Food Structure

Volume 11 | Number 4 Article 4

1992

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FOOD STRUCTURE, Vol. 11 (1992), pp. 323-332
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

1046-705X/92$3.00+.00

ULTRASTRUCTURAL CHANGES IN CHERIMOYA FRUIT INJURED BY CHILLING

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Abstract

Cherimoya (*Annona cherimola Mill.*) is an important fruit crop that is grown in the South of Spain. Ultrastructural modifications of cherimoya fruit were studied after low-temperature storage. When cherimoya was stored at 4°C for 6 days, the starch grains did not suffer degradation and the cell walls remained intact. The membrane systems were severely damaged, resulting in a loss of cell compartmentalization. Cherimoya rewarmed to 22°C after 9 days of low temperature storage is not able to recover, showing the irreversibility of the ultrastructural changes. In addition, disorganization of the internal lamella of chloroplasts, grana unstacking, as well as a general swelling of plastids and mitochondria were also observed. The ultrastructural damage observed is explained in terms of membrane disruption.

Key Words: Cherimoya, ripening, chilling injury, cold, cell disorganization, cell wall, starch, membranes, chloroplasts, mitochondria, Golgi.

Initial paper received May 12, 1992
Manuscript received December 8, 1992
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Introduction

Commercialization of soft fruits is always a problem as the market value of fruit can easily depreciate during transportation. Due to their fast climacteric ripening, cherimoya have a very short shelf-life, lasting no longer than one week, as they become senescent in 5-8 days at 22°C, normal ripening temperature, depending upon cultivar (Fuster and Prestamo, 1980). Unlike other soft fruits, they do not ripen if the harvesting occurs before physiological maturation. Cherimoya, being particularly susceptible to low-temperature damage, are generally harvested and stored as mature-green fruits.

We have been trying to develop a method to delay senescence of cherimoya. Most of the treatments severely compromise the organoleptic characteristics of this fruit. We have been successful only by reducing the storage temperature to 10-12°C, as storage of cherimoya at 8-10°C or below for a few days invariably produces the development of several chilling injury symptoms (Lahoz *et al.*, 1990). Many tropical and subtropical plants, as well as their fruit, when exposed to low, but non-freezing temperatures, in the range 0°C to 15°C undergo physiological damage known as chilling injury (Raison and Lyons, 1986; Jackman *et al.*, 1988; Parkin *et al.*, 1989). *Annona squamosa* "sugar apple" develops chilling injury within 5 days at 4°C (Broughton and Tan, 1979).

The effects on the cell microstructure produced by low temperatures have been investigated in many fruits, a critical temperature (defined as the temperature producing irreversible alterations of the organoleptic characteristics of fruits, deficient ripening, physiological alterations, etc. as a consequence of phase-transitions of lipids within membranes), ranging from 0°C to 15°C for each particular subtropical fruits studied, has been found (Abe, 1990; Marangoni *et al.*, 1989; Miller *et al.*, 1987; Salveit and Cabrera, 1987; Ben-Arie *et al.*, 1979). Storage below the critical temperature has a drastic effect on cellular structure as a consequence of the physiological changes induced. Several effects produced by storage of fruits at low temperatures have been described: The most evident and common are the induction of microvesiculation of the endoplasmic reticulum with loss of ribosomes

(Niki *et al.*, 1978; Ilker *et al.*, 1976), clumping of nuclear chromatin (Ilker *et al.*, 1976; Moline, 1976; Chabot and Leopold, 1985), disruption of tonoplast, (Niki *et al.*, 1978) and disorganization of the matrix and cristae of mitochondria (Platt-Aloia and Thomson, 1976; Murphy and Wilson, 1981). Morphological changes including swelling of plastids, disorganization of internal lamella, and unstacking of grana have also been observed in several plant species (Kimball and Salisbury, 1973; Wise *et al.*, 1983).

Typical macroscopic symptoms of chilling injury include skin darkening, failure to ripen properly, pulp discoloration, and the incidence of pale pink vesicles around the seeds. Although not clearly visible at the chilling temperature, this injury becomes apparent after rewarming the fruits to the normal ripening temperature. Even without the appearance of these symptoms, cherimoya may fail to ripen when they have been stored at chilling temperatures.

The aim of this research was to examine the ultrastructural changes related to chilling injury of cherimoya during storage at refrigerated temperatures, to assess differences in the susceptibility of subcellular structures to chilling, and to correlate morphological, physiological and biochemical changes during injury with microstructural changes.

Material and Methods

Cherimoya fruit used in this study were grown at the Agriculture Tropical Fruit Research Station (C.S.I.C.) "La Mayora" (Málaga). Freshly harvested, mature-green cherimoya (300 $\pm$ 5 g) with no bruising were surface-disinfected to prevent fungal infections during storage by dipping in a wash solution with 0.2% Decosol (Pennwalt, U.K.) for 1 minute followed by washing in a fungicide solution: 0.05% (w/v, weight/ volume) Imazalyl (Fomasa, Spain) and 0.2% Decosol for 1 minute, with stirring, at 22°C. After drying, cherimoya were stored at 4°C and 90% relative humidity. A series of cherimoya was analyzed after 6 days storage. Another series after 9 days at 4°C was transferred to 22°C for 3 more days prior to analysis. Control cherimoya were placed in storage at 22°C and assayed before storage and 6 days after harvesting. Six fruits were analyzed for each series.

