JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

High CO_2 Atmosphere Modulating the Phenolic Response Associated with Cell Adhesion and Hardening of *Annona cherimola* Fruit Stored at Chilling Temperature

ROBERTO MALDONADO, ANTONIO D. MOLINA-GARCIA, MARIA T. SANCHEZ-BALLESTA, MARIA I. ESCRIBANO, AND CARMEN MERODIO*

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frío, Consejo Superior de Investigaciones Científicas, José Antonio Novais 10, E-28040 Madrid, Spain

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5.) activity, tanning ability, and polyphenols levels were measured in cherimoya (*Annona cherimola* Mill.) fruit treated with 20% CO_2 + 20% O_2 + 60% N_2 for 1, 3, or 6 days during chilling temperature (6 °C) storage. The residual effect of CO_2 after transfer to air was also studied. These observations were correlated with texture and cellular characteristics, visualized by cryo-SEM. Tanning ability and the early increase in tannin polyphenols induced by chilling temperature were reduced by CO_2 treatment. Conversely, high CO_2 atmosphere enhanced the nontannin polyphenol fraction as compared with fruit stored in air. Lignin accumulation and PAL activation observed in untreated fruit after prolonged storage at chilling temperature were prevented by high CO_2. Moreover, the restraining effect on lignification was less effective when the CO_2 treatment was prolonged for 6 days. In addition, fruits held at these conditions had greater firmness and the histological characterization of the separation between cells was similar to that in untreated fruits. We conclude that CO_2 treatment modulates the phenolic response that seems to regulate the strength of cell adhesion and so to prevent hardening caused by chilling temperature storage.

KEYWORDS: ***Annona cherimola*; carbon dioxide; chemiroya; chilling injury; low temperature; phenylalanine ammonia-lyase; tannins**

INTRODUCTION

Plants exhibit a range of defense and stress responses to adverse environmental conditions. Prolonged exposure to low temperatures during storage induces metabolic dysfunction in chilling-sensitive plants, which leads to the development of a variety of chilling injury symptoms (*1*). Some of the most common symptoms, specifically in *Annona*s, are related with changes in color and the texture (*2*). Texture changes can result from alterations in the structure of the different polysaccharides present in the cell wall and their covalent cross-linking, which determine the mechanical properties of the walls (*3*). Several cell wall-degrading enzymes are known to catalyze different aspects of the disassembly of polysaccharide polymers (*4*), but less attention has been paid to polymers that may increase cell wall stability by reducing the ability of enzymes to break down the bonds that hold the cell wall structure together or by interacting with pectic polymers and hemicellulose components. In this connection, cell wall properties appear to be modulated by the presence of sterified phenolic components capable of forming heat-stable cross-links (*5*). Tannins are also involved in cell-wall strengthening (*6*) and play an important structural role through their potential cross-linking with wall components. Like tannins, lignin when bound to the cell wall polysaccharide also confers rigidity to the cell wall (*7*). Moreover, some fruits such as cherimoyas are rich in sclereid, a highly lignified cell, and have modified parenchymatous cells with a relatively greater abundance of tannin materials (*8*).

Modifications of cell wall composition and properties of fruit stored at low temperatures have been reported (*9*). Some postharvest technologies, such as controlled atmosphere, have also been shown to modulate membrane deterioration and prevent some physiological disorders related to texture (*10*) caused by chilling temperatures. In cherimoyas, high CO_2 levels maintain fruit quality and improve tolerance of fruit during low-temperature storage (*11*).

The aim of this study was to understand how low temperature and high CO_2 modify fruit texture and modulate the relative amount of polyphenol fractions and tanning ability. Lignification and texture responses were related to the structural features of mesocarp tissues using low-temperature scanning electron microscopy (cryo-SEM), which allows the direct observation of frozen tissues in their hydrated state and thus avoids artifacts associated with preparation procedures (*12*). This study provides information on cell separation in mesocarp tissues of CO_2-treated fruits. In addition, the evidence supports data about the direct

* Corresponding author. Phone: 34 91 549 23 00. Fax: 34 91 549 36 27. E-mail: merodio@if.csic.es.

10.1021/jf020629g CCC: $22.00
Published on Web 11/21/2002

effect of high CO_2 on tannin polyphenols and their ability to link weakly to esterified cell wall components.

