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Water distribution and ionic balance in response to high CO_2 treatments in strawberries (*Fragaria vesca* L. cv. Mara de Bois)

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ABSTRACT

The main organic (acids, amino acids and sugars) and inorganic (mono and divalent cations) solutes associated with changes in water status in response to low temperature and high CO_2 levels were analyzed in untreated and 20% or 40% CO_2 treated strawberries (*Fragaria vesca* L. cv. Mara de Bois) stored at 0 °C. Intercellular water distribution and cellular tissue integrity were visualized using low temperature scanning electron microscopy (LT-SEM). The results indicated that high CO_2 treatments prevented the weight loss and cell structure disorganization observed in untreated strawberries. However, there were differences in water loss regulation dependent on CO_2 levels. In addition to mediating osmotic adjustment, treatment with 20% CO_2 had a protective effect on cellular structure and prevented the movement of water into the intercellular spaces. Specifically, an accumulation in total sugars and proline were detected in 20% CO_2-treated fruit. Moreover, water loss was controlled in these fruit and K^+/Na^+ homeostasis maintained similar to that found in freshly harvested fruit. By contrast, 40% CO_2 controlled water loss, but the intercellular spaces were filled with aqueous solution, possibly as a result of a change in water status. Moreover, these changes were associated with an increase in free soluble Ca^{2+}. In view of the opposite patterns of accumulation in malic, succinic acid, γ-aminobutyric acid (GABA) and glutamate found when comparing 20% and 40% CO_2 treated strawberries, we suggest that their corresponding metabolic pathways play a regulatory role in CO_2 tolerance.

1. Introduction

Given the economic value of the fruit, particularly due to flavour and taste, several cultivars of *Fragaria vesca* L. that undergo repeat flowering are of special interest. However, strawberries are highly perishable and they are sensitive to water loss and fungal decay. Exposure to CO_2-enriched atmospheres is a common postharvest technique to control fungal decay. Indeed, concentrations of 15–20% CO_2 are routinely used for prolonged periods to control decay, with no detrimental effects on soluble solid content, titratable acidity or consumer acceptance. Short-term high CO_2 treatments also effectively increase strawberry fruit firmness (Harker et al., 2000). However, higher CO_2 levels can cause an accumulation of fermentation products that negatively affect fruit acceptability (Watkins et al., 1999). Although a great deal is known about the influence of cultivar, fruit maturity, temperature and length of treatment (Smith and Skog, 1992; Matsumoto et al., 2010) on the specific increase in fruit firmness mediated by high CO_2 levels, less is known about the mechanisms underlying CO_2 tolerance and CO_2-induced damage. Thus, it is necessary to distinguish between the metabolic responses associated with damage and those that are adaptive, favouring fruit quality.

The responses of tolerant fruit to high concentrations of CO_2 involve many complex pathways. Characterization of the roles of specific metabolites in these complex networks may provide insight into the basic mechanism of tolerance to high CO_2. Among the specific metabolites studied, GABA accumulates in CO_2-treated fruit of all cultivars of *Fragaria* × *ananassa* described (Deewatthanawong et al., 2010). In cherimoya, significant increases in GABA levels were also detected after three days of exposure to high concentrations of CO_2, an effect that was reversed by transfer to air (Merodio et al., 1998). The accumulation of GABA (Bouche and Fromm, 2004) plays several important roles, including the regulation of cytoplasmic pH, which varies in fruit treated with high concentrations of CO_2 (Lange and Kader, 1997). Preventing cytoplasmic acidosis in stressful conditions may also involve malate and succinate metabolism, (Roberts et al., 1992), and changes in the levels of malate and succinic acid in CO_2-treated fruit are also well documented (Fernández-Trujillo et al., 1999; Maldonado et al., 2004; Ponce-Valadez and Watkins, 2008). Modifications in the levels of Ca^{2+} contents of the ethanol-insoluble fraction from 100 kPa CO_2 treated strawberries, that exhibited higher firmness values as compared with those stored in air, has also been reported by Hwang et al. (2012).

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Water status is a prominent parameter that indicates plant damage caused by environmental conditions (Vertucci and Stushnuff, 1992; Goñi et al., 2011). Short-term treatment with 20% CO_2 prevented perturbations in the water status and cellular structure of *F. vesca* cv Mara de Bois observed in untreated and 40% CO_2-treated fruit. Moreover, the maintenance of water potential and the unfreezable water fraction or bound water in 20% CO_2-treated fruit was associated with an increase in the levels of fructose-based polymers that exhibited a very high water binding capacity according to their physicochemical properties (Blanch et al., 2012). Fructooligosaccharides (FOS) have been increasingly recognized as protective agents against abiotic stresses in plants and they might serve a role as protectors of macromolecular structures (Valluru and Van den Ende, 2008). The accumulation of these non-structural carbohydrates has also been observed in grapes in response to short-term exposure to 20% CO_2 (Blanch et al., 2011).

Considering the different effect of high CO_2 levels on the water status of *F. vesca* cv Mara de Bois (Blanch et al., 2012), the main objective of the present study was to analyse all the major osmotically relevant metabolites associated with high tolerance to high CO_2 levels, both inorganic (cations) and organic (amino acids, organic acids and soluble sugars) in untreated and 20% or 40% CO_2 treated fruit. In addition, we set out to compare the volume and distribution of the aqueous solution in intercellular spaces in untreated, CO_2-treated and freshly harvested strawberries. In this way, we hope to gain insight into the protective mechanisms activated by high CO_2 treatments during storage at low temperature.

