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Inhibition of ethylene biosynthesis by antisense ACC oxidase RNA prevents chilling injury in Charentais cantaloupe melons

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ABSTRACT

Non-freezing low temperature storage causes injury to melons and most other fruit and vegetables of tropical and subtropical origin. We demonstrate here that ethylene suppression through an antisense ACC oxidase (ACO) gene considerably reduced the sensitivity of Charentais cantaloupe melons to chilling injury. In contrast to wild-type fruit, antisense ACO melons did not develop the characteristic chilling injury of pitting and browning of the rind neither when stored at low temperature (3 weeks at 2 °C) nor upon rewarming. Treating antisense melons with 10 p.p.m. ethylene for more than 1 d prior to cold storage resulted in the restoration of chilling sensitivity. When the ethylene treatment was performed after cold storage, the chilling injury symptoms did not appear. The tolerance to chilling was associated with a lower accumulation of ethanol and acetaldehyde, reduced membrane deterioration and higher capacity of the fruit to remove active oxygen species. The activities of catalase, superoxide dismutase and peroxidase were markedly increased in antisense ACO fruit in comparison with wild-type fruit, particularly upon rewarming and post-storage ethylene treatment. Severe chilling injury symptoms were correlated with a lower activity of activated oxygen scavenging enzymes. These results demonstrate that ethylene acts in conjunction with low temperature to induce metabolic shifts that participate in the development of chilling injury.

Key-words: *Cucumis melo*; antisense ACC oxidase; Charentais cantaloupe melons; chilling injury; ethylene; free radical scavenging enzymes.

INTRODUCTION

Most fruit and vegetables of tropical and subtropical origin develop chilling injury at low non-freezing temperatures that limits their storability and causes significant post-harvest losses (Wang 1989). The cantaloupe melon, like all the other members of the Cucurbitacea family are chilling

sensitive (Tatsumi & Murata 1981; Miccolis & Saltveit 1995). A storage period of over 1 week at temperatures below 2 to 7 °C induces, in particular upon rewarming, pitting and browning of the rind associated with a loss of sensory quality.

Changes in membrane structure and composition are considered as the primary events of chilling injury and lead to a loss of permeability control and metabolic dysfunctioning (Lyons 1973; Whitaker 1992; Marangoni, Palma & Stanley 1996). Chilling injury can also be viewed as an oxidative stress related to a decrease in the activity of enzymes which remove active oxygen species such as catalase, peroxidase and superoxide dismutase (Ju *et al.* 1994; Sala 1998). The emerging model for chilling injury is of membrane lipids undergoing biophysical and chemical changes that lead to a cascade of biochemical reactions culminating in cell death and tissue deterioration (Marangoni *et al.* 1996). Because these events also occur in ripening/senescing tissues (Lester & Stein 1993; Lacan & Baccou 1996, 1998), it has been hypothesized that the plant hormone ethylene could play a role in the triggering of chilling injury. Earlier studies had already documented that some climacteric and non-climacteric fruit were more sensitive to chilling when treated with ethylene (Wang 1993; Yuen *et al.* 1995). However, there are a number of cases where exogenous ethylene was found to be beneficial in reducing chilling injury. For instance, Lipton & Ahroni (1979) showed that treating Honeydew muskmelon with ethylene (1000 p.p.m., 20 °C for 24 h) before cold storage (2.5 °C) reduced the development of chilling injury by at least 75%. In addition, some commodities, including tomatoes and papayas exhibit a decrease in the sensitivity to chilling injury as ripening proceeds and ethylene production increases (Wang 1993).

With the development of biotechnology, it has been possible to control ethylene production in fruit (Hamilton, Lycett & Grierson 1990; Oeller *et al.* 1991; Gray *et al.* 1994; Lelièvre *et al.* 1997). We have generated transgenic cantaloupe melons in which ethylene production was almost completely inhibited (Ayub *et al.* 1996). These melons were used as an experimental model in order to clarify the role of ethylene in chilling injury. In this paper we show that, besides the development of browning and pitting symptoms

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rind, ethylene is responsible, in association with low temperature, for (i) metabolic dysfunctioning of the tissues due to the accumulation of ethanol and acetaldehyde; (ii) acceleration of solute leakage from disc tissues corresponding to the increase of membrane deterioration; and (iii) reduced capacity to remove active oxygen species through lowered activity of peroxidase, catalase and superoxide dismutase.

