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ORIGINAL ARTICLE

Antioxidant enzymatic changes in bell pepper fruit associated with chilling injury tolerance induced by hot water

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Abstract

Green bell pepper is highly susceptible to low temperature. The activation of the enzymatic antioxidant system plays a determining role in tolerance to chilling injury (CI). Immersion in hot water for short time previous to storage at low temperature induces tolerance to this disorder. However, there is a lack of information about the induction of chilling tolerance in bell pepper by hot water and its relationship with the enzymatic antioxidant system. We evaluated the effect of three immersion times (T, 1-, 2-, 3-min) in hot water (HW, 53°C) on the reduction of CI in bell pepper and its relationship with the enzymatic antioxidant system during storage at 5°C and 21°C. The use of hot water for 1-, 2- or 3-min reduced the decay and CI indexes, maintained quality parameters, ascorbic acid, and total phenolics content. The storage at 5°C by itself activated the enzymatic antioxidant system. The use of HWT 1-, 2-, and 3-min helped to increase this effect, especially by HWT_2.

Practical applications

The application of a treatment with hot water for short times in fruit sensitive to chilling injury is undoubtedly a viable alternative to increase their tolerance and commercialization. In this study, the application of a hot water treatment for 1-, 2- or 3-min in bell pepper reduced the deterioration and susceptibility to chilling injury and stimulated the enzymatic antioxidant system. In this sense, agricultural producers can take advantage of this treatment to prolong the storage period of the fruit maintaining its quality and improving its commercialization.

KEYWORDS

bell pepper, chilling injury, enzymatic antioxidant system, hot water, quality

1 | INTRODUCTION

Green bell pepper (*Capsicum annuum* L.) is one of the most important fruit in Mexico both in production and marketing, especially for exportation. Bell pepper fruit is a remarkable source of vitamin C and phenolic compounds (Raffo et al., 2007). However, it is highly perishable due to accelerated dehydration and susceptibility to fungi decay, mainly *Botrytis cinerea* and *Alternaria alternata* (González-Aguilar et al., 1998). The implementation of optimal storage conditions (temperature and relative humidity) protects the fruit from decay and preserves its bioactive compounds. However, when bell pepper is stored at temperatures below 7°C, chilling injury (CI) and

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oxidative stress are triggered, accelerating the fruit decay. Chilling tolerance in horticultural crops allows a reduction of deterioration, a lower respiration rate, greater retention of quality and bioactive compounds and therefore a longer shelf life. Previous studies have shown that membrane damage is the key event producing a cascade of biochemical reactions that culminate in an overproduction of reactive oxygen species (ROS), causing tissue deterioration and loss of membrane integrity (higher electrolyte leaching; Maalekuu et al., 2006; Sánchez-Bel et al., 2012). Among the visual symptoms of CI in bell pepper are superficial pitting, seed browning, pigment degradation, wilting by water loss, and depression in the pericarp that progresses to scald in advanced stages of pathophysiology (Sánchez-Bel et al., 2012). This results in a significant degradation of quality and bioactive compounds that limit its commercialization and health benefits (Wang et al., 2012).

Moderate heat stress can induce chilling tolerance in different fruit (Endo et al., 2019; Ma et al., 2014; Sala & Lafuente, 2000; Zhu et al., 2003). This treatment generates an adaptive response to cold stress, and reduces CI susceptibility and skin damage during cold storage. Heat treatment can activate a system of elimination of ROS that involves antioxidants such as ascorbic acid, phenolics and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (Endo et al., 2019; López-López et al., 2018; Sala & Lafuente, 2000; Sapitnitskaya et al., 2006). The implementation of treatments such as hot water (Fallik et al., 1999; López-Velázquez et al., 2020; Raffo et al., 2007), have been used to reduce CI susceptibility in bell pepper.

The application of HWT for a short period of time (55°C for 1 min; 53°C for 4 min; 45°C for 3 min, 53°C for 2 min) have been successfully used to prevent deterioration of bell pepper, reducing fruit dehydration and maintaining its marketability (Endo et al., 2019; Fallik et al., 1999; González-Aguilar et al., 2000; Rodoni et al., 2016). In addition, this treatment reduced decay caused by *Botrytis cinerea* and *Alternaria alternata* (Fallik et al., 1999; López-Velázquez et al., 2020; Raffo et al., 2007). However, for this to occur, the exposure time must be taken care of, since in some cases HWT for short times can cause little CI tolerance, heat damage, and decay of the fruit.

Nevertheless, there is a lack of information about the effect of different immersion times in hot water on the activation of the enzymatic antioxidant system and CI tolerance in bell pepper. In this way, the aim of this work was to determine the effect of three immersion times in HWT on the induction of CI tolerance, quality parameters and activation of the antioxidant system in bell pepper during storage at 5°C (CI temperature).

2 | MATERIAL AND METHODS

2.1 | Treatments application

Green bell pepper fruit were obtained from a local producer (Agricola Chaparral, Culiacan, Sinaloa, Mexico) and selected based on commercial color ripening (luminosity of 35–37; parameter a^* −11 to −10), size uniformity and absence of defects. The fruit were washed and disinfected with sodium hypochlorite (4 mM) and randomly divided into five groups; each group consisted of 27 fruits (nine fruits for each day of evaluation; each parameter was evaluated in duplicate in each fruit). Three lots received the HWT at 53°C as follows: HWT_1 (fruit immersed for 1 min), HWT_2 (fruit immersed for 2 min), HWT_3 (fruit immersed for 3 min) according to López-Velázquez et al. (2020). Then they were stored at 5°C. The remaining two lots did not receive any treatment and were considered controls; one was stored at 5°C (chilling temperature) and the other at 12°C (simulated commercial storage). All treatments were stored for 21 days. After storage at low temperature, fruits were transferred at 21°C for 7 days (day 21 + 7). After fresh analysis (decay index, CII (%), %WL, %EL, quality parameters, and AsA), the tissue was collected, lyophilized, pulverized, sieved, and stored at −70°C until its subsequent use (MDA, TP, and SOD, CAT, and POD).

2.2 | Decay and chilling injury index

Decay index was determined according to González-Aguilar et al. (2000) with some modifications. This index is based on the surface area with a visible microorganism growth. Grade levels were classified as follows: 1, fruit no decay; 2, less than 25% of fruit surface presented decay; 3, 25%–50% of fruit surface presented decay; 4, 51%–75% of fruit surface presented decay; 5, 76%–100% of fruit surface presented decay. Nine fruit were analyzed per treatment. Decay index was calculated using the following formula:

$$\text{Decay index} = \frac{\sum (\text{grade of decay} \times \text{number of fruit at this level})}{\text{total fruit number}}$$

CI index (%) was visually calculated for each treatment. The fruit was divided in calyx and four equatorial sides (each section represented the 20% of surface fruit). The CI symptoms evaluated were wilting, irregular ripening, superficial pitting, and calyx deterioration. Nine fruits were used per treatment. The percentage of CII was calculated following the formula:

$$\text{CII}(\%) = \frac{\sum (\% \text{ of CI index of each fruit} \times \text{number of fruit at this level})}{\text{total number fruit}}$$

