MOLECULAR REGULATION OF ANTHOCYANIN BIOSYNTHESIS, ASCORBATE PEROXIDASE AND ANTIOXIDANT ACTIVITY IN THE SKIN OF CO_2-TREATED TABLE GRAPE STORED AT LOW TEMPERATURE

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Irene Romero, Mª Teresa Sanchez-Ballesta*, Roberto Maldonado, Mª Isabel Escribano, Carmen Merodio

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frio, IF-CSIC, Ciudad

10 Universitaria, E-28040 Madrid, Spain.

*Author to whom correspondence should be addressed

Summary

A pretreatment with 20 kPa O_2 + 20 kPa CO_2 +60 kPa N_2 for 3 days proved effective in maintaining fruit quality and controlling decay in table grapes (*Vitis vinifera* cv. Cardinal) stored at 0 ºC. To investigate the molecular mechanisms implicated in the beneficial effect of this pretreatment, we isolated partial cDNAs that codified for key enzymes of anthocyanin biosynthesis such as L-phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), and antioxidant enzymes such as ascorbate peroxidase (APX). The pattern of gene expression indicated that the CO_2 pretreatment prevented the high increase in levels of these transcripts observed in non-treated grapes. Also, changes in PAL activity were similar to *VcPAL* mRNA levels. This up-regulation of anthocyanins biosynthesis gene expression and *VcAPX* mRNA in non-treated grape is not enhanced in CO_2-treated grapes, which presented low total decay. Low temperature induced accumulation of total anthocyanins content in the skin of both treated and non-treated grapes, accompanied by a decrease in lightness, chroma saturation and hue values. On the other hand, antioxidant activity decreased in non-treated grapes and did not change in CO_2-treated grapes. These results point out the ability of CO_2-treated grapes to prevent the generation of reactive oxygen species rather than their inactivation by means of induction of studied defense systems.

KEYWORDS: Anthocyanin biosynthesis; Ascorbate peroxidase; Carbon dioxide; Gene expression; Table grapes.

Abbreviations: APX, Ascorbate peroxidase; CA, Controlled atmosphere; CHS, Chalcone synthase; FW, Fresh weight; MAP, modified atmosphere packaging; PAL, L-phenylalanine ammonia-lyase; ROS, reactive oxygen species; TE, Equivalent of trolox;

Introduction

One of the main goals of postharvest technology in table grapes is to delay fungal attack, mainly caused by *Botrytis cinerea*, which causes extensive losses during cold storage. The possibility of safe alternative control methods to assure high quality fruit and control fungal attack is really important because postharvest chemical treatments are restricted in most countries. Among the alternative methods for controlling table grapes postharvest decay, such as modified atmosphere packaging (MAP) (Artés-Hernandez et al., 2006) and controlled atmosphere (CA) (Yahia et al., 1983; Crisosto et al., 2002), high CO_2 pretreatment for shorter storage periods during postharvest handling of table grapes offers interesting possibilities. In previous works, we reported that pretreatment with 20% CO_2 plus 20% O_2 for 3 days reduced fungal decay in table grapes stored at 0 ºC maintaining the fruit quality (Romero et al., 2006; Sanchez-Ballesta et al., 2006). However, little is known about the physiological and molecular events occurring in response to the application of high CO_2 levels in table grapes. Our studies indicated that the efficacy of high CO_2 pretreatment is not mediated by the induction of pathogenesis-related proteins such as class I chitinase and β-1,3-glucanase (Romero et al., 2006). Besides, the stilbene phytoalexin biosynthesis in CO_2-treated fruit was not induced (Sanchez-Ballesta et al., 2006). Stilbene synthase (STS), the key enzyme leading to stilbene synthesis, is closely related to chalcone synthase (CHS), the key enzyme of the biosynthesis of flavonoid-type compounds like anthocyanins. CHS and STS catalyze common condensation reactions of p-coumaroyl-CoA and three C(2)-units from malonyl-CoA but different cyclization reactions to produce naringenin chalcone and resveratrol, respectively. Since the development of protection strategies of CO_2-treated grape is not based on the induction of *STS* gene expression and resveratrol accumulation, this work focuses on the effect of high CO_2 levels on anthocyanin biosynthesis.

