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Relationship between bound water and reserve carbohydrates associated with cellular integrity in *Fragaria vesca* under different storage conditions.

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Abstract

It has been recognized that beneficial high CO_2 delays wilting and reduces weight loss in harvested plant products. Since strawberry fruits have high tolerance thresholds to CO_2, it is of interest to know what changes occur in fruit water status when these thresholds are reached and exceeded. Changes in the content of bound water were analysed in relation to the simple sugars, polyols and fructooligosaccharides (FOS) in Mara des Bois strawberries (*Fragaria vesca* L.) stored at severe low temperature (0 ºC) with 0, 20 or 40 % CO_2. The relationship between cellular water status and these compounds was analyzed at the end of a 3 day treatments and after exposure to air for one day. Unfavourable storage conditions were associated with a significant decrease in the levels of bound water, and in simple sugars, 1-kestose and *myo*-inositol. The 1-kestose and *myo*-inositol content was enhanced when fruit treated with excessive high CO_2 (40 %) were exposed to air at the same time as there was a marked increase in bound water content and a restoration of intracellular water. The ability of fruit to modulate the accumulation of these compounds, and by extension the water binding strength, in conjunction with the pool of the bound water

fraction, appears to be an important mechanism associated with structural cellular integrity. Variations in the percentages of air space and weight loss produced by enhancing the CO_2 treatments during storage at 0 ºC were also analyzed.

Keywords: strawberry; high CO_2; fructooligosaccharides; bound water; air space; *myo*-inositol.

Introduction

Water is the most abundant molecule in fruit, and it has a high impact on its quality and storability, especially in strawberries. The fact that only about 10 % of the solid matter in strawberries has to support a large amount of liquid could explain, in part, their high perishability and susceptibility to textural damage, fungal decay and liquid leakage. Moreover, changes in fruit water status are involved in the regulation of ripening processes (Frenkel and Hartman, 2012) and modifications in the content of the different water fractions have been reported (Goñi et al. 2007). Changes in the bound water fraction are at least partially the result of modifications in the levels of hydrophilic molecules with high water-binding capacity. It was reported that the bound water that represents unfreezable water (Wolfe et al. 2002) plays an important role in tolerance to different environmental stresses such as freezing and drought (Rascio et al. 1998; Sun 2000). Indeed, fruit stored at low temperature under adequate high CO_2 treatment had a larger bound water fraction (Goñi et al. 2011), in association with an increase of inulin series fructans (Blanch et al. 2011). Fructans have been increasingly recognized as protective agents in stress environments (Valluru and Van den Ende 2008). In addition to their protective influence on cell structures, fructooligosaccharides (FOS), which consist of linear β (2-1) linked fructose residues with a terminal glucose residue, participate in carbon storage. Consequently, variations in the levels of sucrose, glucose and fructose are thought to be linked to the changes in FOS. Sugar alcohols, or polyols, are also closely associated with hexose metabolism, and they act as compatible solutes in response to salt and drought stress and play multiple roles in plant protection (Williamson et al. 2002; Merchant and Richter 2011). However, the mechanism by which polyhydroxyalcohols could protect fruits under changing environmental conditions is less well understood.

It has long been recognized that beneficial CO_2 delays wilting (Smith et al. 1966), reduces water loss and incidence of decay in highly perishable fruits (El-Kazzaz et al. 1983; Pretel et al. 2006; Sanchez-Ballesta et al. 2006). However, when the CO_2 concentrations are above the toleration threshold they can also be harmful. Development of off-flavours is a well-known common detrimental effect caused by fermentative metabolite accumulation, especially when high CO_2 concentrations are combined with reduced oxygen levels. Therefore, it would be useful to identify the outstanding metabolic markers indicative of fruit damage associated with excessive CO_2 levels.

Furthermore, by taking advantage of *Fragaria vesca* tolerance to high CO_2 levels, benefits can be obtained if the detrimental effect can be mitigated after transfer to ambient CO_2. Moreover, since commercial harvests last several days, which implies that strawberries are picked from different inflorescences, benefits might be greatly enhanced if effective high CO_2 treatment can be applied to different harvested strawberries produced by different inflorescences.

The aim of the present work was to analyze how changes in bound water fractions were correlated with reserve carbohydrates and cellular structure in strawberry fruit stored under different storage conditions. For this purpose, Mara des Bois strawberries (*Fragraria vesca* L.) from various inflorescences were stored at low temperature (0 ºC) and treated with increased CO_2 concentrations (0, 20 and 40 %). The amount of bound water fraction, simple sugars, short-chain FOS and polyols were analyzed at the end of CO_2 treatments and when strawberries were transferred to air for one additional day. The ability of polyols (mannitol, sorbitol and *myo*-inositol) to modify water behaviour, and the contribution of high CO_2 treatments to weight loss, air space and cellular tissue integrity were also determined.

