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# Ultrastructural and Histochemical Analysis Reveals Ethylene-Induced Responses Underlying Reduced Peel Collapse in Detached Citrus Fruit

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*ABSTRACT* Fruits from many citrus cultivars develop depressed areas in the flavedo (outer part of the peel) and albedo (inner part) following detachment. Although ultrastructural analysis may provide important information about multiple plant responses to stresses and external stimuli at the cell and tissue levels, and despite the proved efficacy of ethylene in reducing peel damage in citrus fruit, cytological responses of this horticultural crop to protective ethylene concentrations have not yet been reported. We show that applying high ethylene levels (2 $\mu L\ L^{-1}$ for 14 days) causes sublethal stress as it favored the alteration of cuticle, vacuole, middle lamella and primary wall, especially in the albedo cells, but reduced peel collapse in detached mature "Navelate" oranges (*C. sinensis*, L. Osbeck) held under nonstressful environmental conditions (22°C and 90−95% RH). Ethylene did not induce relevant changes in lignification but favored the deposition of pectic exudates and the release of sugars from degradation of cell polysaccharides including starch, cellulose, and pectins. In contrast, inhibiting ethylene perception by applying 1-methylcyclopropene (1-MCP) reduced these ethylene-related responses and favored degradation of cell membranes and peel damage. The overall results reflect that mature oranges tolerate high ethylene levels that might favor the activation of defense responses involving oxidative-stress related mechanisms and recycling of nutrients and carbon supply to enable cells to sustain respiration and cope with carbon deprivation stress caused by detachment. *Microsc. Res. Tech. 74:970–979, 2011.* 

## INTRODUCTION

Mature citrus fruits produce small amounts of ethylene and lack an autocatalytic rise in their production. However, ethylene production increases markedly following different biotic and abiotic stress conditions in citrus fruit (Alférez et al., 2003; Martínez-Téllez and Lafuente, 1997). This plant hormone may stimulate senescence but it also plays a protective role against stresses in plants, fresh fruit and vegetables (Saltveit, 1999). Various reports show that ethylene application reduces peel damage caused by stress conditions in citrus fruit (Lafuente and Sala, 2002; Lafuente et al., 2004), but its impact on peel ultrastructure of mature citrus fruit and the potential implications for reducing peel collapse remain unknown. In other fruits, it has been shown that ethylene may activate cell wall hydrolytic enzymes, while cell wall deterioration may be involved in the development of tissue alterations manifested as fruit softening, gelling and mealiness but also as pitting (Han et al., 2006; Lurie and Crisosto, 2005). However, ethylene-induced cell wall structure loosening might not necessarily be the causal factor of physiological disorders (Karakurt and Huber, 2004). The effect of ethylene on cell wall hydrolytic enzymes and on other physiological responses varies among fresh fruits and vegetables and depends on tissue sensitivity and hormone levels (Saltveit, 1999). A comprehensive transcriptomic study performed on mandarin fruit, harvested before color break and treated with high levels (100 $\mu L\ L^{-1}$) of ethylene, revealed that the number of genes involved in cell wall modification down-regulated by the hormone is higher than the number of up-regulated genes (Fujii et al., 2007). It is also noteworthy that ethylene may stimulate biochemical changes, such as the synthesis of lignin-/suberin-like materials, which are essential for cell wall strength in plants (Hatfield and Vermerris, 2001), though the relationship between ethylene and cell wall strengthening in citrus fruit remains almost unknown.

Additional Supporting Information may be found in the online version of this article.

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Environmental factors such as chilling temperatures or water stress may trigger peel disorders in fruit from many citrus cultivars, which are known as chilling injury and nonchilling peel pitting, respectively. Though water stress caused by rehydration of previously dehydrated fruit is an important factor in favoring peel collapse in citrus fruit showing nonchilling peel pitting (Agustí et al., 2001; Alférez et al., 2003), this physiological disorder also occurs in harvested citrus fruit held under nonstressful environmental conditions that do not affect cell water status (Cajuste et al., 2010). This suggests that lack of carbon sources and availability from the parent-plant originated by fruit detachment may lead to peel collapse. Important advances have been made in understanding metabolic control of the carbohydrates pool in response to stresses such as changes in carbon supply and environmental factors that may favor cell energy shortage both in photosynthetic tissues and in detached nonphotosynthetic tubers that contain high levels of carbon respiratory sources (Geigenberger et al., 1994; Smith and Stitt, 2007). However, we know little about the adaptive mechanisms required to balance carbon and energy demand to fuel respiration, biosynthetic activity and cell survival in detached nonphotosynthetic sink organs or plant tissues with fewer carbon sources, such as the peel of mature citrus fruit.

Conditioning mature fruit with ethylene is effective in reducing the incidence of peel collapse in citrus cultivars susceptible to nonchilling peel pitting, though the basis of the efficacy of the hormone is still poorly understood (Lafuente and Sala, 2002). Ultrastructural and histochemical studies are important tools to provide information about stress-related mechanisms and new diagnostic perspectives in plants as they provide a holistic approach and allow differential location of particular responses at the cell and tissue level (Asensi-Fabado et al., 2010; Günthardt-Goerg and Vollenweider; 2007). Electron microscopy of sections from collapsed areas of water-stressed citrus fruit showing nonchilling peel pitting revealed that cells of the transitional zone between the outer (flavedo) and inner (albedo) part of the peel are affected first and show reduced cytoplasm and twisted and squashed walls (Agustí et al., 2001). Examination of epicuticular wax by scanning electron microscopy also suggests that ethylene may induce structural changes in surface wax that might be related to the formation of new waxes (Cajuste et al., 2010). Nevertheless, the structural and histochemical characterization of the albedo and flavedo responses to ethylene levels that noticeably reduce peel collapse in mature citrus fruits has never been performed.

The aim of this work was to examine the effect of ethylene, and of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, on structural and histological characteristics of both flavedo and albedo tissues of mature "Navelate" (*C. sinensis*, *L. Osbeck*) oranges in order to gain an insight into the anatomical responses and tissue sensitivity for reduced peel collapse induced by the hormone. Fruits of this citrus cultivar were used because of its susceptibility to develop nonchilling peel pitting. The ethylene levels were selected according to their efficacy in reducing this disorder and the fruits were kept at constant 22°C and 90–95% RH to avoid stressful environmental conditions.