EXPERIMENTAL MATERIALS AND METHODS

Plant material

Charentais cantaloupe melons (*Cucumis melo* var. cantaloupe; Naud. 'védrantais') expressing an antisense (AS) peroxidase gene (Ayub *et al.* 1996) and wild-type plants were grown in an insect-proof greenhouse in Murcia, Spain. Plants were harvested 2 d before the onset of the climacteric peak in wild-type plants (1–2 p.p.m. ethylene production).

Conditions for cold storage, rewarming and treatment with ethylene and 1-methylcyclopropene

Fruit were placed at 2 °C in a storage room with 90–95% relative humidity for 5, 10 and 15 d (wild-type) or 7, 14 and 21 d (AS) in 1997 and for 2, 8 and 16 d (wild-type and AS) in 1998. Fruit were analysed at the end of the cold storage period and after rewarming at 22 °C for 5 d. Transgenic and wild-type fruit were treated with 10 p.p.m. ethylene for 1, 3 or 5 d at 22 °C before, or 5 d after cold storage, in 32 L vessels. The presence of KOH to eliminate respiratory CO₂. Each vessel containing five fruit was ventilated every 12 h and adjusted for ethylene concentration. The 1 p.p.m. 1-methylcyclopropene (1-MCP) treatment was carried out immediately on wild-type melon immediately after harvest using the same protocol as for the ethylene treatment.

Quantification of the chilling injury symptoms

The intensity of chilling injury was estimated visually by the extent of the surface pitting and browning using a scale ranging from 0 to 4 (0, no symptoms; 1, traces; 2, moderate; 3, moderate; 4, severe). The average chilling index was obtained according to McCornack (1976) using the following formula: CI = $\Sigma(\text{individual rating} \times \text{number of fruit with the same rating}) / \text{total number of fruit}$.

Electrolyte leakage, ethanol, acetaldehyde and ethylene measurements

Discs of rind taken from the equatorial region of the fruit were incubated in 40 mL of 0.4 M mannitol in 50 mL of distilled water. The flasks were shaken at 120 cycles min⁻¹ and the conductivity of solution (EC_0) was measured after 2 h with a digital conductivity meter (Micro CM 2200, Crison SP). The total conductivity (EC_T) was measured after 2 h in the mannitol solution and the tissues at –20 °C for

24 h followed by additional heating at 121 °C for 20 min. Ion leakage was calculated as $(EC_0/EC_T) \times 100$. Ethanol and acetaldehyde were determined according to Davis & Chace (1969) and ethylene according to Guis *et al.* (1997).

Enzyme assays

Rind tissue (3 g fresh weight) stored at –80 °C was homogenized at 4 °C using a PT 10–35 polytron (Kinematica AG), with 0.12 g PVP and 4 mL of cold 50 mM potassium phosphate buffer, pH 7.8. The homogenate was centrifuged at 23500 g for 15 min at 4 °C. The supernatant was used for the determination of enzyme activities. Catalase, peroxidase and superoxide dismutase activities were measured according to Aebi (1984), Ferrer *et al.* (1990) and Foster & Hess (1980), respectively. One unit of enzyme activity corresponds to the decomposition of 1 $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$ at 25 °C for CAT, the inhibition by 50% of the reduction rate of cytochrome *c* for superoxide dismutase and the rate of change in optical density per minute per mg of protein for peroxidase.

