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Expression of antioxidant-related genes in flavedo of cold-stored grapefruit (*Citrus paradisi* Macfad cv. Rio Red) treated with pectic oligosaccharides

Araceli M. Vera-Guzmán[a,b], Emmanuel Aispuro-Hernández[a], Irasema Vargas-Arispuro[a], María A. Islas-Osuna[a], Miguel Ángel Martínez-Téllez[a,*]

[a] Centro de Investigación en Alimentación y Desarrollo, A. C. Ave. Gustavo E. Astiazarán S/N, Col. La Victoria, 83304, Hermosillo, Sonora, México
[b] Instituto Politécnico Nacional, CIIDIR Unidad Oaxaca, Hornos # 1003, 71230, Santa Cruz Xoxocotlán, Oaxaca, México

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ABSTRACT

Cold storage is one of the main postharvest technologies for fruit preservation, however, cold-sensitive citrus fruits develop peel physiological alterations affecting external quality known as chilling injury (CI). An efficient antioxidant system has been associated to an enhanced cold stress tolerance in citrus fruits. The application of pectic oligosaccharides (POs) stimulates the enzymatic antioxidant system in plants and reduces CI development in grapefruit fruit (*Citrus paradisi*) during cold storage. The aim of this study was to contribute to the basic understanding of peel physiological disorders in cold-stressed 'Rio Red' grapefruit fruit at ultrastructural level and to determine whether the POs are involved in the CI reduction by modulating the antioxidant enzymatic system at the transcriptional level. Peel morphology in grapefruit fruit stored at chilling (2 °C) and non-chilling (13 °C) temperatures was analyzed by electronic microscopy and the effect of POs treatment on *manganese superoxide dismutase* (*MnSOD*), *ascorbate peroxidase* (*APX1*), *catalase* (*CAT1*) and *glutathione reductase* (*GR2*) gene expression was investigated by RT-qPCR. Results indicate that a prolonged cold storage promoted the incidence of CI symptoms in 'Rio Red' grapefruit and altered the ultrastructural morphology of flavedo epidermal tissue. POs significantly modulated the *MnSOD, APX1* and *CAT1* expression levels mainly in a storage time- and temperature-dependent manner with regards to controls. By contrast, POs only significantly affected the *GR2* gene expression when grapefruit were stored at non-chilling temperatures. Our results revealed a possible involvement of the *MnSOD, APX1* and *CAT1* genes in the CI susceptibility reduction induced by POs in 'Rio Red' grapefruit stored at chilling temperatures.

1. Introduction

Cold storage has been one of the main strategies for maintaining good quality and increasing the shelf life of citrus fruits (Ladaniya, 2008). Nevertheless, a prolonged cold exposure causes cold stress, which in turn may lead to the occurrence of severe physiological disorders, such as chilling injury (CI), in susceptible citrus varieties (Dou, 2005). In citrus fruits, the main macroscopic CI symptoms include peel pitting and superficial scalding, however, the effect of cold storage on peel ultrastructural features has been much less addressed. Some of the well-known physiologic and metabolic alterations induced by cold stress in plant tissues, include variations on the structure and composition of epidermal tissue (Vercher et al., 1994), decrement of cellular and organelle membrane fluidity and integrity due to up-regulation of genes associated with cell wall and membrane degradation (Lafuente et al., 2017; Kasamo et al., 2000) and quick accumulation of reactive oxygen species (ROS) involved in triggering signaling events (Ruelland et al., 2009). Even though ROS are involved in tolerance to different environmental stresses (Asada, 2006; Xia et al., 2009), their overproduction along with the lack of efficient detoxifying mechanisms may compromise cell viability by causing oxidative damage (Ruelland et al.,2009; Racchi, 2013). To maintain cell homeostasis and minimize undesired physiological disorders induced by low temperatures, plants draw on endogenous antioxidant systems including antioxidant enzymes and small molecules such as ascorbic acid and glutathione (Racchi, 2013; Noctor and Foyer, 1998; Mittler, 2002).

The efficiency of antioxidant enzymatic systems is a determining factor in the tolerance to cold-related oxidative stress in citrus fruits; e.g. cold tolerant cultivars of mandarin and orange fruits presented a higher catalase (CAT) activity than a chilling sensitive cultivar (Sala, 1998; Sala and Lafuente, 1999; Sala et al., 2005). An increased cold stress tolerance was achieved by inducing either the *CAT* gene

* Corresponding author.
E-mail address: norawa@ciad.mx (M.Á. Martínez-Téllez).

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expression levels or its enzymatic activity after different kinds of conditioning treatments (Sala and Lafuente, 1999; Ghasemnezhad et al., 2008; Maul et al., 2011). Similarly, increments in activity of the antioxidant enzymes CAT, ascorbate peroxidase (APX) and glutathione reductase (GR) conferred chilling tolerance to lemon fruit (Siboza et al., 2017). Therefore, modulation of the antioxidant enzymatic systems seems to be an effective approach to induce cold tolerance in citrus fruits with different susceptibility.

