

CHAPTER 2

Ultrastructural Changes in the Walls of Ripening Avocados: Transmission, Scanning, and Freeze Fracture Microscopy

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Chapter 2. Ultrastructural Changes in the Walls of Ripening Avocados: Transmission, Scanning and Freeze Fracture Microscopy

Ultrastructural Changes in the Walls of Ripening Avocados: Transmission, Scanning and Freeze Fracture Microscopy

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ABSTRACT

Avocado fruit at several documented stages of ripening was prepared for transmission, scanning, and freeze fracture electron microscopy. Changes in the ultrastructural organization of the cell wall were studied by each technique and correlated with changes in the activity of wall hydrolytic enzymes. Initial wall breakdown apparently involves degradation of pectins in the matrix and in the middle lamella, corresponding to the reported increase in polygalacturonase activity in the tissue. In later stages of ripening, there is a loss of the organization and density of the wall striations accompanied by an increase in fruit softening. The role of cellulase, which becomes highly active during ripening of avocados and several other fruits, is still somewhat questionable. However, both thin sections and freeze fracture replicas of ripening avocados indicate a loss of fibrillar components of the wall during ripening and, therefore, indicate a possible role for cellulase in fruit softening. No correlation between localized wall degradation and the presence of plasmodesmata could be found.

INTRODUCTION

There have been several ultrastructural investigations on aspects of cell wall degradation in different plant tissues (Sexton and Hall 1974; Sexton, Jamieson, and Allan 1977; Pesis, Fuchs, and Zauberman 1978; Ben-Aire, Kislev, and Frenkel 1979a). Their studies showed a progressive sequence of events, beginning with a decrease in electron density of the middle lamella, followed by a gradual separation of the wall fibrils, and, depending on the tissue, a variable degree of loss of the fibrillar components of the wall.

The initial loss in electron density of the middle lamella was attributed to the action of pectolytic enzymes because pectic materials occur in this region (Albersheim, Mühlethaler, and Frey-Wyssling 1960), pectinase activity increased in some of these

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tissues (Awad and Young 1979; Ben-Aire, Sonogo, And Frenkel 1979b, Yamaki and Kakiuchi 1979), and pectic substances were lost from several ripening fruits (Knee, Sargent, and Osborne 1977; Knee 1978; Ben-Aire et al. 1979b). Additional support for this view was presented by Ben-Aire et al. (1979a), who observed a similar pattern of degradation of the middle lamella in firm apple and pear tissue which had been treated with exogenous polygalacturonase and in untreated tissue which had been allowed to ripen naturally.

Although the apparent breakdown of the wall fibrils has been correlated with an increase in cellulase activity in many of these tissues (Hobson 1968; Lewis and Varner 1970; Sobotka and Stelzig 1974; Awad and Young 1979; Pesis et al. 1978; Awad and Young 1979; Yamaki and Kakiuchi 1979), the role of cellulase in wall loosening and degradation is not completely understood. Ben-Aire et al. (1979a) however, demonstrated a correlation between the ultrastructure of walls treated with cellulase and the changes which occur naturally in tissues with high cellulase activities. In both instances, separation and loss of the fibrillar components of the wall were evident.

The avocado is a climacteric fruit and exhibits a characteristic rise in respiration and ethylene (C₂H₄) production during the ripening process. An increase in the activity of the wall hydrolyzing enzymes polygalacturonase and cellulase has been correlated on a temporal basis with the climacteric (Awad and Young 1979). Therefore, by sampling ripening fruit at known stages of the climacteric, the relative rates of enzyme activities can be correlated with structural changes.

For the present study, we monitored the ripening of avocado fruits by measuring CO₂ and C₂H₄ production, and took samples at several stages during the ripening process. The samples were then prepared for study by transmission (TEM), scanning (SEM), and freeze fracture (FFEM) electron microscopy. Ultrastructural changes in the wall during the ripening process, as revealed by these three techniques, are described and correlated with changes in enzyme activities reported by Awad and Young (1979).