RESULTS AND DISCUSSION

Effect of ethylene on the development of chilling injury symptoms

As already documented for other melon varieties (Tatsumi & Murata 1981; Miccolis & Saltveit 1995), Charentais cantaloupe melons undergo chilling injury when stored at low temperature (Fig. 1). The disorder appears first as thin, pitted and brown areas on the peel, which gradually widen and become more severe. In advanced cases the rind undergoes loosening and decay develops throughout the entire fruit. Two separate experiments performed in 1997 (Fig. 1b) and 1998 (Fig. 1c) show that during cold storage at 2 °C, wild-type fruit developed slight symptoms of chilling injury (chilling index of about 1.5 after 16 d). However, upon rewarming to 22 °C the chilling symptoms became more severe (index 4 within 3 d). As seen in Fig. 1a, the rate of development of chilling injury was correlated with the rate of ethylene production. At low temperature the rate of ethylene production remained low ($< 3.3 \mu\text{L kg}^{-1} \text{ h}^{-1}$) and chilling damage developed slowly. Upon rewarming there was a sharp stimulation of both chilling injury and ethylene production ($> 65 \mu\text{L kg}^{-1} \text{ h}^{-1}$ at 2 d after rewarming). Increasing the length of cold storage led to a widening of chilling damage and an increase in severity upon rewarming. For long duration cold storage (16 d), all fruit exhibited maximum chilling damage (index 4) after 5 d of rewarming whereas for shorter storage periods of 2 and 8 d, damage was less severe (index 2 and 3.5, respectively) after rewarming. This was correlated with a peak of ethylene production that was 1.5 times higher in fruit that had been stored for 16 d than in fruit stored for shorter periods (Fig. 1a). In contrast to wild-type fruit, antisense ACO (AS) fruit produced very low ethylene ($< 0.5 \mu\text{L kg}^{-1} \text{ h}^{-1}$) and exhibited very little damage both during storage at low temperature and

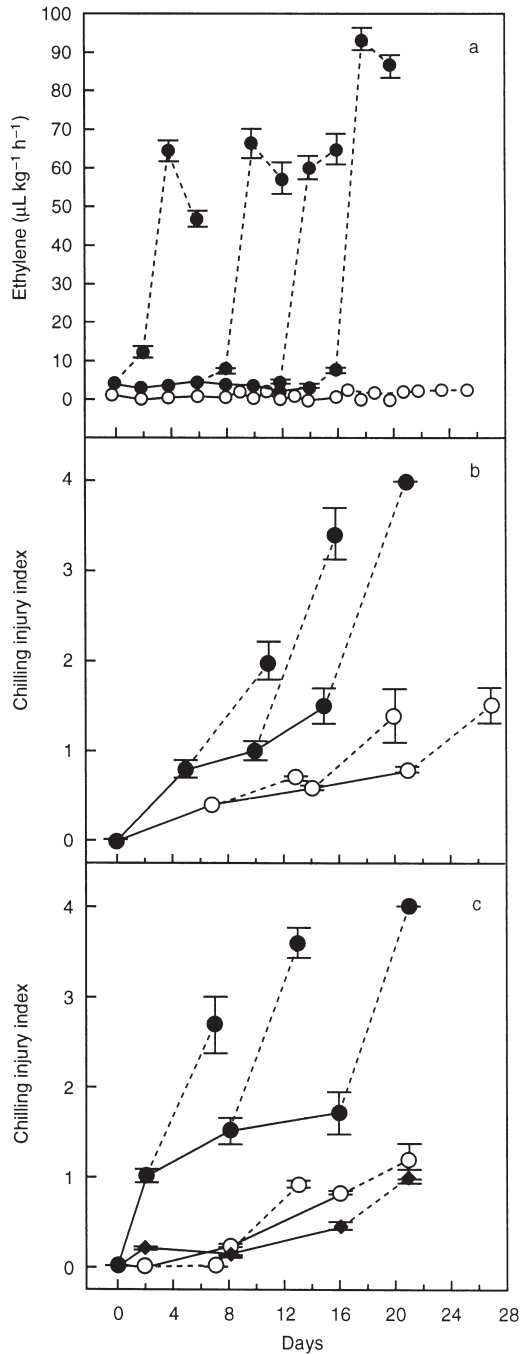


Figure 1. Effects of low temperature storage at 2 °C (-) and rewarming for an additional 5 d at 22 °C (- -) on ethylene production (a) and chilling injury index (b and c) of wild-type (●), wild type treated with 1 p.p.m. 1-MCP (◆) and antisense ACC oxidase (○) Charentais cantaloupe melons. (a) and (b) correspond to 1997 experiments and (c) to 1998. Values are the means of five independent replicates ± SE.

