Involvement of the Redox System in Chilling Injury and Its Alleviation by 1-Methylcyclopropene in ‘Rojo Brillante’ Persimmon

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Abstract. A treatment with 1-methylcyclopropene (1-MCP) is known to reduce softening to the flesh of ‘Rojo Brillante’ persimmon, which is the main chilling injury (CI) symptom that occurs after storage at low temperature. However, very little is known about the mechanism by which 1-MCP confers persimmon tolerance to chilling. The aim of this study was to investigate the changes in the redox system associated with CI and its reduction by 1-MCP during storage at 1 °C and after shelf life period. Our results showed that during cold store, both control and 1-MCP treated fruit underwent gradual oxidative stress (accumulation of H₂O₂, increment in APX, CAT, LOX, and slight increase in SOD activity) but no CI was manifested. During shelf life conditions, ethylene production was slightly higher in control than in 1-MCP treated fruit. Besides, the CI manifestation of control fruit was associated with oxidative burst [major H₂O₂ accumulation and sharp increase in catalase (CAT), peroxidase (POD), and lipoxygenase (LOX) activity], while 1-MCP treatment greatly reduced the CI symptoms. The 1-MCP treated fruit showed down-regulated POD activity and up-regulated CAT activity, which resulted in slower H₂O₂ accumulation. The reduction of the flesh softening as the main manifestation of CI in ‘Rojo Brillante’ persimmon by 1-MCP was associated with the modulation of the redox state of the fruit during the shelf life period that follows low-temperature storage.

Persimmons, like most of tropical and subtropical fruits are sensitive to CI, which is mainly expressed by flesh gelling (development of a gel-like consistency in the flesh), fast fruit softening and flesh browning during and after storage. The CI symptoms as well as their incidence and severity depend upon the cultivar, the storage temperature and duration. Besides, the CI symptoms became most severe after transferring fruit from low to ambient temperatures, although they can also be exhibited during cold storage (Woolf et al., 1997; Zhang et al., 2010).

‘Rojo Brillante’ persimmon has been widely reported to exhibit CI symptoms when stored at temperatures below 15 °C. The main CI symptom of this cultivar is a fast firmness loss. This flesh softening can be exhibited during cold storage at 4–11 °C (Arnal and Del Río, 2004; Orihuel-Iranzo et al., 2010), nevertheless at 0–1 °C the drastic firmness loss only occurs when fruit are transferred to shelf life temperatures (Arnal and Del Río, 2004;

Salvador et al., 2004). Therefore the use of treatments to control CI becomes necessary to cold storage persimmon. In this way 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, has been shown to reduce CI symptoms in a large number of persimmon cultivars, including ‘Rojo Brillante’ (Girardi et al., 2003; Kim and Lee, 2005; Salvador et al., 2004; Tibola et al., 2005).

Studying CI in persimmon and its reduction by 1-MCP has been widely addressed from the changes in flesh structure perspective. Thus Luo and Xi (2005) reported in chilling injured fruit that the primary cell wall and the middle lamella cannot be dissolved normally, while De Souza et al. (2011) reported an increase in the activity of cell wall-degrading enzymes such as endo-1,4-β-glucanase, pectin methylesterase, polygalacturonase, and β-galactosidase. In ‘Rojo Brillante’ persimmon, microstructural studies have shown that 1-MCP preserves the integrity of cell walls and adhesion between adjacent cells (Pérez-Munuera et al., 2009). Moreover, the loss of the cell walls integrity and the flesh breakdown associated with CI development has been linked to increased levels of ethylene (De Souza et al., 2011; Luo and Xi, 2005; Orihuel-Iranzo et al., 2010; Woolf et al., 1997). According to Orihuel-Iranzo et al. (2010), the increase in ethylene production upon transfer from chilling to nonchilling temperature is part of the fruit

chilling response since it does not occur at nonchilling temperatures, and it also appears to be responsible for the collapse of the fruit.

