Impact of postharvest prohexadione calcium treatment on PAL activity in tomato fruit in response to chilling stress

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Abstract

Freshly harvested tomato fruit (*Solanum lycopersicum*) were pre-treated with 0, 50, and 100 µM prohexadione-calcium (Pro-Ca) and then stored at 1 °C for 21 days to investigate the effect of Pro-Ca treatment on electrolyte leakage (EL), malondialdehyde (MDA), proline and total phenols contents, and activity of phenylalanine ammonia-lyase (PAL) in relation to chilling injury (CI). Treatment with Pro-Ca, without significant difference between two applied concentrations, mitigated chilling injury, reduced EL and MDA content and increased proline content. Also, our results indicated that during storage time fruits treated with Pro-Ca exhibited significantly higher PAL activity, but total phenols content was not significantly affected by Pro-Ca treatments. These results suggested that Pro-Ca protects tomato fruit from CI by activation of PAL enzyme, enhancing proline contents and reducing MDA content, and thus maintaining membrane integrity.

Keywords: prohexadione-calcium; tomato; chilling injury; postharvest; phenylalanine; ammonia-lyase

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Introduction

Tomato (*Solanum lycopersicum*) is of prime importance owing to its qualities for human nutrition and its economic value. In order to extend its commercial life, it is usually harvested at unripe mature stages and stored at low temperatures (Re et al., 2012). Storage of tomato as an originally tropical fruit is limited by the risk of chilling injury (CI) (Bourne, 2006).

In general terms it has been accepted by the scientific community that stimulation of phenylpropanoid pathway reflected by the higher PAL activity in fruit stored at chilling temperatures which is part of the response of the fruit in order to alleviate CI (Aghdam and Bodbodak, 2013). PAL activation can be considered as a potential defense mechanism against chilling, as it results in lower peel damage under chilling stress in mandarin fruit (Lafuente et al., 2001). PAL is the initial rate limiting enzyme of the phenylpropanoid pathway and is a key enzyme in the accumulation of phenols that has been reported to protect plants against oxidative stress (Dixon and Paiva, 1995; Lafuente et al., 2003). Phenols as phenylpropanoid pathway products are known to be antioxidant compounds and, interestingly, oxidative stress has been shown to be involved in the chilling tolerance of fruit and vegetables (Lafuente et al.,

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2001). Prohexadione-calcium (Pro-Ca) as a bioregulator affects plant metabolism such as hormonal balance. In apple, the alteration of flavonoid biosynthesis leads to the accumulation of luteoforol, a novel molecule with phytoalexin activity responsible for increased resistance against pathogens (Halbwirth et al., 2002; Spinelli et al., 2005). Application of Pro-Ca for vegetative growth control and/or fireblight suppression is useful (Duyvelshoff and Cline, 2013). In addition, Pro-Ca blocks 1-aminocyclopropane-1-carboxylate-oxidase (ACC oxidase) activity, which is a key enzyme in the ethylene biosynthesis (Rademacher, 2000), and affects fruit quality via improving sugar accumulation in fruits (Rademacher et al., 2004). Recently, Aghdam (2013) reported that the treatment of tomato fruit with Pro-Ca mitigated chilling injury. Tomato fruit treated with Pro-Ca exhibited significantly lower phospholipase D (PLD) and lipoxygenase (LOX) activities as compared with the control fruit. Aghdam (2013) suggested that Pro-Ca might mitigate CI by inhibiting PLD and LOX activities which led to enhancing membrane integrity. The objective of this study was to determine the effects of Pro-Ca on PAL activity and total phenols, MDA and proline contents, electrolyte leakage and their relation to CI in tomato fruit.

