EPICUTICULAR WAX CONTENT AND MORPHOLOGY AS RELATED TO ETHYLENE AND STORAGE PERFORMANCE OF 'NAVELATE' ORANGE FRUIT

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Abstract

The effect of ethylene (2 μL L^{-1}) on total and soft epicuticular wax content and on its morphology has been investigated in mature 'Navelate' (*Citrus sinensis*, L. Osbeck) oranges held under non-stressful environmental conditions (22ºC and constant high relative humidity (90-95% RH)). In addition, the objective of the study was to understand whether the ethylene-induced changes in epicuticular wax might participate in the beneficial effect of ethylene reducing non-chilling peel pitting, by modifying peel water, osmotic or turgor potential, or disease incidence caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. Ethylene increased total and soft epicuticular wax content in 'Navelate' fruit and induced structural changes in surface wax that might be related to the formation of new waxes. Changes in epicuticular wax morphology, but not in its content, might be involved in the protective role of ethylene reducing non-chilling peel pitting, though the beneficial effect of the hormone is not related to water stress. Cell water and turgor potentials in freshly harvested fruit and fruit stored in air under non-stressful conditions suggest that water stress is not a limiting factor leading to the development of this physiological disorder. In addition, the results indicated that formation of new waxes in fruit treated with ethylene may partially cover stomata, cracks or areas lacking wax occurring in stored fruit and is likely to improve physical barriers to *P. digitatum* penetration.

Keywords: citrus fruit peel collapse, epicuticular wax, disease, ethylene, non-chilling peel pitting, Penicillium digitatum, osmotic, water and turgor potentials.

1. Introduction

Epicuticular wax plays important protective roles against biotic and abiotic stresses and are involved in reduction in transpiration and maintenance of water balance as well as in regulation of gas exchange in plants (Jeffree, 2006). In Citrus, it has been shown that the amount, composition and structure of the epicuticular wax vary among cultivars, change with fruit development, maturation and age (Freeman et al., 1979; El-Otmani and Coggins, 1985a; Sala et al., 1992) and have an effect on both water loss (Albrigo 1972a) and transport of gases through the cuticle (Ben-Yehoshua et al., 1985; El-Otmani et al., 1986). Furthermore, redistribution of epicuticular wax layer may improve physical barriers to pathogen penetration and reduce disease incidence caused by *P. digitatum* (Pers.:Fr.) Sacc. in citrus fruit (Schirra and D'hallewin, 1997; Schirra et al., 2000; Dore et al., 2009). A similar behaviour involving changes in epicuticular wax structure was reported in cactus pear fruit (Schirra et al., 1999).

Considerable attention has been paid to understand the effect of growth regulators such as gibberellic acid and 2,4-D (2,4-dichlorophenoxy acetic acid) on epicuticular wax in citrus fruit as these compounds delay peel senescence, may reduce peel softening, the incidence of puffy rinds and of peel disorders related to aging (El-Otmani and Coggins, 1985a, 1985b). The effect of ethylene on the epicuticular wax of horticultural crops has been little studied (Ju and Bramlage, 2001). To our knowledge, there has been no report studying its effect on natural production of wax or its structure in citrus fruit in spite of reducing peel damages caused by abiotic (Lafuente et al., 2001; Lafuente and Sala, 2002) and biotic (Marcos et al., 2005) stresses in this crop.

Fruit from many citrus cultivars are prone to develop non-chilling peel pitting manifested as irregular depressed areas on the peel that may turn brown over time when stored at non-chilling temperatures (Lafuente and Zacarias, 2006). Water status (Agustí

et al., 2001; Ben Yehoshua et al., 2001; Lafuente and Sala, 2002; Alférez et al., 2003; Alférez and Burns, 2004) and modification of internal gas concentrations may influence the incidence of this physiological disorder (Petracek et al., 1998). On the other hand, ethylene is very effective reducing non-chilling peel pitting, though the bases of its efficacy are poorly understood (Lafuente and Sala, 2002; Sala and Lafuente, 2004; Cajuste and Lafuente, 2007). Epicuticular wax has been related to physiological disorders of citrus fruit that cause tissue collapse (Albrigo, 1972a; El-Otmani et al., 1989; Vercher et al., 1994; Sala, 2000), but the involvement of wax in the susceptibility of citrus fruit to non-chilling peel pitting is not well understood. Wax morphology and cuticle permeability of healthy and damaged areas of 'Navelate' fruit with early symptoms of non-chilling peel pitting appear to be similar (Agusti et al., 2001). However, the content of hard wax, which is more prone to crack under stress conditions than soft wax (Albrigo, 1972b), was higher at the time of maximum incidence of the disorder in 'Navelina' oranges harvested along the citrus season (Sala et al., 1992). In addition, the severity of a disorder resembling non-chilling peel pitting in 'Valencia' oranges was related to the degree of amorphousness of the epicuticular wax structure, being more prevalent where this structure had broken down (El-Otmani et al., 1989). Moreover, whether changes in epicuticular wax participate in the lower susceptibility of ethylene-treated citrus fruit to disease remains unknown.