MATERIAL AND METHODS

Avocado fruit (*Persea americana* Mill, var. Hass) were collected from trees at the South Coast Field Station of the University of California. Fruit of uniform size were placed in individual containers at 18 or 22 C. The CO₂ released from each fruit was monitored every 4 h with a Beckman model 215 IR gas analyzer; C₂H₄ was measured at the same time with a Varian model 144D gas Chromatograph equipped with a 300 X 0.16 cm column packed with Porapak Q.

Fruit were removed from their containers at various stages of ripening; in all but the very soft, postclimacteric fruit, samples were taken with a No. 2 cork borer for microscopy. Samples of very soft fruit were taken by removing a thin slice with a sharp knife. Ripening in avocados may not be uniform throughout the fruit (Biale and Young 1971). To avoid accessive variability, we consistently took our samples from the area of largest diameter, on the side of the fruit where the distance from the stem to the blossom end is smallest. After sampling, the fruit was returned to its container for continued monitoring of CO₂ and C₂H₄ production.

Tissue samples for TEM and SEM were placed immediately into 1% glutaraldehyde in

50 mM cacodylate buffer, pH 7.2, and were cut into smaller (~1 x 4 mm) pieces. Samples were fixed in fresh 1% glutaraldehyde for 2-3 h. After a brief rinse in buffer, the tissue was postfixed 3 h or overnight in 1% OsO₄ in 50 mM cacodylate buffer. An excess of OsO₄ for long periods was preferable for optimum fixation, presumably because the high lipid content of the cells sequestered the osmium preferentially, thus apparently reducing its reaction with other cellular components.

The tissue for TEM was dehydrated in acetone and embedded in an epoxy resin (Spurr 1969). Thin sections were cut with a diamond knife using a Porter Blum MT-2 ultramicrotome, stained with 1% aqueous uranyl acetate for 2 h, and with lead citrate (Reynolds 1963) for 1-2 min.

The tissue for SEM was prepared by a modification of the osmium thio-carbohydrazide method (Kelley, Dekkar, and Bluemink 1973; Malick and Wilson 1975). After postfixation in 1% OsO₄ in buffer, tissue was washed 5-6 times in distilled water for 10-20 min, and treated with freshly made, filtered, 1% thiocarbohydrazide for 20-30 min with frequent agitation to dislodge air bubbles. The tissue was again washed 5-6 times for 10-20 min and treated with 1% OsO₄ in distilled water overnight. The entire procedure was repeated the next day. The tissue was then dehydrated in acetone and critical point dried (Anderson 1951) with a Tuisimas CO₂ critical point dryer. After drying, pieces of tissue were broken in half and mounted on aluminum stubs with conductive paint containing colloidal silver. They were oriented so that the freshly broken surface, relatively free from contamination, was exposed. Samples were stored in a desiccator until viewed with a Joelco JSM-U3 scanning electron microscope at 10 or 20 kV.

Samples for FFEM were placed directly into buffer without chemical fixation or cryoprotection, cut into pieces 1-2 mm³, quickly placed into gold-nickel planchets, frozen in Freon 22 near its freezing point, and stored in liquid nitrogen. Fracturing and replication were according to Moor and Mühlethaler (1963) at -120 C and less than 2 X 10⁻⁶ torr with a Balzers BAF 301 equipped with a quartz thin film monitor. The replicas were cleaned with chromic sulfuric acid and 12% KOH in 95% EtOH.

Cytochemical localization of pectin esters was accomplished by modifying the procedures of Albersheim et al. (1960) and Gee, Reeve, and Mccready (1959). Tissue from a freshly picked, mature avocado was fixed in a mixture of glutaraldehyde and paraformaldehyde (Karnovsky 1965) for 2 h, rinsed for 30 min in 0.1 M PO₄ buffer, and placed for 30 min each in 20% and then 60% EtOH. The incubation solutions used for the formation of hydroxamic acids and the subsequent reaction of these with ferric iron were as follows: A, 14% NaOH (wt/vol); B, 14 g NH₂OH.HCl in 100 ml 60% EtOH; C, 1 vol cone HCl in 2 vol 95% EtOH; and D, 2.5 g FeCl₃ in 0.1 N HCl made in 60% EtOH.

