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Effect of Postharvest Calcium and Hot-Water Dip Treatments on Catalase, Peroxidase and Superoxide Dismutase in Chilled Lisbon Lemon Fruit

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Abstract: Lisbon lemons [*Citrus limon* (L.) Brum.] harvested early in the season (green stage) were treated with hot-water dip (HWD, 53°C, 3 min) or vacuum infiltration with 1.5% CaCl₂ (15°C, -33 kPa, 10 min) prior to storage at 1.5°C for up to 8 weeks, to determine the effect of these treatments on the Chilling Injury (CI) and activities of Catalase (CAT; EC 1.11.1.6.), superoxide dismutase (SOD; EC 1.15.1.1.) and Peroxidase (POD; EC 1.11.1.7.), as well as Malondialdehyde (MDA) content. The decrease of CAT and SOD, accompanied with the increase in POD activities during cold storage, might contribute to the development of CI in lemon fruits. HWD and Ca treatments similarly reduced CI and delayed the reduction of CAT, SOD and suppressed the increase in POD activities as well as MDA content compared to the control. CAT activity in fruits exposed to these treatments before cold storage increased and remained higher in the treated fruits than in the non-treated fruits over the storage period. The data indicate that CAT may be a major antioxidant enzyme involved in the defense of lemon fruits against chilling stress. Lemons harvested at the late maturity (yellow) stage had significant higher CAT, SOD and lower POD activities compared to the early maturity (green) stage. Collectively, results indicate that the chilling-tolerant property of lemon fruits may be related to the higher ability of fruits to have or maintain high CAT, SOD and low POD activities during low temperature storage.

Key words: Chilling injury, postharvest treatments, antioxidant enzymes, lisbon lemon

INTRODUCTION

Many citrus cultivars, particularly lemons, are sensitive to chilling injury (CI) and may develop peel injury when exposed to cold treatment, which greatly reduces the fruit marketability (Houck *et al.*, 1990). The beneficial effect of prestorage hot water treatments in increasing chilling tolerance has been reported in different crops (Fallik, 2004). Treatment of lemon fruits with hot water at 53°C for 3 min was found to be effective in alleviating CI after 8 week storage at 1-2°C (Rodov *et al.*, 1995; McLauchlan *et al.*, 1997). It was also known that calcium plays a role in the maintenance of cell stability, particularly membranes, under stress conditions such as postharvest low temperature storage (Roux and Slocum, 1982). Pre-and postharvest calcium applications have been reported to lower the rate of plant senescence, fruit ripening, sensitivity to pathogen and susceptibility to CI in different fruit and vegetables by reducing or delaying cell wall breakdown, maintaining the membrane function and prolonging the capacity for signal transduction (Poovaiah, 1986; Ferguson

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and Drobak, 1988). In a different trial with Fortune mandarin, calcium reduced the level of chilling injury of peel dermatosis as a field treatment or as a postharvest dipping (El-hilali *et al.*, 2003; DAquino *et al.*, 2005). Our previous study showed that vacuum infiltration with 1.5% CaCl₂ for 10 min effectively reduced the incidence of CI in Lisbon lemon fruit following storage at 1.5°C (Safizadeh and Rahemi, 2006).

Oxidative damage is considered to be an early response of sensitive tissues to chilling (Karpinski *et al.*, 2002). Superoxide anion, hydrogen peroxide and hydroxyl radical are Reactive Oxygen Species (ROS) that can result in oxidization of proteins, unsaturated fatty acids and DNA, causing cellular damage and eventually cell death (Mittler, 2002). Several enzymes are involved in the production and scavenging of free radicals in plant systems. Catalase (CAT; EC 1.11.1.6.) can enhance the removal of H₂O₂ without producing dangerously-active free radicals. Peroxidase (POD; EC 1.11.1.7.) also catalyzes the decomposition of H₂O₂, but the mode of action of peroxidase differs from catalase action in that peroxidase liberates free radicals instead of oxygen. These free radicals are highly phytotoxic (Burris, 1960). The damaging effects of superoxide can be modulated if the radicals are scavenged by reacting with superoxide dismutase (SOD; EC 1.15.1.1.). SOD catalyzes the dismutation of the superoxide free radical to H₂O₂ and O₂, removes singlet oxygen and prevents formation of OH·. The combined action of SOD and CAT converts the potentially dangerous superoxide radical and hydrogen peroxide to molecular oxygen and water, thus averting cellular damage (Van Camp *et al.*, 1994). The active oxygen detoxifying enzymes have been shown to participate in the beneficial effect of postharvest heat conditioning treatments protecting Fortune mandarins against chilling (Sala and Lafuente, 2000). However, the effect of postharvest hot-water treatment on ROS detoxifying enzymes in other citrus fruit has not been reported. There is little information available about effects of calcium on cellular protective enzyme activities in citrus fruit.

