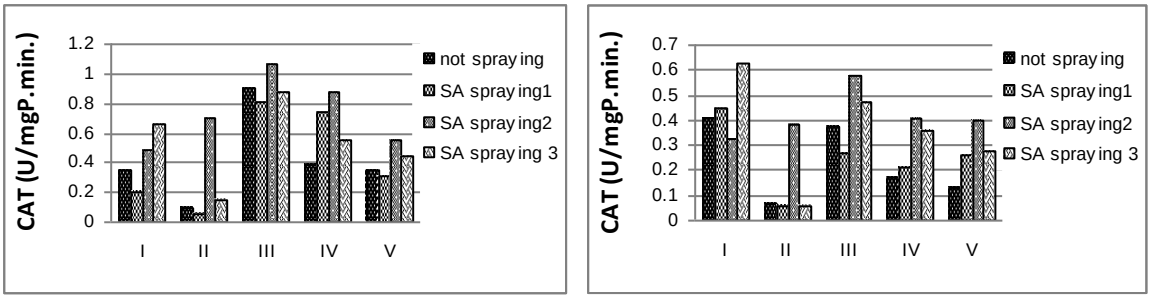

Fig. 4. Effect of exogenous SA pretreatment on CAT in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 ºC); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

The results related to APX activity disclosed the significant (P≤0.1) effects of the main and cold stress × SA and cultivar × SA. However, with this trait, the effects of cold treatment × cultivar and the triple interaction effects were not significant statistically. With the prolonged cold exposure, the APX amount was reasonably decreased and SA treatment (1 hour before the cold treatment) had the most ameliorative effects on the activity of APX (Fig. 5).

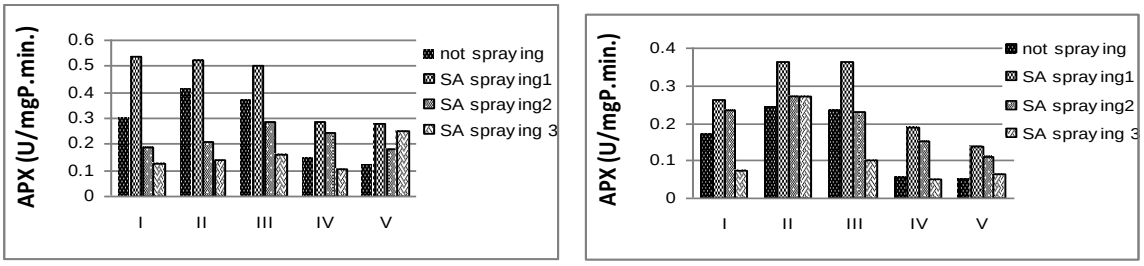

Fig. 5. Effect of exogenous SA pretreatment on APX in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 °C); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

About GPX; the effects for the cultivars and the interaction effects of cold treatment and cultivar were significant (P≤0.1), but all the other main and interaction effects were non-significant. In 'Gizil uzum' GPX content was increased with cold treatment; however, in 'Thompson seedless', the GPX content and activity was dedlined with any increase in cold environment exposure (Fig. 6).

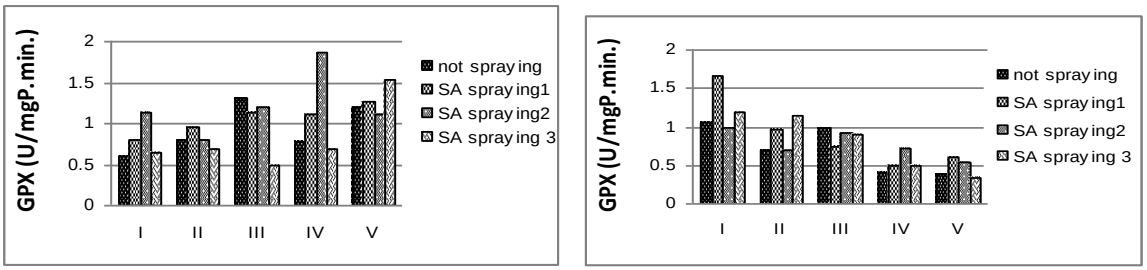

Fig. 6. Effect of exogenous SA pretreatment on GPX in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 °C); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