upon rewarming during the two experimental seasons (Figs 1b & c). In addition, when wild-type fruit were treated with the ethylene perception inhibitor 1-MCP prior to cold storage, they became insensitive to low-temperature damage (Fig. 1c). Furthermore, treating AS fruit prior to

cold storage with 10 p.p.m. ethylene restored the sensitivity to chilling, particularly after rewarming. However the sensitization of the fruit required a duration of 3 or 4 d of ethylene treatment (Fig. 2). Treatment for 1 d at 10 p.p.m. was not sufficient to restore chilling sensitivity. These data clearly indicate that ethylene plays a central role in the development of the chilling injury symptoms in Charentais cantaloupe melons. Interestingly, when AS fruit were first stored at low temperature and then treated with 10 p.p.m. ethylene, the chilling damage remained very low (Fig. 2), indicating that a combination of ethylene and low temperature is required for the development of the disorder.

The chilling disorder generally develops upon rewarming and is correlated with a post-chilling burst of ethylene. For instance, in cucumber, the most chilling-sensitive varieties produce higher amounts of post-chilling ethylene (Cabrera & Saltveit 1992). The absence of post-chilling ethylene in antisense ACO melon can therefore account for the strong reduction of chilling injury symptoms. In addition, slowing down ethylene production in cucumber by heat-shock treatment is known to reduce chilling injury (Hirose 1985; Inaba & Crandall 1988; McCollum *et al.* 1995) and similar observations have been made with mung bean (Collins, Nie & Saltviet 1993). When ethylene was applied exogenously to a number of different fruit, the chilling injury was generally enhanced (e.g. avocado: Chaplin, Wills & Graham 1983; citrus: Hatton & Cubbedge 1981; Yuen *et al.* 1995; Porat *et al.* 1999), consistent with our data for antisense ACO melons. However few cases of reduction of chilling injury by exogenous ethylene have been reported (Wang 1993) but include preclimacteric Honeydew melon submitted to very high levels of ethylene (1000 p.p.m.) for 18 h (Lipton

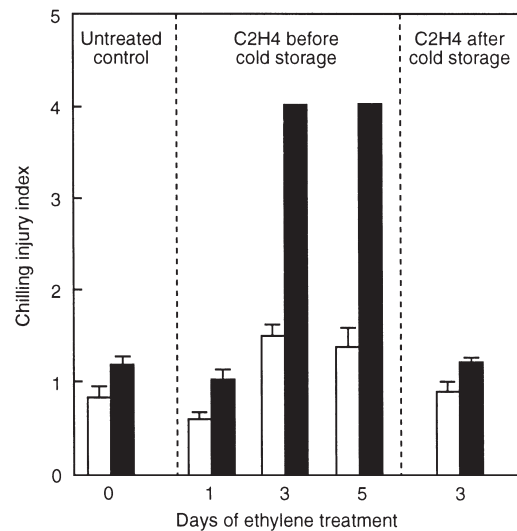


Figure 2. Effect on chilling injury of treating AS fruit with 10 p.p.m. ethylene for 1, 3 and 5 d before and 3 d after cold storage. The chilling injury index was estimated at the end of the 16 d storage period at 2 °C (open histograms) and after an additional 5 d at 22 °C (dark histograms). Values are the means of five independent replicates ± SE.

& Ahroni 1979). However, in these latter conditions, ACC production was considerably reduced during the cold treatment (Lipton & Wang 1987) so that the post-chilling ethylene burst may have been greatly reduced. The inhibition of ACC biosynthesis occurring in that case upon ethylene treatment is known as auto-inhibition of ethylene production (system I) and is well documented in immature climacteric fruit such as sycamore figs (Zeroni, Galil & Ben-Yehoshua 1976) and bananas (Vendrell & McGlasson 1971). Auto-inhibition of ethylene production is also seen in nonclimacteric fruit such as wounded tissues of citrus (Riov & Yang 1982). By taking into account such an effect of exogenous ethylene in Honeydew melons, the discrepancies between the role of ethylene in chilling injury of different melon species become less obvious.