MATERIALS AND METHODS

Cherimoya (*Annona cherimola* Mill. cv. "Fino de Jete") fruit of uniform maturity and size and free from physical and pathological defects were harvested in Almuñecar (Granada, Spain) in October. Selected fruits were randomly divided into two lots of 60 fruits and stored in the dark at 6 °C in separate respiration chambers (20 L) under a continuous flow (100 mL/min) of humidified air (untreated fruit) or a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit). After 1, 3, and 6 days, CO_2-treated fruit were transferred to a continuous humidified air flow. Fruits were sampled at the end of CO_2-treatment and after 13 days of total chilling storage period. Untreated fruit were sampled after 1, 3, 6, and 13 days of storage in air at 6 °C. Six cherimoyas were collected randomly before storage (freshly harvested fruit) and for every subsequent sampling period and used immediately for both cryo-SEM and firmness analysis. Another three cherimoyas were sampled, peeled, frozen in liquid nitrogen, and stored at −80 °C for the different biochemical analyses.

Polyphenol Analysis. Frozen and freeze-dried samples (2.5 g) were extracted four times with 25 mL of 1% (v/v) HCl in methanol, for 1 h each time, under continuous stirring at ambient temperature and centrifuged at 2000*g* for 10 min. The combined supernatant was used for analysis of polyphenols, and the final insoluble-alcohol residue was used for lignin determination.

For the isolation and quantification of tannins, an aliquot of the total polyphenol fraction (15 mL) was dried and redissolved in methanol−ethanol−water (1:3.8:0.2) and centrifuged at 2000*g*, and the supernatant was loaded onto a 1.5 × 20 cm Sephadex LH-20 gel lipophilic filtration column (Sigma-Aldrich, St. Louis, MO) saturated with 95% (v/v) ethanol−water. First, the nontannin polyphenol fraction (NTP) was eluted using 95% (v/v) ethanol−water at a flow rate of 3 mL/min. Fractions of 3 mL were collected, and NTP elution was monitored by following absorbance at 280 nm. Fractions containing NTP were pooled, evaporated, and resuspended in 10 mL of 1% (v/v) HCl in methanol until further analysis. Afterward, the retained tannins were eluted using 50% (v/v) acetone−water; the procedure was as described, and the fractions collected at 400 nm were monitored. Total tannin and nontannin polyphenols were quantified using the Prussian blue method (*13*), which gives less protein interference than other oxidation−reduction reactions such as the Folin−Ciocalteu reagent. Results were expressed as mg of gallic acid/g of dry weight. The standard for gallic acid (Sigma-Aldrich, St. Louis, MO) was used for quantitative analysis. Absorbances were measured using a Perkin-Elmer Lambda-15 UV/vis spectrophotometer (Norwalk, CT).

Lignin (Klason lignin) was determined gravimetrically after acid hydrolysis of the insoluble-alcohol residue under previously established conditions (*14*). The residue was mixed 1:9 (w/v) with 12 M H_2SO_4 and hydrolyzed for 3 h at 20 °C with stirring, diluted with distilled water up to 1 M H_2SO_4, and heated for 2.5 h at 100 °C with continuous shaking. The residue remaining after acid hydrolysis was vacuum-filtered through an acid-treated 0.45 μm HVLP filter (Millipore, Bedford, MA), air-dried at 60 °C overnight, and weighed. Results were expressed as g of lignin/100 g of dry weight. To avoid interference from proteins, Klason lignin values were corrected for nitrogen impurity using a LECO FP-2000 nitrogen/protein determinator (St. Joseph, MO).