Anthocyanins, the main pigments in flowers and fruits, are a flavonoid subclass synthesized from hexose through the shikimate, phenylpropanoid and flavonoid pathways. The anthocyanin biosynthetic pathway is one of the well-known pathways in plants (Holton and Cornish, 1995). The

first committed step is catalyzed by CHS, which condenses the metabolites malonyl-CoA and 4-coumaroyl-CoA formed essentially via the shikimate and/or malonate pathways. It has been suggested that anthocyanins function as photoprotective pigments (Li et al., 1993) and anthocyanin accumulation can reportedly be induced in many plants by biotic and abiotic stresses, such as UV-B radiation, pathogen attack or low temperature (Mancinelli, 1983; Winkel-Shirley, 2001). In view of these findings it is possible that such protective phenolic compounds may be implicated in the efficacy of high CO_2 pretreatment in preventing fungal attack. Most studies of table grapes treated with CO_2, have addressed the quantity and type of anthocyanins but there is still a dearth of knowledge about expression of the anthocyanin biosynthetic genes in relation to these beneficial postharvest treatments.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is the enzyme at the entry-point of the phenylpropanoid pathway, which yields a variety of phenolic compounds with structural and defence-related functions. PAL catalyses the deamination of L-phenylalanine to form *trans*-cinnamic acid, which eventually leads to the production of p-coumaroyl-CoA the common substrate of STS and CHS. Due to the nature and function of the products derived from the phenylpropanoid pathway, PAL activity and the activation of PAL under stress conditions have been considered part of a defense mechanism operating in stress-afflicted cells (Dixon and Pavia, 1995). Therefore, molecular analysis of PAL was also chosen as a mean to understand the effect of high CO_2 levels on table grapes stored at low temperature.

Production of reactive oxygen species (ROS) during so-called "oxidative burst" is thought to be a central event in activation of disease resistance. In plant cells the enhanced generation of ROS in response to both abiotic and biotic constraints has been well documented (Mehdy et al., 1996; Alscher et al., 1997). However, it is not clear whether changes in ROS generation are directly involved in the activation of plant defense response or are a mere consequence of the oxidative stress occurring in the attacked cells. Oxidative events are associated with the different *B. cinerea*–plant interactions, but their precise role in such interactions remains unclear (Grovin and Levine,

2000; Tierens et al., 2002). Hydrogen peroxide (H_2O_2) represents a crucial crossing in the mechanisms of oxidative stress, and its regulation plays an important role in cell life. Therefore, different systems are utilized by cell to balance the levels of H_2O_2. Ascorbate peroxidases (APX) (EC 1.11.1.11) utilize ascorbic acid and its specific electron to reduce H_2O_2 to water. APX genes
5 have been isolated from some plants and their expression has been determined under fruit ripening, oxidative stress, and low temperature, respectively (Kim and Chung, 1998; Yoshimura et al., 2000; Kawakami et al., 2002). In grape, there have been no reports on the involvement of APX in *Botritys* attack or on the effect of a beneficial high CO_2 pretreatment in controlling decay; we therefore isolated an *APX* partial cDNA and analyzed the changes in gene expression in treated and non-
10 treated table grapes stored at low temperature.

In order to further our knowledge of the mode of action of high CO_2 pretreatment and considering the crucial role of the CHS as a key anthocyanin enzyme and PAL in the phenylpropanoid biosynthesis as an initial enzyme of the pathway we have cloned a *VcCHS* and *VcPAL* partial cDNA clones and monitored their expression in treated and non-treated grapes
15 during low temperature storage. We also analyzed total anthocyanin content and antioxidant capacity as they relate to the beneficial effect of the gaseous treatment. This article, then reports our findings on the relationship between *APX* regulation at transcript level and susceptibility to development of natural fungal decay at low temperature.

20 **Materials and methods**

Plant Material

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at random in Camas (Sevilla, Spain) in July. Early-harvesting mature berries were used in this work (12.85% total soluble solids;
25 0.84% tartaric acid). After harvesting, field-packaged bunches were transported to the laboratory, where fruits were immediately forced-air precooled for 14 hours at –1 ºC. After cooling, bunches

free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 ºC and 95% relative humidity in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container in the dark. One lot was stored under normal atmosphere for 33 days (non-treated fruit) and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. The CO_2 concentration was maintained throughout the pretreatment experiment and was measured daily using an automated gas chromatograph system equipped with a thermal conductivity detector and Poraplot Q column (Varian Chrompack CP20033P). After 3 days, CO_2-treated grapes were transferred to air under the same conditions as non-treated fruits until the end of the storage period. Ten clusters were sampled periodically, and berries from five clusters (approx. 300 g each cluster) were peeled and the skin and pulp were frozen in liquid nitrogen, grounded to a fine powder and stored at –80 ºC until analysis. For quality parameters, 45 berries were used, randomly removed from five clusters and distributed in 3 replicates of 15 berries each.

