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Effect of chilling on ethylene production in eggplant fruit

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Abstract

Eggplant is a non-climacteric fruit, with low ethylene production rates after harvest, whose response to storage at low temperature was studied. To this end, fruit were stored for 13 days at 0 and 10 °C. Fruits stored at 10 °C were unaffected, but those maintained at 0 °C suffered severe chilling injury (CI) from day 6. Electrolyte leakage, considered an indirect measure of membrane damage, showed no variation at 10 °C, whereas at 0 °C, leakage increased in parallel to CI. At 0 °C ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) contents increased during the first 6 days, though, from days 9–13, their contents decreased to about the initial levels. ACC oxidase activity decreased considerably during storage at 0 °C, reaching non-detectable values by day 13. At 10 °C, not important changes were observed in ethylene biosynthetic pathway. These results suggest that chilling stress stimulated ethylene, ACC and MACC accumulation in eggplant, and their levels remained high until CI symptoms became severe.

Keywords: Eggplant fruit; Chilling; Electrolyte leakage; Ethylene; ACC oxidase; ACC; MACC

1. Introduction

Eggplant belongs to a family of plants of tropical origin. Its fruit is a non-climacteric large berry and is chilling sensitive. Below 12 °C, eggplants suffer rapid physiological disorders manifested mainly by the appearance of surface injuries such as pitting, and seed browning, especially in the calyx (Fallik, Temkin-Gorodeiski, Grinberg, & Davidson, 1995). Chilling injury (CI) causes cell membrane damage and, as a consequence, affects the effectiveness of membranes as barriers to solute diffusion. Electrolyte leakage is generally considered to provide an indirect measure of this damage, and has been used to determine the extent of CI (Murata, 1989).

Ethylene is a plant hormone involved in different physiological processes, regulating many characteristics of plant growth, development and senescence (Yang & Hoffman, 1984). On the other hand, there is wide evidence that different stress-inducing factors, such as chilling, freezing, pathogen attack, salt stress and wounding, induce ethylene production (Kacperska, 1997). The rate of increase in ethylene synthesis may be observed soon after the stress, depending on the causative factor and the way it is applied. The pathway of ethylene synthesis is well established in higher plants (Yang & Hoffman, 1984), and regulatory control is achieved as two steps: the formation of, 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosyl-L-Methionine (SAM) and the conversion of this intermediate into ethylene (Kende, 1993). Enzyme ACC synthase (ACCs) catalyzes the first step, while ACC oxidase (ACCo) does so with the second.

The effect of cold storage on ethylene biosynthesis has been particularly studied in climacteric fruit, such as tomato and avocado, both specially sensitive to CI (Lyons,

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1973). However, the mechanism by which cold temperature elicits ethylene biosynthesis is not completely understood. However, chilling may stimulate, inhibit or fail to modify ethylene production in these tissues, depending on the species, cultivar, development stage and period of chilling treatment, (Autio & Bramlage, 1986; Lederman, Zauberman, Weksler, Rot, & Fuchs, 1997; Wang & Adams, 1982). Eggplant is a non-climacteric fruit, and no data have been measured on its ethylene evolution during storage at low temperature.

The purpose of this work was to analyze changes in ethylene production, and in the contents of ACC, MACC and ACCo in vivo activity for eggplant fruit stored at low temperatures.

2. Materials and methods

2.1. Plant material and storage conditions

Eggplants (*Solanum melongena* L.) cv Money Maker N°2 (a Japanese variety) were grown in La Plata (Argentine). Fruits were harvested at commercial maturity, and transported to the laboratory, where they were washed with water and dried. Groups of six fruits were packed in low density polyethylene (LDPE) perforated bags and stored for short (5, 10 and 24 h) and long periods (2, 6, 9 and 13 days) at 0 and 10 °C. Two bags were prepared for each combination of temperature and time.

On each sampling day, six fruits were analyzed immediately after being removed from the cold store, whereas six other fruits were transferred to a 20 °C room and kept for 24 h. For each of these conditions, three fruits were analyzed immediately and the other three fruits were peeled, fractionated, frozen by immersion in liquid nitrogen and stored at −80 °C until used.

