The Effect of High Carbon Dioxide at Low Temperature on Ribulose 1,5-Biphosphate Carboxylase and Polygalacturonase Protein Levels in Cherimoya Fruit

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ABSTRACT. The effect of short-term high CO₂ on maintaining postharvest cherimoya (*Annona cherimola* Mill.) fruit quality during storage at low temperature was analyzed. Pretreatment with an atmosphere of 20% CO₂ and 20% O₂ for 3 days retained fruit firmness and prevented an increase in the immunodetected levels of polygalacturonase-related protein during storage at 6 °C. Color scale values, chlorophyll content, and levels of the immunoreactive large subunit of ribulose 1,5-biphosphate carboxylase protein were higher in treated than in air-stored fruit. Short-term high CO₂ treatment had a long residual effect at low temperature and improved storage life by preventing fruit softening and delaying senescence-like responses in green peel of cherimoya fruit tissues.

Many investigations in fruit focus on treatments designed to extend storage life or to increase tolerance to chilling injury. The beneficial effects of elevated CO₂ on the storage life of fresh fruit and vegetables are well established (Wang, 1993). Short-term exposure to high CO₂ before storage at low temperature decreases the development of chilling injury in several commodities (Bertolini et al., 1991; Hatton et al., 1975). In green tissues, chilling temperatures may trigger senescence-like changes as do other stress conditions (Levitt, 1980). Some of these senescent changes involve losses in photosynthetic capacity, which are usually associated with alterations in the activity and levels of ribulose 1,5-biphosphate carboxylase (RuBPCase) protein and changes in chlorophyll content (Wittenbach et al., 1980). Treatment with high CO₂ levels in plants exposed to stressful ambient conditions (such as high O₃ levels) prevents decreases in photosynthetic pigments, soluble proteins, and RuBPCase protein levels (Rao et al., 1995). In green leaves and vegetables, high CO₂ levels maintain the chlorophyll content at a higher level than those stored in air (Aharoni et al., 1989). Controlled-atmosphere (CA) storage represses chlorophyll loss and synthesis of pigments in tomato (Goodenough et al., 1982). These authors also demonstrated that CA storage prevents the synthesis of polygalacturonase (PG), while other enzymes show little inhibition. Kanellis et al. (1991) also observed that the activity and content of cell-wall degrading enzymes in avocado fruit are prevented by CA storage. A higher concentration of total pectins and tannins is retained in CA-stored apricot and peach fruit than in conventional refrigerated stored fruit (Wankier et al., 1970). Small but consistent reductions in the amount of cell-wall turnover have been observed in CA-stored kiwifruit fruit compared to air-stored fruit (Arpaia et al., 1987). These results seem to agree with the well-known effect of high CO₂ in delaying or suppressing fruit and vegetable softening (Kader, 1986). Pretreatment with high CO₂ at ambient temperature delays softening and accumulation of PG protein in cherimoya fruit (Del Cura et al., 1996). At low temperatures, softening of cherimoya

fruit is significantly reduced (Alique et al., 1994), and fruit stored at 6 °C fail to attain the soft stage after 13 d of storage. Ultrastructural analysis of cherimoya fruit stored under several chilling conditions reveal that the major changes take place only in membrane systems, while the cell wall remains intact (Gutiérrez et al., 1992). These authors concluded that low-temperature stress prevents degradation of the cell wall, precluding the action of hydrolytic enzymes, such as PG, pectinesterase, and cellulase. However, no information is available concerning the identification and quantification of specific cell-wall hydrolytic enzymes, such as PG, in cherimoya fruit during storage at low temperature in the presence of high CO₂ levels.

The purpose of this study was to evaluate the effects of short pretreatment with 20% CO₂ on maintaining cherimoya fruit quality during storage at low temperature by 1) reducing the senescence-like responses in peel tissues of cherimoya fruit, namely in the photosynthetic components, and 2) slowing fruit softening through changes in the abundance of PG protein. A better understanding of the action of high CO₂ levels in green tissues of fruit will improve the knowledge of the mechanism by which CAs regulate postharvest quality of fruit and vegetables.

