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Tolerance to chilling injury induced by hot water treatment increases activities of antioxidant enzymes in tomato fruits

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Abstract

Tomato is sensitive to chilling injury (CI) when stored at temperatures below 12°C, and increased evidences showed that CI is associated to oxidative stress. Hot water treatments (HWT) have shown to induce CI tolerance, and it is hypothesised that antioxidant enzymes participate in the induction of CI tolerance. The present work evaluates the effect of a HWT (42°C for 5 min) on CI symptoms, physiological markers related to membrane damage, and the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), in mature-green tomato fruits (cv. Imperial) during 15 d of cold storage at chilling (5°C) and non-chilling (12°C) conditions. The efficiency of HWT to induce CI tolerance in tomato was validated by reductions in CI symptoms severity (CI index). Fruits subjected to HWT and subsequent chilling conditions presented reduced membrane damage (electrolyte leakage) and lipid peroxidation (malondialdehyde content). Furthermore, HWT increased the activities of SOD, CAT, and APX during the storage at 5°C, suggesting that higher antioxidant enzymes activity may be providing protection against lipid membrane peroxidation during cold storage, thus, inducing CI tolerance.

Introduction

Tomato fruit is usually stored under refrigeration during postharvest distribution; however, it is sensitive to chilling injury (CI), a physiological disorder developed when this fruit is exposed at temperatures below 10 - 12°C (Cruz-Mendívil *et al.,* 2015; Gonzalez *et al.,* 2015). Visible CI-symptoms of tomato include uneven ripening, pitting, wilting, decay, and accelerated deterioration which appear once the fruit is transferred to higher temperatures (20 - 22°C) (Vega-García *et al.,* 2010).

CI mechanisms are not completely elucidated, but have been firstly related to the membrane lipids phase transition as primary response, leading to changes in lipid conformation into the cell membranes, and subsequently decreasing in their flexibility and permeability (Lyons, 1973). Nevertheless, recent studies have suggested an important role of oxidative stress in the development of this disorder (Zhao *et al.,* 2009; Zhang *et al.,* 2013; Lado *et al.,* 2016), being mediated by an overproduction of reactive oxygen species (ROS), which could be considered as a secondary response (Sevillano *et al.,* 2009).

Several studies involving antioxidant enzymes in response to CI have been conducted in tomato, but their results are somehow contradictory. Malacrida *et al.* (2006) analysed some antioxidant enzymes in tomato cv. Micro-Tom stored at 4°C, showing the activities of catalase (CAT, EC 1.11.1.6) and glutathione reductase (GR, EC 1.8.1.7) were modified in response to CI, while those of the superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.11) did not show a relation to CI. On the other hand, Gómez *et al.* (2009) reported that SOD and APX responded to CI in tomato cv. Micro-Tom stored at 6°C. Meanwhile, Zhang *et al.* (2013) reported that SOD and APX activities increased in tomato cv. Messina stored at 2°C, while CAT activity decreased. In another study, Zhao *et al.* (2009) analysed two tomato cultivars that differed in CI tolerance, and found that CAT activity increased in the more tolerant cultivar.

Different postharvest treatments have been

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applied prior to storage at low temperatures in order to alleviate CI-symptoms in tomato fruits, including application of methyl jasmonate and methyl salicylate (Ding *et al.,* 2001), gradual cooling (Gálvez *et al.,* 2010), and heat treatments (Imahori *et al.,* 2016). Immersion of the fruits in hot water (hot water treatment, HWT) is one of the most efficient postharvest treatments in terms of protection to CI. In this sense, Yang *et al.* (2009) conducted a microstructural study to evaluate the application of HWT on CI tolerance, showing that tomato fruits with HWT exhibited less damage in cells and organelles of the pericarp tissue during cold storage. Moreover, HWT was effective to reduce electrolyte leakage, respiration rate, and CI-symptoms in tomato fruits cv. Micro-Tom, harvested at mature-green stage, and subjected to cold storage (Luengwilai *et al.,* 2012; Cruz-Mendívil *et al.,* 2015). Recently, Imahori *et al.* (2016) evaluated the effect of HWT and chilling separately on antioxidant enzymes activity of tomato fruits cv. Sanibel harvested at full-red stage, showing that chilling increased the activities of APX, while heating increased the activities of CAT and peroxidase. To our knowledge, the combined effect of HWT and chilling on antioxidant enzymes activity in tomato fruits at early ripening stages has not been studied yet. This is relevant because early ripening stages (mature-green, breaker) are more sensitive to develop CI, and are the most frequently used harvest stages for export.

The aim of the present work was to evaluate the effect of HWT on CI development, membrane damage, and activation of antioxidant enzymes in tomato cv. Imperial stored at low temperatures, in order to improve our understanding about the biochemical mechanisms of CI tolerance acquisition.

