Scientia Horticulturae 234 (2018) 27–35

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

# Short-term high $CO_2$ treatment reduces water loss and decay by modulating defense proteins and organic osmolytes in Cardinal table grape after cold storage and shelf-life

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ARTICLE INFO

*Keywords:*
High $CO_2$
Skin
Pathogenesis-related protein
Dehydrin
Nitrogenous osmolyte
Trehalose

ABSTRACT

Shelf-life quality was improved when Cardinal table grapes (*Vitis vinifera* L.) were pretreated with 20 kPa of $CO_2$ for three days at the beginning of a long-term cold storage. This pretreatment was effective in avoiding postharvest losses of cv. Cardinal grapes in terms of water loss, oxidative damage and disease prevention. To elucidate those physiological and biochemical factors involved in preserving the postharvest shelf-life quality of table grapes, we studied the expression pattern of defense proteins such as pathogenesis-related proteins (PRs) and dehydrins, as well as the profile of protective osmolytes. The efficacy of a short-term high $CO_2$ pretreatment in reducing fungal disease could be mediated by the increase in the low molecular mass chitinase isoform of 16 kDa, which is up-regulated in the skin of $CO_2$-treated grapes in parallel with the shelf-life fungal decay control. In addition, the increasing accumulation of a 22 kDa dehydrin isoform and the endogenous levels of organic osmolytes proline and glycine betaine in fruit stored at 20 °C (mainly in $CO_2$-treated fruit) revealed that these protective biomolecules might play a more effective role in maintaining the structural and cellular homeostasis of table grapes after the shelf-life period, helping to reduce water loss and membrane oxidative damage (malondialdehyde accumulation) associated with the senescence-related disorders of postharvest table grapes.

## 1. Introduction

The aim of adequate postharvest handling is to maintain optimum fruit quality for as long as possible after harvest, to slow down the rate of senescence and the development of any rots, mainly during the shelf-life period, when the metabolic pathways resume their activity, increasing the senescence postharvest losses. These features are particularly relevant in non-climacteric fruit which need to stay on the tree or vine until ready to be consumed to avoid losing their desired characteristics. Postharvest fruit senescence, an oxidative phenomenon that requires a pronounced increase in reactive oxygen species, is eventually associated with the breakdown of cell structures, as well as with important changes in cell wall composition, leading to water loss, fruit softening and greater susceptibility to fungal attack (Tian et al., 2013).

The commercial quality and shelf-life of table grape (*Vitis vinifera* L.), a non-climacteric fruit with a relatively low rate of physiological activity, is considerably diminished after harvest by cluster dehydration, caused by berry water loss and rachis browning, as well as fungal attack (Palou et al., 2010). In addition, although table grapes are cold-tolerant fruit for which storage temperatures of −1.0 to 0.0 °C are recommended, low temperatures can cause structural damage, as well as metabolic and physiological dysfunction, which could increase their susceptibility to water loss and decay (Goñi et al., 2011a; Romero et al., 2006). Consequently, much effort has been focused on developing effective, non-damaging treatments that help extend the shelf life of fruit without negatively impacting its quality, which is crucial for grapes intended for table use. A high $CO_2$ treatment (15 kPa or above) is effective in delaying softening, senescence-related changes and reducing fungal decay during table grape cold storage, and is an effective alternative to $SO_2$ treatments for grey mold control without detrimental effects on table grape quality (Artés-Hernández et al. 2004; Crisosto et al. 2002a; Retamales et al. 2003). Sensitivity to $CO_2$ is, however, dependent on cultivar and maturity (Crisosto et al. 2002a, 2002b). A recent table grape transcriptomic study revealed that most highly represented genes which were differentially expressed in $CO_2$-treated table grapes berries were involved in amino acid, protein and nucleic

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https://doi.org/10.1016/j.scienta.2018.02.020
Received 16 November 2017; Received in revised form 2 February 2018; Accepted 3 February 2018

acid metabolism (Becatti et al., 2010). Moreover, this study demonstrated marked differences in the molecular response of berry tissues to short-term high $CO_2$ treatment, where epicarp cells appear to undergo more pronounced changes in transcriptome profiling when $CO_2$ levels are increased during storage (Becatti et al., 2010). It is important to highlight that the susceptibility to disease and water loss is particularly dependent on the cuticular barrier and the physiological conditions of the underlying epidermal cells in the skin (Boyer et al., 1997; Comménil et al., 1997).

We previously showed the efficacy of a pretreatment with 20 kPa of $CO_2$ for three days in improving the appearance of Cardinal table grapes, reducing withering, browning indices and decay incidence without detrimental effects on table grape quality during prolonged cold-storage (Goñi et al., 2011a; Romero et al., 2006; Sanchez-Ballesta et al., 2006). Several approaches have been followed to understand the biochemical and molecular mechanisms underlying the effectiveness of high $CO_2$ in controlling postharvest losses and to identify those biomarkers associated with its beneficial effect. Preliminary results showed that although the chitinase activity in Cardinal berry skin tissues is consistent with playing a role in total decay, the efficacy of a high $CO_2$ pretreatment in reducing fungal incidence is not mediated by the induction of *Vitis* class I chitinase and 1,3-β-glucanase gene expression (Romero et al., 2006). Furthermore, these studies have revealed new insights into how $CO_2$ treatments influence the tolerance of table grapes to fungal attack. Nevertheless, little is known about the changes in isoform expression or the timing of the accumulation of PR proteins in the skin of table grapes during postharvest storage, nor their implication in the biotic and abiotic stress tolerance mechanism of *Vitis vinifera* fruit.

Likewise, our earlier studies revealed that the gaseous pretreatment is effective in controlling water status in cold-stored fruit (Blanch et al., 2014; Goñi et al., 2011a). In terms of cellular water stress, we recently established that dehydrin and trehalose could be involved in the cold-tolerance mechanism activated by high $CO_2$ levels in Cardinal table grapes (Navarro et al., 2015). However, despite the literature on the application and effects of high $CO_2$ postharvest treatment, little is known about the effect of short-term high $CO_2$ on physiological and biochemical responses and the intrinsic quality of fruit during the postharvest shelf-life period. Plant cell response to dehydration includes an accumulation of osmotically active compounds including hydrophilic proteins such as dehydrins. Several physiological studies focusing on plant stress response have reported a positive relationship between the level of accumulation of dehydrin transcripts or proteins and plant tolerance to environmental stresses which cause cellular dehydration (Kosová et al., 2007). The physicochemical properties of dehydrins make them capable of binding water molecules, as well as proteins, phospholipids, nucleic acids and membranes, maintaining the cell's turgor, while structurally and functionally protecting the biomolecules associated with them from the damage caused by dehydration and freezing (Hara, 2010).