GR activity was meaningful (P≤0.01) for the main effects of cold treatment, SA treatment and cultivar as well as for the interaction effects of cold × cultivar (P≤0.5) and SA × cultivar (P≤0.5). Meanwhile, the results for cold stress × SA and the three-sided interaction effects were not meaningful (table 1). With cold stress treatment (3 hours at 1°C) and in both cultivars, the highest GR was recorded. Also, with the SA treatment; the treatment of 2 hours before the cold stress treatment went to the highest GR activity (Fig. 7).

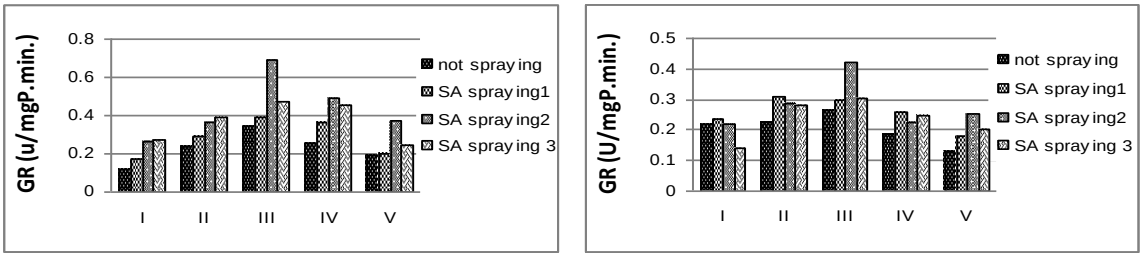

Fig. 7. Effect of exogenous SA pretreatment on GR in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 °C); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

Considering the H_2O_2 content, the results showed that the amount for this compound was different ($P \leq 0.01$) in cultivars × cold treatment interaction treatments (table 1). H_2O_2 amount and accumulation in 'Gizil uzum' was more highlighted than the other cultivar i.e. to 'Thompson seedless' (Fig. 8).

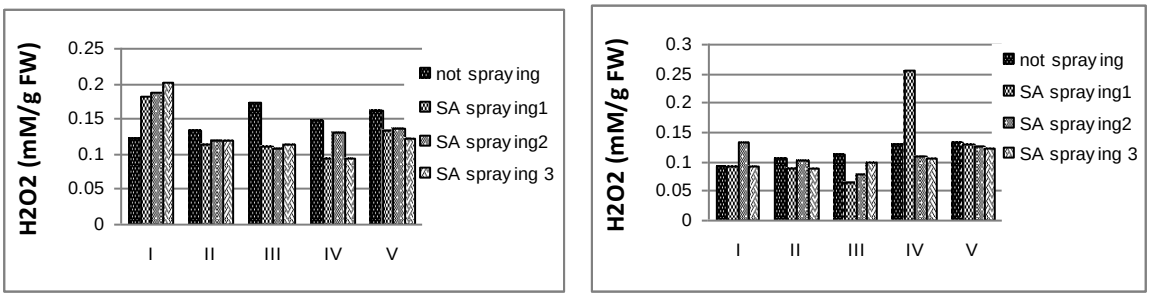

Fig. 8. Effect of exogenous SA pretreatment on H_2O_2 in 'Gizil uzum' and 'Thompson seedless' (from left to right) under cold stress. I: control (22 ºC); II, III, IV, V: 1, 3, 6 and 12 hrs under cold stress respectively.

Discussion

In the current study, exogenous SA application frequently increased the antioxidant enzymes activity in grapevine leaves under or exposed to cold stress treatment (table 1). The idea is that SA induces the cold stress tolerance in the grape plants .Improvements in the tolerance to the stress is commonly due to the increase in the antioxidant system activity like SOD, GR, APX and CAT. These defective enzymes and compounds play a pivotal role in the plants (Feng *et al.*, 2008). Moreover, increase in the activity of GR keeps the GSH/GHS+GSSG ratio at an optimal level and hence improves the defensive mechanism of the cells and later goes to the amended tolerance and the plants to the abiotic and environmental stressors (Sairam *et al.*, 2002; Limone-Pacheco and Gonsebatt, 2009).