Materials and methods

Plant material and postharvest treatments

Tomatoes (*Solanum lycopersicum* cv. Imperial) were obtained from a local producer in Culiacan, Mexico. Fruits were harvested at mature-green stage of ripeness, and selected based on size uniformity, colour, and absence of injuries. A total of 168 tomato fruits were washed, excess of surface water was allowed to drain at room temperature (25°C, 30 min), and these were then randomly divided into four groups. Two groups received a HWT by immersion in water at 42°C for 5 min and air-drying at 25°C for 30 min (surface temperature of the fruit was recorded at all times), while the other two groups did not receive any treatment, and were used as controls. Tomato fruits with and without HWT were stored for 0, 5, 10, and 15 d at 5°C (CI temperature) or at 12°C (non-CI temperature, 85 - 90% relative humidity), followed by a ripening period of 8 d at 21°C (85 - 90% relative humidity) in order to allow ripening and CI symptom development.

Quality parameters: weight loss, firmness, and colour (a, °Hue)*

Weight loss was determined as percentage in respect to initial weight. Firmness was evaluated on the equatorial region using a Chatillon DFE 100 (Ametek Inc, Largo, Fla., U.S.A.), provided with 11-mm diameter probe and registering the maximum penetration force after 5 mm penetration at constant speed (50 mm/min). Results were expressed as Newton (N). CIELAB Colour parameters (a* and °Hue) were performed on three equidistant points on the equatorial region of the fruit, using a Minolta colorimeter (model CR-200; Minolta Co. Ltd., Osaka, Japan).

CI index and physiological markers

CI index was determined after 8 d at 21°C according to Vega-García *et al.* (2010) with some modifications. The severity of the symptoms (U = uneven ripening, P = pitting, and W = wilting) was visually assessed using a rating scale based on the injury level (IL) and the percentage of tissue affected (0 = no injury, 1 ≤ 10%, 2 = 11 - 25%, 3 = 26 - 40%, and 4 ≥ 40%) for each symptom. CI index was calculated using the following equation:

$$(ILU + ILP + ILW)/3$$

The physiological markers, electrolyte leakage (EL) and malondialdehyde (MDA) content were determined after 0, 5, 10, and 15 d of cold storage (5 and 12°C). EL was measured according to Zhao *et al.* (2009) with some modifications, using 0.1 mol/L mannitol solution and pericarp cylinder samples (5 mm diameter and 10 mm length) from tomato fruit tissue. Results of EL were expressed as percentage. MDA content was assessed using the thiobarbituric acid method described by Hodges *et al.* (1999) with some modifications. Briefly, 1 g of frozen tissue was homogenised with 10 mL of ethanol:water (80:20, v:v), and centrifuged at 3,000 g_n for 10 min, where the supernatant was used as the sample solution. The blank consisted in 1 mL aliquot of the sample solution mixed with 1 mL of a solution containing 0.1 g/L butylated hydroxytoluene and 200 g/L trichloroacetic acid. The test solution consisted in 1 mL aliquot of the sample solution mixed in a tube containing the previous solution and 6.5 g/L thiobarbituric acid. Following that, the tubes containing the samples were vigorously mixed, incubated at 95°C for 25 min,

cooled at 25°C, and centrifuged at 3,000 g_n for 10 min. The supernatant was recovered, absorbance was measured at 532 nm and corrected by subtracting the absorbance at 440 and 600 nm. Concentration of MDA was calculated using an extinction coefficient of 1.55 nmol/L•m, and expressed as µmol/kg, in a fresh weight basis.

Extraction and analyses of antioxidant enzymes

All enzymatic activities analyses were carried out after 0, 5, 10, and 15 d of cold storage (5 and 12°C). SOD activity was evaluated as described by Wu *et al.* (2008). Briefly, 1 g of frozen tissue was homogenised with 2 mL of 50 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L EDTA and 50 g/L polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 g_n for 15 min at 4°C. The supernatant was recovered and used as protein extract for the analysis of SOD activity. The assay mixture (200 µL) contained a buffer solution of 0.1 mol/L potassium phosphate (pH 7.8), 10 mmol/L L-methionine, 0.25 g/L Triton X-100, 0.11 mmol/L EDTA, 57 µmol/L nitrotetrazolium blue chloride (NBT), and 10 µL of protein extract. The NBT reduction reaction was simultaneously initiated by adding 1.3 µmol/L riboflavin and exposing the ELISA plates under fluorescent light (26 W) for 10 min. The absorbance was read at 550 nm with a 680 ELISA plate reader (Bio-Rad, Hercules, CA). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction induced by the fluorescent light.

CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm for 3 min at 25°C according to Aebi (1984) with some modifications. Briefly, 2 g of frozen tissue were homogenised with a buffer solution of 50 mmol/L potassium phosphate (pH 7.8) containing 0.5 mmol/L EDTA and 10 g/L polyvinylpolypyrrolidone (PVPP). The mixture was centrifuged at 12,000 g_n for 20 min at 4°C. The supernatant was recovered and used as protein extract for the evaluation of CAT activity. The reaction mixture contained a buffer solution of 50 mmol/L potassium phosphate (pH 7.0), 50 mmol/L H_2O_2, and 50 µL of protein extract. CAT activity was reported as mol of reduced H_2O_2 per kg of protein per s (mol/kg•s).

APX activity was determined according to Nakano and Asada (1987), by monitoring the kinetics of ascorbic acid elimination at 290 nm during 3 min at 25°C. Briefly, 2 g sample of frozen tissue was homogenised with a buffer solution of 50 mmol/L potassium phosphate (pH 7.8) containing 5 mmol/L L-ascorbic acid, 0.5 mmol/L EDTA, and 10 g/L PVPP. The mixture was centrifuged at 12,000 g_n for 20 min at 4°C, then the supernatant was recovered and used as protein extract for the evaluation of APX activity. The reaction mixture contained a buffer solution of 50 mmol/L potassium phosphate (pH 7.0), 5 mmol/L L-ascorbic acid, 0.1 mol/L H_2O_2, and 50 µL of protein extract. APX activity was reported as mol of oxidised ascorbate per kg of protein per s (mol/kg•s).

Statistical analysis

All experiments were performed in triplicate in a completely randomised design. Analysis of variance (ANOVA) were carried out with STATGRAPHICS Plus 5.1 software. Mean comparisons were based on the Fisher's Least Significant Difference test (LSD, $p < 0.05$).

Results

Quality parameters: weight loss, firmness, and colour (a, °Hue)*

Weight loss results (Figure 1) showed increases during the cold storage for all the treatments, reaching about 2% after 15 d. Losses being more marked when fruits were transferred to the ripening temperature where about 8% was reached, showing slight symptoms of dehydration. However, increases in weight loss in fruits stored at 5 and 12°C showed no significant differences ($p > 0.05$) between HWT and non-HWT fruits. Interestingly, after 15 ± 8 d, non-HWT stored at 5°C reached a significantly higher ($p < 0.05$) than non-HWT fruits stored at 12°C.

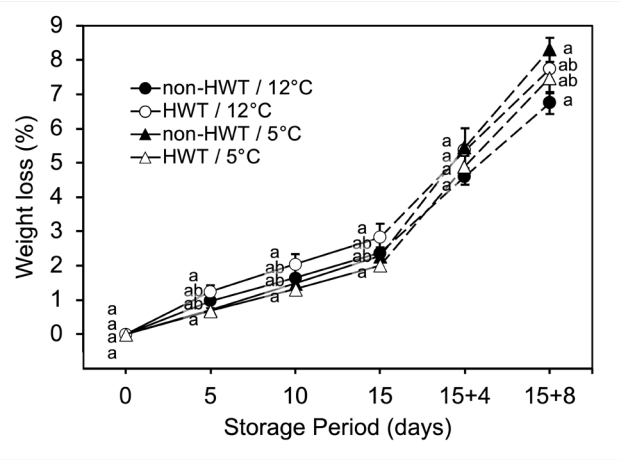

Figure 1. Effect of hot water treatment (HWT) on weight loss in tomato cv. Imperial, stored up to 15 d at 12 or 5°C, followed by a ripening period of 8 d at 21°C. Values are means of triplicates ($n = 3$), and vertical bars indicate standard deviations. Different letters indicate statistical differences ($p < 0.05$).

As expected, fruit firmness (Figure 2A) decreased during the cold storage and the ripening period, this reduction being significantly higher ($p < 0.05$) in the fruits stored at 12°C than at 5°C. Nevertheless, it was observed that HWT failed to retain the firmness in both cold temperatures (5 and 12°C), showing no significant differences ($p > 0.05$) between HTW and non-HWT.

