



Effect of Gradual and Shock Chilling Stress on Abscisic Acid, Soluble Sugars and Antioxidant Enzymes Changes in 'Sultana' Grapevine

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Abstract

Plants may show different behaviours during exposure to low temperature stress through various mechanisms. In this study, the chilling responses of 'Sultana' grapevine were investigated during a gradual chilling (2 °C/h) and also during a shock chilling (5 °C/h) from 24 °C to 4 °C. After artificial chilling stress, electrolyte leakage, lipid peroxidation, hydrogen peroxide production and antioxidant enzymes activities were measured in all plants leaves. Moreover, the potential of plant to accumulate abscisic acid and osmoregulants were compared under both gradual and shock chilling stresses. Based on the results, a significant difference was found between the electrolyte leakage, lipid peroxidation and hydrogen peroxide content of plants under two chilling regimes. These indices were found to be higher in shock chilling stressed plants compared with gradual chilling stressed vines because of the lower catalase, peroxidase and ascorbate peroxidase activities. Moreover, gradual chilling stressed vines exhibited higher accumulation of abscisic acid, proline and total phenolic compounds. The chlorophyll degradation and relative water content were lower in gradual cold stressed plants. Glucose and fructose, unlike sucrose showed higher concentration in gradual cold stressed vines. Totally, under gradual chilling stress grapevine plants showed better cold acclimation by lower oxidative stress and higher accumulation of osmoregulants in compared with shock chilling stressed vines.

Keywords: Chilling regimes, Cold stress, Grapevine, Metabolic changes.

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Introduction

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Chilling stress often affects grapevine growth and productivity in temperate regions. Therefore, improvement in chilling tolerance is one of the main objects in grapevine breeding programs. The study of grapevine plants under different chilling regimes and observation of their

behaviour at these conditions would help to breeders to use these physiological cold-related indices for selection of cold tolerant cultivars. Therefore, comprehensive understanding of mechanisms involved in chilling tolerance has become one of the main objectives of biological researches which would help the development of grapevine cultivars that perform better under cold stress.

Cold acclimation is a favourable trait that has a main role in plant growth, reproduction and survival (Thomashow, 1999). In nature, plant especially woody temperate species such as grapevine may undergo two forms of gradual or rapid chilling stress in late summer and mid-autumn. For example, harsh autumn frost in early November of 2016, while temperature dropped to $-14\text{ }^{\circ}\text{C}$ for three days, damaged at least 5000 ha of vineyards in Malayer, the second grape producing region in Iran, and had serious damages to vineyards. Incomplete development in canes periderm, abnormal or rapid leaf abscission and weak constitution and formation of lignin and suberin in bud's scales are common results of sudden or shock chilling stress during early stage of cold acclimation in autumn (Karimi and Ershadi, 2015). During shock decreasing temperature the genetically potential of trees for coping to cold stress condition may not have been achieved completely compared to gradually chilling stressed plants. Indeed, under gradual decreasing temperature the plants have sufficient time to conduct an array of morphological and physiological change upon exposure to chilling stress (Karimi and Ershadi, 2015).

Woody plants acclimate to low temperature by decreasing day length and non-freezing temperatures of less than $10\text{ }^{\circ}\text{C}$ in late season (Schnabel and Wample, 1987; Keller, 2010). Changes in soluble sugars, proline, proteins, and increase in polyamines, antioxidant capacity, phenolic compounds and abscisic acid (ABA) levels are some of the most important metabolic defences against low temperature stress during cold acclimation process (Compos et al., 2003; Atici and Nalbantoğlu, 2003; Ershadi et al., 2016; Karimi, 2017). Moreover, cold stress has been shown to induce the overproduction of reactive oxygen species (ROS) (Lukatkin et al., 2012; Anjum et al., 2014; Karimi et al., 2016), but grapevine

plants may also activate antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), to eliminate ROS or to alleviate the deleterious effects of ROS (Lukatkin et al., 2012; Anjum et al., 2014; Karimi et al., 2016). The nature of acclimatory responses is complex and differs among species and cultivars, thermal treatments and timing and rating of exposure (Thomashow, 1999; Gusta et al., 2005; Ershadi et al., 2015).

Electrolyte leakage (EL) assay is a commonly used means to measure the amount of plant damage in relation to cold stress (McKay, 1992; Linden, 2002). The assessment is based on the principle that damage to cell membranes results in enhanced leakage of solutes into the intercellular water (Compos et al., 2003). Recording the extent of leakage after chilling stress treatments thus provides an estimate of tissue injury (Linden, 2002; Ershadi et al., 2016).

Chilling stress effects have been widely investigated in both herbaceous and woody plants (Compos et al., 2003; Guo et al., 2012; Karimi and Ershadi, 2015); however, experimental designs frequently have focused on only one type of chilling stress. So far no studies have simultaneously examined the specific contributions of the two distinct forms of chilling stress (i.e. rapid chilling (RC) stress and gradual chilling (GC) stress). The following experiments were carried out to investigate the effect of the two forms of chilling regimes on tolerance of 'Sultana' grapevine to chilling stress conditions. In particular, the physiological and biochemical changes including antioxidant enzymes activities, lipid peroxidation, Hydrogen peroxide (H_2O_2) production, EL, soluble sugars, chlorophyll content, proline and phenolic compounds accumulation in grapevine leaves under both rapidly and gradually applied chilling stress was investigated.

Materials and Methods

Plant material and growth conditions

The cuttings of 'Sultana' grapevine (*Vitis vinifera* L.) were planted in 6 L pots and placed in a greenhouse with maximum irradiance of $1400\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, 12/12 h day/night photoperiod, day/night temperature of 25/ $18\text{ }^{\circ}\text{C}$ and relative

humidity at 60% for four months. The vines were watered three times a week and fertilized using 0.5 g L^{-1} NPK (20–20–20) fertilizer until appearance of 15 functional leaves. The experimental design was completely randomized with three replicates (two vines per replication).

Controlled chilling tests

Potted vines were subjected to chilling stress in a programmable cooling chamber. Chilling was accomplished at two distinct forms including of gradual temperature decrease of $2 \text{ }^{\circ}\text{C/h}$ and shock temperature decrease of $5 \text{ }^{\circ}\text{C/h}$ from $24 \text{ }^{\circ}\text{C}$ to $+4 \text{ }^{\circ}\text{C}$ and kept at $+4 \text{ }^{\circ}\text{C}$ for 12 hours (Karimi and Ershadi, 2015). The plants incubated at $24 \text{ }^{\circ}\text{C}$ were considered as control untreated vines. After chilling exposure, the EL, lipid peroxidation, H_2O_2 , relative water content (RWC), phenolic compounds, proline, soluble sugars (glucose, fructose and sucrose), ABA, soluble protein, ROS-scavenging enzymes (CAT, POD, and APX) and photosynthetic pigments of leaves of 'Sultana' grapevines were measured. For evaluating the changes of above mentioned indices, the first three fully expanded leaves near the shoot apex were taken for further analysis.

