Hydrothermal treatment affects the ascorbic acid content and other quality parameters in 'Ataulfo' mango

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Abstract

L-ascorbic acid is a natural antioxidant produced by plants and their fruits. The abundance of this acid during the development and postharvest ripening of mangoes (*Mangifera indica* L.) depends on genetic and climatic factors. The mangoes undergo a quarantine hydrothermal treatment before being exported to control the fruit fly. A quarantine hydrothermal treatment consists of immersing mangoes in water at 46.1 °C (118 °F) for 60 to 120 min depending on the size, affecting the L-ascorbic acid content and other quality parameters of the fruit. This research aimed to evaluate the effect of QHT on L-ascorbic acid content, firmness, color, and expression of L-ascorbic acid biosynthesis and recycling genes during mango postharvest. 'Ataulfo' mangoes were harvested at physiological ripeness in Escuinapa, Sinaloa in 2019, subjected to QHT (46.1 °C, 75 min), hydrocooled (25 °C, 30 min) and stored at 20 °C for nine days. Mangoes subjected to QHT showed a reduction in L-ascorbic acid content ($p \leq 0.05$). The firmness of the pulp behaved similarly in fruits with QHT and in control fruits, while the external color of the fruits with a quarantine hydrothermal treatment indicated an advanced ripening process compared to the control fruits ($p\# 0.05$). The levels of MiGME1, MiGME2, MiGGP2, and MiMDHAR transcripts increased in response to treatment, suggesting activation of the synthesis and recycling pathway to counteract the abiotic stress caused by the heat to which the mangoes were subjected.

Keywords:

Mangifera indica, heat stress, gene expression, ripening.

Introduction

L-ascorbic acid (L-AA) is an important antioxidant for growth, cell division, flower development, and synthesis of hormonal coenzymes in plants; it is also involved in responses to abiotic stress (Smirnoff, 2018; Fenech *et al.*, 2019).

It has been observed that postharvest ripening and abiotic stress conditions, such as heat, can decrease the L-AA content in fruits (Mditshwa *et al.*, 2017). As a result, there is a growing need to understand the molecular underpinnings of L-AA metabolism to develop more nutritious fruits and abiotic stress-tolerant crops in the context of climate change challenges (Macknight *et al.*, 2017).

The main pathway of L-AA synthesis described in plants is the Smirnoff-Wheeler (SW) pathway (Wheeler *et al.*, 1998), made up of nine enzymatic reactions discovered in *Arabidopsis thaliana* (Dowdle *et al.*, 2007). In fruits such as kiwi, it has been observed that the GDP-mannose epimerase (GME) and GDP-galactose phosphorylase (GGP) enzymes are key points within the SW pathway, in such a way that overexpression or mutation in their genes leads to an increase or decrease in the accumulation of L-AA, respectively (Tao *et al.*, 2018; Liu *et al.*, 2022).

In addition, L-AA consumed during oxidative stress is able to regenerate through the recycling pathway, where the mono (MDHAR) and dehydroascorbate reductases (DHAR) and ascorbate oxidase enzymes participate, maintaining L-AA homeostasis in the plant cell (Paciolla *et al.*, 2019).

The 'Ataulfo' mango fruits are rich in L-AA and are subjected to a hydrothermal treatment (QHT) which consists of immersing the mangoes in hot water (46.1 °C) for 60-120 min depending on the weight of the fruit. This treatment guarantees the quality of the fruit, preventing pest infestation, eliminating the larvae of flies of the genus *Anastrepha* (Hernández *et al.*, 2012; USDA, 2017).

While there is a lot of information on physiological changes due to heat treatments on mangoes, there are limited studies on the metabolism and synthesis of L-AA in fruits in the postharvest stage. There are reports on the molecular response of L-AA metabolic pathways to antioxidant content during a variety of stress conditions, including light (Jiang *et al.*, 2018), heat (Li *et al.*, 2016), drought and salt (Wang *et al.*, 2017; Galli *et al.*, 2019), showing differential expression levels of L-AA-related genes as a result of the stressor.