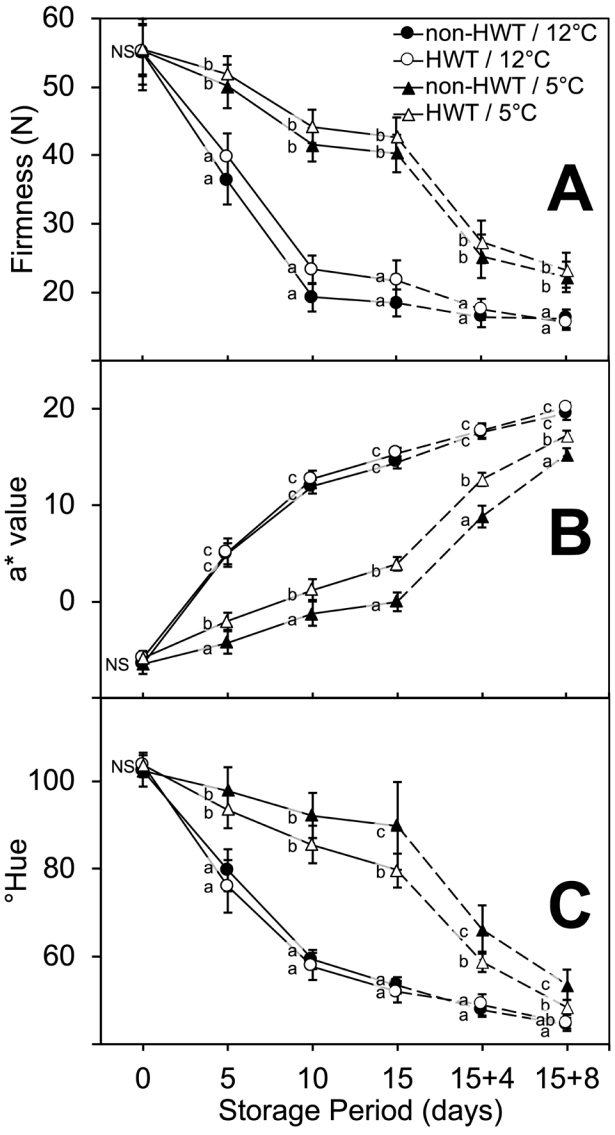

Figure 2. Effect of hot water treatment (HWT) on firmness (A), CIELAB a* (B), and CIELAB °Hue (C) in tomato cv. Imperial, stored up to 15 d at 12 or 5°C, followed by a ripening period of 8 d at 21°C. Values are means of triplicates ($n = 3$), and vertical bars indicate standard deviations. Different letters indicate statistical differences ($p < 0.05$).

Regarding fruit colour parameters, it was observed that a* values of all the treatments (Figure 2B) increased during the cold storage (5 and 12°C) and the ripening period (21°C). As expected, a* value increased more markedly in fruits stored at 12°C as compared to 5°C, but no significant differences between HWT and non-HWT were shown in fruits stored at 12°C. On the other hand, HWT conferred a better development of red colour in fruits stored at 5°C, showing significantly ($p < 0.05$) higher a* values than non-HWT fruits after 5 d stored at 5°C.

Hue values decreased during cold storage and ripening period for all the treatments (Figure 2C). Decrease was significantly ($p < 0.05$) slower in fruits stored at 5°C as compared to those stored at 12°C. No significant differences ($p > 0.05$) were found between HWT and non-HWT stored at 12°C. However, differences between treatments were found at 5°C, where HWT fruits showed lower °Hue values as compared to non-HWT after 10 d storage, up until the end of the experiment (15 ± 8 d).

CI index

CI symptom incidence and severity were observed and evaluated in fruits stored for 5, 10, and 15 d at 5°C, followed by a ripening period of 8 d at 21°C. The major symptoms observed were uneven ripening, pitting, and to a lesser extent, wilting. Severity increased as the storage period increased (Figure 3). Meanwhile, in fruits with HWT stored at 5°C for 10 ± 8 and 15 ± 8 d, CI index values were significantly ($p < 0.05$) reduced (about 36% less) as compared to non-HWT fruits, showing a protective effect against CI provided by HWT application.

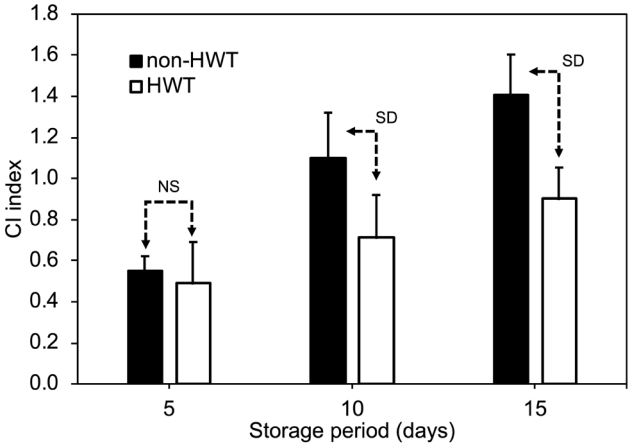

Figure 3. Effect of hot water treatment (HWT) on chilling injury (CI) index in tomato cv. Imperial, stored up to 15 d at 5°C, followed by a ripening period of 8 d at 21°C. Values are means of triplicates ($n = 3$), and vertical bars indicate standard deviations. NS: not significant; SD: statistical differences ($p < 0.05$).

