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Research Article

Interaction of polyamine and proline on the activity of enzymatic and non-enzymatic compounds in the peel of three *Citrus* species under low temperature stress

Soheila Mohammadrezakhani[1*], Farkhondeh Rezanejad[2] and Jafar Hajilou[1]
[1] Department of Horticultural Science, Faculty Agriculture, University of Tabriz, East Azerbaijan, Iran
[2] Department of Biology, Shahid Bahonar University of Kerman, Kerman, Iran
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Abstract

Plants activate antioxidant defense mechanisms under stress, which help maintain the structural integrity of cell components and possibly reduce oxidative damage. Low- temperature stress led to the production of reactive oxygen species and oxidative damage to plants. In this study, the effect of putrescine and proline on reducing the production of reactive oxygen species and increasing the activity of antioxidant enzymes in the peel of three *Citrus* species were investigated. The results showed that with decreasing temperature, the production of reactive oxygen species and activity of antioxidants increased in three *Citrus* species. In both *C. reticulata* and *C. sinensis* species, the activities of antioxidant enzymes were higher compared with *C. paradisi.* However, the production of reactive oxygen species in *C. paradisi* was higher than the other two species. Treatment of fruits with proline and putrescine led to a decrease in the production of reactive oxygen species. The highest amount of glutathione peroxidase and ascorbate peroxidase was observed at -3°C temperature and in fruits treated with proline 20 Mm. Exogenous application proline and putrescine increased the levels of endogenous proline in *Citrus* species. The levels of endogenous proline under both low temperature and exogenous proline and putrescine were higher in *C. reticulata* compared with two other species. Overall, the treatment of putrescine and proline has led to the improvement of defense activities in stressed plants and has significantly increased in the *C. reticulata* species.

Key word: Antioxidant, Proline, Enzyme, Fruit, Polyamine, Stress

Introduction

Temperature is one of the most important environmental factors that has an important impact on the distribution of plants (Avia *et al*., 2013). Under biotic and abiotic stresses, the production of reactive oxygen species increases. These highly reactive molecules can react with many cellular bimolecular and other components and damage DNA, proteins, as well as lipids. To take on the destructive effects of reactive oxygen species, plants have evolved various enzymatic and non-enzymatic defense systems (Teotia and Sing, 2014). To prevent or alleviate cold oxidative injury, plants have evolved several mechanisms which include scavenging by antioxidant systems such as superoxide dismutase, catalase and peroxidase (Fan *et al*., 2014). Antioxidant systems play an important role in maintaining cell homeostasis and in the antioxidant response in plants (Cartea *et al*., 2011). The balance between SOD and APX or CAT activities in cells is a crucial point to maintain the steady-state level of ROS (Kwon *et al*., 2001). Generally, as the main cell antioxidant mechanism, it has been proven that in the first step SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2 molecules. Then, H_2O_2 is detoxified by APX, POD and CAT in different organelles and antioxidant cycles (Mittler, 2002). Several assays show that defensibility against oxidative damages is inhibited by the reduction of antioxidants expression such as SOD, CAT, POD and APX. Cold tolerance is improved when the plant's POD, CAT and SOD levels are enhanced. Proline has been addressed as a unique low molecular weight osmolyte that responds to stresses related to osmosis in wide plant varieties (Hasegawa *et al*., 2000). Proline, an amino acid, accrues during water constraints (Hare *et al*., 1998), salinity (Munns, 2005), low temperature (Naidu *et al*., 1991), heavy metal accumulation (Sharma and Dietz, 2006) among others. Proline, further, is an important variable amino acid in determining protein and membrane structures and scavenge reactive oxygen species (ROS) under drought stress (Ashraf and Foolad, 2007). Hong *et al*. (2000) concluded that the role of proline as a free radical scavenging is more important in alleviating stress than its role as a simple osmolyte (Hong *et al*., 2000). Polyamines are important plant growth regulating substances, regulating plant growth, development and adaptation to environmental stresses (Liu *et al*., 2004). Putrescine may accumulate as a defense response of plants to chilling damage, because

*Corresponding Author, Email: smohammadrezakhani@yahoo.com

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Put accumulation is positively correlated with the cold resistance of plants (Wang *et al*., 2003). This study was aimed to investigate the effects of proline and polyamine (putrescine) on damage reduction in three *Citrus* species (*C. reticulata*, *C. sinensis*and *C. paradisi*) at temperatures of 1C, −1°C and −3°C.