Previous studies on 'Ataulfo' mango fruits subjected to QHT reveal an acceleration of the ripening process and induction of genes related to heat shock and oxidative stress (Dautt-Castro *et al.*, 2018). Javed *et al.* (2022) showed a decrease in antioxidant capacity, phenolic compounds, and L-AA at day 21 of storage in QHT-subjected 'Chenab Gold' mango fruits. These findings suggest that there is a molecular response to an oxidative process triggered by heat, which causes abiotic stress to the fruits.

The aim of this study was to evaluate the effect of QHT on L-AA content, firmness, fruit color, and expression of biosynthesis and recycling genes in 'Ataulfo' mango at different stages of ripeness.

Materials and methods

The 'Ataulfo' mango fruits were collected at the Huerta Díaz in Escuinapa, Sinaloa, Mexico in a state of physiological ripeness (120 days after flowering). Uniformity of color and weight (approximately 250 g) and the absence of apparent damage to the fruits were considered and the fruits were transported to CIAD-Culiacán. They were randomly classified and subjected to QHT conditions (immersion of the fruit in hot water at 46.1 °C for 75 min), followed by hydrocooling in water at 25 °C for 30 min (USDA, 2017).

Fruits with QHT and a control group (without QHT) were stored at 20 °C for nine days (consumption ripeness). We worked in triplicate using one fruit as an experimental unit. The fruits were sampled 1 h after the treatment was applied (day 0), and on days 1, 2, 5 and 9, according to firmness and color parameters. The fruit pulp was immediately frozen and kept at -20 °C until processing for L-AA content analysis and total ribonucleic acid (RNA) extraction for gene expression evaluation.

Determination of L-ascorbic acid content by high-pressure liquid chromatography (HPLC

Total L-AA content was quantified by HPLC according to Doner and Hicks (1981) with modifications. L-AA was extracted from 10 g of pulp sample using an extraction solution of 0.375 mol L^{-1} of metaphosphoric acid dissolved in an 8% glacial acetic acid solution. An NH_2 analytical column (250 x 4.6 mm internal diameter, particle size of 5 μm and loop injector of 10 μl) and a mobile phase of 0.0125 mol L^{-1} of monobasic sodium phosphate dissolved in a 75% acetonitrile solution were used. Total L-AA was determined by UV-visible light at a wavelength of 268 nm and sodium ascorbate was used as standard. L-AA is shown as mg per 100 g fresh weight (mg 100 g^{-1} FW).

Determination of firmness and color of peel and pulp

Pulp firmness was measured in duplicate in each fruit in the equatorial zone using a Lloyd-Ametek LS1 (USA) digital texture meter with an 8 mm diameter tip. The results were expressed in Newtons (N) (Cárdenas-Coronel et al., 2012).

The external (peel) and internal (pulp) color of the fruits were measured with a Konica Minolta CM-700d (USA) portable colorimeter, using the CIE $L^*a^*b^*$ color space. Peel color was measured in the equatorial zone on opposite sides of the fruit, while pulp color was taken at two opposite points around the seed (Siller-Cepeda et al., 2009). The parameters Hue angle (°Hue), luminosity (L^*), and chromaticity were calculated (Konica Minolta, 2007).

Total RNA extraction, purification and synthesis of copy deoxyribonucleic acid (cDNA)

Total RNA was extracted from 0.5 g of mango pulp pulverized in liquid nitrogen using the method proposed by López-Gómez and Gómez-Lim (2019). One milliliter of lysis buffer (2% sodium dodecyl sulfate, 50 mM ethylenediaminetetraacetic acid, 150 mM Tris-Base, 1% β-mercaptoethanol, pH 7.5, adjusted with 1M boric acid) was added to the sample. Chloroform-isoamyl alcohol (49:1) and phenol-chloroform (1:1) were added, it was mixed and centrifuged at 13 000 x g.