Cloning of PAL, CHS and APX partial cDNAs

Total RNA was extracted from the skin of grapes according to the method of Salzman et al. (1999). Partial cDNA clones of PAL, CHS and APX were obtained by RT-PCR. cDNA synthesis was performed with 10 ∝g of a mixture of total RNA from the skin of grapes stored in air and CO_2 for 12 and 33 days. The reaction was carried out in the presence of 500 ng of oligo-dT with 100 units of Reverse Transcriptase (Ecogen). PAL, CHS and APX gene DNA fragments were obtained by PCR amplification using the cDNA as template and the combination of the sense and the antisense primers. A 668 bp fragment of PAL was amplified by combining the degenerate sense primer 5’-CAATGGCTNGGCCCNCAYATHGAA-3’ and the degenerate antisense primer 5’-AARCGNATGTARCTACTGGGGACG-3’. A 634 bp fragment of CHS was amplified using a sense primer 5’-TGATTACTACTTCCGCATCACCA-3’ and the antisense primer 5’-CTTCCTTCTCTTTGACTCTCGTTGA-3’. A 539 bp fragment of APX was amplified using a

degenerate sense primer 5’-GCATGGCACTCTGCTGGWAC-3’, and the degenerate antisense primer 5’-TCATCSGCAGCRTATTTYTC-3’. PCR products were cloned into the pGEMT vector (Promega) and confirmed by sequencing.

RNA gel blot hybridization

Samples of denatured total RNA (10 ∝g) from the skin of grapes were fractionated and blotted as described in Sanchez-Ballesta et al. (2000). Equal loading was confirmed by ethidium bromide staining and by membrane staining with methylene blue. The DNA probe was random-primer labeled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 ºC in 7% sodium dodecyl sulfate, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in 2 x SSC, 0.1% SDS at room temperature and twice in 0.1 x SSC, 0.1% SDS at 65 ºC and exposed to Kodak X-Omat SX film at –80 ºC. Autoradiographs were digitally scanned and band densities quantified by image densitometry using Scion Image software (Scion Corporation, Frederick, MD). The 100% was assigned to the maximum optical density value achieved in each Northern and the rest of optical densities were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

PAL activity assay

PAL was extracted from 5 g of skin according to the method of Faragher and Chalmers (1977). The skin was ground in 25 ml of 100 mM H_3BO_3-NaOH buffer (pH 8.8) containing 25 mM 2-mercaptoethanol and 6% PVP. Afterwards, it was filtered through two layers of gauze and centrifuged for 30 min at 14000 g. The supernatant was saturated with 70% $(NH_4)_2SO_4$ and the pellet was sedimented by centrifuging 14000 g for 30 min, redissolved in the same buffer, and used for the enzyme assay.

The assay was based on the method of Tanaka et al. (1974). The reagent mixture containing 2.5 ml of 100 mM borate-NaOH buffer (pH 8.8) and 0.5 ml of 40 mM phenylalanine was

preincubated at 30 ºC for 5 min and the enzyme was added into the solution. The assay continued for 30 min at 30 ºC and was stopped by adding 0.5 ml of 2 M perchloric acid. Prior to determination of OD at 280 nm, the mixture was centrifuged at 2000 g for 15 min. The control tube included perchloric acid at the start of the assay. Specific enzyme activity was defined as nmol of cinnamic acid/h/mg of protein.

Protein concentration was measured by the method of Bradford (1976) using protein-dye reagent (Bio-Rad, Hercules, CA) and bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard.

Colour

Berry skin colour was measured at three different positions around the equatorial region using the Hunter Lab System and a Minolta CR200™ colorimeter (Minolta Camera Co., Osaka, Japan). Results were given in Commission Internationale de l'Eclairage *L** (Lightness), *a**, and *b** (CIELAB) colour space coordinates. Hue ($h°$) ($\tan^{-1}$ b/a) and chroma (*C**, saturation) $[(a^2 + b^2)^{1/2}]$ were calculated from *a** and *b**.

Analysis of total anthocyanin content

Total anthocyanin contents were determined by the pH differential method as reported by Wrolstad (1976). Briefly, skin samples (0.5 g) homogenized in liquid nitrogen were extracted with 0.01% HCl in 0.5 ml methanol using ultra-sonication with cold water, four times for 10 minutes each. The extracts were centrifuged at 4000 g and the supernatants were removed and filtered. Absorbance was measured in a UV-VIS spectrophotometer (UV-VIS 1601 Shimadzu) at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5 using $A= [(A_{510}-A_{700})pH_{1.0} - [(A_{510}-A_{700})pH_{4.5}]$ with a molar extinction coefficient of malvidin-3-glucoside of 28,000 L mol^{-1} cm^{-1}. The results were expressed as mg of malvidin-3-glucoside equivalent per g fresh weight (FW).