2. Materials and methods

2.1. Plant material

Organic strawberries (*F. vesca* L. cv. Mara de Bois) were harvested by hand on 17 May 2010 at the first flowering at the Monjarama orchard in San Sebastian de los Reyes (Madrid, Spain). Fruit were harvested at full size and when commercially mature (9.8% total soluble solids as° Brix, 0.8% titratable acidity as citric acid, and an external L^*18, a^*40, b^*29 colour). After harvest, fruit were transported to the Institute of Food Science Technology and Nutrition within 2 h. Fruit selected for uniform size and colour were stored at 0 °C (±0.5) and >95% RH in three sealed containers with a capacity of 1 m^3. Fifteen plastic boxes containing approximately 0.5 kg of strawberries per box were stored in each container for three days and exposed to a continuous flow of air (untreated fruit) or a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 or 40% CO_2 + 20% O_2 + 40% N_2. Carbon dioxide and oxygen concentrations were measured using a gas analyzer PBI Dansensor mod. Checkmate 9900. Initially and at the end of the three-day sampling period, 45 strawberries were taken for quality analysis, and another 45 were removed at random from each of the treatment groups and divided into three batches of 15 berries. The 15 strawberries from each batch, used as a biological replicate, were mixed, frozen in liquid nitrogen and stored at −80 °C for further analysis. From each of the three biological replicates, at least two different measurements were taken.

2.2. Extraction and chromatographic determination of the total soluble sugars and organic acids

To determine the total sugars (glucose, fructose and sucrose) and organic acid (oxalic, citric, succinic and malic acids) concentrations, 3 g of frozen fruit sample was homogenized in 10 mL of ultra-pure water, centrifuged at 30,000 × *g* for 20 min, and the supernatants were then filtered through a membrane of 0.45 μm pore size. Sugar determination was carried out by HPAEC-PAD with a Metrosep Carb 1–250 IC column (4.6 mm × 250 mm), as described elsewhere (Bodelón et al., 2010). Organic acids were analyzed by HPAEC using a Metrohm Advanced Compac ion chromatography instrument (867 IC. Metrohm) equipped with a Metrosep Organic Acids column (7.8 mm × 100 mm), an IC-819 conductivity detector, an IC Pump 818 and an IC-837 degasser coupled. Samples were eluted from the column with an isocratic gradient of 0.5 mM $HClO_4$ with 50 mM LiCl suppression over 20 min at a flow rate of 0.5 mL/min. Data were acquired with the ICNet 2.3 Metrohm software.

Oxalic, citric, succinic and malic acids were identified by their retention times and quantified on the basis of calibration curves derived from standards. The content of each sugar and organic acid was expressed as mg/g fresh weight (FW) of the sample, and the data represents the means of the three replicates, two different measurements being made.

2.3. Extraction and determination of free amino acids

A frozen fruit sample (1 g) was homogenized in 2.5 mL of ultra-pure water containing 5 mM chloridric acid and maintained at 4 °C overnight. Samples were centrifuged at 30,000 × *g* for 20 min, after which the supernatants were filtered through a membrane of 0.45 μm pore size, and an aliquot of each sample was injected into an Amino Acid Analyzer (Pharmacia, Biochrom 20). Profile analyses of free amino acids in untreated and CO_2-treated strawberries includes also those of proline and the ubiquitous non-protein amino acid GABA. Free amino acids were identified by comparing the retention times of standard mixtures and the amino acid content, expressed as μmol/g FW of the sample. Data represent the means of the three replicates, two different measurements being made.

2.4. Ion analysis

A frozen fruit sample (1 g) was homogenized for 5 min in 10 mL of ultra-pure water (for Na^+ and K^+) or 10 mL of ultra-pure water slightly acidified with 5 mM chloridric acid (for Ca^{2+} and Mg^{2+}) and analyzed as described previously (Cataldi et al., 2003). Samples were centrifuged at 2000 × *g* for 20 min, after which the levels of soluble ions were determined in the supernatants. Atomic emission spectrometry was used to determine K^+, using a 5100PC atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT, USA) with an air-acetylene flame. Levels of Ca^{2+}, Mg^{2+} and Na^+ were determined by atomic absorption spectrometry with the same instrument, using a multi-element (Ca–Mg–Zn) hollow-cathode lamp. Data represent the means of the three replicates, two different measurements being made.

2.5. Water distribution and microstructural analysis

Initially and at the end of the three-day sampling period, the weight of fifteen boxes of strawberries stored in air, 20% CO_2 or 40% CO_2 were recorded and the weight losses were expressed as a percentage of the initial weight.

The volume of fluid sap supernatant of pre-stored, untreated and CO_2-treated fruit was calculated following centrifugation (Welbaum and Meinzer, 1990) at 350 or 2000 × *g* for 10 min after thawing frozen pieces of strawberries (2.2 g) at 25 °C. Once this intercellular fluid was removed from the intercellular spaces by centrifugation, tissues were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis of microstructure, using low temperature scanning electron microscopy (LT-SEM). With this technique sample components, including water are physically stabilized by freezing in situ. Our LT-SEM studies are prepared as previously described (Goñi et al., 2011), using a Zeiss DSN-960 electron scanning microscope equipped with a cold stage