In addition to the activity of cell wall-degrading enzymes, cell structure can also be disrupted as a result of peroxidation caused by excess reactive oxygen species (ROS) (Blokchina et al., 2003). The ROS metabolism is controlled by an array of interrelated enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD), which act concomitantly with nonenzymatic antioxidants; O₂^{•−} is converted into H₂O₂ by SOD, while H₂O₂ is scavenged predominantly by APX, CAT, and POD. The implication of the oxidative system in CI sensitivity has been studied in depth in other fruits, in which chilling manifestation and its reduction by different treatments have been related to changes in ROS and enzymes of the oxidative system. This is the case of loquats (Cao et al., 2009, 2011; Wang et al., 2005; Xu et al., 2012), apple (Rupasinghe et al., 2000), or mandarins (Sala, 1998; Sala and Lafuente, 1999, 2000).

In ‘Fuyu’ persimmon, Zhang et al. (2010) described changes in the oxidative system during low temperature storage associated with reduced CI by means of 1-MCP. In this study the reduction by 1-MCP of CI symptoms; the external and internal damages exhibited during storage at 4 °C, was attributed to altered oxidative status during cold storage. It must be taken into account that in the case of ‘Rojo Brillante’ the main symptom of CI is the drastic flesh softening, and it is not exhibited while fruit is stored at low temperature (1 °C) but is displayed after transferring fruit to shelf life conditions. Besides this CI symptom is likewise reduced by 1-MCP when it is applied before or after cold storage. Therefore, the involvement of the redox system in CI and its reduction by 1-MCP in ‘Rojo Brillante’ should be approached covering both low temperature storage and subsequent shelf life period.

This study aims to investigate changes during cold storage and shelf life period in the oxidative system [H₂O₂ content, SOD, CAT, APX, POD, and lipoxygenase (LOX) enzymes activity] associated with CI reduction by 1-MCP in ‘Rojo Brillante’.

Material and Methods

Plant material. Persimmon (*Diospyros kaki* Lf.) cv. Rojo Brillante fruit were harvested in l’Alcúdia (Spain) at midseason. After harvest, fruit were taken to the Instituto Valenciano de Investigaciones Agrarias (IVIA), where they were divided into 20 homogenous lots of 15 fruit. One lot of fruit was analyzed to determine the maturity stage of fruit at harvest [color index of 9.42, firmness of 39.25 Newton (N) and soluble tannins content of 0.5% FW]. The remaining lots were submitted to a postharvest deastringency treatment with CO₂ under standard conditions (95% CO₂, 24 h, 20 °C, and 90% RH) in closed containers.

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After the deastringency treatment, fruit were kept at ambient temperature in an air atmosphere for 24 h. After this time, the physiological and redox state of fruit was evaluated with one lot of fruit.

The remaining 18 lots of fruit were divided into two groups of nine lots each to submit one of them to 1-MCP treatment (500 nL·L⁻¹ of 1-MCP for 24 h at 20 °C), while the other group acted as the control. Fruit from each treatment were stored at 1 °C, 85% to 95% relative humidity (RH) for up to 45 d. Periodically (15, 30, and 45 d), three lots of 15 fruit per treatment were removed from the storage room. One lot was analyzed directly and the two other lots were transferred to 20 °C to simulate the shelf life conditions. After 2 and 5 d at 20 °C, one lot of fruit was analyzed.

The evaluation of fruit immediately before storage (24 h after being submitted to CO₂) and periodically during storage and the subsequent shelf life periods involved the determination of physiological parameters (firmness, color, soluble tannins content) and the redox state of fruit [hydrogen peroxide concentration (H₂O₂) and the activity of the SOD, CAT, POD, LOX, and APX enzymes]. CO₂ and ethylene production were also evaluated on days 1, 2, and 5 of each shelf life period.

1-MCP (SmartFresh™), provided by 'AgroFresh' Inc. (Rhom and Haas Inc., Gesate, Italy), is formulated as a powder (0.14% 1-MCP) and it was applied in closed chambers. The calculated quantity of SmartFresh needed to obtain the required 1-MCP concentration in each chamber was placed in a 125-mL tight-sealed bottle, and warm water (16 mL·g⁻¹ product) was added through the septum. It was shaken in a warm water bath until turbidity had disappeared (40 min). The sealed bottles were put inside each 442-L chamber and were opened just before closing it. After 24 h, the chambers were opened and the fruit from each treatment were stored at 1 °C and 85 to 95% RH for up to 45 d.