Tissue slices were incubated in a mixture of 1 ml of A + 1 ml of B for 1 h; then 1 ml of C was added and allowed to react for 1 h. The tissue was transferred to solution D for 1 h. Control sections were de-esterified by treatment with 14% NaOH for 30 min before incubation. After reaction with FeCl₃, the tissue was dehydrated in EtOH and embedded in epoxy resin (Spurr 1969). Sections were viewed and photographed without staining.

All thin sections and replicas were studied with a Philips EM 300 or a Philips EM 400 electron microscope.

OBSERVATIONS

While TEM, SEM, and FFEM provided different types of information the observations in each instance tended to correlate with and complement those of the other two. In thin sections of mesocarp cells from newly picked, preclimacteric, hard fruits, the walls were fairly homogeneous and wall striations (presumably cellulose microfibrils) were difficult to discern (figs. 1, 12). In SEM images of this tissue, essentially all cells on the broken surface were broken (fig. 2), exposing cross fractures of the walls and internal cell organelles. The cell walls were thick, coherent structures (fig. 2). With FFEM, definite striations in the wall were seldom apparent, although in favorable fracture planes a somewhat regular pattern of "ridges" (fig. 4) separated by smooth regions of "matrix" material were observed.

At the point when the fruit began to ripen and started the climacteric rise in respiration, TEM observations revealed that the middle lamella, as marked by an increase in electron density, became more distinct, and wall striations became more apparent throughout the wall (fig. 3). As ripening proceeded to the climacteric peak, there was a loss in the electron density and structure of the middle lamella, and some loosening or separation of the wall striations was evident (fig. 5, ML). Similarly, in FFEM images, the wall striations became more apparent, and the amount of smooth "matrix" material was considerably reduced (fig. 6).

Further breakdown of the middle lamella was apparent in thin sections of the postclimacteric, soft, edible fruit; and the wall striations occurred in rather broad but loosely packed bands adjacent to the plasmalemma (fig. 7). In FFEM micrographs, the loose arrangement of the wall striations was particularly apparent, as it was in thin sections, particularly in regions distant from the plasmalemma (fig. 11). The SEM images corroborated these observations. At low magnification, the location of the zone of separation during breaking changed from through the cells to predominantly between the cells (fig. 8). This presumably was due to the breakdown of the middle lamella and loss of structural unity of the cell walls. At high magnification, the walls of these cells were composed of loosely packed fibrils (fig. 9). Occasionally, in postclimacteric, very soft, overripe, fruit, it was evident that essentially all of the wall material was degraded (fig. 10). In these instances, the electron beam revealed the plasmalemma as a gossamer film, with oil droplets and organelles within the cells.

Plasmodesmata of the preclimacteric mesocarp cells usually occurred in pit fields, often as complex, branching structures, and frequently had an enlarged median cavity (fig. 12). This general pattern of organization remained through the early stage of ripening, and there was no evidence for a differential degradation of the wall near the plasmodesmata (fig. 3). As ripening approached the climacteric peak and wall loosening progressed, the continuity of the plasmodesmata with the plasmalemma was apparently stretched (fig. 13, arrows). In the final stages of fruit softening (postclimacteric), when wall dissolution and separation of the cells occurred, the continuity of the plasmodesmata with the plasmalemma of the cells was lost, and remnants of the plasmodesmatal membranes formed vesicles within the degraded and loosened walls (fig. 7).

Treatment of mature, unripe avocado tissues with hydroxylamine for the localization of esterified pectin resulted in a generalized staining throughout the cell wall (fig. 14).

Controls showed very little or no iron precipitate (fig. 15).