Some pectin-derived biologically active molecules, have been used as elicitors of plant physiological processes and reactions, including enhancement of the antioxidant enzymatic system (Ridley et al., 2001; Zhao et al., 2009; Ochoa-Villarreal et al., 2012; Camejo et al., 2012) and they were also proposed to play a part as early defensive signals for protecting tissues against chilling (Balandrán-Quintana et al., 2002). Recently, our research work demonstrated that a mix of pectic oligosaccharides (POs) decreased CI in cold stored 'Rio Red' grapefruit (Vera-Guzmán et al., 2017), which is a variety of citrus sensitive to cold stress. The molecular mechanism by which POs ameliorate cold stress has not been approached so far, but it is hypothesized that modulation of the antioxidant system may be implicated. Thus, the aim of this study was to evaluate the effect of POs treatment on the expression of genes that code for the antioxidant enzymes manganese superoxide dismutase (MnSOD), APX, CAT and GR in the flavedo of 'Rio Red' grapefruit fruit stored at chilling temperatures.

2. Materials and methods

2.1. Preparation of cell wall derived oligomer

The pectic oligosaccharides (POs) mixture with a 3 to 20 degree of polymerization (DP) was obtained by enzymatic hydrolysis of low methoxyl pectin (Grindsted® LC-950, DANISCO) using a pectinase from *Aspergillus niger* (Sigma, St Louis, MO, USA) for 15 min at 23 °C (Vera-Guzmán et al., 2017). Aliquots were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (Dionex, Sunnyvale, CA, USA). The DP was calculated according to the trigalacturonic acid standard from Sigma (Ochoa-Villarreal et al., 2011).

2.2. Fruit material and experimental design

Grapefruits cv. Rio Red (*C. paradisi*) were harvested at the beginning of November at the breaker maturity stage from adult trees in an experimental orchard located in La Costa of Hermosillo, Sonora, Mexico (Latitude 28°45′0.98″N, longitude 111°27′26.17″O). This is a desert region whose annual average temperature in November is around 21 °C. After harvest, fruit were immediately delivered to the laboratory where they were visually inspected to be free of damage and defects and randomly selected on the basis of uniform size. Fruit were washed and disinfected with a sodium hypochlorite solution (Ballester and Lafuente, 2017) and rinsed with tap water before being allowed to air-dry at room temperature.

The disinfected fruit were divided into four treatment groups, each consisting of three biological replicates, each containing 36 fruit: 16 fruit for gene expression analysis and 20 fruit to follow the development of CI. Treatments consisted in spraying fruit with an aqueous solution of 10 g L^{-1} POs (pH 5.5) or with distilled water as control and, once dried at room temperature, its subsequent storage either at 2 °C (chilling temperature) or 13 °C (control non-chilling temperature that delays fruit senescence) and 90 to 95% relative humidity. The first and second groups were treated with POs and respectively stored at 2 °C and 13 °C; the third and fourth groups were control fruits stored at 2 °C and 13 °C, respectively. Expression levels of the *MnSOD*, *CAT1*, *APX1* and *GR2* genes were evaluated at 0, 7, 21, and 42 days of storage at both temperatures. At each sampling point the flavedo (outer colored part of the peel) was collected from four fruits per replicate, homogenized, frozen in liquid nitrogen, grounded to a fine powder and stored at −80 °C until analysis.

2.3. Peel structural changes associated with postharvest cold storage

Grapefruit peel samples were examined at the beginning and end of storage (0 and 42 days, respectively) by scanning electron microscopy (SEM) to characterize changes on flavedo ultrastructure of grapefruit exposed to chilling and non-chilling conditions. Approximately five mm square peel pieces were excised with a scalpel and frontal flavedo segments were imaged using an environmental microscope model EVO LS 10 (Carl Zeiss, Cambridge, UK) under variable pressure using a secondary electron detector at 8 kV accelerating voltage and 10 Pa chamber pressure.

2.4. Chilling injury (CI) index

The fruit were visually scored to estimate the extent of CI development. Brown, pit-like depressions on the fruit surface is the main symptom of CI; thus, a subjective scale based on necrotic surface and intensity of browning was used as follows: 0 = no pitting, 1 = slight pitting (1–5% of the fruit surface), 2 = medium pitting (6–20% of the fruit surface) and 3 = severe pitting (< 20% of the fruit surface). The average extent of cold damage was expressed as CI index, which was calculated according to Martínez-Téllez and Lafuente (1997) using the following formula:

$$\text{CI index} = \left[\frac{\sum (\textit{pitting scale}\ (0-3))(\textit{number of fruits within each class})}{\text{Total number of fruit}} \right]$$

2.5. Expression levels of the SOD, CAT1, APX1 and GR2 genes

2.5.1. Total RNA isolation

Total RNA was isolated from 0.1 g of frozen grapefruit flavedo tissue using the protocol reported by Reid et al. (2006) with some modifications. Isolated RNA was treated with the DNA-free kit (Ambion, USA) to remove genomic DNA. The quality and integrity of total RNA were evaluated by the spectrophotometric absorbance ratios A260/A280 and A260/A230 using a NanoDrop 2000 (Thermo Scientific NanoDrop, USA) and by electrophoresis on a 1% denaturing agarose gel. Three micrograms of total RNA were reverse transcribed using the SuperScript II Reverse Transcriptase kit (Invitrogen, USA), according to the manufacturer's recommendations.

