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Reduction of Chilling Injury and Peroxide Hydrogen Accumulation in Thompson Grape (*Vitis vinifera* L.) Fruit by Proline and Ascorbic Acid During Storage

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Abstract
This experiment was carried out to determine the effects of proline and ascorbic acid on chilling injury, electrolyte leakage**,** lipid peroxidation, peroxide hydrogen and antioxidant enzymes activity of Thompson grape (*Vitis vinifera* L.) fruit. Grape fruits were treated by 0 (control), 250 and 500 µM proline and 0 (control), 2 and 4% ascorbic acid and their combinations, then stored at 1°C and 90-95 % relative humidity for 28 days. Grapes treated with 500 µM proline and its combination with 2 and 4% ascorbic acid had the lowest amount of lipid peroxidation and peroxide hydrogen and lower chilling injury and electrolyte leakage than non-treated fruits. Moreover, grapes treated with 500 µM proline and its combination with 2 and 4% ascorbic acid significantly increased ascorbate peroxidase, superoxide dismutase, catalase and peroxidase enzyme activity. The present study can be regarded as the first evidence that proline and ascorbic acid enhance grape fruit tolerance to cold stress and therefore fruit quality.

Keywords: Antioxidant enzyme; Grape; Lipid peroxidation; Storage

Introduction

All fresh fruits including grapes are inherently perishable. During the process of distribution and marketing, substantial losses are incurred which ranges from a slight loss of quality to total spoilage. Postharvest losses may occur at any point in the marketing process, from the initial harvest through assembling and distribution to the final consumer. The causes of losses are many factors such as: physical damage during handling and transport, physiological decay, water loss, or sometimes due to glut in the market and there are no customers (Mlikota Gabler 2005). As grape is a non-climacteric fruit with a very short postharvest life, using methods such as pre-cooling and storage in low temperatures is essential to minimize water loss, avoid decay and reduce the physiological and metabolic activities of grape (Ahumada *et al*. 1996). Several promising methods have been developed to increase shelf life of grape fruit. These include postharvest physical treatments (Mlikota Gabler 2005), modified atmosphere packaging (Ahumada *et al*. 1996) temperature conditioning and chemical treatments with plant growth regulators (Crisosto *et al.* 1994). Proline accumulates in higher plants in response to abiotic and biotic stresses such as water and chilling stress (Strizhov *et al.* 1997). Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and is essential for primary metabolism.

It has been reported that proline content was increased in potato hybrids when plants subjected to cold treatment (Verbruggen *et al.* 1993). A frequent reaction of plants exposed to stressful conditions such as dehydration or cold is the production of the amino acid proline (Swaaij *et al.* 1985). Since the first report on proline accumulation in wilting perennial rye grass (*Loliumperenne*) (Kemble and MacPherson 1954),

proline accumulation has been reported during conditions of drought (Chaudhary *et al.* 2005), high salinity (Yoshiba *et al.* 1995), high light and UV irradiation (Saradhi *et al*. 1995), heavy metals (Schat *et al.* 1997), oxidative stress (Yang *et al*. 2009) and in response to biotic stresses (Fabro *et al.* 2004; Haudecoeur *et al.* 2009). An osmo-protective function of proline was discovered first in bacteria, where a causal relationship between proline accumulation and salt tolerance has long been demonstrated (Csonka and Hanson 1991). Such data led to the assumption that proline accumulation in stressed plants has a protective function, which has been emphasized by numerous reviews (Bates *et al*. 1973). Studies using transgenic plants, indicate that proline metabolism has an important effect on development and stress responses, and that proline accumulation is necessary for tolerance to unsuitable environmental conditions (Madan *et al*. 1994). In some plant species such as potato, proline plays a major role in osmotic adjustment (Bussis and Heineke 1998).