Electrolyte leakage

The EL measurements were started by cutting three leaves of cold stressed vines. Samples (0.5 g leaf) were rinsed with distilled water, wiped dry and immersed separately into 50-mL test tubes containing 40 mL of distilled water for 24 h on a shaker at 120 rpm. The electrical conductivity (EC_1) of the bathing solutions was measured using an electro conductivity meter (Cond 720, Germany). Samples were subsequently autoclaved at $120 \text{ }^{\circ}\text{C}$ for 20 min, allowed to cool to room temperature, and electrical conductivity was measured again (EC_2) (Ershadi et al., 2016). Relative electrolyte leakage (REL) was calculated using the following formula: $\text{REL} = (\text{EC}_1 / \text{EC}_2) \times 100$.

Lipid peroxidation

Membrane lipid peroxidation was measured in 200 mg of each leaf sample ($n = 5$ per treatment), which was homogenised in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at $6,000 \times g$ for 5 min. The supernatant

was collected and lipid peroxidation was measured in terms of thiobarbituric acid relative substances (TBARS), according to Heath and Packer (1968).

Hydrogen peroxide

H_2O_2 was measured spectrophotometrically after the reaction with potassium iodide (KI), according to Velikova and Loreto (2005). One g of fresh leaf tissue ($n = 5$ samples per treatment) was ground and homogenised in a mortar containing 10 mL of 0.1% (w/v) TCA. The homogenate was centrifuged at $6,000 \times g$ for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer, pH 7.0 and 0.1 mL of reagent (0.1 M KI in fresh double-distilled water). The absorbance of the supernatant was measured at 390 nm. A blank was prepared using 0.1% (w/v) TCA in the absence of leaf extract. The concentration of H_2O_2 was calculated using a standard curve prepared using known concentrations of H_2O_2 .

Relative water content

Leaf RWC was determined as described by Kirnak et al. (2001). For measurement, small sections of fresh leaves were weighed (4 leaves) to determine fresh weight and plunged in deionized water at $+4 \text{ }^{\circ}\text{C}$ for 20 h and turgid weight was recorded. For dry weights measurement, the samples were placed for 48 h in an oven ($65\text{--}70 \text{ }^{\circ}\text{C}$) and dried. RWC was calculated as following formula: $\text{RWC} = [(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight})] \times 100$.

Soluble carbohydrates

Total soluble carbohydrates content was determined based on anthrone method according to Yemm and Willis (1954). Fresh leaf samples were grounded using liquid nitrogen. For extraction, about 5 mL of ethanol (80 %) was added onto 1 g of ground tissue for three times. After centrifugation for 15 min at $1500 g_n$, about 100 μL of the supernatant (ethanolic extract) was added into test tubes contained 3 mL of 0.2% anthrone solution (2 g anthrone in 1 L of 72% sulfuric acid). The samples were heated in a boiling water bath ($95 \text{ }^{\circ}\text{C}$) for 10 min, and then rapidly transferred into an ice bath and cooled down to

room temperature. Absorbance of the samples was read at 620 nm using a Cary Win UV 100 spectrophotometer (Varian, Australia). Finally, soluble carbohydrates concentration of the samples was calculated through a calibration curve drawn for glucose standard solutions and expressed as mg soluble carbohydrates g⁻¹ fresh weight (FW).

Soluble sugars

The samples of leaves from each replicate were ground into powder in liquid nitrogen. Half gram of the powder was extracted with 10 mL ethanol (80%). After centrifugation at 8000g for 15 min, the supernatants were passed through a 0.2 µm filter (Shin et al., 2002). The filtered supernatants were used to measure fructose, glucose and sucrose using a Crystal 200 series HPLC pump (ATI Unicam, Cambridge, UK) equipped with a SPD UV-Vis detector (Philips, Cambridge, UK) and a Spherisorb ODS-2 Column (0.3 µm, 150 mm × 4.6 mm i.d.) from Hichrom (Berkshire, UK). Sodium Citrate (pH 5.5) and ultrapure acetonitrile (1:99, v/v) at a flow rate of 0.1 mL min⁻¹ was used as the mobile phase. The injection volume was 10 µL (Comis et al., 2001). External standard solution calibrations of sucrose, glucose and fructose (Sigma, Australia) were used to integrate peak. Sugar concentrations were expressed in µmol g⁻¹ FW.

Proline

Fresh leaves samples were grounded using liquid nitrogen and 10 mL of 3% (w/v) aqueous sulfosalicylic acid was added onto 0.5 g of powdered tissue. Then 2 mL of filtered extract was placed into test tubes and 2 mL ninhydrin and 2 mL glacial acetic acid were added. The tubes were heated in a boiling water bath (95 °C) for 60 min, and the reaction was finished in an ice bath. Four mL of toluene was added to the mixture and the organic phase was extracted (Bates et al., 1973). The absorbance of extract was recorded spectrophotometrically at 520 nm, while toluene used as blank. Proline concentration was finally calculated through a calibration curve drawn for proline standard solution and expressed as µmol proline g⁻¹ FW.

Abcisic acid

For ABA analysis, the leaves tissue (1 g) was homogenized in liquid nitrogen and extracted in 10 mL methanol (80%) containing 0.01% polyvinylpyrrolidone and 0.01 g vitamin C, and shaken (120 rpm) overnight in the dark at 4 °C. The homogenate samples were centrifuged at 4000 g_n for 15 min at 4 °C, and the supernatant was collected and its pH was adjusted to 8 with 10% phosphate buffer. Then the homogenates were filtered through 0.45 µm *Whatman filters* and dried in a stream of air. The remained extract pH was adjusted with HCL (0.2 N) approximately to 2.5 and then 10 ml ethyl acetate was added to it. After evaporation of ethyl acetate, the extracts were dissolved in 1 mL methanol (3%) and acetic acid (0.1 M), and then the obtained homogenates were filtered through 0.45 µm *Whatman filters* and analyzed quantitatively by reversed phase high-performance liquid chromatography (HPLC). Chromatography was performed with a Crystal 200 series HPLC pump (ATI Unicam, Cambridge, UK) equipped with a SPD UV-Vis detector (Philips, Cambridge, UK) and a Diamonsic-C₁₈ Column (5 µm, 250 mm × 4.6 mm i.d.) from Hichrom (Berkshire, UK). Elution was achieved at a flow rate of 4 mL min⁻¹ with acid acetic: methanol (97:3 v/v) as the mobile phase. Injection volume was 20 µL. A photo-diode array detector (PDA) was used to monitor the presence of ABA by UV detection (Gilson, Luton, Bedfordshire, UK) at a wavelength of 260 nm (Li et al., 2010).

Total phenolic compounds

Total phenolics were determined colorimetrically using Folin-Ciocalteu reagent as described by Velioglu et al. (1998) with slight modifications. In brief, a volume of 0.3 mL from each diluted methanolic extract (10 %) was mixed with 1 mL Folin-Ciocalteu reagent (10 %) and vortexed. After 5 min, 1 mL of 7% sodium carbonate solution was added to the mixture. The final solution was shaken for 20 min at room temperature and then the absorbance was spectrophotometrically measured at 765 nm. Total phenol concentrations were calculated using a calibration curve drawn for gallic acid standard solution and expressed as mg gallic acid g⁻¹ of FW.