The aim of this study was to investigate the effect of hot water and calcium treatments on changes in the activities of CAT, POD and SOD in Lisbon lemon fruits during storage at chilling temperature. The activities of these enzymes in lemons harvested at the less maturity (green) stage and harvested 10 weeks later at the advanced maturity (yellow) stage was also compared.

MATERIALS AND METHODS

Plant Material, Storage and Treatments

Lisbon lemon [*Citrus limon* (L.) Burm.] were harvested at the green stage from 20-year-old trees grafted onto sour orange (*Citrus aurantium* L.) rootstock and grown at Hajiabad, south of Iran.

Fruits were randomly divided into three lots containing three replicates of 15 fruits to estimate chilling damage and of 5 fruits per storage period to analyze enzyme activity. The first lot was immediately stored at 1.5±0.2°C and 85-90% Relative Humidity (RH) for up to 8 weeks. The second lot was submerged for 3 min at 53±0.1°C in a 12 L recirculating hot water bath with temperature control (Model YCM-04 M, KGIC, Germany). The third lot was treated with calcium at reduced pressure (-33 kPa; Joyce *et al.*, 2001). The 1.5% CaCl₂, 2H₂O (w/v) solution, containing Tween 20 (0.2%) was applied in a 15 L capacity vacuum desiccator (Model B-1834, Gallenkamp, England) at 15°C and applying a reduced pressure of -33 kPa for 10 min. Following treatment, fruits rinsed by dipping for ≈ 2 sec in distilled water at 15±1°C before drying. After treatments of hot-water dip and vacuum infiltration with CaCl₂ all fruits were air-dried and stored as the non- treated fruits.

Three replicates samples of 20 fruits of each lot were sampled after 2, 4, 6 and 8 weeks storage at 1.5°C to determine peel damages and changes in enzyme activities. The colored outer layer of skin (flavedo tissue) was separated from the whole fruit and cut into small pieces and represently 1 g

samples were frozen in liquid nitrogen and stored at -70°C for enzyme assays. Frozen flavedo tissues were grounded to fine powder with a microdismembrator (Model 01M, Raz-Gol, Iran) and immediately were used for enzyme extraction and assay. All optical measurements were performed on a spectrophotometer (UV-160A; Shimadzu, Tokyo, Japan).

Chilling Injury (CI) Evaluation

CI was classified as either peel pitting or surface browning, but these symptoms were combined as a total CI score. CI was rated as 0 (no damage), 1 (slight), 2 (moderate), and 3 (severe). CI index was determined by summing the product of the number of fruit in each category by the score of each category and then dividing this sum by the total number of fruits assessed (Sala, 1998).

Extraction and Assay of CAT

CAT was extracted from 1 g flavedo tissue with 10 mL of 100 mM potassium phosphate buffer, pH 6.8 at 4°C and then centrifuged twice at 27000×g for 15 min at 4°C (Sala and Lafuente, 2000). The supernatant was used to determine CAT activity by the method of Kar and Mishra (1976). Five mL of the assay mixture comprised 60 mM of phosphate buffer, pH 6.8, 20 mM of H₂O₂ and 1 mL enzyme extract. After incubation at 25°C for 1 min, the reaction was stopped by adding 10 mL of 2% (v/v) H₂SO₄ and the residual H₂O₂ was titrated against 0.01N KMnO₄ until a final purple color persisted for at least 15 sec. A control was run at the same time in which the enzyme activity was stopped at “Zero” time. The unit of CAT activity was defined as the amount of enzyme which decomposes 1 mM H₂O₂ per minute at 25°C.