2.3 | Electrolyte leakage and malondialdehyde accumulation

Membrane damage is measured by the percentage of %EL and MDA content. For %EL, bell pepper fruit was cut in cylinders with 7 mm of diameter and 10 mm of height using a stainless-steel corkscrew, obtaining 10 pieces per fruit (Malacrida et al., 2006). The electrolytes leaked by cut were removed with deionized water (three times). 25 ml of 0.4 M mannitol solution were added and

kept in incubation during 2 hr at 25°C under constant shaking. Manual conductivity meter (Hanna Instruments, Model HI98311, Johannesburg, South Africa) was used to measure conductivity in samples (two per fruit). Two measurements were made at 25°C, before and after autoclaving at 121°C for 10 min. %EL was calculated with the following formula:

$$\%\,\mathrm{EL} = \frac{\text{initial electrolytes}}{\text{total electrolytes}} \times 100.$$

MDA content was determined according to Hodges et al. (1999). Two solutions were prepared; solution A contained 20% trichloroacetic acid (TCA), and 0.01% butylated hydroxytoluene (BHT) and solution B (+thiobarbituric acid (+TBA) contained 20% TCA, 0.01% BHT, and 0.65% (TBA). In each solution, one milliliter of supernatant (1 g of frozen tissue, 30 ml ethanol:water (80:20 v/v)) was collocated and centrifuged at 3,000× *g* at 4°C × 10 min. The solutions A and B mixed with the supernatant were shaken, heated (95°C, 25 min), and centrifuged (3,000× *g*, 10 min). All samples were read at three wavelengths (440, 532, and 600 nm) using a spectrophotometer (Unico SQ2800; Unico Inc., San Diego, CA).

MDA equivalents were calculated using three different formulas and were expressed as nmol/g FW:

$$A = \left[\left(\mathrm{Abs}_{532} + \mathrm{TBA}\right) - \left(\mathrm{Abs}_{600} + \mathrm{TBA}\right) - \left(\mathrm{Abs}_{532} - \mathrm{TBA} - \mathrm{Abs}_{600} - \mathrm{TBA}\right)\right].$$

$$B = \left[\left(\mathrm{Abs}_{400} + \mathrm{TBA} - \mathrm{Abs}_{600} + \mathrm{TBA}\right) \times 0.0571\right].$$

$$\text{MDA equivalents} = \left(A - \frac{B}{157.000}\right) \times 10^6$$

2.4 | Weight loss (%)

Nine fruits per treatment were evaluated for weight loss every 3 days during storage at low temperature. Weight loss was determined and expressed as percentage of weight loss (%WL) using the following formula:

$$\%\,\mathrm{WL} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100$$

2.5 | Determination of fruit quality: color and firmness

Fruit color was evaluated with Minolta colorimeter (CR200, Osaka, Japan) using the CIE LAB system, which measures L^* that indicates luminosity (100 indicates white and 0 indicates black), a^* value (+*a* indicates red and –a indicates green), b^* value (+*b* indicates yellow and -b indicates blue), and Hue angle (tan/1 (b^*/a^*); López-López et al., 2013). Three equidistant points were taken of each fruit and nine fruits per treatment.

Firmness fruit was measured using a digital penetrometer (Chatillon DFE100, AMETEK Inc, Largo, FL) with a 11-mm plunger at speed of 50 mm/m and 5 mm penetration. Three equidistant points were obtained per fruit. The results were expressed in newtons (López-López et al., 2013).

2.6 | Determination of antioxidant compounds: ascorbic acid and total phenolics

AsA concentration was determined according to López-Valenzuela et al. (2011) with some modifications. 0.1 g of fresh tissue was homogenized with 15 ml of cold deionized water for 1 min with Ultra-Turrax T18 basic (IKA, Werke, Germany). The homogenized was filtered with organza cloth and 0.45-μm disposable filters (Pall Corp., Port Washington, NY, USA) followed by SEP-PACK C18 cartridges (Waters Corp., Milford, MA, USA), the filtering was collocated in an amber tube. One-ml aliquot was analyzed using a HPLC system (Agilent, Waldbronn, Germany) equipped with a Sphereclone ODS2 column (250 mm × 4.6 mm × 5 μm, Phenomenex, USA) at 16°C. Monobasic potassium phosphate (25 mmol/L) was used like mobile phase (flow 0.7 ml/min). The absorbance was determined at 254 nm during 10 min with an injection of 10 μl. The AsA concentration was determined using a standard curve of AsA (Sigma-Aldrich Co., St Louis, Mo., USA) and the results were expressed as mg AsA/100 g fresh weight (FW).

TPs were determined as reported by Adom and Liu (2005). Methanolic extract (ME) was prepared with 1 g of lyophilized tissue and 5 ml methanol (homogenized for 1 min using a vortex), the mixture was sonicated for 30 min at 25°C, the supernatant was recovered, addition of methanol, and sonication were repeated three times. Supernatant recuperated was filtered through a Whatman No. 1 filter, concentrated under vacuum, and stored at −20°C in darkness. In microplate, 40 μl of ME diluted (1:8 w/v) were mixed with Folin & Ciocalteu's phenol reagent 2 N (1:8 v/v, F9252-100 ml-Sigma Aldrich) and 100 ml Na_2CO_3 (7% w/v). All samples were incubated for 90 min at 25°C. The absorbance was read at 750 nm. A standard curve was prepared using gallic acid (GA), and the results were expressed as mg GAE (GA equivalents)/100 g FW.

2.7 | Antioxidant enzymatic activity

SOD was determined according to Liu et al. (2005) with some modifications. 0.5 g of lyophilized tissue were homogenized with 5 ml of ice-cold 0.05 M phosphate buffer (pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA) + 5% of polyvinylpyrrolidone). The mixture was centrifuged (17,200× *g*, 20 min at 4°C), and the supernatant was collected as enzymatic extract. The reaction mixture consisted of 1.5 μl of enzymatic extract, and 198.5 μl of 0.1 M phosphate buffer (pH 7.8) containing 0.01 M methionine, 0.025% Triton X-100, 0.11 mM EDTA, 57 mM nitro blue tetrazolium chloride (NBT), and 50 μl of 20 mM riboflavin. The microplates were exposed at 25 w for 10 min. The reaction was read at 550 nm before and after light exposition. One unit of SOD activity is the amount of enzyme that

would inhibit 50% of the photoreduction of NBT. SOD activity was expressed in U SOD/ mg of protein.

CAT was determined according to Yimyong et al. (2011). For enzymatic extract, 0.5 g of lyophilized bell pepper were homogenized with 5 ml of 45 mM phosphate buffer (pH 7.0) containing 5 mM of dithiothreitol, 5mM EDTA, and 0.125 g of polyvinylpoly pyrrolidone. The mixture was centrifuged (17,200× *g*, 30 min at 4°C), and the supernatant was used as enzymatic extract. The reaction mixture contained 1 ml of phosphate buffer (40 mM, pH 7.0) containing hydrogen peroxide (H_2O_2; 40 mM), and 25 µl of enzymatic extract. The enzymatic activity was monitored by the disappearance of H_2O_2 for 5 min at 240 nm. The changes in H_2O_2 concentration were calculated based on its extinction coefficient (43.6 M/cm). CAT activity was expressed in U/mg of protein.

POD was determined according to Lin et al. (2011) with some modifications. CAT extract was used for this enzyme. The reaction mixture contained 2.75 ml of 0.1 M phosphate buffer (pH 7.8), 100 µl of 1% guaiacol (Sigma Aldrich), 100 µl of 0.46% H_2O_2 (Sigma Aldrich), and 50 µl of enzymatic extract. The POD activity was read at 470 nm for 5 min. One POD unit was defined as the increase in absorbance of 0.01/min and was expressed in U/mg of protein. Protein content in all enzymes was measured according to Bradford (1976) method using serum albumin for the standard curve.