2. Materials and methods

Plant material

The organic strawberries used in this study (*Fragaria vesca* L. cv. Mara des Bois) were grown in an orchard in San Sebastian de los Reyes (Madrid, Spain) whose coordinates are 40° 32′ 49″ N, 3° 37′ 33″ W. Strawberries belonging to various inflorescences were harvested, immediately transported to the Institute of Food Science Technology and Nutrition and selected for uniform size and colour. The strawberries with 8.9 % total soluble solids as ºBrix, 0.7 % titratable acidity, and an external L^*18, a^*38, b^*28 colour were stored at 0 ºC (±0.5) and >95 % RH in three sealed 1 m^3 containers. Fifteen plastic boxes containing approximately 0.5 kg of strawberries per box were treated for

3 days with increasing amounts of CO_2 (0, 20 and 40 %, balanced with 20 % O_2 and made up to 100 % with N_2). Initially, at the end of the 3 day sampling period and 1 day after exposure to air, 45 strawberries were assessed for quality, 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 were used as biological replicates. Each replicate was mixed, frozen in liquid nitrogen and stored at -80 ºC for further analysis.

Extraction and chromatographic determination of glucose, fructose, sucrose and polyols

Berry samples of approximately 3 g were homogenized in 5 mL of ultra-pure water, centrifuged at 30,000 g for 20 min and the supernatants were filtered through a 0.45 µm pore size membrane. Sugars and polyols were determined by HPAEC-PAD with a Metrosep Carb 1–250 IC column (4.6 mm × 250 mm). Samples were analyzed on an Ion Chromatography 817 Bioscan (Metrohm, Herisau, Switzerland) system equipped with a pulsed amperometric detector (PAD) and a gold electrode. Isocratic elution was carried out with 10 mL/L NaOH 40 % w/w. Samples (1.5 mL) were injected using an autosampler (model, 838 Advanced Sample Processor, Metrohm, Herisau, Switzerland) and the flow rate through the column was 1 mL/min, leading to a sampling time (ts) of 38 min. Appropriate dilutions of solutions containing glucose, fructose, sucrose, *myo*-inositol, D-sorbitol and D-mannitol (Sigma, Steinheim, Germany) were used as calibration standards. Chromatographic peaks were identified by comparing sample retention times with those of known standard mixtures. The content of these sugars was expressed in mg/g fresh weight (FW) of the sample and the content of these polyols was expressed in µg/g FW. The data were expressed as the mean of three analyses of two different biological replicates. The data were acquired using DataApex Clarity software.

Extraction and chromatographic determination of FOS

The quantification of nystose, kestopentaose, and that of 1-kestose (which required pre-treatment with an activated carbon Darco G60, 100 mesh (Sigma, Steinheim, Germany) to remove the mono- and disaccharides), was determined according to the method of Blanch et al. (2011). Briefly, the samples were extracted with 85 % ethanol, boiled under reflux and centrifuged at 5,000 g for 20 min at 4 ºC. The supernatant was evaporated and redissolved in deionised water, filtered through 0.22 µm filters and analysed by HPAEC-PAD using a CarboPac PA1 carbohydrate column. A three-step PAD protocol was employed and the samples (1.5 mL) were injected using an autosampler (model, 838 Advanced Sample Processor, Metrohm, Herisau, Switzerland). FOS identification was performed by comparing the retention

time of standard mixtures 1-kestose and nystose from Sigma (Steinheim, Germany) and kestopentaose from Megazyme (Co. Wicklow, Ireland) and the FOS content was expressed in µg/g FW of the sample.

Determination of water status and water distribution

The unfreezable (bound) water fraction and freezing point were determined using DSC, according to the method of Goñi et al. (2011). The heat flow curves for freezing points (T_f, ºC) were determined in triplicate using a DSC822e apparatus (Mettler-Toledo Inc., Columbus, OH, USA). Samples were placed in sealed aluminium pans that were cooled to -80 ºC at 10 ºC/min, and they were scanned from -80 to 25 ºC at a rate of 10 ºC/min. The resulting thermograms were evaluated paying particular attention to the onset temperature and the enthalpy of the melting endotherm from which the quantities of bound water was calculated. The freezing point was considered as the intersection of the tangent and baseline of the left side of the melting peak.

Microstructure analysis was performed using low temperature scanning electron microscopy (LT-SEM), using a Zeiss DSN- 960 electron scanning microscope equipped with a cold stage (Cryostrans CT-1500, Oxford Instruments, Oxford,UK). Frozen tissue sections were cryofractured at −180 ºC, etched at −90 ºC, gold-coated and subsequently transferred to the microscope where they were analyzed at −150 to −160 ºC. Samples were observed with both secondary and retro-dispersed electrons, and the best images were selected in each case.

Determination of the percentages of air space and weight loss

The percentage of air space was calculated from the ratio of the fruit density and the density of the juice, according to the equation:

$$\text{air space (\%)} = 100\ \frac{V_a}{V_F} = 100\ (1-\frac{\rho_F}{\rho_J})$$

where

$$\rho_F = \frac{M_F}{V_F}$$

and

time of standard mixtures 1-kestose and nystose from Sigma (Steinheim, Germany) and kestopentaose from Megazyme (Co. Wicklow, Ireland) and the FOS content was expressed in µg/g FW of the sample.

$$\rho_J = \frac{M_J}{V_J} = \frac{M_F}{V_F - V_B}$$

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(ρ_F) is fruit density, (ρ_J) is juice density, M_F is fruit mass, V_F is fruit volume, M_J is juice mass, V_J is juice volume, and V_a is air volume.

