High CO₂ alleviates cell ultrastructure damage in Autumn Royal table grapes by modulating fatty acid composition and membrane and cell oxidative status during long-term cold storage

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ABSTRACT

Cell membranes provide a link between postharvest disorders and their integrity is essential for reducing table grape deterioration due to dehydration and susceptibility to fungal infection. We examined the relationship between ultrastructural membrane integrity at the subcellular level (transmission electron microscopy), cell (H_2O_2 generation) and membrane (malondialdehyde accumulation) oxidative status, membrane fatty acid composition (polar lipid fraction) and berry quality in order to determine the critical membrane events associated with long-term storage disorders at 0 °C in table grapes (*Vitis vinifera* L.) cv. Autumn Royal, and evaluate their response to the application of a single or dual short-term 20 kPa CO_2 treatment. The results revealed a progressive disorganization of the cytoplasm and the breakdown of the cell organelle ultrastructure, associated with elevated hydrogen peroxide generation and lipid peroxidation rates and an imbalance in the saturated/unsaturated ratio of polar lipids, mainly due to the decrease in the degree of unsaturation of 18-carbon fatty acids. Conversely, high CO_2 levels maintained the integrity of the microstructure of cell and energy-related organelles that are essential for metabolic damage repair and cell membrane restoration, reduced oxidative damage in cells and membranes, and increased the unsaturation of 18-carbon fatty acids, lipid unsaturation ratio and index of unsaturated fatty acids (IUFA) in the membrane polar lipids. The results also revealed that the more short-term high CO_2 treatment is applied to Autumn Royal grapes, the more cytological evidence there is of enhanced tolerance to long-term cold and prevention of storage disorders in terms of cluster weight loss, rachis browning, berry water loss and decay incidence.

Keywords: Cold storage; Malondialdehyde; Oxidative damages; Polar lipids; Short-term high CO₂; *Vitis vinifera*.

1. Introduction

The goal of adequate postharvest handling is to maintain optimal fruit quality for longer and delay ripening and senescence-associated processes. The main method used to prolong the storage life of fruit is to reduce the fruit temperature to slow metabolism and consequently the rate of ripening and senescence (Thompson et al., 1998; Sevillano et al., 2009). Table grapes are considered as a cold-tolerant fruit with relatively low physiological activity, for which storage temperatures of around 0 °C are recommended to reduce postharvest losses due to cluster dehydration and fungal attack (Artés-Hernández et al., 2004; Palou et al., 2010). However, although the rate of deterioration is affected by different factors such as maturity and skin and pulp structure, postharvest low temperatures can cause structural damage and physiological and metabolic dysfunctions (Romero et al., 2006; Goñi et al., 2011), limiting their storage life and commercial quality.

Postharvest cold cell damage in horticultural products has been attributed to a number of factors such as loss of integrity in membrane systems, lipoxygenase activity and membrane lipid peroxidation, which can lead to altered cell homeostasis and organelle functioning and result in ion leakage, osmotic pressure loss, subcellular compartmentation, interruption of cell signaling routes and loss of functionality in critical integral defense proteins (Lyons and Raison, 1970; Marangoni et al., 1996; Ge et al., 2019). Polar lipids are the major building blocks in biomembranes, and any change in their composition directly affects membrane stability and function (Maalekuu et al., 2006). The modification of membrane fluidity is mediated by changes in

unsaturated fatty acid levels, a function provided in part by the regulated activity of fatty acid desaturases (Upchurch, 2008; Ge et al., 2019). Reactive oxygen species are assumed to play a role in inducing oxidative stress during low-temperature damage (Sharma et al., 2012). For instance, the lipid peroxidation rate gradually increased with longer low-temperature periods, as polyunsaturated fatty acids, of which linoleic and α -linolenic acids are the main substrates for lipoxygenase in plants, were oxidized during cold storage due to attack by free radicals and oxygen (Lukatkin, 2002; Sevillano et al., 2009).

The key to understanding the mechanisms of cold acclimation appears to be the ultrastructural and biochemical alterations in the biomembranes (e.g. changes in lipid concentration and composition) which could preserve fluidity, membrane function and cell compartmentation (Kratsch and Wise, 2000). There also seems to be a correlation between fatty acid composition in membrane lipids and cold tolerance. Changes in the degree of fatty acid unsaturation in lipid components and membrane proteins may play a role in cryoprotection in some plants cells (Watanabe et al., 1990). For instance, it has been shown that chilling resistant cultivars of loquat fruit had lower levels of superoxide radicals and H_2O_2 but higher membrane lipid unsaturation than chilling sensitive fruit (Cao et al., 2011).

The synergistic co-activation of plant stress responses, or cross-stress tolerance, confers a preventive advantage by enabling a general increase in stress resistance following exposure to a single stress condition, indicating extensive overlap and crosstalk between stress/response signaling pathways (Knight and Knight, 2001). Within this framework, different technologies are applied to reduce chilling injury based on this synergistic co-activation of plant responses, whose beneficial effect is associated with the maintenance of a higher unsaturated/saturated fatty acid ratio (Mirdehghan et

al., 2007; Cao et al., 2009). Heat pre-treatments reduce solute and electrolyte leakage in tomato fruit at low temperatures by maintaining the stability of cell membranes, leading to an increase in chilling tolerance (Saltveit, 2001), and low temperatures combined with methyl jasmonate treatment delayed degradation of the cell membrane, reduced malondialdehyde (MDA), and enhanced chilling tolerance in bell pepper (Wang et al., 2019). The beneficial effect of short-term high CO₂ treatment has also been described during fruit cold storage, reduced cell degradation by preserving the integrity of the plasmalemma and increasing lipid-derived aldehyde concentrations in persimmon (Besada et al., 2015), or increased the amount of polyunsaturated fatty acids, mainly α -linolenic acid, from the polar lipid fraction (Blanch et al., 2019), suggesting an effect of the CO₂ treatment on the metabolism of membrane lipids.

We previously highlighted the efficacy of a short-term high CO₂ pre-treatment in maintaining cv. Cardinal and cv. Autumn Royal table grape quality and avoiding postharvest losses in terms of water loss, oxidative damage and disease prevention (Sanchez-Ballesta et al., 2006; Navarro et al., 2015; Vazquez-Hernandez et al., 2017; Vazquez-Hernandez et al., 2018). In this context, it is important to establish the relationship between membrane ultrastructural integrity, cellular and membrane oxidative status and membrane lipid content and composition during long-term postharvest table grape cold storage, and in events associated with the cold-tolerance mechanism activated by high CO₂ levels. It should also be noted that the use of cycles of controlled postharvest abiotic stresses was proposed as a potential solution to enhance successful long-term fruit storage. It has been demonstrated that short intermittent exposures to high CO₂ level atmospheres applied to Hass avocados during cold storage were effective in delaying senescence, reducing chilling injury and controlling decay (Marcellin and Chaves, 1983). However, no new studies until now

have been designed for the purpose of assessing the beneficial effect of postharvest re-exposure to high CO₂ levels for maintaining optimum fruit quality for as long as possible.

The aim of this work is therefore: 1) to establish the relationship between postharvest ultrastructural membrane integrity, cellular and membrane oxidative state and changes in the profile of fatty acid composition in the polar lipid fraction of cv. Autumn Royal table grapes (*Vitis vinifera* L.) during prolonged storage at 0 °C; 2) to analyze the differences in the susceptibility to cell and membrane cold-oxidative stress in fruit pre-treated with 20 kPa of CO₂ for 3 d; and 3) to evaluate the potential of short-term 20 kPa CO₂ re-exposure to lengthen the beneficial effect of the CO₂ treatment during long-term postharvest table grape storage and gain more insight into the CO₂-induced cold-tolerance responses and molecular mechanisms involved in cross-stress tolerance.