## MATERIALS AND METHODS

### Plant Material and Treatments

"Navelate" sweet orange (*C. sinensis*, *L. Osbeck*) fruits were harvested at the mature stage (two months after color change) at a commercial orchard in Valencia, Spain. They were immediately delivered to the laboratory, where those showing visual defects were discarded, and they were divided into three groups which were exposed to the following treatments: (a) continuous flow of air; (b) continuous flow of air after being conditioned for four days with air containing 2 $\mu L\ L^{-1}$ ethylene; (c) continuous flow of air containing 2 $\mu L\ L^{-1}$ ethylene. All the treatments were performed in an equal number of fruits in the presence of $Ca(OH)_2$ to avoid the accumulation of respiratory $CO_2$. The fruits were held at constant 22°C and 90–95% RH during the whole experimental period to avoid stressful environmental conditions. To further understand the role of ethylene in reducing nonchilling peel pitting, the fruits were also treated with 1-MCP, an inhibitor of its action, before being transferred to air. To that end, the freshly harvested fruits were divided at random into two lots containing an equal number of fruits. Fruits included in the first lot were treated with 1 $\mu L\ L^{-1}$ 1-MCP for 12 h at 22°C in a sealed container in the presence of $Ca(OH)_2$; the fruits of the second lot were treated like those of the first lot but incubated in air instead of 1-MCP and used as control. The 1-MCP was donated by Rohm and Haas Spain, S.A, and was prepared following the manufacturer's instructions. After fumigation with 1-MCP, fruits from both lots were ventilated for 2 h at 22°C and transferred to a continuous flow of air at 90–95% RH and 22°C. In addition, three lots of fruit were held in air at 2, 12, and 22°C to examine the effect of lowering the temperature on fruit respiratory rate as compared with the development of peel collapse.

### Evaluation of Nonchilling Peel Pitting Damage

The symptoms of nonchilling peel pitting were collapsed areas of part of the albedo and flavedo that became brown with time. Fruits from each treatment were individually rated for the presence or absence of peel damage, and the incidence of this physiological disorder was expressed as the percentage of fruits showing damage. A visual rating scale from 0 (no damage) to 3 (severe damage) based on surface damage was used to calculate the average peel pitting index (Lafuente and Sala, 2002). Three replicates of 10 fruits were used, and the results are the means of the three replicates ± standard error (SE).

### Textural Analysis

Tissue cohesiveness was determined by performing a texture profile analysis (TPA) (Fiszman et al., 2005). Instrumental parameters were measured with a TA-XT2 Texture Analyser (Stable Micro Systems, Godalming, UK) using the Texture Expert software (Version 1.0) from Stable Micro Systems Ltd Software. A TPA to 50% of the initial height of the sample was carried out on the samples using a 35 mm diameter flat aluminum disc. A test speed of 1.0 mm $s^{-1}$; a resting time of 5 s and a trigger force of 20 g were the selected settings. Measurements were performed on peel discs taken

with a 1.5 cm diameter cork borer from the equatorial zone of 12 fruits (one disc per fruit), placing the albedo facing down. Cohesiveness was calculated by the texturometer software and expressed as the area (arbitrary units) between the first and the second peak. Results are the mean of twelve replicates, and mean comparison using Tukey's test was performed to determine whether the cohesiveness values of fruit peel exposed to the various treatments were significantly different ($P \leq 0.05$).

### Light Microscopy

Healthy peel sections from five individual fruits per treatment and storage period were fixed in situ with F.A.A. (Formaldehyde:ethyl Alcohol:glacial Acetic acid). Healthy tissue areas were taken to identify the changes related to ethylene and 1-MCP, because changes associated with damage could partially mask changes related to these treatments. After washing with phosphate buffer 0.1 M (pH 7.4), the samples were dehydrated in ethanol series (50%, 70%, 96%, followed by 2 × 100%) and infiltrated in Histosec paraffin (Merck) at 56–58°C. Isoamyl acetate was used as an intermediary solvent between the ethanol and the paraffin, the infiltration time was 45 min (60°C), and the blocks were cut into 8-μm sections with an Anglia Scientific microtome. After removing the paraffin with xylene and tissue rehydration in ethanol series, the samples were dyed with safranin and fast green. Other fragments were included in medium grade LR-White (London Resin Co., London, UK) acrylic resin. Semifine cuts (1.5 μm) were made with a Sorvall MT 5000 Ultra Microtome (Girald-Dupont, Wilmington, DE, USA) with glass blades (45°) obtained from a special glass (6.4 mm glass strips from Leica) in a knifemaker (Reichert-Jung, Vienna, Austria). These samples were dyed with toluidine blue at 1%, safranin-fast green and Lugol's solution. An Olympus Provis AX 70 light field microscope was used to observe the preparations, and the pictures were taken with an Olympus Camedia C-2000 Z camera.

### Transmission Electron Microscopy

Samples were fixed in 4% glutaraldehyde buffered with 100 mM sodium phosphate buffer, pH 7.2, for 10 h at 4°C. After three washings in sodium phosphate buffer, samples were postfixed in buffered 2% $OsO_4$ for 2 h and subsequently washed with 100 mM sodium phosphate buffer, pH 7.2, for 15 min. Then they were dehydrated in ethanol series and embedded in LR-White medium-grade acrylic resin (London Resin Co.), as detailed for semithin sections. Ultra-thin sections (70 nm thick) were obtained with an Ultratome Nova LKB Bromma ultramicrotome provided with a DIATOME Ultra 45° diamond knife. Ultra-thin sections were stained with 10% uranyl acetate for 30 min and 0.1% lead citrate for 5 min and observed with a JEOL JEM-1010 (100 kV) transmission electron microscope (JEOL Ltd., Tokyo, Japan). Preparations were photographed with a MegaView III digital camera and image acquisition was performed by using AnalySIS software provided by the Microscopy Service at Universidad Politécnica de Valencia, Spain.

*TABLE 1. Effect of ethylene (2 μL $L^{-1}$) on the incidence of peel damage (%) and cohesiveness (arbitrary units) in mature fruit stored at 22°C and 90–95% RH*

| Treatment | Damage incidence (%) | Cohesiveness |
|---|---|---|
| Freshly harvested (0d) | 0.0[a] | 0.544[a] |
| 4d air | 0.0[a] | 0.551[a] |
| 4d ethylene | 0.0[a] | 0.520[a] |
| 14d air | 75.0[c] | 0.555[a] |
| 4d ethylene + 10d air | 39.6[b] | 0.524[a] |
| 14d ethylene | 27.8[b] | 0.523[a] |

Values labelled with the same letter are not significantly different at the 5% level.