Materials and Methods

PLANT MATERIAL AND TREATMENT. 'Fino de Jete' cherimoya fruit of uniform shape and maturity stage were harvested in Granada, Spain, in January (midseason), shipped by truck, and received at the Instituto del Frío laboratory (Madrid) within 20 h. Selected fruit weighing 180 to 190 g were randomly divided into two groups of 80 fruit and stored in darkness at 20 and 6 °C. At each temperature, two lots of 20 fruit each were placed in separate respiration chambers (20 L) in a continuous flow of air or 20% CO₂ and 20% O₂. The gas flow rate was 180 mL·min⁻¹. After 3 d of treatment with 20% CO₂, fruit were transferred to continuous humidified air flow. Initially, and for every subsequent sampling period, three untreated and treated cherimoyas were randomly collected, peeled, and frozen in liquid nitrogen and stored at -80 °C. The sampling periods were 3, 5, 9, and 23 d for fruit at 6 °C and 3 and 5 d for fruit at 20 °C. Color measurements were made with a HunterLab tristimulus colorimeter (model D25A-9) calibrated with a white standard tile (X = 82.51; Y = 84.53; Z = 101.23). L, a, and b values were assigned as the average for three representa-

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Table 1. Lightness, hue angle, chroma and chlorophyll concentration in cherimoya peel of untreated and CO₂-treated (20% for 3 d) fruit during storage at 6 °C in the dark.

Storage time (d)	Lightness (L)		Hue angle (tan ⁻¹ b/a)		Chroma (a ² + b ²) ^{1/2}		Chlorophyll (μg·g ⁻¹ FM)	
	Air	+CO ₂	Air	+CO ₂	Air	+CO ₂	Air	+CO ₂
0 ²	51.2 ± 1.87 ^y	51.2 ± 1.87	124.0 ± 0.06	124.0 ± 0.06	21.8 ± 0.34	21.8 ± 0.34	106.2 ± 6.36	106.2 ± 6.36
3	52.3 ± 2.63	49.5 ± 1.69	119.3 ± 0.44	122.1 ± 0.47	22.0 ± 0.76	20.4 ± 0.92	ND ^x	ND
5	54.8 ± 3.71	51.8 ± 2.09	124.7 ± 0.78	122.9 ± 0.91	23.6 ± 0.97	22.4 ± 1.26	65.3 ± 2.31	77.5 ± 1.97
9	51.0 ± 3.50	52.7 ± 0.73	112.9 ± 0.75	111.9 ± 2.27	19.8 ± 1.18	18.9 ± 0.61	52.3 ± 2.49	56.9 ± 1.32
23	44.8 ± 2.69	51.2 ± 3.11	109.9 ± 2.36	117.2 ± 1.18	16.0 ± 0.86	19.6 ± 1.11	59.6 ± 3.39	88.05 ± 1.59
Significance	D*, T × D*		D*, T × D*		D*, T × D*		T*, D*, T × D*	

²Fruit after harvest.

^yMeans ± SD.

^xND = not determined.

*Significant at $P \leq 0.05$, where D = days and T = treatment.

tive samples, with three readings taken (in the pedicellar region) for each fruit. The color of the cherimoya peel was described in terms of lightness, hue angle, and chroma. Hue angle was calculated from the arctangent of b/a and chroma was calculated as $(a^2 + b^2)^{1/2}$ (Little, 1975). Firmness was measured in whole fruit at three places on the equator using an Instron (model 1140) testing machine fitted with a double-plate probe.

CHLOROPHYLL DETERMINATION. Total chlorophyll in cherimoya peel was extracted with 80% (v/v) acetone and determined according to Strain et al. (1971). Data were recorded on a fresh-mass (FM) basis (μg·g⁻¹).

PROTEIN EXTRACTION. Ground frozen cherimoya peel tissue (10 g) was homogenized at 4 °C in 30 mL of ice-cold extraction buffer [50 mmol Tris-HCl (Tris(hydroxymethyl)-aminomethane), pH 7.4; 400 mmol NaCl; 20 mmol NaHCO₃; 20 mmol MgSO₄; 10 mmol sodium ethylene-diamino tetraacetic acid; 5 mmol 2-

mercaptoethanol; 0.5 mmol phenylmethyl-sulfonyl-fluoride; 0.01 mmol leupeptin; 10% (v/v) glycerol; and 1% (w/v) Triton X-100]. The resulting homogenate was centrifuged at 20,000× g for 20 min at 4 °C. The filtered supernatant was further purified by using the phenol-ammonium acetate-methanol method (Montero et al., 1995). Protein concentration was measured by the method of Bradford (1976).

ELECTROPHORESIS AND IMMUNOBLOTTING. Protein samples were separated by SDS-PAGE on 14% polyacrylamide slab gels (15 × 11 cm and 1 mm thick) as described by Laemmli (1970). Gels were transferred onto nitrocellulose membranes as described by Towbin et al. (1979). The proteins were immunodetected with rabbit monoclonal antibodies against tomato large subunit of ribulose 1,5-biphosphate carboxylase (LSR) (dilution 1:20,000) or rabbit polyclonal antibodies against tomato PG (dilution 1:60,000), being further incubated with antirabbit IgG horseradish peroxidase conjugate. Development with chemiluminescent substrates and exposure to X-ray films were performed following the manufacturer's (Amersham) recommendations. Levels of antigen on the nitrocellulose filter were quantified using an image analyzer (BioImage and Visage, Millipore Corporation, Ann Arbor, Mich.).