2. Materials and methods

2.1. Plant material and postharvest treatments

Seedless table grapes (*Vitis vinifera* L. cv. Autumn Royal) were harvested at mature stage (12.9 % total soluble solids, 0.46 % tartaric acid) from a commercial farm in Abarán (Murcia, Spain), and transported to the laboratory in Madrid (Spain). Bunches without any physical or pathological defects were randomly divided into three lots containing twenty bunches each and stored in the dark at 0 ± 0.5 °C and 90-95 % relative humidity up to 41 d in three hermetically sealed methacrylate containers of 1 m³ capacity. One lot was stored in air (untreated), a second lot was stored under a gas mixture containing 20 kPa CO₂ + 20 kPa O₂ + 60 kPa N₂ for 3 d at the beginning of cold storage (CO₂-treated), and a third lot was exposed to a second 3d 20 kPa CO₂ treatment

after 13 d of low temperature storage (CO₂/CO₂-treated). After CO₂ treatments, bunches were transferred to air under the same conditions as the untreated lot until the end of storage period. Five clusters of grapes (approximately 400 g each cluster) were sampled periodically and the skin and pulp were collected, frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C until analysis.

2.2. *Quality assessments and total decay*

Berry quality parameters were measured from the juice of 15 berries per triplicate. The soluble solids content (SSC) was determined at 20 °C and expressed in °Brix using a digital refractometer (Atago PR-101, Atago, Japan), the titratable acidity (TA), expressed as percentage of tartaric acid, was determined by titration with 0.1N NaOH to pH 8.1 (Mettler DL-70, Mettler-Toledo, Spain) and the pH was measured in a pH-meter with a glass electrode. To assess berry water loss, 15 berries were cut longitudinally into four pieces with a razor blade and dried at 75-80 °C until a constant dry weight, expressing the results on a percentage fresh weight basis. Skin firmness, expressed in Newtons (N), was determined on the equatorial side of 15 berries, and was measured by peak rupture force required for the 2 mm diameter puncture probe, at a speed of 20 mm min⁻¹, to penetrate 5 mm in the skin. A texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) controlled by the Texture Exponent Software (Texture Technologies and by Stable Micro Systems, Ltd., Scarsdale, NY, USA), and a 30 kg load cell was used.

Decay was assessed on the basis of the total decay after removing the healthy berries and was expressed as the percentage of the decayed berries with respect to the total cluster weight. Browning rachis index for each bunch was determined using five replicates per sample. The browning index was measured using the following subjective

scale: (0) none (entire rachis including the pedicels green-bright), slight (1) (rachis in good conditions and pedicels green-gray), (2) moderate (secondary rachis and pedicels, green-brown), (3) intense (secondary rachis and pedicels, brown, and primary rachis, green with brown areas), (4) severe (pedicels, primary and secondary rachis, brown). For estimation the cumulative bunches weight loss, the weight of five bunches were recorded on the day of harvest and after the different sampling periods and were expressed as a percentage loss with respect of the original weight.

2.3. Determination of malondialdehyde and hydrogen peroxide content

The end product of lipid peroxidation, MDA, was assayed using a thiobarbituric acid method according to Ederli et al. (1997) with modifications. Berries samples (0.5 g) were homogenized in 1.5 mL 5 % cold trichloroacetic acid (TCA) and centrifuged at 10,000 g for 15 min. The supernatant was collected and 150 μ L was mixed with 600 μ L 0.5 % thiobarbituric acid in 15 % TCA. The mixture was heated at 100 $^{\circ}$ C for 30 min and quickly cooled. Absorbance was measured at 532 nm and adjusted for non-specific absorbance at 600 nm. Three independent extractions were made for each sample and extracts were analyzed in duplicate. MDA content was estimated by using a molar extinction coefficient of 155 mmol L⁻¹ cm⁻¹. The results were expressed as the relative increase in μ mol per kg of fresh weight respect to the levels found in prestored fruit. The mean data of two analyses on three different biological samples are presented.

Hydrogen peroxide content in grape berries was measured spectrophotometrically after reaction with KI following the method described by Alexieva et al. (2001). Berries samples (1 g) were homogenized in 3 mL 0.1 % TCA and centrifuged at 12,000 g for 15 min at 4 $^{\circ}$ C. The reaction mixture consisted 0.25 mL 0.1 % TCA berry extract supernatant, 0.25 mL of 100 mM potassium phosphate buffer

pH 7.0 and 1 mL reagent (1 M KI in fresh double-distilled water). The blank probe consisted of 0.1 % TCA in the absence of berry extract. The reaction was developed for 1 h in darkness and absorbance measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve of H₂O₂ from 0 to 150 µM, and the results were expressed as the relative increase in µmol per kg of fresh weight respect to the levels found in prestored fruit. The mean data of two analyses on three different biological samples are presented.

2.4. Microscopic analyses of pericarp cells

Transmission electron microscopy (TEM) was used to examine ultrastructure of berries pericarp tissues (exocarp and outer mesocarp). For microscope analysis, six freshly picked berries were cut transversely into 1 mm thick discs and rectangular pieces (3 mm width × 5 mm length) from the outermost part of the pericarp were collected and immediately fixed by immersion in 1.5 mL of a solution of 4 % paraformaldehyde and 2.5 % glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 6 h at 4 °C. After four washes during 30 min in PBS and stored overnight at 4 °C, were post-fixed with 1 % OsO₄ in PBS for 1 h at room temperature. Following another extensive rinse in PBS, the sample was dehydrated in an ascending series of acetone (30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 2 x 100 %), 15 min each, and embedded in low viscosity Spurr's resin (Dow Chemical Co., USA), following a serial resin: acetone mixtures (1:3 for 1 h, 1:1 for 1 h and 3:1 for 2 h), using plastic molds overnight in the dark at room temperature. Polymerization was conducted at 60 °C for 24 h. The samples were examined by Light Transmission Microscope using a Zeiss Axioplan-2 Microscope (Oberkochen, Germany). For TEM, ultrathin sections (60-70 nm) were cut with a diamond knife on an ULTRACUT E (Reichert-Jung, Austria) ultramicrotome,

collected on thin-bar copper grids (100 mesh) coated with Formvar film, stained with uranyl acetate/lead citrate, and examined using a JEOL JEM 1010 Transmission Electron Microscope (Tokio, Japan) at an accelerating voltage of 100 kV. Three sections were cut from each biological sample and several cells in each section were analyzed (ICTS National Center of Electron Microscopy facility, University Complutense, Madrid, Spain). The micrographs shown are representative of the results obtained from six biological samples.

