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Active Oxygen Detoxifying Enzymes and Phenylalanine Ammonia-lyase in the Ethylene-Induced Chilling Tolerance in Citrus Fruit

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The effects of applying ethylene (2 μL L^{-1}) during cold storage of Fortune mandarins on the development of chilling-induced peel damage and on changes in the activities of the enzymes of the antioxidant system, superoxide dismutase, catalase (CAT), ascorbate peroxidase, guaiacol peroxidase, and glutathione reductase, and on phenylalanine ammonia-lyase (PAL) have been investigated. Chilling damage was reduced by applying ethylene during fruit storage at 1.5 °C. PAL activity increased in response to cold stress and was higher in fruit held under ethylene than under air during the whole storage period, whereas CAT was temporarily higher in ethylene-treated fruit. In contrast, the activities of the other enzymes were not increased by ethylene. The global results suggest that the ethylene-induced chilling tolerance in Fortune mandarins might be due to increased PAL and CAT activities.

KEYWORDS: Ascorbate peroxidase; catalase; citrus; ethylene; glutathione reductase; low-temperature storage; phenylalanine ammonia-lyase; peroxidase; superoxide reductase

INTRODUCTION

The mandarin cultivar Fortune is a cross-pollination of Clementina Fino (*Citrus clementina* Hort. Ex Tan.) × Dancy mandarin (*Citrus reticulata* Blanco). Despite the good quality, fruit marketing is difficult as it is very prone to develop chilling injury (CI) during cold storage at temperatures below 10 °C. Although CI does not affect internal fruit quality, it greatly decreases its fresh market value because of the deterioration of the peel as chilling symptoms develop as small brown necrotic tissue areas (pitting) in the outer colored part of the fruit (flavedo) (*1*).

The mechanisms involved in the tolerance of plants to chilling are still not clear. Oxidative stress has been associated with the appearance of chilling damage in fruits (*2*, *3*), and it has been shown in Fortune mandarins that free active oxygen species (AOS) scavenging enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (POD; EC 1.11.1.7), and glutathione reductase (GR; EC 1.6.4.2) may play a role in the efficacy of postharvest heat-conditioning treatments protecting the fruit against chilling (*4*, *5*). POD may protect plants against stress-induced damage not only by scavenging hydrogen peroxide (*6*) but also by promoting lignification and the cross-linking of primary cell wall via ferrulic acids esterified to carbohydrates (*7*). However, POD has been also related to the deterioration and browning of horticultural crops (*8*, *9*), as this enzyme may oxidize phenolic compounds. Another enzyme involved in the chilling tolerance of citrus fruits is phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) (*10*). This is the initial rate-controlling enzyme in phenolic synthesis and many phenolic compounds in plants possess antioxidant activity (*11*).

The hormone ethylene plays important roles in postharvest processes of fruit and vegetables (*12*). Ethylene has been shown to participate in the defensive responses of plants against stresses (*13*), but it has been also involved in the induction of brown necrotic tissue areas in commercial horticultural crops (*8*). Earlier studies have documented that some climacteric and nonclimacteric fruit were more sensitive to chilling when treated with ethylene (*2*, *14*), but there are a number of cases in which exogenous ethylene was found to be beneficial in reducing CI (*14*, *15*). In Fortune mandarins, it has been shown that the chilling-induced ethylene plays a role in reducing the development of chilling damage, although applying ethylene before fruit exposure to chilling did not reduce its incidence when fruit were subsequently held at a chilling temperature (*10*). Furthermore, it has been shown that the PAL activation occurring in this citrus cultivar in response to chilling is dependent on ethylene and is also an independent cold signal apparently related to the cold-induced peel damage (*10*). The effect of ethylene in the responses of CAT, GR, POD, APX, and SOD to cold stress in citrus fruit remains unknown, and controversial results have been found on the relationship between ethylene and AOS-scavenging enzymes in other crop systems. Thus, application of ethylene during the storage of spinach leaves originated a decrease in the activities of CAT, APX, and GR and favored senescence (*16*), whereas inhibition of ethylene biosynthesis reduced the nitrate-induced activation of the enzymes CAT, APX, and GR

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in chickpea nodules (*17*). POD was also activated by ethylene in lettuce and *Cucurbita maxima* (*9*, *18*). In turn, the deleterious effect of ethylene favoring chilling in cantaloupe melons has been associated with the reduction in SOD, POD, and CAT activities (*3*).