The density of six individual fruits was measured using a balance with 1 mg sensitivity by placing the whole fruit in a pre-tarred steel mesh fully immersed in a glass container with ethanol. Ethanol instead of water was used to avoid the tendency of water to invade the air spaces when the fruit is immersed.

The fresh weight of the fruit was determined gravimetrically and weight loss was expressed as the percentage of the initial weight.

Determination of the amount of unfrozen water of the different polyols

The anti-freeze characteristics of the different polyols were evaluated calorimetrically through the amount of unfrozen water (U_w) defined as described by Furuki (2002) on a DSC822e (Mettler-Toledo Inc., Columbus, OH, USA), with slight differences. Aqueous solutions of polyol at the desired concentrations were prepared by weighing with deionized distilled water. The polyol concentration was gradually changed from 5 weight % up to a maximum of 50 weight %. A series of prepared aqueous solutions were sealed in independent vials and kept for 24 h at 25 °C in an incubator to allow them to reach steady state. Aqueous samples (5-10 µL) were placed in 40 µL hermetically sealed aluminium pans and cooled from 25 ºC to -80 ºC at a rate of 10 ºC/min. The samples were left at this temperature for 5 min and then heated to 25 ºC at 10 ºC/min. The heating scan was used to determine the latent heat of the melting ice (ΔH_m) by integration of the melting endotherm and the T_g' by the deflection midpoint in the heat flow versus temperature curve. U_w was determined by extrapolating ΔH_m to zero, which was linearly dependent on the polyol concentration (weight %) as expressed below:

$$U_w = \frac{\dfrac{100 - C_S'}{M_r(H_2O)}}{\dfrac{C_S'}{M_r(polyol)}}$$

where C_s is the polyol concentration of the freeze-concentrated liquid fraction ($\Delta H_m = 0$) expressed in weight %, M_r (H_2O) the molecular mass of deionized water (18.02 g/mol) and M_r (polyol) the molecular mass of the different polyols.

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Statistical analysis

One-way ANOVA and correlational analyses were performed using SPSS ver. 19.0. The multicomparison of means was assessed by Tukey´s test at a significance level of 0.05. The main effects of CO_2 treatment, storage time and the treatment/time interactions on strawberry fruit were analysed. The data corresponding to the fruit at harvest and the strawberries exposed to the different treatments were analyzed by analysis of variance (ANOVA) and a principal component analysis (PCA).

Results and Discussion

Water status

The unfreezable (bound) water content was assessed in strawberries stored at 0 ºC for 3 days with 0, 20 or 40 % CO_2, and after exposure to air for one day (Fig. 1). In all readings oxygen concentration was kept at 20 %. These changes were compared with those observed in fruit at harvest. There was a significant depletion in the bound water content (65 %) in 40 % CO_2-treated fruit at the end of treatment with respect to the values in freshly harvested strawberries. The decrease in the amount of bound water in 40 % CO_2-treated fruit reverted after transfer to air for 1 day. Since the total water content did not decrease (Fig.1 40 % CO_2 vs 40 % CO_2+1day air), it is clear that the conversion of free water to bound water can be assumed in fruit recovering from excessive high CO_2 treatment. There was also less bound water in strawberries stored at 0 ºC with no CO_2 supplementation (0 % CO_2). Furthermore, in these fruits the lowest total water content was quantified (Fig. 1). Our results indicate that storage at low temperature storage without CO_2 (0 %) or excessively high CO_2 (40 %), produced a drop in the bound water fraction content. On the contrary, in 20 % CO_2-treated strawberries the increase in the amount of bound water fraction was evident at the end of treatment and even more after transfer to air. Changes in bound water content caused by different environmental factors have been observed in different plants (Yoshida et al. 1997; Gusta et al. 2004) and in different tissues of harvested table grape clusters (Goñi et al. 2011). In view of the above results it is reasonable to ask if the changes in water status could be a signal that induces metabolic responses in fruit during storage, in line with that suggested by Passioura and Munns (2000) for leaves, or for the onset of the ripening process in fruit as suggested by Frenkel and Hartman (2012).