Evaluation of physiological parameters. Skin color was determined over 15 fruit using a Minolta colorimeter (Model CR-300; Minolta, Ramsey, NY). Hunter parameters "L," "a," and "b," were measured and the results were expressed as Color Index = 1000a/Lb according to Jiménez-Cuesta et al. (1981). This Color Index, originally developed for citrus fruit, it has been shown to acuity reflect the skin color changes of persimmon fruit. Negative values indicate green tones and positive values indicate orange and red tones (Salvador et al., 2004, 2007). Two measurements were performed on opposite equatorial area in each fruit.

Flesh firmness was evaluated with a Texturemeter Instron Universal Machine model 4301 (Instron Corp., Canton, MA) using an 8-mm plunger. Fruit firmness values were the average of 12 fruit per treatment. The results were expressed as load in Newton (N) to break flesh on one side of fruit after removing peel.

Immediately after the firmness measurement, flesh samples were taken from the opposite side of six fruit and were frozen at

-20 °C until the soluble tannins analyses. The samples from six other fruit were cut into small pieces and frozen with liquid nitrogen to be ground and kept at -80 °C until the analyses of H₂O₂ content and enzymes activity evaluation were done. In both cases, flesh samples were taken together from two fruit (three replicates, two fruit per replicate).

Soluble tannins were evaluated by the Folin-Denis method described by Taira (1995) and modified by Arnal and Del Río (2004). The results were expressed as a percentage of fresh weight (FW).

For determination of CO₂ and ethylene production, three fruit were weighed and individually sealed in 1-L glass jars for 2 h at 20 °C, and 1 mL of headspace was analyzed in a Perkin Elmer gas chromatograph, equipped with a Poropak QS 80/100 column. To determine CO₂, a thermal conductivity detector was used. Helium was the carrier gas used at 9.2 psi. The injector, oven and detector temperatures were 115, 35, and 150 °C, respectively. To determine ethylene, a flame ionization detector was used. Helium was the carrier gas used at 8 psi. The injector, oven, and detector temperatures were 175, 75, and 175 °C, respectively. CO₂ production was expressed as mmol CO₂ per kg⁻¹·h⁻¹ and ethylene production as nmol C₂H₄ per kg⁻¹·h⁻¹.

H₂O₂ content. Hydrogen peroxide was determined from frozen tissue from three replicates each of two fruit. The method used was that according to Novillo et al. (2014). First, 1 g of flesh tissue was homogenized in an ice bath with 1.5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12000 rpm for 20 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). Supernatant absorbance was measured at 390 nm. Hydrogen peroxide content was determined using a standard curve and was expressed as nmol·g⁻¹ FW.

Enzyme activity measurement. All the enzyme extraction procedures were conducted at 4 °C from frozen tissue from three replicates each of two fruit. For CAT, POD, and LOX; 0.5 g of frozen tissue was homogenized with 2.5 mL of 50 mM sodium phosphate buffer (pH 7) containing 1 mM ethylene diamine tetraacetic acid (EDTA), 2 mM dithiothreitol (DTT) and 10 g·L⁻¹ polyvinylpyrrolidone (PVP). For APX, the same buffer was used, but also contained 0.5 mM ascorbic acid (AsA). For SOD, 0.5 g of frozen sample was homogenized with 2.5 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 1.33 mM diethylenetriaminepentaacetic acid (DETAPAC). Homogenates were centrifuged at 12,000 rpm for 25 min at 4 °C. Supernatants were used for the enzymes assays. For all the enzymes, activity was expressed in units per gram of FW per minute.

CAT activity was assayed according to Novillo et al. (2014). This involved monitoring the disappearance of H₂O₂ by recording the drop in absorbance at 240 nm of a reaction

mixture containing 50 mM of sodium phosphate buffer (pH 7), 90 mM of H₂O₂ and 0.5 mL of the CAT extract. The molar extinction coefficient of H₂O₂ at 240 nm was taken as 40 mM⁻¹·cm⁻¹ according to Duan et al. (2011). One unit of CAT activity was defined as the amount of enzyme that decomposed 1 μmol of H₂O₂ per minute.