Applying ethylene at 20−25 °C is a regular commercial practice to degreen citrus fruit. Ethylene concentrations of 5−10 μL L^{-1} were applied initially, but those concentrations have been reduced to 1−2 μL L^{-1} as an excess of ethylene may enhance fruit senescence or provoke deleterious effects on fruit quality (*19*). The effect of treating citrus fruit with ethylene on postharvest physiological disorders has been little studied and appears to be influenced by the fruit physiological stage. Thus, the incidence of rind staining, a nonchilling peel physiological disorder, occurring in Navelina fruits during postharvest management was reduced by treating mature fruit with ethylene (*20*). However, this hormone did not affect the incidence of pitting in Fallglo tangerines harvested before color break (*21*) and favored the incidence of red blotch in degreened lemon fruit at nonchilling temperatures (*22*). As indicated above, applying ethylene before the storage of Fortune mandarins at low temperature does not protect them against chilling, but the induction of endogenous ethylene in citrus fruit in response to cold stress is a defense mechanism against chilling (*10*). Therefore, it could be expected that application of ethylene during cold storage of Fortune fruits may enhance their tolerance to low temperature.

The general objective of this work has been to examine the effect of applying a commercial concentration of ethylene during cold storage of mature Fortune mandarins on changes in the activities of PAL and of the AOS-scavenging enzymes, including SOD, CAT, GR, POD, and APX, and to determine whether this effect is related to the ethylene-induced chilling tolerance in Fortune mandarins.

MATERIALS AND METHODS

Plant Material. Fruits of Fortune mandarin (*Citrus clementina* Hort. Ex Tanaka × *Citrus reticulata* Blanco) were harvested from trees grown in a commercial orchard at Valencia, Spain (latitude, 39° 28′ 48″ N; longitude, 00° 22′ 52″ W). To test the effect of exogenous ethylene on CI and on the activities of the AOS-scavenging enzymes and PAL, freshly harvested Fortune fruits were treated with a continuous flow of 2 μL L^{-1} ethylene at 1.5 °C and 85−90% relative humidity for up to 56 days in 100 L vessels in the presence of lime powder to avoid accumulation of respiratory CO_2. An equal number of fruits were treated with a continuous flow of ethylene-free air under the same conditions and used as control fruits in this experiment. For each treatment, fruits were divided at random into two groups. The first group was used to determine changes in enzyme activities. Three replicates of 10 fruits for enzyme analysis per storage period were included in this group. Flavedo samples were separated from the surface of fruits and ground with a food chopper in liquid N_2 to a fine powder, and the homogenized sample was stored at −70 °C until enzyme assays. The second group contained three replicates of 20 fruits to determine the severity of the chilling-induced peel damage.

Chemical and Reagents. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO), and ethylene was from Abello Linde S.A. (Valencia, Spain).

Estimation of CI Index. Fruits were visually scored to estimate the severity of CI. Brown pit-like depressions in the fruit are the main symptoms of CI. A rating scale from 0 (no injury) to 3 (severe injury) was used to evaluate CI, and an average CI index was calculated by summing the products of the amount of fruit showing CI in each category by the value assigned to this category in the rating scale and dividing the resulting sum by the total number of fruits evaluated. The results are the means of three replicate samples of 20 fruits ± SE.