Another common adaptive response of plants to counteract the damage of environmental stressors is the redirection of metabolism towards the synthesis of various small organic molecules which, in addition to acting as osmoprotectants, in low accumulation directly protect macromolecules either by stabilizing membranes and the tertiary structure of proteins or by scavenging reactive oxygen species, as well as also possibly protecting cells from environmental stresses indirectly *via* their role in signal transduction and ion homeostasis. These include sugars such as the disaccharides sucrose and trehalose, oligosaccharides such as fructans, as well as amino acids such as glycine betaine, γ-aminobutyric acid (GABA) and proline, one of the major organic osmolytes which accumulate in response to stress (Ashraf and Foolad, 2007; Guy et al., 2008; Shelp et al., 1999). Due to the functional implication of dehydrins and organic osmolites in maintaining cellular water and antioxidant status, it would be commercially interesting to know how high $CO_2$ cold-activated defense strategies are regulated during the shelf-life period of table grapes when greater water loss and fungal decay occurs, and the role they play in maintaining the quality of $CO_2$ pretreated berries.

We therefore set out to analyze the effect of a short-term (three day) high $CO_2$ (20 kPa) treatment, applied at the beginning of the cold storage period, on the quality parameters of table grapes (*Vitis vinifera*) cv. Cardinal berry, as well as water loss and decay control after cold-storage and after shelf-life. Consequently, we study the implication of pathogenesis-related proteins, dehydrins and the profile of defense-related metabolites, such as trehalose, GABA, proline and glycine betaine in order to identify those biomolecules that could play a role in the mechanism activated by a high $CO_2$ treatment to overcome cellular water loss, membrane oxidative damage and fungal decay associated with the postharvest handling of table grapes.

## 2. Materials and methods

### 2.1. Plant material and postharvest treatment

Table grapes (*Vitis vinifera* L. cv. Cardinal) from Sevilla (Spain) were harvested at early-harvesting mature stage (12.8% total soluble solids, 0.84% tartaric acid). The field packaged bunches were transported to the laboratory and were immediately forced-air precooled for 14 h at −1 °C (prestored fruit). After cooling, the bunches without any physical or pathological defects were randomly divided into two lots containing ten bunches each and stored in the dark at 0 ± 0.5 °C and 90–95% relative humidity (RH). One lot was stored in air for up to 33 days (untreated), and the other was stored under a gas mixture containing 20 kPa $CO_2$/20 kPa $O_2$/60 kPa $N_2$ ($CO_2$-treated) for three days and then transferred to air under the same conditions as the untreated lot for 30 days. After 33 days, the untreated and $CO_2$-treated grapes were transferred to ventilated storage containers for a further two days at 20 °C and 90–95% relative humidity to simulate their shelf-life during marketing. Clusters of grapes were sampled at the end of the cold storage and their shelf-life. The berries obtained from five clusters (approximately 350 g each cluster) were peeled and the skin was frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C for analysis.

### 2.2. Quality assessments

Berry quality assessments were measured on 15 berries per triplicate and comprised soluble solids contents (SSC), pH, titratable acidity (TA), color and skin firmness. SSC was determined using a digital refractometer Atago PR-101 (Atago Co. Ltd., Japan) at 20 °C and expressed in ºBrix. The pH of the juice was measured in a pH meter with a glass electrode. TA was determined by titration with 0.1N NaOH up to pH 8.1 and results were expressed as % tartaric acid. Berry skin color was measured at three different positions around the equatorial region using the Hunter Lab System and a Minolta CR200TM colorimeter (Minolta Camera Co., Osaka, Japan). Results were given in Commission Internationale de l'Eclairage $L^*$ (lightness), $a^*$, and $b^*$ (CIELAB) color space coordinates. Hue angle (hº) ($\tan^{-1} b/a$) and chroma ($C^*$, saturation) [$(a^2 + b^2)^{1/2}$] were calculated from $a^*$ and $b^*$.

Skin rupture force was measured on the equatorial side of berries using a 2 mm diameter puncture probe at a speed of 20 mm $min^{-1}$, and was determined by measuring the peak force required for the probe to penetrate 5 mm in the skin using 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), using a 30 kg load cell. Skin firmness was expressed in Newtons (N).

### 2.3. Berry water content and total decay

To estimate the water loss during postharvest cold storage and shelf-life period, 15 berries were cut longitudinally into four pieces with a

razor blade and dried at 75–80 °C until a constant dry weight was attained in three consequent weight measurements. The water loss was expressed as the relative content of g of water by 100 g of fresh weight with respect to the levels in prestored fruit. Total decay was assessed on the basis of the total decay after removing and weighing the healthy berries. The weight of the decayed berries was calculated by subtracting the weight of the healthy berries from the total cluster weight. Total decay was expressed as the percentage of the decayed berries with respect to the original cluster weight.

### 2.4. Lipid peroxidation

Malondialdehyde (MDA) concentration, the end product of lipid peroxidation, was assayed using a thiobarbituric acid method according to Ederli et al. (1997) with modifications. Tissue 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 µL was mixed with 600 µL 0.5% thiobarbituric acid in 15% TCA. The mixture was heated at 100 °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 $L^{-1}$ $cm^{-1}$.

### 2.5. Isolation of the protein fraction and inmunodetection

The isolation of total protein fraction and immunodetection of defense proteins in skin tissues was performed as described previously by Navarro et al. (2015). Briefly, the total protein fraction was extracted three times from each sample using a phenol extraction protocol and precipitated with ammonium acetate in methanol. The resulting pellet was dried under nitrogen and resuspended in Laemmli sample buffer in reducing conditions (2.5% β-mercaptoethanol). The protein concentration was determined by the 2D-Quant Kit protocol (GE Healthcare) using bovine serum albumin (BSA) as a standard. Proteins were separated in a SDS-PAGE (13.5% polyacrylamide) and electro-transferred to PVDF membranes (Amersham). The membranes were probed with a polyclonal anti-dehydrin antiserum (1/3000: Agrisera) and with tobacco anti-PR-Q (1/7500), anti-PR-2 (1/7500), anti-PR-5 (1/12500) antisera, which were detected with a horseradish peroxidase conjugated rabbit antiserum against IgG (Amersham). The immuno-complexes were visualized using a chemiluminescence (ECL®) detection system (Amersham) and quantified by densitometry. The results were expressed as the relative fold-change with respect to the levels of accumulation in prestored fruit. Independent experiments were carried out at least twice.

### 2.6. Cryoprotective activity

The *in vitro* cryoprotective activity of the protein fraction of skin tissue was determined using the method described previously by Goñi et al. (2009). To obtain an active protein fraction, 4 g of each sample was homogenized at 4 °C in 0.1 M potassium phosphate buffer pH 7.4 containing 1.5% (w/v) PVPP, 10 mM EDTA and 20 mM sodium ascorbate, centrifuged at 35,000*g* for 30 min and the resulting supernatant was filtered through Amicon 100 kDa cut-off centrifugal filters (Millipore). The filtrates were desalted and concentrated using Amicon 3 kDa cut-off filters. All steps were carried out at 4 °C. A sample of the protein extract and BSA fraction V (Sigma-Aldrich) was diluted to a concentration of 10 µg $mL^{-1}$ and assayed by cryoprotective activity. The data are presented as the lactate dehydrogenase (LDH) activity relative to the unfrozen controls and the mean data of two analyses on three different biological samples are also presented.

