Postharvest Biology and Technology 110 (2015) 33–39

Contents lists available at ScienceDirect

Postharvest Biology and Technology

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

CO_2-driven changes in energy and fermentative metabolism in harvested strawberries

María Blanch[a], Raquel Rosales[a], Francisco Palma[b], María T. Sanchez-Ballesta[a], María I. Escribano[a], Carmen Merodio[a,*]

[a] Department of Characterization, Quality and Security, Institute of Food Science Technology and Nutrition (ICTAN-CSIC), Madrid, Spain
[b] Department of Plant Physiology, University of Granada, Fuentenueva s/n, 18071 Granada, Spain

ARTICLE INFO

Article history:
Received 29 January 2015
Received in revised form 6 July 2015
Accepted 9 July 2015
Available online 26 July 2015

Keywords:
High CO_2
ATP
Energy charge
Fermentative genes
MDA

ABSTRACT

Short postharvest exposure of strawberries to high CO_2 levels provides significant benefits in reducing decay and controlling physiological disorders during storage at 0 °C. To define the different strategies employed by strawberries to tolerate high CO_2 concentrations, the impact of different CO_2 concentrations on energy and fermentative metabolism was studied under the same conditions of O_2 availability. Our data indicate that metabolic depression represents a strategy to effectively adapt to beneficial high CO_2 concentrations, with a decrease in ATP levels and in the energy charge, along with moderate ethanolic fermentation. Moreover, the induction of fermentative genes does not appear to be essential for the accumulation of fermentative metabolites. By contrast, when fruit is stored in air without added CO_2, the metabolism is not directed towards fermentation and is accompanied by a high ATP/ADP ratio and energy charge. However, when exposed to 40 kPa CO_2, the excessively low energy charge and excessive decrease in ATP could not match the ATP requirements, in a process that ultimately causes significant perturbations including a high lipid peroxidation.

1. Introduction

Strawberries have relevant economic, commercial and nutritional benefits that may vary greatly depending on environmental and genetic factors (Tulipani et al., 2011). Considerable effort is currently being expended on strawberry breeding programs in order to develop new cultivars with enhanced flavor and health-related compounds (Vandendriessche et al., 2013). Overall strawberry liking is mainly influenced by sweetness and flavor intensity, which are undermined by environmental pressures that reduce sucrose and the total volatile compounds content (Schwieterman et al., 2014). Indeed, sucrose was proposed to be the metabolite with the single most significant contribution to the overall linking.

Mara des Bois strawberries are highly regarded for their excellent flavor but they have a very short shelf-life. Consequently, an important goal is to maintain its quality by reducing detrimental effect during postharvest storage, applying different conditions of refrigeration (Allais and Létang, 2009) with and without coadjuvant treatments. Interestingly, a short treatment with 20 kPa CO_2 maintained higher sucrose levels than when strawberries were stored in air without added CO_2 (Blanch et al., 2015a). High CO_2 levels (10–30 kPa) also have the potential to reduce fungal decay during postharvest storage of strawberries, without leaving chemical residues in the fruit (Ke et al., 1991), and to increase flesh firmness (Larsen and Watkins, 1995). In addition, short-term 20 kPa CO_2 treatment mitigated certain physiological and structural disorders caused by low temperature storage, and effectively increasing the levels of health-related compounds like proanthocyanidins and those of fructo-oligosaccharides (Blanch et al., 2012a,b).

It is known that energy metabolism plays significant roles in the postharvest physiology of fruit and vegetables, and that ATP, ADP and AMP levels, as well as energy status, are affected by environmental factors, mainly by low oxygen levels (Saquet et al., 2001; Wang et al., 2013; Huang et al., 2014). A reduction of the cellular energy charge together with the accumulation of fermentative metabolites are generally considered to be common responses to hypoxia in aerobic organisms, including plants, and even a slight decrease in oxygen concentration provokes a drop in the cellular energy status (ATP/ADP ratio) (Geigenberger, 2003). Furthermore, it was reported that ethanolic fermentation also had an important function in plant species exposed to environmental stresses at ambient or even at elevated oxygen concentrations (see review Tadege et al., 1999), suggesting that fermentation might be an important switch in regulating carbohydrate metabolism. Fermentative metabolism is

* Corresponding author. Fax: +34 91 5493627.
E-mail address: merodio@ictan.csic.es (C. Merodio).

http://dx.doi.org/10.1016/j.postharvbio.2015.07.003
0925-5214/

essential for the production of ATP, through regeneration of NAD^+ which sustains glycolysis but, due to its inefficiency (Gibbs et al., 2000) a lower yield of ATP per mol of fermentative substrate is produced. This means that a high ethanol production may lead to a carbohydrate decline (Mustroph et al., 2006). However, in first harvest strawberries treated with high CO_2 levels, the increase in the levels of fermentative metabolites was not associated with a decrease in sucrose content. By contrast, lower levels of sucrose and fermentative metabolites were observed in fruit stored in air without added CO_2. Moreover, the expression of genes encoding pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were greatly in excess of the rate of fermentation metabolites in fruit stored in air (Blanch et al., 2015b). Ponce-Valadez and Watkins (2008) had also previously observed that transcript levels of several genes encoding fermentative enzymes were not always positively correlated with the increase in the amounts of their encoded products in different strawberry cultivars.

It has been shown that some stressful conditions lead to changes in energy metabolism, enhancing the production of reactive oxygen species (ROS: Tezara et al., 1999; Blokhina et al., 2001; Dinakar et al., 2012). An imbalance in ROS can induce either adaptive responses or detrimental changes in cell structure and metabolism. To prevent cytotoxic damage associated with the enhanced generation of ROS oxidative stress, fruit have evolved various protective mechanisms that include an enzymatic ROS scavenging system and the use of non-enzymatic antioxidants. Lipid peroxidation is one of the best studied consequences of the action of enhanced ROS levels on membrane structure and function. The oxidative degradation of lipid membranes generates a variety of aldehydes, including malondialdehyde (MDA). MDA is a secondary end product of the oxidation of polyunsaturated fatty acids and it is considered a useful lipid peroxidation marker. Therefore, MDA quantification by high-performed liquid chromatography (HPLC) was performed to estimate lipid peroxidation in strawberries exposed to high CO_2 concentrations.

