

A Treatment Combining Hot Water with Calcium Lactate Improves the Chilling Injury Tolerance of Mango Fruit

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Abstract. ‘Keitt’ mango is one of the most important cultivars, and it is usually stored at a low temperature during its commercialization to extend shelf life and reach distant markets. However, it is susceptible to chilling injury (CI) and some prestorage treatments are required to reduce the incidence of this disorder. This research shows for the first time the protective effect of a combination hot water-calcium lactate (Ca) against CI in mango fruit cv. Keitt. Fruit were subjected to hot water treatment (HWT) (46.1 °C, 75–90 minutes) or treated with 0.5% Ca or with the combination HWT + Ca, stored at 5 °C for 20 days, and ripened at 21 °C for 7 days. CI index (CII), electrolyte leakage (EL), malondialdehyde (MDA) production, bioactive compounds, antioxidant capacity [2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazil (DPPH)], and activity of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)] were analyzed in mango samples after 0, 10, and 20 days of cold storage and after ripening. Hot water treatments (HWT and HWT + Ca) were more effective than Ca in providing protection against CI as evidenced by lower incidence of symptoms and lower EL and MDA. HWT + Ca increased the content of phenolics, flavonoids, and carotenoids during the cold storage, which correlated with the antioxidant capacity by ABTS. SOD and APX showed higher activity in HWT + Ca-treated fruit, whereas CAT activity was higher in fruit with HWT and Ca. These results suggest that HWT + Ca provided CI tolerance of ‘Keitt’ mango by activation of the enzymatic and nonenzymatic antioxidant systems.

According to the international market of tropical fruits, the most important products are mango, pineapple, papaya, and avocado (FAO, 2010). In 2014, the global mango production was 45 million t, and 3.87% of this production was from Mexico (FAOSTAT, 2017). Remarkably, Mexico is the largest mango exporter worldwide and produces the cv. Keitt, which is one of the preferred cultivars in the national and international markets (SAGARPA, 2017).

Mango is climacteric, harvested in physiological maturity, and shows high metabolic activity during postharvest. Consequently, fruit quality declines fast and the postharvest management conditions (e.g., pretreatments, storage, and transport) must be carefully selected (Ngamchuachit et al., 2014). Cold storage is

commonly used to decrease the metabolic activity of climacteric fruits to increase their shelf life and to reach distant markets. However, mango develops a physiological disorder known as CI when it is stored at temperatures below 8 to 12 °C, and it is more susceptible at physiological maturity (Yimyong et al., 2011). If the cold stress is for a long time span, the tissue is affected permanently damaging cell walls, membranes, and cellular compartmentalization, resulting in increased reactive oxygen species (ROS) production (Chidtragool et al., 2011). It has been demonstrated that visual CI symptoms appear after the fruit is removed from the cold storage and transferred to ripening conditions (Ding et al., 2007). The susceptibility to CI also depends on the genotype; preliminary studies showed that mango cv. Keitt was more susceptible to CI than other commercial cultivars in Mexico (Ataulfo, Haden, Kent, and Tommy Atkins) (data not published). The main CI symptoms in mango include uneven ripening, lenticel darkening, pitting, internal browning, off-flavor, and increased incidence to decay (Ketsa et al., 2000).

Fruit under abiotic stress increase the activity of their antioxidant enzymes (e.g.,

SOD, CAT, APX, and glutathione reductase) and the level of antioxidant compounds [e.g., phenolics, ascorbic acid (AA), carotenoids, tocopherols, and glutathione]. Both of them are related to the reduction of oxidative stress and the maintenance of the membrane integrity (Lopes et al., 2016; Wu et al., 2014; Zhao et al., 2006). Different procedures have been applied to reduce the CI of mango, including the treatment with calcium–arabic gum (Khaliq et al., 2015, 2016), hot air (Ketsa et al., 2000; McCollum et al., 1993), thermal shock (Zhao et al., 2006), and hot water (Almeida Miguel et al., 2016; Nyanjage et al., 1999; Talcott et al., 2005). Interestingly, the immersion of mangos in hot water (46.1 °C) for 65–110 min depending on the fruit size and weight is a mandatory quarantine treatment for pest control before exportation (USDA-APHIS, 2014). The hydrothermal treatment of mango reduces CI, but it could decrease fruit firmness and increase the ripening rate (Almeida Miguel et al., 2016; Dea et al., 2010). On the other hand, the treatment of mango fruit with calcium salts improves their firmness, stress tolerance, and ripening. Calcium ions interact with fruit pectins forming calcium pectate that contributes to the cell wall stabilization, membrane integrity, and the maintenance of cell turgor and cell-to-cell contacts (Khaliq et al., 2015, 2016). Consequently, an adequate combination of treatments could improve the CI tolerance of mango. Thus, this research demonstrates for the first time the protective effect of the combination of the HWT and the Ca treatment (HWT + Ca) of mango against CI; moreover, it reports the variations in the content of antioxidant compounds and the activities of antioxidant enzymes during cold storage and ripening of the fruit.

Materials and Methods

Mature-green mango fruit (*Mangifera indica* L. cv. Keitt) were obtained from a local producer in Culiacan, Sinaloa, Mexico. The fruit were selected based on the size (400–600 g), peel color uniformity, and absence of physical damage; then, they were washed with sodium hypochlorite (300 µg·L⁻¹) and divided into four groups (44 fruit/group). The treatments were the following: Control, HWT (46.1 °C, 75 or 90 min); calcium lactate treatment (Ca, 0.5% w/v, 75 or 90 min) (Manganaris et al., 2007; Silveira et al., 2011); and HWT + Ca (same conditions). All HWT were applied according to the USDA-APHIS (2014) standard that establishes the time of immersion in hot water (46.1 °C) based on fruit weight (75 and 90 min for fruit of 375–500 and 501–700 g, respectively). The fruit was air-dried at 21 °C for 1 h and stored at 5 °C for 20 d plus a ripening period of 7 d at 21 °C. The mango pulp of each fruit was homogenized, frozen with liquid nitrogen, and stored at –70 °C until use.