### Respiratory Rate Determination

The respiratory rate of fruits was determined by gas chromatography with TCD detector as previously described by Holland et al. (2002). Three replicates of three fruits were sealed in individual 1.5-L glass containers for 3 h at the temperatures assayed. The concentration of $CO_2$ was determined by injecting 1-mL gas samples, withdrawn from the container headspace using a syringe through a septum into a GC equipped with a 1.5 m × 2.0 mm Chromosorb 102 column from Supelco (Barcelona, Spain).

### Statistical Design

Experimental data are the mean ± SE of three replicate samples. A mean comparison using the Tukey's test was performed to determine whether mean values were significantly different at $P \leq 0.05$.

## RESULTS

### Effect of Ethylene on Nonchilling Peel Pitting and Peel Textural Properties

Irregular depressed areas affecting the inner and outer part of the peel appeared in "Navelate" oranges after four days of storage in air under nonstressful environmental conditions (22°C and 90–95% RH). The effect of ethylene on reducing the number of fruits showing peel collapse was assessed by treating the fruit with different ethylene regimes. The hormone clearly reduced the incidence of peel damage (Table 1). Only 28% of the fruit held continuously in ethylene showed damage by 14 days, while 75% of the control fruit held in air showed tissue collapse. Furthermore, conditioning "Navelate" oranges for four days with 2 μL $L^{-1}$ ethylene before transferring the fruit to air for 10 additional days, without changing temperature and RH conditions, reduced peel damage incidence from 75 to 39%. Similarly, peel pitting indexes were reduced three- to fourfold in fruit conditioned or treated continuously with 2 μL $L^{-1}$ ethylene when compared with air-treated fruit (data not shown). These indexes in the ethylene-treated fruit were very low (<0.38 on a scale up to 3). In contrast, no significant differences in peel texture, measured as cohesiveness, were found between fruit held in air and ethylene for 4 or 14 days at 22°C (Table 1). The hormone did not affect either flavedo or albedo strength as determined by puncture force or fruit firmness (data not shown).

### Effect of Ethylene and 1-MCP on Ultrastructural Changes of Peel Cells

Light (Figs. 1A and 1B) and transmission electron microscopy (TEM) micrographs (Figs. 2A and 2B) show that the anatomical organization of epidermal cells in the flavedo and of the hypodermal parenchymatous cell layers, located immediately below the epidermis, of the freshly harvested mature oranges was typical of healthy citrus fruit. The epidermal tissue was covered by a cuticle of 3–3.2 μm and consisted of a single layer of polygonal epidermal cells (15–20 μm thick), thick-walled and densely packed, with no evidence of disintegration (Figs. 1A and 2A). Hypodermal cells (Figs. 1B and 2B) were larger, isodiametric, spherical to slightly oval (10.5–16 μm), thin-walled (1–1.25 μm), and their size and intercellular spaces increased gradually in deeper tissues (Fig. 1B). Flavedo cells (Fig. 2B) contained a large vacuole compressing the cytoplasm against the cell wall. In the cytoplasm, abundant membranes of the endoplasmic reticulum and numerous amyloplasts were observed (Fig. 2B). Moreover, cell walls of freshly harvested fruit showed numerous pits (Fig. 2B, box).

After 14 days at 22°C in air, degradation of the middle lamella was evident in the flavedo, resulting in some intercellular spaces caused by cell separation (Fig. 1C). Furthermore, degeneration of primary cell wall was evidenced by a lack of uniformity in cell wall thickness both in different cells and within the same cell (Figs. 1C and 2C). Another visible change occurring in these fruits was the deposition of pectic polysaccharide substances (Figs. 1C and 1D). In contrast to freshly harvested fruit, plastids in flavedo cells from air-stored "Navelate" oranges were not distributed uniformly inside the cell, indicating alteration of the vacuole which would lead to the disorganization of cell contents (Figs. 2C and 2D). Degeneration of inner cells in the transitional zone and in the albedo was even more pronounced (Fig. 1D), with a very evident diminution of cell wall thickness and lack of middle lamella in many cells, causing substantial enlargement of intercellular spaces. The deposition of extracellular pectic polysaccharide substances here was larger and shrivelling was already evident (Fig. 1D). In this tissue, wrinkled unbound tonoplast, vacuole damage and disorganization of the cytoplasm were also observed (Figs. 1D).

Holding the fruit continuously for 14 days in 2 μL $L^{-1}$ ethylene enhanced the decomposition of the middle lamella of flavedo cell wall and cell separation (Fig. 1E) with respect to fruit maintained continuously in air (Fig. 1C). Furthermore, it favored cytoplasm disorganization and vacuole damage and increased the presence of degraded tonoplasts. Moreover, most of the cells of the transitional zone between the flavedo and the albedo of ethylene-treated fruits showed a very irregular shape with many angles and little cellulose content. The deleterious effect of treating the fruit continuously with ethylene was even more evident in the albedo cells (Fig. 1F). The intercellular spaces caused by cell separation and the intercellular deposition of pectic polysaccharide substances markedly increased in response to this treatment and cell walls were highly degraded (Figs. 1F and 2E). The treatment also caused a very evident deterioration of the cuticle (Fig. 1E) and of cellulose microfibrils, which were very disorganized in the ethylene-treated samples (Fig. 2E). Moreover, TEM analysis showed electron dense plastids (Fig. 2F), indicating lack of starch in the peel of fruit exposed to the hormone.

The inhibitor of ethylene perception 1-MCP reduced cell wall deterioration in the flavedo, the transitional zone, and in the albedo of fruit stored for 14 days in air (Fig. 1G and 1H). The cell wall was tightly packed and the middle lamella was indistinguishable from the primary walls of adjacent cells in most cases. Furthermore, plastids containing starch in the flavedo cells were distributed uniformly inside the cell (Fig. 2G, white Am), areas with degraded tonoplast or deposition of pectic substances were not observed, and vacuoles remained turgid (Fig. 2H). Disintegration of cells in the transitional zone and in the albedo (Fig. 1H) was also not observed. The primary wall of cells in these layers was thicker and uniform and stained dark blue (Fig. 1G), indicating that cellulose degradation barely occurred. The lack of deposition of substances resulting from pectin degradation was also observed in these samples.