Transmission electron microscopy (TEM)

1 mm^3 cubes from the inner and outer layers of the epicarp, and from different areas of the pericarp, mesocarp and endocarp, were removed from the side of the fruit. The outer layer of epicarp was fixed in a mixture of 2% glutaraldehyde and 6% paraformaldehyde in 50 mM sodium cacodylate buffer, pH 6.8. Other tissues were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 50 mM sodium cacodylate buffer, pH 6.8. Fixation of tissues was carried out for 16 hours at 22°C. Samples were rinsed with 0.1 M sodium cacodylate buffer, pH 6.8, for 2 hours and post-fixed in buffered 2% osmium tetroxide for 2 hours in the dark. Samples were washed three times with 0.1 M sodium cacodylate buffer, pH 6.8, for 1 hour, and dehydrated through a graded ethanol series of 50, 70, 90 and 100% of absolute ethanol, with three changes, at 22°C, for 10 minutes in each ethanol concentration, and embedded in Epon resin. Ultra-thin sections were cut with a Reichert-Jung ultramicrotome, contrasted with Reynolds lead citrate (Reynolds, 1963) and examined in a Zeiss TS 100 electron microscope.

Figure 1. Scanning electron micrographs of epicarp from cherimoya fruit. (**a**) Freshly-harvested fruit. (**b**) Fully ripe fruit after storage for 6 days at 22°C. (**c**) Fruit stored for 6 days at 4°C. (**d**) Fruit stored for 9 days at 4°C and rewarmed to 22°C for 3 additional days. Bars = 50 μm.

Figure 2. Scanning electron micrographs of chilling-injured epicarp from cherimoya fruit. (**a**) Outer layer showing hollow (arrow) and external cell layers (E), and (**b**) inner layers of the epicarp. Bars = 50 μm.

Scanning electron microscopy (SEM)

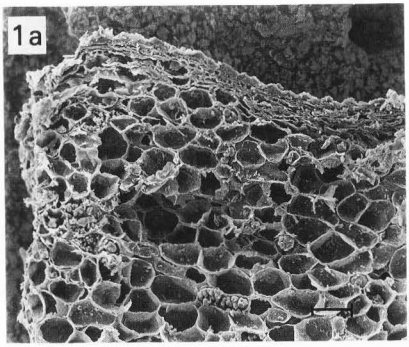
Fixed and dehydrated cubes from epicarp, mesocarp, and endocarp of cherimoya were dried in a critical-point drying apparatus using liquid carbon dioxide (Polaron E-3000), immersed in liquid nitrogen, fractured, mounted on aluminium stubs, and sputter-coated with gold (in Polaron E-5000 coating unit). The cherimoya microstructure was studied using a Zeiss DSM 950 scanning electron microscope (SEM) operated at 15 kV.

Results

Fruit softening is associated with the activity of cell wall-degrading enzymes (Pilnik and Voragen, 1970; Dilley, 1970; Ben-Arie *et al.*, 1979). A decrease in cherimoya firmness is observed throughout ripening. Softening of cherimoya is markedly reduced when the fruits are stored at 4°C.

Fig. 1 shows representative micrographs of cherimoya epicarp. In the freshly harvested cherimoya (Fig. 1a), the individual cells were well defined and the cell walls were observed to be rigid and vertical, indicating the strength of the fiber constituents of the walls. Cherimoya were senescent and no longer edible after 6 days of storage at 22°C (Fig. 1b). Individual cells were difficult to identify due to the structural degradation of the cell walls and the presence of abundant amorphous materials. When cherimoya were stored 6 days at 4°C, some darkened areas covering between 2-10% of the total surface of the fruit were apparent. In the normal green-colored surface of the cherimoya no structural differences with respect to the freshly harvested fruits were apparent (Fig. 1c). However, when cherimoya were stored 9 days at 4°C and transferred for three additional days at 22°C, even in green-colored areas, structural changes, basically consisting in the piling and flattening of several layers of cells, were observed (Fig. 1d). In darkened areas, the integrity of the tissues was lost and hollows were observed within the external cellular layers of the epicarp (Fig. 2a). Thickening of the cell walls was observed in the inner epicarp (Fig. 2b).