Total soluble protein

Total soluble proteins were extracted from leaves (five per treatment), and total soluble

Effect of Gradual and Shock Chilling Stress on physiological characters in 'Sultana' Grapevine

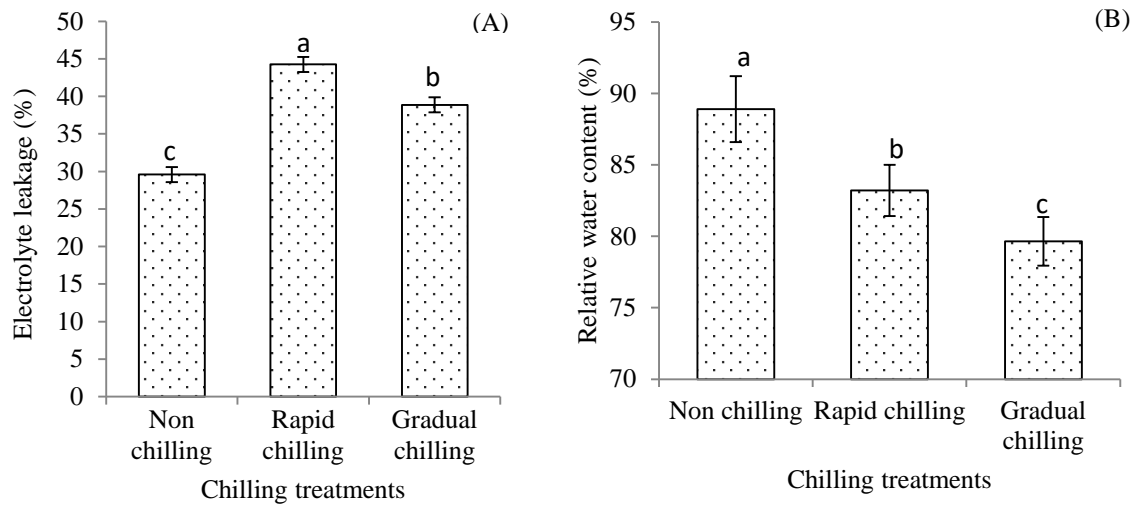


Fig. I. The effect of a gradual temperature decrease and a rapid cold shock on electrolyte leakage (Panel A) and relative water content (Panel B) in leaves of 'Sultana' grapevine

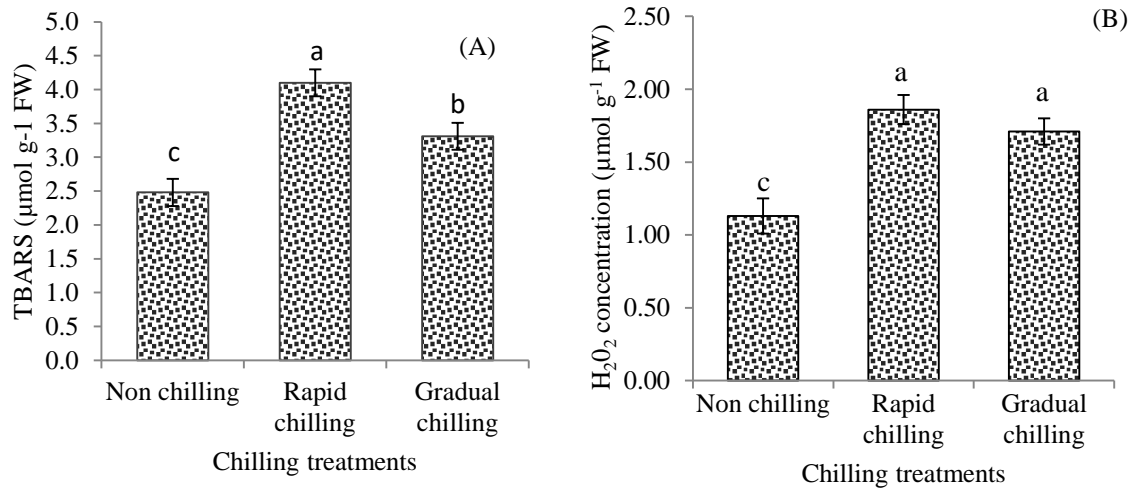


Fig.II. The effect of a gradual temperature decrease and a rapid cold shock on lipid peroxidation (Panel A) and hydrogen peroxide (Panel B) contents in leaves of 'Sultana' grapevine

proteins concentrations was determined using the colorimetric method of Bradford (1976) by measuring the absorbance at 595 nm, with bovine serum albumin as the standard. The values of total soluble proteins were expressed as $\text{mg g}^{-1}\text{ FW}$ of leaf.

Anti-oxidant enzyme activities

One-hundred mg of frozen leaf powder from each grapevine sample was homogenised in 1.0 mL of 0.05 M sodium phosphate buffer (pH 7.8) containing 1.0 mM EDTA and 2% (w/v) polyvinylpyrrolidone. Each homogenate was centrifuged at $10,000 \times g$ for 20 min at 4°C and the supernatant was used for all enzyme activity measurements. All activities were performed at 4°C .

CAT activity was determined by measuring the decrease in absorbance of H_2O_2 at 240 nm (Bergmeyer, 1970). Each 3 mL reaction mixture contained 0.05 M sodium phosphate buffer,

crude leaf enzyme extract was added to a 3 mL reaction mixture containing 0.855 μ L of 25 mM guaiacol and 1.355 μ L of 30% (v/v) H_2O_2 in 3 mL of sodium phosphate buffer (pH 7.0). The reaction