Material and methods

Plant materials: 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, 20mM and putrescine at 0, 5 and 10Mm (Koc *et al*., 2016). After 24 h of spraying, the treated branches were harvested and placed in 15% sucrose solution and then containers containing treated shoots were exposed to temperatures 1°C, −1°C and −3°C for three hours.

Hydrogen peroxide (H_2O_2): 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. H_2O_2 concentration of the supernatant was evaluated by comparing its absorbance at 390 nm to a standard calibration curve.

Enzyme assays, Preparing of enzyme extracts: 1 g of fruits peel were homogenized 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 at 4°C 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 was recorded at 595 nm (Bradford, 1976; Rezanejad *et al*., 2018).

Guaiacol peroxidase (GPX) activity (EC1.11.1.7): GPX activity was measured using guaiacol as a substrate. Reaction mixture (3 ml) contained 25 µl of enzyme extract, 2.77 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of 1% H_2O_2 (V/V), and 0.1 ml of 4% guaiacol (V/V). The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 3 min. One unit of enzyme activity was defined as the amount that caused a change of 0.01 in the absorbance per minute (Zhang *et al*., 2005).

Superoxide dismutase (SOD; EC 1.15.1.1): SOD activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin in light (Giannopolitis and Ries, 1977). The reaction mixture (3 ml) contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 4 mM riboflavin, 0.1 mM EDTA, and 0.25 ml enzyme extract. One unit of enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer. The test tubes were shaken and then placed in a light box of 15 W fluorescent lamps for 10 min. Reaction was stopped by switching off the light and placing the test tubes into dark.

Determination of APX activity (EC 1.11.1.11): The measurement 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 H_2O_2. The decrease in the absorbance of ascorbate was recorded at 290 nm for 3 min against assay solution ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

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 H_2O_2 to initiate the reaction. The decrease in the absorbance H_2O_2 was recorded at 240 nm for 3 min against assay solution ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$).

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.

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 a 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.

The experiment was done as a factorial experiment according to a completely randomized design with three replications. Data were analyzed by analysis of variance (ANOVA) and the means were compared ($P \leq .05$) by Duncan's multiple range test (DMRT). All analyses were performed using a version of the software SAS (SAS Institute, Cary, NC, USA).

Results

Cold stress has led to an increase in the production of hydrogen peroxide in three *Citrus* species (Figure 1 A-C). Treatment of fruits with putrescine and proline has led to a reduction in the production of hydrogen peroxide in *Citrus* species. The lowest amount was

