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Plastic Embeddment of Dry-Processed Tissue[1]

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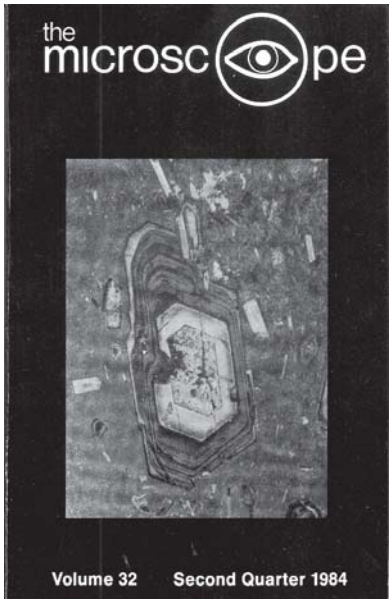
KEYWORDS

Cell walls, dry seeds, peanut, plastic embedding, micrometry, ultrastructure, dehydration

ABSTRACT

Seed tissue is extremely difficult, if not impossible, to embed in plastic when fixed and processed dry; on the other hand, if it is fixed with aqueous fixatives and dehydrated by solvent exchange, seed tissue can be embedded readily. Earlier, I hypothesized that cell walls exclude resin monomers from dry-processed peanut (*Arachis hypogaea L.*) seed tissues. Now I show that seed tissues whose cellular contents have been digested away by a proteolytic enzyme, still are difficult to embed in plastic if air-dried and processed dry. I submit, therefore, that since these specimens consist only of cell walls, air-dried cell walls are indeed the factor that bars passage of resin monomers into seed tissues processed dry.



INTRODUCTION

Ultrastructural studies of seed tissues have been conducted by biologists for a quarter of a century (19, 20, 22). Almost all of the studies have been performed on wetted seeds or seed tissues fixed with aqueous fixatives (1, 9, 10, 11, 15). Intrusion by water, however, is said to cause fine structural (1, 23) and molecular (21) changes in the plasma membranes of dry seeds and at the very least, will cause swelling of dry seed tissue (3). Even chemical fixatives, when prepared in an aqueous solution, will cause dry seed tissues to swell (25).

Perner (1965) was one of the first workers to address this problem. By using OsO_4 fume fixation and dry processing, he obtained valuable information. Unfortunately, his micrographs were not as aesthetically pleasing as those obtained by workers using more traditional, aqueous fixative. Hallam and Capicchiano (1974) and Hallam (1976) fixed dry plant tissues with glutaraldehyde dissolved in an anhydrous solvent. The tissues were post-fixed with OsO_4, also dissolved in anhydrous solvents. Again, the results were relatively poor. More recently, Opik (1980) repeated Perner's (1965) work, but on rice grains. Instead of alcohol and propylene oxide, Opik (1980) used acidified dimethoxypropane as a dehydrating agent and resin vehicle. The results, while slightly better, were still relatively poor. In all of these cases where water was not used, the underlying problem has been poor resin permeation of the specimen.

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In a previous communication (25), I surmised that cell walls of air-dried seed tissues form a barrier that excludes resin from the interior of the specimens. Now I show that enzyme-treated seed tissues, tissues whose cellular contents have digested away, are still extremely difficult to embed in resins if air-dried and processed dry. Therefore, I conclude that the air-dried cell walls are indeed the barriers that restrict resin entry into dry processed seed tissues.

MATERIALS AND METHODS

Specimens about 2 mm^3 were excised from peanut (*Arachis hypogaea L.* cv Jumbo runner) cotyledons and subjected to two treatments: 1) defatting, and 2) defatting plus protein hydrolysis. After the respective treatments, samples were dried either by air-drying or by solvent-exchange dehydration and embedded to Spurr's epoxy resin.

Defatting

Dry peanut specimens were exhaustively extracted with hexane, then further extracted with acetone. The defatted samples were then hydrated in a graded series of aqueous ethanols. Half of the samples were air-dried in 60° oven, then further dried in a vacuum desiccator over P_2O_5. The other was dehydrated in a graded series of aqueous ethanols.

