Assessment of exogenous application of proline on antioxidant compounds in three Citrus species under low temperature stress

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ABSTRACT
Major antioxidants were studied in three Citrus (C. reticulata, C. sinensis, and C. paradisi) under low-temperature stress (1, −1, −3°C). Regulatory effects of exogenous proline (0, 15, 20 mM) were studied as well. The results indicated that treatment of exogenous proline reduced both hydrogen peroxide and lipid peroxidation in three Citrus species. The high-performance liquid chromatography chromatograms at −3°C showed that exogenous proline increases chlorogenic, gallic, p-coumaric, and ferulic acid from phenolic acids as well as naringin, quercetin, and rutin from flavonoids. The amount of oxalic, citric, and ascorbic acid increased with increasing proline concentration but tartaric acid decreased. Exogenous proline increased Gamma-aminobutyric acid although its content showed no significant difference between 15 and 20 mM. Both low-temperature stress and treatment of proline induced the activity of ascorbate peroxidase (APX) and catalase. In contrast, lipoxygenase activity reduced with temperature decrease as well as increasing exogenous proline levels. Also, treatments of low temperature and proline showed that the levels of endogenous proline, catalase and APX in C. reticulata are higher compared with two other species and their amount increases with the reduction of temperature.

Introduction
Iran has always been a major provider of Citrus production in the world. Iran has the seventh ranking production of grapefruit, lemon, and sweet orange, and fifth ranking in mandarin production in the world (FAO 2007). The weather conditions of Iran, especially in the southern provinces, has provided favorable conditions for Citrus cultivation. In these areas, low-temperature stress causes damage to gardeners every few years, which has been a waste of their capital and has a huge economic burden for the country. Currently, low-temperature damages have become one of the major challenges in the Citrus production (Zabihi et al. 2016). Temperature effects are one of the variable environmental factors during maturation and ripening stages. Exposure to low and very low temperature is one of the most common environmental stresses on plants (Haghighi et al. 2014) and has serious impacts on their productivity (Zhang et al. 2005). Various studies have shown that the reaction of different species or cultivars under cold stress is significantly diverse (Sanghera et al. 2011). The production of reactive oxygen species (ROS) is one of the main factors of damage to plants under the condition of stress (Candan and Tarhan 2003). H2O2 is the most stable form of ROS that can be transmitted to the cell membranes and causes oxidative damage in plant cells under stress. Also, H2O2 changes the redox status of the surrounding cells and gives an antioxidant response by acting as a signal of oxidative stress (Sairam et al. 2000). Plants have antioxidant systems that are capable of elimination of active oxygen species and finally can reduce the effects of oxidative stress (Jithesh et al. 2006). MDA is the ultimate product of peroxidation of lipids in plant cells that can represent damage to the cell membranes (Cunhua et al. 2010). The increase in lipid peroxidation under low temperature was observed in coffee (Campos et al. 2003) and olive (Azzarello et al. 2009). Phenolic compounds play a significant role in the response to environmental stresses such as low temperature (Robles et al. 2003). They have antioxidant properties that prevent from the production of active oxygen species (Jakkola and Hohotola 2010). Organic acids accumulate in the vacuoles of Citrus fruit and involve in plant growth processes (Finke-meier et al. 2013). They have the ability to eliminate the toxicity produced by different stresses. Ascorbic acid (AsA) is an antioxidant that plays an important role in the elimination of ROS (Noctor and Foyer 1998). It has been shown that AsA plays multiple roles in cell division, cell wall expansion, and other growth and development processes (Pignocchi and Foyer 2003). Oxalic acid plays an important role in decreasing oxidative stress in various plant species (Lopez-Millan et al. 2009). Citric acid is the source of carbon and cellular energy used in the respiratory cycle and other biochemical pathways (Da Silva 2003). Citric acid as a vital organic acid has been reported to be closely related with stress (Gao et al. 2010). Gamma-aminobutyric acid (GABA) is a non-protein amino acid that exists widely in plants and is involved in processes of growth and development of plants and also in response to environmental stresses (Deewatthanawong et al. 2010; Malekzadeh et al. 2012). It plays an important role in the prevention of expression of genes involved in H2O2 and ethylene production in Caragana intermedia under conditions of stress (Shang et al. 2011). The antioxidant enzymes are one
of the most important systems of defense in plants that scavenge active oxygen species (Scandalias 1994). These antioxidant enzymes including catalase, peroxidase, superoxide dismutase, and ascorbate peroxidase (APX) eliminate ROS to protect plant cells against stress conditions (Orabi et al. 2001). The studies have shown that a correlation exists between low-temperature tolerance and antioxidant enzymes activity in the fruits of satsuma (Citrus unshiu) (Monerri and Guardiola 2001). Lipoxygenase (LOX) is one of the oxidative enzymes that catalyzes the hydroperoxidation of unsaturated fatty acids and accelerates lipid peroxidation (Rosahl 1996; Sadeghian et al. 2008). Increasing LOX activity results in an increase in lipid peroxidation in plants under stress conditions (Aziz and larher 1998). One of the common responses of many plant species exposed to different abiotic stresses is the accumulation of compatible organic solutes such as proline (Serraj and Sinclair 2002). Proline is a highly soluble and non-toxic amino acid which has a low molecular weight (Ashraf and Foolad 2007). This amino acid and other amino acids play the main role as an osmoprotectant in the adaptation to osmotic stress to stabilize cell structures such as membranes, proteins, and DNA and eliminate free oxygen radicals (Orcutt and Nilsen 2000; Verbruggen and Hermans 2008). In addition, it is a source of nitrogen, carbon, and energy during the recovery period after stress (Matysik et al. 2002). Studies of De Carvalho et al. (2013) have shown that the use of exogenous proline reduces the harmful effects of oxidative stress due to its ability to increase the activity of antioxidant enzymes. Several studies have been carried out about the effects of cold stress on Citrus species and changes of their chemical compositions. However, there is no published research on the three studied species. In addition, there is no study on the effect of exogenous proline in the species under study and its role on the reduction of disorders induced by cold stress. The aim of the experiment was to identify the role of proline on biochemical compounds in order to decrease the low-temperature effects as well as to improve the tolerance to this stress in three Citrus species.