### 2.7. Chromatographic determination of organic osmolytes

Extraction of GABA, proline and glycine betaine from the skin tissue (0.5 g) was made in cold methanol (4 mL) for 30 min on ice. The methanol extracts were fractionated by adding 2 mL of chloroform and 2.4 mL of deionized water before being stored at 4 °C overnight. The upper aqueous phase was removed, concentrated to dryness under a stream of nitrogen, dissolved in deionized water and filtered through a 0.45 µm pore size membrane. The GABA, proline and glycine betaine content were determined by Q-TOF LC-MS, which was carried out using an Agilent 1200 series LC coupled to an Agilent 6530 accurate-mass quadrupole time-of-flight (Q-TOF) LC-MS with ESI-Jet Stream Technology (Agilent Technologies). Samples (1 µL) were separated on a Zorbax Eclipse XDB-C8 5 µm, 4.6 × 150 mm column (Agilent Technologies), and were eluted with an isocratic mobile phase made up of a mixture (75:25) of deionized water containing 0.1% heptafluorobutyric acid and 0.1% formic acid (solvent A), and acetonitrile (solvent B), at a flow rate of 1 mL $min^{-1}$ at 30 °C. The Q-TOF acquisition method was high resolution 4 GHz, with an extended dynamic range of 930 *m/z*. Atmospheric pressure electrospray ionization (ESI) was used with a drying gas flow rate of 10 L $min^{-1}$ at 125 °C, a sheath gas flow of 10 L $min^{-1}$ at 300 °C, a nebulizer at 40 psi, a cap voltage of 2500 V, a fragmentor voltage of 90 V, and a skimmer voltage of 65 V. The experiments were carried out at positive polarity with reference masses (*m/z* 121.0509 and 922.0098). Data Acquisition (version B.04.01) and Qualitative Analysis (version B.04.00) were performed on MassHunter Workstation software (Agilent Technologies). GABA, proline and glycine betaine were identified by their retention time and *m/z* data, and were quantified on the basis of a calibration curve derived from standards. The amino acid content was expressed as the relative increase in nmol per g of fresh weight with respect to the levels in prestored fruit. The mean data of two analyses on three different biological samples are presented.

The determination of trehalose levels was performed as described by Navarro et al. (2015). Briefly, 1.5 g of each sample was homogenized in 3 mL of heated ultra-pure water and boiled for 15 min. After centrifugation at 10,000*g* for 20 min at 4 °C and filtration, the trehalose content was determined using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), equipped with a Hamilton RCX30 (250 × 4.6 mm, 5 µm particle size) column and eluted using an isocratic gradient of 150 mM NaOH over 10 min at a flow rate of 1 mL $min^{-1}$. The trehalose content was expressed as the relative increase in nmol per g of fresh weight with respect to the levels in prestored fruit. The mean data of two analyses on three different biological samples are presented.

### 2.8. 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 and discussion

### 3.1. Quality parameters of untreated and $CO_2$-treated table grapes after cold storage and after shelf-life

Sugars (SSC), organic acids (TA) and the SSC/TA ratio are important quality factors which determine whether table grapes are acceptable to consumers. In general, it has been reported that SSC and TA, as well as pH values, remain fairly constant in different grape varieties during long-term cold storage under different high $CO_2$ conditions (Artés-Hernández et al., 2004; Crisosto et al., 2002b). However, to date there has been no description of how the quality parameters of these berries are modified in $CO_2$-treated fruit during a posterior shelf-life period. In the case of untreated Cardinal table grapes, prolonged cold storage

**Table 1**
Quality parameters and units of color space ($L^*$, $C^*$, and h°) of untreated (Air) and $CO_2$-treated ($CO_2$) Cardinal table grapes after storage for 33 days at 0 °C and after transfer to 20 °C for two days.

| Quality parameters | After-harvest | 33 days (0 °C) | | 33 + 2 days (20 °C) | |
|---|---|---|---|---|---|
| | | Air | $CO_2$ | Air | $CO_2$ |
| SSC (%) | 12.8 ± 0.1a | 14.1 ± 0.1b | 14.3 ± 0.04b | 15.1 ± 0.04c | 14.3 ± 0.05b |
| TA (%) | 0.84 ± 0.01c | 0.70 ± 0.01a | 0.77 ± 0.01b | 0.74 ± 0.02ab | 0.78 ± 0.01b |
| SSC/TA | 15.2 ± 0.1a | 20.1 ± 0.3c | 18.6 ± 0.2b | 20.4 ± 0.2c | 18.3 ± 0.1b |
| pH | 3.3 ± 0.01a | 3.7 ± 0.01b | 3.6 ± 0.01b | 3.5 ± 0.01ab | 3.6 ± 0.01b |
| $L^*$ | 36 ± 4b | 25 ± 2a | 23 ± 2a | 24 ± 3a | 23 ± 1a |
| $C^*$ | 17 ± 4c | 2.8 ± 0.5a | 4.1 ± 0.6b | 2.4 ± 0.6a | 3.8 ± 0.4b |
| hº | 346 ± 5a | 351 ± 6a | 347 ± 5a | 334 ± 5b | 347 ± 5a |
| Firmness (N) | 0.89 ± 0.2c | 0.56 ± 0.2a | 0.66 ± 0.2b | 0.58 ± 0.1a | 0.62 ± 0.2b |

The data are presented as the means ± SD of the three biological replicates ($n = 45$) and the different letters within rows indicate significant differences determined by Tukey's test ($P \leq 0.05$).

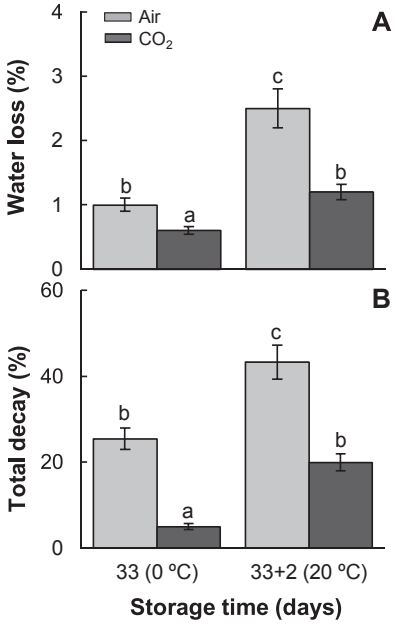


**Fig. 1.** Water loss (A) and total decay (B) in berries from untreated (Air) and $CO_2$-treated ($CO_2$) Cardinal table grapes after storage for 33 days at 0 °C and after transfer to 20 °C for two days. The results were expressed as the relative increase with respect to the levels in prestored fruit (day 0). The water loss data are the average of fifteen berries ± SD ($n = 15$). The total decay data are the average of five bunches ± SD ($n = 5$). The different letters indicate significant differences determined by Tukey's test ($P \leq 0.05$).