Materials and Methods

Fruit and treatment

Tomato fruit (*Solanum lycopersicum* cv. Newton) were harvested at mature green stage in July 2011 from a greenhouse in Ahar, Iran. About 900 fruits were manually picked and immediately transferred to the laboratory. Those with defects were discarded. 810 fruits were selected and divided into 3 lots of 270 for the following treatment in triplicate (90 fruit per replicate): control (0) and Pro-Ca at 50 or 100 µM. Prohexadione-calcium (Pro-Ca) was purchased from Sigma–Aldrich. For each treatment and replicate, the fruit were immersed in a fresh 10 L solution for 5 min. Following treatment, the fruit were allowed to completely dry at room temperature before storage at 1 °C and 85-90% RH for 3 weeks. 15 fruit per replicate of each treatment were removed immediately from cold storage after 7, 14 or 21 days for analyses of electrolyte leakage, MDA, proline and total phenol content and PAL enzyme activity. These samples were mixed and frozen immediately in liquid nitrogen, then stored at −80 °C. For CI evaluation, 15 fruit per replicate of each treatment were sampled weekly from cold storage and held at 25 °C for 3 days. Each treatment was replicated three times.

Chilling injury (CI) index

CI of fruits was evaluated at 25 °C for 3 days after 7, 14 or 21 days in cold storage period. The fruits were returned to ambient temperature (25 °C) for development of CI symptoms. Symptoms were manifested as surface pitting according to the method of Ding et al., (2002), where 0 = no pitting; 1 = pitting covering <25% of the fruit surface; 2 = pitting covering 25% to 50% of the surface; 3 = pitting covering >50% to 75% of the surface, and 4 = pitting covering >75% of the surface. The average extent of cold damage was expressed as the CI index, which was calculated using the following formula:

CI index = ∑ [(CI level) × (number of fruit at the CI level)] / (4 × total number of fruit)]

Electrolyte leakage (EL), malondialdehyde (MDA) and proline content

EL was measured using the method of Jiang et al. (2001). MDA content was measured by the thiobarbituric acid method described by Ding et al. (2007). MDA content was expressed as µmol g^{-1} fresh weight (FW). Proline content was measured using the acid ninhydrin method described by Shan et al. (2007). Proline content was expressed as µg proline g^{-1} FW.

PAL enzyme assays and total phenol content

Measurement of PAL enzyme activity was performed at 290 nm, according to the method of Yao and Tian (2005). PAL activity was defined as nmol cinnamic acid h^{-1} mg^{-1} protein. Protein content was determined according to Bradford (1976) with bovine serum albumin (BSA) as standard. Total phenol content was determined

according to the Folin–Ciocalteu procedure (Chen et al., 2008) and expressed as mg of gallic acid equivalent per 100 g of FW.

Statistical analysis

The experiment was arranged as split plots in time on the basis of completely randomized design with three replications. Analysis of variance (ANOVA) was carried out with SPSS software. Differences between means were assessed by Duncan's multiple range tests with differences being considered significant at $P \leq 0.05$.

Results

Chilling injury and electrolyte leakage

Treatment with the Pro-Ca, without significant differences between two applied concentrations, resulted in a lower CI index ($P \leq 0.01$) (Fig. I). Slight CI symptoms appeared after 7 days at 1 °C plus 25 °C for 3 days in fruit from all treatments, and continued to progress over time. In this study, Pro-Ca was applied which could significantly reduce postharvest CI in tomato fruit (Fig. I). Membrane leakage in fruit, as evaluated by relative EL measurements, was significantly reduced in Pro-Ca treated fruit (Fig. II; $P \leq 0.05$).

MDA and proline contents

As shown in Table 1, Pro-Ca treatments significantly reduced MDA content of tomato fruit ($P \leq 0.05$). The loss of membrane integrity is boosted by oxidative processes, since chilling temperatures increases the levels of reactive oxygen species (ROS) that stimulates lipid peroxidation in cell membranes (Sevillano et al., 2009). Treatment with Pro-Ca resulted in a decrease in MDA content (Table 1), i.e. inhibited lipid peroxidation under chilling stress, which clearly indicated that Pro-Ca could strongly protect plants from oxidative damage and thus enhance chilling tolerance. Also, as shown in Table 1, treatment with Pro-Ca increased proline content in tomato fruit ($P \leq 0.05$; Table 1).