2.5. Fatty acid quantification in different lipid fractions

Total lipids were extracted from frozen and grinded skin and pulp tissues (10 g) per triplicate with 18 mL methanol, 18 mL chloroform and 5 mL ultrapure water after vortexing vigorously for 2 min and stored at 4 °C overnight. After centrifugation to 2,000 g for 15 min at 4 °C, the lower chloroform phase containing the total lipids was collected, concentrated to dryness under a stream of nitrogen and dissolved in 200 µL hexane. Aliquots of the extracts were used for estimation of different lipid fractions following the method described by Blanch et al. (2019). Briefly, a SPE Bond Elut NH2 500 mg column was used for fatty acids fractionating, eluting: neutral lipids, free fatty acids and polar fatty acids, mainly phospholipids, using the internal standards glyceryl tritridecanoate (1,2,3-Tritridecanoylglycerol), nonadecanoic acid and 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine (purchased from Sigma, Germany and Cymit Química, Spain). After methylation of fatty acids from the eluted lipid fraction with sodium methoxide in anhydrous methanol, an aliquot from upper phase containing the fatty acid methyl esters (FAMES) were separated and quantified by gas chromatography (GC, Agilent 7820A) equipped with a flame ionization detector (FID), using a capillary fused silica column (Agilent GC-12 CP-SIL88, 100 m x 250 µm x 0.2

μm), a gradient temperature and helium as the carrier gas. Identification of fatty acids was performed by comparing retention times with those of mixed FAME standards (FAME 37 SUPELCO + PUFA N° 2 Animal Source Sigma + PUFA N° 3 Menhaden oil Sigma), and quantification was made against internal standards. Results were expressed as mg kg^{-1} of fresh weight or as a percentage basis from total fatty acids. The data represent the means of three biological replicates with two different technical measurements for each. Different ratios were used to indicate the degree of unsaturation of the fatty acid in polar lipids: unsaturated/saturated fatty acids (UFA/SFA), index of unsaturated fatty acids (IUFA) calculated by the formula: $[(18:1) \times 1 + (18:2) \times 2 + (18:3) \times 3]$, the degree unsaturation of 18-carbon fatty acids calculated by the formula: $(18:1) + (18:2) + (18:3) / (18:0)$, and the ratio $(18:3) / (18:2)$.

2.6. Statistical analysis

One-way ANOVA was performed using SPSS Statistics ver. 19.0. (IBM Corporation). A multiple comparison of the means was performed using the Tukey's test, with the level of significance at $P \leq 0.05$.

3. Results

3.1. Evaluation of the effect of short-term high CO₂ treatments on quality parameters in Autumn Royal table grapes and total decay during cold storage

Untreated Autumn Royal bunches showed a significant increase in weight loss of up to 7.6 ± 0.1 % during prolonged low-temperature postharvest storage and a maximum value in the rachis browning index (Table 1). The application of one or two short-term treatments with 20 kPa of CO₂ for 3 d was effective in controlling quality loss in the bunches, reaching values that were significantly lower ($P \leq 0.05$) than in

269 untreated grapes after 41 d at 0 °C, and even lower in grapes treated with a second short-
270 term CO₂ treatment. In terms of berry quality, whereas the SSC content remained fairly
271 constant, a significant increase in the SSC/TA ratio ($P \leq 0.05$) and a decrease in pH and
272 TA values were observed up to 28 d of storage in untreated fruit. At this storage time,

Table 1

Quality parameters and total decay of untreated (Air) and treated with 20 kPa CO₂ (CO₂-treated; CO₂/CO₂-treated) Autumn Royal table grapes during storage at 0 °C.

Days at 0 °C	SSC (%)	TA (%)	Maturity index (SSC/TA)	pH	Berry moisture content (%)	Firmness (N)	Decay (%)	Rachis browning index	Weight loss (%)
Prestored	12.9a	0.46b	27.9a	3.88b	89.1e	0.64a	–	–	–
Air									
3 d	12.9a	0.48b	27.1a	3.84b	88.5cd	0.60a	–	0.9b	1.6ab
16 d	13.7b	0.49b	28.1a	3.85b	87.6b	0.53a	–	2.1c	2.8c
28 d	13.0a	0.41a	31.6b	3.77a	87.6b	0.42a	4.4b	3.7d	5.1f
41 d	14.9d	0.43ab	34.5d	3.91bc	87.0a	0.51a	13.7d	4.0e	7.6g
CO₂-treated									
3 d	13.1a	0.48b	27.1a	3.94c	88.4cd	0.56a	–	0.5a	1.0a
16 d	14.5c	0.48b	30.5b	3.86b	88.3 cd	0.54a	–	1.4bc	2.2b
28 d	13.4ab	0.43ab	31.3b	3.81ab	88.1c	0.52a	2.3a	2.7cd	4.3e
41 d	13.4ab	0.41ab	32.6bc	3.85b	88.2c	0.48a	7.0c	3.4d	5.2f
CO₂/CO₂-treated									
16 d	14.0bc	0.49b	28.5a	3.90bc	88.6d	0.54a	–	1.3bc	1.3a
28 d	13.4ab	0.42ab	31.9b	3.80ab	88.7d	0.49a	2.0a	2.0c	3.5d
41 d	13.2ab	0.43ab	30.9b	3.83ab	88.7d	0.52a	4.2b	3.0cd	4.5e

Data represent the mean and each letter within columns indicates the significant differences between means determined with a Tukey's test ($P \leq 0.05$).

whereas TA and pH values remained unchanged compared to prestored fruit, the same trend was observed in SSC and maturity index in untreated and in CO₂ and CO₂/CO₂ treated table grapes (Table 1). However, after 41 d of cold storage, whereas a significant increase ($P \leq 0.05$) in SSC content, SSC/TA ratio and pH values were observed in untreated grapes, CO₂-treated grapes had invariable the berry quality parameter values. Another important characteristic of table grape quality is texture. The puncture test revealed that the initial values obtained in fruit after harvest were maintained throughout postharvest storage, and there were no significant differences ($P \leq 0.05$) between untreated and treated grapes in skin firmness values (Table 1).

The evaluation of the degree of dehydration of Autumn Royal table grapes during postharvest storage revealed a progressive decrease in water content throughout the cold-storage period in untreated fruit (Table 1), reaching water loss of up to 2.4 % at the end of cold storage compared to freshly harvested fruit. However, the berry water loss was less pronounced in grapes treated with a short-term 20 kPa CO₂ treatment. After the application of one CO₂ treatment, water loss was reduced by up to 43 % at the end of the storage period compared to untreated grapes, and the degree of dehydration of the berries fell by up to 80 % when a second CO₂ treatment was applied (Table 1). With regard to decay incidence in postharvest Autumn Royal table grapes, no total decay was observed until 28 d of cold storage, while untreated fruit showed a sharp increase up to 41 d (Table 1). The results also indicated that one and two CO₂ treatments significantly ($P \leq 0.05$) reduced the incidence of rot throughout cold storage, although the effectiveness of the second CO₂ treatment became evident after 41 d when almost 14 % of total decay was quantified in untreated grapes, while only 7 % and 4 % of diseased berries were found in one- and two-time CO₂-treated clusters respectively (Table 1).

3.2. Effect of high CO₂ treatments on prevention against membrane and cellular oxidative damage in Autumn Royal table grapes during cold storage

MDA concentration and hydrogen peroxide were measured in untreated and short-term 20 kPa CO₂-treated Autumn Royal berries at different cold storage times to obtain evidence of membrane and cell oxidative damage respectively. The quantification of these oxidative damage markers revealed that prestored berries contained 52 ± 3 and 28 ± 4 μmol per kg of fresh weight levels of MDA and H₂O₂ respectively.

Figure 1

Fig. 1. Malondialdehyde (A) and hydrogen peroxide (B) in untreated (Air), CO₂-treated (CO₂) and CO₂/CO₂-treated (CO₂/CO₂) Autumn Royal table grapes after storage at 0 °C. The results were expressed as the relative increase with respect to the levels in prestored fruit (day 0). The error bars represent the standard deviation of the mean and each letter indicates the significant differences between the means determined with a Tukey's test ($P \leq 0.05$).