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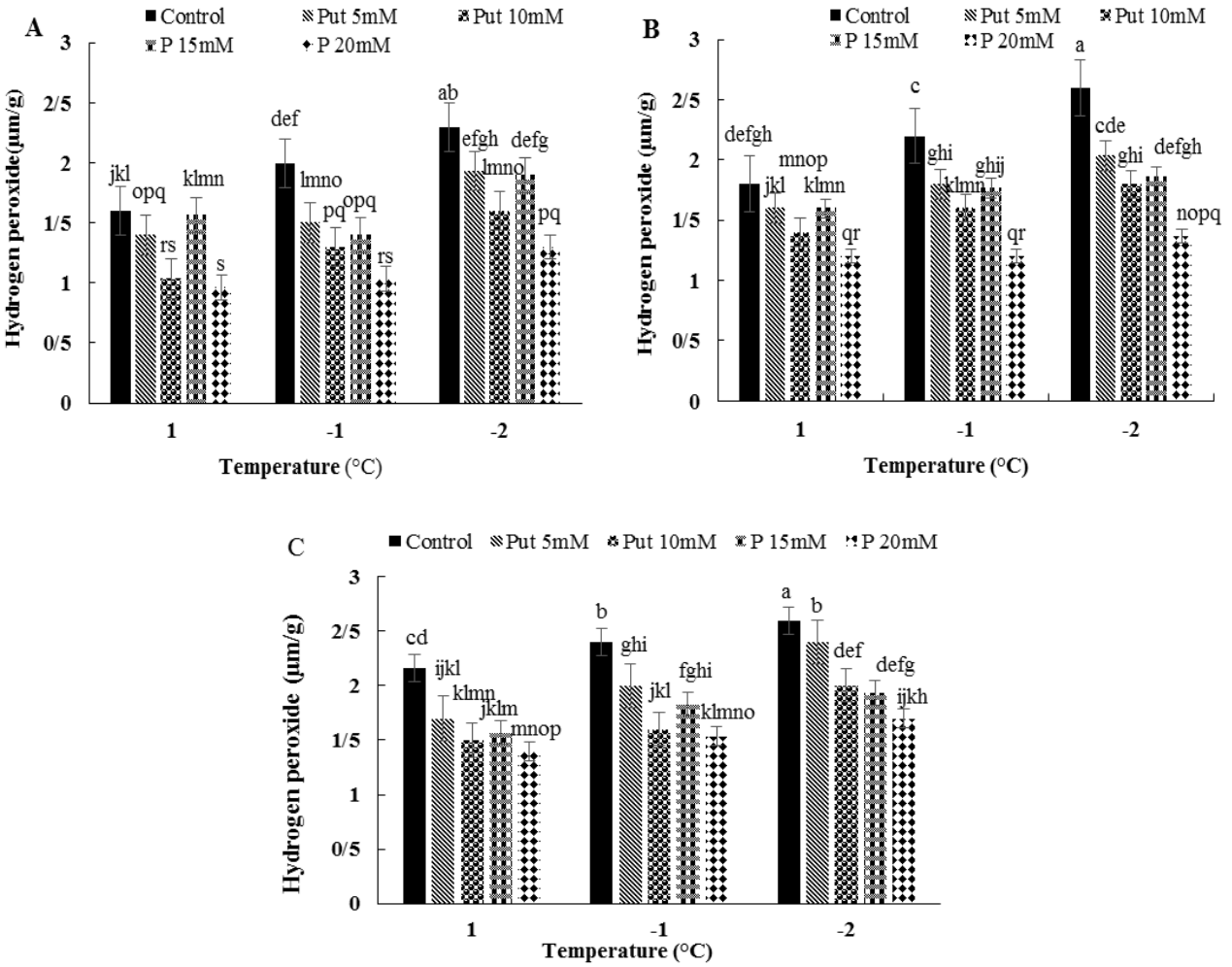

Figure 1 A–C. Effect of temperature and exogenous proline and putrescine on hydrogen peroxidein three *Citrus* species. Means with the same letter are not significantly different from each other (P ≤ .05).

observed in fruits treated with 20 mM proline and 10 mM putrescine (Figure 1 A-C). The production of active oxygen species in *C. reticulata* was less than the other two species.

Enzyme activities: The results showed that with decreasing temperature, the activity of glutathione peroxidase has increased in three *Citrus* species. In both *C. reticulata* and *C. sinensis* species, the activity of this enzyme was higher than that in *C. paradisi* (Table 1). Comparison between treated and the control fruits in all three *Citrus* species at different temperatures showed that with increasing the concentration of treatments compared to the control, glutathione peroxidase activity has increased. The highest amount was observed at -3°C temperature and in fruits treated with proline 20 Mm (Table 1).

In all three *Citrus* species, the activity of superoxide dismutase enzyme was the lowest in the control fruits and the highest in treated fruits. The activity of this enzyme increased with decreasing temperature in the control and treated fruits in all three *Citrus* species. The comparison of the mean between the three citrus species at all three temperatures showed that the highest amount of enzyme was in the fruits treated with proline 20 mM at the lowest temperature (Table 2).

The study of the role of treatment and temperature on ascorbate peroxidase activity showed that at each temperature the highest amount was observed in treated fruits compared to the control fruits. The highest amount was observed in fruits treated with the highest concentration at the lowest temperature in all three *Citrus* species. Differences between species showed that the highest level of enzyme activity was observed in *C. reticulata* species compared to the other species (Table 3).

Tables 4 and 5 demonstrates that both cold stress and exogenous proline and putrescine induced the activity of the antioxidant enzymes including catalase and lipoxygenases. With increasing proline and putrescine level, the activity of CAT (Table 4) and LOX (Table 5) increased. Also, the activity of these enzymes increased, concurrent with temperature reduction. The highest levels of APX and CAT as well as the lowest levels of LOX were observed in fruits of *C. reticulate* (Table 4, 5).

