Phenylalanine Ammonia-lyase As Related to Ethylene in the Development of Chilling Symptoms during Cold Storage of Citrus Fruits

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Low-temperature, nonfreezing, storage induces pitting and necrosis in the flavedo tissue of chilling susceptible citrus fruits. In this study the role of ethylene and phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) in the cold-induced citrus peel damage has been investigated. It has been shown that increasing PAL activity by applying ethylene at a nonchilling temperature did not cause fruit damage or reduce the incidence of this peel disorder when fruits were subsequently held at a chilling temperature (2 °C). The cold-induced peel damage was enhanced by applying inhibitors of PAL activity and ethylene synthesis and action. These results indicate that the induction of PAL and ethylene during fruit cold storage, but not before, plays a role in reducing the development of chilling symptoms. The cold-induced PAL activity was reduced by inhibitors of ethylene production, but inhibitors of ethylene action exerted little effect on the activation of this enzyme. Therefore, the activation of PAL may be dependent on ethylene but also an independent cold signal apparently related to the cold-induced peel damage.

Keywords: *Citrus; cold storage; chilling injury; ethylene; α-aminooxy-β-phenylpropionic acid (AOPP); 2-aminoethoxyvinylglycine (AVG); 1-methylcyclopropene (1-MCP); phenylalanine ammonia-lyase (PAL); silver thiosulfate (STS)*

INTRODUCTION

The storage of citrus fruits at low temperature is necessary to extend their commercial life. However, pitting, necrosis, and staining may occur in the flavedo tissue (the outer colored part of the peel) of many citrus cultivars when exposed to low, nonfreezing, temperatures. An understanding of the biochemical and physiological mechanism underlying this cold-induced peel physiological disorder is important to reduce postharvest losses. Considering the chilling injury (CI) symptoms described above, we undertook in a previous work to study the effect of low-temperature storage on changes in activities of phenolic metabolism enzymes in the flavedo. We showed that there is no relationship between the development of chilling symptoms in Fortune mandarin fruits, a very chilling-sensitive citrus cultivar, and changes in polyphenol oxidase and peroxidase activities but that phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity increased in those fruits stored under chilling temperatures (*1*). Chilling also induced an increase in ethylene production in this citrus cultivar. However, the exact role of PAL and ethylene on the process of CI in not understood. The activity of the enzyme PAL can be stimulated by stressful conditions and has been related to the appearance of physiological disorders that may be induced by very low levels of ethylene in other commercial horticultural crops (*2*, *3*). As CI in Fortune mandarins, these disorders are manifested as brown necrotic tissue areas. On the other hand, the induction of ethylene and PAL activity in response to stressful conditions has been considered to be a defensive mechanism of plants against stress (*4*, *5*). PAL is the enzyme at the entry point of the phenylpropanoid pathway. Phenolic compounds may play a role in necrosis and also in maintaining cellular viability (*6*). Besides those properties, phenolic compounds are known to be antioxidant compounds and, interestingly, oxidative stress has been shown to be involved in the chilling tolerance of citrus fruits (*7*). The activation of PAL in citrus by applying ethylene (*8*) or stresses that may favor the ethylene biosynthesis such as mechanical wounding (*9*) and γ-radiation (*10*) has been also reported. It has not been demonstrated, however, whether the cold-induced ethylene is a signal responsible for the induction of PAL.

Whether the increase in PAL activity is responsible for the development of chilling symptoms, is a consequence of the cold-induced damage, or may play a role in reducing chilling symptoms in citrus has not been studied until now. Some of the mechanisms underlying the chilling-induced ethylene production have been described.

Chilling-induced ethylene biosynthesis in citrus fruits is accompanied by 1-aminocyclopropane-1-carboxylic acid (ACC) accumulation and also by an increase in ACC oxidase activity (*11*). A chilling-inducible ACC synthase gene, CS-ACS1, and a chilling-repressible gene, CS-ACS2, from citrus peel have been also isolated and characterized (*12*). However, as in the case of PAL, little is known about the role of the cold-induced ethylene in

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citrus fruits. In cantaloupe melons, a climacteric fruit, inhibition of ethylene biosynthesis by antisense ACC oxidase RNA prevented CI (*13*).

Our research goal in the present work was to understand how ethylene and PAL are involved in the process of CI in citrus fruits. To elucidate that, we have determined whether (1) the tolerance of fruits to chilling varies by applying ethylene or by increasing their PAL activity before exposing them to chilling; (2) the cold-induced ethylene production and PAL activity are the cause of CI or, on the contrary, physiological responses of the fruits to reduce or repair the peel damage induced by chilling; and (3) the cold-induced ethylene is responsible for the cold-induced PAL activity.