Simple sugars and polyols

In order to identify the causes of the variations in the aforementioned water fractions, we analyzed the changes in sugars (sucrose, glucose and fructose) and their polyol derivatives in strawberries stored at 0 ºC with 0, 20 or 40 % CO_2, keeping a 20 % O_2 concentration in each of them (Table 1). The content of sugars and polyols was assessed in strawberries at the end of the 3-day treatments and after transfer to air for one day. The sucrose concentration was lowest in strawberries stored without added CO_2, reaching values of 13.8 mg/g FW, 25 % lower than in freshly harvested fruit (control fruit). In these fruits, the decrease in fructose (-39 %) or glucose (-31 %) were the highest changes detected. A marked decrease in glucose and fructose was also quantified in strawberries treated with 40 % CO_2. Apart from acting as energy sources and osmoregulators, sugars serve as hydroxyl scavengers in response to oxidative stress and in stabilizing membranes, as well as fulfilling other versatile functions (Couée et al. 2006). Moreover, for glucose and fructose they serve as a substrate for sorbitol and mannitol, respectively. Compared to reducing sugars, these sugar alcohols are chemically less reactive. The maximum absolute value detected for these polyols was significantly lower than the levels of *myo*-inositol that reached values of 581.9 µg/g FW at harvest (Table 1). From this initial level there was a significant decrease in fruit stored without added CO_2 (43 %), which was milder in 40 % CO_2-treated fruit (34 %) and only 8 % in the case of 20 % CO_2-treated fruit. The amount of *myo*-inositol quantified in fruit treated with 40 % CO_2 significantly increased after transfer to air, during the recovery from damage induced by excessively high CO_2 levels. Our data are consistent with a model in which the possible protection afforded by this cyclitol may protect the fruit from severe storage conditions. Moreover, *myo*-inositol plays important structural and signaling roles in animal and plant cells and in the environment (Irvine, 2005), being involved in the phosphate cycle of terrestrial and freshwater ecosystems (Turner et al. 2002). The amount of free *myo*-inositol detected here was significantly higher than that quantified in *Fragaria x ananassa* cv. Camarosa (Bodelón et al. 2010) or from *Fragaria vesca* from the first inflorescence (Blanch et al. 2012). Moreover, when comparing carbohydrates during Camarosa growth under two geoclimatic conditions, no significant differences were reported in the soluble carbohydrate at the two locations, except for inositol (Macías- Rodriguez et al. 2002). From the above, it would seem that interaction with the environment has a direct influence on *myo*-inositol levels. Moreover, the ability of this fruit to maintain high levels of *myo*-inositol is consistent with a possible protection afforded by this cyclitol. The presence of sorbitol and/or mannitol follows a strong taxonomical and ecotypic pattern, sorbitol being a major constituent of the leaves and

fruit of many species from families such as *Rosaceae* (Gao et al. 2003). In Mara des Bois, the sorbitol

content (35.79 µg/g FW) did not change markedly in the different storage conditions compared to the content in fruit at harvest, with the highest content found in 20 % CO_2-treated fruit (+15 %). The low quantities of mannitol (1.8 µg/g FW) only increased in fruit stored without added CO_2, suggesting that interconversion of fructose in mannitol is, to a certain extent, modulated under severe low temperature storage. Polyol accumulation has been correlated with osmoprotection, serving as a reserve carbon source, as well as promoting protection against salt and photoxidative stress (Williamson et al. 2002; Merchant and Richter 2011) however their mechanistic role in fresh fruit remains unclear.

The thermodynamic parameters of polyols

of unfrozen water (g H$_2$O/g solute) in mixtures of these individual polyols in water. These polyols differ in the number of hydroxyl groups and also in their chemical structure. The alcohols, sorbitol and mannitol are chemically reduced forms of the glucose or fructose, with open chains, while *myo*-inositol is a cyclic cyclohexane compound. When the heat of fusion of ice (ΔH_f) measured from aqueous solutions of D-sorbitol (A), D-mannitol (B), and *myo*-inositol (C) was plotted against its concentration (weight fraction, wf, %: Fig. 2), the latter polyol displayed a linear dependence on concentration in this range (Williams et al. 2002; Mc Fadden, 2003; Richter 2011) however their mechanistic role in fresh fruit remains unclear.

In order to understand the possible mechanisms by which polyols exert a protective effect with respect to water status, we analyzed the ability of the polyols quantified in strawberries (mannitol, sorbitol and *myo*-inositol) to modify water behaviour, evaluating the amount of unfrozen water in mixtures of these polyols in water. These polyols differ in the number of hydroxyl groups and also in their chemical structure. The alcohols, sorbitol and mannitol are chemically reduced forms of the glucose or fructose, with open chains, while *myo*-inositol is a cyclic cyclohexane compound. When the heat of fusion of ice (ΔH_f) measured from aqueous solutions of D-sorbitol (A), D-mannitol (B), and *myo*-inositol (C) was plotted against its concentration (weight fraction, wf, %: Fig. 2), the latter polyol displayed a linear dependence on concentration in this range (Williams et al. 2002; Mc Fadden, 2003; Richter 2011) however their mechanistic role in fresh fruit remains unclear.

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ordering decreases in the presence of polyols (Dipaola and Belleau 1977), these compounds may disrupt

the surrounding water molecules, thereby restricting the ability of water to adopt a perfect network structure, and this could influence the maintenance of the cell integrity at aqueous interfaces. A water vapour absorption mechanism in arthropods by accumulation of *myo*-inositol and glucose has been reported by Bayley and Holstrump (1999). These authors also indicated that this type of water vapour absorption confers the capability of meeting the water requirements of a terrestrial arthropod under prolonged drought stress. In spite of the high water binding capacity of *myo*-inositol, it was clearly lower than that of the short-chain FOS (Blanch et al. 2012) but higher than that of sucrose (Furuki 2002 and references therein). Considering the above thermodynamic properties of *myo*-inositol and FOS, we suggest consider that fruit storability and preparedness for environmental change may be reflected in the accumulation of these carbohydrates at harvest.