PAL Activity. PAL (EC 4.3.1.5) activity was determined in samples from flavedo acetone powder as previously described by Martínez-Téllez and Lafuente (*5*). Three replicate samples of representative ground flavedo tissue, processed as described above, were ground in 10 mL of chilled (−20 °C) acetone per gram of flavedo. The homogenates were filtered through a Büchner funnel, the residues washed twice with chilled acetone, and the resulting powders dried for 2 h at room temperature. PAL was extracted from 0.4 g of acetone powder with 15 mL of 100 mM sodium borate buffer, pH 8.8, containing 20 mM β-mercaptoethanol. Proteins were salted out with ammonium sulfate at a final saturation of 46% to purify the extracts, and the precipitated PAL enzyme was dissolved in 4.5 mL of 100 mM ammonium acetate buffer, pH 7.7, containing 20 mM β-mercaptoethanol. The PAL activity was measured by determining the absorbance of cinnamic acid at 290 nm over a period of 2 h at 40 °C. The reaction mixture contained 2 mL of the purified enzyme extract and 0.6 mL of 100 mM L-phenylalanine in a total volume of 6 mL. PAL activity is expressed as nanomoles of cinnamic acid per gram of acetone powder flavedo tissue per hour, and the results are the mean of three replicate samples of 10 fruits each.

SOD Activity. SOD (EC 1.15.1.1) activity was determined in 1 g of fresh weight of frozen flavedo tissue according to the method of Sala and Lafuente (*4*). SOD was extracted at 4°C in a mortar and pestle with 10 mL of cold 50 mM potassium phosphate buffer, pH 7.8, containing 1.33 mM diethylenetriaminepentaacetic acid (DETAPAC). The homogenate was centrifuged twice at 4 °C for 15 min at 27000*g* and the supernatant used to assay SOD activity spectrophotometrically according to the method of Oberley and Spitz (*23*). The superoxide radicals were generated by xanthine−xanthine oxidase, and nitro blue tetrazolium (NBT) was used as an indicator of superoxide radical production. One unit of SOD activity was defined as the amount of enzyme that gave half-maximal inhibition.

CAT Activity. CAT (EC 1.11.1.6) activity assay was performed as previously described (*4*). CAT was extracted by pulverizing in a mortar and pestle 1 g of fresh weight of frozen flavedo tissue with 10 mL of cold 100 mM potassium phosphate buffer, pH 6.8, at 4 °C. The homogenate was centrifuged as described above, and CAT activity was determined at 25 °C in the supernatant according to the method of Kar and Mishra (*24*). One unit of CAT was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per minute at 25 °C.

APX Activity. APX (EC 1.11.1.11) was extracted at 4 °C from 1 g of fresh weight flavedo with 10 mL of cold 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 1 mM ascorbic acid, and 1% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged for 15 min at 27000*g* and the supernatant used to assay APX activity as described by Asada (*25*). The amount of enzyme that oxidized 1 μmol of ascorbate per minute at 25 °C was defined as 1 unit of APX (*4*).

GR Activity. GR (EC 1.6.4.2) was extracted at 4 °C from 1 g of fresh weight flavedo tissue with 10 mL of cold 100 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged twice at 4 °C as described above and the supernatant used to assay GR spectrophotometrically according to the method of Smith et al. (*26*). GR activity was calculated by using a standard curve determined according to the procedure of Carlberg and Mannervik (*27*). One unit of GR was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute (*4*).