Radical cation ABTS scavenging capacity

Extraction of skin samples was performed in the same way as anthocyanin extraction described above. The radical cation 2,2'-azinobis(3-ethylbenzothiazoline 6-sulphonate) ($ABTS^+$) scavenging capacity was measured as described by Rice-Evans et al. (1996), where $ABTS^+$ is
5 oxidized with potassium persulfate. Trolox (Hoffman-La Roche) (6-hydroxy 2,5,7,8-tetramethylchroman- 2-carboxylic acid) (2.5 mM) prepared in ethanol was used as an antioxidant standard and for the calculation of scavenging capacity of grape skin extracts as trolox equivalent. The scavenging activity of grape skins extracts was calculated as mM Trolox equivalent (TE)/g FW.

10 **Statistical analyses**

Data from at least three replicates per sample were subjected to analysis of variance (ANOVA) (Statgraphics program, STSC, Rockville, Md.). Multiple variance analysis was employed to determine the significance of the data at $P \leq 0.05$.

15 **Results**

Effect of high CO_2 levels on anthocyanin genes expression (PAL, CHS) and PAL activity during low temperature storage of grapes

To investigate the pattern of anthocyanin gene expression in response to a 3 days CO_2
20 pretreatment, total mRNA prepared from the skin of CO_2-treated and non-treated grapes stored at 0 ºC for up to 33 days was analyzed by northern hybridization, and PAL activity was determined.

The partial cDNA clones used as probes were obtained by RT-PCR (PAL, GenBank accession no. DQ887093 and CHS, GenBank accession no. DQ887094). The partial *VcPAL* cDNA consisted of 668 bp and encoded a polypeptide of 222 amino acids. The partial amino acid sequence
25 shared about 93% identity with PAL sequences from lemon, mandarin fruit and aspen. As regards *V. vinifera* PAL sequences the deduced PAL amino acid sequence showed 35% identity with a PAL

sequence from the cultivar Cabernet Sauvignon (GenBank accession no. BAA31258) and 92% identity with a PAL from Lambrusco (GenBank accession no. X75967). The partial *VcCHS* cDNA consisted of 634 bp and encoded a polypeptide of 211 amino acids. Comparison of the partial deduced amino acid sequence revealed that it was highly homologous (98% identity) to different
5 CHS sequences from *V. vinifera* (Cv. Cabernet Sauvignon, CHS2 GenBank accession no AB66275, psCH4 GenBank accession no. AB015872; Cv. Lambrusco Foglia Frastagliata, CHS GenBank accession no. X75969).

Storage of grapes at 0 ºC induced the accumulation of *PAL* and *CHS* mRNA levels in the skin of non-treated and CO_2-treated grapes (Fig. 1A). However, both the *VcPAL* and the *VcCHS*
10 mRNA levels in non-treated grapes were higher than in CO_2-treated fruit. In non-treated grapes a sharp increase was observed in the levels of *PAL* mRNA after 12 days at 0 ºC reaching the maximum by day 28 and decreasing thereafter. The accumulation of *CHS* transcript, on the other hand, was slow but progressive, reaching maximum after 28 days at 0 ºC like the *PAL* mRNA and decreasing the levels by day 33. In CO_2-treated grapes, the levels of *VcCHS* transcripts increased
15 after 19 days and did not change until the end of the storage. Also, the *VcPAL* transcript levels increased after 19 days decreasing after 33 days. Transcript levels remained lower than in non-treated grapes throughout storage. Northern analysis revealed that *PAL* and *CHS* gene expression was not detected in the pulp of either treated or non-treated grapes (data not shown).

PAL activity increased in non-treated grapes after 28 days at 0 ºC, paralleled to the higher
20 accumulation in *PAL* transcript and, reaching maximum after 33 days (Fig. 1B). PAL activity was lower in CO_2-treated grapes than in non-treated fruit reaching maximum by day 33, although this maximum was lower than those recorded in non-treated grapes, the same as in PAL mRNA accumulation.

25 **Effect of high CO_2 levels on APX gene expression during low temperature storage of grapes**

A partial *APX* cDNA clone was obtained by RT-PCR using as template a mixture of cDNAs synthesized from total RNA extracted from the skin of CO_2-treated and non-treated grapes stored at 0 ºC. A cDNA fragment of 539 bp was cloned and named *VcAPX* (GenBank accession no. DQ887095). The deduced sequence of 179 amino acid presented the heme-binding domain that shares all APX protein. The deduced VcAPX amino acid sequence presented 82-98% similarity with amino acid sequences of APX genes accessed in GenBank, being the greatest similarity to cytosolic APX sequences.

VcAPX transcript levels increased in non-treated grapes after 12 days, reaching maximum by day 28 and decreasing thereafter (Fig. 2). In CO_2-treated grapes, on the other hand, the increase in the accumulation of the transcript was smaller and did not change during storage at 0 ºC.

