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Research Article

Effect of low- temperature stress on dehydrin protein, GABA, endogenous proline and antioxidant enzymes in *Citrus reticulata* extract in postharvest conditions

Soheila Mohammadrezakhani and Farkhondeh Rezanejad*

Department of Biology, Shahid Bahonar University of Kerman, Kerman, Iran
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Abstract

Low-temperature stress leads to accumulation of proteins, osmolytes and antioxidants, which involve in plant tolerance to stress conditions. This study aimed to evaluate dehydrin, gamma-aminobutyric acid (GABA), endogenous proline and activities of antioxidant enzymes in *Citrus reticulate* under low-temperature stress (1°C, -1°C and -3°C) during postharvest. The results of western blot showed proteins with the molecular weight of 30.19, 36.3 and 62.25 kDa induced in response to low temperature stress. The level of endogenous proline, GABA and activities of antioxidant enzymes (SOD and APX) also increased with decreasing temperature. The amount of GABA and proline in temperatures of -1 and -3°C more than 1°C. With reduction of temperature induces the activity of SOD and APX in *C. reticulata*. As a result, at -3 °C, the highest activity of SOD and APX was observed. Generally, in fruits under the temperature of -3°C, levels of antioxidants and protein accumulation increased.

Keyword: Antioxidant enzymes, Cold stress, Fruit

Introduction

Citrus, such as other tropical and subtropical fruits, is sensitive to low-temperatures stress (Balaguera-Lopez *et al*., 2019). The low-temperature stress reduces various processes, including cell division, photosynthesis, plant growth, development, metabolism, and finally reduce the yield in plants (Zhang *et al*., 2020).

The production of reactive oxygen species is one of the main factors of damage under biotic and abiotic stresses in plants (Chen *et al*., 2018). To avoid hydrogen peroxide accumulation, a compound even more damaging than the superoxide radical, two enzyme activities, catalase (CAT) and ascorbate peroxidase (APX) act detoxifying this compound and yielding water and oxygen. The expression of both enzymes seems to be induced by oxidative products (Sarker and Oba, 2018).

Reports showed that there is a correlation between superoxide dismutase (SOD) enzyme activity and tolerance of Mexican lime (Najafzadeh, 2010) and *Sabina* seedling (Chen *et al*., 2006) to low-temperature stresses. Ascorbate peroxidase (APX) can transform H_2O_2 to H_2O and O_2 (Demiral and Turkan, 2005).

Increasing of antioxidant activity in fruits of *Citrus* under low-temperature has been mentioned (Rapisarda *et al.*, 2008). Plants accumulate certain compounds that play a protective role in adaptation to low-temperature conditions (Sakamoto and Murata, 2002).

These compounds contribute to an antioxidant defense by protecting from the production of free oxygen radicals, detoxifying active oxygen species, protecting the membrane integrity and stability of proteins, enzymes and intracellular macromolecules (Orcutt and Nilsen, 2000).

The accumulation of proline in cells can act as an osmotic protector of the cells and protection of plants from osmotic stress (Hosseinifard *et al.*, 2022).

Dehydrins are proteins that are induced by environmental conditions and act as an essential component of dehydration tolerance (Sun *et al*., 2021). To adapt to environmental condition, plants have developed complex mechanisms that allow them to rapidly respond to abiotic stresses. Dehydrin proteins are considered as stress proteins involved in the formation of plants protective reactions to dehydration (Liu *et al*., 2017).

These proteins may help water retention, membrane stability and also prevention of damage to cellular proteins (Sun *et al*., 2021). Dehydrin protein acts as an antioxidant, which protects plants from oxidative stress and eliminates active oxygen species (Hara *et al*., 2004).

*Corresponding Author, Email: frezanejad@uk.ac.ir

In plants, gamma-aminobutyric acid (GABA) is involved in certian physical and biochemical functions to regulate their growth and increase tolerance to abiotic stresses (Salah *et al.*, 2019).

Low-temperature stress every year causes great damage to gardeners. Therefore, the use of cold stress resistance mechanisms becomes significant. The purpose of this experiment is to investigate the effect of low temperature on biochemical compounds and dehydrin in *Citrus reticulata* at different temperatures in order to prevent oxidative damage.

Material and method

Plant materials: This experiment is in 2018 in Jiroft city with the geographical location 28°40′ 13″ N, 57°44′ 13″ E. Branches containing fruits of desired trees from *C. reticulate* were sprayed with the amino acid proline at concentrations of 0, 15, 20 mM (Gerdakaneh *et al*., 2010). Branches that were approximately the same in length and number of fruits were selected from 15-year-old trees and the branches were placed in containers containing 15% sucrose solution. then fruits after harvest exposed to 1 °C, 1 °C and -3 °C for three hours (Geisel, 2003). The treated fruits were immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analyses.

