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Involvement of fatty acids in the response to high CO_2 and low temperature in harvested strawberries

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ABSTRACT

Fatty acids (FAs) play important roles in membrane fluidity, energy status and the production of specific FA-dependent esters. However, little is known about the modifications in FAs induced by high CO_2 that can improve fruit quality and prevent the disorders associated with storage at low temperature. For this purpose, total, free and esterified FAs in neutral and polar lipid fractions were characterized and quantified and their relationship with straight-chain esters was examined in strawberries treated with high doses of CO_2 and different times of exposure during early storage at 0 °C. In strawberries stored without added CO_2 (T1), storage at 0 °C led to an imbalance in the saturated/unsaturated ratio of polar lipids, mainly due to the decrease in the amount of saturated fatty acids (SFA). In addition, a decrease in the amount of free polyunsaturated fatty acids (PUFA) linked to a prevalence of butanoates and hexanoates esterified to long alcohols was observed. A strong negative correlation ($P \leq 0.01$; $r \geq 0.7$) between butanoate esters and free PUFA was quantified. In contrast, the application of 2 d 20 kPa CO_2 treatment (T2), increased the amount of PUFA from the neutral and polar fractions, with a preference for α-linolenic acid (18:3n3) which results in a rise in the 18:3/18:2 ratio, and which can confer membrane stability and fluidity. Furthermore, our results also showed a strong positive correlation ($P \leq 0.01$; $r \geq 0.75$) between α-linolenic acid (18:3n3) of polar lipids and some ethyl esters that contribute mostly to the aroma in strawberries. However, 3 d 40 kPa CO_2 treatment (T4) depleted the content of FAs from all the lipid fractions in association with deficit ATP levels and an increased lipid peroxidation. These results suggest an active lipid breakdown which could play a causal role in the reported increased leakage of cellular water into intercellular air spaces in stressed-CO_2 fruit. Taken together, our results provide new insights into the beneficial effect of 2 d 20 kPa CO_2 treatment at 0 °C on the enhancement of PUFA in the polar lipid fraction and on the emission of esters other than ethyl acetate, which could improve membrane fluidity and could contribute to enriching aroma in strawberries.

1. Introduction

Short CO_2 treatments are used commercially to reduce fruit decay incidence during low temperature storage in part due to the induction of defense compounds. Some of these compounds are volatile and polymers of (epi)catechin units with the ability to inhibit microbial growth (Alejo-Armijo et al., 2018; Almenar et al., 2006; Blanch et al., 2012a; Prusky et al., 1991). In avocado, the presence of antifungal diene compounds has been reported in CO_2-treated fruit (Prusky et al., 1991) and the prevention of decay appears to be regulated by the presence of epicatechin, an inhibitor of lipoxygenase activity. Other important beneficial effects of high CO_2 treatments include an increase in flesh firmness (Larsen and Watkins, 1995) and the preservation of cellular structure and volume, linked to water status and accumulation of metabolites with an osmolyte function (Blanch et al., 2012b). However, the efficacy of high CO_2 preventing the structural dysfunctions and physiological disorders associated with storage at severe low temperature depends on the concentration of CO_2 used, as well as the length of exposure and other environmental conditions. Indeed, when excess CO_2 is applied to strawberries during low temperature it may produce a sharp decrease in bound water content, in association with intracellular liquid leakage (Blanch et al., 2015b). We hypothesize that

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alterations caused during the early stage of storage at 0 °C and by an excess of CO_2 can be explained by changes in membrane permeability and by extension, through a modification of their lipid composition. In addition to the effect of lipids on the organization of water at the membrane, FAs also play an important role in helping to maintain membrane fluidity (Disalvo et al., 2008; Lee et al., 2008). Membranes must be maintained in the fluid state to function properly at low temperature, and those containing many PUFA will be more fluid and less viscous. Modulating membrane fluidity by altering the relative proportions of different lipid classes in the lipid bilayer, and through the degree of unsaturation of the fatty acyl groups in polar lipids, represents a means of adaptation to different stresses (Lin et al., 2016; Murakami et al., 2000). More specifically, controlling membrane fluidity has been considered an important strategy to adapt to temperature stress (Murata and Los, 1997).

In addition to being essential membrane structural components, FAs and their derivatives play other important roles for energy supply and act as precursors for esters and molecules precursors involved in stress responses (Hamilton-Kemp et al., 1996; Song and Bangerth, 2003). Indeed, it has been reported that the presence of straight-chain esters is largely dependent on an adequate supply of FA-derived precursors (Schwab et al., 2008). Storage conditions like temperature, CO_2 and O_2 are critical factors in determining the predominant esters (Forney et al., 2000; Larsen and Watkins, 1995), which are quantitatively the most important group of volatile compounds responsible for strawberry aroma. In this context, the dynamic changes in FAs and their correlation with volatile esters acquires special relevance to improve flavor and aroma, an important postharvest challenge. This feature is particularly relevant to Mara des Bois strawberry, an aromatic cultivar of *Fragaria vesca* that relies on the accumulation of the esters responsible for its intense aroma. Accordingly, this cultivar represents an excellent system to analyze the effect of low temperature storage and high CO_2 concentrations on the impact of FAs, and on their relationship with volatile esters. Moreover, considering that strawberries can be subjected to intermittent periods of unfavorable conditions during the commercial application of CO_2, much work remains to better understand the metabolic alterations associated with both low temperature and high CO_2 stresses.