RNA was precipitated by adding 3 M lithium chloride and kept at -20 °C overnight. The next day, the sample was centrifuged for 10 min at 20 000 x g. The pellet obtained was washed twice with 75%ethanol and resuspended in water treated with diethylpyrocarbonate. RNA integrity was confirmed by agarose gel electrophoresis under denaturing conditions. Total RNA was purified to remove residual DNA using DNase I (Roche, USA). cDNA was synthesized from 5 μg of total RNA using the SuperScript III RT reagent set (Invitrogen, USA) according to the manufacturer's instructions.

Evaluation of gene expression by quantitative polymerase chain (qPCR) amplification

qPCRs were performed using 40 ng of cDNA from treated and control mango fruits. Each biological replication was measured in triplicate by qPCR using the iTaq Universal SYBR Green reaction mix (Bio-Rad, USA) and the StepOne real-time thermal cycler (Applied Biosystems, USA) (Dautt-Castro et al., 2018).

Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) and normalized with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) constituent gene. Data from days 0, 1, 2, and 5 were compared with data from day 9 of storage (calibrator) using the formula: $\Delta\Delta Ct$= (Ct gene of interest - Ct GAPDH)$_{Day\ 0,1,2,5}$ - (Ct gene of interest - Ct GAPDH)$_{Day\ 9}$.

The relative expression changes of each gene were calculated using the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT$= (Ct gene of interest - Ct GAPDH)$_{treatment}$ - (Ct gene of interest - Ct GAPDH)$_{control}$. If the first ΔCt is greater than the second ΔCt, the value of $2^{-\Delta\Delta CT}$ will be<1, indicating a reduction in gene expression levels due to the effect of the QHT applied. To evaluate the level of reduction in expression, the negative inverse of $2^{-\Delta\Delta CT}$ is obtained. Pairs of primers for MiGME1, MiGME2, MiGGP1, MiGGP2,

MiDHAR, and MiMDHAR were designed using the PrimerQuest tool (Integrated DNA Technologies, USA) (Table 1).

Table 1. Mango L-ascorbic acid synthesis and recycling enzymes and primer sequences to evaluate expression of MiGME1, MiGME2, MiGGP1, MiGGP2, MiDHAR and MiMDHAR mRNAs.

Enzyme	Access key GenBank	Small (aa)	%ID, access key NCBI	Access key ARNm *M. indica* 'Ataulfo'	Starter sequence (5'-3')
MiGME1	UPT49960.1	376	GDP-mannose 3,5-epimerase 2 [*Carica papaya*] (97.1%) XP-021891285.1	MW447158.1	Fw GAGCTTGAGAGGGAACTTTAC Rv TTCAACCGACGAGCAATG
MiGME2	UPT49961.1	355	GDP-mannose 3,5-epimerase 2 [*Acer yangbiense*] (95%) TXG72149.1	MW447159.1	Fw CCCATTCAGCACATTCCT Rv CAGCCCATCCTTCAGTTT
MiGGP1	UPT49962.1	370	GDP-L-galactose phosphorylase 2-like isoform X1[*Pistacia vera*] (81%) XP-031249525.1	MW447160.1	Fw CAGAATCAACCACCCTGAAG Rv GATGGCACCATTAGGTACAG
MiGGP2	UPT49963.1	449	GDP-L-galactose phosphorylase 2 [*Pistacia vera*] (91%) XP-031286515.1	MW447161.1	Fw GAAAAGGAGAATAGCGAGCC Rv CACAGGCAGTGACATCATAG
MiDHAR	UPT49964.1	213	Glutathione S-transferase DHAR2-like [*Citrus sinensis*] (81%) XP-006486019.1	MW447162.1	Fw TTACAAGGCACACCACATC Rv GAGTCAGGCACCCATTTATC
MiMDHAR	UPT49965.1	434	Monodehydroascorbate reductase [*Pistacia vera*] (95%) XP-031249639.1	MW447163.1	Fw GGGTGACACAGTGCTATTT Rv CCAACAACCTTCCCATCTT

The mRNAs were obtained from the transcriptome of Mangifera indica 'Ataulfo' (GenBank PRJNA286253). The primers were designed based on the nucleotide sequence of the coding regions of the genes of interest using the PrimerQuest tool. Length= length of the gene or protein product (amino acid sequence), %ID= percentage of identity.