significantly ($P \leq 0.05$) increased pH and SSC content, as well as decreasing the TA values. The same dynamic was observed in $CO_2$-treated table grapes, although the TA decrease was smaller. Accordingly, the reached SSC/TA ratio was of 20 and 18 for untreated and $CO_2$-treated grapes, respectively (Table 1). After shelf-life, $CO_2$-treated grapes maintained this three parameter value invariable while in the case of untreated grapes there were minimum changes in the pH and TA and a significant ($P \leq 0.05$) increase in the SSC content, although the SSC/TA ratio attained after cold storage (Table 1) did not change. According to a previous study, increases in soluble solids and changes in the SSC/TA ratio could be the result of normal postharvest metabolic activity, and are delayed by the gaseous treatment (Sanchez-Ballesta et al., 2006). In this sense, the significant lower SSC/TA ratio value attained in $CO_2$-treated fruit after two days at 20 °C with respect to untreated ones could be caused by the delay in the metabolic changes associated with the senescence of this fruit provoked by the $CO_2$ pretreatment during cold storage, whose effects are maintained and reflected in the shelf-life period of table grapes.

Other important characteristics of table grape quality are color and texture. In fact, it is remarkable to note that the external color of the berries showed no browning in $CO_2$-treated fruit, with no significant differences between the $L^*$ values in untreated and $CO_2$-treated at the end of a prolonged cold storage and shelf-life. However, a short-term $CO_2$ treatment maintained a higher $C^*$ value than untreated fruit after 33 days at 0 °C, values which were maintained after two days at 20 °C. It is noteworthy that the Hue angle did not change throughout the cold storage period and only in the untreated fruit did the Hue angle decrease during the shelf-life period (Table 1).In addition, a puncture test revealed that the gaseous treatment maintained skin firmness values significantly ($P \leq 0.05$) higher than untreated grapes throughout postharvest storage. These results showed that the pretreatment with 20 kPa of $CO_2$ maintained the firmness and skin color of berries better than those which were untreated, showing no color anomalies after the shelf-life period, one of the most important parameters, together with the ratio between SSC and TA, which determines consumer acceptability of table grapes.

### 3.2. Water loss and total decay in untreated and $CO_2$-treated table grapes after cold storage and after shelf-life

In order to examine the effect of short-term high $CO_2$ treatment on controlling the degree of dehydration and on fungal incidence in Cardinal table berries during the shelf-life period, water loss and total decay were estimated in untreated and $CO_2$-treated fruit after 33 days of storage at 0 °C and after transfer to 20 °C for two days (shelf-life). After cold storage, the percentage of water loss was more accentuated in untreated fruit than in treated ones. When fruit were transferred to 20 °C a significant ($P \leq 0.05$) increase in water loss was detected in both groups. Nevertheless, in three-day $CO_2$ pretreated table grapes the water loss was significantly ($P \leq 0.05$) lower, up to 50% with respect to untreated grapes after the shelf-life period (Fig. 1A). Furthermore, in $CO_2$-treated fruit the total decay was significantly ($P \leq 0.05$) less than in untreated berries throughout the low temperature storage period (Fig. 1B). After 33 days, more than 25% total decay was quantified in untreated fruit, while only 5% of diseased berries were found in $CO_2$-treated clusters (Fig. 1B). When fruit were transferred to 20 °C for two days, the incidence of decay increased sharply and reached the maximum values of untreated fruit, which were in fact twice the values of $CO_2$-treated berries.

Although the rate of deterioration is affected by different factors, the commercial quality and shelf-life of table grapes is considerably diminished by water loss after harvest, which can result in stem browning, berry shatter, wilting, and shriveling of berries (Crisosto and Smilanick, 2007). In addition, gray mold, caused by the fungus *Botrytis cinerea* Pers. (1974), is the most common cause of postharvest decay of table grapes worldwide (Pearson and Goheen, 1988). These results show that a short-term high $CO_2$ treatment is effective in controlling the degree of water loss and the incidence of fungal decay in Cardinal table berries not only during prolonged cold storage, but also after removing

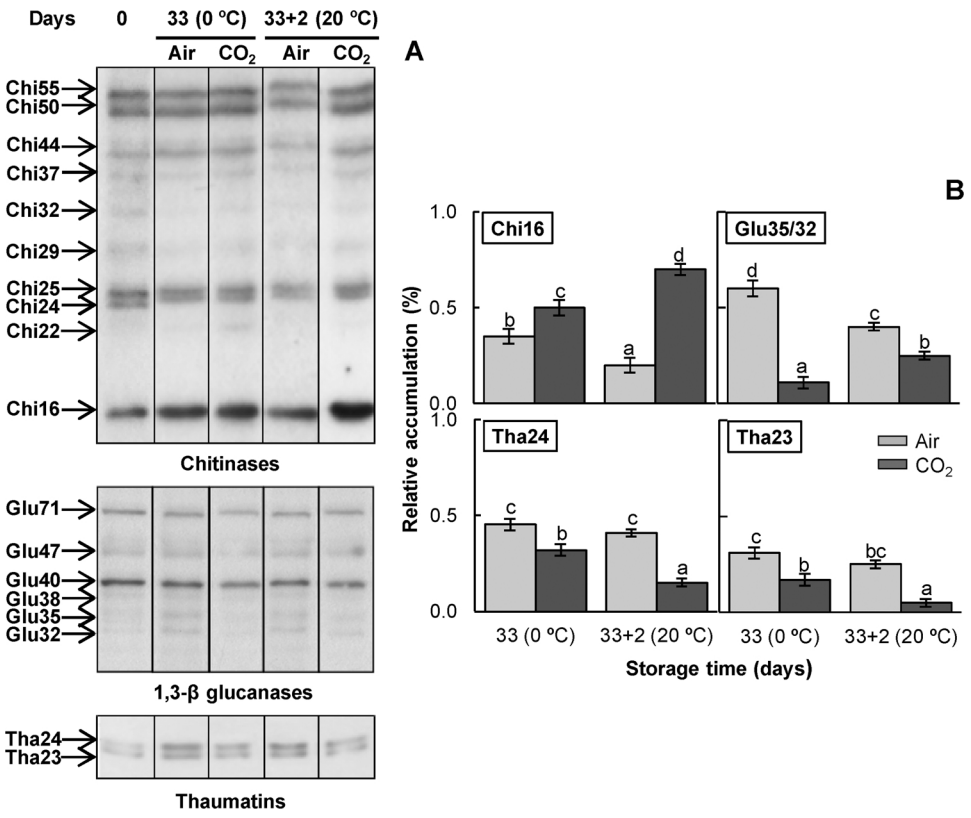


**Fig. 2.** Immunodetection of chitinase, 1,3-β-glucanase and thaumatin isoforms in the skin of untreated (Air) and $CO_2$-treated ($CO_2$) Cardinal table grapes after storage for 33 days at 0 °C, and after transfer to 20 °C for two days. A) Protein extracts (10 µg of protein) were separated by 13.5% SDS-PAGE, transferred to PVDF membranes and probed with antisera raised against tobacco PR-Q, PR-2 and PR-5 proteins. Lane 0: prestored fruit. The results shown are representative of three biological replicates. B) Histograms represent the changes in the levels of immunoreactive PRs bands, quantified by scanning densitometry of the autoradiographs, expressed as the relative fold-change with respect to the levels in prestored fruit (day 0). The data are the average of three biological replicates ± SD ($n = 6$) and the different letters indicate significant differences determined by Tukey's test ($P \leq 0.05$).

the fruit from shelf-life conditions where losses were greater, as was previously seen in other fruit (Barkai-Golan, 2001; Thompson, 2010).

