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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