Short-chain FOS (3-5 sugar moieties)

The profiles of FOS with less than five units were generated from strawberries at the end of treatment with 0, 20 or 40 % CO_2 and after exposure to air for one day. 1-kestose [1^F-(1-β-D- fructofuranosylsucrose)], nystose [6^G (1-β-D-fructofuranosyl)$_2$ sucrose] and kestopentaose [1^F-(1-β-D- fructofuranosyl)$_3$ sucrose] were identified and quantified by HPAEC-PAD (Table 2). 1-kestose constitutes the main FOS, reaching values of 84.6 µg/g FW in freshly harvested fruit, 17 times that found for nystose, the next most abundant FOS. Whereas no significant differences were observed in 20 % CO_2- treated fruit with respect to fruit at harvest, the amount of 1-kestose markedly decreased in fruit stored without added CO_2 and in 40 % CO_2-treated fruit, reaching values of 51.8 and 45.4 µg/g FW, respectively. Interestingly, the levels of 1-kestose in 40 % CO_2-treated fruit were restored after exposure to air, with an increase in 1-kestose of 53 %. The fructose/1-kestose and sucrose/1-kestose ratio also changed after transfer to air, suggesting that conversion of sucrose to FOS did seem to occur during the recovery from excessively high CO_2 levels. Apart from the well-known health benefits for human, there are many positive aspects related to the presence of fructans in plants (Livingston et al. 2009; McIntyre et al. 2012; Dominguez et al. 2014). They provide protection from the damage caused to cytosolic proteins and cell membranes by severe low temperature (Hoekstra et al. 2001). Indeed, it has been reported that fructans can interact strongly with cell membranes through direct hydrogen bonding with phosphatidylcholine lipid bilayers (Hincha et al. 2000; Vereyken et al. 2001). A previous DSC analysis

revealed that 1-kestose, nystose and kestopentaose, exhibit a very high water-binding capacity, with

values of 17.5, 18.9 and 29 mol H_2O/mol solute, respectively (Blanch et al. 2012). Furthermore, the aforementioned PCA (Fig.3) showed that FOS (nystose and 1-kestose) and bound water content are more closely related and they were located in the positive part of PC2. It is reasonable to assume that hydrogen bonds created between water molecules in close proximity to FOS could contribute to the increase in bound water fraction.

Cellular integrity, air space content and weight loss

The LT-SEM micrographs of fractured tissues (Fig. 4) revealed an excess of a surrounding aqueous solution in tissues treated with 40 % CO_2 in part linked to wall disassembly and degradation (Fig. 4D). However, this excess aqueous solution in tissues treated with 40 % CO_2 partially disappeared after exposure to air (Fig. 4E). Translucency is a physiological disorder of unknown cause that occurs when the cell-free spaces become full of liquid, damaging the tissues and giving them a transparent or crystalline aspect. In melon, translucency is diminished by the use of environments rich in CO_2 (15 kPa) (Portella and Cantwell 1998). In micrographs of strawberries treated with 20 % CO_2, empty intercellular gas spaces and a well defined cellular structure was evident at the end of the treatment (Fig. 4B). The structural characteristics visualized were confirmed by our observation that whole fruit treated with 20 % CO_2 exhibited a significantly higher proportion of air space than those exposed to 40 % CO_2 (Fig. 4, inset 1). In fact, fruit treated with 20% CO_2 exhibited the highest proportion of air space at the end of the 3 days of treatment. The connection between the significantly greater percentage air space in fruit exposed to 20 % CO_2 treatment may have functional implications on gaseous diffusion, although this has yet to be determined. It has been stated that an increase in the air space in fruit is expected to lead to easier cell separation and a change in texture (Hatfield and Knee, 1988). It is well known that cell separation, which permits spatial displacement of two adjacent cells, is an important event for fruit softening (Roy et al., 1997). Interestingly, our data showed that air space percentage observed in 20 % CO_2-treated fruit reverted after transfer to air and that this percentage did not increase by increasing the concentration of CO_2. Furthermore, LT-SEM imaging confirmed this increase in air space, specifically, in 20 % CO_2- treated fruit. Treatment with 40 % CO_2 was associated with the lowest rate of weight loss assessed by measuring the weight reduction over the 3-day period (Fig. 4, inset 1). Our data indicate that the reported decrease in bound water content in 40 % CO_2-treated fruit, in part due to the conversion of bound to free

water, could in turn pull water from adjacent cells, thereby generating extra water in the extracellular

spaces that restrains water loss in these fruits. In accordance with this, Wardlaw (2005) suggested that apoplastic water should not be ignored in plant water studies.

Conclusions

The degree of tolerance to severe storage conditions in Mara des Bois strawberries (*Fragaria vesca*) is associated with marked changes in water status, connected with the availability of reserve carbohydrates. Our results show that the decrease in bound water is linked to stress and consequently the re-establishment of initial levels is associated with the ability to recover from unfavourable high CO_2 levels. Evidence for the preferential protection afforded by storage plus 20 % CO_2 was obtained by demonstrating a predominant accumulation of bound water and carbohydrates, with a special emphasis on 1-kestose and *myo*-inositol, in such fruit. The ability of these compounds to reorganize water-hydrogen bonding networks might be the key factor involved in the stabilization of macromolecule aqueous interfaces. These CO_2-driven changes in water status would allow fruit to retain water in the cell more efficiently. Such a beneficial effect is probably more important in the case of strawberries, because these fruit tissues can be easily damaged due to a lack of external protection and softness.