SEM observations of mesocarp (Fig. 3) and endocarp (Fig. 4) tissues show another characteristic feature of cherimoya maturation. In freshly harvested fruits, cherimoya parenchymal cells were fully loaded with starch grains (Figs. 3a, 4a). After 6 days at 22°C, most of the starch was metabolized (Figs. 3b, 4b). However, in cherimoya stored at 4°C for 6 days (Figs. 3c, 4c) or for 9 days and subsequently transferred to 22°C for three additional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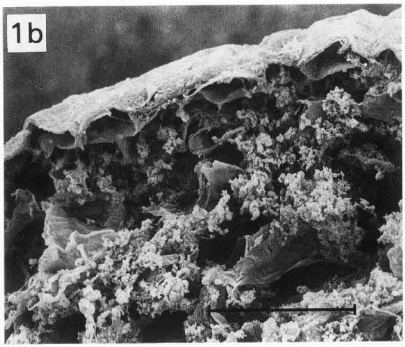
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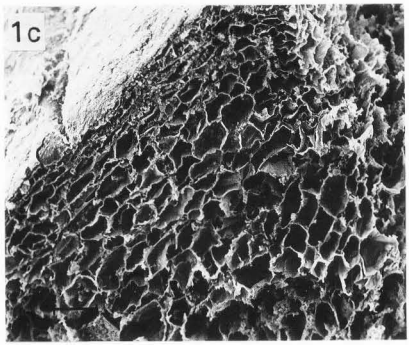
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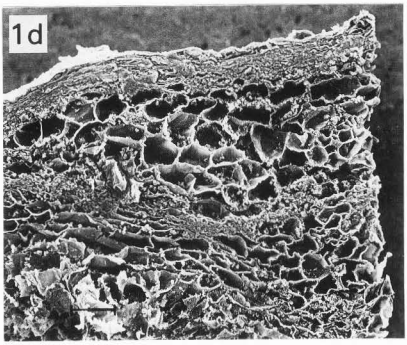
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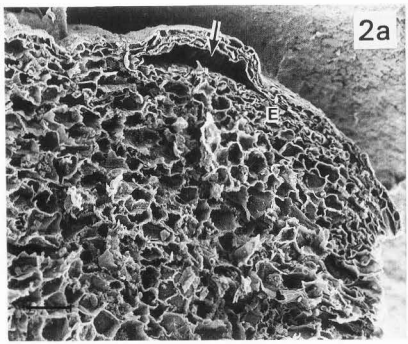
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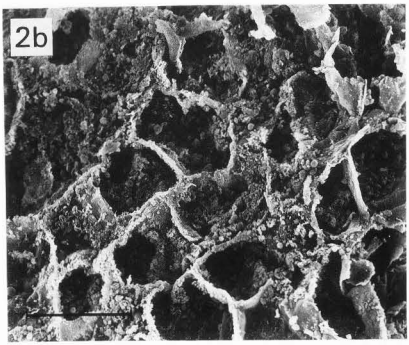
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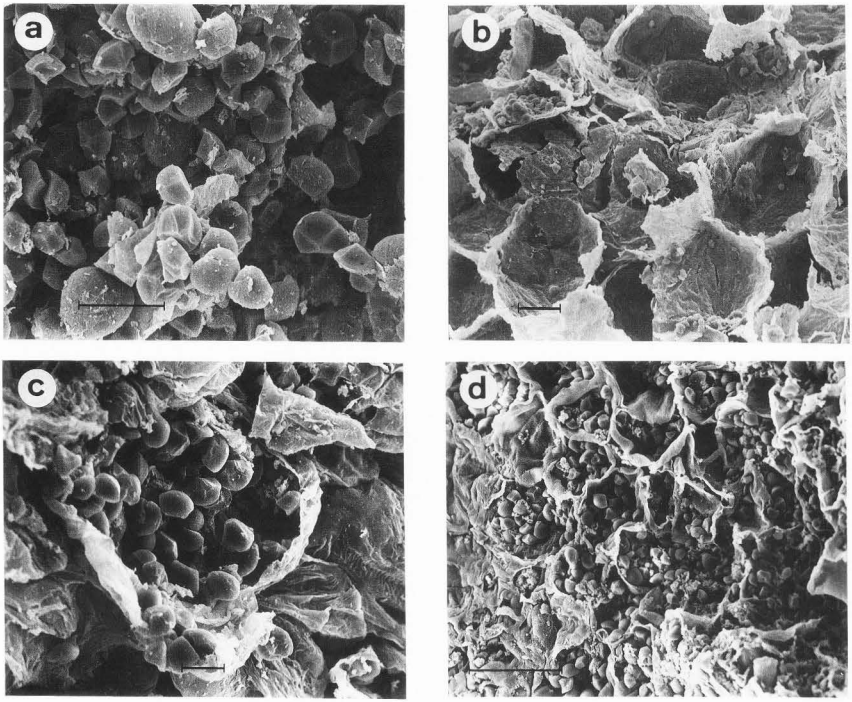


Figure 3. Scanning electron micrographs of mesocarp from cherimoya fruit. **(a)** Freshly harvested fruit. **(b)** Fully ripe fruit after storage for 6 days at 22°C. **(c)** Fruit stored for 6 days at 4°C. **(d)** Fruit stored for 9 days at 4°C and rewarmed to 22°C for 3 additional days. Bars = 20 μm (a, b, d) and 10 μm (c).

days (Figs. 3d, 4d) many starch grains were still evident.

Microstructural changes during normal ripening of cherimoya as well as during chilling injury were observed in greater detail using TEM.

Low magnifications (2,500-5,000 x) were used to obtain a general view of the cells. In freshly harvested cherimoya (Fig. 5a), a large central vacuole limited the cytoplasm to a thin peripheral layer lining the cell wall. Cell walls were intact in the tissues observed. The middle lamella was visible as a markedly electron-dense region between the walls of adjacent cells. In the mesocarp and endocarp tissues, there were many large starch grains embedded in amyloplasts. Both plasmalemma and tonoplast membranes were continuous. After 6 days at 22°C (Fig. 5b), cells were mostly disintegrated. The middle lamella was dissolved and the remaining fibrils of cellulose did not exhibit a clearly defined structure, showing a sinuous appearance. Well-defined membranes were not observed. Spaces previously occupied by vacuoles were irregularly filled with osmiophilic bodies without defined structure. Starch grains were not observed in the mesocarp, although some were still visible in the endocarp. Cell walls were not disrupted in cherimoya stored at 4°C for 6 days (Fig. 5c) and the middle lamella was clearly visible. In spite of the preserved integrity of the cell wall, a high degree of disorganization of the membranes was observed. Amyloplasts membranes were ruptured but still visible. In fruits stored for 9 days at 4°C that was subsequently transferred for three additional days at 22°C (Fig. 5d), the cells walls appeared to be similar to those in cherimoya stored at 4°C. Plasmalemma, tonoplast and amyloplasts membranes were vanished. Starch grains of the same size as in freshly harvested cherimoya were abundant.