Due to the diversity of responses to high CO_2, apart from the genetic background, factors such as harvest time and ripening stage, that affect the levels of soluble saccharides, might be play an important role in the response to high CO_2 levels. Our hypothesis was that the ATP optimization established to preserve carbohydrate pools could be crucial to maintain fruit quality during postharvest storage at low temperature. Thus, the aim of this work was to analyze the energy status and the fermentative metabolism in strawberries at 0 °C treated with different concentrations of CO_2 (20 or 40 kPa), as compared with fruit immediately after harvest. Additionally, we analyzed whether the transfer to ambient CO_2 for one additional day was associated with a decrease in the levels of ethanol or acetaldehyde, and in the expression of PDC, ADH or the energy status In order to assess the changes in energy metabolism, the levels of ATP, ADP and AMP were determined in strawberries at the end of the high CO_2 treatment and after transfer to air. Furthermore, the energy charge, as well as the ATP/ADP ratio and the AMP/ATP ratio, are very sensitive indicators of compromised cellular energy status, and they were also calculated. In the case of fermentation, the levels of ethanol and acetaldehyde detected by gas chromatography, and the expression of *PDC* and *ADH* were evaluated by real-time quantitative RT-PCR. In addition, fruit deterioration was determined by HPLC, analyzing the MDA levels as a measure of lipid peroxidation.

2. Materials and methods

2.1. Plant material and treatments

Strawberries (*Fragaria vesca* L. cv. Mara des Bois) were grown at an organic orchard in San Sebastian de los Reyes (Madrid, Spain). Ripe red strawberries from the second harvest, including fruit from different inflorescences, were collected and transported to the Institute of Food Science Technology and Nutrition within two hours of harvest. The strawberries were then selected for uniform size and color, and those with 8.9% total soluble solids, 0.7% titratable acidity and an external L^*18, a^*38, b^*28 color were stored at 0 °C (±0.5) and >95% RH in three sealed 1 m^3 containers. Fifteen plastic boxes containing approximately 0.4 kg of strawberries per box were treated for three days at 0 °C in the presence of 20 or 40 kPa CO_2. The O_2 concentration was kept constant at 20 kPa, adjusting the N_2 concentration. CO_2 concentration was measured twice using a Check Mate 9900 O_2, O_2/CO_2 Headspace Analyser (Dansensor España, S.L.U.). After a three day exposure to the specific CO_2 concentration, the strawberries were removed from the containers, weighed and transferred to a similar container with a flux of air for a further day under the same temperature and humidity conditions (0 °C and 95% RH). Immediately after harvest, at the end of the three-day sampling period and one day after exposure to air (day four), 45 strawberries were assessed for quality, while another 45 were randomly removed from each of the treatment groups and divided into three batches of 15 berries. Each biological replicate was composed of 15 pooled strawberries and each replicate was mixed, frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Relative gene expression assessed by quantitative RT-PCR (RT-qPCR)

Total RNA was extracted three times from 0.4 g of each sample with CTBA based extraction buffer, according to the protocol of Yu et al. (2012). The quality and purity of the total RNA was evaluated by agarose gel electrophoresis and spectrometry (NanoDrop 2000, Thermo Scientific). The RNA was then treated with DNase (DNase I, RNase-free, Thermo) to remove any genomic DNA and cDNAs were synthesized from 1 μg of each sample using the iScript TM Reverse Transcription Supermix for RT-qPCR (Bio-Rad). RT-PCR amplification was carried out in a 96 well-plate iCycler iQ thermal cycler (Bio-Rad) and quantified using the iCycler iQTM-associated software (Real Time Detection System Software, version 2.0), evaluating each gene in at least two independent runs. The parameters for PCR were: one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 20 s and 60 °C for 1 min. Sequences from the NCBI database and from the available literature were used to design the following gene specific primers using Primer3 software.

The primer pairs used in the RT-qPCR for pyruvate decarboxylase (XM_004302484) were FvPDC_F: GTTGCTTGAGTGGGGGTCTA and FvPDC_R: ATCTGTGAATGCGAATGAAGG; and for alcohol dehydrogenase (XM_004290520), were FvADH_QFw2: GCCCTTCTATACTGTGTCCTC and FvADH_QRv2: ACTGTTCTGGCTGACTGGTT.

The relative expression of the genes studied was assayed by RT-qPCR. To calculate the efficiency of the reaction (optimal range 90–110%) and to establish the most suitable template concentration, the cDNAs synthesized from serial dilutions between 40 ng and 2.5 ng of total RNA were amplified. Standard curves and linear equations were determined by plotting cycle threshold (Ct) values (*y*-axis) against the logs of the total RNA (x-axis). The specificity of the products was validated by analyzing the dissociation curves (evaluated in agarose gels) and by sequencing the products (Genomic Department of the CIB-CSIC). The *actin-97-like* housekeeping gene from *F. vesca* (XM_004307470) was not regulated by low temperature or high CO_2 levels (data not shown) and therefore, it was used as the internal reference gene to normalize the transcript profiles according to the $2^{-\Delta\Delta Ct}$ method and relative to the calibrator sample (fruit at harvest). The a*ctin-97-like* mRNA was

amplified with the primers FvActin_Fw GGGTTTGCTGGAGATGATG and FvActin_Rv CACGATTGGCCTTGGGATTC. Similarly, the specificity of the products was validated by analyzing the dissociation curve in agarose gels and by sequencing.

2.3. Ethanol and acetaldehyde content

Ethanol and acetaldehyde were analyzed from the headspace of the juice of three replicates of 15 strawberries without calyx, immediately after harvest and after each period in storage. An aliquot (5 mL) of juice was transferred to 10 mL vials, closed tightly with crimp-top caps and TFE/silicone septum seals, and frozen at −80 °C. Gas Chromatography (Thermo Trace, Thermo Fisher Scientific) was used to measure the ethanol and acetaldehyde according to the procedure of Valencia-Chamorro et al., (2009), expressing the results as grams per liter of juice.