APX activity was measured according to Novillo et al. (2014). Activity was assayed in a mixture containing 2 mL of sodium phosphate buffer (50 mM, pH 7), 1 mM of AsA and 0.5 mL of H₂O₂ (4 mM). The reaction was initiated by the addition of 0.5 mL of enzyme extract. APX activity was determined by monitoring the drop in absorbance at 290 nm as ascorbate was oxidized. A molar extinction coefficient of 2.8 mM⁻¹·cm⁻¹ according to Duan et al. (2011) was used to calculate activity. One APX unit was defined as the amount of enzyme to cause a decrease in OD290 per min under the assay conditions.

POD activity was determined by measuring the increase in absorption at 470 nm according to Novillo et al. (2014). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7), 90 mM of H₂O₂ and 2% guaiacol. The reaction was initiated by adding 0.3 mL of the POD extract. A molar extinction coefficient of 26.6 mM⁻¹·cm⁻¹ was used to calculate activity (Duan et al., 2011). One POD unit was defined as the amount of enzyme to cause an increase in OD470 per min under the assay conditions.

LOX activity was assayed by monitoring the formation of conjugated dienes from linoleic acid at 234 nm, according to the method of Zheng et al. (2007) with some modifications. Three milliliters of reaction mixture contained 2.425 mL of sodium phosphate buffer (50 mM, pH 7), 75 μL linoleic acid solution (10 mM) and 0.5 mL enzyme extract. The blank contained 2.925 mL of sodium phosphate buffer (50 mM, pH 7) and 75 μL of linoleic acid solution (10 mM). One unit of LOX was defined as the amount of enzyme to produce an OD234 reduction per min under the assay conditions. A molar extinction coefficient of 25 mM⁻¹·cm⁻¹ was used to calculate activity (Quartacci et al., 2001).

SOD activity determination was measured by the method of Beauchamp and Fridovich (1971) with some modifications. The reaction mixture contained 850 μL of 50 mM sodium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid (DETAPAC), 2.24 mM nitrotriazolium blue chloride (NBT) solution, 1.8 mM xanthine solution, 40 U/mL CAT from bovine liver, 50 μL tissue extract. The reaction was initiated by the addition of 100 μL xanthine oxidase and was carried out at 25 °C for 60 min. Assay mixture absorbance was measured at 560 nm. An assay mixture without tissue extract was used as a control. One enzyme activity unit was defined as the amount of enzyme that inhibited the photoreduction of NBT by 50%.

Statistical analysis. Data were subjected to the ANOVA, and multiple comparisons

between means were determined by the least significant difference test ($P \leq 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD).

Results

In the present study, during the first 30 d of cold storage, the control and 1-MCP treated fruit showed no changes in firmness and external color if compared with the values recorded before storing. After 45 d of storage, a very slight decrease in firmness and increase in color were observed in all the fruit (Fig. 1A and B). When the control fruit were transferred to shelf life conditions after 15 d of cold storage, they underwent drastic firmness loss from 35 N to 20 N and to 10 N after 2 and 5 d at 20 °C, respectively. Faster softening was observed during the shelf life period after 30 or 45 d of cold storage when fruit firmness dropped to values below 10 N within the first 2 d at 20 °C. As expected, the 1-MCP treatment significantly reduced firmness loss after the different shelf life periods. Thus, the 1-MCP treated fruit showed firmness close to 25 N after 2 d at 20 °C, which followed at 15, 30, and 45 d of cold storage. After 5 d of shelf life, the firmness value remained constant for the 15 and 30 d of cold storage, but lowered to 17 N when cold storage lasted 45 d (Fig. 1A).

In parallel to firmness loss, fruit exhibited increased external color when transferred from cold storage to the shelf life conditions (Fig. 1B). Color evolution was retarded in the 1-MCP treated fruit if compared with the control fruit, but only during the shelf life that followed the 45-day cold storage.

The measurements of CO₂ and ethylene production taken during shelf life that followed the different cold storage periods, showed that fruit firmness loss throughout storage was associated with an increment of ethylene and respiration rate after 1 d at 20 °C (Fig. 2). The values of both these parameters gradually lowered after 2 and 5 d of shelf life. In general, the fruit treated with 1-MCP showed a lower respiration rate than the control fruit. Ethylene production was also depressed by 1-MCP, which became evident during shelf life after 30 and 45 d of cold storage.