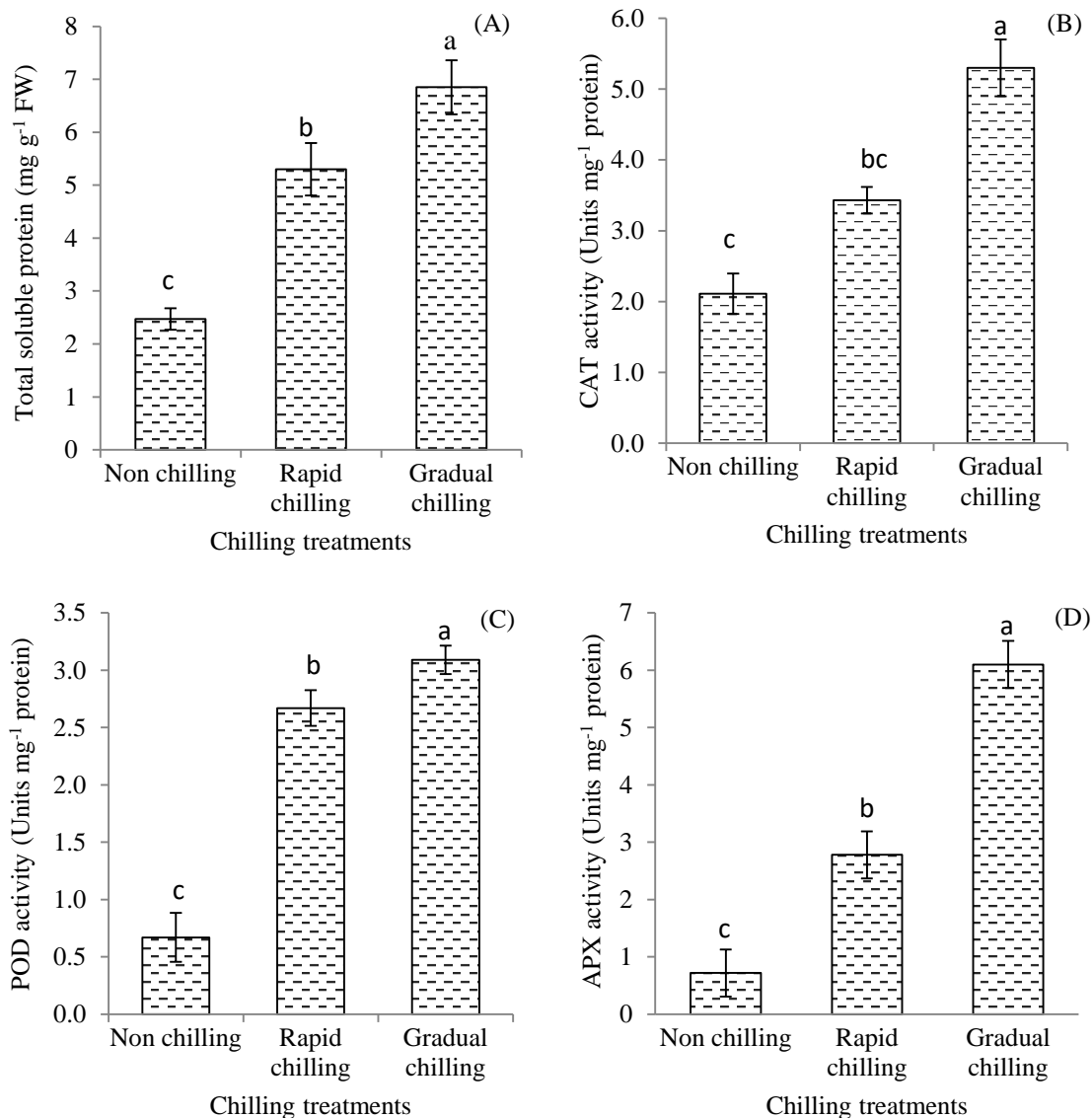


Fig III. The effect of a gradual temperature decrease and a rapid cold shock on total soluble protein (Panel A), catalase (CAT; Panel B), peroxidase (POD; Panel C), and ascorbate peroxidase (APX; Panel D) in leaves of 'Sultana' grapevine. Unit

pH 7.0 with 1.0 mM EDTA and 3% (v/v) H_2O_2 . The decrease in absorption at 240 nm was monitored for 3 min. One unit of CAT activity was defined as the amount of enzyme that resulted in 1.0 μ mol of H_2O_2 degraded $mL^{-1} min^{-1}$.

POD activity was measured by following the oxidation of guaiacol by H_2O_2 at 470 nm according to Herzog and Fahimi (1973). One mL of each

was initiated by adding the H_2O_2 . One unit of POD activity was defined as the amount of enzyme that degraded 1.0 μ mol guaiacol $mL^{-1} min^{-1}$.

APX activity was determined by measuring the decrease in absorbance at 290 nm for 1.0 min as ascorbate was oxidised (Nakano and Asada, 1981). Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM

ascorbate, 0.1 mM Na₂ EDTA, and 1.2 mM H₂O₂. One unit of APX activity was defined as the amount of enzyme that oxidised 1.0 µmol ascorbate mL⁻¹ min⁻¹.

The specific activity of each anti-oxidant enzyme was then expressed in unit's mg⁻¹ protein.

Photosynthetic pigments

Total chlorophyll and carotenoids were extracted from each leaf sample using 80% (v/v) acetone. The absorbance of each supernatant was measured spectrophotometrically using the equations according to Lichtenthaler (1987). Chl a concentrations were determined at 663 nm, Chl b at 645 nm, total Chl at 652 nm, and carotenoids at 470 nm and all were expressed as mg g⁻¹ FW leaf. All spectrophotometric measurements were carried out using a UV-visible spectrophotometer (Cary 100; Varian, Sydney, Australia).

The results were subjected to analysis of variance using GLM procedures of the SAS software package (SAS Institute Inc., Cary, NC, USA), and mean separation was done using Duncan's multiple range test at $P \leq 0.05$.

Results

Electrolyte leakage and Relative water content

Significant increases ($P \leq 0.001$) in leaf EL percentage were observed as a result of chilling stress. However, leaf EL of GC- treated vines was lower approximately 13.8% compared to RC-treated plants (Fig. I A). Totally EL percentage of control non-chilled vines was shown to be 23.1% and 34.2% lower than both GC- and RC- treated plants respectively.

Chilling treatment had significant effect ($P \leq 0.001$) on leaf RWC of grapevine plants (Fig. I B). The lowest RWC was found in plant under GC treatment condition which was 4.9% and 10.5% lower than RC- treated and non-chilled control plants respectively.

Lipid peroxidation and Hydrogen peroxide

Leaf TBARS and H₂O₂ concentrations, as oxidative stress biomarkers, increased noticeably in the leaves of all grapevine plants during exposure to chilling stress, although no significant difference was observed between H₂O₂ concentrations of both GC- and RC- treated plants

(Fig. II A, B). Nevertheless, both TBARS and H₂O₂ were shown to be lower in GC- treated plant compared to RC- treated vines (Fig. II A, B). Leaf TBARS and H₂O₂ concentrations of GC- treated plants exhibited 18.6% and 2.62 % lower oxidative stress respectively compared to those of RC-treated vines.

Total soluble protein and antioxidant enzyme activity

Significant difference ($P \leq 0.001$) was observed in leaf total soluble protein content, CAT, POD and APX activity as a result of low temperature stress. As shown in Fig III, leaf total soluble protein content, CAT, POD and APX activity of both GC- and RC- treated plants increased two to three folds in compared to control non-chilled vines (Fig III). However, leaf soluble protein of GC-treated plants was higher significantly than those of RC- treated grapevine plants. Interestingly, antioxidant enzymes activities of CAT, POD and APX were shown to be higher in GC- treated plants in compared to those of RC-treated vines (Fig III B, C, and D).

Proline and total phenolics content

Proline and total phenolics content increased under chilling stress and differed significantly ($P \leq 0.001$) in the leaf of both GC- and RC -treated plants. The concentration of proline in RC-treated vines was higher than GC-treated plants (Table 1). In contrast to proline changes, the total phenolics content of GC- treated plants shown to be higher than those of RC- treated vines (Table 1).

Total soluble carbohydrates and soluble sugars

Total soluble carbohydrates and soluble sugars including glucose, sucrose and fructose increased in vines leaves under chilling stress condition. In GC- treated plants leaves, total soluble carbohydrates and glucose concentration was shown to be higher than those of RC- treated plants (Fig. IV A, B). Interestingly in RC-treated plants sucrose was significantly ($P \leq 0.001$) higher than GC-treated vines (Fig. IV C). Fructose increased under low temperature stress but did not differed significantly in both GC- and RC-treated plants (Fig. IV D).