Relations with membrane integrity and stimulation of anaerobic metabolism

Membrane structural transition is considered as one of the primary events occurring during low temperature treatment of chilling sensitive species. It results in a loss of membrane semi-permeability (Marangoni *et al.* 1996). Tissue solute leakage has long been used to assess the development of chilling injury in many plant tissues (Lyons, Raison & Steponkus 1979) and is still considered as a simple but valuable means of estimating membrane deterioration (Marangoni *et al.* 1996). Figure 3a shows that skin discs of wild-type fruit exhibiting the low temperature disorder had a higher rate of solute leakage than the corresponding AS tissues, but in both cases the rate of solute leakage increased during low temperature storage. Rewarming of wild-type and AS fruit did not cause significant changes in the rate of solute leakage, although, as documented earlier, it sharply stimulated the development of the chilling injury symptoms in wild-type fruit. Treating AS fruit with 10 p.p.m. ethylene before cold storage resulted in a significant increase in solute leakage only when the ethylene treatment was applied for 3 or 5 d and only in rewarmed tissues (Fig. 4a). When the ethylene treatment was applied after cold storage, solute leakage remained unchanged in comparison with the untreated control (Fig. 4a), indicating again that low temperature and ethylene operate in combination for the stimulation of chilling-induced membrane deterioration.

It has been documented that chilling induces a metabolic shift of tissues towards anaerobic respiration (Schirra 1992). Figures 3b and c show that high levels of ethanol and acetaldehyde accumulated in the skin of wild-type fruit in particular after rewarming at 22 °C. Very little accumulation was observed in AS fruit unless they were treated with 10 p.p.m. ethylene for 3 or 5 d before cold storage (Figs 4b & c). When the ethylene treatment was applied afterwards, there was no stimulation of ethanol and acetaldehyde production. In addition, wild-type fruit treated with 1-MCP displayed low levels of ethanol and acetaldehyde accumulation, similarly to untreated AS fruit (data not shown). The levels of ethanol and acetaldehyde accumulating during the

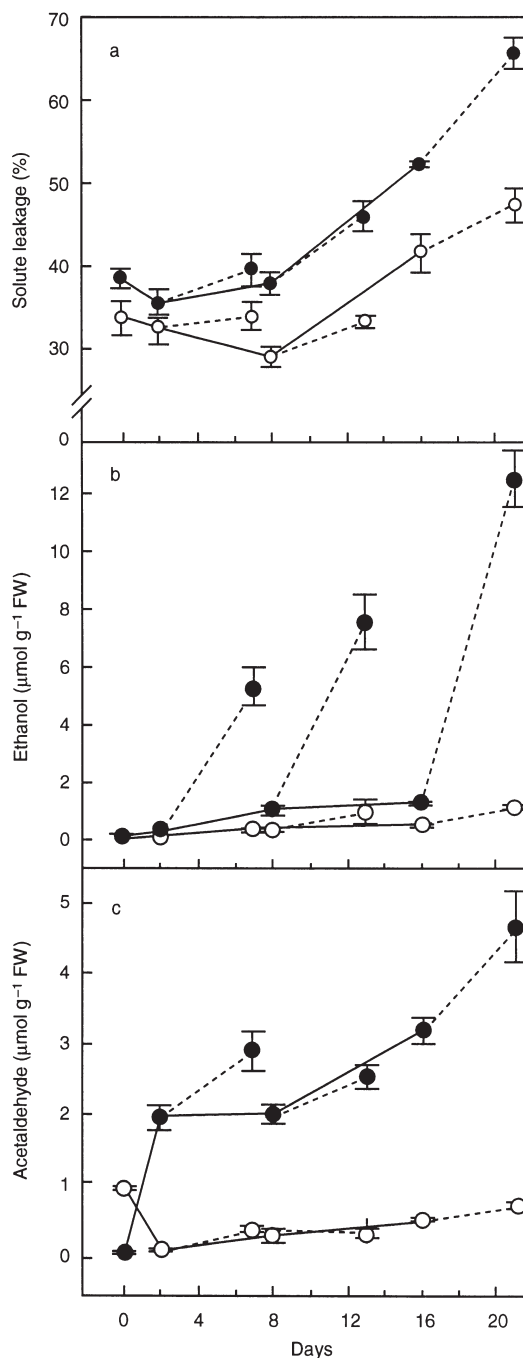


Figure 3. Effects of low temperature storage at 2 °C (-) and rewarming for an additional 5 d at 22 °C (- -) on solute leakage (a), ethanol (b) and acetaldehyde accumulation (c) in the skin of wild-type (●) and antisense ACC oxidase (○) cantaloupe Charentais melons. Values are the means of three independent replicates ± SE.

ripening of unchilled wild-type melon fruit, were about 10 times lower than in chilled wild-type fruit (data not shown).