2.5.2. qPCR analysis of gene expression

cDNA from the different biological replicates and treatments were used as the template for the qPCR reactions with the primers shown in Table 1. For amplification of the *CAT1* gene, primers were designed

Table 1
. Sequences of the primers used for quantitative real-time PCR. Genes *catalase* (*CAT1*), *ascorbate peroxidase* (*APX1*), *glutathione reductase* (*GR2*), *Mn superoxide dismutase* (*MnSOD*), and *β-actin*.

Gene	Primer sequence (5'-3')	GenBank accession number / Reference
CAT1	Fw- AGCCAGTTGGACGCTTGGT Rv- TGGGGGCATTAACTGGAAGCA	MH170285
APX1	Fw- CGAAATGTGCGGCGTCGG Rv- CCCTTCGAGGCCACTCCTC	Lado et al.[24]
GR2	Fw- ACTAATAAAGGAACAGTTGATGGCTTCTC Rv- TCAACCTCTATAGCTCCATTCTTGGTC	Lado et al.[24]
MnSOD	Fw- ACTACAACAAGGCGGTCGAGC Rv- TGAGTGGTTGACATGACCTCCG	XM_006486115; DQ193969; DQ193968
β-Actin	Fw- GCTCCAAGCAGCATGAAGATCAAGG Rv- TGCTGGAAGGTGCTGAGGGA	GQ389668; GU911361

from specific *C. paradisi* cloned partial sequences obtained in this work and deposited in the GenBank of NCBI, primers for *MnSOD* and *β-actin* (internal reference) were designed based on conserved sequences from citrus fruits, and primers reported by Lado et al. (2016) were used for *APX1* and *GR2*. qPCR reactions were run in triplicates in a 48-well StepOne™ Real-Time PCR System (Applied Biosystem Inc., CA, USA). Each 20 µL reaction consisted of 20 ng of cDNA, 3 µL of 5 µM primer mix (1.5 µL of each forward and reverse primer), 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) and H_2O. Amplification conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curves were performed at the end of each reaction run to verify the presence of a single amplification product. Non-template controls were also included.

The optimization of qPCR conditions, standard curves, primer efficiency values and relative gene expression calculations were conducted according to Livak and Schmittgen (2001). Standard curves with serial dilutions of PCR products were constructed for each gene to calculate amplification efficiency according to the equation $E = (10^{-1/slope} - 1) \times 100$. Quantification was achieved by normalizing the number of target gene copies to an internal reference gene by using the comparative C_T method (Schmittgen and Livak, 2008). The formula $2^{\wedge-(\Delta\Delta Ct)}$ was used to calculate the relative fold change between treated samples relative to control samples at each time point. The ΔCt was calculated by subtracting the average Ct value of each treatment sample from the average C_T values of *β-actin*. The ΔΔCt was calculated by subtracting the ΔCt of treated samples from the ΔCt of control samples at every time point. Results were expressed as the mean relative expression levels of three biological replicates.

2.6. Statistical analysis

The data were subjected to analysis of variance to evaluate the effect of POs treatment on CI index and on the relative gene expression levels of *MnSOD*, *CAT1*, *APX1*, and *GR2* using the SAS software (Statistical Analysis System Institute, 9th ed. Cary, North Carolina, USA 2002). A mean comparison using Tukey's test ($P < 0.05$) was performed to determine differences respect to the control at each time.

3. Results and discussion

3.1. Structural characterization of cold-induced peel damage

The peel surface morphology of grapefruit fruit stored at chilling and non-chilling temperatures were studied with environmental scanning electron microscopy (Fig. 1). Just harvested fruit showed an intact slightly wavy surface covered by a smooth continuous cuticle layer and a thin layer of amorphous epicuticular waxes and scattered crystalline wax-like granules (Fig. 1A, E and I). Plant epidermal tissue is involved in regulating multiple relevant processes such as gas exchange, prevent dehydration and protect against mechanical damage and adverse environmental conditions. Prolonged cold exposure in citrus fruits promotes physiological alterations in epidermal cells that, in sensitive varieties, can even cause flavedo tissue necrosis, known as CI (Dou, 2005).

Main macroscopic CI symptoms in grapefruit fruit stored at 2 °C during 42 days include flavedo pitting (Fig. 1B) and scalding (Fig.1C). Small brown-colored depressions were developed randomly on grapefruit peel surface, and they gradually increased in size and severity leading to the formation of bigger collapsed dark areas after 42 days of storage. Micrographs of pitting zones show a rough structure with 20–40 µm cracks (white arrow) (Fig. 1F and J). Additionally, epicuticular wax crystals were lost and the remaining wax layer acquired an irregular morphology. Cold stress may interfere with epidermal cell metabolism compromising the synthesis and secretion of epicuticular waxes. Also, cold temperature may directly influence biophysical features of cuticle components as demonstrated in apple fruit by calorimetric analysis (Aggarwal, 2001).