DISCUSSION

Awad and Young (1979) determined the changes in the cell wall-degrading enzymes cellulase, polygalacturonase and pectinmethylesterase, as a function of the ripening process in avocados. Our study of the ultrastructural changes which occur in the wall during ripening of avocados shows a good correlation with these enzymatic activities. TEM and FFEM reveal a loss of the matrix and middle lamella of the wall, followed by apparent separation and possible shortening of the wall striations which are presumably cellulose. In the SEM, these transitions in structure are manifested by a change in the plane of separation during preparation of the tissue taken at various stages of ripening. All of these ultrastructural changes may be due to the loss of pectins resulting from increased activity of pectinases such as polygalacturonase.

Three related pieces of information support this conclusion: (1) The changes in wall structure coincide with the rise in activity of polygalacturonase (Zauberman and Schiffmann-Nadel 1972; Awad and Young, 1979), and, a breakdown of the middle lamella and cell separation correlates with the high activity of these enzymes. (2) Knee et al. (1977) and Ben-Aire, et al. (1979a) reported that the first evidence of wall dissolution involves a degradation of the middle lamella, attributable to pectinase activity. Also, Ben-Aire et al. (1979a) observed that, with the application of exogenous polygalacturonase to fruit tissues of pears and apples, the middle lamella was broken down. (3) Using a cytochemical technique to localize pectin esters, Albersheim et al. (1960) reported that pectin was distributed throughout the cell walls as well as being localized in the degradation of the pectin or matrix material throughout the wall would tend to bring the visualization of the striations into greater relief, which is consistent with the pattern we have observed.

TEM, SEM, as well as FFEM all indicate that some striations persist even in the very soft fruits. However, all three preparative techniques also indicate degradation or loss of structural components of the cell wall during ripening. Although the precise role of cellulase in wall degradation is not clearly understood, the correlation of increased cellulase activity (Awad and Young 1979) with apparent loss of structural integrity, both in this study, as well as that of Ben-Aire et al. (1979), is consistent with the idea that structural breakdown of the cell wall involves a degradation of cellulose as well as pectins.

Taiz and Jones (1970), Jones (1972), and Juniper (1977), suggested that the plasmodesmata might be a site of release of wall hydrolytic enzymes, based on evidence of earlier and increased wall degradation in the vicinity of the plasmodesmata of barley aleurone layers. On the other hand, Ben-Aire et al. (1979a) observed few degradative changes in the region of the plasmodesmata either in naturally ripening apples or pears or in fruits to which exogenously applied wall hydrolytic enzymes were added. Based on different staining characteristics of the plasmodesmatal region, they suggested that the wall in this area is apparently different in composition from other regions of the wall.

Throughout the ripening process in avocados, we have found no consistent evidence of

differential staining characteristics in the region of the plasmodesmata or different changes in the wall structure of this region compared with other areas of the cell wall. Although the extent of wall degradation varies to some degree throughout a particular sample, even within a single thin section, there does not appear to be a correlation with the location of plasmodesmata. On the contrary, the structure of the plasmodesmata is apparently affected by the breakdown of the wall, rather than the reverse. At the climacteric peak stage, considerable wall hydrolysis has occurred, as evidenced by apparent fibril loss and separation« At this stage of ripening, the membranes of some of the plasmodesmata have apparently broken at the plasmalemma. While it is possible that this initial breakage is due to fixation artifact, a much more pronounced loss of continuity between cells is particularly evident in the postclimacteric stages. At this time apparently all that remains of the plasmodesmata are vesicles in the wall.