The aim of this work has been to study the effect of ethylene on total and soft epicuticular wax content as well as on its morphology in 'Navelate' (*Citrus sinensis*, L. Osbeck) oranges by using scanning electron microscopy (SEM). In addition, the objective was to understand whether the ethylene-induced changes in epicuticular wax might participate in the beneficial effect of ethylene reducing non-chilling peel pitting,

by modifying peel water, osmotic or turgor potential, or the incidence of rots caused by *P. digitatum*.

2. Materials and methods

2.1. Plant material, ethylene treatments and storage conditions

Mature fruit from 'Navelate' sweet orange harvested at a commercial orchard in Valencia, Spain, in February (2 months after fruit colour change) were used in the experiments. Fruit were immediately delivered to the laboratory and sorted on the basis of uniform size and the absence of visual defects. Fruit were divided into three groups, each containing the same number of fruit, and were immediately exposed to the following treatments: (a) continuous flow of air; (b) continuous flow of air after being conditioned for 4 days with air containing 2 μL L^{-1} ethylene; (c) continuous flow of air containing 2 μL L^{-1} ethylene. All the treatments were performed in the presence of $Ca(OH)_2$ to avoid the accumulation of respiratory CO_2 at 22ºC and 90-95% RH to avoid environmental stressful conditions.

2.2. Fungal material and fruit infection with P. digitatum

To examine the influence of ethylene-induced changes in epicuticular wax on disease incidence, fruit were either dipped in a *P. digitatum* conidial suspension or inoculated with the same suspension in wounded peel areas. Fruit were surface-sterilized before being infected with a *P. digitatum* (isolate PHI-26, obtained from a rotten orange) conidial suspension adjusted to 10^4 conidia/mL. The suspension was prepared in sterile distilled water from a 7-day-old culture grown on potato dextrose agar (PDA) at 24ºC and the conidia concentration determined with a haemocytometer. Fruit were infected after being stored for 22 days in: a) air, b) 2 μL L^{-1} ethylene, and c) 18 days in air after being treated for 4 days with 2 μL L^{-1} ethylene. Three replicates of

10 fruit were infected by dipping them in the conidial suspension for 1 min, allowed to dry at ambient temperature, and then arranged separately on plastic boxes and maintained at 90-95% RH and 22ºC. Simultaneously, other three lots of 5 fruit stored under the same conditions were infected by wounding the peel in the equatorial zone of the fruit (four wound per fruit) with a flame-sterilized needle (3 mm depth) and adding 10 µL of the conidial suspension as described by Ballester et al. (2006). Fruit were kept under the same conditions as the dipped fruit to follow disease incidence. Three replicates of 20 inoculated wounds each were evaluated.

2.3. Total cuticular wax determination

Based on Sala (2000), wax extraction was carried out with dichloromethane from three replicates of 10 fruit each. Fruit were immersed and shaken for 1 min in three successive vessels, each containing 400 mL of dichloromethane at 35ºC. The dichloromethane extracts were combined, filtered, evaporated to dryness and oven dried overnight. The total epicuticular wax content was determined gravimetrically and expressed as µg total wax per cm^2 of fruit surface area, which was calculated as previously described by Turrell (1946).

2.4. Yield of soft waxes

The soft wax was determined as that portion of pulverized total wax that would dissolved into petroleum ether (Albrigo, 1972a). The yield of soft waxes from the three replicate samples was determined by evaporating the wax stock solution and refluxing the dry wax with 50 mL of light petroleum ether (35-60ºC) for 1 hr. Following refluxing, solutions were cooled to precipitate insoluble fractions and then filtered, the petroleum-ether soluble fraction was evaporated and the soft wax content determined

gravimetrically and expressed as percentage and μg soft wax per cm^2 of fruit surface area (Freeman et al., 1979).

2.5. Scanning electron microscopy (SEM)

To examine the epicuticular wax of the pericarp by SEM, small sections (20 x 10 mm^2) of the rind of five individual fruit were excised with a razor blade from the equatorial zone of the fruit for each treatment and sampling period. Healthy tissue areas were taken for SEM to identify those changes related to ethylene because changes associated with damages could partially mask those related to ethylene. Fresh tissue sections, fixed to a stub by means of TBS tissue freezing medium (Triangle Biochemical Science, Dunhan, NC, USA), were frozen in slush nitrogen and attached to the specimen holder of a CT-1000C Cryo-transfer system (Oxford Instruments, Oxford, UK) interfaced with a JEOL JSM-5410 SEM (JEOL, Tokyo, Japan). The samples were then transferred from cryostage to the microscope sample stage, where condensed surface water was sublimed at - 90ºC, and transferred again to the cryostage in order to be coated with gold. Finally the sample was put back into the microscope sample stage to be viewed at an accelerating voltage of 15 KeV.