Ascorbic acid is an abundant antioxidant in plant cells and serves as the major contributor to the cell redox state (Smirnoff 2000). It is primarily known for its antioxidant properties, but it also acts as a cofactor for various enzymes and further contributes to the regulation of cell division and expansion (Smirnoff and Wheeler 2000). It is essential for plant growth (Alhagdow *et al.* 2007; Dowdle *et al.* 2007) and seems to control flowering time and the start of senescence (Davey *et al*. 2000). In addition, ascorbic acid could act as signal ingagents (Fotopoulos *et al.* 2008) participating in the interaction with the environment. It is the most effective compound which increases the tolerance of the plants to oxidative stresses. A recent increase of evidences suggests that it may play a role in protection of plant against several environmental stresses such as heavy metal action (Vwioko *et al*. 2008), salinity (Shalata and Neumann 2001) and water loss (Fotopoulos *et al*. 2008). Previous studies have shown that ascorbic acid may alleviate chilling injury at the whole-plant level and also when the fruits are treated by it. When banana fruits were treated with 1% ascorbic acid, the cold tolerance of the fruits increased (Shivashankar 2000).

The objectives of this study were to evaluate the effects of proline and ascorbic acid on chilling injury, lipid peroxidation content, peroxide hydrogen content, and the induction of antioxidant enzymes, such as ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), in Thompson grape (*Vitis vinifera* L.) fruit during storage at 1°C.

Materials and Methods

Thompson grape (*Vitis vinifera* L.) fruit and treatments

Thompson grape (*Vitis vinifera* L.) fruits were harvested at maturity (when total soluble solid (TSS) of the grape fruit was 7-8%) from a commercial orchard in Kerman, Iran, and transported to the laboratory immediately. Grapes were selected based on uniformity of size and absence of physical injury or disease. The harvested fruits were disinfected with 1% sodium hypochlorite (v/v) for 2 min, washed and dried in air. Subsequently, they were randomly divided

into 9 groups. Eight groups were immersed in aqueous solution containing, 250 and 500 µM of proline and 2 and 4% of ascorbic acid and their combinations for 5 min, based on our preliminary experiments. The ninth group was immersed in distilled water for 5 min and served as a control. All fruits were enclosed in plastic boxes with polyethylene film bags to maintain the relative humidity at about 95% and stored at 1°C.

Chilling injury assessment

Chilling injury (%) was calculated by the following formula (Roberts *et al*. 2002):

[(tTotal number of fruits in each treatment - number of fruits with no chilling injury/(total number of fruits in the treatment)]× 100.

Electrolyte leakage assessment

Electrolyte leakage was determined according to Mao *et al*. (2007). Slices of grape tissues were excised with a 10 mm diameter stainless steel cork borer. Two pieces with 4 mm thickness were cut from each slice. After being rinsed three times (2-3 min) with deionized water, 10 pieces were put into 50 ml of deionized water and were shaked at 100 cycles/min for 30 min. Conductivity was measured with a conductivity meter (Model Ec215). Total conductivity was shown after keeping the flasks boiling for 10 min, and the relative electrolyte leakage was expressed as a percentage of total conductivity.

Estimation of lipid peroxidation

Lipid peroxidation was estimated by determining the malon dialdehyde (MDA) content in the leaves according the method of Rajinder *et al*. (1981). A 100 milligram of fruit sample was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000g for 5 min at 4°C. Aliquot of 0.3 ml supernatant was mixed with 1.2 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA 20%, and incubated at 95 °C for 30 min. After stopping the reaction in an ice bath for 5 min, samples were centrifuged at 10000g for 10 min at 25°C. The supernatant absorbance at 532 nm was then measured using a Beckman UV-DU 520 spectrophotometer. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient of 155 $mM^{-1}cm-^{1}$.

Hydrogen peroxide assay

The assay for H_2O_2 content was carried out by the procedure of Patterson *et al*. (1984). Fresh tissues (2g) were homogenized with 10 ml of acetone at 0°C. After centrifugation for 15 min at 6000g at 4°C, the supernatant phase was collected. The supernatant (1 m1) was mixed with 0.1 ml of 5% titanium sulphate and 0.2 ml ammonia, and then centrifuged for 10 min at 6000g and 4°C. The pellets were dissolved in 3 ml of 10% (v/v) H_2SO_4 and centrifuged for 10 min at 5000g. Absorbance of the supernatant phase was measured at 410 nm. H_2O_2 content was calculated using H_2O_2 as a standard and then expressed as $\mu molg^{-1}$ on fresh weight basis (Prasad *et al*. 1994).