Statistical analysis

An Anova was performed considering QHT and ripeness stages as factors, the response variables were L-AA content, firmness, external color and internal color, and relative gene expression. In case of significant differences, Tukey's test was performed with a confidence level of 0.95. NCSS (2022) and GraphPad Prism 7 computer programs were used for statistical analysis and graphs, respectively. The data represent the mean±SE (standard error of the mean).

Results and discussion

L-AA content in 'Ataulfo' mango with QHT

The total L-AA content ($p \leq 0.05$) decreased by approximately 12% due to the effect of QHT on the fruits on day 1 and day 9 (Figure 1). L-AA content ranged from 200.75 to 100.8 mg 100 g^{-1} FW from its peak (day 1, control) to the lowest value (day 9, QHT). The treatment-associated reduction in L-AA content (46.1 °C, 75 min) is consistent with the findings of Djioua *et al.* (2009), under similar conditions for 'Keitt' mango.

Figure 1. Effect of THC on the L-ascorbic acid content (mg 100 g-1 fresh weight) of mango fruit during postharvest ripening at 20°C. Data represent the mean of three biological replicates ± the standard error. Different lowercase letters indicate significant differences between the days of postharvest ripening in the control mangoes. Different capital letters indicate significant differences between the days of postharvest ripening in the treated mangoes. Significant differences between control mangoes and treated mangoes from each day are indicated with an asterisk. Tukey test ($p < 0.05$).
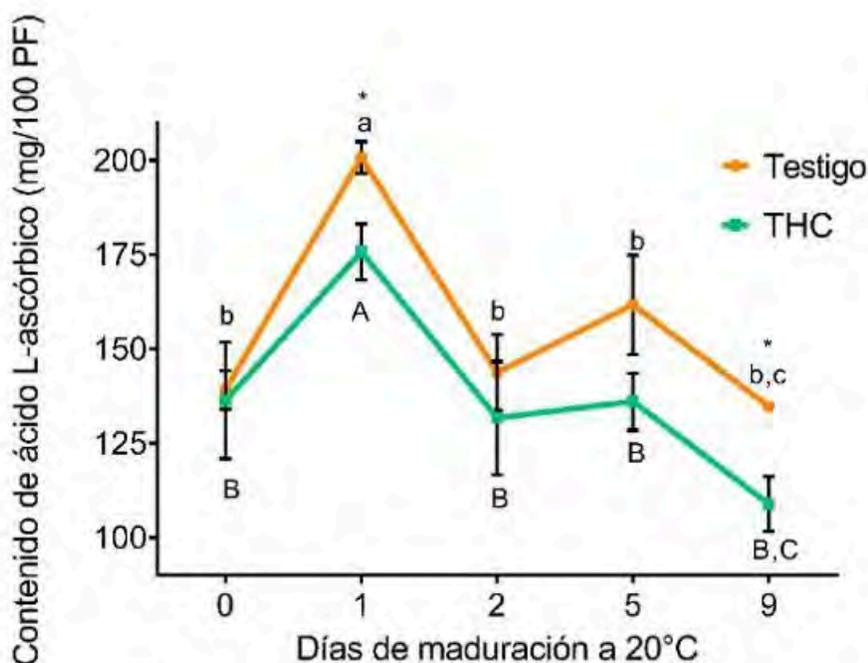
Previous reports show L-AA values of 158.5 mg 100 g^{-1} FW (Robles-Sánchez *et al.*, 2009) for this cultivar. Manthey and Perkins-Veazie (2009) reported values of 125.4 ±6.4, 24.7 ±7.9, 25.6 ±4.9, 19.3 ±4.8, and 31 ±5.2 mg 100 g^{-1} FW, for the cultivars 'Ataulfo', 'Keitt', 'Kent', 'Tommy Atkins' and 'Haden', respectively.