2.6. Determination of fruit weight loss and of water, osmotic and turgor potentials

A subset of 30 fruit from each treatment were used to periodically estimate fruit weight loss and the results were expressed as means of three replicates samples of 10 fruit each ± S.E.M.

Water, osmotic and turgor potentials were measured in four flavedo and albedo disks of 1-2 mm thick and 5 mm diameter excised from the equatorial zone of the fruit by using a cork borer as previously described by Alférez et al., 2005. Disks were placed

into a sample chamber (C-52, Wescor Inc. Logan, UT) connected to a psychrometer switchbox (PS-10, Wescor Inc. Logan, UT) and to a dew point microvoltimeter (HT-33T, Wescor Inc. Logan, UT) and their water potentials measured after 2 h incubation to ensure initial water vapour equilibrium. The disks were then frozen in liquid nitrogen and stored at –20ºC to determine osmotic potential in the same samples. To that end, flavedo or albedo disks were thawed at room temperature to break cell walls and to release solute-binding water, placed into chambers and potential measured as above. Turgor potentials were then calculated as the difference between osmotic and water potentials. The data were expressed as megapascals (MPa).

2.7. Determination of disease incidence

Disease incidence was determined for up to 25 days post-inoculation (dpi) in three replicates of 10 fruit dipped in the *P. digitatum* conidial suspension, and expressed as percentage of infected fruit. The percentage of infected wounds (three replicates of 20 wounds) was estimated for up to 6 dpi in wounded-inoculated fruit as infection progressed much faster than in the dipped fruit.

2.8. Statistical design

A mean comparison using the Tukey´s test was performed to determine if total and soft epicuticular wax content, disease incidence and water, osmotic and turgor potentials of ethylene-treated and non-treated fruit were significantly different ($P \leq 0.05$).

3. Results

3.1. Effect of ethylene on total and soft epicuticular wax content

Ethylene applied as a pre-treatment for 4 days produced a 20% increase in total epicuticular wax content per surface area as compared to fruit maintained for the same period in air (Fig. 1). Nevertheless, no significant difference ($P \leq 0.05$) was found by 4 days between fruit held in air and ethylene. Holding the fruit continuously in ethylene for 24 days produced a greater (≈ 45%) and significant increase in total superficial wax as compared to fruit held in air (Fig. 1). However, no statistical difference in total wax content between fruit held continuously in air and fruit pre-treated with ethylene for 4 days was found after this period.

The percentage of soft wax in mature 'Navelate' oranges (≈ 61%) was higher than that of hard waxes, this effect being more marked in fruit stored for 14 days in ethylene (Table 1). No significant difference was found in both soft wax yield and proportion among fruit exposed to the three treatments by either 4 or 14 day storage. However, soft wax content per surface area increased significantly ($P \leq 0.05$) by 50% in fruit stored for 14 days under a continuous 2 $\mu L\ L^{-1}$ ethylene atmosphere as compared to initial value (freshly harvested fruit).

3.2. Effect of ethylene on epicuticular wax morphology

SEM microscopy observations revealed that the epicuticular wax layer covering freshly harvested mature 'Navelate' fruit consisted of smooth ridges of wax with a few small cracks (Fig. 2A). These fruit showed an amorphous cuticular wax with flattened plates, which is a typical feature in mature citrus fruit. A magnification of the stomata area of these fruit further showed that wax seems smooth and intact before storage and that the surrounding areas are rich in agglomerations of small platelets and smooth ridges arrayed compactly (Fig. 2B). A magnification of cracks in epicuticular wax is shown in Fig. 2C. Ridges on the surface of control fruit stored in air were clearly less

compact. Furthermore, control air-stored fruit showed many wax deficiencies, caused by loss of wax plates, along the fruit surface and also many cracks which were evident in areas of flattened plates and also in areas surrounding or covering the stomata (Fig. 2D, 2E and 2F). Ethylene had a clear impact on wax morphology as fruit held continuously in ethylene for the same period that control fruit showed a smoother and a more compact and homogeneous wax layer surface (Fig. 2G and 2H). Furthermore, ethylene stored fruit showed fewer cracks and zones lacking wax, which in some cases appeared to be covered by new waxes, effect that is highlighted by comparing the stomata of fruit stored in ethylene (Fig. 2I) and air (Fig. 2F). Therefore, wax in fruit kept under constant air showed a greater damage than that of fruit held in ethylene. The wax was also smoother and less damaged in fruit conditioned for 4 days with ethylene and then transferred to air (Fig. 2J, 2K and 2L) than in fruit held continuously in air (Fig. 2D, 2E and 2F). However, wax layers of ethylene-conditioned fruit (Fig. 2J, 2K and 2L) showed a higher trend to crack than epicuticular wax of fruit maintained in an atmosphere were ethylene was not removed, a fact that was very evident when we focused on the stomata, which revealed that wax layer surrounding or covering this zone were cracked if ethylene was not applied continuously (Fig. 2K and L).

