page: 57-64

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 Rezanejad² and Jafar Hajilou¹ ¹Department of Horticultural Science, Faculty Agriculture, University of Tabriz, East Azerbaijan, Iran

² Department of Biology, Shahid Bahonar University of Kerman, Kerman, Iran (Received: 28/11/2021-Accepted: 16/08/2022)

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, H₂O₂ 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

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 (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.

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 μ l of enzyme extract, 2.77 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of 1% H₂O₂ (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 H₂O₂. The decrease in the absorbance of ascorbate was recorded at 290 nm for 3 min against assay solution ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

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 ($\varepsilon = 39.4$ 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 μ l 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 ($P \le .05$) 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



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 ($P \le .05$).

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

Temperature			1°C					-1°C					-3°C		
Treatments Species	С	Put ₁	Put 2	P1	P ₂	С	Put ₁	Put ₂	P1	P ₂	С	Put ₁	Put ₂	P1	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}

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

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	С	Put ₁	Put ₂	P_1	P ₂	С	Put ₁	Put ₂	P_1	P ₂	С	Put ₁	Put ₂	P1	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, Put₁: 5 mM, Put₂:10 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	С	Put ₁	Put 2	P_1	P_2	С	Put ₁	Put ₂	P_1	P ₂	С	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}	$0.36^{\rm hij}$	0.27 ^{pq}	0.25 ^{qr}	0.35 ^{ijkl}	0.28^{pq}	0.33^{imn}	0.28 ^{op}	0.28 ^{op}	0.31no	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, Put₁: 5 mM, Put₂:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 4. Effects of	exogenous	putrescine and	proline on C.	AT enzyme	activity in C.	reticulata
		F	F			

Temperature	1°C					-1°C					-3°C				
Treatments Species	С	Put ₁	Put 2	\mathbf{P}_1	P ₂	С	Put ₁	Put ₂	\mathbf{P}_1	P ₂	С	Put ₁	Put ₂	\mathbf{P}_1	P ₂
C. reticulata	0.31mno	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}	$0.2^{\rm ghi}$	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, Put₁: 5 mM, Put₂:10 mM, P1: proline 15 mM, P2: proline 20 mM.

Table 5.	Effects of	exogenous	putrescine and	proline on LOX	K enzvme activit	v in C	. reticulata
		· · · · · · · · · · · · · · · · · · ·					

Temperature	1°C					-1°C					-3°C				
Treatments Species	С	Put ₁	Put 2	P_1	P ₂	С	Put ₁	Put ₂	P1	P ₂	С	Put ₁	Put ₂	\mathbf{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}	0.360pqr	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 ^{jk1}	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, Put₁: 5 mM, Put₂:10 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



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 ($P \le .05$).

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

or into H₂O₂, which is also damaging, but less so, and is degraded by other enzymes such as APX or CAT. Although both enzymes degrade H₂O₂, 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 growth and regeneration seedling in Vigna radiataexposed 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.

References

- Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G. and Sharma, S. (2010) Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Critical Reviews in Biotechnology 30: 161-175.
- Ashraf, M. and Foolad, M. R. (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany 59: 206-216.
- Awasthi, R., Bhandari, K. and Nayyar, H. (2015) Temperature stress and redox homeostasis in agricultural crops. Frontiers of Environmental Science and Engineering 3: 11.
- Arbona, V., Hossain, Z., Lopez-Climent, M. F., Perez Clemente, R. M. and Gomez-Cadenas, A. (2008) Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. Physiology Plant 132: 452-466.
- Avia, K., Pilet-Nayel, M. L., Bahrman, N., Baranger, A., Delbreil, B., Fontaine, V., Hamon, C., Hanocq, E., Niarquin, M., Sellier, H., Vuylsteker, C., Prosperi, J. M. and Lejeune-Henaut, I. (2013) Genetic variability and QTL mapping of freezing tolerance and related traits in *Medicago truncatula*. Theoretical and Applied Genetics 126: 2353-2366.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. Analytical Biochemistry 72: 248-254.