STATISTICAL ANALYSES. Data from at least three replicates per sampling period were tested by analysis of variance. Two-way analysis of variance was performed using the LSD test procedure

Fig. 1. Effect of short-term high CO₂ on RuBPCase protein levels of cherimoya fruit during storage at low temperature. (a) Coomassie stained gel of peel protein extracted as described in Material and Methods. First lane corresponds to molecular mass markers; lane 0, prestored fruit (0 d); the other lanes correspond to untreated (air) and treated (+CO₂) fruit after 5, 9, and 23 d of storage at 6 °C. 10 μg of total protein were loaded in each lane. (b) Immunodetection of the large subunit of RuBPCase (55 kDa) (LSR) in peel of cherimoya fruit.

Fig. 2. Histogram representing the quantification of changes in RuBPCase (LSR) protein levels of untreated and treated fruit during storage at 6 °C, as described in the legend to Fig. 1. The immunoblots were quantified by densitometry and data are given as relative peak area (percentage of initial value at prestored fruit). Two replicates revealed a similar pattern of protein changes in cherimoya peel.

Fig. 3. Effect of temperature $\times$ CO₂ interaction on RuBPCase protein content of cherimoya fruit. (a) Coomassie stained gel. Lane M = molecular mass markers; lane P = prestored fruit; the other lanes showing untreated (air) and treated (+CO₂) fruit stored 5 d at 20 and 6 °C. (b) Western blot of replicate gel using RuBPCase (LSR) antiserum.

with type III sums of squares and a confidence level of 95% (Statgraphics program, STSC, Rockville, Md). The main effects of high CO₂ treatment, time of storage at 6 °C, and treatment $\times$ time interaction on fruit were analyzed.

Results

CHANGES IN COLOR SCALE VALUES AND CHLOROPHYLL CONTENT. After 23 d of storage, the normal light-green peel color of cherimoyas appeared to be maintained on CO₂-treated fruit, while a decrease in lightness (L) occurred in untreated fruit (Table 1). Carbon dioxide treatment also retained the chroma of cherimoya peel color.

A progressive loss in chlorophyll content was observed for 9 days of storage at low temperature, with CO₂-treated fruit having higher chlorophyll content than untreated fruit (Table 1). Low chlorophyll content was maintained in untreated fruit with further storage times, while an increase in chlorophyll content was recorded in treated fruit.

EFFECT OF HIGH CO₂ TREATMENT ON RUBPCASE PROTEIN CONTENT DURING STORAGE AT LOW TEMPERATURE. The electrophoresis and immunoblotting results showed that the levels of the large subunit of RuBPCase in the peel of untreated and CO₂-treated fruit gradually decreased for 9 d (Fig. 1). Quantitative analysis of these gels (Fig. 2) revealed that this decrease was less in fruit treated with high CO₂ than in untreated fruit. Furthermore, a substantial increase in RuBPCase protein content was observed after 23 d of storage at low temperature in cherimoya peel of treated and untreated fruit (Figs. 1 and 2).

EFFECT OF TEMPERATURE $\times$ CO₂ INTERACTION ON RUBPCASE PROTEIN CONTENT. The western blot and quanti-

Fig. 4. Histogram representing the quantification of changes in RuBPCase (LSR) protein levels of fruit stored at 20 and 6 °C with and without CO₂ treatment, as described in the legend for Fig. 3. The immunoblots were quantified by densitometry and data are given as relative peak area.

tative measurements of LSR levels in the peel of untreated and CO₂-treated fruit of identical chronological ages (5 d) stored at 20 and 6 °C showed that low temperature delayed the loss of RuBPCase protein content while the combination of elevated CO₂ and low temperature had an additive effect on the maintenance of RuBPCase protein levels (Figs. 3 and 4). Consistently, the highest level of RuBPCase protein was present in cherimoya peel of fruit treated with CO₂ and stored at low temperature. Furthermore, the effectiveness of CO₂ was more evident when the loss of RuBPCase protein was higher [Figs. 2 (9 d) and 4].

EFFECT OF HIGH CO₂ ON CHANGES IN FIRMNESS DURING LOW-TEMPERATURE STORAGE. Upon removal from high CO₂ treatment and during 9 d of storage at 6 °C, there was no difference in fruit firmness between treated and untreated fruit (Fig. 5). Softening was later observed in untreated fruit, while the firmness of CO₂-treated fruit remained fairly constant. After 23 d at 6 °C the loss of

Fig. 5. Firmness changes of cherimoya fruit during storage at 6 °C in air (■) or treated with 20% CO₂ (▲). Data are presented as mean values $\pm$ SE.