3.3. Effect of ethylene on fruit weight loss and on water, osmotic and turgor potentials of flavedo and albedo tissues

Fruit weight loss did not exceed the 2% after 22 days storage at 22ºC and high RH (90-95% RH). Under these environmental conditions, weight loss was barely affected by conditioning the fruit for 4 days with 2 $\mu L\ L^{-1}$ ethylene or by treating them cotinuously with the hormone (Fig. 3).

Water potentials of flavedo and albedo did not change significantly in 'Navelate' fruit constantly held at 22ºC and high RH (90-95%) in air and were not significantly affected by ethylene treatments (Fig. 4). Our results also showed that osmotic and turgor potentials of both tissues were not affected by the treatments and that turgor potentials were always positive (data not shown).

3.4. Effect of ethylene on disease incidence caused by P. digitatum

Infection experiments were conducted in air- and ethylene-treated fruit stored for 22 days in order to achieve differences in wax surface before infecting the fruit. The progress of the infection was much slower in fruit dipped in the conidial suspension (Fig. 5A) than in the wounded-infected fruit assay (Fig. 5B). Therefore, disease incidence was measured following fungal inoculation for up to 25 dpi in the dipped fruit and for up to 6 dpi in wounded oranges. The hormone was effective reducing disease incidence in non-wounded fruit dipped in the *P. digitatum* conidial suspension and its efficacy increased with the time that fruit were exposed to ethylene (Fig. 5A). In contrast, no effect of ethylene reducing disease incidence was observed when the pathogen was inoculated into wounds affecting the flavedo and the upper layers of the albedo (Fig. 5B). SEM micrographs in Fig. 6 show *P. digitatum* hyphae and a germinated conidium in the surface of 'Navelate' oranges held at 22ºC. As shown in the micrographs, surface contains stomata and cuticular cracks or areas lacking wax wider than hyphae.

4. Discussion

Ethylene may enhance senescence but it also induces defensive mechanisms alleviating stress-induced damage in plants (Yang and Hoffman, 1984). This hormone

plays a role reducing peel damage in mature citrus fruit exposed to abiotic stresses (Lafuente et al., 2004; Cajuste and Lafuente, 2007) and against the wound pathogen *P. digitatum* (Marcos et al., 2005). As surface waxes regulate water loss and act as the first barrier to pathogen attack, we investigated whether ethylene is able to induce epicuticular wax changes in 'Navelate' oranges. We showed that ethylene increases surface wax when applied for prolonged periods, which is in agreement with previous findings in apple fruit (Ju and Bramlage, 2001) and with the fact that giberellic acid, a growth regulator delaying peel senescence, delayed wax accumulation in oranges (El-Otmani and Coggins, 1985a). The effect of ethylene on wax ultrastructure of citrus fruit had not been investigated. It is noteworthy, however, that the overexpression of an ethylene response transcription factor up-regulates wax production and alter epicuticular wax ultrastructure in Arabidopsis plants (Broun et al., 2004). SEM examination of epicuticular wax morphology revealed that 'Navelate' oranges exposed continuously to ethylene presented the most compact, homogeneous and smoother epicuticular wax layer. This suggests the formation of new waxes onto the cuticle surface that might cover partially wax-lacking areas or cracks occurring as a result of ageing in citrus fruit (Sala 2000), as it was previously indicated in plants (Hall 1967; von-Wettstein-Knowles, 1974). In these reports, it was also suggested that new wax may push the initial wax away, especially the most aged or prone to crack. Interestingly, new waxes in citrus fruit are richer in fatty acids (Freeman et al. 1979), while ethylene has an important impact increasing fatty acids in apples (Ju and Bramlage 2001). Our results also suggest that wax formation in harvested citrus fruit is a dynamic process in which the presence of ethylene would be necessary, as removing ethylene resulted in more cracked waxes as compared to fruit continuously held in ethylene. Significant differences in epicuticular wax content between fruit held continuously in air and

ethylene were only found by 24 days, which might explain the lack of efficacy of ethylene reducing weight loss over the period examined (22 days). Furthermore, though such increase was significant at P 0.05, it might not be high enough (1.4-fold increase) to reduce weight loss.

