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Trisaccharides isomers, galactinol and osmotic imbalance associated with CO_2 stress in strawberries

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ABSTRACT

Treatment with high CO_2 atmospheres is effective in preventing strawberry decay and increasing firmness. Nevertheless, CO_2 stress generates energy disturbances associated with a high rate of fermentation. This study was designed to measure the impact of high CO_2 stress (3 d, 40 kPa CO_2) and its subsequent removal on fruit quality, osmotic balance and water relations. Fructo-trisaccharides isomers and raffinose were characterized and quantified by mass spectrometry (MS and MS^2). CO_2 stress removal was marked by both a rapid upsurge in the ratio of unfreezable water to total water and an osmotic adjustment prompted by the accumulation of soluble sugars, 1-kestose, raffinose and galactinol. Due to strong increase in galactinol concentrations, we propose it as a suitable biomarker for fruit having undergone stresses associated with osmotic imbalance.

1. Introduction

Strawberries are classified as a high CO_2 tolerant fruit. High CO_2 atmospheres have been used successfully to control fungal decay and to maintain or even increase firmness during storage at low temperature, without producing an adverse effect on fruit quality (Smith and Skog, 1992; Larsen and Watkins, 1995; Harker et al., 2000). However, when the CO_2 levels exceed the tolerance threshold, a series of alterations are induced such as: the flesh becoming visually blue-red and water-soaked (Ke et al., 1991), a metabolically harmful accumulation of fermentative metabolites (Ke et al., 1994) and a marked reduction in both ATP and adenylate energy charge (Blanch et al., 2015a). When sustained fermentation is maintained and ATP levels became too low to sustain the energy requirements for cell repair, oxidative damage provokes an increase in lipid peroxidation that is reflected in high levels of malondialdehyde (Blanch et al., 2015a) and a loss of membrane integrity. Our working hypothesis is that given the metabolic energy disturbances sustained by the high ethanolic fermentation that occurs at excessively high CO_2 concentrations, changes in the carbohydrate reserve that acts as an energy source would also be witnessed. Aerobic fermentation can also be induced by other stresses, which is considered an important switch regulating carbohydrate metabolism (Tadege et al., 1999).

Apart from their involvement in energy reserve mobilization, fructo-oligosaccharides (FOS) and raffinose-oligosaccharides (RFOs) are associated with protection during different kinds of plant stress (Hisano et al., 2008; Van den Ende and Valluru, 2009; Rohloff et al., 2012). However, there are significant gaps in our understanding of the protective role of FOS, in part due to their heterogeneous nature. FOS represent complex mixtures of isomers (differing in the bonds between the monomeric sugar units) and oligomers (differing in chain length). As such, it is essential to employ methods suitable for the separation, analysis and unambiguous characterization of FOS isomers.

In the case of trisaccharides, different isomers constitute the templates for further elongation that give rise to inulins, levans and neo-series fructans, such as 1-kestose, 6-kestose and 6G-kestose, respectively. They are all isomers with an identical chemical formula, which in turn means that they produce the same molecular ions. Since each isomer is an extension of sucrose, the differential partitioning of sucrose into each isomer acquires special relevance in growing programs that target greater sucrose content in strawberries.

The use of mass spectrometry (MS^n) to analyze the fragmentation patterns of molecular ions has become a key tool for the structural analysis and characterization of oligosaccharides. The three DP3 fructan isomers can be distinguished by their MS fragmentation patterns without the need for authentic standards (Verspreet et al., 2014). As such, we have employed MS and MS^2 to identify and analyze the accumulation of trisaccharide FOS isomers in strawberries.

As with FOS, an increase in RFOs is often associated with environmental stress, especially that induced by water deficit and temperature

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stress (Taji et al., 2002; Sengupta et al., 2015). The biosynthesis of the raffinose trisaccharide, the smallest RFO, begins with the formation of galactinol from uridine diphosphate galactose (UDP-galactose) and *myo*-inositol. Galactinol, a galactosyl-donor exclusive to the raffinose biosynthetic pathway, plays an important regulatory role in carbon-partitioning between sucrose and RFOs. In raffinose synthesis, uridine diphosphate glucose (UDP-glucose) must be used to produce RFO rather than for glucose and fructose production in order to sustain glycolysis (Nishizawa et al., 2008). Indeed, the levels of galactinol and raffinose increase dramatically during cold acclimatization in the leaves of the woodland strawberry (Rohloff et al., 2012), although only galactinol is clearly associated with cold tolerance (Davik et al., 2013).

Trisaccharides and simple sugars can protect cells osmotically and stabilize biological structures (Hare et al., 1998). They also alter the physicochemical properties of aqueous solutions (Miller and de Pablo, 2000; Furuki, 2002; Blanch et al., 2012), suggesting that carbohydrate-water interactions represent a significant part of their protective functions.

Thus, this study was designed to measure the impact of both high CO_2 stress and its subsequent removal on organic osmolytes, including sucrose-derived trisaccharides, and how this relates to the cellular water status and osmotic balance. For that, the levels of UDP-sugars as well as the total possible trisaccharides of both RFO and FOS family involved in sucrose metabolism have been quantified in strawberries at the end of treatment and after transfer to atmospheric CO_2 for one day. Also assessment of water relations and quality traits related to textural properties and color was assessed. Our data point out to a specific increase in solutes and sucrose related trisaccharides in fruit emerging from high CO_2 stress, associated with water status recovery. Also notable is the firmness enhancement in 40 kPa CO_2 fruit that was not reversed after transfer to atmospheric CO_2.