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). For each experiment, fruits were divided at random into two groups. The first group was used to determine changes in PAL activity and ethylene production. Three replicates of at least 10 fruits for PAL analysis and of 3−4 fruits for ethylene production measurement per temperature and storage period were included in this group. For PAL analysis, flavedo samples were excised from the total surface of fruits and ground with a food chopper in liquid N_2 to a fine powder; the homogenized sample was stored at −70 °C until PAL assay. The second group contained 60 fruits to determine the severity of the chilling-induced peel damage.

Chemicals and Reagents. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except α-aminooxy-β-phenylpropionic acid (AOPP), which was obtained from Cambridge Research Biochemicals (Cambridge, U.K.).

Chilling, Ethylene, and Chemical Treatments. Fortune mandarin fruits were stored at a chilling temperature (2 °C) and 80−85% relative humidity (RH) for at least 21 days in constant darkness.

To test the effect of exogenous ethylene on peel damage and PAL activity, freshly harvested Fortune fruits were treated in a continuous flow of 10 μL L^{-1} ethylene at 20 °C for 6 and 12 h and 1, 4, 10, and 17 days in 20 L vessels in the presence of KOH 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. To estimate whether the tolerance of fruits to chilling may be modified by applying ethylene or by increasing their PAL activity before holding them under cold stress, fruits treated with 10 μL L^{-1} ethylene (ethylene-pretreated fruits) or air (air-pretreated fruits) at 20 °C for the indicated period of time were then exposed to 2 °C for 24 days.

Inhibitors of ethylene synthesis and action and of PAL activity were applied to freshly harvested Fortune mandarins before chilling exposure to study the role of PAL and ethylene in the response of fruits to chilling. Ethylene action was inhibited by 1 mM silver thiosulfate (STS) and by 1 μL L^{-1} 1-methylcyclopropene (1-MCP). The STS aqueous solution was prepared according to the method of Reid et al. (*14*) and applied by dipping the fruits in the solution for 2 min. 1-MCP was prepared as previously described (*15*), and the treatment was carried out for 24 h at 20 °C in a 20 L vessel in the presence of KOH to eliminate CO_2. After fumigation with 1-MCP, fruits were removed from the vessel and left in the dark for 12 h before they were transferred to cold storage at 2 °C. To estimate the effect of 1-MCP, fruits that were treated for 36 h (24 + 12 h) with air under the same experimental conditions were used as control fruits. The cold-induced ethylene production was inhibited by dipping the fruits in aqueous solutions containing 0.25 mM 2-aminoethoxyvinylglycine (AVG) (Sigma Chemical Co., St. Louis, MO) or 1 mM cobalt chloride ($CoCl_2$) (Sigma Chemical Co.) for 2 min before they were exposed to cold stress. To inhibit PAL activity, fruits were dipped for 2 min in an aqueous solution containing 1 mM AOPP. All of the fruits treated with the inhibitors were subsequently exposed to 2 °C for 21 days to evaluate their effect on CI, the cold-induced PAL activity, and ethylene production. An equal number of fruits were exposed at 12 °C (control, nonchilling temperature) after being treated with these inhibitors to test their phytotoxicity in fruits held at a nonchilling temperature.

Estimation of CI Index. Fruits were visually scored to estimate the extent of CI development. Brown pitlike 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 the average CI index determined. The results are means of samples containing 60 fruits.

Assay of PAL Activity. PAL activity was determined in three replicate samples from flavedo acetone powder according to the method of Martínez-Téllez and Lafuente (*1*). A representative ground flavedo tissue sample, processed as described above, was ground in10 mL of acetone, previously chilled to −20 °C, per gram of flavedo. The homogenate was filtered through a Büchner funnel, the residue washed twice with chilled acetone, and the resulting powder dried 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. The extract was purified by salting out proteins with ammonium sulfate at a final saturation of 46%. The precipitated PAL enzyme was dissolved in 4.5 mL of 100 mM ammonium acetate buffer, pH 7.7, containing 20 mM β-mercaptoethanol and the PAL activity 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 on a dry-matter basis as nanomoles of cinnamic acid per gram of acetone powder flavedo tissue per hour. The results are the mean of three replicate samples of 10 fruits each.

Measurement of Ethylene Production. Ethylene production from whole fruits was measured periodically by incubating three replicate samples of four Fortune mandarin fruits in 1 L glass jars at 2 °C. After 4 h of incubation at this temperature, a 1 mL gas sample was withdrawn from the headspace of the jar and injected in a gas chromatograph Perkin-Elmer autosampler (Norwalk, CT), equipped with a 1 m × 2 mm activated alumina column (80/100 mesh) from Supelco (Barcelona, Spain) and a flame ionization detector. Nitrogen was used as carrier gas, and the temperature of the column was maintained at 140 °C. The ethylene standard was obtained from Abello-Oxígeno-Linde, S.A. (Valencia, Spain). The results are the mean of three replicate samples of four fruits each.