Physiological markers of CI

The levels of EL increased as the cold storage time advanced. Tomato fruits stored at 12°C (minimal safe temperature to avoid CI) showed

higher levels of EL than those at 5°C, with no significant ($p > 0.05$) differences between non-HWT and HWT throughout the storage (Figure 4A). On the other hand, HWT fruits stored at 5°C significantly reduced ($p < 0.05$) the EL values as compared to non-HWT, by 21 and 13% for 10 and 15 d of cold storage, respectively.

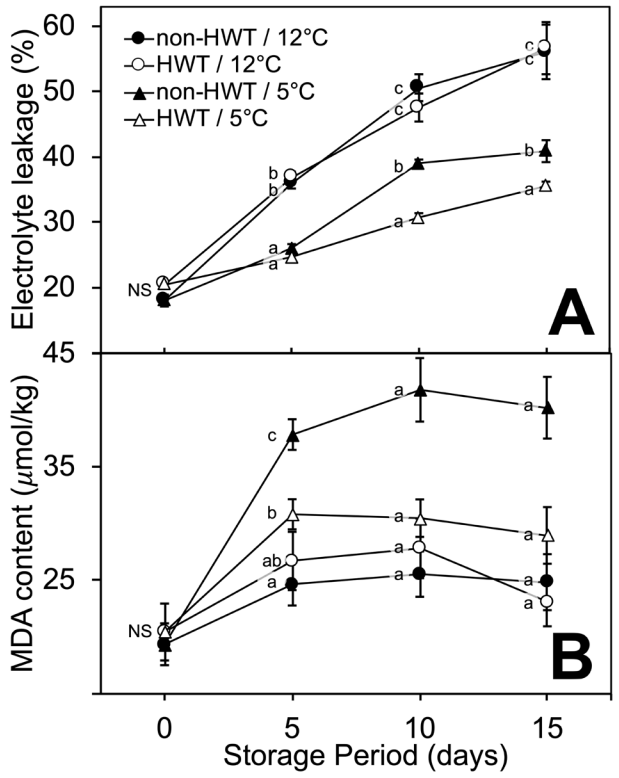

Figure 4. Effect of hot water treatment (HWT) on electrolyte leakage (A) and malondialdehyde (MDA) content (B) in tomato cv. Imperial, stored up to 15 d at 12 or 5°C. Values are means of triplicates ($n = 3$), and vertical bars indicate standard deviations. Different letters indicate statistical differences ($p < 0.05$).

Tomato fruits stored at 12°C showed a slight increase in MDA content after 5 d, and remained stable throughout the storage, without significant ($p > 0.05$) differences between non-HWT and HWT (Figure 4B). Nevertheless, non-HWT fruits stored at 5°C showed a strong increase in MDA content after 5 d of chilling, and remained high until the end of the experiment. Meanwhile, HWT treated fruits stored at 5°C showed significant reductions ($p < 0.05$) in MDA content by 18, 28, and 29% in respect to non-HWT fruits, after 5, 10, and 15 d of cold storage, respectively (Figure 4B). Interestingly, no significant ($p > 0.05$) differences were observed during the storage period between fruits with HWT stored at 5°C and fruits with and without HWT stored at 12°C (Figure 4B).

Antioxidant enzyme activities

In fruits stored at 12°C, SOD activity in non-HWT and HWT remained stable during the first 10 d, presenting a slight increase after 15 d, without significant ($p > 0.05$) differences between treatments (Figure 5A). In the case of fruits stored at 5°C, SOD activity showed a rapid increase in both non-HWT and HWT after 5 d, without significant ($p > 0.05$) differences between them. In the case of fruits stored at 5°C, SOD activity increased in both non-HWT and HWT through the storage. Interestingly, the SOD activity in fruits with HWT showed a significant ($p < 0.05$) increase (by 44%) with respect to non-HWT, after 15 d of cold storage (Figure 5A).