We also focused our attention on understanding whether the beneficial effect of ethylene reducing non-chilling peel pitting in citrus fruit (Lafuente and Sala, 2002) may be related to changes in epicuticular wax content and/or morphology. As previously reported (Cajuste and Lafuente, 2007), conditioning the fruit for 4 days with 2 μL L^{-1} ethylene reduced the incidence of this physiological disorder. In addition, we found that the efficacy of ethylene treatments increased with their duration as 2-. and 3-fold decreases in the percentage of damaged fruit occurred by 14 days in fruit conditioned or treated continuously with ethylene, respectively (data not shown). Although ethylene increased epicuticular wax after prolonged storage, no significant differences in the content of either total or soft waxes between control and ethylene-treated fruit were found by 14 days in spite of the difference found in peel damage. Therefore, it appears that they are not involved in the efficacy of the hormone reducing loss of peel integrity, though soft waxes are less likely to crack or separate than hard waxes and may reduce the development of peel damage related to water loss in citrus fruit (Albrigo 1972a; 1972b). In addition, we demonstrated that water potentials, as well as cell turgor pressure, of flavedo and albedo were not modified by conditioning or continuously treating the fruit with ethylene in spite of the changes induced by the hormone in wax morphology. Therefore, the results suggest that changes in epicuticular wax structure might not be involved in the effect of ethylene reducing loss of peel integrity in 'Navelate' fruit held at high RH by reducing water stress. In concordance with this result, Agustí et al. (2001) found that cuticular permeability to water between healthy

and damaged areas in citrus fruit showing non-chilling peel pitting was similar. Furthermore, our data showing small changes in flavedo and albedo water potentials of detached 'Navelate' oranges maintained in air under high RH confirm more precisely previous suggestions, raised by measuring water content, indicating that non-chilling peel pitting may develop independently of fruit water status (Cajuste and Lafuente, 2007). Likewise, water potential and turgor pressure of freshly harvested fruit reflected turgid non-stressed cells indicating that fruit were not stressed at harvest and, therefore, that they did not develop non-chilling peel pitting as a consequence of re-hydration, which has been shown to induce this disorder (Alférez et al., 2003). Waxes may have a more marked effect on inhibiting transport of gases than transport of water through the cuticle in citrus fruit (Ben-Yehoshua et al., 1985). The mode of action by which external or internal gases may alter the susceptibility of citrus fruit to develop peel damage remains unknown, though postharvest treatments modifying internal gas composition or the O_2 environmental levels may also modify non-chilling peel pitting susceptibility (Petracek, 1998; Ben-Yehoshua et al., 2001; Porat et al., 2004). Our results showing that treating the fruit with ethylene favours exudation of new waxes that may cover in part cracks and stomata and the fact that stomata coverage alters resistance to gas diffusion in citrus tissues (Calatayud et al., 2006) encourages new studies along these lines.

The results also suggested that new waxes covering areas lacking wax or cuticular cracks might participate in the reduction of *P. digitatum*-induced rots in fruit treated with ethylene. Thus, treating the fruit with the hormone reduced rots when fruit were dipped in the conidial suspension after inducing differences in surface waxes, but not when the fungus was inoculated into wounds affecting both flavedo and albedo tissues. The effect of epicuticular wax in the natural incidence of disease in citrus fruit is almost unknown. It is noteworthy, however, that our results showing the effect of

ethylene on epicuticular wax and disease incidence are in agreement with studies showing that heat treatments favouring coverage of peel openings by natural waxes reduced disease incidence in citrus fruit (Schirra and D'hallewin, 1997; Schirra et al., 2000).

In conclusion, the results indicate that: 1) ethylene increased total and soft wax epicuticular content in mature citrus fruit and induced changes in surface wax morphology that might be related to the formation of new waxes; 2) changes in epicuticular wax morphology, but not in its content, might contribute to the protective role of ethylene reducing non-chilling peel pitting although such effect is not related to changes in peel water status; 3) water stress is not a limiting factor originating this physiological disorder as indicated by cell water and turgor potentials both in freshly harvested fruit and in fruit stored in air under non-stressful environmental conditions; and 4) new waxes covering cracks or areas lacking wax occurring during fruit storage might contribute to the beneficial effect of ethylene reducing disease incidence caused by *P. digitatum.*

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FIGURE LEGENDS

Fig.1. Changes in epicuticular wax content, expressed as μg per fruit surface area, in 'Navelate' orange fruit stored for up to 24 days in air (black bars) or air containing 2 μL L^{-1} ethylene (white bars), and in fruit conditioned for 4 days with 2 μL L^{-1} ethylene and then stored for up to 20 days in air (grey bars). Fruit were always maintained at 22ºC and 90-95% RH. Values labelled with the same letter are not significantly different at the 5% significance level according to Tukey's test.