CI index. The CII was determined according to Vega-García et al. (2010) with some modifications. A total of 80 fruit were evaluated,

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20 per treatment. The symptoms evaluated were lenticel darkening (L), pitting (P), uneven color development (U), and decay (D). The severity of the symptoms was measured visually as injury level (IL) using a 5-point scale based on the percentage of tissue affected for each criterion (0 = no tissue injury, 1 = 1% to 25% of tissue injury, 2 = 26% to 50% tissue injury, 3 = 51% to 75% tissue injury, and 4 = $\geq 76\%$ of tissue injury). The CII for each fruit was calculated by the following expression: $CII = (ILL + ILP + ILU + ILD)/4$.

Electrolyte leakage. The method described by Malacrida et al. (2006) was used with some modifications. For each treatment (four mangos), 18 mesocarp tissue cylinders (7 mm) per fruit were cut with a cork borer. The cylinders were washed three times with deionized water to eliminate any electrolyte released during cutting. They were added with 25 mL of 0.4 M mannitol solution and incubated at 25 °C for 2 h under constant shaking; the conductivity of the solution was measured using a manual conductivity meter (HI 98311; Hanna Instruments, Woonsocket, RI). After reading, samples were autoclaved at 121 °C for 10 min to release all the electrolytes. The samples were held at 25 °C and the conductivity was measured again for total EL. The percentage of EL was determined as follows: $\%EL = (\text{initial electrolytes}/\text{total electrolytes}) \times 100$.

Malondialdehyde content. The MDA content was determined according to Hodges et al. (1999) by the thiobarbituric acid (TBA) technique. For each treatment (four mangos), 1 g of homogenized frozen tissue (3 g in total per fruit) was mixed with 30 mL of ethanol: water (80:20 v/v) and centrifuged (3000 g_n , 4 °C) for 10 min. One milliliter of supernatant was mixed with 1 mL of solution A (–TBA) containing 20% trichloroacetic acid (TCA) + 0.01% butylated hydroxytoluene (BHT) or solution B (+TBA) that contained 20% TCA + 0.01% BHT + 0.65% TBA. The solutions were shaken, heated (95 °C, 25 min), and centrifuged. The absorbances were read at 440, 532, and 600 nm using an ultraviolet (UV)–vis spectrophotometer (Unico SQ2800; Unico Inc., San Diego, CA). MDA equivalents were calculated using the following formulas: 1) $A = [(Abs\ 532_{+TBA}) - (Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA})]$, 2) $B = [(Abs\ 440_{+TBA} - Abs\ 600_{+TBA}) \times 0.0571]$, 3) MDA equivalents ($nmol \cdot mL^{-1}$) = $(A - B/157,000) \times 10^6$.

Analysis of Bioactive Compounds

Preparation of methanol extracts (ME). Mango pulp was freeze-dried and 1 g was homogenized with 5 mL methanol, sonicated for 30 min at 30 °C, and allowed to stand for 24 h at 4 °C. The procedure was carried out for 3 d exchanging the solvent every 24 h. The recovered solvent was concentrated under vacuum to obtain the ME, which was stored at –20 °C in darkness until its use.

Total phenolics (TP). Total phenolics content was determined as described by

Adom and Rui (2005) with some modifications. Forty microliters of the ME diluted in methanol (1:10 w/v) were oxidized with 360 μL Folin–Ciocalteu reagent (1:8 v/v) plus 100 μL Na_2CO_3 (7% w/v) for 90 min (21 °C), and the absorbance was measured at 750 nm. A standard curve was prepared using gallic acid (GA) (50–600 $\mu g \cdot mL^{-1}$) and the results were expressed as milligrams of GA equivalents per 100 g of fresh weight [mg GAE/100 g fresh weight (FW)].

Total flavonoids. Total flavonoids were determined according to Moo-Huchin et al. (2014) with some modifications. The ME was diluted in methanol (1:3 w/v) and 0.5 mL of this was mixed with 2 mL of deionized water and 150 μL of 5% $NaNO_2$, allowed to stand for 5 min, added with 150 μL of 10% $AlCl_3$ in methanol, allowed to stand for 1 min (25 °C), and added with 1 mL of 1 M NaOH. Finally, the volume was adjusted to 5 mL with deionized water, shaken, and the absorbance was read at 415 nm.

Ascorbic acid. AA concentration was determined according to Dürüst et al. (1997). Fresh mango pulp (1 g) was homogenized with 10 mL of 0.4% oxalic acid solution for 1 min; an aliquot (1 mL) was mixed with 1 mL of acetate buffer (30 g sodium acetate anhydride + 70 mL water deionized + 100 mL glacial acetic acid) and 8 mL of 2,6-dichlorophenolindophenol disodium salt (DCPI) solution (12 $mg \cdot L^{-1}$) for 15 s, and the absorbance was measured at 520 nm (L_1). For the second absorbance measurement L_2 , the UV–vis equipment was adjusted to zero using the mixture that includes the sample, acetic buffer, and deionized water; L_2 corresponded to the absorbance of the mixture of sample with DCPI solution after 15 s of reaction. Thus, $L_1 - L_2$ values represent the absorbance of the sample. The AA concentration was determined using a standard curve of AA (10–50 $\mu g \cdot mL^{-1}$) and the results were expressed as mg AA/100 g FW.

Total carotenoids. The extraction and quantification of total carotenoids were carried out according to Moo-Huchin et al. (2014). Freeze-dried pulp (0.1 g) was homogenized with 5 mL of a solution containing hexane:acetone:ethanol (70:15:15 by volume) and 0.05% (v/v) of BHT as antioxidant. The mixture was stirred for 1 h in darkness, saponified with 0.5 mL of 40% (w/v) KOH in methanol for 2 h (25 °C darkness), added with 2 mL of hexane, vigorously shaken, and the upper layer was collected. The lower layer was extracted twice more, the three supernatants were mixed and filtered through anhydrous sodium sulfate powder. Total carotenoids content was determined by measuring the absorbance at 450 nm. A calibration curve was prepared using pure β -carotene (BC) in hexane (0–50 $\mu g \cdot mL^{-1}$) and the results were expressed as mg BC/100 g FW.