As shown in Figure 1, the levels of both MDA and H₂O₂ oxidative markers increased with storage time in untreated table grapes, but with a different pattern. The initial level of MDA increased progressively from 16 d of storage at 0 °C, reaching the maximum values in untreated grapes after 41 d (Fig. 1A). CO₂ treatments restrain the level of oxidative stress, and the concentration of MDA in both CO₂-treated fruit was lower than in untreated ones. In terms of lipid peroxidation status, it is worth noting that the differences in the increased levels of MDA between untreated and one- or two-time treated fruit were more pronounced after 41 d of storage at 0 °C. The increase in MDA was 1.8- and 3.3-fold lower than in untreated table grapes in one- or two-time 20 kPa CO₂-treated fruit respectively (Fig. 1A). The level of H₂O₂ increased in Autumn Royal fruit from the beginning of the low-temperature storage and was up-regulated after 28 d to levels that were maintained until the end of storage at 0 °C (Fig. 1B). A similar hydrogen peroxide generation pattern was observed in one-time CO₂-treated berries, although the H₂O₂ increase was smaller. Nevertheless, it is worth noting that the level of cellular oxidative stress at the beginning of cold storage in the two-time CO₂ treatment was significantly lower ($P \leq 0.05$) and that these H₂O₂ levels were maintained up to 41 d of storage at 0 °C. At this time, the increase in hydrogen peroxide generation in two-time CO₂-treated grapes was up to 2.3- and 1.6-fold lower than in untreated and one-time CO₂-treated grapes respectively (Fig. 1B).

3.3. Effect of high CO₂ treatments on cell ultrastructure in Autumn Royal table grape tissues after prolonged cold storage

Micrographs obtained by TEM revealed that the pericarp cells of freshly harvested grape tissue had a high degree of cell wall integrity, with the middle lamellae appearing as a markedly electron-dense region (Fig. 2, day 0 and Fig. 3, day 0). The

plasma membrane was also pressed tightly against the cell wall and the peripheral cytoplasm, confined to a narrow layer adjacent to the cell wall, was homogeneous and rich in organelles. In the nucleus, the nucleoplasm was homogeneous and the double nuclear membrane well preserved (Fig. 2, day 0 and Fig. 3, day 0). The chloroplast/plastid was elongated, about 10 μm in length, with all its membranes intact, forming grana thylakoids stacked and interconnected with each other by stromal thylakoids and containing small osmiophilic globuli.

Figure 2

Fig. 2. Cell pericarp ultrastructure of prestored (day 0), untreated (Air), CO₂-treated (CO₂) and CO₂/CO₂-treated (CO₂/CO₂) Autumn Royal table grapes after storage at 28 d and 41 d at 0 °C. Observations of pericarp micrographs obtained by TEM. The scale bars represent 1 μm . The micrographs shown are representative of six biological samples.

The mitochondria, with well-developed cristae, were numerous and well distributed around the chloroplast or nucleus. Ultrastructural cold damage developed progressively with time, since no significant alterations were observed by TEM in untreated fruit after 28 d at 0 °C. When the cytoplasm and the plasma membrane began to separate from the cell wall in the pericarp, the cell and organelle membranes retained their integrity (Fig. 2, 28 d in Air). However, micrographs of pericarp cells after 41 d at 0 °C showed significant signs of cell damage, with a gradual disruption of the structure of the cell organelles (Fig. 2, 41 d in Air). Although the cell ultrastructure showed no marked differences between CO₂ and CO₂/CO₂-treated and untreated fruit after 28 d, the effect of CO₂ treatments on the maintenance of cell integrity was more noticeable after 41 d (Fig. 2, 41 d).

At the electron microscopy level, and from a general view, no significant alteration was observed in the cell wall of untreated and treated pericarp cells at the end of cold storage. The longitudinal cellulose microfibrils were arranged parallel to the surface and to the plasma membrane, and the different cells presented a continuous and non-disrupted plasmalemma (Fig. 3, PM-CW). However, a cell wall-plasma membrane separation was detected in cells of untreated grapes after 41 d of cold storage. Likewise, but to a lesser extent, detailed membrane-wall separations were located in CO₂-treated fruits, while in CO₂/CO₂-treated fruit the membrane remained completely fixed to the cell wall until the end of the cold storage period (Fig. 3, PM-CW). The nuclei in prestored grapes appeared mainly in the euchromatin form (Fig. 3 NM, day 0). After 41 d of storage, the nucleus of the untreated fruit showed intense condensation of the chromatin in heterochromatin as well as irreversible symptoms of caryolysis. The nuclear study of CO₂-treated fruits revealed a predominance of euchromatin, which was particularly evident in CO₂/CO₂-treated fruit (nuclei with a similar appearance to those

of freshly harvested fruit). The bilayer nuclear membrane remained intact in all the samples analyzed, with no alterations associated with low-temperature storage.

Figure 3

Fig. 3. Transmission electron micrographs of plasmalemma and cell wall (PM+CW), nuclear membrane (NM), mitochondria and chloroplasts in pericarp cell of prestored (day 0), untreated (Air), CO₂-treated (CO₂) and CO₂/CO₂-treated (CO₂/CO₂) Autumn Royal table grapes after storage at 41 d at 0 °C. The scale bars represent 1 μm. The micrographs shown are representative of six biological samples.

Although the organelle is sensitive to low temperature, a large number of mitochondria were observed in all the samples analyzed. “Typical” mitochondria were observed in freshly harvested fruit (Fig. 3 Mitochondria, day 0), with both external and

internal mitochondrial membranes without disruption and organized into mitochondrial cristae arranged in the form of a tube. After 41 d, the outer membrane of the mitochondria in untreated fruit became more osmiophilic and an absolute loss of organization was observed in the mitochondrial cristae, causing the disappearance of a defined matrix space. In contrast, treated fruit (CO_2 and CO_2/CO_2) showed structured mitochondria with no damage at the membrane level. It was thus possible to identify the mitochondrial cristae and the matrix space, and establish their similarity with the morphology of the mitochondria in freshly harvested fruit (Fig. 3 Mitochondria). Finally, the study of chloroplasts/plastids, the most sensitive organelle to low temperature, revealed that after 41 d of cold storage this organelle had acquired a swollen shape, the thylakoid architecture had disappeared and the integrity of the outer membrane was seriously compromised (Fig. 3 Chloroplast). The results indicate that the CO_2 -treated grapes did not develop symptoms of cold damage, which was reflected in the maintained morphology of the chloroplasts, the correct distribution of the thylakoids and the non-appearance of disruptions in their outer membrane. Small continuous vesicles were found within the stroma of the chloroplasts of freshly harvested grapes, and the inner membrane of the chloroplast was identified as peripheral reticulum. It should be noted that although very low levels of peripheral reticulum were identified in the chloroplasts from the start of conservation, the peripheral reticulum was not visible after 41 d of cold storage given the degree of stromal degradation of the untreated fruits. However, while its appearance in the CO_2 -treated grapes was similar to that of freshly harvested fruits, the peripheral reticulum developed primarily with the dual CO_2 treatment.