During cold storage, no differences were exhibited in H₂O₂ content (Fig. 3) in either the pro and antioxidant enzymes activity between the control and 1-MCP treated fruit (Figs. 4 and 5). After 15 d of cold storage, H₂O₂ content was slightly reduced (25 nmol·g⁻¹) when compared with the initial content (40 nmol·g⁻¹). However, a sharp increase to 70 nmol·g⁻¹ of H₂O₂ was observed after 30 d of storage and then H₂O₂ content remained constant as storage advanced (Fig. 3). The trend of changes noted in APX activity ran in parallel to that observed for H₂O₂ content; APX activity declined from 1500 U·g⁻¹ to 1000 U·g⁻¹ during the first 15 d of storage. After 30 d, this activity reached similar values to those observed at the beginning of storage and then remained unchanged

Fig. 1. Flesh firmness (A) and external color (B) of the untreated (CTL) and 1-methylcyclopropene-treated (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 d after deastringency treatment) and after 2 and 5 d of shelf life at 20 °C that followed to deastringency treatment plus 15, 30, and 45 d of cold storage at 1 °C. Vertical bars represent the least significant difference intervals ($P < 0.05$).

after 45 d (Fig. 4A). CAT activity did not undergo major changes if compared with their initial values during 30 d of low-temperature storage, although this enzyme exhibited increased activity after 45 d (Fig. 4B). LOX activity enhanced from 1.1 to 5.4 U·g⁻¹ during the first 15 d of cold storage and slightly increased throughout the storage (Fig. 4C). POD activity remained constant throughout the 45-day cold storage, with similar values to initial ones (25 U·g⁻¹) (Fig. 5A). The SOD activity values did not change during the first 15 d of cold storage, and slightly increased after 30 d to remain at similar levels after 45 d (Fig. 5B). Therefore, the study of the redox system revealed some important changes in H₂O₂ content and pro and antioxidant enzymes activity during cold storage, but no effect of 1-MCP treatment.

During the shelf life that followed the cold storage periods, the 1-MCP treatment had no effect on APX, LOX and SOD activity (Figs. 4A, C, and 5B). Both the control and 1-MCP treated fruit exhibited slightly reduced APX and SOD activity during 5 d at 20 °C if compared with the values recorded after the cold storage periods. LOX enzyme activity sharply increased when fruit were transferred to shelf life. While this increase in the cold-stored fruit over 15 d was observed only after 5 d at 20 °C, when the storage period lasted 30 d and 45 d, this activity was enhanced after 2 d of shelf life.

A major effect of 1-MCP treatment was observed on H₂O₂ content (Fig. 3) and on CAT and POD activity during shelf life (Figs. 4B and 5A). A significant increase in H₂O₂ content was noted in the control fruit during 5 d at 20 °C after the cold storage periods. However this increment was delayed in the 1-MCP treated fruit and even no changes in H₂O₂ content were seen in the 1-MCP treatment during shelf life after 15 d of cold storage. CAT enzyme activity drastically increased during shelf life after cold storage, especially after 15 and 30 d. Although a similar rise took place in both treatments when fruit were stored for 15 d, the 1-MCP treated fruit showed higher CAT activity values than the control fruit after shelf life that follow followed 30 and 45 d of storage.

The effect of the 1-MCP treatment on POD activity during the shelf life period was especially marked (Fig. 5A). While no major changes were observed in the 1-MCP treated fruit, POD activity in the control fruit tripled after shelf life when compared with the values recorded at storage. This increase was observed after 5 d of shelf life when fruit were stored for 15 d, and after 2 d at 20 °C when cold stored for 30 or 45 d.

Discussion

The results obtained in the present study corroborate that the drastic fruit softening is

Fig. 2. Carbon dioxide (CO₂) (A) and ethylene (C₂H₄) (B) production of the untreated control (CTL) and the 1-methylcyclopropene-treated (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 d after deastringency treatment) and after 1, 2, and 5 d of shelf life at 20 °C that followed to deastringency treatment plus 15, 30, and 45 d of cold storage at 1 °C. Vertical bars represent the least significant difference intervals ($P < 0.05$). nd = nondetected.