Micrographs of scalded tissue showed a pronounced wavy morphology and 20 µm cracks, but unlike the pattern in pitted tissue, scattered crystalline epicuticular waxes and some stomatal pores were still present, although lacking the wax layer that covers the stomatal opening (white arrow) (Fig. 1G and K). Cuticular cracks are related to an increased susceptibility to physiological disorders in flavedo of citrus fruits (El-Otmani and Coggins, 1985), while in cherry fruit, cracks are correlated to an enhanced water loss (Knoche et al., 2004). The flavedo ultrastructure of non-cold-stressed grapefruit stored during 42 days at 13 °C exhibited a slight wavy morphology with an increased density of epicuticular waxes (in comparison to just harvested fruit) in the form of crystalline granules and platelets (Fig. 1H and L). This result may be indicative of characteristic postharvest ripening and senescence processes, which are known to alter the content of cuticular wax constituents in orange flavedo (Liu et al., 2015).

3.2. CI index in POs-treated fruit

POs application at a 10 g L^{-1} concentration reduced by 22.7% the CI in the flavedo of 'Rio Red' grapefruit from day 21 to day 42 of storage at 2 °C (Fig. 2); period of time where damage was severely increased in control fruit (Fig. 2A). On the other hand, no significant damage was observed when fruit were stored at 13 °C, a recommended storage temperature for grapefruit (Fig. 2B and D); consequently, the effect of POs treatment did not turn as evident at this temperature. Although the POs effectiveness in reducing CI in citrus fruits is moderate, its contribution could be related to the activation of biochemical and molecular mechanisms after their perception on the cell surface. Among the downstream processes occurring after POs perception, a transient ROS accumulation, along with specific signaling molecules (Ridley et al., 2001; Ferrari, 2010), may stimulate the enzymatic antioxidant system for recovery of cellular homeostasis (Camejo et al., 2012) and protect cells from physiological disorders triggered by cold-induced oxidative stress.

3.3. Expression of the MnSOD, APX1, CAT1 and GR2 antioxidant-related genes in response to POs

In order to determine whether the molecular regulation of the enzymatic antioxidant system is involved to the POs-mediated CI amelioration, expression of the *MnSOD*, *APX1*, *CAT1* and *GR2* genes, encoding for proteins from the antioxidant system responsible of keeping superoxide and peroxide levels from causing cell damage was evaluated (Mittler, 2002; Bowler et al., 1991). It was compared the abundance of these transcripts between the POs-treated and untreated fruits at 7, 21 and 42 post-treatment days for both of the storage temperatures.

3.3.1. MnSOD expression levels

The *MnSOD* relative expression in grapefruit flavedo stored at 2 °C is shown in Fig. 3. The *MnSOD* transcript levels significantly increased (0.4 fold-change) in fruit treated with 10 g L^{-1} POs by day 21 of storage, a time where CI started to decrease on fruit treated with POs. Grapefruit treated with POs doubled the expression of *MnSOD* by day 42 of storage, with respect to the control. On the other hand, *MnSOD* transcript levels were constant in grapefruit stored at 13 °C. These results suggest that low temperature enhanced the POs-induced *MnSOD* expression. Camejo et al. (2012) observed POs were able to induce SOD activity in alfalfa roots. This enzyme is involved in superoxide radical detoxification protecting cells against oxidative stress and conferring cold tolerance (Van Breusegem et al., 1999; Melchiorre et al., 2009).

A storage temperature of 2 °C increases the *MnSOD* expression levels in 'Star Ruby' grapefruit due to the oxidative stress caused by cold exposure (Lado et al., 2016). From our results, we can infer that POs induce the *MnSOD* gene expression in grapefruit stored at low temperatures, since its transcript levels increased in fruit stored at 2 °C from