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Figure captions

Fig.1. Changes in the content of unfreezable (bound) water fraction (g/g dry weight) and in the total water content (g/g dry weight) in strawberries at harvest (control), after 3 days of storage at 0 ºC in 0 %, 20 % or 40 % CO_2 and one day after exposure to air. Dark bars indicate bound water content. The data are presented as the mean ± SE of three replicates (n = 6), and the letters indicate significant differences (P < 0.05).

Fig.2. Relationship between heat of fusion of ice, H_f and the rate of increase in the concentration of D-sorbitol (A), D-mannitol (B) or *myo*-inositol (C), with a correlation coefficient as high as 0.99, for each polyol studied.

Fig.3. Three-dimensional diagram of the principal component analysis (PCA) of 6 strawberry treatments and 10 variables. Percentages of the first 3 principal components (PC1: 37.24; PC2: 24.62 and PC3: 22.44) explained 84.29 % of the total variability.

Fig. 4. LTSEM micrographs showing cells of fractured Mara des Bois strawberries after 3 days of storage at 0 ºC in 0 % CO_2 (A), in 20 % CO_2 (B), and one day after transfer to air (C), or in 40 % CO_2 (D) and one day after transfer to air (E). The extracellular space is indicated by double arrows and the scale bars represent 200 µm. Inset 1 shows the changes in air space and weight loss percentages of strawberries stored under the different treatments. The data are presented as the mean ± SE of three replicates (n = 6), and the letters indicate significant differences (P < 0.05).

Figure
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Fig.1

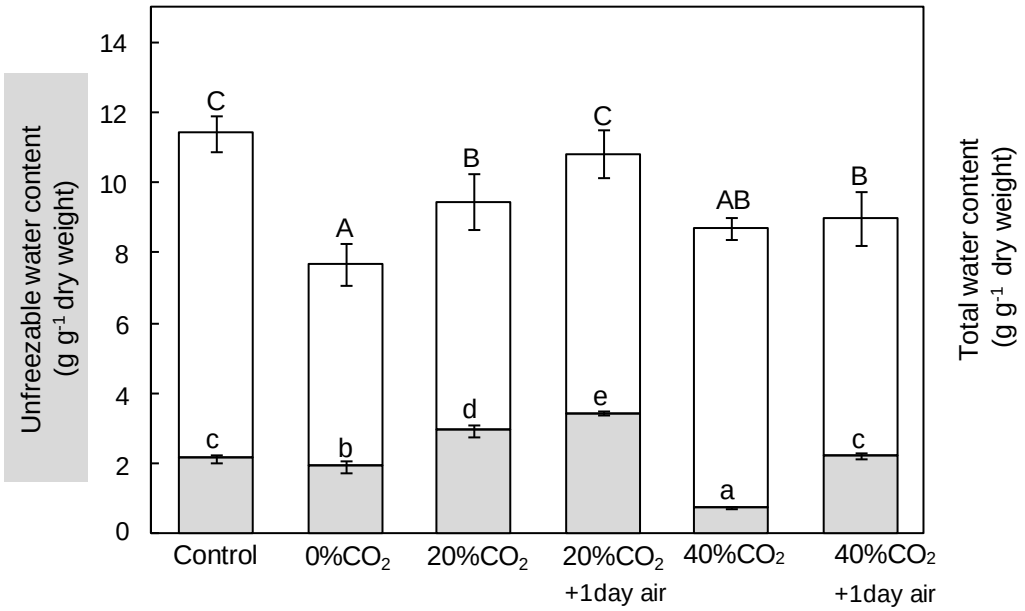
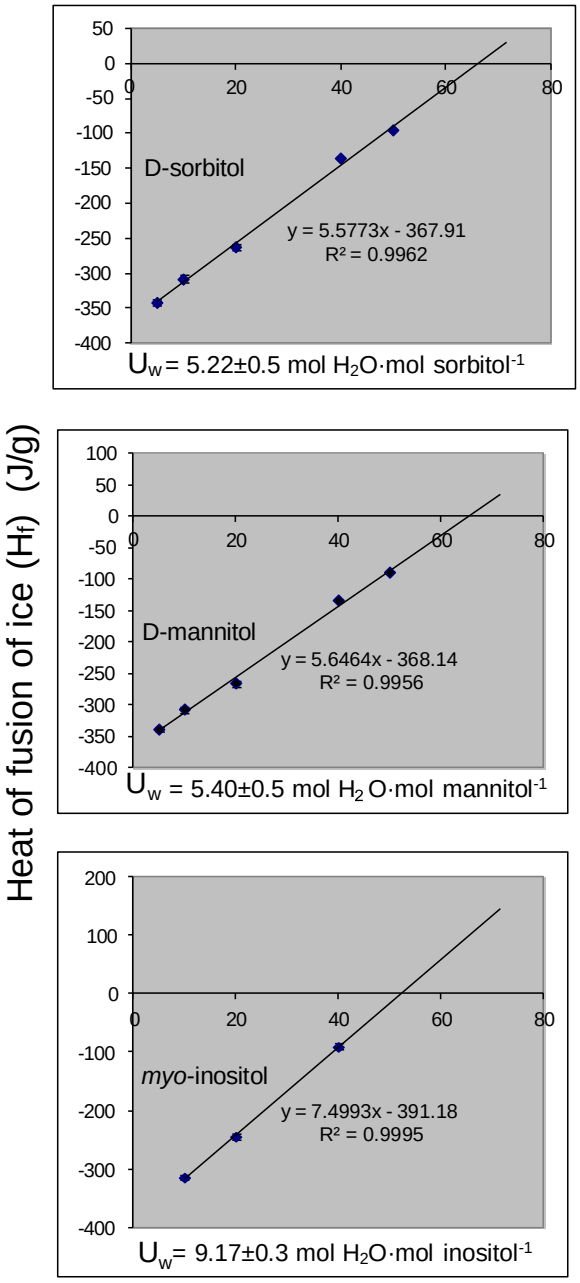