Fig. 3. Hydrogen peroxide (H₂O₂) content of the untreated fruit (CTL) and the 1-methylcyclopropene-treated fruit (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 d after deastringency treatment) and after 2 and 5 d of shelf life at 20 °C that followed to deastringency treatment plus 15, 30, and 45 d of cold storage at 1 °C. Vertical bars represent the least significant difference intervals ($P < 0.05$).

the main CI symptom in 'Rojo Brillante' persimmon and is manifested only after fruit are transferred from low-temperature (1 °C) storage to shelf life conditions. Chilling damage became evident during shelf life that followed to 15 d at 1 °C, but it was aggravated by the cold-storage time. In accordance with previous results (Salvador et al., 2004), the

1-MCP treatment significantly reduced fruit firmness loss during shelf life.

Despite no chilling damage being observed while fruit was maintained at low temperature, the redox stage of fruit underwent several important changes during this cold storage period. It is important to highlight that these changes were similar in

control and 1-MCP treated fruit. Accordingly with numerous reports that support the idea that chilling stress increases ROS production, including O₂⁻, H₂O₂ and OH⁻ (Sevillano et al., 2009), in the present study, after 30 d of cold storage a major H₂O₂ accumulation was observed. This increase in H₂O₂ content would respond to the slight increase in SOD activity detected, an enzyme responsible for converting O₂⁻ into H₂O₂. Sala (1998) reported that chilling stress in cold-stored mandarins was associated with the activation of the antioxidant defense system in response to increasing prooxidant levels. In line with this, our results reveal that after declining the activity of APX at the beginning of the storage, it is up-regulated after 30 d of storage, while CAT activity importantly increases after 45 d if compared with the values recorded for shorter storage periods. The earlier response of the APX enzyme to H₂O₂ accumulation, if compared with the CAT enzyme, is explained by the higher affinity that APX shows for H₂O₂. The APX enzyme is involved in abolishing H₂O₂ in all cellular compartments, while the CAT enzyme is present only in peroxisomes and is essential for detoxifying ROS levels when peroxisome is stressed (Mittler, 2002). Besides the activity of APX and CAT, the activity of LOX was also enhanced during 45 d of storage.

Therefore although any CI symptoms is manifested in the fruit during cold storage, the changes observed in the oxidative system during this period (increased SOD activity, H₂O₂ accumulation and subsequent up-regulation of CAT, APX, and LOX enzymes) reveal that the fruit underwent progressive oxidative stress associated with low-temperature exposure, which was not reduced by the 1-MCP treatment.

Contrarily to that observed at cold storage, during shelf life the changes in the redox system were affected by the 1-MCP treatment. So, transferring fruit from low to shelf life temperatures lead to an oxidative burst, manifested as a major H₂O₂ accumulation and a subsequent drastic increase in the activity of the CAT, POD, and LOX enzymes. Nevertheless in 1-MCP treated fruit the increase of H₂O₂ levels was lower than in control and higher CAT activity and lower POD activity were observed.

Ethylene plays a role in CI, where exogenous exposure aggravates CI symptoms (Besada et al., 2010; Park and Lee, 2005), and ethylene production and respiration rate are higher in chilling injured fruit after removal from cold storage (Besada et al., 2010). The effect of the 1-MCP treatment on attenuating the POD up-regulation observed in the present study is probably mediated by its effect on declining ethylene production during shelf life, which is in accordance with different studies that reports the inductive action that ethylene exerts on POD. So, the induction of POD isoenzymes during ethylene-induced senescence is a common response in cultivars of *Cucumis sativus*, other species of *Cucumis* and other genera of

Fig. 4. Enzyme activity of ascorbate peroxidase (APX) (A), catalase (CAT) (B) and lipoxygenase (LOX) (C) of the untreated fruit (CTL) and the 1-methylcyclopropene-treated fruit (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 d after deastringency treatment) and after 2 and 5 d of shelf life at 20 °C that followed to deastringency treatment plus 15, 30, and 45 d of cold storage at 1 °C. Vertical bars represent the least significant difference intervals ($P < 0.05$).