Protein hydrolysis

Specimens for this procedure were defatted and wetted as above. The wet, defatted samples were placed in a 2-dram screw-cap vial that contained the following: 0.1M tris buffer, pH 8.3. The vial and contents were placed into a 40° oven for 48 hrs. To ensure complete digestion, the procedure was repeated twice more and then the specimen was washed in 10% aqueous ethanol for 24 hrs. Ten percent ethanol was used because Nomoto et al., (1960) showed it to be bacteriostatic. Again, half of the sample was air-dried and the other half "dried" by solvent exchange as above.

Defatted and protein-hydrolyzed samples, both air-dried and dehydrated by solvent exchange, were transferred to acetone and then into Spurr's epoxy resin.

Thick and thin sections were cut from plastic-embedded specimens with a diamond knife on a Sorvall MT-2 ultramicrotome. Thick sections for viewing in the light microscope were stained with toluidine blue. Thin sections for observation in the electron microscope were stained with 1% OsO_4 in 0.05M cacodylate, pH 7.0. The sections were doubly post-stained with aqueous uranyl acetate and lead citrate. The sections were viewed in a Philips EM-200 electron microscope at 60 KV accelerating voltage.

RESULTS

Defatted tissues

Air-dried. These samples failed to embed in Spurr's resin.

Solvent-exchanged. These samples embedded well. Thick sections for light microscopy showed surprisingly good preservation of cytological detail: aleurone grains, nuclei, and occasional starch granules could be seen in the cytoplasmic matrix. However, almost every cell showed a plasmolysis effect; note how the cytoplasm has pulled away from the cell walls (Figure 1).

Thin sections viewed in the electron microscope showed that preservation of cytological detail was even better than light microscopy indicated. Aleurone grains were well preserved, pleomorphic nuclei were seen in the cells, and even spherosomes were well preserved (Figure 2).

Pronase-treated tissues

Air-dried. Again, these samples failed to embed in Spurr's resin.

Solvent-exchanged. As expected, these samples embedded very well.

Thick sections cut very easily. Observation in the light microscope showed that pronase treatment abolished virtually all cellular constituents. Non-proteinaceous elements such as starch granules, cell walls, and some undigested debris remained in the cell lumens (Figure 3).

Thin sections, when examined in the electron microscope, confirmed that the cells were empty. I scrutinized the vicinity of cell walls very carefully for evidence of plasma membranes (Figure 4), but found none. The debris consisted of unfamiliar, fragmented matter.

DISCUSSION

In a previous communication (25), I showed that while it was relatively easy to embed seed tissue that had been wetted — or fixed with an aqueous fixative — and dehydrated by solvent exchange, it was virtually impossible to embed dry-processed, dry seed tissue in epoxy resin. Occasionally it was possible to cut sections from cells at the periphery of poorly embedded specimen but these usually disintegrated in the beam of the electron microscope. Careful observation showed that the tissues fell apart at the cell walls. Since

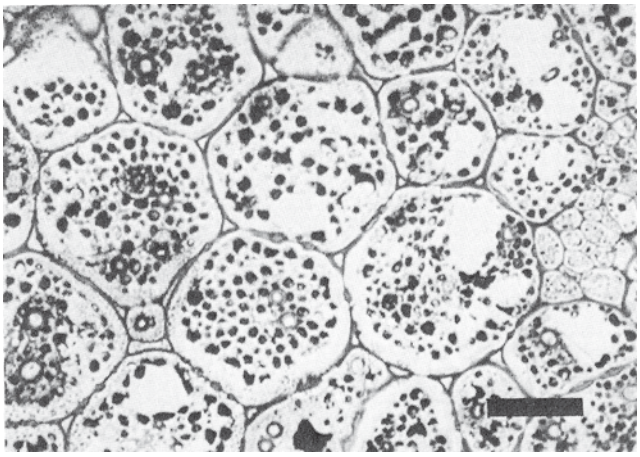

Figure 1. Light micrograph of a thick section of "unfixed" peanut cotyledon tissue. These micrographs were surprisingly similar to those taken of tissue fixed in glutaraldehyde (see text). Note that virtually every cell has an empty space between the cell wall and cytoplasm. Marker indicates 20 μm.