2. Materials and methods

2.1. Plant material

The 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) according to the Regulatory Committee on Organic Production guidelines. Ripe strawberries of uniform size were harvested in the early morning and transported to the Institute of Food Science, Technology and Nutrition within 2 h. Ripe fruit (10.4%) of total soluble solids free of defects were placed in plastic boxes (0.5 kg of fruit per box, approximately 45 per box) and 15 plastic boxes of strawberries were each placed in each 1 m^3 treatment container at 0 °C (± 0.5) and 95% RH. The strawberries were maintained for three days in atmospheric CO_2 or in presence of 40 kPa CO_2, at a constant O_2 concentration of 20 kPa and with a constant flow rate of 100 mL min^{-1}. At the end of the 40 kPa CO_2 treatment, strawberries were transferred to atmospheric CO_2 for one further day keeping at the same temperature (0 °C). The gas composition was measured twice daily using a Check Mate 9900 O_2, O_2/CO_2 headspace analyzer (Dansensor España, S.L.U.). The quality attributes of 45 strawberries from each of the lots was assessed (total soluble solids, pH, titratable acidity and external color), and the texture of another 15 strawberries was analyzed (three replicates of 5 fruit). A further 45 strawberries were removed at random and divided into three batches of 15 berries, frozen in liquid nitrogen and stored at −80 °C for additional analyses. Thus, each biological replicate analyzed here was composed of 15 pooled strawberries.

2.2. Determination of fruit quality

Firmness was assessed in three replicates from each lot by measuring the maximum shear force using a Kramer shear cell of a TA.HD Plus Stable Microsystems Analyser (Stable Microsystems Ltd; Surrey, England). For each assay, five intact strawberries of similar size (adding up 50 g) were horizontally placed in the Kramer shear cell. The crosshead speed was 5 mm/s and the firmness values were expressed in Newtons.

The soluble solids content was determined in the juice obtained from the fresh pulp of strawberries using a digital refractometer (Atago PR-101, Atago, Japan). The titratable acidity was analyzed in homogenized fruit tissues by titration with 0.1 N NaOH to pH 8.1 (Mettler DL-70, Mettler-Toledo, Spain) and the results were expressed in relation to the percentage of malic acid. Triplicate measurements were taken, at least.

External color was measured on three sides of 15 strawberries using a Konica Minolta CM-3500d colorimeter and following the CIE (Commission International de l'Eclairage) system. L^*, a^*, b^* and C^* values were measured to describe a three-dimensional color space, and they were used to calculate the chroma $(C^*) = (a^2 + b^2)^{1/2}$. L^* indicates lightness from 0 (completely opaque or black) to 100 (completely transparent or white), a positive a^* indicates redness ($-a^*$ is greenness) and a positive b^* value yellowness ($-b^*$ is blueness).

2.3. Water status

Strawberry water status was assessed by measuring fruit water potential, total water content, unfreezable and freezable water content, and the ratio of unfreezable to total water.

Fruit water potential was measured using a Dewpoint Potential Meter, WP4 (Decagon Devices, Inc., USA) according to the method of Blanch et al. (2012).

Calorimetric determinations were made using a DSC822eMettler-Toledo differential scanning calorimeter (Mettler-Toledo Inc., Columbus, OH, USA) equipped with a liquid nitrogen cooling accessory and following the method of Goñi et al. (2007). The resulting thermograms were evaluated and the heating scan was used to determine the freezing onset temperature and the melting enthalpy. The melting enthalpy of the samples and that of deionized water, together with the total water (T_W), allowed the freezable (F_W) and unfreezable water (U_W) content to be determined. For differential scanning calorimetry (DSC), at least five measurements were made on each sampling day and the U_W was calculated as:

$$U_W = T_W - F_W$$

where

$$F_W = \Delta H_{sample}/\Delta H_{purewater}$$

Both F_W and U_W were expressed relative to the dry mass and the T_W was determined after the stable weight had been obtained by oven-drying at 65 °C. The ratio of the U_W to T_W was calculated and expressed as a percentage.

2.4. Extraction and analysis of FOS and RFO trisaccharides by MS and MS^2

Frozen berry samples (approximately 3 g) were homogenized in 4 mL of ultra-pure water and the mixture was boiled under reflux in a water bath for 15 min. After cooling, the samples were sonicated for 10 min at 40 °C and the pH of each sample was adjusted to 7.5 with 10% NH_4OH. The samples were then centrifuged for 15 min at 14,700*g* and the supernatants were filtered through a 0.45 µm pore size membrane.

A preliminary identification of the compounds to be studied was made by quadrupole time-of-flight (QTOF) mass spectrometry. These analyses were performed on an Agilent 1200 series Liquid Chromatography system, with a quaternary pump G1311A and an integrated degasser G1322A, a thermostated autosampler G1367B and a thermostated column compartment G1316A. This apparatus was coupled to an Agilent 6530 accurate-mass QTOF LC–MS apparatus