2.2. CI symptoms

On each sampling day, CI symptoms of eggplant fruits were evaluated over six fruits using a numerical scale from 1 to 5. The CI index was calculated according to the following formula:

$$\text{CI Index} = \frac{\Sigma(n_i \times i)}{N},$$

where n_i is the number of fruits receiving the mark "i" (from 1 to 5) and N is the total number of fruits. The numerical scale represents: 5, severe damage; 4, moderate damage; 3, regular damage; 2, low damage; 1, no damage.

2.3. Browning of pulp tissue

The colour parameter L^* indicates the lightness of colour (0 = black and 100 = white) and its values were assayed according to the method of Larrigaudiere, Lentheric, and Vendrell (1998) with little modifications. A Minolta Colorimeter model CR-300 was used to determine L^*, and the readings were taken soon after slicing the central section of each fruit (thickness = 0.5 cm). All measurements were done on three fruits from each condition and by duplicate. The results were expressed as L_0, values higher than 86 denotes whitish pulp and values between 81 and 82 show only seed browning. Lightness near to 78 indicates an incipient browning of seed and pulp, while values below 73 denote considerable browning of seed and pulp.

2.4. Electrolyte leakage

Membrane permeability was measured on the equatorial or central region of three fruits from each condition. Electrolyte leakage was evaluated using 3-mm thick discs of eggplant pulp removed with a 10-mm diameter cork borer from each section. Discs (2 g) were incubated in 20 ml 0.6 M mannitol at 20 °C. The conductivity of the solution was measured at time zero and after 2 h, using a conductivity meter. This suspension was ground with a homogenizer (Dupont Instruments Sorvall® Omni-Mixer), centrifuged at 17,500*g* for 15 min at 20 °C and total electrolytes in the tissue were measured in the supernatant. Results were expressed as a percentage of total electrolytes leaking out of the tissue over 2 h. Determinations were carried out in duplicate and the results averaged.

2.5. Ethylene analysis

The internal ethylene content (IEC) was determined. A glass tube closed at one end with a septum was inserted 3 cm into the eggplant fruit through its peduncle scar and sealed with paraffin. After 2 h, 1 ml of the internal atmosphere sample was withdrawn with a syringe and needle. Ethylene was measured with a Varian Star 3400 CX gas chromatograph fitted with Porapack N column, flame-ionization detector (FID) and using Helium as carrier gas (flow rate, 30 ml min^{-1}). The oven temperature was 100 °C, while injector and detector temperatures were kept at 120 °C. For each condition, results were the average of measurements on three fruits, being expressed as internal ethylene produced per mass unit of fresh tissue (μl kg^{-1}).

2.6. ACC and MACC analysis

To determine free ACC, duplicate samples of tissue from the central section (10 g) from each fruit were frozen in liquid nitrogen and ground with an IKA Labortechnik analytical mill and extracted with boiling 96% ethanol (3:1 v:w) for 15 min. After centrifuging the homogenate at 17,500*g* for 20 min at 4 °C, the pellet was dis-

carded and the supernatant evaporated under vacuum at 42 °C. The residue was resuspended in 2 ml of distilled water. The ACC content was determined by chemical conversion to ethylene using the method of Lizada and Yang (1979). To determine conjugated ACC (MACC), the same type of extraction was done, but the resuspended residue was hydrolyzed into ACC as described by Hoffman, Yang, and McKeon (1982), and determined the ACC total content as described previously. MACC content was calculated from the difference in the ACC content of extracts before and after hydrolysis. The data were expressed as ethylene produced per unit of fresh tissue (μl kg^{-1}).

2.7. ACCo activity

The in vivo ACCo activity was determined by measuring the ethylene produced by pulp discs following exogenous ACC application.

Flesh discs of 10-mm diameter and 3-mm thick were removed from the equatorial section with a cork borer. Discs from 4 eggplants (0.5 g) were incubated in 5 ml of reaction medium containing 5 mM ACC, 0.4 M mannitol in 50 mM phosphate buffer, pH 6. Vials of 10 ml were sealed and incubated for 3 h at 30 °C, and the ethylene produced in the headspace of the vials was determined by gas chromatography as described above. Results are averages of at least 2 replicates from each sample. The ACCo activity was expressed as the ethylene production rate per mass unit of fresh tissue (μl kg^{-1} h^{-1}).

2.8. Statistical analysis

A factorial design was employed. The factors were storage temperature (2 levels) and storage times (8 levels). Data were analyzed by analysis of variance (ANOVA) using the SYSTAT software. All comparisons were carried out at a confidence level of 95%.