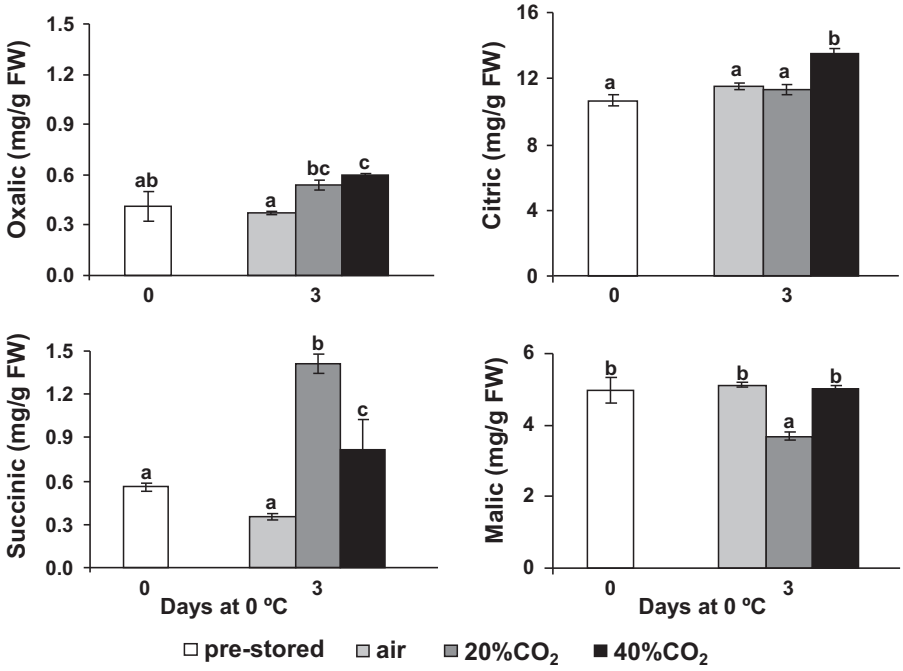

Fig. 1. Oxalic, citric, succinic and malic acid content in *F. vesca* cv Mara de Bois strawberries after harvesting (pre-stored, zero day) and after three days of storage at 0 °C in air (untreated), 20% CO_2 or 40% CO_2. The data are presented as the mean ± SE of three replicates ($n = 6$) and the letters indicate significant differences ($P < 0.05$).

(Cryostrans CT-1500, Oxford Instruments). Frozen tissue sections were cryofractured at −180 °C, etched at −90 °C, gold-coated and subsequently transferred to the microscope where they were analyzed out at −150 to −160 °C. Samples were observed with both secondary and retro-dispersed electrons, and the best images were selected in each case.

2.6. Statistical analysis

One-way ANOVA and correlational analyses were performed using SPSS ver. 19.0. The multicomparison of means was assessed by Bonferroni's test, to determine the level of significance at $P < 0.05$. The main effects of CO_2 treatment, storage time, and treatment time interaction on strawberry fruit were analyzed.

3. Results and discussion

3.1. Effect of high CO_2 treatments on organic solute content

We analyzed the profile of organic acids and free amino acids in strawberries cv Mara de Bois. Upon harvesting, citric acid was the most abundant organic acid (10.68 ± 0.34 mg/g FW), followed by malic acid (4.97 ± 0.34 mg/g FW) (Fig. 1). Succinic acid was detected at 0.56 ± 0.03 mg/g FW and oxalic acid 0.41 ± 0.09 mg/g FW. When compared with freshly harvested fruit, significant increases in the concentrations of both oxalic and citric acid were only observed in fruit treated with 40% CO_2. Citrate accumulation has been reported previously in cherimoya fruit stored in chilled conditions (Alique et al., 1994) and in table grapes after prolonged storage at 2 °C (Pretel et al., 2006). A significant increase in citrate was also reported in strawberries exposed to high salinity (Keutgen and Pawelzik, 2008) and in bananas exposed to 60% CO_2 at 20 °C (Liu et al., 2004) and under oxygen deprivation (Lara et al., 2011). Levels of malic acid decreased significantly in strawberries treated with 20% CO_2, whereas in untreated fruit and those exposed to 40% CO_2 the levels of this metabolite did not differ from those at the time of harvesting. In peel tissues of CO_2-treated fruit, malate levels have been reported to decrease, while they increase in air-treated fruit (Fernández-Trujillo et al., 2001). We previously reported a decrease in malic acid concentration when cherimoyas were treated with CO_2 associated with an increase in cytosolic NADP-malic enzyme (Maldonado et al., 2004). Malic acid accumulation control could be related with its important role in carbon metabolism and ionic homeostasis (Cheffings et al., 1997). Based on the present findings, we propose that the decrease in malic acid content is associated with tolerance to 20% CO_2 concentrations, and indeed no such decrease was observed in fruit treated with 40% CO_2. On the other hand, the levels of succinic acid remained unchanged in fruit stored in air while a marked increase in this acid was found in CO_2-treated fruit, mainly in fruit treated with 20% CO_2. Succinate accumulation is a common response to CO_2 treatment in many plant tissues (Ke et al., 1993), regardless of susceptibility to CO_2 injury, suggesting that succinate accumulation may contribute to stress resistance rather that inducing injury (Fernández-Trujillo et al., 2001).

We suggest that modifications in malic and succinic acid should also be studied in the integrated metabolic processes of amino acid biosynthesis via the GABA shunt and the anaplerotic pathway involving non-photosynthetic CO_2 fixation, respectively. In this respect, although GABA represents a very small fraction of the soluble amino acid pool at harvest, the highest levels of GABA were observed in fruit treated with 40% CO_2. Indeed, GABA accumulation in response to high CO_2 treatment has been reported previously in several fruit types (Ke et al., 1993; Merodio et al., 1998), and this accumulation of GABA after prolonged storage at low temperature in different cultivars of *Fragaria* × *ananassa* did not appear to be consistently associated with fruit sensitivity to CO_2 (Deewatthanawong et al., 2010). It remains unclear whether GABA is associated with stress or if it represents an adaptive response of plant tissues. Moreover, the increase in GABA levels observed in 40% CO_2-treated fruit was linked to a decline in glutamate levels (Table 1), suggesting that higher levels of CO_2 increase the glutamate conversion to GABA via the GABA shunt pathway. The production of GABA from glutamate that uses protons as co-substrate has been reported to be induced by decreasing

Table 1
Content of free amino acids in pre-stored, untreated and 20% CO_2 or 40% CO_2-treated strawberry *F. vesca* cv Mara de Bois stored at 0 °C, calculated in μmol/g FW.