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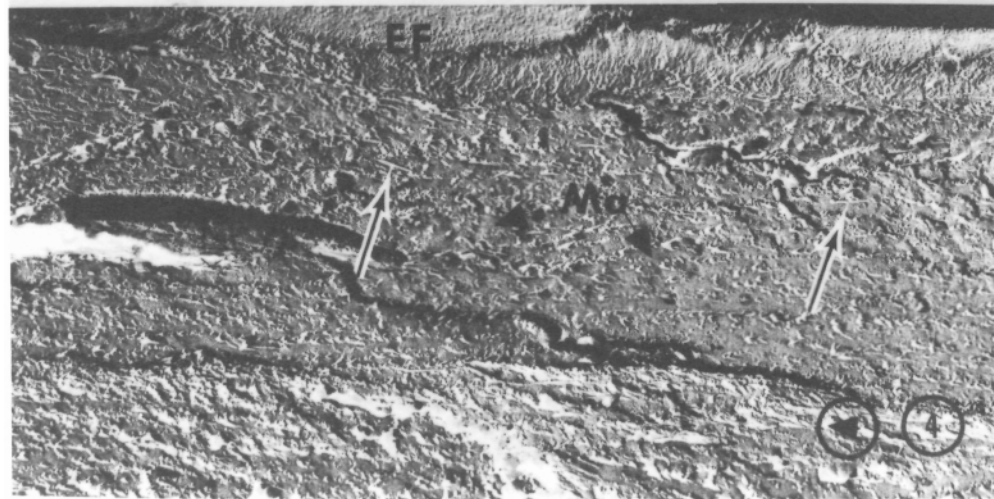
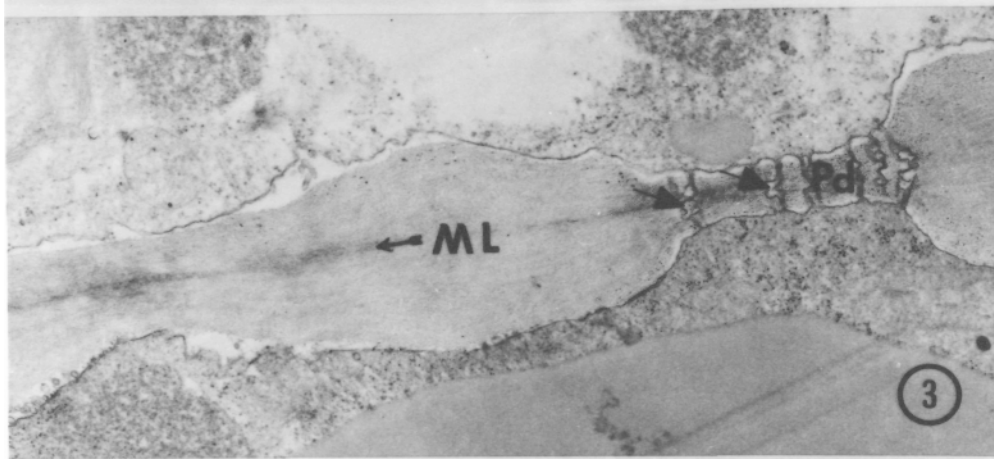
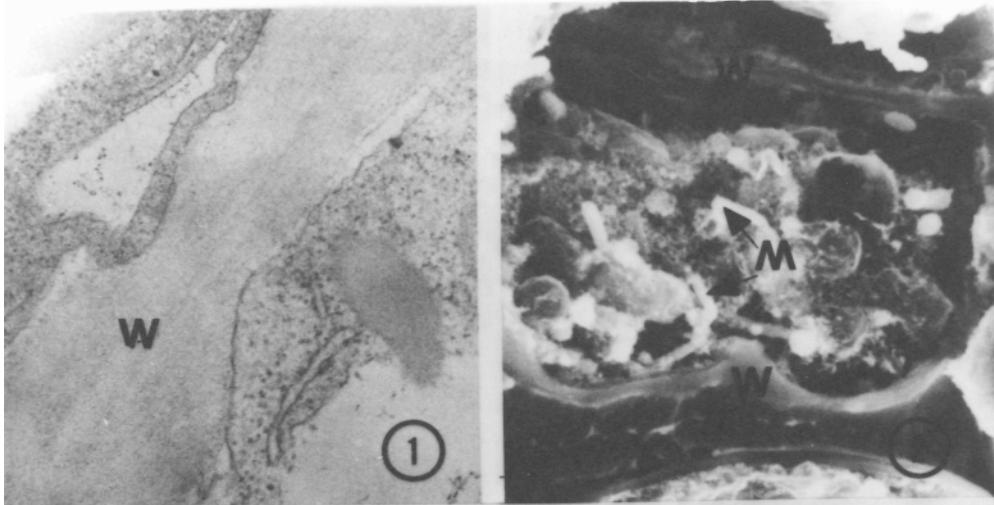