- Bouchereau, A., Aziz, A., Larher, F. and Martin-Tanguy, J. (1999) Polyamines and environmental challenges: Recent development. Plant Science 140: 103-125.
- Bates, L., Waldren, R. P. and Teare, I. D. (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39: 205-207.
- Chen, C. and Dickman, M. B. (2005) Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. Proceedings of the National Academy of Sciences of the United States 102: 3459-64.
- Cuin, T. A. and Shabala, S. (2007) Compatible solutes reduce ROS induced potassium efflux in Arabidopsis roots. Plant, Cell and Environment 30: 875-885.
- Cuevas, J. C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C. and Altabella, T. (2008) Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating ABA levels in response to low temperature. Plant Physiology 148: 1094-1105.
- Corpas, F. J. (2015) What is the role of hydrogen peroxide in plant peroxisomes? Plant Biology (Stuttg) 17: 1099-1103.
- Cartea, M. E. (2011) Phenolic compounds in Brassica vegetables. Molecules 16: 251-280.
- Chance, B. and Mahly, A. C. (1995) Assay of catalases and peroxidases. Method Enzymology 2: 764-817.
- Duan, J. J., Li, J., Guo, S. R. and Kang, Y. Y. (2008) Exogenous spermidine affects polyamine metabolism in salinitystressed *Cucumis sativus* roots and enhances short-term salinity tolerance. Journal Plant Physiology 165: 1620-1635.
- Fan, J., Ren, J., Zhu, W., Amombo, E., Fu, J. and Chen, L. (2014) Antioxidant responses and gene expression in Bermudagrass under cold stress. Journal of the American Society for Horticultural Science 139: 699-705.
- Gupta, D. K., Palma, J. M. and Corpas, F. J. (2016) Redox state as a central regulator of plant-cell stress response. Springer.
- Giannopolitis, C. N. and Ries, S. K. (1977) Superoxide dismutases I. occurrence in higher plants. Plant Physiology 59: 309-314.
- Groppa, M. D., Benavides, M. P. and Tomaro, M. L. (2003) Polyamine metabolism in sunflower and wheat leaf discs under cadmium or copper stress. Plant Science 161: 481-488.
- Hoque, M. A., Banu, M. N., Okuma, E., Amako, K., Nakamura, Y. and Shimoishi, Y. (2007) Exogenous proline and glycinebetaine increase NaCl-induced ascorbate-glutathione cycle enzyme activities, and proline improves salt tolerance more than glycinebetaine in tobacco Bright Yellow-2 suspension-cultured cells. Journal Plant Physiology 164: 1457-1468.
- Hong, Z., Lakkineni, K., Zhang, Z. and Verma, D. P. (2000) Removal of feedback inhibition of delta(1)-pyrroline-5carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiology 122: 1129-1136.
- Hoque, M. A., Banu, M. N., Okuma, E., Amako, K., Nakamura, Y. and Shimoishi, Y. (2007) Exogenous proline and glycinebetaine increase NaCl-induced ascorbate-glutathione cycle enzyme activities, and proline improves salt tolerance more than glycinebetaine in tobacco Bright Yellow-2 suspension-cultured cells. Journal Plant Physiology 164: 1457-1468.
- Hasegawa, P. M., Bressan, R. A., Zhu, J. K. and Bohnert, H. J. (2000) Plant cellular and molecular responses to high salinity. Annual Review of Plant Physiology and Plant Molecular Biology 51: 463-499.
- Hare, P. D., Cress, W. A. and Van Staden, J. (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environmental 21: 535-553.
- Koc, E., Arici, Y. K. and Islek, C. (2016) Pretreatment with spermidine and proline reverses inhibitory effects of Phytophthora capsici stress in pepper. Zemdirbyste-Agriculture 103: 411-418.
- Khedr, A. H. A., Abbas, M. A., Wahid, A. A. A., Quick, W. P. and Abogadallah, G. M. (2003) Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancratium maritimum* L. to salt-stress. Journal of Experimental Botany 54: 2553-2562.
- Kwon, S. Y., Lee, H. S. and Kwak, S. S. (2001) Development of environmental stress-tolerant plants by gene manipulation of antioxidant enzymes. Plant Pathology 17: 88-93.
- Lin, K. H., Huang, H. C. and Lin, C. Y. (2010) Cloning, expression and physiological analysis of broccoli catalase gene and Chinese cabbage ascorbate peroxidase gene under heat stress. Plant Cell Reports 29: 575-593.
- Loreto, F. and Velikova, V. (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiology 127: 1781-1787.
- Liu, H. H., Zhang, Y. Y., Liu, Z. P. and Liu, Y. L. (2004) Relationship between osmotic stress and the levels of free, conjugated and bound polyamines in leaves of wheat seedlings. Plant Science 166: 1261-1267.
- Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Science 7: 405-410.
- Murkowski, A. (2001) Heat stress and spermidine: Effect on chlorophyll fluorescence in tomato plants. Biology Plant 44: 53-57.
- Munns, R. (2005) Genes and salt tolerance: Bringing them together. New Phytologist 167: 645-663.
- Naidu, B. P., Paleg, L. G., Aspinall, D., Jennings, A. C. and Jones, G. P. (1991) Amino acid and glycine betaine accumulation in cold stressed wheat seedlings. Phytochemistry 30: 407-409.