3. Results

3.1. Evolution of CI symptoms

Fruit stored at 10 °C did not exhibit CI symptoms. At the end of storage (13 days), the calyx section presented discoloration and incipient senescence due to the normal aging process. Conversely, storage at 0 °C caused CI on eggplant fruit, so the CI index increased with storage time (Fig. 1). At the second storage day at 0 °C, fruits showed incipient peel discoloration (CI index 1.4). At day 6, CI symptoms such as surface pitting on the calyx section and seed browning were observed (CI index 2.3). Surface scalds and browning of pulp tissue were found on fruit after 9 days of storage, when fruit was considered to be commercially unacceptable (CI index 3.2). At each sampling day, the CI in the fruits transferred from 0 to 20 °C for 24 h became more pronounced (Fig. 1).

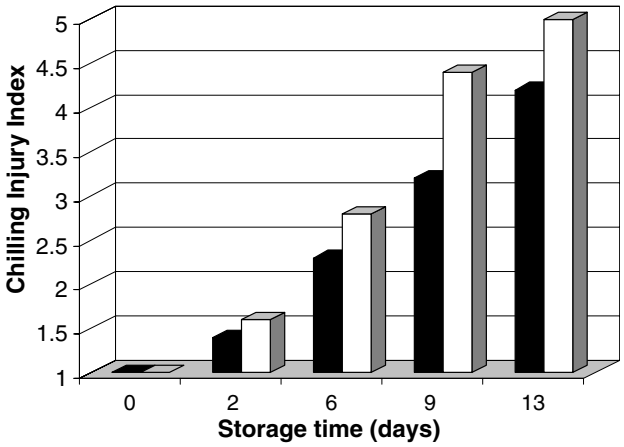

Fig. 1. Chilling injury index of eggplants stored at 0 °C, analyzed immediately after being removed from the cold store (black bar) and after transfer at 20 °C during 24 h (white bar). $LSD_{0.05} = 0.1$; $n = 6$ per treatment combination.

3.2. Changes in pulp browning

L_0 was utilized as a measurement of the browning evolution. At 10 °C, fruit showed L_0 values higher than 87 along the entire storage time (data not shown). Fig. 2 shows the L_0 evolution with storage time at 0 °C. There is a decrease in L_0 reaching a value of 78.9 after 9 days, corresponding to a slight browning of seeds and pulp tissue. After 13 days, a value of L_0 73.9 showed severe browning. After this period, L_0 decreased further after transferring the fruit to 20 °C for 24 h.

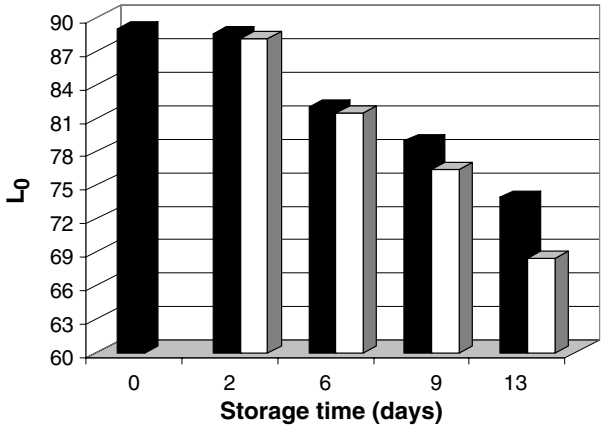

Fig. 2. Browning of pulp tissue measured as L_0 value of eggplants stored at 0 °C and analyzed immediately after being removed from the cold store (black bar) and after transferred at 20 °C during 24 h (white bar). $LSD_{0.05} = 1.86$.

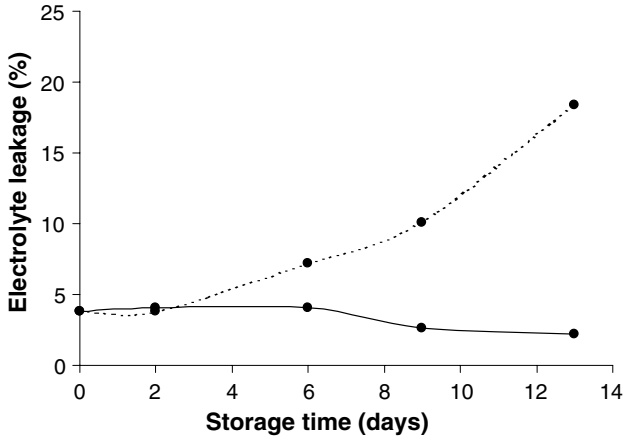

Fig. 3. Electrolyte leakage percentage from pulp tissue of eggplant during storage at 10 °C (solid line) and 0 °C (broken line). Each value is the mean of two replicate samples. $LSD_{0.05} = 0.16$.