Tanning Ability. The radial diffusion method (*15*) was used to determine the capacity of tannins for protein precipitation. Total phenolic fractions were extracted at 4 °C by following the methods described by Brignolas et al. (*16*). Phenolic compounds were obtained, in triplicate, from 0.2 g of frozen and lyophilized mesocarp samples using 80% (v/v) methanol in ultrapure water (5 mL). The mixture was placed in a sonicating water bath for 15 min and centrifuged at 16 500*g* for 20 min at 4 °C. The residue was discarded, and the supernatant, completely dry at 30−40 °C, was collected under a continuous stream of nitrogen. The residue solubilized in 0.25 mL of ultrapure water constituted the final phenolic extract. Tanning ability was determined as the protein-binding efficiency of the extract. Agarose gels (1% (w/v), type I, Sigma) were prepared with 50 mM acetic acid buffer (pH 5) and with 0.1% (w/v) BSA (fatty acid-free fraction V, Sigma) by boiling while stirring in a water bath. Aliquots of 10 mL were dispensed into standard Petri dishes. Five 6-mm-diameter wells were made in each dish. An aliquot of the final phenolic extract (0.04 mL) was placed in each well of the Petri dish. A central well containing 0.02 mL of tannin-containing solution (2 mg/mL) in ultrapure water was used as external standard for all the assays. After 24 h at 25 °C in the dark, the area of each visible BSA precipitation zone was measured. Results were expressed as tanning ability, in mg of tannic acid equiv/g of dried weight, from a standard curve prepared with tannic acid (Sigma).

Phenylalanine Ammonia-Lyase Assay. Extraction and assay of phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.5.) were performed by following the procedure described by Cheng and Breen (*17*), with some modifications. Briefly, frozen pulp was homogenized at 4 °C with chilled 80% (v/v) acetone and placed at −20 °C for 15 min, the homogenate filtered, and the pellet then dried under vacuum. Protein extracts were obtained by homogenizing acetone powder (0.5−0.25 g) at 4 °C in 5 mL of 0.1 M sodium borate buffer, pH 8.8, containing 5 mM β-mercaptoethanol, 2 mM EDTA, and 4% (w/v) poly(vinylpyrrolidone). After 1 h, the homogenate was centrifuged at 27 000*g* for 30 min at 4 °C. The reaction mixtures contained 10 mM of L-phenylalanine, 30 mM sodium borate buffer (pH 8.8), and 1 mL of crude extract in a total volume of 3 mL. The substrate was added after 10 min of preincubation, and the reaction was stopped with 0.1 mL of 6 N HCl. PAL activity was determined by the production of cinnamate for 90 min at 30 °C under continuous shaking, measured by the absorbance change at 290 nm (*18*), using a Perkin-Elmer Lambda-15 UV/vis spectrophotometer (Norwalk, CT). Specific enzyme activity was defined as nmol of cinnamic acid/(h/mg of protein).

Protein concentration was measured by the Bradford method (*19*) using protein-dye reagent (Bio-Rad, Hercules, CA) and bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard.

Firmness Measurement. Flesh firmness was determined as the highest rupture force to penetration with an 8 mm diameter cylindrical flat-surfaced plunger at a crosshead speed of 400 mm/min, using an Instron model 1140 texturometer. Firmness was measured in whole peeled fruits, at three locations on the equator and expressed in newtons (N).

Cryo-SEM Observation. Microscopic observations were carried out using a Zeiss DSN-960 electron scanning microscope, equipped with a cold stage Cryotrans CT-1500 (Oxford). Samples of fruit tissues were introduced in N_2 slush and then transferred to the Cryotrans and the prechamber of the microscope (−180 °C), where they were fractured. After fracturing, samples were placed in the microscope stage for etching at −90 °C, for 2 min. They were then gold-shadowed and subsequently reinserted in the microscope, where observation was carried out at −150 to −160 °C. Samples were observed with both secondary and retrodispersed electrons, and the best images were selected in each case.

An image analysis software (Global Lab, Data Translation, Marlboro, MA) was employed for cell separation quantification. Measurement was performed by manual selection of the corresponding regions on the expanded pictures. The data given are the averages of not less than 25 single measurements.

Statistical Analyses. Data from at least three replicates per sample were subjected to analysis of variance (ANOVA) (Stargraphics program, STSC, Rockville, MD). Multiple variance analysis was employed to determine the significance of the data at $P \leq 0.05$.