- Nakano, Y. and Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate peroxidase in spinach chloroplasts. Plant Cell Physiology 22: 867-880.
- Ndayiragije, A. and Lutts, S. (2006) Do exogenous polyamines have an impact on the response of a salt-sensitive rice cultivar to NaCl? Journal Plant Physiology 163: 506-516.
- Nayyar, H. (2005) Putrescine increases floral retention, pod set and seed yield in cold stressed chickpea. Agronomy and Crop Science 191: 340-345.
- Ouyang, J., Song, C. and Chen, D. (2017) Research progress on heat-tolerance mechanism and transports of polyamfines in plant. Molecular Breeding 15: 3286-3294.
- Okuma, E., Murakami, Y., Shimoishi, Y., Tada, M. and Murata, Y. (2004) Effects of exogenous application of proline and betaine on the growth of tobacco cultured cells under saline conditions. Soil Science and Plant Nutrition 50: 1301–1305.
- Posmyk, M. M. and Janas, K. M. (2007) Effects of seed hydropriming in presence of exogenous proline on chilling injury limitation in *Vigna radiata* L. seedlings. Acta Physiology Plant 29: 509-517.
- Pedersen, A. L., Feldner, H. C. and Rosendahl, L. (1996) Effect of proline on nitrogenase activity in symbiosomes from root nodules of soybean (*Glycine max* L.) subjected to drought stress. Journal of Experimental Botany 47: 1533-1539.
- Rezanejad, F., Shojaei, M., Zamani Bahramabadi, E., Badoei Dalfard, A. and Esmaeili Mahani, S. (2018) Allergenicity of *Acroptilon repens* and *Juglans regia* pollen in rats. Grana 57: 292-297.
- Reddanna, P., Whelan, J., Maddipati, K. R. and Reddy, C. C. (1990) Purification of arachidonate 5-lipoxygenase from potato tubers. Method Enzymol 187: 268-277.
- Rider, J. E., Hacker, A., Mackintosh, C. A., Pegg, A. E., Woster, P. M. and Casero, R. A. Jr. (2007) Spermine and spermidinemediate protection against oxidative damage caused by hydrogen peroxide. Amino Acids 33: 231-240.
- Syvertsen, J. P. (2017) Aspects of stress physiology of citrus. Acta Horticulture 1177: 51-58.
- Sharma, P. and Dubey, R. S. (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: Role of osmolytes as enzyme protectant. Journal Plant Physiology 162: 854-864.
- Teotia, S. and Singh, D. (2014) Oxidative stress in plants and its management. Approaches to Plants Stress and their Management 227-253.
- van Swaaij, A. C., Jacobsen, E. and Feenstra, W. J. (1985) Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum*. Physiology of Plant 64: 230-236.
- Wang, Y., Lu, W. and Zhang, Z. (2003) ABA and putrescine treatment alleviate the chilling damage of banana fruit. Physiology and Molecular Biology of Plants 29: 549-554.
- Wang, X., Shi, G., Xu, Q. and Hu, J. (2007) Exogenous polyamines enhance copper tolerance of *Nymphoides peltatum*. Journal Plant Physiology 164: 1062-1070.
- Yamaguchi, K., Takahashi, Y., Berberich, T., Imai, A., Takahashi, T., Michael, A. J. and Kusano, T. A. (2007) Protective role for the polyamine spermine against drought stress in Arabidopsis. Biochemical and Biophysical Research Communications 352: 486-490.
- Yiu, J. C., Juang, L. D., Fang, D. Y. T., Liu, C. W. and Wu, S. J. (2009) Exogenous putrescine reduces floodinginduced oxidative damage by increasing the antioxidant properties of Welsh onion. Scientia Horticulture 120: 306-314.
- Zeid, I. M. and Shedeed, Z. A. (2006) Response of alfalfa to putrescine treatment under drought stress. Biologia Plantarum 50: 635-640.
- Zhu, J., Dong, C. H. and Zhu, J. K. (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current Opinion in Plant Biology 10: 290-295.
- Zhang, C. Q., Hong, B., Li, J. K. and Gao, J. P. (2005) A simple method to evaluate the drought tolerance of groundcover chrysanthemum rooted cuttings. Scientia Agriculture Sinica 38: 789-796.