Colour evolution, accumulation of total anthocyanins and antioxidant activity in non- treated and CO_2-treated grapes stored at 0 ºC

The highest L^* and C^* values were found in the skin of freshly harvested grapes (Fig. 3). Storage at 0 ºC affected the lightness and chroma parameters; L^* and C^* values decreased in the skin of both treated and non-treated grapes after 12 days, and the decrease was greater by day 22. L^* values at the end of storage (33 days) were similar in CO_2-treated and non-treated grapes, but C^* values were lower in non-treated fruit. In freshly harvested grapes the h^o value was 348º indicating a grape skin colour more closer to magenta. The storage of treated and non-treated grapes at 0 ºC affected the h^o angle showing values around 180º by day 22, indicating a skin colour closer to red.

Changes in lightness, saturation and colour (h^o) were accompanied in general by an increase in total anthocyanin content (Fig. 4). The accumulation increased after 12 days at 0 ºC both in non-treated and CO_2-treated grapes although it was higher in non-treated ones. Thereafter, the accumulation of total anthocyanin decreased in non-treated fruit although at the end of the storage the accumulation was higher in non-treated grape.

The antioxidant capacity in non-treated grapes decreased slightly along the storage reaching minimum by day 22 at 0 ºC (Fig. 5). However, although antioxidant capacity decreased slightly in

CO_2-treated grapes after 12 days, thereafter the levels were similar to those of freshly harvested grapes.

Discussion

5 The use of high CO_2 concentrations to enhance table grapes quality in place of SO_2 fumigation has been a focus of interest in recent years (Yahia et al., 1983; Crisosto et al., 2002; Retamales et al., 2003; Artés-Hernández et al., 2006; Romero et al., 2006; Sanchez-Ballesta et al., 2006). However, there is little understanding of its possible mode of action, although many authors have reported that high CO_2 treatments maintain grape quality by retarding fungal attack, mainly 10 caused by *B. cinerea*. In previous works, we observed that a 3-day high CO_2 pretreatment significantly reduced natural fungal decay in table grapes Cv. Cardinal stored at 0 ºC for up to 33 days but that this beneficial effect was not mediated by the induction of PRs and STS gene expression (Romero et al., 2006; Sanchez-Ballesta et al., 2006). STS and CHS, the key enzymes of resveratrol and anthocyanin biosynthesis respectively, each catalyze the formation of a tetraketide 15 intermediate from a CoA-tethered phenylpropanoid starter and three molecules of malonyl-CoA but use different cyclization mechanisms to produce the end products. Likewise, PAL catalyses the first step of the multi-branched general phenylpropanoid pathway, which supplies substrates for biosynthesis of different phenolic compounds such as the end products of the reactions catalyzed by the STS and CHS enzymes. Since, we observed that high CO_2 pretreatment did not modulate *STS* 20 gene expression to control fungal decay, a new area of research is open to further investigate the molecular mechanisms implicated in the mode of action of high CO_2 concentrations in table grapes by analyzing anthocyanin biosynthesis gene expression.

Using RT-PCR, we have isolated a partial cDNA encoding PAL from the skin of table grapes. The predicted VcPAL amino acid sequence shared significant levels of identity with the 25 published sequence from *V. vinifera* Cv. Lambrusco (Sparvoli et al., 1994) as well as from other plant species. The present work shows that the high CO_2 pretreatment that significantly reduced

natural fungal decay in table grapes stored at 0 ºC, prevented the large increase in *VcPAL* transcript levels observed in non-treated grapes (Fig. 1A). *PAL* gene expression is also elicited in this way in grapevine leaves infected with *B. cinerea* (Bezier et al., 2002). Furthermore, the increase in PAL activity was higher in non-treated grapes (Fig. 1B). PAL was one of the first plant "defense genes" identified, and both PAL mRNA levels and PAL activity could be induced by a wide array of environmental cues, such as pathogen attack, low temperatures, UV irradiation, mechanical wounding, and light (Lawton and Lamb, 1987; Gläβen et al., 1998; Kamo et al., 2000; Sanchez-Ballesta et al., 2000). PAL expression and activity may therefore also be considered a good marker with which to investigate the process whereby high CO_2 concentrations effectively control grape decay. Lignin accumulation and PAL activation observed in untreated cherimoya fruit after prolonged storage at chilling temperature were reduced by an effective high CO_2 pretreatment (Maldonado et al., 2002). Likewise, in mandarin fruit an effective heat treatment prevented the increase in PAL activity that normally occurs around the necrotic zones of fruits when stored at low temperatures (Martínez-Téllez and Lafuente, 1997; Sanchez-Ballesta et al., 2000). The results of the present work, then, reinforce the idea that PAL may be implicated in defense responses against natural fungal infection in non-treated grapes but not in the mechanisms operating in treated grapes to reduce total decay.