Extraction and Assay of POD

The flavedo tissue (1 g) was homogenized in 10 mL 0.2 M phosphate buffer, pH 5.8, containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 1% polyvinyl polypyrrolidone (PVPP) at 4°C. The homogenate was centrifuged at 27000×g for 15 min at 4°C twice and the supernatant was used to determine POD activity by the method of Siegel and Galston (1967). POD activity was determined by the oxidation of guaiacol in the presence of H₂O₂ (extinction coefficient, 26.6 mM⁻¹ cm⁻¹) at 470 nm. The reaction was carried out at 25°C for 60 sec in a 3 mL reaction mixture containing 0.2 M phosphate buffer, pH 5.8, 5 mM guaiacol, 5 mM H₂O₂ and 25-75 µL of enzyme extract. One unit of POD activity represents the amount of enzyme catalyzing the oxidation of 1 mM of guaiacol per minute at 25°C.

Extraction and Assay of SOD

The flavedo tissue (1 g) was homogenized in 10 mL of 50 mM potassium phosphate buffer, pH 7.8, containing 1.33 mM Diethylenetriamine Pentaacetic Acid (DETAPAC) at 4°C and then centrifuged twice at 27000×g for 15 min at 4°C (Sala and Lafuente, 2000). The supernatant was used to determine SOD activity by the method of Beauchamp and Fridovich (1971). A 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 100 µM EDTA and 0-100 µL of enzyme extract. Riboflavin was added last and tubes were shaken and placed in a cylindrical glass water bath at 25°C. Two circular fluorescent lamps (40-w) were attached on the outside wall of the water bath and the entire assembly was fitted in a box lined with aluminium foil. The reaction was started by switching on the light and it was allowed to run for 10 min, after which the lights were switched off and the tubes covered with a black cloth. Absorbance by the reaction mixture was read at 560 nm and the non-irradiated reaction mixture served as blank. The reaction mixture lacking enzyme developed the most color and this decreased with increasing volume

of extract added. Log A_{560} was plotted as a function of the volume of enzyme extract in the reaction mixture. The volume of enzyme extract producing 50% inhibition of the reaction was read from the resultant graph. The unit of SOD activity was defined as that amount of enzyme which caused 50% inhibition of the initial rate of reaction in the absence of enzyme.

Specific activities of all enzymes were expressed as units per mg protein. Protein was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Lipid Peroxidation

Lipid peroxidation in flavedo tissue was determined by estimating the malondialdehyde (MDA) content following the method of Heath and Packer (1968). The supernatant prepared for determination of POD enzyme was used. For every 1 mL of the aliquot of the supernatant 2 mL of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid was added. The mixture was heated at 95°C for 30 min and then cooled quickly on ice bath. The resulting mixture was centrifuged at 10000×g for 25 min and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated by using extinction coefficient of 155 mM⁻¹ cm⁻¹ expressed as mM MDA g⁻¹ Fresh Weight (FW).

Statistical Design

Experimental data are the Mean±standard Error (SE) of three replicates of the determinations for each sample. ANOVA analysis was performed by means of SAS/STAT GLM procedure, using the Duncan multiple range test at 5% level for means comparison.

RESULTS

Severity of Chilling Injury

Low-temperature storage of Lisbon lemon induced CI, peel pitting being the primary symptoms of this physiological disorder. No pitting occurred in stored fruit for up to 2 weeks and thereafter CI increased continuously for all samples as storage period advanced (Fig. 1A). Slight pitting appeared in HWD (CI index = 0.55) or Ca-treated (CI index = 0.6) fruits for up to 6 weeks at 1.5°C, whereas in control fruits significant severe pitting was observed (CI index = 2.37). The CI increased quickly thereafter in HWD or Ca-treated fruits, but significantly less than control fruits up to 8 weeks storage at chilling temperature. Therefore, the development of CI symptoms was similarly delayed by both HWD and Ca treatments.

Activities of POD

Before storage at chilling temperature, POD activities of samples were approximate. Low temperature storage up to 2 weeks induced a decrease in activity of POD, thereafter increased continuously for all samples as storage period advanced (Fig. 2A). After 8 weeks of chilling exposure at 1.5°C, POD activity increased more than 2-fold in the control fruits. POD activity of both HWD and Ca-treated fruits also increased during storage. However, the rate of increase was much less in treated fruits than in the control fruits. No significant differences in POD activity were observed between treated lemons.

POD activity in the flavedo of lemons harvested at the advanced maturity (yellow) stage was lower (2-fold) than those harvested at the less maturity (green) stage (Fig. 3A).