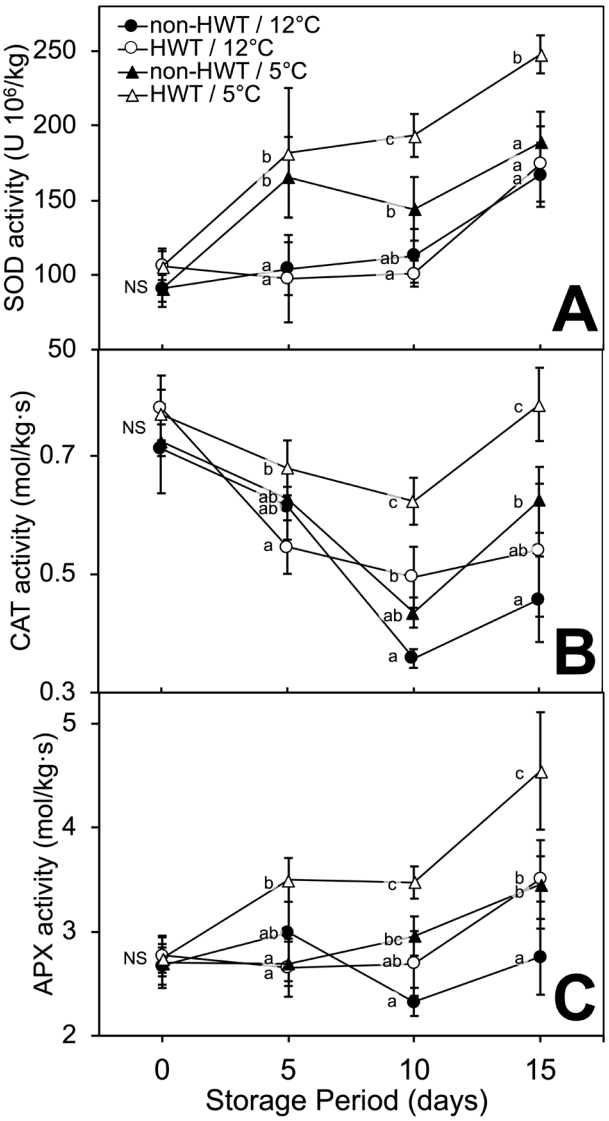

Figure 5. Effect of hot water treatment (HWT) on superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) activities in tomato cv. Imperial, stored up to 15 d at 12 or 5°C. Values are means of triplicates ($n = 3$), and vertical bars indicate standard deviations. Different letters indicate statistical differences ($p < 0.05$).

Except for HWT fruits stored at 12°C, CAT activity decreased gradually in all treatments, during the first 10 d of storage at 5 or 12°C, followed by a moderate increase after 15 d (Figure 5B). In tomato fruits stored at 12°C, CAT activity was only significantly ($p < 0.05$) higher in HWT at day 10 as compared to non-HWT. In the case of fruits stored at 5°C, CAT activity was significantly ($p < 0.05$) higher in HWT after 10 and 15 d of cold storage, being 43 and 24% higher than the registered on non-HWT.

The activity of APX showed different patterns in fruits stored at 5 or 12°C (Figure 5C). APX activity measured in HWT tomato fruits did not change significantly during the first 10 d at 12°C, but after 15 d, it increased significantly ($p < 0.05$). However, in fruits stored at chilling temperature (5°C), APX activity showed increases in both treatments throughout the storage. Nevertheless, higher APX activity ($p < 0.05$) was observed in HWT with respect to non-HWT after 5 and 15 d of storage at 5°C (Figure 5C).

Discussion

Increases in weight loss throughout the cold storage and ripening period could be attributed to loss of water by dehydration (Henríquez *et al.,* 2005; Akbudak *et al.,* 2007). According to Kantola and Helén (2001), tomato fruit loses its freshness when weight loss is about 5 - 6%, meanwhile Nuez (2001) indicates that tomato loses its commercial acceptance when weight loss is about 7% of its original weight. Although it is known that higher temperatures increase respiration and dehydration process, thus starting the loss of water, this phenomenon is observed in environments with low relative humidity conditions; this way, our results of weight loss in cold-stored fruits showed that HWT did not induce a significant increase as compared to non-HWT fruits stored at the same temperature, being in concordance to the studies of Henríquez *et al.* (2005) and Luengwilai *et al.* (2012). However, the higher weight loss values shown by non-HWT fruits stored at 5°C as compared to non-HWT fruits stored at 12°C after 15 ± 8 d of cold storage, could be explained as a consequence of CI, since this physiological disorder causes increases in the respiration rate (Luengwilai *et al.,* 2012), therefore, an increase in fruit metabolism leads to an accelerated conversion of sugars and organic acids to simple molecules, such as water and carbon dioxide, which is the final products of cellular respiration.

Losses in firmness during cold storage and ripening period are due to depolymerisation and solubilisation of polyuronides in lamella media between the cell walls, leading to a progressive loss of cell-cell cohesion, being involved with enzymes such as cellulase, polygalacturonase, and xylanase (Prasanna *et al.,* 2007; Romero and Rose, 2019). Additionally, it has been reported that water loss via transpiration plays a key role in softening of tomato fruits (Saladié *et al.,* 2007). According to several authors, the information about the impact of HWT in firmness of tomato fruits is not consistent. Akbudak *et al.* (2007) and Vega-Espinoza (2010) indicate that HWT confers higher firmness retention during cold storage; however, we did not observe a significant effect on the firmness retention attributable to HWT application in the present work, our result being similar to those reported by Lurie and Sabehat (1997) and Henríquez *et al.* (2005).