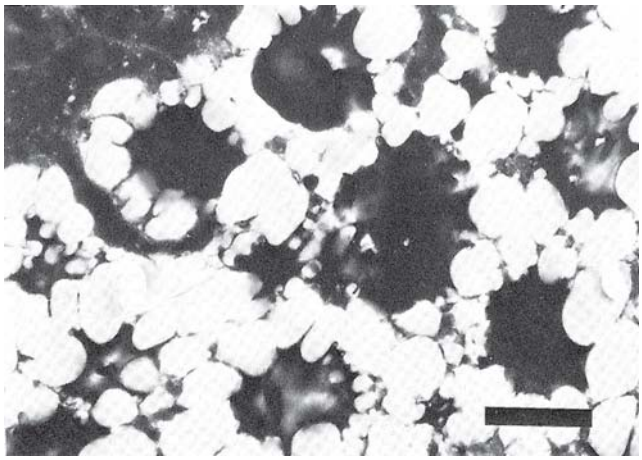

Figure 2. Electron micrograph of a portion of a cell taken from tissue seen in Figure 1. Numerous irregularly shaped aleurone grains (see text), and a portion of a nucleus can be seen. Spherosomes form the general matrix of the cell. It was surprising that so much structure was preserved in this unfixed tissue. Marker indicates 20 μm.

the resin itself is stable to the electron beam, I concluded that the cell walls were not sufficiently permeated with resin.

Attention was directed to a situation somewhat analogous to this in textile research. Reeves et al. (1960) showed a positive correlation between dye uptake by chemically cross-linked cotton fabric and the moisture content of the fabric when it was cross-linked. Interstitial pores in the matrix of the fiber walls caused by the different moisture content at the time of cross-linking were said to be responsible: the larger the pore size (moisture content), the greater the dye uptake. Similarly, I suggested that pores are opened in the cell walls of seeds by moisture and kept open by solvent exchange dehydration: cell walls with open pores being pervious to resin monomers while in the closed position, impervious.

To give an idea of the magnitude of cell wall pore sizes and the effect of moisture on plant cell walls, data were taken from literature and calculations were made.

Carpita et al. (1979) demonstrated pore sizes in living cotton hair cell walls to be about 35 A in diameter. These cell wall pores close upon air-drying due to capillary forces and can open again if the fibers are re-wetted. The pores never again regain their original size, however, even if re-wetted, because of secondary bonding that takes place when pore surfaces come together (24). Thus Goldthwait et al. (1950) showed that never-dried cotton was stainable with Chlorantine Fast Green BLL (a rather large size molecule), but upon drying and re-wetting was no longer stainable. Nirtrogen absorption studies have shown that dry cotton has an internal cell wall surface area of 0.6 to 0.7 m^2/g that expands to 137 m^2/g in the presence of water vapor (18).

Therefore, since the size of pores in the cell walls of never-dried cotton are about 35 A in diameter, and the pores become significantly smaller upon drying and re-wetting, and if the internal surfaces of cotton in the presence of water vapor are almost two orders of magnitude larger than for dry cotton, then the pores in the cell walls of dry cotton must be exceedingly small indeed. So small, in fact, that plastic monomers cannot pass.

To test this hypothesis I tried another approach: removal of certain constituents of the cell to observe the effects of this removal on the ability of resin to penetrate said tissue.

Since peanuts contain about 50% neutral lipids (8), I felt that removal of lipids might facilitate resin permeation of the seed tissues by creating 50% void spaces in the cells through which the resins might flow. I am aware that normal embedding procedures remove much of the lipid from the tissues; however, judging from osmiophilia of the spherosomes, much material remains. Oilseed tissues that are thoroughly extracted with fat solvents do not exhibit osmiophilia in the spherosomes (25).

In spite of exhaustively removing lipids and creating a 50% void space, dry-processed, air-dried peanut tissue failed to embed properly with Spurr's resin. On