	Pre-stored	Untreated	CO_2-treated	
	0 d	3 d air	3 d 20% CO_2	3 d 40% CO_2
Gaba	0.027 ± 0.00[a]	0.057 ± 0.01[b]	0.058 ± 0.01[b]	0.150 ± 0.01[c]
Gln	1.451 ± 0.07[a]	1.570 ± 0.34[a]	1.760 ± 0.26[a]	1.354 ± 0.13[a]
Pro	0.006 ± 0.01[a]	N.D.	0.039 ± 0.01[b]	0.006 ± 0.01[a]
Asp	0.746 ± 0.03[b]	0.711 ± 0.17[b]	0.563 ± 0.06[ab]	0.439 ± 0.03[a]
Thr	0.259 ± 0.02[a]	0.244 ± 0.06[a]	0.278 ± 0.03[a]	0.216 ± 0.02[a]
Ser	5.470 ± 0.13[a]	6.089 ± 1.43[a]	5.835 ± 0.69[a]	5.068 ± 0.29[a]
Glu	0.611 ± 0.03[b]	0.63 ± 0.15[b]	0.937 ± 0.09[c]	0.212 ± 0.03[a]
Gly	0.143 ± 0.01[a]	0.131 ± 0.03[a]	0.185 ± 0.02[a]	0.173 ± 0.02[a]
Ala	0.859 ± 0.03[a]	0.813 ± 0.19[a]	1.284 ± 0.15[b]	1.395 ± 0.10[b]
Cys	0.040 ± 0.00[a]	0.038 ± 0.00[a]	0.038 ± 0.01[a]	0.038 ± 0.00[a]
Val	0.159 ± 0.01[a]	0.156 ± 0.02[a]	0.173 ± 0.00[a]	0.162 ± 0.00[a]
Met	0.072 ± 0.00[a]	0.052 ± 0.01[b]	0.047 ± 0.00[b]	0.038 ± 0.00[b]
Ile	0.044 ± 0.00[a]	0.038 ± 0.01[a]	0.047 ± 0.00[a]	0.043 ± 0.00[a]
Leu	0.050 ± 0.00[b]	0.022 ± 0.02[a]	0.038 ± 0.01[ab]	0.039 ± 0.00[ab]
Tyr	0.016 ± 0.00[a]	0.011 ± 0.01[a]	0.022 ± 0.00[a]	0.020 ± 0.00[a]
Phe	0.045 ± 0.00[a]	0.035 ± 0.01[a]	0.043 ± 0.00[a]	0.037 ± 0.00[a]
His	0.029 ± 0.00[a]	0.026 ± 0.01[a]	0.021 ± 0.02[a]	N.D.
Lys	0.001 ± 0.00[a]	0.008 ± 0.00[b]	0.014 ± 0.00[bc]	0.020 ± 0.00[c]
Arg	0.024 ± 0.01[ab]	0.013 ± 0.01[a]	0.032 ± 0.00[b]	0.039 ± 0.01[b]

The data are presented as the means ± SE of the three replicates ($n = 6$) and the different letters indicate significant differences ($P < 0.05$).

intracellular pH (Crawford et al., 1994) and indeed, higher acidification imposed by 40% CO_2 at 20 °C has been reported (Lange and Kader, 1997).

Not only GABA, but the highest levels of alanine were also quantified in 40% CO_2-treated fruit. Alanine accumulation was confirmed during hypoxia induced by waterlogging (Rocha et al., 2010) and under anoxia (Reggiani et al., 1988). The increase in alanine content was concomitant with the greatest decline in aspartate in strawberries treated with 40% CO_2 for three days. In contrast, proline levels increased significantly after three days of 20% CO_2 treatment while no differences were observed in 40% CO_2-treated fruit, decreasing to undetectable levels in fruit stored in air as compared to freshly harvested strawberries. Free proline has been reported to accumulate in a variety of plants in response to a wide range of biotic and abiotic stressors. Several studies have focused on the ability of proline to mediate osmotic adjustments, stabilize subcellular structures and scavenge free radicals (Hare and Cress, 1997). Given the pool of free proline in strawberries, the increase seen in response to 20% CO_2 is unlikely to have an osmotic effect but rather, it may reflect a metabolic tolerance strategy, and so no such accumulation was observed in 40% CO_2-treated fruit. We suggest that such metabolic divergence in the accumulation of GABA, and proline between 20 and 40% CO_2-treated strawberries may reflect the variation in the degree of tolerance to high CO_2 levels. Levels of the remaining amino acids did not change significantly as a result of CO_2 treatments.

Changes in total sugar content based on the sum of glucose, fructose and sucrose are shown in Fig. 2A. The highest levels of total sugars were detected in fruit treated with 20% CO_2, with a weaker effect observed in that treated with 40% CO_2 and no changes in fruit stored in air at 0 °C. Increased foliar carbohydrate levels have been reported in plants exposed to CO_2 enriched versus CO_2 normal conditions (Hrubec et al., 1985). The changes observed in total solute levels between untreated and CO_2-treated fruit may be attributed to a reduced carbohydrate demand for respiration. However, the effect of 20% CO_2 on the generation, utilization and storage of reserve carbohydrates such as FOS (Blanch et al., 2012) should also be considered.

In addition to their osmotic contribution, sugars and organic acids are also important components of fruit sweetness. In accordance with this, the small increases in the amount of sugars accumulated in 20% CO_2-treated fruit could also indicate a better quality for consumption. Moreover, the ratio between main sugars and organic acids, shown in Fig. 2B, increased only in 20% CO_2-treated strawberries while in untreated fruit such a ratio declined, indicating that 20% CO_2 improved fruit quality in terms of sweetness.