POD Activity. The activities of soluble and insoluble POD (EC 1.11.1.7) were analyzed according to the method of Lagrimini and Rothstein (*28*). Fresh weight flavedo tissue (0.1 g) was extracted twice at 4 °C with 0.75 mL of cold 100 mM potassium phosphate buffer, pH 6.0, the homogenates were centrifuged, and the resulting supernatants were combined to determine the soluble POD. To recover salt-extractable, or ionically bound, POD activity, the pellet was resuspended in 100 mM potassium phosphate buffer, pH 6.0, containing 1 M NaCl, for 1 h in ice and centrifuged, and then the supernatant was assayed to determine cell wall-associated POD. POD (insoluble or soluble) was assayed spectrophotometrically at 30 °C. The reaction mixture included 200 μL of enzyme solution in a total volume of 2 mL, and 20 mM guaiacol and 8 mM H_2O_2 were used as substrates. The progression of

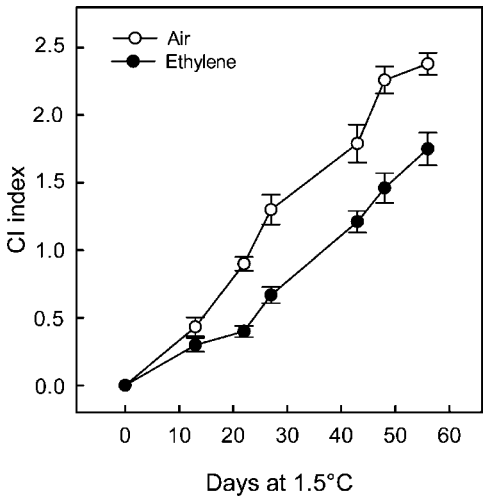

Figure 1. CI index of Fortune mandarin fruits stored for up to 56 days under air (○) or 2 μL L^{-1} ethylene (●) at 1.5 °C and 85–90% relative humidity. Values are the means of three replicate samples ± SE.

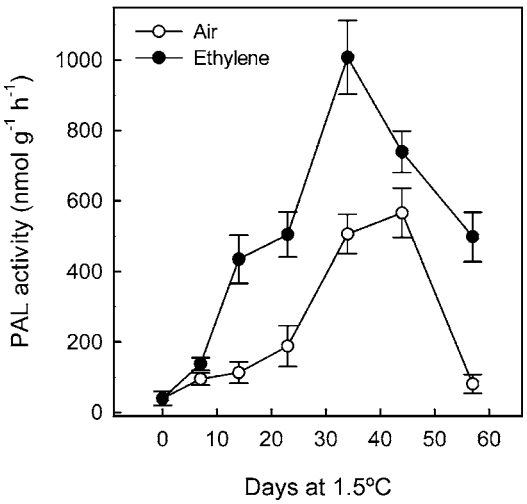

Figure 2. Changes in the activity of PAL in flavedo tissue of Fortune mandarin fruits stored for up to 56 days under air (○) or 2 μL L^{-1} ethylene (●) at 1.5 °C and 85–90% relative humidity. Values are the means of three replicate samples ± SE.

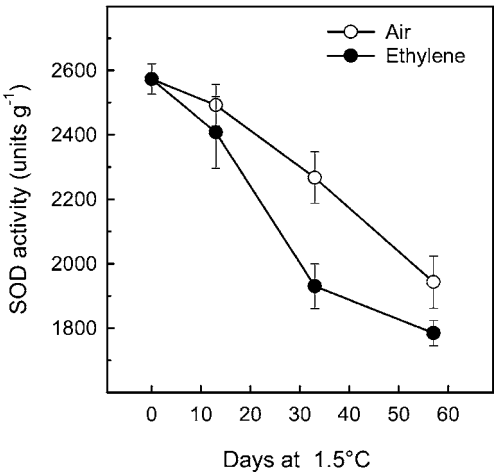

Figure 3. Time course of SOD activity in flavedo tissue of Fortune mandarin fruits stored for up to 56 days under air (○) or 2 μL L^{-1} ethylene (●) at 1.5 °C and 85–90% relative humidity. Values are the means of three replicate samples ± SE.

the reaction was followed by measuring the change in absorbance at 470 nm for up to 5 min. POD activity was expressed as changes in absorbance per gram of fresh weight flavedo per minute.