3.4. Effect of high CO₂ treatments on lipid fatty acid composition in Autumn Royal table grape tissues after prolonged cold storage

Free and esterified fatty acids in neutral (triacylglycerols) and polar (membrane lipids) fractions were extracted, methylated, identified and quantified in skin and pulp tissues of Autumn Royal table grapes. Up to 17 fatty acids were identified in all the lipid fractions of prestored fruit: ten saturated (SFA: C12, C14, C16, C17, C18, C20, C21, C22, C23, C24), four monounsaturated (MUFA: C18:1n9c, C18:1n7c, C20:1n9, C22:1n9) and three polyunsaturated (PUFA: C18:3n3, C18:2n6c, C20:5n3). Compared to the percentage of total lipids (Supplementary Table 1), the main component in neutral and polar lipid fractions was PUFA (51-63 %), whereas in the free fatty acids fraction it was SFA (90 %) in both skin and pulp tissues (Fig. 4A and B).

Figure 4

Fig. 4. MUFA, PUFA and SFA content expressed on percentage basis from the total fatty acids (%), A and B, or mass basis (mg kg⁻¹ fresh weight), C and D, in neutral, free and polar lipid fractions in pulp, A and C, and skin, B and D, from prestored Autumn Royal table grapes. The error bars represent the standard deviation of the mean and each letter indicates the significant differences between the means determined with a Tukey's test ($P \leq 0.05$).

Supplementary Table 1

Composition and content of fatty acids, expressed on a percentage basis from the total fatty acids, in neutral, free and polar lipid fractions from pulp and skin tissues of prestored Autumn Royal table grape cultivar

Fatty acids (%) Lipid fraction	Pulp			Skin		
	Neutral	Free	Polar	Neutral	Free	Polar
Lauric acid (C12:0)	0.72	0.97	0.06	0.80	0.70	0.03
Myristic acid (C14:0)	2.63	2.50	0.46	6.36	1.89	0.30
Palmitic acid (C16:0)	19.03	32.47	27.84	16.87	25.00	24.26
Heptadecanoic acid (C17:0)	0.41	1.17	0.43	0.24	0.86	0.37
Stearic acid (C18:0)	9.86	40.79	2.74	5.23	34.57	3.35
Oleic acid (C18:1n9c)	3.88	5.83	1.40	8.01	2.96	2.49
cis-Vaccenic acid (C18:1n7c)	1.34	0.64	1.00	1.13	0.40	0.98
Linoleic acid (C18:2n6c)	29.02	2.01	40.89	19.53	1.93	40.56
α -Linolenic acid (C18:3n3)	23.69	0.61	18.98	23.30	1.39	23.08
Arachidic acid (C20:0)	1.15	2.89	0.76	1.67	3.08	0.60
cis-Eicosenoic acid (C20:1n9)	0.69	0.41	0.06	0.94	0.30	0.08
cis-Eicosapentaenoic acid (C20:5n3)	2.58	0.91	0.37	6.69	0.63	0.94
Paullinic acid (C21:0)	0.61	2.35	0.70	0.44	2.03	0.51
Behenic acid (C22:0)	1.53	2.47	2.53	3.19	8.20	1.58
Erucic acid (C22:1n9)	0.86	0.39	0.12	2.44	0.71	0.03
Tricosanoic acid (C23:0)	0.37	0.83	0.70	1.34	3.05	0.46
Lignoceric acid (C24:0)	1.63	2.78	0.97	1.83	12.29	0.40

With regard to mass base (fresh weight), although the relative abundance of UFA and SFA was conserved, it is worth noting that the polar fraction is the main lipid fraction extracted from both tissues of Autumn Royal table grapes (Fig. 4C and D). Between 85-89 % of this lipid fraction were PUFA, 61-77 % MUFA and 73-81 % SFA.

Fatty acid composition is known to affect membrane fluidity, stability and function. Changes in the profile of polar lipid fatty acid composition were therefore investigated in pulp and skin tissues in untreated and CO₂-treated fruit after prolonged cold storage, and the index of unsaturated fatty acids (IUFA), UFA/SFA ratio and

degree of unsaturation of C18 were calculated to evaluate the extent of cell membrane damage caused by cold during postharvest storage. The fatty acid composition, structural group and specific ratios are shown in Table 2 (pulp) and Table 3 (skin). Palmitic acid (C16) was predominant among the saturated fatty acids, with up to 75.5 % of the total polar lipid fraction, while linoleic acid (C18:2n6c) was the main unsaturated fatty acid (about 61 %) in both tissues of prestored fruit. Despite the similar fatty acid composition in the polar lipid fraction in skin and pulp tissues of Autumn Royal table grapes, the effect of long-term cold storage and short-term high CO₂ treatments was unlike. Whereas the levels of PUFA and SFA remained unchanged in pulp tissues (Table 2), a significant decrease ($P \leq 0.05$) in the level of MUFA was recorded in untreated grapes, mainly at the end of cold storage. In contrast, MUFA content was maintained or even increased in the pulp of CO₂-treated and CO₂/CO₂ treated grapes respectively. These results were mainly related with changes in the levels of oleic acid (C18:1n9c), which were higher in pulp treated with 20 kPa CO₂, and lower in untreated grapes (Table 2). The different ratios calculated to show the degree of unsaturation of fatty acids in the polar lipid fraction revealed that whereas the IUFA and the 18:3/18:2 fatty acid ratio remained unchanged, during long storage periods there was a significant increase ($P \leq 0.05$) in UFA/SFA and in the degree of unsaturation of 18-carbon fatty acids in the pulp tissues of both untreated grapes and those treated with high CO₂, compared to prestored grapes, due mostly to a decrease in the stearic acid (18:0) content (Table 2).

Table 2

Composition and content of fatty acids, expressed as mg kg⁻¹ fresh weight, in the pulp of prestored (day 0), untreated (Air) and treated with 20 kPa CO₂ (CO₂-treated and CO₂/CO₂-treated) Autumn Royal table grapes after the storage for 28 and 41 days at 0 °C.

Fatty acids	Day 0	Day 28			Day 41		
		Air	CO ₂	CO ₂ /CO ₂	Air	CO ₂	CO ₂ /CO ₂
Lauric acid (C12:0)	0.2b	0.1a	0.1a	0.1ab	0.2ab	0.2ab	0.2ab
Myristic acid (C14:0)	1.5b	1.2ab	1.2a	1.2ab	1.1a	1.3ab	1.3ab
Palmitic acid (C16:0)	91.5a	89.2a	82.7a	83.6a	86.6a	86.8a	87.5a
Heptadecanoic acid (C17:0)	1.4a	1.3a	1.3a	1.3a	1.1a	1.4a	1.4a
Stearic acid (C18:0)	9.0b	8.1a	7.6a	8.0a	7.2a	7.7a	7.8a
Oleic acid (C18:1n9c)	4.6bc	4.1ab	4.5bc	4.4bc	3.8a	4.4bc	5.0c
cis-Vaccenic acid (C18:1n7c)	3.3b	3.1ab	2.8a	2.8a	2.9ab	2.9ab	3.0ab
Linoleic acid (C18:2n6c)	134a	144a	138a	135a	141a	140a	139a
α- Linolenic acid (C18:3n3)	62.4a	63.2a	60.5a	59.9a	61.3a	63.8a	62.0a
Arachidic acid (C20:0)	2.5a	2.5a	2.4a	2.4a	2.5a	2.8a	2.5a
cis-Eicosenoic acid (C20:1n9)	0.2a	0.2a	0.3a	0.3a	0.2a	0.3a	0.2a
cis-Eicosapentaenoic acid (C20:5n3)	1.2b	1.4bc	1.6c	1.6c	1.0a	1.3bc	1.3bc
Paullinic acid (C21:0)	2.3a	2.4a	2.4a	2.4a	2.2a	2.4a	2.4a
Behenic acid (C22:0)	8.3a	8.8a	8.5a	8.3a	8.5a	8.8a	8.8a
Erucic acid (C22:1n9)	0.4a	0.5a	0.5a	0.6ab	0.3a	0.3a	0.5ab
Tricosanoic acid (C23:0)	2.3a	1.6a	1.7a	1.7a	2.2a	1.9a	2.0a
Lignoceric acid (C24:0)	3.2a	3.2a	3.0a	2.9a	2.9a	3.1a	3.0a
Σ MUFA	8.5c	7.9b	8.1bc	8.1bc	7.1a	7.9b	8.6c
Σ PUFA	199a	209a	200a	195a	203a	205a	202a
Σ SFA	122a	118a	111a	112a	115a	116a	117a
Σ UFA/ Σ SFA	1.7a	1.8b	1.9b	1.8b	1.8b	1.8b	1.8b
IUFA	463ab	484ab	465ab	456ab	473ab	478ab	472ab
Degree unsaturation C18	22.7a	26.5bc	27.1bc	25.2bc	29.0c	27.5c	26.8bc
C18:3n3/C18:2n6	0.47a	0.44a	0.43a	0.44a	0.43a	0.46a	0.45a