Materials and methods

Plant materials and stress treatments

Branches containing fruits of desired trees (C. reticulata, C. sinensis var. valencia, and C. paradisi var. redblush) were treated with the amino acid proline at concentrations of 0, 15, 20 mM (Gerdananeh et al. 2010; Koc et al. 2016). The branches which were approximately identical in length and number of fruits were selected from the same age trees. After 24 h of spraying, the treated branches were separated and placed in 15% sucrose solution and then containers containing treated shoots exposed to temperatures 1°C, −1°C and −3°C for three hours. The treated fruits were immediately frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

Biochemical analyses

Endogenous proline

Proline amount was determined according to the method described by Bates et al. (1973). In brief, 0.2 g fruit samples were homogenized in 1 ml of 3% (w/v) sulphosalicylic acid. After centrifugation, 0.1 ml of supernatant was transferred into a solution of 0.2 ml acid ninhydrin, 0.2 ml of 96% (v/v) acetic acid, and 0.1 ml of 3% (w/v) sulphosalicylic acid. Samples were incubated for 1 h at 96°C, and 1 ml of toluene was added. After centrifugation, the upper phase was transferred into quartz cell and the absorbance was recorded at 520 nm. Proline amount was calculated using proline standard curve.

Chromatographic analysis of phenolic compounds by high-performance liquid chromatography