RESULTS

Effect of High CO_2 Atmosphere on the Lignification Response and Tanning Ability in Cherimoya Fruit Stored at Chilling Temperature. Lignin levels increased significantly ($P \leq 0.05$) at the sixth day of storage in air compared to CO_2-treated fruits (**Figure 1A**). Cherimoya PAL activity decreased by 50% in both CO_2-treated and air-stored cherimoyas after 1 day of storage at 6 °C compared to freshly harvested fruits (**Figure 1B**). However, there was a small but progressive increase in PAL activity up to 6 days of storage. CO_2 treatment

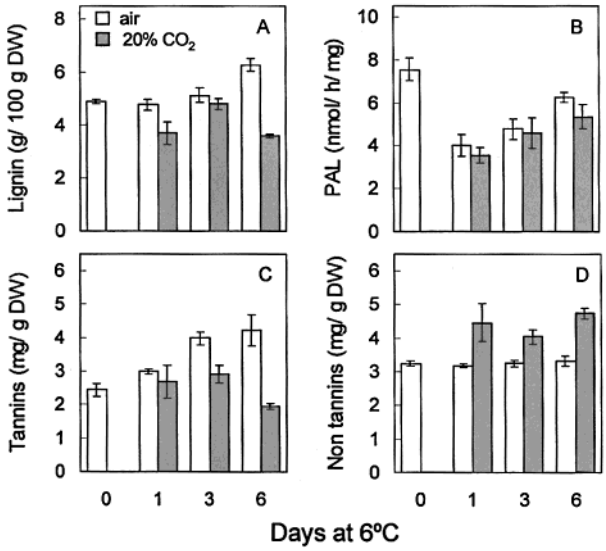

Figure 1. Content of lignin (A), PAL activity (B), tannins (C), and nontannin polyphenols (D) in cherimoya fruit after harvest (0 days) and after 1, 3, and 6 days of storage at 6 °C in air or 20% CO_2. Data are averages of two separate experiments ($n = 6$) ± SE.

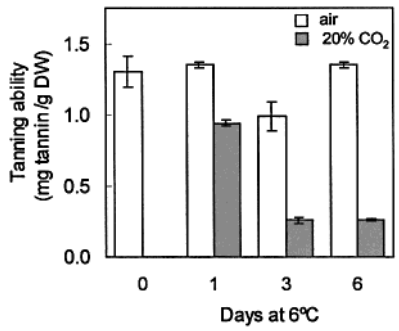

Figure 2. Tanning ability of cherimoya fruit after harvest (0 d) and after 1, 3, and 6 days of storage at 6 °C in air or 20% CO_2. Data are averages of two separate experiments ($n = 6$) ± SE.

did not change the total polyphenol content over the chilled storage period (data not shown), but the phenolic types were modified. CO_2 treatment significantly ($P \leq 0.05$) prevented the increase in tannins observed in air stored fruit (**Figure 1C**), especially in fruit treated for 6 days with 20% CO_2. In contrast, the levels of nontannin polyphenols in CO_2-treated fruits increased while the content found in air stored fruit remained constant (**Figure 1D**).

The tanning ability of CO_2-treated cherimoyas sharply decreased and was significantly ($P \leq 0.05$) lower for fruit treated for 3 and 6 days with 20% CO_2 (**Figure 2**). In contrast, the tanning ability of fruit stored in air did not markedly change throughout low-temperature storage.

Residual Effect of High CO_2 Atmosphere in the Content of the Different Polyphenol Fractions and in the Tanning Ability. To determine the residual effect of high CO_2 on the lignification response, fruits were transferred to air after 1, 3, or 6 days of CO_2 treatment and analyzed at day 13 of storage at chilling temperature (**Figure 3**). After prolonged storage in air at 6 °C, the lignin content reached values of 10.25 ± 0.08 g/100 g of dry weight (**Figure 3A**), 2-fold higher than those in freshly harvested fruit (4.91 ± 0.08 g/100 g of dry weight) (**Figure 1A**). The transfer of CO_2-treated fruit to air was also associated with lignin accumulation. However, the levels reached in the CO_2-treated samples were lower and proportional to the period of treatment applied (6.88 ± 0.32, 7.83 ± 0.23, and 8.77 ± 0.81 g/100 g of dry weight for 1, 3, and 6 days, respectively). The highest lignin levels were quantified in fruit treated with CO_2 for 6 days.