2.8 | 8 Statistical analysis

The effect of hot water immersion time and storage days were evaluated using analysis of variance (ANOVA). Differences between means were tested using the least significant differences (LSDs) by Fisher's test ($\alpha = 0.05$). The statistical analyses were carried out in STATGRAPHICS 5.1 software (Statistical Graphics, Rockville, MD). The data were showed as the means ± standard deviation (*SD*) of determinations made for each sample.

3 | RESULTS

3.1 | Decay and chilling injury index

The application of HWT reduced decay index in bell pepper during the storage at 5°C for 21 days and during shelf life at 21°C (21 + 7 days; Figure 1a). Control 5°C fruit showed the highest decay index throughout the storage at low temperature and 21°C with a value of 3.00 (50% of fruit surface damaged) and 4.33 (more than 75% of fruit surface damaged), respectively. The use of HWT for 1-, 2- or 3-min was effective to reduce decay index in bell pepper during cold storage (21 days) and 21 + 7 days at 21°C, without significant differences among them. Control 12°C fruit showed more serious decay index after 21 days than control 5°C fruit; meanwhile at 21°C, both treatments presented similar decay values ($p > .05$). In the case of CI index, the presence of symptoms like pitting, necrotic areas, and discoloration of the calyx was reduced at least 20% in all HWT-treated fruits compared with control 5°C fruit. CI index was between 31% and 36% for HWT fruit, whereas for control fruit it was 57% (Figure 1b). The three HWT immersion times were enough to reduce the CI index in bell pepper even at 21°C where the symptoms intensified. As it was expected, the fruit stored at 12°C did not present CI symptoms.

3.2 | Electrolyte leakage and malondialdehyde content

The cell membrane permeability increased throughout storage in all treatments (Figure 2a). At the beginning, the fruit treated with hot water (1-, 2-,3-min) presented lower %EL (19.28%, in average) than control (22.75%) but on day 21 at 5°C, HWT_1 maintained the lowest %EL (21.14%) and had significant difference with the other treatments; meanwhile, treatment HWT_3 showed the highest values (30.49%). During storage at 21°C, HWT_1 and HWT_3 showed less leakage than control 5°C and HWT_2; also in addition, these treatments were statistically similar to control 12°C, which remained without significant changes throughout the storage.

As shown in Figure 2b, MDA content increased after 21 days of storage at 5°C in all treatments, the MDA content in these treatments was statistically similar to control 12°C. After 7 days at 21°C, the increase in the MDA content was not significant for all treatments, HWT_3 maintained lower MDA content than control and similar to HWT_1 and HWT_2. For control 12°C, the MDA content was significantly lower than control 5°C, but similar to HWT at 1-, 2- or 3-min.

3.3 | Weight loss (%)

WL is related with loss of quality and deterioration of fruit specially during cold storage (Shi et al., 2020). The percentage of WL showed gradual upward during cold storage (Figure 3). HWT_3 showed lower WL than control 5°C and HWT_1, especially after 12 days. During storage at 21°C, all treatments maintained similar behavior. Fruit immersed during 2- or 3-min in hot water reduced the WL during cold storage and 21°C. The storage at 12°C showed lower dehydration in bell pepper compared with HWT stored at 5°C.

3.4 | Fruit quality parameters

In this study, external color and firmness were used to measure the fruit quality. Luminosity was affected neither by the storage time nor by the application of the treatments (Table 1), in Hue angle a slightly change was observed in HWT_3 regarding storage time while a^* values presented higher changes in HWT for the three times compared with control 5°C. Firmness values were significantly reduced throughout storage. The use of hot water for short time (1-, 2-, or 3-min) maintained the firmness of fruit during cold storage compared with control treatments stored at both 5 and 12°C (Table 1).

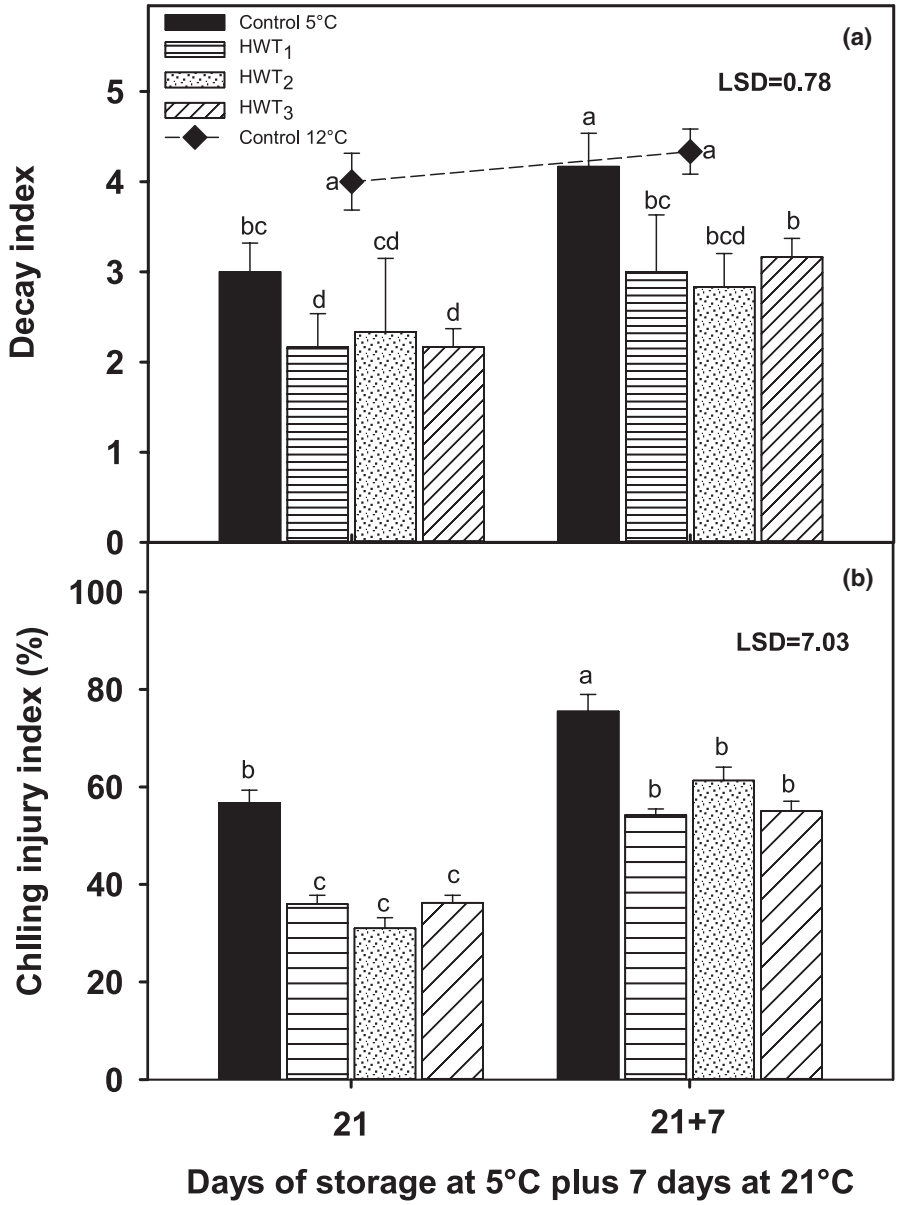

FIGURE 1 Decay index (a) and chilling injury index (b) in bell pepper after 21 days of storage at 5°C plus 7 days at room temperature (21°C). Treatments: control (nontreated; 5 and 12°C), HWT_1 = 53°C, 1 min; HWT_2 = 53°C, 2 min; HWT_3 = 53°C, 3 min. Each data point represents the mean ± *SD* (n = 9). Values with the same letters are not statistically different (least significant difference [LSD] at $p \leq .05$). Vertical bars represent the standard error of the mean

HWT_1 and HWT_3 showed lower firmness loss (24.65% and 20.16%, respectively) than HWT_2 (32.48%) and control 5°C (31.92%) when were transferred at 21°C, whereas control 12°C presented the highest firmness value (99.60 N) with a reduction only of 12.60% and a significant difference with the other treatments.