About 0.5 g of fruits was homogenized in 1 ml of methanol 80%. Extraction was aided by means of vortexing for 30 min intervals. The resultant mix was centrifuged at 12,000g for 10 min at room temperature and the supernatant was used for high-performance liquid chromatography (HPLC) analysis (S2100/Saykam). The mobile phase contained 1% aqueous acetic acid solution (Solvent A) and acetonitrile (Solvent B); the flow rate was adjusted to 0.7 ml/min. The samples were eluted by the following gradient: 90% A and 10% B as initial conditions, 60% A and 40% B for 28 min, 40% A and 60% B for 32 min, finally, 10% A and 90% B for 45 min. HPLC chromatograms were detected using a UVVIS detector at a wavelength of 272 nm according to absorption maxima of analyzed compounds. The column was a C18 column and injection volume was 20 μl (Seal et al. 2016). HPLC profiles were determined for samples exposed to the lowest of temperature (−3°C). The stock solution of concentration 1 mg/ml was prepared by dissolving 1 mg of phenolic acids and the flavonoids in 0.5 ml HPLC-grade methanol and the resulting volume was made up to 1 ml with the solvent for the Mobile phase (acetonitrile and 1% aq. acetic acid). The standard and sample solutions were filtered through 0.45 μm polyvinylidene fluoride (PVDF)-syringe filter and the mobile phase was degassed before the injection of the solutions.

Determination of level of GABA by HPLC

Samples (0.2 g) were homogenized with 1 ml water: Chloroform: methanol (3:5:12 (v/v/v)) solution and centrifuged for 2 min at 10,000g at +4°C. Supernatants was collected, dried, and re-dissolved in 100 μl water. Redissolved samples were mixed with 150 μl Borax buffer and 250 μl of derivatization reagent 2-hydroxynaphthaldehyde (3%w/v in methanol). The mixture was heated in a water bath to 80°C for 20 min and cooled to room temperature. The final volume was adjusted to 1 ml with methanol and samples were filtered before injection into the HPLC column by 0.45 μm PVDF-syringe filters. Flow rate was 0.5 ml min⁻¹, the mobile phase was methanol:water (62:38 v/v) and detection was performed at 330 nm. Retention time was 10 min for assay level of GABA (Bor et al. 2009).

Chromatographic analysis of organic acids by HPLC

Juice extract of three species C. reticulata, C. sinensis, and C. paradisi was prepared. These juices were filtered by 0.45 μm PVDF-syringe filters and then injected. The determination of organic acids was performed using a mobile phase made of 50 mM phosphate solution. The flow rate of the mobile phase was 0.7 ml/min for all the chromatographic separations. The column was a C18 injection
volume was 20 µl and detection was performed at 210 nm. HPLC profiles were determined for samples exposed to the lowest temperature (~3°C). A mixed standard stock solution was prepared for organic acids containing 1 mg/ml citric acid, 2 mg/ml malic acid, 0.3 mg/ml oxalic acid and AsA, 0.7 mg/ml tartaric acid, and 0.5 mg/ml succinic acid (Nour et al. 2010).

**Hydrogen peroxide (H₂O₂)**

Hydrogen peroxide concentration was determined according to the method by Loreto and Velikova (2001). 0.2 g of fruit samples was homogenized in 3 ml of 1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000g for 10 min. Subsequently, 0.75 ml of the supernatant was added to 0.75 ml of 10 mM K-phosphate buffer (pH 7.0) and 1.5 ml of 1 M KI. H₂O₂ concentration of the supernatant was evaluated by comparing its absorbance at 390 nm to a standard calibration curve.

**Lipid peroxidation**

Lipid peroxidation was measured as the amount of MDA determined by the TBA reaction as described by Heath and Packer (1968). 0.2 g of fruits was homogenized in 4 ml of 1% (w/v) TCA, then centrifuged at 10,000g for 10 min. 1.5 ml of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added to 1.5 ml of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. The mixtures were centrifuged at 10,000g for 5 min and the absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted from the 532 nm reading. TBA reacted with MDA, resulting in a color compound, which can be determined spectrophotometrically. The MDA content was calculated using its extinction coefficient of 155 mM⁻¹ cm⁻¹ and expressed as mmol/g.

**Enzyme assays**

**Preparing of enzyme extracts**

Samples extract were prepared for the analyses by homogenizing 1 g of fruit in 4 ml of 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na–EDTA and 1% (w/v) polyvinyl–pyrrolidone (PVP). The experiment was performed on ice cold and then the homogenate was centrifuged at 10,000×g for 10 min. The supernatants were collected and stored at −20°C until using. The total protein content of samples was determined according to Bradford protein assay using bovine serum albumin (BSA) as a standard. The absorbance is recorded at 595 nm (Bradford 1976; Rezanejad et al. 2018).