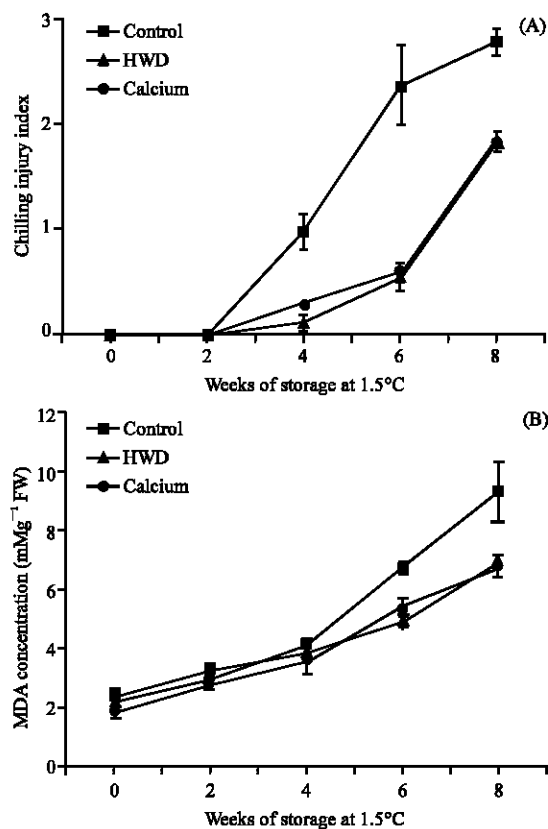


Fig. 1: Effect of a hot-water dip treatment at 53°C for 3 min and a vacuum infiltration treatment with 1.5% CaCl₂ for 10 min on CI index (A) and MDA content (B) of Lisbon lemons during storage at 1.5°C. Values are the mean of three replicate samples±SE

Activities of CAT

Calcium treatment of lemons before they were transferred to 1.5°C induced a 2.43-fold increase in CAT activity, this increase was slightly higher than the induced 2.23-fold by HWD treatment (Fig. 2B). The activity of CAT increased initially for up to 2 weeks at 1.5°C and then declined thereafter. The activity continued to decline in all samples during 4-8 weeks of storage. In lemons treated with HWD or Ca, CAT activity also declined but was about twice that of control fruits for up to 6 weeks of storage. In general, the decline in CAT activity of HWD and Ca-treated fruits during storage followed a similar pattern.

CAT activity in the flavedo of lemons harvested at the advanced maturity (yellow) stage was 2.64-fold higher than those harvested at the less maturity (green) stage (Fig. 3B).

Activities of SOD

Before storage at chilling temperature, treatment with HWD or Ca did not affect SOD activity of lemons (Fig. 2C). SOD activity increased initially for up to 2 weeks of storage, but declined thereafter. The activity continued to decline in all samples throughout 4-8 weeks of exposure to low temperature. In lemons treated with HWD or Ca the activity of SOD was higher than those of the control lemons. The activity afforded by HWD (1-3-fold) or Ca treatment (1-2-fold) was maintained throughout the storage period.