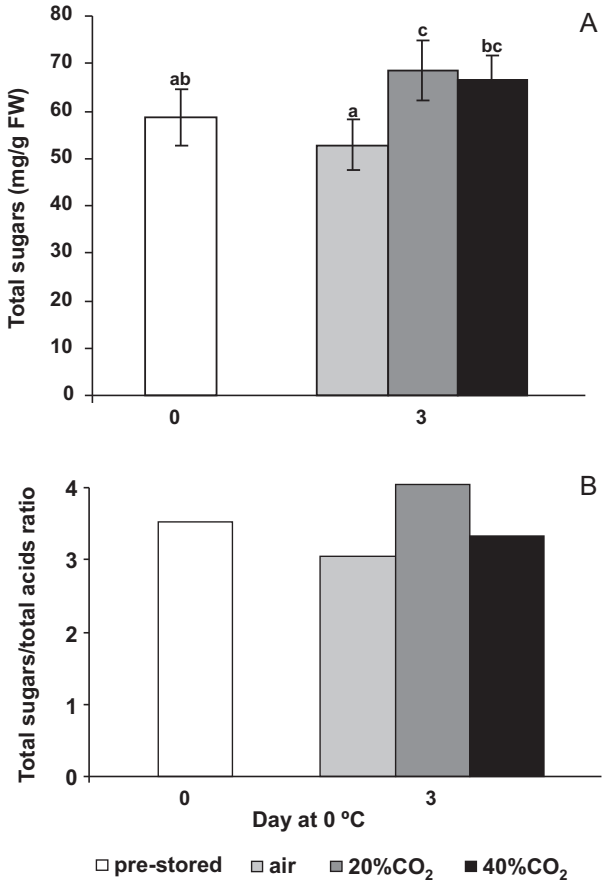

Fig. 2. Total sugars (sum of glucose, fructose and sucrose) (A) and ratio of sum sugars/sum organic acids (B) in *.F. vesca* cv Mara de Bois strawberries after harvesting (pre-stored, zero day) and after three days of storage at 0 °C in air (untreated), 20% CO_2 or 40% CO_2. The data are presented as the mean ± SE of three replicates ($n = 6$) and the letters indicate significant differences ($P < 0.05$).

Table 2
Content of soluble cations in pre-stored, untreated and 20% CO_2 or 40% CO_2-treated strawberry F.vesca cv Mara de Bois stored at 0 °C. Data are expressed as mg/100 g FW.

	Pre-stored	Untreated	CO_2-treated	
	0 d	3 d air	3 d 20% CO_2	3 d 40% CO_2
Monovalent				
Na^+[mg]	2.89 ± 0.39^b	2.26 ± 0.20^a	2.73 ± 0.12^b	3.95 ± 0.17^c
K^+ [mg]	168.90 ± 3.61^{ab}	176.18 ± 1.83^a	171.39 ± 6.34^{ab}	160.66 ± 11.30^b
K^+/Na^+ [relative units]	59.22 ± 8.41^b	78.19 ± 6.50^c	62.83 ± 4.04^b	40.72 ± 3.17^a
Divalent				
Ca^{2+} [mg]	11.01 ± 0.26^b	10.81 ± 0.32^b	9.47 ± 0.27^a	11.93 ± 0.27^c
Mg^{2+} [mg]	12.41 ± 0.15^a	13.42 ± 0.46^b	12.74 ± 0.54^{ac}	13.31 ± 0.15^{bc}

The data are presented as the means ± SE of the three replicates ($n = 6$) and the different letters within rows indicate significant differences at $P < 0.05$.

3.2. Effect of CO_2 treatments on ion homeostasis

Untreated fruit stored in air exhibited the lowest Na^+ values while the highest levels were detected in fruit treated with 40% CO_2 (Table 2). By contrast, the K^+ concentration was significantly lower in the 40% CO_2-treated versus untreated strawberries. As no differences in Na^+ or K^+ were observed between strawberries treated with 20% CO_2 and freshly harvested fruit, we reasoned that the degree of tolerance to high CO_2 (20%) was sufficient to prevent alterations in ion homeostasis, although the underlying mechanism remains unclear. Several stresses significantly decrease K^+ concentrations in plants (You et al., 2011) and mitigation of K^+ loss is strongly correlated with salt tolerance (Shabala and Cuin, 2007).