Superoxide dismutase assay

The activity of superoxide dismutase was assayed according to Misra and Fridovich (1972). About 200 mg fresh tissues were homogenized in 5 ml of

100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) TritonX-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered through muslin cloth and centrifuged at 22000g for 10 min at 4-8°C. Supernatant was dialyzed in cellophane membrane tubings against the cold extraction buffer for 4 h with carbonate bicarbonate buffer and then used for the assay. The assay mixture in a total volume of 3 ml contained 50 mM sodium carbonate bicarbonate buffer (pH 9.8), 0.1 mMEDTA, 0.6 mM epinephrine and enzyme. Epinephrine was the last component to be added. The adrenochrome formation in the next 4 min was recorded at 475 nm in a UV-Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. The specific activity of enzyme is expressed as unit mg protein^{-1}**.**

Ascorbate peroxidase assay

Ascorbate peroxidase was assayed according to Nakano and Asada (1981). Reaction mixture in a total volume of 1 ml contained 50 mM K-phosphate buffer (pH 7.0), 0.2 mM ascorbic acid, 0.2 mM EDTA, 20 mM H_2O_2 and enzyme. H_2O_2 was the last component to be added and the decrease in absorbance was recorded at 290 nm (extinction coefficient of 2.8 mM^{-}1 cm^{-}1) using a UV-Vis spectrophotometer. Correction was made for the low, non-enzymatic oxidation of ascorbic acid by H_2O_2. The specific activity of enzyme is expressed as unit mg protein^{-1}.

Catalase and peroxidase assay

Catalase and peroxidase activity was analyzed according to Xing *et al*. (2011), with a slight modification. The reaction mixture consisted of 2 ml sodium phosphate buffer (50 mM, pH 7.0), 0.5 ml H_2O_2 (40 mM) and 0.5 ml enzyme extract. The decomposition of H_2O_2 was measured by the decline in absorbance (A) at 240 nm. CAT specific activity was expressed as U kg^{-1} of FW, where U= Δ-A at 240 nm per s. For POD determination, 0.5 ml enzyme extract was incubated in 2 ml buffered substrate (100 mM sodium phosphate, pH 6.4 and 8 mM guaiacol) for 5 min at 30◦C and the increase in absorbance was measured at 460 nm every 30 s for 120 s after adding 1 ml of H_2O_2 (24 mM). POD and CAT activity was expressed as U.mg protein^{-1}, where U= ΔA at 470 nm per s.

Protein assay

Protein content in the enzyme extract was estimated using the Bradford (1976) method. Specific activity of the enzyme was expressed as units per milligram protein.

Statistical analysis

The experiment was arranged as factorial based on randomized complete block design with three replications. After analysis of variance the means were compared ($p \leq 0.05$) by Duncan's multiple rang test. All analyses were performed by the SAS program.

Results

Chilling injury (CI) and electrolyte leakage (EL)

The effect of proline and ascorbic acid on CI and EL of the grape fruit during cold storage is shown in Tables 1 and 2. In the untreated control grape fruit, CI symptoms occurred at 7 days after storage, and the CI index was as high as 53% at 28 days in the control grape (Table 1). In grape fruit treated with proline and ascorbic acid, CI symptoms occurred at 14 days after storage (DAS). Moreover, the CI and EL treated with proline and ascorbic acid fruits were significantly lower compared to the control fruit. Among all the treated fruits, 500 µM proline and its combinations with 2 and 4% ascorbic acid were the most effective in alleviating CI and EL (Tables 1 and 2).

Lipid peroxidation and H_2O_2 content

Lipid peroxidation content has been used as the direct indicator of membrane injury, which is often associated with CI and EL. As shown in Table 3, a continuous increase in lipid peroxidation content was observed, both in the control and in the treated grape fruits stored at 1°C, however, the application of proline and ascorbic acid to grapes significantly delayed the increase of lipid peroxidation during storage. At the end of the storage period (Day 28), the lipid

Table 1. Effect of proline and ascorbic acid on chilling injury of grape fruit during storage

Treatment combination		Time (day)			
Proline (mM)	Ascorbic acid (%)	7	14	21	28
		Chilling injury (%)			
0	0	12.66 e	20.33 c	37.00 b	53.00 a+
	2	5.60 i-m	9.33 fg	13.66 pe	21.66 c
	4	4.33 klm	6.33 g-l	8.66 f-i	16.00 d
250	0	6.60 f-k	9.60 f	4.30 de	19.60 c
	2	4.33 klm	5.66 i-m	9.00 fgh	14.00 de
	4	3.33 lm	5.66 i-m	7.33 f-k	15.33 de
500	0	4.33 klm	7.60 f-j	9.00 fgh	14.00 de
	2	3.30 lm	6.00 h-m	7.00 f-k	14.66 de
	4	3.00 m	4.66 jklm	6.66 f-k	13.00 e

+Means with different letters are significantly different ($p \leq 0.05$).