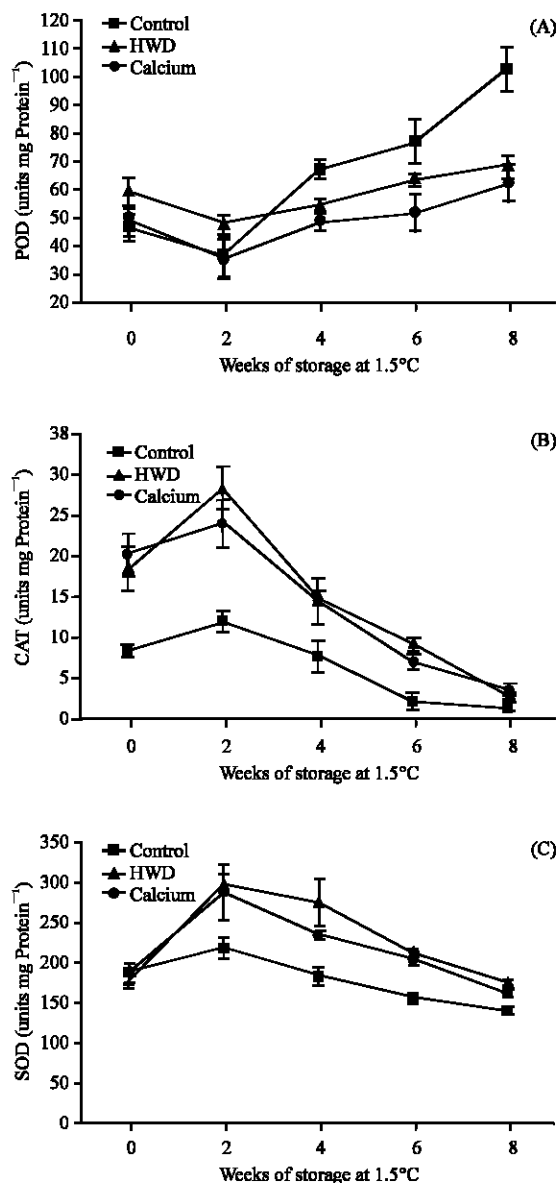


Fig. 2: Effect of a hot-water dip treatment at 53°C for 3 min and a vacuum infiltration treatment with 1.5% CaCl₂ for 10 min on POD (A), CAT (B) and SOD (C) activities of Lisbon lemons during storage at 1.5°C. Values are the mean of three replicate samples±SE

Experiment done to study the effect maturity stage on SOD activity revealed that fruit were harvest at the yellow stage had 2.33-fold more activity than those harvested at the green stage (Fig. 3C).

Malondialdehyde Content

The formation of MDA content was considered as a measure of lipid peroxidation. As the storage period extended, MDA content of all samples increased constantly and reached the highest value up

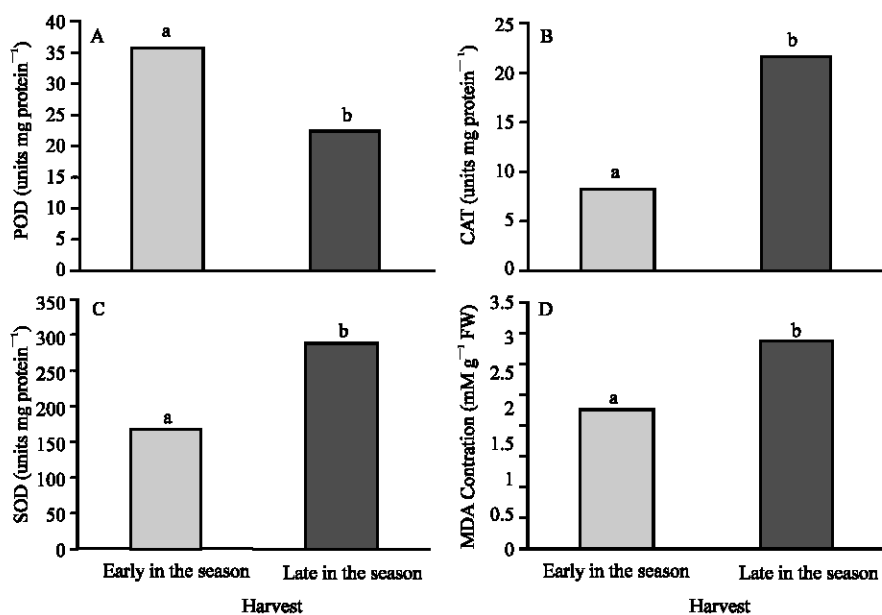


Fig. 3: Influence of maturity stage on activities of POD (A), CAT (B) and SOD (C), as well as MDA content (D) of Lisbon lemons. Columns with different letters were significantly different by the Duncans multiple range test at $p = 0.05$

to 8 weeks storage at 1.5°C (Fig. 1B). A significant difference in MDA content between the treated and control lemons was observed on the 6th week of storage and this difference was maintained for the remained of the storage. HWD and Ca treatments resulted in a significant decrease in MDA content as compared with control. No significant differences in MDA content were observed between treated lemons.

As compared to the early maturity (green) stage, lemons were harvested at the late maturity (yellow) stage had significant higher (1.5-fold) MDA content (Fig. 3D).

DISCUSSION

Free-radical processes are involved in several membrane-associated disorders including CI. Superoxide and hydrogen peroxide produced in plant tissues exposed to low temperature may cause the peroxidation of membrane lipids (Marangoni *et al.*, 1996). The development of CI and the increase in MDA content of Lisbon lemon induced by chilling stress were restrained by both HWD and Ca treatments. Treated lemons consistently had higher SOD activities during storage than the control fruits. SOD from treated fruits may play a protective role by detoxifying free radicals. The superoxide radicals produced during chilling stress might be more efficiently dismutated to H₂O₂ and molecular oxygen in the treated lemons. Thus the stability of cell membranes was maintained by HWD or Ca which lead to an increase in chilling tolerance. These results agree with those for eggplant seedlings (Gao *et al.*, 2004) and grape berries (Zhang *et al.*, 2005). Higher content of MDA in more mature (yellow) lemons may be attributed to the chlorophyll degradation and proteolysis occurs during accelerated of aging and senescence as previously suggested (Meir *et al.*, 1992).