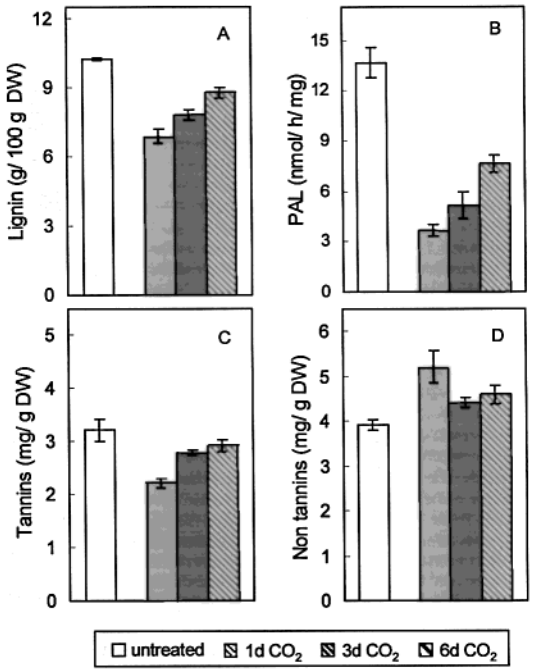

Figure 3. Content of lignin (A), PAL activity (B), tannins (C), and nontannins (D) of untreated and CO_2-treated cherimoyas at day 13 of total storage period at 6 °C. After 1, 3, and 6 days of CO_2 treatment cherimoyas were transferred to air. Data are averages of two separate experiments ($n = 6$) ± SE.

Also, CO_2-treatment largely prevented the sharp increase in PAL activity observed in untreated fruit (**Figure 3B**). Only in fruits treated for 6 days with 20% CO_2 did PAL activity increase to a level similar to that observed in freshly harvested fruits (7.6 ± 0.5 nmol/(h/mg)) (**Figure 1B**).

After transfer to air, there was a slight increase in tannin values and it was proportional to the length of CO_2 treatment, but the maximum absolute content detected in CO_2-treated fruits was always lower than in untreated fruits (**Figure 3C**). Furthermore, considering the tannin levels at the end of the CO_2 treatment (**Figure 1C**), the highest increase in tannin content occurred in fruit treated for 6 days with 20% CO_2 (**Figure 3C**). Meanwhile, after transfer to air the pattern of change in nontannins levels was not altered, and CO_2-treated fruits continued to have higher levels than untreated fruits (**Figure 3D**).

There was no change or even a decrease in the tanning ability of untreated fruit, but a positive trend was observed in CO_2-treated fruit, especially after 6 days (**Figure 4**). These estimations of Δ tanning ability were consistent with the evolution of tannin content after transfer to air. Despite the sharp increase for 6 days of CO_2, higher levels of tanning ability were consistently attained in untreated fruit with values of 0.96 ± 0.03 mg of tannin/g of dry weight.

Residual Effect of High CO_2 Atmosphere on Texture and Morphological Evaluation. The highest flesh firmness values were reached for untreated and 6 days CO_2-treated cherimoyas (66 N) after storage for 13 days at 6 °C (**Figure 5**). SEM micrographs revealed similarities between untreated and 6 days CO_2-treated fruit with respect to intercellular spacing and cell adhesion (**Figure 6**). However, untreated cherimoyas exhibited the highest degree of tissue disorganization with the middle

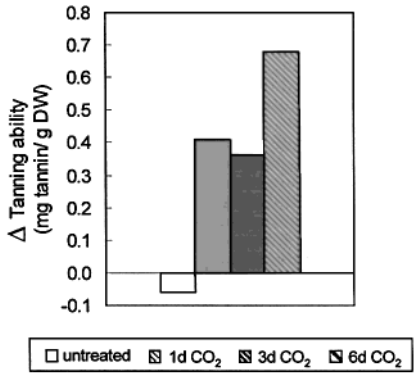

Figure 4. Increment in the tanning ability of untreated and CO_2-treated cherimoyas at day 13 of total storage period at 6 °C. The differences between the results of tanning ability after 1, 3, and 6 days of CO_2 treatment and the values after transfer to air at day 13 were expressed as Δ tanning ability. Data are averages of two separate experiments ($n = 6$).