In grapevine, the copy number of CHS has been estimated at three to four. The predicted partial VcCHS amino acid sequence shared more than 90% identity with the three different grapevine CHS deposited in the databases, showing the closest homology to CHS2 (Goto-Yamamoto et al., 2002). The 3-day CO_2 pretreatment also reduced the increase in the *VcCHS* transcript levels observed in non-treated grapes (Fig. 1A). When compared with *VcPAL* gene expression in non-treated grapes stored at 0 ºC, the increase in *VcCHS* mRNA was later and progressive, but the maximum in both *PAL* and *CHS* gene expression was observed by day 28. The activities of CHS are largely regulated at the transcription level. Expression of CHS genes is controlled by developmental stages of the plant, as well as by biological and environmental stresses

such as low temperature, pathogen attack and wounding (Junghans et al., 1993; Leyva et al., 1995; Seki et al., 1999). Table grapes stored at low temperature respond to natural fungal decay by modulating the phenylpropanoid defense mechanism as indicated by the large increase in *PAL* and *CHS* gene expression and PAL activity. In this respect, the lower *VcPAL* and *VcCHS* mRNA levels in CO_2-treated fruit might constitute cellular signals indicating a lower rate of stress imposition.

The total anthocyanin content increased in both treated and non-treated grapes after 12 days of storage at 0 °C decreasing thereafter (Fig. 4); again as in the case of *VcPAL* and *VcCHS* transcript levels and PAL activity, the accumulation was greater in non-treated grapes. The increase in anthocyanin content was accompanied by a reduction of C^* and h^o values in both CO_2-treated and non-treated grape skins (Fig. 3). In Burlat cherries, CA treatments which resulted in the lowest anthocyanin content being achieved also led to higher C^* and h^o values at the end of storage (Remon et al., 2004). Mori et al. (2005) demonstrated that environmental factors affect anthocyanin synthesis in grape berries through the regulation of PAL activity and *CHS* gene expression. They showed that anthocyanin synthesis, PAL activity and *CHS3* transcript levels in Darkridge grapes were markedly higher in berries grown under low night temperatures. It is already known that anthocyanin synthesis continues after harvesting and also in low temperature storage (Kalt et al., 1999). The application of high CO_2 as a pretreatment in table grapes causes an increase in the total anthocyanin content during storage at 0 °C. By contrast, treatments with high CO_2 applied as CA or MAP inhibit postharvest increase of anthocyanin concentrations in different fruits (Gil et al., 1997; Remon et al., 2004). Nevertheless, Veazie and Collins (2002) reported that total monomeric anthocyanin in Navaho blackberries held in CA storage at 2 °C increased in the first 3 days and decreased thereafter.

The increase of total anthocyanin content in treated and non-treated grapes did not produce any increase of antioxidant activity during low temperature storage (Fig. 5). However, it is interesting to note that whereas antioxidant activity in the treated grapes stored at low temperature was similar to that of freshly harvested fruit, it decreased in the non-treated fruit. No changes in

antioxidant activity were detected in apples stored at low temperature and under CA conditions (van der Sluis et al., 2001), but there was a decrease in antioxidant activity of samples of fresh-cut spinach stored under MAP (Gil et al., 1999). In the present case, on the other hand, analysis of *APX* gene expression revealed that transcript levels increased slightly both in treated and non-treated grapes but a sharp increase was observed after 28 days in non-treated grapes where total decay was evident (Romero et al., 2006) (Fig. 2). These results indicate differences in the mechanisms operating in treated and non-treated grapes suggesting that APX participate in removing H_2O_2 in non-treated grapes. APX seems to be activated during compatible interactions (Garcia-Limones et al., 2002) and many reports indicated that it is even inhibited during resistance or during pathogen-induced programmed cell death (Mittler et al., 1998).

The evidences provided here suggest that the effectiveness of high CO_2 pretreatment to control fungal decay maintaining fruit quality after prolonged low temperature storage is the result of ability to prevent the formation of reactive oxygen species rather than their inactivation once formed by the induction of *APX* gene expression and anthocyanin biosynthesis. Further analysis is needed to ascertain the role of anthocyanins during low temperature storage and to identify responses associated with the mechanisms that participate in CO_2 treatment.

Acknowledgements

This work was supported by research grants (AGL2005-04502/ALI and AGL2002-02308) from the CICYT (Spain). I.R. and MT.S.-B. were supported by a postgraduate and postdoctoral I3P contract from the CSIC, respectively. The authors are grateful to Frutaria SAT for providing tables grapes and to Agrotecnica for industrial assistant.

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Figure captions

Figure 1. Effect of high CO_2 pretreatment on *VcPAL* and *VcCHS* mRNA accumulation in the skin of 'Cardinal' table grapes stored at 0 ºC (A). Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the partial *VcPAL* and *VcCHS* probes. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (B) Time courses of PAL activity in the skin of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 ºC. Data are averages of two separate experiments (n=6) and S.E are shown by vertical bars.