2.4. Chromatographic determination of MDA

Frozen fruit samples (ca. 1 g) were homogenized in 10 mL of ultra-pure water, centrifuged for 20 min at 30,000 × *g* and after filtering through a 0.45 μm pore size membrane, the supernatants were collected to quantify the malondialdehyde (MDA) content. MDA was measured by HPLC as its hydrazone using 2,4-dinitrophenylhydrazine (DNPH) for derivatization and following the method previously described by Mateos et al., (2005), with slight modifications in the chromatographic conditions (Blanch et al., 2015b). The concentrations were expressed as mole MDA per kilogram fresh weight.

2.5. Determination of ATP, ADP and AMP

Frozen fruit samples (ca. 1 g) were homogenized with 5% (v/v) cold perchloric acid (1:2.4; w/v), and the homogenate was centrifuged for 10 min at 6000 × *g* and 4 °C. The supernatant was neutralized to pH 6.5–6.8 with KOH and incubated for 15 min at 4 °C before it was centrifuged for 10 min at 6000 × *g* and 4 °C. After centrifugation, the supernatant was filtered through a 0.22 μm nylon filter, and ATP, ADP and AMP were analyzed by HPLC according to Palma et al., (2015). A relative calibration procedure (0–20 μg mL^{-1}) was used to determine the ATP, ADP and AMP in the samples, and the results were expressed as milligram of ATP, ADP or AMP per kilogram of fresh weight. The adenylate energy charge was calculated according to Pradet and Raymond (1983): ([ATP] + 0.5 × [ADP])/([ATP] + [ADP] + [AMP]).

2.6. Statistical analysis

An analysis of variance (one-way ANOVA) was performed using SPSS v. 19.0 and a multi-comparison of the means was performed using Tukey's test, with the level of significance set at $P < 0.05$.

3. Results

3.1. Effect of different CO_2 concentrations on the PDC and ADH gene transcript profiles

A cDNA fragment was obtained by RT-PCR from Mara des Bois strawberries using specific primers designed from very conservative PDC sequences present in the Rosaceae Genome Database (GDR) and the EST database, and it was then confirmed as a PDC sequence. Specifically, we analyzed the expression of the gene encoding the Fvpdc isoform 2-like (XP_004302532), which is the PDC isoform in *F. vesca* with the highest homology to Fapdc1 (AF333772) from *Fragaria* x *ananassa*, a gene known to be induced by stress conditions (Moyano et al., 2004). The relative expression of *PDC*, as well as changes in acetaldehyde levels were assessed in strawberries stored at 0 °C in the presence of either 20 or 40 kPa CO_2 for three days, maintaining an O_2 concentration of 20 kPa, as well as after transfer to air for one further day (Fig. 1). These results were compared with those from fruit of the same chronological age stored in air for 4 days, and any changes were established relative to the fruit at harvest (day 0).

After three days in the presence of CO_2, the strawberries maintained the same levels of *PDC* transcripts as those seen in the fruit at harvest (day 0), with only a slight decrease in these transcripts after transfer to air. By contrast, a sharp increase in *PDC* expression was observed in fruit stored in air for four days, whereby the fruit stored in air had 2.6 times more *PDC* transcripts than that of the same chronological age previously treated with CO_2. Clearly, both CO_2 treatments (20 kPa and 40 kPa) reduced the accumulation of *PDC* transcripts during storage at 0 °C with respect to the fruit stored in air. While acetaldehyde accumulated in strawberries as the CO_2 concentration increased, the most marked change in acetaldehyde levels was observed in strawberries exposed to 40 kPa CO_2 at the end of treatment (Fig. 1B). These high levels of acetaldehyde diminished on transfer to air, while low acetaldehyde content was quantified in the fruit stored in air for four days. When these data are compared (Fig. 1A and B), there is no clear correspondence between the low acetaldehyde content and the high accumulation of *PDC* transcripts in strawberries stored in air.

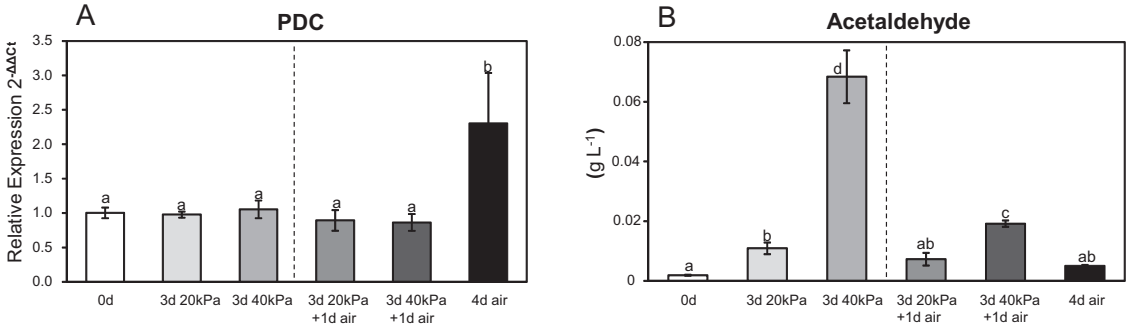

Fig. 1. Relative expression of the PDC gene XM_004302484 (A) and the acetaldehyde content (B) in Mara des Bois strawberries stored at 0 °C with 20 or 40 kPa CO_2 for 3 days, and after transfer to air for one further day. The O_2 concentration was kept at 20 kPa throughout. The results of transfer to air were compared with the fruit of the same chronological age stored in air throughout (four days) and any changes were relative to the fruit at harvest (day 0). The transcripts were measured by quantitative RT-PCR and normalized against those of *actin-97-like* used as a reference gene. The results were calculated relative to a calibrator sample (fruit at harvest, day 0) using the formula $2^{-\Delta\Delta Ct}$ and the values represent three biological replicates per three repeated measures. (B) Changes in acetaldehyde (g L^{-1}) content under the same conditions described above. Each letter indicates significant differences between the means (mean ± standard deviation), as determined with Tukey's test ($P < 0.05$).