Extraction of total proteins and western blot assay: 0.5g of fruits of sample was used for total protein extraction by a P-PER™ Plant Protein Extraction kit (Thermo Scientifc, USA). The extracted materials were stirred for 30 min and centrifuged for 10 min at 10,000g. The supernatants were heated to 85 C for 10 min and centrifuged for 10 min at 10,000g to obtain the heat-stable proteins (Panza *et al*., 2007). The heat-stable protein concentration was estimated using Bradford method and bovine serum albumin as a standard. An amount of 5 µg of each protein sample was resolved by 12.5% SDS-PAGE at 90V using a Mini-PROTEAN 3 electrophoresis Cell (Bio-Rad, Italy). A prestained protein ladder was also used (Thermo scientifc, Lithuania). The resolved proteins were then transferred from gel onto a polyvinylidene fluoride (PVDF) membrane (Amersham, UK) at 100 V for 50 min using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Italy). The membranes were blocked with 5% w/v nonfat dried milk for 3h at 4C. In one experiment, the membrane was incubated with an anti-actin primary polyclonal serum (Agrisera, AS132640, Sweden) at a 1:2500 dilution as loading control and in a separate experiment with an anti-dehydrin primary polyclonal serum (Agrisera, AS07 206A, Sweden) at a 1:1000 dilution each for 1h at 4 °C. The membranes were then incubated with a goat anti-rabbit IgG (HandL) horseradish peroxidase (HRP) conjugated secondary antibody (Agrisera, AS09 602, Sweden) at a 1.10,000 dilution for 1 h at room temperature. (Nonfat dried milk and antibodies were diluted in TBST: 50mM Tris–HCl, 150 mM NaCl, 0.1% v/v Tween 20, pH 7.5) Subsequently, the membranes were exposed to enhanced chemiluminescence (ECL) substrate (Amersham, UK) and the luminescence signals were documented on X-ray films (Fuji, Japan). The films were photographed, and the densitometry of bands was measured using the ImageJ software (Zamani Bahramabadi *et al*., 2019).

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).

Endogenous proline: Proline amount were determined according to 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 (Merck), 0.2 mL of 96 % (v/v) acetic acid, and 0.1 mL of 3 % (w/v) sulphosalicylic acid. Samples were incubated for a 1 h at 96 C, and 1 mL of toluene was added. After centrifugation, upper phase was transferred into quartz cuvettes and absorbance was recorded at 520 nm against toluene. Proline amount was calculated by using proline standard curve (Bates *et al*., 1973).

Enzyme assays: Samples extraction were prepared for the analyses by homogenizing 1g of fruit in 4 ml of 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na–EDTA and 1% (w/v) polyvinyl–polypirrolidone (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).

Determination of superoxide dismutase (SOD): Superoxide dismutase activity was measured by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm. The reaction mixture (1 ml consisted 75 µM NBT, 13 mM L-methionine, 0.1 mM EDTA and 2 µM riboflavin in 50 mM potassium phosphate buffer (pH 7). The reaction and control mixture were incubated for 15 min in 300 $\mu molm^{-1}s^{-1}$ irradiance at 25 °C. A non-irradiated reaction mixture was used as blank. One unit of SOD activity was expressed as the quantity of SOD required to produce a 50% inhibition of NBT photochemical reduction. The specific enzyme activity was said as units per mg of leaf

fresh wet (Giannopolitis and Ries, 1997).

Determination of ascorbate peroxidase (APX) activity: 0.2 g samples were homogenized in 50 mM Tris–HCl buffer (pH 7.2) containing 2% (w/v) polyvinylpoly pyrrolidone, 2 mM ascorbate (Sigma) and 1 mM EDTA. After centrifugation, total protein amount in supernatants was determined according to Bradford (1976). 100 µg of total protein was added to assay solution (50 mM potassium phosphate buffer with 2.5 mM ascorbate; 1 ml total assay medium), and reaction was initiated by the addition of 10 mM H_2O_2. Decrease in the absorbance of ascorbate was recorded at 290 nm for 3 min against assay solution (e = 2.8 $mM^{-1}cm^{-1}$) (Nakano and Asada, 1987).