**Determination of APX activity (EC 1.11.1.11)**

The measure of APX activity using spectrophotometer was determined as described by Nakano and Asada (1981). The assay mixture consisted of 100 µg of the enzyme extract added to assay solution (50 mM K-phosphate buffer (pH 6.6) with 2.5 mM ascorbate) and the reaction was initiated by the addition of 10 mM H₂O₂. The decrease in the absorbance of ascorbate was recorded at 290 nm for 3 min against assay solution (ε = 2.8 mM⁻¹ cm⁻¹).

**Determination of CAT activity (EC 1.11.1.6)**

Catalase activity was determined as described by Chance and Mahly (1995). The assay mixture consisting of 100 µg of the enzyme extract was added to 50 mM K-phosphate buffer (pH 7.0) and 200 mM H₂O₂ to initiate the reaction. The decrease in the absorbance H₂O₂ was recorded at 240 nm for 3 min against assay solution (ε = 39.4 mM⁻¹ cm⁻¹).

**Determination of LOX activity (EC 1.13.11.12)**

LOX activity was measured by monitoring the increase in the absorbance over a 2 min period of time at 234 nm (Reddanna et al. 1990). The typical reaction mixture contained 2.8 ml of 50 mM Na-phosphate buffer in pH 6.4 and 100 µl of the enzyme extract. The reaction was started by adding 250 ml of linoleic acid to the reaction mixture.

**Statistical analysis**

The experimental design was a completely randomized factorial with three replications. Data were analyzed by analysis of variance (ANOVA) and the means were compared (p ≤ .05) by Duncan’s multiple range test (DMRT). All analyses were performed using a version of the software SAS (SAS Institute, Cary, NC, USA).

**Results**

**Endogenous proline**

The results showed that the amount of endogenous proline increased with a decreasing temperature in three *Citrus* species (Figure 1(A–C)). Exogenous application of proline increased the levels of endogenous proline in *Citrus* species, so that with an increasing in its concentration, the amount of endogenous proline also increased (Figure 1A–C). The levels of endogenous proline under both low temperature and exogenous proline were higher in *C. reticulata* compared with two other species (Figure 1A–C).

**Hydrogen peroxide and lipid peroxidation**

Cold stress induced a significant increase in the production of hydrogen peroxide and ultimately was led to lipid peroxidation in three *Citrus* species. Also, the amount of hydrogen peroxide and lipid peroxidation increased with decreasing temperature (Figures 2(A–C) and 3(A–C)). The application of different concentrations of exogenous proline was led to the reduction of levels of hydrogen peroxide and lipid peroxidation in all species (Figures 2(A–C) and 3(A–C)). However, the lowest levels of production of ROS and peroxidation of lipids were observed in fruits treated with proline 20 mM. Also, the data showed that the amounts of both hydrogen peroxide and lipid peroxidation in fruits treated with proline were lower in *C. reticulata* than two other species.
HPLC analyses of phenolics, organic acids, and GABA

HPLC chromatography was applied in order to determine phenols and flavonoids in the extracts of Citrus fruits. Used standards were contained chlorogenic, gallic, tannic, p-coumaric, ferulic acid, and salicylic acid from phenolic acids as well as rutin, quercetin, and naringin from flavonoids. The HPLC chromatograms of Citrus species at −3°C showed that exogenous application of proline led to increasing phenolic compounds including chlorogenic, gallic, p-coumaric, and ferulic acid from phenolic acids and also quercetin and rutin from flavonoids. However, with increasing exogenous proline concentration to 20 mM, no significant increase was observed in the amount of these components compared to 15 mM. The amount of tannic and salicylic acid was negligible and was recorded as undetectable (ND). The contents of phenolics were lower in C. paradisi than two other species (Table 1). The results of HPLC of organic acids showed that the amount of oxalic, citric, and AsA in three Citrus species increased with increasing proline concentration. However, the amount of tartaric acid decreased with the treatment of exogenous proline; also succinic and malic acid were not observed in the studied species. The highest and lowest organic acids were AsA and tartaric acid, respectively; further, similar to phenolics, the levels of organic acids were recorded lower in C. paradisi than two other species (Table 1). The GABA fingerprints showed that exogenous proline was led to an increase in the amount of this free amino acid although...
its content showed no significant difference between concentrations of 15 and 20 mM of exogenous proline. Also, obvious differences were not observed in GABA amount between species (Table 1).

