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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.

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 (Lafoz 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 ± 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 mm³ 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.

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, 1979; 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 outer 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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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.

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,
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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. Dicytosomes 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 = plasmodesma; 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) freshly-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 = plasmodesma; 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-plasmodesma 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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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, 1990). 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.
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In conclusion, changes in membrane structures were observed both during ripening of cherimoya at 22°C (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.

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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 in 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 mitochondria 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 liperoxidation 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: phosphatidyl-choline, phosphatidyl-ethanolamine, two different sphingolipids, lysophosphatidyl-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.