3.5 | Bioactive compounds (AsA and TPs)

The use of HWT in bell pepper reduced the AsA content on the initial day compared with both controls (Figure 4a). At day 21 (5°C), HWT_1 showed an increase of 40.93% in the AsA content; meanwhile, HWT_2 significantly reduced (13.58%) the AsA content, and HWT_3 remained constant. Control fruit stored at 12°C showed an increase of 7.11%, whereas, control at 5°C showed a decrease. When fruits were transferred to 21°C, the storage caused a significant loss of AsA on fruit treated with HWT_1 and controls at 5 and 12°C, meanwhile, on HWT_2 and HWT_3 treatments there was an increase of AsA in 19.81 and 8.48%, respectively. HWT_3 maintained an AsA content similar to control 12°C, without presenting significant difference between them.

At the beginning of the storage, bell pepper immersed in HWT showed less TP content than control (Figure 4b). After 21 days of storage, only fruit treated with HWT (1-, 2-, and 3-min) presented a significant increase in TP (33.56%, 25.33%, and 34.19%, respectively). The storage of bell pepper at 21°C did not cause significant changes in TP regardless of the treatment applied.

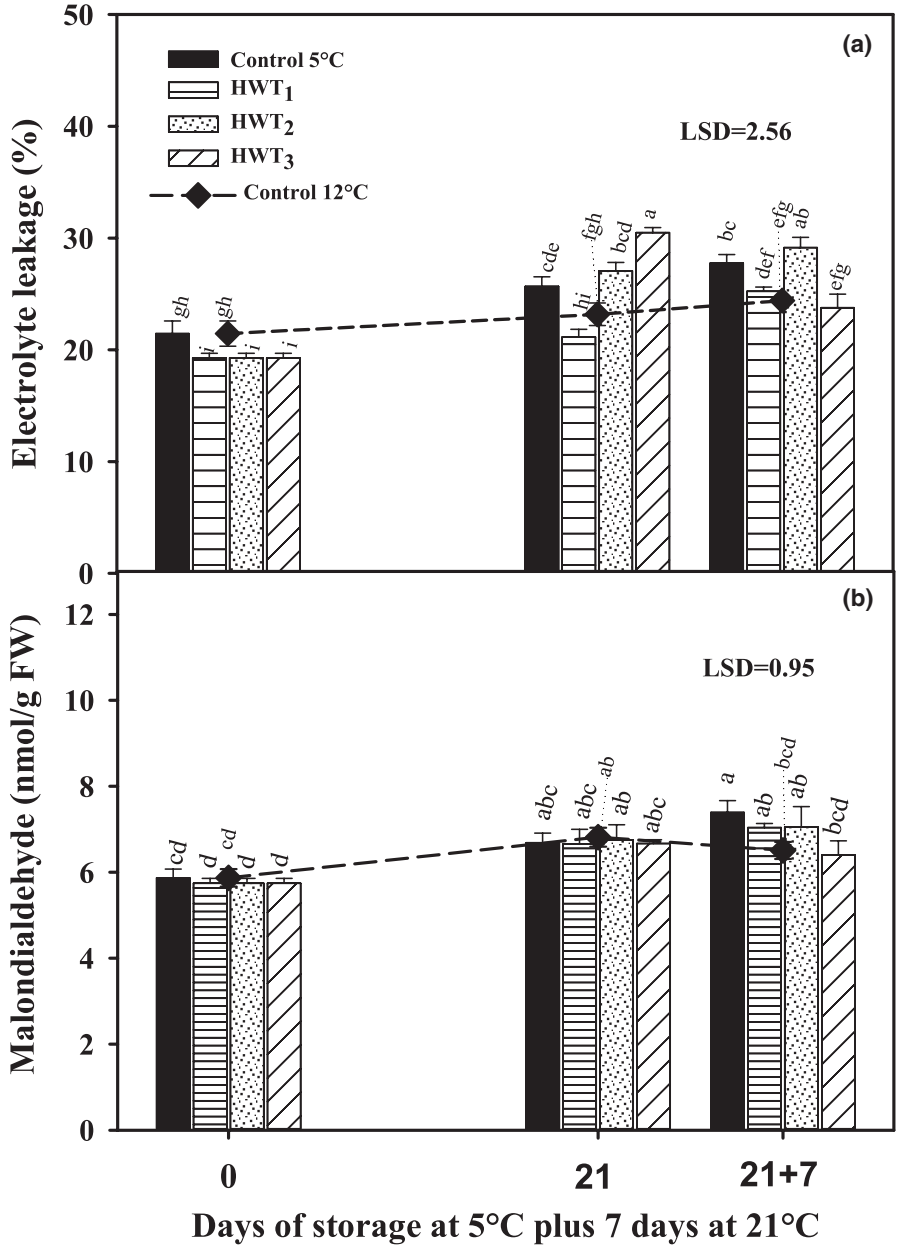

FIGURE 2 Effect of dipping time in hot water on electrolyte leakage (a) and malondialdehyde content (b) in bell pepper fruit during storage at 5°C plus 7 days at 21°C. Treatments: control 12°C (dashed lines), control 5°C (nontreated), HWT_1 = 53°C, 1 min; HWT_2 = 53°C, 2 min; HWT_3 = 53°C, 3 min. Each data point represents the mean ± *SD* (n = 15). Values with the same letter are not statistically different (least significant difference [LSD] at $p \leq .05$). Vertical bars represent the standard error of the mean

3.6 | Antioxidant enzyme activity

The SOD activity showed an increase after 21 days at 5°C only for the fruit dipped in hot water for 1-min (1.12 U/mg protein) or 2-min (1.24 U/mg protein), showing both treatments higher SOD activity than the other treatments ($p \leq .05$; Figure 5a). At day 21 + 7, the SOD activity increased for all treatments. HWT (1-, 2-, and 3-min) and control 12°C (1.11 U/mg protein) showed the highest and the lowest SOD activity, respectively.

In CAT activity, treatments and days of storage significantly affected this parameter (Figure 5b). The use of HWT increased CAT activity on the initial day compared with both controls. After 21 days at 5°C, only HWT_2 (0.67 U/mg protein) maintained the CAT activity, whereas HWT_1, HWT_3, and control 12°C showed a reduction. When fruits were transferred to 21°C, CAT activity increased for all treatments, except for control 12°C. HWT_2 maintained higher activity than HWT_1 and HWT_3. Control treatments previously stored at 5°C and 12°C showed the highest and lowest activity (1.71 and 0.10 U/mg protein), respectively.