ABTS assay: The ABTS radical scavenging was measured using the method of Re *et al*. (1999). The ABTS radical cations ($ABTS^{+}$) were produced by reacting aqueous solution of ABTS (7 mM) with aqueous solution of potassium persulfate (2.45 mM). The mixture was allowed to stand in the dark at room temperature for 12–16 h before use and then was diluted with distilled water to obtain an absorbance of 0.700± 0.005 at 734 nm. 30 µL of the sample added to 3 ml of the ABTS radical solution was allowed at room temperature for 6 min and the absorbance at 734 nm was recorded immediately. Percentage of inhibition of the ABTS radical was calculated according to the following equation: % inhibition of ABTS = [(Abs control - Abs sample)/ Abs control] x 100 where Abs control is the absorbance of ABTS solution without extracts (Re *et al*., 1999).

Statistical analysis: The experimental a completely randomised design with temperature being the single factor with three replications. Data were analysed by analysis of variance (ANOVA) and the means were compared ($P \leq 0.05$) by Dancan's multiple rang test (DMRT). All analyses were performed using a version of the software SAS (SAS Institute, Cary, NC, USA).

Results

Dehydrin protein: The different temperatures have caused the appearance of dehydrin bands in *C. reticulate*. The results of Western blot revealed that three major bands of 30.19, 36.3 and 60.25 kDa were observed in fruits of *C. reticulate.* All three dehydrin bands were observed at all temperatures -3, -1 and 1 °C (Figure 1).

Gamma-aminobutyric acid: The results of analysis table of variance showed that the effect of different temperatures on the amount of GABA in *C. reticulata* fruits is significant at the level of 5% (Table 1). The results of comparison of means showed that the GABA levels in *C. reticulata* were increased in the samples under -1 and -3 °C, although they were not statistically significant. The lowest level is also found in samples under 1 °C (Figure 2).

Endogenous proline: The amount of proline is significant as a result of the effect of different temperatures at the level of 5% in *C. reticulata* fruits (Table 1).

The amount of endogenous proline increased with decreasing of temperature in *C. reticulata,* so that, the highest amount was observed in the fruits exposed to -3 °C. However, no statistically significant differences were observed between temperatures 1 and -1 °C (Figure 3).

SOD and APX: The results of analysis of variance showed that the effects of temperatures on SOD and APX activity in *C. reticulata* fruits were significant (Table 1).

Figures 4 and 5 demonstrate that the reduction of temperature induces the activity of SOD and APX in *C. reticulata*. As a result, at -3 °C, the highest activity of SOD and APX was observed (figures 4, 5).

ABST: Different temperatures have shown significant effects on the amount of ABST in *C. reticulata* fruit (Table 1). The results of the effect of different temperatures on levels of ABST showed that the amount of ABST at -3 °C was higher than other temperature. The lowest amount of ABST has been observed at 1 °C (figure 6).

Discussion

Cold stress can directly influence the fluidity of plant cell membrane and enzyme activity, resulting in metabolic disturbance, photosynthesis inhibition, material transportation disorder and, finally, damage to plants (Yu *et al*., 2018).

Low-temperature lead to the production of reactive oxygen species (ROS), that can damage to cellular components including proteins, pigments, membrane, lipids, and nucleic acids (Hernandez *et al*., 2001). The tolerance level of plant cells may be determined by the number of the dehydrin bands (Panza *et al*., 2007), as well as their expression levels (Sun *et al*., 2021). The results of this study showed that *C. reticulata* fruits have produced dehydrin proteins in order to resist cold stress and its damages.

Dehydrins (DHNs) proteins play a fundamental role in plant response and adaptation to abiotic stresses. They accumulate typically in maturing seeds or are induced in vegetative tissues following salinity, dehydration, cold and freezing stress (Hanin *et al*., 2011).

In this research, the synthesis of antioxidant compounds and osmolytes including GABA and proline in *C. reticulata* fruits under different temperatures has been seen. The decrease in temperature has led to the increase of these compounds and in this way it has reduced the oxidative damage in plants.