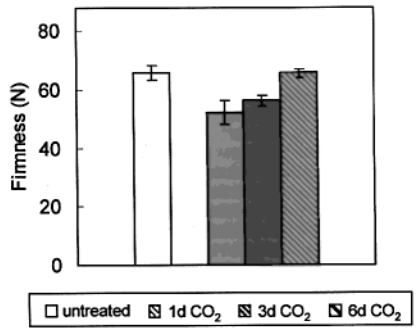

Figure 5. Fruit firmness of untreated and CO_2-treated cherimoyas at day 13 of total storage period at 6 °C. After 1, 3, and 6 days of CO_2 treatment cherimoyas were transferred to air. Data are averages of two separate experiments ($n = 6$) ± SE.

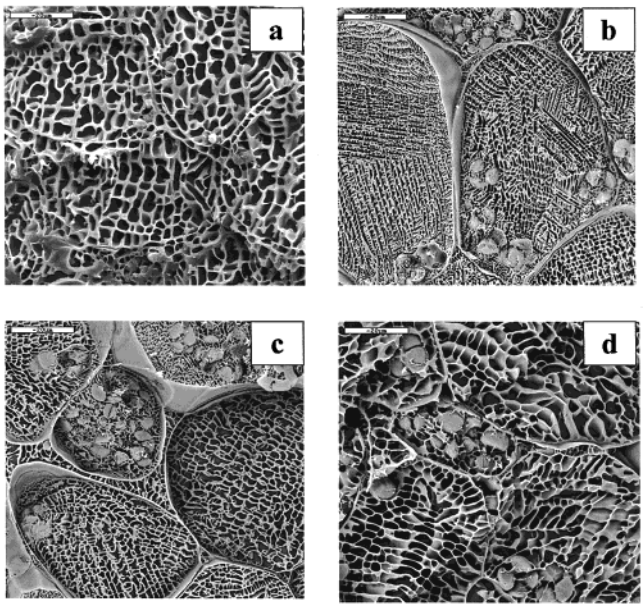

Figure 6. Cryo-SEM micrographs showing cells of mesocarp tissues from untreated (a) and CO_2-treated cherimoyas for 1, 3, and 6 days (b–d, respectively) at day 13 of total storage period at 6 °C. After 1, 3, and 6 days of CO_2 treatment cherimoyas were transferred to air.

lamella mostly degraded. A reduction in cell compaction was observed in fruit treated with CO_2 for 1 and 3 days. CO_2 treatment seems to increase the amount of material surrounding the cells. Fruit treated with CO_2 for 6 days, on the other hand, appeared more compact although the middle lamella was still visible. Image analysis of cherimoya tissues cryo-SEM micrographs showed that untreated and 6 days CO_2-treated cherimoyas exhibited a gap separating cells of 0.50 ± 0.03 μm, while the gap in 1 and 3 days CO_2-treated cherimoyas was larger (0.80 ± 0.05 and 0.70 ± 0.04 μm, respectively). Also, examination of the fracture surfaces showed that modification of cell adhesion was mainly located at the edges of the cell faces rather than across the entire cell surface.

DISCUSSION

In previous work (*20*) we observed that cherimoyas stored at chilling temperature lost their ability to ripen and exhibited several chilling injury symptoms including severe rigidity. Because lignin and tannins play a role in structural strength and their accumulation confers high rigidity and compression resistance to the cell wall, their involvement in texture was analyzed in this study. We also sought to determine whether the phenolic response at low temperatures can be modulated by high CO_2 levels. We observed an accumulation of lignin in response to low temperature after relatively long storage periods. It was also observed that CO_2 treatment modified the time course of this response, delaying and reducing the magnitude of this particular response. Additionally, in all cases of enhanced accumulation of lignin, increased PAL activity was detected. PAL activation has been generally reported in response to several kinds of stress (*21*, *22*). However, treatments used to reduce chilling injury in chilling-sensitive fruit have been associated with a decrease in PAL activity (*23*). In cherimoya, 20% CO_2 decrease PAL activation observed after approximately 2 weeks of low-temperature storage. Moreover, the restraining effect on PAL activity was less effective when the CO_2 treatment was prolonged for 6 days, although the level of PAL activity was always lower than in untreated fruits. Further experimental work is required to explain the observed initial decrease in PAL activity at low temperature. In a previous report (*24*) it was suggested that PAL activity decreased in fruit exhibiting a low rate of ammonia reassimilation.