Fig.2. Changes in epicuticular wax morphology during storage of 'Navelate' orange fruit stored in air or air containing 2 μL L^{-1} ethylene, and in fruit conditioned for 4 days with 2 μL L^{-1} ethylene and then stored in air, as revealed by SEM. Fruit were maintained at 22ºC and 90-95% RH. Surface wax of freshly harvested fruit (A,B,C); fruit held continuously in air (D,E,F) or in ethylene (G,H,I); fruit conditioned for 4 days with ethylene and transferred to air (J,K,L). Annotations (black letters): Stomata (S); cracks (C); absence of epicuticular waxes (A). Bar sizes in micrographs (labelled with white letters): C = 5μm; B, F, I, and L = 10 μm; A, D, E, H, J and K = 20μm; G = 50 μm.

Fig.3. Percentage of weight loss of 'Navelate' orange fruit conditioned for 4 days with 2 μL L^{-1} ethylene and then stored in air for 17 days (■), and in fruit stored continuously for 21 days in air (○) or air containing 2 μL L^{-1} ethylene (□). Fruit were always maintained at 22ºC and 90-95% RH. Values are the means of three replicates of ten fruit each ± S.E.M. The same fruit were assessed each time for weight loss evaluation.

Fig.4. Changes in water potential in the flavedo and albedo of 'Navelate' orange fruit stored for up to 21 days in air (black bars) or air containing 2 μL L^{-1} ethylene (white bars), and in fruit conditioned for 4 days with 2 μL L^{-1} ethylene (grey bars) and then stored in air for up to 17 days. Fruit were always maintained at 22ºC and 90-95% RH. Values labelled with the same letter are not significantly different at the 5% significance level according to Tukey's test.

Fig.5. Incidence (%) of green mold disease caused by *P. digitatum* on 'Navelate' oranges infected after being stored for 22 days in air (black bars), 2 μL L^{-1} ethylene (white bars), and 18 days in air after being treated for 4 days with 2 μL L^{-1} ethylene (grey bars). Inoculation of the pathogen was conducted by dipping the fruit in an aqueous *P. digitatum* suspension containing 10^4 conidia per mL (A) or by inoculating wounded fruit with 10 μL of the same suspension (B). Disease incidence was measured for up to 25 days of inoculation (dpi) at 22ºC and 90-95% RH following inoculation in fruit dipped in the conidial suspension and expressed as percentage of rotten fruit; and for up to 6 dpi in wounded-inoculated fruit and expressed as percentage of infected wounds. Different letters in the same dpi indicate significant differences in the treatments according to Tukey's test with a *p*-value of 0.05. Values are the means of three replicates of ten fruit each (dipping treatment) or of three replicates of twenty wounds (wound-inoculation).

Fig.6. SEM micrographs showing *P. digitatum* hyphae (H), germinating conidium (G), stomata (S) and cracks or absence of epicuticular waxes (C) in the surface of 'Navelate' oranges held at 22ºC. Micrographs were done 5 days after fruit dipping in a conidial suspension containing 10^4 *P. digitatum* conidia per mL. Bar size in the left panel

micrographs: 10 μm; in the right upper corner: 100 μm, and in the right lower corner: 50 μm.

Table 1

Table 1. Effect of ethylene (2 μL L^{-1}) on the proportion (%) and content of epicuticular soft wax, expressed as μg per fruit surface area, in mature 'Navelate' fruit stored at 22ºC and 90-95% RH. Values labelled with the same letter are not significantly different at the 5% level.

Treatment	Soft wax (%)	Soft wax (μg cm^{-2})
Freshly harvested (0d)	61.55[ab]	60.93[b]
4d air	61.22[ab]	62.65[b]
4d ethylene	55.37[b]	67.55[b]
14d air	69.76[ab]	78.74[ab]
4d ethylene + 10d air	67.65[ab]	74.83[ab]
14d ethylene	77.03[a]	91.56[a]

Figure 1

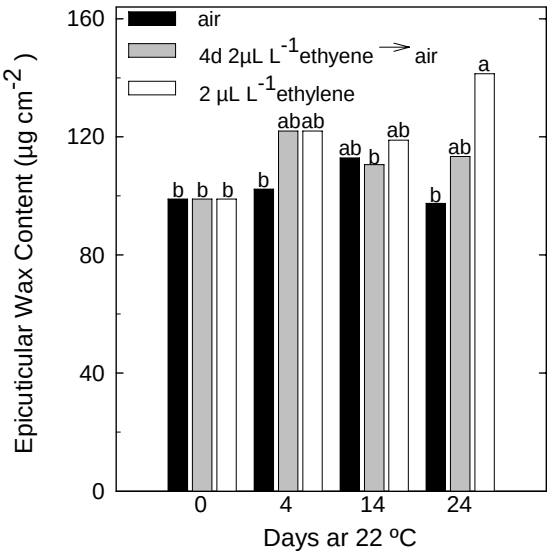
air
4d 2µL L-1ethyene → air
2 µL L-1ethylene
Epicuticular Wax Content (µg cm-2)
160
120
80
40
0
b b b
b
abab
ab b
ab
b
ab
a
0
4
14
24
Days ar 22 ºC