GABA levels were increased in fruits of *C. reticulata* under low-temperature. Among the numerous compounds that seem to serve as osmoprotectants in plants is the four-carbon, non-protein amino acid c-aminobutyric acid (GABA), which under stress conditions may represent a significant fraction of the free amino acid pool (Ramesh *et al*., 2015). Intracellular levels of GABA are typically low, but they rapidly

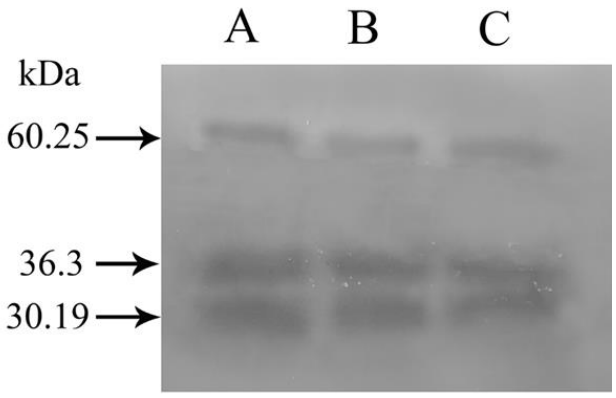

Figure 1. The profile of dehydrin protein in *C. reticulata* at different temperatures, A= 1 °C, B= -1 °C, C= -3 °C

Table 1. Analysis table of variance related to the effect different temperatures on level of GABA, proline, SOD, APX and ABST in *Citrus reticulata*.

Source	df	Mean of squar				
		GABA	Proline	SOD	APX	ABST
Treatment	2	0.1*	0.4*	0.04*	0.11**	2.8**
Error	6	0.02	0.003	0.004	0.007	0.007
CV	-	2.5	5.5	2.5	7.8	8

*** P<0.05, ** P<0.01, ns not significant**

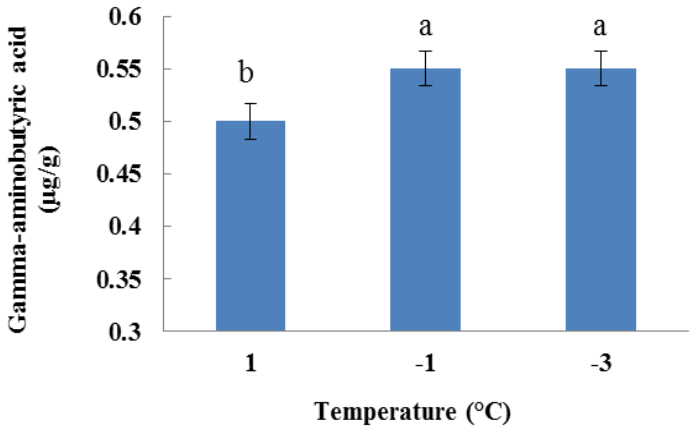

Figure 2. Effect of different temperatures on level of GABA in *Citrus reticulata.* Means with the same letter are not significantly different (P < 0.05).

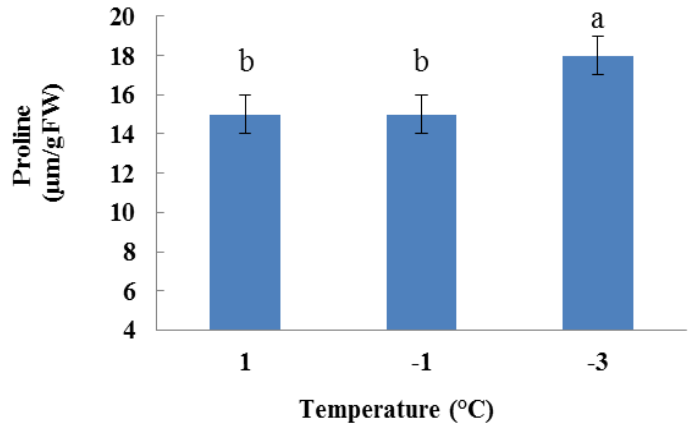

Figure 3. Effect of different temperatures on level of endogenous proline in *Citrus reticulata.* Means with the same letter are not significantly different (P < 0.05).

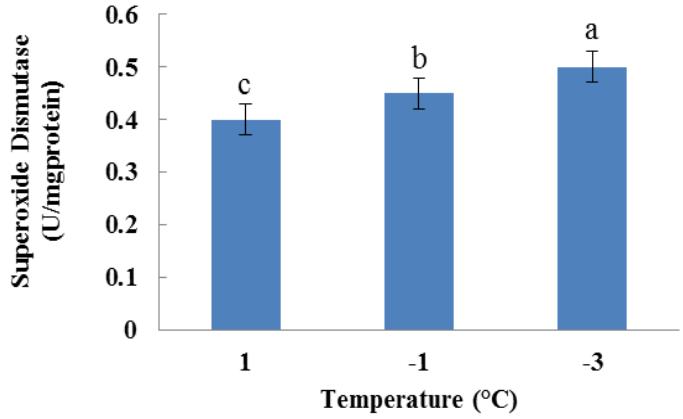

Figure 4. Effect of different temperatures on activity of SOD in *Citrus reticulata.* Means with the same letter are not significantly different (P < 0.05).

