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Cell wall modifications and ethylene-induced tolerance to non-chilling peel pitting in citrus fruit

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ABSTRACT

Non-chilling peel pitting (NCPP), a storage disorder resulting in the formation of depressed areas in the peel of many citrus cultivars, is reduced by ethylene treatments. We hypothesized that this effect may be associated with biochemical changes of cell wall components. Therefore, we extracted cell wall material from albedo and flavedo tissues of 'Navelate' oranges stored in air, conditioned with ethylene ($2\ \mu L\ L^{-1}$) for 4 days and subsequently transferred to air, or continuously stored in an ethylene-enriched atmosphere ($2\ \mu L\ L^{-1}$). Uronic acids and neutral sugars were extracted into five fractions enriched in specific wall polymers namely water-, CDTA-, Na_2CO_3-, and 1 and 4 M KOH-soluble fractions. Pectin insolubilization was found in control fruit at long storage times. Ethylene treatments, alleviating NCPP, increased polyuronide solubility in the albedo and had a slight effect on the flavedo. Ethylene-treated fruit showed greater content of water-soluble neutral sugars and a larger proportion of hemicelluloses readily extractable with 1 M KOH, with a concomitant reduction in the 4 M KOH-soluble fraction. This suggests that the protective role of ethylene on NCPP is associated with an increased solubilization of the wall of albedo cells.

1. Introduction

The plant hormone ethylene is usually associated with fruit ripening regulation, senescence and it is involved in the development of some physiological disorders such as blackheart of pineapple [1], toughening of asparagus [2] and apple scald [3]. However, it is well established that it may protect other horticultural commodities from stresses causing pathological and physiological disorders and that the responses to ethylene are affected by hormone concentrations and treatment durations and also by tissue susceptibility and by the organ physiological stage [4–7].

Mature citrus fruits, in which the chloroplast to chromoplast transition has been completed, tolerate ethylene levels that may reduce infection caused by *Penicillium digitatum* [8], chilling injury [9] as well as peel collapse occurring in fruit held under non-chilling conditions (22 °C, 90–95% RH) [7]. This disorder, manifested as collapsed areas of the flavedo and part of the albedo (Supplementary data, Fig. S1), is known as non-chilling peel pitting (NCPP). A transcriptomic approach on mature oranges highlighted the molecular basis of the ethylene-induced resistance of citrus fruits to *P. digitatum* infection [8]. However, the biological basis of the ethylene-induced tolerance to NCPP in citrus fruits is still unknown [7]. Water stress may favour this disorder [10,11], but NCPP may be also developed in harvested citrus fruit held under non-stressful environmental conditions [12]. Given that fruit detachment induces fast sucrose depletion [13,14] and changes in proteins related to starvation-induced ageing in citrus peel [15], it has been suggested that the lack of carbon sources originated by fruit detachment may be involved in peel collapse [16]. Moreover, on view of ultrastructural changes, it was suggested that ethylene-induced modifications in cell wall might participate in the beneficial effect of the hormone reducing NCPP [16].

The plant cell wall provides mechanical support to individual cells, tissues, and organs. However, its perception as a solely rigid structure providing mechanical support is long past and it is now accepted that it has a key role in a plant's interactions with pathogens and responses to abiotic factors. Dynamic changes in wall polymers occur during normal development and in response to hormonal and environmental conditions [17]. These modifications may have large effects on tissue biomechanical properties

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[18,19]. In addition, wall turnover may generate biologically active oligogalacturonides (OGs) able to induce defensive and developmental responses [20] and to induce ethylene biosynthesis in climacteric fruits like tomato [21]. Solubilized pectin and/or other carbohydrate pools derived from the bulk degradation of cell wall have been considered as alternative sources for fuelling metabolism and maintaining cellular homeostasis under shortage conditions [17]. Although we have recently found that ethylene treatments reducing NCPP caused cell wall ultrastructural modifications [16], the cell wall changes induced by these treatments in the flavedo and albedo tissues of citrus fruit are still unknown. The aim of this study was to characterize compositional changes induced by ethylene treatments that reduce NCPP in cell wall of the flavedo and albedo of mature 'Navelate' (*Citrus sinensis*, L. Osbeck) sweet orange.

2. Materials and methods

2.1. Fruit material, ethylene treatments and storage

Mature 'Navelate' orange fruits were harvested by the end of February (2 months after fruit colour change) from a commercial orchard at Valencia, Spain and immediately delivered to the laboratory. A total of 540 oranges free of visual defects were sorted on the basis of uniform size and divided into three groups, each containing 180 fruit, which were stored under: (a) a continuous flow of air for up to 16 days (control); (b) a continuous flow of air after being conditioned for 4 days with air containing 2 $\mu L\,L^{-1}$ ethylene (ethylene conditioned, EC); and (c) a continuous flow of air containing 2 $\mu L\,L^{-1}$ ethylene (ethylene, E). All treatments were performed at 20 °C and 90–95% RH to avoid stressful environmental conditions. Calcium hydroxide was added to the storing trays to prevent the accumulation of respiratory CO_2. Three replicates of 20 fruit per treatment were used to determine the NCPP index. Additional replicate samples of 10 fruit per treatment and storage period were used for determining changes in cell wall materials (CWM) in both the flavedo and albedo tissues.