Total Antioxidant Capacity

ABTS method. This assay was performed as described by Re et al. (1999) with some modifications. The ABTS stock solution was

prepared by mixing 5 mL of ABTS reagent (7 mM) with 88 μL of potassium persulfate (140 mM) and kept in darkness at 25 °C for 12–16 h. The ABTS solution was diluted with 7 mM phosphate buffer solution (pH 7.4) to obtain an absorbance of 0.75 ± 0.02 at 734 nm. In assay tubes, 10 μL of ME diluted in methanol (1:7 w/v) were mixed with 200 μL of ABTS solution. The mixture was allowed to stand for 30 min at 27 °C in darkness and its absorbance was measured at 734 nm with a Microplate Reader (SynergyTM HT Multi-Detection; Biotek, Inc., Winooski, VT). Trolox (Sigma-Aldrich-238813, St. Louis, MO) was used to prepare a calibration curve (0–225 $\mu g \cdot mL^{-1}$) and the antioxidant capacity was expressed as Trolox equivalents (TE) ($\mu mol\ TE/100\ g\ FW$).

DPPH assay. The DPPH assay was carried out according to Brand-Williams et al. (1995) with minor modifications. ME was diluted in methanol (1:3 w/v); an aliquot (20 μL) was mixed with 180 μL of 150 mM DPPH in methanol, incubated for 30 min (27 °C/darkness), and the absorbance was measured at 550 nm. Results were reported as $\mu mol\ TE/100\ g\ FW$.

Activity of Antioxidant Enzymes

Superoxide dismutase. SOD was determined according to Liu et al. (2005) with some modifications. Freeze-dried pulp (0.5 g) was homogenized in a cold mortar with 5 mL of ice-cold 0.05 M phosphate buffer (pH 7.5) containing 1 mM of ethylenediaminetetraacetic acid (EDTA) and 5% of polyvinylpyrrolidone. The mixture was centrifuged (17,200 g_n , 4 °C) for 15 min and the supernatant was used to determine the enzymatic activity. The reaction mixture consisted of 1 μL enzyme extract and 199 μL of 0.1 M phosphate buffer (pH 7.8) that contained 0.01 M methionine, 0.025% Triton X-100, 0.11 mM EDTA, 57 μM nitro blue tetrazolium chloride (NBT), and 50 μL of 20 μM riboflavin. The reaction was read in a microplate reader at 550 nm before and after light exposition (25 W) for 10 min. One unit of SOD activity was defined as the amount of enzyme that would inhibit 50% of the photoreduction of NBT.

Catalase. Freeze-dried mango pulp (0.5 g) was homogenized with 5 mL of extraction buffer containing 45 mM potassium phosphate buffer (pH 7.0), 5 mM of dithiothreitol, 5 mM EDTA, and 0.125 g of polyvinylpyrrolidone. The mixture was centrifuged (17,200 g_n , 4 °C) for 30 min and the supernatant was used to determine the enzymatic activity. The CAT activity was determined by measuring the disappearance of H_2O_2 for 5 min at 240 nm, according to Yimyoung et al. (2011) with some modifications. The reaction mixture contained 1 mL of phosphate buffer (40 mM, pH 7.0) + H_2O_2 (40 mM) and 25 μL enzymatic extract. The changes in H_2O_2 concentration were calculated based on its extinction coefficient ($43.6\ M^{-1} \cdot cm^{-1}$) at 240 nm. CAT activity was expressed in units per milligram of protein.

Ascorbate peroxidase. The APX activity was determined with the same extract used for evaluation of CAT activity and following the protocol of Yimyong et al. (2011) with some modifications. The reaction mixture contained 969 μL of phosphate buffer (40 mM, pH 7.0), 5 μL of AA (0.1 M), 1 μL of H_2O_2 (0.1 M), and 25 μL of enzymatic extract for a total reaction volume of 1 mL. APX activity was expressed as units of activity per milligram of protein. The changes in AA concentration were calculated based on its extinction coefficient ($2.8 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) at 290 nm.

Statistical analysis. Data were analyzed by two way analysis of variance; the factors were treatments (control, HWT, Ca, and HWT + Ca) and days of storage (with or without ripening). Significant differences ($P < 0.05$) between means were established using the least significant difference by Fisher's test. Pearson correlation coefficients (r) were calculated between data sets ($P < 0.05$). All analyses were performed using the software Statgraphics Plus 5.1 (Statistical Graphics, Rockville, MD).

Results

CI index. The storage of mango at 5°C induced CI as registered by the increasing values of the CII; the symptoms were better observed after the ripening period at 21°C . Remarkably, HWTs (HWT and HWT + Ca) of mangos induced better protection against CI than Ca treatment (Fig. 1A) ($P < 0.05$). At the end of ripening after removal of cold storage, the CII for the HWT and HWT + Ca fruit were 42.8% and 30.4% lower than the corresponding control, but there were no significant ($P > 0.05$) differences between both treatments. The main CI symptoms in control- and Ca-treated mangos were lenticel darkening and pitting in the peel, as well as decay in peel and pulp (Fig. 1B), whereas HWT mangos only showed lenticel darkening and slight pitting in peel, reflecting the protective effect of HWT and HWT + Ca against CI.

Electrolyte leakage and MDA content. The EL values increased during the first 10 d of cold storage, especially in the control fruit that showed significantly ($P < 0.05$) higher values than the other treatments, which could be related with a protective effect provided to the fruit by the treatments (Fig. 2A). HWT + Ca was the best treatment showing the lowest EL values at the end of ripening after the removal of cold storage; the EL value of HWT + Ca mangos (57.21%) was 1.30, 1.09, and 1.09 times lower than those of control-, HWT- and Ca-treated mangos, respectively, and showed significant differences ($P < 0.05$) with respect to the other treatments (Fig. 2A). On the other hand, the MDA content increased with the storage time at 5°C and was higher after ripening ($P < 0.05$) (Fig. 2B). Analyzing the general patterns throughout the storage and ripening periods, both HWT and Ca treatments showed smaller MDA content than the control. At the end of ripening after the

removal of cold storage, the Ca-treated fruit showed the lowest MDA values followed by HWT + Ca and HWT mangos ($P < 0.05$). During cold storage, HWT mangos were better protected than those with HWT + Ca. However, the combination HWT + Ca was always better for mangos cold stored and ripened.