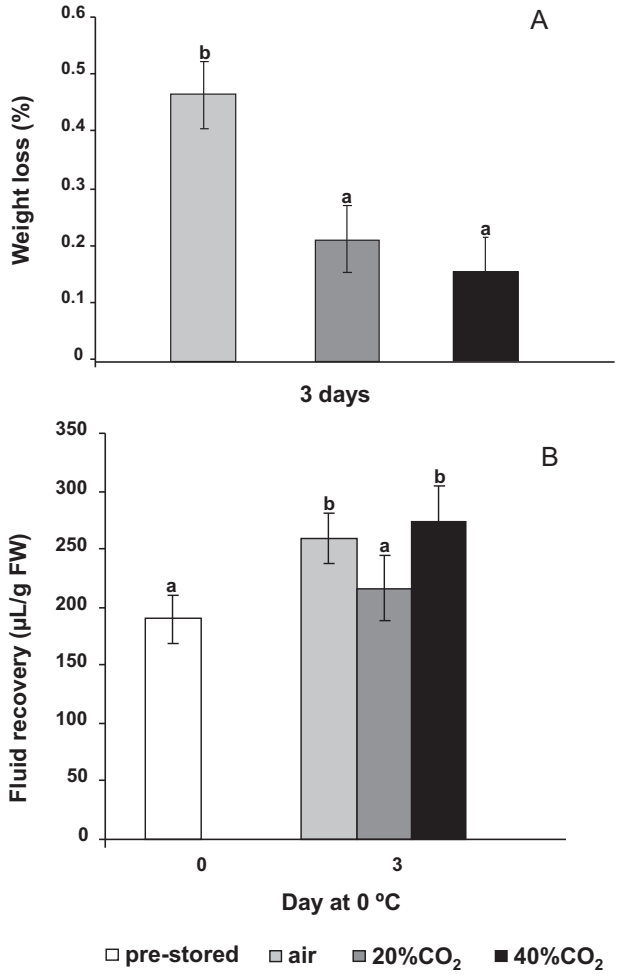

Fig. 3. Weight loss (%) (A) and fluid recovery (μL/g FW) (B) in untreated, 20% CO_2 or 40% CO_2 strawberries. The data are presented as the mean ± SE of three replicates ($n = 6$) and the letters indicate significant differences ($P < 0.05$).

Indeed, Orsini et al. (2011) confirmed the importance of inorganic ions for osmotic adjustment under high salinity conditions. Based on our data, we propose that rather than the absolute quantity of Na^+ and K^+ per se, the cytosolic K^+/Na^+ ratio better reflects damage to high levels of CO_2 and to severe low temperature. Our results suggest that treatment with 20% CO_2 maintains a K^+/Na^+ ratio similar to that found in freshly harvested fruit, whereas a significant decrease in this ratio was provoked when fruit is treated with 40% CO_2. This contrasts with the significantly increased K^+/Na^+ ratio observed at low temperature (0 °C) in the absence of CO_2 treatment. Thus, untreated fruit stored in air at 0 °C exhibited the highest K^+/Na^+ ratio.

With regard to divalent cations, tissues from 20% CO_2-treated fruit showed significantly lower free soluble levels of Ca^{2+} compared with freshly harvested fruit, however no differences were found in the case of Mg^{2+} levels (Table 2). Cation levels play an important role in cross-linking with cell-wall polysaccharides and by extension, in determining fruit tissue integrity (Jarvis, 1982; Tieman and Handa, 1994). The pectin structure is suitable for interactions with positively charged molecules such as calcium ions, forming supra-molecular structures of Ca^{2+} pectate (Morris et al., 2011). In the present study we observed a significant increase in free soluble Ca^{2+} in 40% CO_2-treated fruit, possibly due to the loss of calcium cross-links and the consequent migration of calcium to the water-soluble fraction. In contrast, a reduction of the free soluble Ca^{2+} in the 20% CO_2-treated fruit that led us to suggest its involvement in Ca^{2+} pectate binding and may indicate greater cell wall stability. This mechanism has already been pointed out by Harker et al. (2000) when explaining strawberry firmness enhancement by high CO_2 levels. Moreover, consistent with this, Hwang et al. (2012) reported a reduction in Ca^{2+} efflux and an increase in the Ca^{2+} content of ethanol-insoluble solids from 100 kPa CO_2-treated strawberries that exhibited higher firmness values as compared with those stored in air.

3.3. Water loss, distribution and micro-structural characteristics of untreated and CO_2-treated strawberry tissues

As shown in Fig. 3A, 20% and 40% CO_2 treatments attenuated weight loss by 58% and 68% respectively, indicating that the overall water loss by transpiration was less in CO_2-treated fruit than in untreated fruit stored in air. In fact, the lowest values of water loss were observed in fruit treated with 40% CO_2 for three days. To examine the water distribution in response to high CO_2 concentrations, the volume of the fluid recovered was carried out following centrifugation (Fig. 3B) and further illustrated using LT-SEM. The fluid volume in tissues of freshly harvested strawberries (pre-stored fruit, day 0) was 189.77 μL/g FW and this value increased significantly in untreated fruit stored in air for three days to 259.09 μL/g FW (+36%). Exposure to 20% CO_2 for three days had no effect on the volume recovered (Fig. 3B), whereas 40% CO_2 induced a significant increase with respect to pre-stored