Studying the differences behind the accumulation of this antioxidant in L-AA-rich cultivars, varieties, and species is of interest (Mellidou *et al.*, 2012; Zhang *et al.*, 2016; Chiaiese *et al.*, 2019). The 'Ataulfo' mango, being a high-value cash crop, is a prospective candidate for this purpose as it shows high concentrations of L-AA despite the effect of QHT, covering the recommended daily amount for women and men (75 and 90 mg day^{-1}, respectively) (Castillo-Velarde, 2019).

Effect of QHT on the firmness and color of 'Ataulfo' mangoes

Figure 2 shows the same behavior in the loss of firmness throughout postharvest storage in control and treated mangoes. Firmness was maintained in fruits on days 0 and 2, with a particular peak on day 1. After day 2 of postharvest storage, firmness decreased by about 95% between fruits on day 1 and fruits on day 9 ($p < 0.05$).

Figure 2. Effect of THC on the firmness (Newton) of mango fruit during postharvest ripening at 20°C. Data represent the mean of three biological replicates ± the standard error. Different lowercase letters indicate significant differences between the days of postharvest ripening in the control mangoes. Different capital letters indicate significant differences between the days of postharvest ripening in the treated mangoes. Significant differences between control mangoes and treated mangoes from each day are indicated with an asterisk. Tukey test ($p \leq 0.05$).
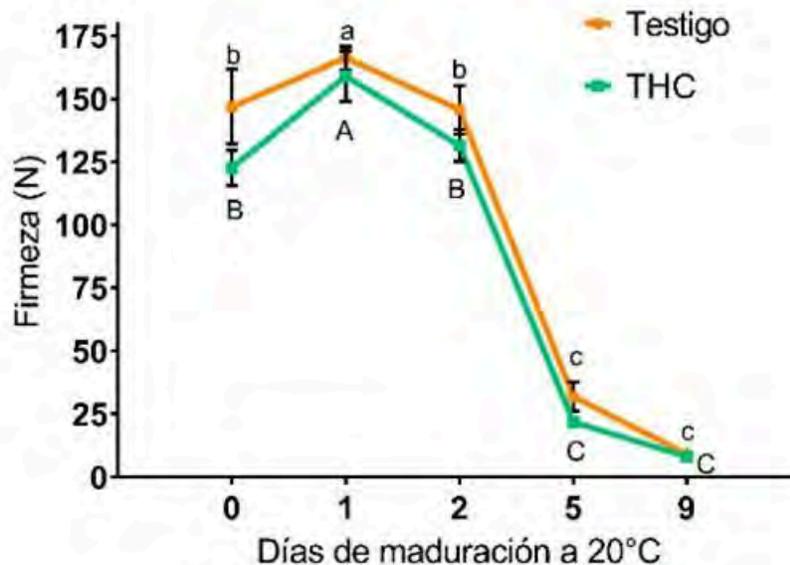
The loss of firmness is consistent with previous findings (Luna-Esquivel *et al.*, 2006); however, Dautt-Castro *et al.* (2018) reported differences in the loss of firmness in the treated fruits (QHT) compared to the control, where the firmness of the QHT fruits decreased two days before the decrease in the controls.

Possibly, these differences with respect to our results are due to the fact that in this study, the mangoes with QHT were immersed in hot water under the conditions proposed by the United States Department of Agriculture (USDA) in a controlled laboratory environment. In contrast, the results reported above come from QHT in commercial packaging plants, possibly with less control over treatment conditions.