Relations with the activity of free radical scavenging enzymes. Oxidative damage is considered to be an early response of sensitive tissues to chilling injury (Hariyadi & Parkin 1991). We found that, during low temperature

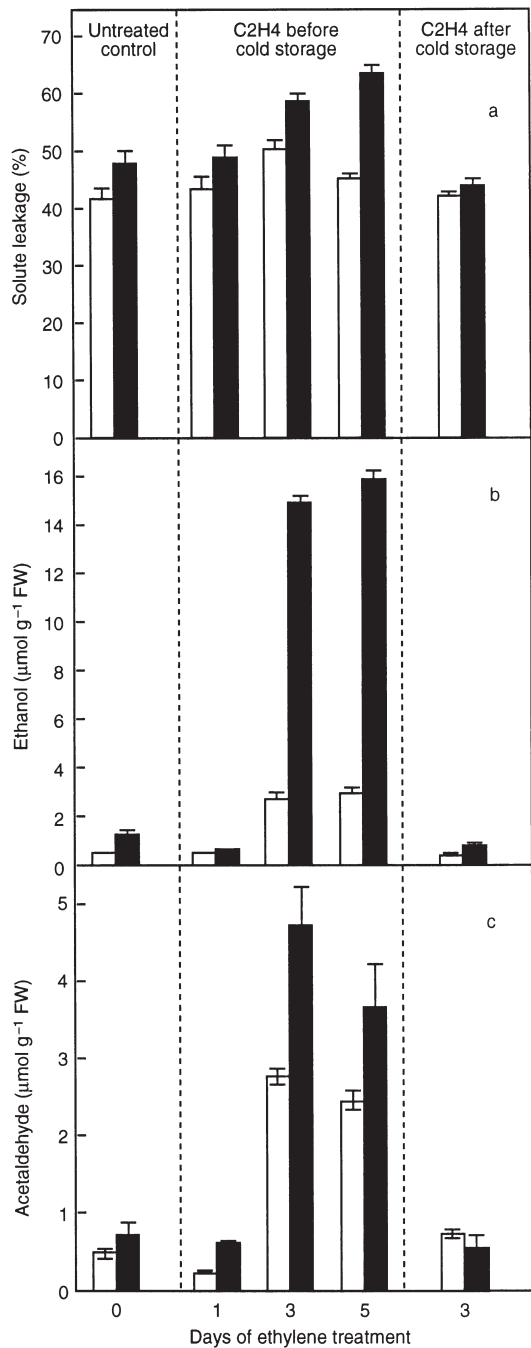


Figure 4. Effect of treating AS fruit with 10 p.p.m. ethylene for 1, 3 and 5 d before and 3 d after cold storage on: solute leakage (a), ethanol (b) and acetaldehyde accumulation (c) at the end of the 16 d storage period at 2 °C (open histograms) and after an additional 5 d at 22 °C (dark histograms). Values are the means of three independent replicates \pm SE.

storage, catalase (CAT) activity steadily decreased in chilling-sensitive wild-type fruit while it was increasing in chilling-resistant AS fruit. After rewarming, CAT activity continued to increase in AS fruit that were rewarmed for 5 d at 22 °C after 16 d of storage to reach a level that was around 35-fold higher than in the corresponding wild-type