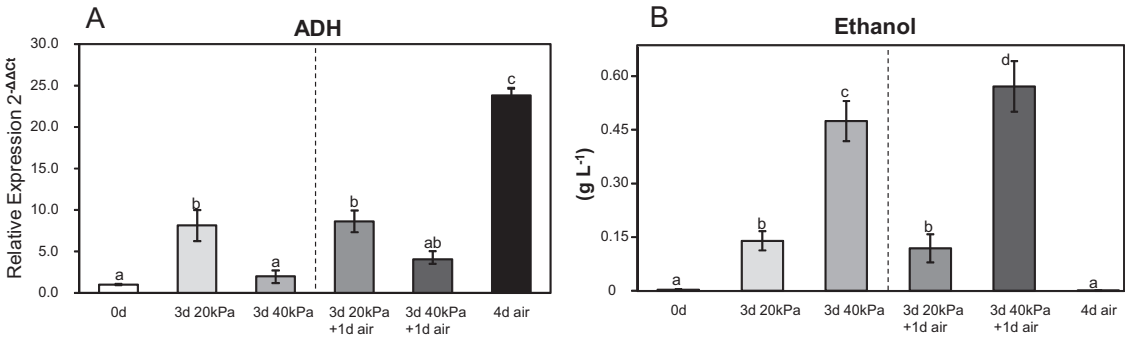

Fig. 2. Relative expression of the ADH gene XM_004290520 (A) and the ethanol content (B) in Mara des Bois strawberries stored at 0 °C for 3 days with 20 or 40 kPa CO_2 and after exposure to air for 1 further day. The O_2 concentration was kept at 20 kPa throughout. The results of the transfer to air were compared with the fruit of the same chronological age stored in air throughout (four days) and any changes were relative to the fruit at harvest (day 0). The transcripts were measured by quantitative RT-PCR and normalized against those of *actin-97-like* used as a reference gene. The results were calculated relative to a calibrator sample (fruit at harvest, day 0) using the formula $2^{-\Delta\Delta Ct}$ and the values represent three biological replicates per three repeated measures. (B) Changes in ethanol ($g\,L^{-1}$) content under the same conditions described above. Each letter indicates significant differences between the means (mean ± standard deviation), as determined with Tukey's test ($P < 0.05$).

Fig. 2 shows the relative quantification of *ADH* expression levels (A) and the changes in ethanol levels (B) in Mara des Bois strawberries. We studied the expression of the gene coding for the predicted *Fragaria vesca* ADH isoform XP_004290568, the sequence with the highest homology to the ADH from *Fragaria* x *ananassa* (P17648) involved in cold storage (Koehler et al., 2012). *ADH* expression increased irrespective of the CO_2 concentration to which the fruit was exposed, with more transcripts quantified in fruit maintained in 20 kPa CO_2 than in 40 kPa CO_2. Like *PDC* and its corresponding metabolite, the strongest expression of *ADH* was

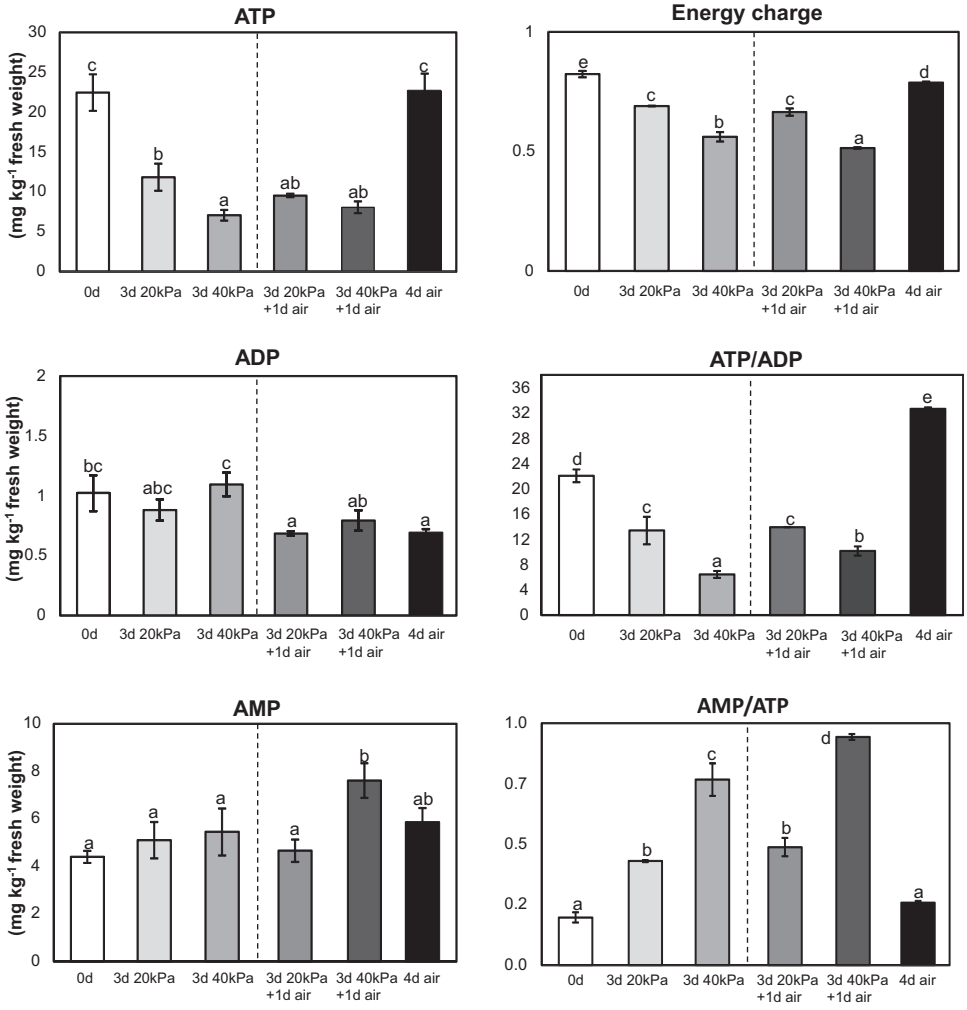

Fig. 3. The ATP, ADP and AMP levels in Mara des Bois strawberries stored at 0 °C for 3 days in the presence of 20 or 40 kPa CO_2 and after exposure to air for 1 further day. The O_2 concentration was kept at 20 kPa throughout. The results of transfer were compared with the fruit of the same chronological age stored in air throughout (four days) and any changes were relative to the fruit at harvest (day 0). The data represent two repeated measures from three biological replicates and the letters indicate significant differences between the means (mean ± standard deviation), as determined with Tukey's test ($P < 0.05$).

detected in fruit stored in air, in conjunction with the lowest levels of ethanol (Fig. 2B). In addition, an inverse relationship between ethanol and *ADH* expression was observed in fruit exposed to 40 kPa CO_2. The marked ethanol content in fruit maintained in 40 kPa CO_2 was therefore associated with weaker *ADH* expression, with the ethanol levels even increasing after transfer to air. Again, there was apparently no correspondence between the ethanol content and *ADH* expression in strawberries during storage at 0 °C in air.