Table 2. Effect of proline and ascorbic acid on electrolyte leakage of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Electrolyte leakage (%)				
0	0	6.78 opq	10.85 fjk	15.89 fg	29.25 b	51.08 a
	2	7.42 opq	8.16 mno	11.79 i	15.64 fgh	23.67 d
	4	6.21 q	7.13 opq	10.00 jkl	12.01 i	16.47 g
250	0	7.41 opq	7.87 nop	11.07 ij	15.87 fg	21.59 e
	2	8.83 lmn	7.01 opq	10.03 jkl	11.09 ij	16.73 f
	4	7.14 opq	6.82 opq	9.17 lm	10.69 ijk	15.09 gh
500	0	6.73 pq	7.00 opq	9.61 kl	11.54 i	15.50 fgh
	2	7.18 opq	6.50 pq	9.55 kl	11.38 i	14.35 h
	4	7.06 opq	7.09 opq	9.36 lm	10.96 ij	14.47 h

+Means with different letters are significantly different ($p \leq 0.05$).

Table 3. Effect of proline and ascorbic acid on lipid peroxidation of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Lipid peroxidation (mg/g Fresh Weight)				
0	0	15.25 o	17.55 l	25.07 e	28.98 b	42.30 a
	2	15.34 o	16.34 m	19.54 i	21.35 g	27.40 c
	4	15.30 o	15.47 o	16.28 m	18.54 jk	22.10 f
250	0	15.24 o	16.32 m	18.92 j	22.07 f	26.38 d
	2	15.25 o	15.53 o	15.57 o	18.24 k	20.37 h
	4	15.28 o	15.34 o	15.71 no	18.33 k	20.51 h
500	0	15.16 o	15.41 o	16.14 mn	18.32 k	21.22 g
	2	15.24 o	15.55 o	15.56 o	18.45 jk	20.33 h
	4	15.23 o	15.41 o	15.62 k	18.09 k	20.20 h

[+]Means with different letters are significantly different ($p \leq 0.05$).

peroxidation content of samples treated with proline and ascorbic acid at 500 µM proline and its combination with 2 and 4% ascorbic acid were significantly lower compared to the lipid peroxidation of the control samples (Table 3).

Changes in H_2O_2 content of grape fruit are presented in Table 4. The initial H_2O_2 content was low in the grape fruits. In general, H_2O_2 content increased as storage time increased. At the end of the storage period (28 DAS), the H_2O_2 content of proline- and ascorbic acid-treated samples were lower than the control (Table 4). The increase of H_2O_2 content in the control groups were much higher than that in the proline- and ascorbic acid-treated grapes.

Induction of antioxidant enzymes

As illustrated in Tables 5 to 8, proline and ascorbic acid treatments (especially 500 µM proline plus 2 and 4% ascorbic acid) induced the activity of APX, SOD, CAT and POD enzymes in the grape fruit stored at 1°C. APX, SOD, CAT and POD activity showed a similar pattern, during storage (Tables 5-8). In the control treatment, activity of APX, SOD, CAT and POD enzymes peaked at the end of the storage period (28 DAS).

Table 4. Effect of proline and ascorbic acid on hydrogen peroxide of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Hydrogen peroxide (µg/g Fresh Weight)				
0	0	10.51 u	15.91 l	23.87 e	30.12 b	38.12 a
	2	10.39 u	14.02 n	17.15 k	22.62 f	26.60 c
	4	10.23 u	11.43 rst	13.11 o	17.63 j	21.14 g
250	0	10.52 u	13.16 o	16.30 l	21.14 g	25.34 d
	2	10.22 u	11.08 t	11.99 q	23.85 e	19.67 i
	4	10.25 u	10.53 u	11.67 qrs	15.88 l	19.31 i
500	0	10.18 u	11.29 st	12.52 p	16.32 l	20.43h
	2	10.21 u	10.31u	11.84 qr	15.21m	19.35i
	4	10.17 u	10.54 u	11.44 rst	15.12 m	19.38 i

[+]Means with different letters are significantly different ($p \leq 0.05$).