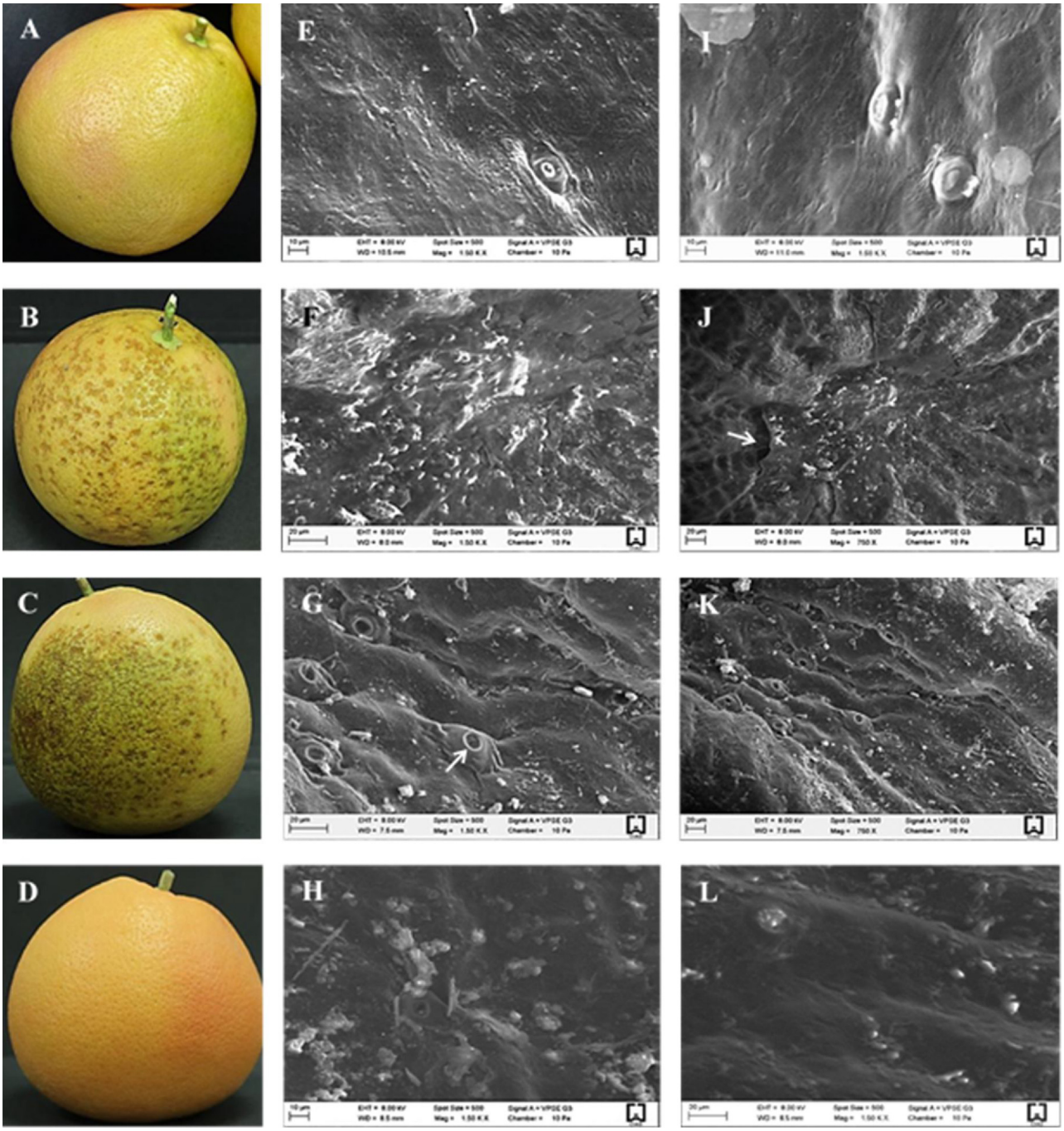

Fig. 1. Micrographs of healthy and chilling-injured flavedo tissue from 'Rio Red' grapefruit visualized by environmental scanning electron microscopy. Just harvested fruit (A, E, I). Storage at a chilling temperature of 2 °C for 42 days: pitting (B, F, J) and scalding (C, G, K). Storage at an optimal non-chilling temperature of 13 °C for 42 days (D, H, L). Images were at spot size 500 and up to 1500× amplification.

day 21 onwards, while they remained at constant levels in fruit stored at 13 °C. Accumulation of high levels of *MnSOD* transcripts could be related to cold tolerance in citrus fruits, as suggested from recent microarray studies (Lafuente et al., 2017). Therefore, the POs-induced *MnSOD* expression agrees with the decreased CI levels observed from day 21 onwards in fruit stored at 2 °C. *MnSOD* was found to be a low-temperature-responsive gene in 'Marsh' grapefruit flavedo, where the application of conditioning treatments increased its transcript abundance and reduced CI levels (Maul et al., 2011). Recent work reported that increments in SOD enzymatic activity improved resistance to chilling damage on the peel of banana fruit (Lo'ay and EL-Khateeb, 2018). Also, *MnSOD* overexpression in transgenic maize and tobacco was associated to cold tolerance (Bowler et al., 1991; Van Breusegem et al., 1999).