Cucurbitaceae (Abeles et al., 1989). Similarly in climacteric fruit, POD and indoleacetic acid (IAA) oxidase isoenzymes have been reinforced with progressing maturity. However in nonclimacteric fruits, where ethylene did not notably change during ripening, only the IAA oxidase isoenzyme concentration increased, while the POD isoenzyme concentration decreased (Vamos-Vigyazo,

1981). Furthermore in bamboo shoots, ethylene has been reported to increase POD activity, while 1-MCP treatments retarded it (Luo et al., 2008).

Moreover, the increased H_2O_2 concentration observed during shelf life while SOD activity remained stable, or even declined, if compared with the values at cold storage, suggests that when fruit are transferred to

moderate temperatures, a source of H_2O_2 not linked to SOD activity may exist. Several studies have reported H_2O_2 formation under different stresses as a result of peroxidase activity through a complex reaction with NADH which is oxidized using trace amounts of H_2O_2 first produced by the nonenzymatic breakdown of NADH (Bestwick et al., 1998; Blokhina et al., 2003; Kim et al., 2010; Šimonovičová et al., 2004). This justify the unexpected results obtained in the present study where, under shelf life conditions, control fruit showed an up-regulation of POD activity in parallel to an increase in H_2O_2 content, while the 1-MCP treatment delayed H_2O_2 accumulation, which is associated with lower POD activity levels.

Therefore, in 'Rojo Brillante' persimmon, development of CI symptoms during shelf life is associated with a high POD activity and the fast H_2O_2 accumulation occurred when fruit is transferred from low to moderate temperature. In contrast, the slower H_2O_2 accumulation detected in the 1-MCP treated fruit, as result of greater CAT activity and the inhibition of POD activity, probably confers chilling tolerance to tissues. Similar results have been found by Wang (1995), who reported that the temperature-preconditioning treatment of zucchini squash reduced declining CAT activity and suppressed increased POD activity during cold storage, thus contributing to CI tolerance.

Moreover, the changes observed by Zhang et al. (2010) when studying the reduction in CI symptoms by 1-MCP on 'Fuyu' persimmon also support the fact that POD and CAT are key enzymes involved in the chilling sensitivity of persimmon. So, in this study, CI is manifested in 'Fuyu' during low-temperature storage and throughout cold storage POD activity increased by 4-fold in control fruit, while it was significantly inhibited in the 1-MCP treated fruit; besides, CAT activity was enhanced in 1-MCP treated fruit when compared with control fruit (Zhang et al., 2010). In the present study, neither POD nor CAT activity in 'Rojo Brillante' showed differences between control and 1-MCP treated fruit during cold storage. Both enzymes were up-regulated when fruit were transferred to shelf life and chilling symptoms were manifested; the CI reduction by 1-MCP was related to declined POD activity and enhanced CAT activity during shelf life if compared with control fruit.

The results of the present study allow us to understand why in 'Rojo Brillante' persimmon the CI reduction by 1-MCP treatment, which occurs during shelf life, is similar when the 1-MCP application is performed before cold storage or immediately before transferring fruit to shelf life conditions (Salvador et al., 2004).

In conclusion, our results demonstrate that the low-temperature storage of 'Rojo Brillante' persimmon leads to gradual oxidative stress, which is aggravated after 30 d of cold exposure and is not alleviated by 1-MCP. The manifestation of CI symptoms

Fig. 5. Enzyme activity of peroxidase (POD) (A) and superoxide dismutase (SOD) (B) of the untreated fruit (CTL) and the 1-methylcyclopropene-treated fruit (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 d after deastringency treatment) and after 2 and 5 d of shelf life at 20 °C that followed to deastringency treatment plus 15, 30, and 45 d of cold storage at 1 °C. Vertical bars represent the least significant difference intervals ($P < 0.05$).

when control fruit were transferred from low temperatures to moderate ones is associated with an oxidative burst, with major H_2O_2 accumulation, and also with a sharp increase in CAT, POD, and LOX activity. The reduction of CI symptoms by the 1-MCP treatment is linked to lower POD activity levels and enhanced CAT enzyme activity, which result in slower H_2O_2 accumulation.

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