Bioactive compounds. Total phenolics values in control, HWT, and Ca-treated fruit decreased during cold storage, whereas an increase in the content of these compounds was observed in HWT + Ca fruit ($P < 0.05$) (Table 1). HWT + Ca mangos showed the highest TP (61.92 mg GAE/100 g FW) after 20 d at 5°C ($P < 0.05$). The ripening period increased the TP in all treatments except at the end of storage where HWT + Ca showed a reduction; TP contents were higher in HWT mangos (54.20–71.41 mg GAE/100 g FW) than control and HWT + Ca fruit ($P < 0.05$).

The total flavonoids content of mango stored at 5°C decreased in the control and HWT fruit ($P < 0.05$) and increased in the Ca-treated fruit (Table 1). After 20 d of cold storage, HWT + Ca- and Ca-treated fruit showed about the same content of flavonoids (68.30 and 68.53 mg quercetin equivalents (QE)/100 g FW, respectively). Ripening of Ca-treated mango improved their total flavonoids reaching the highest values (67.07 and 70.85 mg QE/100 g FW) after 0 and 20 d of cold storage, respectively. On the other hand, fruit with the HWT + Ca treatment showed the lowest total flavonoids content (60.02 mg QE/100 g FW) after 20 d of cold storage and 7 d of ripening.

Clear tendencies were not observed in the changes of the AA content of mango fruit stored at 5°C for different periods (Table 1). The HWT fruit showed the highest values (16.44–19.58 mg AA/100 g FW), whereas the lowest values (8.27–16.14 mg AA/100 g FW) were observed in the HWT + Ca fruit. Ripening after the removal of cold storage decreased the AA content in all treatments. In general, the control fruit were the least affected (7.43–7.91 mg AA/100 g FW) and the HWT fruit were the most affected (6.05–9.53 mg AA/100 g FW). At the end of the cold storage time and ripening, all-treated mangos showed similar AA contents ($P > 0.05$) with values ranging from 5.87 mg (HWT + Ca) to 7.43 mg AA/100 g FW (control).

Storage time at 5°C affected the total carotenoid content of mango, showing a general increase during the first 10 d and then a decrease after 20 d, with significant differences among treatments ($P < 0.05$) (Table 1); the lowest carotenoid contents were observed in the control fruit (7.15–8.93 mg BC/100 g FW; $P < 0.05$). After 20 d of cold storage, Ca-treated mangos showed the highest value (11.21 mg BC/100 g FW) followed by HWT + Ca (10.96 mg BC/100 g FW). In mangos without cold storage (0 d), ripening increased ($P < 0.05$) the carotenoid content in all treatments, especially in the HWT fruit (12.83 mg BC/100 g FW). Considering the storage time, the carotenoid content of control, HWT, and HWT + Ca-treated fruit decreased but that of Ca-treated

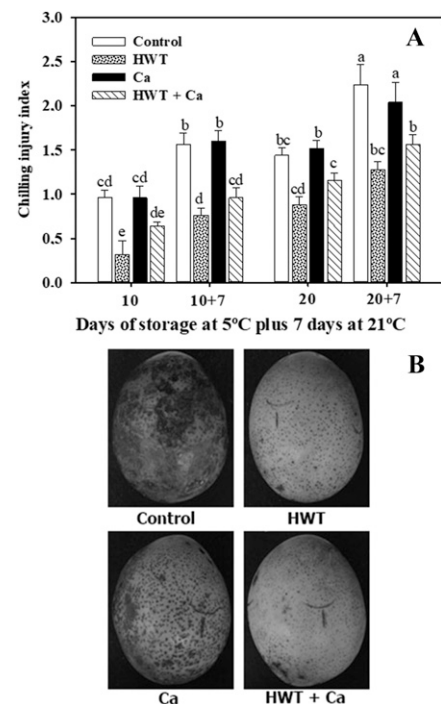


Fig. 1. Chilling injury (CI) index in mango cv. Keitt after 10 and 20 d of storage at 5°C + 7 d at 21°C (A). Symptoms of CI at the end of storage (20 d at 5°C + 7 d at 21°C) (B). Data are the mean of five replicates with SD as vertical bars. Different letters show significant differences among treatments ($P < 0.05$) based on the least significant differences (LSD = 0.36) by Fisher's test.

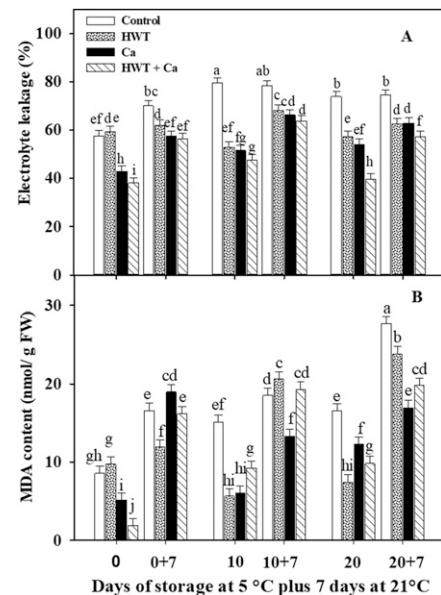


Fig. 2. Electrolyte leakage (%) (A) and malondialdehyde (MDA) content (B) in mango cv. Keitt after 20 d of storage at 5°C + 7 d at 21°C . Data are the mean of five replicates with SD as vertical bars. Different letters show significant differences among treatments ($P < 0.05$) based on the least significant differences (LSD = 4.42 and 1.85, respectively) by Fisher's test.

fruit increased. At the end of ripening, the highest carotenoid contents were for Ca and HWT + Ca fruit with values of 9.70 and

Table 1. Effect of different postharvest treatments and storage conditions on bioactive compounds and antioxidant capacity of mango 'Keitt'.