**Enzyme activities**

Generally, the plants activate the antioxidant enzymes when are exposed to cold stress to eliminate active oxygen species. Figure 4 demonstrates that both cold stress and exogenous proline induce the activity of the studied antioxidant enzymes including APX and catalase (CAT). Further, with increasing of proline level to 20 mM, the activity of APX and CAT increases too. Also, the activity of these enzymes increased, concurrent with temperature reduction (Figures 4(A–C) and 5(A–C)). LOX activity reduces with increasing exogenous proline levels (Figure 6(A–C)). Therefore, proline with the highest concentration was led to an increase in the activity APX and CAT and decreasing LOXs activity. The highest levels of APX and CAT as well as the lowest levels of LOX were observed in fruits of *C. reticulata*.

**Discussion**

Low-temperature stress causes the reduction of the growth and development processes in plants and finally led to their death (Zhu et al. 2007). Chanishvili et al. (2007) indicated that various abiotic stresses including low temperature,
Figure 3. (A–C) Effect of temperature and exogenous proline on lipid peroxidation in three Citrus species. Means with the same letter are not significantly different from each other (p ≤ 0.05).

Table 1. Effects of exogenous proline on level of phenolics, organic acids, and GABA in Citrus species at −3°C.

| Parameters                  | C. reticulata | C. sinensis | C. paradisi |
|-----------------------------|---------------|-------------|-------------|
| Phenolics (µg/g)            |               |             |             |
| Phenols                     |               |             |             |
| Chlorogenic acid            | 0.5<sub>b</sub> | 0.55<sub>b</sub> | 0.63*        |
| Gallic acid                 | 0.38<sub>b</sub> | 0.45<sub>b</sub> | 0.51*        |
| P-Coumaric acid            | 0.19<sub>b</sub> | 0.25<sub>b</sub> | 0.3*         |
| Ferulic acid acid           | 0.18<sub>b</sub> | 0.3*        | 0.16*        |
| Salicylic acid              | ND            | ND          | ND          |
| Tannic acid                 | ND            | ND          | ND          |
| Flavonoids                  |               |             |             |
| Quercetin                   | 0.15<sub>b</sub> | 0.28<sub>b</sub> | 0.34*        |
| Rutin                       | 0.18<sub>b</sub> | 0.26<sub>b</sub> | 0.3*         |
| Naringin                    | 0.3<sub>a</sub>  | 0.28<sub>a</sub> | 0.24<sub>ab</sub> |
| Organic acids (µg/g)        |               |             |             |
| Oxalic acid                 | 0.53<sub>a</sub> | 0.61*        | 0.4<sub>b</sub> |
| Tartaric acid               | 0.21<sub>b</sub> | 0.2<sub>b</sub> | 0.3*         |
| Ascorbic acid               | 0.55<sub>b</sub> | 0.65*        | 0.51<sub>b</sub> |
| Citric acid                 | 0.45<sub>b</sub> | 0.52<sub>b</sub> | 0.43*        |
| Succinic acid               | ND            | ND          | ND          |
| Malic acid                  | ND            | ND          | ND          |
| Free amino acid (µg/g)      |               |             |             |
| Gamma-aminobutyric acid     | 0.55<sub>a</sub> | 0.65*        | 0.4<sub>b</sub> |