All enzymes in the grapes treated with 500 µM proline plus 2 and 4% ascorbic acid showed higher levels compared to control treatments during the entire storage period. All proline and ascorbic acid treatments induced a higher APX, SOD, CAT and POD activity than that the control fruits (Tables 5-8).

Discussion

Chilling injury (CI) is a major factor in reducing the quality and shortening the storage time of temperate fruits, including grape. To prevent CI progress and extend shelf life, a number of strategies, including physical and chemical treatments, have been evaluated (Verbruggen *et al*. 1993; Hughes and Dunn 1996; Shivashankar 2000; Yadegari *et al*. 2007). In the present study, proline and ascorbic acid were applied and the results indicated that proline and ascorbic acid significantly reduces CI and EL of grape fruits during storage at 1°C (Tables 1-2).

Our finding was consistent with the previous reports that proline and ascorbic acid are effective in protecting seedlings and plants (Ait and Audran 1987; Rhodes 1999; Fotopoulos *et al*. 2008; Koc *et al*. 2010) against cold stress and other stresses. However, to the best of our knowledge, this is the first report that proline and ascorbic acid were shown to have beneficial effects against stresses such as chilling and peroxide hydrogen. CI and EL occurrence are often accompanied by oxidative damage, which can be followed through lipid peroxidation content, since they are the final product of lipid peroxidation (Hon *et al*. 1994; Shivashankar 2000; Fotopoulos *et al*. 2008). In this study, there was a continuous increase in lipid peroxidation content in all fruits, but the application of proline and ascorbic acid significantly delayed the increase of lipid peroxidation (Table 3). Moreover, the change in membrane permeability (revealed by H_2O_2 content) showed trends similar to lipid peroxidation content; that is, fruit H_2O_2 content increased with storage duration, but proline and ascorbic acid markedly delayed the increase (Table 4). Proline and ascorbic acid have been considered to be involved in a network of interacting signal transduction pathways, which regulate defense responses to abiotic stress (Yadegari *et al.* 2007; Yang *et al*. 2009). Dowdle

Table 5. Effect of proline and ascorbic acid on superoxide dismutase activity of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Superoxide dismutase enzyme (µ/mg protein)				
0	0	20.23 uv	20.38 uv	20.43 tuv	20.75 t	21.59 tu
	2	20.27 uv	23.12 s	26.91 q	33.10 l	37.13 i
	4	20.26 uv	25.16 r	30.45 n	38.01 g	42.95 e
250	0	20.21 uv	23.35 s	27.24 p	33.31 kl	37.54 h
	2	20.21 uv	26.78 q	33.31kl	38.37 g	43.90 c
	4	20.27 uv	27.29 p	33.45 kl	38.30 g	44.30 b
500	0	20.27 uv	25.45 r	31.14 m	38.26 g	43.52 d
	2	20.27 uv	27.35 p	33.61 k	38.28 g	44.33 b
	4	20.20 v	28.37 o	34.35 j	41.39 f	48.42 a

[+]Means with different letters are significantly different ($p \leq 0.05$).

Table 6. Effect of proline and ascorbic acid on ascorbate peroxidase activity of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Ascorbate peroxidase enzyme (u/mg protein)				
0	0	24.22 p	25.25 n	26.18 m	27.02 l	28.07 k
	2	24.34 p	26.21 m	28.92 j	34.10 f	37.03 e
	4	24.53 op	28.21 k	32.34 h	37.12 e	39.95 c
250	0	24.70 o	26.26 m	29.20 j	34.20 f	37.29 e
	2	24.24 p	29.22 j	34.11 f	38.41 d	41.19 b
	4	24.34 p	29.24 j	34.40 f	38.29 d	41.30 b
500	0	24.28 p	28.38 k	33.32 g	37.34 e	40.29 c
	2	24.30 p	29.15 j	34.19 f	38.22 d	41.36 b
	4	24.28 p	30.05 i	36.03 q	40.19 c	44.12 a

[+]Means with different letters are significantly different ($p \leq 0.05$).

et al. (2007) and Szabados and Savoure (2010) demonstrated that proline- and ascorbic acid-mediated stress tolerance in *Arabidopsis* shows interactions with abscisic acid, ethylene and salicylic acid pathways.