Abscisic acid

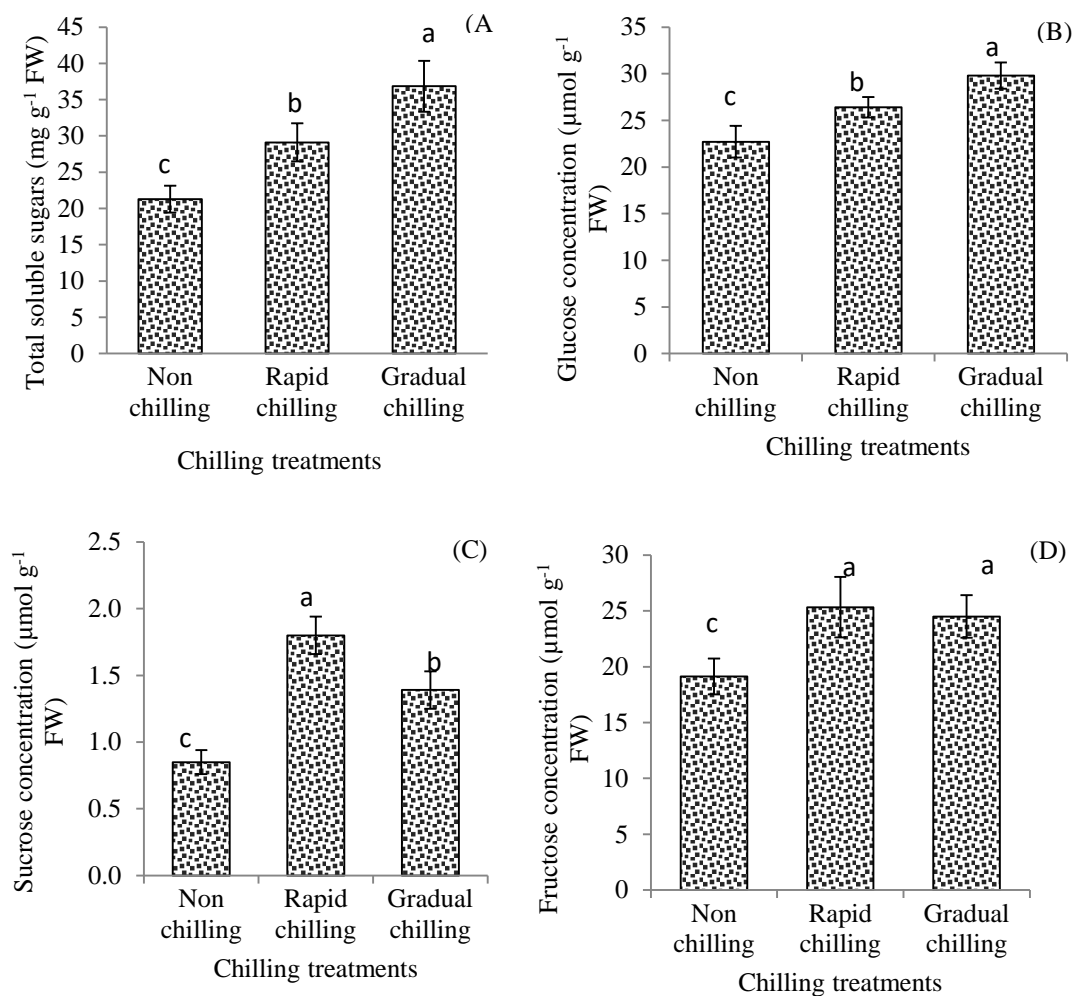


Fig IV. The effect of a gradual temperature decrease and a rapid cold shock on total soluble carbohydrate (Panel A), Glucose (Panel B), Sucrose (Panel C) and Fructose (Panel D) concentrations in leaves of 'Sultana' grapevine.

Significant difference ($P \leq 0.001$) in leaf ABA concentration was observed as a result of low temperature stress. Chilling stress dramatically increased ABA concentration of both GC- and RC-treated grapevine plants (Fig. V). Under the gradually chilling temperature, the leaf ABA concentration was shown to be approximately 18% higher than RC- treated plants (Fig. V).

Photosynthetic pigments

Contrary to carotenoids, leaf chlorophyll content of grapevine plants decreased under two chilling regimes compared to non-chilled vines. The chlorophyll content of GC- treated plants was higher than those of RC- treated plants (Table 1). Contrastingly, carotenoids content of RC- treated plants was higher than those of GC- treated vines (Table 1).

Discussion

In this study the response of 'Sultana' grapevines were investigated under two chilling temperature regimes. The plants showed different behaviour in front to these temperatures decreasing patterns. Solutes leakage recording after chilling stress is one of the common and reliable methods for membranes damages estimation in different woody and herbaceous plants (McKay, 1992; Linden et al., 2000; Ershadi et al., 2016). The rate at which the temperature is lowered is important in determining the plant response (Zhang et al., 2008). Based on our results, leaf EL as a membrane injury index in RC-treated vines was shown to be higher than GC-treated plants. This suggested the better cold acclimation and chilling tolerance adaptation of

grapevine plants through exposure to moderate temperature decreasing compared to vines which were treated by severe chilling stress. In other word, gradually chilling stress may cause mild injury to cell membrane and an increase in EL perhaps is resulted from ROS accumulation in leaf tissues (Karimi et al., 2016).

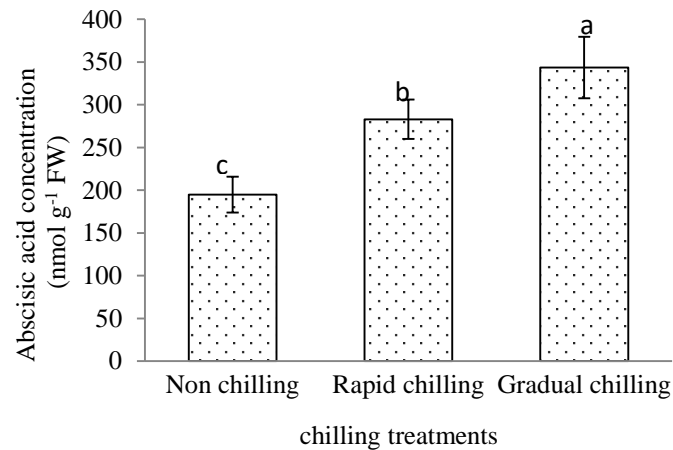


Fig V. The effect of a gradual temperature decrease and a rapid cold shock on abscisic acid concentrations in leaves of 'Sultana' grapevine.

TBARS is another cell membrane injury indicator that has been used for cold tolerance measurement in plants (Guo et al., 2012; Karimi et al., 2016). In the present study, TBARS levels were found to be lower in grapevine leaves under GC temperature regime. The lower lipid peroxidation upon exposure to GC-treated plants was associated with higher anti-oxidant activities in their leaves. Indeed, solutes exudation occurrence from damaged biomembranes usually is result of higher ROS accumulation that observed by higher TBARS production in RC-treated plants compared to others. EL and TBARS are among the main consequences of ROS accrued impact on biomembranes (Halliwell and Gutteridge, 2000; Anjum et al., 2014).

In the present study, chilling stress caused a dramatic increase in H₂O₂ concentrations in both RC- and GC- treated vines. Although, H₂O₂ was found to be higher in RC-treated grapevine leaves than GC- treated vines but did not significantly differed from each other. ROS, especially H₂O₂, are thought to be involved as secondary messengers in cellular signalling to induce anti-oxidant defences under abiotic stress (Foyer and Noctor, 2003; Karimi et al., 2016). The higher

accumulation of H₂O₂ in chilled leaves was reported in previous works (Said et al., 2014; Karimi et al., 2016).