Results obtained by TEM further showed that membranes such as those of the endoplasmic reticulum were abundant but slightly disorganized in fruit held in air for 14 days (Figs. 2C and 2D) when compared with those of freshly harvested fruits (Figs. 2A and 2B). In contrast, 1-MCP had a marked impact in promoting the degradation of membranes, such as those of the endoplasmic reticulum, which were barely detected on day 14 in the 1-MCP-treated fruits (Figs. 2G and 2H). Ethylene also affected membranes (Fig. 2F), but to a much lesser extent than 1-MCP (Figs. 2G and 2H).

The effect of ethylene on lignification of wall layers was examined by using safranin-fast green staining (see Supporting Information Fig. S1 available on line). Our results showed that safranin only stained walls of the occlusive cells in the stomata and walls of conductive cells from xylem, but no substantial differences were observed among tissue sections taken from freshly harvested fruit and from fruit stored for up to 14 days in air or ethylene (data not shown).

Using Lugol's reagent (Figs. 3A–3G), we observed that starch grain degradation occurred during fruit storage at 22°C and that it was favored by ethylene. Starch grain content in fruit treated for four days with ethylene (Fig. 3C) was slightly lower than in fruit held for the same period in air (Fig. 3B). However, no starch was detected in the ethylene-conditioned fruit when transferred to air for 10 additional days (Fig. 3E) or in fruit continuously held in ethylene (Fig. 3F), while amyloplasts were still abundant in the flavedo of nonconditioned fruit maintained for 14 days in air (Fig. 3D). Treating the fruit with 1-MCP barely affected starch grain degradation (Fig. 3G) as compared to fruit maintained in air for 14 days.

### Effect of Temperature and 1-MCP on Respiratory Rate and Peel Collapse

On the basis of the ultrastructural observations and to further understand whether the availability of carbon sources to sustain respiration and cell viability may have an impact on peel collapse in detached citrus