The color of the peel and pulp are essential quality parameters for consumers and producers. The °Hue, L* and chromaticity values that were measured are illustrated in Figures 3A (external color) and 3B (internal color).

Figure 3. A) and B) effect of THC on external and internal color parameters (Hue angle, luminosity, chromaticity) of mango fruit during ripening at 20°C. Data represent the mean of three biological replicates ± the standard error. Different lowercase letters indicate significant differences between the days of postharvest ripening in the control mangoes. Different capital letters indicate significant differences between the days of postharvest ripening in the treated mangoes. Significant differences between control mangoes and treated mangoes from each day are indicated with an asterisk. Tukey test ($p \leq 0.05$). C) visual appearance of the mango fruit during its ripening at 20 °C.
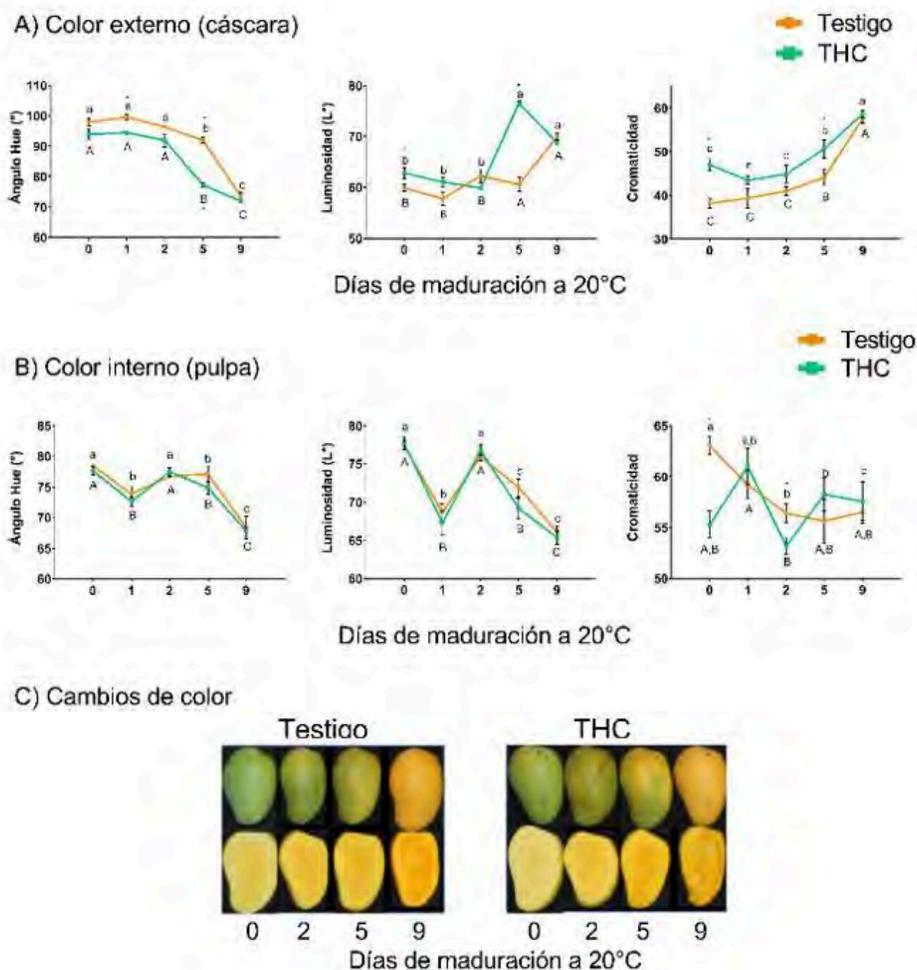
In general, the peel and pulp of treated and control mangoes changed from dark green to light green and finally to a lighter, more intense shade of yellow during postharvest storage. QHT affected all color parameters in the peel ($p \leq 0.05$) on day 5 of storage, that is, in the climacteric. Regarding chromaticity, mangoes with QHT showed values higher than the control group, indicating a more vigorous intensity of color ($p \leq 0.05$).