Statistical Design. Experimental data are the mean ± SE of three replicate samples of the determinations for each sample. A mean comparison using Student's *t* test was performed to determine if the CI index and the activities of the enzymes analyzed in the ethylene-treated fruit were significantly different ($p < 0.05$) from those of the air-treaed fruit.

RESULTS

The development of CI symptoms in Fortune mandarins was reduced by applying a continuous flow of 2 μL L^{-1} ethylene during fruit cold storage (**Figure 1**). Peel damage increased continuously for up to 56 days in both ethylene- and air-treated fruit held at 1.5 °C, but it was higher in fruit stored under an ethylene-free atmosphere. Very slight chilling damage appeared in fruits treated with ethylene for 22 days at 1.5 °C (CI index = 0.4). However, after this period, the CI index of the control air-treated fruit was ~2.3-fold higher and already detectable from the commercial point of view. At the end of the storage period (56 days), the CI index of fruit kept under ethylene was still ~70% of that of fruit held under air (**Figure 1**).

Little changes in PAL activity were found in the flavedo of fruit maintained under air for up to 14 days at 1.5 °C. The activity of this enzyme greatly increased thereafter, reaching a maximum in fruit kept for 44 days at 1.5 °C (**Figure 2**). A 5-fold increase in the activation of PAL occurred during this period. Ethylene exerted a marked effect on the induction of PAL in fruit held at low temperature. PAL activity sharply increased in the flavedo of the ethylene-treated fruits from the beginning of the storage at 1.5 °C. Thus, the activity of this enzyme in the flavedo of fruit stored under air was ~25% that of the ethylene-treated fruit by 14 days of storage. Fewer differences were found thereafter, but PAL was always higher in fruit held under ethylene than in fruits kept at the same temperature and for the same period of time under air (**Figure 2**).

SOD activity in fruit stored at low temperature under ethylene was, in general, lower than in fruit stored under an ethylene-free atmosphere (**Figure 3**). The activity of this enzyme declined in response to cold stress, and such decline was higher in fruit kept under ethylene. Thus, after 13 days of storage, SOD activity was significantly lower ($P \leq 0.05$) in the ethylene-treated fruit, which showed less CI (**Figure 1**).

The effect of ethylene on the cold-induced changes in the activities of the hydrogen peroxide detoxifying enzymes CAT, APX, and GR is shown in **Figure 4**. CAT activity decreased during storage of fruits at 1.5 °C in air (**Figure 4A**). Applying continuously 2 μL L^{-1} ethylene at 1.5 °C exerted little effect on the activity of this enzyme at the beginning of the storage period, but by 33 days the CAT activity of fruit held under ethylene was significantly higher than that of fruit held under air. Cold stress, in the absence of ethylene, produced little changes in the activity of the enzyme APX (**Figure 4B**). The activity of this enzyme increased in Fortune mandarin fruit maintained under ethylene for up to 33 days of cold storage, but the differences found between air- and ethylene-stored fruit were significant only at $P = 0.3$. As it occurred with PAL (**Figure 2**), the activity of the enzyme APX sharply decreased in the ethylene-treated fruit stored for prolonged periods (2 months) at low temperature (**Figure 4B**). No differences in GR activity were found between fruit stored under air or ethylene. As shown in **Figure 4C**, GR remained nearly constant for up