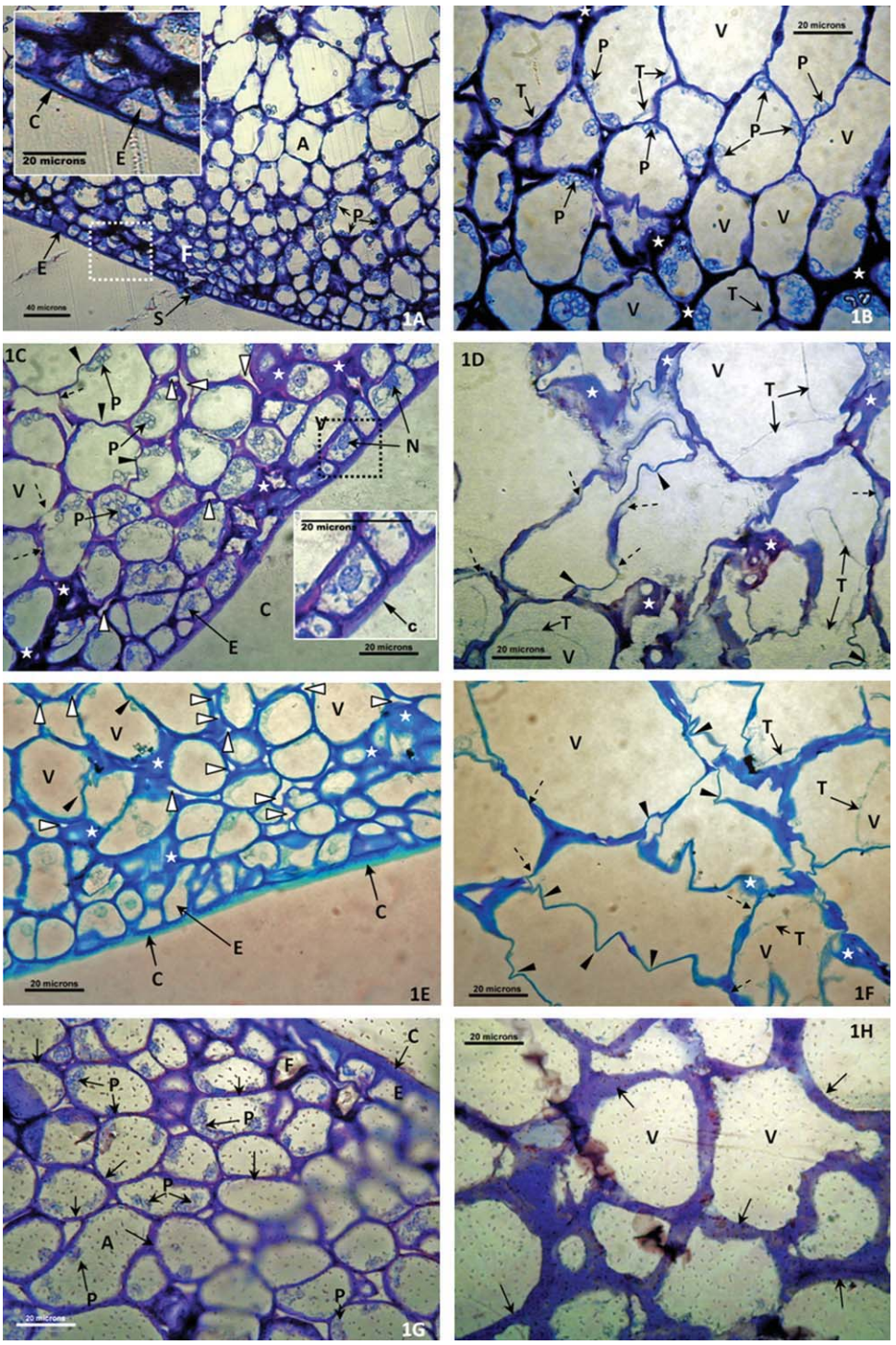


Fig. 1. Effect of ethylene and 1-MCP on peel ultrastructure of mature 'Navelate' orange fruits stored at 22°C and 90–95% RH as shown by light microscopy of semifine sections stained with toluidine blue. Figures 1**A** and 1**B**: Freshly harvested mature orange fruit. A: Epidermis (E), flavedo (F), and transition zone to albedo (A). Left upper corner: Close-up of the epidermis and cuticle layer. Bar: 40 μm. B: Detail of the transition zone to albedo. Diffuse pectinaceous substances (white stars). Bar: 20 μm. Figures 1**C** and 1**D**: Fruits held for 14 days in air, without ethylene pretreatment: C: Detail of epidermis, flavedo and transition zone to albedo showing loss of primary wall (black dashed arrows), increases in the number of intercellular spaces (white arrow heads), deposits of diffuse pectinaceous substance (white stars) and deformed walls (black arrow heads) in some cells. The cuticle (C) is thin. Bar: 20 μm. Box: detail of the epidermis with cuticle. D: Detail of the transition zone to albedo showing loss of primary wall (black dashed arrows), deposits of diffuse pectinaceous substance (white stars) and deformations of the wall (black arrow heads). Bar: 20 μm. Figures 1**E** and 1**F**: Fruits treated for 14 days with 2 μL $L^{-1}$ ethylene. E: The treatment favors loss of middle lamella, increase of intercellular spaces (white arrow heads), accumulations of intercellular pectinaceous substances (white stars), and alterations in the cell wall (black arrow heads) and cuticle (C). Bar: 20 μm. F: Detail of the transition zone and the proximal albedo. Angular deformations (black arrow heads) of the cells by destruction of the primary wall (black dashed arrows) and deposits of diffuse pectinaceous substance (white stars) are observed. Bar: 20 μm. Figures 1**G** and 1**H**: Fruits treated with 1-MCP and thereafter held for 14 days in air. G: Epidermis, flavedo and part of the transition zone to albedo. Cell walls were not altered (black arrow), cuticle was heavy (C) and amyloplasts (P) were functional. Bar: 20 μm. H: Detail of the albedo with uniform thickness walls without marked degenerate areas (black arrows). Bar: 20 μm. N, nucleus; S, stomata; T, tonoplast; V, vacuoles.