Days of storage at 5 °C	Samples without ripening				Samples ripened for 7 d at 21 °C			
	Control	HWT	Ca	HWT + Ca	Control	HWT	Ca	HWT + Ca
0	54.88 ± 2.89 b,C	52.74 ± 0.69 a,D	49.16 ± 0.53 a,E	44.44 ± 2.06 c,F	44.86 ± 1.19 b,F	71.41 ± 1.05 a,A	53.79 ± 1.12 b,CD	57.55 ± 1.65 a,B
10	58.37 ± 0.43 a,B	48.84 ± 1.19 b,E	44.19 ± 2.38 b,F	50.90 ± 1.23 b,E	45.00 ± 0.77 b,F	62.05 ± 1.36 b,A	57.03 ± 1.51 a,BD	55.77 ± 1.96 a,D
20	31.75 ± 0.57 c,F	33.64 ± 1.38 c,E	43.27 ± 0.82 b,D	61.92 ± 2.38 a,A	46.12 ± 0.61 a,C	54.20 ± 1.33 c,B	56.18 ± 2.32 a,BB	48.04 ± 4.83 b,C
Total phenolics (mg GAE/100 g FW)								
0	64.38 ± 1.01 a,B	58.72 ± 1.54 b,F	60.49 ± 1.44 b,C	45.76 ± 0.81 b,E	64.50 ± 1.53 a,B	45.54 ± 0.90 c,E	67.07 ± 1.13 b,A	63.89 ± 1.33 a,B
10	58.80 ± 1.71 b,C	66.11 ± 2.79 a,B	40.61 ± 0.65 c,D	70.17 ± 1.82 a,A	65.31 ± 2.32 a,B	56.68 ± 1.83 b,C	60.75 ± 1.97 c,C	59.07 ± 1.91 b,B
20	46.66 ± 1.04 c,E	39.17 ± 3.61 c,F	68.53 ± 2.21 a,AB	68.30 ± 2.20 a,ABC	65.79 ± 1.54 a,BC	65.54 ± 0.80 a,C	70.85 ± 0.32 a,A	60.02 ± 3.41 b,D
Ascorbic acid (mg AA/100 g FW)								
0	15.00 ± 0.32 a,A	16.44 ± 1.52 a,A	11.27 ± 0.99 b,B	16.14 ± 1.68 a,A	7.91 ± 0.37 b,D	9.53 ± 0.99 a,C	10.97 ± 1.84 a,B	8.59 ± 1.67 b,CD
10	6.65 ± 1.17 b,E	18.91 ± 0.39 a,A	14.87 ± 1.90 a,B	8.27 ± 3.33 b,DE	9.18 ± 0.34 c,CD	9.21 ± 0.53 a,CD	7.57 ± 1.32 b,DE	10.90 ± 1.20 a,C
20	14.02 ± 1.75 a,B	19.58 ± 4.99 a,A	14.68 ± 1.41 a,B	10.40 ± 3.43 b,C	7.43 ± 1.10 b,D	6.05 ± 1.09 b,D	6.68 ± 1.22 b,D	5.87 ± 1.04 c,D
Total carotenoids (mg BC/100 g FW)								
0	7.76 ± 0.09 b,F	8.72 ± 0.08 c,DE	8.54 ± 0.15 c,E	8.55 ± 0.25 c,E	10.91 ± 0.34 a,C	12.83 ± 0.42 a,A	8.83 ± 0.19 b,D	11.43 ± 0.10 a,B
10	8.93 ± 0.10 a,E	11.50 ± 1.02 a,B	10.23 ± 0.19 b,C	12.02 ± 0.09 a,A	9.34 ± 0.19 b,DE	11.14 ± 0.29 b,B	9.44 ± 0.16 a,D	10.54 ± 0.10 b,C
20	7.15 ± 0.03 c,F	9.97 ± 0.65 b,B	11.21 ± 0.06 a,A	10.96 ± 0.04 b,A	7.75 ± 0.15 c,E	9.25 ± 0.14 c,D	9.70 ± 0.38 a,B	9.95 ± 0.15 c,B
ABTS (μmol TE/100 g FW)								
0	219.33 ± 4.54 a,A	148.64 ± 4.96 a,E	173.13 ± 5.82 a,C	183.30 ± 6.64 b,B	150.79 ± 6.62 a,E	144.1 ± 7.74 a,EF	139.91 ± 8.98 c,F	164.56 ± 9.58 a,D
10	211.00 ± 6.67 b,A	150.89 ± 5.33 a,C	134.57 ± 2.79 c,D	179.9 ± 3.99 b,B	150.63 ± 6.16 a,C	137.9 ± 5.70 b,D	154.7 ± 7.13 b,C	154.9 ± 4.26 b,C
20	216.5 ± 7.62 ab,A	103.41 ± 8.61 b,F	157.54 ± 2.63 b,C	221.35 ± 6.32 a,A	147.87 ± 5.76 a,D	122.26 ± 8.27 c,E	165.63 ± 2.85 a,B	143.29 ± 2.96 c,D
DPPH (μmol TE/100 g FW)								
0	179.69 ± 3.45 a,A	152.49 ± 2.95 a,D	161.91 ± 2.56 a,C	133.09 ± 4.78 b,E	60.21 ± 5.62 c,G	134.99 ± 3.80 a,E	87.22 ± 1.23 a,F	169.25 ± 5.36 a,B
10	180.69 ± 2.79 a,A	137.22 ± 2.74 b,C	127.93 ± 1.96 c,D	147.92 ± 4.29 a,B	67.21 ± 5.42 b,G	110.52 ± 2.98 b,E	87.95 ± 6.46 a,F	149.79 ± 2.95 b,B
20	173.38 ± 4.11 b,A	108.73 ± 2.92 c,E	147.01 ± 2.24 b,B	116.27 ± 3.13 c,D	82.31 ± 4.26 a,FG	80.16 ± 4.53 c,G	85.51 ± 8.52 b,F	131.15 ± 3.12 c,C

Different letters (a, b, c) in same column (days of storage and parameter) and row (A, B, C, D, etc.) (treatments) represent statistic difference according to LSD test ($P < 0.05$) by Fisher's test. AA = ascorbic acid; BC = β -carotene; Ca = calcium lactate; FW = fresh weight; GAE = GA equivalents; HWT = hot water treatment; TE = Trolox equivalents.