The activity of POD was higher for HWT_2 (12.16 U/mg protein) after 21 days at 5°C (Figure 5c), whereas HWT_3 and control 12°C did not present significant changes. During storage at 21°C,

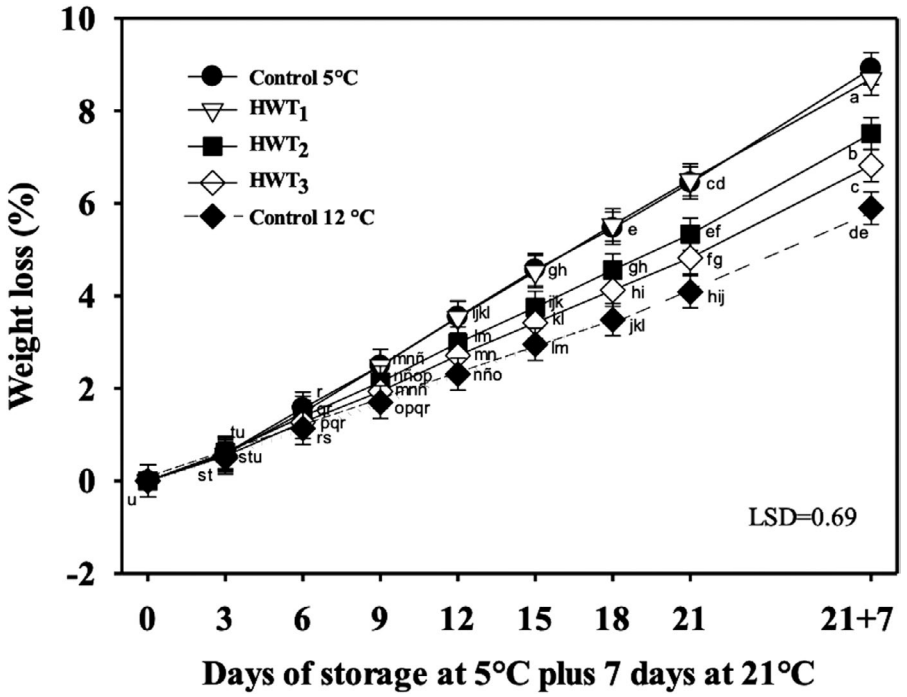

FIGURE 3 Effect of dipping time in hot water on weight loss of bell pepper fruit during storage at 5°C plus 7 days at 21°C. Treatments: control 12°C (non-treated, dashed lines), control 5°C (non-treated), HWT_1 = 53°C, 1 min; HWT_2 = 53°C, 2 min; HWT_3 = 53°C, 3 min. Each data point represents the mean ± *SD* (*n* = 9). Values with same letter are not statistically different (least significant difference [LSD] at $p \leq .05$). Vertical bars represent the standard error of the mean

all fruit presented a significant increase in POD activity except the control 12°C, which presented a reduction. HWT_2 (38.97 U/mg protein) maintained the highest activity followed by HWT_3 and HWT_1 ($p \leq .05$). Complete results of antioxidant enzymes activity showed that HWT_2 was more effective to increase the activity of SOD, CAT, and POD during cold storage and in the 21°C period.

4 | DISCUSSION

CI is a major physiological disorder that reduces the quality of different fruit and vegetables including bell pepper (Wang et al., 2012). The storage of bell pepper at low temperature and relative humidity reduces its decay index. In the present study, it was found that hot water treatment with three different times of immersion, successfully reduced the degree of deterioration and CI symptoms, promoting the inhibition of microbial growth and CI tolerance in bell pepper during storage at low temperature (Figure 1). Different treatments (physical or chemical) in bell pepper seeks to reduce CI susceptibility and promote the use of low temperatures to increase the shelf life of this fruit (González-Aguilar et al., 2000; Wang et al., 2012). The use of hot water with a temperature above 50°C for a short time weakens or kills spores and limits their development (Fallik et al., 1999). Moreover, HWT causes moderate stress in the fruit activating the antioxidant system and causing tolerance to low temperatures (López-López et al., 2018). Similar with our study, López-Velázquez et al. (2020) reported that the use of hot water at 53°C for 1 min was effective to reduce the development of fungal decay (*Botrytis cineria*) and CI symptoms in bell pepper during storage at 5°C. In addition, previous studies have shown that HWT for short or long time in mandarin (Ghasemnezhad et al., 2008; Sala & Lafuente, 2000), banana (Wang et al., 2012), green and red bell peppers (Rodoni et al., 2016), cucumber (Nasef, 2018), and mature green mume (Endo et al., 2019) changes the fruit response to CI, protecting it from cold stress.

The MDA content is the result of the lipid peroxidation, which increases during CI, and provokes a severe oxidative stress and a premature senescence. Therefore, the CI symptoms are related to membrane deterioration (lipid peroxidation and production of MDA), cellular breakdown (increase in electrolyte leakage) as well as the loss of epicuticular wax, increasing water loss, and promoting tissue wilting, pitting, and decay (Lim et al., 2007). In this first response to CI, the use of HWT for 1 min allowed lower electrolyte leakage than the other immersion times (2- and 3-min), but similar MDA production with control fruit during cold storage. Rodoni et al. (2016) related this behavior to the fact that the tissue or metabolic activity is not altered with 1 min in hydrothermal treatment. Whereby, the integrity of the tissue is improved or maintained, reducing susceptibility to deterioration. When fruit were transferred to 21°C the CI symptoms increased, and with it, the lipid peroxidation, loss of membrane integrity, and weight loss. However, fruit immersion in hot water for 3-min was enough to reduce %EL and MDA production, maintaining less damage in membrane during shelf life. This behavior should

TABLE 1 Effect of different immersion times in hot water on color and firmness quality parameters during storage of bell pepper at chilling temperature

Treatments	Days of storage at 5°C plus days at 21°C		
	0	21	21 + 7
Luminosity			
Control 12°C	35.93 ± 0.81^{aA}	36.64 ± 1.83^{aA}	35.72 ± 0.62^{aA}
Control 5°C	35.93 ± 0.81^{aA}	35.13 ± 0.21^{aA}	35.52 ± 0.43^{aA}
HWT_1	36.76 ± 1.68^{aA}	35.79 ± 0.45^{aA}	35.60 ± 0.55^{aA}
HWT_2	36.81 ± 0.68^{aA}	35.28 ± 1.01^{aA}	36.24 ± 1.33^{aA}
HWT_3	36.22 ± 0.46^{aA}	35.66 ± 3.53^{aA}	35.99 ± 0.11^{aA}
a^* value			
Control 12°C	-10.90 ± 0.81^{aA}	-10.00 ± 1.29^{aA}	-9.68 ± 0.59^{bA}
Control 5°C	-10.90 ± 0.81^{aB}	-9.92 ± 0.47^{aAB}	-9.08 ± 0.45^{abA}
HWT_1	-11.49 ± 1.50^{aB}	-9.58 ± 1.18^{aA}	-8.65 ± 0.70^{abA}
HWT_2	-11.33 ± 0.42^{aC}	-9.59 ± 0.49^{aB}	-8.18 ± 0.95^{aA}
HWT_3	$-11–12 \pm 0.11^{aB}$	-9.14 ± 0.81^{aA}	-7.96 ± 0.20^{aA}
Hue angle			
Control 12°C	130.66 ± 1.00^{aA}	129.77 ± 1.14^{aA}	131.21 ± 1.49^{aA}
Control 5°C	130.66 ± 1.00^{aA}	130.62 ± 0.47^{aA}	130.96 ± 0.52^{aA}
HWT_1	131.08 ± 1.36^{aA}	129.82 ± 0.75^{aA}	131.05 ± 0.45^{aA}
HWT_2	131.04 ± 1.13^{aA}	130.27 ± 1.47^{aA}	130.34 ± 1.40^{aA}
HWT_3	131.40 ± 0.57^{aA}	129.67 ± 0.51^{aB}	130.75 ± 0.51^{aAB}
Firmness			
Control 12°C	113.97 ± 2.97^{aA}	98.94 ± 2.09^{cB}	99.6 ± 5.08^{aB}
Control 5°C	113.97 ± 2.97^{aA}	104.26 ± 1.84^{bcB}	77.59 ± 5.70^{bcC}
HWT_1	105.89 ± 4.46^{bcA}	109.24 ± 1.54^{abA}	79.78 ± 5.05^{bcB}
HWT_2	109.76 ± 5.65^{abA}	112.51 ± 1.16^{aA}	74.11 ± 2.80^{cB}
HWT_3	102.55 ± 4.89^{cA}	104.78 ± 2.95^{bcA}	81.87 ± 4.52^{bB}