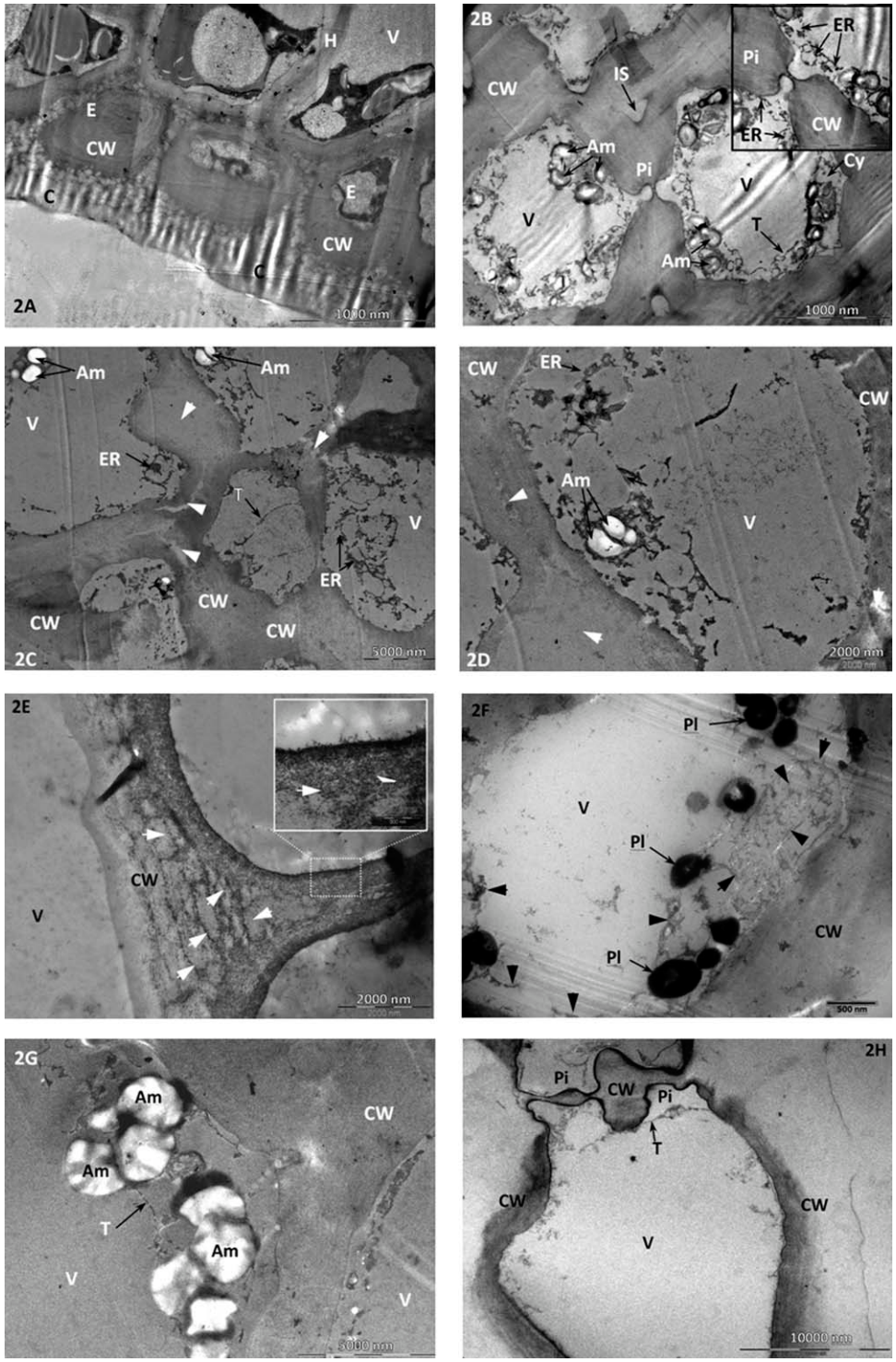


Fig. 2. Effect of ethylene and 1-MCP on peel ultrastructure of mature "Navelate" orange fruits stored at 22°C and 90–95% RH as shown by TEM of ultrafine sections. Figures 2**A** and 2**B**: Freshly harvested mature orange fruit. A: Detail of the epidermis (E) with the cuticle (C) and the hypodermal cells (H). The epidermal cells show a very thick outer tangential cell wall (CW). Bar: 10,000 nm. B: Detail of flavedo area. Cell walls (CW) were not altered. Some small pits (Pi) and intercellular spaces (IS) are shown. The tonoplasts (T) are not damaged and limit very turgid vacuoles (V) compressing the cytoplasmic (Cy) contents into the wall. Numerous amyloplasts (Am) appear in the cell periphery. Bar: 10,000 nm. Right upper corner: Close-up of a pit showing numerous membranes of the endoplasmic reticulum concentrated in the cell periphery. Bar: 5000 nm. Figures 2**C** and 2**D**: Fruits held for 14 days in air, without ethylene pretreatment. C: Detail of flavedo zone. These cells have slightly degraded cell walls (CW) and show clear signs of degradation of the middle lamella (white arrow heads). In some cells the tonoplast (T) is altered and vacuoles are partially collapsed, the amyloplasts (Am) are not evenly distributed, and the reticulum membranes (ER) are disorganized. Bar: 5000 nm. D: Detail of albedo cells. A slightly altered cell wall (CW) with degraded middle lamella (white arrows heads) is observed. Tonoplast (T) is altered, the vacuole (V) collapsed and the amyloplasts (Am) are not evenly distributed. Disorganization of the reticulum membranes (ER) was noticeable. Bar: 2000 nm. Figures 2**E** and 2**F**: Fruits treated for 14 days with 2 $\mu L\ L^{-1}$ ethylene. E: Detail of cell walls (CW) in flavedo cells. Cell walls show evident signs of degradation (white arrow heads) with cellulose microfibrils very disorganized and partially degraded. Bar: 2000 nm. Box: Detail of degraded cell wall. Bar: 500 nm. F: Cell walls (CW) in flavedo zone showing slight signs of inner membrane degradation (black arrows). Plastid (Pl) without starch. Bar: 500 nm. Figures 2**G** and 2**H**. Fruits treated with 1-MCP and thereafter held for 14 days in air. **G**: Detail of cell walls (CW) in flavedo zone. The cell wall is not altered. Tonoplast (T) is not damaged, the vacuole (V) is turgid and amyloplasts (Am) in the cell periphery are compressed against the cell wall but endoplasmic reticulum membranes are not observed. Bar: 5000 nm. H: Detail of cell walls (CW) showing pits (Pi) in albedo zone. No cell wall alterations are observed. Near pits, endoplasmic reticulum membranes are not observed. Bar: 10,000 nm.

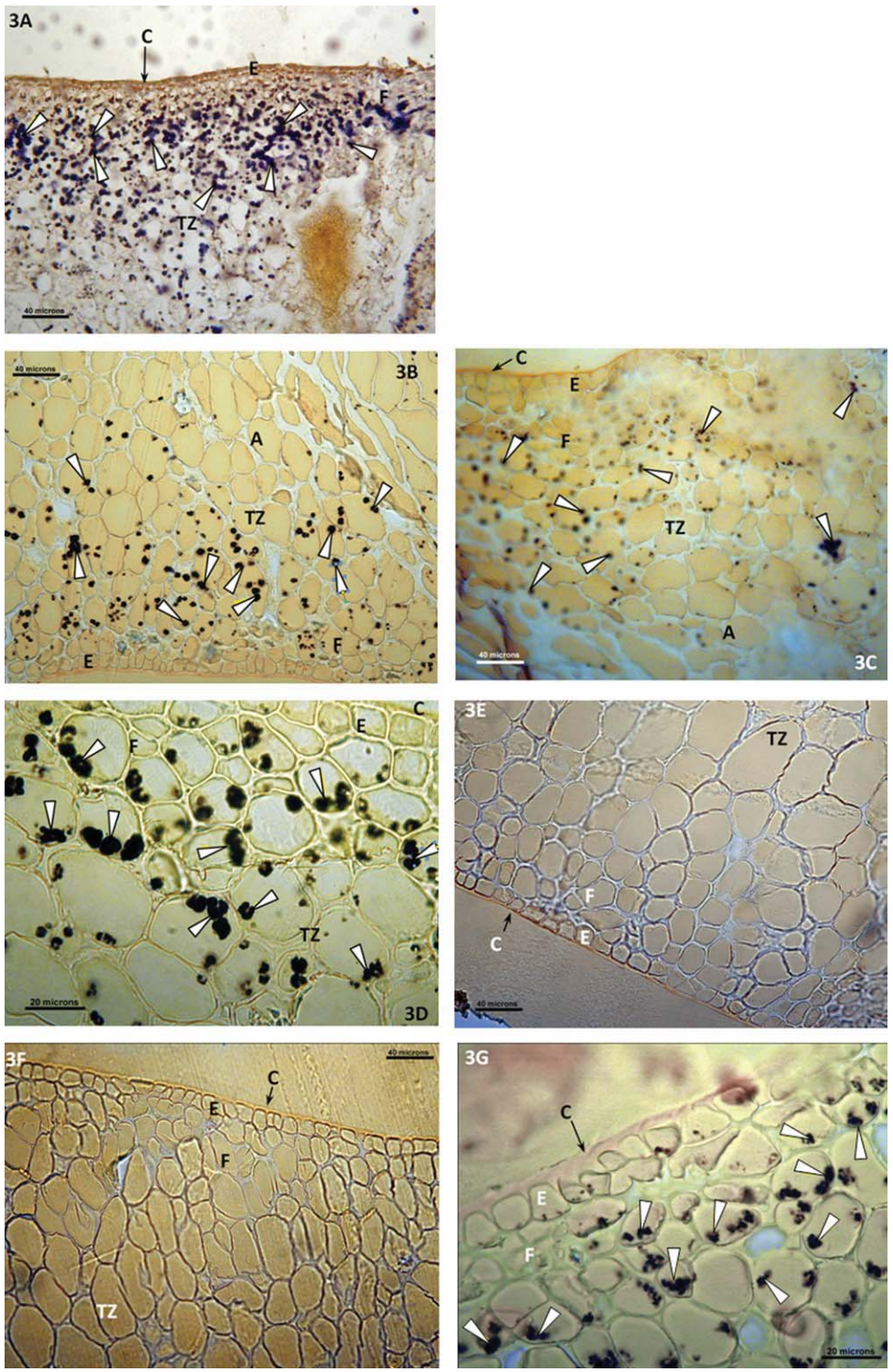


Fig. 3. Effect of ethylene (2 μL $L^{-1}$) and 1-MCP on starch grains of mature "Navelate" orange fruits stored at 22°C and 90–95% RH as shown by light microscopy of semifine sections stained with Lugol's solution. **A**: Freshly harvested mature orange fruit. Amyloplasts (white arrow heads) were of considerable size and abundant in the flavedo (F) and transition zone (TZ). Bar: 40 μm. B: After four days in air. Amyloplasts were slightly less abundant in the area of flavedo and transition zone and barely detected in the albedo. Bar: 40 μm. C: After four days of ethylene treatment. Ethylene enhanced amyloplasts degradation. Bar: 40 μm. D: Fruits held for 14 days in air. Amyloplasts (white arrow heads) are still detected in the flavedo. Bar: 20 μm. E: Fruits treated for four day with ethylene and held for 10 additional days in air. No amyloplasts were detected. Bar: 40 μm. 3: Fruits treated for 14 days with ethylene. No amyloplasts were detected either in the flavedo or in the transition zone. Bar: 40 μm. G: Fruits treated with 1-MCP and thereafter held for 14 days in air. Amyloplasts (white arrow heads) were detected in the flavedo and in the transition zone to albedo. Bar: 20 μm. A, albedo; C, cuticle; E, epidermis; F, flavedo; P, amyloplasts; Ph, phloem; S, stomata; TZ, transition zone; V, vacuoles; X, xylem. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