Several active free-radical scavenging enzyme systems exist in plant tissues as defenses against free-radical attack. CAT uses hydrogen peroxide both as a donor of hydrogen and as a substrate in the catalytic decomposition of hydrogen peroxide to form oxygen and water (Burris, 1960). However, CAT activity was found to decrease in Lisbon lemons after 2 weeks and during subsequent chilling period. Decrease in CAT activity during chilling has also found in mandarin fruit (Sala and Lafuente, 2000), orange fruit (Sala *et al.*, 2005) and grape berries (Zhang *et al.*, 2005). On the other hand, POD activity increased steadily in lemons after 2 weeks of storage. Chilling temperature also enhanced POD activity in mango (Zauberman *et al.*, 1988), Fortune mandarin (El-hilali *et al.*, 2003) and Unshiu mandarin fruits (Lepedus *et al.*, 2005). Peroxidases are ubiquitous enzymes that have diverse biochemical functions in higher plants and are involved in the response of plants to stress (Gaspar *et al.*, 1981). For example in citrus fruit, an increase in POD activity has been observed in response to *Penicillium digitatum* infection (Ballester *et al.*, 2006). Decreasing CAT activity and increasing POD activity could lead to slow removal H₂O₂ in the tissue and may contribute to the development of CI. Both HWD and Ca treatments reduced the decline of CAT activity and suppressed the increase of POD activity. These effects may contribute to increase tolerance of lemons to CI. This result is in accordance with observations that HWD (Lepedus *et al.*, 2005) or calcium (El-hilali *et al.*, 2003) decreased POD activity and similarly HWD or heat-conditioning treatment (Sala and Lafuente, 2000) delayed the reduction of CAT activity in mandarin fruits during chilling stress.

Previous investigations suggested that CAT may be a major antioxidant enzyme participating in the defense mechanism of citrus fruit against chilling conditions (Sala and Lafuente, 2000; Sala *et al.*, 2005). In the study work, treating Lisbon lemon with HWD or Ca resulted the induction of CAT activity, but not involved POD and SOD activities. Furthermore, the maintenance of increased CAT activity during cold storage affects the effectiveness of these treatments. Both HWD and Ca increased chilling tolerance and CAT activity in the fruits, but after 6 weeks of cold storage CAT activity declined at the same time as resistance to chilling. Considering these results further support this idea that CAT might play a major role in the tolerance of citrus fruit to CI.

Reduction of damaging effects of chilling in resistant plants may be related to their ability to reduce and to scavenge free radicals through increase antioxidant enzyme activity, as has been reported for example in mandarin (Sala, 1998) and orange fruit (Sala *et al.*, 2005). This further confirmed from observed increase in CAT, SOD and decrease in POD activities of Lisbon lemons during 2 weeks storage at chilling temperature, which showed no chilling peel pitting. In addition, the lemon fruit harvested at the yellow stage had higher CAT, SOD and lower POD activities than those harvested at the green stage. It is well documented that lemon fruit harvested early in the season were more susceptible to CI than fruit harvested later (Underhill *et al.*, 1995). The overall results may indicate that the linked action of CAT, SOD and POD may contribute, at least to some extent, to the tolerance of Lisbon lemon fruit to CI and the chilling-tolerant property of fruits may be related to the higher ability of fruits to have or maintain high CAT, SOD and low POD activities during low temperature storage.

As the main objective of this study was to examine the role of HWD and Ca on CI and antioxidant enzymes activity, it is interesting to note that the pattern of alleviating chilling symptoms and the changes of antioxidant enzyme activity in the treated fruits were similar during storage. Gong *et al.* (1998) reported that heat stress caused a transient increase in cytosolic Ca²⁺ concentration and this increase may alleviate heat injury and enable plant cells to better survive. The regulatory role of calcium (Ca²⁺) on antioxidant enzyme to induce tolerance to various stresses has also been reported earlier (Karpinski *et al.*, 2002; Agarwal *et al.*, 2005). These results suggest a connection among heat, cytosolic Ca²⁺ and antioxidant enzymes in the reduction of chilling injury. However, further work is needed to define the exact relationship of heat and Ca on antioxidant enzymes.

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