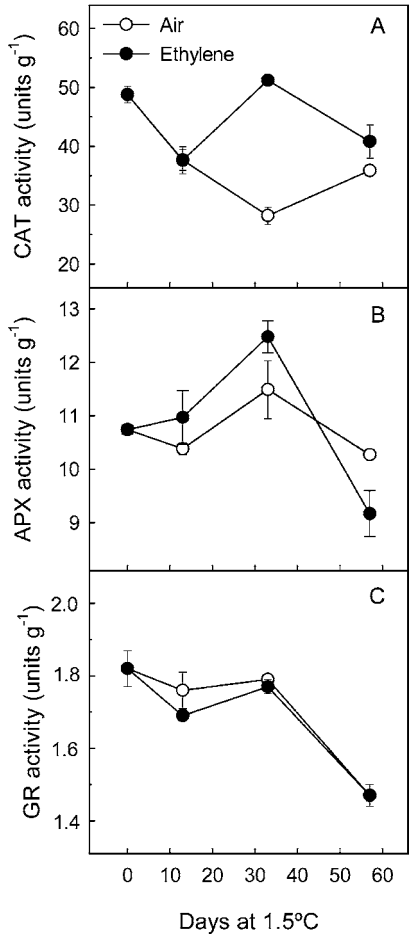

Figure 4. Changes in the activities of CAT (**A**), APX (**B**), and GR (**C**) in flavedo tissue of Fortune mandarin fruits stored for up to 56 days under air (○) or 2 μL L^{-1} ethylene (●) at 1.5 °C and 85–90% relative humidity. Values are the means of three replicate samples ± SE.

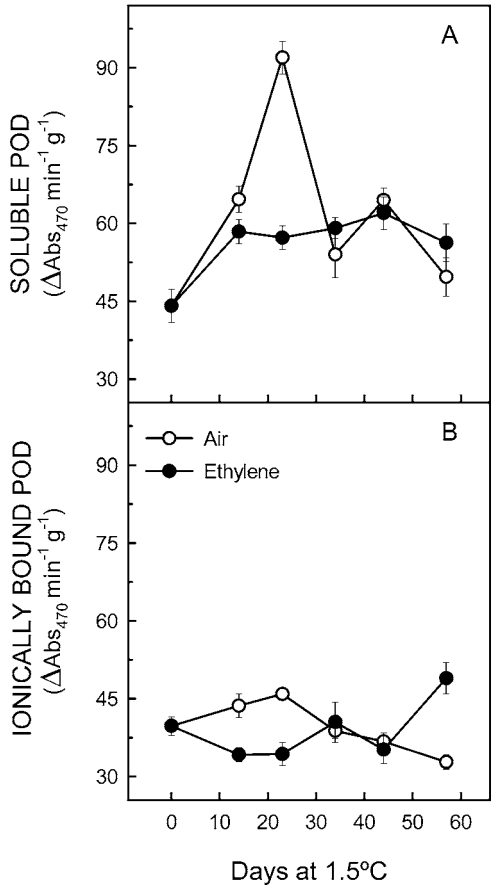

Figure 5. Changes in the activities of soluble (**A**) and insoluble (**B**) POD in flavedo tissue of Fortune mandarin fruits stored for up to 56 days under air (○) or 2 μL L^{-1} ethylene (●) at 1.5 °C and 85–90% relative humidity. Values are the means of three replicate samples ± SE.

to 33 days at 1.5 °C, and it was considerably reduced by prolonging fruit exposure to low temperature under both air or ethylene atmospheres.

Soluble POD activity increased for up to 22 days in response to cold stress in air-treated Fortune fruit. Thereafter, it decreased and by the end of the storage period was similar to that of freshly harvested mandarins (**Figure 5A**). The activity of this enzyme was lower in fruit held at low temperature under the ethylene atmosphere than in the air-treated fruit for up to 22 days of cold storage. Similar levels of soluble POD were found thereafter between fruit exposed to both treatments (**Figure 5A**). In contrast, insoluble POD, which it is thought to be localized in the cell wall, showed little change in fruits kept for up to 22 days at 1.5 °C in air and slightly decreased thereafter, being by the end of the experiment (56 days) lower than that of freshly harvested fruit (**Figure 5B**). In fruit kept under ethylene, the activity of the ionically bound POD decreased by 13 days storage and remained lower than in the air-treated fruit for up to 33 days of storage at low temperature (**Figure 5B**).