Note: Different lowercase letter (a,b,c) in the same column indicate significant differences among treatments and different capital letter (A,B,C) in the same row indicate statistical difference among days of storage according to least significant difference test ($p \leq .05$) by Fisher's test.

be attributed to HWT for short time, presents higher activation of antioxidant enzymes such as SOD and POD, which are responsible for producing and reducing H_2O_2, respectively. The H_2O_2 produces hydroxyl radicals that are highly reactive on the cellular membrane, lipids, proteins, and DNA structure, causing cell death. Therefore, HWT reduced the degradation of plastids and the disappearance of peroxisomes and maintained higher metabolites redox (Sánchez-Bel et al., 2012). The change in membrane permeability related to EL and lipid peroxidation was previously reported in sweet pepper treated with hot water treatment (Raffo et al., 2007; Rodoni et al., 2016) and hot water treatment plus polyethylene bag packaging (Endo et al., 2019), where lipid peroxidation increased with storage time, and changes in membrane compounds directly affected membrane stability and function. Therefore, the use of moderate stress previous to cold storage reduced change in membrane composition, limiting membrane degradation, lipid peroxidation, and electrolyte leakage (Maalekuu et al., 2006). In this sense, hot water for short time (1-, 2-, or 3-min) helps in reducing the degradation of the cellular membrane during chilling stress and ambient temperature where deterioration and senescence increase fast.

Chilling stress increases cellular damage, and WL is related with lipid peroxidation and %EL. Immersion time of 2- and 3-min in hot water allowed less weight loss percentage during cold storage and at ambient temperature, whereas HWT_1 presented a similar behavior with control 5°C. Moreover, HWT for 1 and 3-min reduced the %EL during storage at 21°C; however, HWT for 1-, 2, and 3 min presented similar MDA content, and only HWT_3 allowed least value than control 5°C. HWT in general reduced membrane deterioration, likely, the hot water stimulates the synthesis of wax in the fruit covering cracks produced during cold storage or it melts the epicuticular wax, covering the cracks caused by cell damage, limiting water loss (Ayón-Reyna et al., 2017; Fallik et al., 1999); also, reduces cell membrane degradative enzymes activity such as lipoxygenase and their negative effect on membrane lipid compounds (Maalekuu et al., 2006). Therefore, the water loss is related with loss cell membrane integrity, saturation of fatty acids, and enzymatic activity that increases physiological disorders. In previous study, bell pepper presented high susceptibility to water loss showing high ion leakage and membrane damage that was related with a strong effect on membrane integrity in the fruit during storage (Maalekuu

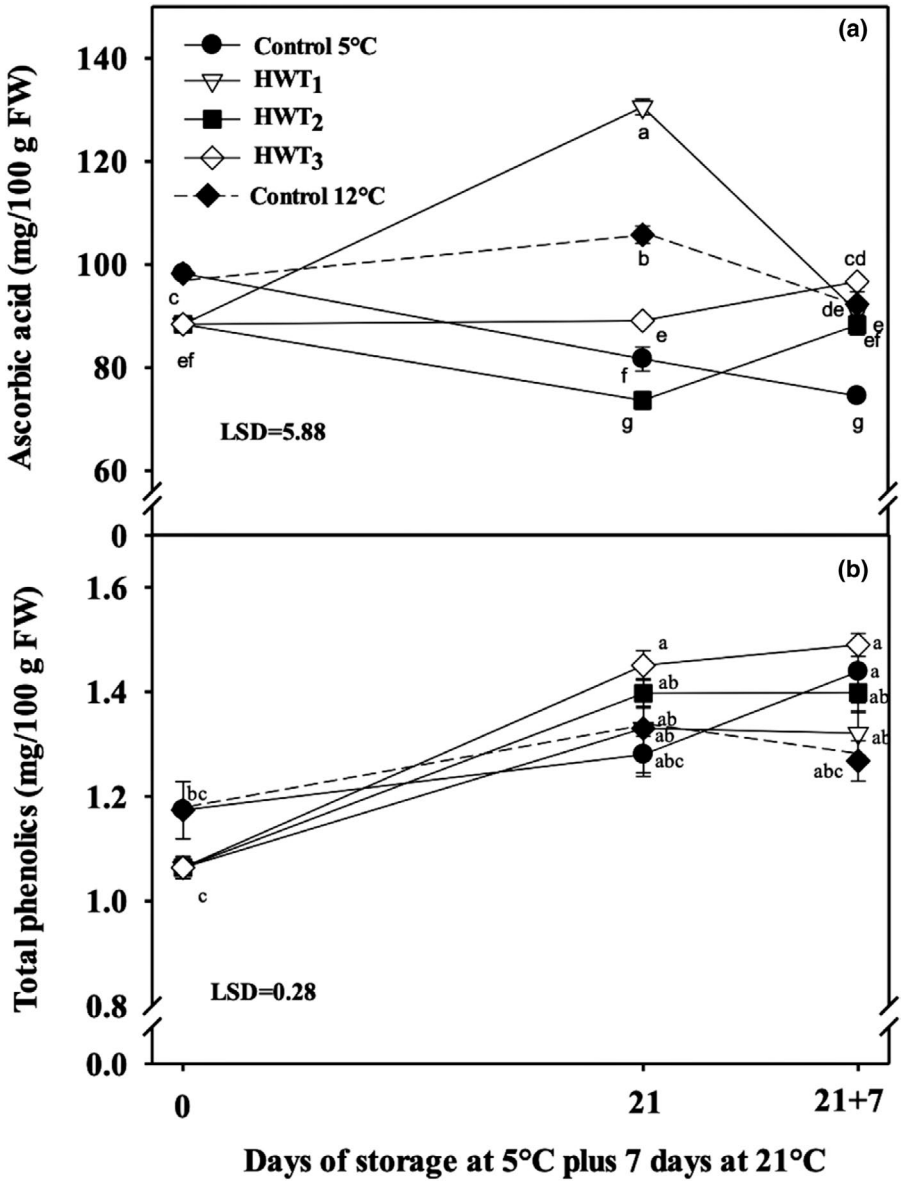

FIGURE 4 Effect of dipping time in hot water on ascorbic acid (a) and total phenolics (b) of bell pepper fruit during storage at 5°C plus 7 days at 21°C. Treatments: control 12°C (nontreated, dashed lines), control 5°C (nontreated), HWT_1 = 53°C, 1 min; HWT_2 = 53°C, 2 min; HWT_3 = 53°C, 3 min. Each data point represents the mean ± *SD* (*n* = 9). Values with the same letter are not statistically different (least significant difference [LSD] at $p \leq .05$). Vertical bars represent standard error of the mean