Anti-oxidant systems are thought to play a critical role upon exposure of plant to chilling stress. The major ROS-scavenging enzymes in plants, (SOD, CAT, POD, and APX) are the first

defence against the deleterious effects of chilling stress. In the current study, the antioxidant enzymes system of chilled leaves exhibited higher activity in GC- treated vines than RC- treated plants. This finding confirmed the lower solute leakage and lipid peroxidation observed in GC-treated vines in current study and the results of other relevant studies (Yang et al., 2011; Guo et al., 2012; Karimi et al., 2016). On the other words, higher ROS-scavenging enzymes activities of GC-treated vines could have explained the biomembranes stability and subsequent photosynthetic capacity which were approved by lower H₂O₂ production in these plants. ROS-scavenging enzymes may be protecting the photosynthetic apparatus from oxidative stress (Van Breusegem et al., 1999; Karimi et al., 2016). ROS accumulation can induce damaging biomembranes by oxidizing membrane biomolecules such as lipids and proteins if not scavenged by antioxidant enzyme activities (Lukatkin et al., 2012; Anjum et al., 2014). Therefore, the efficiency of antioxidant enzymes activities for scavenging ROS mainly determined the plant sensitivity to chilling stress (Lukatkin et al., 2012; Anjum et al., 2014) as confirmed in

Table 1.

The effect of a gradual temperature decrease and a rapid cold shock on proline, total phenolic compound, chlorophyll and carotenoid content of 'Sultana' grapevine

Chilling treatments	Proline ($\mu\text{g g}^{-1}$ FW)	Total phenolic (mg g^{-1} FW)	Total chlorophyll (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
Rapid chilling	3.54 \pm 0.08 a	4.62 \pm 0.35 b	1.16 \pm 0.34 c	0.78 \pm 0.14 c
Gradual chilling	3.24 \pm 0.12 b	5.33 \pm 0.19 a	1.93 \pm 0.24 b	1.52 \pm 0.12 b
Non chilling	1.53 \pm 0.13 c	2.27 \pm 0.33 c	2.41 \pm 0.09 a	1.64 \pm 0.26 a

current study. An increased anti-oxidant capacity is one approach used by plants to improve their tolerance to cold stress (Yang et al., 2011; Guo et al., 2012). The current study indicates that the activities of POD, CAT and APX obviously enhanced when plants were treated by GC temperature regime, which was consistent with the findings reported by Karimi et al. (2016).

Tissues water content is a key factor affecting susceptibility and resistant of grapevine canes and buds to cold stress (Karimi and Ershadi, 2015). In the current study, grapevine plants exposed to chilling stress exhibited a reduction in leaf RWC. It is assumed that decreasing in water uptake and transportation through roots and stems due to reduction of metabolic activity and disruption of leaf transpiration is the reason of tissues dehydration in chilling stress affected plants (Turan and Ekmekci, 2011). Nevertheless, the RWC of GC-treated vines were lower than RC-treated plants. Tissues dehydration during cold acclimation is one of the basic strategies of plant to concentrate their cells sap for overcoming harsh temperatures (Pogosyan et al., 1975).

Tissues dehydration during cold acclimation plays a key role in chilling tolerance of vines (Karimi and Ershadi, 2015). During gradually lowering of temperature, leaf RWC was shown to be lower compared to the amounts of this parameters in RC-treated vines. This suggested that tissue dehydration occurred in better condition under mild decline in temperature. In other words, cold acclimation helped the plant to decline their tissues water content to the levels that is essential for enduring of chilling temperatures.

The chlorophyll stability of GC-treated plants was higher than those of RC-treated plants. The chlorophyll concentration is regulated by its biosynthesis and degradation under environmental stresses including chilling stress (Turan and Ekmekci, 2011; Mohanty et al., 2006). Under chilling stress, the rate of temperature decreasing is critical for chlorophyll stability and photosynthetic efficiency. We did not measure the photosynthetic capacity but it is assumed that step by step decline in temperature might have provided better condition for continuing the photosynthesis than shock chilling stress which is documented by lower EL and TBARS of cell biomembranes and higher antioxidant activity in this study and previous works (Karimi et al., 2016). On the other hand, the lower chlorophyll content of RC-treated vines may be related with lower enzymes activities and higher ROS production which could damage cellular, chloroplast and thylakoid membranes of these plants (Shu et al., 2012).

Abscisic acid mediates plant responses to adverse environmental stimuli since the level of ABA in plants usually increases during abiotic stress conditions (Koussa et al., 1998), and exogenous application of ABA can enhance plant adaptation to cold stresses (Karimi and Ershadi, 2015; Karimi et al., 2016). In the present study, under the gradually chilling temperature, the ABA content was shown to be higher approximately 18% compared to RC-treated plants. It is possibly due to higher expression of certain genes related to ABA synthesis under gradually decreasing temperature condition. Abscisic acid activates a wide array of genes associated with low temperatures and other abiotic stresses (Giraudat

et al., 1994; Busk and Pages, 1998). Abscisic acid is also postulated to mediate cold acclimation through induction of a family of cold-inducible transcription factors referred to as "C-repeat binding factors (CBFs)," (Bark and Pages, 1998). Therefore, higher accumulation of ABA in GC-treated vines indicated greater adaptation of these plants under chilling stress condition.

Plants change their osmolytes concentration such as soluble sugars, proline, and soluble protein during exposure to low temperature stress. In the current study, increased levels of total carbohydrates and soluble sugars including sucrose, glucose and fructose were exhibited under chilling stress regimes with higher accumulation of these sugars (except of sucrose) in GC-treated vines than those of RC-treated plants. Calderon and Pontis (1985) studied sucrose biosynthesis in wheat (*Triticum aestivum*) plants subjected to a chilling shock of 4°C. This temperature shift increased the level of sucrose and fructans in leaf tissue. The only enzyme, related to sucrose metabolism, affected by the temperature treatment was sucrose synthase, which activity rose within 1h of shift. The lower concentration of sucrose in GC-treated vines possibly is associated with increased conversion of this disaccharide to other monosaccharides such as glucose and fructose as documented by higher accumulation of these simple sugars under GC-treated plants. Soluble sugars have a main role in biomembranes stability by interacting with the phosphate in their lipid headgroups, decreasing membrane permeability (Strauss and Hauser, 1986; Tarkowski et al., 2015) under chilling stress which confirmed lower leaf EL of GC-stressed vines in current study. Moreover, carbohydrates were suggested as main components of the cellular ROS scavenging system with higher oxidizing ability against OH^\cdot than other radicals, such as superoxide (O_2^\cdot) (Couée et al., 2006; Nishizawa et al., 2008). Therefore, this potential of soluble carbohydrate has a key role in scavenging of highly deleterious hydroxyl radical and subsequent protection of biomembranes during exposure to chilling stress which could explain lower lipid peroxidation of GC-stressed vine than RC-treated vines. Furthermore, abscisic acid is also known to interact with sugars (Kang et al., 2004) and numerous results have been obtained with

transcriptional mutants that provide evidence that ABA and sugar can act either in parallel or in intersecting pathways (Finkelstein et al., 2002). Considering the signaling and cryoprotective involvement of sugars and its interaction with ABA in cold acclimation (Gusta et al., 2005), higher accumulation of these compounds in GC-treated vines, could explain the lower chilling injury of these plants.