Statistical Design. Experimental data are the mean ± SE of three replicate samples of the determinations for each sample. A variance analysis using the Tukey test at the 5% level was performed to determine if the CI index, ethylene production, and PAL activity induced by cold stress in fruits treated with the different inhibitors of ethylene synthesis and action and of PAL activity showed significant differences ($p < 0.05$). The Tukey test was also performed to determine if the chilling susceptibility and the chilling-induced PAL activity of fruits pretreated with air or ethylene were different.

RESULTS

Increasing PAL Activity before Cold Storage by Applying Ethylene Does Not Cause Peel Damage or Increase the Tolerance of the Fruits to Chilling. A sharp increase in PAL activity was induced in Fortune mandarins by applying continuously 10 μL L^{-1} ethylene at 20 °C, whereas the activation of PAL did not occur in the air-treated samples at this temperature (Figure 1). PAL activity greatly increased for up to 4 days of ethylene treatment and subsequently declined for up to 17 days of ethylene exposure. After 1 day of ethylene

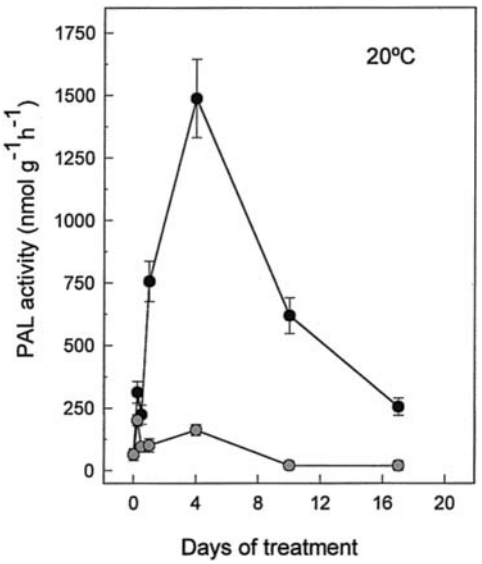

Figure 1. Time course of PAL activity in flavedo tissue of Fortune mandarin fruits treated at 20 °C with 10 μL L^{-1} ethylene (black circles) or air (gray circles) for 0, 6, and 12 h and 1, 4, 10, and 17 days. Values are the means of three replicate samples ± SE.

treatment at 20 °C (Figure 1), PAL activity in the flavedo was higher than the cold-induced PAL activity in fruits stored for 24 days at a chilling temperature (time 0 h pretreatment, Figure 2B) that induced peel damage (time 0 pretreatment, Figure 2A). However, those fruits treated with ethylene at a nonchilling temperature (20 °C) did not show peel damage (data not shown).

The PAL activity of the ethylene-treated fruits (Figure 1) decreased in general after the fruits had been transferred to an ethylene-free atmosphere at 2 °C (Figure 2B). After the pretreated fruits had been held at 2 °C for 24 days, the PAL activity of the fruits exposed to the ethylene pretreatment, either for short periods or for long periods (17 days), was similar to or even higher than that of their control air-pretreated fruits (Figure 2B). The tolerance of Fortune mandarins to chilling was not increased by increasing PAL activity before fruits were exposed to chilling (Figure 2A). Fruits pretreated with ethylene for more than 1 day showed a higher chilling damage than the air-treated fruits during cold storage (Figure 2A).

Effect of Inhibitors of Ethylene and PAL on CI and Cold-Induced PAL Activity and Ethylene Production. The specific competitive inhibitor of PAL activity, AOPP, favored the development of CI symptoms (Figure 3). After 21 days at 2 °C, the CI index of the AOPP-treated fruits was 2.2 and that of the nontreated fruits 1.8, indicating that the cold-induced increase in PAL activity is not the cause of peel damage. To inhibit ethylene production, fruits were treated with inhibitors of the two enzymes involved in the ethylene biosynthesis. Thus, we used AVG, and inhibitor of ACC synthase, or $CoCl_2$, which inhibits ACC oxidase. Both compounds showed an effect on CI similar to that of the inhibitor of PAL activity (AOPP). After 21 days of cold storage, the CI index of fruits treated with AVG or $CoCl_2$ was 2.3 (Figure 3). Ethylene action was inhibited by applying the ethylene perception inhibitors, silver ions (STS) or 1-MCP, to the fruits before they were exposed to cold storage. Both inhibitors exerted a more marked