3.3. Changes in electrolyte leakage

Electrolyte leakage from pulp of eggplant fruit was used as an indirect measure of membrane damage (Fig. 3). The initial value was 3.9%. Negligible variation of electrolyte leakage percentage was observed over the entire storage time at 10 °C. In contrast, fruits stored at 0 °C showed an increase of electrolyte leakage with time. After 6 days of storage, the electrolyte leakage was twice the initial value and, at day 13 it was 5 times as high as the starting measurement ($p < 0.05$).

3.4. Changes in internal ethylene content

IEC was measured during 13 days of storage at 10 (Fig. 4(a)) and 0 °C (Fig. 4(b)). In order to observe whether ethylene quickly responds to low temperature, IEC was also determined after a short period of storage (5, 10 and 24 h). However, no detectable IEC was observed at initial time. No IEC was detected in fruit during first 9 days at 10 °C, and only low levels were observed after day 13 at this temperature. At 0 °C, no IEC was observed in fruit during first 10 h of storage, although a level of 0.04 μl kg^{-1} was found after 24 h of storage. This level remained unchanged until day 6 and then decreased to almost undetectable values at day 9.

3.5. Changes in ACCo activity

In previous experiments, the ACCo in vivo activity was analyzed in presence of cofactors (sodium ascorbate, SO_4Fe and CO_2), as it was suggested by Vioque and Castellano (1998) and Moya-León and John (1994). The results obtained showed that the enzymatic activity was unaffected (Fe^{+2} and CO_2) or decreased

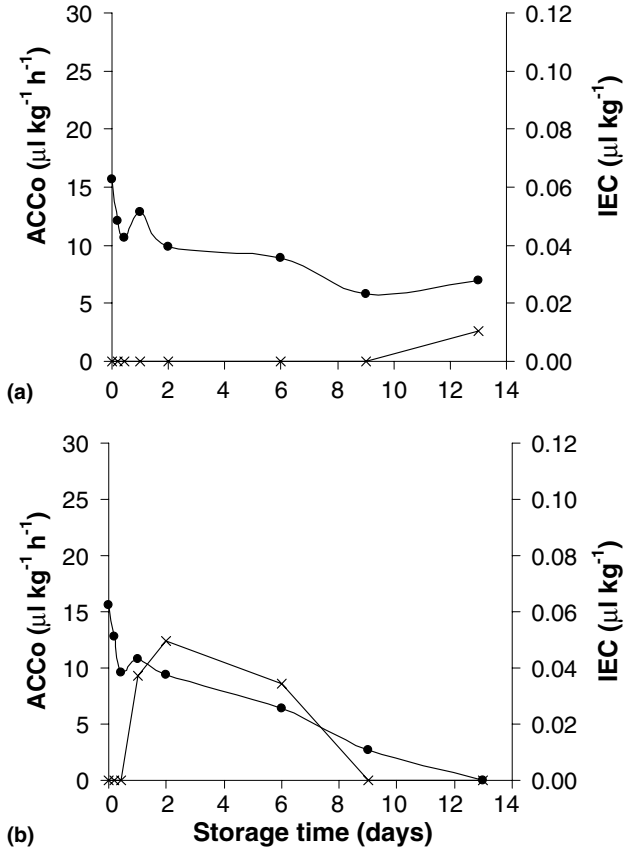

Fig. 4. Internal ethylene content (IEC, ×) and ACCo activity in vivo (•) during storage of eggplant cv Money Marker N°2 at 10 °C (a) and 0 °C (b). Each value is the mean of three replicates for the IEC and at least two replicates for ACCo activity. $LSD_{0.05} = 0.02$ for IEC, and $LSD_{0.05} = 1.2$ for ACCo activity.

(ascorbate). Therefore, the later experiments were performed in absence of cofactors as it was detailed in Section 2.7.