3.2. Effect of different CO_2 concentrations on ATP, ADP and AMP levels

The levels of ATP, ADP and AMP were assessed in strawberries at the end of the 3-day exposure to either 20 or 40 kPa CO_2 (maintaining an O_2 concentration of 20 kPa), and after transfer to air for one additional day (Fig. 3). The results after transfer were compared with those from fruit stored in air of the same chronological age (4 days) and any changes were considered relative to the fruit at harvest (day 0). There was a progressive and significant depletion in ATP as the concentration of CO_2 increased. Consequently, a stronger decrease in ATP was seen in fruit exposed to 40 kPa CO_2 than that treated with 20 kPa CO_2. Conversely, high ATP levels were detected in strawberries stored at 0 °C in air for four days. It is interesting to note that the high levels of ATP in fruit stored in air were associated with low levels of ADP. By contrast, ADP levels were higher in CO_2-treated fruit, mainly in those maintained at 40 kPa CO_2. With respect to AMP, the highest levels were quantified in 40 kPa CO_2-treated fruit after transfer to air for one day. The energy charge, and the ratio of both ATP/ADP and AMP/ATP, was also evaluated (Fig. 3), and the energy charge was lower in CO_2-treated fruit than in fruit at harvest. Interestingly, the depletion in the energy charge was as significant as the increase in the levels of CO_2. Thus, the energy charge was significantly higher in fruit exposed to 20 kPa CO_2 than to 40 kPa CO_2, with the lowest energy charge quantified at the end of the 40 kPa CO_2 treatment. A high energy charge was detected in fruit stored in air, with values above those of the fruit previously treated with CO_2. Moreover, these fruit stored in air presented the highest ATP/ADP ratio. When the AMP/ATP ratio was calculated it was highest in the fruit maintained in 40 kPa CO_2, with this value increasing after transfer to air.

3.3. Effect of different CO_2 concentrations on lipid peroxidation

The impact of high CO_2 levels on lipid peroxidation was measured in terms of the MDA content, detecting the DNPH derivative by HPLC-UV. MDA levels were assessed in strawberries at the end of the 3-day treatment with either 20 or 40 kPa CO_2 and after transfer to air for one additional day (Fig. 3). The level of MDA was significantly higher in fruit stored at 0 °C without added CO_2 than that detected in strawberries at harvest (33.2 g/kg fresh weight), reaching values of 53.4 g/kg fresh weight (Fig. 4). The increase in MDA was 23% higher in fruit maintained in 20 kPa CO_2 at the end of the 3-day treatment, reaching values of 45.8 g/kg fresh weight. Moreover, the MDA in the strawberries increased after transfer to air. When maintained in 40 kPa CO_2 the MDA levels in the fruit were as high as 64.7 g/kg fresh weight, increasing even further after transfer to air.

4. Discussion

Cultivar variation has been reported in reference to the tolerance of strawberries to high CO_2 concentrations and in the accumulation of fermentation products (Watkins et al., 1999; Pelayo et al., 2003). Furthermore, differences in ATP and ADP content have been attributed to different physiological states of

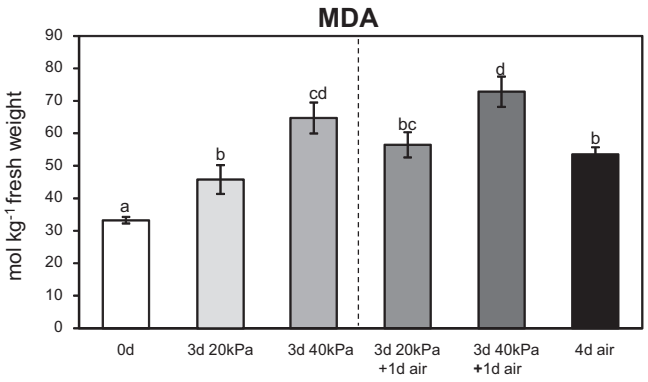

Fig. 4. Change in MDA determined by HPLC in Mara des Bois strawberries stored at 0 °C for 3 days in the presence of 20 or 40 kPa CO_2 and after exposure to air for 1 further day. The O_2 concentration was kept at 20 kPa throughout. The results of transfer were compared with the fruit of the same chronological age stored in air throughout (four days) and any changes were relative to the fruit at harvest (day 0). The data represent two repeated measures from three biological replicates and the letters indicate significant differences between the means (mean ± standard deviation), as determined with Tukey's test ($P < 0.05$).

avocados (also a fruit tolerant to high CO_2) and to the length of CO_2 exposure (Lange and Kader, 1997). Here, the impact of maintaining Mara des Bois strawberries from the second harvest in different high concentrations of CO_2 were evaluated in terms of fermentation and energy metabolism.

The data indicate that the most significant changes in *PDC* expression were evident in fruit stored at 0 °C in air without added CO_2, although low acetaldehyde content was quantified. By contrast, there was no increase in *PDC* transcripts detected in strawberries stored in 20 and 40 kPa CO_2, either at the end of the treatment or after transfer to air for one day, although a rise in acetaldehyde levels was evident. CO_2 was reported to inhibit *PDC* expression in Jewel strawberries during the first days of storage at 2 °C (Ponze-Valadez and Watkins, 2008). Our results also indicate that there is no correlation between *ADH* transcript accumulation and ethanol levels, showing that fruit stored in air underwent the strongest changes in the expression of *ADH* and that it accumulated the lowest levels of ethanol, even lower than at harvest. *ADH* expression in Jewel strawberries also increased after storage at low temperature, although it was not correlated with the accumulation of ethanol (Ponze-Valadez and Watkins, 2008). Elevated acetaldehyde and ethanol concentrations have been reported in fruit held for extended periods in high CO_2 conditions (Wszelaki and Mitcham, 2000). Changes in fermentative gene expression have been detected in strawberries and in cultured cells subjected to anoxia and stress conditions (Moyano et al., 2004). Moreover, the induction of *ADH* and *PDC* expression appears to be low-temperature specific, as reported for *ADH* mRNA accumulation in several plant species (Christie et al., 1991; Jarillo et al., 1993). In terms of acetaldehyde and ethanol accumulation, it seems that fermentative metabolism is activated by high CO_2 concentrations, while fruit stored in air at low temperature do not support such rates of ethanol and acetaldehyde production. The induction of chilling tolerance by endogenous and applied ethanol has been reported (Frenkel and Erez, 1996), and our data show that a higher degree of fermentation occurs in 40 kPa CO_2 than in 20 kPa. Indeed, ethanol content in fruit exposed to 40 kPa CO_2 was not depressed even after transfer to air for one further day.