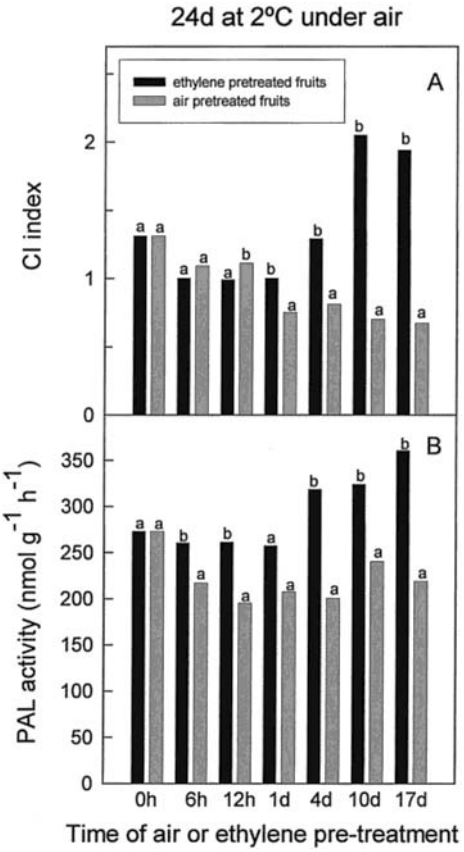

Figure 2. Effect of air and ethylene pretreatment on chilling susceptibility of Fortune mandarins (A) and on chilling-induced PAL activity in flavedo tissue of Fortune fruits (B). Fruits were pretreated with 10 μL L^{-1} ethylene (black bars) or air (gray bars) for 0, 6, and 12 h and 1, 4, 10, and 17 days at 20 °C and subsequently held for 24 days at 2 °C in an ethylene-free atmosphere. Results of CI index are means of 60 fruits and those of PAL activity of 3 replicate samples. For the same time of air or ethylene pretreatment, values labeled with the same letter are not different at the 5% significance level.

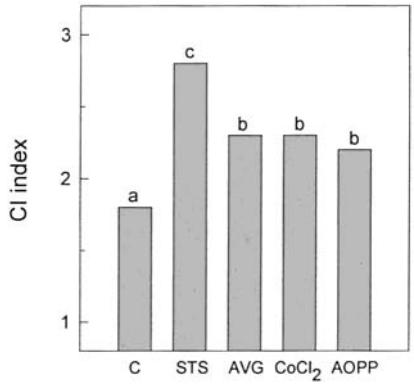

Figure 3. Effect of inhibitors of ethylene action and synthesis and of PAL activity on chilling susceptibility of Fortune mandarins. Fruits were dipped for 2 min in aqueous solutions containing 1 mM STS to inhibit ethylene action, 0.25 mM AVG or 1 mM $CoCl_2$ to inhibit ethylene synthesis, or 1 mM AOPP to inhibit PAL and then exposed for 21 days at 2 °C. Values labeled with the same letter are not different at the 5% significance level.

effect in increasing the cold-induced peel damage than AVG or $CoCl_2$. The CI index of fruits treated with STS was 2.8 (Figure 3). Fruits treated with 1-MCP showed a CI index of 1.9, whereas that of their respective control

Table 1. Cold-Induced Peel Damage, PAL Activity, and Ethylene Production As Affected by 1-MCP

treatment	CI index	PAL activity (nmol h^{-1} g^{-1})	ethylene production (nL g^{-1} h^{-1})
+ 1-MCP	1.91 ± 0.05^b	390.2 ± 15.6^a	0.23 ± 0.03^b
− 1-MCP	1.23 ± 0.04^a	360.5 ± 24.3^a	0.12 ± 0.02^a

[a] Fortune fruits were incubated with 1-MCP for 24 h at 20 °C and subsequently ventilated in air for 12 h at 20 °C before they were exposed for 21 days at 2 °C. Control fruits (−MCP) were incubated for the same period of time in air (36 h) and then exposed for 21 days to cold stress. Values represent means ± SE. Values labeled with the same letter within the same column are not different at the 5% significance level.