ACCo in vivo activity was analyzed in pulp of fruit stored at 10 (Fig. 4(a)) and 0 °C (Fig. 4(b)) during 13 days. Short storage times were also analyzed (5, 10 and 24 h). Initial activity was 15.6 μl kg^{-1} h^{-1}. During the first 24 h at 10 °C, ACCo activity decreased by 20% ($p < 0.05$) and then a gradual decrease to about 40% of the initial enzyme activity at day 13 was detected (Fig. 4(a)). ACCo activity declined by 30% after 24 h of storage at 0 °C ($p < 0.05$) and more progressively during the remainder of the storage (Fig. 4(b)). There was no ACCo activity at day 13.

3.6. Changes in ACC content

The initial ACC content was 0.16 μmol kg^{-1} and the observed changes during storage at 10 (Fig. 5(a)) and 0 °C (Fig. 5(b)) were clearly different. During the first 24 h at 10 °C ACC content did not change (Fig. 5(a), inset), it decreased by 90% after day 2 to remain thereafter at

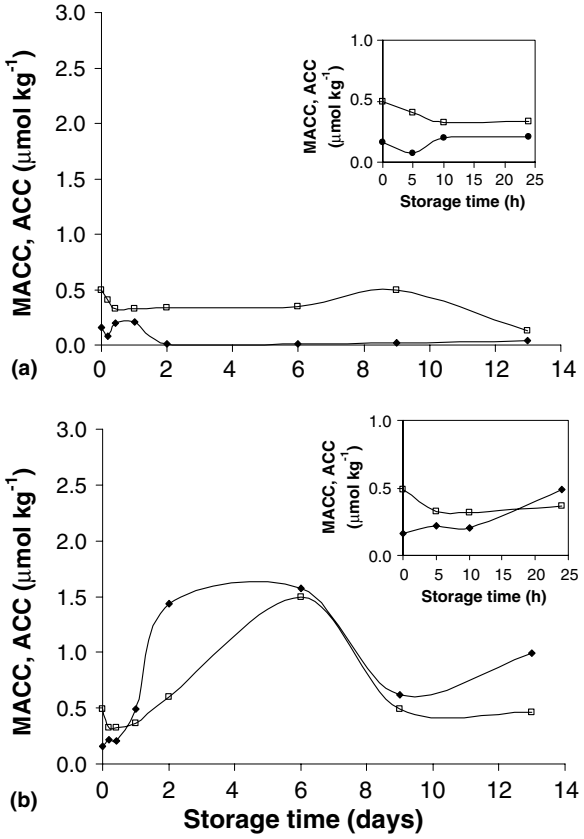

Fig. 5. Evolution of ACC (♦) and MACC (□) content in pulp tissue of eggplant during storage at 10 °C (a) and 0 °C (b). Inset diagrams show the initial portion of both curves. Each value is the mean of two replicate samples. $LSD_{0.05} = 0.12$ for ACC content, and $LSD_{0.05} = 0.46$ for MACC content.

such low levels during the rest of the storage. ACC remained mostly unchanged during the first 10 h of storage at 0 °C (Fig. 5(b), inset), but a sharp accumulation was observed after days 1 and 2 of 3- and 9-fold. ACC levels continued high until day 6 and then decreased reaching a value still 6 times as high as the initial concentration at day 13 ($p < 0.05$).

3.7. Changes in MACC content

The initial MACC content (0.49 μmol kg^{-1}) was 3 times as high as the initial ACC level. Little variation in MACC content was observed (Fig. 5(a)) over the entire storage period at 10 °C, even during first hours of storage (5, 10 and 24 h). MACC remained unchanged during the first 24 h of storage at 0 °C (Fig. 5(b), inset), then increased gradually to reach a peak at day 6, 3 times above the initial value (Fig. 5(b)) and similar to the ACC level reached at the same time and temperature of storage. At day 9 and 13, MACC concentrations decreased almost to the initial value.

4. Discussion

The results described above show that storage at 10 °C causes no damage to eggplant fruit, also showing invariable levels of electrolyte leakage. At 0 °C, on the contrary, symptoms of CI were detected after 6 days, intensifying with storage time. Electrolyte leakage levels increases in parallel to these symptoms, whereas the observed L_0 values also denote intense browning of seeds and pulp after prolonged exposure in chilling storage conditions.