Higher magnifications (30,000-80,000 x) were used to study the effects of the treatments on several cytoplasmic structures such as chloroplasts, mitochondria,

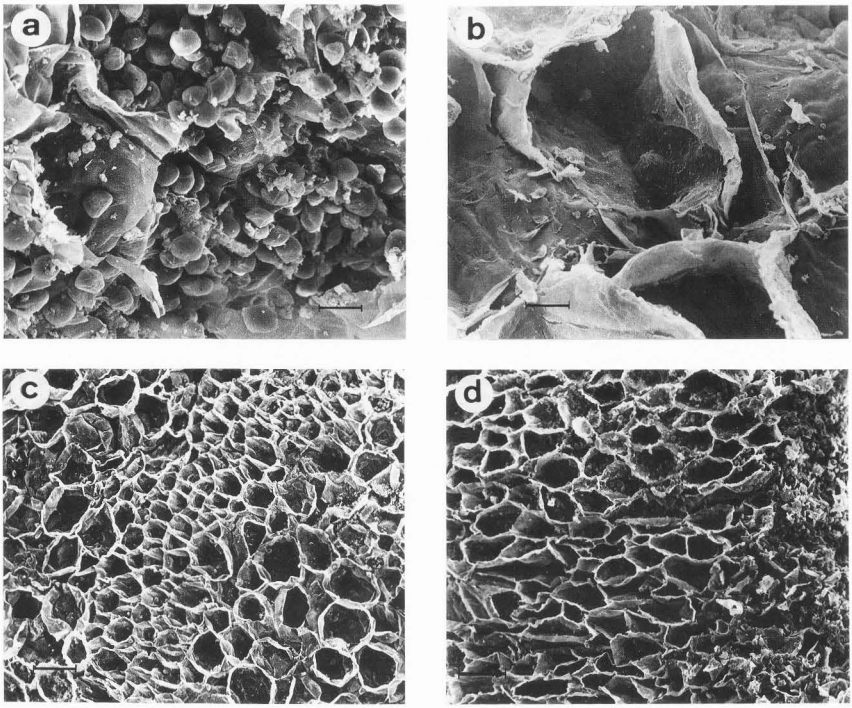


Figure 4. Scanning electron micrographs of endocarp from cherimoya fruit. (**a**) Freshly harvested fruit. (**b**) Fully ripe fruit after storage for 6 days at 22°C. (**c**) Fruit stored for 6 days at 4°C. (**d**) Fruit stored for 9 days at 4°C and rewarmed to 22°C for 3 additional days. Bars = 10 μm (a, b) and 50 μm (c, d).

endoplasmic reticulum and the Golgi apparatus. In cherimoya stored at 22°C for 6 days, none of these cytoplasmic structures could be observed except in some preparations from endocarp tissues, where highly disorganized mitochondria were noticeable.

In epicarp tissues from freshly-harvested cherimoya, chloroplasts containing numerous grana, some plastoglobuli, and a small number of starch grains were observed (Fig. 6a). After 6 days storage at 4°C chloroplasts appeared swollen and grana had begun to unstack (Fig. 6b). In refrigerated cherimoya rewarmed for three days at 22°C, drastically disorganized chloroplasts were observed, the grana were unstacked and tilakoids adopted a random arrangement (Fig. 6c).

In freshly-harvested cherimoya, mitochondria have well developed cristae (Fig. 7a). After storage at 4°C the number of cristae diminished and swollen mitochondria were observed in all fruit tissues (Fig. 7b). Rewarming at 22°C for three additional days produced a more severe disorganization of organelles (Fig. 7c). The observation of disorganized membranes and lack of cristae in mitochondria suggests that the organelles were probably no more functional.

Endoplasmic reticulum was abundant in freshly-harvested cherimoya, as well as Golgi apparatus (Figs. 7a, 8a). Refrigeration produces swelling of the endoplasmic reticulum vesicles with loss of ribosomes, as well as a reduction on the size of Golgi apparatus. Dictyosomes were smaller, formed only by two or three cisterns, in the very low percentage of cells containing Golgi apparatus (Fig. 8b).

Discussion

When fruits ripen under normal physiological conditions, cell wall disintegration, starch degradation, and a

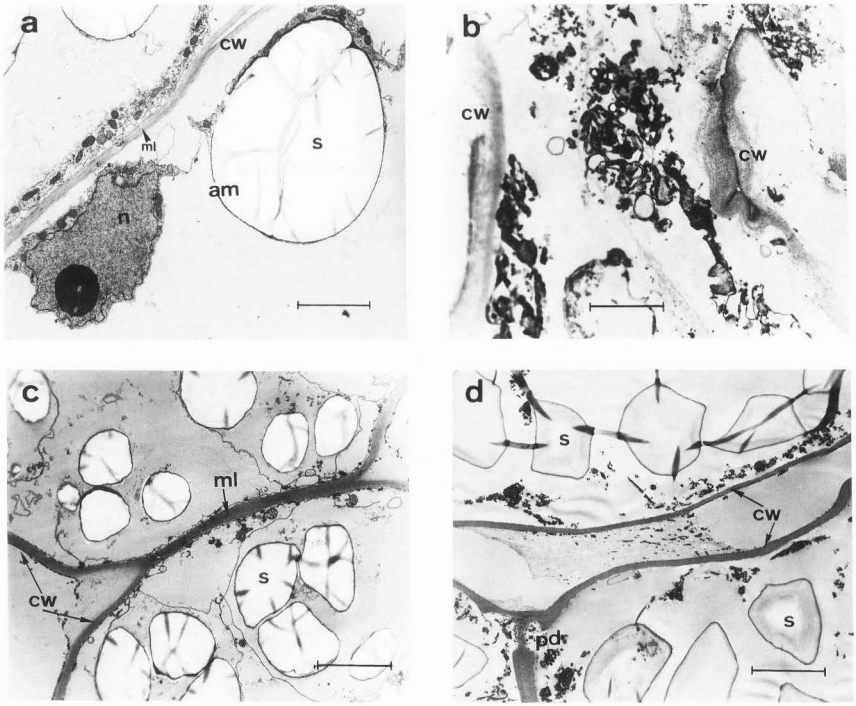
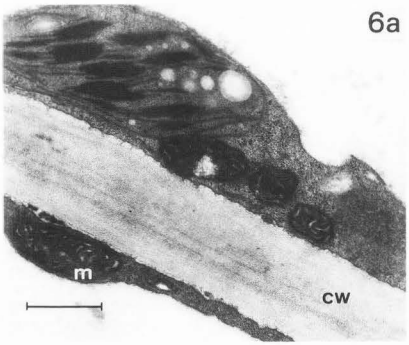