3.3.2. *APX1 expression levels*

The expression levels of *APX1* in the flavedo of grapefruit treated with POs and stored at 2 °C and 13 °C are shown in Fig. 4. It can be observed that at 2 °C *APX1* levels are similar in control and treated fruit stored for 7–21 days. Nevertheless, its expression became significantly increased (2 fold-change) by day 42 in POs-treated fruit with regards to the control. Recently, it was reported that *APX1* transcript levels remained unchanged in 'Star Ruby' grapefruit stressed with low temperature (Lado et al., 2016). However, in this study application of 10 g

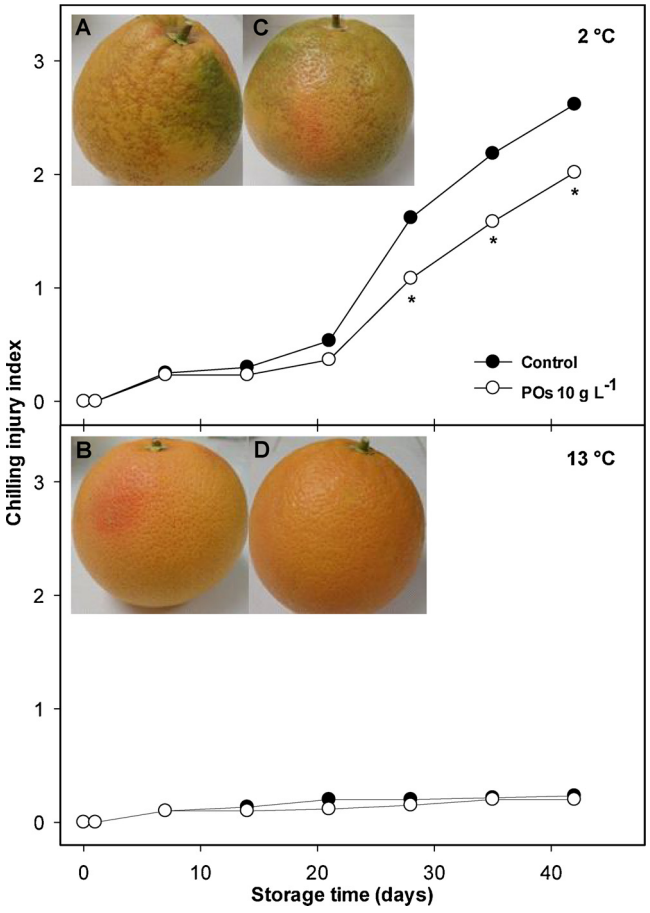

Fig. 2. Chilling injury index of 'Rio Red' grapefruit fruit treated with water (A and B) or 10 g L^{-1} POs (C and D) and stored at 2 °C (up) or 13 °C (down) at 90–95% RH. Mean values of three replications are presented. Asterisks indicate significant differences (P < 0.05) between POs-treated fruit and their respective control samples for the same storage period.

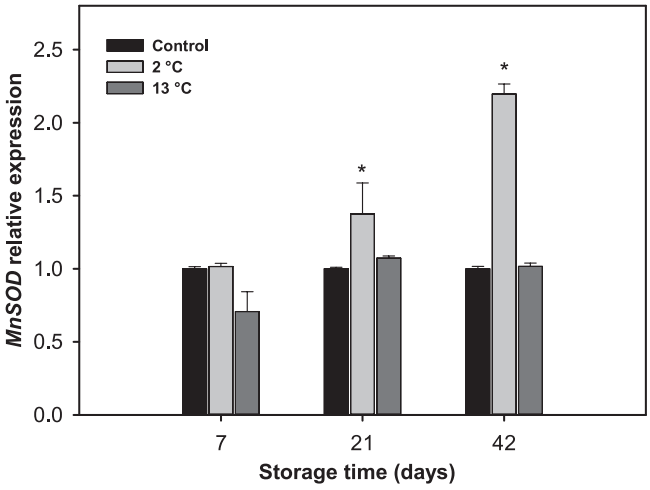

Fig. 3. Relative expression of *MnSOD* in the flavedo of 'Rio Red' grapefruit treated with 10 g L^{-1} POs and stored at 2 °C or 13 °C. Mean values of three replications are presented. Asterisks indicate significant differences ($P < 0.05$) between POs-treated fruit and their respective control samples at each storage period.

L^{-1} POs induced the *APX1* transcript accumulation in grapefruit by day 42 of storage at 2 °C. Transgenic rice plants overexpressing *APX1* under cold stress maintained a higher APX activity and increased cold

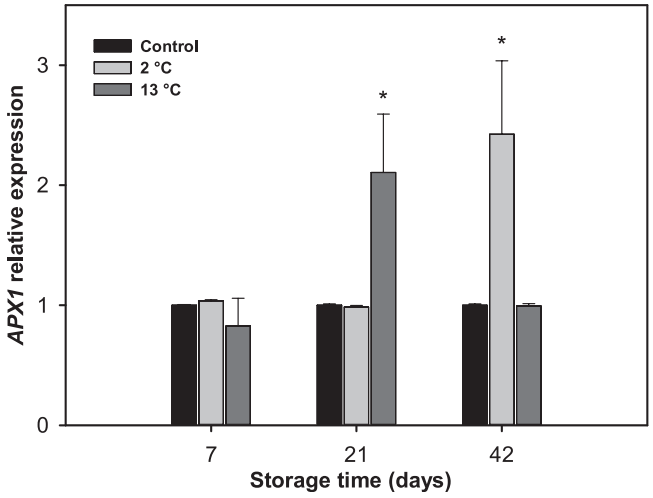

Fig. 4. Relative expression of *APX1* in the flavedo of 'Rio Red' grapefruit treated with 10 g L^{-1} POs and stored at 2 °C or 13 °C. Mean values of three replications are presented. Asterisks indicate significant differences ($P < 0.05$) between POs treated fruit and their respective control samples at each storage period.

tolerance levels (Sato et al., 2011). Likewise, an increased APX enzymatic activity enhanced chilling tolerance in lemon fruit (Siboza et al., 2017). APX catalyzes reduction of hydrogen peroxide to water using ascorbate as an electron donor (Mittler, 2002). Hence, the *APX* up-regulation observed in this study suggests that APX may also be implicated to the decreased CI levels induced by POs in cold-stored grapefruit, by promoting the H_2O_2 elimination process.