Figure 2. Effect of high CO_2 pretreatment on *VcAPX* mRNA accumulation in the skin of 'Cardinal' table grapes stored at 0 ºC (A). Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the partial *VcAPX* probe. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical densities values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining.

Figure 3. Changes in colour (L^*, C^* and h^o) of non-treated and CO_2-treated Cardinal tables grapes stored at 0 ºC. Values are mean of 20 determinations and S.E are shown by vertical bars.

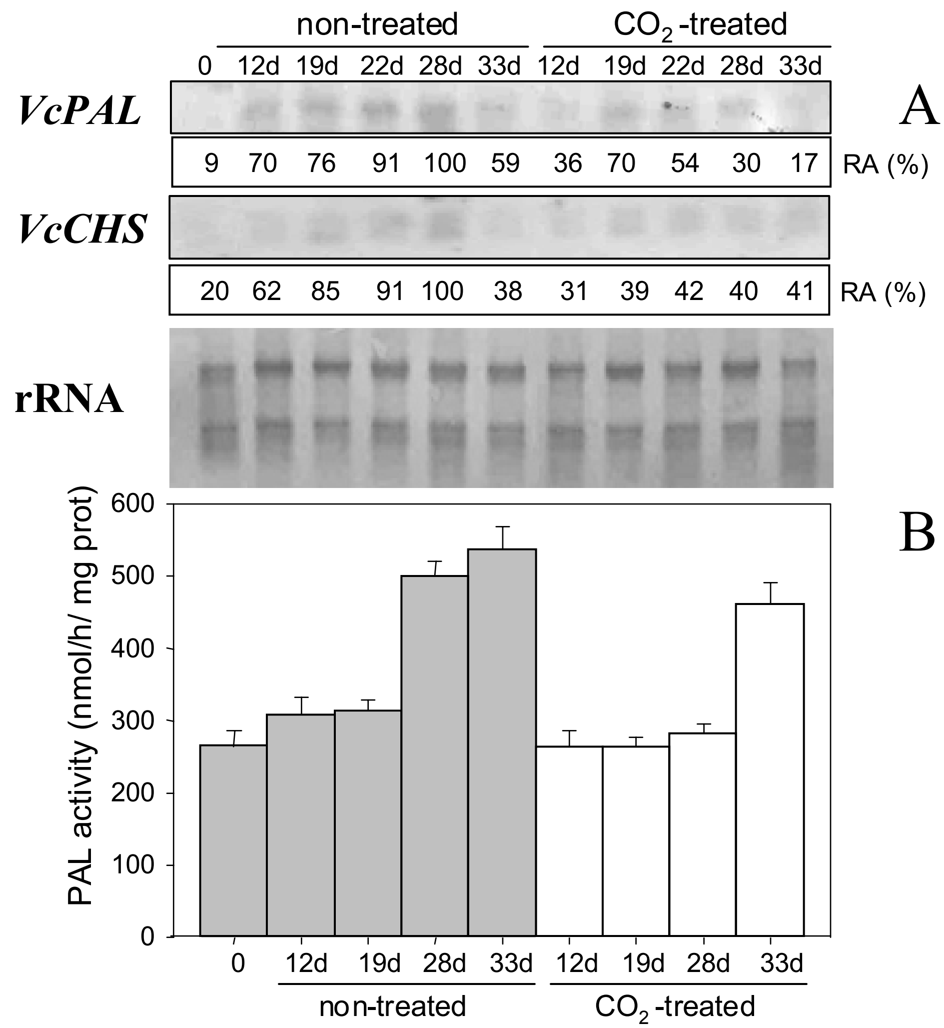
Figure 4. Changes in total anthocyanins content in the skin of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 ºC. Values are the means of three replicates and S.E are shown by vertical bars.

Figure 5. Changes in antioxidant activity expressed as TE (mM/g FW) of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 ºC. Values are the means of three replicates and S.E are shown by vertical bars.

5

Figure

Figure 1



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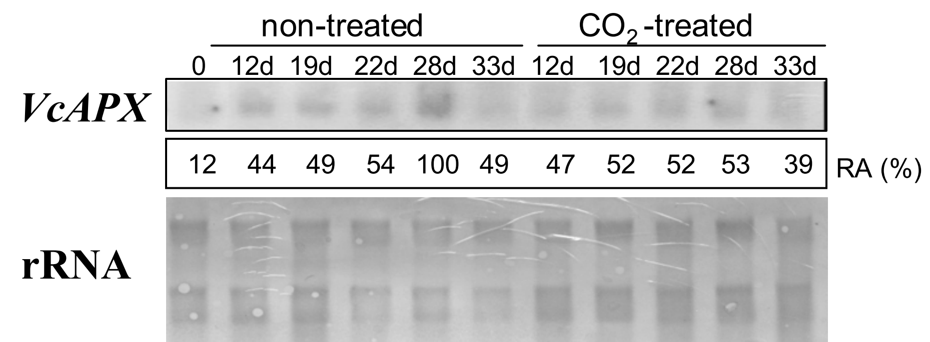