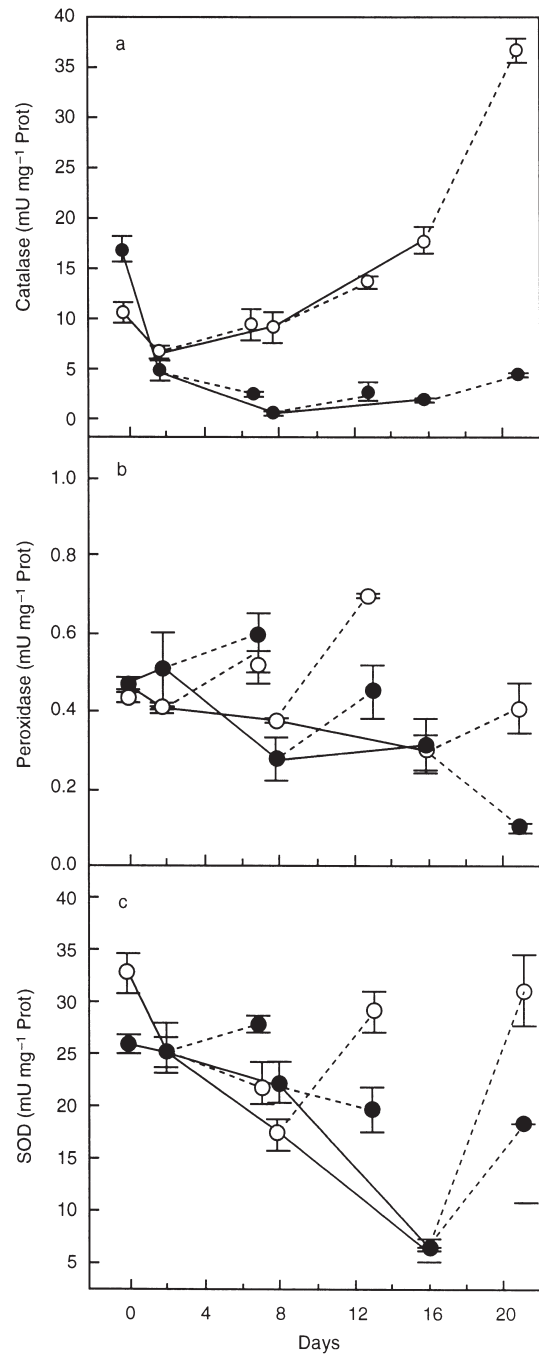


Figure 5. Effects of low temperature storage at 2 °C (-) and rewarming for 5 additional days at 22 °C (- -) on the activity of: catalase (a), peroxidase (b) and superoxide dismutase (SOD) (c) in the skin of wild-type (●) and antisense ACC oxidase (○) Charentais cantaloupe melons. Values are the means of three independent replicates \pm SE. The units of enzyme activities are defined in the Experimental protocol section.

fruit (Fig. 5a). Rewarming did not cause any increase in the CAT activity of wild-type fruit. The treatment of transgenic fruit with exogenous ethylene before the cold treatment resulted in a decrease in CAT activity when ethylene was applied for 3 or 5 d (Fig. 6a). In contrast, when the ethyl-