Figure 2

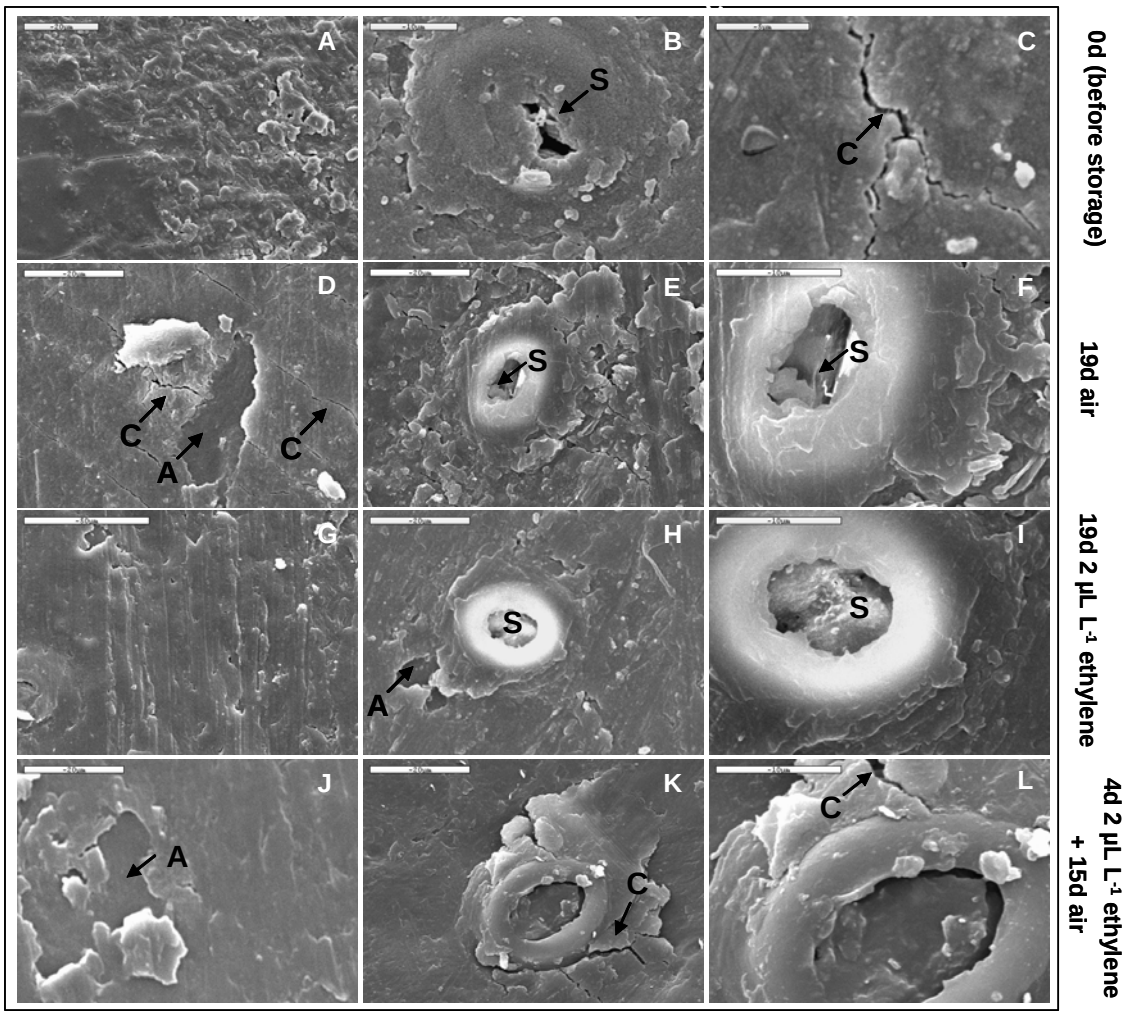
A
B
S
C
C
D
C
A
C
E
S
F
S
G
H
S
A
I
S
J
A
K
C
L
C
0d (before storage)
19d air
19d 2 µL L-1 ethylene
4d 2 µL L-1 ethylene
+ 15d air