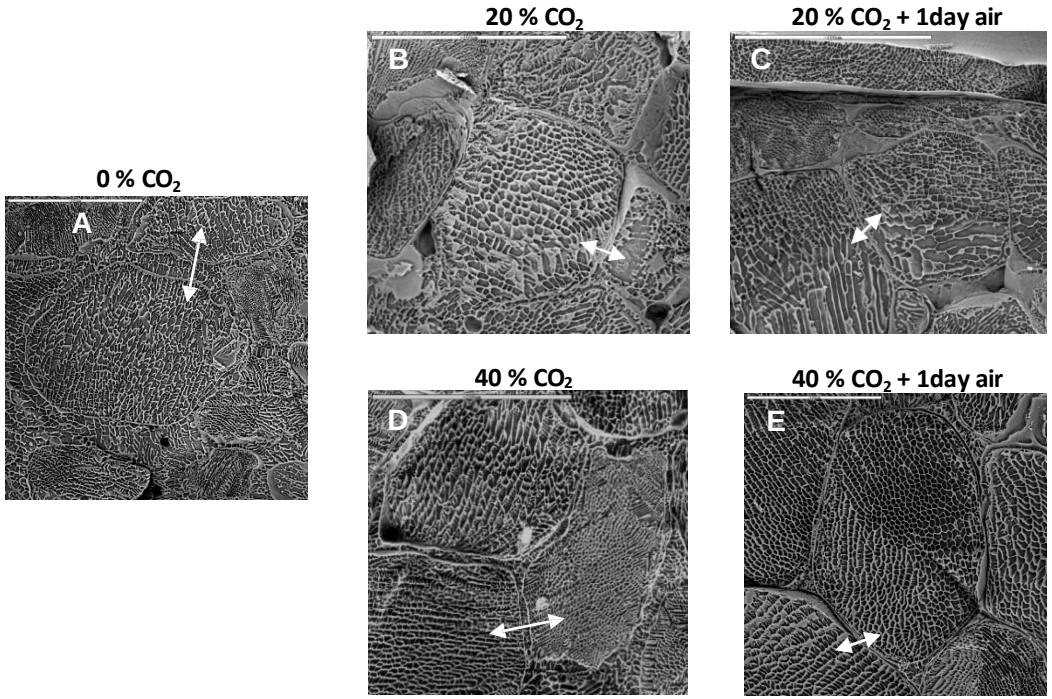
Figure
Fig. 2
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Figure

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Fig. 4



1

Treatments	Air space (%)	Weight loss (%)
0% CO_2	3.48±1.68a	0.3631±0.09b
20% CO_2	7.63±1.25b	0.3395±0.04ab
20% CO_2+1d air	2.72±0.75a	0.3350±0.02ab
40% CO_2	2.32±1.15a	0.2722±0.07a
40% CO_2+1d air	2.54±0.81a	0.3312±0.05ab

Figure
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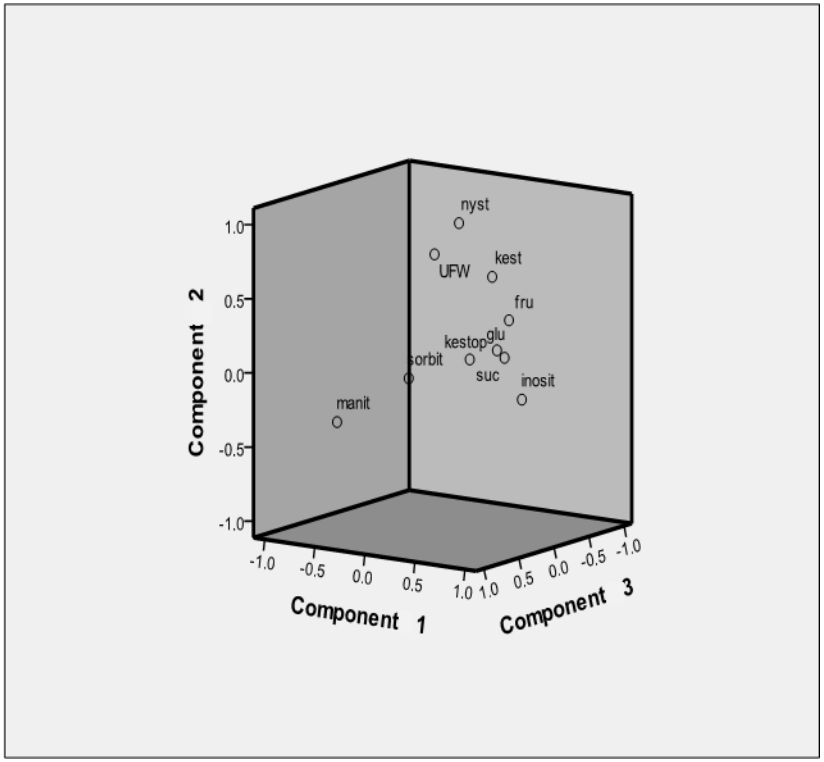
Fig. 3