9.95 mg BC/100 g FW, respectively ($P < 0.05$).

Antioxidant capacity. The time of cold storage (5 °C) did not affect the ABTS radical scavenging capacity of control mangos (211.00–219.33 μmol TE/100 g FW; $P > 0.05$), but it decreased the scavenging capacity of the HWT and Ca-treated fruit (Table 1). Interestingly, HWT + Ca-treated fruit showed an increase in ABTS antioxidant capacity during cold storage, reaching the highest value after 20 d at 5 °C (221.35 μmol TE/100 g FW), which was similar to that registered for control mangos (216.5 μmol TE/100 g FW). The ABTS antioxidant capacity of mango showed positive correlation with TP, flavonoids, carotenoids, and AA (Table 2). Ripening after the removal of cold storage decreased the ABTS antioxidant capacity, except for the Ca-treated fruit that showed an increase (139.91–165.63 μmol TE/100 g FW). The ABTS radical scavenging capacity of the ripened fruit showed positive correlation with TP, total carotenoids, and AA, whereas in the case of total flavonoids this correlation was weaker and in the limit of significance ($r = 0.548$; $P = 0.065$) (Table 2).

During cold storage, HWT, Ca, and HWT + Ca decreased the DPPH radical scavenging capacity in 28.7%, 21.0%, and 21.4%, respectively, whereas this effect was less pronounced in the control (4.0%) (Table 1). The DPPH values of cold-stored mangos showed the strongest positive correlation with TP and total flavonoids, followed by a moderate correlation with total carotenoids and AA (Table 2). Considering the fruit with both cold storage and ripening, the DPPH antioxidant capacity of the HWT + Ca fruit increased with ripening, but it decreased for all other treated mangos ($P < 0.05$). After fruit ripening and considering the time of cold storage, the DPPH antioxidant capacity of the control mangos was unaffected ($P > 0.05$) but decreased in the HWT and HWT + Ca mangos ($P < 0.05$); nevertheless, at the end of storage, the HWT + Ca mangos showed the highest DPPH radical scavenging capacity (131.15 μmol TE/100 g FW; $P < 0.05$). Cold-stored and ripened mangos showed DPPH antioxidant values that were strongly correlated with TP and total carotenoids, followed by moderate correlations with total flavonoids and AA (Table 2).

Activity of antioxidant enzymes. The activity of SOD increased significantly in mango fruit stored for 20 d at 5 °C, showing significant differences ($P < 0.05$) among treatments (Fig. 3A). The highest increments were observed in the hot water-treated fruit, 1.24 times for the HWT (15.01 U·mg⁻¹ of protein) and 1.46 times for the HWT + Ca-treated fruit (17.68 U·mg⁻¹ of protein). The ripening of fruit not exposed to cold storage affected the SOD activity; HWT + Ca induced a significant reduction ($P < 0.05$), whereas Ca treatment induced a large increment (14.39 U·mg⁻¹ of protein) ($P < 0.05$). In the case of fruit stored at 5 °C for 20 d, ripening decreased significantly the SOD activity (3.83–5.33 U·mg⁻¹ of protein) and the highest value was observed in the HWT mangos.

Table 2. Linear correlation coefficients between bioactive compounds and antioxidant capacity.

	Samples without ripening				Samples ripened for 7 d at 21 °C			
	ABTS		DPPH		ABTS		DPPH	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>R</i>	<i>P</i>	<i>r</i>	<i>P</i>
Total phenolics	0.844	0.001	0.928	0.00	0.803	0.002	0.913	0.00
Total flavonoids	0.820	0.001	0.848	0.00	0.548	0.065	0.645	0.023
Total carotenoids	0.717	0.009	0.668	0.018	0.748	0.005	0.857	0.00
Ascorbic acid	0.711	0.010	0.618	0.032	0.725	0.007	0.700	0.011

r = correlation coefficient of Pearson; *P* = probability level; ABTS = 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; DPPH = 2,2-diphenyl-1-picrylhydrazil.

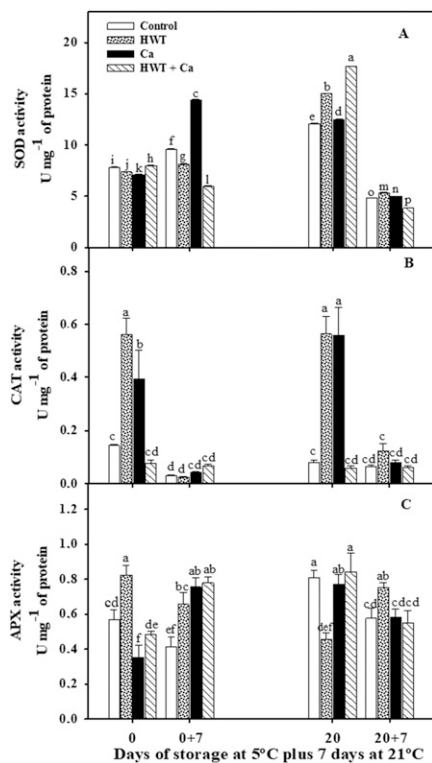


Fig. 3. Antioxidant enzymatic activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) in mango cv. Keitt after 0 and 20 d of storage at 5 °C + 7 d at 21 °C. Data are the mean of six replicates with SD represented by vertical bars. Different letters indicate significant differences among treatments ($P < 0.05$) based on the least significant difference (LSD) (SOD, $LSD = 0.08$; CAT, $LSD = 0.11$; and APX, $LSD = 0.15$, respectively) by Fisher's test.

Contrasting the effect of cold storage in mango CAT activities, the initial values (0 d) ranged from 0.07 to 0.56 U·mg⁻¹ of protein, and those of fruit with 20 d at 5 °C varied from 0.06 to 0.56 U·mg⁻¹ of protein (Fig. 3B). In both cases, the HWT and Ca-treated mangos showed higher values than the control and HWT + Ca-treated fruit; the main difference is that CAT activity of the Ca-treated mangos increased after 20 d of cold storage (0.56 U·mg⁻¹ of protein), whereas that of HWT fruit remained constant (0.56 U·mg⁻¹ of protein) ($P > 0.05$). Ripening significantly decreased the CAT activity, which was more pronounced in fruit without cold storage. The highest CAT activity after 20 d of cold storage plus 7 d of ripening was for the HWT mangos (0.12 U·mg⁻¹ of protein), but

without significant differences ($P > 0.05$) with respect to the other treatments.