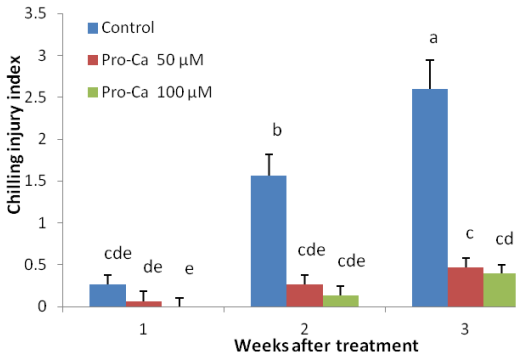

Fig. I. Effects of Pro-Ca treatment at 0, 50, and 100 µM on the CI index of tomato fruit. Tomato fruit were sampled after 1 °C storage for 7, 14, and 21 days plus 3 days at 25 °C. Data shown are Mean values ± SD (n=3), Duncan's test at $P \leq 0.05$ level.

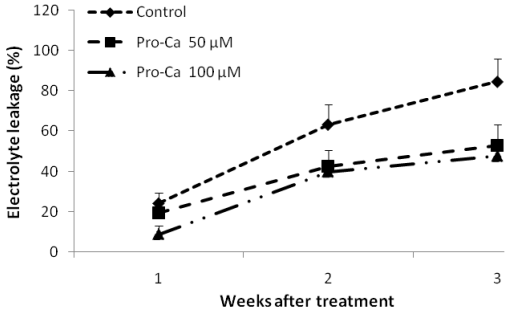

Fig. II. Effects of Pro-Ca treatment at 0, 50 and 100 µM on the electrolyte leakage of tomato fruit storage at 1 °C. Data shown are Mean values ± SD (n=3), Duncan's test at $P \leq 0.05$ level.

PAL enzyme activity

As shown in Table 1, during storage time, PAL activities were significantly higher ($p \leq 0.01$) in Pro-Ca treated fruit than in controls.

Discussions

Cell membrane is the primary cell structure affected by CI (Rui et al., 2010). Cell membrane phase transition from a flexible liquid-crystalline to a solid-gel structure that occurs at chilling temperature increments the risk of loss of controlled cell membrane semi-permeability (Lyons, 1973). If the fruit is exposed to chilling temperatures for too long, cell membranes rupture takes place, causing leakage of intracellular water, ions and metabolites, which

Table 1
Effects of Pro-Ca on MDA, proline contents, and PAL activity in tomato fruit during storage at 1 ° C

Storage time (days)	Treatment	MDA content (µmol g^{-1} FW)	Proline content (µg g^{-1} FW)	PAL activity (nmol cinnamic acid h^{-1} mg^{-1} protein)
0		45.72±36	10.25±1.22	20.42±2.85
7	Control	64.84±22.69b	11.51±3.03c	21.27±3.66e
	Pro-Ca 50 µM	58.85±15.54b	11.74±1.08c	22.65±3.42de
	Pro-Ca 100 µM	35.44±5.96c	12.88±2.38c	23.96±3.70cd
14	Control	71.23±16.78ab	17.71±1.52b	20.98±2.27cd
	Pro-Ca 50 µM	60.52±11.82b	24.72±1.31a	26.84±3.55cde
	Pro-Ca 100 µM	30.54±7.34c	26.84±0.81a	27.72±2.88b
21	Control	94.86±12.93a	17.63±1.26b	26.90±5.27cd
	Pro-Ca 50 µM	65.20±6.87b	25.23±0.84a	47.58±3.85c
	Pro-Ca 100 µM	56.45±10.61bc	27.58±0.44a	51.84±6.42a
Significance				
Treatment (T)	df2	*	* *	*
During (D)	df2	ns	**	* *
T×D	df4	*	*	* *

[a] Mean values ± SD (n=3). Different letters indicate significant differences at significance level $P \leq 0.05$.

can be monitored by determination of electrolyte leakage (Sharom et al., 1994). Electrolyte leakage is an effective parameter to assess membrane permeability and therefore is used as an indicator of membrane integrity (Marangoni et al., 1996). Zhao et al. (2009) have found that the correlation coefficient between CI index and electrolyte leakage was high irrespective of differences in chilling susceptibility between tomato cultivars. Accordingly, both the CI index (Fig. I) and electrolyte leakage (Fig. II) were significantly lower in Pro-Ca treated fruit than in control fruit.