In the absence of oxygen, the ability to maintain an active fermentative metabolism, by fuelling the glycolytic pathway with readily fermentable carbohydrates, is certainly crucial (Magneschi and Perata, 2009). Furthermore, it has been reported by Tadege et al., (1999) that under different stress conditions, which damages

the mitochondrial ATP-generating machinery, the cells resort to ethanolic fermentation to regenerate NAD^+ for the support of glycolytic ATP production. These authors suggest that fermentation might be an important switch in regulating carbohydrate metabolism. In Mara des Bois strawberries maintained in a high CO_2 environment, the activation of fermentative metabolism was associated with a marked decrease in the ATP/ADP ratio and a low energy status, which favors ATP-generating catabolic pathways. A lower ATP/ADP ratio in low O_2-treated pear discs was also reported (Nanos and Kader, 1993), indicating a lower energy charge. By contrast, in strawberries stored in air without added CO_2 there was a sharp increase in the ATP/ADP ratio, coupled to a significant decrease in soluble sugars (Blanch et al., 2015a). Similarly, a decrease in sucrose content was reported in different varieties of strawberries during cold storage at 6 °C (Cordenunsi et al., 2003). Considering that respiration in heterotrophic plant tissues is mostly regulated by intracellular ADP levels (or the ATP/ADP ratio) and the supply of substrates (Shugaev and Bukhov, 1997), the decrease in sucrose content coupled to the high ATP levels and a high ATP/ADP ratio could suggest higher respiration and metabolic activity in fruit stored at 0 °C in air than in that stored in high CO_2 conditions. In this sense, the ATP content of avocado fruit was reported to be closely connected to the increase in the respiration rate (Bennett et al., 1987), and a positive correlation between energy status and the respiration rate was shown (Huang et al., 2014). An increase in the respiration rate immediately after removal from extended cold storage, possibly indicating chilling injury, has also been reported in other fruit (Galli et al., 2009). Moreover, chilled fruit exhibit higher ATP levels than ripe fruit, as quantified by phosphorus NMR (Muñoz et al., 2001). Here, a decrease in the ATP/ADP ratio and the adenylate energy charge was observed in strawberries as the CO_2 concentration increases. Thus, while there are numerous examples where low O_2 conditions effectively suppress the intensity of respiration, there are quite varied responses in terms of the effect of elevated CO_2 (Kubo et al., 1990). Blanke (1991) indicated that fruit subjected to CO_2 shock had a progressive reduction in respiration.

The decrease in energy charge in CO_2-treated strawberries, determined in our results, may reflect the inhibition of a wide range of ATP-consuming processes. It is clear that by reducing the demand for ATP to a threshold level, 20 kPa CO_2-treated fruit not only diminish the depletion rate of fermentative substrates but also, they reduce the rate of excessive anaerobic end product formation. Accordingly, the reported perturbation due to the direct or indirect effects of enhanced ethanol and/or acetaldehyde could be minimized, including those on the membrane (Slater et al., 1993; Pesis, 2005). However, significant differences in the adenylate pools were observed in strawberries exposed to 20 and 40 kPa CO_2. Consequently, exposure to 40 kPa in the absence of any additional adjustments provoked an excessively low energy charge, and the fruit was not able to avoid harmful fermentation

The excessively low energy charge and the excessive decrease in ATP in fruit exposed to 40 kPa CO_2 could not match the ATP requirements for anabolic synthesis of critical antioxidant compounds, a process that ultimately increases ROS formation and the successive peroxidation of lipids. In this sense, a threshold in ATP synthesis has been found, below which potato cells under anoxia become committed to hydrolysis of their membrane lipids (Rawyler et al., 1999). Since the detrimental changes in the cell structure and metabolism as a consequence of oxidative stress are manifested through enhanced lipid peroxidation, the significant formation of MDA in 40 kPa CO_2-treated fruit indicates that oxidative damage is part of the stress induced by high CO_2 concentrations. Moreover, the increase in MDA levels was higher after transfer to air, further indicating that the anabolism of the radical-scavenging system is impaired and membrane lipids are attacked more easily by oxidative stress than in the fruit at the end of treatment. The increase in AMP/ATP ratio in stressed high CO_2-treated fruit might act as a signal of the activation of specific metabolic pathways that should be further studied.

It has been reported that re-aeration of highly reduced anoxic tissues leads to the formation of harmful oxygen radicals and toxic oxidative products, resulting in rapid peroxidative damage (Biemelt et al., 1998). The effects of controlled or modified atmosphere on oxidative stress appear to be commodity specific, yet if applied correctly they could greatly reduce or suppress oxidative stress (Hodges et al., 2004). At the cellular level, the increase in lipid peroxidation is responsible for the alterations to the physical properties of the membrane. Moreover, these results confirm our previous data showing that when the concentration and/or length of exposure to high CO_2 was above the tolerance threshold, excess of ethanolic fermentation potentially caused a loss of the bound water fraction, which resembles cellular water stress (Blanch et al., 2015a). Our results also indicate that excessively high CO_2 (40 kPa) accelerates the loss of membrane integrity, provoking oxidative damage, concomitant with the appearance of higher levels of fermentative volatiles, as mentioned above.