Endogenous proline: The results showed that the amount of endogenous proline increased with a decreasing temperature in three *Citrus* species (Figure 2

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Table 1. Effects of exogenous putrescine and proline on GPX enzyme activity three *Citrus* species

Temperature	1°C					-1°C					-3°C				
Treatments / Species	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2
C. reticulata	0.26^{mn}	0.3^{jk}	0.34^{efg}	0.33^{fgh}	0.37^{cd}	0.29^{ki}	0.33^{fgh}	0.35^{de}	0.36^{de}	0.43^{b}	0.32^{ghij}	0.37^{cd}	0.42^{b}	0.41^{b}	0.49^{a}
C.sinensis	0.19^{s}	0.3^{jk}	0.34^{efg}	0.33^{fgh}	0.31^{ij}	0.23^{op}	0.33^{fgh}	0.42^{b}	0.41^{b}	0.34^{efg}	0.28^{im}	0.37^{cd}	0.42^{b}	0.41^{b}	0.36^{de}
C. paradisi	0.11^{v}	0.21^{qr}	0.27^{lm}	0.25^{no}	0.2^{rs}	0.13^{uv}	0.27^{m}	0.31^{hij}	0.32^{ghi}	0.25^{no}	0.2^{rs}	0.34^{ef}	0.38^{c}	0.36^{de}	0.36^{de}

Values in the same column with different superscript letters represent significant differences between Citrus species at P <.05 by Duncan's test. C: control, Put $_1$: 5 mM, Put $_2$:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 2. Effects of exogenous putrescine and proline on SOD enzyme activity three *Citrus* species,

Temperature	1°C					-1°C					-3°C				
Treatments / Species	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2
C. reticulata	0.43^{u}	0.47^{qrs}	0.5^{imno}	0.47^{pqr}	0.54^{efg}	0.48^{opqr}	0.51^{jklm}	0.55^{de}	0.52^{h-i}	0.57^{cd}	0.5^{klmn}	0.57^{cd}	0.65^{a}	0.59^{b}	0.64^{a}
C.sinensis	0.4^{v}	0.45^{stu}	0.47^{qrs}	0.48^{opqr}	0.51^{ijkl}	0.46^{rs}	0.49^{mnop}	0.54^{fgh}	0.53^{fghi}	0.55^{def}	0.51^{ijkl}	0.53^{fghij}	0.58^{cd}	0.63^{a}	0.58^{bc}
C. paradisi	0.38^{w}	0.44^{u}	0.44^{i}	0.46^{rst}	0.49^{nopa}	0.43^{u}	0.47^{pqr}	0.52^{h-i}	0.48^{opqr}	0.55^{def}	0.5^{imno}	0.52^{g-k}	0.57^{bc}	0.56^{cde}	0.55^{def}

Values in the same column with different superscript letters represent significant differences between Citrus species at P < .05 by Duncan's test. C: control, Put_1: 5 mM, Put_2:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 3. Effects of exogenous putrescine and proline on APX enzyme activity in *C. reticulata*

Temperature	1°C					-1°C					-3°C				
Treatments / Species	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2
C. reticulata	0.27^{pq}	$o.4^{ef}$	0.43^{de}	0.42^{de}	0.49^{ab}	0.38^{fgh}	0.4^{ef}	0.38^{fgh}	0.43^{de}	0.52^{b}	0.42^{de}	0.46^{c}	0.41^{def}	0.46^{c}	0.56^{a}
C.sinensis	0.24^{v}	0.33^{imn}	0.33^{klmn}	0.36^{hijk}	0.42^{de}	0.26^{pqr}	0.35^{ijkl}	0.42^{de}	0.31^{mno}	0.44^{cd}	0.37^{ghi}	0.42^{de}	0.39^{fq}	0.43^{de}	0.44^{cd}
C. paradisi	0.17^{s}	0.25^{qr}	0.34^{jklm}	0.33^{klmn}	0.36^{hij}	0.27^{pq}	0.25^{qr}	0.35^{ijkl}	0.28^{pq}	0.33^{imn}	0.28^{op}	0.28^{op}	0.3^{1no}	0.33^{iml}	0.36^{hij}

Values in the same column with different superscript letters represent significant differences between Citrus species at P < .05 by Duncan's test. C: control, Put_1: 5 mM, Put_2:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 4. Effects of exogenous putrescine and proline on CAT enzyme activity in *C. reticulata*