Figure 5 (above). Transmission electron micrographs of mesocarp from cherimoya fruit. **(a)** Freshly-harvested fruit. **(b)** Fully ripe fruit after storage for 6 days at 22°C. **(c)** Fruit stored for 6 days at 4°C. **(d)** Fruit stored for 9 days at 4°C and rewarmed to 22°C for 3 additional days. am = amyloplast; cw = cell wall; ml = middle lamella; n = nucleus; pd = plasmodesmata; s = starch grains. Bars = 5 μm (a, c, d) and 2 μm (b).

Figures 6-7 (on the facing page). Transmission electron micrographs of chloroplasts (**Figure 6**) and mitochondria (**Figure 7**) from cherimoya fruit: **(6a, 7a)** freshley harvested fruit; **(6b, 7b)** fruit stored for 6 days at 4°C; and **(6c, 7c)** fruit stored for 9 days at 4°C and rewarmed to 22°C for 3 additional days. cw = cell wall; m = mitochondria; ml = middle lamella; pd = plasmodesmata; s = starch grains. Bars = 1 μm (Figure 6) and 0.5 μm (Figure 7).

progressive disorganization of the membrane systems are observed. Cell compartmentalization is necessary throughout ripening as a way to control fruit metabolism in a coordinated manner leading to senescence.

In fully ripe cherimoya, most of the fibrillar organization in the cell wall is lost. Degradation of the middle lamella as well as the fibrillar material was observed, consistent with other observations in different fruits (Grumet *et al.*, 1981; Ben-Arie *et al.*, 1979; Pesis *et al.*, 1978). Cell wall-plasmodesma complexes were observed at all stages of ripeness. These complexes were reported earlier in senescing apples and pears (Ben-Arie *et al.*, 1979; Bain and Mercer, 1964). The material surrounding the cell wall-plasmodesmata complex appears to be in the form of amorphous electron-dense structures persisting in the fully ripe fruits. Ben-Arie *et al.* (1979) suggested that this material is probably resistant to the action of the hydrolytic enzymes, such as polygalacturonase, pectinesterase, and cellulase. This resistance would explain the presence of these structures at a time when the rest of the cell walls are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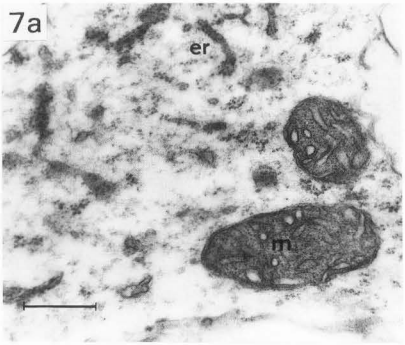
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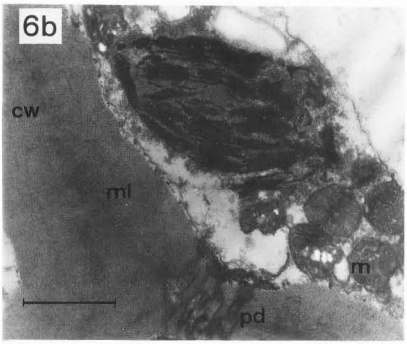
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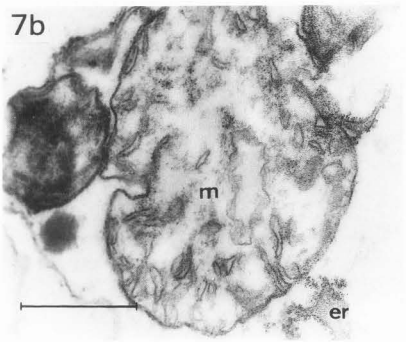
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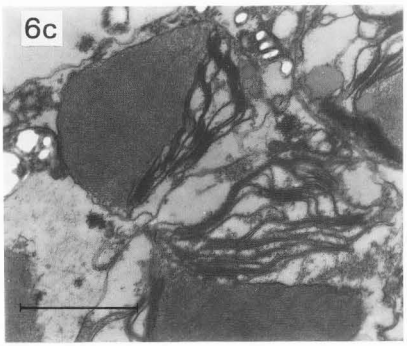
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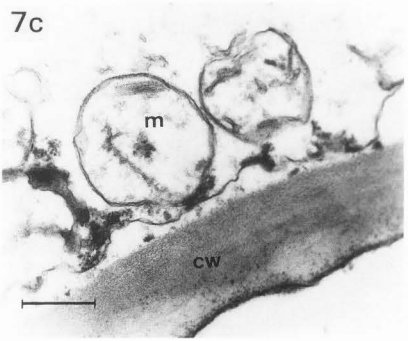
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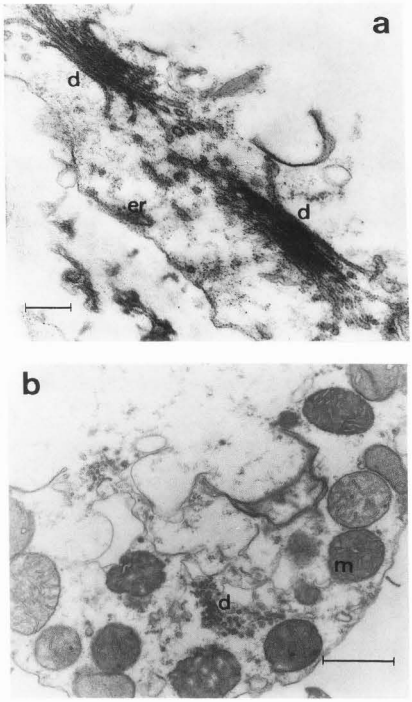

Figure 8. Transmission electron micrographs of Golgi apparatus from cherimoya fruit. (**a**) Freshly harvested fruit. (**b**) Fruit stored for 6 days at 4°C. d = dictyosomes; er = endoplasmic reticulum; m = mitochondria. Bars = 1 μm.

mostly degraded.