In conclusion, the changes to fruit energy metabolism can be interpreted as adaptations in order to tolerate high CO_2 concentrations levels regardless of the O_2 present. These adaptations include lowering the adenylate status to a desirable level, the activation of fermentative metabolism and the depression of metabolism giving priority to ATP-generating catabolic pathways. In terms of the activation of fermentative metabolism, the induction of fermentative genes does not seem to be essential. By contrast, there is a marked increase in the ATP/ADP ratio and a lack of fermentation metabolism in fruit stored in air, associated with a high energy charge, which favors ATP-consuming anabolic pathways. However, in the presence of excessively high concentrations of CO_2 (40 kPa), the accumulation of fermentative products above a threshold and the overly low energy status that is associated with a strong depression of the anabolic processes requiring ATP (such as an excessive reduction of defense strategies), could explain why the oxidative damage and formation of MDA increases markedly in such fruit.

Acknowledgments

This work was financed by CICYT grant AGL2014-53081-R from the MICINN of the Spanish government. R.R. was supported by a postdoctoral JAE contract from the CSIC.

References

Allais, I., Létang, G., 2009. Influence of mist-chilling on postharvest quality of fresh strawberries Cv. Mara des Bois and Gariguette. Int. J. Refrig. 32, 1495–1504.
Bennett, A.B., Smith, G.M., Nichols, B.G., 1987. Regulation of climacteric respiration in ripening avocado fruit. Plant Physiol. 83, 973–976.
Biemelt, S., Keetman, U., Albrecht, G., 1998. Re-aeration following hypoxia or anoxia leads to activation of the antioxidative defence system in roots of wheat seedlings. Plant Physiol. 116, 651–658.
Blanch, M., Sanchez-Ballesta, M.T., Escribano, M.I., Merodio, C., 2012a. Water distribution and ionic balance in response to high CO_2 treatments in strawberries (*Fragaria vesca* L. cv. Mara des Bois). Postharvest Biol. Technol. 73, 63–71.
Blanch, M., Alvarez, I., Sanchez-Ballesta, M.T., Escribano, M.I., Merodio, C., 2012b. Increasing catechin and procyanidin accumulation in high CO_2-treated *Fragaria vesca* strawberries. J. Agric. Food Chem. 60, 7489–7496.
Blanch, M., Sanchez-Ballesta, M.T., Escribano, M.I., Merodio, C., 2015a. The relationship between bound water and carbohydrate reserves in association with cellular integrity in Fragaria vesca stored under different conditions. Food Bioprocess Technol. doi:http://dx.doi.org/10.1007/s11947-014-1449-9.
Blanch, M., Rosales, R., Mateos, R., Perez-Gago, M.B., Sanchez-Ballesta, M.T., Escribano, M.I., Merodio, C., 2015b. Effects of high CO_2 levels on fermentation,