Figure 3

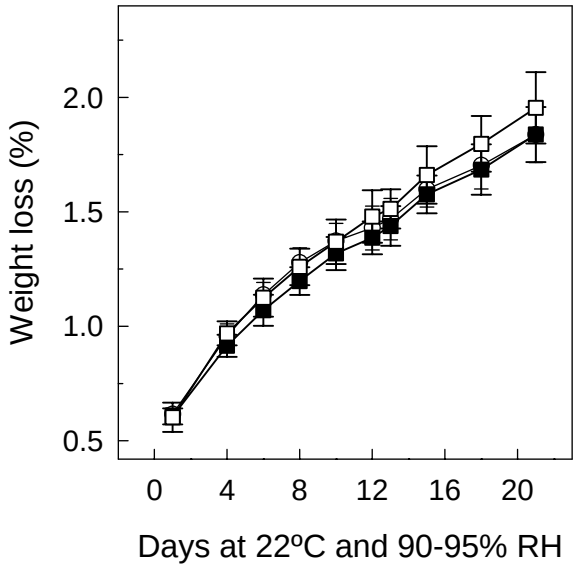

Figure 4

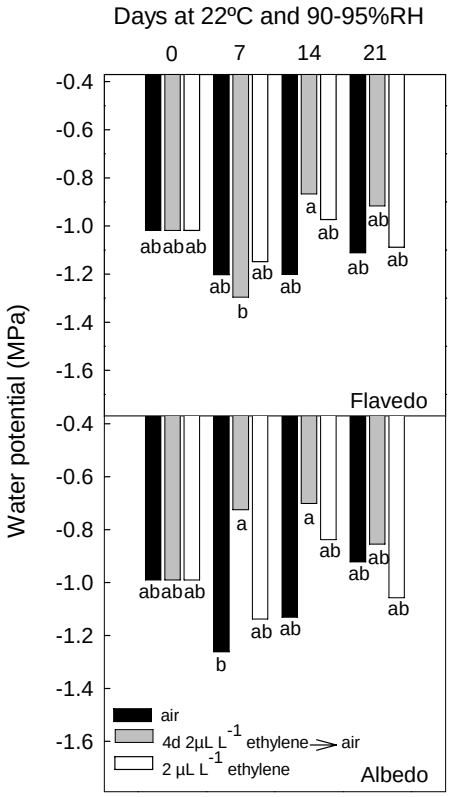
Days at 22ºC and 90-95%RH
0
7
14
21
Water potential (MPa)
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
ab ab ab
ab
b
ab
a
ab
ab
ab
Flavedo
ab ab ab
b
a
ab ab
a
ab
ab
ab
air
4d 2µL L-1 ethylene → air
2 µL L-1 ethylene
Albedo

Figure 5

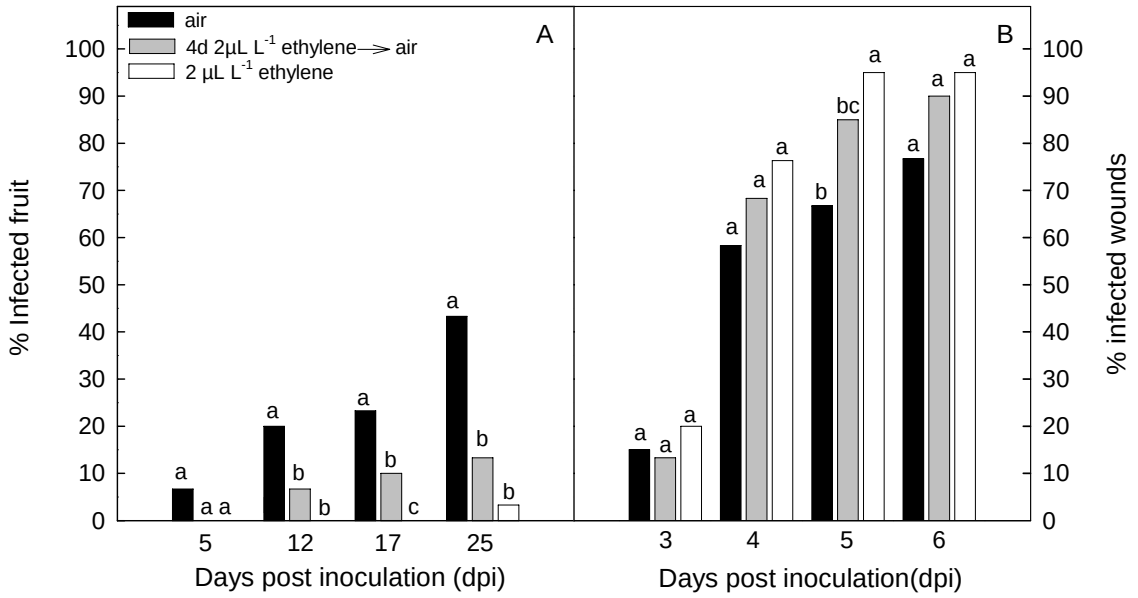
air
4d 2µL L-1 ethylene→ air
2 µL L-1 ethylene
A
B
% Infected fruit
% infected wounds
Days post inoculation (dpi)
Days post inoculation(dpi)
5
12
17
25
3
4
5
6

Figure 6