Storage of cherimoya at 4°C prevents the degradation of the cell walls precluding the action of the hydrolytic enzymes mentioned above. The reason is the deceleration of enzymatic reactions as the consequence of lowering the temperature by chilling.

ATP levels remain high during maturation due to an increased rate of glycolysis (Brady, 1987). The high concentration of ATP supports the induction of cell-wall hydrolytic enzymes during ripening (Sato *et al.*, 1985; Slater *et al.*, 1985). Synthesis of sucrose from starch is also ATP-dependent (Kanellis *et al.*, 1989; Hubbard *et al.*, 1990). Carbohydrate metabolism is severely suppressed in cherimoya stored at 4°C as evidenced by the presence of abundant undigested starch grains in the fruits. In contrast, extensive starch degradation takes place in cherimoya stored at 22°C.

ATP concentration depends on the intact nature of intracellular compartmentalization. Several observations suggest that alteration of the cell membranes is responsible for chilling injury. We have shown that all the cellular structures are completely disorganized in senescent cherimoya. However, fruit stored at 4°C reveal that major changes take place only in membrane systems, while the cell wall remain intact.

Membranes may undergo transitions from the normal fluid-phase state to a more ordered gel-phase state when temperature is reduced below the critical value (Murata and Yamaya, 1984; Quinn, 1985). Phase transitions as a consequence of refrigerated storage of fruits may be responsible for the chilling injury (Quinn, 1985). Phase transitions may be accelerated by oxidative reactions after thermal stress (McKersie *et al.*, 1988; Thompson, 1988), including lipoperoxidation of polyunsaturated fatty acids, which increases the critical temperature. Membrane rigidification and subsequent degradation lead to ultrastructural changes associated with chilling injury in tomatoes (Marangoni and Stanley, 1989). The unstacking of grana is the first chilling-injury related symptom observed with electron microscopy, thus pointing to the relationship between lipoperoxidation and chilling injury.

Disruption of the tonoplast may be the key factor responsible for irreversible chilling damage in callus tissue of *Cornus stolonifera* (Niki *et al.*, 1978; 1979). Furthermore, a breakdown of the tonoplast releases phenolic substances as well as a variety of lytic enzymes such as acid nucleases, non-specific acid phosphatases and proteases from the vacuole into the cytosol, leading to the degradation of subcellular organelles and membranes. Disintegration of the tonoplast may affect ionic distribution within the cells and thus induce over-activity of ionic carriers which, in turn, reduce ATP concentration (Davies, 1980). High cytosolic calcium concentration leads to the dissociation of tubulins and consequent deceleration of protoplasmic streaming (Minorsky, 1985); ripening is prevented by hardening of the cell walls (Ferguson, 1984).

Other symptoms of membrane damage during chilling stress consist of vesiculation of membranes, accumulation of lipidic material in the cytoplasm, and loss of ribosomes. Symptoms of membrane damage were also observed in grapefruit, episcia, and tomato (Platt-Aloia and Thomson, 1976; Murphy and Wilson, 1981; Marangoni *et al.*, 1989).

Depression or impairment of the mitochondrial oxidative metabolism by chilling stress was also reported (Yamawaki *et al.*, 1983a, b; Graham and Patterson, 1982; Wade *et al.*, 1974; Lyons and Raison, 1970). Disruption of mitochondrial membranes leads to metabolic imbalance and injurious secondary reactions (Niki *et al.*, 1978). Although mitochondria are the primary site of chilling damage in grapefruit rind and soybean radicle cells (Platt-Aloia and Thomson, 1976; Chabot and Leopold, 1985), the mitochondria generally appeared less severely damaged after prolonged chilling than other organelles (Platt-Aloia and Thomson, 1976; Ilker *et al.*, 1976). Our observations confirm these results since intact mitochondria were present even in the most severely affected fruits. Nonetheless, swelling took place in the organelles as a consequence of the impaired osmotic regulation because of disintegration of the tonoplasts.

In conclusion, changes in membrane structures were observed both during ripening of cherimoya at 22°C (as the normal temperature for this subtropic fruit) and during storage at the chilling temperature of 4°C. In fruits which ripen within their normal temperature range, disruption of membranes may occur after the enzymes leading to senescence had been synthesized. In contrast, morphological changes in the membranes of fruits stored at 4°C occur before the protein mechanism, needed for complete senescence, is synthesized. Cell walls are not degraded and profound biochemical and physiological alterations take place which modify the genetically established ripening pattern, impairing the appearance of the normal organoleptic characteristics of the mature cherimoya.

Acknowledgements

This work was supported by a grant from Caja Rural de Granada, Spain. We thank Dr. D. Okar for the critical reading of the manuscript.

References

Abe K (1990) Ultrastructural changes during chilling stress. In: Chilling Injury of Horticultural Crops. Wang CY (ed.), CRC Press, Boca Raton, FL, 71-84.