Lipid peroxidation, which can be evaluated by the content of MDA, is the principal process responsible for loss of cell membrane integrity (Wise and Naylor, 1987). MDA is the end product of the peroxidation of membrane fatty acids and the level of this compound is used as a marker of oxidative stress, since a rise in MDA indicates damage on cell membrane integrity (Hodges et al., 1999). The principal consequence of electrolyte leakage and MDA accumulation is the loss of the biomembrane functionality (Sevillano et al., 2009). Wise et al. (1987) reported that, electrolyte leakage and MDA content, well known physiological markers of loss of membrane semi-permeability and membrane lipid peroxidation, were significantly reduced in Pro-Ca treated fruits (Wise and Naylor, 1987; Sharom et al., 1994). Albrecht et al., (2004) reported that the application of Pro-Ca mitigated the frost injury in apple flowers and leaves. The application of Pro-Ca induces alteration of the flavonoid metabolism in the plant tissue compared to the control (Halbwirth et al., 2002; Roemmelt et al., 2002). Albrecht et al. (2004) suggested that the mitigation of frost injury in apple flowers and leaves treated with Pro-Ca can possibly be explained by an alteration of flavonoids, which may reduce oxidative stress, decrease the freezing point or scavenge ROS production under frost stress.

Proline, as a multifunctional amino acid, plays key roles in the osmotic regulation between cytoplasm and vacuole, the redox regulation of the NAD^+/NADH ratio, membrane stabilizer, and finally promoting ROS scavenging systems (Sharp et al. 1990; Bohnert and Jensen, 1996). Bekheta et al. (2009) reported that the application of Pro-Ca enhanced the contents of proline, photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids), total carbohydrates and total soluble sugars in *faba bean* seedlings grown under salt stress. Bekheta et al. (2009) suggested that the growth retardant Pro-Ca may mitigate the harmful effects of

salinity on the growth and development of *Viciafaba* seedlings.

PAL as a key enzyme in the phenyl propanoids pathway catalyze the conversion of phenylalanine to trans-cinnamic acid. PAL connected primary metabolism (shikimic acid pathway) with secondary metabolism (phenyl propanoids pathway) (Dixon and Paiva, 1995). In general terms it has been accepted by the scientific community that an increase of PAL activity in fruit stored at chilling temperatures is part of the response of the plant organ in order to alleviate CI (Rinaldo et al., 2010). Schlangen et al. (2003) reported that the Pro-Ca treatment improved rose leaves tolerance to disease by enhancement of PAL enzyme activity. Also, Fischer et al. (2006) reported that the application of Pro-Ca led to an increase in PAL gene expression and enzyme activity, which led to enhanced tolerance of apple to fire blight and scab. It is generally accepted that PAL activity increases in fruits under chilling temperature, the enhancement being proposed as a mechanism to alleviate CI symptoms (Lafuente et al., 2003), since pre-treatments reducing CI, such as heat (Chen et al., 2008) can lead to higher increases in PAL activity in banana fruit. The increase in PAL activity of Pro-Ca treated fruit during storage time along with the amelioration of CI in this study confirms this finding.

Conclusion

To summarize, the present study has proven that the beneficial effects of Pro-Caon reducing CI are valid also in tomato fruits during low temperature storage. Our results suggest that the applications of Pro-Camight be used in order to reduce CI in tomato fruits during low temperature storage by increasing PAL activity and proline content, and reducing MDA content and thus maintaining membrane integrity.

Acknowledgements

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