*TABLE 2. Effect of temperature and 1-MCP (1 μL $L^{-1}$) on the respiratory rate (mL $CO_2$ $kg^{-1}$ $h^{-1}$) and the severity of peel damage (rating scale from 0 to 3) of detached "Navelate" oranges held for 14 days in air*

| | Temperature | | | Effect of 1-MCP | |
|---|---|---|---|---|---|
| | 2°C | 12°C | 22°C | Air | 1-MCP |
| mL $CO_2$ $kg^{-1}$ $h^{-1}$ | 2.63 ± 0.11 | 5.92 ± 0.15 | 10.29 ± 0.36 | 8.85 ± 0.29 | 24.37 ± 0.57 |
| Damage severity | 0.00 ± 0.00 | 0.23 ± 0.04 | 0.53 ± 0.04 | 0.57 ± 0.08 | 1.20 ± 0.14 |

Both effects were examined in independent experiments and the effect of 1-MCP studied at 22°C. Significant differences ($P \leq 0.05$) were found among values at the different temperatures or between air and 1-MCP treated fruits.

fruit, the effect of lowering temperature and of 1-MCP on the extension of visual damage was compared. These treatments were selected because they may alter carbon consumption or demand by modifying respiratory rate in citrus fruit. As mentioned above, 1-MCP inhibits ethylene action but also increases the respiratory rate of citrus fruit (McCollum and Maul, 2007). Data in Table 2 show that the lower the temperature, the lower the respiratory rate and the severity of peel damage. In agreement with this result, 1-MCP markedly increased both the respiratory rate and peel collapse in fruit held at 22°C.

## DISCUSSION

Ethylene is usually associated with senescence, but it may protect plants and fruits of agronomic interest from stresses (Saltveit, 1999). The mechanistic basis of the ethylene-induced tolerance to stresses in fruits is still little understood. Mature citrus fruits, in which conversion of chloroplast to chromoplasts has been completed, is a good model for this purpose as they may tolerate high levels of ethylene that are very effective in reducing peel collapse in fruit held under stressful (Lafuente et al., 2004) and nonstressful environmental conditions (22°C and constant 90-95% RH) (Cajuste and Lafuente, 2007). Physiological and molecular mechanisms induced by ethylene in citrus fruit have mostly been studied in fruits harvested before color change as this hormone is mainly used for degreening purposes. Furthermore, citrus fruit responses to ethylene are influenced by concentration (Cajuste and Lafuente, 2007) and fruit physiological stage (Lisker et al., 1983). Although histochemical and ultrastructural studies may provide important information about plant responses to external stimuli at the cell and tissue level, and despite the proved efficacy of ethylene treatments in reducing peel damage in mature citrus fruit, no information is available on cytological responses of this horticultural crop to ethylene concentrations producing this beneficial effect. In view of this and the fact that microscopic analysis can lead to a better understanding of the specialized contribution of each tissue to fruit physiology, we have focused on studying changes in peel ultrastructure caused by ethylene levels that reduce peel collapse in citrus fruit and by 1-MCP, which inhibits ethylene perception.

We have shown that holding "Navelate" fruit continuously in ethylene for 14 days favored the dissolution of the middle lamella and cell separation with respect to fruit maintained in air. Furthermore, ethylene enhanced primary cell wall and vacuole damage, disorganization of the cytoplasm, deposition of pectic polysaccharide substances, and degradation of cellulose microfibrils (Figs. 1E, 1F and 2E, 2F). Nevertheless, these cellular changes, which were more marked in the albedo, were not high enough to produce significant differences in peel texture and strength properties between air- and ethylene-treated fruits and reduced visual peel damage in colored fruits held under nonstressful environmental conditions. The evident cell wall alteration and the deposition of pectinaceous exudates observed in fruit treated continuously with ethylene might be an effect of the hormone on cell wall hydrolytic enzymes, but we cannot rule out the contribution of these exudates to the beneficial effect of ethylene in decreasing loss of peel integrity in detached citrus fruit. In this regard, it is noteworthy that oligogalacturonate fragments from the pectic components may induce a broad spectrum of defense signaling pathways in plants (Farmer et al., 1991), while a signaling role for pectic oligosaccharides in the acclimation of zucchini fruit to chilling, manifested as peel damage and altered membrane permeability, has been reported (Balandrán-Quintana et al., 2002).

Another interesting observation is that applying 2 μL $L^{-1}$ ethylene for short or prolonged periods did not induce substantial changes in lignin deposition, which was very low in both the albedo and the flavedo cells. Therefore, histochemical analysis indicates that lignification as a means of cell wall reinforcement is not relevant in the protective role of the hormone in reducing peel collapse. In contrast, ethylene had an important impact on starch grain degradation, a symptom characteristic of stress and/or senescence in plants, which was especially evident after prolonged storage. Fruit detachment may cause stress because of water and nutrient deprivation. We have shown in previous works that water stress or changes in cell water and turgor potentials are not determinant factors in the development of nonchilling peel pitting and in the protective effect of ethylene in reducing visual damage in fruit held at 90–95% RH (Cajuste et al., 2007; Lafuente and Sala, 2002). This, together with the information obtained here from structural analysis, suggests that starch mobilization as well as solubilized pectin and other pools of carbohydrates derived from the bulk degradation of cell wall might act as storage reserves and participate in the effect of ethylene in reducing peel collapse by providing additional energy sources for cell homeostasis and viability (Alonso et al., 2005; Yu, 1999). Reports indicating that carbon starvation may increase the activity of hydrolases leading to released sugar residues from cell wall polysaccharides to sustain respiration and other metabolic processes (Lee et al., 2007) and that detachment causes changes in proteins related to starvation-induced ageing (Lliso et al., 2007) and fast sucrose depletion (Holland et al., 2002, 2005) in the peel of citrus fruits would support

this hypothesis. Likewise, studies by Purvis and Yelenosky (1983) showed that sucrose does not interchange between different tissues in citrus fruit but instead is translocated into the fruit from the leaves. According to structural data shown here, ethylene causes stress to albedo and flavedo cells. Therefore, ethylene might produce cross protection in detached fruit by providing carbon sources and compensating for lack of sucrose translocation from the leaves in detached citrus fruit. In this context, it is noteworthy that carbon starvation and cell perception of energy shortage may be sufficient to induce catabolism of proteins and membrane phospholipids (Alférez et al., 2005; Rawyler et al., 1999; Smith and Stitt, 2007; Yu, 1999), while lipolysis has been associated with the development of nonchilling peel pitting symptoms in citrus fruit (Alférez et al., 2008).