Proline concentration changes upon exposure to chilling stress in higher plants (Ghasemi et al., 2012; Ershadi et al., 2016) and plays as an osmoprotectant and antioxidant, which improves stress adaptation (Delauney, and Verma, 1993). In present study, proline concentration increased in both GC- and RC-treated vines and interestingly, proline was higher in RC-treated vine than GC-treated plants. Proline is thought to play a main role in plant subjected to low temperature stress but its higher concentration did not necessarily ensure lower chilling injury (i. e., lower EL and MDA levels) as shown in current study. It has also been suggested that this accumulation is merely a general symptom of stress (Hanson and Hiz, 1982). The lower correlation between proline and cold hardiness was reported in other studies under cold and freezing stresses (Ghasemi et al., 2012; Ershadi et al., 2016).

The increased levels of soluble proteins in the vines leaves under the GC treatment were higher than those in the RC-treated plants leaves, which suggest that vines under GC-treatment could acclimate to chilling stress at least through higher accumulation of soluble proteins. Our results were consistent with a more recent study by Karimi and Ershadi (2015) who concluded that protein protection plays an important role in maintaining cold tolerance in grapevine. The anti-freeze effects of proteins on cell membranes and several cell structures also have been well documented (Atici and Nalbantoğlu, 2003).

Acclimation to low temperature has been shown to increase concentrations of phenolic compounds in several fruit tree species including grapevine (Said et al., 2014; Karimi and Ershadi, 2015) and Pistachio (Pakkish et al., 2009). The data presented in this study indicate that GC-treated plant exhibited higher total phenol content than those of RC-treated vines in their leaves which may be an adaptation to surviving

chilling stress conditions. Biosynthesis of secondary metabolites such as phenolic compounds is partially affected by regulating genetic expression and mainly by environmental factors such as chilling stress (Dixon and Paiva, 1995). Besides their role as free radical scavengers, these compounds increase membranes stability by cellular osmotic adjustment (Pennycooke and Stushnoff, 2005). Our results suggest the importance of chilling regime or acclimation rate in regulating the production of secondary metabolites in grapevines.

In conclusion low temperature evoked different metabolic compounds in the leaves of 'Sultana' grapevine plants. During two chilling regimes of GC and RC, plants changed metabolic pathways to endure this condition. However, the adaptability of plants at GC treatment was shown to be better than RC treatment. Indeed, the efficiency of GC- treated vines for acclimation to chilling stress was better because of higher accumulation of ABA, soluble sugars and protein as well as phenolic compound which resulted in lower cell free water as documented by lesser RWC. Moreover, higher ROS scavenging activity in GC-treated vines beside the above mentioned metabolic changes resulted in lower chilling injury as exhibited by lower cell biomembranes lipid peroxidation and solute leakage than those of RC-treated vines. Studying the expression pattern of CBF genes in 'Sultana' grapevine under both gradual and shock chilling temperature is worthy to be considered in future researches.

References

- Anjum N, A., A. Sofu, A. Scopa, A. Roychoudhury, S. S. Gill, M. Iqbal, A. S. Lukatkin, E. Pereira, A. C. E Duarte and I. Ahmad.** 2015. 'Lipids and proteins—major targets of oxidative modifications in abiotic stressed plants'. *Environ. Sci. Pollut. Res.* 22, (6): 4099–4121.
- Atici, O. and B. Nalbantoğlu.** 2003. 'Antifreeze proteins in higher plants'. *Phytochemistry* 64: 1187–1196.
- Bates, L.S., R. P. Waldren and I. D. Teare.** 1973. 'Rapid determination of free proline for water stress studies'. *Plant Soil* ,39: 205-207.
- Bergmeyer, N.** 1970. 'Methoden der Enzymatischen Analyse'. Volume 1. AkademieVerlag, Berlin, Germany. 636–647.
- Bradford, M.M.** 1976. 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding'. *Anal. Biochem.* 72: 248–254.
- Busk, P.K. and M. Pages.** 1998. 'Regulation of abscisic acid-induced transcription'. *Plant Mol. Biol.* 37: 425–435.
- Calderon, P. and H. G. Pontis.** 1985. 'Increase of sucrose synthase activity in wheat plants after a chilling shock'. *Plant Sci.* 42: 173-176.
- Campos, P.S., V. Quartin, J. C. Ramalho and M. A. Nunes.** 2003. 'Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea sp* plants'. *J. Plant Physiol*, 160: 283–292.
- Comis, D.B., D. M. Tamayo and J. M. Alonso.** 2001. 'Determination of monosaccharides in cider by reversed-phase Liquid Chromatography'. *Anal. Chem. Acta.* 436: 173- 178.
- Couée, I., C. Sulmon, G. Gouesbet and A. El, Amrani.** 2006. 'Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants'. *J. Exp. Bot.* 57: 449–459.
- Delauney, A.J. and D. P. S. Verma.** 1993. 'Proline biosynthesis and osmoregulation in plants'. *Plant J.* 14: 215–223.
- Dixon, R.A. and N. Paiva.** 1995. 'Stress-induced phenylpropanoid metabolism'. *Plant Cell* ,7:1085–1097.
- Ershadi, A., R. Karimi and K. N. Mahdei.** 2016. 'Freezing tolerance and its relationship with soluble carbohydrates, proline and water content in 12 grapevine cultivars'. *Acta Physiol. Plant.* 38: 1-10.
- Finkelstein, R. and S. I. Gibson.** 2002. 'ABA and sugar interactions regulating development: “cross-talk” or “voices in a crowd”?' *Curr. Opin. Plant. Biol.* 5: 26–32.
- Foyer, C.H. and G. Noctor.** 2003. 'Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria'. *Physiol, Plant* ,119, 355–364.