Note: Values in the same column with different superscript letters represent significant differences between Citrus species at p < .05 by Duncan’s test. C: control, P<sub>1</sub>: proline 15 mM, P<sub>2</sub>: proline 20 mM.
mineral deficiency, and UV radiation increase the production of free radicals and the other oxidative species. Results of the current study indicated that the production of hydrogen peroxide and lipid peroxidation increased with decreasing temperature to $-3^\circ C$. Further, pre-treatment with proline was led to decreasing hydrogen peroxide and lipid peroxidation at different temperatures. The least amount of hydrogen peroxide and lipid peroxidation were observed in *C. reticulata* than other species. Production of hydrogen peroxide and lipid peroxidation in plants occurs immediately after exposing to cold stress (Lyons 1973). When the production of ROS exceeds the capacity of the plant to scavenge, lipid peroxidation increases in the membrane and thus affects the physiological process of the cell. MDA is one of the final products of oxidative modification of lipids, which is responsible for cell membrane damage including changes in the intrinsic properties of the membrane such as fluidity, ion transport, loss of enzyme activity and protein cross-linking, inhibition of protein synthesis, DNA damage and so forth ultimately resulting in cell death (Sharma et al. 2012). *Citrus* fruits are very sensitive to low-temperature damages and especially at the ripening stage, can only tolerate 2–3 h decreasing of temperature from 2 to $-3^\circ C$. However, cold and freezing damages also depend on species type, growth condition, and age (Zabihi et al. 2016).

**Figure 4.** (A–C) Effect of temperature and exogenous proline on APX in three *Citrus* species. Means with the same letter are not significantly different from each other ($p \leq .05$).
Studies have shown that proline is a reactive oxygen scavenger; therefore it has an antioxidant effect and reduces lipid peroxidation (Hossain and Fujita 2010). Sanchez-Rodriguez et al. (2012) have shown that pre-treatment with proline led to decreasing MDA and H₂O₂ contents and consequently increasing the antioxidant defense system. Also, its accumulation has been shown in different abiotic stresses. It has been suggested that proline protects plants by functioning as a cellular osmotic regulator between cytoplasm and vacuole. Also, it supports membrane integrity and stabilizes antioxidant enzymes by detoxifying of ROS (Ozden et al. 2009). Results of this research showed that exogenous application of proline was led to increasing endogenous proline at different temperatures. In *C. reticulata*, the levels of endogenous proline were higher than two other species. The plants resort to defense mechanisms through enhancing the process of osmoregulation such as accumulation of different metabolites, particularly amino acids (Szabados and Savoure 2010). These osmolytes maintain the structure of endogenous proline and photosynthetic apparatus and detoxify ROS through cellular osmoregulation in response to abiotic stresses (Ashraf and Foolad 2007). Higher levels of proline in comparison with other amino acids have been observed under environmental stresses. Generally, there is a strong positive relationship between stress tolerance and proline accumulation in higher plants (Ashraf and Foolad 2007). Therefore, increasing level of endogenous proline in fruits exposed to exogenous proline can be attributed to its osmoprotectant function.

In the present study, pre-treatment with proline increased some phenolic compounds at −3°C. However, with increasing exogenous proline concentration to 20 mM, no significant

![Figure 5](image-url)

**Figure 5.** (A–C) Effect of temperature and exogenous proline on catalase enzyme in three *Citrus* species. Means with the same letter are not significantly different from each other (*p* ≤ .05).
increase was observed in the amount of these components. There are reports showing that proline, effectively stimulates total phenolic content and specific phenolic metabolites, suggesting that phenolics are the main compounds responsible for radical-scavenging activity (Shen et al. 2009). Phenols protect the cells from potential oxidative damage and increase the stability of cell membrane (Burguieres et al. 2006). Some organic acids (oxalic, citric, and AsA) increased under exogenous application of proline but the reduction of tartaric acid was observed. The highest and lowest of organic acids were AsA and tartaric acid, respectively; further, similar to phenolics, the levels of organic acids were recorded lower in *C. paradisi* than two other species. Oxalate quantities greatly vary based on plant family and organs, growing conditions, climate, and growth periods. Role of proline was mentioned in ascorbate-glutathione cycle including AsA (Aggarwal et al. 2011). Researchers also reported significant correlations between total phenols and AsA content with antioxidant activity in peppers (Mohamed and Akladious 2014).