Our data also revealed that lowering temperature decreased visual damage and respiratory rate (Table 2), which further suggests that favoring carbohydrate consumption and the subsequent energy shortage in detached fruit may favor nonchilling peel pitting. In this context it is interesting to note that the higher the temperature, the faster the decline in starch and in sucrose occurring in detached citrus fruit to fuel a higher rate of respiration (Holland et al., 2002). Although lowering temperature may alter other metabolic pathways, we also found that 1-MCP reduced the release of sugar residues and the deposition of pectic polysaccharides and starch grain degradation and markedly increased respiration and peel damage (Table 2). Interestingly, 1-MCP had a marked effect on degrading intracellular membranes such as those of the endoplasmic reticulum, which were barely detected by 14 days storage in the peel of fruits exposed to this treatment (Figs. 2G and 2H). This effect was more evident in the albedo tissue, characterized by numerous intercellular air spaces as compared to flavedo, which is formed by smaller, densely packed, thick-walled cells. Whether the release of available carbohydrates in ethylene-treated fruits is related to its protective effect on reduced peel damage deserves further investigation. Studying the further consequences of ethylene application at the biochemical level goes beyond the scope of this study. It is noteworthy, however, that ongoing investigations in our laboratory on the basis of the results reported here indicate that treating oranges with different carbon sources delays nonchilling peel pitting development in fruits kept under the nonstressful environmental conditions used in the present study (Lafuente et al., unpublished results). This would be in concordance with previous reports indicating that high external $O_2$ levels, which might enhance carbohydrate reserves consumption in detached fruit by stimulating respiratory rate, favor nonchilling peel pitting in citrus fruits (Porat et al., 2004). Thus, one possibility worth considering is that starch and carbohydrate reserves in cell wall of citrus peel, which is very rich in pectins, might function as a carbon reservoir that helps the fruit to cope with starvation, while ethylene might favor carbon supply by stimulating cell wall degradation and remobilization of carbohydrates, which would help the fruit to sustain respiration, metabolic processes and cell viability. The situation may not be simple, as ethylene stimulates respiration in citrus fruit (McCollum and Maul, 2007) and therefore might enhance sucrose consumption (Holland et al., 2002). Nevertheless, it should be kept in mind that under our experimental conditions the ethylene-induced rises in respiration and membrane deterioration are noticeably lower than those induced by 1-MCP, and that 1-MCP inhibits ethylene perception and hence may block other protective mechanisms induced by ethylene (Establés-Ortíz et al., 2009; González-Candelas et al., 2010) that would not be easily detected by ultrastructural analysis. Interestingly, oxidative stress has been related to ethylene-induced acclimation of plants to stress (Lee et al., 1998; Munné-Bosch et al., 2004), and previous observations highlight the importance of the antioxidant enzymatic system in the protective effect of ethylene in reducing peel collapse in citrus fruit (Cajuste and Lafuente, 2007; Sala and Lafuente, 2004). Likewise, ethylene treatments that are effective in reducing damage activate phenylpropanoid metabolism (Cajuste and Lafuente, 2007; Establés-Ortíz et al., 2009), while a number of *Citrus* flavonoids act against harmful reactive oxygen species (Benavente-García et al., 1997). In this context, it is interesting to note that the ethylene treatments assayed in the present work favored not only the deposition of pectic polysaccharides substances in the peel, which indicates a cell wall reaction to a persistent slow intercellular oxidative process in plants (Günthardt-Goerg and Vollenweider, 2007), but also other oxidative stress-related mechanisms, such as starch mobilization and the alteration of cuticle and vacuole (Asensi-Fabado et al., 2010; Günthardt-Goerg and Vollenweider, 2007; Reig-Armiñana et al., 2004). Considering these results and the fact that inhibiting ethylene perception by applying 1-MCP preserved cell ultrastructure in albedo and flavedo tissues, reduced loss of starch grains and enhanced peel damage severity, an attractive possibility is that the ethylene levels used in the present study may be high enough to cause sublethal stress and/or senescence-induced cascade responses, especially in the albedo, which might generate cross-protective responses participating in the reduction of peel damage in detached citrus fruit. According the structural characterization performed here, such responses may involve: 1) oxidative stress-related mechanisms; 2) pectic oligosaccharides, which induce a broad spectrum of defense signaling pathways in plants and protect membranes from stress conditions that cause peel damage in horticultural crops, and 3) the release of sugar residues from bulk degradation of cell polysaccharides, which would provide additional carbon and energy sources needed to cope with nutrient deprivation and long-term cell survival in detached fruit.

In conclusion, our structural analysis provides new information indicating that cell wall deterioration is not the causal factor of the development of peel collapse in detached citrus fruit kept under nonstressful environmental conditions, since ethylene favored structure deterioration of flavedo and albedo cells and the release of pectinaceous exudates and starch grain degradation but reduced nonchilling peel pitting in oranges held at 22°C and 90–95% RH. In contrast, 1-MCP reduced these ethylene-related changes but markedly increased respiration and membrane degradation. Results also indicate that lignification, as a means of cell wall

strengthening, was not relevant in the protective role of ethylene reducing peel collapse. The overall results from this study, using an ultrastructural approach, reflects that the ethylene levels used in the present study cause sublethal stress, especially in the albedo tissue, and provide information suggesting that this stress may favor the activation of defensive responses involving oxidative-stress related mechanisms and recycling of nutrients and carbon supply to enable cells to cope with stress caused by fruit detachment and subsequent nutrient deprivation.

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