Data represent the mean and each letter within rows indicates the significant differences between means determined with a Tukey's test ($P \leq 0.05$). MUFA= monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, SFA = saturated fatty acid, IUFA = Index of unsaturated fatty acid.

The analysis of the fatty acid composition of membrane lipids in skin tissues during postharvest storage shows a notable decrease in MUFA and PUFA levels in untreated table grapes, mainly associated with a significant ($P \leq 0.05$) drop in oleic

(C18:1n9c), *cis*-vaccenic (C18:1n7c), linoleic (C18:2n6c) and α -linolenic (C18:3n3) acids. Treatment with 20 kPa CO₂ for 3 d at the beginning of cold storage maintained the initial unsaturated fatty acid levels in skin tissues, which even increased in two-time treated berries. Both CO₂ treatments raised the PUFA values after 41 d of low-temperature storage, mainly due to the elevated linoleic (C18:2n6c) and α -linolenic (C18:3n3) acid content. The lower SFA content, due to a significant decrease ($P \leq 0.05$) in palmitic acid (16:0), explained the elevated UFA/SFA ratio in the CO₂/CO₂-treated fruit, which was maintained in CO₂-treated grapes and decreased in untreated grapes (Table 3). The index of unsaturated fatty acids (IUFA) and the degree of unsaturation of 18-carbon fatty acids in the polar lipid fraction were significantly ($P \leq 0.05$) higher in the skin of CO₂-treated berries after 28 d, and especially after 41 d of cold storage. It is worth noting that these cold-tolerance markers increased noticeably in CO₂/CO₂-treated fruit after 41 d of storage at 0 °C (Table 3). It is also significant that in spite of the effect of CO₂ treatments on polar lipid fatty acid composition, the C18:3n3/C18:2n6c ratio did not change in skin tissue. In relation to the effect of low temperature and short-term high CO₂ levels on the profile of long-chain fatty acids, whereas after 28 d the amount of the omega-3 fatty acid eicosapentaenoic (C20:5) increased in the skin of untreated and treated berries, by the end of cold storage it declined to the level of prestored fruit, except in two-time CO₂-treated fruit where high levels were maintained (Table 3).

Table 3

Composition and content of fatty acids, expressed as mg kg⁻¹ fresh weight, in the skin of prestored (day 0), untreated (Air), and treated with 20 kPa CO₂ (CO₂-treated and CO₂/CO₂-treated) Autumn Royal table grapes after the storage for 28 and 41 days at 0 °C.

Fatty acids	Day 0	Day 28			Day 41		
		Air	CO ₂	CO ₂ /CO ₂	Air	CO ₂	CO ₂ /CO ₂
Lauric acid (C12:0)	0.2a	0.2a	0.2a	0.2a	0.2a	0.1a	0.1a
Myristic acid (C14:0)	2.3a	2.7a	2.8a	2.5a	2.8a	2.6a	2.2a
Palmitic acid (C16:0)	186cd	164ab	163ab	155ab	195d	172bc	150a
Heptadecanoic acid (C17:0)	2.8a	2.7a	2.6a	2.5a	2.6a	2.7a	2.4a
Stearic acid (C18:0)	25.7b	22.7b	21.9b	20.4b	22.7b	23.8b	19.0a
Oleic acid (C18:1n9c)	19.1c	14.5a	16.8b	16.0b	13.9a	16.0b	19.0c
cis-Vaccenic acid (C18:1n7c)	7.5b	7.7ab	8.1b	8.2bc	7.1a	8.5bc	8.6bc
Linoleic acid (C18:2n6c)	311c	267ab	302bc	287bc	284b	328d	356e
α-Linolenic acid (C18:3n3)	177b	158a	168ab	164ab	159a	184c	213d
Arachidic acid (C20:0)	4.6a	5.2a	4.8a	4.5a	5.4a	5.6a	4.8a
cis-Eicosenoic acid (C20:1n9)	0.6a	0.7a	0.8b	0.8b	0.7a	0.8b	1.1c
cis-Eicosapentaenoic acid (C20:5n3)	7.2ab	8.9c	9.4c	8.7c	6.6a	7.1a	8.8c
Paullinic acid (C21:0)	3.9a	4.0a	3.9a	3.8a	4.2a	4.1a	3.5a
Behenic acid (C22:0)	12.1a	16.0a	12.7a	13.2a	15.6a	14.9a	14.2a
Erucic acid (C22:1n9)	0.2a	0.7bc	0.8cd	0.7bc	0.6b	0.7bc	0.9cd
Tricosanoic acid (C23:0)	3.5a	4.2a	3.5a	3.7a	3.9a	3.7a	3.3a
Lignoceric acid (C24:0)	3.1a	3.3a	3.0a	2.9a	3.3a	3.4a	3.0a
Σ MUFA	27.4cd	23.5ab	26.5bc	25.8bc	22.3a	26.1bc	29.6d
Σ PUFA	495c	434ab	480bc	460bc	449ab	519d	577e
Σ SFA	244bc	225ab	218ab	208a	256c	233b	203a
Σ UFA/ Σ SFA	2.1bc	2.0ab	2.3c	2.3c	1.8a	2.3c	3.0d
IUFA	1180b	1030ab	1132b	1090ab	1066a	1232c	1379d
Degree unsaturation C18	20.0a	19.7a	22.6b	23.3b	20.4a	22.5b	31.4c
C18:3n3/C18:2n6c	0.57a	0.59a	0.58a	0.57a	0.56a	0.56a	0.60a

Data represent the mean and each letter within rows indicates the significant differences between means determined with a Tukey's test ($P \leq 0.05$). MUFA= monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, SFA = saturated fatty acid, IUFA = Index of unsaturated fatty acid.

4. Discussion

The postharvest quality of table grapes for fresh consumption is seriously compromised by fungal infection and cluster dehydration caused by berry water loss and rachis browning. As in the case of other highly perishable fruit, there is growing

interest worldwide in finding safe and natural alternatives to synthetic chemicals to control decay and improve grape quality during prolonged cold storage, mostly for distant markets. Among the approaches for maintaining the postharvest quality of table grapes the use of controlled atmosphere, and particularly high CO₂ treatment, is a non-damaging and effective technology for delaying softening and senescence-related changes and reducing fungal decay without compromising produce quality (Retamales et al., 2003; Artés-Hernández et al., 2004; Sanchez-Ballesta et al., 2006; Vazquez-Hernandez et al., 2018). However, the efficacy of CO₂ treatment depends on the concentrations, timing and duration of the treatment and storage length, as well as the cultivar and maturity (Crisosto et al. 2002a, 2002b; Romero et al., 2009; Terry et al., 2009). High CO₂ levels can be applied for short periods of time as a pre-treatment or as intermittent exposure to reactivate the metabolic responses, and is a potential solution to enhance successful long-term fruit storage.