DISCUSSION

Treating Fortune mandarin fruits with ethylene during cold storage reduced the chilling-induced damage, which reinforces the idea that ethylene plays a role in the enhancement of the tolerance of citrus fruits to low temperature (*10*). The involvement of ethylene in the development of brown necrotic tissue areas, as those found in chilled Fortune mandarins, and in the chilling responses of fruit and vegetables may differ among species and organs (*8*, *10*, *12*). Those differences should be explained by the effect of ethylene on the mechanisms operating in the development of these kinds of physiological disorders in the different species. Thus, for example, the incidence of chilling-induced damage can be reduced by inhibiting ethylene action in avocado fruits because this hormone favored the induction of the enzyme polyphenol oxidase (PPO), which appears to be responsible for the development of chilling symptoms in this fruit (*29*), whereas PPO is not involved in CI of citrus fruit (*5*). We have previously reported that increased PAL activity before exposure of citrus fruit to chilling does not reduce their cold-induced peel damage, but the induction of PAL in response to low temperature plays a role in reducing the development of chilling symptoms in this horticultural crop (*10*). Furthermore, it has been shown that the low-temperature-induced PAL activation is concomitant with the rise in ethylene occurring during fruit exposure to cold stress (*5*). The results presented here demonstrate that exogenous ethylene applied at low temperature (1.5 °C) was able to induce a rapid and great increase in PAL activity in the flavedo of Fortune mandarins (**Figure 2**) and reduced the incidence of chilling-induced damage (**Figure 1**). In addition, PAL was always higher in fruit held under ethylene than under air (**Figure 2**). Therefore, the present results reinforce the idea that the increase in PAL activity during exposure of chilling-sensitive citrus fruit to low temperature is important to reduce cold-induced peel damage and demonstrate that this can be achieved by applying ethylene levels (2 μL L^{-1}) similar to those used under commercial conditions for citrus

fruit degreening. Thus, PAL may play a role in regulating the rate of substrate supply for polyphenol synthesis in response to CI in citrus fruit. This response of citrus fruit differs from other horticultural crops such as lettuce, in which ethylene and PAL activity are important factors for the development of small brown blemishes (*8*, *9*) such as those induced by chilling in Fortune mandarins.

Besides studying the effect of applying ethylene on PAL activity during cold storage of Fortune mandarins, we focused our attention on the effect of ethylene on the oxygen-scavenging enzymes, because oxidative stress appears to participate in the development of chilling symptoms in citrus fruit (*1*, *4*). The relationship between ethylene and these enzymes has been studied in different plant systems, but controversial results have been reported (*3*, *9*, *16*, *17*). Our results indicate that the ethylene treatment did not enhance the ability of Fortune mandarins stored at low temperature to dismutate superoxide radicals, because the activity of the enzyme SOD was lower in the ethylene-treated fruit (**Figure 3**). This result is in accordance with observations indicating that ethylene, in association with low temperature, lowered the SOD activity in melon fruit, although ethylene favored the chilling-induced necrosis in this horticultural crop (*3*). The deleterious effect of ethylene favoring CI in melon fruit was also related to a reduction in CAT activity (*3*). The results found in the present work show that ethylene, applied during fruit cold storage, may increase the capacity of Fortune mandarins to remove an excess of hydrogen peroxide as CAT activity of fruit stored for 33 days under ethylene was significantly ($P \leq 0.05$) higher than that of fruit stored under air (**Figure 4A**). Thus, ethylene may increase the ability of Fortune fruit to cope with oxidative stress. The affinities of CAT (millimolar range) and APX (micromolar range) for hydrogen peroxide are very different. They belong to two classes of hydrogen peroxide-scavenging enzymes (*30*). Thus, although CAT is involved in the removal of excess AOS during stress conditions in plants, the enzyme APX, which has been reported to be one of the most important antioxidant enzymes in defense against low-temperature injury (*31*), appears to be responsible for the fine modulation of AOS for signaling (*30*). Our results showed that the activity of the enzyme APX increases in Fortune mandarins held under ethylene for up to 33 days of cold storage (**Figure 4B**), but no significant differences ($P \leq 0.05$) between fruit exposed to air and ethylene were observed. Our data suggest then that this enzyme appears not to play an important role in the beneficial effect of ethylene increasing the chilling tolerance of Fortune mandarins by modulating hydrogen peroxide. GR, another enzyme of the Halliwell−Asada cycle participating in the detoxification of hydrogen peroxide in plant tissues, was not regulated by ethylene in fruit exposed to low temperature (**Figure 4C**). By the end of the storage period the APX activity sharply decreased in the ethylene-treated fruit and was lower than that of the air-treated ones. This could be the result of aging of the flavedo tissue because such decline occurred in fruit stored for 56 days and ethylene could enhance fruit senescence (*16*). In fact, GR and PAL activities also showed a sharp decrease in air- and ethylene-treated fruit after 33 days storage, and it has been reported that PAL increases with the development of CI symptoms (*32*). Therefore, it could be speculated that the ability of Fortune mandarins to stimulate defense mechanisms against chilling diminished in aged fruit that had been stored for prolonged periods (2 months) at low temperature.