At the beginning of storage (0 d), the activity of APX increased in response to the HWT and decreased in Ca-treated fruit compared with the control, whereas at 20 d of cold storage the activity of this enzyme decreased in HWT fruit ($P < 0.05$) and increased in the rest of the treatments ($P < 0.05$) (Fig. 3C). Ripening of fruit non-exposed to cold storage significantly decreased the APX activity of the control and HWT fruit in 27.3% and 20.5%, respectively, whereas in the case of HWT + Ca and Ca-treated mangos, this activity increased in 60.6% and 121.7%, respectively; however, under this storage condition only the control showed significant lower APX activity with respect to the other treatments. On the other hand, ripening of fruit with 20 d of cold storage increased the APX activity in the HWT mango, whereas the opposite was observed in the other treatments.

The best treatments to induce CI tolerance in mango cv. Keitt were HWT and HWT + Ca. In particular, the HWT + Ca-treated mangos showed the lowest %EL and MDA content. The better CI tolerance of the HWT + Ca mangos could be associated with the high activities of antioxidant enzymes and the high content of phenolics, flavonoids, and carotenoids, although, HWT mangos showed high SOD, CAT, and APX activities.

Discussion

The CI symptoms of mango cv. Keitt were more clearly observed when the cold-stored fruit was transferred to ripening conditions. Compared with the control fruit, HWTs (HWT and HWT + Ca) induced better protection against CI characterized by lower CIL. However, the lowest tissue damage was observed in the HWT + Ca mangos that showed lower EL and MDA content values. Similar results have been reported for mangos 'Zhihua' (Zhu et al., 2003), 'Kent' (Dea et al., 2010), and 'Tommy Atkins' (Almeida Miguel et al., 2016) with HWT. Yimyoung et al. (2011) reported less shriveling of the HWT mango 'Okong' (50 °C, 10 min) by the effect of cold storage (8 and 12 °C) and during ripening, reporting changes in metabolic processes that reduced the damage in cell walls, increased the protein stability and the activity of antioxidant enzymes. Up to date, the protective effect of the combination of HWT and Ca treatments against CI in mango has not been studied. However, the

HWT + CaCl₂ treatment of lettuce and kiwi resulted in better cold-stress protection than any individual treatment, maintaining cellular integrity and higher unsaturated/saturated fatty acid ratios and decreasing the stress associated with ROS accumulation (Martín-Diana et al., 2006; Shahkoomahally and Ramezani, 2015).

The CI of fruits and vegetables has been associated with oxidation of membrane lipids and an increased membrane permeability (Zhao et al., 2006); phenomena that could explain the reduced EL and MDA production of the HWT, Ca-, and HWT + Ca-treated mangos (Fig. 2). Based on these parameters, the best treatment of mango 'Keitt' against CI was the HWT + Ca. Previous studies have shown that thermal-shock treatment (cold or heat) of different mango cultivars reduced the effect of cold stress, characterized by lower EL and lipid peroxidation (MDA production) (Dea et al., 2010; Nyanjage et al., 1999; Zhao et al., 2006). Dea et al. (2010) and Nyanjage et al. (1999) related this behavior with an increase in the content of soluble solids that led to higher osmotic potential in the membrane and reduction of ion leakage; they also observed that mango fruit with higher calcium content showed lower EL and improved membrane integrity. The calcium ions interact with membrane phospholipids, stabilizing the membrane and maintaining a gradient of protons (Ca²⁺, H⁺). Also, calcium interferes with the catalytic activity of lipid enzymes and reduces the overproduction of ROS (Mao et al., 2007). The calcium protective effect of membranes was also observed in cold-stressed fruit such as mangos 'Choke Anan' (Khaliq et al., 2016), 'Tommy Atkins', and 'Kent' (Ngamchuachit et al., 2014); as well as in tomato (Aghdam, 2013) and lemon (Safizadeh et al., 2007).

All postharvest treatments of cold-stressed 'Keitt' mango increased the content of phenolics and flavonoids (Table 1), but only the HWT and Ca treatments increased these bioactive compounds during ripening. Aghdam et al. (2015) suggested that HWT produces a moderate stress in the tissue that is associated with the synthesis of antioxidant compounds involved in the neutralization of ROS excess; whereas calcium stabilizes and protects cellular walls and membranes from oxidative stress and reduces the polyphenol oxidase (PPO) activity avoiding the oxidation of phenolic compounds (Leyva-López et al., 2011). Contrasting with our results, the levels of phenolics in 'Choke Anan' and 'Nam Dok Mai' mangos cold stored (4 °C, 30 d) and ripened (27 to 28 °C, 6 d) did not change (Chidtragool et al., 2011); whereas 'Ataulfo' variety stored for 15 d at 5 °C showed a minimum reduction (Robles-Sánchez et al., 2009). In this regard, the application of hot water (Talcott et al., 2005) or calcium salts (Khaliq et al., 2016; Zhao et al., 2006) resulted in the accumulation or retention of different compounds during cold storage. Chidtragool et al. (2011) indicated that greater phenolic contents in cold-stored fruit were associated with increased activities of phenylalanine ammonia lyase and PPO, and with inhibition of

enzymes involved in cell-wall degradation, providing tolerance also to CI.