### 3.3. PR protein isoforms in the skin of untreated and $CO_2$-treated table grapes after cold storage and after shelf-life

To better understand PR protein isoforms functions in postharvest table grapes, we analyzed the temporal expression pattern of these proteins at the end of low-temperature storage and shelf-life in the skin of Cardinal table grapes. In addition, we evaluated how pretreatment with high $CO_2$ levels affects the profile of these PR isoforms. The proteins recognized by the tobacco PR-Q (chitinase), PR-2 (1,3-β-glucanase) and PR-5 (thaumatin) antisera reflected a complex mixture of these defense protein families in the skin of prestored Cardinal table grapes (Fig. 2A). At harvest, almost nine polypeptides were recognized as chitinases, which showed a wide $M_r$ range, determined by SDS-PAGE, from 16 to 55 kDa, while there were six 1,3-β-glucanases with a $M_r$ from 32 to 71 kDa and two thaumatin-like proteins of 23 and 24 kDa.

During prolonged low temperature storage and after transfer to 20 °C, the expression of most chitinase isoforms did not change significantly ($P \leq 0.05$), while the relative abundance of Chi32 and Chi29 decreased after 33 days at 0 °C in untreated and $CO_2$-treated fruit, maintaining low steady state levels for two days at 20 °C (Fig. 2A). However, the expression of Chi16 isoform was cold and $CO_2$-regulated, with their expression level increasing after 33 days of storage at 0 °C, mainly in $CO_2$-treated fruit (Fig. 2B). Afterwards, during the shelf-life period the level of this low molecular chitinase decreased in untreated grapes, while a further increase was obtained in those pretreated with high levels of $CO_2$. It is noteworthy that the gaseous treatment induced a new chitinase of 22 kDa, although this isoform was only present in the skin of cold storage berries.

Regarding 1,3-β-glucanases, most of the skin isoforms do no change their expression during the different postharvest storage conditions (Fig. 2A). By contrast, the low molecular mass Glu35 and Glu32 isoforms were up-regulated at low temperature in fruit stored continuously under atmospheric conditions. In addition, when the fruit were transferred to 20 °C, the relative abundance of the low molecular mass 1,3-β-glucanases was further down-regulated. However, in $CO_2$-treated grapes a noticeable reduction in the expression level of these 1,3-β-glucanase isoforms was observed, maintaining similar values to those observed in the skin of prestored berries throughout the postharvest storage (Fig. 2B). However, Glu35 and Glu32 levels increase when $CO_2$-treated table grapes are transferred to 20 °C, although the levels reached were significantly ($P \leq 0.05$) lower than in the skin of untreated grapes after shelf-life (Fig. 2B). Thaumatin-like protein isoforms showed a similar pattern which was consistent in an increase of isoform values in both untreated and, albeit at a lower level, in $CO_2$-treated fruit after cold storage. After the shelf-life period, the expression of these isoforms was maintained in untreated grapes, while there was a significant ($P \leq 0.05$) decrease in the thaumatin-like proteins accumulation in the skin of treated grapes (Fig. 2B).

In grape berries, PR proteins are produced constitutively as a normal part of the ripening process (Monteiro et al., 2007; Robinson et al., 1997; Tattersall et al., 1997), and it is assumed that these defense proteins contribute to basal resistance against environmental stress and pathogen attack. Chitinase (group 3), 1,3-β-glucanase (group 2) and thaumatin-like (group 5) proteins are the three major PR protein families present in grape skin tissue from the onset of ripening in the absence of pathogen infection, and they become over-expressed as this tissue matures (Deytieux et al., 2007; Negri et al., 2008). Nevertheless, there is only limited data available concerning PR isoform expression in table grape cultivars and its modification during postharvest storage, as well as the role it plays in biotic and abiotic stress tolerance. Our results show that despite the complex pattern of pathogenesis-related proteins constitutively expressed in the skin of Cardinal table grapes after harvest, only some isoforms are differentially regulated by both temperature and atmospheric storage conditions. Although prolonged storage at low temperature maintained or even decreased the initial abundance of most PR isoforms, it led to an increase in the expression level of one chitinase (16 kDa), two 1,3-β-glucanases (35 and 32 kDa) and two

thaumatin-like proteins (23 and 24 kDa), indicating that these PR isoforms are cold responsive. Moreover, these cold-regulated PR isoforms were down-regulated after the shelf-life period, when a high level of decay occurs (Fig. 1B). As previously described for grapes (Comménil et al., 1997), the abundance of constitutive PR isoforms in the skin of Cardinal table grapes is not sufficient to totally protect berries from pathogen infection during postharvest cold storage, nor during the subsequent shelf-life period.

Short-term exposure to high levels of $CO_2$ also modified the PR defense response in table grapes. In general, gaseous treatment showed a residual effect on the expression of PR isoforms, maintaining similar levels to those in fruit after harvest, and therefore restraining the up-regulation of cold-regulated PR isoforms and those 1,3-β-glucanases associated with maximal fungal decay at 20 °C. These results are consistent with previous studies into the regulation of class I chitinase and 1,3-β-glucanase gene expression during postharvest storage of Cardinal table grapes (Romero et al., 2006). Although high $CO_2$ induced a new chitinase isoform of 22 kDa in the skin of prolonged cold storage Cardinal table grapes, the effectiveness of the short-term elevated $CO_2$ treatment to control postharvest decay seems to be mainly associated with the up-regulation of the expression of a 16 kDa chitinase isoform at the end of cold storage and mainly after shelf-life period. This low molecular mass chitinase could play a role in the active defense mechanism induced by the gaseous treatment which renders grape berries less susceptible to fungal infection. The accumulation of PR proteins is one of the most common markers for active plant defense. However, certain PR isoforms may be induced by a particular elicitor and only particular isoforms show antifungal activities (Goñi et al. 2010; Sela-Buurlage et al., 1993).