In the case of colour parameters, the increases in a* and °Hue values during storage were expected since this behaviour is due to the natural changes of colour from green to red, occurring during the ripening process of tomato. Vega-Espinoza (2010) reported higher a* values in tomato fruits cv. Imperial subjected to HWT in comparison to fruits without HWT stored for 2 w at 12°C. Nevertheless, no significant differences in colour parameters were observed in the present work between HWT and non-HWT fruits during storage at 12°C. On the other hand, the less red colour evidenced by the lower a* and higher °Hue values observed in fruits stored at 5°C as compared to 12°C, may be due to the slower rates of the ripening processes, such as the degradation of chlorophyll and synthesis of lycopene, as a consequence of tomato fruits' low temperature exposition as reported by López-Espinoza (2008) and Yahia *et al.* (2007). Soto-Zamora *et al.* (2005) indicate that 4°C storage of tomato fruits cv. Rhapsody caused incomplete degradation of chlorophyll, and at the same time, a minimal synthesis of lycopene showing values from 105 to 85 for °Hue, meaning in a colour change from yellow-green to yellow. A better colour development from green (a* = -5.87, °Hue = 103.76) to red (a* = 17.24, °Hue = 48.41) was observed in the HWT tomato fruits stored at 5°C as compared to non-HWT, which is in concordance to the reduction of CI visual symptoms, such as uneven ripening. Yang *et al.* (2009) reported higher a* value in tomato fruits subjected to HWT and stored for 19 d at 5°C, and at the same time, a higher conversion of chloroplasts into chromoplasts was observed in the HWT tomato fruits, unlike the untreated ones.

Based on several studies, appearance of CI symptoms in tomato fruits occurs from 5 to 15 d of

cold storage, depending on temperature, which becomes more severe as the storage time progresses (Ding *et al.* 2001; Zhao *et al.* 2009; Vega-García *et al.,* 2010). In concordance to those results, in the present work, for non-HWT fruits, we observed CI symptoms after 10 d of storage at 5°C with a corresponding CI-index of 0.55, reaching values of 1.5 times higher after 15 d of cold storage. Nevertheless, the application of HWT to the fruits prior to cold storage provided tolerance to CI, resulting in lower CI index values than those of non-HWT fruits. Our results are in agreement with several studies (Yang *et al.,* 2009; Luengwilai *et al.,* 2012; Cruz-Mendívil *et al.,* 2015) which reported the effectiveness of different combinations of temperature and time of HWT to decrease CI symptoms in tomato fruits. Although our results showed a reduction in the severity of CI symptoms in fruits stored at 5°C, it is important to note that this does not imply a total elimination of them. Therefore, it can be inferred that HWT could help to reduce the losses caused by CI symptoms and the economic impact by lowering the number of tomato fruits that cannot be commercialised.

Although EL is a marker that indirectly reflects the membrane damages caused by several factors such as CI (Zhao *et al.,* 2009), it has been suggested that it has a limited application since is not a reliable measure of membrane damages at non-chilling conditions (Côté *et al.,* 1993). In the present work, tomatoes stored at 12°C (non-CI temperature) obtained the highest EL values (Figure 4A) which may be associated to a membrane permeability increase related to a normal ripening process of fruits, that could be caused by modifications in membrane lipids composition, and not by a CI development (Malacrida *et al.,* 2006). Based on EL results of non-HWT fruits stored at 5°C, it may be inferred that significant damage to the membranes occurred between 5 and 10 d at low temperature; hence, it is possible that within this lapse occurred the major events that caused CI. On the other hand, the HWT induced decreases in EL levels during cold storage, suggesting that protection provided to CI could be due, in part, to preventing damage to the membranes.

The importance of membrane damage on CI development was stated by Yang *et al.* (2009) who reported that tomato fruits with HWT showed less damage in organelles' membranes of pericarp tissues such as endoplasmic reticulum, vacuoles, and mitochondria. Previous reports have shown that HWT reduced the EL of tomato fruits cv. Micro-Tom during cold storage (Luengwilai *et al.,* 2012; Cruz-Mendívil *et al.,* 2015). Also, lower levels of EL have been observed in tomato fruits from a CI-tolerant cultivar (Santiam) as compared to a sensitive cultivar (Lichun) (Zhao *et al.,* 2009). The loss of membrane function (i.e., loss of permeability or enzymatic activity) has been proposed as one of the primary events that lead to CI (Lyons, 1973). Therefore, it appears that the protection provided to the membranes (evidenced by a lower EL) plays an important role in the acquisition of CI tolerance in tomato with HWT.