Flavedo and albedo samples were taken from the whole surface of fruit from each treatment at harvest, and after 4, 8, 12 and 16 days storage, and immediately frozen in liquid nitrogen, ground to a fine powder in a mill, and stored at −80 °C until use.

2.2. Estimation of non-chilling peel pitting

The NCPP symptoms were manifested as collapsed areas of the flavedo and part of the albedo (Supplementary data, Fig. S1). A visual rating scale from 0 (no damage) to 3 (severe damage), based on surface damage, was used to estimate NCPP severity. The average NCPP pitting index was calculated as previously reported [22] by using the following formula:

NCPP index = Σ(damage scale (0–3) × number of fruit in each class)/total number of fruit.

Three replicates of 20 fruits were used, and results were expressed as the mean NCPP index ± standard error (SE).

2.3. Isolation of cell walls

Five grams of albedo and flavedo tissues from either untreated (control) fruit or subjected to ethylene conditioning or ethylene treatments were placed in 95% (v/v) ethanol and subsequently were homogenized using a Polytron (Kinematica, Swiss) with 20 mL of 95% ethanol and boiled for 30 min to ensure the inactivation of cell wall modifying enzymes and the extraction of low molecular weight solutes. The insoluble material was vacuum filtered and sequentially washed with 40 mL of ethanol, 40 mL of chloroform:methanol (1:1, v/v), and 40 mL of acetone and dried at 37 °C, yielding the alcohol insoluble residue (AIR). The dried residue was weighed, and the yield of AIR was calculated. Three independent extractions were made from fruits exposed to each treatment and storage time.

2.4. Cell wall fractionation

Fractions of different cell wall components were obtained by sequential chemical extraction of the AIR as elsewhere described. [23]. Forty milligrams of AIR from each sample were suspended in 10 mL of water and stirred at room temperature for 3 h under continuous shaking, then centrifuged at 6000 × *g* and vacuum filtered. The filtrate was taken to 14 mL with water and designated as water-soluble fraction (WSF).

The residue was then extracted with 10 mL of 50 mM trans-1,2-diaminocyclohexane-tetraacetic acid (CDTA), pH 6.5, for 3 h under continuous shaking. The slurry was centrifuged and the supernatant was collected, taken to 14 mL with water and designated as CDTA-soluble fraction (CSF). The CDTA-insoluble pellet was then extracted with 10 mL of 50 mM Na_2CO_3 at 4 °C for 1 h. After centrifugation and volume adjustment (as mentioned above), the extracted solution was designated as Na_2CO_3-soluble fraction (NSF). Subsequently, the pellet was extracted with 10 mL of 1 M KOH at 4 °C for 1 h under continuous shaking. After centrifugation, the supernatant was adjusted to 14 mL with the addition of distilled water and designated as 1 M KOH-soluble fraction (1KSF). Finally the pellet was re-extracted with 4 M KOH to yield the 4 M KOH-soluble fraction (4KSF). Samples of the different fractions obtained were assayed in duplicate for uronic acids (UA) and neutral sugars (NS) contents as described below. The residue after extraction with 4 M KOH was subjected to three successive washes with ethanol 50% (v/v) in order to remove the KOH, leaving a residue which was dried at 60 °C, weighed and designated as α-cellulose.

2.5. Uronic acids

The UA contents were measured according to Blumenkrantz and Asboe-Hansen [24]. Aliquots of the different cell wall fractions were poured into test tubes and taken to 200 μL with water. Subsequently, 1 mL of 98% (w/w) H_2SO_4 containing 75 mM sodium borate was added in an ice water bath. Samples were shaken and incubated at 100 °C for 10 min. After boiling, the reaction mixtures were cooled in a water ice bath and 20 μL of 0.15% (w/v) *m*-phenylphenol in 0.5% (w/v) NaOH were added. The mixture was gently mixed and 300 μL of each sample was loaded in 96-well plates and the absorbances at 520 nm were measured in a plate reader (model Infinite 200 PRO, Tecan GmpH, Austria). The calibration curve was established using galacturonic acid in the range of 0–50 $\mu g\,mL^{-1}$ and results were expressed as milligrams of galacturonic acid equivalents per gram of AIR. Three independent samples were analysed for each treatment and storage condition and measurements were done in duplicate.

2.6. Neutral sugars

Anthrone method in 96-well plate format was used to measure NS [25]. Aliquots from the different cell wall fractions were pipetted into test tubes and diluted to 500 μL with distilled water. Subsequently, 1 mL of 2 $g\,L^{-1}$ anthrone (in 98%, w/w H_2SO_4) was added in a water-ice bath. The samples were then incubated for 10 min at 100 °C. The reaction mixtures were cooled in a water-ice bath; 300 μL of each sample was loaded in 96-well plates and the absorbances at 620 nm were measured. The calibration curve was established using glucose in the range of 0–30 $\mu g\,mL^{-1}$. Three independent samples were analysed for each treatment and