### 3.4. Dehydrin isoforms in the skin of untreated and $CO_2$-treated table grapes after cold storage and after shelf-life

The study of dehydrin accumulation patterns in untreated and $CO_2$-treated fruit after 33 days of low temperature storage and after transfer to 20 °C revealed that the four isoforms detected in the skin of table grapes differed in their expression level, which was regulated by environmental factors like temperature and high $CO_2$ exposure (Fig. 3A). At the end of a prolonged cold-storage period (33 days), an increase in the relative abundance of the four dehydrin isoforms (DHN44, DHN27, DHN22 and DHN17) was observed, mainly in those $CO_2$ pretreated grapes. Among the Cardinal table grape dehydrin isoforms constitutively expressed in skin tissues, are those of the molecular mass of 44, 22 and 17 kDa which reached the highest accumulation levels in the skin of untreated berries after cold storage. Although the gaseous pretreatment decreased the relative abundance of DHN44 with respect to those obtained in cold-storage untreated berries, a significant ($P \leq 0.05$) increase was found in the expression levels of the other three dehydrin isoforms. It should be noted that the skin of $CO_2$-treated fruit showed a 3-fold higher DHN17 expression level than untreated ones, and this low molecular dehydrin isoform was the most strongly $CO_2$-regulated during cold storage (Fig. 3B). When fruit which were stored continuously under atmospheric conditions were transferred to 20 °C for two days, most of the dehydrin isoforms decreased (such as DHN44 and DHN17) or maintained a steady state level (such as DHN27) whereas a significant ($P \leq 0.05$) rise in the 22 kDa isoform abundance was detected. Similarly, when $CO_2$-treated fruit were transferred to 20 °C, the abundance of the 44 and 27 kDa dehydrin isoforms were mostly unmodified, while there was a 4-fold decrease in DHN17. On the contrary, the expression levels of DHN22 underwent a noticeable increase in the skin tissues of treated berries, being 2-fold higher than in untreated ones (Fig. 3B).

There is little data about dehydrins during grape development and senescence or the role they play in the beneficial effect of postharvest storage technologies. It has been previously described that several grapevine dehydrin members are differentially expressed during plant

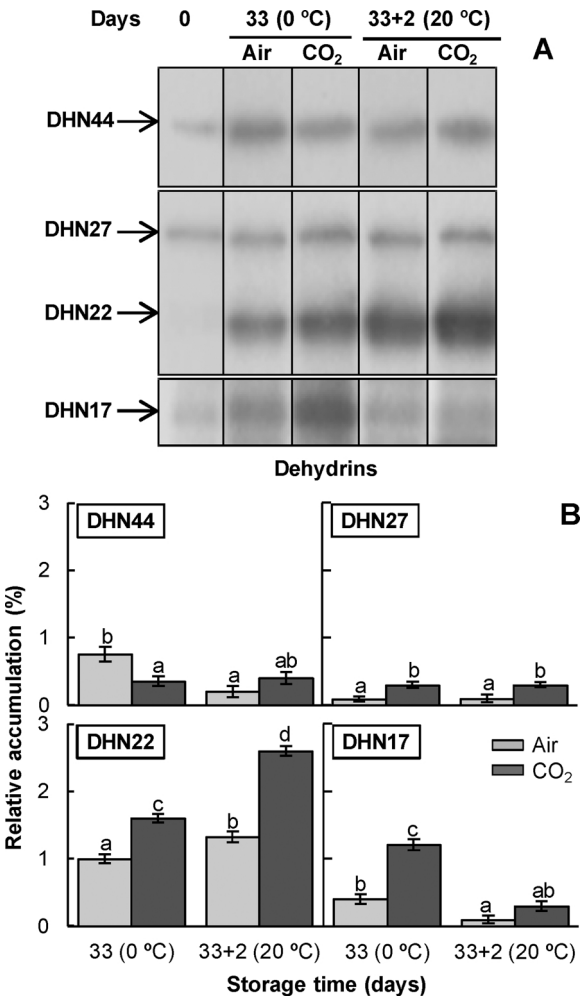


**Fig. 3.** Immunodetection of dehydrin isoforms in the skin of untreated (Air) and $CO_2$-treated ($CO_2$) Cardinal table grapes after storage for 33 days at 0 °C, and after transfer to 20 °C for two days. A) Protein extracts (10 µg of protein) were separated by 13.5% SDS-PAGE, transferred to PVDF membranes and probed with antisera raised against the K-segment. Lane 0: prestored fruit. The results shown are representative of three biological replicates. B) Histograms represent the levels of immunoreactive dehydrin bands, quantified by scanning densitometry of the autoradiographs, expressed as the relative fold-change with respect to the levels in prestored fruit (day 0). The data are the average of three biological replicates ± SD ($n = 6$) and the different letters indicate significant differences determined by Tukey's test ($P \leq 0.05$).

growth and development, or under different environmental stresses (Yang et al., 2012). In addition, we recently established that the expression of dehydrin isoforms in the skin of Cardinal table grapes are differentially and highly regulated by both the length of exposure to low temperature and atmosphere storage conditions (Navarro et al., 2015). Proteomic studies revealed that in wine grapes (*Vitis vinifera*) there is a pulp-specific expression pattern of abiotic stress response proteins such as dehydrins, which were associated with water-deficit stress tolerance (Grimplet et al., 2009). It is noteworthy that some dehydrin genes are induced specifically by cold stress, whereas others are drought-induced (Tommasini et al., 2008), which may suggest different protective roles. Indeed, some cold-regulated dehydrins display *in vitro* cryoprotectant and antifreeze activity (Wisniewski et al., 1999).

The differential accumulation of the dehydrin isoforms associated with postharvest storage in Cardinal table grapes also suggests different protective roles which could be related to their physicochemical characteristics and post-translational modifications (Hara, 2010; Navarro et al., 2015). In this sense, our results established that three out of the four dehydrin isoforms constitutively expressed in the skin of Cardinal grapes (DHN44, DHN27 and mainly DHN17) are cold and $CO_2$

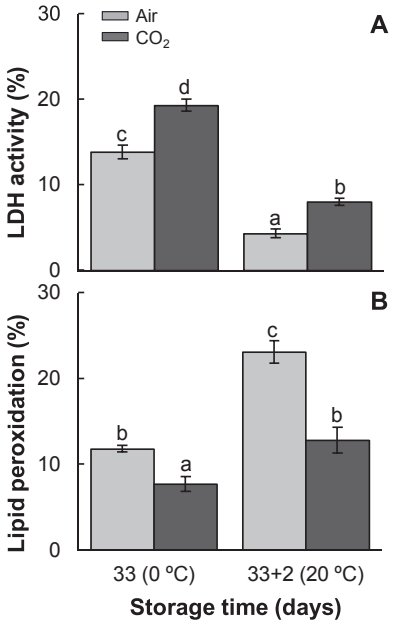


**Fig. 4.** Cryoprotection of LDH by protein extracts (A) and lipid peroxidation levels (B) in the skin of untreated (Air) and $CO_2$-treated ($CO_2$) Cardinal table grapes after storage for 33 days at 0 °C and after transfer to 20 °C for two days. *In vitro* cryoprotective activity assessed as the LDH activity remaining after freeze-thaw deactivation and relative to the unfrozen controls in skin protein extracts (10 μg $mL^{-1}$). Malondialdehyde (MDA) content, the end product of lipid peroxidation, was assayed using a TBARS method. The results were expressed relative to the activity or levels in prestored fruit (day 0). The data are the average of three biological replicates ± SD ($n = 6$) and the different letters indicate significant differences determined by Tukey's test ($P \leq 0.05$).