Fig. 6. Immunodetection of PG protein in cherimoya fruit stored at 20 and 6 °C with and without CO₂ treatment. Total peel protein was extracted and resolved by SDS-PAGE electrophoresis and then transferred to nitrocellulose sheets. Air = unripe (prestored) fruit, 3 d at 20 °C (ripe) and after 9 and 23 d at 6 °C. CO₂ treatment = unripe fruit and treated for 3 d with high CO₂ levels and stored at 20 °C (3 d) and at 6 °C (9 and 23 d).

Fig. 7. Histogram representing the quantification of changes in peel PG protein of untreated and CO₂-treated cherimoya as described in the Fig. 6 caption. Immunoblots were quantified by densitometry and data are given as relative peak area.

firmness of treated and untreated fruit was 14% and 52%, respectively.

EFFECT OF TEMPERATURE AND CO₂ ON PG PROTEIN LEVELS. In a previous work (Del Cura et al., 1996) the PG polypeptide of 46 ku (unified atomic mass unit) of molecular mass showed the most noticeable changes during cherimoya ripening at ambient temperature. The immunoblotting and electrophoresis results in this study (Figs. 6 and 7) revealed that the level of 46 ku polypeptide in air-stored fruit after 9 d at 6 °C was similar to that of unripe (prestored) fruit. An increase in the abundance of this polypeptide was quantified after 23 d of storage at 6 °C, but the level was lower than in ripe fruit (3 d at 20 °C). High CO₂ treatment prevented the increase in the level of 46 ku polypeptide throughout the trial period (23 d) at 6 °C, with levels quite similar to unripe fruit and lower than those achieved in CO₂-treated fruit at 20 °C for 3 d.

Discussion

The storage quality of some fruit and vegetables at low temperature is a function of the progress of chilling symptoms. In the peel of green fruit, the amount of chlorophyll is inversely correlated with the severity of chilling injury (Pesis et al., 1994). In cherimoyas, which are susceptible to chilling injury, a process of chloroplast dismantling has been associated with severe chilling conditions (Gutiérrez et al., 1992). This process, similar to senescence, may involve, among other factors, a decline in chloroplast function (Wardley et al., 1984). In this sense, chlorophyll content and RuBPCase protein levels could be reflecting changes in chloroplasts of cherimoya green tissues, associated with low-temperature storage in the presence or absence of high CO₂ levels. The decrease observed in cherimoya RuBPCase protein levels during cold storage could be in line with the reported decreases in the amounts of plastid proteins during senescence. Enhanced rates of protein degradation or altered rates of RuBPCase subunit synthesis

have been proposed (Brady, 1988; Jiang et al., 1993). Even though there is not enough information available to provide us with a coherent picture regarding protein synthesis and degradation rate in cherimoya, the transient rise in proteolytic activity and the induction of specific chilling-proteins in cherimoya stored at 6 °C (Montero et al., 1995) could be involved in the variation of RuBPCase protein levels. The higher immunoreactive RuBPCase protein level, chlorophyll content, and green color in peel of CO₂-treated cherimoyas, compared with air-stored fruit, agree with the general beneficial effect attributed to high CO₂ atmosphere described for green vegetables (Aharoni et al., 1989; Barth et al., 1993).

The delay or suppression of fruit and vegetable softening is another well-known effect of high CO₂ treatment (Irving, 1992; Kader, 1986). Our results indicate that firmness and the immunodetected levels of PG-related protein content of cherimoya were maintained at low temperature if fruit received an initial treatment of high CO₂ for a short period of time. Goodenough et al. (1982) observed that red pigmentation and the appearance of PG are prevented in tomato by CA storage conditions, while other biochemical changes are not affected. In avocado fruit, the rate of increase in PG activity is appreciably slower under CA storage (Kanellis et al., 1991). Retardation of changes of protopectin to

soluble pectin by CA storage has been mentioned (Wang, 1990). Also, insoluble pectin content remains higher and PG activity increases more slowly in CO₂-treated fruit than in control peach fruit (Lurie and Pesis, 1992). Ultrastructural analyses in cherimoya tissues revealed that the middle lamella is dissolved and remaining fibrils of cellulose do not exhibit a clearly defined structure in ripe fruit, whereas the middle lamella of cherimoyas stored at 4 °C for 6 d is clearly visible and the cell wall is not disrupted (Gutiérrez et al., 1992). Previously observed differences between untreated and high CO₂-treated fruit stored at ambient temperature (Del Cura et al., 1996), and the results of this study at low temperature, indicate that PG protein is related to cherimoya fruit softening. Moreover, the present results indicate that high CO₂ pretreatment has an additional repressive effect on the low PG protein accumulation observed at low temperature.

We conclude that a short-term high CO₂ treatment at low temperature has a long residual effect and improves cherimoya fruit quality by preventing fruit softening and delaying senescence-like responses in green peel tissues.

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