Regarding the internal color (Figure 3B), only the effect of QHT ($p < 0.05$) was observed in the chromaticity parameter. Mango fruits changed from a bright, intense yellow color to a darker, more subdued shade of orange throughout postharvest storage for both groups.

Mango fruit peel color developed faster in fruits with QHT (Figure 3C), consistent with previous work in 'Ataulfo' mango subjected to QHT (Luna-Esquivel *et al.*, 2006; Dautt-Castro *et al.*, 2018) and 'Keitt' mango (Ibarra-Garza *et al.*, 2015). A series of reactions occur during ripening, including chlorophyll degradation, a phenomenon that accelerates when fruits are subjected to QHT due to

photosynthesis inhibition (Wang *et al.*, 2018; Zhang *et al.*, 2018). Carotenoids that are responsible for the yellow-orange color characteristic of the 'Ataulfo' mango are also synthesized (Contreras-Vergara *et al.*, 2022).

Expression changes in L-AA synthesis and recycling pathway genes and effects of QHT

Figure 4 shows the effect of QHT on the relative expression of the MiGME1, MiGME2, MiGGP1, MiGGP2 mRNAs of the L-AA synthesis pathway and of MiDHAR and MiMDHAR, of the L-AA recycling pathway, in mango fruits during postharvest ripening. In most genes, QHT stimulated mRNA levels, especially in fruits at day 2 (Figures 4A and 4B). The MiGME1 mRNA was stimulated during postharvest ripeness, similar to L-AA content, which remained constant.

Figure 4. Effect of THC on the abundance of L-ascorbic acid metabolism transcripts of mango fruit during postharvest ripening at 20°C. A) L-ascorbic acid biosynthesis transcripts: MiGME1, MiGME2, MiGGP1 and MiGGP2. B) L-ascorbic acid recycling transcripts: MiMDHAR AND MiDHAR. Data represent the mean of three biological replicates ± the standard error. Different lowercase letters indicate significant differences between postharvest ripening days. Tukey test (*p*< 0.05).
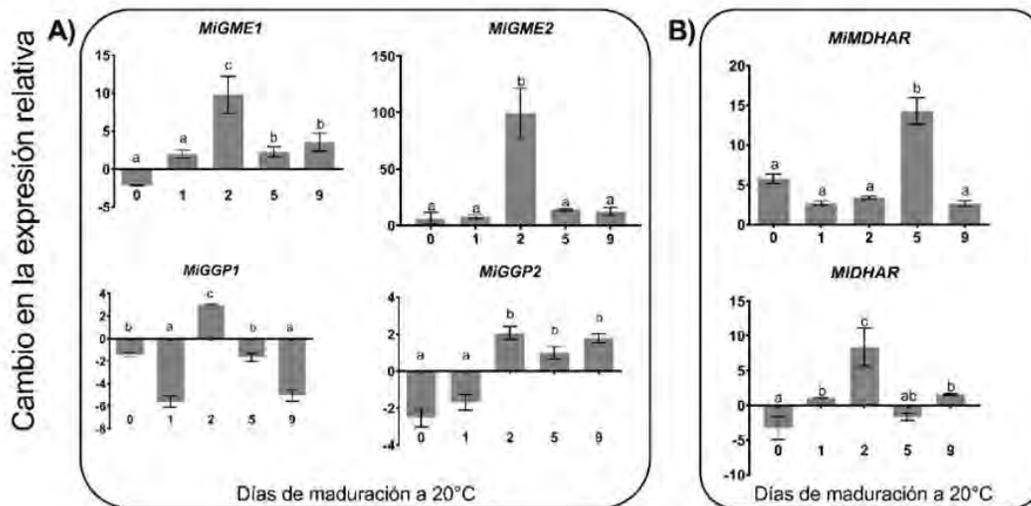
The MiGME2 mRNA was stimulated from day 0 (immediately after QHT was applied), showing the greatest increase in fruits at day 5 (100 times more than the control fruits), indicating an apparent greater sensitivity to heat. The MiGGP1 mRNA was only induced in fruits on day 2, while MiGGP2 was induced in fruits on days 2, 5 and 9.