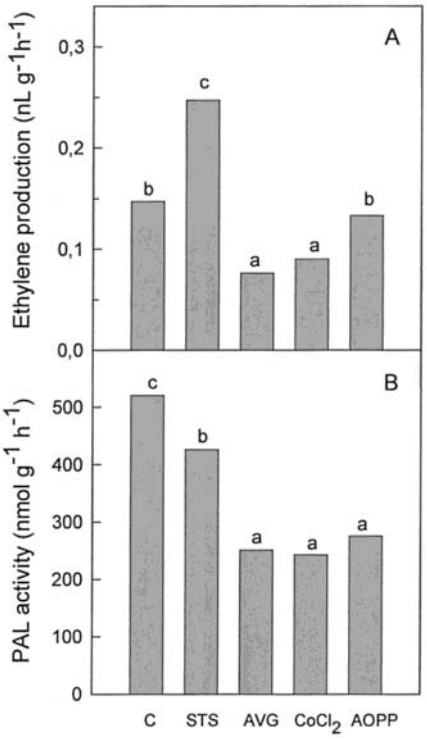

Figure 4. Effect of inhibitors of ethylene action and of inhibitors of the synthesis of ethylene and PAL on chilling-induced ethylene production of Fortune fruits (A) and on chilling-induced PAL activity in the flavedo tissue (B). Fruits were dipped in aqueous solutions containing 1 mM STS, 0.25 mM AVG, 1 mM $CoCl_2$, or 1 mM AOPP for 2 min and then exposed for 21 days at 2 °C. Results are means of three replicate samples, and values labeled with the same letter are not different at the 5% significance level.

fruits treated only with air was 1.2 (Table 1). Peel damage was not induced by any of these inhibitors in fruits held at the nonchilling temperature (data not shown).

After 21 days at 2 °C, the PAL activity of fruits treated with AOPP was ~53% that of untreated control fruits (Figure 4B). The cold-induced ethylene production was barely affected by AOPP, and it was reduced to about 52 and 61% by the inhibitors of ethylene synthesis, AVG and $CoCl_2$, respectively (Figure 4A). Both ethylene inhibitors were also effective in inhibiting the cold-induced PAL activity, which was about 48% (AVG) and 47% ($CoCl_2$) that of control fruits. When ethylene action was inhibited by STS (Figure 4A) or 1-MCP (Table 1), a 1.7- or 1.9-fold increase in the cold-induced ethylene production was found, respectively, with respect to their control fruits. STS was scarcely effective in inhibiting the cold-induced PAL activity (Figure 4B), whereas 1-MCP even enhanced the activation of the enzyme in fruits held at the chilling temperature (Table 1).

DISCUSSION

Whether the rise in ethylene production and PAL activity during cold storage of chilling-sensitive citrus cultivars is caused by or a consequence of or may play a role in reducing the development of CI symptoms in citrus fruits is not well understood. Moreover, if the PAL increase following chilling could be ascribed to the triggering effect of ethylene is unknown. In response to other stresses such as mechanical wounding controversial results have been found; PAL activity may be dependent on but also independent of ethylene production in wounded melon fruits (*16*). In lettuce, however, wound-induced PAL activity is not due to the wound-induced ethylene (*17*). To elucidate (a) if the cold-induced ethylene and the cold-induced PAL activity were the cause of CI symptoms or, on the contrary, physiological responses of the fruit to reduce or heal the peel damage induced by chilling and (b) if ethylene is a signal for the cold-induced PAL, we manipulated ethylene and PAL in fruits of Fortune mandarin, which are very sensitive to chilling.

In the first approach we applied exogenous ethylene for up to 17 days at a nonchilling temperature just to ensure that ethylene by itself, or by increasing PAL and subsequently the levels of phenolics which could be oxidized to brown compounds, did not cause peel damage in Fortune mandarins. The sharp increase in PAL activity induced by ethylene (Figure 1) was much higher than the cold-induced PAL activity in fruits stored at a chilling temperature that induced peel damage (time 0 h pretreatment, Figure 2B). However, Fortune fruits treated with ethylene at 20 °C did not show peel damage. This result suggests that ethylene and the increase in PAL activity appear not to be the cause of the development of CI symptoms. Thus, citrus fruit differs from other horticultural crops such as lettuce in which ethylene and PAL activity by themselves are important factors for russet spotting development (*2*, *3*). This physiological disorder may be induced by low levels of ethylene (0.1 μL L^{-1}) and, like CI in Fortune mandarins, is characterized by the appearance of numerous small brown spots.

PAL is the initial rate-controlling enzyme in phenolic synthesis, and phenolic compounds are known to have antioxidant properties (*18*). In previous papers we suggested that oxidative stress may be involved in the chilling tolerance of citrus fruits (*7*) and that the efficacy of a heat pretreatment delaying CI symptoms in cold-stored fruits could be related to its ability to stimulate a transient induction of PAL, via phenylpropanoid metabolite intermediates (*19*). Considering the sharp increase in PAL activity induced by the ethylene treatment (Figure 1), we have examined the chilling response of the fruits previously treated with ethylene as compared with that of fruits treated with air (control fruits). Interestingly, fruits pretreated with ethylene for more than 1 day showed a higher chilling damage upon cold exposure. Therefore, we might conclude that the induction of a higher level of PAL activity in the flavedo before fruits are exposed to chilling stress (2 °C), which could increase the ability of the tissue to cope with oxidative stress, does not increase their tolerance to chilling. However, the idea that the lack of efficacy of the ethylene pretreatment could be due to the fact that