Bain JM, Mercer FV (1964) Organization resistance and the respiration climacteric. Australian J. Biol. Sci. **17**, 78-85.

Ben-Arie R, Kislev N, Frenkel C (1979) Ultrastructural changes in the cell walls of ripening apple and pear fruit. Plant Physiol. **64**, 197-202.

Brady CJ (1987) Fruit ripening. Ann. Rev. Plant Physiol. **38**, 155-178.

Broughton WJ, Tan G (1979) Storage conditions and ripening of the custard apple fruit (*Annona squamosa*) L. Scientia Hortic. **10**, 73-82.

Chabot JF, Leopold AC (1985) Ultrastructural aspects of chilling injury in the soybean radicle. Amer. J. Bot. **72**, 1120-1126.

Davies DD (1980) Anaerobic metabolism and the production of organic acids. In: The Biochemistry of Plants, Vol. 2. Davies DD (ed.), Academic Press, 581-611.

Dilley DR (1970) Enzymes. In: The Biochemistry of Fruits and Their Products. Hulme AC (ed.), Academic Press, 179-207.

Ferguson IB (1984) Calcium in plant senescence and fruit ripening. Plant Cell. Environ. **7**, 477-489.

Fuster C, Prestamo G (1980) Variation of cherimoya (*Annona cherimola*) texture during storage, as determined with and Instron food testing instrument. J. Food Sci. **45**, 142-145.

Graham D, Patterson BD (1982) Responses of plants to low, nonfreezing temperatures, proteins, metabolism, and acclimation. Rev. Plant Physiol. **33**, 347-372.

Grumet R, Fobes JF, Herner RC (1981) Ripening behavior of wild tomato species. Plant Physiol. **68**, 1428-1432.

Hubbard NL, Pharr DM, Huber SC (1990) Role of sucrose phosphate synthase in sucrose biosynthesis in ripening bananas and its relationship to the respiratory climateric. Plant Physiol. **94**, 201-208.

Ilker R, Waring AJ, Lyons JM, Breidenbach RW (1976) The cytological responses of tomato-seedling cotyledons to chilling and the influence of membrane modifications upon these responses. Protoplasma **90**, 229-252.

Jackman RL, Yada RY, Marangoni AG, Parkin KL, Stanley DW (1988) Chilling injury, a review of quality aspects. J. Food Qual. **11**, 253-278.

Kanellis AK, Solomos T, Matoo AK (1989) Changes in sugars enzymic activities and acid phosphatase isoenzyme profiles of bananas ripened in air or stored in 2.5% O_2 with and without ethylene. Plant Physiol. **90**, 251-258.

Kimball SL, Salisbury FB (1973) Ultrastructural changes of plants exposed to low temperatures. Amer. J. Bot. **60**, 1028-1033.

Lahoz J, Gutiérrez M, Martínez-Cayuela M, Pascual L, Vargas AM (1990) Efecto de la refrigeración sobre la maduración del fruto del chirimoyo (Effects of refrigeration on ripening of cherimoya fruit) Ars Pharm. **31**, 321-329.

Lyons JM, Raison JK (1970) Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol. **45**, 386-389.

Marangoni AG, Smith AK, Yada RY, Stanley DW (1989) Ultrastructural changes associated with chilling injury in mature-green tomato fruit. J. Amer. Soc. Hort. Sci. **114**, 858-962.

Marangoni AG, Stanley DW (1989) Phase transitions in microsomal membranes from chilling sensitive and chilling resistant tomato plants and fruit. Phytochem. **28**, 2293-2301.

McKersie BD, Senaratna T, Walker MA (1988) Deterioration of membranes during aging in plants: Evidence for free radical mediation. In: Senescence and aging in plants. Nooden LD, Leopold AC (eds.), Academic Press, 441-464.

Miller AR, Dalmaso JP, Kretchman DW (1987) Mechanical stress, storage time and temperature influence cell wall-degrading enzymes, firmness and ethylene production by cucumbers. Amer. Soc. Hort. Sci. **112**, 666-671.

Minorsky PV (1985) An hemistic hypothesis of chilling injury in plants: a role for calcium as the primary physiological transducer of injury. Plant Cell Environ. **8**, 75-94.

Moline HE (1976) Ultrastructural changes associated with chilling of tomato fruit. Cytol. Histol. Phytopathol. **6**, 617-624.

Murata N, Yamaya J (1984) Temperature-dependent phase behavior of phosphatidylglycerols from chilling-resistant plants. Plant Physiol. **74**, 1016-1024.

Murphy C, Wilson JM (1981) Ultrastructural features of chilling injury in *Episcia reptans*. Plant Cell Environ. **4**, 261-265.

Niki T, Yoshida S, Sacai A (1978) Studies on chilling injury in plant cells, I. Ultrastructural changes associated with chilling injury in callus tissues of Cornus stolonifera. Plant Cell Physiol. **9**, 139-148.

Niki T, Yoshida S, Sacai A (1979) Studies on chilling injury in plant cells, II. Ultrastructural changes in cells rewarmed at 26°C after chilling treatment. Plant Cell Physiol. **20**, 899-908.

Parkin KL, Marangoni AG, Jackman RL, Yada RY, Stanley DW (1989) Chilling injury, a review of possible mechanisms. J. Food Biochem. **13**, 127-153.

Pesis E, Fuchs Y, Zauberman G (1978) Cellulase and softening in avocado. Plant Physiol. **61**, 416-419.

Pilnik W, Voragen AGJ (1970) Pectic substances and other uronides. In: The Biochemistry of Fruits and Their Products. Hulme AC (ed.) Academic Press, London, 53-87.

Platt-Aloia KA, Thomson WW (1976) An ultrastructural study of two forms of chilling-induced injury to the

rind of grapefruit (*Citrus paradisi*, Macfed) Cryobiol. **13**, 95-103.