Exogenous proline improves cell growth and prevents antioxidant damage by activating antioxidant systems and consequently increasing resistance to environmental stresses (Morita et al. 2002; Tian et al. 2016). The GABA levels increased by external proline but its content showed no significant differences with its concentration. There is no published paper on the role of proline in the amount of GABA in plants. Proline, by the elimination of active oxygen species, leading to an increase in the level of antioxidants, including

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**Figure 6.** (A–C) Effect of temperature and exogenous proline on LOXs in three *Citrus* species. Means with the same letter are not significantly different from each other (p ≤ .05).
the production of GABA in plants under stress conditions. Also, obvious differences were not observed in GABA amount between species. The studies have shown that amount of GABA rapidly increase during abiotic stresses including drought, cold, heat shock, and mechanical stimulation (Bown and Shelp 1989). Wang et al. (2014) reported that low temperatures induce plant defense responses, and GABA accumulation is a plant stress response to abiotic stress. Under low-temperature conditions, plants can accumulate GABA by increasing glutamic acid decarboxylase (GAD) activity and reducing c-aminobutyrate aminotransferase (GABA-T) activity. Mazzucotelli et al. (2006) found that the GABA content in barley seedlings after low-temperature exposure was 30 times higher than the control group, whereas the content of glutamic acid decreased. This suggests that exposing plants to low temperatures for a short period during cultivation is beneficial for GABA accumulation and can induce high GABA content in functional nutrient crops (Yoon et al. 2017).

The results of this experiment showed that both low-temperature stress and proline increased the activity of antioxidant enzymes including CAT and APX. To avoid the potential damage caused by ROS to cellular components, as well as to maintain growth, metabolism, development, and overall productivity, the balance between production and elimination of ROS at the intracellular level must be tightly regulated and/or efficiently metabolized. This equilibrium between the production and detoxification of ROS is sustained by enzymatic and non-enzymatic antioxidants (Mittler et al. 2004). Tolerance to low temperature in plants has been reported with increasing antioxidant enzymes activity such as APX in Citrus aurantiifolia seedlings (Najafzadeh 2010), CAT in Fortune mandarin (Sala and Lafuente 2000), APX and CAT n the olive trees (Ortega-Garcia and Peragon 2009). The enzymes APX and CAT are able to scavenge H2O2 with different mechanisms. Banu et al. (2009) also observed that exogenous application of proline in tobacco cells submitted to salt stress-induced distinct transcript activity of two CAT isoforms. APX is the key enzyme of the ascorbate–gluthione cycle that has an important role in providing resistance to oxidative stresses caused by different stressors such as drought, heavy metals, high light, salinity, and low temperature (Pang and Wang 2010). It has been suggested that osmoproteancts, such as proline, may increase the tolerance of plants to various types of abiotic stresses acting in signal transduction pathways through regulation of stress-responsive genes (Banu et al. 2009). LOX activity decreased with increasing exogenous proline levels. The LOX enzyme is a category of non-homogeneous iron proteins responsible for the peroxidation of unsaturated fatty acids in plants (Sadeghian et al. 2008). Fatty acids are rapidly degraded into metabolites which produce volatile aldehydes, such as MDA (Bird and Draper 1984; Sofo et al. 2015). Proline prevents the degradation of fatty acid molecules and decreases LOX enzyme activity (Matsyik et al. 2002). It has also been proposed that proline can act as an electron acceptor, avoiding damage of photosystems due to their photoinhibition by activated oxygen species (Hare et al. 1997). Proline accumulates in a wide range of plants in response to various stresses. In addition, some studies have shown a direct relationship between the increased activity of enzymes linked to defense against oxidative stress and proline levels (Okuma et al. 2000). The results of this experiment showed that the levels of endogenous proline, catalase, and APX in Citrus reticulata are higher compared with two other species and their levels increase with the reduction of temperature and different concentrations of exogenous proline. Also, the amounts of hydrogen peroxide, lipid peroxidation, and LOX enzyme in fruits treated with proline were lower in Citrus reticulata than two other species under low-temperature stress. The effect of exogenous proline on the level of phenolics, organic acids, and GABA was not significantly different between Citrus species at −3°C.

Disclosure statement

No potential conflict of interest was reported by the authors.

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