ethylene may alter another mechanism favoring CI cannot be ruled out. Additionally, ethylene could enhance fruit senescence and consequently favor its sensitivity to stress conditions (*4*). This result is in contrast to that found in tomato plants, in which ethylene application led to morphological and physiological changes which could alleviate chilling (*20*, *21*). This different behavior may be associated with the fact that ethylene does not control all processes of chilling tolerance (*22*) and those processes may differ between plant species or organs. The activity of PAL in the ethylene-treated fruits may sharply decrease when fruits are transferred to an ethylene-free atmosphere at 2 °C, but in general it was still slightly higher than in the air-pretreated Fortune mandarin fruits (Figure 2). The higher PAL activity of the cold-stored fruits pretreated with ethylene could be a consequence of the higher cold-induced damage and also of the higher initial levels of PAL activity. From the results of this experiment we cannot discriminate whether the induction of PAL in response to cold stress is playing a protective role against CI development or is a consequence of the cold-induced peel damage.

More conclusive results about the role of PAL and ethylene in the response of citrus fruits to chilling were obtained when the levels of the cold-induced PAL activity and ethylene production were decreased by the addition of specific inhibitors. These inhibitors did not cause peel damage in fruits held at a nonchilling temperature (12 °C). The specific inhibitor of PAL activity, AOPP, significantly favored the development of CI symptoms, as indicated by the CI index (Figure 3), and did not affect the cold-induced ethylene production (Figure 4A). This result supports the idea that the induction of PAL in the flavedo is a defense mechanism against chilling rather than the cause of peel damage. The inhibition of PAL activity by AOPP in other plants exposed to different stressful conditions also favored the stress-induced necrosis (*23*). In citrus, the participation of this enzyme in the healing process in mechanically wounded fruits has been suggested, although the effect of a specific inhibitor of PAL activity was not studied (*9*). The inhibition of the cold-induced PAL activity during exposure of Fortune fruits to chilling stress enhanced CI, but increasing its activity before the fruits were exposed to chilling did not protect them from this stress condition. Our results may then indicate that the induction of PAL in the cold-exposed Fortune mandarins could be a protective response occurring in the fruits, once the damage originated by chilling would have happened, to repair or heal the injury. Thus, lignification could be stimulated or some specific phenolics could be formed in response to chilling stress mediated by the induction of ethylene and PAL, which would efficiently reduce the development of chilling symptoms.

Decreasing the level of cold-induced ethylene production by addition of inhibitors of the two enzymes involved in ethylene biosynthesis, ACC synthase (AVG) and ACC oxidase ($CoCl_2$), reduced the cold-induced PAL and also enhanced chilling. Therefore, we can conclude that ethylene is also a defense mechanism of citrus fruits to cope with chilling stress and that ethylene appears to be responsible for the cold-induced PAL. These results reinforce the idea that ethylene and the ethylene-induced PAL activity are important during, but not before, the development of the cold-induced damage. Both mechanisms appear to be necessary to reduce peel damage but not sufficient to avoid it. The involvement of ethylene in the chilling response may differ among species. Ben-Amor et al. (*13*) showed, using transgenic cantaloupe melons in which ethylene production was almost completely inhibited by an antisense-ACO transgene, that ethylene acts in conjunction with low temperature to induce metabolic shifts that participate in the development of CI. STS and 1-MCP inhibit ethylene perception by competitively binding to the ethylene receptors (*24*). Exposure of ixora plants to 1-MCP completely blocked the chilling-induced leaf abscission even in the presence of exogenous ethylene, which enhanced abscission (*25*). However, in tomato plants, chill hardening was more effective in the ethylene-sensitive phenotype than in the ethylene-insensitive mutants (*22*). The application of inhibitors of ethylene action such as STS and 1-MCP did not induce peel damage during storage of Fortune mandarins at a nonchilling temperature but, as in other chilling-sensitive cultivars, favored CI during holding of the fruits at 2 °C (*26*, *27*). The STS and 1-MCP concentrations used in the present study were effective in inhibiting ethylene action in Fortune mandarins because they enhanced the cold-induced ethylene production (Figure 4A; Table 1). In citrus fruits, ethylene exerts an inhibitory feedback mechanism of its own biosynthesis. Thus, for an effective inhibition of ethylene perception, STS or 1-MCP should shut down the ethylene feedback system and an increase in ethylene production should be expected (*28*, *29*). We have further demonstrated that when ethylene action was inhibited by STS or 1-MCP, CI was increased even more than by inhibitors of ethylene biosynthesis. Inhibitors of ethylene action exerted little effect on the cold-induced PAL, which increased with CI symptom development. The cold-stored fruits treated with these competitive inhibitors showed a noticeably higher PAL activity than fruits treated with the inhibitors of ethylene synthesis (Figure 4B; Table 1). Therefore, we cannot rule out the idea that the cold-induced PAL activity in the fruits may be also an independent ethylene cold signal, apparently related to the cold-induced peel damage. This is in agreement with previous results showing that regulation of PAL gene expression in melon is a coordinated process in response to both ethylene and an ethylene-independent wound signal (*16*). Results from Ke and Saltveit (*17*) imply that wound-induced PAL activity is not due to the wound-induced ethylene production in lettuce. In other stress conditions, such as pathogenic inoculation, it has been also suggested that the increases in ethylene production and PAL activity are independent responses (*30*, *31*).