et al., 2006). The reduction of weight loss has been reported in hot water–treated cucumber (Nasef, 2018), orange (Shi et al., 2020), sweet pepper (Endo et al., 2019), and guava (Killadi et al., 2021) where the HWT for short time could improve the barrier functions in the membrane and cell wall–reducing water loss and susceptibility to CI. On the other hand, in bell pepper (González-Aguilar et al., 1998), mango (Osuna-Gacía et al., 2007), and rambutan (Hafiz et al., 2017), the use of HWT increased the water loss due to higher respiration and transpiration caused by increased stomal density, cell breakage, and destruction of mesocarp. Hot water treatment for short time can reduce the fruit ripening, this treatment limits damage to the respiratory mechanism, reduced ripening, and extended shelf life (Fallik et al., 1999).

In this study, the use of HWT in bell pepper did not present a negative effect on the color; nevertheless, it had an effect on its firmness, especially during ambient temperature. According with different authors, these results imply that the HWT did not affect chlorophyll; however, the structure integrity and function of cell wall are injured by the heat treatment and cold storage due to internal collapse caused for the deterioration of protopectin of the middle lamella and primary cell wall as result of the cellulase, pectinmetilesterase, and polygalacturonase activities (Liu et al., 2018; Silva et al., 2012). On the other hand, in rambutan, the HWT was unsuccessful to retain the bright red color during storage (Hafiz et al., 2017). The firmness in bell pepper decreased during storage specially in ambient temperature. The use of HWT_1 or HWT_3 generated a better firmness retention

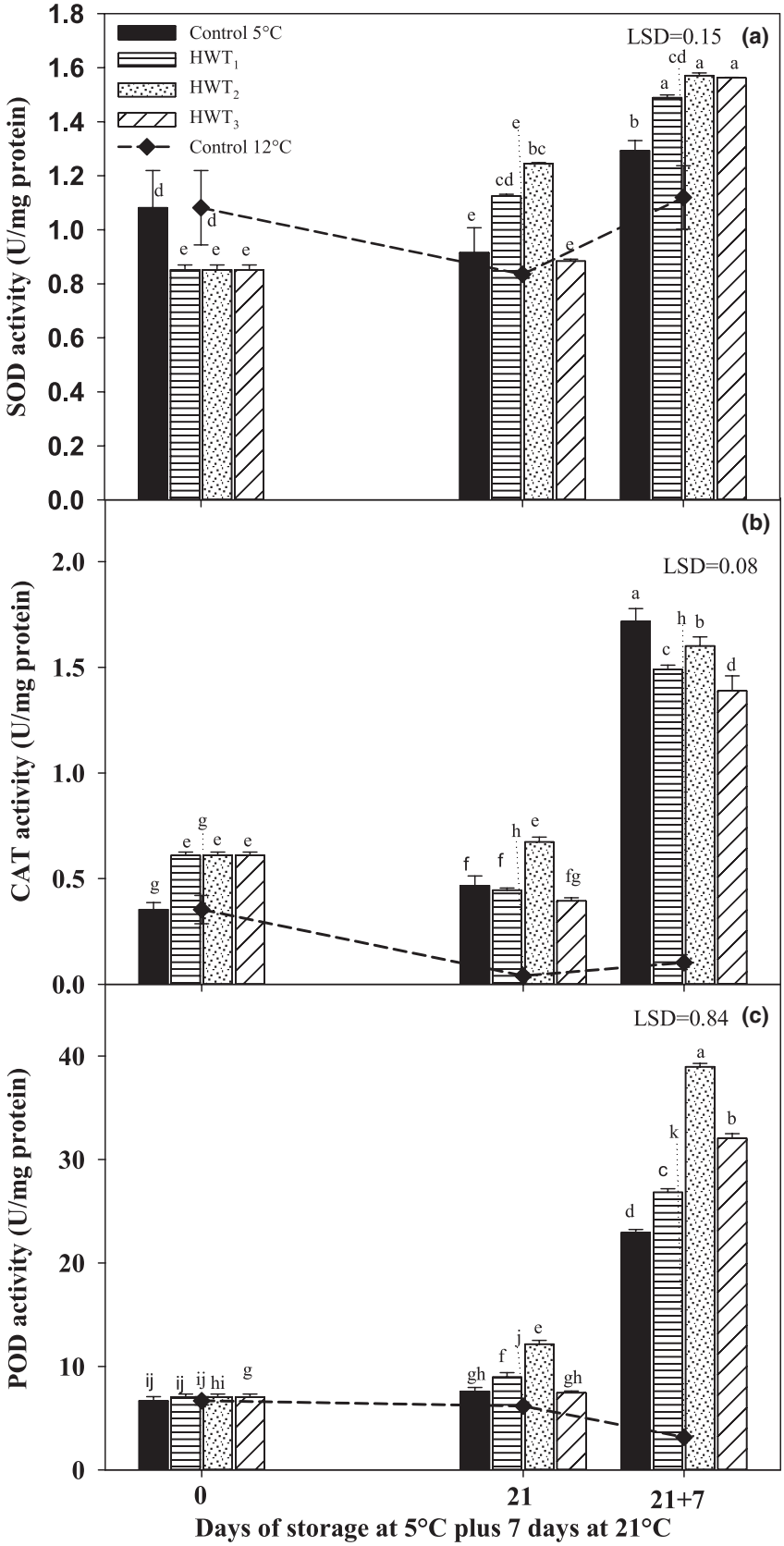

FIGURE 5 Effect of time immersion in hot water on the activity of superoxide dismutase (SOD) (a), catalase (CAT) (b) and peroxidase (POD) (c) of bell pepper fruit during storage at 5°C plus 7 days at 21°C. Treatments: control 12°C (dashed lines), control 5°C (nontreated), HWT_1 = 53°C,1 min; HWT_2 = 53°C, 2 min; HWT_3 = 53°C, 3 min. Each data point represents the mean ± *SD* (*n* = 9). Values with the same letter are not statistically different (least significant difference [LSD] at $p \leq .05$). Vertical bars represent the standard error of the mean