POD plays also a role in the breakdown of hydrogen peroxide, and therefore a chilling-induced increase in POD could be related to a defense mechanism of fruit to cope with oxidative stress. This enzyme has been shown to be activated by ethylene in horticultural crops such as lettuce and *Cucurbita maxima* (*9*, *18*). However, the rate of increase in soluble POD activity of Fortune mandarins stored under air at low temperature was significantly ($P \leq 0.05$) higher than that of fruit held under an ethylene atmosphere for up to 22 days of cold storage (**Figure 5A**). This is in agreement with previous results showing that ethylene reduces POD activity in chilled cantaloupe melons (*3*). As ethylene-treated Fortune mandarins showed lower CI indices, it appears that the initial higher induction of this enzyme in the flavedo of the air-treated fruit might be a consequence of the major cold-induced oxidative stress occurring in fruit treated with air, although such induction was not sufficient to avoid the chilling-induced peel damage. Results from Hyodo et al. (*18*) imply that wound-induced POD activity may led to stimulation of polymerization of monolignols to form lignin macromolecules in the mesocarp tissue of *C. maxima*, and heal the injury, and that the induction of POD is closely dependent on ethylene. Our results may indicate that the induction of soluble POD in the air-cold-stored Fortune mandarins could be a protective response occurring in the fruits to reduce the development of chilling symptoms, whereas the beneficial effect of ethylene increasing the chilling tolerance of citrus fruit appears to be related to other physiological and morphological processes (*33*) rather than to changes in the activity of this enzyme. Ionically bound POD may alter the cell wall properties, by promoting the cross-linking between molecules such as lignin, suberin, proteins, hemicelluloses, and ferulic acid, potentially important for plants in order to cope with stress conditions (*7*, *34*). Ethylene induced ionically bound POD activity in lettuce that correlated with development of russet spotting symptoms (*9*). However, the ionically bound POD was not stimulated by ethylene in the flavedo of Fortune mandarins (**Figure 5B**) and, therefore, the beneficial effect of ethylene increasing its chilling tolerance appears not to be related to its effect on changes in POD-related cell wall properties. In addition, ionically bound POD did not increase with peel damage development, further supporting the lack of its involvement in the response of mandarins to chilling during low-temperature storage.

In conclusion, the beneficial effect of applying ethylene during cold storage of Fortune mandarins in reducing the incidence of CI appears to be mainly related to its effect on increasing the activity of the enzyme PAL. In addition, our results imply that ethylene applied at low temperature may increase the flavedo tissue antioxidant potential by favoring the induction of CAT.

ABBREVIATIONS USED

APX, ascorbate peroxidase; CAT, catalase; CI, chilling injury; GR, glutathione reductase; PAL, phenylalanine ammonia-lyase; POD, peroxidase; SOD, superoxide reductase.

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