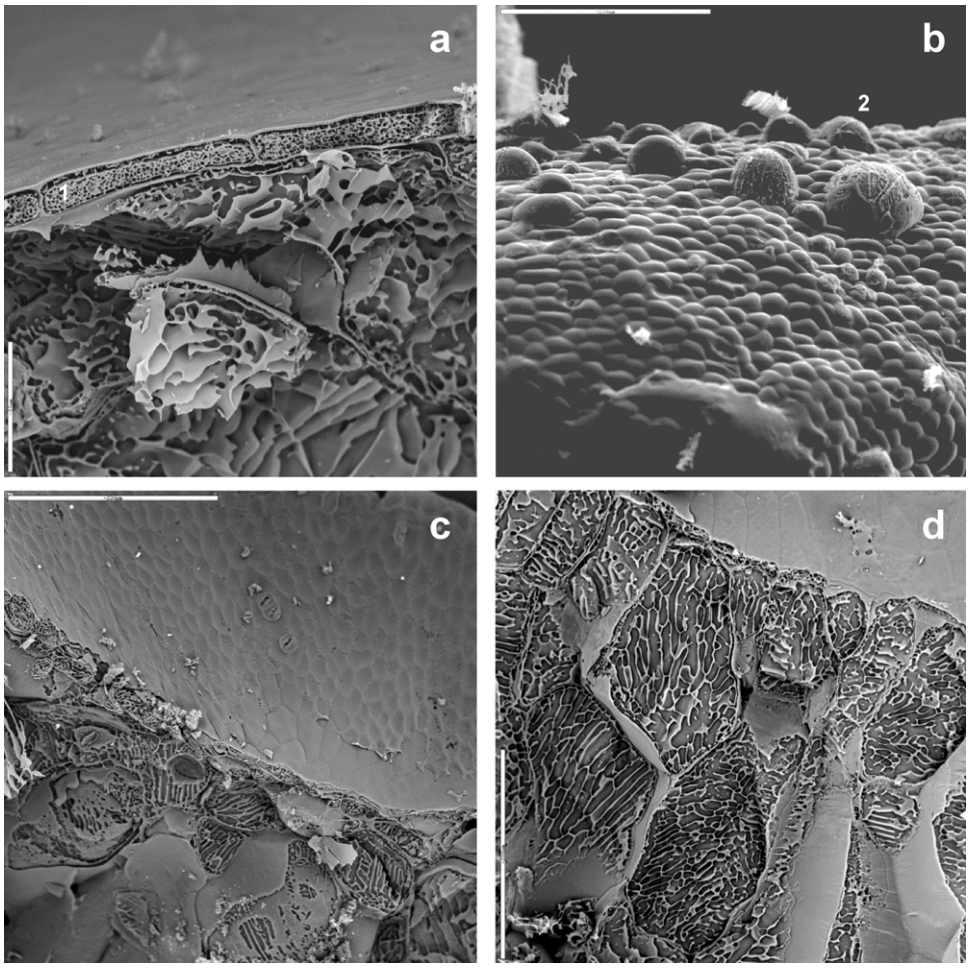

Fig. 4. LT-SEM micrographs showing anatomy of freshly harvested strawberries *F. vesca* cv Mara de Bois. (a) Image showing single layer of rectangular epidermal cells (1). (b) Image showing the distribution of achenes (2). (c) External receptacle parenchyma region with irregular cells. (d) Internal receptacle parenchyma region with large cells.

tissues (+44%). As the equivalent centrifugation values to those used here previously resulted in retention at the surface of substantial volumes of sugar solution in pockets of the cut cells due to surface tension (Dong et al., 1994), we therefore carried out additional studies using higher centrifugation. The fluid recovery values were clearly reinforced by the micro-structural data (Figs. 4 and 5) which showed differences in juice leakage and the amount of space between cells.

Fig. 4 enables us to visualize the contrast in size and shapes of cells between the epidermis (skin) and the parenchyma tissues (flesh) in ripe strawberry fruit at harvest. Epidermis (Fig. 4a) consists of a single cell layer fitting closely together, where the achenes (real one-seeded fruit) are embedded (Fig. 4b) and covered with special outer wall layers (cuticle and wax, Fig. 4c). From this anatomy, it can be deduced that the uniform epidermis cells without intercellular spaces and with close contact between cells could explain the significantly higher firmness values reported by Hwang et al. (2012). These authors reported that fruit firmness values at harvest time were nearly twofold higher for intact fruit compared with fruit whose epidermis had been removed. The underlying flesh cells are larger with different shapes between the receptacle cortical parenchyma cells, and those of the flesh located in the interior of receptacle tissue (Fig. 4c). The vertical middle section of the parenchyma is made up of larger cells (between 110 and 160 μm) (Fig. 4d).

To illustrate the effect of low temperature and high CO_2 levels on the aforementioned fluid volume, micro-structural characteristics of parenchyma cells (1.7–2 mm deep) in untreated and CO_2-treated strawberry tissue is shown in Fig. 5. Images showing the differences between freshly harvested (Fig. 5A), untreated (Fig. 5B), 20% CO_2 (Fig. 5C) and 40% CO_2) treated tissue (Fig. 5D) after centrifugation were compared with the corresponding non-centrifuged tissues (Fig. 5a–d). When compared with parenchyma cells of freshly harvested fruit (Fig. 5a), the most striking feature of the untreated fruit stored in air (Fig. 5b) is the layer of cell sap covering the cells, indicating that storage in air produced a high degree of cell degradation. The micrographs of CO_2-treated fruit tissues revealed a better-maintained structure conserving cell integrity with clear high free intercellular spaces in the case of 20% CO_2-treated fruit (Fig. 5c), as opposed to that in 40% CO_2-treated fruit (Fig. 5d) surrounded by fluid.

Using nuclear magnetic resonance microscopy, strawberry parenchyma tissues infected with *Botrytis cinerea* were shown to have a much longer transverse relaxation time (T_2) value than healthy tissues, due to the inundation of intercellular gas spaces with intracellular fluid released following cell wall damage (Goodman et al., 1996). These authors also reported that healthy strawberry parenchyma showed short T_2 values that were not seen in studies with other soft berries, possibly due to the gas spaces surrounding the parenchyma cells. The image of strawberries treated with 20% CO_2 (Fig. 5c) exhibited less fluid sap and large, empty intercellular spaces. It is known that air-filled intercellular spaces are necessary and ubiquitous in higher plants (Woolley, 1983). With regard to the centrifuged microstructural tissue images, it can be seen that the cells of 20% CO_2-treated fruit (Fig. 5C) and those of pre-stored fruit (Fig. 5A) were found to retain their cell shape and