- Ghasemi, S.A.A., A. Ershadi and E. Fallahi.** 2012. 'Evaluation of cold hardiness in seven Iranian commercial pomegranate (*Punica granatum* L.) cultivars'. *Hortscience* 47: 1821-1825.
- Giraudat, J., F. Parcy, N. Bertauche, F. Gosti and J. Leung.** 1994. 'Current advances in abscisic acid action and signalling'. *Plant Mol. Biol.* 26: 1557–1577.
- Guo, W.L., R. G. Chen, Z. H. Gong, Y. X. Yin, S. S. Ahmed and Y. M. He.** 2012. 'Exogenous abscisic acid increases anti-oxidant enzymes and related gene expression in pepper (*Capsicum annuum*) leaves subjected to chilling stress'. *Genet. Mol. Res.* 11: 4063–4080.
- Gusta, L.V., R. Trischuk and C. J. Weiser.** 2005. 'Plant cold acclimation: the role of abscisic acid'. *J. Plant Growth Regulat.* 24: 308-318.
- Halliwell, B. and J.M.C. Gutteridge.** 2000. 'Free radicals in biology and medicine'. Oxford: Oxford University Press.
- Hanson, A.D. and W. D. Hitz.** 1982. 'Metabolic responses of mesophytes to plant water deficits'. *Annu. Rev. Plants Physiol.* 33: 163-203.
- Herzog, V. and H. D. Fahimi.** 1973. 'Determination of the activity of peroxidase'. *Anal Biochem.* 55: 554–562.
- Jiang, M.Y. and J. H. Zhang.** 2004. 'Abscisic acid and anti-oxidant defence in plant cells'. *Acta Bot. Sin.* 46: 1–9.
- Kalberer, S.R., M. Wisniewski and R. Arora.** 2006. 'Deacclimation and reacclimation of cold-hardy plants: Current understanding and emerging concepts'. *Plant Sci.* 171: 3–16.
- Kang, J., S. Mehta and F. J. Turano.** 2004. 'The putative glutamate receptor 1.1 (AtGLR1.1) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signalling to control development and water loss'. *Plant Cell Physiol.* 45, 1380–1389.
- Karimi, R.** 2017. 'Potassium-induced freezing tolerance is associated with endogenous abscisic acid, polyamines and soluble sugars changes in grapevine'. *Sci. Hort.* 215, 184-194.
- Karimi, R. and A. Ershadi.** 2015. 'Role of exogenous abscisic acid in adapting of 'Sultana' grapevine to low temperature stress'. *Acta Physiol. Plant.* 37(8):1-11.
- Karimi, R., A. Ershadi, A. Rezaeinezhad and S. Khanizadeh.** 2016. 'Abscisic acid alleviates the deleterious effects of cold stress on 'Sultana' grapevine (*Vitis vinifera* L.) plants by improving the anti-oxidant activity and photosynthetic capacity of leaves'. *J. Hortic. Sci. Biotechnol.* Doi:10. 1080/ 14620316. 2016. 1162027.
- Keller, M.** 2010. 'The Science of Grapevines: Anatomy and Physiology'. Burlington, MA: Academic Press.
- Kirnak, H., C. Kaya, I. Tas and D. Higgs.** 2001. 'The influence of water deficit on vegetative growth, physiology, fruit yield and quality in egg plants'. *Plant Physiol.* 27, 34-46.
- Koussa, T., D. Zaoui and M. Broquedis.** 1998. 'Relationship between the levels of abscisic acid in latent buds, in leaves and in internodes of *Vitis vinifera* L. cv. Merlot during the dormancy phase'. *J. Int. Sci. Vigne. Vin.* 32:203-210.
- Li, Z., X. Zhao, A. K. Sandhu and L. Gu.** 2010. 'Effects of exogenous abscisic acid on yield, antioxidant capacities, and phytochemical contents of greenhouse grown lettuces'. *J. Agric, Food Chem.* 58, 6503-6509.
- Lichtenthaler, H.K.** 1987. 'Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes'. *Methods Enzymol.* 148, 350–382.
- Linden, L.** 2002. 'Measuring cold hardiness in woody plants'. Ph.D. Dissertation, University of Helsinki.
- Lukatkin, A. S.** 2002. 'Contribution of oxidative stress to the development of cold-induced damage to leaves of chilling-sensitive plants: 2. The activity of antioxidant enzymes during plant chilling'. *Russ. J. Plant Physio.* 49, 782–788.
- McKay, H.M.** 1992. 'Electrolyte leakage from fine roots of conifer seedlings: a rapid index of plant vitality following cold storage'. *Can. J. For. Res.* 22: 1371–1377.
- Mohanty, S., B. Grimm and B. C. Tripathy.** 2006. 'Light and dark modulation of chlorophyll biosynthetic genes in response to temperature'. *Planta*, 224: 692–699.
- Nakano, Y. and K. Asada.** 1981. 'Hydrogen peroxide is scavenged by ascorbate-specific

- peroxidase in spinach chloroplasts'. *Plant Cell Physiol.* 22:867–880.
- Nishizawa, A., Y. Yabuta and S. Shigeoka.** 2008. 'Galactinol and raffinose constitute a novel function to protect plants from oxidative damage'. *Plant Physiol.* 147, 1251–1263.
- Pakkish, Z., M. Rahemi and A. Baghizadeh.** 2009. 'Seasonal changes of peroxidase, polyphenol oxidase enzyme activity and phenol content during and after rest in pistachio (*Pistacia vera* L.) flower Buds'. *World Appl. Sci.* 6: 1193-1199.
- Pennycooke, J.C., S. Cox and C. Stushnoff.** 2005. 'Relationship of cold acclimation, total phenolic content and antioxidant capacity with chilling tolerance in petunia (*Petunia x hybrida*)'. *Environ. Exp. Bot.* 53: 225-232.
- Pogosyan, K.S., I. A. Sklyarova and Z. G. Karapetyan.** 1975. 'Relation between vine frost-resistance and tissue water content'. *Biol Zh Armenii*,28: 23-28.
- Said, Q., I.F. Ez-zohra, F. Mohamed and K. Tayeb.** 2014. 'Changes in levels of hydrogen peroxide and phenolic compounds in grapevine latent buds during the annual cycle'. *Int. J. Sci. Research Public.* 4, 5.
- Schnabel, B. J. and R. L. Wample.** 1987. 'Dormancy and cold hardiness of *Vitis vinifera* L cv. white riesling as influenced by photoperiod and temperature'. *Am. J. Enol. Vitic.* 38, 265–272.
- Shin, K.S., D. Chakrabarty and K. Y. Paek.** 2002. 'Sprouting rate, change of carbohydrate contents and related enzymes during cold treatment of Lily bulblets regenerated in vitro'. *Sci. Horticult.* 96: 195-204.
- Shu, S.,S. R. Guo, J. Sun and L.Y. Yuan.** 2012. 'Effects of salt stress on the structure and function of the photosynthetic apparatus in *Cucumis sativus* and its protection by exogenous putrescine'. *Physiol.Plant.* 146, 285–296.
- Strauss, G. and H. Hauser.** 1986. 'Stabilization of lipid bilayer vesicles by sucrose during freezing'. *Proc. Natl. Acad. Sci. U.S.A.* 83: 2422–2426.
- Tarkowski, L.P. and W. Van den Ende.** 2015. 'Cold tolerance triggered by soluble sugars: a multifaceted countermeasure'. *Front. Plant Sci.* 6, 203.
- Thomashow, M.F.** 1999. 'Plant cold acclimation: freezing tolerance genes and regulatory mechanisms'. *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* 50:571–599.
- Turan, O. and Y. Ekmekci.** 2011. 'Activities of photosystem II and antioxidant enzymes in chickpea (*Cicer arietinum* L.) cultivars exposed to chilling temperatures'. *Acta. Physiol. Plant.* 33, 67–78.
- Van Breusegem, F., L. Slooten, J. M. Stassart , J. Botterman, T. Moens, M. Van Montagu and D. Inzé.** 1999. 'Effects of overproduction of tobacco Mn SOD in maize chloroplasts on foliar tolerance to cold and oxidative stress'. *J. Exp. Bot.* 50, 71–78.
- Velikova, V. and F. Loreto.** 2005. 'The relationship between isoprene emission and thermo-tolerance in *Phragmites australis* leaves exposed to high temperatures and during the recovery from a heat stress'. *Plant Cell. Environ.* 28, 318–327.
- Velioglu, Y.S., G. Mazza, L. Gao and B. D. Oomah.** 1998. 'Antioxidant activity and total phenolics in selected fruits, vegetables and grain products'. *J. Agric. Food. Chem.* 46: 4113-4117.
- Yang, H., H. Li. L. Rao, G. Long, G. Shi and G. Peng.** 2011. 'Effects of exogenous ABA on antioxidant enzymes in detached citrus leaves treated by rapid freezing'. *Afr. J. Biotechnol.* 10: 9779–9785.
- Yemm, E.W. and A. J. Willis.** 1954. 'The estimation of carbohydrates in plant extracts by anthrone'. *Biochem. J.* 57: 508–514.
- Zrenner, R. and M. Stitt.** 1991. 'Comparison of the effect of rapidly and gradually developing water-stress on carbohydrate metabolism in spinach leaves'. *Plant Cell Environment*, 14: 939–946.