than control 5°C and HWT_2 with respect to initial day, which may be due to higher cell organization and less degradation of the pectin. Control 12°C presented the lowest firmness loss, which is associated with a low water loss and a predominant membrane integrity in the fruit. The reduction of softening during cold storage in treated fruit was previously reported in sweet pepper (Raffo et al., 2007), kiwifruit (Ma et al., 2014), bell pepper (Liu et al., 2018), cucumber (Nasef, 2018) and guava (Killadi et al., 2021). Hafiz et al. (2017) reported that HWT (56°C) for 1 min in rambutan was adequate to maintain the firmness of the fruit compared to the control, HWT can reduce the activity of enzymes such as polygalacturonase and pectin methyl esterase, delaying the degradation of pectin in the cell wall, inhibit the activity of enzymes such as desmolase, dehydrogenase and carboxylase, maintaining starch levels (Ullah et al., 2018), and sometimes interrupting or blocking the production of ethylene (Killadi et al., 2021). Accumulation of ROS during cold stress is directly involved with susceptibility to CI, decay tissue, senescence and death cell (Liu et al., 2005). HWT provoked a reduction of AsA at day 0, which could be related to the stability of this compound because AsA is highly labile, hence the use of thermic treatment and storage time impact its stability (Yahia et al., 2000). The use of hot water treatment for 1-min in bell pepper increased significantly AsA content at the end of storage at low temperatures, which could be related to the stability provided by the immersion time that prevents the oxidation of this compound, in addition to not allowing the activation of the enzyme ascorbate peroxidase that degrade this compound (Shahkoomahally & Ramezanian, 2013). The dipping in HWT for 2- or 3-min presented a reduction in AsA content, a behavior that some authors related with the activity of enzymes that catalyze oxidation reactions such as ascorbate oxidase and ascorbate peroxidase that use AsA as substrate in response to cold stress and water loss during storage (Killadi et al., 2021; Raffo et al., 2007; Yahia et al., 2000). At the end of storage, the fruit treated with HWT presented higher AsA content than control fruit that presented significative losses in AsA, especially with the immersion for 3 min; according with Yahia et al. (2000) the hot water treatment allow that AsA undergoes continuous oxidation and reduction. The products of oxidation such as ascorbic and dehydroascorbic acids are transformed newly into AsA, maintaining higher levels at the end of storage (Yahia et al., 2000). Similar results were previously reported in sweet pepper (Endo et al., 2019) and guava fruit (Killadi et al., 2021) treated with HWT at 45 and 50°C, respectively. Both temperatures presented retention and an increase in AsA content during cold storage, Therefore, the use of HWT help to maintain low levels of hydrogen peroxide during cold storage, so it is considered as an important indicator of quality and reflects tolerance to cold stress. AsA shows an important role in conserving the cellular redox state of fruit; therefore, the exposure of the fruit to stress prior to storage at low temperatures increases AsA content, maintaining the ROS in equilibrium (Mustafa et al., 2016). Phenolic compounds are unstable at high temperatures and storage time. The use of heat temperatures caused a similar synthesis of phenolic compounds at the beginning of storage. On the other hand, during storage at cold temperature the use of HWT at different immersion time allowed higher synthesis of TP (compared with the initial day) as a defense mechanism in response to cold stress; serving these compounds as substrates for antioxidant enzymes like CAT and POD, providing higher tolerance to CI and membrane integrity. In addition, this protective effect of moderate heat stress can cause the interruption of the normal metabolic process of the fruit, inhibiting the synthesis of oxidative enzymes. In addition, the retention of these compounds during ambient temperature for HWT may be related to a reduction of enzymatic browning and interruption of normal metabolic process (Killadi et al., 2021). In this way, Shen et al. (2013) reported that the use of hot water at 50°C in mandarin and Nasef (2018) in cucumber treated with HWT at 55 and 45°C, the TP content increased due to higher integrity of vacuoles in response to tolerance to low temperatures and reduction in polyphenol oxidase and peroxidase activity. Therefore, the HWT for short time has an important effect to maintain the synthesis of AsA and TP content that actively participate in providing tolerance to cold stress in bell pepper maintaining redox system in cell.

The equilibrium among SOD, CAT, APX, and POD in the cell is very important to maintain normal levels of super oxide anion (O_2^-), H_2O_2, and hydroxyl (OH^-). In this study, the SOD activity increased significantly with HWT especially during 1 and 2-min of immersion. The use of HWT increased SOD activity during ambient temperature compared with control 12°C. The SOD enzyme increases oxygen and H_2O_2 levels by dismutating O_2^-. Although CAT and POD reduced H_2O_2 levels by limiting the production of OH^-, molecule involved in lipid peroxidation and loss of membrane integrity. Liu et al. (2018) reported that induction of SOD and CAT enzymes is a vital mechanism which protects cell during any stress exposition. Likewise, POD plays an important role in reduction of H_2O_2, but also this enzyme can be related to deterioration generated by microorganism like fungi, bacteria, and viruses, a more reducing state of ascorbic acid and glutathione (which increases peroxide production), and phenols oxidation during stress (Liu et al., 2018; Mustafa et al., 2015). In this study, CAT and POD presented higher activity in HWT_2 during cold stress and ambient temperature, where presented the highest activity. According with Endo et al. (2019), an increase in CAT and POD activities is a sign of an adaptive process about accumulation of H_2O_2 and production of H_2O and $O_{2;}$ this behavior could be related with the increase observed in SOD activity (Figure 5a). Therefore, a balance among antioxidant enzymes is necessary to preserve cell structure. During storage at 12°C, bell pepper fruit presented a reduction in SOD and CAT activities, and retention of POD activity generating an accumulation of H_2O_2 and OH^- in the cell, causing a reduction in ascorbic acid, total phenol and higher decay index. Therefore, CAT and POD enzymes can be highly sensitive to stress caused by storage temperatures, and peroxide removal mechanisms predominate under these stress conditions. Authors like Sánchez-Bel et al. (2012) and Endo et al. (2019) reported that low activity of CAT during storage at low temperatures is related with peroxisome disintegration and accumulation of H_2O_2. Therefore, to restore cell homeostasis is important to keep CAT activity and protein associated with ascorbate-glutathione cycle. Similar results were reported in cucumber and sweet pepper (Endo et al., 2019; Nasef, 2018), the

authors showed that hot water treatment for a short time could induce heat shock proteins, activate antioxidant enzymes, and maintain membrane cell reducing weight loss (Nasef, 2018). On the other hand, in mandarin (Ghasemnezhad et al., 2008) and daylily flowers (Liu et al., 2018), it was only observed an increase in CAT activity due that POD activity was suppressed. Nasef (2018) reported that CAT participates in the cell giving protection versus any stress. Therefore, the reduction of CI index in bell pepper treated with hot water is closely related to SOD, CAT, and POD activity.

In conclusion, the results in this work indicated that heat treatment for 1-, 2-, or 3-min reduced decay index, CI symptoms and maintained the fruit quality. At the end of storage, HWTs showed higher AsA content than control 5°C. Only the fruit subjected to a hot water treatment increased the TP content during cold storage. The storage under CI temperature (5°C) increased notably the activity of the evaluated enzymes. In general, the HWT at different immersion times contributed as well to activate the SOD and POD enzymes on fruit stored at CI temperatures; however, HWT_2 presented better activation. Therefore, the use of any of the three times of immersion in hot water reduces the susceptibility to CI, maintains the quality, and it is associated with the activation of the antioxidant enzymes in bell pepper.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Gabriela López-Angulo: Conceptualization; Methodology; Supervision. **Jordi Lopez:** Investigation; Writing-review & editing. **Misael Vega:** Project administration; Supervision; Validation; Writing-review & editing. **Wendy Denisse Bojórquez Acosta :** Data curation; Investigation; Methodology. **Francisco Delgado-Vargas:** Formal analysis; Investigation; Writing-review & editing. **Lidia Ayon:** Formal analysis; Investigation; Writing-review & editing. **Martha Edith López López:** Conceptualization; Investigation; Methodology; Project administration; Writing-original draft; Writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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