Temperature	1°C					-1°C					-3°C				
Treatments / Species	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2
C. reticulata	0.31^{mno}	0.31^{fg}	0.33^{de}	0.31^{efg}	0.38^{b}	0.26^{kl}	0.31^{fg}	0.33^{de}	0.32^{def}	0.4^{a}	0.3^{fgh}	0.33^{de}	0.36^{c}	0.36^{c}	0.41^{a}
C.sinensis	0.19^{s}	0.21^{opqr}	0.24^{m}	0.23^{mn}	0.2^{ghi}	0.21^{opq}	0.24^{m}	$o.28^{ij}$	0.26^{kl}	0.31^{fg}	0.26^{k}	0.3^{gh}	0.32^{ef}	0.3^{gh}	0.34^{d}
C. paradisi	0.18^{s}	0.19^{s}	0.2^{qrs}	0.21^{pqr}	0.22^{nop}	0.19^{rs}	0.22^{mnop}	0.24^{lm}	0.23^{mn}	0.26^{kl}	0.22^{nop}	0.24^{lm}	0.26^{jk}	0.27^{jk}	0.26^{ki}

Values in the same column with different superscript letters represent significant differences between Citrus species at P < .05 by Duncan's test. C: control, Put_1: 5 mM, Put_2:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 5. Effects of exogenous putrescine and proline on LOX enzyme activity in *C. reticulata*

Temperature	1°C					-1°C					-3°C				
Treatments / Species	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2	C	Put_1	Put_2	P_1	P_2
C. reticulata	0.34^{ts}	0.38^{imn}	0.43^{gh}	0.39^{klm}	0.45^{def}	0.39^{klm}	0.42^{hi}	0.47^{d}	0.43^{fgh}	0.5^{c}	9.43^{hi}	0.45^{def}	0.52^{b}	0.46^{de}	0.56^{a}
C.sinensis	0.31^{u}	0.34^{rst}	0.360^{pqr}	0.36^{opqr}	0.38^{klmn}	0.34^{rst}	0.38^{imn}	0.39^{klm}	0.39^{jkl}	0.42^{hi}	0.39^{klm}	0.43^{fgh}	0.46^{def}	0.45^{efg}	0.44^{fgh}
C. paradisi	0.3^{v}	0.33^{tu}	0.36^{pqrs}	0.35^{qrs}	0.37^{nopq}	0.33^{tu}	0.35^{qrs}	0.38^{imno}	0.37^{mnop}	0.39^{jkl}	0.37^{mnop}	0.39^{jkl}	0.41^{ji}	0.42^{hi}	0.4^{jk}

Values in the same column with different superscript letters represent significant differences between Citrus species at P < .05 by Duncan's test. C: control, Put_1: 5 mM, Put_2:10 mM, P1: proline 15 mM, P2: proline 20 mM.

A–C). Exogenous application of proline and putrescine ramped up the levels of endogenous proline in *Citrus* species too, so that with increasing their concentration, the amount of endogenous proline also increased (Figure 2 A–C). The levels of endogenous proline under both low temperature and exogenous proline and putrescine were higher in *C. reticulata* compared with two other species (Figure 2 A–C).