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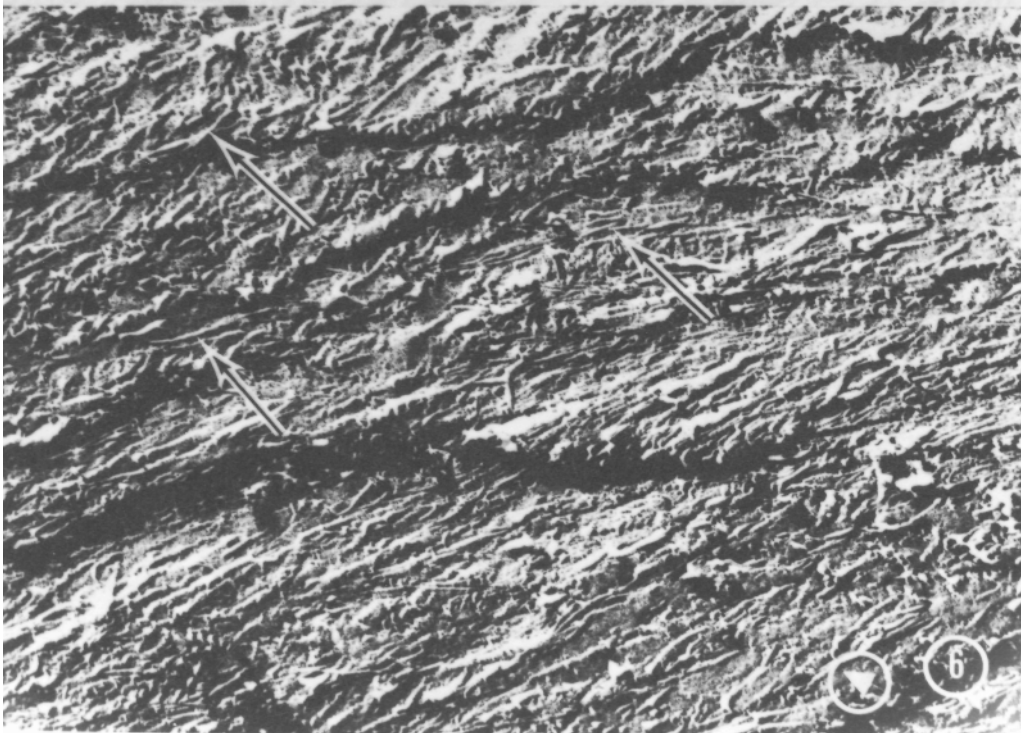
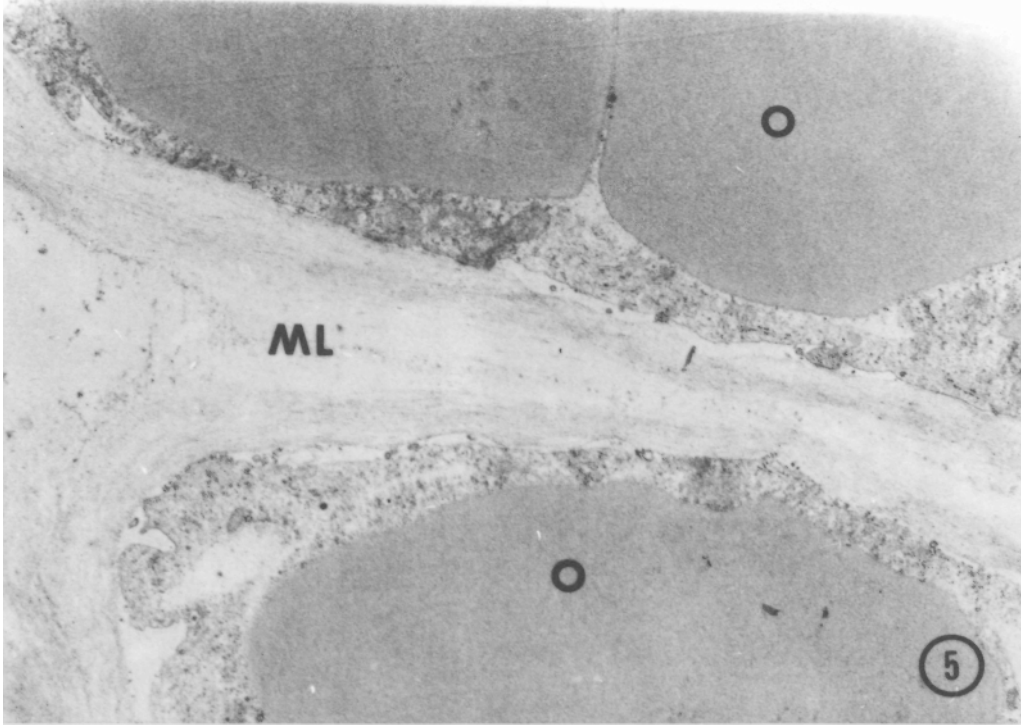
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FIGURES AND FIGURE LEGENDS