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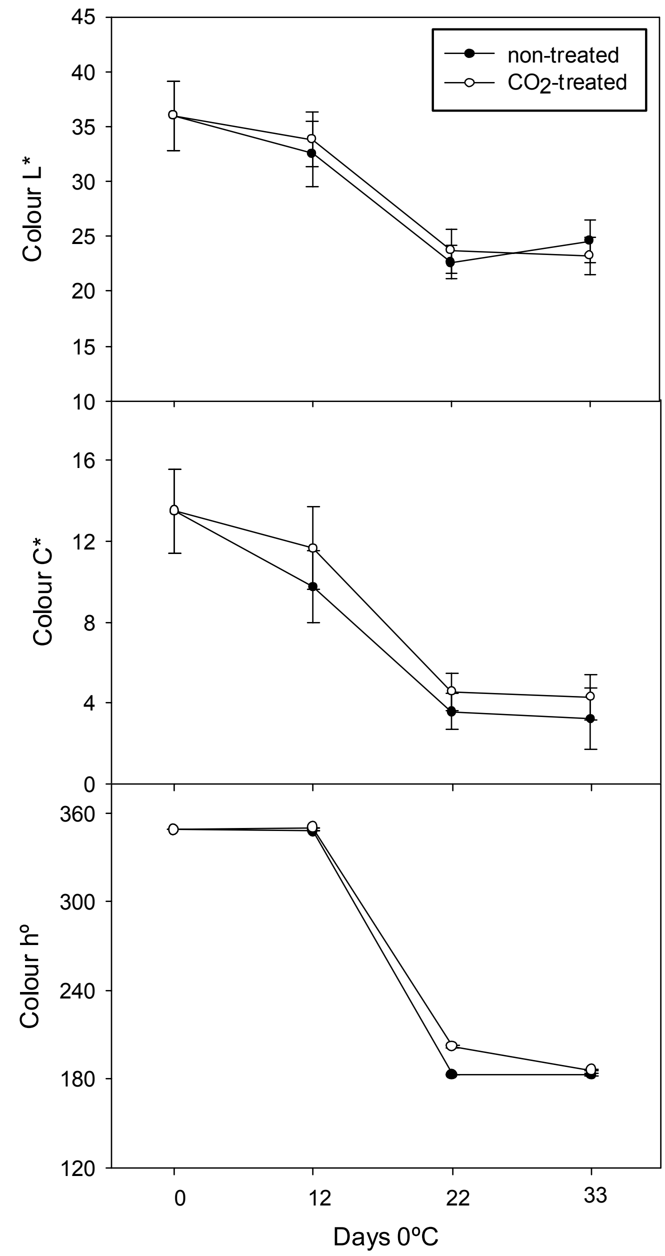

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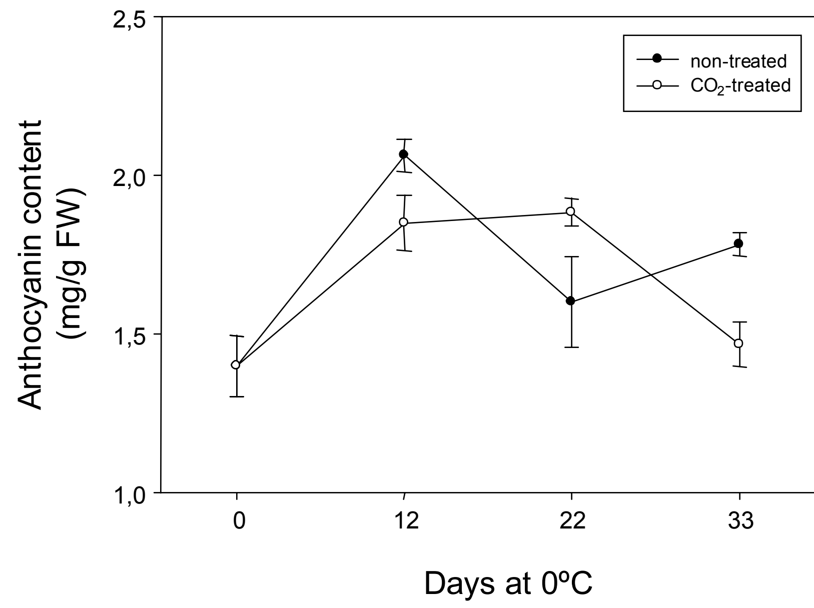

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Figure 5