Discussion

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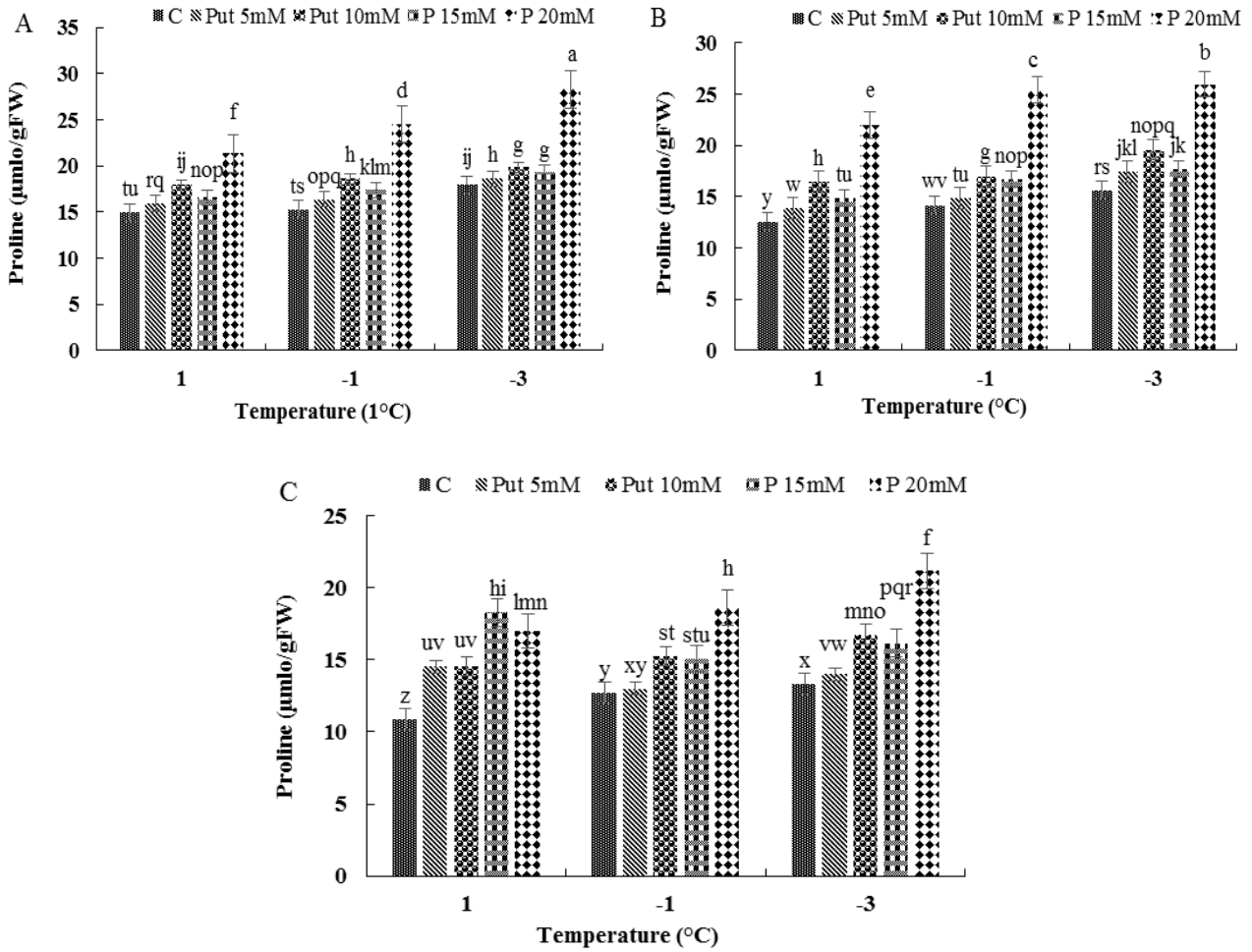

Figure 2 A–C. Effect of temperature and exogenous proline and putrescine on endogenous proline in three Citrus species. Means with the same letter are not significantly different from each other (P ≤ .05).

Citrus, one of the most important fruit tree crops in the world, is sensitive to many environmental stresses including drought, salinity, nutrient deficiency, high irradiance, high temperature, as well as high atmospheric evaporative demand. Cold resistance is different in *Citrus* species. The negative effects of stresses usually reduce tree growth, fruit yield and quality. Under natural conditions, long-lived evergreen *Citrus* trees often experience multiple stresses at the same time, so there are direct and indirect interactions between almost all abiotic and biotic stresses (Syvertsen, 2017). Among environmental stresses, temperature fluctuations can have devastating effects on plants and lead to significant economic losses in agriculture (Awasthi *et al*., 2015). Low temperature reduces the biosynthetic activity of plants and inhibits their normal physiological processes and may cause permanent damage, eventually leading to death (Zhu *et al*., 2007). *Citrus*, a cold-sensitive plant, often suffers from low temperatures, which seriously affects its production. Thus, plants have developed several mechanisms whereby the endogenous content of antioxidant enzymes provides protection against the harmful effects of oxidative stress generated by abiotic/biotic sources (Gupta *et al*., 2016). The results of previous research confirm this study that cold stress has activated chemical compounds and internal antioxidants in plants leading to resistance in plants.

High concentrations of ROS are very harmful to organisms, and if symptoms persist, irreversible damage is done to the cells, resulting in loss of physiological capacity and eventual cell death. Thus, defense mechanisms against oxidative damage are activated during stress to regulate the toxic level of ROS (Lin *et al*., 2010). The balance between ROS production and inhibition may be disturbed by a number of biotic and non-biotic agents, which may increase the intracellular level of ROS. When the level of ROS increases and exceeds the defense mechanisms, the cells are in a state of oxidative stress (Mittler, 2002).