Interestingly, an increase in the levels of *APX1* was observed in POs-treated grapefruit stored at 13 °C by day 21 of storage. Suggesting that accumulation of the *APX1* mRNA is induced by POs, regardless of the storage temperature.

3.3.3. *CAT1* expression levels

The *CAT1* expression in POs treated fruit stored at 2 °C showed an oscillatory behavior, which fluctuated depending on time of storage. Fruit treated with 10 g L^{-1} POs induced a significant decrease in the *CAT1* transcript levels by day 21 with regards to the controls (Fig. 5). It was observed that CI incidence is low at this storage time, less than 17.8%, without differences between controls and POs-treated fruit (Fig. 2). However, by day 42 of storage, a significant increase in *CAT1*

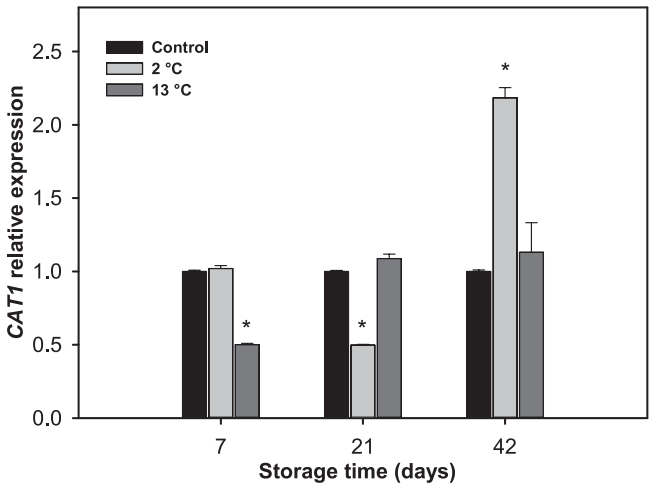

Fig. 5. Relative expression of *CAT1* in the flavedo of 'Rio Red' grapefruit treated with 10 g L^{-1} POs and stored at 2 °C or 13 °C. Mean values of three replications are presented. Asterisks indicate significant differences ($P < 0.05$) between PO treated fruit and their respective control samples at each storage period.

expression was observed in POs-treated fruit, which is coincident with the time of highest CI severity in control fruit with respect to those treated with POs.

Reduction of the *CAT1* transcript levels observed at day 21 in fruit treated with POs could contribute to a transient increase of the H_2O_2 concentration, triggering an inductive response, along with a subsequent increase in its expression, as observed by day 42, which could be part of the cellular homeostasis restoring mechanism (Orozco-Cardenas and Ryan, 1999). A modulator effect of POs on *CAT1* expression, at temperatures that promote CI in citrus fruits, is suggested by such variation of the transcript levels observed in the study hereby. Catalase may be responsible for removing ROS excess during stress, thus preventing oxidative damage (Mittler, 2002). The H_2O_2 level modulation can be decisive for inducing several biochemical and physiological responses in cells. CAT activity can be induced by POs in plant tissues (Camejo et al., 2012); increments in *CAT* transcript levels were associated with a higher cold tolerance in 'Marsh' grapefruit (Maul et al., 2011), lemon (Siboza et al., 2017) and banana (Lo'ay and EL-Khateeb, 2018).

The increase in CAT activity by a heat conditioning treatment was associated with a lower CI incidence (Sala and Lafuente, 1999; Ghasemnezhad et al., 2008). Therefore, it is suggested that the more cold-tolerant the fruits are, the better enzymatic antioxidant system they have with regards to sensitive fruits, as observed in citrus fruit varieties (Sala, 1998; Sala and Lafuente, 1999). Also higher CAT, APX, and SOD activities were related to cold tolerance in tomato fruit (Zhao et al., 2009). Conversely, in fruit stored at 13 °C, the POs had the *CAT1* expression transiently reduced by day 7, showing no subsequent changes in the *CAT1* transcript levels between treated fruit and controls during the rest of the experiment (Fig. 5).

According to these results, it could be inferred that POs modulate the *CAT1* expression levels under low-temperature stress conditions. Furthermore, a time-dependent differential transcriptional regulation between the *APX* gene and those genes coding for the SOD-CAT antioxidant system may also be suggested; the increase in SOD activity can improve the flavedo capacity to dismutate superoxide radicals, whereas CAT and APX shall contribute to hydrogen peroxide removal at a different extent (Mittler, 2002; Sala and Lafuente, 1999).