The highest AA contents in the cold-stressed mangos were registered for HWT and Ca-treated fruit. The AA content decreased during ripening for all treatments (Table 1). Contrasting with our results, Djoua et al. (2009) and Zhao et al. (2006) found a retention and reduction of AA in cold-stored mangos pretreated by cold- and heat treatment, respectively. On the other hand, our results were similar to those registered by Dea et al. (2010) who reported that AA increased in heat-treated mangos; this result was related with higher activity of L-galactose-γ-lactone dehydrogenase, which is responsible for the synthesis of ascorbate in tissue damaged by thermal stress, as well as with the presence of dehydroascorbic acid included in the quantification. In the present study, the combination HWT and Ca (HWT + Ca) maintained the AA content in 'Keitt' mangos. Calcium lactate could improve the cell-wall firmness by favoring the formation of a larger net of cross-linked pectin; thus, cellular turgor and membrane integrity may be better maintained avoiding the AA oxidation (Abd-Elhady, 2014). Khaliq et al. (2015, 2016) treated mango with CaCl₂ and observed less AA reduction during cold stress compared with the combination of CaCl₂ with arabic gum. Another study demonstrated that HWT (46.1 °C, 75–90 min) combined with AA–citric acid–CaCl₂ in minimally processed mangos reduces the loss of AA during cold storage of 'Ataulfo', 'Kent', and 'Keitt' mangos (González-Aguilar et al., 2008).

All postharvest treatments of 'Keitt' mangos increased the total carotenoids, and the highest values at the end of the storage and ripening were registered for the Ca- and HWT + Ca-treated fruit (Table 1). Similar results were registered for 'Tommy Atkins' mango pretreated with hot water (50 °C, 60 min) during cold storage, although this cultivar did not show an increase in total carotenoids during ripening (Talcott et al., 2005). Djoua et al. (2009) observed that HWT (50 °C for 30 min) of 'Keitt' mango increased its total carotenoids, but the treatment at 46 to 50 °C for 75 min caused a reduction in these compounds. It has been proposed that heat treatment promotes cell membrane disruption, chemical extractability, and depending on the time and temperature, oxidation of carotenoids (Djoua et al., 2009). On the other hand, González Aguilar et al. (2008) showed that the combination of HWT + antioxidant solution increased the synthesis of carotenoids in minimally processed mangos during the first days of cold storage, with the highest rate observed during ripening. By contrast, the combination of HWT and CaCl₂ in cold-stored papaya (Ayón-Reyna et al., 2015), carrots, and eggplants (Chepango et al., 2016) reduced the BC content.

Mango pulp is rich in bioactive compounds with reducing power and free radical-scavenging capacities (Ma et al., 2011). In our research, the antioxidant capacity of the control fruit was significantly higher than

all the treated fruit during the first days of cold storage (Table 1). The increased content of phenolics and flavonoids in the control fruit improved its antioxidant properties. These results agree with previous studies where the antioxidant capacity of different cold-stored mango cultivars was correlated with the polyphenols, flavonoids, carotenoids, and AA contents, providing tolerance to CI (Barman and Asrey, 2014; Chongchatuporn et al., 2013). However, the better balance of bioactive compounds and antioxidant capacity of the cold-stored control fruit did not correspond with the CI sensitivity. After ripening, Ca- and HWT + Ca-treated mangos showed higher antioxidant capacity than the control and HWT fruit. This pattern could be explained by a reduced metabolic activity of heat-treated mangos that showed an impaired synthesis of phenolics and flavonoids (Table 1), limiting their potential for ROS scavenging (Ummarat et al., 2011), whereas the increase for the Ca-treated mangos could be due to higher contents of phenolics and flavonoids. Contrasting with our results, Kim et al. (2007) and Talcott et al. (2005) showed that heat treatment does not affect the polyphenolic content and antioxidant capacity of cold-stored mangos. Khaliq et al. (2016) reported that cold-stored mangos pretreated with a combination of arabic gum–CaCl₂ showed a CI reduction, a phenomenon associated with an increased synthesis of phenolic compounds, flavonoids, and vitamins, as well as with a higher antioxidant capacity.

Belhadj et al. (2016) correlated the cold-stress sensitivity of mango with both the accumulation of bioactive compounds and the activity of antioxidant enzymes. The coordinated actions of SOD, CAT, and APX, among other antioxidant enzymes, are very important for ROS scavenging, cell membrane protection, and CI reduction (Wu et al., 2014). Different pretreatments of mango have improved its antioxidant system and cold-stress response (Lopes et al., 2016; Ren et al., 2017; Yimyoung et al., 2011). In the present study, HWT + Ca mangos showed increased SOD and APX activities during cold storage, whereas CAT activity was higher in the HWT and Ca-treated fruit. During ripening, SOD and APX activities were higher in HWT mangos, and all-treated mangos showed similar CAT activity (Fig. 3). Thus, the better protection of hot water-treated 'Keitt' mangos (HWT and HWT + Ca) against CI seems to be mainly associated with increased activities of SOD and APX. Wang et al. (2008) and Wu et al. (2014) have shown that high activity of fruit antioxidant enzymes decrease the ROS production and CII. Mango fruit with increased SOD, CAT, and peroxidase activities and reduced lipoxigenase and PPO activities showed a reduced concentration of MDA, superoxide anion, and hydroperoxides, as well as higher CI tolerance (Ren et al., 2017). Yimyoung et al. (2011) demonstrated that heat treatment increased the CAT activity and CI tolerance of mango; however, the activities and transcription of APX and Mn-SOD were reduced. Considering the Ca

treatment, similar results were reported for grafting eggplant seedlings that showed induction of SOD, CAT, and APX activities during cold storage (Gao et al., 2004); the authors suggested that calcium treatment increases the activities of protective enzymes and reduces CI. However, calcium-treated pear fruit showed a reduction in the enzyme activities which resulted higher along with the time, even than the control, and a reduction in their CII (Kou et al., 2015). The effect of the combination HWT + Ca on the activities of antioxidant enzymes has not been previously reported; thus, this is an important contribution for the fresh market of mango by considering that the HWT and HWT + Ca treatment improves the activities of antioxidant enzymes, mainly SOD and APX, and reduces the CI.

The HWT, alone or combined with calcium lactate treatment (HWT + Ca), induced CI tolerance of mango fruit cv. Keitt during storage at 5 °C for up to 20 d and ripening. The HWT + Ca treatment provided CI tolerance, which seems to be associated with lower membrane permeability, highest content of some bioactive antioxidant compounds and the highest activities of SOD and APX during cold storage. Remarkably, the activities of the same two enzymes were higher during ripening of HWT mangos, showing also increased levels of TP and total flavonoids.

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