Before storage at low temperatures, MACC content was higher than ACC, but this relation was not maintained during storage. The same was observed in mature-green and mature-red peppers (also a non-climacteric fruit) (Serrano, Martínez-Madrid, Pretel, Riquelme, & Romojaro, 1997), and the initial levels were also similar to those detected in eggplant.

Unimportant changes in both ACC and MACC contents were observed at 10 °C. On the other hand, although ACCo activity decreased, it kept relatively high values during storage.

Exposure to low temperature (0 °C) induced an increase in ACC, during the first 24 h, while MACC did so after 2 days. (Wang & Adams (1982) have reported that the chilling-stimulated pathway was not the conversion of ACC to ethylene but the ACC synthesis, probably due to ACCs activation as a stress response. Eggplant seems to comply with this hypothesis since after the first 24 h it responded to chilling stress. Therefore, at short storage times (24 h) the ACC–ethylene pathway was active so low temperatures would induce ACCs activity. In Fortune mandarin, Zacarias, Lafuente, Marcos, Saladie, & Dupille (2003) have reported low storage temperature as responsible for the activation of ACCs transcript at first time and ACCo transcript, later. So, ACCs gene induction appears to be the primary signal of chilling stress. Zacarias et al. (2003) have also found that the induction of ACCs and ACCo transcripts by chilling preceded the appearance of chilling symptoms. This effect may be possibly caused by a direct induction of low temperature on the expression of ethylene biosynthesis genes or to initial cellular damage occurring before manifestation of morphologically detectable peel pitting. Lelièvre et al. (1997) found in Passe–Crassane pears that the ACCs and ACCo activities were increased after fruit exposure to 0 °C. In our work the ACCo activity decreased during either storage at 10 or 0 °C, being more pronounced at lower temperature. ACCo maintained a 70% of activity with respect to the initial value during the first 24 h, this level being sufficiently high to produce an increase of the IEC. Likewise, Lin, Hall, & Saltveit (1993) have found that, either in mature-green or in mature-red peppers, the ACCo activity decreases during storage at 1 °C, while ethylene production increases.

ACC, MACC and ethylene contents kept high until day 6 at 0 °C. At day 9 ACC decreased, though still keeping higher than the initial value. The effects of prolonged chilling storage on ACC content in plant tissues constitutes a controversial issue in the literature. Some previous observations have indicated that ACC keeps increasing during long chilling exposure in apples (Larrigaudiere & Vendrell, 1993), pears (Gerasopoulos & Richardson, 1997) or mandarins (Zacarias et al., 2003). ACC content increased in mature-green peppers stored at 2 °C, while no changes were observed in mature-red peppers (Serrano et al., 1997). Decrease in ACC content obtained in this study may result from depletion of ACCs protein due to rapid degradation and/or inhibition of synthesis, inactivation of ACCs activity in vivo, conversion of ACC into ethylene and/or MACC, or any combination of these processes. However, as IEC was not detectable and MACC also decreased, the reduction in ACC content might be mainly related, to an alteration of ACCs content or activity.

On the other hand, the conversion of ACC into ethylene is known to be altered by prolonged storage at low temperatures (Wang & Adams, 1982) probably because ACCo activity generally depends on membrane integrity and properties (Andersen, 1986; Antunes & Sfakiotakis, 2002; Porter, Borlakoglu, & John, 1986). Although Ververidis & John (1991) found that ACCo was a soluble enzyme. Our results indicate at day 9 that CI was severe, that electrolyte leakage was increased. It must be pointed out that determination of ACC in vivo activity was carried on in the presence of exogenous ACC, so the loss of capacity to convert ACC into ethylene and the depressed ethylene production after prolonged chilling storage is not caused by absence of ACC in the tissue.

5. Conclusions

In summary, our results indicate that under chilling conditions cells of Money Maker N°2 eggplant, a non-climacteric fruit, is able to accumulate ACC, MACC and ethylene. When cold-induced severe symptoms of damage appeared, ethylene, ACC and MACC contents decrease. Under these conditions browning of pulp tissue is high as shown by L_0 values, and loss of cell integrity is represented by higher values of electrolyte leakage. ACCo activity decrease with exposition to 0 °C and it did not have a clear relationship with ACC content and ethylene production, so additional experiments are required to obtain more evidence on this point.

Acknowledgements

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