3.3.4. GR expression levels

No significant changes in *GR2* expression were revealed in POs-treated fruit stored at 2 °C with regards to controls (Fig. 6). Nonetheless, GR plays a relevant role in protecting plants from oxidative stress (Kornyeyev et al., 2003), and it has been shown that *GR* gene expression increases in 'Ruby Star' grapefruit under stress conditions due to low temperatures (Lado et al., 2016). The involvement of GR in cold stress is still controversial in citrus fruits; studies in 'Fortune' mandarin showed that a conditioning treatment aimed to reduce CI caused a decrease on its activity, but an increase on the CAT, APX, and SOD activities (Sala and Lafuente, 1999). Furthermore, treatment with 10 g L^{-1} POs did not set a defined trend in fruit stored at 13 °C (Fig. 6). The *GR2* transcript levels decreased at days 7 and 42, but they were significantly higher than the control by day 21, suggesting POs treatment modulates *GR2* gene expression at temperatures that do not cause CI in citrus fruits. Considering that POs also induced the increment of *APX1* transcript levels in fruit stored at 13 °C, APX and GR (main enzymatic actors in the glutathione-ascorbate cycle) could be involved in other functions not necessarily related with thermal stress. The GR uses NADPH as a reducing agent to maintain the levels of reduced glutathione (GSH) in the glutathione-ascorbate cycle (Gill et al., 2013). Ascorbate is regenerated by GSH to keep the APX active and to prevent accumulation of toxic H_2O_2 levels (Uzilday et al., 2015). However, the study hereby showed POs did not influence *GR2* expression in fruit stored at 2 °C, suggesting that modulation of *GR2* transcript levels may likely not be a constraint for ascorbate regeneration (Foyer and Noctor, 2011; Asada, 1999).

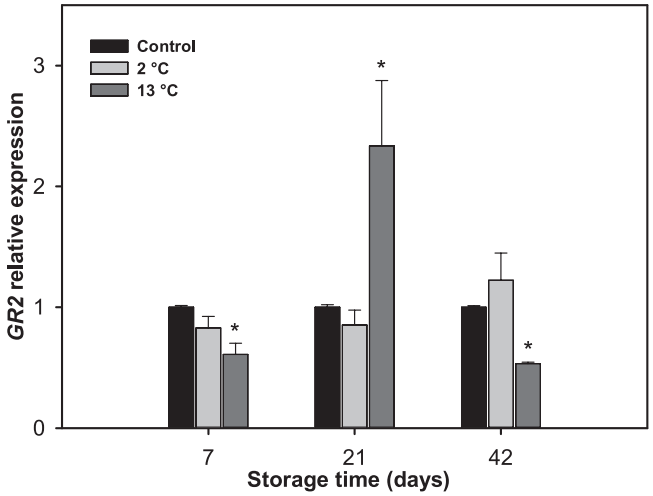

Fig. 6. Relative expression of *GR2* in the flavedo of 'Rio Red' grapefruits treated with 10 g L^{-1} POs and stored at 2 °C or 13 °C. Mean values of three replications are presented. Different letters indicate significant differences ($P < 0.05$) between POs-treated fruit and their respective control samples at each storage period.

Finally, the POs involvement in the differential *MnSOD*, *APX1*, and *CAT1* response was revealed, suggesting POs may be implicated in modulating H_2O_2 levels at the transcriptional level and consequently with the overall antioxidant capacity and the state of the cellular redox balance (Melchiorre et al., 2009). Therefore, considering that by day 21 of storage at 2 °C the *MnSOD* expression increased but *CAT1* expression decreased, it is suggested that the mechanism by which POs reduce CI in grapefruit flavedo could involve a differential and coordinated regulation in the *MnSOD*, *APX1* and *CAT1* expression, which could lead to a transient increase of the H_2O_2 levels followed by a gradual detoxification promoted by a subsequent increase in the *CAT1* and *APX1* expression, as observed by day 42 of storage. Additionally, it is suggested that the POs-mediated CI susceptibility reduction in grapefruit does not require an increase in *GR2* expression, leading to an H_2O_2 decomposition mainly through the activity of CAT and APX (Foyer and Noctor, 2011; Mhamdi et al., 2010).

4. Conclusion

In summary, a prolonged cold storage alters the ultrastructural morphology of flavedo epidermal tissue and promotes the incidence of chilling injury (CI) symptoms in 'Rio Red' grapefruit. The application of pectic oligosaccharides (POs) modulates, at the transcriptional level, the enzymatic antioxidant system in 'Rio Red' grapefruit stored at chilling and non-chilling temperatures, suggesting that POs-mediated CI susceptibility reduction involves a coordinated expression of the *Mn superoxide dismutase*, *ascorbate peroxidase*, and *catalase* genes. Moreover, at non-chilling temperatures, POs induce the transcriptional modulation of the glutathione-ascorbate cycle.

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