In the current experiment, with any increase in the MDA level; as an index for the oxidative deteriorative effect, membranes lipid peroxidation was significantly increased (table 1). Low levels of MDA in the leaves of ‹Gizil uzum› demonstrate that this cultivar was more tolerant to the oxidative damages caused by low temperature. Regarding, in a previous study with two sensitive and tolerant cultivars and maize the results reported was that same as our experiment (Janda *et al.*, 2005). MDA amount was drastically increased with sensitive cultivar. Lipid peroxidation is one of the major cell targets directly affected by free radicals (Soumen, 2005). Low temperament stress is in line with the direct accumulation of MDA and ROS molecules in the plat cell; which they triggers the generation and accumulation of another types of ROSs (Zhou *et al.*, 2005).

Cold stress treatment significantly increased the H_2O_2 content in 'Gizil uzum' leaves. The overall idea is that under stress conditions due to the unbalanced equilibrium in the generation and scavenging of ROS molecules, their concentration in the cells increases profoundly. From these, H_2O_2 is a predominant messenger molecule that initiates a cascade of events for the protection of plant cells and whole organism against diverse stressors (Wan etal, 2009). For example; H_2O_2 stimulates and activates the Ca^{2+} canals in plant systems. SA as a protective agent induces the production of H_2O_2 and hence the running of other protective pathway to maintain the cell integrity and to preserve the whole plant life cycle (Hayat and Ahmad, 2007).

APX with aiming the ASA cycle serves as an electron donor for the scavenging of H_2O_2. In a study on pea plants, SA external application under warm conditions led to the increase in the activity of peroxidase and ascorbate peroxidase (Chakraborty and Tongden, 2005).

Cold treatment goes to the reduction in SOD activity in 'Thompson seedless'. However, SOD activity in 'Gizil uzum' was increased with the SA application exposed to cold stress. Moreover, some researchers reported the meaningful decrease in SOD activity under salinity condition in pepper (Turhan *et al*., 2006) and under cold stress in barley (Mutlu *et al*., 2013). In an experiment on bean and tomato plants, the exogenous application of SA under stressful conditions reduced the activity of SOD and peroxidase (Senaratna *et al*., 2000). In a similar study, the same increase in the activity of those two enzymes was reported under the cold stress conditions (Janda *et al*., 1999). Meanwhile, the exogenos effects of the SA application has not been approved on SOD apoplastic activity with cold stressful situations, but, the new data reveals that under salinity conditions the SOD activity was increased with SA application (Turhan *et al*., 2006; Mutlu *et al*., 2009).

SA treatment before the cold stress treatment led to the significant increase in the CAT activity with both cultivars. The increase in 'Gizil uzum' was higher than 'Thompson seedless'. CAT activity induced by biotic and abiotic stressors has been frequently reported in several plant species (Hernandez *et al*., 2000; Patykowski and Urbanek, 2003; Tasgin *et al*., 2006; Mutlu *et al*., 2009). CAT biosynthesis is the most efficient mechanism in scavenging the H_2O_2 in apoplastic media (Patykowski and Urbanek, 2003).

Some researchers reported that the plant genotypes tolerant to stresses have the most efficient potential in the scavenging of harmful radical (Mutlu *et al*., 2009; Mallik *et al*., 2011; Zhang *et al*., 2011).

Conclusion

Overall, the results revealed that stress treatment had the prominent impact on ROS content and activity and the ROS accumulation in the vegetation initiates the antioxidant defensive system in plants. ROS over production oxidizes the lipid molecules in phospholipids bilayer membrane and hence has a damaging effect on cell structure and function. Enzymatic defense system for the prevention of cell deterioration and keeping the homeostatic optimal environment and situation is the first option for the plant cell to keep the living system alive. If the stressful situation is strict and prolonged, the defense system is not enough potentiate to combat with the ROS molecules. In these situations, the activity of antioxidant enzymes drastically declines and the side-effects derived from the enlarged pool ROS molecules affects the living system and eventually goes to the cell and plant damage and death.

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