Therefore, we set out to analyze whether the early stage of storage at 0 °C and different high CO_2 treatments directly induce qualitative and quantitative changes in FAs in the neutral, free and polar lipid fractions of Mara des Bois strawberries, and their impact on the emission of esters, energy status and membrane oxidative damage. In addition, we try to distinguish if the reported structural and physiological alterations observed in strawberries during low temperature storage and those induced by excessive high CO_2 concentrations can be attributed to changes in the proportion of saturated and unsaturated FAs or to a specific unsaturated FAs.

2. Materials and methods

2.1. Plant material and treatments

Strawberries (*Fragaria vesca* L. cv. Mara des Bois) were grown in an organic orchard in San Sebastián de los Reyes (Madrid, Spain) according to the guidelines of the Regulatory Committee on Organic Production. Ripe, fully red strawberries from the second harvest were collected and transported to the Institute of Food Science Technology and Nutrition (ICTAN-CSIC) within two hours of harvest. The ripe fruit, free of defects, was placed in plastic boxes (0.5 kg of fruit per box, approximately 45 per box), and 15 plastic boxes of strawberries were placed in a 1 m^3 treatment container at 0 °C (± 0.5) and 95% relative humidity (RH). Four different treatments were analyzed: 3 d 0.03 kPa CO_2 + 20 kPa O_2 in N_2 (T1); 2 d 20 kPa CO_2 + 20 kPa O_2 in N_2 (T2); 3 d 20 kPa CO_2 + 20 kPa O_2 in N_2 (T3); 3 d 40 kPa CO_2 + 20 kPa O_2 in N_2 (T4), applied at a constant flow rate of 100 mL min^{-1}. The four treatments were compared with strawberries harvested at a commercial stage (CH). The CO_2 concentration was measured with a Check Mate 9900 O_2, O_2/CO_2 Headspace Analyzer (Dansensor España, S.L.U.). At the end of the 2- or 3 d sampling period, 15 strawberries were assessed for firmness, while another 45 were removed at random from each of the four treatments and divided into three batches of 15 berries. The 15 strawberries from each batch were used as biological replicates and each replicate was mixed, frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Determination of firmness

Firmness was assessed by measuring the maximum shear force using a Kramer shear cell of a TA.HD Plus Stable Microsystems Analyzer (Stable Microsystems Ltd; Surrey. England). Firmness was calculated dividing the maximum shear force value by the total weight of strawberries, according to Larsen and Watkins (1995). Strawberries were sheared at a speed of 4 mm per second at ambient temperature. For each assay, five intact strawberries were placed in the Kramer shear cell. Measurements were made in triplicate, expressing the results as N per gram of product.

2.3. Determination of phosphorylated nucleotides

ATP, ADP and AMP were quantified by HPLC performed as described previously (Blanch et al., 2015a). The adenylate energy charge was calculated according to Pradet and Raymond (1983): ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]). Measurements were made in triplicate, expressing the results as mg kg^{-1} fresh weight.

2.4. Ethanol and acetaldehyde content

Ethanol and acetaldehyde were quantified in the headspace of 5 mL of juice from three replicates of 15 strawberries without calyx in 10 mL vials. Gas Chromatography was performed as described previously (Blanch et al., 2015a) and triplicate measurements were taken, expressing the results as mg L^{-1} of juice.

2.5. Fatty acid analysis

Frozen berry samples (approximately 10 g) were homogenized in 18 mL of MeOH and 18 mL of $CHCl_3$, and the mixture was then vortexed for two min. 5 mL of deionized water was added, vortexed for 2 min and cooled over 24 h. The homogenate was then centrifuged at 1684 *g* for 15 min at 4 °C to separate the methanolic, aqueous and chloroform phases. The total lipids were recovered in the chloroform phase and 15 mL of this phase was concentrated in an evaporator under nitrogen gas at 40 °C. A SPE Bond Elut NH2 500 mg column was used for FA fractionating, eluting: neutral lipids with 10 mL chloroform:isopropanol (2:1); free FAs with 10 mL diethyl ether containing 2% acetic acid; and polar FAs, mainly phospholipids, with 10 mL methanol containing 2% HCl. To each sample, 300 µg of glyceryl tritridecanoate (1,2,3-Tritridecanoylglycerol), 50 µg of nonadecanoic acid and 700 µg of 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine were added as internal standards (purchased from Sigma, Germany and Cymit Química, Spain).

After evaporating their respective eluents, the samples were methylated for 15 min at 50 °C with 2 mL (neutral and polar FAs) or 1 mL (free FAs) of 0.5 M sodium methoxide in anhydrous methanol. Acetyl chloride in anhydrous methanol (1:10 v/v) 2 mL (to neutral and polar FAs) or 1 mL (to free FAs) was added before mixing thoroughly and heating for 1 h at 60 °C. Hexane 1.8 mL (neutral and polar FAs) and 0.8 mL (free FAs), distilled water (1 mL) and anhydrous sodium sulfate (0.2 g) were added, mixing and then centrifuging for 5 min at 277 *g*. An aliquot of the organic solvent (top layer) was collected in an amber vial for subsequent gas chromatography.