Quinn PJ (1985) Phases of membrane polar lipids in aqueous systems. Cryobiol. **2**, 128-146.

Raison JK, Lyons JM (1986) Chilling injury, a plea for uniform terminology. Plant Cell Environ. **9**, 685-686.

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208-212.

Salveit ME, Jr., Cabrera RM (1987) Tomato fruit temperature before chilling influences ripening after chilling. HortScience **2**, 452-454.

Sato T, Kusaba S, Nakagawa H, Ogura N (1985) Polygalacturonase mRNA of tomato: Size and content in ripe fruit. Plant Cell Physiol. **26**, 211-214.

Slater A, Maunders MJ, Edwards K, Schuch W, Grierson D (1985) Isolation and characterization of cDNA clones for tomato polygalacturonase and other ripening-related proteins. Plant Mol. Biol. **5**, 137-147.

Thompson JE (1988) The molecular basis for membrane deterioration during senescence. In: Senescence and Aging in Plants. Nooden LD, Leopold AC (eds.) Academic Press, 51-83.

Wade NL, Breidenbach RW, Lyons JM (1974) Temperature-induced phase changes in the membranes of glyoxysomes, mitochondria and proplastids from germinating castor bean endosperm. Plant Physiol. **4**, 320-327.

Wise RR, McWilliam JR, Naylor AW (1983) A comparative study of low temperature-induced ultrastructural alterations of three species with differing chilling sensitivities. Plant Cell Environ. **6**, 525-535.

Yamawaki K, Tomiyana M, Chachin K, Iwata T (1983a) Relationship between change in mitochondrial function and chilling of cucumber fruit. J. Jpn. Soc. Hortic. Sci. **52**, 332-339.

Yamawaki K, Yamauchi N, Chachin K, Iwata T (1983b) Relationship of mitochondrial enzyme activity to chilling injury of cucumber fruit. J. Jpn. Soc. Hortic. Sci. **2**, 93-98.

Discussion with Reviewers

F. Escher: Is it really acceptable that physiology of ripening, storage behavior, and cold-injury is discussed only on the basis of a morphological analysis and of physiological data which were not collected on comparable specimens of cherimoya fruits?

Authors: Unfortunately, there is not much information available on the physiology of cherimoya ripening. We are doing a great deal of work on this topic and more papers will be published. In any case, we discuss our observations on the basis of similar findings obtained with other tropical or subtropical fruits. It seems that microstructural damage during chilling injury has a common pattern in most fruits studied so far.

E. Kovács: How and in which step could the temperature influence the biosynthesis of ethylene? What kind of effect do the degraded ribosomes have on the chilling injury?

Authors: Ethylene concentration measured in the whole cherimoya fruit rises over 1,000 fold three days after harvesting when fruits are stored at 22°C. Fruit stored at 12°C have a climacteric peak of about the same magnitude 10-12 days after harvesting. When fruits are stored at 4°C the increase in ethylene concentration is less than 100 fold and occurs three weeks later. We have not studied the steps responsible for this effect, but we do believe that retardation of protein biosynthesis as well as alterations ion the oxidative metabolism are involved.

We have not studied whether the ribosomes actually suffer any degradative process during chilling injury. What we have observed, is that endoplasmic reticulum is transformed mostly into smooth vesicles after release of ribosomes. This observation may be related to the deficit in the biosynthesis of proteins needed for ripening.

E. Kovács: How can you explain the sensitivity of mitochondrium to chilling injury?

Authors: From an analysis of the lipid content in cherimoya fruit stored under chilling conditions, we have found an increase in free fatty acids as well as in the molar ratio esterified-sterols/phospholipids, data that confirm microstructural observations in membrane damage. We have not studied these changes in isolated mitochondria, but as mitochondria have a much lower content of sterols than other membrane systems, they may be more resistant to chilling injury than other organelles.

B.G. Swanson: What is osmiophilic material? How do you identify, characterize, and observe these materials?

Authors: We define osmiophilic material as one that is specially susceptible to staining with OsO_4. The material that we referred to in the previous manuscript may be worth studying. It aligns perfectly with the undigested cell wall after chilling injury of cherimoya. Even at the highest magnifications available, we could not observe any structure in these materials. By refraction studies, we can only say that they are amorphous.

B.G. Swanson: What evidence do you have that thermal stress increases lipid peroxidative reactions in cherimoya? What lipids were identified in cherimoya? How was accumulation of lipids detected? How can you see difference between amylose and lipidic material?

Authors: The fact that thermal stress increases lipoperoxidation reactions is well documented. We have studied lipid composition of cherimoya. Using bidimensional chromatography, we have isolated 16 different lipid species. We identified some of them as: phosphatidil-choline, phosphatidil-ethanolamine, two different sphingolipids, lysophosphatidil-choline, sulphatides, two different fractions of esterified sterols. Our results of studies of fatty acid composition in each band, as well as in the isolated free fatty acids, show a decrease in the unsaturation index for fruits stored under chilling conditions. Malondialdehyde was also detected.

Accumulation of lipid-like material in the cytoplasm coincides in time with the breakdown of membranes. We have not differentiated between amylose and lipidic material, but under conditions of chilling storage very little starch is degraded; consequently, amylose accumulation should not account for the observed material.

B.G. Swanson: What evidence do you have to support the statement in **Discussion** "... swelling was produced in the organelles as a consequence of the impairment of osmotic regulation ... "?

Authors: The only evidence is that tonoplast is broken. After the breakage of the tonoplast membrane, mitochondria usually appear swollen. We have measured neither swelling in isolated mitochondria nor osmotic pressures.