regulated as well as possibly contributing to the high cryoprotective activity found in the protein extracts from this tissue (Fig. 4A). In these sense, whereas the high levels of cryoprotective activity recorded in skin protein extracts of freshly harvested fruit (more than 3-fold than the activity of the standard cryoprotective protein BSA) increased up to 13% in untreated fruit and 19% in $CO_2$-treated fruit at the end of cold storage, a sharp decrease in the protection of LDH activity against freeze-inactivation was measured when fruit were transferred to 20 °C, regardless of the atmospheric storage conditions (Fig. 4A). Recently, a functional analysis of the 17 kDa recombinant protein encoded by the cold-induced *VvDHN1a* gene from Cardinal table grapes revealed that this dehydrin has a potent cryoprotective effect on LDH activity (Rosales et al., 2014).

By contrast, the up-regulation of the DHN22 after shelf-life associated with the degree of berry water loss control (Figs. 1A and Fig. 3B) is noticeable, suggesting that it could be associated with a large amount of water molecules and also solute ions, such as metal ions, enabling this dehydrin to retain water and to buffer the increased ion concentrations during dehydration stress, contributing to maintaining cellular homeostasis. This is consistent with our previous findings which show that high $CO_2$ treatment prevented bound water loss and water-soluble $K^+$ accumulation in the skin tissues of Cardinal grapes, indicative of a homeostatic sequestering of water molecules and $K^+$ ions (Blanch et al., 2014). Bearing in mind the capacity of dehydrins for interaction with membrane phospholipids and their membrane localization (Allagulova et al., 2003), the 22 kDa dehydrin isoform could likewise be involved in the stabilization of cell membrane structures, contributing to the maintenance of membrane fluidity during postharvest senescence. In this context, we analyzed the level of membrane oxidative damage (measured by the grade of lipid peroxidation) at the end of cold storage and after the shelf-life period by estimating the MDA content, a direct indicator of membrane injury. As can be seen in Fig. 4B, the gaseous treatment restrains the oxidative damage prompted during cold-storage, showing 4% lower MDA levels than untreated berries. Moreover, it is noteworthy that after shelf-life, when a disorganization of membrane systems is characteristic of senescence in fruit and an increase in lipid peroxidation occurs (Salunkhe et al., 1991; Tian et al., 2013), the percentage of MDA in $CO_2$-treated grapes was up to 10% lower than in untreated ones (Fig. 4B). Thus, the gaseous treatment restrains the oxidative damage, maintaining the stability of cell membranes, which led to an increased dehydration tolerance. It is noteworthy that the expression of the DHN22 isoform was likewise associated with less cell membrane oxidative damage, mainly in $CO_2$-pretreated berries stored for two days at 20 °C. Recently, it has been shown that His-rich domains present in some dehydrins contain double His sequences which contribute to their metal binding capacity, and this radical-scavenging activity may reduce metal toxicity in plant cells under conditions of water stress (Hara et al., 2005). This antioxidant activity is a crucial function of dehydrins because dehydration stresses which promote dehydrin accumulation produce reactive oxygen species. These multifunctional protective biomolecules might play a more effective role in counteracting structural and cellular damage prompted by dehydration and oxidative senescence-related disorders, maintaining cellular homeostasis. Our findings might also indicate that, in addition to their particular physicochemical and structural properties, different dehydrins most likely fulfil an essential protective function in fruit stress conditions.

### 3.5. *Organic osmolytes in the skin of untreated and $CO_2$-treated table grapes after cold storage and after shelf-life*

In Cardinal skin tissues, low temperature triggered an increase in endogenous trehalose levels (80 ± 1.5 nmol $g^{-1}$ fresh weight), a non-reducing disaccharide of glucose, mainly in fruit pretreated with 20 kPa $CO_2$ for three days (Fig. 5A). The transfer of fruit to 20 °C produced a sharp decrease in the trehalose content of skin tissues in all the cases, but particularly in those fruit stored under atmospheric conditions where the levels of this disaccharide decreased up to 89% with respect to that found after 33 days at 0 °C, reaching levels similar to those present in prestored fruit. With respect to $CO_2$-treated fruit, the decrease in trehalose was significantly less pronounced ($P \leq 0.05$).

The quantification of endogenous levels of glycine betaine, proline and GABA using Q-TOF LC–MS, revealed that the skin of prestored Cardinal table grapes contains the order of 17 ± 0.5, 221 ± 8 and 51 ± 3 nmol per g of fresh weight, respectively. During prolonged cold storage a significant ($P \leq 0.05$) increase in these nitrogenous solutes was reached in the skin of untreated berries, mainly in those of proline (164 ± 13 nmol $g^{-1}$ fresh weight) and GABA (75.5 ± 5.4 nmol $g^{-1}$ weight). The pretreatment with 20 kPa $CO_2$ for three days increased by 2-fold the endogenous levels of organic osmolytes such as trehalose, proline and GABA and up to 8.5-fold those of the quaternary ammonium with respect to those stored continuously under atmospheric conditions and with the same chronological age (Fig. 5B–D). However, the transference of fruit to 20 °C clearly modified the profile of these organic osmolytes. It is noteworthy that after the shelf-life of untreated fruit the GABA content was maintained at a similar level to that of fruit over a 33-day storage at 0 °C, even decreasing in $CO_2$-treated grapes (Fig. 5D). On the contrary, after the shelf-life period there was a significant ($P \leq 0.05$) increase in the levels of glycine betaine and proline, being more pronounced in $CO_2$-treated grapes where levels were up to 1.5-fold higher than those found in untreated fruit (Fig. 5B, C).