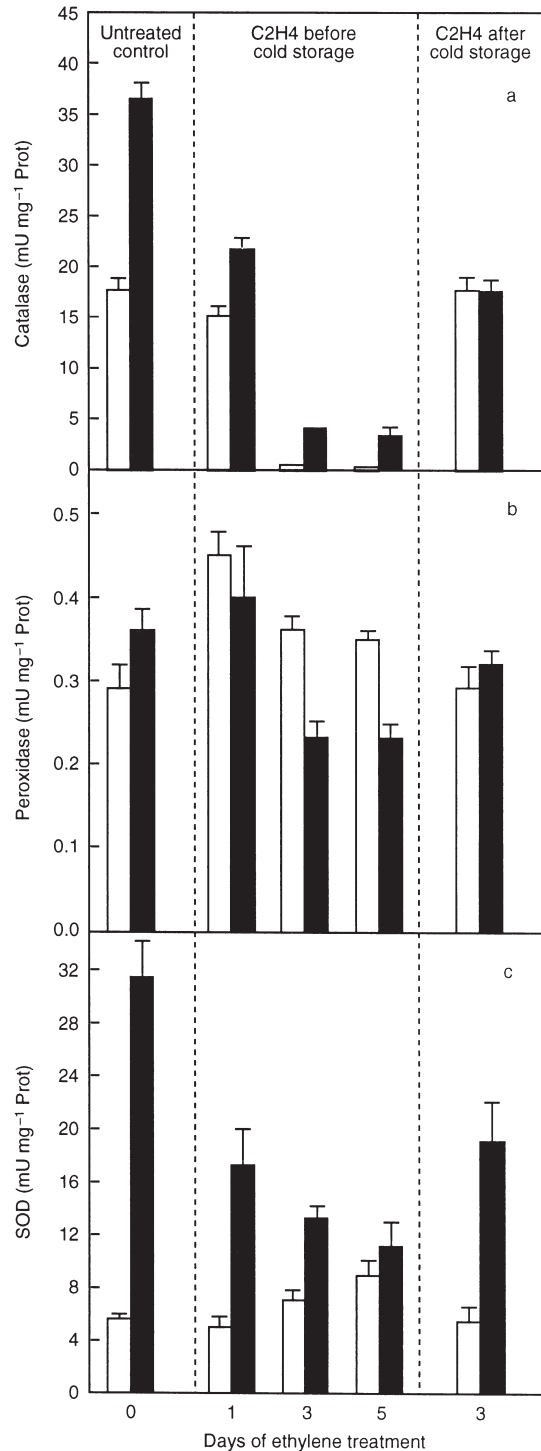


Figure 6. Effect of treating AS fruit with 10 p.p.m. ethylene for 1, 3 and 5 d before and 3 d after cold storage on the activity of: catalase (a), peroxidase (b) and superoxide dismutase (SOD) (c) at the end of the 16 d storage period at 2 °C (open histograms) and after an additional 5 d at 22 °C (dark histograms). Values are the means of three independent replicates \pm SE.

ene treatment was performed after cold storage the CAT activity remained high (Fig. 6a).

Peroxidase (PER), like catalase, plays a role in the breakdown of hydrogen peroxide. However, our studies showed no significant changes in PER activity during cold storage and no substantial differences between AS and wild-type fruit. During rewarming, the PER activity was, in general, stimulated in both types of fruit although this stimulation was higher in AS fruit, particularly when rewarming was performed after 8 and 16 d of cold storage (Fig. 5b). Treating AS fruit with ethylene for 3 or 5 d (but not for 1 d) before the cold storage resulted in a decrease in PER activity whereas treating after cold storage did not change activity compared with untreated control fruit (Fig. 6b).

The activity of superoxide dismutase (SOD) was sharply decreased by more than 75% in both wild-type and AS fruit during cold storage. Upon transfer to 22 °C, SOD activity was stimulated, but to a lesser extent in wild-type than in AS fruit (Fig. 5c). The pattern of changes induced by ethylene treatment of AS fruit before or after cold storage is not clear. Figure 6c indicated higher SOD activity in fruit that had been treated for 3 and 5 d before storage, immediately upon removal from cold, and a decrease after 5 d of rewarming at 22 °C. Therefore, SOD activity was lower during rewarming of fruit that had been previously sensitized to chilling by ethylene.

The deleterious effect of ethylene on chilling is therefore associated with a reduction in the activity of free radical scavenging enzymes (catalase, superoxide dismutase, peroxidase). Ethylene has also been found to stimulate the activity of free-radical-producing enzymes (lipoxygenases, NADH oxidase, etc.) either directly or through the stimulation of the senescence process (Paliyath & Droillard 1992). It is noteworthy that overexpression of catalases in fruit tissues results in higher chilling resistance (Kerdnaimongkol & Woodson 1999).

In previous work we showed that the inhibition of ethylene synthesis in the melon, through the expression of an antisense ACO cDNA, results in a reversible slow-ripening character (Guis *et al.* 1997). In this work, we have shown that the same strategy results in an increased resistance to chilling injury. As the sensitivity to chilling injury is restored by treating fruit with ethylene prior to cold storage, but not after, it is clear that ethylene acts in conjunction with low temperature to induce metabolic shifts that participate in the development of chilling injury. From the applied point of view, the decreased rate of ripening of antisense ACO fruit and their ability to be ripened on command have already been shown to enhance the post-harvest life of melons. By combining this with cold temperature storage, the post-harvest handling of the melon can be revolutionized.

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