The mechanism by which proline- and ascorbic acid-induced cold resistance in grape was investigated in this study. When horticultural crops are exposed to severe abiotic stresses, including cold stress, large amounts of intracellular ROS are generated (Gualanduzzi *et al*. 2009; Wagstaff *et al*. 2010). The detoxification of ROS is dependent on antioxidant enzymes such as APX, SOD, CAT and POD (Wang 1993; Yang *et al*. 2009; Zhang and Tian 2010). The increase in these enzymes' activity contributes to the adaptation of plants to cold stress and ameliorates oxidative damage such as lipid peroxidation (lipid peroxidation increase as the indicator) and H_2O_2 content (Shivashankar 2000; Yadegari *et al*. 2007; Yang *et al*. 2009). In the present study, we found that the activity of the four enzymes in grape was induced by proline and ascorbic acid treatment during the storage at 1°C (Tables 5-8). Similar results have been reported in the previous studies. Shivashankar (2000) and Szabados and Savoure (2010) demonstrated that proline and ascorbic acid were effective in promoting in vitro growth of plants by elicitation of CAT, POD, APX and polyphenol oxidase. Bayat *et al*. (2013) and Claussen (2005) reported that proline and ascorbic acid enhanced the activity of CAT, APX and SOD in plants and reduced H_2O_2. Therefore, proline and ascorbic acid induced antioxidant defense system. We argue that the proline- and ascorbic acid-induction of antioxidant enzyme activity in grape fruits may be a key factor in lowering oxidative damage caused by cold stress, thus improving the cold tolerance and alleviating CI of grape stored at 1°C.

Table 7. Effect of proline and ascorbic acid on catalase activity of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Catalase enzyme (u/mg protein)				
0	0	35.19 l	35.32 l	35.56 l	36.26 k	37.06 ij
	2	35.28 l	36.42 kj	38.19 g	39.96 f	43.03 d
	4	35.19 l	37.24 hi	39.66 f	42.17 e	45.07 c
250	0	35.16 l	36.49 jk	38.31 g	40.21 f	42.03 e
	2	35.27 l	37.38 hi	39.59 f	43.06 d	47.02 b
	4	35.18 l	37.33 hi	39.80 f	43.17 d	47.17 b
500	0	35.21 l	37.45 hi	39.79 f	42.27 e	45.32 c
	2	35.19 l	37.45 hi	39.60 f	43.22 d	47.28 b
	4	35.21 l	37.88 gh	40.17 f	45.08 c	50.00 a

[+]Means with different letters are significantly different ($p \leq 0.05$) .

Table 8. Effect of proline and ascorbic acid on peroxidase) activity of grape fruit during storage

Treatment		Time (day)				
Proline (mM)	Ascorbic acid (%)	0	7	14	21	28
		Peroxidase enzyme (u/mg protein)				
0	0	30.22 u	30.30 u	30.71 t	31.32 s	31.95 r
	2	30.17 u	35.11 q	38.16 o	41.13 l	45.08 f
	4	30.15 u	37.74 p	41.62 k	44.29 g	47.81 d
250	0	30.15 u	35.21 q	38.29 o	41.34 l	45.57 e
	2	30.13 u	39.09 n	43.30 i	45.21 f	50.42 b
	4	30.17 u	39.29 mn	43.28 i	45.25 f	50.48 b
500	0	30.13 u	38.26 o	42.25 j	45.16 f	48.99 c
	2	30.16 u	39.37 m	43.54 h	45.18 f	50.46 b
	4	30.15 u	42.47 j	45.20 f	49.03 c	53.82 a

[+]Means with different letters are significantly different ($p \leq 0.05$).

Conclusion

Application of proline and ascorbic acid reduced CI of grape stored at 1°C and maintained grapes quality as well. The chilling injury, electrolyte leakage, lipid peroxidaion, peroxide hydrogen were significantly reduced by proline and ascorbic acid treatment especially at 500 µM proline in combination with 2 and 4% ascorbic acid. Proline and ascorbic acid treatment that induced cold resistance, may be due to stimulation of antioxidant enzymes and protection against membrane oxidative damage, decreased lipid peroxidation and H_2O_2 content in the grape fruits. These results may have implications for the use of proline and ascorbic acid in managing postharvest CI of other temperate fruits stored at low temperatures.

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