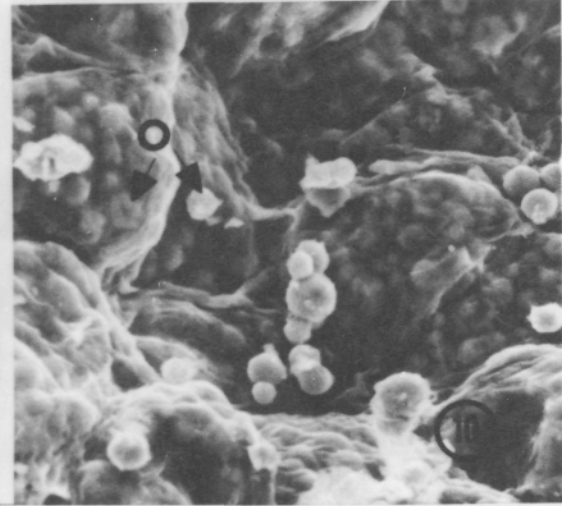
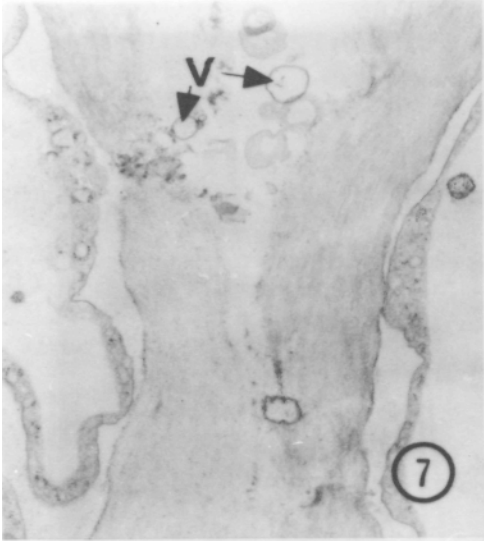
FIGS. 1-4.—Fig. 1, Thin section of the mesocarp of a hard, unripe avocado. The cell wall (W) appears fairly homogeneous and striations or fibrils are difficult to discern (X 24,500). Fig. 2, SEM of a mesocarp cell of an unripe avocado. When the tissue was broken after critical point drying, most cells were fractured open, as this one, revealing the organelles and cross fractures of the cell walls (W) which appear to be electron dense, coherent structures. M = mitochondrion, O = oil droplet. X 2,600. Fig. 3, Thin section of the wall of a mesocarp cell of an avocado which has begun the climacteric rise in respiration. The middle lamella (ML) appears more electron dense than the rest of the wall, and striations or fibrils are more apparent in the wall than in earlier stages (see fig. 1). Plasmodesmata (Pd) have slightly enlarged median cavities (arrows). There does not appear to be a differential degree of wall degradation in the region of the plasmodesmata, compared with other regions of the wall. X 17,600. Fig. 4, FFEM replica of the wall of a mesocarp cell from an unripe avocado fruit. Definite striations (arrows) are only apparent in some regions, and there appears to be a considerable amount of smooth matrix material (Ma) between "ridges" of striations. EF = external fracture face of the plasmalemma of a mesocarp cell. The arrow in the lower right indicates the direction of shadowing. X 37,000.