peroxidation and cellular water stress in *Fragaria vesca* stored at low temperature in conditions of unlimited O2. J. Agric. Food Chem. doi:http://dx.doi.org/10.1021/jf505715s.
Blanke, M.M., 1991. Respiration of apple and avocado fruit. Postharvest News Inform. 2, 429–436.
Blokhina, O.B., Chirkova, T.V., Fagerstedt, K.V., 2001. Anoxic stress leads to hydrogen peroxide formation in plant cells. J. Exp. Bot. 52, 1179–1190.
Cordenunsi, B.R., Nascimiento, J.R.O., Lajolo, F.M., 2003. Physico-chemical changes related to quality of five strawberry fruit cultivars during cool-storage. Food Chem. 83, 167–173.
Christie, P.J., Hahn, M., Walbot, V., 1991. Low-temperature of *alcohol dehydrogenase-1* mRNA and protein activity in maize and rice seedlings. Plant Physiol. 95, 699–706.
Dinakar, C.D., Djilianov, D., Bartels, D., 2012. Photosynthesis in desiccation tolerant plants: energy metabolism and antioxidative stress defense. Plant Sci. 182, 29–41.
Frenkel, C., Erez, A., 1996. Induction of chilling tolerance in cucumber (*Cucumis sativus*) seedlings by endogenous and applied ethanol. Physiol. Plant. 96, 593–600.
Galli, F., Archbold, D.D., Pomper, K.W., 2009. Pawpaw fruit chilling injury and antioxidant protection. J. Am. Soc. Hort. Sci. 134, 466–471.
Geigenberger, P., 2003. Response of plant metabolism to too little oxygen. Curr. Opin. Plant Biol. 6, 247–256.
Gibbs, J., Morrell, S., Valdez, A., Settter, T.L., Greenway, T., 2000. Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. J. Exp. Bot. 51, 785–796.
Hodges, D.M., Lester, G.E., Munro, K.D., Toivonen, P.M.A., 2004. Oxidative stress: importance for postharvest quality. HortScience 39, 924–929.
Huang, Z., Guo, L., Wang, H., Qu, H., Ma, S., Liu, Y., Huang, H., Jiang, Y., 2014. Energy status of kiwifruit stored under different temperatures or exposed to long-term anaerobic conditions or pure oxygen. Postharvest Biol. Technol. 98, 56–64.
Jarillo, J.A., Leyva, A., Salinas, J., Martínez-Zapater, J.M., 1993. Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling-tolerant plant. Plant Physiol. 101, 833–837.
Ke, D., Golstein, L., O'Mahony, M., Kader, A.A., 1991. Effects of short-term exposure to low O_2 and high CO_2 atmosphere on quality attributes of strawberries. J. Food Sci. 56, 50–54.
Koehler, G., Wilson, R.C., Goodpaster, J.V., Sønsteby, A., Lai, X., Witzmann, F.A., You, J. S., Rohloff, J., Randall, S.K., Alsheikh, M., 2012. Proteomic study of low-temperature responses in strawberry cultivars (*Fragaria* × *ananassa*) that differ in cold tolerance. Plant Physiol. 159, 1787–1805.
Kubo, Y., Inaba, A., Nakamura, R., 1990. Respiration and C_2H_4 production in various harvested crops held in CO_2-enriched atmospheres. J. Am. Soc. Hort. Sci. 115, 975–978.
Lange, D.L., Kader, A.A., 1997. Elevated carbon dioxide exposure alters intracellular pH and energy charge in avocado fruit tissue. J. Am. Soc. Hort. Sci. 122, 253–257.
Larsen, M., Watkins, C.B., 1995. Firmness and aroma composition of strawberries following short-term high carbon dioxide treatments. Hort. Science 30, 303–305.
Magneschi, L., Perata, P., 2009. Rice germination and seedling growth in the absence of oxygen. Ann. Bot. 103, 181–196.
Mateos, M., Lecumberri, E., Ramos, S., Goya, L., Bravo, L., 2005. Determination of malondialdehyde (MDA) by high-performance liquid charomatography in serum and liver as a biomarker for oxidative stress. Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants form fruits. J. Chromtogr. B. 827, 76–82.
Moyano, E., Encinas-Villarejo, S., López-Ráez, J., Redondo-Nevado, J., Blanco-Portales, R., Bellido, M.L., Sanz, C., Caballero, J.L., Muñoz-Blanco, J., 2004. Comparative study between two strawberry pyruvate decarboxylase genes along fruit development and ripening, post-harvest and stress conditions. Plant Sci. 166, 835–845.
Mustroph, A., Boamfa, E.I., Laarhoven, L.J.J., Harren, F.J.M., Albrecht, G., Grimm, B., 2006. Organ-specific analysis of the anaerobic primary metabolism in rice and wheat seedlings. I: dark ethanol production is dominated by the shoots. Planta 225, 103–114.
Muñoz, T., Ruiz-Cabello, J., Molina-García, A.D., Escribano, M.I., Merodio, C., 2001. Chilling temperature storage changes the inorganic phosphate pool distribution in cherimoya (*Annona cherimola*) fruit. J. Am. Soc. Hort. Sci. 126, 122–127.
Nanos, G.D., Kader, A.A., 1993. Low O_2-induced changes in pH and energy charge in pear fruit tissue. Postharvest Biol. Technol. 3, 285–291.
Palma, F., Carvajal, F., Ramos, J.M., Jamilena, M., Garrido, D., 2015. Effect of putrescine application on maintenance of zucchini fruit quality during cold storage: Contribution of GABA shunt and other related nitrogen metabolites. Postharvest Biol. Technol. 99, 131–140.
Pelayo, C., Ebeler, S.E., Kader, A.A., 2003. Postharvest life and flavor quality of three strawberry cultivars kept at 5 °C in air or air + 20 kPa CO_2. Postharvest Biol. Technol. 27, 171–184.
Pesis, E., 2005. The role of the anaerobic metabolites, acetaldehyde and ethanol in fruit ripening, enhancement of fruit quality and fruit deterioration. Postharvest Biol. Technol. 37, 1–9.
Ponce-Valadez, M., Watkins, C.B., 2008. Fermentation and malate metabolism in response to elevated CO_2 concentrations in two strawberry cultivars. Physiol. Plant. 134, 121–133.
Pradet, A., Raymond, P., 1983. Adenine-nucleotide ratios and adenylate energy-charge in energy-metabolism. Annu. Rev. Plant Physiol. 34, 199–224.
Rawyler, A., Pavelic, D., Gianinazzi, C., Oberson, J., Braendle, R., 1999. Membrane lipid integrity relies on a threshold of ATP production rate in potato cell cultures submitted to anoxia. Plant Physiol. 120, 293–300.
Saquet, A.A., Streif, J., Bangerth, F., 2001. On the involvement of adenine nucleotides in the development of brown heart in 'Conference' pears during delayed controlled atmosphere storage. Eur. J. Hort. Sci. 66, 140–144.
Slater, S.J., Ho, C., Taddeo, F.J., Kelly, M.B., Stubbs, C.D., 1993. Contribution of hydrogen bonding to lipid–lipid interactions in membranes and the role of lipid order: effects of cholesterol, increased phospholipid unsaturation, and ethanol. Biochemistry 32, 3714–3721.
Schwieterman, M.L., Colquhoun, T.A., Jaworki, E.A., Bartoshuk, L.M., Gilbert, J.L., Tieman, D.M., Odabasi, A.Z., Moskowitz, H.R., Folta, k.m., Klee, H.J., Sims, C.A., Whitaker, V.M., Clark, D.G., 2014. Strawberry flavor: diverse chemical compositions, a seasonal influence, and effects on sensory perception. PL0S One 9, 1–12.
Shugaev, A.G., Bukhov, N.G., 1997. Opposite trends of seasonal changes in ADP content and respiration rate in sugar beet roots. J. Plant Physiol. 150, 53–56.
Tadege, M., Dupuis, I., Kuhlemeier, C., 1999. Ethanolic fermentation: new functions for an old pathway. Trends Plant Sci. 8, 320–325.
Tezara, W., Mitchell, V.J., Driscoll, S.D., Lawlor, D.W., 1999. Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature 401, 914–917.
Tulipani, S., Marzban, G., Herndl, A., Laimer, M., Mezzetti, B., Battino, M., 2011. Influence of environmental and genetic factors on health-related compounds in strawberry. Food Chem. 124, 906–913.
Valencia-Chamorro, S.A., Pérez-Gago, M.B., del Río, M.A., Palou, L., 2009. Effect of antifungal hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings on postharvest decay development and quality attributes of cold-stored 'Valencia'oranges. Postharvest Biol. Technol. 54, 72–79.
Vandendriessche, T., Vermeir, S., Mayayo Martinez, C., Hendrickx, Y., Lammertyn, J., Nicolaï, B.M., Hertog, M.L.A.T.M., 2013. Effect of ripening and inter-cultivar differences on strawberry quality. LWT-Food Sci. Technol. 52, 62–70.
Wang, H., Qian, Z., Ma, S., Zhou, Y., Patrick, J., Duan, X., Jiang, Y., Qu, H., 2013. Energy status of ripening and postharvest senescent fruit of lichi (*Litchi chinensis* Sonn.). BMC Plant Biol. 13, 55.
Watkins, C.B., Manzano-Mendez, J.E., Nock, J.F., Zhang, J., Maloney, K.E., 1999. Cultivar variation in response of strawberry fruit to high carbon dioxide treatments. J. Sci. Food Agric. 79, 886–890.
Wszelaki, A.L., Mitcham, E.J., 2000. Effects of superatmospheric oxygen on strawberry fruit quality and decay. Postharvest Biol. Technol. 20, 125–133.
Yu, D., Tang, H., Zhang, Y., Du, Z., Yu, H., Chen, Q., 2012. Comparison and improvement of different methods of RNA isolation from strawberry (*Fragraria* × *ananassa*). J. Agr. Sci. 4, 51–56.