While the total amount of all phenolic compounds expressed on the basis of dry matter was fairly constant throughout cold storage, the relative amounts of polyphenol fractions changed. Therefore, the average tannin content increased early in fruit stored at chilling temperature and occurred earlier than lignin accumulation, supporting the idea that phenolic complexes in the wall act as nucleation sites for lignin deposition (*25*). Conversely, in CO_2-treated fruit the increase of tannins was prevented and only the nontannin fraction rose. Tannins have been defined as "any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyl and other suitable groups (i.e. carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied" (*26*). Considering the pH dependence of the aggregation between tannin and protein (*27*), the significantly ($P \leq 0.05$) lower tanning ability of treated fruit at the end of 20% CO_2 treatment (**Figure 2**, specifically for those treated for 3 and 6 days) could indicate that the CO_2 treatment had interfered with the cellular pH. Moreover, the inverse trend observed in the tannin content and in their binding ability when the fruit was transferred from the high CO_2 atmosphere to air suggests a direct effect of CO_2. The duration of CO_2 treatment was shown to be also crucial for the modification of phenol quality and for further stimulation of the phenylpropanoid pathway. Biolley et al. (*28*) reported

that the elicitation and amount of the ozone-induced phenolics were closely connected with both exposure time and ozone concentration. In this respect, 6 days of CO_2 may be too long, given that there was a sharp increase in tannin ability and higher accumulation of lignin when fruits were transferred to air. Moreover, such deposition of lignin could account for the higher texture values of these fruits and also the modified cell wall physical properties, as detected by cryo-SEM.

A detailed histological characterization revealed that treatment with a high CO_2 concentration results in an increase in cell separation in the corners of intercellular spaces. Parker et al. (*29*) concluded that the domains at the corners of intercellular spaces correspond to the edges of adjacent cell faces and are enriched with weakly esterified polygalacturonic acid. The authors further concluded that adhesion of these domains must be overcome during fruit ripening, cooking, or chemical treatments to allow cell separation. According to our results, it is possible that tannin cross-linking of polymers, mainly concentrated in the corners of intercellular spaces, could explain the greater cell adhesion of untreated fruit stored at chilling temperature. The CO_2 treatment by altering the concentration and the timing of accumulation of the phenolic compounds could be modulating the cross-linking of cell wall polymers and allowing cell separation and lower hardening during the early stage of storage at chilling temperature. This idea is further supported by the change in the mechanical properties and the corresponding increase in polyphenol fractions and tanning ability after 6 days of CO_2 treatment. Further work on the ability to manipulate the texture of fruit and vegetables should consider the role of these compounds in the biochemical and molecular mechanisms involved in cell adhesion.

ABBREVIATIONS USED

CI, chilling injury; cryo-SEM, low-temperature scanning electron microscopy; NTP, nontannin polyphenols; PAL, phenylalanine ammonia-lyase.

ACKNOWLEDGMENT

This work was supported by a CICYT (Spain) grant (ALI99-0954-C03-01). Thanks also are due to Joston S. Assis for his helpful support.

LITERATURE CITED

(1) Wang, C. Y. Chilling injury of tropical horticultural commodities. *HortScience* **1994**, *29*, 986−988.

(2) Merodio, C.; De La Plaza, J. L. Cherimoya. In *Postharvest Physiology and Storage of Tropical and Subtropical Fruits*; Mitra, S. K, Ed.; CAB International: Wallingford, U.K., 1997; pp 269−293.

(3) Iiyama, K.; Bach-Tuyet Lam, T.; Stone, B. A. Covalent cross-links in the cell wall. *Plant Physiol.* **1994**, *104*, 315−320.

(4) Fry, S. C. Polysaccharide-modifying enzymes in the plant cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1995**, *46*, 497−520.

(5) Waldron, K. W.; Ng, A.; Parker, M. L.; Parr, A. J. Ferulic acid dehydrodimers in the cell walls of *Beta vulgaris* and their possible role in texture. *J. Sci. Food Agric.* **1997**, *74*, 221−228.

(6) Chalker-Scott, L.; Krahmer, R. Microscopic studies of tannin formation and distribution in plant tissues. In *Chemistry and Significance of Condensed Tannins*; Hemingway, R. W., Karchesy, J. J., Eds.; Plenun Press: London, U.K., 1989; pp 345−368.