FIGS. 5, 6.—Fig. 5, Electron micrograph of a thin section taken from the mesocarp of an avocado fruit at the climacteric peak of respiration. A loss of material from the middle lamella (ML) is evident, and wall striations are more clearly apparent. 0 = oil droplet. X 10,000. Fig. 6, FFEM replica of the cell wall of an avocado fruit at the climacteric peak. Wall striations (arrows) are very apparent and stand out in greater relief than at earlier stages of ripening, apparently because of a loss of matrix pectins. Arrowhead in the lower right indicates the direction of shadowing. X 37,000.



FIGS. 7-11.—Fig. 7, Thin section of a wall of a postclimacteric, soft avocado fruit. Cell separation due to loss of the middle lamella is evident. The membranes of plasmodesmata have evidently broken and formed vesicles (V) in the loosened, degrading wall. X 12,000. Fig. 8, SEM of the broken surface of a critical point dried, postclimacteric avocado fruit. The zone of separation which formed when the tissue was broken passed primarily between cells as opposed to through cells as it did in the hard fruit (cf. fig. 2). One cell (lower right) was broken open to reveal oil droplets (O) and organelles. X 600. Fig. 9, Higher magnification of SEM of the cell wall of a soft avocado fruit. The loosely arranged fibrous nature of the wall can be seen. The openings (arrows) could be former pit fields. X 4,500. Fig. 10, SEM of the broken surface of a very soft, postclimacteric avocado fruit. In this overripe fruit the cell walls are apparently entirely degraded, leaving only the plasma- membrane surrounding the cells. Oil droplets (O) can be seen through the membrane, and some, which have apparently been released from broken cells, lie on the surface of the tissue. X 600. Fig. 11, FFEM replica of the wall of a soft, postclimacteric avocado fruit. Wall striations and other components are loosely arranged. The mosaic pattern of separation of the striations may indicate ice damage, however, similar patterns were also occasionally seen in thin sections (not shown). The plasmamembrane is situated toward the top of the figure. The striations (arrows) become more loosely arranged as the distance from the plasmalemma increased. The arrowhead in the lower right indicates the direction of shadowing. X 33,000.



FIGS. 12-15.—Fig. 12, Thin section of the wall of a hard, preclimacteric avocado fruit. The plasmodesmata are complex, with an enlarged median cavity (arrows). Wall striations are difficult to discern. O = oil droplet. X 25,000. Fig. 13, Thin section of the wall of an avocado at the climacteric peak, illustrating an apparent stressing of the connection between the plasma membrane and the plasmodesmata (arrows). C = Chloroplast. X 25,000. Fig. 14, Thin section of the cell wall of an unripe avocado fruit which has been treated with hydroxylamine and FeCl₃ to localize esterified pectins. The generalized staining indicates the ubiquity of pectins throughout the wall. No osmium or stains other than FeCl₃ were used in either fig. 14 or 15. Pd = Plasmodesmata. X 20,000. Fig. 15, Thin section of control tissue for pectin localization. The pectins were de-esterified with 14% NaOH before reaction with hydroxylamine and FeCl₃. Almost no iron precipitation is present. X 21,000.