In Autumn Royal grapes the progression of cluster quality parameters was slowed in 3-d 20 kPa CO₂-treated bunches, as has previously been described for the Cardinal table grape cultivar (Rosales et al., 2013), and was particularly effective when a second 3-d 20 kPa CO₂ treatment was applied after 13 d of cold storage. The values for berry quality parameters of titratable acidity, pH and firmness remained fairly constant during long-term cold storage, as has been reported for other cultivars (Crisosto et al., 2002b ; Artés-Hernández et al., 2004). However, prolonged storage at 0 °C increases solid soluble content and SSC/TA ratio in untreated berries. According to a previous study, in an early harvested state grapes (12.9 %), the increase in soluble solids and the maturity index could be the result of normal postharvest metabolic activity, and is delayed by CO₂ pre-treatment (Sanchez-Ballesta et al., 2006; Vazquez-Hernandez et al., 2018). The lower maturity index values in one- and two-time short-term 20 kPa CO₂-

treated berries could be caused by the delay in senescence-related physiological changes, and were more effective when a second CO₂ treatment was applied, contributing to prolonging a fresh-like quality in Autumn Royal table grapes. The effectiveness of high CO₂ treatments in slowing the senescence rate of Autumn Royal table berries is associated with a reduction in berry water loss and fungal decay incidence, physiological and pathological disorders that were mostly controlled after re-exposure to 20 kPa CO₂ for 3 d. It has been recently shown that prestorage application of high carbon dioxide combined with controlled atmosphere storage or 1-MCP combined with high CO₂ levels are novel dual approaches to control decay incidence in grape berries without affecting visual or sensory quality (Teles et al., 2014; Wang et al., 2019). The application of a second short-term 20 kPa CO₂ treatment could thus be a promising low-cost and low-waste commercial alternative technology for controlling dehydration and grey mold incidence without any loss of cluster quality during long-term table grape cold storage.

Cell membranes also provide a link between storage disorders, since fruit senescence – as an oxidative phenomenon –, and low temperature can cause a marked increase in reactive oxygen species, affecting membrane lipid organization and the integrity of cell and organelle membranes (Mittler, 2002; Sevillano et al., 2009; Tian et al., 2013), which are closely related to fruit quality. The degree of lipid membrane peroxidation and oxidative stress in untreated Autumn Royal berries reveals a loss of integrity in membrane systems that leads to altered membrane properties and a loss of subcellular compartmentation during the cold storage period, leading water loss and greater susceptibility to fungal attack. Increased peroxidation of membrane lipids has been noted in chilling-sensitive crops at chilling temperatures, which could reduce the degree of lipid unsaturation and induce phase transition of membrane lipids from a

liquid-crystalline to a solid-gel state (Marangoni et al., 1996). The application of short-term CO₂ treatments is associated with reduced cellular and membrane oxidative damage during long-term Autumn Royal berry cold-storage, restrained the degree of intracellular oxidative stress and peroxidation of unsaturated fatty acids in the polar lipids responsible for damage to the cell membranes (Gutteridge and Halliwell, 1990; Sairam and Srivastava, 2002).

To date, few studies have reported the effect of environmental conditions such as cold stress on fruit at the cellular and subcellular level (Luza et al., 1992; Yang et al., 2009), and none have focused on the effect of short-term high CO₂-treatment. Cell ultrastructure analysis of Autumn Royal grapes during cold storage revealed that ultrastructural symptoms of damage developed progressively with storage time at 0 °C. The ultrastructural changes observed were similar to those described for mature-green tomatoes, where organelles are severely damaged under cold stress and only plasmalemma are reasonably membrane resistant (Yang et al., 2009). These symptoms are largely similar across species under chilling stress, and also resemble those seen in programmed cell death (Kratsch and Wise, 2000). In addition to the response to cold stress, the ultrastructural damage observed could also be related to the berries' dehydration status, another stressful postharvest environmental condition that induces oxidative stress, and which could be a potential cause of the cytomembrane damage associated with long-term storage disorders (Ben-Yehoshua and Rodov, 2002; Sharma et al., 2012). Moreover, as has been described for table grapes (Zhang et al., 2005) and fruit (Gutiérrez et al., 1992; Liu et al., 2015), chloroplasts are the first and most severely impacted organelle. Oxidative damage is particularly severe in chloroplasts and mitochondria, since a greater reactive oxygen species imbalance occurs during photorespiration, photosynthesis and mitochondrial respiration when the plants are

exposed to stress situations (Sharma et al., 2012). Although the nucleus and mitochondria are generally less susceptible to cold stress, the changes manifested in the ultrastructural organization of these organelles are related to the disruption of cell metabolic and transcriptional processes induced by environmental factors, which reduce the defense responses to abiotic and biotic stress (Kratsch and Wise, 2000), mainly in non-photosynthetic tissues.

Unlike untreated berries, fruit under short-term 20 kPa CO₂ treatment primarily retained an integrated cell ultrastructure throughout long-term cold storage. The results revealed that the more short-term high CO₂ treatment is applied to Autumn Royal grapes, the more the cell ultrastructure is maintained, and the cell organelles are not adversely affected during long-term storage at low temperature. The beneficial effect of cycle applications of intermittent warming to enhance successful long-term fruit storage has also been previously described (Cabrera and Saltveit, 1990; Biswas et al., 2012; Liu et al., 2015). The molecular, microscopic and macroscopic tissues features are usually paralleled with functional properties (Sila et al., 2008) which are directly associated with the health of the cell organelles. The conservation of mitochondria, chloroplast and nuclear integrity and functionality thus leads to the transcription of genes involved in the cell's biotic and abiotic stress defense responses (Navarro et al., 2015; Rosales et al., 2016; Romero et al., 2018) and the preservation of the energy status essential to maintain membrane integrity and control metabolic damage repair (Sevillano et al., 2009; Yadav, 2010). Plant mitochondria, in addition to being an energy-producing organelle, may control stress-reactive oxygen species generation by means of energy-dissipating systems and therefore play a central role in cell adaptation to abiotic stresses, which are known to induce oxidative damage at the cellular level (Gill and

Tuteja, 2010). These results provide the first cytological evidence of postharvest storage disorders tolerance induced by CO₂ treatment.

The maintenance of the integrity of the organelle membrane may be a consequence of the lower production rate of reactive oxygen species and the rate of lipid peroxidation in cells mediated by high CO₂ levels, as mentioned above. It has been suggested that the induction of antioxidant enzymes to scavenge hydrogen peroxide and superoxide anion by UV-C treatment is associated with the maintenance of mitochondrial membrane integrity, which also plays a key role in delaying senescence in peach fruit (Yang et al., 2014). It is thought that the main cause of senescence in postharvest fruit is irreversible mitochondrial dysfunction due to the accumulation of reactive oxygen species (Qin et al., 2009). It has also been suggested in Cardinal table grapes that the antioxidant enzyme ascorbate peroxidase may participate in H₂O₂ low-temperature induced removal, and could be unleashed by 3-d CO₂ pre-treatment (Romero et al., 2008).