To survive cold stress, plants have antioxidant mechanisms that are divided into two components: Nonenzymatic antioxidants and enzymatic antioxidant systems to scavenge ROS and mitigate their toxic effects (Ahmad *et al*., 2010).

The most active enzymes in response to environmental stressful conditions are CAT and SOD. SOD is usually considered as the first line of defense against oxidative stress. This enzyme catalyzes the partitioning of O^{2-} into either an ordinary molecular O_2

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or into H_2O_2, which is also damaging, but less so, and is degraded by other enzymes such as APX or CAT. Although both enzymes degrade H_2O_2, the role of CAT is mainly focused on neutralizing the excess of ROS during stressful conditions, whereas APX is more involved in the fine modulation of ROS for signaling (Lin *et al*., 2010). In general terms, the activity of these enzymes was significantly higher under cold than under control conditions. Our results showed that activity of antioxidant enzymes including SOD, CAT, APX, and GR have generally increased with decreasing of temperature. Recently, plant growth regulators and osmolytes have been used to reduce the damage caused by cold stress and increasing antioxidants. In this study, proline and putrescine were used to activate antioxidant enzymes and amino acids in three *Citrus* species. Antioxidant enzymes can scavenge ROS to prevent membrane lipid peroxidation and stabilize membrane structure (Ouyang *et al*., 2017). To date, few studies have focused on the physiological functions of PAs in plants under high temperature stress. It has been suggested that PAs play an important role in modulating the defense response of plants to diverse environmental stresses (Bouchereau *et al*., 1999), which includes metal toxicity (Groppa *et al*., 2003), oxidative stress (Rider *et al*., 2007), drought (Yamaguchi *et al*., 2007), salinity (Duan *et al*., 2008) and chilling stress (Cuevas *et al*., 2008). It has been reported that exogenous application of PAs is also an effective approach for enhancing stress tolerance of crops for enhanced crop productivity. Exogenous application of putrescine has been successfully used to enhance salinity (Ndayiragije and Lutts, 2006), cold (Nayyar, 2005), drought (Zeid and Shedeed, 2006), heavy metals (Wang, 2007), osmotic stress (Liu *et al*., 2004), high-temperature (Murkowski, 2001), water logging (Arbona *et al*., 2008) as well as flooding tolerance of plants (Yiu *et al*., 2009). Exogenous proline treatment also increases proline content, thereby alleviating chill-induced stress. Besides, acting as a free radical scavenger and stabilizing membranes, exogenous proline also acted as a source of nitrogen and carbon, thereby improving seedling growth and regeneration in *Vigna radiata*exposed to chilling stress (Posmyk and Janas, 2007). Furthermore, exogenous proline application, enhancing the activity of antioxidative enzymes (CAT, POX and SOD) (Hoque *et al*., 2007) has also been known to enhance the activity of other enzymes. Nitrogenase activity in drought-stressed soybean nodules was significantly enhanced when proline (an osmolyte) was applied exogenously. However, when other osmolytes such as malate, were tested there was no significant enhancement in drought-stressed nodule nitrogenase activity (Pedersen *et al*., 1996). Proline is known to act as an enzyme protectant during abiotic stress conditions (Sharma *et al*., 2005). This effect is further supported by the finding that exogenous proline application alleviates salt stress by upregulating the stress protective proteins in *Pancratium maritinmum* (Khedr *et al*., 2003) and reducing oxidation of lipid membranes in tobacco (Okuma *et al*., 2004). Exogenous proline acted as an active oxygen scavenger thereby overcoming the oxidative stress induced by chilling (Posmyk and Janas, 2007). Van Swaaij *et al*. (1985) showed that exogenous proline application resulted in increased frost tolerance in leaves of *Solanum*. Reports indicate that proline is responsible for scavenging the ROS and other free radicals (Chen and Dickman, 2005). Proline, when applied exogenously to roots of *Arabidopsis*, resulted in a reduced level of ROS, indicating the ROS scavenging potential of proline (Cuin and Shabala, 2007). Furthermore, exogenous proline application also reduced ROS-induced K^+ efflux (Cuin and Shabala, 2007). Hoque *et al*. (2007) reported that the activities of antioxidative enzymes including. catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) were significantly enhanced when proline was applied exogenously in tobacco suspension cultures exposed to salinity stress (Hoque *et al*., 2007).

Conclusion

Cold stress in plants causes the production of reactive oxygen species which damages lipid membrane. Putrescine and proline increase antioxidant activity and activate the defence systems in plants.

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