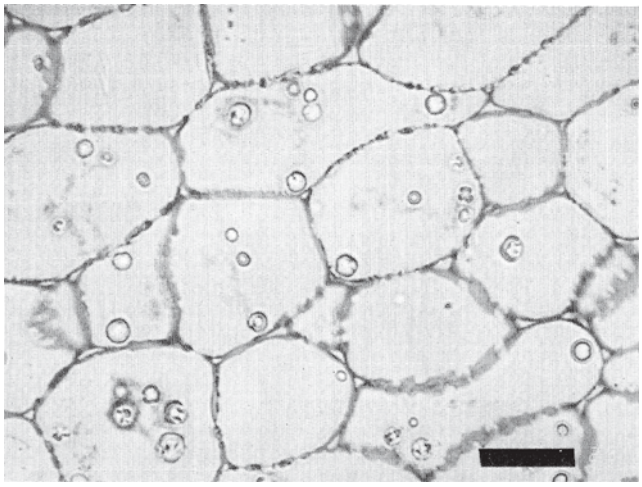

Figure 3. Light micrograph of pronase-treated peanut cotyledon tissue that was "dried" by solvent exchange. Note that the cells are virtually empty (compare with Figure 1). Some starch granules and bits of debris are left in the cells. Marker indicates 20 μm.

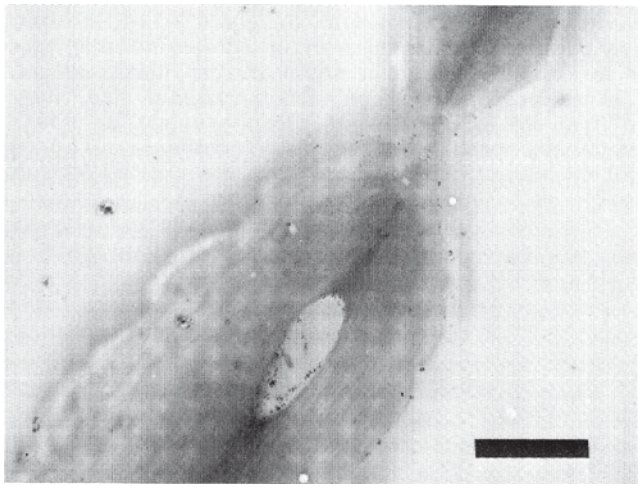

Figure 4. Higher magnification electron micrograph of pronase-treated peanut showing a portion of a cell wall that separates two cells. Note that there is nothing visible on the surface of the cell walls such as membranes or debris that could be interpreted as a possible barrier for the passage of plastic monomers. Marker indicates 20 μm.

the other hand, if dehydrated by solvent exchange, they embedded very well. The samples showed good preservation of cellular structure (Figure 1), particularly in view of the fact that they had not been fixed with some sort of cross-linking agent, such as an aldehyde. However, these tissues were soaked in alcohol and acetone during processing, and it will be recalled that alcohol (2) and acetone (13) are considered fixatives.

It will be noted that almost all of the cells exhibited a plasmolysis effect. This was the result of two phenomena: 1) water causes wrinkled cell walls of dry tissues (3, 7, 16) to extend and swell, while 2) alcohol causes hydrated tissues to shrink (2).

Approximately 30% of the dry weight of peanuts consist of proteins (8). Since practically all cellular entities have protein as an important part of their make-up, e.g., membranes, cytosol, nucleoproteins, ergastic substances, etc., I felt that treatment of the seed tissues with a wide-spectrum bacterial protease should remove much of the remaining cellular constituents. Figure 3 is a light micrograph of peanut tissue that was treated with pronase — a wide-spectrum bacterial protease. As can be seen, virtually all of the cellular contents have been hydrolyzed away by this treatment; only non-proteinaceous cell walls, starch granules, and some debris that was not resolvable in the light microscope remained. Examination of the cells in the electron microscope failed to contribute much more information; it merely confirmed that the cells were empty. Inspection of the "debris" proved unfruitful. I was unable to recognize any familiar profiles and concluded that the debris is made up of remnants of membranes, ribosomes, etc., that contain substances not affected by pronase, e.g., phospholipids, nucleic acids, and polysaccharides.

Since no provisions for removal of phospholipids were made, there was a possibility that plasma membranes could have survived pronase treatment and hinder passage of resins through the cell walls; but no membrane profiles were seen in the tissues examined. Figure 4 is an electron micrograph of cell walls where plasma membranes should be located; there is no sign of membranes, apparently they have been abolished by the pronase treatment. Aside from starch granules (and possibly debris), there is nothing else left in the tissues that can hinder passage of resins — except the cell walls. Therefore, I am forced to conclude that dry-processed, air-dried cell walls *are* the barriers that exclude resins from seed tissues.

These results confirm my earlier hypothesis that the cell wall of dry seed tissue is the limiting factor that bars permeation of seed tissue by resins when processed dry.

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