From the overall results obtained in this work we can conclude that (1) the induction of PAL activity during the exposure of chilling sensitive citrus fruits to cold stress is important to reduce cold-induced peel damage, but increasing the activity of the enzyme before they are stored at low temperature does not protect the fruits against chilling; (2) the inductions of ethylene and PAL in citrus fruits in response to cold stress are defense mechanisms against chilling rather than the cause of pitting; and (3) the activation of the enzyme PAL is a coordinated process in response to both ethylene and an ethylene-independent cold signal.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Martínez-Téllez, M. A.; Lafuente, M. T. Effect of high-temperature conditioning on ethylene, phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase activities in flavedo of chilled 'Fortune' mandarin fruit. *J. Plant Physiol.* **1997**, *150*, 674−678.

(2) Hyodo, H.; Kuroda, H.; Yang, S. F. Induction of phenylalanine ammonia-lyase and increase in phenolics in lettuce leaves in relation to the development of russet spotting caused by ethylene. *Plant Physiol.* **1978**, *62*, 31−35.

(3) Ke, D.; Saltveit, Jr., M. E. Plant hormone interaction and phenolic metabolism in the regulation of russet spotting in iceberg lettuce. *Plant Physiol.* **1988**, *88*, 1136−1140.

(4) Yang, S. F.; Hoffman, N. E. Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* **1984**, *35*, 155−189.

(5) Dixon, R. A.; Paiva, N. L. Stress-induced phenylpropanoid metabolism. *Plant Cell.* **1995**, *7*, 1085−1097.

(6) Tamagnone, L.; Merida, A.; Stacey, N.; Plaskitt, K.; Parr, A.; Chang, C. F.; Lynn, D.; Dow, J. M.; Roberts, K.; Martín, C. Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. *Plant Cell* **1998**, *10*, 1801−1816.

(7) Sala, J. M.; Lafuente, M. T. Catalase in the heat-induced chilling tolerance of cold-stored hybrid Fortune mandarin fruits. *J. Agric. Food Chem.* **1999**, *47*, 2410−2414.

(8) Riov, J.; Monselise, S. P.; Kahan, R. S. Ethylene-controlled induction of phenylalanine ammonia-lyase. *Plant Physiol.* **1969**, *44*, 631−635.

(9) Ismail, M. A.; Brown, G. E. Postharvest wound healing in citrus fruit: Induction of phenylalanine ammonia-lyase in injured 'Valencia' orange flavedo. *J. Am. Soc. Hortic. Sci.* **1979**, *104*, 126−129.

(10) Riov, J.; Monselise, S. P.; Kahan, R. S. Effect of gamma radiation on phenylalanine ammonia-lyase activity and accumulation of phenolic compounds in citrus fruit peel. *Radiat. Bot.* **1968**, *8*, 463−466.

(11) Zacarías, L.; Dupille, E.; Latche, A.; Lafuente, M. T. Cold-induced ethylene biosynthesis in fruits of Fortune mandarin. Presented at the Congress of Biology and Biotechnology of the Plant Hormone Ethylene, Crete, Greece, 1996; Abstract P14.

(12) Wong, W. S.; Ning, W.; Xu, P. L.; Kung, S. D.; Yang, S. F.; Li, N. Identification of two chilling-regulated 1-aminocyclopropane-1-carboxylate synthase genes from citrus (*Citrus sinensis* Osbeck) fruit. *Plant Mol. Biol.* **1999**, *41*, 587−600.

(13) Ben-Amor, M.; Flores, B.; Latché, A.; Bouzayen, M.; Pech, J. C.; Romojaro, F. Inhibition of ethylene biosynthesis by antisense ACC oxidase RNA prevents chilling injury in Charentais cantaloupe melons. *Plant Cell Environ.* **1999**, *22*, 1579−1586.