Polar lipids (phospholipids, glycolipids and glycerylphospholipids) are the main structural blocks in membranes, and any change in fatty acid lipid composition directly affects membrane stability and function (Maalekuu et al., 2006). In prestored Autumn Royal grapes, the composition of fatty acids in skin and pulp tissues was similar, and the levels were of the order described for skin and pulp tissues in other table grape cultivars, with the majority – in percentage or base mass – being linoleic and palmitic acids (Pereira et al., 2011). Nevertheless, in our case a greater total number of up to 17 fatty acids were detected by gas chromatography in both Autumn Royal pericarp tissues. These were mainly long-chain unsaturated omega-3 fatty acids such as eicosapentaenoic (C20:5n3), omega-9 fatty acids such as erucic acid (C22:1n9) and saturated fatty acids such as heneicosanoic (C21:0) and tricosanoic acid (C23:0), with

the highest levels in the CO₂-treated grape tissues. Long-chain fatty acids formed by the elongation of C16 and C18 fatty acids are involved in lipid molecules deposited at the primary plant surfaces that serve as a barrier and prevent excess water loss and pathogen attack (Jenks et al., 1994).

It is significant that the constitutive levels of PUFA in the polar lipid fraction of pericarp tissues in Autumn Royal grapes were more than 1.5 and 2 times higher than those of SFA. This high proportion of polyunsaturated fatty acids could be positively correlated with the relative cold tolerance of table grapes. Higher membrane lipid unsaturation was beneficial in enhancing the resistance of loquat fruit to chilling injury (Cao et al., 2011). This feature could also explain the relative maintenance of cell microstructure in the pericarp of Autumn Royal table grapes for up to 28 d at 0 °C. However, an extension of the cold storage period led to a decrease in the main unsaturated fatty acid composition in the polar lipid fraction, predominantly in the degree of unsaturation of 18-carbon fatty acids, with a concomitant reduction in the UFA/SFA ratio and MUFA levels in skin and pulp tissues respectively.

Fatty acid desaturation is one factor involved in conferring cold-tolerance in fruit during chilling acclimation (Wang et al., 1992; Zhang and Tian, 2009). Short-term high CO₂ treatments led to an increase in the content of PUFA omega-6 linoleic and omega-3 α -linolenic acid in the membrane of exocarp cells, concomitant with a decrease in SFA palmitic and stearic acid, reflecting a shift in polar lipid metabolism and a more efficient antioxidant system that may contribute to helping to reduce water loss and membrane oxidative damage and renders grapes berries less susceptible to natural postharvest fungal infection. It has recently been suggested that the application of a 2-d 20 kPa treatment in strawberries induces an adjustment of fruit metabolism resulting in α -linolenic acid accumulation that could confer membrane stability during low-

temperature storage (Blanch et al., 2019). These features could be associated with the regulation of fatty acid desaturases due to the effect of high CO₂. It has been reported that a higher level of omega-3 fatty acid desaturase gene mRNA and α -linolenic acid were found in peach fruit stored at 0 °C (Zhang and Tian, 2009). Likewise, the integrity and functionality of energy-related organelles are essential for this cold-tolerance mechanism, since cell energy directly affects the biosynthesis of membrane lipids and cell membrane restoration (Zhou et al., 2014; Wang et al., 2015;), and because *de novo* fatty acid biosynthesis in plant cells occurs mainly in the plastids, after which the lipids are desaturated to highly unsaturated forms by the membrane-bound desaturases of the chloroplast and endoplasmic reticulum (Ohlrogge and Browse, 1995).

The higher polar lipid UFA/SFA ratio, IUFA and the degree of 18-carbon fatty acid unsaturation, associated with the significant increase in linoleic and α -linolenic acid levels, mainly in the twice-treated short-term high CO₂ table grapes, could contribute to preserving biomembrane fluidity and function, subcellular compartmentation and energy status during the period of long-term cold storage. It has been reported that appropriate intermittent warming cycles alleviated cold injury and are associated with an increase in polar lipid unsaturation of fatty acids (Liu et al., 2015; Wang and Baker, 1979). A higher unsaturation ratio of fatty acids has been previously reported in cold-resistant mitochondria and chloroplast membranes (especially in the envelope) as opposed to cold-sensitive membranes, due to an increase in the proportion of linoleic and α -linolenic acids (Alberdi and Corcuera, 1991; Upchurch, 2008). Further, the analysis of the composition of the polar lipid fraction reveals that the induced-CO₂ cold-tolerant molecular responses are tissue-dependent in Autumn Royal grapes. Marked differences in the transcriptome profiling of berry tissues have been reported previously, with more pronounced changes in exocarp cells (Becatti et al., 2010).

In conclusion, the application in Autumn Royal table grapes of a second treatment with 20 kPa CO₂ for 3 d was very effective in delaying the decline in harvested cluster quality caused by senescence and long-term postharvest cold-storage disorders in term of cluster weight loss, rachis browning, maturation index, berry water loss and decay incidence. The application of a second short-term high CO₂ treatment can enhance fruit tolerance to long-term cold storage disorders, and contribute to prolonging fresh-like quality in Autumn Royal table grapes. This work also establishes that at the microstructural level, CO₂ postharvest treatments counteract ultrastructural cell damage such as the disorganization of cytoplasm and the breakdown of cell organelle ultrastructure, leading to cellular biotic and abiotic stress defense gene transcription and the energy status essential for membrane integrity and control of metabolic damage repair. These results provide the first cytological evidence of postharvest storage disorders tolerance induced by CO₂ treatment. At the metabolic level, short-term CO₂ treatments significantly reduced the severity of long-term cold damage in pericarp tissues, restraining intracellular oxidative stress and lipid peroxidation, and leading to lipid metabolic adjustment improving the polar fatty acid lipid unsaturation levels responsible for maintaining the integrity and physiology of berry cell membranes.

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Figure Captions

Fig. 1. Malondialdehyde (A) and hydrogen peroxide (B) in untreated (Air), CO₂-treated (CO₂) and CO₂/CO₂-treated (CO₂/CO₂) Autumn Royal table grapes after storage at 0 °C. The results were expressed as the relative increase with respect to the levels in prestored fruit (day 0). The error bars represent the standard deviation of the mean and each letter indicates the significant differences between the means determined with a Tukey's test ($P \leq 0.05$).

Fig. 2. Cell pericarp ultrastructure of prestored (day 0), untreated (Air), CO₂-treated (CO₂) and CO₂/CO₂-treated (CO₂/CO₂) Autumn Royal table grapes after storage at 28 d and 41 d at 0 °C. Observations of pericarp micrographs obtained by TEM. The scale bars represent 1 µm. The micrographs shown are representative of six biological samples.

Fig. 3. Transmission electron micrographs of plasmalemma and cell wall (PM+CW), nuclear membrane (NM), mitochondria and chloroplasts in pericarp cell of prestored (day 0), untreated (Air), CO₂-treated (CO₂) and CO₂/CO₂-treated (CO₂/CO₂) Autumn Royal table grapes after storage at 41 d at 0 °C. The scale bars represent 1 µm. The micrographs shown are representative of six biological samples.

Fig. 4. MUFA, PUFA and SFA content expressed on percentage basis from the total fatty acids (%), A and B, or mass basis (mg kg⁻¹ fresh weight), C and D, in neutral, free and polar lipid fractions in pulp, A and C, and skin, B and D, from prestored Autumn Royal table grapes. The error bars represent the standard deviation of the mean and each

letter indicates the significant differences between the means determined with a Tukey's test ($P \leq 0.05$).

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