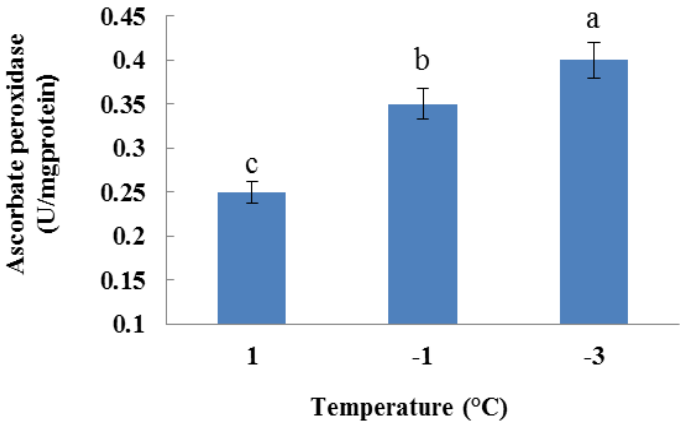

Figure 5. Effect of different temperatures on level of APX in *Citrus reticulata.* Means with the same letter are not significantly different (P < 0.05).

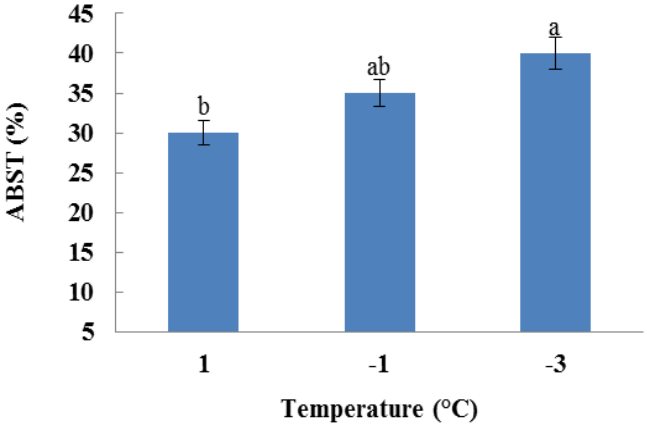

Figure 6. Effect of different temperatures on levels of ABST in *Citrus reticulata.* Means with the same letter are not significantly different (P < 0.05).

increase in response to several abiotic stresses, such as drought, cold, heat shock, and mechanical stimulation (Shelp *et al*., 2012). Proline, through its antioxidant properties, can overcome active oxygen species and prevent their production (Hosseinifard *et al.*, 2022). The accumulation of compatible solutes, such as amino acids especially proline, prevents the development of osmotic stress in plants (Rai, 2002). Proline and other amino acids plays a main role as an osmoprotectants in the adaptation to osmotic stress (Verbruggen and Hermans, 2008). The results of the research indicate that proline can well remove hydroxyl radicals, thus protecting the essential macromolecules of cells such as proteins, lipids and DNA from the dangers of free radicals. Proline plays a role in radical scavenger and stabilizer of macromolecules and a cell wall component (Hayat *et al*., 2012). Proline and other free amino acids content increased in *Citrus* fruits under cold stress.

Environmental stresses cause plants to have high levels of proline compared to other amine acids (Ashraf and Foolad 2007). Plants have various protective mechanisms to eliminate ROS through both enzymatic and non-enzymatic defense mechanisms (Beak and Skinner, 2003). In the present study, activity of SOD and APX was increased with decreasing of temperature. Similar increase has been reported in the activity of SOD enzyme in Mexican lime under low-temperature stress (Najafzadeh, 2010) and *Sabina* seedlings (Chen *et al*., 2006). The result of this experiment indicated that low-temperature has significantly affects antioxidant capacity ($P < 0.05$). Antioxidant capacity of fruits of *C. reticulata* at -3 °C was higher than other temperature treatments. Previous studies have shown that the decreasing of oxidative damage is often correlated with an antioxidant capacity under low-temperature stress (Ruth, 2002). This result was similar to previous reports by Rapisarda *et al*. (2008), who showed the increase antioxidant capacity during low-temperature stress in *Citrus sinensis* fruit.

Conclusion

During stress, plants synthesize chemical compounds that reduce the production of reactive oxygen species and oxidative stress. In this study, low temperature stress in *Citrus reticulata* species led to the production of endogenous proline, antioxidant enzymes and GABA, which moderated the low-temperature damage.

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