Storage at 12°C is safe to avoid CI in tomato fruits; therefore, fruits stored under these conditions showed lower MDA values than those stored at 5°C (Figure 4B). In the case of fruits stored at 5°C, the lower MDA content found in HWT with respect to non-HWT suggests that the treatment prevented, to some extent, the lipid peroxidation. Furthermore, the MDA content of HWT fruits stored at 5°C did not significantly increase with respect to fruits stored at safe temperature (12°C). Lipid membranes are highly susceptible to peroxidation caused by abiotic stress, such as low temperature, and it can be evaluated by measuring the content of MDA, one of the final products of the oxidation process of unsaturated fatty acids. The extent of lipid peroxidation depends on the level of stress suffered by the fruits, and the time of exposure (Pongprasert *et al.,* 2011).

Since the integrity and survival of membranes are fundamental for the cell and many organelles (including mitochondria, plasmatic membranes, endoplasmic reticulum, and chloroplasts), the damage caused by lipid peroxidation is highly deteriorative on their functions when the tissues are subjected to conditions causing CI (Devasagayam *et al.,* 2003). In this sense, reduced lipid peroxidation has been previously related to CI tolerance; for instance, Zhao *et al.* (2009) reported a lower MDA content in CI-tolerant cultivar (Santiam) as compared to a non-tolerant-CI cultivar, during low temperature storage (2°C). Therefore, the deterioration of the membranes results in loss of selective permeability (Zhang and Tian, 2010). This may also explain the EL results, where non-HWT fruits stored at 5°C resulted in higher membrane damage as compared to fruits with HWT.

Tomato fruits stored at 5°C showed higher levels of SOD activity as compared to those stored at safe temperature (12°C), which could be attributed to an increase in oxidative stress and ROS production caused by CI. Since SOD is part of the antioxidant enzyme system, and catalyses the conversion of superoxide radicals, producing hydrogen peroxide and molecular oxygen, higher SOD activity observed

in HWT fruits stored at 5°C may be an important role in ROS detoxification, thus providing protection against oxidative stress. In this sense, Zhang *et al.* (2013) reported that SOD activity was increased in tomato fruits cv. Messina as the cold storage progressed, showing higher values of SOD activity in fruits pre-treated with hot air. Since chilling increases ROS accumulation in plants, the ability to scavenge ROS during and after chilling determines the resistance and adaptation to low temperature (Ding *et al.,* 2015). Thus, an increase in SOD activity may have reduced the potential damage to the membranes caused by ROS.

The increase in CAT activity observed in HWT fruits stored at 5°C could be related to its role in H_2O_2 decomposition in order to attenuate the possible oxidative stress suffered by tomato tissue, since CAT works in conjunction with SOD by removing the H_2O_2 produced by the latter, catalysing the decomposition reaction of H_2O_2 into water and molecular oxygen. Consequently, a higher CAT activity may have contributed to CI-symptoms reduction in fruits subjected to HWT. Similarly, Zhao *et al.* (2009) reported a higher CAT activity in a CI-tolerant tomato cultivar (Santiam). Furthermore, Zhang *et al.* (2013) reported CAT activation in tomato cv. Messina subjected to hot air treatment and cold storage.

Our results showed that APX activity increased over the 15 d of storage at 5°C, which coincided to the behaviour of the physiological markers related to membrane damage (lower EL and lipid peroxidation). Considering that the elimination of H_2O_2 by APX plays an important role in ROS detoxification (Mittler, 2002), higher APX activity values in fruits with HWT throughout the 5°C storage may have influenced the acquisition of CI tolerance, as part of a strategy of the tissues to counteract the oxidative stress produced.

Although it has been reported that SOD and CAT work together to maintain the redox balance in plant tissues (Mittler, 2002), our results showed high concordance between SOD and APX activities, which could be due to the existing relationship between their catalytic functions involved in ROS elimination. Despite the H_2O_2 produced by SOD reaction can be used as a substrate for CAT and APX, our results suggest a greater participation of APX. It is likely that HWT conferred preconditioning to stress in tomato tissue, which addressed to activation of the antioxidant enzymatic system to counteract the overproduction of ROS during the storage at 5°C. Due to the fact that ROS are responsible for membrane damage via lipid peroxidation, the acquired CI tolerance of tomato fruits attributed to HWT might be related to their increased capacity to remove those harmful species.

Conclusion

Application of HWT in tomato cv. Imperial prior to 5°C storage resulted in lower CI index and better colour parameters at the end of the ripening period (15 d at 5°C and 8 d at 21°C). The results of CI index, EL, and MDA content suggest that CI initiation occurs after 5 d of storage at 5°C. The protective effect of HWT during 5°C storage was evidenced by reduction in membrane damages and lipid peroxidation in addition to increases in SOD, CAT, and APX activities, which were associated with a greater tolerance to CI. It is likely that the coordinated action of the antioxidant enzymes in scavenging ROS helped to reduce the cell oxidative damage and CI. The results of the present work suggest that HWT increases the tolerance to CI in tomato cv. Imperial, perhaps by the activation of antioxidant enzymes, thereby protecting the cell membrane against oxidative stress.

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