(14) Reid, M. S.; Paul, J. L.; Farhoomand, M. B.; Kofranek, A. M.; Staby, G. L. Pulse treatments with silver thiosulfate complex extend the vase life of cut carnations. *J. Am. Soc. Hortic. Sci.* **1980**, *105*, 25−27.

(15) Magid, R. M.; Clarke, T. C.; Duncan, C. D. An efficient and convenient synthesis of 1-methylcyclopropene. *J. Org. Chem.* **1971**, *36*, 1320−1321.

(16) Diallinas, G.; Kanellis, A. K. A phenylalanine ammonia-lyase gene from melon fruit: cDNA cloning, sequence and expression in response to development and wounding. *Plant Mol. Biol.* **1994**, *26*, 473−479.

(17) Ke, D.; Saltveit, M. E., Jr. Wound-induced ethylene production, phenolic metabolism and susceptibility to russet spotting in iceberg lettuce. *Physiol. Plant.* **1989**, *76*, 412−418.

(18) Larson, R. A. The antioxidants of higher plants. *Phytochemistry* **1988**, *27*, 969−978.

(19) Sanchez-Ballesta, M. T.; Zacarias, L.; Granell, A.; Lafuente, M. T. Accumulation of *PAL* transcript and PAL activity as affected by heat-conditioning and low-temperature storage and its relation to chilling sensitivity in mandarin fruits. *J. Agric. Food Chem.* **2000**, *48*, 2726−2731.

(20) Liptay, A.; Phatak, S. C.; Jaworski, C. A. Ethephon treatment of tomato transplants improves frost tolerance. *HortScience* **1982**, *17*, 400−405.

(21) Woodrow, L. Effects of ethylene on photosynthesis and partitioning in tomato *Lycopersicon esculentum* Mill. *J. Exp. Bot.* **1988**, *39*, 667−684.

(22) Ciardi, J. A.; Deikman, J.; Orzolek, M. D. Increased ethylene synthesis enhances chilling tolerance in tomato. *Physiol. Plant.* **1997**, *101*, 333−340.

(23) Mayr, U.; Michalek, S.; Treutter, D.; Feucht, W. Phenolic compounds of apple and their relationship to scab resistance. *J. Phytopathol.* **1997**, *145*, 69−75.

(24) Sisler, E. C.; Serek, M. Inhibitors of ethylene responses in plants at the receptor level: Recent developments. *Physiol. Plant.* **1977**, *100*, 577−582.

(25) Michaeli, R.; Philosoph-Hadas, S.; Riov, J.; Meir, S. Chilling-induced leaf-abscission of *Ixora coccinea* plants. I. Induction of oxidative stress via increased sensitivity to ethylene. *Physiol. Plant.* **1999**, *107*, 166−173.

(26) Martínez-Téllez, M. A.; Zacarías, L.; Lafuente, M. T. Ethylene and phenylalanine ammonia-lyase in relation to chilling injury of Fortune mandarins. In *Postharvest Physiology, Pathology and Technologies for Horticultural Commodities*: *Recent Advances*; Ait-Oubahou, A., El-Otmani, M., Eds.; Institut Agronomique et Vétérinaire Hassan II: Agadir, Marruecos, 1995; pp 257−262.

(27) Porat, R.; Weiss, B.; Cohen, L.; Daus, A.; Goren, R.; Droby, S. Effect of ethylene and 1-methylcyclopropene on the postharvest qualities of 'Shamouti' oranges. *Postharvest Biol. Technol.* **1999**, *15*, 155−163.

(28) Riov, J.; Yang, S. F. Effects of exogenous ethylene on ethylene production in citrus leaf tissue. *Plant Physiol.* **1982**, *70*, 136−141.

(29) Mullins, E. D.; McCollum, T. G.; McDonald, R. E. Ethylene: A regulator of stress-induced ACC synthase activity in nonclimateric fruit. *Physiol. Plant.* **1999**, *107*, 1−7.

(30) Meravy, L.; Machackova, I.; Cvikrova, M.; Eder, J. The effect of aminoethoxyvinylglicine and silver on ethylene synthesis and activity of phenylalanine ammonia-lyase in alfalfa cell suspension culture. *Plant Physiol. Biochem.* **1991**, *29*, 165−170.

(31) Boller, T. Ethylene and pathogenesis and disease resistance. In *The Plant Hormone Ethylene*; Mattoo, A. T., Suttle, J. C., Eds.; CRC Press: Boca Raton, FL, 1991; pp 293−314.

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