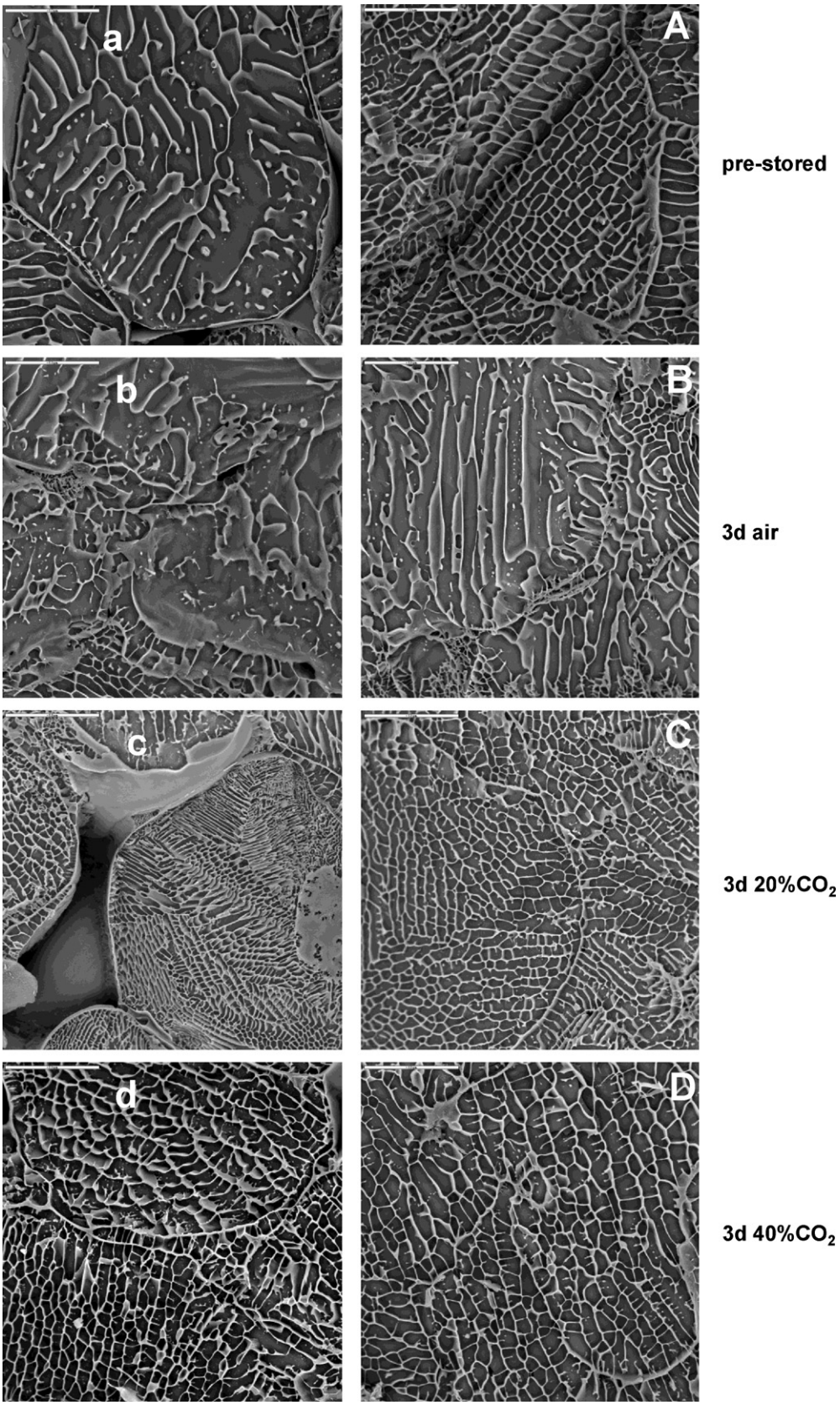

Fig. 5. Microstructural analysis of parenchyma cells of *F. vesca* cv Mara de Bois strawberries after harvesting (pre-stored, zero day) and after three days of storage at 0 °C in air (untreated), 20% CO_2 or 40% CO_2. Each photograph shows a cross-fractured face of one cell in the center. Small letters (a, b, c and d) correspond to the samples before centrifugation while the capital letters (A, B, C and D) indicate samples after centrifugation. Scale bars represent 50 μm.

integrity to a greater extent, followed by those treated with 40% CO_2 (Fig. 5D). In contrast, the micrographs of untreated tissues revealed a high degree of cell degradation (Fig. 5B), concomitant with an excess of aqueous solution and the changes in free cation levels (Table 2). 40% CO_2-treated fruit exhibited an opposite pattern of accumulation of free soluble cations than untreated fruit (Table 2) and better cell structure (Fig. 5d and D). Moreover, the larger volume of fluid in the intercellular spaces was consistent with previous results (Blanch et al., 2012), that showed a marked increase in free water content concomitant with an increase in total water potential in 40% CO_2-treated fruit, and a steady-state level of compounds such as FOS that exhibited a high water binding capacity.

We conclude that short-high CO_2 treatments are beneficial for preventing water loss and cell structure disorganization observed in untreated strawberries stored in air. However there were differences in water loss regulation dependent on CO_2 levels. Treatment with 20% CO_2, led to an increase in cellular water retention that was associated with an accumulation of osmolytes, some of which exerted cell protection and regulated ion homeostasis, in addition to mediating osmotic adjustments and quality improvement in terms of sweetness. Specifically, an increase in total sugar levels and proline content were quantified in 20% CO_2-treated fruit. In contrast, treatment with 40% CO_2 facilitated water movement into the intercellular spaces possibly as a result of modifications in cellular water status consistent with our previous findings. Moreover, we found that treatment with 40% CO_2 modified the K^+/Na^+ balance and increased the free soluble Ca^{2+} levels. Furthermore, this divergent pattern between fruit treated with 20% and 40% CO_2 was also observed in the accumulation levels of several metabolites including malic and succinic acid, γ-aminobutyric acid (GABA) and glutamate. We propose that their corresponding metabolic pathways may provide insight into the mechanisms of tolerance/damage to CO_2 levels and to a range of other environmental factors. Further studies will be necessary to determine whether water reabsorption from the intercellular spaces occurs after transfer of 40% CO_2-treated fruit to air, and to identify the mechanisms underlying the increase in the protective agents in the tissues of 20% CO_2-treated fruit.

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