Similar results were reported by Li *et al.* (2013) in kiwi leaves subjected to heat stress (42 °C), which showed repression of the GGP mRNA at 12 and 24 h after exposure; nevertheless, at 48 h, an increase in expression was observed. In the tea plant (*Camellia sinensis*), both GME and GGP are induced during heat stress (Li *et al.*, 2016), although with different responses during the first hours after treatment.

As shown in Figure 1, the L-AA content decreased due to the effect of QHT; nonetheless, in fruits at days 2, 5 and 9, the concentration of L-AA was similar to that of fruits at day 0 (*p*< 0.05), which can be related to the increase in expression of L-AA synthesis genes (Figure 4A). As mentioned above, L-AA biosynthesis is finely regulated, also involving stress-sensitive transcription factors and L-AA-

related gene regulators such as ethylene response factor 98 (ERF98), ascorbic acid regulator 1 (AMR1), and HD-ZIP1 (Bulley and Laing, 2016; Mellidou and Kanellis, 2017).

Regarding the expression of genes of the recycling pathway, the MiMDHAR mRNA increased in fruits at days 0, 1, 2, 5, and 9 (Figure 4B). The highest levels are observed in fruits at day 5 (climacteric), with a 15-fold increase due to QHT. For its part, the MiDHAR mRNA increased in fruits on days 1, 2, and 9. Their highest expression levels were 8-fold in fruits at day 2. The development of mRNAs from the recycling pathway due to QHT suggests that it is possible to conserve a constant amount of L-AA to counteract the heat stress to which the fruit is subjected added to the stress that comes with ripening *per se*.

In acerola, there was a positive correlation between changes in the gene expression of MDHAR and DHAR and their enzymatic activities under stress and ripening conditions (Eltelib *et al.*, 2011). The importance of MDHAR and DHAR enzymes lies in their ability to recycle their respective oxidized forms of L-AA (DHA and MDHA), preventing further irreversible oxidation into 2,3-diketogulonic acid (Paciolla *et al.*, 2019).

In this context, MDHAR and DHAR could be the first responders in maintaining L-AA homeostasis during abiotic stress conditions. QHT could likely have tipped the balance towards a greater accumulation of reactive oxygen species (ROS), resulting in a reduction in total L-AA content and stimulation of genes related to both L-AA biosynthesis and recycling. In this research, the focus was on gene expression levels, in the future it will be possible to determine the activity of enzymes of L-AA metabolism in mango fruits.

Conclusions

QHT caused a reduction in L-AA in the treated mangoes, indicating an effect of the heat or active oxidative process, so further work could focus on oxidative stress. This reduction did not alter the overall nutritional quality of the fruit. Likewise, QHT affected parameters such as external color, indicating early ripening, without damaging the quality of the fruit, which showed a more vigorous intensity of color. Changes in the expression levels of genes related to L-AA metabolism are evident.

In general, QHT stimulated MiGME1, MiGME2, MiGGP2, and MiMDHAR mRNAs, especially after day 2 of postharvest ripening. So, these genes are considered more sensitive to heat, making them potential candidates for future research. Therefore, it is possible to propose that regulation in response to QHT occurs at the transcriptional level in mango fruit tissue due to the abiotic stress caused by heat treatment and the ripening process during postharvest storage. This work can serve as a basis for the current landscape of horticultural crop research to develop stress-tolerant L-AA-rich crops in response to the challenges posed by climate change.

Acknowledgements

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Hydrothermal treatment affects the ascorbic acid content and other quality parameters in 'Ataulfo' mango