(7) Lewis, N. G.; Yamamoto, E. Lignin: occurrence, biogenesis and biodegradation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990**, *41*, 455−496.

(8) Schroeder, C. A. Fruit morphology and anatomy of the cherimoya. *Bot. Gaz. (Chicago)* **1951**, *6*, 436−446.

(9) Bauchot, A. D.; Hallett, I. C.; Redgwell, R. J.; Lallu, N. Cell wall properties of kiwifruit affected by low temperature breakdown. *Postharvest Biol. Technol.* **1999**, *16*, 245−255.

(10) Deschene, A.; Paliyath, G.; Lougheed, E. C.; Dumbroff, E. B.; Thompson, J. E. Membrane deterioration during postharvest senescence of broccoli florets: modulation by temperature and controlled atmosphere storage. *Postharvest Biol. Technol.* **1991**, *1*, 19−31.

(11) Merodio, C.; Muñoz, M. T.; Del Cura, B.; Buitrago, M. D.; Escribano, M. I. Effect of high CO_2 level on the titres of γ-aminobutyric acid, total polyamines and some pathogenesis-related proteins in cherimoya fruit stored at low temperature. *J. Exp. Bot.* **1998**, *49*, 1339−1347.

(12) Wergin, W. P.; Erbe, E. F. Introduction to the advantages and problems associated with low-temperature scanning electron microscopy. *Scanning* **1991**, *13*, 14−26.

(13) Price, M. L.; Butler, L. G. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* **1977**, *25*, 1268−1273.

(14) Saura-Calixto, F.; Goñi, I.; Mañas, E.; Abia, R. Klanson lignin, condensed tannins and resistant protein as dietary fibre constituents: determination in grape pomaces. *Food Chem.* **1991**, *39*, 299−309.

(15) Hagerman, A. E. Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* **1987**, *13*, 437−449.

(16) Brignolas, F.; Lacroix, B.; Lieutier, F.; Sauvard, D.; Drouet, A.; Claudot, A.; Yart, A.; Berryman, A. A.; Christiansen, E. Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. *Plant Physiol.* **1995**, *109*, 821−827.

(17) Cheng, G. W.; Breen, P. J. Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J. Am. Soc. Hortic. Sci.* **1991**, *116*, 865−869.

(18) Zucker, M. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.* **1965**, *40*, 779−784.

(19) Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(20) Alique, R.; Zamorano, J. P.; Calvo, M. L.; Merodio, C.; De La Plaza, J. L. Tolerance of cherimoya (*Annona cherimola* Mill.) to cold storage. *J. Am. Soc. Hortic. Sci.* **1994**, *119*, 524−528.

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

(22) Ke, D.; Salveit, M. E. Carbon dioxide-induced brown stain development as related to phenolic metabolism in iceberg lettuce. *J. Am. Soc. Hortic. Sci.* **1989**, *114*, 789−794.

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

(24) Maldonado, R.; Escribano, M. I.; Merodio, C. Relationship between the levels of ammonia and coordination of phenylalanine ammonia-lyase and phospho*enol*pyruvate carboxylase in *Annona cherimola* stored under different conditions. *Postharvest Biol. Technol.* **2002**, *25*, 301−309.

(25) Whettene, R. W.; MacKay, J. J.; Sederoff, R. R. Recent advances in understanding lignin biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 85−609.

(26) Horvarth, P. J. The nutritional and ecological significance of hacer-tannins and related polyphenols. M.S. Thesis, Cornell University, Ithaca, NY, 1981.
(27) Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* **1981**, *259*, 4494−4497.
(28) Biolley, J. P.; Kanoun, M.; Goulas, P. The response of vacuolar phenolic content of common bean (*Phaseolus vulgaris* cv. Bergamo) to a chronic ozone exposure: questions and hypotheses. *Funct. Plant Biol.* **2002**, *29*, 1−11.
(29) Parker, C. C.; Parker, M. L.; Smith, A. C.; Waldron, K. W. Pectin distribution at the surface of potato parenchyma cells in relation to cell−cell adhesion. *J. Agric. Food Chem.* **2001**, *49*, 4364−4371.

Received for review June 5, 2002. Revised manuscript received September 24, 2002. Accepted September 24, 2002.

JF020629G