It has been previously described that all these low molecular weight organic solutes play a key role in maintaining cell osmotic equilibrium given that these substances are compatible with the cellular metabolism that accumulates in the cytoplasm to balance external osmotic pressure (Alberdi and Corcuera, 1991). In addition, low levels of organic osmolytes play a role in protecting membranes and macromolecules by either stabilizing their tertiary structure or by scavenging reactive oxygen species produced in response to cell dehydration (Zhu, 2001). The trend of changes in these protective metabolites during postharvest

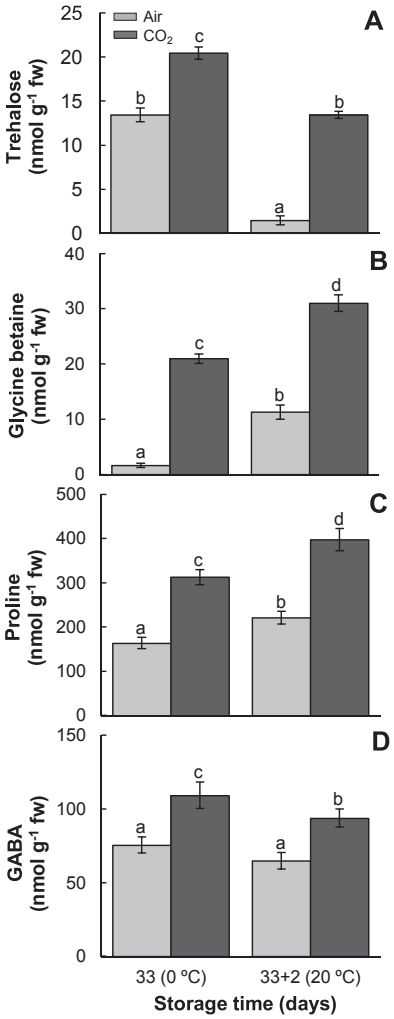


**Fig. 5.** Trehalose (A), glycine betaine (B), proline (C) and GABA (D) accumulation in the skin of untreated (Air) and $CO_2$-treated ($CO_2$) Cardinal table grapes after storage for 33 days at 0 °C, and after transfer to 20 °C for two days. Trehalose levels were analyzed and quantified by HPAEC-PAD. Nitrogenous osmolyte levels were analyzed and quantified by Q-TOF LC-MS. The results were expressed relative to the levels in prestored fruit (day 0). The data are the average of three biological replicates ± SD ($n = 6$) and the different letters indicate significant differences determined by Tukey's test ($P \leq 0.05$).

storage of Cardinal table grapes revealed that trehalose and GABA are cold-regulated, and that together with cold-responsive dehydrin isoforms they might provide cytosolic proteins and cell membranes protection from cold damage. Therefore, these compounds could contribute to maintaining metabolic activity during low temperature storage, and even more so in $CO_2$-treated berries where physiological and cellular damage is minimized (Blanch et al., 2014; Guy et al., 2008). The disaccharide trehalose, on account of its physicochemical properties, has been described as a better stabilizer than other sugars for protecting membranes and biomolecules (Elbein et al., 2003). The effectiveness of trehalose has been attributed to several of its properties such as replacing water by establishing hydrogen bonds with membranes or the ability to modify the solvation layer of proteins, protecting them from denaturation during dehydration or low temperature (Fernandez et al., 2010).

In table grapes, the trend in GABA biosynthesis might also be associated with the cytoplasmic acidification-related stress control (biochemical pH-stat: Shelp et al., 1999). In this sense, it was previously reported that GABA accumulation is induced by $CO_2$ treatment in low temperature storage fruit, in association with the prevention of cytoplasmic acidosis (Deewatthanawong et al., 2010; Merodio et al., 1998). As well as contributing to the carbon-nitrogen balance (GABA-shunt) and to the regulation of cytosolic pH (pH-stat), GABA also plays a role in osmoregulation and protection against oxidative stress, as has been proposed for other metabolites and signaling molecule in plants (Kinnersley and Turano, 2000; Shelp et al., 1999).

With respect to the profile of protective metabolites such as proline and glycine betaine, the results show the relevant role that these organic osmolytes may play in the beneficial effect of a gaseous pretreatment in maintaining table grape postharvest quality during shelf-life. The protective molecules identified could contribute to the stabilization and consolidation of both cell integrity and tissue permeability, and to maintain cellular homeostasis, thereby controlling water loss, as well as scavenging intracellular reactive oxygen species, reducing the oxidative damage to biological macromolecules and the rate of postharvest senescence. In this sense, it is remarkable that a short-term high $CO_2$ treatment nearly triples the endogenous levels of proline, one of the most abundant free amino acids in table grape berries which accumulate during berry ripening (Kliewer, 1968), reflecting their accumulation in the skin of Cardinal grapes in response to developmental and environmental cues. In the same way that has been described for other plants (Vendruscolo et al., 2007), stress-induced synthesis of proline confers tolerance to water deficit in grapevine (Hatmi et al., 2015). Moreover, drought-tolerant plants also exhibited up-regulation of PR genes, especially 1,3-β-glucanase and chitinase, compared with sensitive plants, suggesting a possible connection between water loss tolerance and immune response in grapevine (Hatmi et al., 2015). It is noteworthy that among organic osmolytes, proline is one of the most effective free radical scavengers (Smirnoff and Cumbes, 1989).

We previously suggested that glycine betaine could help protect the cellular machinery from cold-imposed cellular damage in $CO_2$-treated chilling-sensitive fruit like cherimoyas (Goñi et al., 2011b). In general, accumulation of this quaternary amine appears to play an important role in enhancing plant cold tolerance. However, the enhanced glycine betaine accumulation in transgenic maize favors the integrity of the cell membrane and enzyme activity in conditions of drought stress (Quan et al., 2004). In addition, it has been reported that an exogenous glycine betaine treatment retained quality and increased the antioxidant defense of El-Bayadi table grapes after cold storage and shelf life, being suggested as natural alternatives to synthetic chemicals (Awad et al., 2015).

In conclusion, the results show evidence that the pretreatment with 20 kPa of $CO_2$ for three days is an effective technology to counteract the loss of water, oxidative damage and decay incidence during the shelf-life postharvest period of Cardinal table grapes. These features are correlated with an elevation in the berry skin tissues of the endogenous levels of specific organic osmolites and defense proteins involved in water and redox cellular homeostasis as well as controlling decay. After shelf-life, the expression of a chitinase of 16 kDa and a dehydrin of 22 kDa isoforms along with the proline and glycine betaine profile are differentially and highly regulated, mainly in $CO_2$ pretreated fruit. When fruit were transferred to 20 °C, the metabolic pathways resume their activity enhanced senescence postharvest losses due to the increase in reactive species that decrease membrane fluidity, resulting in water loss, deterioration in berry quality and greater susceptibility to fungal attack. In this context, the identified protective biomolecules could modify the physiological response of skin tissues, increasing their capacity to counteract senescence-related disorders and improving fruit postharvest quality. In addition, this could contribute to the stabilization and consolidation of both cell integrity and tissue permeability, and to maintaining cellular homeostasis, thereby controlling water loss, as well as scavenging intracellular reactive oxygen species, and thus reducing the oxidative damage to biological macromolecules and the rate of postharvest senescence.

## Acknowledgements

The authors are grateful to E. de Vega and I. Alvarez from the Analysis Service Unit facilities (ICTAN) for their technical assistance in the Ion Chromatography and Mass Spectrometry analysis, and to M. Legrand (Strasbourg, France) for his generosity in providing PR-Q, PR-2 and PR-5 antisera. This research was supported by the CICYT Projects AGL2014-53081-R (MINECO) and AGL2017-85291-R (MINECO/AEI/FEDER, UE). M. Vazquez-Hernandez acknowledges the support of the FPI program financed by the MICINN.

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