Rotated Component Matrix

	Component		
	1	2	3
myo-Inositol (inosit)	.917	-.109	.180
Fructose (frut)	.835	.427	.247
Glucose (glu)	.829	.247	.409
Sucrose (suc)	.718	.147	.143
Mannitol (manit)	-.641	-.359	.591
Nystose (nyst)		.935	-.204
UFW (bound water)	.215	.835	.424
Kestose (kest)	.613	.686	.168
Sorbitol (sorbit)	.240		.828
Kestopentaose (kestop)	-.306	-.107	-.824

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalization.



Table

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Table 1: Sugars (mg/g FW) and polyols (µg/g FW) in strawberries *Fragaria vesca* cv. Mara des Bois at harvest, 3 days of storage at 0 ºC with 0, 20 % or 40 % CO_2 and after exposure to air for 1 day. In all readings oxygen concentration was kept at 20 %.

	At harvest	Treatments				
	Control	0 % CO_2	20 % CO_2	20 % CO_2+1d air	40 % CO_2	40 % CO_2+1d air
Sugars						
glucose (mg/g FW)	27.20±2.2b	18.74±1.7a	25.39±1.2b	22.27±4.5ab	19.36±2.6a	23.56±0.6ab
fructose (mg/g FW)	26.54±2.6c	17.85±1.8a	24.32±1.4c	25.59±1.9c	19.09±3.0ab	23.15±1.2bc
sucrose (mg/g FW)	18.05±0.6ab	13.80±1.2a	19.61±2.0b	17.67±1.9ab	16.93±2.6ab	18.35±2.8ab
Polyols						
myo -inositol (µg/g FW)	581.92±34.3d	332.33±17.9a	534.25±39.8cd	436.77±70.0bc	383.89±58.3ab	485.92± 12.2c
sorbitol (µg/g FW)	35.79±2.15b	33.89±0.9ab	42.29±1.2c	32.57±0.8ab	29.67±2.7a	31.97±1.6ab
mannitol (µg/g FW)	1.85±0.2b	2.90±1.1c	2.07±0.4b	1.27±0.1a	1.41±0.2a	2.11±0.1b

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

Table

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Table 2: Fructo-oligosaccharides (µg/g FW) in strawberries *Fragaria vesca* cv. Mara de Bois at harvest, 3 days of storage at 0 ºC with 0, 20 % or 40 % CO_2 and after exposure to air for 1 day. In all readings oxygen concentration was kept at 20 %.

	At harvest	Treatments				
	Control	0%CO_2	20%CO_2	20%CO_2+1d air	40% CO_2	20%CO_2+1d air
FOS						
1-Kestose	84.65±1.4b	51.88±4.7a	79.27±2.9b	85.98±3.4b	45.40±0.5a	79.44±5.16b
Nystose	4.82±0.1c	4.43±0.5c	5.02±0.2c	11.05±0.6d	3.50±0.1b	1.29±0.2a
Kestopentaose	1.05±0.1a	2.26±0.5bc	1.15±0.5ab	2.54±0.5c	0.82±0.5a	2.92±0.4c
Fructose/1-kestose	0.31	0.34	0.31	0.30	0.42	0.29
Sucrose/1-kestose	0.21	0.26	0.25	0.20	0.38	0.23

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

Abstract

It has been recognized that beneficial high CO_2 delays wilting and reduces weight loss in harvested plant products. Since strawberry fruits have high tolerance thresholds to CO_2, it is of interest to know what changes occur in fruit water status when these thresholds are reached and exceeded. Changes in the content of unfreezable (bound) water were analysed in relation to the simple sugars, polyols and fructooligosaccharides (FOS) in strawberries (*Fragaria vesca* L. Mara des Bois) stored at severe low temperature (0 ºC) with 0, 20 or 40 % CO_2. The relationship between cellular water status and these compounds was analyzed at the end of a 3 day treatments and after exposure to air for one day. Unfavourable storage conditions were associated with a significant decrease in the levels of bound water, and in simple sugars, 1-kestose and *myo*-inositol. The 1-kestose and *myo*-inositol content was enhanced when fruit treated with excessive high CO_2 (40 %) were exposure to air at the same time as there was a marked increase in bound water content and a restoration of intracellular water. The ability of fruit to modulate the accumulation of these compounds, and by extension the water binding strength, in conjunction with the pool of the bound water fraction, appears to be an important mechanism to avoid damage. Variations in the percentages of air space and weight loss produced by enhancing the CO_2 treatments during storage at 0 ºC were also analyzed.